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Purposeful scientific research is a basic pillar for the advancement of Iraq

Determination the optimal conditions for the production of antibiotics from some species of Streptomyces bacteria that locally isolated

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ABSTRACT

The current study was conducted in the postgraduate laboratories of Education College for Pure Sciences at Tikrit-University for the period from the first of December 2019 until the end of March 2020, The study aimed to isolate and identify Streptomyces spp. from soil and optimize cultural growth conditions for Antibacterial Metabolites (including antibiotic) Production. Thirteen soil samples were collected from different locations in Tikrit city. Iraq ,Twenty-eight Streptomyces isolates were purified and testing their antibacterial activity using primary screening against pathogenic bacteria by perpendicular streaking method and the result showed that all isolates had antibacterial activity in different spectra, The active Streptomyces A11 isolate witch exhibited high activity against pathogenic bacteria was identified using several morphological and biochemical methods. Moreover, 16srRNA gene analysis confirmed that this isolate shbowed 99% identity to *Streptomyces vinaceusdrappus*. Ethyl acetate was used to extract the antibacterial compound from fermentation medium, the optimum growth conditions for antibacterial metabolite production by Streptomyces A11 isolates were incubation period in 6 days, pH 8 and temperature at 30°C. the GC-MS analysis revealed 12 chemical compounds that may have biological activity .

Keywords: Streptomyces, Soil, antibacterial activity, GC-MS.

1. Introduction

Members of the genus Streptomyces are one of individual group of Actinomycetes known for their abundant production of antibiotics and numerous biologically active secondary metabolic compounds (1). They are Gram positive, high GC-content (2), they spreads by making thread-like hyphae that enable them to obtain nutrients and when nutrient deficient, Streptomyces produce air filaments known as aerial hyphae that split and produce spores witch are resistant to unfavorable conditions and can easily spread to new environments (3).

During the growth stage, Streptomyces produce secondary metabolic compounds (3), These metabolic by-products aid vegetative bacterial cells to obtain minerals like iron (siderophore), and enabling it to communication with other species and also protect them from UV light by producing pigments (4). The medical importance of Streptomyces is that they possess secondary metabolic pathways that produce more than half of the active compounds, including surfactants, antibiotics, anticancer, antiparasitics, antifungals and antibacterial, as well as immunosuppressant's and herbicides (5).

Secondary metabolites production usually occurs at the stationary phase. The nature of secondary metabolites is genetically determined; However, gene expression can be affected by environmental condition. As a consequence, secondary metabolism usually occurs by supplementation with inducer, nutrient depletion, decreased growth, or by various environmental signals delivered by different organisms in soil niche. (4), the types and quantities of compounds produced by microorganisms are influenced by Many factors, including the nature of the carbon source, nitrogen, pH and aeration, as well as the temperature and the type of strain (6).

2. Material and methods

2.1. Sample collection and Isolation of Streptomyces spp.

Soil samples were collected in sterile polyethylene bags from different locations in Tikrit city. soil sample was taken from a depth of 5-20 cm after removing 2 cm from the top layer of soil to avoid surface contamination(7), samples were then dried in electric air oven at 37°C, for four days with the addition of calcium carbonate CaCO3 in a ratio of 10:1 w/w, One gram from each sample was weighed and added to 10 ml of sterile distilled water, and a serial dilution was performed (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}), 0.1 ml of dilution 10^{-4} , 10^{-5} was spreading on a plate of yeast extract –tryptone agar medium with the following composition (g/l): trypton ,5; yeast

extract, 3;Agar,15; pH 7.3. (8),This was followed by incubation in inverted position at 28-30°C an incubator for 7-14 days, Separate colonies were picked up and re inoculated on plates of the same agar medium by streaking method to ensure the purity of the selected colonies(9).

2.2. Primary screening for antibacterial activity

The antibacterial activity was investigated by using the perpendicular streak plate method (10) against number of pathogenic bacteria which included species of gram-positive bacteria such as *Staphylococcus aureus, Staphylococcus saprophyticus, Staphylococcus epidermidis, Streptococcus pyogenes, Enterococcus faecium*, and species of gram-negative bacteria such as *Pseudomonas aeruginosa، Klebsiella pneumoniae، Serratia marcescens، Pseudomonas putida، Enterobacter cloacae ، Proteus mirabilis، Escherichia coli* .

