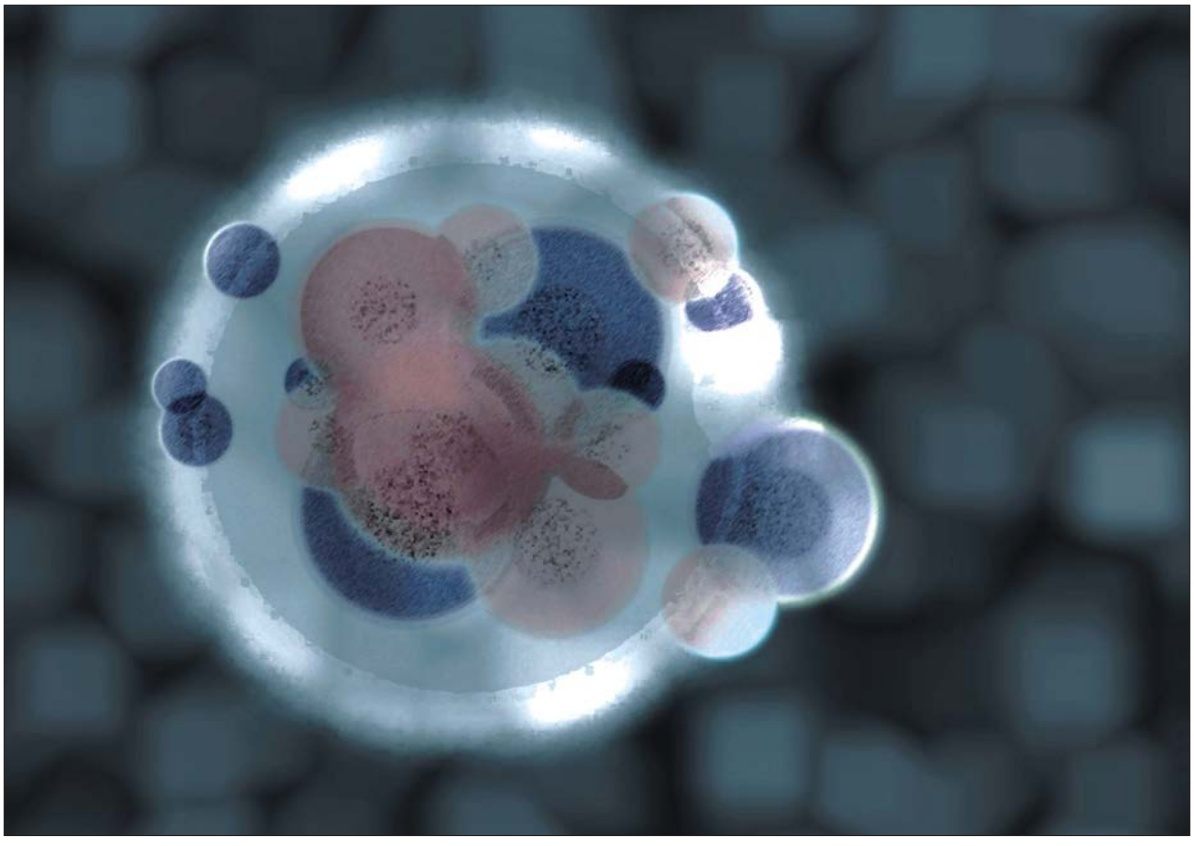

Journal of Biological Sciences

ISSN 1727-3048

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Journal of Biological Sciences 7 (6): 904-910, 2007
ISSN 1727-3048

Antifungal and Antibacterial Activity of the Microalgae Collected from Paddy Fields of Iran: Characterization of Antimicrobial Activity of *Chroococcus dispersus*

