Secondary metabolites of mangrove-derived endophytic fungus, Pseudopestalotiopsis species investigated for antimicrobial and antioxidant activities

Virginia N. Ajah[1], Chukwubuikem C. Okolo[1], Ugochukwu M. Okezie[2], Cletus Anes Ukwubile[3], Anita Kelechi Asekunowo[4], Blessing Ogechukwu Umeokoli*[1], Festus B.C. Okoye[1]

[1]Nnamdi Azikiwe University, Faculty of Pharmaceutical Sciences, Department of Pharmaceutical and Medicinal Chemistry, Awka, Nigeria.
[2]Nnamdi Azikiwe University, Faculty of Pharmaceutical Sciences, Department of Pharmaceutical Microbiology and Biotechnology, Awka, Nigeria.
[3]University of Maiduguri, Faculty of Pharmacy, Department of Pharmacognosy, Maiduguri, Nigeria.
[4]University of Lagos, Faculty of Science, Department of Chemistry, Yaba, Nigeria.

Submitted: 4th Sept., 2023; Accepted: 10th Oct., 2023; Published online: 31st Oct., 2023
DOI: https://doi.org/10.54117/jcbr.v3i5.2
**Corresponding Author: Blessing Umeokoli; bo.umeokoli@unizik.edu.ng*

Abstract

Attention is being diverted toward the bioprospecting of newer bioactive compounds from marine endophytic fungi. This is because marine fungi have shown a large chemo-diversity of untapped important secondary metabolites needed for drug development. In the present study, the secondary metabolites of a mangrove-derived endophytic fungus *Pseudopestalotiopsis species* isolated from the root of *Rhizophora racemosa* were investigated for antimicrobial and antioxidant activities. The fungal isolation, taxonomic identification, fermentation, extraction, and isolation of the fungal secondary metabolites were carried out using standard techniques. The fermentation product was subjected to fractionation. The crude extract and its fractions were screened for antimicrobial and antioxidant assays. The active extracts and fractions exhibited good antimicrobial activities against *Staphylococcus aureus*, *Bacillus subtilis, Escherichia coli*, *Pseudomonas oleaginous*, *and Candida albicans* with MIC values that ranged between 0.06 to 1 mg/mL. The Gram negatives were the most susceptible bacteria while *C. albicans* was the most susceptible fungi. Moderately low antioxidant activities were recorded in the DPPH and FRAP assays. The chromatographic separation and HPLC analysis of the fungal secondary metabolites yielded compounds: Palitantin (A), Cytosporin D (B), Cytosporin K (C), and Fusarielin (D). These compounds have been previously reported to possess varying pharmacological activities which include antimicrobial and antioxidant activities. *Thus,* The results of this study show that these compounds may, in part, account for the anti-microbial and antioxidant *effect of* the root of *Rhizophora racemosa* ethno medically.

Keywords: Marine fungi, Natural Products, Secondary Metabolites, Endophytic fungi, antimicrobial activity, Antioxidant activity.

Introduction

The emergence of new microbial pathogenic strains and recurrent epidemics brought on by drug-resistant microbes require the development of novel antibiotics (Miethke *et al*., 2021). Until recently, when it was reported that microorganisms associated with plants can also provide materials and products with high therapeutic potentials (Subbulakshmi *et al*., 2012), plants have been a source of bioactive compounds for use as medicines against many types of illnesses (Gouda *et al.,* 2016). Fungi are significantly enhancing structurally new secondary metabolites with promising bioactivities, which is expanding their repertory of biosynthetic and medicinal chemistry (Kianfé, et al., 2023). Marine fungi frequently develop in environments with unusual conditions, which prompts the activation of metabolic pathways and the synthesis of distinctive unidentified molecules (Waters et al., 2010) that support the fungi's ability to adapt to their environment and survive in marine ecosystems (de Souza Sebastianes *et al*., 2013; Knowles *et* al., 2022; Fox & Howlett, 2008). Particularly in the context of drug discovery, marine fungi have been acknowledged as a sustainable supply of secondary metabolites (Waters *et al.,* 2010).The Rhizophora genus is a salt-tolerant mangrove plant that can be found in tropical and subtropical intertidal coastal areas, including Nigeria. It is worth noting that of the nearly 300,000 plant species that exist on Earth, any given plant is colonized by several to a few hundred endophytic fungal species. Only a few of these plants have ever been completely studied as regards their endophytic biology (Strobel & Daisy, 2003). The *Pseudopestalotiopsis* genus belongs to the Sporocadaceae family and is a rich source of novel and diverse bioactive metabolites (Liu *et al*.**, 2019).** In previous studies, 195 metabolites were discovered in Rhizophora-derived endophytic fungi, and their structures were reported within a biogenetic context. Bioassays of previous studies showed antitumor, antimicrobial, as well as anti-H1N1 activities to be the most notable bioactivities of the secondary metabolites (Zhou & Xu, 2018). Also, it appears more feasible economically to produce secondary metabolites on a large scale from microorganisms than from plants, and this makes endophytes an interesting source of new valuable compounds (Gimenez *et al*., 2007). Hence, this study was carried out to investigate the antimicrobial and antioxidant activities of secondary metabolites isolated from a mangrove-derived endophytic fungus,

Pseudopestalotiopsis species, and also to identify the bioactive secondary metabolites present in the fungal extract using high performance liquid chromatography Diode Array Detector (HPLC- DAD).

Materials and Methods

Isolation of Endophytic fungi, fermentation and extraction of secondary metabolites

The fresh root of *Rhizophora racemosa* was harvested from a mangrove forest in Lagos Nigeria. Using the conventional procedure outlined by Arnold *et al*. (2000), endophytic fungi were isolated from the plant root.

Identification and isolation of fungi

Fresh roots *of the plant (Rhizophora racemosa)* were harvested from the mangrove forest in Lagos, Nigeria Nigeria, and inoculated in malt extract agar plates, where they were cultured for three (3) days at 25℃. To create a pure culture, the observed fungal growths were subcultured onto freshly prepared malt extract agar plates to obtain a pure culture. The molecular identification methodology of DNA amplification and sequencing of the internal transcribed space (ITS) region previously described (Kjer et al., 2010) was used to identify one of the fungi. The DNA sequence was deposited to BLASTN and assigned the accession number KY597581.1 in the NCBI nucleotide database.

Vacuum Liquid Chromatography

The crude extracts were subjected to vacuum liquid chromatography using 500 mL of different ratios of hexane and ethyl acetate (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10) as well as dichloromethane and methanol (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10). All of the fractions obtained were concentrated *in vaccuo* using rotary evaporator set at a revolution of 70 RPM and a temperature of 50°C.

