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Research Article

Antimicrobial, Antioxidant Activity of Ethyl Acetate Extract of *Streptomyces* sp. PERM2, its Potential Modes of Action and Bioactive Compounds

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Abstract

Background: Microorganisms belonging to *Streptomyces* sp. are Gram-positive bacteria known for their unsurpassed capacity for the production of secondary metabolites with diverse biological activities. The aim of this study was to evaluate the antimicrobial and antioxidant properties of ethyl acetate *Streptomyces* sp. PERM2 extract, its potential modes of action and bioactive secondary metabolites.

Results: The ethyl acetate PERM2 extract showed antimicrobial activity more pronounced on both Gram-positive and Gram-negative bacteria and fungi with a Minimum Inhibitory Concentration value (MIC) of 0.5 mg/mL and Minimum Bactericidal Concentration (MBC) of 2 - 4 mg/mL against bacterial pathogens. MIC value against pathogenic fungi was 2 mg/mL and Minimum Fungicidal Concentration (MFC) of 0.01 - 0.05 mg/mL against pathogenic fungi. PERM2 crude extract showed the ability to inhibit bacteria cell wall synthesis at 0.5 and 1 MIC. The extract was found to possess dose-dependent 2,2-Diphenyl-picrylhadrazyl (DPPH) free radical scavenging and Ferric reducing activity. The gas chromatography-mass spectrometry (GC-MS) analysis revealed the presence of three major compounds identified as 9,12-octadecadienoic acid (Z, Z) (29.75%), tridecyl trifluoroacetate (24.82%) and 1-(+)-ascorbic acid 2, 6-dihexadecanoate (22.34%). The liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis revealed the presence of 22 non-volatile metabolites in PERM2 extract and only the compound 3, 30-O-dimethylellagic acid was identified.

Conclusion: The results of this study indicate that ethyl acetate *Streptomyces* sp. PERM2 extract possesses antibacterial, antifungal, and antioxidant activities; inhibits bacteria cell wall and protein synthesis; and contains significant bioactive secondary metabolites which could be used as an alternative to multi-resistance antibiotics.

More Information

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Keywords: *Streptomyces* sp. PERM2; Ethyl acetate extract; Antimicrobial; Modes of action; Antioxidant; GC-MS; LC-MS/MS; Bioactive secondary metabolites

Abbreviations: MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; MFC: Minimum Fungicidal Concentration; DPPH: 2,2-Diphenylpicrylaldrazyl; GC-MS: Gas Chromatography-Spectrometry; LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry; MHB: Mueller Hinton Broth; MHA: Mueller Hinton Agar; PDA: Potato Dextrose Agar; YMEA: Yeast Malt Extract Agar; DMSO: Dimethoxyl sulphoxide

Introduction

Microbial natural products are a source of several important drugs of high therapeutic value. The majority of commercially available pharmaceutical products are secondary metabolites or their derivatives produced by bacteria, fungi, and actinobacteria [1]. Microbial secondary metabolites are one of the immense reservoirs of natural chemical diversity with potent biological activity [2]. Among all living organisms, the actinobacteria phylum currently represents the most prospective group of microorganisms for the discovery of bioactive compounds such as antimicrobials, antitumor agents, antiparasitics, anticancer agents, enzymes, and some other endogenous metabolites with free radicals scavenging

activities properties [3-5]. Out of 22,500 biologically active compounds that have been extracted from microbes, 45% are from actinobacteria, 38% are from fungi and 17% are from unicellular bacteria [6].

Actinobacteria are a diverse group of Gram-positive bacteria and filamentous bacteria that have high Guanine-Cytosine (GC) content ranging from 50 to 70 mol% [7]. They are characterized by complex morphological differentiation and are considered as an intermediate group of bacteria and fungi [8]. Their presence in various ecological habitats has enabled researchers to exploit their tremendous potential as the richest source of pharmaceutical and biologically active products [9]. Therefore, actinobacteria are considered the most economical and biotechnological important prokaryotes which produce several secondary metabolites with significant biological activities. Out of these actinobacteria, *Streptomyces* is an important industrial group of organisms that is widely explored for a wide range of biologically active compounds [10]. Nearly 75% of all the known industrial antibiotics [11] and numerous economically important compounds [12] were obtained from streptomyces.

Secondary metabolites are organic compounds that have no direct role in the vegetative growth and development of the organism. Microbial secondary metabolites are organic compounds that have the potential for the discovery of new drugs to fight against antibiotic resistance [13]. Among the microorganisms, the phylum Actinobacteria (order- *Actinomycetales*), represents a notable source for the production of new bioactive secondary metabolites including antibiotics [14,15]. The phylum alone accounts for the production of approximately 75% of the total bioactive compounds including antibiotics with more than 70% produced by members of the genus *Streptomyces* [10,16]. Genus *Streptomyces* is the most dominant and prolific source of bioactive metabolites with a variety of biological activities including antimicrobial, anti-cancer agents, and other pharmaceutically useful compounds [17-19]. Of 10,000 known compounds, the genus *Streptomyces* alone accounts for nearly 7500 compounds, while the rare actinobacterial genera including *Nocardia*, *Micromonospora*, *Streptosporangium*, *Actinomadura*, *Saccharopolyspora,* and *Actinoplanes* represent 2500 compounds [17]. *Streptomyces* spp. is widely distributed in various habitats like soils, marine environments, fresh waters, and rhizosphere [20-23].

Bio-prospecting studies on actinobacteria are mostly confined to soils, marine environments, freshwater, and rhizosphere and with less interest given to plant tissues. However, there are few reports about the presence of actinobacteria in plant tissues (as endophytes) [3]. Moreover, the possibility of finding a novel bioactive molecule from the soil, rhizosphere, and marine environments habitats has diminished over the years. Plant tissues are underexploited sources for the discovery of novel metabolites. With the increase in resistance among pathogens and the unavailability of novel metabolites from the rhizosphere and soil habitats, endophytic-derived drugs could be of great importance. Our previous studies showed that, the potential *Streptomyces* sp. PERM2 has been isolated from cocoyam roots from the Kumba locality around Mount Cameroon [24]. Mount Cameroon's locality has been found unexplored region for actinobacterial research. The present study is intended to investigate the antimicrobial and antioxidant activities of the ethyl acetate crude extract of *Streptomyces* sp. PERM2; to determine its modes of action and characterize its bioactive secondary metabolites production for further use as potential therapeutic agents.

