Ann Microbiol (2012) 62:55–59
DOI 10.1007/s13213-011-0226-5

ORIGINAL ARTICLE

The characterisation of bioactive compounds from an Egyptian *Leptolyngbya* sp. strain

Nermin Adel El Semary

Received: 25 August 2010 / Accepted: 7 February 2011 / Published online: 26 February 2011

Abstract An investigation into the bioactive metabolites from a benthic, mat-forming strain dominating a polluted wastewater canal in Egypt was conducted. Phytochemical screening revealed the presence of saponins, flavonoids and alkaloids; vitamin C was also found at high concentrations. The isolate was investigated as a source of antimicrobial compounds. The lipophilic fraction was extracted using chloroform/methanol and bioassays for antimicrobial compounds were performed using strains of pathogenic bacteria. The fraction that showed the highest bioactivity was purified and its structure elucidated using UV, FTIR, proton-NMR and GC-MS. The compound's molecular weight was 220 and it was identified as butylated hydroxytoluene which has both antimicrobial and antioxidant activities. On the ecological front, this compound, and the other metabolites detected, seem to enable the isolate to dominate its niche and protect it from adverse conditions. On the commercial front, this compound is used as a food additive and was recently discovered in different cyanobacteria, and can be used as a lead compound for both drug and food industries to substitute for the expensive and hazardous synthetic analogs. Therefore, this strain can be considered as a potential source of bioactive compounds that should be further explored.

Keywords Phytochemical screening · UV · FTIR · Proton-NMR · GC-MS · Butylated hydroxytoluene

N. A. El Semary (✉)
Department of Botany and Microbiology, Faculty of Science,
Helwan University,
Helwan, Egypt 11795
e-mail: nerminel_semary@yahoo.co.uk

N. A. El Semary
e-mail: nerminel_semary@Science.helwan.edu.eg

Introduction

Cyanobacteria are oxygenic phototrophs that represent a rich source of food (Herrero et al. 2006), fuel (Chisti 2007) and bioactive compounds (Skulberg 2000). Their ability to tolerate the harshest growth conditions is mainly a result of their remarkable metabolic activities that enable them to dominate their habitats and out-compete other microorganisms (Whitton and Potts 2000). Although cyanobacteria are considered as a prolific source of secondary metabolites with a wide spectrum of bioactive effects (Thajuddin and Subramanian 2005), they are still not thoroughly investigated and have been poorly exploited (Ehrenreich et al. 2005; Barrios-Llerena et al. 2007). Extracts from cyanobacteria from India proved to be active against multidrug-resistant *Mycobacterium tuberculosis*, the causative agent of tuberculosis (Rao et al. 2007). Similarly, Chauhan et al. (2010) showed that extracts from *Anabaena* possessed antimicrobial activity against several pathogenic bacteria, and new antibacterial agents were obtained from those extracts. In order to allow for more new bioactive compounds to be discovered, new taxa from unusual habitats need to be screened for their bioactive metabolites (Skulberg 2000).

Despite the fact that several studies have focused on the characterisation of bioactive compounds from different cyanobacterial strains from different geographical locations (Falch et al. 1995; Doan et al. 2000; Ghasemi et al. 2004; Asthana et al. 2006), antimicrobial compounds derived from Egyptian cyanobacterial flora have rarely been characterised, except for some limited efforts such as those by Issa (1999), El-Sheekh et al. (2006), El Semary et al. (2009) and El Semary and Abd El Naby (2010).

To begin to address this deficiency, the current project examined the antimicrobial activity of a mat-forming,

benthic *Leptolyngbya* sp. strain isolated from a wastewater canal called "Teraat El Khashaab" in Helwan, Egypt. This canal is being used as a sink for industrial, domestic and agricultural wastewater and is polluted with heavy metals (Abd El-Hady 2007). The isolate attracted attention as it dominated the benthos of this canal with massive mat formations. Also, it was observed to grow on a solid medium, with no obvious bacterial contamination, i.e. no apparent bacterial colonies were observed to grow on the solid medium on which this cyanobacterium was grown. Therefore, this study aimed at investigating the bioactive compounds produced by this benthic cyanobacterium.

Materials and methods

Culture conditions

Cultures of the *Leptolyngbya* sp. strain, originally isolated from a wastewater canal, Teraat El Khashaab, Helwan (29º50′N, 31º20′E, Egypt), were kept in *Oscillatoria* medium (Feuillade 1994) as both liquid and solid (1.5% agarose, w:v) cultures. Liquid cultures were grown without agitation to allow mat formation at 35°C and incident radiation of 14 μmol photon m^{-2} s^{-1} (15:9 h, light/dark cycle).

