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Study on the effect of metabolites secondary of Streptomyces on the bacterium Pseudomonas aeruginosa isolated from dog's wound

Estudo do efeito dos metabólitos secundários de Streptomyces na bactéria Pseudomonas aeruginosa isolada em ferida de cão

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ABSTRACT

In Baghdad, Iraq, private veterinary clinics provided one hundred samples between February and December 2022. The study included 72 female and 28 male dogs with wound infections. Thirty isolates of *P. aeruginosa* were identified using biochemical assays and cultural traits. At Tikrit University, ten isolates of *Streptomyces* bacteria were discovered in soil samples collected from various locations in 2022. Biochemical testing and microscopic analysis were used to identify the isolates. *Streptomyces* secondary metabolites were subjected to an antibacterial activity assay at doses of 1024, 512, and 256µg/mL. The results illustrated that raw bacterial extract (*Streptomyces* spp.) exhibited considerable antibacterial activity against all isolates of *P. aeruginosa*. The results demonstrated that a concentration of 1024µg/mL was more efficient than 256µg/mL in combating *P. aeruginosa*. The susceptibility test of all *P. aeruginosa* isolates to 10 antibiotics was carried out by Kirby-Bauer method. The results demonstrated that all isolates were resistant to Ampicillin and Erythromycin (96.66%) followed by Ceftraxone (63.33%), Azithrofomycin (50%), Levofloxacin (46.66%), Cefotaxime, Ciprofloxacin and Gentamycin (43.33%), Ceftaziaime (26.66%) and Amikacin (6.66%). The results of the current study indicated that the majority of isolates with high resistance to antibiotics could be affected by *Streptomyces* secondary metabolites to expedite the healing of infected wounds.

KEYWORDS: *Pseudomonas aeruginosa; Streptomyces;* secondary metabolites.

RESUMO

Em Bagdá, Iraque, clínicas veterinárias privadas forneceram cem amostras entre fevereiro e dezembro de 2022. Foram incluídos neste estudo 72 cães fêmeas e 28 cães machos com infecções de feridas. Trinta isolados de *P. aeruginosa* foram identificados usando ensaios bioquímicos e características culturais. Na Universidade de Tikrit, dez isolados de bactérias *Streptomyces* foram descobertos em amostras de solo coletadas em várias localidades em 2022. Testes bioquímicos e análise microscópica foram utilizados para identificar os isolados. Metabólitos secundários de *Streptomyces* foram submetidos a um ensaio de atividade antibacteriana nas doses de 1024, 512 e 256µg/ml. Os resultados mostraram que o extrato bacteriano bruto (*Streptomyces* spp.) exibiu considerável atividade antibacteriana contra todos os isolados de *P. aeruginosa*. Os resultados demonstraram que a concentração de 1024µg/mL foi mais eficiente do que 256µg/ml no combate a *P. aeruginosa.* O teste de suscetibilidade de todos os isolados de *P. aeruginosa* a 10 diferentes antibióticos foi realizado pelo método de Kirby-Bauer. Os resultados demonstraram que todos os isolados foram resistentes à Ampicilina e Eritromicina (96,66%), seguidos por Ceftriaxona (63,33%), Azitromicina (50%), Levofloxacina (46,66%), Cefotaxima, Ciprofloxacina e Gentamicina (43,33%), Ceftazidima (26,66%) e Amicacina (6,66%). Os resultados do presente estudo indicaram que a maioria dos isolados com alta resistência a antibióticos poderia ser afetada por metabólitos secundários de *Streptomyces* para acelerar a cicatrização de feridas infectadas.

