The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)
ISSN: 2791-8033

The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS), 2023

Volume 12, Pages 1-12

ICMeHeS 2023: International Conference on Medical and Health Sciences

Investigation of Metabolites Isolated from *Sphingomonas egypticus* DM, A Rhizosphere of *Datura metel*

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Abstract: Using naturally occurring bioactive compounds has been crucial in progressing contemporary medical practices. The rhizosphere, which refers to the narrow region of soil surrounding plant roots, serves as a highly dynamic environment for soil bacteria owing to the substantial quantity of organic compounds released into the soil by plant roots. This paper outlines the fermentation and subsequent processing of rhizosphere *Sphingomonas egypticus* DM. The culture media of *Sphingomonas* was cultivated and subsequently subjected to propagation. The ethyl acetate extract was then obtained and subjected to fractionation and purification utilizing various chromatographic techniques. This process led to the isolation of a compound under investigation that exhibited a distinct spot on thin-layer chromatography (TLC) plates, with a calculated retention factor (R_f = 0.56) using a mobile phase composed of methylene chloride and methanol (9:1). Upon spraying with anisaldehyde/sulphuric reagent and subsequent heating for a brief period, the compound displayed a violet colour. This compound was investigated in vitro to assess its antimicrobial and minimum inhibitory concentration (MIC) capabilities. The results demonstrated a significant inhibitory effect against phytopathogenic fungi, specifically *Rhizoctonia solani* (21.3 mm) and *Alternaria alternate* (18.3 mm). Additionally, a moderate inhibitory effect was observed against *Pseudomonas aeruginosa* (12.7 mm), whereas a somewhat less inhibitory effect was shown against *Streptococcus mutans* (9.3 mm). The study also demonstrated that (MIC) against *Fusarium oxysporum* was seen at a concentration of 125 µg/mL compared to various conventional antibiotics. Finally, the strain underwent PCR screening to detect PKS and lipopeptide 4'-phosphopantetheinyl transferase *sfp* genes. The PCR amplification assay demonstrated the presence of genes encoding the KS domain and Surfactin. Furthermore, the sequences of *Sphingomonas egypticus* DM have been officially recorded in the NCBI GenBank database and can be accessed using the accession codes OR469907 and OR499756.

Keywords: *Sphingomonas*, *Datura metel*, Antimicrobial activity, Polyketide synthase, Lipopeptide gene

Introduction

Despite the prevalence of synthetic products, bioactive natural products continue to exert a considerable influence on modern medical practice. Antibiotics have been essential to the development of modern medicine, yet they are currently losing some of their effectiveness. As a result, finding new natural products is a direct consequence of the research into new biodiversity niches (Radić & Štrukelj, 2012). This is where bio products come in, such as bacteria, which have shown that they successfully prolong the amount of time that food may be stored while also conserving the environment and keeping the nutritional value of the food intact (Huang et al., 2003). Biological control agents are also important in drug discovery because of the variety of their structures and the range of their biological activities (Newman & Cragg, 2016; Pastor et al., 2012). *Datura metel* has a long history of use in traditional medicine to treat many diseases, including those related to the nervous and cardiovascular systems, fever, catarrh, pain, diarrhea, skin diseases, chronic bronchitis, asthma, digestive disorders, etc. It is rich in a wide range of essential phytochemicals, such as withanolides, daturaolone, datumetine, daturglycosides, ophiobolin A, baimantuoluoline A, and a lot of other similar compounds (Islam et al., 2023). In addition, the seeds of *Datura metel* plant contain various combinations, the concentration of which rises as the plant matures. These components include alkaloids, tannins, cardiac glycosides, flavonoids, carbohydrates, amino acids, and phenolic substances (Afsharypuor et al., 1995; Ratan et al., 2011).

The rhizosphere is home to a rich microbial community that substantially influences plant development (Philippot et al., 2013; Raaijmakers et al., 2009; Zhou et al., 2021). The antimicrobial activity of rhizobacterial communities is due to specific metabolites, such as phenazines, phenolics, and pyrrole-type compounds (Kennedy et al., 2015). *Sphingomonas* is a genus of rod-shaped bacteria well-known for its ability to digest environmental toxins and industrial pollutants and persist in hostile conditions (Fegatella & Cavicchioli, 2000; Leys et al., 2004; Seo et al., 2009; Zhang et al., 2020). *Sphingomonas* strains have significant quantities of dihydrosphingosine, long-chain sphingolipid, and polyamines (Takeuchi et al., 2001). Extensive research has been conducted on this genus, and 139 species have been identified and described (Busse et al., 2003; Chen et al., 2012; Feng et al., 2017; Shen et al., 2022; Takeuchi et al., 2001; Yabuuchi et al., 2002). The phyllosphere and the rhizosphere are just two plant-based environments that have yielded *Sphingomonas* (Chung et al., 2011; Rivas et al., 2004; Zhu et al., 2015).

