



TLC Bioautographic Detection and Characterization of Antibacterial Compound from the Cyanobacterium *Anabaena oryzae*

**Mahmoud A. Al-Saman¹, Ragaa A. Hamouda², Asmaa Abdella^{1*},
Sabha M. El-Sabbagh³ and Ghada W. Abo El-Seoud³**

¹Department of Industrial Biotechnology, Genetic Engineering and Biotechnology Research Institute,
University of Sadat City, Sadat City, Egypt.

²Department of Microbial Biotechnology, Genetic Engineering and Biotechnology Research Institute,
University of Sadat City, Sadat City, Egypt.

³Department of Botany, Faculty of Science, Menoufia University, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Author MAAS performed the analytical tasks, statistical analysis and wrote the first draft of manuscript. Authors RAH, SMES and GWAES designed the study, did the microbiological works and wrote the first draft of manuscript. Author AA wrote the manuscript and managed the literature search. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJB2T/2018/39097

Editor(s):

(1) K. Vijaya Ramesh, Assistant Professor, Plant Biology and Plant Biotechnology, Quaid-e-Millath Government College for Women, India.

Reviewers:

(1) Rachid Drissi El Bouzaidi, University Ibnou Zohr, Morocco.

(2) Bushra Uzair, International Islamic University, Pakistan.

(3) Milena Kalegari, Faculdades Pequeno Príncipe, Brazil.

Complete Peer review History: <http://prh.sdiarticle3.com/review-history/23266>

Original Research Article

Received 9th November 2017
Accepted 25th January 2018
Published 21st February 2018

ABSTRACT

Background: Microalgae have the potential to produce natural bioactive compounds, which are difficult to be produced by chemical synthesis. These compounds also possess diverse biological activities, such as antibacterial, anti-inflammatory, anti-atherosclerotic and anti-carcinogenic activities.

Objective: The purposes of the current study were to use a simple and accurate Thin layer chromatography (TLC) bioautographic method for detection and isolation of antibacterial compound from the complex cyanobacterial extract and the characterization of the isolated compound.

*Corresponding author: E-mail: asmaa.abdelaa@gebri.usc.edu.eg, dr_aasmmaa@yahoo.com;

Materials and Methods: Polar and non-polar extracts of cyanobacterium *Anabaena oryzae* were screened for antibacterial activity against various Gram-positive and Gram-negative bacterial species using agar well diffusion method. The mode of action of methanolic extract on pathogenic bacteria was examined using scanning electron microscopy, Thin layer chromatography-direct bioautography (TLC-DB) was applied for the first time in detection and isolation of the antimicrobial compound in cyanobacterial extract. The target band was purified by silica gel column chromatography and antibacterial compound was isolated. The isolated compound was characterized using IR and mass spectroscopy.

Results: The methanolic extract had the highest biological activity against *Escherichia coli*, MIC 0.05 µg/ml. Scanning electron microscopy revealed a shortening and swelling of the *E. coli* cells, and multiple blisters and bubbles formed on their surface. One compound with antibacterial activity was isolated using (TLC-BD) and column chromatography. Infrared spectra (IR) indicated that the isolated compound was phenolic compound. Mass spectroscopy indicated that its molecular weight was 443.38.

Conclusion: It was concluded that cyanobacteria *Anabaena oryzae* are metabolically very diverse group and a promising group of organisms for research on medicines discovery. It was also concluded that (TLC-DB) is very useful in the isolation and identification of compounds with antimicrobial activity from biological extract.

Keywords: Antibacterial; cyanobacterium; *Anabaena oryzae*; direct bioautography; phenolic compound.

1. INTRODUCTION

Blue-green algae (Cyanobacteria) are a group of Gram-negative photoautotrophic prokaryotes containing a blue-green colored pigment (c-phycocyanin) [1]. They can be found in nearly all habitats such as fresh and salty water, lakes, ponds, soil, rocks and trees [2]. Their morphological form may be unicellular, filamentous or colonial forms [3].

Cyanobacteria are one of the richest sources of bioactive compounds which are widely applied in therapeutic applications [4-6]. Various strains of cyanobacteria have the ability to produce intracellular and extracellular metabolites with diverse biological activities. The acetone extract of *Nostoc commune* was having antibacterial activity on *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* [7]. Antifungal compounds such as fisherellin A, hapalindole, carazostatin, a phytoalexin, palytoxin, scytophycin, toyocamycin, thiamazole, nostocyclamide, and nostodione were produced by cyanobacteria belonging to Stigonematales, Nostocales and Oscillatoriales [8]. The algicidal phenolic compound 4,4' dihydroxybiphenyl and the indol alkaloid norharmane were found in *Nostoc insular* and *Nodularia harveyana*, respectively [9]. There are also metabolites with antiviral [10], immunosuppressant, anticancer, anticoagulant, antimalarial, antiprotozoal, antituberculosis, and antitumor activities [11].

Bacterial resistance towards antibiotics is responsible for the increase in mortality from bacterial infections. So, there is rising interest in the discovery of new antimicrobial agents recently. Secondary metabolites with antibacterial activity are widely produced by cyanobacteria. They are effective against both Gram-positive and Gram-negative bacteria [12]. Five new ambiguines from *Fischerella* sp. were isolated, which showed antibacterial activity [13]. Two new norbietane compounds, showing antibacterial activity against various Gram-positive and Gram-negative bacteria, were isolated from *Micrococcus lacustris* [14].

