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ACL-4, an Endophytic Fungus Isolated from *Ageratum conyzoides* Leaves Possesses the Unique Potential of Generating Low Molecular Weight Bioactive Lead Compounds

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ABSTRACT

Over the years, endophytic fungi have generated novel bioactive lead molecules for drug development. A detailed chemical investigation of ACL-4, an endophytic fungus previously isolated from *Ageratum conyzoides*, led to the isolation of eight low molecular weight bioactive secondary metabolites. The metabolites generated by the axenic fungus, when grown on rice medium using solid fermentation, were extracted using ethyl acetate. Further chromatographic separation of the crude extract led to the isolation of compounds **1-8.** The chemical structures of these compounds were determined using a combination of HPLC-DAD, and NMR analyses. The fractions from which the compounds were isolated were also subjected to antimicrobial screening using Agar well dilution techniques. The chemical structures of the compounds were elucidated as 2-hydroxy-6- (1'-hydroxyethyl) benzoic acid (**1**), 2-(4'-hydroxyphenyl) ethanol (**2**), 4-hydroxybenzoic acid (**3**), 2-(4'-methylphenyl) ethanol (**4**), 3-methoxy-4-hydroxybenzoic acid (**5**), epiguaymasol (**6**), 2- {4'- (4"hydroxyphenyl) methylphenyl) ethanol (**7**), and protocatechuic acid (**8**). The tested fractions displayed varying degrees of antimicrobial activity, with MICs ranging from 0.125 – 0.5 mg/mL. The antimicrobial activities shown by the fungal extract and fractions may be as a result of the presence of some of these compounds, which have been previously reported as antimicrobial agents.

***Keywords*:** Secondary metabolites, Structure elucidation, Endophytic fungus, *Ageratum conyzoides*.

Introduction

Endophytes have shown immense potential as sources of novel bioactive secondary metabolites and as such have stimulated significant interest in drug discovery in the recent times.[1-6] In our previous reports, we have also observed close biological association between the host plants and their associated endophytes, which results in the production of diversity of biologically active secondary metabolites.[7,8] The observed symbiotic relationship between the host plants and their associated fungi endophytes will be responsible for the presence of less toxic bioactive secondary metabolites, which can be harnessed as therapeutic drug molecules. The significance of this is that therapeutic molecules derived therefrom will be potentially less toxic to human cells. [9] There are many recent reports of isolation of plant-derived anticancer agents like paclitaxel (Taxol®) from *Taxomyces* and *Pestalotiopsis species*.[10-11] Other examples are camptothecin,[12] podophyllotoxin,[13] vinblastine,[14] and vincristine,[15] which have been isolated from endophytes in relatively small amounts.

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In our continued search for novel bioactive secondary metabolites from fungal endophytes associated with Nigerian medicinal plants, we further investigated the chemistry of an endophyte ACL-4, which we previously described from *Ageratum conyzoides*.[16] This fungal endophyte was observed to be a rich source of low molecular weight bioactive phenolic compounds. Low molecular weight compounds have contributed to the great structural variabilities and diversities of secondary metabolites from fungal endophytes. Apart from playing major roles in understanding the biochemistry and biogenic routes of the varied bioactive compounds from plants and their associated fungi endophytes, they also play major roles in the observed biological functions attributed to endophytic fungi of medicinal plant origins.
This current study, therefore, further elucidates these low molecular weight bioactive natural compounds from an endophyte of *Ageratum conyzoides* thus providing new frontiers for effective management of diseases that affect mankind.

Materials and Methods

Isolation, identification and fermentation of endophytic fungus
The isolation, identification and fermentation of the endophytic fungi was previously described.[16]

Vacuum Liquid Chromatography (VLC)
Vacuum liquid chromatography was carried out on 2 g of the crude extract obtained from the fungal fermentation. The glass column (4x50 cm) was packed to the height of 15 cm with silica gel (230-400 mesh size). The extract was adsorbed on silica gel in the column, which was connected to a vacuum pump and was eluted by step gradient of

combinations of n-hexane: ethyl acetate and then dichloromethane: methanol. Successive fractions (ACL-4-1 to ACL-4-19) were collected, with the column allowed to run dry with each collection. The fractions, each were collected in a different flask and concentrated using the vacuum rotary evaporator at reduced pressure.

