Anti-Multidrug resistant activity of endophytic fungi isolated from the stem of *Tradescantia pallida* against Gram-positive pathogens

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Abstract

The present study aimed at the isolation and in vitro anti-MRSA and antioxidant properties of endophytic fungi derived from the stem of Tradescantia pallida. The fungal endophytes were isolated in a PDA agar medium. A total of seven isolates were recovered. Sequential solvent extraction was done using different polarity solvents and the crude extracts obtained were tested for their antagonistic activity against drug-resistant bacteria. In vitro, the antioxidant activity of the potent fungus was carried out by DPPH assay.

Crude extract from TPS2 isolate displayed maximum inhibition towards Methicillin-resistant Staphylococcus aureus ATCC 43300 and 700699. It also exhibited free radical scavenging activity in the range of 30 to - 85%. The potential isolate was identified as Aspergillus niger. Thus, we conclude that the dichloromethane crude extract of A.niger fungal can produce antibacterial and antioxidant compounds. Hence the bioactive metabolites can be probed as antibacterial agents in treating infectious diseases.

Keywords: *Aspergillus niger*, endophytes, symbionts, resistant bacteria, gram-positive pathogens, radical scavenging.

Introduction

Antimicrobial resistance is a significant threat to the environment, humans and animals globally, as the resistant microorganisms have spread worldwide because of their genetic plasticity[15].

In microorganisms, antimicrobial resistance development is a natural phenomenon which is enhanced by the selective pressure exercised by misuse of antimicrobial agents in animals and humans and eventually reflects the severe problem in treating pathogenic microbes[3]. Drug resistance is a major global problem, predominantly in hospital settings contributing to the renaissance of intricate infections[5].

Currently, resistant *Staphylococcus aureus* and *Enterococcus* species are the primary threats among gram-positive pathogens. In addition, MRSA (Methicillin-resistant *Staphylococcus aureus*) has become a chief source of nosocomial and community-associated MRSA infections[21].

The development of resistant bacteria to the available antibiotics has demanded an urgent need for new antibacterial agents[18]. Plants are one of the untapped reservoirs of new bioactive molecules[14]. They produce many biological metabolites such as saponins, quinones, alkaloids, terpenoids, tannins, flavonoids and coumarins[8]. Plant crude extracts and their phytochemicals as antimicrobial properties have great significance in treatments[17]. Plants can aid as reservoirs for abundant microorganisms. They are classified based on their location and function in the plant. Since ancient times, plants and extracts have been used to treat various conditions as they are considered safe, are readily available and cost-effective[5,19].

Endophytes are microbes that reside within the inner tissues of their hosts without causing apparent symptoms and damage[1]. Almost all parts of the plants were known to harbor endophytes[12]. Regardless of their habitat differences, their metabolites offer one or more healing properties[10]. They are an outstanding source of biologically active natural compounds as they can synthesize similar functional products to their host plant[20]. They produce a wide range of bioactive compounds with unique mechanisms that protect their host from biotic and abiotic stress[13]. Besides, the novel chemical structure of secondary metabolites has antibacterial, antifungal, anti-inflammatory, neuroprotective, antioxidant, antiparasitic, antitumor and cytotoxic activities[16].

Currently, in therapeutics, endophytic fungi have become one of the significant sources of bio-actives. Among bioactive compounds isolated from all the available biosources, 38% of biologically active metabolites originated from fungi[2]. Secondary metabolites from endophytes can inhibit many pathogens in humans, plants and animals. Thereby isolation of endophytes from medicinal plants is considered a resource for novel compounds and has become more enjoyable to be explored[16]. *Tradescantia pallida* (Rose) D.R. Hunt var. purpurea Boom is a perennial plant in the family Commelinaceae. It originated in American countries and was distributed from the southern part of Canada to northern Argentina.

It has been reported that leaf extracts demonstrated anticancer activity against cervical cancer cell lines and antioxidant properties. It also showed antagonism against gram-positive and gram-negative bacteria[11]. The study investigated fungal endophytes associated with the *T.*

pallida stem and their antibacterial activity against selected drug-resistant pathogens.

Material and Methods

Chemicals: All the chemicals and media were procured from HiMedia and SRL analytical grade.

Sampling: The healthy stems of *Tradescantia pallida* were collected from the Vellore district and transported to the laboratory in a sterile polythene bag. The samples were stored at 4°C until further processing.

