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In vitro evaluation of antimicrobial activities from aqueous and methanolic extracts of cyanobacteria

Moein Safari[1]*, Salman Ahmady-Asbchin[2], Pantea Zamanifar[3]

[1] Department of Biology, Faculty of Basic Science, Ilam University, Ilam, Iran

[2] Department of Molecular and Cell Biology, Faculty of Basic Science, University of Mazandaran, Babolsar, Iran

[3] Department of Biology, Faculty of Basic Science, Islamic Azad University, Varamin-Pishva branch, Tehran, Iran

* Correspondence: Tel. +98-9367263245; E-mail: safari_moein@yahoo.com

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ABSTRACT: In this present study, antimicrobial activities of aqueous and methanolic extracts of cyanobacteria against some of fungi and pathogenic bacteria were investigated. Cyanobacteria strains *Fischerella ambigua* ISC67 and *Schizothrix vaginata* ISC108 were cultured in BG-11 medium. Extraction was performed by adding the solvent to cyanobacterial biomass and then filtering and drying of the mixture. The antimicrobial activity was evaluated by disc diffusion method and broth microdilution method was applied to determine the minimum inhibitory concentration. The results show that the aqueous and methanolic extracts of *F. ambigua* has a significant antimicrobial effect while, the tested extracts of *S. vaginata* was no significant antibacterial and antifungal activity. Highest antibacterial activity from aqueous extract of *F. ambigua* was against *S. aureus* (PTCC 1112) which the average zone diameter around it was 33.33 mm. The antibacterial effect of aqueous extracts against Gram-positive bacteria was more than Gram-negative bacteria significantly. Antifungal activity showed that methanolic extract of *F. ambigua* have significant antifungal activity. Minimum inhibitory concentration of active extract against most tested bacterial and fungal was 125 mg/ml. The present study has proved that the aqueous and methanolic extracts of *F. ambigua* possessed strong antibacterial and antifungal properties against the pathogenic microorganism. Therefore, cyanobacteria can be a rich source for natural products with antimicrobial activity.

Keywords: Cyanobacteria; Antimicrobial activity; Disc diffusion; Broth microdilution; *Staphylococcus aureus*.

1. INTRODUCTION

Natural products play a great role in the discovery and development of new drug. These products have been isolated from a wide variety of natural sources and tested for various biological activities [1]. The screening of extracts or isolated compounds from different natural sources is a common way to discover biological active metabolites. In such research activities microalgae like cyanobacteria were found to be a rich source for various products of commercial, pharmaceutical or toxicological interest: primary metabolites, such as proteins, fatty acids, vitamins or pigments, various secondary metabolites with different bioactivities

(antifungal, antiviral, antibiotic and other) or cyanotoxins like the hepatotoxic microcystins and nodularins or the neurotoxic anatoxins and saxitoxins were isolated from cyanobacteria [2]. Cyanobacteria are the Gram-negative photosynthetic bacteria, morphologically very diverse, and inhabiting a multiplicity of environments worldwide [3]. Also, cyanobacteria adapt to our changing environmental conditions, and thrive in water sources impacted by development and climate change [4]. These microorganisms are known to produce metabolites with diverse biological activities such as antibacterial, antifungal, antiviral, anticancer, algaecide activities [5-7]. Screening of cyanobacteria for antimicrobial and other pharmacologically active compounds, has received ever-increasing interest as a potential source for new drugs [8]. Cyanobacteria from local habitats seem to be a source of potential new active substances that could contribute to reduction of the number of bacteria, fungi, viruses and other microorganisms [9].

