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Mycosporine-like amino acid composition of the edible red alga, *Palmaria palmata* (dulse) harvested from the west and east coasts of Grand Manan Island, New Brunswick

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ABSTRACT

The oxygen radical absorbance capacity (ORAC), antiproliferative activities and mycosporine-like amino acid (MAA) profiles of methanol extracts from two grades of dulse harvested from locations varying in UV-exposure (west vs east coasts of Grand Manan Island, NB) were determined in the present study. MAAs confirmed by LC/MS in both grades 1 (low-UV) and 2 (high-UV) dulse were palythine, shinorine, asterina-330, palythinol and porphyra-334; usujirene was present only in grade 2 dulse. ORAC values of grade 1 and 2 dulse extracts were 36.42 and 38.78 μmol Trolox/g extract. B16-F1 murine skin melanoma cell proliferation was inhibited ($p < .05$) by 68.5% and 91.9% by grade 1 and 2 dulse extracts at 6.0 mg/mL. The antiproliferative efficacy of grade 2 dulse was greater ($p < .05$) than grade 1 from 0.375 to 6.0 mg/mL. MAA differences between the grade 1 and 2 dulse extracts likely influenced the antiproliferative efficacies, despite the similar ORAC values.

1. Introduction

Red (*Rhodophyceae*; R), brown (*Phaeophyceae*; B) and green (*Chlorophyceae*; G) seaweeds have an ancient history as foodstuffs in the diets of Pacific (Indonesia, Philippines, Maori of New Zealand, Hawai'i) and Asian cultures (China, Japan, Korea) and even to some extent in Iceland, Wales as well as the Canadian and U.S. Maritimes as recently reviewed (Yuan, 2008). This is not surprising since many edible marine algae can be good sources of protein (i.e. red algae such as *Grateloupia filicina, Porphyra tenera*, *Palmaria palmata*); long-chain polyunsaturated fatty acids such as eicosapentaenoic acid (*P. tenera*, *Porphyridium* sp. (R)); soluble and insoluble dietary fibers (i.e. *G. filicina*, *Chondrus crispus* (R), *Ulva lactuca* (G), *Eisenia bicyclis* (B), *Hijikia fusiformis* (B)) and minerals, in particular iodine (*P. palmata*, *Laminaria* sp. (B), *Fucus vesiculosus* (B); Athukorala et al., 2003; Yuan, 2008). Moreover, a medicinal or functional food role for edible algae in the treatment of chronic disease, namely breast cancer, can be found as early as approx. 1534 B.C. in the ancient Egyptian 'Ebers Papyrus' (Teas, 1981). Indeed, animal model studies have demonstrated inhibitory effects of edible red, green and brown algae against mammary (Teas, Harbison, & Gelman, 1984; Yamamoto, Maruyama, & Moriguchi, 1987), intestinal (Yamamoto & Maruyama, 1985) and skin carcinogenesis (Higashi-Okai, Otani, & Okai, 1999; Yamamoto, Maruyama, Takahashi, & Komiyama, 1986). Mechanisms underlying algal anticarcinogenic effects are thought to include: antimutagenicity of brown and red algal extracts against cancer inducing agents (Cho, Rhee, & Park, 1997); enhanced antioxidant enzyme activity and reduced lipid peroxidation in livers of rats given a breast carcinogen and fed on brown algae (Maruyama, Watanabe, & Yamamoto, 1991). Moreover, antioxidant and/or antimutagenic effects of edible algae have been demonstrated in rodent models of colon and skin carcinogenesis via suppression of tumor initiation (Higashi-Okai et al., 1999). The antioxidant capacity of algae is comprised of not only labile molecules such as L-ascorbate and glutathione (GSH) but also, more stable constituents such as carotenoids, tocopherols, flavan-3-ols, phenolic acids, lignans, phlorotannins as well as mycosporine-like amino acids (MAAs; Athukorala et al., 2003; Dunlap, Masaki, Yamamoto, Larsen, &

Abbreviations: 4-DG, 4-deoxygadusol; AAPH, 2,2′-azo-bis(2-amidinopropane)dihydrochloride; ACN, acetonitrile; DMEM, Dulbecco's modified Eagle's medium; ESI-MS, electrospray ionisation mass spectrometry; FBS, foetal bovine serum; GSH, glutathione; LC/MS, liquid chromatography/mass spectrometry; MAA, mycosporine-like amino acid; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; ORAC, oxygen radical absorbance capacity; PAR, photosynthetically active radiation; PBS, phosphate-buffered saline

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Karube, 1998; Dunlap & Yamamoto, 1995; Yuan, Bone, & Carrington, 2005).

