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RESEARCH ARTICLE

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Screening of metabolites from endophytic fungi of some Nigerian medicinal plants for antimicrobial activities

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Abstract

Endophytic fungi associated with Nigerian plants have recently generated significant interest in drug discovery programmes due to their immense potential to contribute to the discovery of new bioactive compounds. This study was carried out to investigate the secondary metabolites of endophytic fungi isolated from leaves of *Newbouldia laevis, Ocimum gratissimum,* and *Carica papaya.* The plants were collected from Agulu, Anambra State, South-East Nigeria. Endophytic fungal isolation, fungal fermentation; and extraction of secondary metabolites were carried out using standard methods. The crude extracts were screened for antimicrobial activities using the agar well diffusion method, and were also subjected to high performance liquid chromatography (HPLC) analysis to identify their constituents. A total of five endophytic fungi was isolated, two from *N. laevis* (NL-L1 and NL-L2), one from *O. gratissimum* (SL-L1), and two from *C. papaya* (PPL-LAC and PPL-LE2). In the antimicrobial assay, the extracts of NL-L2, SL-L1, and PPL-LE2 displayed mild antibacterial activity against both Gram negative and Gram positive test bacteria. PPL-LAC extract showed mild activity only against *S. aureus,* while no antimicrobial activity was recorded for NL-L1 extract. All the endophytic fungal extracts showed no activity against the test fungi *C. albicans* and *A. fumigatus.* HPLC analysis of the fungal extracts revealed the presence of ethyl 4-hydroxyphenyl acetate and ferulic acid in NL-L1; ruspolinone in NL-L2; protocatechuic acid, scytalone, and cladosporin in SL-L1; indole-3-acetic acid and indole-3-carbaldehyde in PPL-LE2; and indole-3-acetic acid in PPL-LAC. The findings of this study revealed the potentials possessed by these plants as source of endophytes that express biological active compounds. These endophytes hold key of possibilities to the discovery of novel molecules for pharmaceutical, agricultural and industrial applications.

Keywords: Endophytic fungi, *Newbouldia laevis, Ocimum gratissimum, Carica papaya,* secondary metabolites, antimicrobial activity

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Introduction

Due to the relationships that endophytes seem to have with their host plants, they make a myriad of biologically active compounds. These compounds can be classified as antibiotics, antioxidants, anticancer agents, volatile antimicrobial agents, immunosuppressive compounds, plant growth promoting agents, and insecticides (1). It has been reported that endophytes possess the ability to produce the same or similar chemicals as those originating from their host plants (2, 3). This presents endophytic microorganisms as potential alternatives to plants in the search for biologically active molecules.

Newbouldia laevis (Bignoniaceae), *Ocimum gratissimum* (Lamiaceae), and *Carica papaya* (Caricaceae) are medicinal plants commonly found in Nigeria. These plants have been used ethnomedicinally in the treatment of several disease conditions including ulcers, burns, diarrhea, bleeding, haemorrhoids, dysentery, abdominal pains, cough, colds, pruritus, stress, headache, pneumonia, conjunctivitis, diabetes mellitus, epilepsy and convulsions (4-12).

Various scientific studies have shown that *N. Laevis* possesses antimicrobial, antidiabetic, antioxidant, and antimalarial activities (11-14). *O. gratissimum* has been reported to show antidiabetic, antioxidant, anxiolytic, sedative, anti-inflammatory, hepatoprotective, antitumor, gastroprotective, hypolipidemic, insecticidal, nematicidal,

and antibacterial activities (4,5,15-27). Also, scientific reports have revealed that *C. papaya* possesses immunomodulatory, antimicrobial, anti-inflammatory, antihelmintic, anticancer, wound healing, anti-fertility, abortifacient, diuretic, anti-hypertensive and antimalarial properties (10, 28-39).

In our search for biologically active molecules from endophytic fungi associated with Nigerian plants, this present study was carried out to investigate the secondary metabolites of endophytic fungi isolated from three Nigerian medicinal plants *N. laevis*, *O. gratissimum* and *C. papaya* for antimicrobial activities, and also identify the constituents of the fungal extracts using high performance liquid chromatography (HPLC).

