

## ABSTRACT

KELLOGG, JOSHUA JAMES. Multifaceted Bioactivity of Alaskan Marine Algae Against Metabolic Syndrome Targets. (Under the direction of Dr. Mary Ann Lila.)

Coastal seaweeds are fundamental components of traditional diet and medicine for indigenous cultures worldwide, especially among Native American and Alaska Native (NA/AN) tribes and First Nations. Indigenous communities have consumed seaweed for generations, and these marine resources form an important part of the communities' traditional ecological knowledge, providing important nutrients as well as a source of novel bioactive phytochemicals. For this project, six species of coastal Alaskan seaweed were evaluated in multiple *in vitro* assay systems for their potential to attenuate underlying causes of diabetes and obesity, and the underlying phytochemistry that might play a role in the observed bioactivity was investigated.

Chapter 1 presents an overview of the potential role of Alaskan seaweeds in metabolic syndrome, summarizing the relevant literature and studies of the underlying causes of metabolic syndrome. A review of seaweed phlorotannins and their traditional ecological uses by NA/AN are briefly described in Chapter 2.

The first study (Chapter 3) examined the chemical antioxidant capacity of these macroalgae samples. The antioxidant capability was assessed by measuring radical quenching and ferrous iron chelation in a series of colorimetric assays. The highest radical scavenging activity was exhibited by *Fucus distichus* and *Saccharina groenlandica* fractions (IC<sub>50</sub> 4.29 – 5.12 µg/mL). *F. distichus* and *Alaria marginata* demonstrated the greatest

inhibitory potential of nitric oxide (NO) inhibition and radical oxygen species (ROS) generation in an *in vitro* macrophage culture. These results suggest that Alaskan seaweed extracts contain powerful antioxidant phytochemicals that could offset oxidative stress-related chronic health conditions.

A second study (Chapter 4) explored the inhibition of the carbohydrate-hydrolyzing enzymes,  $\alpha$ -glucosidase and  $\alpha$ -amylase. *F. distichus* and *A. marginata* significantly reduced enzyme activity, even more potent than the known pharmaceutical acarbose. *F. distichus* subfractions were potent mixed-mode inhibitors of  $\alpha$ -glucosidase and  $\alpha$ -amylase, with IC<sub>50</sub> values of 0.89 and 13.9  $\mu$ g/mL, respectively. The observed bioactivity of the *F. distichus* fractions was associated with the presence of fucophloroethol oligomers with degrees of polymerization up to 18 monomer units. These findings suggest that coastal Alaskan seaweeds are sources of  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitory phlorotannins, and thus have potential to limit the degradation of carbohydrates and alleviate postprandial hyperglycemic spikes.

Finally, investigations into the anti-inflammatory potential of Alaskan seaweed were carried out using multiple *in vitro* cell models (Chapter 5). Extracts from the brown seaweeds were most effective at reducing gene expression of five preliminary inflammatory markers. *F. distichus* was selected for further study, and subfractions FD-18 and FD-24 significantly inhibited an array of 12 inflammatory markers in LPS-induced macrophage cells. This phenomenon appeared to proceed via reduction in expression of toll-like receptor 4 (TLR4). In 3T3-L1 adipocytes, the active subfractions FD-18 and FD-24 lowered lipid

levels to 48.5% and 45.9% of the untreated control, respectively. The two subfractions also reduced inflammatory gene markers in a dose-dependent manner, while increasing adipogenic genes. The fraction FD-24 was found to contain higher phlorotannin oligomers (> 9 DP), while FD-18 contained a monoglycosyldiacylglycerol possessing two eicosatetraenoic acid (C20:4  $\omega$ -3) fatty acid residues. These results suggest that brown Alaskan seaweeds, especially *F. distichus*, contain multiple phytochemicals capable of ameliorating inflammation and are able to reduce the potential lipid accumulation in mature adipocyte cell systems.

Collectively, these findings suggest that Alaskan seaweeds classes of phytochemicals that modulate multiple pathways associated with the development of metabolic syndrome. The bioactive phytochemicals were capable of combatting oxidative damage, reducing metabolic overload through lowered digestive enzyme activity, and lowering the expression of chronic inflammation makers and inhibiting lipid accumulation in mature adipocytes. Thus, this thesis highlights the potential of Alaskan seaweed as a dietary agent to support human health and protect against insulin resistance and obesity.

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Multifaceted Bioactivity of Alaskan Marine Algae Against Metabolic Syndrome Targets

by  
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## DEDICATION

This work is dedicated to my wife Sarah, for her continued support, love, and the joy she brings to my life every day.

## BIOGRAPHY

Joshua James Kellogg was born in 1982 in Los Angeles, California. As a result of his parent's outdoorsy influence, Josh developed a passion and curiosity of the natural world, reveling in the annual family camping trips across the western United States and frequent hiking excursions around the LA area.

In high school, the first time he encountered chemistry, Josh knew he was hooked. It was a way of describing the entire world, from how a battery worked to how a plant's leaves changed colors in the fall to how the minute workings of a human cell all interact. With this in mind, Josh attended the University of California, Berkeley, which transformed his outlook on chemistry and future career decisions. While progressing to a Bachelor's Degree in chemistry, Josh also worked in Dr. John Arnold's organometallic chemistry lab. However, it was the class he took in his senior year, Medicinal Ethnobotany with Dr. Thomas Carlson, which changed his course a few years later.

Post-graduate, Josh worked at Bio-Rad Labs, but the call of academia brought him back to grad school, and in considering which programs and projects to pursue, the realm of ethnobotany came to the forefront of his mind. Blending botany, medicine, and chemistry, it seemed to have abundant potential for interesting, innovative research. Josh found Dr. Mary Ann Lila at the University of Illinois at Urbana-Champaign, and began a project looking at wild berries in Alaska, their chemistry and potential to affect diabetes and obesity.

As the berry project came to completion, and Dr. Lila was offered a new position at North Carolina State University, Josh jumped at the chance to tackle a new project for his

doctoral work. He remained centered on Alaska, working instead with marine algae and their more complex phytochemical composition, while also maintaining the work to evaluate the seaweed on their activity against diabetes and obesity. This project has yielded numerous fruitful collaborations, publications, and future directions for additional research.

During his graduate career, Josh has also been a part of the Global Institute of BioExploration, which works with indigenous cultures and scientists in developing nations to explore natural products and potential bioactive medicines in an ethical and reciprocal manner. He taught workshops to implement a research system of field bioassays in Alaska, North Dakota, Ecuador, Chile, Kenya, and Bhutan. This blend of ethnobotany, science education and outreach, and natural product discovery has left an indelible imprint on his research direction and passions.

Josh's move to North Carolina also brought about the serendipitous discovery of his future wife Sarah. Since 2011 they have embarked on a number of adventures together, canvassing the United States and Caribbean above and below the waves as they pursue diving, hiking, and natural science museums together. In 2014 they welcomed their daughter Isabelle (affectionately termed "little turkey") to their family, and Josh is looking forward to the next chapter of life's winding road.

## ACKNOWLEDGEMENTS

No scientific endeavor, however small, is brought to fruition in a vacuum. As scientists we are always searching with our eyes, reaching out with our hands and taking new strides with our feet, but there is always someone helping shed light to illuminate those eyes, point those hands in the right direction or supporting those steps when the path becomes twisted and barely perceptible. These words are a small gesture of thanks and appreciation to those who have made my journey possible.

First, to my love Sarah. One would scarcely dream to have such an amazing partner to accompany me through life, but I am lucky beyond belief to have found Sarah. Her unwavering love, patience, and encouragement always kept me going through the difficult times of graduate school, while her humor brightens my days and brings immeasurable fun and joy she brings to my life. I am forever grateful to have her by my side.

My mentor Dr. Mary Ann Lila. She epitomizes the sentiment of Ray Bradbury, who said, “The best scientist is open to experience and begins with ... the idea that anything is possible.” She encouraged my out-of-the-box project idea, and throughout the long duration she has been fully supportive of my projects, through thin funding and logistical nightmares, to conferences, publications, and the approaching culmination of my graduate work. I owe her a great debt of gratitude for her influence and guidance, and I have become the scholar I am today because of her.

The Lila lab. The members of this hard-working lab have been an invaluable source of knowledge, wisdom, and assistance throughout the project’s many iterations. Dr. Mary

Grace and her NMR and LC-MS expertise, Dr. Debora Esposito with her vast understanding of cell culture and gene expression, Dr. Gad Yousef and his HPLC wizardry. Lorie Solomon-Beale and Renetta Roberts for keeping the cells healthy and growing during the *in vitro* assays and endlessly extracting mRNA, Dr. Ivette Guzman and Nathalie Plundrich for being constant good friends, and awesome scientific sounding boards and idea factories when I hit roadblocks.

Our collaborators in Alaska have brought immense support and enthusiasm to this project throughout its duration. Dr. Gary Ferguson from the Alaska Native Tribal Health Consortium, and Renae Matheson of the Southeast Alaska Regional Health Consortium helped set up logistics and collection of the materials necessary. My committee members and their patience and wisdom in planning out the experiments and keeping the project on track. Other partners who deserve recognition include Carol Ann McCormick of the University of North Carolina Herbarium, and Janet Dow from the Flow Cytometry Facility at NCSU.

To everyone here, and to the multitudes who escape my notice at the present, I humbly thank you for everything you have done for this project, and for my growth as a scientist and a person.

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## LIST OF ABBREVIATIONS

ABTS	2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin-I converting enzyme
AMPKa	AMP-activated protein kinase a
ANOVA	Analysis of variance
ASCVD	Atherosclerotic cardiovascular disease
C/EBP	CCAAT-enhancer-binding proteins
CCAAT	Cytidine-cytidine-adenosine-adenosine-thymidine
CDC	Centers for disease control and prevention
CoA	Acetyl coenzyme A
COX	Cyclooxygenase
CRP	C-reactive protein
DAG	Diacylglycerol
DCF	2,7-dichlorofluorescein
DEX	Dexamethasone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
DPPH	Degrees of polymerization
ESI	Electrospray ionization
FBS	Fetal bovine serum

GLUT	Glucose transporter
H2DCFDA	2,7-dichlorodihydrofluorescein diacetate acetyl ester
HPLC	High performance liquid chromatography
IBMX	1-methyl-3-isobutylxanthine
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRS	Insulin receptor substrate
IT-TOF	Ion trap-time of flight analyzer
JAK	Janus kinase
JNK	C-Jun N-terminal protein kinase
LC-MS	Liquid chromatography-mass spectrometry
LDC	Low-digestable carbohydrate
LPL	Lipoprotein lipase
MCP-1	Monocyte chemoattractant protein-1
MGDG	Monoglycosyldiacylglycerol
NA/AN	Native american / alaska native
NEFA	Non-esterified fatty acid
NF- $\kappa$ B	Nuclear factor- $\kappa$ b
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NPLC	Normal phase liquid chromatography

NPLC-MS	Normal-phase liquid chromatography-mass spectrometry
PBS	Phosphate buffered saline solution
PDH	Pyruvate dehydrogenase
PFK	Phosphofructose kinase
PGE	Phloroglucinol equivalents
PI3-kinase	Phosphatidylinositide 3-kinase
PKC	Protein kinase C
PNPG	P-nitrophenyl- $\alpha$ -d-glucopyranoside
PPAR	Peroxisome proliferator-activated receptor
PTPase	Protein tyrosine phosphatase
ROS	Radical oxygen species
STAT	Signal transducer and activator of transcription
T2DM	Type 2 diabetes mellitus
TLR	Toll-like receptor
TNF- $\alpha$	Tumor necrosis factor alpha
TPC	Total polyphenolic content
UCP-1	Uncoupling protein-1
VCAM-1	Vascular-cell adhesion molecule-1

# CHAPTER 1 SEAWEED AND METABOLIC SYNDROME

## **Introduction**

Investigation into natural sources of pharmaceuticals, nutraceuticals, and cosmeceuticals and their impacts on human health has increased over the past couple decades due to heightened interest in achieving human wellness through natural means (Barnes *et al.*, 2004; Craig, 1999). The traditional ecological knowledge (TEK) of Native American and Alaska Native (NA/AN) communities provides a rich source of information to help identify and explore botanical sources of food and medicines.

The research presented herein was conducted in cooperation with a particular indigenous group – Alaska Natives (AN) – who have exhibited a strong predisposition towards diabetes and obesity in modern times. The central tenet of this thesis is to address whether the consumption of traditional resources, specifically marine seaweed, could serve as a potential candidate to address the dramatic rise in obesity and diabetes in these communities. In order to address this question, the biochemical mechanisms that modulate metabolic syndrome and the identity and structural characterization of phytochemicals responsible for the observed bioactivity were investigated.

### **Metabolic syndrome: a global health threat**

Metabolic syndrome was first described by Reaven (1988), who suggested that the risk factors of atherosclerotic cardiovascular disease (ASCVD) tended to cluster together and should thus be addressed as a coherent condition called *Syndrome X*. Since then, the concept of *Syndrome X* has matured into what is now commonly described as *metabolic syndrome*, the term used today to describe the five dominant clinical risk factors for ASCVD:

atherogenic dyslipidemia, raised blood pressure, elevated glucose levels, a proinflammatory state, and a prothrombotic state. Two major underlying conditions that contribute to metabolic syndrome are obesity and insulin resistance (also known as Type 2 diabetes) (NCEP, 2002).

The incidence of these two metabolic conditions has increased exponentially over the past several decades across the globe, but especially in the United States. Diabetes incidence has risen from 5.6 million cases in 1980 to 18.8 million in 2010 (CDC, 2011a), while obesity rates have increased to 35.7% of the total adult US population in 2010. These statistics result mainly from a dramatic shift in caloric consumption patterns, particularly with the rise of carbohydrates as a fundamental source of energy in the diet (Ogden *et al.*, 2012).

## **Role of macromolecular metabolism**

### **Fatty acid accumulation**

A diet high in fat and carbohydrates (Patel *et al.*, 2007) can lead to higher circulating concentrations of non-esterified fatty acids (NEFA) derived from lipoprotein lipase (LPL) action on very low-density lipoproteins (Lee *et al.*, 2006) and from lipolytic function on digested triglycerides (Carriere *et al.*, 1993). Adipocytes enlarge and become hypertrophic in an attempt to accommodate the influx of digested NEFA, yet the increased lipolysis associated with hypertrophic adipocytes translates into high NEFA levels in obese tissue via inhibited blocking of hormone-sensitive lipase (HSL) (Bjorntorp *et al.*, 1969; Milosavljevic *et al.*, 2003). The higher levels of NEFA in serum contribute directly to oxidative stress and inflammation, and are an initiator of insulin resistance (Boden, 2008).

Acute elevation of plasma NEFA can activate the proinflammatory NF $\kappa$ B pathway in multiple cell systems. By binding with toll-like receptor 4 (TLR4) on the cell surface of adipocytes and macrophages (Shi *et al.*, 2006), NEFA increased expression of several proinflammatory cytokines including tumor necrosis factor-alpha (TNF $\alpha$ ), interleukins 1 $\beta$  and 6 (IL-1 $\beta$  and IL-6), and increased circulating monochemoattractant protein 1 (MCP-1) (Boden *et al.*, 2005). Increases in serum NEFA results in intramyocellular accumulation of diacylglycerol (DAG) and activation of several protein kinase C (PKC) isoforms, leading to activation of I $\kappa$ B kinase, which is involved in the activation of the pro-inflammatory nuclear factor kappa-B (NF- $\kappa$ B) (Gao *et al.*, 2004). Thus, high NEFA concentrations yield DAG-mediated PKC activation, and may be an upstream effector of NF- $\kappa$ B activation and a culprit in the initiation of inflammation in obese tissue.

High plasma NEFA levels also contribute to the development of insulin resistance. In insulin-mediated cells (adipocytes, liver cells, and muscle cells), increased NEFA raises intracellular concentrations of DAG, which activates c-Jun N-terminal kinases (JNK) and serine phosphorylates the insulin receptor-1 (IRS-1), blocking the activity of phosphoinositol-3 (PI3) kinase and decreasing translocation of the glucose transporter 4 (GLUT4) to the cell surface for glucose absorption (Boden & Shulman, 2002; Shulman, 2000). Lower GLUT4 concentrations on the cell membrane result in higher circulating glucose levels, which consequently increase insulin synthesis and contribute to resistance development. In addition, an increased uptake of NEFA by cells inhibits glucose oxidation. According to the hypothesis put forward by Randle *et al.* (1994), there are two regulatory enzymes where inhibition occurs. First, an increase in acetyl coenzyme A (CoA) from

incorporation and oxidation of NEFA inhibits pyruvate dehydrogenase (PDH) via a feedback inhibitory loop, reducing the transportation of the end products of glycolysis (pyruvate) into the citric acid cycle. The accumulation of pyruvate, in turn, decreases the activity of glucose-6-phosphatase and halts glycolysis. The second process occurs in the oxidation of acetyl CoA to citrate, which is a negative regulator of phosphofructose kinase (PFK), the second step in the glycolytic pathway. These inhibitors slow down pathways of glucose oxidation, leading to higher concentrations of circulating glucose in the blood stream and hyperglycemia-induced insulin resistance (Savage *et al.*, 2005).

### **Gastric and pancreatic lipase**

The hydrolysis of dietary triglycerides to monoglycerides and NEFA initiates the metabolism of lipids in the digestive system, and is catalyzed by both gastric and pancreatic lipases. Pancreatic lipase accounts for 50-70% of the total gastrointestinal lipolysis *in vivo*, with gastric lipase representing an additional 10-30% (Carriere *et al.*, 1993). However, it has been discovered that the concentration and activity of lipase enzymes present in the human gastrointestinal tract is sufficient to break down a much larger quantity of triglyceride than is normally consumed (Carriere *et al.*, 2000), and the excess of lipases translates into rapid digestion of normal or high-fat meals. Digestion of dietary lipids represents the greatest contribution to serum concentrations of FFA and monoglycerides, which is positively correlated to the hypertrophy of adipocytes and onset of obesity (Verger, 1997); reduction of intestinal lipid digestion is related to a decrease in intra-abdominal fat content and obesity markers (Birari & Bhutani, 2007; Garza, 2005; Sternby *et al.*, 2002), making lipase a key target for anti-obesity prophylactic development (de la Garza *et al.*, 2011).

## **Carbolytic enzymes**

Postprandial enzymatic carbohydrate metabolism and the associated hyperglycemic spike are positively correlated with the development of insulin resistance via increased glucose oxidation and insulin secretion (Shulman, 2000). Two carbolytic enzymes include  $\alpha$ -glucosidase and  $\alpha$ -amylase.  $\alpha$ -Glucosidase is a membrane-bound enzyme that completes the final step in carbohydrate digestion by hydrolyzing disaccharides and small carbohydrate molecules to glucose and related monosacharides (Israili, 2011), while  $\alpha$ -amylase begins the process in the oral cavity by cleaving starch molecules into smaller polysaccharides for further digestion (Kwon *et al.*, 2008). Inhibitors of  $\alpha$ -glucosidase and  $\alpha$ -amylase can retard the cleavage of glucose from complex carbohydrates, delaying glucose absorption in the small intestine and consequently lowering the postprandial blood glucose spike. Therefore, such inhibitors can be an important strategy in the management of hyperglycemia linked to type II diabetes, (Lordan *et al.*, 2013), and can also decrease incidence of obesity, adipokine dysregulation, and cardiovascular diseases (Arakawa *et al.*, 2008). Several carbohydrate-targeting pharmaceuticals (such as acarbose) have been approved for anti-diabetic therapy by the FDA (Lordan *et al.*, 2013).

## **Chronic oxidation and inflammation in metabolic disorders**

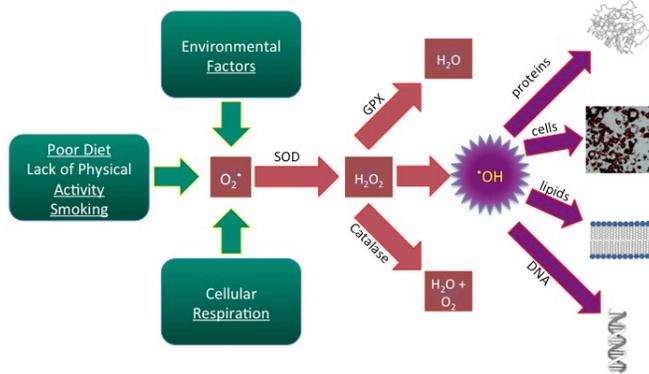
### **Oxidative stress**

Radical oxygen species (ROS) are ubiquitous, highly reactive derivatives of oxygen elicited by environmental stress, UV light exposure, and as a byproduct of regular oxidative cell function. While they are short-lived, the resulting oxidative damage is detrimental to multiple human tissues, including lipid membranes, proteins, and DNA. The human body

counteracts accumulation of ROS via quenching mechanisms such as superoxide dismutase, catalase, and the glutathione system (Figure 1.1), and uses exogenous phytochemical antioxidants like carotenoids, vitamins, and polyphenols from dietary sources as a second tier of defense against oxidative stress (Kaliyora *et al.*, 2006).

Oxidative stress is one mechanism through which obesity is induced in adipose tissue. The higher levels of circulating NEFA result in greater peroxisomal fatty acid metabolism, in which  $H_2O_2$  is formed as a byproduct, and, despite peroxisomes containing high catalase activity, the metabolic pathway increases ROS production and associated oxidative stress (Patel *et al.*, 2007).

There is also growing evidence that oxidative stress may contribute to the development of insulin resistance and type 2 diabetes mellitus (T2DM) (Evans *et al.*, 2005).



**Figure 1.1.** Generation, endogenous degradation, and toxicity of Radical Oxygen Species (ROS).

Chronic generation of ROS from lipid and glucose metabolism can lead to the activation of multiple serine kinase cascades and inhibition of protein tyrosine phosphatases (PTPases) (Salmeen *et al.*, 2003). These activated cascades increase the serine

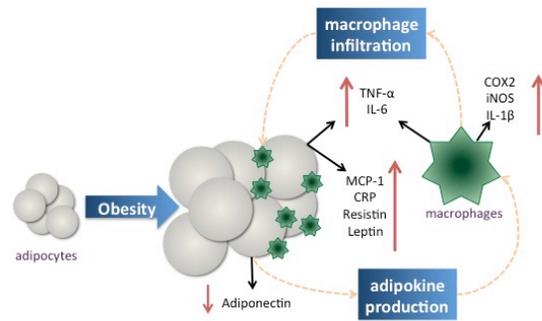
phosphorylation of IRS-1 and IRS-2, which are less able to associate with the membrane-bound insulin receptor as well as downstream target enzymes, such as PI3-kinase, ultimately leading to reduced insulin action (Hemi *et al.*, 2002; Paz *et al.*, 1997). These processes are ubiquitous across glucose-metabolizing cells, and thus oxidative stress can inhibit insulin action in muscle cells, adipocytes, and hepatic cells (Roberts & Sindhu, 2009).

### **Chronic inflammation in obese tissue**

Once thought to be a static depot for storage of triglycerides and fatty acids, adipose tissue is currently understood to be an active endocrine organ, secreting a variety of cytokines (“adipokines”) or lipid mediators (“lipokines”) that have systematic regulatory functions (Johnson *et al.*, 2012). As higher levels of lipid metabolism and storage induce adipocyte hypertrophy and hyperplasticity, proinflammatory adipokines are upregulated, resulting in a chronic inflammatory state.

The increase in adipocyte activity and pro-inflammatory adipokine secretions from obese tissue and the development of insulin resistance is intricately linked with the functionality of macrophage cells (Hsu *et al.*, 2013; Xu *et al.*, 2003). Increased lipid metabolism overloads adipocytes with triglycerides, resulting in hypertrophy of adipocytes and upregulation of MCP-1, which recruits macrophages to adipose tissue (Sartipy & Loskutoff, 2003; Weisberg *et al.*, 2003) (Figure 1.2).

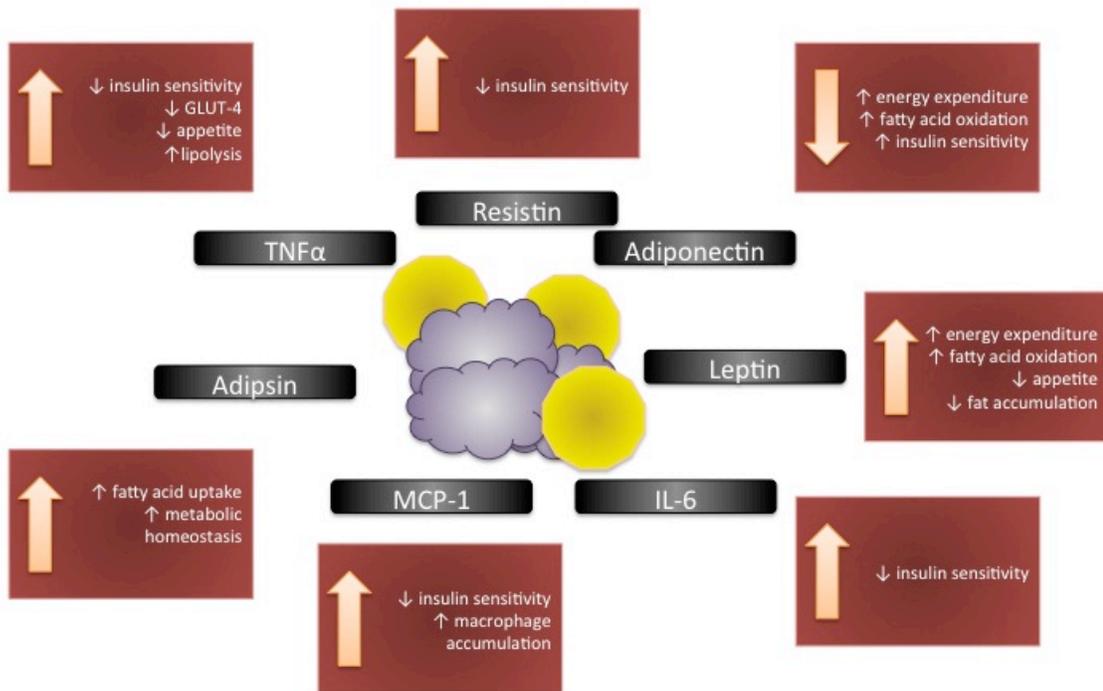
Macrophage accumulation in turn results in a paracrine cycle of inflammation in obese adipose tissue; secretion of TNF- $\alpha$  impairs triglyceride deposition (via blocking the effect of PPAR $\gamma$  in esterifying NEFA) and increases lipolysis (Hotamisligil *et al.*, 1993). TNF- $\alpha$  also activates NF- $\kappa$ B and increases expression of adhesion molecules to the cell surface of adipocytes. Adiponectin, which is a powerful anti-inflammatory adipokine inhibiting TNF- $\alpha$ , decreasing C-reactive protein (CRP) levels, and inhibiting adhesion of monocytes to endothelial cells, is decreased in the obese state (Sikaris, 2004). The confluence of macrophage and adipocyte proinflammatory cytokines leads to the increased dysregulation of insulin sensitivity mechanisms and leads to resistance.



**Figure 1.2.** Obese adipocytes initiate a paracrine cycle of inflammation, including the infiltration of macrophages into adipose tissue.

There are a host of adipokines secreted by adipocytes with multi-functional bioactivity in regulation of obesity and insulin resistance (Figure 1.3) (Fernández-Sánchez *et al.*, 2011). Leptin was the first adipokine primarily produced by adipocytes to be discovered, and is positively correlated with triglyceride accumulation. Higher circulating concentrations of leptin are associated with decreases in appetite (via activation of dopamine reuptake in the hypothalamus), stimulated lipolysis, and inhibition of lipogenesis via the Jak-STAT pathway

(Fonseca-Alaniz *et al.*, 2007). However, long-term exposure to high leptin concentrations is thought to lead to resistance to its energy homeostasis effects (Fonseca-Alaniz *et al.*, 2007). TNF- $\alpha$  is involved in increasing the release of NEFA in adipocytes by blocking triglyceride synthesis via PPAR $\gamma$ , and adiponectin increases the accumulation of triglycerides and NEFA in adipocytes by activating lipoprotein lipase (LPL) (Lastra *et al.*, 2006). Counteracting the effects of these pro-obese, pro-inflammatory adipokines, adiponectin inhibits endothelial NF- $\kappa$ B signaling by the c-AMP-protein kinase A-dependent mechanism, blocking inflammation pathways and increasing weight loss and fatty acid oxidation (Goldstein & Scalia, 2004; Sikaris, 2004). Adiponectin, however, is inhibited by higher concentrations of TNF- $\alpha$  and IL-6, both of which are secreted by inflamed adipocytes and macrophages (Figure 1.3) (Goldstein & Scalia, 2004; Haluzik *et al.*, 2004; Pyrzak *et al.*, 2010; Sikaris, 2004).



**Figure 1.3.** Summary of dominant adipokine secretion from adipocytes in an obese state. Large arrows indicate up/downregulation in obese tissue, with associated systemic effects listed adjacent.

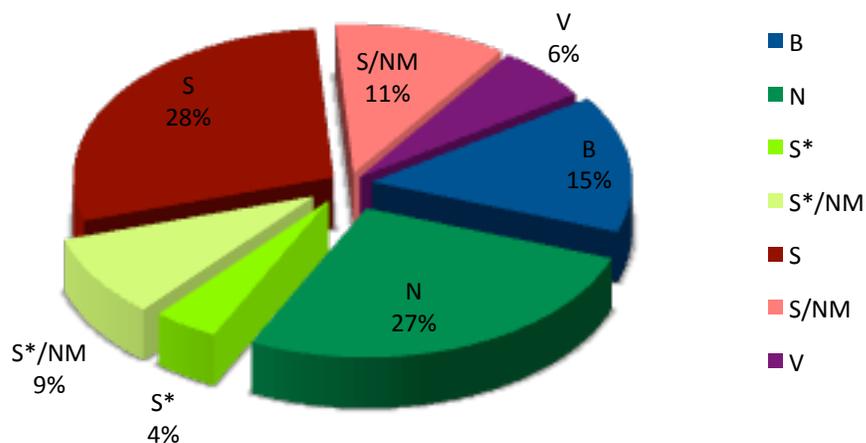
The development of insulin resistance is initiated and accelerated by many of the same regulatory adipokines in energy metabolism and lipid balance. In adipocyte and muscle cells, TNF- $\alpha$  blocks the tyrosine-phosphorylation of IRS-1 via the JNK pathway (Savage *et al.*, 2005), preventing the intracellular insulin signal cascade by blocking activation of PKC and interrupting the translocation of GLUT4 to the cell surface (Hotamisligil *et al.*, 1993). Resistin is produced by mature adipocytes, and is found to be a powerful link in the decrease of insulin sensitivity, though the mechanism remains to be fully elucidated (Dulloo *et al.*, 2010). IL-6 decreases insulin sensitivity via the Janus kinase (JAK) and Signal Transducer

and Activator of Transcription (STAT) mechanism, also blocking the phosphorylation of IRS-1. Adiponectin improves insulin sensitivity through increasing IRS-1 and PI3 kinase activity, though its activity is reduced by the secretion of increasing concentrations of other pro-inflammatory cytokines (Goldstein & Scalia, 2004; Pyrzak *et al.*, 2010). The process of chronic inflammation, and the resultant development of insulin resistance, is a complicated interchange between adipocytes and macrophages involving multiple signaling pathways.

### **Relevance of natural products and ethnobotanical bioexploration**

Despite the advances of synthetic and combinatoric chemistry, natural products and their derivatives remain the dominant paradigm in pharmaceutical, nutraceutical, and cosmeceutical development. Upwards of 66% of all approved pharmaceuticals in the three decades spanning from 1981 – 2010 had at their foundation a natural product, whether it was an isolated natural product, a large peptide or protein, or a combinatoric, semi-synthetic, or synthetic preparation based upon natural product inhibitors or substrates (Figure 1.4) (Newman & Cragg, 2012). Ganesan commented that combinatorial chemistry's greatest successes have, "come from improving an existing lead, rather than from discovering the initial lead" (Ganesan, 2004). These drugs represent agents capable of treating all known categories of medical conditions, and highlight the continued need to explore natural sources of chemicals that might serve as leads or scaffolds for future pharmaceutical candidate development.

There are four main methods through which natural resources can be sampled for screening: (1) random selection of plant material followed by chemical screening for classes of secondary metabolites (alkaloids, cardiac glycosides, etc.); (2) random selection with subsequent assaying of all gathered plant portions against test pathogens or cellular targets



**Figure 1.4.** Sources of pharmaceuticals 1981-2010 (adapted from Newman and Cragg, 2012). Codes are as follows: B-“biologics” (a large peptide or protein isolated from an organism or produced via biotechnological means); N-“natural product” (including natural product derivatives); S\*-“semi-synthetic” (made by total synthesis, but the active pharmacore is of natural origin); S\*/NM-“semi-synthetic natural mimic” (obtained from total synthesis of combinatorics, but based upon natural product inhibitors or substrates); S-“synthetic” (totally synthetic drug found by random screening or modification of existing agents); S\*/NM-“synthetic natural mimic” (totally synthetic drug based upon natural product inhibitors or substrates); V-“vaccine”.

for potential activity; (3) literature-based screening with biochemical or *in vitro* testing to confirm bioactivity; and (4) prioritization based upon historical or current documentation of ethnomedical or traditional uses of bioactive plants against human health ailments (Fabricant & Farnsworth, 2001). In fact, ethnobotanical knowledge can serve as a guide for elucidating

novel compounds with comparable or superior activity, offering enhanced pre-selection of lead candidates to pursue in a laboratory setting. In Farnsworth and Kaas' seminal paper, the authors screened 750,000 botanical samples for potential anti-tumor activity. They demonstrated that if a sample was linked to a traditional use such as "good for cancer", the probability of finding an active sample was twice that of random sampling (Farnsworth & Kaas, 1981). Similarly, a Brazilian study in 1988 tested plant species for *in vivo* antimalarial activity, and ethnobotanical information again increased the likelihood of discovering an active lead. Random selection yielded a 3% hit rate; when ethnopharmacological criteria were included in selecting the plant samples, the hit rate rose to 18%, orders of magnitude above the rates generated by large-scale HTS studies (Brandao *et al.*, 1992).

Thus, drug discovery programs have increasingly sought natural sources for pharmaceutical candidate leads, whether to obtain new treatments (Dias *et al.*, 2012), or decrease the deleterious effects of current therapy regimens (Etxeberria *et al.*, 2012). These investigations have been carried out not only for infectious agents, but for debilitating chronic health conditions, such as inflammatory diseases and metabolic syndrome (Newman & Cragg, 2012).

## **Alaskan seaweed and NA/AN communities**

### **Metabolic syndrome and NA/AN populations**

Native American and Alaskan Native (NA/AN) populations suffer disproportionate rates of the two dominant risk factors for metabolic syndrome, insulin resistance and obesity. While incidence rates vary between tribal groups, the CDC reports that NA/AN, with a 16.1% incidence rate of type 2 diabetes, are over two times more likely to have diabetes as

non-Hispanic Whites (CDC, 2011b), and rates have increased 68% from 1994-2004 (CDC, 2009). Obesity rates are also substantially higher for NA/AN populations; 40.8% of NA/AN adults are classified as obese, a rate 60% higher than non-Hispanic Whites (CDC, 2012). These higher rates of obesity are present in children as well; one in three NA/AN children is overweight or obese by the age of five, with 48.3% of 10-17 year olds, a rate that has increased nearly 2% since 2003 (CDC, 2009; Ness *et al.*, 2012).

Several different socio-economic influences have been suggested as underlying causes for the dramatic increase in obesity and diabetes incidence among AN populations. One is the transition away from a traditional subsistence diet toward an increasingly commercial and commodity-based Western diet. The traditional diet of many AN populations emphasized marine sources of protein remarkably high in omega-3 fatty acids, yet the historic prevalence of chronic disease was comparatively low (Adler *et al.*, 1994; Ebbesson *et al.*, 2005; Parkinson *et al.*, 1994). Into the 1980s, AN consumed more kilocalories, protein, fat, carbohydrates, iron, vitamin A, and vitamin C than the general U.S. population, and also six times more fish (Nobmann *et al.*, 1992). However, over the past several decades, AN dietary patterns have shifted. Many traditional foods have been replaced by processed food items, leading to an increasingly inadequate nutritional intake: 63% of Yup'ik Eskimos surveyed had poor quality diets (Bersamin *et al.*, 2006). This dietary evolution favoring a more Western pattern has generally led to diets with higher carbohydrate intake, higher saturated and trans fatty acids, and lower poly-unsaturated fatty acids (Bersamin *et al.*, 2008), and yielded a more risky nutritional profile favoring the development of obesity, diabetes, and the metabolic syndrome. Evidence to support this

theory can be found in remote, isolated AN communities, which eschew commodity diets and remain faithful to traditional foodstuffs, and do not experience the same rise in chronic disease incidence rates (Gahagan & Silverstein, 2003).

A second factor suggested for the sudden rise in obesity and diabetes in NA/AN populations is the shift to a more sedentary lifestyle and lowered physical activity. There is substantial evidence in NA/AN samples of a positive association between physical activity and overall health (Coble & Rhodes, 2006; Poltavski *et al.*, 2010), yet as NA/AN communities have become increasingly exposed to Western culture, they have lowered their physical activity levels; currently, NA/AN communities engage in less leisure-time physical activity (only 54% are physically active regularly) than their nonminority counterparts (62%) (CDC, 2000; Duncan *et al.*, 2009). While youth generally had higher levels of physical activity than their elders (Coble & Rhodes, 2006), lowered physical activity has been demonstrated to be prevalent in the newest generations, with decreased neighborhood support, lower sports participation, and higher television viewership (Ness *et al.*, 2012). The shifts in physical activity paralleling the introduction and dominance of Western influence on NA/AN communities is thus another risk factor that has led to the increased levels of diabetes and obesity in the overall population.

### **Traditional consumption by NA/AN**

One potential antidote for this increasing trend toward metabolic syndrome in AN populations may be encouraging a renewed interest in traditional marine resources for food and medicine. Marine macroalgae have been harvested as a source of food, livestock fodder, and pharmaceuticals by traditional cultures across the globe, especially communities situated

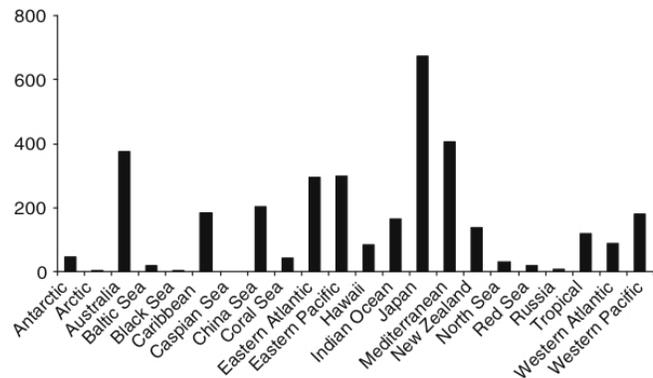
around the Pacific Rim (Jiménez-Escrig *et al.*, 2012; Widjaja-Adhi Airanthi *et al.*, 2011). From Arctic Alaska to the Pacific Northwest, seaweeds have played a large role in the traditional cultures of multiple NA/AN and First Nations communities, as a ubiquitous source of macro- and micronutrients (Garza, 2005; Turner & Bell, 1973; Turner, 2003). Seaweeds have evolved into an important part of the communities' traditional ecological knowledge, evident in the variety of ways they are incorporated into traditional diets. First Nations in British Columbia cook species of the red alga *Porphyra* with clams, salmon eggs, or fish in soups, toast the thalli as a snack, or sprinkle dried seaweed over other foods (Turner, 2003), and nearly 60% of the households of the Inuit community of the Canadian Arctic's Belcher Islands regularly consume *Rhodomyenia* spp. and *Laminaria* spp. (Wein *et al.*, 1996).

## **Seaweeds as a source of natural products**

### **Chemical constituents**

Algae are one of the most biologically diverse groups of organisms on the planet, with approximately 50,000 species of algae worldwide, and yet only 5-10% have been systematically explored (Li & Chen, 2001). Due to the competitive environment in which algae exist, they have developed unique defensive and adaptive bioactive compounds. Over 3,300 of these metabolites have been isolated since 1975 from waters across the world (Figure 1.5). However, bioexploration of algae from northern waters of the Pacific and Arctic remain under-represented (Ioannon & Roussis, 2009).

Seaweeds contain high levels of essential vitamins and minerals, especially trace elements and minerals, which are in greater abundance in seaweeds compared to terrestrial foodstuffs (MacArtain *et al.*, 2007). In addition to dietary macronutrient components,



**Figure 1.5.** Geographic distribution of algal metabolites discovered since 1975 (from Ioannon & Roussis, 2009).

algae species synthesize bioactive secondary metabolites, with representation from all major natural product classes. Seaweed produce polysaccharides (phycocolloids) (Ale & Meyer, 2013), carotenoids (Miyashita *et al.*, 2011; Takaichi, 2011), alkaloids (Güven *et al.*, 2010), and peptides (Fitzgerald *et al.*, 2011). The dominant class of compounds, however, is polyphenols, which can account for as much as 20% of the dry weight of the seaweed (Ragan & Glombitza, 1986). The myriad compounds present in algae have demonstrated to possess numerous bioactive properties, including efficacy against inflammation and metabolic syndrome.

### **Antioxidant capacity**

Tidal seaweeds are endemic to a highly competitive and stressful environment, and have evolved a diverse antioxidant arsenal of defenses to combat the constantly changing abiotic conditions. Fluctuating ocean levels, common to intertidal ecosystems, repeatedly subject

seaweed to exposed and submerged conditions, with alternative high/low ultraviolet (UV) light and oxygen levels that necessitate adaptive defense mechanisms (Blanchette, 1997; Peteiro & Freire, 2011). Algal phlorotannins provide UV-protection, with biosynthesis of the metabolites up-regulated by high levels of incident radiation; phlorotannins are even exuded into the surrounding sea medium to further bolster protection against UV radiation (Bischof *et al.*, 2006; Pavia *et al.*, 1997; Schoenwaelder *et al.*, 2003).

The same bioactive phytochemicals accumulated by the seaweed to resist environmental stressors, once ingested, are relevant to human health maintenance (Bischof *et al.*, 2006; Bunsom & Prathep, 2012). Seaweed extracts demonstrate strong antioxidant properties (O'Sullivan *et al.*, 2011; Wang *et al.*, 2012; Wang *et al.*, 2009), leading to a growing interest for their use in food technology applications to prevent oxidation of lipids and proteinaceous tissue (Wang *et al.*, 2009), and to reduce the effects of radical oxygen degradation in chronic diseases (Kim *et al.*, 2008b; Zhang *et al.*, 2007).

