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Research Article

Mycosporine-Like Amino Acids (MAAs) Profile of Two Marine Red Macroalgae, *Gelidium* sp. and *Ceramium* sp.

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Abstract

Macroalgae have evolved different strategies to mitigate the damaging effects of solar ultraviolet radiation (UVR), including accumulation of photoprotective compounds such as mycosporine-like amino acids (MAAs). MAAs are secondary metabolites, synthesized by a large variety of organisms including macroalgae, phytoplanktons, cyanobacteria, lichen, fungi and some marine animals. MAAs act as photoprotectants and antioxidants. In the present investigation, MAAs profile of methanolic extracts of two marine red algae *Gelidium* sp. and *Ceramium* sp., collected from their natural environment, was studied. High-performance liquid chromatography (HPLC) and UV-Vis spectrometry analyses were used to reveal different MAAs profile in the extracts obtained from these two red algae. The MAAs isolated by HPLC were identified as shinorine (λ_{max}= 333.5 nm), porphyra-334 (λ_{max}=332.3 nm) and palythine (λ_{max}=317.9 nm) having retention times (RT) 1.26, 2.12 and 3.64 min, respectively, in the extract obtained from *Gelidium* sp. Similarly, shinorine (λ_{max}=332.3 nm), porphyra-334 (λ_{max}=333.5 nm) and palythinol (λ_{max}=332.5 nm) with RT 1.27, 2.13 and 4.61 min, respectively, were identified in the extract obtained from *Ceramium* sp. This study revealed that *Gelidium* sp. and *Ceramium* sp. could serve as potential source for economical exploration of MAAs in pharmaceutical sciences.

Keywords: *Gelidium*; *Ceramium*; Mycosporine-like amino acids; Shinorine; Porphyra-334; UVR.

Abbreviations: HPLC: High-performance liquid chromatography; MAAs: Mycosporine-like amino acids; UVR: Ultraviolet radiation; UV-A: Ultraviolet-A; UV-B: Ultraviolet-B, AS: Asterina-330, MG: Mycosporine-glycine, PE: Palythene, PL: Palythinol, PR: Porphyra-334, PT: Palythine, SH: Shinorine

Introduction

The continuous accumulation of anthropogenically released atmospheric pollutants, such as chlorofluorocarbons (CFCs), chlorocarbons (CCs), organobromides (OBs) and reactive nitrogen species (RNS) including nitric oxide (NO), nitrous oxide (N_2O) and peroxynitrite (ONOO) in the stratosphere has resulted in the depletion of ozone layer which provides shield against ultraviolet radiation (UVR; 100-400 nm) (Crutzen, 1992; Lubin and Jensen, 1995; Ravishankara *et al*., 2009). The process of ozone depletion has been reported at mid latitude and especially in the Antarctic where ozone levels declined by more than 70% during late winter and early spring in the polar vortex (Smith *et al*., 1992). The decreased density of ozone layer results in increased intensity of harmful ultraviolet-B radiation (UV-B; 280-315 nm) reaching on the Earth's surface (Hofmann and Deshler, 1991; Madronich *et al*., 1998; Sahoo *et al*., 2005). UV-B radiation fluence reaching temperate and equatorial latitudes could be 1.5-2 Wm^{-2}, as compared with 50-60 Wm^{-2} of ultraviolet-A radiation (UV-A; 315-400 nm) and 500 Wm^{-2} of photosynthetically active radiation (PAR; 400-700 nm) under clear weather conditions (Castenholz, 1997).

Although very small proportion of UV-B radiation reaches on the Earth (< 1% of total irradiance), it is highly energetic and has the potential to affect all living

organisms by damaging a number of physiological, biochemical and molecular processes (Gao *et al*., 2007; Lesser, 2008; Sinha *et al*., 2008). Phototrophic organisms are especially prone to negative effects of UVR due to their obligation for harvesting light energy to produce chemical energy and reducing power for fixation of carbon. UVR may reach upto 70 meter into sea water column depending on the organic matter, flora and fauna of aquatic ecosystems, and can negatively affect the benthic photosynthesizers including cyanobacteria, phytoplankton and macroalgae (Smith *et al*., 1992; Häder *et al*., 2007). Thus, UVR has the potential to exert deleterious effects on photosynthetic terrestrial and aquatic organisms, thereby affecting the productivity of ecosystems (Karentz *et al*., 1991a; Vincent and Roy, 1993; Williamson, 1995; Sinha and Häder, 1996).

