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Characterization of Photoprotective and Pharmaceutically Important Compounds from Cyanobacteria and Algal Assemblages from Historical Kunds of Varanasi, India

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ABSTRACT

Various cyanobacterial (Microcystis aeruginosa, Anabaena sp., Merismopedia sp., Aulosira fertilissima, Oscillatoria sp. and Gloeocapsa sp.) and algal samples were collected from four historical Kunds of Varanasi, India, and were investigated for the presence of photoprotective and pharmaceutically important compound such as mycosporine-like amino acids (MAAs). High-performance liquid chromatography (HPLC) and UV-Vis spectrophotometry analyses reveal the presence of five different MAAs, identified as porphyra-334 (λ_{max}=334 nm), palythine (λ_{max}=320 nm), palythenic acid (λ_{max}=337 nm), mycosporine-glycine (λ_{max}=310 nm) and mycosporine-methylamine-serine (λ_{max}=327 nm) having retention times (RT) of 3.62, 1.65, 4.15, 2.55 and 1.51 min respectively. These MAAs were further characterised by using Fourier Transform Infrared (FTIR) analysis. To the best of our knowledge this is the first report of its kind from different historical Kunds of Varanasi. Photoprotective MAAs may help cyanobacteria and other algal assemblage to protect themselves from deleterious radiations and in turn to colonize the historical Kunds. These MAAs containing organisms could be a valuable source of photoprotective compounds of pharmaceutical importance in developing natural sunscreens.

Keywords: Kunds; Mycosporine-like amino acids (MAAs); High-performance liquid chromatography (HPLC); Fourier Transform Infrared (FTIR) spectroscopy; Ultraviolet radiation

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INTRODUCTION

Cyanobacteria are treated as extensive biomass producers having an important role in retaining the trophic energy dynamics of an ecosystem [1,2]. They are a valuable source of several treasure natural product of economic and ecological importance. In the past few decades, increase in UV-A (315-400) and UV-B (280-315) radiations on the Earth's surface [3] have triggered enormous concern about its harmful effects on aquatic as well as terrestrial life

forms including cyanobacteria and other algae [4]. These organisms have certain defence mechanisms against harmful UV radiations which are predominantly associated with the biosynthesis of photoprotective compounds such like mycosporine-like amino acids (MAAs) and scytonemin [5-7].

MAAs and their allied group of organic compounds termed mycosporines are low molecular weight (<400 Da) and colourless water soluble compounds. These are composed of either aminocyclohexenone or an aminocyclohexinimine chromophore conjugated with the nitrogen substituent of an amino acid or its imino alcohol. There are around 40 MAAs known so far with an absorption maxima ranging from 309-365 nm [8-13]. In addition to the members of cyanophyceae, MAAs have also been reported in some Chlorophyceae [14], Bacillariophyceae [15] and Chrysophyceae [16]. MAAs have high molar extinction coefficients (ε=28,100-50,000 M^{-1} cm^{-1}) and the capacity to prevent UV-induced formation of thymine dimers [2,17]. MAAs are supposed to have antioxidant properties due to their ability to disperse absorbed energy as heat into their surroundings without producing reactive oxygen species (ROS) [18]. The link between UV radiation and nascent life is an inseparable evidence, strengthen with the corroboration for biogenicity of ~3.5 Ga old euphotic stromatolitic formations [19-25] of seemingly photosynthetically active, still UV-C bathed, microbial colonies of cyanobacteria like organisms [26,27]. Under extreme UV-radiation, oldest stromatolite fossil (~3.7 Ga) from an Eoarchean shallow marine environment, is an oldest evidence for life flourish on Earth [28]. Aquatic organisms are also affected by temperature and pH. At high temperature and low pH organisms tend to degrade before the synthesis of photoprotective compounds. In this way photoprotective compounds are affected indirectly at different temperature and pH. In the present study, various cyanobacterial and algal organisms collected from four historical Kunds of Varanasi, India, were examined for their ability to synthesise MAAs. Furthermore, these MAAs were characterised by using various techniques.

EXPERIMENTAL SECTION

Sampling Sites and Collection

Our sampling sites were located in Varanasi (25°28' N, 82°96' E), Uttar Pradesh, India. Collection of samples was done from four different ancient Kunds (water bodies) such as Pisachmochan Kund, Lolark Kund, Kri Kund and Durga Kund (Figures 1 and 2). All of these ancient and religious Kunds have their own importance and belief. Samples were filtered by using planktonic net of 10 µm mesh size (Plankton Sampling Kit, Fieldmaster®, Wildco) and were collected from January 2019 to February 2019 in gallon. These water samples were rich in cyanobacteria and other algal organisms. We collected the samples again in the month of June 2019 to check the effects of high intensity solar radiation on the organisms. Sampling sites were taped by using digital camera (Figure 2). UV-A and UV-B radiations, temperature, pH and relative humidity of the sites were recorded.

