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Evaluation of free radical scavenging activity and skin whitening potential of cyanobacteria

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ABSTRACT

In day-to-day life, our skin is exposed to various chemicals, environmental pollution and UV radiations which results in early onset of photoaging and skin darkening. There is increased demand of skin whitening cosmetics for skin protection as well as aesthetic looks. The current skin whitening agents used in cosmetic industries have adverse effects on skin. There is increased demand for natural active agents as skin whitening agent. Cyanobacteria are ubiquitous in nature and quickly adopt different environments.[1] Algae produce various secondary metabolites like carotenoids, phenolics, flavonoids, MAA (mycosporine like amino acids) which are antioxidants in nature and have therapeutic potential in pharmaceutical and cosmetic industry[2].. This study focuses on isolation and characterization of cyanobacteria from different environmental niche. The antioxidant potential of whole cell extracts of cyanobacteria was evaluated using DPPH inhibition assay and ABTS assay. Their potential application as skin whitening agent was studied by anti-tyrosinase activity using mushroom tyrosinase enzyme. The tyrosinase inhibition activity of cyanobacterial isolates was observed in the range of 30 to 40%. They have good antioxidant potential. They are rich carbohydrate, protein and lipid content indicate potential of the cyanobacterial extracts so they have potential in cosmetic and pharmaceutical industry.

Keywords— *Antioxidant, Cyanobacteria, Skin Whitening, Tyrosinase Inhibition*

1. INTRODUCTION

Healthy skin has major impact on quality of life as well as it boosts self-confidence. Nowadays, desire of unblemished and radiant skin is seen in all age groups not only in women but also in men leading to increased demand of cosmetics. The cosmetic industry is also evolving day by day with the help of new technologies and scientific research. Cosmeceuticals are emerging products which generally contain biologically active compounds with therapeutic potential[3]. Skin lightening cosmetics include natural or synthetic active agents to reduce the melanin content resulting in lighter the skin tone or to provide flawless skin complexion. Skin whitening products have great demand in Asian countries like China, India, Japan, Korea, and Indonesia. The global market for skin lightening products may reach to 23000 million US $ by December 2022[4].

In day-to-day life, human skin is exposed to various stress factors like environmental pollution, UV radiations etc. which induce generation of reactive oxygen species (ROS) leading to oxidative stress. This leads to various skin disorders like dry, wrinkled skin, hyperpigmented skin indicating onset of photoaging. In worst situations it leads to skin cancer. ROS accumulation accelerates activity of tyrosinase enzyme resulting in overproduction and accumulation of melanin pigment. Melanogenesis results in generation of ROS like hydrogen peroxide in melanocytes inducing oxidative stress.

Desire for flawless and radiant skin is demand of all types of consumers irrespective of age and sex especially in Asian countries. The main molecular mechanism of skin lightening agents is to reduce content of melanin pigment which is responsible for skin color. Melanin is synthesized in melanocytes in response to UVB radiation, which is transported to epidermal keratinocytes. Under physiological conditions, melanin plays important role in photoprotection, protection against oxidative stress and DNA damage. The abnormal loss of melanin and depigmentation lead to abnormal aesthetic look and dermatological condition. Excess melanin concentrations result in dermatological conditions like freckles, dark spots and melasma, Acanthosis nigricans, neurodegeneration associated with Parkinson's disease and skin cancer[5].

To treat hyperpigmentation, active agents are used which have having potential for scavenging free radicals and inhibition of tyrosinase enzyme which in turn decelerate melanogenesis. The active agents having ability to retain moisture balance of skin or have a moisturizer property as well as sun protection ability will have added advantage for skin-lightening cosmeceuticals[6]. Hence, biological active agents are more preferred than chemical ones. Moreover, synthetic chemicals like arbutin, hydroquinone have side effects like dermatitis, toxicity and carcinogenicity. Hydroquinone is banned in cosmetic products by European union and Kojic acid is also not preferred due to carcinogenicity. Hence, there is demand for safe and effective active agent in cosmetic world.

