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Molecular Identification of Some Selected Cyanobacteria and Their Antioxidant Activities

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Abstract

Cyanobacteria, photosynthetic prokaryotes, are promising sources of biologically active compounds with antioxidant properties, attracting interest in their potential applications in the food, pharmaceutical, and cosmetic sectors. This present study aimed to use 16S rRNA sequences for identification of some selected strains of cyanobacteria which were previously morphologically identified. The investigation of their antioxidant activities was also performed by ABTS radical scavenging assay, ORAC-fluorescein assay and metal chelating assay in this study. Molecular identification of 8 isolates revealed 1 *Calothrix* sp., 2 *Nostoc* sp., 2 *Amazonocrinis* sp., 2 *Aulosila* sp., and 1 from the Hapalosiphonaceae family. *Aulosila* sp. exhibited the highest ABTS inhibition (IC_{50}: 18.87 µg/µL), followed by a Hapalosiphonaceae cyanobacterium (19.11 µg/µL) and *Calothrix* sp. (21.74 µg/µL). The ORAC-fluorescein assay showed *Nostoc* sp. TUBT05 and a Hapalosiphonaceae cyanobacterium had the highest peroxyl radical scavenging with TE values of 0.22 and 0.21 µg/µL, respectively. *Nostoc* sp. TUBT01 demonstrated the highest metal chelating activity (IC_{50}: 36.48 µg/µL). The combined analysis of selected cyanobacteria revealed insights into the relationship between their taxonomy and antioxidant profiles, highlighting their potential as natural antioxidant sources.

Keywords: Cyanobacteria, Antioxidant activities, 16S rRNA sequences, Molecular identification, ABTS radical scavenging assay, ORAC-fluorescein assay, Metal chelating assay, Taxonomic relationships

Introduction

Cyanobacteria are known for producing diverse bioactive compounds, including potent antioxidants like pigments and phenolic compounds [1,2]. These antioxidants help neutralize reactive oxygen species (ROS), which can cause oxidative damage and are linked to diseases such as cardiovascular issues, cancer, diabetes, and neurodegenerative disorders [3]. Hydrophobic pigments like chlorophylls and carotenoids are common in cyanobacterial biomass, and cyanobacteria also produce phenolic compounds (polyphenols), a class of antioxidants with high *in vitro* activity and health benefits [4,5]. These phenolics include hydroxybenzoic acids, hydroxycinnamic acids, flavan-3-ols, flavonoids, glycosides, and proanthocyanidins [5]. These compounds can act as free radical scavengers, metal chelators, and inhibitors of lipid peroxidation, thus protecting cells from oxidative damage. The variety of phenolic compounds produced by cyanobacteria, along with their potent antioxidant properties, makes them a promising source for the development of natural antioxidant therapies. Furthermore, certain cyanobacterial strains have been shown to produce unique phenolic compounds not found in other organisms, highlighting their potential as a novel source of bioactive compounds. The exploration of natural antioxidants from sources like cyanobacteria has gained significant attention due to the growing preference for natural alternatives over synthetic antioxidants, which may exhibit toxicity and carcinogenic or mutagenic effects [6].

Rice fields offer a unique habitat for diverse cyanobacterial strains, many yet unexplored for their antioxidant potential [7,8]. These strains, adapted to fluctuating paddy conditions, likely produce antioxidants in response to environmental stressors like light, temperature, and nutrient limitations [2,8,9]. Their antioxidant production is influenced by factors such as genetic makeup, growth conditions, and nutrient availability [7,10]. Identifying high-antioxidant strains from rice fields shows promise for agriculture as biofertilizers to enhance crop yield and stress tolerance [11,12], and for the pharmaceutical/nutraceutical industries as sources of natural antioxidants for combating oxidative stress-related diseases [1,13]. Moreover, understanding the antioxidant mechanisms of these strains could provide knowledge into the complex interplay between cyanobacteria and their environment.

Molecular identification of cyanobacteria has become increasingly feasible through the use of 16S rRNA gene sequencing. This method allows for precise identification and phylogenetic analysis of cyanobacterial strains, enabling researchers to link specific strains to their bioactive potential [14]. The 16S rRNA gene is highly conserved among prokaryotes, but it also contains hypervariable regions that provide species-specific signature sequences, making it an ideal marker for microbial identification [15]. By sequencing the 16S rRNA gene, researchers can accurately identify and classify cyanobacteria, facilitating the discovery of species with significant antioxidant activity and other valuable properties.

This study evaluated the antioxidant activity of cyanobacterial strains isolated from rice fields using the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging, Oxygen Radical Absorbance Capacity (ORAC), and metal chelating assays to assess their free radical neutralization abilities. Additionally, 16S rRNA gene sequencing was also used to identify the strains and link their genetic identity to their antioxidant potential.

Materials and methods

Samples

Eight isolates of cyanobacteria were obtained from the Algae and Plankton Research Group, Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Thailand. These isolates were previously morphologically identified and nominated *Calothrix* N5, *Nostoc* sp. TUBT05, *Fischerella* sp., *Nostoc* sp. TUBT04, *Nostoc* sp. TUBT02, *Nostoc* sp. TUBT01, *Anabaena* sp. N2, and *Tolypothrix* sp. TUBT031 [16]. Cyanobacteria were cultured in BG (Blue Green)-11 medium with constant shaking (120 rpm) under a 12-hour light/dark cycle at 25 °C for 30 days. Cells were harvested by centrifugation (8,000 rpm, 10 min, 4 °C), the supernatant discarded, and the pellet weighed.

PCR amplification, cloning and sequencing of 16S rRNA gene from cyanobacteria

DNA extraction was performed using a modified Cetyltrimethylammonium bromide (CTAB) method [17]. The 16S rRNA gene fragment was amplified with primers 16S rRNA-F1 (5'-CGGACGGGTGAGTAACGCGTGA-3') and 16S rRNA-R02 (5'-GCTGACCTGCAATTACTAGCG-3') in a 25 µL PCR reaction containing 12.5 µL of 2X Taq PCR Master Mix (Thermo Scientific, USA), 0.5 µM of each primer, 1 µL of template DNA (20 ng/µL), and nuclease-free water. The thermal cycling conditions were 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 52 - 56 °C for 45 s, and 72 °C for 90 s, with a final extension at 72 °C for 7 min. PCR product (approximately 1,200 bp) was analyzed on 1 % agarose gel, purified with QIAquick Kit (QIAGEN, Germany), cloned into pGEM-T vector (Promega, USA), transformed into *Escherichia coli* DH5α, and sequenced by Macrogen Inc., South Korea.