The increasing clinical incidence of antibiotic-resistant bacteria is a major global health care issue [10]. Resistance to antibiotics and drugs in pathogenic bacteria has progressively become a clinical-annoyance, since patients admitted to hospitals carry drug resistant bacteria, which have nosocomial spreads [11]. This has led to a development of natural antimicrobials compounds [12]. Cyanobacteria secondary metabolites can be a good candidate for inhibition of many pathogenic bacteria. Recently, researchers at several institutions have started to screen extracts of cyanobacteria for various biological activities and about 4000 species of cyanobacteria have been screened for novel biological active compounds. This indicates that cyanobacteria are a rich source of potentially useful natural products [13]. Cyanobacteria of Iran have not yet been many studied for antimicrobial activity and little work has been done to screen cyanobacteria to their production of bioactive compounds. The general objective of this study was evaluation of antibacterial and antifungal activity of aqueous and methanolic extract of cyanobacteria against some of fungi and pathogenic bacteria.

2. MATERIAL AND METHODS

2.1. Cultivation of cyanobacteria

In this experimental study, the cyanobacteria strains *Fischerella ambigua* ISC67 and *Schizothrix vaginata* ISC108 were obtained from the algal culture collection of research institute of applied science, ACECR, Tehran, Iran. These microorganisms were cultured in a 500 ml flask containing 150 ml of BG-11 medium without shaking, for 30 days. The incubation temperature was 28°C ± 2 and illumination at 3000 lux with a white continuous light [5].

2.2. Extraction procedure

Extraction was carried out using Val method [14]. Briefly, at the stationary phase of growth (30 days), cultures were harvested by centrifugation at 5000 rpm for 15 min. The supernatant was collected and the culture pellet was extracted with 30 ml of methanol, with shaking 150 rpm for 20 min. The culture supernatants and solvent extracts were dried under reduced pressure at 60°C and were stored at -20°C for further studies. The aqueous and methanolic extracts obtained from cyanobacteria were analyzed for the presence of antimicrobial activity.

2.3. Preparation of bacterial and fungal strains

Five Gram-positive phatogenic bacteria including; *Staphylococcus aureus* (PTCC 1112), *Staphylococcus epidermidis* (PTCC 1114), *Bacillus cereus* (PTCC 1247), *Enterococcus faecalis* (PTCC 1237), *Streptococcus pyogenes* (PTCC 1447) and five Gram-negative phatogenic bacteria including; *Escherichia coli* (PTCC 1338), *Pseudomonas aeruginosa* (PTCC 1430), *Proteus vulgaris* (PTCC 1312), *Salmonella paratyphi*

B (PTCC 1231) and *Klebsiella pneumonia* (PTCC 1053) obtained from the Persian Type Culture Collection, Tehran, Iran (PTCC). The plant fungal pathogen species including; *Fusarium oxysporum*, *Rhynchosporium secalis*, *Fusarium solani* and *Botrytis cinerea* obtained from Laboratory of Plant Pathology, University of Ilam, Iran. Bacterial strains were inoculated on nutrient broth and incubated at 37°C for 24 h. The fungal strains were inoculated on potato dextrose broth and incubated at 30°C for 5 days. To antimicrobial test all strains of bacteria and fungal were adjusted to 0.5 Mc-Farland standard by optical density (OD) method at 620 nm (1 x 10^8 cfu/ml for bacteria and 1 x 10^6 cfu/ml for fungal) [15].

2.4. Antibacterial and antifungal bioassay

The antibacterial activities of cyanobacteria extracts was assayed by agar disc diffusion method [16]. Briefly, different concentrations of each dry extract, (125, 250, 500 and 1000 mg/ml), prepared in dimethyl sulfoxide (DMSO). Bacterial and fungal suspensions that were adjusted to 0.5 Mc-Farland standard individually were swabbed on Muller-Hinton agar and potato dextrose agar (PDA) plates at three directions according to CLSI [17]. Then, filter paper discs (6.0 mm) were saturated with each extracts, dried under laminar air flow and placed on the Muller-Hinton agar plate for bacteria and potato dextrose agar (PDA) for fungi. Plates were incubated at 37°C for a period of 18 to 24 h for bacteria and at 25°C for a period of 24 to 48 h for fungi. Discs treated with 50 µl DMSO was used as negative controls and commonly used antibiotics was used as positive controls. The aqueous and methanolic extracts containing antibacterial components produced distinct, clear, circular zones of inhibition around the discs and the diameters of clear zones were determined and used as an indication of antibacterial activity.

