Original Research Article

Antimicrobial activities of the Endophytic fungus,*Fusarium equiseti*,isolated from the leaves of *Ocimum gratissimum*.

Abstract

Introduction

Fungal endophytes of medicinal plants origin are gaining increasing interest as sources of novel bioactive agents with therapeutic capacity. This study was designed to identify and evaluate secondary metabolites isolated from*Fusarium equiseti*; an endophytic fungus isolated from leaves of *Ocimum gratissimum* for their antimicrobial potentials.

Method

The isolation and molecular identification of the endophytic fungus, as well as fermentation, and extraction of secondary metabolites were carried out following standard laboratory procedures. The crude extract was partially purified by partitioning into different fractions using column chromatographic techniques and the fractions were tested for antimicrobial activity. The phytoconstituents in the bioactive fractions were detected by dereplication using High-Performance Liquid Chromatography linked withDiode Array Detection (HPLC-DAD).

Results

Our findings showed that fraction 4 (DCM/ Methanol fraction 95:5) exhibited moderate to strong inhibition against the test micro-organisms namely, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* with MIC values in the range of 0.03 to 1 mg/mLbut had no inhibition against *Aspergillus niger* and *Salmonella typhi*. The compounds detected in the HPLC assay include Phomoxanthone A, Scalarolide, equisetin, epi-equisetin an episomer of equisetin, Naamine A, Carbonarone A, destruxin B, cytosporin G, and WLIP (β-hydroxydecanoyl-Leu1-D Glu-De-Thr-Dval-DLeu3 DSer-Leu-Dser-Ile). These compounds have been reported to possess antimicrobial activity.

Conclusion

The detection of these metabolites further confirms the potential of the endophytic fungus, *Fusarium equiseti* isolated from leaves of *Ocimum gratissimum*as a potential source of bioactive molecules for treatment of infectious diseases.

Keywords: Endophytic fungi; *Ocimum gratissimum*; *Fusarium equiseti*; Secondary metabolites; antimicrobial.

Introduction

Antimicrobial resistance has remained a public health threat due to its implications for achieving the millennium goal of health for all. The growing trend in antimicrobial resistance is due to misuse, overuse, and abuse of antibiotics by users leading to a huge economic burden because diseases are now harder to treat using the available conventional antibiotics. Among the consequences of antimicrobial resistance are higher medical costs, prolonged stays in hospital, increased mortality and morbidity [1]. Due to the problems associated with antimicrobial resistance, the world is gradually becoming a “post-antibiotic era” consequently antimicrobial resistance is becoming a global challenge with the presence of emerging diseases. Despite the huge pool of about 252 antimicrobial agents in preclinical trials being developed to treat WHO’s priority pathogens [2], very few target the most critical resistant Gram-negative bacteria, therefore, there is an urgent need for antimicrobial agents with a wide broad spectrum for gram-negative and gram-positive bacteria. Several alternative approaches to conventional antibiotics

have been extensively studied and currently receiving world-wide attention for the treatment of antibiotic resistant microorganisms. The natural product has received the attention of the world due to the great potential it has as a reservoir for potent antimicrobial agents among other drugs.
Endophytic fungus belonging to a group of fungi that live within the tissue of living organisms symbiotically without causing any hazard to the host plant [3] has recently received the attention of natural product researchers because of its huge potential for bioactive compounds.
This is because it is believed that endophytes possess the ability to produce similar chemicals as those originating from their host plants [4-5]. Although endophytes are associated with living tissues, they do not cause harm to the host plants but assist the host plant to fight biotic and abiotic stresses by scavenging and regulating the damaging reactive oxygen species (ROS) [6-7] and therefore there is need to harness their potentials as an alternative source of plant metabolites for development and synthesis of novel drugs for the treatment of diseases associated resistant micro-organism that affects living organism.
Ocimum gratissimum L is an aromatic herb usually found in the tropics and is commonly known as Camphor basil or Ram Tulsi with a characteristic clove-like flavor belonging to the family of Lamiaceae. It has wide pharmacological uses folklorically as anti-carcinogenic, free radical scavenging, and radio defensive [8] with numerous other therapeutic uses across different cultures where they are found. It was previously reported to have antimicrobial activities against *Escherichia coli* [9], *Botryosphaeria rhodina, Rhizoctonia* specie, *Alternaria* specie. [10], *Staphylococcus aureus, Salmonella typhi*, *Salmonella typhimurium*, [11], and *Cryptococcus neoformans* [12] among others. Previous researchers reported numerous bioactive compounds of therapeutic importance such as thymol, thymol p-cymeme, γ-terpene,t-sabiene hydrate, β-phellandrene, limonene, eugenol, eugenol spathulenol, geraniol, eugenol γ-muurolene, 1,8-cineole, gratissimol, germacrene-D and β-caryophyllene and external flavones mainly xantomicrol and cirsimaritin [13-16] were all reported from the essential oil the whole plant. Our recent reports haveshownthat Nigerian Medicinal plants are unquestionable sources of novel endophytic fungi with enormous potentials of generating novel bioactive lead molecules[17-20]. This presentstudy, therefore, evaluated the antimicrobial potential of the endophytic fungus isolated from the fresh leaf of *Ocimum gratissimum* for selected strains of resistant microbes on the premise that the endophytes produce similar compounds to their host. This makes them a potential source of antibiotics with a wide spectrum for both gram-positive and gram-negative bacteria because endophytes help the host plant in defense and therefore should be exploited.

Materials

Chemicals and Solvents.

Culture media used include Mueller-Hinton Agar (Oxoid Ltd., Basingstoke, UK), Sabouraud Dextrose Agar (Oxoid Ltd.), Silica gel (200-400μm), Sephadex LH 20 prep grade, Acetic acid (Merck, Germany), Formic acid (Merck, Germany), Chloramphenicol (Mediphenicol, China), Ciprofloxacin (Basic Pharma, India), and Miconazole (Basic Pharma, India). General solvents used for purificationare all analytical grade (Sigma Aldrich, Germany) and include: ethyl acetate, methanol, n-hexane, and Dichloromethane, Dimethyl sulphoxide (DMSO) was used as a solubilizing buffer in cytotoxicity assay and negative control in antimicrobial studies. Distilled water and methanol of HPLC grade were used as HPLC solvents for separation.

