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Research Article

ASSESSMENT OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF A SPECIES OF *ASPERGILLUS:* AN ENDOPHYTIC FUNGUS OF *SCHIMA WALLICHII* (DC.) KORTH. LEAVES

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ABSTRACT

Objective: The main goals of this study were to check the antimicrobial and antioxidant potentials of an endophytic fungal strain isolated from the leaves of *Schima wallichii* (DC.) Korth.

Methods: The antibacterial and antifungal activities of the isolated fungal endophyte Visva-Bharati endophyte fungal (VBEF2) were checked by disc diffusion and agar well diffusion methods, respectively, against six pathogenic bacteria and four pathogenic fungi. The minimum inhibitory concentration (MIC) values and the mode of action of VBEF2 against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* were determined following colony-forming units (CFU) counting method. Antioxidant activity of the isolate was studied following 2, 2-diphenyl-2-picrilhydrazyl (DPPH) reduction assay.

Results: The cell-free supernatants (CFS) of VBEF2 exhibited excellent antibacterial activity against all the bacteria used. The ethyl acetate extract of the endophyte was found to have the MIC of 50 µg/ml and 150 µg/ml against *S. aureus* and *E. coli*, respectively. It showed bactericidal mode of action against both of them. The CFS of the strain VBEF2 also showed excellent activities against two animal and two plant pathogenic fungi by producing zones of inhibition in the range of 10-20 mm. In the DPPH scavenging antioxidant assay, the ethyl acetate extract of VBEF2 was found with a low IC_{50} value of 19.01 µg/ml. The strain VBEF2 was identified as a species of *Aspergillus* based on its colony morphology and structural features observed under a compound light microscope.

Conclusion: The strain VBEF2 can be implemented in various fields of pharmaceutical industry as it showed multidimensional beneficial attributes such as excellent antimicrobial and antioxidant activity.

Keywords: *Schima wallichii*, Endophyte, Antimicrobial activity, Minimum inhibitory concentration, Bactericidal, Antioxidant activity.

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INTRODUCTION

Endophytes are microorganisms that colonize the plant tissue without causing any adverse effect or producing symptoms on the plant [1]. Several fungi, bacteria, and actinomycetes have been reported as endophytes previously [2], and the fungal members are most common and frequently isolated from several plants [3-7]. Endophytes play several beneficial roles for their host plants like they can produce innumerous secondary metabolites [8] which shield the host from the pathogens, involve in the mineralization process, and add nutritive value. Endophytes can also produce many precursor molecules of plant metabolism and bioactive compounds which are generally found in the crude drugs like 'Taxol' [9]. Isolation of an endophyte with specific function thus definitely helps in the production of a desired compound or drug in a large scale and the practice is also a less time-consuming procedure. Till now, several endophytes are reported having excellent antimicrobial and antioxidant activities [10-12]. Tripura is a small state and located in the North Eastern part of India. The state represents huge floral diversity with different ethnic groups [13]. *Schima wallichii* (Theaceae) is an indigenous plant of Tripura whose different parts specially leaf is traditionally used by the local people in many medical problems. The leaf paste of it is generally used by the ethnic people for healing cuts and injuries. They also use the leaf decoction for curing flatulence. In our previous study, we reported the leaves of *S. wallichii* (Theaceae) to show excellent antimicrobial as well as antioxidant activities which were collected from Udaipur, Tripura [14]. The present study describes the isolation of endophytic fungi from the leaves of *S. wallichii* and their antimicrobial and antioxidant potentials.

