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Orthosporin, a major component of the fermentation product of *Lasiodiplodia theobromae* - an endophytic fungus of *Musa paradisiaca* as a potential antimicrobial agent

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Abstract

Endophytic fungi remain an unexplored reservoir of chemical diversity, and have become the primary focus is several bio-prospecting programs. This study was carried out to detect the constituents and bioactivity of the secondary metabolites of an endophytic *Lasiodiplodia theobromae* associated with *Musa paradisiaca.* Following standard protocols, the axenic fungus was isolated from healthy leaves of *Musa paradisiaca* harvested from Agulu, Anambra State, Nigeria. The isolated fungus was characterized using a standard taxonomic identification procedure involving the amplification and sequencing of the ITS region of the DNA. The fungus was subjected to solid state fermentation in rice medium, followed by extraction, chromatographic separation and chemical characterization of its secondary metabolites. The antimicrobial, antioxidant, antiviral and cytotoxic activities of fungal extract was evaluated using standard methods. At a 1 mg/mL, the fungal extract demonstrated inhibitory potentials against *Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli* with inhibition zone diameters that ranged from 3-7 mm. The extract, at 100 µg/mL, exhibited antioxidant effect having an IC_{50} of 65.8 µg/mL. The extract showed dose-dependent antiviral properties during the *in vitro* antiviral assay with 54% and 60% inhibition of reverse transcriptase observed at 0.5 and 1 mg/mL concentrations respectively. Following chromatographic separation and chemical analyses of the fungal metabolite, orthosporin was isolated. This widely known phytotoxic compound, together with other constituents of the fungal secondary metabolites, may have exhibited the observed bioactivities of the fungal extract recorded. Thus, our findings provide additional data on the potentials of endophytic fungi as producers of interesting bio-molecules.

Keywords: antimicrobial; antioxidant; antiviral; endophytic fungi; *Lasiodiplodia theobromae*; *Musa paradisiaca*; orthosporin

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Introduction

Plants have been extensively screened for new bioactive pharmaceutical molecules. However, this approach involving the use of large quantities of plant parts for extraction of specific lead molecules pose a setback in the isolation of novel lead compounds from plants. Fungal endophytes have shown untold level of unexplored reservoir of chemical diversity and have become the primary focus of several advanced secondary metabolites screening programs (Newman and Cragg, 2012; Deepika *et al.*, 2015; Okoye *et al.*, 2015; Eze *et al.*, 2019). These groups of organisms exist in a mutually beneficial relationship with their host plants (Rodriguez *et al.*, 2009) and are also capable of producing plant-associated primary metabolites with their host (Uzor *et al.*, 2015; Ebada *et al.*, 2016).

According to Eze *et al.* (2018), in the search for natural bioactive products of fungal origin, Nigeria's rich plant community provides a huge foundation for natural products researchers to explore avoiding the destructive harvesting of plants, but rather by harnessing their associated endophytes for important pharmaceutical and industrial molecules.

In our continued search for bioactive natural products produced by fungal endophytes, this study was carried out to screen the metabolites of an endophytic fungus isolated from leaves of a Nigerian plant, *Musa paradisiaca,* commonly known as plantain.

M. paradisiaca is a tropical plant that is popular in West and Central Africa, and its fruit is a staple food that is especially sought after due to the palatable variations in its stages of ripeness and cooking methods (FAO, 2005; Oladele and Khokhar, 2011). Nigeria contributes over 2.11 million metric tons of plantain yearly, which significantly make available nutrition for her local populations (FAO, 2005).

M. paradisiaca is known to have medicinal properties, as it is used in the folkloric management of a variety of disease conditions due to its anti-ulcerogenic, antifungal, antibacterial analgesic and anti-urolithiatic properties (Kumar *et al.*, 2012). The peels are high in polyphenols, caroteinoids, dietary fibers, and other bioactive compounds that have a variety of health benefits (Wolfe *et al.*, 2003).

Considering the medicinal properties of *M. paradisiaca,* the current study was undertaken to explore the plant's endophytic fungal populations for secondary metabolites with pharmaceutical and medicinal applications.

