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Full Length Research Article

In Vitro Antioxidant Activity of Secondary Metabolites of *Aspergillus fumigatus* Isolated from *Piliostigma thonningii*

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ABSTRACT

Plant endophytic fungi are novel reservoirs of bioactive compounds. This study aims to explore the antioxidant activity of the secondary metabolites (SM) of an endophytic fungus isolated from the leaves of *Piliostigma thonningii* plant. An endophytic fungus (coded EDP 1) was isolated, purified, and identified. The pure endophyte was fermented, its crude SM extracted and screened for antioxidant activities using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. With an IC_{50} value of 18.84, the SM of the endophytic fungus (*Aspergillus fumigatus*) demonstrated significant antioxidant activity compared to 5.79 µg/mL recorded for ascorbic acid. The SM and ascorbic acid showed free radical scavenging activity of 77.67; 85.67% and 68.50; 88.76% in the DPPH and FRAP assays, respectively. Using gallic acid (R^2 = 0.991) and quercetin equivalent (R^2 = 0.986) standard plots, the total phenolic and flavonoid contents were found to be 228.88 ± 1.12 mg GAE/g and 152.56 ± 1.27 mgQE/g, thus suggesting phenolic and flavonoid-related antioxidant activity of the SM. This study showed that the SM of *A. fumigates* has promising antioxidant properties, which could be explored further for their pharmacological applications.

KEYWORDS: Antioxidant, *Aspergillus fumigatus*, Endophytic fungus, *Piliostigma thonningii*

INTRODUCTION

There is an increasing desire for new and safer bioactive compounds in all aspects of medicine (Gouda *et al.*, 2016). Nature, particularly plants, fungi, bacteria, and endophytes, has continually offered humans a wide range of pharmacologically active compounds that can be investigated as pharmaceutical targets for treating both novel and established diseases or as foundational molecules for developing synthetic drugs (Cragg and Newman, 2013; Nwachukwu *et al.*, 2018). Endophytes residing within medicinal plants have been shown to be a consistent origin of novel lead molecules with antimicrobial, anti-inflammatory, anticancer, and antiviral properties, among others, which have aided drug discovery (Zhao *et al.*, 2011; Okoye *et al.*, 2015).

In Africa, *Piliostigma thonningii* Schum (family Fabaceae; common names, monkey bread and camel's foot) is a perennial tree with diverse ethnomedicinal and economic applications (Adjanahoun *et al.*, 1991; Igoli *et al.*, 2005; Jimoh *et al.*, 2005; Togola *et al.*, 2005). In Nigeria, the plant is known commonly as "*Abafe*" in Yoruba, "*Kalgo*" in Hausa, and "*Okpoatu*" in Igbo (Togola *et al.*, 2005; Ogbiko *et al.*, 2017). The plant is used traditionally as an antipyretic (Igoli *et al.*, 2002; Igoli *et al.*, 2005; Togola *et al.*, 2005) and has been shown to also have antimicrobial (Ibewuike *et al.*, 1997; Ogundaini, 1999), antihelmintic (Fakae *et al.*, 2000) and anti-inflammatory (Ibewuike *et al.*, 1997) properties.

Nigeria's abundant plant biodiversity has been documented to host a variety of endophytic microbial communities, offering the potential for discovering biologically significant compounds valuable in pharmaceutical and industrial applications (Okoye *et al.*, 2005; Abba *et al.*, 2016; Ebada *et al.*, 2016; Okezie *et al.*, 2017; Eze *et al.*, 2018). Literature on the isolation or screening of secondary metabolites from endophytes associated with *P. thonningii* is scanty. Thus, our research aim to extract and examine the antioxidant activity of the secondary metabolites of an endophytic fungus isolated from the leaves of *P. thonningii* grown in Sokoto State, North-Western Nigeria.

MATERIALS AND METHODS

Chemicals

The chemicals and pharmaceuticals used for the research were of analytical grade supplied by Sigma Chemical Company, St. Louis, USA.

