

Original Research Article

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Mycosporine-like Amino Acids from Biological Integuments of Historical Monuments

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ABSTRACT

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Biological crusts collected from seven historical monuments in and around Varanasi, India, were screened for the presence of photoprotective mycosporine-like amino acids (MAAs). Nine strains of cyanobacterial genera such as *Lyngbya* sp., *Nostoc* sp., *Anabaena* sp., *Scytonema* sp., *Phormidium* sp., *Westiellopsis* sp., *Aphanocapsa* sp., *Hapalosiphon* sp. and *Aphanothece* sp. were found to be present in the samples collected from various monuments apart from other group of organisms. Cyanobacteria, the dominant population growing on all the monuments had a maximum diversity of 5 species in Brahaspati temple, 4 species in Ramnagar fort and LalKhan's tomb, 3 species in Manikarnika ghat and Sanskrit University and only 1 species in Sarnath and Bharat Mata temple. Pigment profile of the crusts from the seven monuments showed peaks at 665, 470, 310 and 386 nm that correspond to the presence of chlorophyll *a*, carotenoids, MAAs and scytonemin respectively. High content of chlorophyll *a* was recorded in the crusts from Sarnath and the Brahaspati temple, whereas carotenoids content was higher in the crust sample of LalKhan's tomb. In all the collected samples from various monuments, photoprotective MAAs were found to be predominant than the photosynthetic pigments (chlorophyll *a* and carotenoids).

Introduction

Cyanobacteria are Gram-negative prokaryotes having a cosmopolitan distribution ranging from hot-springs to the Arctic and Antarctic regions (Stanier and Cohen-Bazire, 1977). They were the first photosynthetic oxygen-evolving prokaryotes which appeared during the Precambrian era (Brocks *et al.*, 1999). Fossil records, organic biomarkers and genomic sequence analyses indicate the presence of cyanobacteria on the

early Earth, when there was no ozone layer (Schopf, 2000; Hedges *et al.*, 2001; Häder *et al.*, 2015). The ancient monument all over the world are deteriorating due to the deposition of cyanobacteria, resulting in the loss of basic structure and creates pits, cracks and fissures (Ortega-Calvo *et al.*, 1991; Dhama *et al.*, 2014).

On ancient monuments, cyanobacteria played an important role as pioneer for

establishing life on bare inorganic rocks and produced considerable biomass. Most of these cyanobacterial crusts growing on the ancient monuments were colonial or filamentous and occurred in association with other algae, fungi, bacteria, lichen and moss *Protonema* (Lüttge *et al.*, 1995). Several studies on the cyanobacterial crust residing on the ancient monuments have been done in South-West USA and Mexico (Friedmann, 1972), monuments of Northern Transvaal, South Africa, marbles of Parthenon (Acropolis-Athens) of Greece (Anagnostidis *et al.*, 1983), Roman frescoes (Grilli-Caiola *et al.*, 1987; Albertano and Grilli-Caiola, 1989), Inferniglio cave of Italy (Abdelahad, 1989), Lund cathedral of Sweden (Ortega-Calvo *et al.*, 1991), Roman Necropolis (Albertano *et al.*, 1994) and Goldengate highlands National Park, South Africa (Wessels and Büdel, 1989) etc.

In Varanasi (India) region, so far no prior studies have been done on the presence of photoprotective MAAs in the crust forming cyanobacteria of the monuments. Some of these monuments are situated in the warm temperate region having a very suitable condition for the growth and development of cyanobacteria which is responsible for the defragmentation of the monuments. Though such problems are important in humid and tropical climates, there is little information about the cyanobacteria growing on the monuments of India (Tripathy *et al.*, 1999; Pattanaik and Adhikary, 2002). Some historical monuments of Varanasi such as, Sarnath temple is about three hundred years old and a holy place for Buddhists, LalKha ka Rauza build by Mughal emperor is situated at the bank of sacred river Ganges, Sanskrit University is about 150 years old, Ramnagar fort was manufactured by the King of Varanasi about five hundred years ago and Manikarnika ghat temple is about 600 hundred years old, have wonderful

artistic work and are important part of glorious traditions and rich cultural heritage of Varanasi.

Photoprotective MAAs are <400 Da, colorless, water-soluble compounds composed of a cyclohexenone or cyclohexenimine chromophore conjugated with the nitrogen substituent of an amino acid or its imino alcohol (Carreto *et al.*, 2005; Richa and Sinha, 2015) and have absorption maxima in the range of 310-362 nm. Generally, the ring system contains a glycine subunit linked to the third carbon atom. Some MAAs also contains sulfate esters or glycosidic linkages through the imine substituents. Differences between the absorption spectra of MAAs are due to the attached side groups and nitrogen substituent. The biosynthesis of MAAs has been predicted to occur *via* the first part of the shikimate pathway but concluding evidences are lacking. It has been found that 3-dehydroquinate, which is formed in the centre of the shikimate pathway, acts as a precursor for the synthesis of fungal mycosporines and MAAs *via* gadusols (Shick *et al.*, 2002).

