ABSTRACT

LIU, XIN. Extraction and Antioxidant Activity of Phlorotannins from Edible Brown Algae. (Under the direction of Dr. Wenqiao Yuan).

Bioactive compounds of marine organisms have gained attention because of their various bioactivities and abundance in ecosystems. Among these organisms, marine brown algae are a potent source of bioactive compounds such as phlorotannins, polysaccharides, unsaturated fatty acids, and proteins. Phlorotannins are polyphenolic compounds that are only found in brown algae and are known for various bioactivities, e.g. antioxidant, antimicrobial, anticancer, anti-diabetes, anti-HIV, etc. Thus they have a great potential to be applied as medicine or nutraceuticals. Solvent extraction is the most widely used method for phlorotannin extraction in the literature and different solvent extraction methods have been applied in previous research. However, the effectiveness for phlorotannin extraction and antioxidant activity has not been studied among different extraction methods. Phlorotannins can be divided into three groups, soluble phlorotannins (SPs), membane-bound phlorotannins (MPs) and exuded phlorotannins based on their location in brown algae cells. The SPs have been extensively investigated but the MPs were only reported in a few studies. Meanwhile, the five edible brown algae species *Ascophyllum nodosum, Fucus vesiculosus, Alaria esculenta, Laminaria digitata,* and *Saccharina latissima* investigated in this project were not fully studied. In this project, two widely used solvent extraction methods were applied and compared for their effectiveness in phlorotannin extraction and effects on the antioxidant activity of the extracts. Total Phlorotannin Content (TPC) and antioxidant activity of the three phlorotannin groups were also evaluated and compared. This project was focused on understanding the effect of extraction method as well as the algal species and phlorotannin groups on antioxidant production from brown algae.

The objectives of the first part of this project were to evaluate the contents of soluble and membrane-bound phlorotannins in five brown macroalgae and examine the influence of extraction methods on phlorotannin yield. Two solvent-extraction methods were first utilized to obtain SPs. The leftover algae powder was then treated with sodium hydroxide to release the MPs. Folin-Ciocalteu method using phloroglucinol (1,3,5-benzenetriol) as the standard was applied to quantify SP and MP contents. MP samples were further purified with solvents to obtain the extracted MP (eMP). *F. vesiculosus* and *A. nodosum* showed the highest SP content (4.66 and 4.90 mg/g algae, respectively) by the first extraction method. The highest MP content was observed in *A. nodosum* (68.55 mg/g algae) followed by *F. vesiculosus* (62.58 mg/g algae). For every algal species tested, the content of MP was greater than eMP, suggesting the great potential for harvesting value-added products from the leftover algae powder after SP extraction. The two algal species were then extracted by the second extraction method. SP yields from *A. nodosum* and *F. vesiculosus* were 6.59 $\pm$ 0.77 and 3.07 $\pm$ 0.48 mg/g algae, respectively. Method 1 was better on F. vesiculosus but method 2 was better on *A. nodosum* in terms of SP yield, indicating that the effectiveness of extraction methods was algal species dependent.

The objective of the second part of this project was to evaluate the antioxidant activity of the phlorotannins extracted from the five algae species. The DPPH radical scavenging activity was applied to evaluate the antioxidant activity of phlorotanins. The yields and antioxidant activity of *F. vesiculosus* (yield = 14.83 $\pm$ 3.67 mg-extract/g-algae, IC_{50} = 0.0038 $\pm$ 0.0002 mg/ml) and *A. nodosum* (yield = 12.80 $\pm$ 1.37 mg-extract/g-algae, IC_{50} = 0.0072 $\pm$ 0.0010 mg/ml) were the highest among the five algae species. Moreover, the SP, MP and eMP extracts of *F. vesiculosus* and *A. nodosum* showed equal or higher DPPH

radical scavenging activity than commercial antioxidants butylated hydroxytoluene (BHT) (IC_{50} = 0.051 ± 0.0005 mg/ml) and ascorbic acid (IC_{50} = 0.0063 ± 0.0002 mg/ml). Different extraction processes also showed effects on the antioxidant activity of the phlorotannin extracts.

Extraction and Antioxidant Activity of Phlorotannins from Edible Brown Algae

by
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DEDICATION

This work is dedicated to my parents, Mr. Xiaonan Liu and Mrs. Tongxiu Zhang, for their genuine love, encourage and support. And to my grandparents in heaven, Mr. Jingtang Liu and Mrs. Binghua Zhang, who gave me the most precious love and encouraged me to pursue my dream of studying abroad.

BIOGRAPHY

Xin Liu was born in Zibo, Shandong, China in 1990. She received her bachelor's degree from Harbin Institute of Technology at Weihai in 2012, majored in Biotechnology. In August 2013 she joined the research group of Dr. Wenqiao Yuan in Department of Biological and Agricultural Engineering, North Carolina State University, and began her graduate study as a master student.

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CHAPTER 1 Introduction and Literature Review

1.1 Introduction

Searching natural compounds with health-beneficial properties is always a hot issue in biological and medical science. Many terrestrial phytochemicals have been approved to have health-promoting functions such as antioxidant, antimicrobial, anticancer, anti-diabetes, etc. However, the bioactive agents of terrestrial ecosystems have been investigated and applied for a long time; the marine ecosystem is expected to be a new source of therapeutic and nutraceutical agents. The ocean is an important source of nutritious foods, e.g. fish, shellfish, and seaweed. Marine organisms, which comprise half of the total biodiversity, are exposed to more extreme conditions than terrestrial organisms due to the larger surface of the ocean (Li et al., 2011). In order to survive the extreme conditions, e.g. ultraviolet, extreme salinity levels, temperature variations, low light intensities and nutrient deficient habitats (Steevensz et al., 2012), marine organisms generate a variety of bioactive compounds to protect themselves. Since the 1970s, over 21,000 natural compounds with diverse bioactivities have been found from marine algae, microbes, and invertebrates ([a]Eom et al., 2012). Recently, values of bioactive compounds from marine organisms have been revealed and emphasized.

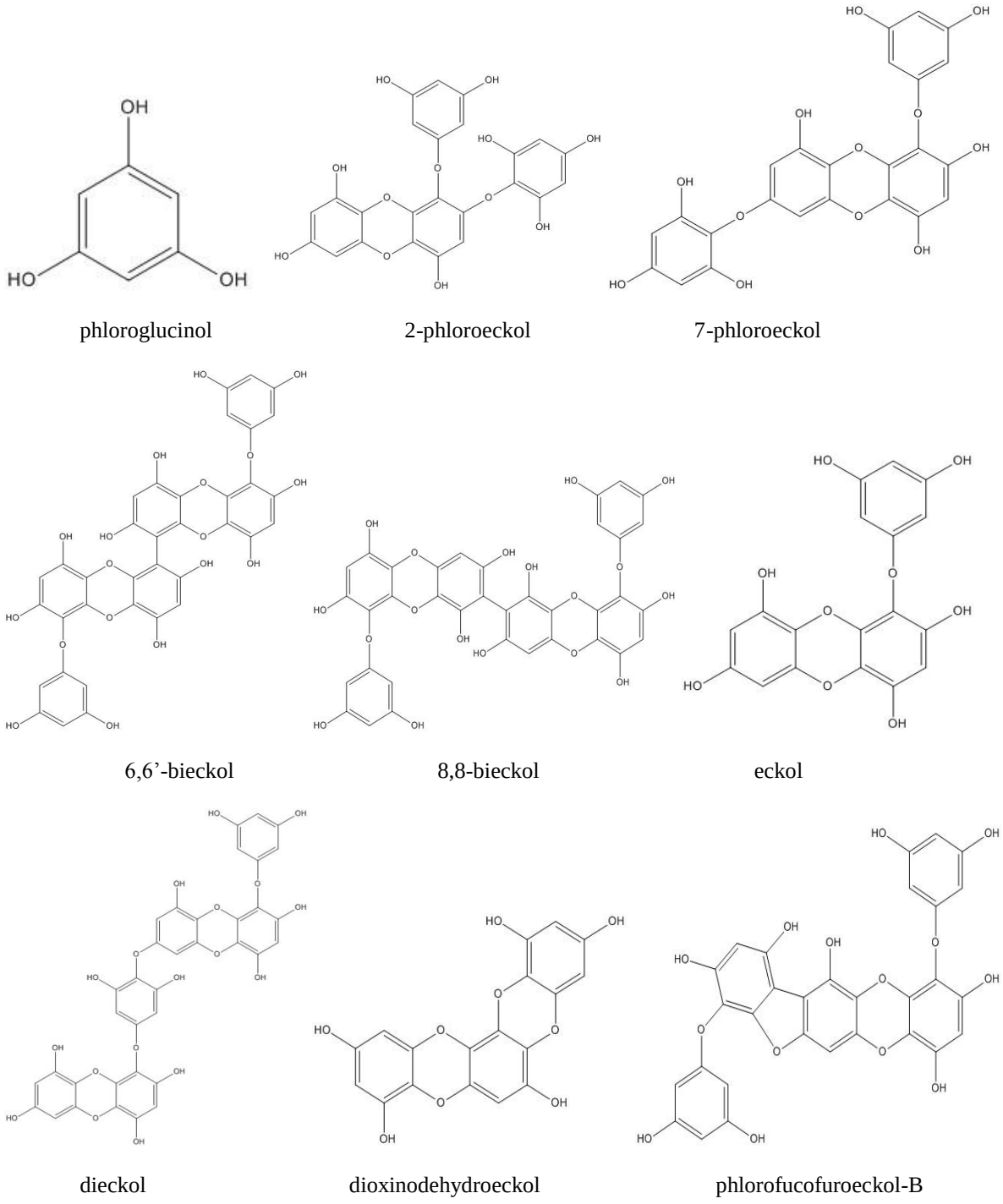
Among all the marine organisms, edible seaweeds are considered as under-exploited sources of functional food and pharmacologically active metabolites ([a]Eom et al., 2012). Seaweeds are macroscopic, multicellular marine macroalgae that grow on the seabed. They have been widely consumed in Asia, Europe and Hawaii as food (McDermid et al., 2003). They are of

nutritional interest because of their low calorie and high amount of vitamins, minerals, proteins, polyphenols, polysaccharides and dietary fibers (Ruperez, 2002). Major industrial products of seaweeds are thickeners and gelling agents, while they can also be applied as ingredients in cosmetical, pharmaceutical, animal feed, and fertilizer industries. Seaweeds can be classified into brown (*Phaeophyceae*), green (*Chlorophyte*), and red (*Rhodophyta*) algae based on their pigments (Li et al., 2011). Brown algae have been reported to contain higher content of active compounds than green and red algae. Major bioactive compounds from brown algae include phenolic compounds, polysaccharides, polyunsaturated fatty acids, proteins, peptides, pigments, vitamins, terpenoids and sterols (Balboa et al., 2013).

Consumption of polyphenol-riched foods, such as fruits and vegetables, tea, red wine, and soy bean products, have been confirmed by recent epidemiological and clinical studies to have negative relationship with occurrence of oxidative damage related diseases and other chronic diseases such as heart diseases and diabetes (Thomas et al., 2011). Polyphenols are compounds having one or more hydroxyl groups attached to a benzene ring (Sies, 2010). Natural polyphenols range from simple molecules such as phenolic acids to highly polymerized compounds like tannins (Bravo, 1998). The two main types of dietary polyphenols are flavonoids and phenolic acids (Manach et al., 2004). Phlorotannins are polyphenolic secondary metabolites that are found only in brown algae (Shibata et al., 2008). They are highly hydrophilic components with a wide range of molecular sizes from 400 to 400,000 Da and occur in variable content (0.5-20%) in brown algae (Balboa et al., 2013).

They have been reported to present pharmaceutical activities such as antibacterial, antioxidant, antifungal, anti-HIV, anti-diabetes, anti-inflammation, and anti-allergic functions ([a]Eom et al., 2012).

Several phlorotannins found in brown algae in previous studies are shown in Figure 1.1. Based on the type of linkages between phlorolucinol subunits and the number of hydroxyl groups on the aromatic skeletons, phlorotannins can be divided into these subclasses: fuhalols and phloroethols (with ether linkages), fucols (with a phenyl linkage), fucophloroethols (with ether and phenyl linkages) and eckols (with dibenzodioxin linkages) (Ferreres et al., 2012). They consist of polymers of phloroglucinol (1,3,5-tryhydroxybenzene) units and are formed in the acetate-melonate pathway, also known as the polyketide pathway (Li et al., 2011; Thomas et al., 2011; Steevensz et al., 2012). Terrestrial polyphenols are polymers based on flavonoids or gallic acids, which are structurally different from phlorotannins (Shibata et al., 2002).



phloroglucinol 2-phloroeckol 7-phloroeckol

6,6’-bieckol 8,8-bieckol eckol

dieckol dioxinodehydroeckol phlorofucofuroeckol-B

Figure 1. 1 Phlorotannins isolated in brown algae

1.2 Different groups of phlorotannins

Phenolic compounds in brown algae are found in physodes, membrane-bound vesicles and cell walls and most of them are phlorotannins (Budhiyanti et al., 2011). Phlorotannins are produced inside brown algae cells as secondary metabolites and some of them are secreted outside the cells. They have been divided into three groups, soluble phlorotannins (SP), membrane-bound phlorotannins, and exuded phlorotannins based on their location and function in brown algae cell (Budhiyanti et al., 2011; Swanson et al., 2002). The three groups of phlorotannins are described in the following paragraphs.

