Research Article

Antibacterial activity of metabolites isolated from local soil *Actinomycetes* spp.

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ABSTRACT

Background: Microbial diseases have increased in recent years and have become a threat to public health. The most threatening threat to the world is the resistance of microbes to drugs. The study aims to isolate some *Actinomycetes* that produce antimicrobial metabolites from different soil. **Materials and Methods:** A total of 150 samples of different soils were randomly collected from different parts of Hilla city and studied soils properties (pH, temperature, elemental carbon, organic carbon, and relative humidity). *Actinomycetes* were isolated using serial dilution and plated on the International Streptomyces Project type-2 media medium. A total of 33 *Actinomycetes* strains were isolated and studied their morphology and cross streaked against various human microbial pathogens. **Results:** Only 13 isolates showed high antibacterial against Gram-positive and Gram-negative pathogenic bacteria through primary screening. Secondary metabolic products were extracted from extracellular and intracellular and tested for its activity against pathogenic bacteria using the well diffusion method. The diameters of the inhibition zone were measured for extracellular and intracellular extracts. The diameters of the inhibition zone ranged from 13 to 33 against Gram-positive bacteria and 6.3–39.2 mm against Gram-negative bacteria for extracellular extracts and ranged from 18 to 30.5 mm against Gram-positive bacteria using intracellular extracts and from 9.5 to 32.3 mm against Gram-negative bacteria. **Conclusion:** The current study concludes the antibacterial activity of *Actinomycetes* metabolites with different spectrum and inhibition zone like those resulted from carbapenems.

KEY WORDS: *Actinomycetes*, Antibacterial, Metabolites, Soli bacteria

INTRODUCTION

Actinomycetes spp. is a diverse group of heterotrophic prokaryotes forming hyphae at some stage of their growth,[1,2] hence referred to as filamentous prokaryotes. *Actinomycetes* are a successful group of bacteria that occur in a multiplicity of natural and man-made environments.[3-5] The vast majority of cultures of aerobic *Actinomycetes* have originated from the soil. Viable counts of over four million/gm may be obtained from fertile soils.[6,7] *Actinomycetes* are frequently documented as producers of antibiotics and other useful secondary metabolites as explained by Khanna *et al*.[8] Comprehensively, till now about 70–80% of the commercially obtained antibiotics and antimicrobial secondary metabolites have been isolated and characterized from numerous *Actinomycetes* species.[9] *Actinomycetes* are frequently documented as producers of antibiotics and other useful secondary metabolites as explained by Khanna *et al*.[8] The Vast majority of antibiotics and antimicrobial secondary metabolites has been isolated from numerous actinomycetes species.[9] *Actinomycetes* isolation and their antimicrobial potency have been explored from different parts of the globe, both from land and marine soils. Isolates reported from different locality/sites with varied inhibitory effect to the pathogens vehemently supports hope for finding novel antibiotic producer strains with promising inhibitory effect that could help fight growing antimicrobial resistance.[9,10]

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MATERIALS AND METHODS

Samples Collection

All samples were collected randomly depending on clay, agricultural, sandy, and river sediment soil from different locations in Hilla city summarized in Table 1 during the period from March 2, 2018, to April 20, 2018. The total collected samples were 150 soil samples.

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Table 1: Site, number and soil conditions were collected for the isolation of locally *Actinomycetes* spp. in Hilla city

Site	Sample number	Soil condition
Al-Mahaweel	3, 4, 2 and 7	Clay
	5, and 11	Sandy
	2, 10, 4, 5, and16	Agricultural
Al-Kothar	17, 8, 9, 0, 46	Agricultural
	22, 23, 1, 2, 49	Clay
	27, 9, 3, 6, 8	River sediment
	39, 0, 8, 0, 1, 2	Sandy
Al-Kothar	21, 5, 4, 6, 7	Agricultural
	59, 0, 2, 3, 4	Clay
	65, 7, 8, 9, 0	River sediment
	73, 5, 3	Sandy
Al-Hilla	72, 76, 7, 9, 0, 5	Agricultural
	0, 1, 2, 4, 8	Clay
	1, 1, 2, 3	River sediment
	7, 8, 9	Sandy
Al-Amam	34, 4, 5, 05, 07, 2	Agricultural
	113, 15, 21, 02	Clay
	124, 26, 04	River sediment
	106, 08, 18, 16	Sandy
Al-Hamza	101, 09, 10, 11, 17	Agricultural
	37, 20, 22, 25, 27	Clay
	28, 5, 21, 23	River sediment
	133, 32, 36	Sandy
Al-Kasim	74, 30, 31, 34, 12	Agricultural
	29, 00, 78, 3	Agricultural
	135, 37, 03, 28, 1	River sediment
	41, 19, 3, 5	Sandy
Al-Mashrwia	42, 39, 38, 7, 42	Agricultural
	80, 3, 26, 0	Clay
Al-Mussiab	46, 9, 8, 6, 40	Agricultural
	114, 43, 96	River sediment

