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Biologically active secondary metabolites of an endophytic fungus of *Psidium guajava*

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Abstract

Endophytes possess several phytohormones and bioactive metabolites of medicinal importance and thus, continue to generate research interest as candidates in drug discovery programmes. This study was carried out to investigate the secondary metabolites of an endophytic fungus isolated from leaves of *Psidium guajava*. Endophytic fungal isolation, fungal fermentation; and extraction of secondary metabolites in ethyl acetate were carried out using standard methods. The crude extract was subjected to Vacuum Liquid Chromatography (VLC) using binary combinations of Hexane:Ethyl acetate and Dichloromethane:Methanol to obtain fourteen sub-fractions designated PG55-1 to PG55-14. The fungal crude extracts and VLC sub-fractions were screened for antimicrobial activity and were also subjected to high-performance liquid chromatography-diode-array detection (HPLC-DAD) analysis for the identification of bioactive compounds. An endophytic fungus, PG55 was isolated from the leaf of *Psidium guajava*. The fungal secondary metabolites showed antibacterial properties, with minimum inhibitory concentrations ranging from 0.0625 – 1 mg/ml. No antifungal activity was observed. HPLC-DAD analysis of the extract suggested the presence p-hydroxybenzoic acid, pentenedioic acid and palitantin in one of the fractions of PG55. Some of these compounds are known antimicrobial agents and may be responsible for the antimicrobial activities recorded for the fungal extracts. The results of this study, suggests the many potentials possessed by Nigerian plants as hosts of endophytes that could be reservoirs for excellent sources of pharmacologically active compounds.

Keywords: Metabolites; Structure elucidation; Endophytic Fungus; *Psidium guajava*

1. Introduction

Endophytes have recently generated significant interest in the microbial chemistry community due to their immense potential to contribute to the discovery of new bioactive compounds. It has been suggested that the close biological association between endophytes and their plant host results in the production of a greater number and diversity of biological molecules compared to epiphytes or soil-related microbes [1]. Moreover, the symbiotic nature of this relationship indicates that endophytic bioactive compounds are likely to possess reduced cell toxicity, as these chemicals do not kill the eukaryotic host system. This is of significance to the medical community as potential drugs may not adversely affect human cells [2].

The production of bioactive substances by endophytes is directly related to the independent evolution of these microorganisms, which may have incorporated genetic information from higher plants, allowing them to better adapt

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to plant host and carry out some functions such as protection from pathogens, insects, and grazing animals [1]. Endophytes are chemical synthesizer inside plants [3], in other words, they play a role as a selection system for microbes to produce bioactive substances with low toxicity toward higher organisms [1].

Recent reports of the isolation of important plant-derived anticancer drugs from endophytic fungi have served to focus attention on these sources. Examples are paclitaxel (Taxol®) from *Taxomyces* [4] and many *Pestalotiopsis* species [5], as well as camptothecin [6], podophyllotoxin [6], vinblastine [7], and vincristine [8], all produced in relatively small amounts by endophytic fungi isolated from the producing plants.

Bioactive natural compounds produced by endophytes show promising potentials in safety and human health concerns, although there is still a significant demand of the drug industry for synthetic products due to economic and time-consuming reasons [9]. Problems related to human health such as the development of drug resistance in human pathogenic bacteria, fungal infections, and life-threatening virus demand for new therapeutic agents for effective treatment of diseases in humans, plants, and animals that are currently unmet [10,11].

Psidium guajava Linn. (Family Myrtaceae) is a fructiferous tree widely used as food and in folk medicine in different parts of the world [12]. Pharmacological activities attributed to extracts of *P. guajava* include antimicrobial, hepatoprotective, antioxidant, cytotoxic, cardioprotective, antidiabetic activities among others [13].

The present study was carried out to isolate endophytic fungi from the leaves of *P. guajava,* assay the fractions for antimicrobial activity and also identify the constituents of the fungal extracts using high performance liquid chromatography – diode array detector (HPLC-DAD) assay.

2. Material and methods

2.1. Culture media

Culture media used in this study include: fungal isolation and purification medium [Malt Extract Agar (MEA) (Oxoid, UK)], fermentation medium [Rice medium (Rice-100 g **+** 110 mL of distilled water)], antimicrobial assay media [Mueller Hinton Agar (MHA) and Sabouraud Dextrose agar (SDA) (Oxoid, UK)].

2.2. Test microorganisms

Laboratory strains of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger* and *Candida albicans* obtained from the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria, were used in the antimicrobial assay of the endophytic fungal extract.