1.2.1 Soluble phlorotannins (SP)

Soluble phlorotannins (SP) are the phlorotannins stored in vesicles inside brown algae cells and they have been fully studied in literature. Usually SP can be extracted from dried algae powder with water, methanol, or ethanol, and then with hexane, chloroform, n-butanol, ethyl acetate, ethyl ether, or acetone. The ethyl acetate and acetone fraction contained the SP while the other fractions were used to remove non-phenolic compounds. It has been demonstrated that over 82% of the crude SP extracts are phlorotannins (Kindleysides et al., 2012). The content of SP in brown algae varied from 0.5 to 20% based on the algae species and the extraction methods used (Balboa et al., 2013). However, large quantity of algae powder residue obtained from the extraction process can be a big problem and some value-added products can be isolated from the leftover, such as membrane-bound phlorotannins.

1.2.2 Membrane-bound phlorotannins (MP)

Phlorotannins are an important part of cell walls of brown algae. They are known to be involved in secondary roles such as chemical defense, protection against UV irradiation, interactions with other organisms or the abiotic environment. It has been suggested that phlorotannins become part of cell walls and membranes when physodes containing phlorotannins fuse with cell membrane (Budhiyanti et al., 2011). Factors such as stereochemistry, conformational flexibility, molecular weight or percentage of galloylation of the phlorotannins can affect their retention in cell wall. MP are usually bound to polysaccharides by hydrophobic interactions and hydrogen bonds (Pinelo et al., 2006). In order to obtain MP from the leftover of solvent extraction processes, cell wall structure of brown algae cells has to be broken to release MP. Budhiyanti et al. (2011) reported that the MP content of brown seaweed *Sargassum hystrix* was higher than SP. However, different result was observed from *Fucus vesiculosus* that the SP content was higher than MP (Koivikko et al., 2005).

1.2.3 Exuded phlorotannins

Many seaweeds defend themselves against bacterial fouling by production of secondary metabolites that preventing attachment and growth of bacterial colonies (Cox et al., 2010). One important group of these secondary metabolites is the exuded phlorotannins, which are the phlorotannins secreted outside cells and into the surrounding environment (Budhiyanti et al., 2011; Koivikko et al., 2005).

1.3 Extraction processes

Solvent extraction is the most commonly used extraction method to obtain extracts from plant materials because of their ease of use, efficiency, and wide applicability (Dai et al., 2010). The extraction procedure, such as type of solvents with varying polarities, extraction time and temperature, sample to solvent ratio as well as chemical and physical properties of samples can affect the phlorotannin yield and antioxidant activities (Lopez et al., 2011; Dai et al., 2010). Polar solvent are more efficient at extracting phlorotannins from than water and apolar solvents (Wang et al., 2012). Two extraction methods widely found in literature for SP extraction were applied and compared in this project. The two extraction methods were described in the following paragraphs.

1.3.1 SP extraction-Method 1

In this extraction process, the algae powder was treated with methanol or ethanol, chloroform, deionized water, finally with ethyl ether or ethyl acetate. Ethyl acetate was applied as the final solvent in some articles and contained the phlorotannins. Different results were observed from a range of articles using ethyl ether. Some reported that the methanol-water fraction contained the phlorotannins while the others reported phlorotannins were concentrated in ethyl ether fraction. Research articles using this method are summarized in Table 1.1.

Table 1. 1 Application of extraction method 1 in literature

Algae species	**Solvent used**	**Fraction containing phlorotannins**	**Bioactivity**	**Phlorotannins isolated**	**Reference**
Ecklonia cava	Ethanol, water, ethyl acetate	Ethyl acetate	Anti-diabetes in *db/db* mouse model	Dieckol	Kang et al., 2013
Ecklonia kurome	Methanol, chloroform, water, ethyl acetate	Ethyl acetate	Algicidal effect on red tide microalgae	Phloroglucinol, eckol, phlorofucofuroeckol A, bieckol and 8,8'-bieckol	Nagayama et al., 2003
Ecklonia kurome	Methanol, chloroform, water, ethyl acetate	Ethyl acetate	Bactericidal activity against MRSA	Phloroglucinol, eckol, phlorofucofuroeckol A, dieckol, 8,8'-bieckol, etc	Nagayama et al., 2002
Ecklonia kurome and Eisenia bicyclis	Methanol, chloroform, water, ethyl ether	Ethyl ether	Inhibition on hyaluronidase	Phloroglucinol, eckol, phlorofucofuroeckol A, and dieckol	Shibata et al., 2002
Ecklonia stolonifera	Methanol, water, ethyl acetate	Ethyl acetate	Inhibition on matrix metalloproteinase-1 in human dermal fibroblasts	Eckol and dieckol	Joe et al., 2006
Ecklonia cava, Ecklonia stolonifera, and Eisenia bicyclis	Methanol, chloroform, water, ethyl ether	Water	Not investigated	Dieckol and phlorofucofuroeckol-A	Chowdhury et al., 2014

1.3.2 SP extraction-Method 2

In this extraction procedure, the algae powder was firstly treated with methanol or ethanol, then the liquid fraction was separated between upper and lower layers in turn with hexane, CH_2Cl_2, ethyl acetate, and n-butanol. Hexane and CH_2Cl_2 were applied to remove nonpolar components and pigments (Lee et al., 2010). The ethyl acetate fraction was the phlorotannin-riched fraction and showed better bioactivities than others. Research articles using this method are summarized in Table 1.2.

Table 1. 2 Application of extraction method 2 in literature

Algae species	Bioactivity	Phlorotannins isolated	Reference
Eisenia bicyclis	Pancreatic lipase inhibitory activity	Eckol, 7-phloroeckol, dioxindehydroeckol, phlorofucofuroeckol A, dieckol	Eom et al., 2013
Ecklonia cava	Antibacterial activity against MRSA and *Salmonella*. spp	Eckol	Choi et al., 2010
Eisenia bicyclis	Inhibition effects on α-glucosidase and α-amylase	Fucofuroeckol-A, dioxinodehydroeckol	[b]Eom et al., 2012
Ecklonia cava	Lipopolysaccharide induced inflammatory inhibition	N/A	Kim et al., 2010
Sargassum siliquastrum	Inhibition of red blood cell hemolysis		Lim et al., 2002
Ecklonia cava	Anti-HIV-1 activity	6,6'-bieckol	Artan et al., 2008
Ecklonia cava	Antiallergic effects on histamine release and binding inhibition between IgE and FcεRI	Fucodiphloroethol G, eckol, etc.	Li et al., 2008
Ishige okamurae	Protection effect against radiation-induced cell damage	Diphlorethohydroxycarmalol	Ahn et al., 2011
Ishige okamurae	Inhibitory activity on HIV-1 reverse transcriptase	Diphlorethohydroxycarmalol	Ahn et al.,2006
Ecklonia cava	Antioxidant activity	Eckol, dieckol, 6,6'-bieckol, etc.	Li et al., 2009

1.3.3 MP extraction-Alkali and acid treatment

This process was applied to treat the algae powder residue obtained from the two extraction processes. Budhiyanti et al. used this method to obtain membrane bound extract from the reside of brown algae *Sargassum hystrix* and found that this extract showed higher phlorotannin yield than the cytoplasmic extracts, the SP (Budhiyanti et al., 2011). Same method was applied to obtain cell-wall-bound phlorotannins from *Fucus vesiculosus* but the content of MP was lower than SP (Koivikko et al., 2005). However, a similar method was used to extract fucoidan, a polysaccharide, from brown algae *Turbinaria turbinata, Sargassum filipendula, Dictyota caribaea* and *Padina perindusiata,* suggesting that this method can also extract non-phenolic compounds thus further purification is needed to obtain phlorotannins from the extract.

1.4 Total Phlorotannin content (TPC) and antioxidant activity

1.4.1 TPC test: Folin-Ciocalteu method

The content of total phlorotannins was estimated using the Folin-Ciocalteu assay in almost all researches regarding total polyphenol content. This method is an electron transfer based assay and is a modified method of the Folin-Denis assay. It was first developed in 1927 for measuring tyrosine (Walker et al., 2010). Folin-Ciocalteu reagent determines total phenols, producing blue color by reducing yellow heteropolyphosphomolybate-tungstate anions. The reactions below show the general chemistry of Folin-Ciocalteu method (Prior et al., 2005):

$Na_2WO_4/Na_2MoO_4 \rightarrow (phenol\text{-}MoW_{11}O_{40})^{-4}$

$Mo(VI)\ (yellow) + e^- \rightarrow Mo(V)\ (blue)$

A standard compound such as phloroglucinol or garlic acid was dissolved and diluted in series, and a standard curve was made with the standard compound between its concentration and optical density (OD) value. The results were usually expressed as mg gallic acid or phloroglucinol equivalent per gram of dried algae (mg GAE/g algae or mg PHG/g algae). It's a measure of total phenolic compounds and other oxidation substrates owing to the general nature of the Folin-Ciocalteu chemistry (Dai et al., 2010). The assay is a measure of total antioxidant capacity but because phenols are the most abundant antioxidants in most extracts thus F-C method can give a rough approximation of TPC in most cases (Everette et al., 2010).

1.4.2 Antioxidant activity test: DPPH radical scavenging activity

Antioxidants play an important role in reducing oxidative reactions and protecting human body from oxidative damage. The internal antioxidant system of human body, which consists of endogenous antioxidant enzymes (e.g. superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymetic antioxidants (e.g. ascorbic acid, tocopherol and selenium), protects internal organs and tissues from oxidative damage. However, serious health issues and chronic diseases such as cancer, cardiovascular disease, hypertension, diabetes mellitus, inflammation, neurodegenerative diseases and aging will occur due to the imbalance between the internal antioxidant system and reactive oxygen species (ROS) (Samarakoon et al., 2012).

ROS are a class of highly reactive molecules formed during aerobic life, which include superoxide anion radical, hydroxyl radical, nitric oxide, single oxygen and hydrogen peroxide (Chew et al., 2008). Some synthetic antioxidants like butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone and propyl gallate have been applied as food, cosmetic and drug compositions (Ahn et al., 2007). However, the side effects and toxicity of these synthetic antioxidants have been queried and researchers are looking for natural antioxidants that can be safely used in food and medicine (Wang et al., 2009). Although synthetic antioxidants are more efficient at low doses, natural antioxidants may replace synthetic antioxidants in some cases because they are generally recognized as safe (GRAS) (Balboa et al., 2013).

Marine brown macroalgae have been considered as good sources of natural antioxidants because of their high phenol content. Phlorotannins from brown algae have up to eight interconnected benzene rings and thus are more potent radical scavengers than polyphenols of terrestrial plants (Balboa et al., 2013). Polypheonls can inactivate free radicals such as lipid peroxides and reduce the oxidative potential of metal ions such as Fe^{3+} *in vitro*. They are also able to inhibit pro-oxidant enzymes and induce antioxidant enzymes *in vitro*. Evidence shows that polyphenols can protect other antioxidants and work with them synergistically (Weichselbaum et al., 2010). Addition of phlorotannins to food products can improve the oxidative stability and provide additional health-promoting effects (Wang et al., 2010).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Fig 1.2) radical is a stable free radical that has been widely used for evaluating antioxidant activity of brown algae extracts and other natural antioxidants (Balboa et al., 2013). The DPPH radical absorbs at around 517 nm and antioxidant activity can be determined by monitoring decrease in absorbance. Results were usually expressed as IC_{50} or EC_{50} value, which was the amount of antioxidants necessary to scavenge 50% of the initial DPPH radicals (Antolovich et al., 2001). A low IC_{50} or EC_{50} value for an antioxidant indicates that the antioxidant behaves as a strong radical scavenger (Liu et al., 2010). This assay is based on the principle that DPPH ·accept a hydrogen atom from the scavenger molecule, e.g. antioxidants, resulting into reduction of DPPH ·to $DPPH_2$ with a color change from purple to yellow (Mishra et al., 2012). Phenols can transfer a hydrogen atom into DPPH radicals and quench the radical propagation process in a dose-dependent manner (Heo et al., 2008). Several phlorotannin components have been isolated and purified from edible brown algae and their antioxidative activities were investigated and compared to those of commercial antioxidants. Three phlorotannins isolated from *Ecklonia stolonifera,* phlorofucofuroeckol A, dieckol, and dioxinodehydroeckol, were reported to have stronger DPPH radical scavenging activity than L-ascorbic acid (Kim et al., 2009). Four phlorotannin compounds, 6,6'-bieckol, 8,8'-bieckol, dieckol, and phlorofurokukoeckol presented lower IC_{50} values in DPPH radical scavenging than synthetic antioxidants BHA and butylated hydroxytoluene (BHT) (Kang et al., 2003). The 2,7''-phloroglucinol-6,6'-biedkol and pyrogallol-phloroglucinol-6,6'-bieckol isolated from *Ecklonia cava* have shown stronger antioxidant activity than ascorbic acid (Kang et al., 2012).