Materials and methods

Streptomycete strain

Streptomyces sp. PERM2 (KY400013) used in this study was isolated from cocoyam root from Kumba locality around Mount Cameroon and characterized in our previous study [24]. Pure cultures were kept in 20% glycerol at -80 °C storage.

Microbial organisms

The following Gram-positive and Gram-negative bacteria and some fungi were used for the experiment. Gram-positive: *Streptococcus pneumoniae* (ATCC 6465), *Haemophilus influenza* (ATCC 49247), *Staphylococcus aureus* (ATCC 43300) and *Bacillus cereus*. Gram-negative: *Salmonella typhi*, *Escherichia coli (*ATCC 25922), *Pseudomonas aeruginosa*, *Salmonella enteritidis*. Fungi: *Pythium myriotylum, Fusarium solani* and *Phytophthora megakarya.* The reference bacterial cultures and fungal pathogens strains were obtained from the Laboratory of Clinical Microbiology of the University of Yaoundé I and the Laboratory of Phytoprotection and Valorization of Genetic Resources of the Biotechnology Center of the University of Yaoundé I respectively. Bacterial inocula were prepared by growing cells in Mueller Hinton broth (MHB) for 24 h at 37 °C. The filamentous fungi were grown on Potato Dextrose Agar (PDA) slants at 28 °C for 14 days and the spores were collected using sterile double distilled water and homogenized. These microorganisms were maintained on an agar slant in a refrigerator at 4 °C.

Antimicrobial activity of *Streptomyces* sp. PERM2

The antibacterial activity of *Streptomyces* sp. PERM2 was performed by using the disc diffusion method [25]. The 24 hours on Mueller-Hinton Agar (MHA) of test bacteria were inoculated into tubes containing 5 mL of sterile distilled water and the concentration was calibrated at 1.0×10^5 CFU/mL using Mac Farland cell [26]. Using sterile swabs, 100 µL of calibrated broth cultures of test bacteria were swabbed on sterile Mueller Hinton agar plates followed by deposition of the 6 mm bacterial plug of 14 days old grown on yeast malt extract agar. The plates were incubated at 4 °C for 4 hours, then at 37 °C for 24 hours. The zone of inhibition formed was

measured using a ruler. The experiment was carried out in triplicate and the average value was recorded.

The antifungal activity of *Streptomyces* sp. PERM2 was performed by using dual culture *in vitro* assay [27]. *Streptomyces* sp. PERM2 was transferred to the center of the Petri dishes with PDA, with a flamed inoculating loop, forming a straight line across the plate, and was incubated at room temperature (28 ± 2 ºC), for five days. After this incubation period, two discs (6 mm in diameter) of the 8-day-old fungus culture were transferred to both halves of the plate, at a distance of 1.5 cm from the line of growth of the actinomycete colonies. The fungi were grown as described above. The control treatments consisted of Petri plates with PDA, without the actinomycete cultures, but with the fungus discs transferred to the plates at the same distance as de ones for the other treatments. The assay was incubated at room temperature, and the mycelium growth was daily measured with a ruler, for a period of five days (period in which the fungal cultures from the control treatment reached the plate margins). The percentage inhibition was calculated as follows:

$$I = \frac{C - T}{C} \times 100$$

Where:

I: Inhibition percentage (%).

C: Distance done by fungal in Petri plates with PDA, without the actinomycete cultures (mm).

T: Distance done by fungal in Petri plates with PDA, with the actinomycetes cultures (mm).

Preparation of the crude extract

In this study, solid-state fermentation was adopted for the production of the crude extract [28]. For the preparation of inoculum, the streptomycete strain PERM2 was streaked on the yeast extract malt extract agar (ISP2 medium) plates and incubated at 28 °C for 7 days. The spores were scrapped from the plate and inoculated into 25 mL of yeast extract malt extract broth (ISP2) medium and incubated in a rotary shaker for 48 h with 150 rpm at 28 °C. After incubation, the inoculum was prepared by transferring the 2-day cultures in 250 mL of ISP2 broth contained in a 1,000 mL conical flask and incubated at 28 °C for 7 days. About 50 g of wheat bran was added into a 1,000 mL conical flask with 50 mL of distilled water and sterilized. Then 10% of inoculum was added into a conical flask containing sterile wheat bran. The flasks were incubated at 28 ± 2 °C for 30 days. After incubation, the fermented biomass of the PERM2 strain was mixed with ethyl acetate and macerated (3 × 24 h). The crude extract was collected and concentrated by evaporation. The quantity of crude extract was measured by adding the crude into the dried 100 mL preweighed beaker. After evaporation of the solvent, the weight of the crude extract was measured and stored in sterile vials.

Antibacterial activity of ethyl acetate extract of *Streptomyces* sp. PERM2

The efficacy of ethyl acetate crude extract of PERM2 to inhibit bacteria was tested against four Gram-positive including *Streptococcus pneumoniae* (ATCC 6465), *Haemophilus influenza* (ATCC 49247), *Staphylococcus aureus (*ATCC 43300) and *Bacillus cereus* and four Gram-negative: *Salmonella typhi*, *Escherichia coli (*ATCC 25922), *Pseudomonas aeruginosa*, *Salmonella enteritidis* by discs diffusion assay [29]. The 24 hours on Mueller-Hinton Agar (MHA) of test bacteria were inoculated into tubes containing sterile distilled water. Using sterile swabs, 100 µL of broth cultures of test bacteria calibrated at 1.0×10^5 CFU/mL using Mac Farland cell [26], were swabbed on sterile Mueller Hinton agar plates followed by depositing the sterile discs (6 mm) impregnated with 30 µL of ethyl acetate extract (20 mg/mL of 10% DMSO (Dimethyl sulphoxide). Antibiotics (Streptomycin, 1 mg/mL) along with bacteria cultures were used as a positive control, and DMSO (10%) containing bacterial cultures was used as the negative control. The plates were incubated at 37 °C for 24 hours. The zone of inhibition formed was measured using a ruler. The experiment was carried out in triplicate and the average value was recorded.

