ORIGINAL ARTICLE

Isolation and screening of endophytic fungi from three plants used in traditional medicine in Nigeria for antimicrobial activity

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Background: Endophytes represent a promising source of biologically active metabolites for pharmaceutical and agricultural applications. **Objective:** This study was aimed to investigate the endophytic fungi diversity and the antimicrobial potential of three popular medicinal plants (*Alstonia boonei*-Ahun, *Enantia chlorantha*-Awopa and *Kigelia africana*-Pandoro) that have ethnobotanical history in Nigeria. **Materials and Methods:** The stem barks were used for isolation of endophytic fungi and fermented, and the cell free fermentation broths were subjected to antimicrobial screening against six human pathogens; *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi* and *Candida albicans* by using standard agar well diffusion method. **Results:** A total of ten endophytic fungi were isolated from the stem bark of the plants. Seven of these fungi were identified, which include; *Aspergillus niger*, *Macrophomina* spp., *Trichoderma* spp. and four different *Penicillium* species, while three of the isolated endophytes remained unknown. Furthermore, nine of the isolated endophytes showed potential antimicrobial activity against at least one of the six tested pathogens. **Conclusion:** This study shows that endophytic fungi inhabiting the inner tissue of medicinal plants studied may be the source of the curative properties of the plants.

Key words: Agar well diffusion, antimicrobial activity, endophytes, medicinal plant

INTRODUCTION

The need for new and useful compounds to provide assistance and relief in all aspects of the human condition is ever growing. Drug resistance in bacteria, the appearance of life-threatening viruses, the recurring problems with disease in persons with organ transplants, and the tremendous increase in the incidence of fungal infections in the world's population each only underscore our inadequacy to cope with these medical problem.[1] The success of several medicinal drugs from microbial origin such as the antibiotic penicillin from *Penicillium* sp., the immunosuppressant cyclosporine from *Tolypocladium inflatum* and *Cylindrocarpon lucidum*, the antifungal agent griseofulvin from *Penicillium griseofulvum*, the cholesterol biosynthesis inhibitor lovastatin from *Aspergillus terreus*, and ß-lactam antibiotics from various fungal taxa, has shifted the focus of drug discovery from plants to microorganisms.[2] The probability, of obtaining a novel compound, is higher from a novel source. Therefore, endophytic fungi are considered as potential sources of antimicrobial compounds.

Endophytes are microbes that colonize the internal plant tissues beneath the epidermal cell layers without causing any apparent harm or symptomatic infection to their host. Endophytic fungi are known to contribute to their host plants by producing excessively substances that provide protection and survival values to the plant.[3]

Of the 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes, thus providing a rich reservoir of microorganisms.[4,5]

The functional metabolites produced by endophytes include alkaloids, terpenoids, steroids, quinones, isocoumarin derivatives, flavanoids, phenols and phenolic acids, and peptides. Some species produced novel antimicrobial agents (e.g. Cryptocandin from *Cryptosporiopsis quercina*), others produced potent anti-cancer compounds (e.g. Taxol from *Taxomyces andreanae*), and yet others produced compounds that can be utilized industrially, such as enzymes and solvents.[6]

Among the new bioactive molecules discovered are: Novel wide-spectrum antibiotics, kakadumycins, isolated from the endophytic streptomycete associated

Access this article online	
Quick Response Code:	Website: www.greenpharmacy.info
	DOI: 10.4103/0973-8258.150929

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Received: 25-08-2014; **Accepted:** 27-01-2015

with the fern-leafed grevillea (*Grevillea pteridifolia*) from the Northern territory of Australia;[7] ambuic acid, an antifungal agent from several isolates of *Pestalotiopsis microspora;*[8] and subglutinols A and B, immunosuppressive compounds produced by *Fusarium subglutinans*, an endophyte of Tripterygium *wilfordii*.[9]

The plant genetic resources of Nigeria are a veritable source of pharmaceuticals and therapeutics though the plants are not adequately documented and the following plants are often used as medicinal plants in Nigeria namely; *Aframonum melegueta* (atare, ataliya, itaye); *Khaya ivorensis*; *Morinda lucida* (oruwo); *Enantia chlorantha* (awopa); *Alstonia boonei* (ahun), *Azaradichta indica* (dongoyaro) and *Kigelia africana* (pandoro).