PALAVRAS-CHAVE: *Pseudomonas aeruginosa*; *Streptomyces*; metabólitos secundários**.**

INTRODUCTION

Actinomycetes were first observed by Colin in 1875 in the tear glands of the human eye. Currently referred to as *Streptomyces*, they were also isolated from soil, straw, hay, and air by Rossi-Doria in 1891

(OYEDOH et al. 2023). Studies have demonstrated the importance of actinomycetes in the fields of medicine and as a source of soil fertility. Scientific advances in the production of many types of antibiotics, such as antibacterial, antifungal, and antiviral, have played an important role in limiting the growth of some pathological microorganisms (WILLIAM & PAUL 2006). It has been observed that actinomycetes create the majority of antibiotics that are therapeutically effective for treating infections (RAJARAM et al. 2020). It has been suggested that most of the new antibiotics were discovered through soil isolates screening (BANIYA et al. 2018).

Gram-positive filament bacterium Streptomycin is a member of the actinomycetes group, which comprises nearly all soil bacterial species. In addition, the main commercial attraction of these bacteria is the wide variety of antibiotics and anti-cancer compounds they produce (HOPWOOD 2019). Given the current health problems caused by multi-resistant bacteria, finding new antibiotics is a top priority activity. Consequently, bacteria belonging to this genus represent a valuable source for the development of new medicines. To encourage the development of these mysterious routes, various techniques were employed, and some of them had positive results. (AL-AJEELI 2012, BECERRIL et al. 2018, ZHANG et al. 2022).

The Gram-negative bacterium Pseudomonas *aeruginosa* is an opportunistic disease that can infect people and animals (ALHAZMI 2015). In animals, it causes metritis, mastitis, pneumonia, enteritis, and dermatitis (QUINN et al. 1998). Pseudomonas *aeruginosa* cells exhibit significant resistance to widely used antibacterial substances, such as penicillins and tetracyclines (SHAEBTH 2018, VINGOPOULOU et al. 2018). *Pseudomonas aeruginosa isolates are associated with multidrug resistance (MDR) against more than three distinct classes of antibiotics* (HAYATI et al. 2019). Antibiotic-resistant strains of Pseudomonas aeruginosa, especially those observed in animals, are becoming increasingly prevalent (WU et al. 2019). The skin acts as a barrier to prevent the entry of bacteria, fungi, and viruses; any breakage of this barrier allows simple microbial infiltration (AL SHAMMAA 2016).

A wound is, by definition, a violation of the integrity of the epithelium of the skin and can affect the architecture, physiology, and functions of the skin. Acute and chronic wounds are two different types of wounds. A common public health problem worldwide is acute or mild wounds. The acute wounds need four to six weeks to heal completely at a physiological level. However, if the healing process continues for more than six weeks without showing any resolution symptoms, the wound may be chronic or more severe wound (CHE SOH et al. 2020). The complicated underlying physiology of each individual makes it difficult to diagnose and treat chronic wounds, which are defined by compromising or interrupting the natural process of wound healing. Thus, the research identified the main causes of infections in canine wounds, and those affected by secondary Streptomyces metabolites. Many virulence factors are present in *Pseudomonas aeruginosa*, including enzymes that break down the effector molecules of the immune system and the components necessary for the cellular and tissue structure of the host. (MATHUR et al. 2006, MARQUES 2015). Nosocomial infections *related to P. aeruginosa* have generated health concerns, mainly due to the high prevalence of antibiotic resistance (ROSENTHAL et al. 2016).

The aim of this study was to: *In vitro*: study of the effects of secondary metabolites of *Streptomyces* spp. Pseudomonas *aeruginosa*

By:

- Isolation and identification of *Pseudomonas aeruginosa* from wound infections in dogs.
- Detecting the virulence factors of *Pseudomonas aeruginosa* using biochemical and molecular methods.
- Determine antibiotic susceptibility and select the most resistant isolate.
- Isolation and identification of filamentosus bacteria *(Streptomyces*spp.) from soil samples.
- Extraction of secondary metabolites from filamentosus bacteria*(Streptomyces* spp.).

MATERIALS AND METHODS

The project of this study was conducted in Baghdad's province between February and December 2022, with dogs of various ages, genres, and species. The samples were promptly collected and sent to the Faculty of Veterinary Medicine laboratory for molecular analysis and bacterial culture.

