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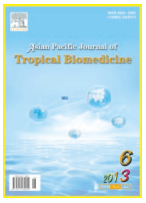

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Cyanobacteria, *Lyngbya aestuarii* and *Aphanothece bullosa* as antifungal and antileishmanial drug resources

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PEER REVIEW

Peer reviewer

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Comments

It is very interesting that the authors explored both marine and freshwater cyanobacteria as drug resource. There are few reports regarding antifungal and antileishmanial activity from cyanobacteria therefore such screening effort is commendable.
Details on Page 462

ABSTRACT

Objective: To investigate two cyanobacteria isolated from different origins *i.e. Lyngbya aestuarii (L. aestuarii)* from brackish water and *Aphanothece bullosa* (*A. bullosa*) from fresh water paddy fields for antifungal and antileishmanila activity taking *Candida albicans* and *Leishmania donovain* as targets. **Methods:** Biomass of *L. aestuarii* and *A. bullosa* were harvested after 40 and 60 d respectively and lyophilized twice in methanol (100%) and redissolved in methanol (5%) for bioassay. Antifungal bioassay was done by agar well diffusion method while antileishmanial, by counting cell numbers and flageller motility observation of promastigotes and amastigotes from *L. donovani*. Fluconazole and 5% methanol were used as control. **Results:** Both the cyanobacteria were found to be potent source of antifungal activity keeping fluconazole as positive control, however, methanolic crude extract (15 mg/mL) of *A. bullosa* was found more potent (larger inhibition zone) over that of methanolic crude extract of *L. aestuarii*. Similarly antileishmanial activity of crude extract (24.0 mg/mL) of *A. bullosa* was superior over that of methanolic crude extract of *L. aestuarii* (25.6 mg/mL). **Conclusions:** Antifungal and antileishmanial drugs are still limited in the market. Screening of microbes possessing antifungal and antileishmanial activity drug is of prime importance. Cyanobacteria are little explored in this context because most of the drugs in human therapy are derived from microorganisms, mainly bacterial, fungal and actinomycetes. Thus in the present study two cyanobacterial strains from different origins showed potent source of antifungal and antileishmanial biomolecules.

KEYWORDS

Antifungal, Antileishmanial, Secondary metabolite, Cyanobacteria

1. Introduction

In spite of emergence of modern approaches to drug discovery, the pace of drug development has slowed down because of lack of proper lead in biomolecules, which is crucial to designing newer drug[1]. Therefore, such newer natural bioresources could act as the reservoir for such molecules, and led us to opt for the little explored cyanobacterial flora. However, microbial metabolites produced by actinomycetes, fungi and unicellular bacteria along with cyanobacteria contributed as 45%, 38% and 17%, respectively[2]. Secondary metabolites produced from

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cyanobacteria are found to be antibacterial, antifungal, antialgal, anticancerous and antimalarial with many more activitys[3,4]. Microbial productions of metabolites are greatly influenced by factors such as pH, temperature, and light intensity[5-8]. However, species specificity of secondary metabolites is well established[9].

Literature survey indicated that there are limited numbers of drugs available against fungi and *Leishmania* in contrast to bacteria. In recent years fungal disease has been more difficult to treat in immunocompromised HIV patient. Food and Drug Administration of US described 10 antifungal drugs only[10]. Leishmaniasis is a tropical affliction that constitutes one of the six entities on the World Health Organisation tropical disease research list of most important disease[11,12]. The concern has been reflected by the Panama International Cooperative Biodiversity Group investigating Panamanian microorganism for led compounds with antileishmanial activity[13,14]. Although various species of *Lyngbya* is known to produce more than 200 compounds including antifungal such as Lobocyclamide A-C, Lyngbyabellin B and antileishmanial such as Almiramide A-C, Dragonamide E[15-19]. Fresh water *Aphanothece pallida* and *Aphanothece stagnina* were found to possess antifungal activity[20]. Although many fresh water cyanobacteria were screened for antialgal, antibacterial as well as antifungal activity such as *Nodularia harveyana* for Norharmane (9H-pyrido-3,4-b-indole), *Nostoc insulare* for 4,4′-dihydroxy biphenyl[21], *Fischerella musciola* for herbicidal and antifungal, Fischerellin A and *Synechocystis* for partially purified AK3 as antifungal[22,23]. In the present investigation we have selected two cyanobacterium from different habitats, *Leishmania aestuarii* (*L. aestuarii*) from brackish water in Chilka Lake, Orissa and *Aphanothece bullosa* (*A. bullosa*) from paddy field around Banaras Hindu University (fresh water) for their antifungal and antileishmanial activities.