Plant Collection and Endophytic Fungus Isolation

Fresh leaves of *P. thonningii* were initially collected, authenticated by a plant taxonomist then documented with the specimen voucher number: UDUH/ANS/0137. The isolation of endophytic fungi from the collected leaves was performed following the methods described by Eze *et al.* (2018) and Abba *et al.* (2016). The leaves of the plant were cleaned thoroughly with running tap water to eliminate any attached debris and were subsequently rinsed with double-distilled deionized water. The samples were sectioned into segments measuring 1 cm² prior being sterilized using 2% sodium hypochlorite solution for 2 minutes and 70% ethanol for 2 minutes, followed by rinsing with distilled water and then allowed to air dry under sterile conditions. The effectiveness of the surface sterilization process was verified using the imprint method as described by Schulz *et al.* (1993). The sterilized sample was introduced into broth media as a control to assess growth in liquid culture. The dissected tissues were placed onto malt extract agar (MEA) enhanced with 50 mg/L chloramphenicol in Petri dishes. These Petri dishes were incubated for 5 days at 28 °C. Hyphal tips from distinct colonies appearing on leaf

segments were transferred to fresh MEA plates to achieve pure colonies through the single spore technique (Strobel *et al*., 2004; Kharwar *et al*., 2008).

Endophytic Fungus Cultivation, Induction, and Extraction of Secondary Metabolites

A 1-liter Erlenmeyer flask filled with autoclaved, sterile solid rice medium (comprising 100 grams of rice and 200 milliliters of distilled water) was inoculated using 3 mm diameter agar blocks taken from the malt extract agar plates that contained pure cultures of each fungus. The inoculated flasks were kept at a temperature range of 27-30 °C for 21 days to promote the production of secondary metabolites. After fermentation was complete, the fungal secondary metabolites were extracted with ethyl acetate, and the crude extracts were concentrated with a rotary evaporator set at 40 °C under reduced pressure (Akpotu *et al*., 2017).

Determination of DPPH Free Radical Scavenging Activity

The extracted endophytic fungi secondary metabolites were assayed for anti-oxidant activity using 1,1-diphenyl-2-picryl-hydrazyl (DPPH) following the method described by Jain *et al.* (2008). A 3 mL sample of the extract with five different concentrations - 10.0, 25.0, 50.0, 100.0, and 200 µg/mL each was mixed with 1 mL of a 0.1 mM DPPH solution in methanol. The absorbance readings were taken using a spectrophotometer at 517 nm, with absorbance taken every 30 minutes against a blank sample of 0.1 mM DPPH solution and pure methanol. The percentage of radical scavenging activity was determined using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{A0 - A1}{A0} \times 100$$

Where: A_0 = absorbance of DPPH radical + methanol (blank); A_1 = absorbance of DPPH radical + secondary metabolites.

Ferric Reducing Power Assay

The ferric reducing power was assessed following the method described by Ebrahimzadeh *et al*. (2008). Briefly, 100 µL of the extract (ranging from 100 to 500 µg/mL) was combined with 2.5 mL of a 200 mmol/L phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 minutes. Next, 2.5 mL of 10% trichloroacetic acid was added, and the samples were centrifuged at 10,000 rpm for 10 minutes. Following this, 5 mL of the supernatant was combined with 5.0 mL of distilled water and 1 mL of 0.1% ferric chloride. The absorbance of the resultant mixtures was recorded at 700 nm, using ascorbic acid as the positive control. The ferric reducing antioxidant power was calculated using the relationship:

$$\text{Ferric Reducing Antioxidant Power (\%)} = \frac{\text{A sample}}{\text{A control}} \times 100$$

Determination of Total Phenolic Content

The total phenolic content of the extracted secondary metabolites was assessed using the Folin-Ciocalteu colorimetric method. A 0.5 mL aliquot of the secondary metabolites at a concentration of 1000 µg/mL was combined with 4.5 mL deionized distilled water and 0.5 mL of Folin-Ciocalteu's reagent, which had been previously diluted with deionized water in a 1:10 (v/v) ratio. The resulting mixture was left at room temperature for 5 minutes, after which 5 mL of 7% sodium carbonate and 2 mL of deionized distilled water were added. The well-mixed solution was then incubated for 90 minutes at 23 °C. The absorbance was measured using a spectrophotometer set to a wavelength of 750 nm. The total phenolic content (TPC) was quantified as milligrams of gallic acid equivalents (GAE) per gram of the extract. Gallic acid served as the positive control (Ebrahimzadeh *et al*., 2008).