Antimicrobial assay

Preliminary antimicrobial screening of the fungal extract was carried out using the agar well diffusion assay described by Aida *et al*. (2001). The test was conducted using four standardized broth cultures of the test bacteria (S. aureus, B. subtilis, P. aeruginosa, and E. coli) and two clinical isolates of the fungi (*Aspergillus niger* and *Candida albicans*). Each test isolate's 0.5 McFarland standard bacterial and fungal suspension was applied using a sterile swab stick to sterile Mueller-Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) (LS BIOTECH, USA), respectively. Then, a sterile cork borer was used to make five wells (8 mm in diameter) on each of the MHA and SDA plates. Aliquots of 80 µL of each extract dilution, reconstituted in DMSO at concentrations of 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL was applied in each

of the wells in the culture plates previously inoculated with the test organisms. The positive controls utilized were ciprofloxacin (5 g/mL) and miconazole (50 g/mL), whereas DMSO was used as the negative control. The cultures in MHA and SDA plates were incubated for 24 and 48 hours, respectively, at 37 and 27 ℃. The antimicrobial potential of each extract was determined by measuring the zone of inhibition around each well (excluding the diameter of the well). The Minimum Inhibitory Concentration (MIC) of the active endophytic fungal extract was determined for each of the test organisms that were sensitive in the preliminary screening. Stock solution of 10 mg/mL for each of the eight active samples in the preliminary assay was prepared, followed by a two-fold serial dilution in other to get graded (working) concentrations including 5, 2.5, 1.25, 0.625 mg/mL. Then, 1 mL from each of this dilution (concentration) was transferred into sterile petri dishes and 9 mL of molten agar cooled to 40℃ was added to it and the mixture stirred clockwise and anticlockwise to ensure proper mixing [The addition of the agar diluted the concentration of the extract to a final concentration of (1, 0.5, 0.25, 0.125, and 0.0625 mg/mL)]. Thereafter, the base of each plate was divided according to the number of the test organisms then a loopful of each of the test organisms previously standardized to McFarland turbidity was streaked on its respective segment. Also, for negative control, the organisms were streaked on the sterile molten agar that did not contain the extract. The culture plates were then incubated for the bacterial and fungal isolates at 37°C for 24 hours and 25°C for 3 days, respectively. After incubation, the plates were examined for microbial growth by checking for visible growth, using a plus sign (+) indicating growth (resistance) while a negative sign (-) indicates no growth (inhibition / susceptible)

Antioxidant Assay

DPPH scavenging assay

The free radical scavenging activity of the fungal extracts and fractions was evaluated using the method described by Patel and Patel (2011) with some modifications. DPPH solution (0.6 mMol) was freshly prepared by adding 25 mL of methanol to 6.0 mg of DPPH. This solution (0.25 mL) was mixed with 0.5 mL of different dilutions (500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 µg/mL) of the crude extract and fractions. The volume of the solution was adjusted with methanol to a final volume of 2.5 mL. After incubation in the dark for 30 minutes at room temperature, the absorbance was measured at 520 nm using a UV-VIS spectrophotometer (Model 752, China). The Ascorbic acid solution was used

as a positive standard. The experiment was carried out in duplicates and the control (containing 0.25 mL of DPPH solution and 2.25 mL of methanol) was used to calculate the free radical scavenging activities of the fractions as shown in the relationship below;

DPPH Scavenging activity equals 100 [(AC - AS)/AC].

Where; AS stands for absorbance of sample, while AC denotes absorbance of control.

Ferric Reducing Antioxidant Power (FRAP) assay

The ferric-reducing antioxidant power of the extract and fractions were determined using the method described by Kumar *et al*., 2005 with little modifications. In duplicate, 0.25 mL of the various fraction dilutions (500, 250, 125, 62.5, 31,.25, 15.625, and 7.8125 g/mL) and 0.25 mL of water (as a control) were put into test tubes with labels. After adding 0.62 mL of phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide, the mixture was incubated for 20 min at 50 °C. After adding 0.62 mL of 10% trichloroacetic acid (TCA), the mixture was centrifuged at 5000 rpm for five minutes. After combining 0.62 mL of the solution's upper layer with 0.62 mL of distilled water, 0.12 mL of ferric chloride solution at a concentration of 0.1% was added. The absorbance was measured using a UV-VIS spectrophotometer (Model 752, China) at 710 nm. The ferric-reducing antioxidant power of the fractions was obtained using the relationship below;

Ferric reducing antioxidant power = 100 [(AS – AC)/ AC]

AC = Absorbance of control and AS = Absorbance of sample

High Performance Liquid Chromatography (HPLC)

The High-Performance Liquid Chromatography (HPLC) was carried out using the method described by Okolo *et al*., 2021.Two milliliters of HPLC-grade methanol were used to reconstitute a 2 mg weight of each of the eight fractions. The mixture was sonicated for 10 minutes, followed by centrifugation at 3000 rpm for 5 minutes. Then, a volume of 100 µL of the dissolved sample was transferred into HPLC vials containing 500 µL of the HPLC grade methanol to make a final concentration of 600 µL. 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). The 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 compounds were detected by comparing the retention times and UV spectra with inbuilt library.

Results

Fungal isolation and identification

The sequence obtained was deposited to BLASTN in the NCBI nucleotide database with accession number KY597581.1. The BLAST showed a 100% sequence similarity belonging to Pseudopestalotiopsis species. The BLAST result showed a 100% sequence similarity belonging to Pseudopestalotiopsis species.

Antimicrobial assay

The results of the antimicrobial assay are shown in Tables 1- 8.

Table 1: Preliminary antimicrobial assay of crude extract

Test organisms	**Concentration (mg/mL) / Inhibition zone diameter (mm)**					
	E1 (Crude extract)					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	0±0	0±0	0±0	0±0	0±0	5
B. s	0±0	0±0	0±0	0±0	0±0	6
E. c	0±0	0±0	0±0	0±0	0±0	0
P. a	11.5±0.7	9±1.4	7±0	5±1.4	0±0	5
A. n	0±0	0±0	0±0	0±0	0±0	0
C. a	0±0	0±0	0±0	0±0	0±0	0

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; A. n: Aspergillus niger; C. a: Candida albicans*. Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL.

Table 2: Preliminary antimicrobial assay of 3W1-HE11

Test organisms	**Concentration (mg/mL) / Inhibition zone diameter (mm)**					
	3W1-HE11					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	6±1.4	4.5±	3±1.4	0±0	0±0	5
B. s	4.5±0.7	3.5±0.7	0±0	0±0	0±0	6
E. c	4.5±0.7	0±0	0±0	0±0	0±0	0
P. a	7.5±0.7	6±0	5.5±0.7	5.5±0.7	5.5±0.7	5
A. n	0±0	0±0	0±0	0±0	0±0	0
C. a	0±0	0±0	0±0	0±0	0±0	0

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; A. n: Aspergillus niger; C. a: Candida albicans*. Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL. 3W1-HE11: Hexane –ethyl acetate VLC fraction (ratio 0:10)

Table 3: Preliminary antimicrobial assay of 3W1-HE3

Test organisms	**Concentration (mg/mL) / Inhibition zone diameter (mm)**					
	3W1-HE3					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	5±0	3±0	0±0	0±0	0±0	5
B. s	0±0	0±0	0±0	0±0	0±0	6
E. c	3.5±0.7	0±0	0±0	0±0	0±0	0
P. a	9±0	7±0	5±1.4	4.5±0.7	0±0	5
A. n	0±0	0±0	0±0	0±0	0±0	0
C. a	0±0	0±0	0±0	0±0	0±0	0

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; A. n: Aspergillus niger; C. a: Candida albicans*. Antimicrobial

potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL.
3W1-HE3: Hexane –ethyl acetate VLC fraction (ratio 8:2)

Table 4: Preliminary antimicrobial assay of 3W1-HE5

Test organisms	**Concentration (mg/mL) / Inhibition zone diameter (mm)**					
	3W1-HE5					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	4±0	0±0	0±0	0±0	0±0	5
B. s	5±1.4	4±1.4	0±0	0±0	0±0	6
E. c	4.5±0.7	3.5±0.7	0±0	0±0	0±0	0
P. a	9±0	7±1.4	6.5±7	6±1.4	4.5±0.7	5
A. n	0±0	0±0	0±0	0±0	0±0	0
C. a	4.5±0.7	4±0	0±0	0±0	0±0	0