Melanogenesis is a complex process involving various enzymatic and chemical reactions. Tyrosinase enzyme and other tyrosinase related proteins (Tyr-1 and Tyr-2) are key regulators of melanogenesis. Tyrosinase is a binuclear copper enzyme. This metalloenzyme catalyses rate limiting reactions in melanogenesis: tyrosinase hydroxylation and formation of o-dopaquinone by oxidation of L-DOPA (3,4-dihydroxyphenylalanine). The skin color depends on type and concentration of melanin and its distribution in keratinocytes. Therefore, skin lightening products mainly focus on tyrosinase inhibition[7].

The tyrosinase enzyme inhibition is studied using spectrophotometric technique with mushroom tyrosinase enzyme. The enzyme derived from mushroom viz. *Agaricus bisporus* is highly similar and homologous to human tyrosinase enzyme. Mushroom being rich and cheaper source, this enzyme is widely used in tyrosinase study. The tyrosinase activity is studied in in of the two reactions i.e., either in monophenolase activity wherein L tyrosine substrate is hydroxylated to L-Dopa (3,4 dinitrophenyl alanine) or in diphenolase activity in which L-Dopa is oxidized to o-Dopaquinone.

Different natural and synthetic compounds possess different types of tyrosinase inhibition mechanism. Copper chelators such as aromatic acids, phenolics, polyphenols compound mimic substrate and inhibit competitively. Noncompetitive inhibitors bind enzyme substrate complex. Mixed inhibitors like pthalic acid or cinnamic acid bind to enzyme or enzyme substrate complex for monophenolase activity. Phthalic acid, tetrapthalic acid, D-(-) arabinose , brazeilein inhibit diphenolic activity by mixed type inhibition. Phenolic substances, flavanones, tannins, amides possess inhibitory activity against mushroom tyrosinase enzyme[4,5]. These phytochemicals are generally observed in cyanobacteria. Moreover, cyanobacteria are also rich sources of antioxidants, proteins and polysaccharides which are beneficial in skin cosmetics. They are also known for their photoprotective properties[8]. Therefore, this study mainly focuses on isolation of cyanobacteria from various ecosystems and screening their potential as an antioxidant agent and skin whitening agent.

2. MATERIALS AND METHODS

Chemical used: DPPH, ABTS, Mushroom tyrosinase enzyme, L-tyrosine were purchased from Sigma. All other chemicals were used from Merck.

2.1 Isolation and biochemical characterization of algal isolates

2.1.1 Isolation of cyanobacteria: Soil, water and tree bark samples were collected in sterile container from various regions of Maharashtra. The samples were enriched and isolation of cyanobacteria was done using Bold Basal Medium (pH 7-7.5). at 25 ℃ for 15 days[9].Unialgal cultures were obtained by repeated subculturing and screening. The isolates were identified using microscopy.

2.1.2 Harvesting of cyanobacteria: The cyanobacterial isolates were harvested at stationary phase by filtration using nylon cloth filter. Repeated washing was done using distilled water to remove traces of media, if any. The algal mass was dried completely at 30 ℃ to 35 ℃ and stored in refrigerator at 4℃ for further use.

2.1.3 Biochemical characterization of algae: The cyanobacterial isolates were studied for their protein, carbohydrate, lipid, content.

2.1.4 Estimation of intracellular proteins: Intracellular proteins were extracted from 10 mg of dry powder of cyanobacterial isolates using 10 ml freshly prepared lysis buffer. (100mM Tris buffer, 10mM EDTA, 150 mM Triton-X-100, 0.09 mM PMSF, 0.65 mM Dithiothreitol). Pellet was separated by centrifugation at 13500 rpm for 20 min. The supernatant was used for protein estimation by Folin-Lowry method. 1mg/ml BSA was used as standard. 2 ml of alkaline $CuSO_4$ reagent was added in 0.2 ml of test solution and 0.2 ml lysis buffer was added in the blank. After incubation at room temperature for 10 min, 0.2 ml of 50 % Folin-ciocalteu reagent was added. The reaction mixture was incubated at room temperature for 30 min and absorbance was measured at 585 nm on Kanad Vidyut 0392 spectrophotometer[10].

