Original Research Article

Synthesis and Determination of Antimicrobial Efficacies of Secondary Metabolite of *Streptomyces longisporoflavus* and its 3D-Protein Structural Prediction

ABSTRACT

Aims: The need for new drug molecules is of high significance considering the rate at which pathogenic bacteria evolve into drug-resistant pathogens. Actinomycetes have been reported as valuable biological agents that possess potent bioactive molecules. This work aims to isolate local strains of actinomycetes in the environment and determine their antimicrobial activities against some clinical isolates

Study design: This was an in-vitro study.

Place and Duration of Study: The research was carried out at the Microbial Resources Research Laboratory, Department of Pure and Applied Biology, Ladoke Akintola University of Technology Ogbomoso, Nigeria between January 2020 and December, 2021.

Methodology: Phenotypic and molecular methods of identification of the isolated microorganisms were done. A secondary metabolite of the isolate was obtained. An antibiotic sensitivity test of its metabolites was performed using ten (10) clinical isolates; inhibition zones were measured and recorded for each test organism. Gas chromatography-mass spectrometry (GC-MS) was used to determine the probable bioactive molecules present in the metabolite. The nucleotide sequences of the isolate were translated using Phyre2 and viewed with PyMOL.

Results: The isolate was identified as *Streptomyces longisporoflavus.* Various zones of inhibition were recorded for each of the tested pathogenic organisms. Five (5) bioactive molecules were identified in the metabolites, with butane-1,1-dibutoxy-2 (1H)-quinolone having the highest peak area. The PYMOL result shows that the protein structure has a 64% identity as a binding molecule.

Conclusion: This study reveals that the local isolates of *S. longisporoflavus* showed promising antimicrobial potential with bioactive molecules that are potent inhibitors of pathogenic organisms.

Keywords: Streptomyces longisporoflavus, metabolites, antimicrobial potential, bioactive molecules

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1. INTRODUCTION

Microbes are developing resistance to conventional antibiotics, so screening actinomycetes for antimicrobial compounds is critical [1]. Actinomycetes have proven to be very valuable microorganisms from an economic and biotechnological standpoint. They have very high metabolic possibilities and produce many substances that are needed for health [2, 3]. They are responsible for the production of more than 50% of the bioactive microbial compounds known today, which include and are not limited to antimicrobials, immune suppressants, and enzymes. Actinomycetes are reported to account for approximately 45% of all biologically active substances obtained from microorganisms, while fungi are associated with about

38%, and the rest is produced by the various forms of bacteria known. The various species of *Streptomyces* have been used in the industrial production of nearly 75% of all known antibiotics in the world today [4].

The most well-known actinomycetes products are antibiotics [5, 6]. Natural products from microbes are the origin of the antibiotics used today, and they remain the major source of novel antibiotics. It has also been reported that there is a startling shortage of new antibiotics presently being developed in the pharmaceutical industry; however, actinomycetes have produced significant bioactive compounds of great commercial value [7]. *Rhodococcus, Corynebacterium* and *Mycobacterium* have a potential advantage in the synthesis of secondary metabolites of industrial and clinical significance among actinomycetes [8]. They are believed to have exceptional metabolic and enzymatic varieties. In this research, a local isolate of *Streptomyces* was isolated from soil samples and the antimicrobial potentials of its metabolites were investigated.

2. MATERIALS AND METHODS

2.1 Collection of Soil Samples

Two (2) grams of soil samples were collected from different locations at the Botanical Garden of Ladoke Akintola University of Technology (LAUTECH) in Ogbomoso with a hand trowel (15 cm) [10]. It was then transferred immediately into sterile containers and transported to the Microbial Resources Research Laboratory located in the Department of Pure and Applied Biology at LAUTECH, Ogbomoso [11].

2.2 Preparation of Culture Media

One (1) gram of yeast extract, 2.5 grams of malt extract, 1 gram of dextrose (YMD), and 1.5 grams of agar were homogenized in 100 ml of distilled water before autoclaving at 121 °C for 15 min [12]. The media was allowed to cool down after sterilization (0.05 g/100 ml). Chloramphenicol was added to the media aseptically to inhibit bacterial growth. The medium was corked, agitated, allowed to cool, and then dispensed into Petri dishes to solidify [13].