2.5. Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration of active crude extracts was determined by broth microdilution method [18]. The test was performed using polystyrene 96well plates. Two fold serial dilutions of all active extracts were made in Cation-Adjusted Muller-Hinton Broth ranging from 1000 to 125 mg/ml. Each inoculum was prepared with 50 µl Muller-Hinton Broth for bacteria and potato dextrose broth for fungi, then 50 µl of the diluted active extracts and 50 µl of the prepared bacterial and fungal suspension was added for the assay. After 24 h of incubation at 37°C for bacteria and 48 h of incubation at 25°C for fungi. MIC was defined as the lowest concentration of the extracts at which the microorganisms showed no visible growth.

2.6. Statistical analysis

All the experiments were done in triplicate. The SPSS software version 20 was used for data analysis. The results are expressed as the mean ±SD of three experiments. The experimental data obtained were analyzed for multiple comparisons using one-way ANOVA and when the results were significant, the Duncan test was also used.

3. RESULTS

In this study, the antibacterial and antifungal activities of two strains of cyanobacteria, *Fischerella ambigua* ISC67 and *Schizothrix vaginata* ISC108, were determined by disc diffusion method. The result show that from the tested extracts, the aqueous and methanolic extracts of *F. ambigua* have a significant antimicrobial effect while, the tested extracts of *S. vaginata* has no significant antibacterial and antifungal activity (p-value $\leq$ 0.01). The results of aqueous and methanolic extracts of *F. ambigua* that demonstrated antibacterial activity are shown in Table 1. As shown in Table 1, methanolic extract of *F. ambigua* has no considerable effect against tested bacteria, however, this extract had significant effect against *Bacillus cereus*

(PTCC 1247), *Proteus vulgaris* (PTCC 1312) and *Klebsiella pneumonia* (p-value ≤ 0.01). The results also clearly showed that aqueous extract of this cyanobacterium strains had significant antibacterial activity against most pathogenic bacteria, so that maximum antibacterial activity was against *Staphylococcus aureus* (PTCC 1112) which the average zone diameter around it was 33.33 mm (Figure 1). The results indicated that both extract had antibacterial activity against gram positive and gram negative bacteria. The antibacterial effect of aqueous extracts against gram-positive bacteria was more than gram-negative bacteria significantly (p-value ≤ 0.01), however, compression of antibacterial activity of methanolic extract against Gram-positive bacteria and Gram-negative bacteria was not significantly according to statistical analysis (p-value ≤ 0.02). Among the tested bacteria only two strains of the bacteria, *Bacillus cereus* (PTCC 1247) and *Proteus vulgaris* (PTCC 1312), were inhibited by both aqueous and methanolic extracts of *F. ambigua*. Also the result show that extracts obtained from *F. ambigua* has not considerable antibacterial activity against *Streptococcus pyogenes* (PTCC 1447), *Escherichia coli* (PTCC 1338), and *Pseudomonas aeruginosa* (PTCC 1430). Among the commonly used antibiotics, Penicillin was the minimum effective antibiotic against tested bacteria. Results indicated that antibacterial activity of aqueous and methanolic extracts of *F. ambigua* against some of bacteria was more than commonly used antibiotics. For example, the antibacterial activity of aqueous extract of *F. ambigua* against *S. aureus* (PTCC 1112) and *S. epidermidis* (PTCC 1114) and also antibacterial activity of methanolic extract of *F. ambigua* against *P. vulgaris* (PTCC 1312) were more than commonly used antibiotics against these bacteria.