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; A. n: Aspergillus niger; C. a: Candida albicans*. Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL.
3W1-HE5: Hexane –ethyl acetate VLC fraction (ratio 6:4)

Table 5: Preliminary antimicrobial assay of 3W1-DM3

Test organisms	**Concentration (mg/mL) / Inhibition zone diameter (mm)**					
	3W1-DM3					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	4.5±0.7	3.5±0.7	0±0	0±0	0±0	5
B. s	5±0	3±1.4	0±0	0±0	0±0	6
E. c	4.5±0.7	2±0	0±0	0±0	0±0	0
P. a	11.5±0.7	10±1.4	9±0	8±0	8±0	5
A. n	0±0	0±0	0±0	0±0	0±0	0
C. a	5.5±0.7	0±0	0±0	0±0	0±0	0

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; A. n: Aspergillus niger; C. a: Candida albicans*. Antimicrobial

potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL. 3W1-DM3: dichloromethane –methanol VLC fraction (ratio 5:5)

Table 6: Preliminary antimicrobial assay of 3W1-HE8

Test organisms	**Concentration (mg/mL) / Inhibition zone diameter (mm)**					
	3W1-HE8					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	4±0	0±0	0±0	0±0	0±0	5
B. s	0±0	0±0	0±0	0±0	0±0	6
E. c	0±0	0±0	0±0	0±0	0±0	0
P. a	2±0	0±0	0±0	0±0	0±0	5
A. n	0±0	0±0	0±0	0±0	0±0	0
C. a	0±0	0±0	0±0	0±0	0±0	0

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; A. n: Aspergillus niger; C. a: Candida albicans*. Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL. **3W1-HE8:** Hexane –ethyl acetate VLC fraction (ratio 3:7)

Table 7: Preliminary antimicrobial assay of 3W1-HE9

Test organisms	**Concentration (mg/mL) / Inhibition zone diameter (mm)**					
	3W1-HE9					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	0±0	0±0	0±0	0±0	0±0	5
B. s	0±0	0±0	0±0	0±0	0±0	6
E. c	0±0	0±0	0±0	0±0	0±0	0
P. a	5±0	4±0.7	0±0	0±0	0±0	5
A. n	0±0	0±0	0±0	0±0	0±0	0
C. a	0±0	0±0	0±0	0±0	0±0	0

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; A. n: Aspergillus niger; C. a: Candida albicans*. Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL. 3W1-HE9: Hexane –ethyl acetate VLC fraction (ratio 2:8)

Table 8: Preliminary antimicrobial assay of 3W1-HE10

Test organisms	**Concentration (mg/mL) / Inhibition zone diameter (mm)**					
	3W1-HE10					
	1	0.5	0.25	0.13	0.06	Positive control
S. a	0±0	0±0	0±0	0±0	0±0	5
B. s	0±0	0±0	0±0	0±0	0±0	6
E. c	6±0	4±0.7	0±0	0±0	0±0	0
P. a	6±0	3±0	0±0	0±0	0±0	5
A. n	0±0	0±0	0±0	0±0	0±0	0
C. a	0±0	0±0	0±0	0±0	0±0	0

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichia coli; P. a: Pseudomonas aeruginosa; A. n: Aspergillus niger; C. a: Candida albicans*. Antimicrobial potential was indicated by zone of inhibition produced around the well and measured in millimeters. The positive controls used were Ciprofloxacin 5 µg/mL and miconazole 50 µg/mL. 3W1-HE10: Hexane –ethyl acetate VLC fraction (ratio 1:9)

Table 9: Minimum Inhibitory Concentration (MIC)

Extract/fractions	Test Organisms	MIC (mg/mL)
Crude extract(3W1)	*S.a*	> 1
	B.s	> 1
	E.c	0.13
	P.a	> 1
	A.n	> 1
	C.a	> 1
3W1- HE 11	*S.a*	0.13
	B.s	0.5
	E.c	1
	P.a	0.06
	A.n	> 1
	C.a	> 1
3W1- HE 3	*S.a*	1
	B.s	> 1
	E.c	> 1
	P.a	0.13
	A.n	> 1
	C.a	> 1
3W1- HE 5	*S.a*	1
	B.s	0.5
	E.c	0.13
	P.a	0.13
	A.n	> 1
	C.a	0.5
3W1- DM 3	*S.a*	1
	B.s	0.5
	E.c	1
	P.a	0.06
	A.n	> 1
	C.a	0.5

Key: *S. a: Staphylococcus aureus; B. s: Bacillus subtilis; E. c: Escherichial coli; P. a: Pseudomonas aeruginosa; A. n: Aspergillus niger; C. a: Candida albicans*

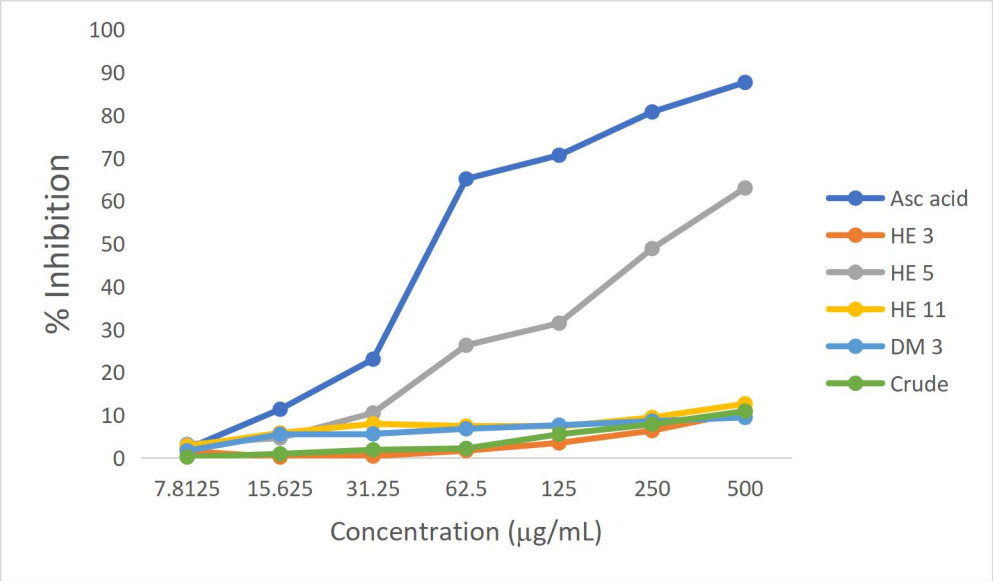


Figure 1: DPPH radical scavenging antioxidant assay of the crude extract and fractions of the secondary metabolites

Key: Asc: HE 3: hexane –ethyl acetate VLC fraction (ratio 8:2); HE5: Hexane –ethyl acetate VLC fraction (ratio 6:4); HE 11: Hexane –ethyl acetate VLC fraction (ratio 0:10); DM3: dichloromethane –methanol VLC fraction (ratio 5:5)