MAAs provide protection from UV radiation not only in their producers but also to primary and secondary consumers through the food chain. MAAs has been reported in diverse organisms, such as, bacteria, cyanobacteria, macroalgae, phytoplankton and various animals such as arthropods, rotifers, molluscs, fishes, cnidarians, tunicates, poriferans, nematodes, echinodermates, platyhelminthes, polychaetes, bryozoans and protozoans (Sinha *et al.*, 2007; Kannaujiya *et al.*, 2014; Pathak *et al.*, 2015), but not in animals as they lack the shikimate pathway, but these compounds may be accumulated either *via* the food chain or synthesized by their symbiotic algal partner (Shick and Dunlap,

2002). Presently, about 22 MAAs have been reported from terrestrial, freshwater and marine organisms. MAAs have high molar extinction coefficient which favours them as strong photoprotectant. In addition, to their photoprotective role in various organisms, MAAs are also of immense importance for humans as these compounds have been found to effectively block thymine dimer formation by UVR *in vitro*, have antioxidant as well as growth stimulation activity in human cells and were recently found to reduce UV-induced aging (Oyamada *et al.*, 2008; Singh *et al.*, 2010). In the present study, for the first time, we have reported the diversity of cyanobacteria and the presence of photoprotective MAAs from the exposed surfaces of different monuments of Varanasi, India.

Materials and Methods

Collection sites

The cyanobacterial samples were collected from various monuments such as, LalKhan's tomb, Manikarnika ghat, Brahaspati temple, Sarnath, Ramnagar fort, Sanskrit University and Bharat Mata temple. These monuments are situated between 25° 20' 0" North, 83° 0' 0" East in Varanasi district of Uttar Pradesh, India. These historical places are very popular and some are protected by the Archaeological survey of India. Since the cyanobacterial colonization occurs in the upper surface of the crust, we have collected the crust growing over these monuments by a non-destructive double layered adhesive tape method and then transferred to pre-sterilized screw-cap bottles and transported to the laboratory for further analyses.

Identification of organisms

The collected crusts from the monuments were soaked in sterile distilled water for 12-

48 h. To examine the presence of cyanobacteria, a pinch of the rehydrated crusts were observed under compound microscope (Getneroptik KFS4 (I) 5582) and photographed using Dewinter image microscope fitted with digital camera. Various cyanobacteria, such as, *Anabaena* sp., *Nostoc* sp., *Lyngbya* sp. and *Scytonema* sp. etc. were found to be present in the collected crusts.

Mycosporine-like amino acids (MAAs) extraction and spectroscopic analysis

For extraction of MAAs, equal amount of crust (0.5 mg) collected from various monuments were dissolved in 2 mL of 100% methanol (HPLC-grade) and incubated at 4 °C for overnight. The methanol extracts were centrifuged at 10,000 rpm for 10 min, and the supernatant was subjected to spectroscopic analysis between 250-700 nm wavelengths, using a double beam spectrophotometer (UV-VIS 2900, Hitachi, Japan). The raw spectra (peaks) were analyzed using UV Probe version software (Hitachi, Japan).

Purification and estimation of MAAs

After initial characterization of MAAs by spectroscopic analysis, methanolic extracts were evaporated to dryness at 45°C, and the dried product was dissolved in 1ml Milli Q water in a microcentrifuge tube. After adding a few drops of chloroform, the suspension was subjected to centrifugation, and the water phase was carefully transferred into a fresh microcentrifuge tube to remove contaminating lipophilic compounds.

Finally, the resulting suspension was filtered through a 0.2 µm pore size syringe filters (Axiva SicheM Biotech., New Delhi) and further subjected to high performance liquid chromatography (HPLC; Waters,

Elstree,UK) analysis using a reverse phase semi preparative column (symmetry prep C18, 7 µm particle size, 7.8mm × 300 mm long) connected to an asymmetry guard column, outfitted with a Waters Photodiode array detector. 0.2 % acetic acid was used as mobile phase and detection of MAAs was done at 330 nm. Identification and characterization of MAAs was done on the basis of the retention time and their corresponding absorption spectra.