Materials and Methods

Isolation of endophytic fungus, fermentation and extraction of metabolites

Fresh leaves of *Newbouldia laevis, Ocimum gratissimum,* and *Carica papaya* were collected from Agulu, Anambra State, Nigeria. Isolation of endophytic fungi from the plant leaves was carried out as described by Eze *et al.* (40). The plant leaves were washed thoroughly in running tap water and then cut into small fragments (about 1 cm^2). The leaf fragments were surface-sterilized by immersion in 2% sodium hypochlorite solution for 2 min, 70% ethanol for nearly 2 min, before a final rinse in sterile water for 5 min. The leaf fragments were transferred into malt extract (ME) agar plates supplemented with chloramphenicol. The Petri plates were then incubated at a temperature of 28°C and fungal growths from the leaf fragments were monitored. Hyphal tips from several distinct colonies emerging from the leaf segments were sub-cultured onto fresh ME plates to obtain pure colonies. Solid state fermentation of the endophytic fungi was carried out in 1L Erlenmeyer flasks containing sterile solid rice medium (100 g of rice + 100 mL of distilled water, autoclaved at 121 °C at 15 psi for 1 h). The flasks were inoculated with 3 mm diameter agar blocks containing the respective fungi and incubated at 28°C for 21 days. At the completion of fermentation, the secondary metabolites (contained in the fermentation medium) were extracted with ethyl acetate, and the extracts concentrated under vacuum at 40°C using a rotary evaporator.

Antimicrobial assay

Preliminary antimicrobial screening of the endophytic fungal extracts was carried out using the agar well diffusion assay method as described by Akpotu *et al.* (41). Working concentrations (1 mg/mL) of the fungal extracts were prepared by dissolving the extracts in dimethyl sulphoxide (DMSO 100% v/v). Standardized broth cultures of test bacterial isolates (*S. aureus, S. typhi, B. subtilis, and E. coli*) and fungal isolates (*Candida albicans* and *Aspergillus fumigatus*) were spread aseptically onto the surface of Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) plates respectively using sterile cotton swabs. All culture plates were allowed to dry for about 5 min and agar wells were made by using a sterile cork-borer (6 mm in diameter). These wells were respectively filled with 20 µL of the fungal extracts and controls. The plates were then kept at room temperature for 1 h to allow the agents to diffuse into the agar medium and incubated accordingly. Gentamincin (10 µg/mL) and ketoconazole (50 µg/mL) were used as positive controls in the antibacterial and antifungal evaluations respectively; while DMSO (100% v/v) was used as the negative control. The MHA plates were then incubated at 37°C for 24 h, and the SDA plates were incubated at 25-27°C for 2-3 days. The inhibition zones diameters (IZDs) were measured and the size of the well (6 mm) was deducted from the values obtained to get the actual IZDs. This was conducted in triplicate and the mean IZDs were calculated and recorded.

High performance liquid chromatography (HPLC) analysis

HPLC analysis was carried on the fungal extracts was carried out as described by Eze *et al.* (40). A Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany) was used in the analysis. The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany) and a linear gradient of nanopure water

Table 1. Result of antimicrobial assay of endophytic fungal extracts showing the inhibition zone diameters (IZDs) produced against test isolates

Test Organisms	IZDs (mm)						
	NL-L1	NL-L2	SL-L1	PPL-LAC	PPL-LE2	Positive control Gentamicin (10 µg/ml)	Negative control DMSO
S. aureus	0	9	7	3	4	17	0
S. typhi	0	0	5	0	3	21	0
B. subtilis	0	6	6	0	0	22	0
E. coli	0	6	8	0	0	16	0
						Ketoconazole (50 µg/ml)	DMSO
C. albicans	0	0	0	0	0	17	0
A. fumigatus	0	0	0	0	0	4	0

