Antimicrobial, Cytotoxic, Antiviral Effects, and Spectroscopic Characterization of Metabolites Produced by *Fusarium oxysporum* YP9B

Gözde Kılıç, Gonca Tosun, Arif Bozdeveci, İshak Erik, Elif Öztürk, Rengin Reis, Hande Sipahi, Merve Cora, Şengül Alpay Karaoğlu and Nurettin Yaylı

1Institute of Health Sciences, Karadeniz Technical University, Trabzon, Türkiye
2Department of Pharmacognosy, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, Türkiye
3Department of Biology, Faculty of Science, Recep Tayyip Erdoğan University, Rize Türkiye
4Departments of Nutrition and Dietetics, Faculty of Health Sciences, Karadeniz Technical University, Trabzon, Türkiye
5Department of Toxicology, Faculty of Pharmacy, Yeditepe University, İstanbul, Türkiye
6Department of Toxicology, Faculty of Pharmacy, Acıbadem Mehmet Ali Aydınlar University, İstanbul, Türkiye
7Department of Medical Microbiology, Karadeniz Technical University School of Medicine, Trabzon, Türkiye

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**Abstract:** The goal of the work is to determine the bioactive pharmaceutical metabolites produced by the *Fusarium oxysporum* YP9B isolate. Ten new natural compounds were isolated from the ethyl acetate extract of the *F. oxysporum* YP9B strain. Their structures were elucidated by spectroscopic methods using 1D and 2D NMR, UV, FT-IR, and mass spectra (LC-QTOF MS and GC-FID/MS). Identified compounds were named as; (1-benzyl-2-methoxy-2-oxoethyl)-2-hydroxy-3-methylbutanoate (1), 2-oxo-8-azatricyclo[9.3.1.1^{7,11}]-hexadeca-1(15),3(16),4,6,11,13-hexaen-10-one (2), 2,3-dihydroxypropanoic, hexadecanoic anhydride (3a), 2,3-dihydroxypropanoic (9Z)-octadecenoic anhydride (3b), 2,3-dihydroxypropanoic (9Z,12Z)-octadecadienoic anhydride (3c), 2,3-dihydroxypropanoic (11Z)-octadecenoic anhydride (4a), 2,3-dihydroxypropanoic, (9E,12E)-octadecadienoic anhydride (4b), 3-hydroxy-1,2,6,10-tetramethyleneundecyl hexadecanoate (5a), 3-hydroxy-1,2,6,10-tetramethyleneundecyl (9E)-octadecenoate (5b), and 3-hydroxy-1,2,6,10-tetramethyleneundecyl octadecanoate (5c). Antimicrobial activities of the isolates obtained from the YP9B strain were determined. Cytotoxic and antiviral activities were tested for the isolates against VERO, MCF-7, PC-3, and A549. Compounds 5a-c, 1, and 3a-c showed bacteriostatic activity at low concentrations, and 4a-b and 2 were found to be bactericidal. MIC and MBC values against *Mycobacterium smegmatis* for the compounds 5a-c and 1 were determined to be <0.5 µg/mL and 0.46 µg/mL, respectively. The experimental result showed that compounds 2, 5a-c, and 1 have strong cytotoxic (7.51±1.38 and 19.13±0.68 (µM) IC_{50}) activity. The antiviral activity against HSV type-1 was determined to be 1.25 µM for compounds 4a-c and 0.312 µM for compound 1.

**Keywords:** *Fusarium oxysporum* YP9B; secondary metabolite; antimicrobial; cytotoxic; antiviral. © 2020 ACG Publications. All rights reserved.

*Corresponding authors: E-mail address: yayli@ktu.edu.tr (N. Yaylı); Phone: 05333153187; sengualpay@yahoo.com (Ş.A.Karaoğlu)
1. Introduction

The use of fungi for commercial production is ancient, but it has increased rapidly over the last 50 years [1-3]. The pathogenic/nonpathogenic species of fungi are known to release volatile or non-volatile metabolites in their presence. Numbers of Fusarium species with and without plant pathogens have been identified [2]. The majority of them have been found to secrete mycotoxins, and different chemical structures [4] of these toxins continue to be elucidated. Mushrooms have broad biodiversity. Various interdisciplinary studies were ongoing on the secondary metabolites secreted by nonpathogenic Fusarium species such as F. oxysporum, Fusarium graminearum, and Fusarium moniliforme [4-19]. Many studies have focused on the toxicity and diversity of Fusarium species on cereals [20-22]. However, there is little information about secondary metabolites produced by Fusarium that infects fruit-bearing plants rather than cereals [23-24]. In the literature, Alternaria sp., Sinopodophyllum, and F. oxysporum were endophyte fungi isolated from Sabirna recurva [14, 25]. In a study, camptothecin type two analogs (9-methoxycamptothecin and 10-hydrocycamptothecin) were reported as anti-cancer microbial products [26]. These microbial products were determined to be produced by the endophyte fungus Fusarium solani, isolated from Camptotheca acuminata. Many secondary metabolites produced by many Fusarium species cause different physiological and pharmacological responses in plants and animals. It is well known that Fusarium species produce trichothecene mycotoxins [4], but these species are also known to have as other compounds; pigments, antibiotics, phytotoxins, etc. [5-19, 27].

In our study, F. oxysporum YP9B is isolated from garden soil where vegetables are grown in Pazar-Rize (Eastern Black Sea region of Türkiye). YP9B isolate was determined to produce metabolites containing antimicrobial, cytotoxic and antiviral activities. Details of the isolation, structure elucidation of ten new compounds, and biological activities are presented herein.

2. Materials and Methods

2.1. General

Optical rotations were measured on Automatic AA-5 Series polarimetry. UV spectra were obtained with a Spectrostar nano BMG labtech spectrometer. Infrared spectra were obtained with a PerkinElmer 1600 FT-IR (4000-400 cm\(^{-1}\)) spectrometer. The mass spectral analyses were carried out on an Agilent 6230A LC-Q-TOF-Q-MS. Shimadzu QP2010 ultra GC-FID/MS was used to identify the FAMEs. Melting points were determined using the Thermo-var apparatus fitted with a microscope and are uncorrected. \(^1\)H and \(^13\)C NMR, along with 2D NMR spectra, were obtained on a Bruker 400 MHz NMR spectrometer (400 MHz for \(^1\)H, 100 MHz for \(^13\)C), using TMS as an internal standard. CDCl\(_3\) and acetone-d\(_6\) were used as NMR solvent. ACD NMR program was used for the elucidation of isolated compounds. Some of the carbon peaks may exchange in the NMR spectra of compounds 3, 4, and 5 series, which were overlapped due to each series' mixture. Chemical shifts were expressed in \(\delta\) (ppm), and coupling constants (\(J\)) were reported in hertz (Hz). TLC was carried out on Silica gel 60F\(_{254}\), and the spots were visualized by spraying with 20% H\(_2\)SO\(_4\) and heating. Silica gel was used for column chromatography. Routine laboratory equipment's used were: Refrigerated Centrifuge (Sigma), pH meter (Hanna), Shaker (GFL), Thermoblok (Nosheng), Incubator (Memmert), Shaking oven (Nüve), Gel Imaging System (Uvp), Centrifugal (Sartorius), Bidistilled water (Gfl), Device Security cabinet (Nüve), Power supply (Owl), Autoclave (Nüve), Analytical balance (Denver), and Horizontal Shaker (Nüve).

2.2. Substrate and Chemicals

The substrate agar, PDA, yeast extract, Müller Hilton agar, Müller Hilton broth, peptone, corn meal, malt extract agar, Mg\(_2\)SO\(_4\), Na\(_2\)SO\(_4\), NaCl, sodium acetate, NaOH, KH\(_2\)PO\(_4\), 3H\(_2\)O, K\(_2\)HPO\(_4\),
glucose, dichloran, rose bengal, chloramphenicol (DRBC), agar, HCl, glycerol, ethanol, ethyl acetate, chloroform, acetone, n-hexane, methanol, silica gel, agarose, yeast nitrogen base, and yeast carbon base were purchased from by Sigma-Aldrich, Fluka or Merck unless otherwise stated. They were in analytical grade unless otherwise stated.

