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Research Article

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Antimicrobial, Antioxidant and Cytotoxic Properties of Secondary Metabolites from Endophytic Fungi of Psidium Guajava

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Abstract This study was undertaken to investigate the biological properties of secondary metabolites of two endophytic fungi (code names: GA and GC) isolated from *Psidium guajava* leaves. Isolation of the endophytic fungi, fermentation and extraction of fungal secondary metabolites was carried out using standard procedures. The fungal extracts were screened for antimicrobial, antioxidant and cytotoxic activities using the agar well diffusion, DPPH and MTT assay methods respectively. Also, the extracts were subjected to high performance liquid chromatography (HPLC) analysis for the identification of the constituents. Result of the antimicrobial assay revealed that at 1 mg/mL, the crude extract of GA displayed broad spectrum antibacterial activity against all the test bacteria - *E. coli, S. aureus, B. subtilis* and *S. typhi* with inhibition zone diameter (IZD) of 5, 6, 5 and 4 mm respectively. Extract of GC showed antibacterial activity only against *S. aureus*, with IZD of 7 mm. In the DPPH antioxidant assay, GA and GC extracts at 100 µg/mL displayed average antioxidant activity with inhibitions of 56.7 and 55.2% respectively, which is comparable to the 63.7% inhibition recorded for the positive control ascorbic acid. At 10 µg/mL, GA and GC extracts showed poor cytotoxic activities against mouse lymphoma cells L5178Y, with inhibitions of 38.5 and 12.4% respectively. HPLC analysis of GA extract revealed the presence of citreoisocoumarinol, while acropyrone and citreoisocoumarin were detected in the extract of GC. The results of this study indicate that endophytic fungi isolated from leaves of *P. guajava* produced secondary metabolites that can play vital role in the development of drugs for effective treatment of diseases.

Keywords *Psidium guajava*, endophytic fungi, secondary metabolites, antioxidant, antimicrobial, cytotoxicity, HPLC analysis

Introduction

Endophytic fungi have been proven useful for novel drug discovery as revealed by the chemical diversity of their secondary metabolites. Studies have shown that endophytic microorganisms have the ability to produce the same or similar bioactive chemicals as those originated from their host plants, thus presenting these organisms as potential alternatives to plants for the large-scale production of valuable natural products [1-4].

Psidium guajava Linn. (Myrtaceae), popularly known as guava, is an important food crop and medicinal plant grown in tropical and subtropical countries around the world [5]. The plant contains important phytoconstituents such as tannins, phenolic compounds, β-sitosterol, flavonoids, triterpenoids, volatile oils, and many other biological

important phytocompounds [5-7]. Traditionally, many parts of *P. guajava* have been used to manage conditions like malaria, gastroenteritis, vomiting, diarrhea, dysentery, wounds, ulcers, toothache, coughs, sore throat, inflamed gums, and a number of other conditions [8,9]. Extracts and phytochemicals from *P. guajava* leaves have been shown to have useful pharmacological activities such as antioxidant, hepatoprotection, anti-allergy, antimicrobial, antigenotoxic, antiplasmodial, cytotoxic, antispasmodic, cardioactive, anticough, antidiabetic, antiinflamatory and antinociceptive activities, supporting its traditional uses [5,7].

In the course of our ongoing research on endophytic fungi associated with Nigerain medicinal plants (3,10-24), this study was aimed at investigating the secondary metabolites of a endophytic fungi isolated from the leaves *P. guajava* for possible antioxidant, antimicrobial and cytotoxic properties, and identifying some of its metabolites.

Materials and Methods

Plant collection, isolation of endophytic fungi, fermentation and extraction of metabolites

The fresh leaves of *P. guajava* were collected in the month of September, 2013 from Ngwo, Enugu North Local Government Area of Enugu State, Nigeria. Isolation of the endophytic fungi from the plant leaves, solid state fermentation of the fungi and extraction of the fungal metabolites were carried out using methods previously described by Eze *et al.* [10]. The plant leaves were washed clean tap water and then cut into small fragments of about 1 cm^2. Surface-sterilization of the leaf fragments was carried out by immersion in 2% sodium hypochlorite solution for 2 min, 70% ethanol for 2 min, before a final rinse in sterile water for 5 min. The sterilized leaf fragments were then transferred to Petri dishes containing malt extract agar (MEA) supplemented with chloramphenicol, after which the Petri dishes were incubated at 28°C. Fungal growths from the leaf fragments were monitored and hyphal tips from distinct colonies emerging from leaf fragments were sub-cultured onto fresh MEA plates to obtain pure colonies. Solid state fermentation of the isolated fungi was carried out in 1L Erlenmeyer flasks containing autoclaved rice medium (100 g of rice and 100 mL of distilled water). About 3 mm diameter agar blocks containing the fungi were transferred into respective flasks and incubated at 28°C for 21 days. At the completion of fermentation, the fungal secondary metabolites were extracted with ethyl acetate and then concentrated under vacuum at 40°C.