Instruments.

The instruments used in this study include a vacuum pump machine (Vaccubrand, Germany), glass column (300 × 40 mm), analytical balance (Model- Scout Pro SP401, Ohaus®, USA,), refrigerator, TLC plate (silica gel coated on aluminum sheet F254 nm), TLC glass chamber, UV Transilluminator (254, 366 nm, Visible spectrum), incubator (Genlab, UK), autoclave

(EQUITRON, Partially Automatic Autoclave, by Medica Instrument Manufacturing Co., India), and 5% CO_2incubator,

Analytical HPLC components include: pump (P580A LPG. Dionex), autosampler (AS1-100, Dionex), photodiode array detector (UVD 340S, Dionex) column oven (STH 585 Dionex), column (Eurosphere 100-C18; 5 µM; 125 x 4 mm: with integrated pre column), and software (Chromeleon 6.30). Semi preparative HPLC components include: pump (L-7I00. Merck Hitachi), photodiode array detector UV-L7400, Merck/Hitachi), column (Eurosphere 100-C18: 10 µm, 300 mm: Knauer), and pre-column (Eurosphere 100-C18: 10 µm; 30 x 8 mm; Knauer.

TEST MICROORGANISMS

Clinically cultured laboratory resistant strains of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Aspergillus niger*, and *Candida albicans* were obtained and authenticated at the Department of Pharmaceutical Microbiology and Biotechnology(Nnamdi Azikiwe University, Awka, Nigeria).

Isolation and Identification of Endophytic Fungi

Isolation and molecular identification of endophytes fungal strain from leaves of *O. gratissimum* was done at the Institute of Pharmaceutical Biology(Henrich University, Dusseldorf, Germany)according to the methods described by [21]. In brief, fresh leaves of *O. gratissimum* were harvested from the botanical garden of the faculty of Pharmaceutical Sciences (Nnamdi Azikiwe University, Anambra State). The leaves were washed thoroughly under running water to remove dirt before processing. Epiphytic microorganisms were eliminated by surface sterilization in 70% ethanol for 2min, and in sodium hypochlorite solution (4%) for 3 min, after which they were finally rinsed in sterile distilled water for 2 min. The samples were left to dry in the laminar flow on a sterile filter paper. A sterile knife was used to cut the samples to approximately 1 cm in length and inoculated them on prepared Malt Extract Agar (MEASigma Aldrich, Germany) plates. The cut end of the plant material was made to come in contact with the media using sterile forceps. Petri dishes were properly sealed using parafilm and then incubated at 30°C. After 7 days, hyphae tips of actively growing fungi from the plant material were removed and sub-cultured in fresh sterile MEA plates and incubated for 7 days. Purification of the fungi was done by subculturing the hyphal tips of fungi, by carefully picking the tips with the aid of sterile forceps and placing them on a freshly prepared MEA aseptically. Incubation was done at 30°C for 7 days. The identification of pure fungus was done through DNA amplification by Polymerase Chain Reaction (PCR) (Hot StarTag Master Mix Taq polymerase and primer pairs ITS1 and ITS4), sequencing and subsequent BLAST searches of the NCBI GenBank data. The identified pure fungus was subsequently subjected to fermentation for production of bioactive fungi metabolites. The fermentation medium comprised 100 g of local (Nigerian) rice, which was placed in a 1L Erlenmeyer flask. A volume of 200 ml of distilled water was poured into the flask and sterilized at 121°C for 30 min and was allowed to cool. Thereafter, pure segments of the endophytic fungi were aseptically cut from the actively growing pure isolates on MEA and inoculated onto the fermentation medium contained in the 1L Erlenmeyer flask. The flask was properly sealed and kept on the shelf. The fermentation process was allowed for 21 days at 30°C under static conditions leading to the production of fungal biomass from the pure isolates. The fungal biomass, including the fermenting medium, was cut into small lumps using a sterile glass rod and the mixture was homogenized with 500 mL of ethyl acetate in 1L Erlenmeyer flasks. This was placed on a rotary shaker for 18 h and then filtered using Whatman filter paper (size: 188 mm). The filtrate was air-dried at room temperatureat 25 ^{o}C (what temperature) in order to preserve the constituent of the crude extract.

Antimicrobial Assay

The antimicrobial screening was done according to the method described by [22]. Antimicrobial screening of fractions of the fungal extracts was carried out using the agar well diffusion assay

method[22]. A concentration of 1 mg/mL of the samples was prepared by dissolving the extracts in DMSO. Standardized broth cultures of test bacterial isolates (*Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella typhi*) 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 samples and controls. The plates were then kept at room temperature(25 ^{o}C) for 1 hour 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°C for 24 h, and the SDA plates were incubated at 25-27°Cfor 3days. The inhibition zone 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 were calculated and recorded.

Determination of Minimum Inhibitory Concentrations (MICs)

The Minimum Inhibitory Concentrations (MICs) of fractions of the endophytic fungal extracts were determined according to the method described by [22] against the test organisms on which the extracts showed activity in the preliminary antimicrobial screening. The MICs of the fungal extracts were determined using the agar dilution method according to the guidelines of the CLSI (2018). A stock solution of 5 mg/ml was prepared for each sample and these were further diluted in a 2-fold serial dilution to obtain the following concentrations: 2.5, 1.25, 0.625, 0.3125, and 0.15625 mg/mL. Agar plates were prepared by pouring 4 mL of molten double strength MHA and SDA (for bacterial and fungal isolates, respectively) into sterile Petri plates containing 1mL of the various dilutions of the sample making the final plate concentrations become 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 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 extract. The MHA plates were then incubated at 37°C for 24 hours and the SDA plates were incubated at 27°C for 3days, after which all plates were observed for growth. The minimum dilution (concentration) of the samples completely inhibiting the growth of each organism was taken as the minimum inhibition concentration (MIC). This procedure was conducted in triplicate.