High Performance Liquid Chromatography (HPLC)-Diode Array Detection (DAD) Analysis
The isolated compounds (0.5 mg) or the dried VLC fractions (2 mg) were separately reconstituted in HPLC grade methanol (2 mL), sonicated for 10 min, and thereafter centrifuged for 5 min at 3000 rpm. The supernatant (100 µL) of the dissolved samples were transferred into HPLC vials prefilled with 500 µL of the HPLC grade methanol. These samples were subjected to HPLC analysis with a Dionex P 580 HPLC system, which was coupled to a photodiode array detector (UVD340S, DionexSoftron GmbH, Germering, Germany) and detection was done at 235 nm. Eurospher-10 C18 (Knauer, Germany) was used to pre-fill the separation column which has the dimensions of 125 mm × 4 mm; length × internal diameter. The eluent was a linear gradient of nano pure water (adjusted to pH 2 by the addition of formic acid) and HPLC grade methanol. The HPLC analyses were used to determine the optimum condition for further separation using semi preparative HPLC and also to ascertain the purity of the isolated compounds. Some compounds were also detected by dereplication using HPLC-ultraviolet (UV)/visible database, which contains over 1600 registered compounds.

Semi preparative HPLC
The solvent systems used for the semipreparative HPLC were determined from the analytical HPLC initially carried out on the VLC fractions. Gradient mixtures of methanol and nanopure water was used as the mobile phase in each purification. About 50-100 µL containing 1–3 mg of the fraction dissolved in HPLC grade methanol was injected at each run. The flow rate of 5 mL/min was used and the eluted peaks, which were detected by UV detector were manually collected in clean test tubes. Further purification of ACL-4-4 led to the isolation of compounds **1** and **2**, purification of ACL-4-5 led to the isolation of compound **3**, purification of ACL-4-6 led to the isolation of compounds **4**, **5**, **6** and **7**, while purification of ACL-4-7 led to the isolation of compound **8.**

Determination of Nuclear Magnetic Resonance (NMR) Spectra.
The chemical structures of the isolated compounds were determined by proton NMR measurements using the Bruker DPX 300 NMR spectrometer. All 1-dimensional NMR spectra were obtained using the standard Bruker software. The samples were dissolved in deuterated methanol (MeOD). The internal standards (reference signals) were determined based on the residual solvent signals and observed chemical shift (δ) values and the coupling constants (*J*) were given in ppm and in Hz, respectively.

Antimicrobial Assay
Using Agar well dilution technique, the minimum inhibitory concentration (MIC) of the test fractions was determined against laboratory strains of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger* and *Candida albicans*. The test solutions of 5, 2.5, 1.25, 0.625 mg/mL were obtained from two-fold serial dilutions of the stock solution of 10 mg/mL of the fractions. A 10-fold dilution of each of the concentration was made by adding 1 mL of the test solution into 9 mL sterile molten agar, and allowed to solidify. The microbial inoculums were standardized to 0.5 McFarland turbidity and thereafter streaked on the solidified Agar. The plates were incubated at 37ºC for 24 h for the bacteria plates and at 25ºC for 48 h for the fungi plates. The plates were examined for microbial growth after the prescribed incubation periods. Ciprofloxacin (CIPRO) and miconazole (MICON) were used as positive controls for bacteria and fungi respectively.

Results and Discussion

The separation of the crude extract of ACL-4 by VLC and further purification of the resulting fractions by semi-preparative reverse phase HPLC led to the isolation and structure elucidation of compounds **1** to **8**.

*2-hydroxy-6- (1'-hydroxyethyl) benzoic acid (**1**)*
White solid (1.5 mg); UV λ_{max} (PDA): 206.6, 346.0, 313.9 nm; NMR (600MHz, MeOH-d4) δ_H: 6.94 (d, *J* = 8.4, H-3), 7.57 (t, *J* = 8.0, H-4), 7.07 (d, *J* = 7.6, H-5), 4.56 (q, H-1'), 1.46 (d, H-2').