The structural characteristics of natural products have shown that most are members of a significant chemical family. This family is known as polyketides, composed of compounds manufactured by multifunctional enzymes known as polyketide synthases (PKSs) (Hildebrand et al., 2004). In symbiotic microbes, the biosynthetic sources of natural products have been conclusively proven in various studies based on the genomic method. This comes from the rapid growth of molecular genetics connected to polyketides in microorganisms (Davidson et al., 2001; Piel et al., 2004; Schmidt et al., 2005). Identifying the authentic microbial origin responsible for the biosynthesis of the desired natural compound from the host organism is essential. Discodermolide is commonly biosynthesized using bacterial type I modular polyketide synthase (Staunton & Weissman, 2001). Antimicrobial substances known as lipopeptides are often generated by *Bacillus* and *Pseudomonas* species and have a cyclic structure with a low molecular weight (Peypoux et al., 1978; Xun-Chao et al., 2013). These primarily comprise a hydrophilic head consisting of seven to ten amino acids coupled to a hydrophobic fatty acid tail (Vanittanakom et al., 1986). Surfactins comprise a chain of seven α-amino acids connected to a distinct β-hydroxy fatty acid. Surfactins have a range of fatty-acid chain lengths from C13 to C16 (Lang, 2002). Surfactins have been shown to exhibit potent antibacterial activity (Gond et al., 2015). It is necessary to research microorganisms' host specificity to determine whether they create particular secondary metabolites with potential ecological or therapeutic significance. In addition, the efficient selection of a specific strain of a bioactive microorganism is based on the presence of a gene responsible for the biosynthesis of a secondary metabolite. Combining the screening for bioactivity with the screening for genes involved in the biosynthesis of secondary metabolites is possibly one appropriate technique (Zhu et al., 2009).

Sphingomonas egypticus DM, a rhizosphere strain, has been shown to secrete a promising secondary metabolite, and this has sparked a lot of interest in the possibility of exploring the biological consequences of this metabolite. Moreover, using PKS and lipopeptide *sfp* phylogenies, this study attempted to present molecular evidence of the host specificity of *Sphingomonas egypticus* DM.

Materials and Methods

Collection of Bacterial Strain

Sphingomonas egypticus strain DM (OQ269457), previously isolated from the rhizosphere of *Datura metel* by the current authors, was obtained from the laboratory collection of the current authors.

Large-Scale Fermentation and Working Up for Extraction of Secondary Metabolites

As a seed culture, the bacterial suspension containing the selected effective strain was inoculated into 100 mL of tryptone soy broth medium and cultured at 37°C for two days. For propagation, 5 mL of the seed culture was transferred in an aseptic manner to inoculate a total of ten 1 L Erlenmeyer flasks. The flasks were incubated at 37°C for 14 days while continuously shaken. Following incubation, bacterial cultures were sonicated for 30 minutes to rupture the cells. After harvesting, the broth was macerated in methanol (5 L). Following filtration and vacuum concentration, the methanol extract was prepared. The remaining residue was extracted using ethyl acetate until exhaustion after suspending it in water. The ethyl acetate extract was vaporized until completely dry and preserved for subsequent purification (Hamed et al., 2021).

Purification and Identification of the Isolated Secondary Metabolites

2.5 grams of ethyl acetate fraction was subjected to normal phase silica gel column chromatography (60 x 2 cm). A stepwise elution of the column with DCM–MeOH gradient [DCM, DCM: MeOH (95:5)] was monitored by analytical TLC and preparative TLC (0.5mm thick) performed on pre-coated silica gel plates (Merck, Germany). R_f values of the bioactive compounds and visualization of their chromatograms were carried out under UV light (254 and 365 nm) and further by spraying with anisaldehyde/sulfuric acid reagent and heating (Springer-Verlag, 1984). Furthermore, the use of Dragendorff reagent is employed for the purpose of detecting the presence of alkaloids (Linch et al., 1973).

Screening of the Antimicrobial Activities

Test Microorganisms

Gram-positive bacteria (*Staphylococcus aureus ATCC:13565* and *Streptococcus mutans ATCC:25175*) and Gram-negative bacteria (*Escherichia coli ATCC:10536, Pseudomonas aeruginosa ATCC:27853* and *Klebsiella pneumonia ATCC:10031*) were provided by Micro Analytical Center, Faculty of Science, Cairo University. Additionally, Fungal Phytopathogens (*Alternaria alternate SCUF0000310, Rhizoctonia solani SCUF0000317,* and *Fusarium oxysporum SCUF000091*) were received from the Agricultural Genetic Engineering Research Institute, Cairo University, Cairo.

