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Extracts from dulse (*Palmaria palmata*) are effective antioxidants and inhibitors of cell proliferation in vitro

Yvonne V. Yuan *, Meshell F. Carrington, Natalie A. Walsh

School of Nutrition, Faculty of Community Services, Ryerson University, 350 Victoria Street, Toronto, ON, Canada M5B 2K3

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Abstract

Previously, we reported that a 1-butanol soluble extract of the edible red alga *Palmaria palmata*, known as dulse, exhibited hydroxyl and stable free radical scavenging activity as well as inhibition of lipid peroxidation, attributed to the reducing activity and polyphenol content of the dulse extract. In the present study, we evaluated the antioxidant and antiproliferative activities of two grades of dulse harvested from Canadian Maritime locations differing in UV radiation exposure (i.e. west versus east coasts of Grand Manan Island, New Brunswick). The 1-butanol soluble extract from Grade 1 dulse (reduced UV-exposure) exhibited lower reducing activity versus Grade 2 dulse (greater UV exposure) reflecting a lower requirement for endogenous antioxidant protection. Grade 1 and 2 dulse extracts both inhibited ($p \leqslant 0.03$) AAPH-induced lipid peroxidation, but had no effect on AMVN-induced lipid peroxidation, demonstrating the aqueous nature of the antioxidants involved. The Grade 1 and 2 dulse extract inhibition ($p < 0.05$) of HeLa cell proliferation was dose-dependent over 0.5–5.0 mg/mL and maximal at 48 and 72 h incubation. The antiproliferative effects of the Grade 1 and 2 dulse extracts in the present study likely reflect the bioactivity of the polyphenol content of these extracts.

Keywords: *Palmaria palmata*; Dulse; Antioxidant activity; Lipid oxidation; AAPH; AMVN; Linoleic acid emulsion; HeLa cells; Cell proliferation

1. Introduction

Dietary seaweeds, such as algae from brown (*Phaeophyta*), green (*Chlorophyta*) and red (*Rhodophyta*) taxonomies such as the *Laminariales*, *Ulvales* and *Porphyridiales*, respectively, are common in the diets of Asian countries, particularly China, Japan, Korea and Indonesia (Anggadiredja et al., 1997; Chan et al., 1997; Yan et al., 1998; Nakayama et al., 1999; Nagata et al., 2003; Skibola, 2004); but less so in European diets (e.g. France, Iceland, Ireland and Norway) (Mabeau and Fleurence, 1993; Le Tutour, 1990; Le Tutour et al., 1998) and the Canadian Maritimes (Morgan et al., 1980; Yuan et al., 2005). These seaweeds, or sea vegetables, are often used as wrappers such as the Japanese sushi seaweed 'Nori' or Korean 'Kim' derived from the roasted or toasted red alga, *Porphyra tenera*; condiments and seasonings in soups and salads such as the brown algae in Japanese cuisine: 'Hijiki' (*Hijikia fusiformis*), 'Wakame' (*Undaria pinnatifida*) or 'Makonbu'

Abbreviations: AAPH, 2,2′-azo-bis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); BHA, butylated hydroxyanisole; CD, conjugated dienes; EGC, epigallocatechin; FBS, foetal bovine serum; GSH, glutathione; MAA, mycosporine-like amino acid; MDA, malondialdehyde; MEM, minimum essential medium; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; ·OH, hydroxyl radical; PBS, phosphate buffered saline; ROS, reactive oxygen species; TBARS, 2-thiobarbituric acid reactive substances; TCA, trichloroacetic acid.

* Corresponding author. Tel.: +41 6979 5000x6827; fax: +41 6979 5204.
E-mail address: yyuan@ryerson.ca (Y.V. Yuan).

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(*Laminaria japonica*); as well as health food snacks such as the European/North American 'Dulse' derived from the red alga, *Palmaria palmata*.

Estimates of seaweed intake in Japanese populations range from 4.6 g/day (0.4–29.2 g) from food-frequency questionnaire data (Nagata et al., 2003) to 10–25% of food intake, or approximately 3–13 g/day (Skibola, 2004), with the highest intakes attributed to the Japanese of Okinawa (Sho, 2001). Conversely, seaweed intakes are vastly decreased outside of Japan from an estimated 100% consumption of seaweed by Japanese living in Japan to approx. 12–20% by migrant Japanese living in Hawaii (Teas, 1981). Similarly low to zero seaweed intakes can be expected in Western, particularly North American, populations. These estimates of seaweed intakes in different cultures and populations highlight one clear environmental (i.e. dietary) difference between populations which are also known to differ in chronic disease rates; for example, breast cancer with 42.2 and 13.1 one-year prevalence cases per 100,000 in Japan and China (including Korea, Mongolia and Hong Kong), respectively versus 125.9 cases in North America (NA); and prostate cancer with 10.4 and 0.7 one-year prevalence cases in Japan and China, respectively versus 117.2 cases in NA using 1990 data (Pisani et al., 2002).