UVR can directly affect macromolecules such as DNA and proteins whereas indirect damages are caused due to production of reactive oxygen species (ROS) (Karentz *et al*., 1991b; Vincent and Neale, 2000). However, many photosynthetic microorganisms have developed certain mitigation strategies such as avoidance, scavenging of UV-induced ROS by enzymatic and non-enzymatic antioxidants (Middleton and Teramura, 1994; Singh *et al*., 2013), and screening by certain UV-absorbing/screening compounds like MAAs and scytonemin that counteract the deleterious effects of UVR (Karentz *et al*., 1991c; Dunlap and Shick, 1998; Sinha *et al*., 1998). Besides this, repair of UV-induced damage of DNA by photoreactivation and excision repair and resynthesis of proteins are also important mechanisms to prevent UV-induced photodamage (Britt, 1995; Kim and Sancar, 1995).

In this context, MAAs have obtained much consideration for their photoprotective role as these compounds have been reported in cyanobacteria, macroalgae, phytoplankton and various marine animals such as arthropods, rotifers, molluscs, fishes, cnidarians, tunicates, poriferans, nemertineans, echinodermates, platyhelminthes, polychaetes, bryozoans and protozoans (Sinha *et al*., 2007). This also indicates the evolutionary significance of these compounds during the course of evolution by conserving them. It has been found that MAAs provides protection from UVR not only in their producers but also to primary and secondary consumers through the food chain (Helbling *et al*., 2002). MAAs are small (<400 Da), colorless, water-soluble compounds composed of a cyclohexenone or cyclohexenimine chromophore conjugated with the nitrogen substituent of an amino acids or its imino alcohol (Singh *et al*., 2008; Richa and Sinha, 2015). Generally, the ring system contains a glycine subunit at the third carbon atom. Some MAAs also contains sulfate esters or glycosidic linkages through the imine substituent (Böhm *et al*., 1995; Wu *et al*., 1997).

The strong UV-absorption maxima of MAAs between 310 and 362 nm (Cockell and Knowland, 1999), their high molar extinction coefficients (ε = 28,100- 50,000 M^{-1} cm^{-1}) and photostability in both fresh and sea water in the presence of photosensitizers support the hypothesis that MAAs have a photoprotective role (Whitehead and Hedges, 2005). Differences in the absorption spectra of MAAs are due to variations in the attached side groups and nitrogen substituent. They are present intracellularly in many marine and freshwater organisms (Bandaranayake, 1998; Gröniger *et al*., 2000; Shick and Dunlap, 2002; Rezanka *et al*., 2004). UV exposure is essential for biosynthesis of MAAs, but some studies have suggested that other factors, including osmotic stress may also be involved in regulation of MAAs synthesis (Portwich and Garcia-Pichel, 1999).

In the cyanobacterium, *Cylindrospermum stagnale* sp. PCC 7417, a new gene cluster (*mylA-E*) putatively responsible for MAAs biosynthesis has been reported which is homologous to MAAs gene clusters from *Anabaena variabilis* sp. ATCC 29413 (*ava_3855-3858*) and *Nostoc punctiforme* (*NpR5598-5600* and *NpF5597*) (Katoch *et al*., 2016). The two main enzymes involved in MAAs biosynthesis are *O*-methyltransferase (*O*-MT) encoded by genes Ava_3857, NpR5599, Myl B and 2-demethyl-4-deoxygadusol (DDG) synthase encoded by genes Ava_3858, NpR5600, Myl A, respectively. Heterologous expression of *mylA-E* in *Escherichia coli* resulted in the production of mycosporine-lysine and the novel compound mycosporine-ornithine (Katoch *et al*., 2016).

MAAs are true 'multipurpose' secondary metabolites and due to potent UV-screening and antioxidant properties, it has widespread cosmeceuticals and pharmaceutical applications (Carreto and Carignan, 2011). Besides UV-photoprotective activity, these compounds also act as osmoprotectants, and, probably, anti-desiccant (Oren and Gunde-Cimerman, 2007). To date, more than 35 MAAs have been reported from marine organisms such as cyanobacteria, algae, and vertebrate and invertebrate animals (Becker *et al*., 2016). MAAs are most abundant in Rhodophyta (red algae) compared to Chlorophyta (green algae) and Phaeophyta (brown algae) macroalgal species (Karentz *et al*., 1991c; Hoyer *et al*., 2001; Kräbs *et al*., 2002; Sinha *et al*., 2007).