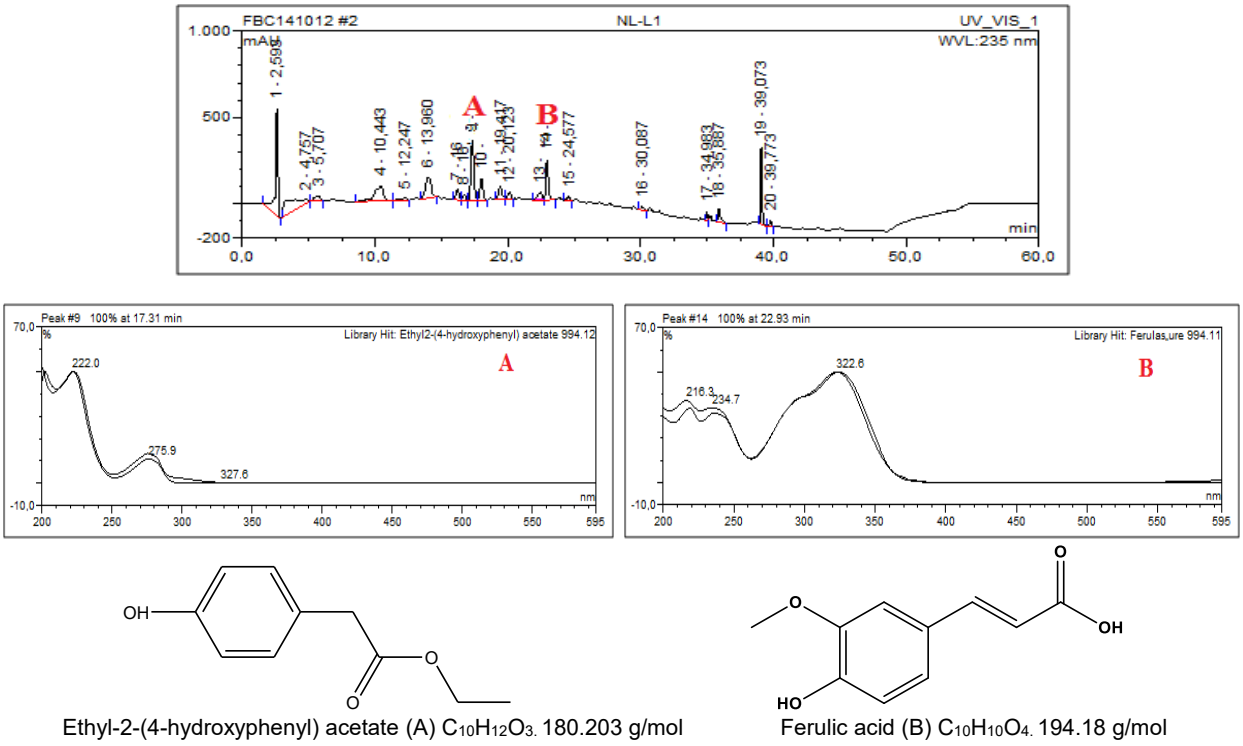


Figure 1. HPLC chromatogram of NL-L1 extract showing ethyl-2-(4-hydroxyphenyl) acetate (A) and ferulic acid (B); their UV spectra; and structures.

(adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. A weight of 2 mg of each fungal extract was reconstituted with 2 mL of HPLC grade methanol, and the mixture sonicated for 10 min, and thereafter centrifuged at 3000 rpm for 5 min. A volume of 100 µL of the dissolved sample was then transferred to a vial containing 500 µL of HPLC grade methanol, and vial was put in the HPLC machine for analysis. Detection was at 235 nm. The absorption peaks of the fungal extracts were analyzed by comparing with those in the HPLC-UV/Vis database.

Results

A total of five endophytic fungi was isolated, two from *N. laevis* (NL-L1 and NL-L2), one from *O. gratissimum* (SL-L1), and two from *C. papaya* (PPL-LAC and PPL-LE2). In Table 1, it can be observed that the extracts of NL-L2, SL-L1, and PPL-