Materials and Methods

Plant material

Fresh and healthy leaves of *Musa paradisiaca* were harvested from a farmland in Nneogidi a village in Agulu, Anambra State, Nigeria. The plant material was identified and authenticated by a plant taxonomist at the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University Awka, Nigeria, where a voucher specimen with the identification code (PCG/474/A/065) was deposited.

Isolation and identification of endophytic fungus

An endophytic fungus was isolated from healthy leaves of *M. paradisiaca* following a previously described protocol (Okezie *et al.*, 2017). Axenic fungal cultures of the isolate were obtained by way of multiple sub-culturing on fresh malt extract agar. Identification of the axenic fungus taxonomy was achieved by DNA amplification and sequencing of fungal ITS region (Kjer *et al.*, 2010).

Fermentation and extraction of secondary metabolites

Fungal fermentation was carried out adopting the solid-state fermentation technique described by Eze *et al.* (2019). Axenic fungus was cultivated in 1 L Erlenmeyer flasks containing sterilized rice medium (100 g of rice + 200 mL distilled water, autoclaved at 121 °C at 15 psi for 1 h). The flasks were properly sealed and incubated at 25 ± 2 °C for 21 days (Okezie *et al.*, 2015). At the end of fermentation, the fungal secondary metabolites were extracted using ethyl acetate. The filtrate was transferred to the rotary evaporator, then the ethyl acetate content was evaporated at 40 °C.

Bioassays

Antimicrobial assay

The fungal extract was tested for possible inhibitory potentials against some selected laboratory strains such as *Staphylococcus aureus*, *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albicans* using the agar-well diffusion method described by Okezie *et al.* (2017). The test organisms were provided by the Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, Nigeria. The test organisms were standardized to 0.5 McFarland turbidity reference standard in sterile water and inoculated onto previously sterilized 90 mm Mueller-Hinton (for the bacterial test isolates) and Sabouraud dextrose agar plates (for the fungal test isolate) using sterile cotton swabs. The fungal crude extract was reconstituted in dimethyl sulfoxide (DMSO, 100% v/v) and diluted appropriately to a concentration of 1 mg/mL. A volume of 80 µl of the extract solution was transferred into the wells made in the agar using a 6 mm sterilized cork borer. For the positive controls, 80 µl of Ciprofloxacin (5 µg/mL) and Miconazole (50 µg/mL) were tested against the test bacteria and fungus respectively. The bacterial cultures were incubated at 37 °C for 24 h, and the fungal test culture was incubated at 25 °C for 48 h. After incubation, the resulting inhibition zone diameters (IZD) were measured in mm using a meter rule. This assay was carried out in triplicate for each test organism and the mean IZD values calculated.

Antioxidant assay

The propensity of the fungal crude extract to inactivate free radicals such as 2,2-diphenyl-1-picryl hydrazyl (DPPH) was determined using a previously described procedure (Okezie *et al.*, 2021). In order to prepare a concentration of 100 µg/mL of both the fungal extract and positive control (quercetin), 300 µg of each was weighed and dissolved in 3 mL of methanol. A reaction mixture comprising 0.25 mL of the stock, 0.25 mL of DPPH (0.6 mMol) and 2 mL of methanol solution was formed. These were incubated at 27 °C for 30 min. Then, the absorbance which is a measurement of the antioxidant capacity was determined at 517 nm using a UV-vis spectrophotometer (model 721, ANENG, China). The measurement for the extract and control was carried out in triplicate. The capacity to disrupt free radicals were calculated using the formula below and expressed as percentage inhibition.

$$\%\ Inhibition = \frac{A0 - A1}{A0}\ x\ \frac{100}{1}$$

where A0: Absorbance of blank; A1: Absorbance of test sample

Antiviral assay (Reverse Transcriptase Assay)

In vitro antiviral activity of the fungal crude extract was determined using the Roche colometric reverse transcriptase assay kit (Cat. No. 11468120910, Merck KGaA, Germany). The assay protocols contained in the kit's manual was used. Concentrations of 1 and 0.5 mg/mL of the crude extract of the fungus under study were prepared in DMSO. Measure of absorbance of the samples were determined at a wavelength of 405 nm using a microplate reader (UV06452, Thermo max, USA). Each concentration was tested in triplicate. A mixture of lysis buffer, HIV-1-RT and RT inhibitor served as the positive control. For the negative control, a mixture of lysis buffer, RT inhibitor and reaction mixture (solution 3a) was used. 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.