Antifungal activity of ethyl acetate extract of *Streptomyces* sp. PERM2

The antifungal activity of crude extract was determined using the agar well diffusion method [30]. Antifungal bioassay was done on a 9 cm Petri plate with 20 mL of PDA. A 6 mm fungal plug was cut from the leading edges of a seven-day-old pure culture of *P. myriotylum*, *F. solani*, *P. megakarya*, and was put in the center of the plate. A 6 mm diameter well was made at 1.5 cm from the fungal plug and another well at the opposite side, followed by pipetting 30 µL aliquot of each crude extract (20 mg/mL) into the two wells on each plate. Control plates contained wells of DMSO 10% and nystatin (2 mg/mL). Radial growth was recorded after incubation for 10 days. The radial growth of the fungal colony was recorded with a meter ruler along two diagonal lines drawn on the reverse side of each plate. The experiment was carried out in triplicate and the average value was recorded. The Percent Inhibition of Radial Growth (PIRG) of each treatment compared to control was computed utilizing the formula below:

$$\text{PIRG (\%)} = \frac{\text{Radial growth of control} - \text{Radial growth of treatment}}{\text{Radial growth of control}}$$

Minimum Inhibitory Concentration (MIC) of ethyl acetate extract of *Streptomyces* sp. PERM2

The MIC of the extract was determined by NCCLS microbroth dilution methods [31]. Bacterial and fungal pathogens were grown in sterile broth and 10 µL of log phase culture was added into 96 well micro titre plates. The crude extract was dissolved in 10% DMSO and diluted in different concentrations

(16,000, 8,000, 4,000, 2,000, 1,000, 500, 250, 125, 62.5, 31.25, 15.62.and 7.81 µg/mL). Then, 10 µL of the bacterial and fungal suspension (1.0 × 10^5 cells/mL) was inoculated into each well of a 96-well microplate, each containing a different concentration of the test agents. Diluted extracts and sterile broth were added into pre-coated microbial cultures, making up a total volume of 200 µL. Streptomycin (200 µg/mL) and nystatin (400 µg/mL) were used as the positive control for bacteria and fungi respectively, and culture medium (200 µL) was used as the negative control. The plates were sealed and incubated at 37 °C during 24h for bacteria and at 30 °C during 48 h for fungi. After incubation, MIC of extracts was revealed with 10 µL of iodonitrotetrazolium (2 mg/mL) by adding in each well and incubated for 30 minutes at room temperature. The wells where there was no coloration are considered as concentrations that possess inhibitory activity against pathogens and wells containing the smallest concentration uncolored were considered as the MIC.

Minimum Bactericidal Concentration (MBC) of ethyl acetate extract of *Streptomyces* sp. PERM2

The MBC values of extract were determined by inoculating into Yeast Malt Extract Agar (YMEA) plates, 10 mL of medium from each of the wells from the MIC test which showed no turbidity. The plates were incubated at 37 °C for 24 h. Minimum bactericidal concentration (MBC) was defined as the lowest concentration of the test agent at which no microbial growth was observed on the plates [31].

Antioxidant assays of ethyl acetate extract of *Streptomyces* sp. PERM2

DPPH free radical scavenging activity: The 2,2-Diphenyl-1-picrylhydrazyl free radical scavenging activity of the crude ethyl acetate extract of PERM2 strain was measured according to the procedure described by [32]. Briefly, 500 µL of DPPH solution (0.0016% in methanol) was mixed with 500 µL of different concentrations (200, 400, 800, and 1,600 µg/mL) of ethyl acetate extract and reference standard (ascorbic acid) in separate tubes. The tubes were incubated in the dark at room temperature for 20 minutes and the optical density was measured at 515 nm using a UV-visible spectrophotometer (SHIMADZU, Japan). The absorbance of the DPPH control (without extract/standard) was noted. The scavenging activity was calculated using the formula:

Scavenging activity (%) = [(A – B) / A] x 100

Where A is the absorbance of DPPH control and B is the absorbance of DPPH in the presence of extract/standard.

Ferric reducing activity

The reducing potential of the ethyl acetate extract was determined by a Ferric reducing assay [33]. In this assay, 0.2 ml of different concentrations (100, 200, 400, 600, 800, and 1,000 µg/mL) of ethyl acetate extract of PERM2 and ascorbic acid (reference standard) in 1 mL of methanol were mixed separately with 0.5 mL of phosphate buffer (200 mM, pH 6.6) and 0.5 mL of 1% potassium ferricyanide. The tubes were incubated at 50°C for 20 minutes in a water bath, cooled rapidly, and mixed with 0.5 mL of 10% trichloroacetic acid and 0.05 mL of 0.1% ferric chloride. After 10 minutes in the dark, the amount of iron (II)-ferricyanide complex formed was determined by measuring the formation of Perl's Prussian blue at 700 nm. An increase in absorbance on an increase in concentration indicates increased reducing power.

Metal chelating activity (%) = [(A – B) / A] x 100

Where A is the absorbance of the control and B is the absorbance of the sample. EDTA was used as a positive control.

Determination of modes of action of ethyl acetate extract of *Streptomyces* sp. PERM2

Effect of ethyl acetate extract of *Streptomyces* sp. PERM2 on inhibition of bacteria cell wall synthesis: The modified protocol of [34] was used to conduct this experiment. For this purpose, a standardized bacterial suspension (*E. coli*) at the 0.5 McFarland scale in 0.9% NaCl was prepared. A volume of 100 µL of extract was introduced into different tubes containing this suspension in order to have concentrations of the suspension equivalent to the minimum inhibitory concentration (1 MIC) and half minimum inhibitory concentration (½ MIC) in the medium. The suspensions obtained were incubated at 37 °C while stirring. At times 0 h, 2 h, 4 h, and 6 h, the absorbance was measured at 620 nm. The absorbance at zero hours (0 h) was used to evaluate the relative absorbance (Ar) at different times in order to draw the Ar = f (t) curve.

Effect of ethyl acetate extract of *Streptomyces* sp. PERM2 on inhibition of bacteria protein synthesis: In five tubes each containing 9 mL of suspension Mueller Hinton, 0.5 mL of bacterial suspension (*E. coli*) (standardized to McFarland's 0.5 scale) was added to each tube. The extract was then added to each preceding mixture to give the concentrations: 1 MIC and ½ MIC. The control tube was treated under the same conditions and received 0.5 mL of PDB instead of the extract. The tubes were incubated at 37 °C with a rotation of 80 rpm. After 24 hours of incubation, centrifugation at 13,000 rpm for 2 minutes was used to recover the bacterial cells, which were weighed and mixed with the lysis buffer at a rate of 40 mg of bacteria per 500 mL of buffer. After 1 hour of incubation, centrifugation at 13,000 rpm for 3 minutes allowed the recovery of the supernatant containing the proteins, which were then assayed by the Bradford reagent (Sigma-Aldrich) using the microplate reader (FLUOstar Omega Microplate Reader). The lysis buffer constituted the blank [35].