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Abstract: Antifungal and antibacterial activity of the microalgae from paddy fields in the south of Iran were studied. Soil samples were collected from paddy fields of Fars province and were cultured in BG11 medium. Supernatants, methanolic and hexane extracts from biomass of 60 strains of microalgae were isolated and screened against six strains of bacteria and four strains of fungi. The culture supernatants of 21 strains of microalgae and methanolic extracts of 8 strains exhibited significant antibacterial effect and 17 strains showed antifungal effect. No antimicrobial activity was detected in the hexane extracts and no methanolic extracts inhibited the growth of fungi. In present screening, *Chroococcus dispersus*, *Chlamydomonas reinhardtii* and *Chlorella vulgaris* appeared to be the most promising strains and it was shown that they excreted a broad spectrum of antimicrobial substances in the culture medium. Among all of the species studied in this investigation for antibacterial and antifungal activity, *Chroococcus dispersus* PTCC 1677 indicated widespread spectrum of antimicrobial activities. Bioautography and Bioassay-guided fraction of culture medium of the *Chroococcus dispersus* exhibited a polar substance in the culture medium as well.

Key words: *Chroococcus dispersus*, microalgae, antimicrobial, paddy fields

INTRODUCTION

Microalgae are a diverse group of photosynthetic microorganisms found in the soil and fresh water environments (Metting and Pyne, 1986). They are able to produce a wide range of active substances with antibacterial, antiviral, antifungal, enzyme inhibiting, immunostimulant, cytotoxic and antiplasmodial activities (Ghasemi *et al*., 2004). Most of the isolated substances belong to groups of polyketides, amides, alkaloids and peptides (Ghasemi *et al*., 2004). Pratt *et al*. (1944) were the first to isolate an antibacterial substance from *Chlorella*. A mixture of fatty acids, named chlorellin, exhibited inhibitory activity against both Gram positive and Gram negative bacteria (Pratt *et al*., 1944). Research to identify antimicrobial compounds produced by microalgae has recently received considerable attention as a new source of novel antimicrobial substances (Ghasemi *et al*., 2004).

A few studies have been done to screen microalgae for production of antimicrobial substances from paddy fields. Synthesis of highly active toxins is probably a defense strategy of microalgae in these environments against other organisms like bacteria, fungi and viruses (Mundt *et al*., 2001). In one study the culture media of cyanobacteria belonging to Nostocaceae, Microchaetaceae and Scytonemaceae isolated from the Argentine paddy fields were found to be active against *Staphylococcus aureus* and *Candida albicans* (De Caire *et al*., 1993). In another study it was shown that cyanobacteria from the paddy fields of Northern Thailand produce bioactive substances with antibiotic activity against *Bacillus subtilis* (Chetsumon *et al*., 1993).

In our previous studies we reported the antimicrobial activity of the microalgae of Northern Iran (Ghasemi *et al*., 2003). Also, a novel antimicrobial substance named parsiguine has been reported (Ghasemi *et al*., 2004). Therefore in a continuing investigation, the antimicrobial activities of various species and strains of terrestrial microalgae in the south of Iran were studied.

Antimicrobial activities were tested against Gram positive and negative bacteria and fungi. The

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antimicrobial activity of one strain, *Chroococcus dispersus*, with ability to produce broad-spectrum of antimicrobial substances was examined further. For this purpose bioassay-guided fractionation and bioautographic test for antimicrobial effect were employed. This species was identified as *Chroococcus dispersus* PTCC 1677 and deposited in Persian Type Culture Collection (PTTC) with code 1677.

MATERIALS AND METHODS

Isolation of microalgae: The microalgae were isolated during a screening program from soil samples collected from paddy fields of Marvdasht in the south of Iran (Fars province) from April to December 2004. Primary culturing was done in BG-11 medium. After colonization, pure cultures of the living specimens were prepared using subculturing with agar plate method in BG-11 medium (Allen, 1968). Preserved specimens were prepared and the living specimens were incubated in 50 mL-conical flasks. Constant illumination was used at 60 μE m^{-2} sec^{-1} intensity with white fluorescent lamps. Temperature was 28±2°C. The resulted cultures were identified based on morphology following taxonomic schemes of Desikachary (1959), Prescott (1962), Anand (1990) and Sant'Anna (2004). Semi-permanent slides were prepared from each specimen and were coded and preserved in Microalgal Culture Collection of Shiraz University of Medical Sciences.