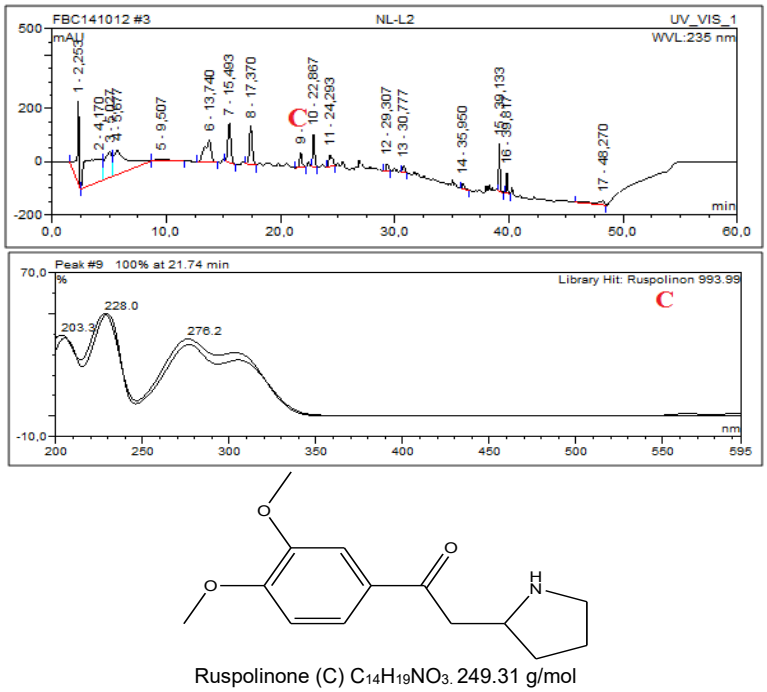


Figure 2. HPLC chromatogram of NL-L2 extract showing ruspolinone (C); its UV spectrum; and structure.

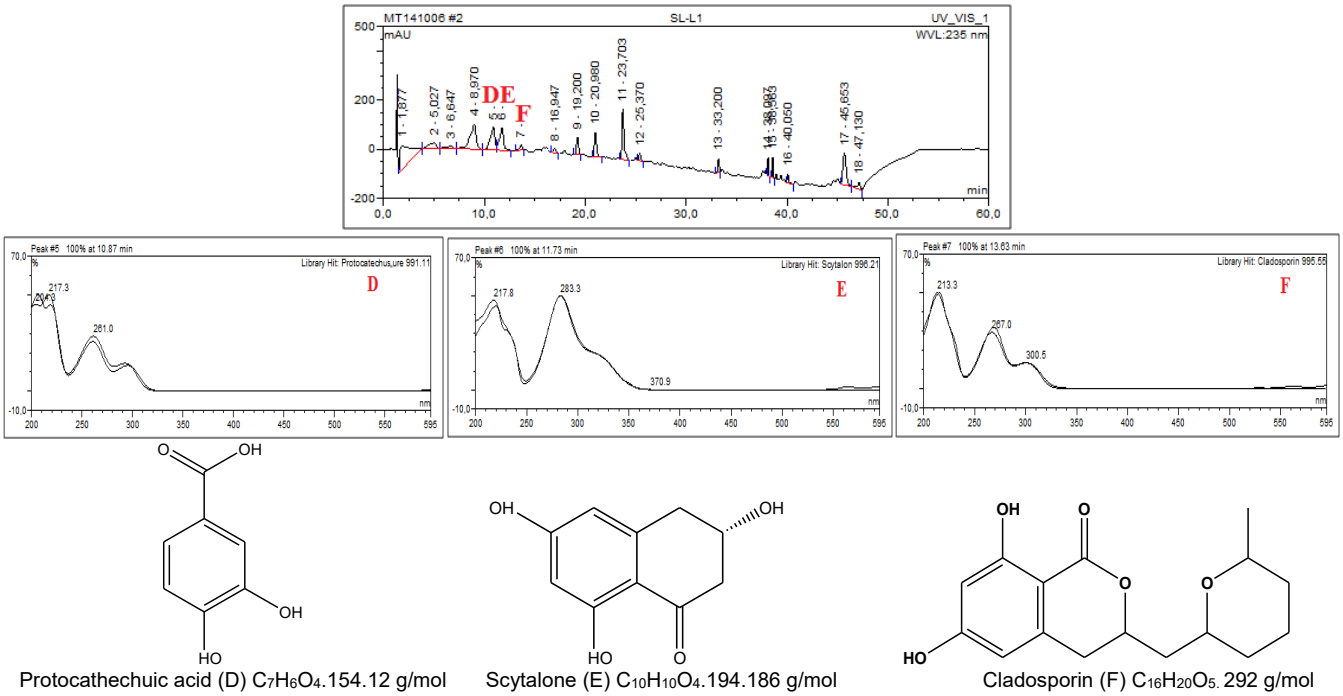


Figure 3. HPLC chromatogram of SL-L1 extract showing Protocathechuic acid (D), Scytalone (E) and Cladosporin (F); their UV spectra; and structures.

