

Antibacterial potential of fungal endophytes isolated from *Boerhaavia diffusa* L.

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ABSTRACT

Boerhaavia diffusa L. is widely used for its ethno-medicinal properties. The present study assessed the antibacterial activity of endophytic fungi harbored in this plant. The endophytic fungi isolated belonged to various genera like *Aspergillus*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Fusarium*, *Pestalotiopsis* and *Zygorhynchus* and the overall colonization frequency of endophytic fungi was found to be 24%. Among the isolated fungi *Aspergillus fumigatus*, *Cladosporium sphaerospermum*, *Fusarium* sp. and *Zygorhynchus* sp. showed antibacterial activity in preliminary screening against one or all the test bacteria. Further, after fermentation, the ethyl acetate extracts of the selected endophytic fungi displayed variable antibacterial activity with maximum 54.75 mm and a minimum 27.50 mm zone of inhibition against test bacteria in disc diffusion assay. The lowest minimum inhibitory concentration (MIC) was 0.156 mg ml⁻¹ in the broth dilution assay against a *B. subtilis*, *S. typhi* and *Staph. aureus*, while it was 0.312 mg ml⁻¹ against *E. coli* which was offered by the ethyl acetate extracts of *A. fumigatus*.

INTRODUCTION

Endophytic fungi are an interesting group of microorganisms associated with the tissues of plants and are often regarded as plant-defending mutualists (Saikkonen *et al.*, 2004). Plant endophytic fungi are defined as the fungi that spend the whole or part of their life cycle colonizing inside the healthy tissues of the host plant, typically causing no apparent symptoms of disease (Rodriguez *et al.*, 2009) and these fungal endophytes lie under the widely neglected component of plant communities. Endophytic fungi are distributed world-wide and have been isolated from a variety of plant types including mosses, liverworts, ferns and higher plants (Higgins *et al.*, 2007; Rosa *et al.*, 2010; U'Ren *et al.*, 2012). Endophytic fungi have diverse application as they may play role in plant growth (Mucciarelli *et al.*, 2003); plant defense (Gao *et al.*, 2010); affect

plant resistance under stressful conditions (both biotic and abiotic stresses) (Marquez *et al.*, 2007; Rodriguez *et al.*, 2008); decompose plant litter (Sun *et al.*, 2011). Apart from these, endophytic fungi are also known to produce many valuable bioactive metabolites including anti-microbial, anti-insect, anti-cancer, anti-diabetic and immunosuppressant compounds along their great potential applications in agriculture, medicine and food industry (Verma *et al.*, 2009; Zhou *et al.*, 2010; Zhao *et al.*, 2011; Hema *et al.*, 2015). There are various reports on fungal endophytes that produce biologically active compounds including fungicidal and herbicidal properties (Petersen *et al.*, 2004).

The probability of sampling specific medicinal plants that harbor antibiotic producing endophytes might increase significantly if one is guided by the medicinal role of native people (Raviraja *et al.*, 2006). Hence, there is need to isolate new antimicrobial compounds from endophytic fungi that can overcome the difficulties related to the treatment of infections caused by resistant pathogens. Thus, in the present study endophytic fungi associated with *Boerhaavia diffusa* L.- a medicinal plant with high medicinal properties was screened for their antibacterial potential against test pathogens.

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MATERIALS AND METHODS

Collection of plant material

Healthy (showing no visual disease symptoms) mature stem of *Boerhaavia diffusa* L. were collected from Mysore region, Karnataka. For sampling selection, the plant was randomly collected from different sites in the same vicinity for the study. The healthy stems of the plant were collected and brought to the laboratory and processed immediately to reduce the risk of contamination.