Preparation of supernatant and cell extracts: The cultures were harvested after 15 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 for each extraction. The culture supernatants and solvent extracts were dried under reduced pressure and stored in -20°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, *Bacillus subtilis* PTCC 1023, *Escherichia coli* PTCC 1047, *Salmonella typhi* PTCC 1609, *Pseudomonas aeruginosa* PTCC 1074, *Candida kefyr* ATCC 38296, *Candida albicans* ATCC 14053, *Aspergillus niger* PLM 1140 and *Aspergillus fumigatus* PLM 712. Dried extracts and supernatants were dissolved in 4 mL of their extraction solvents and antimicrobial activity was determined by the disc method (Ghasemi *et al*., 2004). 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 Sabouraud's dextrose agar plate for fungi, which had been inoculated with a lawn of the test microorganisms. The plates were incubated at 37°C for a period of 18-24 h for bacteria and 25°C for 24-48 h for fungi. The discs treated with 50 μL methanol were used as negative controls and gentamicin, ampicillin and amphotericin B discs were used (10 μg) as positive controls. The extracts and supernatants containing antibacterial and antifungal components produce 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 (Ghasemi *et al*., 2004).

Bioassay-guided fractionation of the culture medium of *Chroococcus dispersus*: The antimicrobial activity of aqueous supernatant of *Chroococcus dispersus* 039 was examined further. The culture was harvested after 15 days by centrifugation at 5000 rpm for 15 min. The aqueous supernatant was collected and concentrated under reduced pressure. The dried supernatant was dissolved in methanol and loaded on a column (2×40 cm) of silica gel (Kieselgel 60; 35-70 mesh, Merck), equilibrated in $CHCl_3$. The column was eluted with chloroform using stepwise (20 mL) increasing of methanol (each step 10%). The fractions (each of 20 mL) were collected and the aliquots of each fraction were assayed for antimicrobial activity against *E. coli* PTCC 1047, *S. epidermidis* PTCC 1114 and *C. kefyr* ATCC 38296 in a soft-agar disc-diffusion assay.

The fractions that were active against the test organisms were assayed for more evaluation about the localization of antimicrobial compounds on TLC by bioautographic method (Betina, 1973). The active fractions were combined and loaded on silica gel plates for preparative TLC with methanol/chloroform (90:10). The bands with antimicrobial activity thus obtained were further purified by re-chromatography. The bands were visualized with UV light (254 and 366 nm). The quantity of the compound with antimicrobial activity was evaluated against the *S. epidermidis* PTCC 1114, *E. coli* PTCC 1047 and *C. kefyr* ATCC 38296, by a minimum inhibitory concentration (MIC) of μg mL^{-1}, according to procedure of Baron and Finegold (1990).

RESULTS

All of the microalgae for this study were isolated from paddy fields of Marvdasht in the south of Iran (Fars province) due to high distribution of paddy fields in these

regions. Sixty strains of microalgae were isolated and cultured in BG11 medium. The supernatants were isolated and the biomasses were extracted with methanol and hexane. The results of culture supernatants and methanolic extracts of the isolated microalgae that demonstrated antibacterial and antifungal activity are shown in Table 1-3. Supernatant and methanolic extract of 21 strains from the 60 microalgal strains showed significant antibacterial activity against at least one Gram-positive or Gram-negative bacterium. Nine of them were identified as *Chlorella* species, where three *Chroococcus* species, two *Nostoc* and *Anacystis* species and one *Oscillatoria*, *Phormidium*, *Chlamydomonas*, *Oocystis* and *Scenedesmus* species were also among those showing antibacterial activity.