Isolation of Endophytic fungi: Surface sterilization was carried out by treating the sample surface with 1% sodium hypochlorite for a minute followed by 70 % ethanol for 30 seconds and washing it with distilled water two to three times. Then the segments were dried thoroughly and placed on a potato dextrose agar medium and the plates were incubated at 27°C for 7 days. Post incubation, the emerging endophytic fungus was selected, subcultured on a PDA plate and incubated to get pure colonies.[9]

Colony morphology: Macroscopically the colony morphology of the fungus was identified based on size, shape and color. Isolates were further identified microscopically by the lactophenol cotton blue method.

Fungal Cultivation and solvent extraction: The fungal isolates were cultivated by inoculating 3 mm agar blocks of actively growing pure endophytic fungi in an Erlenmeyer flask containing 100 mL of PDB (potato dextrose broth). The flasks were incubated for 21 days at 27°C. After incubation, the mycelia mat and broth were separated using filter paper. Then the filtrate was extracted with different polarity solvents (Dichloromethane, Ethyl acetate, Butanol) using a separating funnel in a 1:1 ratio. All the collected extracts were evaporated using a rotatory evaporator and the resultant compound was used for further studies.[6]

Inoculum preparation: The antibacterial property of the fungal crude extracts was determined against gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* 43300 and 700699, *S.auerus* (ATCC 25923) and (MTCC 3160). The bacterial suspension was prepared by inoculating it in a tryptic soy broth medium.

Antibacterial susceptibility *in vitro* testing: The agar well diffusion method tested the *in vitro* antibacterial activity of crude extracts from the endophytic fungi *Aspergillus niger* against resistant bacterial pathogens. Muller Hinton agar plates were prepared and fresh microbial lawn cultures were made using a cotton swab.

Wells of 5mm diameter were bored using a sterilized cork borer and 100 µL of different concentrations (25, 50, 75, 100 µg/mL) prepared from 1mg/mL of extracts were added. Then the plates were incubated overnight at 37°C and the zone of inhibition around the wells was recorded in mm. Oxacillin and streptomycin were used as positive and DMSO as the negative control.[15]

Determination of Minimum Inhibitory Concentration: Minimum inhibitory concentrations of the crude extracts were determined using Micro-broth dilution. The extract concentration was prepared in 50 to 0.39 µg/mL. About 100 µL of Muller Hinton broth was added to flat bottom 96 well plates. Two-fold extract dilutions were loaded and 10 µL of bacterial culture was added. The well with broth alone is a negative and oxacillin is a positive control. The plates were incubated overnight at 37°C. The lowest concentration of the extract that shows no visible growth or turbidity, was considered MIC.[19]

Free radical scavenging activity: The free radical scavenging activity of the fungal extracts was examined *in vitro* using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. About 1mg of crude extracts was dissolved in dimethyl sulfoxide (DMSO). Various concentrations (50, 100, 150 µg/mL) of the extract were prepared and added to the test tube containing 2mL of DPPH. The mixture was shaken vigorously and left to stand in a dark condition for 30 min. After the incubation period at 517 nm, absorbance was measured on a UV-Visible spectrophotometer against a reagent blank. Control was prepared by adding all the solutions without the sample. The reference standard used was gallic acid. The reaction was carried out in triplicate. The percentage inhibition for scavenging DPPH radical was calculated according to the equation:[T4]

$$\%\text{decolorization} = [(Abs\ of\ control - Abs\ sample)/ Abs\ of\ control]\times 100$$

Phytochemical screening: A standard phytochemical screening protocol was carried out to analyze the presence of the group of secondary metabolites (alkaloids, flavonoids, terpenoids, saponins and tannins) in the dichloromethane fungal extract.[4]

Results and Discussion

Endophytic fungi isolation and Identification: A total of seven morphologically different endophytic fungi were isolated from the healthy stems of *T. pallida* and coded as TPS1 to TPS7. The most potent fungal isolate against bacterial pathogens was subjected to morphological and microscopic identification. The colony morphology and lacto phenol study identified it as *Aspergillus niger* (Fig. 1).

Anti-MRSA activity: The crude extracts from seven endophytic fungi were evaluated for their antibacterial activity against test pathogens. Among them, the maximum antagonism of 14 and 15 mm was observed in the dichloromethane extract of *Aspergillus niger* against MRSA 43300 and 700699 respectively at 100 µg/mL concentration. The ethyl acetate crude extract exhibited moderate activity of 12 and 13 mm. There was no activity against any test pathogens at 25 µg/mL and butanol against MRSA. No zone

of inhibition was seen in positive control oxacillin (1mcg) against MRSA and about 15 mm zone was exhibited by streptomycin control used against *S. aureus*. The data relating to the antibacterial potential of the fungal extracts are shown in table 1 and fig. 2.