Antimicrobial Activity

The antimicrobial activity of the purified secondary metabolite was investigated against various human and phytopathogenic microorganisms using the agar well diffusion method to observe the zones of inhibition in comparison with conventional antibiotics. Using Mueller–Hinton agar media, the chemical was evaluated for its potential to inhibit the growth of bacteria *in vitro*. In addition, the antifungal activity was assessed using a sabouraud dextrose agar medium. For Gram-positive and Gram-negative bacteria, the antibiotics ampicillin and gentamicin, respectively, were used as standard drugs. For fungal strains, nystatin was considered as a standard drug. As a solvent control (a negative control), DMSO was utilized. A sterilized cork borer was used to create wells in the solidified media with a diameter of 6 mm. After that, a total volume of 100 µL of the tested compound was added to each well. Regarding antibacterial activity, the plates were kept at 37°C for 24 hours, but for antifungal activity, they were kept at 28 °C for three to five days. This experiment was performed in triplicate, and the inhibition zones were determined on a millimeter scale (Scott, 1989).

Determination of MICs

A single colony was selected from each strain and placed into a tube containing 3-4 mL of sterile broth medium. The mixture was incubated at 37°C for 2-6 hours, and the turbidity of the suspension was then compared to a McFarland Standard 0.5. Once the turbidity was equal to or greater than the standard, the antimicrobial agent was dissolved in 1 mL of DMSO, and two-fold serial dilution was done using a broth medium. A predetermined quantity of the bacterial inoculum was introduced into each tube and allowed to incubate for 16-20 hours (or 24-48 hours in the case of fungal inoculum). The Minimum Inhibitory Concentration (MIC) represents the minimum concentration of antimicrobial agent that effectively curtails the visible growth of the tested isolate, ascertained through naked-eye observation (Chudáčková et al., 2010).

Extraction and Purification of DNA

To transfer the freshly enriched *Sphingomonas* culture, a micro-centrifuge tube with a 1.5 mL capacity was utilized, with one milliliter of the culture being transferred. The cell suspension was centrifuged for 10 minutes at 14,000 × g. The pellet was then resuspended in 300 µL of DNase-RNase-free distilled water and centrifuged once more for 5 minutes at 14,000 × g. After carefully discarding the supernatant, the pellet was resuspended in 200 µL of DNase-RNase-free distilled water. The suspension was then incubated for 15 minutes at 100°C and rapidly chilled on ice. Finally, it centrifuged for 5 minutes at 14,000 × g at 4°C, with 5 µL of the supernatant used as the PCR template DNA (De Medici et al., 2003).

PCR Amplification

The Taq polymerase relied on DNA samples as its template, and all PCRs were conducted using a T1 Thermal Cycler from Biometra, Germany. The resulting products underwent screening via 1% agarose gel electrophoresis. To further analyze *Sphingomonas egypticus* DM, which exhibited notable antimicrobial activity, we searched for the 4'-phosphopantetheinyl transferase *sfp* gene for surfactin lipopeptide. The lipopeptide gene was amplified through PCR, starting with an initial denaturation of 5 min at 95°C, followed by 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 52°C), extension (1 min at 72°C), and a final extension at 72°C for 10 min. For detecting modular PKS genes, degenerate oligonucleotide primers were utilized. The reactions involved the following steps and cycles: 3 min at 94°C, 35 cycles at 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, followed by 7 min at 72°C (Gond et al., 2015; Zhu et al., 2009). The primers used for respective genes are listed in Table 1.

Table 1. PCR primers for amplification of lipopeptide *sfp* and PKS genes.

Target gene	Primers	Sequences (5' - 3')	Amplicon size
sfp gene	*sfp*-f	ATGAAGATTTACGGAATTTA	675
	sfp-r	TTATAAAAGCTCTTCGTACG	
KS domain	KSF	GCGATGGATCCNCAGCAGCG	680
	KSR	GTGCCGGTNCCGTGNGYYTC	

DNA Sequencing

The PCR product purification from 1% agarose gel was accomplished using a GeneDireX gel extraction kit (Taiwan). After that, the Macrogen facility in Korea performed sequencing using the *sfp*-f and KSF forward primers with the Big TriDye sequencing kit (ABI Applied Biosystems) (Zhu et al., 2009).

Sequence Analysis and GenBank Accession Numbers

In conjunction with the National Center for Biotechnology Information (NIH, MD, U.S.A.), the BLAST tool was utilized to establish the similarity percentage of the nucleotide sequences compared to other sequences. To create a phylogenetic tree, the nucleotide sequences were aligned with different sequences sourced from GenBank, utilizing Clustal X (Thompson et al., 1997). From there, MEGA version 3.1 was used to construct neighbor-joining phylogenies (Kumar et al., 2002). Lastly, the sequences presented in this study from *Sphingomonas egypticus* DM have been deposited in the NCBI GenBank database.

Statistical Analysis

One-way analysis of variance (ANOVA) is used to thoroughly analyze the antimicrobial study data. After that, a Duncan multiple comparisons test was carried out with the SPSS software version "22" for Windows.

