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ORIGINAL RESEARCH PAPER

Endophytic fungi from *Vitex payos*: identification and bioactivity

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Abstract
Endophytic fungi isolated from medicinal plants have an important role to play in the search for new bioactive natural compounds. However, despite their potential as repositories of bioactive compounds, the endophytes of African medicinal plants are largely underexplored. The aim of this study was to isolate and identify the endophytic fungi associated with *Vitex payos* and evaluate their antimicrobial and antioxidant potential. The surface sterilization technique was used to isolate the endophytic fungi that were identified by rDNA sequencing of the ITS region. Crude methanol and ethyl acetate extracts were screened for antimicrobial activity using the agar diffusion method and evaluated for antioxidant activity using a commercial total antioxidant capacity assay kit. The total phenolic content of the extracts was determined using the Folin–Ciocalteu method and functional groups present in the extracts were predicted using Fourier-transform infrared spectroscopy. Seven endophytic fungi isolates identified as *Glomerella acutata*, *Epicoccum nigrum*, *Diaporthe species*, *Penicillium chloroleucon*, *Diaporthe endophytica*, *Mucor circinelloides*, and *Epicoccum nigrum* were isolated from the tissues of *Vitex payos*. None of the extracts exhibited antimicrobial activity and the crude ethyl acetate extract obtained from *E. nigrum* demonstrated both the highest total phenolic content (2.97 ±0.13 mg GAE g^{-1} dry weight) and total antioxidant capacity (231.23 ±2.03 µM CRE). Fourier-transform infrared spectral analysis of the crude extracts from *E. nigrum* confirmed the presence of molecules carrying bonded hydroxyl functional group characteristic of phenolic compounds. These preliminary results indicate that most of the isolated fungal endophytes from *V. payos* belong to the phylum Ascomycota and that the isolated *E. nigrum* strain has potential as a source of natural antioxidants.

Keywords
diversity; antimicrobial; antioxidant; bioprospecting; Africa; endophyte

Introduction

There is heightened interest in bioprospecting for natural compounds with potential use as therapeutics owing to several factors that include the rapid development of antimicrobial resistant pathogenic microbes and emergence of new life-threatening diseases [1,2]. Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease [3]. Endophytic fungi have been shown to produce a broad variety of bioactive secondary metabolites and bioprospecting of endophytes is considered a new frontier in the search for natural products with potential agricultural, pharmaceutical, and industrial applications [4,5].

Medicinal plants are rich sources of bioactive natural compounds and studies have shown that some medicinal properties of these plants may be related to the endophytic fungi that they host [2]. The endophytic fungi may participate in some of the plant metabolic pathways or may gain some genetic information to produce specific biologically active compounds, such as those produced by the host plant [6]. Therefore, endophytic fungi isolated from medicinal plants have an important role to play in the search for new bioactive natural compounds. Despite this potential of medicinal plants and the rich plant biodiversity in Africa, only a tiny fraction of African medicinal plant species have been studied with regard to their endophytic fungi diversity. *Vitex payos* is an African ethnomedicinal plant used to treat several ailments in Zimbabwean traditional medicine [7]. As part of our contribution to the ongoing efforts to understand the diversity of endophytic fungi, we isolated and identified endophytic fungi found in the leaf and stem tissues of *V. payos*. Furthermore, we determined the antibacterial activity and antioxidant potential for some of the identified endophytic fungi strains. In addition, Fourier-transform infrared (FT-IR) spectroscopy was used predict the presence of various functional groups in the endophytic fungi crude extracts.

Material and methods

Collection and identification of plant materials

Fresh leaf and stem tissue of endophytic fungi were isolated from five leaf tissue samples and two stem tissue samples obtained from a single *V. payos* plant grown under natural (wild) conditions at the National Herbarium and Botanical Gardens (Harare, Zimbabwe). The tissue samples were pretreated by cleaning them under running water to remove dirt and soil particles, and then air dried to remove any surface moisture before they were packaged into labeled sterile sample collecting bags. The samples were then transported to the laboratory and stored at 4°C until endophyte isolation procedures could be instituted [8].

