

EVALUATION OF ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF CYANOBACTERIA, *LIMNOTHRIX* SP. AND *LEPTOLYNGBYA* SP. FROM ARABIAN SEA

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ABSTRACT

The hexane fractions of the marine cyanobacteria: *Leptolyngbya* sp. and *Limnothrix* sp., collected from Arabian Sea were found to display promising antioxidant properties than their ethyl acetate fraction during radical scavenging ABTS/DPPH assays (IC₅₀=30/330 and 60/260 µg ml⁻¹ respectively). Phytochemical analyses of the fractions indicated presence of carbohydrates, flavanoids and steroids in both organisms. The hexane fraction of *Limnothrix* sp exhibited higher UV-Vis (300–450 nm) absorption, indicating presence of aromatic antioxidant compounds like flavanoids. This fraction was relatively non-toxic towards mouse fibroblast 3T3 cells (IC₅₀ 400 µg ml⁻¹) and in fact, protected live cells from UV induced intracellular reactive oxygen species (ROS) stress. On the contrary, the hexane fraction of *Leptolyngbya* sp. had lower UV-Vis absorption, richer in alkaloids and also more cytotoxic (IC₅₀ 200 µg ml⁻¹) towards the mouse fibroblast 3T3 cells. In short the hexane fraction of *Limnothrix* sp. is a safe antioxidant additive for food and healthcare products. On the other hand, the hexane fraction of *Leptolyngbya* sp. is rich in alkaloids and was cytotoxic, with potential application in cancer therapy.

Keywords: Marine cyanobacteria, Arabian Sea, antioxidant, UV, ROS, Cytotoxicity

INTRODUCTION

In recent years, marine cyanobacteria are being recognized as a potential source for novel antibacterial, anticancer (cytotoxic) and antioxidant molecules (Dixit & Suseela, 2013). It is possible that antioxidant compounds are produced by these organisms as an adaptive response to mitigate the ill effects of high exposure to solar radiations. Within the solar UV radiations, the higher energy UV-B (315–280 nm) cause direct cellular damage while the lower energy UV-A (400–315 nm) act indirectly via reactive oxygen species (ROS) mediated pathway (Armstrong & Krickler, 2001; Brash *et al.*, 1991; Maltzman & Czyzyk, 1984). UV-A radiation convert intracellular triplet oxygen into reactive singlet state (³O₂ → ¹O₂), which, then produce ROS through a cascade of intracellular energy transfer reactions (Banks, Board, Carter, & Dodge, 1985; Hader, Kumar, Smith, & Worrest, 2007). Increased ROS levels lead to several harmful biochemical changes viz., lipid peroxidation, protein denaturing / damage, breakage of genetic materials (Asok *et al.*, 2012; Maisch, Bosl, Szeimies, Lehn, & Abels, 2005) and even destruction of biochemical components vital for physiological metabolisms (Halliwell, 1996). Antioxidants help organisms to mitigate ROS stress and protect food/cosmetics from oxidative decay (Halliwell, 1996; Rastogi, Richa, Sinha, Singh, & HÅnder, 2010). Many of these compounds also prevent the progression of major human degenerative diseases (Ames, Shigenaga, & Hagen, 1993; Takamatsu *et al.*, 2003). The repertoire of natural antioxidants include polyphenols, flavonoids, vitamins, melatonin, pycocyanins and phlorotannins (Chu, Lim, Radhakrishnan, & Lim, 2010; Gantar & Svircev, 2008; Kang *et al.*, 2004; Pandey & Rizvi, 2009; Phang, Malek, & Ibrahim, 2013), while butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA) and tetra-butylated hydroquinones (TBHQ) are some of the synthetic compounds in market today. However, high cytotoxicity and lack of broad spectrum activity still limits their widespread usage (Kahl & Kappus, 1993; Pandey & Rizvi, 2009).

