
Antifungal Activity of *Eleutherine Bulbosa* Bulb against Mycelial Fungus

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Abstract The present study was undertaken to find out antifungal activity of *Eleutherine bulbosa* bulb (Iridaceae). Agar cup, MIC, TLC bioautography studies were performed against seven fungal isolates of clinical origin using various solvent extracts from the bulb of *E. bulbosa*. Butanol and ethanol extracts showed promising antifungal activity. The diameter of inhibition of zones varies in between 12-24 mm. MIC of all test extracts ranged in between 0.375-3.0 mg/ml. TLC bioautography was set up with butanol extract against *Trichophyton* sp. and result found that butanol extract have clear zone of inhibition all over the entire TLC plate. The results justify that the bulb extract of *E. bulbosa* can be used as potent antifungal agent.

Keywords: natural antifungal agent, medicinal herb, alternative control, dermatophytes, Aspergillosis, MIC, Iridaceae

Introduction

Fungal infections have been gaining major importance because of the morbidity of hospitalized patients. In India, due to increase in immune-compromised patients, lack of awareness and specialized expertise fungal infections are in rise. The spectrum of fungal infection is very wide. Approximately 90% of human fungal infections are caused by *Aspergillus*, *Candida*, *Cladosporium*, *Epidermophyton*, *Microsporum* and *Trichophyton* species (Dubey *et al.*, 2005). Among the pathogenic fungi, emerging infection due to filamentous fungi like *Fusarium* sp., *Acremonium* sp., *Dermatophytes* such as *Trichophyton* sp. are of great concern. *Trichosporon* sp., causing fungemias in immune-compromised patients host are often challenging. Other fungal infections include aspergillosis due to *Aspergillus flavus*, *A. fumigatus* and *A. niger* (Musial *et al.*, 1988). In addition to this, fungal species such as *Aspergillus*, *Fusarium* and *Penicillium* sp. are associated with heavy loss of

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grains, fruits, vegetables and other products during pickling, transit and storage, as a result unfit for human consumption.

On the other hand antifungals availability to clinicians is limited either by their low effectiveness or by their toxicity. Also, upon prolonged exposure to antifungals, pathogenic fungi become resistant to drugs resulting in a failure of treatment. Rapid drug extrusion mediated by efflux pump proteins of either ATP binding cassette or major facilitator's super families represents one of the major mechanisms of resistance mastered by pathogenic fungi (Prasad, 2009). Therefore, it is essential to search new antifungals which are less toxic, inexpensive and without problems of resistance. One of the most promising targets in the search for new biological active compounds is from plants. *Eleutherine bulbosa* (Miller) Urban is herb of Iridaceae family. This plant is native of South America and present in tropical countries. It is used by some populations as a vermifuge (Schultes and Raffauf, 1990), intestinal disorders (Lin *et al.*, 2002) and as an abortive and antifertility agent (Wenigeret *et al.*, 1982). To the best of our knowledge this plant has not reported as antifungal agent. To assessing the ability of *E. bulbosa* against clinical fungal pathogens, this research was concentrated on evaluationg antifungal properties.

Materials and methods

Collection, identification and extraction of plant material

The bulb of *Eleutherine bulbosa* were collected in the month of June, 2008 from forest guest house, Salabani, Baripada, Odisha (Figure-1a). Identification and voucher specimen deposition of this plant was done at the Post Graduate Department of Botany, North Orissa University, Baripada. Solvent extraction (chloroform, ethyl acetate, ethanol, butanol) was carried out using maceration method by soaking 500g of dried plant material in cleaned airtight bottles at a temperature of 28 °C for a period of 2 d. After proper incubation, the solvent was filtered using a filter paper. Filtration process was repeated twice and the extracts obtained were pooled for evaporation for obtaining residue. For aqueous extraction, 100 g of powdered bulb sample taken separately in a conical flask containing 400 ml distilled water followed steam distillation for 30 m to reduce the concentration up to 1/5th of the total volume.

Fungal cultures and growth condition

Test fungal strains include seven species of mycelia fungi viz. *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *Fusarium oxysporium*, *Pencillium* sp., *Rhizopus*

sp., *Trichophyton* sp. and *Trichosporon rubrum*. All the isolates were clinical and obtained from S.C.B. Medical College, Cuttack, India. Cultures were maintained in Sabouraud dextrose agar (Himedia, Mumbai) at 4 °C. To find out the resistance pattern the fungal strains were screened with antifungal agents by disc diffusion method and zone of inhibition was calculated using Himedia antibiotic zone reader (Figure-1b).

Screening for antifungal activity

Antifungal activity of plant extracts was conducted using the hole-plate (agar well) diffusion method (Leven *et al.*, 1979). 0.2 ml (5.0×10^5 spores/ml) of the inoculum was mixed with 20 ml of sterile SDA (maintained at 45-50 °C in molten state), and then poured into sterilized petridishes (90 mm in diameter) and set aside. After congealing, the seeded agar was punched out with a sterile hole borer at spaced out positions in order to make 4 holes (6 mm in diameter). Three holes were filled with 0.1 ml of test sample solution (20 mg/ml) while the fourth one was filled with Clotrimazole/Nitrofurantoin (0.2 mg/ml) dissolved in sterile water. The plates were incubated at 28 °C for 4 days to observe clear zone of inhibitions. The results (mean value, n=3) were recorded by measuring zone of growth of inhibition surrounding the cup.