Vitamin C

One gram of lyphilised cyanobacterial filaments was extracted in 80% methanol at room temperature. Vitamin C was detected in the extract using reverse phase HPLC (C18 Column). The mobile phase was a mixture of methanol:water (97:3), added under isocratic conditions. The flow rate was 0.5 ml/min, using UV detector at 254 nm (Jaffe 1984). The vitamin was identified by co-chromatography of authentic standards (Sigma). By comparing peaks of both the standard sample of known concentration and the sample of unknown concentration and relating this to the weight of the cyanobacterial mass from which the sample of unknown concentration was derived, we were able to calculate the amount of vitamin C.

Phytochemical screening

One gram of cyanobacterial biomass was lypholised then extracted in 80% for 2 days. The extract was used for the following qualitative tests. In the test for terpenes, 2 ml of the alcoholic extract was added to 2 ml distilled water and filtered using Whatmann filter paper number 2. One ml of 5% ferric chloride was added to the filtrate. The production of a yellowish green colour is evidence for the presence of tannins (Claus 1967). In the test for saponins, 2 ml of alcoholic extract were added to 1 ml of distilled water then filtered. The filtrate was vigorously shaken. Sapnoins are detected by their ability to develop a froth that is stable for a period of 30 min and longer (Wall et al. 1954). In the test for alkaloids, the lyphilised sample was boiled in water with 5 ml 2M HCl solution and the filtrate was treated with Mayer's reagent which precipitates alkaloids (Scholz and Liebezeit 2006). The test for flavonoids (Wall et al. 1954) involved the dropwise addition of 1 ml of concentrated hydrochloric acid to 1 ml of algal alcoholic extract which contained a fragment of magnesium ribbon; a pinkish colour forms if flavonoids are present.

Antimicrobial screening

Extraction and column chromatography of lipophilic fractions

One gram of lyophilised cyanobacterial biomass was homogenised with a mixture of methanol: chloroform (2:1, v:v) and centrifuged at 14,000 *g* for 30 min (Hettich-R200 microcentrifuge, Germany). The lower layer was collected and re-extracted twice with the same solvent mixture. The lipophilic phase was collected, and the sample

Table 1 Column fractionation of the lipophilic extracts from the cyanobacterial isolate and the antimicrobial activity of the fractions indicated by the diameter of the inhibition zone in cm

Fraction	Eluting agent (Methanol:Chloroform)	*Bacillus cereus*	*Pseudomonas aeruginosa*	*Salmonella typhi*	*Bacillus subtilis*
I	(0:100)	_	_	_	_
II	(5:95)	1.1±(0.3)	_	_	_
III	(10:90)	_	_	_	1.1±(0.2)
IV	(15:85)	_	_	1.1±(0.1)	_
V	(20:80)	_	_	1.2±(0.1)	_
VI	(25:75)	1.1±(0.2)	1.2±(0.1)	1.1±(0.2)	1.3±(0.3)
VII	(30:70)	1.1±(0.1)	_	1.2±(0.2)	_
VIII	(35:65)	1.1±(0.2)	1.7±(0.2)	1.1±(0.2)	1.6±(0.3)

The volume of each fraction is 50 ml;_– designates null inhibition. Standard deviation is given in parentheses. The average values of three inhibition zone diameters and standard deviation were calculated using Excel, standard functions

Table 2 FTIR absorption sections and the functional groups bands

Analysis	Region of absorption spectrum (cm^{-1})	Bands (cm^{-1})	Functional groups indication
FTIR	4,000–2,800	3,660–3,300	–OH group
		3,796 and 3,724	C-H aromatic stretching
		2,929 and 2,886	C-H aliphatic stretching
	2,800–1,000	1,464 and 1,636	aromatic C = C stretch
		1,378 and 1,340	vibration of CH_3
	1,000-400	815	C-H of aromatic alkane (out of plane bending)
		760	Substitution of the aromatic ring