Testing Soil Properties

Testing soil pH

Weigh out about 10 g (to the nearest half gram), of soil into the contrary. Add 50 ml of distilled water to the soil. Any rough measurement ensuring a 1:5 diluted will suffice. Shake the container for about 2–3 min then allow the soil to settle for 2 min measure the pH value on the water above the soil in the container.[10]

Testing soil temperature

Using soil thermometer in depth 10 cm through is as follows:

1. Measure 12 cm up from the tip of the soil thermometer and mark this spot.
2. Measure the distance from the base of the soil thermometer dial to the 12 cm mark
3. Make a spacer by cutting a piece of plastic tubing or wood to this length (if using wood, drill a hole through the center of the block)
4. Insert the soil thermometer through the spacer. 12 cm of the thermometer should be sticking out of the bottom of the spacer
5. Label this spacer 10 cm measurement.

Testing soil relative humidity

Calculation of the results for soil analysis is done on the basis of oven – dry soil. The moisture should be determined shortly before soil analysis as in this procedure.[10]

1. Transfer approximately 5 g fine earth to yard moisture tin and weight it as A gram
2. Dry overnight at 1050°C
3. Remove the tin from the oven and but its cover and weight it as B gram.

The moisture content is obtained by: Moist = (A – B/B – tare tin) × 100.

Treatment of the Collected Samples

About 100 g of soil samples was collected from the regions mentioned previously at a depth ranged from 10 to 15 cm and they were kept in polyethylene bags 20 cm × 40 cm. Soil samples pretreated with $CaCO_3$ at a ratio of 10:1 soil: $CaCO_3$ and kept at ambient temperature for a week to enrich *Actinomycetes* which usually prefer alkaline conditions and also to reduce the contamination with molds and yeast, as described by El-Nakeeb and Lechevalier,[11] Abdulhameed.[12]

Preparation of (International Streptomyces Project type-2 Media) Medium

Chemical compounds of International Streptomyces Project type-2 media (ISP-2) prepared as the following: Yeast extract 4.0 g, malt extract 10.0 g. dextrose.4.0 g, agar 20.0 g, and distilled water 1000 ml. The pH of this medium adjusts from 7.0 to 7.3, then the components liquefy by heating at 100°C. After this, sterilized by autoclaved at 121°C for 15 min.[13]

Isolation and Purification of *Actinomycetes* spp. from Soil Samples

After homogenizing each sample, it was passed through the 2 mm opening to remove gravel, large stone, and debris. Then, 1 g of each soil samples was added to 9 ml of D. W and successive dilutions were made up to 10–5. Each of the serial dilution was spread and placed on the surface (ISP-2) agar medium. Nystatin antibiotic was added to minimize fungal contamination. All plates were incubated at 28°C for 5–7 days. After incubation, *Actinomycetes* growing colonies were selected and purified by sub culturing on ISP-2 agar medium plates according to type and forms of these colonies. Then, the purified colonies were examined under the oil immersion objective at the light microscope. After this, the typical growing colonies of *Streptomyces* were cultured on ISP-2 agar slants and stored at 4°C for further uses.[14]

Morphological Identification of *Actinomycetes* spp.

Actinomycetes spp. isolates were identified by the study of morphological characteristics for colonies shape, aerial mycelia, substrate mycelia, and cell shape.[15] Characteristics of *Streptomyces spp*. isolates were done by cultured of these bacteria on different agar media of the ISP, such as ISP-2[16] in accordance

with the ISP and noted their characteristics.[17] All were Gram-positive and oxygen requirement.