It is readily apparent that the rapid and efficient detection of bioactive compounds such as antimicrobial drugs in the extract of microorganism is a critically important aspect. The principal problem in their detection emerges from the interference of the structurally diverse compounds in the algal extract. TLC bioautographic methods combine chromatographic separation and in situ activity determination facilitating the localization and target-directed isolation of active constituents in a mixture. Traditionally, the bioautographic method has used growth inhibition of microorganisms to detect anti-microbial components of extracts chromatographed on a TLC layer. This methodology has been considered as the most efficacious assay for the detection of anti-microbial compounds [15]. For the natural product, the separation process is not

easy, and the separated amount is very little in most cases, so it is necessary to develop a process which can detect the active compounds in a small amount and biological activity can also be measured successively. Considering these problems, we can say that bioautographic method would create a new era in separation science [16].

There are three types of the bioautographic assays: The direct bioautography; where the microorganisms grow directly on thin-layer chromatography (TLC) plates, contact bioautography; where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, and agar overlay or immersion bioautography; where a seeded agar medium is applied to the TLC plate. The latter technique can be considered as a hybrid of direct and contact bioautography [17].

In this particular study *Anabena oryzae* is used because few previous types of research on *Anabena oryzae* have been already carried out (Hamouda *et al*) and for the further conclusion, the piece of study has to be continued. This study applied the TLC bioautographic method for the first time on a cyanobacterial extract that can be beneficial for detection and isolation of antibacterial compounds from them. This study will help the researcher to uncover the critical areas of isolation of active constituents from complex biological extracts that many researchers were not able to explore. Thus a new theory on -active constituents isolation may be arrived at. It also aimed to characterize the structure of the isolated antibacterial compounds.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Cyanobacterium

The study was carried out at Microbial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute, Univ. of Sadat City, Egypt. The blue-green alga used in this study was collected from the soil surface of rice fields at Kafrelsheikh governorate in Egypt during March 2015. The samples were dried at 25°C, ground to pass a 2-mm sieve and diluted in sterilized double distilled water. The diluted samples were cultured on agar plates containing BG-11 medium and incubated at a fluorescent light with an intensity of 2500 lux for three weeks. The best colonies were picked up, re-cultured

several times on new solid BG-11 medium (Thermo Fischer Scientific)(g/L): 1.5 NaNO₃, 0.0314 K₂HPO₄, 0.036 MgSO₄·7H₂O, 0.036 CaCl₂·2H₂O, 0.02 Na₂CO₃, 0.006 Citric acid, 0.001 Na₂Mg EDTA, 0.006 Ferric ammonium citrate, 10 agar) [18]. They were incubated under continuous illumination at 28±1°C. Isolated cyanobacterium was identified as *Anabena oryzae* on the basis of its morphological characteristic under a light microscope (*Leica* DM300) at 100x oil immersion lens and on the taxonomical approaches according to Desikachary [19], Prescott [20], Anagnostidis and Komarek [21].

2.2 Culture Conditions

The isolate was cultured in 250 ml Erlenmeyer flasks containing 100 ml of the liquid BG-11 medium under aerobic conditions, at 25°C and illumination at 2500 lux with a white continuous light and a regime of 14 h light 10 h dark. The cultures were shaken every day to prevent algal cell clumping and adherence of algal cells to the containers. They have been harvested after two weeks by centrifugation (Centurion Scientific LTD Model 1020 series) at 1000 rpm for 10 min.

2.3 Preparation of Algal Extracts

One hundred mg of the dried alga was extracted by 10 ml petroleum ether at (60-80)°C for 48 h and filtrated; the precipitate was re-extracted by an equal volume of dichloromethane for the same period and the extraction process was repeated with both ethyl acetate and methanol. All filtrates were concentrated to dryness in a rotary evaporator, re-dissolved in the minimum amount of dimethyl sulfoxide (DMSO) to final concentrations of 10% (W/V) and then sterilized using a 0.45 µm filter.

2.4 Test Microorganisms

The microorganisms used in this study were Gram-positive bacteria (*Bacillus cereus*, ATCC 14579) and Gram-negative bacteria (*Escherichia coli*, ATCC 13529, *Klebsiella pneumonia*, ATCC 13883). They were obtained from Microbial Type Culture Collection (MTCC), Cairo, Egypt. The bacterial strains were grown and maintained on nutrient agar slants at 37°C.

2.5 Antibacterial Assays

The different cyanobacterial extracts were tested at a concentration of (50,100,150) µg/ml against both Gram-positive and Gram-negative bacteria

using agar well diffusion bioassay adopted by Valgas et al. [22]. Nutrient agar, plates were inoculated with 100 μ l of a 24 h broth culture of the test bacteria. Two wells (6 mm) were made and filled with 100 μ l extract. The inoculated plates were incubated for 24 h at 37°C. The diameter of the inhibition zone was measured with calipers and the results were recorded. All experiments were conducted in triplicates. Minimum inhibitory concentrations of active crude extracts were determined by broth microdilution method as recommended by National Committee for Clinical Laboratories Standards. Streptomycin was used as positive control [23].

2.6 Scanning Electron Microscopy

Scanning electron microscopy (SEM) examination was performed in order to visualize the morphological changes in the bacterial cells after treatment with 0.2 ml of methanolic extracts of *A. oryzae*. The bacterial cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C, washed with phosphate buffered saline (PBS), pH 7.4 and fixed with 2% glutaraldehyde followed by 1% osmium tetroxide (OsO₄). After completion of fixation, the samples were washed with PBS and dehydrated in ascending order of ethanol concentrations. The fixed bacterial cells were then dried completely and finally coated with thin layer of gold. All bacterial samples were examined under scanning electron microscope (JSM 5300 SEM; JEOL, Japan) [24].

