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Original Article

UV Induced Biosynthesis of Cyano-sunscreen "Scytonemin" by *Leptolyngbya mycodia* and its Effectual Antioxidant Activity

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Abstract

The indole-alkaloid scytonemin, an ecologically and pharmaceutically important secondary metabolite, is exclusively biosynthesized by some cyanobacteria. Due to its photoprotective function and valuable antioxidant capacity, this cyano-sunscreen may be of great value for production of natural sunscreen in cosmetic and other pharmaceutical industries. As scytonemin is only produced by some cyanobacterial species, identification of novel strains and verification of synthesis induction factors are important research areas. In the present study, using the high-performance liquid chromatography and mass spectrometry analyses, scytonemin was characterized ($UV\lambda_{max}$) at 370 nm; m/z 545) in two filamentous cyanobacteria: *Leptolyngbya mycodia* and *Phormidium* sp. Under photosynthetic active radiation (PAR), *L. mycodia* revealed superior growth as well as scytonemin specific content of 0.0427 (Aλ/mg d.w.). As one of the physicochemical stressors on regulation of scytonemin biosynthesis, the role of UV irradiation in synthesis induction was examined. A remarkable change was observed on scytonemin specific content under PUAB regime (280-700 nm) compared to PAR (400–700 nm). UV-B (280–315 nm) significantly induced scytonemin synthesis up to 4.25 Aλ /mg d.w. while synthesis (2.31 Aλ/mg d.w.) to a lesser extent was observed under UV-A (315-400 nm). Moreover, present study confirmed the role of extracted scytonemin as an active antioxidant, indicating its strong radical scavenging (IC50=48.84%) with relatively high (61%) antioxidant rate at concentration of 200 µg/l. This is the first report on the UV-induced scytonemin biosynthesis by cyanobacterium *L. mycodia* and its remarkable antioxidant activity is of great value for future biotechnological research and development of natural sunscreens.

*Keywords***:** Cyanobacteria, Natural sunscreen, Photoprotection, Radical scavenging activity, Secondary metabolite, UV radiation.

1. Introduction

Depletion of the stratospheric ozone has led to the increased level of harmful ultraviolet radiation (UVR) reaching the earth's surface. The highly energetic UV-C (100-280 nm) of the incoming solar radiation can be absorbed by oxygen and ozone and lost through atmospheric scattering, while UV-B (280–315 nm) and UV-A (315–400 nm) may affect normal state of life either through direct effects on cellular DNA and proteins or indirectly by the production of reactive oxygen species (ROS) [1, 2]. UVR is

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lethal for most sun-exposed organisms ranging from prokaryotes to eukaryotes, including humans [3, 4]. Therefore, to protect humans' skin from the UV-induced damage and aging, the market has offered various inorganic (mineral/physical blocker) and organic sunscreen filters to absorb the UV-radiation [5, 6]. However, these filters produce highly oxidizing radicals causing toxicity in human and ecosystems, in addition to showing side effects such as endocrine disruptor effects [7, 8] and contact dermatitis in children [9]. Accordingly, during the last two decades, investigations on natural UV protecting compounds with effective roles for skin benefit have become the focus of cosmetic research [10].

From biotechnological and industrial point of views, cyanobacteria, rich producers of secondary metabolites, are considered as appropriate microorganisms with interesting bioactive potential for cosmetics and cosmeceuticals [11]. Cyanobacteria, the oxygenic photoautotrophic gram-negative prokaryotes, have contributed to life development in earth when there was no ozone layer by evolving several lines of defensive mechanisms to counteract the deleterious effects of UVR during the Precambrian era [12, 13]. These include the avoidance, scavenging of ROS by antioxidants, repair and re-synthesis and programmed cell death (PCD) as well as the synthesis of UV-absorbing/screening compounds such as mycosporine-like amino acids (MAAs) and scytonemin [14].

MAAs are small (<400 Da), colorless, water-soluble compounds composed of cyclohexanone/cyclohexenimine chromophores with various substituents produced by taxonomically diverse organisms ranging from heterotrophic bacteria, lichens, cyanobacteria, fungi, microalgae/macroalgae to several animals [15, 16]. Production of MAAs as osmolytes and/or antioxidants as well as screening UVR has been well studied among cyanobacteria [17, 18]. In contrast, scytonemin, a lipophilic, yellow-brown exopolysaccharide sheath pigment with a molecular mass of 544 Da and a symmetrical heterocyclic skeleton structure, has exclusively been isolated from cyanobacteria.

