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GC-MS ANALYSIS AND ANTIOXIDANT ACTIVITY OF TWO SPECIES OF CYANOBACTERIA ISOLATED FROM DRANG SALT MINE OF DISTRICT MANDI, HIMACHAL PRADESH, INDIA

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Abstract

The present study was undertaken to explore the possibilities of antioxidant compounds present in two halophilic cyanobacterial species *Cylindrospermum muscicola* HPUSD12 and *Phormidium* sp. HPUSD13 isolated from Drang salt mine of Distt. Mandi Himachal Pradesh. These compounds can be used by human to treat some diseases such as cancer, hypercholesterolemia and oxidative damage. Methanol extracts of *Cylindrospermum muscicola* HPUSD12 and *Phormidium* sp. HPUSD13 at 21 day of growth were prepared to study preliminary phytochemical screening, gas chromatography and mass spectrometry (GC-MS) analysis and antioxidant activity by DPPH free radical scavenging assay. Preliminary phytochemical screening of methanol extracts of *Cylindrospermum muscicola* HPUSD12 and Phormidium sp. HPUSD13 showed the presence of alkaloids, saponins and terpenoids and GC-MS analysis detected 9 and 24 compounds respectively. Phthalic acid (82.52%) and hexadecanoic acid methyl ester (15.67%) were the major compounds in *Cylindrospermum muscicola* HPUSD12 and phthalic acid (27.69%) and octadecenoic acid methyl ester (22.05) were the major compounds in *Phormidium* sp. HPUSD13. The antioxidant activity of methanol extract of *Phormidium* sp. HPUSD13 is better than *Cylindrospermum muscicola* HPUSD12. Cyanobacteria inhabiting extreme environment produce biologically active compounds which help them to adapt and survive under harsh environment. Further, these compounds can be used in pharmaceutical science to treat major diseases.

Key words: Cyanobacteria, Salt mine, GC-MS, Antioxidant, DPPH, Pharmaceutical.

Introduction

Cyanobacteria are gram negative photosynthetic autotrophs with well known nitrogen fixing ability. They are found almost in all types of habitats ranging from bare rocks to soil and from water to air. They are the potential source of food and pharmaceuticals (Parikh and Madamwar, 2006). At present there is intense demand of drugs produced by screening of bioactive compounds from natural resources. Thus researchers have started use of microorganisms like cyanobacteria as a drug producer organism. Cyanobacteria have various natural compounds which lead to the development of biopharmaceutical drugs (Wase and Wright, 2008). The secondary metabolites present in cyanobacteria have antibacterial, antifungal, antiviral and antioxidant activities. They are rich in proteins, vitamins, lipids having fatty acids like linoleic acid. The bioactive compounds present in cyanobacteria such as phthalic acid, hexadecanoic acid, ethyl ester, hexadecanoic acid, methyl ester, 9-octadecanoic acid (Z)- ethyl ester have antimicrobial, antioxidant, hypocholesterolemic and anticancer activities respectively by (Ananya and Kamal, 2013).

Cyanobacteria contain pigments and phytoconstituents which have antioxidant and antimicrobial properties that help in protecting the cells against free radical damage. During biological activities reactive oxygen species (ROS) are generated within the cells which leads to oxidative damage of the cells (Cerutti, 1991). ROS and free radical formation during biological reactions lead to many diseases in human beings such as ageing, diabetes, cancer, cardiovascular diseases (Halliwell, 1997). Our body is prevented from oxidative damage by scavenging activity of antioxidants. The antioxidants scavenge the free radicals as well as inhibit peroxidation. In recent years, much attention has been paid towards study of plant based natural antioxidants specially phenolics and tocopherols by (Ozsoy *et al.,*

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2008). Natural antioxidants neutralize reactive oxygen species and help in the treatment of certain diseases. Screening of cyanobacteria for pharmaceutically important bioactive compounds and antioxidant activity has received much attention during past few decades. Antioxidant property of some cyanobacteria of extreme environment such as sulphur spring of western ghats of Karnataka have been investigated by (Sharathachandra and Rajashekhar , 2013).