### **Lipid-lowering capabilities**

The biochemical constituents of seaweed have been shown, in both *in vitro* and clinical studies, to possess the ability to alter the profile and metabolism of lipids and regulate homeostasis of adipose tissue. Working to inhibit the digestion and breakdown of dietary lipids, ethanolic extracts of the tropical red alga *Kappaphycus striatus* reduced lipase activity by 92 % (Balasubramaniam *et al.*, 2013), and a screening of methanolic extracts 54 species of Phaeophyta, Rhodophyta, and Chlorophyta by Bitou *et al.* (1999) revealed lipase inhibitory levels of up to 100% for *Hypnea charoides*, *Asparagopsis taxiformis*, and *Caulerpa toxifolia*. Caulerpenyne, isolated from the active extract of *C. toxifolia*, reduced

plasma triglyceride levels in a rat model by >60 % (Bitou *et al.*, 1999). Extracted phlorotannins fucofuroeckol A and 7-phloroeckol, from the brown alga *Eisenia bicyclis*, also showed potent inhibition of pancreatic lipase with IC<sub>50</sub> values of 37.2 ± 2.3 and 12.7 ± 1.0 μM, respectively (Eom *et al.*, 2013), demonstrating how phytochemicals from all three major phyla of seaweed are capable of reducing lipase activity.

The few performed clinical studies have also shown positive benefits on lipid profiles from seaweed supplementation; one study found that adding 48 g/day of powdered seaweed decreased serum concentrations of triglycerides while increasing high-density lipoprotein cholesterol (p<0.05) (Kim *et al.*, 2008b). More evidence has been obtained using 3T3-L1 cellular models for study of differentiation mechanisms from preadipocytes to adipocytes and fatty acid accumulation in mature adipocytes. Seaweeds have numerous positive effects on adipocyte cells affecting their lipid accumulation and adipokine secretion. Analysis of seaweed effects on adipocytes revealed an increase in circulating monounsaturated fatty acids, without affecting saturated fatty acid concentrations, in a dose dependent manner (He *et al.*, 2009), and an overall decrease in accumulated lipids within adipocyte vesicles (Seo *et al.*, 2013). Extracts of the red algae *Gracilaria verrucosa* and *Grateloupia lanceolata* inhibited lipid accumulation by reducing mRNA levels of differentiation transcription factors PPARγ and C/EBPα (Seo *et al.*, 2013; Woo *et al.*, 2012); these transcription factors were also reduced by fucoidan, a polysaccharide found in the brown alga *Undaria pinnatifida* (Kim & Lee, 2012). *U. pinnatifida* also contains the carotenoid fucoxanthin, which was shown to significantly decrease weight of the white adipose tissue (WAT) in a mouse model, and clearly express mitochondrial uncoupling protein 1 (UCP-1) which regulates fatty acid

oxidation (Maeda *et al.*, 2005). Thus, algal bioactives are capable of regulating lipid metabolism and homeostasis, at the *in vitro* and clinical level.

### **Hyperglycemia modulation potential**

Seaweed extracts and supplements have demonstrated the ability to alleviate diabetic symptoms, improve response to insulin, and decrease hyperglycemia in several clinical and animal studies. Zhang *et al.* (2007) found a crude extract and polyphenol-enriched fraction from *Ascophyllum nodosum* improved fasting serum glucose levels in a diabetic mouse model. The enriched fraction also lowered the spike of postprandial serum glucose. This result was also found in another study using supplementation with diphlorethohydroxycarmalol, isolated from *Ishige okamurae*, which significantly reduced the insulin incremental area under the curve in streptozotocin-induced mice (Heo *et al.*, 2009). Human clinical trials have evidenced similar results: compared against a placebo, consumption of *A. nodosum* and *Fucus vesiculosus* was associated with a 12.1% reduction in the insulin incremental area under the curve, and a 7.9% increase in insulin sensitivity (Paradis *et al.*, 2011). Kim *et al.*'s study, noted above, also determined that the 48 g/day seaweed supplementation significantly decreased fasting blood glucose levels and 2-hour postprandial blood glucose measurements (Kim *et al.*, 2008b).

The mechanisms underlying these antidiabetic properties are still under investigation, but several different hypotheses have been proposed. One main hypothesis is the translation of ecological functions of seaweed phytochemicals into mammal systems. Seaweeds have been shown to combat herbivorous predation by constitutive and inducible defensive cocktails designed to reduce consumption of damaged tissues or to increase the exposure of

assaulting herbivores to predators (Bischof *et al.*, 2006; Oliveira *et al.*, 2013). In particular, macroalgae are capable of preventing herbivorous destruction by inhibiting the digestive enzymes of their predators, including glucanases of marine invertebrates (Agarkova *et al.*, 2007; Ermakova *et al.*, 2001), and  $\alpha$ -fucosidase,  $\beta$ -galactosidase and  $\beta$ -mannosidase from the shell *Turbo cornutus* (Shibata *et al.*, 2002). These phytochemicals are also responsible for significant inhibition of mammalian digestive enzymes, most notably  $\alpha$ -glucosidase and  $\alpha$ -amylase. Multiple species of red and brown algae have demonstrated inhibitory effects against these carbolytic enzymes (Eom *et al.*, 2012; Kim *et al.*, 2008a; Lordan *et al.*, 2013), and a recent study by Kawamura-Konishi *et al.* (2012) isolated a phlorotannin from *Sargassum patens* that had a significantly lower 50% inhibitory concentration (IC<sub>50</sub>) for  $\alpha$ -amylase compared to the commercial inhibitor acarbose. Correlation between enzymatic and animal studies has demonstrated a positive relationship between inhibiting  $\alpha$ -glucosidase and increasing basal uptake of glucose and lowering post-prandial glucose levels (Kim *et al.*, 2008b; Zhang *et al.*, 2007). More limited evidence is also present for the ability of seaweed extracts to affect glucose transporter systems at the cellular level; an extract from the red alga *Gracilaria verrucosa* increased glucose uptake, GLUT4 expression, and AMPKa phosphorylation compared in 3T3-L1 adipocytes (Woo *et al.*, 2012). Thus, there is increasing evidence that seaweed extracts have the ability to augment the absorption and digestion of carbohydrates, which can lower postprandial serum glucose spikes in the blood stream.

## Objectives

This project aims to explore the bioactive properties of edible, traditionally consumed Alaskan coastal seaweed species and a coastal plant species and their efficacy in modulating multiple cellular and biochemical targets to offset diabetes and obesity.

Oxidative stress is a dominating factor in the development of insulin resistance and hypertrophy in adipocytes and macrophages. The chemical antioxidant capacity was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferrous ion chelating, and nitric oxide (NO) inhibition assays. *In vitro* inhibition of radical oxygen species (ROS) generation and NO synthesis was evaluated in a RAW 264.7 macrophage culture.

Regulation of metabolism was investigated through a series of high-throughput assays to ascertain inhibition of enzymes that promote digestion of high-energy macronutrients, such as the carbohydrate-hydrolyzing enzymes  $\alpha$ -glucosidase and  $\alpha$ -amylase. In addition, insight into the molecular interaction of inhibitors with inhibitor kinetic data yielded greater information as to the mechanism by which these compounds impact digestion and metabolism.

In an *in vitro* adipocyte cell system, the ability of seaweed extracts and phytochemicals to lower the lipid accumulation in mature adipocyte cells and the production of adipokines that promote obesity and inflammation was assayed, as well as their activity in depressing inflammatory pathways in macrophage cells by monitoring expression of biomarkers for inflammation-related cytokines that regulate prostaglandin synthesis, ROS production, and systemic and chronic inflammation. Relative levels of gene expression were

correlated with relevant physiological biomarkers to evaluate their impact upon the pathways of inflammation and obesity using adipocytes stressed with TNF- $\alpha$ .

In order to fully investigate candidates for clinical efficacy and implementation into drug discovery programs, the structural determination of any active constituents is a key aspect of natural product bioexploration and research. It is through the characterization of bioactive phytochemicals of seaweed that evidence for mechanisms of action are uncovered, such as enzyme inhibition, cellular receptor interference, or other downstream processes. Thus, a final objective of the project was the determination of the chemical composition of the selected seaweed species, including the specific characterization of any active metabolites.

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## CHAPTER 2 PHLOROTANNINS IN SEAWEED

## **Ethnobotany and ethnopharmacology**

Marine seaweeds have been traditionally harvested as a source of food, livestock fodder, and pharmaceuticals by coastal communities across the globe, especially those situated around the Pacific Rim (Widjaja-Adhi Airanthi *et al.*, 2011). From arctic Alaska to the temperate Pacific Northwest, seaweeds have played a large role in the traditional cultures of multiple Native American/Alaska Native (NA/AN) and First Nation communities, including the Haida, Inuit, Tlingit, Tsimshian, Eyak, and Alutiiq peoples, as a ubiquitous source of macro- and micronutrients (Garza, 2005; Turner & Bell, 1973). Indigenous populations have harvested and consumed red, brown, and green seaweeds for generations, and these marine resources form an important part of the communities' traditional ecological knowledge.

Communities in Alaska's Bristol Bay region consumed *Fucus* spp. (Figure 2.1) and *Macrocystis* spp. covered with herring eggs as a springtime delicacy. First Nations in British Columbia prepare soups with species of the red alga *Porphyra* and clams, salmon eggs, or fish. The thalli of red and brown algae, including *Porphyra* spp. and *Laminaria* spp., are dried and toasted to eat as a snack or sprinkled over other foods (Turner, 2003). Seaweeds are also gathered and fermented to improve their shelf life for long-term use, then reconstituted as a soup flavored with oulachen oil (Turner & Bell, 1973). To this day, nearly 60% of Inuit households among the Canadian Arctic's Belcher Islands regularly consume *Rhodomenia* spp. and *Laminaria* spp. (Wein *et al.*, 1996).

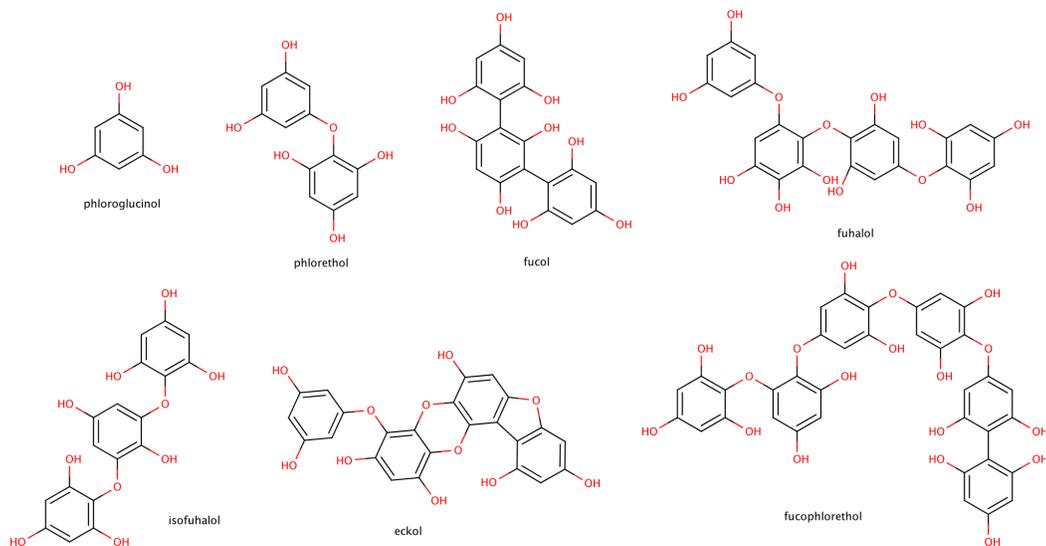


**Figure 2.1:** Fresh *Fucus distichus* ("bladder wrack" or "popweed") harvested near Whittier, Alaska, USA.  
Photo by author © 2013.

In addition to their consumption as a part of traditional diets, brown and red seaweeds have been utilized as part of many communities' ethnomedical pharmacopeia. For example, hot baths of *Fucus* spp. were employed with yellow cedar boughs (*Chamaecyparis nootkatensis*) in order to create a therapeutic steam to help cure bronchial infections and rheumatism (Boas, 1966). In addition, *Fucus* were chopped and heated in conjunction with dried tobacco (*Nicotiana* spp.), alder bark (*Alnus rubra*), and twinberry cuttings (*Lonicera involucrata*) and applied as a poultice compress to relieve inflammation, aches, and pains (Turner & Bell, 1973). Similarly, children with sores or itchy scabs were rubbed with *Fucus* thalli followed by catfish oil and burnt red ochre, or with strips of the brown seaweed *Nereocystis luetkeana* (Boas, 1966). The *N. luetkeana* was also applied externally to the stomach of a pregnant woman to ease childbirth, as that the child would become as slippery as the seaweed. The green seaweed *Ulva lactuca* was mixed with twinberry bark and applied to a woman's breasts after delivery to relieve soreness and inflammation (Turner & Bell, 1973).

## Chemistry and bioactivity

Seaweeds, especially brown algae, have been shown to contain high levels of polyphenols, which can account for as much as 20% by dry weight of the seaweed (Ragan & Glombitza, 1986). In brown seaweeds, the predominant polyphenols are a family of tannin-like structures known as phlorotannins, with nearly 150 unique phlorotannin structures identified which range from 126 to over 500,000 Da (Martínez & Castañeda, 2013). Phlorotannins are oligomeric constructs based upon the monomer phloroglucinol (1,3,5-trihydroxybenzene) which is biosynthesized via the polyketide pathway (Meslet-Cladière *et al.*, 2013). The polymers are primarily stored in the thalli and their composition and quantity exhibit internal, geographic and temporal variability. The monomeric units are linked through aryl-aryl bonds and diaryl-ether bonds forming four phlorotannin subgroups differentiated by their means of linkage: phlorotannins with an ether bridge (fuhalols and phlorethols), with an aryl-aryl linkage (fucols), those with ether and phenyl links (fucophlorethols), and phlorotannins with a dibenzodioxin linkage (eckols and carmalols) (Glombitza & Pauli, 2003) (Figure 2.2).



**Figure 2.2:** Structural classes of phlorotannins, oligomers of phloroglucinol (1,3,5-trihydroxybenzene).

The myriads of phlorotannin structures in brown algae have demonstrated numerous bioactive properties. They have strong antioxidant activities against free radical mediated oxidation by scavenging radicals and inhibiting peroxidation (Shibata *et al.*, 2008). Like terrestrial polyphenolic compounds, phlorotannins are also ubiquitous enzyme inhibitors that have been shown to modulate the activity of carbohydrate-hydrolyzing enzymes  $\alpha$ -glucosidase and  $\alpha$ -amylase, thereby decreasing hyperglycemia (Eom *et al.*, 2012), inhibiting the angiotensinogen-I-converting enzyme to regulate blood pressure (Jung *et al.*, 2006), blocking the digestive enzyme lipase and lowering dyslipidemia (Eom *et al.*, 2013), and arresting tyrosinase activity, preventing the synthesis of melanin and subsequent hyperpigmentation (Yoon *et al.*, 2009). Furthermore, phlorotannins have exhibited strong anti-inflammatory properties, blocking production of pro-inflammatory cytokines such as

prostaglandins and nitric oxide (Kim *et al.*, 2009). In addition, phlorotannins have reduced growth of certain cancers, including MCF-7, HeLa, HT1080, A549, and HT-29 cells (Li *et al.*, 2011). More recently, evidence has emerged that phlorotannins may reduce allergic reactions by blocking histamine release from basophils (Sugiura *et al.*, 2006).

## **Modern uses**

Brown seaweed consumption around the globe remains a dietary source of phlorotannins. However, concentrated phlorotannin preparations have begun to be commercialized as ingredients within various health and beauty products. Commercial extracts of the brown algae prepared from *Ascophyllum nodosum* and *Fucus vesiculosus* are marketed to reduce postprandial serum glucose levels (Roy *et al.*, 2011), while phlorotannins have also been incorporated into cosmeceutical formulations for their tyrosinase inhibiting properties (Thomas & Kim, 2013). Marine-based phlorotannins hold great potential for continued development as nutraceutical supplements and other therapeutic formulations that impact multiple chronic human conditions.

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CHAPTER 3 CHEMICAL AND *IN VITRO* ASSESSMENT  
OF ALASKAN COASTAL VEGETATION  
ANTIOXIDANT CAPACITY

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## **Abstract**

Alaska Native (AN) communities have utilized tidal plants and marine seaweeds as food and medicine for generations, yet the bioactive potential of these resources has not been widely examined. This study screened six species of Alaskan seaweed (*Fucus distichus*, *Saccharina latissima*, *Saccharina groenlandica*, *Alaria marginata*, *Pyropia fallax*, and *Ulva lactuca*) and one tidal plant (*Plantago maritima*) for antioxidant activity. Total polyphenolic content (TPC) was determined, and chemical antioxidant capacity was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferrous ion-chelating, and nitric oxide (NO) inhibition assays. *In vitro* inhibition of radical oxygen species (ROS) generation and NO synthesis was evaluated in a RAW 264.7 macrophage culture. Greatest TPC (557.2 µg phloroglucinol equivalents (PGE)/mg extract) was discovered in *Fucus distichus*' ethyl acetate fraction, and highest DPPH scavenging activity was exhibited by *F. distichus* and *Saccharina groenlandica* fractions (IC<sub>50</sub> 4.29 – 5.12 µg/mL). These results support the potential of Alaskan coastal vegetation, especially the brown algae, as natural sources of antioxidants for preventing oxidative degeneration and maintaining human health.

## **Keywords**

Alaska, seaweed, antioxidant, RAW 264.7 macrophages, radical oxygen species, phlorotannin, nitric oxide, total phenolic content

## **Introduction**

Marine macroalgae have been harvested as a source of food, livestock fodder, and pharmaceuticals by traditional cultures across the globe, especially communities situated

around the Pacific Rim (Jiménez-Escrig *et al.*, 2012; Widjaja-Adhi Airanthi *et al.*, 2011). From Arctic Alaska to the Pacific Northwest, seaweeds have played a large role in the traditional cultures of multiple Native American/Alaskan Native (NA/AN) and First Nation communities, as a ubiquitous source of macro- and micronutrients (Garza, 2005; Turner & Bell, 1973; Turner, 2003). Seaweeds have evolved into an important part of the communities' traditional ecological knowledge, evident in the variety of ways they are incorporated into traditional diets. First Nations in British Columbia cook species of the red alga *Porphyra* with clams, salmon eggs, or fish in soups, toast the thalli as a snack, or sprinkle dried seaweed over other foods (Turner, 2003), and nearly 60% of the households of the Inuit community of the Canadian Arctic's Belcher Islands regularly consume *Rhodomenia* spp. and *Laminaria* spp. (Wein *et al.*, 1996).

Seaweed's role in traditional diets has encouraged research into the multifunctional nutraceutical potential of these marine macroalgae. Small peptides from seaweed have demonstrated angiotensin-I converting enzyme (ACE) inhibition (Sato *et al.*, 2002), while seaweed fractions enriched in polyphenolics and polysaccharides exhibited activity against diabetes, by inhibiting  $\alpha$ -glucosidase, increasing basal uptake of glucose and lowering post-prandial glucose levels at both the cellular and human clinical levels (Kim *et al.*, 2008; Zhang *et al.*, 2007). In recent years, some studies have reported that seaweed extracts demonstrate strong antioxidant properties (O'Sullivan *et al.*, 2011; Wang *et al.*, 2012; Wang *et al.*, 2009), leading to a growing interest for their use in food technology applications to prevent oxidation of lipids and proteinaceous tissue (Wang *et al.*, 2009), and to reduce the effects of radical oxygen degradation in chronic diseases (Kim *et al.*, 2008; Zhang *et al.*,

2007). Indeed, seaweeds contain a wide variety of bioactive components with potential antioxidant capacity. The polyphenolic phlorotannins found in brown seaweed can comprise 1%-20% of the dry weight of the algae (Ragan & Glombitza, 1986) and are often more potent antioxidants than analogous polyphenols derived from terrestrial sources due to their interlocking phenol ring structure (Heo *et al.*, 2009). Seaweed also possess non-polyphenolic compounds, including tocopherols, carotenoids, terpenoids, and alkaloids, which have demonstrated antioxidant activity in a variety of *in vitro* studies (Hu *et al.*, 2008).

Radical oxygen species (ROS), elicited by environmental stress, UV light exposure, and as a byproduct of regular oxidative cell function, are detrimental to multiple human tissues, including lipid membranes, proteins, and DNA. Damage can result in cellular dysfunction, and ROS are implicated in the development of several chronic disease conditions, including cardiovascular disease, metabolic syndrome, cancer, and some neurodegenerative diseases (Aruoma, 1998). The human body counteracts accumulation of ROS via quenching mechanisms such as superoxide dismutase, catalase, and the glutathione system, and uses exogenous phytochemical antioxidants like carotenoids, vitamins, and polyphenols from dietary sources as a second tier of defense against oxidative stress (Kaliora *et al.*, 2006).

Terrestrial botanical resources have been evaluated for their potential in offsetting the risk of chronic disease (Scalbert *et al.*, 2005), although less attention has been paid to marine sources of antioxidants, especially seaweeds, which are endemic to a highly competitive and stressful environment, and have evolved a diverse antioxidant arsenal of defenses. Often located in intertidal zones, coastal seaweeds are exposed to a constantly changing

environment. Fluctuating ocean levels, common to intertidal ecosystems, repeatedly subject seaweed to exposed and submerged conditions, with alternative high/low ultraviolet (UV) light and oxygen levels that necessitate adaptive defense mechanisms (Blanchette, 1997; Peteiro & Freire, 2011). Algal phlorotannins provide UV-protection, with biosynthesis of the metabolites up-regulated by high levels of incident radiation; phlorotannins are even exuded into the surrounding sea medium to further bolster protection against UV radiation (Bischof *et al.*, 2006; Pavia *et al.*, 1997; Schoenwaelder *et al.*, 2003). Furthermore, seaweed have been shown to combat herbivorous predation by constitutive and inducible defensive cocktails designed to reduce consumption of damaged tissues or to increase the exposure of assaulting herbivores to predators (Bischof *et al.*, 2006; Oliveira *et al.*, 2013). These abiotic and biotic stresses have elicited the complex phytochemical composition of seaweeds. The same bioactive phytochemicals accumulated by the seaweed to resist environmental stressors, once ingested, are relevant to human health maintenance (Bischof *et al.*, 2006; Bunsom & Prathep, 2012). Increasing water temperatures have pushed seaweed communities beyond their ability to acclimate, leading to shifts in distribution patterns as species are forced into more polar waters while new, previously non-indigenous species expand their geographic ranges (Díez *et al.*, 2012; Harley *et al.*, 2012). Indeed, the degree of shifting has raised some alarm that continued oceanic warming could force seaweed species, and their genetic and phytochemical biodiversity, beyond the point where sustained retreat is possible, raising the potential for global extinctions (Wernberg *et al.*, 2011).

The waters around Alaska hold an abundant diversity of macroalgae (Lindberg & Lindstrom, 2010) used by AN cultures for generations, but little research has demonstrated

their mechanisms for health protection. In this study, six species of seaweed and one tidal terrestrial plant harvested from the Southeastern coast of Alaska in early summer were surveyed for their antioxidant potential.

## **Materials & Methods**

### **Chemicals**

Unless otherwise noted, all chemicals were of reagent or microbiological grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

### **Sample material**

Several algal species and one coastal perennial plant were collected from the coastal area surrounding Sitka, Alaska in June 2012. These samples included four Phaeophyta (brown algae) species: *Fucus distichus* (commonly known as “bladderwrack”), *Saccharina latissima* (“sugar wrack”), *Saccharina groenlandica* (“kelp”), and *Alaria marginata* (“winged kelp”); one species of Rhodophyta (red algae): *Pyropia fallax* (“laver”); a species of Chlorophyta (green algae): *Ulva lactuca* (“sea lettuce”); and the terrestrial Plantaginaceae species *Plantago maritima* (“goosetongue”) (Garza, 2005). Identifications were verified by the authors in the field with assistance from the Sitka Sound Science Center (Sitka, AK). Freshly collected seaweeds (at least three different individual plants per species) were washed with fresh seawater to remove particulates, salts, and epiphytes that might have been attached to the surface of the thalli, frozen, and transported overnight to the laboratory, where the samples were frozen at -80 °C, lyophilized, and kept at -80 °C until extract preparation.

### **Extract preparation**

Four grams of each freeze-dried sample were powdered using a grinding mill (IKA, Wilmington, NC, USA) and the powder was suspended in 200 mL 80% aqueous methanol and shaken for 24 hours on an orbital shaker at 250 rpm and 23 °C in the dark. The extract liquid was filtered through Whatman #1 filter paper and the powder re-extracted a second time. The two extracts were combined and evaporated under reduced pressure to remove excess solvent. The resulting aqueous residue was diluted to 200 mL with deionized water and sequentially partitioned with hexane, ethyl acetate, 1-butanol (3 x 200 mL), yielding 4 crude fractions including the aqueous residue (H, E, B, and W, respectively). Solvents were removed via rotary evaporation, and all fractions were lyophilized and held at -80 °C until analysis. For all chemical and *in vitro* assays, samples were re-constituted in an aqueous methanol or ethanol solvent system to the appropriate concentration for testing.

### **Microplate assay for total phenolics**

The total phenolic content (TPC) of seaweed fractions was quantified using a microplate-adapted Folin-Ciocalteu protocol (Herald *et al.*, 2012). Briefly, each well of a 96-well plate was charged with 75 µL deionized water, 25 µL of the sample or standard, and 25 µL of Folin-Ciocalteu reagent, diluted 1:1 with water. The reaction mixture was mixed and held for 6 minutes, after which 100 µL 7.5% Na<sub>2</sub>CO<sub>3</sub> were added to each well. The plate was incubated in the dark for 90 minutes, and then absorbance was read at 765 nm on a Molecular Devices M3 microplate reader (Molecular Devices Inc., CA). Phloroglucinol (15.5 – 500 µg/mL) was used to create a standard curve for calculation of the phenolic content, in phloroglucinol equivalents (PGE, R<sup>2</sup> = 0.999).

## **Chemical antioxidant analysis**

### **Field antioxidant screening**

Fractions were first analyzed for potential antioxidant activity using a qualitative assay protocol developed for field screening (Kellogg *et al.*, 2010a; Kellogg *et al.*, 2010b). Each well of a 96-well plate was charged with 200  $\mu$ L of an ABTS solution (7 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and 200 mM potassium persulfate), and 10  $\mu$ L of sample was introduced to each well. The degree of color loss was analyzed visually and ascribed a qualitative value ranging from 0 (no color change from the negative control, no antioxidant activity) to 3 (completely colorless solution, high antioxidant activity). All assays were run in triplicate.

### **DPPH activity**

To determine the scavenging activity of the fractions against the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), a rapid microplate assay was adapted from Herald *et al.* (2012) In summary, 200  $\mu$ L of DPPH solution (150  $\mu$ M in 80% methanol, prepared fresh daily) was charged to the wells of a 96-well plate. Serial dilutions of the fractions (from an initial concentration of 1 mg/mL) were prepared in 80% methanol, 25  $\mu$ L added to the well, and the plate was incubated at room temperature in the dark for 2 hours, after which the absorbance was read at 515 nm on a microplate reader.

A calibration curve was made with DPPH, from 4-150  $\mu$ M, which was used to calculate the inhibition percentage of DPPH in the reaction mixture ( $R^2 = 0.999$ ). The percentage inhibition of DPPH was plotted against the  $\log_{10}$  sample concentration to obtain

an IC<sub>50</sub> value, which represented the seaweed fraction concentration required to scavenge 50% of the DPPH radical in the reaction.

#### **Ferrous ion chelation**

The ferrous ion-chelating ability was assayed according to the method of Wang et al. (2012) with minor modifications. A working solution of 135 µL distilled water and 5 µL FeCl<sub>2</sub> (2 mM) was prepared fresh, and 100 µL of extract or sample (1 mg/mL in 80% methanol) was introduced. The reaction was initiated by adding 10 µL of ferrozine (5 mM) and incubating 10 minutes at room temperature. The absorbance at 562 nm was measured on a microplate reader.

#### **Nitric oxide radical inhibition**

The ability of seaweed fractions to inhibit nitric oxide radical formation was determined according to the procedure of Oliviera et al. (2010). In a 96-well plate, 100 µL of 20 mM sodium nitroprusside was incubated with 100 µL of sample (prepared at 1 mg/mL in 80% methanol) for 60 minutes at room temperature, under light. Subsequently, 100 µL Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 2% phosphoric acid) was added, and the mixture was incubated at room temperature for 10 minutes, then read on a microplate reader at 562 nm. Absorbances were compared against a calibration curve created with serial dilutions of sodium nitrite ( $R^2 = 0.998$ ).

#### ***In vitro* ROS and NO inhibition**

##### **Macrophage cell culture**

RAW 264.7 macrophages (American Type Culture Collection, Rockville, MD) were maintained at a subconfluent density at 37 °C in a 5% CO<sub>2</sub> atmosphere during culturing and

treatment. All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 100 units/mL penicillin-streptomycin, 10 mM sodium pyruvate, and 10% fetal bovine serum (FBS).

#### **Cytotoxicity assay**

All extracts were assayed for decreases in cell vitality. CellTiter 96AQueous One Solution (Promega, Madison, WI) was used to quantify the number of viable cells according to manufacturer's recommendations. Briefly, 20  $\mu$ L of CellTiter 96AQueous One Solution was charged to each well containing 100  $\mu$ L of DMEM without FBS, and the plates were incubated at 37 °C and 5% CO<sub>2</sub> atmosphere for 2 hours. The absorbance was measured on a microplate reader at 515 nm and compared against a vehicle-treated control. None of the seaweed fractions significantly decreased cell viability (defined as cell counts <80% of control, data not shown).

#### **Fluorescent ROS assay**

For determining *in vitro* ROS generation, a fluorescent dye protocol was adapted from Choi et al. (2007). RAW 264.7 macrophage cells were seeded at a concentration of  $4 \times 10^5$  cells/well into a 24-well plate, and incubated overnight at 37 °C. Cells were charged with 500  $\mu$ L of 50  $\mu$ M 2',7'-dichlorodihydrofluorescein diacetate acetyl ester (H<sub>2</sub>DCFDA, Molecular Probes, OR), prepared fresh daily in sterile phosphate-buffered saline (PBS) for 30 minutes. Fluorescent medium was aspirated, and cells were exposed to 25  $\mu$ L of extract/fraction (100  $\mu$ g/mL final concentration) and 1  $\mu$ L of lipopolysaccharide (LPS, from *Escherichia coli* 026:B6) and incubated for 24 hours, after which the fluorescence of 2',7'-dichlorofluorescein (DCF) was measured at 485 nm (excitation) and 515 nm (emission) on a

microplate reader. The known antioxidant dexamethasone (DEX) was used as a positive control. The experiments were performed with three independent replications, each replication assayed at least in duplicate.

#### **Nitric oxide assay**

The production of nitrite, the stable end-product of NO generation in activated macrophages, was assayed by a colorimetric assay. To 100  $\mu$ L of cell culture medium, 100  $\mu$ L of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) was added, and the mixture incubated at room temperature for 10 min. The absorbance at 540 nm was read on a microplate reader. The nitrite concentration was calculated using a sodium nitrite standard curve.

#### **Statistics**

All assays were performed at least in triplicate. Results are presented as mean of triplicate runs  $\pm$  SEM. Statistical analysis was conducted using repeated measures ANOVA followed by Tukey's test (Prism 6.0, GraphPad Inc., La Jolla, CA), with statistical significance determined at the  $P < 0.05$  or  $P < 0.01$  level. Half maximal inhibitory concentration ( $IC_{50}$ ) data was compiled after logarithmic transformation and expressed as the geometric mean with 95% confidence intervals.

## Results

### Total phenolic content

The total phenolic content of the fractionated marine vegetation, determined by the Folin-Ciocalteu colorimetric analysis, is given in Table 3.1. The green algae *U. lactuca* demonstrated the lowest TPC across the four fractions (56.8-113.4 µg PGE/mg), compared to the four species of Alaskan brown algae (123.4-557.2 µg PGE/mg). *Fucus distichus*' ethyl acetate ( $557.2 \pm 9.7$  µg PGE/mg) and butanol ( $420.1 \pm 15.2$  µg PGE/mg) fractions had the greatest TPC content of all the samples, higher than a previous study of the related species *F. vesiculosus*, which was measured at  $37.4 \pm 0.6\%$  TPC(Wang *et al.*, 2012). The terrestrial goosetongue, *P. maritima*, showed levels of phenolics (111.8-316.6 µg PGE/mg) that fell in the middle range of values for the species tested.

**Table 3.1.** Total phenolic content (TPC) of Alaskan coastal vegetation ( $\mu\text{g PGE}/\text{mg fraction}$ ).<sup>a</sup>

Species	Fraction	TPC		Species	Fraction	TPC	
<i>A. marginata</i>	H	158.6 $\pm$ 8.90	cb	<i>S. latissima</i>	H	150.8 $\pm$ 13.6	cb
	E	330.5 $\pm$ 6.50	e		E	265.8 $\pm$ 7.40	ed
	B	227.8 $\pm$ 11.7	d		B	326.8 $\pm$ 17.8	e
	W	152.1 $\pm$ 14.4	cb		W	317.9 $\pm$ 13.4	e
<i>F. distichus</i>	H	272.9 $\pm$ 18.1	ed	<i>U. lactuca</i>	H	68.2 $\pm$ 6.20	ba
	E	557.2 $\pm$ 9.40	g		E	113.4 $\pm$ 8.80	b
	B	420.1 $\pm$ 15.2	f		B	56.8 $\pm$ 4.70	a
	W	140.5 $\pm$ 9.70	cb		W	81.7 $\pm$ 5.10	ba
<i>P. fallax</i>	H	180.0 $\pm$ 11.2	dc	<i>P. maritima</i>	H	111.8 $\pm$ 2.20	b
	E	188.5 $\pm$ 12.2	dc		E	222.4 $\pm$ 13.0	d
	B	112.6 $\pm$ 8.70	ba		B	316.6 $\pm$ 18.8	e
	W	129.8 $\pm$ 3.50	cb		W	309.9 $\pm$ 15.9	e
<i>S. groenlandica</i>	H	123.4 $\pm$ 13.2	cb				
	E	160.2 $\pm$ 8.80	dc				
	B	222.2 $\pm$ 10.1	d				
	W	194.9 $\pm$ 9.60	e				

<sup>a</sup> – All values are expressed as the mean  $\pm$  SEM (n=3). Values in the same column followed by different letters are significantly different ( $P < 0.05$ ).

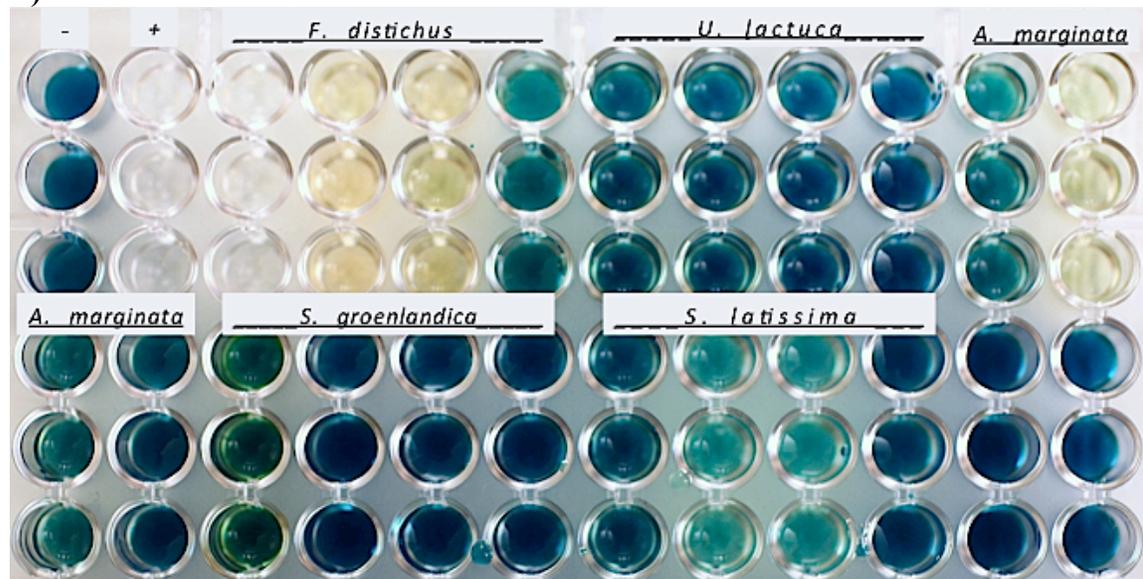
### Antioxidant capacity - chemical assays

The antioxidant capacity of the marine vegetation extracts, based upon the preliminary screening assay, were assigned qualitative values from 0 to 3 based on color development, comparing each fraction against positive and negative controls (1 mM ascorbic acid and 60% ethanol, respectively). All seven crude extracts exhibited antioxidant activity (Figure 3.1), and subsequent fractions were also assayed under the same conditions (Figure 3.1 displays representative fractions from several species). All crude extracts demonstrated some degree of antioxidant activity. The brown algae generally exhibited higher levels of radical quenching. The majority of fractions displayed antioxidant activity by quenching the

A)

Species	<i>A. marginata</i>	<i>F. distichus</i>	<i>P. fallax</i>	<i>S. groenlandica</i>	<i>S. latissima</i>	<i>U. lactuca</i>	<i>P. maritima</i>
Antioxidant value	2	3	1	1	2	1	3

B)



**Figure 3.1.** Antioxidant activity assessed using a field bioassay for *F. distichus*, *U. lactuca*, *A. marginata*, *S. groenlandica*, and *S. latissima*. A) Table represents qualitative antioxidant values for maritime vegetation based upon ABTS screening assay. Levels were scored from 0 (no activity) to 3 (highest activity) based upon visual inspection and comparison against controls. See Kellogg et al.(2010b) B) Four fractions of each species were tested: hexane, ethyl acetate, butanol, and aqueous fractions, arranged in sequential columns (each fraction tested in triplicate). Lighter color represents a greater degree of quenching of the ABTS radical, qualitatively evaluated against the positive control (+), ascorbic acid.

ABTS radical; the ethyl acetate and butanol fractions showed higher activity than hexane or aqueous fractions.

As shown in Table 3.2, the fractionated extracts of marine vegetation varied in their ability to scavenge the DPPH radical. Two seaweed species, the red alga *P. fallax* and the green *U. lactuca*, had no detectable antioxidant activity in the DPPH assay. Congruent with the screening assay, fractions from the four brown algae – *A. marginata*, *F. distichus*, *S. groenlandica*, and *S. latissima* – each demonstrated radical scavenging in a dose-dependent manner over a concentration range of 1-100 µg/mL. Of the four, *F. distichus* was the most active, with ethyl acetate-soluble and butanol-soluble fractions at an IC<sub>50</sub> of  $5.12 \pm 0.77$  and  $4.76 \pm 0.39$  µg/mL, respectively. *S. groenlandica*'s butanol fraction ( $4.29 \pm 0.19$  µg/mL) and the ethyl acetate fraction of *A. marginata* ( $7.59 \pm 0.83$  µg/mL) also exhibited capacity for scavenging DPPH radicals. These values were similar in magnitude to that of the commercial antioxidant ascorbic acid ( $3.28 \pm 0.11$  µg/mL), and were also comparable to those of the closely-related *F. vesiculosus* (Wang *et al.*, 2012), with ethyl acetate and butanol fractions at IC<sub>50</sub> of  $3.76 \pm 0.22$  and  $4.77 \pm 0.25$  µg/mL, respectively. *Plantago maritima*'s ethyl acetate ( $20.78 \pm 0.83$  µg/mL) and butanol ( $11.26 \pm 0.47$  µg/mL) fractions also demonstrated moderate antioxidant efficacy. The mild activity of some aqueous fractions (*F. distichus*, *P. fallax*, and *S. groenlandica* had IC<sub>50</sub> of 37.91, 50.84, and 39.81 µg/mL, respectively) could be attributed to the presence of sulfated polysaccharides that are found in all three major taxa of algae (Rocha de Souza *et al.*, 2007; Rupérez *et al.*, 2002; Shao *et al.*, 2013).

**Table 3.2.** Antioxidant values for maritime vegetation fractions based upon DPPH\* scavenging, ferrous chloride chelating, and nitric oxide (NO) development<sup>a</sup>.

Species	Fraction	DPPH IC <sub>50</sub> (µg/mL)		FeCl <sub>2</sub> chelating (%)		NO inhibition (%)	
<i>A. marginata</i>	H	13.83 ± 0.67	d	24.28 ± 0.62	k	30.6 ± 3.1	ed
	E	7.59 ± 0.83	b	22.87 ± 0.85	k	33.1 ± 2.6	ed
	B	35.09 ± 0.92	h	14.88 ± 0.38	i	10.9 ± 2.0	cba
	W	nd		2.19 ± 0.07	bc	19.7 ± 1.5	dcb
<i>F. distichus</i>	H	14.69 ± 0.64	d	14.10 ± 0.69	hi	27.9 ± 2.8	d
	E	5.12 ± 0.77	a	11.14 ± 0.09	h	37.9 ± 2.1	e
	B	4.76 ± 0.39	a	9.17 ± 0.27	eg	32.4 ± 2.1	ed
	W	37.91 ± 0.58	i	nd		26.1 ± 2.7	d
<i>P. fallax</i>	H	nd		11.64 ± 1.61	g	39.2 ± 2.1	e
	E	nd		29.03 ± 0.86	l	29.7 ± 2.2	ed
	B	nd		8.95 ± 0.18	ef	23.6 ± 1.7	dc
	W	50.84 ± 0.03	k	18.21 ± 0.85	j	19.9 ± 1.8	dcb
<i>S. groenlandica</i>	H	nd		6.07 ± 0.87	de	50.0 ± 1.7	f
	E	24.85 ± 0.13	f	12.28 ± 0.56	gh	58.6 ± 2.4	g
	B	4.29 ± 0.19	a	0.09 ± .012	a	25.8 ± 0.9	dc
	W	39.81 ± 0.03	i	4.43 ± 0.21	d	27.4 ± 2.2	d
<i>S. latissima</i>	H	31.46 ± 0.20	g	19.95 ± 0.99	j	37.5 ± 2.5	e
	E	41.44 ± 0.19	j	nd		39.6 ± 3.8	e
	B	nd		7.44 ± 0.14	e	13.5 ± 0.9	ba
	W	nd		nd		16.4 ± 3.2	cb
<i>U. lactuca</i>	H	nd		nd		25.2 ± 1.3	dc
	E	nd		1.32 ± 0.26	ab	15.5 ± 1.2	cb
	B	nd		nd		22.5 ± 2.7	dcb
	W	nd		9.63 ± 1.06	fg	17.7 ± 1.0	cb
<i>P. maritima</i>	H	nd		nd		27.7 ± 2.8	d
	E	20.78 ± 0.83	e	nd		17.9 ± 1.8	cb
	B	11.26 ± 0.47	c	10.68 ± 0.15	fg	14.9 ± 1.9	b
	W	92.8 ± .45	l	3.56 ± 0.23	cd	6.3 ± 1.9	a

<sup>a</sup> – All values are expressed as the mean ± SEM (n=3). Values in the same column followed by different letters are significantly different (P < 0.05). “nd”=not detected

In addition, the TPC levels of Alaskan seaweed had a strong positive correlation with DPPH radical scavenging capability ( $R^2 = 0.662$ ,  $P < 0.001$ ). Very high and significant positive correlations, up to  $R^2 = 0.99$ , have been documented between polyphenolic content

and antioxidant capability of seaweeds in previous studies (Jiménez-Escrig *et al.*, 2001; Wang *et al.*, 2009). The moderate correlation found in the current study may suggest that other active, non-polyphenolic components could have synergistic effects on radical scavenging, as shown in Heo *et al.*'s 2005 study, where fractions obtained from the brown algae *Ecklonia cava* and *Sargassum coreanum* possessed low DPPH radical scavenging activity despite testing for comparative TPC levels as other, higher-activity extracts (Heo *et al.*, 2005).