The potential beneficial association between consumption of seaweed and breast cancer can be traced back to the ancient Egyptian 'Ebers Papyrus' dating from approx. 1534 B.C., which mentioned that seaweed was administered as a treatment to breast cancer patients (Teas, 1981). Indeed, a growing body of research in rodent models has demonstrated the anticarcinogenic effects of dietary kelps, such as *Laminaria angustata*, and other red and green seaweed species against mammary (Maruyama et al., 1991; Yamamoto et al., 1987), intestinal (Yamamoto and Maruyama, 1985) and skin carcinogenesis (Higashi-Okai et al., 1999). Mammalian cell culture and cell-free studies have begun to elucidate the underlying mechanisms of the potential protective effects of seaweed constituents against carcinogenesis, including antimutagenic effects of kelp extracts against breast and colon cancer inducers (Reddy et al., 1985); inhibition of telomerase activity (Eitsuka et al., 2004) and anti-inflammatory and anti-proliferative activities by a variety of red algal extracts (Bergé et al., 2002).

One of the mechanisms thought to contribute to the inhibitory effect of dietary kelp against chemically-induced mammary carcinogenesis was the enhancement of antioxidant enzyme activity (i.e. glutathione peroxidase) and reduction in lipid peroxides in the livers of treated rats (Maruyama et al., 1991). A similar antioxidant effect was observed with a sulfoglycolipid fraction isolated from the red alga, *Porphyridum creuntum*, which inhibited the production of superoxide anion radicals by cultured peritoneal activated leukocytes (Bergé et al., 2002). Edible seaweeds contain labile antioxidant molecules such as ascorbate and glutathione (GSH) when fresh (Burritt et al., 2002; Morgan et al., 1980), as well as more stable molecules including carotenoids (Yan et al., 1999; Okai et al., 1996; Morgan et al., 1980), mycosporine-like amino acids (MAA, mycosporine–glycine and –taurine; Dunlap et al., 1997; Nakayama et al., 1999; Sekikawa et al., 1986), catechins (Yoshie et al., 2000), phlorotannins (Jiménez-Escrig et al., 2001) and the tocopherols (Morgan et al., 1980). In fact, extracts from several brown, green and red alga harvested in France (Le Tutour et al., 1998; Le Tutour, 1990), Spain (Jiménez-Escrig et al., 2001), Indonesia (Anggadiredja et al., 1997), Korea (Han et al., 1999), China (Yan et al., 1998) and Japan (Yan et al., 1999; Sekikawa et al., 1986) have demonstrated antioxidant activity in vitro. Moreover, algae have been reported to vary in antioxidant composition due to environmental factors such as water depth and UV-exposure (Burritt et al., 2002; Karsten and Wiencke, 1999). We recently reported hydroxyl and stable free radical scavenging activities by a *P. palmata* extract (Yuan et al., 2005). Interestingly, this alga is known to vary in pigmentation (R-phycoerythrin) depending on its UV-exposure during growth (Galland-Irmouli et al., 2000); however, it is not known whether this also influences the antioxidant capacity of *P. palmata*.

Taken together, these lines of evidence suggest a potential for edible seaweeds as sources of dietary antioxidants and anticarcinogens. There is a paucity of data however, characterizing the antioxidant and anticarcinogenic potential of North American algae such as *P. palmata* (order *Palmariales*, family *Palmariaceae*). Therefore, the objectives of this study were to characterize the antioxidant activity of dulse extracts from two different locations in the Canadian Maritimes, known to vary in UV-exposure, and to assess the influence of these extracts on epithelial cancer cell (HeLa cells) proliferation in vitro.

2. Materials and methods

2.1. Materials

Two grades of certified organic (Organic Crop Improvement Association International, OCIA) dulse (*P. palmata*) harvested in Spring 2001 were obtained from Atlantic Mariculture Ltd. (Grand Manan, New Brunswick (NB), Canada). Grade 1 dulse was harvested from Dark Harbour on the western side of Grand Manan Island, NB and consisted of intact dulse fronds (whole leaf); whereas the Grade 2 dulse was harvested from the 'Passage' area on the eastern side of Grand Manan Island and consisted of comminuted dulse flakes. Grade 1 'Dark Harbour' dulse is a dark reddish-purple, thicker and more flavourful than the Grade

2 'Passage' dulse, which is noted to be a bit saltier tasting, lighter in colour and lighter in texture (Atlantic Mariculture 2004, personal communication). HeLa cells were purchased from American Type Culture Collection (ATCC CCL-2, Manassas, VA). Linoleic acid, 1-butanol, ethyl acetate, hexane, methanol, ethanol, chloroform and malondialdehyde (MDA) were purchased from Fisher Scientific (Mississauga, ON). 2-Thiobarbituric acid (TBA), L-ascorbic acid, monolaurate Tween 20, gallic acid, Folin-Ciocalteau's phenol reagent, trichloroacetic acid (TCA) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich Canada (Oakville, ON). 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were purchased from Wako Chemicals USA Inc. (Richmond, VA). Minimum essential medium (MEM), L-glutamine, sodium bicarbonate, non-essential amino acids, sodium pyruvate, foetal bovine serum (FBS) and phosphate buffered saline (PBS) were obtained from Invitrogen Corporation (Burlington, ON). Water (H_2O) used in all assays was purified using an E-pure Barnstead system (VWR Canlab, Mississauga, ON). All solvents used were of ACS or HPLC grades. Sample absorbances were read using a Lambda 20 UV/Vis Spectrometer (Perkin–Elmer, Norwalk, CT).