2.3 Isolation and Maintenance of Cultures

In a test tube, 1 gram of soil sample was diluted in 10 ml of distilled water using a serial dilution protocol. The serial dilution protocol was done using the 10-fold dilution method. Serial diluents of 10^{-1}, 10^{-2}, 10^{-3}, 10-4, and 10^{-5} were prepared. Aliquot dilution (0.1 ml) of the diluents was transferred aseptically into Petri dishes containing solidified dextrose medium, malt extract, and yeast extract, respectively. Each plate was labeled according to the dilution factor and incubated for 7 days at 28 °C [14], after which sub-culturing was done by selecting distinct colonies that developed from the incubated plates onto freshly prepared plates. The pure colonies obtained were transferred into a slant bottle and soured at 4 °C for further use [13].

2.4 Characterization of the Isolates

The morphology and physiological characterization of the isolate were done to study its forms, structure, and specific structural features. The isolates were inoculated on ISP4 media, and the morphology of the developed colonies was visually observed. Other standard characterization analyses were carried out to identify the isolated colonies [15].

2.5 Synthesis of Microbial Metabolites by Submerged Fermentation

A submerged fermentation procedure was used for the synthesis of secondary metabolites of the actinomycetes. Yeast-malt-dextrose (YMD) broth was prepared following the manufacturer's description. The flasks contained 250 ml of YMD broth and were sterilized by

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autoclaving for 15 minutes at 121 °C. The sterilized media were allowed to cool. A sterile inoculating needle was used to cut a small piece of the isolate and then transfer it into the prepared broth. Aseptically, the isolates were inoculated into the YMD broth at room temperature for optimum yield over 21 days. A filtration technique was used to collect the cell biomass. This process ensured a batch biomass system, which was expressed in terms of total wet weight, total dry weight, and mean weight (XSD). The extraction of the metabolite from the medium was done by mixing the spent medium with ethyl acetate in a ratio of 1:2. The extract was centrifuged for 15 minutes at 4000 rpm, and the cell-free supernatant was decanted aseptically into labeled tubes [13, 16, 17]. The metabolite was kept in the refrigerator overnight at 4 °C.

2.6 Collection and Culture Maintenance of Clinical Isolates

Ten (10) clinical microbes, namely *Proteus vulgaris, P. mirabilis, Hemophilusinfluenzae, Klebsiella sp., K. pneumoniae, Escherichia coli, Staphylococcus aureus, Streptococcus pyogenes, Coliform sp.,* and *Pseudomonas aeruginosa,* were obtained from the microbial culture bank of Bowen Teaching Hospital (BUTH) Ogbomoso. MaConkey agar (measured according to the manufacturer's specification) was used to plate it out for clarification to know its physiological structure and to get pure colonies. A loop of pure colony was aseptically inoculated in a test tube containing 9 ml of sterile peptone water and incubated for 24 hours at 37 °C. About 1 ml aliquot was then pipette into 5 ml of sterile water and compared with McFarland to adjust the turbidity of the bacterial suspension. The step was done for each of the test organisms and labeled accordingly [18].

2.7 Determination of Antimicrobial Potentials of Metabolites

To test the metabolite's antimicrobial potential, Mueller Hinton Agar media plates were prepared, specific test bacteria were inoculated on each plate using sterile swab sticks, three 5 mm diameter holes were bored into the Mueller Hinton Agar on each plate using a cork borer, and the plates were labeled appropriately. An insulin pipette (the smallest unit) was used to pipette the metabolite (10 mg/ml) into the hole that corresponded to the label on the plate and incubated at 37 °C for 24 hr to assess the antimicrobial property of each metabolite by the appearance of a clear zone around the hole, called the zone of inhibition. Conventional antibiotics such as Ciprofloxacin (5 g) and Gentamycin (10 g) were used as controls. This was observed and measured in millimeters [19].

2.8 Detection of Bioactive Molecules in the Metabolites

Gas chromatography-mass spectrometry (GC-MS) was carried out to detect and identify the probable bioactive compounds present in the metabolites of *Streptomyces longisporoflavus* [20].Gas Chromatography Model: 7890A A system hyphenated to a mass spectrometer (5975 C) having a triple axis detector and a 10 ml syringe auto-injector was used. Helium gas was used as the carrier phase. The column temperature was raised from 35 °C to 150 °C for 5 min at a rate of 4 °C/min; the temperature was raised to 250 °C at a rate of 20 °C/min and held for 5 min. The elution time was 47.5 minutes. Solution software provided by the supplier was used for system control and data retrieval. Compound identification was accomplished by comparing GC-MS results to those from the NIST library (NISTII).