Marine cyanobacteria are a rich source for several novel toxins, natural vitamins, carotenoids, phycobiliproteins, *etc.* (Beltron & Nielan, 2000; Burja, Banaigs, Abou-Mansour, Grant Burgess, & Wright, 2001; Carmichael Wayne, Mahmood Nik, & Hyde Edward, 1990; Dixit & Suseela, 2013; Jha & Zi-

rong, 2004). These organisms are ubiquitous in all niches of marine environments and contribute more than 70 % of the productivity of the open ocean (Muhling *et al.*, 2005). Nearly 300 alkaloids, constituting more than 24 % of all marine natural products used in biomedical research today, are of cyanobacterial origin (Tan, 2013). Antioxidants constitute yet another group of promising metabolites from these organisms (Takamatsu *et al.*, 2003). Most of these compounds were reported from organisms belonging to the order Oscillatoriales, Nostocales, Chroococcales, Pleurocapsales and Stigonimatales (Dixit & Suseela, 2013; Leao *et al.*, 2013). Despite the abundance of bioactive metabolites, commercial exploitation of these organisms is still limited, mainly due to their slow growth rate in laboratory conditions and other practical problems associated with their mass culture (Leao *et al.*, 2013). Thus, though about 750 botanically identified cyanobacterial strains are known from Indian waters (Van Baleen, 1962), systematic chemical investigation of only few of them have been accomplished till date (Kosta, Jain, & Tiwari, 2010; Sundararaman, Averal, Akbarsha, & Subramanian, 1994). Here we report the phytochemical composition of two cyanobacterial strains, *Limnothrix* sp and *Leptolyngbya* sp from Arabian Sea and assayed *in vitro* antioxidant properties, cytotoxicity in mouse fibroblast 3T3 cells and ability to protect live 3T3 cells from UV induced reactive oxygen stress for the first time.

MATERIAL AND METHODS

Cyanobacterial isolates and culture condition

Filamentous cyanobacteria, *Limnothrix* sp. and *Leptolyngbya* sp., were isolated from surface waters of Arabian Sea in Artificial Seawater Nutrient –III (ASN-III) medium and were identified by morphological and molecular tools (Nubel, Garcia-Pitchel, & Muyzer, 1997; Rippka, Deruelles, Waterbury, Herdman, & Stanier, 1979). The 16S rRNA gene sequences of the isolates are submitted to NCBI Genbank (JF4288391: *Limnothrix* sp. and KF793929: *Leptolyngbya* sp). The isolates were purified by serial dilution and maintained in ASN-III medium at room temperature with light: dark cycle of 12:12 hr (40 µmol photon m⁻² s⁻¹

light). The purity of the culture was monitored regularly using optical microscopy and pH of the media was maintained at 7.5 ± 0.5 using 0.1N HCl or NaOH.

Extraction of bioactive molecules

The cells were separated from a month old (i.e. stationary phase) culture by centrifugation at 5000 rpm for 10 min, and pellets were washed with Phosphate Buffered Saline (PBS). The crude extracts of cyanobacteria were prepared by extracting 10 g wet weight of cyanobacteria biomass in 100 ml methanol with occasional shaking for 24 hr at room temperature. The residual cells were separated by passing through Whatmann No11 filter paper and crude extracts were fractionated into hexane and ethyl acetate fractions. The organic fractions were concentrated to dryness using rotary evaporator at 40 °C, dissolved in DMSO to a final concentration of 1000 μgml^{-1} and stored at -20°C until used. The absorbance spectrum of the extracts from 300 to 700 nm was measured in a multimode microplate reader (Biotek instruments, USA).

Phytochemical analysis of extracts

Phytochemical analysis were carried out following standard protocols to qualitatively analyze the presence of various compounds such as alkaloids, carbohydrates, proteins, terpenoids, phenols, tannins, flavanoids, steroids and saponins in the extract (Antony *et al.*, 2011). Here the indicator reagents were added to separate glass tubes containing 1 ml aliquots of extracts (100 μgml^{-1}), and the presence of phytochemicals were recorded as color change. Based on the intensity of color formation, the hexane and ethyl acetate fractions were classified into negative (-), low (+) and high (++) for the presence of different phytochemicals. The presence of alkaloids was confirmed with the formation of reddish-brown precipitate on addition of few drops of Wagner's reagent. Wagner's reagent was prepared by dissolving one gram of iodine and three grams of potassium iodide in 50 ml of distilled water. Carbohydrates were confirmed by the formation of brick-red precipitate on boiling the extract with equal volume of Fehling's reagents A and B for one minute. Proteins in the extract formed violet coloration on addition of equal volume of Ninhydrin solution (0.1% in methanol). Phenols and tannins formed dark green color on addition of few drops of 5 % FeCl_3 to the extract. Formation of reddish-brown precipitate upon addition of concentrated H_2SO_4 to a solution of the extract in chloroform confirmed presence of terpenoids. In the same assay, formation of greenish yellow in the lower H_2SO_4 layer and reddish brown ring in the upper layer indicated the presence of steroids. Flavanoids form yellow color on addition of few drops of NaOH (1N) solution, which becomes colorless upon acidification with HCl (1N). Formation of froth on addition of few drops of water, oil or Na_2HCO_3 to the extract confirmed presence of saponins.

