INHIBITORY EFFECTS OF BROWN ALGAE (*FUCUS VESICULOSUS*) EXTRACTS AND ITS CONSTITUENT PHLOROTANNINS ON THE FORMATION OF ADVANCED GLYCATION ENDPRODUCTS

By

HAIYAN LIU

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2011

© 2011 Haiyan Liu

To my family and friends

ACKNOWLEDGMENTS

I want to express my gratitude to my major advisor, Dr. Liwei Gu, for his patience, continuous encouragement and mentorship. Without his guidance and support, this research could not be accomplished. I am grateful for my committee members, Dr. Maurice R. Marshall, Dr. W. Steven Otwell, and Dr. Edward J. Phlips, for their valuable time and suggestions.

I cherished the friendship created with my lab group members, Keqin Ou, Wei Wang, Hanwei Liu, Amandeep K. Sandhu, Zheng Li and Timothy Buran. They were always willing to offer helping hands. The laughter we shared brought abundant joy and made our lives memorable. I also want to give my thanks to Sara Marshall, who kindly offered help to proofread the present thesis.

Most importantly, I want to express my deepest gratitude to my parents for their constant love and great support of my education. Without their love, patience and unconditional support I would not be able to successfully accomplish my graduate studies.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AGEs	Advanced Glycation Endproducts
BSA	Bovine serum albumin
DAD	Diode array detector
HPLC	High performance liquid chromatogram
M	Molar
Mg	Milligram
Min	Minute(s)
mL	Milliliter
mM	Millimolar
m/z	Mass to charge ration
ORAC	Oxygen radical absorbance capacity
Trolox	6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid
µg	Microgram
µL	Microliter

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

INHIBITORY EFFECTS OF BROWN ALGAE (*FUCUS VESICULOSUS*) EXTRACTS AND ITS CONSTITUENT PHLOROTANNINS ON THE FORMATION OF ADVANCED GLYCATION ENDPRODUCTS

By

Haiyan Liu

December 2011

Chair: Liwei Gu
Major: Food Science and Human Nutrition

Advanced glycation endproducts (AGEs) are a group of complex and heterogeneous compounds generated during protein glycation. Accumulation of AGEs is associated with aging, diabetes, Alzheimer's disease, renal failure, etc. Reactive carbonyls produced during lipid peroxidation or sugar glycoxidation play an important role in protein glycation. Carbonyl compounds rapidly react with amino groups in biological molecules to form AGEs. Previous studies showed that polyphenols scavenged reactive carbonyls and had inhibitory effects on the formation of AGEs. Brown algae *Fucus vesiculosus* contains a wide range of bioactive phytochemicals including phlorotannins, which are the oligomers and polymers of phloroglucinol found exclusively in brown algae. We hypothesized that *F. vesiculosus* may inhibit the formation of AGEs by scavenging reactive carbonyl compounds.

F. vesiculosus phlorotannins were extracted using 70% acetone. The resultant extract was fractionated into dichloromethane, ethyl acetate, butanol and water fractions using a liquid-liquid partition method. The ethyl acetate fraction was further fractionated into four subfractions (Ethyl-F1 to F4) using a Sephadex LH-20 column. The antioxidant

capacities of *F. vesiculosus* acetone extract or fractions were evaluated. The inhibitory effects of *F. vesiculosus* extracts on the formation of AGEs were investigated in bovine serum albumin (BSA)-glucose model and BSA-methylglyoxal model. The capacity of *F. vesiculosus* extracts to scavenge methylglyoxal was examined. Phloroglucinol, the constituent unit of phlorotannins, was incubated with methylglyoxal and glyoxal at pH 7.4. Phloroglucinol-carbonyl adducts were detected, and their structures were tentatively identified using HPLC-ESI-MS^n.

Liquid-liquid partition of *F. vesiculosus* acetone extract caused phlorotannins to be concentrated in butanol and ethyl acetate fractions. HPLC-ESI-MS^n analyses showed that Ethyl-F1 and F2 contained phlorotannin oligomers and polymers. Etnyl-F3 and F4 contained exclusively phlorotannin polymers. Phlorotannin trimers through pentamers were identified in Ethyl-F1. Phlorotannin pentamer, heptamer and octamers were identified in Ethyl F-2.

F. vesiculosus acetone extract or fractions significantly displayed high capacities to scavenge free radicals. All the extracts inhibited the formation of AGEs mediated by glucose and methylglyoxal. The inhibitory effects increased in a concentration-dependent manner. The concentrations of *F. vesiculosus* extracts required to inhibit 50% of albumin glycation (EC_{50}) in 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 BSA-glucose assay, *F. vesiculosus* extracts inhibited glycation of bovine serum albumin more or as effectively as aminoguanidine, except for Ethyl-F3 and F4. Ethyl acetate fraction and its four subfractions scavenged more than 50% of methylglyoxal in two hours. The reaction

between phloroglucinol with glyoxal or methylglyoxal led to the generation of adducts. The structures of phloroglucinol-carbonyl adducts were tentatively identified using HPLC-ESI-MS^n.

Our study showed that *F. vesiculosus* phlorotannins inhibited the formation of AGEs by scavenging reactive carbonyls. *F. vesiculosus* in diet may provide benefits in preventing carbonyl or AGE-related chronic diseases.

CHAPTER 1
INTRODUCTION

Background

The advance glycation endproducts (AGEs) are a class of compounds with brown, fluorescent, or cross-linked characteristics. Accumulation of AGEs is associated with aging, diabetes, Alzheimer's disease, renal failure, and many other chronic diseases. Reactive carbonyls such as glyoxal and methylglyoxal are formed during glycoxidation. They play a pivotal role in the formation of AGEs. Reactive carbonyls cause protein dysfunctions and tissue damage, leading to pathological consequences such as inflammation and apoptosis that contribute to the progression of diseases *(1)*.

Marine Algae

Marine algae, classified as *Laminariales* (brown), *Chlorophyta* (green) and *Rhodophyta* (red), have a long history of use in the Asian diet and are considered underexploited resources (*2-3*). They are known to contain a wide range of bioactive natural substances with diverse health benefits.

Health Benefits

Previous epidemiological studies have demonstrated that marine algae provided protective effects against mammary (*4-5*), intestinal (*6-7*) and skin carcinogenesis (*8-9*). Rodents fed algae exhibited suppression of tumor initiation and mutagenic inhibition in the colon and skin (*7, 9*). Red and brown algae extracts have been shown to have inhibitory effects on breast and colon cancer induction (*10*). Anti-inflammatory and proliferative activities by a variety of red algal and kelp extracts were also documented (*11*). Brown algae was effective in reducing blood cholesterol and lowering blood pressure, as well as preventing arteriolosclerosis (*12-13*). Blood pressure control,

constipation prevention and improvement of various gastroenteric disorders have been attributed to the polysaccharide alginic acid in the algae (*14*). Moreover, a number of studies have shown that marine algae have potent antioxidant activities (*15-19*).

Phytochemicals in Marine Algae

Marine algae contain a variety of phenolic phytochemicals. Flavonols and flavonol glycosides have been identified in the methanol extracts of red and green algae (*20*). Phlorotannins are oligomers and polymers of phloroglucinol that exist exclusively in brown algae (*21-22*). They have been identified from several brown algal families such as *Alariaceae, Fucaceae* and *Sargassaceae* (*23*). Three types of phlorotannins including fucols, fucophlorethols and phlorethols were isolated and identified in brown algae *F. vesiculosus.* Fucols contain phloroglucinol units which are linked by aryl-aryl bonds. Phloroglucinol units in fucophlorethols are connected with ether and aryl-aryl bonds, and only ether bonds are present in phlorethols (*24*). The structures of *F. vesiculosus* phlorotannins are illustrated in Figure 1-1.

Three fucophlorethols isolated form *F. vesiculosus* scavenged free radicals and inhibited cytochrome P450 enzyme (*24*). A polyhydroxylated fucophlorethols from *F. vesiculosus* showed antibacterial effects (*25*). Phlorofucofuroeckol-B isolated from a brow algae *E. arborea* had anti-allergic effects (*26*). Phloroglucinol derivatives such as dioxinodehydroeckol, eckol, eckstolonol, phlorofucofuroeckol-A, dieckol, triphlorethol-B, 2-phloroeckol and 7-phloroeckol were isolated from *E. stolonifera* (*27-28*). These compounds possessed antibacterial, antitumor, nitric oxide inhibitory and reverse transcriptase inhibition activities (*29-32*). Oligomeric phlorotannins including eckol, phlorofucofuroeckol A, dieckol and 8, 8'-bieckol were identified in the Japanese Laminariaceous brown algae *Eisenia bicyclis Setchell* and *Ecklonia kurome.* These

compounds exhibited inhibitory effects on phospholipid peroxidation and radical scavenging activities *(33)*. The 7-phloro eckol, 6,6'-bieckoll of phloroglucinol derivatives and fucodiphloroethol G from *Ecklonia cava* showed antioxidant properties (*34*). Phlorofucofuroeckol-A from brown algae had inhibitory effects on the formation of AGEs mediated by glucose (*35*).

Besides phlorotannins, brown algae also contain carotenoid pigments such as β-carotene, astaxanthin and fucoxanthin (*23*). Carotenoids (*36*) protect cells and tissues from damaging effects of free radicals and singlet oxygen (*37*). Fucoxanthin is a unique marine carotenoid that possesses excellent antioxidant (*38-39*), anti-inflammatory (*40*) and anti-obesity effects (*41*). Fucoxanthinol and halocynthiaxanthin are two metabolites of fucoxanthin in marine organisms (*42-43*). Fucoxanthin could be hydrolyzed to fucoxanthinol during absorption by Caco-2 human and mouse intestine cells (*44*).

Reactive Carbonyls

Reactive carbonyl compounds generated endogenously from sugar glycoxidation and lipid peroxidation rapidly react with amino groups in biological molecules to form advanced glycation end products (AGEs) and advanced lipid peroxidation end products (ALEs) (*45*). Their accumulation *in vivo* is associated with aging, diabetes, Alzheimers, renal failure and other chronic disease (*46-47*).

Exogenous Sources of Reactive Carbonyl Compounds

The structures of reactive carbonyl compounds are depicted in Figure 1-2. Reactive carbonyls including methylglyoxal and glyoxal are found in various foods, including sugar-sweetened beverages, high lipids content foods, and thermally processed products. Methylglyoxal and glyoxal were detected in cookies ranging from 3.7 to 81.4 mg/kg, and 4.8 to 26.0 mg/kg, respectively (*48*). In toasted bread,

methylglyoxal and glyoxal content ranged from 0.5 to 2.5 mg/kg (*49*). Beverages such as carbonated soft drinks are important exogenous sources of dicarbonyls due to the high fructose corn syrup content (*50*). Other drinks such as coffee, wine, and beer were also found to contain carbonyl compounds (*51*). Dicarbonyl compounds found in commercial soybean, olive and corn oil products were attributed to lipid peroxidation during manufacturing and storage (*52*). Soy sauce and brewed coffee contained the highest amount of methylglyoxal in food products and beverages (*53*).

Formation of Reactive Carbonyl Compounds

Carbonyl compound are generated under oxidative conditions *in vivo* and *in vitro.* Lipid peroxidation produces a number of reactive carbonyl compounds. For example, oxidation of polyunsaturated fatty acid leads to the formation of α, β-unsaturated hydroxyalkenals, such as 4-hydroxynonenal and 4-hydroxyhexenal (*45*). Methylglyoxal and glyxoal can also be generated from sugar glycoxidation(*45*). During protein glycation, degradation of Schiff bases and oxidation of Amadori products produce reactive carbonyls such as methylglyoxal, glyoxal, and 3-deoxyglucosone (*54*). Besides the non-enzymatic pathway, reactive carbonyls can be generated via enzymatic catalyzed metabolism. The catabolism of lipids and amino acid produces methylglyoxal (*55*). Methylglyoxal can also be generated from ketone bodies by cytochrome P450 enzymes *(56).* Enzymatic oxidation of theronine by myeloperoxidase produces acrolein *(57).*

Advanced Glycation Endproducts (AGEs)

The Formation of AGEs

Advanced glycation endproducts (AGEs) are a group of complex and heterogeneous compounds generated during protein glycation (*58*). Protein glycation

has three stages: the initial stage, intermediate stage, and the late stage (*54*). In the initial stage, reducing sugars such as glucose react with the primary amino groups of a lysine residue to form a Schiff base, which is reversible. N-substituted glycosylamine is formed from the cyclization of the Schiff base (*59*). This unstable N-substituted glycosylamine undergo Amadori rearrangement to generate relatively stable Amadori products (*60*). Such reactions can be catalyzed by either iminium ion or transition metal ions (*54*). On the other hand, the unstable Schiff base may degrade to generate carbonyls including glyxoal and glycoaldehyde via the Namiki pathway (*61*). In the intermediate stage, Amadori products decompose to generate the dicarbonyls such as 3-deoxyglucosone. In addition to the degradation of Schiff base and oxidation of Amadori products, α-dicarbonyls such as glyxoal, methylglyoxal and 3-deoxyglucosone can also be generated from sugar glycoxidation (*45*). The late stage of protein glycation is mediated primarily by these dicarbonyls. Glyoxal reacts with proteins to form several AGEs including (carboxymethyl)lysine, (carboxymethyl)arginine, glyoxal-lysine dimer, arginine-lysine-glyoxal crosslink, and pentosidine (*54*). Methylglyoxal reacts with amino group of lysine residue in proteins to form (carboxymethyl)lysine, N^{ε}-(carboxyethyl)lysine (*62-63*) and methylglyoxal-lysine dimer (*64*). 3-deoxyglucosone modifies lysine residue in proteins to generate AGEs such as labile pyrraline and imidazolone (*65-66*). The structure of pentosidine, pyrraline and (carboxymethyl)lysine are illustrated in Figure 1-3.

Inhibition of AGEs Formation

Studies have shown that the formation of AGEs is a major pathogenic factor in diabetes (*67*), advanced aging (*68*), Alzheimer's disease (*68*), renal failure (*69*) and other chronic diseases. The current approach to prevent these conditions is to use

antiglycation agents. Antiglycation agents suppress the formation of AGEs by either scavenging reactive carbonyls or alleviating oxidative stress (*54*). Aminoguanidine, metformin and pyridoxamine are antiglycation drug candidates that inhibit AGE inhibition by scavenging reactive dicarbonyls (*70*). Pyridoxamine also prevents the conversion of Amadori product to (carboxymethyl)lysine (*71*). But, the application of these drugs in clinical trial was not successful due to toxicity. It was reported that phenolic compounds from plants had antiglycation effects. Polyphenols from cinnamon bark and mung bean inhibited protein glycation and the formation of AGEs *(72-73).* Catechin, epicatechin and epigallocatechin from green tea had inhibitory effects on the protein glycation *(74-75).* It was found that these phenolic compouds scavenged reactive carbonyls.

Research Objectives

The investigation of carbonyl scavenging agents and AGEs inhibitors provides a novel approach to prevent diabetic complications and other pathological diseases. We hypothesized that phytochemicals from edible marine algae are able to inhibit the formation of AGEs by scavenging reactive carbonyls. There were three research objectives in this thesis.

1. To extract, fractionate and identify phlorotannins from *F. vesiculosus.*
2. To investigate the antiglycation effects and carbonyl scavenging activities of *F. vesiculosus* extracts.
3. To identify phloroglucinol-carbonyl adducts formed as a result of carbonyl scavenging.

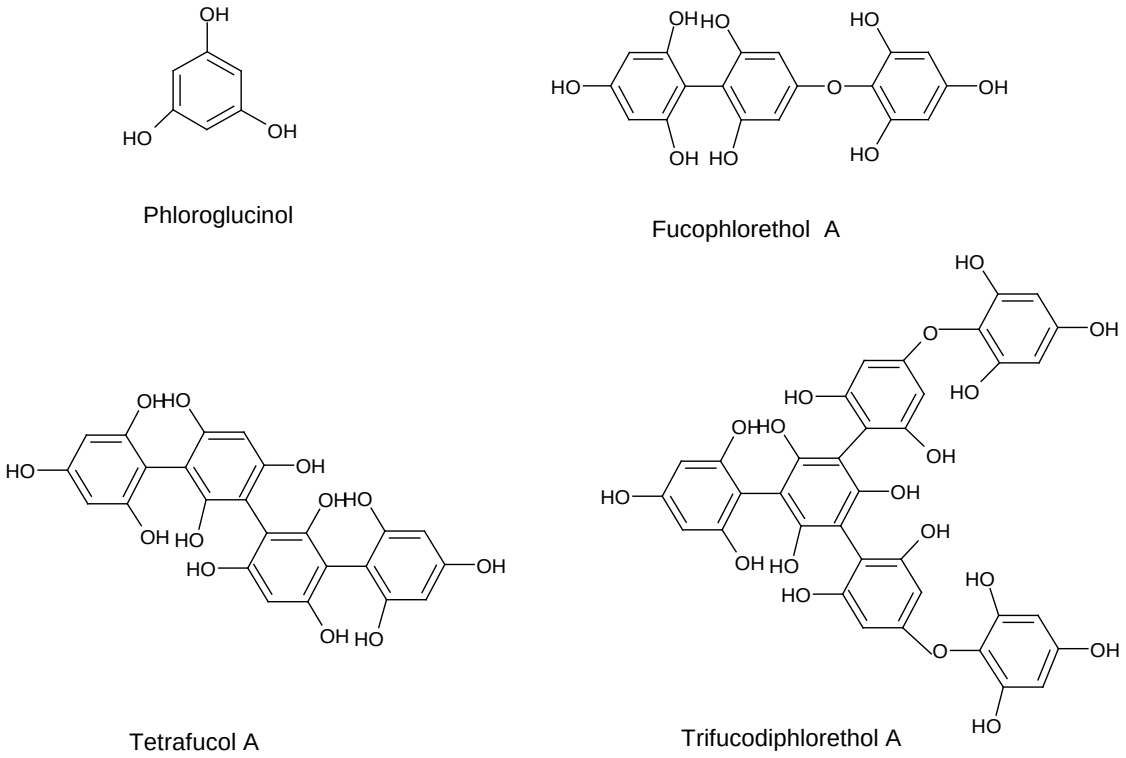


Figure 1-1. Structures of phlorotannins from *F. vesiculosus*.