2.9 Genomic DNA Extraction of the Isolates

A total of 750 l of Lysis Solution was added to a ZR Bashing TM Lysis tube containing 200 l of water, and the tube was secured in a bead fitted with a 2 ml tube holder assembly. The mixture was processed at maximum speed for more than 5 minutes, and the ZR Bashing TM Lysis tube was centrifuged in a micro-centrifuge for one minute. The supernatant of up to 400 l was decanted into a Zymo-Spin^{TM} IV Spin Filter with orange top in a Collection Tube, the tube was centrifuged at 7,000 rpm for 1 minute, after centrifugation, the base of the Zymo-Spin TM IV Spin Filter was snapped off before 1,200 l of fungal or bacterial DNA

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binding buffer was added. 800 l of the mixture was decanted into a Zymo-Spin^{TM} IIC Column in a collection tube; this was also centrifuged at 10,000 rpm for 1 minute. The flow was discarded and added to 200 l of DNA pre-wash buffer for the Zymo-Spin^{TM} IIC column in a new collection tube and centrifuged at 10,000 rpm for one minute [21]. After centrifugation, 500 l of fungal/bacterial DNA wash buffer was introduced into the Zymo-Spin TM IIC column; this was centrifuged at 10,000 rpm for 1 minute, and the Zymo-SpinTM IIC column was decanted to a clean 1.5 ml micro-centrifuge tube into which 100 l of DNA elution buffer was introduced directly to the column matrix, and the DNA was eluted. This makes the DNA suitable for PCR and other downstream applications [22].

2.10 Purification of PCR Products

Two volumes of 20 l of absolute ethanol were introduced into the product of PCR; this was incubated at ambient temperature for 15 minutes before centrifugation at 10000 rpm for 15 minutes. The supernatant was decanted and spun again at 10000 rpm for 15 minutes, decanted, and air-dried, after which ultra-pure water was mixed into the precipitate and the amplicon was analyzed on 1.5 % agarose. The purified product was loaded into the ABI 3500 genetic analyzer [22] with the following mix and conditions for the PCR (Table 1).

2.11 DNA Sequencing

An ABI 3500 Genetic Analyzer was used to sequence the PCR, and an amplicon was recovered. Isolates with more than 95% sequence similarity were thought to be of the same species. The FASTA algorithm was used to search for the sequence-based identification in the EMBL/Gen-bank database of the actinomycetes nucleotide sequences. The criterion for species identification was a > 99 % identity to the database sequence when the sequence match reached 99–100 %. The tree view program was employed to view the phylogenetic tree [23, 24].

2.12 Homology Modeling and 3D Protein Structure Prediction

The nucleotide sequence was blasted in the GenBank in order to look for the existing ones in the NCBI, after which the nucleotide sequence was translated to an amino acid by using EMBOSS [25], and the amino acid was then translated to a protein with the use of Phyre2 software. The protein structure was then viewed with the use of PyMOL software in order to see the position of the segments, elemental symbols and residues [26].

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Table 1: PCR Cocktail Mix

PCR Mix	Conc.
2.5Mm DNTPs	0.8
25mM $MgCl_2$	1.0
DMSO	1.0
5pMol reverse primer	0.5
10ng/µl DNA	2.0
5pMol forward primer	0.5

Taq 5u/ µl	1.0
H_2O	3.1
10× PCR buffer	1.0
TOTAL	10.9µL

16s F: GTGCCAGCAGCCGCGCTAA
16s R: AGACCCGGGAACGTATTCAC

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Microorganism

From the four (4) samples of soil collected from the LAUTECH botanical garden, only one species of *Streptomyces* was isolated using starch-casein agar supplemented with 0.05 g of chloramphenicol. The isolates were characterized based on proper identification of their morphology and physiology, [27]. The isolate's phenotype (fig. 1) revealed that it is bright orange or bright yellow vegetative mycelium, occasionally green, and produced diffusible pigments on the medium, such as the Actinomycin X complex. The presence of green vegetative mycelium agreed with existing findings [27]. However, some actinomycetes produce white aerial mycelium, which is inconsistent with this study [28]. Molecular identification of the isolated actinomycetes using the pair wise sequence alignment tool (fig. 2) and phylogenetic relationship (fig. 3) at the National Centre for Biotechnological Information (NCBI) showed 100% similarity at GenBank to *Streptomyces longisporoflavus.* Analysis on the evolutionary relatedness of the actinomycetes was carried out using MEGA 7 [13].

