



Phenolic Compounds from Endophytic *Pseudofusicoccum* sp. Isolated from *Annona muricata*

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ABSTRACT

Endophytic fungal populations of Nigerian medicinal plants have been shown to possess enormous potentials as sources of biologically active compounds of pharmaceutical and industrial importance. Our study was carried out to investigate the secondary metabolites of an endophytic fungus *Pseudofusicoccum* sp. isolated from the leaves of *Annona muricata* growing in Ifite Dunu, Anambra State, South-East Nigeria. Isolation of the endophyte, identification by DNA amplification and sequencing of the internal transcribed spacer (ITS) region were carried out using standard methods. Solid state fermentation of the endophyte was carried out in rice medium and the metabolites were extracted with ethyl acetate. The crude ethyl acetate extract was subjected to antioxidant and antimicrobial assays. Fractionation of the fungal extract, and the isolation and characterization of compounds were carried out using various chromatographic and spectroscopic methods. The fungal crude extract showed mild antimicrobial activity against *B. subtilis*, *S. typhi* and *C. albicans*, with inhibition zone diameters (IZDs) of 2, 3, and 2 mm respectively. The extract showed good antioxidant activity with an inhibition of 96%. Chromatographic purification of the fungal extract afforded three known phenolic compounds (1-3) that are known for their antioxidant properties. This study shows that *A. muricata* harbor endophytes that could be excellent sources of pharmacologically active compounds.

Keywords: *Annona muricata*, *Pseudofusicoccum* sp., endophytes, secondary metabolites.

Introduction

Annona muricata is widely distributed throughout the tropical and subtropical parts of the world, including Nigeria.¹ Ethnomedicinally, the plant has been used in the treatment of disease conditions like dysentery, fever, neuralgia, arthritis, rheumatism, cystitis, diabetes, headaches, insomnia, abscesses, tumors and cancer.^{1,2,3,4}

Phytochemical evaluations of the plant have shown the presence of alkaloids,^{5,6} flavonol triglycosides,⁷ phenolics,⁸ cyclopeptides and essential oils.⁹ Gas chromatography- mass spectroscopy (GC-MS) and GC analyses of the leaf oil of *A. muricata* showed the presence of sesquiterpenes, with β -caryophyllene being the most abundant.¹⁰ Similar studies on *A. muricata* leaf oil identified volatile oil constituents such as β -pinene, germacrene D, ρ -mentha-2,4 (8)-diene, α -pinene and β -elemene, δ -cadinene, epi- α -cadinol and α -cadinol.^{11,12} High concentrations of mono- and sesquiterpenes, including β -caryophyllene, 1,8-cineole and linalool, were also isolated from *A. muricata* fruit pulp.¹³

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A. muricata have been studied for the bioactive natural products produced by its endophytic fungal populations.¹⁴⁻²³ Several compounds have been reported from the endophytic fungus *Periconia* sp. associated with *A. muricata*. These compounds include polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) hybrid metabolites: pericoannosins A and B;^{15,16} polyketide-terpenoid hybrid molecules: periconone A-E;¹⁸⁻²⁰ monoterpenoids: 2-carene-5,8-diol, 2-carene-8,10-diol, 2-carene-8-acetamide, 8-hydroxy-1,7-epoxy-2-menthene, and anethofuran;²¹ cytochalasins: Periconiasins A-H;^{14,15,17} and sesquiterpenes: periconianones C-K.¹⁹

Secondary metabolites of endophytic fungi of *A. muricata* showed anticancer activities.^{22,23} Periconiasins A and B showed significant cytotoxicity against human colon cancer cells (HCT-8).¹⁴ Pericoannosins A and Periconiasins F, periconone B were shown to have anti-HIV activity.^{15,20} Periconianones D, G and K showed antioxidant activity due to lipopolysaccharide-induced nitric oxide (NO) inhibition.¹⁹

Nigeria's rich plant biodiversity presents the opportunity to discover a plethora of biologically important compounds expressed by the millions of endophytic microbial communities inherent in these plants.²⁴ Investigations of the endophytic fungal populations of some Nigerian medicinal plants have revealed the enormous potentials possessed by these organisms as sources of compounds of pharmaceutical and industrial importance.²⁴⁻³¹ There is, therefore, the need to further explore these plants for endophytes producing biologically important molecules. Hence, our study seeks to investigate the secondary metabolites of an endophytic fungus isolated from leaves *A. muricata* growing in Anambra State, South-East Nigeria.

