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Metabolites of endophytic *Colletotrichum gloeosporioides* isolated from leaves of *Carica papaya*

Dominic O Abonyi, Peter M Eze, Chika C Abba, Chidimma R Chukwunwejim, Chika P Ejikeugwu, Festus BC Okoye and Charles O Esimone

Abstract
Endophytic fungi associated with Nigerian plants have recently generated significant interest in drug discovery programmes due to their immense potential to contribute to the discovery of new bioactive compounds. This study was carried out to investigate the secondary metabolites of endophytic *Colletotrichum gloeosporioides* isolated from leaves of *Carica papaya*. The plants were collected from Agulu, Anambra State, Nigeria. Endophytic fungal isolation; identification by DNA amplification and sequencing of the fungal internal transcribed spacer (ITS) region; fungal fermentation; and extraction of secondary metabolites were carried out using standard methods. The crude extract was tested for antimicrobial, antioxidant and cytotoxic activities. Also, the extract was subjected to high performance liquid chromatography (HPLC) analysis to identify its constituents. In the bioassay, *C. gloeosporioides* extract only exhibited antibacterial activity against *Klebsiella pneumoniae.* The extract showed moderate antioxidant activity in the 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay with an inhibition of 28.2% at a concentration of 500 µg/mL. At a concentration of 100 µg/mL, the fungal extract showed moderate cytotoxic activity against cisplatin-sensitive ovarian cancer cell line 2780 (sens) and cisplatin-resistant ovarian cancer cell line 2780 (CisR) with growth inhibitions of 35.42 and 15.7% respectively. HPLC analysis of the extract revealed the presence of 3 bioactive compounds Aureonitol, protocathechuic acid, and Glucobrassicin in the crude extract of *C. gloeosporioides*. The findings of this study revealed the potentials possessed by *C. papaya* as source of entophytes that express biological active compounds. These endophytes hold key of possibilities to the discovery of novel molecules for pharmaceutical, agricultural and applications.

Keywords: *Endophytic fungi, Colletotrichum gloeosporioides, Carica papaya,* secondary metabolites

1. Introduction

Since Nigeria is rich in plant biodiversity, there is need to explore her enormous plant resources, with particular reference to their endophytic microbial communities, for novel biologically active molecules of pharmaceutical, agricultural and industrial importance. Some recent studies have revealed the enormous potentials possessed by endophytes isolated from Nigerian plants, with several interesting pharmacological activities recorded by secondary metabolites expressed by these fungi [1-3-4-6-7-10].

Carica papaya (Caricaceae) also known as papaya, papaw, or pawpaw is indigenous to most tropical regions of the world, including Nigeria. The plant is used traditionally for the treatment of a wide range of ailments including wounds, ulcers, burns, diarrhea, bleeding, haemorrhoids, whooping cough, dysentery, and skin diseases [11-13]. *C. papaya* has been reported to show anti-inflammatory, antihelmintic, anticancer, antimicrobial, wound healing, anti-fertility, abortifacient, diuretic, immunomodulatory, anti-hypertensive and antimalarial activities [12-14-15-17-18-20-21-24]

In our study, we isolated an endophytic fungus from *C. papaya* and screened its secondary metabolites for biological activities. We also identified some of the constituents of the fungal culture extract using HPLC analysis.

2. Materials and Methods

2.1 Isolation and Identification of Endophytic Fungi

Fresh leaves of *C. papaya* were collected from Agulu, Anambra State, and South-Eastern Nigeria. Isolation of endophytic fungus from leaves of *C. papaya* was carried out as previously

Described [10]. Taxonomic identification of the endophytic fungus was achieved by DNA amplification, sequencing of the fungal ITS region [25].

2.2 Fermentation of Endophytic Fungi, Extraction and isolation of metabolites

Solid state fermentation was carried out by growing the fungus in 1 L Erlenmeyer flasks containing sterile solid rice medium (100 g of rice + 100 mL of distilled water, autoclaved at 121 ºC at 15 psi for 1 h) under static conditions at 22 °C for 14 days. At the end of fermentation, the fungal cultures were extracted with ethyl acetate and the crude extract concentrated under reduced pressure. The fungal crude extract was then fractionated by vacuum liquid chromatography (VLC) using silica gel (200-400 mesh size). Stepwise gradient elution with a non-polar solvent (dichloromethane) and subsequent increase in the amounts of a polar solvent (methanol) gave successive fractions (ECB4, ECB5, and ECB8).