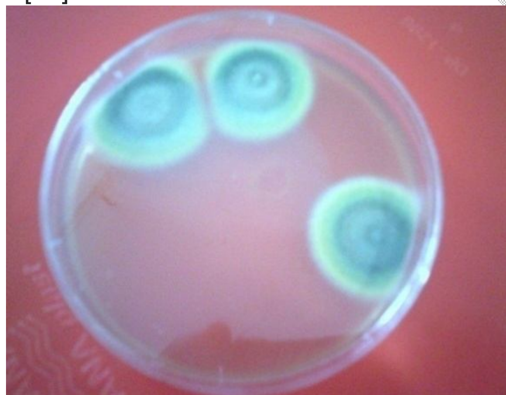

Fig. 1: Phenotypic view of *Streptomyces longisporoflavus* on culture media

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Fig. 2: Pair-wise Sequence Alignment of unknown isolate (MAJFB) with *Streptomyces longisporoflavus* (Accession No. KY657268.1)

Streptomyces longisporoflavus, a bacterium species from the genus *Streptomyces* that has been isolated from soil [29], is the closest to the isolated organism. *S. longisporoflavus* cultured in yeast-malt-dextrose (YMD) broth were monitored for 21 days for the synthesis of metabolites containing active biomolecules that can serve as antimicrobial drugs or drug precursors against microorganisms [20]. The growth patterns of the isolate and the biomass wet and dry weights are shown in fig. 4 and fig. 5 respectively.

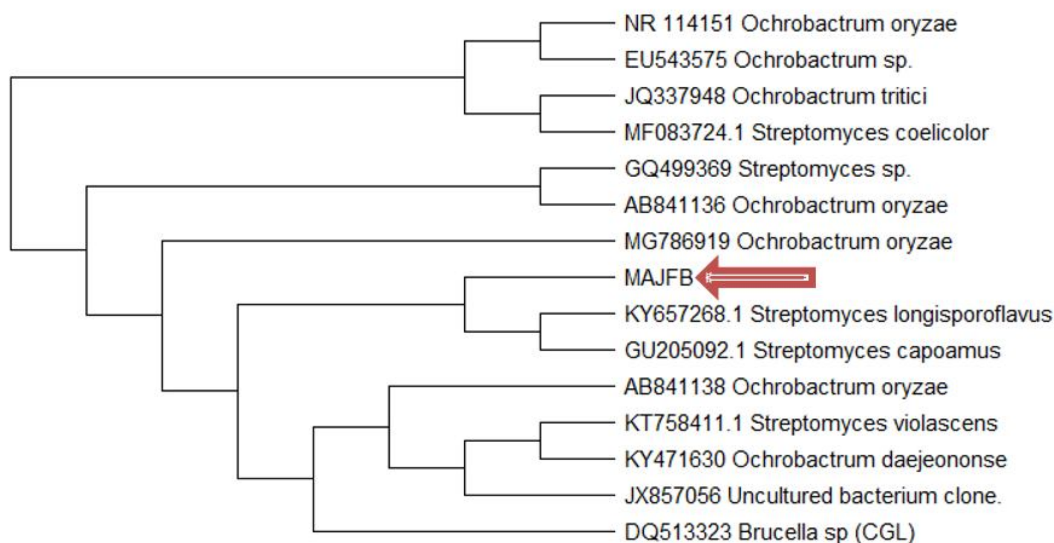
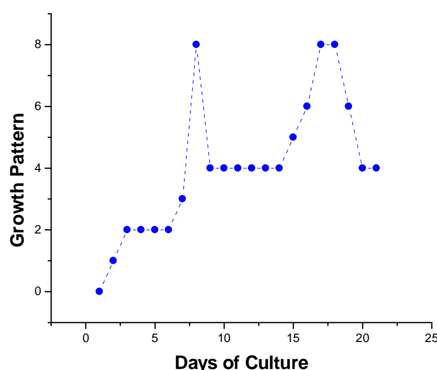


