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Antibacterial Activity of Soil Bacteria against *Escherichia coli* and GC-MS Analysis of their Organic Compounds

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Abstract

Antimicrobial agents encompass a wide range of compounds, including antibiotics, bacteriocins, and lipopeptides, which play a crucial role in combating infectious diseases. Antibiotics, in particular, are secondary metabolites of low molecular weight predominantly synthesized by soil-dwelling microorganisms. These microbial metabolites have long served as a vital source of clinically important therapeutic agents. Members of the genus ***Bacillus*** and other rhizosphere-associated bacteria are especially known for producing diverse antimicrobial substances. In this study, ten rhizosphere soil samples were collected from different sites in Sana'a city, Yemen. From these samples, 50 antibiotic-producing soil bacteria were isolated. Bioactive metabolites were extracted using the solvent extraction method with chloroform and Ethanol. The crude extracts were analyzed by Gas Chromatography–Mass Spectrometry (GC–MS) to identify their organic composition. Among the 50 bacterial isolates, 14 showed antibacterial activity against resistant ***Escherichia coli*** using the agar well diffusion method. Further secondary screening revealed that the filtrates of four isolates ***Pseudomonas fluorescens, Bacillus subtilis***, ***Acinetobacter baylyi***, and ***Azotobacter vinelandii*** exhibited the strongest antibacterial effects. GC–MS analysis showed that each isolate produced more than eighty organic compounds; however, only a subset demonstrated antibacterial activity. The most notable bioactive compounds detected included Phenol, 4-(2-aminoethyl)- (CAS: tyramine), 2,4-di-tert-butylphenol, and n-hexadecanoic acid. This study highlights the potential of rhizosphere soil bacteria as promising sources of novel bioactive compounds. The identification of active metabolites and their antibacterial properties against resistant ***E. coli*** underscores their possible application in the development of alternative therapeutic strategiesto address antibiotic resistance

Article Info

Keywords:
Secondary metabolites, GC-MS spectrophotometer, ***Escherichia coli***, Soil bacteria, Organic compounds, Chloroform, Ethanol.

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1. INTRODUCTION

In response, innovative strategies, including machine learning-based resistance prediction and enhanced bacteriophages and, therapies, are under investigation to tackle these resistant strains [1]. Additionally, a common metabolite is present in soil 50% of ***Bacillus*** species and over 88% of ***Pseudomonas*** strains inhabiting rhizospheric soil and plant root nodules [2], While the microbial communities vary across different environments, soils are recognized as hosting an urgent need for robust antibiotic stewardship and comprehensive global monitoring systems [3], The microbial communities vary across different environments, and soils are recognized as hosting the most complex and diverse microbiomes on Earth [4].

Bacillus subtilis is a gram-positive, endospore-forming bacterium that produce a diverse array of secondary metabolites with important ecological and biotechnological roles. Although these compounds are not essential for bacterial growth, they play vital roles in microbial competition, communication, and defence. Notably, ***B. subtilis*** generates various lipopeptides such as surfactins, iturins, and fengycins, which possess strong antimicrobial and antifungal activities [5]. creating pores

in the cell membranes [6]. Besides lipopeptides, ***B. subtilis*** produces polyketides including bacillaene, difficidin, and macrolactin, which exhibit antibacterial effects by inhibiting protein synthesis [7].

Pseudomonas fluorescens is a metabolically adaptable gram-negative bacterium renowned for producing a wide variety of secondary metabolites that support its ecological versatility and beneficial interactions with plants. A prominent metabolite is pyoverdine, which is a high-affinity siderophore that facilitates iron acquisition under nutrient-limited conditions. Pyoverdine synthesis is strongly affected by environmental factors, including the type of available carbon source [8]. Beyond siderophores, P. fluorescens also emits various volatile organic compounds (VOCs) which not only suppress phytopathogens but also promote plant growth and activate plant defense mechanisms [9].

Acinetobacter baylyi, especially the ADP1 strain, has attracted interest because of its natural competence and extensive metabolic versatility, making it a valuable model for environmental and synthetic biology research [10].