Isolation and identification of endophytic fungi

The collected plant samples were washed thoroughly 2-3 times in running tap water to remove the dust and debris present on the surface of explants, followed by repeated washing in distilled water. After proper washing, stem samples were cut into small pieces (1 to 2 cm length) under aseptic conditions using sterile scalpel. Further, they were subjected to surface sterilization by immersing the plant samples in 70% ethyl alcohol for one minute, followed by immersion in 4% sodium hypochlorite solution for three minutes and then rinsed with deionized sterile distilled water three times to remove the sterilants present on the explants and blot dried on sterile blotter sheets. The efficiency of surface sterilization procedure was ascertained for every segment of tissue following the imprint method of Schulz *et al.* (1993). About 08 to 10 stem segments were placed on Petri plates containing 20 ml of potato dextrose agar (PDA) medium supplemented with antibiotic chloramphenicol to avoid the emergence of endophytic bacteria. Then the Petri plates were sealed using para film and incubated at $25 \pm 2^\circ \text{C}$ for 15 days. Endophytic fungal colonies emerging from their host were picked with sterile fine tip needle and sub cultured on to Petri plates containing PDA devoid of antibiotic to obtain pure cultures. The fungi were identified based on their morphological, conidial and cultural characters (Barnett and Hunter, 1998). All the fungal isolates were maintained in test tubes and Petri plates on PDA media.

Evaluation of antibacterial activity

Test organisms

All the isolated endophytic fungal isolates were screened for antibacterial activity. The indicator bacteria included both Gram-positive (*Staphylococcus aureus* MTCC 7443 and *Bacillus subtilis* MTCC 121) and Gram-negative (*Escherichia coli* MTCC 7410 and *Salmonella typhi* MTCC 733) bacteria obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India and used throughout the study. All the microbial cultures were adjusted to 0.5 McFarland standards, which is visually comparable to a microbial suspension of approximately $1.5 \times 10^8 \text{ cfu ml}^{-1}$.

Preliminary screening

The preliminary screening of antibacterial activity was carried out done following agar plug method (Devaraju and Satish,

2011). The nutrient agar (NA) medium were poured into Petri plates and inoculated with 100 μl of the bacterial suspension and spread uniformly. The mycelial discs (6 mm) of each endophytic fungal isolate (15 day-old) grown on PDA were obtained from actively growing margins using a sterile cork borer and placed on the surface of the NA medium previously seeded with test organisms. PDA media (6 mm discs) devoid of any fungal colony served as negative control. The plates were sealed using Para film and incubated at 37°C for 24 h. After incubation, antibacterial activity was confirmed by the visualization and measurement of inhibition zones. Each experiment was carried out in triplicates and measured diameters of zone of inhibition surrounding the agar disc were averaged and expressed in milli meter (mm). The mean value of the zone of inhibition was recorded.

Fermentation of endophytic fungi

The endophytic fungal isolates that offered antibacterial activity in primary screening were subjected to fermentation for metabolite production. Each endophytic fungi (5 -10 discs) were picked from actively growing margins and were fermented in 500 ml Erlenmeyer flasks containing 250 ml of potato dextrose broth (PDB) for 4-6 weeks at $25 \pm 2^\circ \text{C}$ under static conditions devoid of antibiotic. After incubation, the culture broth was filtered through Whatmann No. 1 filter paper to separate fungal mat and the resulting culture filtrate was extracted with equal volume of ethyl acetate in total three times and evaporated to dryness using flash evaporator.

Secondary screening

The fungal isolates with relatively broader antibacterial spectrum or stronger activities shown in the preliminary assay were selected for secondary assay. The secondary antibacterial screening was done by following disc diffusion method (Elcycynmi, 2007). The resulting crude extract of the fungal isolates were dissolved in ethyl acetate and tested for secondary assay. The test bacteria were seeded onto the surface of NA media and uniformly spread. Each sterile disc (6 mm) were loaded with 50 μl of fungal extract (concentration 1 mg disc^{-1}) and 50 μl of ethyl acetate and equidistantly placed on NA plates. Streptomycin discs were also placed as standard. The inoculated plates were incubated at $25 \pm 2^\circ \text{C}$ for 24 h and measured for its antibacterial activity by measuring the inhibition zones.

