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Antimicrobial activities of secondary metabolites of endophytic fungi isolated from *Catharanthus roseus*

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ABSTRACT

Introduction: Recently, several endophytes have been shown to possess the potentials to synthesize novel bioactive compounds that have found use for drug discovery. We isolated endophytic fungi associated with *Catharanthus roseus* collected from the river banks of Amassoma in Southern Nigeria, and identified some of their bioactive secondary metabolites.

Methods: The fungi were subjected to solid-state fermentation on rice medium and the metabolites were extracted using ethyl acetate. The fungal crude extracts were screened for antimicrobial activity and were also subjected to high-performance liquid chromatography-diode-array detection (HPLC-DAD) analysis for the identification of the bioactive compounds.

Results: The fungal extracts showed both antibacterial and antifungal activities with minimum inhibitory concentrations ranging from 0.0625 to 1 mg/mL. The HPLC-DAD analysis of the extracts suggested the presence of citreoisocoumarin, citreoisocoumarinol, questinol, hydroxyemodin, acropyrone, methyl 2-(4-hydroxyphenyl) acetate, nigricinol, and cladosporin.

Conclusion: The results of this study suggest that endophytic fungi associated with *C. roseus* could be a promising source of novel bioactive compounds with pharmaceutical and industrial importance.

Keywords: *Catharanthus roseu;* endophytic fungi; antimicrobial activity; HPLC-DAD

INTRODUCTION

Natural products from microbial origin have been a consistent source of novel lead molecules and recently several endophytes have been shown to possess the potentials to synthesize novel bioactive compounds that have found great use for drug discovery (1). It is estimated that there might be as many as one million different endophytic fungal species, however, only a handful of them have been described, which means investigating the metabolites of these endophytes can increase the chance of finding novel bioactive natural products (2,3).

Catharanthus roseus L. (*Apocynaceae*) is a herbaceous medicinal plant native to the Indian ocean

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of Madagascar but also found in Nigeria and other tropical and subtropical areas of the world (4). The leaf is used traditionally to treat wasp sting, sore throat, diabetes, malaria, and Hodgkin's lymphoma (5). Extracts of *C. roseus* have demonstrated significant anticancer and antiviral activities (6-8).

Endophytic fungi associated with Nigerian medicinal plants have been poorly investigated for their potentials as sources of novel bioactive compounds. Results of a few of such studies have revealed the enormous potentials which abound in endophytes associated with Nigerian plants as sources of novel molecules of pharmaceutical or industrial importance (1,9-16). In our effort to further explore Nigeria's biodiversity for novel biologically important molecules, we isolated endophytic fungi associated with *C. roseus* collected from the river banks of Amassoma in Southern Ijaw Local Government Area of Bayelsa State, Southern Nigeria, and identified some of their bioactive secondary metabolites.

METHODS

Isolation of endophytic fungi, fermentation, and extraction of metabolites

Isolation of endophytic fungi from healthy leaves of *C. roseus* was carried out using standardized methods (17). The isolated pure fungal strains were maintained in malt extract agar. Solid-state fermentation was carried out in 1 L Erlenmeyer flasks containing autoclaved rice medium (100 g of rice and 200 mL of distilled water). The flasks were inoculated with 3 mm diameter agar blocks cut out from malt extract agar plates containing pure cultures of the each fungus. The inoculated flasks were incubated at 27-30°C for 28 days. At the completion of fermentation, the secondary metabolites were extracted with ethyl acetate and then concentrated using a rotary evaporator at 40°C.

Antimicrobial assay

Primary antimicrobial evaluation of the fungal extracts

Preliminary antimicrobial screening of the fungal extracts was carried out using the agar well diffusion method described by Akpotu et al. (16). The extracts were tested against laboratory strains of *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Escherichia coli, Aspergillus fumigatus*, and *Candida albicans* which were obtained from the Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. A concentration of 1 mg/mL was prepared for all the fungal extracts by dissolving in 100% dimethyl sulfoxide (DMSO). 20 mL of molten Mueller-Hinton agar (MHA) and sabouraud dextrose agar (SDA) (for bacterial and fungal isolates, respectively) were poured into sterile Petri dishes (90 mm) and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of test isolates were swabbed aseptically on the agar plates and holes (6 mm) were made in the agar plates using a sterile metal cork borer. 20 µl of the extracts and controls were put in each hole under aseptic condition, kept at room temperature for 1 hour to allow the agents to diffuse into the agar medium and then incubated accordingly. Gentamicin (10 µg/mL) and fluconazole (50 µg/mL) served as positive control for bacteria and fungi, respectively, while DMSO was used as the negative control. The MHA plates were then incubated at 37°C for 24 hours, and the SDA plates were incubated at room temperature (25-27°C) for 2 days. The inhibition zone diameters (IZDs) were measured and recorded. The size of the cork borer (6 mm) was deducted from the values recorded for the IZDs to get the actual diameter. This procedure was repeated in triplicate and the mean value for IZDs was calculated and recorded.