Estimation of Chlorophyll *a* and carotenoids

Crust (1g) collected from different monuments of Varanasi was homogenized in 95% methanol with the help of motor and pestle. The homogenate was centrifuged at 10,000 rpm for 10 min using Spinwin 650 and the collected supernatants were subjected to spectroscopic analysis between 400-700 nm wavelengths using a double beam spectrophotometer (UV-VIS 2900, Hitachi, Japan). Chlorophyll *a* showed the maximum absorbance at 665 nm and total carotenoids at 470 nm. The amount of these pigments was calculated according to the formula of Lichtenthaler and Wellburn (1985).

Results and Discussion

Nature of the crust and algal colonization

The crust collected from all the monuments were blackish to brownish in color, predominately growing at the ledges of the temples, roof of the fort and on the domes of the monuments, which were exposed to wide spectrum of solar and UV radiation (Polo *et al.*, 2012; Pathak *et al.*, 2015). There were almost no variations in the climatic conditions of all the monuments and their environment. Nine species of cyanobacteria (Table 1) viz., *Lyngbya* sp.,

Nostoc sp., *Anabaena* sp., *Scytonema* sp., *Phormidium* sp., *Hapalosiphon* sp., *Westiellopsis* sp., *Aphanocapsa* sp. and *Aphanothece* sp. were found to be present in the crust samples of various monuments. The 16S *rRNA* gene sequences analyses indicate the presence of similar cyanobacteria in sub-aerial habitats of monuments, sculptures and archeological sites (Bruno *et al.*, 2009).

Figure 1 shows the microphotographs of the cyanobacterial filaments collected from various monuments. Among the algal group, cyanobacteria were the dominant population growing over all the monuments with maximum diversity of 5 species in Brahaspati temple, 4 species in Ramnagar fort and LalKhan's tomb, 3 species in Manikarnika ghat and Sanskrit university and only 1 species in Sarnath and Bharat Mata temple.

Pigment analysis

Pigment profile of the crusts collected from the monuments are shown in Fig. 2. The absorption spectra of 100 % (v/v) methanolic extract showed peaks at 665, 470, 310 and 386 nm that correspond to the presence of chlorophyll *a*, carotenoids, MAAs and scytonemin respectively. In all the crust samples MAAs was found to be quite predominant than the scytonemin, chlorophyll *a* and carotenoids (Pathak *et al.*, 2015).

Chlorophyll *a* content was recorded to be highest in the samples collected from Sarnath and Brahaspati temple. The level of chlorophyll *a* in the crust samples of five other monuments of Varanasi (LalKhan's tomb, Ramnagar fort, Bharat Mata temple, Manikarnika ghat, Sanskrit University) were found to be very close to each other. However, crust samples of Ramnagar Fort

and Sanskrit University showed a slightly higher content of Chl *a* in comparison to other three monuments (Fig. 3). Carotenoids level was highest in crust sample of LalKhan's tomb followed by Sarnath and Brahaspati temple (Fig. 4). The levels of carotenoids in crust samples of remaining monuments were negligible.

Analysis of photoprotective compound

The absorption spectra of methanolic extract showed the presence of a photoprotective compound, mycosporine-like amino acids peaking around 307-330 nm (Fig. 2). HPLC chromatogram of the aqueous solution of Manikarnika ghat sample showed the presence of eight MAAs having retention times (RT) ranging from 2.19-13.2 min and

a corresponding absorption maxima (λ_{max}) between 307-330 nm (Fig. 5), whereas, in case of crust from Sarnath temple the presence of five MAAs having RT 2.0 -14.4 min (λ_{max} : 310-330 nm) (Fig. 6) were recorded.

The aqueous solution of crust from Sanskrit university (Fig. 6), Brahaspati temple and Bharatmata temple showed the presence of six (RT-2.0-4.4 min; λ_{max} : 307-330 nm), three (RT- 2.3-4.1 min; λ_{max} : 310-330 nm) and eight (RT- 2.2-7.3 min; λ_{max} : 313-330 nm) MAAs respectively (Fig. 7). The crust from Ramnagar fort and LalKhan's tomb showed the presence of five (RT- 2.0-20.0min; λ_{max} : 307-330 nm) and nine (RT- 2.13-7.2 λ_{max} : 307-330 nm) (Fig. 7) MAAs respectively.

Table.1 Cyanobacterial strains inhabiting different historical monuments of Varanasi, India

Historical monuments							
Cyanobacteria	Ramnagar Fort	Bharat Mata Temple	Brihaspati Temple	LalKhan's Tomb	Sanskrit University	Sarnath	Manikarnika Ghat
<i>Lyngbya</i> sp.	+	-	-	+	+	+	+
<i>Nostoc</i> sp.	+	-	+	-	+	-	+
<i>Anabaena</i> sp.	+	-	+	-	-	-	-
<i>Scytonema</i> sp.	-	+	+	+	+	-	+
<i>Phormidium</i> sp.	+	-	-	-	-	-	-
<i>Hapalosiphon</i> sp.	-	-	+	-	-	-	-
<i>Westiellopsis</i> sp.	-	-	+	-	-	-	-
<i>Aphanocapsa</i> sp.	-	-	-	+	-	-	-
<i>Aphanothece</i> sp.	-	-	-	+	-	-	-

+ = Present; - = Absent

Fig.1 Cyanobacteria isolated from different monuments. (A) *Lyngbya* sp., (B) *Westiellopsis* sp. (C) *Hapalosiphon* sp., (D) *Nostoc* sp., (E) *Aphanothece* sp. (F) *Scytonema* sp., (G) *Phormidium* sp. (H) *Anabaena* sp. and (I) *Aphanocapsa* sp.