Effect of ethyl acetate extract of *Streptomyces* sp. PERM2 on the inhibition of ATPase /H+ proton pumps: The evaluation of the inhibitory effect of the crude extract on proton pumps was carried out by controlling the pH of the

spore suspension medium according to the protocol described by [36]. Inhibition of the acidification of the medium in the presence of the extracts was attributed to an inhibitory effect of the functioning of the H+ ATPase pumps by the extracts. 4 mL for the bacterial (*E. coli*) suspension was prepared and 0.5 mL of each extract was added to obtain suspension concentrations equal to ½ MIC and 1 MIC. After 10 min of pre-incubation at 37 °C, acidification of the medium was initiated by adding 0.5 mL of 20% glucose solution whose rapid catabolism will be accompanied by the release of protons into the medium. Thereafter, the pH of the medium was measured every 15 min for 1h30 min. For the negative control, the extract was replaced by water; the pH values noted made it possible to draw the curve of pH variation as a function of time.

Gas chromatography-mass spectrometry (GC-MS)

The analysis of the volatile constituents in the extract was determined by GC-MS technique. In brief,

PERM2 crude extract was subjected to a Shimadzu GC-17A attached to a Shimadzu GC-MS-QP5050A system. The column used was a Phenomenex Zebron ZBFFAP ultra-low-bleed Bonded Polyethylene Glycol fused capillary column of 30 mL × 0.25 mm I.D × 0.25 µm film thickness. Split ratio 20 injection was performed. Helium was the transporter carrier gas with a stream flow rate of 0.7 mL/ min. The column temperature was kept at 70 °C for 3 min, then modified at 10 °C/min to 90 °C via programming and finally modified at 5 °C/min to 230 °C. The inlet and detector temperatures were 230 °C and 250 °C, respectively, while the dissolvable deferral (solvent delay) was 5.75 min [30].

Liquid chromatography-mass spectrometry (LC-MS/MS)

The analysis of the non-volatile constituents in the extract was determined by LC-MS/MS technique. An AB Sciex 5500QTrap (Linear Quadrupole Hybrid Ion Trap Mass Spectrometer, AB Sciex, Toronto, Canada) mass spectrometer operating in Electrospray Ionization (ESI) negative mode and hyphenated with an Agilent 1290 ultra-high performance liquid chromatography system was used. The high-purity nitrogen gas for the mass spectrometer was set at 40 psi for source gas, 40 psi for the heating gas, and HIGH for collision gas with a source temperature of 500 °C. The setting for electrospray ionization voltage was set to 4500 kV. The collision energy to attain fragmentation was set at 35 eV with a spread of ±15 eV. The mass range for MS/MS scan was set from 50 - 1000 m/z while the mass range for full scan was set from 100 - 1000 m/z while scan speed was set at 1000 m/z per second. A Phenomenex Synergi Fusion RP (100 mm × 2.1 mm i.d., 3 um particle size, Phenomenex, CA, USA) was used to obtain separation. The mobile phase was made up of aqueous ammonium formate (5 mmol/l) with 0.1% formic acid (solvent A) and acetonitrile with ammonium formate (5 mmol/l) with 0.1% formic acid (solvent B). The compounds were separated with the following linear-programmed solvent gradient: 0 min (10% B), 10 min (95% B), 2 min (95% B) then equilibrating back to 10% B for 3 min. The flow rate for the column was set at 0.25 mL/min while the column temperature was set at 40 °C and the injection volume at 10 µL [30].

Statistical analysis

All experiments were conducted in triplicate and the readings were taken as the mean ± the standard deviation of the mean of three replicates, which were calculated using Microsoft Excel XP 2013. Data were analysed using GraphPad Prism software version 8.0.1. One-way analysis of variance (ANOVA) followed by Tukey's test was performed to determine differences between the biological activities of the ethyl acetate PERM2 crude extract. Differences were considered significant at a probability level of 5% ($p < 0.05$).

Results

Antimicrobial activity of *Streptomyces* sp. PERM2

Based on antimicrobial activity using the disc diffusion method, *Streptomyces* sp. PERM2 showed potent activity (> 10 mm diameter inhibition zones) against the growth of five pathogenic bacteria (*H. influenza* (ATCC 49247), *E. coli* (ATCC 25922), *S. enteritidis*, *S. aureus,* and *B. cereus*). The minimum inhibition diameter was obtained against *S. pneumonia* (10.00 ± 0.5 mm). *Streptomyces* sp. PERM2 showed stronger antagonistic activity against all the pathogenic fungi (Table 1).

Antimicrobial assays of ethyl acetate extract of *Streptomyces* sp. PERM2

Antibacterial and antifungal activities: Antagonistic characteristics of the ethyl acetate crude extract of *Streptomyces* sp. PERM2 showed potent antagonistic activity against bacterial and fungal pathogens (Table 2). Of eight bacterial pathogens, the highest inhibition activity was manifested against *P. aeruginosa* (26.00 ± 0.3 mm). Susceptibility of *H. influenza* (14.00 ± 0.3 mm), *S. aureus* (16.00 ± 0.5 mm), *B. cereus* (17.00 ± 0.4 mm), *S. typhi* (16.65 ± 0.5 mm), *E. coli* (15.66 ± 0.5 mm) and *S. enteritidis* (14.00 ± 0.4 mm) to PERM2

Table 1: Antimicrobial activity of *Streptomyces* sp. PERM2.

Test microorganisms	Zone of inhibition diameter (mm)
Bacteria	
Streptococcus pneumoniae (ATCC 6465)	10.00 ± 0.5d
Haemophilus influenza (ATCC 49247)	26.70 ± 0.6a
Staphylococcus aureus (ATCC 43300)	20.66 ± 0.4b
Bacilus cereus	20.33 ± 0.4b
Salmonella typhi	17.00 ± 0.5c
Escherichia coli (ATCC 25922)	26.60 ± 0.9a
Pseudomonas aeruginosa	12.00 ± 0.3d
Salmonella enteritidis	21.60 ± 0.8b
Fungi	**Inhibition percentage (%)**
Pythium myriotylum	100a
Fusarium solani	95.85a
Phytophthora megakarya.	100a

ATCC: American Type Culture Selection.