2.3 Antimicrobial Assay

Preliminary antimicrobial screening of the endophytic fungal extract and fractions was carried out using the agar well diffusion assay method as described by Akpotu *et al.* [8]. A stock concentration of 4 mg/mL of the fungal extract was prepared by dissolving the extract in dimethyl sulphoxide (DMSO). The stock solution was further diluted in a 2-fold serial dilution process to obtain 2, 1, and 0.5 mg/mL. Standardized broth cultures of test bacterial isolates (*S. aureus, B. subtilis, S. pneumoniae, P. aeruginosa, E. coli,* and *K. pneumoniae*) and fungal isolates (*Aspergillus niger* and *Candida albicans*) were spread aseptically onto the surface of Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) plates respectively by using sterile cotton swabs. All culture plates were allowed to dry for about 5 min and agar wells were made by using a sterile cork-borer (6 mm in diameter). These wells were respectively filled with 20 µL of the fungal extract and controls. The plates were then kept at room temperature for 1 h to allow the agents to diffuse into the agar medium and incubated accordingly. Ciprofloxacin (5 µg/mL) and miconazole (50 µg/mL) were used as positive controls in the antibacterial and antifungal evaluations respectively; while DMSO was used as the negative control. The MHA plates were then incubated at 37 ºC for 24 h, and the SDA plates were incubated at 25-27 ºC for 2-3 days. The inhibition zones diameters (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 conducted in triplicate and the mean IZDs calculated.

2.4 Cytotoxicity Assay against Ovarian Cancer Cell Line A2780

MTT assay was performed following the method described by Mueller *et al.* [26] and Engelke *et al.* [27]. A2780 cells were cultivated in RPMI-1640 medium supplemented with 10% FBS, 120 µg/ml streptomycin and 120 U/ml penicillin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. The cisplatin (cDDP)-resistant sub clone A2780CisR was obtained by intermittent treatment of A2780 cells with cDDP for 24 weekly cycles. The cell types A2780Resv and A2780Ellag were generated by weekly cDDP treatment (IC50) of A2780 cells for 26 cycles in permanent presence of 10 µM RV or 3.2 µM EA. Furthermore, the cDDP-resistant cell line A2780CisR was permanently cultivated with 10 µM EA or 10 µM RV for 6 months, respectively. This treatment generated the cell lines termed A2780CisR+Ellag and A2780CisR+Resv. Cells were grown to 80-90% confluence, washed with 1x PBS, and treated with trypsin- EDTA before subculturing.

In the MTT assay, cells were plated into 96-well microtiter plates (approximately 9,000 cells/well) and pre-incubated with growth medium overnight. Then, cells were incubated with increasing concentrations of test compounds for 72 h. After 72 h, 25 µL of a solution of MTT were added to each well. After approximately 10 min, formazan crystals occurred, and medium was removed. Formazan crystals were then dissolved in 75 µl DMSO. Absorption was measured at 544 nm (test wavelength) and 690 nm (reference wavelength) using the BMG FLUO star (BMG Lab technologies, Offenburg, Germany). Absorption of the reference wavelength was subtracted from the absorption of the test wavelength.

2.5 Antioxidant Assay

The antioxidant activity of the fungal extract was carried out using the 1, 1-diphenyl-2-picryl-hydraxyl (DPPH) free radical assay as described by Sheen *et al.* [28], but with modification. A concentration of 100 µg/mL of the fungal extract and control (ascorbic acid) were prepared in methanol. A 0.1 mM solution of DPPH was also prepared in methanol. The samples of the fungal metabolites were reacted with the stable DPPH free radical in a methanol 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 radical solution. Absorbance was measured at 490 nm wave length after 30 min of incubation in the dark, using a UV/VIS spectrophotometer. The 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.

$$\text{DPPH scavenging effect (\% inhibition)} = \frac{\text{Abs of blank } (A_0) - \text{Absorbance of sample } (A_1)}{\text{Abs of blank } (A_0)} X \frac{100}{1}$$

2.6 High performance liquid chromatography (HPLC)

HPLC analysis of the fractions was carried out as described by Eze *et al.* [10] using a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany) and a linear gradient of Nandpuri 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 samples were analyzed by comparing with those in the HPLC-UV/Vis database.