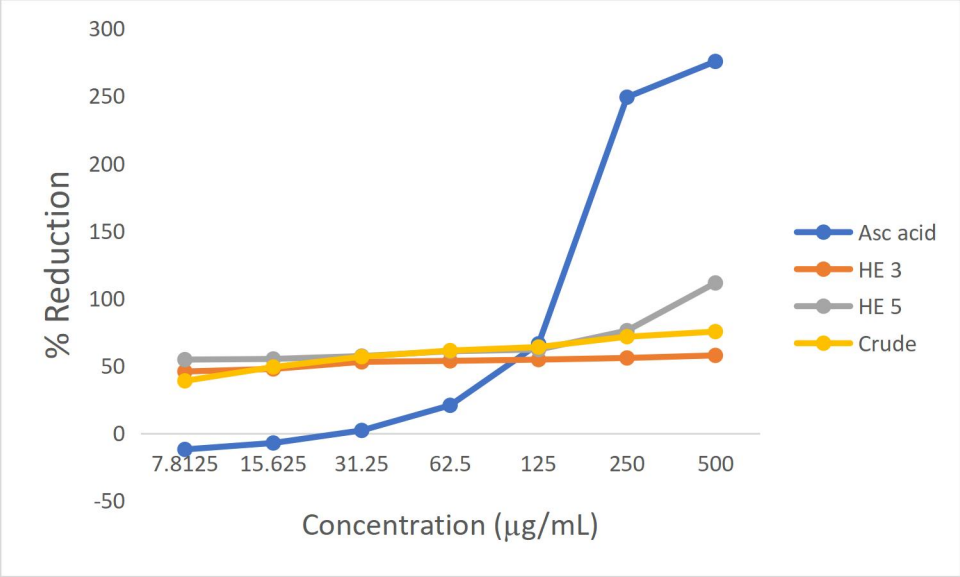
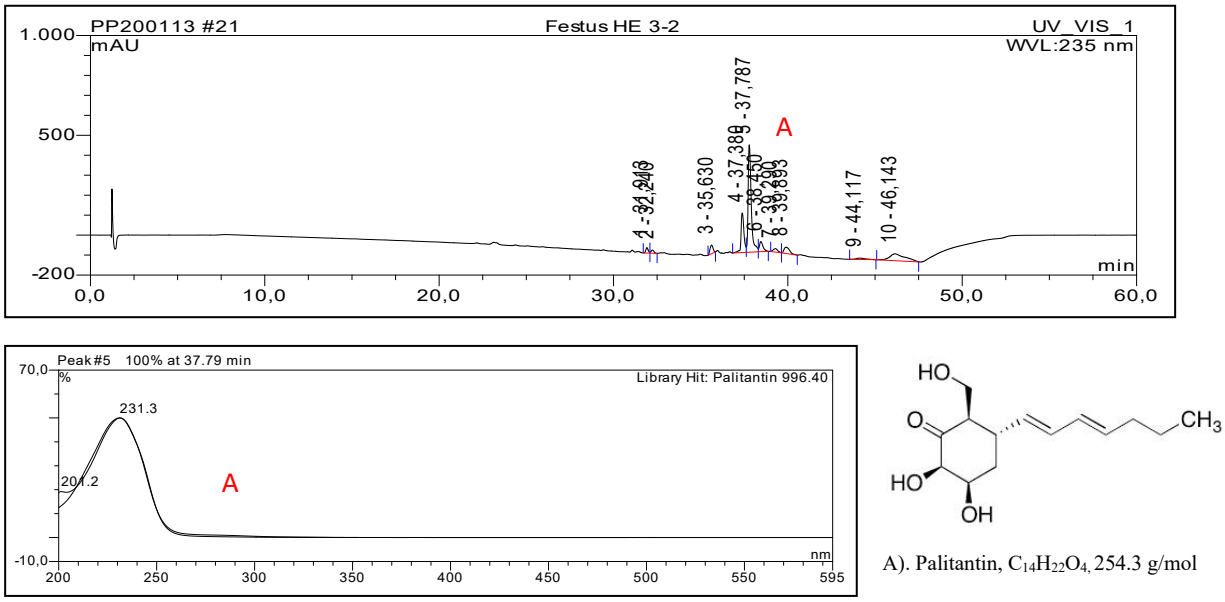


Figure 2: Ferric Reducing Antioxidant Power (FRAP) assay of the crude extract and fractions of the secondary metabolites

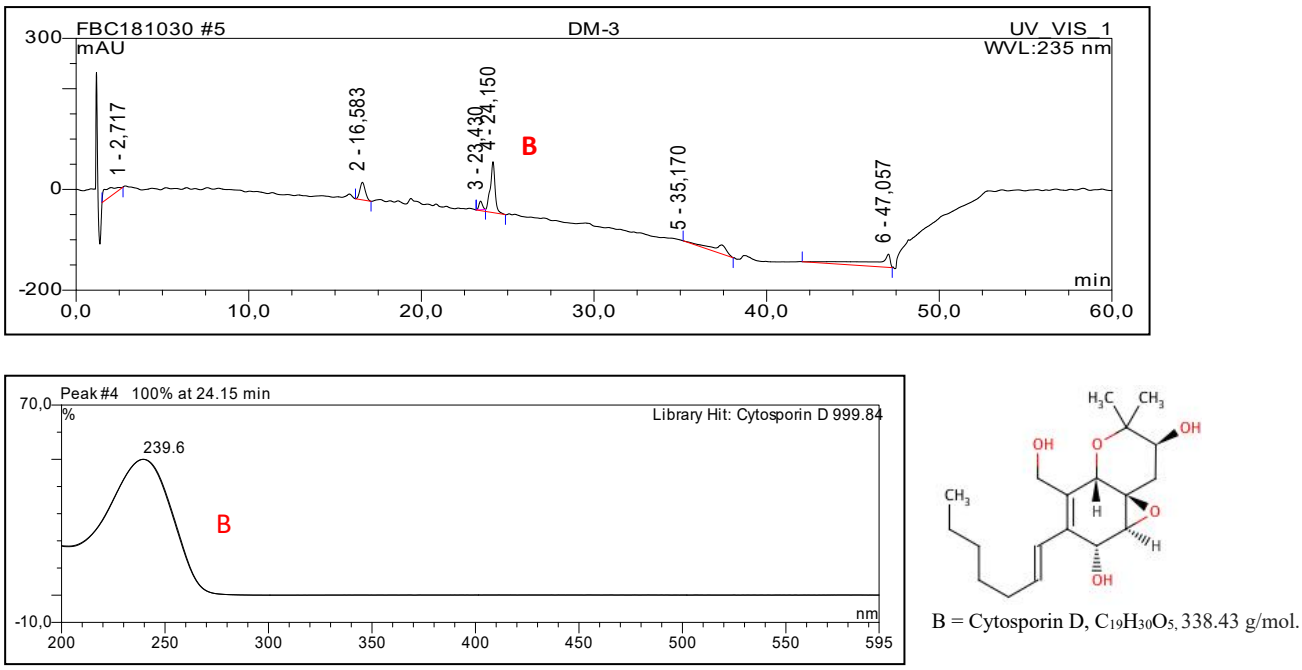
Key: Asc: Ascorbic acid; HE 3: 3: hexane –ethyl acetate VLC fraction (ratio 8:2)

HE5: Hexane –ethyl acetate VLC fraction (ratio 6:4)



