SUPPLEMENTARY MATERIAL

Antioxidant potential of indigenous Cyanobacterial Strains in relation with their phenolic and flavonoid contents.

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Abstract

Antioxidant activities of eight indigenous cyanobacterial strains belonging to the genera *Oscillatoria*, *Chroococcidiopsis*, *Leptolyngbya*, *Calothrix*, *Nostoc* and *Phormidium* were studied in relation with their phenolic and flavonoid contents, ranging from 3.9-12.6 mg GAE g⁻¹ and 1.7-3.44 mg RE g⁻¹. The highest activities were shown by *Leptolyngbya* sp. SI-SM (EC₅₀ = 63.45 and 67.49 µg ml⁻¹) and *Calothrix* sp. SI-SV (EC₅₀ = 65.79 and 69.38 µg ml⁻¹) calculated with ABTS and DPPH assays. Significant negative correlations were seen between total phenolic and flavonoid contents and the antioxidant activities in terms of EC₅₀ values. Furthermore, HPLC detected fifteen phenolic compounds with total concentrations ranging from 277.3-829.7 µg g⁻¹. The prevalent compounds in most of the strains were Rutin, Tannic acid, Orcinol, Phloroglucinol and Protocatechuic acid. Cyanobacterial strains showed high potential as a good source of phenolic compounds with potent antioxidative potential which could be beneficial for food, cosmetic and pharmaceutical industries.

Keywords: Antioxidation; Phenolics; Flavonoids; ABTS; DPPH; HPLC.

Experimental

1. *Growth and Cultivation of Cyanobacteria*

To study the antioxidation potential of phenolic compounds produced by cyanobacteria, eight different strains of cyanobacteria: *Oscillatoria* sp. *SI-SA* (GenBank accession no. **KJ755069.1**) *Oscillatoria* sp. *SI-SF* (GenBank accession no. **KJ755070.1**), *Chroococcidiopsis thermalis* *SI-ST* (GenBank accession no. **KJ755066.1**), *Leptolyngbya* sp. *SI-SM* (GenBank accession no. **KJ755064.1**), *Calothrix* sp. *SI-SV* (GenBank accession no. **KJ755062.1**), *Nostoc* sp. *SI-SN* (GenBank accession no. **KJ755063.1**), *Oscillatoria* sp. *SI-SK* (GenBank accession no. **KJ690262.1**) and *Phormidium* sp. *SI-SC* (GenBank accession no. **KJ755065.1**) were selected, previously isolated and purified from different environmental sources. They were identified morphologically and with 16s rRNA gene sequencing. The sequence was then analyzed with BLASTN database (the details will be discussed in a subsequent study). The strains were grown in 500ml Erlenmeyer flasks containing 250ml of sterilized BG-11 media having pH of 7.6. Cyanobacterial strains were grown for 60 days in an Infors HT Multitron shaking incubator under continuous low intensity white fluorescent light of $80 \mu\text{E m}^{-2} \text{s}^{-1}$ measured with a scalar PAR meter (Biospherical Instruments, Inc. QSL-2100) at 28°C and 50 rpm.

2. *Harvest and Extraction*

The cyanobacterial biomass obtained was harvested through high speed centrifugation (Sigma 3K30) at 25,000xg for 30 min at 4°C. The biomass was washed twice with sterile distilled water and then quickly frozen with liquid nitrogen. The frozen biomass was then lyophilized (Christ Alpha 2-4 LD) under reduced pressure of 0.040 mbar at -83°C and then stored at -20°C until further use. 0.1 g freeze dried biomass was homogenized and ultra-sonicated on ice for 15 minutes with 5 breaks in 10 ml of 70% methanol. The extracts were kept at 4°C overnight in the dark for complete extraction after which they were centrifuged two times to remove the cell debris. They were then passed through 0.45 μm sterile filter.

