Research Article

Metabolites of Endophytic Fungi Isolated from *Euphorbia hirta* growing in Southern Nigeria

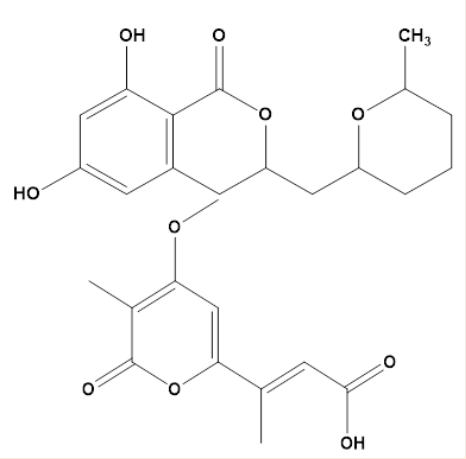
Mark O. Akpotu[1], Peter M. Eze[2]*, Chika C. Abba[3], Charles U. Nwachukwu[3], Festus B. C. Okoye[3]*, and Charles O. Esimone[2]

[1]Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu Campus, Ogun State, Nigeria
[2]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria
[3]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria

Abstract

Two endophytic fungi LA(1) and LC(2) were isolated from the leaves of a Nigerian plant *Euphorbia hirta*. The fungi were subjected to solid state fermentation on rice medium and metabolites were extracted using ethyl acetate. The fungal crude extracts were screened for antimicrobial and antioxidant activities. The bioactive compounds of the extracts were detected using HPLC-DAD analysis. The fungal extracts showed both antibacterial and antifungal activities, but exhibited poor antioxidant activity. The HPLC-DAD analysis of the extracts revealed the presence of citreoisocoumarinol and cladosporin in LA(1), and acropyrone in LC(2) as the major components of the extracts. Results of this study suggest that endophytic fungi associated with Nigerian plants could be a promising source of novel bioactive compounds with pharmaceutical and industrial importance.

Keywords: *Euphorbia hirta*, Endophytic fungi, Secondary metabolites, HPLC-DAD analysis



***Correspondence**
Author: Peter M. Eze, Festus B. C. Okoye
Email: ezep2004@hotmail.com, fb.okoye@unizik.edu.ng

Introduction

Globally, the threat posed by cancer and infectious diseases is ever-increasing, so is the emergence and increasing incidence of antibiotic resistance. Thus, the discovery of new drugs with novel mechanism of action is highly imperative to tame these ugly trends. Natural products from microbial origin have been a consistent source of novel lead molecules and recently several endophytes have been shown to possess the capacity to synthesize bioactive compounds that have found great use for novel drug discovery [1]. A single endophyte could produce several novel bioactive compounds, hence they have been receiving increased considerations in recent times [2-4].

Euphorbia hirta L. (Euphorbiaceae) is an herb that is distributed in many parts of the world, including Nigeria [5]. *E. hirta* is well documented for its biological activities such as anthelminthic, lactagogue, antipyretic, anti-inflammatory [6], antioxidant, anticancer [7], antibacterial [8], and antifungal [9] activities. The plant has been used for female disorders, but it has found greater use in treating respiratory ailments such as cough, coryza, bronchitis and asthma. It is also used to treat worm infestations in children [10]. Latex of the plant is used as ear drops and in the treatment of boils, sore and promoting wound healing [11].

Nigerian medicinal plants have been poorly investigated for their endophytic fungal population. A few of such studies carried out [1, 12-16] however, have lent credence to the enormous potentials which abound in endophytes associated with Nigerian medicinal plants as sources of novel therapeutic molecules. The aim of this research

therefore, was to isolate endophytic fungi from *Euphorbia hirta* collected from the river banks of Amassoma in Southern Ijaw Local Government Area of Bayelsa State, Southern Nigeria, and identify their bioactive secondary metabolites.

Experimental

Isolation of Endophytic Fungi, Fermentation and Extraction of metabolites

Using standardized methods [17] two endophytic fungi LA(1) and LC(2) were isolated from healthy leaves of *E. hirta.* Solid state fermentation was carried out in 1L 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 containing the fungi and incubated at 25-27°C for 21 days. At the completion of fermentation, the secondary metabolites were extracted in ethyl acetate and then concentrated under vacuum at 40^{o}C using a rotary evaporator.