Fig. 3: Phylogenetic tree showing the evolutionary relationship between the unknown isolate (MAJFB) and *Streptomyces longisporoflavus* (Accession No. KY657268.1)

3.2 Cell Growth and Biomass Production

The microorganisms grew in suspension in a liquid medium, and the liquid broth contained nutrients that resulted in the production of enzymes, antibiotics, and other products. The growth of the organism occurred in different phases, as no growth was observed in the first day, which is the lag phase (the organism adapts itself to the growth condition). The growth rate increases from day 2 to day 14 (log-exponential phase), and the growth curve appears sloppy, indicating that the number of divisions per cell unit time indicates the organism's specific growth rate [30]. The highest growth rate was observed on the 17th day and then became steady for 2 days, after which a declination in the growth rate occurred between days 20 and 21 (fig. 4) due to a lack of nutrients, fluctuations in environmental temperature, and the accumulation of toxic waste. This finding corroborates reports that the declination of the growth rate of organisms may be due to the depletion of nutrients and the accumulation of toxic wastes [31]. It has also been reported that while the total number of organisms remains constant, the viable count decreases.



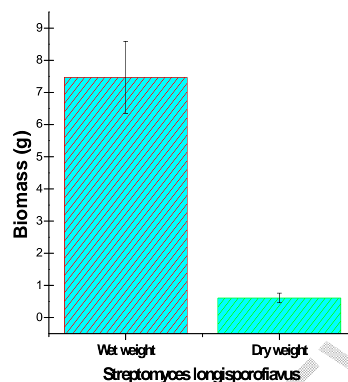


Fig. 4 Fig. 5

Fig. 4: Growth Pattern of *Streptomyces longisporoflavus* at 21 days of incubation

Fig. 5: Biomass wet and dry weight of *Streptomyces longisporoflavus*

3.3 Antimicrobial Susceptibility Test (AST)

Screening for antimicrobial susceptibility was done using the test pathogenic isolates acquired from the culture bank of Ladoke Akintola University Teaching Hospital, Ogbomoso. A cloudy growth of pathogenic organisms was seen on each plate. A clear zone around each hole was measured on each plate, and this was tagged as the inhibition zone (ZI). The inhibition zone shown for each isolate can be seen in figure 5 as follows: *Staphylococcus aureus* (11.10±1.2mm), *Proteus vulgaris* (13.00±1.1mm), *Pseudomonas aeruginosa* (9.0±1.0mm), *P. mirabilis* (10.0±1.1mm),*Hemophilus influenza* (9.0±1.0mm),*Klebsiella*spp(9.0±1.0mm), *Escherichia coli* (10.0±1.2mm), *Staphylococcus pyrogenes*(9.0±1.2mm),*coliforms* (9.0±1.1mm) and *K. pneumonia* (8.0±1.1mm).

From this work, it can be deduced that the metabolites of *S. longisporoflavus* are promising antimicrobial agents, and further improvements can be made for novel applications in producing vital pharmaceutical compounds for the treatment of diseases. Our findings aligns with earlier reports that three actinomycetes have antimicrobial properties against gram-positive bacteria with inhibition zones as large as 20 mm [32]. A similar report showed that actinomycetes metabolites had an inhibition zone of 12 mm against *Salmonella typhi*and *K. pneumonia* [33].Our results showed that *Streptomyces longisporoflavus* metabolites exhibited broad-spectrum antimicrobial properties, which is consistent with previous findings that the genus *Streptomyces* contains a large number of microorganisms with the potential to produce antimicrobial biomolecules [6]. In addition, a similar report showed that *Streptomycessaburaviensis* exhibited broad-spectrum activity and has maximal antimicrobial production on the 5th day of incubation at 30 °C [34-35].