Determination of minimum inhibitory concentrations (MICs)

The MICs of the plant extracts on the test isolates were determined by the agar dilution method described by Akpotu et al. (16). The stock solution (20 mg/mL) was further diluted in a 2-fold serial dilution to obtain the following concentrations: 10, 5, 2.5, 1.25, and 0.625 mg/mL. Agar plates were prepared by pouring 9 mL of molten double strength MHA and SDA (for bacterial and fungal isolates, respectively) into sterile Petri plates containing 1 mL of the various dilutions of the extract making the final plate concentrations to become 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mg/mL.

The test isolates which were grown overnight in broth were adjusted to McFarland 0.5 standard and streaked onto the surface of the agar plates containing dilutions of the extract. The MHA plates were then incubated at 37°C for 24 hours and the SDA plates were incubated at room temperature (25-27°C) for 2 days, after which all plates were observed for growth. The minimum dilution (concentration) of the extracts completely inhibiting the growth of each organism was taken as the MIC.

High-performance liquid chromatography-diode-array detection (HPLC-DAD) assay

Each of the dried fungal metabolite extract (2 mg) was reconstituted with 2 ml of HPLC grade methanol. The mixture was sonicated for 10 minutes, followed by centrifugation at 3000 rpm for 5 minutes. Then, 100 µL of the dissolved samples were transferred into HPLC vials containing 500 µL of the HPLC grade methanol. The HPLC-DAD analysis was carried out on the samples with a Dionex P 580 HPLC system coupled to a photodiode array detector (UVD340S, DionexSoftron GmbH, Germering, Germany). Detection was at 235, 254, 280, and 340 nm. The separation column (125 mm × 4 mm; length × internal diameter) was pre-filled with Eurospher-10 C18 (Knauer, Germany) and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. The absorption peaks for each of the 10 dried fungal metabolite extract were analyzed by comparing with those in the HPLC-ultraviolet (UV)/visible database, which contains over 1600 registered compounds.

RESULTS

A total of five fungal endophytes labeled CR-MR1B, CR-MR1, CR-LC, CR-MR3, and CR-MRB2 were isolated from the leaves of *C. roseus*. Results of the antimicrobial assay of the fungal extracts (Tables 1 and 2) reveal that at 1 mg/mL, the extracts showed activity against the bacterial and fungal test isolates with inhibition zones ranging from 2 to 16 mm. The MIC values of the extract against the test organisms ranged from 0.0625

TABLE 1. Results of the antimicrobial evaluation of the fungal extracts showing the IZD (mm) produced against test organisms

Test organisms	1 mg/mL					Positive control	Negative control
	CR-MR1	CR-MR1B	CR-MRB2	CR-MR3	CR-LC	Gentamicin (10 µg/ml)	DMSO
S. aureus	0	2	0	6	14	23	0
B. subtilis	0	2	2	7	16	21	0
S. typhi	4	2	0	5	8	25	0
E. coli	3	0	0	3	9	20	0
						Fluconazole (50 µg/ml)	DMSO
A. fumigatus	3	2	0	0	0	14	0
C. albicans	5	4	0	7	0	9	0

S. aureus: Staphylococcus aureus; B. subtilis: Bacillus subtilis; S. typhi: Salmonella typhi; E. coli: Escherichia coli;
A. fumigatus: Aspergillus fumigatus; C. albicans: Candida albicans; IZD: Inhibition zone diameters; DMSO: Dimethyl sulfoxide

TABLE 2. The MICs of fungal extracts against the test organisms

Fungal extracts	MICs (mg/mL)					
	S. aureus	*B. subtilis*	*S. typhi*	*E. coli*	*A. fumigatus*	*C. albicans*
CR-MR1	-	-	0.5	0.5	0.5	0.5
CR-MR1B	1	1	1	-	1	0.5
CR-MRB2	-	1	-	-	-	-
CR-MR3	0.25	0.25	0.25	0.5	-	0.25
CR-LC	0.0625	0.03125	0.25	0.125	-	-