2.3. Molecular Diagnosis, Revitalization and Extraction from Fusarium sp. YP9B Strain

The YP9B strain used in the study was isolated from the tomato plant root in Pazar-Rize, Turkey, in August 2014 [28]. The isolated Fusarium sp. is coded as YP9B regarding the region it was isolated (Highway edge in town of Pazar-Rize) [29]. YP9B isolation and characterization were done in Recep Tayyip Erdoğan University Microbiology and Molecular Biology Research Laboratory [30]. The most crucial criterion in the identification of Fusarium species is morphological features, and traditional diagnosis has been made according to the literature information [28,30]. To confirm the conventional diagnosis, F. oxysporum YP9B has been identified by molecular diagnosis using ITS sequence analysis (18S rRNA ITS1-5.8S-ITS2 intragenic gene regions) and NCBI database (version 5) [31]. Fusarium sp. is defined as YP9B strain, which was before characterized according to the molecular method [31]. F. oxysporum YP9B was determined to be 99% similarity (GB code MT539140) compared to the sequences existing in GenBank (Table 1).

| Isolate                  | ITS 1-5.8S-ITS 2 | Overlap (%) | Similarity (%) | Genbank no   |
|-------------------------|------------------|-------------|----------------|--------------|
| YP9B                    | F. oxysporum     | 93%         | 99.45%         | MT447537.1   |
|                         | strain GFR32     |             |                |              |
|                         | F. oxysporum     | 93%         | 99.45%         | KP942940.1   |
|                         | strain EECC-643  |             |                |              |
|                         | Fusarium sp.     | 93%         | 99.45%         | KT268977.1   |
|                         | strain P1704     |             |                |              |
|                         | F. oxysporum     | 93%         | 99.45%         | MH879861.1   |
|                         | isolate FUS-33   |             |                |              |
|                         | F. oxysporum     | 94%         | 99.27%         | KU847855.1   |
|                         | isolate 107      |             |                |              |

F. oxysporum YP9B isolate was cultured on a plate of potato dextrose agar at 28 °C for ten days. Potato dextrose agar (PDA) included of Potato Infusion 4.0 G/L (Nitrogen Content 3.0-3.4 % and Amino-N 2.0-2.2%), D-(-)-Glucose 20.0 G/L and Agar-Agar 15.0 G/L). The media's final pH was adjusted to 5.6±0.2 (25°C) before sterilization [32]. The YP9B strain was taken from the freezer (at -80°C) was cultivated at 28°C for a week by sowing 100 µL to the potato dextrose agar (PDA) medium. Fresh cultivation (inoculation) was performed in spot cultivation (3-4 dots) in the plates obtained from PDA agar prepared as a total volume of 10 L. Cultures were incubated for ten days at 28 °C in an incubator. Later agar plates were cut into small pieces with a sterile scalpel and taken into an Erlenmeyer flask (2 L) with a maximum of 500 g isolate with ethyl acetate (1:1 ratio). The media of F. oxysporum YP9B was then incubated at 25°C on a rotary shaker at 200 rpm for 48 hours. After the extraction, it was filtered with sterile filter paper and kept at -20°C until it was used.

2.4. Secondary Metabolite Isolation

The ethyl acetate extract (6 L) was completely evaporated under vacuum to afford the crude extract (5 g). The crude extract (4.5 g) was subjected to Silica gel (230-400 mesh) column chromatography using n-hexane (100 mL), n-hexane-chloroform (1:1 100 mL), chloroform (100 mL), chloroform-ethyl acetate (1:1, 100 mL), ethyl acetate (100 mL), ethyl acetate-methanol (9:1; 8:2, 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; and 1:9; 100 mL, each), and methanol (100 mL) gradient elution to afford 30 fractions (~50 mL each). Fractions were control by TLC and were visualized by UV lamp (254 nm) and spraying with 20% H2SO4 and heating. In this context, isolated 10 new compounds were obtained from the fractions as follows; compound 1 (80.6 mg) from the fraction 11, compound 2 (17.8 mg) from the fraction 5, compounds 3a-c (194.2 mg) from the fraction 2, compounds 4a-b (61.7 mg) from the fraction 3, and compounds 5a-c (151.0 mg) from the fraction 9 within the scope of the work.
New metabolites produced by *Fusarium oxysporum* YP9B

2.5. Preparation of Fatty Acid Methyl Esters (FAMEs)

Compounds 3a-c, 4a-b, and 5a-c (~15 mg each) were heated with 5% sodium hydroxide solution in methanol (2 mL) at 70°C for 3 h. The solutions were cooled, and the aqueous mixture was neutralized with 2 N HCl and extracted with diethyl ether (2 mL x 3). The organic layer was separated and washed with water (2 mL), dried over anhydrous Na₂SO₄, filtered, and the solvent evaporated. Approximately 5-6 mg of each sample was dissolved in methanol (2 mL) in a test tube, and the solutions were cooled in an ice bath; an excess of BBr₃ was then added, dropwise. The tube was heated in a boiling water bath at 100°C for three h and cooled. Then water (3 mL) was added, and methanol evaporated. The aqueous layer was extracted with HPLC grade n-hexane (2 mL x 2 times), shaking briefly. The n-hexane layers were washed with potassium bicarbonate solution (2 mL, 2%), dried over anhydrous Na₂SO₄, and filtered. The organic solvent was removed under reduced pressure in a rotary evaporator to give FAMEs.

2.6. Gas Chromatography-Mass Spectrometry (GC-FID/MS)

FAMEs analysis was carried out on a Shimadzu QP2010 ultra GC-MS, Shimadzu 2010 plus FID, fitted with a PAL AOC-5000 plus autosampler Shimadzu Class-5000 Chromatography Workstation software. The separation was analyzed using a Restek Rxi-5MS capillary column (30 m x 0.25 mm x 0.25 μm) (USA). FAMEs injections to GC-FID/MS was performed in split mode (1:30) at 230°C. The FAMEs solution (1 μL) in n-hexane (HPLC grade) were injected and analyzed with the column held initially at 60°C for 2 min and then increased to 240°C with a 3°C/min heating ramp. The oven program was as follows: the initial temperature was 60°C for 2 minutes, which was increased to 240°C at 3 minutes, the final temperature of 250°C was held for 4 minutes. Helium (99.999 %) was used as carrier gas with a constant flow-rate of 1 mL/min. Detection was implemented in electronic impact mode (EI); ionization voltage was fixed at 70 eV, scan mode (40-450 m/z) was used for mass acquisition. Each sample was analyzed and mean reported.

2.7. Identification of FAMEs

Retention indices of the FAMEs were determined by the Kovats method using n-alkanes (C₆-C₃₂) as standards. FAMEs were identified by comparisons with literature RI [33-37] and MS compared to existing analytical standards and matching mass spectral libraries (NIST, Wiley7NL, FFNSC1.2, and W9N11).

2.8. Antimicrobial Activity Assessment (Agar-well Diffusion Method)

All test microorganisms were obtained from the Hifzisihha Institute of Refik Saydam (Ankara, Turkey) and were as follows: *Escherichia coli* ATCC25922, *Yersinia pseudotuberculosis* ATCC911, *Klebsiella pneumonia* subsp. *pneumonia* ATCC13883, *Pseudomonas aeruginosa* ATCC27853, *Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC29212, *Streptococcus mutans* RSKK07038, *Lactobacillus casei* RSK591, *Bacillus cereus* 702 Roma, and *Mycobacterium smegmatis* ATCC607 and also *Candida albicans* ATCC60193, *C. tropicalis* ATCC 13803 and *Saccharomyces cerevisiae* RSKK251 were used to determine their antifungal activities. Mueller Hinton agar-liquid (MHB, MHA) media for Gram-negative and positive bacteria, Brain Heart Infusion agar-liquid (BHIB, BHIA) media for *M. smegmatis*, MRS agar for lactobacilli and Potato dextrose agar and Malt extract liquid (PDA, MEB) media for fungi were used for the antimicrobial screening.

Isolates were tested against microorganisms for antimicrobial activity by agar well diffusion method [38-39]. From the overnight cultures of the bacteria to be tested, dilutions of approximately
McFarland at 0.5 turbidities (about $10^{6-7}$ cfu/mL bacteria) (cfu: colony-forming unit) were prepared in MHB, and widespread cultivation was carried out on pre-prepared MHA plates with a sterile swab. For yeast-like fungi, McFarland 2.0 dilutions were made using ME broth, and sterile swab smear was applied to the surface of the previously prepared PDA media. On the medium whose cultivation is completed, wells with a 5 mm diameter were opened at 2 cm intervals with a sterile glass pipe. 50 µL of the filtrate was added to each well from the isolate of the metabolites with their control. Petri dishes containing bacteria were incubated for 24 hours, and Petri dishes containing yeast and M. smegmatis for 48 hours at 35°C. Lactobacilli and S. mutans were incubated for 48 hours in a 5% CO₂ medium. After incubation, their effectiveness was determined by measuring the inhibition zone diameters with a ruler. Ampicillin for bacteria, streptomycin, and fluconazole for fungi was used as standard control drugs. Standard solvents were also used as controls.

2.8.1. **MIC and MBC assay**

The antimicrobial properties of isolated compounds 1-5 were investigated quantitatively in respective broth media by using double microdilution, and the minimal inhibition concentration (MIC) values (µg/mL) were examined [38-39] and used in our previous work [35-37]. The antibacterial and anti-tuberculosis assays were carried out in Mueller-Hinton and Brain Heart Infusion broths at pH 7.2. The microdilution test plates were incubated for 18-48 h at 35°C. The anti-lactobacilli and anti-fungal assays were carried out in MRS and Malt extract broths (Merck, Germany) at pH 6.2, respectively. The microdilution test plates were incubated for 48 h at 35°C in 5% CO₂. The MIC was defined as the lowest concentration that showed no growth. Ampicillin (10 mg/ mL), streptomycin 10 mg/mL and fluconazole (2 mg/mL) were used as standard antibacterial and antifungal drugs, respectively. Dimethyl sulphoxide, with a dilution of 1:10, was used as solvent control. Concentrations (dilutions without microorganism growth) above the MIC value were used to determine the minimal bactericidal concentration values of the extracts. By taking all dilutions above the MIC value (100 µL), passages were passaged at suitable agar media and incubated under appropriate conditions. Dilutions without microorganism development were determined as MBC values.

