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Evaluation of the antimicrobial and antiviral potentials of extracts of endophytic fungi from *Azadirachta indica*

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Abstract

Endophytic fungi are known to inhabit the internal tissues of plants. The neem plant is one of the medicinal plants cultivated in Nigeria on large scale for its therapeutic potential. Endophytic fungi are producers of important bioactive compounds. In the present study, endophytic fungi were isolated from healthy leaves of *Azadirachta indica*, fermented and ethyl acetate were used to extract the secondary metabolites produced. The crude extracts were subjected to antimicrobial and antiviral assays adopting standard procedures. Following the isolation and purification stages, five (5) AIE1, AIE2, AIE3, AIE4, and, AIE5 endophytic fungi were differentiated using morphological characteristics. The ethyl acetate crude extract of the fungi exhibited microbial growth-inhibitory potentials when tested at a concentration of 1 mg/mL. Secondary metabolites of AIE1, AIE2, AIE4, and, AIE5 exhibited broad-spectrum antimicrobial potentials, inhibiting at least one Gram-positive and Gram-negative bacteria respectively. At 1 mg/mL, AIE1 extract showed good microbial growth inhibitory effects against *Staphylococcus aureus, Bacillus subtilis*, *Escherichia coli, Pseudomonas aeruginosa*, and, *Candida albicans*. Low antiviral activity was recorded for each of the fungal crude extracts. When tested at 1 mg/mL, AIE1 and AIE2 crude extract recorded 21 and 15% reverse transcriptase inhibition respectively *in vitro*. Our findings highlight the bioactivity of secondary metabolites and also validate the chemo-biosynthetic capacities of fungal endophytes associated with *Azadirachta indica.*

Keyword: *Azadirachta indica;* Antimicrobial; Anti-HIV; Endophytic fungi; Neem; Secondary metabolites

1. Introduction

Natural products have continued to provide novel bioactive metabolites, impacting significantly on modern medicine. Over the years endophytic fungi have been shown to be producers of bioactive metabolites with huge chemo-diversity [1]. Studies on bioprospecting of bioactive compounds from *Azadirachta indica* have revealed diverse fungal endophytes associated with this plant with metabolic pathways leading to the biosynthesis of novel compounds [2]. In Nigeria, different parts of the "*Neem*" plant are used for the treatment of various diseases such as malaria, intestinal worms, piles, diabetes, respiratory disorders, constipation, treatment of rheumatism, and chronic syphilitic sores [3] making it a potential source of new bioactive compounds. The development of newer antimicrobial and antiviral agents is of global health importance due to the increase in the spread of multi-drug resistant microorganisms and the emergence of newer viral infections. Several bioactive compounds have been isolated from fungal endophytes isolated from *Azadirachta indica* collected *from Asia* [2]. This study was carried out to evaluate the secondary metabolites produced by endophytic fungi isolated from healthy leaves of *Azadirachta indica*, collected from Nigeria.

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2. Material and methods

2.1. Plant Sample Collection

Fresh *Azadirachta indica* leaves were collected from Agulu, Anaocha Local Government Area of Anambra State (when), and were identified by a plant taxonomist Mrs Amaka Onwunyili at the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria.

2.2. Isolation, Fermentation, and Extraction of Fungal Secondary Metabolites

Axenic cultures of endophytic fungi isolated from healthy leaves of *Azadirachta indica* (Figure 1) were prepared following previously described protocol [4; 5]. Multiple sub-culturing of isolates on fresh malt extract agar was carried out in order to obtain pure isolates.

Solid-state fermentation technique which involved aseptically transferring each fungus into 1 L Erlenmeyer flasks containing sterilized rice medium (100 g of rice + 200 mL distilled water, autoclaved at 121 ℃ at 15 psi for 1 h) was employed [6; 4]. Each fermentation flask was properly sealed and incubated at 28 ℃ for 21 days. Extraction of fermentation products was achieved using 500 mL of ethyl acetate followed by concentration of the filtrate at a reduced temperature of 40 ℃ using a rotary evaporator.

A B C

Figure 1 Endophytic fungi isolation; A: Cultivation of plant segments (mid rib and leaf-blade), B: Mixed endophytic fungi growth, C: Axenic cultures