While much is known about the phytochemistry of these plants, there is no information available about the endophyte biology. Hence, the objective of our study was to isolate the endophytic fungi from three of such plants and determine their antimicrobial potential.

MATERIALS AND METHODS

Chemicals and Media

Absolute ethanol, 95% ethanol, 2% sodium hypochlorite, lactophenol cotton blue (LPCB) and immersion oil. While the media used include Potato dextrose agar (PDA), malt extract agar (MEA), mueller hinton agar, and nutrient agar.

Plant Collection and Identification

Healthy (showing no visual disease) barks of medicinal plants; *A. boonei* (Ahun), *E. chlorantha* (Awopa) and *K. africana* (pandoro) were obtained from the local herbal market, Mushin, Lagos. The plants were identified and authenticated at the University of Lagos Herbarium, Nigeria.

Surface Sterilization and Isolation of Endophytic Fungi

The collected plants were subjected to surface sterilization procedures.[10] Briefly, Plant materials were first washed several times under running tap water, followed by washing in distilled water. Surface sterilization was then done by sequentially rinsing the plant materials with 70% ethanol for 30 s, followed by 0.5% sodium hypochlorite (NaOCl) for 2–3 min, and then rinsing in 70% ethanol for nearly 2 min, and finally with sterile distilled water 2–3 times. Plant materials were then dried in between folds of sterile filter papers, and each sample was then dried under aseptic conditions. The efficiency of surface sterilization procedure was ascertained for every segment of tissue.[11]

After sterilization, the plant materials were further cut (aseptically) to expose the interior surface to the nutrient media. For each plant, three segments were placed in petri dishes containing PDA amended with chloramphenicol 500 mg/l.[12] The dishes were sealed with parafilm and incubated at 27°C for 3–6 days. The incubation period for each fungus was recorded, and this was taken as the day the first visual growth was observed from the plating date and was considered as an incubation period of growth.

Purification, Selection and Preservation of Endophytic Fungi

Isolation from the master plates was done by the transfer of the hyphal tips to fresh PDA plates without the addition of antibiotics to obtain pure cultures for identification. Nonsterilized plant tissues were cultured as a positive control. The purified endophytic isolates were then transferred separately to PDA slants and maintained at 4°C till further use.

Identification

Macroscopic Study

Morphological study was done by plating the fungi on PDA and MEA and incubating for 7 days. The growth appearance was then noted by observing both the back and front views of the plates.

Microscopic Study

Unknown endophytic fungi were identified by studying their cultural characteristics, spore formations and mycelium. Slides were prepared by tease mount method using LPCB reagent and observed at ×40 and ×100 magnifications.

Fermentation

Thirty milliliters of potato dextrose broth was distributed in 100 ml conical flasks and autoclaved at 121°C for 15 min. The isolated endophytic fungal strains were inoculated aseptically into all flasks. The flasks were kept on the shaker at 27°C for 9 days for growth. The flasks were examined periodically for any contamination. After 9 days, culture media were centrifuged at 5,000 rpm for 30 min. After centrifugation, the culture supernatants were collected and subjected to antimicrobial screening by agar diffusion technique.[13]

Screening for Antimicrobial Activity by Agar Well Diffusion Technique

Six tests microorganisms were obtained from Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria and used for the antimicrobial assay. These include three Gram-negative bacteria-*Pseudomonas aeruginosa, Escherichia coli and Salmonella typhi*, two Gram-positive bacteria-*Staphylococcus aureus* and *Enterococcus faecalis and* one yeast-*Candida albicans*.

Antimicrobial activity of culture supernatants of endophytes was tested by agar well diffusion method using Mueller Hinton agar medium for the bacteria and

Sabouraud Dextrose agar for *C. albicans*. All the overnight culture (Turbidity adjusted to 0.5 McFarland Standard) of test microbes were inoculated into Mueller Hinton agar plates using sterile cotton swab. About 5 mm size well was made using sterile cork borer and 200 µl of culture supernatant was added into it. All the plates were observed for zone of inhibition after incubation at 37°C for 24 h. The antimicrobial activities were assessed by the presence or absence of inhibition zone[14] and the degrees of sensitivity of the isolates to the antibiotics (culture supernatants) were determined by measuring the diameter of zone of inhibition in millimeter. The result obtained was then compared with that obtained with standard antibiotic discs used as control.

