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ANTIOXIDANT, ANTI-INFLAMMATORY, AND HEP G2 CELL GROWTH-INHIBITORY EFFECTS OF AQUEOUS-ETHANOL EXTRACTS OBTAINED FROM NON-PUFFED AND COMPRESSIONAL-PUFFED SARGASSUM CRASSIFOLIUM

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ANTIOXIDANT, ANTI-INFLAMMATORY, AND HEP G2 CELL GROWTH-INHIBITORY EFFECTS OF AQUEOUS-ETHANOL EXTRACTS OBTAINED FROM NON-PUFFED AND COMPRESSIONAL-PUFFED *SARGASSUM CRASSIFOLIUM*

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Key words: anti-inflammation, antioxidant, Hep G2 hepatoma cells, *Sargassum crassifolium*.

ABSTRACT

There is growing interest in the bioactive components of marine algae due to their biomedical importance. In this study, a compressional-puffing (CP) process was adopted to pretreat algal samples of *Sargassum crassifolium* (SC), and the bioactive components of non-puffed and compressional-puffed SCs were extracted using aqueous-ethanol solution. Two extracts, namely, NPSC (non-puffed) and CPSC (compressional-puffed) were obtained, and their compositions were analyzed and compared. It was found that the composition of CPSC extract was different from that of NPSC. Further biological activity studies revealed that extracts of both NPSC and CPSC exhibited antioxidant activity, but only NPSC extract showed anti-inflammatory activity. In growth inhibition experiments, it was found that CPSC extract had a more cytotoxic effect in Hep G2 hepatoma cells and induced more DNA fragmentation of Hep G2 hepatoma cells compared with the effects exerted by NPSC extract. Taken together, both NPSC and CPSC extracts exhibited antioxidant activity. NPSC extract was investigated to determine its potential as a natural anti-inflammatory ingredient. CPSC extract had higher inhibitory activity in liver cancer cell growth and may have potential as an effective agent for the prevention or adjuvant treatment of hepatocellular carcinoma.

I. INTRODUCTION

The prevalence of numerous types of human cancers is high in many countries, and is one of the leading causes of death. In Taiwan, cancer is becoming an increasingly important public health issue due to its aging population and the widespread adoption of Western lifestyle habits (Chiang et al., 2010). According to statistical data provided by Taiwan's Ministry of Health and Welfare, cancer was the leading cause of death in 2016. In addition, the top three causes of cancer death were lung, liver, and colon cancer, respectively (Ministry of Health and Welfare, 2016). As a general rule, anticancer drugs are used to treat tumors. Unfortunately, these drugs are commonly toxic and affect not only cancer cells but also normal cells and tissues. Moreover, chemotherapy and radiation therapies for cancer frequently induce severe side effects and thus their efficacy is limited (Komarov et al., 1999). Consequently, nontoxic agents from natural sources are critically needed for the prevention or auxiliary treatment of cancer.

Marine algae have been the focus of growing interest in the production of various therapeutic compounds and a number of them have been found to exhibit great antibiotic, antioxidant, anticoagulant, anti-inflammatory, antimicrobial, antiviral, and antitumoral properties (Mariya and Ravindran, 2013). A variety of studies have been conducted with aqueous extracts of

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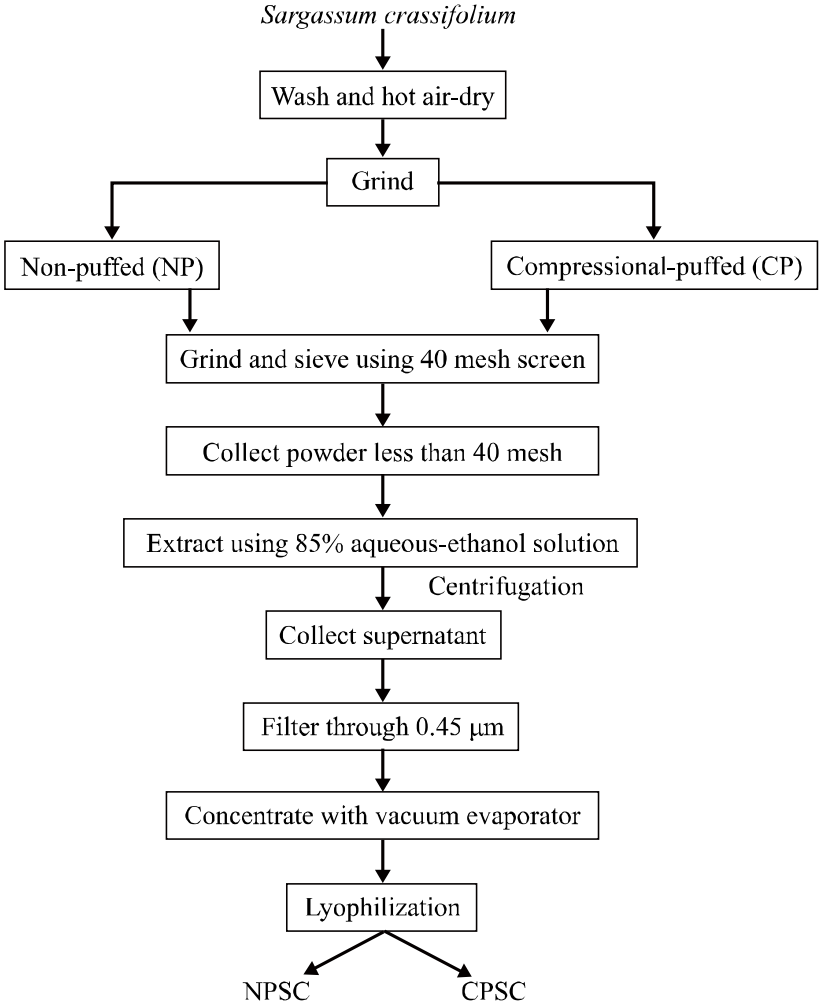

Fig. 1. Procedure for the compressional-puffing of *S. crassifolium* and extraction of aqueous-ethanol extracts from non-puffed and compressional-puffed *S. crassifolium*