2.9. **Determination of Cytotoxic and Antiviral Activity**

Isolated compounds (1-5) showed potent antibacterial activity and were then examined for cytotoxic and antiviral activity.

2.9.1. **Cytotoxicity Activity**

VERO culture was used for the cytotoxicity activity of the isolated compounds (1-5). VERO cells, which were 80-90% confluent in Erlenmeyer, were tripinized and counted using trypan blue. $10^5$ cells were cultivated in each well of the 96-well plate with 100 µL of growth medium. The cells were incubated for 5-6 hours at 37°C in an oven containing 5% CO₂ to hold the cells. At the end of the incubation, 96-well plate dilutions were prepared, with specific substances and three wells from each concentration. As a negative control, only wells containing cells were used. Prepared plates were incubated at 37°C in an oven containing 5% CO₂ for 96 hours. 10 µL MTT was added to each well by removing the expired plate. The plate was incubated at 37°C in an oven containing 5% CO₂ for 3.5 hours. At the end of incubation, 100 µL sterile DMSO was added to the wells by removing the wells’ medium. The plates were wrapped in aluminum foil not to see the light and were shaken at room temperature and shaker at low speed for 30 minutes. Plates were read on the spectrophotometer at a wavelength of 570 nm, and the results were evaluated in the Microsoft Excel program regarding the control wells. The viability rate of the cells in the control well was determined to be 100%, and the viability rate of the cells in the wells added to the substance was defined as %.
New metabolites produced by *Fusarium oxysporum* YP9B

2.9.2. Antiviral Activity

VERO cells, which were 80-90% confluent in the flask, were counted using trypan blue. 10⁴ cells were cultivated in each well of the 96-well plate with 100 µL of growth medium. The cells were incubated for 5-6 hours at 37°C in an oven containing 5% CO₂ to hold the cells. The medium in the wells was evacuated. The number of viruses was adjusted on the medium to be 1 MOI based on the number of infections (MOI), and 100 µL of the virus was added to the plate. Plates were removed at 37°C for one hour in an oven containing 5% CO₂, with an interval of 10 minutes, and incubated by shaking. The wells’ media were emptied, and the concentrations of the substances specified in table 2 were prepared with maintenance medium and placed in the wells with 100 µL and three replicates. Acyclovir at a concentration of 25 µg/mL was used as a positive control, and only wells containing virus were used as a negative control. Plates were incubated for three days at 37°C in an oven containing 5% CO₂. At the end of the period, 10 µL of MTT was added to the wells and incubated for 3.5 hours at 37°C in an oven containing 5% CO₂. At the end of the incubation, 100 µL DMSO was added to the wells by removing the medium in the wells. The plates were shaken in a dark environment at room temperature for 30 minutes at low speed. Absorbance values of wells were read at 570 nm in a spectrophotometer. The results were evaluated in the Microsoft Excel program, and the viability rates of the cells were calculated as a percentage. The project was committed to testing against a DNA and an RNA (HSV and polio) virus to determine antiviral activity. Still, only the DNA virus HSV-I (Human herpes simplex virus Type-I) was examined due to a lack of budget.

2.9.3. Cell Viability Assay

Cell viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MCF-7 (human breast cancer), PC-3 (human prostate cancer), and A549 (human lung adenocarcinoma) (ATCC, USA) were seeded in a 48-well plate and incubated for 24 h to form a semi-confluent layer [40-41]. After 24 h, cells were exposed to different concentrations of compounds dissolved in DMSO. After 24 h incubation, MTT was added to all wells at 0.5 mg/mL of concentration and incubated an additional 2 h at 37°C. After discarding the medium from plates, 100 µL of isopropanol was added to the wells. The absorbance of the MTT formazan was determined at 570 nm by a UV-spectrophotometric plate reader. Viability was defined as the ratio (expressed as a percentage) of the cells’ absorbance exposed to compounds to the cells treated with 0.5% DMSO (v/v). As a reference standard, doxorubicin HCl was used. All measurements were done in triplicates.

**Compound 1**: Colorless semi-solid, m.p. 35-38°C; Rf: 0.90 (ethyl acetate-methanol, 9:1); [α]D²⁵ -54.86 (c 0.0056, Ethyl acetate); UV (Ethyl acetate) λmax nm (log ε): 290 (2.36); FT-IR (ATR) 3392 (-OH), 3007 (=C-H), 2919 (CH), 1659 (C=O), 1437(C=C), 1407 (C=O), 1315, 1016, 901, 705 cm⁻¹; C₁₅H₂₀O₅, Positive LC-QTOF-MS: m/z (%) [M-OCH₃+Na+2H]⁺ 274.2987 (100), calc. 274.2985; for ¹H and¹³C NMR spectroscopic data see table 2.

**Compound 2**: Light yellow solid, m.p. 125-127°C; Rf: 0.78 (ethyl acetate); [α]D²⁵ -4.0 (c 0.0025, Ethyl acetate); UV (Ethyl acetate) λmax nm (log ε): 320 (2.88); FT-IR (ATR) : 3355 (-NH), 2926, 2855 (CH), 1673 (C=O), 1588, 1450 (C=C), 1282, 1233 (C-O), 998, 769, 692 cm⁻¹; C₁₄H₁₃NO₂. Positive LC-QTOF-MS: m/z (%) [M+CH₃OH+H₂O]⁺ 274.2815 (100), calc. 274.2813; for ¹H and¹³C NMR spectroscopic data see table 3.

**Compounds 3a-c**: m.p. (mix.) 165-169°C; Rf: 0.86 (chloroform-methanol, 7:2); [α]D²⁵ +3.89 (c 0.0077, CHCl₃); UV (CHCl₃) λmax nm (log ε): 285 (1.95), 290 (3.55); FT-IR (ATR) : 2922, 2853 (CH), 1742 (C=O), 1464, 1377 (C=C), 1161, 1097 (C-O), 972, 722, 699 cm⁻¹; 3a (C₁₀H₁₈O₃), Positive LC-QTOF-MS: m/z (%) [M+CH₃OH+Na]⁺ 399.2561 (8), calc. 399.2576; 3b (C₁₂H₁₈O₃), Positive LC-QTOF-MS: m/z (%) [M+2H+Na]⁺ 395.2750 (6), calc. 395.2762, [M-H₂O+H]⁺ 353.2693 (100), calc. 353.2641; 3c
(C_{31}H_{52}O_3), Positive LC-QTOF-MS: \( m/z \) (%) [M+2Na+H]^+ 415.2449 (10), calc. 415.2435; for \(^1\)H and \(^{13}\)C NMR spectroscopic data see tables 4 and 5.

**Compounds 4a-b:** m.p. 60-64°C; Rf: 0.82 (ethyl acetate); [\( \alpha \)]\(_D\)^{25} +8.33 (c 0.0024, CHCl\(_3\)); UV (CHCl\(_3\)) \( \lambda_{max} \) nm (log e): 292, 2854 (CH), 1744 (C=O), 1464 (C=C), 1164 (C-O), 721, 617 cm\(^{-1}\); 4a (C_{21}H_{38}O_5), Positive LC-QTOF-MS: \( m/z \) (%) [M+Na]^+ 393.2643 (15), calc. 393.2616, [M-H\(_2\)O+H]^+ 353.2689 (100), calc. 353.2651; 4b (C_{21}H_{36}O_5), Positive LC-QTOF-MS: \( m/z \) (%) [M+H\(_2\)O]^+ 386.2692 (6), calc. 386.2699, [M+CH\(_3\)OH]^+ 400.2959 (8), calc. 400.2962; for \(^1\)H and \(^{13}\)C NMR spectroscopic data see tables 4 and 5.

**Compounds 5a-c:** Semi-solid m.p. 36-40°C; Rf: 0.80 (ethyl acetate-methanol, 9:0.5); [\( \alpha \)]\(_D\)^{25} +73.33 (c 0.0025, Ethyl acetate); UV (Ethyl acetate) \( \lambda_{max} \) nm (log e): 300 (2.52); FT-IR (ATR): 3394 (OH), 2964, 2929, 2856 (CH), 1737 (C=O), 1458, 1466 (C=C), 1188 (C-O), 1007, 618 cm\(^{-1}\); 5a (C_{31}H_{62}O_3), Positive LC-QTOF-MS: \( m/z \) (%) [M+CH\(_3\)OH]^+ 515.8619 (5), calc. 515.8610, [M+2H\(_2\)O]^+ 518.8499 (65), calc. 518.8415; 5b (C_{33}H_{64}O_3), Positive LC-QTOF-MS: \( m/z \) (%) [M+H\(_2\)O]^+ 525.8546 (60), calc. 525.8531; 5c (C_{33}H_{66}O_3), Positive LC-QTOF-MS: \( m/z \) (%) [M]^+ 510.8655 (4), calc. 510.8649, [M+Na]^+ 533.8541 (4), calc. 533.8546; for \(^1\)H and \(^{13}\)C NMR spectroscopic data see tables 6 and 7.