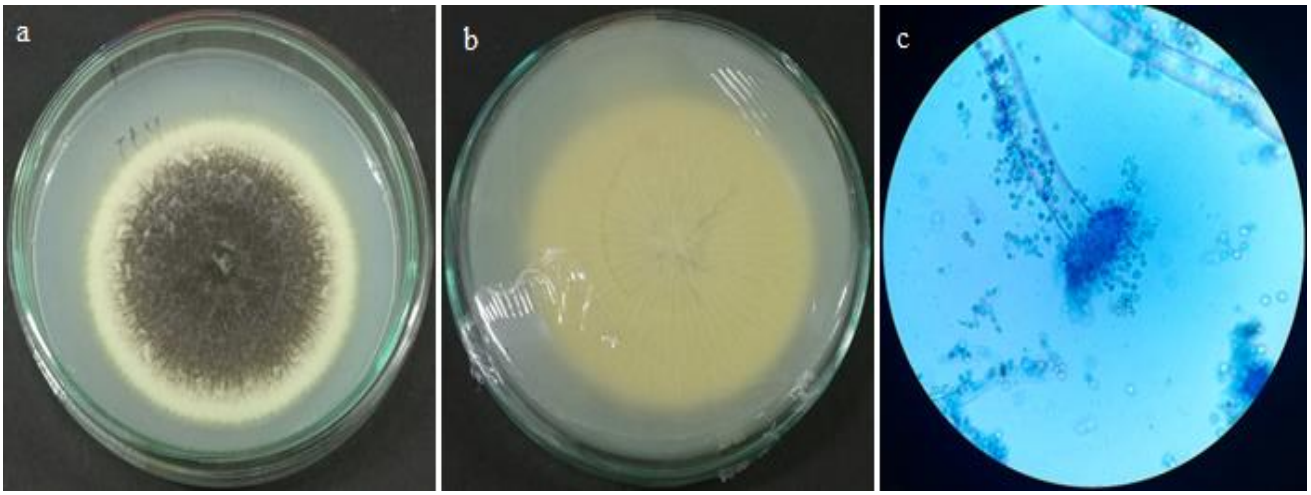

Fig. 1: (a) Pure endophytic fungal colony, (b) Reverse position of the culture plate and (c) Microscopic view

Table 1
Antagonistic activity of *A. niger* crude extracts

Pathogens	Concentration of extracts (µg/mL)	Zone of inhibition (measured in mm)		
		DCM	EA	Butanol
MRSA (ATCC 43300)	50	-	-	-
	75	12	-	-
	100	14	12	-
MRSA (ATCC700699)	50	-	-	-
	75	13	-	-
	100	15	13	-
S. aureus (ATCC 25923)	50	17	12	11
	75	19	18	15
	100	21	21	18
S. aureus (MTCC 3160)	50	15	11	12
	75	19	16	14
	100	20	19	17

DCM= Dichloromethane, EA= ethyl acetate, (-) = Not activity.