HPLC-DAD Analysis

Each of the dried fungal metabolite extract (2 mg) was reconstituted with 2 ml of HPLC grade methanol. The mixture was sonicated for 10 min, followed by centrifugation at 3000 rpm for 5 min. Then, 100 µL of the dissolved samples were each transferred into HPLC vials containing 500 µL of the HPLC grade methanol.

The HPLC analysis was carried out on the samples with a Dionex P 580 HPLC system coupled to a Photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). Detection was at 235 nm. The separation column (125 × 4 mm) was prefilled 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 fungal extracts were analyzed by comparing with those in the HPLC-UV/Vis database (spectral library), which contains over 1600 registered compounds.

Bioassay of Fungal Extracts

Primary Antimicrobial Assay

The primary antimicrobial screening of the fungal extracts was carried out using the agar well diffusion method described by Onyegbule *et al*. [18]. A concentration of 1 mg/mL was prepared for all the fungal extracts by dissolving the extracts in DMSO. Twenty (20) mL of molten Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) (for bacterial and fungal isolates respectively) were poured into sterile Petri plates (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. Twenty (20µl) of the extracts and controls were put in each hole under aseptic condition, kept at room temperature for 1 h 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^{o}C for 24 h, and the SDA plates were incubated at 25-27^{o}C for 2-3 days. The inhibition zones diameters (IZDs) were measured and recorded. The size of the cork borer (6mm) was deducted from the values recorded for the IZDs to get the actual diameter. This procedure was conducted in triplicate and the mean IZDs calculated and recorded.

Determination of the Minimum Inhibitory Concentrations (MICs)

The MICs of the plant extracts on the test isolates were determined by the agar dilution method described by Onyegbule *et al*. [18]. Stock solutions (20 mg/mL) of the fungal extracts were prepared by dissolving the extracts in DMSO. The stock solutions were 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^{o}C for 24 h and the SDA plates were incubated at 25-27^{o}C for 2-3 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.

Antioxidant Assay

The antioxidant activities of the metabolites were determined using the 1, 1- diphenyl-2-picrylhydraxyl (DPPH) assay method described by Shen *et al.* [19] with modifications. Several dilutions (100, 80, 60, 40 and 20 µg/mL) of the fungal extracts and positive control (ascorbic acid) were prepared, and these were mixed with equal volume of 0.1 mM solution of DPPH. The negative control (DPPH + methanol) was also prepared. These were carefully distributed in a 96-wells microtitre plate. The microtitre plates were covered with a foil and incubated in the dark at room temperature for 30 min, and their absorbance were then measured at 517 nm using a Thermomax® microtitre plate reader (Global Medical Instrumentation, Inc., USA). The parameter for the interpretation of the results from DPPH assay method is the IC_{50} value, which is the concentration of the substrate that causes 50 % loss of the DPPH activity.

The capacity of the extracts to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH Scavenging effect (\%)} = \frac{A_o - A_1}{A_o} \times \frac{100}{1}$$

Where, A_o= absorbance of the negative control, A_1 = absorbance of the tested samples.

Results and Discussion

Results

Two endophytic fungi with codes LA(1) and LC(2) were isolated from the leaves of *Euphorbia hirta*. 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 0 − 16 mm. The MICs of the extract against the test organisms ranged from 0.125 − 1 mg/mL. Of the two fungal extracts, LA(1) showed broad spectrum antimicrobial activity against both test bacteria and test fungi. LA(1) exhibited highest antimicrobial activity against *S. aureus, S. typhi* and *C. albicans* with MIC of 0.25 mg/mL. LC(2) inhibited only the Gram-negative bacteria *S. typhi* and *E. coli* with an MIC of 1 mg/mL. The fungal extracts exhibited poor antioxidant activities with IC_{50} of 115 and 112 µg/ml recorded for LA(1) and LC(2) respectively, compared to the positive control ascorbic acid that recorded an IC_{50} of 18 µg/ml (**Table 3**).