Specimens

The specimens were collected with sterile wound coats that had been cleaned with normal saline before collection and then immersed in 5 mL of sterile nutritional broth before being immediately transported to the laboratory.

Isolation of *Pseudomonas aeruginosa* from dog's wound infection

The swabs were incubated for 24 h at 37°C in MacConkey agar, blood agar, and nutrient agar and subcultured in chromogenic Pseudomonas agar.

Biochemical tests

All biochemical analyses were performed, including the growth in Nutrient agar at 42 °C, the fermentation of lactose in MacConkey, the oxidase, catalase, and urease tests, and the production of enzyme protease in skimmed milk agar (FORBES et al. 2016).

Antibiotic Susceptibility testing for *Pseudomonas aeruginosa*

Using the agar disc diffusion method, 10 antibiotics were selected according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2022) (Table 1) (BAUER et al. 1966) and modified Kirby-Bauer method (OLIVER et al. 2015, SHEHAB & JASSIM 2019, AL ABOOD 2022).

Table 1. The antibiotic disks used in this research.

No.	Antibiotics discs	Code	Disc potency (µg/disc)	Company	Origin
1	Levofloxacin	LEV	5	Bioanalysis	Turkey
2	Cefotaxime	TX	30	CONDA	Spain
3	Ceftraxone	CRO	10	CONDA	Spain
4	Azithrofomycin	AZM	15	Bioanalysis	Turkey
5	Ciprofloxacin	CIP	30	Bioanalysis	Turkey
6	Gentamycin	GN	10	Bioanalysis	Turkey
7	Ampicillin	AM	25	Bioanalysis	Turkey
8	Ceftaziaime	AZ	30	CONDA	Spain
9	Erythromycin	E	10	Bioanalysis	Turkey
10	Amikacin	AK	10	Bioanalysis	Turkey

Biochemical and molecular techniques for *detecting virulence factors in Pseudomonas aeruginosa*

Capsule detection: This technique uses nanum ink and Triple Sugar Iron to measure motility as described by DAVIS et al. (1990) and QUINN et al. (2004). The production of amilase was determined using a starch medium according to the protocol described by MacFADDIN et al. (2000). Using blood agar, the synthesis of the he

molysin enzyme was measured. Using skim milk agar, enzyme protease synthesis was performed using the procedure described by BENSON (2005). We used nutritional agar, naphthalene, and egg yolk to identify the production of the enzyme phospholipase according to the method described by ATLAS (1995). The production of the enzyme urease was detected using urea medium; the DNA medium was used to monitor DNAase synthesis according to the protocol described by QUINN et al. (2004).

Genome DNA extraction

Pseudomonas aeruginosa was reactivated using genetic techniques by being grown in nutrient agar for 24 h at 37 °C, as well as bacterial DNA isolated using the techniques described by NIKBIN et al. (2012) and WASAN (2021) and using the AccuPower®PCR PreMix, according to the manufacturer's instructions.

Thermocycling programs

Table 2. The thermocycler was adjusted in three stages for the genes (PslA, toxA1, lasA, algD), respectively.

No.	Steps	Temperature (°C)	Time	Number of cycles
1	Initial denaturation	95	5 min.	1
2	Denaturation	95	30 sec.	
3	Annealing	57.2	30 sec.	30
4	Extension	72	1 min.	
5	Final Extension	72	5 min.	1
	Store		8°C	

Isolation and identification of *Streptomyces* spp. Sampling of *Streptomyces* spp.

The first step

Ten soil samples were collected from different locations at the College of Veterinary Medicine at the University of Baghdad and the University of Tikrit. As the weight of each sample ranged between (200-250) g of soil, which was extracted from a depth of (10-100) centimeters after removing (2) centimeters of the upper layer, soil samples were placed in sterile containers and completely mixed with $_{CaCO3}$ (10% p/p). Ten grams of soil sample were mixed with 100 milliliters of sterilized distilled water ($^{10-1}$) in a 250 milliliter conical vial and stirred for 10 min. Each vial contained 90 ml of sterile de-ionized water, and each vial contained 10-ml

suspension aliquots. This was done to dilute the sample in series. This final dilution (10^{-5}) used for inoculation (ZHANG et al. 2000). Incubated at 30 °C for a period of 10 days, as mentioned in the development of a formalized method (LEE & HWANG 2002).