Scytonemin, derived from the intermediates of aromatic amino acid biosynthesis [2, 19], is known to be the earliest developed mechanism of ultraviolet protection, more ancient than the flavonoids or melanin [20]. Scytonemin (oxidized/reduced forms) and its four other derivatives have been reported in various cyanobacteria growing in brightly lit habitats with desiccation cycle, nutrient limitation, and high temperature [21]. This highly stable pigment was first reported by Nägeli in 1849 in some terrestrial cyanobacteria and later it was termed as scytonemin [22, 23]. Garcia-Pichel and Castenholz were the pioneer workers who studied scytonemin in several cyanobacterial species from laboratory cultures to natural populations, including crusts, mats, and colonies [24].

The induction of scytonemin biosynthesis in numerous cyanobacterial strains such as *Aphanocapsa* sp., *Chlorogloeopsis* sp., *Chroococcus* sp., *Diplocolon* sp. [25], *Calothrix* sp. [9], *Anabaena* sp., *Tolypothrix* sp. [25], *Lyngbya* sp. [24-26] and *Scytonema* sp.

[27-29] was examined in response to UV treatment and various abiotic stresses. Pentecost reported correlations between the UV flux and scytonemin content in *Scytonema* and *Rivularia* sp., where the correlation was negative for the former and positive for the later [30]. Considerably high amount of scytonemin was required for continued photosynthesis under high UV flux in *Calothrix* sp. [31]. *Tolypothrix byssoidea* showed a prominent absorption at 260 and 384 nm, denoting the presence of scytonemin [32]. Additionally, scytonemin was reported to prevent the entry of about 90% of UV-A radiation in cyanobacterial cells [20, 33]. Moreover, Rastogi *et al*. studied scytonemin synthesis in diverse cyanobacteria and indicated the effective role of UV radiation on synthesis induction under varying environmental conditions [34-36].

Recent studies have established the importance of scytonemin in scavenging of ROS, revealing its potential application in cosmetics and cosmeceuticals [11, 37, 38]. Additionally, scytonemin is well known for its anti-inflammatory and anti-proliferative activities. Moreover, the unique chemical structure of scytonemin offers a potential scaffold for further chemical modification to develop a new class of therapeutically useful drugs in treating hyperproliferative disorders as well as in controlling the growth of cancerous cells. Also, the fat-soluble nature of scytonemin would allow them to easily blend with oil-based foundations, water-in-oil moisturizers, anhydrous creams, or waxes, many of which tend to have a higher SPF and are waterproof [21, 39].

Scytonemin production due to its role as a photo-protectant particularly against UV radiation and its application as a potent anti-cancerous drug, has therefore become a striking target in biotechnology and biomedical research. This valuable natural product is only produced by some but not all cyanobacterial species, and therefore, identification and development of novel strains are required for scytonemin production at large scale. In this study, we first examined two filamentous cyanobacteria for their potential scytonemin production. Scytonemin was then partially characterized and measured by HPLC and LC/MS methods and the superior strain was chosen. Subsequently, the complex stress response pathway of this strain was stimulated under different wavelength bands of UV radiation to enhance scytonemin production. Finally, the antioxidant activity of the extracted scytonemin was assessed in terms of radical scavenging capacity against free radicals as a photoprotective criteria.

2. Materials and Methods

2.1. Cyanobacterial Strains: Maintenance and Inoculum Preparation

Strain isolation was not the aim of this work and both studied cyanobacteria were selected based on previous reports on scytonemin synthesis in filamentous strains. Axenic cultures of two filamentous cyanobacterial strains *Leptolyngbya mycodia* (IBRC-M-5200) in solid and *Phormidium* sp. (IBRC-M-5032) in liquid phases were obtained from Iranian Biological Resource Center.

For secure and stable maintenance, long-term preservation techniques were employed. Briefly, centrifuged cells at mid-log phase

cultures were aseptically transferred to the medium containing dimethyl sulfoxide (DMSO) as a cryoprotectant and mixed thoroughly to give a final concentration of 5% (v/v) DMSO and then transferred in sealed vials to freeze at -70 °C [40].

Both strains were routinely stored at 4 °C. To provide fresh inoculum, cells were sub-cultured in defined media. At mid-log phase, this culture was centrifuged (4,000 × g, 15 min, 20 °C) and after discarding the upper phase, cells were re-suspended in BG11 medium (1:3 (v/v)). This fresh cell suspension was used to inoculate the medium at 5% (v/v) in all experiments.

2.2. Culture Medium

Cultures were grown in an autoclaved liquid medium BG-11 containing (in g/L each): $NaNO_3$, 1.5; $MgSO_4 \cdot 7H_2O$, 0.075; Na_2CO_3, 0.02; K_2HPO_4, 0.04; Citric acid, 0.06; $FeNH_3$-Citrate, 0.06; $CaCl_2.2H_2O$, 0.036 and $Na_2EDTA.2H_2O$, 0.001 and 1 ml/L trace metal solution containing (in g/L each): H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.222; $Na_2MoO_4.2H_2O$, 0.39; $CuSO_4.5H_2O$, 0.079 and $Co(NO_3)_2.6H_2O$; 0.0494 was used in all experiments [41]. This medium was supplemented with F/2 vitamin solution (1 ml/L) containing (in mg/L each): Thiamine HCl (B1), 200; Biotin (H), 1 and Cianocobalamin (B12), 1 [42].