Primary Screening Test of *Actinomycetes* spp. against Pathogenic Bacteria

This antibacterial activity of *Actinomycetes* spp. isolates was tested by perpendicular streak plate method.[18,19] This method was prepared by cultured of *Actinomycetes* spp. isolates separately through made cross lines from these isolates in the middle of Muller-Hinton agar media and incubated at 28°C for 3 days. After this, columns from the pathogenic test bacteria were streaked at right angles on each side of the *Actinomycetes* spp. isolates at straight line colony.[20,21] The media then incubated at 37°C for 24 h. and the results were recorded as positive through inhibition growth of pathogenic test bacteria.[22]

Secondary Screening Test of *Actinomycetes* spp.

The positive results were obtained from the primary screening protocol. Besides, it was ready-to-use the batch culture fermentation as well as agar well diffusion assay was used to determining the antimicrobial activities of the isolated *Actinomycetes*.[23]

Fermentation Condition

Secondary screening for the production of antimicrobial metabolites was done by the use of one loop full of spores with their mycelial growth was transferred to a conical flask containing 250 ml of sterilized ISP2 and incubated at 29°C ± 1°C, 150 rpm for 7 days in a shaking incubator. The process was carried out in the biology department.[23,24]

Extraction of Antimicrobial Metabolites

Extraction of extracellular antimicrobial metabolite

Each of the *Actinomycetes* spp. isolates was inculcated in a 500 ml flask containing 250 ml of ISP-2 broth of pH 7.2 and incubated at 28°C for 10 days. After the incubation period, the fermented broth is filtered through a Whatman No.1 filter to separate the cellular components from the culture filtrate, then the broths were centrifuged at 6000 rpm for 15 min and the cell free supernatant was taken. Culture supernatants were extracted with an equal volume (1:1 v/v) of appropriate of ethyl acetate before shaking vigorously for one dryness and the residue obtained was weighted. The residue obtained from an evaporated flask in a water bath is weighed and used for antibacterial analyses as a secondary screening test. This performed by the agar well diffusion method against standard pathogenic organisms.[8] Hour to complete the extraction and formation of two layers (organic layer and aqueous layer). The ethyl acetate phase that contains the antibiotics was separated from the aqueous layer phase; then the ethyl acetate was evaporated in a water bath at 80°C–90°C.

Intracellular Extraction of Antimicrobial Metabolites

Intracellular extracts were achieved after the separation of the extracellular crude extracts by centrifugation. The residual in the tube contained bacterial cells pellet with its intracellular antimicrobial metabolites. These components were used to determine the intracellular antimicrobial activity by agar well diffusion as follows: the pelleted cells were re-suspended in the test tube containing lysis buffer 1 ml TE buffer "Tris 200 ml and 50 ml ethylenediaminetetraacetic acid", 60 ul of 10% sodium dodecyl sulfate and 6 ul of proteinase K, with a gentle shaking, the mixture was incubated at 37°C for 60 min that caused bacterial cell walls disruption as well as intracellular metabolites liberation. 600 µl of the intracellular crude metabolites was taken and mixed with 600 µl of methanol. The mixture was gently mixed and left for 60 min. Then the tubes were spun at 1000 rpm for 10 min at room temperature. The mixture was separated into two phases, the upper phase methanolic phase containing dissolved metabolites was collected and transferred to the sterilized Petri dish, then kept in a hot air oven 45°C for 24 h to dry the dissolved intracellular crude extract. Finally, the dried intracellular crude extracts were dissolved in double volume of sterilized distal water 1200 ul. Agar well diffusion procedures as mentioned previously were used to determine the antimicrobial activity against tested microbial pathogens.[24]

RESULTS AND DISCUSSION

Isolation

All samples were collected randomly depending on clay, agricultural, sandy, and river sediment soil from different locations in Hilla city. The serial dilution technique was used to isolate *Actinomycetes* from 150 soil sources after inoculating the plates with soil suspension on ISP2 agar and incubating the inoculated plates for 7–14 days. Out of 150 soil sources, only 50 soil sources and from this soil sources we obtain 33 colonies Table 2. 33 colonies were obtained from the isolation processes, subcultured on ISP2, to obtain pure colonies culture of locally isolated strains and the process of sub-culturing repeated more than 1 time. Morphologically, on the basis of aerial mycelium color, there are many similarities among a large number of them, especially when purified on plates. However, all 33 purified colonies were kept at 4°C in a refrigerator for further study primary screening programs. The results was in agreement with the finding of both,[25,26] concerning the isolation process that each plate was often contained one or few colony types ranging from two to four colonies, and from similar habitats, the *Actinomycetes* diversity exhibited few different colony types.

