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Molecular Identification and Norharmane Quantification of Cyanobacterium, *Geitlerinema carotinosum*

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ABSTRACT

The cyanobacteria classified as oxygenic photoautotrophs are widely distributed in nature and play a significant role in primer productivity and the global carbon cycle. Norharmane, a significant secondary metabolite existing in cyanobacteria and plant species represents the large group of natural *β*-carboline alkaloids with diverse pharmacological properties. The aim of this study was identification and cultivation of cyanobacterium, *Geitlerinema carotinosum*, and quantification of norharmane excreted by this species. The sample was collected from Tokat-Yesilirmak River and was isolated under an inverted microscope. Identification was executed as morphologically and molecularly. The isolated *G. carotinosum* was cultivated in a BG-11 nutrient medium. The quantification of norharmane was determined by HPLC analysis at first. The amount of norharmane (µg/g) was calculated according to the Gauss method by drawing a calibration curve with the absorbance value at 247 nm wavelength of the standard. Norharmane amount was found to be 1.191 µg/g in *G. carotinosum* methanol extract.

INTRODUCTION

The Recent tendency in drug discovery from natural origins dwells on the investigation of freshwater to produce plentiful, fascinating, highly complex, chemical compounds [1]. Cyanobacteria (blue-green algae) reveal the same structural features with bacteria [2]. Nevertheless, they are classified with algae due to the consisting of chlorophyll-a and related compounds. The atmospheric nitrogen is converted into the ammonia by all prokaryotes that may explain the why nitrogenous compounds occur frequently in blue-green algae.

The cyanobacteria have an interesting secondary metabolism yielding many nitrogenous compounds [3]. The secondary metabolites synthesized by cyanobacteria consist of peptides and depsipeptides which are the most promising active natural compounds. Therefore cyanobacteria have been known as one of the most significant group organisms to generate novel, biochemically active natural compounds [4]. Cyanobacterial metabolites demonstrate a wide range of biological activity such as anti-HIV [5], anticancer [6], antiproliferative [7], antifungal [8], anti-inflammatory , anti-nephrolith, antioxidant [9], antimicrobial, antiviral, cytotoxic [10], cytoskeleton disruption [11], herbicidal [12], enzyme inhibitor [13], antimitotic activities [14].

Norharmane 9H-pyrido (3, 4-b)indole, a derivative of *β*-carboline alkaloids reveals the biological properties including the inhibition of various enzymes such as *monoamine oxidase* [15], *indoleamine 2,3-dihydroxygenase*, and nitric oxide synthesis [16]. *β*-carbolines also displayed the antihypertensive, cardiovascular [17], anti-HIV effects [18]. It was reported that many cyanobacteria species such as *Nodularia harveyana*, *Nostoc insulare* and *Synechocystis* sp. consisted of norharmane [19]. Hence, we scanned the *G. carotinosum* methanol extract whether it contained the norharmane and found out that the norharmane was the main product of *G. carotinosum.*

In this study, after identification and cultivation of cyanobacterium *Geitlerinema carotinosum* (Geitler) Anagnostidi, quantitative analysis of norharmane in *G. carotinosum* was presented by HPLC analyses at first.

MATERIALS AND METHODS

Sample collecting, cultivation and identification

Sample material was collected from Yesilirmak river, Tokat, Turkey (40° 19' 45.655" N, 36° 33' 45.06" E). It was filtered through filter paper (Whatman, Germany) then was streaked onto an agarised BG11 medium (with 1.5% agar). Incubation was kept for 2 weeks at 26 °C ±2 for 12/12 h (light/dark). The light intensity was 155 µmol photon $s^{-1}m^{-2}$. Single filaments were removed from the agarised medium by forceps [20]. Purification and identification of *G. carotinosum* were examined by Olympus light microscopes at 1000 × magnification. Axenic culture of *G. carotinosum* was transferred into 100 ml Erlenmeyer flask and was grown under the same laboratory conditions.

Genetic analysis

Total genomic DNA was extracted with ZRFungal/Bacterial DNA kit according to the manufacturer's instructions. Concentration and purity of DNA sample were determined using a NanoDrop. 16S rRNA gene fragments were amplified using the primers F27 (5'-CTCTGTGTGCCTAGGTATCC-3') and R1492 (5'-CTCTGTGTGCCTAGGTATCC–3') for molecular characterization of this strain [21, 22]. PCR amplification was carried out by Roche FastStart Taq DNA polymerase kit using thermal cycler (Bio-Rad, USA). Thermal cyclic conditions were initial denaturized for 4 min at 95 °C, for 1 min 95 °C, annealing for 45 s at 60 °C, an extension for 1 min at 72 °C for 30 cycles and 72 °C for 7 min for final extension step. The reaction was performed in a volume of 25 ml, containing 50 ng of DNA. PCR product was visualized by 1.5% agarose gel electrophoresis stained with ethidium bromide and photographed under UV transillumination. Sequence analysis of PCR product was sent to REFGEN (Gene Research and Biotechnology Ltd. Co, Ankara, Turkey).