2.3. Cultivation Method

All experiments were carried out in batch culture (500 ml Erlenmeyer flasks containing 100 ml medium) placed in a shaker incubator, manually equipped with internal irradiation sources from the top and a digital lux meter. The culture was incubated at 25 °C and 85 rpm under various irradiation regimes. At each sampling time, two or three flasks were collected and the entire content of each flask was analyzed.

2.4. Sources and Regimes of Irradiation

In this research, three irradiation regimes were tested using various sources. To evaluate cell growth and obtaining cultures at mid-log phase, P regime (PAR) was exploited in which 4-6 cool white 20 W fluorescent tubes (Pars Shahab, T10) were placed at suitable distances leading to 7 and 10 w/m^2 irradiances under light/dark cycle of L:16/D:8 h. To assess scytonemin induction, two combination regimes, namely PUA (P regime + UV-A) and PUAB (P regime + UV-A + UV-B) were exploited. UV-A (315–400 nm) and UV-B (280–315 nm) irradiations were provided by Philips TL/15 w and TL /20 w broadband tubes, respectively.

2.5. Experiments

Scytonemin is exclusively biosynthesized by several but not all cyanobacteria and generally in response to UVR. To investigate the intrinsic potential of the selected strains in scytonemin synthesis in the absence of abiotic stresses, in this work, we first examined the growth and scytonemin production under normal environment (no induction) as control experiments. Afterwards, the synthesis efficiency was examined in response to drastic effect of high energy irradiation as a defensive

mechanism. Ultimately, as an additional protection strategy against ROS, the antioxidant activity of the partially characterized scytonemin was assessed.

2.5.1. Biomass Production

In the first set of experiments, the growth behavior of the two cyanobacterial strains separately exposed to PAR at intensities of 7 and 10 w/m^2 were compared. Hence, BG11 medium was individually inoculated by a loop-full of each strain and the culture was exposed to PAR. In this set, the time course of growth was examined within 15 days of cultivation. At each sampling time, two flasks were collected and the biomass content of each flask was analyzed by gravimetric method.

2.5.2. Scytonemin Characterization and Quantification

Second set of experiments were conducted to study scytonemin synthesis potential. First of all, some flasks were inoculated and cultivated under P regime. After 15 days of incubation, the whole flask content was harvested, dried and extracted for the analyses of scytonemin content according to the analytical methods.

2.5.3. UV Induction Experiments

UV induction experiments were performed to test how irradiation regimes affected scytonemin synthesis. These experiments were conducted by using the superior strain selected in the previous section. In three separated experiments, the growing cultures under P regime at mid-log phase were exposed continuously to P, PUA and PUAB regimes. For each irradiation regime, three flasks were collected at the subsequent time courses of 24, 48 and 72 h. The dried harvested biomass of each flask was prepared for scytonemin analyses.

2.5.4. Antioxidant Function Assessment

The last set of experiments was carried out to examine the free radical scavenging activity of partially extracted samples. Similar to previous set, the growing cultures at mid-log phase were exposed to PUA and PUAB regime for 72 h and the dried harvested cells were analyzed by DPPH assay.

2.6. Analyses

2.6.1. Biomass Measurement

Gravimetric measurement method was used to assess cell growth. Aliquots of 100 mL filamentous culture was centrifuged (at 4000×g for 15 min) and the supernatant was discarded. The settled biomass was washed by distilled water and re-centrifuged. At last, the oven dried biomass (at 45 °C) was recorded as biomass concentration in g d.w. cell/L [43].

2.6.2. Extraction of Scytonemin and Other Pigments

Scytonemin and internal cell pigments are readily extractable with a combination of methanol/ethyl acetate (1:1v/v) as solvent. For extraction, the whole dried harvested biomass was oven dried (45 °C), weighted and ground in solvent (5 ml). Samples were kept overnight at 4 °C under darkness and then placed in ultrasonic water bath (15 min/at 20 °C) to complete the extraction. To remove the solvent insoluble impurities, sample was centrifuged (at 4000×g for 15 min at 4 °C) and the upper phase was transferred to a new vial for

solvent removal (under darkness at ambient temperature) [24].