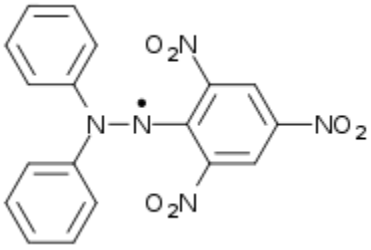


Figure 1. 2 Chemical structure of DPPH

1.5 Applications of phlorotannins

Seaweeds have been consumed in Asian countries for a long tradition due to their nutritional value as rich sources of polysaccharides, proteins, lipids, minerals, vitamins and dietary fibers. They are also a major source of thickening and gelling agents (Samarakoon et al., 2012). Seaweed ingredients are widely used in various industries such as cosmeceutical, pharmaceutical, animal feed, and fertilizer industries (Li et al., 2011; Thomas et al., 2011). However, phenolic extracts of brown algae have not been sold in market yet and some of their potential applications are listed as follows.

1.5.1 Antioxidants

Antioxidants in food systems conventionally refer to compounds that inhibit lipid auto oxidation. Protection against oxidation in oil-containing foods is fundamental for maintaining their nutritional and organoleptic properties (Balboa et al., 2013). Several synthetic antioxidants, e.g., BHT and BHA, are used in food preservation but their safety issue has been doubted and discussed. Natural antioxidants can protect human body from oxidative

damages caused by radicals and retard the progress of chronic diseases, as well as prevent lipid oxidative rancidity in foods (Heo et al., 2009). A key application of phlorotannins could be their usage in food products as preservatives to extend shelf life and maintain quality (Balboa et al., 2013).

1.5.2 Antimicrobial agents

Increasing resistance of clinically important bacteria and fungus to existing antibiotics has become a major problem throughout the world. For instance, is has been reported that increasing proportions of *Staphylococcus aureus* are resistant to penicillin and other antibiotics thus *Staphylococcus aureus* became known as methicillin-resistant *S. aureus* (MRSA) ([a]Eom et al., 2012). One of the methods to prevent antibiotic resistance of microorganisms is to develop new compounds with antimicrobial activity. Developing natural substances with synergistic effect with conventional antibiotics is one of the most effective methods (Choi et al., 2010). In the marine ecosystem, the surfaces of seaweeds are exposed to microbes but are free from biofouling, which suggested that the marine macroalgae may produce natural product with antimicrobial property ([a]Eom et al., 2012). Phlorotannins from brown algae have been investigated for their antimicrobial activity and was suggested to be potential antimicrobial agents. Bactericidal activity of phlorotannin extracted from brown algae *Ecklonia kurome* against MRSA and food-borne pathogenic bacteria was reported (Nagayama et al., 2002). Plant extracts with antimicrobial properties have a potential to prevent bacterial and fungal growth in food production (Cox et al., 2010).

1.5.3 Pharmaceutical, cosmeceutical and nutraceutical application

Oxidative damage is believed to contribute to aging and lifestyle diseases such as diabetes and heart diseases. It is suggested that antioxidant supplements could be an effective inhibiting agent to these diseases. Recent epidemiological and clinical studies indicated consumption of polyphenol-riched plant foods moderates the risk of lifestyle diseases (Shibata et al., 2008). Brown algae may serve as a good source of pharmaceutical compounds due to their high phlorotannin content.

Cosmeceutical applications are cosmetics with potential of pharmaceutical- or drug-like benefits for the human skin, and they can enhance its protection, appearance and anti-aging properties. The skin matrix is responsible for the skin's mechanical properties, therefore protective effect from ROS damaging would be expected from cosmeceuticals to reduce the natural aging process (Samarakoon et al., 2012). Phlorotannins isolated from brown algae *Ecklonia cava* were found to present protective effect against photo-oxidative stress induced by UV-B radiation in human fibroblast cells (Heo et al., 2009). Phlorotannins dieckol and phlorofucofuroeckol-A extracted from *Ecklonia cava* has been shown to inhibit *Propionibacterium acnes*, which is responsible for the development of inflammatory acne (Choi et al., 2014). These researches suggested that phlorotannins had the potential to be applied in the cosmetical application.

Nutraceuticals from seaweeds have been considered as a rich source of health-promoting components (Li et al., 2011). Dried flake of *Padina antillarum* has been used as table salt replacement for high blood pressure patients (Chew et al., 2008). Combination of phlorotannins with fish oil and other marine products is also a good application in nutraceutical market. The major commercial phenolic nutraceuticals are extracts of apple, green tea and grapes (Table 1.3, data obtained from Amazon) and 40-98% of the bioactive compounds of them are polyphenols. The phlorotannins have not been sold as nutraceuticals or functional foods in the authors' knowledge.

Table 1. 3 Information of some commercial polyphenol extracts

Plants extracted	**Brand**	**Percentage of polyphenol (%)**	**Serving per capsule (mg polyphenol/capsule)**	**Serving per container**	**Price**	**Reference**
Apple (especially skin)	Life Extension	50	300	30	$14.99	Amazon.com 2015a
Apple	Swanson ultra	Not shown	125	60	$11.64	Amazon.com 2015b
Green tea	Now Foods	40	160	250	$9.59	Amazon.com 2015c
Green tea	Life Extension	98	710	100	$18.06	Amazon.com 2015d
Grape seed	Now Foods	90	225	90	$15.85	Amazon.com 2015d
Grape	Olympian Labs	95	380	100	$16.11	Amazon.com 2015e
Grape seed	GNC Herbal Plus	95	285	100	$17.03	Amazon.com 2015f

1.6 Summary and objectives

Phlorotannins isolated from brown algae are a potent source of natural antioxidants that can be applied in pharmaceutical, cosmeceutical, and nutraceutical industries. They are more potent antioxidants than terrestrial polyphenols and may replace synthetical antioxidants.

Besides antioxidant activities, phlorotannins showed other health-promoting benefits, e.g. anti-inflammation, anti-cancer, anti-diabetes, etc. Different extraction methods have been applied in previous research but their influence on phlorotannin yield and antioxidant activity were not studied. Among the three phlorotannin groups, the SPs are the most extensively studied for their content, component, and bioactivities. However, only a few studies reported the content the MP and none of them purified the MP extracts or investigated their bioactivities.

In this project, total phlorotannin content and antioxidant activity were investigated on the three phlorotannin groups SP, MP, and eMP obtained from five edible brown algae by two extraction methods. The objectives of this project were to:

1. Evaluate and compare the contents of SP, MP, and eMP in five brown algae, *Ascophyllum nodosum, Fucus vesiculosus, Alaria esculenta, Laminaria digitata*, and *Saccharina latissma*.
2. Examine the influence of extraction method on phlorotannin yield and figure out the better solvent extraction method for phlorotannin extraction.
3. Evaluate the antioxidant activity of the three phlorotannin groups extracted from the five brown algae species.
4. Study the influence of extraction method on the antioxidant activity of phlorotannin extracts.
5. Evaluate the potent of applying the five brown algae species as sources of

phlorotannins and natural antioxidants.

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CHAPTER 2 Extraction and quantification of phlorotannins from edible brown algae

Abstract The objectives of this study were to evaluate the contents of soluble and membrane-bound phlorotannins in five brown macroalgae, *Saccharina latissima, Alaria esculenta, Laminaria digitata, Fucus vesiculosus,* and *Ascophyllum nodosum*, and examine the influence of extraction methods on phlorotannin yield. Two solvent-extraction methods were first utilized to obtain soluble phlorotannins (SPs). The leftover algae powder was then treated with sodium hydroxide to release the membrane-bound phlorotannins (MPs). Folin-Ciocalteu method using phloroglucinol (1,3,5-benzenetriol) as the standard was applied to quantify SP and MP contents. MP samples were further purified with solvents to obtain the extracted MP (eMP). Significant differences in SP and MP contents among the five algae ($p<0.05$) were observed. *F. vesiculosus* and *A. nodosum* showed the highest SP content (4.66 and 4.90 mg/g algae, respectively) by the first extraction method. The highest MP content was observed in *A. nodosum* (68.55 mg/g algae) followed by *F. vesiculosus* (62.58 mg/g algae). The MP content of all algae was higher than the SP content, indicating the great potential for harvesting value-added products from the leftover algae powder after SP extraction. The two algae species with the highest SP content, *A. nodosum* and *F. vesiculosus*, were then extracted by the second extraction method and the yields are 6.59 ± 0.77 and 3.07 ± 0.48 mg/g algae, respectively. The yields of SP by the second method were significantly different from that by the first extraction method ($p<0.05$). Method 1 was better on *F.*

vesiculosus but method 2 was better on *A. nodosum* in terms of SP yield, indicating that the effectiveness of extraction methods was algal species dependent.

Keywords brown algae; phlorotannin; bioseparation; polyphenol; *Ascophyllum nodosum*; *Fucus vesiculosus.*

2.1 Introduction

The ocean covers more than 70% of the earth and regulates the climate. It's also an important source of nutritious foods, e.g. fish, shellfish, and seaweed. Marine organisms, which comprise half of the total biodiversity, are exposed to more extreme conditions than terrestrial organisms due to the larger surface of the ocean (Li et al., 2011). In order to survive extreme conditions, e.g. ultraviolet, extreme salinity levels, temperature variations, low light intensities and nutrient deficient habitats (Samarakoon et al., 2012), marine organisms generate a variety of bioactive compounds to protect themselves. More than 21,000 structurally diverse, bioactive natural products have been discovered from marine microbes, algae and invertebrates since 1970s (McDermid et al., 2003). In addition, the bioactive agents of terrestrial ecosystems have been investigated and applied for a long time; the marine ecosystem is expected to be a new source of therapeutic and nutraceutical agents. Recently, values of bioactive compounds from marine organisms have been revealed and recognized.

Among all the marine organisms, edible seaweeds are considered as under-exploited sources of functional food and pharmacologically active metabolites (McDermid et al., 2003). Seaweeds are macroscopic, multicellular marine algae that grow on the seabed and are widely consumed in Asia, Europe and Hawaii as food or food supplements (McDermid et al., 2003). Seaweeds are of nutritional interest because of their low calorie and high amount of vitamins, minerals, proteins, polyphenols, polysaccharides and dietary fibers (Rajauria et al., 2013; Ruperez, 2002). Major industrial products of seaweeds include thickeners and gelling agents, while they can also be applied as ingredients in cosmetical, pharmaceutical, animal feed, and fertilizer industries (Li et al., 2011;). Seaweeds can be classified into brown (*Phaeophyceae*), green (*Chlorophyte*), and red (*Rhodophyta*) algae based on their pigments (Li et al., 2011; Samarakoon et al., 2012). Brown algae have been reported to contain more active compounds than green and red algae. Major bioactive compounds from brown algae include phenolic compounds, polysaccharides, polyunsaturated fatty acids, proteins, peptides, pigments, vitamins, terpenoids and sterols (Balboa et al., 2013).

Consumption of polyphenol-rich foods, such as fruits and vegetables, tea, red wine, and soybean products, have been confirmed by recent epidemiological and clinical studies to have negative relationship with occurrence of oxidative damage related diseases such as aging, cancer, cardiovascular diseases (Thomas et al., 2011). Chemically, polyphenols are compounds having one or more hydroxyl groups attached to a benzene ring (Fraga et al., 2010). Natural polyphenols range from simple molecules such as phenolic acids to highly

polymerized compounds like tannins (Bravo, 1998). The two main types of dietary polyphenols are flavonoids and phenolic acids (Scalbert et al., 2005). Phlorotannins are polyphenolic secondary metabolites that are found only in brown algae (Li et al., 2011). Phlorotannins can be classified into three primary types: (i) fucols (phlorotannins with only phenyl linkages), (ii) phlorethols (phlorotannins with only arylether bonds), and (iii) fucophlorethols (with both arylether and phenyl bonds) (Martinez et al., 2013). They consist of polymers of phloroglucinol (1,3,5-tryhydroxybenzene) units that are formed in the acetate-melonate pathway ([a]Eom et al., 2012; Balboa et al., 2013; Kim et al., 2009; Bertoni, 2013), also known as the polyketide pathway (Li et al., 2011). Terrestrial polyphenols are polymers based on flavonoids or gallic acids, which are structurally different from phlorotannins (Cox et al., 2010). Phlorotannins are highly hydrophilic compounds with a wide range of molecular sizes from 400 to 400,000 Da and occur in variable contents (0.5-20%) in brown algae (Balboa et al., 2013). They have been reported to have pharmaceutical activities such as antibacterial, antioxidant, antifungal, anti-HIV, anti-diabetes, anti-inflammation, and anti-allergic functions (Li et al., 2011). Phlorotannins can be divided into two groups, soluble phlorotannins (SPs) and membrane-bound phlorotannins (MPs), according to their location in brown algae cells. SPs are stored in cell organelles, physodes, which are round or elliptical, highly mobile, vesicle-like strongly refractive bodies in the cytoplasm of brown algae cells. MPs are believed to transform into components of cell walls when physodes fuse with cell membrane and the phlorotannins are secreted into the cell wall, complexing finally with alginic acid (Bertoi, 2013). Phenolics are components of plant cell walls and may play roles

in signaling and defense against invading bacteria or in response to environmental stress, such as wounding and excessive light or ultraviolet radiation (Balboa et al., 2013; Cox et al., 2010). It has been shown that phlorotannins are the main phenolic compounds detected in brown algae (Steevensz et al., 2012).