Vacuum Liquid Chromatography on Silica

The column was packed using the dry-pack method. A piece of cotton wool was inserted into a clean dry glass column of 30 cm height and 4 cm internal diameter, followed by the addition of 40 g absorbent silica gel of mesh size (200-400 mm) with continuous tapping until a stably packed bed was formed up to a height of 15 cm on the glass column. The ethyl acetate crude extract (6 g) was loaded after triturating with a small amount of the silica gel and covered with a layer of coarse sizes of the silica gel (70-230 Mesh). A piece of cotton wool was placed above the setup to allow for gentle permeation of liquid solvents when poured through the setup. The column was connected to a vacuum to provide the needed negative pressure. A 1000 ml volume of solvent systems comprising a series of mobile phases in a nonpolar to polar direction were eluted through the column resulting in the collection of different fractions of the crude extract in a round bottom flask. The mixtures used in order of increasing polarity are n-hexane 100%, n-hexane/ethyl acetate 70:30, n-hexane/ethyl acetate 50:50 Dichloromethane/methanol 95:5, Dichloromethane/methanol 80:20, and Methanol 100%. The column was connected to a vacuum pump, which increased the elution speed of each fraction. The column was allowed to run dry before another fraction was eluted. The collected fractions were concentrated using rotary evaporator and used for biological analyses.

HPLC

Analytical HPLC was used to identify important peaks in the fractions as well as to evaluate the purity of isolated compounds. The HPLC analysis was performed using a Dionex Ultimate 3000 System coupled to a photodiode array detector (DAD300RS). The separation column used was (125 × 4 mm, L × i.d.) prefilled with Eurospher-10 C18. Each of the dried fungal fractions (2 mg) was reconstituted with 2 mL of HPLC grade methanol. The mixture was sonicated for 10 min and thereafter centrifuged at 3000 rpm for 5 min. Then, 100 µL of the dissolved samples were each transferred into HPLC vials containing 500 µL, of HPLC grade methanol. The gradient elution used started from 10:90% MeOH: nanopure H2O (0.1% formic acid) to 100% MeOH in 60 min. Peaks were detected at 235, 254, 280, and 340 nm, and known substances were identified by a comparison library using the online software.

Results

Isolation and Identification of the Fungi

The isolated pure fungi endophyte was identified by molecular characterization as *Fusarium equiseti*.

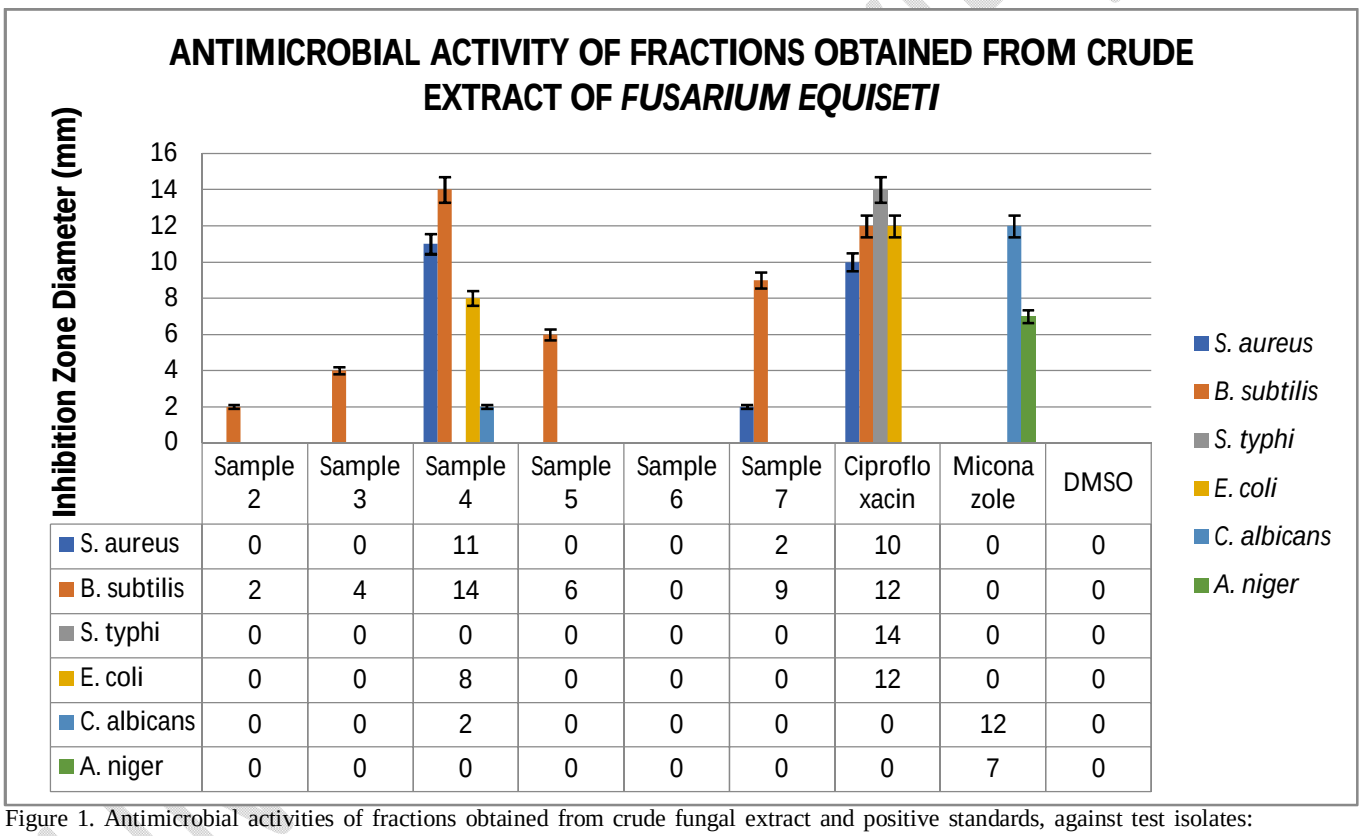
Vacuum Liquid Chromatography on silica

The result of the column chromatography of the crude extract on silica gel yielded six fractions (samples : 2, 3,4,5,6). They include n-hexane/ethyl acetate 70:30, n-hexane/ethyl acetate 50:50 Dichloromethane/methanol 95:5(a), Dichloromethane/methanol 95:5(b), Dichloromethane/methanol 80:20, and Methanol 100%, in their order of increasing polarity.