2.7 Chromatographic Methods for Detection and Isolation of the Antibacterial Substances Produced by *A. Oryzae*

2.7.1 Thin layer chromatography

The components of crude extracts were detected qualitatively on silica gel plates (Fluka, silica gel 60F-254 TLC aluminum sheet 20x20 cm). 20 μ l of each sample (1mg/mL) was spotted manually using the capillary tube on the plate. Different mobile phases with varying concentrations were employed in the screening programme (Table 1) and the one in which good separation of compounds was clear: benzene/acetone (90:10, v/v) was selected. All plates were visualized directly after drying with the help of UV at 365 nm in UV TLC viewer. The R_f value of the different spots was observed and calculated.

Table 1. Running solvent system for Thin layer chromatography development

Solvent mixture	% (v/v)
Benzene: chloroform	50:50
Benzene: acetone	90:10
Chloroform: methanol	50:10
Chloroform: acetic acid	95:05
Ethyl acetate: chloroform	80:20
n-hexane: ethyl acetate	80:20
Methanol: ethyl acetate	50:50

2.7.2 Thin layer chromatography scanning

The developed plates were scanned using a TLC scanner 3 with Wincats software under 254 nm [25]. All plates were visualized directly after drying and a fingerprint profile was photo documented using a Camag Reporter – 3 under 365 nm in UV light. The digital images of the chromatograms were evaluated with the programme CAMAG Video Scan. The captured image was subjected to a visual inspection on the computer screen.

2.7.3 Thin layer chromatography-direct bioautography (TLC-DB)

One hundred μ l of extract was spotted directly to the TLC plate and developed with a benzene/acetone (90:10, v/v). After separation, the developed plate was dried for the complete removal of the remaining solvents and the fluorescent compounds were detected and marked. Two hundred μ l of each of the bacterial suspension was prepared (as mentioned above) and spread on the developed TLC plate with a sterilized glass rod until just wet. The plate inoculated with the target organism was allowed to dry aseptically under laminar flow for minimization of other airborne microorganisms. After that, the TLC plate was incubated for 12 h at 37°C, sprayed with a tetrazolium solution {2 mg/mL (2,3,5-triphenyltetrazolium chloride (TTC)) supplied by Aldrich Chemical Co., UK and incubated again overnight in a sealed container for the color development. Antibacterial compounds were identified as clear inhibition zones (colorless) against a pink colored background that indicated bacterial growth. The experiment was compared with duplicate chromatogram developed under the same conditions [26].

2.7.4 Column chromatography

After inhibitory zones are identified by a bioassay, the corresponding region on a duplicate TLC plate was repeatedly eluted from the silica gel with acetone (analytical grade). The samples were then centrifuged at 6000 rpm for 20 min to remove the silica gel. A glass column (20x2 cm) packed with silica gel was used to purify the obtained antibacterial material. The supernatants were dried, re-dissolved in a small volume of acetone (~5 ml), loaded on the column chromatography and eluted with the solvent mixture which consists of benzene: acetone: methanol (90:10:5, v/v) at a flow rate of 1 ml/3 min. Twenty-five fractions were collected and dried under reduced pressure and tested for purity by using TLC. The purified fraction was tested for its antimicrobial activity and was lyophilized to isolate the active compounds.

2.8 Characterization of Isolated Compound

2.8.1 Infrared spectra

The isolated active compound obtained from column chromatography was analyzed using the Fourier transform infrared (FTIR) instrument (Jasco, Model FTIR 4100, Japan) at Cairo Univ. Micro Analytical Center, Giza, Egypt; in the frequency range of 4000 up to 400 cm^{-1} and the results were compared against characteristic IR absorptions reference.

2.8.2 Mass spectrometry

Mass spectrometry (MS) analysis for the active compound was carried out using (JEOL JMS - AX500); the scan range for the mass spectrum was set at m/z zero to 800.

2.9 Statistical Analysis

The experimental results were expressed as mean \pm SD of three replicates using SPSS version 17.0 statistical software (IBM Corporation). Comparison of means for *in vitro* antibacterial evaluation was carried out using One-way analysis of variance (ANOVA) and Duncan's multiple range tests. *P* value ≤ 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Antibacterial Activity of *Anabaena oryzae* Extracts

Results summarized in (Table 2) show the antimicrobial activity of different crude extracts of *Anabaena oryzae* against various Gram-positive and Gram-negative bacterial species at concentrations ranged from 50 to 150 $\mu\text{g ml}^{-1}$. Antibacterial activity was observed in dichloromethane, ethyl acetate and methanolic extracts of *A. oryzae* against *E. coli* and *K. pneumoniae*. We can conclude that the diameter of the zone of inhibition was related to the type of extract and the type of the tested bacteria. It was also evident that the diameter of zone inhibition increased with the increase in the crude extract concentration regardless of both the type of extract or the bacterial species. Also as seen in (Table 2), all extracts of *A. oryzae* had an effect on the Gram-negative bacteria (*E. coli* & *K. pneumoniae*) while it had no effect on the Gram-positive bacteria (*B. cereus*).

It was also noted the non-polar petroleum ether extract had no inhibitory effect on all the tested strains and by increasing the polarity the inhibitory effect increased till reaching the highest inhibitory effect on all the tested strains using the methanolic extract. The experimental analysis of antibacterial effects indicated that all tested bacterial strains showed the highest sensitivity to the methanolic extract of *A. oryzae* at a concentration of (150 $\mu\text{g ml}^{-1}$) with the highest antibacterial activity against *E. coli* & *K. pneumoniae* (17.3 \pm 0.09 and 15.3 \pm 0.03) mm inhibition zone respectively.

The antibacterial activity of the extract could be attributed to the presence of different chemicals such as phenolic, flavonoids and triterpenoids that may have an effect on the growth and metabolism of bacteria [27]. These results go in harmony with those obtained of Kaushik *et al.* who obtained the highest effective zone of inhibition by using the methanolic extract of *A. variabilis* against *S. aureus*, *E. coli*, *P. aeruginosa* and *Salmonella typhi* [28]. On contrast, Madhumathi *et al.* revealed that acetone and ethanol were the best solvents for extraction of antibacterial compounds from *Lyngbya martensiana* and *Oscillatoria latevirens* while the methanolic extract showed a moderate activity [29].