Materials and Methods

Endophytic Fungal isolation and identification

Fresh and healthy leaves of *A. muricata* were collected in February 2015, from Ifite Dunu in Anambra State, South-East Nigeria. The plant was authenticated by a plant taxonomist at the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka and deposited therein with the specimen voucher number PCG474/A/049. The isolation of an endophytic fungus from the plant leaves was carried out using a previously described method.²⁶ The plant leaves were washed in running tap water and then cut into small fragments of about 1 cm². The leaf fragments were surface-sterilized by immersion in 2% sodium hypochlorite solution for 2 min, 70% ethanol for nearly 2 min, and then rinsed in sterile water for 5 min. The leaf fragments were put into Petri dishes containing malt extract agar (MEA) supplemented with chloramphenicol. The Petri dishes were then incubated at a temperature of 28°C and fungal growths from the leaf fragments were monitored. Hyphal tips from distinct colonies emerging from leaf segments were sub-cultured onto fresh MEA plates to obtain pure colonies. An endophytic fungus was isolated from the plant leaves and taxonomic identification of the fungus was achieved by DNA amplification and sequencing of the ITS region.³²

Fermentation, Extraction, Isolation of Secondary Metabolites

The fungus was grown in sterile solid rice medium (100 g rice and 110 mL distilled water) in 1L Erlenmeyer flasks at 27°C under static conditions for 30 days. At the completion of fermentation, the fungal secondary metabolites were extracted with EtOAc and the crude extract concentrated under reduced pressure. A weight of 6.3 g of the fungal crude extract was obtained. Vacuum liquid chromatography (VLC) of the fungal crude extract was carried out using Silica gel 60 (70–230 mesh, Merck, Germany) as the stationary phase. Stepwise gradient elution was done using non-polar/moderately polar solvent system *n*-hexane:EtOAc in the ratio of 90:10, 80:20, 60:40, 50:50, 40:60, and then increasing the polarity with polar solvent system DCM:MeOH in the ratio of 100:0, 95:5, 90:10, 80:20 and 0:100 to obtain successive fractions from the fungal crude extract.

Size exclusion chromatography on Sephadex LH-20 (Sigma-Aldrich, Germany) as the stationary phase and DCM:MeOH, in the ratio of 1:1 (v/v), as the mobile phase was used for further purification. Semi-preparative HPLC was performed using a Merck/Hitachi HPLC System (UV detector L-7400; pump L-7100), with a Eurosphere 100 C18 column (300 × 8 mm, Knauer, Germany). Gradient mixtures of MeOH:H₂O were used as mobile phase at a flow rate of 5.0 mL/min. The crude and fractions were all subjected to analytical HPLC analyses performed with a Dionex P580 system coupled to a P580A LPG pump and a photodiode array detector (UVD340s, Dionex). The separation column was pre-filled with Eurospher 10 C18 (4 × 125 mm, Knauer, Germany). The wavelengths set for UV detections were 235, 254, 280, and 340 nm. NMR spectra were recorded in CD₃OD using Bruker 300 spectrometer. Spectra were referenced to the residual solvent signals. Proton chemical shifts were compared with corresponding signals in literature data. The chemical shift values (δ) and coupling constants (*J*) are indicated in parts per million (ppm) and Hertz (Hz) respectively. For mass determination, LC-MS measurements were carried out on a LC-MS HP1100 Agilent Finnigan LCQ Deca XP Thermoquest spectrometer and electron impact (EI) mass spectra were measured on a Finnigan MAT 8430 mass spectrometer.

Antimicrobial assay

The antimicrobial assay of the endophytic fungal crude extract (1 mg/mL) was performed using the agar well diffusion assay method as described by Abba *et al.*²⁶ The antibacterial activity of the fungal extract was tested against laboratory strains of *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, while *Aspergillus niger* and *Candida albicans* were used for the antifungal assay. Ciprofloxacin (5 μ g/mL) and miconazole (50 μ g/mL) served as positive control for the antibacterial and antifungal assays respectively, while DMSO was used as the negative control.

Antioxidant Assay

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) antioxidant assay was performed as described by Shen *et al.*,³³ but with modification. A stock solution of 1000 μ g/ml of the extract and positive control (quercetin),

and 0.2 mM DPPH solution were prepared in MeOH. A volume of 2 mL of sample (extract or control) was added to 2 mL of DPPH solution to achieve final concentration of 500 μ g/mL of the sample and 0.1 mM DPPH solution. Each of the mixtures was shaken vigorously, and then incubated in the dark at room temperature for 30 min. The UV absorbance was measured at 517 nm using a UV-VIS spectrophotometer. A 2 mL of MeOH solvent mixed with 2 mL of DPPH solution served as blank which is the negative control. The DPPH free radical scavenging effect of the samples was calculated using the following formula.