The second stage

The soil was treated with a $CaCO_3$ solution (10% p/v) and incubated at 37 °C for four days before being tested. A ringer solution, which should be sterile, was used to suspend the sample. To separate spores from vegetative cells, test tubes containing (10-2) of the sample dilutions were incubated for 16 h at 45°C. After separating the spores from the vegetative cells, the dilutions were placed in a selective medium (KORN & KUTZNER 1992).

Isolation of *Streptomyces* spp.

Serious dilution crops of soil in agar yeast extract and malt extract incubated at 30°C followed growth for 2 to 7 days and studied the nature of growth in the middle, the odor of the colonies, the color and shape on the surface side and the opposite side of the Petri plate (JOO & RHEE 1997).

Identification of *Streptomyces* spp.

The isolates were identified using biochemical tests, such as catalase, oxidase, urease, DNase, enzyme phospholipase synthesis, and starch hydrolysis.

Antibiotic production by *Streptomyces* spp.

For the production of antibiotics, yeast, malt extract broth, and glucose were distributed in the medium in 500 ml glass vials at a rate of 100 ml per vial, sterilized in an autoclave, left to cool, and then inoculated with 2% of trapped spores, placing the vials in a 120 rpm vibratory incubator at 28 °C for 10 to 14 days (SABARATNAM &TRAQUAIR, 2002).

Antibacterial activity of *the secondary metabolites* of Straptomyces against isolated P. aeruginosa

The antibacterial activity of the secondary metabolites of *Streptomyces* against *P. aeruginosa* was determined using the diffusion technique in agar wells at concentrations of 1024, 512 and 256µg/mL (RAHEEM 2014). Table 3 and Fig. 1.

Table 3. Antibacterial activity test of secondary metabolites of *Streptomyces* at concentrations (1024, 512, 256) µg/ml.

Raw bacterial extract of *Streptomyces* spp.									
P. aeruginosa isolated	1024 µg/ml	512 µg/ml	256 µg/ml	Control	*P. aeruginosa* isolated	1024 µg/ml	512 µg/ml	256 µg/ml	Control
P1	26	20	17	R	P11	25	21	16	R
P2	25	24	20	R	P12	26	25	20	R
P3	26	23	19	R	P13	26	18	16	R
P4	24	20	18	R	P14	24	22	20	R
P5	21	19	16	R	P15	26	21	20	R
P6	25	20	17	R	P16	25	22	20	R
P7	24	20	18	R	P17	28	26	21	R
P8	24	22	20	R	P18	23	21	20	R
P9	28	23	20	R	P19	25	22	20	R
P10	30	22	23	R	P20	28	24	20	R
No. of values	20	20	20	4	Multiple comparisons test	Under the Limit		Summary	P-Value
Mean	25.45	21.75	19.05	0.000					
Std. Deviation	2.012	2.023	1.877	0.000	Control vs. 256	Yes		**	<0.0001
Std. Mean Error	0.4500	0.4523	0.4197	0.000	Control vs. 512	Yes		**	<0.0001
					Control vs. 1,024	Yes		**	<0.0001
ANOVA results			<0.0001		256 vs. 512	Yes		**	0.0002
P-value			**		256 vs. 1,024	Yes		**	<0.0001
Significant differences between averages			< 0.05		512 vs. 1,024	Yes		**	<0.0001

