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ANTIFUNGAL AND ANTIBACTERIAL ACTIVITY OF PADDY-FIELDS CYANOBACTERIA FROM THE NORTH OF IRAN

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Abstract

Antifungal and antibacterial activity of some heterocystous cyanobacteria from paddy-fields in the north of Iran was studied. Soil samples were collected from paddy-fields of Gillan, Mazandaran and Golestan Provinces and cyanobacteria were isolated. Supernatants, methanolic and hexane extracts from biomass of 150 strains of cyanobacteria were isolated and screened against six strains of bacteria and eight strains of fungi. Methanolic extracts and culture supernatants of 21 strains of cyanobacteria exhibited significant antibacterial effect and 13 strains showed antifungal effect. No antimicrobial activity was detected in the hexane extracts and no extract inhibited the growth of *E. coli.* According to these results, it is concluded that strains of Stigonemataceae including *Fischerella* and *Stigonema* species, seem to be more potential for producing antimicrobial substances than other strains.

Keywords: Antimicrobial active compound; Blue-green algae; Cyanobacteria; Paddy-field; *Fischerella*; *Stigonema*

Introduction

Cyanobacteria are a very old group of organisms and represent relics of the oldest photoautotrophic vegetation in the world that occur in freshwater, marine and terrestrial habitats [26]. Cyanobacteria have drawn much attention as prospective and rich sources of biologically active constituents and have been identified as one of the most promising groups of organisms to be able of producing bioactive compounds [10,37]. Cyanobacteria are known to produce metabolites with diverse biological activities such as antibacterial [4,18,19], antifungal [20,30], antiviral [32], anticancer [13,23], antiplasmodial, [29], algicide [28], antiplatelet aggregation [36] and immunosuppressive [22] activities.

Screening of cyanobacteria for antibiotics and other pharmacologically active compounds, has received ever-increasing interest as a potential source for new drugs [4,10,27,37]. 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 [25].

Cyanobacteria of Iran have not yet been studied for antimicrobial activity and little work has been done to

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screen cyanobacteria isolated from paddy-fields with regard to their production of bioactive compounds. In order to find the potential of cyanobacteria for production of antibacterial and antifungal compounds in rice-fields of north of Iran, 150 strains of heterocystous cyanobacteria were isolated and their potency were studied. The results are presented in this paper.

Materials and Methods

Isolation of Cyanobacteria

Soil samples were collected from different paddy-fields in Golestan, Mazandaran and Gillan provinces. Soil samples in laboratory were cultured directly in N-free BG-11 media [3], after colonization, cyanobacteria were transferred to the same medium. Unialgal cultures were prepared using subculturing methods [21]. Each isolated cyanobacterium was 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.

Preparation of Supernatant and Cell Extracts

The cultures were harvested after 30 days by centrifugation at 5000 rpm for 15 min. The aqueous supernatant was collected and the algal pellet was extracted with 15 ml of methanol followed by 15 ml hexane, with shaking for 20 min. The culture supernatants and solvent extracts were dried under reduced pressure at 40°C and were stored at −10°C for further studies.

Antibacterial and Antifungal Bioassay

The following bacteria and fungi were used as test organisms: *Staphylococcus aureus* (PTCC 1112), *Staphylococcus epidermidis* (PTCC 1114), *Staphylococcus haemolyticus* (ATCC 29970), *Escherichia coli* (PTCC 1047), *Shigella sonnei* (PTCC 1235), *Proteus vulgaris* (PTCC 1312), *Candida krusei* (ATCC 44507), *Candida kefyr* (ATCC 38296), *Candida albicans* (ATCC 14053), *Candida guilliermondii* (IF 00838), *Cryptococcus neoformans* (PLM 589), *Aspergillus niger* (PLM 1140), *Aspergillus fumigatus* (ATCC 13073) and *Aspergillus fumigatus* (PLM 712).