Antimicrobial Assay

Antimicrobial activity was determined by agar well diffusion assay as described by Akpotu *et al.* [11]. The extracts were tested against laboratory strains of *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Salmonella typhi, Candida albicans* and *Aspergillus fumigatus*. A concentration of 1 mg/mL of the fungal extracts was prepared by dissolving in dimethyl sulfoxide (DMSO, 100% v/v). A volume of 20 mL of molten Mueller-Hinton agar (MHA) and Sabouraud dextrose agar (SDA), for bacterial and fungal isolates respectively, were poured into sterile 90 mm Petri dishes and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of the test isolates were swabbed aseptically on the agar plates, and 6 mm holes were made in the agar plates using a sterile metal cork borer. A volume 20 µL of the extracts and controls were put into respective holes under aseptic condition, and allowed to stand for 1 h for the agents to diffuse into the agar medium. The MHA plates were incubated at 37°C for 24 h, and SDA plates at 28°C for 48-72 h. Gentamicin (10 µg/mL) and fluconazole (50 µg/mL) were used as the positive control against the test bacteria and fungi respectively, while DMSO (100% v/v) was used as the negative control. Antimicrobial activity was indicated by the appearance of inhibition zone diameters (IZDs) in the plates. These 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.

Antioxidant Assay

Using the 1, 1-diphenyl-2-picryl-hydraxyl (DPPH) free radical assay [12], the fungal extracts were screened for antioxidant activity. A concentration of 100 µg/mL of the fungal extracts and positive control (ascorbic acid) was prepared in methanol. Also, 0.1 mM solution of DPPH in methanol was prepared. The samples were reacted with

the DPPH solution in a 96-well micro-titer plate. The reaction mixture consisted of 25 µL of sample, 150 µL of methanol and 25 µL of 0.1 mM DPPH solution. Absorbance was measured using a UV/VIS spectrophotometer at 490 nm after 30 min of incubation in the dark. A mixture of methanol (175 µL) and DPPH radical solution (25 µL) was used as blank. The DPPH free radical scavenging effect of the samples was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of } blank - Absorbance \ \ of \ sample}{Absorbance \ \ of \ blank} \times 100$$

Cytotoxicity assay

The cytotoxic property of the fungal extracts against mouse lymphoma cells (L5178Y) was evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] assay [13]. The cells were grown in Eagle's minimal essential medium supplemented with 10% horse serum, 100 units/mL penicillin and 100 units/mL streptomycin. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. A concentration of 10 µg/mL of crude fungal extracts was prepared by dissolving in ethanol (96% v/v). Exponentially growing cells were harvested, counted and diluted appropriately. Using a sterile pipette, 50 µL of the cell suspension (containing ~3750 cells) was transferred to 96-well microtiter plate. Subsequently, 50 µL of the test samples was added to each well. The test plate was incubated at 37 °C with 5% CO_2 for 72 h. A volume of 20 µL MTT solution (5 µg/mL) prepared in phosphate buffered saline was pipetted into each well. The yellowish MTT penetrated the cells, and in the presence of mitochondrial dehydrogenases, MTT was transformed to its blue formazan complex. For negative controls, media with 0.1% (v/v) ethanol were included in all experiment. After an incubation period of 3 h 45 min at 37°C in a humidified incubator with 5% CO_2, the medium was centrifuged (15 min at 210 x g) with 200 µL DMSO and the cells were lysed to liberate the formazan product. This was thoroughly mixed and the absorbance measured at 520 nm. The color intensity is correlated with the number of healthy living cells, and the cell survival was thus calculated using the formula:

$$\text{Survival \%} = 100 \text{ x } \frac{Absorbance \ \ of \ untreated \ \ cells - Absorbance \ \ of \ culture \ \ medium}{Absorbance \ \ of \ treated \ \ cell - Absorbace \ \ of \ Culture \ \ medium}$$