Norharmane analysis

The cells were centrifuged to separate from BG11 medium then were extracted with methanol. TLC (Thin layer chromatography) and HPLC analyses confirmed the presence of norharmane into the methanol extract. Dry sample (50 mg) was dissolved in methanol and chloroform (1/1). Then, the solution was vortexed for 1 minute and was kept in the ultrasonic bath for 10 minutes, filtrated with polytetrafluoroethylene (PTFE) syringe (Chrom Tech, 0.45 µm 13 mm) for straining. The specimen (20 µl) was taken from the extract after straining and was injected into the column (Meriluoto and Codd, 2005**).** HPLC analysis was performed with HPLC-DAD (high-performance liquid chromatography and diode array detection) (Shimadzu, Japanese) device with C18120A (Thermo, 4.6 x150 mm, 3 µm particle size)

reverse phase column. The flow rate was adjusted to 1 ml/min using a gradient system of A, water with 0.1% formic acid and B, acetonitrile. The gradient program was fixed as follows: 0-14 min, 100% A; 15-29 min, 80% A, 30-32 min, 60% A, 33-34 min, 0% A. UV spectra were measured at 247 nm [6]. The amount of norharmane was calculated by the calibration curve using the Gauss method.

RESULTS

Morphological characterization

Width and length of cyanobacterium were measured by placing micrometer on oculars of the light microscope. The shape of trichomes is smooth and becomes thin toward to the edge. The cells were arranged in bunches in the shape of fascicules (Figure 1). The cells are at 1.5-3 µm width; 3-9 µm length [23].

Figure 1. Imagine of *Geitlerinema carotinosum* under light microscope

Molecular characterization

Cyanobacterial 16S rRNA gene sequences defined in REFGEN were aligned using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) analysis, and phylogenetic tree was formed with MEGA 6 Bootstrap Test of Phylogeny (neighbor joining) software (Figure 2). According to the results of 16S rRNA analysis, it demonstrated in 1289/1295 base pair region (99%) similarity with G*eitlerinema carotinosum* AICB 37.

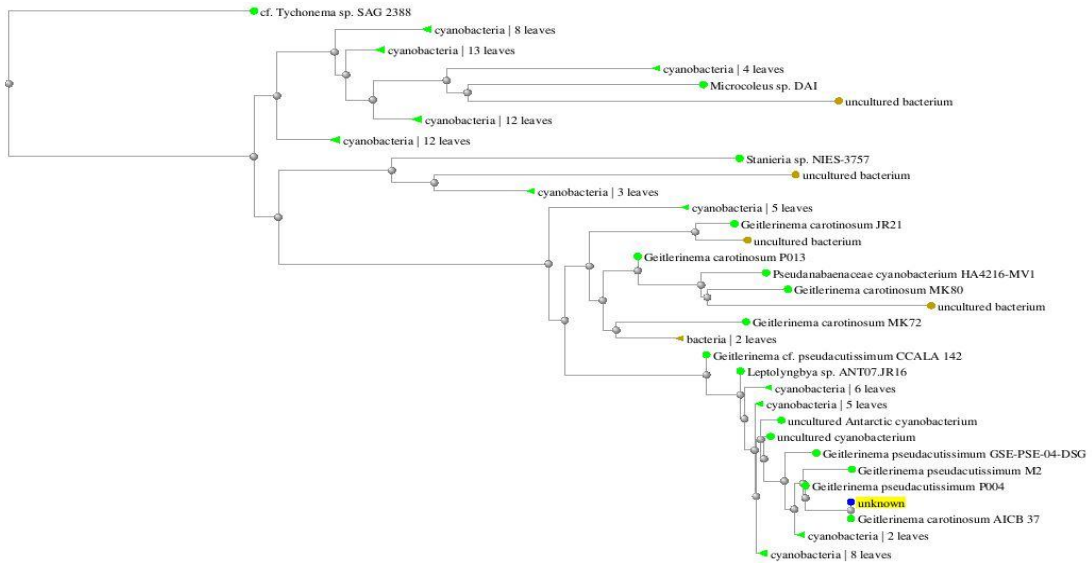

Figure 2. 16S rRNA Neighbour-joining tree based on 1289 nucleotide positions. The percentage of replicate trees in which the associated tax clustered together in the bootstrap test

Norharmane analyses

The quantitative analysis of narharmane was executed by HPC. The standard was taken from Sigma-Aldrich (product numbers; Norharmane N6252). The peaks of norharmane standard and extract appeared in 10th minute (Figure 3). Norharmane amounts of *G.carotinosum* (µg/g) were calculated in accordance with Gaus method (1.19 µg g^{-1}) by plotting calibration curve over absorbance value in 247 nm wavelength of the standard.