Table 2. Antibacterial activity of different crude extracts of *A. oryzae* against *E. coli*, *K. pneumonia* and *B. cereus*; Diameter of zone of inhibition (mm), including the disc diameter of 6 mm; each disc carries 20 µl of extract (1-3 µg/disc)

Bacterial species	<i>Anabaena oryzae</i> extracts prepared with different organic solvents											
	Pet. ether (µg ml ⁻¹)			Dichloromethane (µg ml ⁻¹)			Ethyl acetate (µg ml ⁻¹)			Methanol (µg ml ⁻¹)		
	50	100	150	50	100	150	50	100	150	50	100	150
<i>E. coli</i>	*	*	*	*	7.3±0.03	9.3±0.06	*	*	08.6±0.03	08.3±0.03	14.3±0.19	17.3±0.09
<i>K. pneumonia</i>	*	*	*	7.0±0.03	13.0±0.03	14.0±0.03	*	10.3±0.03	11.0±0.00	07.6±0.03	12.3±0.03	15.3±0.03
<i>B. cereus</i>	*	*	*	*	*	*	*	*	*	*	*	*

*no activity
n= 3, value= mean ± SE

Table 3. Electron impact ionization data by mass spectrometry of the isolated antibacterial Compound

Fragment (m/z)	Relative intensity (%)
54.17	21.32
55.15	100.00
56.15	27.01
57.16	82.28
60.11	46.14
67.14	33.09
69.16	39.14
331.30	0.29
443.38	0.14

3.2 Scanning Electron Microscopy Examination (SEM)

According to (Fig. 1), the bacterial cells were examined before and after treatment with a methanolic extract of *A. oryzae* using scanning electron microscopy to follow the morphological changes of bacterial cultures. As seen in Fig. 1(A and B) the *E. coli* and *K. pneumoniae* cells treated with a methanolic extract of *A. oryzae* ($150 \mu\text{g ml}^{-1}$) for 4 h at 37°C , demonstrated signs of the irregular outer surface, uneven and curled edges, and accumulated debris cells. All observations were compared with the standard morphological shape of untreated cells as a control Fig. 1 (A1 and B1). No clear differences could be marked between the untreated *B. cereus* cells and *B. cereus* cells treated with the methanolic extract of *A. oryzae* Fig. 1 (C and C1). Hartmann et al. [30] used scanning electron microscopy (SEM) to examine the ultrastructural changes in Gram-negative strain *E. coli* ATCC 25922 and Gram-positive strain *Staphylococcus aureus* ATCC 25923, bacteria induced by antimicrobial substances. SEM revealed a shortening and swelling of the *E. coli* cells, and multiple blisters and bubbles formed on their surface.

3.3 Chromatographic Methods for Detection and Isolation of the Antibacterial Substances Produced by *A. oryzae*

3.3.1 Thin layer chromatography

Methanol was the optimum solvent for the extraction of the active antibacterial substances from *A. oryzae* used in this experiment. Results of TLC analysis using solvent mixture benzene:

acetone (9:1, v/v) revealed that the methanolic extract of *A. oryzae* contained three different components with the retention factor (R_f) of 0.65, 0.78 and 0.86, respectively. (Fig. 2A). Scanning of TLC chromatogram confirmed the results obtained by TLC, and R_f values of all compounds coincided with that of TLC (Fig. 3).

3.3.2 Thin layer chromatography-direct bioautography (TLC-DB)

The antimicrobial compounds of cyanobacterial extract were detected using Thin-layer chromatography-direct bioautography assay (TLC-DB). Actively growing microorganisms have the ability to reduce a tetrazolium solution (2,3,5-triphenyl tetrazolium chloride) to red formazan which is directly proportional to the viable active cells [31]. In the presence of antimicrobial compounds in the cyanobacterial extract applied on the chromatograms, the growth of the organism is inhibited resulting in the appearance of clear inhibition zone (with no pink color) at the position of R_f 0.65 (band No. 1) against a pink background on the chromatograms (Fig. 2B).

This bioassay has a couple of potential applications. The size of the zone of inhibition can be used as a rough estimate of the amount of the inhibitory compound since the radius of the inhibitory zone is proportional to the logarithm of the amount of the compound causing the inhibition [32]. Perhaps the most common use of TLC plate bioassays is to narrow the range of possible antimicrobial compounds in the cyanobacterial extract.

3.3.3 Column chromatography

One fraction out of the 25 collected fractions eluted from column (fraction no 11) were tested for the antimicrobial activity using the agar well diffusion method and were found to exhibit high activity against *K. pneumoniae* and *E. coli*. It was concentrated under vacuum, spotted again on TLC to check the purity and by lyophilization one compound was isolated (13.2 mg, greenish crystals).

3.4 Characterization of the Isolated Compound

IR spectrum of the isolated compound was represented in (Fig. 4). Absorption peaks belonging to functional and or structural groups were recorded. The IR spectra revealed a weak band and a shoulder at 2852.2 and 2919.7 cm^{-1} which can be attributed to the stretching

vibrations of the CH aliphatic group and aromatic CH group. A broad band appeared at 3432.67 cm^{-1} represent the presence of stretching vibration of a phenolic compound containing hydrogen bonding. There was band due to the stretching vibration of $\text{C}\equiv\text{N}$ group at 2362.36 cm^{-1} . There were also two bands due to the stretching vibration of the $\text{C}=\text{C}$ group at 1631.48 cm^{-1} and the $\text{C}-\text{O}$ group at 1030.77 cm^{-1} respectively. There was also another peak at 1380.78 cm^{-1} related to isopropyl group. The mass spectrum fragmentation pattern of the compound under investigation was shown in (Fig. 5). It revealed the presence of molecular ion peak at m/z 443.38 of relative abundance characteristic of the parent compound. The fragmentation of the parent compound yield a compound m/z 55.15. According to the obtained data, the isolated compound was a phenolic compound and its molecular weight is 443.38. The presence of phenolic compound was confirmed by Folin-Ciocalteu method [33].