Antimicrobial studies

The result of the antimicrobial activities of the fractions of the crude extract of *Fusarium equiseti* is presented in Figures 1 and 2. The result showed the fractions showed varied levels of antimicrobial activity against the tested organisms. The result showed that sample 4 need to use a term consistently throughout the manuscript) had the best inhibition against the *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* but no inhibition against *Aspergillus niger* and *Salmonella typhi*. It showed that sample 2 had an inhibition zone diameter of 2 mm against *Bacillus subtilis* but showed no activity against *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger*. Sample 3 had an inhibition zone diameter of 4 mm against *Bacillus subtilis* but showed no activity against *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger*. Sample 5 had an inhibition zone diameter of 6 mm against *Bacillus subtilis* but showed no activity against *Staphylococcus aureus*, *Salmonella typhi*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger* while Sample 6 had no activity against all the tested micro-organism. Sample 7 had inhibition zone diameters of 2 and 9 mm against *Staphylococcus aureus* and *Bacillus subtilis* respectively. Ciprofloxacin showed inhibition against *Bacillus subtilis* (12 mm), *Staphylococcus aureus* (10 mm), *Salmonella typhi* (14 mm), *Escherichia coli* (12 mm) but no inhibition *Candida albicans* and *Aspergillus niger* which are fungi. Miconazole had no inhibition against all the bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli*) but had inhibition diameters of 12 and 7 mm against *Candida albicans* and *Aspergillus*, respectively. The result further showed that in all the samples the fractions of the crude extract of *Fusarium equiseti* had no effect against *Salmonella typhi* and *Aspergillus.*

Fractions 2 and 3, which contained oil components in the mixture showed little or no activity. This could be due to the length of the hydrophobic chain, which decreases the polarity of the compounds and makes them less able to penetrate the aqueous gel media. Application of an agar dilution method could bring an improvement in their antimicrobial activity study. *Salmonella typhi* showed complete resistance to all the fractions tested; indicating no potential for this fraction in the treatment of typhoid fever. No antifungal activity was also observed by all fractions against *Aspergillus niger*. Sample 4 showed a broad range of antimicrobial activity against gram-positiveand –negative bacteria, and fungi. Gram-positive organisms were most sensitive to the fractions, suggesting that the antimicrobials may be working by a mechanism involving disrupting the bacterial cell membrane (needs an elaboration here). DMSO showed no inhibitory activity, indicating the antimicrobial neutrality of the solvent used. A lot of bioactive compounds detectedare perceived to be responsible for antimicrobial activities recorded (in this study? or other literatures, clarification needed here).



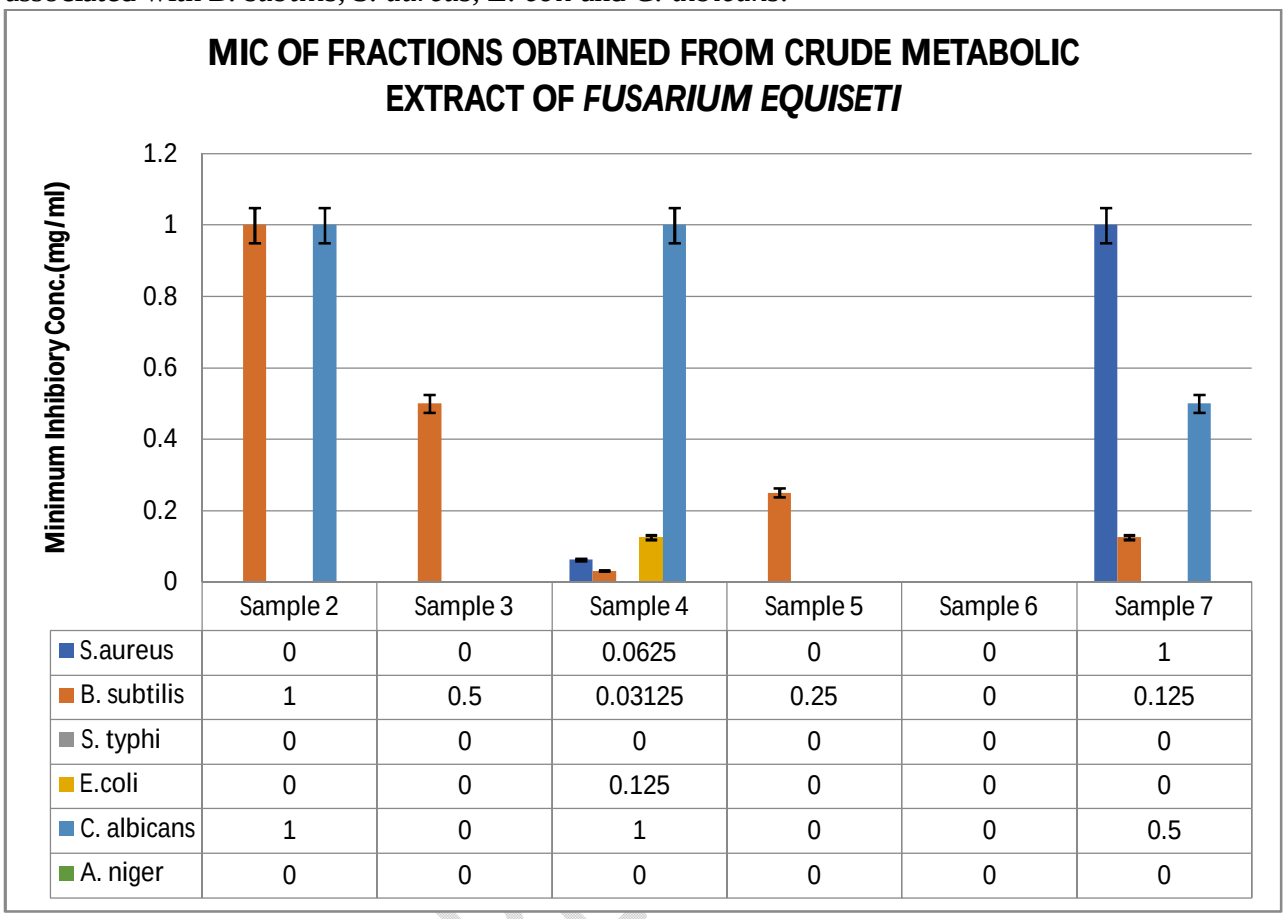
	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Ciproflo xacin	Micona zole	DMSO
S. aureus	0	0	11	0	0	2	10	0	0
B. subtilis	2	4	14	6	0	9	12	0	0
S. typhi	0	0	0	0	0	0	14	0	0
E. coli	0	0	8	0	0	0	12	0	0
C. albicans	0	0	2	0	0	0	0	12	0
A. niger	0	0	0	0	0	0	0	7	0