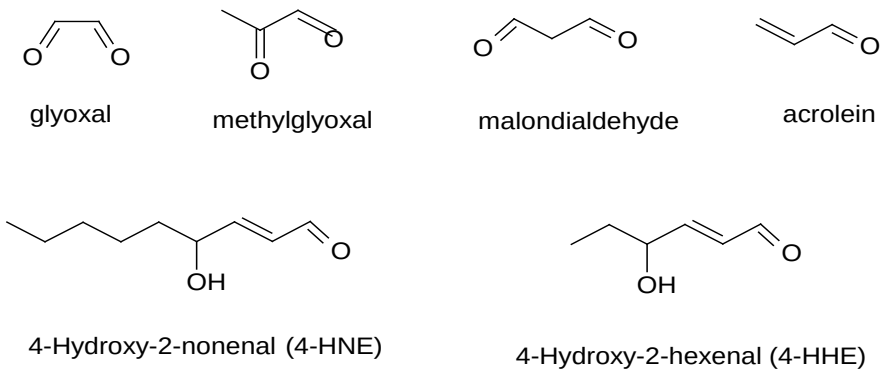
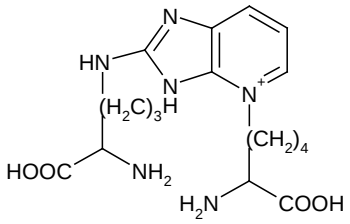
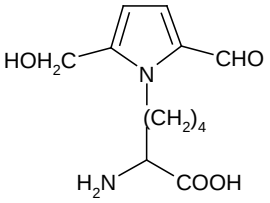


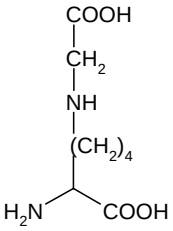
Figure 1-2. Structures of reactive carbonyl compounds.



pentosidine



pyrraline



N-carboxylmethyl lysine

Figure 1-3. Structures of advance glycation endproducts (AGEs).

CHAPTER 2
EXTRACTION, FRACTIONATION AND HPLC-ESI-MS IDENTIFICATION OF PHLOROTANNINS FROM *FUCUS VESICULOSUS*

Background

Phlorotannins are oligomers and polymers of phloroglucinol that exist exclusively in brown algae. The objective of this chapter was to extract phlorotannins from brown algae *F. vesiculosus* and tentatively identify these compounds using HPLC-ESI-MS^n.

Materials and Methods

Chemicals and Materials

Dry *F. vesiculosus* was obtained from Maine Seaweed Co. (Steuben, Maine). Phloroglucinol, N, N-dimethylformamide, and 2, 4-dimethoxybenzaldehyde were products from Acros Organics (Morris Plains, NJ). Sephadex LH-20 was obtained from Sigma-Aldrich (St. Louis, MO). HPLC grade ancetonitrile and other organic solvents were purchased from Fisher Scientific Co. (Pittsburg, PA). AAPH (2, 2'-azotis(2-amidinopropane)) was a product of Wako Chemicals Inc. (Bellwood, RI). 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Sigma-Aldrich (St. Louis, MO).

Phlorotannin Extraction and Fractionation

The phlorotannin extraction and fractionation procedure is depicted in Figure 2-1. Three hundred grams of *F. vesiculosus* materials was ground into fine powder using a blender and extracted with 1200 mL 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 minutes 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 dichloromethane three times in a separation funnel. The dichloromethane phases were combined and evaporated to yield 4.934 g extract. The aqueous phase was then partitioned with ethyl acetate three times (100 mL each) before it was partitioned with butanol an additional three times (100 mL each). The ethyl acetate and butanol phases were evaporated to yield 4.946 g and 4.983 g extracts, 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.

Folin-Ciocalteu Assay

The total phenolic contents of *F. vesiculosus* extracts were determined by Folin-Ciocalteu assay with modification (*76*). *F. vesiculosus* extracts was dissolved in methanol and diluted to the appropriate concentration for analysis. *F. vesiculosus* extracts (100 µL) were mixed with Folin-Ciocalteu reagent (1 ml, 0.2 N) and sodium carbonate (1 ml, 15%). Absorption at 765 nm was measured in a microplate reader (SPECTRAmax 190, Molecular Devices, Sunnyvale, CA) after incubation for 30 min at room temperature. Phloroglucinol solutions with concentrations ranging from 100-400mg/L were used to generate a standard curve. The results were expressed as

milligrams of phloroglucinol equivalents per gram of *F. vesiculosus* extracts (mg of PG/g).

Oxygen Radical Absorbance Capacity Assay

The oxygen radical absorbance capacity (ORAC) assay was used with modification (*77*). *F. vesiculosus* extracts were dissolved in phosphate buffer (50 mM, pH 7.4). Fifty microliters of each extract were mixed with fluorescein solution (100 µl, 20 nM) in a 96-well black microplate. Mixture were incubated at 37 °C for 10 min before the addition of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 50 µl, 0.14 M). Fluorescence was measured using 485 nm excitation and 530 nm emission (Spectra XMS Gemini, Molecular Device, Sunnyvale, CA). Readings were taken at 1 min intervals for 40 min. 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) was used to generate a standard curve. The antioxidant capacities of extracts were expressed as µmol Trolox equivalents (TE) per gram of *F. vesiculosus* extracts (µmol of TE/g).

Total Phlorotannin Content Assay

Total phlorotannins were quantified using 2,4-dimethoxybenzaldehyde (DMBA) assay (*78*). The 2, 4-dimethoxybenzaldehyde (2.0 g/100 mL glacial acetic acid) and HCl (16.0 mL concentrated HCl in 100 mL glacial acetic acid) were used as stock solutions. A working reagent was prepared by mixing equal volumes of these two solutions prior to use. A standard curve was prepared by making a phloroglucinol solution with concentrations of 0 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1.0 mg/mL, and 1.25 mg/mL. Ten µl of phloroglucinol solution were mixed with 10 µl of N, N-dimethylformamide and 2.5 mL of working reagent. The mixtures were incubated at 30 ^{o}C for 60 min. After incubation, the absorbance was measured at 494 nm. Similarly, 10

µl of *F. vesiculosus* acetone extract or fractions were mixed with 10 µl N, N-dimethylformamide and 2.5 mL working reagent. After incubating at 30 ^{o}C for 60 min, the absorbance was measured at 515 nm. The results were expressed as milligrams of phloroglucinol equivalent per gram of *F. vesiculosus* extracts (mg of PG/g).

Phlorotannin Identification by HPLC-DAD-ESI-MS^{n}

Chromatographic analyses were performed on an Agilent 1200 series HPLC system (Agilent, Palo Alto, CA) equipped with an autosampler/injector, a binary pump, a column compartment, and a diode array detector. A Zorbax Stablebond Analytical SB-C18 column (4.6 mm × 250 mm, 5 µm, Agilent Technologies, Palo Alto, CA) was used for separation. Mobile phases consisted of A (0.1% formic acid aqueous solution) and B (0.1% formic acid in acetonitrile). UV−vis spectra were scanned from 220 to 600 nm with a recording wavelength of 280 nm. The flow rate was 1 mL/min. The linear gradient was as follows: 10% B from 0 to 5 min, 10 to 26% B from 5 to 15 min, 26 to 30% B from 15 to 30 min, 30 to 44% B from 30 to 32 min, 44 to 60% B from 32 to 42 min, and 60 to 10% B from 42 to 45 min. Electrospray mass spectrometry was performed on a HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Column effluent was monitored in the positive and negative ion mode of the instrument in an alternative manner during the same run. Other experimental conditions for the mass spectrometer were as follows: nebulizer, 45 psi; dry gas, 10.0 L/min; dry temperature, 350 °C; ion trap, scan from *m/z* 100 to 2200; smart parameter setting (SPS), compound stability, 50%; trap drive level, 60%. The mass spectrometer was operated in Auto MS^2 mode. MS^2 was used to capture and fragment the most abundant ion in full scan mass spectra.

Data Expression and Statistical Analysis

One way ANOVA with Tukey-Kramer HSD test were 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 were expressed as the mean $\pm$ standard deviation.

Results and Discussion

Total Phenolic Content

The total phenolic content of *F. vesiculosus* extracts were determined using Folin-Ciocalteu with modification (*76*). The results of the Folin-Ciocalteu assay indicated that all the *F. vesiculosus* extracts contained various amounts of phenolic compounds, with a range from 24.9 to 594.5 mg phloroglucinol equivalents per gram extract (Figure 2-2). Ethyl-F3 had the highest phenolic content (594.2 ± 0.7 mg phloroglucinol/g extract) among all the extracts. The total phenolic content of ethyl acetate fraction was higher than that of Ethyl-F1 and F2, but was lower than that of Ethyl-F3 and F4. Ethyl-F1 showed comparable amounts of polyphenols as butanol and acetone extracts, and higher phenolic content than that of dichloromethane fraction. The phenolic content of water fractions was the lowest.

Oxygen Radical Absorbance Capacity

All the *F. vesiculosus* extracts showed high antioxidant capacity, with values higher than 539.7 µmol Trolox equivalents per gram extract. Ethyl acetate fraction and its four subfractions showed significantly higher antioxidant capacity than *F. vesiculosus* acetone extract (Figure 2-3). Ethyl-F2 and Ethyl-F3 had higher antioxidant capacities (6578.6 ± 321.6, and 6288 ± 171.4 µmol TE/g extract) than Ethyl-F1 and Ethyl-F4. The antioxidant capacities of four subfractions are comparable to those of ethyl acetate fractions. Dichloromethane and water fractions showed the lowest ORAC values. The

antioxidant activities of *F. vesiculosus* extracts may be attributed to phenolic compounds, especially phlorotannin content. Ethyl acetate fraction and its subfractions, which are concentrated with phlorotannins, showed significantly higher ORAC values than other extracts. This is in agreement with previous research that showed phlorotannis scavenged oxygen species (*79-80*). The antioxidant activity of phlorotannins from brown algae was associated with their unique molecular skeleton (*81*). The phenol rings of phlorotannins acted as electron traps to scavenge peroxy, superoxide-anions and hydroxyl radicals (*23*). Phlorotannins from brown algae have eight interconnected rings, and exhibit greater free radical scavenging activity than other polyphenols like green tea catechin, which only has three to four rings (*82*).

Total Phlorotannin Content

The total phlorotannin content of *F. vesiculosus* extracts were determined using DMBA assay *(78)*. The results were expressed as milligram phloroglucinol equivalent per gram dry weight. The 70% acetone was used for extraction because it was shown as the most effective solvent to extract phlorotannins from brown algae (*83*). Phytochemicals in the crude acetone extract was fractionated according to polarity using liquid-liquid extraction. Dichloromethane was used to obtain lipid compounds from the extract. Both ethyl acetate and butanol were used to concentrated phlorotannins. As a result, ethyl acetate fraction showed the highest concentration phlorotannins. Phlorotannin content was also high in butanol fraction (Figure 2-4). Dichloromethane and water fractions had lower phlorotannins contents than the original extract. All four ethyl acetate subfractions had significantly higher phlorotannin content than original *F. vesiculosus* extract. Ethyl-F1 showed the highest phlorotannin content (133.81± 2.74

mg phloroglucinol/g extract). The phlorotannin content in Ethyl-F3 was comparable to that of ethyl acetate fraction, but was higher than Ethyl-F2 and Ethyl-F4.

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. After sub-fractionation of ethyl acetate fraction on 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.

Phlorotannin Identification by HPLC-DAD-ESI-MS^n

F. vesiculosus butanol and ethyl acetate fractions showed similar HPLC-DAD chromatograms. They both had a predominant peak at 35 min and many smaller peaks between 5 and 20 min (Figure 2-5A,B). The intensities of phlorotannins peaks in butanol fractions were much lower than those in ethyl acetate fraction, which was consistent with their phlorotannin contents. Ethyl-F1 showed group peaks at 5-12 min and a smaller peak at 35 min (Figure 2-5C). Etnyl-F2 showed a profile similar to ethyl acetate fraction, except for absence of a peak between 5 and 10 min (Figure 2-5D). A single peak at 35 min was observed for Ethyl-F3 and F4 (Figure 2-5E,F). Mass spectrometry indicated that phlorotannin trimers through octamers, including a group of structural isomers, eluted between 5 to 15 min. Seven peaks of phlorotannin oligomers in Ethyl-F1 and additional 5 peaks in Ethyl-F2 were determined using a mass spectrometer (Figure 2-5G,H). Only one or two possible structures of each group of structural isomers were proposed since MS data was insufficient to determine the exact structure of each peak. The identification of peak eluted at 35 min was inclusive on a mass spectrometer. However, this peak was deduced to be polymeric phlorotannins for the following reasons. First, the separation mode of tannins on Sephadex LH-20 was adsorption

instead of gel permeation. Phlorotannins of smaller molecular size bind with Sephadex LH-20 with lower affinity, and thus elute earlier than phlorotannins of larger molecular sizes. This was consistent with the chromatographic analysis and mass spectrometric data. Secondly, the peak at 35 min showed a typical UV-vis spectrum that was characteristic for phlorotannins. A similar observation was made by Pent et. al. on the HPLC analysis of procyanidins (*84*). In this previous study, procyanidin dimers through tetramers were separated on a reversed-phase column. Polymers (pentamers and beyond) eluted as a single peak at the end of chromatogram. The mass spectra of tentatively identified phlorotannin peaks are shown in Figure 2-6 and 2-7. Structures of the phlorotannins isomers are depicted in Figure 2-8.

The ESI-MS (positive mode) of peak 1 (retention time 2.8 min) showed pseudomolecular ions at *m/z* 375 $[M+H]^+$, indicating the compound was phlorotannin trimer. The compound gave rise to fragments at *m/z* 232 $[M+H-18-125]^+$, suggesting the loss of one phloroglucinol ring. The fragment at *m/z* 125 was phloroglucinol. The structure of one possible isomer is illustrated in Figure 2-8.

The peak 2 (retention time 5.9 min) and 3 (retention time 7.0 min) gave *m/z* 499 $[M+H]^+$, suggesting these two compounds were isomers of phlorotannin tetramer. Peak 2 produced fragment ions at *m/z* 356 $[M+H-18-125]^+$, 355 $[M+H-18-126]^+$, 358 $[M+H-125-16]^+$, 232 $[M+H-18-125-124]^+$, 250 $[M+H-125-124]^+$. The fragments at *m/z* 356 and 232 were yielded after losing one phloroglucinol ring and hexahydroxybiphenyl, respectively. Peak 3 gave rise to the fragments at *m/z* 374 $[M+H-125]^+$, 356 $[M+H-18-125]^+$, 232 $[M+H-18-125-124]^+$, 234$[M+H-125-124-16]^+$. On the basis of the fragmentation pattern and published data *(25)*, the structure of one isomer is shown in

Figure 2-8(2). The compound with *m/z* 499 has been isolated from *F. vesiculosus* previously (*25*).

Peaks 4, 5, 9 and 10 had *m/z* 747 $[M+H]^+$, suggesting that these compounds were isomers of phlorotannin hexamer. Peak 4 (retention time 8.4 min) yielded fragment ions at *m/z* 585, 586, 570, 462, 446, 338, and 332. Ion at *m/z* 586 $[M+H-36-125]^+$ indicated the loss of two water molecules and a phloroglucinol ring. The fragment at *m/z* 570 $[M+H-36-125-16]^+$ suggested the loss of two water molecules along with a phloroglucinol ring and an ether bond. The ions at *m/z* 462 $[M-H-36-125-124]^+$,and 446 $[M+H-36-125-124-16]^+$, were deduced to be the fragments after loss of two phloroglucinol rings along with two water molecules and two phloroglucinol rings along with a ether bond and two water molecules, respectively. The ion at *m/z* 338 $[M+H-36-125-124-124]^+$, was generated after losing three phloroglucinol rings along with two water molecules. The compound lost three phloroglucinol rings along with one ether bond to yield fragment at *m/z* 322 $[M+H-125-124-124-16]^+$.

