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Research Note

Evaluation and Comparison of Functional Properties of Freshwater-cultivated Glasswort (*Salicornia herbacea* L.) with Naturally-grown Glasswort

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Abstract Glasswort (*Salicornia herbacea* L.) is a halophyte that grows normally in salt marshes and has been used as a seasoned vegetable. Physico-chemical properties, antioxidant, cytotoxic, and anti-inflammatory activities of water extracts from freshwater-cultivated (FWE) and naturally-grown (SWE) glasswort were investigated. Total phenolic and flavonoid contents of FWE were higher than for SWE. The salinity was higher in SWE. Radical scavenging activities and inhibitory effects against lipid peroxidation of FWE were more potent than for SWE. FWE also showed more potent cytotoxic effects with higher potency against cancer cells. FWE was effective for inhibition of protein expression of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide-stimulated RAW264.7 cells. Constituents in FWE were more active than in SWE, and freshwater-cultivated glasswort can be a useful dietary source with health benefits.

Keywords: *Salicornia herbacea*, glasswort, antioxidant, anti-inflammation, cytotoxicity

Introduction

Glassworts, succulent halophytes that usually grow in salt marshes of coastal areas, are a rich source of minerals and the ashes of the plants were formerly used for making glass and soap (1,2). *Salicornia herbacea* L., known as *Tungtungmadi* or *Hamcho* in Korean, is widely distributed in the foreshore area of the Western coast (3). The aerial parts have been consumed as a seasoned vegetable, in salads, and as fermented foods. *S. herbacea* has also been used as a folk medicine for treatment of diabetes, obesity, and cancer (4,5). *S. herbacea* contains minerals and nutrients, particularly sodium, magnesium, calcium, potassium, essential fatty acids, and dietary fiber (6).

A number of studies have reported that *S. herbacea* has beneficial health effects, including antioxidant, immunomodulatory, and anti-carcinogenic activities (7,8). Several biologically-active constituents in *S. herbacea* have also been identified. Tungtungmadic acid isolated from glasswort showed protective effects against lipid peroxidation and DNA damage via scavenging of reactive oxygen species (9). A polysaccharide fraction from *S. herbacea* modulated chemotaxis and activation of inflammatory cells (10). In addition, *S. herbacea* showed anti-hyperlipidemic and anti-hyperglycemic effects *in vivo* (11). Accordingly, the plant has received attention as a functional food and as a source of bioactive materials based on potential health beneficial effects.

S. herbacea, an annual plant, is germinated on the seashore in spring and is usually harvested during the summer season. Accordingly, consumption as a fresh vegetable is limited to only a single season. It was reported that inorganic constituents of *S. herbacea* varied according to different soils and the salinity of provided water (12). Naturally grown *S. herbacea* has shown a large variation in chemical composition due to variations in water salinity. In addition, a massive area of the coastal region is now reclaimed cropland and salt marsh areas and the natural habitat of glasswort has been rapidly reduced. A method of artificial cultivation to achieve a stable supply of *S. herbacea* with a consistent composition is needed.

Glasswort, as a salt tolerant plant, does not require a saline environment for growth. It is reported that freshwater or low saline concentration water was favorable for glasswort germination and the germination rate decreased under high salt conditions (13,14). Several previous studies have reported the effects of soil salinity on growth, germination, and mineral concentration of glasswort (12-15). Yet, reports regarding evaluation of functional properties of *S. herbacea* cultivated using freshwater are unknown. In this study, cultivation of *S. herbacea* using fresh tap water was performed in a greenhouse, and physico-chemical properties, and antioxidant, cytotoxic, and anti-inflammatory effects of water extracts from freshwater-cultivated glasswort were evaluated and compared with effects of naturally-grown *S. herbacea*.