Results and Discussion

Large-Scale Fermentation And Working Up

Large-scale fermentation of the bacterium followed by solvent extraction and purification using various chromatographic methods (see experimental section) led to the isolation of potent secondary metabolite. The obtained fractions from the column chromatography of the bacterial crude culture extract (2.5 gm) were monitored by TLC, and the common fractions were collected. In our previous work (submitted to BMC complementary medicine and therapies), two compounds were identified and tested for their biological activity. Here, we report the isolation of further compound eluted from the column using a mobile phase composed of methylene chloride and methanol (9:1). Testing of this compound on TLC using the same solvent polarity revealed its purity, and it acquired a violet colour on spraying with anisaldehyde/sulphuric acid reagent. The purity of the isolated compound was also verified by preparative thin layer chromatography (PTLC) to eliminate any other contaminations present (Figure 1). The chemical structure of this compound is under investigation. Simultaneously, we continue testing its biological activities.

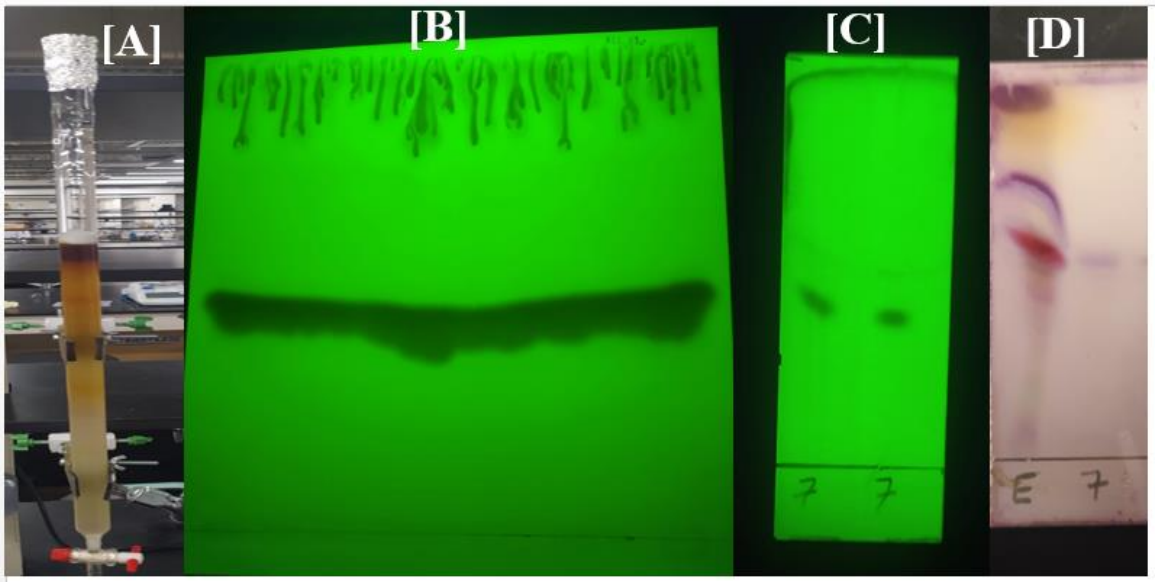

Figure 1. Various chromatographic methods used for isolation and purification of a potent secondary metabolite [A] Bacterial crude extract subjected to column chromatography, [B] Preparative TLC for purification, [C] TLC visualized by UV light (254 nm), [D] Spraying with anisaldehyde/sulphuric acid reagent. Dragendorff reagent gives negative result.

Biological Activities

Antimicrobial Screening

As part of the research, a refined secondary metabolite was derived from *Sphingomonas egypticus* DM and assessed for its ability to combat microbes. Following incubation, clear zones with measurements ranging from 9.3 to 21.3 mm were observed against various bacteria and fungi, as detailed in Table 2. These findings suggest that the isolated compound has the potential to serve as an antibacterial or antifungal agent against certain harmful microbes. Based on the agar well diffusion method, it was determined that the compound effectively inhibits at least three tested pathogens. It showed significant antimicrobial activity against pathogenic fungi, such as *Rhizoctonia solani* (21.3 mm), *Alternaria alternate* (18.3 mm), and *Fusarium oxysporum* (17.0 mm). Moreover, a moderate inhibitory effect has been shown against pathogenic bacteria such as *Pseudomonas aeruginosa* (12.7 mm) and *Klebsiella pneumonia* (12.3 mm). However, the metabolite exhibited the least inhibitory effect against *Streptococcus mutans* (9.3 mm), as illustrated in Figure 2.