Molecular identification of cyanobacteria and phylogenetic construction

Cyanobacterial identification was performed using BLAST and phylogenetic analysis. The 16S rRNA gene sequences (approximately 1,200 bp) of the cyanobacterial isolates were compared with those available in the NCBI database using the BLASTN tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine their closest relatives. For phylogenetic analysis, multiple sequence alignment was conducted using Clustal W. The aligned sequences were then used to construct a phylogenetic tree employing the Neighbor-Joining (NJ) method in MEGA version 11.0 [18], with 1,000 bootstrap replicates to assess the reliability of the tree branches.

Preparation of cyanobacterial crude extracts

The collected cell pellets were resuspended in sterile deionized water and disrupted using ultrasonication. Sonication was performed with a duty cycle of 1 min on and 1 min off for a total of 10 cycles, with the centrifuge tubes immersed in an ice bath to prevent heat damage. The disrupted cells were then centrifuged at 9,000 rpm for 30 min at 4 °C to obtain the supernatant. The protein concentration of the crude extract was measured using a Lowry assay. Based on the measured protein concentration, the crude extract was diluted to various concentrations of its total protein for further analysis.

ABTS radical scavenging assay

The antioxidant activity of crude extracts was evaluated using the ABTS radical cation decolorization [19]. ABTS radical cations were generated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate and incubating in the dark at room temperature for 12 - 16 h. This solution was then diluted with 10 mM phosphate buffer (pH 7.4) to achieve an absorbance of 0.7 ± 0.02 at 734 nm. For the assay, 900 µL of the diluted ABTS solution was mixed with 100 µL of cyanobacteria extract at various protein concentrations. The reaction was incubated in the dark for 5 min, and absorbance at 734 nm was measured using a UV-Vis spectrophotometer (Shimadzu, Japan). Trolox served as the positive control. ABTS radical inhibition (%) was calculated using the following equation:

$$\text{ABTS radical scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}]\times 100 \quad (1)$$

$A_{control}$ is the absorbance of the control (distilled water instead of sample), and A_{sample} is the absorbance for the sample. The IC_{50}, the concentration required to inhibit 50 % of ABTS radicals, was determined from the dose-response curve.

Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay measured peroxyl radical scavenging activity of cyanobacterial extracts following Dávalos *et al.* [20]. A 583 µM fluorescein stock was diluted 1:5,000 in 75 mM sodium phosphate buffer (pH 7.4). The assay, performed in 96-well black plates, contained 120 µL of fluorescein working solution and 20 µL of extract at various concentration of its total protein. After 15 min at 37 °C, 60 µL of 40 mM 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was added, and fluorescence was measured using a microplate reader (Thermo Scientific, USA) every minute for 2 h (excitation: 485 nm, emission: 520 nm). The area under the fluorescence decay curve (AUC) was calculated and compared to the Trolox standard to determine the ORAC value, expressed as Trolox equivalents (TE).

Metal chelation activity

The metal ion chelation activity of cyanobacterial extracts was assessed following Wong *et al.* [21]. Briefly, extract (250 µL) at varying concentrations of its total protein was mixed with 0.1 mM ferrous sulfate solution (250 µL) and incubated at room temperature (10 min). The absorbance (562 nm) was measured after adding with 0.25 mM ferrozine (500 µL) and incubating for 10 min. Ethylenediaminetetraacetic acid (EDTA) serves as standard chelating agent. Metal ion chelation was calculated using the following formula:

$$\text{Metal chelating activity (\%)} = [(A_{control} - A_{sample})/A_{control}]\times 100 \quad (2)$$

$A_{control}$ is the absorbance of the control (distilled water instead of sample), and A_{sample} is the absorbance for the sample. The IC_{50}, the concentration required to chelate 50 % of the ferrous ions, was determined from the dose-response curve.

Statistical analysis

The IC_{50} values from the ABTS radical scavenging assay and Metal ion chelation assay of cyanobacterial extracts were analyzed using clustering analysis via

MetaboAnalyst software (6.0) [22]. Hierarchical clustering grouped the extracts based on their antioxidant activities, with a dendrogram and principal component (PC) plot used to visualize the relationships and patterns among them.

Result and discussion

Eight cyanobacterial isolates were morphologically identified as *Calothrix* N5, *Nostoc* sp. TUBT05, *Fischerella* sp., *Nostoc* sp. TUBT04, *Nostoc* sp. TUBT02, *Nostoc* sp. TUBT01, *Anabaena* sp. N2, and *Tolypothrix* sp. TUBT031 by Chittapun and Charoenrat [16]. In this study, molecular identification of selected isolates was performed using BLAST analysis (**Table 1**) and phylogenetic clustering of their 16S rRNA sequences (**Figure 1**).

Some BLAST analysis results showed consistency with previous morphological identifications. For instance, Isolate-1 (98.3 % identity) aligned with *Calothrix* sp. CCAP1410/13 (HF678491.1), and Isolate-2 and Isolate-5 had identities of 98.04 and 97.28 % with *Nostoc ellipsosporum* CCAP1453/2 (HF678488.1). Two distinct *Calothrix* clusters were identified by phylogenetic analysis, consistent with Komárek [23], which divides *Calothrix* into the Rivulariaceae and Gloeotrichiaceae families. The phylogenetic analysis further confirmed the identities of these isolates, with isolate-1 being placed in the *Calothrix* cluster, consistent with its classification as a member of the family Gloeotrichiaceae and Isolate-2 and 5 are *Nostoc* (**Figure 1**). Additionally, ribosomal intergenic spacer (IGS) analysis provided further support for the identity of Isolate-1 alignment (89.50 % identity with *Calothrix* sp. PCC7507 (CP003943.1)) as member of *Calothrix*. These results demonstrate the power and reliability of molecular techniques in identifying cyanobacterial isolates.