Lipid and cellular oxidation may be amplified by interaction with transition metal cations, which play an important part in ageing and age-related chronic diseases (Salih *et al.*, 1989; Zhang *et al.*, 2012), and the ability of antioxidants to chelate these ions can diminish the cumulative effects of transition metal-mediated free radical and oxidative damage (Hipkiss, 2005). The species' fractions demonstrated varying degrees of ferrous ion chelation capacity (Table 3.2). *P. fallax*'s ethyl acetate fraction ( $29.03 \pm 0.86\%$ ) had the greatest chelating ability, while the hexane ( $24.28 \pm 0.62\%$ ) and ethyl acetate ( $22.87 \pm 0.85\%$ ) fractions of *A. marginata* also exhibited high levels of chelating. These levels were consistent with others previously reported (Wang *et al.*, 2012; Wang *et al.*, 2009) when adjusting for sample concentration. Though the ferrous ion chelation of the fractions was not correlated with TPC ( $R^2 = 0.08$ ), this lack of parallel response has been seen in other seaweed studies (Wang *et al.*, 2009), as well as in extracts of malting barley (Zhou *et al.*, 2008), where TPC had poor correlations with both DPPH as well as cation radical scavenging activities.

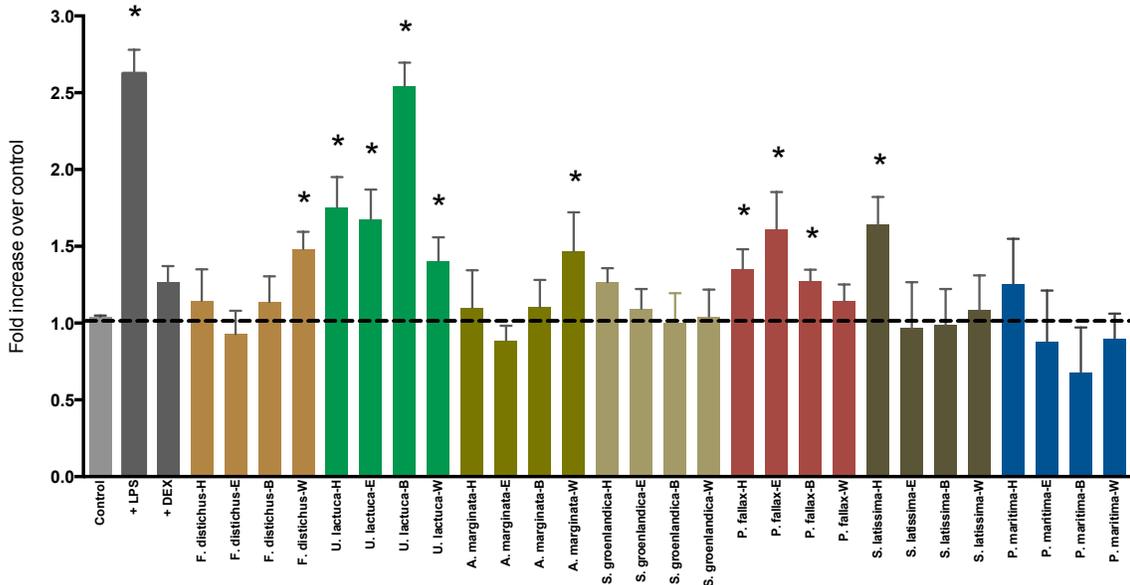
Nitric oxide radical (NO) inhibition levels, as assayed by sodium nitrite production from light-induced sodium nitroprusside decomposition, varied between extract fractions

(Table 3.2). Two fractions derived from *S. groenlandica* demonstrated the greatest NO scavenging ability. The hexane ( $50.0 \pm 1.7\%$  inhibition) and ethyl acetate ( $58.6 \pm 2.4\%$ ) fractions showed more than 50% NO inhibitory activity. Other brown algae fractions, from *F. distichus*, *A. marginata*, and *S. latissima*, also had strong levels of NO inhibition (19.7 - 39.5%). Within each species of brown algae, the ethyl acetate fraction demonstrated the highest degree of NO inhibition (33.1, 37.9, 58.6, and 39.6% inhibition for *A. marginata*, *F. distichus*, *S. groenlandica*, and *S. latissima*, respectively). The red alga *P. fallax* was on the same magnitude as brown algae. The green alga, *U. lactuca*, and tidal plant *P. maritima*, had lower ability to inhibit NO formation than the red or brown algae (6.3 – 27.7%). These species all showed greatest intraspecies inhibition with the hexane fraction (39.2% for *P. fallax*, 25.2% for *U. lactuca*, and 27.7% for *P. maritima*).

### ***In vitro* ROS and NO inhibition**

Cellular antioxidant activities of Alaskan coastal resources were determined using an *in vitro* system to gauge reduction of radical-mediated oxidation in a RAW 264.7 macrophage culture. ROS production in the RAW 264.7 cells was monitored through quantitative fluorescence signaling. Introduced H<sub>2</sub>DCFDA penetrated the outer membrane of viable cells, where it was deacetylated by cytosolic esterases and subsequently reacted with ROS within the cell to yield the highly fluorescent dichlorofluorescein (DCF). Lipopolysaccharide (LPS, 1  $\mu\text{g}/\text{mL}$ ) induced a 2.5-fold overproduction of ROS in the cells compared to non-stimulated cells. Figure 2 shows the effect of Alaskan seaweed and plant fractions on the inhibition of intracellular LPS-induced ROS generation. The positive control dexamethasone (DEX) lowered ROS production back to nominal levels. Examination of

the cytotoxicity of the prepared fractions in RAW 264.7 macrophages by MTT indicated that,

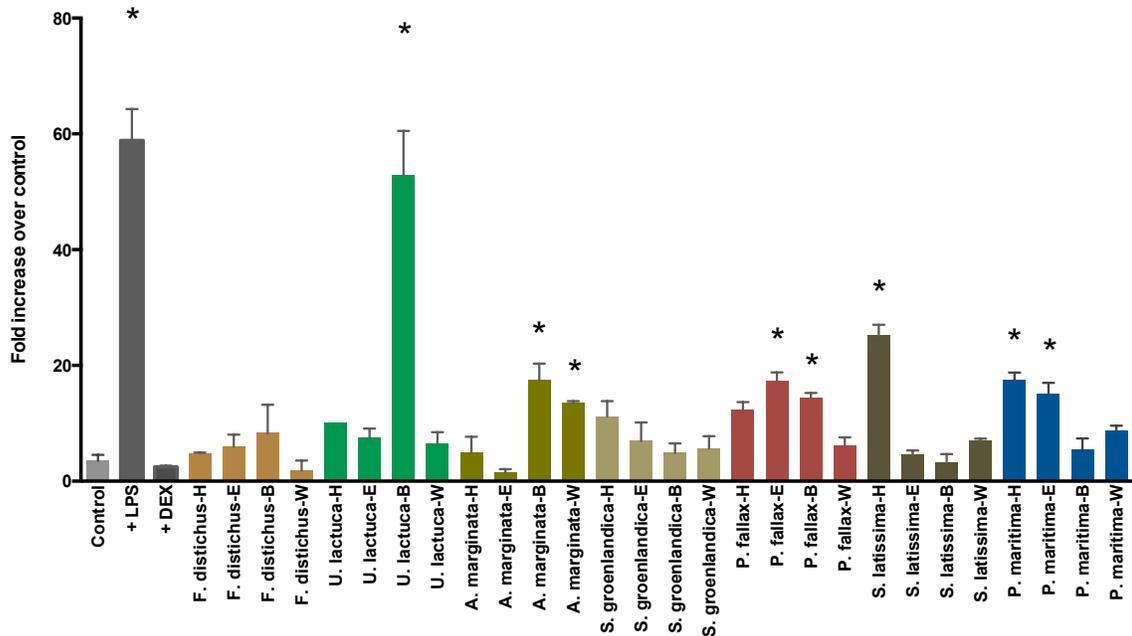


**Figure 3.2.** Radical oxygen species (ROS) production in RAW 264.7 macrophage cells. Cultures were stimulated with LPS (1  $\mu\text{g}/\text{mL}$ ) and treated with 100  $\mu\text{g}/\text{mL}$  fractions of Alaskan coastal vegetation fractions for 24 hours. Samples indicated with an asterisk (\*) are significantly different compared to untreated control ( $P < 0.05$ ). All samples were assayed in triplicate.

at 100 $\mu\text{g}/\text{mL}$ , none of the Alaskan vegetative fractions affected the viability of RAW 264.7 cells (data not shown). Therefore, inhibition of LPS-induced oxidative stress (via ROS or NO production) was not the result of a cytotoxic effect.

Alaskan seaweeds had varying levels of intracellular ROS inhibition (Figure 3.2). ROS levels after treatment by the fractions of the green alga *U. lactuca* remained statistically higher than the control levels, indicating insufficient antioxidant activity at the cellular level to inhibit LPS stimulation. Three of the four fractions of the red alga *P. fallax* showed

significantly higher ROS levels over the control, with only the aqueous fraction (W) having a large inhibition of ROS production. The brown algae, *F. distichus*, *A. marginata*, *S. groenlandica*, and *S. latissima* all affected a significant reduction ( $P < 0.05$ ) on cellular ROS levels; in fact, only three fractions (*F. distichus*-W, *A. marginata*-W, and *S. latissima*-H) did not reduce ROS production back to control levels. The terrestrial goosetongue also had significant ROS inhibition across all four fractions, lowering the ROS generation of the cells to their non-stimulated state. The ethyl acetate fractions appeared to be lower than the other fractions in reducing ROS production, though these comparisons were not statistically significant. These activities were only moderately correlated with total phenolic content ( $R^2 = 0.450$ ,  $P < 0.001$ ), indicating that potentiating interactions and/or synergies between polyphenols and other phytochemicals could have contributed to ROS inhibition.



**Figure 3.3.** Nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophage cells. Cultures were co-treated with 100  $\mu\text{g}/\text{mL}$  fractions of Alaskan coastal vegetation fractions for 24 hours. Samples indicated with an asterisk (\*) are significantly different compared to untreated control ( $P < 0.05$ ). All samples were assayed in triplicate.

The oxidative nitric oxide pathway culminates in the generation of nitrite, which can be quantitatively measured from culture media using the Greiss reagent. NO production was monitored in RAW 264.7 cells stimulated by LPS for 24 h. LPS (1  $\mu\text{g}/\text{mL}$ ) evoked a 16.6-fold induction of nitrite production versus the naive control, and this induction was inhibited by the positive control, dexamethasone, to baseline levels ( $P < 0.001$ ). The Alaskan vegetative fractions reduced NO production to varying degrees (Figure 3.3). All except the butanol fraction of *U. lactuca* reduced NO production by a statistically significant amount ( $P < 0.001$ ); however, several species and fractions were unable to reduce the NO levels back to non-stimulated levels. In general, the brown algae fractions lowered NO production to basal

levels, and the aqueous fraction of *F. distichus*, the ethyl acetate fraction of *A. marginata*, and the butanol fraction of *S. latissima* had the most activity of all fractions tested. Fractions from goosetongue reduced NO levels as well, but not as effectively as the seaweed (comparison  $P < 0.034$ ). The NO reduction levels achieved by Alaskan vegetation exhibited a very strong positive correlation with *in vitro* ROS inhibition capability ( $R^2 = 0.844$ ,  $P < 0.001$ ), supporting the observation that antioxidant activity and NO inhibition share common metabolic pathways. However, there was only moderate correlation with TPC ( $R^2 = 0.192$ ,  $P < 0.019$ ).

## Discussion

This study demonstrated the antioxidant capacities of edible Alaskan intertidal vegetative extracts/fractions based on chemical assays as well as *in vitro* scavenging effects on ROS levels and reduction of NO production by RAW 264.7 cells. The brown algae species, *F. distichus*, *A. marginata*, *S. groenlandica*, and *S. latissima* all demonstrated high levels of chemical antioxidant activities (based upon DPPH, NO, and FeCl<sub>2</sub> assays), as well as high *in vitro* capacities of scavenging ROS and decreasing NO production. Phlorotannins, oligomer derivatives of phloroglucinol, are the predominant polyphenol component of brown algae (Steevensz *et al.*, 2012), and all species tested had high TPC levels. The correlation observed between TPC and chemical (DPPH  $R^2 = 0.662$ ) and *in vitro* ( $R^2 = 0.450$ ) antioxidant activity indicated that polyphenolics are a significant contributor to the antioxidant capabilities of these seaweeds, though this correlation was lower than expected compared against other studies on TPC-enriched seaweed fractions (Kim *et al.*, 2009) suggesting that other non-phenolic compounds could also play a role in antioxidant protection. The sole terrestrial plant, *P. maritima*, had high levels of polyphenolics, and moderate levels of chemical antioxidant activity, but exhibited high levels of *in vitro* ROS and NO inhibition, suggesting that this tidal plant also is a strong potential source of antioxidant phytochemicals.

The red alga *P. fallax* did not contain phlorotannins, had moderate levels of phenolics, and exhibited variable abilities to quench radicals in the chemical assays. With the exception of the aqueous fraction, there was little significant activity in the *in vitro* ROS

assay, or nitric oxide suppression. The green alga *U. lactuca* registered greatly diminished *in vitro* antioxidant capacity compared to the other species tested. This result concurred with the low levels of antioxidant activity observed in the DPPH and NO inhibition assays as well as the low TPC levels for the green alga.

In general, these species, which form part of the traditional diets of Pacific Northwest cultures, exhibited effective protection against oxidation in chemical and *in vitro* systems. As such, they may have the potential to confer protection when incorporated into the diet, offsetting many oxidative-damage related conditions, such as obesity, diabetes, and cardiovascular disease. Nitric oxide is an inflammatory mediator, and thus these seaweed have the potential to also act as anti-inflammatory agents by reducing inflammatory markers and decreasing the production of pro-inflammatory cytokines. This is an arena for future experimentation, as marine polyphenols have not been widely studied for their anti-inflammatory capacity with respect to suppression of NO and other cytokines (Kim *et al.*, 2009). Additional research is being undertaken to determine which specific phytochemicals confer antioxidant protection, including further isolation and structure elucidation to better understand the mechanisms that underpin the observed effects.

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## CHAPTER 4 PHLOROTANNINS FROM ALASKAN SEAWEED INHIBIT CARBOLYTIC ENZYME ACTIVITY

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## Abstract

Global incidence of type 2 diabetes has escalated over the past few decades, necessitating a continued search for natural sources of enzyme inhibitors to offset postprandial hyperglycemia. The objective of this study was to evaluate coastal Alaskan seaweed inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase, two carbolytic enzymes involved in serum glucose regulation. Of the six species initially screened, the brown seaweeds *Fucus distichus* and *Alaria marginata* possessed the strongest inhibitory effects. *F. distichus* fractions were potent mixed-mode inhibitors of  $\alpha$ -glucosidase and  $\alpha$ -amylase, with IC<sub>50</sub> values of 0.89 and 13.9  $\mu$ g/mL, respectively; significantly more efficacious than the pharmaceutical acarbose (IC<sub>50</sub> of 112.0 and 137.8  $\mu$ g/mL, respectively). The activity of *F. distichus* fractions was associated with phlorotannin oligomers. Normal-phase liquid chromatography-mass spectrometry (NPLC-MS) was employed to characterize individual oligomers. Accurate masses and fragmentation patterns confirmed the presence of fucophloroethol structures with degrees of polymerization from 3 to 18 monomer units. These findings suggest that coastal Alaskan seaweeds are sources of  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitory phlorotannins, and thus have potential to limit the release of sugar from carbohydrates and thus alleviate postprandial hyperglycemia.

## Keywords

Alaska; seaweed; diabetes; glucosidase; hyperglycemia; amylase; type 2 diabetes mellitus; phlorotannin; polyphenol; ethnopharmacology

## Introduction

Type 2 diabetes is a chronic metabolic disease characterized by defects in insulin secretion and action, and has developed into a widespread global health threat. Currently, 20.9 million people in the United States are diagnosed with diabetes; nearly 8% of the adult population (Centers for Disease Control & Prevention, 2011a). Postprandial hyperglycemia has been implicated as an important contributing factor in the development of insulin resistance (Ceriello, 2005), shown to be one of the first indicators of deteriorating glucose regulation (Monnier *et al.*, 2007). In addition, postprandial glucose levels, compared against fasting glycemic response, have demonstrated stronger predictive power for diabetes-associated cardiovascular events (Cavalot *et al.*, 2006), as well as other pathophysiological outcomes related to insulin resistance (Heo *et al.*, 2009). Hydrolysis of dietary starch is the foremost source of serum glucose, and the cleavage of glucose from carbohydrates is regulated by the activity of two main enzymes,  $\alpha$ -amylase and  $\alpha$ -glucosidase. Inhibition of these carbolytic enzymes reduces glucose absorption and the associated postprandial hyperglycemic spike.

Control of serum glucose by modulating the activity of  $\alpha$ -amylase and  $\alpha$ -glucosidase is one strategy in the management of diabetes (Perfetti *et al.*, 1998; Roy *et al.*, 2011). Several approved antidiabetic pharmaceuticals – including acarbose, miglitol, and voglibose – exert their effects through this mechanism. However, these pharmaceuticals are non-specific inhibitors of both  $\alpha$ -glucosidase and  $\alpha$ -amylase, and inhibition of the latter releases larger starch fragments to the lower gastrointestinal tract that are not digestible by  $\alpha$ -

glucosidase (Grabitske & Slavin, 2009). Instead, they are digested by gut microbiota, resulting in adverse gastrointestinal effects similar to that of low-digestible carbohydrates (LDCs): diarrhea, abdominal cramping, and, in some cases, liver toxicity (Etxeberria *et al.*, 2012; Hanefeld, 1998; van de Laar, 2008). Thus, there is a continued need for natural  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitors that have greater specificity and fewer adverse secondary effects.

Seaweeds and their organic extracts contain a wide array of bioactive substances with diverse health benefits, including efficacy in counteracting metabolic syndrome and diabetes. *In vivo* assays using diabetic mice have demonstrated the efficacy of seaweed in improving fasting serum glucose levels (Zhang *et al.*, 2007). Similar results have been shown in human clinical studies, where seaweed supplementation was correlated with increased insulin sensitivity (Paradis *et al.*, 2011), lowered glucose and triglyceride levels (Kim *et al.*, 2008b), and improved postprandial glycemic response (Goñi *et al.*, 2000). One of the biochemical mechanisms responsible for the decrease in serum glucose levels is the inhibition of carbolytic enzymes. Seaweeds, especially their polyphenolic constituents, have exhibited strong inhibitory activity against both  $\alpha$ -glucosidase and  $\alpha$ -amylase (Eom *et al.*, 2012; Heo *et al.*, 2009; Kim *et al.*, 2008a; Lee *et al.*, 2009; Lordan *et al.*, 2013).

The traditional diets and pharmacopeia of Native American/Alaska Native (NA/AN) populations have included coastal and benthic seaweeds for generations. Seaweeds have served as a source of macro- and micronutrients (Garza, 2005; Turner & Bell, 1973; Turner, 2003), and are featured in their ethnobotanical knowledge and dietary traditions. Nearly 60% of Inuit households in the Canadian Arctic's Belcher Islands regularly consume *Rhododymenia*

spp. and *Laminaria* spp. for example (Wein *et al.*, 1996), and First Nations in British Columbia combine the red alga *Porphyra* with clams, salmon eggs, or fish into soups, as well as sprinkle dried seaweed over other foods (Turner, 2003). However, over the last few decades, the dietary preferences of AN communities have shifted away from traditional subsistence diets to more commodity-based Western consumption, resulting in a diet that contains fewer traditional marine resources, including marine mammals and seaweeds (Bersamin *et al.*, 2006; Ebbesson *et al.*, 2005; Gahagan & Silverstein, 2003; Nobmann *et al.*, 2005). This dietary evolution has been hypothesized as a contributing factor in the significant rise in diabetes incidence in these communities; AN populations are twice as likely to have diagnosed diabetes as non-Hispanic whites (Centers for Disease Control & Prevention, 2011b).

The cold, temperate oceans around Alaska hold an abundant diversity of macroalgae (Lindberg & Lindstrom, 2010), yet little research has been undertaken to evaluate the ability of Alaskan seaweeds to influence hyperglycemia and carbolytic enzymatic efficacy. In this study, six species of seaweed harvested from the southern coast of Alaska were surveyed in order to identify seaweed extracts that hold potential for diabetic care through their inhibition of carbolytic enzyme activity.

## **Materials & Methods**

### **Chemicals**

Unless otherwise noted, all chemicals were of reagent or spectroscopic grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

## **Instrumentation**

LC-MS analysis was performed using a Shimadzu LC-MS-IT-TOF instrument (Shimadzu, Tokyo, Japan) equipped with a Prominence HPLC system (SIL-20A HT autosampler, LC-20AD pump system, SDP-M20A photo diode array detector). The LC separation was performed using a method adapted from Grace *et al.* (2013) with a Develosil Diol column (250 mm × 4.6 mm × 5 μm, Phenomenex, Torrance, CA, USA) and a binary solvent system comprised of 0.2% acetic acid in acetonitrile (A) and methanol:water:acetic acid (97:3:0.2) (B). Compounds were separated into the ion source at a flow rate of 0.8 mL/min with the following step-wise gradient: 0-40% B, 0-35 min; 40-100% B, 35-40 min; 100% B, 40-45 min; 100-0% B, 45-50 min; 0% B, 50-60 min. Prior to the next injection, the column was re-equilibrated for 5 min at initial conditions. The heat block and curved desolvation line (CDL) were maintained at 200 °C. Nitrogen was used as nebulizer and drying gas with the flow rate set at 1.5 L/min. The ESI source voltage was set at 4.5 kV and the detector was set at 1.5 V. The instrument was calibrated to <5 ppm error in mass accuracy with an external standard of sodium TFA solution. Ionization was performed using a conventional ESI source in positive and negative ionization mode. Data was acquired from *m/z* 150 – 2500. Shimadzu's LCMS Solution software was used for system control and data analysis. NMR spectra were recorded on a Bruker Avance 700 MHz spectrometer (Bruker BioSpin Corporation, Billerica, MA, USA).

## **Sample material**

Seaweed samples of six Alaskan coastal species were harvested from the coastal area surrounding Sitka, Alaska in June 2012, including: four Phaeophyta (brown algae) species

(*Fucus distichus*, *Saccharina latissima*, *Saccharina groenlandica*, and *Alaria marginata*); one species of Rhodophyta (red algae) (*Porphyra fallax*); and a species of Chlorophyta (green algae) (*Ulva lactuca*) (Table 4.1). Freshly collected specimens were rinsed to remove particulates and any epiphytes that might have attached to the surface, and transported to the laboratory. Samples were frozen at -80 °C, lyophilized, and kept at -80 °C until extract preparation.

**Table 4.1.** Alaskan seaweeds collected from Sitka, AK, USA June 19, 2012

Phylum	Classification	Species	Abbreviation	Common name
Phaeophyta	Brown seaweed	<i>Alaria marginata</i>	AM	Winged kelp
		<i>Fucus distichus</i>	FD	Bladder wrack
		<i>Saccharina groenlandica</i>	SG	Kelp
		<i>Saccharina latissima</i>	SL	Sugar kelp
Rhodophyta	Red seaweed	<i>Pyropia fallax</i>	PF	Laver
Chlorophyta	Green seaweed	<i>Ulva lactuca</i>	UL	Sea lettuce

### Extraction and isolation

40 g of each freeze-dried sample were powdered using a grinding mill (IKA, Wilmington, NC, USA) and the powder was suspended in 1 L 80% aqueous methanol and shaken overnight at room temperature, then filtered through Whatman #1 filter paper and the powder re-extracted a second time. The extract was evaporated under reduced pressure to remove excess solvent, and the resulting aqueous residue was diluted to 400 mL with deionized water and sequentially partitioned with hexane, ethyl acetate, and 1-butanol (3 x 400 mL), yielding 4 crude fractions including the aqueous residue (H, E, B, and W,

respectively). Solvents were removed via rotary evaporation, and all fractions were lyophilized and held at -80 °C until analysis and subfractionation.

Active fractions were separated by flash chromatography using either silica gel (50 g, 230-400 mesh, 60Å Merck, column dimensions 20 × 3 cm) eluted with hexane:ethyl acetate:methanol [100:0:0 → 0:50:50] to collect 20 subfractions, or Sephadex LH-20 columns (10 g dry weight, column dimensions 18 × 3 cm) eluted with methanol: chloroform 4:1 followed by 70% aqueous acetone to obtain 24 subfractions. The progress of separation was monitored by TLC (60 F254; Sigma–Aldrich) stained with vanillin-HCl.

## **Biochemical assays**

### ***α*-Glucosidase assay**

A rapid, multi-well plate system was used to assay seaweed extracts and fractions for inhibitory activity against *α*-glucosidase. *α*-glucosidase from *Saccharomyces cerevisiae* (75 U/mg; Sigma-Aldrich) was diluted to a working concentration of 0.005 mg/mL in 100 mM phosphate buffer, pH 7 (PB). To each well of a 96-well plate, 20  $\mu$ L of inhibitor, extract, or solvent (control) was mixed with 100  $\mu$ L of the substrate p-nitrophenyl-*α*-D-glucopyranoside (PNPG; 1 mM in PB) and incubated at 30 °C for 5 min. 100  $\mu$ L of the enzyme working solution was charged to each well, and absorbance was measured at 405 nm for 30 minutes using a Molecular Devices M3 microplate reader at 30 °C (Molecular Devices Inc., Sunnyvale, CA, USA). Blank wells (without enzyme) were subtracted from each well and results were compared versus a control (no inhibition). The commercial pharmaceutical acarbose was utilized as a positive control. Half-inhibitory concentration (IC<sub>50</sub>), where 50% of the enzyme activity was inhibited, was determined using serial dilutions of active extracts

or fractions ( $n \geq 6$ ), and the percentage inhibition was plotted against the  $\log_{10}$  sample concentration.

For each sample, the inhibitory activity was calculated as follows:

$$\% \text{ inhibitory activity} = (A_{\text{con}} - (A_{\text{ext}} - A_{\text{blank}})) / A_{\text{con}} \times 100\% \quad (1)$$

where  $A_{\text{con}}$  is the absorbance of the uninhibited enzyme,  $A_{\text{ext}}$  is the absorbance of the enzyme treated with the extract, and  $A_{\text{blank}}$  is the absorbance of the extract with substrate (no enzyme present).

#### **Kinetics of $\alpha$ -glucosidase inhibitors**

The enzyme-inhibitor reaction was performed using the above method at both 0.25 and 0.125  $\mu\text{g/mL}$  of the inhibitor and at substrate concentrations from 0.25 – 2 mM PNPG. The type of inhibition was determined by Lineweaver-Burk plot, using the double reciprocal of the substrate concentration and velocity of inhibition at  $t = 6$  min. The inhibition constant  $K_i$  of the competitive inhibitor was calculated using the following equation:

$$1/v = K_m (1 + [\text{inhibitor}] / K_i) / (V_{\text{max}}[\text{substrate}]) + 1/V_{\text{max}} \quad (2)$$

#### **$\alpha$ -Amylase assay**

To determine the inhibition of  $\alpha$ -amylase, the  $\alpha$ -amylase from porcine pancreas (Sigma-Aldrich) was dissolved in 20 mM Tris-HCl buffer pH 7.5 (Fisher Scientific, Pittsburgh, PA, USA) to a working concentration of 25  $\mu\text{M}$ . Each well of a 96-well plate was charged with 35  $\mu\text{L}$  of the extract, fraction, or inhibitor, with 5  $\mu\text{L}$  of the starch substrate (1% w/v starch solution; Sigma-Aldrich), and incubated at 37  $^{\circ}\text{C}$  for 5 min. Ten  $\mu\text{L}$  of the working enzyme solution was charged to each well, and the resulting solution was incubated

for 10 min at 37 °C. 150 µL of diluted Lugol's solution (1:1 dilution with distilled H<sub>2</sub>O) was added, and inhibition was determined by reading absorbance at 595 nm on a microplate reader. Blank wells without amylase were subtracted from each well to account for innate color of the sample. As with the α-glucosidase assay above, acarbose was utilized as the positive control, and IC<sub>50</sub> values were generated from serially diluted sample solutions.

For each sample, the inhibitory activity was calculated as follows:

$$\% \text{ inhibitory activity} = (A_{\text{con}} - (A_{\text{ext}} - A_{\text{blank}})) / A_{\text{con}} \times 100\% \quad (3)$$

where A<sub>con</sub> is the absorbance of the uninhibited enzyme, A<sub>ext</sub> is the absorbance of the enzyme treated with the extract, and A<sub>blank</sub> is the absorbance of the extract with substrate (no enzyme present).

### **Statistics**

All assays were performed at least in triplicate. Results are presented as mean of triplicate runs ± SEM. Statistical analysis was conducted using repeated measures ANOVA followed by Tukey's test (Prism 6.0, GraphPad Inc., La Jolla, CA), with statistical significance determined at the P < 0.05 or P < 0.01 level. Half maximal inhibitory concentration (IC<sub>50</sub>) data was calculated after logarithmic transformation and expressed as the geometric mean with 95% confidence intervals. Lineweaver-Burk plots and kinetic data were obtained using biochemical algorithms in Prism.

## Results

### Carbolytic enzyme inhibition

The inhibitory effect of Alaskan seaweed against  $\alpha$ -glucosidase and  $\alpha$ -amylase was determined using p-nitrophenyl- $\alpha$ -D-glucopyranoside (PNPG) and Lugol's solution as colorimetric indicators, respectively. While previous studies to date have focused on a single species or class of macroalgae, this study evaluated the inhibitory effect of six seaweed species from the three distinct phyla of algae: Phaeophyta (*A. marginata*, *F. distichus*, *S. groenlandica*, and *S. latissima*), Rhodophyta (*P. fallax*), and Chlorophyta (*U. lactuca*). At 4 mg/mL, the crude extracts of the brown algae (*A. marginata* (AM), *F. distichus* (FD), *S. groenlandica* (SG) and *S. latissima* (SL)) significantly ( $P < 0.05$ ) reduced both  $\alpha$ -glucosidase and  $\alpha$ -amylase activity (Table 4.2), and the red alga *P. fallax* (PF) only significantly impacted  $\alpha$ -amylase activity. The two species, AM and FD, reduced enzyme activity to  $< 20\%$ , and were selected for subsequent fractionation.

Table 4.2. Inhibitory potential (% control) of Alaskan seaweed crude extracts<sup>a</sup>

Sample	Crude extract yield (g)	$\alpha$ -glucosidase	$\alpha$ -amylase
<i>Alaria marginata</i>	9.537	6.4 $\pm$ 0.8 <sup>a ***</sup>	17.9 $\pm$ 4.1 <sup>a ***</sup>
<i>Fucus distichus</i>	11.198	3.0 $\pm$ 1.2 <sup>a ***</sup>	18.4 $\pm$ 5.3 <sup>a ***</sup>
<i>Saccharina groenlandica</i>	11.595	76.1 $\pm$ 5.0 <sup>b ***</sup>	65.5 $\pm$ 3.2 <sup>b *</sup>
<i>Saccharina latissima</i>	13.011	75.1 $\pm$ 3.1 <sup>b ***</sup>	56.3 $\pm$ 9.8 <sup>b *</sup>
<i>Pyropia fallax</i>	7.780	86.6 $\pm$ 6.1 <sup>c</sup>	62.2 $\pm$ 8.2 <sup>b *</sup>
<i>Ulva lactuca</i>	7.982	88.0 $\pm$ 6.7 <sup>c</sup>	82.4 $\pm$ 9.4 <sup>c</sup>

<sup>a</sup> – Samples were assayed at 4 mg/mL, data represents mean  $\pm$  SEM (n  $\geq$  4).

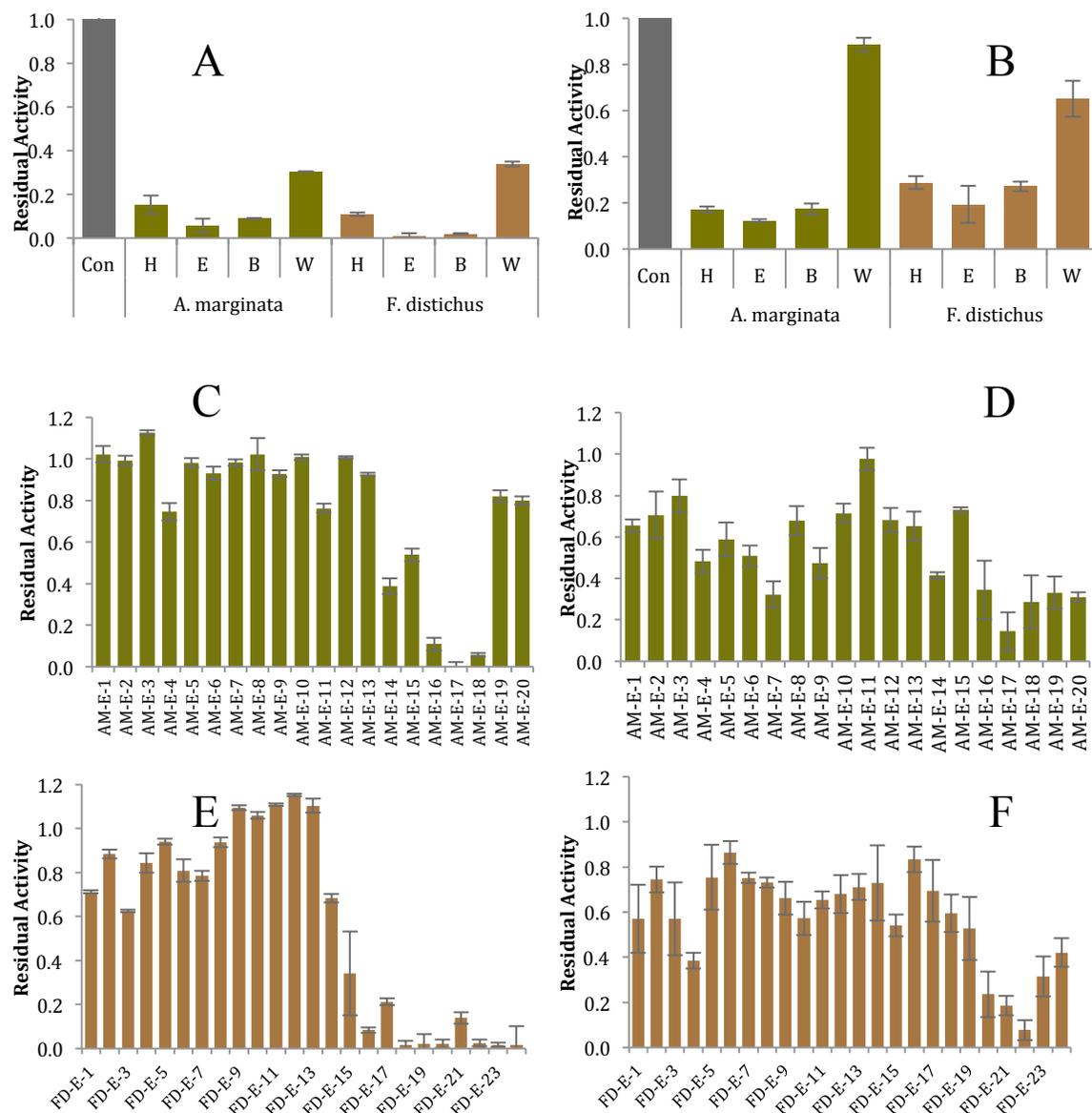
Different letters in same column denote significantly different values ( $P < 0.05$ ).

\*  $P < 0.05$  vs. uninhibited control

\*\*\*  $P < 0.001$  vs. uninhibited control

Analysis of the organic partitions of AM and FD demonstrated that the medium-polar ethyl acetate fractions (AM-E and FD-E) were primarily responsible for the  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitory activity exhibited by the crude extracts (Figure 4.1A, 4.1B). An aliquot of 600 mg of AM-E was separated via flash silica gel chromatography, yielding 20 subfractions, while 1.1 g FD-E was charged to a Sephadex LH-20 column for separation, eluting 24 subfractions. Each subfraction was re-screened for inhibitory activity at an initial concentration of 2 mg/mL. From the AM-E subfractions, AM-E-17 (8.7 mg) displayed the greatest inhibition of  $\alpha$ -glucosidase, reducing activity to  $1.98 \pm 0.14\%$  of the control (Figure 4.1C), yet yielded moderate inhibition of  $\alpha$ -amylase, with a residual activity of  $14.44 \pm 1.27\%$  compared to the uninhibited control (Figure 4.1D).

From *F. distichus*, the polar subfractions demonstrated high activity levels against  $\alpha$ -glucosidase and  $\alpha$ -amylase. FD-E-22 (29.5 mg) evidenced highly effective inhibition of  $\alpha$ -glucosidase, yielding  $1.63 \pm 0.55\%$  of the control's activity (Figure 4.1E). Similar to AM subfractions, FD-E-22 exhibited strong levels of  $\alpha$ -amylase inhibition, reducing enzyme activity to  $7.7 \pm 0.12\%$  of the control (Figure 4.1F).

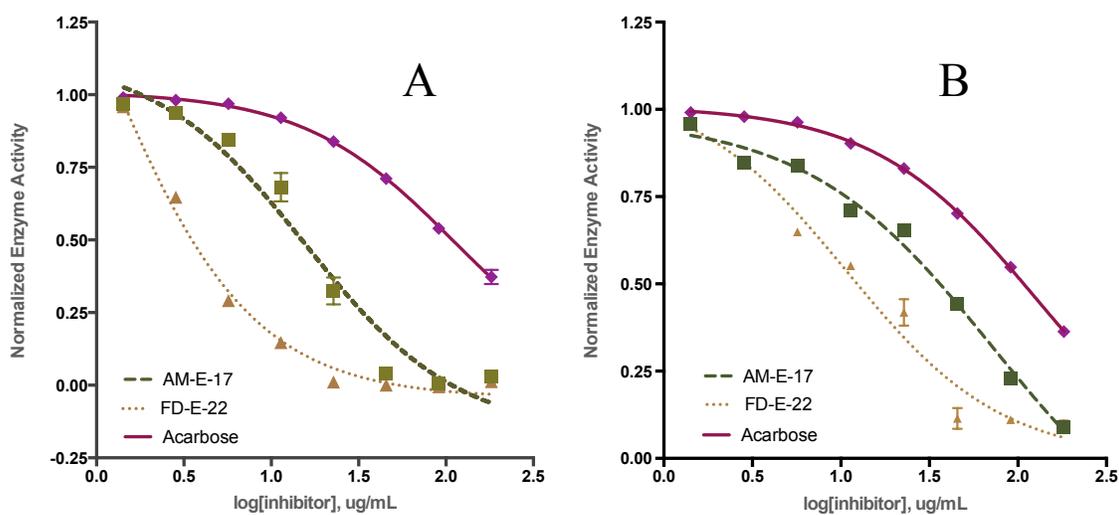


**Figure 4.1.** Inhibition of  $\alpha$ -glucosidase (A) and  $\alpha$ -amylase (B) by enriched partitions (4 mg/mL) of *F. distichus* and *A. marginata*. (C) and (D) inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase, respectively, by subfractions (2 mg/mL) of AM-E. (E) and (F) inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase, respectively, by subfractions (2 mg/mL) of FD-E. Con = control (untreated enzyme), H = hexane fraction, E = ethyl acetate fraction, B = 1-butanol fraction, W = water fraction. Bars indicate mean value  $\pm$  SEM (n = 3).

### Comparison of inhibitory activity with acarbose

The active subfractions of *A. marginata* and *F. distichus* displayed dose-dependent inhibition of  $\alpha$ -glucosidase or  $\alpha$ -amylase (Figure 4.2). The inhibitory activity of AM-E-17 and FD-E-22 was compared to that of acarbose, an oligosaccharide derived from *Actinoplanes* spp. and widely known to inhibit both  $\alpha$ -glucosidase and  $\alpha$ -amylase. Table 4.2 shows the  $IC_{50}$  value for AM-E-17 and FD-E-22 for  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitory activity. The  $IC_{50}$  value for AM-E-17 and FD-E-22 inhibiting  $\alpha$ -glucosidase was  $15.66 \pm 0.82$  and  $0.89 \pm 0.08$   $\mu\text{g/mL}$ , respectively; significantly lower than that for acarbose. For this study, the  $IC_{50}$  of acarbose was determined to be  $112.0 \pm 2.85$   $\mu\text{g/mL}$ , similar in magnitude to other studies (Lordan *et al.*, 2013). The greatly reduced values for the AM and FD fractions indicate they are efficient inhibitors of  $\alpha$ -glucosidase.

Similarly, the two fractions of AM and FD that were most active at inhibiting  $\alpha$ -glucosidase were highly active against  $\alpha$ -amylase, and exhibited significantly lower  $IC_{50}$  values compared to acarbose. AM-E-17 inhibited  $\alpha$ -amylase with an  $IC_{50}$  of  $63.28 \pm 0.87$   $\mu\text{g/mL}$ , while FD-E-22 had an  $IC_{50}$  value of  $13.98 \pm 1.32$   $\mu\text{g/mL}$ , compared to  $138.7 \pm 0.65$   $\mu\text{g/mL}$  for acarbose (Table 4.2). Both fractions evidenced stronger inhibitory activity than was observed with acarbose, implying increased efficiency of inhibition against  $\alpha$ -amylase. However,  $\alpha$ -glucosidase inhibitory activity (defined by each fraction's  $IC_{50}$ ) was greater than for  $\alpha$ -amylase, suggesting that the seaweed have preferential inhibition of  $\alpha$ -glucosidase.



**Figure 4.2.** IC<sub>50</sub> values for seaweed inhibition of  $\alpha$ -glucosidase (A) and  $\alpha$ -amylase (B). Extracts of *Alaria marginata* (AM (---)) and *Fucus distichus* (FD (...)) were evaluated at a range of concentrations (1.5 – 200  $\mu$ g/mL final concentration); acarbose was used as a positive control.

**Table 4.3.** IC<sub>50</sub> values ( $\mu$ g/mL) for Alaskan seaweed inhibitory effect on  $\alpha$ -glucosidase and  $\alpha$ -amylase.

