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Chemical and in Vitro Assessment of Alaskan Coastal Vegetation Antioxidant Capacity

Joshua Kellogg and Mary Ann Lila*

Plants for Human Health Institute, Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, 600 Laureate Way, Kannapolis, North Carolina 28081, United States

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 the ethyl acetate fraction of *F. distichus*, and highest DDPH scavenging activity was exhibited by *F. distichus* and *S. groenlandica* fractions (IC_{50} = 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.[1,2] From Arctic Alaska to the Pacific Northwest, seaweeds have played a large role in the traditional cultures of multiple Native American/Alaska Native (NA/AN) and First Nation communities, as a ubiquitous source of macro- and micronutrients.[3−5] 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,[5] and nearly 60% of the households of the Inuit community of the Canadian Arctic's Belcher Islands regularly consume *Rhodymenia* spp. and *Laminaria* spp.[6]

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,[7] whereas seaweed fractions enriched in polyphenolics and polysaccharides exhibited activity against diabetes, by inhibiting α-glucosidase, increasing basal uptake of glucose, and lowering postprandial glucose levels at both the cellular and human clinical levels.[8,9] In recent years, some studies have reported that seaweed extracts demonstrate strong antioxidant properties,[10−12] leading to a growing interest for their use in food technology applications to prevent oxidation of lipids and proteinaceous tissue[12] and to reduce the effects of radical oxygen degradation in chronic diseases.[8,9] 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[13] and are often more potent antioxidants than analogous polyphenols derived from terrestrial sources due to their interlocking phenol ring structure.[14] Seaweeds also possess nonpolyphenolic compounds, including tocopherols, carotenoids, terpenoids, and alkaloids, which have demonstrated antioxidant activity in a variety of in vitro studies.[15]

Radical oxygen species (ROS), elicited by environmental stress and 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.[16] The human body counteracts accumulation of ROS via quenching mechanisms such as superoxide dismutase, catalase, and the glutathione system and uses exogenous phytochemical antioxidants such as carotenoids, vitamins, and polyphenols from dietary sources as a second tier of defense against oxidative stress.[17]

Terrestrial botanical resources have been evaluated for their potential in offsetting the risk of chronic disease,[18] 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

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conditions, with alternative high/low ultraviolet (UV) light and oxygen levels that necessitate adaptive defense mechanisms.[19,20] 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.[21−23] Furthermore, 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.[22,24] These abiotic and biotic stresses have elicited the complex phytochemical makeup of seaweeds. The same bioactive phytochemicals accumulated by the seaweed to resist environmental stressors, once ingested, are relevant to human health maintenance.[22,25] 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 nonindigenous, species expand their geographic ranges.[26,27] 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.[28]

The waters around Alaska hold an abundant diversity of macroalgae[29] 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 AND 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, AK, 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).[3] 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 of 80% aqueous methanol and shaken for 24 h on an orbital shaker at 250 rpm and 23 °C in the dark. The extract liquid was filtered through Whatman no. 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, and 1-butanol (3 × 200 mL), yielding four 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 reconstituted 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.[30] Briefly, each well of a 96-well plate was charged with 75 μL of 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 min, after which 100 μL of 7.5% Na_2CO_3 was added to each well. The plate was incubated in the dark for 90 min, and then the absorbance was read at 765 nm on a Molecular Devices M3 microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA). Phloroglucinol (15.5 − 500 μg/mL) was used to create a standard curve for calculation of the phenolic content, in phloroglucinol equivalents (PGE, $R^2 = 0.999$).

Chemical Antioxidant Analysis. *Field Antioxidant Screening.* Fractions were first analyzed for potential antioxidant activity using a qualitative assay protocol developed for field screening.[31,32] Each well of a 96-well plate was charged with 200 μL of an ABTS solution (7 mM 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 200 mM potassium persulfate), and 10 μL 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.[30] In summary, 200 μL of DPPH solution (150 μM 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 μL was added to the well, and the plate was incubated at room temperature in the dark for 2 h, after which the absorbance was read at 515 nm on a microplate reader.