2.1.5 Estimation of carbohydrate: For extraction of carbohydrates**,** 250 µl of concentrated sulfuric acid was added in 25 mg of dried powder of test cyanobacterial isolates After incubation at 30℃ for 1h, 7 ml distilled water was added in the reaction mixture and heated in boiling water bath for 10 min. After cooling, estimation of carbohydrates by Anthrone method. For Anthrone assay, 400 µl of 4% sulfuric acid was added in 100µl of test extract. Glucose (50 mg/ml) was used as standard. 100 µl of 4% sulfuric acid was used in blank. 2 ml of Anthrone reagent was added in all the tubes followed by heating in boiling water bath for 10mts. The tubes were cooled and absorbance was measured at 585 nm using kanad Vidyut 0392 spectrophotometer[11].

2.1.6 Estimation of lipid: Extraction of lipids was done using methanol: chloroform (1:2) proportion with overnight incubation at 4℃. The supernatant was separated by centrifugation at 13500 rpm for 20 min. Then 1.3 ml distilled water and 3 ml chloroform was added in the solution and mixed thoroughly. The chloroform layer was separated and used for lipid estimation. After complete evaporation of the extracts, incubation was done at 100℃ for 10 min with 100 µl concentrated sulfuric acid. After cooling, 2.5 ml of sulfovanillin (SPV) reagent was added. After incubation at room temperature for 20 min, absorbance was measured at 530 nm in Kanad Vidyut 0392 spectrophotometer[12].

2.2 Preparation of whole cell extract of algae:

Extraction of dry algae powder was done using tetrahydrofuran and methanol (2:8) with overnight incubation at 4°C. The supernatant was evaporated at room temperature for 18-24 h stored in refrigerator at 4°C for further use[13].

2.3 Determination of antioxidant activity

2.3.1 Determination of antioxidant activity of algal extracts using DPPH assay: The reaction mixture consisted of 2ml 0.15mM of DPPH reagent and 200µl of test algal extracts in methanol with incubation at room temperature for 30mts. 0.5 mM ascorbic acid was used as standard[14]. Absorbance was measured 530nm using Kanad Vidyut 0392 spectrophotometer. Per cent inhibition was calculated using the following formula.

$$\textbf{Percent inhibition of DPPH} = [(Abs_{control} - Abs_{test})/Abs_{control}] * 100$$

Where, Abs control = absorbance of control and Abs test = Absorbance of test

2.3.2 Determination of antioxidant activity of algal extracts using ABTS assay: ABTS (2,2'-Azino-bis (3-ethylbenzothiazioline-6-sulfonic acid) was prepared by mixing 7mM ABTS solution with 2.45 potassium persulfate (1/1 v/v) and incubated at room temperature for 16-20h to get stable radical. The absorbance of ABTS radical was adjusted to 0.7 ± 0.05 using 50% methanol. 200µl of test algal extract in methanol is mixed with 1800µl of ABTS solution followed by incubation at room temperature for 6mts. Ascorbic acid was used as standard. The absorbance is measured at 700 nm on Kanad Vidyut 392 spectrophotometer[15]. Percentage inhibition was calculated using the following formula:

$$\textbf{Percentage inhibition of ABTS} = [(Abs_{control} - Abs_{test})/Abs_{control}] * 100$$

Where, Abs control = absorbance of control and Abs test = Absorbance of test

2.4 Screening of whole cell extract of algae for inhibition of mushroom tyrosinase enzyme

Tyrosine reacts in two steps. In first step, L-tyrosine is converted to L-DOPA and in second step L-DOPA is converted to o - Dopaquinone. L-tyrosine or L-DOPA is used as substrate. Tyrosinase enzyme is incubated with test extracts to check enzyme inhibition, if any. The reaction mixture consisted of 400 µl of 5mM phosphate buffer, 200 µl of test extract, 200 µl of tyrosinase enzyme (200 units/ml)> incubation was done at 37°C for 10 min. Buffer was used instead of sample in blank. Kojic acid was used as standard. Absorbance was measured at 475 nm. Per cent inhibition was calculated as follows[16,17]:

$$\textbf{Percent inhibition of tyrosinase} = [(Abs_{control} - Abs_{test})/Abs_{control}] * 100$$