Sample <sup>a</sup>	$\alpha$ -glucosidase	$\alpha$ -amylase
AM-E-17	15.66 $\pm$ 0.82 ***	63.28 $\pm$ 0.87 ***
FD-E-22	0.89 $\pm$ 0.08 ***	13.98 $\pm$ 1.32 ***
Acarbose	112.0 $\pm$ 2.85	138.7 $\pm$ 0.65

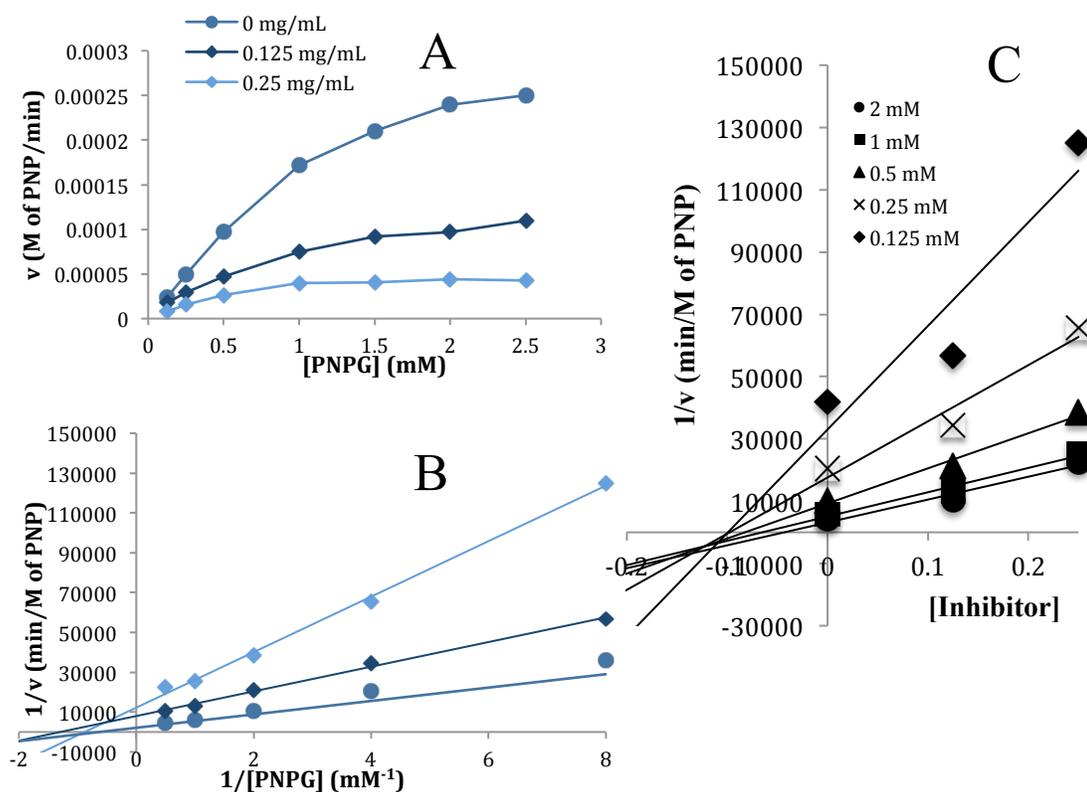
Data represents the mean  $\pm$  SEM (n  $\geq$  4)

<sup>a</sup> – AM-E-17 = *Alaria marginata*'s ethyl acetate fraction, subfraction 17; FD-E-22 = *Fucus distichus*' ethyl acetate fraction, subfraction 22.

\*\*\* P < 0.001 vs. acarbose

### **Inhibition kinetics**

Kinetic studies of the inhibitory effect of FD-E-22 on  $\alpha$ -glucosidase were performed using the same procedure as described above. The initial velocity  $v$  of the enzyme was measured at various concentrations of the substrate PNPG (Figure 4.3A). In the absence of any inhibitor,  $\alpha$ -glucosidase had a Michaelis constant ( $K_m$ ) and  $V_{max}$  of 1.597 and  $4.2 \times 10^{-4}$  M/min, respectively, from the Lineweaver-Burk plot of the kinetic data. As shown in the plot (Figure 4.3B), the presence of FD-E-22 (0.25 mg/mL) lowered  $v$  and decreased  $K_m$  as well as  $V_{max}$  to 0.586 and  $0.64 \times 10^{-4}$  M/min, respectively. This suggested that FD-E-22 exhibited mixed mode inhibition against  $\alpha$ -glucosidase, characterized by a combination of competitive and noncompetitive inhibition, which has been evidenced in other studies (Kim *et al.*, 2008a). The inhibition coefficient  $K_i$  for FD-E-22 was shown to be  $0.116 \pm 0.009$  mg/mL, determined by plotting  $1/v$  against inhibitor concentration at each concentration of substrate (Figure 4.3C).



**Figure 4.3.** Kinetics of  $\alpha$ -glucosidase inhibition in the presence or absence of *Fucus distichus* fraction FD-E-22 ( $\bullet$ , 0 mg/mL;  $\blacklozenge$ , 0.125 mg/mL,  $\blacktriangle$ , 0.25 mg/mL). (A) Velocity  $v$  of carbolytic reaction at various concentrations of the substrate (p-nitrophenyl- $\alpha$ -D-glucopyranoside) [S]. (B) Lineweaver-Burk plots for inhibition of  $\alpha$ -glucosidase, the reciprocal of the velocity versus the reciprocal  $1/[S]$ . (C) Inhibition constant ( $K_i$  value) plot of  $1/v$  versus [FD-E-22] at multiple [S] concentrations. Data points represent the mean value  $\pm$  SEM (n=3).

### Phlorotannin characterization

The ethyl acetate fraction of *F. distichus* exhibited the highest  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitory activity amongst the tested fractions, and had been shown in a previous study to possess the highest phenolic content of all fractions (Kellogg & Lila, 2013). Sub-fractionating the active extract on Sephadex LH-20 yielded the potent extract FD-E-22, eluted with methanol: chloroform (4:1), which was subsequently explored to determine its

chemical constituents. Reverse phase liquid chromatography was unsuited to separate the highly polar phlorotannin units (data not shown). Instead, normal phase liquid chromatography (NPLC) with a diol column and a binary mobile phase consisting of acetonitrile and aqueous methanol allowed for separation of 24 phlorotannin isomers (Figure 4.4), comprising 95.4% of the fraction, determined from HPLC UV absorption at  $\lambda = 254$  nm. These 24 isomers were analyzed via mass spectrometry, acquiring full spectra in both positive and negative ion modes from  $m/z$  150 to 2500. Mass spectrometry analysis revealed an ion chromatogram with protonated molecular ions ( $[M+H]^+$ ) corresponding to fucophloroethol oligomers with three (375 Da) to 18 (2235 Da) phloroglucinol units. Masses corresponded to a single aryl or ether bridge between monomer units, as opposed to the heterocyclic dibenzodioxin and furan ringed phlorotannins, and, unlike fuhalols, there were no additional hydroxyl units on the phenyl rings. Phlorotannins were eluted sequentially by increasing degrees of polymerization (DP), forming successive peaks in the NPLC chromatogram with increasing ion mass.

The fragmentation of the parent peaks in both the positive and negative mass spectrometry mode provided a more comprehensive analysis of the phlorotannins present (Table 4.4). All analyzed compounds showed similar fragmentation patterns characteristic of fucophloroethol fragmentation, with losses of one and two molecules of water ( $-18$  Da and  $-36$  Da, respectively), phloroglucinol ( $-126$  Da), the protonated molecular ion of phloroglucinol ( $-127$  Da), as well as the tandem loss of phloroglucinol and methyl ( $-126$  Da and  $-14$  Da) (Table 4.4). Based on the MS/MS data, the 24 signals were categorized as fucophloroethol isomers ranging from three to 18 monomer units. Fucophloroethols are a

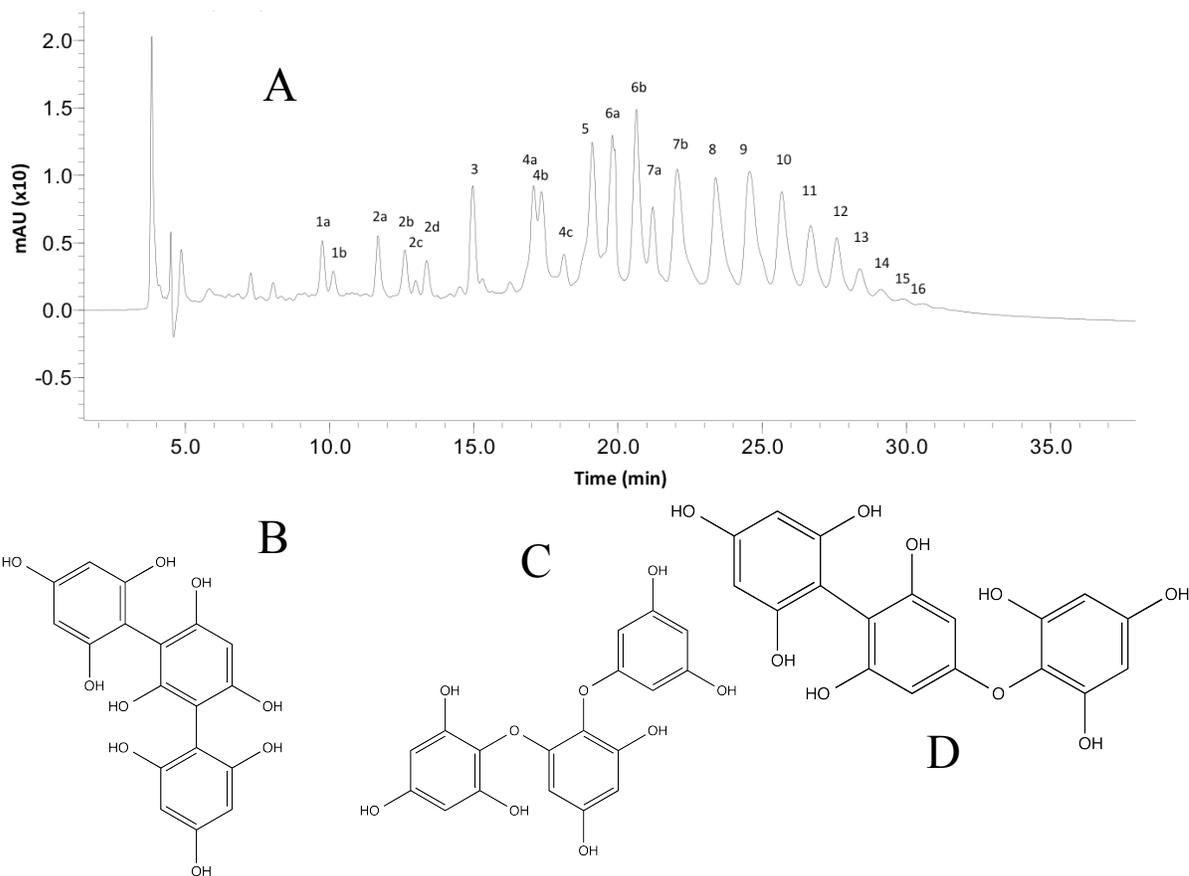
group of phlorotannins exhibiting characteristics of both fucols and phloroethols, and possess combinations of aryl-aryl and ether-linked phloroglucinol units in either linear or branched arrangements; however, fucols, phloroethols, and fucophloroethols all have identical accurate masses and similar fragmentation patterns (Figure 4.4B – 4.4D), and mass spectrometry techniques are not sufficient to differentiate between them.

Several smaller phlorotannins (DPs of 4, 6, 7, 8, and 9) exhibited multiple ion peaks corresponding to the same  $m/z$  ion but differing in retention time on the column. This is possibly due to the presence of varying conformations of fucophloroethols, having the same molecular ion yet differing in the branching position of the aryl and ether linkages of subsequent phloroglucinol additions (Ferrerres *et al.*, 2012; Steevensz *et al.*, 2012).

The larger phlorotannins (DP > 11) manifested distinguishable peaks in the negative mode mass spectra as  $[M - H]^-$  and  $[M - 2H]^{-2}$ . Mass spectrometry identification was achieved through the accurate mass parent ion and daughter ions (Table 4.4). The mass differences from fragmentation of these daughter ions were half of the corresponding mono-ionized fragmentation patterns; a single water loss was recorded as -9 Da, phlorotannin monomer unit loss -63 Da, and methyl group loss -7 Da. Nevertheless, each larger polymer evidenced cleavage into multiple daughter ions (Table 4.4), allowing for the confident characterization of these phlorotannins as fucophloroethols with DP from 11 to 18.

Proton NMR yielded two groupings of signals. One represented the meta-distributed aromatic proton signals directly bound to the aryl rings (5.8 – 6.1 ppm). The second, broader due to proton exchanges, was seen in the hydroxyl proton range (9 ppm). The shift and multiplicity of these proton groupings was similar to other studies of phlorotannins

(Glombitza & Pauli, 2003), providing corroborating evidence to the NPLC-MS data suggesting the presence of fucophloroethol phlorotannins.



**Figure 4.4.** A) LC-MS chromatogram for *Fucus distichus*' FD-E-22 subfraction on a normal phase diol column with a binary mobile phase consisting of acetonitrile and 97:3 methanol:water. Peaks were measured at 254 nm. Peaks are labeled in order of elution (Table 3). (B-D) Representative structures of a trimeric fucol with aryl-aryl bonds (B), phloroethol showing ether bridges (C), and fucophloroethol with a mixture of the two linkages (D); all three possess identical accurate masses (374.0638 Da)..

**Table 4.4.** NPLC-IT-TOF-MS<sup>2</sup> characterization of phlorotannins from FD-E-22\*

Peak No.	RT (min)	Molecular Formula	ES I	Measured Mass ( <i>m/z</i> )	Predicted Mass ( <i>m/z</i> )	$\Delta m$ (ppm)	DP <sup>a</sup>	MS/MS Ions ( <i>m/z</i> )					
1	9.752	C <sub>18</sub> H <sub>14</sub> O <sub>9</sub>	(+)	375.0710; [M + 1] <sup>+</sup>	375.0711	-0.1	3	357	231				
2a	11.860	C <sub>24</sub> H <sub>18</sub> O <sub>12</sub>	(+)	499.0907; [M + 1] <sup>+</sup>	499.0871	3.6	4	481	463	355	337	231	
2b	12.679	C <sub>24</sub> H <sub>18</sub> O <sub>12</sub>	(+)	499.0880; [M + 1] <sup>+</sup>	499.0871	0.9	4	481	463	355	338	231	
2c	12.996	C <sub>24</sub> H <sub>18</sub> O <sub>12</sub>	(+)	499.0880; [M + 1] <sup>+</sup>	499.0871	0.9	4	481	463	355	337	231	
2d	13.259	C <sub>24</sub> H <sub>18</sub> O <sub>12</sub>	(+)	499.0892; [M + 1] <sup>+</sup>	499.0871	2.1	4	481	463	356	337	231	
3	14.822	C <sub>30</sub> H <sub>22</sub> O <sub>15</sub>	(+)	623.1007; [M + 1] <sup>+</sup>	623.1031	-2.4	5	605	587	479	461	355	231
4a	16.255	C <sub>36</sub> H <sub>26</sub> O <sub>18</sub>	(+)	747.1121; [M + 1] <sup>+</sup>	747.1119	0.2	6	729	711	585	571	479	355
4b	16.943	C <sub>36</sub> H <sub>26</sub> O <sub>18</sub>	(+)	747.1135; [M + 1] <sup>+</sup>	747.1119	1.6	6	729	711	585	571	479	355
4c	17.345	C <sub>36</sub> H <sub>26</sub> O <sub>18</sub>	(+)	747.1089; [M + 1] <sup>+</sup>	747.1119	-3.0	6	729	711	585	571	479	355
5a	17.922	C <sub>42</sub> H <sub>30</sub> O <sub>21</sub>	(+)	871.1296; [M + 1] <sup>+</sup>	871.1328	-3.2	7	853	745	601	479		
5b	19.113	C <sub>42</sub> H <sub>30</sub> O <sub>21</sub>	(+)	871.1343; [M + 1] <sup>+</sup>	871.1328	1.5	7	853	745	601	479		
6a	19.809	C <sub>48</sub> H <sub>34</sub> O <sub>24</sub>	(+)	995.1530; [M + 1] <sup>+</sup>	995.1513	1.7	8	977	959	869	729	581	461
6b	20.632	C <sub>48</sub> H <sub>34</sub> O <sub>24</sub>	(+)	995.1532; [M + 1] <sup>+</sup>	995.1513	1.9	8	977	959	869	729	581	461
7a	21.204	C <sub>54</sub> H <sub>38</sub> O <sub>27</sub>	(+)	1119.1647; [M + 1] <sup>+</sup>	1119.1673	-2.6	9	1101	993	853	709	461	
7b	22.050	C <sub>54</sub> H <sub>38</sub> O <sub>27</sub>	(+)	1119.1716; [M + 1] <sup>+</sup>	1119.1673	4.3	9	1101	993	853	709	461	
8	23.390	C <sub>60</sub> H <sub>42</sub> O <sub>30</sub>	(+)	1243.1853; [M + 1] <sup>+</sup>	1243.1834	1.9	10	1225	959	851	469		
9	24.561	C <sub>66</sub> H <sub>46</sub> O <sub>33</sub>	(+)	1367.1960; [M + 1] <sup>+</sup>	1367.2000	-4.0	11	1351	1241	1227	705	683	
10	25.677	C <sub>72</sub> H <sub>50</sub> O <sub>36</sub>	(+)	1491.2123; [M + 1] <sup>+</sup>	1491.2155	-3.2	12	1473	1347	829	807	745	

Table 4.4 continued

Peak No.	RT (min)	Molecular Formula	ES I	Measured Mass ( $m/z$ )	Predicted Mass ( $m/z$ )	$\Delta m$ (ppm)	DP <sup>a</sup>	MS/MS Ions ( $m/z$ )						
11	26.681	C <sub>78</sub> H <sub>54</sub> O <sub>39</sub>	(-)	1613.2214; [M - 1] <sup>-</sup>	1613.2174	4.0	13	806	797	599				
12	27.581	C <sub>84</sub> H <sub>58</sub> O <sub>42</sub>	(-)	1737.2298; [M - 1] <sup>-</sup>	1737.2336	-3.8	14	868	859	806	797			
13	28.385	C <sub>90</sub> H <sub>62</sub> O <sub>45</sub>	(-)	1861.2540; [M - 1] <sup>-</sup>	1861.2496	4.4	15	931	922	868	859	735	643	
14	29.113	C <sub>96</sub> H <sub>66</sub> O <sub>48</sub>	(-)	1985.2614; [M - 1] <sup>-</sup>	1985.2656	4.2	16	993	984	930				
15	29.905	C <sub>102</sub> H <sub>70</sub> O <sub>51</sub>	(-)	2109.2806; [M - 1] <sup>-</sup>	2109.2806	0.0	17	1055	1046	983				
16	30.578	C <sub>108</sub> H <sub>74</sub> O <sub>54</sub>	(-)	2233.2928; [M - 1] <sup>-</sup>	2233.2966	-3.8	18	1116	1107	1044	783	715	540	

\* - FD-E-22 = *Fucus distichus* ethyl acetate fraction, subfraction 22. <sup>a</sup> - degree of polymerization

## Discussion

A main approach of anti-diabetic treatments is the maintenance of circulating glucose concentrations to a near constant level by delaying absorption of glucose via inhibition of the two primary enzymes responsible for carbohydrate digestion,  $\alpha$ -glucosidase and  $\alpha$ -amylase. Due to undesirable side effects of synthetic inhibitors (Etxeberria *et al.*, 2012), there is a need for alternative anti-diabetic compounds, and certain polyphenols have proven to be particularly efficacious inhibitors of these two carbolytic enzymes (Xiao *et al.*, 2013). Polyphenols are known to interact with a variety of proteins via hydrogen bonding and hydrophobic interactions, forming complexes which modulate enzymatic bioactivity (Spencer *et al.*, 1988). Modeling the interaction between the active site of  $\alpha$ -amylase and phenolic compounds, Piparo *et al.* (2008) correlated the relationship between the inhibitory activity of flavonoids and the associated protein-polyphenol interactions. Their study postulated the dependence of inhibitory activity on the generation of hydrogen bonds between the hydroxyl groups of the polyphenols and the carboxylate groups of Asp197 and Glu233 located at the active site of  $\alpha$ -amylase. While this study was based solely upon flavonoids from terrestrial plants, the presence of polyhydroxylated structures in phlorotannins could potentially allow the adoption of a similar conformation upon interaction with the enzyme active site, and thus inhibit  $\alpha$ -amylase activity through a similar mechanism.

In this study, the organic fractions from six different seaweed species harvested from the southeastern coast of Alaska were evaluated for their potential to inhibit carbolytic enzyme activity. All species exhibited stronger inhibitory activity against  $\alpha$ -glucosidase than

$\alpha$ -amylase, which may suggest a more selective inhibition profile by seaweed extracts, with the potential to regulate the digestion and metabolism of complex carbohydrates without incurring the side-effects of non-specific enzyme inhibitors. Milder  $\alpha$ -amylase inhibition has been shown to prevent abnormal gut microfloral fermentation of undigested carbohydrates when they enter the colon (Etxeberria *et al.*, 2012). The four brown seaweed species exhibited inhibition of the enzymes, and two species, *A. marginata* and *F. distichus*, demonstrated sufficiently high activity against  $\alpha$ -glucosidase and  $\alpha$ -amylase to warrant further analysis using bioassay-guided fractionation techniques. From each of these two species, one fraction (AM-E-17 and FD-E-22) displayed efficacy as potential anti-hyperglycemic agents, and IC<sub>50</sub> values for both demonstrated strong potential for the inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase. The IC<sub>50</sub> values for inhibition of  $\alpha$ -glucosidase by FD-E-22 ( $0.89 \pm 0.08 \mu\text{g/mL}$ ) were significantly lower than reported values for isolated phlorotannin derivatives; one published study reported IC<sub>50</sub> values for fucophloroethol and eckol oligomers ranging from 8.0 – 24.5  $\mu\text{g/mL}$  (Lee *et al.*, 2009), while a second study found eckol species inhibiting  $\alpha$ -glucosidase with IC<sub>50</sub> values from 1.2 – 17.8  $\mu\text{g/mL}$  (Moon *et al.*, 2011). FD-E-22 possessed the greater inhibitory activity than AM-E-17, and was selected for more detailed phytochemical characterization.

The dominant phytochemicals in the active extract FD-E-22 were phlorotannins, polyphenolic oligomers of phloroglucinol. These polyphenolic polymers have been revealed in other members of the genus *Fucus*, especially in the widely studied *Fucus vesiculosus* and *F. spiralis* (Ferrerres *et al.*, 2012; Steevensz *et al.*, 2012). Phytochemical analysis of the fraction of *Alaria marginata* that demonstrated significant levels of enzyme inhibitory

activity, AM-E-17, remained inconclusive. Originally hypothesized to also contain phlorotannins, none were observed after NPLC-MS analysis. This could be due to the initial use of silica gel for sub-fractionation of *A. marginata*, potentially leading to the oxidative breakdown of labile phlorotannins (Koivikko *et al.*, 2007; Steevensz *et al.*, 2012), whereas Sephadex LH-20 was employed for the fractionation of the FD-E partition. The inhibitory activity could have arisen from other phytochemicals present in the seaweed matrix, yet the mass spectrometry analysis was unable to provide a dominant molecular ion. Additionally, while the other genera of brown seaweed tested have been known to contain phlorotannins, including *Laminaria* and *Alaria*, it is possible that the concentration, degree of polymerization or configuration of phlorotannins were not sufficient to inhibit the assayed enzymes. Variations in structure of the polysaccharide fucoidan, isolated from both *Ascophyllum* and *Fucus*, displayed marked differences in the resulting enzyme inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase, suggesting the potential for wide variation in these polymeric phytochemicals between genera (Kim *et al.*, 2014).

An NPLC-MS methodology allowed for the separation and subsequent identification of phlorotannins up to 18 monomer units in size. Previous studies had reported the elution of phlorotannins greater than 1,200 Da in size (a degree of polymerization 10 and higher), as a single, broad peak, with many isomers co-eluting simultaneously. This conglomeration of phlorotannins impeded accurate detection and characterization (Koivikko *et al.*, 2007), and it was hypothesized that these large phlorotannins were either bound irreversibly to the column or unable to be individually eluted with the given conditions (Steevensz *et al.*, 2012). The column and solvent system employed in this study was sufficiently sensitive to discern

multiple high molecular weight phlorotannins up to 18 phloroglucinol units, allowing for a more detailed analysis of larger polymer units.

In summary, traditionally consumed Alaskan seaweed, particularly brown kelps, evidenced a pronounced inhibitory effect on carbolytic enzyme activity. The phlorotannins found in *Fucus distichus* were even more effective in their inhibition efficacy than the known pharmaceutical inhibitor acarbose, suggesting their potential to delay the absorption of digested carbohydrates in the gastrointestinal tract post-consumption. Thus, these species have potential as natural sources of anti-diabetic agents that could reduce post-prandial hyperglycemia.

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CHAPTER 5 ALASKAN SEAWEED LOWER  
INFLAMMATION AND DECREASE LIPID  
ACCUMULATION IN RAW 264.7 MACROPHAGES  
AND 3T3-L1 ADIPOCYTES

## Abstract

Chronic inflammation is implicated in the development of multiple metabolic conditions, including insulin resistance and obesity. The accumulation of macrophages in adipose tissue, and the subsequent up-regulation of pro-inflammatory cytokines, precipitate the dysregulation of lipid metabolism and lead to insulin resistance. The aim of this work was to study the inhibitory effects of coastal Alaskan seaweed on the inflammatory response of both macrophages and adipocytes. *F. distichus* demonstrated substantial activity in lowering inflammatory gene expression in RAW 264.7 macrophages and 3T3-L1 adipocytes, as well as improving lipid metabolism in adipocytes. Fractionation yielded two active subfractions, one containing a monoglycosyldiacylglycerol (MGDG) comprised of two eicosatetraenoic fatty acid residues, and the other possessing larger molecular weight fucophloroethol phlorotannins. Utilizing a broad array of inflammatory biomarkers, the MGDG- and phlorotannin-rich fractions from *F. distichus* decreased mRNA expression of acute and chronic inflammatory interleukins, including IL-1  $\beta$ , IL-2, IL-6, IL-10, IL-17, as well as cyclooxygenase 2 (COX2), inducible nitric oxide synthase (iNOS), intracellular adhesion molecule (iCAM), monocyte chemoattractant protein (MCP-1), and tumor necrosis factor alpha (TNF $\alpha$ ). Reductions were also observed in expression of Toll-like receptors 4 and 9 (TLR4 and TLR9, respectively), suggesting a potential mechanism of anti-inflammatory activity via TLR attenuation and NF $\kappa$ B inhibition by the active fractions. *F. distichus* fractions reduced lipid accumulation by 55% and increased free glycerol concentrations by 28-45%, suggesting an increase in lipid metabolism and a decrease for

future potential hypertrophication, which was supported by observed increases in adiponectin and UCP-1 and decreases in leptin mRNA expression levels. Overall, the Alaskan seaweed *F. distichus* inhibited proinflammatory responses and improved lipid metabolism, which suggested the potential for seaweed phytochemicals to serve as therapeutic agents in attenuating inflammatory diseases.

## **Introduction**

Inflammation is a microcirculatory process that occurs in response to physical damage and cellular trauma, exogenous infections, and other irritants. The process is initiated by the activation of macrophages induced after exposure to interferon- $\gamma$ , tumor necrosis factor-alpha (TNF $\alpha$ ), and the microbial lipopolysaccharide (LPS) (Xie *et al.*, 1993). Prolonged or chronic inflammation results in the excess production and up-regulation of multiple inflammatory mediators. These include (a) chemokines, such as interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1); (b) pro-inflammatory cytokines, including IL-6, IL-2, IL-1 $\beta$ , and TNF $\alpha$ ; (c) adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular-cell adhesion molecule-1 (VCAM-1); and (d) inflammatory enzymes, such as inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase-2 (COX-2) (Barnes & Karin, 1997; Cheung *et al.*, 2013). Activated macrophages have been implicated in the pathophysiology of several degenerative conditions, including hepatitis, atherosclerosis (Maskrey *et al.*, 2011), cancer, insulin resistance, and obesity (Xu *et al.*, 2003).

Long-term excessive caloric intake leads to metabolic overload and increased triglyceride uptake by adipose tissue, which results in adipocyte enlargement and eventual hypertrophy. As adiposity increases, hypertrophied adipocytes increase secretion of MCP-1, which functions as a chemoattractant for enhanced macrophage infiltration into adipose tissue (Sartipy & Loskutoff, 2003). As hypertrophied adipocytes apoptose, synthesis of macrophage chemoattractants increases, attracting and activating macrophages to the adipose tissue (Chawla *et al.*, 2011). The infiltration of macrophages initiates a cycle of inflammation (Culberg *et al.*, 2014), characterized by up-regulation of TNF $\alpha$  and interleukins, such as IL-6, which modulate insulin sensitivity of adipocytes. TNF $\alpha$  has been implicated in the direct de-sensitizing of adipocytes and myocytes to insulin signaling via glucose transporter 4 (GLUT4) down-regulation (An *et al.*, 2009; Hotamisligil *et al.*, 1993), while interleukins perpetuate the low-grade chronic inflammation and oxidative stress which disrupts insulin receptor signaling and the synthesis and translocation of the inducible GLUT4 (Choi *et al.*, 2011). Thus, adipocytes cease normal metabolic response to insulin in an effort to limit storage of excess nutrients and relieve cellular stress (Chawla *et al.*, 2011).

Seaweed extracts have demonstrated the ability to improve sensitivity to insulin and decrease hyperglycemia. Several studies have demonstrated that crude extracts and polyphenol-enriched fractions from *Ascophyllum nodosum* and *Ishige okamurae* improved fasting serum glucose levels and lowered postprandial serum glucose levels in a diabetic mouse model (Heo *et al.*, 2009; Zhang *et al.*, 2007). Human clinical trials have evidenced similar results: compared against a placebo, consumption of *A. nodosum* and *Fucus*

*vesiculosis* was associated with a 12.1% reduction in the insulin incremental area under the curve, and a 7.9% increase in insulin sensitivity (Paradis *et al.*, 2011).

Coastal Alaskan seaweed species have served as a source of food, livestock fodder, and pharmaceuticals for generations, evolving into an important part of traditional cultures of multiple Native American/Alaska Native (NA/AN) and First Nation communities, including the Haida, Inuit, Tlingit, Tsimshian, Eyak, and Alutiiq peoples (Garza, 2005; Turner & Bell, 1973). Seaweed provide a ubiquitous source of macro- and micronutrients, and are incorporated into traditional diets in a variety of preparations, including dried, toasted, fermented, and in soups (Turner & Bell, 1973; Turner, 2003). In addition, *Fucus* thalli were chopped and heated with dried tobacco (*Nicotiana* spp.), alder bark (*Alnus rubra*), and twinberry cuttings (*Lonicera involucreta*) and applied as a poultice to relieve inflammation, aches, and pains (Turner & Bell, 1973). The study of seaweeds' biological functions and mechanisms of action has been limited, however one study results revealed powerful antioxidant capabilities of Alaskan seaweed (Kellogg & Lila, 2013).

The present study aims to investigate the effect of coastal marine seaweed species on the inflammatory response in macrophages and adipocytes, including inflammation-related cytokine and adipokine expression levels.

## **Materials & Methods**

### Chemicals

Unless otherwise noted, all chemicals were of reagent or microbiological grade and obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell culture medium and all other

materials required for cell culture were purchased from Gibco (Life Technologies, Grand Island, NY, USA).

### Instrumentation

LC-MS analysis was performed using a Shimadzu LC-MS-IT-TOF instrument (Shimadzu, Tokyo, Japan) equipped with a Prominence HPLC system (SIL-20A HT autosampler, LC-20AD pump system, SDP-M20A photo diode array detector). The LC separation was performed using a method adapted from Grace *et al.* (2013) with a Develosil Diol column (250 mm × 4.6 mm × 5 μm, Phenomenex, Torrance, CA, USA) and a binary solvent system comprised of 0.2% acetic acid in acetonitrile (A) and methanol:water:acetic acid (97:3:0.2) (B). Compounds were separated into the ion source at a flow rate of 0.8 mL/min with the following step-wise gradient: 0-40% B, 0-35 min; 40-100% B, 35-40 min; 100% B, 40-45 min; 100-0% B, 45-50 min; 0% B, 50-60 min. Prior to the next injection, the column was re-equilibrated for 5 min at initial conditions. The heat block and curved desolvation line (CDL) were maintained at 200 °C. Nitrogen was used as nebulizer and drying gas with the flow rate set at 1.5 L/min. The ESI source voltage was set at 4.5 kV and the detector was set at 1.5 V. The instrument was calibrated to <5 ppm error in mass accuracy with an external standard of sodium TFA solution. Ionization was performed using a conventional ESI source in positive and negative ionization mode. Data was acquired from *m/z* 150 – 2500. Shimadzu's LCMS Solution software was used for system control and data analysis. NMR spectra were recorded on a Bruker Avance 700 MHz spectrometer (Bruker BioSpin Corporation, Billerica, MA, USA).

## Sample material

Six seaweed species were collected from the coastal area surrounding Sitka, Alaska in June 2012, including: four Phaeophyta (brown algae) species (*Fucus distichus*, *Saccharina latissima*, *Saccharina groenlandica*, and *Alaria marginata*); one species of Rhodophyta (red algae) (*Porphyra fallax*); and a species of Chlorophyta (green algae) (*Ulva lactuca*) (Table 5.1).

**Table 5.1.** Alaskan seaweeds collected from Sitka, AK, USA June 19, 2012.

Phylum	Classification	Species	Abbreviation	Common name
Phaeophyta	Brown seaweed	<i>Alaria marginata</i>	AM	Winged kelp
		<i>Fucus distichus</i>	FD	Bladder wrack
		<i>Saccharina groenlandica</i>	SG	Kelp
		<i>Saccharina latissima</i>	SL	Sugar kelp
Rhodophyta	Red seaweed	<i>Porphyra fallax</i>	PF	Laver
Chlorophyta	Green seaweed	<i>Ulva lactuca</i>	UL	Sea lettuce

Freshly collected specimens were rinsed to remove particulates and epiphytes, frozen, and transported to the laboratory. Samples were lyophilized and kept at -80 °C until extract preparation. Species identifications were verified by the authors in the field with assistance from the Sitka Sound Science Center (Sitka, AK, USA), and voucher specimens were deposited in the University of North Carolina, Chapel Hill Herbarium (NCU).

## Extract preparation

Crude extracts and fractions were prepared as previously described (Kellogg & Lila, 2013). Briefly, 4 g of freeze-dried sample were powdered using a grinding mill (IKA,

Wilmington, NC, USA) and extracted in  $2 \times 200$  mL of 80% aqueous methanol. The two extracts were combined and evaporated under reduced pressure to remove excess solvent. The resulting aqueous residue was re-constituted in 200 mL dd-H<sub>2</sub>O and sequentially partitioned with  $3 \times 200$  mL hexane, ethyl acetate, 1-butanol, yielding 4 crude fractions including the aqueous residue (H, E, B, and W, respectively), which were dried, lyophilized, and held at -80 °C until analysis.

Active fractions of FD were separated by flash chromatography using either silica gel (50 g, 230-400 mesh, 60Å Merck, column dimensions  $20 \times 3$  cm) eluted with hexane:ethyl acetate:methanol [100:0:0  $\rightarrow$  0:50:50] or Sephadex LH-20 columns (10 g dry weight, column dimensions  $18 \times 3$  cm) eluted with methanol: chloroform 4:1 followed by 70% aqueous acetone to obtain 24 subfractions, which were evaporated to dryness and lyophilized. The progress of separation was monitored by TLC (60 F<sub>254</sub>; Sigma–Aldrich) stained with vanillin-HCl. For all *in vitro* assays, samples were re-constituted in an aqueous DMSO solvent system to the appropriate concentration for testing.

#### Macrophage cell culture

Murine macrophage RAW 264.7 cells (American Type Culture Collection, Rockville, MD, USA) were maintained at a subconfluent density at 37 °C in a 5% CO<sub>2</sub> atmosphere during culturing and treatment. All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 100 units/mL penicillin-streptomycin, 10 mM sodium pyruvate, and 10% fetal bovine serum (FBS).

### **Adipocyte cell culture**

Cultured cells 3T3-L1 cells were maintained in DMEM containing 10% FBS. To induce differentiation, two-day post confluent preadipocytes were cultured in DMEM containing 10% FBS, 170 nM insulin, 0.25 $\mu$ M dexamethasone (DEX) and 0.5 mM 1-methyl-3-isobutylxanthine (IBMX) for two days. Cells were then cultured in DMEM supplemented with 10% FBS and 170 nM insulin for another two days, after which cells were fed every other day with DMEM containing 10% FBS at 37 °C and 5% CO<sub>2</sub>, to a total differentiation process of 10 days. Test samples were diluted in DMSO to the appropriate final concentration before adding to the medium.

### **Cell viability assay and dose range determination**

All extracts were assayed for decreases in cell vitality. CellTiter 96AQueous One Solution (Promega, Madison, WI, USA) was used to quantify the number of viable cells according to manufacturer's recommendations. Briefly, 20  $\mu$ L of CellTiter 96AQueous One Solution was charged to each well containing 100  $\mu$ L of DMEM without FBS, and the plates were incubated at 37 °C and 5% CO<sub>2</sub> atmosphere for 2 hours. The absorbance was measured on a microplate reader at 515 nm and compared against a vehicle-treated control. The concentrations of test reagents that showed no changes in cell viability compared with that vehicle (DMSO) were selected for further studies.

### **Anti-inflammatory *in vitro* assay**

Macrophage cells were seeded in 24-well plates at a density of  $5 \times 10^5$  cells/mL and allowed to grow to confluence overnight. The cells were pretreated for 1 h with seaweed extracts and fractions at 12.5 – 50  $\mu$ g/mL, then elicited with lipopolysaccharide (LPS, from

*Escherichia coli* 026:B6, Sigma-Aldrich) at 1 µg/mL for an additional 4 h. Adipocytes were plated and brought to maturity as noted above, then treated with seaweed extracts and fractions at the same concentrations as macrophages overnight, but were not elicited with LPS. For both cell types, DEX (10 µM) was utilized as the positive control, and the vehicle (DMSO, final concentration 0.5% v/v, Sigma-Aldrich) was included with each plate. Three replicates were performed for both treatments and controls.

### **Lipid accumulation**

Treated mature adipocytes were washed with DPBS and fixed for 1 h with 10% formaldehyde (v/v in DPBS) (Sigma-Aldrich). Cells were washed in 60% isopropanol and air-dried. The Oil Red O solution was prepared by dissolving 0.1 g of Oil Red powder (Sigma-Aldrich) in 20 mL of 100% isopropanol and diluted to a final volume of 50 mL with dd-H<sub>2</sub>O. Each well was stained with 2 mL for 60 min, after which the cells were washed with H<sub>2</sub>O four times and allowed to air-dry. The Oil Red O dye was eluted from the lipid droplets by adding 2 mL of 100% isopropanol for 10 min. The resulting eluant was analyzed on a SpectraMax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 510 nm. Percent accumulation was calculated by the following equation:

$$1 - [(A_{\text{control}} - A_{\text{treatment}}) / A_{\text{control}}] \times 100\% = \% \text{ lipid accumulation} \quad (1)$$

### **Glycerol quantification**

The accumulation of glycerol in the medium was quantified using a Glycerol Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions. In brief, 10 µL of sample was charged to a 96-well plate, to which 150 µL of the diluted Glycerol Enzyme Mixture was added and incubated at room

temperature for 15 minutes. The absorbance was measured at 550 nm and glycerol concentration calculated against a standard curve.

### **RNA extraction and cDNA construction**

Total RNA was isolated from RAW macrophages and adipocytes using TRIzol reagent (Life Technologies) following manufacturer's instructions, and was quantified with a Synergy H1/Take 3 spectrophotometer (BioTek, Winooski, VT, USA). cDNA was synthesized using 2  $\mu$ g of each sample's RNA using the commercial cDNA Reverse Transcriptase kit (Life Technologies), following the manufacturer's instructions on an ABI GeneAMP 970 (Life Technologies).

### **Quantitative PCR analysis**

The synthesized cDNA was amplified in duplicate by real-time quantitative PCR using SYBR green PCR Master Mix (Life Technologies). In order to avoid any genomic DNA contaminant interference, only intron-overlapping primers were selected using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) as listed in Table 5.2. Quantitative PCR (qPCR) amplification was performed on an ABI 7500 Fast real-time PCR (Life Technologies) using the following program: 1 cycle for 2 min at 50 °C, 1 cycle for 10 min at 95 °C, 40 cycles at 95 °C for 15 sec each, and 1 min at 60 °C. The dissociation curve was run for 1 cycle of 1 min at 95 °C, 30 sec at 55 °C, and 30 sec at 95 °C. Expression levels were analyzed via the  $\Delta\Delta$ CT method, normalized against the expression of  $\beta$ -actin using 7500 Fast System SDS v 1.3.0 software (Life Technologies).

**Table 5.2.** Murine primers used in the current study.

<b>Primer</b>	<b>Forward (5'→ 3')</b>	<b>Reverse (5'→ 3')</b>
$\beta$ Actin	AAC CGT GAA AAG ATG ACC CAG AT	CAC AGC CTG GAT GGC TAC GT
COX 2	TGG TGC CTG GTC TGA TGA TG	GTG GTA ACC GCT CAG GTG TTG
iNOS	CCC TCC TGA TCT TGT GTT GGA	TCA ACC CGA GCT CCT GGA A
iCAM	AAC AGT TCA CCT GCA CGG AC	GTC ACC GTT GTG ATC CCT G
IL-1 $\beta$	CAA CCA ACA AGT GAT ATT CTC CAT G	GAT CCA CAC TCT CCA GCT GCA
IL-2	CGC AGA GGT CCA AGT TCA TC	AAC TCC CCA GGA TGC TCA C
IL-6	TAG TCC TTC CTA CCC CAA TTT CC	TTG GTC CTT AGC CAC TCC TTC
IL-10	CCC TCC TGA TCT TGT GTT GGA	TCA ACC CGA GCT CCT GGA A
IL-17	ATC TGG TCC TAC ACG AAG CC	GTC CCG GAC TTC AAG ACC C
MCP-1	CTT CTG GGC CTG CTG TTC A	GCA GCC TAC TCA TTG GGA TCA
TNF $\alpha$	CAT CTT CTC AAA ATT CGA GTG ACA A	TGG GAG TAG ACA AGG TAC AAC CC
TLR 4	GGA CTC TGA TCA TGG CAC TG	CTG ATC CAT GCA TTG GTA GGT
TLR 9	GAA TCC TCC ATC TCC CAA CAT	CCA GAG TCT CAG CCA GCA CT
Leptin	TGA AGC CCA GGA ATG AAG TC	TCA AGA CCA TTG TCA CCA GG
Adiponectin	ACG TCA TCT TCG GCA TGA CT	CTC TAA AGA TTG TCA GTG GAT CTG
UCP-1	AGA AGC CAC AAA CCC TTT GA	TCT CTG CCA GGA CAG TAC CC

## Statistics

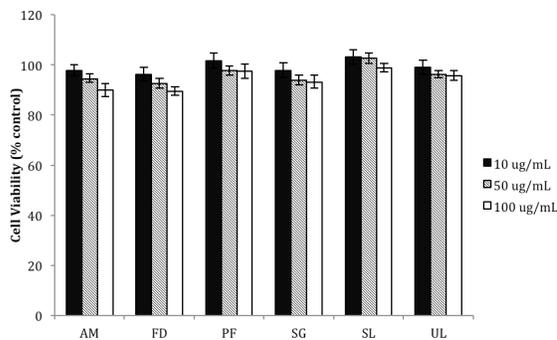
All assays were performed at least in triplicate. Results are presented as mean of triplicate runs  $\pm$  SEM. Statistical analysis was conducted using repeated measures ANOVA followed by Tukey's test (Prism 6.0, GraphPad Inc., La Jolla, CA), with statistical significance determined at the  $P < 0.05$  or  $P < 0.01$  level. Half maximal inhibitory concentration ( $IC_{50}$ ) data was compiled after logarithmic transformation and expressed as the geometric mean with 95% confidence intervals. Gene heat maps were generated using the

Matrix2png web application (<http://www.chibi.ubc.ca/matrix2png/index.html>) (Pavlidis & Noble, 2003).