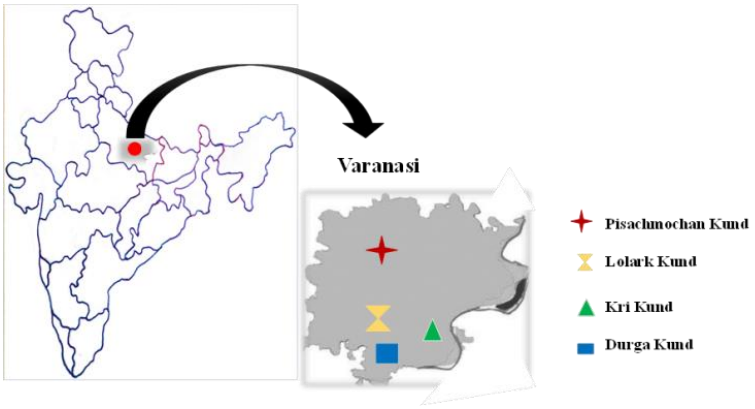

Figure 1. Sampling sites showing the location of different historical Kunds of Varanasi, India

Figure 2. Historical Kunds (sampling sites) of Varanasi, India. (A) Pisachmochan Kund, (B) Lolark Kund, (C) Kri Kund and (D) Durga Kund

Identification of Microorganisms

The collected samples were centrifuged at 6000 rpm for 15 min and pellets were repeatedly washed with sterile distilled water. Thereafter, the pellets were examined for the presence of cyanobacteria and other algal organisms by using light microscope (OLYMPUS, Model number: CX21i-TR). Photographs were taken by using Dewinter-2011 scientific digital camera and analysed with Dewinter software.

Extraction of MAAs

Samples (Water) of different Kunds were centrifuged (6000 rpm for 15 min) and the pellets were dissolved in 2 ml of 100% (v/v) methanol (HPLC-grade) and placed overnight at 4℃. After extraction, aliquots were centrifuged (6000 rpm for 10 min) and methanolic extracts were subjected to spectroscopic analysis between 200 -700 nm in a UV-Vis double beam spectrophotometer (U-2600, Shimadzu, Japan). The raw spectra were transferred to computer and peaks were analyzed by UV-probe software (Version-2, Japan). Thereafter, methanol was evaporated at 45ºC and redissolved in 80% water and 20% chloroform to separate water soluble compounds from organic compounds. It was centrifuged at 10,000 rpm for 10 min and upper layer supernatant was taken (water soluble MAAs). Before being subjected to HPLC analyses, samples were filtered through 0.2 µm pore-sized sterilized microcentrifuge syringe-driven filters (Axiva Sichem Biotech., New Delhi).

HPLC analysis of MAAs

HPLC analysis of partially purified MAAs were carried out by using HPLC system equipped with a Photodiode Array (PDA) Detector, Waters 2998, 515 HPLC pump, Nova-Pak ® C18 reverse phase guard column (4.6 × 150 mm inside diameter). A 20µL sample was injected into the HPLC column with the help of an auto-injector through a Waters 717 plus Autosampler. Wavelength was set at 330 nm in detector and scanning of wavelength from 250 to 400 nm by PDA to see the separated peaks. A 0.02% (v/v) acetic acid was used as mobile phase which eluted isocratically at a flow rate of 1 mL min^{-1}. Identification of MAAs was done by its characteristic absorption maxima and retention time. Eluted samples were collected with the help of Fraction Collector III. These purified fractions of MAAs were further used for the characterization and identification.

Characterization of MAAs by Fourier Transform Infrared (FTIR) spectroscopy

HPLC purified fractions of MAAs were lyophilized to perform FTIR by fused with oven-dried potassium bromide (stored in desiccator) in 1:100 ratio, transparent disk was prepared using hydraulic press and inserted in a Perkin Elmer FTIR/FIR Spectrometer Frontier version 10 (Perkin Elmer, Waltham, MA, USA) to record the spectra.

RESULTS

Environmental Parameters

Nearly all Kunds had stagnant water (pond) system which were dominated by greenish and blue-green mats of cyanobacteria and algae and were exposed to the solar radiation for longer period of time. These mats were capable of producing photoprotective compounds such as MAAs. At study sites, relative humidity was found to be in the range of 75-97%, tropospheric ozone in the range of 60.98-131.67 µg/m³, UV-A in the range of 6-9 KJ/m² and UV-B in the range of 11.297-33.928 KJ/m². Environmental conditions such as temperature (18-28°C) and pH (6.02-8.31) (Figures 3 and 4) were favorable for the growth of cyanobacterial and algal populations in most of the Kunds.