Where, Abs control = absorbance of control and Abs test = Absorbance of test

3. RESULTS AND DISCUSSION

3.1 Sample collection

Samples were collected from different ecological niche of Maharashtra viz, Freshwater rivers like Kalu river from Titwala, Godavari and Kadhava river from Nashik, garden soils in Thane and tree barks of *Albizia julibrissin* and *Syzygium cumini*. The samples were enriched in Bold Basal medium at 25°C for 15 days. After repeated subculturing, 8 unialgal isolates were obtained belonging *Pseudophormidium* sp. and *Leptolyngbya* sp., *Oscillatoria* sp., *Weistellopsis* sp., *Phormidium* sp and *Haphalosiphon* sp. The details of source and isolate is shown in figure I.

3.2 Biochemical characterization of algal isolates

The isolates were studied for protein, carbohydrate, lipid, polyphenols and flavonoid content. Protein content of the 8 algal isolates was observed in the range of 20.69 % to 35.70 % the highest protein content of 35.70% was observed in isolate no GR01A1. Carbohydrate content was observed in the range of 24.34 % to 35.60% and Lipid content was observed in the range of 30.90% to 40.72%. Isolate no KDR01A7 showed highest carbohydrate content (35.61%) and lipid content of 40.722% the results are presented in Table no 1.

The cyanobacterial species produce various secondary metabolites to adopt environmental conditions. Most of these metabolites like flavonoids, mycosporine like amino acids, carotenoids are antioxidant in nature. Antioxidant metabolites play key role in preventing skin damage due to different stressors like UV rays, environmental pollution etc. Therefore, the 8 unialgal isolates were screened for their antioxidant potential by two different assays DPPH inhibition assay and ABTS inhibition assay. The 8 strains showed DPPH inhibition in the range if 72% to 89%. The highest antioxidant activity for DPPH was observed by 89.51 % inhibition by ALB01A5 followed by 89.46 % inhibition by KL01A7. In case of ABTS inhibition assay, per cent inhibition of BATs radical by cyanobacteria was observed in the range of 82% to 97%. The maximum inhibition was observed by GR01A3 (96.05%) followed by GR01A1 (96.03%). KL01A7 found to have higher potential for both DPPH as well as ABTS radical. All the eight strains found to have potential for scavenging free oxygen radicals, thereby protecting skin from various disorders like photoaging, sunburn etc.
All the strains were evaluated for their potential as skin whitening agent using tyrosinase inhibition assay. Kojic acid was used as standard. Tyrosinase inhibition was observed in the range of 17.39% to 39.58%. Kojic acid showed 99.86% inhibition. Cyanobacteria have less efficiency than standard. But they are safe alternatives to current synthetic active agents. Maximum inhibition of tyrosinase enzyme was observed in case of isolate KDR01A7 (39.58%) followed by greater than 30% tyrosinase inhibition in three isolates namely TG01A4 (36.5%), GR01A1(33.82%) and ALB01A1(32.72%).

4. CONCLUSION

Eight cyanobacteria were isolated from different ecosystems of Maharashtra. All the eight isolates showed good antioxidant activity. This property has application in protecting skin from oxidative stress, UV radiations and environmental pollution. The eight isolates were also found to be rich in protein, carbohydrates, and lipids. Proteins help in skin nourishment. Carbohydrates play major role in skin cosmetics as a moisturizer and to retain moisture balance of skin. They also provide skin lubrication, protect the skin from roughness and unblemished skin. (Nowruzi et al 2020) These isolates were also observed to have tyrosinase inhibition property.

The tyrosinase inhibition efficacy is only 30-40% in case of cyanobacteria which is three times less than Kojic acid (99%). However, cyanobacterial extracts are safe active agents. Moreover, they have other benefits like radical scavenging effect, moisturizing effect. They are known to have photo protective effect. Thus, these multiple benefits suggest, great potential of cyanobacterial extracts to be used in skin care cosmetics.