Sargassum spp. to determine their antioxidant activity (Wang et al., 2015), as well as their antibacterial (Lavanya and Veerappan, 2011), immunomodulatory (Wu et al., 2016), and inhibitory effects on cancer cell growth (Wang et al., 2015). However, to date, few studies have conducted compositional analyses and evaluations of the biological functions of aqueous-ethanol extracts from *Sargassum* spp. Taiwan has abundant marine resources, including a plentiful supply of various brown algae species. The production of *Sargassum* spp. is sufficient for the extraction of bioactive compounds on a commercial scale. The present study builds upon on the research reported in our previous investigations (Huang et al., 2018a; Huang et al., 2018c; Huang et al., 2016; Yang et al., 2017). In brief, an oven-dried brown seaweed *S. crassifolium* (SC), which was harvested from the southern coastal area of Taiwan, was either non-puffed or compressional-puffed (CP) at 18.3 kg/cm^2 and then bioactive components were extracted by 85% aqueous-ethanol solution. To the best of the authors' admittedly limited knowledge, this is the first investigation to combine CP pretreatment and aqueous-ethanol extraction to obtain active components from SC and analyze their biological activities. The recovered NPSC and CPSC extracts were further examined to determine their composition, as well as their antioxidant, anti-inflammatory, and Hep G2 cell growth-inhibitory properties. Our aim was to explore naturally derived agents which showed antioxidant effects and were capable of modulating the immune system, and additionally exhibited their potential applicability in preventive or auxiliary treatment of liver cancer.

II. MATERIALS AND METHODS

1. Chemicals

Potassium persulfate and ferrous chloride were purchased from Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), sodium nitrite, sodium carbonate, LPS, Prussian blue, Folin-Ciocalteu's phenol reagent, gallic acid, L-fucose, D-galacturonic acid, bovine serum albumin (BSA), Bradford reagent, dimethyl sulfoxide (DMSO),

3-(2-pyridyl)-5, 6-bis (4-phenylsulfonic acid)-1, 2, 4-triazine (ferrozine), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and 2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Calbiochem (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM), Minimum Essential Medium (MEM), trypsin/EDTA, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco Laboratories (Grand Island, NY, USA). Phenol, sulfuric acid, potassium ferricyanide, trichloroacetic acid (TCA), and iron(III) chloride 6-hydrate were obtained from Nihon Shiyaku Industrial, Ltd. (Tokyo, Japan). All other reagents if not declared were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were all of analytical grade.

2. Materials

A sample of SC, collected from a coastal area of Pingtung, Taiwan, was washed with fresh water soon after collection in order to remove salt and sand, oven-dried at 50 ºC, and then kept in plastic bags at 4 ºC until use.

3. Compressional-Puffing Procedure

A compressional-puffing (CP) method (Huang et al., 2016) with minor modification was adopted to pretreat algal samples before the extraction procedure. In brief, the dried algal samples were ground and sieved using a 20-mesh screen. The portion retained by the screen was collected and puffed using a continuous and patented CP machine with temperature set at 220 ºC. After the CP process, the algal samples were ground into fine particles and stored at 4 ºC for further extraction experiments.

4. Extraction Procedure

The process for preparation of aqueous-ethanol extracts from non-puffed and compressional-puffed SC was performed according to a previous method (Samee et al., 2009) with minor modification. In brief, the non-puffed and compressional-puffed algal samples were ground until the particle size was less than 40 mesh and mixed with 85% ethanol (w/v = 1:25), then placed at room temperature for 6 h with shaking. The mixture was centrifuged at 2,200 × *g* for 30 min and the supernatant was collected and filtered through 0.45 μm. The filtrate was concentrated with a vacuum evaporator and then lyophilized. A detailed summary of the CP and aqueous-ethanol extraction processes is provided in Fig. 1. Extraction yield was calculated using the following equation:

$$\text{Extraction yield (\%)} = (gA / gB) \times 100$$

where gA represents weight of the extracted solid on a dry basis, and gB is the weight of the algal sample on a dry basis.

5. Color Analysis

The algal samples, after being ground and sieved through a 40 mesh screen, were utilized for the determination of color. Tristimulus color values, namely, *L* (lightness), *a* (redness–greenness), and *b* (yellowness–blueness) values, were measured using a spectrophotometer (SA-2000, Nippon Denshoku Industries Co., Ltd., Tokyo, Japan). The data were recorded using at least three separate algal samples for each test point. The browning index was obtained using the following equation (Ureta et al., 2016):

$$\text{Browning index} = \frac{\left[100\left(x-0.31\right)\right]}{0.172}$$

Where

$$x = \frac{\left(a+1.75L\right)}{\left(5.645L+a-0.312b\right)}$$

6. Bulk Density

The volume of the algal samples was determined by filling a container of known volume, and noting the sample weight. The bulk density was calculated as the ratio of the mass of the sample to that of its volume. Bulk density results were based on an average of five measurements.

7. Biochemical Analyses

The phenol-sulfuric acid colorimetric method was utilized to determine the total sugar content, and L-fucose was used as the standard. The fucose content was determined according to the method of Gibbons (Gibbons, 1955) using L-fucose as the standard. Uronic acids were estimated by the colorimetric method using D-galacturonic acid as the standard (Filisetti-Cozzi and Carpita, 1991). Alginate content was measured according to a previously described method (Honya et al., 1993). Protein in the extract was quantified by the Bradford method using BSA as the standard. Polyphenols were analyzed by the Prussian blue assay (Margraf et al., 2015) and gallic acid was utilized as a standard agent. Lipid was determined gravimetrically after Soxhlet extraction with petroleum ether.