3. Results and Discussion

3.1. Structure Elucidation

In our study, it was observed that the strains isolated from garden soil in Pazar (Rize) and identified as *F. oxysporum* YP9B by traditional methods [37-38] produce a significant metabolite against antagonistic fungus species used for the biological struggle but do not show any pathogenic features against germination of tomato seeds and vegetable plants. Seconder metabolites produced from Fusarium YP9B strain were extracted with ethyl acetate. The crude extract was purified by column chromatography, a total of 10 new compounds (1, 2, 3a-c, 4a-b, and 5a-c) were isolated, and their structures were identified by spectroscopic methods using NMR (1D: \(^1\)H, \(^{13}\)C, APT and 2D: COSY, TOCSY, HMBC, and HSQC), FT-IR and mass spectra (LC-QTOF-MS, LC-MS and GC-FID/MS) and their formulas are given in Figure 1.
Compound 1 was obtained as a colorless semi-solid with a negative optical rotation $[\alpha]_D^{25}$-54.86 (c 0.0056, ethyl acetate). Its LC-MS spectrum showed a pseudo-molecular ion peak at $m/z$ 280.21 [M]+, and others as 303.88 [M+Na]+, 319.07 [M+K]+. Its molecular formula was determined to be C$_{15}$H$_{20}$O$_5$ (six degrees of unsaturation) from its LC-MS spectrum. The absorption bands in the FT-IR spectrum of compound 1 indicated the presence of hydroxyl groups (3448 cm$^{-1}$), carbonyl (1659 cm$^{-1}$), and aromatic double bonds (1437 cm$^{-1}$). The $^1$H NMR data (Table 2) for compound 1 exhibited typical signals for an ester with a hydroxyl, methoxy and benzyl type carbon skeleton, including three tertiary methyl groups at $\delta$ 1.97 (3H, $d$, $J=7.7$ Hz, $H_3$), 5.01 (1H, $d$, $J=8.7$ Hz, $H_2$), and 1.97 (1H, octet, $H_5$). Besides, five aromatic protons at $\delta$ 7.26 (2H, $d$, $J=7.7$ Hz, $H_{2,5''}$), 7.30 (2H, $d$, $J=7.7$ Hz, $H_{3,5''}$), and 7.17 (1H, $t$, $J=7.7$ Hz, $H_{4''}$) were also observed in the $^1$H NMR spectrum of compound 1. The 2D $^1$H-$^1$H COSY and TOCSY NMR data of compound 1 showed the $H_2$ to $H_5$, $H_1$ to -CH$_2$Ph and aromatic phenyl $H_{2''}$, $H_{3''}$, and $H_{4''}$ correlation (Table 2).

The $^{13}$C NMR spectrum of compound 1, in combination with the APT,$^{13}$C-$^1$H HSQC, and HMBC spectra, showed 13 carbon signals that were classified into three methyl at $\delta$ 19.65 ($C_4$), 17.71 ($C_5$), and 57.07 (-OCH$_3$), one methylene at $\delta$ 34.54 (-CH$_2$Ph), six methines at $\delta$ 74.78 ($C_7$), 31.78 ($C_8$), 74.54 ($C_7''$), 128.35 ($C_2''$-$C_4''$), 129.07 ($C_3''$-$C_5''$), and 126.54 ($C_4''$), and three quaternary carbons at $\delta$169.79 ($C_1''$), 169.02 ($C_2''$), and 137.27 ($C_1''$) (Table 2).

Table 2. NMR data of compound 1 (400 MHz, Acetone-$d_6$)

| No | $^1$H ($\delta$H: ppm, $J=Hz$) | $^{13}$C ($\delta_c$: ppm) | APT | COSY, TOCSY |
|----|-------------------------------|---------------------------|-----|-------------|
| 1  | -                             | 169.79                    | COO | -           |
| 2  | 5.01, $d$, $J=8.7$            | 74.78                     | CH  | $H_2$-$H_3$-$H_4$-$H_5$ |
| 3  | 1.97, $m$                     | 31.78                     | CH  | -           |
| 4  | 0.97, $d$, $J=6.8$            | 19.65                     | CH$_3$ | - |
| 5  | 0.43, $d$, $J=6.8$            | 17.71                     | CH$_3$ | - |
| $1''$ | 5.44-5.48, $dd$, $J=4.8$, 14.3 | 74.54                     | CH  | $H_1''$-CH$_2$Ph |
| 2'' | -                            | 169.02                    | COO | -           |
| OCH$_2$ | 3.07, $s$                   | 57.07                     | OCH$_3$ | - |
| Ph-CH$_2$ | 3.26-3.31                   | 34.54                     | CH$_2$Ph | - |
| 3.03-3.10, $dd$, $J=4.9$, 11.1 | 34.54                     | CH$_2$Ph | - |
| 1'' | -                            | 137.27                    | C   | -           |
| 2'' | 7.26, $d$, $J=7.7$            | 128.35                    | CH  | -           |
| 3'' | 7.30, $d$, $J=7.7$            | 129.07                    | CH  | ArCH-CH$_2$Ph-H$_1$ |
| 4'' | 7.17, $t$, $J=7.7$            | 126.54                    | CH  | -           |

$^2$2D COSY, HMBC, HSQC, and ACD NMR program is used for interpretation.

Based on the above analysis, the structure of compound 1 was elucidated as (1-benzyl-2-methoxy-2-oxoethyl)-2-hydroxy-3-methyl butanoate. Close similarities of the chemical shifts of significant carbon signals for compound 1 with reported values for similar compounds confirmed that compound 1 is hydroxyl, methoxy, and benzyl substituted ester compound (Table 2, see supporting information for the spectra, Figure S1-6). In the literature review, no compound was found to be in the structure of compound 1. However, screening a similar compound, there is a natural compound in which another terpenic group was replaced instead of the methoxy group of compound 1, which was isolated from Ixeris debilis and I. repens [42].

Figure 1. Structure of compounds 1, 2, 3a-c, 4a-b, and 5a-c isolated from F. oxysporum YP9B strain.
Compound 2 was also obtained as a light yellow solid with the m.p. 125-127°C. The LC-MS indicated a molecular formula to be C_{14}H_{11}NO_2 (226.85 (35) [M+H]^+, and 250.96 (100) [M+Na+2H]^+) for compound 2. The absorption bands revealed the secondary amine, carbonyl, and aromatic double bond groups at 3355 cm\(^{-1}\), 1673 cm\(^{-1}\), and 1450 cm\(^{-1}\) in the FT-IR spectrum of it, respectively.

Table 3. NMR data of compound 2 (400 MHz, Acetone-d_6)

| No | \(^1\)H (\(\delta_H\): ppm, \(J=Hz\))^a, \((\delta_C): ppm)^a | \(^{13}\)C | APT | COSY |
|----|---------------------------------|------|------|-------|
| 1  | -                               | 159.22 | C     |       |
| 2  | 6.78, bd, \(J=2.4\)             | 115.30 | CH    |       |
| 3  | -                               | 140.05 | C     |       |
| 4  | 6.76, d, \(J=8.8\)              | 118.18 | CH    | H_2-H_4-H_5-H_6 |
| 5  | 7.12, t, \(J=7.8\)              | 131.15 | CH    |       |
| 6  | 6.96-6.72, dd, \(J=8.8, 2.3\)   | 116.57 | CH    |       |
| 1' | -                               | 159.42 | C     |       |
| 2' | 7.48, dd, \(J=1.8, 2.1\)       | 121.83 | CH    |       |
| 3' | -                               | 138.51 | C     |       |
| 4' | 7.56, d, \(J=7.7\)              | 122.39 | CH    | H_2'-H_4'-H_5'-H_6' |
| 5' | 7.33, t, \(J=7.4\)              | 131.48 | CH    |       |
| 6' | 7.05-7.08, dd, \(J=0.9, 8.0\)  | 121.74 | CH    |       |
| 1''| -                               | 196.89 | C=O   |       |
| 2''| 4.22, s                         | 45.03  | CH_2  |       |
| C_4H_8-NH | 8.60, bs, 1H | -     | -     |       |

^2D COSY, TOCSY, HSQC, and ACD NMR program is used for interpretation

Signals for one methylene proton at \(\delta 4.22\) (2H, s, H_2'), one -NH- proton at \(\delta 8.60\) (1H, bs, exchanges with D_2O), and eight aromatic phenyl ring A and B protons at \(\delta 6.78\) (1H, bd, \(J=2.4\) Hz, H_2), 6.76 (1H, d, \(J=8.8\) Hz, H_3), 7.12 (1H, t, \(J=7.8\) Hz, H_5), 6.96-6.72 (1H, dd, \(J=2.3\) and 8.8 Hz, H_6) for ring A and at \(\delta 7.48\) (1H, dd, \(J=1.8\) and 2.1 Hz, H_2), 7.56 (1H, d, \(J=7.7\) Hz, H_4), 7.33 (1H, t, \(J=7.4\) Hz, H_5), 7.05-7.08 (1H, dd, \(J=0.9\) and 8.0 Hz, H_6) for ring B were displayed in the \(^1\)H NMR spectrum (Table 3).