Testing Soil Properties

Different properties of soil were measured such as pH, temperature, humidity, EC, OC, and optical density to give an idea about the environment of *Actinomycetes* The data presented in Table 3 summarize all properties of different soils sources of *Actinomycetes*. All soil sources of 33 *Actinomycetes* differ in their properties and this a agreement with the finding of[27] which mentioned that due to their stringent aerobic metabolisms, *Actinomycetes*, especially *Streptomyces*, exist in different types of soil and they are abundant on the surface layer of soils as well as they favor the alkaline soils, compost, river's mud, and riverbeds. Nonoh *et al*.[28] described the physical properties, organic matter content, pH, moisture, soil reactions,

Table 2: Soil condition and collection sites

Number	Site	Sample number	Abbreviated	Soil condition
1	Al-Mahaweel	12	MA-CL12	Clay
2	Al-Mahaweel	16	MA-AG16	Agricultural
3	Al-Kothar	18	KO-AG18	Agricultural
4	Al-Nile	21	NI-AG21	Agricultural
5	Al-Hilla	24	HI-SA24	Sandy
6	Al-Nile	25	NI-AG25	Agricultural
7	Al-Kothar	27	KO-RS27	River sediment
8	Al-Kothar	29	KO-RS29	River sediment
9	Al-Amam	34	AM-AG34	Agricultural
10	Al-Hamza	28	HA-RS28	River sediment
11	Al-Hamza	37	HA-CL37	Clay
12	Al-Kasim	41	KA-SA41	Sandy
13	Al-Mashrwia	42	MA-CL42	Clay
14	Al-Mashrwia	43	MA-CL43	Agricultural
15	Al-Mussiab	46	MU-AG46	Agricultural
16	Al-Amam	52	AM-AG52	Agricultural
17	Al-Hamza	55	HA-RA55	River sediment
18	Al-Kasim	74	KA-AG74	Agricultural
19	Al-Mussiab	79	MU-AG79	Agricultural
20	Ai-Hilla	88	HI-CL88	Clay
21	Ai-Hilla	92	HI-AG92	Agricultural
22	Al-Amam	102	AM-CL102	Clay
23	Al-Amam	105	AM-AG105	Agricultural
24	Al-Kasim	119	KA-SA119	Sandy
25	Al-Hamza	127	HA-CL127	Clay
26	Al-Hamza	132	HA-SA132	Sandy
27	Al-Hamza	136	HA-SA136	Sandy
28	Al-Mashrwia	138	MA-AG138	Agricultural
29	Al-Mashrwia	139	MA-AG139	Agricultural
30	Al-Mashrwia	142	MA-AG142	Agricultural
31	Al-Mussiab	143	MU-RA143	River sediment
32	Al-Kothar	146	KO-AG146	Agricultural
33	Al-Kothar	149	KO-CL149	Clay

Table 3: The properties of the soil

Sample number	Soil condition	pH	Temperature	EC	OC	Humidity (%)
12	Clay	7	22	22.1	3.9	15.8
16	Agricultural	7.3	18.9	22.49	0.4	19.8
18	Agricultural	7.5	30	44.8	0	13.9
21	Agricultural	6.3	31	47.8	0.8	13.5
24	Sandy	7.2	27.8	66.5	3.7	5.2
25	Agricultural	6.5	25.3	31.8	0.9	18.1
27	River sediment	7.2	15.8	41.9	0.3	19.8
29	River sediment	8	27.2	78.8	3.1	16.6
34	Agricultural	7	25.3	41.7	1.2	12
28	River sediment	8.5	26.8	42.3	3.6	16.8
37	Clay	7.3	27	54.2	4	20
41	Sandy	6.5	28.3	61.9	3.6	8
42	Clay	6.9	28	24.7	4.2	17.4
43	Agricultural	8.4	30.1	50.4	1.4	13.9
46	Agricultural	7.4	27.5	75.6	0.8	15.5
52	Agricultural	7	20.5	22	0.7	16
55	River sediment	6.7	28	32	3	20.8
74	Agricultural	7	30	33.9	1.8	18.9
79	Agricultural	7.2	26.9	77	0.9	17
88	Clay	7	28	63	4.4	23.9

