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Original Article

Preliminary Screening for Antibacterial Activity of Endophytic Fungi isolated from *Azadirachta indica* and *Mentha piperita* Phyllosphere against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*

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ABSTRACT

Endophytes have been identified as reservoirs of novel bioactive secondary metabolites that can serve as a potential candidate for the development of new antimicrobial drugs. The aim of the study was to screen for antibacterial activity of endophytic fungi isolated from *Azadirachta indica* and *Mentha piperita* phyllosphere (healthy leaves). The endophytic fungi isolates were screened for their antibacterial activity against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa.* Antibacterial activity of ethyl acetate extracts of the isolates was also determined. A total 35 endophytic fungi were isolated out of which 11 showed antibacterial activity against at least two of the test bacterial isolates. Ethyl acetate extracts of these 11 endophytes had varying degree of antibacterial activity with zones of inhibition ranging from 10±10 mm to 26±0.5 mm. Result of this study revealed that endophytic fungi isolated leaves of *A. indica* and *M. piperita* produce bioactive compounds with antibacterial activity against the test bacterial isolates.

1. Introduction

The emergence of multidrug bacterial strains calls for search of new antibacterial compound. One of the methods can be applied in the development of novel antibiotics is screening for previously undiscovered bioactive compounds in the known resource pool such as endophytes, marine microorganisms and soil microorganisms [1-3].

Azadirachta indica (neem) belonging to the meliaceae family is very important medicinal plant traditionally used to treat difference diseases such as gastrointestinal upset, diarrhea and malaria in Nigeria. The various parts of neem (leaf, bark, steam, and seed) are used as a traditional medicine and it has been shown to exhibit wide pharmacological activities such as antibacterial and antiviral [4].

Mentha piperita (Peppermint) plant is a perennial glabrous strongly scented herb and medical crop. Its oil and tea are used often in the treatment of gas and indigestion. It has been reported to possess antibacterial, antifungal and antiviral activities [5].

Endophytic fungi are fungi that colonize internal plant tissue, where they and their host plants establish very complex relationship without causing any apparent disease [1,6]. They produce plant growth promoter hormones and act as reservoirs of secondary metabolites with biological activities [1]. They constitute an important source for drug discovery as these metabolites serve as potential candidates for the development of new antimicrobial drugs. Novel compounds have been isolated from endophytes and their antimicrobial activities have been reported by any

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researchers [1,7,8]. Endophytic fungi are not considered saprophytes as their associated with living tissues of plants contribute in some way to the well-being of the plant. Plants are provide nutrients to the microbe, while the microbe produce factors that protect the host plant from attack by animals, insects or microbes [9].

Endophytic fungi are diverse and they evolved adapt to special and unusual environments, hence they are considered great source of novel antimicrobial drugs with diverse uses [10-12].

The aim of the study was to determine the antibacterial activity of medicinal plants associated endophytic fungi isolates against clinical isolates of *S. aureus*, *E. coli* and *P. aeruginosa*.

2. Materials and Methods

2.1. Plant collection

Healthy leaves of *Azadirachta indica* and *Mentha piperita* were collected within Ahmadu Bello University, Zaria Nigeria in aseptic conditions to avoid any external contamination, in clean polythene bag and taken to Herbarium, Department of Botany Ahmadu Bello University Zaria for identification.

2.2. Isolation and identification of endophytic fungi

The leaf samples were washed with clean water under running tap water and then surface sterilized by immersing in; 95% ethanol for 1 min, followed by 3.5% (v/v) sodium hypochlorite solution for 3 min and finally 70% (v/v) ethanol for 30s. The surface sterilized leaves were then washed twice in sterile distilled water and allowed to dry on filter papers under sterile condition. The samples were cut into small pieces of dimensions 6mm x 6mm with the help of sterile blade, placed on the surface of Potato Dextrose Agar (PDA) and incubated at room temperature for 7 days. The fungal mycelium growing out from the leaves were subsequently transferred onto fresh PDA plates by hyphal tip transfers and incubated at room temperature for 7 days. The endophytic fungal isolates were maintained in PDA for future studies [13]. The endophytic fungal isolated were identified based on their macroscopic and microscopic characteristics using Larone Atlas of Mycology as guide.

