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(RESEARCH ARTICLE)

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Antimicrobial activity of secondary metabolites produced by *Colletotrichum* species, an endophytic fungus on *Vernonia amygdalina* Del (fam. Asteraceae)

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Abstract

The emergence of antibiotic-resistant strains of microorganisms calls for research and the development of strategies to curb this menace. The screening of antimicrobial compounds from endophytic fungi is a promising way to meet the increasing threat of drug-resistant. This study aims at evaluating the antimicrobial activity of the endophytic fungal extract of *Colletotrichum* sp. from the leaves of *Vernonia amygdalina (*Del). Endophytic fungal isolation, cultivation, and identification were carried out using standard methods. Subsequently, fermentation and extraction of the secondary metabolites were done on a rice medium. About 1.2g of the fungal extract was subjected to vacuum liquid chromatography (VLC) using gradient-based elution; n-hexane and ethyl acetate followed by dichloromethane and methanol. The high-performance liquid chromatography (HPLC) analysis of the crude extract and VLC fractions were also carried out. Gel permeation chromatography using Sephadex LH-20 was carried out on Fraction JT2. Antimicrobial Assays for fraction JT4 and the Sephadex LH-20 fractions (JT2C – JT2E) were also carried out. The results were analyzed using Statistical Package for Social Sciences. The high-performance liquid chromatography (HPLC) analysis of the crude extract and VLC fractions revealed the presence of the five bioactive metabolites (acropyrone, beauvericin, indole-3-carbaldehyde, indolyl-3-acetic acid, and rocaglamid A). The result of the antimicrobial activity of the VLC fraction, JT4, showed that the fraction exhibited a significant inhibitory effect against *Bacillus subtilis, Pseudomonas aeruginosa* at 0.25 – 1 mg/ml (IZD) and antifungal activity against *Aspergillus niger* only at 0.0625 – 1 mg/ml (IZD). The result of the antimicrobial activity of the gel chromatographic fractions revealed a significant effect against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* at 0.0625 – 1 mg/ml and had no inhibitory effect against *Bacillus subtilis, Aspergillus niger* and *Candida albicans.* The results of this study show that endophytic fungi on *V. amygdalina* could be a potential source of bioactive compounds for the development of potential and effective antibiotics.

Keywords: Antimicrobial activity; HPLC-DAD; Endophyte; Fungi; Chromatography

1. Introduction

Plants parts have been a source of remedy from time immemorial [1], [2] and active compounds from medicinal plants are preferred as drug candidates to conventional medicine because of their efficiency, cultural acceptability and little or no side effects [3]. Endophytic organisms have received considerable attention due to their ability to protect their host against insects, pests, pathogens, and even domestic herbivores [4], [5] and almost all plant species harbor one or more

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endophytic organisms [6]. *Vernonia amygdalina* (Del.) is a tropical plant belonging to the family Asteraceae and is used widely as a vegetable and medicinal plant. Extracts of the bitter leaf have been reported to exhibit antimicrobial activity against drug-resistant microorganisms and possess antioxidant, anticancer, antiviral, anthelminthic and anti-inflammatory activities [7], [8], [9]. Researchers ([10], [11], [8], [12]) showed that both aqueous, ethanol and 60% methanol extracts of the leaves exhibited antibacterial activity against pathogenic organisms. Also reported medicinal properties of *V. amygdalina* include antioxidant activity [13], [14], anticancer/tumor properties [15], [16], [17] [18], hepatoprotective and nephron – protective properties [19], oxytocic property [20] [21] and pesticidal property [22], [23]. Ethno-botanically, the leaves are particularly utilized in the treatment of malaria, diabetes mellitus, venereal diseases, wounds, hepatitis and cancer [24], [25], [26], [27]. *Colletotrichum gloeosporioides* is a pathogenic fungus of the Cashew tree, but it was also found as an endophyte in many cases [28]. *Colletotrichum fulcatum* is another species of clinical interest [29]. An antimicrobial tridepside, colletotric acid, has been obtained from *Colletotrichum gloeosporioides,* an endophytic fungus isolated from *Artemisia mongolica,* which displayed antibacterial activity against bacteria as well as against the fungus *Helminthsporium sativum* [30].

2. Materials and methods

2.1. Materials

2.1.1. Plant Material

The collection and authentication of Fresh and healthy leaves of *Vernonia amygdalina were* described in our previous report [27]

2.1.2. Test Organism

The cultures were maintained on Nutrient Agar slants for a period of 48 hours in a refrigerator before they were subcultured into freshly prepared MacConkey Agar, Mannitol salt Agar, Centrimide Agar, Nutrient Agar and Sabouraud Dextrose Agar slants for nutrient replenishment and finally transferred on to the Muller Hinton agar and Sabouraud Dextrose Agar for agar-well diffusion assay method. The bacteria isolates are made up of Gram-positive (*Staphylococcus aureus* and *Bacillus subtillis) and Gram*-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa),* while the fungi isolates are *Aspergillus niger* (mould) and *Candida albicans* (yeast).