The red algae are one of the oldest groups of eukaryotic algae and also one of the largest, with about 5,000-6,000 species of mostly multicellular, macroscopic, marine algae, including many notable seaweeds. Their dominant pigment is phycoerythrin, which gives off rich shade of red color. Due to their sessile nature, macroalgae are exposed to elevated levels of PAR and UV radiation in their natural habitat. They cannot avoid radiation stress by migration to less affected areas like microalgae. The accumulation of

MAAs has been previously reported for red algae as an effective strategy to counteract the damaging effects of UVR (Sinha *et al*., 2000). Some studies have reported synthesis and accumulation of various types of MAAs in *Gelidium* sp. and *Ceramium* sp. MAAs such as PT, AS and SH were found to be present in *Gelidium amansii* (Nakamura *et al*., 1982). Karsten *et al*. (1998a) showed presence of various MAAs in different species of *Gelidium*. AS, PT, PL, PR and SH were found to be present in *Gelidium* sp. (Karsten *et al*., 1998a). MAAs such as PT, AS, PL, PR and SH were reported in *Gelidium pusillium* and PT, AS, PL, PR and SH were screened in *Gelidium sesquipedale* (Karsten *et al*., 1998a). Gröniger *et al*. (2000) reported presence of SH in *Gelidium latifolium*. Similarly Karsten *et al*. (1998) reported various MAAs in different species of *Ceramium.* MG, PT, PL, PR and SH were found in *Ceramium* sp., PT, PR and SH were screened in *Ceramium nodulosum* and MG and SH were reported in *Ceramium rubrum* (Karsten *et al*., 1998a). As bioprospection and screening of MAAs constitute important aspect of its economic utility, the prime objective of the present study was to identify the MAAs present in two marine red macroalgae *Gelidium* sp. and *Ceramium* sp. collected from Western coast (Arabian sea) of India.

Materials and Methods

Geographical Region and Collection of Algal Material

The two marine macroalgae belonging to the division Rhodophyta namely *Gelidium* sp. (Class Rhodophyceae, Order Gelidiales, Family Gelidiaceae) and *Ceramium* sp. (Class Rhodophyceae, Order Ceramiales, Family Ceramiaceae) were collected from their natural habitat, i.e., the rocky sea shore of Arabian sea near sunset point at Dwarka beach (22°23' N, 68°97' E), Gujarat, India (**Fig. 1a, b**). *Gelidium* sp. is a thalloid red alga reaching around 0.79-16 inches in size. Species of *Gelidium* are found both in littoral region and in deeper water. Branching is irregular or occurs in rows on either side of the main stem (**Fig. 1c**). Similarly, *Ceramium* sp. is reddish brown in colour, prostrate and branched with terminal pincer-like structures at the end of each of the branches. Species of *Ceramium* occur abundantly between tide-level, as well as in deeper water. *Ceramium* sp. grows up to 10 inches in size (Fritsch, 1945) (**Fig. 1d**).

Fig. 1: Photographs showing (a) Map of sampling area, (b) Sunset point at Dwarka, Gujarat, India (collection site) (c) Morphology of *Gelidium* sp., and (d) Morphology of *Ceramium* sp.

Extraction and Partial Purification of Mycosporine-Like Amino Acids (MAAs)

Extraction and partial purification of MAAs was done from weighed samples following the method of Sinha *et al*. (1999). The samples were homogenized in 5 ml 100% methanol (HPLC grade, SRL Chem, New Mumbai, India) with the help of mortar and pestle and incubated overnight at 4°C. After extraction, aliquots were centrifuged (5000 *g* for 10 min), and methanolic extracts were subjected to spectroscopic analysis between 200 -700 nm in a UV-Vis double beam spectrophotometer (U-2910, 2J1-0012, Hitachi, Tokyo, Japan). The raw spectra were transferred to computer and peaks were analyzed by UV-solution software (Version-2.2, Hitachi, Tokyo, Japan). After scanning the methanolic extracts, supernatants were evaporated to dryness at 40°C, and the extracts were redissolved in 2 ml double distilled water. After centrifugation at 10000 *g* for 10 min, aqueous solution was filtered through a sterilized syringe driven filter (0.2 µm; Axiva Biotech., New Delhi), and transferred to new Eppendorf tubes. The partially purified MAAs were further subjected to HPLC analysis.