Materials and Methods

Plants, chemicals, and cell lines Fresh, naturally-grown glasswort (*S. herbacea* L.) harvested in August of 2010 was obtained from Daeshinhamcho Co. (Sinan, Korea). Fresh water-cultivated *S. herbacea* was first collected as young plants (<10 cm tall) with surrounding soil from a coastal area of Sinan, Korea during April of 2010. Plants were subsequently grown in a greenhouse at Seoul Women's University with regular doses of fresh tap water for 4 months. Plants were harvested in early August. Both natural and cultivated plants were freeze-dried (Lyph-Lock 6; Labconco Co., Kansas city, MO, USA) and powdered before extraction. 2,2-diphenyl-β-picrylhydrazyl (DPPH), 2,2'-azobis-(3-ethyl-benzothiazoline-6-sulfonicacid) (ABTS), and *N*-1-naphthyl-ethylenediamine dihydrochloride (NED) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 3-(-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Amresco (Solon, OH, USA). Antibodies against inducible nitric oxide synthase (iNOS), cytosolic phospholipase A_2 ($cPLA_2$), cyclooxigenase-2 (COX-2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HCT 116 human colon adenocarcinoma cells, INT-407 human normal immortalized intestinal cells, and RAW264.7 murine macrophage cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Culture media, supplements, and all other chemicals were obtained from Sigma-Aldrich Chemical.

Preparation of Extracts Extraction from the aerial parts of naturally-grown and freshwater-cultivated (FWE) *S. herbacea* was performed using hot distilled water on a hot plate (VS-130SH; Vision Scientific Co., Daejeon, Korea) with stirring at 70-80°C for 3 h. Extracts were filtered, and a second extraction following the same procedure was performed on residues. Filtered extracts were pooled, centrifuged, and dried using a freeze-dryer (Lyph-Lock 6). Extracts were then dissolved at a concentration of 200 mg/mL in dimethyl sulfoxide (DMSO), and stored at −80°C in a freezer (DF8520; IlshinBiobase Co., Dongducheon, Korea) before use.

Analysis of physico-chemical properties Levels of total phenolic and flavonoid compounds were determined based on previous methods (16) and expressed as tannic acid and catechin equivalents, respectively. Extracts were dissolved in deionized water and the salinity was determined using a salinometer (SINAR NS-3P; Merbabu Co., Tokyo, Japan). The zeta potential and other electro-physical properties of 1 mg/mL extracts dissolved in deinonized water were measured using a particle size analyzer (Zetasizer Nano-ZS; Malvern Instruments Ltd., Wercestershire, UK).

Antioxidant activity Antioxidant properties were evaluated based on measurement of scavenging activities of DPPH and ABTS radical and NO following previously reported methods (16). Effects on lipid peroxidation were determined following the method of Ambati *et al.* (17) with slight modification. A 500 μL reaction mixture consisting of 0.2% linoleic acid in 0.8% sodium dodecyl sulfate (SDS), 100 μM ascorbic acid, and 10 μM $FeSO_4$ was incubated with each extract at 37°C for 1 h. Then, 125 μL of 1 M trichloroacetic acid (TCA), 125 μL of 0.8% thiobarbituric acid (TBA), and 20 μL of 5% buthylated hydroxytoluene (BHT) were added to the reaction mixture, followed by boiling for 5 min at 100°C. The developed color was analyzed at 532 nm using a microplate reader (Spectramax M3; Molecular Devices, Sunnyvale, CA, USA).

The Fe^{2+} ion chelating activity was also measured using ferrozine. Each 50 μL extract solution was incubated with 100 μL of 100 μM $FeCl_2$ at 25°C for 10 min. Then, 50 μL of 1 mM ferrozine was added to the reaction mixture, followed by incubation for 10 min, and the color response of the reaction of free $Fe^{2+}$with ferrozine was analyzed at 550 nm using a microplate reader (Spectramax M3).

Cell culture and analysis of cell viability HCT 116 and INT-407 cells were grown in RPMI 1640 and MEM medium, respectively, at 37°C in 95% humidity and 5% CO_2. Both media were supplemented with 10% fetal bovine serum (FBS), 100 unit/mL penicillin, and 0.1 mg/mL streptomycin. For analysis of the cytotoxic effect, approximately 10^4 cells were plated in a 96 well plate in a growth medium and cells were treated the next day with both extracts. After incubation for 24 h, the medium was replaced with a medium containing 0.5 mg/mL MTT and cells were further incubated at 37°C for 1-2 h. The medium was then removed and MTT formazan was dissolved in 100 μL of DMSO and measured at 550 nm using a microplate reader (Triad LT; Dynex Technologies Inc., Chantilly, VA, USA).