The \(^{13}\)C NMR data, together with APT and HSQC experiments, indicated fourteen carbon signals: one methylene at \(\delta 45.03\) (C_2'), eight methines at \(\delta 115.30\) (C_2), 118.18 (C_3), 131.15 (C_5), 116.57 (C_6), 121.83 (C_7), 122.39 (C_8), 131.48 (C_9), and 121.74 (C_10), and five quaternary carbons at \(\delta 159.22\) (C_1), 140.05 (C_3b), 159.42 (C_1'), 138.51 (C_3), and 196.89 (C=O, C_1'). All the proton and carbon signals of compound 2 were assigned unambiguously by analyzing its COSY, TOCSY, and HSQC NMR data (Table 3, see supporting information for the spectra, Figure S10-13). Therefore, the structure of compound 2 was in phenoxazine structure and named as 2-oxo-8-azatricyclo[9.3.1.1^{3,7}]hexadeca-1(15),3(16),4,6,11,13-hexaen-10-one. Compound 2 was isolated in a relatively pure form, which is in the tricyclic phenoxazine form. In the literature search, the same compound was not found. However, by scanning similar compounds, some phenoxazine compounds with different link positions have been reported, and their biological activities were also mentioned [43-46].

Compounds 3a-c and 4a-b series are in hydroxyl substituted 2,3-dihydroxypropanoic alkyl/alkenyl (C16/C18) anhydride, obtained from the different eluate in a mixture of 3a-c and 3a. 4a-b compounds. Due to polarity, it was not possible to purify one by one. However, a mixture of compounds 3a-c was hydrolyzed and methylated then GC-FID/MS analyzes were performed as fatty acid methyl ester (FAMEs). As a result of the GC-MS analysis, oleic acid (49.79\%, 3b), palmitic acid (36.86\%, 3a) and octadecadienoic acid (11.09\%, 3c, 18: 2, \(\Delta^{9,12}\), (E, E)) were characterized. Similarly, mixtures of compounds 3a and 4a-b were hydrolyzed and GC-FID/MS analyzes revealed palmitic acid (3a, 63.07\%), octadecanoic acid (4a, 34.21\%, 18: 1, \(\Delta^{11}\), (Z)) and octadecadienoic acid (4b, 2.47\%, 18: 2, \(\Delta^{9,12}\), (E, E)). Compounds 3a-c and 4a-b series were mixtures, and their molecular formula was assigned to be C_{19}H_{36}O_5 for 3a; C_{21}H_{38}O_5 for 3b; C_{21}H_{36}O_5 for 3c, C_{21}H_{38}O_5 for 4a; and C_{21}H_{36}O_5 for 4b based on LC-QTOF-MS, LC-MS, GC-FID/MS, and NMR data. The \(^1\)H and \(^{13}\)C NMR
spectra of compounds 3a-c and 4a-b (Table 4 and 5, see supporting information for the spectra, Figure S17-21, and S25-29) series were very similar. The 1H NMR data for compound 3a-c showed one CH at δ 5.27 (1H, m, H2), one methylene at δ 4.27-4.31 and 4.11-4.16 (2H, m, H2) for 3a-c, one olefin protons at δ 6.26-5.33 (2H, m, Δ2') for 3b and 3c (Table 4). The 1H NMR spectrum of compounds 4a-b gave one CH at δ 5.35 (2H, t, J=6.28 Hz, H2) and one methylene at δ 4.32-4.36 and 4.15-4.20 (2H, dd, J= 3.88 and 6.28 Hz, H2) for 4a, and 3b, one olefin protons at δ 5.10-5.36 (2H, m, Δ2') for 4a and two double bond protons at δ 5.10-5.36 (4H, m, Δ9,12) for 4b (Table 4). Other alkyl side chain proton NMR peaks for compounds 3a-c and 4a-b were in the upfield of spectra at δ 0.88-2.32 ppm. The 13C NMR spectra of compounds 3a-c and 4a-b (Table 5) commonly showed two carbonyls at δ172.12 (C1) and 172.41 (C1), one methyl at δ 13.55 (C16/C18), one methylene at δ 61.87 (C4; one CH at δ 69.01 (C2) ppm. Other carbon peaks for the alkyl chain are located at δ 22.50 ppm. The planar structure of compounds 3a-c and 4a-b were further confirmed by 2D NMR (COSY, HMQC, and HMBC), LC-QTOF-MS, and GC-FID/MS spectral analysis and named as 2,3-dihydroxypropanoic hexadecanoic anhydride (3a), 2,3-dihydroxypropanoic (9Z)-octadecenoic anhydride (3b), 2,3-dihydroxypropanoic (9Z,12Z)-octadecadienoic anhydride (3c), 2,3-dihydroxypropanoic (11Z)-octadecenoic anhydride (4a), 2,3-dihydroxypropanoic (9E,12E)-octadecadienoic anhydride (4b). According to the literature survey, none of these compounds 3a, 3b, 3c, 4a, and 4b are known.

Compound 5a-c was the ester type compound containing hydroxyl substituted sesquiterpene units isolated as a mixture. Because of their close polarity, they could not be isolated separately. However, the mixture of compounds 5a-c was hydrolyzed and methylated, then GC-FID/MS analyzes gave palmitic acid (5a, 81.25%), octadecenoic acid (5b, 12.62%, 18:1, Δ9, (E)) and stearic acid (5c, 6.12%, 18:0). Compounds 5a-c were a mixture, and their molecular formula was assigned to be C31H60O6 for 5a; C33H60O6 for 5b; and C33H60O6 for 5c LC-QTOF-MS, LC-MS, GC-FID/MS, and NMR data. LC-MS spectrum of 5a-c showed a pseudo-molecular ion peak at m/z 482.88 [M]+, 509.06 [M+H]+, and 510.75 [M]+, respectively. The 1H NMR data for compounds 5a-c showed one CH at δ 5.27 (1H, m, H2), one methylene at δ 4.27-4.31 and 4.11-4.16 (2H, m, H2) for 5a-c, one olefin protons at δ 5.26-5.33 (2H, m, Δ2') for 5b and 5c. The 1H NMR spectrum of compounds 5 gave one CH at δ 5.35 (2H, t, J=6.28 Hz, H2) and one methylene at δ 4.32-4.36 and 4.15-4.20 (2H, dd, J= 3.88 and 6.28 Hz, H2) for 5a and 5b, one olefin protons at δ 5.10-5.36 (2H, m, Δ2') for 5a and two double bond protons at δ 5.10-5.36 (4H, m, Δ9,12) for 5b. Other alkyl side chain proton NMR peaks for compounds 5a-c were in the upfield of spectra at δ 0.88-2.32 ppm (Table 6, see supporting information for the spectra, Figure S34-35, 37-38). Other carbon peaks for the alkyl and the sesquiterpene chain of compound 5a-c are located at the upfield of NMR spectra. The planar structure of compounds 5a-c were further confirmed by 2D NMR (COSY, HMQC, and HMBC), LC-QTOF-MS, LC-MS, and GC-FID/MS spectral analysis and named as 3-hydroxy-1,2,6,10-tetramethyldodecyl hexadecanoate, 5a, 3-hydroxy-1,2,6,10-tetramethyldodecyl (9E)-octadecanoate, 5b, 3-hydroxy-1,2,6,10-tetramethyldodecyl octadecanoate 5c. In the literature review, compounds 5a, 5b, and 5c were not found. A literature survey showed that some of the natural compounds contain sesquiterpene part of compounds 5a-c [47-48].

