

Isolation of Nostocales and Study the Effects of Scytonemin on Growth Profile and Melanogenesis by using Melanoma Cell Line

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ABSTRACT: The purpose of this study was to screen scytonemin, a UV screening pigment, in isolated Nostocales, *Scytonema sp.*, *Plectonema sp.*, *Spirulina sp.*, and *Lyngby sp.* Scytonemin was isolated by silica gel column chromatography and its effects were tested *in vitro* for growth profile and melanogenesis on B16F10 mouse melanoma cell line. In order to study effect of these scytonemin on growth profile- cell viability count, MTT and DCPIP cytotoxicity assay, Comet genotoxicity assay and microscopic observations were considered. To study tyrosinase enzyme activity- diphenolase assay and native SDS PAGE methods were used. Scytonemin isolated from *Scytonema sp.* and *Lyngby sp.* shows decrease in melanogenesis, which inhibit tyrosinase activity, having 30.25 μM , 100.25 μM IC 50 values respectively. While that of scytonemin isolated from *Plectonema sp.* and *Spirulina sp.* was not showing effect on melanogenesis as tyrosinase activity not altered, having 45.5 μM , 60.5 μM IC 50 values respectively.

KEY WORDS: Cytotoxicity, Genotoxicity, Melanogenesis, Scytonemin, Tyrosinase.

I. INTRODUCTION

For continued existence, solar radiation plays important role in evolution and development of life. Solar radiation that reaches to the Earth's surface, the ultraviolet (UV) radiation is of particular importance for the human skin. Skin pigmentation i.e. production of melanin by the process known as melanogenesis is highly depend on solar UV radiations (Del Marmol et al., 1993, Jimbow et al., 2000). At molecular level tyrosinase enzyme regulates process of melanogenesis in response to UV rays, in which it act as key enzyme and regulate conversion of tyrosine to DOPA and DOPA to DOPAquinone. (Hearing V. J. et al., 1980, Hearing and Tsukamoto, 1991, A. slominski et al., 2004).

The UV-region of the electromagnetic spectrum is divided into three different regions: UVC radiation (200-290 nm), UVB radiation (290-320 nm) and UVA radiation (320-400 nm). Of these wavelengths all UVC radiation as well as large quantities of the UVB radiation gets absorbed by ozone layer. Therefore, the UV radiation that reaches the Earth's surface contains about 5% of UVB and 95% of UVA radiation. (Mang R. et al., 2006)

Increased penetration of UV-B (not of UVA) on the surface of the earth is due to the decreasing ozone concentration in the stratosphere (Kerr et al., 1993 and S. Madronich et al., 1998). Thus UV-B, which is deemed deleterious, can penetrate biologically significant depths into the water column (Kuwahara et al., 2000), thereby affecting aquatic ecosystems. The detrimental effects of UV-B on primary producers are varied, mediated primarily by damaging molecular targets such as nucleic acids, proteins and pigments, and indirectly, by producing reactive oxygen species. These can lead to the inhibition of cell division and growth, affecting various physiological and biochemical processes, such as motility, orientation in motile organisms and photosynthesis, which can consequently alter community structure and function. UV-A can have a net damaging influence on photosynthesis (Cullen et al., 1992).

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Although UV rays are harmful, some organisms attempt to cope with UV radiation. Sinha et al. (1998) identified the following four adaptation strategies through organisms tolerate or adapt to UV environment -

- I. DNA repair mechanisms
- II. Production of enzyme systems and induced formation of quenching agents
- III. Behavioral modification to avoid exposure to UV
- IV. Production of UV-absorbing substances

In terrestrial environments, where higher plants are the foremost primary producers, several studies have shown that harmful UV radiation in higher plants is absorbed by epidermally located phenylpropanoids, mainly flavonoid derivatives (Kootstra, 1994). In aquatic environments, where microalgae found abundantly, the presence of UV-absorbing compounds like sporopollenin, scytonemin, and mycosporine-like amino acids (MAAs) have been established.