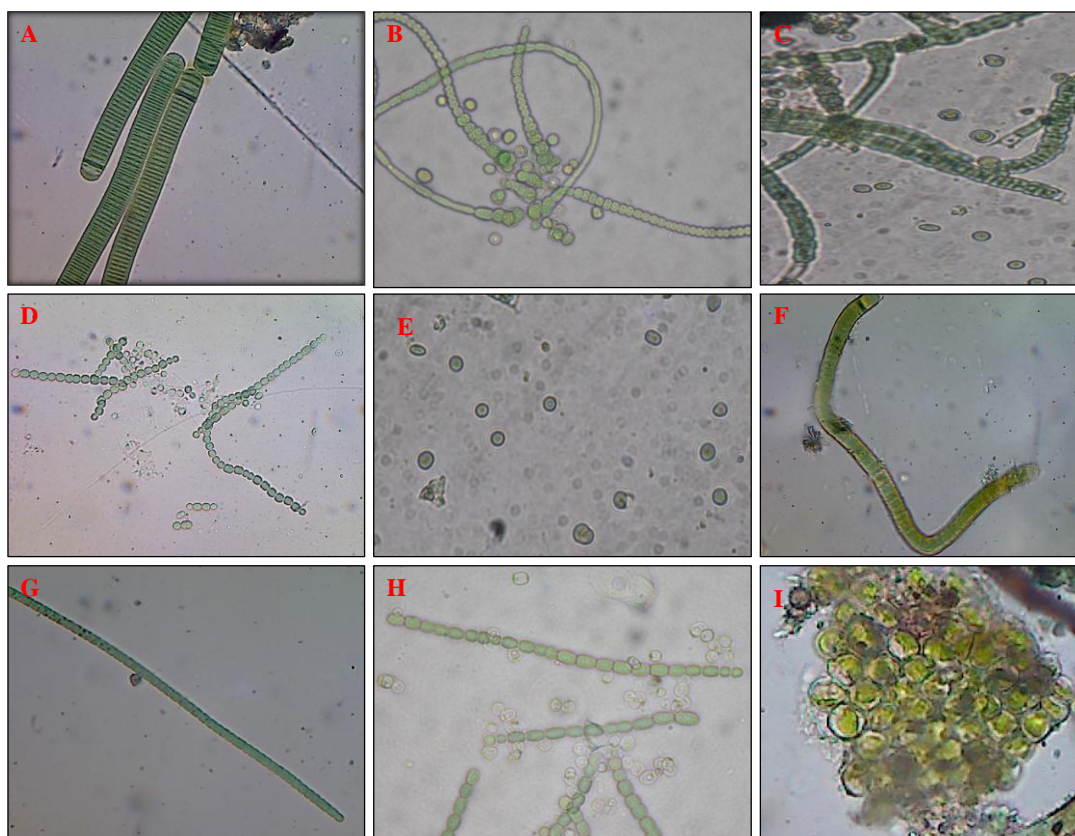


Fig.2 Absorption spectra showing the presence of *Chl a*, carotenoids and the predominant MAAs in biological crusts of different monuments.