Antioxidant properties of cyanobacteria have been least studied as compared to plants. Keeping in mind all these aspects, it was considered worthwhile to study the bioactive compounds and antioxidant properties of cyanobacteria of extreme environments such as saline soils. Hence the present investigations are carried out to determine the GC-MS analysis of cyanobacterial extracts for detection of bioactive compounds of pharmaceutical importance and to study antioxidant activity.

Materials and Methods

Study area

Soil samples were collected from Drang salt mine, Tehsil Padhar, Distt. Mandi, Himachal Pradesh. Geographical location of the area is $31^0$13" to $32^0$04"N, $76^0$37" to $77^0$23" E at an altitude of 754 meters asl. Soil samples were collected from different depths after removing the top soil. Cyanobacterial strains were identified using taxonomic key given by (Desikachary, 1959). The isolated species were grown in BG-11 medium.

Table 1: Preliminary screening of cyanobacterial species for the presence of phytochemicals.

Phyto-chemicals	Test Species	
	Cylindrospermum muscicola HPUSD12	Phormidium sp. HPUSD13
Alkaloids	+	_
Saponins	++	+
Tannins	_	_
Phenols	_	_
Terpenoids	+	+
Flavonoids	_	_

+: Present, - : Absent

Preparation of extracts

The algal extracts were prepared by harvesting the cultures at 21 days. The wet biomass of algae was taken for extraction with methanol in the ratio of 1:10 following the method given by (Kaushik and Chauhan, 2009). The stock solution of concentration 50 mg/ml was prepared.

Preliminary phytochemical screening and GC-MS analysis

The methanol extracts of cyanobacteria were tested for the presence of bioactive compounds like alkaloids, flavonoids, saponins, terpenoids and phenols by using standard method of (Harborne, 1973). Gas chromatography and mass spectrometry (GC-MS) analysis of methanol extracts were done using an electron ionization system with ionization energy of 70 eV. Helium gas was used as a carrier gas at flow rate of 1.2 ml per minute. (Column) Injector and mass transfer line temperature were set as 270 and 280 C. The compounds were identified by comparing mass spectra with library of the National Institute of Standard and Technology (NIST).

Antioxidant activity by DPPH free radical scavenging assay

The 2, 2- diphenyl-1-pycrylhydrazyl (DPPH) test were carried out by following the method of (Burtis and Bucar, 2000). One ml of methanol extract at different concentration was mixed with 1ml DPPH reagent (0.002% w/v). After an incubation period, the absorbance was measured at 517 nm. Ascorbic acid was used as

Table 2: Compounds identified from methanol extract of *Cylindrospermum muscicola HPUSD12* by GC-MS analysis.

Peak No.	Name of Compounds	Compound nature	Molecular formula	Retention time (RT)	Peak area %
1	Phthalic acid, hex-3-yl isobutyl ester	Aromatic compound	$C_{18}H_{26}O_4$	34.59	82.52
2	Phthalic acid, hept-4-yl isobutyl ester	Aromatic compound	$C_{19}H_{28}O_4$	34.59	82.52
3	Phthalic acid, hept-3-yl-isobutyl ester	Aromatic compound	$C_{19}H_{28}O_4$	34.59	82.52
4	7,10-Hexadecadienoic acid, methyl ester	Fatty acid	$C_{17}H_{30}O_2$	34.89	1.81
5	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester 5,8,11-Heptadecatrien-1-ol	_	$C_{22}H_{38}O_2$	34.89	1.81
6	5,8,11-Heptadecatrien-1-ol	Fatty alcohol	$C_{17}H_{30}O$	34.89	1.81
7	Hexadecanoic acia, methyl ester	Saturated fatty acid	$C_{17}H_{34}O_2$	35.20	15.67
8	Pentadecanoic acid, 14-methyl-,methyl ester	Palmitic acid methyl ester	$C_{17}H_{34}O_2$	35.20	15.67
9	Hexadecanoic acid, 15-methyl-, methyl ester	Fatty acid ester	$C_{18}H_{36}O_2$	35.20	15.67

positive control.

% Antioxidant activity = Ac - At/Ac×100

Where At, is absorbance of algal samples extract and Ac is the absorbance of methanol DPPH solution.

Results

Phytochemical screening

The preliminary phytochemical screening showed the presence of different bioactive compounds such as alkaloids, saponins and terpenoids in two species of cyanobacteria Table 1.