Nearly the same results were obtained by El-Sheekh *et al.* who indicated that the IR spectrum

of a chloroformic extract of cyanobacterium *Nostoc muscorum* had absorption bands related to νOH , νCH aromatic, νCH aliphatic, $\nu\text{C}\equiv\text{N}$ $\text{C}=\text{N}$, $\nu\text{C}=\text{C}$, and $\text{C}-\text{O}$ which was related to the phenolic compound. [34]. The presence of phenolic compound was also confirmed in our previous study in methanolic extract of the cyanobacteria *Anabaena oryzae* [35]. The results obtained in this study also corroborate the results obtained by Abd El-Aty *et al.* who found that the methanolic extract of the cyanobacteria *Oscillatoria agardhii* had high antibacterial activity due to the presence of phenolic compounds [36]. Entesar, [37] had the same finding by confirmation of the presence of high percentage of phenolic compounds and flavonoids in the methanolic extract of cyanobacterium (*Nostoc caeruleum*, *Spirulina platensis*, *Cylindrospermum majus*, *Oscillatoria forms*) which had antibacterial activity on three Gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*) and three Gram-negative bacteria (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*).

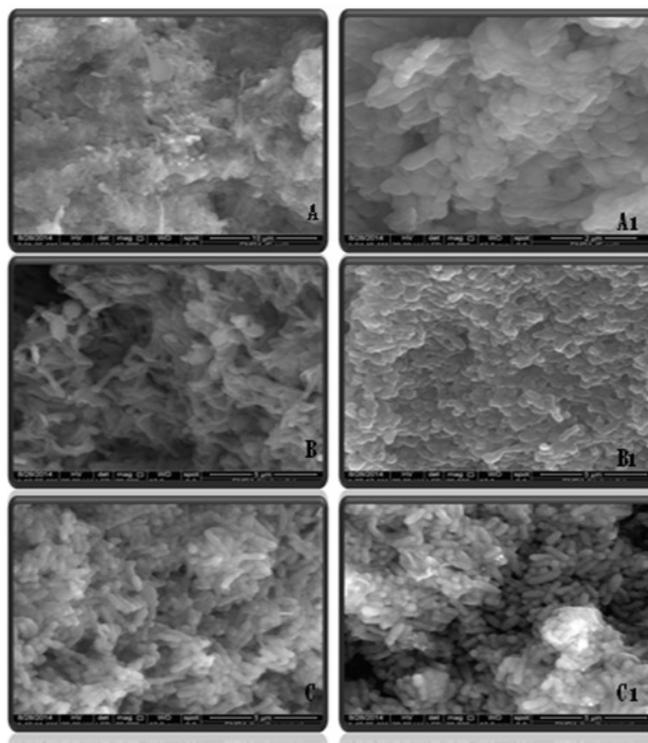


Fig. 1(A-C). Scanning electron microscopy (SEM) of treated and un-treated *E. coli* with methanolic extract of *A. oryzae* (A and A1), SEM of treated and un-treated *K. pneumoniae* with methanolic extract of *A. oryzae* (B and B1), SEM of treated and un-treated *B. cereus* with methanolic extract of *A. oryzae* (C and C1)

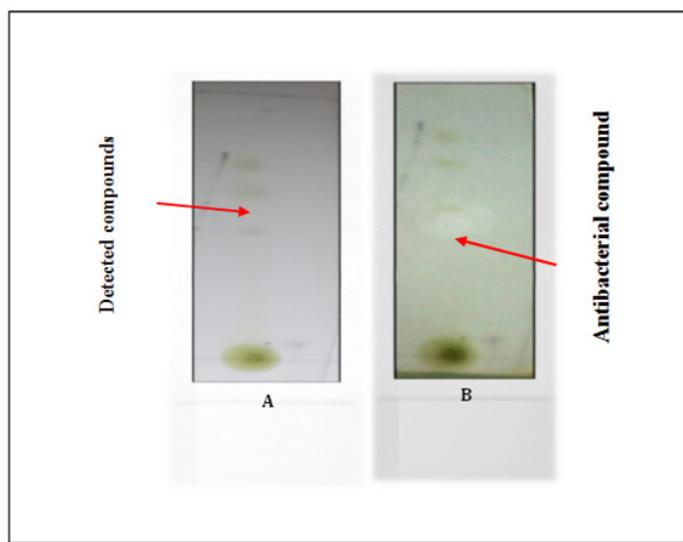


Fig. 2. (A-B). (A) Normal plate developed in solvent mixture benzene: acetone (9:1, v/v) showing the detected components under UV at 365 nm. (B) TLC-DB, developed in solvent mixture benzene: acetone (9:1, v/v) and viewed under UV at 365 nm showing the compound with the antibacterial activity