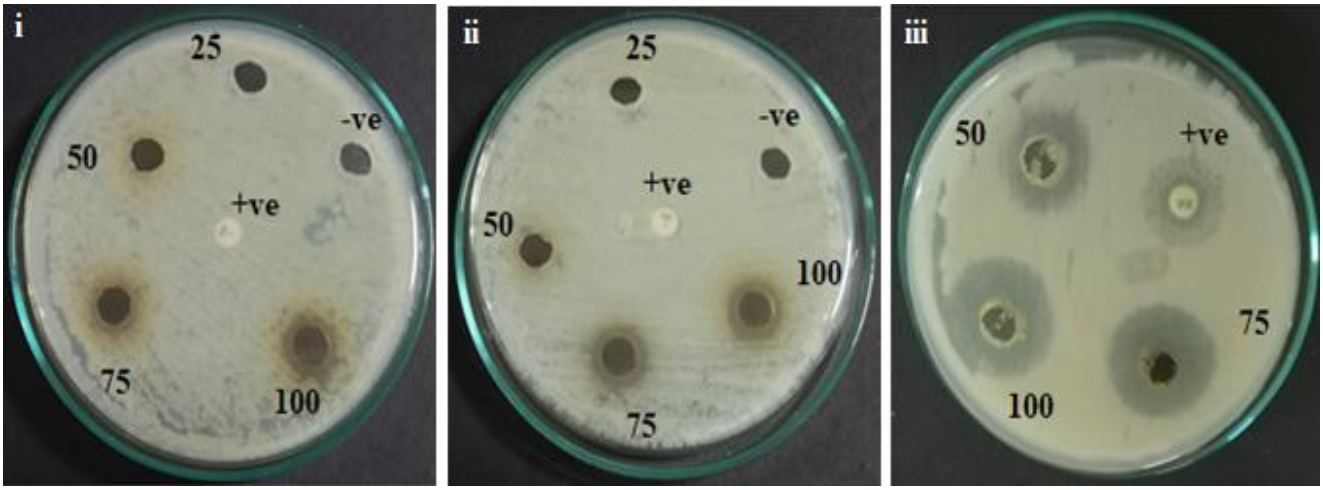

Fig. 2: Antagonistic activity of DCM crude extract against MRSA (i) ATCC 43300; (ii) ATCC700699; (iii) *S.aureus* (ATCC 25923)

Table 2
MIC of *A. niger* crude extracts

Crude extract	DCM	EA	Butanol
	MIC (µg/mL)		
MRSA (ATCC 43300)	50	50	-
MRSA (ATCC 700699)	25	50	-
S. aureus (ATCC 25923)	6.25	3.125	12.5
S. aureus (MTCC 3160)	3.125	6.25	12.5

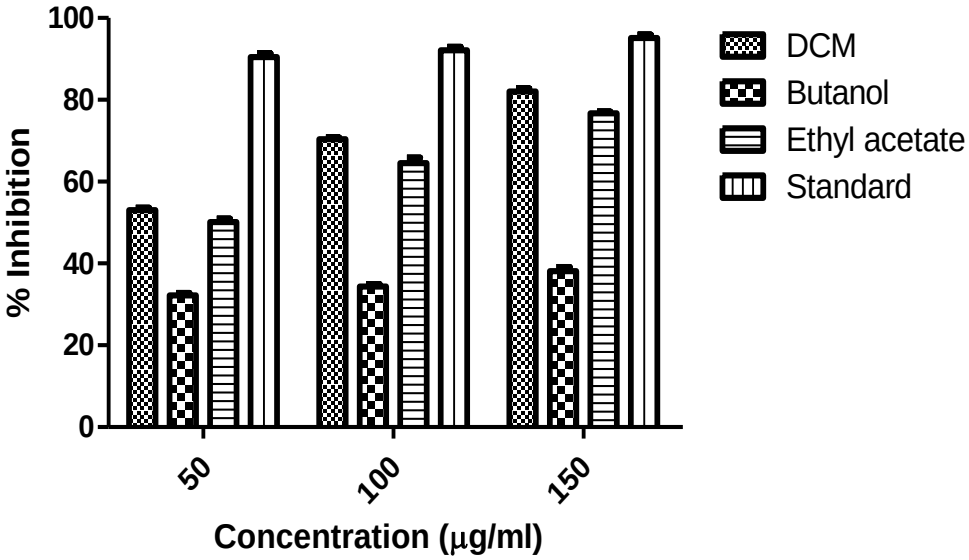

Fig. 3: Free radical scavenging activity

MIC evaluation: Based on the results obtained from the antibacterial study, the potent DCM crude extract of *A. niger* was taken further to determine minimum inhibitory concentration using the broth microdilution method. The extract inhibited MRSA 43300 and 700699 at the lowest MIC of 50 and 25 µg/mL. It inhibited *S.auerus* (ATCC 25923) and (MTCC 3160) at 6.25 and 3.125 µg/mL respectively. The data reporting MIC against test pathogens was tabulated in table 2.

Qualitative phytochemical screening: The phytochemical investigation revealed that the dichloromethane extract of *A.niger* contains alkaloids, flavonoids, tannins, saponins and terpenoids that are well-known for their antibacterial activities.

Determination of antioxidant capacity: Free radical scavenging activity of different extracts obtained from *Aspergillus niger* displays significant antioxidant activity. The study was carried out at 50-150 µg/mL concentration and revealed a dose-dependent increase in inhibitory percentage against the antioxidant activity. In comparison, dichloromethane extract has shown maximum inhibition of 81±0.5 % at 150 µg/mL concentration. The second highest inhibition was in ethyl acetate extract at about 77±0.85 % whereas butanol extract showed minimum inhibitory activity of 38±0.7%. Finally, the standard gallic acid showed an inhibition rate of 97±0.23 % as shown in fig. 3.

Conclusion

The present investigation concludes that the dichloromethane extract of *A.niger* possesses significant antibacterial and antioxidant potency. Hence, it serves as a key platform for further phytochemical and pharmacological studies for developing a new alternative drug for antibiotic-resistant bacterial pathogens at a commercial scale.

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