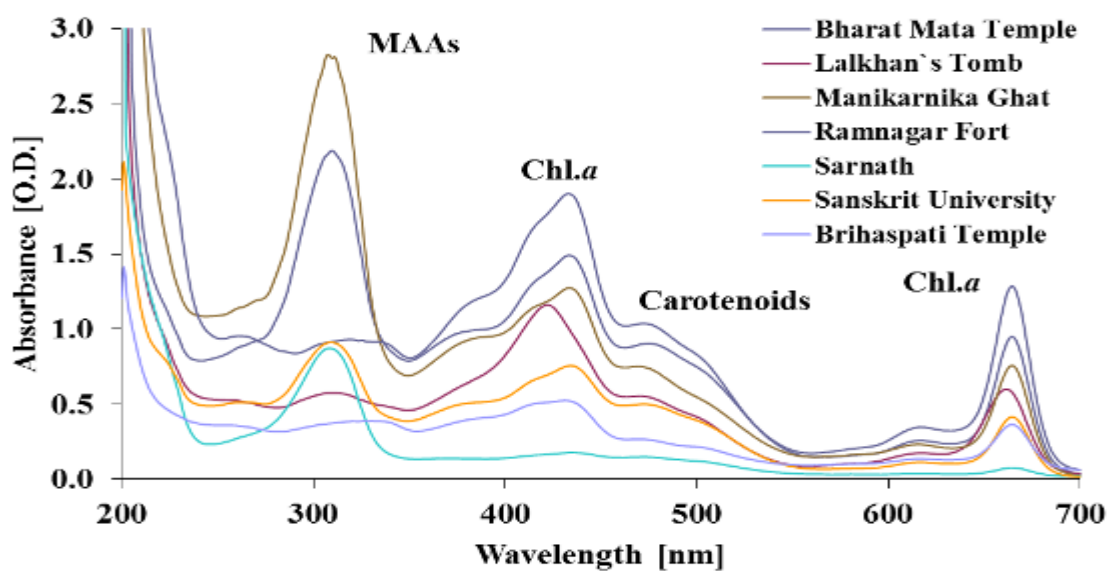


Fig.3 Chlorophyll *a* content in biological crusts collected from different monuments of Varanasi, India

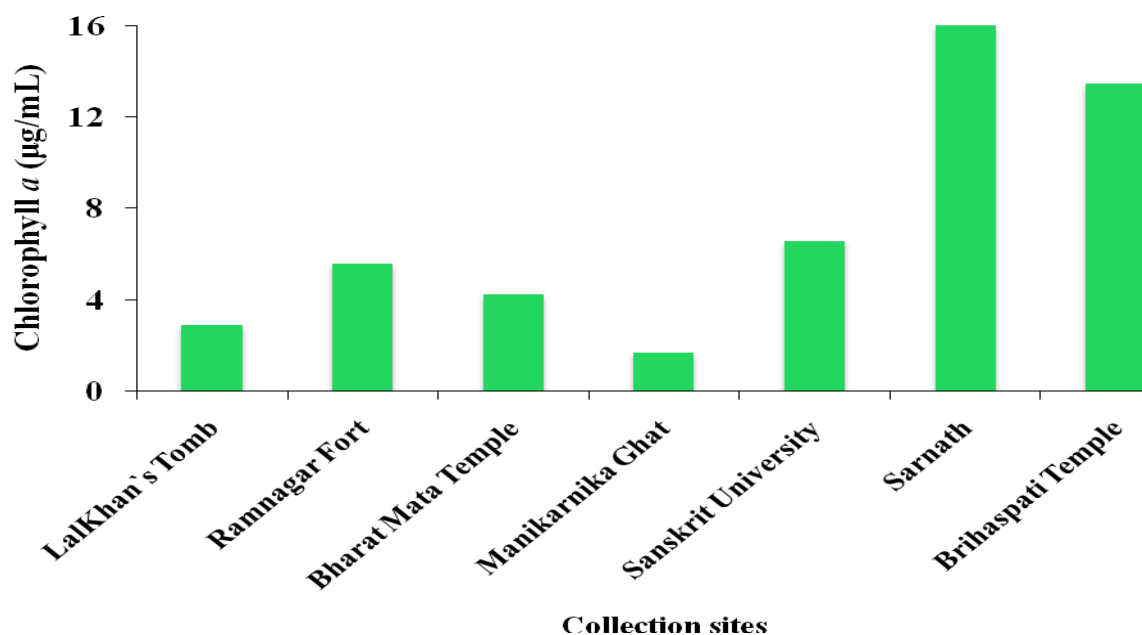


Fig.4 Carotenoids content in biological crusts collected from different monuments of Varanasi, India

