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Antimicrobial and Anticancer Potential of Actinobacteria Newly-Isolated from Caves in Beni-Suef, Egypt

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ABSTRACT

In recent years, actinobacteria have emerged as promising candidates for anti-cancer therapies, garnering significant attention in biomedical research. This study focuses on isolating and characterizing actinobacteria from diverse cave systems within Egypt's Beni-Suef governorate. The aim of the current study was to assess the biological activity, particularly antibacterial and anticancer properties, of the bioactive compounds produced by these actinobacteria. Through selective media and techniques, 112 strains were isolated from cave soil samples, subsequently purifying them for further analysis. Evaluation of secondary metabolites extracted from representative strains revealed significant antimicrobial activity against various pathogenic bacteria, yeast, and fungi, with a notable preference for Gram-positive bacteria. Additionally, five actinobacteria extracts have displayed potential antibacterial properties, while extracts from two strains, Am 139 and Am 152, exhibited substantial cytotoxic effects on multiple cancer cell lines. Further experiments confirmed their promising anticancer potential against MCF7, HepG2, and JURKAT cell lines across various concentrations. Our findings underscore the untapped potential of actinobacteria-derived metabolites from Beni-Suef caves. Moreover, the isolation of actinobacteria from previously unexplored sites such as caves suggests a rich source of bioactive compounds with antimicrobial and anticancer activities, promising new avenues for drug discovery and development.

Keywords: Actinobacteria; Antimicrobial Agents; Anticancer Properties; Biomedical Research; Cave Exploration.

INTRODUCTION

Cave ecosystems with surface openings represent a unique and largely unexplored environment worldwide. Despite their distinctive characteristics, these ecosystems have received limited attention in scientific research. They consist of relatively isolated geological systems that share fundamental physicochemical parameters, including full darkness, constant humidity, and temperature stability (Lange-Enyedi *et al.*, 2023). Cave environments are characterized by harsh conditions such as a lack of sustenance, excessive humidity, low light intensity, and a consistent temperature.

These conditions provide a separate ecological niche for creatures with specialized adaptations. Caves are typically home to dense extremophile colonies and distinct underground animal populations (Cyrier, 2022). These interesting and enigmatic settings have been home to distinct and diverse microbial populations for millions of years. These cave microbiotas are critical to the operation of cave ecosystems, playing vital roles in biogeochemical cycling, mineral production, and organic waste decomposition. One of their principal activities is the breakdown of organic materials, which is required for nutrient cycling and the survival of cave ecosystems (Zhu *et al.*, 2022).

Microorganisms like bacteria and fungi degrade organic materials, releasing nutrients like carbon, nitrogen, and sulfur. These nutrients are subsequently made available to other creatures in the cave environment. Cave microbiotas also contribute to the creation of stalactites and stalagmites, mineral formations that grow over thousands of years. Microorganisms such as bacteria and fungi contribute to the precipitation of minerals like calcium carbonate and calcium sulfate, which serve as the foundation for these structures (Farda *et al.*, 2022).

Actinobacteria are a kind of bacteria that may be found in volcanic and other caves worldwide. Actinobacteria discovered in caves have distinct morphological, physiological, and molecular properties. Filamentous growth, branching, and spore production are their morphological characteristics. These characteristics allow them to colonize a wide range of surfaces and substrates in caves, involving rocks, sediments, and speleothems (Riquelme *et al.*, 2015). Actinobacteria grow slowly, allowing them to live in nutrient-poor settings like caves. They can also withstand harsh environments, including cold temperatures, high humidity, and low oxygen levels.

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Actinobacteria found in caves are prized for their capacity to create a wide range of secondary metabolites, making them a potential bioprospecting target. Caves provide unique prospects for discovering unknown bacterial species and studying their interactions with emerging diseases (Rangseekaew and Pathom-Aree, 2019).

Cave actinobacteria create bioactive compounds with potential use in medicine and industry. Consequently, cave actinobacteria bioactive compounds have several potential uses in medicine and industry, making them a crucial field of study for drug development and innovation (Muazi Alenazi *et al.*, 2023; Xie and Pathom-Aree, 2021).