3.2. Antimicrobial Activities of Compounds 1-5

Antimicrobial activities of isolated compounds 1-5 were investigated, and minimal inhibition (MIC, µg/mL) and minimal bactericidal concentrations (MBC, µg/mL) were detected (Table 8a,b). When looking at the antimicrobial activities in general, it was observed that all of the selected fractions had high antimicrobial activity at low concentrations. It was found that the isolated compounds showed high antimicrobial activities, compounds 1, 2, and 5a-c had an anti-microbial reproductive activity, and compounds 4a-c and 2 showed a microorganism-killing activity. Compound 1 had a strong antimicrobial effect at concentrations of 0.47-1.8 µg/mL against Gram-positive bacteria (S. aureus, E.
faecalis, S. mutans, B. cereus, and M. smegmatis) except L. acidophilus, which is a member of normal flora.
Table 4. $^1$H NMR data of compounds 3a, 3b, 3c, 4a, and 4b (400 MHz)

| Compounds | CDC$_b$ | Acetone-$d_6$ |
|-----------|---------|--------------|
| 3a (16:0) | H$_2$ 1.99-2.02, m | H$_2$ 2.32, t, J= 7.36 |
| 3b (18:1, Z) $^\Delta_9$ | H$_2$ 1.99-2.05, m | H$_3$ 1.25-1.61, m |
| 3c (18:2; E, E) $^\Delta_9\Delta_{12}$ | H$_3$-H$_8$ 1.25-1.60, m | H$_3$-H$_7$ 1.25-1.61, m |
| 4a (18:1, Z) $^\Delta_{11}$ | H$_9$ 5.26-5.33, m | H$_8$ 5.10-5.36, m |
| 4b (18:2; E, E) $^\Delta_9\Delta_{12}$ | H$_9$ 5.26-5.33, m | H$_9$-H$_{10}$ 5.10-5.36, m |

H$_2$ 5.27, m, H$_3$ 4.27-4.31, m, H$_4$ 4.11-4.16, m
### Table 5. $^{13}$C NMR data of compounds 3a, 3b, 3c, 4a, and 4b (100 MHz)

| Compounds ($\delta_c$: ppm)* | CDCl$_3$ | Acetone-d$_6$ |
|-----------------------------|----------|---------------|
| **No** | 3a (16:0) | 3b (18:1, Z $\Delta$) | C | 173.15 | C | 173.18 | C | 172.12 | C | 172.12 |
| 2 | 33.99 | CH$_2$ | 34.15 | CH$_2$ | 33.99 | CH$_2$ | 33.54 | CH$_2$ | 33.75 | CH$_2$
| 3 | 25.61 | CH$_2$ | 24.85 | CH$_2$ | 24.83 | CH$_2$ | 26.96 | CH$_2$ | 24.77 | CH$_2$
| 4 | 29.08 | CH$_2$ | 29.04 | CH$_2$ | 27.18 | CH$_2$ | 29.12 | CH$_2$ | 28.92 | CH$_2$
| 5 | 29.68 | CH$_2$ | 29.28 | CH$_2$ | 29.19 | CH$_2$ | 31.39 | CH$_2$ | 29.12 | CH$_2$
| 6 | 29.54 | CH$_2$ | 29.11 | CH$_2$ | 29.18 | CH$_2$ | 29.51 | CH$_2$ | 29.09 | CH$_2$
| 7 | 29.54 | CH$_2$ | 29.18 | CH$_2$ | 29.67 | CH$_2$ | 29.64 | CH$_2$ | 29.51 | CH$_2$
| 8 | 29.54 | CH$_2$ | 31.52 | CH$_2$ | 27.18 | CH$_2$ | 29.51 | CH$_2$ | 31.39 | CH$_2$
| 9 | 29.76 | CH$_2$ | 130.12 | CH | 129.95 | CH | 29.40 | CH$_2$ | 129.75 | CH
| 10 | 29.54 | CH$_2$ | 129.63 | CH | 127.86 | CH | 28.40 | CH$_2$ | 127.95 | CH
| 11 | 29.54 | CH$_2$ | 31.93 | CH$_2$ | 25.61 | CH$_2$ | 129.73 | CH | 26.96 | CH$_2$
| 12 | 29.63 | CH$_2$ | 29.71 | CH$_2$ | 128.04 | CH | 129.81 | CH | 127.89 | CH
| 13 | 29.34 | CH$_2$ | 29.38 | CH$_2$ | 129.96 | CH | 28.90 | CH$_2$ | 129.73 | CH
| 14 | 31.91 | CH$_2$ | 29.67 | CH$_2$ | 29.04 | CH$_2$ | 29.09 | CH$_2$ | 33.54 | CH$_2$
| 15 | 22.69 | CH$_2$ | 29.54 | CH$_2$ | 29.48 | CH$_2$ | 29.51 | CH$_2$ | 29.64 | CH$_2$
| 16 | 14.10 | CH$_3$ | 31.93 | CH$_2$ | 31.91 | CH$_2$ | 33.75 | CH$_2$ | 31.39 | CH$_2$
| 17 | - | - | 22.58 | CH$_2$ | 22.58 | CH$_2$ | 22.39 | CH$_2$ | 22.50 | CH$_2$
| 18 | - | - | 14.10 | CH$_3$ | 14.06 | CH$_3$ | 13.55 | CH$_3$ | 13.55 | CH$_3$
| 1' | 173.18 | C | 172.75 | C | 172.75 | C | 172.41 | C | 172.41 | C
| 2' | 68.86 | CH | 68.86 | CH | 68.86 | CH | 69.01 | CH | 69.01 | CH
| 3’ | 62.05 | CH$_2$ | 62.05 | CH$_2$ | 62.05 | CH$_2$ | 61.87 | CH$_2$ | 61.87 | CH$_2$

*1D APT, 2D HMBC, and ACD NMR program are used for interpretation.
Table 6. $^1$H NMR data of compounds 5a, 5b, and 5c (400 MHz, acetone-$d_6$)

| Compounds ($\delta_{	ext{H}}$ ppm, $J$ Hz)$^a$ | 5a (16:0) | 5b (18:1, Z) $\Delta^\alpha$ (E) | 5c (18:0) |
|----------------------------------------------|-----------|---------------------------------|-----------|
| $H_2$                                        | 2.27, t, $J = 7.4$ | $H_2$                            | 2.27, t, $J = 7.4$ | $H_2$                            | 2.27, t, $J = 7.4$ |
| $H_2$-$H_{15}$                               | 1.20-1.60, m | $H_2$-$H_7$                        | 1.20-1.60, m | $H_{13}$-$H_{17}$                  | 1.20-1.60, m |
| $H_{16}$                                      | 0.88, t, $J = 7.2$ | $H_8$                            | 1.94-2.04, m | $H_{18}$                          | 0.88, t, $J = 7.2$ |
| $-$                                          | $-$                        | $H_9$-$H_{10}$                       | 5.20-5.39, m | $-$                                | $-$                        |
| $-$                                          | $-$                        | $H_{11}$                           | 1.94-2.04, m | $-$                                | $-$                        |
| $-$                                          | $-$                        | $H_{12}$-$H_{17}$                   | 1.20-1.60, m | $-$                                | $-$                        |
| $-$                                          | $-$                        | $H_{18}$                          | 0.88, t, $J = 7.2$ | $-$                                | $-$                        |
| $H_1'$                                       | 5.24-5.33, m | $H_1'$                            | 5.24-5.33, m | $H_1'$                            | 5.24-5.33, m |
| $H_2'$                                       | 1.60, m                      | $H_2'$                            | 1.60, m                      | $H_2'$                            | 1.60, m                      |
| $H_3'$                                       | 4.67-4.75, m | $H_3'$                            | 4.67-4.75, m | $H_3'$                            | 4.67-4.75, m |
| $H_4'$                                       | 1.40-1.60, m | $H_4'$                            | 1.40-1.60, m | $H_4'$                            | 1.40-1.60, m |
| $H_5'$                                       | 1.48, m                      | $H_5'$                            | 1.48, m                      | $H_5'$                            | 1.48, m                      |
| $H_6'$                                       | 1.50, m                      | $H_6'$                            | 1.50, m                      | $H_6'$                            | 1.50, m                      |
| $H_7'$                                       | 1.45-1.60, m | $H_7'$                            | 1.45-1.60, m | $H_7'$                            | 1.45-1.60, m |
| $H_8'$                                       | 1.45, m                      | $H_8'$                            | 1.45, m                      | $H_8'$                            | 1.45, m                      |
| $H_9'$                                       | 1.45-1.60, m | $H_9'$                            | 1.45-1.60, m | $H_9'$                            | 1.45-1.60, m |
| $H_{10}'$                                    | 1.60, m                      | $H_{10}'$                         | 1.60, m                      | $H_{10}'$                         | 1.60, m                      |
| $H_{11}'$                                    | 0.92, d, $J = 6.4$          | $H_{11}'$                         | 0.92, d, $J = 6.4$          | $H_{11}'$                         | 0.92, d, $J = 6.4$          |
| $H_{12}'$                                    | 0.92, d, $J = 6.4$          | $H_{12}'$                         | 0.92, d, $J = 6.4$          | $H_{12}'$                         | 0.92, d, $J = 6.4$          |
| $H_{13}'$                                    | 0.96, d, $J = 6.5$          | $H_{13}'$                         | 0.96, d, $J = 6.5$          | $H_{13}'$                         | 0.96, d, $J = 6.5$          |
| $H_{14}'$                                    | 0.86, d, $J = 6.6$          | $H_{14}'$                         | 0.86, d, $J = 6.6$          | $H_{14}'$                         | 0.86, d, $J = 6.6$          |
| $H_{15}'$                                    | 1.14, d, $J = 6.4$          | $H_{15}'$                         | 1.14, d, $J = 6.4$          | $H_{15}'$                         | 1.14, d, $J = 6.4$          |

$^a$2D COSY, and ACD NMR program are used for interpretation

When we check the the MBC values, it was determined that S. aureus 30 µg/mL, B. cereus 7.5 µg/mL, and M. smegmatis 0.46 µg/mL were found to have bactericidal activity. Compounds 5a-c is highly effective at the probiotic bacteria group L. casei, low concentrations to other Gram-positive bacteria (8.5-68.8 µg/mL), and very low to the ARB positive bacteria group M. smegmatis has been determined that it has high efficacy in low concentrations (<0.5 µg/mL). Compounds 1 and 5a-c have a killing activity against M. smegmatis, suggesting their potential to become an antituberculosis agent. Compounds 1 and 5a-c generally have similar efficacy but are observed to be effective at lower concentrations against Gram-negative bacteria and yeast fungi. It is thought that isolates carry high efficacy at low concentrations and increase the probability of being a potential drug. It was determined that the compounds 4 and 2 were similar in terms of antimicrobial activity and were effective against all microorganisms (broad-spectrum) at low concentrations (61.2-0.8 µg/mL). When MBC values were examined, it was observed that they had microbicidal (Bacteriocyclic fungicide) activity against all tested microorganisms (broad-spectrum) except for spore bacteria B. cereus and M. smegmatis. MIC value of compound 3 was found to have a 338.8 µg/mL against all tested microorganisms. The lack of antimicrobial activity of compound 3 showed that it could not be used an antimicrobial drug due to the lack of MBC.