Discrepancies were noted between previous morphological identifications and molecular data. Isolate-3, initially classified as *Fischerella* sp. based on morphology, exhibited 100 % identity with *Hapalosiphon welwitschia* (OQ693653.1), *Westiella* intricate (KJ767016.1), and *Hapalosiphon delicatulus* (AB093484.1) in BLAST analysis. Both BLAST and phylogenetic analyses could not clearly differentiate Isolate-3 from *Hapalosiphon*, *Neowestiellopsis*, and *Westiella* species, placing it in the *Hapalosiphonaceae* family (phylogenetic cluster-H). This indicates a close genetic relationship among these genera, highlighting the limitations of relying solely on morphological identification and the need for molecular methods for accurate classification [25].

Table 1 Identification of cyanobacterial isolates based on morphological traits and BLAST analysis of 16S rRNA sequences, with family classification following Komárek [23] and Alvarenga *et al.* [24].

Isolate No.	**Nomination of the Cyanobacterial isolates based on their morphological identification**	**BLAST analysis of 16s rRNA sequence**		
		Closest Match	**Family**	**% Identity**
1	*Calothrix* sp. N5	*Calothrix* sp. CCAP 1410/13 (HF678491.1)	Gloeotrichiaceae	98.30
2	*Nostoc* sp. TUBT05	*Nostoc ellipsosporum* CCAP 1453/2 (HF678488.1)	Nostocaceae	98.04
3	*Fischerella* sp.	*Hapalosiphon welwitschii* PMC1099.19 (OQ693653.1), *Westiella intricata* UH HT-29-1 (KJ767016.1) and *Hapalosiphon delicatulus* IAM M-266 (AB093484.1)	Hapalosiphonaceae	100.00
4	*Nostoc* sp. TUBT04	*Amazonocrinis nigriterrae* CENA238 (MN551905.1)	Nostocaceae	98.98
5	*Nostoc* sp. TUBT02	*Nostoc ellipsosporum* CCAP 1453/2 (HF678488.1)	Nostocaceae	97.28
6	*Nostoc* sp. TUBT01	*Amazonocrinis nigriterrae* CENA238 (MN551905.1)	Nostocaceae	99.00
9	*Anabaena* sp. N2	*Nostoc carneum* (LC215292.1)	Nostocaceae	99.31
		Aulosira sp. CENA272 (MN551913.1)	Fortieaceae	99.31
10	*Tolypothrix* sp. TUBT031	*Aulosira* sp. SG5-PS (PP165366.1)	Fortieaceae	99.75

Our phylogenetic analysis, using 16S rRNA sequences from the family Hapalosiphonaceae (including *Hapalosiphon* and *Westiella*), showed that these genera remain indistinguishable. To resolve this, additional molecular approaches like multi-locus sequence analysis (MLSA), whole-genome sequencing, or metabolic profiling should be considered, along with detailed morphological studies. Previous studies suggest that *Fischerella*, *Hapalosiphon*, and *Westiella* may represent a single species, supported by high nucleotide identity and shared biosynthetic pathways for secondary metabolites such as hapalindoles, ambiguines, and welwitindolinones [26,27].

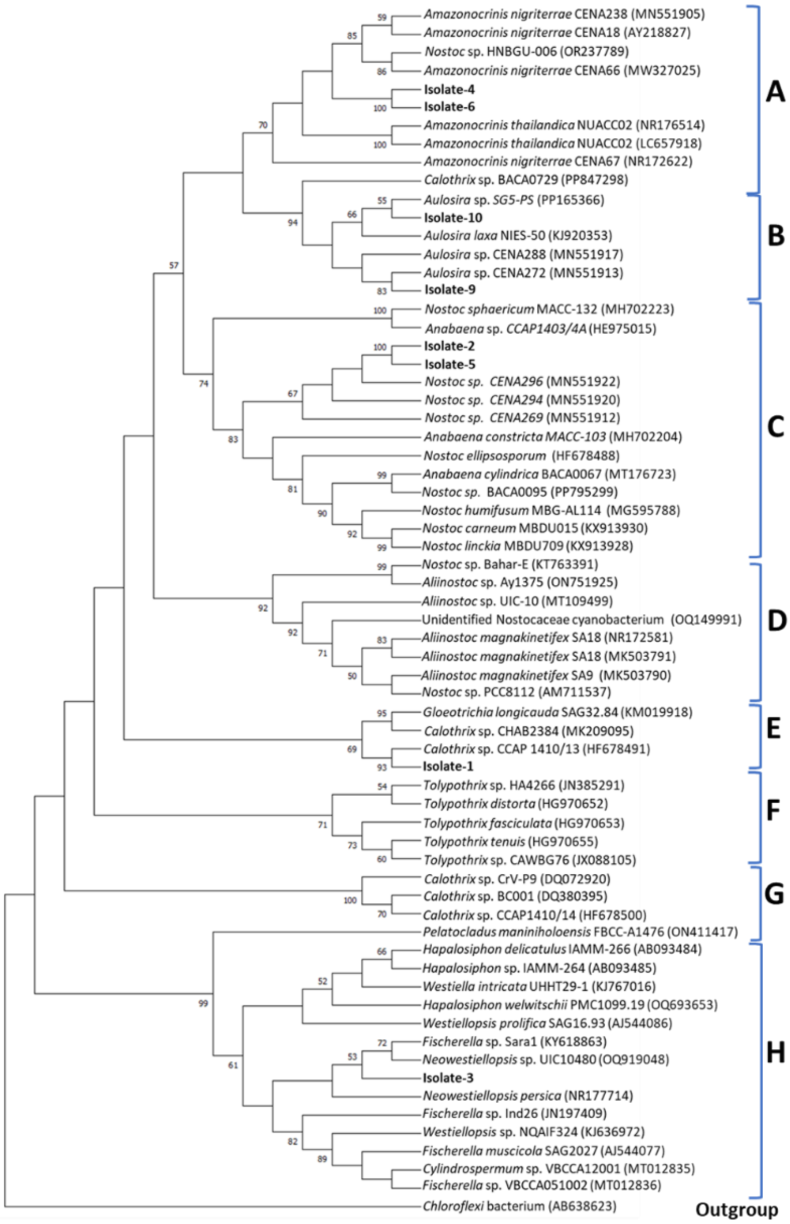


Figure 1 Phylogenetic tree based on partial 16S rRNA gene sequences from selected cyanobacteria (both from this study and GenBank), constructed using the neighbor-joining method with a *Chloroflexi bacterium* as the outgroup. Cyanobacterial isolates from this study are shown in bold with unique numbers, and the groups are labeled (A) to (H).