Minimum Inhibitory Concentration (MIC) by Micro broth dilution assay

Minimal Inhibitory Concentration (MIC) was determined by broth micro-dilution technique according to the method of Sarker *et al.*, (2007). The crude ethyl acetate extracts of selected endophytic fungi were diluted to a concentration of 50 mg ml^{-1} (stock solution). The 96 well plates were prepared by dispensing 100 μl of broth and 100 μl of the extract to the first well. A two-fold serial dilution was made in the row up to 12 well and final concentrations from well one to 12 were 2.5- 0.001 mg ml^{-1} . A 10 μl inoculum suspension of each bacterial strain was added to each

well. The wells containing nutrient broth with inoculum and solvent served as negative control. The plates were incubated at 37 °C for 24 h and the absorbance was measured at 620 nm using micro plate reader. The lowest concentration that inhibited visible growth of the test bacteria was recorded as the MIC based on the readings. The MIC was also detected by adding 10 µl well⁻¹ of TTC (2, 3, 5-triphenyl tetrazolium chloride) (Sigma) dissolved in water (TTC 2 mg ml⁻¹) and incubated under appropriate conditions for 30 min (Qaiyami, 2007). Viable organism reduced the dye to pink color. The lowest concentration at which the colour change occurred was taken as the MIC value. All MIC tests were repeated in triplicates.

Statistical Analysis

Data from three replicates were analyzed for each experiment and analysis of variance (ANOVA) using SPSS Inc. 16.0. Significant effects of treatments were determined by F values ($P \leq 0.05$). Treatment means were separated by Tukey's Honestly Significant Differences (HSD) test.

RESULTS

Isolation and identification of endophytic fungi

A total of 12 endophytic fungi were isolated from healthy stem segments of *B. diffusa*. The overall colonization frequency of

endophytic fungi was found to be 24%. The isolated endophytic fungi were classified into seven different taxa of *Zygorhynchus* sp., *Pestalotiopsis* sp., *Fusarium* sp., *Aspergillus* sp., *Cladosporium* sp., *Colletotrichum* sp., and *Curvularia* sp. Among the isolated fungi, *Cladosporium sphaerospermum* was the dominant fungus showing 25% of colonization frequency, followed by *Fusarium oxysporum* (16.6%) and all the other isolated fungi had a colonization frequency of 8.3% (Table 1).

Evaluation of antibacterial activity

Preliminary screening

All the isolated endophytic fungi were screened for their antibacterial activity against test bacteria by agar plug method. Among the isolated endophytic fungi, *Aspergillus fumigatus*, *Cladosporium sphaerospermum*, *Fusarium sporotrichioides*, *Fusarium* sp., *Zygorhynchus* sp. possessed inhibitory activities against one or more test organisms, while other test endophytic fungi had no antibacterial activity. *A. fumigatus* showed a maximum inhibition zone of 25 mm against *Staph. aureus* followed by *Fusarium* sp. which offered 16 mm zone of inhibition. Likewise, maximum inhibition zone of 13, 15 and 15 mm against *B. subtilis*, *E. coli* and *S. typhi*, respectively was observed in *Fusarium* sp. (Table 2).

Table 1: List of endophytic fungi isolated from of *B. diffusa*.

Endophytic fungi	No. of isolates	Colonization rate* (%)	Dominant fungi (%)
<i>Aspergillus fumigatus</i>	01	02	8.3
<i>Cladosporium sphaerospermum</i>	03	06	25.0
<i>Colletotrichum</i> sp.	01	02	8.3
<i>Curvularia eragrostidis</i>	01	02	8.3
<i>Fusarium oxysporum</i>	02	04	16.6
<i>Fusarium sporotrichioides</i>	01	02	8.3
<i>Fusarium</i> sp.	01	02	8.3
<i>Pestalotiopsis guepini</i>	01	02	8.3
<i>Zygorhynchus</i> sp.	01	02	8.3
No. of isolates	12	24%	

*Based on 50 plant segments plated.

Table 2: Antibacterial activity of isolated endophytic fungi against test bacteria by agar plug method (Zone of inhibition in mm).