The results indicated that the MICs value of aqueous and methanolic extracts against most sensitive pathogenic bacteria was 125 mg/ml, while it was 250 mg/ml against *P. vulgaris* (PTCC 1312) (Table 1). Evaluation of antifungal activity showed that methanolic extract of *F. ambigua* has significant antifungal activity against most pathogenic fungal (p-value ≤ 0.01), so that maximum antifungal activity was against *Rhynchosporium secalis* which the average zone diameter around it was 32.33 mm. The results also indicated that aqueous extract of *F. ambigua* has no considerable effect against tested fungal, however, this extract had significant effect against *Fusarium oxysporum* (Table 2). Minimum inhibitory concentration of aqueous and methanolic extracts of *F. ambigua* against pathogenic fungal has been shown that the MICs of active extract against tested fungal was 125 mg/ml, whereas it was 250 mg/ml against *Fusarium solani* affected by extract (Table 2).

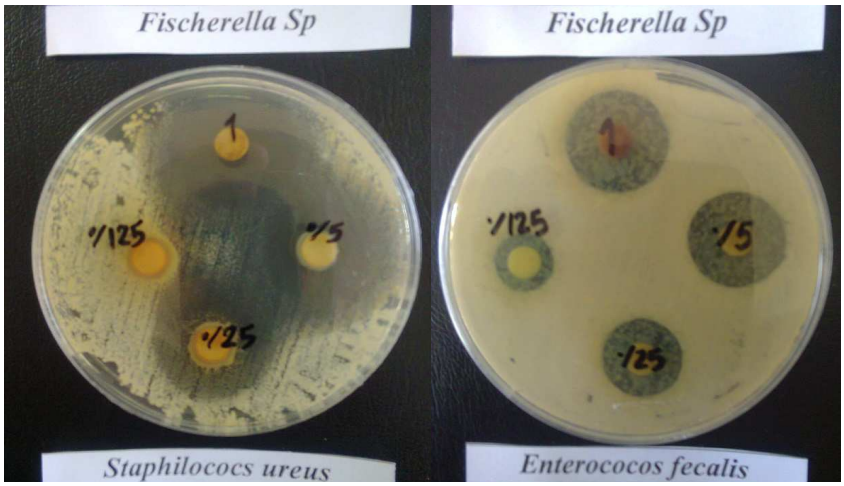

Figure 1. Inhibitory effects of different concentrations of aqueous extracts from *F. ambigua* against *Staphylococcus aureus* (PTCC 1112) and *Enterococcus faecalis* (PTCC1237).

Table 1. The average diameter of inhibition zone and standard deviation of antibiotics and aqueous and methanol extract of *Fischerella ambigua* against pathogen bacteria (mm).

