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Phlorotannins from Brown Algae (*Fucus vesiculosus*) Inhibited the Formation of Advanced Glycation Endproducts by Scavenging Reactive Carbonyls

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Supporting Information

ABSTRACT: Accumulation of advanced glycation end products (AGEs) in vivo is associated with aging, diabetes, Alzheimer's disease, renal failure, etc. The objective of this study was to investigate the inhibitory effects of brown algae *Fucus vesiculosus* phlorotannins on the formation of AGEs. *F. vesiculosus* phlorotannins were extracted using 70% acetone. The resultant extract was fractionated into dichloromethane, ethyl acetate, butanol, and water fractions. The ethyl acetate fraction was further fractionated into four subfractions (Ethyl-F1 to -F4) using a Sephadex LH-20 column. *F. vesiculosus* acetone extract or fractions significantly inhibited the formation of AGEs mediated by glucose and methylglyoxal in a concentration-dependent manner. The concentrations of *F. vesiculosus* extracts required to inhibit 50% of albumin glycation (EC_{50}) in the bovine serum albumin (BSA)−methylglyoxal assay were lower than those of aminoguanidine (a drug candidate for diabetic complication), except for *F. vesiculosus* acetone extract and dichloromethane fraction. In the BSA−glucose assay, *F. vesiculosus* extracts inhibited BSA glycation more than or as effectively as aminoguanidine, except for Ethyl-F3 and -F4. The ethyl acetate fraction and its four subfractions scavenged more than 50% of methylglyoxal in two hours. The hypothesis whether *F. vesiculosus* phlorotannins scavenged reactive carbonyls by forming adducts was tested. Phloroglucinol, the constituent unit of phlorotannins, reacted with glyoxal and methylglyoxal. Five phloroglucinol−carbonyl adducts were detected and tentatively identified using HPLC−ESI-MS^n.

KEYWORDS: *Fucus vesiculosus, advanced glycation endproducts, methylglyoxal*

INTRODUCTION

The advanced glycation end products (AGEs) are a class of compounds with brown, fluorescent, or cross-linked characteristics which are generated during protein glycation.[1] Accumulation of AGEs is associated with aging,[2] diabetes,[3] Alzheimer's disease,[2] renal failure,[4] and many other chronic diseases. The process of protein glycation has three stages.[5] In the initial stage, reducing sugars such as glucose react with the primary amino groups to form a reversible Schiff base. N-Substituted glycosylamine is formed from the cyclization of the Schiff base.[6] Then the unstable N-substituted glycosylamine undergoes Amadori rearrangement to generate relatively stable Amadori products.[7] In the intermediate stage, Amadori products decompose to generate the reactive dicarbonyls. In addition to the oxidation of Amadori products and degradation of the Schiff base, reactive carbonyls such as glyoxal, methylglyoxal, and 3-deoxyglucosone can also be generated from sugar glycoxidation.[8] In the late stage, protein glycation is mediated primarily by these dicarbonyls. Methylglyoxal or glyoxal reacts with proteins to form several AGEs, leading to pathological consequences such as inflammation and necrosis that contribute to the progression of diseases.[9]

Marine algae, classified as *Laminariales* (brown), *Chlorophyta* (green), and *Rhodophyta* (red), are known to contain a wide range of bioactive natural substances with diverse health benefits. Brown algae were effective in reducing blood cholesterol and lowering blood pressure, as well as preventing arteriolosclerosis.[10,11] Phlorotannins are oligomers and polymers of phloroglucinol that exist exclusively in brown algae.[12,13] They have been identified from several brown algal families such as *Alariaceae*, *Fucaceae*, and *Sargassaceae*.[14] A number of phlorotannin oligomers, including fucophlorethol A (a trimer), tetrafucol A (a tetramer), and trifucodiphlorethol A (a hexamer), had been isolated from *F. vesiculosus*[15,16] (Figure 1). They have been shown to have antioxidant,[16] anti-inflammatory,[16] and antibacterial activities.[17] No study has been conducted to investigate the capacity of *F. vesiculosus* phlorotannins to scavenge reactive carbonyls and inhibit protein glycation. On the basis of the high phlorotannin content and antioxidant capacities of *F. vesiculosus,* we hypothesized that *F. vesiculosus* phlorotannins may inhibit the formation of AGEs by scavenging reactive dicarbonyls. The present study was to test these hypotheses.

MATERIALS AND METHODS

Chemicals and Materials. Dry *F. vesiculosus* was obtained from Maine Seaweed Co. (Steuben, ME). Sephadex LH-20, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (St. Louis, MO). Glyoxal (40 wt % solution), *N,N*-dimethylformamide, 2, 4-dimethoxybenzaldehyde, aminoguanidine, phloroglucinol, and *o*-phenylenediamine were products from Acros Organics (Morris Plains, NJ). AAPH (2,2′-azobis(2-amidinopropane))