2.1.3. Culture Media

All media components and chemicals used in the studies were of analytical grade. Culture media used were Mueller-Hinton Agar (Titan, Biotech) and Sabouraud Dextrose Agar (Oxiod Ltd, UK), Mannitol salt Agar, Centrimide Agar, MacConkey Agar (Oxoid Ltd, UK). Culture media used in this study include fungal isolation and purification medium [Malt Extract Agar (MEA) (Oxiod Ltd, UK)], fermentation medium [Rice medium (Rice-100 g + distilled water- 110 mL of distilled water)]. Culture media were prepared according to the instructions of the manufacturer.

2.1.4. Chemicals, Solvents and Drugs

The chemicals and solvents used for this study were of analytical grade. They include methanol, n-hexane, ethyl acetate, dichloromethane (Sigma Aldrich, Germany), dimethylsulphoxide (DMSO) (Uvasol Merck, Germany), silica gel (mesh size: 200-400) (Merck, Germany), Sephadex LH-20 (Merck, Germany). All laboratory reagents were freshly prepared and freshly distilled water was used as required. ZR fungal/bacterial DNA MiniPrep™ Kit (Zymo ResearchCorp, USA), Hot Star Taq Master Mix Kit (Qiagen, Germany), agarose gel (Biozym LE Agarose, Biozym Scientific GmbH), Zymoclean™gel DNA recovery kit (Zymo Research Corp, USA).

2.2. Methods

2.2.1. Fungal Isolation and Cultivation

Fresh healthy leaves of *V. amygdalina* Del. were rinsed in sterile distilled water and subjected to surface sterilization based on the method described by Petrini and Muller [34]. The surface sterilized leaves were cut into smaller pieces of approximately 2 mm segments from the lamina and placed on Petri dishes containing malt extract agar (MEA) supplemented with chloramphenicol. The plates were kept on laboratory benches at room temperature with ambient light. Periodically, fungal growth from the leaf segments was monitored and hyphal tips from distinct colonies emerging from leaf segments were sub-cultured on fresh MEA plates to obtain pure colonies.

2.2.2. Identification

Taxonomic identification of all fungal strains was achieved by DNA amplification and sequencing of the fungal ITS region. Total fungal genomic DNA was extracted and purified directly from fresh, axenic mycelia using fungal DNA extraction and purification kits. DNA amplification by PCR was performed using fungal Hot Star Tag Master Mix Taq polymerase and the primer pair ITS 1 and ITS4 in a thermocycler. The polymerase chain reaction (PCR) products were visualized on a 1% agarose gel, and amplified DNAs were recovered from the gel using appropriate kits. Fungal DNAs were sequenced and resulting sequences were subjected to BLAST searches of the NCBI GenBank database for identification.

2.2.3. Fermentation and Extraction of Metabolites

Solid state fermentation was carried out in Erlenmeyer flasks containing Rice Medium (i.e., 100g of rice +110 mL of distilled water), which was autoclaved at 121°C at 15 psi for 1 hour and allowed to cool. The flasks were inoculated with 3 mm diameter agar blocks containing test fungi and incubated at 22°C for 21 days and extracted exhaustively with ethyl acetate. The organic phase was then vacuum-concentrated using the rotary evaporator at 40°C to obtain the concentrated extracts.

2.2.4. Vacuum Liquid Chromatography (VLC) of the Crude Fungi Extract

The column was packed using the protocol described in our previous report [27]. The column was eluted gradually with increasing order of polarity of solvent from non-polar to highly polar solvent system. The column was developed with a gradient mixture of 500 ml of n-hexane and ethyl acetate solvent system in the ratio of 10:0, 9:1, 8:2, 6:4, 4:6, 3:7, 2:8, 0:10 using negative pressure created by the vacuum pump. Furthermore, the column was developed with a gradient mixture of 500 ml of dichloromethane and methanol solvent system in the ratio of 10:0, 9:1, 8:2, 6:4, 4:6, 2:8, and 0:10 to give successive fractions. The column was allowed to run dry after each fraction was collected. The eluate was collected separately with a round bottom flask, evaporated using a rotary evaporator and labeled appropriately.

2.2.5. Analytical High-Pressure Chromatography (HPLC)

The High-Pressure Liquid Chromatography (HPLC) of the crude extract and each of the VLC fractions were carried out following the method described in our previous report [27].