2.6.3. Spectrophotometric Analysis of Scytonemin and Other Pigments

For qualitative analysis, the extracted samples were dissolved in 100% acetone (5 ml) and the UV-visible spectra were obtained using spectrophotometer (UVS-2800). Scytonemin (Scyt), total carotenoid (T. Car) and chlorophyll a (Chl a) were quantified using a set of trichromatic equations. Garcia-Pichel and Castenholz developed these equations by recording the ratios of absorbance at three different wavelengths (384, 490 and 663 nm) for the purified acetone solution of scytonemin, Chla and T. Car as given in Eqs. (1-3) [24]:

$$A^*_{384}(Scyt) = 1 \cdot 04A_{384} - 0 \cdot 79A_{663} - 0 \cdot 27A_{490} \quad (1)$$

$$A^*_{490}(Car) = 1 \cdot 04A_{490} - 0 \cdot 08A_{384} - 0 \cdot 026A_{663} \quad (2)$$

$$A^*_{665}(Chla) = 1 \cdot 02A_{665} - 0 \cdot 027A_{384} + 0 \cdot 01A_{490} \quad (3)$$

where A_λ = the measured absorbance at λ and A^* = the absorbance contributed to each pigment. The specific contents are expressed in units of absorbance normalized to dry weight (Aλ /mg). These units represent the absorbance of 1 mg dry weight of material extracted in 1ml acetone in a cuvette with 1-cm path length. Also, the extinction coefficient of scytonemin was assumed 112.6 L/g.cm and the scytonemin content was estimated using Eq. (4), [20].

$$C = A^*/\varepsilon \quad (4)$$

A^* and ε are scytonemin absorbance and its extinction coefficient, respectively.

2.6.4. HPLC and Mass Spectrometry Analysis of Scytonemin

UV-absorbing/screening scytonemin was isolated and partially characterized for both strains. First, the remaining extract was re-dissolved (in 500 µL of 100% methanol) and filtered (0.22 µm pore-sized syringe filter) before HPLC analysis. Partially purified scytonemin was analyzed in Shimadzu HPLC system equipped with UV-detector (LC-10A) with detection wavelength at 370 nm. The samples (20 µL) were injected into the RP-18 HPLC column (5 µm, 250 × 4 mm) and elution was at a flow rate of 1.5 mL/min with the 30-min gradient program through 0-15 min linear increase from 10% solvent A (ultra-pure water) to 100% solvent B (acetonitrile-methanol-tetrahydrofuran, 75:15:10, v/v) and 15-30 min at 100% solvent B [35].

Mass spectrum of extracted sample was recorded using Agilent LC-MS chromatography (6410 Triple-Quad) equipped with a gas nebulizer probe and separation was achieved using Shimadzu Inertsil ODS3 cartridge columns protected by an ODS3 guard column. The injection volume was 10µL and flow rate was 0.4 mL/min. The MS analysis was performed in the positive electrospray ionization (ESI) mode with a scan range from m/z 200–700. Identification of scytonemin was made from its online UV/Vis spectra and mass spectra as described earlier [34, 44].

2.6.5. DPPH Photometric Assay

The radical scavenging capacity of the extracted scytonemin was measured using a DPPH (2,2-diphenyl-1picrylhydrazyl) free

radical scavenging assay as described by Mensor *et al.* with slight modifications [37]. Briefly, stock solution of each scytonemin sample (0.8 mg/mL) were serially diluted in methanol (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 mg/mL) in duplicate set. One mL of a 0.3 mM DPPH methanol solution was added to 2.5 mL of sample solution at different concentrations, and after 30 min dark incubation at room temperature, the absorbance values were measured at 518 nm using a spectrophotometer (UVS-2800) and converted into the percentage of antioxidant activity (AA) using Eq. (5):

$$AA\,\% = 100 - \left\{\left[\left(Abs_{\,sample} - Abs_{\,blank}\right) \times 100\right]/Abs_{control}\right\} \quad (5)$$

Methanol (1.0 mL) plus scytonemin extracted solution (2.5 mL) and DPPH solution (1.0 mL; 0.3 mM) plus Methanol (2.5 mL) were used as a blank and control, respectively. Moreover, ascorbic acid (Merck -100468) was used as a standard antioxidant in comparison with samples.

The antioxidant capacity of each sample was determined using the inhibition concentration (IC50). The IC50 values (concentration in µg/mL required to inhibit DPPH radical by 50%) were estimated from the percentage of inhibition versus log of sample concentration plot, using a Graph Pad Prism software (9.2.0.332).

2.6.6. Statistical Analysis

The results were presented as the mean values of two or three replicates (mean ± SD; n = 2 or 3) and the error bars indicated the deviations. Paired sample test in compared means analysis was applied to evaluate the significance of the data with SPSS statistics software (27, Chicago, IL, USA) to assay the differences among various treatments (the level of significance was set at 0.05).