Scytonemin [(3E,3'E)-3,3'-bis(4-hydroxybenzylidene)-[1,1'-bi(cyclopenta [b] indole)]-2,2'-(3H,3'H)-dione] is a UV-screening pigment that is commonly produced in populations of sheathed cyanobacteria that live in different habitats and geographic locations, where solar radiations are very intense (Garcia-pichel et al., 1991). The yellow-green pigment scytonemin was first reported by Nageli as early as 1849 and the chemical structure was provided by Proteau et al., 1993. It is a lipid soluble alkaloid that is synthesized in response to UVA radiation and accumulates within the extracellular sheaths of cyanobacteria. The organisms are thereby protected from cell damage by this natural UV-filter that absorbs the harmful solar radiation. (Fleming and Castenholz, 2007 and Stevenson et al., 2002) Scytonemin absorbs mostly in the UVA (325-425 nm, $\lambda_{max} = 370$ nm) and UVC region ($\lambda_{max} = 250$ nm), but it also absorbs substantially in the UVB region (280-320 nm). The maximum absorption wavelength of scytonemin is 370 nm *in vivo*. However, it shifts towards a longer wavelength of 384 nm in a solvent after isolation. It is reported that the molar extinction coefficient of scytonemin is large (250 l/g/cm) at wavelength 384 nm (Vincent et al., 1993), it is calculated to be 136,000 l/mol/cm based on a molecular weight of 544 Da. Therefore, scytonemin is an efficient photo-protective compound due to its large extinction coefficient (Bultel-Poncef et al., 2004). It is a dimer composed of indolic and phenolic subunits having a molecular mass of 544 g/mol. The linkage between two subunits in scytonemin is an olefinic carbon atom that is unique among natural products. Hence, scytonemin possess a new ring system in nature for which Proteau et al. (1993) have proposed the trivial name 'the scytoneman skeleton'. Scytonemin exists in oxidized (green) and reduced (red) form. In an oxidized state, the two chromophores are connected by a single bond. Therefore, they can freely rotate and prevent steric repulsion. This steric repulsion between two bulky chromophores makes a dihedral angle of about 90 degrees, so electronic interaction between them becomes very weak. On the other hand, in a reduced state, the indole ring and the benzene ring, which have 10- π and 6- π electron aromaticity, respectively, are alternately connected by double bonds. Therefore, π -conjugation is expanded by increase of planarity. Due to this structural and electrical change, the color of the compound changes from brown (oxidized state) to red (reduced state) (Saman M. et al., 2014).

During this study naturally occurring UV screening compound is isolated from Nostocales and tested *in vitro* for melanogenesis using mouse melanoma cells.

II. METHOD

1. Isolation of cyanobacteria

A. Collection and Enrichment of samples

Water and Soil samples were collected from Old swimming pool in Savitribai Phule Pune University, in autoclaved glass bottle. Algal blooms or mats were collected by using mesh net (pore size 25-30 μ m). pH and salinity of samples were recorded using pH strips and salinometer respectively. Samples were centrifuged and pellet was suspended in BG 11 media with 1 mg/L Germanium Dioxide and 0.05 mg/ml Cycloheximide. Samples were kept for enrichment by providing 12 hours light and dark conditions at 28°C for 10-15 days. Bacterial and fungal contamination was reduced by adding 0.05 mg/L streptomycin, 10 U/ml penicillin-G, 0.05 mg/L amphotericin B in the media. (Algal culture techniques, by Robert Andersen)

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B. Preparation of Axenic culture

Unialgal cultures were obtained by using serial dilution method for enriched samples. Enriched samples were diluted as 10^{-2} - 10^{-10} in BG 11 media and spread on BG 11 agar plates (1.5% agar) and incubated for 10-15 days by providing 24 hours light condition at 28°C. Green colored colonies were streak on fresh BG 11 agar plates (1.5% agar with 5 µg/L streptomycin, 10 U/ml penicillin-G, 5 µg/L amphotericin B in BG 11 media). Plates were incubated for 15-20 days by providing 24 hours light condition at 28°C. Mixed algal culture was separated under stereo microscope. (Algal culture techniques, by Robert Andersen)