GC-MS analysis

The methanol extracts of two cyanobacterial species were characterized by gas chromatography-mass spectrometry (GC-MS) technique. Table 2 and table 3 shows the major constituents present in methanol extracts of *Cylinrospermum muscicola* HPUSD12 and *Phormidium* sp. HPUSD13 respectively with their name, nature, retention time and peak area percentage. Fig. 1 and Fig. 2 represents the GC-MS chromatogram of methanol extract of *Cylindrospermum muscicola* HPUSD12 and Phormidium sp. HPUSD13, respectively.

Antioxidant activity by DPPH free radical scavenging assay

The free radical scavenging activity of these cyanobacteria were determined by DPPH method Table 4. *In-vitro* screening for antioxidant activity of methanol extracts of cyanobacteria were carried out at different concentrations (20, 40, 60, 80, 100 µg/ml). The free radical scavenging activity was compared with standard antioxidant (Ascorbic acid). Among two species of cyanobacteria Phormidium sp. HPUSD13 displayed higher antioxidant activity (17.18 %) at a concentration of 100 µg/ml as compared to *Cylindrospermum muscicola* HPUSD12 where it was 6.98 % at 100 µg/ml concentration.

Relative % antioxidant activity of *Cylindrospermum muscicola* HPUSD12 and Phormidium sp. HPUSD13

Table 3: Compounds identified from methanol extract of *Phormidium* sp. *HPUSD13* by GC-MS analysis.

Peak No.	Name of Compounds	Compound nature	Molecular formula	Retention time (RT)	Peak area %
1	9,12-Octadienoic acid, methyl ester	Fatty acid ester	$C_{19}H_{34}O_2$	33.46	22.45
2	9,12-Octadecadienoic acid (Z,Z)-,methyl ester	Linoleic acid	$C_{19}H_{34}O_2$	33.46	22.45
3	Methyl 9-cis, 11-trans-octadecadienoate	Linoleic acid	$C_{19}H_{34}O_2$	33.46	22.45
4	Trans-13-Octadecenoic acid, methyl ester	Linoleic acid	$C_{19}H_{36}O_2$	33.64	22.05
5	6-Octadecenoic acid, methyl ester, (Z)-	Fatty acid ester	$C_{19}H_{36}O_2$	33.64	22.05
6	cis-13-Octadecenoic acid, methyl ester	Linoleic acid	$C_{19}H_{36}O_2$	33.64	22.05
7	13,16-Octadecadiynoic acid, methyl ester	Linolelaidic acid	$C_{19}H_{30}O_2$	34.24	2.98
8	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl)methyl]cyclopropyl] methyl]-,methyl ester	Fatty acid	$C_{25}H_{42}O_2$	34.24	2.98
9	17-Octadecynoic acid, methyl ester	Fatty acid ester	$C_{19}H_{34}O_2$	34.24	2.98
10	Oleic acid	Fatty acid	$C_{18}H_{34}O_2$	34.37	3.74
11	Cis-Vaccenic acid	Fatty acid	$C_{18}H_{34}O_2$	34.37	3.74
12	Trans-13-Octadecenoic acid	Linoleic acid	$C_{18}H_{34}O_2$	34.37	3.74
13	Phthalic acid, hex-3-yl isobutyl ester	Aromatic compound	$C_{18}H_{26}O_4$	34.59	27.69
14	Phthalic acid, hept-4-yl isobutyl ester	Aromatic compound	$C_{19}H_{28}O_4$	34.59	27.69
15	Phthalic acid, isobutyl 2-pentyl ester	Aromatic compound	$C_{17}H_{24}O_4$	34.59	27.69
16	6,9,12-Octadecatrienoic acid, phenyl methyl ester, (ZZZ)	Fatty acid ester	$C_{25}H_{36}O_2$	34.80	0.65
17	3H-naphth[1,8a-b]oxirene,3-bromo-1a-chlorooctahydro-, trans –(3a)	_	$C_{10}H_{14}BrClO$	34.80	0.65
18	1,2-Ethanediol, 1,2-dimyrtenyl	Glycol	$C_{20}H_{30}O2$	34.80	0.65
19	7-Hexadecenoic acid, methyl ester, (Z)	Fatty acid Ester	$C_{17}H_{32}O_2$	35.01	9.61
20	Methyl hexadec-9-enoate	Fatty acid	$C_{17}H_{32}O_2$	35.01	9.61
21	9-Hexadecenoic acid, methyl ester, (Z)	Fatty acid ester	$C_{17}H_{32}O_2$	35.01	9.61
22	Pentadecanoic acid, 14-methyl-, methyl ester	Palmitic acid methyl ester	$C_{17}H34O_2$	35.21	10.83
23	Hexadecanoic acid, methyl ester	Palmitic acid ester	$C_{17}H34O_2$	35.21	10.83
24	Hexadecanoic acid, 15-methyl-, methyl ester	Palmitic acid methyl ester	$C_{18}H_{36}O_2$	35.21	10.83