An effective endogenous antioxidant system is of vital importance to intertidal organisms such as algae, to provide protection against oxidative stress associated with daily tidal fluctuations and thereby, tissue desiccation, as well as UV-irradiation which would vary with water depth and turbidity (Karsten & Wiencke, 1999), geographic location (Karsten et al., 1998), time of year (Aguilera, Bischof, Karsten, & Hanelt, 2002) and topographic features such as steep cliffs (Yuan, Carrington, & Walsh, 2005). Thus, it is not surprising that algal levels of antioxidants such as GSH, ascorbate, carotenoids and tocopherols are typically low during Winter and Spring months, and increase during Summer and Fall (Aguilera et al., 2002; Burritt, Larkindale, & Hurd, 2002; Yuan, 2007) coincident with an increase in photosynthetically active radiation (PAR, 400–700 nm) and thereby UVB (280–320 nm) and UVA (320–400 nm) exposure. The MAAs, characterised by a cyclohexenone or -hexenimine core conjugated with the nitrogen moiety of an amino acid (Fig. 1), are synthesised by the shikimic acid pathway via 3-dehydroquinic acid and 4-deoxygadusol (4-DG), a known strong antioxidant (Dunlap, Masaki, Yamamoto, Larsen, & Karube, 1997; Shick & Dunlap, 2002). These algal secondary metabolites, which are unique to the *Rhodophyceae*, have absorbance maxima between 310 and 360 nm (Karsten & Wiencke, 1999; Shick & Dunlap, 2002). A UV-absorbing sunscreen protective role for these compounds in red algae (e.g. *P. palmata*, *P. tenera* and *Devaleraea ramentacea*), corals (e.g. *Palythoa tuberculosa*) and marine species such as sea squirts (*Lissoclinum stellatus*) and krill (*Euphausia superba*) can be inferred from the overlap of the absorbance maxima with UVA and B wavelengths as well as the absorbance maxima for DNA. Further evidence of a UV-absorbing sunscreen function for MAAs was reported by Karsten and Wiencke (1999), that total MAA levels in *P. palmata* samples from shallow waters (1.5 m depths) were greater than those from deeper waters (3 m depths); moreover, the MAA contents of *P. palmata* and *D. ramentacea* samples collected prior to the Spring break-up of ice-cover were reduced compared to those of samples collected in the Summer (Aguilera et al., 2002).

Previously, this laboratory reported that butanol extracts of *P. palmata* were effective hydroxyl and hydrophilic stable free radical scavengers, as well as inhibitors of linoleic acid emulsion lipid peroxidation and HeLa cell proliferation in vitro (Yuan, Bone et al., 2005; Yuan, Carrington et al., 2005). These *P. palmata* extracts also demonstrated reducing activity, which was enhanced in samples exposed to greater amounts of UV-irradiation during growth (Yuan, Carrington et al., 2005). Support for the hypothesis that MAAs play a role in the endogenous antioxidant activity of red algae, such as *P. palmata*, is derived from an earlier study by Dunlap and Yamamoto (1995) demonstrating that mycosporine-glycine was consumed in a reaction with the hydrophilic stable free radical 2,2′-azo-bis(2-amidinopropane)dihydrochloride (AAPH), whereas the imino-carbonyl MAAs such as shinorine, porphyra-334, palythine, asterina-330 and palythinol remained at initial levels in a *P. tuberculosa* extract. A major impediment to further research investigating the potential bioactivities of MAAs is the absence of commercially available standards to confirm the identity of MAAs based on LC retention times and molar extinction coefficients alone, or LC/MS methods. More recent reports have elucidated the MS spectra of the principal MAAs by electrospray ionisation mass spectrometry (ESI-MS; Whitehead & Hedges, 2002). While data exists on the MAA profiles of *P. palmata* harvested from the northern latitudes of Norway (78°55.5′N, 11°56.0′E; Aguilera et al., 2002; Karsten & Wiencke, 1999; Karsten et al., 1998), there are no data for samples further south, such as from Grand Manan Island, New Brunswick (44°40.0′N, 66°45.0′W). Thus, the objectives of the present study were to elucidate the MAA profiles of two grades of dulse harvested from Grand Manan Island locations differing in UV-irradiation during growth; and to evaluate the antioxidant activity and influence of the dulse extracts on murine skin melanoma (B16-F1) cell proliferation in vitro.

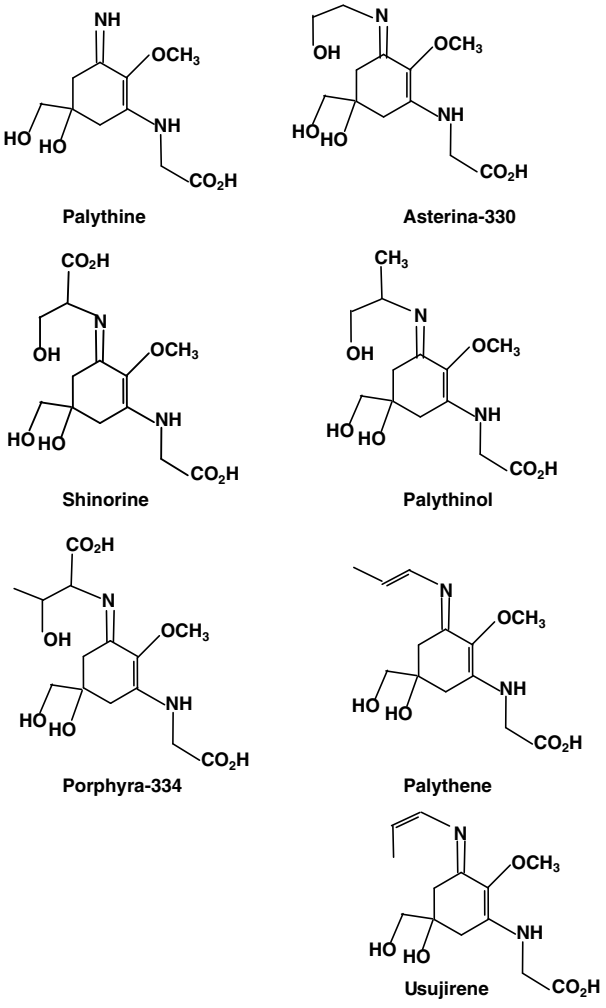

Fig. 1. Mycosporine-like amino acids (MAAs) identified in the grade 1 and 2 *P. palmata* (dulse) specimens harvested from the west and east coasts of Grand Manan Island, NB.