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

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

Cytotoxicity assay

The antiproliferative property of the fungal extract was evaluated against L5178Y mouse lymphoma cells using MTT assay. Briefly, 50 µl containing 3750 cells of L5178Y were aseptically transferred into 96-microtiter plates. Cell attachment was allowed for 24 h and then treated with 50 µL of the fungal crude extract at a concentration of 1 mg/mL. The assay plates were subjected to incubation at 37 °C with 5% CO_2 for 72 h. The formation of a colored formazan product from the tetrazolium was used to assess cell viability as previously described by (Okezie *et al.*, 2015). The test was performed in triplicate. Proper mixing was ensured, then the absorbance was measured at 520 nm. Cytotoxic activity was evaluated through the number of healthy living cells and cell survival was calculated using the formula below.

$$\%\ Survival = \frac{ABu - ABm}{ABt - ABm}\ x\ \frac{100}{1}$$

where ABu: Absorbance of untreated cells; ABm: Absorbance of culture medium;
ABt: Absorbance of treated cells

Chromatographic separation of fungal extract and general chemical analyses

Vacuum liquid chromatography (VLC) was carried out as described by Ebada *et al.* (2008) with slight modifications using Silica gel 60 (70-230 mesh, Merck, Germany). First, "*n*-hexane (100%) was used to de-fat the extract. Fractionation of the extract was then carried out using gradient mixtures of dichloromethane and methanol (DCM:MeOH) to yield 11 fractions (F1 to F11). Orthosporin (code: PM1-MeOH-10%-1) was isolated from semi-preparative HPLC of fraction F2 [DCM (90%):MeOH (10%)]. Analytical HPLC analysis was employed to monitor the chromatographic process, as well as the purity of the isolated compound. The HPLC instrument consists of a Dionex P580 system coupled to a P580A LPG pump, a photodiode array detector (UVD340s, Dionex Softron, Germany), and a separation column (125 x 4 mm) pre-filled with Eurosphere-10 C18 (Knauer, Germany) with MeOH:H_2O mixtures as the gradient solvent system. Also, pre-coated TLC plates (silica gel 60 F254, 20× 20 cm, 0.25 mm thick, Merck, Germany) were used to monitor fractions under UV detection (Camag UV cabinet, Germany) at 254 and 366 nm. Semi-preparative HPLC was performed using a Merck-Hitachi HPLC System comprising of a UV detector (L- 7400), pump (L-7100), and a Eurosphere column (100 C18, 300 × 8 mm, Knauer, Germany). Gradient MeOH:H_2O mixtures were used as the mobile phase at a flow rate of 5.0 mL/min. The molecular weight of the isolated compound was measured using electrospray Ionisation mass spectrometry (ESI-MS) with a UHR-QTOF maXis 4G (Bruker Daltonik, Germany) mass spectrometer. Distilled solvents were used for column chromatography and spectral-grade solvents were used for spectroscopic measurements" Ebada *et al.*, (2008).

Statistical analysis

Measurements were done in triplicate (n = 3) and the results expressed as mean values. One way analysis of variance (ANOVA) and SPSS (version 20) was used as the statistical program.

Results

Following taxonomic identification protocol for DNA amplification accompanied by sequencing of the fungal ITS region, the endophytic fungus was identified as *Lasiodiplodia theobromae*. The resultant data of the fungal DNA sequenced was deposited in the NCBI database (GenBank) with accession number MZ573183.

The fungal axenic culture in malt extract agar plate and its photomicrographic appearance are presented in Figure 1.