3. Results and Discussion

3.1. Growth Comparison of Strains

The prime objective was to achieve a high level of viable biomass in a suitable physiological state as an inoculum. Since light intensity as a critical environmental factor affects the photosynthetic rate, growth of each strain was examined under P regime using two intensities of 7 and 10 w/m^{2}s. Growth trends of cyanobacterial cultures over a time course of 2 weeks are shown in Figure 1.

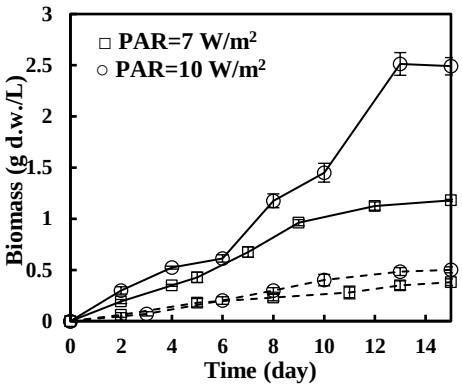

Figure 1. Time courses of growth for *L. mycodia* (—) and *Phormidium* sp. (----) under PAR at two intensities of 7 and 10 W/m^2

Results revealed a significant difference between the two cultures in terms of rate and final harvested biomass. Under both irradiation intensities, *L. mycodia* exhibited higher specific growth rate compared to *Phormiduim* sp. With increased PAR intensity from 7 to 10 w/m^2, there was not a significant difference in harvested biomass of *Phormidium* sp. (P-value=0.878>0.05). However, biomass

synthesis of *L. mycodia* significantly enhanced under the exposure to 10 W/m^2 PAR (P-value=0.033<0.05). At the intensity of 10 w/m^2, *L. mycodia* showed a final biomass concentration of 2.5 g d.w./L, being five times higher than that of *Phormidium* sp. Therefore, *L. mycodia* was selected as a superior cyanobacterial strain for production of growth associated products. Figure 1 also shows that the cultures reached their mid-log phase after 8 days, therefore, 8-day old inoculum was used for inoculation in further experiments.

3.2. Scytonemin Characterization

Preliminary assessment of scytonemin in samples of the two cultures under PAR was separately carried out using the UV-VIS absorption spectra of the acetone extract. The scytonemin absorption was mostly reported at 370 and 386 nm for in-vivo extracted samples and purified forms, respectively [24, 44]. In present study, the absorption was specified at 370 nm related to scytonemin pigment, however the sophisticated serial- extraction procedures to purify scytonemin was not exactly followed [24, 44]. Although the revealed absorption peak could be allocated to scytonemin, more accurate analysis was necessary.

Scytonemin characterization was also followed through the chromatography analyses methods. The HPLC and LC-MS chromatograms were exhibited in Figure 2 for both partially extracted strains. Accordingly, a prominent peak was identified at 370 nm (RT ~18.6 min) which was identical to those reported previously for scytonemin [35, 45-47]. Moreover, the LC-MS obtained peaks in the scan range from m/z 100 to 600 revealed obvious ion peaks for protonated molecules at m/z 545.1 and 545.7 for *L. mycodia* and *Phormidium* sp., respectively. These results are consistent with the earlier MS spectra of scytonemin isolated from different cyanobacteria species [35, 44, 45].

3.3. Scytonemin Synthesis Assessment

After confirmation of scytonemin existence in the cultures of the two strains (see Section 3-2),

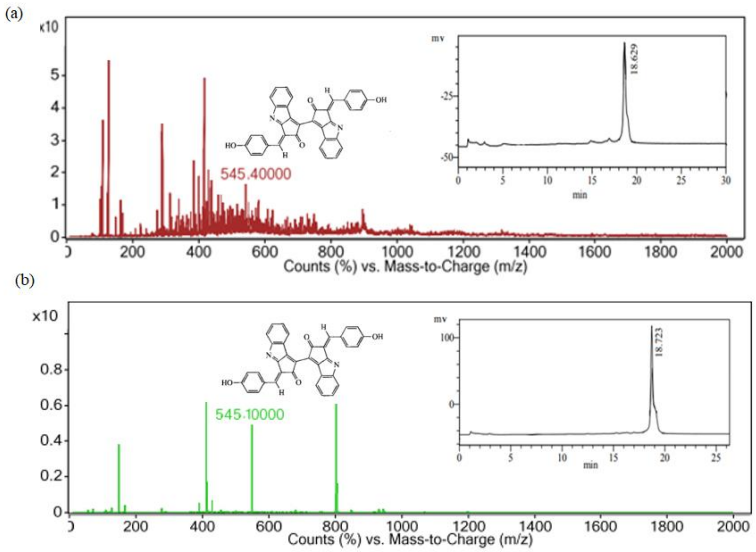

Figure 2. HPLC and LC-MS analyses of partially purified samples showing the scytonemin peak in chromatogram for (a) *L. mycodia* and (b) *Phormidium* sp.

cell content of scytonemin was estimated based on the absorbance normalized to dry weight cell (Aλ/mg d.w.) in trichromatic equations. The scytonemin specific contents of 0.0427 and 0.0131 for *L. mycodia* and *Phormidium* sp., respectively, were obtained. The absorbance results were comparable to those reported by Mushir *et al*., reporting the highest scytonemin content of 0.053 for *A. fertilissima* among 9 screened cyanobacteria cultivated under P regime [48].