Table 1 Results of the Antimicrobial Evaluation of the Fungal Extracts

Test Organisms	Inhibition Zone Diameters (IZD) (mm)			
	LA(1) (1 mg/mL)	**LC(2) (1 mg/mL)**	**Gentamicin (10 µg/ml)**	**DMSO**
S. aureus	8	0	23	0
B. subtilis	4	0	21	0
S. typhi	8	2	25	0
E. coli	6	1	20	0
			Fluconazole (50 µg/ml)	**DMSO**
A. fumigatus	4	0	14	0
C. albicans	7	0	9	0

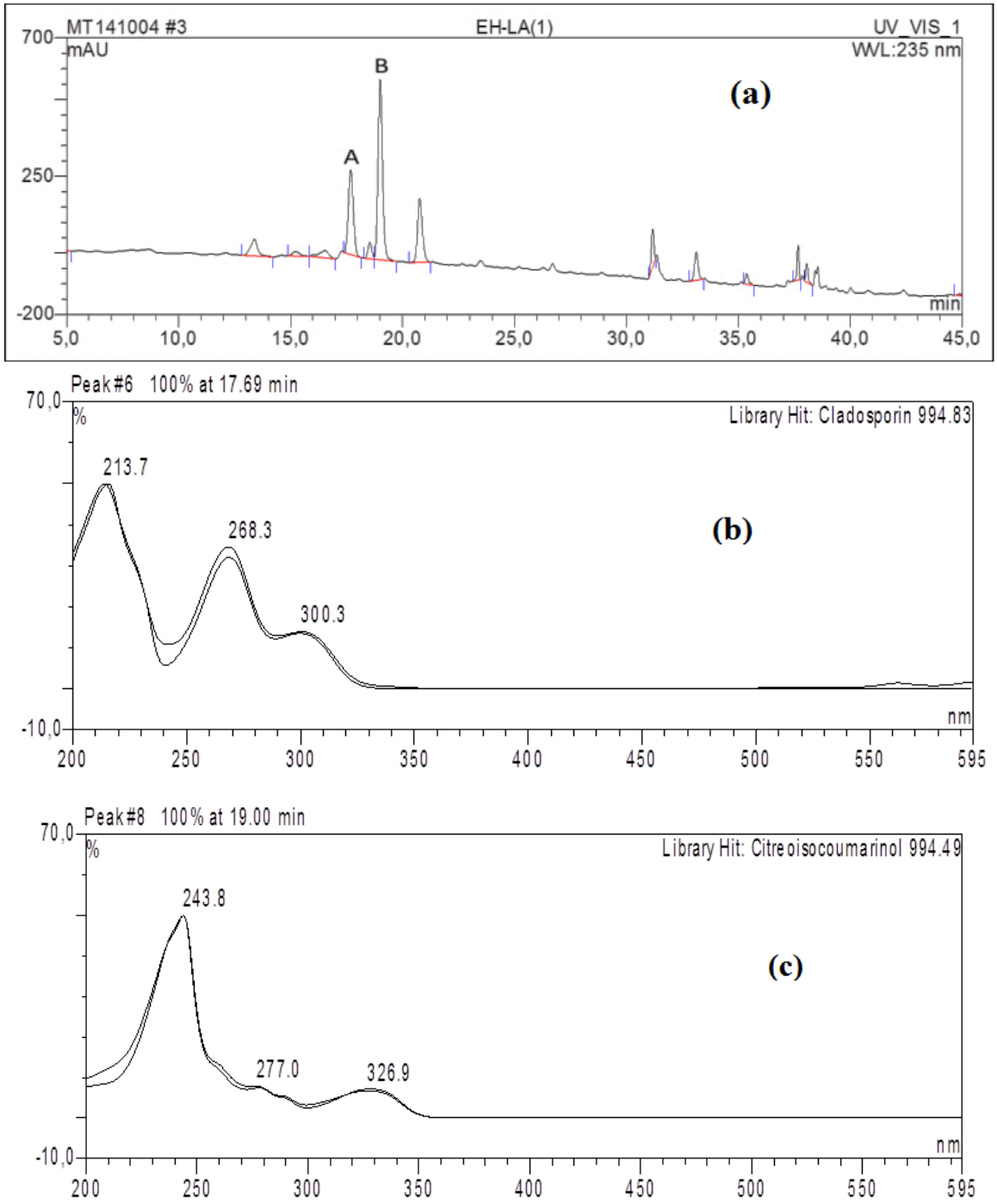
Table 2 The minimum Inhibitory Concentrations (MICs) of Fungal Extracts against the test organisms