2.2. Preparation of dulse extract

Two hundred and fifty grams ground, freeze-dried Grade 1 or Grade 2 dulse were each extracted with methanol overnight 3 × at room temperature, filtered and concentrated by rotary evaporation (Buchi R-200V, Brinkmann Instruments, Mississauga, ON), 40 °C, as previously described (Yuan et al., 2005). The concentrated extract was further washed with hexane and the lower methanol phase then extracted with H_2O + ethyl acetate. The lower H_2O–methanol layer was then extracted with 1-butanol and the upper butanol layer concentrated by rotary evaporation to obtain a light brown residual powder. The Grade 1 and 2 dulse extracts were solubilized in 0.1% ethanol for use in assays.

2.3. Reducing activity

The reducing activities of the dulse extracts were evaluated according to the method of Yen and Chen (1995) with modifications (Yuan et al., 2005). L-ascorbic acid was prepared in degassed H_2O for use as the standard to quantify reducing activity. To 0.5 mL of reducing agent were added the following: 1.25 mL 0.2 M phosphate buffer, pH 6.6 + 1.25 mL 1% $K_3Fe(CN)_6$ followed by incubation at 50 °C in a water bath for 20 min. Samples were then cooled and mixed with 1.25 mL 10% TCA and an 1.25 mL aliquot removed to a fresh tube. For the final reaction, the sample aliquot was mixed with 1.25 mL of H_2O + 0.25 mL 0.1% $FeCl_3 \cdot 6H_2O$ and left to react at room temperature for 10 min. Sample absorbances were read at 700 nm. Reducing activities of the dulse extracts were expressed as ascorbic acid equivalents from the ascorbic acid calibration curve.

2.4. Total polyphenols

The polyphenol contents of the dulse extracts were quantified according to the method of Taga et al. (1984) as modified by Yuan et al. (2005). Briefly, aliquots of test samples (100 μL) were mixed with 2.0 mL 2% Na_2CO_3 and allowed to react at room temperature, 2 min. At this time, 100 μL 50% Folin-Ciocalteau's phenol reagent was added, and the reaction tube allowed to react at room temperature, 30 min prior to reading the absorbance at 720 nm. Gallic acid was used as the standard for a calibration curve and the total polyphenol content of the dulse extracts expressed as gallic acid equivalents.

2.5. Inhibition of AAPH- or AMVN-initiated lipid oxidation in a linoleic acid emulsion

The linoleic acid emulsions were prepared by homogenizing 2.5 mL linoleic acid + 22.5 mL 5 mM phosphate buffer, pH 7.4 + 125 μL Tween 20 on ice 2 × 10 s (Powergen 700, Fisher Scientific). The emulsions were incubated at 38 °C with shaking and aliquots removed at 0, 30, 60, 90, 120 and 240 min followed by phase separation using $CHCl_3$:CH_3OH 2:1 and 2-thiobarbituric acid reactive substances (TBARS) measured in the aqueous fraction at 532 nm (Coupland et al., 1996). Briefly, 2.0 mL of the aqueous phase + 0.5 mL 1% TBA in 0.05 M NaOH + 0.5 mL 10% TCA were heated in sealed test tubes in a boiling water bath for 15 min. After the tubes were cooled, 1.5 mL $CHCl_3$ was added to the samples, followed by centrifugation (8000 rpm, 3 min). The aqueous layer was then removed for determination of TBARS at 532 nm.

For the emulsions in which lipid oxidation was initiated by the hydrophilic radical AAPH, the AAPH was first solubilized in the 22.5 mL 5 mM phosphate buffer, pH 7.4 for a concentration of 10 mM AAPH in the final emulsion. For the emulsions in which lipid oxidation was initiated by the lipophilic radical AMVN, the AMVN was solubilized in the 2.5 mL of linoleic acid + 125 μL Tween 20 + 2 mL $CHCl_3$. The $CHCl_3$ was subsequently removed by rotary evaporation prior to addition of the phosphate buffer and homogenization of the emulsion for a concentration of 10 mM AMVN in the final emulsion.