A calibration curve was made with DPPH, from 4 to 150 μM, 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_{50} 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.[11] with minor modifications. A working solution of 135 μL of distilled water and 5 μL of $FeCl_2$ (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 for 10 min 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.[33] 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 min at room temperature, under light. Subsequently, 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 2% phosphoric acid) was added, and the mixture was incubated at room temperature for 10 min and 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, USA) were maintained at a subconfluent density at 37 °C in a 5% CO_2 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, USA) was used to quantify the number of viable cells according to the manufacturer's recommendations. Briefly, 20 μL of CellTiter 96AQueous One Solution was charged to each well containing 100 μL of DMEM without FBS, and the plates were incubated at 37 °C and 5% CO_2 atmosphere for 2 h. The absorbance was measured on a microplate reader at 515 nm and compared against a vehicle-treated

Table 1. Total Phenolic Content (TPC) of Alaskan Coastal Vegetation[a]

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

[a]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).

A)

Species	*A. marginata*	*F. distichus*	*P. fallax*	*S. groenlandica*	*S. latissima*	*U. lactuca*	*P. maritima*
Antioxidant value	2	3	1	3	2	1	3

B)

Figure 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) on the basis of visual inspection and comparison against controls; see Kellogg et al.[31]]; (B) four fractions (hexane, ethyl acetate, butanol, and aqueous fractions) of each species were tested, 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].

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.[34] RAW 264.7

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Table 2. Antioxidant Values for Maritime Vegetation Fractions Based upon DPPH Scavenging, Ferrous Chloride Chelating, and Nitric Oxide (NO) Development[a]

species	fraction	DPPH IC_{50} (μg/mL)		$FeCl_2$ chelating (%)		NO inhibition (%)	
A. marginata	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
F. distichus	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
P. fallax	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
S. groenlandica	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 ± 0.012	a	25.8 ± 0.9	dc
	W	39.81 ± 0.03	i	4.43 ± 0.21	d	27.4 ± 2.2	d
S. latissima	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
U. lactuca	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
P. maritima	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 ± 0.45	l	3.56 ± 0.23	cd	6.3 ± 1.9	a

[a]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.

macrophage cells were seeded at a concentration of 4×10^5 cells/well into a 24-well plate and incubated overnight at 37 °C. Cells were charged with 500 μL of 50 μM 2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (H_2DCFDA, Molecular Probes, Eugene, OR, USA), prepared fresh daily in sterile phosphate-buffered saline (PBS) for 30 min. Fluoresecent medium was aspirated, and cells were exposed to 25 μL of extract/fraction (100 μg/mL final concentration) and 1 μL of lipopolysaccharide (LPS, from *Escherichia coli* 026:B6) and incubated for 24 h, 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 μL of cell culture medium was added 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid), and the mixture was 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 the 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, USA), with statistical significance determined at the P < 0.05 or P < 0.01 level. Half-maximal inhibitory concentration (IC_{50}) data were 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 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). The ethyl acetate (557.2 ± 9.7 μg PGE/mg) and butanol (420.1 ± 15.2 μg PGE/mg) fractions of *F. distichus* 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 ± 0.6% TPC.[11] 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.

Antioxidant Capacity: Chemical Assays. The antioxidant capacity of the marine vegetation extracts, based upon the preliminary screening assay, were assigned qualitative values