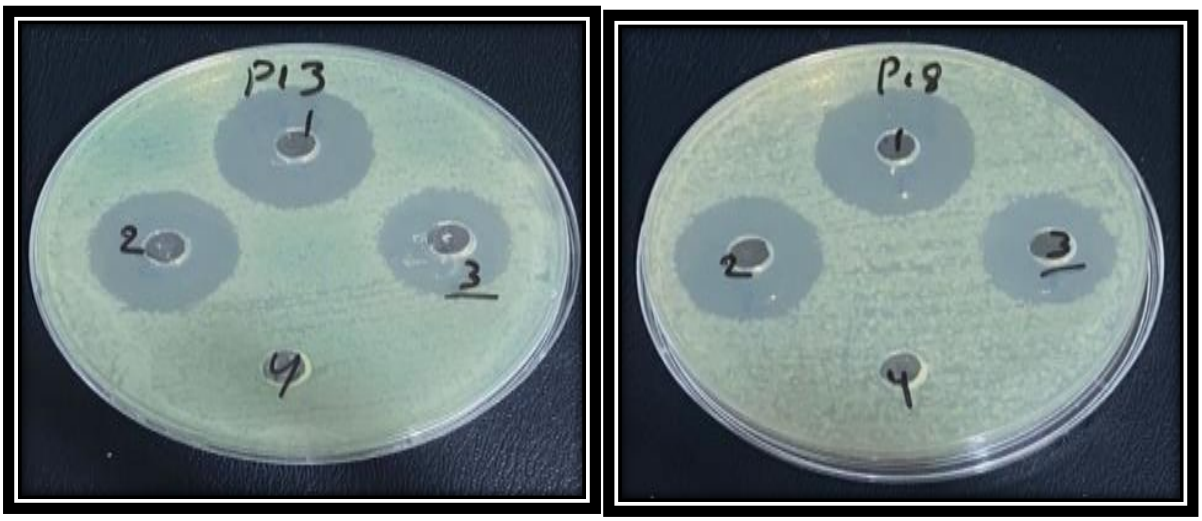

Figure.1. Antibacterial activity of the secondary metabolites of *Streptomyces* against *P. aeruginosa* in (1) 1024 µg/ml, (2) 512 µg/ml, (3) 256 µg/ml and (4) control.

RESULTS AND DISCUSSION

Isolation of *P. aeruginosa* from dog's wound infections

Of the 100 samples (28 males and 72 females) of canine wound infections included in this study, only 30 samples had a positive culture; most samples were identified mainly by morphological characteristics and biochemical tests. Most bacterial isolates developed hemolysis (B) after 24 h of incubation. These huge, flat bacterial colonies with grape smell grew up in the middle of Blood Agar. *P. aeruginosa* was identified as tiny, clear yellow, spherical, and convex colonies in MacConkey agar (non-lactose fermentant). The chromogenic agar Phonomonas was used. The green hue of the colony of *P. aeruginosa* and the change of color from pale or neutral to pale green are the parameters that influence colony color. (ALFRED 2005). The isolates *of P. aeruginosa* were grown on MacConkey agar, chromogenic Pseudomonas agar, and blood agar. You will see red/rosed bastos of *P. aeruginosa*. They fail to preserve their primary color (violet crystal), suggesting that they are Gram-negative bacteria. This result differed from the NOOMI (2018) results for enzyme amylase (3.3%, hemolysin%), enzyme 70%), protease (10%), enzyme phospholipase 83.3%, enzyme urease 100%, enzyme DNAase 80%, pigments 93.3%, and growth in agar nutrient at 42℃.

Genetic techniques for identifying virulence factors

Table 4 shows that, of all Pseudomonas isolates, algD and lasA were present in approximately 86.6% of the isolates, toxA1 in 16.6%, and pslA in 96.6%. Figures 2–5 show the results of the PCR experiments. These results differ from those recorded by (ALORNAAOUTI 2015, SCHROEDER et al. 2017, ABEDAL-NABIE 2021).

Table 4. Genes of *Pseudomonas aeruginosa* (virulence factors) identified in infections of canine wounds.