Cave microbiotas, such as actinobacteria, have the potential to address medical issues like antidrug-resistant bacteria and cancer therapy. Streptomyces, a genus of actinobacteria, produces bioactive compounds like Actinomycins, Aminoglycosides, Anthracyclines, Macrolides, and Polyketides, which are effective against Gram-positive and Gram-negative bacteria, as well as fungus and yeast. However, more research is needed to fully explore their potential in this area (Ghosh *et al.*, 2017; Riquelme *et al.*, 2017).

The dearth of comprehensive studies on caves hosting actinobacteria globally, particularly in the Middle East, presents a fertile ground for exploration. This research endeavors to fill this gap by isolating and characterizing actinobacteria from diverse cave systems in the Beni-Suef governorate, Egypt. The aim of this study is to shed light on previously undiscovered microbial diversity and harness their multifaceted bioactive potential for biomedical applications, marking a significant stride toward drug discovery and innovation.

MATERIALS AND METHODS

Sample collection and isolation of actinobacteria

The cave soil samples were collected using sterile sampling tools such as trowels and spoons from various locations surrounding the Meidum Pyramid in El Wasta, Beni-suef governorate (29.3241° N latitude and 30.9953° E longitude), Egypt (Figure 1). The soil dilution plate technique was used to selectively isolate actinobacteria (Johnson *et al.*, 1959). The air-dried soil samples were serially diluted up to four times before being inoculated in triplicate onto two selective media indicated for actinobacteria isolation: yeast extract/malt extract (ISP2) (Pridham *et al.*, 1957), and MM (Hozzein and Goodfellow, 2007b).

All media were adjusted to pH 7.4 and supplemented with nystatin at a concentration of 100 units/mL as an antifungal antibiotic and nalidixic acid at a concentration of 20 µg/mL to inhibit the growth of other bacterial colonies. Moist and dry heat pretreatment of the materials was also used as a selective isolation strategy to favor actinobacteria strain isolation. After two weeks of incubation at 30°C, the plates were examined, and 112 isolates were selected and purified by streaking on the same isolation medium. The isolates were subsequently subcultured for further investigation. The purified isolates were stored at -80°C in sterile 20% glycerol. To exclude duplicate strains, the purified actinobacteria were de-replicated by evaluating their cultural characteristics, including the color of aerial and substrate mycelia as well as soluble pigments, on the isolation medium and oatmeal agar medium (ISP3) (Küster and Williams, 1964). Thirty-seven different representative organisms were chosen for screening based on their color differences.

Figure (1): The location map shows the site of collected samples.

Secondary metabolites extraction

A total of 37 representative strains out of the 112 obtained isolates were selected based on their preliminary antimicrobial and anticancer activities, as well as their morphological diversity. These selected strains were fermented in their respective isolation media, specifically ISP2 and ISP3, in liquid form on a shaking incubator at 180 rpm and 30°C for 4 to 6 days immediately after selection. The secondary metabolites were extracted using conventional methods (Abutaha *et al.*, 2015). The fermentation broth was collected and filtered through Whatman No. 1 filter paper, and the mycelia were extracted with 100% methanol. The methanolic extracts were then evaporated to dryness using a rotary evaporator, and the residue was dissolved in 5 mL of methanol (designated as extract A). The culture broth was mixed in a 1:1 (v/v) ratio with ethyl acetate and vigorously shaken for 10 minutes. The concentration of ethyl acetate used was 100%. After vigorous shaking, the organic phase was separated from the water phase using a separating funnel. The organic phase was then evaporated to dryness, and the residue was dissolved in 5 mL of methanol (designated as extract t B).

Both extracts A and B were measured and subsequently combined. The crude extracts were stored in the refrigerator at 4°C until they were used in the subsequent screening experiments.

Antimicrobial activities of actinobacterial extracts

Using the disc diffusion method (De Beer and Sherwood, 1945), the antibacterial properties of the extracts were evaluated against representative pathogenic Gram-positive bacteria, Gram-negative bacteria, as well as unicellular and filamentous fungi.

Gram-positive test strains included *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, and *Staphylococcus aureus* ATCC 6538, whereas Gram-negative test strains included *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 10031, *Pseudomonas aeruginosa* ATCC 25668, and *Salmonella typhi* ATCC 9992. *Candida albicans* ATCC 90028 was utilized as the test unicellular fungal strain, and *Aspergillus niger* ATCC 16404 was employed as the test filamentous fungal strain. All pathogenic strains were courteously obtained from Faculty of Medicine, Beni-Suef University, Egypt.