*2-(4'-hydroxyphenyl) ethanol (**2**)*
White solid (1.8 mg); UV λ_{max} (PDA): 220.1, 276.5 nm; NMR (300MHz, MeOH-d4) δ_H: 3.68 (t, *J* = 7.2, H_2-1), 2.7 (t, *J* = 7.2, H_2-2), 7.03 (d, *J* = 8.4, H-2'/6'), 6.70 (d, *J* = 8.4, H-3'/5').

*4-hydroxybenzoic acid (**3**)*
White solid (1.5 mg); UV λ_{max} (PDA): 255.6 nm; NMR (300MHz, MeOH-d4) δ_H: 7.85 (d, *J* = 8.0, H-2/6), 6.78 (d, *J* = 8.0, H-3/5).

*2-(4'-methylphenyl) ethanol (**4**)*
White solid (2.0 mg); UV λ_{max} (PDA): 221.2, 276.7 nm; NMR (300MHz, MeOH-d4) δ_H:4.19 (t, *J* = 7.1, H_2-1), 2.81 (t, *J* = 7.1, H_2-2), 7.03 (d, *J* = 8.4, H-2'/6'), 6.70 (d, *J* = 8.4, H-3'/5').

*3-methoxy-4-hydroxybenzoic acid (**5**)*
White solid (2.1 mg); UV λ_{max} (PDA): 202.0, 216.9, 260.8, 290 nm; NMR (300MHz, MeOH-d4) δ_H: 7.56 (d, J = 1.9 Hz, H-2), 6.82 (d, J = 8.2, H-5), 7.54 (dd, J = 8.2, 1.9 Hz, H-6), 3.89 (s, H_3-3-OMe).

*Epiguaymasol (**6**)*
White solid (2.0 mg); UV λ_{max} (PDA): 223.7, 276.7 nm; NMR (300MHz, MeOH-d4) δ_H: 2.02 (m, H_A-1), 2.49 (dq, *J* = 11.3, 7.1 Hz, H_B-1), 4.25 (m, H-2), 4.20 (dt, *J* = 11.7, 5.8 Hz, H-3), 1.23 (dd, *J* = 16.1, 7.1 Hz, H_A-4), 1.44 (m, H_B-4), 0.95 (m, H-5), 1.34 (d, *J* = 6.2 Hz, H_3-6), 1.17 (d, *J* = 7.0 Hz, H_3-7), 7.03 (d, *J* = 8.4, H-2'/6'), 6.73 (d, *J* = 8.4, H-3'/5').

2- (4'- (4''hydroxyphenyl) methylphenyl) ethanol (7)
White solid (1.9 mg); UV λ_{max} (PDA): 222.5 and 277.0 nm; NMR (300MHz, MeOH-d4) δ_H: 4.21 (t, *J* = 6.9, H_2-1), 2.79 (t, *J* = 6.9, 2H H_2-2), 7.02 (d, *J* = 8.4, H-2'/6'), 6.71 (d, *J* = 8.4, H-3'/5'), 3.48 (s, H_2-4'), 6.79 (d, *J* = 8.0, H-2"/6"), 6.68 (d, *J* = 8.0, H-3"/5")

*3,4-dihydroxybenzoic acid (**8**)*
White solid (2.2 mg); UV λ_{max} (PDA): 205.0, 215.0, 259.8 nm; NMR (300MHz, MeOH-d4) δ_H: 7.43 (d, J = 2.1 Hz, H-2), 6.78 (d, J = 8.2, H-5), 7.41 (dd, J = 8.2, 2.1 Hz, H-6)