DPPH scavenging effect (% inhibition)

$$= \frac{\text{Abs of blank (A0)} - \text{Absorbance of sample (A1)}}{\text{Abs of blank (A0)}} \times 100$$

Statistical analysis

In the antimicrobial and antioxidant assays, inhibition zone diameters (IZDs) and UV absorbance were measured in triplicates respectively, and the average calculated and recorded. The result of the antimicrobial assay of the endophytic fungal extract is presented as mean \pm standard error of mean IZDs.

Results and Discussion

The endophytic fungus isolated from *A. muricata* was identified as *Pseudofusicoccum sp.* The DNA sequence data of the fungus was deposited in the NCBI database with GenBank accession number - MH022721. The crude EtOAc extract of *Pseudofusicoccum sp.* was subjected to antimicrobial and antioxidant assays. In the antimicrobial assay (Table 1), the extract showed mild activity against *B. subtilis*, *S. typhi* and *C. albicans*, with inhibition zone diameters (IZDs) of 2, 3, and 2 mm respectively. No antimicrobial activity was recorded against *S. aureus* and *A. niger*. The antioxidant result (Table 2) showed that the fungal crude extract produced inhibition of 96% (which is higher than 93% recorded for the positive control, quercetin). Chemical characterization of the fungal extract afforded three known phenolic compounds - tyrosol, protocatechuic acid and *p*-hydroxyphenyl acetic acid.

Compound 1

Compound 1 was isolated from ethyl acetate extract of rice cultures of *Pseudofusicoccum sp.* as cream coloured oil. It showed a retention time of 17.85 min in HPLC analysis and UV absorption maxima at 220 and 276.9 nm. Compound 1 exhibited a pseudo-molecular ion peak at *m/z* 138 in the positive mode of EI-MS, which is consistent with molecular mass of 138 and molecular formula of C₈H₁₀O₂. The base peak was found at *m/z* 107, which shows loss of CH₂OH from the compound. The ¹H-NMR spectrum indicated the presence of *ortho*-coupled aromatic protons of the AA'BB' pattern at δ_H 7.02 (*J* = 8.49 Hz, 2H) and 6.70 (*J* = 8.49 Hz, 2H) assignable to 2'/6' and 3'/5' respectively, which is consistent with para-substituted benzene ring. The ¹H-NMR also showed the presence of AB coupled protons in the aliphatic regions at δ_H 3.68 (*J* = 7.2 Hz, 2H) and 2.71 (*J* = 7.2 Hz, 2H) each integrating to two protons assignable to 1 H_{a/b} and 2 H_{a/b} respectively and corresponded to two coupled -CH₂ group. The de-shielded position of the methylene group at δ_H 3.68 suggested attachment to an electronegative group, in this case -OH, while the slightly de-shielded position of the methylene group at δ_H 2.71 indicated attachment to an aromatic ring. Taking into consideration the molar mass, molecular formula, the observed fragments from ¹H-NMR analysis and comparison with literature data,³⁴⁻³⁶ compound 1 was elucidated as 2-(4-hydroxyphenyl) ethan-1-ol, commonly known as tyrosol.

Compound 2

Compound 2, was isolated as an off white tiny crystal from crude ethyl acetate extract of *Pseudofusicoccum sp.* It showed HPLC retention time at 5.39 mins and UV absorption maxima (MeOH) at 222, 258 and 294 nm which suggested conjugated chromophores. Compound 2 exhibited a pseudo-molecular ion peak at *m/z* 153 [M-H]⁻ negative ion mode of ESI-MS, which is consistent with molar mass of 154 g/mol and molecular formula of C₇H₆O₄. The pseudo-molecular ion peak observed at *m/z* 199 and 307 showed [M + CHOO]⁻ and [2M-H]⁺ respectively. The ¹H-NMR spectrum showed three aromatic proton peaks of the ABX pattern at δ_H 7.45 (s, 1H), 7.43 (*J* = 7.4 Hz, m, 1H) and 6.80 (*J* = 7.5 Hz, m, 1H), assignable to H-2, H-6 and H-5 respectively. Taking into

consideration the observed coupling pattern, the molecular formula and comparison with literature data,³⁷ compound **2** was elucidated as 3,4-dihydroxybenzoic acid (protocatechuic acid).