2.3.Phenotypic Characterization of selected A11 isolate

Standard Gram stain was carried out and visualized under a compound microscope at X100.Microscopic features like of the substrate and aerial mycelium were observe by using the slide culture or coverslip culture technique (11)(12). The growth ability, color of both substrate and aerial mycelium and production of diffusible pigment of the selected isolate A11 which showed broad spectrum activity antibacterial activity against pathogenic bacteria was determined using different culture media; Yeast extract-malt extract agar, Inorganic salts-starch agar, glucose -asparagine agar , Tyrosine agar , glycerol -asparagine agar and Nutrient agar ,and it was subjected to the following biochemical tests: Urease test, catalase test, citrate utilization test, Starch hydrolysis test , Casein hydrolysis test , Gelatin hydrolysis test , Lethinase test, Lipase test, Blood heamolysis test, Tyrosine hydrolysis test and melanin production, In addition to fermenting different types of sugars as a carbon source (Xylose, Inositol ,Raffinose ,Rhamnose, glucose, sucrose, fructose, mannitol) .

2.4.Molecular identification of A11 isolate

The DNA was extracted using Promega Genomic DNA Purification Kit according to the manufacturer instructions, Amplification of 16srRNA was carried out using the two primers, 27f (5′-AGAGTTTGATCCTGGCTCAG -3′) and 1392r (5′-GACGGGCGGTGTGTAC -3′). (13)(14),The PCR amplification included initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 35 s for denaturing, at 55°C for 60 s for annealing, at 72°C for 60s of extension and at 72°C for 5 min for complete extension (13) . The PCR amplification products with the forward and reverse primers were sent to the Micro gene Laboratory in Korea. The 16s rRNA gene base sequences were determined, The results of sequencing were then identified by using the BLAST program (National Centre for Biotechnology Information).

2.5. Fermentation and Extraction of Secondary Metabolites and study its biological activity

The selected isolate A11 was inoculated into flask contain Glycerol -yeast extract broth (5g glycerol, 25g peptone, 2g yeast extract, 0.1 g of K2HPO4) (15), and then the flasks were placed in the shaking incubator at 30°C and a rotation speed of 150 rpm for 7 days, After that, bacterial biomass was isolated from the production medium using a centrifuge at 10,000rpm for 20 minutes and then the purification of the production medium was completed using Millipore filter(Whatman No.1.) .The cell-free supernatant was extracted with an equal volume of ethyl acetate, 1:1 (v/v) and shaken hard for 1 h to complete the extraction process, The aqueous layer was separated from the solvent layer that contain the antibiotic using a separating funnel, a rotary evaporator was used for evaporating the solvent and obtaining a crude precipitate of the antibiotic (16).

Agar diffusion method was used to test the sensitivity of pathogenic bacteria to the antibiotic extract, wells 6 mm in diameter were made on Moller Hinton agar medium which was inoculated with the pathogenic test bacteria and each well was filled with 0.1 ml of the residue obtained after dissolved in DMSO (17)(18), then the dishes were incubated at 37 °C for 24 hours and diameters of the inhibition zones were measured (19).

2.6. Optimizing Cultural Conditions for antimicrobial compounds Production

2.6.1. Effect of Incubation Time

To study the effect of incubation period, flasks contain 50 ml of broth glycerol –yeast extract medium inoculated with selected isolate and the flasks incubated at 30°C in shaking incubator at 150 rpm for 7 days. After incubation, the metabolites were extracted and antibacterial activity was tested daily starting from the second day reaching to the seven day and inhibition zones were measured by using agar diffusion method to determine the optimum period (20).

2.6.2. Effect of pH

To study the optimal pH for antibiotic production, flasks contain 50 ml of broth glycerol –yeast extract medium was set to pH values ranged from 4 to 9 using 1N HCl or NaOH. The medium were inoculated with selected isolate and placed in shaking incubated at 30°C and 150 rpm for optimam incubation period 6 days, and after that the metabolites were extracted and antibacterial activity was tested(20).