2.2.6. Sephadex LH-20 Separation of Endophytic Fraction JT2

About 10 g of Sephadex LH-20 (0.25-0.1 mm mesh size) as stationary phase was dispersed in a 100 ml of dichloromethane and methanol (1:1 (v/v)) solvent system as mobile phase. The mixture was shaken for 10 min and the slurry was transferred into a glass column (30 cm x 1.5 cm; length x internal diameter). The gel was allowed to stabilize for about 5 hours before use. Approximately 78 mg of fraction JT2 was reconstituted in 2 ml of dichloromethane and methanol (1:1). The solution was sonicated for 5 min and centrifuged at 1000 rpm for 10 min to remove undissolved solid particles. The supernatant was collected using a pipette and used for the separation/purification process. The supernatant was introduced into the Sephadex LH-20 column and adjusted to the flow rate of approximately 1 ml/min. About 24 fractions were collected with the aid of test tubes. After the elution, the fractions were subsequently subjected to thin layer chromatography and antimicrobial assay.

2.2.7. Analytical Thin Layer Chromatography

The fractions in each test tube were spotted with the aid of a capillary tube on an aluminum pre-coated silica gel TLC plate as a stationary phase and allowed to air-drying. The spotted plate was developed in a TLC tank using a dichloromethane: methanol solvent system as the mobile phase. After the development, the plates were allowed to dry and visualized at 254 nm (short UV) and visible region using UV/visible lamb and similar fractions were collected and bulked together before evaporation.

2.3. Antimicrobial Assay

2.3.1. Agar Well Diffusion Assay of Fungi Fractions

Antimicrobial Assays for fraction JT4 and the Sephadex LH-20 fractions (JT2C – JT2E) were carried out using the agar well diffusion assay as described by Aida *et al.* [31] with little modification. The antimicrobial activities of the fractions were tested against four standard clinical bacteria isolates namely: *Staphylococcus aureus, Bacillus subtillis, Pseudomonas aeruginosa* and *Escherichia coli* and two fungi isolate namely *Aspergillus niger* and *Candida albicans.*

Subsequently, 0.5 McFarland standard bacteria and fungi suspensions of each of the test isolates were prepared and these formed the bacteria and fungi stock solutions used in the agar well diffusion assay.

2.3.2. Agar dilution Assay of Fungi Fractions

The media, Mueller-Hinton Agar (MHA) (38 g) and Sabouraud Dextrose Agar (SDA) (65 g) were prepared according to the manufacturer's specification and mixed with 1 L of sterile distilled water and sterilized at 121°C for 15 minutes. The sterile MHA and SDA plates were inoculated with the test culture by seeding method. About 0.1 ml of each of the standardized cultures were transferred into the sterilized MHA and SDA plates; 20 ml of the molten agar that has been cooled to 50°C was added and then mixed thoroughly by swirling clockwise and anticlockwise to ensure even growth of the organisms. This was done to obtain uniformity of the inoculums. Sterile cork was used to make six wells (8 mm in diameter) on each of the MHA and SDA plates. A stock concentration of 1 mg/ml of the samples was prepared by dissolving the extracts in dimethylsulphoxide (DMSO). Aliquots of 80µl of each extract dilution, reconstituted in DMSO at concentrations of 1, 0.5, 0.25, 0.125 and 0.065 mg/ml for each of the extracts were applied in each of the wells in the culture plates previously seeded with the test organisms. Ciprofloxacin (5 µg/ml) and miconazole (50 µg/ml) served as the positive control in the antibacterial and antifungal evaluations respectively, while DMSO was used as the negative control respectively. The culture plates were incubated at 37°C for 24 hours for bacterial and 25±2°C for 48 hours for fungi respectively to allow the growth of microorganisms. The antimicrobial potential for each extract was determined by measuring the zone of inhibition around each well (excluding the diameter of the well). The procedure was conducted in triplicate for each fraction and the results were recorded as the mean of the zone of inhibition in milimetre. The minimum inhibitory concentrations (MIC) of the fungal fractions were determined using the agar dilution method [32]. A stock solution of 1 mg/ml was prepared were prepared by pouring 4 ml of the molten double-strength MHA and SDA, for bacterial and fungal isolates respectively, into the sterile Petri dish containing 1 ml of the various dilutions of the fungal fractions to give the final plate concentrations of 1, 0.5, 0.25, 0.125 and 0.0625 mg/ml. the test isolates which were grown overnight in broth were adjusted to McFarland 0.5 standard and streaked onto the surface of the agar plates containing dilutions of the fungal fractions. The plates were then incubated at 37°C for 24 hours and 25±2°C for 3 days, after which the plates were visually observed for growth. The minimum dilution (concentration) of the fraction that completely inhibits the growth of each test organism was taken as the MIC.

2.4. Statistical Analysis

The results of the antimicrobial assay in triplicate were analyzed with statistical Package for Social Sciences (SPSS) version 16.0 and presented as mean ± standard error of mean (SEM) inhibition zone diameters (IZD) using one-way ANOVA and further subjection to Turkey's post hoc test. Differences between means were accepted at a significance of $p< 0.05$.

3. Results

3.1. Percentage Yields of Fractions from *Colletotrichum* Sp

The result of the percentage yield of the fractions (VLC fractions and Sephadex LH-20 fractions) from *Colletotrichum* sp. are shown in table 1 below.