Evaluation of MIC

The MIC is carried out with strains those show zone of inhibition higher than 10 mm against the test fungi by the agar cup method. Plates were prepared in sterilized Petriplate by mixing 20 ml of molten SDA with 1 ml of the extract dilution to give final concentrations of 6, 3, 1.5, 0.75, 0.37, 0.18, 0.09, 0.045 and 0.022 mg/ml in the agar media. After solidification, the surface of each plate was inoculated with liquid fungal mycelial cultures. All the plates were incubated at room temperature (25 ± 1 °C) for four days. Each test was done in triplicates. Inhibition of growth was judged by comparison with growth in control plate prepared without plant extracts, which were set up at the same time in a separate plate.

TLC Bioautography

TLC bioautography assay was performed by agar overlay bioautography technique. Plant extracts (5 µl) were applied simultaneously in two different silica gel-G coated plate (Merck 60). After drying, the plates were developed using solvent chloroform: methanol (9: 1) as mobile phase. One of the

developed TLC plates were carefully dried for complete removal of solvents and kept in a sterilized petriplate. 0.2 ml (5.0×10^5 spores/ml) of the inoculum was mixed with 20 ml of sterile SDA (maintained at 45-50 °C in molten state), and then poured into sterilized petridishes containing TLC plate under aseptic condition in laminar airflow. The plates were incubated at room temperature (25 ± 1 °C) for four days to find out the zone of inhibition. The other TLC plate is subjected to UV ray (264) and sprayed with iodine, 0.36% vanillin-sulphuric acid to observe the separated component.

Results

The zones of inhibition of all extracts along with standard antifungal agent Clotrimazole and Nitrofurantoin as controls are presented in Table 1. Butanol and ethanol extracts showed potent antifungal activity against fungi in the present study. Ethyl acetate and aqueous extracts showed moderate zone of inhibition against *A. flavus*, *Trichosporon rubrum* and *Trichophyton* species. Chloroform extracts did not show any activity against mycelial fungi.

Table 1. Screening of antifungal activity of *Eleutherine bulbosa* bulb

Name of the strain	Zone of inhibition in mm (MIC in mg/ml)					
	EtAc	BT	ET	AQ	Cc	Nf
<i>Aspergillus flavus</i>	13.3±0.7 (1.5)	-	12.6±1.5 (3.0)	13.3 ±1.15 (1.5)	14.3±0.7	-
<i>Aspergillus fumigatus</i>	-	13.0 ±1.4 (1.5)	12.3 ±0.7 (1.5)	-	20.3±1.52	15.6±1.52
<i>Aspergillus niger</i>	-	13.0 ±1.4 (1.5)	13.3 ±1.15 (3.0)	-	15.3±0.57	18.3±1.57
<i>Fusarium oxysporium</i>	-	-	-	-	29.6±1.1	-
<i>Pencillium</i> sp.	-	-	-	-	16.3±1.15	26.6±1.15
<i>Rhizopus</i> sp.	-	24.0 ±2.6 (1.5)	16.3±0.57 (3.0)	-	13.0±1.73	21.6±0.57
<i>Trichosporon rubrum</i>	15.0 ±2.0 (0.75)	12.3 ±2.5 (0.75)	15.0±2.0 (0.75)	16.0 ±1.7 (0.75)	14.3±0.7	12.6±1.5
<i>Trichophyton</i> sp.	14.3 ±0.5 (1.5)	17.0 ±3.2 (0.375)	19.6±1.52 (0.75)	14.0 ±2.6 (0.75)	9.6±2.08	18.6±0.57

All values are mean zone of inhibition ± SD; Zone of inhibition including 6 mm borer; Extract concentration (2 mg/ml); Chloroform extract has no activity; (-); No zone of inhibition; Cc-Clotrimazole (0.2 mg/ml), Nf-Nitrofurantoin (0.2 mg/ml); EtAc-Ethyl acetate; BT-Butanol; ET-Ethanol; AQ-Aqueous; Value within () indicates MIC in mg/ml.

