

Antimicrobial Potentials of *Leptolyngbya* sp. and Its Synergistic Effects With Antibiotics

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Background: Resistance to antibiotics is a clinical problem. Cyanobacteria have many more antibiotic productions. Nowadays, combinations of antimicrobial compounds are used.

Objectives: The current research studied antimicrobial properties of *Leptolyngbya* sp.

Materials and Methods: Chloroform extracts of *Leptolyngbya* sp. were prepared. Antimicrobial effects of these extracts were evaluated singly and in combination with antibiotics against various microorganisms. Growth optimization, antimicrobial activity and their correlations were studied.

Results: *Leptolyngbya* sp. had antimicrobial activity individually and in synergy with antibiotics against Gram-positive and Gram-negative bacteria. The highest activity of the chloroform extract was on Gram positive strains and its effects on fungi were moderate, but it had a little effect against Gram negative ones. Phosphate salt was the most important factor for growth and antibacterial activity in *C. leptolyngbya*.

Conclusions: Optimization of medium composition and synergistic antimicrobial activity on the cyanobacteria could lead to production of effective antimicrobials.

Keywords: Cyanobacteria spp.; *Leptolyngbya* sp.; Synergism

1. Background

Resistance to antibiotics is a ubiquitous relentless clinical problem. New antibiotic development by pharmaceutical sector has aggravated the challenge of widespread resistance and signals a critical need for innovation (1). Cyanobacteria have a wide range of secondary metabolites including antibiotics (2). Researches to identify antimicrobial compounds produced by cyanobacteria, have recently received attention as a new source of novel antimicrobial substances. Ethyl acetate extract of *Spirulina platensis* can inhibit some Gram positive and Gram negative bacteria and *Candida albicans* (3). *Oscillatoria* spp. can produce derivatives with antimicrobial activity (4). Nowadays, combinations of antimicrobials are usually used in medicine to broaden antimicrobial spectrum and generate synergistic effects. Drug combinations have potentials for counteracting drug resistance (1). The economical and oligotrophic growth (5-7) of *Leptolyngbya* sp. form the aim of this research, whereas arriving at new antimicrobial compounds is of prime importance.

The current research studies the antimicrobial effects of

oligotrophic cyanobacterium *Leptolyngbya* sp. independently and in synergy with antibiotics. This taxonomic cluster was originally named as "LPP -group B" by bacteriologists (8), and has been recently supported and accepted by molecular analysts (9). This cyanobacteria is moderately a new thermophilic halophile or halotolerant genus (10, 11). The basic and fundamental requirements for cyanobacterial biotechnology are the availability of natural cyanobacterial sources and understanding their ecobiological properties. Therefore, a detailed study of its habitats was made to identify the available cyanobacterial species and subsequently to isolate, purify and establish a culture collection which could be used for further studies (12).

The successful cultivation and growth of cyanobacteria require modification in media composition (9). Several liquid media have been modified for the culture of marine and freshwater species e.g., artificial seawater nutrients III (ASN-III), Marine Medium (MN), and Blue-green 11 (BG-11) (8). The modification to find a desirable culture related to requirements of cyanobacteria may differ from species to species (13). In the current research,

Implication for health policy/practice/research/medical education:

This is the first report on antimicrobial activity of hot benthic waters *Leptolyngbya* strains from East Azerbaijan of Iran and its synergistic properties.

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the optimum growth conditions of *Leptolyngbya* sp. were studied. BG-11 medium is a general culture medium for cyanobacteria. BG-11 medium was subject to some modifications in Nitrogen, phosphorus, magnesium, iron and copper contents. The results are discussed.

2. Objectives

The current study aimed to access new natural antimicrobial compounds; therefore, the current research studied antimicrobial properties of *Leptolyngbya* sp.

3. Materials and Methods

3.1. Sampling and Culturing

The unexplored fresh or hot benthic waters in East Azerbaijan province of Iran were the sampling regions. Samples were taken directly by immersion in water of sampling bottles by "grab" or "snap" sampling methods (14). The samples were stored at a temperate range of 10-17°C under dark conditions in a storage box in order to decrease metabolism and to avoid the growth interfering factors during sampling and transportation.