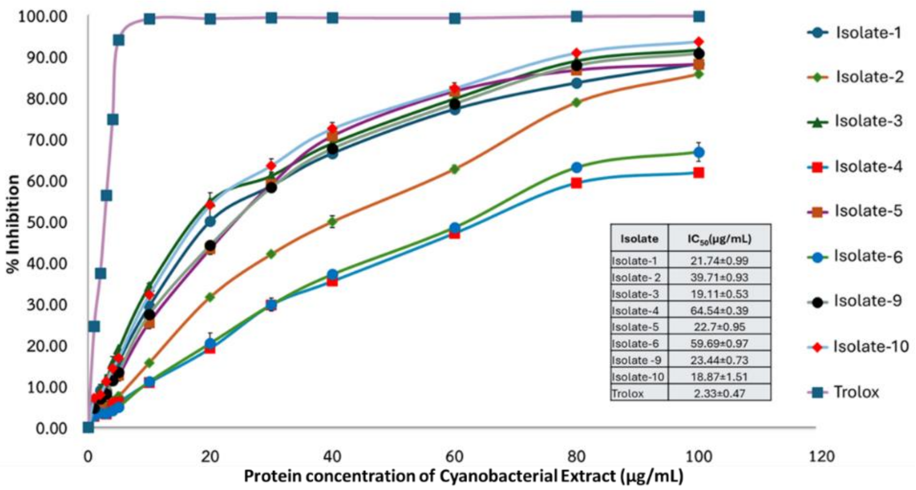
Phylogenetic analysis showed that Isolate-4 and Isolate-6 formed a distinct cluster closely related to *Amazonocrinis* within Cluster-A (**Figure 1**), confirming BLAST results and suggesting a stronger affinity with *Amazonocrinis* than *Nostoc*, as initially identified [16]. This highlights the limitations of morphological classification in distinguishing closely related taxa within the Nostocaceae family [28]. *Amazonocrinis*, recently assigned to Nostocaceae [24], shares morphological traits with *Nostoc*, including heterocystous filaments and uniseriate trichomes [29,30].

Phylogenetic analysis revealed that Isolates-9 and -10 clustered within a distinct clade (phylogenetic cluster-B), sharing close evolutionary relationships with *Aulosira* species. BLAST analysis indicated that Isolate-9 and -10 were most closely related to *Aulosira* sp. CENA272 (MN551913.1) and *Aulosira* sp. SG5-PS (PP165366.1), with identities of 99.31 and 99.75 %, respectively. This suggests that previous identifications of Isolate-9 as *Anabaena* sp. and Isolate-10 as *Tolypothrix* sp. may not accurately reflect their taxonomic positions. Further investigations, including detailed morphological characterization and broader 16S rRNA gene sampling from *Aulosira* and *Tolypothrix* species, are warranted to definitively classify Isolates-9 and -10. Additionally, genomic data could offer valuable insights into the genetic basis of the observed phylogenetic relationships.

The combined results from BLAST and phylogenetic analyses gave a thorough understanding of the cyanobacterial isolates. Although molecular methods confirmed many of the morphological identifications, they also exposed cases of misclassification and showed the genetic complexity within cyanobacterial groups. These findings emphasize the need to combine molecular techniques with traditional morphological methods to achieve accurate and reliable cyanobacterial classification.

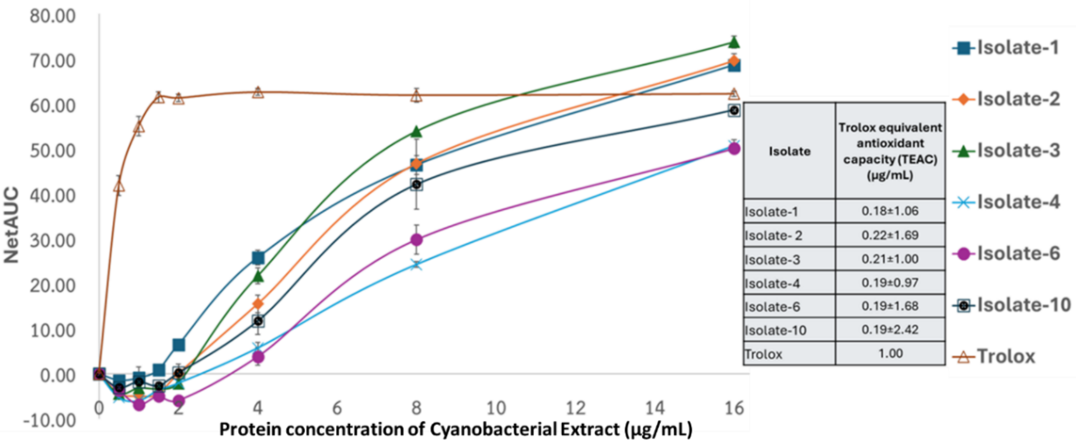
This study also comprehensively evaluated the antioxidant properties of cyanobacterial crude extracts using 3 distinct assays: The ABTS radical cation decolorization assay, the ORAC-fluorescein assay, and the metal chelating activity assay. These assays provide valuable insights into the ability of cyanobacterial extracts to scavenge free radicals and chelate pro-oxidant metal ions, which are crucial mechanisms in combating oxidative stress.

The ABTS radical cation decolorization assay evaluated the antioxidant capacity of selected cyanobacterial crude extracts by measuring their ability to scavenge the ABTS radical. This assay is based on a single-electron transfer (SET) mechanism, where antioxidants donate electrons to neutralize the free radical. The results, shown in **Figure 2**, indicated that the crude extract from Isolate-10 exhibited the highest ABTS radical scavenging activity, with an IC_{50} value of 18.87 ± 1.51 µg/mL, the lowest among the strains tested. The IC_{50} values of the other extracts were as follows: Isolate-3 (19.11 ± 0.53 µg/mL), Isolate-1 (21.74 ± 0.99 µg/mL), Isolate-5 (22.7 ± 0.95 µg/mL), Isolate-9 (23.44 ± 0.73 µg/mL), Isolate-2 (39.71 ± 0.93 µg/mL), Isolate-6 (59.69 ± 0.97 µg/mL), and Isolate-4 (64.54 ± 0.39 µg/mL). These findings highlight Isolate-10 and Isolate-3 as potential sources of potent antioxidants.