2. Materials and methods

2.1. Cultivation of cyanobacteria and extraction of biomass

The cyanobacterial strains, *L. aestuarii* (a brackish water strain from Chilka lake, Orissa, India) and *A. bullosa* (a fresh water strain from paddy field around Banaras Hindu University, Varanasi, India), were grown in ASN III and BG 11 medium, respectively for screening of biological activity[24]. Cultures were maintained at (28±2) °C, at a light intensity of 14.40 W/m^2 provided by cool white fluorescent tubes with a light/dark cycle of 18/6 h. Biomass of *L. aestuarii* and *A. bullosa* were harvested after 40 and 60 d respectively. The harvested biomass was centrifuged at 10 000 r/min for 15 min (Remi, India) and lyophilized. Lyophilized cyanobacterial biomass (5 g) was extracted twice in 300 mL methanol (100%). By keeping it on shaker (150 r/min for 48 h) and centrifuged at 15 000 r/min for 15 min. Supernatant was dried in a rotary evaporator (Prefit, India) at 40 °C redissolved in 3 mL methanol (100%) to be used for bioassay against *Candida albicans* (*C. albicans*) and *Leishmania donovain*[25]. The final concentration of methanol was kept as 5% in bioassay experiment.

2.2. Cultivation of fungi and bioassay

C. albicans was grown on Sabouraud Dextrose agar (4%, SDA) at 22 °C for 48 h. For antifungal bioassay sterile petri plates having 4% SDA were prepared and after solidification 4 mm wells were made with sealing off bottom by soft agar (0.8%). *C. albicans* was suspended in 0.91% NaCl and turbidity was adjusted to 10^7-10^8 CFU/mL, corresponding to 0.5 Mac Farland standard according to NCCLS 1997 guidelines (now CLSI)[26]. SDA plate was now swabbed with testing fungal strain aseptically. The cyanobacterial extract (20 μL) was filled in each well along with methanol (5%) and fluconazole (100 μg/mL) as controls. The petri plates were kept aseptically in laminar hood for about 20 min and incubated at 22 °C for 24 h. After completion of incubation period, the diameters of the zones of inhibition were measured in mm.

2.3. Culture of the L. donovani promastigotes and amastigotes and bioassay

A cloned line of *L. donovani* (MHOM/IN/80/Dd8) promastigotes were grown in a biochemical oxygen demand (BOD) incubator at 26 °C in complete RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and penicillin (100 U/mL), gentamycin (20 μg/mL), streptomycin (100 μg/mL) (pH 7.2). The promastigotes were harvested in the late log phase, counted in Neubauer's chamber and adjusted to a concentration of 1×10^6 cell/mL for the *in vitro* assay of the methanolic crude extracts of cyanobacteria. Axenic amastigotes were grown in complete RPMI-1640 medium supplemented with FBS and antibiotics having the pH 5.5 and at 37 °C in a humidified atmosphere containing 5% CO_2 in air in a CO_2 incubator[27,28]. The cellular morphology of these parasites was evaluated using phase-contrast light microscopy (Nikon Corporation, Japan).

The culture of promastigotes (1×10^6 cell/mL) were inoculated in 24-well plates containing complete RPMI-1640 medium supplemented with 10% FBS, antibiotics and different concentrations of crude extract of *L. aestuarii* (0.8, 1.6, 3.2, 6.4, 12.8 and 25.6 mg/mL) and *A. bullosa* (0.750, 1.500, 3.75, 5.625, 7.500 and 15.000 mg/mL). Promastigotes were incubated at 26 °C for 48 h in a BOD incubator while amastigotes at 37 °C in a CO_2 incubator with 5% CO_2. The survival percentage of the parasites was evaluated at 1, 6, 12, 24, 32, 40 and 48 h intervals by counting motile promastigotes and by using tryptan blue in amastigotes.

The percentage inhibition or killings was investigated through the counting of cells in phase contrast compound microscope after staining with Trypan blue dye, which is used for differentiation between dead and live cells. Methanol (5%) was used as control. All the experiments were done in triplicate.