Peak 5 (retention time 10.3 min) showed a major ion in MS^2 at *m/z* 711 $[M+H-36]^+$. This compound gave rise to fragments at *m/z* 586 $[M+H-36-125]^+$, 570 $[M+H-36-125-16]^+$, 446 $[M+H-36-125-16-125]^+$, suggesting the loss of a phloroglucinol ring and two water molecules, the loss of a phloroglucinol ring along with the ether bond and two water molecules, the loss of two phloroglucinol rings along with the ether bond and two water molecules, respectively.

Peaks 9 (retention time 13.1 min) and 10 (retention time 13.8 min) had similar fragment patterns. The major fragment ions in MS^2 were at *m/z* 622 $[M+H-125]^+$, 462 $[M+H-36-125-124]^+$,356 $[M+H-18-125-124-124]^+$,338 $[M+H-36-125-124-124]^+$,

suggesting the loss of a phloroglucinol ring, the loss of two phloroglucinol rings with two water molecules, the loss of three phloroglucinol rings with one water molecule, and the loss of three phloroglucinol rings with two water molecules. Since MS data is insufficient to identify the exact structure of each peak, the structures of two possible isomers were given in Figure 2-8. The fifth structure illustrated in Figure 2-8 has been identified in *F. vesiculosus* according to previous research (*24*).

Peaks 6 and 7 had the same $[M+H]^+$ ion at *m/z* 623, suggesting that these compounds are the isomers of phlorotannin pentamer. Peak 6 (retention time 10.7 min) generated fragments at *m/z* 480 $[M+H-18-125]^+$ after losing a phloroglucinol ring and one water molecule. The fragment at *m/z* 464$[M+H-18-125-16]^+$, was due to the loss of a phloroglucinol ring along with an ether bond and one water molecule. The compound gave the fragment ions at *m/z* 356 $[M+H-18-125-124]^+$, 340 $[M+H-18-125-124-16]^+$, suggesting the loss of two phloroglucinol rings and one water molecule, and the loss of two phloroglucinol rings along with an ether bond an one water molecule. Peak 7 (retention time 10.7 min) had the fragment ions in MS^2 as 480 $[M+H-18-125]^+$, 356$[M+H-18-125-124]^+$, and 340$[M+H-18-125-124-16]^+$. Based on the fragmentation pattern, the structure of one possible isomer was illustrated in Figure 2-8.

Peak 8 (retention time 12.6 min) had a $[M+H]^+$ ion at *m/z* 871, suggesting it is phlorotannin heptamer. The major fragments in MS^2 included *m/z* 710 $[M+H-36-125]^+$, 586$[M+H-36-125-124]^+$, 462 $[M+H-36-125-124-124]^+$. The fragment was produced after losing one phloroglucinol ring along with two water molecules, two phloroglucinol rings and two water molecule, and three phloroglucinol rings with two water molecules,

respectively. A possible structure of the phlorotannin heptamer was illustrated in Figure 2-8.

Peaks 11 (retention time 14.4 min) and 12 (retention time 15.1) gave *m/z* 995 $[M+H]^+$, suggesting these compounds were phlorotannin octamer. Mass spectrometric data of peak 12 showed major ions in MS^2 at *m/z* 977 $[M+H-18]^+$, 834 $[M+H-36-125]^+$, 710 $[M+H-36-125-124]^+$,694 $[M+H-36-125-124-16]^+$, 586 $[M+H-36-125-124-124]^+$, 462 $[M+H-36-125-124-124-124]^+$,338 $[M+H-36-125-124-124-124-124]^+$. The fragments were generated after losing one water molecule, one phloroglucinol ring with two water molecules, two phloroglucinol rings along with two water molecules, two phloroglucinol rings along with one ether bond and two water molecules, three phloroglucinol rings and two water molecules, four phloroglucinol rings and two water molecules, five phloroglucinol rings with two water molecules, respectively. Peak 11 showed a similar fragment pattern. The structure of one possible isomer is illustrated in Figure 2-8.

In conclusion, *F. vesiculosus* acetone extract was fractioned into dichloromethane, ethyl acetate, butanol and water fractions using liquid-liquid extraction. All the extracts showed high phenolic content and antioxidant capacities. The ethyl acetate fraction was found to be enriched with phlorotannins. This fraction was separated into four subfractions on a Sephadex LH-20 column. The Ethyl-F1 and F2 contain a mixture of oligomers and polymers, while the Ethyl-F3 and F4 contained exclusively polymers. Phlorotannin trimer, teramer, pantamer and hexamer were found in Ethyl-F1. Hexamer, heptamer and octamer were found in Ethyl-F2.

Table 2-1. Retention times and mass spectrometric data of phlorotannins in *F. vesiculosus* determined by HPLC-ESI-MS^n.

Peaks	Phlorotannin compound	Retention time(min)	Molecular weight	MS^1 (*m/z*)	MS^2 (*m/z*)
1*	Trimer	2.8	374	375$[M+H]^+$	357,232,231
2*	Tetramer	5.9	498	499$[M+H]^+$	481,358,356,355,250,232
3*	Tetramer	7.1	498	499$[M-H]^-$	374,373,358,357,356, 355,234,233,232,231
4*	Hexamer	8.4	746	747$[M+H]^+$	729,586,585,570,462, 461,462,446,337,338,322
5*	Hexamer	10.3	746	747$[M+H]^+$	711,586,585,570,462, 446,445
9*	Hexamer	13.1	746	747$[M+H]^+$	729,622,462,356,338,231
10*	Hexamer	13.8	746	747$[M+H]^+$	729, 622, 621,462, 356,338
6	Pentamer	10.7	622	623$[M+H]^+$	605,480,464,356, 340,231,179
7	Pentamer	11.6	622	623$[M+H]^+$	605,480,479,356,340,231
8	Heptamer	12.6	870	871$[M+H]^+$	853,710, 586,462
11	Octamer	14.4	994	995$[M+H]^+$	977,834,710,694,586,462,338
12	Octamer	15.1	994	995$[M+H]^+$	977,834,710,586,462,338

[a] Compounds followed by an asterisk were isolated from *F. vesiculosus* previously.

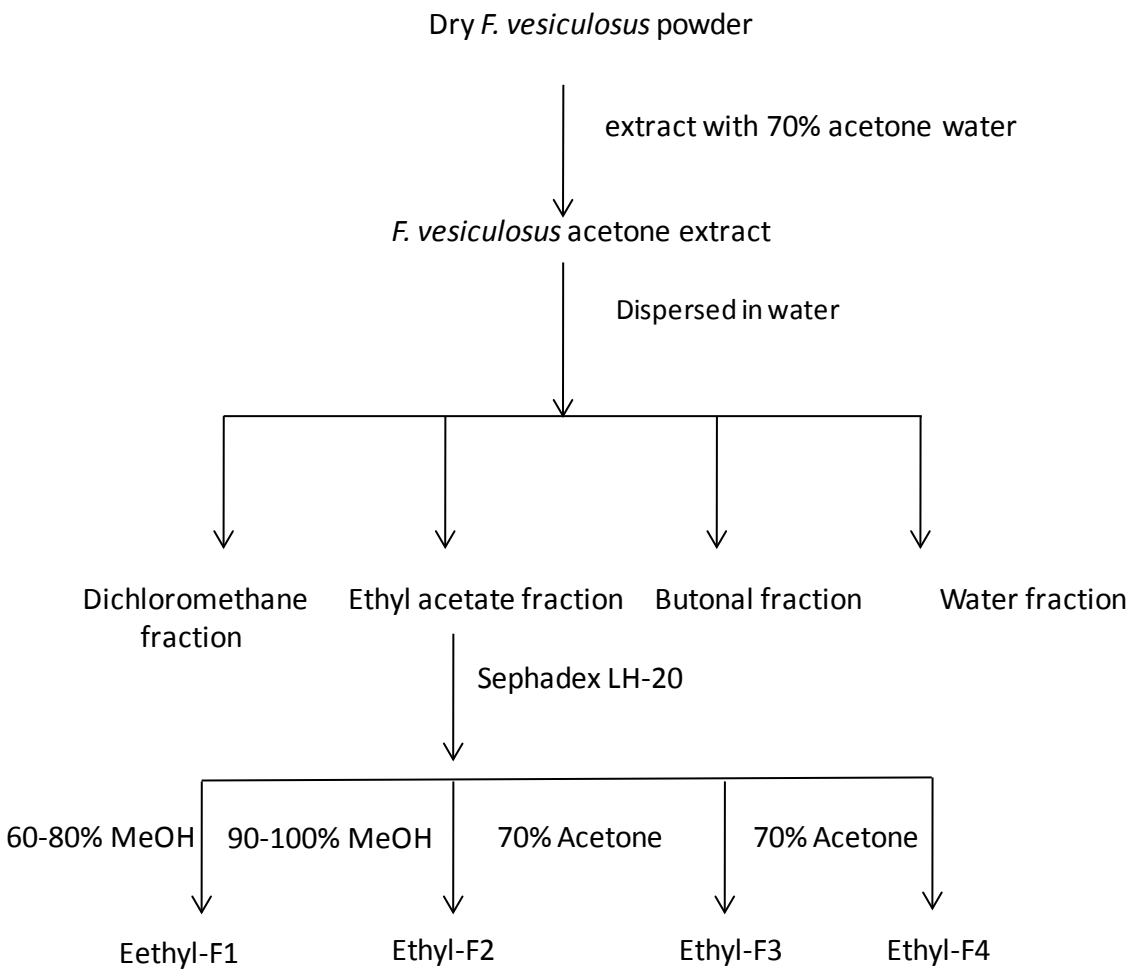


Figure 2-1. Extraction and fractionation of phlorotannins from *F. vesiculosus*.

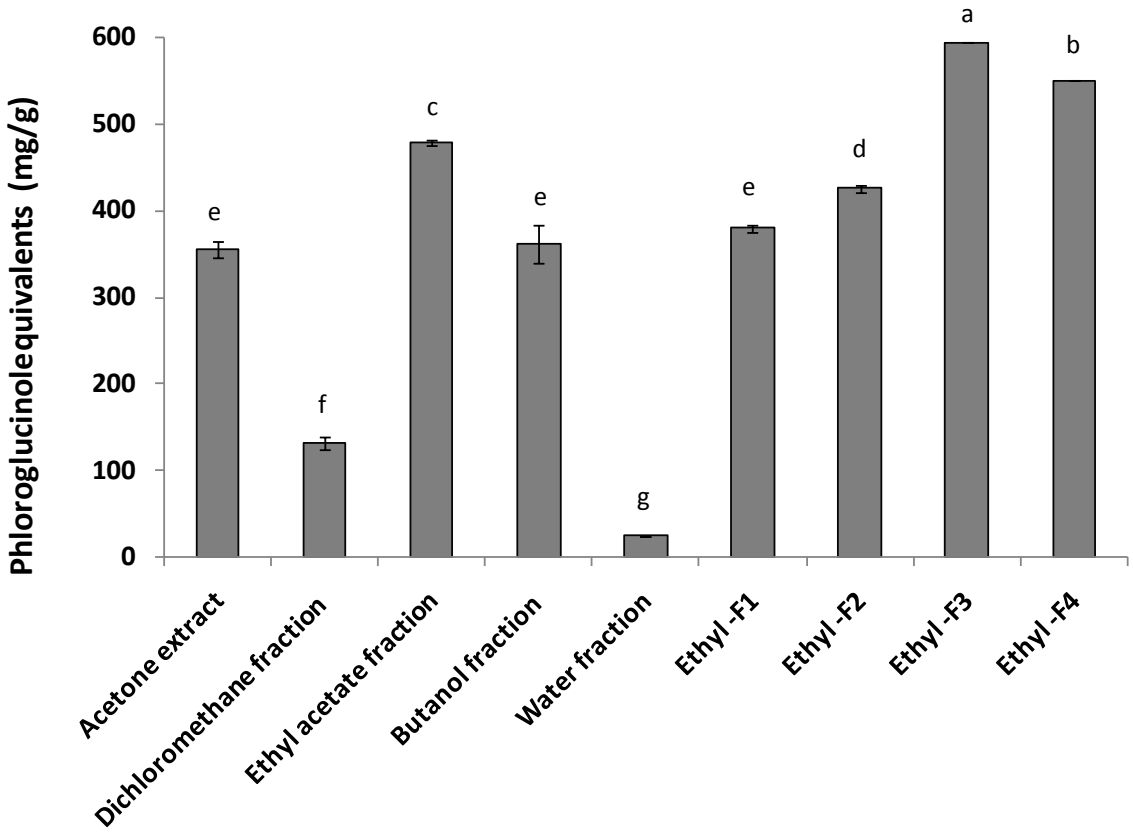


Figure 2-2. Total phenolic content of *F. vesiculosus* extracts determined by Folin-Ciocalteu assay. Results are means ± standard deviation of duplicate assay. Bars with different letters were significantly different ($P < 0.05$) from each other.

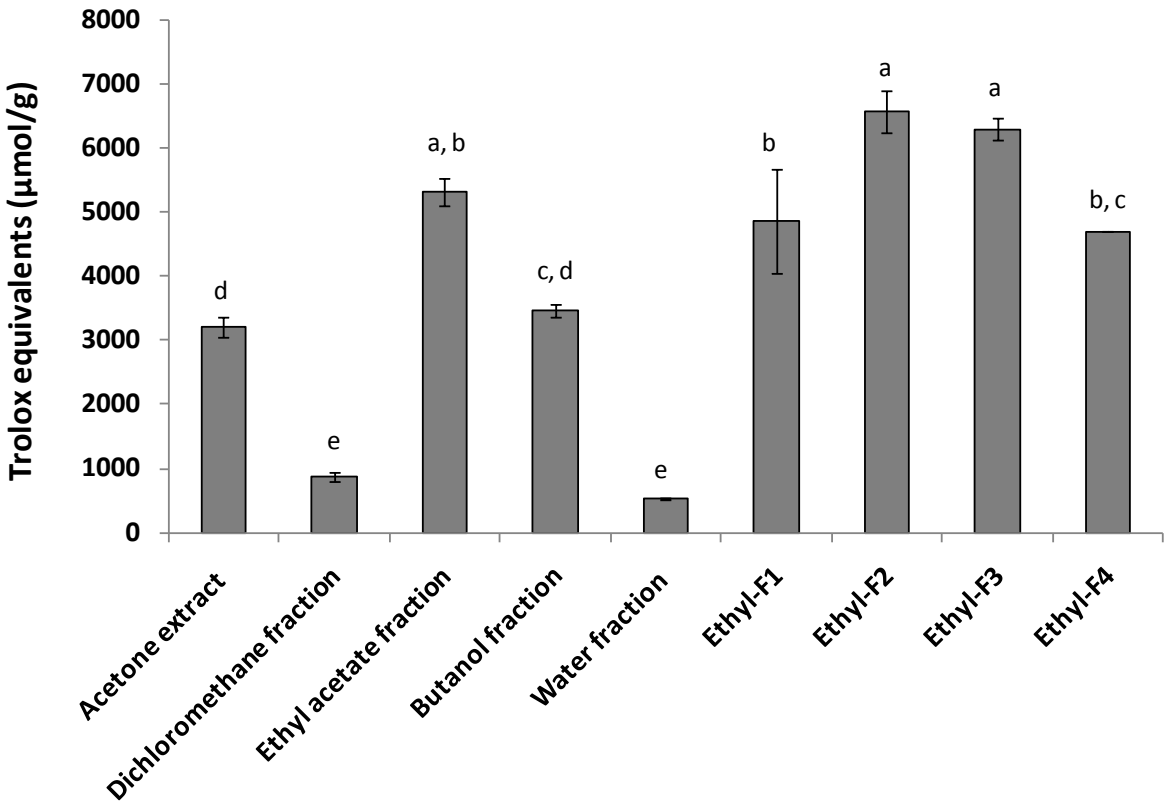


Figure 2-3. Antioxidant capacities of *F. vesiculosus* extracts determined by oxygen radical absorbance capacity assay. Results are means ± standard deviation of duplicate assay. Bars with different letters were significantly different ($P < 0.05$) from each other.