Isolate	IC_{50}(µg/mL)
Isolate-1	21.74±0.99
Isolate- 2	39.71±0.93
Isolate-3	19.11±0.53
Isolate-4	64.54±0.39
Isolate-5	22.7±0.95
Isolate-6	59.69±0.97
Isolate -9	23.44±0.73
Isolate-10	18.87±1.51
Trolox	2.33±0.47

Figure 2 ABTS radical scavenging capacities of selected cyanobacterial crude extracts at concentrations from 0 to 100 µg/mL. IC_{50} values, indicating the concentration required to inhibit 50 % of ABTS radicals, were determined for each isolate, with Trolox as the standard.

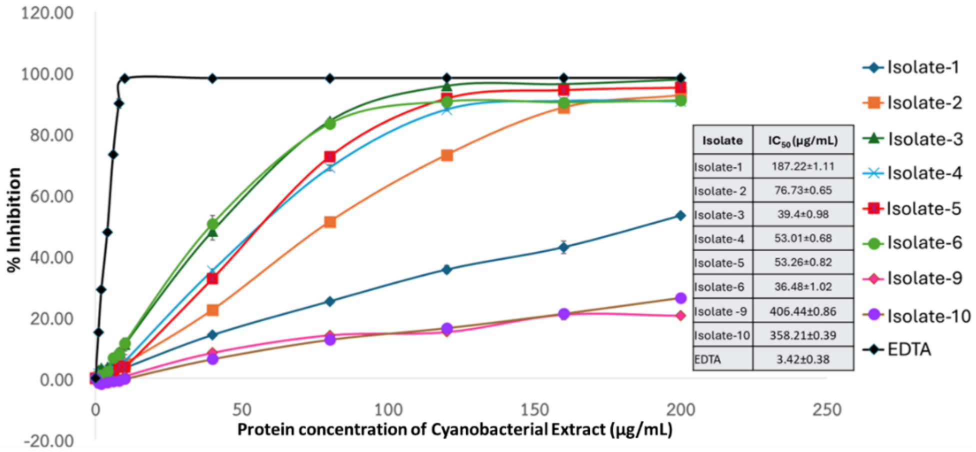


Isolate	Trolox equivalent antioxidant capacity (TEAC) (µg/mL)
Isolate-1	0.18±1.06
Isolate- 2	0.22±1.69
Isolate-3	0.21±1.00
Isolate-4	0.19±0.97
Isolate-6	0.19±1.68
Isolate-10	0.19±2.42
Trolox	1.00

Figure 3 Antioxidant activity of cyanobacterial crude extracts (0 to 16 µg/mL) measured using the ORAC-fluorescein assay, expressed as Trolox equivalents, with Trolox as the standard.

The ORAC-fluorescein assay assessed the peroxyl radical scavenging capacity of cyanobacterial crude extracts by measuring their ability to protect fluorescein from degradation by AAPH-generated peroxyl radicals. Antioxidant activity was quantified through Net AUC (**Figure 3**). Isolate-3 and Isolate-2 showed higher Net AUC values than other strains. Linear regression of Net AUC values (within the concentration range of 4 - 16 µg/mL) revealed that Isolate-2 had the highest Trolox equivalent (TE) value (0.22 ± µg/mL), indicating superior peroxyl radical scavenging capacity.

Ferrous ion (Fe^{2+}) chelating assay evaluated the ability of cyanobacterial extracts to chelate Fe^{2+}. Isolate-6 showed the highest chelating activity with an IC_{50} of 36.48 ± 1.02 µg/mL, followed by Isolate-3 (39.4 ± 0.98 µg/mL). Isolates 10 and 9 had the lowest activity (358.21 ± 0.39 and 406.44 ± 0.86 µg/mL, respectively). Isolate-6 provided high chelating activity indicates its potential as a natural chelating agent for managing oxidative stress in various applications [31,32].



Isolate	IC_{50} (µg/mL)
Isolate-1	187.22±1.11
Isolate- 2	76.73±0.65
Isolate-3	39.4±0.98
Isolate-4	53.01±0.68
Isolate-5	53.26±0.82
Isolate-6	36.48±1.02
Isolate -9	406.44±0.86
Isolate-10	358.21±0.39
EDTA	3.42±0.38

Figure 4 Metal chelating activities of cyanobacterial crude extracts at concentrations from 0 to 200 µg/mL. IC_{50} values indicate the concentration needed to chelate 50 % of metal ions. EDTA served as the standard chelating agent.

Cyanobacterial isolates exhibited diverse antioxidant profiles across 3 assays. Isolate-10 excelled in ABTS radical scavenging, Isolate-2 and Isolate-3 in ORAC assay, and Isolate-6 in Fe^{2+}chelation. These results highlight cyanobacterial extracts as versatile sources of antioxidants and chelating agents, with varying mechanisms suggesting diverse bioactive compounds for potential oxidative stress management.

Our PCA analysis of the selected cyanobacteria based on their ABTS scavenging and Fe^{2+} chelating activity classified them into 3 major groups (**Figure 5**). Additionally, it revealed correlations between the chemometric clusters of the antioxidant profiles and their taxonomic classification (**Figure 6**).

Cluster-1, identified through chemometric PCA analysis (**Figure 5**), showed high Fe^{2+} chelating activity. This cluster includes Isolates 2, 4, 5, and 6 from Nostocaceae (phylogenetic clusters A and C), and Isolate-3 from Hapalosiphonaceae (cluster H). These findings align with previous research on *Nostoc* species and Hapalosiphonaceae cyanobacteria's Fe^{2+} chelation abilities [2,33,34]. The strong Fe^{2+} chelating activity suggests these cyanobacteria may effectively mitigate iron-induced oxidative stress, a key environmental adaptation.

Furthermore, chemometric cluster-1 shows diverse ABTS scavenging activity, including *Nostoc* and *Amazonocrinis* genera (Nostocaceae family) and unidentified Hapalosiphonaceae cyanobacteria. *Amazonocrinis* isolates (4 and 6) exhibited lower ABTS scavenging but significant iron chelating activity compared to *Nostoc* isolates (2 and 5), suggesting distinct antioxidant mechanisms. *Amazonocrinis*, a separate genus within Nostocaceae, remains understudied. Varying ABTS scavenging levels between *Nostoc* isolates highlight intra-genus differences. These findings emphasize the importance of strain-level investigations in cyanobacterial research, as antioxidant capabilities may be influenced by ecological niches, genetic backgrounds, and environmental adaptations.