8. DPPH Radical Scavenging Activity

The scavenging activity of the DPPH radical in the samples was determined using a method previously described (Huang et al., 2018d). In brief, 50 μL of sample was added to 200 μL 0.1 mM DPPH solution (in methanol). The mixture was shaken vigorously for 1 min and left to stand for 30 min in the dark at room temperature. After the reaction, the absorbance of all sample solutions was then measured at 517 nm using an ELISA reader (PowerWave 340, Bio-Tek Instruments, Winooski, VT, USA). The radical-scavenging activity was calculated as the percentage inhibition using the following equation:

$$DPPH_{radical\text{-}scavenging}\ (\%) = (1 - \frac{A_{sample}}{A_{control}}) \times 100$$

where A_{sample} is the absorbance of the methanol solution of DPPH with tested samples, and $A_{control}$ represents the absorbance of the methanol solution of DPPH without the sample.

9. ABTS Radical Cation Scavenging Activity

The ABTS radical cation scavenging activity was performed according to the previously described method (Huang et al., 2018d). The $ABTS^{\bullet+}$ solution was produced by mixing 5 mL of 7 mM ABTS solution with 88 μL of 140 mM potassium persulfate and allowing the mixture to stand in the dark for 16 h at room temperature before use. The $ABTS^{\bullet+}$ solution was diluted with 95% ethanol so that its absorbance at 734 nm was adjusted to 0.70 ± 0.05. To determine the scavenging activity, 100 μL of diluted $ABTS^{\bullet+}$ solution was mixed with 100 μL of various sample solutions and the mixture was allowed to react at room temperature for 6 min. After the reaction, the absorbance of all sample solutions was then measured at 734 nm using an ELISA reader (PowerWave 340, Bio-Tek Instruments, Winooski, VT, USA). The blank was prepared in the same manner, except that distilled water was used instead of the sample. The scavenging activity of $ABTS^{\bullet+}$ was calculated using the following equation:

$$ABTS_{cation\ radical\text{-}scavenging}\ (\%) = (1 - \frac{A_{sample}}{A_{control}}) \times 100$$

where A_{sample} is the absorbance of ABTS with tested samples, and $A_{control}$ represents the absorbance of ABTS without the sample.

10. Ferrous Ion-Chelating Activity

The ferrous ion-chelating activity of extracts was measured using the previously described method (Wang et al., 2015). Briefly, 200 μL of sample, 740 μL of methanol, and 20 μL of $FeCl_2$ solution (2 mM) were mixed. The mixture was incubated for 30 s followed by the addition of 5 mM ferrozine (40 μL). After allowing the reaction to continue for 10 min at room temperature, the absorbance of the mixture was measured at 562 nm using an ELISA reader (PowerWave 340, Bio-Tek Instruments, Winooski, VT, USA). The chelating activity of ferrous ion was calculated as follows:

$$Chelating\ activity\ (\%) = (1 - \frac{A_{sample}}{A_{control}}) \times 100$$

where A_{sample} is the absorbance with tested samples, and $A_{control}$ represents the absorbance without the sample.

11. Reducing Power Assay

The reducing power of samples was measured according to the previously described method (Wang et al., 2015). In brief, 0.5 mL of the sample was mixed with 0.5 mL of phosphate buffer (0.2 M; pH 6.6) and 0.5 mL of potassium ferricyanide (1%). The mixture was incubated at 50 ºC for 20 minutes, and 0.5 mL of TCA (10%) was added to the reaction followed by a centrifugation step (970 × *g* for 10 min). Finally, 0.5 mL of the supernatant solution was mixed with 0.5 mL of double-distilled water and 0.1 mL of $FeCl_3$ (0.1%), and then the resultant solution was left to stand for 10 minutes. The absorbance was measured at 700 nm using an ELISA reader (PowerWave 340, Bio-Tek Instruments, Winooski, VT, USA).

12. Cell Line and Culture

Murine macrophage cell lines RAW 264.7 (BCRC no. 60001) were obtained from the Biosource Collection and Research Center, the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan). The cells were grown in DMEM supplemented with 10% FBS and 100 U/mL penicillin-streptomycin solution at 37 °C in a humidified chamber with 5% CO_2. The human Hep G2 hepatoma cell line (BCRC no. RM60025) was obtained from FIRDI, which was cultured in MEM supplemented with 10% of FBS, 100 U/mL penicillin-streptomycin solution, and 2 mM glutamine, and then incubated at 37 °C in a humidified chamber with 5% CO_2.

13. Measurement of Nitrite Oxide in Culture Media

RAW 264.7 cells (2×10^5 cells/ml) were seeded in a 96-well flat bottom plate for 24 h at 37 °C with 5% CO_2. The culture medium was removed and replaced with fresh medium containing tested samples at various concentrations (0–20 μg/mL) prior to challenging with 1 μg/mL of LPS. The nitrite concentration was measured in the culture supernatant after 24 h of co-incubation. In brief, 50 μL of the cultured supernatants was added in the 96-well plate and 100 μL of Griess reagent was added to each well and allowed to stand for 10 min at room temperature. The absorbance at 540 nm was measured using an ELISA reader (PowerWave 340, Bio-Tek Instruments, Winooski, VT, USA), and the quantification of nitrite was standardized with $NaNO_2$ at 0–100 μM concentrations (Kobuchi et al., 1997).

14. Measurement of Cell Viability

The MTT assay was used to evaluate cell viability. Briefly, RAW 264.7 cells (2×10^5/ml in a 96-well plate) or human Hep G2 hepatoma cells (1×10^5/ml in a 96-well plate) were plated with culture medium and incubated for 24 h at 37 ºC, with 5% CO_2 in a humidified atmosphere. The medium was removed and fresh serum-free medium containing different concentrations of the tested compounds were added. After 24 h (for RAW 264.7 cells) or 48 h (for human Hep G2 hepatoma cells) of incubation at 37 ºC, with 5% CO_2, the MTT reagent (0.1 mg/mL) was added. After incubating at 37 ºC for 4 h, the MTT reagent was removed and DMSO (100 μL) was added to each well and thoroughly mixed by pipetting to dissolve the MTT-formazan crystals. The absorbance was then determined by an ELISA reader (PowerWave 340, Bio-Tek Instruments,