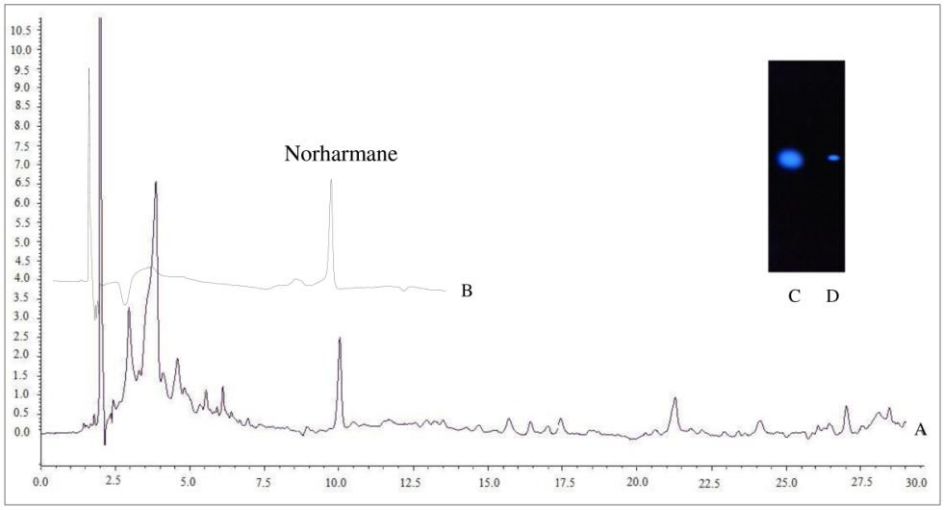

Figure 3. HPLC chromatogram of extract (A) and standard, norharmane (B). TLC (UV_{254} nm) of *G. carotinosum* (C) and norharmane (D), EtOAc as a mobile phase

DISCUSSION

Taxonomy and classification of cyanobacteria have been performed morphologically according to cell structures since mid-19th century. However, many species defined morphologically are not clear, and some genus needs significant revision. Researchers have emphasized in recent years that characterization of these bacteria at molecular aspect has been important. The latest development in taxonomy of cyanobacteria is the multi-perspective approach combining morphologic-ecologic combination and molecular characterization described as an innovative system. Therefore, genetically defined species must be in compliance with phenotype and ecologic data [24, 25]. With use of molecular methods such as 16S rRNA analysis, morphologic features are required to be compared genetically. 95% 16S rRNA sequence similarity is accepted as the criterion for distinguishing genus of cyanobacteria [26]. Species under filament cyanobacteria such as *Geitlerinema, Microcoleus* and *Phormidium* are recognized both with molecular characters and taxonomically. Especially these geniuses and the species belonging to genus are confused since they are very similar to each other morphologically. In this case, the ones having high incidence of similarity in 16S rRNA sequences were verified by comparing through morphological features [23]. According to the results of NCBI-BLAST analysis, the cyanobacterium *Geitlerinema carotinosum* AICB 37 demonstrated a high rate of similarity and got accession number (AY423710.1) from NCBI.

Cyanobacteria are inevitable sources of natural compounds used in biotechnology and attract attention in medical and agricultural practices, due to the synthesis of great numbers of bioactive secondary metabolites [27-29]. The research on presence of cyanobacterial norharmane is quite a few. The presence of norharmane and bis-acetamide into the *Nostoc insulare* was introduced by HPLC analyses [19]. The presence of norharmane in more than eight cyanobacteria, *Anabaena cylindrica*l, *Anabaena inaequalis*, *Anabaenopsis siamensis*, *Chroococcus minutes*, *Nostoc carneum*, *Nostoc commune*, *Nodularia harveyana* and *Phormidium foveolarum* was detected [30]. Herein, we determined presence of norharmane in *G.carotinosum* as well as quantification at first. *G. carotinosum* was isolated and cultivated then quantitative analysis of norharmane in *G. carotinosum* was presented. Norharmane was the main product of *G. carotinosum.* Due to the medicinal and pharmaceutical significance of norharmane, *G. carotinosum* could be the promising source of norharmane production in large scale.

CONCLUSION

G. carotinosum was isolated and identified by morphologically and molecularity. Quantitative analysis of norharmane in *G. carotinosum* was presented. Norharmane was the main product of *G. carotinosum*. Due to the medicinal and pharmaceutical significant of norharmane, *G. carotinosum* could be promising source of norharmane production in large scale.

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