Table 1: Antibacterial activity of microalgal supernatants against Gram-positive and Gram-negative bacteria as presented by inhibition zone diameter (in mm)

Sample	*S. aureus*	*S. epidermidis*	*B. subtilis*	*E. coli*	*S. typhi*	*P. aeruginosa*
Control (Ampicillin)	16	14	20	-	-	-
Control (Gentamicin)	-	-	-	10	10	16
Chroococcus dispersus 013	9	11	8	10	9	-
Chroococcus dispersus 034	20	20	22	-	26	24
Chroococcus dispersus 039	26	20	22	18	26	20
Anacystis nidulans 005	10	10	7	-	10	-
Anacystis nidulans 006	-	-	8	8	12	-
Phormidium sp. 016	20	-	12	16	20	-
Nostoc muscorum 002	-	9	-	9	-	-
Nostoc muscorum 017	12	9	12	-	13	-
Oscillatoria splendida 010	10	-	-	13	-	-
Chlorella ellipsoidea 006	-	10	8	13	11	15
Chlorella vulgaris 012	16	15	15	14	13	14
Chlorella vulgaris 046	20	10	16	-	-	-
Chlorella vulgaris 025	9	8	12	-	8	-
Chlorella vulgaris 036	8	-	10	10	-	-
Chlorella ellipsoidea 032	20	9	8	-	10	-
Chlorella ellipsoidea 024	14	-	8	-	10	10
Chlorella ellipsoidea 031	8	14	9	-	8	8
Chlorella sp. 030	18	12	16	-	20	10
Chlamydomonas reinhardtii 008	18	18	24	14	24	12
Scenedesmus obliquus 019	8	-	12	-	9	-
Oocystis sp. 047	8	8	12	-	10	9

Table 2: Antibacterial activity of microalgal methanolic extracts against Gram-positive and Gram-negative bacteria as presented by inhibition zone diameter (in mm)

Sample	*S. aureus*	*S. epidermidis*	*B. subtilis*	*E. coli*	*S. typhi*	*P. aeruginosa*
Control (Ampicillin)	16	14	20	-	-	-
Control (Gentamicin)	-	-	-	10	10	16
Chroococcus dispersus 039	10	8	10	8	10	8
Chlamydomonas reinhardtii 008	8	9	11	8	-	-
Oocystis sp. 047	8	8	10	-	-	7
Chlorella sp. 030	8	-	9	-	-	-
Chlorella ellipsoidea 006	10	9	10	-	-	-
Chlorella vulgaris 012	10	8	8	-	-	7
Chlorella vulgaris 029	12	8	-	-	7	8
Chlorella vulgaris 016	12	8	-	-	-	8

Table 3: Antifungal activity of microalgal supernatants as presented by inhibition zone diameter (in mm)

Sample	*C. albicans*	*C. kefyr*	*A. fumigatus*	*A. niger*
Control (Amphotericin B)	9	11	10	11
Chlamydomonas reinhardtii 008	-	11	8	9
Chroococcus dispersus 034	-	14	8	11
Chlorella vulgaris 012	-	13	9	14
Anacystis nidulans 006	-	8	10	11
Chlorella ellipsoidea 040	-	9	8	7
Chlorella ellipsoidea 031	-	14	8	9
Scenedesmus obliquus 019	-	9	7	8
Chlorella sp. 030	-	16	10	12
Oocystis sp. 047	-	13	12	10
Chlorella vulgaris 025	-	11	11	11
Chlorella ellipsoidea 006	-	9	-	-
Anacystis nidulans 005	-	11	-	-
Chlorella vulgaris 030	-	8	-	-
Chroococcus dispersus 039	12	16	10	9
Chlorella ellipsoidea 032	-	8	8	-
Chroococcus dispersus 013	-	-	8	-
Phormidium sp. 016	-	-	7	10

-: No anti microbial activity

The Supernatant of 17 isolated strains showed antifungal activity against at least one of the fungal strains. Eight of them were *Chlorella* species, 3 *Chroococcus* species, 2 *Anacystis* species and one strain of each of the *Phormidium*, *Chlamydomonas*, *Oocystis* and *Scenedesmus* species were also identified. Among the fungi, *Candida albicans* was more resistant. It was inhibited by only *Chroococcus dispersus* 039. No antimicrobial activity was detected in the hexane extracts and no methanolic extracts inhibited the growth of fungi. The culture media of 15 strains such as *Chroococcus*, *Chlamydomonas*, *Chlorella*, *Anacystis*, *Scenedesmus* and *Oocystis* species showed antifungal activities against *C. kefyr*. The results showed that antibacterial and antifungal activity was seen predominantly from the *Chlorella* species.