2. Materials and methods

2.1. Materials

Certified organic (Organic Crop Improvement Association International) dulse (*P. palmata*) harvested in Spring 2005 was obtained from Atlantic Mariculture Ltd. (Grand Manan Island, New Brunswick, Canada). Grade 1 dulse originated from Dark Harbour, Grand Manan Island, NB, while grade 2 dulse was harvested from the 'Passage' side of Grand Manan Island as previously described by Yuan, Carrington et al., 2005. Solvents (formic acid, methanol, acetonitrile (ACN)) were purchased from Fisher Scientific (Mississauga, ON) and were all of HPLC grade. Murine skin melanoma (B16-F1) cells were obtained from American Type Culture Collection (ATCC CRL-6323, Manassas, VA). Cell culture medium components (Dulbecco's modified Eagle's medium (DMEM), L-glutamine, sodium bicarbonate, foetal bovine serum (FBS) and phosphate-buffered saline (PBS)); 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT); 6-hydroxy-

2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and fluorescein Na-salt were purchased from Sigma–Aldrich Canada (Oakville, ON). 2,2′-Azo-bis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Wako Chemicals USA Inc. (Richmond, VA). Water (H_2O) used as a solvent or in mobile phases was purified using a Millipore Super-Q Water System (Millipore, Bedford, MA).

2.2. Preparation of algal extracts

Freeze-dried samples of each grade of sun-dried dulse were ground to pass through a 40 mesh screen using a Wylie Mill. Samples (0.250 g) were soaked overnight, 4 °C, in 3 mL H_2O according to Carreto, Carignan, and Montoya (2005) prior to the addition of 14 mL methanol and sonication for 5 min in an ice-bath (Branson 5210 sonicator, 40 kHz, VWR International, Mississauga, ON). Following centrifugation (4 °C, 15 min, 3000 rpm) and collection of the supernatant, the residue was extracted twice more with 17 mL methanol and the supernatants collected and pooled. Extracts were then concentrated in 12 mL aliquots by rotary evaporation, 40 °C, and freeze-drying in 25 mL flasks. Dulse extracts were solubilised in 400 μL H_2O followed by sonication (5 min), and passed through a 0.20 μ syringe filter into a glass vial with an insert (Chromatographic Specialties Inc., Brockville, ON) for HPLC and LC/MS analyses, or use in the ORAC and cell proliferation studies below.

2.3. HPLC analyses

Mobile phases A and B were 0.2% formic acid in H_2O and 0.2% formic acid in ACN, respectively, which had been passed through a 0.20 μ nylon membrane filter. HPLC analyses were conducted using an Agilent 1100 Series LC equipped with a diode array detector (Agilent Technologies, Mississauga, ON) using HP Chemstation Software (Agilent Technologies). MAA separations were performed using an Inertsil ODS-3 column, 5 μ, 4.6 × 250 mm (GL Sciences, Inc. USA, Torrance, CA) with 2 μL injections of the dulse extracts and elution using a gradient of 100% mobile phase A from zero to 7 min, 30% A + 70% mobile phase B from 7 to 15 min, and 20% A + 80% B from 15 to 20 min, with a flow rate of 1.0 mL/min and column temperature at 30 °C. Detection was monitored at 330 nm and absorption spectra (191–400 nm) recorded for preliminary identification of compounds in the absence of commercially available standards (Karsten & Wiencke, 1999; Shick & Dunlap, 2002).

2.4. MS analyses

Positive ion electrospray ionisation (ESI) was used to determine the molecular masses of the separated MAAs. ESI-MS conditions were optimised to produce protonated molecules $[M+H]^+$ using an Agilent LC/MSD XCT Plus ion trap mass spectrometer (Agilent Technologies) with LC/MSD Trap Security Pack 1.0 Software. The ESI source was operated at a capillary temperature of 350 °C and a capillary voltage of 113.5 V. N_2 was used as the sheath gas at a flow rate of 12.0 mL/min. MS scans were performed from *m/z* 50 to 600. MAAs were identified using published data for formula weights and $[M+H]^+$ values (Whitehead & Hedges, 2002).

2.5. Oxygen radical absorbance capacity (ORAC-fluorescein) assay

The oxygen radical absorbance capacity (ORAC) of the grade 1 and 2 dulse extracts was determined according to Dávalos, Gómez-Cordovés, and Bartolomé (2004) as modified by Kitts and Hu (2005). Briefly, aliquots of dulse extract or the reference Trolox antioxidant (0.1–0.8 nmol) were mixed with 60 nM fluorescein in a black 96-well plate (Nunc, Fluorescent microtitre plate) in the presence of 75 mM phosphate buffer, pH 7.0, and incubated at 37 °C, 10 min. AAPH was then added to make a final concentration of 12 mM in individual wells, and the plate shaken for 10 s prior to the initiation of fluorescence readings with an excitation wavelength of 485 nm and emission of 527 nm (Fluorskan Ascent FL, Labsystems); fluorescence readings were taken every 60 s up to 1 h. Fluorescence data were transformed according to the method of Dávalos et al. (2004); dulse extract ORAC values were expressed as μmol Trolox/g extract.