Table 4: Antioxidant activity of two species of cyanobacteria isolated from salt mine.

Antioxidant assay	Extract	Extract concentration (µg/ml)	*Cylindrospermum muscicola* HPUSD12	*Phormidium* sp. HPUSD13	Ascorbic acid
DPPH free	Methanol	20	0.698±0.24678	3.315±1.974596	57.97±0.18
radical		40	1.57±0.24678	6.98±2.8383327	65.63±0.46
scavenging		60	3.7515±0.802213	9.5105±1.789334	72.31±0
activity		80	5.061±0	10.2085±1.295773	80.12±0.18
		100	6.98±0.987121	17.1895±4.874441	85.34±0.37

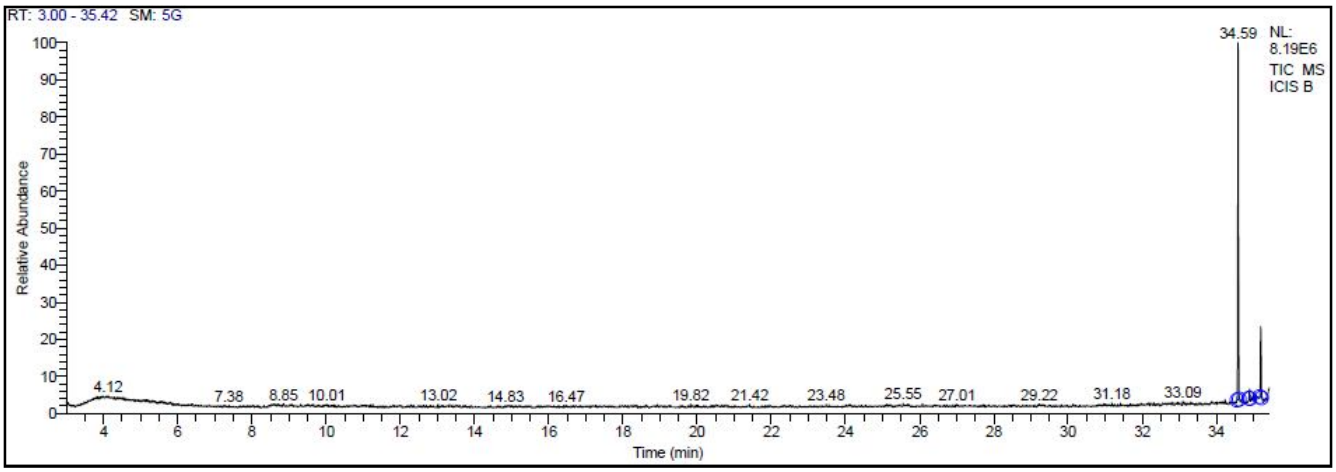

Fig. 1: GC-MS chromatogram of methanol extract of *Cylindrospermum muscicola* HPUSD12.