Determination of antioxidant activity

Three different bioassays, viz., ABTS [2, 2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) decoloration assay, DPPH (1-diphenyl-2-picrylhydrazyl (α , α -diphenyl- β -picrylhydrazyl) radical scavenging assay and total antioxidant activity by FRAP (Ferric reducing antioxidant power) method were employed. In ABTS decoloration assay, the ABTS radical cations (ABTS*) were generated by treating ABTS stock solution (7mM) with 2.45 mM potassium persulfate at room temperature, in dark for 12–16 hr. Varying concentrations of hexane and ethyl acetate fractions (20, 40, 60, 80, 100 μgml^{-1}) were added separately to one ml of diluted solution containing ABTS* (A_{734} 0.706) and incubated at room temperature for seven minutes. Absorbance was measured at 734 nm using BHT as positive control. Negative control without any extract was also maintained. The percentage ABTS* scavenging activity was calculated using the formula:

$$\text{ABTS* Scavenging activity (\%)} = \left\{ \frac{N_1 - N_2}{N_1} \right\} \times 100$$

Where N_1 and N_2 are the absorbance of control and sample groups.

In DPPH assay, different concentrations of the organic fractions (100-300 μgml^{-1}) were mixed with 1.0 ml of 0.135 mM DPPH in methanol, made up to a final volume of 2.0 ml and kept in dark for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as positive control. The radical scavenging activity was determined based on percentage inhibition of absorbance, which was calculated using the following formula:

$$\text{Inhibition of absorbance (\%)} = \left\{ \frac{N_1 - N_2}{N_1} \right\} \times 100$$

Where N_1 and N_2 are the absorbance of control and sample groups.

For FRAP assay different concentrations of the organic fractions (100-500 $\mu\text{g ml}^{-1}$) were allowed to react with 2.85 ml of substrate solution in a final volume of 3.0 ml for 30 min under dark. The substrate solution was prepared by mixing acetate buffer (300 mM, pH 3.6): TPTZ (2,4,6-tripyridyl-s-triazine 40 mM): $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (20 mM) in a ratio of 10:1:1. Absorbance of the coloured product (ferrous tripyridyltriazine complex) was measured at 593 nm. Ascorbic acid was

used as a standard and activity values were calculated from slope of the standard curve. Results were expressed in $\mu\text{g ml}^{-1}$ ascorbic acid.

Cytotoxicity assay

Cytotoxicity of the hexane fractions of *Leptolyngbya* sp and *Limnothrix* sp, having higher antioxidant activity, were estimated using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium chloride (MTT) assay. Here, $\sim 1 \times 10^6$ mouse embryonic fibroblast cells (3T3) were inoculated into 96 well tissue culture plates containing Dulbecco's modified Eagle's medium [DMEM] supplemented with 10 % fetal bovine serum [FBS] and incubated for 48 hr at 37 °C. The cells were washed with phosphate-buffered saline [PBS], and the medium was exchanged with DMEM containing different sample concentrations (100, 200, 300, 400 and 500 $\mu\text{g ml}^{-1}$) of hexane fractions of cyanobacteria. The cells were incubated for 24 hr at 37 °C and subjected for MTT assay following standard protocol. Briefly, cells were washed and supplemented with 50 μl of MTT solution (5 mg ml^{-1}) prepared in PBS and kept for incubation under dark at 37 °C for 3 hr. Subsequently, the viability of the cells was measured as a function of reduction of MTT to insoluble formazan by mitochondrial dehydrogenase enzyme of healthy cells. Formazan crystals were dissolved in dimethyl sulphoxide and the absorbance was recorded at 570 nm using a multimode microplate reader (Biotek instruments, USA). All tests and analyses were run in triplicate.