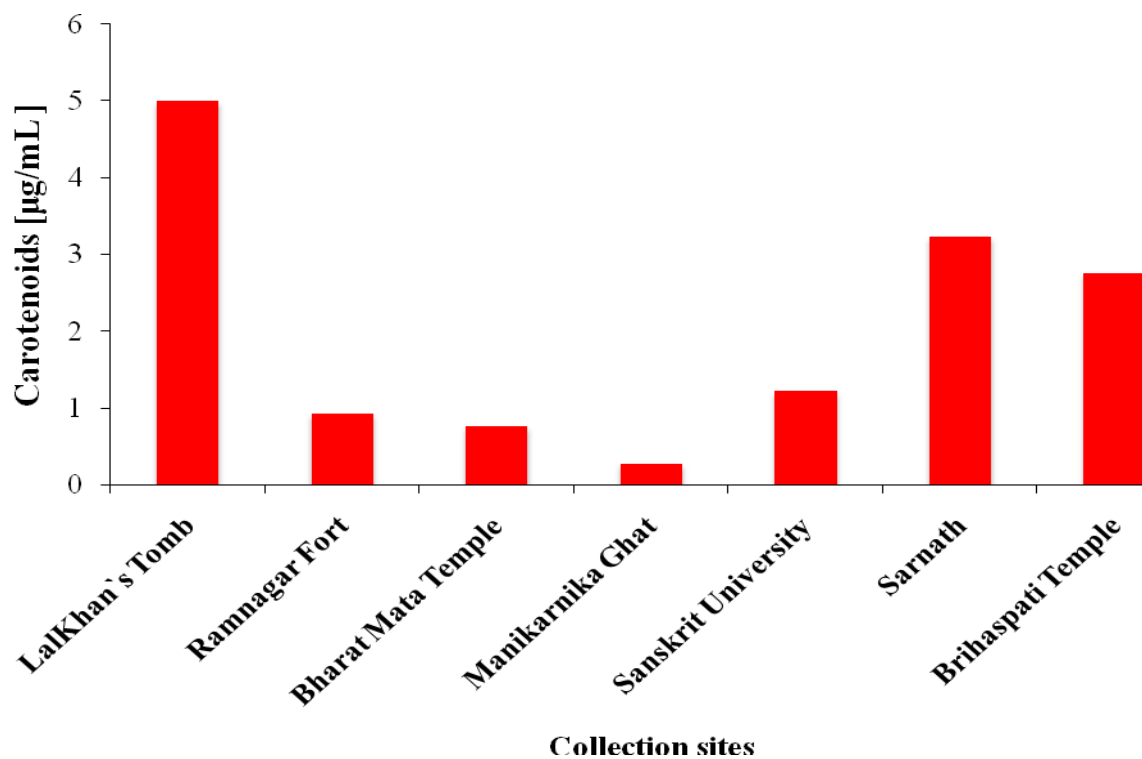


Fig.5 Photographs showing different collection sites of Manikarnika Ghat (A-C). The HPLC chromatograms (D-F) and the corresponding absorption maxima (G-I) of MAAs present in the biological crusts. Red arrows show the point of crust collection.

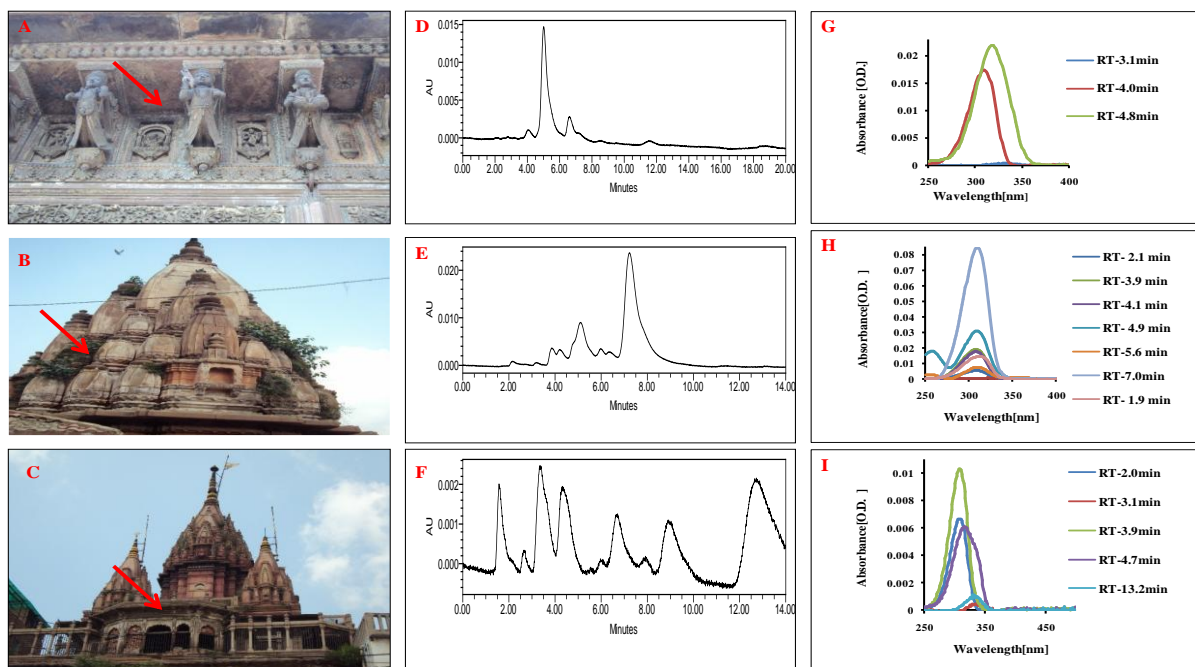


Fig.6 Photographs showing different collection sites of Sarnath (A and B) and Sanskrit University (C). The HPLC chromatograms (D-F) and the corresponding absorption maxima (G-I) of MAAs present in the biological crusts. Red arrows show the point of crust collection.