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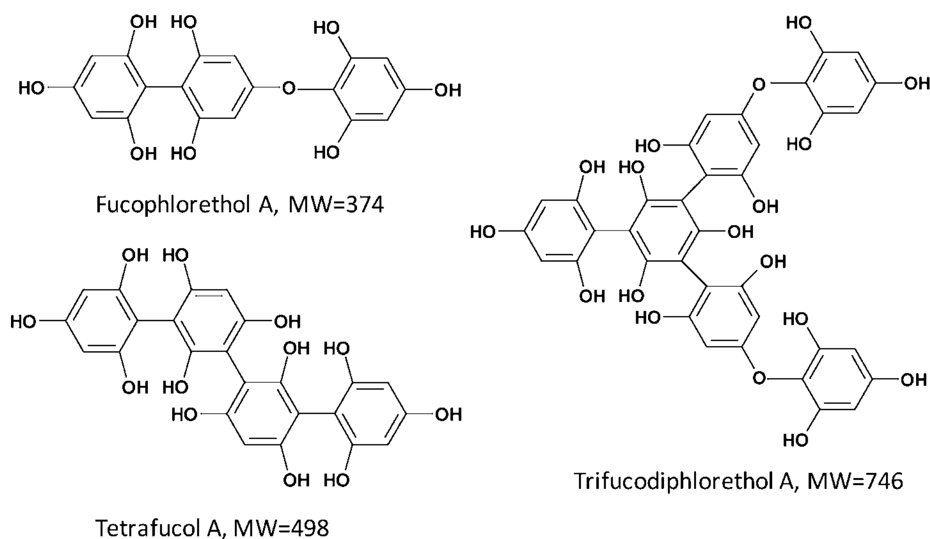

Figure 1. Structure of phlorotannin oligomers isolated from *F. vesiculosus.*

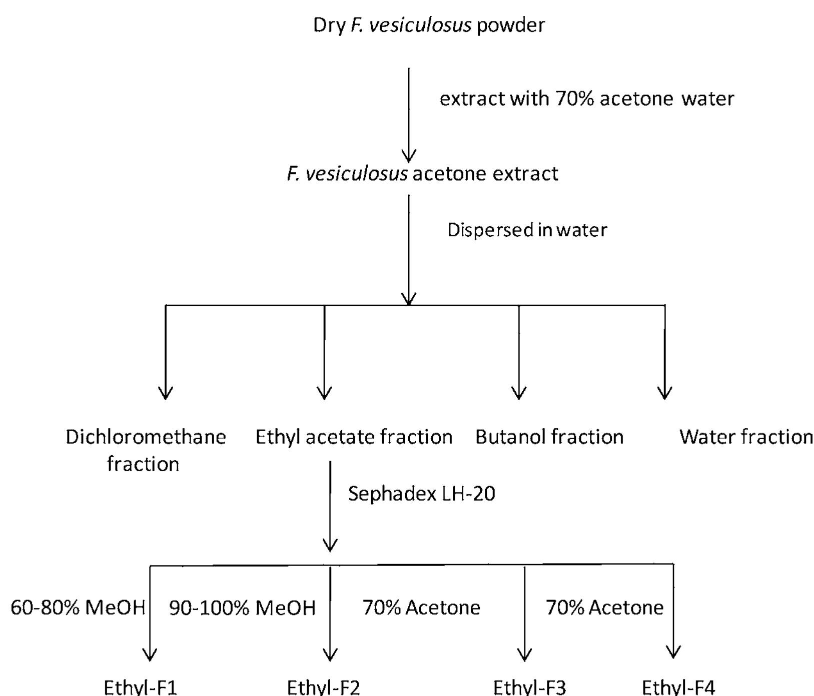

Figure 2. Extraction and fractionation of phlorotannins from *F. vesiculosus.*

was a product of Wako Chemicals Inc. (Bellwood, RI). Methylglyoxal (40% aqueous solution) and glucose were obtained from MP Biomedicals, LLC (Solon, OH). Bovine serum albumin (BSA), sodium azide, monobasic and dibasic sodium phosphate, 96-well plates with clear bottom wells, and acetone and other organic solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

***F. vesiculosus* Phlorotannin Extraction, Fractionation, and Characterization.** The phlorotannin extraction and fractionation procedure is depicted in Figure 2. Three hundred grams of *F. vesiculosus* materials were ground into fine powder using a blender and extracted with 1200 mL of acetone:water:acetic acid (70:29.7:0.3, v/v/v). The mixture was sonicated in a water-bath sonicater (FS30, Fisher Scientific) for 30 min and then kept at room temperature for two hours. The extraction was repeated once. Extracts obtained after vacuum filtration were combined and concentrated under partial vacuum using a rotary evaporator. Fifty-four grams of crude extract was obtained. Part of this extract (30 g) was suspended in 100 mL of water and partitioned with 100 mL of dichloromethane three times in a separation funnel. The dichloromethane phases were combined and evaporated to yield 4.934 g of extract. The aqueous phase was then partitioned with ethyl acetate three times (100 mL each) before it was partitioned with butanol for an additional three times (100 mL each). The ethyl acetate and butanol phases were evaporated to yield 4.946 and 4.983 g of extract, respectively. The aqueous phase was dried in a rotary evaporator to yield the water fraction (2.787 g). An extra sample ethyl acetate fraction (5.231 g) was obtained by repeating the previous fractionation steps. Part of the ethyl acetate fraction (7.622 g) was dissolved in 60% methanol and loaded onto a Sephadex LH-20 column (5.8 × 28 cm). The column was eluted with 60% methanol (3 L), 80% methanol (2 L), 90% methanol (2.5 L), 100% methanol (2 L), and 70% acetone (4 L). Ethyl acetate subfraction I (Ethyl-F1, 0.823 g) was obtained by combining 60% and 80% methanol eluent. Ethyl-F2 (0.834 g) was obtained by combining 90% and 100% methanol eluent. Ethyl-F3 (3.492 g) and Ethyl-F4 (0.669 g) were from 70% acetone eluent.