The results indicated that all of the supernatants and methanolic extracts of *Chlorella vulgaris* 012, *Chlamydomonas reinhardtii* 008 and *Chroococcus dispersus* 039 had a high activity against Gram positive bacteria. Antifungal activity assays showed a good activity against *C. kefyr* and minimum activity against *C. albicans*.

Among all of the species studied in this investigation for antibacterial and antifungal activity, *Chroococcus dispersus* 039 exhibited widespread spectrum of antimicrobial activities. The classification of the isolate alga was performed by Persian Type Culture Collection (PTCC), Tehran, Iran, as a strain of *Chroococcus dispersus* (Keissl.) Lemmermann PTCC 1677. *Chroococcus dispersus* PTCC 1677 was selected and examined further for isolation and purification of antimicrobial substances. The culture medium of *Chroococcus dispersus* PTCC 1677 (4 L) was concentrated in vacuum to give a dry brown solid (1.25 g). Dried supernatant was dissolved in methanol and fractionated over a silica gel column. Ten fractions (each of 20 mL) were collected in which fractions 9-10 were active against the test microorganisms. Fractions 9-10 were pooled and localization of antimicrobial compound was assayed on TLC. The visualization of the spots with UV light (254 and 366 nm) and bioautographic assay showed only one antimicrobial substance with $R_f = 0.76$. The combined fractions were repurified on preparative silica gel plates with methanol/chloroform (90:10). The band with antimicrobial activity was cut from the plates and eluted from the adsorbent by washing in a mixture of methanol/chloroform (40:50). The residue was concentrated and led to the isolation of 2 mg purified substance. The purified antimicrobial substance was isolated as a yellow powder (2 mg), in yield of 0.16 % on the basis of dry weight of the supernatant (1.25 g). The antimicrobial substance presented MIC values of 10 μg mL^{-1} against *S. epidermidis* and *E. coli* and 20 μg mL^{-1} against *C. kefyr*.

DISCUSSION

The microalgae such as *Chlorella* spp., *Scenedesmus* ssp. (Ördög *et al*., 2004), *Chlamydomonas* spp. (Kellam and Walker, 1989), *Euglena viridis* (Das *et al*., 2005), *F. ambigua* (Ghasemi *et al*., 2004), *Nostoc* spp. (Jaki *et al*., 2000), *Scytonema hofmannii* (Pignatello *et al*., 1983), *Hapalosiphon fontinalis* (Moore *et al*., 1987), *Anabaena* spp. (Frankmolle *et al*., 1992), *Microcystis aeruginosa* (Ishida *et al*., 1997), *Phormidium* sp. (Fish and Codd, 1994), have been reported as the main groups of microalgae to produce antimicrobial substances. The ability to produce antimicrobial agents may be significant not only as a defensive instrument for the algal strains but also as a good source of the new bioactive compounds from a pharmaceutical point- of-view.

Screening efforts aimed to identify antimicrobial agents in microalgae have revealed several promising lead compounds. Some of the substances identified include Chlorellin (Metting and Pyne, 1986), Parsiguine (Ghasemi *et al*., 2004), Nostocyclyne A (Ploutno and Carmeli, 2000), Nostofungicidine (Kajiyama *et al*., 1998), Kawaguchipeptin B (Ishida *et al*., 1997), Nostocin A (Hirata *et al*., 1996), Ambigol A and B (Falch *et al*., 1993), Hapalindoles (Moore *et al*., 1987) and Scytophycins (Ishibashi *et al*., 1986).

Most of the studies have only used *in vitro* assays and, it is likely that most of these compounds have little or no application in medicine as they are either too toxic or inactive *in vivo* (Borowitzka, 1995). They may however serve as useful lead compounds for the 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 rost wheat infections (Borowitzka, 1995).