was applied to a silica gel G60 (Merck) column (1.5×25 cm) prepared from a slurry of 30 g of precipitated Silica gel G60 (Doan et al. 2000). The column was developed using the solvent system sequence in Table 1. The pathogenic bacterial strains were *Bacillus subtilis* HMCCBs1, *Pseudomonas aeruginosa* HMCCPa2, *Bacillus cereus* HMCCBc1 and *Salmonella typhi* HMCCSt1 (Helwan Microbial Culture collection). The concentrated lipophilic fractions were applied to 6-mm paper disks (Difco) and left to dry. The sensitivity of these bacterial strains to the extracted fractions was assessed by using the Disk Diffusion Susceptibility method (Bauer et al. 1966). Disks containing cholroform/methanol were left to evaporate and then used as negative controls. Disks containing the antibiotics ampicillin (6 μg/ml) and kanamycin (20 μg/ml), were used as positive controls. The bioactive fractions, the pathogens affected and the average values of three inhibition zone diameters are recorded in Table 1.

Elucidation of the structure of the bioactive compound in fraction VIII

The GC-MS spectra were recorded on a Shimadzu-QP2010-plus with library search at electron voltage 70 ev and EI ionisation mode. The UV-spectrum was recorded with a UV-VIS spectrophotometer (Shimadzu UV-160) and the FTIR spectrum was recorded on a Jasco FT/IR 4100 as a liquid sample. For proton NMR, 5 mg of the lypholised sample was dissolved in DMSO and measured at 300 MHz using microtube and NMR analyser (Oxford).

Results

Vitamin C and phytochemical screening

Vitamin C was found at a considerable concentration (10 μg/mg dry weight) using reverse phase C18-HPLC. The phytochemical screening confirmed the presence of saponins (formation of persistent froth), flavonoids (formation of pinkish colour) and alkaloids (formation of a precipitate with Mayer's reagent), whereas tannins were absent (no formation of yellowish-green colour).

Elucidation of the chemical structure of the bioactive compound in fraction VIII

I-UV analysis The UV spectrum of the bioactive compound showed two bands of medium intensity indicating the presence of an aromatic system. Bands were detected at 245 and 264 nm in the UV range of 200-400 nm (Pavia et al. 1996).

II-FTIR analysis The spectrum (Table 2) was subdivided into four absorption sections mainly; 4,000–2,800, 2,800–2,000, 2,000–1,000 and 1,000-400 cm^{-1}. The identification of functional groups was performed according to guidelines detailed in Pavia et al. (1996). Absorption bands in the 4,000–2,800 cm^{-1} region indicated the presence of –OH group that is bonded when dissolved in alcohol. To the left of the broad band a C-H aromatic group was detected. Whereas to the right of the broad band a C-H aliphatic group was detected, as well as stretching vibration due to aliphatic side substitution. Absorption bands in the 2,800–1,000 cm^{-1} region indicated the presence of aromatic C = C as well as a CH_3 group. Absorption bands in the 1,000-400 cm^{-1} region indicated the presence of CH of aromatic alkane and the presence of a substituted aromatic ring.

II-The proton–NMR analysis The proton-NMR spectrum showed signals indicative of: β-CH_3 group, methine group,

Table 3 Proton-NMR signals and their indications

Analysis	Signals (ppm)	Indication
Proton-NMR	0.86	β-CH_3 group
	1.5	Methine group
	2.3	α-C H_3 group
	2.8	OH group
	7.2	CH in a benzene ring
	8.5	Aromatic protons

α-C H_3 group, OH group, CH in a benzene ring and aromatic system (Table 3).

IV- Gas chromatography-mass spectrometry analysis The mass spectrum of the compound under investigation revealed that the molecular weight of the compound was 220. A literature search confirmed the identity as butylated hydroxytoluene.

Discussion

The quenching of harmful free radicals in an environment rich in inducers of those radicals (e.g. heavy metals in industrial wastes) may explain the high concentration of vitamin C in the studied organism. Vitamin C is a powerful antioxidant and functions primarily to protect lipid membranes and structures from peroxidation caused by free radicals (Buettner 1993). Moreover, it has antimicrobial effects; for example, vitamin C from the green alga *Haematococcus* inhibits *Helicobacter pylori* infection in humans (Wang et al. 2000). This vitamin thereby provides the organism with protection that enables it to cope with the environmental stress of this unusual niche and to dominate other microorganisms that are not as protected. Meanwhile, the phytochemical screening revealed the presence of saponins, flavonoids and alkaloids and the absence of tannins. Some of these substances were detected in cyanobacterial extracts and reported to have antimicrobial effects (Kulik 1995). Similarly, it was found from an extensive phytochemical screening of microalgae that those bioactive compounds not only play a major biological role in cellular metabolism but also play a role as a defensive mechanism where they inhibit co-existing microorganisms (Scholz and Liebezeit 2006). Consistent with this, fraction VIII proved to be most effective against all bacterial pathogens tested in the antimicrobial screening.