In the study, the antimicrobial activity of the endophyte Fusarium sp. obtained from the leaves of the honeysuckle plant was investigated and compared with 1% streptomycin sulfate [49]. It was mentioned that endophyte Fusarium solani isolated from Taxus baccata (Yew) has antimicrobial activity on many microorganisms [13]. In the literature, the raw extract of a Fusarium species isolated from the sea showed the antibacterial (Bacillus subtilis, Streptococcus mutans, Staphylococcus epidermidis, E. coli, P. aeroginosa, and K. pneumoniae) and antifungal (Candida rugosa, F. oxysporum, S. cerevisiae, Rhizopus oryzae and Aspergillus flavus) activities [11]. The best activity was observed against E. coli at a concentration of 200 µg/mL.
Table 7. $^{13}$C NMR data of compounds 5a, 5b, and 5c (100 MHz, acetone-d$_6$)

| Compounds (δ: ppm)$^a$ | No | 5a (16:0) | 5b (18:1, Z) Δ$^7$ (E) | 5c (18:0) |
|-------------------------|----|-----------|-------------------------|-----------|
| 1                       | 174.18 C | 168.94 C | 170.19 C |
| 2                       | 31.74 CH$_2$ | 31.74 CH$_2$ | 31.74 CH$_2$ |
| 3                       | 25.12 CH$_2$ | 29.69 CH$_2$ | 24.83 CH$_2$ |
| 4                       | 29.44 CH$_2$ | 29.03 CH$_2$ | 29.40 CH$_2$ |
| 5                       | 29.59 CH$_2$ | 29.24 CH$_2$ | 29.59 CH$_2$ |
| 6                       | 29.63 CH$_2$ | 29.12 CH$_2$ | 29.63 CH$_2$ |
| 7                       | 29.55 CH$_2$ | 29.03 CH$_2$ | 29.55 CH$_2$ |
| 8                       | 29.55 CH$_2$ | 31.74 CH$_2$ | 29.55 CH$_2$ |
| 9                       | 29.24 CH$_2$ | 129.70 CH | 29.24 CH$_2$ |
| 10                      | 29.63 CH$_2$ | 127.92 CH | 29.55 CH$_2$ |
| 11                      | 29.55 CH$_2$ | 31.74 CH$_2$ | 29.51 CH$_2$ |
| 12                      | 29.51 CH$_2$ | 29.60 CH$_2$ | 29.55 CH$_2$ |
| 13                      | 29.51 CH$_2$ | 29.26 CH$_2$ | 29.63 CH$_2$ |
| 14                      | 31.74 CH$_2$ | 29.63 CH$_2$ | 29.28 CH$_2$ |
| 15                      | 22.51 CH$_2$ | 29.40 CH$_2$ | 31.74 CH$_2$ |
| 16                      | 14.09 CH$_3$ | 31.98 CH$_2$ | 31.94 CH$_2$ |
| 17                      | - | - | 24.83 CH$_2$ |
| 18                      | - | - | 13.63 CH$_3$ |
| 1'                      | 74.65 CH | 73.31 CH | 73.31 CH |
| 2'                      | 36.24 CH | 36.24 CH | 36.24 CH |
| 3'                      | 62.17 CH$_2$ | 61.90 CH$_2$ | 62.03 CH$_2$ |
| 4'                      | 31.74 CH$_2$ | 31.74 CH$_2$ | 31.74 CH$_2$ |
| 5'                      | 29.63 CH$_2$ | 29.63 CH$_2$ | 29.63 CH$_2$ |
| 6'                      | 28.93 CH | 28.93 CH | 28.93 CH |
| 7'                      | 31.74 CH$_2$ | 31.74 CH$_2$ | 31.74 CH$_2$ |
| 8'                      | 25.12 CH$_2$ | 25.12 CH$_2$ | 25.12 CH$_2$ |
| 9'                      | 36.24 CH$_2$ | 36.24 CH$_2$ | 36.24 CH$_2$ |
| 10'                     | 29.63 CH | 29.63 CH | 29.63 CH |
| 11'                     | 19.61 CH$_3$ | 19.61 CH$_3$ | 19.61 CH$_3$ |
| 12'                     | 18.86 CH$_3$ | 18.86 CH$_3$ | 18.86 CH$_3$ |
| 13'                     | 17.94 CH$_3$ | 17.94 CH$_3$ | 17.94 CH$_3$ |
| 14'                     | 10.98 CH$_3$ | 10.98 CH$_3$ | 10.98 CH$_3$ |
| 15'                     | 17.98 CH$_3$ | 17.98 CH$_3$ | 17.98 CH$_3$ |

$^a$ID APT, 2D HMBC, HSQC, and ACD NMR program are used for interpretation.

Table 8a. Minimal Inhibition (MIC) concentration values of the isolated compounds 1-5 (µg)

| Comp. | Minimal Inhibition Concentration Values (MIC) | Stock (µg/mL) | Gram (+) | Gram (+) | ARB+ Yeast Like Fungi |
|-------|-----------------------------------------------|---------------|----------|----------|-----------------------|
| No    | Ec | Yp | Kp | Pa | Sa | Ef | Sm | Lc | Bc | Ms | Ca | C1 | Sc |
| 1     | 4800 | 60 | 60 | 60 | 60 | 0.94 | 1.8 | 1.8 | 30 | 0.94 | 0.47 | 60 | 60 | 120 |
| 2     | 2000 | 1.6 | 1.6 | 1.6 | 1.6 | 0.8 | 1.6 | 6.3 | 12.5 | 0.8 | 3.1 | 3.1 | 3.1 | 6.3 |
| 3a-c  | 27100 | 338.8 | 338.8 | 338.8 | 338.8 | 338.8 | 338.8 | 338.8 | 338.8 | 338.8 | 338.8 | 338.8 | 338.8 | 338.8 |
| 4a-b  | 9800 | 7.8 | 7.7 | 7.7 | 7.7 | 3.8 | 7.7 | 30.6 | 61.2 | 7.7 | 30.6 | 30.6 | 30.6 | 30.6 |
| 5a-c  | 22000 | 550 | 550 | 550 | 550 | 4.3 | 4.3 | 4.3 | 1100 | 2.1 | <0.5 | 1100 | 1100 | 1100 |
| Cont. | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Amp.  | 10 | 18 | 18 | >128 | 10 | 35 | NT | NT | 15 | 4 | <8 | <8 | <8 |
New metabolites produced by *Fusarium oxysporum* YP9B

### Table 8b. Minimal Bactericidal (MBC) concentration values of the isolated compounds 1-5 (µg)

| Comp. | Stock | Ec | Yp | Kp | Pa | Sa | Ef | Sm | Lc | Bc | Ms | Ca | Ct | Sc |
|-------|-------|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 1     | 4800  | -  | -  | -  | 30 | -  | -  | -  | 7.5| -  | -  | -  | -  | -  |
| 2     | 2000  | 3.1| 3.1| 3.1| 3.1| 6.3| 12.5| 12.5| 25 | -  | -  | 12.5| 12.5| 12.5|
| 3a-c  | 27100 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| 4a-b  | 9800  | 15.3| 61.3| 15.3| 15.3| 30.6| 61.3| 61.3| 245| -  | -  | 61.3| 61.3| 61.3|
| 5a-c  | 22000 | -  | -  | -  | -  | 68.8| 34.3| 8.5 | -  | 17.1| <0.5| -  | -  | -  |
| Control| -     | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

Ec.: *Escherichia coli*, Yp.: *Yersinia pseudotuberculosis*, Kp.: *Klebsiella pneumonia*, Pa.: *Pseudomonas aeruginosa*, Sa.: *Staphylococcus aureus*, Ef.: *Enterococcus faecalis*, Sm.: *Streptococcus mutans*, Lc.: *Lactobacillus casei*, Bc.: *Bacillus cereus* 702 Roma, and Ms.: *Mycobacterium smegmatis*, Ca.: *Candida albicans*, Ct.: *Candida tropicalis*, and Sc.: *Saccharomyces cerevisiae*. (-): Activity not observed; Amp.: *Ampicillin*, Strep.: *Streptomycin*, (-): Fluc.: *Fluconazole*.