3. Results

In the present study we have observed antifungal potential of the methanolic crude extracts of *A. bullosa* and *L. aestuarii*. Three replicates of methanolic crude extracts of *A. bullosa* (A1, A2 and A3) produced 20, 12 and 16 mm (mean 16.00±2.30) of inhibition zones on the lawn of *C. albicans* while replicates of *L. aestuarii* (L1, L2 and L3) produced 18, 15 and 10 mm (mean 14.33±2.33) of inhibition zones. Bioassay was done along with methanol and fluconazole (100 μg/mL) as control. It was clear from the data (Table 1) that antifungal element containing crude extracts (15 mg/mL) of cyanobacteria has produced half of the inhibition zone over that of pure fluconazole. Therefore, the inhibition zone size obtained after the crude extracts, also justify the antifungal potential inspite of its being crude extracts.

Different concentrations of methanolic crude extracts of *A. bullosa* and *L. aestuarii* were also screened for antileishmanial activity in term of percentage killings of amastigotes and promastigotes of *L. donovani*. The microphotograph (20×) of *L. donovani* promastigotes and amastigotes without cyanobacterial extract and in presence of the extract revealed reductions in the number and flegeller motility (Figure 1). A complete lethality in promastigotes was obtained in 24.0 mg/mL of *A. bullosa* crude extract started from 24 to 48 h while *L. aestuarii* crude, was lethal at 48 h and higher concentration (25.6 mg/mL) (Figure 2). However, 100% killing promastigotes suggested that this impact was related wtih dose and time dependent. Similarly 100% killing of amastigote was observed by methanolic crude of *A. bullosa* only at 48 h at 24.0 mg/mL while *L. aestuarii* crude did not kill 100% amastigote at any concentration (Figure 3). The data indicated resistance of amastigotes towards crude extract compare to promastigotes. Crude from both cyanobacteria has antileishmanial activity at higher concentration. A negative control corresponded to the number of parasites in methanol (5%) with no detectable inhibition of their growth, alterations in cell morphology or even motility was observed. Thus methanolic crude extract of *A. bullosa* was found more potent against *L. donovani* compared to *L. aestuarii*.

Table 1
Inhibition zone on the lawn of *C. albicans* by replicates of *L. aestuarii* (L1, L2 and L3) and *A. bullosa* (A1, A2 and A3) along with methanol and fluconazole as control.

Samples and controls	Inhibition zone (mm)
L1	18.0
L2	15.0
L3	10.0
A1	20.0
A2	12.0
A3	16.0
Methanol (5%)	0.0
Fluconazole (100 μg/mL)	30.0

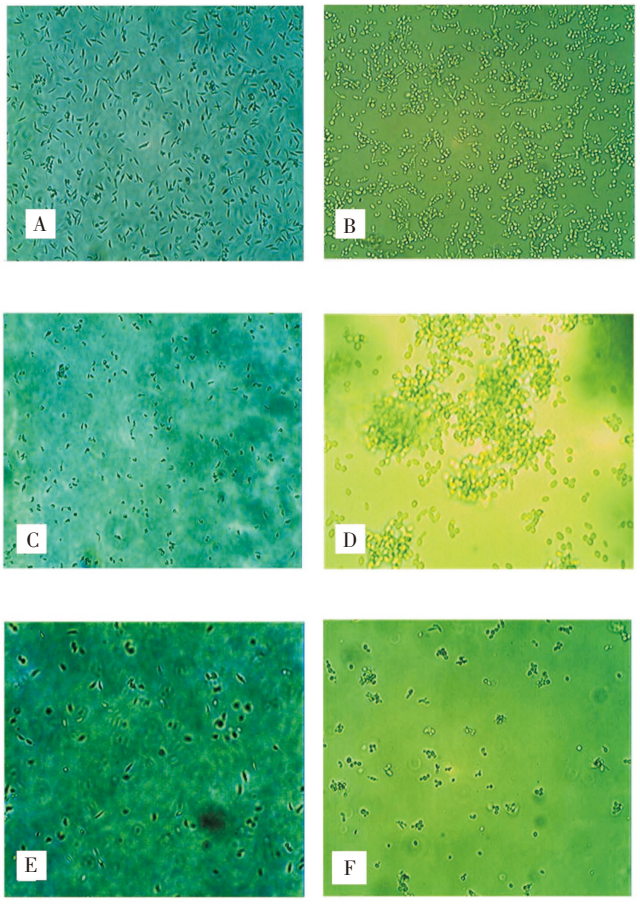

Figure 1. Photomicrograph of *L. donovani* (20×).
A: promastigote control; B: amastigote control in presence of *L. aestuarii* methanolic crude extracts (25.6 mg/mL); C: promastigote; D: amastigotes, while in presence of *A. bullosa* methanolic crude extract (24 mg/mL); E: promastigote; F: amastigote after 48 h interaction.