		***Staphylococcus aureus* (PTCC 1112)**	***Staphylococcus epidermidis* (PTCC 1114)**	***Enterococcus faecalis* (PTCC 1237)**	***Bacillus cereus* (PTCC 1247)**	***Streptococcus pyogenes* (PTCC 1447)**	***Escherichia coli* (PTCC 1338)**	***Pseudomonas aeruginosa* (PTCC 1430)**	***Proteus vulgaris* (PTCC 1312)**	***Salmonella paratyphi***	***Klebsiella pneumoniae***
Gentamycin		28.67±1.15	10.67±0.57	25.33±0.57	21.67±1.52	23.33±1.52	13±1	19.33±0.57	20.33±0.57	21.33±0.57	22±0
Streptomycin		23.33±0.57	10.33±0.57	20.67±0.57	18.33±0.57	12±0	14.67±1.15	21±1.73	10±0	15±0	15.68±0.57
Chloramphenicol		30.33±0.57	31.33±0.57	23.67±0.57	23.67±2.3	15.67±0.57	23.33±0.57	19.33±0.57	16.33±0.57	8.33±0.57	30.33±0.57
Nalidixic acid		15.33±0.57	R*	16.67±1.15	19.33±0.57	16.33±1.52	R	12.33±0.57	14.33±0.57	25.33±1.15	23.67±0.57
Penicillin		27.33±0.57	R	16±1	R	R	R	R	R	R	R
Methanolic extract of *Fischerella* sp. (mg/ml)	1000	6±0	6±0	6±0	20.67±1.15	6±0	6±0	6.33±0.57	27.33±0.57	6±0	23.33±0.57
	500	6±0	6±0	6±0	19±0	6±0	6±0	6±0	23.67±0.57	6±0	21±1
	250	6±0	6±0	6±0	18±1	6±0	6±0	6±0	20.67±0.57	6±0	20±0
	125	6±0	6±0	6±0	14.33±0.57	6±0	6±0	6±0	19.33±0.57	6±0	17.33±0.57
	MIC**	ND	ND	ND	125	ND	ND	ND	125	ND	125
Aqueous extracts of *Fischerella* sp. (mg/ml)	1000	33.33±0.57	32.33±0.57	24±1	20.33±0.57	6±0	6±0	6.33±0.57	19.33±0.57	21±1	6±0
	500	30±0	27.33±0.57	21.33±0.57	19.33±0.57	6±0	6±0	6±0	16.67±0.57	16.33±0.57	6±0
	250	28.67±1.15	25±1	19.67±0.57	17.33±0.57	6±0	6±0	6±0	13.67±1.15	12.67±0.57	6±0
	125	22±0	17.33±0.57	13.67±0.57	12.33±0.57	6±0	6±0	6±0	8±0	10.33±0.57	6±0
	MIC	125	125	125	125	ND***	ND	ND	250	125	ND

* R; Resistant

**MIC; Minimum inhibitory concentration value expressed in mg/ml

***ND; Not determinated

Table 2. The average diameter of inhibition zone and standard deviation of antibiotic and aqueous and methanol extract of *Fischerella ambigua* against pathogenic fungi (mm).

		Fusarium oxysporum	***Rhynchosporium secalis***	***Fusarium solani***	***Botrytis cinerea***
Nystatin		22.67±1.15	35.67±0.57	17±0	28±1
Methanolic extract of *Fischerella* sp. (mg/ml)	1000	15.33±0.57	32.33±0.57	14.33±0.57	20±1
	500	14.67±1.15	25.67±0.57	12.67±0.57	18.33±0.57
	250	13.33±0.57	22.67±0.57	11.67±1.15	15.67±0.57
	125	11.33±0.57	18.67±0.57	9.33±0.57	14.33±0.57
	MIC*	125	125	125	125
Aqueous extracts of *Fischerella* sp. (mg/ml)	1000	10.67±0.57	6±0	6±0	6±0
	500	9.67±0.57	6±0	6±0	6±0
	250	8.33±0.57	6±0	6±0	6±0
	125	6±0	6±0	6±0	6±0
	MIC	250	ND**	ND	ND

*MIC; Minimum inhibitory concentration value expressed in mg/ml

**ND; Not determinated

4. DISCUSSION

In this present study, cyanobacterium *F. ambigua* shown antibacterial and antifungal activity against pathogenic microorganism, while *S. vaginata* was no significant antimicrobial activity. Cyanobacteria are known to have wide variety of secondary metabolites with antimicrobial properties. Antibacterial and antifungal effects of curd extracts from many cyanobacteria strains, such as *Fischerella* sp., *Oscillatoria angustissima*, *Spirulina platensis*, *Synechococcus* and other species have been reported [19, 20]. In the resent years, several reports have been published of antibacterial compounds isolated from cyanobacteria, such examples as ambiguine, isonitriles, aoscomin, comnostins A-E, norharmane, lyngbyazothrins, and carbamidocyclophanes [2]. Terpenoids and phenolic compounds were thought to inhibit microorganisms by membrane disruption [21]. Also, flavonoid activity is probably due to their ability to complex with extracellular and soluble proteins and with bacterial cell wall which inhibition of the porin on the cell membrane and by alteration of the membrane permeability leads to the cell destruction [22]. Thus, it has been suggested that the antimicrobial activity of these extracts could be attributed to the high contents of this natural compounds such as terpenoids, phenolics, flanonoeids, ambiguine and etc.