2.6. Cell proliferation studies

B16-F1 murine skin melanoma cells (ATCC CRL-6323) were grown in 75 cm^2 flasks in DMEM containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose and 10% FBS. Cells were seeded into 96-well plates at a density of 2×10^4 per well and allowed to attach overnight at 37 °C, 5% CO_2 with a fully humidified atmosphere. The grade 1 and 2 dulse extracts in H_2O were diluted with PBS prior to addition to plated cells at final concentrations of 0.188, 0.375, 0.75, 1.5, 3.0 and 6.0 mg/mL; cells were incubated in the dulse extract-containing medium for 24 or 48 h. A set of solvent controls were included for each 96-well plate. Following the incubations, traces of seaweed extract were removed by washing the cells twice with PBS; then 100 μL of fresh medium introduced + 10 μL of 5 mg/mL MTT dissolved in PBS to determine the effects of the dulse extracts on B16-F1 cell proliferation (Mosmann, 1983). Cells were then incubated for 4 h, followed by the addition of 100 μL 10% (w/v) SDS to each well in order to solubilise the product of MTT cleavage, and incubated once more overnight. The following day, the absorbance at 570 nm, with a reference wavelength of 690 nm, was read using a Multiskan Ascent Microplate Reader (Thermo Labsystems, Franklin, MA). The percent inhibition of cell proliferation was calculated as follows:

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

Each concentration of the respective dulse extracts was assayed in triplicate.

3. Statistics

All data are expressed as means ± SD of triplicate experiments. One-way analysis of variance (ANOVA; SPSS 10.0 for Windows; SPSS Inc., Chicago, IL) was used to test for differences between *P. palmata* extract concentrations. Where differences did exist, the source of the differences at a $p \leqslant .05$ significance level was identified by the Student–Newman–Keuls multiple range test. Student's *t*-test for independent samples was used to test for differences between *P. palmata* grades at a significance level of $p \leqslant .05$ where appropriate (SPSS).

4. Results

The presence of five MAAs was confirmed in the grade 1 dulse methanol extract: palythine, shinorine, asterina-330, palythinol and porphyra-334 from the LC peak UV-absorbance maxima and ESI/MS positive ion spectra (Table 1 and Fig. 2A). The grade 1 dulse extract peak which eluted at 11.28 min exhibited a λ_{max} of 324 nm and an $[M+H]^+$ ion of 200 which did not correspond to any known MAA in the literature (Fig. 2A). In comparison to the grade 1 dulse extract, the grade 2 dulse exhibited an additional MAA at 11.33 min with a λ_{max} at 356 nm and $[M+H]^+$ ion of 285.1 identifying this sixth MAA as usujirene, the *cis*-isomer of palythene (Table 1 and Fig. 2B).

Table 1
Retention times, UV-absorbance maxima and ESI/MS values for the mycosporine-like amino acids identified in the grade 1 and 2 *P. palmata* (dulse) specimens[a]

Peak	Retention time (min)	Mycosporine-like amino acid	λ_{max}	$[M+H]^+$	Mass
1	4.16	Palythine	320	245.0	244.11
2	4.60	Shinorine	332	333.1	332.12
3	4.86	Asterina-330	330	289.1	288.13
4	8.18	Palythinol	330	303.1	302.15
5	9.64	Porphyra-334	332	347.1	346.14
6	11.33	Usujirene	356	285.1	284.0

[a] Palythine, shinorine, asterina-330, palythinol and porphyra-334 were present in both grades 1 and 2 dulse, whereas usujirene was present in grade 2 dulse only.

Table 2
HPLC peak area percentages of grade 1 and 2 *P. palmata* (dulse) mycosporine-like amino acid (MAA) profiles monitored at 330 nm

Peak	Mycosporine-like amino acid	% peak area of total MAAs	
		Grade 1 dulse	Grade 2 dulse
1	Palythine	26.6	24.1
2	Shinorine	5.6	7.0
3	Asterina-330	5.8	2.2
4	Palythinol	6.4	4.2
5	Porphyra-334	55.6	58.9
6	Usujirene	–	3.6

The LC peak area percentages of individual MAAs for the two grades of dulse, recorded at 330 nm, indicated that for palythine, shinorine and palythinol, grade 1 = 2; for asterina-330, grade 1 > 2; for prophyra-334, grade 1 < 2; while usujirene was present in grade 2 dulse, but absent from grade 1 dulse (Fig. 2 and Table 2). The predominant MAA in both grade 1 and 2 dulse was porphyra-334 at >55% of total MAA peak area; while palythine was the next most predominant at ca. 25% in grade 1 and 2 dulse; with shinorine, asterina-330, palythinol and usujirene each present at <10% total MAA peak area at 330 nm. It should be noted that using the peak area values recorded at 330 nm likely underestimated the amount of usujirene in the dulse 2 extract since the observed absorbance is a function of concentration and molar extinction coefficient (ε). Moreover, λ_{max} and ε are both noted to increase with double-bond conjugation as observed with usujirene (λ_{max} 356 nm; Fig. 1). The data obtained from monitoring the LC chromatograms at 330 nm does enable comparisons between the grade 1 and 2 dulse specimens for the MAAs which are common to both algae: palythine, shinorine, asterina-330, palythinol and porphyra-334.

The ORAC values of the grade 1 and 2 dulse methanol extracts were 36.42 ± 1.92 and 38.78 ± 1.40 μmol Trolox/g extract, respectively; thus, there was no difference between grade 1 and 2 dulse ORAC values.