2.3. Isolation, identification and fermentation of endophytic fungus

Fresh leaves of *Psidium guajava* were collected in June, 2015 from Akegbe-Ugwu, Enugu State, South-Eastern Nigeria. The plants were identified and authenticated by a plant technologist in the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State. Samples of the plants were deposited at the herbarium of Department of Pharmacognosy and Traditional Medicine as voucher specimen Psidium guajava: PCG474/A/045. Isolation of endophytic fungi from the plant leaves was carried out according to the method described by [14]. Fresh leaves of the plant were rinsed under running tap water to remove debris. The leaves were then cut into small segments (approximately 1 x 1 cm). Surface sterilization of the leaf fragments was achieved by immersion in 1 % sodium hypochlorite for 2 min, after which the leaves were soaked in 70 % ethanol for 2 min, and then rinsed with sterile water for 5 min. Using sterilized forceps, the leaf segments were placed into Malt Extract Agar (MEA) plates supplemented with chloramphenicol to suppress bacterial growth. The plates were then incubated at room temperature (25 °C) and monitored periodically until mycelial growths were observed from the leaves. Hyphal tips of the distinct fungal growth were transferred onto fresh agar plates to obtain a pure culture of the fungal isolate.

2.4. Fermentation, extraction and isolation of fungal secondary metabolites

Fermentation and extraction of fungal secondary metabolites was carried out according to the method described by Abba [14]. The endophytic fungi were subjected to solid state fermentation in Erlenmeyer flasks containing 100 g of rice and 110 ml of tap water. These were autoclaved at 121 °C at 15 psi for 1 h and allowed to cool. The flasks were inoculated with 3-5 mm diameter agar blocks containing test fungi and incubated at 25 °C for 21 days and extracted

with EtAOc. The EtAOc extract was concentrated under vacuum at 400 °C to yield crude extract. The extract was stored as stock materials at 4 °C for further studies.

2.5. Vacuum Liquid Chromatography (VLC)

A weight of 2 g (2000 mg) of the crude extracts obtained from the fungal fermentation were subjected to vacuum liquid chromatography (VLC). The extracts were adsorbed on silica gel in a glass column 4x50 cm packed with silica gel (230-400 mesh size) to a bed height of 15 cm and connected to a vacuum pump. The column was eluted by step gradient elution with 500 mL each of a mixture of n-hexane:ethyl acetate (8:2, 6:4, 5:5, 4:6, 2:8, 1:9) and thereafter with dichloromethane:methanol (10:0, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 1:9).). The fractions obtained were collected separately and concentrated using the rotary evaporator to produce fractions PG 55-1 to PG 55-14 respectively.

2.6. High Performance Liquid Chromatography (HPLC)-Diode Array Detection (DAD) Analysis

The dried fungal fractions (2 mg) were reconstituted with 2 ml of HPLC grade methanol. The mixture was sonicated for 10 minutes, followed by centrifugation at 3000 rpm for 5 min. Then, 100 µL of the dissolved samples were 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, DionexSoftron GmbH, Germering, Germany). Detection was at 235 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 fungal extract were analyzed by comparing with those in the HPLC-ultraviolet (UV)/visible database, which contains over 1600 registered compounds. All extracts, fractions and pure compounds were monitored by analytical HPLC to determine the composition of the fractions, the purity of the isolated substances and the optimum conditions for semi preparative HPLC. Additionally, known substances were identified by comparison of the obtained UV spectra with the internal UV-spectra library using the online software. For the routine analytical HPLC detection, a solvent system of nanopure water (adjusted to pH 2.0 by addition of phosphoric acid) (eluent A) and methanol (eluent B) with a flow rate of 1 mL/min employing the standard gradient system, was used.

2.7. Semi preparative HPLC

This technique was used for isolation and purification of compounds from fractions pre-purified using column chromatographic separation. The most appropriate solvent systems were determined by analytical HPLC before running the preparative HPLC separation. The mobile phase consisted of methanol and nanopure water. Each injection consisted of 1–3 mg of the fraction dissolved in 100 µL of the solvent system. The solvent system was pumped through the column at a rate of 5 mL/min. The eluted peaks were detected by the online UV detector and collected separately in clean test tubes.