Table 1. Total Phenolic Content, Total Phlorotannin Content, and Antioxidant Capacity of *F. vesiculosus* Extracts[a]

F. vesiculosus extracts and fractions	total phenolic content (mg gallic acid equiv/g)	total phlorotannin content (mg phloroglucinol equiv/g)	antioxidant capacity (μmol Trolox equiv/mg)
F. vesiculosus acetone extract	286.6 ± 20.0 b	42.29 ± 0.59 e	3214.5 ± 161.9 d
dichloromethane fraction	92.1 ± 13.1 c	26.06 ± 1.17 f	879.4 ± 66.6 e
ethyl acetate fraction	352.9 ± 20.4 b	115.46 ± 4.44 b	5320.8 ± 224.6 ab
butanol fraction	298.8 ± 18.9 b	75.55 ± 1.98 d	3466.2 ± 87.4 cd
water fraction	24.6 ± 0.5 c	7.48 ± 0.07 g	539.7 ± 12.0 e
Ethyl-F1	277.4 ± 2.6 b	133.81 ± 2.74 a	4861.2 ± 820.8 b
Ethyl-F2	340.4 ± 25.1 b	76.75 ± 3.80 d	6578.6 ± 321.6 a
Ethyl-F3	459.7 ± 39.5 a	109.91 ± 5.36 b	6288.0 ± 171.4 a
Ethyl-F4	447.0 ± 26.8 a	96.77 ± 2.62 c	4704.0 ± 310.0 bc

[a]Data are mean ± standard deviation of duplicate tests. Values with different letters differed significantly at $p \leq 0.05$.

The total phenolic content was determined using the Folin–Ciocalteu method.[18] Total phlorotannins were quantified using the 2,4-dimethoxybenzaldehyde (DMBA) assay.[19] The oxygen radical absorbance capacity (ORAC) assay was used to evaluate the antioxidant capacity of *F. vesiculosus* extracts.[20]

Bovine Serum Albumin (BSA)–Glucose Assay. This assay followed a published method.[21] Bovine serum albumin and glucose were dissolved in phosphate buffer (100 mM, pH 7.4) to a concentration of 100 mg/mL and 188 mg/mL, respectively. *F. vesiculosus* acetone extract or fractions were dissolved in the same phosphate buffer. One milliliter of the BSA solution was mixed with 1 mL of glucose solution and 1 mL of *F. vesiculosus* extracts. The mixtures were incubated at 37 °C. Sodium azide (0.2 g/L) was used as an aseptic agent. Phosphate buffer was used as blank. Aminoguanidine and phloroglucinol were used as positive controls. After seven days of incubation, fluorescence of the samples was measured using an excitation of 330 nm and an emission of 410 nm, respectively. The % inhibition of AGE formation = [1 − (fluorescence of the test group/fluorescence of the control group)] × 100%.

BSA–Methylglyoxal Assay. This assay was modified based on a published method.[22] The assay evaluates the middle stage of protein glycation. BSA and methylglyoxal were dissolved in phosphate buffer (100 mM, pH 7.4) to a concentration of 20 mg/mL and 60 mM, respectively. *F. vesiculosus* acetone extract or fractions were dissolved in the same phosphate buffer. One milliliter of the BSA solution was mixed with 1 mL of methylglyoxal solution and 1 of mL *F. vesiculosus* extracts. The mixture was incubated at 37 °C. Sodium azide (0.2 g/L) was used as an aseptic agent. Phosphate buffer was used as a blank. Aminoguanidine and phloroglucinol were used as positive controls. After seven days of incubation, fluorescence of the samples was measured using an excitation of 340 nm and an emission of 420 nm, respectively. The % inhibition of AGE formation = [1 − (fluorescence of the test group/fluorescence of the control group)] × 100%.

Methylglyoxal Scavenging Assay. Methylglyoxal scavenging assay followed a published method with modifications.[23] Methylglyoxal (5 mM) and *o*-phenylenediamine (derivatization agent, 20 mM) were freshly prepared in phosphate buffer (100 mM, pH 7.4). *F. vesiculosus* acetone extract or fractions and phloroglucinol were dissolved in the same buffer to a concentration of 1 mg/mL. Aminoguanidine (5 mM) was used as a positive control. Methylglyoxal solution (0.25 mL) was mixed with 0.25 mL of phosphate buffer (blank) or test samples. The mixtures were incubated at 37 °C for 0, 5, 10, 20, 40, 60, 120 min, respectively. After incubation, 0.125 mL of *o*-phenylenediamine was added to each test solution. The mixtures were kept at room temperature for 30 min for derivatization to complete. The mixture after derivatization was injected for HPLC analysis (Agilent Technologies, Palo Alto, CA). Compound separation was carried out on a Zorbax SB-C18 column (4.6 × 250 mm, 5 μm, Agilent Technologies, Palo Alto, CA). Mobile phases were composed of 0.1% formic acid in water (phase A) and methanol (phase B). The flow rate was set as 1 mL/min, and the injection volume was 15 μL. The linear gradient for elution was as follows: 0–3 min, 5–50% B; 3–16 min, 50–50% B; 16–17 min, 50–90% B; 17–19 min, 90–90% B; 19–19.5 min, 90–5% B; followed by 1 min of re-equilibration. Methylglyoxal reacted with *o*-phenylenediamine to form 1-methylquinoxaline, which eluted at 12.9 min using a detection wavelength of 315 nm.