The grade 1 and 2 dulse methanol extracts inhibited B16-F1 cell proliferation in a dose-dependent manner during the 24 and 48 h incubation periods (Fig. 3A and B). For the grade 1 dulse extract, after 24 h incubation, inhibition of B16-F1 cell proliferation was observed with only the highest concentration, 6.0 mg/mL ($p < .001$); after 48 h, inhibition of cell proliferation was observed at 3.0 and 6.0 mg/mL ($p < .001$) concentrations (Fig. 3A). For the grade 2 dulse extract, after 24 h incubation, inhibition of B16-F1 cell proliferation was observed from 1.5 to 6.0 mg/mL ($p < .001$) in a clear dose-dependent manner; after 48 h, inhibition was observed starting from 0.375 mg/mL and was maximal at 6.0 mg/

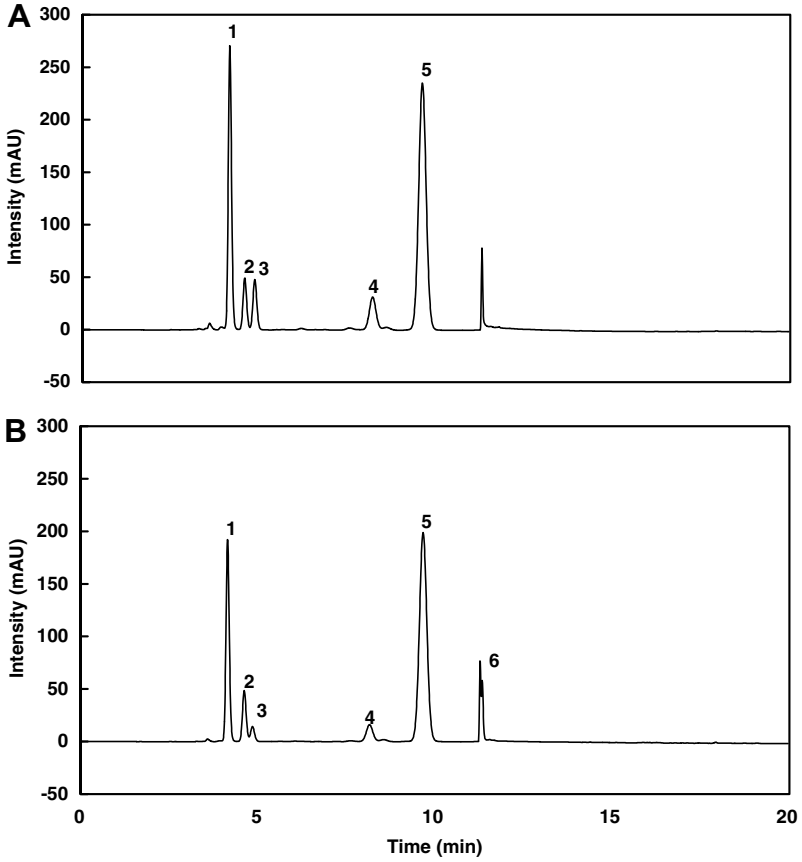

Fig. 2. HPLC-DAD chromatograms of mycosporine-like amino acid profiles of grade 1 (A) and 2 (B) *P. palmata* (dulse) specimens monitored at 330 nm. 1 = palythine, 2 = shinorine, 3 = asterina-330, 4 = palythinol, 5 = porphyra-334, 6 = usujirene.

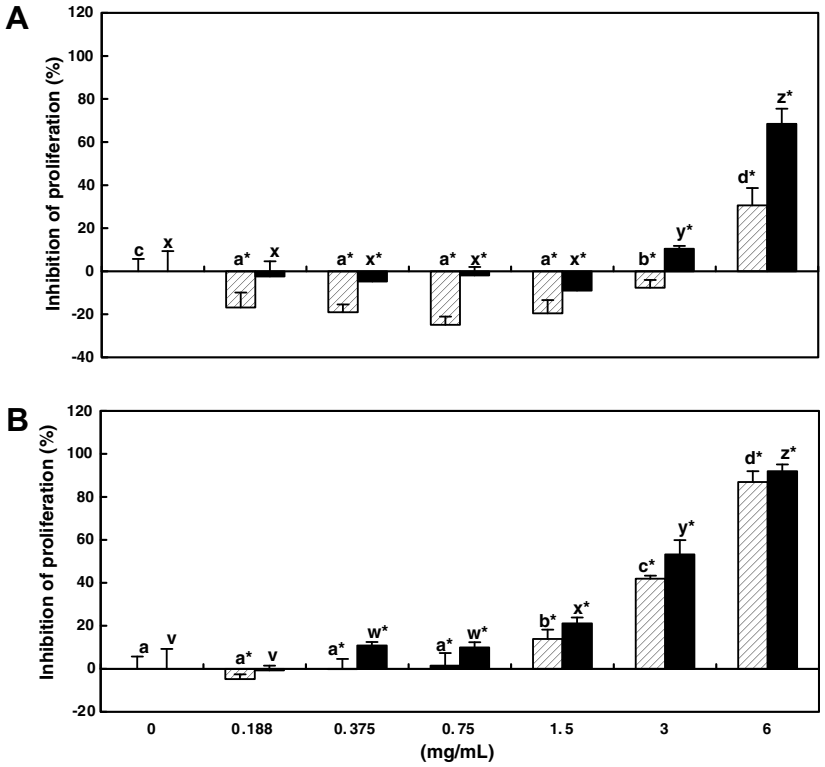

Fig. 3. Influence of grade 1 and 2 *P. palmata* (dulse) methanol extracts on cell proliferation of B16-F1 murine skin melanoma cells. (A) Grade 1 dulse; (B) grade 2 dulse. ▨ 24 h, ■ 48 h incubation. [a,b,c,d]Significant difference ($p < .05$) between concentrations of *P. palmata* extracts after 24 h incubation; [v,w,x,y]Significant difference ($p < .05$) between concentrations of *P. palmata* extracts after 48 h incubation; [*]Significant difference ($p < .05$) between grade 1 and 2 *P. palmata* extracts at a particular dose and incubation period.

mL ($p < .001$) concentrations (Fig. 3B). The inhibitory effect of the grade 2 dulse extract on B16-F1 cell proliferation was greater ($p < .001$) than that of the grade 1 extract from 0.375 to 6.0 mg/mL (Fig. 3A and B).