Numerous types of bacteria, such as *Sphingomonas, Bacillus, Pseudomonas, Aeromonas, Marinobacter, Nocardia,* and *Methylobacterium,* have been researched for their ability to create antimicrobial substances that relate to the rhizosphere and phyllosphere (Bodenhausen et al., 2013; Buedenbender et al., 2017; Chen et al., 2019). Studies have indicated that *Sphingomonas* sp. is the primary organism in healthy dicotyledonous plants and can lessen disease symptoms while hindering the growth of the foliar plant pathogen *Pseudomonas syringae* (Innerebner et al., 2011). Studies indicate that *Sphingomonas* spp. has the potential to shield plants against Xcc, a notorious bacterium known for causing plant diseases (Buell, 2002). This suggests that they may be able to combat a broader spectrum of foliar pathogens. As Ji and Wilson explain, bacterial strains with a higher

nutritional resemblance to the pathogen are more likely to be effective against plant diseases (Ji & Wilson, 2002). Natural compounds, such as secondary metabolites synthesized by plants and microbes, have been extensively researched as a sustainable substitute for synthetic chemicals (Atanasov et al., 2015).

Table 2. Antimicrobial activities of pure secondary metabolite recovered from *Sphingomonas egypticus*.

Sample / Microorganism	Zone of inhibition (mm)	
	Pure Secondary metabolite	Standard antibiotic
Gram-negative bacteria		Gentamicin
Escherichia coli (ATCC:10536)	NA	27±1.0
Klebsiella pneumonia (ATCC:10031)	12.3±0.6	25.3 ±0.7
Pseudomonas aeruginosa (ATCC:27853)	12.7±0.6	28±1.0
Gram-positive bacteria		Ampicillin
Staphylococcus aureus (ATCC:13565)	NA	21.3±0.7
Streptococcus mutans (ATCC:25175)	9.3±0.6	28.3±0.7
Fungal phytopathogens		Nystatin
Alternaria alternate (SCUF0000310)	18.3±0.6	21±0.5
Rhizoctonia solani (SCUF0000317)	21.3±0.6	19±0.5
Fusarium oxysporum (SCUF000091)	17.0±1.0	15±0.5

Values are means of three replicates ± standard deviation (SD)

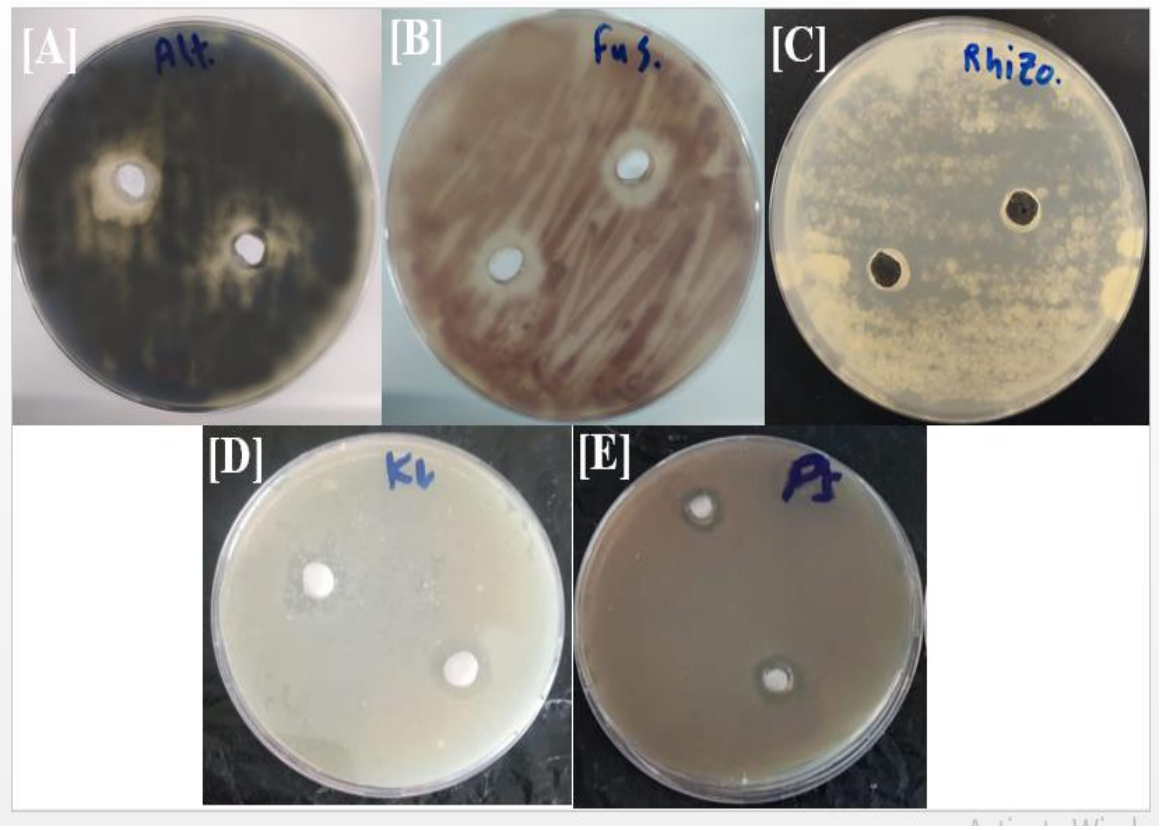

Figure 2. Antimicrobial activities of pure metabolite isolated from *Sphingomonas egypticus* DM against [A] *Alternaria alternate*, [B] *Fusarium oxysporum*, [C] *Rhizoctonia solani*, [D] *Klebsiella pneumonia*, [E] *Pseudomonas aeruginosa*.