Phloroglucinol–Carbonyl Reaction and Adduct Identification. The phloroglucinol–carbonyl reaction was conducted in the phosphate buffer saline (pH 7.4). Phloroglucinol (10 mM) was incubated with methylglyoxal or glyoxal at a concentration of 1 mM at 37 °C. After two hours of incubation, the adducts were analyzed using the HPLC–ESI-MS^n technique. An Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA) equipped with a diode array detector and HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA) was used for adduct identification. Compound separation was carried out on a Zorbax SB-aqueous column (3.0 × 250 mm, 5 μm, Agilent Technologies). Mobile phases were composed of 0.1% formic acid in water (phase A) and 0.1% ancetonitrile (phase B). The linear gradient was as follows: 0–20 min, 0–10% B; 20–22 min, 10–17% B; 22–37 min, 17–30% B; 37–40 min, 30–70% B; 40–42 min, 70–0% B; followed by 5 min of re-equilibration. The flow rate was 0.25 mL/min. The detection wavelength on the diode array detector was 270 nm. Electrospray ionization at both positive and negative modes was performed using nebulizer 50 psi, drying gas 10 L/min, and drying temperature 300 °C. One precursor ion with the highest intensity was isolated and fragmented to obtain the product ion spectra of adducts.

Statistical Analyses. Half inhibition concentrations (EC_{50}) were determined using Probit analysis function of SPSS software (Version 13, SPSS Inc., Chicago, IL.) One way ANOVA with the Tukey–Kramer HSD test was done using JMP software (Version 8.0, SAS Institute Inc., Cary, NC), and a difference of $p \leq 0.05$ was considered as significant. All data was expressed as the mean ± standard deviation.

RESULTS AND DISCUSSION

***F. vesiculosus* Phlorotannin Extraction, Fractionation, and Characterization.** The 70% acetone was used for extraction because it was shown as the most effective solvent to extract phlorotannins from brown algae.[24] Phytochemicals in the crude acetone extract were fractionated according to polarity using liquid–liquid partition. Dichloromethane was used to obtain lipid and other nonpolar compounds from the extract. Both ethyl acetate and butanol were used to concentrate phlorotannins. After solvent partition, about 10%, 45%, 29.7%, and 1.6% of total phlorotannins were distributed in the dichloromethane, ethyl acetate, butanol, and water fractions, respectively. Ethyl acetate fraction showed the highest phlorotannin concentration, followed by the butanol fraction (Table 1). Total phenolic content and ORAC values of ethyl acetate were also higher than those of other fractions. Dichloromethane and water fractions had the lowest total phenolic and phlorotannin contents and antioxidant capacities (Table 1).

The ethyl acetate fraction was further fractionated into four subfractions on a Sephadex LH-20 column. All four ethyl

acetate subfractions had significantly higher phenolic and phlorotannin content than the original *F. vesiculosus* extract. Ethyl-F1 showed higher phlorotannin content than the ethyl acetate fraction. The phlorotannin content in Ethyl-F3 was comparable to that of the ethyl acetate fraction. Ethyl-F2 and Ethyl-F4 showed lower phlorotannin content than the ethyl acetate fraction. After subfractionation of the ethyl acetate fraction on the Sephadex column, about 5.6, 3.3, 19.6, and 3.3% of initial phlorotannins were distributed in Ethyl-F1, Ethyl-F2, Ethyl-F3, and Ethyl-F4, respectively. The phlorotannin profiles of these fractions were assessed using HPLC−MS^n (Supporting Information). The predominate amount of phlorotannins in butanol or ethyl acetate fractions appeared to be polymers. Sephadex LH-20 separates phlorotannins on the basis of adsorption partition. Smaller phlorotannins adsorbed with low affinity and eluted first. As a result, trimers to pentamers were detected in Ethyl-F1 along with small amounts of polymers. Hexamers to octamers were detected in Ethyl-F2 with higher amounts of polymers. Ethyl-F3 and -F4 contained exclusively polymers.

The antioxidant capacities of four ethyl acetate subfractions were significantly higher than that of *F. vesiculosus* acetone extract (Table 1). Ethyl-F2 and Ethyl-F3 had higher antioxidant capacities than Ethyl-F1 and Ethyl-F4. The antioxidant capacities of four subfractions are comparable to that of the ethyl acetate fraction. The antioxidant activities of *F. vesiculosus* extracts may be attributed to their phenolic compounds, especially their phlorotannin content. Ethyl acetate fraction and its subfractions, which concentrated with phlorotannins, showed significantly higher ORAC values than other extracts. This is in agreement with previous research that showed phlorotannins scavenged oxygen species.[25,26] The antioxidant activity of phlorotannins from brown algae was associated with their unique molecular skeleton.[27] The phenol rings of phlorotannins acted as electron traps to scavenge peroxy, superoxide anions, and hydroxyl radicals.[14]

BSA−Glucose and BSA−Methylglyoxal Assays. *F. vesiculosus* acetone extract and its fractions significantly inhibited protein glycation mediated by glucose, and the antiglycation effects increased with concentration. EC_{50} was defined as the concentrations of *F. vesiculosus* extracts required to inhibit 50% of BSA glycation. Ethyl acetate fraction was a more potent antiglycation agent than the acetone extract and other fractions (Table 2). Dichloromethane fraction had much lower phlorotannin content than butanol fraction, yet they showed similar antiglycation activities in the BSA−glucose assay. Ethyl-F1 and -F2 were similar to phloroglucinol. Ethyl-F3 and -F4 had much lower activity compared to all other fractions except for water fraction in the BSA−glucose assay. It is probably due to the differences in the phlorotannin composition in ethyl acetate subfractions. Oligomeric phlorotannins were reported to have strong inhibitory activity on glycation.[28] HPLC chromatograms of Ethyl-F3 and -F4 indicated that these two ethyl acetate subfractions contained exclusively phlorotannin polymers (Figure S1 in the Supporting Information) and therefore had lower activity.