2.6.3. Effect of Incubation Temperature

The optimum temperature for antibiotic production was determined by using different incubation temperatures (26, 28, 30, 32, 34 and 36) °C. The media at pH 8 were inoculated with selected isolate and placed in shaking incubator at 150 rpm for 6 days(20).

2.7.Identification of bioactive compounds

The antibacterial compounds of the biologically active crude extracts (extracted by ethyl acetate) were identified by using Gas Chromatography-Mass Spectrometer technique (GC MS). The spectrum of the raw ingredients was compared with the spectrum of the recognized ingredients in the National Institute Standard and Technology (NIST) library from which the compound name was determined for the components of the test materials (21). The analysis was carried out using the GC-MS device model 7820A from Agilint company, and the analysis conditions were as follows:

- Colum: Hp-5ms ultra lneit (30m length x)
- 250 nm diameter x 0.25 um in side diameter
- Injection volume: 1 cup
- Pressure: 11.933 psi
- GC inlet line temperature : 250 C
- Aux heaters temperature : 310 C
- Carrier Gas : He 99.99%
- Injection : Temperature : 250 C
- Injection type : Split less
- Scan range : m/z 50-500
- Oven Program:
- Ramp1: 50 C Hold 1 min., Ramp1: 50 C to 150 C, 5 C/ min
- Ramp2: 150 C to 280 C, 8 C/min
- Time amounted to about 37 min

3. Results and Discussion

The development of resistance pathogenic bacteria to antibiotics increased the require to search for new sources of antimicrobial agent to fight these pathogenic bacteria. Secondary metabolites produced by bacteria yet interested, because of highly specific antimicrobial activities and their complicated chemical structures. The soil bacteria especially Streptomyces spp. are rich sources for many bioactive natural products.

3.1. Isolation of Streptomyces spp. and Primary Screening for their Antibacterial Activities

A total of 28 Streptomyces spp. were isolated from soil samples collected from different place in tikrit province, Iraq. Several studies indicate that the soil niches were a rich in many critical actinomycetes (22)(23).All collected isolates were characterized depens on morphology , color of mycelia and diffusible pigment. The results of the preliminary screening by perpendicular streaking method showed that all isolates had antibacterial activity in different spectra, Several previous studies indicate the isolation of Streptomyces spp. from soil with antimicrobial activities at a high rate(24)(25).

In this study, A11isolate showed the highest inhibitory activity against gram positive and gram negative pathogenic bacteria So, these talented isolate were selected for the production of antibiotics in liquid cultures.

3.2. Phenotypic Characterization and Molecular identification of A11 isolate

The selected isolate were found as long filamentous gram-positive bacteria when examined by light microscope at X100 magnification after staining with Gram stain, When using the slide culture technique Substrate mycelium and aerial mycelium of selected isolate showed clearly.

The Phenotypic characters like color of substrate and aerial mycelia, production of soluble pigment was determined after growing in different growth media Table(1), The physiological ,biochemical characters and utilization of different carbon sources are illustrated in Table 2. Based on these morphological and biochemical characteristics of A11, it was presumptively classified into the genus Streptomyces .

Moreover, the partial 16srRNA gene of the selected isolate A11 was sequencing for successful identification, The selected isolate sequence result was matched with previously reported actinobacterial sequences available in the NCBI databases and the result revealed a 99% similarity with *Streptomyces vinaceusdrappus*, The selected isolate 16srRNA sequence was submitted in Genbank (NCBI) and received accession number as MZ618754.1.

Table 1: Cultural characteristics of A11 isolate on different culture media

Characterization	Culture Medium			
	Growth	Color of substrate mycelium	Color of aerial mycelium	diffusible pigment
Yeast extract-malt extract agar	good	yellowish cream	yellowish cream	Absent
Inorganic salts-starch agar	good	cream-gray	white-grey	Absent
glucose -asparagine agar	good	yellowish cream	cream	Absent
Tyrosine agar	good	Orange-yellowish brown	Powdery white -light gray	Absent
glycerol -asparagine agar	good	Orange-light brown	Powdery white	Absent
Nutrient agar	good	Cream	Powdery white	Absent