The elucidation of the pure bioactive compound structure was performed using several chemical analyses. UV analysis showed that the compound has an aromatic ring, and FTIR confirmed this aromatic nature. The proton-NMR showed the presence of a phenolic ring as well as aliphatic substitution. Finally, the GC mass coupled with a literature search identified the compound as butylated hydroxytoluene of molecular weight 220. This compound is commonly used as a food additive due to its antioxidant activity (Sikkema et al. 1995); however, it is reported to also have antimicrobial activity (Davidson and Branden 1981). The mechanism of toxicity to microorganisms, of this and other hydrocarbons, was discussed in the comprehensive review by Sikkema et al. (1995). In their review, these authors reported that the interactions of lipophilic compounds with the cell membrane of microorganisms can cause loss of membrane integrity, leading to an increase in permeability to protons and the dissipation of the proton motive force. Both lipids and proteins of the membrane are affected. It was speculated that lipophilic compounds may also affect membrane-bound enzymes due to alteration in the hydrophobic environment around the membrane. Thus, the compound discovered herein can be used as an antimicrobial/antioxidant agent that can be easily obtained from a natural source. In line with this, Babu and Wu (2008) were able to isolate and produce this compound from naturally-occurring freshwater phytoplankton, including the filamentous cyanobacteria *Oscillatoria* and *Cylindrospermopsis raciborskii*. They also investigated some of the conditions that may trigger greater production of this substance and highlighted the possibility of substituting the chemical synthesis of this substance with natural production using those microalgae. Such clean technology is increasingly important and future research should focus on studying factors that maximise the production of this compound and other useful natural substances.

Acknowledgement The author is very grateful to Dr Dave G. Adams, Faculty of Biological Sciences, University of Leeds, United Kingdom, for proofreading of the manuscript.

References

Abd El-Hady BA (2007) Compare the effect of polluted and River Nile irrigation water on contents of heavy metals of some soils and plants. Res J Agri Biol Sci 3: 287-294. Available from: http://www.insipub.com/rjabs/2007/287-294.pdf. Accessed 24 August 2010

Asthana RK, Srivastava A, Singh AP, Deepali MK, Singh SP, Nath G, Srivastava R, Srivastava BS (2006) Identification of an antimicrobial entity from the cyanobacterium *Fischerella* sp. isolated from bark of *Azadirachta indica* (neem) tree. J Appl Phycol 18:33–39. doi:10.1007/s10811-005-9011-9.C

Babu B, Wu J-T (2008) Production of natural Butylated Hydroxytoluene as antioxidant by freshwater Phytoplankton. J Phycol 44:1447–1454. doi:10.1111/j.1529-8817.2008.00596.x

Barrios-Llerena ME, Burja AM, Wright PC (2007) Genetic analysis of polyketide synthase and peptide synthetase genes in cyanobacteria as a mining tool for secondary metabolites. J Ind Microbiol Biotechnol 34:443–456. doi:10.1007/s10295-007-0216-6

Bauer AW, Kirby WM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Amer J Clin Pathol 45: 493-496. Available from: http://www.garfield.library.upenn.edu/classics1985/A1985ANC2900001.pdf. Accessed 24 August 2010

Buettner GR (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. Arch Biochem Biophy 300: 535-543. Availablefrom http://www.ncbi.nlm.nih.gov/pubmed/8434935. Accessed 24 August 2010

Chauhan A, Chauhan G, Gupta PC, Goyal P, Kaushik P (2010) In vitro antibacterial evaluation of *Anabaena* sp. against several clinically significant microflora and HPTLC analysis of its active crude extracts. Indian J Pharmacol 42:105–107. doi:10.4103/0253-7613.64490

Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306. doi:10.1016/j.biotechadv.2007.02.001

Claus ER (1967) Pharmacognosy, 5th edn. Henry Kimpton, London

Davidson PM, Branden AL (1981) Antimicrobial activity of non-halogenated phenolic compounds. J Food Prot 44:623–632

Doan NT, Rickards RW, Rothschild JM, Smith GD (2000) Allelopathic actions of the alkaloid 12-epi-Hapalindole E isonitrile and calothrixin A from cyanobacteria of the genera *Fischerella* and *Calothrix*. J Appl Phycol 12:409–416. doi:10.1023/A:1008170007044