3. *Total Phenolic Content*

The total phenolics were estimated in a 96-well flat bottom micro-titer plate by a method described by Herald et al. (2012) with some modifications. Briefly, to each of the 96 wells, 75 μl of milli-Q water was added followed by 25 μl of sample, standard or blank. After mixing, 25 μl

of diluted (1:1) Folin-Ciocalteu reagent was added to the mixture. After 6 minutes of incubation in the dark, 100 μl of 75 mg ml^{-1} sodium carbonate solution was added to each well. The solutions were mixed and the plates were covered and left in dark for 90 minutes. The absorbance of the colored product formed was measured with a micro-well plate reader at 765nm. The concentrations of total phenolics were then analysed with the linear regression equation obtained from the means $\pm$ SEM of standard calibration curve of Gallic acid (Figure S4). The results were expressed as mg GAE g^{-1} dry weight.

4. Total Flavonoid Content

The flavonoids were estimated in a 96-well flat bottom micro-titer plate by a method described by Herald et al. (2012) with slight adjustments. Firstly, 150 μl of 95% ethanol was added to the wells followed by 50 μl of extracts, standard or blank. After that 10 μl of 100 mg ml^{-1} aluminum chloride solution was added followed by 10 μl of 1M potassium acetate solution. 95% ethanol was used as a blank. The mixture was then incubated at room temperature in dark for 40 minutes after which absorbance was recorded at 415nm with the help of a micro-well plate reader. The concentrations of total flavonoids were then analysed with the linear regression equation obtained from the means $\pm$ SEM of standard calibration curve of Rutin (Figure S5). The results were expressed as mg RE g^{-1} dry weight.

5. Antioxidation Assay

The free radical scavenging activity of phenolic extract was analyzed by ABTS and DPPH assays in 96-well plates using the methods described by Re et al. (1999) and Fukumoto and Mazza (2000) respectively. Trolox and Ascorbic acid were used as standard antioxidants. The percentage scavenging effect was calculated as:

$$\% \text{ Scavenging Activity} = [(A_{\text{control}} - A_{\text{sample or standard}}) / A_{\text{control}}] \times 100.$$

The antioxidation activities were expressed in terms of EC_{50} values which is a concentration of cyanobacterial extracts or standards at which 50% of the free radical was scavenged. EC_{50} values were calculated with non-linear regression analysis.

6. RP-HPLC UV/VIS Analysis of Phenolic and Flavonoid Content.

The analysis of phenolic and flavonoid compounds in cyanobacterial extract was done with HPLC system (Sykam) equipped with UV/VIS detector (Sykam S3210), dual pump system (Sykam S1122), temperature control module (Sykam S4011), pump control module (Sykam), and RP-C18 column (250 x 4.6mm Hypersil ODS 5 µm particle size). The peaks were analysed with integrated software clarity. The mobile phase consisted of methanol and water (95:5) and the flow rate was adjusted to 1ml min⁻¹. The analysis was run at ambient temperature. The crude phenolic extracts and standard phenolic and flavonoid compounds were diluted with absolute methanol (HPLC grade) and 25µl of each sample was loaded with a micro syringe. Each sample was run for 20 min and the absorbance was recorded at 280nm. The phenolic compounds were detected by comparing the peaks in the crude extracts with those of standards.

7. Statistical Analysis

The experiments included three technical replicates with three independent experiments (n=3). The standard calibration curves were made with Microsoft Excel 2010. The EC₅₀ and regression analysis and correlations were determined by using GraphPad prism version 5.01. The correlations were analysed with one-way analysis of variance (ANOVA) under Tukey's multiple comparison test. Significant difference was expressed at $p < 0.05$.

References

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Table S1. Total Phenolic, Flavonoid contents and Antioxidation Potential of Cyanobacterial Extracts.