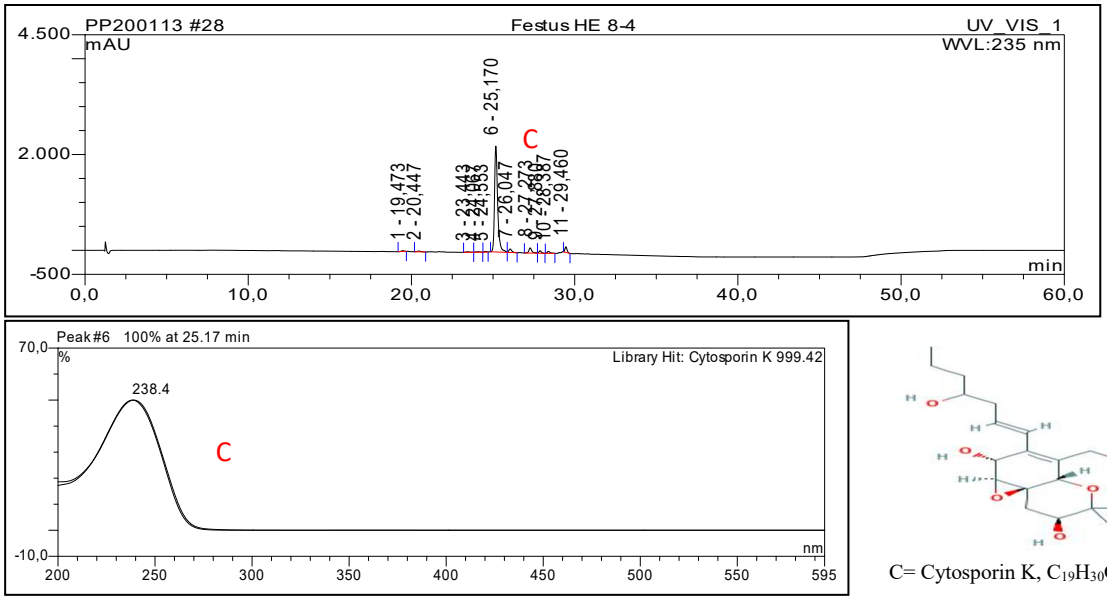
A). Palitantin, $C_{14}H_{22}O_4$, 254.3 g/mol

Figure 3: HPLC Chromatogram and UV- Spectra of major compounds detected from fractions of *Pseudopestalotiopsis sp.* A= Palitantin (RT=37.79, 996.40)



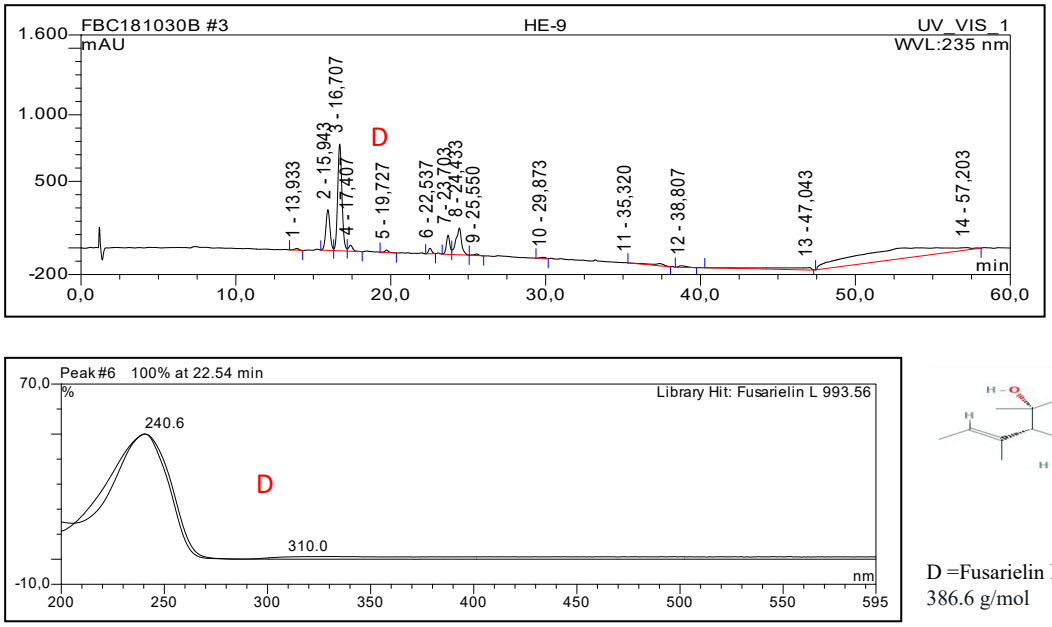
B = Cytosporin D, $C_{19}H_{30}O_5$, 338.43 g/mol.

Figure 4: HPLC Chromatogram and UV- Spectra of major compounds detected from fractions of *Pseudopestalotiopsis sp.* B= Cytosporin D (RT=24.15, 999.84)



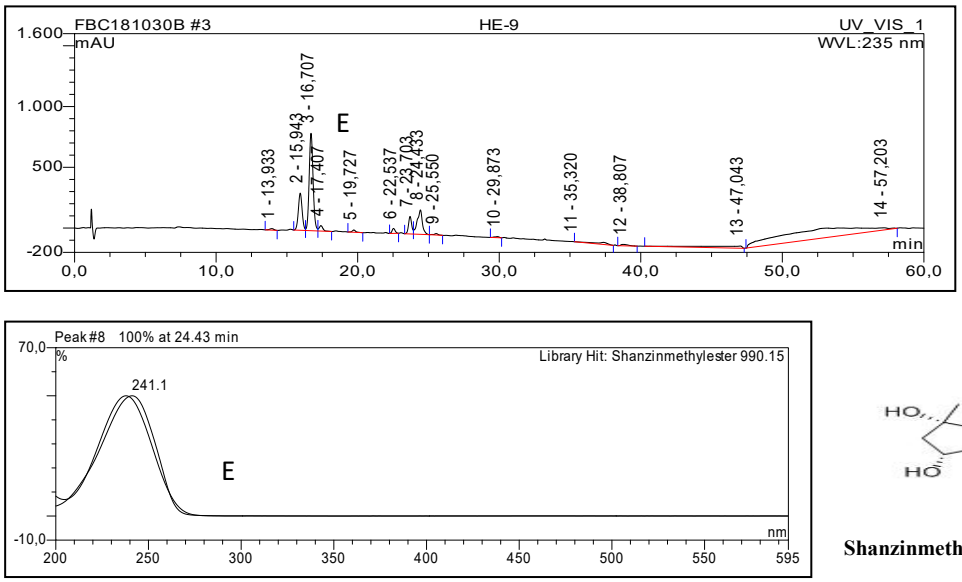
C= Cytosporin K, $C_{19}H_{30}O_6$; 354.4g/mol

Figure 4: HPLC Chromatogram and UV- Spectra of major compounds detected from fractions of *Pseudopestalotiopsis sp.* C= Cytosporin K (RT=25.17, 999.42)



D =Fusarielin L, $C_{25}H_{38}O_3$, 386.6 g/mol

Figure 5: HPLC Chromatogram and UV- Spectra of major compounds detected from fractions of *Pseudopestalotiopsis sp.* D =Fusarielin (RT=22.54, 993.56)



Shanzinmethylester: $C_{17}H_{26}O_{11}$, 406.38g/mol

Figure 6: HPLC Chromatogram and UV- Spectra of major compounds detected from fractions of *Pseudopestalotiopsis sp.* E =Shanzinmethylester (RT=24.43, 990.15)