Comparison of scytonemin synthesis potential in terms of scytonemin specific content and HPLC-peak area was shown in Figure 3. Results revealed that higher scytonemin (more than three times) was identified in *L. mycodia* compared to *Phormidium* sp. under P regime. Additionally, it can be seen that both measuring methods confirmed the superiority of *L. mycodia* with respect to scytonemin cell content. Considering the higher biomass production (see Figure 1) and scytonemin cell content by this strain, selection of *L. mycodia* for further studies can be justified by the higher scytonemin productivity.

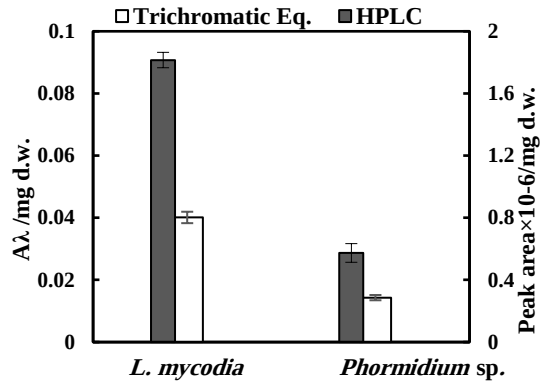

Figure 3. Comparison of scytonemin content in cyanobacterial strains cultivated under P regime in terms of specific content and peak area revealed in spectroscopic and HPLC analyses, the error bars denote standard deviations of means (means ± SD, n = 3).

3.4. UVR Induction Role

Syntheses of scytonemin, total carotenoids and chlorophyll a as well as biomass production were investigated in response to various UVR regimes for *L. mycodia* culture, the results of which are given in Figure 4-a, b.

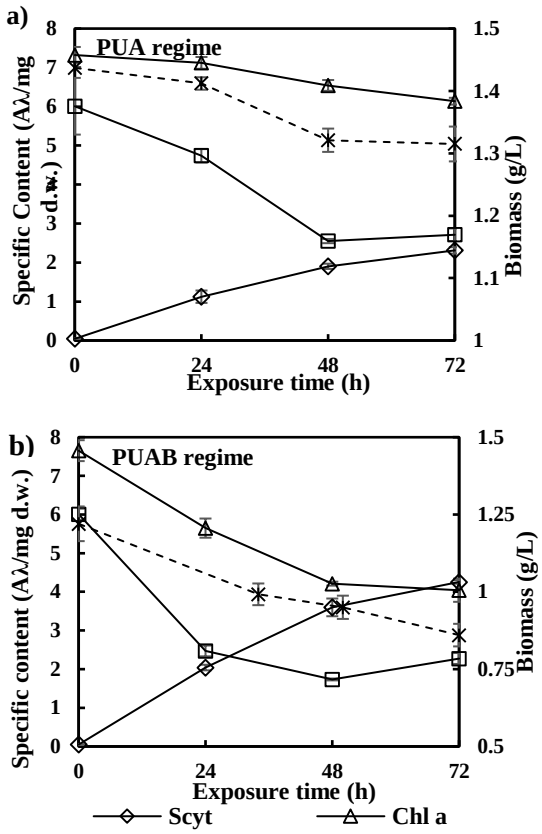

Figure 4. The effect of (a) PUA (PAR+UV-A) and (b) PUAB (PUA+UV-B) regimes on specific contents of scytonamin (Scyt), chlorophyll a (Chl a) and total carotenoids (T. Car.) as well as biomass concentration of *L. mycodia* during the 72 h exposure period. The error bars denote standard deviations of means (means ± SD, n = 3).

Figure 4-a shows the time courses of growth and pigmentation during the 3-day exposure to PUA regime. Despite the slight decrease during the first day, biomass concentration sharply decreased on day two due to the destructive

effect of UV-A irradiation and remained almost constant thereafter possibly due to the protective role of scytonemin synthesis. Overall, 8.5% decrease was observed in biomass concentration. Accordingly, chlorophyll a and total carotenoid contents diminished over time. However, scytonemin synthesis showed a positive correlation with UV-A incident time, showing a significant increase during the first day followed by the gradual increase in scytonemin specific content of up to 2.3 (A_λ/mg d.w.) after 3 days of PUA exposure.