Cyanobacterial Strains and Standard	Phenolic and Flavonoid Contents ^a		Antioxidation Potential EC ₅₀ Values ^b	
	TPC mg GAE g ⁻¹	TFC mg RE g ⁻¹	EC ₅₀ ABTS μg ml ⁻¹	EC ₅₀ DPPH μg ml ⁻¹
<i>Oscillatoria</i> sp. SI-SA (KJ755069.1)	5.0 ± 0.008	1.71 ± 0.004	98.59 ^{**}	103.9 ^{**}
<i>Oscillatoria</i> sp. SI-SF (KJ755070.1)	3.90 ± 0.02	2.82 ± 0.01	91.57 ^{**}	92.23 ^{**}
<i>Chroococcidiopsis thermalis</i> SI-ST (KJ755066.1)	4.32 ± 0.01	2.44 ± 0.004	94.01 ^{**}	99.36 ^{**}
<i>Leptolyngbya</i> sp. SI-SM (KJ755064.1)	12.60 ± 0.03	3.44 ± 0.003	63.45 ^{***}	67.49 ^{**}
<i>Calothrix</i> sp. SI-SV (KJ755062.1)	12.40 ± 0.02	3.23 ± 0.004	65.79 ^{**}	69.38 ^{***}
<i>Nostoc</i> sp. SI-SN (KJ755063.1)	10.32 ± 0.01	2.71 ± 0.004	75.99 ^{***}	78.51 ^{**}
<i>Oscillatoria</i> sp. SI-SK (KJ690262.1)	4.0 ± 0.01	2.1 ± 0.006	112.1 ^{**}	119.6 ^{***}
<i>Phormidium</i> sp. SI-SC (KJ755065.1)	8.50 ± 0.03	2.6 ± 0.01	81.17 ^{***}	83.08 ^{**}
Ascorbic Acid	N.A	N.A	N.A	28.16 ^{**}
Trolox	N.A	N.A	25.48 ^{**}	N.A

TPC= Total Phenolic content, TFC= Total Flavonoid content, GAE= Gallic acid equivalent, RE= Rutin equivalent.

^a The data is expressed as a mean ± SEM of triplicate experiments. Significant difference is expressed as ^{**} $p < 0.05$ ^{***} $p < 0.01$. ^b EC₅₀ values were obtained by interpolation from non-linear regression analysis.

Table S2. HPLC UV/VIS Analysis of Phenolic and Flavonoid Content in Cyanobacterial Strains.

Phenolic Compounds	RT min	Cyanobacterial Strains ($\mu\text{g g}^{-1}$ dw)							
		SI-SA	SI-SF	SI-ST	SI-SM	SI-SV	SI-SN	SI-SK	SI-SC
Rutin	2.83	N.D	138.1 ^d	116.0 ^a	176.2 ^b	148.2 ^{cd}	122.3 ^c	96.3 ^a	N.D
Tannic acid	2.61	40.2 ^b	N.D	13.4 ^c	56.2 ^a	42.1 ^e	75.0 ^d	28.3 ^c	56.4 ^{de}
Orcinol	3.0	146.1 ^b	N.D	120.5 ^{ab}	154.1 ^c	123.4 ^a	152.2 ^{de}	71.2 ^{bc}	167.4 ^c
Phloroglucinol	2.80	28.3 ^c	N.D	13.3 ^{ac}	21.3 ^c	20.2 ^{ac}	16.4 ^{ab}	N.D	12.0 ^a
Resorcinol	2.91	N.D	18.0 ^d	N.D	34.0 ^c	30.4 ^b	25.3 ^b	6.2 ^a	81.3 ^c
Gallic acid	2.53	N.D	N.D	25.6 ^a	205.4 ^{de}	N.D	61.4 ^{bc}	18.2 ^c	43.8 ^b
Caffeic acid	3.1	N.D	28.2 ^a	N.D	12.0 ^a	10.3 ^b	5.2 ^b	N.D	N.D
Ferulic acid	4.4	N.D	N.D	N.D	98.1 ^c	92.2 ^{de}	N.D	35.5 ^b	N.D
Vanillic acid	6.12	N.D	N.D	6.3 ^a	19.1 ^d	15.3 ^a	N.D	N.D	21.7 ^d
Vanillin	3.8	N.D	N.D	N.D	N.D	17.2 ^a	N.D	17.7 ^b	16.6 ^b
Salicylic acid	3.9	23.5 ^c	N.D	N.D	12.0 ^b	15.1 ^c	10.1 ^c	N.D	8.2 ^a
Benzoic acid	6.9	N.D	17.5 ^d	N.D	N.D	14.1 ^d	N.D	N.D	12.0 ^a
Syringic acid	3.3	N.D	N.D	N.D	N.D	N.D	N.D	8.4 ^b	13.4 ^c
Acetyl Salicylic acid	4.5	13.2 ^c	16.1 ^c	8.3 ^c	N.D	N.D	20.1 ^{de}	N.D	N.D
Protocatechuic acid	2.3	94.0 ^a	74.4 ^{de}	91.1 ^{de}	41.3 ^{de}	45.2 ^{de}	N.D	35.6 ^b	85.3 ^{de}
Total		345.3	277.3	394.5	829.7	590.9	531	317.4	518.1