High Performance Liquid Chromatography Analysis

The partially purified MAAs were analyzed by HPLC system (Water 2998 with PDA detector, 515 PUMP, autoinjector 717 plus, Milford, USA) equipped with Empower-2 software. The system has an ODS-2 (RP18) column (Water, Spherisorb analytical column, 5µm, 4.6 × 250 mm diameter, Ireland) fitted with guard (4.6 × 10 mm). A 50 µl sample was injected into the column and separation of MAAs was achieved by using 0.02% acetic acid as mobile phase with a flow rate of 1 ml min^{-1}. The identification of MAAs were performed as described earlier (Sinha *et al*., 1999, Kannaujiya *et al*., 2014).

Results

The present study investigated the presence of photoprotective compound, i.e., MAAs in two red algae *Gelidium* sp. and *Ceramium* sp. by spectroscopic analysis. Once the presence was confirmed, MAAs were further characterized by HPLC analysis based on the similarity of retention times (RT) and absorption spectrum of individual MAA with known standards.

Spectroscopic Analysis

The absorption spectra of methanolic extracts of both red algae showed a peak between 300-350 nm, which suggested the presence of MAAs in these organisms. The absorption maxima for MAAs were found to be at 320 nm and 332 nm for *Gelidium* sp. and *Ceramium* sp., respectively. In addition to the peaks for MAAs, methanolic extracts of *Gelidium* sp. and *Ceramium* sp. also showed the peaks for chlorophyll *a* (420 and 660 nm), carotenoids (460 nm) and phycobiliprotein (620 nm) (Fig. 2a, b).

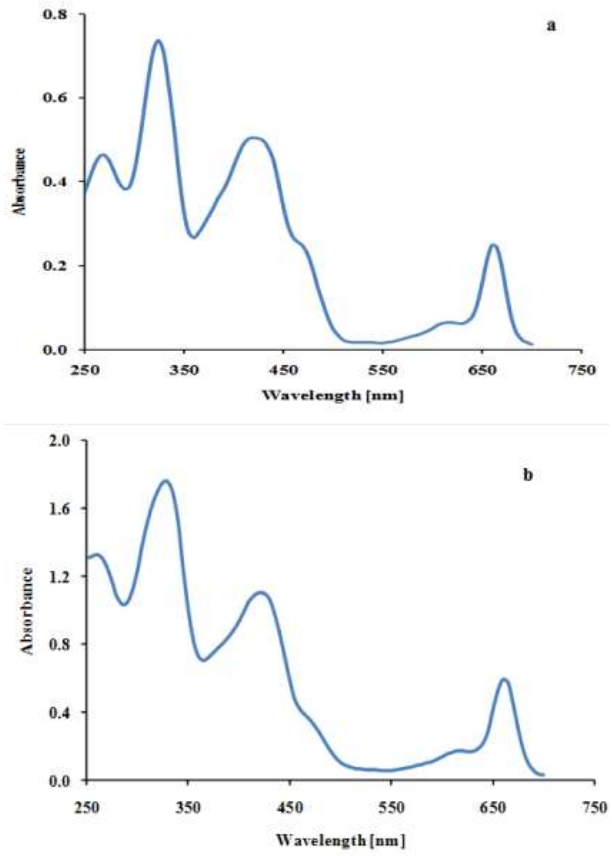

Fig. 2: Absorption spectra showing the presence of MAAs, chlorophyll *a*, carotenoids and biliproteins in (a) *Gelidium* sp. and (b) *Ceramium* sp.

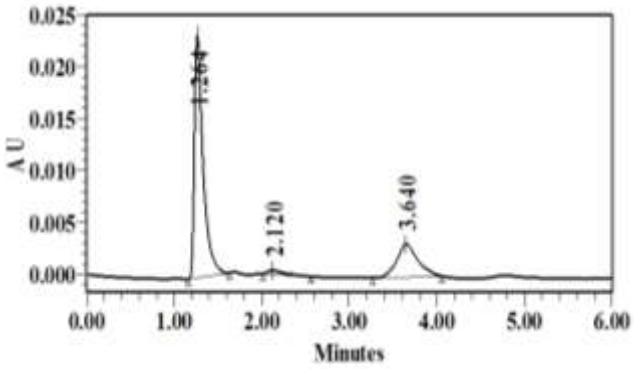

Fig. 3: HPLC chromatogram of partially purified MAAs from *Gelidium* sp., showing the typical peak at retention times of 1.26 min (shinorine), 2.12 min (porphyra-334), and 3.64 min (palythine).