RESULTS

Isolation of Endophytic Fungi

Ten fungal endophytes belonging to different species and genus were isolated from the bark stems of the plants [Table 1 and Figure 1] and were named as: AA, AB, AD, AG BA, BB, CBP, CBG, CC and CD.

Table 1: Number of organisms isolated per plant

Plant	Part of plant	Number of fungi isolated
Alstonia boonei	Bark stem	4
Enantia chlorantha	Bark stem	2
Kigelia africana	Bark stem	4

Morphological and Microscopical Characterization of the Isolated Endophytic Fungi

The colony morphology of endophytic fungal isolate AA showed white, fluffy growth that darkens with age and eventually becomes black. Microscopically, the hyphal strands are thin, septate and nonsporulating. Based on macroscopic and microscopic characteristics, the endophytic fungal isolate AA was identified as *Macrophomina* spp. [Table 2].

Endophytic fungal isolate AD showed fast growing colonies as white and downy, later developing yellowish-green to deep green compact tufts. Conidiophores are repeatedly branched, irregularly verticillate, bearing clusters of divergent, irregularly bent, flask-shaped phialides. Conidia are green, with rough walls and are formed in slimy conidial heads (gloiospora) clustered at the tips of the phialides. Based on macroscopic and microscopic characteristics, the endophytic fungal isolate AD was identified as *Trichoderma* spp. [Table 2].

The colony morphology of fungal isolate AG was initially white but soon turns black on the top side, while the reverse side remains white or pale yellow. Microscopically, the Hyphae are septate and hyaline. Conidial heads are radiate initially and split into several loose columns at maturity. Conidial heads are biseriate (vesicles produce sterile cells known as metulae that support the conidiogenous

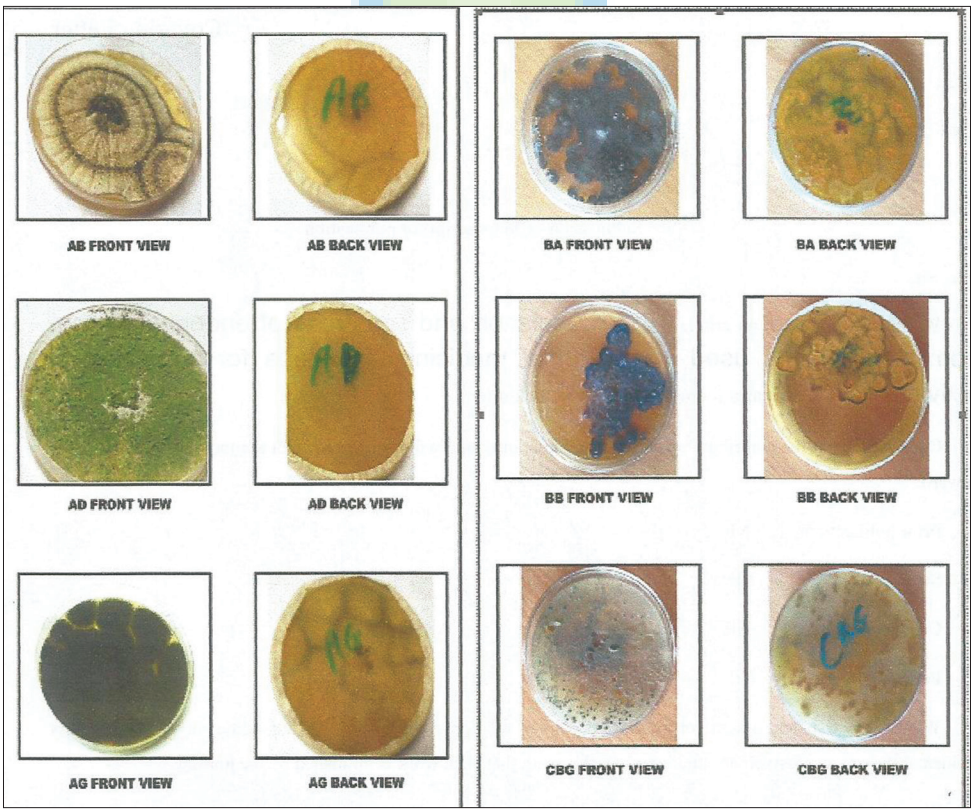

Figure 1: Some of the isolated endophytic fungi

phialides). Conidiophores are long (400–3000 µm), smooth, and hyaline, becoming darker at the apex and terminating in a globose vesicle (30–75 µm in diameter). Metulae and phialides cover the entire vesicle. Conidia are brown to black, very rough, globose, and measure up to 6 or 7 µm diameter. Based on macroscopic and microscopic characteristics, the endophytic fungal isolate AG was identified as *Aspergillus niger* [Table 2].