To the best knowledge of the authors, phlorotannins of the five algae species in this study, *Ascophyllum nodosum, Fucus vesiculosus, Laminaria digitata, Saccharina latissima,* and *Alaria esculenta,* were not fully studied. In most of the previous research on phlorotannins, contents and bioactivities of SPs were investigated but knowledge about the MPs was very limited. In addition, various extraction methods were reported in the literature but their effectiveness for obtaining phlorotannins were not compared. Thus, the objectives of this study were to evaluate the contents of soluble and membrane-bound phlorotannins in five brown macroalgae, *S. latissima*, *A. esculenta*, *L. digitata*, *F. vesiculosus*, and *A. nodosum,* and examine the influence of two commonly used solvent extraction methods on phlorotannin yields.

2.2 Materials and methods

Five brown algae, *S. latissima* (kelp*)*, *A. esculenta* (alaria), *L. digitata* (digitata), *F. vesiculosus* (bladderwrack*)*, and *A. nodosum* (rockweed) were investigated in this study. They were obtained from Maine Coast Sea Vegetables (Franklin, ME, USA), all in dried powder except for *S. latissima* which was in dried whole leaf. The samples were sealed in

airtight bags and had a greenish brown color. The whole leaf of *S. latissima* was ground and sieved with a 1-mm sieve before extraction. All samples were sealed and stored under -20℃ until experiments.

2.2.1 Extraction

2.2.1.1 SP extraction

SP was extracted with two methods commonly found in the literature. In Method 1, algae powder was extracted using a modified method of previous study (Shibata et al., 2002). Briefly, 20-g powder was immersed in 80-ml methanol in a flask, shaken on an orbital shaker at 100 rpm for 3 h at room temperature. Then 160-ml chloroform was added, and the mixture was shaken for 0.5 h. During the shaking process the flasks were covered with foil to avoid exposure to light. Then the mixture was filtered to obtain algae powder residue and liquid fraction. After filtration, algae powder residue was dried at room temperature in vacuum oven for 24 h and preserved for experiments later. The liquid fraction was separated between the upper and lower layer with 60-ml deionized water. The upper layer was extracted with 60-ml ethyl acetate (EtOAc) twice. The EtOAc fraction contained phlorotannins. This method was also used in other studies (Ahn et al., 2004; Budhiyanti et al., 2011; Nagayama et al., 2002; Nagayama et al., 2003).

The other extraction method, which is called Method 2, was a modified method of Eom et al. (2006). Algal species with high phlorotannin content by Method 1 were extracted by Method

2. Briefly, 20-g powder was mixed with 80-ml methanol in a flask and shaken in the dark for 3 h at room temperature. The solvent was then evaporated *in vacuo*. The crude extract was dissolved in 20-ml 10% methanol and then partitioned in turn with n-hexane (20-ml × 3), dichloromethane (20-ml × 3), EtOAc (20-ml × 3) and n-butanol (20-ml × 3). The EtOAc fraction contained phlorotannins. This method was widely applied in phlorotannin extraction too (Kim et al., 2009; Choi et al., 2010; Lim et al., 2002; Artan et al., 2008; [b]Eom et al., 2012; Li et al., 2008).

2.2.1.2 MP extraction

Algae powder residue was extracted with the method of Budhiyanti et al. (2011) to evaluate the content of MP. First, 200-mg algal residue after SP extraction by Method 1 was dissolved in 8-ml 1 M NaOH, stirred for 2 h, and concentrated under 2400 g for 5 min. Then, the supernatant was neutralized with phosphoric acid and its MP content was tested.

2.2.1.3 eMP extraction

After measurement of the total phlorotannin content (TPC), the supernatant was extracted using a modified version of Method 1, in which ethyl acetate was replaced with ethyl ether (Nagayama et al., 2003), to obtain the extracted MP (eMP). The methanol-water mixture fraction was tested to contain eMP. Each extraction was repeated three times.

2.2.2 Total phlorotannin content test

TPC was determined according to a modified version of Folin-Ciocalteu method, using phloroglucinol (PHG) as the standard (Chowdhury et al., 2014). Samples were diluted taking into account the range of the standard curve. A 0.04-ml aliquot of the sample was mixed in a 1.5-ml centrifugation tube with 0.4-ml 1 N Folin-Ciocalteu reagent and 0.8-ml 20% Na_2CO_3. After standing for 3 min, the sample was incubated in the dark at room temperature for 45 min and centrifuged at 1600 g for 8 min. Optical density (OD) of the supernatant was measured at 730 nm using a BioTek 96-well microplate reader (Winooski, VT, USA). TPC test was performed in triplicate and the result was expressed as mg phloroglucinol equivalent per gram algae (mg PHG/g algae) using the following calibration equation: $Y = 2.3356X - 0.0544$ ($r^2 = 0.996$), where Y is the OD at 730 nm and X is the concentration of phloroglucinol as the standard (mg/ml). Folin-Ciocalteu phenolic reagent and phloroglucinol were obtained from Sigma-Aldrich CO. LLC. (St. Louis, MO, USA). All other chemicals and organic solvents were obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.2.3 Statistical analysis

The results of the present study were expressed as mean ± standard deviation. Statistical analysis was performed by one-way ANOVA and Tukey test with SAS (Cary, NC, USA). A p-value of 0.05 or less was considered significant.

2.3 Results and discussion

2.3.1. Soluble phlorotannin content

The SP contents of the five algae by Method 1are shown in Figure 2.1. Significant variations were observed in TPC of the five algae ($p<0.05$). The highest TPC was observed in *A. nodosum* with 4.90 ± 0.53 mg PHG/g algae. *F. vesiculosus* represented the second highest TPC of 4.66 ± 0.70 mg PHG/g algae, but no significant differences in TPC were noticed between these two algae. TPC of the other three algae, *L. digitata, A. esculenta, S. latissima* were 0.20 ± 0.02, 0.65 ± 0.05, and 0.19 ± 0.01 mg PHG/g algae, respectively. Contents of SP of the five algae have been reported in several other studies. TPC in water and 70% acetone extracts of these five algae were in this order: *F. vdsiculosus* > *A. nodosum* > *A. esculenta* > *S. latissima* > *L. digitata* (Kim et al., 2006), which roughly agrees with the present study. It was reported that *L. digitata* and *S. latissima* showed total phenolic content of 37.66 and 66.75 mg gallic acid (GAE)/g algae (Cox et al., 2010), which was higher than the TPC in the present study. The difference was probably because of the different standard compounds used in TPC measurement. TPC of *A. nodosum* was reported to be about 2.5% of total dry weight of algae (Zubia et al., 2007; Audibert et al., 2010) and was higher than the present study. However, their crude phlorotannin was not purified with ethyl acetate, which is commonly used to obtain phlorotannins. Other possible reasons for variations in reported TPC of the same algae species might be the insoluble complex formed by phlorotannins and proteins (Wang et al., 2012) and differences in algae habitats.

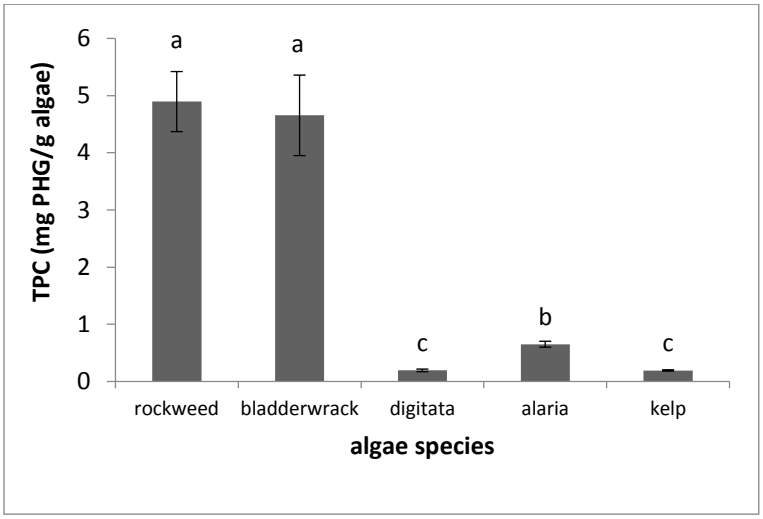

Figure 2. 1 Soluble phlorotannin contents of the five brown algae. Different letters indicate significant differences ($p<0.05$).

2.3.2 Membrane-bound phlorotannin content

Membrane-bound phlorotannin contents by Method 1 are shown in Figure 2.2 and significant differences were observed. *A. nodosum* presented the highest TPC of 68.55 ± 4.93 mg PHG/g algae followed by *F. vdsiculosus* of 62.58 ± 1.57 mg PHG/g algae. The MP content of *L. digitata* was the lowest among the five algae. Only a few articles have studied membrane-bound phlorotannins. The fractioned membrane bound extracts (1.16-18.58 g phloroglucinol/100 g dried extract) showed higher TPC than fractionated cytoplasmic extracts (0.15-4.78 g phloroglucinol/100 g dried extract) in brown algae *Sargassum hystrix* (Budhiyanti et al., 2011). However, a study on *F. vesiculosus* reported an opposite trend that the concentrations of cell-wall-bound phlorotannins were lower than that of soluble phlorotannins (Koivikko et al., 2005). This was probably because the algal residue was

treated in alkali under a higher temperature (80℃) and phlorotannins might degrade at such high temperature.

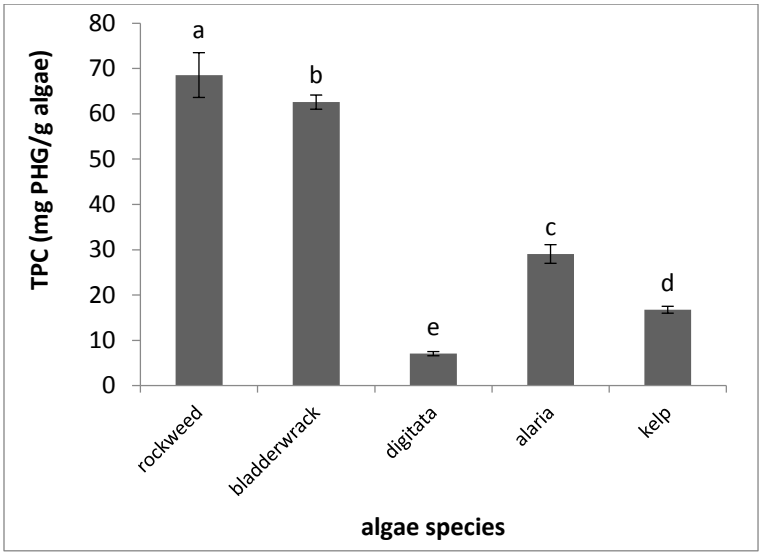

Figure 2. 2 Membrane-bound phlorotannin contents of the five brown algae. Different letters indicate significant differences ($p<0.05$).

2.3.3. Extracted membrane-bound phlorotannin content

According to a previous study, polysaccharides embedded in cellulose microfibrils of brown algae cell walls can be released using acid and alkali treatments and then extracted with ethanol (Garcia-Rios et al., 2012). Significant amounts of uronic acids or fucose and sulphate were found in acid and alkali extracts of *F. vesiculosus* and *L. digitata* (Mabeau et al., 1987). Thus MP samples in the present study were further purified again with organic solvents to obtain eMP, and tested with Folin-Ciocalteu method to compare the content of MP and eMP. Figure 2.3 shows that *A. nodosum* and *F. vesiculosus* had higher eMP, 17.56 ± 1.45 and

17.06 ± 0.68 mg PHG/g algae, respectively, than *L. digitata* (3.54 ± 0.14 mg PHG/g algae), *A. esculenta* (9.49 ± 0.88 mg PHG/g algae), and *S. latissima* (10.50 ± 0.93 mg PHG/g algae) by Method 1. The eMP content of every algae was much lower than MP, which suggested that the MPs contained non-phenolic components and thus need to be purified. Some antioxidants might be easier to dissolve in ethyl ether than in EtOAc, which resulted in lower TPC in eMP extracts. Interestingly, the content of eMP in every algae was higher than that of SP ($p<0.05$).