5. Discussion

The present study is the first to report the MAA profiles of two commercially available *P. palmata*, dulse, specimens harvested from two temperate zone locations varying in UV-radiation exposure on Grand Manan Island, New Brunswick, Canada. We extend these findings by reporting the oxygen radical absorbance capacities (ORAC) and B16-F1 murine skin melanoma cell antiproliferative efficacies of the aqueous methanol extracts from these dulse specimens. We have previously reported that 1-butanol soluble extracts of dulse specimens collected from these same two locations differed in reducing activities, but exhibited similar polyphenol contents, antioxidant activities against AAPH-induced linoleic acid emulsion oxidation as well as antiproliferative efficacies in HeLa cell culture (Yuan, Carrington et al., 2005). However, in order to study aqueous, water-soluble constituents such as the MAAs, the extraction procedure requires an aqueous methanol solvent system in contrast to our previous protocol which was focused on flavonoid, hydroquinone and lignan glycoside extraction (Yuan, Bone et al., 2005; Yuan, Carrington et al., 2005). An aqueous methanol extraction of lyophilised dulse tissue, coupled with reverse-phase HPLC allowed the separation and identification of the highly polar MAAs palythine, shinorine, asterina-330, porphyra-334, medium polarity palythinol, and the low polarity usujirene in the present study.

The MAA profile of dulse harvested in Spitsbergen, Norway (78°55.5′N, 11°56.0′E) has been reported to contain (in descending order): porphyra-334, an unknown absorbing at 357 nm (possibly usujirene), palythine, palythene, mycosporine-glycine, palythinol, shinorine and asterina-330 (Karsten et al., 1998). In other reports, the principal MAAs of dulse harvested from the same location in Norway have been (in descending order) palythine, porphyra-334 and shinorine (Karsten & Wiencke, 1999) and shinorine, palythine and porphyra-334 (Aguilera et al., 2002). More recently, Carreto et al. (2005) reported that the MAA profile of *Palmaria decipiens* (R) harvested in the South Shetland Islands, Antarctica (62°49.5′S, 59°42.5′W) consisted of porphyra-334, shinorine, usujirene, palythinol, palythine, palythene and an MAA absorbing at 335/360 nm. In the present study, for both the grade 1 and 2 dulse specimens, when monitored at 330 nm, the predominant MAAs were porphyra-334 at 55% and 59% total peak area and palythine at 27% and 24% total peak areas, respectively, with much lower amounts of shinorine (6–7% total peak area), palythinol (4–6%) and asterina-330 (2–6%); usujirene (4%) was present only in the grade 2 dulse. As mentioned above, we have likely underestimated the concentration of usujirene in the grade 2 dulse extract, due to the greater λ_{max} and ε associated with the double-bond conjugation of this compound. Exposure of red algae to PAR-, UVA- and UVB-irradiation affects not only tissue antioxidant capacity (Aguilera et al., 2002; Yuan, Carrington et al., 2005) and MAA profiles (Karsten & Wiencke, 1999), but also tissue light harvesting photosynthetic pigments, namely chlorophyll a, R-phycoerythrin and phycocyanin, which are noted to be present in concentrations inversely proportional to the UV-radiation exposure of samples during ice-cover vs sea-ice break-up (Aguilera et al., 2002), or high cliff-shade vs low lying sunny shores, as was the case with the dark reddish-purple grade 1 and paler purple 2 dulse specimens from the east and west coasts of Grand Manan in the present and previous studies (Yuan, Carrington et al., 2005). Moreover, in transplan-

tation studies, Karsten and Wiencke (1999) demonstrated that dulse tissue levels of porphyra-334 were enhanced with increased PAR exposure, which was further increased by UVA-irradiation, which overlaps with the UV-absorbance maxima of this MAA at 334 nm. On the other hand, dulse tissue levels of shinorine were only slightly influenced by increased PAR, but were greatly increased with the addition of UVA-irradiation which overlaps with the UV-absorbance maxima of this MAA at 332 nm. Similarly, dulse tissue levels of palythine were only slightly influenced by increased PAR and PAR + UVA, but were increased with the inclusion of UVB-irradiation overlapping with the UV-absorbance maxima of this MAA at 320 nm (Karsten & Wiencke, 1999). In the present study, the grade 2 dulse exhibited a slightly higher porphyra-334 content vs grade 1 dulse, which coincided with the greater exposure of the grade 2 dulse to PAR + UV-irradiation during growth on the sunny, low lying shores of east Grand Manan Island. Moreover, the MAA profile of the grade 2 dulse alone included usujirene, which is characterised by an UV-absorbance maximum at 356 nm, in the UVA range. Thus, differences in the PAR and UV-irradiation exposure between the grades 1 and 2 dulse likely resulted in the different MAA profiles observed in the present study.