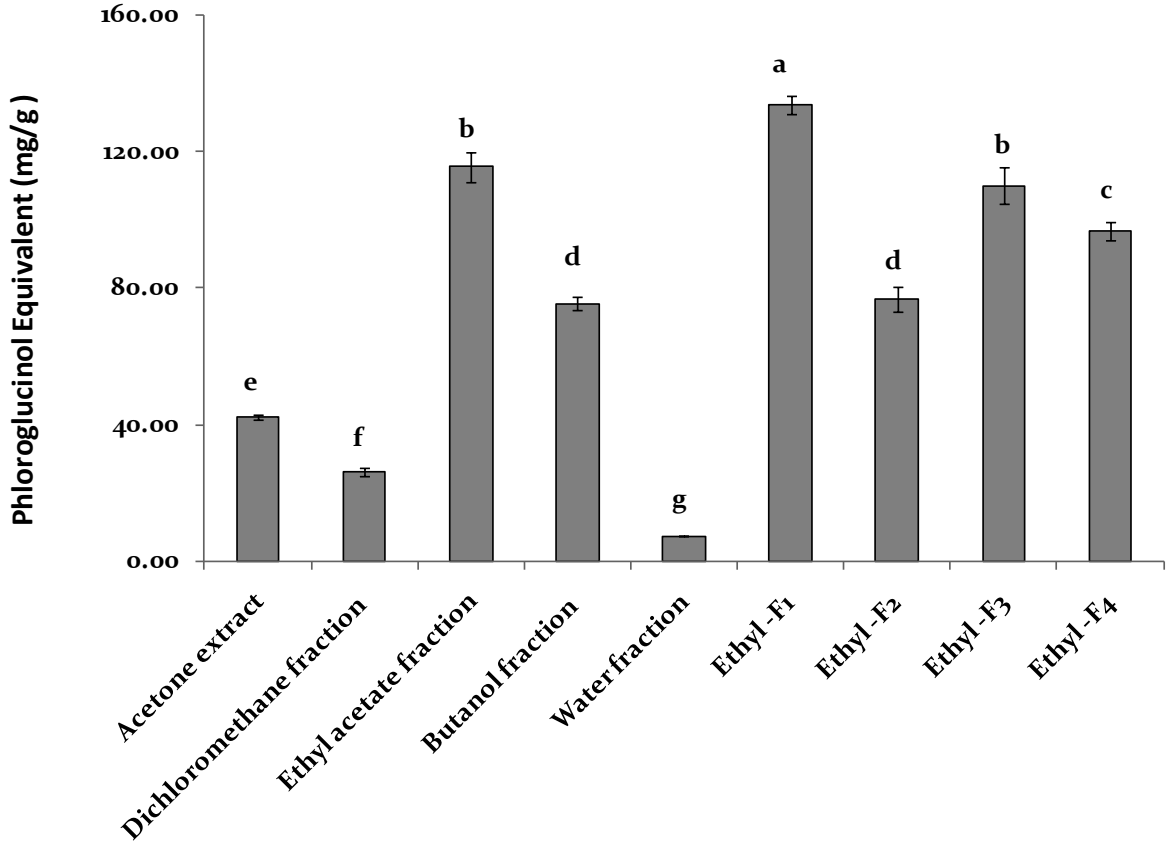


Figure 2-4.Total phlorotannin content of *F. vesiculosus* extracts determined by DMBA assay. Results are means ± standard deviation of triplicate assay. Bars with different letters were significantly different ($P < 0.05$) from each other.

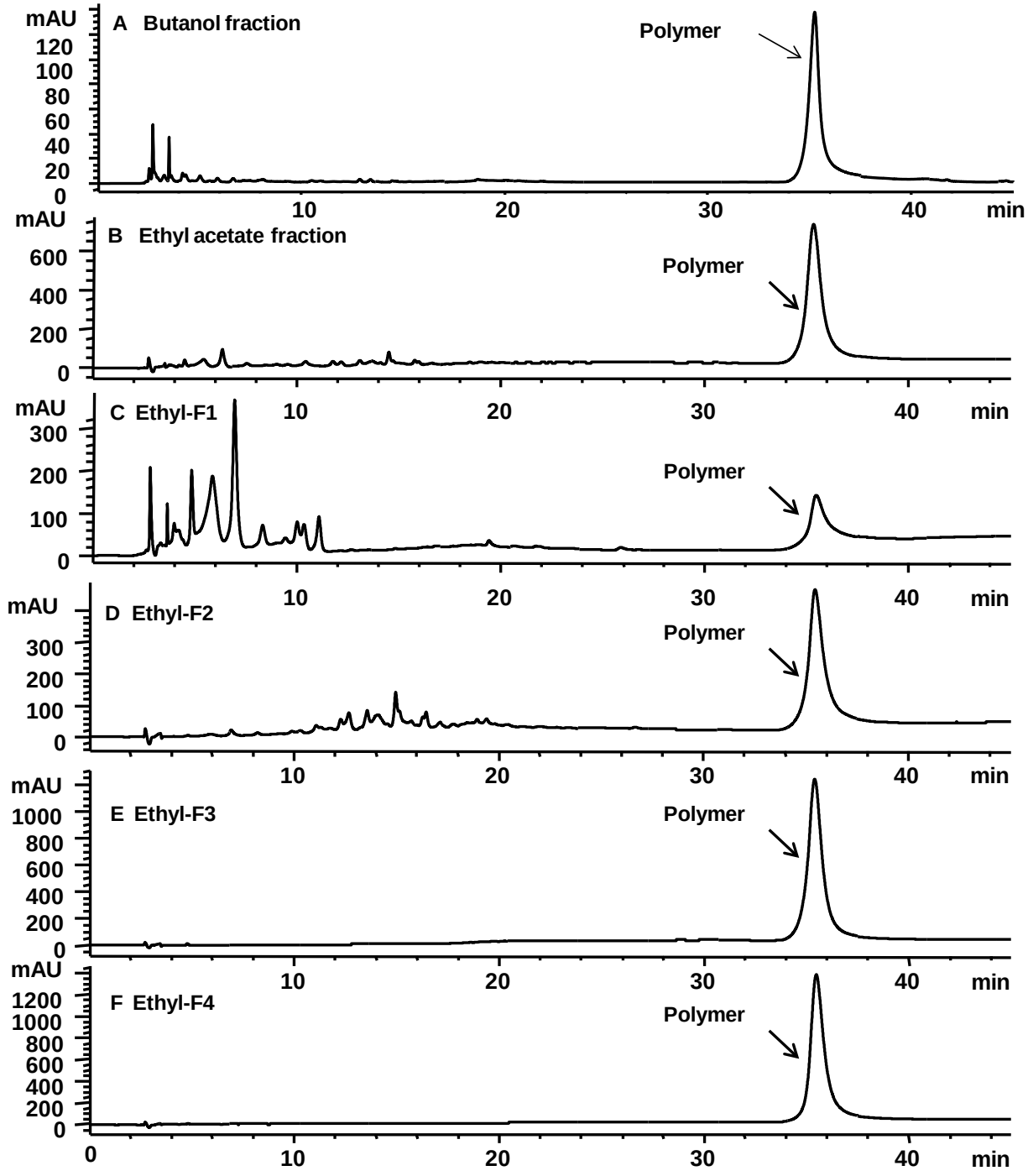


Figure 2-5. HPLC-DAD chromatograms of *F. vesiculosus* fractions. A) Butanol fraction. B) Ethyl acetate fraction. C) Ethyl-F1. D) Ethyl-F2. E) Ethyl-F3. F) Ethyl-F4. G) Magnified chromatogram of Ethyl-F1. H) Magnified chromatogram of Ethyl-F2. Peaks marked with numbers were tentatively identified using HPLC-ESI-MS^n.

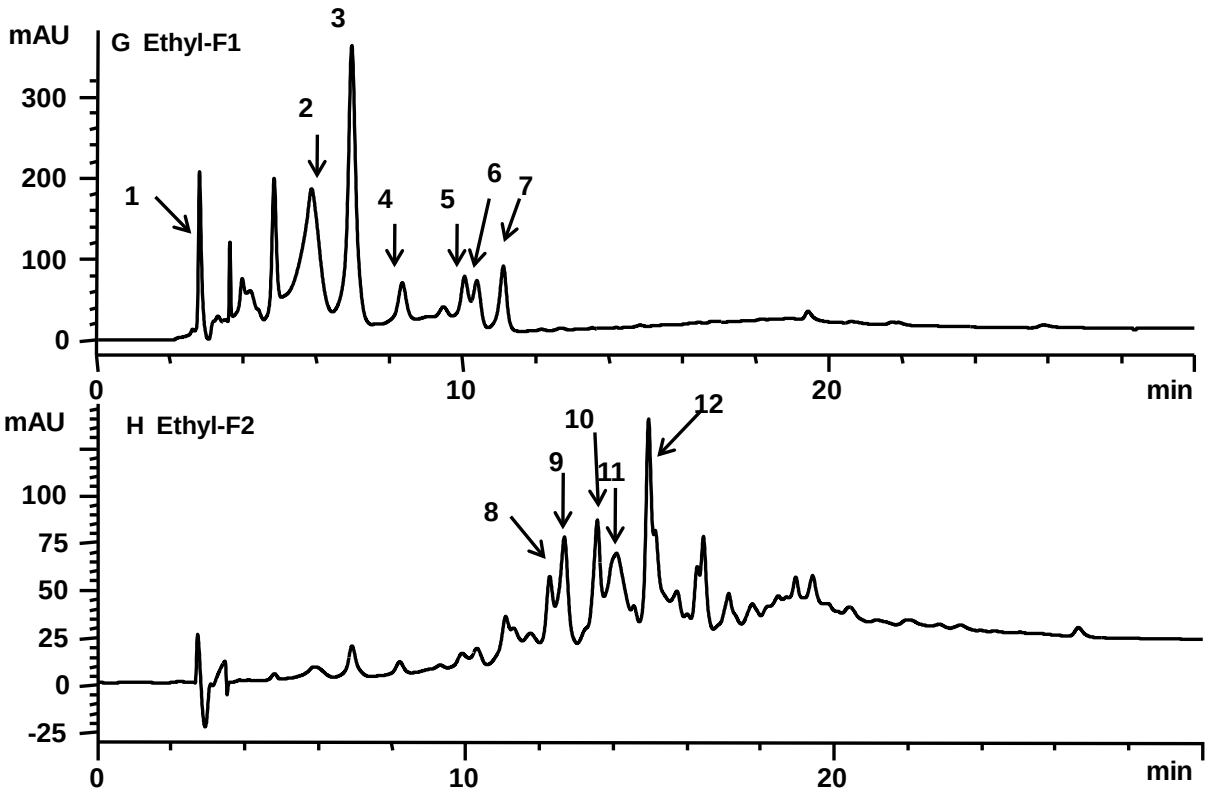


Figure 2-5. Continued.

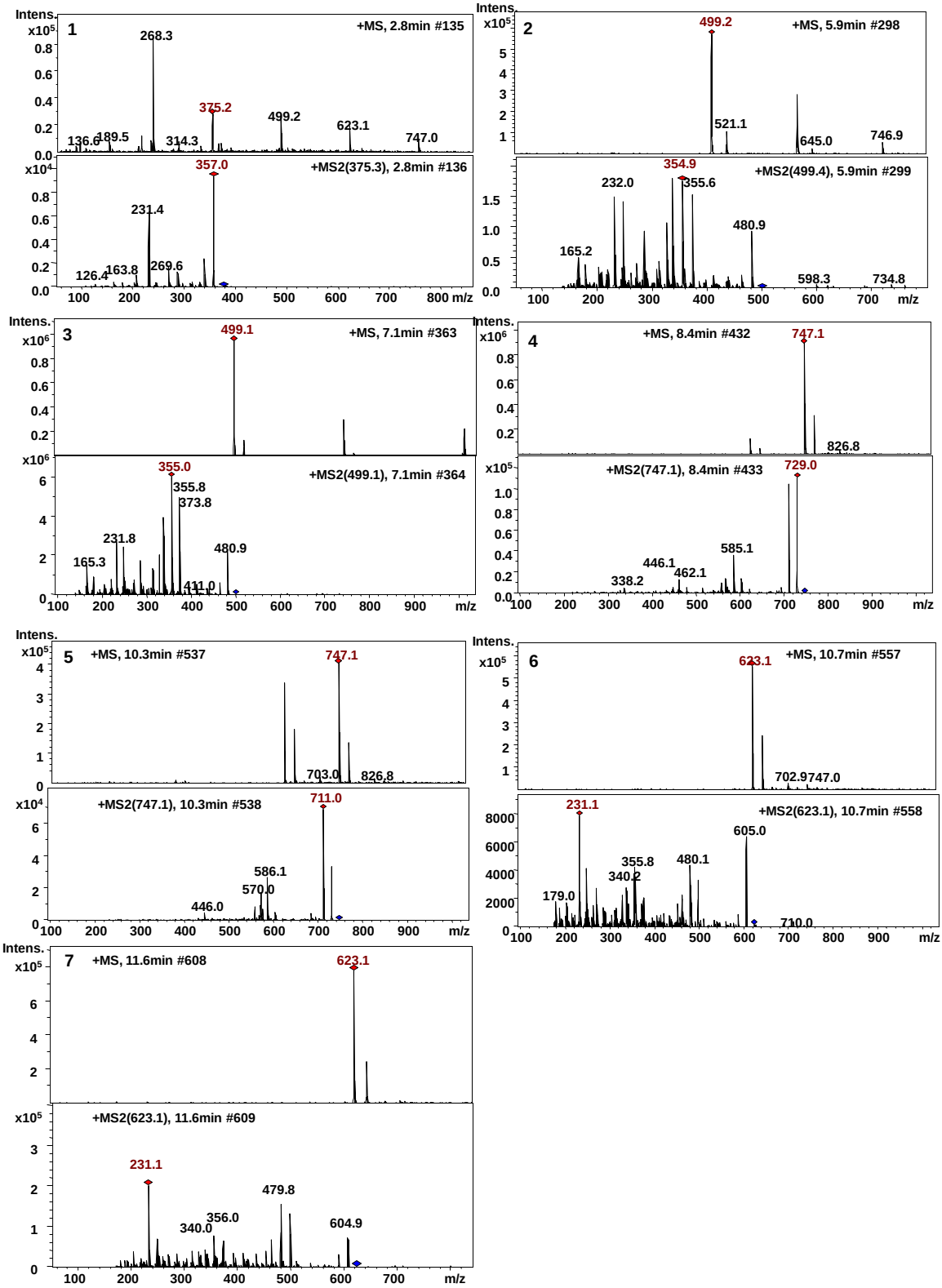


Figure 2-6. MS and MS^2 spectra of phlorotannin peaks in Ethyl-F1. Number 1-7 on the figures match those in the chromatogram of Ethyl-F1 in Figure 2-5G.

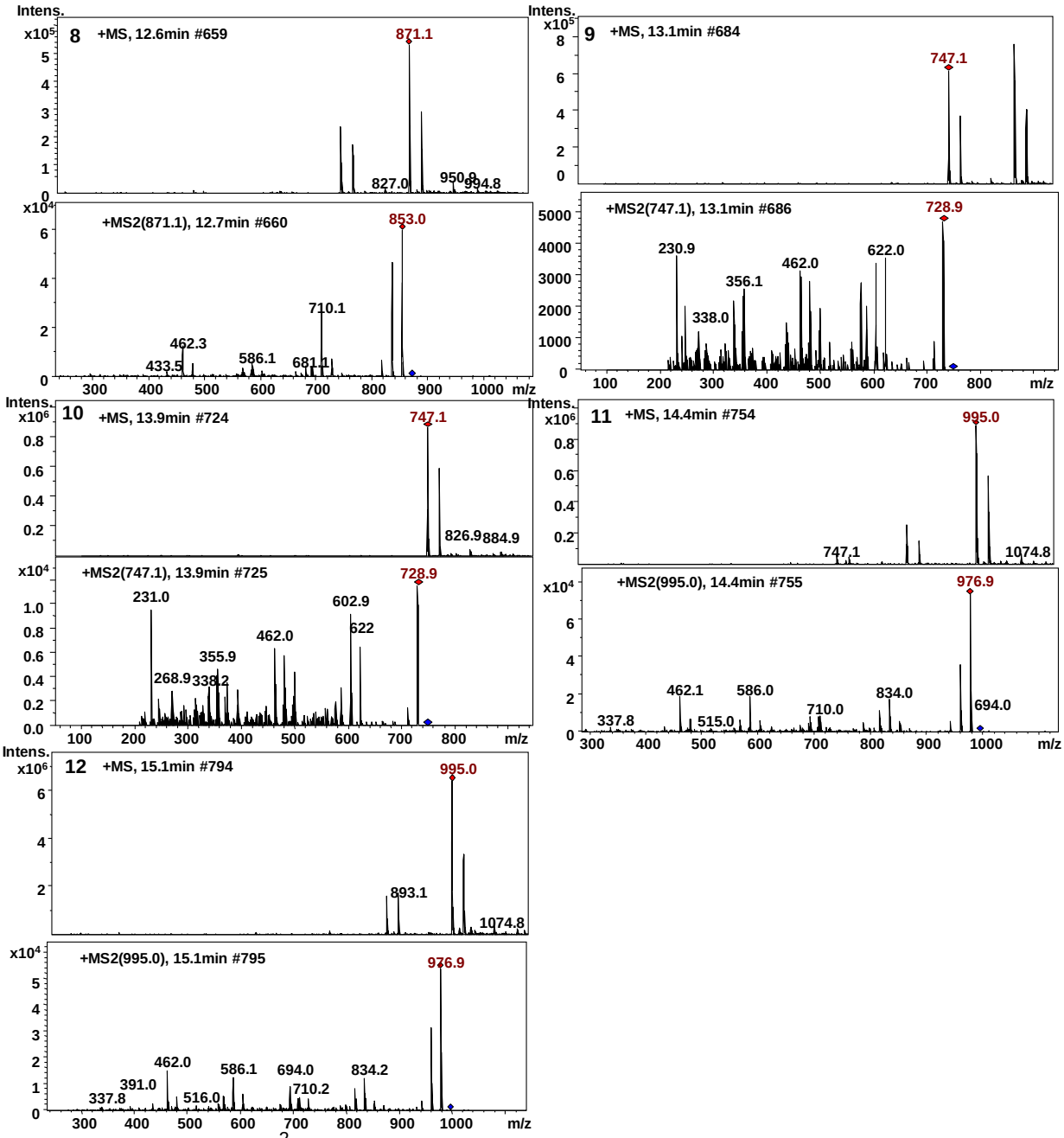


Figure 2-7. MS and MS^2 spectra of phlorotannin peaks in Ethyl-F2. Number 8-12 on the figures match those in the chromatogram of Ethyl-F2 in Figure 2-5H.