3.2. Growth Conditions

The samples were cultured batch wise in BG-11 agar and broth media (15) and illuminated at a light intensity of 3500 lux (16 hours light: 8 hours dark) at 22°C and shaking at 100 rpm. The samples were screened for *Leptolyngbya* sp. by different purification methods according to the degree of contamination of samples.

3.3. Molecular Analysis

DNA extraction with boiling method For PCR test was performed and then was amplified and electrophoresis (16). PCR products were sequenced and aligned with the cited sequences in NCBI.

3.4. Oligotrophic and Halophilic or Halotolerant Aspects of the *Leptolyngbya* sp.

Growth of *Leptolyngbya* sp. in oligotrophic state was studied in the CHU NO.10 modified medium. The medium contains very low amounts of nutrients and salts and is therefore only suitable for oligotrophic strains. Halophilic or halotolerant properties of *Leptolyngbya* sp. were tested with van Rijn and Cohen (RC) Medium (17). RC modified media were prepared at 1/3 and 2/3 reduced salt concentrations compared to the original.

3.5. Antibacterial and Antifungal Bioassay

3.5.1. Extract Preparation

Leptolyngbya sp. strains grown in the liquid medium

were harvested at late exponential phase. The biomass was extracted with hexane, methanol, diethyl ether, ethyl acetate, chloroform organic solvents. Aqueous crude extracts were also used.

3.5.2. Antimicrobial Activity on Test Microorganisms

The following bacteria and fungi were used as test organisms: *Staphylococcus aureus* (PTCC 1112), *Micrococcus luteus* (PTCC 1625), *Escherichia coli* (PTCC 1329), *Erwinia carotovora* (PTCC 1675), *Salmonella typhi* (PTCC 1609) and *Candida kefyr* (ATCC 38296).

Minimum inhibitory concentrations (MIC), Minimum bactericidal inhibition (MBC) and minimum fungicidal inhibition (MFC) were assayed by broth dilution methods (18, 19). The antibacterial effects of a combination of the chloroform extract, which exhibited the highest antimicrobial activity with antibiotics, were assessed by the checkerboard test and fractional inhibitory concentration (FIC) (20). Where more than one combination resulted in a change in the MICs value of the extract or antibiotic, the FICI was described as the total of the individual FIC values (20).

3.6. Evaluation of the Effects of BG-11 Medium Components on the Optimum Growth and Antimicrobial Activity of *C. Leptolyngbya* BG-11 medium components

(0.75 mg) MgSO₄.7H₂O, (36 mg) CaCl₂.2H₂O, (1500 mg) NaNO₃, (30 mg) K₂HPO₄, (20 mg) Na₂CO₃, (1 mg) Na₂EDTA.2H₂O, (6 mg) Ferric ammonium citrate, (1 ml) Micronutrient solution (A5+CO) (8), final volume 1000 ml with deionized water and pH after filtrate ~7.1.

(A5+CO) components: (286 mg) H₃BO₃, (181 mg) MnCl₂.4H₂O, (22 mg) ZnSO₄.7H₂O, (39 mg) NaMoO₄.2H₂O, (79 mg) CuSO₄.5H₂O in the final volume 100 ml with deionized water. Culture media was prepared in three forms: 0, 1.5 and 2 fold of BG-11 salts. Incubation conditions were as described earlier. After 13 days, the culture media were taken for growth measurement, determination of dry weight and chloroform extracts antibacterial assay.

3.7. Growth Measurements

Chlorophyll-a, from each sample was extracted in 80% acetone and the content of the pigments was determined using the procedure of Myers and Kratz at an absorbance of 663 nm. Specific growth rate was calculated using the formula: $\mu = [\ln (B_f/B_i)]/t_i$. Where B_i is the initial chlorophyll-a, and B_f is the chlorophyll-a, at the end of incubation. Productivity was calculated from the formula: $P = (X_i - X_0)/t_i$, where P = productivity (mg L⁻¹day⁻¹), X₀ = initial biomass density (mg L⁻¹), X_i = biomass density at time i (mg L⁻¹) and t_i = time interval (h) between X₀ and X_i (21).

3.8. Determination of Dry Weight

10 ml from different cultures were washed several times with distilled water to remove soluble salts. The cyanobacteria cells were then dried at 80°C for 30 min and weighed (22).

4. Results

4.1. Isolation

Overall, 35 samples were isolated out of which only 2 samples were *Leptolyngbya* spp.