Anti-inflammatory activity RAW264.7 macrophages were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 unit/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C in 95% humidity and 5% CO_2. RAW264.7 cells were plated in a 6 well plate at approximately 1.5×10^6/well. After 24 h, the complete medum was replaced with serum free DMEM containing 2 mg/mL lipopolysaccharides (LPS) (Sigma-Aldrich Chemical Co.) for 1 h, and cells were further incubated with fresh serum free DMEM containing *S. herbacea* extracts for 24 h. Cells were then washed with ice cold PBS twice and lysed using a cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA). (1 mM phenylmethanesulfonylfluoride, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, and 1 mM Na_2VO_4 in 20 mM Tris(hydroxymethyl) aminomethane, pH 7.4). Cell lysates were centrifuged at 10,000×*g* for 10 min at 4°C. The supernatant containing 20 μg of protein was loaded on to 7% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane and probed with primary antibodies for iNOS, $cPLA_2$, COX-2, and GAPDH. Western blots were analyzed and quantified using a luminescent image analyzer (LAS-4000mini; Fujifilm, Tokyo, Japan). Protein concentrations

in cell lysates were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Data analysis Statistical significance was evaluated using Student's *t*-test. A one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test were used for comparison of multiple results. IC_{50} (concentrations that caused 50% inhibition) values were calculated based on corresponding linear regression equations using at least 4 points for a line. Each point was obtained as a mean value from triplicate experiments. Statistical analysis was performed at $p<0.05$ using the SAS software package of the SAS Institute (Cary, NC, USA).

Results and Discussion

Evaluation of physico-chemical properties *S. herbacea* cultivated with freshwater grew normally up to a >30 cm height during 4 months. When harvested, there were no abnormal features observed, compared with naturally-grown plants from the seashore, consistent with a previous report that *S. herbacea* grown using tap water did not exhibit any significant differences in weight, plant height, and number of branches per plant, compared with plants grown using salt water (15). Physico-chemical properties were compared based on preparation of hot water extracts from both cultivated and naturally grown *S. herbacea* plants (Table 1). Yields of SWE and FWE from freeze-dried plant samples were 42.4 and 37.0%, respectively. Total polyphenol and flavonoid contents of FWE were 39.0 and 36.5 μg/mg of extract based on tannic acid and catechin equivalents, respectively. Levels of polyphenolic compounds in FWE were 2-3x higher than levels in SWE. Protein contents of FWE and SWE were 479 and 220 μg/mg of extract, respectively.

FWE contained more water soluble proteins or peptides than SWE. The salinity of SWE was higher than the salinity of FWE (6.3 vs 4.7%). Kim *et al*. (14) reported that *S. herbacea* grown using a high proportion of seawater in supplied water contained more Na. In this study, the higher salinity of SWE was probably because SWE contained higher levels of Na, the most abundant mineral in glasswort, than FWE. It was also reported that levels of Ca and K were higher in *S. herbacea* grown using tap water, compared to plants grown using 30 and 90% seawater, and the K/Na ratio in glasswort decreased under cultivation using a higher seawater content (14). Accordingly, freshwater-cultivated glasswort may cause a desirable effect for lowering the sodium content in the human diet. Effects of freshwater cultivation on overall mineral contents and balance among minerals in *S. herbacea* need further investigation.

No significant ($p<0.05$) differences in zeta potential or particle size (Z-average) between SWE and FWE were observed. The zeta potential is influenced by the ionic concentration formed around solutes and is an indicator of the stability of colloidal dispersions. The stability of both extracts in this study in aqueous solutions was not significantly ($p>0.05$) different. The suitability of FWE may not be different from SWE when applied to beverages or other aqueous food products. Conductivity was significantly ($p<0.05$) higher in SWE solutions than in FWE solutions, consistent with the observation of a higher salinity for SWE (Table 1).

Antioxidant and cytotoxic properties Antioxidant activities are one of the most commonly investigated functional properties of natural dietary products. In this study, antioxidant activities of both extracts from *S. herbacea* were evaluated based on analysis of scavenging activities against DPPH and ABTS radicals, and NO. IC_{50} values of FWE for scavenging DPPH and ABTS radicals were 0.25 and 0.22 mg/mL, respectively. The potency of FWE was more than 2x higher than for SWE (Fig. 1A). FWE also showed a significantly ($p<0.01$) higher NO scavenging activity than SWE with IC_{50} values of 0.13 vs 0.23 mg/mL, respectively (Fig. 1A).