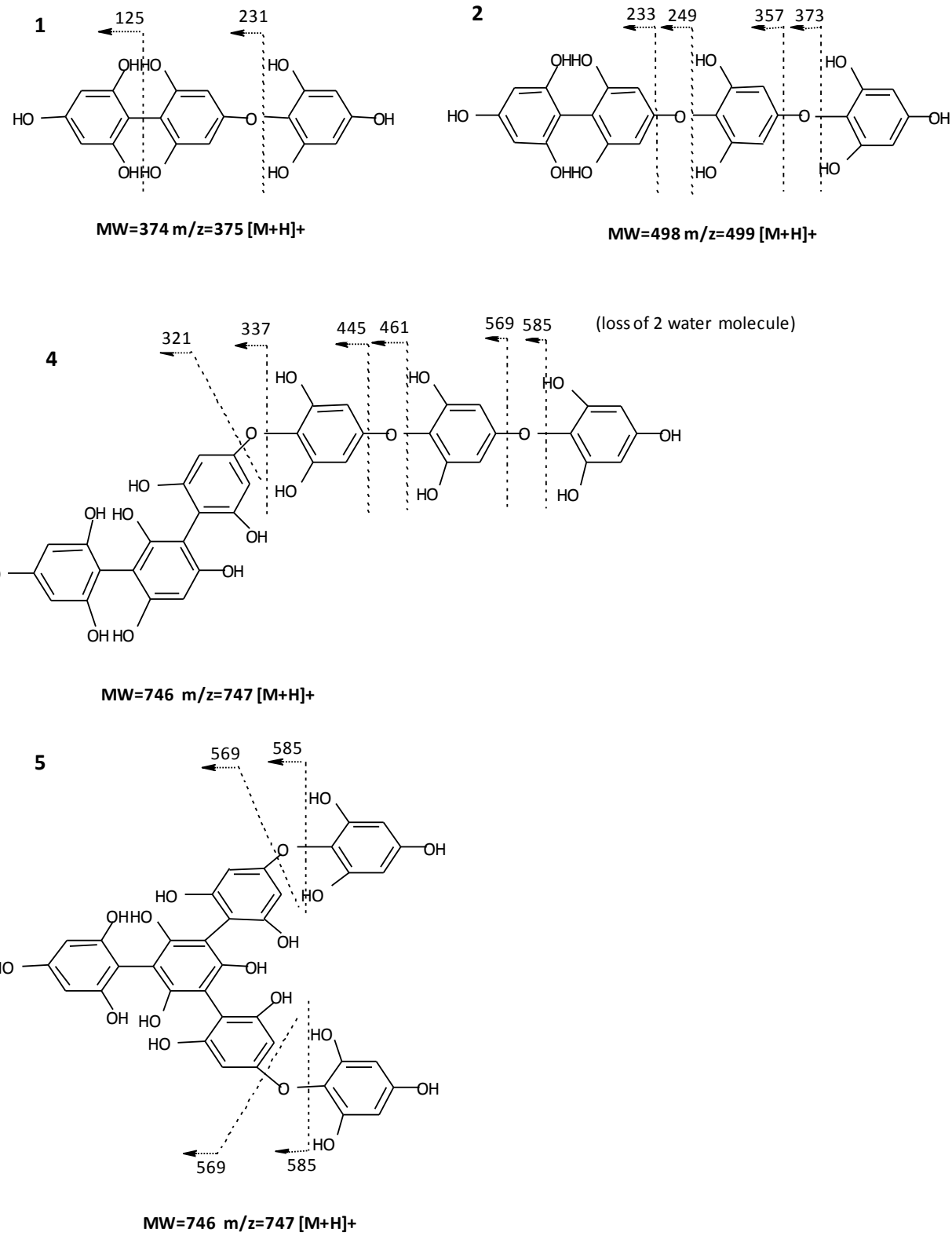


Figure 2-8. The proposed structures and fragmentation of phlorotannins in Ethyl-F1 and Ethyl-F2. Numbers on the figures match those in the chromatograms and mass spectra.

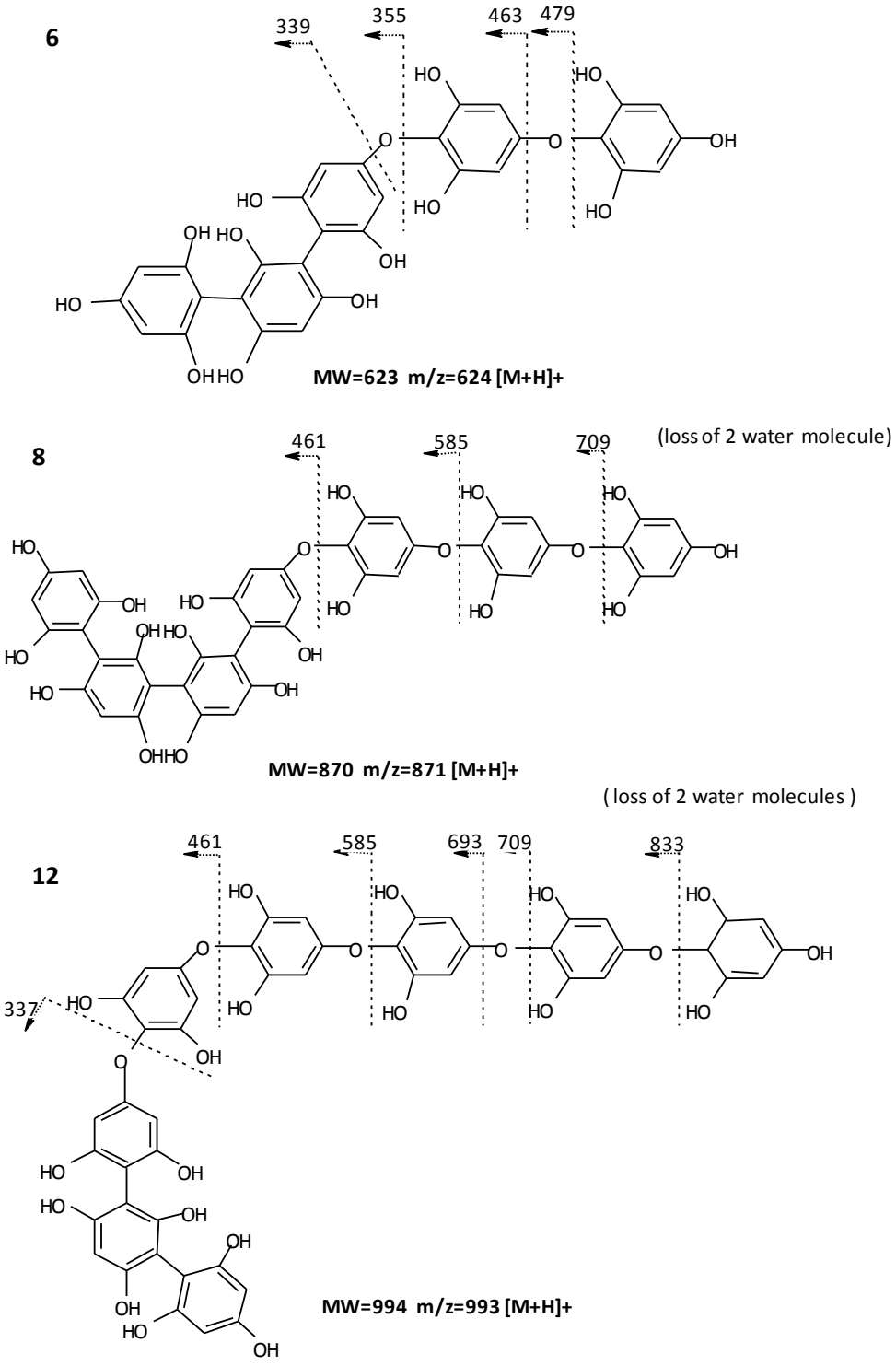


Figure 2-8. Continued.

CHAPTER 3
INHIBITORY EFFECTS OF *FUCUS VESICULOSUS* ON THE FORMATION OF ADVANCED GLYCATION ENDPRODUCTS

Background

Reactive carbonyls accelerate the formation of advance glycation endproducts (AGEs). Scavenging reactive carbonyls is an effective approach to inhibit protein glycation. *F. vesiculosus* acetone extract and its fractions showed high phlorotannin content. However, it is not known whether *F. vesiculosus* extracts are effective in inhibiting protein glycation. The objective of this chapter was to investigate the inhibitory effects of *F. vesiculosus* extracts on the formation of AGEs, as well as their capacities to scavenge reactive carbonyls.

Materials and Methods

Chemicals and Materials

Aminoguanidine, o-phenylenediamine, were products from Acros Organics (Morris Plains, NJ). Methylglyoxal (40% aqueous solution) and glucose was 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, acetone and other organic solvent were purchased from Fisher Scientific Co. (Pittsburg, PA).

Bovine Serum Albumin (BSA)-Glucose Assay

This assay evaluates protein glycation mediated by glucose. Bovine serum albumin and glucose were dissolved in phosphate buffer (100 mM, pH 7.4) to a concentration of 100 mg/mL and 1.6 M, respectively. *F. vesiculosus* acetone extract or fractions were dissolved in the same phosphate buffer. One mL 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 ^{o}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, the fluorescence of samples was measured using an excitation of 330 nm and an emission of 410 nm, respectively. The % inhibition of AGEs formation= [1 - (fluorescence of the test group/fluorescence of the control group)] x 100%.

BSA-Methylglyoxal Assay

This assay evaluates the middle stage of protein glycation. BSA and methylglyoxal were dissolved in phosphate buffer (100 mM, pH 7.4) to concentrations of 20 mg/mL and 60mM, respectively. *F. vesiculosus* acetone extract or fractions were dissolved in the same phosphate buffer. One mL of the BSA solution was mixed with 1 mL of methylglyoxal solution and 1 mL *F. vesiculosus* extracts. The mixture was incubated at 37 ^{o}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, the fluorescence of samples was measured using an excitation of 340 nm and an emission of 420 nm, respectively. The % inhibition of AGEs formation= [1 - (fluorescence of the test group/fluorescence of the control group)] x 100%.

Methylglyoxal Scavenging Assay

Methylglyoxal scavenging assay followed a published method with modifications *(72).* Methylglyoxal (5 mM), 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 ^{o}C for 0, 5, 10, 20, 40, 60, and 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 at 1 mL/min, and the injection volume was 15 µl. The linear gradient for elution was: 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 detection wavelength of 315 nm.

Data Expression and Statistical Analysis

Half inhibition concentrations (EC_{50}) were determined using Probit analysis function of SPSS software (Version 13, SPSS Inc., Chicago, IL.). One way ANOVA with 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 were expressed as the mean $\pm$ standard deviation.

Results

Anti-Glycation Effects in BSA-Glucose Assay

F. vesiculosus acetone extract and its fractions significantly inhibited protein glycation mediated by glucose, and the antiglycation effects increased with concentration (Figure 3-1). 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. Dichloromethane fraction had much lower phlorotannin content than butanol fraction, yet it showed similar antiglycation activities. Ethyl-F1 and F2 were similar to phloroglucinol. Ethyl-F3 and F4 had the much lower activity compared to all other fractions except that of water fraction.

Anti-Glycation Effects in BSA-Methylglyoxal Assay

F. vesiculosus acetone extract and its fractions inhibited the formation of fluorescent AGEs mediated by methylglyoxal. The inhibitory effects increased in a concentration-dependent manner (Figure 3-2). The ethyl acetate fraction had lower EC_{50} (0.169 mg/mL), therefore was more effective than other *F. vesiculosus* fractions. Subsequently, antiglycation activities of four subtractions showed similar antiglycation effects to ethyl acetate fraction and aminoguanidine (Table 3-1). 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 phlorotannin contents.

Methylglyoxal Scavenging Capacity

Methylglyoxal content decreased significantly after incubation with *F. vesiculosus* extract and fractions for 120 min (Figure 3-3). After incubating with butanol fraction, 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. Ethyl acetate fraction and its four subfractions showed the strongest capacity to scavenge methylglyoxal, followed by butanol fraction, acetone extract, dichloromethane fraction and water fraction.

Discussion

The antiglycation activities of *F. vesiculosus* extracts were evaluated in selected models. In BAS-glucose assay, Ethyl-F1 and F2 were more effective than Ethyl-F3 and F4 in preventing protein glycation, whereas no differences were observed among these four subfractions in BSA-MGO assay. Ethyl acetate fraction had high phlorotannin content, which explained its significant inhibitory effects on the formation of AGEs. Butanol fraction had lower phlorotannins content than ethyl acetate fraction, which was consistent with lower antiglycation activity in the BSA-MGO system. BSA-glucose evaluates all stages of protein glycation, while BSA-MGO assay assesses the protein glycation only in the middle stage. The data suggested that phlorotannins of lower molecular weights were more effective than phlorotannins of high molecular weights 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* (*85*).

Methylglyoxal was stable in phosphate buffer. Its level was significantly decreased by *F. vesiculosus* extracts. Ethyl acetate fraction showed the highest methylglyoxal scavenging activity comparing to other fractions. Dichloromethane and water fractions showed the least activities. This data was consistent with their antiglycation activities. Interestingly, Ehtyl-F1 to F4 showed similar methylglyoxal scavenging capacity, 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 in 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 *(86)*. Alternatively, these key intermediates can be generated by the glucose glycoxidation and lipid peroxidation (*45*). 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.

F. vesiculosus phlorotannins had showed reactive oxygen species scavenging capacity. It has been reported that the free radical scavenging activity of phenolic compounds and their inhibitory effects on the formation of AGEs are positively correlated in many plant extracts (*87*). Oxidative stress would accelerate the formation of AGEs. Phlorotannins may inhibit protein glycation by scavenging free radicals.

In conclusion, *F. vesiculosus* acetone extract and its fractions inhibited the formation of AGEs in BSA-glucose and BSA-methylglyoxal models. Ethyl acetate fraction showed the highest antiglycation activities in BSA-glucose, and BSA-methylglyoxal models. Ethyl-F1 and F2 were better antiglycation agents than F3 and F4 in BSA-glucose assay. They appeared to equally active in BSA-methylglyoxal assay. *F. vesiculosus* acetone extract and its fractions scavenged methylglyoxal, suggesting carbonyl scavenging was a major mechanism for protein glycation inhibition.

Table 3-1. EC_{50} of *F. vesiculosus* extracts inhibiting protein glycation in two assays.

F. vesiculosus extracts	EC_{50} (mg/mL)	
	BSA-MGO assay	BSA-Glucose assay
F. vesiculosus acetone extract	0.393±0.0127 b	0.338±0.0146 c, d
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 c, d
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 c, d	0.310±0.0607 c, d

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

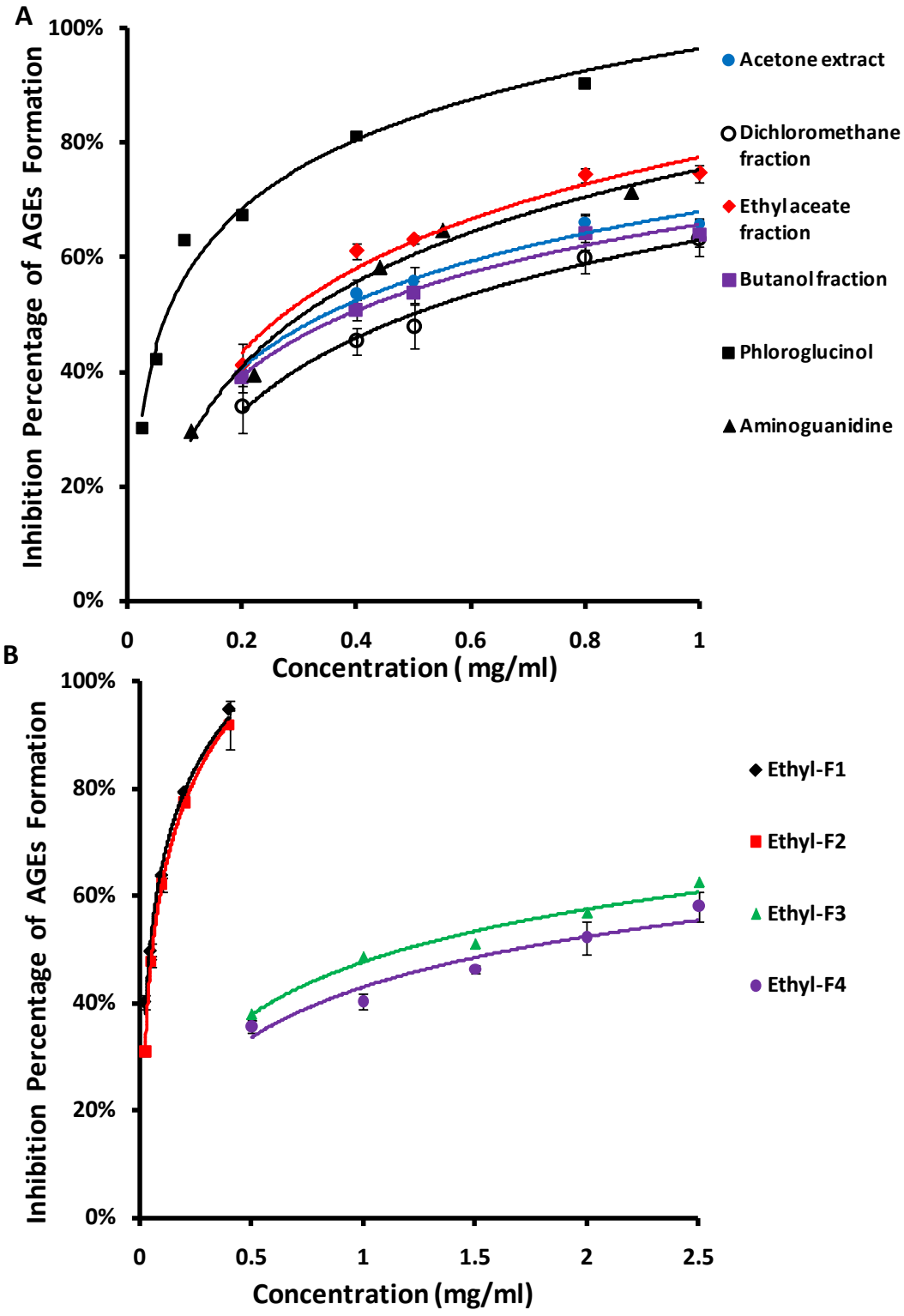


Figure 3-1. Inhibitory effects of *F. vesiculosus* extracts on the formation of AGEs in BSA-glucose assay. Results are means ± standard deviation of triplicate assay.