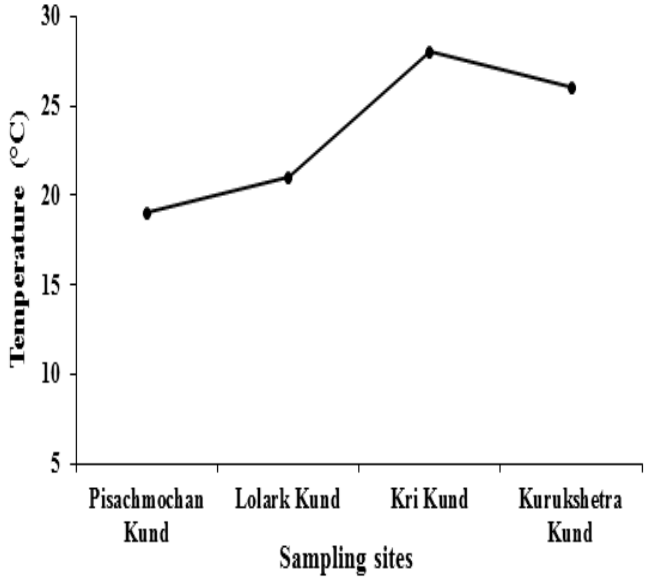

Figure 3. Temperature at different historical Kunds of Varanasi, India

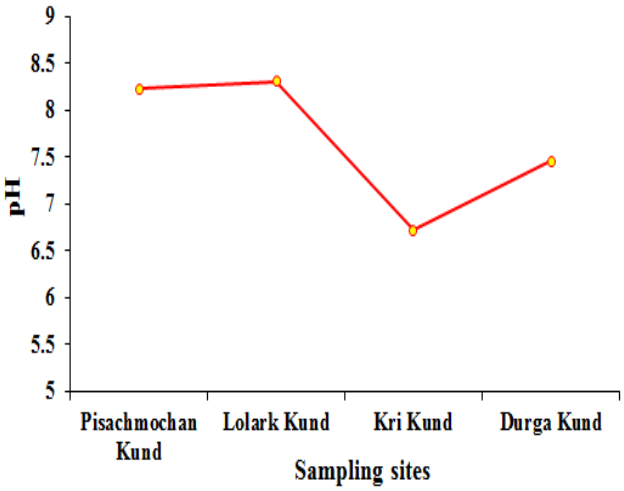

Figure 4. pH at different historical Kunds of Varanasi, India

Screening and Identification of Microorganisms

Six species of cyanobacteria and several other algal groups were recorded from the four ancient and religious Kunds of Varanasi, India (Figures 5 and 6). Kunds were dominated by the cyanobacteria such as *Anabaena* sp., *Oscillatoria* sp., *Aulosira fertilissima* and *Gloeocapsa* sp. Other notable algal groups present were *Chlorella* sp., *Chlamydomonas* sp. *Ankistrodesmus* sp., *Scenedesmus* sp. and *Nitzschia* sp. All the Kunds had stagnant water (pond) system due to which bloom-forming cyanobacteria such as *Microcystis aeruginosa* and *Merismopedia* were also present but in small number. Organisms identified belong to the class Cyanophyceae, Chlorophyceae, Chrysophyceae and Bacillariophyceae (Table 1).