C. Identification of cyanobacteria

Isolated cultures were identified by colony characteristics, presence of pigments (and), cellulose and pectin, microscopic observations as per given in "The freshwater algal flora of the British Isles: an identification guide to freshwater and terrestrial algae." edited by D. M. John et al. (2002) and "Cyanophyta" by T. V. Desikachary. 80% acetone extract of sample was used to detect presence of β-carotene (450 nm) and chlorophyll a (430 nm and 662 nm) while that of phosphate buffer extract of sample was used to detect presence of phycocyanobillin (620 nm 652 nm or 562 nm). Presence of cellulose and pectin in sample was detected by Anthrone method (E. P. Samsel and J. C. Aldrich, 1957) and Carbazole test (Mc Comb et al., 1957 and S. Nurdjanah et al., 2013) respectively.

2. Screening of scytonemin from cyanobacterial isolates

Presence of scytonemin was detected by following techniques (Proteau et al., 1993).

A. TLC for scytonemin

5 ml acetone was added in 10 mg of cyanobacterial sample and kept in dark at 4°C for about 10-12 hours. 0.1 µl of sample was spotted on TLC plates (Sigma-Aldrich, silica coated on aluminum support with pore size of 60 Å and thickness of 200µm) and run by using chloroform: methanol (9: 1) as mobile phase. Yellowish brown colored spot was detected and Rf value was calculated. (Rf value for scytonemin is around 0.8)

B. Spectrophotometric analysis

5 ml acetone was added in 10 mg of cyanobacterial sample and kept in dark at 4°C for about 10-12 hours. Sample was centrifuged at 5000 rpm for 10 min at 4 °C and supernatant was scanned from 350 nm to 450 nm on spectrophotometer (Shimadzu UV 1601 UV-Visible). Peak at 384 nm (or 380nm -390 nm) shows presence of scytonemin. (Shailendra et al. 2010). Scytonemin containing cyanobacteria were used for further experimentations.

3. Isolation of Scytonemin

Biomass was oven dried at 60 °C. Dry weight was recorded and scytonemin was isolated by column chromatography. (Naoki W., 2013). About 2.5 gram of dried biomass were suspended in 50 ml of acetonitrile and kept at 4 °C for 1 hr. biomass was separated by centrifugation and supernatant containing pigments was collected. This step was repeated twice. Acetonitrile was removed by using Rota-vapor (Buchi R-210). Dried pigments were suspended in 50 ml of ethyl ether to remove chlorophyll and other pigments. Un-dissolved pigments were collected and dissolved in acetone. Silica gel (Hi-media) column was prepared in acetone and samples were resolved on 50 cm silica column using acetone as mobile phase. Procedure was repeated twice for further purification. Collected samples were screened on spectrophotometer (Shimadzu UV 1601) for scytonemin.

4. Study effect of scytonemin on growth profile and melanogenesis

A. Cell line maintenance -

B16F10 mouse melanoma cell line was purchased from NCCS, Pune. Cell line was maintained in DMEM with 10% serum and 50 µg/ml streptomycin by providing 5% of CO₂ atmosphere. Sub-culturing was done after every 5 days (Culture of Animal cells by Ion Freshney).

B. Addition of scytonemin-

Purified scytonemin stock 10 mM (5.5 mg/ml) was prepared in 100 % acetone. For testing, 1µm, 10 µm, 100 µm, 1000 µm and 10000 µm concentration was added in cell culture (initial cells concentration was 3×10^5 cells/well).

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C. Cell viability count-

Cell viability was counted with the help of 0.4% Trypan blue vital stain using haemocytometer. Stained and non-stained cells counted under microscope and recorded as cells/ml. (Culture of Animal cells by Ion Freshney)

D. Protein estimation

Protein estimation was done by modified Bradford's assay (1976). Unknown proteins were estimated by dissolving cell pellets in protein extraction buffer containing 50 mM Tris-HCl, 5 mM EDTA, 0.1% Triton X-100 and 1 mM PMSF. 0.2 ml of 5x Bradford's reagent (500 µg/ml CBB G-250, 25% of ethanol and 50% of ortho-phosphoric acid) were added in 0.8 ml diluted sample by vortexing and OD was taken at 595 nm. OD was compared with BSA standard graph and unknown concentrations of proteins were estimated.