Endophytic fungi	Test Bacteria			
	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Salmonella typhi</i>
<i>Aspergillus fumigatus</i>	0.00 ± 0.00 ^e	25.00 ± 0.40 ^b	11.00 ± 0.40 ^c	13.00 ± 0.40 ^d
<i>Cladosporium sphaerospermum</i>	12.75 ± 0.47 ^b	0.00 ± 0.00 ^d	15.25 ± 0.25 ^b	0.00 ± 0.00 ^e
<i>Colletotrichum</i> sp.	0.00 ± 0.00 ^e	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^e
<i>Curvularia eragrostidis</i>	0.00 ± 0.00 ^e	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^e
<i>Fusarium oxysporum</i>	0.00 ± 0.00 ^e	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^e
<i>Fusarium sporotrichioides</i>	0.00 ± 0.00 ^e	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^e
<i>Fusarium</i> sp.	13.25 ± 0.62 ^b	16.00 ± 0.40 ^c	15.00 ± 0.40 ^b	15.00 ± 0.40 ^b
<i>Pestalotiopsis guepini</i>	0.00 ± 0.00 ^e	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^e
<i>Zygorhynchus</i> sp.	12.25 ± 0.25 ^b	0.00 ± 0.00 ^d	11.00 ± 0.40 ^c	14.00 ± 0.40 ^c
Positive control (Streptomycin 50 µg/ disc)	32.00 ± 0.40 ^a	26.00 ± 0.40 ^a	28.00 ± 0.40 ^a	29.00 ± 0.40 ^a
Negative control (PDA Agar plug)	0.00 ± 0.00 ^e	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^e

Values are means of three independent replicates. ± indicate standard error. Means followed by the same letter(s) within the same column are not significantly different according to Tukey's HSD.

Table 3: Antibacterial activity of ethyl acetate extracts of selected endophytic fungi against test bacteria by disc diffusion assay (Zone of inhibition in mm).

Endophytic fungi	Test Bacteria			
	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Salmonella typhi</i>
<i>Aspergillus fumigatus</i>	37.75 ± 0.47 ^a	34.75 ± 0.25 ^a	32.00 ± 0.40 ^a	44.25 ± 0.47 ^c
<i>Cladosporium sphaerospermum</i>	30.25 ± 0.47 ^c	28.25 ± 0.47 ^c	27.50 ± 0.50 ^b	27.75 ± 0.47 ^c
<i>Fusarium</i> sp.	28.50 ± 0.64 ^c	31.50 ± 0.50 ^b	28.00 ± 0.57 ^b	54.75 ± 0.62 ^a
<i>Zygorhynchus</i> sp.	30.25 ± 0.47 ^c	28.75 ± 0.47 ^c	28.50 ± 0.28 ^b	48.75 ± 0.47 ^b
Streptomycin	32.25 ± 0.47 ^b	28.00 ± 0.40 ^c	29.00 ± 0.40 ^b	29.50 ± 0.28 ^d
Negative control	0.00 ± 0.00 ^d	0.00 ± 0.00 ^d	0.00 ± 0.00 ^c	0.00 ± 0.00 ^f

Values are means of three independent replicates. ± indicate standard error. Means followed by the same letter(s) within the same column are not significantly different according to Tukey's HSD.

Table 4: MIC of ethyl acetate extracts of selected endophytic fungi against test bacteria (mg ml⁻¹).

Endophytic fungi	Test Bacteria			
	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Salmonella typhi</i>
<i>Aspergillus fumigatus</i>	0.156	0.156	0.312	0.156
<i>Cladosporium sphaerospermum</i>	0.625	0.625	0.625	0.625
<i>Fusarium</i> sp.	0.625	0.625	0.625	0.312
<i>Zygorhynchus</i> sp.	0.312	0.625	0.625	0.156
Control (Streptomycin)	0.156	0.007	0.156	0.007