## Results

### Macrophage inflammation inhibition

The cell viability of RAW macrophages is shown in Figure 5.1. Crude extracts from all six species were evaluated at 10 µg/mL, 50 µg/mL, and 100 µg/mL. None of the extracts



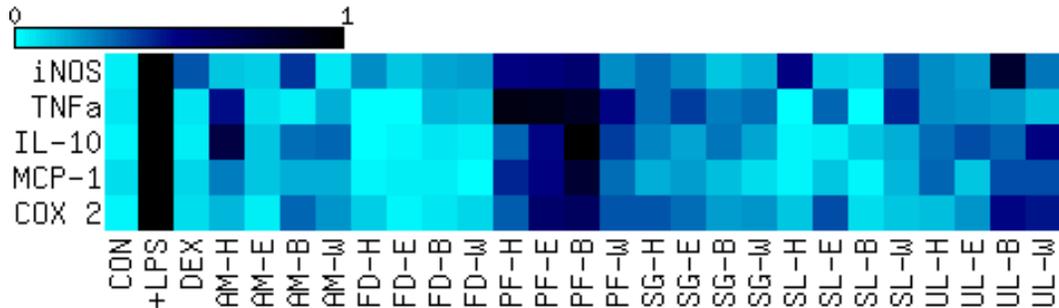
**Figure 5.1.** Cell viability of RAW 264.7 macrophages after treatment with crude Alaskan seaweed fractions at 10 µg/mL, 50 µg/mL, and 100 µg/mL

reduced cell viability below 85% of the control, and thus were not considered cytotoxic at any concentration.

The crude fractions of Alaskan seaweed were assayed for their inhibitory potential against five common inflammatory markers, COX2, iNOS, TNF $\alpha$ , IL-10, and MCP-1. Figure 5.2 shows

the heat map of the relative mRNA expression of the five genes. The horizontal rows of the map represent genes, whereas the columns represent applied samples. Each pixel represents the mean expression of one gene; the shades depict mRNA expression normalized against that of LPS-induced cells. The lighter the cyan coloration, the larger decrease in expression relative to the positive (LPS) control. From the resulting distribution, the red alga *Pyropia fallax* (PF) and green alga *Ulva lactuca* (UL) possessed limited anti-inflammatory

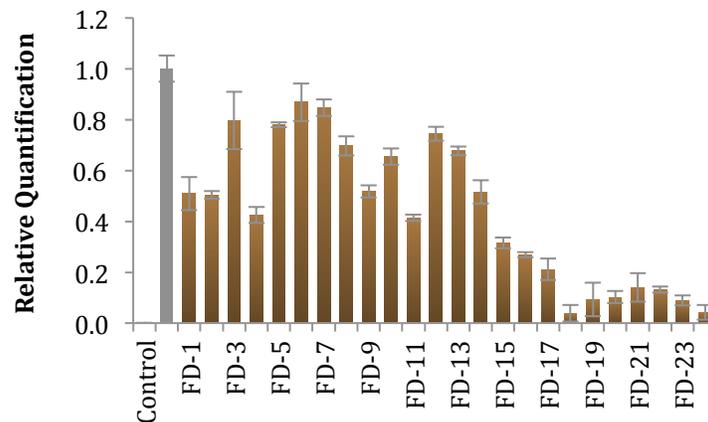
capabilities against the five markers. However, the fractions of the brown seaweed species *Alaria marginata* (AM), *Fucus distichus* (FD), and *Saccharina latissima* (SL) demonstrated significant inhibitory activity against all five inflammatory genes. Of these three, FD reduced expression of TNF $\alpha$ , IL-10, MCP-1, and COX2 to basal levels, and reduced iNOS



**Figure 5.2.** Heat map of mean gene expression from multi-gene analysis of macrophage inflammatory markers iNOS, TNF $\alpha$ , IL-10, MCP-1, and COX2. Cyan represents an expression level of 0, whereas black represents the normalized expression of the +LPS control (1). Crude fractions (hexane, “H”, ethyl acetate, “E”, butanol, “B”, or aqueous, “W”) of six Alaskan seaweed (*Alaria marginata*, “AM”, *Fucus distichus*, “FD”, *Pyropia fallax*, “PF”, *Saccharina groenlandica*, “SG”, *Saccharina latissima*, “SL”, and *Ulva lactuca*, “UL”) were applied to RAW 264.7 macrophages at a final concentration of 50  $\mu$ g/mL.

significantly below the LPS-induced cells (Figure 5.2). The hexane and ethyl acetate fractions (FD-H and FD-E, respectively) provided the most significant reductions in inflammatory gene expression levels. These two fractions were selected for further fractionation and analysis.

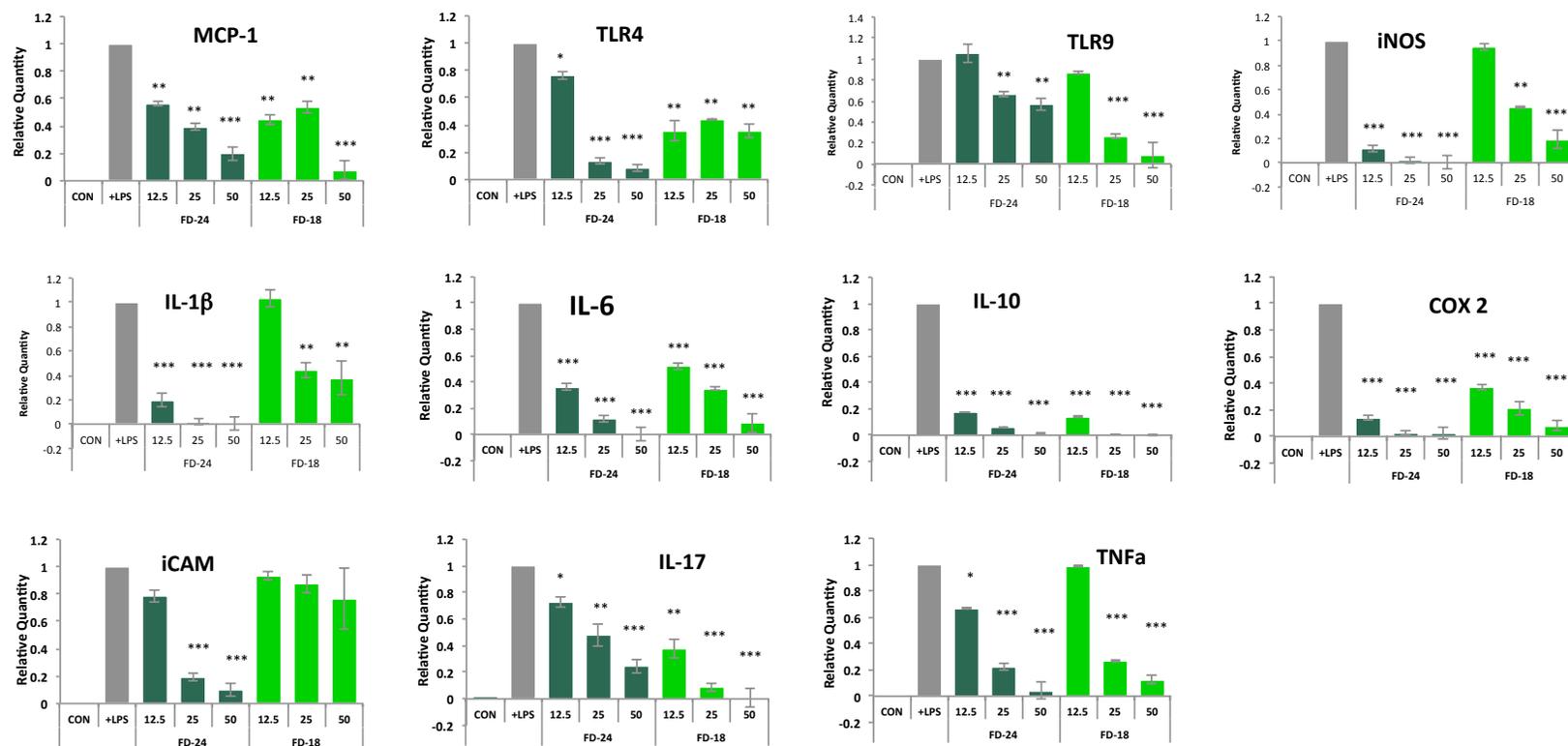
*F. distichus*' hexane and ethyl acetate fractions were combined prior to fractionation with silica gel and Sephadex LH20 flash chromatography columns, and the resulting separation yielded 24 subfractions. The isolated fractions were re-evaluated utilizing the same inflammatory *in vitro* assay system (Figure 5.3) at a concentration of 50  $\mu\text{g}/\text{mL}$  to quantify COX2 expression levels. Two subfractions, FD-18 and FD-24, were most active in the *in vitro* model, reducing expression of COX2 to  $5.1 \pm 0.03 \%$  and  $4.2 \pm 0.02 \%$  of the LPS-stimulated control, respectively. These subfractions were selected for further investigation.



**Figure 5.3.** Inhibition of COX2 expression in a RAW 264.7 macrophage system by the 24 subfractions of *F. distichus*' non-polar fractions (FD-1 to FD-24). Cells were treated at a standard concentration of 50  $\mu\text{g}/\text{mL}$  sample and 1  $\mu\text{L}$  LPS.

To ascertain the breadth of the anti-inflammatory effect of FD-18 and FD-24, their activity was evaluated against an array of inflammation gene markers (Figure 5.4). The two subfractions were assayed at multiple concentrations to show any potential dose dependency

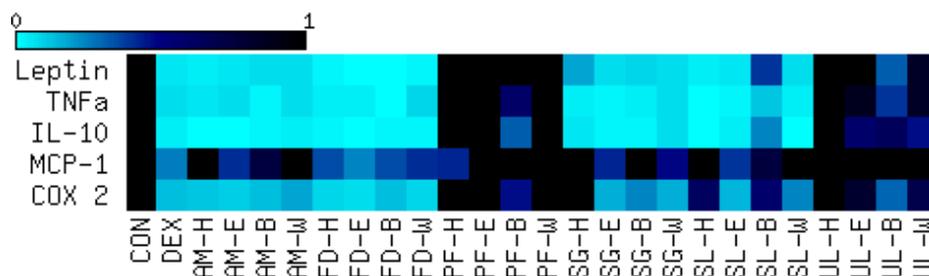
in the resulting gene expression levels. Both FD-24 and FD-18 showed high levels of activity against multiple pro-inflammatory biomarkers (Figure 5.4). COX2 and IL-10 expression were the most strongly affected by all treatments, with significant inhibition at all dose ranges (12.5 – 50  $\mu\text{g/mL}$ ) of FD-24 and FD-18. The subfraction FD-24 also demonstrated high levels of inhibition against IL-1 $\beta$ , iNOS, whereas only the higher concentrations (25 – 50  $\mu\text{g/mL}$ ) of FD-18 yielded any significant decreases in expression levels. Expression of TNF $\alpha$ , IL-17, and MCP-1 were highly dose-dependent, with the middle and high dose levels producing more significant decreases in biomarker expression. For each of these three genes, FD-24 was more efficacious in reducing expression compared against FD-18. For the intercellular adhesion molecule-1 (iCAM), FD-24 reduced expression at the higher doses, while FD-18 was unable to reduce expression in a significant manner across the entire dose range. The two Toll-like receptors (TLRs) that were measured, TLR4 and TLR9, showed decreased expression in response to the two subfractions. TLR4 was lowered in the presence of FD-24, whereas TLR9 was more susceptible to the effect of FD-18 in reducing expression levels.



**Figure 5.4.** Effect of *F. distichus* subfractions 24 and 18 (FD-24 and FD-18, respectively) on pro-inflammatory gene expression. RAW macrophages were pretreated with fractions as specified and inflammatory response was induced with 1  $\mu$ g/mL LPS for 4 h. Fold changes in gene expression are reported relative to induced (+LPS) controls as mean response of triplicate runs  $\pm$  SEM. \* p < 0.05 vs control; \*\* p < 0.01 vs control; \*\*\* P < 0.001 vs control.

## Adipocyte inflammation inhibition

The crude fractions of Alaskan seaweed were assayed to evaluate their ability to reduce pro-inflammatory biomarkers in a 3T3-L1 adipocyte cell line. The brown kelp fractions (*F. distichus*, *A. marginata*, *S. groenlandica*, and *S. latissima*) were all powerful modulators of inflammatory and adipokine expression levels as shown in the gene heat map (Figure 5.5), reducing expression of leptin, TNF $\alpha$ , COX2, and IL-10 to basal levels. Their effect on MCP-1 expression was limited, however, with only a few crude fractions from *F. distichus* demonstrating any discernable activity. The red alga *Pyropia fallax* (PF) and green alga *Ulva lactuca* (UL) did not exhibit any significant anti-inflammatory capabilities against the five markers and were excluded from further study.

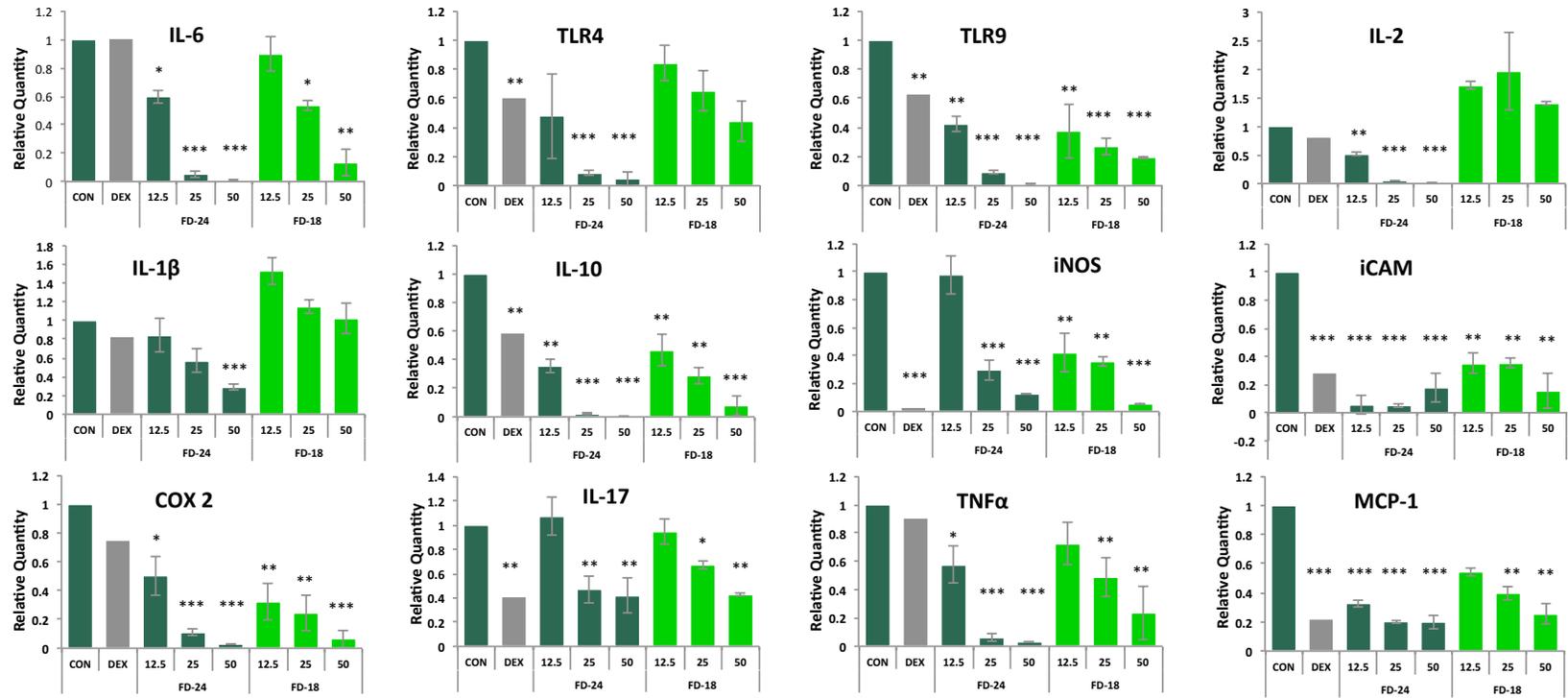


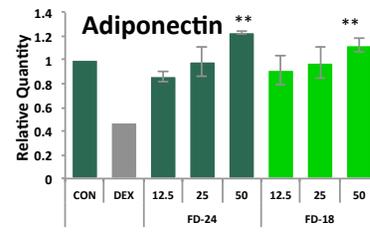
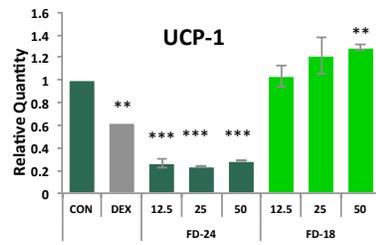
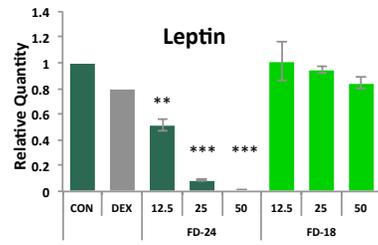
**Figure 5.5.** Heat map of mean gene expression from multi-gene analysis of adipocyte inflammatory markers leptin, TNF $\alpha$ , IL-10, MCP-1, and COX2. Light cyan represents an expression level of 0, whereas black represents the normalized expression of the untreated control. Crude fractions (hexane, “H”, ethyl acetate, “E”, butanol, “B”, or aqueous, “W”) of six Alaskan seaweed (*Alaria marginata*, “AM”, *Fucus distichus*, “FD”, *Pyropia fallax*, “PF”, *Saccharina groenlandica*, “SG”, *Saccharina latissima*, “SL”, and *Ulva lactuca*, “UL”) were applied to 3T3-L1 adipocytes at a final concentration of 50  $\mu$ g/mL

Investigation of the bioactive subfractions of *F. distichus* against an array of inflammatory genes yielded multiple sites of reduced expression (Figure 5.6). TLR4 and

TLR9 were both reduced after treatment with FD-24, and TLR9 was inhibited by FD-18, similar to the effects seen in the macrophage cell model (Figure 5.4). Interleukins IL-1 $\beta$ , 2, 6, 10, and 17 were inhibited to varying degrees by FD-24, with IL-10, 2, and 6 exhibiting a dose-response in expression levels and near-complete inhibition at the mid-range dose. FD-18 was only mildly effective in reducing interleukin expression, having inhibitory activity against IL-6, 17, and 10. Both FD-24 and FD-18 reduced levels of the inducible synthases iNOS and COX2, as well as iCAM and MCP-1, while FD-24 was more effective than FD-18 in inhibiting TNF $\alpha$  expression.

**Figure 5.6.** Effect of *F. distichus* subfractions 24 and 18 (FD-24 and FD-18, respectively) on pro-inflammatory gene expression levels in 3T3-L1 adipocyte cells. Adipocytes were treated with fractions as specified. Fold changes in gene expression are reported relative to untreated controls as mean response of triplicate runs  $\pm$  SEM. \*  $p < 0.05$  vs control; \*\*  $p < 0.01$  vs control; \*\*\*  $P < 0.001$  vs control

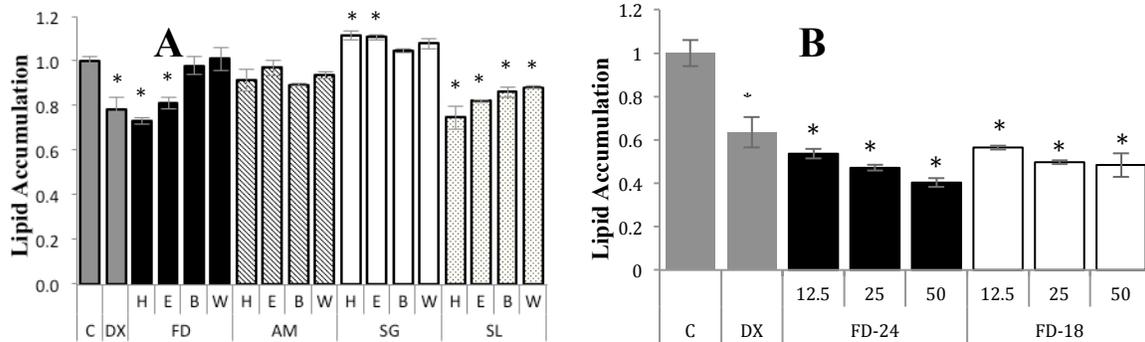




*F. distichus* subfractions also modulated lipid metabolism gene expression in the adipocyte model. FD-24 significantly reduced mRNA expression of leptin. UCP-1 was increased after treatment with FD-18, and both FD-24 and FD-18 demonstrated a dose-dependent increase in mRNA expression of adiponectin.

### **Lipid accumulation in 3T3-L1 adipocytes**

The four brown seaweeds (*F. distichus*, *A. marginata*, *S. groenlandica*, and *S. latissima*) were assayed to determine their effects on lipid accumulation in mature murine 3T3-L1 adipocytes. As seen in Figure 5.7A, the hexane and ethyl acetate fractions of *F. distichus* and *S. latissima* were most active in reducing lipid droplet size. *F. distichus*' hexane and ethyl acetate fractions (FD-H and FD-E) lowered the lipid accumulation levels to  $73.0 \pm 0.01\%$  and  $77.5 \pm 0.03\%$  of the untreated control, respectively. Post-fractionation, the active subfractions FD-18 and FD-24 showed significant inhibition of lipid accumulation, greater than that of the cruder fractions FD-H or FD-E. Both reduced lipid droplet size in a dose-dependent manner: FD-24 reduced lipid accumulation to  $45.9 \pm 0.01\%$  of the control, while FD-18 attained a minimum lipid content of  $48.5 \pm 0.05\%$  of control (Figure 5.7B).

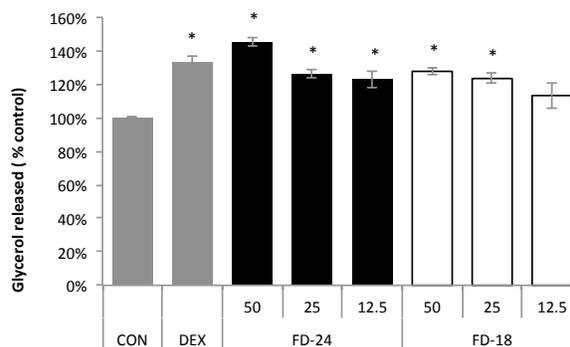


**Figure 5.7.** Effect of Alaskan seaweed on lipid accumulation by 3T3-L1 adipocytes. Mature adipocytes were treated with fractions overnight, then stained using the Oil Red O assay to quantify lipids present in cellular lipid droplets. Data were normalized to the negative control (“C”); dexamethasone (10 µM) “DX” was used as a positive control. (A) Crude fractions of the four brown kelps (hexane, “H”, ethyl acetate, “E”, butanol, “B”, or aqueous, “W” from *Alaria marginata*, “AM”, *Fucus distichus*, “FD”, *Saccharina groenlandica*, “SG”, and *Saccharina latissima*, “SL”), treated at a final concentration 100 µg/mL and incubated overnight. (B) Dose-response curves for active subfractions from *F. distichus*, FD-24 and FD-18. Adipocytes were treated with each subfraction at a final concentration of 12.5 – 50 µg/mL. Data are presented as average ± SEM (n = 3) \* designates statistically significant result versus control, p < 0.01.

The decreases in lipid accumulation by *F. distichus* subfractions occurred in parallel with an observed increase in lipolysis of the treated cells, as measured by the release of glycerol into the culture media (Figure 5.8). At 50 µg/mL, FD-24 increased free glycerol content to  $45 \pm 2.7\%$  greater than the untreated control, while FD-18 increased glycerol concentrations to  $28 \pm 2.2\%$  over the control. While the differences between treatments were not statistically significant, both subfractions appeared to behave in a dose-dependent manner.

## Composition of *F. distichus* active fractions

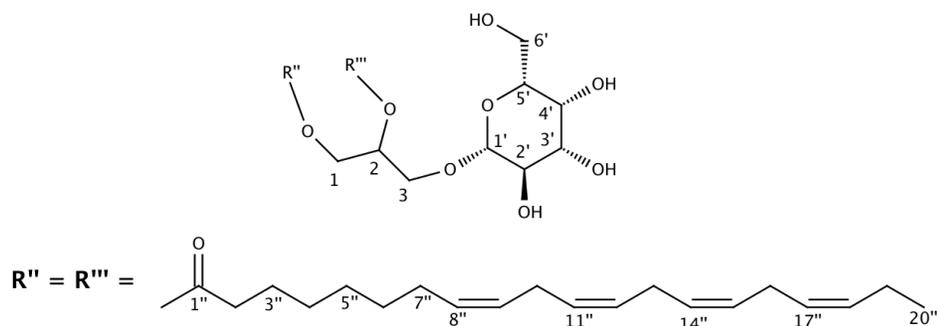
The hexane and ethyl acetate fractions of *F. distichus* exhibited the highest anti-inflammatory activity amongst the tested fractions, consistent with their high bioactivity in previous studies (Kellogg *et al.*, 2014; Kellogg & Lila, 2013). Combining and sub-fractionating the active fractions via flash chromatography yielded the potent subfractions FD-18 and FD-24. These two were explored to determine phytochemical constituents.



**Figure 5.8.** Free glycerol released into cell medium by 3T3-L1 cells after treatment with active fractions from *F. distichus*, FD-24 and FD-18. Adipocytes were treated with each subfraction at a final concentration of 12.5 – 50  $\mu\text{g/mL}$ . Data are presented as average  $\pm$  SEM (n = 3) \* designates statistically significant result versus control,  $p < 0.01$ .

Mass spectrometry analysis of the subfraction FD-18 revealed an ion chromatogram with a protonated molecular ion ( $[\text{M}+\text{H}]^+$ ) with  $m/z$  827.5684 Da, corresponding to the molecular formula  $\text{C}_{49}\text{H}_{78}\text{O}_{10}$ . Fragmentation of the parent ion yielded peaks at  $m/z$  645 indicating the loss of the glycosyl unit ( $-\text{C}_6\text{H}_{12}\text{O}_6$ ) and  $m/z$  357 the loss of the glycosyl unit and one of the eicosatetraenoic acid chains ( $-\text{C}_6\text{H}_{12}\text{O}_6 - \text{C}_{20}\text{H}_{31}\text{O}$ ). NMR spectra confirmed the presence of a monoglycosyldiacylglycerol (MGDG) featuring two eicosatetraenoic acids ( $\text{C}_{20:4} \omega\text{-3}$ ) (Figure 5.9). The spectrometric data for the MGDG for the glycerol and glycosyl segments was as follows:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 600 MHz): 4.38 (1H, dd,  $J = 11.4, 3.3$  Hz, H-1a), 4.18 (1H,

dd, J = 11.4, 6.4 Hz, H-1b), 5.27 (1H, m, H-2), 3.86 (1H, dd, J = 11.0, 5.6 Hz, H-3a), 3.69 (1H, dd, J = 11.0, 6.4 Hz, H-3b), 4.23 (1H, d, J = 7.4 Hz, H-1'), 3.61 (1H, dd, J = 9.5, 7.4 Hz, H-2'), 3.58 (1H, dd, J = 9.5, 2.5 Hz, H-3'), 4.00 (1H, br d, J = 2.5 Hz, H-4'), 3.49 (1H, br t, J = 4.6 Hz, H-5'), 3.86 (1H, dd, J = 12.5, 5.8 Hz, H-6a'), 3.80 (1H, dd, J = 12.5, 4.6 Hz, H-6b') and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 600 MHz): 63.05 (C-1), 70.38 (C-2), 68.41 (C-3), 104.25 (C-1'), 71.45 (C-2'), 73.49 (C-3'), 69.40 (C-4'), 74.66 (C-5'), 62.20 (C-6'). For the eicosatetraenoic acids, spectrometric data was:  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 600 MHz): 5.31 (16H, m, H- 8'', 9'', 11'', 12'', 14'', 15'', 17'', 18'', 8''', 9''', 11''', 12''', 14''', 15''', 17''', 18'''), 2.80 (12H, m, H-10'', 13'', 16'', 10''', 13''', 16'''), 2.30 (4H, m, H-2'', 2'''), 2.05 (8H, m, H-7'', 19'', 7''', 19'''), 1.61 (4H, m, H-3'', 3'''), 1.36 (12H, m, H-4'', 5'', 6'', 4''', 5''', 6'''), 0.94 (3H, t, J = 7.5 Hz, H- 20''), 0.86 (3H, t, J = 7.5 Hz, H-20''') and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 600 MHz): 173.83 (C-1''), 173.56 (C-1'''), 34.27 (C-2''), 32.12 (C-2'''), 24.91 (C-3''), 24.67 (C-3'''), 26.72 – 29.96 (C-4'', 5'', 6'', 4''', 5''', 6'''), 132.23 (C-7''), 129.75 (C-7'''), 127.22-129.74 (C-8'', 9'', 11'', 12'', 14'', 15'', 17'', 18'', 8''', 9''', 11''', 12''', 14''', 15''', 17''', 18'''), 25.77 (C-10'', 13'', 16'', 10''', 13''', 16'''), 27.05 (C-19'', 19'''), 14.48 (C-20'''), 14.30 (C-20''). These assignments were congruent with those reported in the literature for similar MGDG structures isolated from both marine (Lopes *et al.*, 2014) and terrestrial (Grace *et al.*, 2012) sources.



**Figure 5.9.** Bioactive constituent from the Alaskan seaweed *Fucus distichus* subfraction 18: a monoglycosyldiacylglycerol (MGDG) with eicosatetraenoic acid side chains (C20:4  $\omega$ -3).

The second active fraction, FD-24, was separated via normal phase liquid chromatography (NPLC) with a diol column and binary mobile phase of acetonitrile and aqueous methanol resulting in the visualization of 12 isomers. These 12 isomers were analyzed via mass spectrometry, acquiring full spectra in the negative ion mode from  $m/z$  150 to 2500. Analysis revealed an ion chromatogram with pseudomolecular ions corresponding to fucophloroethol oligomers with 9 (1117 Da) to 20 (2480 Da) phloroglucinol units (Table 5.3). Masses corresponded fucophloroethol oligomers with single aryl or ether bridges between monomer units. All analyzed peaks showed fragmentation patterns characteristic of fucophloroethol fragmentation, similar to those seen in a previous study (Kellogg *et al.*, 2014).

The subfraction FD-24 contained only larger phlorotannin oligomers ( $DP \geq 9$ ), and manifested peaks in the negative mode mass spectra as  $[M - H]^-$  and  $[M - 2H]^{-2}$ . Identification was achieved through the accurate mass spectrometry of parent ion and daughter ions (Table 5.3), with fragmentation mass differences corresponding to half of the

corresponding mono-ionized fragmentation patterns. The larger polymers evidenced cleavage into multiple daughter ions (Table 5.3), allowing for the characterization of these phlorotannins as fucophloroethols with DP from 9 to 20.

**Table 5.3.** Normal-phase liquid chromatography-mass spectrometry (NPLC)-IT-TOF-MS<sup>2</sup> characterization of phlorotannins from FD-24 \*.

Peak No.	RT (min)	Molecular Formula	Measured Mass ( <i>m/z</i> )	Predicted Mass ( <i>m/z</i> )	$\Delta m$ (ppm)	DP <sup>a</sup>	MS/MS Ions ( <i>m/z</i> )		
1	21.790	C <sub>54</sub> H <sub>38</sub> O <sub>27</sub>	1117.1484; [M – H] <sup>-</sup>	1117.1528	-4.4	9	1109	993	709
2	22.583	C <sub>60</sub> H <sub>42</sub> O <sub>30</sub>	1241.1610; [M – H] <sup>-</sup>	1241.1653	-4.3	10	1223	993	931
3	24.578	C <sub>66</sub> H <sub>46</sub> O <sub>33</sub>	1365.1879; [M – H] <sup>-</sup>	1365.1849	3.0	11	1347	993	931
4	25.681	C <sub>72</sub> H <sub>50</sub> O <sub>36</sub>	1489.2050; [M – H] <sup>-</sup>	1489.2009	4.1	12	1471	1347	993
5	26.457	C <sub>78</sub> H <sub>54</sub> O <sub>39</sub>	806.1006; [M – 2H] <sup>2-</sup>	806.1048	-4.1	13	797	599	541
6	27.740	C <sub>84</sub> H <sub>58</sub> O <sub>42</sub>	868.1179; [M – 2H] <sup>2-</sup>	868.1129	5.0	14	851	806	797
7	28.370	C <sub>90</sub> H <sub>62</sub> O <sub>45</sub>	930.1181; [M – 2H] <sup>2-</sup>	930.1209	-2.8	15	922	868	
8	29.280	C <sub>96</sub> H <sub>66</sub> O <sub>48</sub>	993.1317; [M – 2H] <sup>2-</sup>	993.1362	-4.5	16	984	931	759
9	29.677	C <sub>102</sub> H <sub>70</sub> O <sub>51</sub>	1054.1378; [M – 2H] <sup>2-</sup>	1054.1369	0.9	17	1046	993	931
10	30.642	C <sub>108</sub> H <sub>74</sub> O <sub>54</sub>	1116.1443; [M – 2H] <sup>2-</sup>	1116.1449	-0.6	18	1107	1054	931
11	31.170	C <sub>114</sub> H <sub>78</sub> O <sub>57</sub>	1178.1522; [M – 2H] <sup>2-</sup>	1178.1530	-0.8	19	1160	1054	931
12	31.532	C <sub>120</sub> H <sub>84</sub> O <sub>60</sub>	1240.1660; [M – 2H] <sup>2-</sup>	1240.1610	5.0	20	1231	1054	931

\* FD-24 = *Fucus distichus*' subfraction 24; <sup>a</sup> Degree of polymerization.

## Discussion

Once thought to be a static depot for storage of triglycerides and fatty acids, adipose tissue is now understood to be an active endocrine organ, secreting and responding to a variety of chemokines that have systematic regulatory functions (Johnson *et al.*, 2012). Higher levels of lipid metabolism and storage induce up-regulation of proinflammatory adipokines, resulting in a chronic inflammatory state linked with the recruitment and activation of macrophage cells (Hsu *et al.*, 2013; Xu *et al.*, 2003), eventually leading to insulin resistance and imbalances in lipid metabolism (Sartipy & Loskutoff, 2003; Weisberg *et al.*, 2003).

Toll-like receptors function as integral members of host immune and inflammatory responses to lipopolysaccharide stimulation. TLRs enervate multiple pathways, such as NF $\kappa$ B, c-Jun kinase (JNK), and mitogen-activated protein kinase (MAPK), which activate regulators of downstream inflammatory processes, cytokine/chemokine mRNA expression and/or protein secretion. Down-regulation of TLRs has been associated with lower inflammatory responses (Chahal *et al.*, 2013; Lee *et al.*, 2012a). The phlorotannin-rich subfraction FD-24 demonstrated significant inhibition of TLR4 expression levels in the macrophage and adipocyte *in vitro* systems, while the glycolipid MGDG inhibited TLR9 gene expression (Figure 5.4, 5.6). This decrease in TLR expression levels provides one potential mechanism through which the *F. distichus* subfractions could lower inflammation in macrophage and adipocyte models. TLRs have been shown to be a target by

phytochemicals, including polyphenols and  $\omega$ -3 fatty acids, which can lower downstream inflammatory markers through attenuation of TLRs (Chahal *et al.*, 2013).

These subfractions also lowered expression of inflammatory biomarkers downstream of TLR activation. Markers of acute inflammation, including IL-10, COX2, iNOS, and IL-1 $\beta$  were highly inhibited, as well as chronic inflammatory biomarkers, such as TNF $\alpha$ , IL-6, and IL-17. These inflammatory genes are controlled predominantly via the NF $\kappa$ B and JNK pathways after activation by TLRs. Additionally, lower levels of iNOS expression after treatment by both FD-18 and FD-24 correlated with a previous study which found *F. distichus* crude partitions reduced nitric oxide levels in LPS-stimulated macrophages (Kellogg & Lila, 2013). These downstream effects support the hypothesis of TLR attenuation and inhibition of the NF $\kappa$ B and JNK pathways by *F. distichus*. In contrast, the expression of iCAM, which progresses via the p38 MAPK path, had lower levels of expression inhibition.

Lipid accumulation is an indicator of energy balance and regulation within adipocytes, which is dysregulated under chronic inflammatory conditions and after hypertrophy occurs (Guilherme *et al.*, 2008). Inflammatory cytokines such as TNF $\alpha$ , IL-1 $\beta$ , and IL-6 suppress adiponectin expression and secretion (Simons *et al.*, 2007), increase leptin synthesis, and reduce UCP-1 expression and activation (Sakamoto *et al.*, 2013). *F. distichus* subfractions reversed many of these effects at the gene expression level. Increased UCP-1 expression levels recorded after treatment with subfraction FD-18 could indicate an increase in energy expenditure as regulated by UCP-1, which would account for the observed decreased lipid accumulation and increase in free glycerol concentration after treatment.

Other phytochemicals found in brown algae, especially the carotenoid fucoxanthin, have also been observed to activate UCP-1 and increase energy expenditure at the cellular level (D'Orazio *et al.*, 2012; Maeda *et al.*, 2005). FD-24 lowered leptin expression in a dose-dependent manner, but had no significant effect on adiponectin synthesis. FD-18, however, increased adiponectin expression levels, which could in turn exert its own anti-inflammatory effects on multiple pathways (Ajuwon & Spurlock, 2005)

The bioactive constituents responsible for the activity observed in this study were of two distinct natural product classes. Subfraction FD-18 contained a MGDG comprised of eicosatetraenoic acid fatty acid residues (C20:4  $\omega$ -3). Multiple genera of brown seaweed, including *Fucus*, contain species that contain long chain mono and polyunsaturated fatty acids with unsaturation beginning at the  $\omega$ -3 carbon (Lopes *et al.*, 2014). These glycosylated glycerolipids possess anti-inflammatory properties; a monoacylglycerol and a MGDG mixture (C20:5  $\omega$ -3 fatty acid combined with either C18:4  $\omega$ -3 or C18:3  $\omega$ -3 fatty acids) isolated from *F. spiralis* both inhibited nitric oxide production in a macrophage cell system, the  $\omega$ -3 fatty acid chains of the MGDG providing greater anti-inflammatory activity than the  $\omega$ -9 monoacylglycerol (Lopes *et al.*, 2014). Other diacylglycerols and long-chain  $\omega$ -3 fatty acids from algal and botanical sources have also demonstrated anti-inflammatory capabilities (Banskota *et al.*, 2013; Oliver *et al.*, 2012), or are metabolized by cells into more bioactive constituents (Balvers *et al.*, 2010).

*F. distichus*' second bioactive subfraction, F-24, possessed multiple phlorotannin oligomers ranging from DP 9 to 20. Polymeric phloroglucinol derivatives have inhibited inflammatory markers, including NO and prostaglandin synthesis (Kim *et al.*, 2009), acting

through the NF $\kappa$ B pathway (Lee *et al.*, 2012b). It is significant to note the absence of lower degree of polymerization phlorotannins in the most active subfraction FD-24. While oligomers ranging from 3 to 18 in were present in similar subfractions, including FD-22, they were not as active in the *in vitro* anti-inflammatory assays. Previous studies have also shown that higher DP phlorotannin oligomers are more potent in modulating inflammatory effects (Lee *et al.*, 2012b), though the exact mechanisms underlying this behavior are not well understood.

In the current investigation, Alaskan seaweed demonstrated multiple anti-inflammatory effects in adipose and macrophage cells. Through an array approach of inflammatory marker expression analysis involving 12-15 inflammatory and metabolic genes, *F. distichus* demonstrated significant inhibition of both acute and chronic inflammatory biomarkers. There was considerable overlap in the expression levels for many of the cytokines and inflammatory biomarkers examined, which are produced by both cell systems (MCP-1, TNF- $\alpha$ , IL-10, IL-1 $\beta$ , TLR4, TLR9). Thus, considering the potential cellular signaling capabilities of polyphenols, phlorotannins may not merely exert their effects as free radical scavengers as seen in previous studies (Kellogg & Lila, 2013), but may also modulate inflammatory signaling receptors such as TLR4 and TLR9, as well as downstream protein pathways, including NF $\kappa$ B, JNK, and p38 MAPK mechanisms. Verification and additional investigation of anti-inflammatory pathways will provide a clearer understanding of the mechanisms underlying the anti-inflammatory activity observed in the current study. Through these gene-level effects, seaweeds like *F. distichus* hold

potential to provide beneficial effects against inflammatory-mediated diseases as potent anti-inflammatory functional foods.

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## CHAPTER 6 CONCLUSIONS AND FUTURE DIRECTIONS

## Concluding remarks

Edible seaweeds have formed a fundamental part of traditional diets for indigenous cultures across the globe, especially in the Eastern Pacific region of Alaska and the Pacific Northwest. These seaweeds have provided coastal communities with important macro- and micro-nutrients, and have served as a source of novel bioactive phytochemicals to maintain human health. Seaweeds have been one facet in NA/AN communities' historically low rates of cardiovascular disease, resistance to insulin (type 2 diabetes), and obesity. The absence of such dietary inputs is hypothesized to have contributed to the significant rise in chronic metabolic conditions that have plagued NA/AN communities over the recent past few decades.

Through the course of this project, Alaskan coastal seaweeds have demonstrated multiple complimentary mechanisms of action against insulin resistance, obesity, and metabolic syndrome. Seaweeds were efficacious in reducing oxidative stress, capable of scavenging organic radicals, chelating metal ions, and reducing nitric oxide generation in chemical assays while also showing *in vitro* scavenging effects on ROS levels and reduction of NO production by RAW 264.7 cells. *F. distichus* and *A. marginata* contributed to reduced carbohydrate hydrolysis by the enzymes  $\alpha$ -glucosidase and  $\alpha$ -amylase, ameliorating carbohydrate digestion and metabolism. Brown seaweeds were also capable of attenuating inflammatory responses in the two principal cell types involved in the development of metabolic syndrome: macrophages and adipocytes, lowering multiple pathways of inflammatory markers, while also reducing lipid accumulation in mature adipocyte cells.