2.3. Screening of the antibacterial activity

The endophytic fungi were screened for antibacterial activity for rapid and qualitative selection of bioactive compound producing fungal isolates. Clinical isolates of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* used as test bacterial isolates in this study were gotten from the main teaching laboratory of Microbiology department. Endophytic fungi were grown on PDA for 7 days after which their mycelia plugs were transferred onto the surface of Mueller Hinton agar previously inoculated with the test clinical bacterial isolates. The plates were incubated at 37ºC for 24 hours and then observed for antibacterial activity indicated by presence of zones of inhibition around the fungal isolates [13].

2.4. Extraction of bioactive substances from endophytic fungi

The endophytic fungi that showed antibacterial activity were inoculated on Malt Extract Agar (MEA) and incubated at 30°C for 10 days. Five fungal discs (6 mm diameter each) from the edges of growing cultures were inoculated into 500 mL Erlenmeyer flasks containing 100 mL of Malt Extract Broth (MEB) and incubated at 220 rpm in a shaker incubator for 14 days. The broths were then filtered and extracted exhaustively with ethyl acetate. The extracts were evaporated under vacuum in a rotary evaporator to yield ethyl acetate extracts. The extracts were dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C as stock solution [13].

2.5. Screening for antimicrobial activity of ethyl acetate extract of endophytic fungi

Antibacterial activity of ethyl acetate extract of the endophytic fungal isolates was determined by Agar Well Diffusion Method. Briefly, sterile swabs were dipped into 0.5 McFarland standardized inocula and then excess fluid was removed by rotating the swab against the side of the tube. Mueller Hinton agar was then inoculated by spreading the swab stick three times over the surface of the agar, rotating the plate approximately 60º each time to ensure even distribution of the inoculums. The plates were then allowed to sit at room temperature for 3-5minutes for the surface of the agar to dry. Using a sterilized cork borer (5 mm), four wells of equal distance were bored in each plate and filled with the extracts. The extracts were allowed to diffuse prior to incubation at 37ºC for 24 hours. Inhibition zones were indicated by clear area around the wells which was measured to the nearest millimeters using a transparent ruler [14].

3. Results and Discussion

Sixteen (16) and nineteen (19) endophytic fungi were isolated respectively from healthy leaves of *Azadirachta indica* and *Mentha peperita* giving a total of 35 endophytic fungi isolates. The fungal isolates belong to the genera: *Trichoderma* (10), *Rhizoctonia* (1), *Penicillium* (6), *Aspergillus* (6) and *Fusarium* (12) (Figure 1).

Isolation of endophytic fungi from health of these medical plants indicated probably a symbiotic relationship between the endophytes and the medicinal plants. The antimicrobial properties of medicinal plant are partly due to the ability of its endophytic microorganisms to produce bioactive secondary metabolites [15, 16].

Members of the genus *Fusarium* were the endophytes with

the highest isolation rate; this is likely due to the fact that they are widely found in plants, this is similar to the report of Du *et al.* [1].

Result of the preliminary screening for antibacterial activity of the endophytic fungi against *Staphylococcus aureus*, *Escherichia coli* and *P. aeruginosa* showed that 11 (31.4%) of the endophytic fungi had antibacterial activity (Table 1 and Figure 2). Antibacterial activities were observed against all the three bacterial isolates with the exception of *Trichoderma* sp. AL1, *Aspergillus nidulans* AL15 *Aspergillus nidulans* ML12 and *Aspergillus fumigatus* ML19 that had activity against two isolates.

Broad spectrum of activity i.e. activity against all the test bacterial isolates was exhibited by 63.6% (7/11) of the endophytes. This implies that they produce bioactive compounds with antibacterial potential.

The percentage of bioactive compound producing endophytic fungi isolated (31.4%) in this study is comparable to 33.6% reported by Santos *et al.* [12] for endophytic fungi isolated from leaves of *Indigofera suffruticosa.* However, lower percentages were reported by Lin *et al*. [17], Gong and Guo [18] and An *et al.* [19] where 27.6% of endophytes isolated from *Camptotheca acuminate*, 8.3% of endophytes isolated *Dracaena cambodiana* and *Aquilaria sinensis* and 10.0% of endophytic fungi isolated from *Chloranthus japonicus* showed antimicrobial activity against pathogens respective.