Fungal Extracts	MICs (mg/mL)					
	S. aureus	***B. subtilis***	***S. typhi***	***E. coli***	***A. fumigatus***	***C. albicans***
LA(1)	0.25	0.5	0.25	0.5	0.5	0.25
LC(2)	-	-	1	1	-	-

Table 3 Antioxidant activity of the extracts showing their IC_{50}

Samples	IC_{50} (µg/ml)
LA(1)	115
LC(2)	112
Ascorbic acid	18

The HPLC-DAD analysis of the fungal extracts revealed the presence of compounds which may be responsible for the biological activities they elicit. Two compounds citreoisocoumarinol and cladosporin were identified as the major compounds in LA(1), and acropyrone was detected as the major compound in LC(2). The HPLC-UV data and structures of compounds detected in fungal extracts are presented in **Figures 1** and **2**.



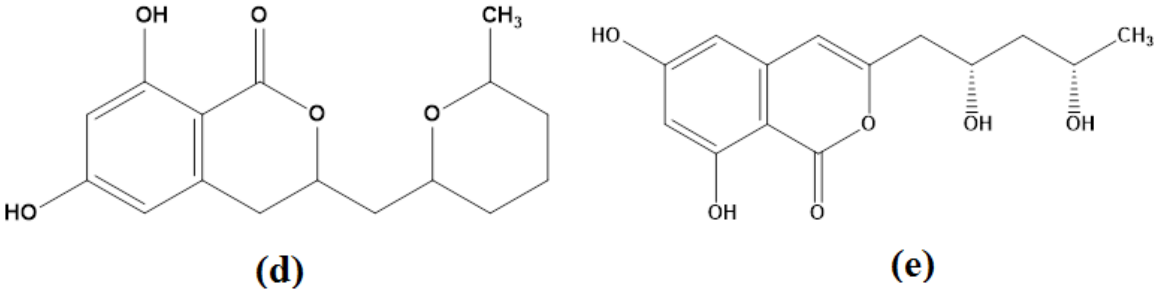
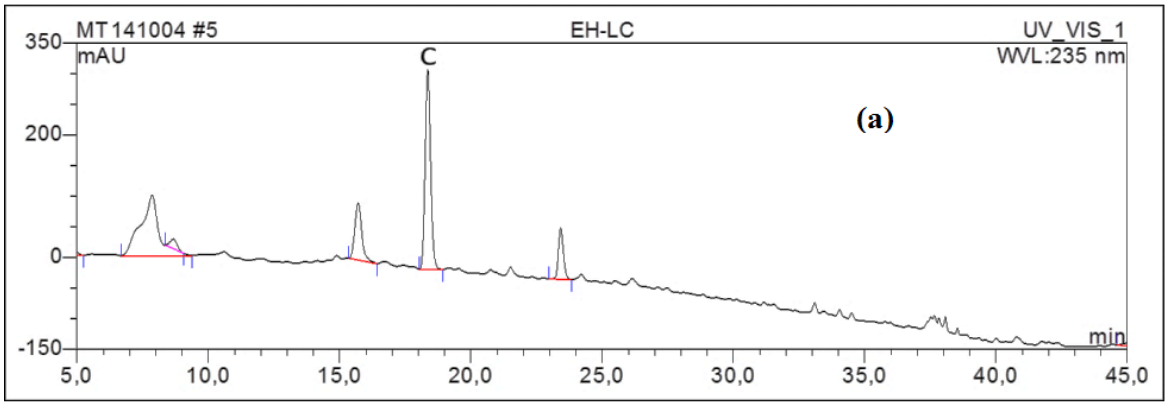


Figure 1 The HPLC-UV data and structures of compounds detected in LA(1) extract: (a) HPLC chromatogram showing the detection of A (cladosporin) and B (citreoisocoumarinol) in LA(1) extract; (b) UV-spectrum of cladosporin; (c) UV-spectrum of Citreoisocoumarinol; (d) Chemical structure of cladosporin ($C_{16}H_{20}O_5$, 292 g.mol^{-1}); (e) Chemical structure of citreoisocoumarinol ($C_{14}H_{16}O_6$, 280 g.mol^{-1}).