METHODS

Isolation of the endophytes

Fresh leaves of *S. wallichii* were collected from Tripura and were carefully brought to the mycology-plant pathology laboratory, Visva-Bharati. Healthy leaves were washed in the sterilized water, and the laminas were cut into small pieces with sterilized scissor under aseptic condition. Next, the leaf pieces were surface sterilized using 4% sodium hypochlorite (NaOCl) for 5-7 minutes followed by 70% ethyl alcohol for 4-5 seconds. After washing in sterilized water and mild drying, the leaf pieces were placed in the malt extract agar (MEA) plates containing 2% agar-agar for the isolation of the endophytic fungi. Streptomycin (100 µg/ml) was mixed in the isolating medium for avoiding the bacterial contamination. The plates were then incubated at 28°C for the appearance of fungal mycelium.

Identification of the endophyte

The endophytic strain VBEF2 was identified both by noticing its colony nature in MEA plate and its morphological characteristics under compound light microscope. VBEF2 was properly grown on MEA Petri plate till the prominent mycelial growth. Next, the mycelia were stained with cotton blue and lactophenol and identified by observing its structural morphology under both low-power (×10) and high-power (×40) magnifications.

Antibacterial potential of the endophyte

Antibacterial range of the endophytic fungal isolate Visva-Bharati endophyte fungal (VBEF2) was carried out using disc diffusion method [15]. Its antibacterial efficiency was checked against three Gram-

negative pathogenic bacteria, namely, *Escherichia coli* MTCC1667, *Salmonella typhimurium* MTCC98, and *Pseudomonas aeruginosa* MTCC741 and three Gram-positive bacteria, namely, *S. aureus* MTCC96, *Bacillus subtilis* MTCC121, and *Listeria monocytogenes* MTCC657. The strains were procured from Microbial Type Culture Collection (MTCC), IMTech, Chandigarh, India. VBEF2 was cultured in ME broth at 28°C and after its proper growth, paper discs (6mm in diameter) of Whatman No. 1 filter paper soaked with the cell-free supernatants (CFS) of the VBEF2 culture were placed over the pathogenic bacterial lawn in the nutrient agar (NA) plates. In this assay, positive and negative controls used were ciprofloxacin (10 µg/ml) and sterilized dimethyl sulfoxide (DMSO), respectively. The plates were incubated at 35-37°C for 24 hrs.

Minimum inhibitory concentration (MIC) of the ethyl acetate fraction of the endophyte

As VBEF2 showed good antibacterial efficiency, its MIC values against one Gram-positive and one Gram-negative bacteria, i.e., *S. aureus* and *E. Coli,* respectively, were determined. The CFS of VBEF2 was extracted with 40% ethyl acetate. After proper drying of the extract, different concentrations of it, namely, 25, 50, 100, 150, and 200 µg/ml were prepared in DMSO. These different concentrations were added to respective test tubes containing NB and fixed volume of bacterial culture. Next, the test tubes were incubated at 37°C for overnight. After proper incubation, 100 µl of each bacterial culture were spread on the NA plates with proper dilution. Finally, the plates were again incubated at 37°C for overnight and the MICs were calculated by CFU counting method.

Mode of action of the endophyte

The mode of action of the ethyl acetate extract of VBEF2 against *S. aureus* and *E. coli* was determined following time-kill study. Two simultaneous experimental sets for each bacterium were run as control and treated set. The ethyl acetate extract of VBEF2 was added in the actively growing cultures of *S. aureus* and *E. coli* at its MIC values in the treated sets. CFU for successive 10 hrs from both sets of each bacterium was recorded to determine the mode of actions of the extracts [16].

Antifungal efficiency of the endophyte

The antifungal activity of VBEF2 was checked by agar well diffusion method [17] against two plant pathogenic fungi, namely, *Helminthosporium compactum* MTCC351 and *Alternaria alternata* VBAV007 and two animal pathogenic fungi, namely, *Candida albicans* MTCC1644 and *Aspergillus parasiticus* MTCC2796. The fungal strains were taken either from the Mycology and Plant Pathology Laboratory, Visva Bharati, India or procured from MTCC, IMTech, Chandigarh, India. Pathogenic fungal cultures (100 µl) were spread on the respective MEA plates, in which wells were prepared by cork borer. 50 µl of CFS of fully grown VBEF2 was applied in the wells. Fluconazole (200 µg/ml) and sterilized DMSO were used as positive and negative controls. Experimental plates were incubated at 28°C for 3-5 days.