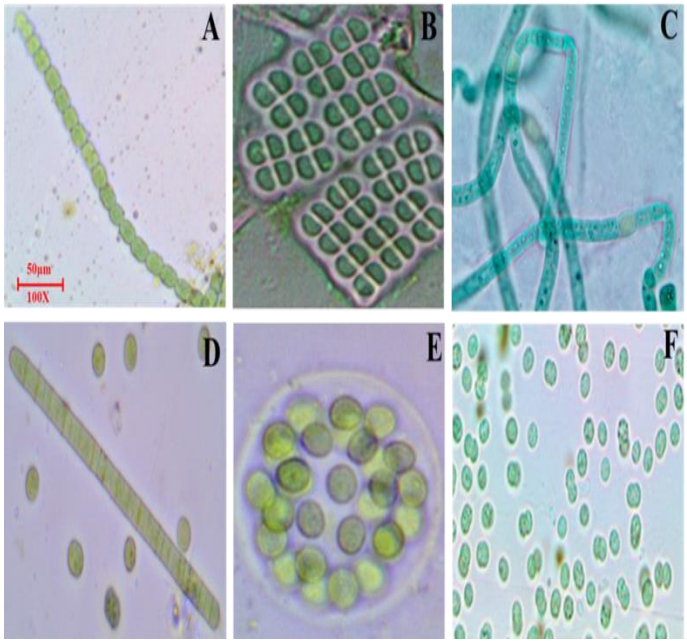

Figure 5. Cyanobacterial strains identified from different historical Kunds of Varanasi, India. (A) *Anabaena* sp., (B) *Merismopedia* sp., (C) *Aulosira fertilissima*, (D) *Oscillatoria* sp., (E) *Gloeocapsa* sp. and (F) *Microcystis aeruginosa*

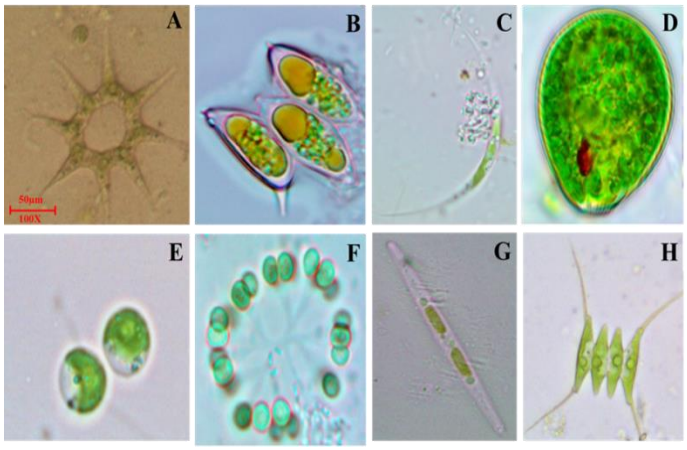

Figure 6. Algal strains identified from different Kunds of Varanasi, India. (A) *Pediastrum* sp., (B) *Scenedesmus* sp., (C) *Ankistrodesmus* sp., (D) *Chlamydomonas* sp., (E) *Chlorella* sp., (F) *Dictyosphaerium* sp., (G) *Nitzschia* sp. and (H) *Desmodesmus opoliensis*

Table 1. Cyanobacteria and other algal colonization (+ shows the presence and – shows the absence of organisms) in the different historical Kunds of Varanasi, India.

	Kunds (Water bodies)			
Organisms	Pisachmochan kund	Lolark Kund	Kri Kund	Durga Kund
Cyanophyceae				
Anabaena sp.	+	+	-	+
Oscillatoria sp.	+	+	+	-
Aulosira fertilissima	+	+	+	-
Merismopedia sp.	-	-	+	-
Gloeocapsa sp.	+	+	-	+
Microcystis sp.	-	+	-	+
Chlorophyceae				
Chlamydomonas sp.	+	+	-	+
Chlorella sp.	+	+	+	+
Scenedesmus sp.	-	+	+	-
Ankistrodesmus sp.	+	-	-	+
Dictyosphaerium sp.	-	+	+	-
Desmodesmus opoliensis	-	-	+	+
Pediastrum sp.	+	+	-	-
Chrysophyceae				
Mallomonas sp.	-	+	-	+
Bacillariophyceae (Diatom)				
Nitzschia sp.	+	+	+	-
Fragilaria sp.	-	-	+	+
Aulacoseira sp.	-	+	-	-
Cyclotella meneghiniana	+	-	-	+

UV-Vis Spectroscopic analysis

The absorption spectra of methanolic extracts of all samples showed a peak in the range of 310-337 nm which suggested the presence of MAAs in these organisms. In addition to the peaks for MAAs, methanolic extracts of all these samples also showed the peaks for chlorophyll *a* (420 and 660 nm), carotenoids (474 nm) and phycobiliproteins (620 nm) (Figure 7).

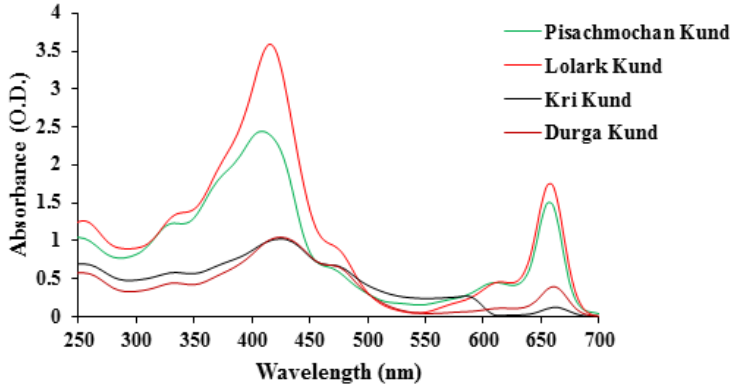

Figure 7. Absorption spectra of methanolic extracts of cyanobacterial and algal assemblages from historical Kunds of Varanasi, India