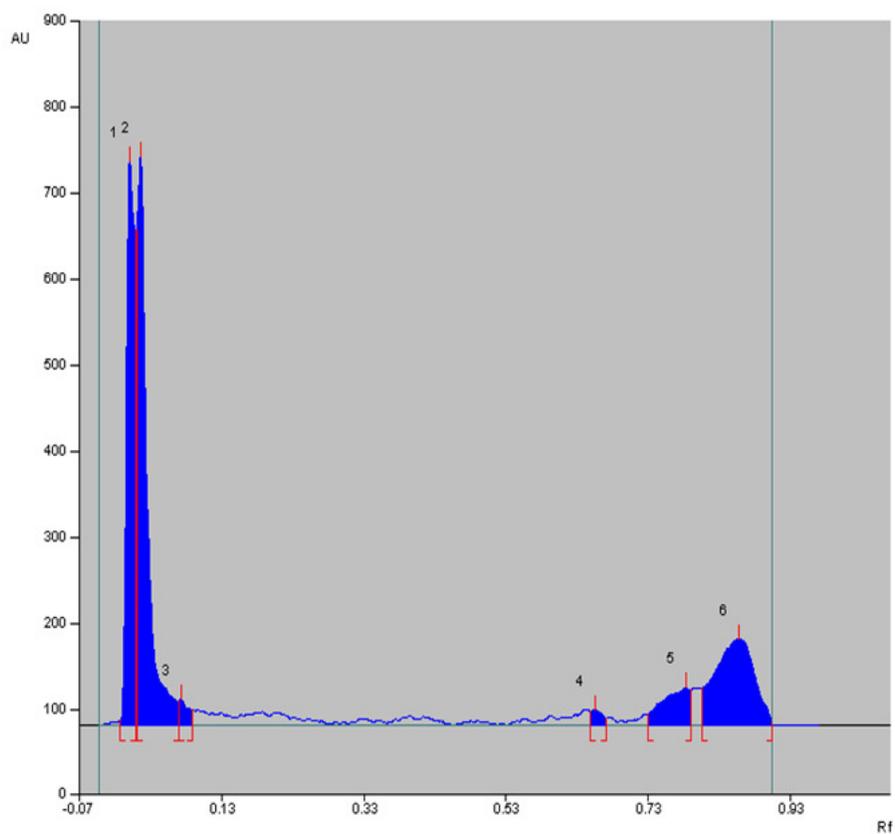


Fig. 3. Scanning of TLC chromatogram using CAMAG TLC Scanner 3

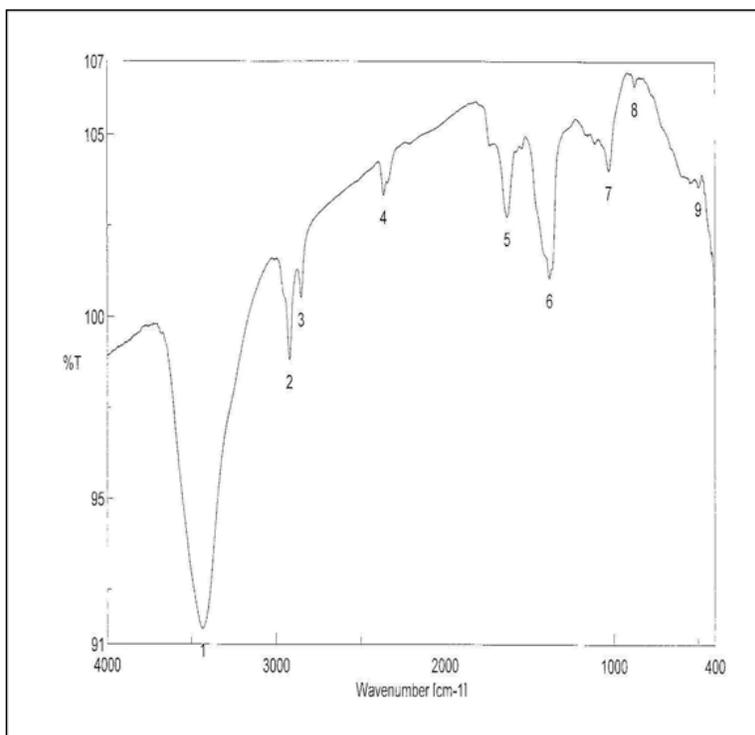


Fig. 4. Infrared spectra of the isolated antibacterial compound produced by *A. oryzae*

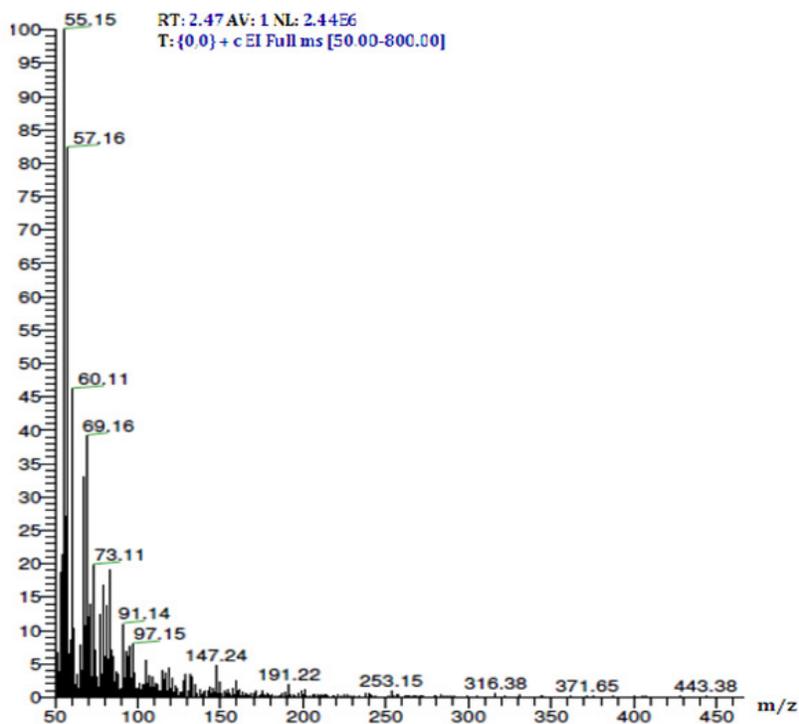


Fig. 5. Mass spectra of the isolated antibacterial compound produced by *A. oryzae*