EC: Electrical conductivity, OC: Organic carbon

Table 4: Morphological and cultural characteristics of *Actinomycetes* spp., growing on International Streptomyces Project type-2, after 7–14 days at 28°C±1°C

Isolate number	Growth state (mycelium)	Colony texture	Aerial mycelium color	Substrate (reverse) color	Soluble pigments		Culture shape	
					Presence	Color	Rear	Front
12	Moderate	Powder	Green	Brown	-	-		
16	Very good	Filamentous	White	Yellow	-	-		
l17	Good	Filamentos	White	Yellow	-	-		
19	Very good	Powder	Brown	Black	+	Brown		
18	Very good	Filamentous	White	Yellow	+	Yellow		
24	Very good	Filamentous	Yellow	Brown	+	Yellow		
25	Good	Powder	Black	Dark black	-	-		
27	Very good	Powder	Greenish	Brown	+	Yellow		
28	Good	Filamentous	White	Black	-	-		
34	Very good	Filamentous	Brown	Orange	+	Greenish		
41	Very good	Powder	Grey	Yellow green		Greenish		
42	Very good	Filamentous	White	Dark red	+	Red		
43	Very good	Powder	Green	Brown	+	Green		
37	Good	Filamentous	Brown	Black	+	Yellow		
46	Very good	Filamentous	Green	Brown	+	Green		
52	Very good	Powder	White	Purple	+	Purple		
55	Very good	Filamentous	Green	Brown	-	-		

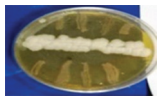
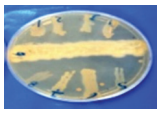
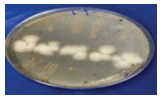
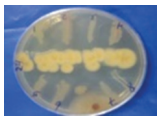
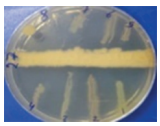
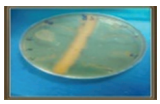
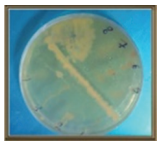
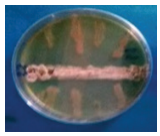
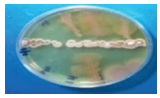
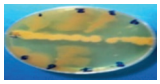
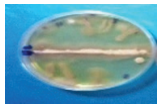
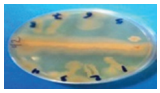
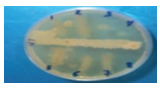
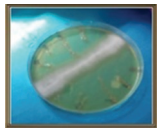
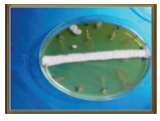
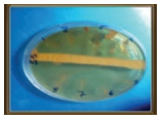
+: Soluble pigment present; -: Soluble pigment absent

and soil texture which were considered as the main factors that the concentration (distribution) of *Streptomyces* depended on.

Morphological and Cultural Characteristics

The present results in Table 4 of colonies characteristics for 17 colonies which choice from 33 colonies were

Table 5: Antibacterial activity of *Actinomycetes* spp. against pathogenic Gram-positive and negative bacteria (primary screening)

Sample number	Isolate symbol	Primary screening	
		Front shape	Rear shape
16	MA-Ag16		
18	KO-AG18		
24	HI-SA24		
27	KO-RS27		
28	HA-RS28		
34	AM-AG34		
37	HA-CL37		
41	KA-SA41		
42	MA-CL42		
43	MA-CL43		
46	MU-AG46		
52	AM-AG52		
55	HA-RA55		

agreed with the results of Ganesan *et al*.,[1] Zin *et al*.[29] who isolated the *Actinomycetes* spp. from the soil of peripheral area at the University of Putra in Malaysia to study the shapes of colonies and aerial with substrate mycelia colors on ISP-2 media.[1,29,30] Furthermore, the presented results were agreed with the results of Balasubramaniam *et al*.,[31] Sheik *et al*.[32] who isolated the *Streptomyces* ssp. from soils in India and study the morphological characteristics for these bacteria.[31]

Primary Screening Results for *Actinomycetes* Isolates against Pathogenic Bacteria

The 13 *Streptomyces* isolates were showed that antimicrobial activity against Gram-positive bacteria (*Staphylococcus albus*, *Staphylococcus aureus,* and *Streptococcus pyogenes*) and Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens,* and *Aeromonas hydrophila*) were tested by perpendicular streak method. The present results of primary screening test were agreed with Pan *et al*., (2013)[33]. Rana and Salam (2014)[34] are summarize the primary screening, if the microbial pathogens were susceptible to the antimicrobial produced by the isolated *Actinomycetes*; it would not allow their growth near the *Actinomycetes* as presented in plate in Table 5.