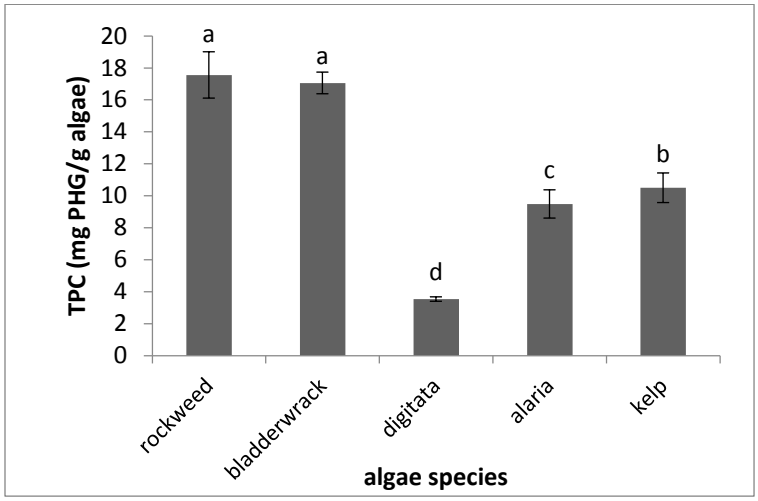

Figure 2. 3 Extracted membrane-bound phlorotannin contents of the five brown algae. Different letters indicate significant differences ($p<0.05$).

2.3.4. Correlation among SP, MP and eMP of five brown algae species

Strong positive correlations between SP and MP ($r^2 = 0.95$), and between SP and eMP ($r^2 = 0.81$) were obtained (Fig 2.4), indicating that the algae species with higher SP content also had higher contents of MP and eMP. It implied that a species with high SP content may also have high MP content, which makes extraction of both phlorotannins possible. These

correlations also suggested that the phlorotannins were produced inside the brown algae cells and transported to cell walls and membranes. The ratios of eMP to MP in the five brown algae species are shown in Table 3.1. The results indicated that polyphenols may not be the major antioxidants in some brown algae species since only *L. digitata* and *S. latissima* exhibited eMP to MP ratios greater than 0.5. Surprisingly, *A. nodosum* and *F. vesiculosus* had the lowest ratios of eMP to MP though they had the highest SP, MP and eMP contents. It suggested that although the two algae contained greater amounts of antioxidants on their cell membranes and cell walls, only a small fraction were phlorotannins. However, the other three algae with relatively lower SPs, MPs and eMPs exhibited higher eMP to MP ratios, which meant that phlorotannins are the major antioxidants bound to their cell membranes and cell walls. A negative correlation between eMP to MP ratio and MP content of the five algae was found (Fig 3.5), which indicated that phlorotannins might be a stronger defender especially for the algae species with lower amount of antioxidants exhibited on cell walls and membranes and that the algal species with lower eMP to MP ratio might live in harsher habitats so they also need large amount of non-phenolic compounds to protect themselves.

Table 2. 1 The ratio of eMP to MP in the five algae. Different letters following the values indicate significant differences ($p<0.05$).

Algae species	eMP to MP ratio
A. nodosum (rockweed)	0.256d
F. vesiculosus (bladderwrack)	0.273d
L. digitata (digitata)	0.501b
A. esculenta (alaria)	0.327c
S. latissima (kelp)	0.627a

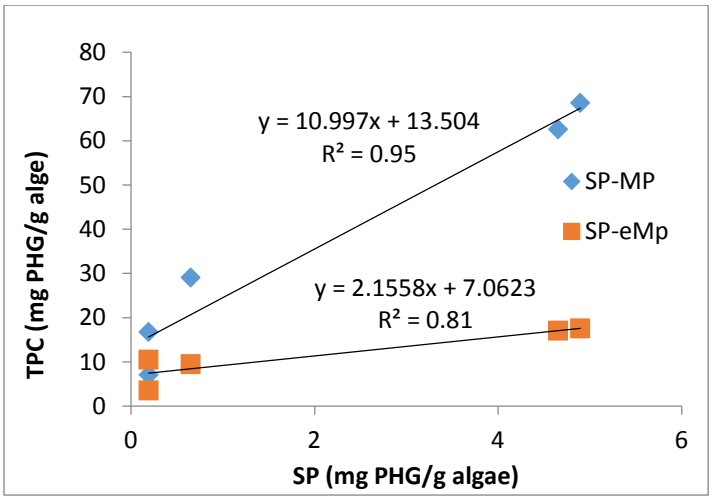

Figure 2. 4 Correlations between SP and MP, and between SP and eMP.

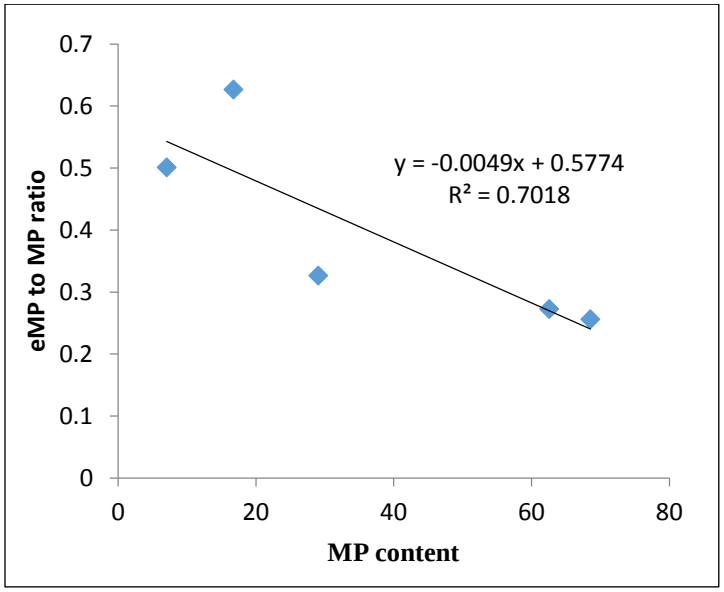

Figure 2. 5 The correlation between MP content and eMP to MP ratio.

2.3.5 Effects of the extraction methods on phlorotannin yield

The two algae species with the highest TPC, *A. nodosum* and *F. vesiculosus* were extracted by Method 2 to study the effect of extraction method on phlorotannin yield. The SP yields of these two algae species by the two extraction methods are presented in Table 2.2. Phlorotannin yields of *A. nodosum* and *F. vesiculosus* showed no significant differences in Method 1 ($p>0.05$). However, yield of phlorotannin differ significantly between the two extraction methods in the two algae species. The second method resulted in more phlorotannins from *A. nodosum*, but less from *F. vesiculosus* than the first extraction method. This result suggested that the extraction process had significant effects on phlorotannin yield and there might be a unique extraction process for specific algae to obtain more phlorotannins.

Table 2. 2 Soluble phlorotannin yields from the two extraction methods

Algae	Yield of Method 1 (mg phlorotannin/g algae)	Yield of Method 2 (mg phlorotannin/g algae)	p-value between the two methods
A. nodosum (rockweed)	4.90 ± 0.53	6.59 ± 0.77	****
F. vesiculosus (bladderwrack)	4.66 ± 0.70	3.07 ± 0.48	****

****: p-value<0.0001, compared between the two extraction methods.

The TPC distributions of different solvent fractions in both extraction methods were also studied. Chloroform and dichloromethane were applied to remove most of the pigments and lipids. Ethyl acetate fraction contained phlorotannins while non-polar components such as lipophilic compounds and chlorophyll were removed by hexane (Koivikko et al., 2007). The n-butanol fraction might contain polysaccharides and their derivatives. TPC of each fraction is shown in Table 2.3. The ethyl acetate fraction of *A. nodosum* in both extraction methods presented the highest TPC among all fractions. Similar results were observed in *Eisenia bicyclis* with the highest TPC and strongest α-glucosidase and α-amylase inhibitory effects in ethyl acetate fraction ([b]Eom et al., 2012). However, the highest TPC from *F. vesculosos* was found in the methanol-water fraction by method 1 and n-butanol fraction by method 2. It suggested that *F. vesiculosus* may contain some non-phenolic compounds that may also react with the Folin-Ciocalteu reagent and thus be included in the TPC. Identification and quantification of non-phenolic components needs further investigation.

Table 2. 3 TPC of each solvent fraction

Extraction method	**Solvent fraction**	***A.nodosum* TPC (mg PHG/g algae)**	***F.vesiculosus* TPC (mg PHG/g algae)**
Method 1	Chloroform	0.68 ± 0.48	0.23 ± 0.03
	Ethyl acetate	4.90 ± 0.53	4.66 ± 0.70
	Methanol-water	3.73 ± 0.46	7.17 ± 0.27
Method 2	Hexane	0.06 ± 0.00	0.08 ± 0.01
	CH_2Cl_2	0.09 ± 0.03	0.08 ± 0.01
	Ethyl acetate	6.59 ± 0.77	3.07 ± 0.48
	n-Butanol	1.53 ± 0.23	4.40 ± 0.67
	Methanol	0.66 ± 0.01	3.12 ± 0.53

2.4 Conclusions

The present study showed that the effectiveness of solvent-extraction methods of brown algae phlorotannins depended on the algae species. Method 1 was better on *F. vesiculosus* but method 2 was better on *A. nodosum* in terms of SP yield. *F. vesiculosus* and *A. nodosum* presented the highest SP, MP and eMP contents among the five algae species investigated, indicating their great potential as phlorotannin sources. The TPC of SP and MP of the two algae species were around 70 mg/g algae and could satisfy production as phenolic nutraceuticals. The content of MP and eMP were higher than but positively correlated with SP in all algae, which suggested that obtaining phlorotannins from the leftover residue after

SP extraction could be worthwhile. However, the existence of non-phenolic compounds in MPs may be problematic and need further purifications.

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CHAPTER 3 The antioxidant activity of phlorotannins from edible brown algae

Abstract The objective of this study was to evaluate the antioxidant activity of the phlorotannins extracted from five algae species (*Saccharina latissima, Alaria esculenta, Laminaria digitata, Fucus vesiculosus*, and *Ascophyllum nodosum*). The antioxidant activity of the three phlorotannin groups, including soluble, membrane-bound, and extracted membrane-bound phlorotannins obtained by two extraction methods were evaluated with DPPH radical scavenging activity. The yields and antioxidant activity of *F. vesiculosus* (yield = 14.83 ± 3.67 mg-extract/g-algae, IC_{50} = 0.0038 ± 0.0002 mg/ml) and *A. nodosum* (yield = 12.80 ± 1.37 mg-extract/g-algae, IC_{50} = 0.0072 ± 0.0010 mg/ml) were the highest among the five algae species. Moreover, the SP, membrane-bound phlorotannin (MP) and extracted membrane-bound phlorotannin (eMP) extracts of *F. vesiculosus* and *A. nodosum* all showed equal or higher DPPH radical scavenging activity than commercial antioxidants, butylated hydroxytoluene (BHT) and ascorbic acid. A parameter called Antioxidant Potential (AP) was introduced to evaluate the potential of the phlorotannin extracts as antioxidant sources. The AP values of the MP extracts of *F. vesiculosus* and *A. nodosum* (5890 and 5278 ml/g algae, respectively) were higher than those of SP and eMP, suggesting that the MPs of *F. vesiculosus* and *A. nodosum* had great potential to be used as antioxidants. Different extraction processes also showed effects on the antioxidant activity of the phlorotannin extracts.

Keywords: brown algae, phlorotannin, bioseparation, polyphenol, antioxidant, antioxidant activity

3.1 Introduction

Reactive Oxygen Species (ROS) are the major causes of oxidative damage and related diseases including atherosclerosis, rheumatoid arthritis, muscular dystrophy, cataracts, some neurological disorders and some types of cancer as well as aging. ROS are a class of highly reactive molecules formed during aerobic life, which include superoxide anion radical, hydroxyl radical, nitric oxide, single oxygen and hydrogen peroxide (Chew et al., 2008). Normal level of ROS may be essential for many cellular functions such as killing phagocytes, bacterial ingestion and redox regulation of signal transduction. However, overproduction of ROS in living organisms can cause harm to DNA, cell membrane, proteins and consequently induce degeneration, destruction and toxicity of various molecules in cells (Choi et al., 2009).

Natural antioxidants in terrestrial plants and their applications in food preservation and nutraceuticals have been studied in numerous studies. Some synthetic antioxidants like butylated hydroxyanisol (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone and propyl gallate have been applied as food, cosmetic and drug compositions (Ahn et a., 2007). However, the side effects and toxicity of these synthetic antioxidants has been questioned and researchers are looking for natural antioxidants that can be safely used in food and medicine (Wang et al., 2009). Polyphenols are the most abundant dietary

antioxidants and exert antioxidant properties through various mechanisms including scavenging free radicals and inhibition of the generation of ROS during cell metabolism (Kim et al., 2009). Phlorotannins in brown algae are prominent natural antioxidants that may replace synthetic antioxidants. Some phlorotannins isolated from edible brown algae have shown stronger antioxidant activity than commercial antioxidants (Kim et al., 2009; Kang et al., 2012).