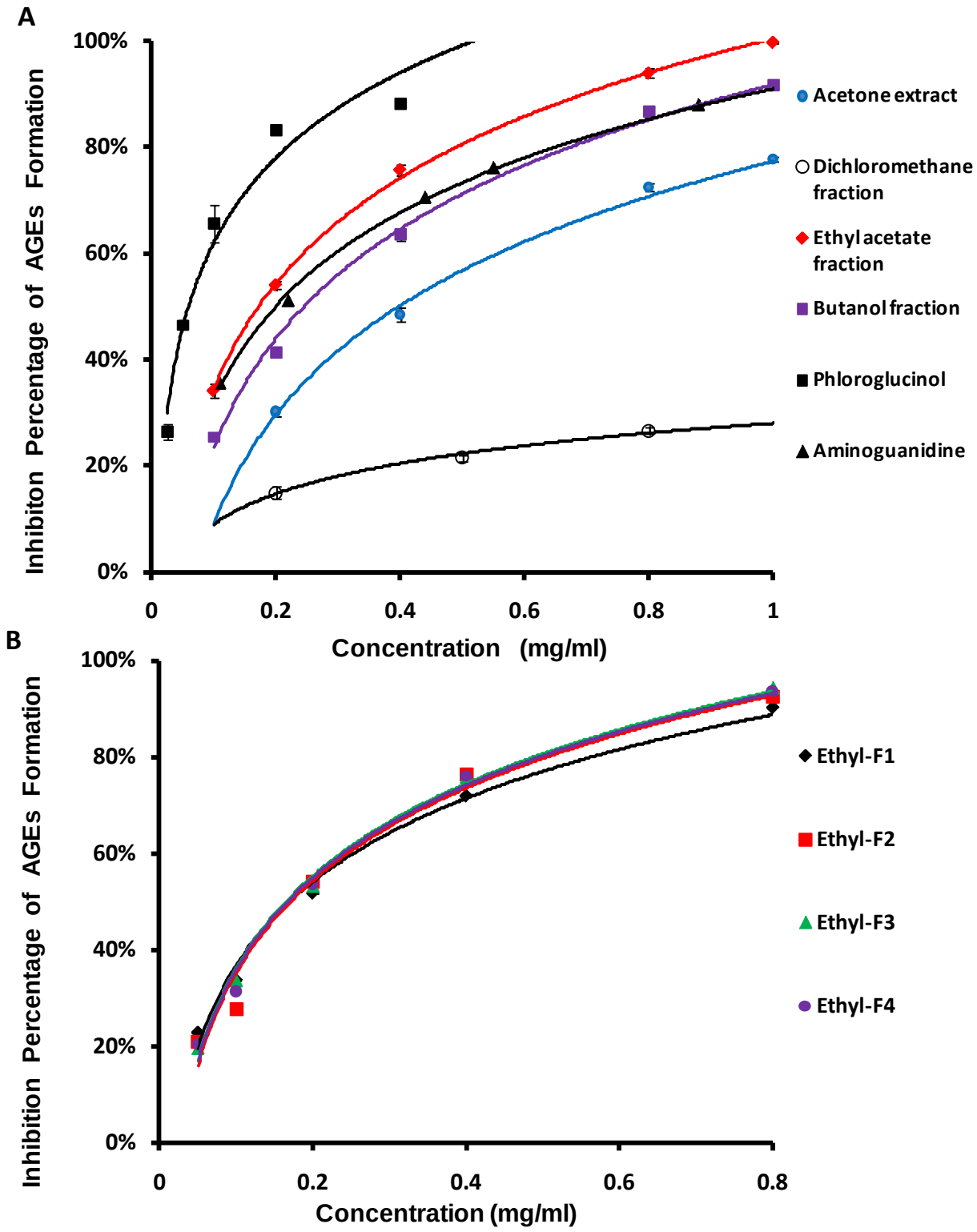


Figure 3-2. Inhibitory effects of *F. vesiculosus* extracts on the formation of AGEs in BSA-methylglyoxal assay. Results are means ± standard deviation of triplicate assay.

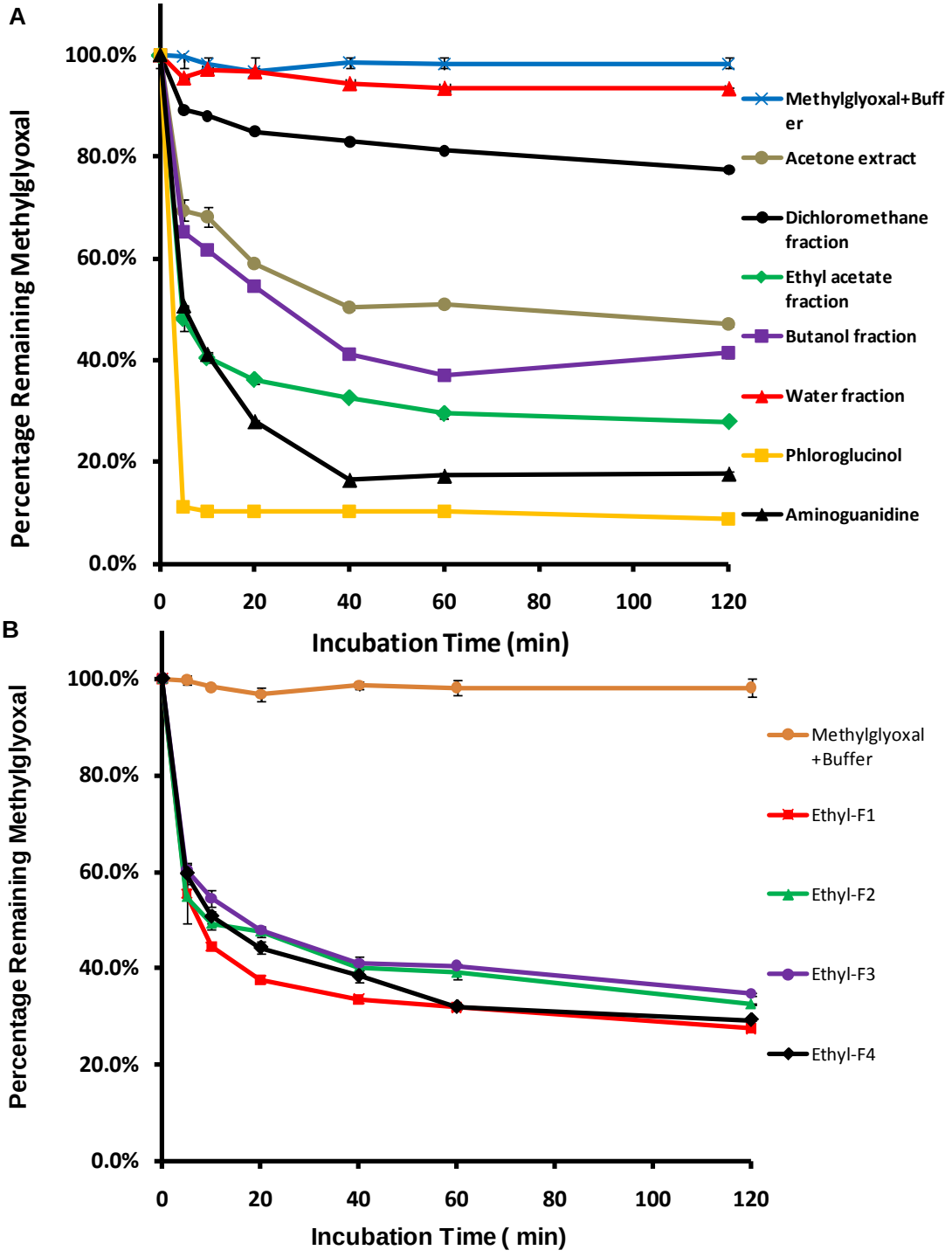


Figure 3-3. The capacity of *F. vesiculosus* extracts to scavenge methylglyoxal. Methylglyoxal (5mM) was incubated with *F. vesiculosus* extracts (1.0 mg/ml). Aminoguanidine and phloroglucinol (5 mM) were used as positive controls. Results are means ± standard deviation of duplicate assay.

CHAPTER 4
HPLC-ESI-MS IDENTIFICATION OF PHLOROGLUCINOL-CARBONYL ADDUCTS

Background

F. vesiculosus extracts showed significant antiglycation activities. Such activities were attributed in part to phlorotannins and their abilities to scavenge reactive carbonyls. Phlorotannins are complex oligomers and polymers that consist of phloroglucinol. The objective of this c hapter was to use phloroglucinol as a model compound to explore the reaction mechanisms and tentatively identify phytochemical-carbonyl adducts on HPLC-ESI-MS^n.

Materials and Methods

Chemicals

Phloroglucinol, glyoxal (40% wt solution) was obtanied from Acros Organics (Morris Plains, NJ). Methylglyoxal (40% aqueous solution) was purchased from MP Biomedicals, LLC (Solon, OH). Other chemicals and materials were described in previous chapters.

Phloroglucinol-Glyoxal/Methylglyoxal Reaction and Adduct Identification

The phloroglucinol-carbonyls reaction was conducted in the phosphate buffer saline (pH 7.4). Phloroglucinol (10 mM) was incubated with methylglyoxal or glyoxal at the concentration of 1 mM at 37°C. After two hours incubation, the adducts were analyzed using HPLC-ESI-MSn 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, Palo Alto, CA). Mobile phases were composed of 0.1%

formic acid in water (phase A) and 0.1% ancetonitrile (phase B). The linear gradient was: 0-20 min, 0-10% B; 20-22 min, 10-17% B; 22-37 min, 17-30% B; 37-40 min, 30-70% B; 40-42min, 70-0% B, followed by 5 min of re-equilibration. The flow rate was 0.25 mL/min. The detection wavelength on 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 ^{o}C. One precursor ion with the highest intensity was isolated and fragmented to obtain the product ion spectra of adducts product ion spectra of adducts.

Results

Phloroglucinol-Glyoxal/Methylglyoxal Adducts Identification. After phloroglucinol was incubated in phosphate buffer for two hours, no apparent degradation was observed (Figure 4-1A). 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 4-1B,C), and their mass spectra are shown in Figure 4-2. Proposed structures of adducts and fragments are illustrated in Figure 4-3.

The first phloroglucinol-glyoxal adduct eluted at 11.2 minutes and had *m/z* 183 $[M-H]^-$. It yielded a product ion at *m/z* 125 that was consistent with phloroglucinol moiety (Figure 4-2A). 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 4-3A. The second adduct at 16.5 minutes gave rise to m*/z* 349 $[M-H]^-$ and product ions at *m/z 331*, 291, 183, and 125 (Figure 4-2B). 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 tentatively identified as a diphloroglucinol-diglyoxal adduct (Figure 4-3B). 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 (*88*). 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 minutes) showed *m/z* 291 $[M-H]^-$ (Figure 4-2C). 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 4-3C).

The first methylglyoxal-phloroglucinol adduct eluted at 20.4 minutes and yielded *m/z* 197 $[M-H]^-$. The fragments at *m/z* 179 and 125 were due to water elimination and phloroglucinol, respectively (Figure 4-2D). It was tentatively identified as monophloroglucinol-monomethylglyoxal adduct (Figure 4-3D). The second methylglyoxal-phloroglucinol adduct eluded at 22.2 minutes and produced *m/z* 269 $[M-H]^-$ (Figure 4-2E). 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 4-3E. 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 ketone group and it also has less steric hindrance during reaction (*82*). Similar observations had been made in methylglyoxal and epicatechin reactions (*88*).

Discussion

It is difficult to study the scavenging capacity of phlorotannins due to its structural complexity. We hypothesized that the antiglycation activities of phlorotannins can be attributed to its constituent unit, the phloroglucinol (1, 3, 5-trihydroxybenzene). Investigating the reaction between phloroglucinol and carbonyls will provide insight about the functions of phlorotannins in carbonyl scavenging.

The electronegativity character of phloroglucinol leads to the nucleophilic addition reaction between phloroglucinol and the aldehyde group. One or two glyxoal molecules attached on to one phloroglucinol molecule to form a monophloroglucinol-monoglyoxal, or monophloroglucinol-diglyoxal adduct. Two phloroglucinol rings were cross-linked by one glyoxal molecule. It has been reported that epicatechin, which includes one phloroglucinol ring, could react with glyoxal at the C-6 or C-8 position (*89*). The cross-linking of two epicatechin molecules by aldehydes has also been reported. We speculated that the reaction began with protonation of glyoxal, which was then attacked by one phloroglucinol molecule through nucleophilic addition to form a monophloroglucinol-monoglyoxal adduct. Then the monomer glyoxal-phloroglucinol intermediate lost one water molecule, leading to a new carbocation (*90-91*). The new carbocation attacked another phloroglucinol molecule to generate an ethyl-linked diphloroglucinol-monoglyoxal adduct.

In the phloroglucinol-methylglyoxal reaction, instead of ketone group, the aldehyde groups of methylglyoxal molecules attached on phloroglucinol molecule to form a

monophloroglucinol-monomethylglyoxal, or monophloroglucinol-dimethylglyoxal adduct. One explanation is that the aldehyde group is more reactive than the ketone group due to its less steric hindrance. The carbon of ketone group is less partial positive charge than that of aldehyde group, which makes the carbonyl carbon as a weaker nucleus *(82)* methylglyoxal reacted with phloroglucinol to generate a series of stereoisomers.

In conclusion, phloroglucinol rapidly reacted with methylglyoxal and glyoxal by forming adducts at pH 7.4. One to three glyoxal molecules attached on one or two phloroglucinol molecules to form six phloroglucinol-glyoxal compounds with different structures. Up to two methylglyoxal molecules attached on one phloroglucinol to generate two phloroglucinol-methylglyoxal adducts. The reaction between phloroglucinol and carbonyls provided insight in the carbonyl scavenging mechanism of phlorotannins.