Azotobacter vinelandii produces antimicrobial compounds, including siderophores, ammonia, and hydrogen cyanide, which inhibit the growth of pathogens such as ***Escherichia coli, Staphylococcus aureus,***and ***Pseudomonas aeruginosa*** [11]. These metabolites create an inhospitable environment for pathogens by sequestering vital nutrients such as iron and generating toxic substances that compromise bacterial cell integrity [11].

Escherichia coli (E.coli) is a well-studied bacterium commonly found in the intestinal tracts of humans and animals. Although many strains are harmless, some cause serious infections including urinary tract infections, sepsis, and gastroenteritis. Over recent decades, the rise and spread of antibiotic-resistant E. coli strains have emerged as a significant global Public health threat [12, 13]. ***E.coli*** employs various mechanisms to develop resistance, such as producing extended-spectrum β-lactamases (ESBLs), utilizing efflux pumps, and forming biofilms, all of which reduce the efficacy of many commonly prescribed antibiotics [14].

E.coli employs various mechanisms to develop resistance, such as producing extended-spectrum ß-lactamases (ESBLs), utilizing efflux pumps, and forming biofilms, all of which reduce the efficacy of many commonly prescribed antibiotics [14]. Surveillance data indicate a growing prevalence of multidrug-resistant ***E. coli*** in both healthcare settings and communities, highlighting the urgent need for robust antibiotic stewardship and comprehensive global monitoring systems [3].

The aim of this study was to isolate soil bacteria with antibacterial activity against ***Escherichia coli,*** evaluate their inhibitory potential through in vitro assays, and identify the bioactive organic compounds responsible using gas chromatography–mass spectrometry (GC-MS).

2. MATERIALS AND METHODS

Collection of samples

In a systematic screening program for the isolation of bacteria, 10 soil samples were collected from different locations within Sana'a city from **July 6, 2024** to **July 19, 2024**. These samples were collected from the rhizosphere area where most of the microbial activity occurs ,and thus, where most of the bacterial population is concentrated [15].

Soil samples (approximately 5g) were collected in a clean, dry, sterile lastic bag with a sterile spatula [16].

Table 1. Sampling Sites and Corresponding Dates of Collection

Sample No.	Name of Site	Date of collection
1	Science collage	July6, 2024 11:46AM
2	Art collage	July6, 2024 11:56AM
3	Al_ahsan garden	July7, 2024 3:41PM
4	New Sana'a university	July8, 2024 1:33PM
5	Altahrer street	July9, 2024 12:45PM
6	Alhasbah street	July12, 2024 2:43PM
7	Sawan garden	July13, 2024 12:45PM
8	Home's Soil (from plant)	July16, 2024 12:45PM
9	Almadenah street	July18, 2024 4:21PM
10	Motaher taqi street	July19,2024 12:21PM

Isolation of Soil Bacteria

1g of the soil samples was dissolved in 10 ml of sterile distilled water to make soil suspensions [16] to solidify. This method followed standard microbial culture procedures as described in recent study [17] In this study , nutrient agar medium was used for bacterial cultivation. A total of 28 g of nutrient agar **(Himedia)** was weighed and dissolved in 1000 mL of distilled water. The solution was vigorously stirred and heated on a hot plate until it was fully dissolved. It was then sterilized in an autoclave at 121ºC for 15 min. After sterilization, the medium was allowed to cool to approximately 45–50ºC before being poured into sterile Petri dishes, where it was left Portions of the suspension were inoculated onto nutrient agar using the plate dilution method and incubated at 37°C for 24 hours.Subsequently , colonies of different bacterial isolates were observed the broth culture inoculum, and in the pure isolates were preserved on **nutrient agar slants** at the refrigeration temperature [18].

The identification of bacterial isolates

Soil isolates identified as pure were grown on a nutrient agar plate for 24 h, after which, for each isolate, a single isolated colony was picked from the plate and suspended in 5 ml Eppendorf tube containing 3 ml of nutrient broth using the loop, after which the plates and tubes were incubated at 37 °C for 24h for plates and 72h

for tubes [19].

Gram's staining

Colonies that were grown on nutrient agar were Gram-stained by the standard Gram taining procedure described by [20].