Effects of both extracts on lipid peroxidation induced using $FeSO_4$/ascorbic acid were also analyzed. FWE significantly ($p<0.05$) decreased the level of TBA-reactive substances (TBARS) produced during lipid peroxidation at 0.25 mg/mL. SWE was not effective. At all concentrations tested, FWE showed more potent inhibitory effects than SWE (Fig. 1B). Metal chelating activities also contribute to inhibitory effects against lipid peroxidation. Therefore, the Fe^{2+} chelating activity of each extract was investigated using ferrozine. Both SWE and FWE were effective for chelation of Fe^{2+} at 0.15 mg/mL and showed concentration-dependent effects. FWE was more potent in chelation of Fe^{2+} (Fig. 1C).

Both extracts exhibited radical scavenging activities with IC_{50} levels of 0.13-0.65 mg/mL for the DPPH and ABTS radicals, and NO, mainly attributable to actions of phenolic and flavonoid compounds in extracts. FWE contained >2x higher contents of these compounds

Table 1. Physico-chemical characteristics of extracts from naturally-grown (SWE) and freshwater-cultivated (FWE) glasswort plants

	Total polyphenols[1)] (μg/mg of solids)	Total flavonoids[2)] (μg/mg of solids)	Protein[3)] (μg/mg of solids)	Salinity (g/kg of solids)	Zeta potential (mV)	Z-average (d.nm)	Conductivity (mS/cm)
SWE	16.13±0.21	11.72±0.33	219.9±5.36	63.0±5.0**	-24.45±3.01	330.1±44.2	0.895±0.024**
FWE	39.03±0.19**[4)]	36.50±1.38**	479.0±21.7**	47.0±3.0	-26.25±1.12	300.2±55.4	0.706±0.015

[1)]Microgram (μg) of contents/mg of solid based on tannic acid as a standard
[2)]Microgram of contents/mg of solid based on catechin as a standard
[3)]Microgram of contents/mg of solid based on bovine serum albumin as a standard
[4)]Significantly different based on Student's *t*-test ($p<0.01$)