Compound 3

Compound **3**, which was isolated as a light brown solid had a HPLC retention time at 8.86 min and UV maxima at 222.1 and 276.2 nm. Compound **3** exhibited a molecular ion peak at m/z 152 in the positive mode of an EI-MS, which is consistent with molecular mass of 152 g/mol and molecular formula of $C_8H_8O_3$. The base peak at m/z 107 shows loss of COOH and m/z 77 showed deprotonated benzene with loss of CH_3OH . The 1H -NMR spectra indicated the presence of ortho-coupled aromatic protons of the AA'BB' pattern at δ_H 7.09 ($J = 8.5$ Hz, 2H) and 6.72 ($J = 8.5$ Hz, 2H) assignable to 2'/6' and 3'/5' respectively, which is consistent with para-substituted benzene ring. The 1H -NMR also showed the presence of a singlet in the aliphatic region at δ_H 3.47 indicating the $-CH_2$ group without a neighboring proton. The deshielded position of the methylene group suggested closeness to an electron withdrawing group. From the information generated by the EI-MS, 1H -NMR and comparison with literature data,³⁸ compound **3** was elucidated as 2-(4-hydroxyphenyl) acetic acid, also known as 4-hydroxyphenylacetic acid or *p*-hydroxyphenylacetic acid.

Pseudofusicoccum is a fungal genus in the family Botryosphaeriaceae. *Pseudofusicoccum* sp. exists as an endophyte in plant tissues and has been reported to also cause canker disease in some plants.^{39,40,41} *Pseudofusicoccum* sp has been isolated from several plants including *Adansonia gibbosa*, *Acacia synchronica*, *Eucalyptus* sp., *Ficus opposita*, *Mangifera indica*, *Pterocarpus angolensis*, *Jatropha podagrica*.^{39,40,42,43} *Pseudofusicoccum* sp. has been reported to express the bioactive compound lasiodiplodin.⁴⁴

In this study, *Pseudofusicoccum* sp. crude extract showed mild antimicrobial and excellent antioxidant activities. When fractionated, *Pseudofusicoccum* sp. crude extract yielded three simple phenolic compounds. The leaves of *Annona muricata* from which the endophytic *Pseudofusicoccum* sp. was isolated are known to be abundant in Annonaceous acetogenins such as muricoreacin and murihexocin C,⁴⁵ murihexocins A and B.^{46,47} Phenolic compounds exhibiting various pharmacological activities are found mostly in the fruit of this plant. These compounds include cinnamic acid derivatives, coumaric acid hexose, 5-caffeoylquinic acid, dihydrokaempferol-hexoside, *p*-coumaric acid, caffeic acid derivative, dicaffeoylquinic acid, feruloylglycoside, 4-feruloyl-5-caffeoylquinic acid and *p*-coumaric acid methyl ester.⁸

In animals, phenolics are reported to show antioxidant, anti-inflammatory and other biological properties, and may protect from oxidative stress and some diseases.⁴⁸ Simple phenolics are bactericidal, antiseptic and anthelmintic and phenol itself is a standard for other antimicrobial agents.⁴⁹ Phenols and phenolic acids from fungal endophytes usually have pronounced biological activities.⁵⁰ The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. In addition, they have a metal chelation potential.^{51,52}

Our study reports the isolation of three phenolic compounds expressed by the fungus *Pseudofusicoccum* sp. These compounds include tyrosol, protocatechuic acid and *p*-hydroxyphenyl acetic acid (Figure 1). These compounds have been reported to possess diverse pharmacological activities.^{56-65, 67-78}

Tyrosol, also known as 2-(4-Hydroxyphenyl) ethanol, is a well-known phenolic compound present in different dietary sources that can exert mild antioxidant properties based on *in vitro* and *in vivo* studies.⁵³ It has been produced by many fungi including *Phialocephala fortinii* Rac56,³⁶ *Diaporthe eres*,⁵⁴ and *Glomerella cingulata*.⁵⁵ The compound has been shown to be effective in inhibiting the oxidation of low-density lipoprotein (LDL) and preventing the modification of the apoprotein moiety.⁵⁶ It has also been effective in inhibiting leukocyte 5-lipoxygenase, and protecting the Caco-2 intestinal mucosa cells against the cytostatic and cytotoxic effects produced by oxidized LDL.⁵⁷⁻⁵⁹ Tyrosol was reported to have scavenging effects on reactive oxygen and nitrogen species that are implicated in human pathologies such as cardiovascular and thrombotic diseases,^{55, 60,61} and has been reported as a candidate to be used in stroke therapy due to its neuroprotective effect in rats.⁶² It was identified as an auto-regulatory molecule with important implication on the dynamics of growth and morphogenesis in *Candida albicans*, a process known as quorum-sensing, which is characterized by a cellular density-dependent phenomenon.⁶³ The antifungal activity of tyrosol against *Lagenidium callinectes* and *Gibberella pulicaris* has also been reported.^{64,65}