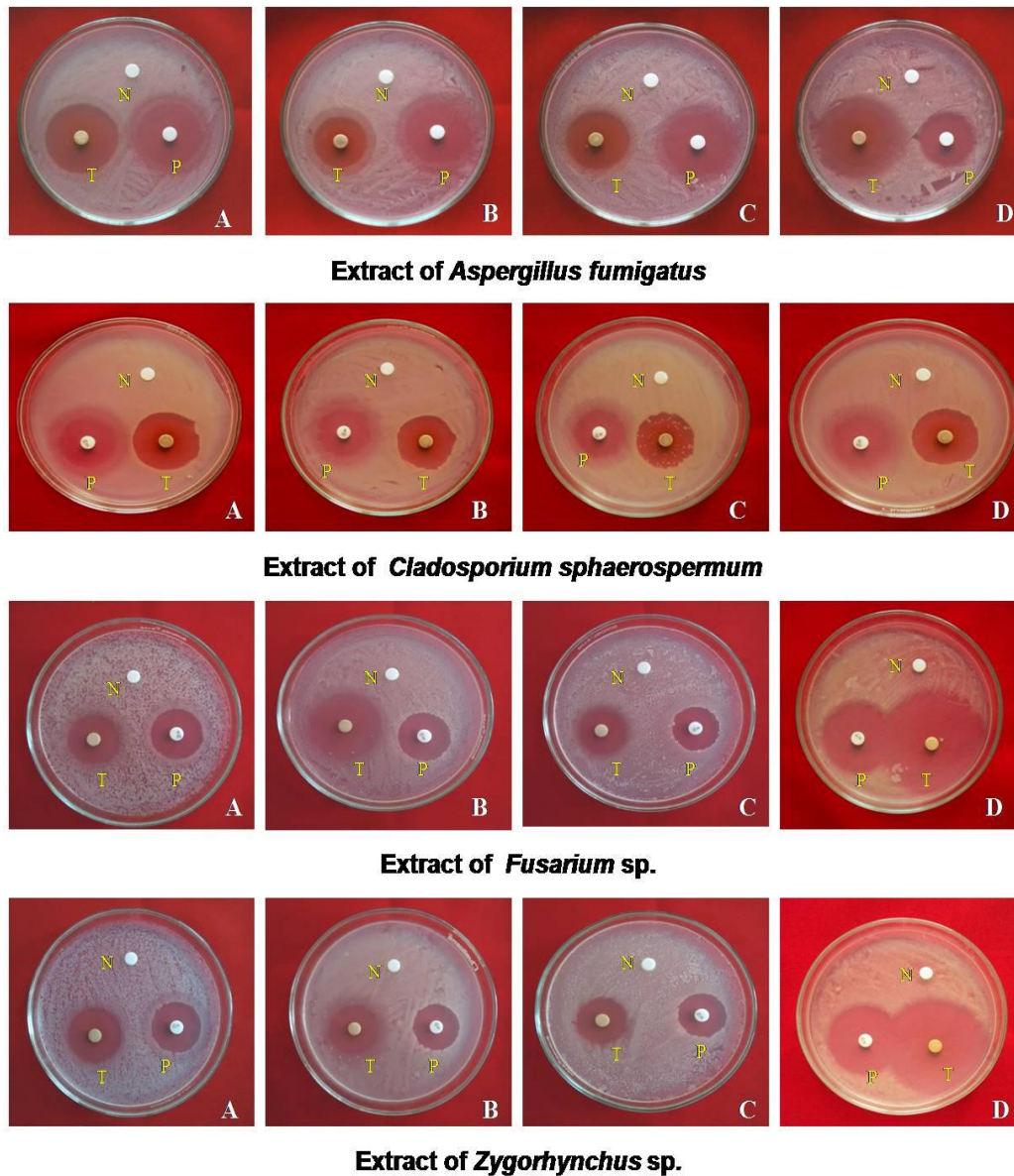


Fig. 1: Antibacterial activity of ethyl acetate extracts of selected endophytic fungi against test pathogens by disc diffusion method. A: *B. subtilis*; B: *Staph. aureus*; C: *E. coli*; D: *S. typhi*; N: Negative control; P: Positive control; T: Treatment.

Secondary screening

The fungal isolates that showed antibacterial activity in primary screening were subjected to aerobic liquid fermentation and tested for its potential against test pathogens by disc diffusion method. The selected endophytic fungi that had offered antibacterial activity against one or all test pathogens in primary screening, but when subjected for secondary screening by using ethyl acetate extract showed significant antibacterial activity against all the test pathogens. Among the test endophytic fungi maximum inhibition zone against all the test pathogen was offered by the crude ethyl acetate extracts of *A. fumigatus*, followed by *Zygorhynchus* sp. and *C. sphaerospermum* extracts, respectively (Table 3).

Minimum Inhibitory Concentration (MIC) by Micro broth dilution assay

All the ethyl acetate extracts of selected fungi were further subjected for MIC as they offered significant antibacterial activity against test pathogens. The MIC of the crude ethyl extracts varied in the range of 0.156 to 0.625 mg ml⁻¹ (Table 4). The results of the study revealed that 0.156 mg ml⁻¹ of the extract was more than enough to inhibit *B. subtilis*, *S. aureus* and *S. typhi*, while 0.312 mg ml⁻¹ of the extract was required to inhibit *E. coli*. Among the test ethyl extracts, *A. fumigatus* had minimum MIC value of 0.156 mg ml⁻¹, while the extracts of *Fusarium* sp. showed highest MIC values with 0.625 mg ml⁻¹ against test bacteria.