The induction role of PUAB regime in synthesis of scytonemin and other pigments within 3 days was demonstrated in Figure 4-b. There was considerable induction in scytonemin synthesis during the first 48h of PUAB exposure. The content of other pigments decreased in response to PUAB regime similar to PUA regime. However, the total carotenoid content slightly increased in the last day of PUAB exposure. Scytonemin specific content in *L. mycodia* culture finally reached 4.25 (A_λ/mg d.w.) in response to 72 h PUAB exposure. Also, upon exposure to PUAB, 30% decrease was observed in biomass concentration.

Garcia-Pichel and Castenholze reported the values of scytonemin specific content in a range of 0.05-6.53 (A_λ /mg d.w.) by various cyanobacteria under UV irradiation [49]. Moreover, Rastogi and Incharoensakdi reported that the induction of *Lyngbaya* sp. CU2555 by UV-A and UV-B radiations led to significant synthesis of scytonemin up to 3.5 A_λ/mg d.w. [36]. While, *Rivularia* sp. HKAR-4 produced about 1.6 A_λ/mg d.w. scytonemin in UV irradiated samples [35].

Figure 5 compares scytonemin content in *L. mycodia* cultivated under P, PUA and PUAB regimes at various exposure times. In comparison to P regime, maximum induction of scytonemin synthesis was observed in the UV-AB irradiated cultures. Results also showed that PUAB was more efficient than PUA regime in scytonemin synthesis (P-value=0.02<0.05) for all examined exposure times. After 3 days of exposure, scytonemin content of 0.39 mg/g d.w cell was estimated for PUAB, which is 30% higher than that under PUA regime. Our obtained scytonemin content for *L. mycodia* was similar to 0.4 mg/g d.w. cell reported for *Nostoc commune* [44] and 0.42 mg/g d.w. cell for *Leptolyngbya cf. fragilis* [38].

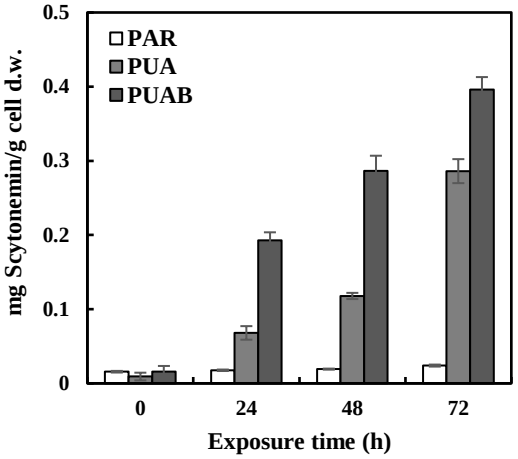

Figure 5. Effect of irradiation regimes (P, PUA and PUAB) on scytonemin synthesis by *L. mycodia*, the error bars denote standard deviations of means (means ± SD, n = 3).

UV radiation plays a vital role in inducing the synthesis of scytonemin. Under natural conditions, increasing UV radiation was found

to stimulate the synthesis and accumulation of high scytonemin concentrations in the microbial mat of *Lyngbya* sp. [50]. Pentecost *et al*. showed that UV uptake rate had positive effect on scytonemin synthesis in *Rivularia* sp., whereas a negative correlation was reported between pigmentation and UV irradiance in *Scytonema* sp. [30]. In a more comprehensive study on *Scytonema* sp. R77DM, PUA irradiation showed more stimulative effects on the scytonemin synthesis in comparison with PUAB [34]. Similarly, the positive effect of UV on scytonemin synthesis by *Rivularia* sp. was confirmed by Rastogi *et al*. [35]. Also, Ehling-Schulz *et al*. revealed that scytonemin was induced only slightly by UV-B (<315 nm), very strongly by near UV-A (350 to 400 nm), and not at all by far UV-A (320 to 350 nm) in nitrogen-fixing cyanobacterium *Nostoc commune* [51]. Moreover, Bernawitz and Kastenholz studied the long-term effects of UV and visible light on natural population of *Calothrix* sp. and established the correlation between UV protection and presence of scytonemin under solar radiation [31]. Moreover, in a research conducted by Kokbi *et al*., scytonemin biosynthesis was induced under long time UV exposure in cyanobacterium *Leptolyngbya cf. fragilis*, however, the content increased only during the first 24 h and insignificant change was observed for longer exposure times [38]. These diverse response patterns against long wavelength of UVR, revealed that scytonemin synthesis are triggered by different UV photoreceptors in various cyanobacteria.