3. Results

An endophytic fungus was isolated from the leaves *C. papaya* and was identified *Colletotrichum gloeosporioides*. The fungal DNA sequence data was deposited in the NCBI database (Gen Bank) with accession number MH333065.

The crude ethyl acetate extract from solid state fermentation of the endophytic fungus was subjected to antimicrobial, cytotoxicity and antioxidant assays. The results of the preliminary antimicrobial screening revealed that at the concentrations analysed (1-4 mg/mL), *C. gloeosporioides* extract showed antibacterial activity only against *K. pneumoniae* with inhibition zone diameters (IZDs) ranging from 1-5 mm. *C. gloeosporioides* extract showed no antifungal activity against the fungal test isolates (Table 1).

The results of the cytotoxicity assay showed that at 100 µg/mL, the crude extract showed fair cytotoxic activity against the ovarian cancer cell lines 2780 (sens) and 2780 (CisR) with growth inhibitions of 35.42 and 15.7 % respectively (Table 2). In the DPPH antioxidant assay, at 500 µg/mL, the crude extract of *C. gloeosporioides* displayed poor DPPH scavenging activity with an inhibition of 28.2% (Table 3).

Identification of the constituents of *C. gloeosporioides* crude extract was achieved using HPLC analysis. The HPLC chromatograms of the VLC fractions (ECB4, ECB5, and ECB8) of *C. gloeosporioides* extract showing the detected compounds, their UV spectra and structures are presented in Figures 1-3.

Table 1: Result of Antimicrobial Assay of *C. gloeosporioides* Crude Extract

Test Organisms	Mean Inhibition Zone Diameters (IZDs)(mm)					
	Concentration (mg/mL)				Positive control	Negative control
	4	2	1	0.5	Ciprofloxacin (5 µg/mL)	DMSO
S. aureus	0	0	0	0	6	0
B. subtilis	0	0	0	0	8	0
S. pneumoniae	0	0	0	0	10	0
P. aeruginosa	0	0	0	0	4	0
E. coli	0	0	0	0	24	0
K. pneumoniae	5	3	1	0	8	0
					Miconazole (50 µg/mL)	DMSO
C. albicans	0	0	0	0	16	0
A. niger	0	0	0	0	8	0

Table 2: Result of DPPH Antioxidant Assay of *C. gloeosporioides* Crude Extract

Samples	Concentration (µg/ml)	% Inhibition
C. gloeosporioides extract	500	28.2
Quercetin (control)	500	91.7

Table 3: Result of Cytotoxicity Assay of *C. gloeosporioides* Extract on Ovarian Cancer Cell Lines

Ovarian Cancer Cell Lines	Concentration (µg/mL)	Growth inhibition (%)
2780 sens	100	35.42±13.32
2780 CisR	100	15.7±7.02

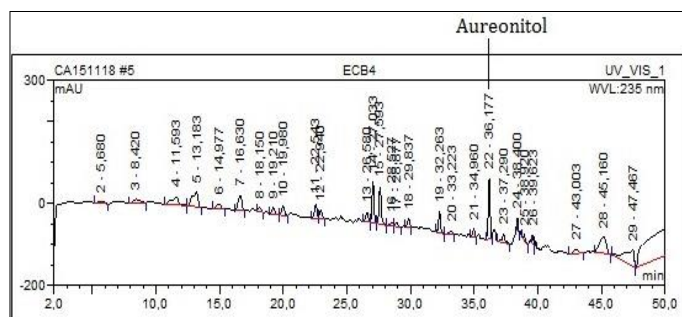


Fig (A)

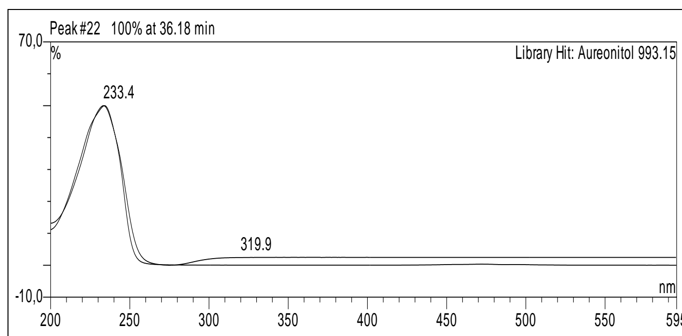
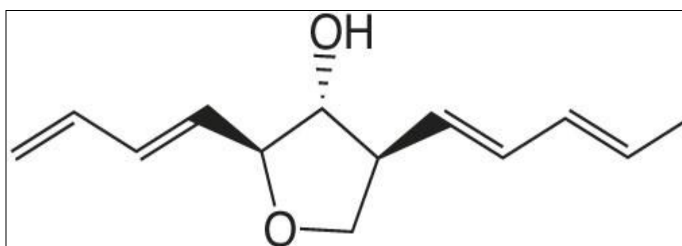