Similarly, in the BSA−methylglyoxal assay, *F. vesiculosus* acetone extract and its fractions inhibited the formation of AGEs in a concentration-dependent manner. The ethyl acetate fraction had lower EC_{50} (0.169 mg/mL) and, therefore, was more effective than other *F. vesiculosus* fractions. Subsequently, antiglycation activities of four subtractions showed similar antiglycation effects to ethyl acetate fraction and amino-

Table 2. EC_{50} of *F. vesiculosus* Extracts To Inhibit Protein Glycation in Two Assays[a]

extracts and fractions	EC_{50} (mg/mL) BSA−methylglyoxal assay	BSA−glucose assay
F. vesiculosus acetone extract	0.393 ± 0.0127 b	0.338 ± 0.0146 cd
dichloromethane fraction	1.776 ± 0.0536 a	0.489 ± 0.0692 c
ethyl acetate fraction	0.169 ± 0.0050 d	0.278 ± 0.0186 d
butanol fraction	0.237 ± 0.0057 c	0.386 ± 0.0364 cd
water fraction	>6.0	>2.0
Ethyl-F1	0.166 ± 0.007 d	0.045 ± 0.001 e
Ethyl-F2	0.166 ± 0.012 d	0.057 ± 0.003 e
Ethyl-F3	0.159 ± 0.005 d	1.157 ± 0.046 b
Ethyl-F4	0.162 ± 0.012 d	1.526 ± 0.161 a
phloroglucinol	0.058 ± 0.0036 e	0.068 ± 0.0056 e
aminoguanidine	0.197 ± 0.0095 cd	0.310 ± 0.0607 cd

[a]Data are mean ± standard deviation of triplicate tests. Values with different letters differed significantly at $p \leq 0.05$.

guanidine (Table 2). Phloroglucinol, the constituent unit of phlorotannins, appeared to be the most effective antiglycation agent with an EC_{50} value of 0.058 mg/mL. Dichloromethane and water fraction had the least antiglycation activities, which was consistent with their low activities in BSA−glucose assay.

Ethyl-F1 and -F2 were more effective than Ethyl-F3 and -F4 in preventing protein glycation in the BSA−glucose assay, whereas no differences were observed among these four subfractions in the BSA−methylglyoxal assay. The ethyl acetate fraction had high phlorotannin content, which explained its significant inhibitory effects on the formation of AGEs mediated by methylglyoxal. The butanol fraction had lower phlorotannin content than the ethyl acetate fraction, which was consistent with a lower antiglycation activity in the BSA−methylglyoxal system. BSA−glucose evaluates all stages of protein glycation, while the BSA−methylglyoxal assay assesses the protein glycation in the middle stage. The phlorotannin profiles of *F. vesiculosus* fractions and their EC_{50} value in the BSA−glucose assay suggested that phlorotannins of lower molecular weights were more effective than phlorotannins of higher molecular weight in inhibiting protein glycation mediated by glucose. This was consistent with the potent antiglycation effects observed for phloroglucinol. On the other hand, molecular weight had little impact on methylglyoxal-mediated protein glycation. Our results were consistent with a previous study where several phloroglucinol derivatives from brown algae were found effective in inhibiting the formation of AGEs in vitro.[28]

Methylglyoxal Scavenging Capacity. Methylglyoxal content did not change during incubation in phosphate buffer. Its content decreased significantly after incubation with *F. vesiculosus* extract and fractions for 120 min (Figure 3). After incubation with the butanol fraction, the ethyl acetate fraction, and its four subfractions, less than 50% of methylglyoxal remained at 120 min. Aminoguanidine and phloroglucinol scavenged 82.6% and 77.2% of methylglyoxal after 120 min of incubation. The ethyl acetate fraction showed the highest methylglyoxal scavenging activity as compared to other fractions. The dichloromethane and water fractions showed the least activity (Figure 3A). This observation was consistent with their antiglycation activities in the BSA−methylglyoxal assay. Interestingly, Ethyl-F1 to -F4 showed similar methyl-