Biochemical Tests

Biochemical tests, including catalase, oxidase, indole, methyl red, citrate utilization, and triple sugar iron tests, were performed following the standard procedures described previously [21, 22].

Extraction of antimicrobial agents from bacteria

Bacterial isolates were inoculated in Erlenmeyer flasks containing nutrient broth and incubated for 3 - 4 days. The fermentation flask was incubated at 110 rpm on a rotary shaker at room temperature for 7 days. After fermentation, the culture broth was filtered and the filtrates were separately mixed with solvents, chloroform, and ethanol, in a ratio of 1:1. Chloroform was added to the culture broth, to form two layers, and the solvent layer was separated using a separating funnel and stored in sterile vials. The ethanol-added culture broth was retained as an aqueous extract [23].

Confirmation of Antibacterial Activity

To evaluate the antibacterial activity of the bacterial isolates with potential antibiotic production, the agar well diffusion method was employed. In this technique, a standardized bacterial suspension was spread over the surface of a Muller Hinton agar plate to form a uniform lawn, after which wells (6 μm) were aseptically created and filled with bacterial extract(10 μl). The plates were then incubated to allow diffusion of substances and interaction with the test organisms. The formation of clear inhibition zones around the wells indicated antibacterial activity , and the inhibition zone was measured in mm [19].

GC-MS Analysis of Bacterial Metabolites

Secondary Metabolites were analyzed by Gas chromatography coupled mass spectrometry (GC-MS) (YSMO) Yemen Standardization, Metrology and Quality Control Organization in Sana'a city –Yemen Model **(GCMS QP2020 NX-SHIMADZU JUPAN)** was used to identify the compounds. GC-MS analysis was performed using a Perkin–Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with an Elite-5MS (5% biphenyl /95% dimethyl poly siloxane) fused capillary column (30 × 0.25 μm ID × 0.25 μm df). For GC-MS detection, an electron ionization system was operated in the electron impact mode with an ionization energy of 70 eV. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 ml/min, and an injection volume of μl was employed (split ratio of 10:1). The injector temperature was maintained at 250 ℃, the ion-source temperature was 200 ℃, the oven temperature was programmed from 110 ℃ (isothermal for 2 min), with an increase of 10 ℃/min to 200℃, then 5 ℃/min to 280 ℃, ending with a 9 min isothermal at 280 ℃. Mass spectra were recorded at 70 eV, and the component was calculated by comparing its average peak area to the total area. Turbo-Mass Gold-Perkin-Elmer- mass detector was used, and Turbo-Mass ver-5.2 software was used to handle mass spectra and chromatograms scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 - 2 min, and the total GC-MS run time was 36 min. The relative percentage of each component was calculated by comparing the average peak area to the total area. A Turbo-Mass Gold-Perkin-Elmer- mass detector was used, and Turbo-Mass ver-5.2 software was used to handle mass spectra and chromatograms [24].

Description of components

The mass spectrum of GC-MS was interpreted using the database of the National Institute of Standards and Technology (NIST), which contain more than 62,000 patterns. The mass spectrum of the unknown components was compared with the spectrum of the known components stored in the NIST library. Name and molecular weight, the structure of the components of the test materials was confirmed as in [24]

3. RESULTS

The results revealed that 50 bacterial isolates were recovered from cultivated soils collected from ten locations in Sana'a city-Yemen, as shown in Al-Ahsa, Saudi Arabia (Figure 1)

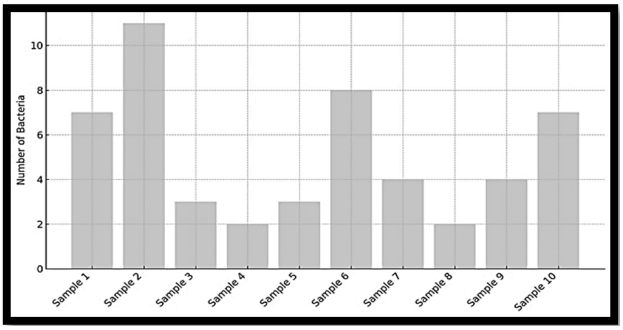


Figure 1. Number of Bacterial isolates recovered from 10 Soil samples

Out of 50 bacterial isolates, only 14 isolates showed variable degrees of variable degrees against ***Escherichia coli*** ,with different inhibition zones ranging from 10 mm to 20 mm, as shown in (table 2)

Fourteen antagonistic bacterial isolates were identified at the species level, of which four demonstrated strong antimicrobial activity based on biochemical characterization."

