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.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.7,15 Several endophytic bacterial species have however been isolated from Citrus jambhiri.19,20,21

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.

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

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.13 and Akpotu et al.14 Fresh healthy leaves of C. jambhiri were collected in June 2014 from Ezima-Uli, Anambra state, Nigeria. The plant leaves were washed thoroughly in running tap water and then cut into small fragments (about 1 cm3). 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 28°C 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
extracted with ethyl acetate and then concentrated under vacuum at 40°C 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. 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 out on the crude fungal extract with a Dionex P580 HPLC system coupled to a photodiode array detector (UV340S, 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 was carried out using the agar well diffusion method (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 protocatechuic 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.

**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 protocatechuic 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.

**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) | Ketoconazole (50 µg/mL) |
| *S. aureus*    | 3                | 17               | 0                |
| *S. typhi*     | 0                | 21               | 0                |
| *B. subtilis*  | 0                | 22               | 0                |
| *E. coli*      | 0                | 16               | 0                |
| *C. albicans*  | 0                | 17               | 0                |
| *A. fumigatus* | 0                | 4                | 0                |

**Figure 1:** HPLC chromatogram of the endophytic fungal extract showing the detected compounds - (A) Protocatechuic acid, (B) Indole-3-acetic acid and (C) Acropyrone.
According to Akpotu et al., 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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