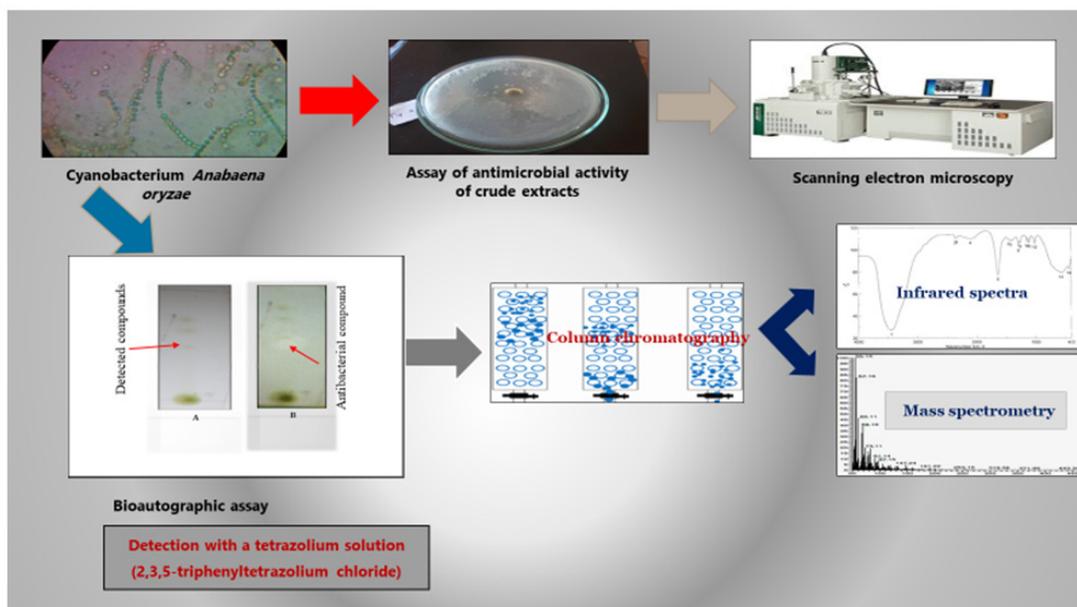


Fig. 6. Graphical abstract

The characterization of the antimicrobial compound as phenolic compound explains the high antimicrobial activity of the methanolic extract compared to the other extracts which were due to the high solubility of phenolic compounds in highly polar solvents [38].

This study can be potentially applied in the isolation of active constituents in biological extracts even at very low concentration and that saves time and effort needed for fractionation of biological extract.

4. CONCLUSION

The present study is an endeavor towards the isolation of antibacterial compound from the cyanobacteria *Anabaena oryzae* using both TLC-DB and column chromatography. It was concluded that cyanobacteria *Anabaena oryzae* are a metabolically very diverse group which makes them as a promising group of organisms for research on medicines discovery. Thin layer chromatography-direct bioautography (TLC-DB) is very useful in isolating compounds with antimicrobial activity because the R_f of the active compound can be used in bioassay guided fractionation instead of requiring labor-intensive determination of activity of fractions. This also helps in ensuring that the compound isolated at the end is the same compound that was present in the extract and is not an artifact of the isolation procedure. The characterization of the isolated

antimicrobial compound revealed that it was a phenolic compound. More chemical analyses and phytochemical studies must be carried out to elucidate the complete structure of the isolated antibacterial compound.

ACKNOWLEDGEMENT

The work was in part supported by the Genetic Engineering and Biotechnology Research Institute, Sadat City University, Egypt.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ananya AK, Ahmad IZ. Cyanobacteria "the blue-green algae" and its novel applications: A brief review. *Int J Innovation Appl Stud*. 2014;7(1):251-261
2. Issa AA, Adam MS, Fawzy MA. Alterations in some metabolic activities of *Scenedesmus quadricauda* and *Merismopedia glauca* in response to glyphosate herbicide. *J Biol Earth Sci*. 2013;3(1):17-28
3. Singh SP, Montgomery BL. Determining cell shape: Adaptive regulation of cyanobacterial cellular differentiation and