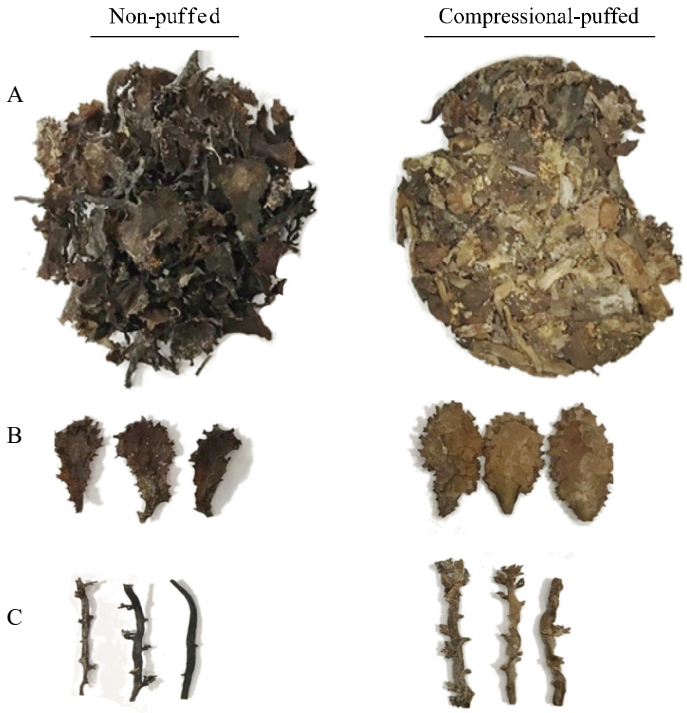

Fig. 2. Morphologies of non-puffed and compressional-puffed *S. crassifolium*. (A) Algal sample; (B) Blade; (C) Stem

Winooski, VT, USA) at a wavelength of 570 nm. The cell viability (%) was calculated using the following equation (Huang et al., 2018b):

$$\text{Cell viability (\%)} = (\frac{T}{C}) \times 100$$

where T is the absorbance in the test, and C is the absorbance for the control.

15. Cell Cycle Analysis

Human Hep G2 hepatoma cells were plated at 1×10^6 cells/ml in 10 cm dish with MEM growth medium and incubated for 24 h at 37 °C, with 5% CO_2 in a humidified atmosphere. Thereafter, cells were treated with tested compounds at 500 μg/mL for 48 h. Floating and adherent cells were then collected and washed with 1× ice-cold phosphate-buffered saline (PBS) twice. The cell pellets were collected and fixed with 70% ice-cold ethanol and then stored in the freezer for at least 2 h. After washing with staining buffer twice, cells were then stained with 50 μg/mL propidium iodide (PI) in the presence of 25 μg/mL RNase A at 37 °C for 15 min. Flow cytometric analysis was completed using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) and a minimum of 10,000 cells per sample were collected. To estimate the percentage of each phase in the cell cycle, the data were analyzed by BD Accuri C6 software.

16. Statistical Analysis

Experiments were performed at least three times. Values represent the means ± standard deviation (SD). Statistical evaluation of data was calculated by Student's t-test or one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range tests. The differences were considered to be statistically significant if $p < 0.05$.

III. RESULTS AND DISCUSSION

1. Proximate Compositions of *S. crassifolium*

An algal sample of SC was collected from Pingtung, Taiwan. The proximate composition of SC was found to be 2.36% protein, 0.98% lipid, 33.98% ash, and 62.67% carbohydrate (dry basis) (Yang et al., 2017). In another study, SC collected from North Jakarta, Indonesia contained 9.73% protein, 0.72% lipid, 24.04% ash, and 65.51% carbohydrate (dry basis) (Kawaroe et al., 2013). The variations in the proximate composition of these two samples may be attributed to the growth, maturity, and harvest season of algae (Murakami et al., 2011). However, both findings provide evidence that the most abundant element in the proximate composition of SC is carbohydrate, followed by ash and then protein, with lipid having the least amount. Therefore, SC may serve as a good source of certain bioactive carbohydrate molecules such as alginate, fucoidan, and laminarin (Percival, 1979). In addition, a previous report suggested that some plant-derived polyphenols appear to be twisted together with cell wall polysaccharides via tight hydrophilic and hydrophobic bonds (Wijesinghe and Jeon, 2012), and thus co-extraction of phenolic compounds from brown

Table 1. Characteristics of puffed algal samples and extraction yields of aqueous-ethanol extracts for NPSC and CPSC.

Characteristics of puffed algal samples	NPSC[b]	CPSC[b]	*p* value
L	41.7 ± 0.0	40.7 ± 0.0	***
a	4.44 ± 0.00	4.00 ± 0.02	***
b	3.55 ± 0.04	3.13 ± 0.07	**
Browning index	16.4 ± 0.1	14.9 ± 0.2	***
Bulk density (g/ml)	0.60 ± 0.00	0.51 ± 0.00	***
Extraction yield (%)[a]	17.1 ± 0.5	17.7 ± 0.4	

[a]Extraction yield (%) = ($g_{solid\ extract,\ dry\ basis}$ /$g_{algal\ sample,dry\ basis}$) × 100

[b]Values are mean ± SD (n = 3); **, $p < 0.01$; ***, $p < 0.001$.

Table 2. Compositional analyses of aqueous-ethanol extracts for NPSC and CPSC.

Compositional analyses of aqueous-ethanol extracts	NPSC[b]	CPSC[b]	*p* value
Total sugar (%)[a]	22.9 ± 2.3	20.0 ± 0.9	
Fucose (%)[a]	6.48 ± 0.51	5.70 ± 0.00	
Uronic acid (%)[a]	7.61 ± 0.11	5.48 ± 0.59	**
Alginate (%)[a]	10.5 ± 0.1	7.31 ± 0.31	**
Protein (%)[a]	1.12 ± 0.22	3.94 ± 0.32	***
Polyphenols (%)[a]	0.50 ± 0.01	0.57 ± 0.02	**
Lipid (%)[a]	10.82	8.66	

[a]Total sugar (%), fucose (%), uronic acid (%), alginate (%), protein (%), polyphenols (%), lipid (%) = ($g/g_{extracts,\ dry\ basis}$) × 100

[b]Values are mean ± SD (n = 3); **, $p < 0.01$; ***, $p < 0.001$.

algae during the extraction process commonly occurs.

