ORIGINAL ARTICLE

Purification and Identification of Extracellular Secondary Metabolites from Actinomycetes and Study Its Antibacterial Activity Against Pathogenic Bacteria

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ABSTRACT

Background: *Actinomycetes are Gram-positive, spore -forming filamentous bacteria known for producing a broad spectrum of antibiotics compounds.* ***Objective:*** *This study aimed to isolate Actinomycetes from soil samples collected across Babylon province and assess their antibacterial potential against multidrug resistant bacteria.* ***Methodology:*** *A total 100 soil samples were treated with calcium carbonate (CaCO3), serially diluted, and cultured on ISP2 medium selective for Actinomycetes. The spore morphology was examined use electron microscope. Secondary metabolites were extracted using ethyl acetate, partially purified by thin-layer chromatography (TLC), and analyzed by high -performance liquid chromatography (HPLC) with a photodiode array detector.* ***Results****: The chromatographic profile revealed multiple peaks in the crude extract, with highest peaks intensity observed between 2.00 to 5.30 minutes. Antibacterial activity was evaluated using the agar well diffusion method against multidrug -resistant bacterial isolates, including 13 strains of Staphylococcus aureus, 13 strains of Klebsela pneumoniae and 14 strains of Escherichia coli.The extract showed inhibition zone from 4.00 to43.00 mm across concentrations of 25,50,100 and 200 mg/ml. Statistically significant results ($p<0.05$) were observed, with the highest efficacy against S.aureus.* ***Conclusion****: This findings confirm that secondary metabolites were derived from Actinomycetes possess significant antibacterial properties especially against multidrug-resistant S.aureus, while showing less activity against K.pneumoniae.*

INTRODUCTION

Actinomycetes are diverse group of gram positive filamentous bacteria widely distributed in soil and known for their ability to produce a wide range of bioactive secondary metabolites, including antibiotics[1].It is considered one of the most important natural source of antimicrobial compounds. Approximately 70-80% of commercially available antibiotics have been isolated from Actinomycetes, particularly from the genus streptomycin [2]. Due to the rise of multidrug-resistant (MDR) bacteria,there is a growing need to discover new antimicrobial agents.[3]This study aims to isolate actinomycetes from soil samples, extract and partially purify their extracellular secondary metabolites, and evaluate their antibacterial activity against clinically isolated pathogenic bacteria, including *staphylococcus aureus*, *Escherichia coli*, and *klebsela pneumoniae*.[4]

METHODOLOGY

Samples collection and preparation:

Fifty soil samples were randomly collected from various locations in Babylon, Iraq, including clay, sandy, and agricultural soils. Samples were treated with CaCO3(10:1 Raito) and incubated for one week at 28 C to enhance Actinomycetes growth, which usually prefer alkaline conditions and also to reduce the contamination with molds and yeast [5,6]

Isolation and morphological identification:

Actinomycetes were isolated using ISP2 agar medium and purified by sub-culturing colonies were characterized morphologically by observing mycelial structure, pigmentation, and spore formation under light and electron microscope.[7,8]

Extraction of Extracellular secondary metabolites

Selected isolates were cultured in ISP2 broth for 10 days. Filtrates were extracted with ethyl acetate, and the organic phase was evaporated to obtain crude extract.[9,10]

Partial purification (TLC & Bioautography);

Crude extracts were applied on TLC phase using different solvent system. Bioautography was used to detect antibacterial spots by overlaying TLC strips on agar plates inoculated with pathogens [11,12]

High-Performance Liquid Chromatography (HPLC)

HPLC was performed on ethyl acetate extract and active TLC spots to analyze compound purity and retention times using a photodiode array detector.[13]

Isolation of Pathogenic Bacteria:

A total of 135 clinical specimens were collected from patients in Babylon Hospitals. *S.aureus*, *E.coli*, and *K .pneumoniae* were identified using standard cultural and biomedical methods[14]

The antibacterial activities and Antibiotic susceptibility test

The agar well diffusion method was used to assess antibacterial activity of extract at various concentrations [15]. MIC and MBC were determined using broth diffusion methods[16]

RESULTS

Identification of Actinomycetes by morphological and cultural characteristics

The features of Actinomycetes isolates showed different mycelia shapes, different substrates color and different in presence or non-soluble pigments.