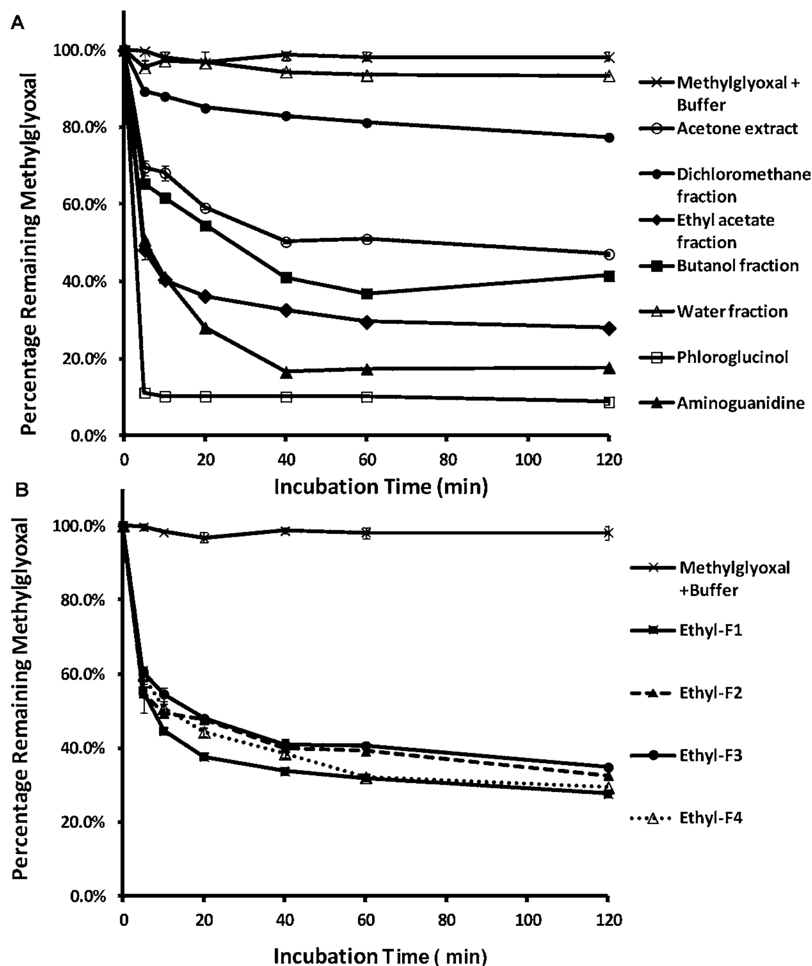

Figure 3. The capacity of *F. vesiculosus* extracts to scavenge methylglyoxal. Methylglyoxal (2.5 mM) was incubated with *F. vesiculosus* extracts (1.0 mg/mL). Aminoguanidine and phloroglucinol (2.5 mM) were used as positive controls. Results are means ± standard deviation of duplicate assay.

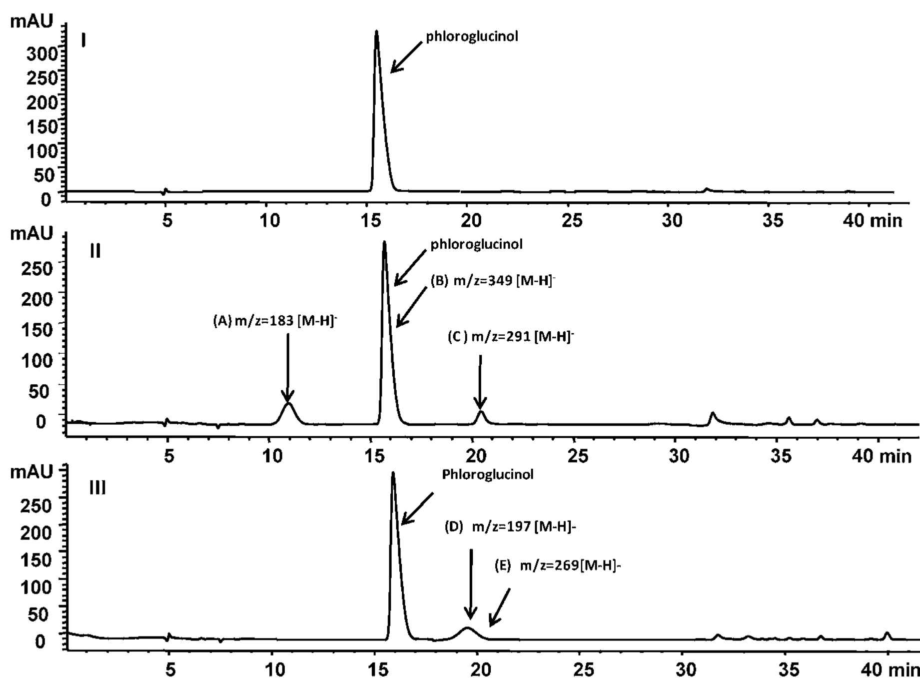

Figure 4. HPLC−DAD chromatograms of the phloroglucinol (10 mM) after incubation with phosphate buffer (I), 1 mM glyoxal (II), and 1 mM methylglyoxal (III). Peaks of identified adducts were labeled with their molecular weight.