Minimum Inhibitory Concentrations (MICs)

According to the study, the growth of pathogenic microorganisms was effectively inhibited by potent secondary metabolite. The pure secondary metabolite exhibited a minimum inhibitory concentration (MIC) range of 125 to 500 µg/mL when tested against various pathogens. The pure metabolite demonstrated the highest efficacy against *Fusarium oxysporum* with a MIC value of 125 µg/mL. At the same time, it exhibited the lowest efficacy against *Klebsiella pneumonia*, indicating a MIC value of 500 µg/mL (Table 3).

Table 3. MICs of pure secondary metabolite recovered from *Sphingomonas egypticus* against the tested organisms.

Sample / Microorganism	MICs (µg/mL)	
	Pure Secondary metabolite	Standard antibiotic
Gram-negative bacteria		Gentamicin
Escherichia coli (ATCC:*10536*)	-	31.25
Klebsiella pneumonia (ATCC:10031)	500	62.5
Pseudomonas aeruginosa (ATCC:27853)	250	125
Gram-positive bacteria		Ampicillin
Staphylococcus aureus (ATCC:13565)	-	62.5
Streptococcus mutans (ATCC:25175)	-	62.5
Fungal phytopathogens		Nystatin
Alternaria alternate (SCUF0000310)	250	25
Rhizoctonia solani (SCUF0000317)	250	22.3
Fusarium oxysporum (SCUF000091)	125	32.4

A recent study examined different metabolites from the same strain, *Sphingomonas,* to determine their Minimum Inhibitory Concentration (MIC) values. The results showed that two metabolites, sattabacin-like and xenocyloin B, had MIC values below 32 µg/mL when isolated from a co-culture of *Paenibacillus* and *Sphingomonas* strains against various clinical strains of *Acinetobacter baumannii* (Qi et al., 2021). This study underscores the significance of developing drugs with lower MIC values, as they would be more cost-effective and productive.

Screening of Lipopeptide *sfp* and PKS Genes

The presence of the lipopeptide 4'-phosphopantetheinyl transferase *sfp* gene in *Sphingomonas egypticus* DM was confirmed through PCR amplification, indicating the strain's antimicrobial activity. Moreover, the results showed that the PKS KS domain was successfully amplified in the DNA template from the rhizospheric strain, as depicted in Figure 3. Subsequent annealing-temperature gradient PCRs were conducted to verify the initial PCR results. The amplified fragments were sequenced to validate that they were indeed the lipopeptide *sfp* gene and PKS KS domain. Sequencing was performed on all unique clones of the appropriate size (approximately 675 bp for the lipopeptide *sfp* gene and 680 bp for the PKS KS domain). A phylogenetic tree was generated to compare the KS domains of various microorganisms. The PKS KS fragments from *Sphingomonas egypticus* DM exhibited the highest similarity to the KS fragments of *Salmonella enenterica* and *Escherichia coli* (Figure 4). Meanwhile, the lipopeptide *sfp* fragments from the same strain revealed sequence similarity to the fragments of *Bacillus sibtilis* (Figure 5). The sequences presented in this study have been deposited in GenBank under the accession numbers OR469907 and OR499756.