Underlying this bioactivity is a diverse array of phytochemicals present in the brown seaweeds. While all seaweeds examined possessed moderate to high levels of phenolic content, *F. distichus* had multiple oligomers of the polyhydroxylated phlorotannin group, with degree of polymerization from 3 to >20. In addition, the same species contained a monoglucosyldiacylglyceride (MGDG) containing two eicosatetraenoic fatty acid residues (C20:4  $\omega$ -3). These two diverse chemical classes both demonstrated anti-inflammatory potential and lipid-lowering characteristics.

In general, these species, which form part of the traditional diets of Pacific Northwest cultures, exhibited effective protection against the underlying causes of metabolic syndrome in both chemical and *in vitro* systems. As such, they have potential to confer protection, when incorporated into the diet, against many oxidative- and inflammatory-dependent physiopathologies, such as obesity, diabetes, and cardiovascular disease.

### **Future directions**

During the course of this project, more potential questions and avenues of inquiry have arisen than have been resolved. Alaskan seaweeds are diverse and rich subjects of study, with numerous paths upon which one could expand on the work presented here to better characterize the algae and their bioactivity.

In the research presented here, the sampled seaweeds were treated as samples of any other botanical source would be: harvested raw, immediately frozen, and lyophilized until extraction and bioactivity testing. However, this processing does not represent the normal route by which seaweeds have traditionally been eaten. As part of the culinary traditions of

Alaskan Natives, seaweeds were consumed in a variety of dishes and prepared many different ways. Kelps were boiled into soups, fermented and pressed into cakes, or dried and toasted to be sprinkled over other foods. As such, the heat, fermentation microorganisms, or drying technique could impact the phytochemical constituents and their bioactive potential. Polyphenols are known to be sensitive to heat processing, and thus the potential of processing to affect the phytochemistry could be essential to determine whether the bioactive constituents are altered or degraded and could lose their health-promoting properties. Future research could logically examine how processing and culinary preparation impacts on the health beneficial properties of seaweeds as food and medicine.

Ingested polyphenols and other natural products are limited in their ability to cross the brush border in the intestine and enter into the bloodstream. If the bioactivity is predicated on passing the intestinal tract, this could lower the potential clinical efficacy of natural products, which would contradict any apparent *in vitro* bioactivity. However, potential metabolism by gut microbiota as part of the digestion process could augment structural properties and alter absorption to the intestine, or attenuate the microflora directly to affect *in vivo* efficacy. A study involving human participants would be the logical continuation. A placebo-controlled, double-blind study could monitor metabolic markers during seaweed consumption over the short to medium time frame, while allowing measurement of any potential phase 1 or phase 2 metabolites derived from seaweed phytochemicals.

Finally, the information and results stemming from this project could have the greatest impact with the communities along the coast of Alaska who have a history of

seaweed consumption. The chemical and in vitro results, coupled with a potential future clinical study, could directly inform the population who could most directly benefit from seaweed consumption. Thus, moving forward, it is imperative to develop a comprehensive plan for public dissemination and engagement. This will help bring about awareness of the potential impact of traditional foods and diets in maintaining personal health.

## APPENDICES

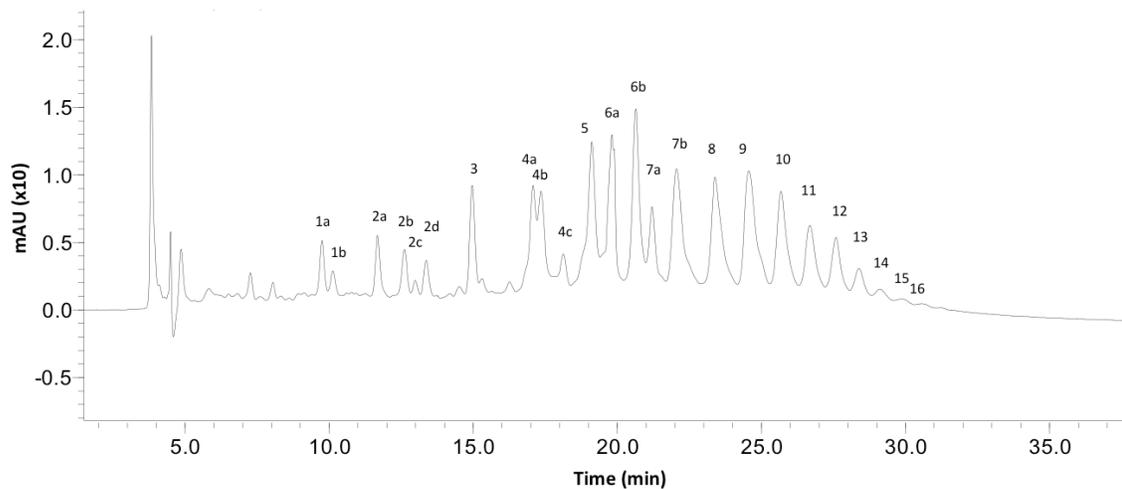
## APPENDIX A. COLLECTION DATA

<b>Date Collected</b>	6/17/2012	6/19/2012	6/19/2012	6/19/2012	6/19/2012	6/19/2012	6/19/2012
<b>Scientific Name</b>	<i>Fucus distichus</i>	<i>Ulva lactuca</i>	<i>Alaria marginata</i>	<i>Saccharina groenlandica</i>	<i>Pyropia fallax</i>	<i>Saccharina latissima</i>	<i>Plantago maritima</i>
<b>Common Name</b>	Bladderwrack ; popweed	Sea lettuce	Ribbon kelp	Split kelp; kelp	False laver	Sugar kelp	Goosetongue
<b>Laboratory ID</b>	FD	UL	AM	SG	PF	SL	PM
<b>Latitude</b>	57° 02.917' N	57° 03.350' N	57° 03.350' N	57° 05.917' N	57° 05.917' N	57° 05.917' N	57° 05.917' N
<b>Longitude</b>	135° 19.250'	135° 22.317'	135° 22.317'	135° 24.000'	135° 24.000'	135° 24.000'	135° 24.000'
<b>Elevation</b>	0'	0'	0'	0'	0'	0'	15'
<b>Herbarium</b>	NCU	NCU	n/a*	NCU	NCU	NCU	NCU
<b>Accession #</b>	633751	633750	n/a*	633749	633748	633752	633753
<b>Amount Collected</b>	48 g	41 g	29 g	71 g	33 g	18 g	19 g
<b>Habitat</b>	Rocky coast	Rocky coast	Rocky coast	Rocky coast	Rocky coast	Rocky coast	Rocky coast
<b>Temperature</b>	64°F	60°F	60°F	60°F	60°F	60°F	60°F
<b>Herbivory/Fungus</b>	None	None	None	None	None	None	None

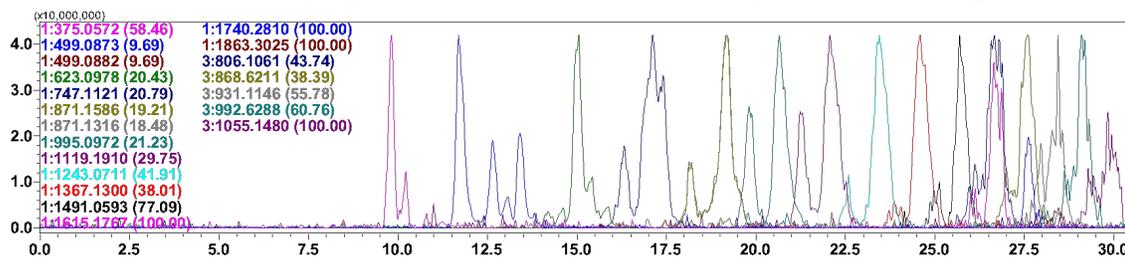
\* Insufficient material for accession to herbarium.

# APPENDIX B. MASS SPECTROMETRY DATA FOR FD-E-22

## NPLC chromatogram (labeled as in Chapter 4)

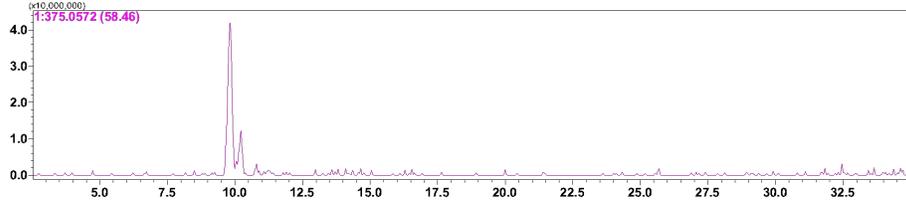


## Extracted ion chromatographs from NPLC-MS for each phlorotannin oligomer

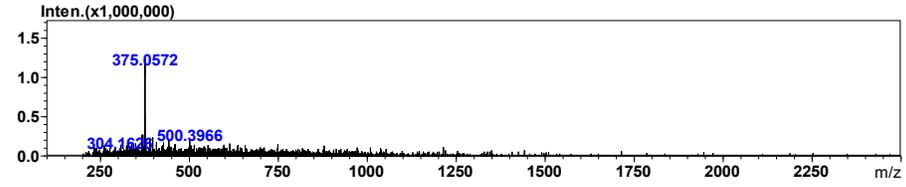


$m/z$  375  
DP = 3  
RT = 9.752 min

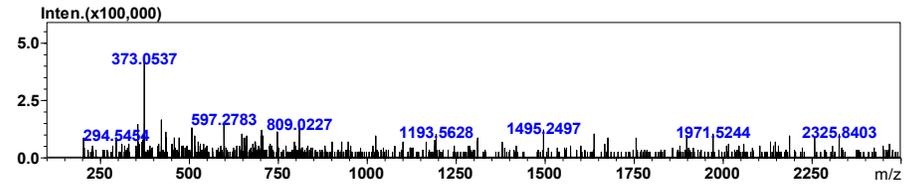
Extracted  
ion current



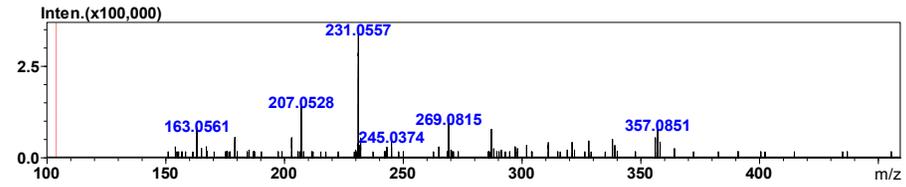
$[M + H]^+$



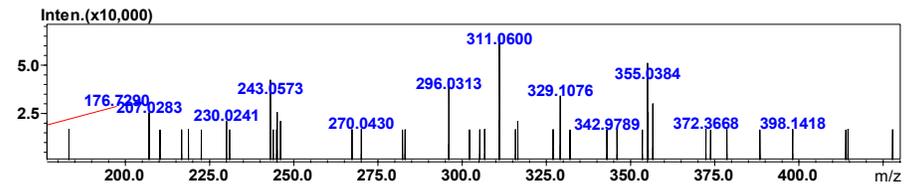
$[M - H]^-$



MS<sup>2</sup>  $[M + H]^+$

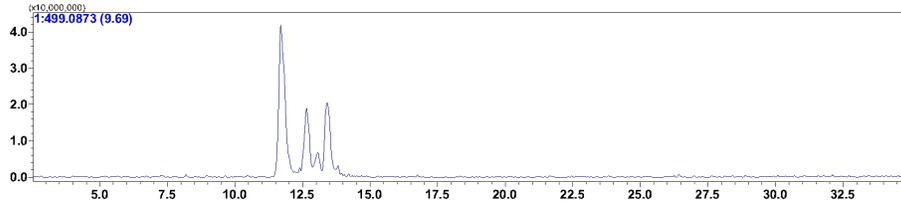


MS<sup>2</sup>  $[M - H]^-$

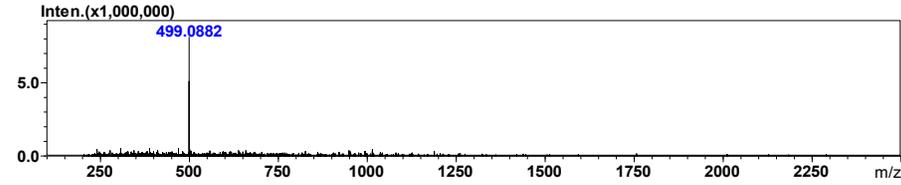


$m/z$  499  
DP = 4  
RT = 11.860, 12.679, 12.996, 13.259 min

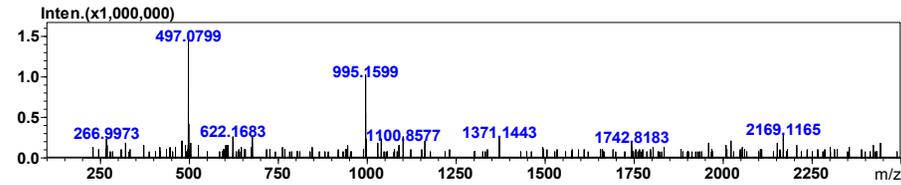
Extracted  
ion current



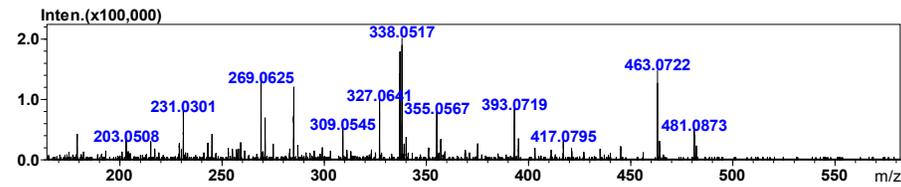
$[M + H]^+$



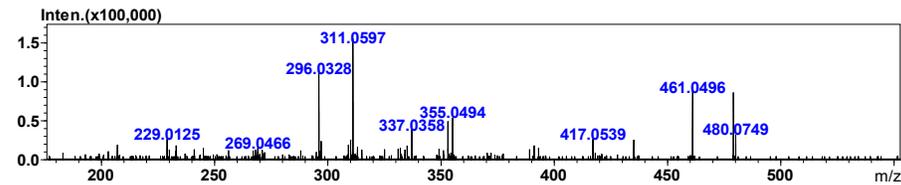
$[M - H]^-$



$MS^2 [M + H]^+$

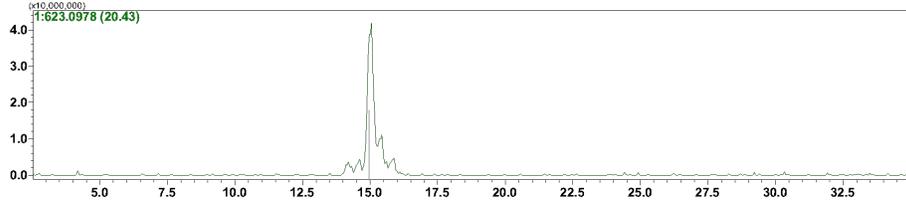


$MS^2 [M - H]^-$

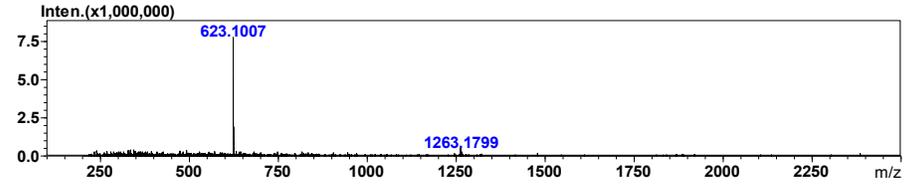


$m/z$  623  
DP = 5  
RT = 14.822 min

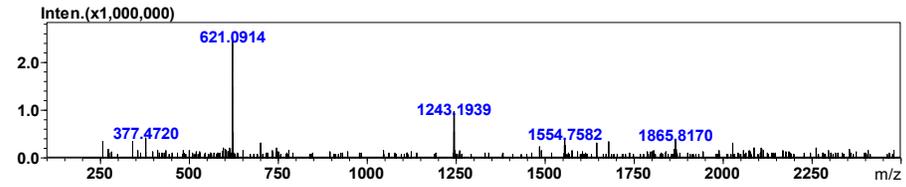
Extracted  
ion current



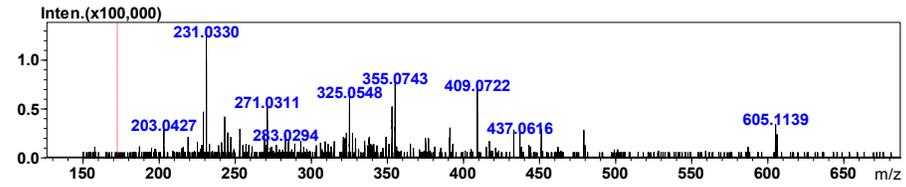
$[M + H]^+$



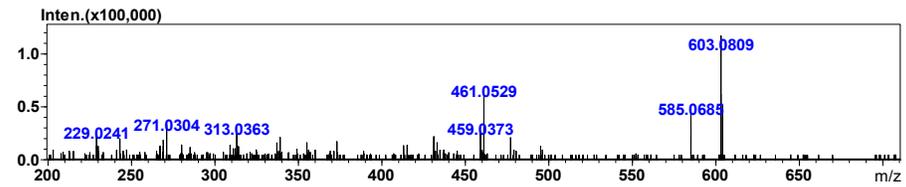
$[M - H]^-$



$MS^2 [M + H]^+$

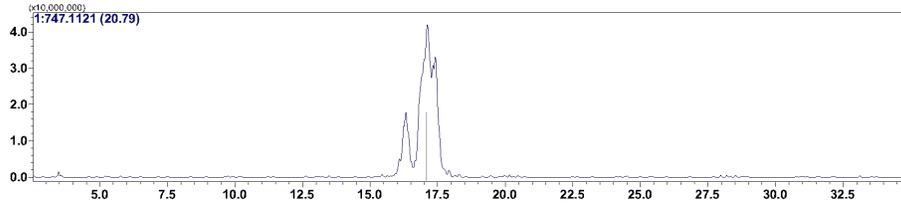


$MS^2 [M - H]^-$

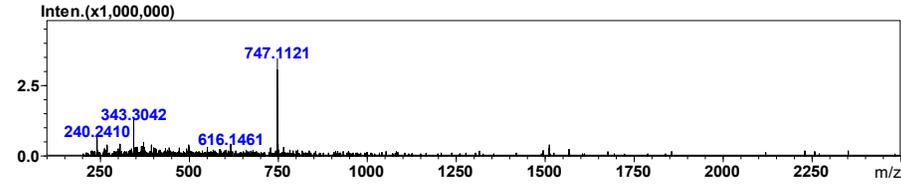


$m/z$  747  
DP = 6  
RT = 16.255, 16.943, 17.345 min

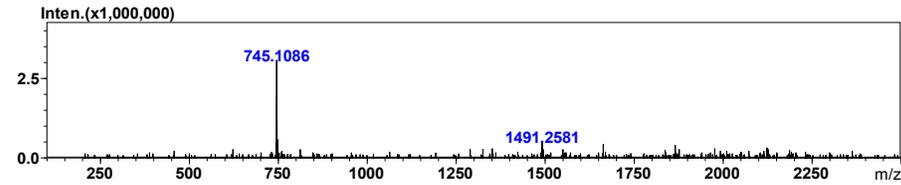
Extracted  
ion current



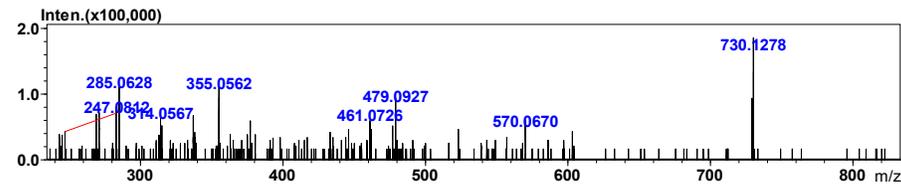
$[M + H]^+$



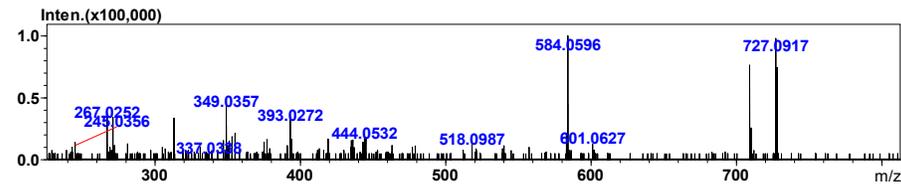
$[M - H]^-$



$MS^2 [M + H]^+$

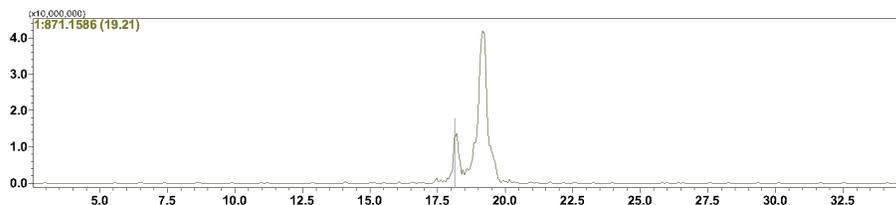


$MS^2 [M - H]^-$

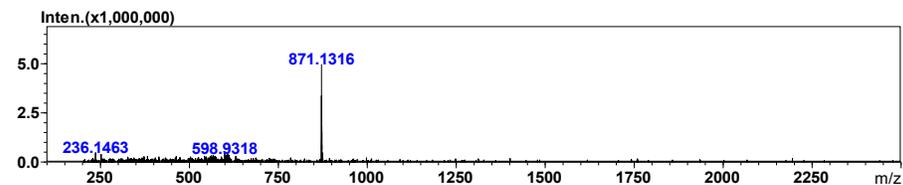


$m/z$  871  
DP = 7  
RT = 17.922, 19.113 min

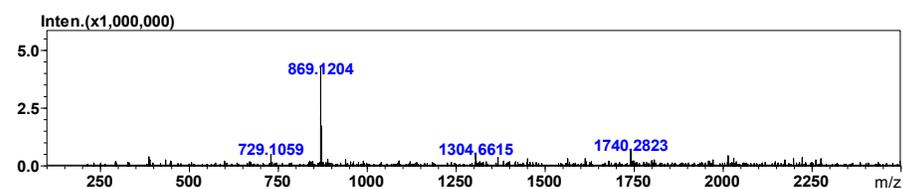
Extracted ion current



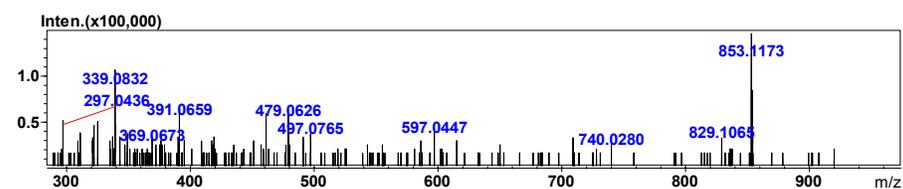
$[M + H]^+$



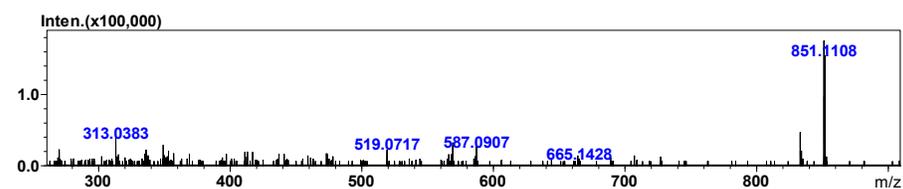
$[M - H]^-$



$MS^2 [M + H]^+$

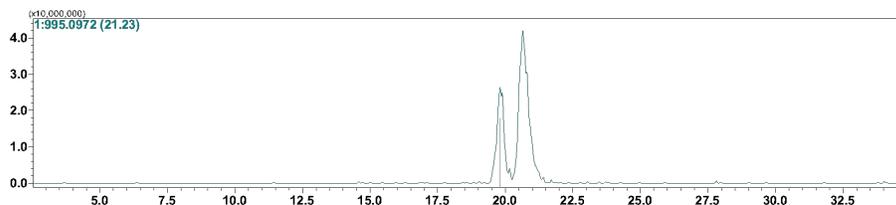


$MS^2 [M - H]^-$

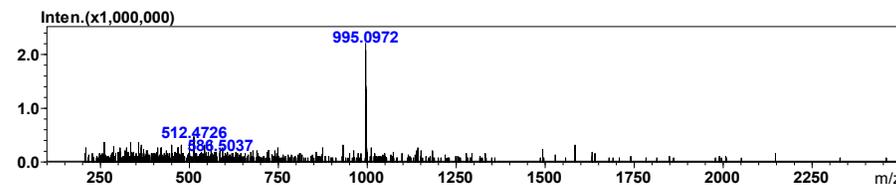


$m/z$  995  
DP = 8  
RT = 19.809, 20.632 min

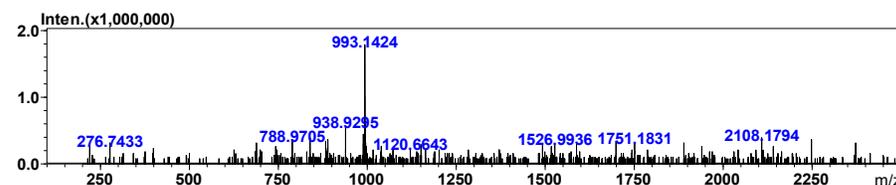
Extracted  
ion current



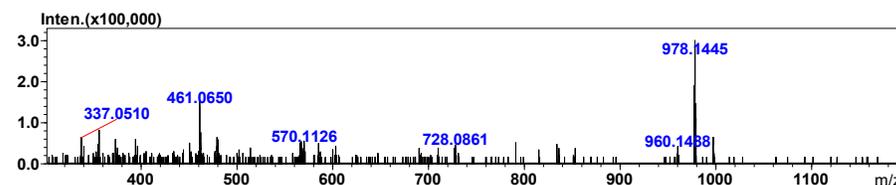
$[M + H]^+$



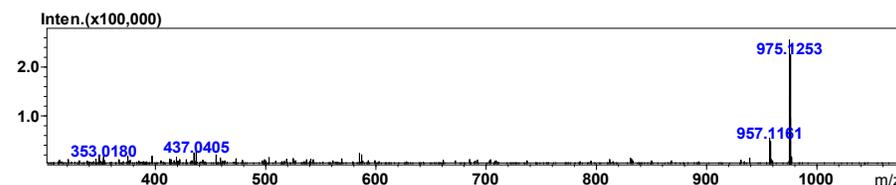
$[M - H]^-$



$MS^2 [M + H]^+$

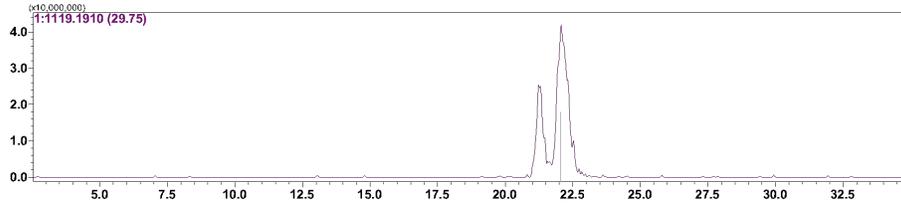


$MS^2 [M - H]^-$

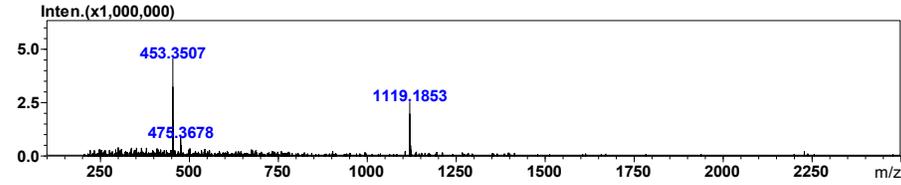


$m/z$  1119  
DP = 9  
RT = 21.204, 22.050 min

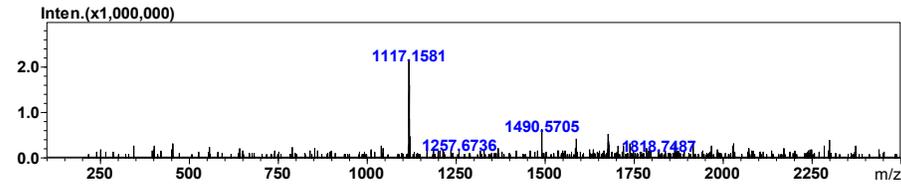
Extracted  
ion current



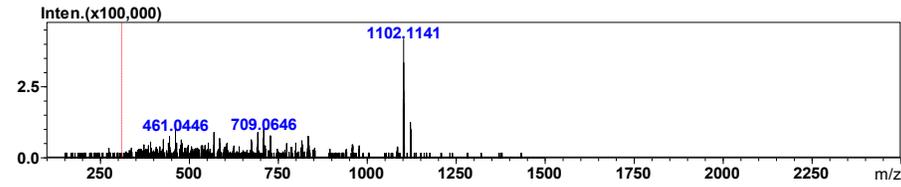
$[M + H]^+$



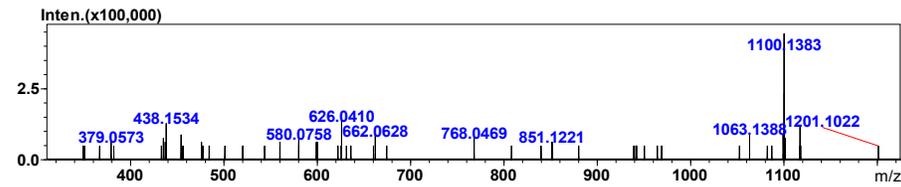
$[M - H]^-$



$MS^2 [M + H]^+$

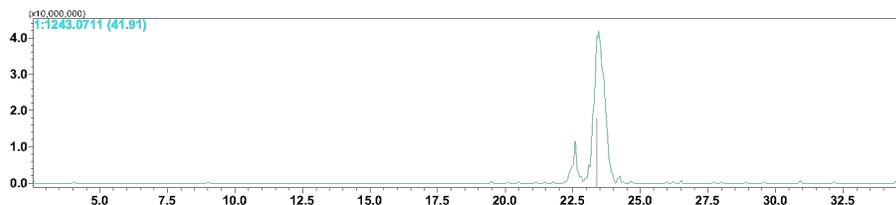


$MS^2 [M - H]^-$

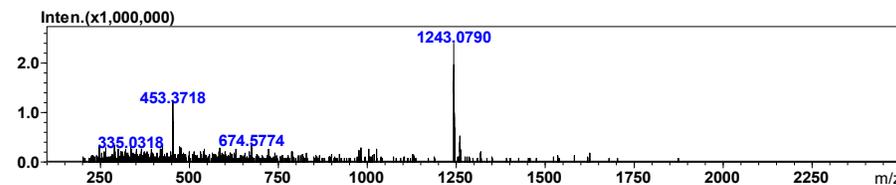


$m/z$  1243  
DP = 10  
RT = 23.390 min

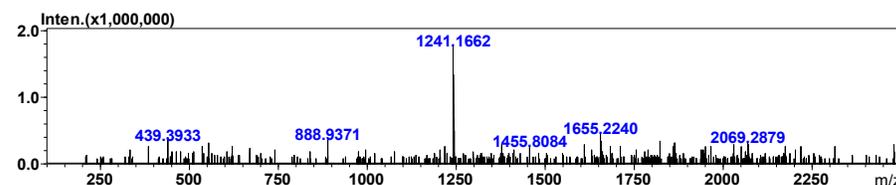
Extracted  
ion current



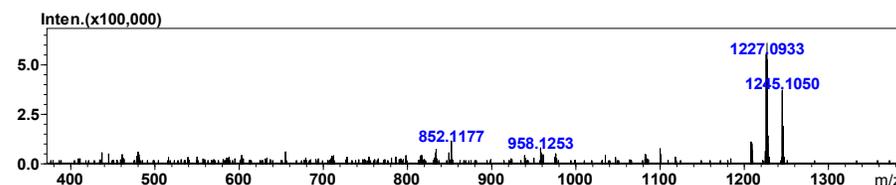
$[M + H]^+$



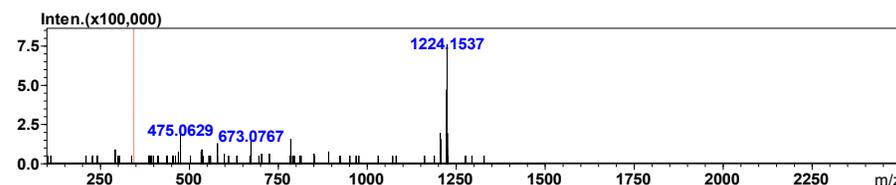
$[M - H]^-$



$MS^2 [M + H]^+$

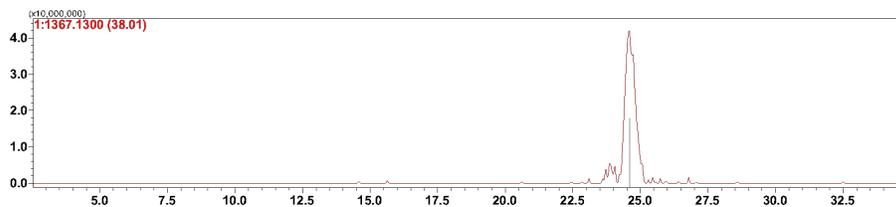


$MS^2 [M - H]^-$

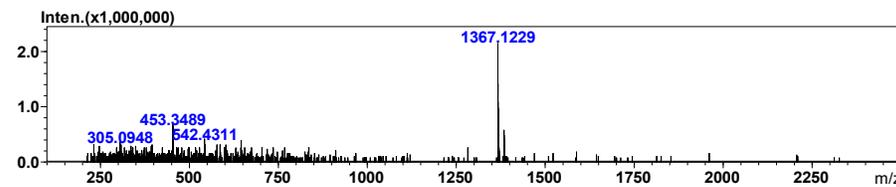


$m/z$  1367  
DP = 11  
RT = 24.561 min

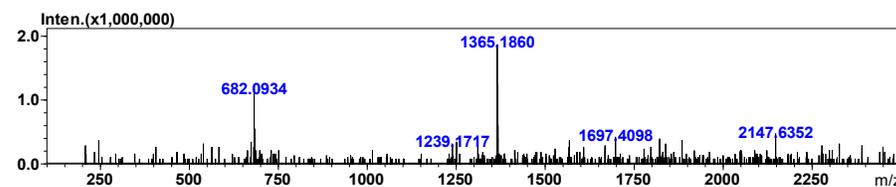
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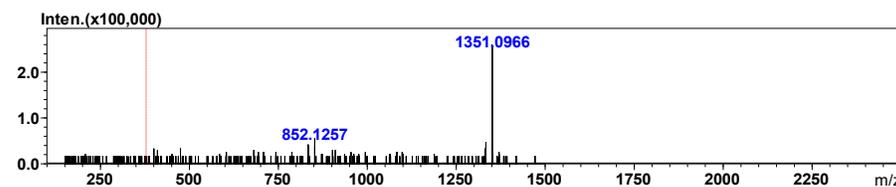
$[M + H]^+$