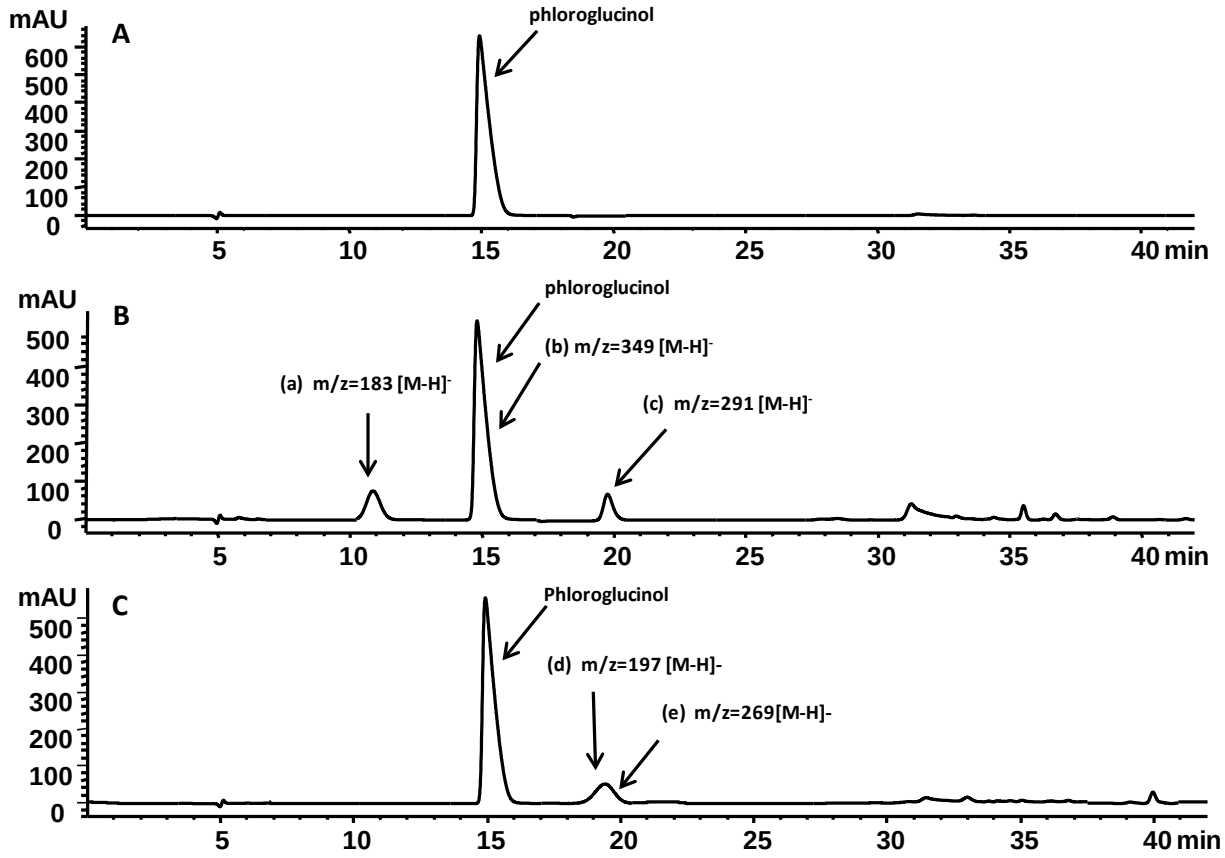


Figure 4-1. HPLC-DAD chromatograms of the phloroglucinol after incubation for two hours. A) Phloroglucinol (10 mM) was incubated with phosphate buffer. B) Phloroglucinol (10 mM) was incubated with 1 mM glyoxal for two hours. C) Phloroglucinol (10 mM) was incubated with 1mM methylglyoxal. Peaks of identified adducts were labeled with their molecular weight.

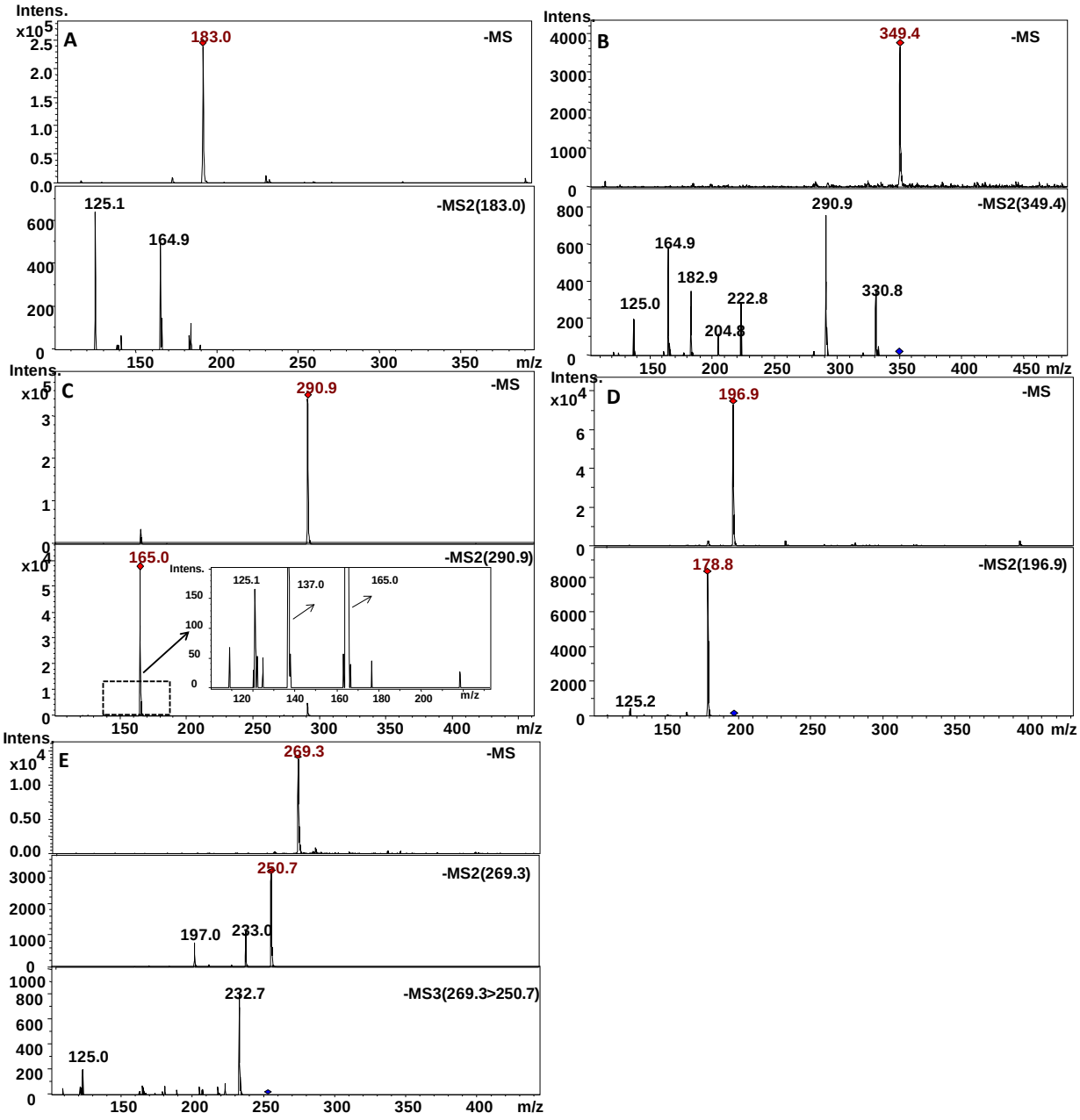


Figure 4-2. MS and MS^2 spectra of phloroglucinol-glyoxal/methyglyoxal adducts peaks. Letter A-E on the figures match those in the Figure 4-1.

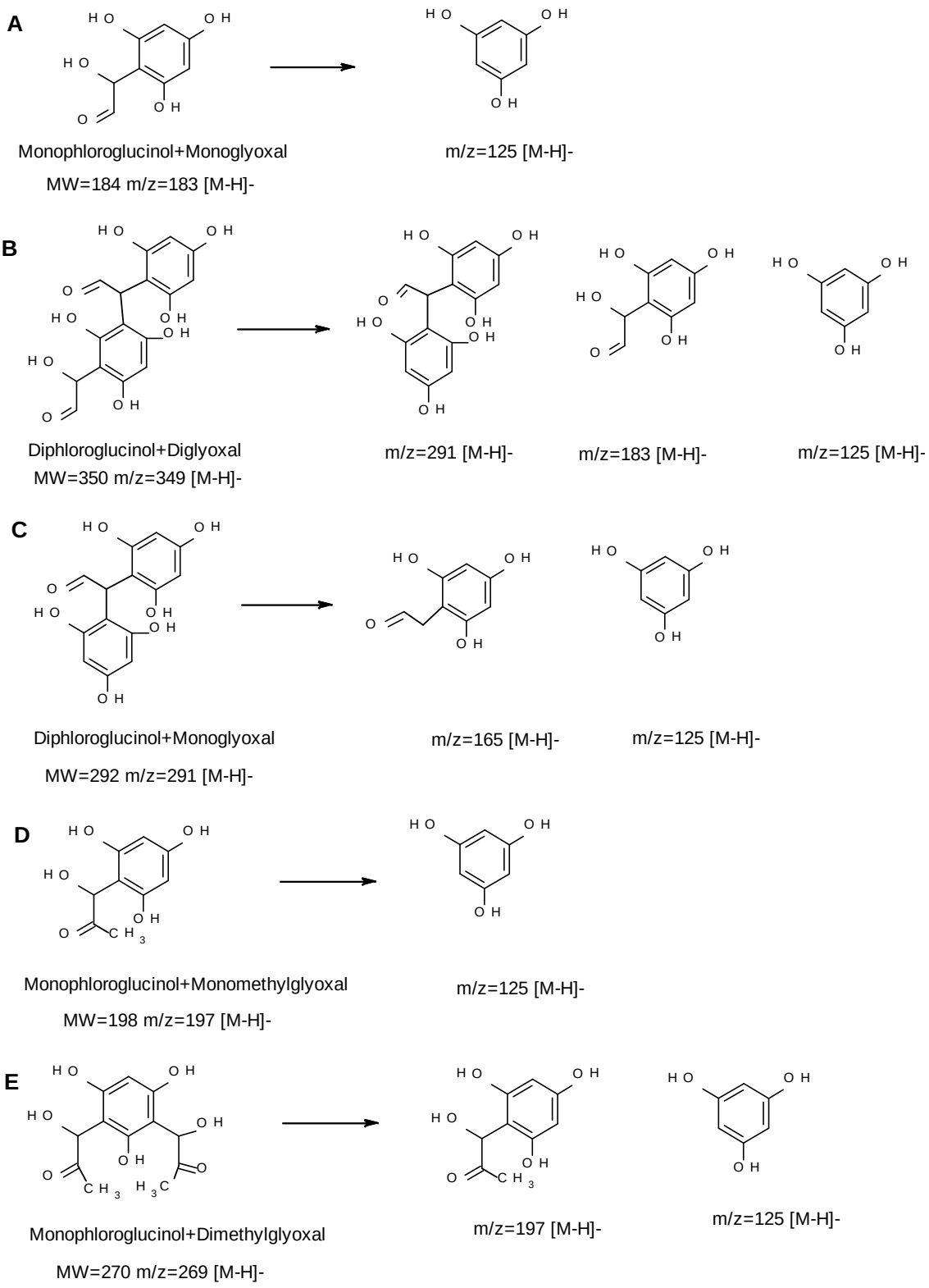


Figure 4-3. The proposed structures of phloroglucinol-glyoxal/methylglyoxal adducts and their product ions. Letter A-E on the figures match those in the Figure 4-1 and Figure 4-2.

CHAPTER 5
CONCLUSION

F. vesiculosus phytochemicals were extracted and fractioned into four fractions. Phlorotannins were concentrated in ethyl acetate and its subfractions. Ethyl-F1 and F2 were found to contain a mixture of oligomers and polymers. Ethyl-F3 and F4 had exclusively polymers. All the *F. vesiculosus* extracts showed high antioxidant capacities, antiglycation activities in BSA-glucose and BSA-methylglyoxal models, and directly scavenged methylglyoxal. The ethyl acetate fraction and its subfractions showed the highest antioxidant, antiglycation and reactive carbonyl scavenging activities. Phloroglucinol, the constitute unit of phlorotannins, rapidly reacted with reactive carbonyls by forming adducts, indicating that the ability of phlorotannins to react with carbonyls was the major mechanism for protein glycation inhibition.

LIST OF REFERENCES

1. Petersen, D. R.; Doorn, J. A. Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radical Biology and Medicine.* **2004,** 37, 937-945.

2. Yuan, Y. V.; Walsh, N. A. Antioxidant and antiproliferative activities of extracts from a variety of edible seaweeds. *Food and Chemical Toxicology.* **2006,** 44, 1144-1150.

3. Jiménez-Escrig, A.; Jiménez-Jiménez, I.; Pulido, R.; Saura-Calixto, F. Antioxidant activity of fresh and processed edible seaweeds. *Journal of the Science of Food and Agriculture.* **2001,** 81, 530-534.

4. Teas, J.; Harbison, M. L.; Gelman, R. S. Dietary Seaweed (*Laminaria*) and Mammary Carcinogenesis in Rats. *Cancer Res.* **1984,** 44, 2758-2761.

5. Yamamoto, I.; Maruyama, H.; Moriguchi, M. The effect of dietary seaweeds on 7,12-dimethylbenz[a]anthracene-induced mammary tumorigenesis in rats. *Cancer Letters.* **1987,** 35, 109-118.

6. Yamamoto, I.; Maruyama, H. Effect of dietary seaweed preparations on 1,2-dimethylhydrazine-induced intestinal carcinogenesis in rats. *Cancer Letters.* **1985,** 26, 241-251.

7. Lee, E. J.; Sung, M. K. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by seatangle, a fiber-rich seaweed. *Plant Foods for Human Nutrition (Formerly Qualitas Plantarum).* **2003,** 58, 1-8.

8. Yamamoto, I.; Maruyama, H.; Takahashi, M.; Komiyama, K. The effect of dietary or intraperitoneally injected seaweed preparations on the growth of Sarcoma-180 cells subcutaneously implanted into mice. *Cancer Letters.* **1986,** 30, 125-131.

9. Hiqashi-Okaj, K.; Otani, S.; Okai, Y. Potent suppressive effect of a Japanese edible seaweed, Enteromorpha prolifera (Sujiao-nori) on initiation and promotion phases of chemically induced mouse skin tumorigenesis. *Cancer Letters.* **1999,** 140, 21-25.

10. Reddy, B. S.; Sharma, C.; Mathews, L. Effect of Japanese seaweed (Laminaria angustata) extracts on the mutagenicity of 7,12-dimethylbenz[a]anthracene, a breast carcinogen, and of 3,2'-dimethyl-4-aminobiphenyl, a colon and breast carcinogen. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis.* **1984,** 127, 113-118.

11. Berge, J. P.; Debiton, E.; Dumay, J.; Durand, P.; Barthomeuf, C. In Vitro Anti-inflammatory and Anti-proliferative Activity of Sulfolipids from the Red Alga Porphyridium cruentum. *Journal of Agricultural and Food Chemistry.* **2002,** 50, 6227-6232.

12. Awad, N. E.; Selim, M. A.; Saleh, M. M.; Matloub, A. A. Seasonal variation of the lipoidal matters and hypolipidaemic activity of the red alga *Corallina officinalis* L. *Phytotherapy Research.* **2003,** 17, 19-25.

13. Brussaard, J.; van Raaij, J.; Stasse-Wolthuis, M.; Katan, M.; Hautvast, J. Blood pressure and diet in normotensive volunteers: absence of an effect of dietary fiber, protein, or fat. *Am J Clin Nutr.* **1981,** 34, 2023-2029.

14. Fujihara, M.; Nagumo, T. An influence of the structure of alginate on the chemotactic activity of macrophages and the antitumor activity. *Carbohydrate Research.* **1993,** 243, 211-216.

15. Duan, X.-J.; Zhang, W.-W.; Li, X.-M.; Wang, B.-G. Evaluation of antioxidant property of extract and fractions obtained from a red alga, Polysiphonia urceolata. *Food Chemistry.* **2006,** 95, 37-43.

16. Huang, H.-L.; Wang, B.-G. Antioxidant Capacity and Lipophilic Content of Seaweeds Collected from the Qingdao Coastline. *Journal of Agricultural and Food Chemistry.* **2004,** 52, 4993-4997.

17. Kuda, T.; Tsunekawa, M.; Hishi, T.; Araki, Y. Antioxidant properties of dried '*kayamo-nori*', a brown alga Scytosiphon lomentaria (*Scytosiphonales, Phaeophyceae*). *Food Chemistry.* **2005,** 89, 617-622.

18. Zhang, W.-W.; Duan, X.-J.; Huang, H.-L.; Zhang, Y.; Wang, B.-G. Evaluation of 28 marine algae from the Qingdao coast for antioxidative capacity and determination of antioxidant efficiency and total phenolic content of fractions and subfractions derived from Symphyocladia latiuscula (*Rhodomelaceae*). *Journal of Applied Phycology.* **2007,** 19, 97-108.

19. Zhang, Q.; Li, N.; Liu, X.; Zhao, Z.; Li, Z.; Xu, Z. The structure of a sulfated galactan from Porphyra haitanensis and its in vivo antioxidant activity. *Carbohydrate Research.* **2004,** 339, 105-111.

20. Yoshie-Stark, Y.; Hsieh, Y. P.; Suzuki, T. Distribution of flavonoids and related compounds from seaweeds in Japan. *Journal-Tokyo University of Fisheries.* **2003,** 89, 1-6.

21. Koivikko, R.; Loponen, J.; Pihlaja, K.; Jormalainen, V. High-performance liquid chromatographic analysis of phlorotannins from the brown alga *Fucus Vesiculosus*. *Phytochemical Analysis.* **2007,** 18, 326-332.

22. Jormalainen, V.; Honkanen, T. Variation in natural selection for growth and phlorotannins in the brown alga *Fucus vesiculosus*. *Journal of Evolutionary Biology.* **2004,** 17, 807-820.

23. Wang, T.; Jónsdóttir, R.; Ólafsdóttir, G. Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chemistry.* **2009,** 116, 240-248.

24. Parys, S.; Kehraus, S.; Krick, A.; Glombitza, K. W.; Carmeli, S.; Klimo, K.; Gerhäuser, C.; König, G. M. In vitro chemopreventive potential of fucophlorethols from the brown alga Fucus vesiculosus L. by anti-oxidant activity and inhibition of selected cytochrome P450 enzymes. *Phytochemistry.* **2010,** 71, 221-229.

25. Sandsdalen, E.; Haug, T.; Stensvåg, K.; Styrvold, O. B. The antibacterial effect of a polyhydroxylated fucophlorethol from the marine brown alga, Fucus vesiculosus. *World Journal of Microbiology and Biotechnology.* **2003,** 19, 777-782.