Dried extracts and supernatants were dissolved in 4 ml of their extraction solvent, and antimicrobial activity was determined by the disc method. Filter paper discs (6.4 mm) were saturated with 50 µl of the test solution, dried under laminar air flow and placed on the Muller-Hinton agar plate for bacteria and Saubouraud's dextrose agar plate for fungi, which had been inoculated with a lawn of the test microorganisms. Plates were incubated at 37°C, for a period of 18-24 h for bacteria and at 25°C, for 24-48 h for fungi. Discs treated with 50 µl methanol or hexane were used as negative controls and gentamycine and nystatin discs were used (10 µg) as positive controls. The extracts and supernatants containing antibacterial and antifungal 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 and antifungal activity.

The following formula was used for comparison of the antimicrobial activity of the sample with that of the standard (antimicrobial index):

$$\frac{\text{inhibition zone of sample}}{\text{inhibition zone of the standard}} \times 100$$

Identification of Cyanobacteria

Identification was done using morphological variation studies and taxonomical approaches according to Anagnostidis and Komarek [1], Desikachary [7], Prescott [35] and Anand [2].

Semipermanent slides were prepared from each specimen and were coded and preserved in algal herbarium of Reaserch Institute of Applied Science, Shahid Beheshti University.

Results

The results of culture supernatants and methanolic extracts of the isolated cyanobacteria that demonstrated antibacterial and antifungal activity are shown in Tables 1, 2, 3, and 4. Supernatant and methanolic extract of 21 strains from the 150 cyanobacteria strains, showed significant antibacterial activity against at least one Gram-positive or Gram-negative bacterium. Thirteen of them were identified as *Fischerella* species, where 5 *Stigonema* species, two *Nostoc* species and one *Hapalosiphon* species, were also among those showing antibacterial activity.

Supernatant and methanolic extract of 13 isolated strains showed antifungal activity against at least one of the fungal strains. Seven of them were *Fischerella* species, 4 were *Stigonema* species, and one strain of each of the *Nostoc* and *Hapalosiphon* species were also identified.

No antimicrobial activity was detected in the hexane extracts and none of the methanolic extracts or culture supernatants showed antibacterial activity against *E. coli*.

Table 1. Antibacterial activity of cyanobacteria supernatants against Gram-positive and Gram-negative bacteria as presented by inhibition zone diameter (in mm) and antimicrobial index (in parentheses)

Sample	*S. epidermidis* (PTCC 1114)	*S. aureus* (PTCC 1112)	*S. haemolyticus* (ATCC 29970)	*P. vulgaris* (PTCC 1312)	*S. sonnei* (PTCC 1325)	*E. coli* (PTCC 1047)
Control (gentamycine)	16 (100)	14 (100)	15 (100)	20 (100)	20 (100)	10
Fischerella sp. YG 0077	12 (75)	14 (100)	16 (106)	11 (55)	10 (50)	–
Stigonema sp. YG 0841	12 (75)	12 (85)	14 (93)	10 (50)	10 (50)	–
Stigonema sp. YG 0104	14 (87)	12 (85)	16 (106)	13 (65)	12 (60)	–
Fischerella sp. YG 0050	14 (87)	12 (85)	12 (80)	14 (70)	14 (70)	–
Fischerella sp. YG 0842	10 (62)	12 (85)	13 (86)	12 (60)	11(55)	–
Stigonema sp. YG 0117	14 (87)	12 (85)	12 (80)	14 (70)	12 (60)	–
Fischerella sp. YG 0115	12 (75)	13 (92)	12 (80)	12 (60)	12 (60)	–
Fischerella sp. YG 0147	14 (87)	13 (92)	12 (80)	15 (75)	13 (65)	–
Fischerella sp. YG 0139	14 (87)	12 (85)	18 (120)	13 (65)	14 (70)	–
Nostoc sp. YG 0159	9 (56)	8 (57)	–	–	8 (40)	–
Fischerella sp. YG 0118	14 (87)	13 (92)	16 (106)	12 (60)	14 (70)	–
Fischerella sp. YG 0070	16 (100)	12 (85)	18 (120)	14 (70)	14 (70)	–
Fischerella sp. YG 0137	10 (62)	12 (85)	16 (106)	14 (70)	13 (65)	–
Stigonema sp. YG 0100	14 (87)	10 (71)	16 (106)	10 (50)	12 (60)	–
Fischerella sp. YG 0116	15 (93)	12 (85)	12 (80)	13 (65)	12 (60)	–
Fischerella sp. YG 0052	14 (87)	12 (85)	16 (106)	12 (60)	12 (60)	–
Fischerella sp. YG 0148	12 (75)	14 (100)	14 (93)	14 (70)	14 (70)	–
Hapalosiphon sp. YG 0072	7.5 (46)	–	–	–	7 (35)	–
Fischerella sp. YG 0047	12 (75)	12 (85)	20 (133)	14 (70)	12 (60)	–
Nostoc sp. YG 0156	8.5 (53)	18 (128)	–	–	7 (35)	–
Stigonema sp. YG 0146	14 (87)	12 (85)	16 (106)	12 (60)	12 (60)	–