Table 2: Physiological and biochemical characteristics of A11selected isolate

Test	Result
Catalase activity	
Urease activity	+
Starch hydrolysis	+
Casein hydrolysis	+
Gelatin hydrolysis	-
Citrate utilization	-
Lethinase activity	+
Lipase activity	-
Blood heamolysis	+
Tyrosine hydrolysis	+
Melanin production	-
Carbon source utilization	
Glucose	+
Sucrose	+
Fructose	+
Xylose	+
Mannitol	+
Arabinose	+
Rhamnose	+
Inositol	+
Raffinose	+

(+)positive reaction and (-) negative reaction

3.3. Antibacterial Activity of A11 isolate Secondary Metabolites

Among the Streptomyces isolates that obtained from soil and were screened primarily for their antibacterial activity, only the more active isolate were submitted to secondary screening for the production of metabolic products (include antibiotics) in a liquid culture method depending on antibacterial activity against 12 indicator pathogenic bacteria by the agar diffusion methods, Ethyl acetate has been widely used for secondary metabolites extraction (26) indicated that the secondary metabolites extracted by used ethyl acetate in a 1:1. ratio showed good activity against pathogenic bacteria than other solvents.

Streptomyces A11 isolate Secondary Metabolites exhibited antimicrobial activities against Gram-positive and Gram-negative pathogenic bacterial isolates.this means that isolate have broad spectrum antibacterial potential (Table 3), The highest inhibition zone against gram negative bacteria was 21 mm against *K.pneumonia* and the lowest inhibition zone of 13 mm against *Entero.cloacae*, while the highest inhibition zone against gram positive bacteria was 26 mm against *Staph.epidermidis* and the lowest inhibition zone of 16 mm against *Staph. aureus* and *Strep.pyogenes*. These results were agree with the previous study conducted by Ahmed *et al* (27) that reported that *S. vinaceusdrappus* has distinct inhibitory effects against Gram-positive and Gram-negative bacteria including *Staph. aureus* and *E. coli* besides antifungal activity and also antitumor against human colon and liver cell lines; and HCT 116and HepG 2 .

Table 3: Extraction of secondary metabolite by Ethyl acetate solvents

Organic solvents	**test bacteria**											
	K.pneumoniae	***Ser.marcescens***	***Pseudo.aeroginosa***	***Pseudo.putida***	***Entero.cloacae***	***P.mirabilis***	***E.coli***	***Staph.aureus***	***Staph.saprophyticus***	***Staph.epidermidis***	***Strep.pyogenes***	***E.faecium***
	Zone of inhibition (mm)											
Ethyl acetate	21	18	19	20	13	16	14	16	25	26	16	20

3.4. Effect of incubation period

The results showed, as in the table 4, that the highest inhibitory activity against tested pathogenic bacteria by selected isolate A11 was observed at 6 days of incubation, the inhibition zone against pathogenic bacteria ranged between 16mm and 28mm. This is similar to the observation of Thakur *et al* (28) that appropriate incubation period to obtain maximum antibiotic production from Streptomyces spp. 201 was at 6 days later, but differed from earlier reports by BSSN *et al* (29) which indicated that secondary metabolites produced by *S. lavendulocolor* VHB-9 showed the highest antimicrobial activity after 4 days of incubation. The difference could be due to the type of media used in production and varying growth rate of the strains used.

Table 4: Effect of incubation period

incubation period	test bacteria											
	K.pneumoniae	*Ser.marcescens*	*Pseudo.aeroginosa*	*Pseudo.putida*	*Entero.cloacae*	*P.mirabilis*	*E.coli*	*Staph.aureus*	*Staph.saprophyticus*	*Staph.epidermidis*	*Strep.pyogenes*	*E.faecium*
	Zone of inhibition (mm)											
2	14	8	11	9	8	11	11	8	15	11	8	13
3	19	10	15	15	10	13	13	10	21	21	14	18
4	20	11	13	16	11	14	13	13	21	20	15	20
5	26	24	16	23	14	15	14	19	27	25	17	25
6	23	25	18	23	15	16	15	19	28	28	17	25
7	21	18	16	19	14	15	14	15	25	27	15	21