LE2 displayed antibacterial activity against both Gram negative and Gram positive test bacteria. PPL-LAC extract showed activity only against *S. aureus*, while no antimicrobial activity was recorded for NL-L1 extract. All the endophytic fungal extracts had no activity against the test fungi *C. albicans* and *A. fumigatus*. HPLC analysis of the fungal extracts revealed the presence of ethyl 4-hydroxyphenyl acetate and ferulic acid in NL-L1 (**Fig. 1**); ruspolinone in NL-L2 (**Fig. 2**); Protocatechuic acid, scytalone, and cladosporin in SL-L1 (**Fig. 3**); indole-3-acetic acid and indole-3-carbaldehyde in PPL-LE2 (**Fig. 4**); and indole-3-acetic acid in PPL-LAC (**Fig. 5**).

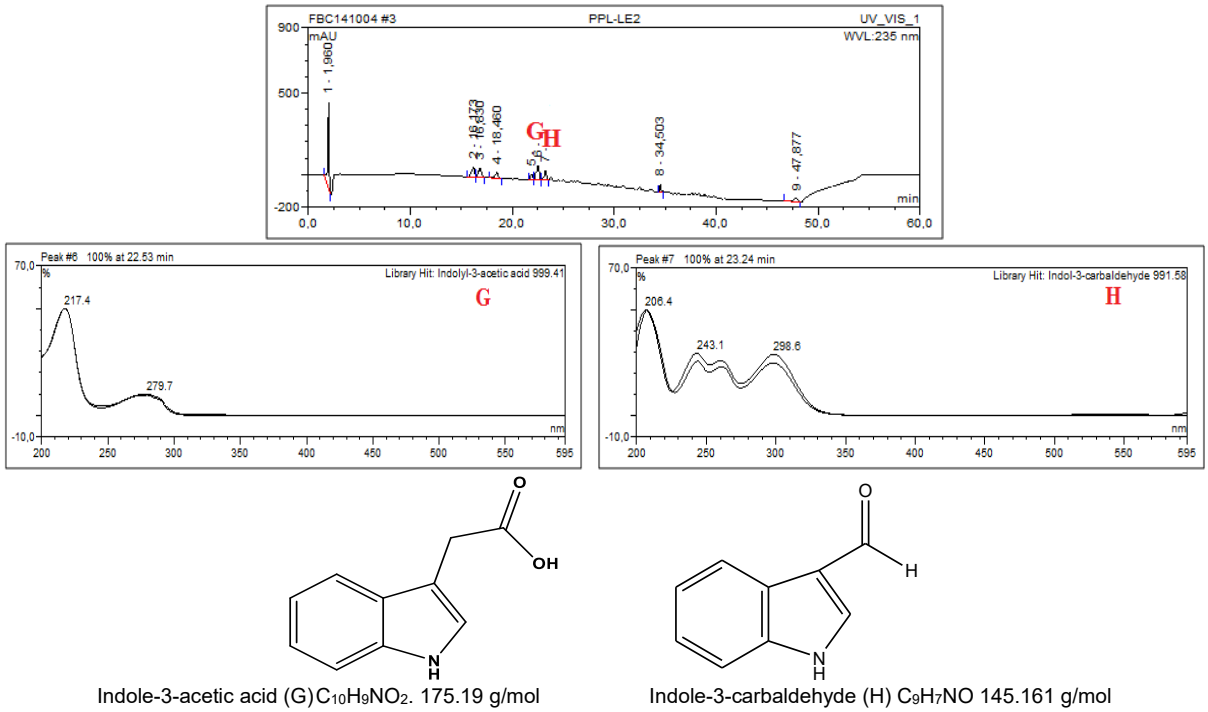
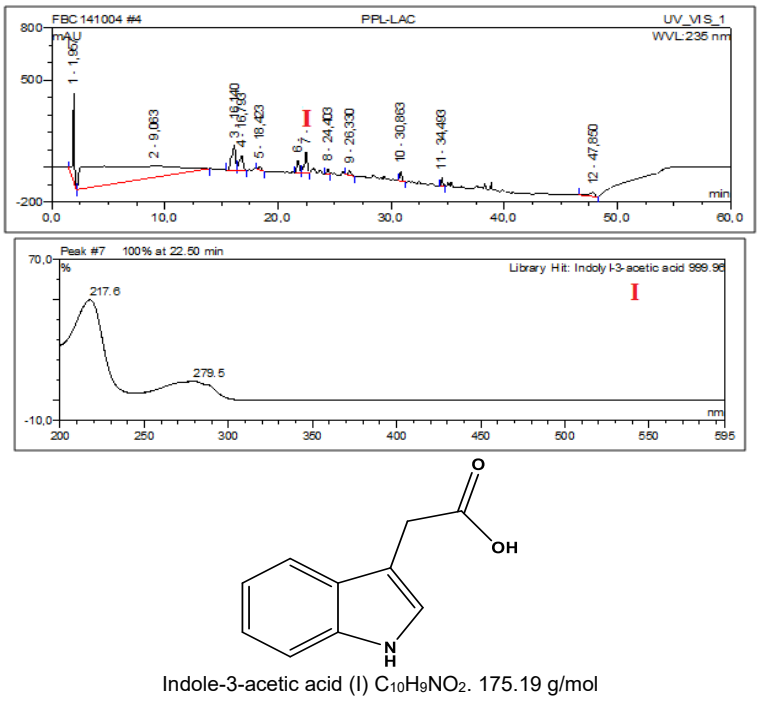