HPLC Analysis of MAAs

HPLC chromatograms showed various peaks with different retention time (RT) corresponding to respective MAAs (Figure 8A-8D). Identification of different MAAs was done on the basis of retention time and absorption maxima (Table 2). Results indicate that MAAs porphyra-334 (λ_{max}-334 nm; RT-3.62 min); palythine (λ_{max}-320 nm; RT-1.63 min); palythenic acid (λ_{max}-337 nm; RT-4.15 min); mycosporine-glycine (λ_{max}-310 nm; RT-2.55 min) and mycosporine-methylamine-serine (λ_{max}-327 nm; RT-1.51 min) were present in the samples. Porphyra-334 was present in almost all the samples except Kri Kund. Again sampling was done in the period of June 2019 (temperature 37°C). Thereafter centrifugation was done to take supernatant (water) and checked for the presence of photoprotective compound without adding any solvent. After all these, presence of photoprotective compound mycosporine-glycine at absorption maxima 310 nm and retention time 2.55 min (Figure 9) was confirmed by HPLC. This result shows that at high intensity of UV-radiation, organisms degrade (after synthesis of photoprotective compound) and release their synthesized photoprotective compounds in water bodies. Concentration of these compounds was low due to mixing in water but these photoprotective compounds protect other organisms from degradation.

Concentration of all MAAs which was found in various Kunds has been shown in Figure 10. Altogether five different types of Kund specific MAAs were present in our samples. Palythenic acid was present only in Pisachmochan kund. Porphyra-334 was present in all Kunds except Kri Kund and its content was highest in Durga Kund. Palythine was present in Pisachmochan, Lolark, Kri and Durga Kund, in which higher concentration was observed in Kri Kund. Mycosporine-glycine and Mycosporine-methylamine-serine were present only in Lolark Kund (Figure 10). These concentrations of MAAs depend on several conditions such as exposure to the UV radiation for longer period and other component as nutrient at the time of rituals near these ancient and religious

Kunds. Figure 11 represents the percentage distribution of MAAs in these samples. The results indicate that porphyra-334 was contributing maximum (58 %) in Durga Kund and minimum (27 %) in Lolark Kund to the total MAAs. Palythine was contributing maximum (100 %) in Kri Kund and minimum (20 %) in Lolark Kund. Palythenic acid was contributing 50% in Pisachmochan Kund. Mycosporine-glycine and mycosporine-methylamine-serine were contributing 20 % and 33%, respectively in Lolark Kund.