Isolation and establishment of in vitro culture of endophytes

Endophytic fungi were isolated from the leaf and stem tissues collected from the medicinal plant using a modified surface sterilization procedure as described by Kjer et al. 2010 [9]. The plant tissue samples were removed from storage and thawed by washing them using running tap water. The thawed plant tissue samples were then washed using 0.1% (v/v) Tween-80 for 15 minutes followed by another wash for 1.5 hours using running water. The cleaned plant tissues were then transferred to a laminar airflow cabinet where the surface sterilization and endophyte isolation was conducted under aseptic conditions. The plant tissue samples were cut into 5-cm segments and surface-sterilized with 70% ethanol (30 seconds for leaf samples and 2 minutes for stem samples), soaked in 2% NaOCl solution for 15 minutes, rinsed four times with sterile double distilled water, and finally blot-dried using sterile paper towels. The effectiveness of the sterilization procedure was tested by plating 0.1 mL of the final sterile water rinse onto Petri dishes containing potato dextrose agar (PDA) and rolling the sterilized sample onto Petri dishes containing PDA. The surface-sterilized tissues were cut into smaller segments (1–2 cm) using sterile razor blades and placed onto Petri dishes containing PDA supplemented with 200 U mL^{-1} Penicillin-Streptomycin (Lonza, Basel, Switzerland; cat. No. 17-602E) and incubated at 28 ±2°C until fungal growth was initiated. The fungal mycelia growing out of the sample segments were subcultured and maintained on Petri dishes containing PDA.

Identification of the endophytic fungi

Genomic DNA was extracted from the cultures using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA; cat. No. D6005). The internal transcribed

spacer (ITS) target region was amplified using EconoTaq PLUS GREEN 2X Master Mix (Lucigen, Madison, WI, USA) using the universal primers ITS1 and ITS4. The PCR reactions were carried out under the following conditions: initial denaturation at 95°C for 15 minutes, 35 cycles at 95°C (denaturation) for 1 minute, 56°C (annealing) for 30 seconds, 72°C (extension) for 1 minute, and a final extension for 10 minutes at 72°C. The PCR products were run on a gel and extracted (Zymo Research, Irvine, CA, USA; Zymoclean Gel DNA Recovery Kit; cat. No. D4001). The extracted fragments were sequenced in the forward and reverse directions using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit; cat. No. D4050). The purified fragments were run on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). CLC Bio Main Workbench v7.6 was used to analyze the .ab1 files generated by the ABI 3500xl Genetic Analyzer and endophytic fungi isolates were identified on the basis of similarity of amplified sequence with those found in the U. S. National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (nBLAST).

Secondary metabolite extraction

The small-scale fermentations and solvent extractions were carried as previously described [9] with modifications. Briefly, the fungi were cultivated in 500 mL of potato dextrose broth and were incubated at 28 ±2°C for 30 days in a shaker at 180 rev min^{-1}. The fungal mycelia were then separated from the culture broth by filtration and extracted with analytical grade methanol (MeOH) solvent (100 mL). The filtrate was then extracted three times at the liquid-liquid partition with an equal volume of analytical grade ethyl acetate (EtOAc) solvent (1:1 v/v). The resulting crude extracts were collected and concentrated to dryness in a vacuum rotary evaporator at 40–45°C, then dissolved in 1 mL of MeOH solvent, followed by drying under vacuum to obtain the EtOAc and MeOH extracts. The crude extracts were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg mL^{-1} and kept at 4°C [10].

Determination of the antimicrobial activity of the crude extracts

The microorganisms were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The extracts were tested against the gram-positive bacteria *Staphylococcus aureus* (ATCC 11632), gram-negative bacteria *Escherichia coli* (ATCC1056) and *Klebsiella pneumoniae* (ATCC 13883) using the agar disk diffusion method using the Clinical and Laboratory Standards Institute protocol [11]. Briefly, the test microorganisms were grown in nutrient broth medium until 1×10^8 colony-forming units were attained and then were used to inoculate sterile 90-mm Petri dishes filled with nutrient agar medium using the spread-plate technique. Dried and sterile filter paper disks (6.0 mm diameter) were impregnated with 40 µL of the extracts (containing 500 µg fungal extracts), air dried under aseptic conditions in a laminar airflow cabinet, and placed on plates inoculated with the test microorganism. The plates were incubated at 37°C for 24 hours. Three sets of controls were used. The organism control consisted of a seeded Petri dish with no sample. In the second control, samples were introduced to the unseeded Petri dishes to check for sterility. Disks impregnated with 40 µL DMSO were run simultaneously as a third control. Standard antibiotics were also run simultaneously as reference agents to understand the comparative antimicrobial efficacy. Three replicates per extract were used for the antimicrobial activity assays. The antimicrobial potency of the extracts was measured by their ability to prevent the growth of the microorganisms surrounding the disks.

Determination of total phenolic content of the crude extracts

The total phenolic content of the extracts was determined by the Folin–Ciocalteu method. Briefly, 0.2 mL of each crude extract (dissolved in DMSO at a concentration

of 1 mg mL^{-1}) was added to 2.8 mL of distilled water and mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 minutes, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was left to stand for a further 60 minutes in the dark and absorbance was measured at 650 nm using a model I-290 spectrophotometer (Lasany, Panchkula, India). The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight. Three replicates per extract were used for the determination of total phenolic content and the mean values (±*SD*) were calculated.