Phlorotannins are the polyphenolic compounds that are only found in marine brown macroalgae. They consist of polymers of phloroglucinol (1,3,5-tryhydroxybenzene) units that are formed in the acetate-melonate pathway as secondary metabolites. They are highly hydrophilic compounds with a wide range of molecular sizes from 400 to 400,000 Da and occur in variable contents (0.5-20%) in brown algae. They have been reported to have pharmaceutical activities such as antibacterial, antioxidant, antifungal, anti-HIV, anti-diabetes, anti-inflammation, and anti-allergic functions (Eom et al., 2012). Phlorotannins can be divided into two groups, soluble phlorotannins (SPs) and membrane-bound phlorotannins (MPs), according to their location in brown algae cells. SPs are stored in cell organelles, physodes, which are round or elliptical, highly mobile, vesicle-like strongly refractive bodies in the cytoplasm of brown algae cells. MPs are believed to transform into components of cell walls when physodes fuse with cell membrane and the phlorotannins are secreted into the cell wall, complexing finally with alginic acid (Budhiyanti et al., 2011).

The 2, 2 - diphenyl -1- picrylhydrazyl (DPPH) radicals are stable organic nitrogen radicals bearing a deep purple color (Prior et al., 2005). They may be neutralized by either direct reduction via single electron transfer or by radical quenching via hydrogen atom transfer. During reactions, color of the solution changes from purple to yellow when antioxidant eliminate the DPPH radicals (Rajauria et al., 2013). The DPPH assay has been widely used due to its stability, simplicity, rapidity and reproducibility. The other reason for applying DPPH test is that both phlorotannins and DPPH radicals are easily dissolved in methanol, which is the reaction condition for DPPH assay. The most widely used parameter evaluating the property of scavenging DPPH radicals is the IC_{50} value, which is the phlorotannin amount that can eliminate half of given DPPH radicals (Dawidowica et al., 2012; Villano et al., 2007). The extracts with lower IC_{50} value indicate stronger antioxidant activity.

To the best knowledge of the authors, the DPPH radical scavenging activity of the MP and eMP extracts has not been studied. Radical scavenging activity of the SP has been reported in some articles but the five algae species, *Saccharina latissima* (kelp), *Alaria esculenta* (alaria), *Laminaria digitata* (digitata), *Fucus vesiculosus* (bladderwrack), and *Ascophyllum nodosum* (rockweed) have not been fully investigated. Also, the effects of different extraction processes on the antioxidant activity of the phlorotannin extracts were not fully studied. Thus the objectives of this study were to evaluate the antioxidant activity of soluble and membrane-bound phlorotannins of *S. latissima*, *A. esculenta*, *L. digitata*, *F. vesiculosus*, and

A. nodosum, and examine the influence of two commonly used solvent extraction methods on the antioxidant activity of the phlorotannin extracts.

3.2 Materials and Methods

3.2.1 Materials

Five brown algae *S. latissima* (kelp), *A. esculenta* (alaria), *L. digitata* (digitata), *F. vesiculosus* (bladderwrack), and *A. nodosum* (rockweed) investigated in this study were obtained from Maine Coast Sea Vegetables (Franklin, ME, USA), all in dried powder except for *S. latissima* which was in dried whole leaf. The samples were sealed in airtight bags and had a greenish brown color. The whole leaf of *S. latissima* was ground and sieved with a 1-mm sieve before extraction. All samples were sealed and stored under -20℃ until experiments.

3.2.2 Methods

3.2.2.1 Phlorotannin extraction and sample preparation

The SP was extracted with two solvent extraction methods commonly found in the literature. The extraction processes are shown in Fig 3.1 and Fig 3.2. Algal species with high phlorotannin content and antioxidant activity by Method 1 were extracted by Method 2. In the first extraction method, methanol (MeOH), chloroform ($CHCl_3$), deionized water and ethyl acetate (EtOAc) were applied in sequence. In the second extraction method, MeOH, dichloromethane (CH_2Cl_2), EtOAc, and n-butanol were used. The EtOAc fraction in both

extraction methods contained phlorotannins and was analyzed for TPC and DPPH radical scavenging activity.

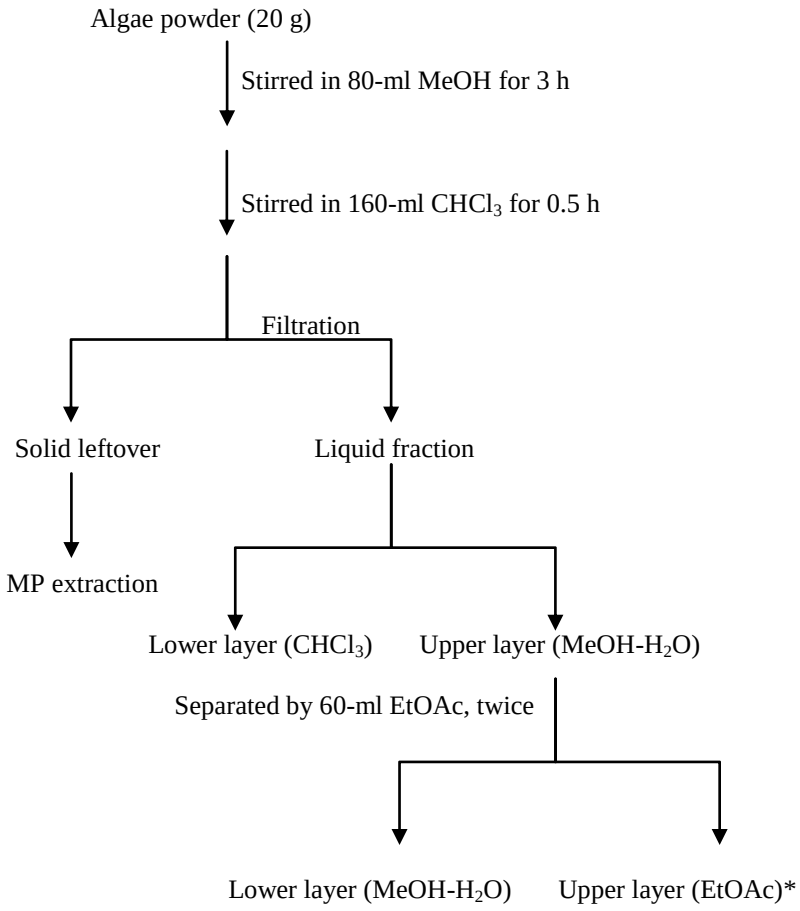

Figure 3. 1 The first extraction method for SP extraction (*represents the fraction containing phlorotannin) (Ahn et al., 2004; Artan et al., 2008; Kang et al., 2013)

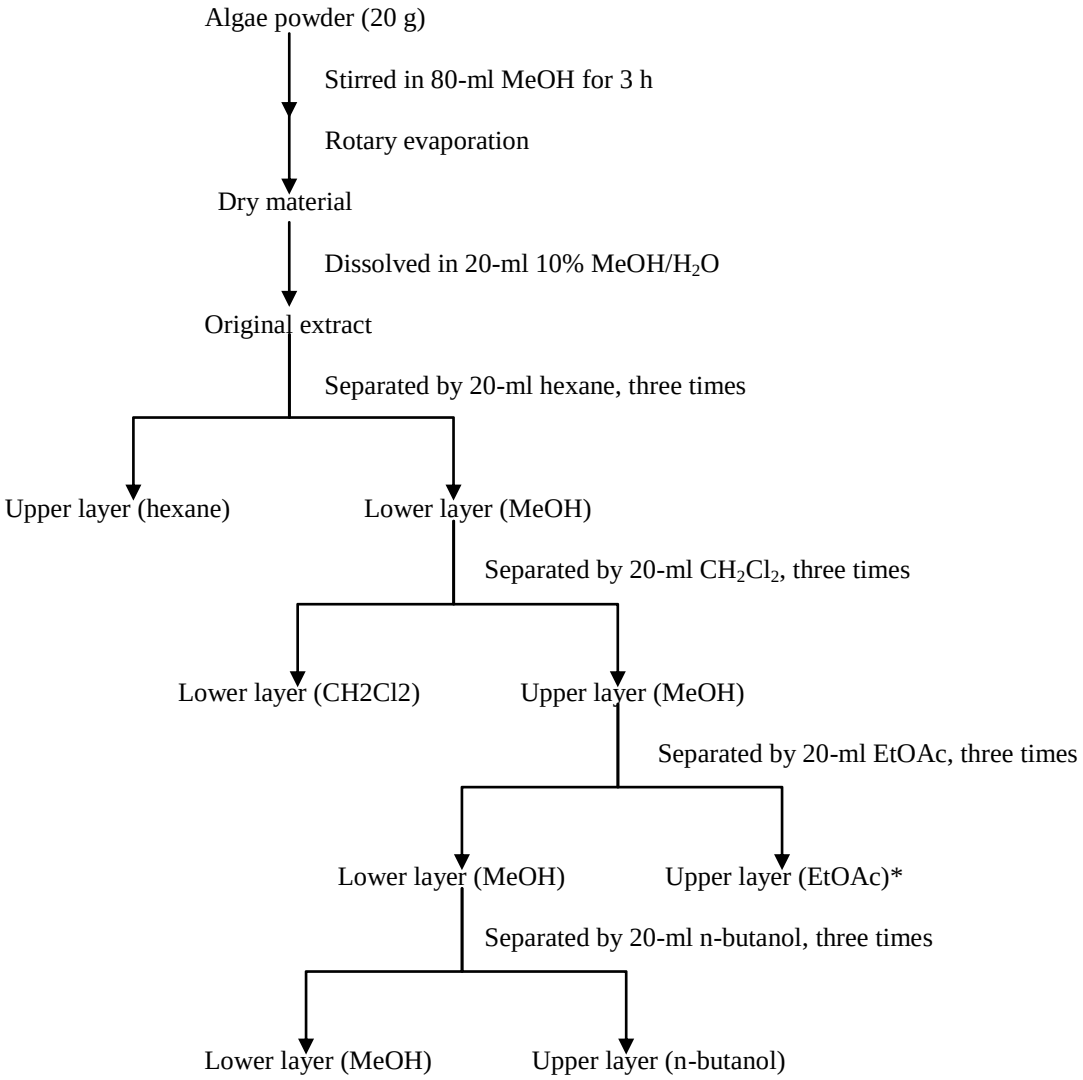

Figure 3. 2 The second extraction method for SP extraction (*represents fraction containing phlorotannin) (Eom et al., 2011; Kim et al., 2010; Nakai et al., 2006)

Algae powder residue left from solvent extraction was extracted with the method of Budhiyanti et al. (2011) to obtain the MP. First, 200-mg alga residue after SP extraction was dissolved in 8-ml 1 M NaOH, stirred for 2 h, and concentrated under 2400 g for 5 min. Then, the supernatant was neutralized with phosphoric acid and its MP content was tested.

The eMP extracts were obtained by extracting the MP extracts with a modified version of the first extraction method, in which ethyl ether was applied in place of ethyl acetate (Iwai, 2008). The liquid fractions containing phlorotannins obtained by these processes were evaporated with rotary evaporator and dried *in vacuum* under 60 ℃ for 24 hours to obtain the dried extracts of phlorotannins. The mass of dried extracts were measured to evaluate the yields of phlorotannin extracts. All samples were freshly prepared and tested within 24 hours. All chemicals and organic solvents were of analytical grade and purchased from Fisher Scientific (Pittsburgh, PA, USA).

3.2.2.2 DPPH radical scavenging activity test

DPPH free radical scavenging activity was measured using the method of Cox et al. (2010) with minor modifications. A 1 mg/ml phlorotannin solution was made by dissolving dried phlorotannin extracts in deionized water. Then the solution was diluted with deionized water by 10, 100, 1000, and 10,000 times to make a series of phlorotannin samples with various concentrations. A 152 μM DPPH radical solution was made by dissolving DPPH radicals in methanol. Then 0.1-ml phlorotannin sample was added to 0.1-ml of 152 μM DPPH radical solution. The reaction mixtures were incubated in the dark for 30 min at room temperature, and the optical density (OD) was measured at 517 nm using a BioTek 96-well microplate reader (Winooski, VT, USA). The DPPH test was performed in triplicate and the result was expressed as half maximum inhibitory concentration (IC_{50}) value (mg-extract/ml-water),

which was the phlorotannin concentration whose radical scavenging capacity was 50%. The ability to scavenge the DPPH radical was calculated with the following equations:

$$\text{Scavenging capacity (\%)} = 1 - \frac{A_{sample} - A_{sampleblank}}{A_{control}}$$

where $A_{control}$ is the OD of the DPPH solution, A_{sample} is the OD of DPPH solution with sample, $A_{sampleblank}$ is the OD value of the sample only. The DPPH radicals were purchased from Sigma-Aldrich CO. LLC. (St. Louis, MO, USA). Commercial antioxidants BHT and ascorbic acid were purchased from Fisher Scientific and their antioxidant activities were also measured for comparison purpose.