2. Effects of Compressional-Puffing Variables on Characteristics of Puffed Algal Samples and Compositions of Aqueous-Ethanol Extracts for NPSC and CPSC

The dried algal samples were ground and sieved using a 20-mesh screen, and the portion retained by the screen was collected and subjected to either non-puffing or compressional-puffing. For the CP process, the algal sample was put into a round chamber and a mechanical compression force of approximately 5 kg/cm^2 was applied to the algal sample three times before puffing. Afterward, the chamber was heated to the puffing temperature. Generally, a high puffing temperature and a long heating time results in a higher puffing pressure (Yoon et al., 2005). The puffing temperature was set at 220 ºC, and the corresponding pressure level inside the chamber was approximately 18.3 kg/cm^2. The total reaction time for the operation was about 10 s. After the puffing process, the degree of moisture loss in CPSC was high and reached 35.5% ± 1.1%. The mechanical compression force presses and compacts the structure of the algal sample before puffing, which distinguishes the CP process from the traditional rotary puffing gun. After the CP process, the compressional-puffed algal sample possessed a flattened and pressed surface morphology (Fig. 2A). The blade and stem of compressional-puffed algal samples showed a change in color and an expanded volume as compared to the non-puffed algal sample (Figs. 2B and 2C). Table 1 shows the characteristics of non-puffed and compressional-puffed algal samples. The color change between non-puffed and compressional-puffed algal samples was characterized by measurements of the Hunterlab *L*, *a*, and *b* values, and browning index. The *L*, *a*, and *b* values, and browning index of CPSC significantly differed from those of NPSC (Table 1). The CPSC had a smaller browning index than NPSC, indicating that the heat and pressure generated by the puffing treatment resulted in puffed algal samples with a lighter color. The volume expansion of compressional-puffed algal samples was characterized by analyzing the bulk density. It was found that the algal sample of CPSC had a lower bulk density (0.51±0.00 g/ml) than that (0.60±0.00 g/ml) of NPSC, which suggests that puffing treatment may expand the volume of algal samples while simultaneously decreasing its bulk density (Table 1). The non-puffed and compressional-puffed algal samples were then ground to fine particles and sieved through 40 mesh. The resulting samples were utilized to extract the bioactive components using 85% aqueous-ethanol solution at 25 ºC for 6 h. The extraction yields of extracts for NPSC and CPSC were 17.10± 0.50 and 17.66±0.35 g/100g, respectively (Table 1). Although the extraction yields between NPSC and CPSC do not significantly differ, both NPSC and CPSC had relatively high extraction yields, which would be advantageous in terms

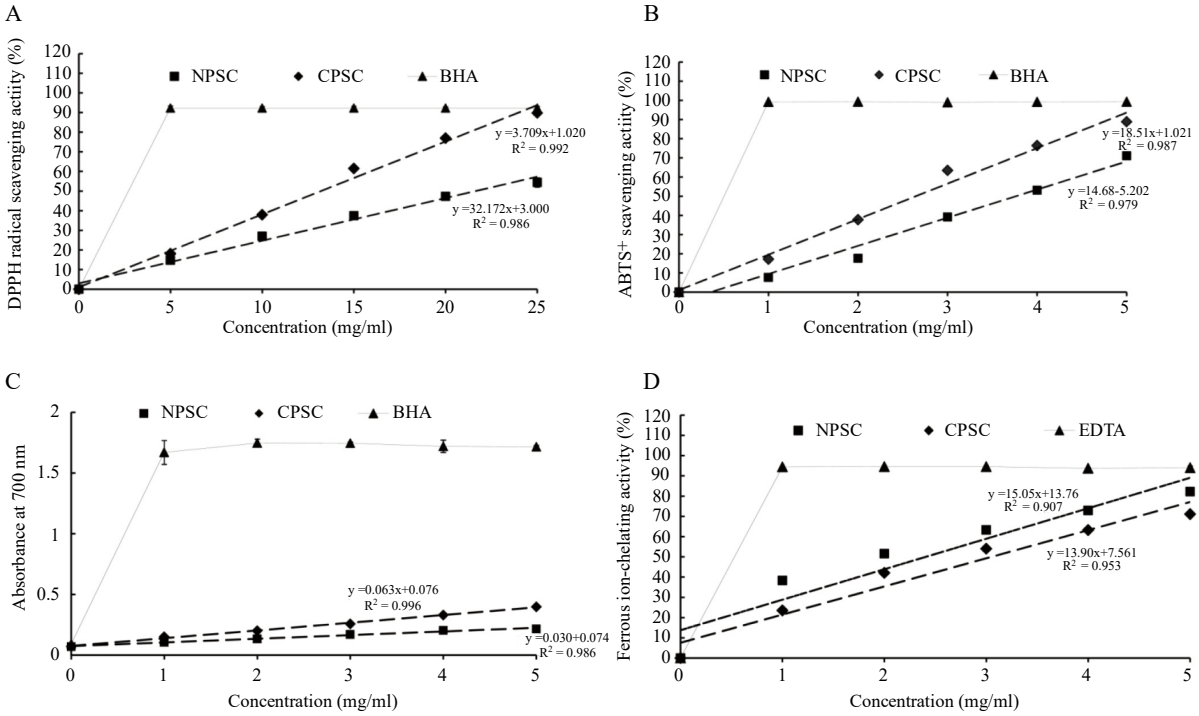

Fig. 3. Antioxidant activities of NPSC and CPSC. (A) DPPH radical scavenging activity for NPSC, CPSC, and BHA. (B) ABTS$^{\bullet+}$ scavenging activity for NPSC, CPSC, and BHA. (C) Reducing power for NPSC, CPSC, and BHA. (D) Ferrous ion-chelating activity for NPSC, CPSC, and EDTA. Each value represents the mean ± SD of three determinations. The linear regression curve and linear regression equation for NPSC and CPSC are shown on each graph.