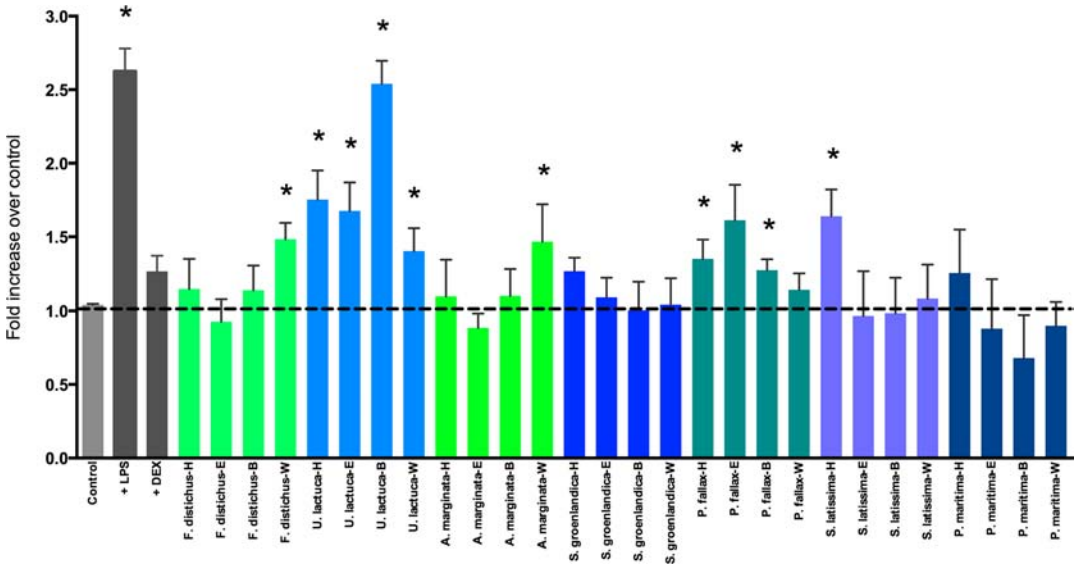

Figure 2. Radical oxygen species (ROS) production in RAW 264.7 macrophage cells. Cultures were stimulated with LPS (1 μg/mL) and treated with 100 μg/mL fractions of Alaskan coastal vegetation fractions for 24 h. Samples marked with an asterisk (∗) are significantly different compared to untreated control ($P < 0.05$). All samples were assayed in triplicate.

from 0 to 3 on the basis of 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 1), and subsequent fractions were also assayed under the same conditions (Figure 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 ABTS radical; the ethyl acetate and butanol fractions showed higher activity than hexane or aqueous fractions.

As shown in Table 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 IC_{50} values of 5.12 ± 0.77 and 4.76 ± 0.39 μg/mL, respectively. The butanol fraction of *S. groenlandica* (4.29 ± 0.19 μg/mL) and the ethyl acetate fraction of *A. marginata* (7.59 ± 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 ± 0.11 μg/mL) and were also comparable to those of the closely related *F. vesiculosus*,[11] with ethyl acetate and butanol fractions at IC_{50} values of 3.76 ± 0.22 and 4.77 ± 0.25 μg/mL, respectively. The ethyl acetate fraction (20.78 ± 0.83 μg/mL) and butanol fraction (11.26 ± 0.47 μg/mL) of *Plantago maritima* also demonstrated moderate antioxidant efficacy. The mild activity of some aqueous fractions (*F. distichus*, *P. fallax*, and *S. groenlandica* had IC_{50}values 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.[35−37]

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.[12,38] The moderate correlation found in the current study may suggest that other active, nonpolyphenolic components could have synergistic effects on radical scavenging, as shown in Heo et al.'s 2005 study, in which 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.[39]

Lipid and cellular oxidation may be amplified by interaction with transition metal cations, which play an important part in aging and age-related chronic diseases,[40,41] and the ability of antioxidants to chelate these ions can diminish the cumulative effects of transition metal-mediated free radical and oxidative damage.[42] The species' fractions demonstrated various degrees of ferrous ion chelation capacity (Table 2). The ethyl acetate fraction of *P. fallax* (29.03 ± 0.86%) had the greatest chelating ability, whereas the hexane (24.28 ± 0.62%) and ethyl acetate (22.87 ± 0.85%) fractions of *A. marginata* also exhibited high levels of chelating. These levels were consistent with others previously reported[11,12] when sample concentration is adjusted for. Although 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,[12] as well as in extracts of malting barley,[43] for which TPC had poor correlations with both DPPH and 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 2). Two fractions derived from *S. groenlandica* demonstrated the greatest NO scavenging ability. The hexane (50.0 ± 1.7% inhibition) and ethyl acetate (58.6 ± 2.4%) fractions showed >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 alga, the ethyl acetate fraction demonstrated the highest degree of NO