DISCUSSION

Endophytes are garnering increased attention because they are ubiquitous and possess to have varied roles including protection to host plants against various pathogens and herbivores and potential to produce bioactive compounds (Arnold *et al.*, 2003; Strobel, *et al.*, 2004; Schulz and Boyle, 2005; Saikkonen *et al.*, 2006; Rudgers *et al.*, 2007). The present day research is focused on isolating novel compounds of biological importance from endophytic fungi as they are unexplored to a greater extent. In the present study, the diverse endophytic population was detected to colonize *B. diffusa*. A total of twelve endophytic fungi belonging to six different genera were isolated. The overall colonization frequency was determined as 24% in surface sterilized tissues. Among the endophytic fungal population, *Cladosporium sphaerospermum* was the most dominant endophyte in *B. diffusa*. Likewise, many endophytic fungi have been isolated from other plant species (Saikkonen *et al.*, 2006; Rudgers *et al.*, 2007).

Microbial endophytes are considered as a promising source for producing a variety of antimicrobial compounds and numerous attempts have been made to isolate and identify bioactive metabolites from endophytic fungi by researchers (Strobel *et al.*, 2004; Saikkonen *et al.*, 2006; Rudgers *et al.*, 2007). In the present study, all the isolated endophytic fungi were subjected for preliminary antibacterial screening by agar plug

method against four potent pathogens. The results of the study revealed that a total of four endophytic fungi were able to inhibit the growth of one or all the test pathogens (Table 1) which is significantly higher as 33% of isolated endophytic fungi offered antibacterial activity. The results corroborate with that of Gong and Guo (2009) where a total of 8.3% of endophytic fungi isolated from *Dracaena cambodiana* and *Aquilaria sinensis* showed antimicrobial activity. It is interesting that four endophytic fungi, *Aspergillus fumigatus*, *Cladosporium sphaerospermum*, *Fusarium* sp. and *Zygorhynchus* sp. inhibited the growth of at least two of the four pathogens (50%), showing their broad spectrum antibacterial activity. There are reports on antimicrobial and cytotoxicity potential of various groups of endophytic fungi isolated from different hosts (Kharwar *et al.*, 2011).

Further, the ethyl acetate extract of the selected fungi were subjected for their antibacterial potential by disc diffusion method. The results offered greater antibacterial potential when the crude ethyl extracts were used. The results obtained may be ascribed for their antimicrobial potential of the extract or due to the high concentration of unidentified active principle present in them. The results are in agreement with earlier studies, wherein the crude extracts of endophytic fungi have yielded more potent compounds that offer greater antimicrobial activity once they had undergone some purification (Fabry *et al.*, 1998; Devi *et al.*, 2012).

From the MIC study, it has been observed that the endophytic fungi extract exhibited activity against tested bacteria (both Gram-positive and Gram-negative). All the crude extracts of endophytic fungi presented significant antibacterial activity against the test pathogens. The MIC value of the extracts ranged from 0.156 to 0.625 mg ml⁻¹. Among the test endophytic extracts, *A. fumigatus* was highly active against all the test pathogens when compared to other extracts (Table 4). However, many studies have shown that several endophytic fungi isolated from *Paris polyphylla* var. *yunnanensis*, *Acanthus ilicifolius* and *Acrostichum aureum* showed broad spectrum antimicrobial activity (Maria *et al.*, 2005; Zhao *et al.*, 2010). The results of the study have highlighted that the ethyl acetate extracts of selected endophytic fungi may have a relatively high proportion of active antibacterial compounds against test bacterial pathogens. The overall findings of this study denote that *Boerhaavia diffusa* was sampled for the first time to isolate endophytic fungi, and interestingly the plant harbored good diversity with greater antibacterial activity. The endophytic fungi isolated from the host *B. diffusa* and their promising antibacterial activity against test pathogens may lead to identify active principle present in them.

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