The compounds were detected at 280 nm. RT= retention Time, N.D= Not detected. Means in the same rows representing different letters are significantly different ($p<0.05$).

Figure S1. Free radical scavenging activity of 70% methanolic extracts of cyanobacterial strains. (A). Percentage radical scavenging by cyanobacterial strains measured by ABTS assay. (B). Percentage radical scavenging by cyanobacterial strains measured by DPPH assay. The doses of the extracts are presented as log transformed concentrations ranging from 10-800 $\mu\text{g ml}^{-1}$.

Figure S2. Free radical scavenging activity of standards Trolox and Ascorbic acid (A). Percentage radical scavenging by Trolox measured with ABTS assay. (B). Percentage radical scavenging by Ascorbic acid measured with DPPH assay. The doses of the extracts are presented as log transformed concentrations ranging from 10-200 $\mu\text{g ml}^{-1}$. Error bars indicate mean $\pm$ SEM of three independent experiments with three technical replicates.

Figure S3. Correlation between the antioxidant capacity and phenolic and flavonoid content of 70% methanolic extracts of cyanobacterial strains (A). Correlation between total phenolic content and EC₅₀ values obtained from ABTS and DPPH assays. **(B).** Correlation between total flavonoid content and EC₅₀ values obtained from ABTS and DPPH assays.

Figure S4. Standard calibration curve of Gallic acid. Error bars indicate mean $\pm$ SEM of three independent experiments with three technical replicates.

Figure S5. Standard Calibration curve of Rutin. Error bars indicate mean $\pm$ SEM of three independent experiments with three technical replicates.

RP-HPLC PROFILES OF STANDARDS AND CYANOBACTERIAL CRUDE PHENOLIC EXTRACTS

STANDARDS

Figure S6a. RP-HPLC of Standard Phenolic compounds. Peaks: 1-Resorcinol, 2-Syringic acid, 3-Salicylic acid, 4-Ferulic acid, 5-Acetyl Salicylic acid, 6-4-Tert-Butylphenol, 7-Vanillic acid, 8-Benzoic acid.

Figure S6b. RP-HPLC of Standard Phenolic compounds. Peaks: 1-Protocatechuic acid, 2-Gallic acid, 3-Tannic acid, 4-Phloroglucinol, 5-Rutin, 6-Orcinol, 7-Caffeic acid, 8-Vanillin.

CYANOBACTERIAL CRUDE PHENOLIC EXTRACTS

Figure S7. HPLC Profile of *Oscillatoria* sp. SI-SA crude Phenolic extract.

Figure S8. HPLC Profile of *Oscillatoria* sp. SI-SF crude Phenolic extract.

Figure S9. HPLC Profile of *Chroococidiopsis thermalis* SI-ST crude Phenolic extract.

Figure S10. HPLC Profile of *Leptolyngbya* sp. SI-SM crude Phenolic extract.

Figure S11. HPLC Profile of *Calothrix* sp. SI-SV crude Phenolic extract.

Figure S12. HPLC Profile of *Nostoc* sp. SI-SN crude Phenolic extract.

Figure S13. HPLC Profile of *Oscillatoria* sp. SI-SK crude Phenolic extract.

Figure S14. HPLC Profile of *Phormidium* sp. SI-SC crude Phenolic extract.