S. aureus: Staphylococcus aureus; B. subtilis: Bacillus subtilis; S. typhi: Salmonella typhi; E. coli: Escherichia coli;
A. fumigatus: Aspergillus fumigatus; C. albicans: Candida albicans; MIC: Minimum inhibitory concentration

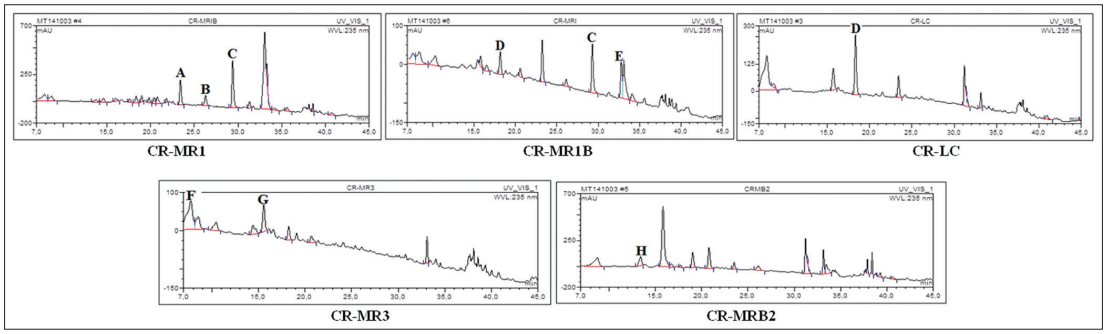

FIGURE 1. HPLC chromatograms showing the detection of, (A) Citreoisocoumarin, (B) Citreoisocoumarinol, (C) Questinol, (D) Acropyrone, (E) Hydroxyemodin, (F) Methyl 2-(4-hydroxyphenyl) acetate, (G) Nigricinol and Cladosporin in the fungal extracts.

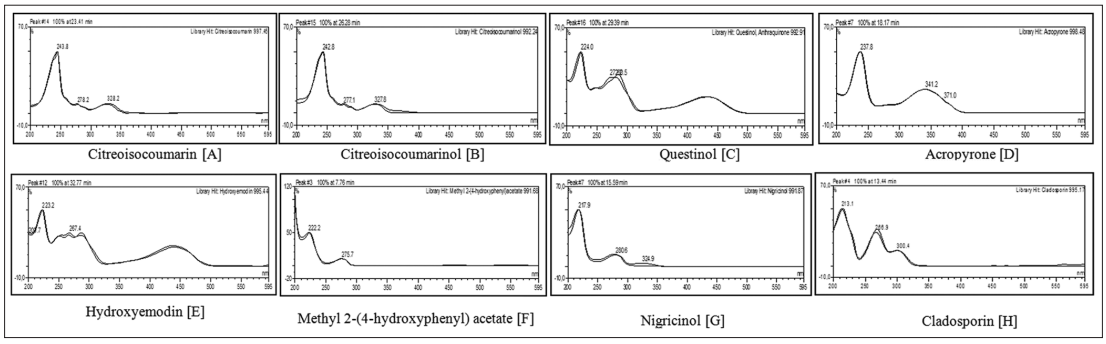

FIGURE 2. UV-spectra of detected compounds, (A) Citreoisocoumarin, (B) Citreoisocoumarinol, (C) Questinol, (D) Acropyrone, (E) Hydroxyemodin, (F) Methyl 2-(4-hydroxyphenyl) acetate, (G) Nigricinol, (H) Cladosporin.

TABLE 3. Bioactive compounds detected in endophytic fungal extracts by HPLC-DAD analysis

Fungal extracts	Detected compounds
CR-MR1B	Citreoisocoumarin, citreoisocoumarinol, questinol
CR-MR1	Acropyrone, questinol, hydroxyemodin
CR-LC	Acropyrone
CR-MR3	Methyl 2-(4-hydroxyphenyl) acetate, nigricinol
CR-MRB2	Cladosporin

HPLC-DAD: High-performance liquid chromatography-diode-array detection

to 1 mg/mL. Three of the five fungal extracts, CR-MR1B, CR-MR1, and CR-MR3 showed broad-spectrum antimicrobial activity against both test bacteria and test fungi. Although CR-LC and CR-MRB2 showed no antifungal activity, the highest antibacterial activity was exhibited by CR-LC, especially against *S. aureus* and *B. subtilis* (with IZD of 14 and 16 mm; and a MIC of 0.0625 and 0.03125 mg/mL, respectively).