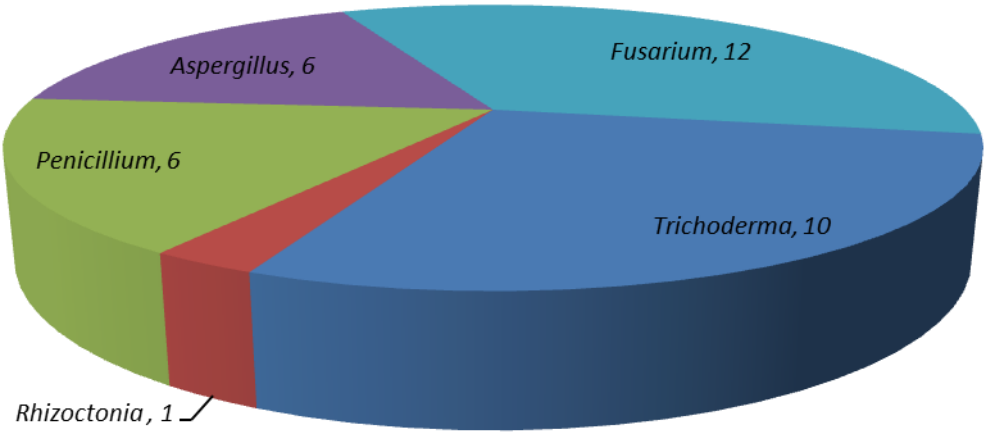
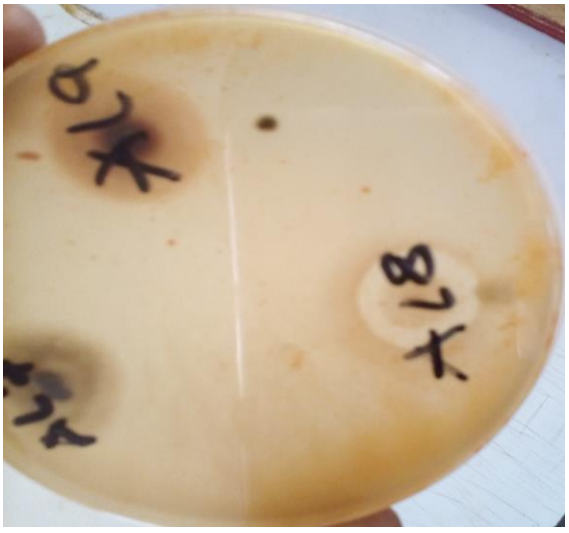


Fig 1. Genus distribution of the endophytic fungi isolated from phyllosphere of *Azadirachta indica* and *Mentha piperita*.

Fig 2. Antibacterial activity of endophytic fungi against test bacterial isolates.



Endophytic fungi belonging to the genus *Fusarium* and *Penicillium* exhibited broad spectrum of antibacterial activity. Highest antibacterial activity against *P. aeruginosa* were exhibited by *Penicillium* sp. ML17 (26.0±0.0 mm) and *Fusarium oxysporum* ML1 (26.0±0.5 mm). This is in agreement with the report of endophytic belonging to the genus *Fusarium* sp. demonstrating the strongest antibacterial activity against *Pseudomonas aeruginosa* by Zhang *et al.* [20] and Manganyi *et al.* [21].

Table 2 shows the antibacterial activity of ethyl acetate extract of the endophytic fungi isolated against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa.* The zones of inhibition of the endophytic fungi against the test isolates ranged between 10±10 mm and 26±0.5 mm. *Fusarium oxysporum* ML1 exhibited the highest zone of inhibition (21.0±0.5 mm) against *S. aureus* while *Penicillium* sp. ML17 exhibited the highest zone of

inhibition (23.5±0.5 mm) against *E. coli*. Highest zones of inhibition against *P. aeruginosa* were exhibited by *Penicillium* sp. ML17 (26.0±0.0 mm) and *Fusarium oxysporum* ML1 (26.0±0.5 mm). Broad spectrum of activity against all the isolates was exhibited by isolates of *Fusarium* species and *Penicillium* species.