UV treatment and ROS assay

The efficiency of the hexane fraction of *Limnothrix* sp in protecting 3T3 cells from UV induced ROS toxicity was measured using CellROx deep red oxidative stress detection reagent (Molecular probes, Life Technologies USA). Here, 3T3 cells prepared in 96 well microplate were supplemented with PBS (pH 7.4) containing 100 $\mu\text{g ml}^{-1}$ hexane fraction and exposed to 200 Jm^{-2} UV light (254 nm UV-C) using a UV gene linker apparatus equipped with an energy output control. Control cells without extract under UV and without UV exposure were also maintained. Cells were washed copiously with PBS and stained with CellROX Deep red reagent following the protocol of the manufacturer. CellROX Deep red reagent is a fluorogenic probe designed to reliably measure ROS in live cells. CellROX Deep Red Reagent was added at a final concentration of 25 μM to the cells, and then incubated for 30 min at 37 °C. Subsequently, the cells were washed with PBS and the fluorescent intensities were measured at excitation/emission maxima at 640/665 nm using a multimode microplate reader (Biotek instruments USA).

RESULTS AND DISCUSSION

Globally, demand for non-cytotoxic antioxidant molecules as food preservatives and in health management are currently on the rise. Earlier studies have established antioxidant properties from crude extracts and fractions of several cyanobacteria, viz., *Oscillatoria* sp, *Microcystis aeruginosa*, *Cylindrospermopsis raciborskii* (Babu & Wu, 2008), *Synechococcus* sp, *Chlamydomonas nivalis* and *Nostoc ellipsosporum* (Li *et al.*, 2007). Chemical examination had indicated phenols and microsporine like aminoacids to be the prominent antioxidant metabolites of cyanobacteria (Takamatsu *et al.*, 2003). Another interesting group of compounds are pycocyanins from *Oscillatoria tenuis* (Thangam *et al.*, 2013). Flavanoids, the prominent antioxidants in terrestrial plants have also been reported from marine cyanobacteria (Booiji-James, Dube, Jansen, Edelman, & Mattoo, 2000; Rastogi *et al.*, 2010; Wada, Sakamoto, & Matsugo, 2013). The phytochemical analysis showed that the hexane extract of the *Leptolyngbya* sp and *Limnothrix* sp were rich in flavanoids, steroids and terpenoids (Table 1). The UV-Vis absorption spectra of the hexane and ethyl acetate fractions (Figure 1) were comparable to that of flavanoids, the well known antioxidant molecules found in terrestrial plants and cyanobacterial sources (Rastogi *et al.*, 2010; Wada *et al.*, 2013). Flavanoids are a class of low molecular weight natural polyphenolic compounds that have the ability to donate a hydrogen atom or an electron in order to form stable radical intermediates, which renders them antioxidant properties (Hajimahmoodi *et al.*, 2010). Their chemical diversity, absorption properties and biotechnological potentials as antioxidant, antibacterial, antioncogenic and antiinflammatory agents are reviewed (Rastogi *et al.*, 2010; Ververdis *et al.*, 2007).

The antioxidant activity of hexane fractions of *Limnothrix* sp and *Leptolyngbya* sp were confirmed using ABTS decoloration assay, DPPH radical scavenging activity and total antioxidant activity by FRAP assay. The superoxide anion radical scavenging properties of the fractions were measured as its ability to decolorize ABTS*. The maximum percentage of decolorization was observed with hexane fraction of *Leptolyngbya* sp. (IC_{50} =30 $\mu\text{g ml}^{-1}$) while the other fractions showed comparable activity at relatively higher concentrations (IC_{50} 60-80 $\mu\text{g ml}^{-1}$, Figure 2a). The DPPH assay (Figure 2b) revealed a dose-dependent activity, with highest being shown by the hexane fraction of *Limnothrix* sp (IC_{50} =260 $\mu\text{g ml}^{-1}$), followed by the two ethyl acetate fractions, while the hexane fraction of *Leptolyngbya* sp had low activity (IC_{50} =330 $\mu\text{g ml}^{-1}$). FRAP assay revealed the reducing potential of these fractions. Here too, the hexane fractions of *Limnothrix* sp. was found the most promising (IC_{50} =117 $\mu\text{g ml}^{-1}$), followed by