The colony morphology of fungal isolate BA on PDA showed white surface initially but turns dark green later. Colonies are powdery and the reverse side is pale yellow with rapid growth. While on MEA, the colonies initially were white but turns olive green with time. Colonies are velvety. Microscopically, conidiophores biverticillate and Philiades flask shaped. Conidia spherical to subsperical, smooth-walled or finely roughened, 2.2–3.0 µm diameter septate hyphae. Based on macroscopic and microscopic characteristics, the endophytic fungal isolate BA was identified as *Penicillium citrinum* [Table 2].

Endophytic fungal isolate CBP colonies on PDA are dark green in color, scattered, marked by fairly prominent, but very narrow concentric zones, and with light brown colour on the reverse. Colonies on MEA are olive green in color, of velvety texture, heavy sporing with conidiophores long, up to 400 or even 500 µm, arising primarily from the substratum and bearing comparatively large and strongly divaricate penicilli, consisting of several diverging, fairly definite columns of conidia up to 100 µm in length. Based on macroscopic characteristics, the endophytic fungal isolate CBP was identified as *Penicillium nigricans* [Table 2].

Endophytic fungal CBG colonies were greenish, velvety growth on the agar surface while reverse side is pink. Based on macroscopic characteristics, the endophytic fungal isolate CBG was identified as *Penicillium* spp. [Table 2].

However, fungal isolates AB, CC and CD were unidentified as they did not produce sporing structures on PDA, but showed dirty white cotton growth that darkens with age differently in each isolate [Table 2].

Table 2: Identification of endophytic fungi

Plant	Isolates	Identification
Alstonia boonei	AA	*Macrophomina* spp.
	AB	Unidentified
	AD	*Trichoderma* spp.
	AG	*Aspergilus niger*
Enantia chlorantha	BA	*Penicillium citrinum*
	BB	*Penicillium* sp 1.
Kigelia africana	CBP	*Penicillium* sp 2.
	CBG	*Penicillium nigricans*
	CC	Unidentified
	CD	Unidentified

Antimicrobial Activities of Extracts

Results showed that, of the ten isolates tested, nine isolates showed inhibitory effect towards the test organisms. Two of the nine isolates (AG and CBG) showed weak inhibitory effect towards *E. faecalis* with inhibition less than 6 mm, while nine isolates (AA, AG, BB, BA, CC, CD, CBP, CBG and AD) produced inhibition more than 10 mm on *E. coli*, *P. aeruginosa*, *S. aureus and C. albicans*. The strongest inhibitory effect was produced by fungal endophyte CBG with 40 mm [Table 3]. A control experiment was conducted using standard antibiotics disc [Table 4].

DISCUSSION

A rich diversity of endophytic fungi associated with the medicinal plants: *A. boonei*, *E. chlorantha* and *K. africana* were observed in this study. A total of ten endophytic fungal strains were isolated from the bark stem of the plants when cultured in the laboratory. The diversity of endophytic fungi diversity was highest in *A. boonei*, containing four different species of fungi, higher in *K. africana*, containing a minimum of two different genera and lowest in *E. chlorantha* having just two different species of the same genus of fungi [Table 2]. Fungal growth was initiated mostly within 3 days of inoculation (the day at which first visual growth was observed from plating date was considered as an

Table 3: Antimicrobial activity of endophytic fungi (zone of inhibition in mm)

Fungi isolate	Endophytic fungi	*E. coli* (mm)	*P. aeruginosa* (mm)	*E. faecalis* (mm)	*S. aureus* (mm)	*S. typhi* (mm)	*C. albicans* (mm)
AA	*Macrophomina* spp.	10	12	N*	N*	N*	N*
AG	*Aspergillus niger*	14	16	6	N*	N*	N*
BB	*Penicillium* sp 1.	N*	25	N*	20	N*	N*
BA	*Penicillium citrinum*	N*	28	N*	N*	N*	N*
CC	Unidentified	N*	N*	N*	20	N*	38
CD	Unidentified	12	N*	N*	N*	N*	30
CBP	Penicillium sp 2.	N*	N*	6	N*	N*	40
CBG	*Penicillium nigricans*	N*	N*	N*	N*	N*	35
AD	*Trichoderma* sp.	N*	N*	N*	N*	N*	26