Antioxidant activity

The antioxidant assay of the ethyl acetate extract of VBEF2 was carried out using stable 2, 2-diphenyl-2-picrilhydrazyl (DPPH) [18]. A stock solution of DPPH (0.004%) was prepared for this assay. Another stock solution of ethyl acetate extract of VBEF2 was prepared by dissolving 0.01 g ethyl acetate extract in 1 ml of methanol. Next, different concentrations (5-150 µg/ml) were made by mixing specific volume of endophyte stock solutions and fixed volume of DPPH stock solution and incubated in dark condition for 30 minutes. Finally, after incubation, optical density of each set was measured using spectrophotometer at 517 nm. Methanol with DPPH solution (0.004%) was used as the blank set in this assay. Ascorbic acid was used as the control in this experiment. Next, percentage of inhibition (POI) of each concentration set was calculated, from which the IC_{50} value of the ethyl acetate extract of VBEF2 was determined. POI of each set was calculated using the formula given below:

POI of DPPH activity (%) = [(a−b)/a] × 100; a = O.D. of blank set and b = O.D. of each set.

RESULTS AND DISCUSSION

Antibacterial activity of the endophytic isolates

Among the 17 endophytic fungal strains isolated from the leaves of *S. wallichii* (Table 1), only VBEF2 exhibited very good antibacterial potentials. Its CFS produced clear zone of inhibition ranging from 10 to 20 mm against the Gram-negative as well as Gram-positive pathogenic bacteria. It showed more antibacterial efficiencies against the Gram-positive bacteria compared to Gram-negative bacteria on the basis of the diameter of zone of inhibition it produced (Fig. 1). This was found may be due to the production of any antibacterial compounds that interfere with the peptidoglycan layers of cell wall of Gram-positive bacteria. The VBEF2 showed highest activity against *S. aureus* and lowest against *P. aeruginosa*. The test bacteria were found to be sensitive to ciprofloxacin and resistant to DMSO.

MIC and the mode of action of VBEF2

By counting the CFU of the test bacteria, the MIC values of ethyl acetate extract of VBEF2 were found to be 50 µg/ml and 150 µg/ml against *S. aureus* and *E. coli*, respectively. These results also attest the fact about the more antibacterial efficiency of the endophyte against Gram-positive bacteria (Table 2). The mode of action of the ethyl acetate extract of VBEF2 against both *S. aureus* and *E. coli* was also determined by counting CFU of the both control and treated sets at every hour. Based on the growth pattern of the bacteria observed in the treated set, the activity of the VBEF2 extract was found to be bactericidal against both the bacterial strains. In the treated sets, the growth of the bacteria declined sharply due to the bactericidal activity of the extract (Fig. 2a and b).

Antifungal activity of VBEF2

The CFS of the endophytic strain VBEF2 was found to show excellent antifungal range as it produced clear zones of inhibition against all the four pathogenic fungi used. The antifungal potential of VBEF2 was determined based on the diameter of zones of inhibition produced by it. The antifungal potential of the endophyte was much higher against the plant pathogenic fungi, i.e., *H. compactum* and *A. alternata*. Moderate antifungal activity of VBEF2 was also found against *C. albicans* and *A. parasiticus* (Fig. 3). All the test fungi were sensitive to fluconazole and resistant to DMSO. *C. albicans* is the causal organism for oral and vaginal candidiasis, and *A. parasiticus* is the pathogen responsible for aspergillosis. Hence, the ability of VBEF2 in controlling these pathogens