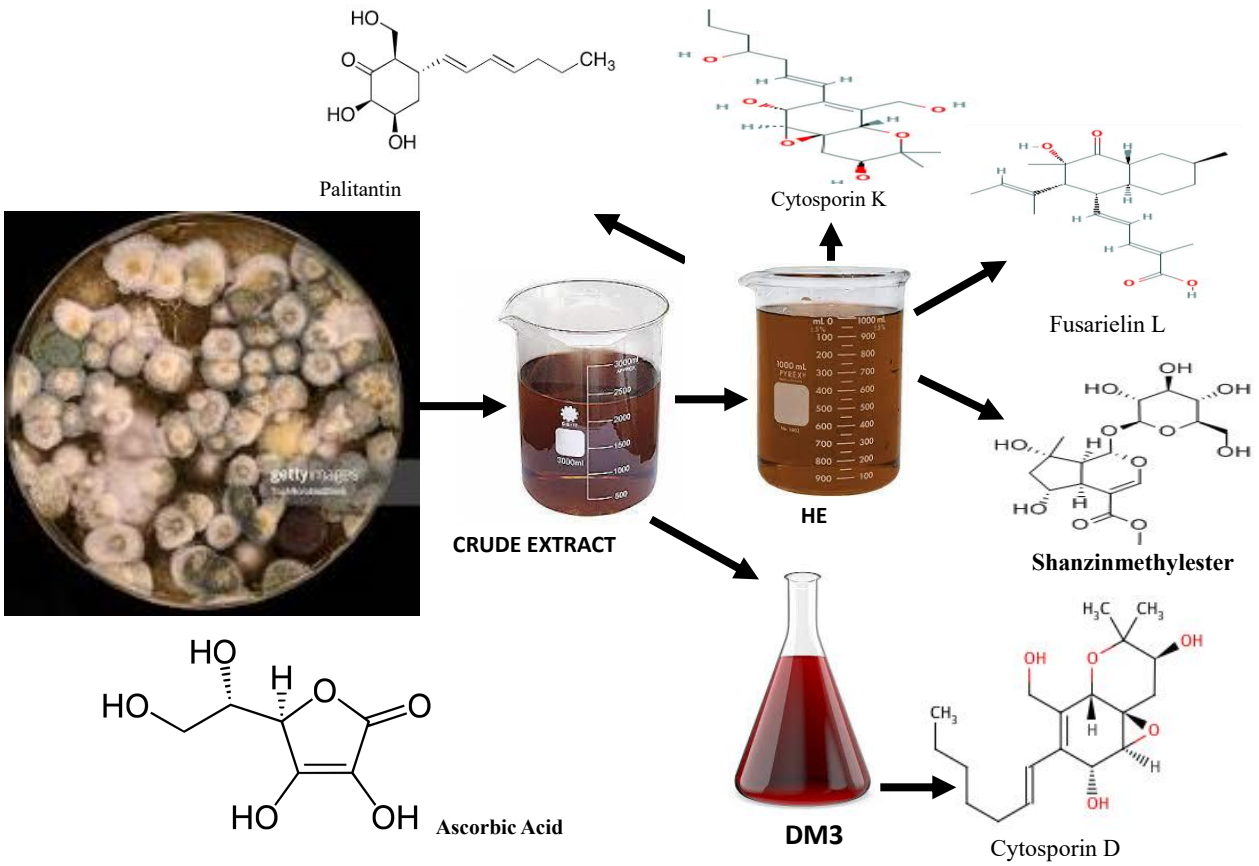


Figure 7: A pictorial diagram of the detected compound from the HPLC –DAD

Keys: HE-Hexane and ethyl acetate VLC fractions DM: Dichloromethane and methanol VLC fractions

According to the determined inhibitory zone diameter (IZD), the preliminary antibacterial assay against the clinical isolates of bacteria and fungus revealed a significant inhibition. Additionally, it was found that the active secondary metabolites from the endophytic fungi, *Pseudopestalotiopsis species* inhibitory effects on the test organisms were concentration-dependent tables 1–8. As indicated in Tables 4 and 5, respectively, fractions 3W1-HE5 and 3W1-DM3 were shown to be the only fractions efficacious against *Candida albicans*. Furthermore, it was discovered that Faction 3W1-HE5 was the most active, inhibiting all of the test bacteria isolates with MICs of 1, 0.5, 0.5, and 0.06 mg/mL against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, respectively. Moreover, other active fungal samples (crude extract and VLC fractions) displayed a range of concentration-dependent microbial growth inhibitory effects. This could be due to the presence of the compound, Cytosporin D detected in the fractions by HPLC dereplication. An array of biological activities has been linked to cytosporin D, a previously isolated from Cytosporarhizophorae (Liu *et al.,* 2019). It possesses a novel benzo[b] [1, 5] dioxocane skeleton. According to previous researchers (Jayasuriya *et al*., 2003; Sadorn *et al*., 2018; Ciavatta *et al*., 2008), secondary metabolites of the genus *Cytospora* are reputed to be a prolific source of potent antimicrobial compounds. Fraction 3W1-HE3 was observed from the result above (table 3) to be active against *Escherichia coli*, *Staphylococcus aureus (SA),* and Pseudomonas aeruginosa (PA) with MICs of 1 mg/mL, 0.5 mg/mL, , and 0.13 mg/mL respectively. It showed a concentration-dependent activity against the tested organisms. It showed a broader spectrum of antibacterial activity covering *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However, it was inactive against the fungal isolates at the observed concentrations. This may be due to the presence of Palitantin detected in the HPLC chromatogram. Palitantin is a cyclohexane metabolite first isolated from *Penicillium sp.* reported to have antiprotozoal effects and moderate antimycobacterial activity (Fuska *et al*., 1970,

Chigozie *et al.,* 2020). The antibacterial and HIV-1 integrase inhibitory activities of Palitantin were described in a prior study (Torres, 2013). It has also been reported to have been isolated from apple-associated endophytic bacteria cultivated in a solid rice medium (Ahmed *et al*., 2015). Fraction 3W1-HE 8 had poor antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* at doses of each of 1 mg/mL, as seen from the results above (table 8). Cytosporins are a family of hexahydrobenzopyran metabolites that are generated from fungi and have a distinctive heptene side chain residue. They are known to be angiotensin II binding inhibitors. To date, nearly 30 natural cytosporins of this structural class have been predominantly isolated from four genera of fungi: Cytospora sp. (Stevens-Miles *et al.,* 1996), Pestalotiopsis sp. (Akone et al., 2013), Eutypella sp. (Ciavatta *et al*., 2008; Zhang *et al*., 2019), and Pseudopestalotiopsis sp. (Yu, *et al*., 2020). The cytosporin family of compounds has a variety of bioactive effects, including cytotoxic, antibacterial, and antagonistic activity (Stevens-Miles *et al*., 1996; Ciavatta *et al*., 2008). Cytosporin K is an analogue of Cytosporins having a C7 side chain substituted with hydroxyl at C5. When compared to the Cytosporin D analogue without the Oxygen function, the agent's lipophilicity may be reduced by the OH polar function at the C7 side chain, which may result in a modest reduction in its antibacterial effectiveness. The oxygen function could always have an impact on the octanol-water partition coefficient as well as other physicochemical properties. It may also reduce membrane permeability, which would then result in a decrease in the bioactivity of the antimicrobial agent (Tokuyama *et al*., 2001). There was no antimicrobial activity detected for fraction 3W1-HE9 as it showed no activity against the bacterial and fungal isolates. There is no reported antimicrobial result for the HPLC-detected compounds: Fusarielin and Shanzinmethyl ester even though, Shanzinmethyl ester has been reported to have hepatoprotective activity (Gaurav *et al*., 2009). Figures 1 and 2 above show the results of the DPPH radical scavenging antioxidant assay of the crude extract and VLC fractions (HE 3, HE5, HE 11, DM3) as well as the ferric reducing antioxidant power (FRAP) assay of the crude extract and VLC fractions (HE 3 and HE5) in comparison to the ascorbic acid standard. Moderate antioxidant activity was found in the *two in-vitro* antioxidant assay models. DPPH (2, 2-diphenyl-1-picrylhydrazyl) is considered an accurate, valid, and easy