3.2. Results of the HPLC-DAD Analysis of Crude Extract (VAL3) and Fractions JT1-15

The chromatograms of the crude extract and fractions JT1-15 are shown in Figures 1 and 2 below.

3.3. Results of Antimicrobial Activities of the Fungal Fractions (JT4, JT2C-E) of *Colletotrichum* species

3.3.1. In vitro Antibacterial Studies

The result of the *In-vitro* Antibacterial Studies of VLC fraction (JT4) and Sephadex chromatographic sub-fractions (JT2C, JT2D, and JT2E) of *Colletotrichum* species extract against four standard clinical bacteria isolates: *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli* are shown in tables 2 – 6 below.

3.3.2. In-vitro Antifungal Studies

The antifungal screening studies of JT4 and JT2C, JT2D, and JT2E were performed by the standard agar dilution method against two fungi isolates: *Aspergillus niger* and *Candida albicans* and are as presented in Tables 2-6 below.

Table 1 Percentage Yield of the Vacuum Liquid Chromatography (VLC) and Gel Chromatography (Sephadex LH-20) Fractions

VLC Fractions	**Weight (g)**	**Percentage Yield (%w/w)**
JT1	0.0989	8.242[a]
JT2	0.0779	6.492[a]
JT3	0.0193	1.608[a]
JT4	0.0093	0.775[a]
JT5	0.0101	0.842[a]
JT6	0.0068	0.567[a]
JT7	0.0241	2.008[a]
JT8	0.0055	0.458[a]
JT9	0.0015	0.125[a]
JT10	0.0624	5.200[a]
JT11	0.0010	0.083[a]
JT12	0.0015	0.125[a]
JT13	0.0757	6.308[a]
JT14	0.0553	4.608[a]
JT15	0.0256	2.133[a]
Sephadex LH-20 Fractions	Weight (g)	Percentage Yield (% w/w)
JT2A	0.0011	1.412[b]
JT2B	0.0030	3.851[b]
JT2C	0.0104	13.35[b]
JT2D	0.0088	11.296[b]
JT2E	0.0317	40.693[b]

aPercentage yield calculated from 1.2 g of crude extract; bPercentage yield calculated from 0.0779 g, Sephadex LH-20 (JT2) fraction.

Table 2 Result of the Antimicrobial Activity of JT4 Fungal Fraction

Concentration (mg/ml)/IZD (mm)							
Test Organisms	**1**	**0.5**	**0.25**	**0.125**	**0.0625**	**Ciprofloxacin (50 µg/ml)**	**DMSO**
Staphylococcus aureus	-	-	-	-	-	15.0±0.0	-
Bacillus subtilis	4.5*±0.29	3.0*±0.0	-	-	-	11.0±0.0	-
Escherichia coli	-	-	-	-	-	13.0±0.0	-
Pseudomonas aeruginosa	4.0*±0.0	2.5*±0.25	2.0±0.0	-	-	14.0±0.0	-
						Miconazole (50 µg/ml)	DMSO
Aspergillus niger	10.0*±0.0	6.0*±0.0	5.0*±0.0	4.0*±0.00	2.0*±0.0	23.0±0.0	-
Candida albicans	-	-	-	-	-	21.0±0.0	-

All values are presented as Mean±SEM, n = 3, * = $p < 0.05$ compared to negative control (One-way ANOVA; Tukey's post hoc), - = No Inhibition, IZD = Inhibition zone diameter, JT4 = n-Hexane: ethyl acetate (4:6) fraction.

Table 3 Result of the antimicrobial activity of JT2C Fungal Fraction

Concentration (mg/ml)/IZD (mm)							
Test Organisms	**1**	**0.5**	**0.25**	**0.125**	**0.0625**	**Ciprofloxacin (50 µg/ml)**	**DMSO**
Staphylococcus aureus	3.5*±0.29	1.0±0.58	-	-	-	7.0*±0.0	-
Bacillus subtilis	-	-	-	-	-	11.0±0.0	-
Escherichia coli	4.0±0.0	-	-	-	-	19.0±0.0	-
Pseudomonas aeruginosa	6.0*±0.0	2.0±1.0	-	-	-	16.0*±0.0	-
						Miconazole (50 µg/ml)	DMSO
Aspergillus niger	-	-	-	-	-	23.0±0.0	-
Candida albicans	-	-	-	-	-	21.0±0.0	-

All values are presented as Mean±SEM, n = 3, * = $p < 0.05$ compared to negative control (One-way ANOVA; Tukey's post hoc), - = No Inhibition, IZD = Inhibition zone diameter, JT2C = Sephadex LH-20 sub-fraction of JT2