Separation and Identification of MAAs by High-Performance Liquid Chromatography (HPLC)

The separated MAAs were identified based on the similarity of RT and absorption spectrum with standards. The chromatogram obtained from HPLC analysis confirmed the presence of three MAAs in *Gelidium* sp. (Fig. 3). Three MAAs in *Gelidium* sp. were identified as shinorine (λ_{max}= 333.5 nm; Fig. 4a), porphyra-334 (λ_{max}= 332.3 nm; Fig. 4b) and palythine (λ_{max}=317.9 nm; Fig. 4c)

with RT of 1.26, 2.12 and 3.64 min, respectively. Similarly, aqueous solution of MAAs obtained from *Ceramium* sp. was found to contain three MAAs (Fig. 5) which were identified as shinorine (λ_{max}= 332.3 nm; Fig. 6a), porphyra-334 (λ_{max}= 333.5 nm; Fig. 6b) and palythinol (λ_{max}= 332.5 nm; Fig. 6c) with RT of 1.27, 2.13 and 4.61 min, respectively.

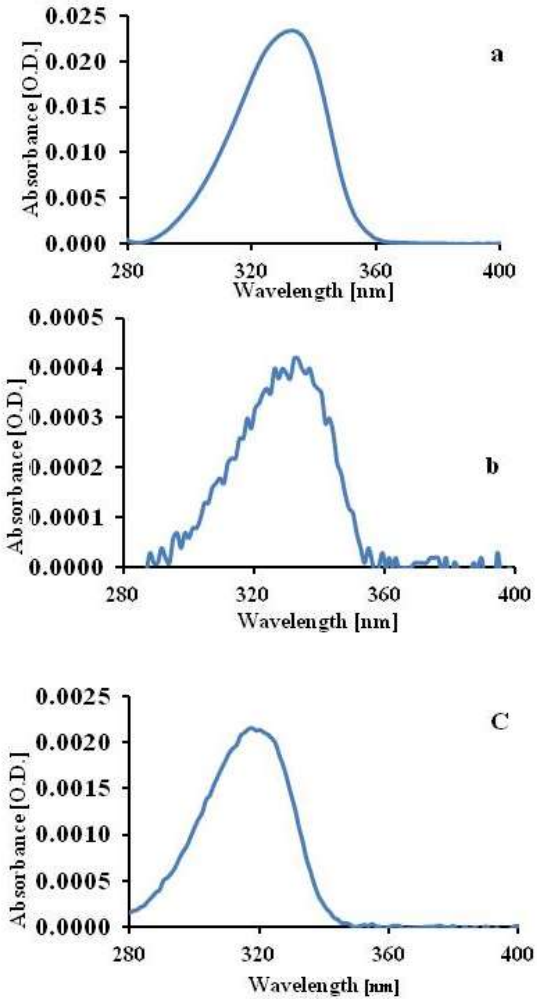

Fig. 4: Absorption spectra of purified MAAs from *Gelidium* sp. (a) shinorine, (b) porphyra-334 and (c) palythine, as separated by HPLC.

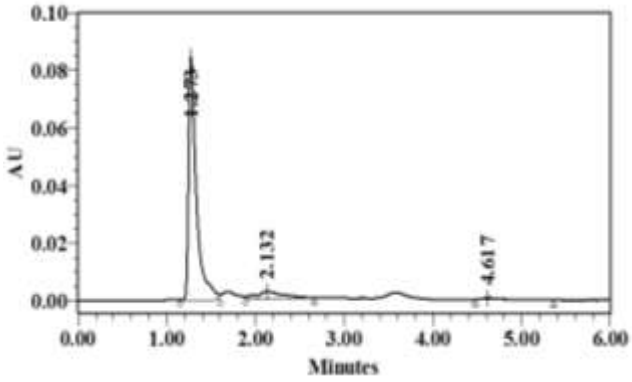

Fig. 5: HPLC chromatogram of partially purified MAAs from *Ceramium* sp., showing the typical peak at retention times of 1.27 min (shinorine), 2.13 min (porphyra-334), and 4.61 min (palythinol).