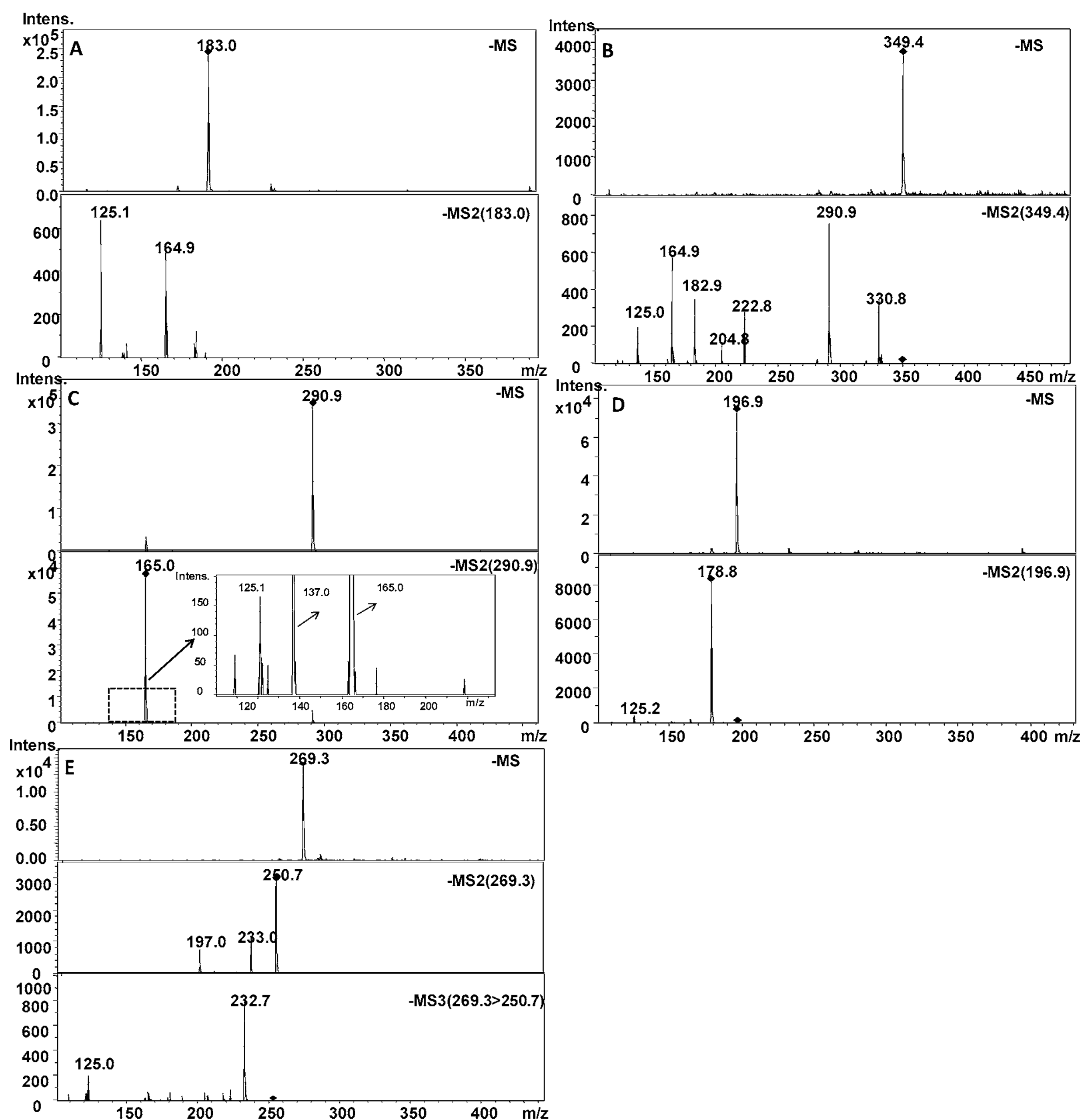

Figure 5. MS and MS^n spectra of phloroglucinol−carbonyl adducts. Letters A to E match those in Figure 4.

glyoxal scavenging capacity (Figure 3B), which explains their similarity in inhibiting methylglyoxal-mediated protein glycation.

Scavenging of reactive carbonyls appeared to be a major mechanism for algae extract to inhibit protein glycation. Reactive carbonyls such as methylglyoxal, glyoxal, and 3-deoxyglucosone are formed from the degradation and oxidation of Amadori products in the middle stage of protein glycation.[29] Alternatively, these key intermediates can be generated by glucose glycoxidation and lipid peroxidation.[8] Since methylglyoxal is an active intermediate of AGE formation, the inhibitory effect of *F. vesiculosus* phlorotannins was attributed in part to their abilities to scavenge reactive carbonyls.

Phloroglucinol−Carbonyl Reaction and Adduct Identification. Phloroglucinol remained stable in phosphate buffer saline (pH 7.4) (Figure 4I). Its content decreased after incubating with glyoxal or methylglyoxal at molar ratio of phloroglucinol:glyoxal/methylglyoxal = 10:1 for two hours. Three phloroglucinol−glyoxal and two phloroglucinol−methylglyoxal adducts were detected (Figure 4II,III), and their mass spectra are shown in Figure 5. Proposed structures of adducts and fragments are illustrated in Figure 6.

The first phloroglucinol−glyoxal adduct eluted at 11.2 min and had m/z 183 $[M-H]^-$. It yielded a product ion at m/z 125 that was consistent with phloroglucinol moiety (Figure 5A). Fragment m/z 165 was due to water elimination from 183 $[M-H]^-$. This adduct was tentatively identified as a monophloroglucinol−monoglyoxal adduct. Its structure is depicted in Figure 6A. The second adduct at 16.5 min gave rise to m/z 349 $[M-H]^-$ and product ions at m/z 331, 291, 183, and 125 (Figure 5B). Fragment m/z 331 was due to water elimination from 349 $[M-H]$. Fragments with m/z 291 and 183 were produced after losing a glyoxal, a glyoxal moiety and one phloroglucinol moiety, respectively. This adduct was