Ehrenreich IM, Waterbury JB, Webb EA (2005) Distribution and diversity of natural product genes in marine and freshwater cyanobacterial cultures and genomes. Appl Environ Microbiol 71 (1):17401–17413. doi:10.1128/AEM.71.11.7401-7413.2005

El Semary NA, Ghazy SM and Abd El Naby M (2009) Investigating the taxonomy and bioactivity of an Egyptian *Chlorococcum* isolate. Australian Journal of Basic and Applied Sciences 3(3): 1540-1551. http://www.insipub.com/ajbas/2009/1540-1551.pdf

El Semary N and Abd El Naby M (2010) Characterization of a *Synechocystis* sp. from Egypt with the potential of bioactive compounds production. World J Microbiol Biotechnol 26 (6): 1125-1133. doi 10.1007/s11274-009-0280-3. http://www.springerlink.com/content/2620w7h267341h56/

El-Sheekh MM, Osman MEH, Dyab MA, Amer MS (2006) Production and characterization of antimicrobial active substance from the cyanobacterium *Nostoc muscorum*. Environ Toxicol Pharmacol 21(1):42–50. doi:10.1016/j.etap.2005.06.006

Falch BS, Konig GM, Wright AD, Sticher O, Angerhofer CK, Pezzuto JM, Bachmann H (1995) Biological activities of cyanobacteria: evaluation of extracts and pure compounds. Planta Med 61:321–328. doi:10.1055/s-2006-958092

Feuillade J (1994) The cyanobacterium (blue-green alga) *Oscillatoria rubescence* D.C.: ecological significance. Arch Hydrobiol Beih Ergebn Limnol 42:77–93

Ghasemi Y, Yazdi MT, Shafiee A, Amini M, Shokravi S, Zarrini G (2004) Parsiguine, a novel antimicrobial substance from *Fischerella ambigua*. Pharm Biol 42:318–322. doi:10.1080/13880200490511918

Herrero M, Cifuentes A, Ibañez E (2006) Sub- and supercritical fluid extraction of functional ingredients from different natural sources: plants, food-by-products, algae and microalgae: a review. Food Chem 98:136–148. doi:10.1016/J.foodchem.2005.05.058

Issa A (1999) Antibiotic production by the cyanobacteria *Oscillatoria augustissima* and *Calothrix parietina*. Environ Toxicol Pharmacol 8:33–37

Jaffe GM (1984) Ascorbic acid. In: Grayson M (ed) Kirk-Othmer encyclopedia Of chemical technology, vol 24. Wiley, New York

Kulik MM (1995) The potential for using cyanobacteria (blue-green algae) and algae in the biological control of plant pathogenic bacteria and fungi. Eur J Plant Pathol 101:585–599

Pavia DL, Lampman GM, Kriz GS (1996) Introduction to spectroscopy, 2nd edn. Harcourt Brace, New York pp 28–30, 465–467

Rao M, Malhotra S, Fatma T, Rattan A (2007) Antimycobacterial activity from cyanobacterial extracts and phytochemical screening of methanol extract of Hapalosiphon. Pharm Biol 45:88–93. doi:10.1080/13880200601105319

Scholz B, Liebezeit G (2006) Chemical screening for bioactive substances in culture media of microalgae and cyanobacteria from marine and brackish water habitats: first results. Pharm Biol 44:544–549. doi:10.1080/13880200600883114

Sikkema J, De Bont A, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201–222

Skulberg OM (2000) Microalgae as a source of bioactive molecules – experience from cyanophyte research. J Appl Phycol 12:341–348. doi:10.1023/A:1008140403621

Thajuddin N, Subramanian G (2005) Cyanobacterial biodiversity and potential applications in biotechnology. Curr Sci 89(1):47–57. doi:10.1023/A:1013840025518

Wall ME, Kreider MM, Kemson CF, Eddy CR, Williaman JJ, Corell DS, Gentry HS (1954) Steroidal sapogenins. VII-Survey of plants for steroidal sapogenins and other constituents. J Am Pharm Soc 43:1–3

Wang X, Willen R, Wadström T (2000) Astaxanthin-rich algal meal and vitamin C inhibit *Helicobacter pylori* infection in BALB/cA Mice. Antimicrob Agents Chemother 44:2452–2457. doi:0066-4804/00/$04.00+0

Whitton BA, Potts M (2000) The ecology of cyanobacteria: their diversity in time and space. Kluwer, Dordrecht