E. Tyrosinase enzyme activity staining *in situ* on SDS polyacrylamide gel –

Assay was done as described by Jimenez-Cervantes et al. (1993). Cell pellets were lysed in lysis buffer containing 50 mM TrisHCl, 5 mM EDTA, 0.1% Triton X-100 and 1 mM PMSF. The melanoma protein samples were mixed with sample buffer without β-mercaptoethanol (0.18 M Tris- HCl pH 6.8, 15% glycerol, 0.075% bromophenol blue, 9% SDS) in a 2:1 ratio without giving heat treatment. The samples were then resolved by 12% SDS-PAGE at 30mA. After electrophoresis gel were equilibrated in 50 mM sodium phosphate buffer (pH 6) for 15 min at 37°C. Tyrosinase activity was detected by immersing the equilibrated gel in 50 mM sodium phosphate buffer (pH 6.8) containing 1.5 mM L-DOPA and 4 mM MBTH at 37°C with gentle shaking until dark pink-brown coloured bands were developed.

F. Tyrosinase enzyme assay-

The tyrosinase activity was determined by a spectrophotometric method, as described by (Celia et al. 2001). The 10 µl enzyme extract was added to the substrate containing 1.5 mM L-DOPA, 1 mM DMF and 4 mM MBTH in phosphate buffer (pH 6.8). L-DOPA is the main substrate that converts to dopaquinone by enzyme activity. MBTH is a strong nucleophile that is attached to produce a pink complex. DMF is added to the reaction mixture in order to keep the resulting complex in solution state during the course of investigations.

The progress of the reaction was followed by measuring the intensity of the resulting pink color at 505 nm. A typical reaction mixture with a total volume of 1 ml contained 100 µl enzyme solution, 850 µl substrate solution and 50 µl phosphate buffer (pH 6.8). To estimate tyrosinase activities in, the molar extinction coefficient of the product was 3700 per mole per centimetre.

G. Superoxide dismutase (SOD) assay

SOD assay was done as given by Adriana M C et al. (2004). Enzyme extract was prepared by dissolving cell pellet (containing 10³ cells/ml) in lysis buffer and supernatant were used after centrifugation at 12000 rpm. Following components were added in two sets of tubes. 1.5 ml of 0.1 M potassium phosphate buffer (pH 7.8), 0.6 ml of 65 mM L- methionine, 0.3 ml of 750 µM NBT, 0.3 ml of 2 mM Riboflavin, 1µl of 10 mM EDTA, 0.1 ml of Enzyme extract (for control 0.1 ml water was added) and volume was adjusted to 3.5 ml with Distilled water. One set of tube was illuminated under the light source for 30 min at a distance of 30 cm and another set of tube was kept in dark for 30 min. Absorbance was read at 560 nm after 30 min of incubation at room temperature. Amount of SOD is directly proportional to the amount of reactive oxygen species generated during reaction which are measured at 560 nm and calculated by formula.

H. MTT and DCPIP assay for cell cytotoxicity

Cell cytotoxicity was tested as given by Karl B. et al. (2012). On 1st day, 1 x 10⁵ cells/well allow to attach in 96 well plate with 200 µl of complete medium in CO₂ incubator.

On 2nd day, compounds to be tested was added in respective wells with fresh medium and incubated for 24 hr.

On 3rd day, medium were removed and monolayer were washed with PBSA 3-4 times.

For MTT assay - Fresh medium containing 5 µg/ml MTT were added in each well and incubated for 4 hr in CO₂ incubator.

After 4 hr. medium was removed and crystals were dissolved in 200 µl of DMSO. To this 50 µl of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) was added.

Immediately absorbance at 570 nm was recorded using ELISA plate reader. And IC 50 value was calculated.

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For DCPIP assay - 200 μ l fresh medium were added in each well, to this 5 μ l of 2 mM DCPIP reagent is added in each well and incubated for 4 hour in CO₂ incubator.