Table 2: Antibacterial and antifungal activities of ethyl acetate crude extract of *Streptomyces* sp. PERM2.

Test microorganisms	Zone of inhibition (mm)		
Bacteria	**Extract**	**Streptomycin**	**DMSO**
Streptococcus pneumoniae (ATCC 6465)	11.00 ± 0.4	23.66 ± 0.6	0.00
Haemophilus influenza (ATCC 49247)	14.00 ± 0.3	23.66 ± 0.6	0.00
Staphylococcus aureus (ATCC 43300)	16.00 ± 0.5	24.66 ± 0.6	0.00
Bacillus cereus	17.00 ± 0.4	21.33 ± 0.5	0.00
Salmonella typhi	16.65 ± 0.5	18.33 ± 0.4	0.00
Escherichia coli (ATCC 25922)	15.66 ± 0.5	20.66 ± 0.6	0.00
Pseudomonas aeruginosa	26.00 ± 0.3	32.66 ± 0.5	0.00
Salmonella enteritidis	14.00 ±0.4	17.33 ± 0.5	0.00
Fungi	**Percentage of inhibition (mm)**		
	Extract	**Nystatin**	**DMSO**
Pythium myriotylum	30	56.67	0.00
Fusarium solani	36.11	52.78	0.00
Phytophthora megakarya	28.57	51.43	0.00

ATCC: American Type Culture Selection; DMSO: Dimethylsulfoxide.

crude extract was noticeable. *Streptococcus pneumonia* was less susceptible to PERM2 crude extract (11.00 ± 0.4 mm). However, the inhibitory effect of the extract was lesser than that of standard antibiotics. DMSO (10%) did not show any inhibition of bacteria. Among fungal pathogens, a reduction in mycelial growth was significantly observed against *F. solani* (36.11%) (Table 2).

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

Based on the 96-well microliter assay, the MIC values of the ethyl acetate crude extract of *Streptomyces* sp. PERM2 is shown in Table 3. The MIC value of the crude extract of PERM2 was determined as 0.5 mg/mL against *H. influenza*; 1 mg/mL against *B. cereus*, *P. aeruginosa,* and *S. enteritidi*; 2 mg/mL against *S. aureus*, *S. thyphi,* and *E. coli*. The lowest MIC was observed against *H. influenza* at 0.5 mg/mL. MIC against fungal pathogens was determined as 2 mg/mL against *P. myriotylum* and 4 mg/mL against *F. solani* and *P. megakarya* (Table 3). The MBC value of the crude extract of PERM2 was 2 mg/mL against 6 pathogenic bacteria (*P. pneumoniae*, *H. influenza*, *B. cereus*, *S. thyphi*, *E. coli*, and *S. aeruginosa*) while the MFC value was 0.01 mg/mL against *F. solani* and 0.05 mg/mL against *P. myriotylum* and *P. megakarya* (Table 3).

Modes of action and resistance of ethyl acetate extract of *Streptomyces* sp. PERM2

Inhibition of bacteria cell wall synthesis: The 0.5 MIC and 1 MIC destroyed bacterial cell walls or inhibited cell wall bacteria of the crude extract of *Streptomyces* sp. PERM2 with compared to negative control (Figure 1a). However, this bacterial cell wall lysis increased with time.

Inhibition of bacteria protein synthesis: The 0.5 MIC and 1 MIC of the ethyl acetate crude extract of *Streptomyces* sp. PERM2 did not significantly inhibit the *E.coli* proteins compared to the negative control (Figure 1b).

Table 3: Minimum inhibitory concentration and Minimum bactericidal concentration of *Streptomyces* sp. PERM2 ethyl acetate crude extract.

Bacteria	MIC (mg/mL)	MBC (mg/mL)
Streptococcus pneumoniae (ATCC 6465)	2	2
Haemophilus influenza (ATCC 49247)	0.5	2
Staphylococcus aureus (ATCC 43300)	2	4
Bacillus cereus	1	2
Salmonella typhi	2	2
Escherichia coli (ATCC 25922)	2	2
Pseudomonas aeruginosa	1	2
Salmonella enteritidis	1	4
Fungi	**MIC (mg/mL)**	**MFC (mg/mL)**
Pythium myriotylum	2	0.05
Fusarium solani	4	0.01
Phytophthora megakarya.	4	0.05

ATCC: American Type Culture Selection; MIC: Minimum Inhibition concentration; MBC: Minimum Bactericidal Concentration; MFC: Minimum Fungicidal Concentration.

Inhibition of ATPase/H+ proton pumps: The 0.5 MIC and 1 MIC of the ethyl acetate crude extract of PERM2 have significantly inhibited the ATPase pump compared to the negative control (Figure 1c).

Antioxidant properties

DPPH radical scavenging assay: Both PERM2 extract and ascorbic acid exhibited dose-dependent scavenging of DPPH radicals (Figure 2a). The maximum DPPH radical scavenging effect of the extract was 44.41 ± 2.63% at 1,600 µg/mL. Though the extract was able to scavenge DDPH* (free radical) and convert it into DPPHH, the scavenging effect of the extract was lesser than that of ascorbic acid.

Ferric reducing power: The reducing power was determined by the reduction of Fe^{3+} to Fe^{2+} in the presence of different concentrations of ethyl acetate extract and ascorbic acid. As shown in Figure 2b, the absorbance of the reaction mixture at 700 nm increased with the increase in concentration of extract indicating the reducing potential of extract. The maximum reducing activity of the extract was observed at 1,000 µg/mL. However, the reducing potential of the extract was lesser than the reference standard.

GC-MS analysis of ethyl acetate extract of *Streptomyces* sp. PERM2

The GC-MS chromatogram of the ethyl acetate crude extract of *Streptomyces* sp. PERM2 is shown in Figure 3. A total of 112 volatile compounds are detected in the ethyl acetate crude extract. The three major compounds identified in the ethyl acetate crude extract of *Streptomyces* sp. PERM2 were 9, 12-octadecadienoic acid (Z, Z) ($C_{18}H_{32}O_2$), with a concentration of 29.75% and a retention time of 18.003; tridecyl trifluoroacetate ($C_{15}H_{27}F_3O_2$), with concentration 24.82% and the retention time 18.034 and 1-(+)-ascorbic acid 2,6-dihexadecanoate ($C_{38}H_{68}O_8$), with concentration 22.34% and the retention time 16.849. The identified compounds in the ethyl acetate crude extract of *Streptomyces* sp. PERM2 in GC-MS is represented in Table 4.