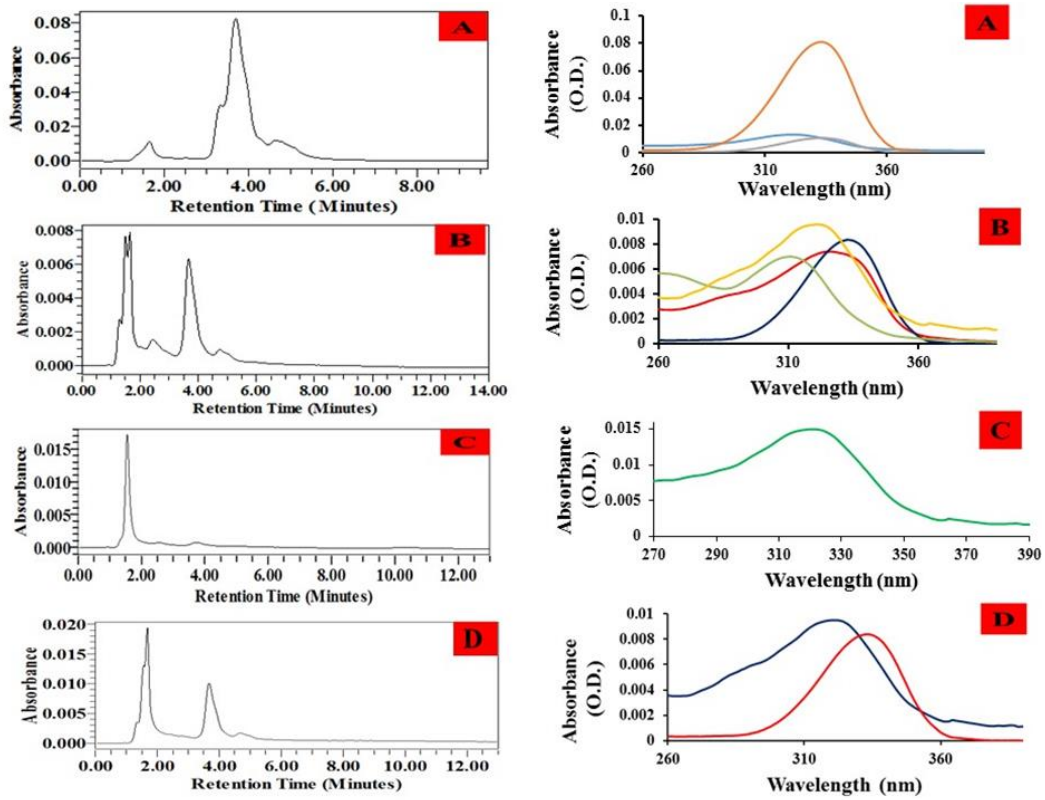

Figure 8. HPLC chromatograms of the purified MAAs and their corresponding absorption spectra. (A) Pisachmochan Kund, (B) Lolark Kund, (C) Kri Kund and (D) Durga Kund

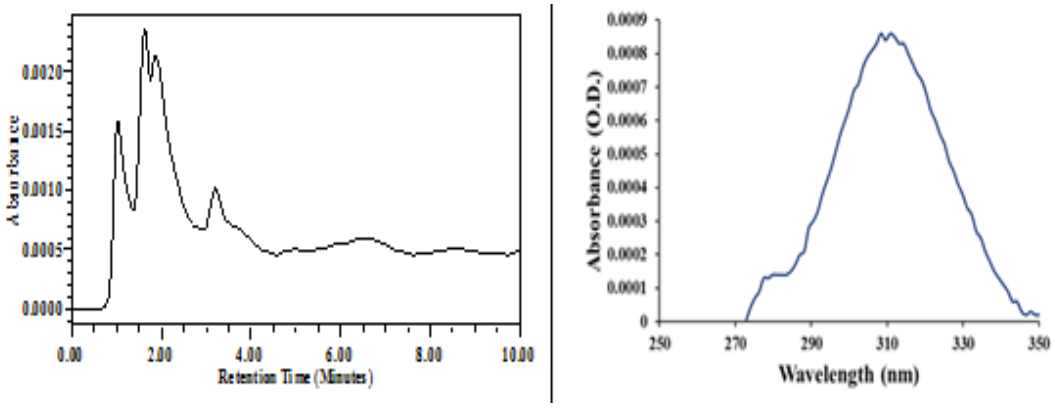

Figure 9. HPLC chromatogram and its corresponding absorption spectrum of purified fraction of water sample after centrifugation have Mycosporine-glycine at 310

Table 2. Photoprotective compounds from historical Kunds of Varanasi, India, with their corresponding absorption maxima and retention times.

Kunds	Mycosporine-like amino acids (MAAS)		
	λ_{max} (nm)	Retention time (min)	MAAs
	334	3.78	Porphyra-334
Pisachmochan Kund	320	1.65	Palythine
	337	4.15	Palythenic acid
	334	3.62	Porphyra-334
Lolark Kund	320	1.61	Palythine
	310	2.55	Mycosporine-glycine
	327	1.51	Mycosporine-methylamine-serine
Kri Kund	320	1.63	Palythine
Durga Kund	334	3.52	Porphyra-334
	320	1.68	Palythine