After 4 hr. absorbance at 660 nm was recorded using ELISA plate reader. And IC 50 value was calculated.

I. Comet assay for genotoxicity

Assay was performed as given by Stevenson C. S. et al. (2002). Glass slide is covered with thin layer of 1.5% melted agarose. To the 200 μ l of 0.6% melted agarose total of 20 μ l of cell suspension (1×10^6 /ml) was added. This was spread on coated slide using coverslip. Slide was kept at 0 °C for 2-3 min. and slide was submerged in lysis buffer (0.25 M NaCl, 0.6 % NaOH, 100 mM EDTA, 2% DMSO, 0.2% Triton X 100, pH 10) for 2 hours in dark. Ice cold running buffer (300 mM NaOH, 0.5 M EDTA, pH 13) was added in electrophoresis tank and sample was run at 300 mA for 30 min in dark conditions.

After electrophoresis, slide was kept in neutralization buffer (0.4 M tris HCl pH 7.5) and then washed with distilled water. EtBr stain was sprinkled on slide and slides were observed under fluorescence microscopy for comets.

III. RESULTS AND DISCUSSION

1. Isolation of Nostocales and scytonemin

11 algae were isolated from collected samples, among those only 6 were cyanobacteria. All 6 cyanobacteria were characterized and screen for scytonemin, only four of them shows presence of scytonemin namely *scytonema sp.*, *Plectonema sp.*, *Spirulina sp.* and *Lyngbya sp.* (Fig. 1) Separation of scytonemin on TLC shows R_f values as 0.82 (green) for *Scytonema sp.*, 0.80 (brownish purple) for *Plectonema sp.*, 0.82 (brown) for *Lyngbya sp.*, 0.84 (yellowish brown) for *Spirulina sp.* and peak at 384 nm. Due to presence of variable side chains and electric charge on Scytoneman skeleton, scytonemin can exist in different colors in nature. As Scytonemin pigment is highly hydrophobic and exist in different color, it can be used in textile industries for coloration.

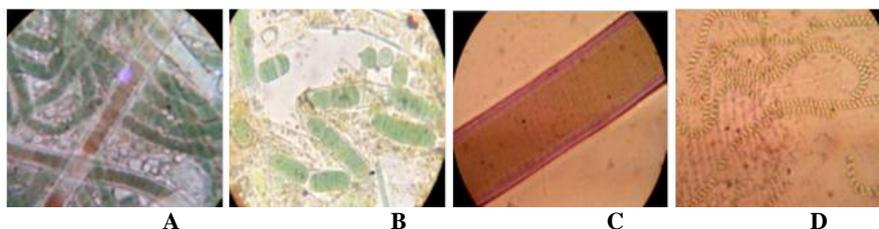


Fig 1. Isolated and selected Nostocales A – *Scytonema sp.*, B – *Plectonema sp.*, C – *Spirulina sp.* and D – *Lyngbya sp.*

2. Effect of Scytonemin on melanoma cells

Scytonemin isolated from all 4 Nostocales shows similar effects on B16F10 mouse melanoma cells under microscope. (Fig. 2) As compare to control (with and without acetone), scytonemin up to 1 μ M concentration does not show any effect on melanoma cells, while at 10 μ M concentration cell growth is reduced. At 100 μ M concentration melanoma cells become apoptotic, while at 1000 μ M concentration cells do not attached and dead. Viable cell count decreases as concentration of scytonemin increases (Graph 1A).