2.6. Cell proliferation studies

HeLa cells (ATCC CCL-2) were grown in 75 cm^2 flasks in MEM containing 2 mM L-glutamine, 1.5 g/L

sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10% FBS. Cells were seeded into 96-well plates at a density of 5×10^3 per well and allowed to attach overnight in 300 μL medium at 37 °C and 5% CO_2.

The Grade 1 and 2 Dulse extracts in 0.1% ethanol were each sonicated for 30 min and sterile-filtered prior to addition to plated cells. Dulse extracts were added at a final concentration of 0.5, 1, 2 or 5 mg/mL of media, and the cells were left to incubate in the seaweed extract-containing medium for 24, 48 or 72 h, 37 °C and 5% CO_2. A set of solvent controls (0.1% ethanol) was included for each microtitre plate. Following incubation, traces of seaweed extract were removed by washing cells twice with 200 μL PBS and applying 100 μL of fresh medium plus 10 μL of a 5 mg/mL stock solution of MTT dissolved in PBS to determine the effects of the dulse extracts on cell proliferation (Mosmann, 1983). Cells were then incubated for 4 h at 37 °C and 5% CO_2. To solubilize the product of MTT cleavage, 100 μL of isopropanol containing 0.04 N HCl was added to each well and thoroughly mixed using a multichannel pipettor. Within 1 h of HCl–isopropanol addition, the absorbance at 570 nm, with a reference wavelength of 630 nm, was read using a BioRad 550 Microplate Reader (Mississauga, ON). The percent inhibition of cell proliferation was calculated as follows:

$$\%\ \text{Inhibition} = \frac{(\text{Abs. 570 nm Control} - \text{Abs. 570 nm sample})}{\text{Abs. 570 nm Control}} \times 100$$

Each concentration of the respective dulse extracts was assayed in triplicate for an $n = 3$.

2.7. Statistics

All data are expressed as means ± SEM. One-way analysis of variance (ANOVA; SPSS 10.0 for Windows; SPSS Inc., Chicago, IL) was used to test for differences between different treatment concentrations. Where differences did exist, the source of the differences at a $p \leqslant 0.05$ significance level was identified by the Student–Newman–Keuls multiple range test. Two-way analysis of variance (MANOVA; SPSS 10.0) was used to test for interactions between grade of dulse and treatment concentration. Student's *t*-test for independent samples was used to test for differences at a $p \leqslant 0.05$ significance level where appropriate.

3. Results

Table 1 summarizes the reducing activity and total polyphenol content of the dulse extracts. The reducing activity (expressed as ascorbic acid equivalents) of the Grade 2 dulse extract was 1.68 fold greater than that of the Grade 1 dulse extract. However, the total polyphenol contents (expressed as gallic acid equivalents) of the extracts from the two grades of dulse were very similar.

Table 1
Reducing activity and total polyphenol content of Grade 1 and 2 dulse extracts[a]

	Reducing activity (μg Ascorbic acid equivalents)	Polyphenols (μg Gallic acid equivalents)
Dulse extract		
Grade 1	4.48	12.8
Grade 2	7.51	12.7

[a] Data are expressed as equivalents for 1 mg of the respective dulse extracts.

In the AAPH-initiated linoleic acid emulsion model of lipid oxidation, both Grade 1 and 2 dulse extracts were effective in inhibiting TBARS production (Fig. 1A and B). The Grade 1 dulse extract inhibited ($p \leqslant 0.03$) TBARS production at 60, 90, 120 and 240 h; albeit, there was no dose dependent difference between 1 and 2 mL dulse extract treatment levels (Fig. 1A). Similarly, the Grade 2 dulse extract inhibited ($p < 0.001$) TBARS production at 60–240 h; again, there was no dose dependent difference between 1 and 2 mL dulse extract treatment levels (Fig. 1B). Neither the

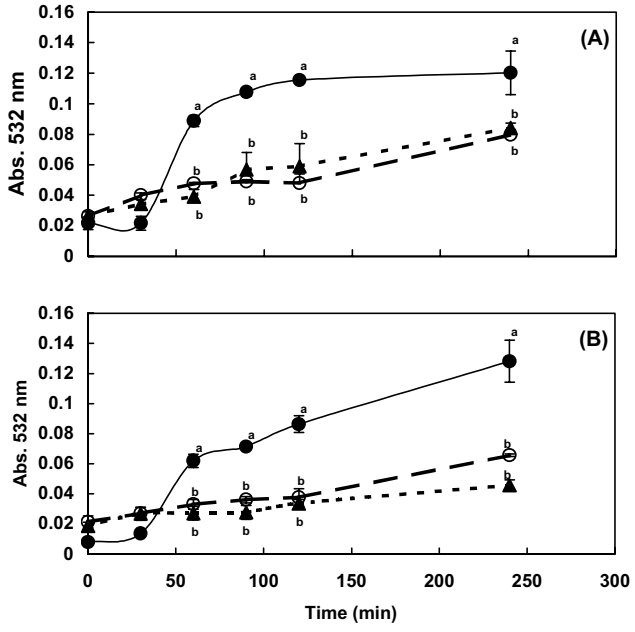

Fig. 1. Effect of Grade 1 and Grade 2 dulse extracts on AAPH-initiated lipid oxidation, as TBARS, in linoleic acid emulsions incubated with shaking at 38 °C. (upper panel, A) Grade 1 dulse; (lower panel, B) Grade 2 dulse. (●) control; (▲) 1 mL dulse extract; (○) 2 mL dulse extract. a, b Indicate a significant difference ($p < 0.05$) between control and 1 and 2 mL dulse treatments by ANOVA.