Determination of the antioxidant activity of the crude extracts

The total antioxidant capacity (TAC) of the endophytic fungal extracts at 1 mg mL^{-1} concentration was determined using the OxiSelect Total Antioxidant Capacity Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA) using the manufacturer's instructions. The method is based on the single electron transfer (SET) mechanism and involves the reduction of Cu^{2+} to Cu^{+} by the endogenous antioxidants and by other reducing equivalents in the sample. The Cu^{+} interacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. The absorbance value is proportional to the total antioxidant (respectively reducing) capacity of extracts. The samples were analyzed spectrophotometrically at 490 nm using the SPECTROstar Nano microplate reader (BMG LABTECH, Ortenberg, Germany). TAC was determined using a calibration curve based on uric acid standards. The results were expressed as μM copper reducing equivalents (CRE). Three replicates per extract were used for the determination of the antioxidant activity of the crude extracts and the mean values (±*SD*) were calculated.

FT-IR analysis

Infrared spectra were collected on a Nicolet 6700 FT-IR spectrometer (Thermo Fisher Scientific) equipped with a diamond crystal attenuated total reflectance sampling accessory (Thermo Fisher Scientific). A few grams of the samples were placed on the attenuated total reflectance unit and scanned from 500 to 4,000 cm^{-1} with a resolution of 4 cm^{-1}. Each recorded spectrum was the result of 36 coadded scans. FT-IR was performed to predict the presence of various functional groups in the isolates.

Data analysis

The colonization rate of endophytes was determined as the total number of segments yielding ≥ 1 isolates divided by the total number of segments from which endophytes were isolated (expressed as a percentage). The results for the antimicrobial and antioxidant activity and total phenolic content determination assays are expressed as the mean ±*SD* of triplicates ($n = 3$). Difference analysis (one-way ANOVA) of the antioxidant activity results was conducted using Microsoft Excel 2013 (Microsoft, USA). A p value <0.05 was considered to indicate statistically significant differences between groups.

Results

Isolation of endophytic fungi

A total of seven endophytic strains were isolated from *V. payos* and the endophytic colonization rate was 100%. The isolated strains were identified (Tab. 1) and all found to belong to the phylum Ascomycota, except for *Mucor circinelloides*, which is a member of the phylum Zygomycota.

Tab. 1 Endophytic fungi isolated from the stems and leaves of *V. payos*.

Isolate code*	Sequence similarity with	% sequence similarity	Accession number
TL1	*Glomerella acutata*	99	AM991137
TL2	*Epicoccum nigrum*	99	KX869952
TL3	*Diaporthe* sp.	98	KU671340
TL4	*Penicillium chloroleucon*	99	KP016813.
TL5	*Diaporthe endophytica*	96	AB899789
TS1	*Mucor circinelloides*	100	KC461495
TS2	*Epicoccum nigrum*	99	KX869952

* TL – endophyte isolated from leaf tissues; TS – endophyte isolated from stem tissues.

Tab. 2 Total phenolic content and antioxidant capacity of extracts obtained from *E. nigrum* and *Diaporthe* species*.

Endophyte	Isolate code	Total phenolic content (TPC) (mg GAE/g extract[a])	Total antioxidant capacity (TAC) 1mg/mL [CRE[b] (µM)]
		Ethyl acetate extracts	
Epicoccum nigrum	TS2	2.97 ±0.13	231.23 ±2.03
		Methanol extracts	
Epicoccum nigrum	TS2	0.50 ±0.01	32.28 ±2.96
Diaporthe species	TL3	2.76 ±0.07	60.97 ±2.53

* Results are represented as means ±*SD* (n = 3). [a] Gallic acid equivalent; [b] copper reducing equivalent.

Antimicrobial activity

None of the extracts exhibited antibacterial activity against the test microorganisms: *E. coli* (ATCC1056), *K. pneumoniae* (ATCC 13883), and *S. aureus* (ATCC11632) as well as strains of the same bacteria obtained from clinical samples.

Antioxidant capacity and total phenolic content

The antioxidant activity of EtOAc and MeOH extracts of the two representative isolates obtained from stem (*E. nigrum*; isolate TS2) and leaf (*Diaporthe species*; isolate TL3) tissues was determined. The EtOAc and MeOH extracts of the endophytic fungi *E. nigrum* both exhibited antioxidant activity, while for the *Diaporthe* species of endophytic fungi only the MeOH extract showed some antioxidant activity (Tab. 2). Data analysis revealed significant differences [$F(2, 6) = 8{,}429.77$, $p < 0.00001$ at the 0.05 alpha level] in the antioxidant activities of the studied extracts, whereas there was no significant difference [$F(1, 4) = 5.495$, $p = 0.07902$ at the 0.05 alpha level] in the total phenolic content between the EtOAc extract from *E. nigrum* and the MeOH extract from the *Diaporthe* species.