Determination of Total Flavonoid Content (TFC)

The aluminum chloride method was used to assess the total flavonoid content in the secondary metabolite of the endophytic fungus. Exactly 0.5 mL solution of the secondary metabolites with a concentration of 5000 µg/mL was mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The mixture was incubated at room temperature for 30 min. The absorbance was taken at 415 nm. The results were expressed as milligrams quercetin equivalents per gram of extract (mg QE/g dry extract). Quercetin served as the positive control (Ebrahimzadeh *et al*., 2008).

Endophytic Fungus Identification

The endophytic fungi were identified through morphological, microscopic, and molecular methods, including DNA amplification and sequencing of the fungal ITS region. The fungal DNA was extracted using the Zymo fungal/bacteria DNA extraction kit (Zymo Research Corp., South Africa), following the manufacturer's protocols. Polymerase chain reaction (PCR) was performed to amplify the ITS gene from the fungus's specific DNA utilizing the primer pair ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'). The amplified product was purified using ExoSAP, and Sanger sequencing was conducted with Nimagen's Brilliant DyeTM Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000. To identify the endophytic fungus, the Basic Local Alignment Search Tool (BLAST) was employed to match the amplified sequence with ITS sequence data from strains in the US National Centre for Biotechnology Information (NCBI) database.

Statistical Analysis

All assessments were conducted in triplicate, and the results were expressed as mean ± standard error of the mean (SEM). The difference between means of samples was determined using one-way analysis of variance (ANOVA) and Duncan's multiple range test with a level of significance set at 95%. SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA) was used to statistically analyze the results.

RESULTS

Preliminary Identification of Isolated Endophytic Fungi

Eight strains of endophytic fungi were extracted from the leaves of *P. thonningii* and classified as shown in Table 1

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Table 1: Endophytic fungi (EDP) isolated from the leaves of *P. thonningii*

CODE	PRELIMINARY IDENTIFICATION
EDP 1	*Aspergillus fumigatus*
EDP 2	*Curvularia* sp.
EDP 3	*Curvularia* sp.
EDP 4	*Alternaria alternata*
EDP 5	*Bipolaris* sp.
EDP 6	*Rhizoctonia* sp.
EDP 7	*Rhizoctonia* sp.
EDP 8	*Pleospora* sp.

Characterization of the Most Active Endophytic Fungus EDP 1

The morphological and microscopic characterization of EDP 1 is presented in Tables 2 and 3, while Figure 1 shows the gel electrophoresis and BLAST ITS sequence for the fungal species identification.

Table 2: Morphological characteristics of endophytic fungus EDP 1

CHARACTERISTICS	OBSERVATION
Surface color	Green to dark green
Growth	Rapid
Margins	Entire
Reverse side	Yellow

Table 3: Microscopic characteristics of endophytic fungus EDP 1

CHARACTERISTICS	OBSERVATION
Hyphae	Branched septate
Conidiophore	Present
Vesicle	Dome shaped
Conidia	Present
Phialides	Uniseriate
Fruiting body	Cleistothecia

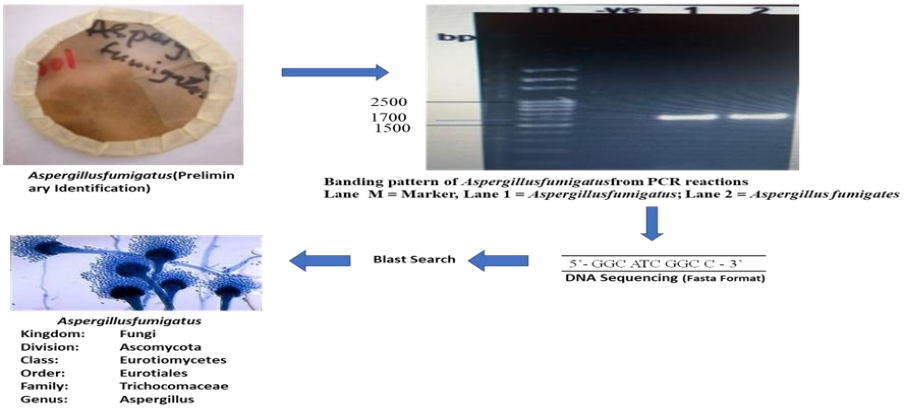

Figure 1: Molecular characterization of *A. fumigatus*

Total Phenolic and Flavonoid Contents

The total phenolic and flavonoid contents of the secondary metabolites of *A. fumigatus* extract, calculated from the gallic acid calibration curve (R^2 = 0.991) and quercetin calibration curve (R^2 = 0.986) are presented in Table 4.

