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Antimicrobial Activity of Metabolites of an Endophytic Fungus Isolated from the Leaves of *Citrus jambhiri* (Rutaceae)

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ABSTRACT

Some few studies on the endophytic fungal populations of Nigerian medicinal plants have confirmed the enormous potentials which abound in these organisms as sources of novel bioactive molecules. These studies highlight the need to further explore Nigeria's plant biodiversity for endophytes producing biologically important molecules. In our study, an endophytic fungus was isolated from the leaves of *Citrus jambhiri* growing in South-East Nigeria. The fungus was subjected to solid state fermentation on rice medium and the metabolites were extracted using ethyl acetate. The fungal extract was screened for antimicrobial activity and some of the bioactive compounds of the extract were detected using high-performance liquid chromatography (HPLC) analysis. The antimicrobial assay was carried out using the agar diffusion method against several bacterial and fungal strains. The fungal extract, at a concentration of 1 mg/mL, showed antibacterial activity only against *Staphylococcus aureus* with an inhibition zone diameter (IZD) of 3 mm. No activity against the test fungi was recorded. The HPLC analysis of the extract revealed the presence of three bioactive compounds: protocathechuic acid, indole-3-acetic acid, and acropyrone. Results of this study suggest that endophytic fungi associated with *C. jambhiri* could be a promising source of novel compounds with pharmacological importance.

Keywords: *Citrus jambhiri*, endophytes, HPLC analysis, secondary metabolites.

Introduction

Citrus species are one of the most important fruit trees grown in Nigeria, as well as, globally due to their high nutritional value. The citrus industry is considered to be a major industry for the production of fruits and fruit products.[1,2]

Citrus fruits (Rutaceae) possess high amounts of bioactive compounds which can influence human health, these include: vitamin C, carotenoids (*β*-carotene), flavonoids, limonoids, essential oils, coumarins, acridone alkaloids, high quality soluble fibre, minerals, vitamin-B complex and related nutrients such as thiamine, riboflavin, nicotinic acid/niacin, pantothenic acid, pyridoxine, folic acid, biotin, choline, and inositol.[3] Health promoting effects of citrus include antioxidant, cardioprotective, anticarcinogenic, anti-allergic, antiplatelet, antiviral, antibacterial and antifungal activities.[4-6]

Nigeria is rich in plant biodiversity. These plants, which are hosts to millions of endophytic microbial communities, present the opportunity to discover a plethora of biologically important compounds and offer a renewable source of natural products. Recent studies on the endophytic fungal populations of Nigerian medicinal plants have confirmed the enormous potentials which abound in these organisms as sources of novel bioactive molecules.[7-15] Only a few studies on the presence of endophytes in citrus plants have been performed and little is known about the microbial endophytic community of the citrus plants.[16-19] Several endophytic bacterial species have however been isolated from *Citrus jambhiri*.[18,20,21]

Our study, therefore, seeks to further explore Nigeria's plant biodiversity for biologically important molecules by isolating an endophytic fungus from the leaves of *C. jambhiri* and identifying some of its bioactive metabolites.

Materials and Methods

Isolation of endophytic fungus, fermentation and extraction of metabolites

The isolation of the endophytic fungus, fermentation and extraction of metabolites were carried out as described by Abba *et al*.[12] and Akpotu *et al*.[14] Fresh healthy leaves of *C. jambhiri* were collected in June 2014 from Eziama-Uli, Anambra state, Nigeria. 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 put into Petri dishes containing malt extract agar (MEA) supplemented with chloramphenicol. The Petri dishes were then incubated at a temperature of 28ºC 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. Solid state fermentation of the endophytic fungus was carried out in 1L Erlenmeyer flask containing autoclaved rice medium (100 g of rice and 100 mL of distilled water). The flask was inoculated with 3 mm diameter agar blocks containing the fungi and incubated at 28°C for 21 days. At the completion of fermentation, the secondary metabolites (contained in the fermentation medium) were

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extracted with ethyl acetate and then concentrated under vacuum at 40ºC using a rotary evaporator.

Antimicrobial assay

Antibacterial and antifungal screening of the fungal extract was carried out using the agar well diffusion method described by Abba *et al*.[12]A concentration of 1 mg/mL of the extract was tested against laboratory strains of *Staphylococcus aureus, Salmonella typhi, Bacillus subtilis, Escherichia coli, Candida albicans and Aspergillus fumigatus.* Gentamicin (10 µg/mL) and ketoconazole (50 µg/mL) were used as positive controls in the antibacterial and antifungal tests respectively, while DMSO was used as the negative control in both tests. The inhibition zone diameters (IZDs) produced against the test isolates were measured and recorded.