4.2. Identification

The 16S rRNA sequences of two isolates from Heravi location of Mehranrud and Bostan Abad underground hot spring were subject to NCBI blast. The former corresponded to *Leptolyngbya nodulosa* UTEX 2910 with accession number (gi|119367623|EF122600.1), 99% identity and 0% gap. The latter corresponded to *Leptolyngbya* spp. 0418 with accession number (gi|15842033|EU068735.1), 99% identity and 0% gap.

4.3. Growth Ability

B-110 culture medium is nitrogen limited medium. The isolated strains of *Leptolyngbya* sp. were grown in the culture medium and therefore may be considered as putative nitrogen fixing species for *Leptolyngbya* sp. isolates. These strains relieve suitable growth in the CHU-10 modified medium. Bottle-green and glucose pigments in nitrogen limited media, namely BG-110 and CHU-10 modified culture did not occur. Among the different dilutions of saline RC culture medium, the best growth was observed at 1:3 dilution. Available strains have growth ability in the RC 1/3 modified.

4.4. Antimicrobial Results

Chloroform extracts compared to other solvents, showed better results in antimicrobial testing. Therefore, further investigation of the antimicrobial activity with chloroform extract was performed. The Screening tests showed that Gram positive bacteria were more susceptible than Gram negative bacteria. The antimicrobial activities are given in Table 1.

Table 1. Antimicrobial Activity of the Chloroform Extracts of *Leptolyngbya* sp. 1 For Bacteria and *Leptolyngbya* sp. 2 for Fungi Alone and Different Antibiotics Combined.

Strains	Agent	MIC, µg/mL	FIC	FICI	Outcome	Type
		Alone	Combined			
<i>Micrococcus luteus</i>	<i>Leptolyngbya</i> sp. 1 Gentamicin	80/>80 ^a 1.2	20 (0.3)	0.25 (0.25)	0.5	Synergistic
<i>Micrococcus luteus</i>	<i>Leptolyngbya</i> sp. Erythromycin	80/>80 (0.3)	20 (0.15)	0.25 (0.5)	0.75	Additive
<i>Staphylococcus aureus</i>	<i>Leptolyngbya</i> sp. 1 Gentamicin	40/80 (0.6)	20 (0.15)	0.5 (0.25)	0.75	Additive
<i>Staphylococcus aureus</i>	<i>Leptolyngbya</i> sp. 1 Erythromycin	40/80 (0.3)	20 (0.15)	0.5 (0.5)	1	Additive
<i>Salmonella typhi</i>	<i>Leptolyngbya</i> sp. 1 Gentamicin	Resistance (1.2)	20 (0.6)	0 (0.5)	0.5	Synergistic
<i>Salmonella typhi</i>	<i>Leptolyngbya</i> sp. 1 Ceftazidime	Resistance (12.5)	20 (6.2)	0 (0.5)	0.5	Synergistic
<i>Candida kefyri</i>	<i>Leptolyngbya</i> sp. 2 Amphotericin B	40/60 (0.6)	20 (0.3)	0.5 (0.5)	1	Additive

^a The fractions are related to the MBC data of

Table 2 shows the values of maximum specific growth rate (μ_{max}) and productivity (P312) 13 days after culturing two strains of *Leptolyngbya* genera at different salt concentrations of BG-11 culture media. Their antibacterial effects were evident against *M. luteus*. Due to the importance of nitrogen, phosphorus, iron, magnesium and copper ions in the NaNO₃, K₂HPO₄, Ferric ammonium citrate, MgSO₄, CuSO₄ salts, have been studied in the current research (Table 2). With the exception of phosphorus and copper salts, high concentrations of other elements did not lead to increased antibacterial activity. None of the isolated *Leptolyngbya* strains showed signs of growth at two-fold increased micro-nutrients solution (A5+CO) (Table 2).

5. Discussion

Leptolyngbya strains grew well in the CHU-10 modified medium. The findings were in support of those of

Douterelo *et al.* (5), Mateo *et al.* (6) and Perona *et al.* (7) in which *Leptolyngbya* sp. was oligotrophic. In the modified RC, saline concentration was approximately equivalent to 34 psu. This means that cyanobacteria have ability for growth in the average saline sea waters. These observations are in agreement with those of Abed *et al.* (10), and Chatchawan *et al.* (11). According to the antimicrobial test, about 42.85% of all the interactions were synergistic, while indifferent and antagonistic interactions were not observed. Additive activity was against the *Salmonella typhi*. Synergistic activity between *Leptolyngbya* sp.2 and Amphotericin B against *C. kefyri* shows that this strain possesses antifungal activity. Over all, strain 2 has more antifungal activity than strain 1. Antibacterial activity of strain 1 is higher than that of strain 2. Gram positive bacteria were more susceptible than Gram negative bacteria confirming Dixon *et al.* report (23).