-MS Analysis of Bacterial Metabolites

The GC-MS chromatogram of the chloroform extract (Figure 2) showed four peaks, indicating the presence

Table 2. Antimicrobial activity of soil bacterial isolates against *E. coli:*

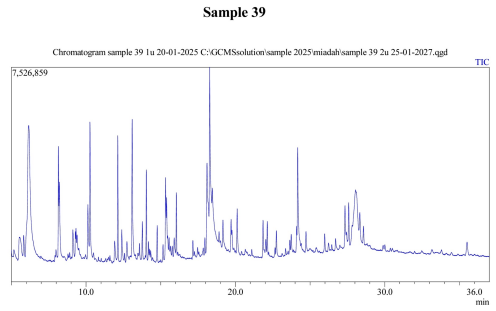
Bacteria species	Inhibition zones against *E.coli*
Bacillus subtilus	15mm
Pseudomonas fluorescens	16mm
Bacillus subtilis	13mm
Pseudomons fluorescens	18mm
Bacillus subtilis	12mm
Pseudomons fluorescens	14mm
Bacillus subtilis	13mm
Acinetobacter baylyi	10mm
Acinetobacter baylyi	10mm
Bacillus subtilis	13mm
Acinetobacter baylyi	10mm
Azotobacter vinelandii	15mm
Azotobacter vinelandii	15mm
Acinetobacter baylyi	10mm

of four bacterial constituents. By comparing the mass spectra of the constituents with the NIST library, four microbial constituents were characterized and identified.

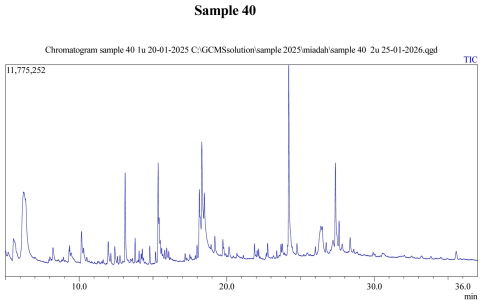
4. DISCUSSION

The present study demonstrated that rhizospheric soils from Sana'a City, Yemen, harbor diverse bacterial isolates with significant antimicrobial potential. Of the 50 isolates, 14 showed inhibitory activity against ***Escherichia coli***, with inhibition zones ranging from 10 to 20 mm. These findings are consistent with earlier studies reporting that soil-derived bacteria, particularly those from agricultural soils, frequently produce secondary metabolites with antibacterial activity [25, 26].

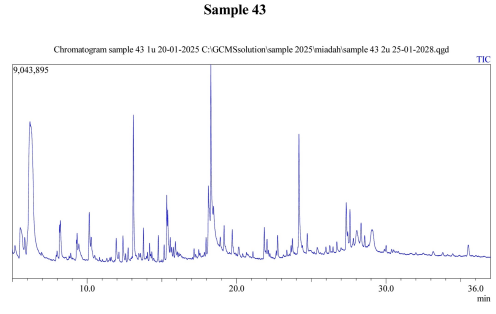
Among the antagonistic isolates, ***Bacillus subtilis*** and ***Pseudomonas fluorescens*** exhibited the strongest inhibition zones. This result agrees with previous reports where Bacillus species were shown to produce lipopeptides such as surfactin, iturin, and fengycin, which disrupt bacterial cell membranes [27]. Similarly, ***Pseudomonas fluorescens*** synthesizes phenazines, pyoluteorin, and other volatile organic compounds that possess broad-spectrum antimicrobial properties [28]. The identification of ***Azotobacter vinelandii*** and ***Acinetobacter baylyi*** as antagonistic strains further supports the role of non-traditional soil bacteria in producing metabolites with potential therapeutic value, as both genera have been linked to fatty acid and ester derivatives with antimicrobial effects [29, 30]. GC–MS analysis revealed the presence of various bioactive compounds, including phenolic derivatives (e.g., 2,4-di-tert-butylphenol), fatty acids (e.g., n-hexadecanoic acid and, octadecanoic acid), esters, and hydrocarbons. Similar compounds have been reported in soil bacterial extracts and have demonstrated antibacterial activity through mechanisms such as the disruption of lipid bilayers, inhibition of energy metabolism, and alteration of cell permeability [31, 32]. For example, n-hexadecanoic acid and octadecanoic acid have been reported to exhibit bacteriostatic effects against Gram-negative pathogens [33]. The detection of squalene and tris(2,4-di-tert-butylphenyl) phosphate also suggests antioxidant and membrane-interfering properties that may indirectly contribute to the antibacterial activity [34].