Compound **1,** which was isolated as a white solid showed a retention time of 14.80 min in reverse phase HPLC analysis thus indicating that the compound is polar. The UV spectrum showed maxima at 206.6, 346.0 and 313.9 nm characteristic of a simple phenolic acid. NMR analysis showed a characteristic ABC coupling pattern in the aromatic region which is suggestive of a 1,2,3 – trisubstituted benzene nucleus. These signals appeared at δ_H 7.57 (t, *J* = 8.0, 1H) assigned to H-4, 7.07 (d, *J* = 7.6, 1H) assigned to H-5 and 6.94 (d, *J* = 8.4, 1H) assigned to H-3. The ^{1}HNMR spectrum also showed a quartet at δ_H 4.56 (q) assigned to H-1' and a methyl doublet at 1.46 (d) assigned to H_3-2'. The latter two signals are suggestive of the presence of 1'-hydroxyethyl moiety. Compound **1** was thus elucidated as 2-hydroxy-6- (1'-hydroxyethyl) benzoic acid.
Compound **2** was isolated as a white solid. It has a retention time of 7.003 min in HPLC suggesting a strongly polar compound. The UV spectrum showed maxima at 220.1 and 276.5 nm characteristic of the presence of a 1,4-disubstituted benzene moiety. NMR analysis showed a characteristic AA'BB' coupling pattern in the aromatic nucleus which further confirmed the 1,4 – disubstituted benzene moiety. The aromatic signals appeared at δ_H 7.03 (d, *J* = 8.4, 2H) assigned to H-2'/6' and 6.70 (d, *J* = 8.4, 2H) assigned to H-3'/5'.

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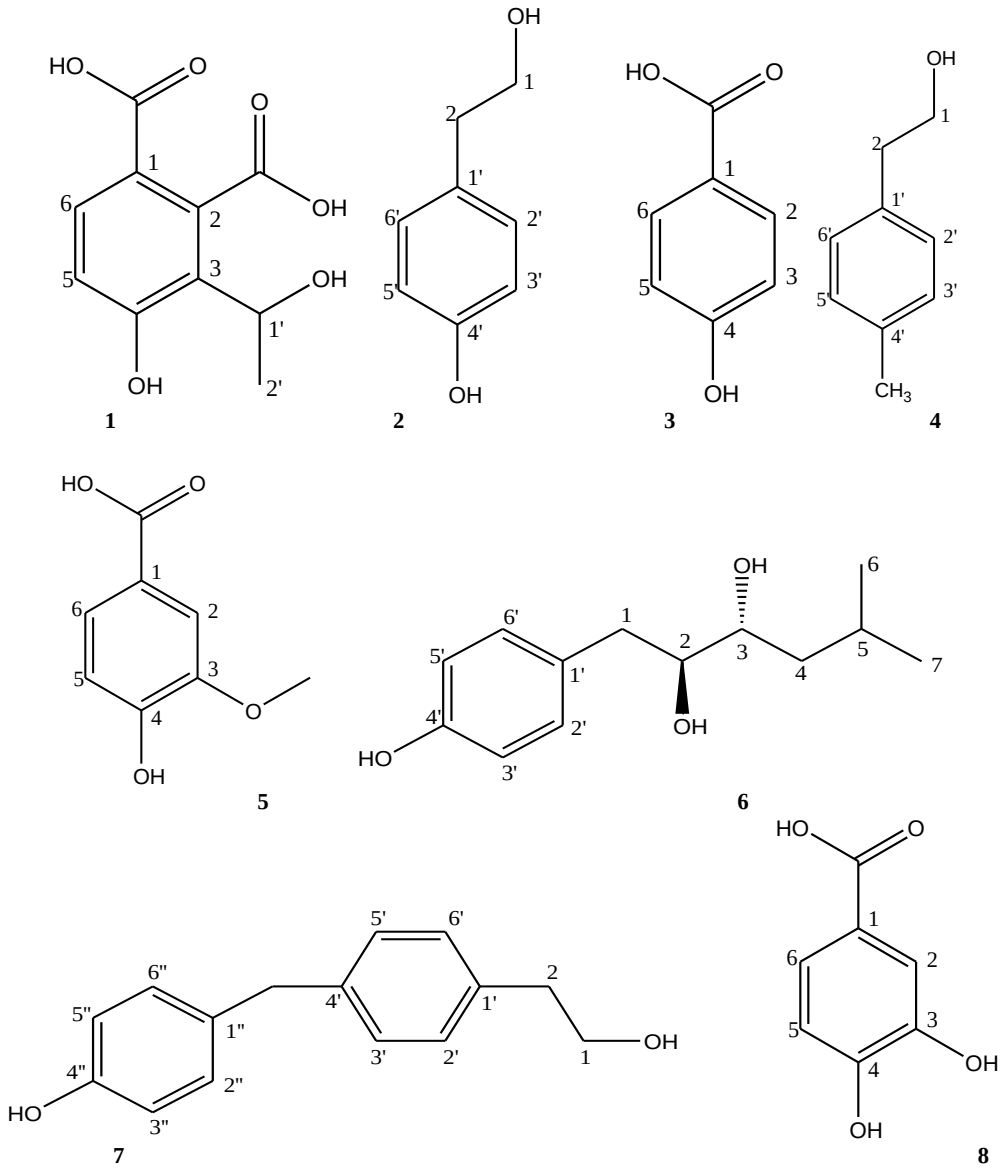