Table 4 Result of the Antimicrobial Activity of JT2D Fungal Fraction

Concentration (mg/ml)/IZD (mm)							
Test Organisms	**1**	**0.5**	**0.25**	**0.125**	**0.0625**	**Ciprofloxacin (50 µg/ml)**	**DMSO**
Staphylococcus aureus	5.0 ±0.0	4.0 ±0.0	-	-	-	7.0±0.0	-
Bacillus subtilis	-	-	-	-	-	11.0±0.0	-
Escherichia coli	-	-	-	-	-	19.0±0.0	-
Pseudomonas aeruginosa	4.0 ±0.0	-	-	-	-	16.0±0.0	-
						Miconazole (50 µg/ml)	DMSO
Aspergillus niger	-	-	-	-	-	23.0±0.0	-
Candida albicans	-	-	-	-	-	21.0±0.0	-

All values are presented as Mean ± SEM, n = 3, * = $p < 0.05$ compared to negative control (One-way ANOVA; Tukey's post hoc), - = No Inhibition, IZD = Inhibition zone diameter, JT2D= Sephadex LH-20 sub-fraction of JT2.

Table 5 Result of the Antimicrobial Activity of JT2E Fungal Fraction

Concentration (mg/ml)/IZD (mm)							
Test Organisms	**1**	**0.5**	**0.25**	**0.125**	**0.0625**	**Ciprofloxacin (50 µg/ml)**	**DMSO**
Staphylococcus aureus	4.0* ±0.0	2.5* ±0.26	-	-	-	7.0*±0.0	-
Bacillus subtilis	-	-	-	-	-	11.0±0.0	-
Escherichia coli	2.0 ±0.0	-	-	-	-	19.0±0.0	-
Pseudomonas aeruginosa	8.0* ±0.58	7.0* ±0.58	5.5* ±0.25	5.5* ±0.23	4.5* ±0.17	16.0±0.0	-
						Miconazole (50 µg/ml)	DMSO
Aspergillus niger	-	-	-	-	-	23.0±0.0	-
Candida albicans	-	-	-	-	-	21.0±0.0	-

All values are presented as Mean±SEM, n = 3, * = $p < 0.05$ compared to negative control (One-way ANOVA; Tukey's post hoc), - = No Inhibition, IZD = Inhibition zone diameter, JT2E= Sephadex LH-20 sub-fraction of JT2.

Table 6 Result of the Minimum Inhibitory Concentrations of Fungal Fractions against Test Organisms

	MICs (mg/ml)			
Test organisms	**JT2C**	**JT2D**	**JT2E**	**JT4**
Staphylococcus aureus	0.5	0.5	0.5	-
Bacillus subtilis	-	-	-	0.5
Escherichia coli	1.0	-	1.0	-
Pseudomonas aeruginosa	0.5	1.0	-	0.125
Aspergillus niger	-	-	-	-
Candida albicans	-	-	-	-