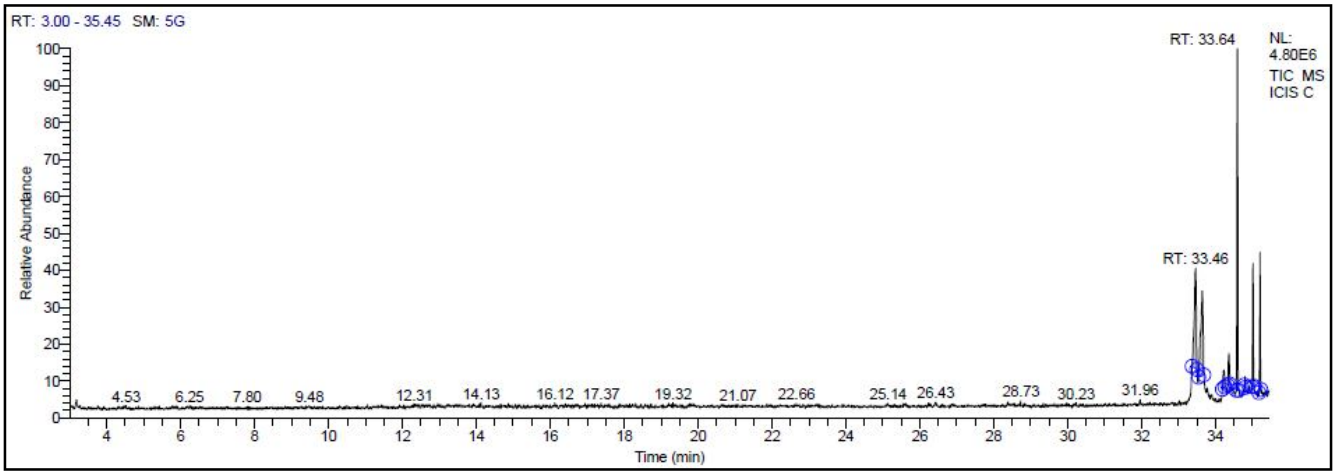

Fig. 2: GC-MS chromatogram of *Phormidium* sp. HPUSD13.

was compared with control (Ascorbic acid) at different concentrations (20, 40, 60, 80, 100 µg/ml) by taking respective activity at 20 µg/ml as 100. There is an increase in percentage antioxidant activity with increasing extract concentration. The relative % increase of antioxidant activity of *Cylindrospermum muscicola* HPUSD12 was maximum (124% to 900%) as compared to *Phormidium* sp. HPUSD13 (100% to 500%) and ascorbic acid (13% to 46%) with increase in concentration from 20 to 100 µg/ml Fig. 3.

Discussion

The phytochemical screening showed the presence of alkaloids, saponins and terpenoids in *Cylindrospermum muscicola* HPUSD12 while tannins, phenols and flavonoids were absent. On the other hand, in *Phormidium* sp. HPUSD13 only saponins and terpenoids were found. The phytochemicals were found in large amount in *Cylindrospermum muscicola* HPUSD12. The alkaloids like hapalindole isolated from *Fischerella* sp. has been found to possess antibacterial activity. The carotenoids are terpenoids which have antioxidant activity. The saponins present in cyanobacteria help in lowering serum lipid level and lowering body weight.

GC-MS analysis helped in retrieving many important

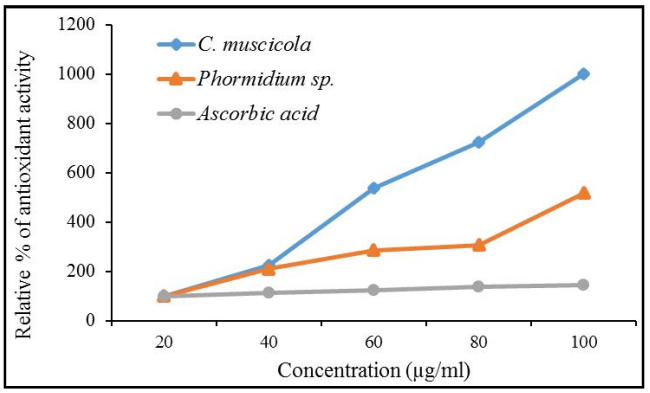

Fig. 3: Relative antioxidant activity in methanol extracts of *Cylindrospermum muscicola* HPUSD12 and Phormidium sp. HPUSD13 in comparison with control (Ascorbic acid) [Absorbance at 20 µg/ml taken as 100 for each].

organic volatile compounds of pharmaceutical importance in our study. Our findings match with the study done on fatty acid profiling and antioxidant potential of cyanobacterium *Nostoc muscorum* by (Ananya and Kamal, 2016). The compounds such as phthalic acid has antimicrobial activity. 9-Octadecanoic acid, ethyl ester has anti-inflamatory, anticancer, hypocholesterolemic, 5-alpha reductase inhibitor and anti-androgenic activity while hexadecanoic acid ethyl ester and hexadecanoic acid methyl ester have antioxidant, hypocholesterolemic, nematicide and hemolytic 5-alpha reductase inhibitor activity (Ananya and Kamal, 2016).