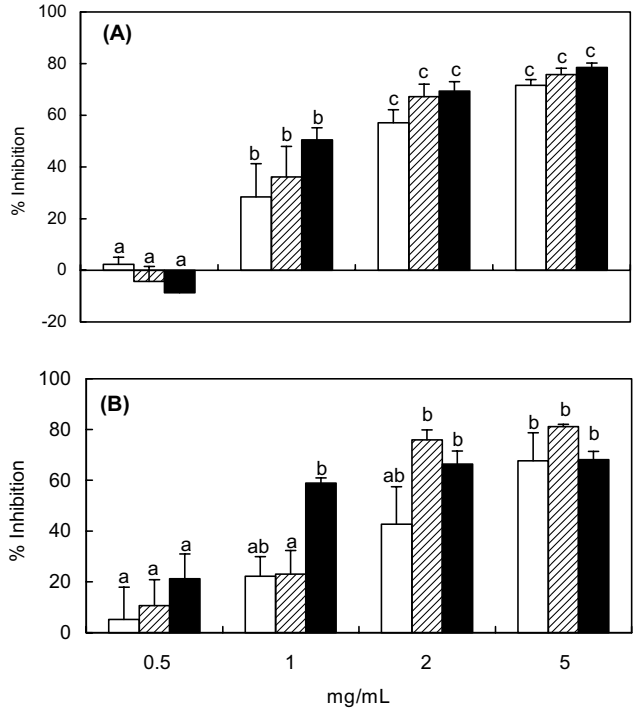

Fig. 2. Influence of Grade 1 and Grade 2 dulse extracts on inhibition of cell proliferation of HeLa cells. (upper panel, A) Grade 1 dulse; (lower panel, B) Grade 2 dulse. (□) 24 h; (▨) 48 h; (■) 72 h incubation. a, b, c Indicate a significant difference ($p < 0.05$) between doses, within each incubation period, by ANOVA.

Grade 1, nor 2, dulse extracts had any influence on TBARS production in the AMVN-initiated linoleic acid emulsion model of lipid oxidation (data not shown).

The Grade 1 and 2 dulse extracts inhibited HeLa cell proliferation in a dose-dependent manner over the 24, 48 and 72 h incubation periods (Fig. 2A and B). For the Grade 1 dulse extract, after 24, 48 or 72 h of incubation, inhibition of HeLa cell proliferation was greatest ($p \leqslant 0.001$) with 2.0 and 5.0 mg/mL dulse extract treatments and least with the 0.5 mg/mL dose (Fig. 2A). For the Grade 2 dulse extract, after 24 h of incubation, inhibition of HeLa cell proliferation was greatest ($p < 0.05$) with the 5.0 mg/mL dulse extract treatment and least with the 0.5 mg/mL dose (Fig. 2B). After 48 h of incubation, inhibition of HeLa cell proliferation was greatest ($p < 0.001$) with the 2.0 and 5.0 mg/mL Grade 2 dulse extract treatments. Inhibition of HeLa cell proliferation after 72 h of incubation was greatest ($p = 0.001$) with the 1.0, 2.0 and 5.0 mg/mL treatments. There were no differences between the effects of Grade 1 versus Grade 2 dulse extracts on HeLa cell proliferation after either 24 or 48 h of incubation. However, after 72 h of incubation, there was an interaction between grade of dulse and dose ($p = 0.004$) in that with the 0.5 mg/mL dulse extract treatments, the Grade 2 dulse extract was inhibitory, while the Grade 1 dulse extract had little effect at this dose (Fig. 2A and B). Conversely, with the 5.0 mg/mL dulse extract treatments, the Grade 1 dulse extract exhibited a greater inhibitory effect compared to the Grade 2 dulse extract due to a reduction in inhibition of HeLa cell proliferation between 48 and 72 h of incubation with the Grade 2 dulse extract.