The extracts of the endophytic fungi from *C. roseus* represent a dependable source of bioactive compounds, evidenced by the wide range of compounds with diverse biological properties present in these extracts. The HPLC-DAD analysis of the extracts suggested the presence of citreoisocoumarin, citreoisocoumarinol, questinol, hydroxyemodin, acropyrone, methyl 2-(4-hydroxyphenyl) acetate, nigricinol, and cladosporin (Table 3). The HPLC chromatograms, UV-spectra, and chemical structures of detected compounds are presented in Figures 1-3, respectively.

DISCUSSION

The antimicrobial activity shown by the endophytic fungal extracts may be due to the presence of the detected compounds with known antimicrobial activity. These compounds include: Hydroxyemodin, citreoisocoumarin, citreoisocoumarinol, and cladosporin (18-22). The biological properties of these

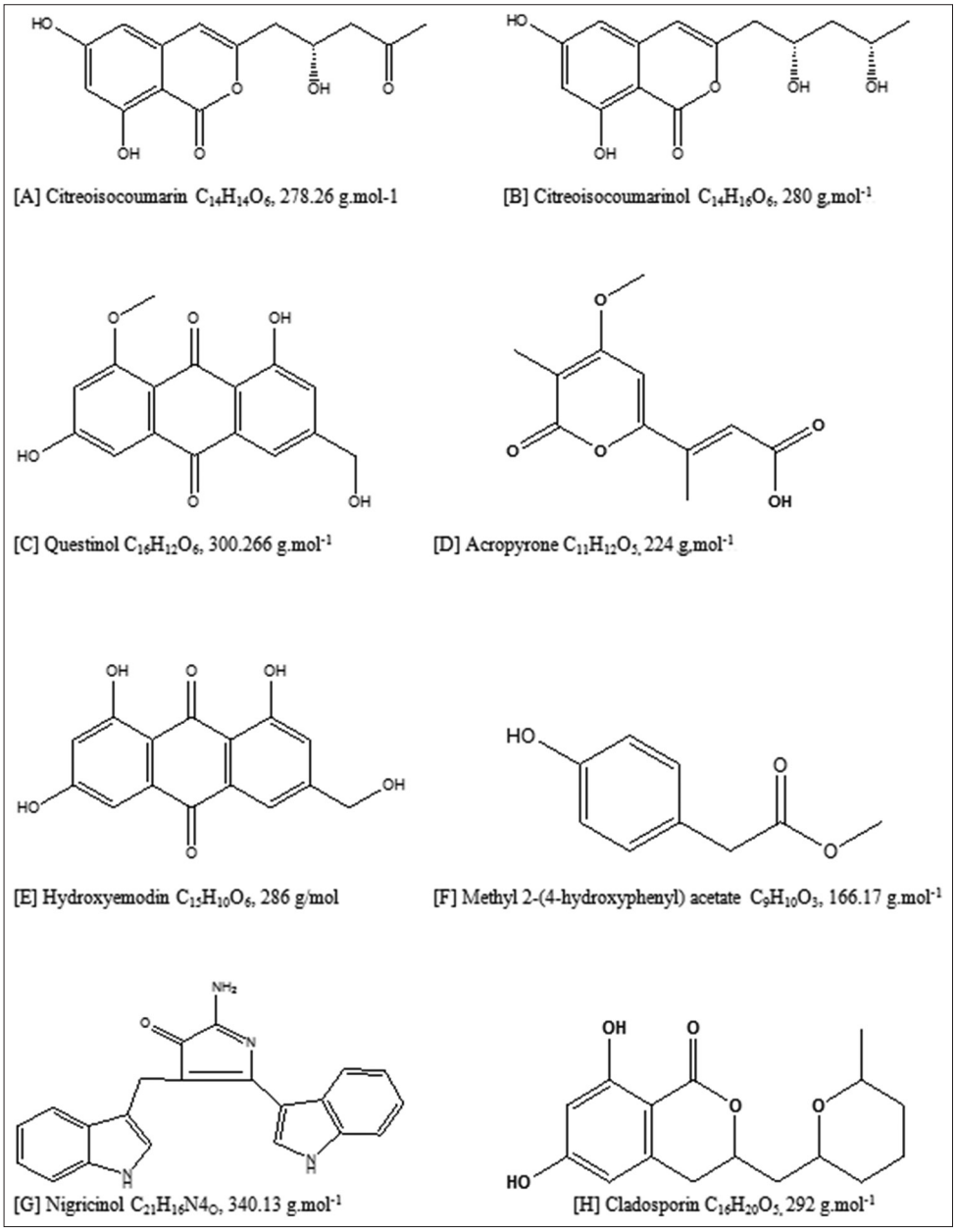

FIGURE 3. Chemical structures of detected compounds, (A) Citreoisocoumarin $C_{14}H_{14}O_6$, 278.26 g.mol^{-1}, (B) Citreoisocoumarinol $C_{14}H_{16}O_6$, 280 g.mol^{-1}, (C) Questinol $C_{16}H_{12}O_6$, 300.266 g.mol^{-1}, (D) Acropyrone $C_{11}H_{12}O_5$, 224 g.mol^{-1} , (E) Hydroxyemodin $C_{15}H_{10}O_6$, 286 g/mol, (F) Methyl 2-(4-hydroxyphenyl) acetate $C_9H_{10}O_3$, 166.17 g.mol^{-1}, (G) Nigricinol $C_{21}H_{16}N4_O$, 340.13 g.mol^{-1}, (H) Cladosporin $C_{16}H_{20}O_5$, 292 g.mol^{-1}

compounds, as well as other detected compounds, are explained below.

Acropyrone, an acetophenone dimer with cytotoxic activity, has been isolated from *Acronychia pedunculata* (23,24). Hydroxyemodin is a derivative of emodin, which is a naturally occurring anthraquinone biosynthesized by fungi (25). Hydroxyemodin has been isolated from *Penicillium* sp. and was

shown to have antimicrobial activity (18). Methyl 2-(4-hydroxyphenyl) acetate is known to possess antiviral property (26). It has previously been isolated from *Penicillium chrysogenum* (27) and *Cordyceps sinensis* (28). Nigricinol was previously isolated from a marine sponge *Petrosia nigricans* and was reported to show cytotoxic activity (29).