of scaling up to the levels required for commercial production. A previous study suggested that pigments, fats, proteins, polysaccharides, and other minor bioactive components could possibly be extracted from seaweeds by 85% aqueous-ethanol solution (Samee et al., 2009). Therefore, we analyzed the composition of NPSC and CPSC extracts and the results are presented in Table 2. It was found that the total sugar and fucose contents for NPSC and CPSC did not significantly differ. CPSC extract had higher protein and polyphenol contents than those of NPSC. In contrast, NPSC extract had higher lipid, alginate, and uronic acid contents than those of CPSC. These findings suggest that the CP process altered the composition of aqueous-ethanol extract of CPSC as compared to NPSC. Among the components which exhibited different contents in NPSC and CPSC extracts, alginate (mainly consisting of mannuronic acid and guluronic acid) isolated from brown algae exhibited anti-inflammatory and antioxidant potentials (Borazjani et al., 2017). Polyphenols have been reported to exhibit strong antioxidant (Shahidi et al., 1992) and anti-cancer (Namvar et al., 2013; Pallela et al., 2010) properties. In addition, fatty acids and lipid derivatives possessed anti-inflammatory activity (Fernando et al., 2016). Since the NPSC and CPSC extracts were composed of different bioactive components, we therefore further investigated their antioxidant, anti-inflammatory, and cancer cell growth inhibition properties.

3. Effects of NPSC and CPSC extracts on Antioxidant Activities

The antioxidant activities for NPSC and CPSC were examined by DPPH radical scavenging activity, ABTS$^{\bullet+}$ scavenging activity, reducing power, and ferrous ion-chelating activity. Fig. 3A shows the DPPH radical scavenging properties of NPSC, CPSC, and butylated hydroxyanisole (BHA) (as a reference). It can be seen that NPSC and CPSC displayed DPPH radical scavenging activity in a dose-dependent pattern. Moreover, CPSC showed higher DPPH radical scavenging activity than that of NPSC. Consistent with previous investigations, the higher DPPH radical scavenging activity of CPSC was positively correlated with its high polyphenol content (Table 2) (Augusto et al., 2014). The ABTS$^{\bullet+}$ scavenging properties of NPSC, CPSC, and BHA (as a reference) are presented in Fig. 3B. The NPSC and CPSC showed ABTS$^{\bullet+}$ scavenging activity in a dose-dependent manner. In addition, CPSC had a higher ABTS$^{\bullet+}$ scavenging property than that of NPSC. The higher ABTS$^{\bullet+}$ scavenging activity of CPSC was also positively correlated with its high polyphenol content, which is in line with previously reported results (Table 2) (Augusto et al., 2014). Reducing power is also an indicator of antioxidant activity. In the reaction system, antioxidant components in samples cause a reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+} form, and Fe^{2+} can be monitored by measuring the formation of Prussian blue at 700 nm (Wang et al., 2010). Fig. 3C shows the reducing power of

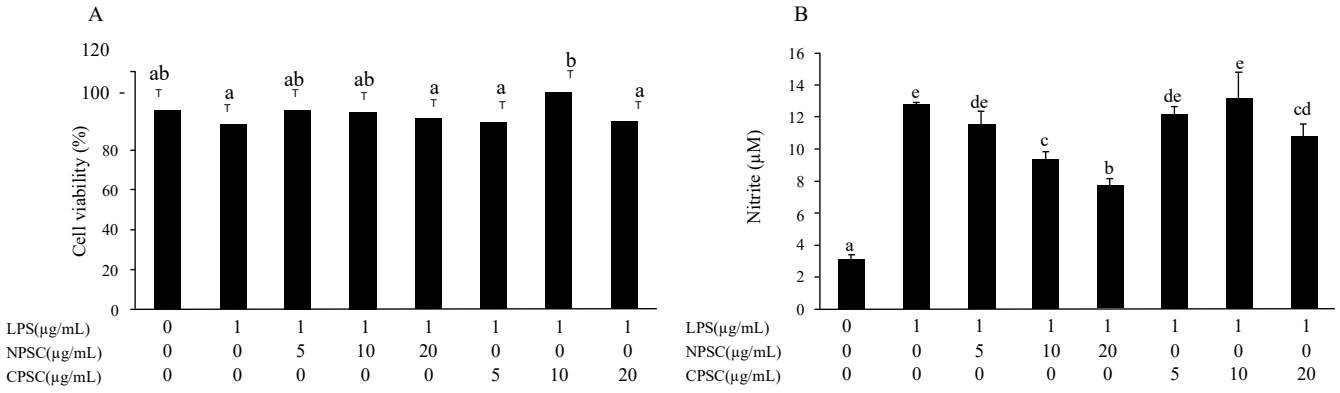

Fig. 4. (A) Effects of NPSC, CPSC, and LPS on cell viability of RAW 264.7 cells. (B) Effects of NPSC, CPSC, and LPS on NO secretion in RAW 264.7 cells. The data are the means ± SD of triplicate samples. Bars with different letters are significantly different ($p < 0.05$)