Figure 1: Chemical Structures of Compounds 1 to 8

The proton NMR spectrum also showed signals in the aliphatic region corresponding to two AB coupled methylene groups at δ_H 3.68 (t, J = 7.2, 2H) assigned to H_2-1 and 2.7 (t, J = 7.2, 2H) assigned to H_2-2. The deshielded position of the signal of H_2-1 (3.68 ppm) supported the attachment of -OH group at this position, while the slightly deshielded position of the signals of H_2-1 (2.71 ppm) suggested proton vicinal to a sp2 hybridized carbon, thus supporting attachment to the benzene nucleus. Compound **2** was thus identified as 2-(4'-hydroxyphenyl) ethanol.

Compound **3** was isolated as a white solid. It showed a retention time of 9.27 min in reverse phase HPLC analysis indicating that the compound is strongly polar. The UV spectrum exhibited maxima at 255.6 nm characteristic of a simple phenolic acid. The HPLC-DAD analysis predicted p-hydroxybenzoic acid. This compound was further confirmed by the NMR analysis which showed aromatic proton peaks of AA'BB' coupling pattern at δ_H 7.85 (d, J = 8.0, 2H) assigned to H-2/6 and 6.78 (d, J = 8.0, 2H) assigned to H-3/5. Compound **3** was thus identified as 4-hydroxybenzoic acid.

Compound **4,** which was also isolated as a white solid showed a retention time of 17.56 min in reverse phase HPLC analysis thus suggesting that the compound is weakly polar. The UV spectrum showed maxima at 221.2 and 276.7 nm, which is very similar to that of compound **2** and as such characteristic of presence of a 1,4-disubstituted benzene moiety. The ^{1}HNMR spectrum of compound **4** also showed signals very similar to that of compound **2**. These included the aromatic signals of AA'BB' coupling pattern which appeared at δ_H 7.03 (d, J = 8.4, 2H) assigned to H-2'/6' and 6.70 (d, J = 8.4, 2H) assigned to H-3'/5'. There are also signals in the aliphatic region corresponding to two AB coupled methylene groups at δ_H 4.19 (t, J = 7.1, 2H) assigned to H_2-1 and 2.81 (t, J = 7.1, 2H) assigned to H_2-2. Just as in compound **2**, these signals suggested the presence of 2-monosubstituted ethanol moiety. The major difference, however, is the presence of a slightly deshielded methyl singlet signal at δ_H 2.00 (s, 3H) assigned to 4'-Me. The deshielded position of this methyl signal (2.00 ppm) supported a methyl group vicinal to a sp2 hybridized carbon (hence attachment to benzene nucleus). Compound **4** was thus identified as 2-(4'-methylphenyl) ethanol.

Compound **5** was isolated as a white solid. It showed a retention time of 11.40 min in reverse phase HPLC analysis suggesting that the compound is polar. The UV spectrum showed maxima at 202.0, 216.9, 260.8 and 290 nm, which is very characteristic of presence of a 3,4-disubstituted benzoic acid nucleus. The NMR spectrum of **5** also showed a characteristic ABX coupling pattern in the aromatic nucleus, which appeared at δ_H 7.56 (d, J = 1.9 Hz, 1H) assigned to H-2; 7.54 (dd, J = 8.2, 1.9 Hz, 1H) assigned to H-6; 6.82 (d, J = 8.2, 1H) assigned to H-5. There is also the presence of a methoxy singlet signal

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at δ_H 3.89 (s, 3H) assigned to 3-OMe. Compound **5** was thus identified as 3-methoxy-4-hydroxybenzoic acid.