Isolate-3, an unidentified Hapalosiphonaceae cyanobacterium (phylogenetic cluster-H), exhibited the highest Fe2+ chelating activity and significant ABTS scavenging capabilities (**Figure 5**). This highlights Isolate-3 and potentially other cyanobacteria in phylogenetic cluster-H as promising antioxidant and metal chelating sources, meriting further study of their oxidative stress responses.

Chemometric cluster-2, containing Isolate-1, exhibited lower Fe^{2+} chelating activity than cluster-1 (**Figure 5**). This cluster, aligned with the genus *Calothrix* and phylogenetic cluster-E, which includes *Calothrix* sp. CCAP1410/13, *Calothrix* sp. CHAB2384, and *Gloeotrichia longicauda* SAG32.84, places Isolate-1 within the family Gloeotrichiaceae. While extensive data exists on the antioxidant activity of *Calothrix* in the family Rivulariaceae, information on *Calothrix* and *Gloeotrichia* in Gloeotrichiaceae remains limited, necessitating further research on these strains.

Chemometric cluster-3 (Isolate-9 and Isolate-10) demonstrated significant ABTS scavenging activity but displayed the lowest Fe^{2+} chelating capacity among the cyanobacteria studied (**Figure 5**). Their clustering highlights a close genetic relationship, suggesting they may belong to the same genus, corresponding to phylogenetic cluster-B and the genus *Aulosira* within the Fortieaceae family. This aligns with Singh *et al.* [2], who also found *Aulosira fertilissima* exhibited lower metal chelating ability compared to other cyanobacteria, but had strong ABTS scavenging activity, similar to *Calothrix geitonos*, *Nostoc ellipsosporum*, and *Hapalosiphon fontinalis*.

The significant ABTS scavenging activity observed in this study and by Singh *et al.* [2] underscores the potential of *Aulosira* spp. in biotechnological applications aimed at developing natural antioxidants, as suggested by recent studies on cyanobacterial antioxidants [35,36]. The low Fe^{2+} chelating capacity of *Aulosira* may be attributed to specific metabolic pathways and regulatory mechanisms that differ from those in other cyanobacteria [37]. Understanding these mechanisms can provide insights into cyanobacterial adaptation strategies in varying metal ion environments [38]. Furthermore, many reports demonstrate that *Aulosira* possess enzymatic and non-enzymatic antioxidant defense systems, which are crucial for their survival in diverse and often harsh environment [1,35,36].

The overall clustering analysis also indicates that there are likely significant differences in metal chelating activity among the cyanobacterial families, except for the Nostocaceae and the unidentified cyanobacterium within the Hapalosiphonaceae in this study.

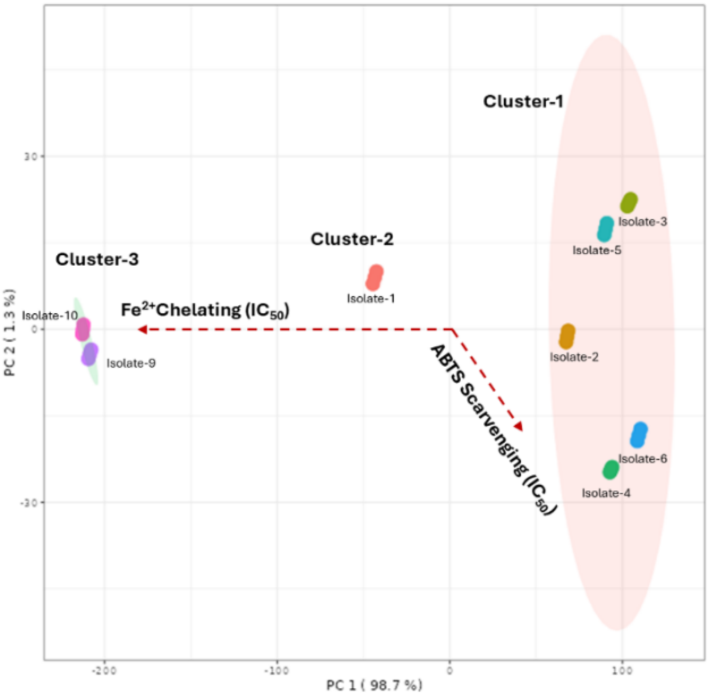


Figure 5 Principal component analysis (PCA) of selected cyanobacterial crude extracts and their antioxidant activities. PC1 and PC2 represent the total variance. The circle highlights a chemometric cluster with distinct antioxidant activity characteristics.