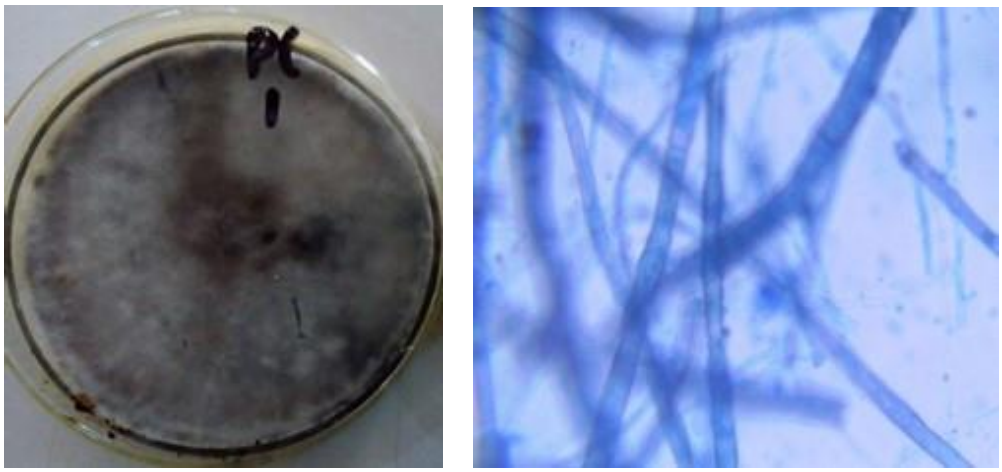


Figure 1. *Lasiodiplodia theobromae* axenic culture in malt extract agar plate, and its photomicrographic appearance after lactophenol cotton blue staining (X40)

Biological investigations of *L. theobromae* crude extract revealed its antimicrobial, antioxidant and antiviral properties (Tables 1-3). At a concentration 1 mg/mL, the extract demonstrated a broad-spectrum activity, inhibiting *B. subtilis* (a Gram positive); *P. aeruginosa,* and *E. coli* (Gram negatives), with an inhibition zone diameter (IZD) that ranged from 3-7 mm. However, no activity was observed against the test fungus, *C. albicans*. Also, the fungal crude extract, at a concentration of 100 µg/mL, exhibited moderate capacity to scavenge and/or disrupt free radicals during it reactivity with DPPH antioxidant assay having an IC_{50} of 65.8 µg/mL (Table 2). The extract showed dose-dependent antiviral properties with 54% and 60% inhibition of reverse transcriptase recorded at concentrations of 0.5 and 1 mg/m respectively during the *in vitro* antiviral assay (Table 3). The fungal crude extract did not show cytotoxic effect on the L5178Y mouse lymphoma cells at 10 µg/mL.

Following chromatographic separation and mass spectrometric analysis, orthosporin was isolated and characterized from the crude fermentation product of *L. theobromae*. Orthosporin showed UV (MeOH) λ_{max} at 243.2, 278.1 and 327.8 nm. The ESIMS of orthosporin revealed pseudo-molecular ion at m/z 235 $[M-H]^-$ in the negative mode revealing a molecular weight of 236 g/mol. The UV and MS data of the compound was confirmed by comparing with previous reports (Harris and Mantle, 2006; Lee *et al.*, 2006). The HPLC chromatogram, UV spectrum and chemical structure of the isolated compound are presented in Figure 2.

Table 1. Result of antimicrobial assay

Samples	Inhibition zone diameters (mm)				
	S. aureus	*B. subtilis*	*P. aeruginosa*	*E. coli*	*C. albicans*
L. theobromae crude extract (1 mg/mL)	0	4	7	3	0
Ciprofloxacin (5 µg/mL)	0	14	14	12	-
Miconazole (50 µg/mL)	-	-	-	-	19

Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL.