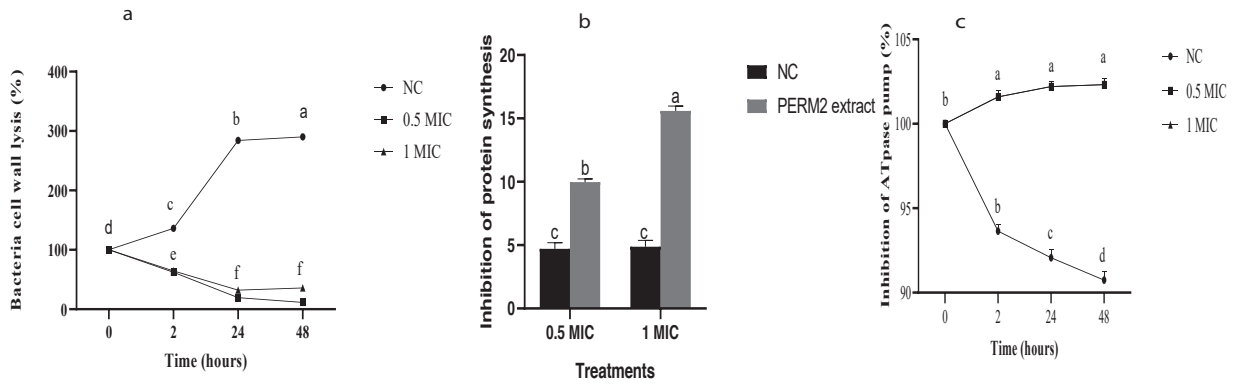

Figure 1: Different modes of action of ethyl acetate extract of *Streptomyces* sp. PERM2: (a) Inhibition of bacteria cell synthesis; (b) inhibition of bacteria protein synthesis; (c) inhibition of ATPase/H+ proton pumps.

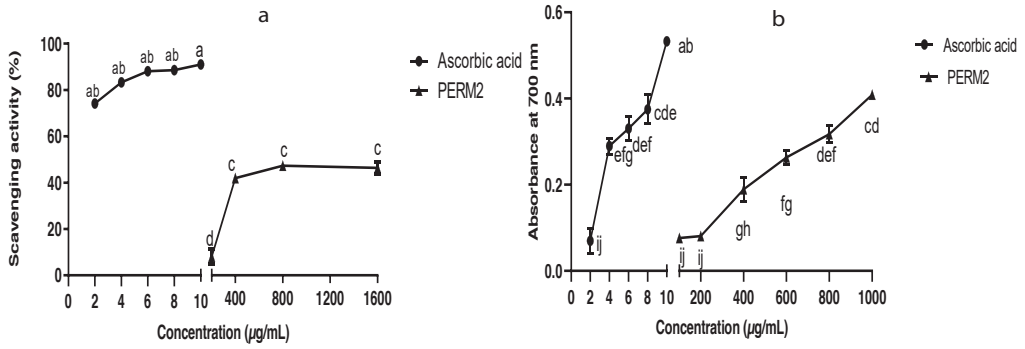

Figure 2: DPPH radical scavenging activity (a) and ferric reducing activity (b) of ethyl acetate extract of *Streptomyces* sp. PERM2 and ascorbic acid.

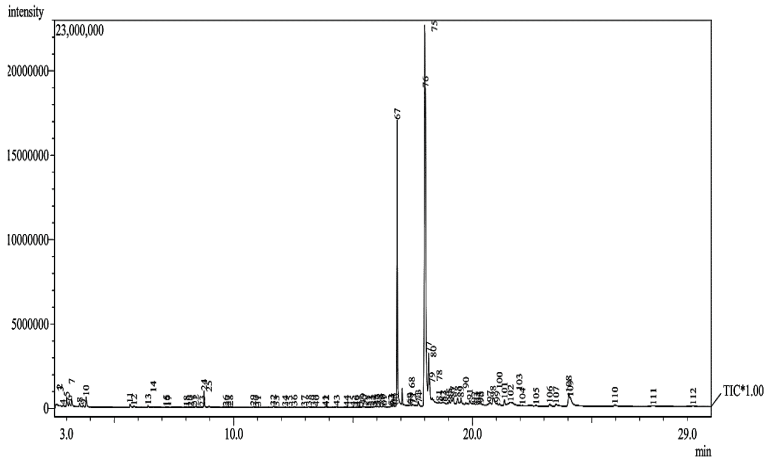

Figure 3: GC-MS chromatogram of ethyl acetate extract of *Streptomyces* sp. PERM2.

Table 4: Major compounds identified in the ethyl acetate crude extract of *Streptomyces* sp. PERM2 in GC-MS.

Retention time (min)	Area (%)	Name of the compound	Molecular weight	Molecular formula	Nature of compound	Biological activity
18.003	29.75	9, 12-octadecadienoic acid (Z, Z)	280	$C_{18}H_{32}O_2$	Polyunsaturated Fatty acid	Antioxidant, anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, antihistaminic antieczemic, antiacne, antiandrogenic, antiarthritic, anticoronary, insectifuge
18.034	24.82	tridecyl trifluoroacetate	296	$C_{15}H_{27}F3O_2$	Ester compound	Antibiofilm
16.849	22.34	1-(+)-ascorbic acid 2, 6-dihexadecanoate	652	$C_{38}H_{68}O_8$	Ascorbic acid (Vitamin C)	Antioxidant, antiscorbutic, anti-inflammatory, antinociceptive, anti-mutagenic, wound healing property

LC-MS/MS analysis of ethyl acetate extract of *Streptomyces* sp. PERM2

The results of LC-MS/MS analysis showed the presence of 25 different non-volatile compounds from ethyl acetate crude extract (Table 5). One of the compounds in the extract was identified as 3, 30-di-methyl ellagic acid ($C_{16}H_{10}O_8$), with a peak at retention time of 8.274 min and molecule weight 429.2.