26. Sugiura, Y.; Matsuda, K.; Yamada, Y.; Nishikawa, M.; Shioya, K.; Katsuzaki, H.; Imai, K.; Amano, H. Isolation of a new anti-allergic phlorotannin, phlorofucofuroeckol-B, from an edible brown alga, Eisenia arborea. *Bioscience, biotechnology, and biochemistry.* **2006,** 70, 2807-2811.

27. Jung, H.; Yoon, N.; Woo, M.-H.; Choi, J. Inhibitory activities of extracts from several kinds of seaweeds and phlorotannins from the brown alga Ecklonia stolonifera on glucose-mediated protein damage and rat lens aldose reductase. *Fisheries Science.* **2008,** 74, 1363-1365.

28. Kang, H.; Chung, H.; Kim, J.; Son, B.; Jung, H.; Choi, J. Inhibitory phlorotannins from the edible brown alga ecklonia stolonifera on total reactive oxygen species (ROS) generation. *Archives of Pharmacal Research.* **2004,** 27, 194-198.

29. Mazumder, S.; Ghosal, P.; Pujol, C.; Carlucci, M.; Damonte, E.; Ray, B. Isolation, chemical investigation and antiviral activity of polysaccharides from Gracilaria corticata *(Gracilariaceae, Rhodophyta*). *Int J Biol Macromol.* **2002,** 31, 87 - 95.

30. Kapadia, G. J.; Tokuda, H.; Konoshima, T.; Takasaki, M.; Takayasu, J.; Nishino, H. Anti-tumor promoting activity of Dryopteris phlorophenone derivatives. *Cancer Letters.* **1996,** 105, 161-165.

31. Nakane, H.; Arisawa, M.; Fujita, A.; Koshimura, S.; Ono, K. Inhibition of HIV-reverse transcriptase activity by some phloroglucinol derivatives. *FEBS Letters.* **1991,** 286, 83-85.

32. Ishii, R.; Horie, M.; Saito, K.; Arisawa, M.; Susumu, K. Inhibitory effects of phloroglucinol derivatives from Mallotus japonicus on nitric oxide production by a murine macrophage-like cell line, RAW 264.7, activated by lipopolysaccharide and interferon-[gamma]. *Biochimica et Biophysica Acta (BBA) - General Subjects.* **2001,** 1568, 74-82.

33. Shibata, T.; Ishimaru, K.; Kawaguchi, S.; Yoshikawa, H.; Hama, Y. Antioxidant activities of phlorotannins isolated from Japanese Laminariaceae. *Journal of Applied Phycology.* **2008,** 20, 705-711.

34. Li, Y.; Qian, Z.-J.; Ryu, B.; Lee, S.-H.; Kim, M.-M.; Kim, S.-K. Chemical components and its antioxidant properties in vitro: An edible marine brown alga, *Ecklonia cava*. *Bioorganic & Medicinal Chemistry.* **2009,** 17, 1963-1973.

35. Jung, H. A.; Yoon, N. Y.; Woo, M. H.; Choi, J. S. Inhibitory activities of extracts from several kinds of seaweeds and phlorotannins from the brown alga *Ecklonia stolonifera* on glucose-mediated protein damage and rat lens aldose reductase. *Fisheries Science.* **2008,** 74, 1363-1365.

36. Akoh, C. C.; Min, D. B., *Food lipids: chemistry, nutrition, and biotechnology*. CRC press: 2002.

37. Sachindra, N. M.; Sato, E.; Maeda, H.; Hosokawa, M.; Niwano, Y.; Kohno, M.; Miyashita, K. Radical Scavenging and Singlet Oxygen Quenching Activity of Marine Carotenoid Fucoxanthin and Its Metabolites. *Journal of Agricultural and Food Chemistry.* **2007,** 55, 8516-8522.

38. Yan, X.; Chuda, Y.; Suzuki, M.; Nagata, T. Fucoxanthin as the Major Antioxidant in Hijikia fusiformis, a Common Edible Seaweed. *Bioscience, Biotechnology, and Biochemistry.* **1999,** 63, 605-607.

39. Zhao, X.; Xue, C.-H.; Li, B.-F. Study of antioxidant activities of sulfated polysaccharides from Laminaria japonica. *Journal of Applied Phycology.* **2008,** 20, 431-436.

40. Shiratori, K.; Ohgami, K.; Ilieva, I.; Jin, X.-H.; Koyama, Y.; Miyashita, K.; Yoshida, K.; Kase, S.; Ohno, S. Effects of fucoxanthin on lipopolysaccharide-induced inflammation in vitro and in vivo. *Experimental Eye Research.* **2005,** 81, 422-428.

41. Maeda, H.; Hosokawa, M.; Sashima, T.; Funayama, K.; Miyashita, K. Fucoxanthin from edible seaweed, Undaria pinnatifida, shows antiobesity effect through UCP1 expression in white adipose tissues. *Biochemical and Biophysical Research Communications.* **2005,** 332, 392-397.

42. Ookubo, M.; Matsuno, T. Carotenoids of sea squirts--II. Comparative biochemical studies of carotenoids in sea squirts. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.* **1985,** 81, 137-141.

43. Partali, V.; Tangen, K.; Liaaen-Jensen, S. Carotenoids in food chain studies--III. Resorption and metabolic transformation of carotenoids in Mytilus edulis (Edible mussel). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.* **1989,** 92, 239-246.

44. Sugawara, T.; Baskaran, V.; Tsuzuki, W.; Nagao, A. Brown Algae Fucoxanthin Is Hydrolyzed to Fucoxanthinol during Absorption by Caco-2 Human Intestinal Cells and Mice. *J. Nutr.* **2002,** 132, 946-951.

45. Negre Salvayre, A.; Coatrieux, C.; Ingueneau, C.; Salvayre, R. Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors. *British journal of pharmacology.* **2008,** 153, 6-20.

46. Smit, A. J.; Lutgers, H. The clinical relevance of advanced glycation endproducts (AGE) and recent developments in pharmaceutics to reduce AGE accumulation. *Current medicinal chemistry.* **2004,** 11, 2767-2784.

47. Dalle-Donne, I.; Giustarini, D.; Colombo, R.; Rossi, R.; Milzani, A. Protein carbonylation in human diseases. *Trends in molecular medicine.* **2003,** 9, 169-176.

48. Arribas-Lorenzo, G.; Morales, F. J. Analysis, Distribution, and Dietary Exposure of Glyoxal and Methylglyoxal in Cookies and Their Relationship with Other Heat-Induced Contaminants. *Journal of agricultural and food chemistry.* **2010,** 58, 2966-2972.

49. Nagao, M.; Fujita, Y.; Sugimura, T.; Kosuge, T. Methylglyoxal in beverages and foods: its mutagenicity and carcinogenicity. *IARC scientific publications.* **1986**, 283.

50. Tan, D.; Wang, Y.; Lo, C. Y.; Sang, S.; Ho, C. T. Methylglyoxal: its presence in beverages and potential scavengers. *Annals of the New York Academy of Sciences.* **2008,** 1126, 72-75.

51. Hayashi, T.; Shibamoto, T. Analysis of methyl glyoxal in foods and beverages. *Journal of agricultural and food chemistry.* **1985,** 33, 1090-1093.

52. Fujioka, K.; Shibamoto, T. Formation of genotoxic dicarbonyl compounds in dietary oils upon oxidation. *Lipids.* **2004,** 39, 481-486.

53. O'Brien, P. J.; Siraki, A. G.; Shangari, N. Aldehyde sources, metabolism, molecular toxicity mechanisms, and possible effects on human health. *CRC Critical Reviews in Toxicology.* **2005,** 35, 609-662.

54. Cho, S. J.; Roman, G.; Yeboah, F.; Konishi, Y. The road to advanced glycation end products: a mechanistic perspective. *Current medicinal chemistry.* **2007,** 14, 1653-1671.

55. Peyroux, J.; Sternberg, M. Advanced glycation endproducts (AGEs): pharmacological inhibition in diabetes. *Pathologie Biologie.* **2006,** 54, 405-419.

56. Bondoc, F. Y.; Bao, Z.; Hu, W.-Y.; Gonzalez, F. J.; Wang, Y.; Yang, C. S.; Hong, J.-Y. Acetone catabolism by cytochrome P450 2E1: studies with CYP2E1-null mice. *Biochemical Pharmacology.* **1999,** 58, 461-463.

57. Podrez, E. A.; Abu-Soud, H. M.; Hazen, S. L. Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radical Biology and Medicine.* **2000,** 28, 1717-1725.

58. Singh, R.; Barden, A.; Mori, T.; Beilin, L. Advanced glycation end-products: a review. *Diabetologia.* **2001,** 44, 129-146.

59. Neglia, C. I.; Cohen, H.; Garber, A.; Ellis, P.; Thorpe, S.; Baynes, J. 13C NMR investigation of nonenzymatic glucosylation of protein. Model studies using RNase A. *Journal of Biological Chemistry.* **1983,** 258, 14279.

60. Burn, J. Advances in Pharmacology. *British Medical Journal.* **1957,** 1, 149.

61. Glomb, M. A.; Monnier, V. M. Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. *Journal of Biological Chemistry.* **1995,** 270, 10017.

62. Ahmed, M. U.; Frye, E. B.; Degenhardt, T. P.; Thorpe, S. R.; Baynes, J. W. N-epsilon-(carboxyethyl) lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochemical Journal.* **1997,** 324, 565.

63. Degenhardt, T.; Thorpe, S.; Baynes, J. Chemical modification of proteins by methylglyoxal. *Cellular and molecular biology (Noisy-le-Grand, France).* **1998,** 44, 1139.

64. Brinkmann, E.; Wells-Knecht, K. J.; Thorpe, S. R.; Baynes, J. W. Characterization of an imidazolium compound formed by reaction of methylglyoxal and N [small alpha]-hippuryllysine. *Journal of the Chemical Society, Perkin Transactions 1.* **1995**, 2817-2818.

65. Nakayama, T.; Hayase, F.; Kato, H. Formation of -(2-Formyl-5-hydroxy-methyl-pyrrol-1-yl)-L-norleucine in the Maillard Reaction be-tween D-Glucose and L-Lysine. *Agricultural and Biological Chemistry.* **1980,** 44, 1201-1202.

66. Hayase, F.; Nagaraj, R.; Miyata, S.; Njoroge, F.; Monnier, V. Aging of proteins: immunological detection of a glucose-derived pyrrole formed during maillard reaction in vivo. *Journal of Biological Chemistry.* **1989,** 264, 3758.

67. Ahmed, N. Advanced glycation endproducts--role in pathology of diabetic complications. *Diabetes research and clinical practice.* **2005,** 67, 3-21.

68. Münch, G.; Thome, J.; Foley, P.; Schinzel, R.; Riederer, P. Advanced glycation endproducts in ageing and Alzheimer's disease. *Brain Research Reviews.* **1997,** 23, 134-143.

69. Raj, D.; Choudhury, D.; Welbourne, T.; Levi, M. Advanced glycation end products: a nephrologist's perspective. *American Journal of Kidney Diseases.* **2000,** 35, 365-380.

70. Thornalley, P. J.; Yurek-George, A.; Argirov, O. K. Kinetics and mechanism of the reaction of aminoguanidine with the [alpha]-oxoaldehydes glyoxal, methylglyoxal, and 3-deoxyglucosone under physiological conditions. *Biochemical pharmacology.* **2000,** 60, 55-65.

71. Voziyan, P. A.; Khalifah, R. G.; Thibaudeau, C.; Yildiz, A.; Jacob, J.; Serianni, A. S.; Hudson, B. G. Modification of proteins in vitro by physiological levels of glucose. *Journal of Biological Chemistry.* **2003,** 278, 46616.

72. Peng, X.; Cheng, K.-W.; Ma, J.; Chen, B.; Ho, C.-T.; Lo, C.; Chen, F.; Wang, M. Cinnamon Bark Proanthocyanidins as Reactive Carbonyl Scavengers To Prevent the Formation of Advanced Glycation Endproducts. *Journal of Agricultural and Food Chemistry.* **2008,** 56, 1907-1911.

73. Peng, X.; Zheng, Z.; Cheng, K.-W.; Shan, F.; Ren, G.-X.; Chen, F.; Wang, M. Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation endproducts. *Food Chemistry.* **2008,** 106, 475-481.

74. Tan, D.; Wang, Y.; Lo, C. Y.; Sang, S.; Ho, C. T. Methylglyoxal: its presence in beverages and potential scavengers. *Ann N Y Acad Sci.* **2008,** 1126, 72-5.

75. Zhu, Q.; Liang, C. P.; Cheng, K. W.; Peng, X.; Lo, C. Y.; Shahidi, F.; Chen, F.; Ho, C. T.; Wang, M. Trapping effects of green and black tea extracts on peroxidation-derived carbonyl substances of seal blubber oil. *J Agric Food Chem.* **2009,** 57, 1065-9.

76. Singleton, V.; Rossi Jr, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture.* **1965,** 16, 144.

77. Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of agricultural and food chemistry.* **2002,** 50, 4437-4444.

78. Parys, S.; Rosenbaum, A.; Kehraus, S.; Reher, G.; Glombitza, K. W.; König, G. M. Evaluation of quantitative methods for the determination of polyphenols in algal extracts. *Journal of Natural Products.* **2007,** 70, 1865-1870.

79. Wu, C. H.; Yen, G. C. Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. *Journal of agricultural and food chemistry.* **2005,** 53, 3167-3173.

80. Kang, H. S.; Chung, H. Y.; Kim, J. Y.; Son, B. W.; Jung, H. A.; Choi, J. S. Inhibitory phlorotannins from the edible brown alga Ecklonia stolonifera on total reactive oxygen species (ROS) generation. *Archives of pharmacal research.* **2004,** 27, 194-198.

81. Ahn, G.-N.; Kim, K.-N.; Cha, S.-H.; Song, C.-B.; Lee, J.; Heo, M.-S.; Yeo, I.-K.; Lee, N.-H.; Jee, Y.-H.; Kim, J.-S.; Heu, M.-S.; Jeon, Y.-J. Antioxidant activities of phlorotannins purified from Ecklonia cava on free radical scavenging using ESR and H_2O_2-mediated DNA damage. *European Food Research and Technology A.* **2007,** 226, 71-79.

82. March, J., *Advanced organic chemistry*. Wiley New York:: 1992; Vol. 283.

83. Koivikko, R. Brown algal phlorotannins: Improving and applying chemical methods. **2008**.

84. Peng, Z.; Hayasaka, Y.; Iland, P. G.; Sefton, M.; Høj, P.; Waters, E. J. Quantitative analysis of polymeric procyanidins (tannins) from grape (Vitis vinifera) seeds by reverse phase high-performance liquid chromatography. *Journal of agricultural and food chemistry.* **2001,** 49, 26-31.

85. Okada, Y.; Ishimaru, A.; Suzuki, R.; Okuyama, T. A New Phloroglucinol Derivative from the Brown Alga Eisenia bicyclis: Potential for the Effective Treatment of Diabetic Complications. *Journal of Natural Products.* **2003,** 67, 103-105.

86. Thornalley, P.; Langborg, A.; Minhas, H. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochemical Journal.* **1999,** 344, 109.

87. Peng, X.; Zheng, Z.; Cheng, K. W.; Shan, F.; Ren, G. X.; Chen, F.; Wang, M. Inhibitory effect of mung bean extract and its constituents vitexin and isovitexin on the formation of advanced glycation endproducts. *Food Chemistry.* **2008,** 106, 475-481.

88. Liu, H.; Liu, H.; Wang, W.; Khoo, C.; Taylor, J.; Gu, L. Cranberry phytochemicals inhibit glycation of human hemoglobin and serum albumin by scavenging reactive carbonyls. *Food & Function.* **2011,** 2, 475-482.

89. Totlani, V. M.; Peterson, D. G. Epicatechin carbonyl-trapping reactions in aqueous maillard systems: identification and structural elucidation. *Journal of agricultural and food chemistry.* **2006,** 54, 7311-7318.

90. Es-Safi, N. E.; Cheynier, V.; Moutounet, M. Role of aldehydic derivatives in the condensation of phenolic compounds with emphasis on the sensorial properties of fruit-derived foods. *Journal of agricultural and food chemistry.* **2002,** 50, 5571-5585.

91. Timberlake, C.; Bridle, P. Interactions between anthocyanins, phenolic compounds, and acetaldehyde and their significance in red wines. *American Journal of Enology and Viticulture.* **1976,** 27, 97.

BIOGRAPHICAL SKETCH

Haiyan Liu was from Xi'an, China. She received her bachelor's degree in food safety and security from China Agricultural University in 2008. After that she was admitted as a master's student in the Food Science and Human Nutrition Department at the University of Florida. In graduate school, Haiyan presented her research at the IFT annual meeting in 2010. Her most recent research has resulted in two abstracts that were submitted to Experimental Biology and IFT annual meeting in 2011. Further, she received the William L. and Agnes F. Brown Graduate Scholarship from UF in 2011. Upon her completion of the master's degree in 2011, Haiyan plans to continue her study in the food science major and hopefully pursue the doctorate degree in the future.