Extraction of Extracellular secondary metabolites

Extraction of Extracellular secondary metabolites from actinomycetes by ethyl acetate, according to the quantity of the product and the speed of its production.

TLC and Bioautography

TLC showed multiple spots, but only one per isolate was bioactive .These spots were visualized under UV and confirming through Bioautography as having antimicrobial activity, figure (1)

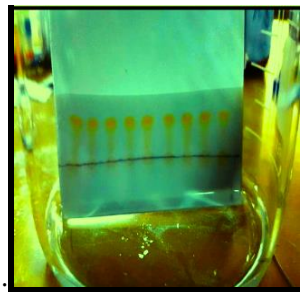


Fig. 1: TLC and bioautography of extracellular crude extract of actinmycetes extract

The extracellular crude extract, as illustrated in Figure 1, exhibited numerous spots on the TLC plate. However, only one of these spots exhibited antimicrobial activity during bioautography testing to determine the solubility of the bioactive region and its Rf in a variety of mobile phases.

High-Performance Liquid Chromatography

Chromatograph revealed several peaks in both crude and TLC -isolated samples**.**The extract showed the most intense peaks between 2.00-5.3 minutes retention time, indicating strong bioactive content.

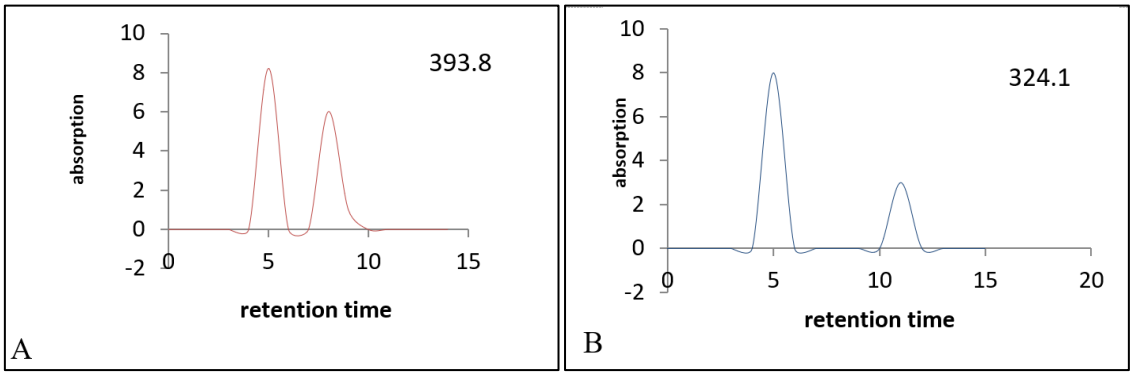


Fig. 2: HPLC spectrum of a secondary metabolisms extracted from: A-ethyl acetate extract, B- scraped spot extract

Table 1: Profile of using HPLC to diagnose secondary metabolites

Type Of extract	No. Of peak	Reten. Time [min]	Area [mAU.s]	Height [mAu]	Area%	Height %	W05 (min)
ethyl acetate extract	1	5.2	52360.89	590.24	85.00	75.00	0.25
	2	8.6	22147.63	360.44	15.00	25.00	0.15
		Total	74508.52	950.44	100.00	100.00	
scraped spot 1extract	1	5.1	52360.89	590.24	95.00		
	2	11.8	32761.09	489.77	5.00		
		Total	85121.98	1080.01	100.00		

Pathogenic Bacteria isolation and identification

From 135 hospital samples, 13 strains were *S.aureus*, 14 *E.coli*, and 13 *K.pneumoniae* were isolated and identified by standard culturing and VITEK2 system.

Antibiotic Resistance

MDR patterns were observed; 61.5% of *S.aureus*, 57.1% of *E.coli*, and 38.5% of *K.pneumoniae* were resistant to multiple antibiotics.