Discussion

Bioactive metabolites produced by microbes have gained attention recently, due to their sophisticated chemical structure and highly specific biological activities. Actinobacteria are a potential candidate to fight against multidrug-resistant organisms, are well-known producers of antimicrobial compounds, and have been found in different habitats worldwide [29,37]. They are intensively used in pharmaceutical and agrochemical industries. These bacteria produce about 75% of commercially and medically useful antibiotics [38]. *Actinomyces* are useful biological tools in the production of antimicrobials against bacteria and fungi [39]. *Streptomyces* sp. PERM2 showed good antimicrobial activity in solid medium and fermented state. Our results indicated that the antimicrobial metabolites were extracellular. Most of the secondary metabolites and antibiotics are extracellular in nature and extracellular products of actinomycetes show potent antimicrobial activities [40]. From the results, it appears that the antimicrobial action of *Streptomyces* sp. PERM2 and the ethyl acetate crude extract were more pronounced on both Gram-positive and Gram-negative bacteria and fungi. These results are different from the reports of Rammali, et al. [41] and Anavadiya, et al. [42]. The ethyl acetate crude extract of *Streptomyces* sp. PERM2 was tested for its MIC level against pathogenic bacteria and fungi. The MIC value of the ethyl acetate crude extract of PERM2 ranges from 0.5 - 2 mg/mL against pathogenic bacteria and from 2 - 4 mg/mL against pathogenic fungi. These values were higher than the values obtained from ethyl acetate crude extract of *Streptomyces* sp. Strain FR7 which ranged from 0.005 - 0.1 mg/mL against pathogenic bacteria and fungi [43].

The ethyl acetate crude extract of *Streptomyces* sp. PERM2 was investigated for the scavenging abilities on DPPH and ion-reducing power. DPPH is the most common and reasonably simple approach for measuring the radical scavenging activity of active biological particles [44]. The ethyl acetate crude extract of *Streptomyces* sp. PERM2 was able to reduce the stable radical DPPH to the yellow–colored diphenylpicrylhydrazine. Similar results have been found with the ethylacetate extract of *Streptomyces* sp. Strain FR7 [43]. On the other hand, the reducing power increased with increasing concentration of the ethyl acetate crude extract of *Streptomyces* sp. PERM2. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [45].

Table 5: Bioactive metabolites obtained by LC-MS/MS from the ethyl acetate extract of *Streptomyces* sp. PERM2.

Peaks	Retention time (min)	Fragments	Molecular weight	Identification
1	7.168	97.029; 99.009; 110.038; 125.097; 163.111; 191.122; 199.133; 207.024; 225.113; 243.124	-	unknown
2	7.696	79.959; 191.073; 263.095;399.199		unknown
3	8.274	127.114; 139.114; 171.105; 199.132; 201.116; 211. 134 ; 275.203; 293. 215 ; 311.225; 329.239	$C_{16}H_{10}O_8$	3,30-di-O- methyl ellagic acid
4	9.171	58.008; 99.093; 127.076; 129.093; 171.102; 189.141; 195.139; 201.14 ; 279.119; 295.231; 313.243	-	unknown
5	9.648	59.017; 129.092; 171.102; 183.139; 195.139; 277.218; 295.231; 309.207; 313.240; 356.250	-	unknown
6	9.913	171.103; 195.139; 211.170; 277.220; 295.232; 341.271	-	unknown
7	10.072	123.118; 171.105; 195.140; 223.228; 259.207; 277.222; 295.232	-	unknown
8	10.337	152.937; 171.007; 402.245; 476.297; 477.216		unknown
9	10.974	399.220; 413.233; 416.221; 431.247; 446.271; 461.296		unknown
10	11.821	130.89 ; 349.329;392.322	-	unknown
11	12.509	171.101; 277.216; 279.232; 291.196; 293.212; 295.227; 309.207; 311.2323; 313.287, 327.217; 589,453	-	unknown
12	13.096	171.102; 279. 236 ; 293.214; 311.227; 313.239; 329.235; 591.473	-	unknown
13	13.682	279.232; 281.250; 293.212; 311.225; 313.235; 329.233 ; 593.485	-	unknown
14	14.319	211.134; 255.296; 296.213; 311.226; 329.234; 567,472	-	unknown
15	14.425	277.219; 279.236; 295.231; 313.239; 575.467	-	unknown
16	14.532	333.213; 574.434; 575.450; 575.484; 575.656; 576.014	-	unknown
17	15.062	156.137; 256.237; 266.296; 278.220; 296.232; 313.299; 314.241;552.477	-	unknown
18	15.275	361.349; 365.340; 403.363; 429.340; 447.354	-	unknown
19	15.328	241.216; 275.200; 276.204, 277.216, 279.232; 296.230; 360.220; 404.361, 5543.444; 556.514, 556.449, 584.444; 602.391; 602.477	-	unknown
20	15.915	346.287; 581. 466 ; 607.513; 625.524	-	unknown
21	17.194	374.321; 635.550; 563.565	-	unknown
22	17.992	402.351, 663.579; 681.592	-	unknown
23	19.002	663.579 ; 681.596	-	unknown
24	20.007	635. 543 ; 708.606 ; 710.578 ; 754.510 ; 607	-	unknown
25	20,915	562. 522; 580.533; 742. 592; 788.639	-	unknown