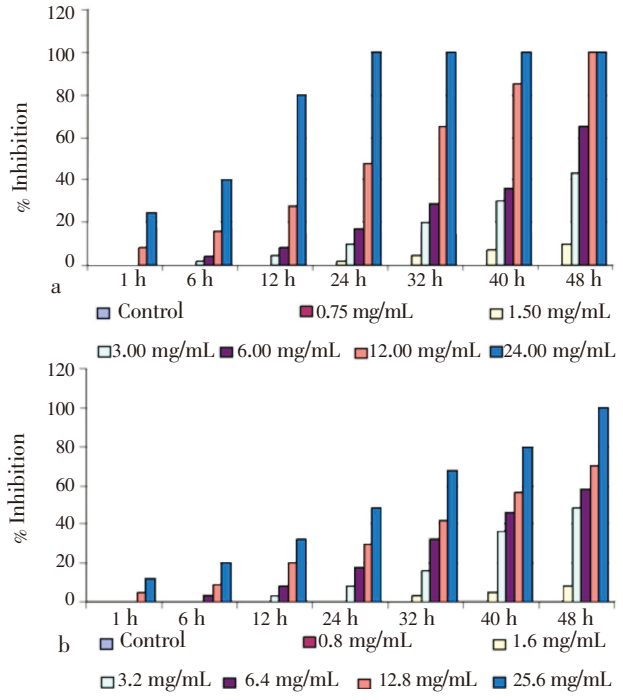

Figure 2. Dose and time dependent effect of methanolic crude extracts of a: *A. bullosa*; b: *L. aestuarii* on promatigote of *L. donovani*.

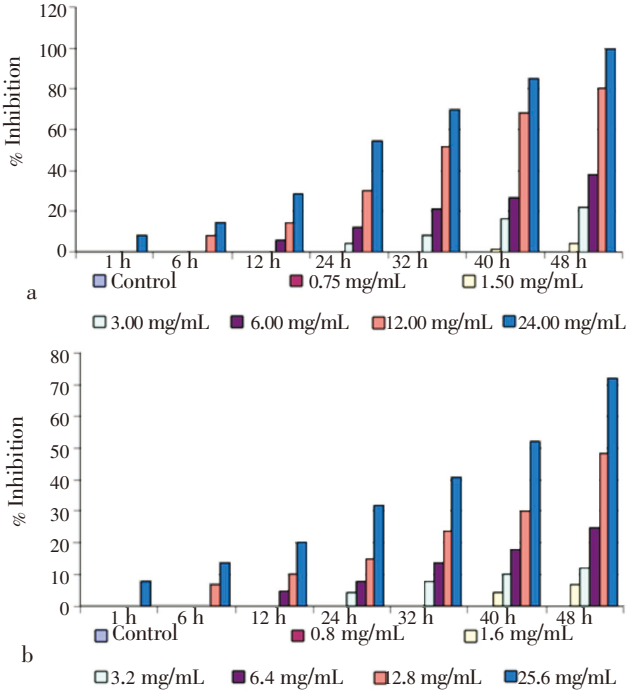

Figure 3. Dose and time dependent effect of crude extracts of a: *A. bullosa*; b: *L. aestuarii* on amastigote of *L. donovani*.

4. Discussion

Cyanobacterial genera are known to possess various sort of bioactive molecules active against prokaryotes as well as eukaryotes[29,30]. There are frequent reports of screening of cyanobacteria as a source of antibacterial molecules, in contrast to infrequent report of antifungal as well as antileishmanial activity and/biomolecules. Fungi, as a major disease causing agent, were realized after 1980, especially among the immunocompromised and other serious diseases[31]. Among other diseases of concern to scientists, leishmaniasis is caused by species of related genus of *Leishmania*[32-34]. There are limitations with regards to antifungal and antileishmanial drugs because of their price and side effects[35].