Antioxidant Activity

The results of the DPPH and FRAP antioxidant assay of the endophytic fungi are shown in Figures 2a and b.

Table 4: Total phenolic and flavonoid content of the secondary metabolites of *A. fumigatus*

PHYTOCHEMICAL	CONCENTRATION
Total phenolic content (mgGAE/g extract)	228.88 ± 1.12
Total flavonoid content (mgQE/g extract)	152.56 ± 1.27

Values are expressed as mean ± the standard error of the mean based on three measurements.

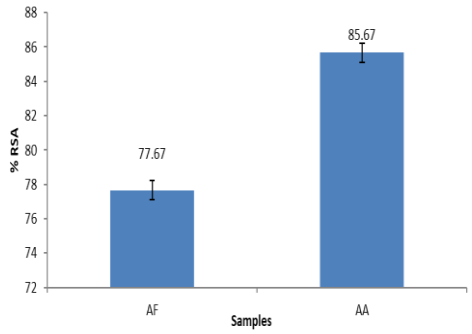

Figure 2a: DPPH radical scavenging activity (RSA) of the secondary metabolites of EDP 1.
Each point is a mean from triplicate measurement, AF = *Aspergillus fumigatus*; AA = Ascorbic acid

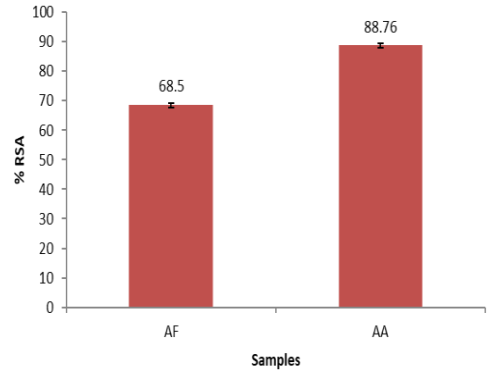

Figure 2 (b): FRAP antioxidant activity of the secondary metabolites of EDP 1.
Each point is the mean of triplicate measurements. AF = *Aspergillus fumigatus*; AA = Ascorbic acid

DISCUSSION

External factors such as climatic conditions, nutrient availability, plant species, geographical location, soil composition, and water supply have been identified as determinants of the microbiome composition within plants (Eyre et al., 2019). Although morphological and microscopic characterization of fungi are widely used methods, they are insufficient for fungal identification since many fungi share similar morphological characteristics, highlighting the necessity of adopting molecular techniques for accurate species identification (Klich and Pitt, 1988; Shalini and Amutha, 2014). Molecular characterization is a quick approach that aids in differentiating morphologically similar fungal species through the use of PCR technology and the internal transcribed spacer (ITS) (Henson and French, 1993). The characterization of EDP 1 confirms the fungus as *Aspergillus fumigatus*, aligning with the findings of Shalini and Amutha (2014). This marks the first documented instance of isolating any endophytic fungus from the genus Piliostigma. Free radicals have been implicated in various pathological conditions ranging from cancer, cardiovascular disorders, arthritis, inflammation, and liver diseases (Martin *et al*., 1993). The secondary metabolites of *A. fumigatus* demonstrated a notable ability to scavenge free radicals, which was not statistically significant in comparison to the ascorbic acid standard. Phenolic compounds have been documented to play a key role in stabilizing lipid oxidation and are associated with antioxidant activity (Yanishlieva-Maslarova, 2001). The appreciable value of the total phenolic and flavonoid contents (Table 4) could be attributed to being responsible for its antioxidant activity, as the antioxidant capacities of endophytic fungal cultures are significantly correlated with their total phenolic content (Huang *et al*., 2007; Fernandes *et al*., 2009).

CONCLUSION

The secondary metabolites found in the leaves of *P. thonningii* contain significant levels of phenolic and flavonoid compounds that exhibit antioxidant activity. Therefore, the secondary metabolites of *P. thonningii* could serve as a viable natural source for creating a functional ingredient that may aid in preventing and treating free radicals associated with degenerative diseases.

DECLARATION OF COMPETING INTEREST

The authors declare no conflict of interest.

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