3.2.2.3 Total phlorotannin content test

Total phlorotannin content (TPC) was determined according to a modified version of Folin-Ciocalteu method, using phloroglucinol (PHG) as the standard (Cox et al., 2010). Samples were diluted taking into account the range of the standard curve. A 0.04-ml aliquot of the sample was mixed in a 1.5-ml centrifugation tube with 0.4-ml 1 N Folin-Ciocalteu reagent and 0.8-ml 20% Na_2CO_3. After standing for 3 min, the sample was incubated in the dark at room temperature for 45 min and centrifuged at 1600 g for 8 min. Optical density (OD) of the supernatant was measured at 730 nm using a BioTek 96-well microplate reader (Winooski, VT, USA). TPC test was performed in triplicate and the result was expressed as mg phloroglucinol equivalent per gram algae (mg PHG/g algae) using the following

calibration equation: Y = 2.3356X - 0.0544 (r^2 = 0.996), where Y is the OD at 730 nm and X is the concentration of phloroglucinol as the standard (mg/ml). Folin-Ciocalteu phenolic reagent and phloroglucinol were obtained from Sigma-Aldrich CO. LLC.. All other chemicals and organic solvents were obtained from Fisher Scientific.

3.2.3 Statistical analysis

The results of the present study were expressed as mean ± standard deviation. Statistical analysis was performed by one-way ANOVA and Tukey test with SAS (Cary, NC, USA). A p-value of 0.05 or less was considered significant.

3.3 Results and Discussion

3.3.1 DPPH scavenging activity of SP

The DPPH scavenging activity, expressed as IC_{50} value, is shown in Table 3.1. *A. nodosum* and *F. vesiculosus* had the strongest DPPH scavenging activity among the SP extracts of the five algae species by Method 1. Meanwhile, significant differences in antioxidant activity were observed between the two extraction methods for *A. nodosum* and *F. vesiculosus.* Method 1 was better on *F. vesiculosus* while Method 2 was better on *A. nodosum* in terms of antioxidant activity of SP. It indicated that extraction process had effects on the antioxidant activity of the extracts obtained, which agreed with the results of Turkmen et al. (2007), who reported that the antioxidant activity of black tea extracts were dependent on the solvent used

and length of the extraction processes. The IC_{50} values of *S. latissima,* 0.62 ± 0.06 mg/ml, was significantly lower than *L. digitata,* 0.74 ± 0.10 mg/ml. It suggested that SP extract of *S. litissima* was a better antioxidant than *L. digitata*. However, Cox et al. reported that the IC_{50} value of SP from *L. digitata* was much lower than *S. latissima* (Cox et al., 2010). A study investigating the DPPH radical scavenging activity of extracts from brown algae *Sargassum marginatum, Padina tetrastomatica* and *Turbinaria conoides*, used a method that was similar to the present study (Chandini et al., 2008). The IC_{50} values reported in their study were higher than those of *A. nodosum* and *F. vesiculosus* investigated in the present study, which suggested that *A. nodosum* and *F. vesiculosus* were better sources of antioxidants than *S. marginatum, P. tetrastomatica* and *T. conoides*. The DPPH radical scavenging activity of SP extracts from the two algae species were equal to or better than BHT (0.051 ± 0.0005 mg/ml) and ascorbic acid (0.0063 ± 0.0002 mg/ml).

Table 3. 1 DPPH radical scavenging activity of the SP extracts from the two extraction methods

Algae species	**IC_{50} value of Method 1 (mg/ml)**	**IC_{50} value of Method 2 (mg /ml)**
Ascophyllum nodosum	0.0072 ± 0.0010 (d, p)	0.0063 ± 0.0004 (q)
Fucus vesiculosus	0.0038 ± 0.0002 (d, s)	0.0077 ± 0.0001 (r)
Laminaria digitata	0.74 ± 0.010 (a)	
Alaria esculenta	0.09 ± 0.0024 (c)	
Saccharina latissima	0.62 ± 0.06 (b)	

The letters a, b, c, d indicate significant difference among the SP of the five algae species obtained by Method 1 (a>b>c>d); Letters p and q indicate significant difference between the SP of *Ascophyllum nodosum* obtained by the two extraction methods (p>q); Letters r and s indicate significant difference between the SP of *Fucus vesiculosus* obtained from the two extraction methods (r>s). A p-value of 0.05 or less was considered to indicate significant difference.

The correlation between TPC and IC_{50} values of SP extracts obtained by the first extraction method is shown in Fig 3.3. A strong correlation coefficient (r^2=0.98) was observed among the four algae species *A. nodosum, F. vesiculosus, S. latissima* and *L. digitata*, suggesting the algae species with higher SP content also had stronger antioxidant activities. Interestingly, when *Alaria esculenta* was considered in this correlation, the coefficient decreased to r^2=0.61. It indicated that some nonphenolic compounds, e.g. polysaccharides, with strong antioxidant activity were in the SP extract of *A. esculenta* thus further identification and antioxidant activity test is needed. Correlation between TPC and antioxidant activity of extracts from both macroalgae and terrestrial plants were reported in the literature. High correlation

coefficient (r^2=0.99) was found between TPC and DPPH scavenging activity between SPs of Icelandic seaweeds (Wang et al., 2009). Total phenols of wild berry fruits grown in southeast Serbia were found to correlate negatively with the IC_{50} values (Radovanovic et al., 2013). Similar results were also reported in the enzyme-assisted extract from grape residues (Gomez-Garcia et al., 2012). These studies suggested that polyphenols were the major antioxidants in macroalgae and terrestrial plants.

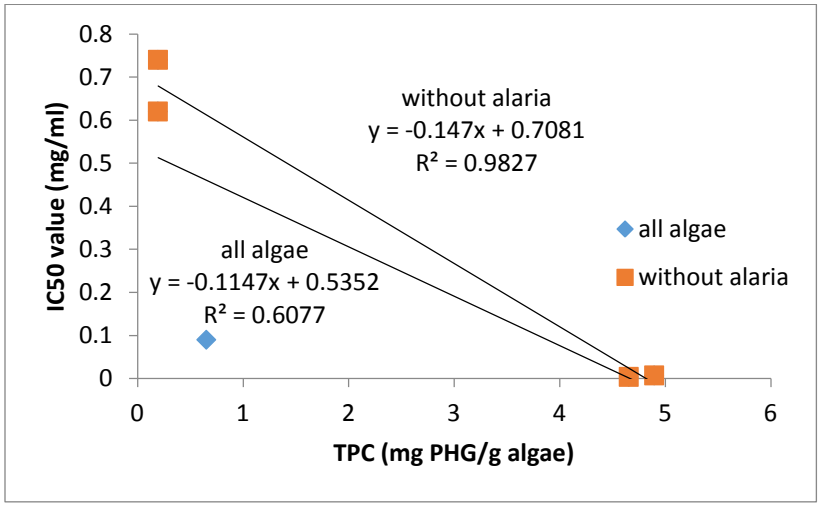

Figure 3. 3 The correlation between IC_{50} and SP content

The yields of SP extracts are shown in Table 3.2. The highest yield, 26.34 ± 3.54 mg extract/g algae, was obtained in *A. nodosum* by Method 2. However, the yield of the same algae by Method 1 was just 12.80 ± 1.37 mg extract/g algae. Interestingly, *F. vesiculosus* showed no differences in the yield between the two extraction methods. The yields of the three algae *A. esculenta, S. latissima* and *L. digitata,* were significantly lower than the other

two. Wang et al. reported that the solvents used had an impact on the yield of phenolic compounds from marine macroalgae. They also found that the yield of *S. Latissima* was low because of the high content of alginate in the extracts (Wang et al., 2009).

The Antiradical Power (ARP), which was $1/IC_{50}$, was used in the literature as the parameter evaluating antioxidant activity instead of IC_{50}. Wang et al. found that the ARP of *A. nodosum* and *F. vesiculosus* were higher than that of *A. esculenta, L. digitata* and *S. latissima* (Wang et al., 2009). However, ARP does not reflect the amount of extracts from the source material. In order to evaluate the potential of the brown algae extracts as natural antioxidant sources, a new parameter named Antioxidant Potential (AP), which considered both the yield and antioxidant activity of the extracts was applied to evaluate the potential of brown algae extracts as natural antioxidants.

$$\text{Antioxidant Potential (AP)} = \text{yield of extracts} \times \frac{1}{IC_{50}}$$

As shown in Table 3.2, the SP extracts of *F. vesiculosus* by Method 1 and *A. nodosum* by Method 2 had the highest AP values of 3903.51 ± 996 and 4180.42 ± 562 ml/g algae, respectively. The AP values of *A. nodosum* and *F. vesiculosus* were much higher than *A. esculenta, S. latissima* and *L. digitata,* which indicated their greater potential of usage as natural antioxidant sources.

Table 3. 2 The yield and antioxidant potential of SP extracts by both extraction methods

Extraction method	Algae species	Yield (mg-extract/g-algae)	Antioxidant Potential (ml-DPPH solution/g-algae)
Method 1	*Ascophyllum nodosum*	12.80±1.37b	1777.78±190.31s
	Fucus vesiculosus	14.83±3.67b	3903.51±996.04r
	Alaria esculenta	4.10±0.46c	45.56±5.09t
	Laminaria digitata	1.78±0.16c	2.41±0.22t
	Saccharina latissima	1.83±0.15c	2.96±0.25t
Method 2	*Ascophyllum nodosum*	26.34±3.54a	4180.42±561.96r
	Fucus vesiculosus	15.25±1.13b	1980.52±147.08s

*The letters a, b, c, and d indicate significant differences among SP yields in the order of a>b>c>d while r, s, and t indicate significant differences among the Ap in the order of r>s>t ($p<0.05$).

3.3.2 DPPH scavenging activity of MP and eMP

The DPPH scavenging activities of the MP extracts of *A. nodosum* and *F. vesiculosus*, which had the strongest antioxidant activities in their SP extracts, are shown in Fig 3.4. The IC_{50} values of MP extracts from *A. nodosum* and *F. vesiculosus* by Method 1 were lower than Method 2. The lowest IC_{50} value, which was 0.0047 ± 0.0002 mg/ml, was observed from *F. vesiculosus* by Method 1. However, the largest IC_{50} value, 0.0092 ± 0.0003 mg/ml, was also observed in the same algae by Method 2. It indicated that extraction methods had effects on the antioxidant activity of MP extracts. The chloroform applied in Method 1 may aid to preserve the antioxidants in the alga powder and resulted in stronger antioxidant property.

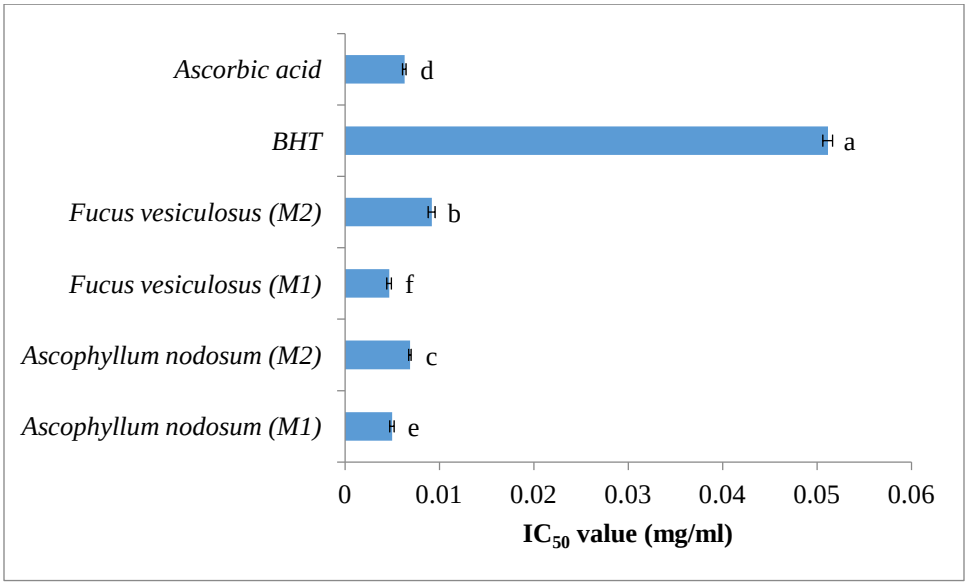

Figure 3. 4 IC_{50} values of the MP extracts (M1-Method 1, M2-Method 2) and commercial antioxidants. Different letters indicate significant differences ($p<0.05$) in the order of a>b>c>d>e>f. Lower IC50 values mean higher antioxidant activity.