Figure 4. HPLC chromatogram of PPL-LE2 extract showing indole-3-acetic acid (G) and indole-3-carbaldehyde (H); their UV spectra; and structures.



Indole-3-acetic acid (I) $C_{10}H_9NO_2$. 175.19 g/mol

Figure 5. HPLC chromatogram of PPL-LAC extract showing indole-3-acetic acid (I); its UV spectrum; and structure.

Table 2. Biological activities/applications of detected compounds

Plants	Endophytic fungi	Compounds detected by HPLC analysis	Biological activities/applications	References
Newbouldia laevis	NL-L1	Ethyl 4-hydroxyphenyl acetate	An intermediate for the preparation of anti-inflammatory agents	(42)
		Ferulic acid	Antioxidant anti-diabetic, antihypertensive, anticancer, anti-inflammatory, hepatoprotective, antimicrobial, anti-allergic, increase sperm viability, antithrombotic, antiviral and vasodilatory actions, metal chelation, modulation of enzyme activity, activation of transcriptional factors, gene expression and signal transduction activities	(43-54)
	NL-L2	Ruspolinone	Organocatalysts and building blocks in organic synthesis	(55,56)
Ocimum gratissimum	SL-L1	Protocatechuic acid	Antioxidant, antimicrobial, anticancer, anti-ulcer, antidiabetic, anti-ageing, antifibrotic antiviral, anti-inflammatory, analgesic activity, anti-atherosclerotic, cardiac, hepatoprotective, neurological and nephroprotective activities	(57-71)
		Cladosporin	Antiplasmodial, antimicrobial, insecticidal, and antitumor activities	(72-76)
		Scytalone	Plant growth promotion, intermediate in the biosynthesis of melanin in fungi	(77-80)
Carica papaya	PPL-LAC	Indole-3-acetic acid	Cytotoxic/anticancer, antioxidant, anti-inflammatory activities	(81-83)
	PPL-LE2	Indole-3-acetic acid		
		Indole-3-carbaldehyde	Cytotoxic and antibacterial activities, associated with the innate immunity to microbial pathogen infections in plants	(84-86)

Discussion

The antibacterial activity displayed by the endophytic fungal extracts can be attributed to the antimicrobial compounds present in the extracts. As can be observed in Table 2, compounds with antimicrobial properties detected by HPLC analysis of the fungal extracts include: ferulic acid, cladosporin, indole-3-carbaldehyde, and protocatechuic acid. The other detected compounds (ethyl 4-hydroxyphenyl acetate, ruspolinone, scytalone, and indole-3-acetic acid) possess diverse biological activities that include cytotoxic/anticancer, antioxidant, anti-inflammatory activities, etc. (42-86).