Dog wound infection samples (100)			
Isolates *of Pseudomonas* (30)			
	Virulence factors	No.	%
1	algD	26	86.6
2	lasA	26	86.6
3	toxA1	5	16.6
4	pslA	29	96.6

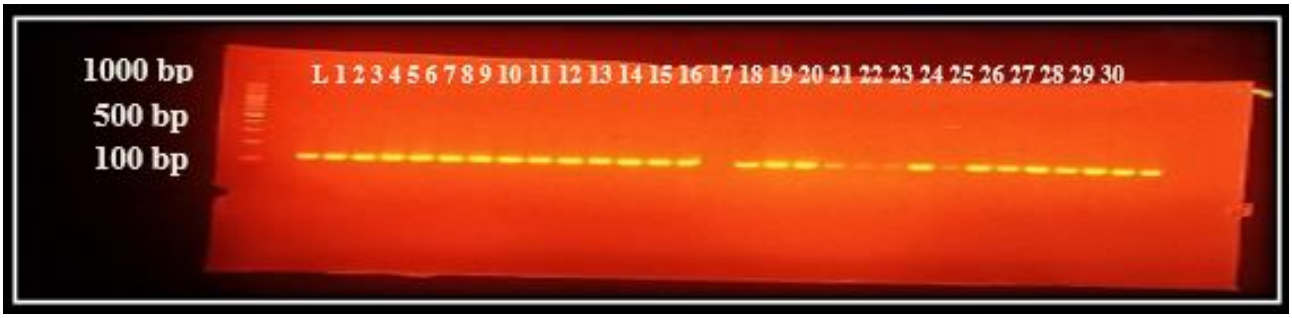

Figure 2. PCR product electrophoresis in agarose gel. L: molecular scale of 100 pb, lines (1-30), a positive result for the gene (algD) *of P. aeruginosa* at 127 pb.

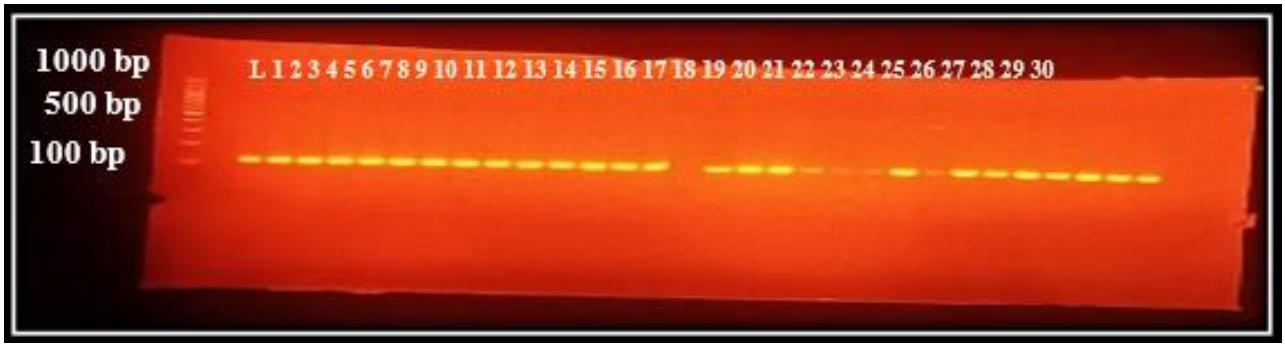

Figure 3. PCR product electrophoresis in an L agarose gel: a molecular scale of 100 pb, lines 1-30; positive result for the gene *of P. aeruginosa* (lasA) at 107 pb.

Figure 4. Electrophoresis of PCR products in agarose gel. L: molecular scale of 100 pb, lines 1-30; positive result for the gene toxA1 of P. aeruginosa at 106 pb.