2.3. Bioassays

Preliminary antimicrobial screening of the endophytic fungal extract was carried out using the agar well diffusion assay as described by [7]. Five (5) standardized broth cultures of each of the test bacteria isolates (*S. aureus, B. subtilis, Salmonella typhi, P. aeruginosa,* and *E. coli*) and two fungal isolates (*Aspergillus niger,* and *Candida albicans*) were used. "A 0.5 McFarland standard bacteria and fungi suspensions of each of the test organism was applied on sterile Mueller-Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) (LS BIOTECH, USA) respectively, using sterile swab stick. Then, a sterile cork borer was used to make five wells (8 mm in diameter) on each of the MHA and SDA plates. Aliquots of 80 µL of each extract dilution, reconstituted in DMSO at concentrations of 1, 0.5, 0.25, 0.125, 0.0625 mg/mL was applied in each of the wells in the culture plates previously inoculated with the test organisms. Ciprofloxacin (5 µg/mL) and Miconazole (50 µg/mL) served as the positive controls while DMSO served as a negative control. The cultures in MHA plates and SDA plates were incubated at 37 ℃ for 24 hr and 27 ℃ for 48 hr respectively. The antimicrobial potential of each of the extracts was determined by measuring the zone of inhibition around each well (excluding the diameter of the well) [8; 7; 5].

2.4. Evaluation of reverse transcriptase activity

The potential for the endophytic extracts to inhibit viral replication was evaluated *in vitro* adopting the protocols contained in Roche colorimetric reverse transcriptase assay kit (Cat. No. 11468120910, Merck KGA, Germany). "At concentrations of 1 and 0.5 mg/mL of the fungal extracts reconstituted in DMSO, the antiviral potential of each fungal extract was measured by taking the absorbance of each sample at a wavelength of 405 nm using a microplate reader (UV06452, Thermo max, USA). The reaction mixture consisted of a lysis buffer, HIV-1-RT, and RT inhibitor which served as the positive control. While the negative control consisted of a mixture of lysis buffer, RT inhibitor, and reaction mixture (solution 3a). This was reconstituted following the manufacturer's direction. Percentage inhibitory potential of

each concentration against RT was evaluated by comparing their inhibition (%) with the negative control. Each concentration was tested in triplicate" [9].

$$\%\ Inhibition = \frac{ABn - ABt}{ABt}\ x\ \frac{100}{1}$$

Where ABn: Absorbance of negative control; ABt: Absorbance of the test sample

2.5. Statistical Analysis

Data obtained were presented as a mean for experiments carried out in triplicates. The mean inhibition zones diameter of the fungal extracts against the various test microbes was compared using one-way ANOVA. Statistical significance was considered at $p \leq 0.05$. Analysis of data and graphs were made using Microsoft Excels 2013 software and SPSS version 20.

3. Results and discussion

3.1. Isolation and Bioactivity of Fungal endophytes

Healthy leaves of *Azadirachta indica* yielded five (5) endophytic fungi (Table 1).The different ethyl acetate crude extract yield is an indication of the different metabolic abilities of each of the fungal endophytes (Table 1) at the prevailing temperature.

The ethyl acetate crude extract of each of the fungus *AIE1, AIE2, AIE3, AIE4, and, AIE5* exhibited antimicrobial potentials (Table 2). Amongst the active fungal secondary metabolites, AIE1, AIE2, AIE4, and, AIE5 exhibited broad-spectrum antimicrobial potentials.

AIE1 exhibited good growth inhibitory effects against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and, *Candida albicans.* While other active fungal extracts inhibited at least one test organism. In addition, only AIE1 exhibited antifungal activity specifically anti-candida activity (Table 2).

AIE1 and *AIE2* fungal crude extracts showed inhibition of reverse transcriptase activity at 1 mg/mL with percentage inhibition of 21 and 15% respectively. In contrast, the positive control recorded a percentage inhibition of 80% (Table 3).

Table 1 Endophytic fungi isolates and Secondary metabolites produced

Unidentified fungi	Segment of isolation	Actual yield (Net wt in g)
AIE1	Leaf blade	1.42
AIE2	Leaf blade	1.40
AIE3	Leaf blade	2.21
AIE4	Mid rib	1.29
AIE5	Mid rib	1.98

Table 2 Antimicrobial assay

Test organisms	Unidentified fungal crude extract (1 mg/mL) / IZD (mm)					
	AIE1	**AIE2**	**AIE3**	**AIE4**	**AIE5**	**Pos. Ctrl.**
S. aureus	5	0	3	0	0	17
B. subtilis	5	3	0	3	2	16
E. coli	3	0	0	2	3	20
P. aeruginosa	0	3	0	0	0	0
C. albicans	5	0	0	0	0	23
A.niger	0	0	0	0	0	21

Key: *S. a: Staphylococcus aureus; P. a: Pseudomonas aeruginosa; E. c: Escerichia coli; B. s: Bacillus subtilis; C.a: Candida albicans;A.n: Aspergillus niger*; Pos. Ctrl positive control: Ciprofloxacin 5 µg/mL; and, Miconazole 50 µg/mL.

Antimicrobial inhibition zone diameter values of endophytic fungi extracts of *Azadirachta indica* against the test microorganisms.