Figure 1. Antimicrobial activities of fractions obtained from crude fungal extract and positive standards, against test isolates: (Note sample.3 means n-Hexane /ethyl-acetate fraction 50:50; sample.4 means DCM/ Methanol fraction 95:5; sampl.5 means DCM/ Methanol fraction 80:20;sample.6 means Methanol fraction 100;sample.4 means DCM/ Methanol fraction 5:95)

Minimum Inhibition Concentration

The result of the minimum inhibition concentration (MIC) is presented in Fig 2. The result showed that fraction 4 (again, does the authors mean fraction 4?, need to use a term consistently throughout the manuscript) had the 0.06 mg/mL MIC for *S. aureus*, 0.03mg/mL for *B. subtilis*, 0.13mg/mL for *E. coli*, and 1mg/mL for *C. albicans*. *B. subtilis* was susceptible to all the samples except Sample 6 with MICs of 0.03mg/mL for sample 4, 0.125mg/mL for sample 7, 0.25 mg/mL for sample 5, 0.5 mg/mL for sample 3 and 1 mg/mL for sample 2. *C. albicans* was inhibited by samples 2, 4, and 7 with MICs of 1, 1, and 0.5 mg/mL, respectively. Sample 6 showed no inhibition against all the tested organisms. This implied that sample 4 had the best

antimicrobial activity against all the tested micro-organisms except for *A niger* and *S. typhi* and could be harnessed as a potent source of antimicrobial agent for the treatment of diseases associated with *B. subtilis, S. aureus, E. coli* and *C. albicans.*



	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
S.aureus	0	0	0.0625	0	0	1
B. subtilis	1	0.5	0.03125	0.25	0	0.125
S. typhi	0	0	0	0	0	0
E.coli	0	0	0.125	0	0	0
C. albicans	1	0	1	0	0	0.5
A. niger	0	0	0	0	0	0

Figure 2. Minimum inhibition concentration of the fractions obtained from crude fungal extract of *O. gratissimum* endophytic fungus *Fusarium equiseti*and positive standards, against test isolates. (Note sampl.3 means n-Hexane /ethyl-acetate fraction 50:50; sampl.4 means DCM/ Methanol fraction 95:5; sampl.5 means DCM/ Methanol fraction 80:20; sampl.6 means Methanol fraction 100; sampl.4 means DCM/ Methanol fraction 5:95).

Analytical HPLC

The result of the analytical HPLC of the most active fraction led to the detection of numerous bioactive compounds. The chromatogram, UV spectrum and molecular structures of the compounds detected are presented in Figures 3-5.

Figure 3 showed the presence of a compound called Phomoxanthone A. Figure 4 showed the presence of three compounds, which include (A) scalarolide a sesterterpene γ-butenolide, (B) equisetin an N-methyl tetramic acid derivative and (C) 5'- epi-equisetin an episomer of equisetin.

Also, Figure 5 showed the presence of three (3) compounds A,B, and C with 3 different peaks which include Naamine A (an Imidazole alkaloid), Carbonarone A (a benzo-y-prrone), and destruxin B (a cyclic hexadepsipeptide), While Figure 6 (figure4) revealed the presence of 2 compounds such as cytosporin G (an epoxyquinol derivatives), and WLIP called β-hydroxydecanoyl-Leu1-D Glu-De-Thr-Dval-DLeu3 DSer-Leu-Dser-Ile (a lactone ring derivative).