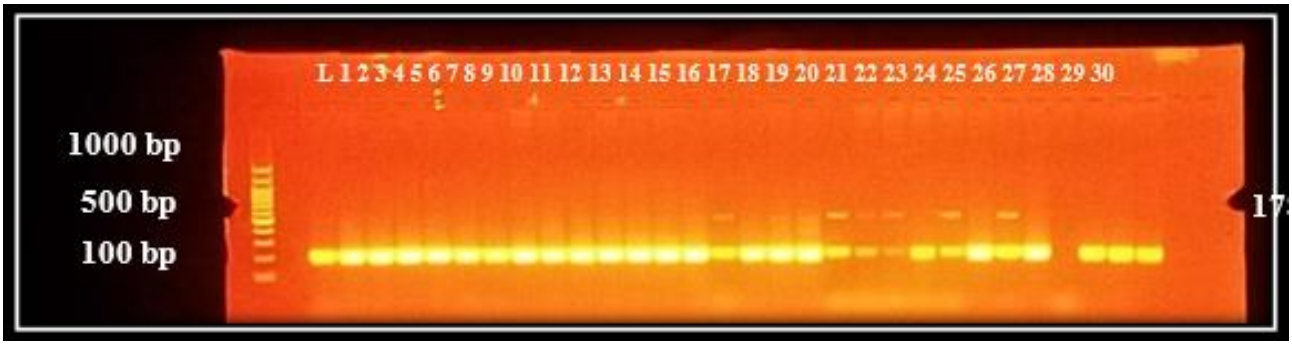

Figure 5. PCR electrophoresis products in agarose gel. L: molecular scale of 100 pb, lines 1–30; positive result at 178 pb for the gene (pslA) of P. aeruginosa.

Antibiotic susceptibility of *P. aeruginosa*

According to a recent study, *P. aeruginosa* developed resistance to conventional antibiotics because of drug use. The antibiotic susceptibility test in *P. aeruginosa* showed that 43.33% of the isolates were resistant to cefotaxime (TX), gentamicin (GN), and ciprofloxacin (CIP). Next, 46.66% went to Levofloxacin (LEV), 63.33% to Ceftraxone (CRO), 50% to Azithromycin (AZM), and 96.66% to Ampicillin (AM) and Erythromycin (E).26.66% of the isolates were resistant to Ceftaziaime (AZ) and 6.66% were resistant to Amikacin (AK), which is consistent with the 2022 ABD (6.67%) (Table 5).

Table 5. Antibiotic susceptibility test results.

Antibiotics	Antibiotic Disk Test			Chi-Square	Sig.	*P*- Value
	Sensitive	Intermediary	Resistant			
Levofloxacin	7	9	14	2.600	NS	0.2725
Cefotaxime	8	9	13	1.400	NS	0.4966
Ceftriaxone	5	6	19	12.20	**	0.0022
Azithromycin	12	3	15	7.800	*	0.0202
Ciprofloxacin	15	3	12	7.800	*	0.0202
Gentamycin	11	6	13	2.600	NS	0.2725
Ampicillin	1	0	29	54.20	**	<0.0001
Ceftazidime	2	20	8	16.8	**	0.0002
Erythromycin	0	1	29	54.20	**	<0.0001
Amikacin	9	19	2	14.60	**	0.0007
Chi-Square	32.00	57.42		41.71		
Sig.	**	**		**		
P-Value	0.0002	<0.0001		<0.0001		

Strain of *Streptomyces* spp. isolation.

After incubation for 2 to 7 days at a temperature of 30 °C, the results of activation of the isolate in agar yeast extract and malt extract demonstrated the formation of huge colonies up to 10 to 20 mm in diameter, flat, smooth, and cream-colored, and these results agree with (WAKSMAN, 1976). The results showed that the isolation of *Streptomyces* spp. did not produce the enzyme DNase (Table 6). The enzyme DNase analyzes the phosphodiester bond that connects the primary building blocks of DNA, known as nucleotides, to each other, causing the decomposition zones around the colonies that grow in the middle to look purple (NICIEZA et al. 1999, KEMPF et al. 2015, EXNER et al. 2017).