Table 1: List of endophytic fungi isolated from the leaves of *S. wallichii*

Leaf S. No.	Endophytic fungal isolate
Leaf1	VBEF1, VBEF2, VBEF3, VBEF4
Leaf2	VBEF5, VBEF6, VBEF7, VBEF8, VBEF9
Leaf3	VBEF10, VBEF11, VBEF12
Leaf4	VBEF13, VBEF14, VBEF15
Leaf5	VBEF16, VBEF17

S. wallichii: Schima wallichii, VBEF: Visva-Bharati endophyte fungal

Table 2: MICs of the ethyl acetate extract of VBEF2 CFS against *S. aureus* and *E. coli*

Concentration (µg/ml)	CFU/ml	
	S. aureus	*E. coli*
Control	1.2×10^{9}	2.1×10^{9}
25	2.2×10^{8}	1.7×10^{8}
50	1.8×10^{5}	1.1×10^{8}
100	1.7×10^{3}	2.1×10^{7}
150	3.1×10^{2}	3.4×10^{4}
200	1.8×10^{2}	2.1×10^{2}

MIC: Minimum inhibitory concentration, VBEF: Visva-Bharati endophyte fungal, CFS: Cell-free supernatants, *S. aureus: Staphylococcus aureus, E. coli: Escherichia coli*

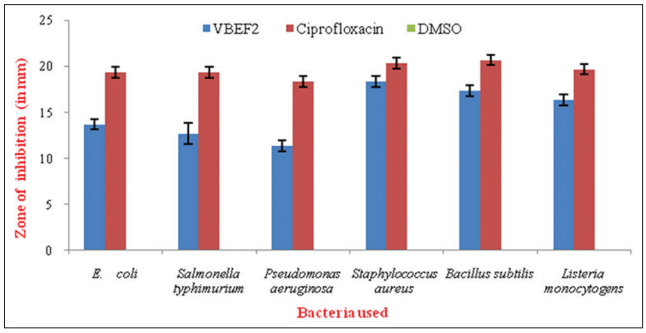

Fig. 1: Antibacterial activity of the cell-free supernatants of VBEF2 against six pathogenic bacteria

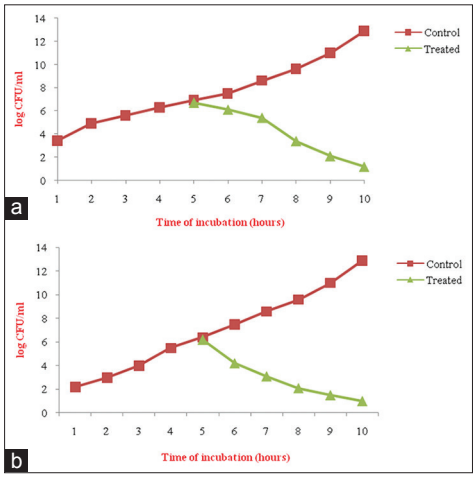

Fig. 2: Mode of action of the ethyl acetate extract of VBEF2 cell-free supernatants against (a) *Staphylococcus aureus* and (b) *Escherichia coli*

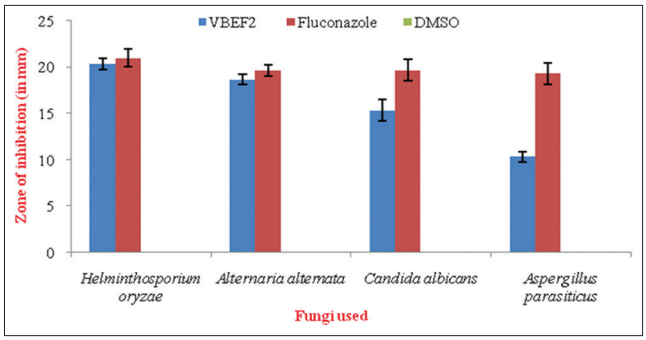

Fig. 3: Antifungal potentials of the cell-free supernatants of VBEF2 against four pathogenic fungi antioxidant activity of VBEF2

as well as the plant pathogenic fungi attests its applicability in the pharmaceutical as well as agriculture sector.