- morphology. Trends Microbiol. 2011;19(6): 278-285.
4. Tan LT. Bioactive natural products from marine cyanobacteria for drug discovery. Phytochem. 2007;68:954-79.
 5. Gademann K, Portmann C. Secondary metabolites from cyanobacteria: Complex structures and powerful bioactivities. Curr Org Chem. 2008;12:326-341.
 6. Martins RF, Ramos MF, Herfindal L, Sousa JA. Antimicrobial and cytotoxic assessment of marine cyanobacteria *Synechocystis* and *Synechococcus*. Mar Drugs. 2008;6:1-11.
 7. Abedin RMA, Taha HM. Antibacterial and antifungal activity of cyanobacteria and green microalgae: Evaluation of medium components by plackett – Burman design for antimicrobial activity of *Spirulina platensis*. Global J Biotechnol Biochem. 2008;3(1):22-31.
 8. Abed RMM, Dobretsov S, Sudesh K. Applications of cyanobacteria in biotechnology. J Appl Microbiol. 2009;106: 1-12.
 9. Volk RB, Furkert FH. Antialgal, antibacterial and antifungal activity of two metabolites produced and excreted by cyanobacteria during growth. Microbiol Res. 2006;161:180—186.
 10. Zainuddin E, Mundt S, Wegner U, Mentel R. Cyanobacteria a potential source of antiviral substances against influenza virus. Med Microbiol Immunol. 2002;191: 181–182.
 11. Mayer AMS, Rodríguez AD, Berlinck R, Hamann MT. Marine compounds with anthelmintic, antibacterial, anticoagulant, antifungal, anti-inflammatory, antimalarial, antiprotozoal, antituberculosis, and antiviral activities; affecting the cardiovascular, immune and nervous systems, and other miscellaneous mechanisms of action. Comp Biochem Physiol. 2007;145(4):553-581.
 12. Burja AM, Banaigs B, Abou-Mansour E, Burgess JG. Marine cyanobacteria – a prolific source of natural products. Tetrahedron. 2001;57:9347–9377.
 13. Mo S, Kronic A, Chlipala G, Orjala J. Antimicrobial ambiguanine isonitriles from the cyanobacterium *Fischerella ambigua*. J Nat Prod. 2009;72(5):894–899.
 14. Gutierrez RMP, Flores AM, Solis RV, Jimenez JC. Two new antibacterial norabietane diterpenoids from cyanobacteria *Microcoleus lacustris*. J Nat Med. 2008;62:328–331.
 15. Shahverdi AR, Abdolpour F, Monsef-Esfahani HR, Farsam H. A TLC bioautographic assay for the detection of nitrofurantoin resistance reversal compound. J Chromatogr B. 2007;850: 528–530.
 16. Dewanjeea S, Gangopadhyay M, Bhattacharya N, Khanra R. Bioautography and its scope in the field of natural product chemistry. J Pharm Anal. 2015;5(2):75–84.
 17. Suleimana MM, McGaw LI, Naidoo V, Eloff J. Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected south african tree species. African J Tradit Complement Altern Med. 2010;7(1):64–78.
 18. Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G. Purification and properties of unicellular blue green algae (order Chroococcales). Bacteriol Rev. 1971;35:171-205.
 19. Desikachary TV. Cyanophyta. Indian Council of Agricultural Research: New Delhi. 1959;187-198.
 20. Prescott GW. Algae of the western Great Lake area. W.M.C. Dubuque, Brown Company. 1962;543-551.
 21. Anagnostidis K, Komarek J. Modern approaches to the classification of cyanobacteria. Stigonematales. Arch Hydrobiol. 1990;4:224-286.
 22. Valgas C, de Souza SM, Smânia EFA, Smânia Jr. A. Screening methods to determine antibacterial activity of natural products. Braz J Microbiol. 2007;38:369–380.
 23. National committee for clinical laboratory standards. Clinical and laboratory standards institute: Performance standards for antimicrobial susceptibility testing”; twenty-second informational supplement. 11th Edn. M100-S22. Standards. 2012;32(3).
 24. Borgers M, Van De Ven MA, Van Cutsen J. Structural degeneration of *Aspergillus fumigatus* after exposure to saperconazole. J. Med. Vet. Mycol. 1989; 27:381-389.
 25. Wagner H, Bladt. Plant drug analysis: A thin layer chromatography. Atlas, 2nd Edn. Springer-Verlag, Berlin Heidelberg London, New York. 1996;349–354.
 26. Hamburger MO, Cordell GA. A direct bioautographic TLC assay for compounds

- possessing antibacterial activity. J. Nat. Prod. 1987;50:19-22.
27. Yu H, Jia S, Dai Y. Growth characteristics of the cyanobacterium *Nostoc flagelliforme* in photoautotrophic, mixotrophic and heterotrophic cultivation. J Appl Phycol. 2009;21:127-133.
28. Kaushik P, Chauhan A. In vitro antibacterial activity of laboratory grown culture of *Spirulina platensis*. Ind J Microbiol. 2008;48:348-352.
29. Madhumathi V, Deepa P, Jeyachandran S, Manoharan C, Vijayakumar S. Antimicrobial activity of cyanobacteria isolated from freshwater lake. Int J Microbiol Res. 2011;2(3):213-216.
30. Hartmann M, Berditsch M, Hawecker J, Ardakani MF. Damage of the bacterial cell envelope by antimicrobial peptides Gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. Antimicrob Agents. 2010; 54(8):3132-3142.
31. Eloff JN. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med. 1998;64(8):711-713.
32. Kagan IA, Hammerschmidt R. Arabidopsis ecotype variability in camalexin production and reaction to infection by *Alternaria brassicicola*. J. Chem. Ecol. 2002;28(11): 2121-2140.
33. Slinkard K, Singleton VL. Total phenol analyses: Automation and comparison with manual methods. Am. J. Enol. Vitic. 1977; 28:49-55.
34. El-Sheekh MM, Osman MEH, Dyab MA, Amer MS. Production and characterization of antimicrobial active substance from the cyanobacterium *Nostoc muscorum*. Environ Toxicol and Pharmacol. 2006; 21:42-50.
35. Hamouda RA, Al-Saman MA, El-Sabbagh SM, Abo El-Seoud GW, Hendawy AN. Approach to improve the productivity of bioactive compounds of the cyanobacterium *Anabaena oryzae* using factorial design. Egypt. J Bas. App. Sci. 2017;4:190-195.
36. Abd El-Aty AM, Mohamed AA, Samhan FA. *In vitro* antioxidant and antibacterial activities of two fresh water cyanobacterial species, *Oscillatoria agardhii* and *Anabaena sphaerica*. J App Pharm Sci. 2014;4(7):69-75.
37. Entesar AA. Antimicrobial activity of microalgal extracts isolated from Baharia Oasis, Egypt. Glob Adv Res J Microbiol. 2016;5(3):33-41.
38. Pietta PG, Gardana C., Pietta AM. Analytical methods for quality control of propolis. Fitoterapia. 2002;73(1):7-20.

© 2018 Al-Saman et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:

The peer review history for this paper can be accessed here:
<http://prh.sdiarticle3.com/review-history/23266>