Table 6. Biochemical and enzymatic test results for *Streptomyces Spp.*

	Tests	Results
1	Oxidase	+
2	Catalase	+
3	Urease	+
4	DNase	-
5	phospholipase enzyme	+
6	Starch hydrolysis	+

Antibacterial activity of *Streptomyces secondary metabolites* against *P. aeruginosa* in vitro

The secondary metabolites *of Streptomyces* were tested for their antibacterial activity at concentrations of 1024, 512, and 256 g/mL (Figure 6). The results illustrated that in contrast to the control, the raw bacterial extract *(Streptomyces* spp.) significantly inhibited the growth of all *P. aeruginosa* isolates. The results indicate that the concentration of 1024 g/ml was higher than 256 g/ml in terms of the efficacy against *P. aeruginosa*.

It is believed that the raw bacterial extracts of Streptomyces species are natural substances with a number of biological functions, such as antioxidants, anticancer agents, and antimicrobials (RIAZ-RAJOKA et al. 2020, MAHDI et al. 2021).

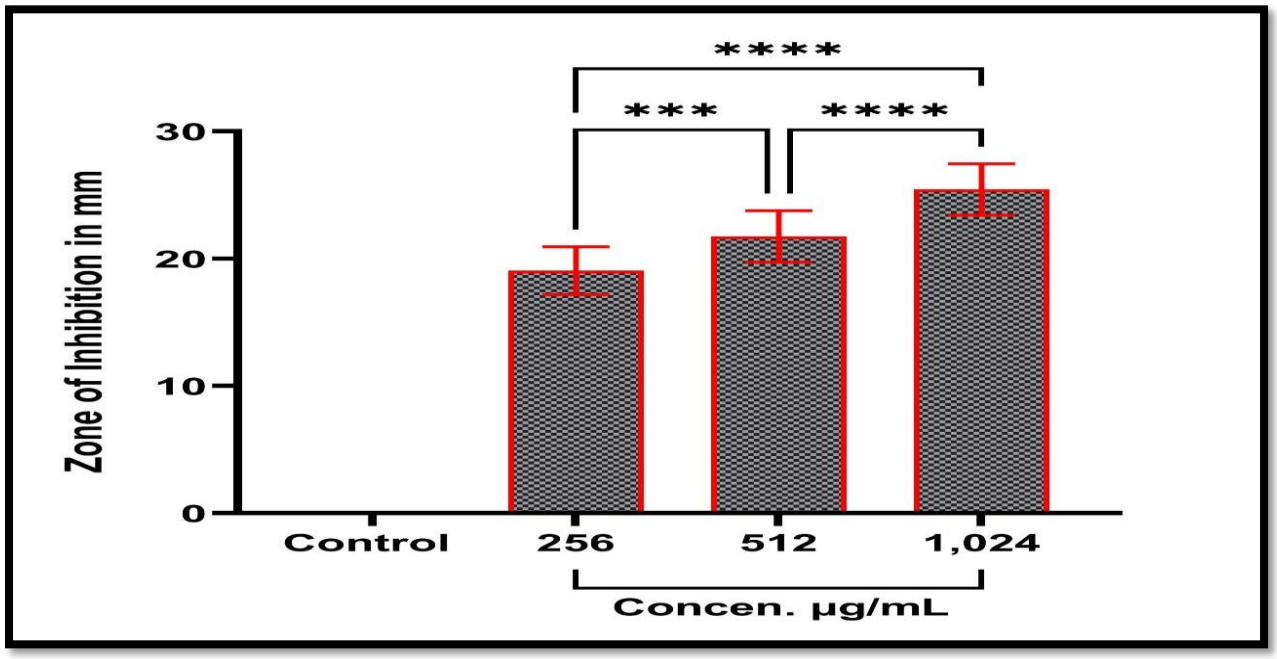

Figure 6. Antibacterial activity of secondary metabolites of Streptomyces against P. aeruginosa in (1) 1024 µg/mL, (2) 512 µg/mL, (3) 256 µg/mL, and (4) control.

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