High Performance Liquid Chromatography (HPLC) Analysis

HPLC analysis was carried on the crude fungal extract with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). The fungal extract (2 mg) was reconstituted with 2 mL of HPLC grade methanol. The mixture was sonicated for 10 min and thereafter centrifuged at 3000 rpm for 5 mins. Then, 100 µL of the dissolved sample was transferred into a vial containing 500 µL of HPLC grade methanol. The vial was then put in the HPLC machine for analysis. Detection was at 235 nm. The separation column (125 × 4 mm; length × internal diameter) 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 of the fungal extract were analyzed by comparing with those in the HPLC-UV/Vis database, which contains over 1600 registered compounds.

Results and Discussion

An endophytic fungus CJ-MR2 was isolated from the leaves of *C. jambhiri*. The result of the antimicrobial assay of the fungal extract revealed that at 1 mg/mL, the extract showed antibacterial activity only against *S. aureus* with an IZD of 3 mm (Table 1). The extract showed no antifungal activity against the test fungi *C. albicans* and *A. fumigatus*.

The extract of the endophytic fungus from *C. jambhiri* represents a dependable source of bioactive compounds, evidenced by the wide range of compounds with diverse biological properties present in these extracts. The HPLC analysis of the extract revealed the presence of protocathechuic acid, indole-3-acetic acid, and acropyrone**.** The HPLC chromatogram of the fungal extract, as well as the UV-spectra and chemical structures of detected compounds are presented in Figures 1 and 2.

Fungi are well known for producing many novel biologically active chemicals, and are among the most important groups of eukaryotic organisms that are being explored for therapeutic molecules. The compounds detected in the extract of the endophytic fungus isolated from *C. jambhiri* possess biological activities that are either antimicrobial, cytotoxic, anti-inflammatory or antioxidant.

The fungal extract showed mild antibacterial activity against *S. aureus* and this activity may be attributed to antimicrobial compounds present in the extract. Protocathechuic acid and acropyrone are known to exhibit antimicrobial activity,[22,23] and these compounds may have contributed greatly to the antimicrobial activity shown by the endophytic fungal extract.

Protocatechuic acid is a type of widely distributed naturally occurring phenolic acid and is widely distributed and present in most edible and medicinal plants.[24-26] Protocathechuic acid has been reported to show antioxidant,[27] antibacterial,[22] anticancer,[28] anti-ulcer,[29] antidiabetic,[30] anti-ageing,[31] antifibrotic,[32] antiviral,[33] anti-inflammatory,[34] analgesic activity,[34] anti-atherosclerotic,[35] cardiac,[36] hepatoprotective,[37] neurological,[38] and nephroprotective[39] activities.

Indole-3-acetic acid is a known plant compound, and the most common plant hormone of the auxin class which regulates various aspects of plant growth and development.[40-42] Many fungal species have been reported to be able to produce indole-3-acetic acid.[40] The compound has been reported to possess cytotoxic/anticancer, antioxidant, anti-inflammatory activities.[43-45] In this study, it was observed that indole-3-acetic acid was the most abundant compound in the fungal extract, as it showed the most prominent peak (B) in the HPLC chromatogram of the extract (Figure 1).

Acropyrone is an α-pyrone compound with cytotoxic[46,47] and antibacterial[23] activities. It has been previously isolated from *Acremonium strictum*[23] and *Acronychia pedunculata.*[46,47]

Indole-3-acetic acid and acropyrone have also been previously reported to be present in extracts of some endophytic fungi associated with Nigerian plants.[13-15] These endophytes can serve as a ready source for large-scale production of these bioactive compounds for pharmaceutical or industrial applications.

Nigeria's rich plant biodiversity presents an enormous platform for researchers to explore in the area of bioprospecting without the destructive harvesting of plants, but by exploring their associated endophytic organisms for pharmaceutically and industrially important molecules. The rapid depletion of rainforests, which hold a potential for endophytes and their promising products, is one of the major problems facing the future of natural product discovery. It is understood that when a plant species disappear, so does its associated endophytes. It is, therefore, necessary that steps be taken now to secure and conserve this plant biodiversity before they are completely lost.

Table 1: Results of the antimicrobial evaluation of the fungal extract showing the inhibition zone diameters (IZD) (mm) produced against test organisms.