Extracts from the endophytic fungi isolates showed a wide variety of antibacterial activities against the tested bacterial isolates. This implies that the endophytic fungi secrete bioactive compound with antibacterial activity. Antibacterial activities of natural bioactive compounds produced by endophytic fungi against bacteria such as *Staphylococcus aureus, Escherichia coli* [1,12,23], *Pseudomonas aeruginosa* [1,12], *Enterococcus faecalis* [1], *Bacillus subtilis, Klebsiella pneumoniae* [12] and *Mycobacterium tuberculosis* [22] have been reported.

Similar results were observed by Santos *et al.* [12] in a study of antibacterial activity of endophytic fungi from leaf of *Indigofera suffruticosa* Miller (Fabaceae), where 18 out of 65 endophytic fungi showed antibacterial activity against at least 2 of the test bacteria.

Table 1. Screening for antibacterial activity of the endophytic fungi against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*

Endophtic Fungal isolate	**Source**	**Antibacterial activity against**		
		S. aureus	***E. coli***	***P. aeruginosa***
***Trichoderma* sp. AL1**	***A. indica***	+	+	-
***Fusarium* sp. AL8**	*A. indica*	+	+	+
***Penicillium* sp. AL10**	*A. indica*	+	+	+
***Aspergillus nidulans* AL15**	*A. indica*	+	-	+
***Fusarium oxysporum* AL16**	*A. indica*	+	+	+
***Fusarium oxysporum* ML1**	*M. peperita*	+	+	+
***Fusarium* sp. ML2**	*M. peperita*	+	+	+
***Aspergillus nidulans* ML12**	*M. peperita*	+	+	-
***Penicillium* sp. ML17**	*M. peperita*	+	+	+
***Trichoderma* sp. ML18**	*M. peperita*	+	+	+
***Aspergillus fumigatus* ML19**	*M. peperita*	+	-	+

Key: + = presence of antibacterial activity ; - = absence of antibacterial activity

Table 2. Antibacterial activity of ethyl acetate extract of endophytic fungi against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*

Endophtic Fungal isolate	**Source**	**Mean zones of inhibition (mm) ± SE**		
		S. aureus	***E. coli***	***P. aeruginosa***
***Trichoderma* sp. AL1**	*A. indica*	10.0±1.0	17.0±0.0	NT
***Fusarium* sp. AL8**	*A. indica*	14.5±1.0	18.0±0.0	19.0±0.0
***Penicillium* sp. AL10**	*A. indica*	16.0±2.0	20.5±1.0	17.0±1.5
***Aspergillus nidulans* AL15**	*A. indica*	13.0±0.5	NT	12.0±0.0
***Fusarium oxysporum* AL16**	*A. indica*	12.5±0.5	22.0±0.0	19.5±0.5
***Fusarium oxysporum* ML1**	*M. peperita*	21.0±0.5	17.5±0.5	26.0±0.5
***Fusarium* sp. ML2**	*M. peperita*	20.0±0.0	19.0±0.5	19.5±0.5
***Aspergillus nidulans* ML12**	*M. peperita*	13.0±0.0	10.5±0.5	NT
***Penicillium* sp. ML17**	*M. peperita*	14.0±0.5	23.5±0.5	26.0±0.0

***Trichoderma* sp. ML18**	*M. peperita*	18.0±0.5	17.5±0.5	18.0±0.0
***Aspergillus fumigatus* ML19**	*M. peperita*	20.0±0.0	NT	21.0±0.5

Key: NT – not tested ; SE = Standard Error

4. Conclusion

Findings of this study revealed that isolates the endophytic fungi exhibited promising potential since they produce bioactive compounds with antibacterial activity against test bacteria. Isolation and identification of the bioactive compounds produced by endophytic fungi will guide in the search and discovery of new drugs.

Conflict of Interest

The authors declare that they have no conflict of interest

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