Compound **6** was isolated as a white solid. It showed a retention time of 18.40 min in reverse phase HPLC analysis suggesting that the compound is not very polar. The UV spectrum showed maxima at 223.7 and 276.7 nm, which is very similar to that of compounds **2** and **4** and as such characteristic of a 1, 4-disubstituted benzene nucleus. The ^{1}HNMR spectrum of **6** showed aromatic proton signals of AA'BB' coupling pattern which appeared at δ_H 7.03 (d, J = 8.4, 2H) assigned to H-2'/6' and 6.73 (d, J = 8.4, 2H) assigned to H-3'/5'. The HNMR spectrum also showed a long spin system in the aliphatic region suggestive of the attachment of long aliphatic side chain. The spin system started from a slightly desheided diasotropic methylene protons signals at δ_H 2.49 (dq, J = 11.3, 7.1 Hz, 1H) assigned to H-1B and 2.02 (m, 1H) assigned to H-1A. These protons showed correlation to a deshielded proton signal at δ_H 4.25 (m, 1H) assigned to H-2 and the later to another deshielded proton signal at δ_H 4.20 (dt, J = 11.7, 5.8 Hz, 1H) assigned to H-3. The signals of H-3 showed correlation to the signals of a shielded diasotropic methylene proton at δ_H 1.44 (m, 1H) assigned to H-4B and 1.23 (dd, J = 16.1, 7.1 Hz, 1H) assigned to H-4A. These also showed correlation to a shielded proton multiplet signal at δ_H 0.95 (m, 1H) assigned to H-5. The latter showed correlation to two shielded methyl doublets at δ_H 1.34 (d, J = 6.2 Hz, 3H) assigned to H_3-6 and 1.17 (d, J = 7.0 Hz, 3H) assigned to H_3-7. The HNMR signals of **6** are very similar to those previously reported for guaymasol and epiguaymasol.[17] The compound was, however, identified as epiguaymasol based on the observed large coupling constant $J_{2/3}$ (11.7 Hz), which suggest trans-orientation of H-2 and H-3 and differences in chemical shift positions of the two methyl doublet signals.

Compound **7** was isolated as a white solid. It showed at a retention time of 21.69 min in reverse phase HPLC analysis suggesting that the compound is weakly polar. The UV spectrum showed maxima at 222.5 and 277.0 nm, which is very similar to that of Compounds **2** and **4** and **6** and as such characteristic of presence of a 1, 4-disubstituted benzene nucleus. The NMR spectrum of **7** also showed signals very similar to that of **2**. These included the aromatic signals of AA'BB' coupling pattern which appeared at δ_H 7.02 (d, J = 8.4, 2H) assigned to H-2'/6' and 6.71 (d, J = 8.4, 2H) assigned to H-3'/5'. There are also signals in the aliphatic region corresponding to two AB coupled methylene groups at δ_H 4.21 (t, J = 6.9, 2H) assigned to H_2-1 and 2.79 (t, J = 6.9, 2H) assigned to H_2-2. These signals also suggested the presence of 2-monosubstituted ethanol moiety. The major difference, however, is the presence of an extra aromatic signals of AA'BB' coupling pattern which appeared at δ_H 6.79 (d, J = 8.0, 2H) assigned to H-2"/6" and 6.68 (d, J = 8.0, 2H) assigned to H-3"/5" and a deshielded methylene singlet signal at δ_H 3.48 (s, 2H) assigned to 4'-CH_2. The deshielded position of this methyl signal (3.48 ppm) supported a methylene group vicinal to two sp2 hybridized carbons (hence attachment to two benzene nuclei). Compound **7** is thus identified as 2- (4'- (4"hydroxyphenyl) methylphenyl) ethanol.