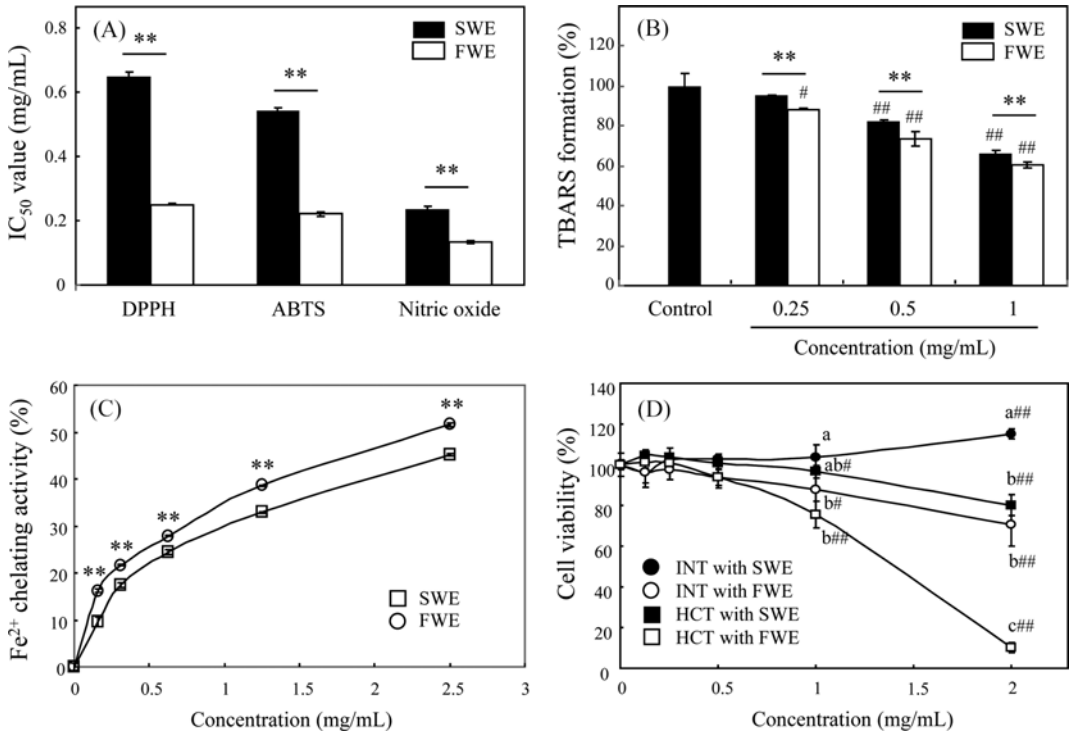

Fig. 1. Antioxidant and cytotoxic effects of SWE and FWE from glasswort. Radical scavenging effects (A), effects on lipid peroxidation (B), Fe^{2+} chelating activities (C), and cytotoxic effects (D) of extracts were evaluated. Each value represents the mean±standard deviatrion (SD) (n=3 in A-C or n=8 in D). $^{\#, \#\#}$significantly different from control ($^{\#}p$<0.05; $^{\#\#}p$<0.01 in B and D); **significantly different between SWE and FWE (**p<0.01) based on Student's t-test. Different letters indicate a significant difference (p<0.05) based on a one-way ANOVA and Tukey's HSD test (D).

than SWE (Table 1). Differences in radical scavenging activities of FWE and SWE against both DPPH and ABTS radicals were comparable to differences in contents of these active compounds. A previous study also reported that tungtungmadic acid (3-caffeoyl-4-dihydrocaffeoylquinic acid), a phenolic compound in *S. herbacea*, displayed a strong antioxidant activity based on protection against oxidative damage (9). FWE inhibited lipid peroxidation at <1 mg/mL with a significantly (p<0.01) higher potency than SWE. Transition metals, including iron, stimulate many oxidation processes based on generation of hydroxyl radicals through Fenton reactions, and accelerate lipid peroxidation based on decomposition of lipid hydroperoxides (18). The Fe^{2+} chelating activities of both extracts could also have contributed to the overall antioxidant potential.

Cytotoxic effects of FWE and SWE were also evaluated in HCT 116 human colon cancer and INT-407 human normal immortalized intestinal cells using the MTT assay. After 24 h of incubation, both extracts did not show any significant (p>0.05) cytotoxic effect at concentrations <0.5 mg/mL. SWE stimulated INT 407 cell growth slightly at >1 mg/mL (Fig. 1D). FWE showed a higher cytotoxicity in HCT 116 colon cancer cells, based on induction of >90% cell death at 2 mg/mL. However, >70% of normal cells survived at the same concentration. The selective cytotoxic effect of FWE against cancer cells and related mechanisms, and physiological relevance need further exploration.

Anti-inflammatory activities Anti-inflammatory activities of *S. herbacea* extracts were investigated in an LPS-stimulated RAW264.7 murine macrophage model. No cytotoxic effect for either extract in RAW 264.7 cells was observed up to 100 μg/mL (Fig. 2A). After stimulation with LPS, the NO level in cells was elevated by approximately 2x over 24 h, which was decreased significantly (p<0.05) in the presence of 50 μg/mL FWE, but not SWE, compared with controls. (Fig. 2B). Stimulation of RAW cells with LPS induced iNOS expression, and FWE at 50 μg/mL decreased the iNOS protein level significantly (p<0.05), compared with controls. SWE did not show a significant (p>0.05) inhibition of iNOS expression (Fig. 2C and 2D). LPS stimulation also increased the $cPLA_2$ level, but neither extract affected the protein level. The level of COX-2 induced by LPS was, however, significantly (p<0.05) reduced in the presence of 50 μg/mL *S. herbacea* extracts. Repetitive experiments showed significant (p<0.05) reductions in the COX-2 protein level with FWE, but not with SWE, compared with controls (Fig. 2C and 2D).