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APPENDIX

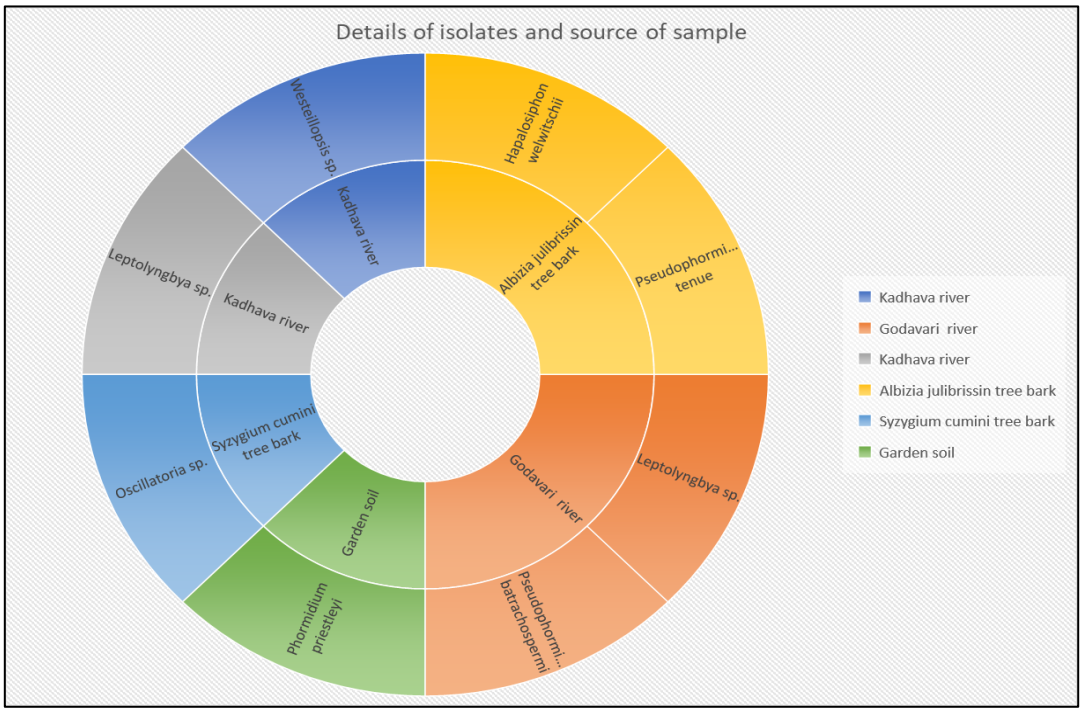

Chart 1: Details of isolates and their source of origin

Table 1: Estimation of protein carbohydrate and lipid content of cyanobacterial isolaes

Isolate ID	Genus	Proteins	Carbohydrates	Lipids
KL01A7	*Westeillopsis sp.*	32.84 ± 1.48	29.60 ± 0.09	30.90 ± 0.88
GR01A1	*Pseudophormidium sp.*	35.71 ± 4.48	29.94 ± 0.03	33.76 ± 1.13
GR01A3	*Leptolyngbya sp.*	31.91 ± 3.53	26.15 ± 0.06	40.36 ± 0.39
KDR01A7	*Leptolyngbya sp.*	20.69 ± 3.25	35.61 ± 0.07	40.72 ± 0.46
ALB01A4	*Pseudophormidium sp.*	32.13 ± 5.30	31.86 ± 0.07	40.19 ± 0.37
ALB01A5	*Hapalosiphon sp.*	28.91 ± 3.08	27.01 ± 0.17	38.84 ± 0.30
JB01A2	*Oscillatoria sp.*	25.03 ± 1.44	24.34 ± 0.05	38.63 ± 0.87
TG01A4	*Phormidium sp.*	32.39 ± 5.82	43.79 ± 0.13	24.14 ± 1.93