4. Discussion

Previous work from this laboratory indicated that a 1-butanol soluble extract from whole leaf dulse (*P. palmata*) was capable of scavenging hydroxyl radicals and quenching the stable free radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) and 2,2′-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS$^{\bullet+}$) as well as inhibiting lipid peroxidation in separate model systems (Yuan et al., 2005). In the present study, we demonstrated for the first time, the antiproliferative activity of extracts from dulse using an epithelial adenocarcinoma cell line (HeLa cells) model. Further, we extended the findings of our previous work by demonstrating that these extracts, from two grades of commercially available dulse, inhibited lipid peroxidation in a linoleic acid emulsion initiated by the aqueous azo dye AAPH, which could be related to the reducing activity and total polyphenol content of these extracts. Antioxidant activity of algae can be inferred from the fact that intertidal organisms require endogenous protection against UV radiation (Karsten and Wiencke, 1999) as well as the effects of dehydration due to daily tidal fluctuations (Burritt et al., 2002). Moreover, beneficial health effects from seaweed consumption can be linked not only with the soluble and insoluble dietary fibres (i.e. carregeenans, agar, β-1,3-glucan, floridean starch; Lahaye et al., 1993; Mabeau and Fleurence, 1993; Han et al., 1999), but also the nutritional value of the proteins (Chan et al., 1997) as well as the nutritive antioxidant constituents such as ascorbate, glutathione, α- and β-carotene and α-, γ-, and δ-tocopherols (Morgan et al., 1980; Burritt et al., 2002) in these algae.

Dulse harvested in the Canadian Maritimes, Norway and Japan have been reported to contain several hydrophilic antioxidant compounds, namely ascorbate, GSH (Aguilera et al., 2002a; Morgan et al., 1980) and a variety of MAAs such as mycosporine–glycine, shinorine and palythine (Aguilera et al., 2002b; Karsten and Wiencke, 1999; Sekikawa et al., 1986). However, while fresh dulse may contain between 200 and 500 μg ascorbic acid/g wet wt., sun drying and subsequent storage of the dulse will drastically reduce the levels of this labile antioxidant (Aguilera et al., 2002a; Burritt et al., 2002; Morgan et al., 1980). When the red alga, *Stictosiphonia arbuscula*, was subjected to differing levels of dehydration, samples harvested from the lower tidal zone exhibited decreased GSH/GSSG (reduced/oxidized GSH)

ratios at 12 h which remained low at 24 and 48 h; whereas samples from the higher tidal zone experienced a temporary increase in the GSH/GSSG ratio at 24 h which declined again at 48 h (Burritt et al., 2002). A similar phenomenon with *S. arbuscula* levels of reduced/oxidized ascorbate, ASA/DHA (dehydroascorbate) ratios between lower and higher tidal zone samples occurred with dehydration. Algae are exposed to differing amounts of UV radiation depending on their tidal zone habitat; *P. palmata* is classified as an upper sublittoral alga inhabiting depths of 0–3 m in the tidal zone and is subject to tidal fluctuations and thereby, considerable photooxidative stress (Aguilera et al., 2002a). Thus, an endogenous antioxidant protective system is necessary in these intertidal organisms for survival of periodic dehydration at low-tide such as stable ascorbate and GSH pools. In the present study, the two dulse extracts exhibited weak reducing activity with 1 mg of each of the Grade 1 and 2 extracts equivalent to microgram quantities of L-ascorbic acid, with the Grade 2 dulse extract exhibiting 1.7 fold the reducing activity of the Grade 1 extract. This difference in reducing activity was likely associated with the harvest location of the two grades of dulse: the Grade 1 dulse was harvested from Dark Harbour on the western coast of Grand Manan Island, an area noted for its high cliffs shading the intertidal zone decreasing exposure of the alga to UV radiation, whereas the Grade 2 ''Passage' dulse was harvested from the low lying eastern shores of the island with increased exposure to UV radiation. Indeed, this difference in UV-exposure between these two grades of dulse is corroborated by the difference in the intensity of the red–purple colouration of these plants in that tissue levels of the main light-harvesting pigment in red algae, R-phycoerythrin, are inversely proportional to UV radiation exposure (Galland-Irmouli et al., 2000). Taken together, this evidence suggests that the Grade 2 dulse, exposed to greater amounts of UV radiation, was characterized by an enhanced reducing capacity required to help protect the alga against greater photooxidative stresses compared to the Grade 1 dulse.

The reducing activity, discussed above, likely played a role in the present study wherein the two dulse extracts exhibited antioxidant protection against lipid peroxidation initiated by AAPH. However, these same dulse extracts had no effect on lipid peroxidation initiated by the non-polar azo dye AMVN. Azo compounds (diazenes) have been used as free radical initiators in the study of antioxidant compounds due to the predictable thermal decomposition of azo compounds to yield N_2 and two carbon radicals, R$^{\cdot}$ (Niki, 1990). These radicals may then either react with each other to yield a stable non-radical end product, or react with molecular O_2 to yield peroxyl radicals, ROO$^{\cdot}$ which can then participate in the peroxidation of a polyunsaturated lipid emulsion model system. AAPH is a hydrophilic radical initiator which would generate its radicals in the aqueous region of an oil-in-water emulsion as used in the present study. Whereas, AMVN as a lipophilic radical initiator would generate its radicals within the linoleic acid micelle droplets of the emulsion herein. The $T_{1/2}$ of AAPH and AMVN are 175 and 100 h, respectively (Niki, 1990; Yoshida et al., 2003); therefore, the rate of generation of radicals (R_i) would have been constant during the 240 min period studied. The rate of generation of AAPH and AMVN radicals at 37–38 °C at a neutral pH is given by the following equations: R_i (mol/L/s) = 1.36×10^{-6} [AAPH] (Niki, 1990) and R_i (mol/L/s) = 3.88×10^{-6} [AMVN] (Shi et al., 1999). Thus, the total amounts of radicals formed in the linoleic acid emulsions over the 240 min period initiated by AAPH and AMVN were 1.96 and 5.59×10^{-4} moles/L, respectively.