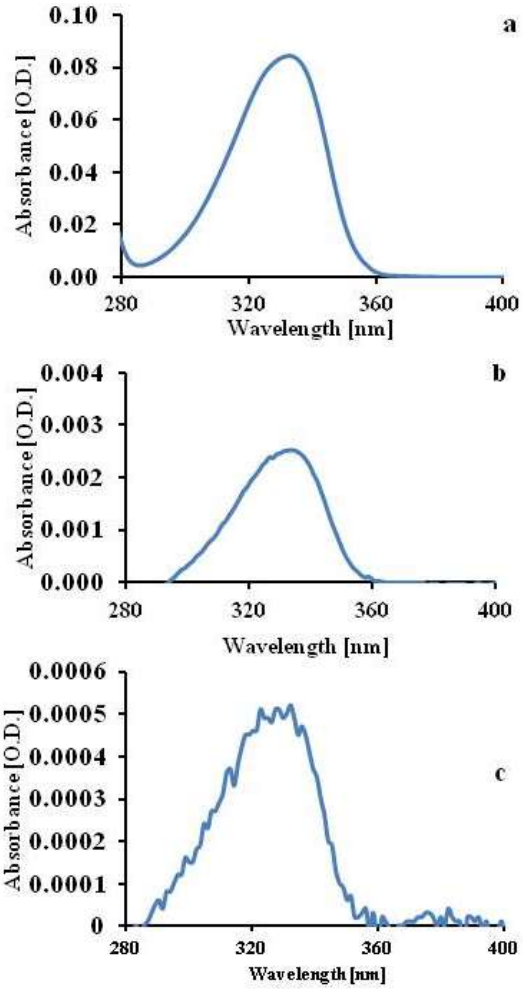

Fig. 6: Absorption spectra of purified mycosporine-like amino acids from *Ceramium* sp. (a) shinorine, (b) porphyra-334, and (c) palythinol, as separated by HPLC.

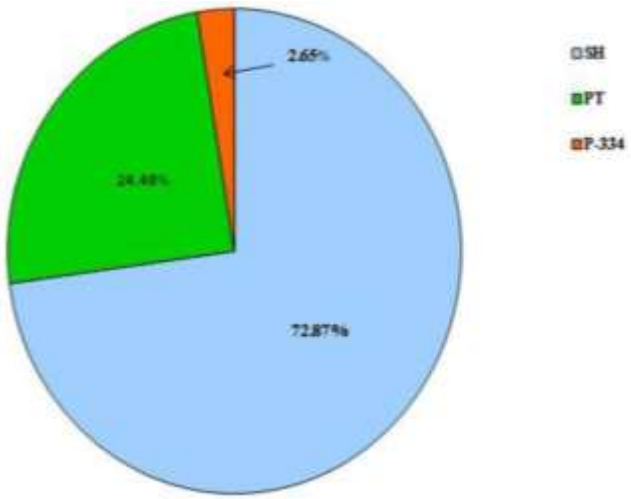

Fig. 7: Percentage distribution of MAAs (shinorine, palythine and porphyra-334) in *Gelidium* sp.

Once it became evident that these two red macroalgae produces MAAs, we calculated the percentage distribution of shinorine, porphyra-334 and palythine in *Gelidium* sp. and shinorine, porphyra-334 and palythinol in *Ceramium*

sp., respectively. Fig. **7** represents the percentage distribution of MAAs in *Gelidium* sp. The results indicate that shinorine was contributing maximum (72.87%) and porphyra-334 was contributing minimum (2.65%) to the total MAAs in *Gelidium* sp. However, percentage distribution of palythine was found to be 24.48% to the total MAAs in *Gelidium* sp. Fig. 8 represents the percentage distribution of MAAs in *Ceramium* sp. with maximum contribution of shinorine (89.30%) and minimum contribution of palythinol (1.50%) to the total MAAs. Porphyra-334 was contributing 9.21% to the total MAAs in *Ceramium* sp. We quantified different MAAs in the two algal species in terms of the peak areas from their respective chromatograms as shown in Fig. 9 and Fig. 10.

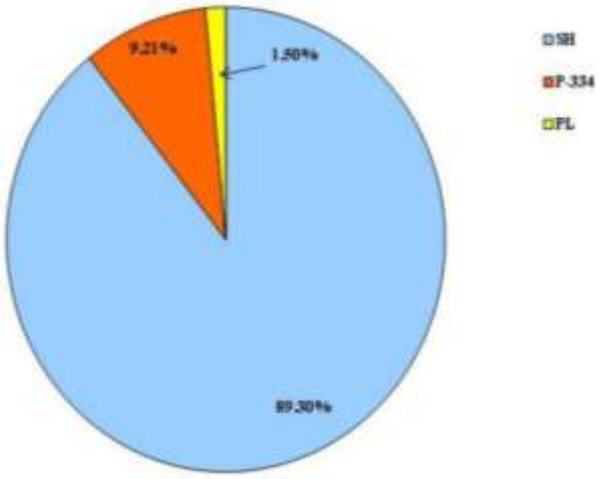

Fig. 8: Percentage distribution of MAAs (shinorine, porphyra-334 and palythinol) in *Ceramium* sp.