2.8. Antimicrobial assay

The Minimum Inhibitory Concentration (MIC) of the extracts was determined for each of the test organisms in triplicate petri dishes. Stock solutions of 10 mg/mL of the various fractions were prepared. Then, two-fold serial dilutions were carried out to achieve 5, 2.5, 1.25, 0.625 mg/mL, thereafter 10-fold dilutions of each of the concentration was made using 9 mL sterile molten agar, which was allowed to solidify. The microbial inoculums which had been standardized to 0.5 McFarland turbidity were streaked on the agar appropriately. The plates were incubated at 37 ^{0}C and 25 ^{0}C for 24 and 48 hrs for the bacteria and fungi plates respectively. After incubation the plates were examined for microbial growth by checking for growths.

3. Results

An endophytic fungus (PG 55) was isolated from the leaves of *P. guajava.* The antimicrobial assay (Figure 1) revealed that at concentration of 1 mg/mL, PG55-10 showed best antimicrobial activity against *B. subtilis* and *P. aeruginosa* with an IZD of 4mm and 6mm respectively. Fraction 8 in addition to showing antibacterial activity against *B. subtilis* and *P. aeruginosa* also recorded an MIC of 0.5mg/ml against *E. coli*. No fraction showed any activity against *A. niger and C. albicans.* The results of minimum inhibitory concentration (Figure 2) revealed that only fractions 4 and 9 inhibited *S. aureaus* with MIC of 1 mg/mL respectively. Fractions 8 and 10 inhibited growth of *B. subtilis.* Fractions 4, 8 and 9 inhibited the growth of *E. coli* with MICs of 1mg/mL, 0.5mg/mL and 0.5mg/mL respectively. Fraction 4, 8, 9 and 10 showed activity against *P. aeruginosa* with MICs of 0.25 mg/mL, 0.125mg/mL, 0.5 mg/mL and 0.125 mg/mL.

The HPLC-DAD analysis of the fungal extract revealed the presence of three compounds including p-hydroxybenzoic acid, pentenedioic acid (trans-Glutaconic acid) and palitantin. The UV-spectra and chemical structures of the detected compounds are presented in Figures 3 to 6 below.