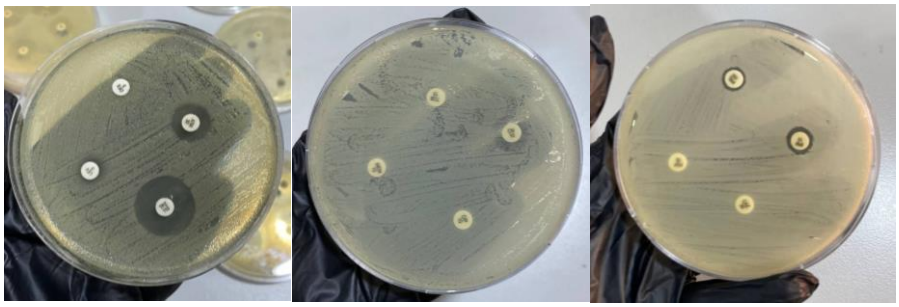


Fig 3: Zones of inhibition demonstrating antibiotic resistante pattern in selected bacterial isolated

Biological Activity Against Pathogenic Bacteria

The antibacterial activity of crude extracts from the Actinomycetes isolates was evaluated against more resistance isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* using the agar well diffusion method at various concentrations (25, 50, 100, 200 mg/mL). The results showed concentration-dependent inhibition zones..

Additionally, the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the extracts were determined. The extract displayed the lowest MIC and MBC values against all tested pathogens, confirming its strong antibacterial potential.

Table 2: The antibacterial activity of extract against more resistant isolates of *S. aureus*

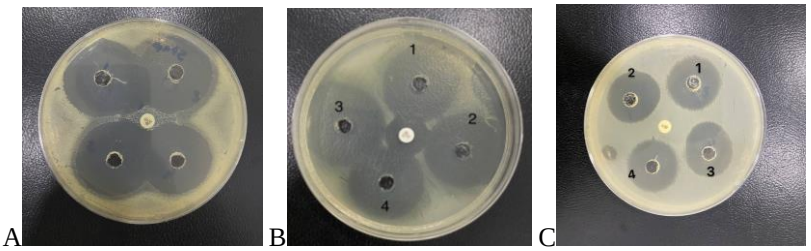
concentration (mg/ml)	(Mean ± SE)	p-value
25 mg/ml	34.62 ± 2.05	1.000
50 mg/ml	38.75 ± 1.13	0.134
100 mg/ml	40.75 ± 1.29	0.021
200 mg/ml	43.00 ± 0.91	0.002

Table 3: The antibacterial activity of extract against more resistant isolates of *e E.coli*

Concentration (mg/ml)	(Mean ± SE)	p-value
25 mg/ml	22.43 ± 4.53	1.000
50 mg/ml	28.29 ± 3.07	0.776
100 mg/ml	29.86 ± 3.04	0.592
200 mg/ml	31.71 ± 3.23	0.865

Table 4: The antibacterial activity of extract against more resistant isolates of *K. pneumoniae*

Concentration (mg/ml)	(Mean ± SE)	p-value
25 mg/ml	7.00 ± 4.64	1.000
50 mg/ml	14.60 ± 3.19	0.055
100 mg/ml	18.20 ± 3.84	0.020
200 mg/ml	19.20 ± 4.15	0.019



A B C

Fig. 4: Show the results of antibacterial activity of extract against more resistance isolates. A-*S.aureus*, B-*E.coli*, C-*K. pneumonia*

Table 5: MIC and MBC of crude extract of isolate was tested against microbial pathogens

Pathogenic Bacteria	Concentration of crude extract of isolates					
	200µg/ml	100µg/ml	50µg/ml	25µg/ml	12.5µg/ml	6.26µg/ml
S.aureus	-	-	-	MIC	MBC	
K.pneumoniae	-	-	MIC	MBC		
E.coli	-	-	-	-	MIC	MBC

DISCUSSION

Actinomycetes are known to produce a number of secondary metabolites, including lytic enzymes and antibiotics. Of these, isolates have been found to possess traits that make them effective antibacterial agents against pathogens[14]

TLC revealed three spots, but only one showed antibacterial activity of extract. The bioactive compound had Rf values between 0.3-0.92 cm and was soluble in most organic solvents except n-hexane. These findings algin with earlier reports identifying a single active metabolite using similar solvents system [15-20]