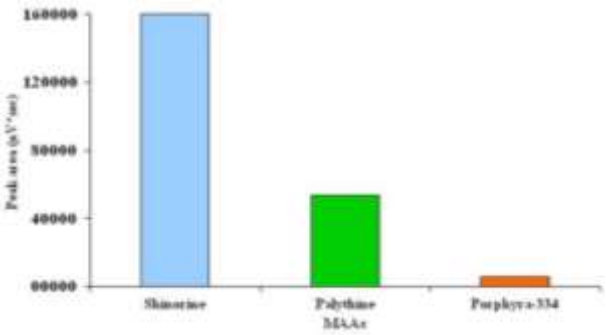

Fig. 9: Quantification of MAAs in *Gelidium* sp. in terms of peak area of chromatogram.

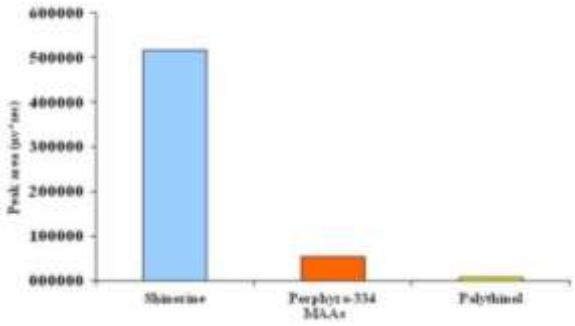

Fig. 10: Quantification of MAAs in *Ceramium* sp. in terms of peak area of chromatogram.

Discussion

Macroalgae, inhabiting the coastal areas of the world, are exposed to varying intensities of solar radiation, including harmful UV-B and UV-A radiation (Lüning, 1985). UV-B radiation is highly detrimental to all living organisms, including photosynthetic organisms inhabiting in marine, freshwater and terrestrial ecosystems. There is an evidence that UV-B irradiation results in the formation of thymine dimers in macroalgae such as *Porphyra umbilicalis* and *Ceramium rubrum* (= *Ceramium virgatum*), which results in several mutations in the genome of organisms (Sinha *et al*., 2001). UV-B radiation was found to induce mutation in tetraspores of the Rhodophyte *Gracilariopsis lemaneiformis* (Fu *et al*., 2013).

Phycobilisomes (PBSs), which are the major light harvesting complexes in red algae, were found to be sensitive to UVR (Schmidt *et al*., 2010a, b). Among different phycobiliproteins, phycocyanin was found to be most sensitive and it is rapidly destroyed by UV-B irradiation (Rinalducci *et al*., 2006). Thus, UVR negatively affects the process of carbon fixation which is reflected in terms of decreased photosynthetic efficiency. The passive screening of UVR by MAAs is one of the photoprotective mechanisms developed by several macroalgae against UVR (Sinha *et al*., 2007; Häder, 1997; Sinha *et al*., 1998, 2000; Carreto and Carignan, 2011; Kannaujiya *et al*. 2014; Richa *et al*., 2016). MAAs are known to absorb UVR and dissipate energy as harmless heat, and were estimated to prevent 3 out of 10 photons of UV-B radiation from hitting cytoplasmic targets in a cell (Garcia-Pichel *et al*., 1993).

The concentration of MAAs in macroalgae reflects the dose of UVR which is received in their natural habitats (Karsten *et al*., 1998b), and based on the levels of MAAs in various macroalgae, three patterns of MAAs distribution is commonly seen among macroalgae: (a) high initial MAA content and no increase during light treatment, (b) low MAA content with an increase during light treatment, and (c) no initial MAA and no induction during light treatment. However, in this study we did not test the induction of MAAs after light treatment due to limitation of the sample, and therefore, it is not possible to report the MAAs pattern for studied organisms. The red macroalga *Chondrus crispus*, harvested from the subtidal zone showed an increase in the number and amount of MAAs (Karsten *et al*., 1998b). Initial high levels but no significant increase in the MAAs content was found in the upper intertidal rhodophyte *Porphyra umbilicalis* (Gröniger *et al*., 1999). Similarly, no *in vivo* induction of MAAs was detected after exposure to either UV alone or in combination with PAR in the marine red alga *Gracilaria cornea* (= *Hydropuntia cornea*), which possesses a very high amount of naturally occurring MAAs having an absorption maximum at 334 nm (Sinha *et al*., 2000). Shinorine and porphyra-334 are the most

common MAAs reported in macroalgae in species collected from tropical to polar waters (Karentz *et al*., 1991c; Karsten *et al*., 1998a; Helbling *et al*., 2004; Sinha *et al*., 2007), and in this study these two MAAs were also found in studied algae.