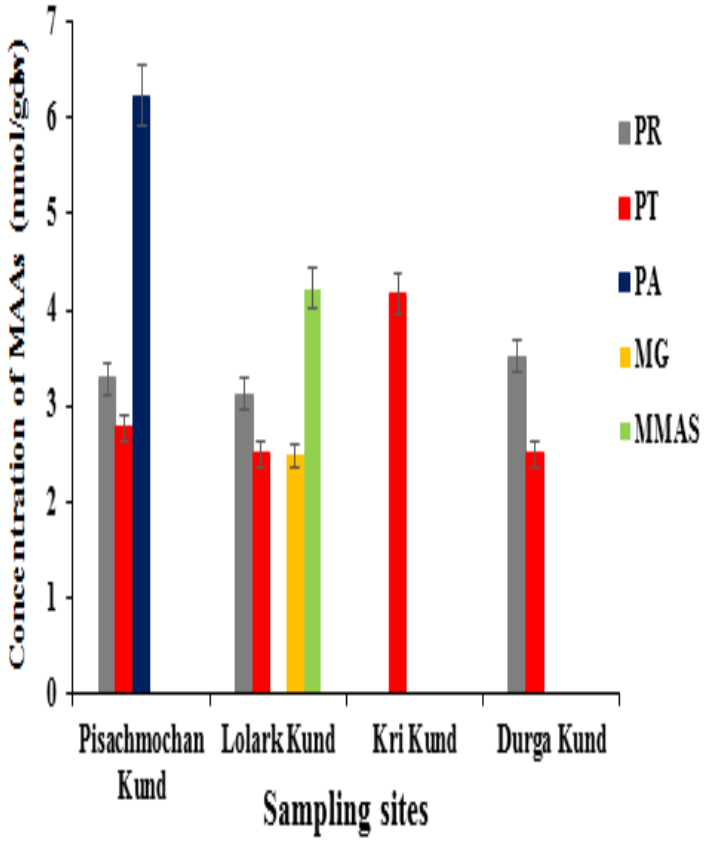

Figure 10. Concentration of MAAs (nmol/gdw) in different historical Kunds of Varanasi, India. (PR: Porphyra-334, PT: Palythine, PA: Palythenic acid, MG: Mycosporine-glycine, MMAS: Mycosporine-methylamine-serine). The error bar represents standard deviation of mean (means ± S.D., n=3)

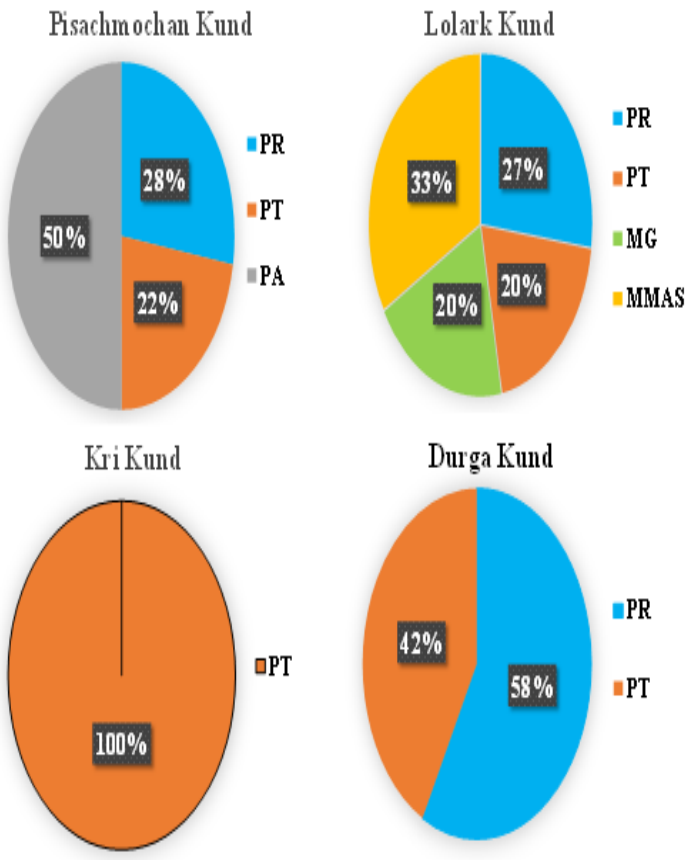

Figure 11. Percentage distribution of MAAs (PR: Porphyra-334, PT: Palythine, PA: Palythenic acid, MG: Mycosporine-glycine, MMAS: Mycosporine-methylamine-serine) at various Kunds)

Effect of Temperature and pH on MAAs in Natural Environment

Temperature and pH does not directly affect MAAs but they affect them indirectly by affecting cyanobacteria. High temperature (above 32°C) and low pH (less than 6.5) are not favourable for organisms. At this high temperature and low pH, solubility of oxygen decreases and heavy metals in water become more soluble to increase toxicity and therefore the death of organisms. This effect of temperature and pH in natural environment are same as in laboratory conditions. Organisms grow luxuriantly at the temperature ranging from 19-28°C (Figure 3) and pH ranging from 6.72-8.31 (Figure 4). In general the optimum growth temperature for cyanobacteria is higher than that for most of the algae.

Characterization of MAAs by Fourier Transform Infrared Spectroscopy (FTIR)

Collected fractions of FTIR containing KBr pellets revealed the presence of four prominent bands (shown by arrows) in all the four samples (Figure 12). Band of 3353 cm^{-1} may be assigned to OH group, 2947 cm^{-1} for side chain vibrations consisting of CH stretching and indicating the presence of NH_2^+ and band of 2836 cm^{-1} indicating the presence of OH stretching in COOH (carboxylic group) and band of 1650 cm^{-1} may be assigned for the presence of an NH_2 group.