The ethyl acetate fraction of the CFS of VBEF2 showed very low IC_{50} value (19.01 µg/ml) in the DPPH scavenging antioxidant assay. This observation was considered as a good result as its IC_{50} value was found to be close with the IC_{50} value of the control ascorbic acid used (8.5µg/ml). DPPH scavenging pattern of the ethyl acetate fraction of VBEF2 is represented graphically (Fig. 4). Plant extracts contain several phenolic compounds such as flavonoids, phenolic acids, tannins, and phenolic diterpenes which are responsible for the antioxidant effects [19]. Previous reports revealed that the phytochemicals with antioxidant activity may reduce the risk of cancer and also improve health conditions. Till now, many reports

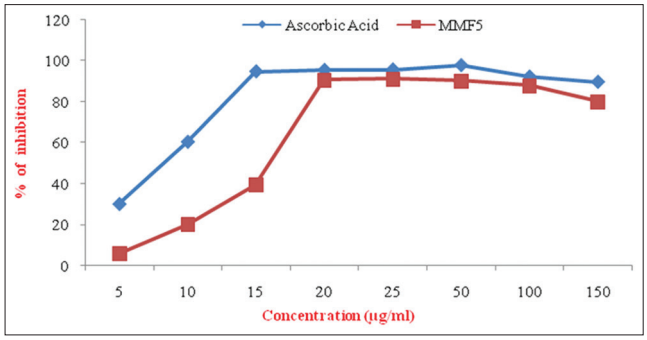

Fig. 4: Antioxidant potential of VBEF2 in DPPH scavenging assay identification of the isolate VBEF2

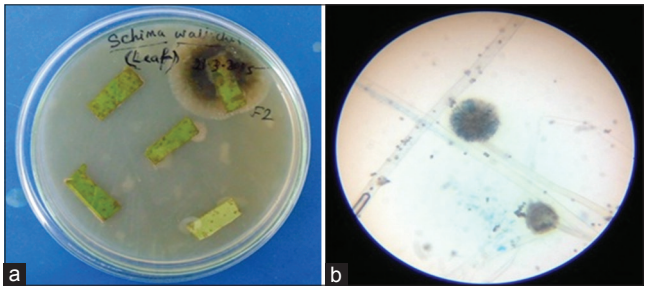

Fig. 5: (a) Colony nature and (b) microscopic view of V0BEF2 under compound light microscope

have been published regarding the antioxidant potentials of several isolates of *Aspergillus* [20].

The colony of the isolate VBEF2 was observed with blackish mycelia with grayish border in MEA plates which are very common features of *Aspergillus* colony. Besides, the presence of unbranched conidiophores with terminal globose vesicles covered by chains of conidia and simple septation in the vegetative mycelia is observed under microscope also helped to identify the fungus as a species of *Aspergillus* (Fig. 5a and b). Previously, an endophytic species of *Aspergillus* isolated from the leaf of *Justicia adhatoda* showed antibacterial activities against *S. aureus*, *P. Aeruginosa,* and *E. coli* [21].

CONCLUSION

17 endophytic fungi were isolated from the leaves of *S. wallichii,* and among them, only VBEF2 exhibited its antibacterial potentials against all the six pathogenic bacteria used. Its low MIC values with bactericidal mode of action against both *S. aureus* and *E. coli* also indicate its antibacterial efficiencies against Gram-positive as well as Gram-negative bacteria. The endophyte was also found to show excellent antifungal potentials against both plant pathogenic as well as animal pathogenic fungi. In the DPPH scavenging antioxidant assay, VBEF2 also showed very good antioxidant activity by showing low IC_{50} value. Hence, the *Aspergillus* isolate can be a great prospect in the pharmaceutical sector for its excellent antimicrobial as well as antioxidation potentials.

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