MIC = Minimum Inhibitory Concentration

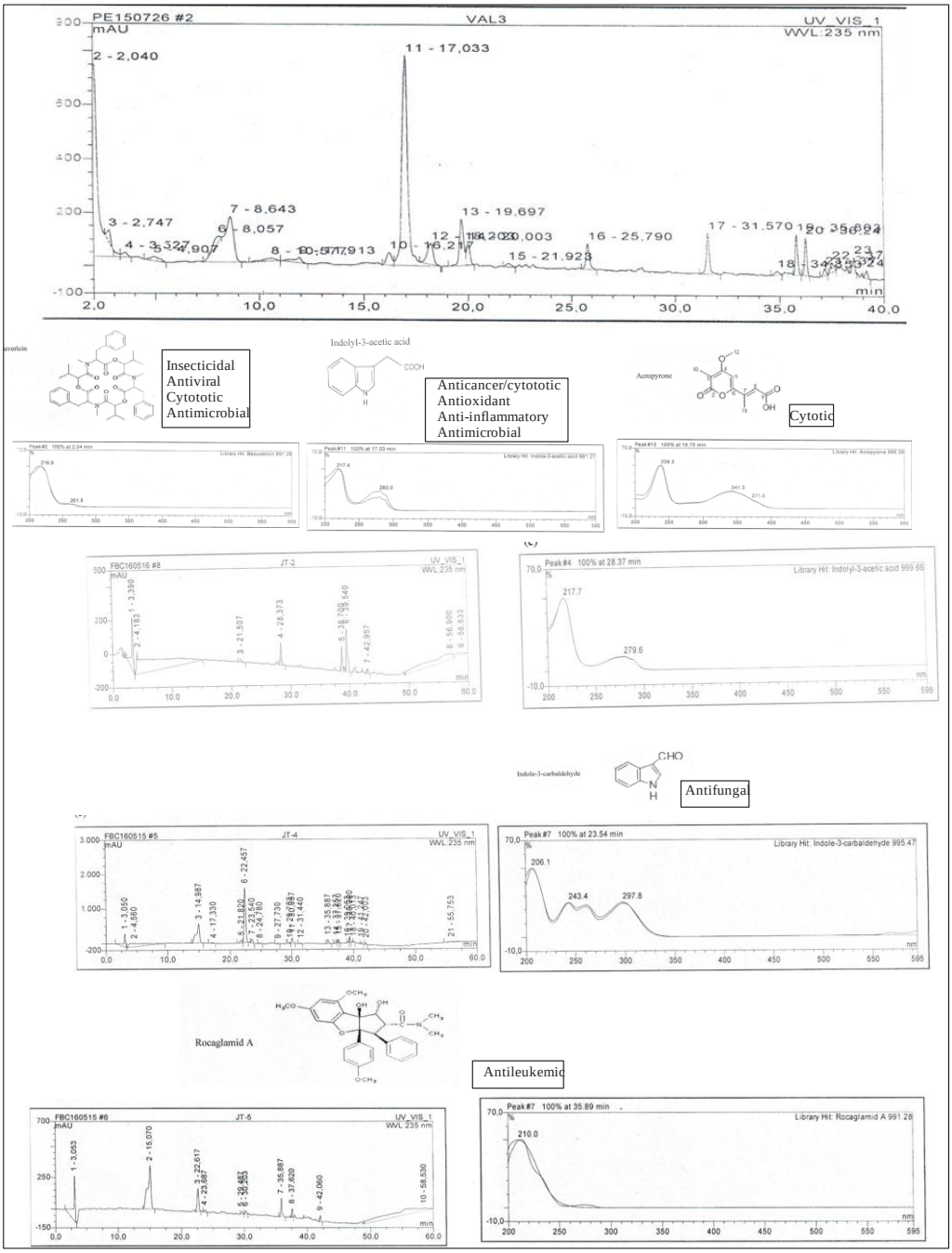

Figure 1 HPLC Chromatogram and ultraviolent spectra showing detected compounds and some reported bioactivities

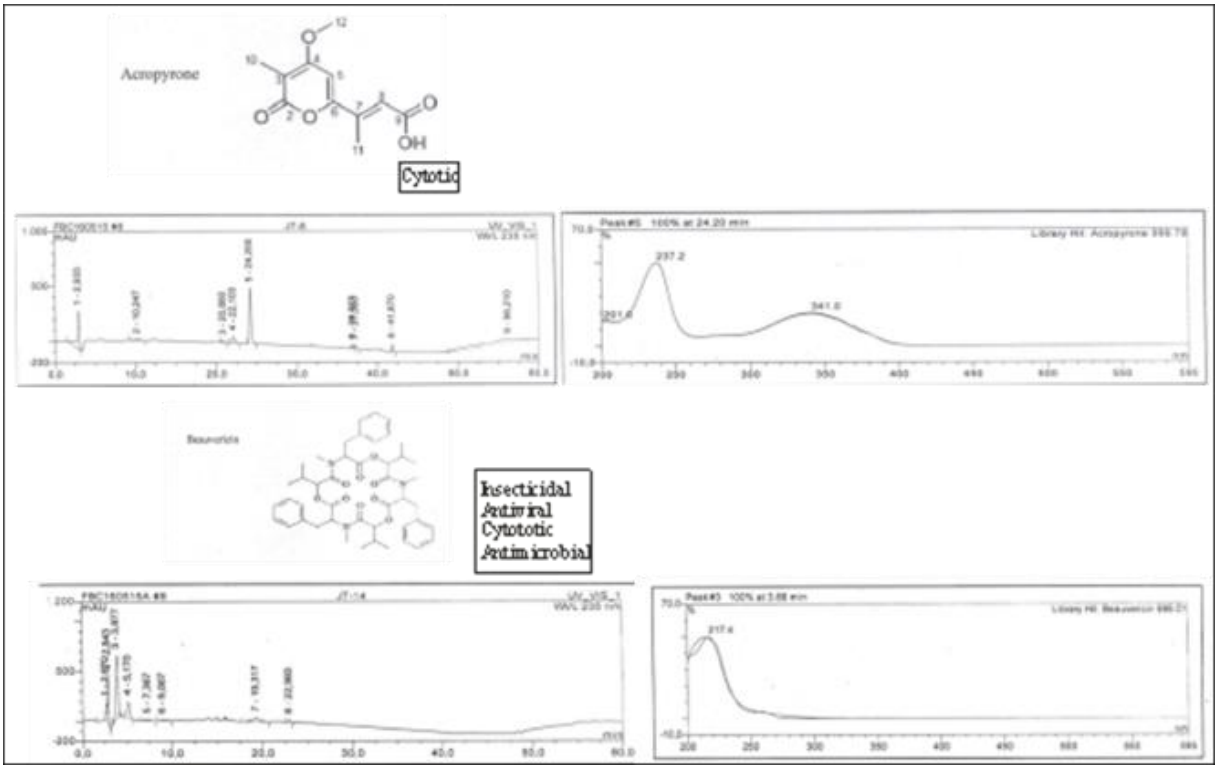

Figure 2 HPLC Chromatogram and ultraviolent spectra showing detected compounds and some reported bioactivities