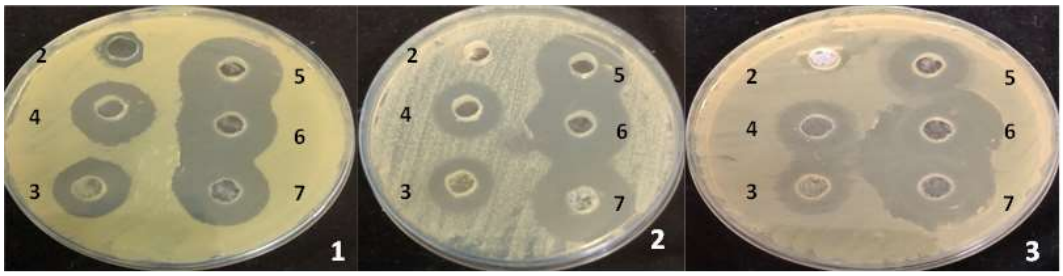

Fig. 1: effect of different incubation period on the inhibitory activity of the extract prepared from the selected isolate A11

1- *E.faecium* 2- *Staph.epidermidis* 3- *K.pneumoniae*

3.5. Effect of pH

The results revealed that selected isolate A11 produced the antibacterial metabolite (including antibiotic) in different range of pH values (6-9). The highest antibiotic productivity of the selected isolate A11 obtained at pH 8, the diameters of the inhibition zones ranged between15mm and 28mm (Table5).

The pH of the culture medium is an important factor affecting the action of cellular enzymes, nutrient ionization, transport across cell membranes, and this will effect on the rate of bacterial growth and the biosynthesis of secondary metabolites.

This result is in agreement with a previous study by Vasavada *et al* (30) which mention that optimum pH for antibiotic production from *Streptomyces sannanensis* RJT-1 was pH 8. Another study by Adeyemo *et al* (31) reported that co-cultured strains of *S. xinghaiensis*-OY62 and *S. rimosus*-OG95 showed excellent antimicrobial activity against different pathogenic bacteria at pH 8.0.

This might be due to the existence of active enzymes for the synthesis of antimicrobial metabolites at this pH, and that hydrogen ion concentration plays a major role in enzyme activity and production of secondary metabolites (32).

Table 5: Effect of value

pH	test bacteria											
	K.pneumoniae	***Ser.marcescens***	***Pseudo.aeroginosa***	***Pseudo.putida***	***Entero.cloacae***	***P.mirabilis***	***E.coli***	***Staph.aureus***	***Staph.saprophyticus***	***Staph.epidermidis***	***Strep.pyogenes***	***E.faecium***
	inhibition zone (mm)											
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	15	14	15	19	9	13	11	11	20	21	16	22
7	16	14	17	18	13	13	11	11	19	20	11	20
8	25	26	20	26	15	17	16	19	27	28	19	26
9	12	18	11	16	8	11	10	11	20	19	14	19

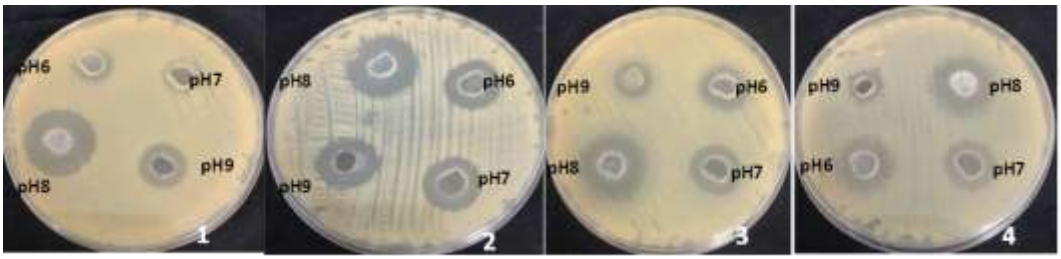

Fig. 2: effect of different values of pH on the inhibitory activity of the extract prepared from the selected isolate A11 against pathogenic bacteria

1- *Ser.marcescens* 2 - *Staph.saprophyticus* 3- *Pseudo.putida* 4-− *K.pneumoniae*

3.6. Effect of Temperature

Temperature is one of the factors that have been studied for its effect on the productivity of antibiotics, as it has a direct effect on all metabolic processes, including enzymes activity and active metabolites production (33).The results showed That highest antibiotic yield from selected isolate A11 was obtained at 30°C and the inhibition zone against pathogenic bacteria ranging between 17mm and 35mm (Table 6), These results agreement with previous report (34)(35). This might be because the metabolic enzymes that enabled the production of antimicrobials were stable at this temperature.