Results showed that the ethyl acetate extract contain more chromatographic peaks than in TLC-scraped spot, this indicate broader range of bioactive compounds in the crude extract. The presence of multiple distinct peaks, especially within the 2.00-5.30.8 minutes retention time, confirms the efficacy of TLC separation process and highlights the chemical of the extract. These findings are consistent with reports from WHO and recent studies that classify Gram-positive bacteria as particularly *S. aureus* as leading contributors to hospital-acquired infections due to their ability to rapidly acquire resistance genes.[21]

The high resistance rate in *E.coli* likewise reflects its established role as a model Gram- negative MDR organism, often exhibiting resistance through plasmid-mediated B-lactamases and efflux pumps[22]. The relatively lower MDR rate in *K.pneumoniae*, through still clinically relevant be due to sampling variations or environmental factors influencing resistance gene acquisition. However, the emergence of carbapenem -resistant strains of *K.pneumoniae* globally remains a critical threat [23]

In this study the antibacterial activity of actinomycetes isolates was tested against some pathogenic bacteria were isolated clinically. The result showed effect of actinomycetes to inhibit some gram positive pathogenic bacteria (*S.aureus*) and some gram negative bacteria (*E.coli and K. pneumoniae*),but the gram positive was more effected than gram negative bacteria .The observed variation in sensitivity is primarily linked to their structural differences where gram-negative bacteria are characterized by an additional outer membrane composed of lipopolysaccharide compounds. This renders the cell wall impermeable to lipophilic solutes. Gram-positive bacteria are more susceptible due to possessing only an exterior peptidoglycan layer, which is not an effective permeability barrier [24,25] .The findings of the present investigation demonstrated a robust agreement with prior researches [26,27]

Among all tested pathogens, *S.aureus* exhibited the highest sensitivity to the extract, which demonstrated an inhibition zone of up to 43 mm at 200 mg/ ml with statistical significance($p<0.05$) across all concentrations for support its robust antibacterial activity .These findings are agree with previous studies indicating that gram positive bacteria are more susceptible to natural product derived from actinomycetes due to absence of outer membrane that otherwise restricts the entry of antimicrobial agents.[28]

The extract showed strong activity against *E.coli*, with inhibition zones reaching 31 mm .This effect was concentration-dependent and statistically significant ($p<0.05$), reflecting the efficacy of this extract despite the known resilience of gram negative bacteria[19]

K. pneumoniae exhibited the highest resistance among the tested pathogens. The maximum inhibition zone observed for extract was 19 mm at 300 mg/ml, and the lack of significant p-values ($p<0.05$) suggests a limited efficacy at the tested concentration. The resistance of *K.pneumonia* may be attributed to its protective polysaccharide capsule and presence e of extended spectrum of B -lactamase (ESBLs) which can inactivate wide range of antibiotic. These findings are consistent with previous reports [27] which described the inherent resistance mechanisms in *Klebsiela* species including protective capsule and multiple resistance enzymes**.**

CONCLUSION

Our Findings emphasize the need for enhanced purification, synergistic combination, or structural modification of the active compounds to increase their efficacy against encapsulated and ESBLs -producing strains

Declarations

Ethical Approval

This study was conducted in accordance with the ethical standards of the institutional and national research committees. Ethical approval for the collection and handling of clinical specimens was obtained from the Ethical Committee of the College of Science, University of Babylon, Iraq (Approval No.: M240905). All patient-derived bacterial isolates were collected anonymously with no personally identifiable information recorded, ensuring the confidentiality and privacy of subjects. The study adhered to the principles outlined in the Declaration of Helsinki.

Journal Publication Statement

The authors declare that the content of this manuscript is original and has not been published or submitted elsewhere. All authors have read and approved the final version of the manuscript and have consented to its submission to the journal. The manuscript complies with ethical standards and institutional approval has been granted (Approval No.: M240905).

Conflict of Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions
Istbraq Abbas Hassan: Conceptualization, methodology, investigation, supervision, writing – original draft.
Yazi Abdullah Jassim: Sample collection, laboratory work, data analysis, writing – review & editing.
Noor Salman Kadhim AL-Khafaji: TLC and HPLC analysis, statistical analysis, visualization, writing – review & editing.

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