FT-IR analysis

The EtOAc extracts from *E. nigrum* that exhibited both the highest antioxidant and total phenolic content were further analyzed using FT-IR spectroscopy, which is a well-established tool for the characterization and identification of functional groups present in extracts. The FT-IR spectral analysis of the EtOAc extract of *E. nigrum* revealed the presence of multiple functional groups, including –OH, –CHO, –COOH,

Tab. 3 Major bands observed in the FT-IR spectra of the *E. nigrum* EtOAc extracts.

Wave number (cm^{-1})	Vibration band/group	Probable compound classes
3,600–3,300	Hydrogen-bonded O–H stretch	Phenols, alcohols
3,000–2,500	H–C–H asymmetric and symmetric stretch	Saturated aliphatic
	O–H stretch	Carboxylic acid
	S–H stretch	Thiols
1,820–1,680	C=O stretch	Carbonyls, lactones
1,600–1,550	C–C=C symmetric stretch	Aromatics
	–C=N–	Thiols and thio-substituted compounds
	C=O	Carbonyls
	N–H bend	Amines
	Nitrogen-oxy	Aromatic nitro compounds
1,500–1,450	C–C=C asymmetric stretch	Aromatics
1,420–1,300	C=O	Carbonyls
	Aliphatic nitro compounds	Hetero-oxy compounds
	Dialkyl/aryl sulfones	Sulfur-oxy compounds
1,250–1,000	C–O stretch	Alcohols, Phenols
	C–N	Aliphatic amines
	N–O	Aromatic amine oxide
	C=S	Thiocarbonyl
	Φ–O–H	Aromatic ethers

and –COOR [12]. Important IR absorption frequencies obtained from the extracts are tabulated in Tab. 3.

Discussion

The observation that most of the isolated endophytic fungi belonged to genera in the phylum Ascomycota is in line with observations from other studies [13–15]. It is important to note that all the genera of endophytic fungi isolated from *V. payos* in this study have previously been isolated from a wide range of other different plant hosts in diverse environments, suggesting that these genera are not host and environment specific.

The obtained preliminary antimicrobial activity results suggest that the studied endophytic fungi do not have potential as sources of new antimicrobial agents against the assessed bacterial species. While this is disappointing, the results support the findings from other studies that not all endophytic fungi strains have antibacterial activity [16]. However, there is a need to assess the antibacterial activity of these strains against other test microorganisms, screen them for antifungal activity, and vary the fermentation conditions before we totally discount these strains as potential sources of new antimicrobial agents.

The EtOAc extract of the endophytic fungus *E. nigrum* exhibited both the highest antioxidant activity and total phenolic content. Endophytic and marine-derived strains of *E. nigrum* have been shown to have antioxidant activity [17,18]. This suggests that this species might be generally predisposed to produce natural antioxidants. Phenolic compounds have redox properties and are antioxidants owing to their hydroxyl groups that confer to them their free radical scavenging ability [15,19]. The total phenolic content and antioxidant activity results collected for the extracts obtained from the stem isolate of *E. nigrum* (TS2) suggest that phenolic content influenced antioxidant activity. However, there is a need for further studies to confirm this observation. Endophytic species of the genus *Diaporthe* have been reported to have antioxidant activity; our results for the MeOH extract of our *Diaporthe* leaf isolate tally with those observations.

Biological activity of extracts is influenced by the functional groups present in the extracts. Hence, functional group analysis plays an important role in understanding the biological activity of extracts [20]. In the present study, the FT-IR spectral analysis of the *E. nigrum* crude EtOAc extracts suggests the presence of molecules carrying the bonded hydroxyl (–OH) functional group. The hydroxyl group is an integral part of most of the phenolic compounds, such as flavonoids and tannins. Therefore, the FT-IR results serve to provide further supporting evidence for the presence of phenolic compounds in the crude EtOAc extracts. The FT-IR spectra analysis also suggests that diverse groups of functional groups (and hence diverse classes of compounds) are potentially present in the extracts. This is in line with previous findings by other researchers that endophytic fungi produce diverse classes of metabolites [21–23]. However, it is important to note that further investigations using different techniques are required to identify the compounds present in the *E. nigrum* crude EtOAc extracts. To the best of our knowledge, this is the first reported work on the identification, antibacterial and antioxidant activities of fungal endophytes isolated from *V. payos*. The results of this study suggest that while fungal endophyte isolates obtained from *V. payos* have potential as sources of natural antioxidants, further research is required to isolate and identify the bioactive molecules from the crude extracts and evaluate in vivo their biological activities.

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