Fig (B)



Aureonitol $C_{13}H_{18}O_2$. 206.28 g/mol

Fig 1(ABC): HPLC chromatogram of fraction ECB4 of *C. gloeosporioides* extract showing the compound Aureonitol, its UV spectrum and structure

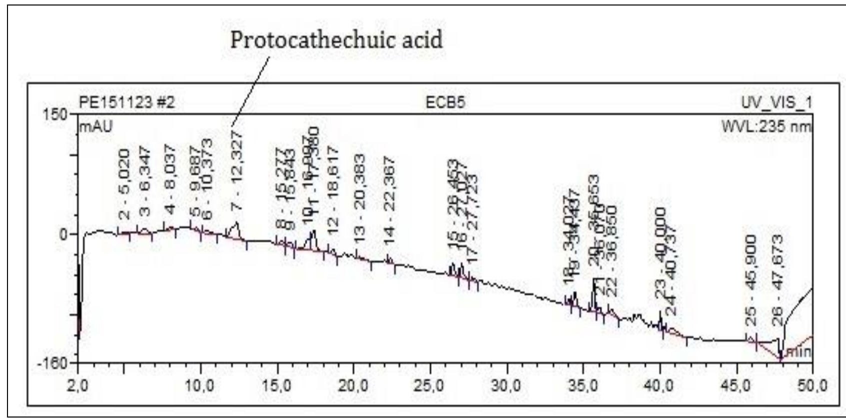


Fig (A)

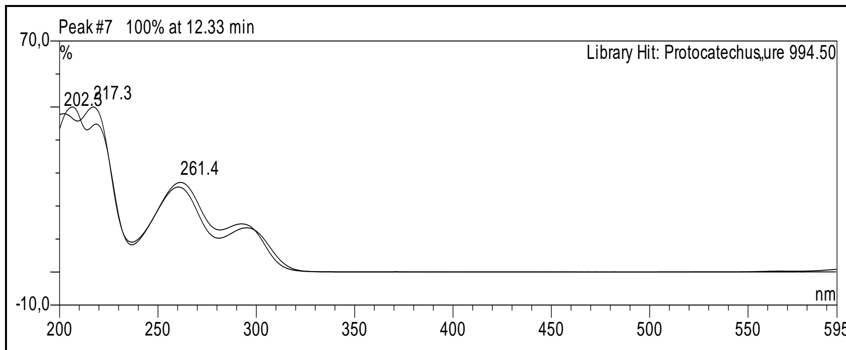
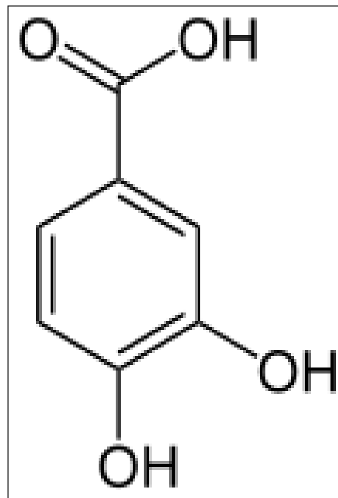


Fig (B)



Protocathechuic Acid $C_7 H_6 O_4$: 154.12 g/mol

Fig 2 (ABC): HPLC chromatogram of fraction ECB5 of *C. gloeosporioides* extract showing the compound protocathechuic acid, its UV spectrum and structure

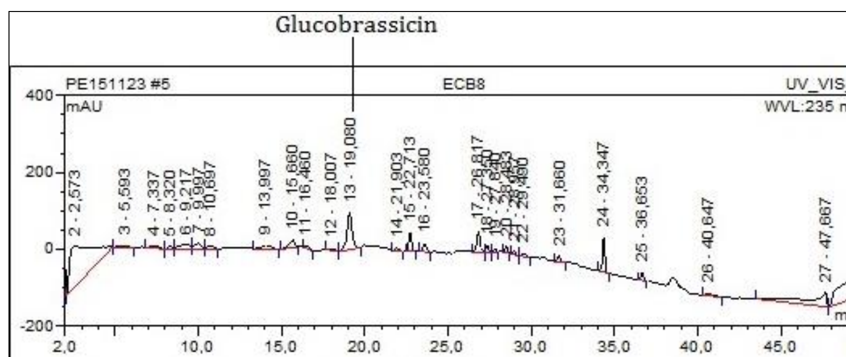


Fig (A)