Protection against UVR ensures the cyanobacterium to continue its normal metabolic function by absorbing/screening the lethal doses of UVR during harsh environmental conditions of ambient solar intensities. In present work, UV radiation played a vital role in inducing the scytonemin synthesis in cyanobacterium *L. mycodia*. Although scytonemin was greatly affected under UV-B stress, there was significant positive correlations between PUA and PUAB irradiances and scytonemin synthesis.

3.5. Free Radical Scavenging Capacity

The high-energetic UV-B radiation may affect normal state of life by generation of ROS, leading to cellular damage. The sunscreen pigment scytonemin has demonstrated the radical scavenging activity in its photoprotection function and may act as an antioxidant and can prevent cellular damage caused by UV-induced ROS production [44, 52]. The decolorization of DPPH radicals in presence of antioxidant was known as an easy and rapid way to evaluate scavenging capacity and has been used to detect antioxidant activity of the synthesized scytonemin by *L. mycodia*. Therefore, free radical scavenging activity of different concentrations of extracted samples was directly monitored and compared with ascorbic acid as a standard in Figure 6. Decrease of DPPH radicals was detected for all examined concentrations, revealing the radical scavenger capacity of scytonemin synthesized by *L. mycodia*. In PUAB exposure samples, the dose-dependent antioxidant activity of 35, 54, and 61% were obtained at concentrations of 50, 100, and 200 μg/mL, respectively, while more than 96% anti-oxidation rate was obtained for

ascorbic acid at the same concentrations and this difference was statistically significant (P value=0.0379<0.05). Also, anti-oxidant activity rate was less than 10% in the non-UV-irradiated extracts and confirms the effective role of UV irradiation on induction of scytonemin synthesis (P value=0.042<0.05)). Results of DPPH assay in Figure 6 was also used to calculate the antioxidant activity as the inhibition concentration (IC50). It was found that IC50 of cyanobacterium extract (48.84 µg/ml) was lower than that for vitamin C (6.43 µg/ml) as a strong antioxidant and this specified scytonemin as a moderate radical scavenger.

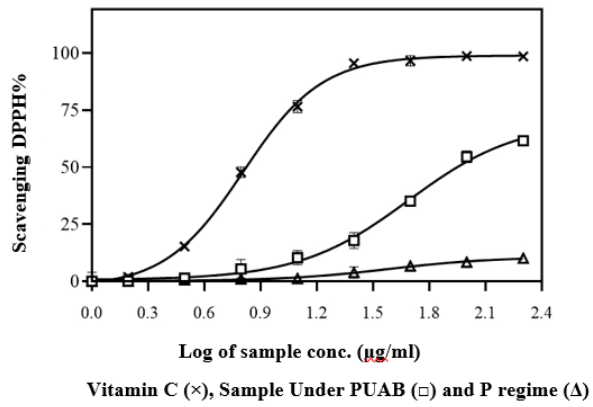

Figure 6. Decolorization of DPPH radicals as percentage of radical scavenging capacity (mean ± SD, n=3) at different levels of partially purified scytonemin extracts from *L. mycodia* as well as ascorbic acid as standard.

The radical-scavenging activity of scytonemin isolated from *N. commune* was also monitored by Matsui *et al*. [44] by means of DPPH assay where scytonemin was incapable of scavenging DPPH radicals in vitro. In contrast, Rastogi *et al*. (36) showed a relatively moderate antioxidant activity, 57% scavenging of DPPH at sample concentration of 200 µg/mL for scytonemin extracted from *Lyngbya* sp. Recently, Kokabi *et al*. [38] indicated antioxidant activity of scytonemin extracted from *Leptolyngbya cf. fragilis* and showed the maximum total antioxidant activity (IC50=71.73 µg/mL) for ethyl acetate extract after 72 h UV treatment in comparison with butylated hydroxyl toluene BHT (IC50 = 21.22) as a synthetic pure antioxidant.

4. Conclusion

Scytonemin production is a normal metabolic function in some cyanobacteria to protect cells against the lethal dose of UV radiation. Due to its UV-absorbing/screening potential as well as antioxidant activity to cope with oxidative stress under intense solar radiation, scytonemin may be biotechnologically exploited by pharmaceutical and cosmetic industries. The present study identified this biomolecule in cultures of two filamentous cyanobacteria, *Leptolyngbya mycodia* and *Phormidium* sp. with the former showing a higher scytonemin production efficiency. Induction of scytonemin was very low under PAR and short-wavelength UV-A, while UV-B extremely enhanced scytonemin synthesis yield. Moreover, this study strongly supports the role of extracted scytonemin as an active antioxidant preventing cellular damage caused by UV-induced oxidative stress. Our obtained results are of great importance, providing a basis for exploration of UV-protective small biomolecules and efficiency development offering a new opportunity to present alternative routes for commercial production of natural sunscreen, beyond the traditional cosmetics and pharmaceuticals.

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