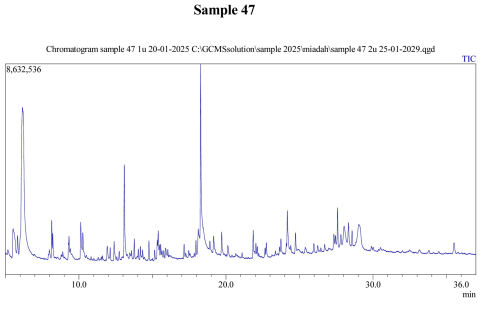
(a) *Pseudomonas fluorescens*



(b) *Bacillus subtilis*



(c) *Acinetobacter baylyi*



(d) *Azotobacter vinelandii*

Figure 2. GC-MS chromatogram of bacterial extract

Table 3. Biochemical test for the Antagonistic bacteria

Isolates NO.	Gram Stain	Shape	Catalase test	Oxidase test	Citrate Utilization	Glucose Fermentation	Sucrose Fermentation
Pseudomonas fluorescens	Negative	Bacilli	+	+	+	+	-
Bacillus subtilis	Positive	Bacilli	+	-	+	+	+
Acinetobacter baylyi	Negative	Coccobacilli	+	-	+	-	-
Azotobacter vinelandii	Negative	Ovoid	+	+	-	(Oxidative)	+

Table 4. Compounds Characteriazation

Table 4A: GC-MS Chromatogram of Bacterial Extract and There Compounds Names For Sample NO. 39 *Pseudomonas fluorescens*

S.NO.	Retention Time	Compound	Molecular Formula	Molecular Weight	Peak Area %
1	9.286	Phenol,2,4-bis(1,1-dimethylenthyl)-(CAS)2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206.32	0.42
2	11.599	Eicosane	$C_{20}H_{42}$	282.55	0.09
3	12.125	Cyclononasiloxane,octadececamethyl-	$C_{18}H_{54}O_9Si_9$	667.2	2.28
4	14.254	Caffeine	$C_8H_{10}N_4O_2$	194.19	0.25
5	15.326	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	1.99
6	17.470	9-Octadecenoic acid,methyl ester,(E)-	$C_{19}H_{36}O_2$	296.49	0.28
7	17.560	8-Octadecenoic acid,methyl ester,(Z)-	$C_{19}H_{36}O_2$	296.49	0.12
8	18.454	Octadecanoic acid	$C_{18}H_{36}O_2$	284.48	3.36
9	22.134	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667.2	0.79
10	24.099	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667.2	0.56
11	24.415	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (CAS) Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.56	0.10
12	28.050	Tris(2,4-di-tert-butylphenyl) phosphate	$C_{42}H_{63}O_4P$	646.9	7.38
13	28.576	Squalene	$C_{30}H_{50}$	410.72	0.60

Table 4B: GC-MS Chromatogram of Bacterial Extract and There Compounds Names For Sample NO.40 *Bacillus subtilis*

S.NO.	Retention Time	Compound	Molecular Formula	Molecular Weight	Peak Area %
1	8.226	Phenol, 4-(2-aminoethyl)- (CAS) Tyramine	$C_8H_{11}NO$	137.18	1.03
2	9.352	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206.32	0.88
3	15.352	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	5.65
4	18.313	Ethanol, 2,2'-(dodecylimino)bis-	$C_{26}H_{56}N_2O_2$	428.73	7.71
5	18.481	Octadecanoic acid	$C_{18}H_{36}O_2$	284.48	6.67
6	22.265	Octadecanamide	$C_{18}H_{37}NO$	283.49	0.05
7	23.925	Myristic acid glycidyl ester	$C_{17}H_{32}O_3$	284.44	0.21
8	27.367	Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	358.56	5.02
9	28.612	Squalene	$C_{30}H_{50}$	410.72	0.15
10	35.556	beta.-Sitosterol acetate	$C_{31}H_{52}O_2$	456.75	0.54