method to evaluate the radical scavenging activity of antioxidants (Torres, 2013). From the results, weak scavenging activities were exhibited by the fractions as depicted by their poor percentage inhibitions in both DPPH and FRAP analysis compared to the positive control. The DPPH scavenging and Reducing Powers (FRAP) were based on different mechanisms of antioxidant assay and results from both methods provided a basis for comparison. Furthermore, it provides more reason to believe the extract possessed weak antioxidant activity since it has been suggested that antioxidant activity should not be concluded based on a single test model (Amudha & Rani, 2016). It is pertinent to say that the detected compounds cannot act as primary/chain-breaking antioxidants or secondary/ preventative antioxidants (usually acting by transition metal ion chelation) (Madhavi *et al*., 1995). The causes may be obvious from a deeper examination of the structure and pharmacophore of the substances that the HPLC detected. According to researchers (Charlton *et al*., 2023), there are pharmacophore features, structural motifs, and substituents that are important contributors to the antioxidant potentials of small molecules. These include highly conjugated (e.g., aromatic) hydroxyl groups, amino groups, sulfhydryl group (thiol), and isoprenoid groups. Hence antioxidant compounds contain one or more of these pharmacophoric features. The structures of OH-based antioxidants vary greatly, ranging from simple molecules like ascorbic acid (Padayatty& Levine, 2016) to polyphenols (Rice-Evans*, et al*., 1997), or compounds with long alkyl chains such as in vitamin E. Nitrogen-based antioxidants possess a conjugated active NH group as their pharmacophore with mechanisms, similar to that of OH-based compounds (Horton *et a*l., 2018). Some compounds combine the above-described pharmacophoric features (Oyewole& Birch-Machin, 2015) in their antioxidant mechanisms. Structurally (figure 7), some of the suggested bioactive and detected compounds contain an alcoholic hydroxyl group as seen in Palitantin, cytosporin D, cytosporin K, and fusarielin L; however they lack the pharmacophoric phenolic hydroxyl function (highly conjugated hydroxyl groups) that largely characterize antioxidant agents like Ascorbic acid (Karasawa & Mohan, 2018). Also comparing, the structure of the two cytosporin analogues- the benzophenone derivative (Liu *et al.,* 2019) or hexahydrobenzopyran metabolites (Stevens-Miles *et al.,* 1996); cytosporin D in DM fraction and cytosporin K in HE fraction, one can quickly assert that the

oxygen function (Hydroxyl) in C5 side chain of cytosporin K plays a role in the difference observed in the antioxidant properties. This could be the possible explanation of the reason for the mild antioxidant activity observed in the fractions. This may be one explanation for the fraction's low level of antioxidant activity.

Conclusion

The present study investigated the antimicrobial and antioxidant activities of secondary metabolites isolated from a mangrove-derived endophytic fungus, *Pseudopestalotiopsis species*

The observed antimicrobial activity may, in part, be accounted for by the presence of the detected compounds in the HPLC assay. The active extracts and fractions exhibited good antimicrobial activities against *Staphylococcus aureus*, *Bacillus subtilis, Escherichia coli*, *Pseudomonas aeruginosa*, *and Candida albicans* with MIC values that ranged between 0.06 to 1 mg/mL. The Gram negatives were the most susceptible bacteria while *Candida albicans* was the most susceptible fungi. Moderately-low antioxidant activities were recorded in the DPPH and FRAP assays. The limited antioxidant potential of the extract and fractions may be due to the lack of pharmacophoric phenolic hydroxyl function (highly conjugated hydroxyl groups) features that largely and primarily characterize antioxidant agents like Ascorbic acid in the detected compounds. The detected compounds can serve as lead structures for the development of better therapeutic molecules targeted towards some specific oxidative stress related diseases and for curbing antimicrobial resistance.

Acknowledgement

The authors are grateful to Prof. Dr. Peter Proksch of the Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University, Düsseldorf in whose laboratory the HPLC-DAD analysis was carried out.

Conflict of interest

The authors declare no conflict of interest

References

Ahmed, H. A., Ebrahim, W., Mikhailovna, P. A., Henrich, B., Proksch, P. (2015). Extraction and Identification of some metabolites produced by antagonistic apple plant bacteria Brevibacteriumhalotolerans. *International Journal* of *Advanced* Research, 3, 1208-17.

Aida, P., Rosa, V., Blamea, F., Tomas, A., & Salvador, C. (2001). Paraguyan plants used in traditional medicine. *Journal of Ethnopharmacology*, *16*, 93-98.

Akone, S. H., El Amrani, M., Lin, W., Lai, D., &Proksch, P. (2013). Cytosporins F–K, new epoxyquinols from the endophytic fungus Pestalotiopsistheae. Tetrahedron Letters, 54(49), 6751-6754.

Amudha, M., & Rani, S. (2016). Evaluation of in vitro antioxidant potential of Cordia retusa. Indian journal of pharmaceutical sciences, 78(1), 80.

Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D., &Kursar, T. A. (2000). Are tropical fungal endophytes hyperdiverse? *Ecology letters*, *3*(4), 267-274.

Charlton, N. C., Mastyugin, M., Török, B., &Török, M. (2023). Structural features of small molecule antioxidants and strategic modifications to improve potential bioactivity. Molecules, 28(3), 1057**.**

Chigozie, V., Okezie, M., Ajaegbu, E., Okoye, F., & Esimone, C. (2020). Isolation, identification, and evaluation of biological activities of Daldiniaeschscholtzii, an endophytic fungus isolated from the leaves of Musa paradisiaca. *GSC Biological and Pharmaceutical Sciences*, *12*(1), 216-228.

Ciavatta, M. L., Lopez-Gresa, M. P., Gavagnin, M., Nicoletti, R., Manzo, E., Mollo, E., ... & Cimino, G. (2008). Cytosporin-related compounds from the marine-derived fungus Eutypella scoparia. *Tetrahedron*, *64*(22), 5365-5369.

de Souza Sebastianes, F. L., Romao-Dumaresq, A. S., Lacava, P. T., Harakava, R., Azevedo, J. L., de Melo, I. S., &Pizzirani-Kleiner, A. A. (2013). Species diversity of culturable endophytic fungi from Brazilian mangrove forests. Current genetics, 59, 153-166.

Fuska, J., Kuhr, I., Nemec, P., &Fusková, A. (1970). The effect of palitantin, a metabolite of Penicillium frequentans on Leishmania brasiliensis. *Folia microbiologica*, *15*, 111-116.