Differences between the MAA profiles of the grade 1 and 2 dulse specimens harvested in a temperate zone herein, compared to dulse harvested from polar regions in Norway, may also relate to the stronger solar radiation at lower latitudes. For example, the UVA and B irradiances in Norway range from 15 to 20 W m^{-2} and 0.8 to 1.2 W m^{-2}, respectively; in Bremerhaven (70 km south) from 20 to 35 W m^{-2} and 1.5 to 2.0 W m^{-2} and Spain (warm temperate) from 35 to 50 W m^{-2} and 2.0 to 2.5 W m^{-2}, respectively (Karsten et al., 1998). Thus, at lower latitudes, the light path for solar radiation is not only shorter, but UVA-irradiation predominates, which may influence the predominance of one MAA over the others. Other environmental factors which may influence dulse MAA profiles include the presence or absence of ice-cover (Aguilera et al., 2002) and water depth (Karsten & Wiencke, 1999; Karsten et al., 1998); i.e. MAA profiles of polar region dulse samples have been reported to contain a majority of shinorine in specimens from 3 to 4 m depths under ice-cover or after sea-ice break-up (Aguilera et al., 2002), palythine in specimens from 2 to 3 m depths (Karsten & Wiencke, 1999) and porphyra-334 in specimens from 0 to 2 m depths (Karsten et al., 1998). Thus, similar to the latter report, the MAA profiles of the temperate zone dulse samples from 0 to 2 m depths herein contained a majority of porphyra-334. The absence of mycosporine-glycine from the grade 1 and 2 dulse specimens in the present study, as well as the relatively low levels of shinorine, may be due to the conversion of these two molecules into other MAAs via the shikimic acid pathway (Carreto et al., 2005; Shick & Dunlap, 2002). 4-DG is the main precursor in MAA biosynthesis, as its conjugation with glycine results in the formation of mycosporine-glycine which can then be reacted with serine to yield shinorine, as proposed by Carreto et al. (2005). If mycosporine-glycine is reacted with threonine, the product is porphyra-334; mycosporine-glycine can be converted into palythine via more than one pathway; shinorine can be converted into palythinol and subsequently into asterina-330; whereas, usujirene may be derived from porphyra-334 (Carreto et al., 2005).

The aqueous methanol extracts of the grade 1 and 2 dulse specimens exhibited similar oxygen radical absorbance capacity (ORAC) values, indicating roughly equivalent efficacies in quenching peroxyl radicals derived from the hydrophilic azo dye AAPH as a free radical initiator. Moreover, an earlier report from our lab demonstrated that 1-butanol soluble extracts of grade 1 and 2 dulse specimens harvested from the same locations also exhibited equivalent inhibitory effects against AAPH-initiated lipid oxidation of a linoleic acid emulsion system (Yuan, Carrington et al., 2005). In other work to elucidate the free radical scavenging efficacy of MAA-containing extracts, Dunlap and Yamamoto (1995) reported that extracts containing an 8:1 ratio of asterina-330:palythine exhibited little inhibition of AAPH-induced phosphatidylcholine peroxidation (0.9 mM total MAAs), whereas those containing a 9.6:1 ratio of porphyra-334:shinorine (0.9 mM) or 2.4:1 ratio of shinorine:mycosporine-glycine (1.2 mM) exhibited moderate inhibition, and that containing a 27.9:9:4.3:1 ratio of mycosporine-glycine:palythine:palythinol:asterina-330 (1.6 mM total MAAs) exhibited the greatest inhibition of AAPH-induced phosphatidylcholine peroxidation in vitro. When the UV-absorbance and HPLC profiles of these MAA-containing extracts were further investigated from 0 to 10 h incubation, asterina-330, porphyra-334, shinorine, palythine and palythinol exhibited little to no decreases, whereas, mycosporine-glycine decreased steadily over time (Dunlap & Yamamoto, 1995). Further studies with purified MAAs have indicated weak, but dose-dependent inhibition of AAPH-induced phosphatidylcholine peroxidation by shinorine, and strong, dose-dependent inhibition by mycosporine-glycine and the shikimic acid pathway MAA precursor, 4-DG (Dunlap et al., 1998). Others have determined that usujirene strongly inhibits oxidation of a linoleic acid emulsion over several days at 40 °C (Nakayama, Tamura, Kikuzaki, & Nakatani, 1999). Taken together, these lines of evidence suggest that the ORAC antioxidant activities of the grade 1 and 2 dulse extracts herein are attributable to the efficacy of shinorine and usujirene in our extracts.

Mechanistic studies have hypothesised that the greater susceptibility of mycosporine-glycine to oxidation, and thereby, its efficacy as an antioxidant is related to its lower redox potential and greater ability to act as a reducing agent in donating an electron to stabilize a free radical (Dunlap & Yamamoto, 1995). More recently, mycosporine-glycine was reported to inhibit singlet oxygen ($^1O_2$) mediated type II photosensitization in not only eosin Y-mediated red blood cell hemolysis, but also methylene blue-mediated lipid peroxidation of soybean hypocotyl microsomes (Suh, Lee, & Jung, 2003). The rate constant for $^1O_2$ quenching by mycosporine-glycine was reported as 5.6×10^7 M^{-1} s^{-1}, greater than that of α-tocopherol (15.4×10^7 M^{-1} s^{-1}, Suh et al., 2003). Moreover, these workers reported that direct incubation of mycosporine-glycine with eosin Y under visible light irradiation degraded the MAA into at least three major and several minor unidentified products. The $^1O_2$ quenching efficacy of mycosporine-glycine may conceivably be associated with the carbonyl group of the cyclohexenone chromophore and some resonance stabilization by the carbon ring double-bond structure. On the other hand, the inhibition of AAPH-derived peroxyl radical-induced lipid peroxidation by mycosporine-glycine may be associated with hydrogen abstraction from the cyclohexenone ring at C-4 or C-6 with resonance stabilization provided as described above. For usujirene, Nakayama et al. (1999) hypothesised that the strong antioxidant activity of this MAA may be associated with hydrogen abstraction from the cycloheximine ring at C-4, C-6 or the methylene group at C-9 of the glycine residue with resonance stabilization provided by the conjugated double-bonds of the *cis*-unsaturated chain at C-11 on the double-bonded nitrogen in series with the carbon ring double-bond structure. Likewise, it is plausible that the weak antioxidant activity of shinorine against AAPH-derived peroxyl radical lipid oxidation is associated with hydrogen abstraction from the cycloheximine ring at C-4, C-6 or the methylene group at C-9 of the glycine residue with some resonance stabilization from the carbon ring double-bond structure.