The DPPH scavenging activities of eMP extracts of *A. nodosum* and *F. vesiculosus* are presented in Fig 3.5. *A. nodosum* had stronger antioxidant activity by both extraction methods than *F. vesiculosus.* It suggested that the radical scavenging activity of eMP from *F. vesiculosus* was not as strong as that of *A. nodosum.* Significant differences between the eMP extracts of *F. vesiculosus* obtained from the two extraction methods also suggested that extraction method had effects on the antioxidant activity of eMP extracts and Method 1 was the better method in terms of *F. vesiculosus* but Method 2 was better for *A. nodosum.*

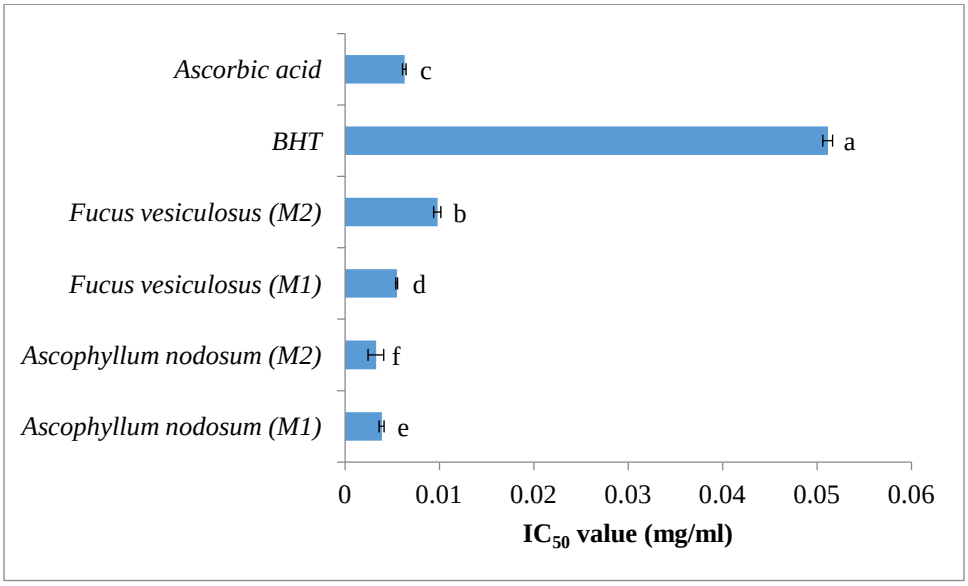

Figure 3. 5 IC_{50} values of the eMP extracts (M1-Method 1, M2-Method 2). Different letters indicate significant differences ($p<0.05$) in the order of a>b>c>d>e>f. Lower IC50 values mean higher antioxidant activity.

Yields and AP values of the MP and eMP extracts of *A. nodosum* and *F. vesiculosus* are shown in Table 3.3. Unlike the DPPH scavenging activity, yields of MP extracts were not very different from each other except for *F. vesiculosus*, whose yield by Method 1 was greater than by Method 2. Similar result was also observed in its eMP yields, with the eMP yield by Method 1 much greater than Method 2. These results suggested that Method 1 was better than Method 2 in terms of phlorotannin yields from *F. vesiculosus*. Surprisingly, the yields of MP and eMP extracts from *A. nodosum* were not significantly different between the two extraction methods, suggesting that extraction processes had no effects on the yields of MP and eMP of *A. nodosum.* For every algae and both extraction methods, the AP value of the eMP was lower than that of the MP extract, indicating better potential of MP than eMP as natural antioxidants.

Table 3. 3 The yield and antioxidant potential of MP and eMP extracts

Extraction method	**Algae species**	**Yield of MP (mg-extract/g-algae**	**Antioxidant Potential of MP (ml-DPPH solution/g-algae)**	**Yield of eMP (mg-extract/g-algae)**	**Antioxidant Potential of eMP (ml-DPPH solution/g-algae)**
Method 1	*A. nodosum*	26.39±0.24ab	5278.03±47.82o	7.68±0.50t	1969.53±128.73y
	F. vesiculosus	27.68±1.71a	5890.13±364.36o	24.11±1.56r	4382.64±283.22x
Method 2	*A. nodosum*	26.03±0.74ab	3772.91±107.80p	7.30±0.48t	2212.21±144.59y
	F. vesiculosus	25.45±0.73b	2766.02±79.03q	13.63±0.74s	1391.23±75.69z

*Different groups of letters, abcd, opq, rst, and xyz indicate significant difference ($p<0.05$) among yield of MP, AP value of MP, yield of eMP, and AP value of eMP, respectively, in the order of a>b>c>d, o>p>q, r>s>t, and x>y>z.

3.3.3 Comparison of the antioxidant activity among SP, MP, and eMP

The antioxidant activities of the three phlorotannin groups, SP, MP, and eMP, are presented in Table 3.4. In *A. nodosum*, the eMP extracts showed the strongest antioxidant activity while the SP extracts of *F. vesiculosus* were the strongest antioxidants by both extraction methods. It has been demonstrated in a previous study that high molecular weight phlorotannins had more potent antioxidant activities than the monomers (Gomez-Garcia et al., 2012). Thus phlorotannins with higher degree of polymerization and higher molecular weight might be in the SP of *F. vesiculosus* and eMP of *A. nodosum*. Surprisingly, the antioxidant activity of MP was stronger than eMP for *F. vesiculosus*, indicating that the purification process for

obtaining eMP might have removed non-phenolic compounds with strong radical scavenging activities.

Table 3. 4 Comparison of IC_{50} values among the three phlorotannin groups SP, MP and eMP obtained from the same extraction method and algae species

Phlorotannin group	**IC_{50} of Method 1**		**IC_{50} of Method 2**	
	Ascophyllum nodosum	***Fucus vesiculosus***	***Ascophyllum nodosum***	***Fucus vesiculosus***
SP	***	*	**	*
MP	**	**	***	**
eMP	*	***	*	***

Different numbers of "*" indicates the significant difference ($p<0.05$) among the SP, MP, and eMP groups in a column. Fewer "*" indicates smaller IC50 (stronger antioxidant activity) in the column. Comparisons were made among SP, MP, and eMP obtained from one algae by one extraction method.

Table 3.5 shows the AP values of the three phlorotannin groups. MP extracts were the highest among the three phlorotannin groups except for *A. nodosum* by Method 2. From this table it can be concluded that the MP was the phlorotannin group with the best potential to be applied as natural antioxidants. Compared with Table 3.4 it can be found that the phlorotannin group with strong antioxidant property may not be the best source of natural antioxidants when the yield of that phlorotannin is taken into consideration.

Table 3. 5 Comparison of AP values among three phlorotannin groups SP, MP and eMP obtained from the same extraction method and algae species

Phlorotannin group	**AP of Method 1**		**AP of Method 2**	
	Ascophyllum nodosum	***Fucus vesiculosus***	***Ascophyllum nodosum***	***Fucus vesiculosus***
SP	*	*	***	**
MP	***	***	**	***
eMP	**	**	*	*

Different number of "*" indicates the significant difference ($p<0.05$) among the SP, MP, and eMP groups in a column. The AP value with more "*" indicate greater AP in the column. Comparisons were made among SP, MP, and eMP obtained from one algae by one extraction method.

3.4 Conclusions

The SP extracts of *A. nodosum* and *F. vesiculosus* showed better antioxidant activity than that of *S. latissima, A. esculenta, and L. digitata*, indicating that there existed significant differences in the phlototannin contents among different algal species. The MP extracts showed the highest antioxidant potential among the three phlorotannin groups of SP, MP, and eMP, which suggested that obtaining phlorotannins from leftover residue after SP extraction could be worthwhile for isolation of natural antioxidants. The antioxidant activity and yield of phlorotannins were also found to be affected by the solvent extraction methods used. Method 1 was better than Method 2 to obtain phlorotannins with stronger antioxidant activity and higher yield.

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CHAPTER 4 Summaries and recommendations for future work

4.1 Summaries

The contents of soluble, membrane-bound, and extracted membrane-bound phlorotannins from five brown algae, *Ascophyllum nodosum, Fucus vesiculosus, Alaria esculenta, Laminaria digitata*, and *Saccharina latissima* were evaluated in this project. The effectiveness of extraction methods on phlorotannin yield was also examined. The following conclusions were drawn:

1. Significant differences in SP, MP, and eMP contents among the five algae ($p<0.05$) were observed, indicated that the phlorotannin content was algal species dependent. *F. vesiculosus* and *A. nodosum* showed the highest SP content (4.66 and 4.896 mg/g algae, respectively) by the first extraction method.
2. The highest MP content was observed in *A. nodosum* (68.55 mg/g algae) followed by *F. vesiculosus* (62.58 mg/g algae). Strong correlation between contents of SP and MP ($r^2=0.95$), and between SP and eMP ($r^2=0.81$) were found, which suggested that the algae species with high SP content also had high MP and eMP content, indicating these algae species could be worthwhile for harvesting value-added products from the leftover algae powder after SP extraction.
3. The effectiveness of extraction methods was algal species dependent. Method 1 was

better on *F. vesiculosus* but method 2 was better on *A. nodosum* in terms of SP yield, indicating that the effectiveness of extraction methods was algal species dependent.

The antioxidant activity of the three phlorotannin groups were also evaluated in this project. The following conclusions were drawn:

1. The SP of *F. vesiculosus* and *A. nodosum* showed the highest yield and the strongest DPPH radical scavenging activities among the five algae species. The two algae species could be worthwhile for SP production in the five algae species investigated.
2. The SP, MP and eMP extracts of *F. vesiculosus* and *A. nodosum* all showed equal or higher DPPH radical scavenging activity than commercial antioxidants butylated hydroxytoluene and ascorbic acid, indicating the great potential of replacing synthetic and commercial antioxidants with phlorotannins, which are natural antioxidants.
3. The MPs of *F. vesiculosus* and *A. nodosum* had great potential to be used as antioxidants due to the highest AP values (5890 and 5278 ml/g algae, respectively) among the three phlorotannin groups, SP, MP, and eMP.

4.2 Contributions to the field

This work was the first to compare the effectiveness of commonly used extraction methods and the first to investigate contents and antioxidant activity of the MP and eMP extract.

Yields of phlorotannins from one algae species can vary with the extraction methods used. The two solvent extraction methods have been widely used in previous research but their effectiveness on specific algal species has not been evaluated. This work revealed the fact the influence of extraction methods on phlorotannin yields and antioxidant activity was depending on the algae species, suggesting that specific extraction method should be developed for certain brown algae species to improve the yields and antioxidant activities of the phlorotannins, thus increase their value as potential nutraceuticals or medicine.

The large amount of algal powder leftover after solvent extraction for obtaining SP is a big problem in phlorotannin extraction. Obtaining value-added products, such as MPs, can serve as a good method to treat and make value from these leftovers. Contents of MP have been investigated in a few studies but their antioxidant activity and the more purified MP extract, which was the eMP in this project, were not investigated. From this work it can be concluded that both MP and eMP showed considerable contents and antioxidant activity, and had great potential to be applied as nutraceuticals or drugs. For certain algae species, the MPs might have even greater potential as antioxidant sources than eMP due to their stronger antioxidant activity and higher yield, which could save time and energy for purifying the MP extracts.

Antioxidant activity was the only parameter considered for evaluating extracts as potential antioxidants. However, the yield of the extract was not considered in evaluation of potential antioxidants. In this work, a new parameter named Antioxidant Potential was introduced to

combine the yield and antioxidant activity of extract in antioxidant evaluation, which can guide the selection of algal species and extraction methods.

4.3 Recommendations for future work

Some recommendations were made for the future research of phlorotannins:

1. Further identification and quantification of the compounds in the phlorotannin extracts is needed. For example, the eMP was supposed to be more pure and show stronger bioactivity than MP extract. However, the antioxidant activity of eMP of *F. vesiculosus* was not as strong as MP. Thus further analysis on the compounds is desired. Reverse-phase HPLC, mass spectrometer and nuclear magnetic resonance are needed for further isolation and identification of the compounds obtained.
2. More algae species should be investigated on their SP, MP and eMP. In this project, five algae species were studied but other algae species with considerable phlorotannin contents, e.g. *E. cava* and *E. bicyclis*, are deserved to be studied on their usage as MP sources by the alkali and acid treatment used in this project.
3. Other antioxidant activity such as ferric-reducing antioxidant power, ABTS radical scavenging activity, and oxygen radical reducing capacity could be tested together with DPPH radical scavenging activity to obtain a through profile of the antioxidant activity of phlorotannins.
4. Application of phlorotannin as food preservation and antimicrobial agents is

worthwhile. Further investigating of the antimicrobial effect on food spoilage microorganisms is recommended.

5. Phlorotannins have been reported to have health-benefiting effects such as anti-diabetes, anti-cancer, etc. Animal test and clinical trials are recommended in future studies to develop new drugs made of phlorotanins from brown algae.