hexane fraction of *Leptolyngbya* sp. ($IC_{50}=176 \mu\text{g ml}^{-1}$, Table 2). These values are promising and comparable to the antioxidant extracts from other terrestrial (Antony et al., 2011; Boonchum et al., 2011; Rodrigues, Mariutti, & Mercadante, 2012; Sajeesh, Arunachalam, & Parimelazhagan, 2011) and cyanobacterial sources (Shanab, Mostafa, Shalaby, & Mahmoud, 2012). Previously, the antioxidant properties of the hexane extracts of *Anabaena* sp., *Chroococcus* sp., *Nostoc* sp., *Microchaete* sp., *Tolypothrina* sp. and *Fischerella* sp were evaluated and found positive correlation with the level of phenolic compounds (Hajimahmoodi et al., 2010). The aqueous extract of *Anabaena flos-aquae* and *Nostoc humifusum* reported the antioxidant properties ($IC_{50} \leq 100 \mu\text{g ml}^{-1}$), while they were toxic at this concentrations (Shanab et al., 2012). Interestingly, the non-cytotoxic fractions such as ethanol extracts of *Phellinus merrillii*, methanol extract of *Agelas oroides* and aqueous extract of *Saccharomyces cerevisiae* showed antioxidant property at very high concentrations (Heng-yuan CHANG, 2007; Orhan et al., 2012; Phonnok, Tanechpongamb, & Wongsatayanon, 2010). Although an ideal antioxidant molecule for application in food and health care products are expected as noncytotoxic and active in cellular environment, many of the synthetic as well as natural antioxidants were reported to be cytotoxic too (Kahl & Kappus, 1993; Shanab et al., 2012). *In vitro* assays indicate the ability of an antioxidant molecule to donate electrons to stabilize free radicals, but do not indicate its cytotoxicity or efficiency in scavenging intracellular ROS. It is possible that some antioxidant molecules, after donating electrons, might become toxic to host cells. Interestingly the hexane fraction of *Limnothrix* sp showed antioxidant properties without inducing any cytotoxicity. Here the cytotoxicity of hexane fractions to mouse fibroblas 3T3 cells were studied using standard MTT assay. In MTT assay, the viability of animal cells were measured as their ability to produce mitochondrial dehydrogenase enzyme which reduce yellow MTT into purple formazan. A decrease in the reduction of MTT is an index of mitochondrial damage and cell death. During our studies, *Leptolyngbya* sp (IC_{50} : $200 \mu\text{g ml}^{-1}$) was found more toxic than *Limnothrix* sp. (IC_{50} : $400 \mu\text{g ml}^{-1}$) (Figure 3). Presence of alkaloids, as revealed in the phytochemical analysis of the hexane fraction of *Leptolyngbya* sp. could be the reason for its higher toxicity. Our result is also in agreement with earlier reports on toxicity of this organism (Costa et al., 2014). Recent studies reported that roughly one third of terrestrial and half of marine cyanobacteria are potentially exploitable for bioactive molecules with cytotoxic potential (Hrouzek et al., 2016; Raja, Hemaiswarya, Ganesan, & Carvalho, 2015).

The ability of hexane fraction of *Limnothrix* sp to protect 3T3 cells from UV induced ROS stress was assayed using CellROX Deep red reagent (Molecular probes by Life Technologies, USA). Here, the cell permeable deep red reagents are non-fluorescent in reduced state, but exhibit strong fluorogenic signals upon oxidation by UV induced ROS. Exposure of 3T3 cells to UV radiation for 5 minutes increased ROS production by 1.6 times while it was comparable with control levels in cells supplemented with $100 \mu\text{g ml}^{-1}$ of the above hexane fraction of *Limnothrix* sp (Figure 4). Under normal conditions, the UV radiations induce direct or indirect toxicities to animal cells. High energy carrying UV-B photons (280 – 315 nm) cause skin reddening and sunburns, damage the superficial epidermis and plays a key role in the development of skin cancer. On the other side, UV-A radiation (315 – 400 nm) transfers energy to molecular oxygen (3O_2), leading to the generation of singlet oxygen (1O_2) and initiate a cascade of energy transfer reactions leading to the production of reactive oxygen species (ROS). A minimum concentration of ROS is essential for maintaining the regular metabolism of cells, and antioxidants are available in cells for self protection from ROS mediated toxicities. The elevated levels of ROS induce damage to DNA and protein leading the the development of extreme chronic conditions like ageing, arthritis, diabetes, tissue damage, muscular dystrophy and neurological disorders. Aslo, ROS are known to be the prominent cause of food decay, leading to rancidity, toxicity and destruction of biochemical components important in physiological metabolism. ROS stress assay results in live 3T3 cells indicates that the hexane fraction of *Limnothrix* sp can scavenge intracellular ROS and thus could be a potential antioxidant for application at cellular level.