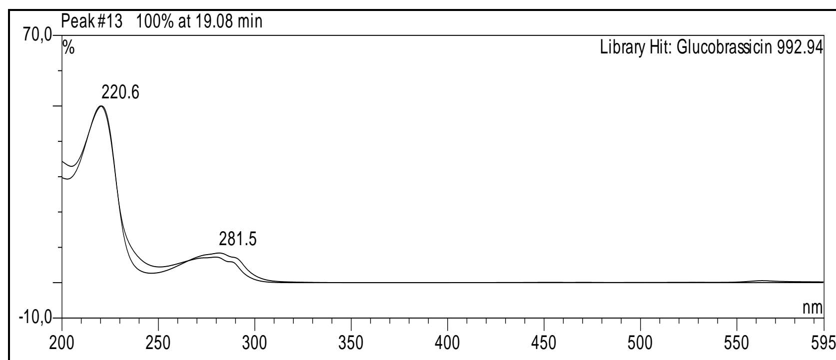
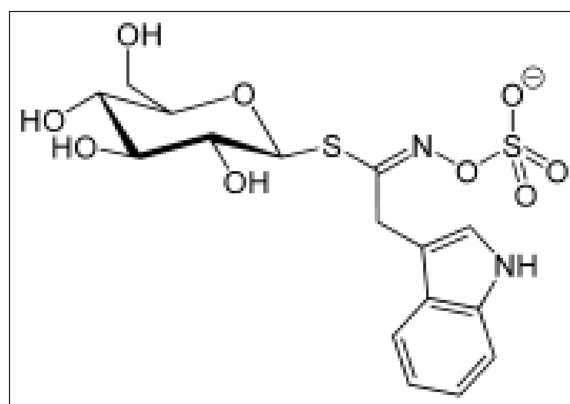


Fig (B)



Glucobrassicin $C_{16}H_{19}N_2O_9S_2^-$. 447.46 g/mol

Fig 3: HPLC chromatogram of fraction ECB8 of *C. gloeosporioides* extract showing the compound glucobrassicin, its UV spectrum and structure

4. Discussion

C. gloeosporioides (anamorph), also known as *Glomerella cingulata* (teleomorph) is a plant pathogenic fungus that causes disease on many different hosts and has been reported to cause anthracnose on several tropical plants including papaya [29]. More than 700 species of *Colletotrichum* comprise the genus *Colletotrichum* Corda having similar morphological characteristics which may be endophytic, pathogenic and saprophytic in nature [30]. *C. gloeosporioides* is very frequently isolated as endophytic fungus from the tissues of healthy leaves and branches from a range of plant species [31-33]. Colonies of *C. gloeosporioides* are pale brown or greyish white, consisting of hyaline, septate, branched mycelium. Conidiomata are acervular, separate, composed of hyaline to dark brown septate hyphae. In culture the fungus produces sclerotia, which are dark brown, occasionally setose. Setae are long, brown, septate. Conidiogenous cells are enteroblastic, phialidic, and hyaline. Conidia are hyaline, one celled, straight, cylindrical, and obtuse at apices and measuring 9-24 × 3-4.5 µm [34].

Like in our study, Mello *et al.* [35] reported the isolation of *C. gloeosporioides* from tissues of healthy leaves, branches and fruits of *C. papaya*. Endophytic *C. gloeosporioides* is also reported to be associated with several other medicinal plants. These include *Salacia chinensis, Theobroma cacao*, *Phlogacanthus thyrsiflorus, Justicia gendarussa*, *Virola michelli* and *Piper nigrum* [36-38-39-40].

C. gloeosporioides has been extensively investigated, particularly for the production of secondary metabolites [41]. Devi and Singh [38] reported the antibacterial and antioxidant activity exhibited by crude fermentation extract of *C. gloeosporioides* which was isolated from *Phlogacanthus thyrsiflorus*. Several compounds of pharmaceutical or industrial value have also been isolated from this fungus. Carvalho *et al.* [39] reported on the isolation of sitosterol, stigmasterol, sitostenone, squalene, ergosterol and ergosterol peroxide from *C. gloeosporioides.* Chithra *et al.* [40] reported the production of piperine by *C. gloeosporioides* which was isolated from *Piper nigrum*. The anticancer agent paclitaxel was isolated from *C. gloeosporioides* associated with leaves of *Justicia gendarussa* [34].