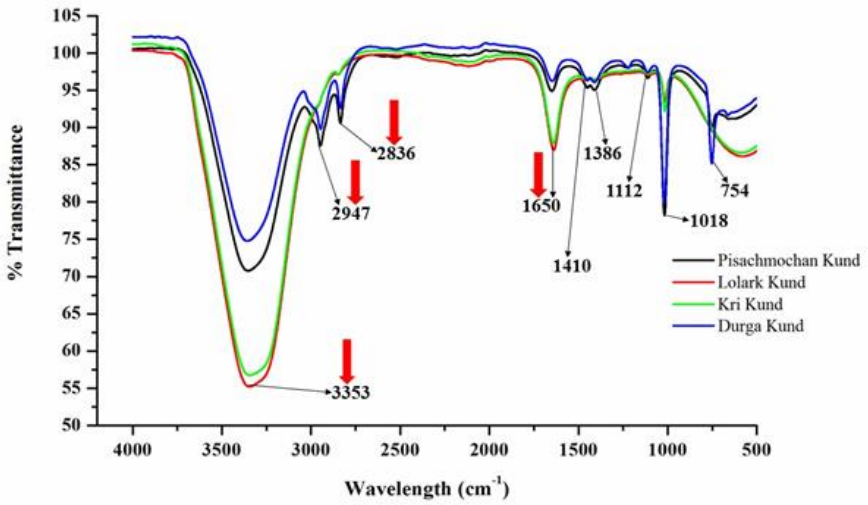

Figure 12. Fourier Transform Infrared (FTIR) spectroscopy showing four prominent spectral bands (marked by arrows) revealing the presence of MAAs

DISCUSSION AND CONCLUSION

Cyanobacteria and other algal groups are worthwhile and beneficial source of natural products with potential biological activities. Members of Chlorophyceae such as *Chlamydomonas* sp., *Chlorella* sp., *Scenedesmus* sp. and *Ankistrodesmus* sp. are known to produce MAAs viz., porphyra-334, mycosporine-glycine and shinorine [29]. *Chlorella* sp. has been reported to produce porphyra-334, palythine, mycosporine-glycine and shinorine by their symbiotic association with ciliates [30]. Small mass of 44 Da (CO_2) and 18 Da (H_2O) misplaying repeatedly discern commonly in porphyra-334 [31,32]. MAAs such as MAA-322 (322 nm) and MAA-324 (324 nm) have also been reported to be produced by the green alga *Chlorella* [33]. *Scenedesmus* sp. is known to produce MAAs such as porphyra-334, palythine, shinorine, asterina-330 and MAA-302 [34]. Production of asterina-330 and shinorine has been reported earlier in *Ankistrodesmus* sp. [34]. The Bacillariophycean member *Nitzschia* sp. has also been reported to produce MAAs [35]. These different types of MAAs were identified on the basis of varying absorption maxima and their respective retention times by HPLC followed by FTIR spectroscopy. Organisms which are able to synthesize or accumulate MAAs are supposed to provide protection to the internal organelles and components from harmful UV radiations [36]. In comparison to other MAAs, palythine and asterina-330 have been reported in a limited number of microalgae or macroalgae [37-39].

Our study suggests that changing temperature and pH are responsible for the optimum growth and assemblage of cyanobacteria and other algal groups [40]. Cyanobacterial growth rate increases at higher temperature (up to 25°C) due to change in climate and therefore there is dominance of cyanobacteria in temperate water bodies [41-43]. They are the main source of photoprotective compound MAAs, which have several other pharmaceutical importances such as antiaging, anticancerous, anti-inflammatory and anti-immunosuppressive. Erythema, edema and sunburn are acute UV-damages which have been shown to reduce in the presence of MAAs [44]. DNA lesions have also been reported to be eliminated in the presence of MAAs [45]. MAAs has the ability to inhibit the overexpression of biomarker of skin inflammation and in this way may protect from psoriasis, a chronic condition caused by overcome immune system [46]. Composition and occurrence of MAAs may vary within the species [30]. MAAs especially

mycosporine-glycine and its precursor gadusol act as antioxidants to avert cellular damage arising from UVR-induced ROS [47]. In conclusion, cyanobacteria together with algal assemblages are useful in producing natural photoprotective compounds even under various stress conditions and therefore could be a valuable source for the development of natural sunscreens and antioxidants.

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Author Contributions

RPS: conceived and designed the experiments, analyzed and interpreted the data and edited the MS; SM: Performed the experiments and drafted the paper; AP: Helped in performing the experiments and critically revised the MS. All authors read and approved the final MS.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

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