The Grade 1 and 2 dulse extracts in the present study demonstrated the phenomenon of the 'polar paradox' whereby polar compounds exhibit weak antioxidant activity in emulsions due to the dilution of these compounds in the aqueous phase (Koleva et al., 2002). Conversely, non-polar compounds would be expected to exhibit strong antioxidant activity due to the concentration of the antioxidant at the lipid–air interface allowing strong protection of an emulsion against oxidation. Both the Grade 1 and 2 dulse extracts inhibited the AAPH-induced production of TBARS in the linoleic acid emulsions, albeit there were no dose-dependent effects with the increased concentrations of dulse extract. These observations were likely associated with the considerable dilution of the dulse extracts when added to the buffer volume of the oil-in-water emulsions we studied herein. On the other hand, the absence of an antioxidant effect of the dulse extracts on the AMVN-induced lipid peroxidation could be attributed to not only the polarity of the compounds in the extracts which exhibited reducing activity and the presence of polyphenol compounds, but also the concentration of the non-polar radical initiator in the lipid phase of the emulsion. The AMVN azo dye would be expected to be concentrated in the linoleic acid micelle droplets of the emulsion in a total volume of 2.5 mL. Moreover, due to the greater rate of generation (R_i) of AMVN radicals compared to that of AAPH radicals, the generation of peroxyl and lipid free radicals likely exhausted the dulse extract antioxidant capacity in the vicinity of the linoleic acid emulsion droplets.

The Grade 1 and 2 dulse extracts exhibited not only antioxidative reducing activity, but also the presence of polyphenolic functional groups soluble in alcohol, namely 1-butanol, methanol and aqueous ethanol, used to solubilize the dulse extracts herein. Polyphenols represent a diverse class of compounds including flavonoids (i.e. flavones, flavonols, flavanones, flavononols, chalcones and flavan-3-ols), lignans, lignins, tocopherols, tannins and phenolic acids (Shukla et al., 1997). The

antioxidant activity of plant-derived polyphenols is known to include a variety of mechanisms including reactive oxygen species scavenging, inhibition of lipid peroxidation as well as metal ion chelation (Shahidi, 1997). However, we previously reported that a 1-butanol soluble dulse extract did not possess transition metal ion chelation activity in vitro (Yuan et al., 2005). While we have not determined the identity of the chemical species in our extracts in the present study, a review of the literature can provide some valuable insights for this future work. For example, Takano et al. (1979) isolated and determined the structure of several UV-absorbing mycosporine-like amino acids (MAAs) from the red alga *P. tenera*. Moreover, several MAAs, characterized by a glycine residue sidechain, an aromatic methyl ester core and a hydroxylated sidechain function, have been identified in dulse harvested from Japanese and Norwegian waters (Sekikawa et al., 1986; Karsten and Wiencke, 1999; Aguilera et al., 2002b). These intracellular alcohol- and water-soluble secondary metabolites are thought to function as UV-absorbing sunscreens, since concentrations of MAAs were lowest in dulse samples from deeper waters (3–10 m) and increased when dulse was transplanted to shallower depths (Karsten and Wiencke, 1999). The biosynthesis of MAAs is derived from the shikimic acid pathway via 3-dehydroquinic acid and 4-deoxygadusol (4-DG; Dunlap et al., 1997). Interestingly, 4-DG has been noted to possess strong antioxidant activity (Dunlap et al., 1997). Moreover, the shikimic acid pathway is the biosynthetic route for various phenolic acids during the metabolism of the aromatic acids phenylalanine and tyrosine: quinic acid is converted to 3-dehydroquinic acid, then to 3-dehydroshikimic acid and finally to protocatechuic acid. Phenolic acids such as protocatechuic and caffeic acids are noted to function as free radical quenchers and oxidation chain-breakers associated with the *o*-dihydroxylation of these compounds (Shukla et al., 1997). Other flavonoids, such as the prenylated and non-prenylated chalcones and flavanones isolated from beer and hops, do not chelate transition metal ions in vitro (Miranda et al., 2000). Taken together, this evidence suggests that the Grade 1 and 2 dulse extracts likely contained a mixture of compounds with phenolic functional groups including non-chelating polyphenols and MAAs derived from the shikimic acid pathway.