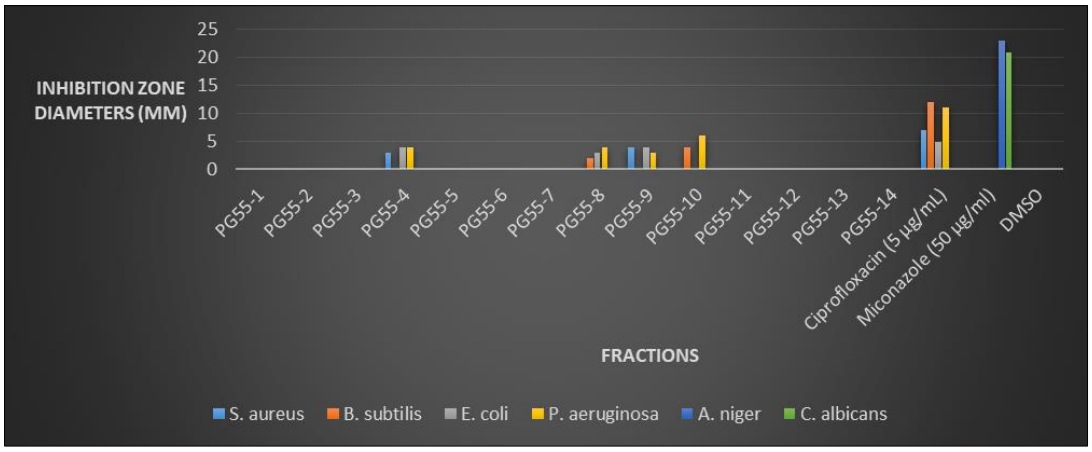

Figure 1 Antimicrobial activity of PG 55 fractions at 1 mg/mL

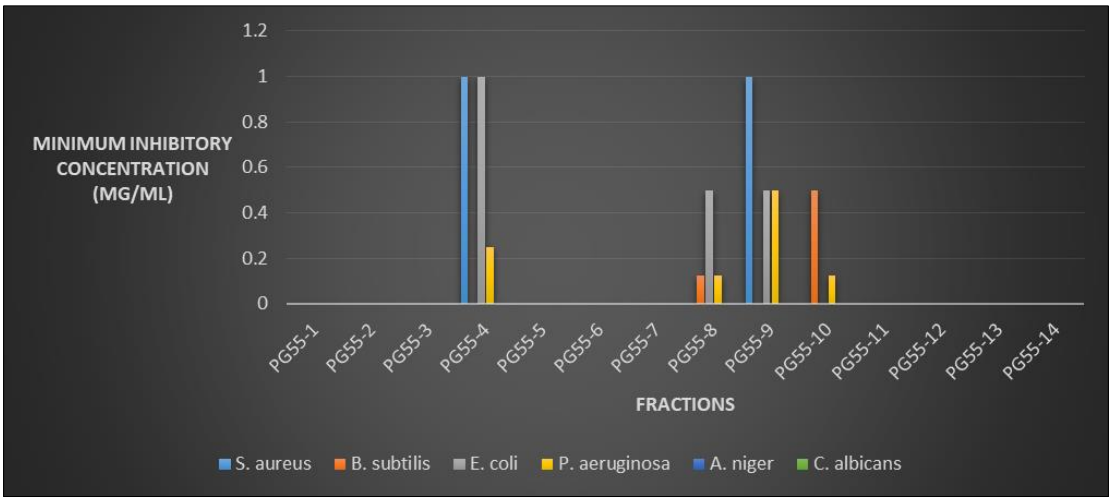

Figure 2 Minimum Inhibitory Concentrations of PG55 fractions

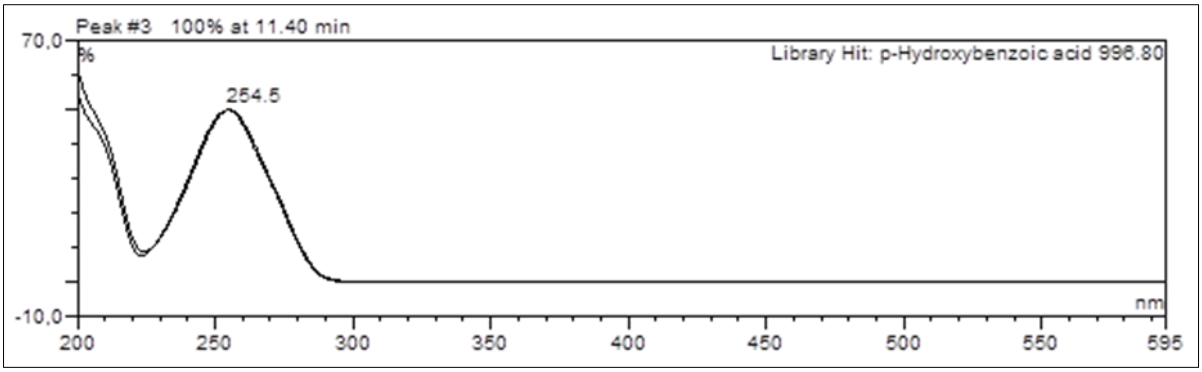

Figure 3 UV-Spectrum of p-Hydroxybenzoic acid

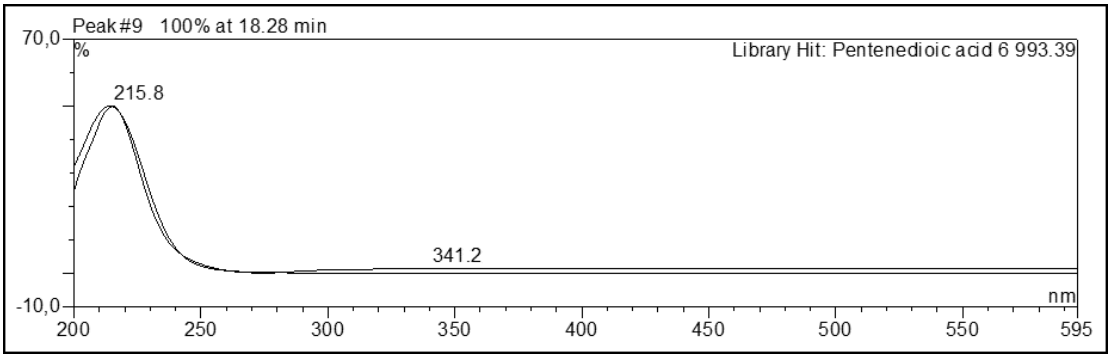

Figure 4 UV-Spectrum of Pentenedioic acid

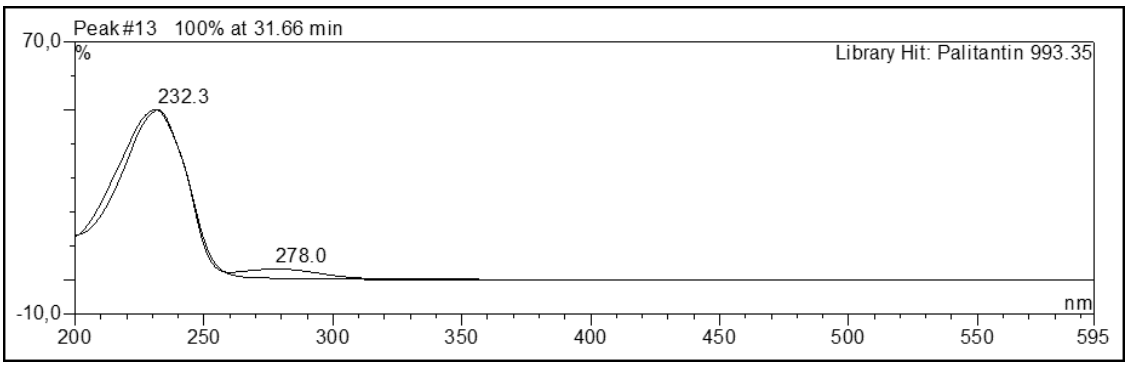

Figure 5 UV-Spectrum of Palitantin

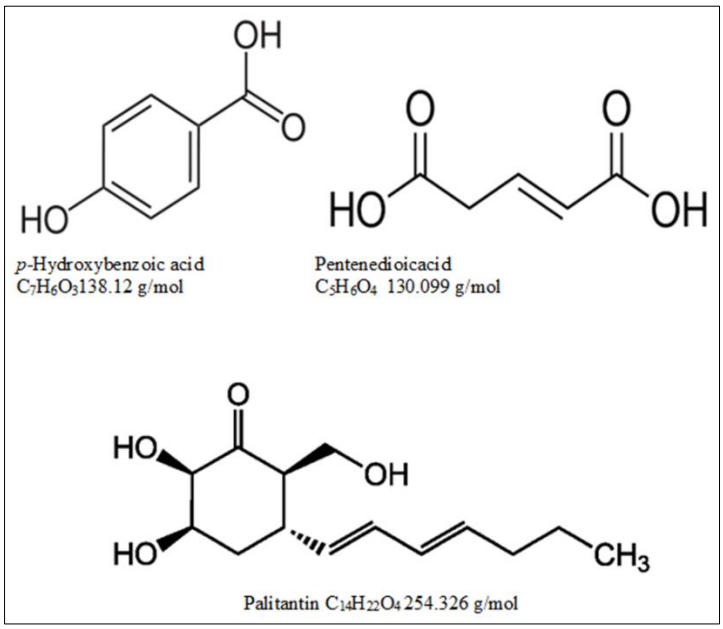

Figure 6 Chemical structures of detected compounds

4. Discussion

After solid-state fermentation in rice medium, the secondary metabolites of PG 55 were isolated using ethyl acetate. The fungal crude extract was further fractionated and these fractions were subjected to antimicrobial screening.

These fractions displayed varying degrees of antibacterial activities, IZDs ranging from 2 – 6 mm, and Minimum Inhibitory Concentrations (MICs) ranging from 0.125 – 1 mg/ml. (Figures 1 and 2). The fungal secondary metabolites inhibited the growth of the bacterial test isolates (*S. aureus, B. subtilis, E. coli,* and *P. aeruginosa*), but not the fungal test isolates (*A. niger* and *C. albicans*).

The various fractions of PG55 were subjected HPLC-DAD analysis which revealed the presence of three (3) bioactive compounds in fraction in fraction PG55-1. The compounds are *p*-hydroxybenzoic acid, pentenedioic acid and palitantin. These compounds possess diverse biological properties such as antioxidant and antimicrobial properties.

p-Hydroxybenzoic acid is reported to show antioxidant and antimicrobial properties [15,16]

Pentenedioic acid also referred to as trans-glutaconic acid, is a dicarboxylic acid that exists as a colorless solid and is related to the saturated chemical glutaric acid. The biological beneficial use of this compound has not been confirmed.

Palitantin is known to possess antifungal and antiprotozoal properties [17, 18]

5. Conclusion

Fungi are among the most important groups of eukaryotic organisms that are being explored for generation of novel therapeutic molecules [19]. They are well known for producing many pharmacologically-active metabolites that are directly used as drugs or function as lead structures for synthetic modifications. The compounds identified in fractions of the fungal endophyte may be connected to the anti-microbial activity observed. The results compare quite favorably with those found in literature.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The author declares no conflict of interest.

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