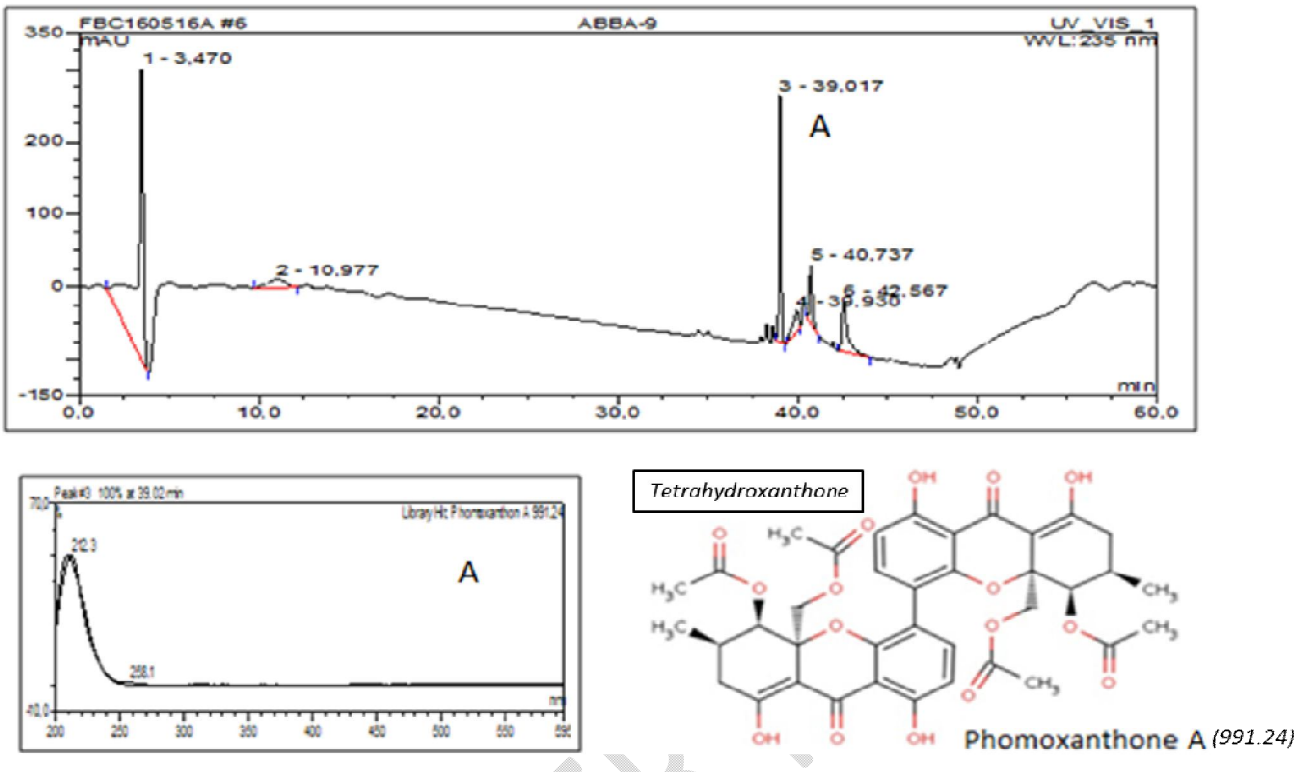


Figure 3: HPLC Chromatogram and UV absorption spectra of the endophytic fungus *Fusarium equiseti* showing the presence of Phomoxanthone A.

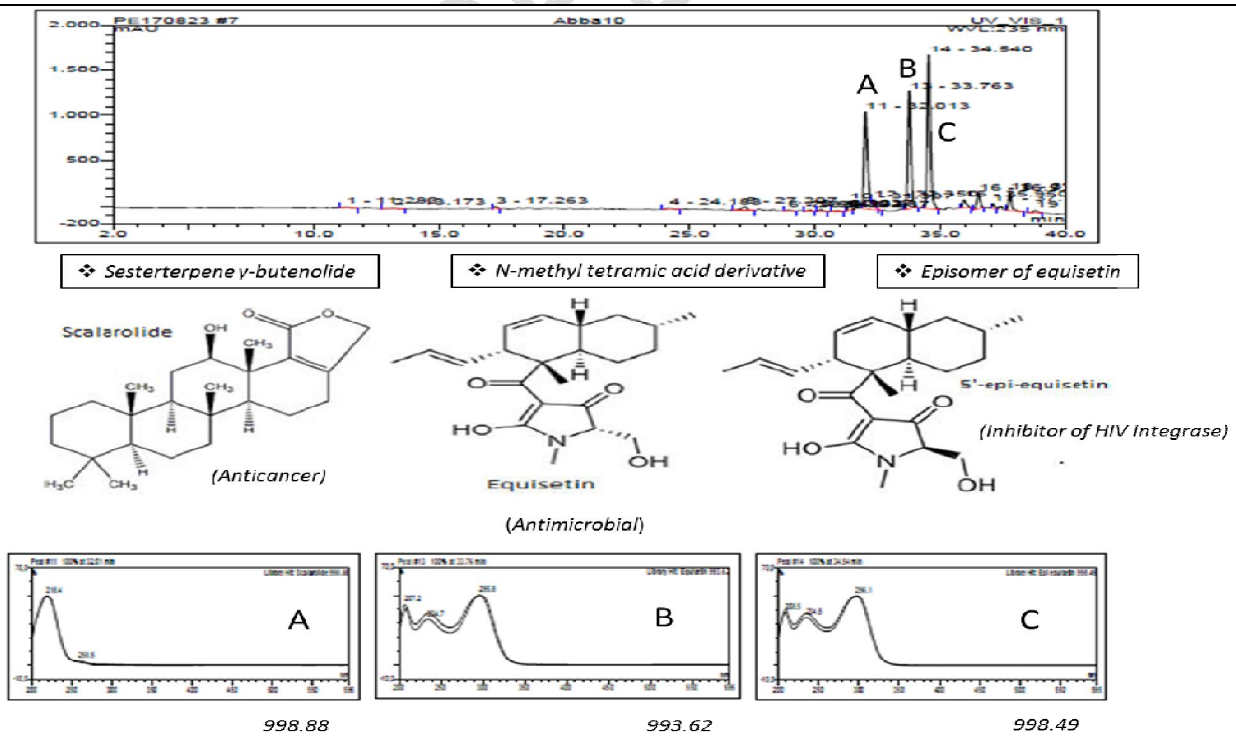


Figure 4: HPLC Chromatogram and UV absorption spectra of the endophytic fungus *Fusarium*

equiseti showing three compounds such as (A) scalarolide, (B) equisetin, and (C) 5' - epi-equisetin.