High performance liquid chromatography (HPLC)

The fungal extracts were subjected to HPLC analysis using the Dionex P580 HPLC system coupled to a UVD 340S photodiode array detector (Dionex, Germany) [10]. A weight of 2 mg of each extract was reconstituted with 2 mL of HPLC grade methanol. The mixture was sonicated for 10 min and thereafter, centrifuged at 13000 rpm for 5 min. The supernatant was collected and about 100 µL of the dissolved sample was then transferred into HPLC vial containing 500 µl of HPLC grade methanol, and vial was put in the HPLC machine for analysis. The separation column (125 x 4 mm; length x 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. Detection was at 235 nm and the absorption peaks of the fungal extracts were analyzed by comparing with those in the HPLC-UV/Vis database.

Results

Two endophytic fungi (GA and GC) were isolated from the leaves of *P. guajava.* The result of the antimicrobial assay (Table 1) revealed that at 1 mg/mL, the extract of GA showed broad spectrum antibacterial activity against the Gram-positive and Gram-negative test bacteria - *E. coli, S. aureus, B. subtilis* and *S. typhi* with IZD of 5, 6, 5 and 4 mm respectively. Extract of GC showed antibacterial activity only against *S. aureus*, with IZD of 7 mm. Both fungal extracts showed no antifungal activity against *C. albicans* and *A. fumigatus*.

Table 2 shows the result of the DPPH antioxidant assay of the fungal extracts. At 100 µg/mL, GA and GC extracts displayed average antioxidant activity with inhibitions of 56.7 and 55.2% respectively, which is comparable to the 63.7% inhibition recorded for the positive control ascorbic acid. Result of the cytotoxicity assay reveals that at 10 µg/mL, crude extracts of GA and GC showed poor cytotoxic activities against mouse lymphoma cells L5178Y, with inhibitions of 38.5 and 12.4% respectively (Table 3).

HPLC analysis of the crude extract of GA revealed the presence of citreoisocoumarinol, while GC showed the presence of acropyrone and citreoisocoumarin. The HPLC chromatograms of the fungal extracts, as well as the UV-spectra and chemical structures of the detected compounds are presented in Figures 1 and 2.

Table 1: Antimicrobial activity and the zone of inhibition of the endophytic fungal extracts

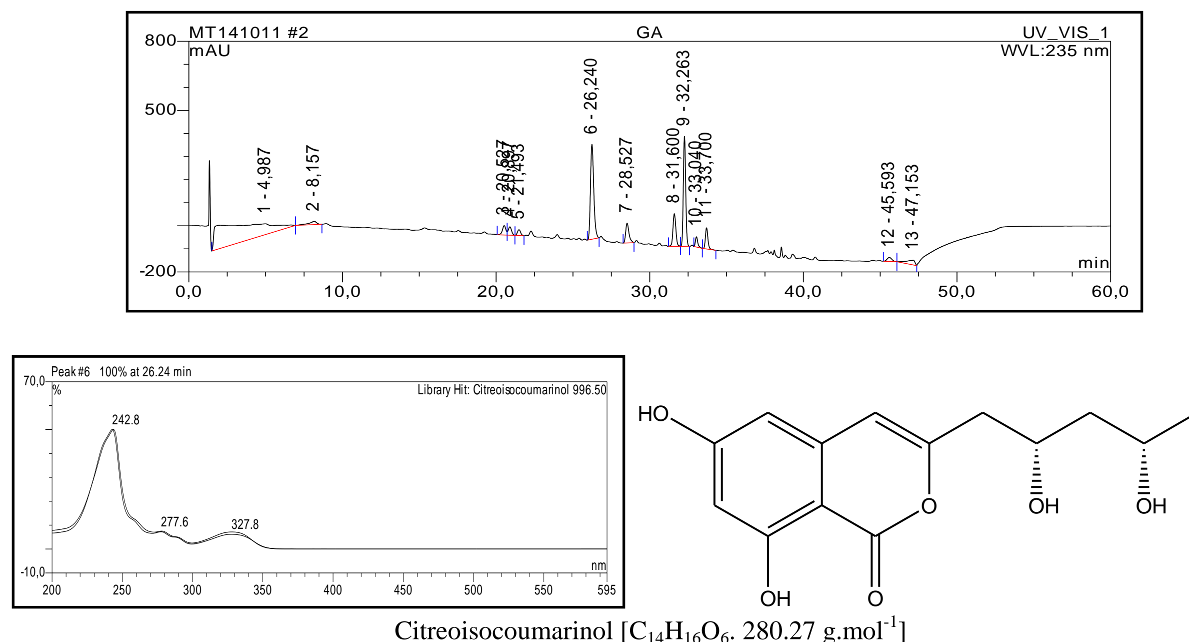
Test Organisms	Endophytic Fungal Extracts (1 mg/mL)		Positive control	Negative control
	GA	**GC**	**Gentamicin (10 µg/ml)**	**DMSO**
E. coli	5	0	16	0
S. aureus	6	7	17	0
B. subtilis	5	0	22	0
S. typhi	4	0	21	0
			Fluconazole (50 µg/ml)	**DMSO**
C. albicans	0	0	17	0
A. fumigatus	0	0	4	0