The compounds such as heptadecatriene has also been reported from other microalgae extract by (Salem *et al.*, 2014). This compound has antimicrobial, antioxidant and anticancer activity. Studies have been shown that all these volatile compounds have antibacterial activity (Lee *et al.,* 2007). The present results are in agreement with the reports cited by earlier workers where compounds have been detected which are fatty acids such as oleic acid, vaccenic acid, hexadecenoic acid, terpenes and aromatic compounds like phthalic acid and which have antimicrobial activity (Kannan *et al.,* 2010). The compound methyl hexadec-9-enoate detected has been used for the production of bioethanol, biodiesel and biohydrogen has been investigated recently from mangrove environment (Armstrong *et al.,* 2019). All these fatty acid compounds detected from cyanobacteria have antioxidant properties (Yamuna *et al.,* 2017). The transformation of all these secondary metabolites according to the cell requirement is the key feature of cyanobacteria that helps during their adaptations (Zyszka-Haberecht *et al.*, 2018).

The antioxidant activity of methanol extracts of cyanobacteria showed that *Phormidium* sp. HPUSD13 has better antioxidant activity than *Cylindrospermum muscicola* HPUSD12. The reducing capacity of a compound acts as a significant indicator of antioxidant capacity. The reducing activity depends on the reductones, which shows antioxidant activity by breaking the free radical chain and donating hydrogen ion (Huang *et al.*, 2005).

Recently much attention has been given to cyanobacteria due to the presence of various types of bioactive compounds such as phenols, alkaloids, saponins, tannins, terpenoids, flavonoids and polysaccharides (Abd El-Baky *et al.*, 2008). The antioxidant activity of carotenoids extracted from *Spirullina maxima* has been studied by (Miranda *et al.,* 1998). The bioactive compounds like alkaloids and terpenoids reported from cyanobacteria have potential antioxidant property to overcome free radicals which are harmful to the cell. The carotenoids are terpenoids which have antioxidant activity. There are another compounds like flavonoids which are polyphenolic compounds with free radical scavenging activity and help to overcome oxidative stress (Frankel, 1995).

In the present investigation relative percentage of antioxidant activity of methanol extracts of *Cylindrospermum muscicola* HPUSD12 is greater than *Phormidium* sp. HPUSD13 and ascorbic acid which may be due to the presence of alkaloids, phenols and terpenoids. The antioxidant activity of methanol extract of cyanobacteria has been investigated and found that *Plectonema boryanum* and *Scytonema* sp. showed greater antioxidant activity *i.e.* 30% and 27% as compared to ascorbic acid where it was 25% at 50 mg/ml by (Shazia *et al.,* 2011). The antioxidant activity of crude extracts of *Spirullina maxima* was studied by Miranda *et al.*, 1998). The potential for antioxidant property in cyanobacteria may be due to the presence of phenols, alkaloids, terpenoids and phycocyanins in the extracts (Rojas *et al.,* 1992). Nowadays synthetic antioxidants like ascorbic acid and BHA (Beta Hydroxy Acid) are immense in use. But due to their side effects to natural systems, natural antioxidants are preferred over them as they are easily available and with less side effects.

Conclusion

It can be concluded from this study that the two cyanobacterial species *Cylindrospermum muscicola* HPUSD12 and *phormidium* sp. HPUSD13 contain many volatile compounds which have antimicrobial, antioxidant, anti-inflamatory, anticancer and hypocholesterolemic properties. Thus GC-MS analysis has helped us to detect various compounds of pharmaceutical importance. The

compounds reported from cyanobacteria such as alkaloids and terpenoids have potential antioxidant property thus help in overcoming free radical oxidative damage to cells. Further studies can be done on cyanobacteria of unusual habitats like saline soil, sulphur springs, arid and semi arid soils. This study would help in pharmaceutical sciences for determining cyanobacteria as potential antioxidants and potential compounds of utilization.

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