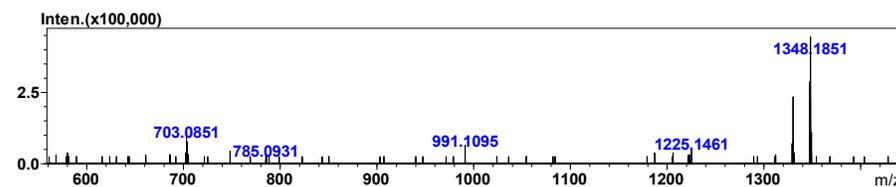
$[M - H]^-$



$MS^2 [M + H]^+$

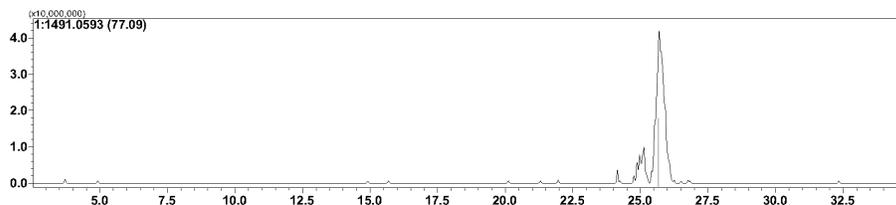


$MS^2 [M - H]^-$

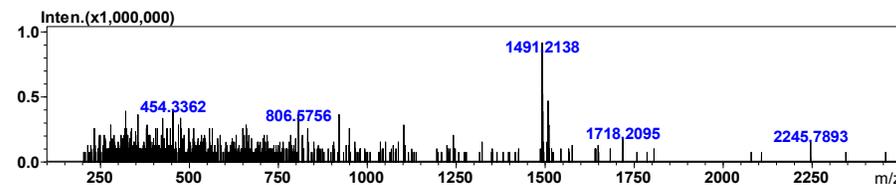


$m/z$  1491  
DP = 12  
RT = 25.677 min

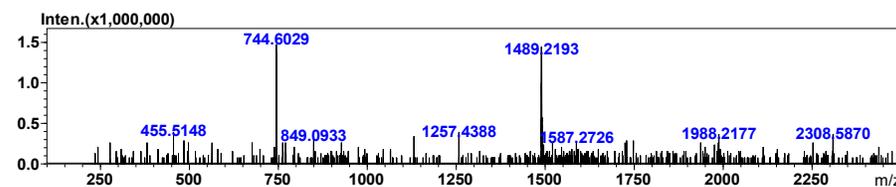
Extracted  
ion current



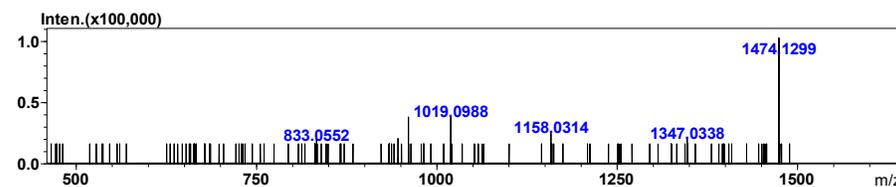
$[M + H]^+$



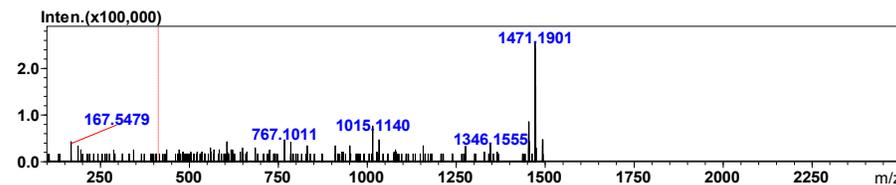
$[M - H]^-$



$MS^2 [M + H]^+$

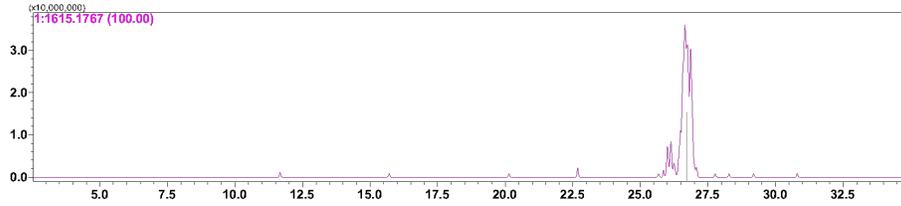


$MS^2 [M - H]^-$

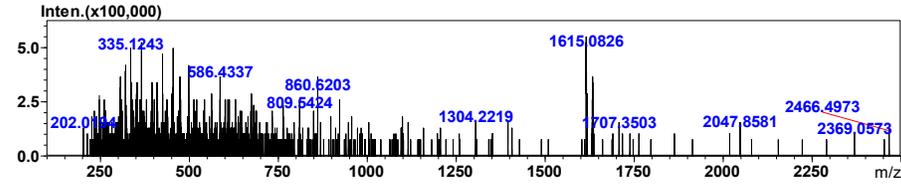


$m/z$  1615  
DP = 13  
RT = 26.681 min

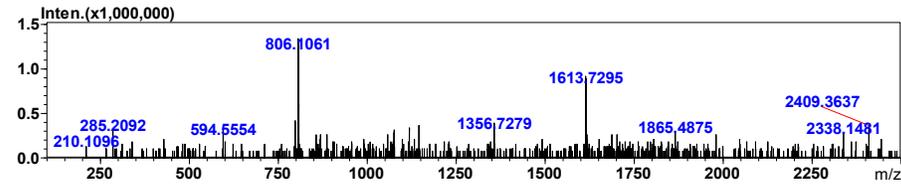
Extracted ion current



$[M + H]^+$



$[M - H]^-$



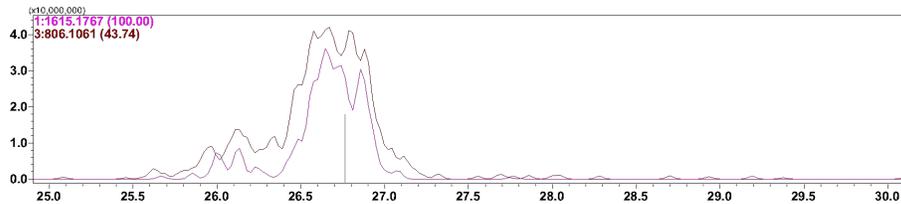
$MS^2 [M + H]^+$

n/a

$MS^2 [M - H]^-$

n/a

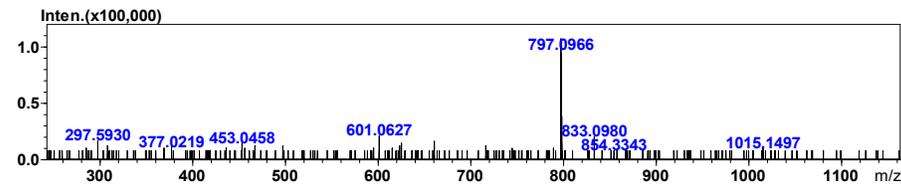
Extracted ion current



$MS^2 [M + 2H]^{+2}$

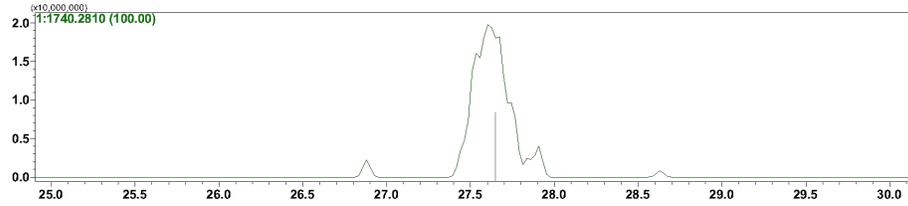
n/a

$MS^2 [M - 2H]^{-2}$

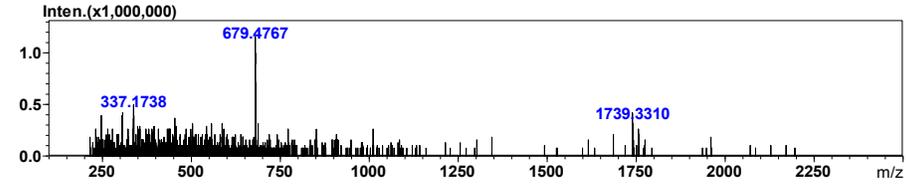


$m/z$  1729  
DP = 14  
RT = 27.581 min

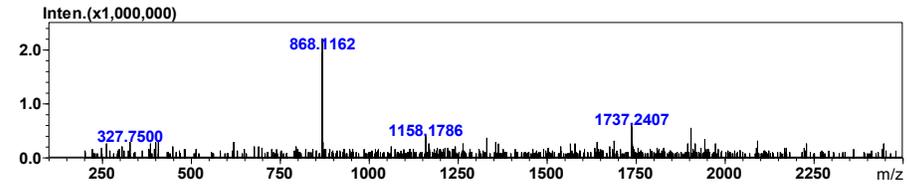
Extracted ion current



$[M + H]^+$



$[M - H]^-$



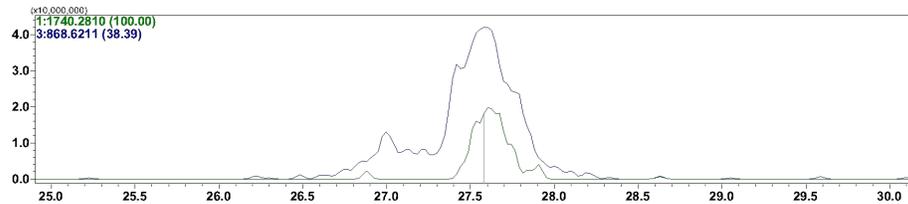
$MS^2 [M + H]^+$

n/a

$MS^2 [M - H]^-$

n/a

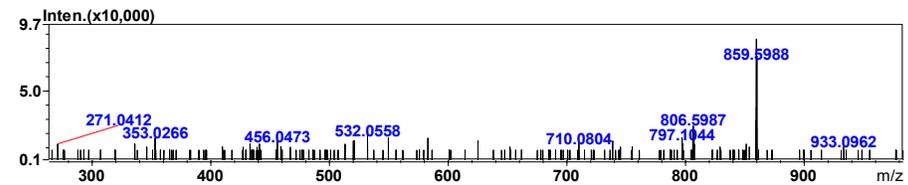
Extracted ion current



$MS^2 [M + 2H]^{+2}$

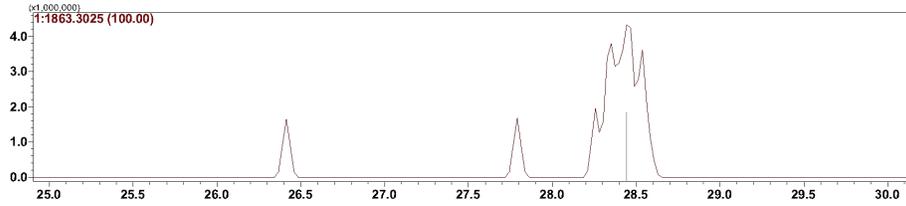
n/a

$MS^2 [M - 2H]^{-2}$

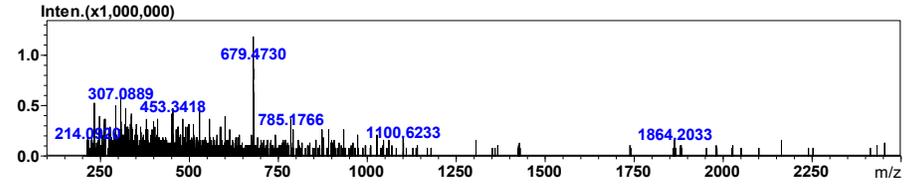


$m/z$  1863  
DP = 15  
RT = 28.385 min

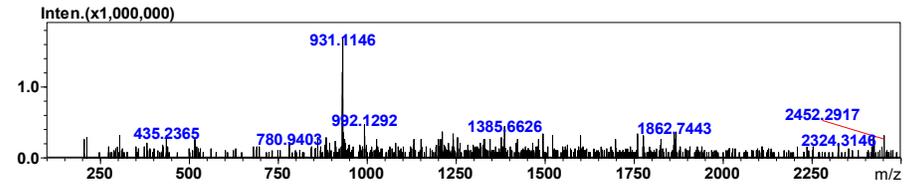
Extracted ion current



$[M + H]^+$



$[M - H]^-$



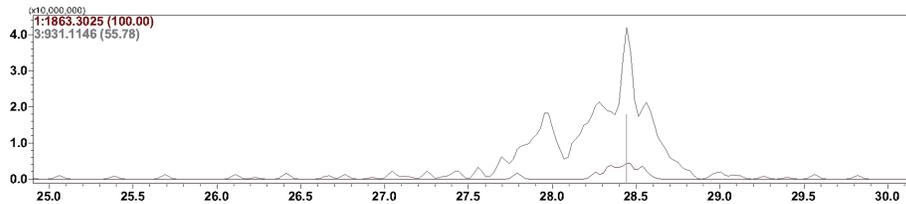
$MS^2 [M + H]^+$

n/a

$MS^2 [M - H]^-$

n/a

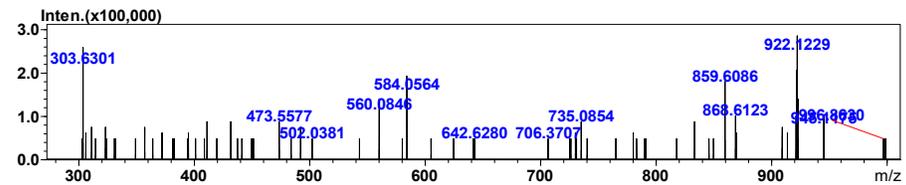
Extracted ion current



$MS^2 [M + 2H]^{+2}$

n/a

$MS^2 [M - 2H]^{-2}$

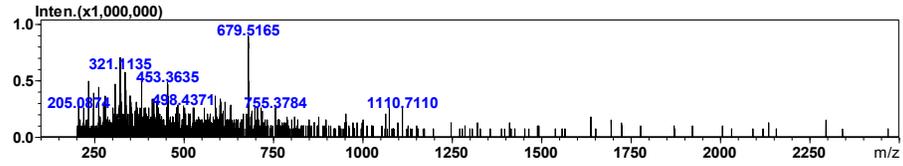


$m/z$  1987  
DP = 16  
RT = 29.113 min

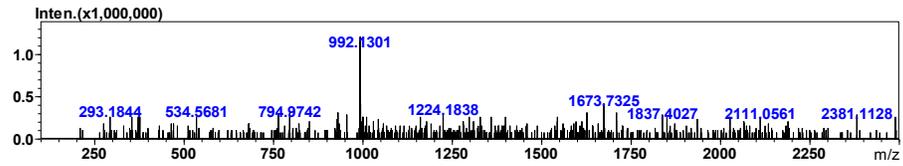
Extracted  
ion current

n/a

$[M + H]^+$



$[M - H]^-$



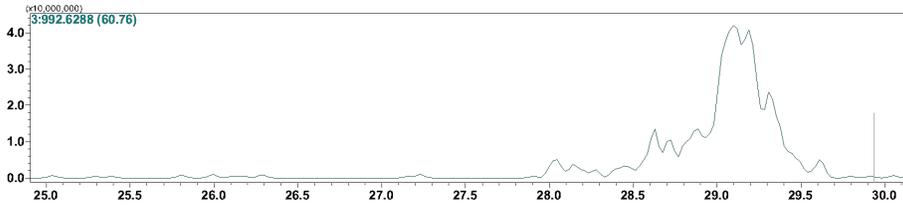
$MS^2 [M + H]^+$

n/a

$MS^2 [M - H]^-$

n/a

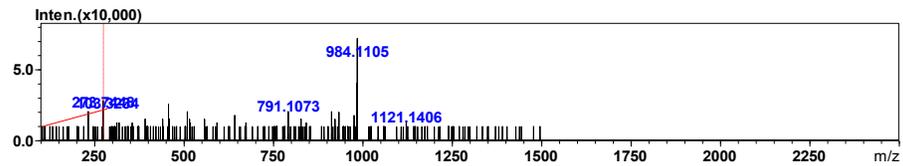
Extracted  
ion current



$MS^2 [M + 2H]^{+2}$

n/a

$MS^2 [M - 2H]^{-2}$

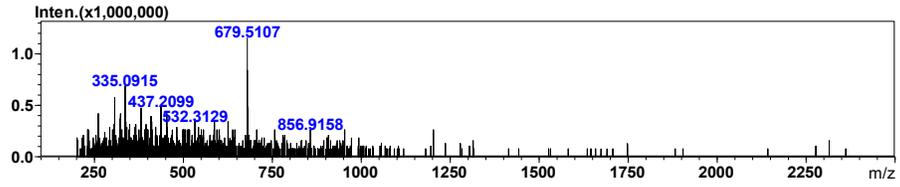


$m/z$  2111  
DP = 17  
RT = 29.905 min

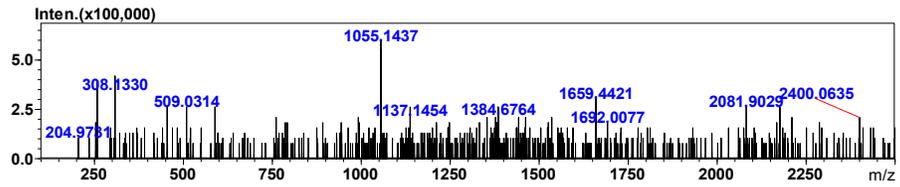
Extracted  
ion current

n/a

$[M + H]^+$



$[M - H]^-$



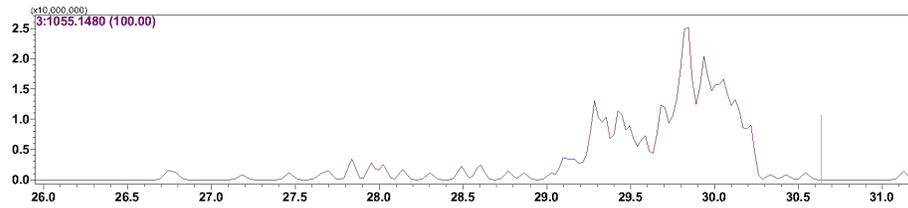
$MS^2 [M + H]^+$

n/a

$MS^2 [M - H]^-$

n/a

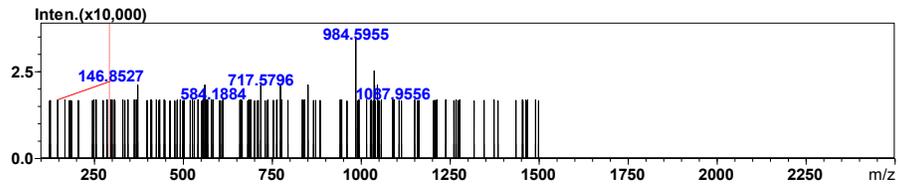
Extracted  
ion current



$MS^2 [M + 2H]^{+2}$

n/a

$MS^2 [M - 2H]^{-2}$

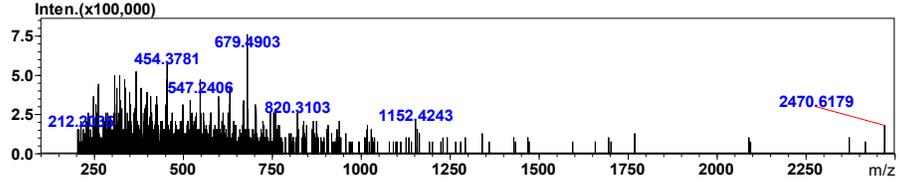


$m/z$  2335  
DP = 18  
RT = 30.578 min

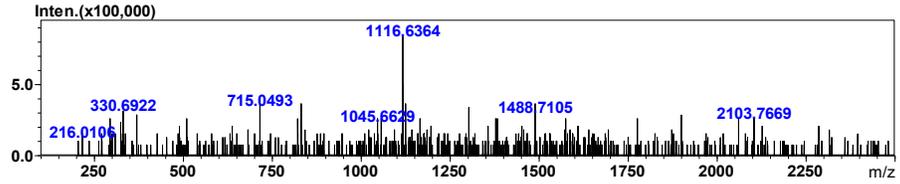
Extracted  
ion current

n/a

$[M + H]^+$



$[M - H]^-$



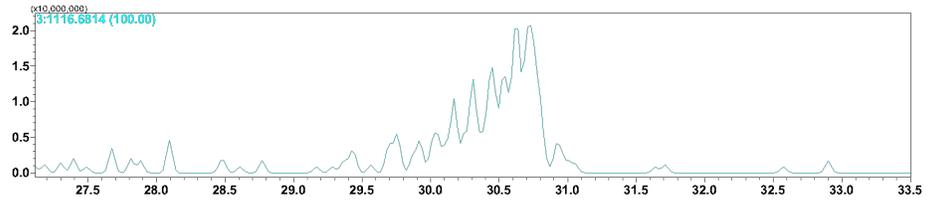
$MS^2 [M + H]^+$

n/a

$MS^2 [M - H]^-$

n/a

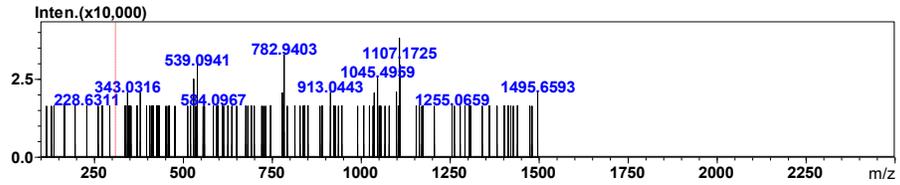
Extracted  
ion current



$MS^2 [M + 2H]^{+2}$

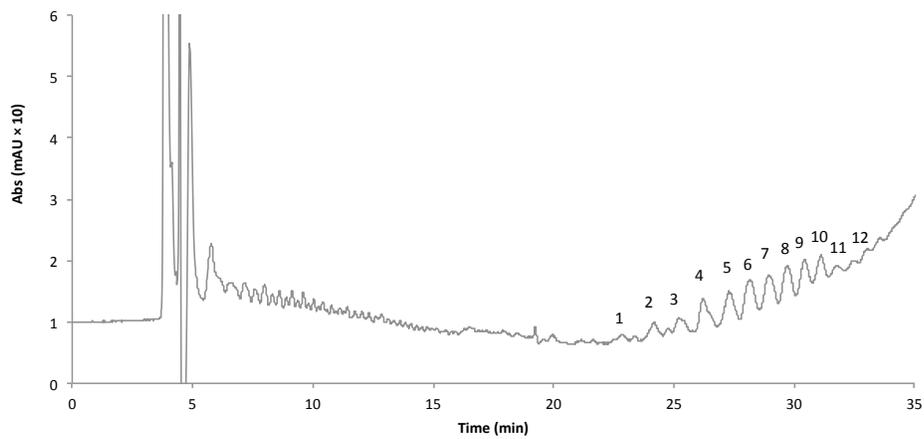
n/a

$MS^2 [M - 2H]^{-2}$



# APPENDIX C. MASS SPECTROMETRY DATA FOR FD-24

NPLC chromatogram (labeled as in Chapter 5)



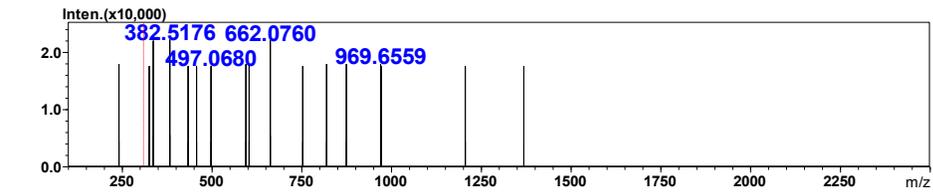
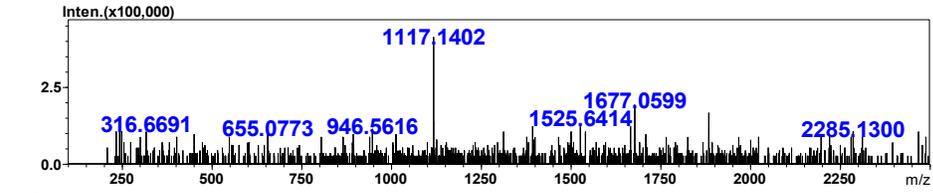
$m/z$  1117  
DP = 9  
RT = 21.790 min

$[M + H]^+$  n/a

$[M - H]^-$

$MS^2 [M + H]^+$  n/a

$MS^2 [M - H]^-$



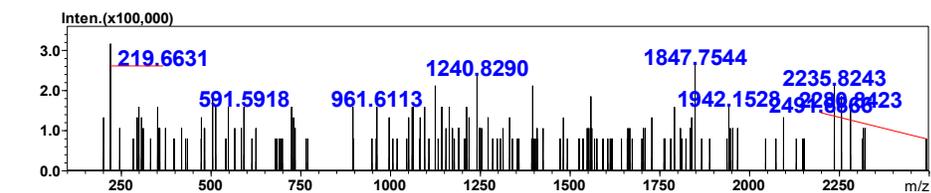
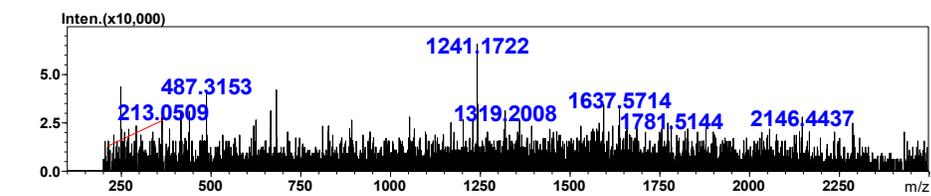
$m/z$  1241  
DP = 10  
RT = 22.583 min

$[M + H]^+$  n/a

$[M - H]^-$

$MS^2 [M + H]^+$  n/a

$MS^2 [M - H]^-$



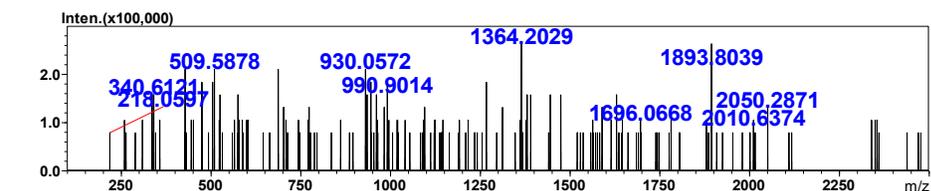
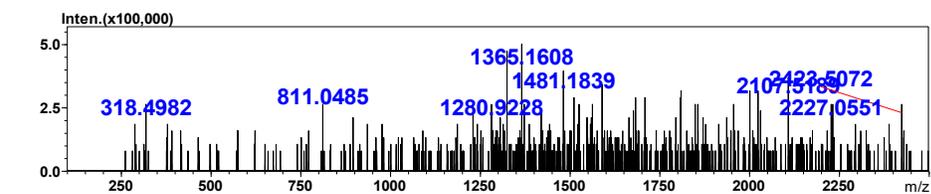
$m/z$  1365  
DP = 11  
RT = 24.578 min

$[M + H]^+$  n/a

$[M - H]^-$

$MS^2 [M + H]^+$

$MS^2 [M - H]^-$



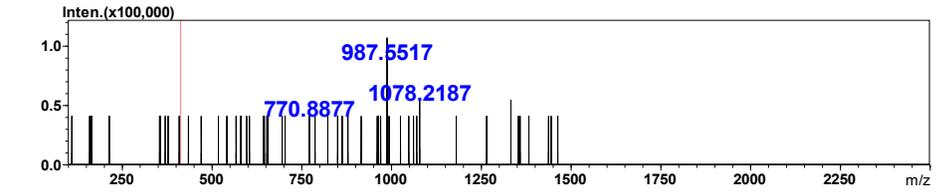
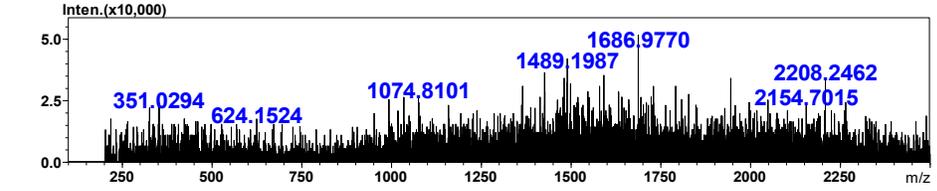
$m/z$  1489  
DP = 12  
RT = 25.681 min

$[M + H]^+$  n/a

$[M - H]^-$

$MS^2 [M + H]^+$  n/a

$MS^2 [M - H]^-$



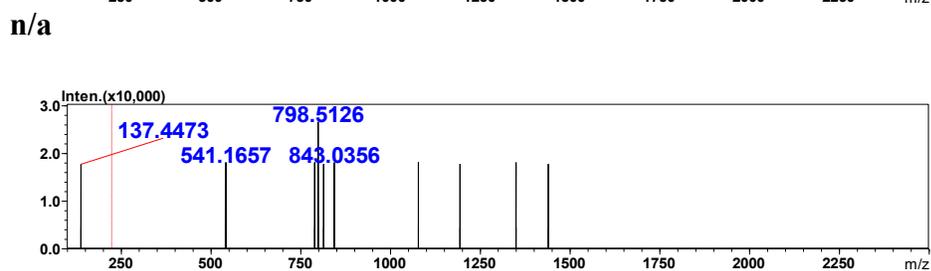
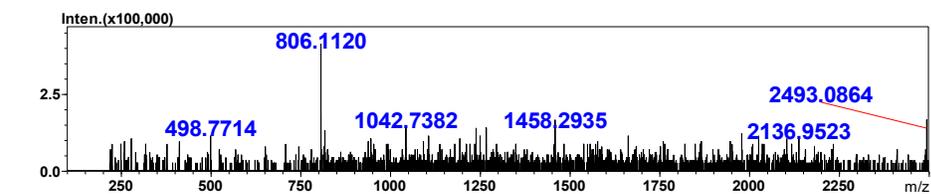
$m/z$  806  
DP = 13  
RT = 26.457 min

[M + H]<sup>+</sup> n/a

[M - H]<sup>-</sup>

MS<sup>2</sup> [M + H]<sup>+</sup>

MS<sup>2</sup> [M - H]<sup>-</sup>



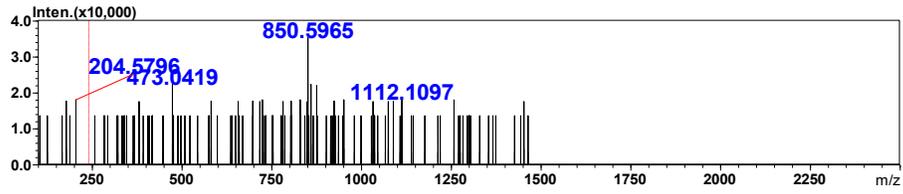
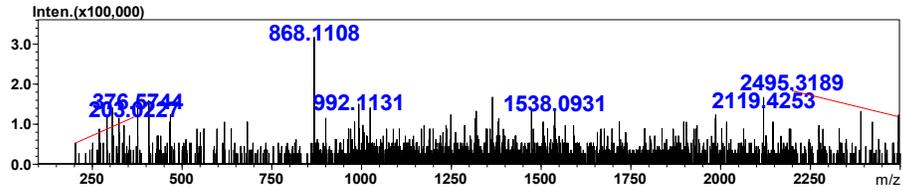
$m/z$  868  
DP = 14  
RT = 27.740 min

$[M + 2H]^{2+}$  n/a

$[M - 2H]^{2-}$

$MS^2 [M + 2H]^{2+}$  n/a

$MS^2 [M - 2H]^{2-}$



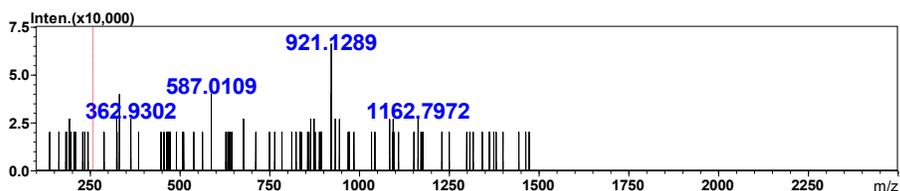
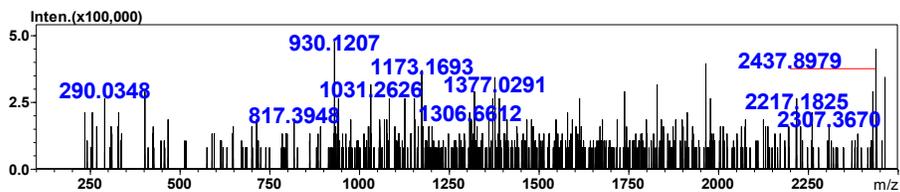
$m/z$  930  
DP = 15  
RT = 28.370 min

$[M + 2H]^{2+}$  n/a

$[M - 2H]^{2-}$

$MS^2 [M + 2H]^{2+}$  n/a

$MS^2 [M - 2H]^{2-}$



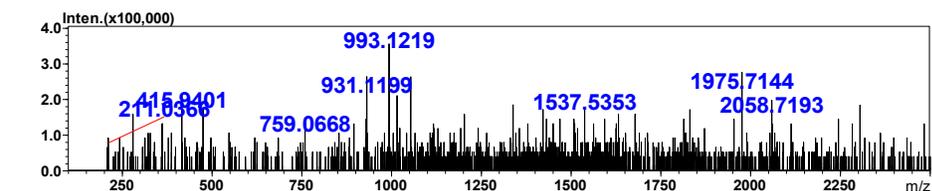
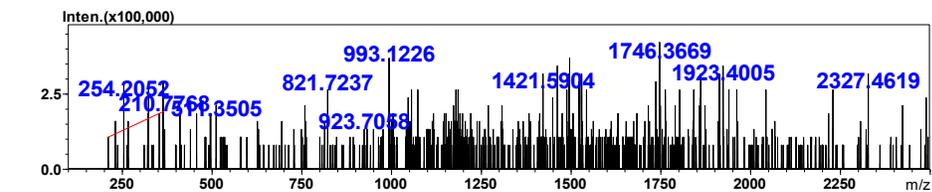
$m/z$  993  
DP = 16  
RT = 29.280 min

$[M + 2H]^{2+}$  n/a

$[M - 2H]^{2-}$

$MS^2 [M + 2H]^{2+}$  n/a

$MS^2 [M - 2H]^{2-}$



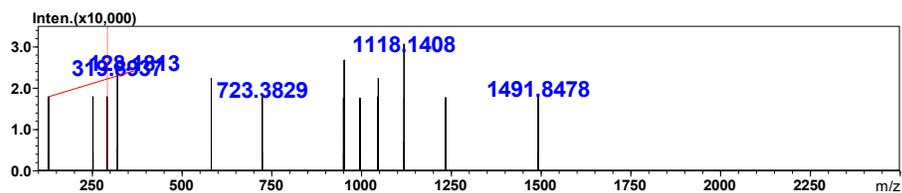
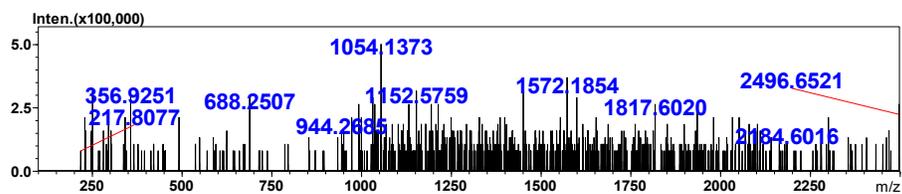
$m/z$  1054  
DP = 17  
RT = 29.677 min

$[M + 2H]^{2+}$  n/a

$[M - 2H]^{2-}$

$MS^2 [M + 2H]^{2+}$  n/a

$MS^2 [M - 2H]^{2-}$



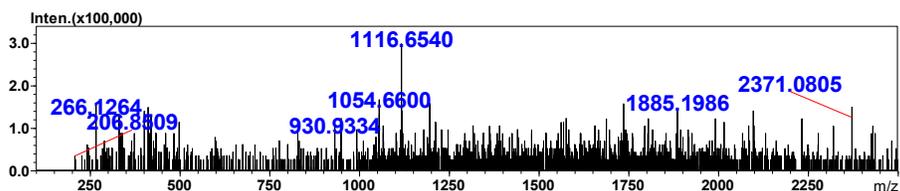
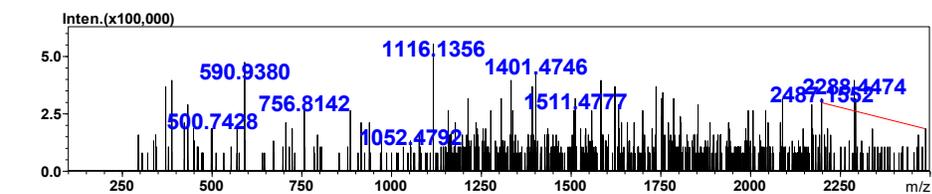
$m/z$  1116  
DP = 18  
RT = 30.642 min

$[M + 2H]^{2+}$  n/a

$[M - 2H]^{2-}$

$MS^2 [M + 2H]^{2+}$  n/a

$MS^2 [M - 2H]^{2-}$



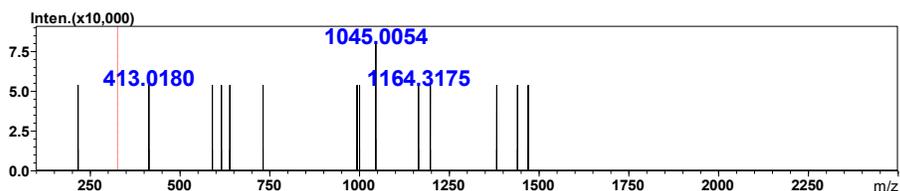
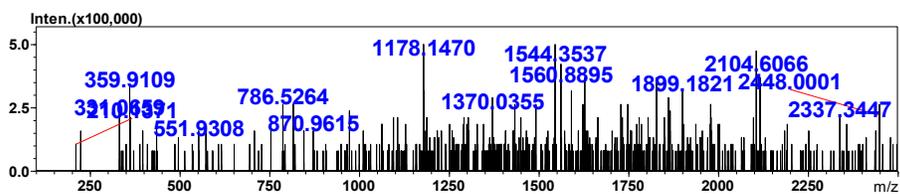
$m/z$  1178  
DP = 19  
RT = 31.170 min

$[M + 2H]^{2+}$  n/a

$[M - 2H]^{2-}$

$MS^2 [M + 2H]^{2+}$  n/a

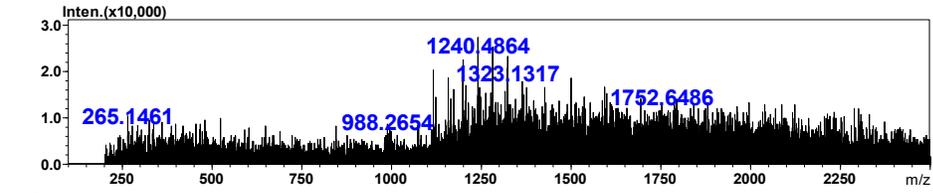
$MS^2 [M - 2H]^{2-}$



$m/z$  1240  
DP = 20  
RT = 31.532 min

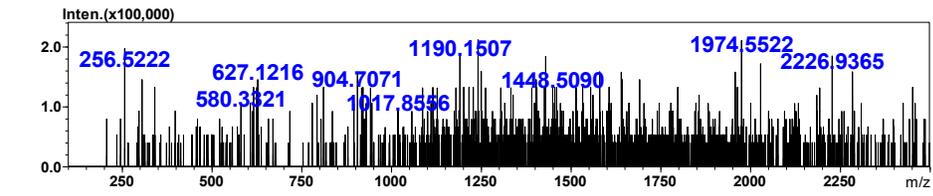
$[M + 2H]^{2+}$  n/a

$[M - 2H]^{2-}$



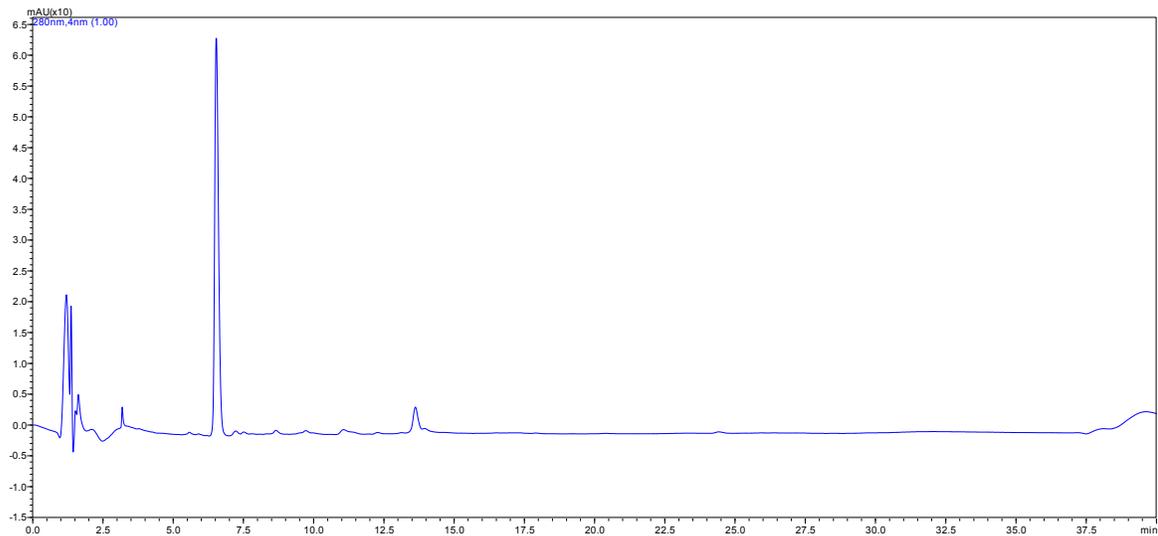
$MS^2 [M + 2H]^{2+}$  n/a

$MS^2 [M - 2H]^{2-}$

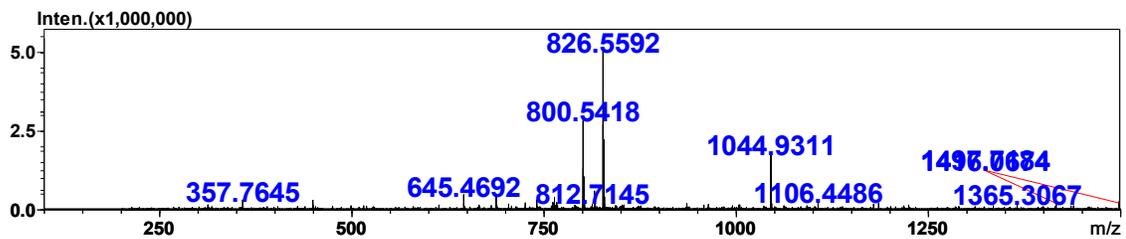


# APPENDIX D. MASS SPECTROMETRY AND NMR DATA FOR FD-18

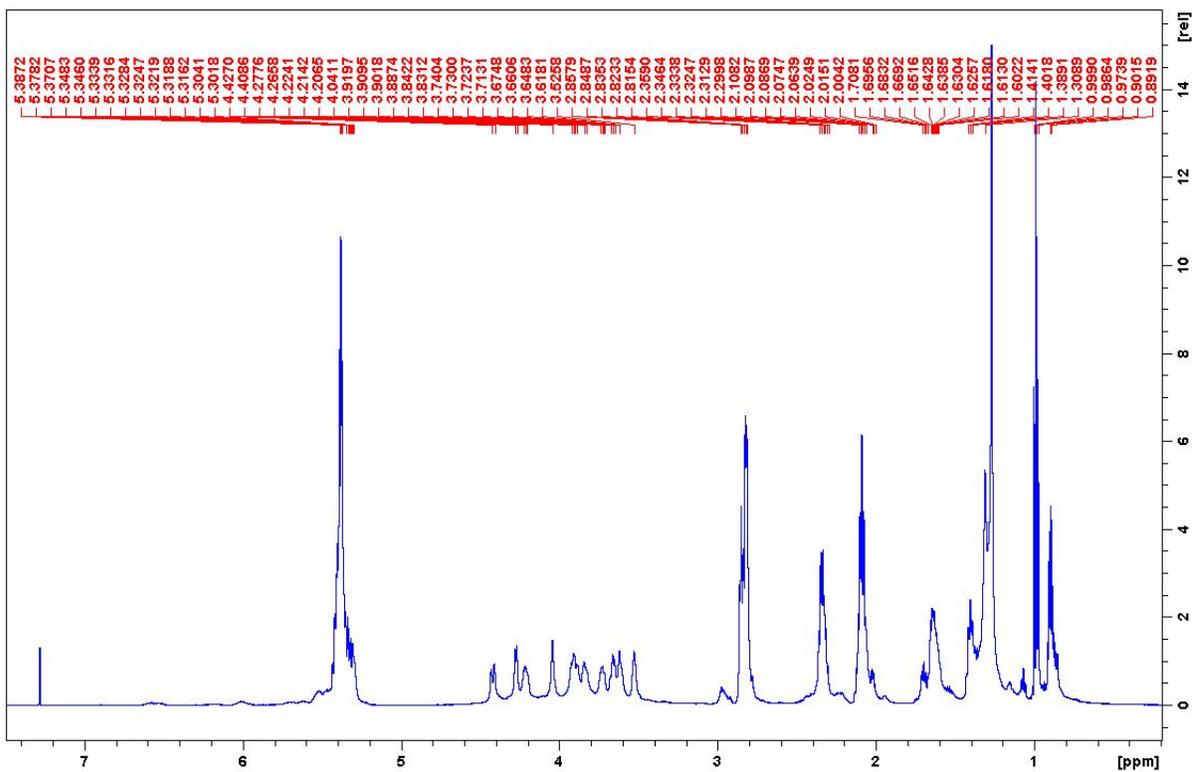
## LC-Chromatogram



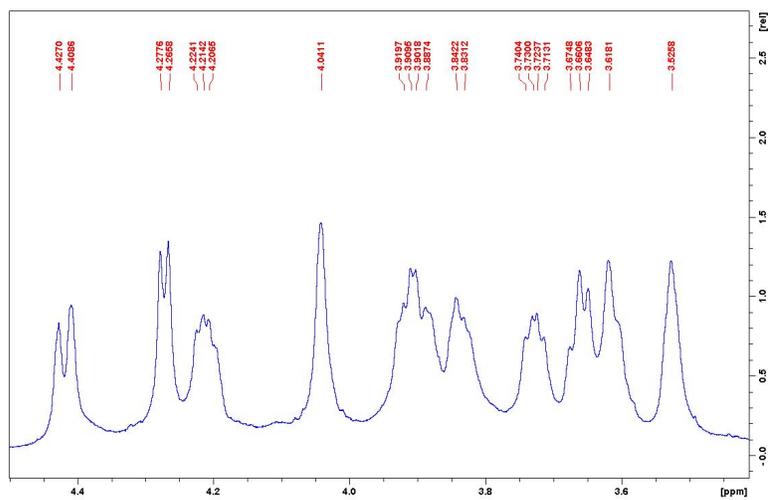
## Mass Spec Fragmentation for $[M + H]^+$ ion

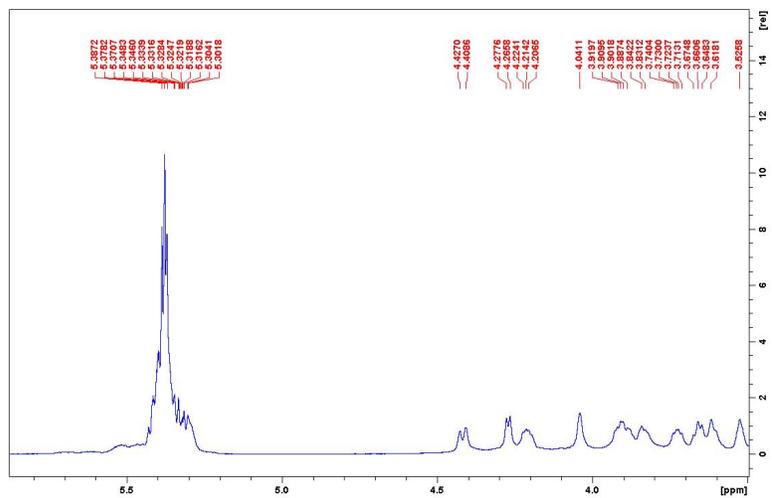
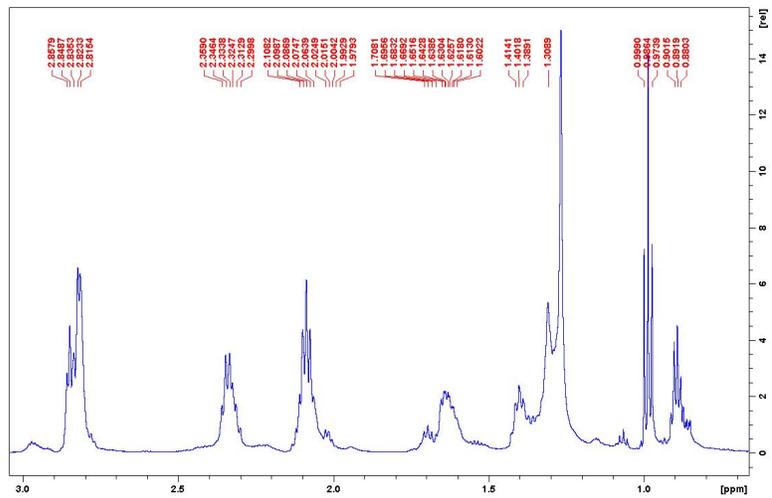