NO synthesis and arachidonic acid metabolism are involved in inflammatory and carcinogenic processes (19-21). Although NO, an endogenous free radical, is involved in beneficial events, including vasodilation, inhibition of platelet function, synaptic neurotransmission, and host defense, the bulk of NO production due to iNOS in macrophages causes inflammatory damage (19). FWE decreased NO levels in LPS-stimulated RAW264.7 macrophages with inhibition

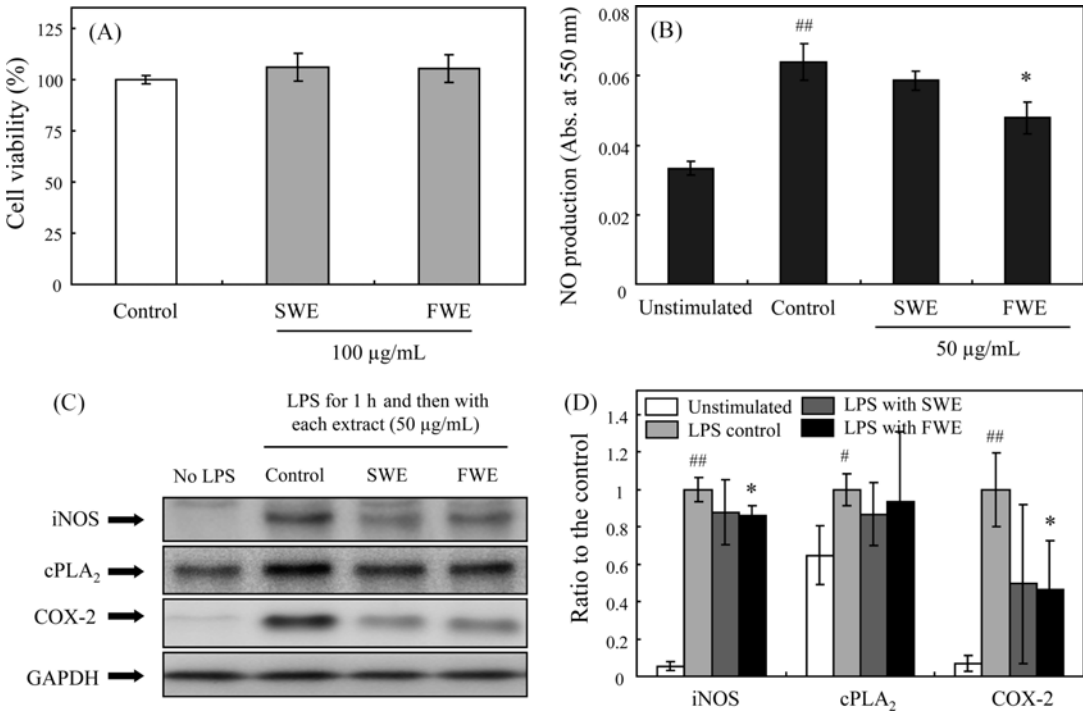

Fig. 2. Anti-inflammatory effects of SWE and FWE from glasswort. Cytotoxic effect (A), effects on NO level (B), and protein levels of iNOS, $cPLA_2$, and COX-2 (C) of extracts were evaluated in LPS-stimulated RAW264.7 cells after 24 h incubation. Densitometry quantification of each protein level normalized by GAPDH is also presented (D). Each value represents the mean±SD (n=8 in A, n=3 in B and D). **Significantly different from control (*p<0.05); $^{\#, \#\#}$significantly different from unstimulated cells ($^{\#}p$<0.05; $^{\#\#}p$<0.01) based on Student's t-test.

probably due to modulation of iNOS protein levels and the direct scavenging activity of NO.

Arachidonic acid is released by $cPLA_2$ from membrane phospholipids, and arachidonic acid is further metabolized by COX and lipoxygenases to potent inflammatory mediators, such as prostaglandins and leukotrienes (20). Modulation of the arachidonic acid metabolism based on inhibition of these enzymes has been suggested as an effective target for anti-inflammation and cancer chemoprevention (20,21). In the experimental system used herein, protein levels of $cPLA_2$ and COX-2 were elevated in LPS-stimulated macrophages and only the COX-2 level was down-regulated by FWE. Since phosphorylation of $cPLA_2$ plays a major role in modulation of $cPLA_2$ action (22), post-translational modulation of $cPLA_2$ by *S. herbacea* needs to be further studied. Results presented herein indicate that FWE contained higher levels of polyphenols and showed more potent antioxidant and anti-inflammatory effects than SWE. Freshwater-cultivated *S. herbacea* can be used as a dietary supplement or as a health food that exerts better health beneficial effects than naturally-grown plants.

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Disclosure The authors declare no conflict of interest.

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