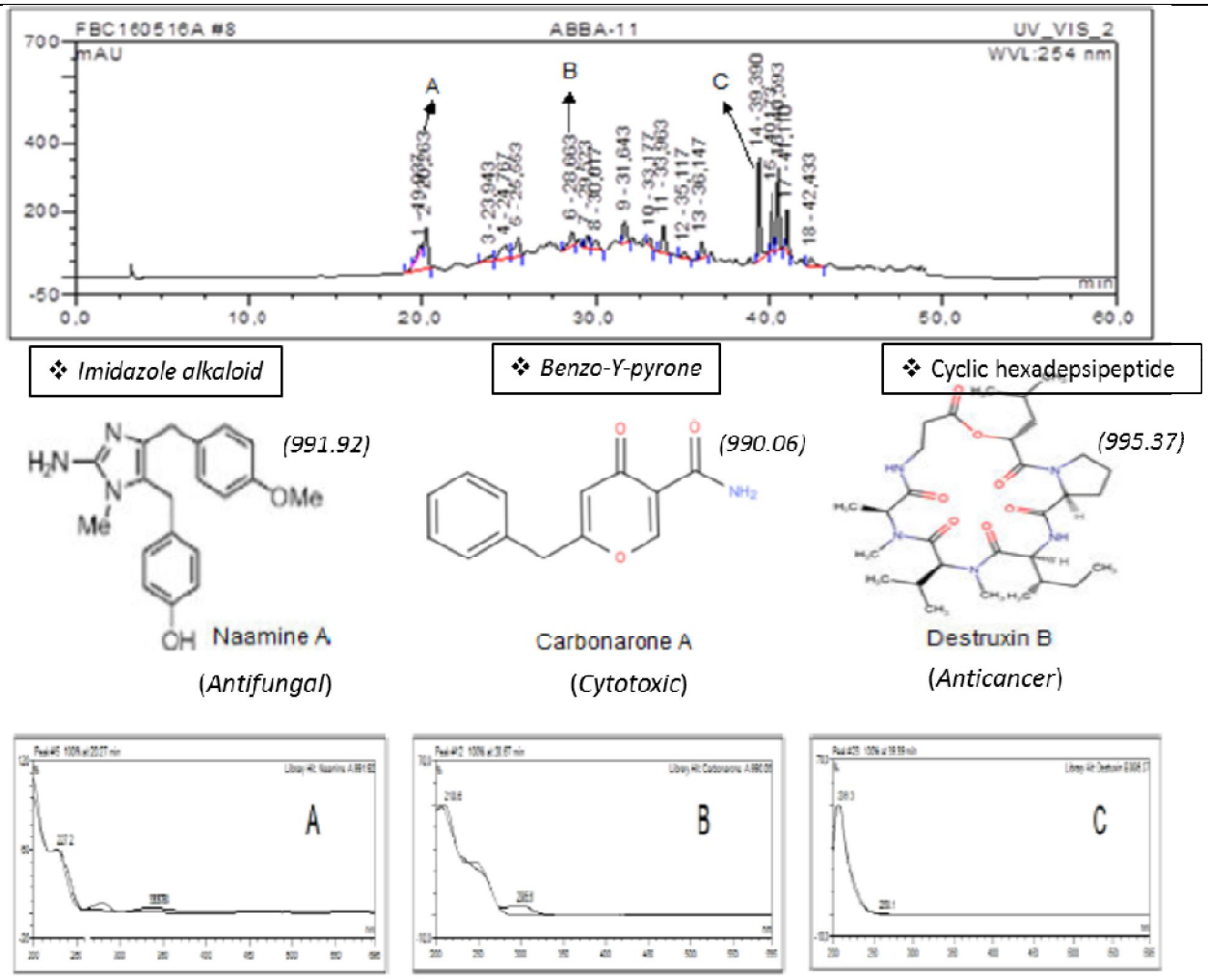


Figure 5: HPLC Chromatogram and UV absorption spectra of the endophytic fungus *Fusarium equiseti* showing 3 different compounds (A) Naamine A, (B) Carbonarone A, and (C) destruxin B.