### 3.3. Determination of Cytotoxic, and Antiviral Activity

The cytotoxic activities of an isolate obtained from *F. oxysporum* YP9B were tested against the VERO cell line, the kidney green monkey (*Cercopithecus aetiops*) epithelial cell, and the results are given in Table 9 and Figure 2.

### Table 9. Cytotoxic effect on VERO cells for compounds 1-5 isolated from *F. oxysporum* YP9B

| Comp. | Control cell | Fraction Concentration (µM) and Live Cell Count |
|-------|--------------|-----------------------------------------------|
|       |              | 40   | 20   | 10   | 5    | 2.5  | 1.25 | 0.625 |
| 1     | 100          | -    | -    | 6.758| 31.594| 58.720| 85.677| 145.925|
| 2     | 100          | -    | 9.931| 8.209| 44.789| 65.974| 72.471| 87.215|
| 3a-c  | 100          | 6.488| 7.368| 53.510| 91.782| 93.783| 98.913| 109.516|
| 4a-b  | 100          | -    | -    | 7.157| 59.346| 95.046| 97.089| 97.974|
| 5a-c  | 100          | -    | -    | 7.021| 6.7166| 6.330| 6.523| 6.457|

**Figure 2.** Cytotoxic effect of substances on VERO cells
Table 10. Antiviral effect of compounds 1-5 on HSV type-1

| Comp. | Cell | Virus | Acyclovir | Antiviral activity tests studied concentrations (µM) |
|-------|------|-------|-----------|-----------------------------------------------|
|       | 5    | 2.5   | 1.25      | 0.63  | 0.31  | 0.16  | 0.08  | 0.04  |
| 1     | 100  | 33.70 | 92.83     | -     | -     | 38.46 | 39.90 | 48.88 | 44.24 | 42.30 | 42.74 |
| 2     | 100  | 33.70 | 92.83     | -     | -     | 37.36 | 43.46 | 37.11 | 36.56 | 35.52 | 23.69 |
| 3a-c  | 100  | 33.70 | 92.83     | 44.18 | 39.93 | 39.25 | 54.75 | 27.73 | 28.65 | -     | -     |
| 4a-b  | 100  | 33.70 | 92.83     | -     | -     | 50.11 | 54.96 | 39.68 | 40.03 | 30.77 | 45.92 |

According to the results obtained, compounds 3a-c at five µM and below, compounds 4a-b at 2.5 µM and below, and compounds 1 and 2 at 1.25 µM and below were not showed cytotoxic to VERO cell lines. But, compound 5a-c had strong cytotoxic activity against the VERO cell line. Thus, antiviral activity tests for compounds 5a-c could not be performed. The antiviral activity of the isolated compounds 1-4 was determined using the HSV type-1 DNA virus that lysed VERO cells. The VERO cell line concentrations that remained alive compared to the virus control were higher than the virus control, as the antiviral activity value (Table 10, 4a-b ≤ 2.5 µM, and 1 and 2 ≤ 1.25 µM was effective in the antiviral activity that they could be used for antiviral activity tests. Thus two of the total five isolates showed antiviral activity. The result showed that compound 4a-b at 1.25 µM and compound 1 at 0.312 µM concentrations produced a partial increase in cell viability, and it was found to have antiviral activity for HSV Type-1.

The cytotoxic activities of the isolated compounds (1-5) were tested against the VERO cell line with the concentration of 5 µM and below dilutions for compound 3, 2.5 µM and below dilutions for compound 4, 1.25 µM and below dilutions for compounds 1 and 2 (total six-fold dilution) was effective. Thus, they were used for antiviral activity tests. Compound 5 has a strong cytotoxic activity to VERO cells (Table 9, Figure 3). HSV type I, which is the DNA virus, was used for antiviral activity. HSV type-1 is a virus that lyses VERO cells. In determining antiviral activity, the number of cells that survive according to the VERO cell line’s virus control and higher concentrations than the virus control is determined as the value of the antiviral activity. Accordingly, it was determined that compound 4 produced a partial increase in cell viability at concentrations of 1.25 µM, and compound 1, 0.312 µM. The increase in cell viability of compound 3 at a concentration of 0.625 µM was not considered significant since it could not be demonstrated in repeated experiments.
Cytotoxic activities of five isolate from *Fusarium oxysporum* YP9B were tested using MCF-7, PC-3, and A549 cell lines. The compounds tested showed brou cytotoxic activity (IC$_{50}$) in the cell lines used (MCF-7, PC-3, and A549) (Table 11 and Figure 4).

**Table 11.** Cytotoxic activity values (IC$_{50}$ values) of the five isolate obtained from *F. oxysporum* YP9B on three cancer cell lines

| Comp.  | MCF-7 (µM) ± SD | PC-3 (µM) ± SD | A549 (µM) ± SD |
|--------|-----------------|----------------|----------------|
| 1      | 15.01± 4.55     | 19.13± 0.68    | 17.06± 1.69    |
| 2      | 790.33± 32.56   | 917.06± 84.66  | 905.35± 82.24  |
| 3a-c   | ni              | ni             | ni             |
| 4a-b   | ni              | ni             | ni             |
| 5a-c   | 7.75± 1.40      | 17.75± 0.65    | 7.51± 1.38     |
| Doxorubicin | 0.053± 0.004  | 0.09± 0.014    | 17.75 ± 5.61  |

ni: no inhibitions

**Figure 4.** Cytotoxic activity of compounds at 100 µM in MCF-7, PC-3, and A549 cell lines compared to the control group. The significance level of the differences between the groups and the control is defined by * p <0.05, **p <0.01, and ***p <0.0001

As seen in Table 11, compounds 5a-c has shown the most potent anti-proliferative activity on all tested cell lines, notably. Comp compound 1 has exerted second potent activity on each cell line, while compounds 4a-b have the weakest inhibitory activity on MCF-7, PC-3, and A549 cell lines. Compared to other isolates, compounds 3a-c and 4a-b did not exert cytotoxicity on these cell lines. Furthermore, compounds 3a-c and 4a-b lead to an increase in tested cell lines' viability in a dose-dependent manner (2-1000 µM). Anti-cancer activity on MCF-7, PC-3, and A549 exposed to 100 µM of test substances has been shown in Figure 4. Compounds 1, 5a-c to 2 were found to have anti-cancer activity against the breast cancer line MCF-7, the prostate cancer cell line PC-3, and the lung cancer cell line A549. Compounds 3a-c and 4a-b do not show cytotoxicity even at doses up to 1000 µM against tested MCF-7, PC-3, and A549 cell lines. It has been determined that cell lines increase the viability in a dose-dependent manner. Therefore, these compounds (3a-c and 4a-b) did not show anti-cancer activities.
*F. oxysporum* is an important phytopathogenic fungus species that infects about 150 plant species and has a wide host range with biological activities [50-57]. Secondary metabolites obtained from *Fusarium* sp. have been found to vary from species to species [13-28], depending on the medium. Different secondary metabolites were isolated from the *F. oxysporum* strain in the literature, and their cytotoxic activities were tested against three cancer cell lines (PC-3, PANC-1, and A549) using the MTT method [40-41]. The efficacy of extracted Beauvericin to PC-3, PANC-1, and A549 cell lines was reported as IC\textsubscript{50}: 49.5 ± 3.8, 47.2 ± 2.9, and 10.4 ± 1.6 μM, respectively [5]. Beauvericin is also reported to have antibacterial activity against methicillin-resistant to *S. aureus* (MIC = 3.125 μg/mL) and *Bacillus subtilis* (MIC = 3.125 μg/mL) strains [5]. In another study, it was reported that ethyl acetate extract of *F. oxysporum* SS46 and n-hexane extracts of *F. oxysporum* SS50 isolates from solid rice medium was effective against HCT-8, MDA-MB435, and SF295 cancer cell lines *in vitro* [8]. In another study using the present technique, extracts from 14 different *Fusarium* species were fractionated by HPLC, aurofusarin, and bikaverin was observed as red pigments. They reported the antibacterial activity against *Lactobacillus acidophilus* at eight micrograms and against *Bifidobacterium breve* at 64 micrograms. However, there are no broad spectrums of antimicrobial, cytotoxicity, and antiviral activity for the secondary metabolites or strains producing metabolites related to the existing *F. oxysporum* strains [7]. The absolute configurations of the chiral carbon on the isolated compounds could not be established. Thus, no studies have been found in the literature regarding all these new compounds related to all of these works done in this manuscript.

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**Supporting Information**

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**ORCID**

Gözde Kılıç: 0000-0003-1248-7912
Gonca Çelik: 0000-0002-4634-3354
Arif Bozdeveci: 0000-0002-0729-9143
İshak Erik: 0000-0002-9729-1013
Elif Öztürk: 0000-0002-0809-6145
Rengin Reis: 0000-0002-3484-2201
Hande Sipahi: 0000-0001-6482-3143
Merve Cora: 0000-0002-5956-9133
Şengül Alpay Karaoğlu: 0000-0003-1047-8350
Nurettin Yaylı: 0000-0003-4174-3014

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