Table 2. Growth Ability With μ max, P 312 Tests and Antibacterial Activity Research With Kirby-Bauer Disk Diffusion Method

Treatments	μ max, day ^a	P312, mgL ^a day ^a	Inhibition Zones ^b
BG-11 plus 0.5 fold of K ₂ HPO ₄ salt to <i>Leptolyngbya</i> sp.1	0.178 ± 0.004	9.93 ± 0.16	Resistance
BG-11 plus 1 fold of K ₂ HPO ₄ salt to <i>Leptolyngbya</i> sp.1	0.224 ± 0.002	10.57 ± 0.27	9 ± 0.63
BG-110 plus 1 fold of K ₂ HPO salt to <i>Leptolyngbya</i> sp.1	0.088 ± 0.003	4.8 ± 0.75	8 ± 1.09
No K ₂ HPO ₄ salt BG-11 to <i>Leptolyngbya</i> sp.1	No growth	No growth	14 ± 0
BG-11 plus 0.5 fold of K ₂ HPO ₄ salt to <i>Leptolyngbya</i> sp.2	0.162 ± 0.004	6.89 ± 0.33	Resistance
BG-11 plus 1 fold of K ₂ HPO ₄ salt to <i>Leptolyngbya</i> sp.2	0.193 ± 0.003	4.32 ± 0.22	Resistance
BG-110 plus 1 fold of K ₂ HPO ₄ salt to <i>Leptolyngbya</i> sp.2	0.098 ± 0.004	6.25 ± 0.18	9 ± 1.09
No K ₂ HPO ₄ salt BG-11 to <i>Leptolyngbya</i> sp.2	No growth	No growth	15 ± 0.89
BG-11 plus 0.5 fold of NaNO ₃ salt to <i>Leptolyngbya</i> sp.1	0.169 ± 0.003	7.37 ± 0.9	Resistance
BG-11 plus 1 fold of NaNO ₃ salt to <i>Leptolyngbya</i> sp.1	0.194 ± 0.008	8.01 ± 0.1	Resistance
BG-11 plus 0.5 fold of NaNO ₃ salt to <i>Leptolyngbya</i> sp.2	0.157 ± 0.005	4.8 ± 0.24	Resistance
BG-11 plus 1 fold of NaNO ₃ salt to <i>Leptolyngbya</i> sp.2	0.168 ± 0.006	5.6 ± 0.17	Resistance
BG-11 plus 0.5 fold of Ferric ammonium citrate to <i>Leptolyngbya</i> sp.1	0.131 ± 0.001	6.08 ± 0.79	Resistance
BG-11 plus 1 fold of Ferric ammonium citrate to <i>Leptolyngbya</i> sp.1	0.155 ± 0.003	5.44 ± 0.18	Resistance
No Ferric ammonium citrate BG-11 to <i>Leptolyngbya</i> sp.1	0.148 ± 0.005	5.44 ± 0.14	Resistance
BG-11 plus 0.5 fold of Ferric ammonium citrate to <i>Leptolyngbya</i> sp.2	0.13 ± 0.001	3.04 ± 0.58	Resistance
BG-11 plus 1 fold of Ferric ammonium citrate to <i>Leptolyngbya</i> sp.2	0.179 ± 0.006	3.04 ± 0.35	Resistance
No Ferric ammonium citrate BG-11 to <i>Leptolyngbya</i> sp.2	0.145 ± 0.002	1.76 ± 0.9	Resistance
BG-11 plus 0.5 fold of CuSO ₄ . 5H ₂ O salt to <i>Leptolyngbya</i> sp.1	No growth	No growth	14 ± 0
BG-11 plus 1 fold of CuSO ₄ . 5H ₂ O salt to <i>Leptolyngbya</i> sp.2	No growth	No growth	10 ± 1.26
BG-11 plus 0.5 fold of CuSO ₄ . 5H ₂ O salt to <i>Leptolyngbya</i> sp.1	No growth	No growth	11 ± 0
BG-11 plus 1 fold of CuSO ₄ . 5H ₂ O salt to <i>Leptolyngbya</i> sp.2	No growth	No growth	9.5 ± 0
BG-11 plus 0.5 fold of MgSO ₄ . 7H ₂ O salt to <i>Leptolyngbya</i> sp.1	0.144 ± 0.003	4.16 ± 0.33	9 ± 0.63
BG-11 plus 1 fold of MgSO ₄ . 7H ₂ O salt to <i>Leptolyngbya</i> sp.1	0.184 ± 0.005	4.807 ± 0.1	7.5 ± 0.37
BG-11 plus 0.5 fold of MgSO ₄ . 7H ₂ O salt to <i>Leptolyngbya</i> sp.2	0.133 ± 0.008	2.4 ± 0.11	8 ± 0
BG-11 plus 1 fold of MgSO ₄ . 7H ₂ O salt to <i>Leptolyngbya</i> sp.2	0.188 ± 0.004	3.044 ± 0.98	7.5 ± 0.31
BG-11 plus 0.5 fold of micronutrients [A5+CO] to <i>Leptolyngbya</i> sp.1	0.118 ± 0.001	2.24 ± 0.8	Resistance
BG-11 plus 1 fold of micronutrients [A5+ CO] to <i>Leptolyngbya</i> sp.1	0.13 ± 0.005	1.76 ± 0.19	Resistance
BG-11 plus 0.5 fold of micronutrients [A5+CO] to <i>Leptolyngbya</i> sp.2	Cell death	Cell death	-
BG-11 plus 1 fold of micronutrients [A5+CO] to <i>Leptolyngbya</i> sp.2	Cell death	Cell death	-
Routine BG-11 (Control) to <i>Leptolyngbya</i> sp.1	0.135 ± 0.004	2.24 ± 0.28	8 ± 0.7
Routine BG-11 (Control) to <i>Leptolyngbya</i> sp.2	0.13 ± 0.001	1.76 ± 0.37	7.5 ± 0.31