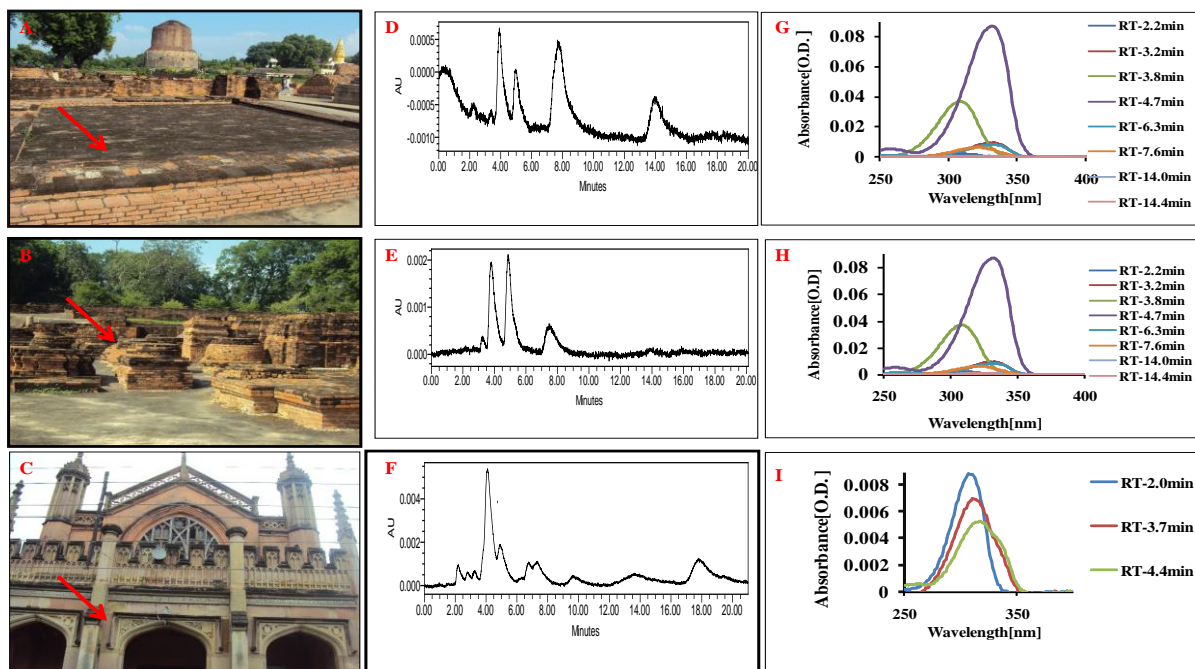
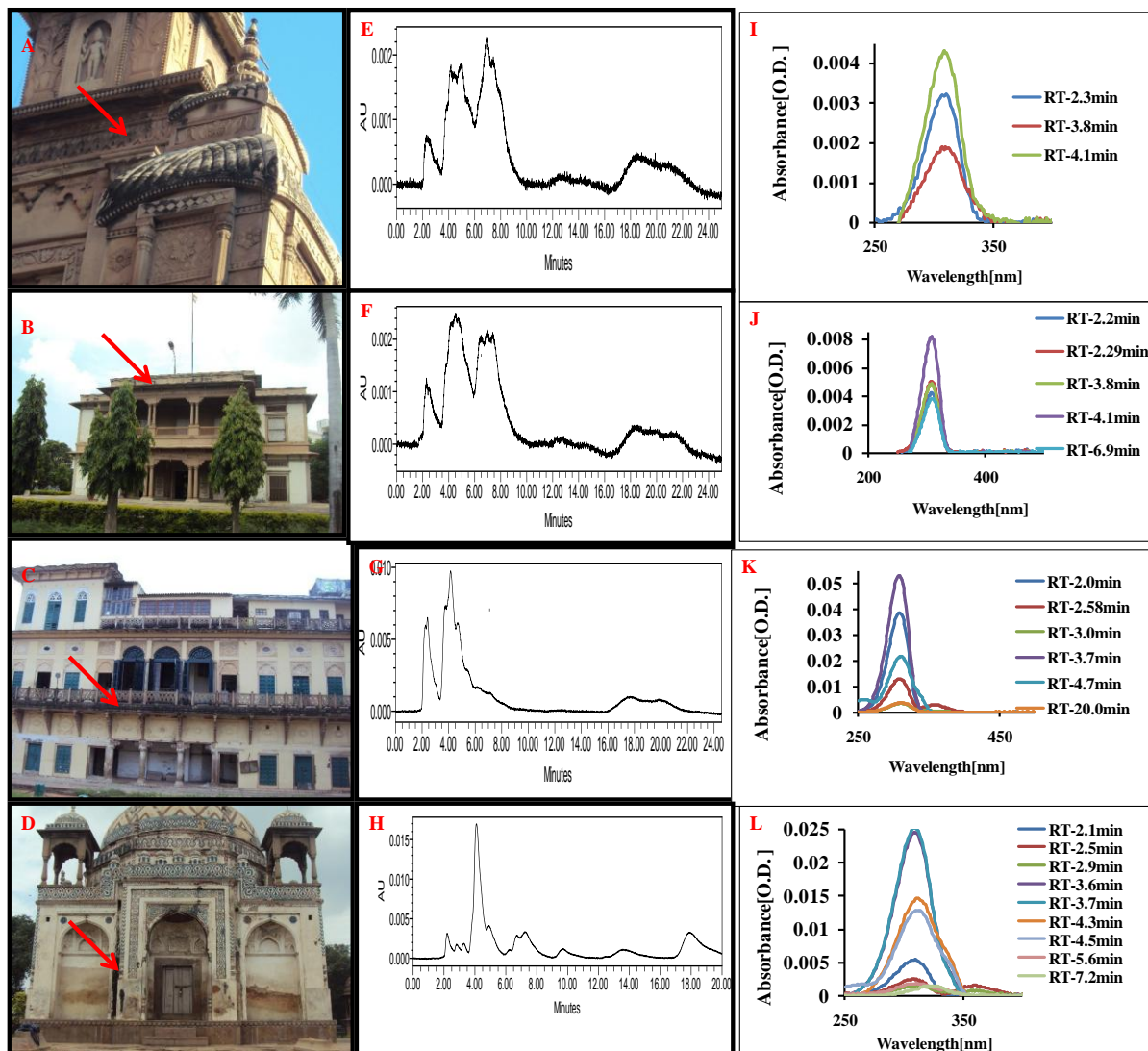