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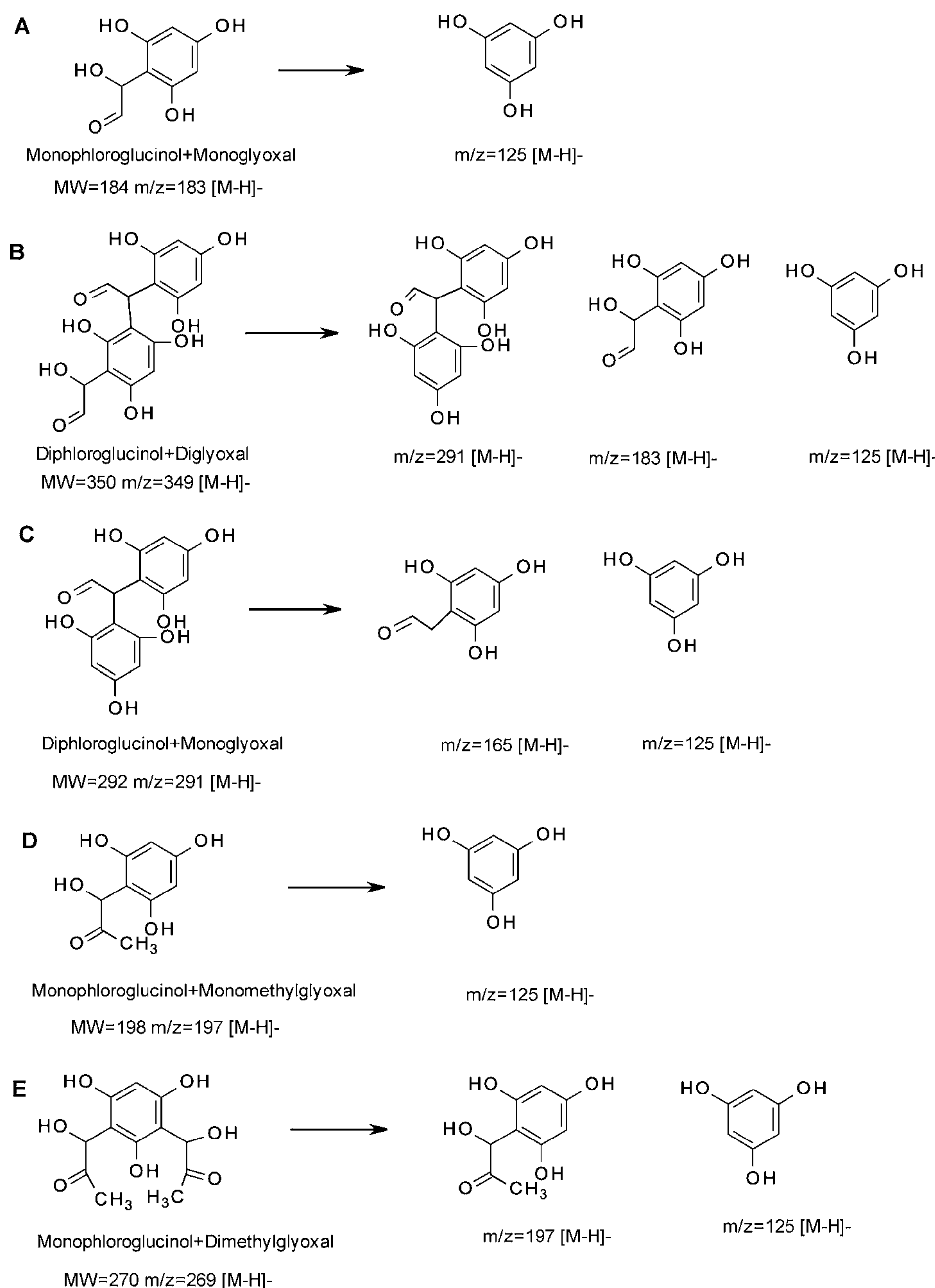

Figure 6. The proposed structures of phloroglucinol−carbonyl adducts and their product ions. Letters A to E match those in Figure 4 and Figure 5.

tentatively identified as a diphloroglucinol−diglyoxal adduct (Figure 6B). In this adduct, two phloroglucinol molecules were crossed linked by the aldehyde group of the glyoxal. A similar reaction had been reported for glyoxal and epicatechin.[30] We postulated that the reaction started from protonation of glyoxal, which was attacked by one phloroglucinol through nucleophilic addition to form a glyoxal−phloroglucinol intermediate. The intermediate continued to lose a water molecule to form a carbocation. The new carbocation then attacked another phloroglucinol to yield an ethyl-linked adduct. The third glyoxal−phloroglucinol adduct (21.4 min) showed m/z 291 $[M - H]^-$ (Figure 5C). This adduct lost one phloroglucinol moiety to yield a fragment at m/z 165 $[M - H]^-$. The fragment at m/z 125 $[M - H]^-$ was phloroglucinol. It was consistent with an adduct composed of two phloroglucinol molecules and one glyoxal molecule in between (Figure 6C).

The first methylglyoxal−phloroglucinol adduct eluted at 20.4 min and yielded m/z 197 $[M - H]^-$. The fragments at m/z 179 and 125 were due to water elimination and phloroglucinol, respectively (Figure 5D). It was tentatively identified as monophloroglucinol−monomethylglyoxal adduct (Figure 6D). The second methylglyoxal−phloroglucinol adduct eluted at 22.2 min and produced m/z 269 $[M - H]^-$ (Figure 5E). It lost one methylglyoxal moiety to generate a product ion at m/z 197. Fragment m/z 251 was due to water elimination from $[M - H]^-$, which continued to fragment to produce m/z 125. The structure of this adduct is illustrated in Figure 6E. Glyoxal has two aldehyde groups. The methylglyoxal has a ketone group and an aldehyde group. In the phloroglucinol−methylglyoxal reaction, the aldehyde group of methylglyoxal molecules attacked phloroglucinol to form the adduct. This occurred because an aldehyde group has greater electrophilicity than a

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ketone group and it also has less steric hindrance during reaction.[31] Similar observations had been made in methylglyoxal and epicatechin reactions.[30]

Bioactivity of phlorotannins in vivo depends on its bioavailability. Phloroglucinol is known to be absorbable.[32] However, little is known about the bioavailability of oligomeric and polymeric phlorotannins present in marine algae. We speculate that the bioavailability of phlorotannins may be similar to that of proanthocyanidins.[33]The low oligomers (dimer, trimer, and tetramer) may have limited bioavailability in the gastrointestinal tract. Polymers cannot be absorbed, and they are degraded by microflora in the large intestine into monomer (phloroglucinol) and simple phenolic acids. The absorbed phlorotannins and phenolic acids may serve as carbonyl scavengers in vivo.

In conclusion, our study showed that *F. vesiculosus* phlorotannins were effective in inhibiting the formation of AGEs. The antiglycation activities of *F. vesiculosus* phlorotannins were attributed in part to their abilities to scavenge reactive carbonyls. The ability of phlorotannins to react with carbonyls was the major mechanism for protein glycation inhibition. These results suggested that phlorotannins from brown algae may prevent or improve the AGE associated chronic conditions.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as discussed in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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