NPSC, CPSC, and BHA (as a reference). It can be seen that NPSC and CPSC displayed reducing power in a dose-dependent pattern. In addition, CPSC showed a higher reducing power than that of NPSC. Consistent with previous reports, the higher reducing power of CPSC may also be attributed to its high polyphenol content (Table 2) (Eslami et al., 2016). Polyphenol content has been reported as a critical factor contributing to antioxidant activity (Beratto-Ramos et al., 2019). The polyphenol contents of NPSC and CPSC were 0.50 ± 0.01 and 0.57 ± 0.02 g gallic acid equivalents per 100 g dried plants, respectively (Table 2), which were relatively low values compared with other reported data (Cai et al., 2004). NPSC and CPSC are crude extracts and possess only tiny amounts of polyphenols, whereas BHA is a pure organic compound and a chemical derivative of phenol. This difference may explain why the antioxidant activities of NPSC and CPSC were very low compared to BHA. Ferrous ions were revealed to stimulate lipid peroxidation and have been recognized as an effective pro-oxidant in food systems (Yamauchi et al., 1988). The ferrous ions generate free radicals from peroxides via Fenton reactions. Ferrozine can form a complex with ferrous ions. In the presence of other chelating agents, this complex is less formed and the red color of the complex is diminished (Eslami et al., 2016). The ferrous ion-chelating activities of NPSC, CPSC, and EDTA (as a reference) are presented in Fig. 3D. The NPSC and CPSC showed ferrous ion-chelating activities in a concentration-dependent fashion. NPSC showed higher ferrous ion-chelating activity than that of CPSC. Although polyphenols were shown to have ferrous ion-chelating activity (Eslami et al., 2016), it was reported that alginates have a high affinity for divalent cations (Chen et al., 2002; Khoo and Ting, 2001). Table 2 shows that NPSC had a higher alginate content than that of CPSC. Thus, it is reasonable to postulate that the alginate content in extracts may play a more important role in the ferrous ion-chelating activity than polyphenols. Taken together, we found that NPSC and CPSC exhibited antioxidant activities and both could serve as natural antioxidant agents in the food and pharmaceutical industries. The CPSC had higher DPPH radical scavenging activity, $ABTS^{\bullet+}$ scavenging activity, and reducing power, which may be due to its high polyphenol content. In contrast, NPSC had higher ferrous ion-chelating activity, which may be attributed to its higher alginate content. A previous study suggested that antioxidants may enhance immune defenses and lower the risk of cancer and degenerative diseases (Valko et al., 2006), and therefore the anti-inflammation and anti-cancer activities of NPSC and CPSC were further examined.

4. Effects of NPSC and CPSC extracts on Anti-inflammatory Properties

NO is an inflammatory mediator induced by inflammatory cytokines or bacterial LPS in various cell types including macrophages (Swindle and Metcalfe, 2007). Samples with NO inhibitory activity thus have the potential to possess anti-inflammatory activity. NPSC and CPSC extracts were tested for their anti-inflammatory activities by investigating their effects on NO production in LPS-induced RAW264.7 macrophages. Neither NPSC nor CPSC obviously affected the viability of RAW264.7 cells at the 5-20 μg/mL concentrations that were tested, in the presence of 1 μg/mL LPS (Fig. 4A). As shown in Fig. 4B, when RAW264.7 cells were treated with 1 μg/mL LPS, the NO production was increased from 3.11±0.25 μM to 12.75±0.14 μM. Moreover, when RAW264.7 cells were treated with 1 μg/mL LPS in the presence of various concentrations of NPSC, it was found that NO production was significantly decreased from 12.75±0.14 μM to 7.73±0.44 μM, whereas in the presence of various concentrations of CPSC, NO production was not obviously affected. Previous studies indicated that potential anti-inflammatory components from marine algae may include polysaccharides (containing alginate), fatty acids and lipid derivatives, and other compounds (Fernando et al., 2016). An analysis of the data presented in Table 2 showed that CPSC possessed low amounts of uronic acids, alginate, and lipid, which may account for the low anti-inflammatory activity in CPSC. However, further research is necessary to obtain a better understanding of the composition, structural heterogeneity, and anti-inflammatory activity in NPSC and CPSC. Overall, these results indicate that NPSC

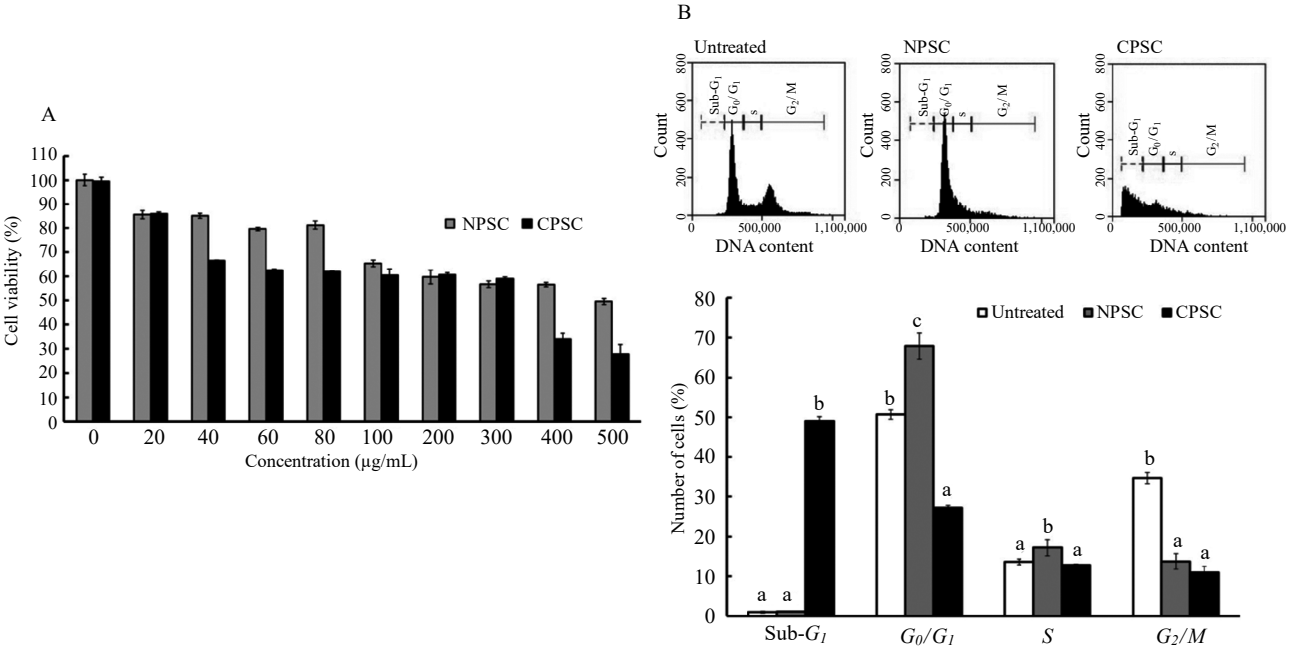

Fig. 5. The growth inhibitory effects of NPSC and CPSC on human Hep G2 hepatoma cells. (A) Cytotoxic effects of NPSC and CPSC on human Hep G2 hepatoma cells. The data are the means ± SD of triplicate samples. (B) The cell cycle profiles of Hep G2 hepatoma cells after treatment of NPSC and CPSC at 500 μg/mL for 48 h. The bar graph summarizes the three cell cytometry experiments and shows the percentage of cells in the sub-G_1, G_0/G_1, S, and G_2/M phase of the cell cycle according to treatments after analysis using BD Accuri C6 software. The data are the means ± SD of triplicate samples. In each group of columns related to each cell cycle phase, the means which have at least one common letter do not differ significantly ($p < 0.05$).