Protocatechuic acid (PCA) (3,4-dihydroxybenzoic acid), a natural phenolic compound found in many edible and medicinal plants, is a major benzoic acid derivative with a strong antioxidative effect.⁶⁶ PCA is one of the main metabolites of complex polyphenols such as anthocyanins and procyanidins that are normally found at high concentrations in vegetables and fruits, and are absorbed by animals and humans. It has been shown that PCA possesses antioxidant,^{67,66} anti-inflammatory,⁶⁸⁻⁷⁰ hypoglycemic,⁷¹ anticancer,⁷² antiulcer,⁷³ antiaging,⁷⁴ antiviral,⁷⁵ chemopreventive,⁷⁶ and neuroprotective⁶⁶ activities.

p-Hydroxyphenylacetic acid or 4-hydroxyphenylacetic acid (4-HPA) is a phenolic compound and a metabolite of tyrosine in humans that has been isolated from other fungi like *Oidiodendron* sp.⁷⁷ and endophytic fungus HP-1 of Chinese eaglewood.⁷⁸ 4-HPA has shown nematicidal activities against the root-lesion nematode *Pratylenchus penetrans* and the pine wood nematode *Bursaphelenchus xylophilus*⁷⁷ and also antibacterial activity against *S. aureus*.⁷⁸

There are lots of tropical medicinal plants in Nigeria with proven pharmacological activities, yet their endophytes have not been studied for production of new secondary metabolites. According to Eze *et al.*,²⁴ Nigeria's rich plant biodiversity presents an enormous platform for researchers to explore in the search for biologically active molecules without the destructive harvesting of plants, but by exploring their associated endophytic organisms for pharmaceutically and industrially important molecules. This present study has also confirmed that many Nigerian plants harbor endophytes that could be excellent sources of pharmacologically active compounds. This study gives the first report of an endophyte associated with *A. muricata* from Nigeria, and the first report of the isolation of *Pseudofusicoccum* sp. from *A. muricata*.

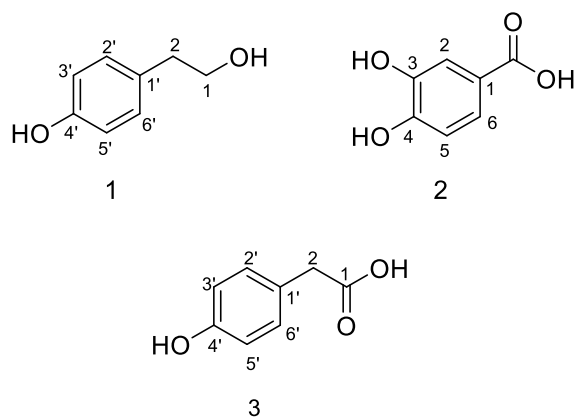
Table 1: Antimicrobial Assay of *Pseudofusicoccum* sp. Crude Extract.

Test Organisms	IZD (mm) of Fungal Crude Extract at 1 mg/mL	Positive control	Negative control
		Ciprofloxacin (5 µg/mL)	DMSO
<i>S. aureus</i>	0 ± 0.00	5 ± 0.33	0 ± 0.00
<i>B. subtilis</i>	2 ± 0.00	8 ± 0.00	0 ± 0.00
<i>S. typhi</i>	3 ± 0.33	7 ± 0.33	0 ± 0.00
<i>E. coli</i>	0 ± 0.00	8 ± 0.33	0 ± 0.00
<i>A. niger</i>	0 ± 0.00	12 ± 0.89	0 ± 0.00
<i>C. albicans</i>	2 ± 0.33	14 ± 0.33	0 ± 0.00

Table 2: DPPH Antioxidant Assay of *Pseudofusicoccum* sp

Crude Extract.

Sample	% Inhibition
<i>Pseudofusicoccum</i> sp. extract	96
Quercetin	93

**Figure 1:** Phenolic compounds isolated from *Pseudofusicoccum* sp; compounds **1** (tyrosol), **2** (protocatechuic acid) and **3** (*p*-hydroxyphenyl acetic acid).

Conclusion

The fungus *Pseudofusicoccum* sp. from *A. muricata* afforded three known phenolic compounds that are well known for their antioxidant properties; tyrosol, protocatechuic acid and *p*-hydroxyphenyl acetic acid. The pharmacological activities of these compounds are already well known.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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