Table 2. Antibacterial activity of cyanobacteria methanolic extract against Gram-positive and Gram-negative bacteria as presented by inhibition zone diameter (in mm) and antimicrobial index (in parentheses)

Sample	*S. epidermidis* (PTCC 1114)	*S. aureus* (PTCC 1112)	*S. haemolyticus* (ATCC 29970)	*P. vulgaris* (PTCC 1312)	*S. sonnei* (PTCC 1325)	*E. coli* (PTCC 1047)
Control (gentamycine)	16 (100)	14 (100)	15 (100)	20 (100)	20 (100)	10
Fischerella sp. YG 0077	12 (75)	12 (85)	12 (80)	13 (65)	13 (65)	–
Stigonema sp. YG 0841	10 (62)	10 (71)	10 (66)	14 (70)	10 (50)	–
Stigonema sp. YG 0104	10 (62)	12 (85)	12 (80)	12 (60)	16 (80)	–
Fischerella sp. YG 0050	16 (100)	10 (71)	13 (86)	10 (50)	16 (80)	–
Fischerella sp. YG 0842	12 (75)	10 (71)	12 (80)	12 (60)	12 (60)	–
Stigonema sp. YG 0117	18 (112)	10 (71)	10 (66)	8.(40))	10 (50)	–
Fischerella sp. YG 0115	12 (75)	12 (85)	11 (73)	14 (70)	14 (70)	–
Fischerella sp. YG 0147	16 (100)	12 (85)	12 (80)	14 (70)	12 (60)	–
Fischerella sp. YG 0139	12 (75)	14 (100)	12 (80)	12 (60)	12 (60)	–
Nostoc sp. YG 0159	8 (50)	7 (50)	7 (46)	8.(40)	8 (40)	–
Fischerella sp. YG 0118	14 (87)	12 (85)	14 (93)	14 (70)	12 (60)	–
Fischerella sp. YG 0070	12 (75)	10 (71)	12 (80)	15 (75)	11 (55)	–
Fischerella sp. YG 0137	9 (56)	7 (50)	10 (66)	12 (60)	10 (50)	–
Stigonema sp. YG 0100	9 (56)	10 (71)	7 (46)	14(70))	7 (35)	–
Fischerella sp. YG 0116	8 (50)	8 (57)	8 (53)	12 (60)	12 (60)	–
Fischerella sp. YG 0052	12 (75)	10 (71)	14 (93)	14 (70)	10 (50)	–
Fischerella sp. YG 0148	14 (87)	8 (57)	14 (93)	14 (70)	13 (65)	–
Hapalosiphon sp. YG 0072	7 (43)	–	–	–	–	–
Fischerella sp. YG 0047	14 (87)	10 (71)	12 (80)	12 (60)	13 (65)	–
Nostoc sp. YG 0156	8 (50)	7 (50)	8 (53)	7 (35)	8 (40)	–
Stigonema sp. YG 0146	12 (75)	10 (71)	12 (80)	12 (60)	12 (60)	–

Table 3. Antifungal activity of cyanobacteria supernatants as presented by inhibition zone diameter (in mm), and antimicrobial index (in parentheses)