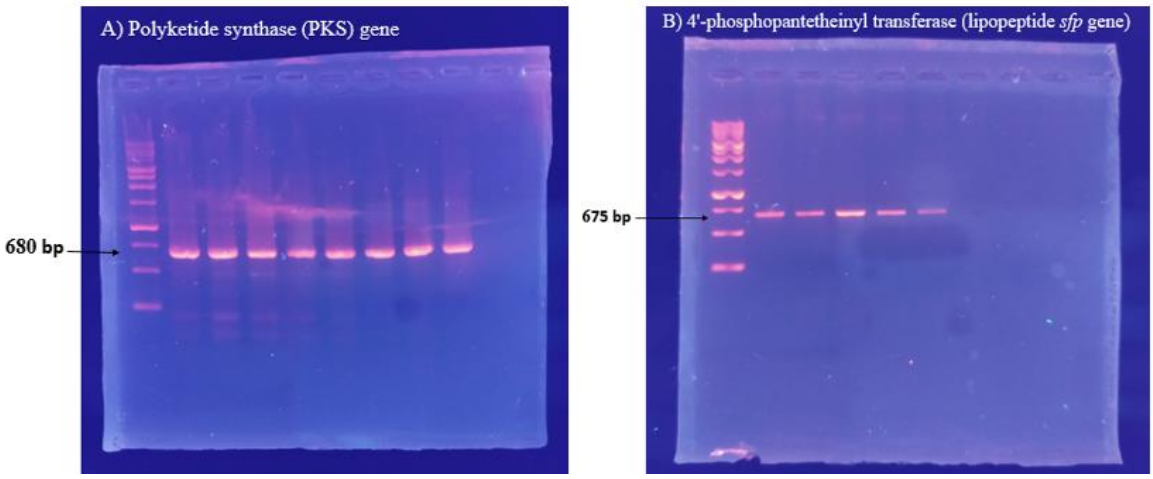

Figure 3. PCR amplification shows polyketide synthase (**A**) and lipopeptide *sfp* (**B**) genes.

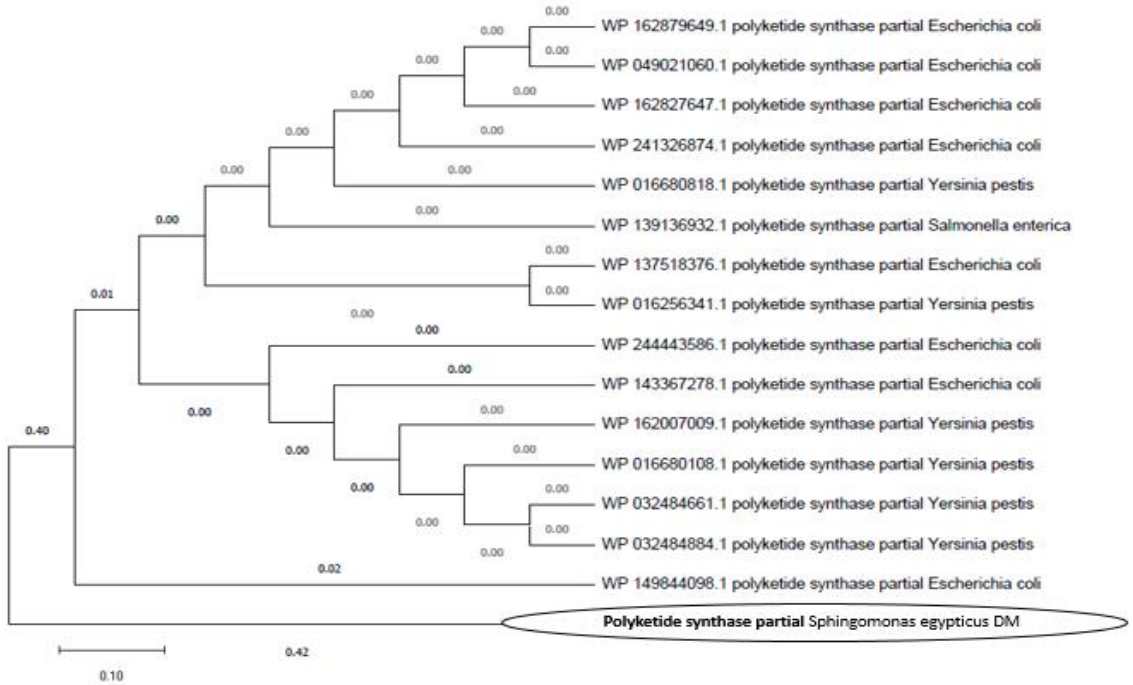

Figure 4. Phylogenetic analysis of KS domain from Sphingomonas egypticus DM compared to other microorganisms' diverse KS fragments

The production of lipopeptides in *Bacillus* spp. is dependent on the activity of a pivotal factor, the 4′-phosphopantetheinyl transferase (PPTase), which is encoded by the *sfp* gene (Jin et al., 2017). Lipopeptides are considered to be major antimicrobial compounds that are grouped under surfactins. It has been discovered that surfactin is a highly effective antibacterial agent through gene amplification screening (Gond et al., 2015). The genome of *Sphingomonas egypticus* DM has confirmed the presence of the antimicrobial surfactin gene (as shown in Figure 3). This exciting discovery suggests that *Sphingomonas egypticus* DM may secrete this antimicrobial lipopeptide in the rhizosphere of *Datura metel* to protect the plant from various pathogens. Other lipopeptides, such as surfactin-like bamylocin A from *Bacillus amyloliquefaciens*, kurstakin from *Bacillus thuringiensis*, maltacines from *Bacillus subtilis*, and polymyxins from *Bacillus polymyxa*, have also been reported (Hagelin et al., 2007; Hathout et al., 2000; Lee et al., 2007; Storm et al., 1977). Polyketide Synthase is a biosynthetic system in microorganisms synthesizing numerous biologically active compounds. To determine whether this system exists in the isolated bioactive bacteria, PCR primers were created to amplify the KS domains of PKS. After screening, the results confirmed the presence of PKS in the bacterial strain. Therefore, this study supports the hypothesis that bioactive bacteria should contain PKS-specific genes. To pinpoint the biosynthetic gene in bioactive bacteria, combining bioactivity screening with secondary metabolite biosynthetic gene screening is crucial while incorporating conserved sequences from other biosynthetic pathways for PCR screening (Zhu et al., 2009).