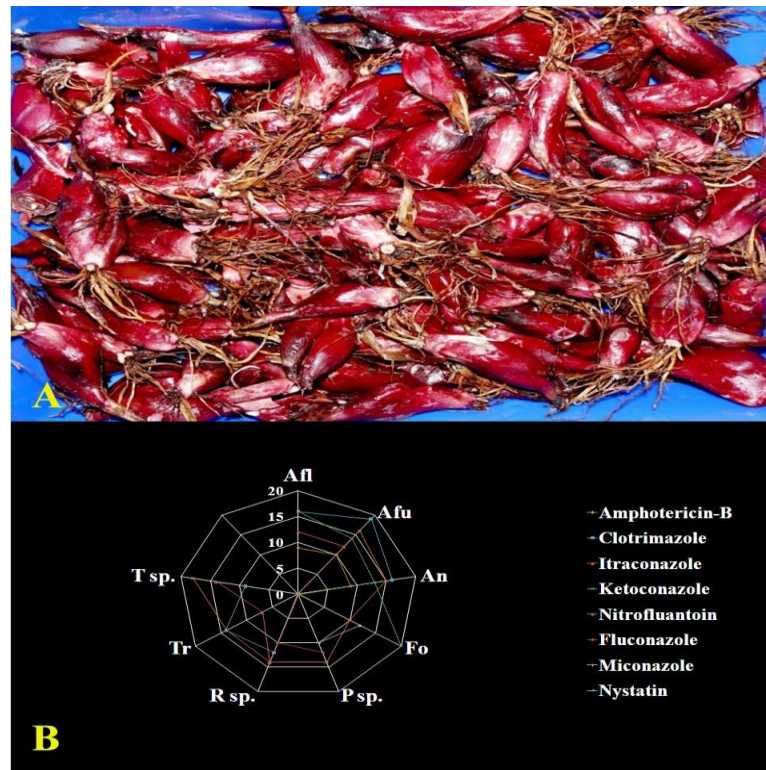


Fig. 1. A. *Eleutherine bulbosa* bulb; B. Antifungal resistance among fungal strains

Consequently, chloroform extract and species such as *F. oxysporium*, *Pencillium* sp., *Rhizopus* sp., *A. fumigatus*, *A. niger* and *A. flavus*, which were not inhibited by the respective extracts were removed from next stages of experiment (MIC). Species like *T. rubrum*, *Trichophyton* sp. and *A. flavus* were completely inhibited by respective extracts with zone of inhibition higher than standard antifungal agents. From MIC results, among the mycelial fungi, ethanol extracts showed excellent inhibitory effect (range 0.375-3.0 mg/ml).

The corresponding value for butanol extract was recorded against *A. fumigatus*, *A. niger*, *Rhizopus* sp., *Trichosporon rubrum* and *Trichophyton* sp. Similar results were also obtained for aqueous and ethyl acetate extracts against selected fungal isolate such as *A. flavus*, *T. Rubrum* and *Trichophyton* sp. The TLC study detected several spots using chloroform: methanol (9: 1) solvent in UV detector and vanillin spray. Most of the spot having identical R_f value 0.90, 0.77, 0.51 and 0.42. TLC bioautography was set up in butanol extract against *Trichophyton* sp. since this strain showed reliable zone of inhibition against all text extracts. Result found that butanol extract have clear zone of inhibition all over the TLC plate against *Trichophyton* species.

Discussions

The potential of *E. bulbosa* bulb extracts to inhibit growth of fungal species will differ with the extractant used. Among the all extractant, butanol is the most effective while chloroform did not inhibit the growth of fungal strains in the present study. This may be due to presence of different constituents having antifungal activity in the butanolextractant. The fact that *E. bulbosa* exhibited inhibitory activity against some of the fungal implicated in the pathogenesis of human diseases (Aspergillois by *Aspergillus* sp. and skin diseases by *Trichophyton* and *Trichosporon* sp.) provide some scientific basis for the utilization of this plant in the treatment of fungal diseases. MIC value recoded in between concentration (0.375-3.0 mg/ml), the bulb extracts (except chloroform) inhibited the growth of *Aspergillus* sp., *Rhizopus* sp., *Trichophyton* and *Trichosporon* species of fungi.

Over the past 2-3 decades the number of systematic and deep-seated fungal infection has dramatically increased. It appears that fungi are flourishing human body especially due to extensive administration immunosuppressive therapy and use of broad spectrum antibiotics. Systematic mycoses are respiratory disease caused by mycelia fungal pathogens. *Aspergillus*, *Penicillium*, *Cladosporium* and *Fusarium* species are often associated with lower respiratory infection, bronchitis and chronic pulmonary disease.

Recent study found that most of these fungal strains are resistant to standard antifungal agents. This is mainly due to the non-availability of effective antifungal drugs for systemic fungal infections and toxicity of available drugs such as Amphotericin-B (Saral, 1991). However, few authors reported Amphotericin-B has been the drug of choice for the most invasive fungal infections (Prasad, 2009). The major advantages of this agent are its broad spectrum and its concentration dependent fungicidal action against most susceptible fungi. Nevertheless, in the present study, strains such as *A. flavus*, *A. fumigatus*, *A. niger* and *Rhizopus* sp. illustrate zone of inhibition ≤ 10 mm while the plant have higher zone of inhibition in compare to Amphotericin B. Similarly, *F. oxysporium*, *Pencillium* sp., *Trichophyton*sp. and *Trichosporon rubrum* are resistant to Ampotericin B while the bulb extract confirmed excellent zone of inhibition against *Trichophyton*sp. and *Trichosporon rubrum*.

The increased rate of fungal infection resulted in the availability of new compounds for antifungal therapy. The best way to finding such compounds is from plants because plant products are without any side effects, do not add any physiological pressure on the pathogens for the development of drug resistance and easily degradable. Based on test data, further study on chemical and pharmacological investigation is recommended.

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