Table 2. Result of antioxidant assay

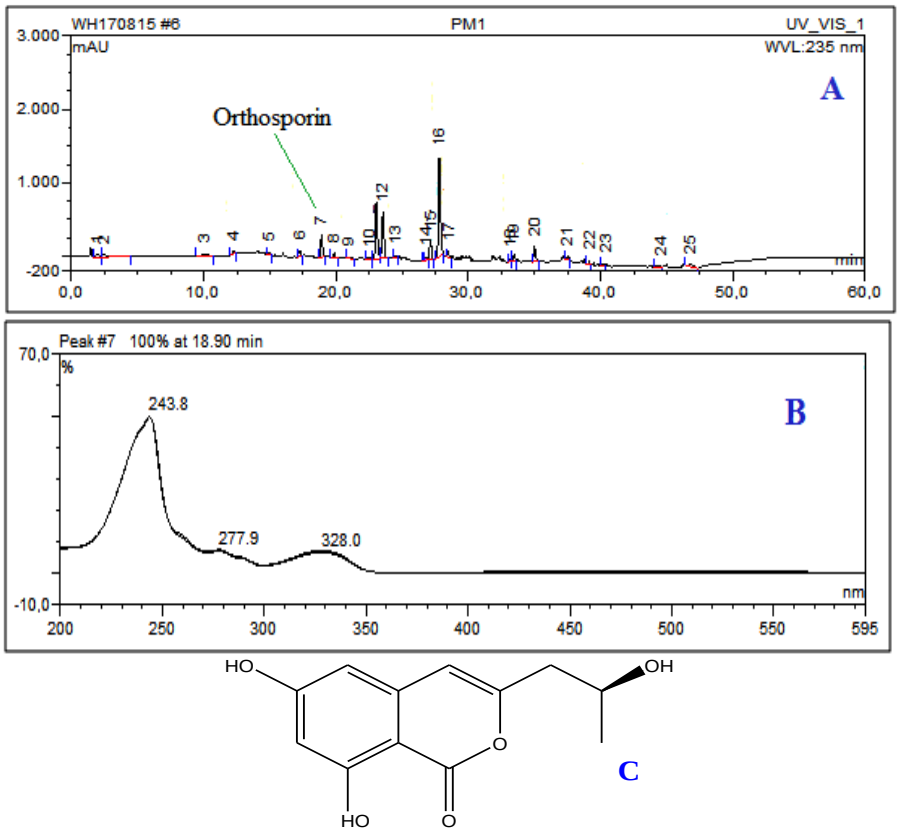
Samples	IC_{50} (µg/mL)
L. theobromae crude extract (100 µg/mL)	65.8
Quercetin (100 µg/mL)	48.0

Antioxidant potential was indicated by the degree of decolorization of the purple color of DPPH which was measured by the UV-Spectrophotometer. Quercetin 100 µg/mL served as the positive control.

Table 3. Result of *in vitro* antiviral assay

Samples	% Inhibition
L. theobromae crude extract (1 mg/mL)	60.0
L. theobromae crude extract (0.5 mg/mL)	54.0
Positive Control	80.0

The *in vitro* inhibition of reverse transcriptase activity by *L. theobromae* crude extract was tested at 1 and 0.5 mg/mL



$C_{12}H_{12}O_5$. 236 g/mol

Figure 2. HPLC chromatogram (A) of *Lasiodiplodia theobromae* crude extract indicating orthosporin Peak; UV spectrum of orthosporin (B) and its chemical structure (C)

Discussion

In this study, *Lasiodiplodia theobromae* an endophytic fungus was isolated from fresh and healthy leaf samples of *Musa paradisiaca* (plantain). *L. theobromae* is a member of the *Botryosphaeriaceae* family characterized by varying morphological features including having an aseptate ascospores. The aseptate are

observed to be pigmented usually with multi-septate and thick-walled conidia having longitudinal striations (Begoude *et al.*, 2009).

Several reports show members of *Botryosphaeriaceae,* especially *Lasiodiplodia* spp., to be disease-causing (Jiuxu, https://www.sciencedirect.com/science/article/pii/B9780124115521000107 - !2014). *L. theobromae* has been linked to some plant diseases notably Botryophaeria dieback (BD), a trunk disease affecting grapevines (Abdullah *et al.*, 2012). Diplodia stem-end rot, a disease observed in both citrus and mango fruits which develops from a latent infection is known to be caused by *L. theobromae* (Zhang and Swingle, 2007; Zhang, 2014*)*. Similarly, durian (*Durio zibethinus*) fruit rot has been linked to *L. theobromae* (Jingtair, 2011). Jasmonic acid methyl ester, a fungal metabolite detected in the fermentation extract of *L. theobromae*, expressed inhibitory effect against plants (Teruhiko, 1999). Also in humans, fungal infections such as mycotic keratitis has been linked to *L. theobromae* (Deanna, 2009).