Citreoisocoumarin and citreoisocoumarinol are derivatives of isocoumarin. They have been reported to show mild α-glucosidase inhibitory and antimicrobial activities (30,31). Isocoumarins are prevalent in most natural products that exhibit a wide range of pharmacologic activities including antidiabetic (32), antimicrobial (19), insecticidal (33), antiparasitic (34,35), cytotoxic (36), anti-inflammatory (37), and antiangiogenic (38). Several reports have been made on the isolation of citreoisocoumarin and citreoisocoumarinol from different endophytic fungi such as *Nectria* sp. (31), *Microdochium bolleyi* (19), *Fusarium tricinctum* (39), *Ampelomyces* sp. (40), *Penicillium corylophilum* (41), *Aspergillus* sp. (42,43), and *Penicillium* sp. (44).

Cladosporin, another isocoumarin derivative, has been previously isolated from *Cladosporium cladosporioides* (20,21) and *Eurotium* sp. (22). Cladosporin has been shown to possess antiplasmodial (45), antifungal (21), antibacterial (20,22), insecticidal (33), and antitumor properties (21).

Questinol, an anthraquinone, with reported anti-inflammatory activity (46), has been previously isolated from *Polygonum* spp. (47), and *Cassia* spp. (48). This compound has also been isolated from culture extracts of strains of *Eurotium rubrum* (49) and the marine-derived fungus *Eurotium amstelodami* (46).

The extracts of endophytic fungi isolated from *C. roseus* showed antimicrobial activity. The detected compounds with known antimicrobial activity which include hydroxyemodin, citreoisocoumarin, citreoisocoumarinol, and cladosporin may have contributed greatly to the activity shown by the endophytic fungal extract against the test microorganisms.

The HPLC analysis is a major analytical tool for the identification of the various constituents of crude mixtures, such as the endophytic fungal extracts. The use of HPLC as the sole tool of identifying the bioactive metabolites in the crude fungal extracts is limited, as only compounds whose UV-spectra are already in the HPLC spectral library can be detected. As a result of this limitation, the undetected compounds or compounds whose spectra had no library hit/match may represent important or novel bioactive compounds with interesting pharmaceutical or industrial applications. It is therefore recommended that further studies be carried out employing other more sensitive analytical tools such as mass spectrometry and/or NMR to validate the findings of this research.

CONCLUSION

The results of this study suggest that endophytic fungi associated with *C. roseus* could be a potential source of novel compounds for pharmaceutical and industrial applications.

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COMPETING INTERESTS

Authors declare no conflict of interest.

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