Table 2 shows that combination of antibiotics and extracts had better results than their single use. According to the first row of this table, MIC of the extract alone is 80 μ g/mL; while MIC in combination with antibiotic has decreased to 20 μ g/mL. MIC for antibiotic alone in this case is 1.2 μ g/mL, while in combination with the extract it decreased to 0.3 μ g/ml. The results demonstrated that the extracts combined with antibiotics significantly increased antimicrobial inhibition effects. Biomass increased with increasing concentrations of NaNO₃, K₂HPO₄, Ferric ammonium citrate in μ max and productivity tests were considerable (24). Table 2 shows the importance of salts, particularly phosphate salt in the BG-11 culture medium

for biomass production and growth of *Leptolyngbya* sp. This is probably applicable to all Cyanobacteria. K₂HPO₄ is so important that its absence is quite clear.

The importance of phosphorus and potassium content along with the buffering capacity of K₂HPO₄ indicate that Cyanobacteria will not be able to grow in the absence of K₂HPO₄. This condition is also true with the lower concentration of nitrogen in the culture medium. The nitrogen limited BG-110 culture media was used. Yellowish pigments produced in this medium show a major reduction in μ max with chlorophyll-a test calculation. Contrary to this phenomenon, higher values in μ max test were observed as a result of high MgSO₄ concentration showing

increased chlorophyll-a pigment. Absence of growth at two-fold increased micronutrients solution (A5+CO) indicates toxic effect of the higher concentrations of the trace elements. Increased antimicrobial activity at higher concentration of MgSO₄, could be attributed to its sulfur content (25).

Importance of phosphorus, and nitrogen for cyanobacteria growth is in agreement with the reports by Downing et al. (26), Reynolds (27), Rolland et al. (28), and Xie et al. (29). Increased concentration of copper salt and micronutrients solution (A5+CO) resulted in no growth promotion confirming Ruitter et al. work (30). According to antimicrobial test results, *Leptolyngbya* species have more antimicrobial effects against the Gram positive bacteria and *Candida* genera than the Gram negative or fungi; this could be due to hydrophobic property of Gram-negative bacteria outer membrane lipopolysaccharides. The results indicate that the increased antimicrobial activity and biomass production are reversely related.

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