The Grade 1 and 2 dulse extracts exhibited inhibition against proliferation of the epithelial adenocarcinoma HeLa cell line. Moreover, the antiproliferative activity of the dulse extracts was dose-dependent and followed a temporal pattern with the greatest effects observed at 48 and 72 h incubation. The calculated EC_{50} values after 72 h incubation of HeLa cells with Grade 1 and 2 dulse extracts were approximately 2.3 and 1.6 mg/mL, respectively. The similar antiproliferative effects of the Grade 1 and 2 dulse extracts parallelled the polyphenol and antioxidant, inhibition of lipid peroxidation, results above. Anticarcinogenic effects of red alga were demonstrated by Yamamoto and Maruyama (1985) who reported that powdered *P. tenera*, at 2% of the diet, reduced intestinal tumour incidence in rats treated with 1,2-dimethylhydrazine. Similarly, powdered *P. tenera* in the diet and an hot water extract of this alga injected *i.p.* inhibited the growth of implanted sarcoma-180 cells in mice by 24.5 and 37.9%, respectively (Yamamoto et al., 1986); as well, powdered *P. tenera* at 2% of the diet inhibited the incidence of mammary tumours in rats treated with 7,12-dimethylbenz[α]anthracene (Yamamoto et al., 1987). These workers attributed the anticarcinogenic effects of dietary algae to the moderate sulfated ester content of the polysaccharide fractions from *P. tenera* and other seaweeds. More recently, a variety of antimutagenic compounds have been identified from a methanol–acetone extract of *P. tenera* such as the carotenoids β-carotene and lutein as well as chlorophyll a (Okai et al., 1996). Clearly, the well established antioxidant capacity of carotenoid pigments as quenchers of singlet oxygen and peroxyl or alkoxyl free radicals may play a role in the antimutagenic and/or anticarcinogenic effects of these compounds in vitro and in vivo. Indeed, Cho et al. (1997) demonstrated that methanol extracts from a variety of Korean seaweeds, including *P. tenera*, reduced the mutagenicity of aflatoxin in the Ames assay and inhibited the growth of human gastric and colon carcinoma cell lines in culture. We previously reported that a 1-butanol soluble dulse extract exhibited hydroxyl and stable free radical scavenging activities, as well as inhibition of TBARS and conjugated diene production in a linoleic acid emulsion system as indicators of the efficacy of the dulse extract to provide protection against oxidative stress in vitro, and potentially against the proliferation of cancer cells as demonstrated in the present study (Yuan et al., 2005).

The underlying mechanisms of the antiproliferative effects of dietary seaweeds are likely as varied as the chemistry of the various secondary metabolites involved (e.g. products of the shikimic acid pathway) as well as reflecting the metabolism of the particular cell line under study. For example, flavonoids such as the flavan-3-ols, which include the catechins found in green tea as well as a variety of edible red Japanese algae (e.g. epigallocatechin-gallate (EGCG) and epicatechin; Yoshie et al., 2000) are noted to inhibit telomerase activity in a cell free system as well as HT29 colon cancer cells and monoblastoid leukemia cells, resulting in cell death (Naasani et al., 1998). Telomerase activity is vital for the maintenance of the 3′ ends of telomeres and is present in 80–90% of human cancers, but is absent from normal cells (Eitsuka et al., 2004). Thus, if the 3′ ends of telomeres are not maintained, cell death will eventually occur. In studies with HeLa cells, Cutter et al. (2001) reported that EGCG inhibited cell growth associated with

the inhibition of cell surface NADH oxidase, a hydroquinone oxidase which is absent or decreased on the surface of normal cells. Thus, given the demonstrated reducing activity and polyphenol content of the Grade 1 and 2 dulse extracts in the present study as well as the presence of secondary metabolites of the shikimic acid pathway in dulse from other locations, the antiproliferative activity of the dulse extracts against HeLa cells may potentially involve the mechanisms discussed above.

In conclusion, the greater reducing activity of the grade 2 dulse confirmed the requirement for increased endogenous antioxidant activity due to greater UV-exposure during growth versus grade 1 dulse. That the two grades of dulse were similar in total polyphenol content, inhibition of AAPH-induced lipid peroxidation and inhibition of HeLa cell proliferation, suggests that the antioxidant and antiproliferative activities of dulse consist of more than reducing activity alone. The AAPH-induced lipid peroxidation results confirmed that the antioxidant capacity of dulse is hydrophilic in nature. Further studies will identify if the antioxidant and antiproliferative activities of dulse were associated with MAAs derived from the shikimic acid pathway, or if these effects were due to other shikimic acid pathway products such as phenolic acids or flavonoids. Confirming the presence of shikimic acid pathway derived antioxidant molecules in Canadian dulse will contribute to the further study of natural antioxidants, and the potential use of MAAs in cosmetics and functional foods for their sunscreen/UV-protection, antioxidant and antiproliferative activities.

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