Compound **8** was isolated as a white solid. It showed a retention time of 5.26 min in reverse phase HPLC analysis suggesting that the compound is strongly polar. The UV spectrum showed maxima at 205.0, 215.0 and 259.8 nm very similar to that of compound 5, which is characteristic of presence of a 3,4-disubstituted benzoic acid nucleus. NMR analysis also showed a characteristic ABX coupling pattern in the aromatic nucleus which supported a 1,3,4 – trisubstituted benzene nucleus thus confirming the 3,4-disubstituted benzoic acid nucleus. The signals occur at δ_H 7.43 (d, J = 2.1 Hz, 1H); 7.41 (dd, J = 8.2, 2.1 Hz, 1H) assigned to H-6; 6.78 (d, J = 8.2, 1H) assigned to H-5. The presence of methoxy group would have contributed to the less polar nature of Compound **5** (HPLC RT = 11.40 min) compared to **8** (HPLC RT = 5.26 min). Compound **8** is thus identified as 3,4-dihydroxybenzoic acid (protocatechuic acid).

The fractions from which these compounds were isolated displayed varying degrees of antibacterial activity, with MICs in the range of 0.125 – 1 mg/mL (Table 1).

Table 1: Minimum Inhibitory Concentrations of ACL4 fractions

Fractions	Minimum Inhibitory Concentrations (mg/mL)					
	S. aureus	***B. subtilis***	***E. coli***	***P. aeruginosa***	***A. niger***	***C. albicans***
ACL4-1	-	-	-	-	-	-
ACL4-2	-	-	0.5	0.25	-	-
ACL4-3	-	-	-	-	-	-
ACL4-4	-	-	-	-	-	-
ACL4-5	-	-	-	-	-	-
ACL4-6	-	0.125	-	0.125	-	-
ACL4-7	-	-	-	-	-	-
ACL4-8	-	-	-	-	-	-
ACL4-9	-	-	-	-	-	-
ACL4-10	-	-	-	-	-	-
ACL4-11	-	-	-	-	-	-
ACL4-12	-	-	-	-	-	-
ACL4-13	-	-	-	-	-	-
ACL4-14	0.25	0.5	0.25	0.25	-	-
ACL4-15	-	-	-	0.25	-	-
ACL4-16	-	-	-	-	-	-
ACL4-17	-	-	-	-	-	-
ACL4-18	-	-	-	-	-	-
ACL4-19	-	-	0.25	0.125	-	-
CIPRO	0.007	0.012	0.005	0.011	-	-
MICON	-	-	-	-	0.023	0.021

The growth of the bacterial strains (*S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa*) were inhibited by the secondary metabolites present in the endophytic fungus. The fungal strains (*A. niger* and *C. albicans*) were, however not inhibited. Some of the isolated compounds have also been previously reported to possess diverse biological properties that are either antioxidant, antidiabetic, anti-ageing, antifibrotic, anticancer or antimicrobial.

Compound **2** (2-(4'-hydroxyphenyl) ethanol), also known as Tyrosol, has been previously isolated from other endophytic fungi and shown to possess anti-oxidant property through its scavenging effects on the free radicals (reactive oxygen and nitrogen species), which have been implicated in human pathologies associated with oxidative stress.[18,19] It is also used as an anti-arrhythmic and as a cardiovascular drug.[20]

Compound **4** (2-(4'-methylphenyl) ethanol), also known as 2-p-tolylethanol, is used as a food additive/flavoring agent.[21] Compound **3 (**4-hydroxybenzoic acid) is known to possess anti-microbial and anti-oxidant activities.[22,23] The compound is also used as a flavoring agent,[21] as a food preservative,[24] as an intermediate for synthesis of drugs, dyes and fungicides.[25] It is also used as a corrosion inhibitor, anti-oxidant and emulsifier,[26] and as a component in the production of polyester.[27]

Compound **8** (3,4- dihydroxybenzoic acid), also known as protocatechuic acid, is known to exhibit diverse pharmacological activities, including antioxidant, antibacterial, anticancer, antiulcer, antidiabetic, antiviral, anti-inflammatory, analgesic, and hepatoprotective properties. [28,29]

The biological activities and beneficial uses of compounds, **1, 5, 6** and **7** have not been reported elsewhere.

Conclusion

The antimicrobial activities shown by the fungal extracts and fractions may be as a result of the presence of some of these compounds, which have been previously reported as antimicrobial agents. The results of this study, further confirm the potentials of Nigerian medicinal plants and their associated endophytes as excellent sources of pharmacologically active compounds.

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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