Gaurav L., Hemant K.S., Kamlesh K.P., Zeashan H. (2009).Hepatoprotective effects of Calotropisgigantea extract against carbon tetrachloride induced liver injury in rats. ActaPharm. ;59:89-96.

Gimenez, C., Cabrera, R., Reina, M., Gonzalez-Coloma, A. (2007). Fungal endophytes and their role in plant protection. Current Organic Chemistry, 11(8), 707-20.

Gouda, S., Das, G., Sen, S. K., Shin, H. S., & Patra, J. K. (2016). Endophytes: a treasure house of bioactive compounds of medicinal importance. *Frontiers in microbiology*, *7*, 1538.

Horton, W., Peerannawar, S., Török, B., &Török, M. (2019). Theoretical and experimental analysis of the antioxidant features of substituted phenol and aniline model compounds. Structural Chemistry, 30, 23-35.

Jayasuriya, H., Guan, Z., Polishook, J. D., Dombrowski, A. W., Felock, P. J., Hazuda, D. J., & Singh, S. B. (2003). Isolation, structure, and HIV-1 integrase inhibitory activity of Cytosporic acid, a fungal metabolite produced by a Cytospora sp. Journal of natural products, 66(4), 551-553.

Karasawa, M. M. G., & Mohan, C. (2018). Fruits as prospective reserves of bioactive compounds: a review. Natural products and bioprospecting, 8, 335-346.

Kianfé, B. Y., Tchamgoue, J., Narmani, A., Teponno, R. B., Njouonkou, A. L., Stadler, M., &FogueKouam, S. (2023). Bioactive Secondary Metabolites from Fungi of the Genus CytosporaEhrenb.(Ascomycota). Molecules, 28(7), 3120.

Kumar, S., Taneja, R., & Sharma, A. (2005). The genus turnera.: A review

update. *Pharmaceutical biology*, *43*(5), 383-391.

Liu, H. X., Tan, H. B., Chen, K., Zhao, L. Y., Chen, Y. C., Li, S. N., ... & Zhang, W. M. (2019). Cytosporins A–D, novel benzophenone derivatives from the endophytic fungus Cytosporarhizophorae A761. Organic & Biomolecular Chemistry, 17(9), 2346-2350.

Liu, H. X., Tan, H. B., Chen, K., Zhao, L. Y., Chen, Y. C., Li, S. N., ... & Zhang, W. M. (2019). Cytosporins A–D, novel benzophenone derivatives from the endophytic fungus Cytosporarhizophorae A761. Organic & Biomolecular Chemistry, 17(9), 2346-2350.

Madhavi, D. L., Deshpande, S. S., &Salunkhe, D. K. (1995). Food antioxidants: Technological: Toxicological and health perspectives. CRC Press.

Miethke, M., Pieroni, M., Weber, T., Brönstrup, M., Hammann, P., Halby, L., ... & Müller, R. (2021). Towards the sustainable discovery and development of new antibiotics. Nature Reviews Chemistry, 5(10), 726-749.

Okolo, C. C., Mbachu, N. A., Anyanwu, O. O., Ngwoke, K. G., & Okoye, F. B. (2021). Screening for anti-inflammatory activity of extract and fractions of Morinda lucida stem bark and detection of bioactive secondary metabolite. *GSC Biological and Pharmaceutical Sciences*, *16*(1), 031-039.

Oyewole, A. O., & Birch-Machin, M. A. (2015). Mitochondria-targeted antioxidants. The FASEB Journal, 29(12), 4766-4771.

Padayatty, S. J., & Levine, M. (2016). Vitamin C: the known and the unknown and Goldilocks. Oral diseases, 22(6), 463-493.

Patel Rajesh, M., & Patel Natvar, J. (2011). In vitro antioxidant activity of coumarin compounds by DPPH, Super oxide and nitric oxide free radical scavenging methods. *Journal of advanced pharmacy education & research*, *1*(1), 52-68.

Rice-Evans, C., Miller, N., &Paganga, G. (1997). Antioxidant properties of phenolic compounds. Trends in plant science, 2(4), 152-159.

Sadorn, K., Saepua, S., Boonyuen, N., Boonruangprapa, T., Rachtawee, P., &Pittayakhajonwut, P. (2018). Antimicrobial activity and cytotoxicity of xanthoquinodin analogs from the fungus Cytosporaeugeniae BCC42696. Phytochemistry, 151, 99-109.

Stevens-Miles, S., Goetz, M. A., Bills, G. F., Giacobbe, R. A., Tkacz, J. S., Chang, R. S., ... & Huang, L. (1996). Discovery of an angiotensin II binding inhibitor from a Cytospora sp. using semi-automated screening procedures. The Journal of Antibiotics, 49(2), 119-123.

Stevens-Miles, S., Goetz, M. A., Bills, G. F., Giacobbe, R. A., Tkacz, J. S., Chang, R. S., ... & Huang, L. (1996). Discovery of an angiotensin II binding inhibitor from a Cytospora sp. using semi-automated screening procedures. The Journal of Antibiotics, 49(2), 119-123.

Strobel, G., & Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. Microbiology and molecular biology reviews, 67(4), 491-502.

Subbulakshmi, G. K., Thalavaipandian, A., Ramesh, V., & Rajendran, A. (2012). Bioactive endophytic fungal isolates of Biota orientalis (L) Endl., Pinus excelsa Wall. and Thuja occidentalis L. *International Journal of Advanced Life Sciences (IJALS)*, *4*, 9-15.

Tokuyama, R., Takahashi, Y., Tomita, Y., Tsubouchi, M., Yoshida, T., Iwasaki, N., ... & Nagata, O. (2001). Structure-activity relationship (SAR) studies on oxazolidinone antibacterial agents. 2.1) Relationship between lipophilicity and antibacterial activity in 5-thiocarbonyl oxazolidinones. Chemical and pharmaceutical bulletin, 49(4), 353-360.

Torres, R. R. (2013). *Stereoselective organocatalysis: bond formation methodologies and activation modes*. John Wiley & Sons.

Waters, A. L., Hill, R. T., Place, A. R., & Hamann, M. T. (2010). The expanding role of marine microbes in pharmaceutical development. Current opinion in biotechnology, 21(6), 780-786.

Waters, A. L., Hill, R. T., Place, A. R., & Hamann, M. T. (2010). The expanding role of marine microbes in pharmaceutical development. *Current opinion in biotechnology*, *21*(6), 780-786.

Waters, A. L., Hill, R. T., Place, A. R., & Hamann, M. T. (2010). The expanding role of marine microbes in pharmaceutical development. *Current opinion in biotechnology*, *21*(6), 780-786.

Yu, X., Müller, W. E., Meier, D., Kalscheuer, R., Guo, Z., Zou, K., ... &Proksch, P. (2020). Polyketide derivatives from mangrove derived endophytic fungus Pseudopestalotiopsistheae. Marine Drugs, 18(2), 129.

Zhang, H., Rothwangl, K., Mesecar, A. D., Sabahi, A., Rong, L., & Fong, H. H. (2009). Lamiridosins, hepatitis C virus entry inhibitors from Lamium album. *Journal of natural products*, *72*(12), 2158-2162.

Zhou, J., & Xu, J. (2018). Chemistry and biodiversity of Rhizophora-derived endophytic fungi (Vol. 8, pp. 165-186). London, UK: IntechOpen**.**