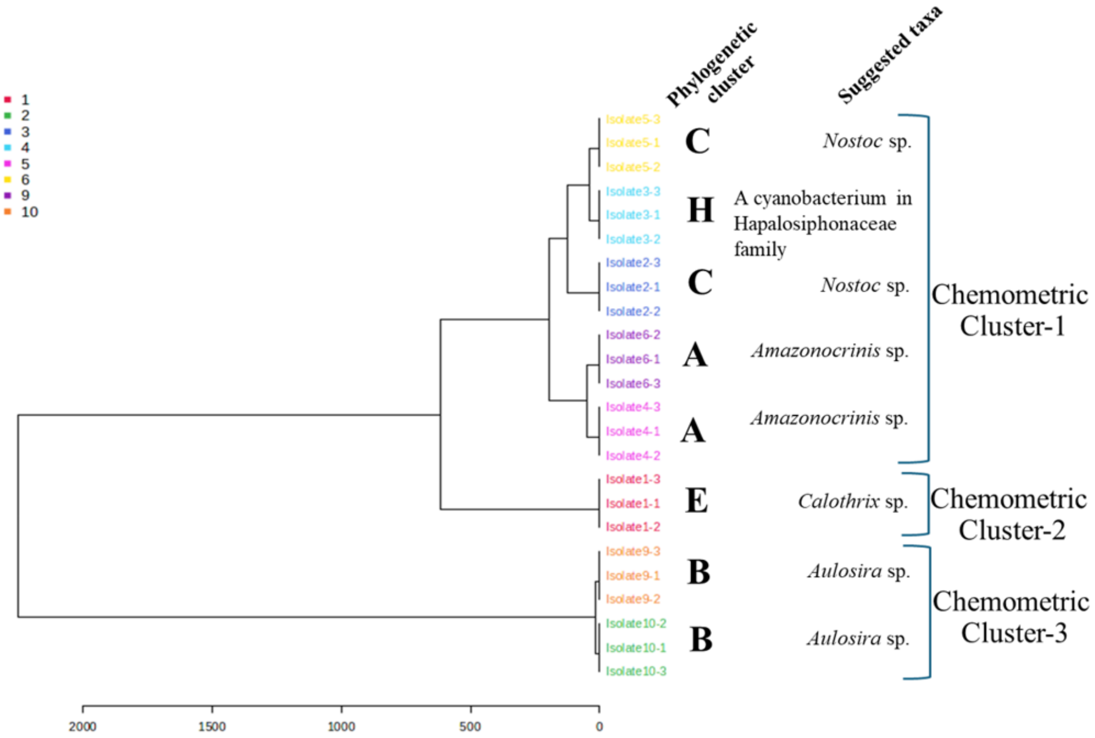


Figure 6 The relationship between phylogenetic and chemometric clusters based on antioxidant activities.

In this present study, some closely related organisms exhibited similar antioxidant profiles, while others differed, suggesting independent evolution of antioxidant properties in cyanobacteria, potentially due to specific environmental pressures [39,40]. The variation in Fe^{2+} chelating and ABTS scavenging activities across genera reflects diverse antioxidant strategies in cyanobacteria, offering potential for biotechnological applications in environmental remediation and novel antioxidant development. Further research is needed to identify the compounds responsible for these antioxidant properties and their biological roles. Expanding the study to include more cyanobacterial genera and species could enhance our understanding of antioxidant evolution and their potential as sources of dual-function antioxidant and metal chelating compounds. These compounds may protect against oxidative stress and metal toxicity in aquatic environments. Exploring biosynthetic pathways and linking antioxidant profiles to habitat preferences could offer insights into their ecological roles and biotechnological applications.

Conclusions

This study presents a comprehensive molecular and biochemical analysis of 8 cyanobacterial isolates, demonstrating their diverse antioxidant activities and taxonomic identities. Molecular reclassification through 16S rRNA sequencing and phylogenetic analyses revealed limitations in morphological identification. Notably, *Aulosira* sp. exhibited the highest ABTS scavenging, while *Nostoc* sp. TUBT01 showed the strongest Fe^{2+} chelation. The clear link between taxonomy and antioxidant potential emphasizes cyanobacteria as promising candidates for natural antioxidants. Future studies should explore the specific bioactive compounds and their potential biotechnological applications.

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References

[1] A Guerreiro, MA Andrade, C Menezes, F Vilarinho and E Dias. Antioxidant and cytoprotective properties of cyanobacteria: Potential for biotechnological applications. *Toxins* 2020; **12(9)**, 548.

[2] DP Singh, R Prabha, S Verma, KK Meena and M Yandigeri. Antioxidant properties and polyphenolic content in terrestrial cyanobacteria. *3 Biotech* 2017; **7(2)**, 134.

[3] K Jomova, R Raptova, SY Alomar, SH Alwasel, E Nepovimova, K Kuca and M Valko. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: Chronic diseases and aging. *Archives of Toxicology* 2023; **97(10)**, 2499-2574.

[4] Y Kumar, A Tarafdar, D Kumar, C Saravanan, PC Badgujar, A Pharande, S Pareek and OA Fawole. Polyphenols of edible macroalgae: Estimation of *in vitro* bio-accessibility and cytotoxicity, quantification by LC-MS/MS and potential utilization as an antimicrobial and functional food ingredient. *Antioxidants* 2022; **11(5)**, 993.

[5] S Lomakool, K Ruangrit, I Jeerapan, Y Tragoolpua, C Pumas, S Srinuanpan, J Pekkoh and K Duangjan. Biological activities and phytochemicals profiling of different cyanobacterial and microalgal biomass. *Biomass Conversion and Biorefinery* 2021; **13**, 4195-4211.

[6] R Liu and SA Mabury. Synthetic phenolic antioxidants: A review of environmental occurrence, fate, human exposure, and toxicity. *Environmental Science & Technology* 2020; **54(19)**, 11706-11719.

[7] HTL Pham, LTT Nguyen, TA Duong, DTT Bui, QT Doan, HTT Nguyen and S Mundt. Diversity and bioactivities of nostocacean cyanobacteria isolated from paddy soil in Vietnam. *Systematic and Applied Microbiology* 2017; **40(8)**, 470-481.

[8] C Akoijam, AK Singh and AN Rai. Characterization of free-living cyanobacterial strains and their competence to colonize rice roots. *Biology and Fertility of Soils* 2012; **48(6)**, 679-687.

[9] N Wada, T Sakamoto and S Matsugo. Mycosporine-like amino acids and their derivatives as natural antioxidants. *Antioxidants* 2015; **4(3)**, 603-646.

[10] IY Massey, MA Osman and F Yang. An overview on cyanobacterial blooms and toxins production: Their occurrence and influencing factors. *Toxin Reviews* 2022; **41(1)**, 326-346.

[11] B Ramakrishnan, NR Maddela, K Venkateswarlu and M Megharaj. Potential of microalgae and cyanobacteria to improve soil health and agricultural productivity: A critical view. *Environmental Science: Advances* 2023; **2(4)**, 586-611.

[12] D Chittora, M Meena, T Barupal, P Swapnil and K Sharma. Cyanobacteria as a source of biofertilizers for sustainable agriculture. *Biochemistry and Biophysics Reports* 2020; **22**, 100737.

[13] MF Hossain, RR Ratnayake, K Meerajini and KLW Kumara. Antioxidant properties in some selected cyanobacteria isolated from freshwater bodies of Sri Lanka. *Food Science & Nutrition* 2016; **4(5)**, 753-758.

[14] U Nübel, F Garcia-Pichel and G Muyzer. PCR primers to amplify 16S rRNA genes from cyanobacteria. *Applied and Environmental Microbiology* 1997; **63(8)**, 3327-3332.

[15] JM Janda and SL Abbott. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology* 2007; **45(9)**, 2761-2764.

[16] S Chittapun and T Charoenrat. Isolation and growth of N_2-fixing cyanobacteria from organic agricultural areas in Sanamchaikate, Chachoeng-Sao Province, Thailand. *Thammasat International Journal of Science and Technology* 2015; **20(2)**, 27-32.