# <sup>1</sup>H NMR in d6-DMSO

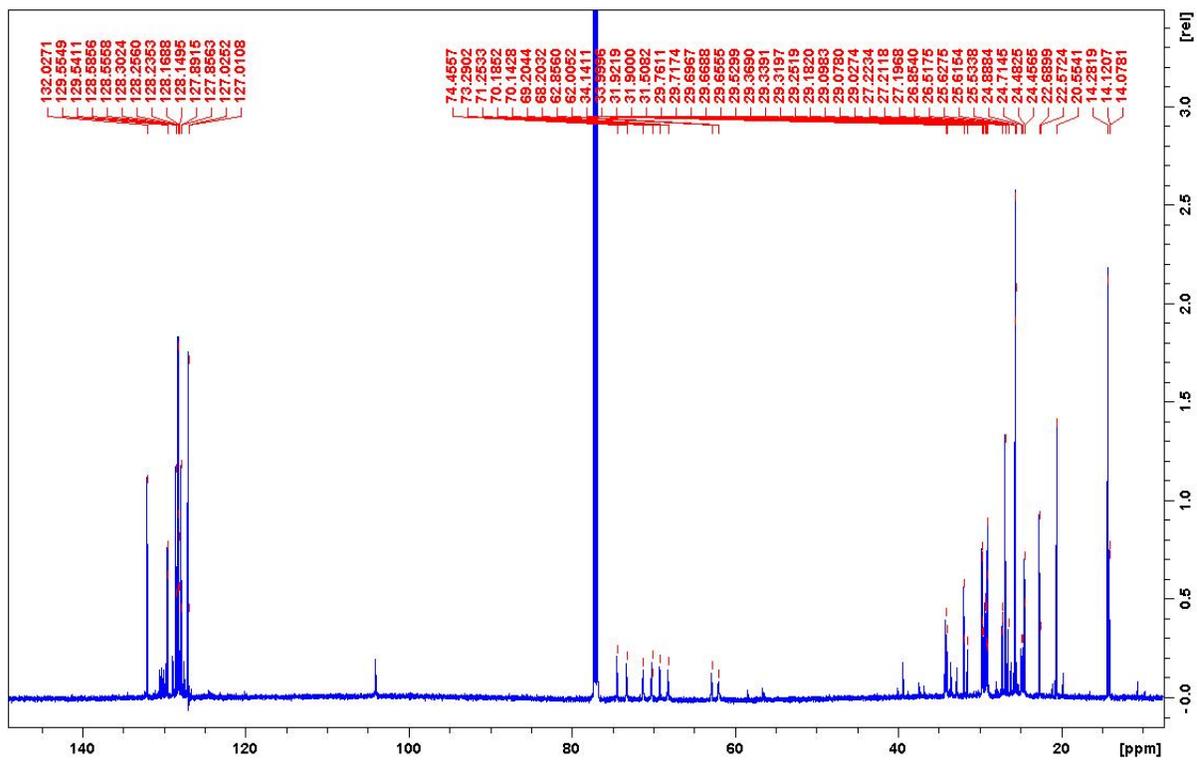


## Magnified regions

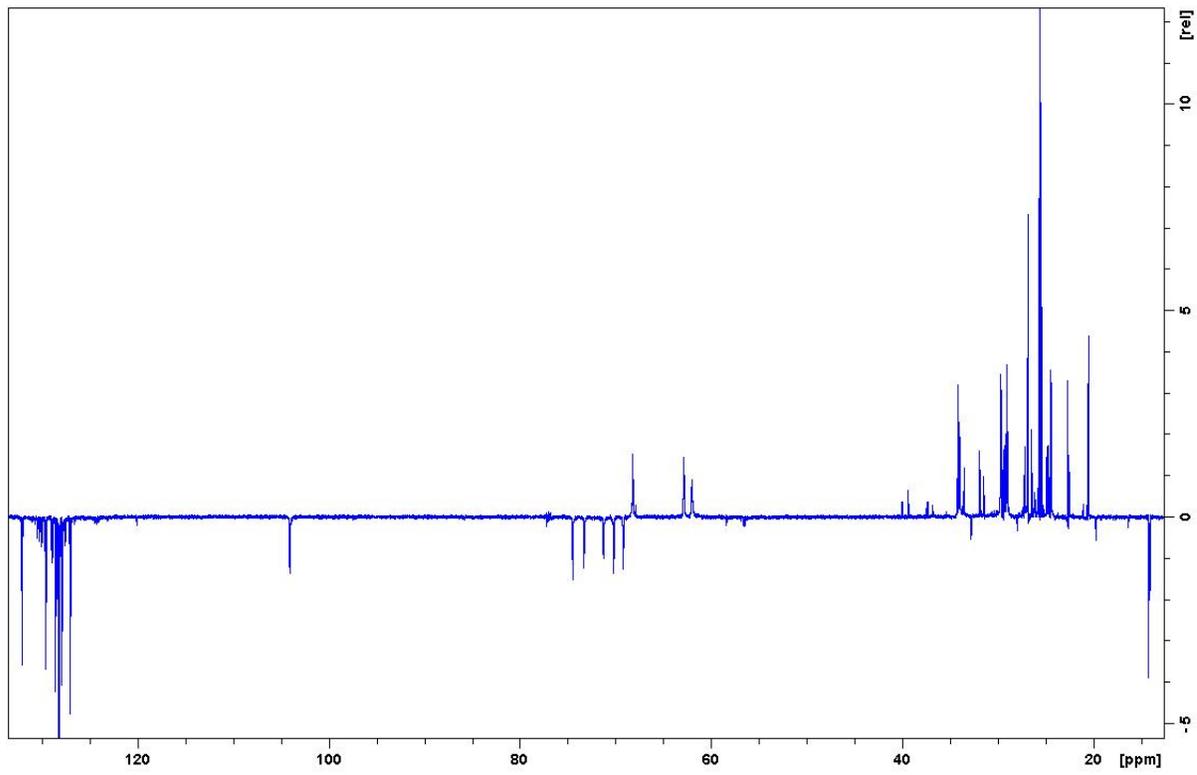




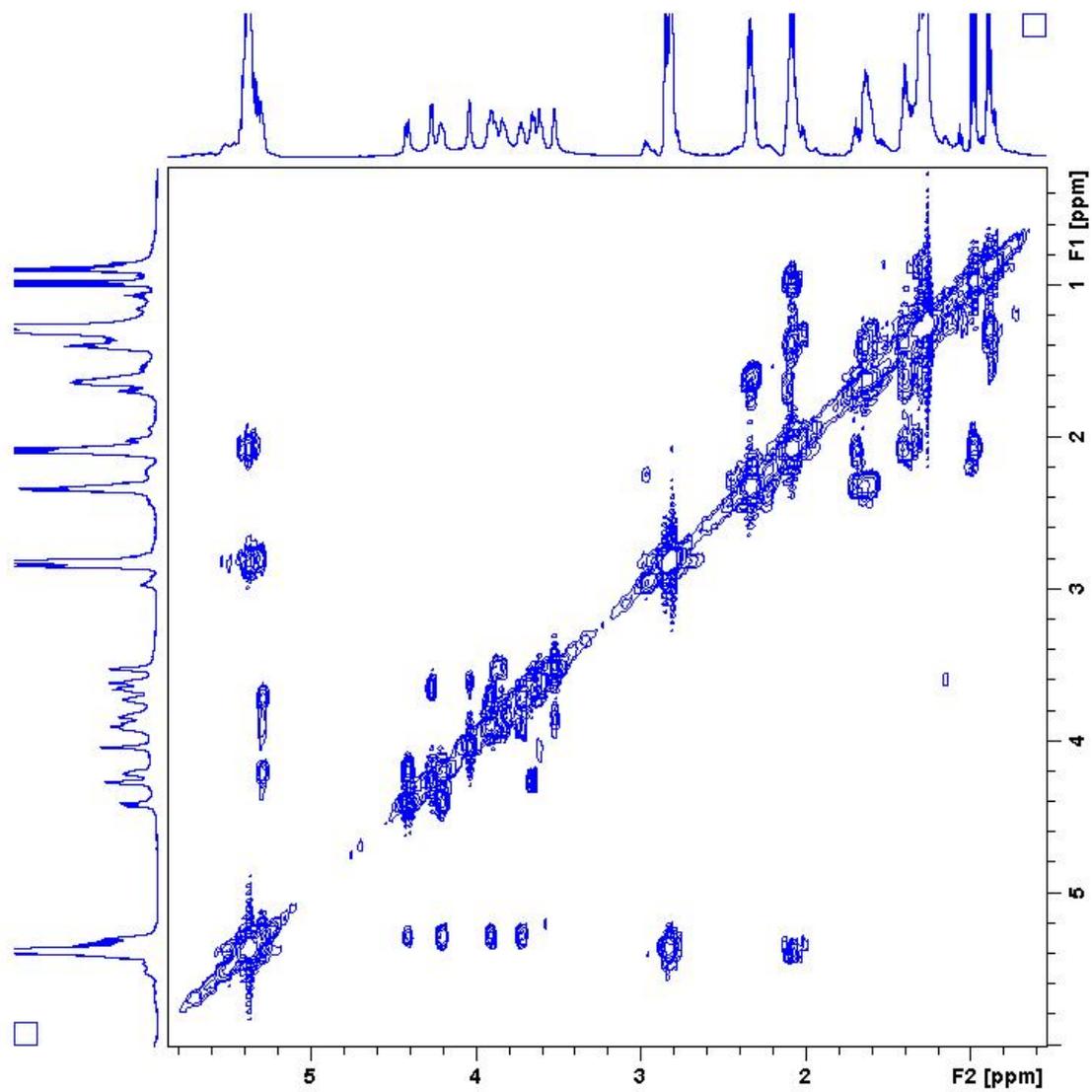
# <sup>13</sup>C NMR in d6-DMSO



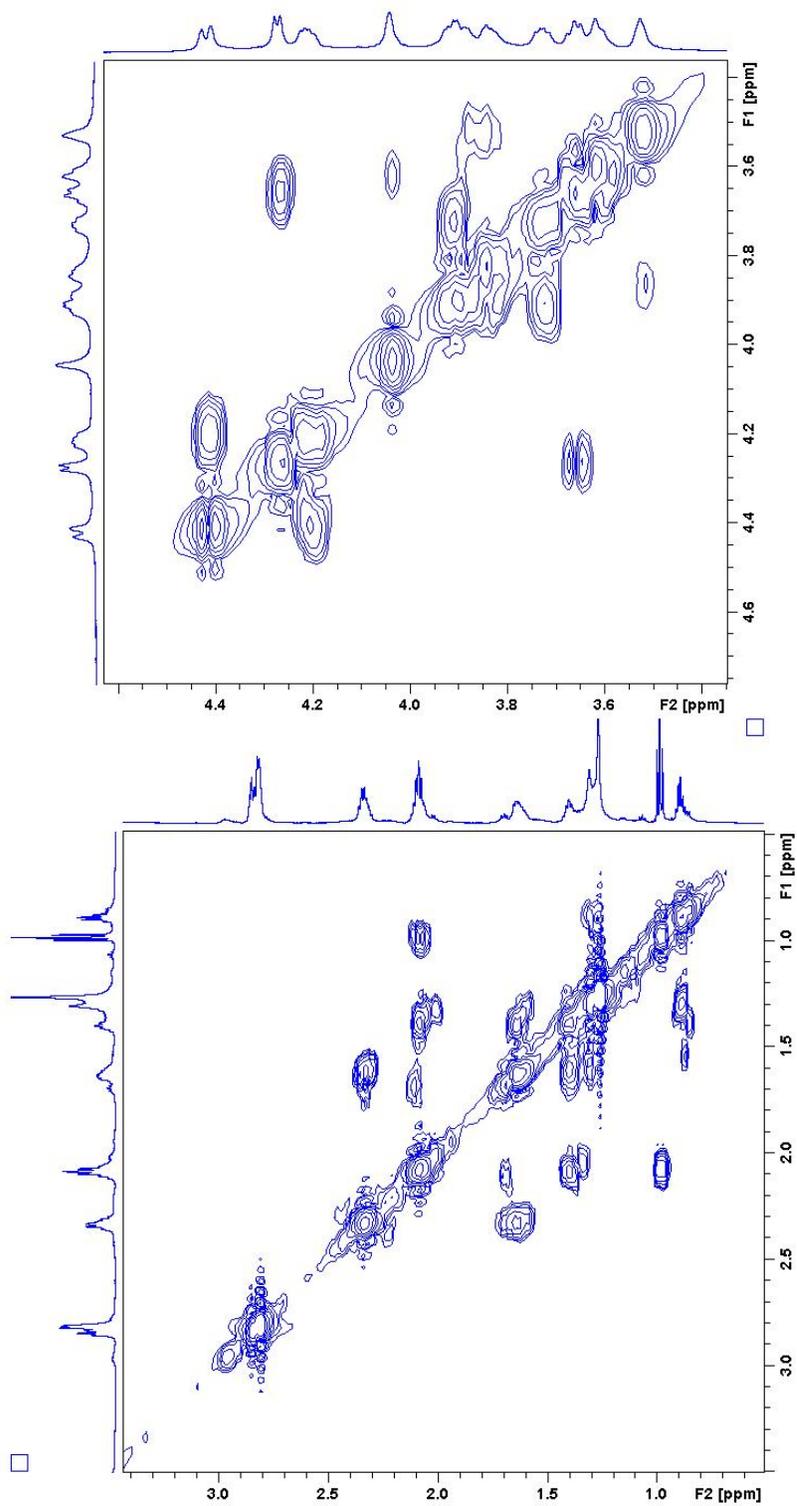
# DEPT-135



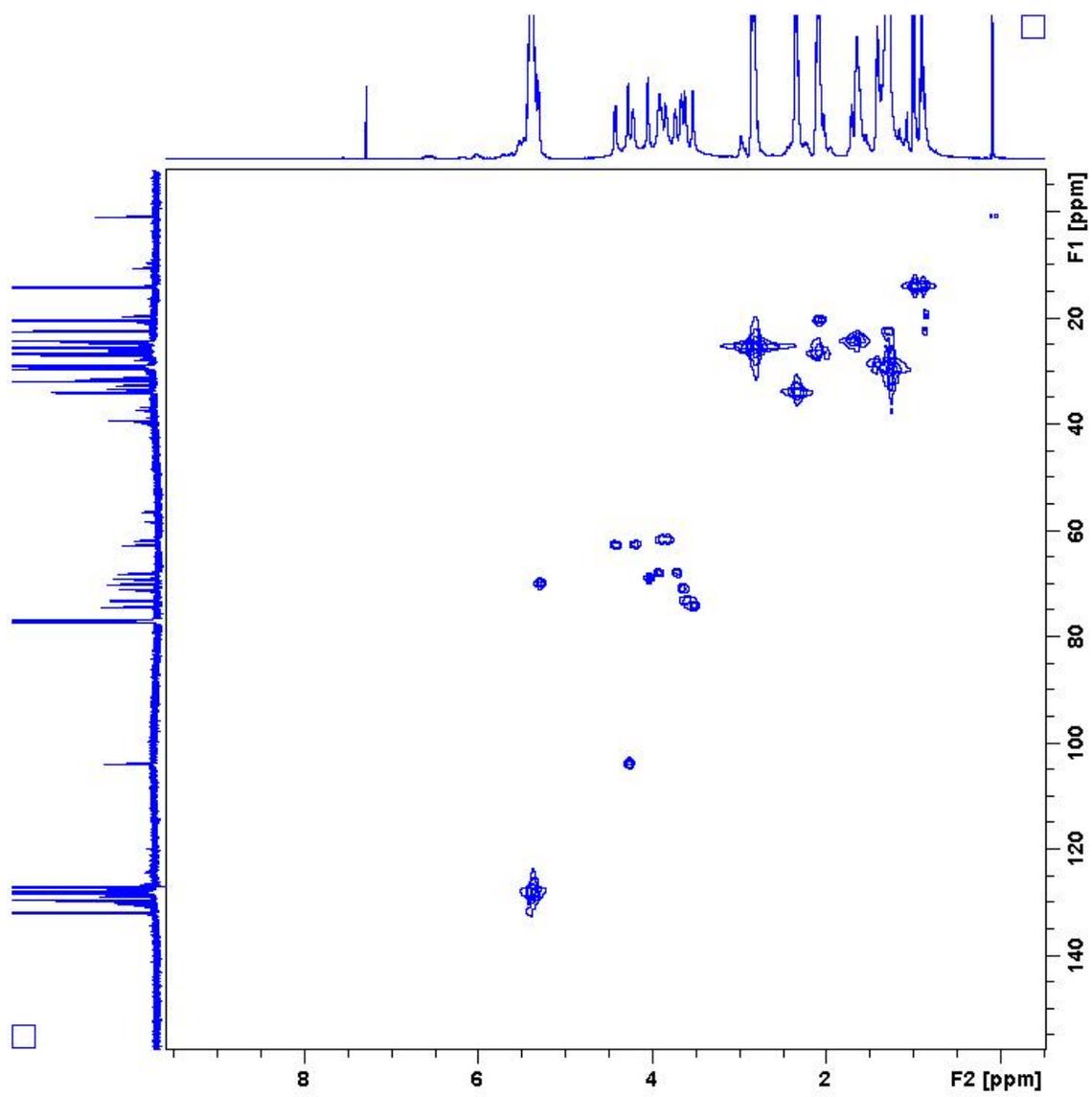
# $^1\text{H}$ - $^1\text{H}$ COSY



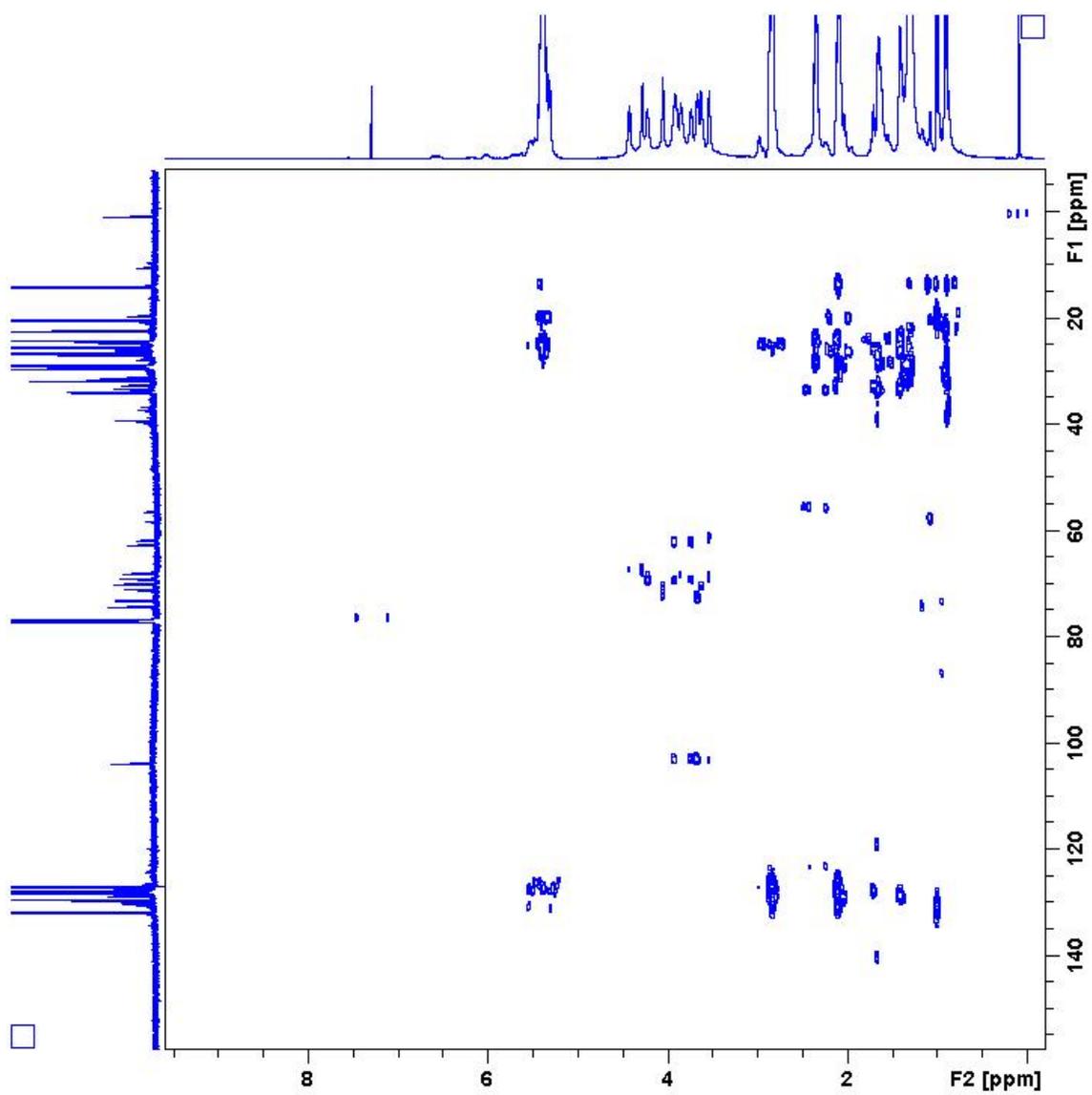
## Magnified regions



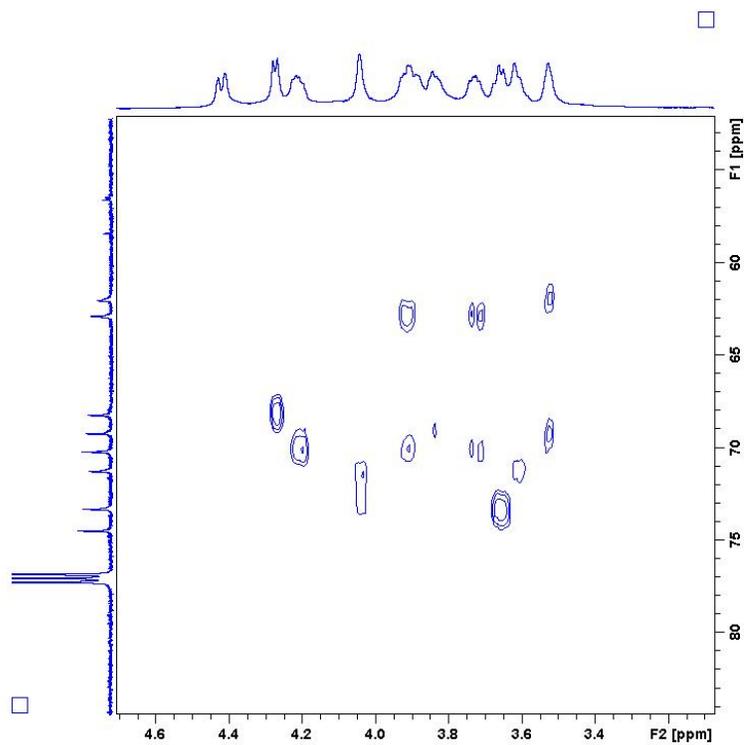
# HMQC

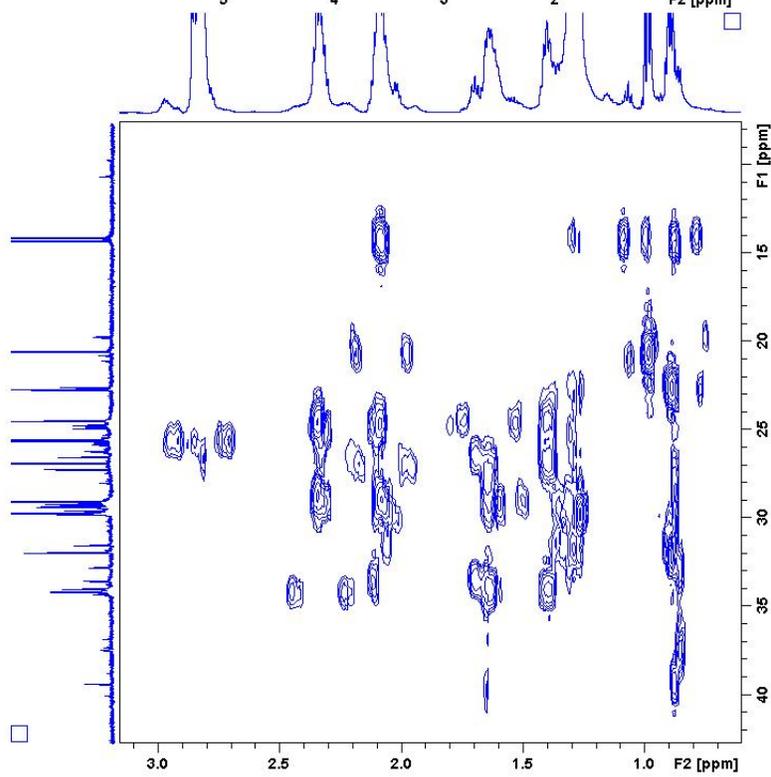
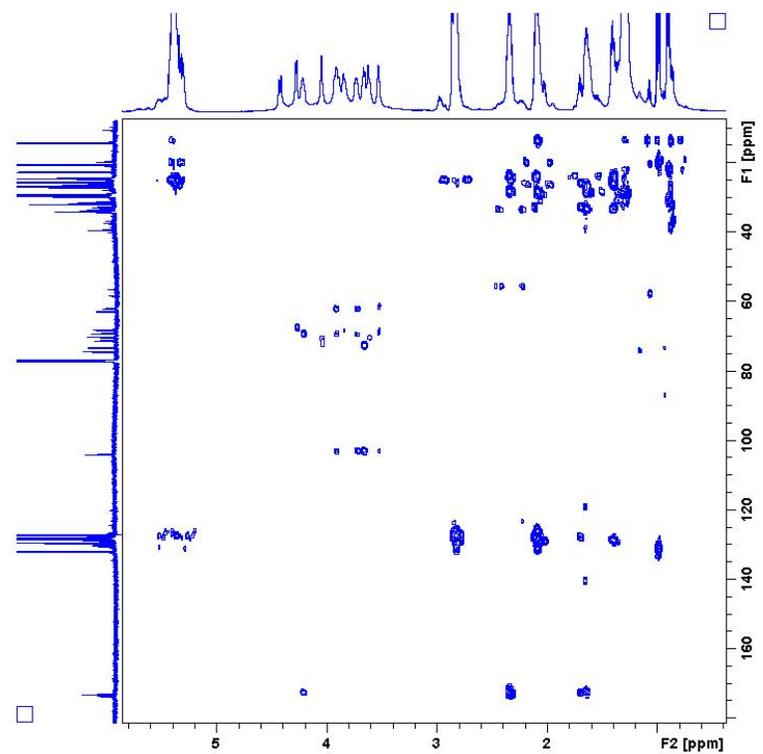


# HMBC



## Magnified regions





## APPENDIX E. DISSEMINATION OF RESEARCH

### North Carolina State University Graduate Research Symposium

March, 2014  
Poster

**Title:** Multi-target Functionality of Alaskan Seaweed in Combatting Hyperglycemia and Type 2 Diabetes

**Authors:** Kellogg, J., Lila, M.A.

**Abstract:**

Two dominant factors underlying the development of type 2 diabetes is prolonged hyperglycemia due to increased carbohydrate consumption and metabolism and chronic inflammation in adipose tissue and macrophages. Alaska Native populations, who suffer disproportionately high rates of diabetes, have shifted away from traditional dietary foods, like seaweeds, that are a rich source of phytochemicals with potential to counteract diabetes and its complications. In this work, Alaskan seaweeds *Fucus distichus* (FD), *Saccharina latissima* (SL), *Saccharina groenlandica* (SG), *Alaria marginata* (AM), *Pyropia fallax* (PF), and *Ulva lactuca* (UL) were evaluated for their potential to decrease metabolism of carbohydrates and ameliorate symptoms of hyperglycemic-linked inflammatory pathways. High levels of phenolics were discovered in ethyl acetate and butanol fractions of AM, FD, and SG (326.8 - 557.2  $\mu\text{g}$  phloroglucinol equivalents (PGE)/mg extract). The medium-polarity fractions of AM and FD significantly inhibited the activity of two primary carbolytic enzymes,  $\alpha$ -glycosidase and  $\alpha$ -amylase, with lower  $\text{IC}_{50}$  concentrations compared to the known inhibitor acarbose. Fractions of AM, FD, SG, and SL reduced nitric oxide levels in LPS-induced RAW 264.7 macrophages to basal levels, and mRNA expression assays demonstrated that organic fractions of AM, SL, and UL reduced expression levels for the

inflammatory cytokines IL-10, MCP-1, iCAM, and TNF- $\alpha$  by 92%, 89%, 82%, and 85%, respectively. These results support the potential of Alaskan algae to provide two activities in reducing hyperglycemia and type 2 diabetes, by lowering inflammation and regulating carbohydrate digestion.

# Multi-Target Functionality of Alaskan Seaweed in Combatting Hyperglycemia and Type 2 Diabetes

NC STATE UNIVERSITY

Joshua J. Kellogg & Mary Ann Lila

Plants for Human Health Institute, Kannapolis, NC

Department of Food, Bioprocessing, and Nutrition Sciences, Raleigh, NC



## Abstract

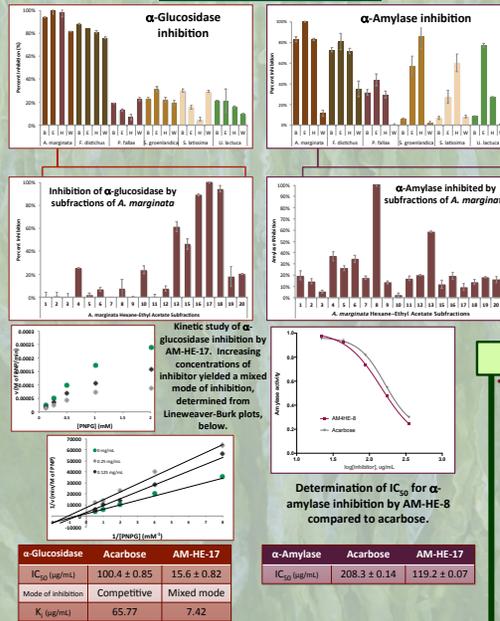
Two dominant factors underlying the development of type 2 diabetes is prolonged hyperglycemia due to increased carbohydrate consumption and metabolism and chronic inflammation in adipose tissue and macrophages. Alaskan Native populations, who suffer disproportionately high rates of diabetes, have shifted away from traditional dietary foods, like seaweeds, that are a rich source of phytochemicals with potential to counteract diabetes and its complications. In this work, Alaskan seaweeds *Fucus distichus* (FD), *Saccharina latissima* (SL), *Saccharina groenlandica* (SG), *Alaria marginata* (AM), *Pyropia fallax* (PF), and *Ulva lactuca* (UL) were evaluated for their potential to decrease metabolism of carbohydrates and ameliorate symptoms of hyperglycemic-linked inflammatory pathways. High levels of phenolics were discovered in ethyl acetate and butanol fractions of AM, FD, and SG (226.9–557.2 µg phenolugluconol equivalents (PGE)/mg extract). The medium-polarity fractions of AM and FD significantly inhibited the activity of two primary carbolytic enzymes,  $\alpha$ -glucosidase and  $\alpha$ -amylase, with lower  $IC_{50}$  concentrations compared to the known inhibitor acarbose. Fractions of AM, FD, SG, and SL reduced nitric oxide levels in LPS-induced RAW 264.7 macrophages to basal levels, and mRNA expression assays demonstrated that organic fractions of AM, SL, and UL reduced expression levels for the inflammatory cytokines IL-10, MCP-1, iNOS, and TNF- $\alpha$  by 92%, 89%, 82%, and 85%, respectively. These results support the potential of Alaskan algae to provide two activities in reducing hyperglycemia and type 2 diabetes, by lowering inflammation and regulating carbohydrate digestion.

## Introduction

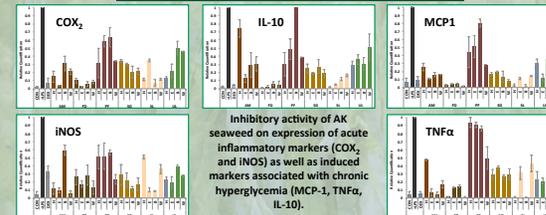
- Diabetes and Native Americans and Alaskan Natives
  - 16.1% incidence rate of type 2 diabetes
  - Over twice the national average
  - Rates have increased 68% from 1994-2004 alone
- Underlying factors of diabetes incidence
  - Carbohydrate metabolism
    - $\alpha$ -glucosidase and  $\alpha$ -amylase cleave glucose from starch
    - glucose oxidation
    - insulin secretion
  - Chronic inflammation
    - Metabolism-induced adipocyte hypertrophy
    - Accumulation of macrophages
    - Up-regulates production of pro-inflammatory cytokines
      - TNF- $\alpha$
      - MCP-1
      - COX2
      - IL-10
      - iNOS
    - glucose transport and insulin signaling
- Alaskan seaweed
  - Play a large role in traditional NA/AN cultures
  - Source of macro- and micronutrients
  - Key part of traditional ecological knowledge
  - Little research on the possible mechanisms for protection against diabetes.

## Results

### ENZYME INHIBITION



### ANTI-INFLAMMATORY ACTIVITY



### TOTAL PHENOLIC CONTENT

Species	Extraction Solvent	Total Phenolic Content (µg PGE/mg fraction)
<i>A. marginata</i>	A	156.5 ± 0.90
	B	395.5 ± 0.90
	W	155.1 ± 0.44
<i>F. distichus</i>	F	272.9 ± 0.84
	E	102.2 ± 0.40
	W	140.5 ± 0.20
<i>S. latissima</i>	S	404.1 ± 0.2
	B	110.0 ± 0.36
	W	317.0 ± 0.24
<i>P. fallax</i>	P	188.5 ± 0.22
	B	116.4 ± 0.70
	W	320.8 ± 0.10

Total phenolic content of Alaskan seaweed ranged from <75 to 3500 µg PGE/mg fraction, brown seaweeds possessed highest concentrations.

## Discussion

- Brown Alaskan seaweed are active against multiple targets associated with diabetes
- Brown seaweeds AM, FD, SL, and SG yielded highest phenolic content
- AM and FD possessed greater activity against carbolytic enzyme activity compared to acarbose
  - AM subfraction 17 inhibited  $\alpha$ -glucosidase ( $IC_{50}$ : 15.6 µg/mL)
  - AM subfraction 8 inhibited  $\alpha$ -amylase ( $IC_{50}$ : 119.2 µg/mL)
  - Reduced carbohydrate metabolism is associated with lowered serum hyperglycemic spikes.
- FD, AM, and SL reduced expression of inflammatory markers in macrophages
  - Near-complete suppression of MCP-1, TNF $\alpha$ , and IL-10
  - Correlated with development of insulin resistance
- Traditionally-consumed seaweed have potential to lower chronic inflammation and hyperglycemia
- Yield possible lower risk factors and lower incidence of diabetes development

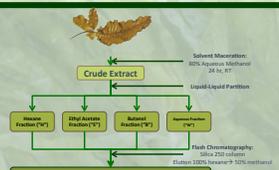
## Methods

### Collection & Extraction

Algal species were collected from the coastal area surrounding Sitka, Alaska in June 2012. Fresh seaweeds were washed to remove particulates, salts, and attached epiphytes, frozen at -80 °C and lyophilized until extract preparation.

Phylum	Species	Abbreviation	Common name
Ochrophyta	<i>Alaria marginata</i>	AM	Winged kelp
	<i>Fucus distichus</i>	FD	Bladder wrack
Rhodophyta	<i>Saccharina groenlandica</i>	SG	Kelp
	<i>Saccharina latissima</i>	SL	Sugar wrack
Chlorophyta	<i>Ulva lactuca</i>	UL	Sea lettuce
	<i>Pyropia fallax</i>	PF	Laver

Freeze-dried samples were powdered using a grinding mill and suspended in 80% aqueous methanol and shaken for 24 h. The aqueous residue was sequentially partitioned with hexane, ethyl acetate, and butanol, yielding four crude fractions. Solvents were removed via rotary evaporation, and fractions were lyophilized and held at -80 °C. Active fractions were loaded onto a 230-40 mesh silica column and separated using gradient elution of hexane:ethyl acetate:methanol.



### Total Phenolic Content

The total phenolic content (TPC) of seaweed fractions was quantified using a microplate-adapted Folin-Ciocalteu protocol. Each well was charged with 75 µL water, 25 µL sample, and 25 µL Folin-Ciocalteu reagent. The reaction mixture was held for 5 min, 100 µL 7.5% Na<sub>2</sub>CO<sub>3</sub> were added to each well and incubated for 90 min, after which the absorbance was read at 765 nm. Phenolugluconol equivalents were used as the standard curve (PGE, R<sup>2</sup> = 0.999).

### Enzyme Assays

#### $\alpha$ -Glucosidase Assay

$\alpha$ -Glucosidase from *Saccharomyces cerevisiae* was dissolved in 100 mM phosphate buffer (pH 7.0) to a final concentration of 0.005 mg/mL. 20 µL of sample was added to each well, along with 100 µL of 1 mM p-nitrophenyl- $\alpha$ -D-glucopyranoside, and incubated at RT for 5 min, and then 100 µL of enzyme solution was added to each well and incubated at 25 °C for 6 min before reading absorbance at 405 nm.

#### $\alpha$ -Amylase Assay

$\alpha$ -Amylase was dissolved to a final concentration of 10 µM (5.5 µg/mL) in 20 mM Tris-HCl (pH 7.5). To a 96-well plate, 35 µL of extract/fraction was added, and the reaction mixture allowed to react for 10 min at RT. For visualization, 150 µL of 0.1% w/v I<sub>2</sub>, 1.0% w/v KI in ethanol was added to each well, and the absorbance read at 595 nm.

### Cell Inflammation

#### Cell Culture

RAW 264.7 macrophage cells were maintained at a subconfluent density at 37 °C in a 5% CO<sub>2</sub> atmosphere during culturing and treatment. All cells were cultured in DMEM with 100 units/mL penicillin, 10 mM sodium pyruvate, and 10% FBS. Cells were pre-incubated with fractions (100 µg/mL) for 1 h, then induced with 1 µL

lipopolysaccharide (LPS, from *Escherichia coli* 026:B6) and incubated for 4 h, after which cells were harvested for gene expression analysis.

#### Quantitative PCR Analysis

mRNA was extracted using Trizol reagent and quantified spectrophotometrically. The cDNA was synthesized using 2 µg of isolated mRNA and a high-capacity reverse transcription kit. Complementary DNA sequences were quantified using a real-time PCR design with SYBR Green fluorescence. The forward and reverse PCR primers and the SYBR Green dye, with 5 µL of cDNA. The mixture was cycled on an ABI PCR system with the following cycling parameters: 95°C for 15 min for enzyme activation, followed by denaturing at 95 °C for 15 min, and annealing and elongation at 60 °C for 1 min, with a total of 40 cycles. Relative expression of gene expression were quantified using the  $\Delta\Delta C_T$  method.

#### Statistics

All assays were performed at least in triplicate. Results are presented as mean of triplicate runs  $\pm$  SEM. Statistical analysis was conducted using repeated measures ANOVA followed by Tukey's test, with statistical significance determined at P<0.05. Half maximal inhibitory concentration ( $IC_{50}$ ) data was expressed as the geometric mean with 95% confidence intervals. Michaelis-Menten kinetics of enzyme inhibition was determined using Prism software.

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## Experimental Biology Conference

April, 2014

Poster

**Title:** Anti-inflammatory bioactives in Alaskan seaweed: implications in metabolic syndrome

**Authors:** Kellogg, J., Esposito, D., Komarnytsky, S., Lila, M.A.

**Abstract:**

A dominant factor underlying the development of metabolic syndrome is abdominal obesity, which is associated with chronic inflammation in adipose tissue. Alaska Native populations, who suffer disproportionately high rates of obesity, have shifted away from traditional dietary foods, like seaweeds, that are a rich source of polyphenols with potential to counteract metabolic syndrome. In this work, Alaskan seaweeds *Fucus distichus* (FD), *Saccharina latissima* (SL), *Saccharina groenlandica* (SG), *Alaria marginata* (AM), *Pyropia fallax* (PF), and *Ulva lactuca* (UL) were evaluated for their potential to ameliorate symptoms of obesity-linked inflammatory pathways. High levels of phenolics were discovered in ethyl acetate and butanol fractions of AM, FD, and SG (326.8 - 557.2  $\mu\text{g}$  phloroglucinol equivalents (PGE)/mg extract). Fractions of AM, FD, SG, and SL reduced nitric oxide levels in LPS-induced RAW 264.7 macrophages to basal levels, and assays with 3T3-L1 adipocytes demonstrated that organic fractions of AM, SL, and UL decreased triglyceride accumulation 13.3 - 24.7%. Subsequent analysis of AM fractions revealed reductions in mRNA expression levels for the inflammatory adipokines IL-10, MCP-1, iCAM, and TNF- $\alpha$  by 92.6%, 50.7%, 67.3%, and 65.4%, respectively. These results support the potential of Alaskan algae to reduce inflammation and obesity in adipose tissue.



## Phytochemical Society of North America Conference

August, 2014

Oral Presentation

**Title:** Multi-target Functionality of Alaskan Seaweed in Combatting Hyperglycemia and Type 2 Diabetes

**Authors:** Kellogg, J., Lila, M.A.

**Abstract:**

Two factors underlying the development of type 2 diabetes are prolonged hyperglycemia due to increased carbohydrate consumption, and chronic inflammation in adipose tissue and macrophages. Alaska Native populations, who suffer disproportionately high rates of diabetes, have shifted away from traditional dietary foods, including seaweeds, which are rich sources of phytochemicals with potential to counteract diabetes. In this work, Alaskan seaweeds *Fucus distichus* (FD), *Saccharina latissima* (SL), *Saccharina groenlandica* (SG), *Alaria marginata* (AM), *Pyropia fallax* (PF), and *Ulva lactuca* (UL) were evaluated for their potential to decrease carbohydrate metabolism and ameliorate symptoms of hyperglycemic-linked inflammatory pathways. High levels of polyphenolics were discovered in medium-polarity fractions of AM, FD, and SG (326.8 - 557.2  $\mu\text{g}$  phloroglucinol equivalents (PGE)/mg extract). AM and FD demonstrated selective inhibition of  $\alpha$ -glycosidase and  $\alpha$ -amylase, with significantly lower  $\text{IC}_{50}$  concentrations compared to acarbose. Fractions of AM, FD, SG, and SL reduced nitric oxide levels in LPS-induced RAW 264.7 macrophages, and mRNA expression assays demonstrated that AM, SL, and UL reduced levels for inflammatory cytokines IL-10, MCP-1, COX2, and TNF- $\alpha$  by 92%, 89%, 82%, and 85%, respectively. These results suggest that Alaskan algae may alleviate

hyperglycemia and other type 2 diabetes biomarkers by depressing inflammation and regulating carbohydrate digestion.

## Alaskan Plants as Food and Medicine Symposium

August, 2014

Keynote Talk

**Title:** Anti-Inflammatory Potential of Edible Brown Alaskan Seaweed in Combatting Metabolic Syndrome

**Authors:** Kellogg, J., Lila, M.A.

**Abstract:**

Inflammation of adipose tissue and increased metabolism of carbohydrates are two dominant factors underlying the development of insulin resistance and metabolic syndrome, a condition that disproportionately impacts Alaska Native populations. However, traditional dietary foods, like seaweeds, are a rich source of polyphenols with potential to counteract metabolic syndrome. In this work, Alaskan seaweeds *Fucus distichus* (FD), *Saccharina latissima* (SL), *Saccharina groenlandica* (SG), *Alaria marginata* (AM), *Pyropia fallax* (PF), and *Ulva lactuca* (UL) were evaluated for their potential to ameliorate symptoms of obesity-linked inflammatory pathways and inhibition of carbohydrate metabolism. The organic extracts of FD, SL, AM, and SG all contained high levels of polyphenolics, and these four species possessed heightened bioactivity against a number of diabetes- and obesity-linked targets. Fractions of AM, FD, SG, and SL reduced biochemical oxidative markers as well as nitric oxide levels in stressed macrophages to background levels. Seaweed extracts reduced the expression of inflammatory markers in macrophages by >90%. Analysis of FD fractions on adipocyte cell expression revealed reductions in mRNA levels for both inflammatory and metabolic adipokines. Furthermore, AM and FD demonstrated selective inhibition of the carbohydrate-hydrolyzing enzymes  $\alpha$ -glycosidase and  $\alpha$ -amylase, which represent two main digestive enzymes present in the gastrointestinal system. These two species possessed

significantly lower inhibitory concentrations compared to the known pharmaceutical acarbose. Taken together, these results support the potential of traditionally consumed Alaskan algae to ameliorate the potential development of insulin resistance and metabolic syndrome by acting on multiple targets, reducing inflammation in macrophages and adipose tissue and lowering the activity of metabolic enzymes.