Interestingly, not all the detected metabolites demonstrated antibacterial activity, which highlights the complexity of microbial secondary metabolism. This aligns with previous observations that only a fraction of microbial metabolites is bioactive against pathogens, while others may serve ecological roles such as signaling, competition, and stress tolerance [35].

Overall, the results of this study underscore the potential of Yemeni soils as reservoirs for novel antimicrobial-producing bacteria. Comparative findings from Saudi Arabia, India, and Egypt further support the global significance of soil-derived metabolites in combating antimicrobial resistance [36, 37].

5. CONCLUSION

This study demonstrated that rhizospheric soils from the city of Sana'a , Yemen, harbor diverse bacterial isolates with significant antimicrobial activity against ***Escherichia coli***. Fourteen isolates, representing four bacterial species, exhibited inhibition zones ranging from 10 to 20 mm, with ***Bacillus subtilis*** and ***Pseudomonas fluorescens*** showing the strongest effects. GC–MS anal-

Table 4C: GC-MS Chromatogram of Bacterial Extract and There Compounds NamesFor Sample NO.43 *Acinetobacter baylyi*

S.NO.	Retention Time	Compound	Molecular Formula	Molecular Weight	Peak Area %
1	8.153	Cycloheptasiloxane, tetradecamethyl-	$C_{14}H_{42}O_7Si_7$	518.99	0.69
2	11.604	Eicosane	$C_{20}H_{42}$	282.55	0.12
3	13.445	2-(Dodecylamino)ethanol	$C_{14}H_{31}NO$	229.41	0.33
4	14.260	Caffeine	$C_8H_{10}N_4O_2$	194.19	0.18
5	15.327	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	1.87
6	23.645	Palmitic Acid, TMS derivative	$C_{19}H_{40}O_2Si$	328.62	0.30
7	23.737	Retinol, acetate	$C_{22}H_{32}O_2$	328.49	0.60
8	26.710	Stearic acid, TMS derivative	$C_{21}H_{44}O_2Si$	356.67	0.41
9	27.336	Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	358.56	1.88
10	27.438	n-Octanoic acid, ethyldimethylsilyl ester	$C_{12}H_{26}O_2Si$	230.43	0.59

Table 4D: GC-MS Chromatogram of Bacterial Extract and There Compounds Names For Sample NO.47 *Azotobacter vinelandii*

S.NO.	Retention Time	Compound	Molecular Formula	Molecular Weight	Peak Area %
1	7.920	Dodecanal	$C_{12}H_{24}O$	184.32	0.20
2	7.982	Dodecanal	$C_{12}H_{24}O$	184.32	0.56
3	11.603	Eicosane	$C_{20}H_{42}$	282.55	0.13
4	15.326	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.43	0.84
5	17.848	Methyl stearate	$C_{19}H_{38}O2$	298.50	0.11
6	18.118	Oleic Acid	$C_{18}H_{34}O_2$	282.47	1.43
7	18.907	Hexadecanamide	$C_{16}H_{33}NO$	255.44	0.33
8	27.339	Octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	358.56	0.78
9	28.577	Squalene	$C_{30}H_{50}$	410.72	0.68
10	29.034	Tris(2,4-di-tert-butylphenyl) phosphate	$C_{42}H_{63}O_4P$	646.90	3.64

ysis revealed that bioactive metabolites included fatty acids, hydrocarbons, and phenolic compounds such as n-hexadecanoic acid, octadecanoic acid, and 2,4-di-tert-butylphenol, all of which have been reported to interfere with bacterial membranes and metabolic pathways. These findings highlight Yemeni soils as underexplored reservoirs of bioactive metabolites and support their potential as sources for the development of novel antimicrobial agents against drug-resistant pathogens.

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