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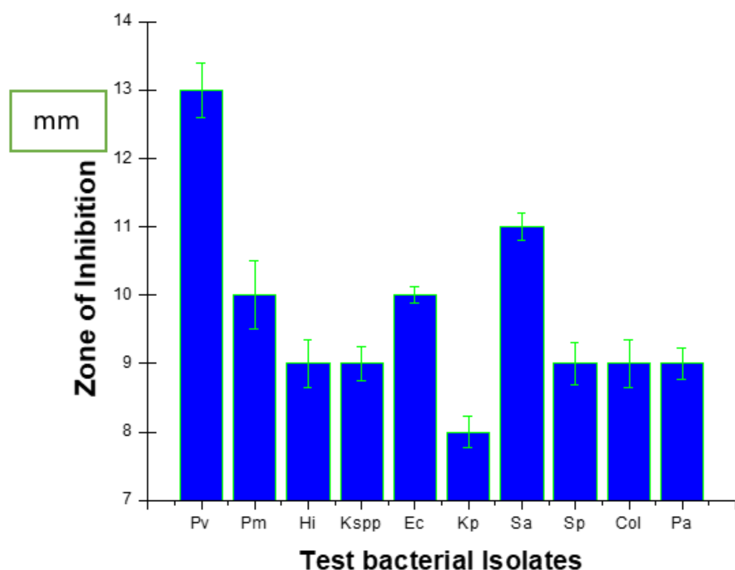

Fig. 6: Antimicrobial activities of *S. longisporoflavus* metabolites of against pathogenic microorganisms and their zones of Inhibition
Proteus vulgaris (Pv), P. mirabilis (Pm), Heamophilus influenza (Hi), Klebsiellaspp (Kspp), Escherichia coli (Ec), Klebsiella pneumonia (Kp), Staphylococcus pyrogenes (Sp), Coliforms (col), Pseudomonas aeruginosa (Pa).

3.5 Identification of Compounds Present in the Metabolites of *Streptomyces longisporoflavus*

A GC-MS was carried out to screen and identify the probable bioactive compounds present in the metabolite (fig. 6).The GC-MS examination identified the bioactive compounds present in the metabolite and their peak areas (fig.7). The identified compounds were confirmed based on the peak area and retention time. There were five (5) compounds present, and the first and third peaks' compounds occur in a fragment that is a resemblance of the same compound.Butane1,1-dibutoxy-2 (1H) was the most prominent compound in the analysed sample because it has the highest percentage area of 51.86% at 19.32min, followed by 5-cholestene-3-ol with a percentage peak area of 33.6% at 44.4min, butanoic acid hexyl ester with a 10.88% peak area at 9.4min, Dronabinol with a 2.34% peak area at 2.3min, and Ergost-5-en-3-ol with 1.87% peak area at 43.8 (table 2).

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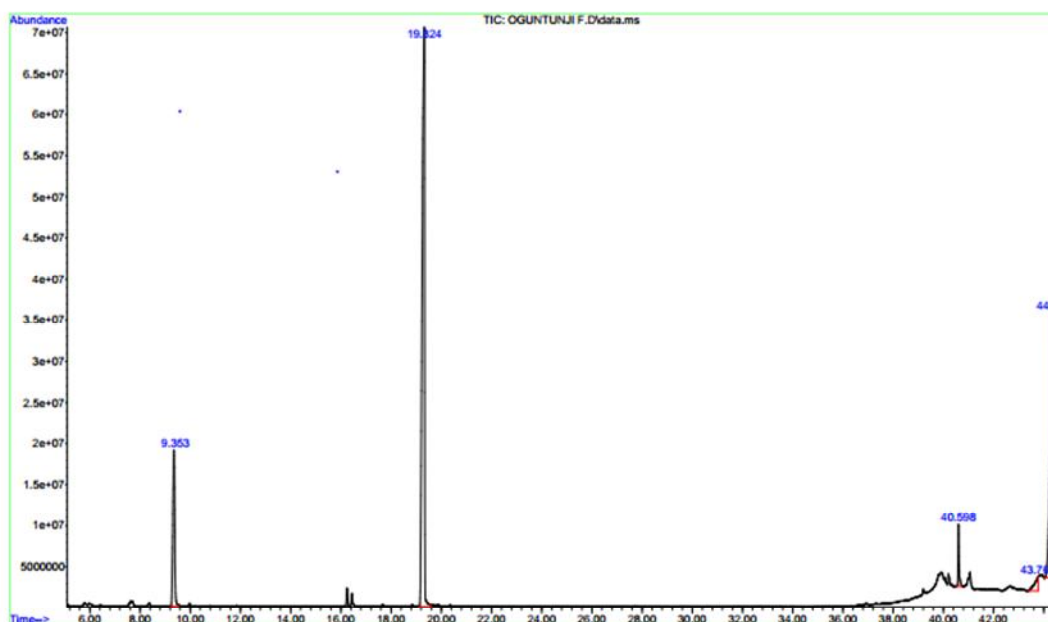