Modes of action of ethyl acetate crude extract of *Streptomyces* sp. PERM2 such as bacteria cell wall synthesis and inhibition of bacteria protein synthesis were evaluated against *E. coli*, one of the most sensitive bacteria pathogens. Bacteria cells are surrounded by a cell wall made of peptidoglycan, which is a particular biological structure, present predominantly in the Gram-positive bacteria wall, made from polysaccharide chains consisting of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), cross-linked together by short peptides containing modified amino acids, such as aminopimelic acid (DAP) and native L-or D-amino acids. From a functional point of view, the bacterial cell wall is the most important structure that permits bacteria to maintain their shape and respond efficiently to environmental stresses, maintaining the osmotic balance [46]. ß-lactams and glycopeptides are molecules recognized to inhibit cell wall synthesis. In this study, the 0.5 MIC and 1 MIC of the crude extract of Streptomyces sp. PERM2 has inhibited cell wall synthesis. This result showed that the ethyl acetate crude extract of *Streptomyces* sp. PERM2 could contain Beta-lactam and/or Glycopeptide molecules. Streptomyces sp. PERM2 could be a good candidate to be used in the formulation of ß-lactams antibiotics whose biological actions are correlated to the inhibition of cell wall biosynthesis. Protein biosynthesis is catalyzed by ribosomes and cytoplasmic factors. The bacterial 70S ribosome is composed of two ribonucleoprotein subunits, the 30S and 50S subunits [47]. Antimicrobials inhibit protein biosynthesis by targeting the 30S or 50S subunit of the bacterial ribosome [48,49]. Among these antimicrobials, Aminoglycosides and Tetracyclines are recognized to respectively interact with the 16S r-RNA of the 30S subunit near the A site through hydrogen bonds or act upon the conserved sequences of the 16S r-RNA of 30S ribosomal subunit to prevent binding of t-RNA to the A site [47,48]. Antimicrobials belonging to the class of macrolides affect the early stage of protein synthesis (translocation), by targeting the conserved sequences of the peptidyl transferase center of the 23S rRNA of the 50S ribosomal subunit [47,50]. While oxazolidinones interfere with protein synthesis at several stages as follows: (i) inhibit protein synthesis by binding to 23Sr RNA of the 50S subunit and (ii) suppress 70S inhibition and interact with peptidyl-t-RNA [51,52]. In this study, 0.5 MIC and 1 MIC of the ethyl acetate crude extract of *Streptomyces* sp. PERM2 has not significantly inhibited the *E. coli* proteins compared to the negative control. The results reveal that the ethyl acetate crude extract of *Streptomyces* sp. PERM2 does not contain antimicrobials able to interact on the 30S and 50S subunits of the 70S ribosome of bacteria. Determination of bacterial resistance to antibiotics of all classes is helpful. A better understanding of the mechanisms of antibiotic resistance will help clinicians regarding the usage of antibiotics in different situations [53]. From the results, it appeared that 0.5 MIC and 1 MIC of the ethyl acetate crude extract of *Streptomyces* sp. PERM2 has significantly inhibited bacteria ATPase pumps. Thus, this extract could significantly contribute to overcoming multidrug-resistant organisms.

Secondary metabolite profiling based on GC-MS is becoming a foundation in the field of biological sciences and has been successfully employed to determine Volatile Organic Compounds (VOCs) from various samples [54,55]. The actinobacteria phylum has been reported as a prolific producer of thousands of bioactive secondary metabolites. The present investigation revealed the presence of 112 VOCs from the ethyl acetate crude extract of *Streptomyces* sp. PERM2 and the 3 major compounds were indicated by the highest peaks. The most notable compounds identified with GC-MS were 9, 12-octadecadienoic acid (Z, Z), a polyunsaturated fatty acid. Fats are known to be vital sources of energy however, fats, as dietary intakes have more roles in the physiological system. Earlier studies have shown that unsaturated fatty acids have more health benefits than saturated fatty acids [54,56]. Observations made on the intake of dietary fats have shown a steady relationship between polyunsaturated fatty acids and reduced risk of heart disease [57]. This fatty acid compound is recognized to possess bioactive properties which include: antioxidant, anti-inflammatory, hypocholesterolemic, cancer preventive, antifungal, antibacterial, anti-acne, anti-coronary, anti-eczemic, insecticidal properties [57,58]. Tridecyl trifluoroacetate has the second-highest content from the ethyl acetate crude extract of *Streptomyces* sp. PERM2 belongs to the family of ester compounds. This compound was also identified from the methanol extract of *Halimeda* sp., a macroalga isolated from the Red Sea, and had been demonstrated to possess antibiofilm activities [59].

The compound 1-(+)-ascorbic acid 2, 6-dihexadecanoate, the third high content from the ethyl acetate crude extract of *Streptomyces* sp. PERM2 is a vitamin C compound. Ascorbic acid (Vitamin C) is required for the synthesis of collagen, a substance necessary for the healing of wounds. It is a highly effective antioxidant protecting cells from damage by free radicals. Natural antioxidants are potentially safe as they have limited side effects, efficient and inexpensive, and are obtained from renewable sources. Studies have shown that the vitamin can help speed up the healing process of wounds [60]. The compound l-(+)-ascorbic acid 2,6-dihexadecanoate is reported to possess antibacterial, antioxidant, antiscorbutic, anti-inflammatory, anti-nociceptive, antimutagenic, antitumor, and wound healing properties [61-63].

Based on LC-MS/MS analysis, only one chemical compound, the 3, 30-dimethylellagic acid was identified from ethyl acetate PERM2 crude extract. This identified compound is an ellagic acid derivative. The compound 3, 30-dimethylellagic acid is recognized to possess antioxidant, antibacterial, antiviral, anti-inflammatory, antidiabetic, cytolytic, and neuroprotective properties [64,65].

In-vitro antimicrobial activity of the ethyl acetate extract of *Streptomyces* sp. PERM2 against the most critical group of multidrug-resistant bacteria (*Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Pseudomonas*

aeruginosa) Thus, the antimicrobial endophytic *Streptomyces* derivatives could be useful alternatives for the treatment of fungal and bacterial infections. The advantages of using these bioresources and their natural compounds may reduce the risk of side effects and lower the cost. Based on antimicrobial activities, modes of action, antioxidant activities, and the profile of compounds present in the ethyl acetate crude extract of *Streptomyces* sp. PERM2, it will be necessary to complete the chemical identification and purification of unknown compounds present in this extract for recovery in pharmaceutical industries, then contribute to reducing antibiotic resistance.

Conclusion

The ethyl acetate extract of *Streptomyces* sp. PERM2 displayed significant antimicrobial activities against Gram-negative and Gram-positive bacterial pathogens, and pathogenic fungi, and exhibited DPPH and ion-reducing power antioxidant activities *in vitro*. The ethyl acetate extract of PERM2 significantly showed a great effect on the inhibition of bacteria cell wall synthesis and ATPase pumps. GC-MS showed the presence of three major compounds 9, 12-octadecadienoic acid (Z, Z) (29.75%), trifluoroacetic acid (24.82%), and 1-(+)-ascorbic acid 2, 6-dihexadecanoate (22.34%) while the LC-MS/MS showed the presence of 3,30-di-methyl ellagic acid which are recognized to possess diverse biological activities which mostly are antibacterial, antifungal, antioxidant and anti-inflammatory properties.

Authors' contributions

Conceived and designed the experiments: PFDK and TB. Performed the experiments: EGG and PFDK. Analysed the data: PFDK and EGG. Prepared the manuscript: PFDK. Reviewed and edited the draft: PFDK, MFT, WMY, CB, and TB. All authors have read and approved the final version of the manuscript.

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Competing interest

The authors declare that they have no competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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