Ferulic acid, a derivative of cinnamic acid, is a predominant natural phenolic compound first isolated from the plant *Ferula foetida* (45). It is present in plant seeds and leaves as a component of lignocelluloses, conferring rigidity to the cell wall by making the crosslink between polysaccharides and lignin (45, 87). Ferulic acid has been detected in and/or isolated from culture extracts of endophytic fungi like *Annulohypoxylon stygium* (88) and *Aspergillus austroafricanus* (89). Ferulic acid has been reported to show several pharmacological activities including antioxidant anti-diabetic, antihypertensive, anticancer, anti-inflammatory, hepatoprotective, and antimicrobial activities. Other reported biological activities of ferulic acid are anti-allergic, increase sperm viability, antithrombotic, antiviral and vasodilatory actions, metal chelation, modulation of enzyme activity, activation of transcriptional factors, gene expression and signal transduction activities (43-54).

Protocatechuic acid (3,4-dihydroxybenzoic acid) is a widely distributed naturally occurring phenolic acid present in most edible and medicinal plants (53, 90-92). The compound has also been reported to be produced by several species of bacteria and fungi (40, 57, 93-95). Protocatechuic acid has been reported to show antioxidant, antimicrobial, anticancer, anti-ulcer, antidiabetic, anti-ageing, antifibrotic antiviral, anti-inflammatory, analgesic activity, anti-atherosclerotic, cardiac, hepatoprotective, neurological and nephroprotective activities (57-71).

Cladosporin is an isocoumarin derivative previously isolated from *Cladosporium cladosporioides* (72, 75) and *Eurotium* sp. (76). Cladosporin has been reported to show antiplasmodial, antimicrobial, insecticidal and antitumor activities (72-76).

Indole-3-carbaldehyde (indole-3-carboxaldehyde), an indole alkaloid, had been isolated from an endophytic *Lasiodiplodia* sp. associated with *Viscum coloratum* (96), from endophytic actinomycetes (85, 97), from *Pseudomonas* sp. (98), and from a Red Sea sponge *Hyrtios erectus* (86). Indole-3-carbaldehyde showed moderate cytotoxic (86) and antibacterial activities (85). In plants, indole-3-carbaldehyde is associated with the innate immunity to microbial pathogen infections (84,85).

Indole-3-acetic acid is the most abundant and well known plant hormone of the auxin class which regulates various aspects of plant growth and development (99-101). Many bacterial and fungal species have been reported to be able to produce indole-3-acetic acid (40, 99, 101, 102). Indole-3-acetic acid has been reported to possess cytotoxic/anticancer, antioxidant, anti-inflammatory activities (81-83).

Ethyl-2-(4-hydroxyphenyl) acetate (ethyl *p*-hydroxyphenyl acetate) has been isolated from *Ixeris chinensis* (103), *Osmanthus fragrans* (104). Ethyl *p*-hydroxyphenyl acetate has been used as an intermediate for the preparation of anti-inflammatory agents (42).

Ruspolinone, a pyrrolidine alkaloid, was isolated from the plant *Ruspolia hypercrateriformis* (105, 106). Pyrrolidine alkaloids are hazardous for humans and animals, but exhibit a host of biological activities, and are used as organocatalysts and building blocks in organic synthesis (55, 56).

Scytalone, a tetralone derivative, has been previously isolated from several endophytic fungi which include *Penicillium* cf. *glaucoalbidum* (107), *Cladosporium tenuissimum* (108), *Annulohypoxylon* sp. (109), *Phomopsis* sp. (78), and *Scytalidium* sp. (110). Scytalone has been known as an intermediate in the biosynthesis of melanin, the dark pigment of many phytopathogenic fungi which aid their hyphal (appresoria) penetration into leaves and cell walls (78-80). Scytalone was also reported to promote plant growth (77).

Studies of the endophytic fungal population of Nigerian plants have revealed the potentials possessed by these plants as host to endophytes that express important biological active compounds (2, 3, 40, 41, 111-121). These endophytes also hold key of possibilities to the discovery of novel molecules for pharmaceutical, agricultural and applications.

Conclusion

Endophytic fungi isolated from with three Nigerian plants *Newbouldia laevis, Ocimum gratissimum,* and *Carica papaya* produced compounds with diverse biological activities. These plants and their associated endophytes could be an excellent source of novel biologically active compounds with pharmaceutical or industrial importance.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

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