In the course of present research on the microalgae from paddy fields in the north of Iran, (Ghasemi *et al*., 2003, 2004), a screening program was carried out for isolation and identification of antimicrobial-producing species from paddy fields in the south of Iran. In this investigation, out of 60 strains of microalgal isolates, 21 showed significant *in vitro* antibacterial activity and 17 of them had antifungal effect.

The proportion of the isolates with antibacterial and antifungal activities were approximately 35 and 28%, respectively, which is comparable with those published

earlier in other screening programs: 14% (Ghasemi *et al*., 2003), 11% (Flores and Wolk, 1986), 7% (Patterson *et al*., 1993) and 10% (Schlegel *et al*., 1999).

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 probably was because of polar nature of the active components. It shows that the chance of finding antimicrobial activity is higher in culture supernatants and methanolic extracts.

Antifungal activity assays showed a good activity against *C. kefyr*, *A. niger* and *A. fumigatus* and the minimum activity against *C. albicans*. *C. albicans* was inhibited by only *Chroococcus dispersus* 039.

Among the isolated microalgae, *Oscillatoria* species had the minimum activity against the test organisms. Among all of the species studied in this investigation for antibacterial and antifungal activity, it seems that *Anacystis nidulans* is being reported for the first time as the producer of antibacterial substances. This strain showed a good activity against the test microorganisms. Although it was found that antibacterial activity of the supernatants and methanolic extracts were higher against Gram positive bacteria in comparison with Gram negative bacteria, the same results have been observed in our previous studies (Ghasemi *et al*., 2003 and 2004). Generally antibiotics are less effective against Gram negative bacteria because of their more complex multilayered cell wall structure, which makes it more difficult for the active compound to penetrate (Ördög *et al*., 2004).

In previous study, microalgae that produce potent antimicrobial substances from the north of Iran belong to filamentous cyanobacteria, Stigonemataceae and Nostocaceae and they did not show inhibition against Gram negative bacterium, *E. coli* PTCC 1047 (Ghasemi *et al*., 2003). In spite of that, the data obtained from this research demonstrated antimicrobial activity against *E. coli* PTCC 1047 in 2 methanol and 11 aqueous extract. This is in agreement with Falch (1995) and Hirata (1996) that reported active compound against *E.coli* in the petroleum ether fraction of *Fischerella ambigua* and supernatant of *Nostoc spongiaeforme*, respectively.

Also, *Chlorella* and *Chroococcus* species from Chlorophyceae and Chroococcaceae had the greatest frequency among the species that showed antibacterial and antifungal activity and exhibited the most prominent effect. The effect of antimicrobial activity of *Chlorella* species has been reported in other studies such as Kellam and Walker 1989, Ördög *et al*., 2004; Debro and Ward 1979. Ördög *et al*. (2004), reported that antibacterial and antifungal activity was seen predominantly from the *Chlorella* species. This result is in agreement with that of our current experiment.

There is only little research regarding the biological effects of *Chroococcus* spp. In one study, the methanolic extract of *Chroococcus* sp. was found to be biologically active against *Staphylococcus epidermidis* (Soltani *et al*., 2005). In another study it was shown that *Chroococcus* sp. produced bioactive substances with antibiotic activity against *Bacillus cereus*, *S. aureus* and *S. epidermidis* (Mian *et al*., 2003).

This study confirms that the supernatant and methanolic extract of *Chroococcus dispersus* PTCC 1677 have high activities against all of the test microorganisms. The result was further confirmed by individual fractions of supernatant of *Chroococcus dispersus* PTCC 1677 separated through silica gel column chromatography and bioautography. The purified antimicrobial substance presented MIC values of 10 μg mL^{-1} against *S. epidermidis* and *E. coli* and 20 μg mL^{-1} against *C. kefyr*. This compound, which exhibited a polar substance and tentatively named Pars A, has interesting antifungal and antibacterial activity with good MIC (10-20 μg mL^{-1}). Therefore, it is expected that our experiments may reveal a novel antimicrobial substance from *Chroococcus dispersus* PTCC 1677 that have not yet described.

ACKNOWLEDGMENT

This research was supported by a grant from the Research Council of Shiraz University of Medical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran.

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