Test Organisms	CJ-MR2 (1 mg/mL)	Positive control	Negative control
		Gentamicin (10 µg/mL)	DMSO
S. aureus	3	17	0
S. typhi	0	21	0
B. subtilis	0	22	0
E. coli	0	16	0
		Ketoconazole (50 µg/mL)	DMSO
C. albicans	0	17	0
A. fumigatus	0	4	0

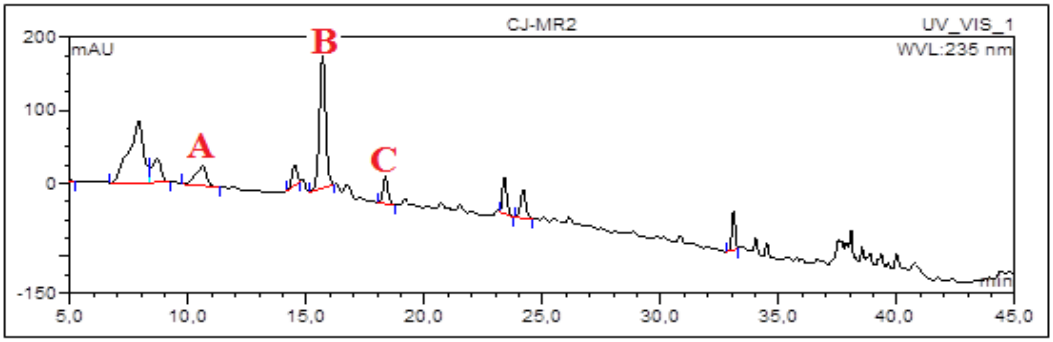

Figure 1: HPLC chromatogram of the endophytic fungal extract showing the detected compounds - (A) Protocathechuic acid, (B) Indole-3-acetic acid and (C) Acropyrone.

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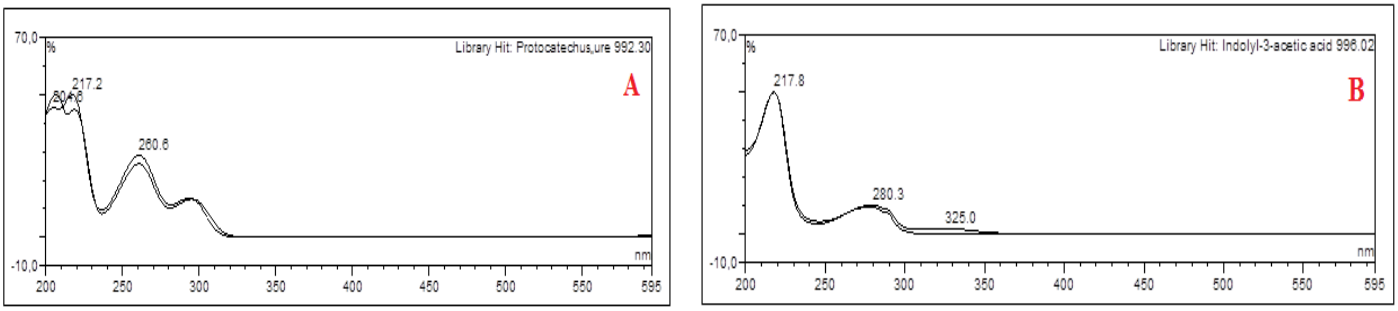
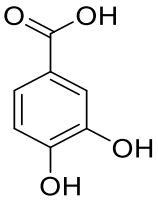
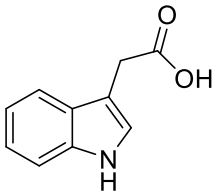
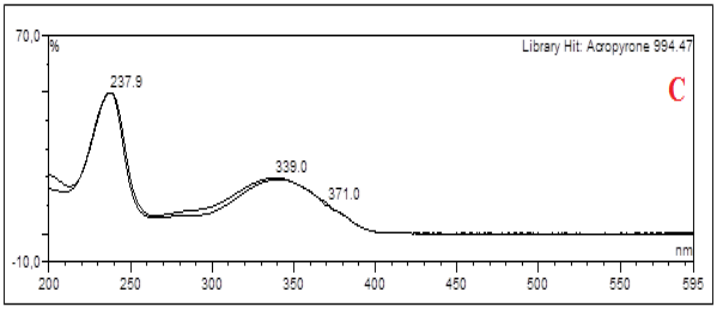


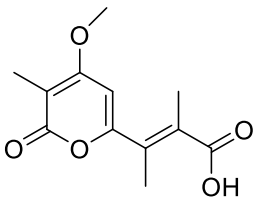


(A) Protocathechuic acid $C_7H_6O_4$, 154.12 g.mol^{-1}



(B) Indole-3-acetic acid $C_{10}H_9NO_2$, 175.19 g·mol^{-1}





(C) Acropyrone $C_{11}H_{12}O_5$, 224 g.mol^{-1}

Figure 2: UV Spectra and chemical structures of detected bioactive compounds: (A) Protocathechuic acid, (B) Indole-3-acetic acid and (C) Acropyrone**.**

According to Akpotu *et al.*,[14,15] the HPLC analysis has limitations as only compounds whose UV-spectra are already in the spectral library can be detected. Consequently, in the endophytic fungal extract, the undetected compounds or compounds whose spectra had no library hit may represent important or novel bioactive compounds. 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. jambhiri* could be a potential source of novel compounds for pharmaceutical and industrial applications.

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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