Sample	*C. krusei* (ATCC44507)	*C. guilliermondii* (IF00838)	*C. albicans* (ATCC14053)	*A. niger* (PLM 1140)	*C. kefyr* (ATCC13073)	*A. fumigatus* (PLM712)	*A. fumigatus* (ATCC13073)	*C. neoformans* (PLM589)
Control (nystatin)	12 (100)	12 (100)	14 (100)	12	16 (100)	13	12 (100)	16 (100)
Fischerella sp. YG 0077	–	–	–	–	7 (43)	–	–	10 (62)
Stigonema sp. YG 0841	–	–	–	–	7 (43)	–	–	–
Fischerella sp. YGS0050	10 (83)	–	8 (57)	7 (58)	14 (87)	8 (61)	8 (66)	15 (93)
Fischerella sp. YG0842	–	–	–	–	10 (62)	–	–	8 (50)
Stigonema sp. YG 0117	–	–	–	–	8 (50)	–	–	7 (43)
Fischerella sp. YG 0147	8 (66)	–	7 (50)	7 (58)	10 (62)	8 (61)	–	12 (75)
Fischerella sp. YG 0139	7 (58)	–	7 (50)	–	7 (43)	–	8 (66)	12 (75)
Stigonema sp. YG 0100	–	8 (66)	7 (50)	–	–	–	–	8 (50)
Fischerella sp. YG0148	8 (66)	–	7 (50)	–	8 (50)	8 (61)	–	10 (62)
Hapalosiphon sp. YG 0072	10 (83)	–	–	–	11 (68)	–	–	12 (75)
Fischerella sp. YG 0047	8 (66)	–	–	–	8 (50)	–	–	12 (75)
Nostoc sp. YG 0156	10 (83)	–	–	–	9 (56)	–	–	10 (62)
Stigonema sp. YG 0146	8 (66)	7 (58)	7 (50)	7 (58)	12 (75)	8 (61)	–	8 (50)

Table 4. Antifungal activity of cyanobacteria methanolic extracts as presented by inhibition zone diameter (in mm), and antimicrobial index (in parentheses)

Sample	*C. krusei* (ATCC44507)	*C. guilliermondii* (IF00838)	*C. albicans* (ATCC14053)	*A. niger* (PLM 1140)	*C. kefyr* (ATCC13073)	*A. fumigatus* (PLM712)	*A. fumigatus* (ATCC13073)	*C. neoformans* (PLM589)
Control (nystatin)	12 (100)	12 (100)	14 (100)	12 (100)	16 (100)	13 (100)	12 (100)	16 (100)
Fischerella sp. YG 0077	–	–	–	–	7 (43)	–	–	9 (56)
Stigonema sp. YG 0841	–	–	–	–	10 (62)	–	–	10 (62)
Fischerella sp. YGS0050	7 (58)	–	8 (57)	8 (66)	12 (75)	8 (61)	7 (58)	13 (81)
Fischerella sp. YG0842	–	–	–	–	7 (43)	–	–	10 (62)
Stigonema sp. YG 0117	7 (58)	–	7 (50)	–	8 (50)	–	–	–
Fischerella sp. YG 0147	7 (58)	–	7 (50)	7 (58)	8 (50)	7 (53)	–	10 (62)
Fischerella sp. YG 0139	7 (58)	–	–	–	–	–	–	8 (50)
Stigonema sp. YG 0100	–	7 (58)	8 (57)	–	10 (62)	–	–	10 (62)
Fischerella sp. YG0148	7 (58)	–	7 (50)	–	–	–	–	–
Hapalosiphon sp. YG 0072	8 (66)	–	–	–	8 (50)	–	–	10 (62)
Fischerella sp. YG 0047	7 (58)	–	–	–	8 (50)	7 (53)	7 (58)	10 (62)
Nostoc sp. YG 0156	7 (58)	–	–	–	8 (50)	–	–	10 (62)
Stigonema sp. YG 0146	8 (66)	7 (58)	–	7 (58)	8 (50)	–	7 (58)	10 (62)

Results showed that antibacterial and antifungal activity were seen predominantly from the algal family Stigonemataceae and *Fischerella* and *Stigonema* genera.