A factor which may play a role in whether an MAA exhibits antioxidant activity is the relative acidity of these compounds, and thereby, the degree of dissociation in solution. For example, mycosporine-glycine, palythine, palythinol, asterina-330 and usujirene are only weakly acidic, while both shinorine and porphyra-334 are strong acids. Thus, antioxidant activity, or the absence

thereof, observed at pH 8.0 in the presence of 0.1 mM EDTA (Dunlap & Yamamoto, 1995), may not be equivalent to studies conducted at pH 7.0, as in the present and in other studies (Nakayama et al., 1999; Suh et al., 2003). Moreover, existing reports evaluating the antioxidant efficacy of MAAs have not studied whether or not synergistic interactions occur between different MAAs in solution, or if intermolecular interactions occur between specific MAAs. Thus, the ability to synthesise specific MAAs from precursor compounds, or to use preparative LC methods to isolate MAAs in larger quantities is key to the further progress of these areas of inquiry.

The grade 1 and 2 dulse extracts exhibited dose-dependent inhibition against proliferation of the murine skin melanoma B16-F1 cell line in the present study. The EC_{50} values calculated for grade 1 and 2 dulse extract inhibition of B16-F1 cell proliferation after 48 h incubation were 5.3 and 3.2 mg/mL, respectively. Similarly, we previously reported that the 1-butanol soluble extract of grade 2 dulse exhibited greater efficacy than that from grade 1 dulse in the inhibition of human epithelial cervical cancer HeLa cell proliferation (Yuan, Carrington et al., 2005). Studies evaluating the biological activity of MAAs have documented UV-protective roles for MAAs in not only micro- and macroalgae, but also coral reefs, as well as in the eyes of teleost Japanese medaka fish (*Oryzias latipes*) and in the eggs of sea urchins (*Strongylocentrotus droebachiensis*; Mason, Schafer, Shick, & Dunlap, 1998; Shick & Dunlap, 2002). Few studies have been conducted to date with mammalian cell culture or in vivo feeding trials to determine the uptake, absorption, tissue deposition or potential bioactivity of dietary MAAs. When *Mastocarpus stellatus* (R; rich in shinorine) was fed at 5% w/w to SKH-1 hairless mice (*Mus musculus*), dietary MAAs were absent from the eyes, skin and liver tissues, but were present in the small and large intestinal tissues as well as in the feces (Mason et al., 1998). On the other hand, these workers were able to demonstrate the concentration dependent and apparently saturable, active-transport of shinorine by human skin carcinoma A431 cells in culture. While the albino hairless, euthymic and immunocompetent SKH-1 mouse is an animal model used in many studies evaluating the effects of phytochemicals on carcinogen- or UV-induced skin cancer, this rodent model has also been demonstrated to exhibit unique regional distribution and frequency of gastrointestinal endocrine cells which may influence the physiological and absorptive functions of the digestive tract (Ku, Lee, Lee, & Park, 2002). Thus, it remains unclear whether mammals absorb dietary MAAs or if particular MAAs are better absorbed than others depending on their polarity or charge.

Previous studies demonstrating anticarcinogenic activity of edible red algae include the work of Yamamoto and coworkers which demonstrated that diets containing 2% w/w of *P. tenera* inhibited not only 1,2-dimethylhydrazine-induced intestinal tumors in male Sprague–Dawley rats (Yamamoto & Maruyama, 1985), but also 7,12-dimethylbenz[α]anthracene-induced mammary tumors in female Sprague–Dawley rats (Yamamoto et al., 1987). These same workers reported that dietary *P. tenera* as well as the i.p. injection of the hot water extract from this alga inhibited the growth of implanted sarcoma-180 ascites cells in mice (Yamamoto et al., 1986). More recently, Cho et al. (1997) reported that methanol extracts of the red algae *P. tenera* and *Gelidium amansii* exhibited dose-dependent inhibition of the growth of AGS human gastric adenocarcinoma and HT-29 colon cancer cells. The methanol extracts of *P. tenera* and *G. amansii* were also demonstrated to inhibit aflatoxin B_1 and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenicity in the Ames assay (*Salmonella typhimurium* TA100; Cho et al., 1997). Taken together, these lines of evidence suggest that the water-soluble MAAs identified in the grade 1 and 2 dulse extracts may have a role in the antiproliferative effects of these extracts when incubated with the murine skin melanoma B16-F1 cells. The greater efficacy of the grade 2 dulse extract was likely associated with the presence of the least polar MAA, usujirene, which was unique to this specimen; the lower polarity of usujirene may have allowed the passive uptake of this MAA into the murine skin melanoma cell membranes enhancing the antiproliferative effects of this extract.

In conclusion, the aqueous methanol extracts from grade 1 and 2 dulse specimens exhibited differences in their MAA profiles attributable to the high cliff-shade vs low lying sunny shores, respectively, during growth in a temperate zone. The similar ORAC activities of the grade 1 and 2 dulse extracts likely reflected the efficacy of the MAAs shinorine and usujirene as quenchers of AAPH-derived peroxyl radicals. The greater antiproliferative effects of the grade 2 dulse extract likely reflects the absorption and bioactivity of the least polar MAA usujirene into murine skin melanoma cell membranes. Further work with single and combinations of purified MAAs will elucidate the antioxidant activities of these unique UV-sunscreen molecules to further our understanding of the bioactivities of these red algal constituents. Moreover, studies of the digestion, absorption and tissue deposition of dietary MAAs in a well characterised rodent animal model will help to determine whether mammals are able to benefit from the potential bioactivities of these compounds in the processed and/or functional food industries.

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