Secondary Screening Results for Extracellular Crude

Actinomycetes spp. are widely recognized as important organisms for their ability to produce different kinds of novel secondary metabolites.[35] More than 70–80% of all known antibiotics have been isolated from *Actinomycetes* and are used in medicine and agriculture[36,37] who isolated of *Actinomycetes* from different locations in Muthupettai Mangrove areas in India and found the diameters of inhibition zones ranged between 0 and 23 mm against pathogenic Gram-positive bacteria and between 0 and 18 mm against pathogenic Gram-negative bacteria and this return to the antimicrobial activity of *Actinomycetes* secondary metabolites [Tables 6 and 7].

Table 6: Antimicrobial activity of external crude extract on pathogenic gram positive bacteria (secondary screening)

Sample number	Isolate symbol	Diameter (mm)		
		Staphylococcus aureus	*Staphylococcus albus*	*Streptococcus pyogenes*
1	KO-AG18	27	19.5	6.3
2	HA-RS28	18.5	18.3	27.3
3	AM-AG34	24.5	18.3	0
4	KA-SA41	23	20.5	0
5	MA-CL42	18.2	21.5	0
6	MA-CL43	0	13	18.3
7	MU-AG46	12.3	20.3	17
8	HA-RA55	13.3	25	27
9	HI-SA24	17	35	34
10	KO-RS27	33	13	35

Table 7: Antimicrobial activity of external crude extract on pathogenic gram negative bacteria (secondary screening)

Sample number	Isolate symbol	Diameter (mm)				
		Escherichia coli	*Pseudomonas aeruginosa*	*Klebsella pneumoniae*	*Salmonella typhi*	*Aeromonas hydrophila*
1	KO-AG18	18	18.3	18	0	6.3
2	HA-RS28	19.5	19	20	20.8	12.3
3	AM-AG34	20.5	13	24.5	13.3	0
4	KA-SA41	17.5	9.5	17.2	28.5	17
5	MA-CL42	20.3	27	0	30.5	15.2
6	MA-CL43	28	0	0	6.3	25.3
7	MU-AG46	18.5	33	23	0	24.5
8	HA-RA55	0	0	0	17.3	30
9	HI-SA24	12.2	0	27	30.5	32
10	KO-RS27	33.5	23	39.2	24.5	33.7

Table 8: Antimicrobial activity of internal crude extract on pathogenic gram positive bacteria (secondary screening)

Sample number	Isolate symbol	Dimater		
		Staphylococcus aureus	*Staphylococcus albus*	*Streptococcus pyogenes*
1	MA-Ag16	27	29.5	32.5
2	HA-CL37	25.3	27.5	2703
3	HI-SA24	30	30.7	33
4	AM-AG52	20.5	18.5	18
5	MA-CL43	0	16.3	0

Table 9: Antimicrobial activity of internal crude extract on pathogenic gram negative bacteria (secondary screening)

Sample number	Isolate symbol	Dimater				
		Escherichia coli	*Pseudomonas aeruginosa*	*Klebsella pneumoniae*	*Salmonella typhi*	*Aeromonas hydrophila*
1	AM-AG52	0	30.5	0	30	18.5
2	HA-CL37	9.5	27	28.5	19	17.3
3	HI-SA24	20	18.5	13.3	20	20.5
4	MA-Ag16	13	20.5	32.3	18.5	19.3
5	MA-CL43	0	16.3	12.5	0	0

Secondary Screening Results for Intracellular Crude *Actinomycetes* Isolates against Pathogenic Bacteria

Further, in the present study, the intracellular methanol extract of *Actinomycetes* showed maximum zone of inhibition of 32.5 mm against *S. pyogenes* and lowest zone of inhibition of 9.5 mm against *E. coli* this return to the antimicrobial activity of internal crude extract of *Actinomycetes* (1 and 37) as shown in Tables 8 and 9.

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