The biological evaluations of the extract of *L. theobromae* revealed the antibacterial, antioxidant and antiviral activities of the fungal secondary metabolites. The extract demonstrated a broad-spectrum activity against the bacteria test isolates including *B. subtilis, P. aeruginosa,* and *E. coli,* with an inhibition zone diameter (IZD) that ranged from 3 – 7 mm (Table 1), and a moderate antioxidant property having an IC_{50} of 65.8 µg/mL during the DPPH antioxidant assay (Table 2). Also, the fungal extract showed dose-dependent antiviral properties with 54% and 60% inhibition of reverse transcriptase recorded at concentrations of 0.5 and 1 mg/m respectively during the *in vitro* antiviral assay (Table 3).

Although the controls had higher activities in the assays performed in the study, the activities and/or inhibitions by the fungal extract can be said to be comparable to the controls.

Previous studies have reported the identification of important bioactive compounds in fermentation broth of *L. theobromae.* These include aranosinol B, *p*-hydroxybenzoic acid, cyclohexenes, cyclohexenones, 3-indolacetic acid, 3-indolcarbaldehyde, lasiolactol A, simplicildone A (Salvatore *et al.*, 2020; Ujam *et al.*, 2020). Also in our study, orthosporin, a naturally occurring and pytotoxic isocoumarin, was isolated from the fermentation extract of *L. theobromae.*

Orthosporin, has been isolated from both plants such as *Daldinia concentrica* (Lee *et al.*, 2006) and microbes including *Aspergillus ochraceus, Scytalidium thermophilum, Ceratocystis minor, Daldinia eschscholtzii, Diaporthe terebinthifoli, Mucor sp., Cadophora sp., Rhynchosporium orthosporum, etc* (Hemingway *et al.*, 1977; Ichihara *et al.*, 1989; Harris and Mantle, 2001; Feng *et al.*, 2014; Rusman *et al.*, 2015; de Medeiros *et al.,* 2018; Hai *et al.*, 2019). The compound has been detected in the fermentation broth of several endophytic fungi including *Botryosphaeria sp.*, a fungus isolated from a mangrove plant *Kandelia candel* (Ju *et al.*, 2016) and *Diaporthe terebinthifoli* a fungal endophyte isolated from *Schinus terebinthifolius* a medicinal plant (de Medeiros *et al.,* 2018).

The phytotoxic properties of orthosporin have been reported by several authors including Hallock *et al.* (1988) who isolated orthosporin from *Drechslera siccansa,* a phytopathogenic fungus implicated in the necrotic disease conditions of soya bean, crabgrass, maize and barnyard grass; and Ichihara *et al.* (1989), who detected orthosporin in a phytotoxic culture filtrate of *Rhynchosporum orthosporum.* Hemingway *et al.* (1977) identified a methyl derivative of orthosporin as a major phytopathogenic metabolite of Ceratocystis minor, which infects chestnut trees, as a major phytopathogenic metabolite.

Despite being a phytotoxin, orthosporin is known to possess several beneficial biological properties including antimicrobial, antidiabetic, and antioxidant (Rusman *et al.*, 2015; Tanapichatsakul *et al.*, 2017; Hai *et al.,* 2019).

This study describes the isolation of *L. theobromae* from the internal tissue of healthy leaves of *M. paradisiaca,* the biological properties of the fungal secondary metabolites, accompanied by the isolation of orthosporin, a well-known metabolic product, from the fungal fermentation extract.

Conclusions

This study reports the biological activities of the secondary metabolites of endophytic *L. theobromae* associated with a Nigerian plant, *M. paradisiaca.* Orthosporin, a phytotoxic isocoumarin compound, was isolated from the fungal fermentation extract. The compound is well-known to possess several beneficial biological properties including Antimicrobial: Rusman *et al.* (2015); Antioxidant: Tanapichatsakul *et al.* (2017) and Anti-diabetic: Hai *et al.* (2019) activities.

This study highlights the potentials of endophytic fungi as producers of interesting bio-molecules.

Authors' Contributions

COE and FBCO designed and supervised the study; UMO performed the laboratory experiments and bioassays; FBCO and PME performed the chemical analyses; UMO Wrote the first draft; PME and FBCO reviewed and edited the draft. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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