Discussion

The cyanobacteria such as *Fischerella ambigua* [9], *Fischerella musciola* [14], *Nostoc commune* [18], *Scytonema hofmanni* [33], *Hapalosiphon fontinalis* [24], *Anabaena* spp. [12], *Nostoc spongiaeforme* [15], *Microcystis aeruginosa* [17], *Phormidium* sp. [10], have been reported as the main cyanobacteria to produce antimicrobial substances.

Screening efforts aimed to identify antimicrobial agents in cyanobacteria have revealed several promising lead compounds. Some of these substances identified including Nostocyclyne A [34], Nostofungicidine [20], Kawaguchipeptin B [17], Nostocin A [15], Ambigol A and B [9], Hapalindoles [24] and Scytophycins [16].

Most of the studies have only done as *in vitro* assays and, it is likely that most of these compounds will have little or no clinical application as they are either too toxic or inactive *in vivo* [4]. They may however serve as useful lead compounds for synthesis of antibiotics or may find application in agriculture. For example Tjipanazoles isolated from the cyanobacterium, *Tolypothrix tjipanensis*, showed appreciable fungicidal activity against rice blast and leaf rust wheat infections [4].

A few studies have been done to screen cyanobacteria for production of antimicrobial substances from paddy-fields. Possibly the synthesis of highly active toxin is a defence option of cyanobacteria in these environments against other organisms like bacteria, fungi, viruses and eukaryotic microalgae [25].

In one study, the culture media of cyanobacteria belonging to Nostaceae, Microchaetaceae and Scytonematacaea isolated from the Argentinian paddy-fields, were found to be active against *S. aureus* and *Candida albicans* [6].

In another study, it was shown that cyanobacteria from the paddy-fields of northern Thailand produce bioactive substances with antibiotic activity against *Bacillus subtilis* [5].

Cyanobacteria of Iran have not yet been studied for antimicrobial activity and this screening program is among the first studies done for assessment of antibacterial and antifungal activity of Iranian paddy-field cyanobacteria.

In this investigation, out of 150 strains of cyanobacterial isolates, 21 showed significant *in vitro* antibacterial activity and 13 of them had antifungal effect.

The proportion of the isolates with antibacterial and antifungal activities were approximately 14% and 9%, respectively, which is comparable with those published earlier in other screening programs: 11% [11], 7% [31], and 10% [37].

As depicted in Tables 1, 2, 3, and 4, *Fischerella, Stigonema, Nostoc* and *Hapalosiphon* species produce bioactive substances which may have potential for antibacterial and antifungal activity.

A variety of solvents with different polarities, were used for the extraction of algal bioactive materials. No antimicrobial activity was detected in the hexane extracts. This is probably because of polar nature of the active components. It shows that the chance of finding antimicrobial activity is higher in culture supernatants and in methanolic extracts. The results indicated that all of the supernatants and methanolic extracts of *Fischerella* and *Stigonema* species had high activity against Gram-positive bacteria.

Although some of the cyanobacteria produce active compounds against Gram-negative bacteria such as *S. sonnei* and *P. vulgaris* but no activity was found against *E. coli*. Different results have been reported by other authors in this case. Falch *et al.* [9] and Hirata *et al.* [15] reported active compounds against *E. coli* in the petroleum ether fraction of *Fischerella ambigua* and supernatant of *Nostoc spongiaeforme*, respectively.

Antifungal activity assays showed a good activity against *C. krusei*, *C. kefyr* and *C. neoformans* and the minimum against *Aspergillus* spp.

Among the isolated cyanobacteria, *Hapalosiphon* species had the minimum activity against the test organisms.

Fischerella and *Stigonema* species from Stigonemataceae had the greatest frequency among the species that showing antibacterial and antifungal activity and exhibited the most prominent effect. The effect of antimicrobial activity of Stigonemataceae has been reported in other studies such as Patterson *et al*. [32], Falch *et al*. [9], and Smitka *et al*. [38].

Among all of the species studied in this investigation for antibacterial and antifungal activity, it seems *Stigonema* strains are being reported for the first time as producer of antibacterial substances.

The results of this work indicate that this group of organisms displays a potential that warrants further investigations.

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