4. Discussion

From the *In vitro* Antibacterial Studies of Fractions (JT4, JT2C-E), the minimum inhibitory concentration (MIC) calculated for fraction JT4 was 0.5 mg/ml and 0.25 mg/ml against *Bacillus subtilis and Pseudomonas aeruginosa respectively*. Also, the MIC calculated for fraction JT2C were 0.5, 0.5 and 0.25 mg/ml against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* respectively. The MIC calculated for fraction JT2D was 0.5 mg/ml and 1.0 mg/ml for Staphylococcus *aureus* and *Pseudomonas aeruginosa respectively* and 0.25, 0.5 and 0.0625 mg/ml against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* for fraction JT2E respectively. This suggests that the fraction exhibited broad-spectrum antimicrobial activities. From the result of the *In-vitro* Antifungal Studies, The MIC value calculated was 0.0625 mg/ml against *Aspergillus niger* for fraction JT4 only. The data obtained from the antimicrobial activities of fraction JT4 showed that the fungi extract exhibited a significant effect against *Bacillus subtilis, and Pseudomonas aeruginosa* and antifungal activity against *Aspergillus niger* only. *Bacillus subtilis* was the most sensitive organism to the JT4 fraction of *Colletotrichum* species extract of *V. amygdalina* with the zone of inhibition of 4.5 mm (MIC of 0.25 mg/ml) and the least sensitive organism to the same fungi extract was *Pseudomonas aeruginosa* with the zone of inhibition of 4.0 mm (MIC of 0.125 mg/ml) at the same concentration (1 mg/ml) respectively. From the HPLC-DAD Analysis result, five bioactive metabolites with known antimicrobial, anticancer, antioxidant, anti-inflammatory, antiviral, antileukemic, and insecticidal activities were revealed. The VLC fraction, JT2 revealed indolyl-3-acetic acid with a retention time of 28.37 min while the indole derivative with a retention time of 17.03 min was also identified in the crude extract. A previous report [33] isolated indolyl-3-acetic acid (IAA), an indole derivative containing a carboxymethyl substituent from the terrestrial *Streptomyces baarnensis,* MH4. Activities reported for the indole derivative Includes anti-inflammatory [34], antioxidant, anticancer [35], [36] and antimicrobial properties [37]. The fraction, JT4, of the VLC fractions revealed indole-3-carbaldehyde with a retention time of 23.54 min previously isolated from a marine sponge, *Halichondria* species [38] and from the terrestrial *Streptomyces baanensis,* MH4 [33]. Li *et al.* [38] reported the antifungal activity of Indole-3-carbaldehyde against *Mortierella ramannianus.* From the VLC fraction (JT5), Rocaglamid A with a retention time of 35.89 min was identified. Rocaglamid A is a flavagline that was reported by King et al. [39] as a novel antileukemic 1H-cyclopenta[b]benzofuran isolated from *Aglaia elliptifolia.* Janprasert et al. [40] reported Rocaglamid as a highly substituted benzofuran isolated and identified it as the active insecticidal constituent in the twigs of the Chinese rice flower bush, *Aglaia odorata*. Also, previous research [41] stated that Rocaglamid is a potent natural anti-cancer phytochemical that inhibits cancer growth at nanomolar concentrations. However, the compound Rocaglamid A isolated was previously isolated from *Guignardia* species from *Undaria pinnatifida,* a type of seaweed in Changdao Sea [42] and *Neosartorya* species of mature plants *Cyperus malaccensis* that dominates about one-