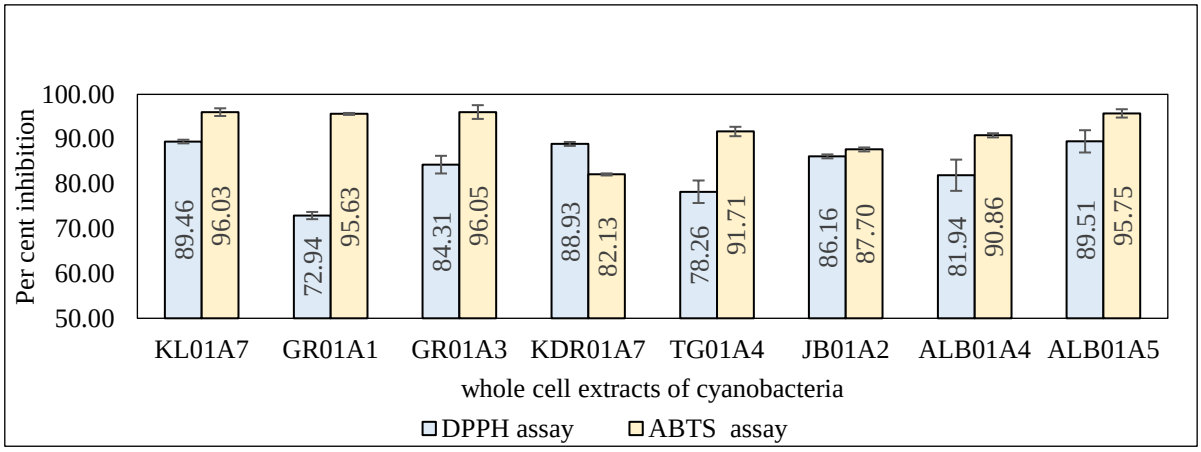

Chart 2: Estimation of antioxidant activity of whole cell extracts of cyanobacteria using DPPH and ABTS assay.

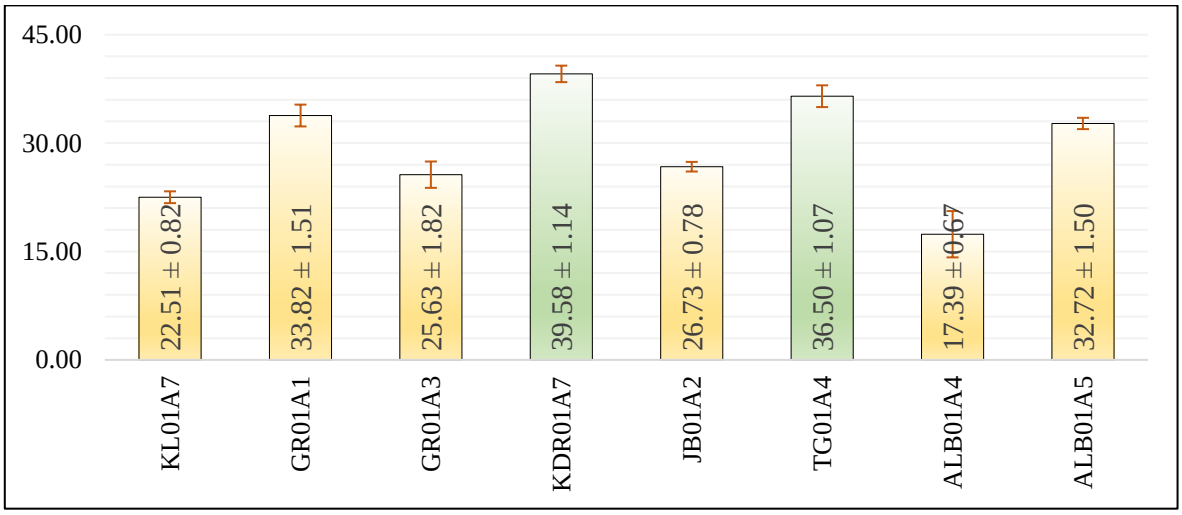

Figure 3: Estimation of tyrosinase inhibition activity of whole cell extracts of cyanobacteria.