In present study methanolic crude extract of *A. bullosa* crude was found more potent antifungal in comparision to *L. aestuarii*. However, antifungal activity is also reported with crude extract and pure compounds from other freshwater cyanobacteria such as *Anabaena, Nostoc, Aphanocapsa, Synechocystis, Synechococcus, Oscillatoria, Nodularia, Calothrix*[20,21,36-39], while marine cyanobacteria are little explored with regard to antifungal activity except various species of as *Leishmania confervoides, Leishmania majuscula*[12,13]. This activity was caused due to species specific biomolecules as specific secondary metabolite produces in specific organism and in specific habitat[5-7]. In antifungal study pure compound fluconazole was also used as control but its inhibition zone was double time bigger than crude although its concentration was 100 μg/mL. However, fluconazole was found to inhibit *C. albicans* at a lower concentration≤0.125 to 0.250 μg/mL[36]. It is clear that selected cyanobacteria are potent antifungal agents.

Crude extracts of *L. aestuarii* and *A. bullosa* are not abnormal with sense that there may contain meager amount of potent antileishmanial compounds. There are few drugs like miltefosine, kill promastigote of *L. donovani* at 120 μmol/L concentration[40]. Both the cyanobacterial crude extracts killed amstigotes but the potency of *A. bullosa* is greater than *L. aestuarii*. The similar results of inhibiting amastigotes by pure compounds were observed as amphotericin B and paromomycin were at 1 mg/mL and 0.03 mg/mL respectively[41]. As many structurally diverse antileishmanial compound show activity against amstigotes at 0.06 to 14.00 μmol/L[42]. Maytansine, taxol and the electrophiles block the growth of *L. donovani* amastigote like form *in vitro* at low (<1 μmol/L) concentration[43,44]. The activity of methanolic crude extract is similar but their concentration was higher compared to pure compounds. The methanolic crude extracts of both cyanobacterial species were to be antifungal as well as antileishmanial with *A. bullosa* as more potent in comparision to *L. aestuarii*. It seemed that biomolecules are niche specific in its origin. Thus, India, a tropical country, has many diverse niches, so it can act as a source of many biomolecules of biotechnological significance, if screened.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Secondary metabolites produced from cyanobacteria are found to be antibacterial, antifungal, antialgal, anticancerous, antimalarial with many more activity. The present work is limited to two cyanobacterial strains *L. aestuarii* and *Aphanothece bullosa* derived from brackish as well as paddy fields respectively. However, both the strains were active against the target microbes as antifungal and antileishmanial in nature.

Research frontiers

The present study was carried out because antifungal and antileishmanial drugs are still limited in the market. Screening of such little explored cyanobacteria possessing antifungal and antileishmanial activity drug seemed to be fundamentally important.

Related reports

Antibacterial and antifungal activity are also reported with crude extract and pure compounds from other freshwater cyanobacteria, such as *Anabaena, Nostoc, Aphanocapsa, Synechocystis, Synechococcus, Oscillatoria, Nodularia, Calothrix* (Ghazala *et al*. 2010; Volk and Furkert 2005), while marine cyanobacteria are little explored with regard to antifungal activity except species of *L. confervoides*, *L. majusculae* (Mac Millan *et al*. 2002). Different concentrations of methanolic crude extracts of *A. bullosa* and *L. aestuarii* were also screened for antileishmanial activity in term of percentage killings of amastigotes and promastigotes of *L. donovani*. Methanolic crude extract of *A. bullosa* was found more potent against *L. donovani* compared to *L. aestuarii*. The data showed that antifungal element containing crude extracts of cyanobacteria has produced half of the inhibition zone as compared with the zone by fluconazole.

Innovations and breakthroughs

There are limitations with regards to antifungal and antileishmanial drugs because of their price and side effects. Therefore such screening from natural microbial resource may lead a clue towards proper biomolecules of interest.

Applications

The methanolic crude extracts of both cyanobacterial species were found antifungal as well as antileishmanial with *A. bullosa* as more potent in comparision to *L. aestuarii*. It seemed that biomolecules are niche specific in its origin. Thus, tropical countries having diverse niches may be explored for source of biomolecules of biotechnological significance.

Peer review

It is very interesting that the authors explored both marine and freshwater cyanobacteria as drug resource. There are few reports regarding antifungal and antileishmanial activity from cyanobacteria therefore such screening effort is commendable.

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