[17] J Doyle. *DNA protocols for plants*. *In*: Molecular techniques in taxonomy. Springer, Germany, 1991, p. 283-293.

[18] K Tamura, G Stecher and S Kumar. MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution* 2021; **38(7)**, 3022-3027.

[19] R Re, N Pellegrini, A Proteggente, A Pannala, M Yang and C Rice-Evans. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine* 1999; **26(9-10)**, 1231-1237.

[20] A Dávalos, C Gómez-Cordovés and B Bartolomé. Extending applicability of the oxygen radical absorbance capacity (ORAC- fluorescein) assay. *Journal of Agricultural and Food Chemistry* 2004; **52(1)**, 48-54.

[21] FC Wong, AL Yong, EP Ting, SC Khoo, HC Ong and TT Chai. Antioxidant, metal chelating, anti-glucosidase activities and phytochemical analysis of selected tropical medicinal plants. *Iranian Journal of Pharmaceutical Research* 2014; **13(4)**, 1409-1415.

[22] Z Pang, Y Lu, G Zhou, F Hui, L Xu, C Viau, AF Spigelman, PE MacDonald, DS Wishart, S Li and J Xia. MetaboAnalyst 6.0: Towards a unified platform for metabolomics data processing, analysis and interpretation. *Nucleic Acids Research* 2024; **52(W1)**, W398-W406.

[23] J Komárek. A polyphasic approach for the taxonomy of cyanobacteria: Principles and applications. *European Journal of Phycology* 2016; **51(3)**, 346-353.

[24] DO Alvarenga, APD Andreote, LHZ Branco, E Delbaje, RB Cruz, ADM Varani and MF Fiore. *Amazonocrinis nigriterrae* gen. nov., sp. nov., *Atlanticothrix silvestris* gen. nov., sp. nov. and *Dendronalium phyllosphericum* gen. nov., sp. nov., nostocacean cyanobacteria from Brazilian environments. *International Journal of Systematic and Evolutionary Microbiology* 2021; **71(5)**, 4811.

[25] TT Ngo, BLT Nguyen, TA Duong, THT Nguyen, TL Nguyen, KT Kieu, MHT Do, SV Nguyen, ND Thang and HTL Pham. Polyphasic evaluation and cytotoxic investigation of isolated cyanobacteria with an emphasis on potent activities of a *Scytonema* strain. *Frontiers in Microbiology* 2022; **13**, 1025755.

[26] BF Tan, SH Te, CY Boo, KYH Gin and JR Thompson. Insights from the draft genome of the subsection V (Stigonematales) cyanobacterium *Hapalosiphon* sp. Strain MRB220 associated with 2-MIB production. *Standards in Genomic Sciences* 2016; **11(1)**, 58.

[27] B Nowruzi, L Hutarova, D Vešelenyiova and JS Metcalf. Characterization of *Neowestiellopsis persica* A1387 (Hapalosiphonaceae) based on the *cpc*A, *psb*A, *rpo*C1, *nif*H and *nif*D gene sequences. *BMC Ecology and Evolution* 2024; **24(1)**, 57.

[28] P Rajaniemi, P Hrouzek, K Kaštovska, R Willame, A Rantala, L Hoffmann, J Komárek and K Sivonen. Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *International*

Journal of Systematic and Evolutionary Microbiology 2005; **55(Pt 1)**, 11-26.

[29] W Tawong, P Pongcharoen, P Pongpadung, S Ponza and W Saijuntha. *Amazonocrinis thailandica* sp. nov. (Nostocales, Cyanobacteria), a novel species of the previously monotypic *Amazonocrinis* genus from Thailand. *Algae* 2022; **37(1)**, 1-14.

[30] F Cai, G Yu, Y Liu, Y Sun and R Li. Description of two new species of *Nostoc* from China based on the polyphasic approach. *Fottea* 2021; **21(2)**, 259-271.

[31] ISS Pinto, IFF Neto and HMVM Soares. Biodegradable chelating agents for industrial, domestic, and agricultural applications—a review. *Environmental Science and Pollution Research* 2014; **21**, 11893-11906.

[32] M AAlikhani, M Khalili and M Jahanshahi. The natural iron chelators' ferulic acid and caffeic acid rescue mice's brains from side effects of iron overload. *Frontiers in Neurology* 2022; **13**, 951725.

[33] MMS Ismaiel, YM El-Ayouty and MD Piercey-Normore. Antioxidants characterization in selected cyanobacteria. *Annals of Microbiology* 2014; **64**, 1223-1230.

[34] WSM Aly and SC Andrews. Iron regulation of growth and heterocyst formation in the nitrogen fixing cyanobacterium *Nostoc* sp. PCC 7120. *Journal of Ecology of Health & Environment* 2016; **4(3)**, 103-109.

[35] R Rai, S Singh, KK Rai, A Raj, S Sriwastaw and LC Rai. Regulation of antioxidant defense and glyoxalase systems in cyanobacteria. *Plant Physiology and Biochemistry* 2021; **168**, 353-372.

[36] Y Kumar, A Tarafdar, D Kumar, C Saravanan, PC Badgujar, A Pharande, S Pareek and OA Fawole. Polyphenols of edible macroalgae: Estimation of *in vitro* bio-accessibility and cytotoxicity, quantification by LC-MS/MS and potential utilization as an antimicrobial and functional food ingredient. *Antioxidants* 2022; **11(5)**, 993.

[37] C Kranzler, H Lis, Y Shaked and N Keren. The role of reduction in iron uptake processes in a unicellular, planktonic cyanobacterium. *Environmental Microbiology* 2011; **13(11)**, 2990-2999.

[38] A Al-Amin, F Parvin, J Chakraborty and YI Kim. Cyanobacteria mediated heavy metal removal: A review on mechanism, biosynthesis, and removal capability. *Environmental Technology Reviews* 2021; **10(1)**, 44-57.

[39] JS Boden, KO Konhauser, LJ Robbins and P Sánchez-Baracaldo. Timing the evolution of antioxidant enzymes in cyanobacteria. *Nature Communications* 2021; **12**, 4742.

[40] M Bernroitner, M Zamocky, PG Furtmüller, GA Peschek and C Obinger. Occurrence, phylogeny, structure, and function of catalases and peroxidases in cyanobacteria. *Journal of Experimental Botany* 2009; **60(2)**, 423-440.