third of the areas of estuaries and mangroves [43]. The VLC fraction, JT8 revealed acropyrone with a retention time of 24.20 min, which was also identified in the crude extract with a retention time of 19.70. Acropyrone is an acetophenone dimer and a polyketide derivative that was previously isolated from *Acronychia pedunculata* and reported to exhibit cytotoxic activity [44], [45]. This study however is the first report of isolation of acropyrone from the endophytic fungi, *Colletotrichum* species on leaves of *Vernonia amygdalina* Del. The crude extract and VLC fraction, JT14 revealed Beauvericin with retention times of 2.04 and 3.88 min respectively. Beauvericin is a cyclic hexadepsipeptide that belongs to the enniatin antibiotic family. Beauvericin is a famous mycotoxin produced by many fungi, such as *Beaveria bassiana.* The antimicrobial activity of the Sephadex (JT2C, JT2D and JT2E) fractions against the tested pathogenic organisms showed that they are effective against *Staphylococcus aureus* at 1 – 0.5 mg/ml, *Escherichia coli* at 1 mg/ml and *Pseudomonas aeruginosa* at 1 – 0.0625 mg/ml of minimum inhibitory concentration. Fraction JT2C was moderately active against the bacterial strains of *Staphylococcus aureus, Pseudomonas aeruginosa* strains only as shown in tables 3 and 6. Additionally, fraction JT2E showed pronounced efficacies, particularly against the human pathogenic bacterial strains of *Pseudomonas aeruginosa* with a maximum inhibition zone of 8.0 mm, 4.0mm against *Staphylococcus aureus* and 2.0 mm against *Escherichia coli* at 1 mg/ml. The activities of the fractions are concentration dependent which shows that increase in concentration will give rise to a wider zone of inhibition. However, the results showed that all the Sephadex fractions tested had no inhibitory effect against *Bacillus subtilis, Aspergillus niger* and *Candida albicans.* The values of the minimum inhibitory concentrations of the fungal fractions on the test organism evaluated were observed to range from 0.0625 – 0.25 mg/ml. Fraction JT2C recorded MIC of 0.5, 1.0 and 0.5 mg/ml against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* respectively while fraction JT2D had an MIC of 0.5 and 1.0 mg/ml for *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively. The MIC values of fraction JT2E against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* were 0.5 and 1.0 mg/ml respectively. MIC values were not recorded against *Bacillus subtilis, Aspergillus niger* and *Candida albicans* for all the fungal Sephadex fractions. The failure of the tested fractions to inhibit some of these microorganisms may be due to inactivation which is a mechanism by which microorganisms may circumvent the inhibitory action of antimicrobial agents. Similarly, it has been reported that metabolites of the endophytic fungus, *Colletotrichum* species had strong antimicrobial activity [46]. Developing countries are usually faced with a problem of fungal infestation and they cause disease in man, plants and animals [47]. Akinpelu [8] has reported that a 60% methanolic extract of the leaves of *V. amygdalina* had no effect against *Candida albicans* – a popular opportunistic pathogen of humans while Erasto, *et al.* [48] reported the activity of *V. amygdalina* against the fungi, *Aspergillus niger.* The result of this work showed inhibitory activity against both Gram-positive and Gram-negative bacteria and fungi indicating its broad spectrum of activity. From the results, it can be inferred that there is a high concentration of an active principle in the extracts of *Colletotrichum* species which also is in concordance with the HPLC analysis which revealed indole-3-carbaldehyde and indolyl-3-acetic acid known for their antifungal and antimicrobial activities respectively [37], [38]. According to a previous report [49], naturally found alkaloids have antimicrobial properties, and the indole derivatives (known to be a plant hormone) could be responsible for the antimicrobial activities of the plant. In addition, since the fungi endophyte was isolated from the leaves of *V. amygdalina* Del, it is suggestive of the fact that it provides some form of metabolite-mediated chemical defense to the host plant against invading pathogens. Studies [50], [51] implicated *S. aureus, E. coli* and *P. aeruginosa* as the leading causative agents of community infections which makes this study an important treatment option for infections caused by these organisms. Hence, justify the use of *V. amygdalina* in the treatment of wounds and some gastrointestinal problems in traditional medicine. The insecticidal activity of beauvericin was first reported by Hamill *et al.* [52] and subsequently, its insecticidal effect on a microgram level was investigated on *Calliphora erythrocephala, Aedes aegypti, Lygus* spp., *Spodoptera frugiperda* and *Schizaphis graminum* [53]. The cytotoxicity of beauvericin to human leukemia cells has been reported [54]. According to Shin *et al.* [55], beauvericin is the most effective inhibitor of the cyclic hexadepsipeptides (that inhibit HIV-1 integrase). Beauvericin has strong antibacterial activity against human, animal and plant pathogenic bacteria [56], with no selectivity between Gram-positive and Gram-negative bacteria and lacks antifungal activity. However, Zhang *et al.* [57] and Fukuda *et al.* [58] reported the antifungal activity of beauvericin in combination with ketoconazole or miconazole which may indicate its ability to enhance the activity of the azoles.

5. Conclusion

Natural sources have yielded many important new pharmaceuticals and utilizable products for a wide range of applications in the past and continue to be one of the most important sources of pharmacologically active compounds in the quest for drugs against life-threatening diseases on the leaves of *Vernonia amygdalina* Del., a medicinal, plant from Nigeria include acropyrone (cytotoxic activity), beauvericin (antimicrobial, antiviral, cytotoxic and insecticidal activities), indole-3-carbaldehyde (antifungal activity), indolyl-3-acetic acid (antimicrobial, anti-inflammatory, antioxidant, and cytotoxic activities), and rocaglamid A (antileukemic, cytotoxic, and insecticidal activities). In this study, we observed that the endophytic fungi, *Colletotrichum* spp., isolated from *Vernonia amygdalina,* produce some metabolites (fractions JT2C-E and JT4) that possess antimicrobial potentials, especially against human pathogenic bacterial strains and this, however, this can justify the use of the *Vernonia amygdalina* leaf in traditional medicine

practice as a therapeutic agent and can explain the traditional use of this plant in the treatment of a range of infections caused by these microorganisms. The present findings indicate that the antimicrobial properties of endophytic fungi associated with medicinal plants are considered a potential source of diverse bioactive metabolites that can be exploited for the development of new and effective antibiotics.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript. We certify that the submission is original work and is not under review at any other publication.

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