Fig.7 Photographs showing different collection sites of Brihaspati temple (A), Bharat Mata temple (B), Ramnagar Fort (C) and LalKhan's Tomb (D). The HPLC chromatograms (E-H) and the corresponding absorption maxima (I-L) of MAAs present in the crusts. Red arrows show the point of crust collection



Analysis of the result showed that species composition in the crusts of temples, ghats, mosque and old building varied with the nature of the substratum (solid rock, bare rocks, bare bricks, cement, etc.). The exposed roof top part of the monument which is composed of bricks without any uneven sculptures is mostly covered by black colored crust dominated by *Lyngbya* sp., *Nostoc* sp., *Anabaena* sp., *Scytonema* sp.

and *Phormidium* sp. whereas the decorated walls were dominated by unicellular/colonial forms of cyanobacterial species such as *Westiellopsis* sp., *Aphanocapsa* sp. and *Aphanothece* sp., etc. Cyanobacteria are the dominant species colonizing on the sub aerial surfaces in warm temperate to tropical regions (Videla *et al.*, 2000; Couradeau *et al.*, 2016). During summer season, the temperature of different

monuments and Ghats goes beyond 65⁰C, coupled with high light intensity, UV radiation and extreme dryness and cyanobacteria can survive in such extreme environment in the surfaces of these monuments as blackish-brownish crust. Since the Ramnagar fort and Sarnath monument is fully exposed to UV radiation, hence probably had maximum diversity of cyanobacteria. Cyanobacterial species exposed to strong UV radiation have also been found to contain one or more UV-absorbing mycosporine-like amino acid compounds and/or extracellular sunscreen pigment scytonemin which have been suggested as a protective/defensive strategy against high UV radiation (Garcia-Pichel and Castenholz 1991; Keshari and Adhikary, 2013).

In conclusion, we studied the presence of photoprotective compound mycosporine-like amino acid, cyanobacterial diversity, chlorophyll *a* and carotenoids content in the crust samples collected from several monuments of Varanasi. We observed that 9 species of cyanobacteria such as *Lyngbya* sp., *Nostoc* sp., *Anabaena* sp., *Scytonema* sp., *Phormidium* sp., *Westiellopsis* sp., *Aphanocapsa* sp., *Hapalosiphon* sp. and *Aphanothece* sp. were present in the crust samples. There was almost no variation in the climatic conditions of all the monuments and their environment. All the cyanobacterial samples inhabiting the monuments showed high content of MAAs, followed by Chl *a* and carotenoids. The higher MAAs concentration could probably be responsible for providing protection to these organisms which are continuously exposed to intense UV radiation on the monument surfaces.

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