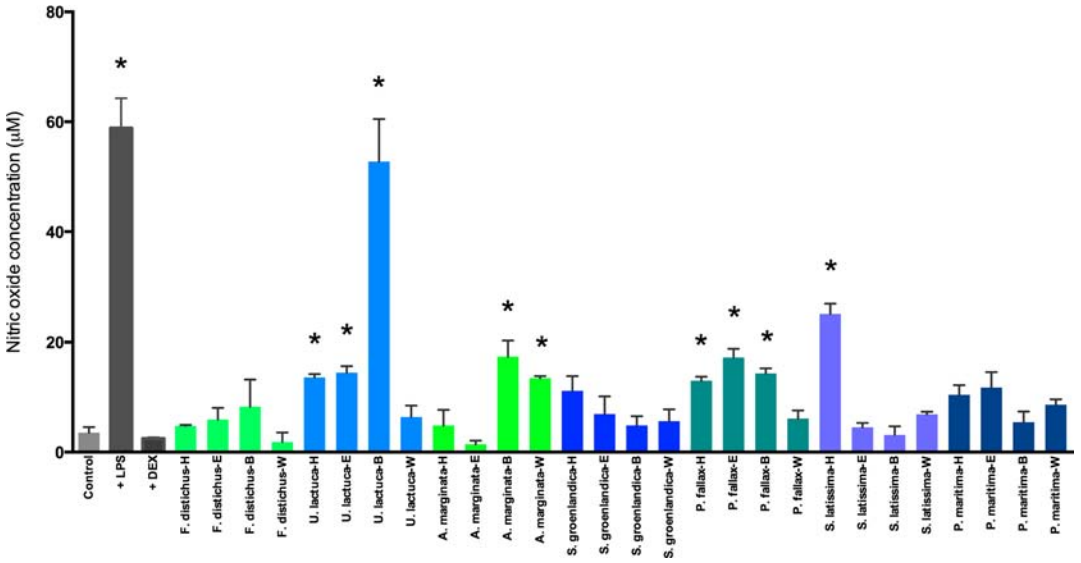

Figure 3. Nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophage cells. Cultures were cotreated with 100 μg/mL fractions of Alaskan coastal vegetation fractions for 24 h. Samples marked with an asterisk (∗) are significantly different compared to untreated control ($P < 0.05$). All samples were assayed in triplicate.

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_2DCFDA 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 fluoresecent DCF. Lipopolysaccharide (LPS, 1 μg/mL) induced a 2.5-fold overproduction of ROS in the cells compared to nonstimulated cells. Figure 2 shows the effect of Alaskan seaweed and plant fractions on the inhibition of intracellular LPS-induced ROS generation. The positive control 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, at 100 μg/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 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 effected 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 nonstimulated state. The ethyl acetate fractions appeared to be lower than the other fractions in reducing ROS production, although 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.

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 μg/mL) evoked a 16.6-fold induction of nitrite production versus the naive control, and this induction was inhibited by the positive control, DEX, to baseline levels ($P < 0.001$). The Alaskan vegetative fractions reduced NO production to various degrees (Figure 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 nonstimulated 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 $FeCl_2$ 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,[44] 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, although this correlation was lower than expected compared against other studies on TPC-enriched seaweed fractions,[45] suggesting that other nonphenolic 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 seaweeds 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.[45] 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.

AUTHOR INFORMATION

Corresponding Author

*(M.A.L.) E-mail: maryann_lila@ncsu.edu. Phone: (704) 250-5407. Fax: (704) 250-5409.

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