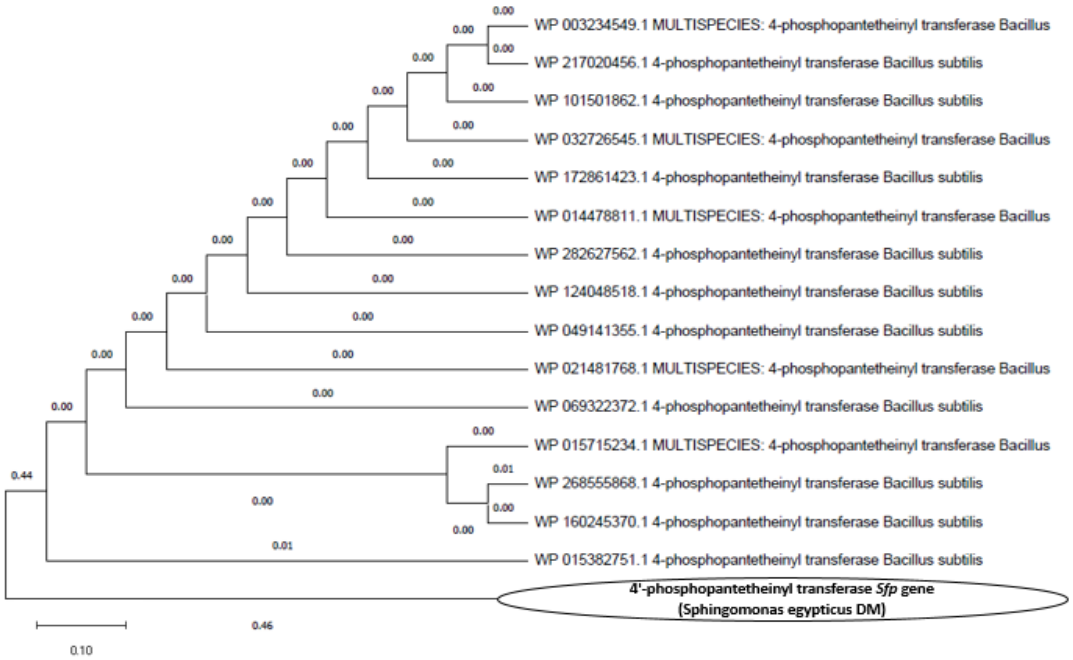

Figure 5. Phylogenetic analysis of lipopeptide *sfp* gene from *Sphingomonas egypticus* DM compared with various lipopeptide *sfp* fragments of *Bacillus subtilis*.

Conclusion

Using various chromatographic methods, we successfully extracted a pure secondary metabolite from the crude extract of rhizospheric *Sphingomonas egypticus* DM. Our findings suggest that the isolated compound exhibits promising biological activity against pathogenic fungi, specifically *Rhizoctonia solani* and *Alternaria alternate*. Notably, this strain demonstrated the lowest minimum inhibitory concentrations (MICs) against *Fusarium oxysporum*. Further PCR amplification and sequencing analysis revealed the presence of PKS and lipopeptide *sfp* genes in the strain. We have deposited the obtained sequences in the NCBI GenBank database under OR469907 and OR499756.

Recommendations

It is necessary to develop various techniques that combine bioactivity screening with screening for genes related to secondary metabolite biosynthesis to determine the factors causing the biological effect.

Scientific Ethics Declaration

The authors declare that the scientific, ethical and legal responsibility of this article published in EPHELS journal belongs to the current authors. The manuscript contains original work, and all authors mutually agree for submission.

Acknowledgements or Notes

* This article was presented as an oral presentation at the International Conference on Medical and Health Sciences (www.icmehes.net) held in Antalya/Turkey on November 16-19, 2023.

*The authors are thankful to the Ministry of Higher Education and Scientific Research (MOHE), Egypt for supporting this work. The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

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To cite this article:

Awad, M. A., Soliman, H. S., El-Mashtoly, S. F., El-Deeb, B., & Hammad, S. F. (2023). Investigation of metabolites isolated from *Sphingomonas egypticus* DM, a rhizosphere of *Datura metel*. *The Eurasia Proceedings of Health, Environment and Life Sciences (EPHELS)*, *12*, 1-12.