Recently, two novel MAAs named as catenelline and prasiolin were reported in marine red macroalgae *Catenella repens* and terrestrial green macroalga *Prasiola calophylla*, respectively (Hartmann *et al*., 2015, 16). A higher content of porphyra-334 in several red algae plays an important role in photoprotection (Conde *et al*., 2000). The algae of median tidal area are more efficient in producing UV-B screening compounds to cope up with its deleterious effect (Van De Poll *et al*., 2003). Algal extracts are valuable source of bioactive compounds such as antioxidants (polyphenols), tocopherols (vitamin E), ascorbate (vitamin C), glutathione (GSH), MAAs, pigments (carotenoids, chlorophylls, phycobilins, scytonemin), carbohydrates, proteins, lipids, minerals, and natural plant growth compounds (auxins, gibberellins, cytokinins) which possess antibacterial, antiviral, antifungal, antioxidative, anti-inflammatory, and antitumor properties and various potential of industrial applications (Rastogi and Sinha, 2009; Rajneesh *et al*., 2016).

MAAs are considered as natural photoprotectants that may be biotechnologically exploited in various ways. There is evidence that presence of MAAs (such as shinorine, porphyra-334 and mycosporine-glycine) protect human fibroblast cells from UV irradiation and it was observed that all the three examined MAAs protect the cells from UV-induced cell death (Oyamada *et al*., 2008). However, mycosporine-glycine was found to be more effective. MAAs has potential applications in cosmetics and toiletries as a UV protectors and activators of cell proliferation. MAAs have been commercially exploited as suncare products for protection of skin and other non-biological materials, e.g., as photostabilising additives in plastics, paint and varnish (Bandaranayake, 1998).

A database has been developed on photoprotective compounds in micro- and macroalgae that can be responsible for the skin protection against solar UV radiation induced premature skin aging (photoaging). Different MAAs (mycosporine-glycine, palythine, asterina-330, palythinol, porphyra-334, shinorine, palythene) extracted from different species of macroalgae are listed in (Gröniger *et al*., 2000). A human study showed that a cream containing MAA from red alga *Porphyra umbilicalis* efficiently protects the skin against UVA-induced lipid oxidation (Schmid *et al*., 2006). A skin care product called Helioguard ® 365 that contains MAAs from the red alga *Porphyra umbilicalis* has been commercialized. These compounds may be of great value in the development of artificial sunscreens and a source for future biotechnological research. Misonou *et al*. (2003) has observed that MAAs can block the production of both 6-4 photoproduct and cyclobutane pyrimidine dimer (CPD) formation. MAA extracted from red alga *Porphyra umbilicalis* demonstrated in *in-vitro* studies a DNA-protecting effect and the viability enhancing properties (Schmid *et al*., 2006).

MAAs may also act as antioxidants. For example, the oxo-carbonyl mycosporines and porphyra-334 have antioxidant activity and prevent cellular damage resulting from UV-induced production of ROS (Carreto and Carignan, 2011). MAAs were extracted and purified from a marine red alga *Bryocladia* sp. (Kannaujiya *et al*., 2014). *In vitro* effects of hydrogen peroxide on algal MAAs with a view to illustrate their level of stability and scavenging properties against ROS were studied and it was found that isolated MAAs from *Bryocladia* sp. acted as an efficient peroxide scavenger (Kannaujiya *et al*., 2014). Antiproliferative activities of MAAs extracted from marine red macroalgae have studied. MAAs are suggested to exert pharmaceutical relevant bioactivities in the human system. Immunomodulatory effects of MAAs, shinorine and porphyra-334 have been investigated in human myelomocytic cell lines (Becker *et al*., 2016). The results from present investigation indicate that two types of MAAs shinorine and porphyra-334 could be present as photoprotective compounds in two marine red algae *Gelidium* sp. and *Ceramium* sp. Palythine and palythinol could only be present in *Gelidium* sp. and *Ceramium* sp. respectively in trace concentration.

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Conflict of interest

Authors declare no conflict of interest.

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