Table 6: Effect of temperature

Temperature	**test bacteria**											
	K.pneumoniae	***Ser.marcescens***	***Pseudo.aeroginosa***	***Pseudo.putida***	***Entero.cloacae***	***P.mirabilis***	***E.coli***	***Staph.aureus***	***Staph.saprophyticus***	***Staph.epidermidis***	***Strep.pyogenes***	***E.faecium***
	inhibition zone (mm)											
24	0	0	0	0	0	0	0	0	0	0	0	0
26	17	15	8	17	14	11	11	12	19	26	10	20
28	18	14	10	20	11	14	13	15	17	18	12	18
30	25	30	19	26	17	22	23	21	29	35	20	28
32	24	28	16	22	15	20	22	20	24	31	15	25
34	23	27	14	23	14	20	21	19	26	30	14	24

36	20	25	11	20	13	19	20	10	23	34	12	23

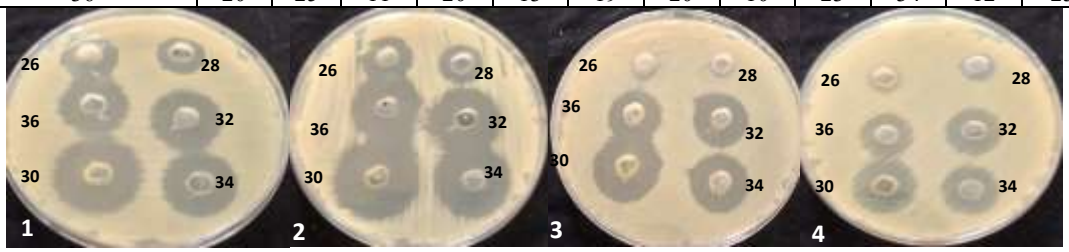

Fig. 3: effect of different incubation temperature on the inhibitory activity of the extract prepared from the selected isolate A11 against pathogenic bacteria

1- *E.faecium* 2-*Staph.saprophyticus* 3-*E.coli* 4-*P.mirabilis*

3.7. Identification of bioactive compounds

The GC -MS analysis showed the presence of 19 chemical compounds in the extract of selected isolate A11, Among these compounds are 12 chemical compounds that may have biological activity, namely: 2-Piperidinone(36)(37),Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-(37)(38), Hexadecanoic acid, methyl ester(39)(40), n-Hexadecanoic acid (41), cis-Vaccenic acid (42),2-Methyl-Z,Z-3,13-octadecadienol(43),oleic acid(44),n-Propyl 11-octadecenoate(45),11-Octadecenoic acid, methyl ester(46),9-Octadecenamide, (Z)-, 13-Octadecenal (Z)-(44), Heptadecanoic acid, 16-methyl-, methyl ester(47),The presence of the above-mentioned compounds in the extract singly or their synergistic activity with other components may show antimicrobial activity against pathogenic bacteria.

Table 7: Chemical compounds present in the extract of A11isolate using GC-Mass technology

No.	Compound name	R-Time	Area %
1	2-Piperidinone	10.459	1.58
2	3-Methyl-1,4-diazabicyclo[4.3.0]no nan-2,5-dione, N-acetyl-	18.216	0.41
3	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	18.853	1.62
4	Hexadecanoic acid, methyl ester	20.795	4.74
5	n-Hexadecanoic acid	21.291	0.8
6	2-Methyl-Z,Z-3,13-octadecadienol	22.629	0.33
7	9-Octadecenoic acid, methyl ester, (E)-	22.942	53.78
8	Heptadecanoic acid, 16-methyl-, methyl ester	23.179	4.1
9	Oleic Acid	23.416	19.8
10	cis-Vaccenic acid	23.643	2.35
11	9,12-Octadecadienoic acid, methyl ester, (E,E)-	23.880	2.99
12	cis-11-Eicosenoic acid, methyl ester	25.056	0.93
13	Eicosanoic acid, methyl ester	25.326	0.46
14	9-Octadecenamide, (Z)-	25.693	1.57
15	13-Octadecenal, (Z)	26.836	1.78
16	Docosanoic acid, methyl ester	27.365	0.42
17	Difluoro(methylamino)phosphine sulfide	28.530	0.56
18	n-Propyl 11-octadecenoate	28.724	0.51
	11-Octadecenoic acid, methyl ester	28.983	1.26