Table 3 *In vitro* reverse transcriptase assay

Unidentified fungi extract (1 mg/mL)	% inhibition
AIE1	21
AIE2	15

In vitro inhibition of reverse transcriptase activity by *AIE1* and *AIE2* fungal crude extracts

Several reports have shown the presence of diverse endophytic fungi associated with the leaves of *Azadirachta indica*. Some examples include: *Chaetomium sp., Curvularia sp., Colletotrichum sp*. and *Trichoderma sp* [10]; *Periconia, Stenella*, and *Drechslera, Phomopsisoblonga, Cladosporiumclado sporioides, Pestalotiopsis sp., Trichoderma sp.,* and *Aspergillus sp* [11]; *Lasiodiplodia theobromae* [12]. Also, Tenguria and Khan [13] isolated *Chaetomium globosum, Pestalotiopsis spp., Phoma sp., Aspergillus flavus, Aspergillus niger, Alternaria alternata (Fr.) Keissl, Fusarium spp., Penicillium spp., Trichoderma spp.*, and Sterile mycelia from fresh leaves of *Azadirachta indica*. Furthermore*, Geotrichum sp.* AL4 an endophytic fungus was isolated from the leaf of *Azadirachta indica*. Chemical analysis on its fermentation broth revealed the presence of novel active components with nematicidal activities [14].

In this study, five (5) fungal endophytes were isolated from the leaves of *Azadirachta indica.* These unidentified endophytic fungi *AIE1, AIE2, AIE3, AIE4, and, AIE5* produced different amounts of extracts when fermented on a local rice medium for 21 days under static conditions. These extracts exhibited moderate antimicrobial activities with antiviral activity comparable to the positive control in the test kit. When tested at 1 mg/ml, *AIE1* crude extract exhibited good antimicrobial potentials, inhibiting *S. aureus, B. subtilis, E. coli, and, C. albicans* with inhibition zones of 5, 5, 3, and 5mm respectively. Also, at 1 mg/mL*, AIE2* extract inhibited the growth of both *B. subtilis* (3 mm) and *P. aeruginosa* (3 mm), while *AIE4 and, AIE5* inhibited the growths of *B. subtilis* and *E. coli* with inhibition zones of 3, 2, and 2, 3 mm respectively. In contrast, Ciprofloxacin (5 µg/mL) and Miconazole (50 µg/mL) used as the standard positive controls, inhibited all the test microbial species. The antimicrobial activities exhibited by *AIE1, AIE2, AIE4, and, AIE5* were observed to be broad-spectrum. This finding is an indication that these active fungal endophytes can be valuable producers of bioactive secondary metabolites for the development of new agents for antimicrobial therapy.

Similarly, secondary metabolites of a fungus *Clostridium sp*. isolated from the root of *A. indica* by Kharwar *et al*. [15] was observed to be active against *C. albicans, E. coli, Bacillus. Sp* at MIC values that ranged between 20 and 40 µg/mL. Also, secondary metabolites produced by *Penicillium species* isolated from the leaf of neem plant exhibited some degree of antimicrobial potentials [16]. Chemical analysis revealed that 8R-acetoxymultiplolide A is a compound biosynthesized by *Phomopsis sp.* YM 311483, obtained from the stem of *Azadirachta indica*. This compound when tested against *Aspergillus niger, Botrytis cinerea, Fusarium avenaceum, Fusarium moniliforme, Helmintho sporiummaydis, Penicillium islandicum*, and *Ophiostoma minus* showed antifungal activities with MIC values in the range of 31.25–500 µg/mL [17].

The *in vitro* antiviral assay showed low reverse transcriptase inhibition potentials exhibited by both *AIE1* and *AIE2* at a maximum concentration of 1 mg/mL. Both fungal crude extracts produced 21 and 15% reverse transcriptase inhibition respectively *in vitro* (Table 3). However, Awah *et al*. [3] reported the anti-HIV activity of the extract *A. indica* by acting as fusion or reverse transcriptase inhibitors. Also, *A. indica* plant extract has been reported to exhibit antiviral activities against other viruses such as HSV-1 [18] and coxsackievirus virus B-4 [19]. The bioactivity of each endophytic fungal extract as observed in this study may be attributed to the presence of several metabolic constituents contained in each crude extracts. These secondary metabolites have been detected in the fungal crude extract from *A. indaca* [20] and selected indian medicinal plants [21]. Thus, further shows that endophytic fungi hold untapped possibilities required for the discovery of novel molecules and their development into chemotherapeutic agents.

4. Conclusion

Our findings further validate endophytic fungi associated with the leaves of *Azadirachta indica* a medicinal plant as a hub of bioactive secondary metabolites and also shows that continuous bioprospecting for endophytic fungal secondary metabolites increases the prospect of isolating lead molecules.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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