In this study, the crude ethyl acetate extract of *C. gloeosporioides* was tested for antimicrobial, cytotoxicity, anti-tubercular, and antioxidant activities. From the results of the bioassay, it was observed that *C. gloeosporioides* extract exhibited antibacterial activity against the Gram-negative bacterium *K. pneumoniae* (Table 1). At a concentration of 100 µg/mL, the crude extract showed fair cytotoxic activity against cisplatin-sensitive ovarian cancer cell line (2780 sens) and cisplatin-resistant ovarian cancer cell line (2780 CisR) with growth inhibitions of 35.42 and 15.7%, respectively (Table 2). The extract showed a mild antioxidant activity in the DPPH assay with an inhibition of 28.2% at a concentration of 500 µg/mL (Table 3).

From the results of HPLC analysis, three compounds Aureonitol, protocathechuic acid, and glucobrassicin were identified in the crude extract of *C. gloeosporioides.* These compounds are well known to possess diverse biological properties that can be applied pharmaceutically or industrially.

Aureonitol, a tetrahydrofuran (THF) derivative and an antiviral agent, has been isolated from different fungal species of the genus *Chaetomium* [42, 43]. Sacramento *et al.* [43] investigated the anti-influenza activity of the compound and found that it inhibited influenza A and B virus replication. Although it has been demonstrated that other THF derivatives

are endowed with antiviral activity [44-47], the effects of aureonitol on influenza replication have not been characterized. Sacramento *et al*. [43] revealed that aureonitol inhibited influenza replication by targeting conserved residues on the viral hem agglutinin (HA), and that aureonitol inhibited influenza replication at Nano molar concentrations, with very low cytotoxicity. They stated that the compound presented a very safe range to be used *in vitro* and was approximately 100-times more potent than other fungi-derived natural products previously studied against influenza. They indicated that aureonitol chemical structure may be of interest for further development of anti-influenza drugs [43].

Protocatechuic acid (PA) is a type of widely distributed naturally occurring phenolic acid. It is widely distributed and present in most edible and medicinal plants [48-51]. PA is chemically known as 3, 4-dihydroxybenzoic acid and has structural similarity with gallic acid, caffeic acid, vanillic acid, and syringic acid which are well-known antioxidant compounds. PA is a gray to tan solid crystalline powder, with a 221°C melting point and 410°C boiling point at 760 mmHg. It has a mild phenolic odour, sparingly soluble in water (1:50), soluble in alcohol, ether and discolors in air. The compound is generally stable but incompatible with strong oxidizing agents and strong bases. It irritates lungs, eyes, and skin [52]. PA has been reported to show antioxidant, antibacterial, anticancer, anti-ulcer, antidiabetic, anti-ageing, antifibrotic, antiviral, anti-inflammatory, analgesic activity, anti-atherosclerotic, cardiac, hepatoprotective, neurological and nephron-protective activities [53-56,57-60,61-64,65-67]. A significant yield of PA and other phenolic compounds was reported to be achieved when rice bran containing low levels of the compound was fermented by the fungus *Rhizopus oryzae* [68]. PA is reported to be safer at its therapeutic dose of 100 mg/kg. However, further series of studies are required to prove its clinical reliability, safety, and efficacy [52].

Glucobrassicin, an indole glucosinolate, has been isolated from *Brassica* vegetables such as broccoli, cabbage, cauliflower, and kale. Several derivatives of glucobrassicin are known. The compound itself was first isolated from *Brassica* plants, hence the ending of the name. When a second, similar natural product was discovered, it was named Neoglucobrassicin [69,70]. The compound has been reported as a secondary metabolite from a fungus *Alternaria alternata* [71]. Glucobrassicin, like other glucosinolates, has been known to possess anticancer activity [72].

5. Conclusion

The findings of this study revealed the potentials possessed by *C. papaya* as source of endophytes that express biological active compounds. These endophytes hold key of possibilities to the discovery of novel molecules for pharmaceutical, agricultural, and industrial applications.

6. Acknowledgments

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7. Conflicts of Interest

The authors declare no conflicts of interest.

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