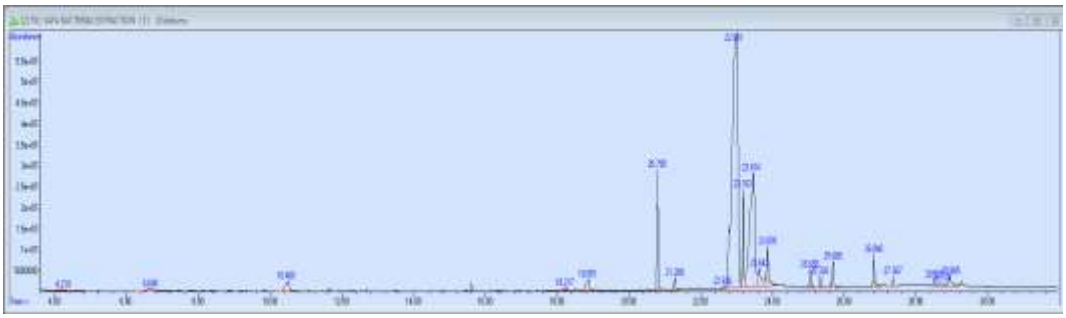

Fig. 4: GC–MS spectrum of antimicrobial compounds produced by A11 isolate.

Conclusion

Soil Streptomycetes are the main source of bioactive secondary metabolites. In the current study, 13 soil samples were collected from different location in Tikrit province. Iraq. The active isolate was identified as *S.vinaceusdrappus*, which exhibited broad

spectrum antibacterial activity. Improving culture conditions may effect on the production of secondary metabolites. The GC-MS is one of newer method and the most efficient for identification and characterization of secondary metabolites.Moreover, this study may be useful for the development of potential antibacterials agents against various pathogens.

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تحديد الظروف المثلى لإنتاج المضادات الحيوية من بعض أنواع بكتريا Streptomyces المعزولة محليا

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الملخص

أجريت هذه الدراسة في مختبرات الدراسات العليا في قسم علوم الحياة– كلية التربية للعلوم الصرفة–جامعة تكريت لعزل انواع مختلفة من بكتريا Streptomyces للفترة من الاول من كانون الاول 2019 ولغاية نهاية شهر اذار 2020، هدفت الدراسة إلى عزل وتشخيص بكتريا Streptomyces spp. من التربة وتحديد الظروف المثلى للنمو لإنتاج المؤيضات المضادة للبكتيريا (بما في ذلك المضادات الحيوية). جمعت ثلاثة عشر عينة ترابية من مواقع مختلفة في مدينة تكريت. العراق ، تم تنقية 28 عزلة من بكتريا Streptomyces واختبر نشاطها المضاد للبكتيريا بواسطة التحري الأولي ضد البكتيريا المسببة للأمراض بطريقة الخطوط المتعامدة وأظهرت النتائج أن جميع العزلات لها نشاط مضاد للبكتريا بمديات مختلفة. تم تشخيص العزلة A11 التي اظهرت اعلى فعالية ضد البكتريا المرضية اعتماداً على الاختبارات الكيموحيوية والصفات المظهرية بالإضافة لذلك أكد تحليل الجين 16srRNA أن هذه العزلة أظهرت تطابق بنسبة 99 ٪ لـ *Streptomyces vinaceusdrappus*، استخدمت خلات الاثيل لاستخلاص المركبات المضادة للبكتريا الموجودة في وسط التخمير ، كانت ظروف النمو المثلى لإنتاج المؤيضات المضاد للبكتيريا بواسطة العزلة Streptomyces A11 هي فترة الحضانة 6 أيام ، ،الاس الهيدروجيني pH8 ودرجة الحرارة 30م. كشف تحليل GC-MS وجود 12 مركب كيميائيًا قد يكون لها نشاط بيولوجي.