Susceptibility of the tested microorganisms to the cyanobacteria extracts was different. The susceptibility varied according to strains and species which were also in agreement with our results. The extract with low activity against a particular organism gave high MIC, while the highly reactive extract gave low MIC value. The MIC technique is used to evaluate the efficiency of antimicrobial agents [18]. The aqueous extract of *F. ambigua* have significant antibacterial activity against most pathogenic bacteria, These results was corresponded with Madhumathi et al. [23] and Chauhan et al. [24], which found that some cyanobacteria had high antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermis*, *Salmonella paratyphi*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Streptococcus enteritidis*, *Bacillus cereus* and *Proteus vulgaris*. According to the result, aqueous

extracts of *F. ambigua* was most effective against Gram-positive bacteria as compared to Gram-negative strains, These results was corresponded with Safari et al. [19] and Madhumathi et al. [23] that showed the effective of extracts from cyanobacteria against gram positive bacteria was more than gram negative bacteria. A possible explanation for these observations may be attributed to the significant differences in the outer layers of gram- negative and positive bacteria. Gram negative bacteria possess an outer membrane and a unique periplasmic space not found in Gram positive bacteria. The resistance of Gram negative bacteria towards antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide molecules, presenting a barrier for the penetration of numerous antibiotic molecules. The membrane is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside [25]. Antifungal activity of *F. ambigua* indicated that methanol was best solvent for extracting antifungal compound from this microorganism, so that methanolic extract was effective against even four tested pathogenic fungal. This result has corresponded with previously study that indicated methanol is best solvent for extracting antifungal compound from cyanobacteria [26], but has not corresponded with Ghasemi et al. [27] investigations. Smithka et al. [28] reported that ambiguine produced by cyanobacteria possess antifungal activity and probably antifungal activity of *F. ambigua* methanolic extract in this study due to their ability to production of ambiguine. A variety of solvents (water, methanol, ethanol, acetone, petroleum ether, and hexane) used for extracting the antibacterial agents and methanol was best than other solvents [29]. In contrast to the Challouf et al. [29], in this study, methanol were not the best solvents for extracting the antibacterial agents from *Fischerella* sp. and culture supernatants (aqueous extract) gave the highest inhibition zone than other solvents that these results go in harmony with Ghasemi et al. [27]. However, in present study it was found that methanol was best solvent for extracting of antifungal compound.

5. CONCLUSION

The present study has proved that aqueous and methanolic extracts of *F. ambigua* possessed strong antibacterial and antifungal properties against the pathogenic microorganism. Antibacterial activity of aqueous extract of *F. ambigua* was more than methanolic extract and it can be concluded that methanol were not the best solvents for extracting the antibacterial agents from cyanobacteria. According to our knowledge, this is the first study about antimicrobial activity of *Fischerella ambigua* ISC67 and *Schizothrix vaginata* ISC108 and antifungal activity of these cyanobacteria. According to the results of this study, cyanobacteria extract can be a rich source for natural products with antimicrobial activity to development of new drug. Improvement knowledge of the composition, analysis, and the properties of these cyanobacteria extract with respect to antimicrobial compounds would encourage emphasis for search of drug molecules. Also Further studies are needed to isolate the active principles and bioactive compounds from the extract and to elucidate the exact mechanism of action of the free radical scavenging effect and antibacterial activity.

Conflict of Interest: The authors declare no conflict of interest.

Authors Contributions: MS: conceived and designed the experiments, carried out the experiment, processed the experimental data, performed the analysis, wrote the manuscript, contributed to the interpretation of the results. SA-A: involved in planning and supervised the work, contributed to the interpretation of the results, other contribution. PZ: contributed to sample preparation, wrote the manuscript, drafted the manuscript and designed the figures. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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