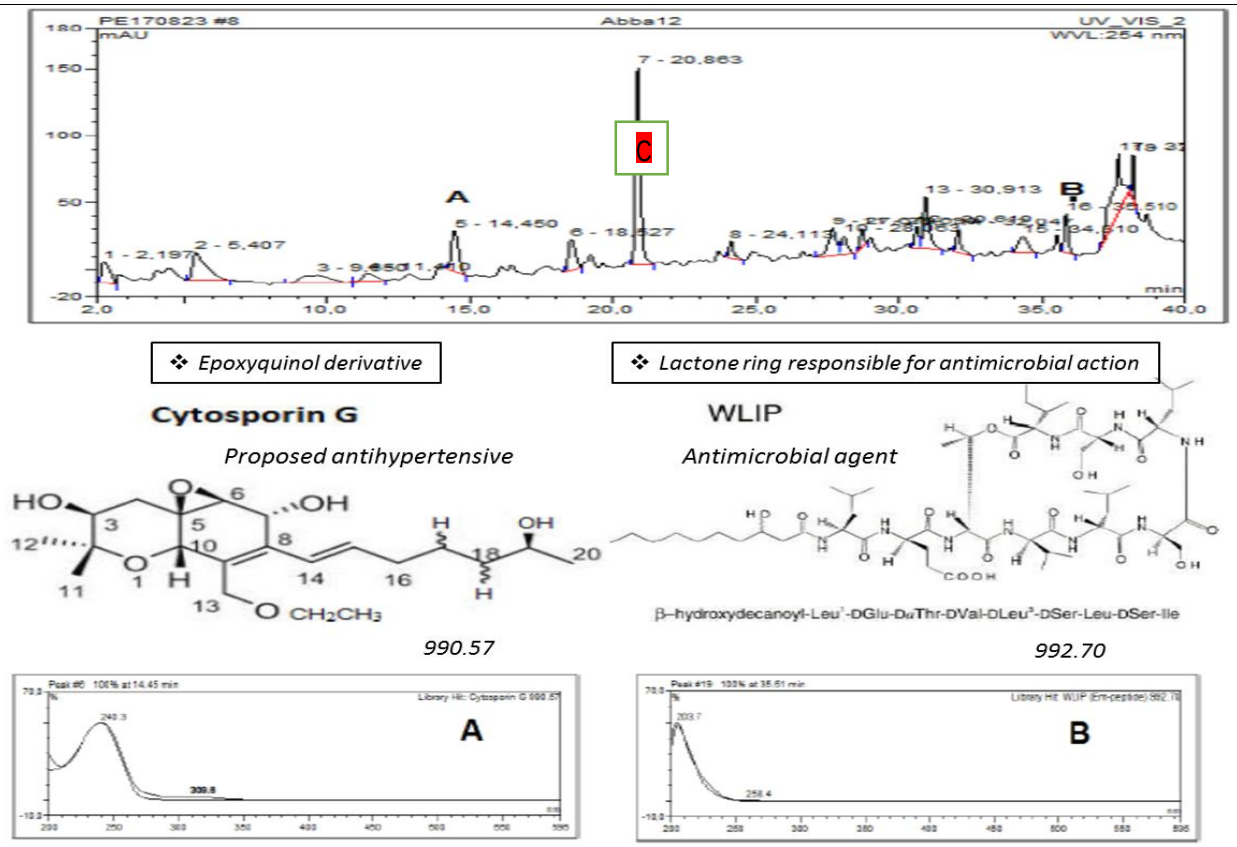


Figure 6: HPLC Chromatogram and UV absorption spectra of the endophytic fungus *Fusarium equiseti* showing two compounds (A) Cytosporin G and (B) WLIP called β-hydroxydecanoyl-Leu[1]-D Glu-De-Thr-Dval-DLeu[3] DSer-Leu-Dser-Ile.

DISCUSSION

The result showed that the fractions obtained from the crude extract of the endophytic fungus (*Fusarium equiseti*), which was isolated from *O. gratissimum*, had varying levels of antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* but no inhibition against *Aspergillus niger* and *Salmonella typhi*. The antifungal activity observed for this fraction could be attributed to the compound naamine detected in fractions 4 and 7. Sample (Fraction?) 5 showed a moderate but specific antibacterial activity against *B. subtilis*. Therefore, this implied that extracts of the endophytic fungus, *Fusarium equiseti*, which was isolated from *O. gratissimum* could represent a potential source of bioactive compounds, due to the presence of compounds previously reported for antimicrobial properties present in this extract.

The result of the inhibition zone diameters is supported by the minimum inhibition concentration and still suggest that sample 7 and 4 had the best antimicrobial activity against most of the tested resistant micro-organism.

All the fractions (except for sample 6) showed MIC value of ≤1 mg/mL but samples 4 and 7 showed a broad spectrum of activity against gram-negative and gram-positive resistant (against what?) micro-organisms tested.

Phomoxanthone A, shown in figure 3 is a chemical compound previously isolated from Phomopsis (move this sentence to somewhere else for better read of the manuscript). Numerous compounds including Phomoxanthone A, equisetin, epi-equisetin, Naamine, and WLIP compounds have shown antimicrobial activity in previous work respectively. In detail, Phomoxanthones A [23] and Phomoxanthones B [24] were reported to exhibit significant activity against clinical strains of *Plasmodium falciparum* and *Mycobacterium tuberculosis*, and also was reported to have a cytotoxic effect on two cancer cell lines [25]. Scalarolide shown in figure 4 is a marine sesterterpene first isolated from the Okinawan Sponge *Hyrtios erectus* [26]). The scalarolide, among other acyclic triterpenes, is known for anticancer activity [27]. Equisetin and epi-equisetin (Figure 4) are stereoisomeric derivatives of the same compound that was first isolated and characterized as an antibiotic compound from *Fusarium equiseti* [28] and were reported to have activity against human immunodeficiency virus type 1 (HIV-1) integrase [29]. Equisetin (figure 4) has also been shown to exhibit activity against several gram-positive bacteria (*Mycobacterium phlei, M. smegmatis, M. rhodochrus, Bacillus subtilis, Staphylococcus aureus*) and a gram-negative bacterium called *Neisseria perflava* but had no activity against other gram-negative bacteria [30].

Destruxin B shown in figure 5 is a cyclic hexadepsipeptide mycotoxin first isolated from *Oospora destructor* and is believed to contribute to the virulent nature of these entomopathogenic fungi reported to exhibit insecticidal [31] and phytotoxic activities. Additionally, it is prone to metabolization by plants [32]. Destruxin B has also been reported to induce apoptosis in human non-small cell lung cancer cells in culture [33] and suppresses the expression of the hepatitis B viral surface antigen (HBsAg) gene in human hepatoma cells [34].

Carbonarone A (figure 5) identified in chromatogram 11 is a bioactive gamma-Pyrone isolated previously from the marine fungus *Aspergillus carbonarius* [35]. Carbonarone A (Figure 5) is a monocarboxylic acid amide that was previously reported for strong cytotoxicity against K562 cells and therefore could be responsible for the cytotoxicity effect observed with the crude endophytic fungal extract.Naamine A (figure 5) belongs to imidazole alkaloids isolated from a red sea sponge *Leucetta chagosensis* [36] and was previously reported to possess strong antifungal and anti-tumor properties [37].Also seen in figure 6 corresponds to a library compound named Cytosporin G, a new Epoxyquinols, previously reported by [38] to have shown no activity against mouse lymphoma cells and therefore proposed to be an anti-hypertensive regulatory agent. Another compound identified in figure 6 is the *W*hite *L*ine *I*nducing *P*rinciple (WLIP), a lipodepsipeptide (LDP) with a molecular weight of 1125 Da composed of an N-terminal β-hydroxydecanoic acid and a peptide moiety of nine amino acids. WLIP is produced from the bacteria *Pseudomonas reactans and has been* reported to possess antimicrobial, antibacterial, and antifungal properties [39].

Figure 6 also showed the most prominent peak at 20.86 min rt, which corresponds to a compound whose library hit was undetected and labeled as C. The compound is under development but switching to a more enriched library or doing a comparison with an online database would help identification of this compound which seems to be very important in the bioactivity of the fraction.

Conclusion

The results of this study suggest that endophytic fungi associated with *O. gratissimum* produced compounds that have great antimicrobial activity and could be a potential source of novel compounds for pharmaceutical application with limited potency for the treatment of clinically important micro-organisms (because the compounds were not effective against *Aspergillus* and *Salmonella*).

Data Availability Statement

Data generated or analyzed during this study are provided in full within the published article and its supplementary materials.

Reference

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