extract showed anti-inflammatory activity, and thus it may have application as a natural anti-inflammatory ingredient. It has been reported that NO is an important biological mediator of the non-specific host defense against invading microbes and tumors (Diouf et al., 2009). Although CPSC extract did not possess obvious NO modulatory functions, certain molecules such as L-fucose (Tomsik et al., 2011) and phenolic compounds (Hussain et al., 2016) in CPSC may also contribute to anti-cancer activity. Therefore, the anti-cancer functions of both NPSC and CPSC extracts were further characterized.

5. Effects of NPSC and CPSC extracts on Liver Cancer Cell Growth Inhibition

Since the antioxidant and anti-inflammatory activities of NPSC and CPSC have been examined previously, we subsequently evaluated the effects of NPSC and CPSC on the inhibition of human Hep G2 hepatoma cell growth using a tetrazolium-based colorimetric assay (MTT test) and cell cycle analysis. Fig. 5A shows that Hep G2 hepatoma cell growth can be inhibited by NPSC and CPSC, and the latter showed a more potent inhibitory effect than the former. Phenolic compounds from brown seaweeds are thought to be linked to receptor sensitivity, cell signaling pathways in gene regulation or in inflammatory enzyme activity, and inhibition of cancer cell growth (Hussain et al., 2016). Hence, higher polyphenol content in CPSC may account for its higher growth inhibitory effect on Hep G2 hepatoma cells. However, more *in vitro* and *in vivo* evidence is still needed to elucidate the mechanisms involved. The treatment of Hep G2 hepatoma cells with 500 μg/mL of NPSC and CPSC also resulted in a marked reduction of the number of viable cells by more than 50%, and the percentage of viable cells reached 49.52% ± 1.25% (for NPSC) and 27.83% ± 3.91% (for CPSC), respectively (Fig. 5A). Therefore, the concentration of 500 μg/mL for NPSC or CPSC was employed for determination of cell cycle distribution by flow cytometry. Flow cytometry is a rapid technique for identifying compounds capable of selective or preferential elimination of cancer cells by influencing the cell cycle regulation and/or causing apoptosis. After treatment of cells with an apoptosis-inducing agent, DNA fragmentation can take place and that can also be detected by flow cytometry (Ma et al., 2013). As shown in Fig. 5B, when Hep G2 hepatoma cells treated with 500 μg/mL of NPSC or CPSC for 48 h, CPSC exhibited the highest percentage of cells in the sub-G_1 phase (49.06% ± 1.12%), followed by NPSC (1.14% ± 0.13%) and untreated cells (1.00% ± 0.24%). As shown in previous studies, after induction of apoptosis in cells, DNA fragmentation can occur and the small fragments of the DNA can be eluted by washing with PBS. Cells that have lost DNA take up less PI stain and will appear to the left of the G_1 peak (so-called sub-G_1 peak). The cell population in the sub-G_1 phase is directly proportional to the induced DNA fragmentation, which is a hallmark of apoptosis (Ma et al., 2013). Thus, CPSC exhibited by far the most DNA fragmentation (also

known as sub-G_1 cell cycle arrest), followed by NPSC, and untreated cells. The retardation of cell growth can be visualized by examining the cell cycle phase distribution. In Fig. 5B, a significant increase ($p < 0.05$) of the cell population in the G_0/G_1 phase was found after treatment with NPSC (67.92% ± 3.37%) as compared to that of the untreated cells (50.71% ± 1.17%). Moreover, a significant increase ($p < 0.05$) of the cell population in the *S* phase was also detected after treatment with NPSC (17.20% ± 2.07%) as compared to that of the untreated cells (13.63% ± 0.72%). This was accompanied by a significant decrease ($p < 0.05$) of the cell population in the G_2/M phase after treatment with NPSC (13.73% ± 1.95%) as compared to that of the untreated cells (34.67% ± 1.37%). The results clearly suggest that the accumulation of cells in the G_0/G_1 phase and *S* phase might account for the induction of cell cycle arrest in Hep G2 hepatoma cells by NPSC. In summary, both NPSC and CPSC extracts exhibited cytotoxicities and growth inhibitory effects on Hep G2 hepatoma cells. NPSC inhibited the growth of Hep G2 hepatoma cells via induction of G_0/G_1 and *S* cell cycle arrest; whereas CPSC inhibited the growth of Hep G2 hepatoma cells via induction of sub-G_1 cell cycle arrest. Compared to NPSC, CPSC had a more cytotoxic effect on Hep G2 hepatoma cells and induced more DNA fragmentation of Hep G2 hepatoma cells. Thus, CPSC is recommended for use as a natural agent in the prevention or adjuvant treatment of hepatocellular carcinoma.

IV. CONCLUSIONS

In this paper, two aqueous-ethanol extracts, namely NPSC and CPSC, were extracted from non-puffed and compressional-puffed SC, respectively, and both extracts had relatively high extraction yields. Both NPSC and CPSC extracts exhibited antioxidant activities dose-dependently. NPSC extract had apparent anti-inflammatory activity, and thus it may have potential as a natural anti-inflammatory ingredient. In addition, CPSC extract had a more cytotoxic effect on Hep G2 hepatoma cells and induced more DNA fragmentation of Hep G2 hepatoma cells. Thus, CPSC may have potential in the development of a novel agent for the prevention or adjuvant treatment of hepatocellular carcinoma. Future *in vivo* investigations on the anti-inflammatory effects of NPSC and *in vivo* studies on the anti-liver cancer effects of CPSC are needed.

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