Fig. 7: Chromatogram of metabolite of *Streptomyces longisporoflavus*

Table 2: Notable compounds present in the metabolite of *Streptomyces longisporoflavus*

S/No. (min)	Probable Compound	% Peak Area	Retention time
1	Butane1,1-dibutoxy-2 (1H)	51.86	19.32
2	5- Cholestene -3-ol	33.6	44.40
3	Butanoic acid hexyl ester	10.88	9.36
4	Dronabinol	2.34	40.59
5.	Ergost-5-en-3-ol	1.87	43.76

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3.6 3D Protein Structure Prediction

The protein structure of *Streptomyces longisporoflavus* was viewed with pyMOL Molecular Graphics Systems (fig. 8 a-b) in order to know the properties of the predicted 3D protein structure, revealing the position of segments and types of residues present, which include: Lysine, Serine, Asparagine, Valine, Glutamine, Tyrosine, Glycine, Histidine, Arginine, Methionine, Proline, Trypsin, Isoleucine, Glutamine, Alanine, and Cytosine. The presence of amino acid residues in the metabolite, can be further explored for probiotic activities. The predicted 3D protein structure of the isolate can be effectively utilized to generate rich polypeptide structural information from adequately large and diverse protein family

alignments. The identified compounds present in the metabolites (Butane 1.1-dibutoxy-2 (1H) Quinolinone, 5-Cholestene-3-Ol, Dronabinol, Ergost-5-en-3-Ol, and Butanoic Acid Hexyl Ester) possess antimicrobial properties that can serve as bio-control agents for pathogenic organisms. Therefore, the secondary metabolite extracted from *S. longisporoflavus* has antimicrobial potential for the discovery of new drugs to fight antibiotic-resistant microbes.

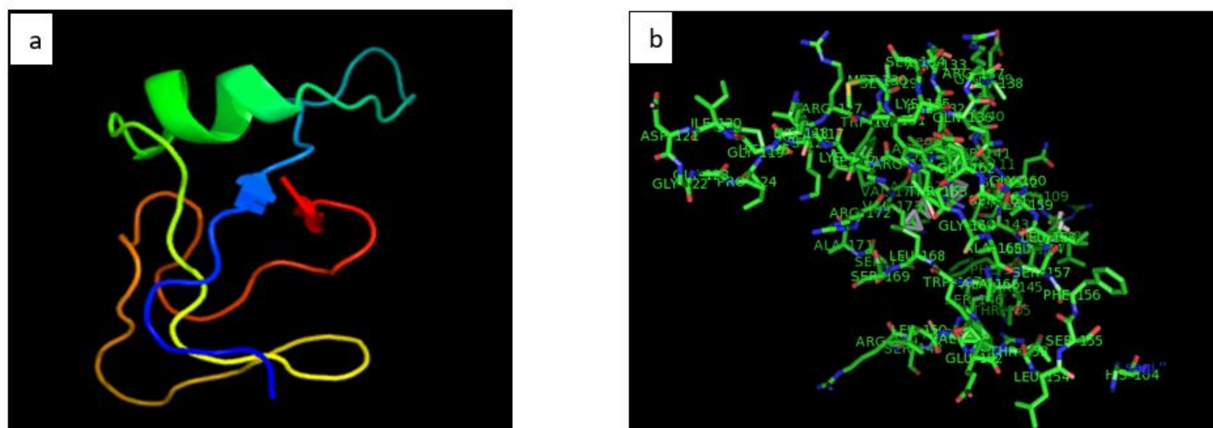

Fig. 8 (a-b): 3D protein structure of *S. longisporoflavus* showing the amino-acid residues present

4.0 CONCLUSION

This study has revealed that a secondary metabolite synthesized from a local isolate of *Streptomyces longisporoflavus* has promising antimicrobial potential and can be used as a drug or drug precursor for combating infectious microbial agents. Therefore, the soil is a good reservoir of microbes, which are natural agents that can be harnessed and utilized in the pharmacy industry for drug synthesis.

CONSENT AND ETHICAL APPROVAL

This was not necessary for this work

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