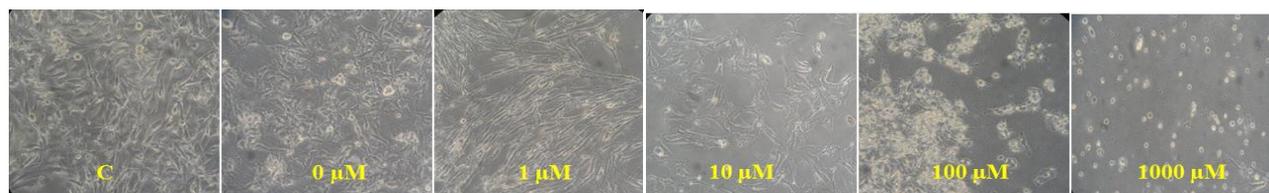
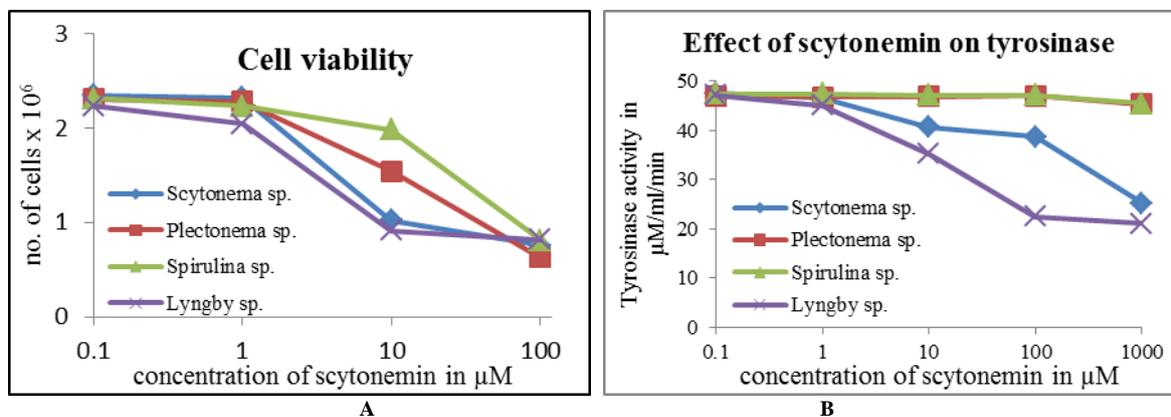


Fig 2. Effect of scytonemin on B16F10 mouse melanoma cell line.



Graph 1. (A) Decrease in cell viability (B16F10 cells) due to higher concentrations of Scytonemin isolated from 4 cyanobacteria. (B) Effect of scytonemin on tyrosinase enzyme activity in B16F10 melanoma tyrosinase.

3. Effect of Scytonemin on tyrosinase activity

As per results for in situ staining on gel (Fig. 3) and tyrosinase activity by spectrophotometric analysis (Graph 1B) shows similar effects. Scytonemin isolated from *Scytonema sp.* and *Lyngbya sp.* shows reduction in tyrosinase activity, while Scytonemin isolated from *Plectonema sp.* and *Spirulina sp.* does not shows any effect on tyrosinase activity. Enzyme activity reduces from 47 µM/ml/min to 23 µM/ml/min due to 1 mM of Scytonemin.

As per our results scytonemin from certain Nostocales shows inhibitory tyrosinase activity, hence it can be used to treat disorders related to melanogenesis such as – Melisma, over pigmentation and deposition of melanin in patches. As tyrosinase activity inhibited by scytonemin it may regulate melanin biosynthesis and hence melanin production can be regulated.



Fig 3. In situ staining of tyrosinase activity on gel. Effect of Scytonemin isolated from A – *Scytonema sp.*, B – *Plectonema sp.*, C – *Spirulina sp.* and D – *Lyngbya sp.* on tyrosinase enzyme.

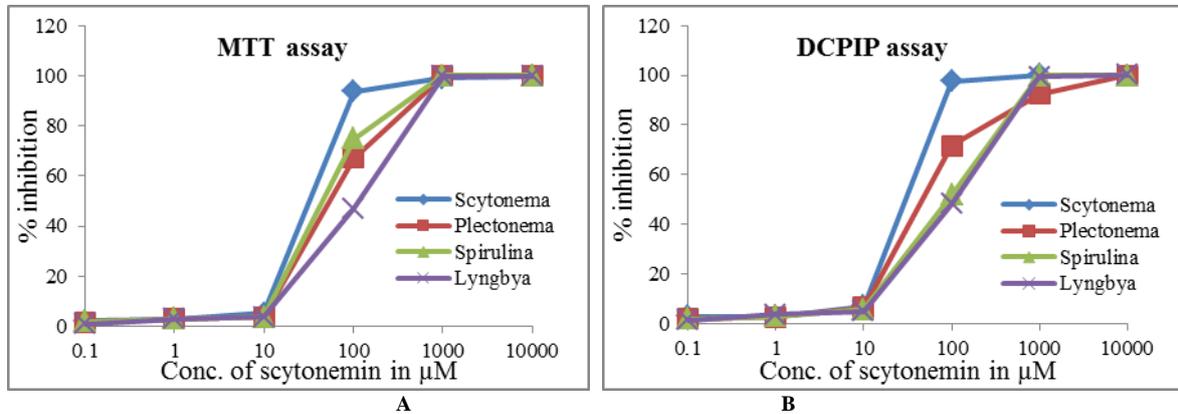
4. Effect of scytonemin on growth profile of melanoma cells