Table 2: Antioxidant activity of the endophytic fungal extracts

Endophytic fungal extracts	Concentration (µg/ml)	% inhibition
GA	100	56.7
GC	100	55.2
Ascorbic acid	100	63.7

Table 3: Cytotoxicity of endophyte extracts against L5178Y mouse lymphoma cell line

Endophytic fungal extracts	Concentration (µg/ml)	% inhibition
GA	10	38.5
GC	10	12.4



Citreoisocoumarinol [$C_{14}H_{16}O_6$. 280.27 g.mol^{-1}]

Figure 1: HPLC chromatogram of the endophytic fungal extract (GA), UV spectrum and chemical structures of the detected compound - citreoisocoumarinol

Acropyrone [$C_{11}H_{12}O_5$, 224 g.mol^{-1}]

Citreoisocoumarin [$C_{14}H_{14}O_6$. 278.26 g.mol^{-1}]

Figure 2: HPLC chromatogram of the fungal extract (GC), UV Spectra and chemical structures of the detected compounds - acropyrone and citreoisocoumarin

Discussion

Endophytic fungi have been proven to be rich sources of chemical compounds with important biological properties. Screening of secondary metabolites of endophytes for biological activities is necessary in exploring their potentials in the discovery of novel bioactive molecules.

The results of this present study revealed a broad spectrum antimicrobial activity exhibited by the secondary metabolites of an endophytic fungus (GA) isolated from *P. guajava*. The fungal extract exhibited potent antibacterial activity against both Gram-positive and Gram-negative bacteria (Table 1). Secondary metabolites of the two endophytic fungi (GA and GC) investigated in this study displayed considerable antioxidant activity (Table 2), but poor cytotoxic activity against mouse lymphoma cells L5178Y (Table 3). The biological activities exhibited by the endophytic fungal extracts could be said to be due to the different constituents of the extracts. HPLC analysis of the fungal extracts revealed the presence of citreoisocoumarinol in GA, and acropyrone and citreoisocoumarin in GC (Figures 1 and 2).

Citreoisocoumarin and citreoisocoumarinol are isocoumarin derivatives that have been reported to show α-glucosidase inhibitory and antimicrobial activities [25,26]. These compounds have been previously isolated from several different endophytic fungi such as *Nectria* sp. [26], *Microdochium bolleyi* [27], *Fusarium tricinctum* [28], *Ampelomyces* sp. [29], *Penicillium corylophilum* [30], *Aspergillus* sp. [31,32], and *Penicillium* sp. [33].

Acropyrone is an alpha-pyrone compound reported to possess cytotoxic [34,35] and antibacterial [36] properties. It has been previously isolated from *Acremonium strictum* [36] and *Acronychia pedunculata* [34,35].

The three detected compounds - citreoisocoumarin, citreoisocoumarinol and acropyrone have also been previously reported to be present in extracts of some endophytic fungi associated with Nigerian plants [10,11,12,14]. These endophytes can serve as a ready source for large-scale production of these bioactive compounds for pharmaceutical or industrial applications.

The discovery of novel therapeutic molecules from endophytes is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens and the declining number of potent, safe and nontoxic drugs available against infectious diseases and cancer.

Conclusion

The study demonstrated that endophytic fungi of *Psidium guajava* produce secondary metabolites with biological properties. These endophytic fungi can be a good source of novel therapeutic compounds which may play a vital role in the development of drugs for the effective treatment of diseases.

Conflict of Interests

The authors declare no conflict of interest.

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