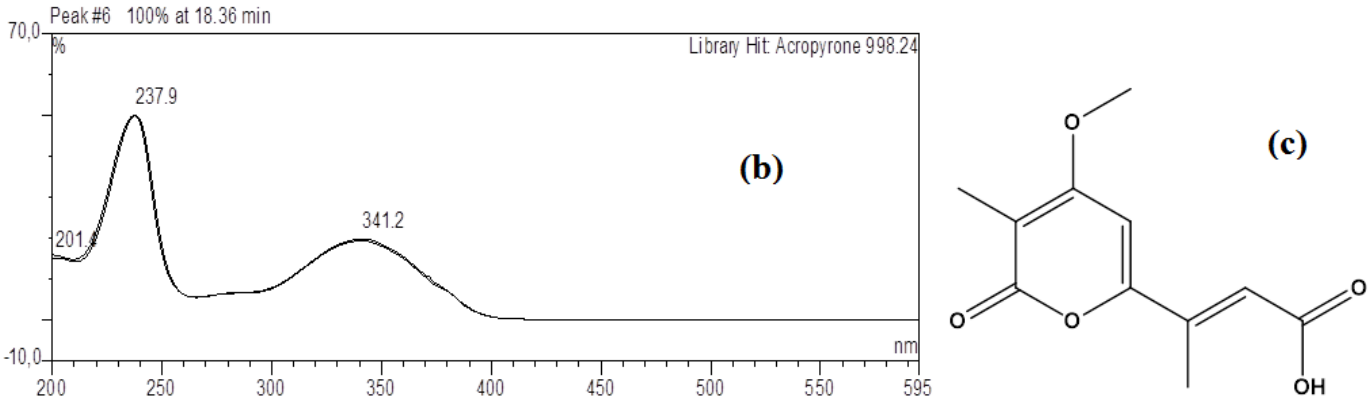


Figure 2 The HPLC-UV data and structure of compound detected in LC(2) extract: (a) HPLC chromatogram showing the detection of C (acropyrone) in LC(2) extract; (b) UV-spectrum of acropyrone; (c) Chemical structure of acropyrone **(**$C_{11}H_{12}O_5$, 224 g.mol^{-1}).

Discussion

Citreoisocoumarinol is an isocoumarin derivative and has been reported to show α-glucosidase inhibitory activity [20]. Isocoumarins are prevalent in most natural products that exhibit a wide range of biological activities [21]. These naturally occurring lactone compounds are known to show several pharmacologic activities including anti-diabetic [22], antimicrobial [23], insecticidal [24], antiparasitic [21, 25], cytotoxic [26], anti-inflammatory [27], and anti-angiogenic [28]. Several reports have been made on the isolation of citreoisocoumarinol from different endophytic fungi such as *Penicillium corylophilum* [29], *Nectria sp*. [20], and *Fusarium tricinctum* [30].

Cladosporin, another isocoumarin derivative detected with citreoisocoumarinol in LA(1), has been isolated from *Cladosporium cladosporioides* [31, 32], *Eurotium sp.* [33], etc. It has been reported to show antiplasmodial [34],

antifungal [32], antibacterial [31, 33], insecticidal [24], and antitumor properties [32]. Acropyrone, an acetophenone dimer, has been isolated from *Acronychia pedunculata* and has been reported to show cytotoxic activity [35, 36].

Although the two endophytic fungal extracts showed poor antioxidant activity, they both exhibited antibacterial and antifungal activity. The two isocoumarins - citreoisocoumarinol and cladosporin which were predominant in LA(1) may be responsible for the antimicrobial activity recorded by the endophytic fungal extract against the test bacterial and fungi.

The limitation associated with the HPLC-DAD analysis is that only compounds whose UV-spectra are already in the spectral library can be detected. Therefore, in the endophytic fungal extracts, the undetected compounds, or compounds whose spectra had no library hit may represent important or novel bioactive compounds.

Conclusion

The extracts of the endophytic fungi from *Euphorbia hirta* represent a dependable source of bioactive compounds. The results of this study suggest that endophytic fungi associated with Nigerian plants could be a potential source of novel compounds for pharmaceutical and industrial applications.

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