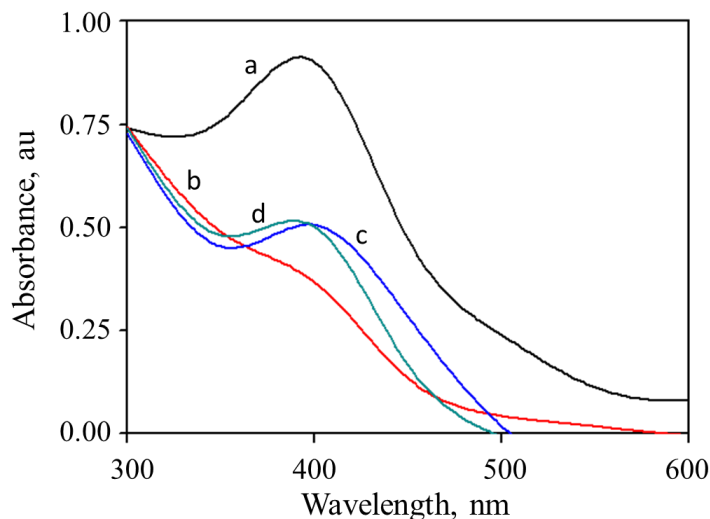


Figure 1 Absorption spectra of hexane (a,c) and ethyl acetate (b,d) fractions of *Limnothrix* sp (a,b) and *Leptolyngbya* sp (c,d)

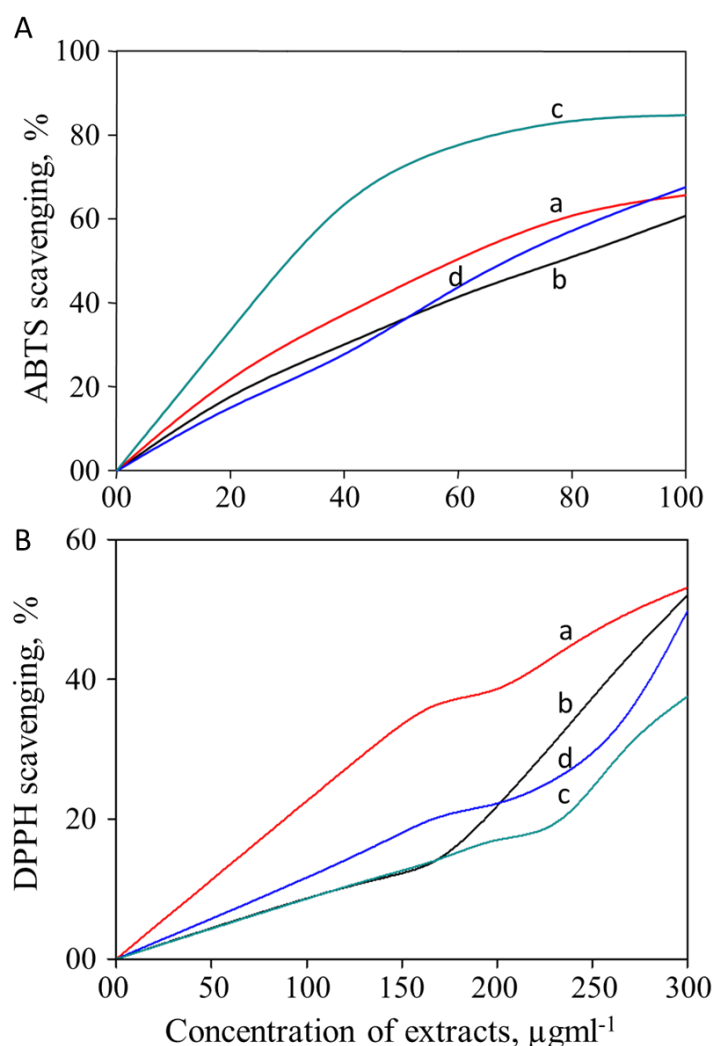


Figure 2 Antioxidant properties of hexane and ethyl acetate fractions of the samples expressed as their ability to scavenge ABTS (A) and DPPH (B) radicals. Hexane (a,c) and ethyl acetate (b,d) fractions of *Limnothrix* sp (a,b) and *Leptolyngbya* sp (c,d) are labelled