As concentration of scytonemin isolated from 4 different cyanobacteria increases, % reduction of GI decreases (Graph 3A). Higher concentrations of scytonemin i.e. above 1mM are toxic to the melanoma cells. Amount of SOD increases, as concentration of scytonemin increases (Graph 3B).

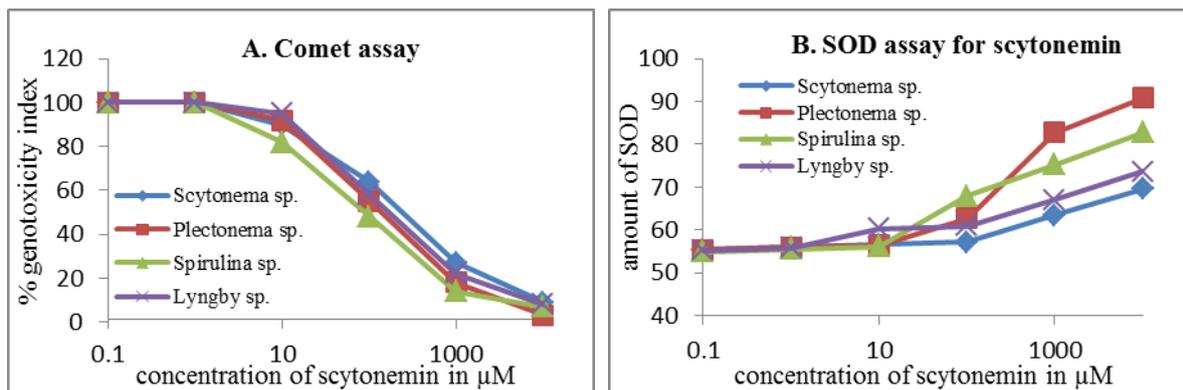
As per MTT assay and DCPIP assay (Graph 2), IC 50 values of Scytonemin (isolated from 4 Nostocales) ranges from 30 µM to 100 µM which are mentioned in Table 1.

IC 50 value	Concentration of Scytonemin in µM		
	MTT assay	DCPIP assay	Average
<i>Scytonema sp.</i>	30	30.5	30.25
<i>Plectonema sp.</i>	50	40.5	45.5
<i>spirulina sp.</i>	40.5	90	60.5
<i>lyngbya sp.</i>	100	100.5	100.25

Table 1. IC 50 values of Scytonemin isolated from 4 Nostocales for B16F10 cell line, cell cytotoxicity MTT and DCPIP assay.



Graph 2. % inhibition of B16F10 melanoma cells due to Scytonemin, cell cytotoxicity
A. MTT and B. DCPIP assay



Graph 3. A. Comet assay and B. SOD assay for Scytonemin isolated from 4 cyanobacteria.

IV. CONCLUSION

Major role of scytonemin in cyanobacteria is to protect cells from UV rays. As it is a natural UV screening compound having radical scavenging activity, it can be used in cosmetic industries. As per our results scytonemin isolated from *Scytonema sp.* and *Lyngbya sp.* inhibit tyrosinase activity, hence it can be used to treat disorders related to melanogenesis such as – Melisma, over pigmentation and deposition of melanin in patches. As tyrosinase activity inhibited by scytonemin it may regulate melanin biosynthesis and hence melanin production can be regulated.

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