*No inhibition. *E. coli* – *Escherichia coli*; *P. aeruginosa* – *Pseudomonas aeruginosa*; *E. faecalis* – *Enterococcus faecalis*; *S. aureus* – *Staphylococcus aureus*; *S. typhi* – *Salmonella typhi*; *C. albicans* – *Candida albicans*

Table 4: Antimicrobial activity of standard antibiotics (zone of inhibition in mm)

Standard antibiotics	*E. coli* (mm)	*P. aeruginosa* (mm)	*S. typhi* (mm)	*S. aureus* (mm)	*E. faecalis* (mm)
Augmetin	N*	N*	N*	†	†
Cetriazone	N*	N*	N*	†	†
Nitrofuranton	N*	N*	N*	†	†
Gentamycin	12	N*	N*	16	17
Cotrimozazole	N*	N*	N*	20	N*
Ofloxacin	35	20	14	25	15
Amoxycillin	N*	N*	N*	N*	16
Ciprofloxacin	12	N*	N*	20	N*
Tetracycline	N*	N*	N*	†	†
Perfloxacin	N*	N*	16	N*	14
Streptomycin	†	†	†	N*	N*
Chloramphenicol	†	†	†	20	10
Ceftriazone	†	†	†	N*	N*
Erythromcin	†	†	†	20	10

*No inhibition; †Test not relevant. *E. coli* – *Escherichia coli*; *P. aeruginosa* – *Pseudomonas aeruginosa*; *E. faecalis* – *Enterococcus faecalis*; *S. aureus* – *Staphylococcus aureus*; *S. typhi* – *Salmonella typhi*

incubation period for growth). The difference in diversity of endophytes observed might be due to physiological differences in the interior of the stem parts.

Morphological investigations, using both macroscopic and microscopic features, have resulted in the identification of seven fungal species: *Macrophomina* sp., *Trichoderma* sp., *Aspergillus sp, Penicillium citrinum, Penicillium* sp1., *Penicillium nigricans, Penicillium* sp. 2. Three fungal species, although subjected to the same morphological investigations remain unidentified. These results thus confirm the fact that these medicinal plants; *A. boonei, E. chlorantha* and *K. africana* host many species of endophytic fungi.

Also, from our findings the crude extracts from the culture of endophytic fungi displayed varying degrees of antimicrobial activity. Some extracts were effective against at least one bacterial strains used as test organisms in the study [Table 3]. The varying degrees of antimicrobial potency displayed by the various extracts could be as a result of the number and concentration of the active compounds they contain. The extracts with high antimicrobial potency could therefore be said to probably contain quite a number of active compounds or contain high concentration of the available active compounds. Other endophytic fungal extracts, which showed low anti-microbial activity, may have active compounds, but probably in smaller amounts. However, of the six tested microbes, *S. typhi* was the only one that was not susceptible to any of the antimicrobial substances produced by the endophytic fungi as no zone of inhibition was noticed after incubation [Table 3], but was susceptible [Table 4] to two standard antibiotics discs (ofloxacin and perfloxacin).

CONCLUSION

This study has shown that endophytic fungi isolated from medicinal plants; *A. boonei, E. chlorantha* and *K. africana* commonly used in Nigeria (South west in particular) as local herbs may be a potential natural resource to yield useful biologically active compounds and for development of antimicrobial drugs. Further investigation will focus on the bioactive secondary metabolites of these fungi.

ACKNOWLEDGMENT

The authors are thankful to Mr. Oyebamiji of the Herbarium, University of Lagos, Nigeria for providing all the necessary facilities for the successful completion of the research work.

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How to cite this article: Tolulope RA, Adeyemi AI, Erute MA, Abiodun TS. Isolation and screening of endophytic fungi from three plants used in traditional medicine in Nigeria for antimicrobial activity. Int J Green Pharm 2015;9:58-62.

Source of Support: Nil, **Conflict of Interest:** None declared.