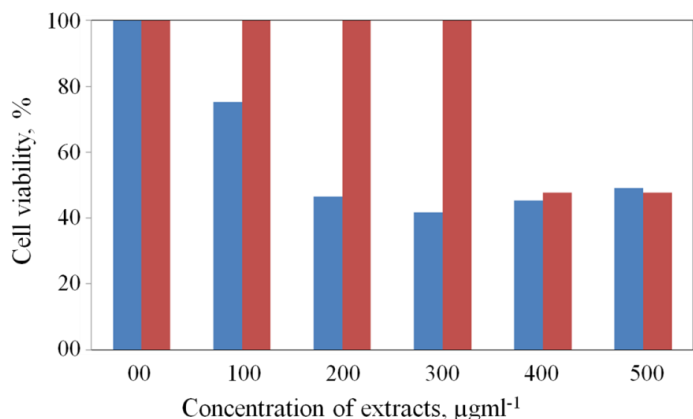


Figure 3 Viability of mouse fibroblast 3T3 cells in the presence of increasing concentrations of hexane extracts from *Limnothrix* sp (red) and *Leptolyngbya* sp (blue)

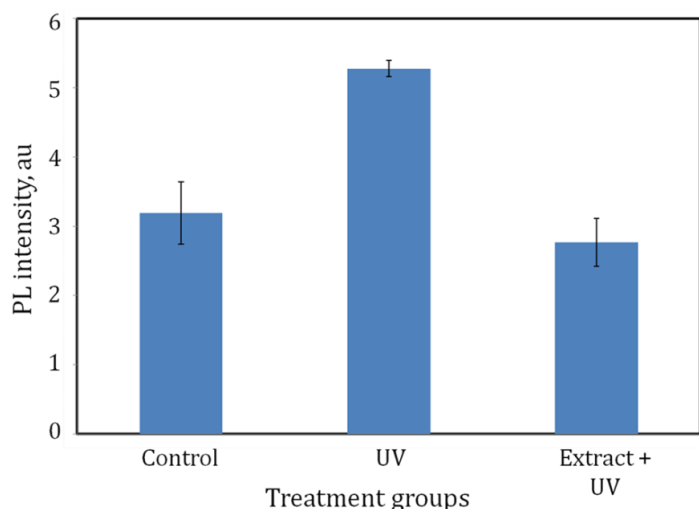


Figure 4 Efficiency of hexane extract ($100 \mu\text{g ml}^{-1}$) of *Limnothrix* sp in scavenging UV induced intracellular ROS measured by CellROX deep red reagent. The photoluminescence (PL) intensity indicated the mean concentration of ROS in 3T3 cells, without UV (control), with (UV) and in the presence of extract (extract +UV)

Table 1 Phytochemical analysis of hexane and ethyl acetate fractions of *Leptolyngbya* and *Limnothrix* sp. The results are expressed as negative (-), positive at low (+) and high (++) levels.

Phytochemical	<i>Leptolyngbya</i> sp		<i>Limnothrix</i> sp	
	Hexane	Ethyl acetate	Hexane	Ethyl acetate
Alkaloids	++	++	+	-
Carbohydrate	-	+	-	+
Aminoacids	-	+	-	+
Phenols & Tannins	-	+	-	+
Terpenoids	++	+	++	++
Saponins	-	-	-	-
Flavonoids	++	-	++	++
Steroids	++	+	++	++

Table 2 Antioxidant properties of hexane and ethyl acetate fractions of samples expressed as their ability to reduce ferric ions (FRAP assay)

Extracts	FRAP values ($\mu\text{g/Ascorbic acid equivalent}$)	
	<i>Leptolyngbya</i> sp	<i>Limnothrix</i> sp
Hexane	176	117
Ethyl Acetate	260	225

CONCLUSION

Though cyanobacteria are known to be a rich source for antioxidants, toxicity prevents their widespread usage as antioxidants in food/cosmetics. We were particularly interested in identifying safe antioxidants which could be used directly, without any cumbersome de-toxification processes. To evaluate these contrasting properties, we selected two filamentous cyanobacteria ubiquitous in

Arabian Sea and evaluated their toxicity and antioxidant property using specific assays. At the same time, a qualitative idea, regarding different types of compounds present in them were obtained through specific phytochemical analyses. The hexane fraction of *Limnothrix* sp was found to be rich in non-cytotoxic antioxidant molecules which are also capable of protecting animal cells from UV induced ROS stress. The above benevolent properties make this organism a good choice for non-toxic antioxidant products for use in beverages and other food products and in cosmetics. At the same time, the higher cytotoxicity of the hexane fraction of *Leptolyngbya* sp. may be useful in cancer therapy and is to be explored further.

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