Three agar media were prepared and sterilized for the disc diffusion method: nutritional agar (Williams and Cross, 1971) for bacteria, Sabouraud agar (Odds, 1991) for yeast, and Czapek Dox agar (Atlas, 2004) for fungal strains. After solidification, the agar media was poured and streaked with bacterial and fungal strains. The actinobacteria extracts were placed onto sterile filter paper discs (5 mm diameter; 50 µg g per disc), which were then inserted aseptically into the inoculated agar plates alongside commercially available tetracycline (30 µg) and erythromycin (25 µg) discs as control for comparison. For homogeneous diffusion into the agar, the plates were maintained at 4°C for 1 hour. The plates were then incubated at 37°C for 24 hours for bacteria and 30°C for 48-72 hrs for yeast and fungi. Following incubation, the diameters of the inhibition zones around the paper discs were measured in millimeters to determine antimicrobial activity.

Anticancer activities of actinobacterial extracts

The MTT assay was used to test the anti-cancer effects of actinobacteria extracts against the cancer cell lines MCF-7 (DSMZ ACC-115, human breast adenocarcinoma), JURKAT (DSM ACC-282, human T cell leukaemia), and HepG-2 (DSM ACC-180, human hepatocellular carcinoma). The cell lines were grown and propagated in RPMI-1640 media with 10% FBS and $NaHCO_3$. The cells were cultivated in an incubator with 5% CO_2 at 37°C and 90% relative humidity. The anti-cancer properties were assessed using Mosmann's cytotoxicity MTT test (Mosmann, 1983).

MTT and Cell viability

The MTT test is based on the reduction of MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] (Sigma) to the violet formazan product by living cell dehydrogenases. The actinobacteria extracts were treated in MeOH to make 1 mg/ml stock solutions. A serial dilution set was created using the stock solution. The experiment was carried out in 96-well plates, with aliquots of 60 µL of these serial dilutions of the examined extract mixed with 120 µL of a cell suspension containing approximately 5×10^4 cells/ml.

The plates were subsequently incubated for four days in the CO_2 incubator under the aforementioned conditions. The assay was carried out by adding 20 µL of MTT (0.5 mg/ml in phosphate-buffered saline, PBS) and incubating the plates for 2 hrs at 37°C. After centrifuging the plates to precipitate the produced formazan crystals, the supernatant was discarded. In 100 µL of isopropanol, the precipitate was dissolved. After shaking the plates for 10 minutes, they were measured at 595 nm with a microplate reader (Zenyth 200 ST, Biochrom, UK). The extracts were serially diluted and evaluated in triplicate. Cell growth inhibition was determined using the following formula:

$$\text{Inhibition\%} = (1\text{-OD/OD0})\ 100$$

Where, OD/OD0 represents the absorbance of treated and untreated cells, respectively. The cytotoxicity activity curve for each extract was also plotted using the obtained results.

Purification and characterization of the most potent bioactive metabolite

The crude extracts were partially purified, and their chemical properties were examined using thin-layer chromatography (TLC) with a solvent system of CH_2Cl_2-MeOH (9:1, v/v). The activity was evaluated at the partial purification stage using the bioautography method, which involved placing the generated chromatogram strip on the surface of a *B. subtilis*-seeded agar plate. The plate was then incubated for 24 hours at 37°C. To prepare for characterization, the active areas were scratched from the TLC plate and extracted with the least amount of methanol possible.

The TLC plates were treated with ninhydrin, Dragendorff reagent, and vanillin-sulfuric acid to characterize the functional chemical groups of the active metabolites; however, we concentrated on those that create alkaloid compounds and had the highest ratio of active compounds. TLC easily detected the alkaloids following spraying with the Dragendorff reagent, which produced an orange or red color on a yellow background. In addition, HPLC-UV profiles were created to investigate the chemical diversity in the extracts identified by HPLC (Waters HPLC 1525) over an RP-8 chromatographic column eluted using a gradient elution system (1-100% H_2O-MeOH, v/v).

Characterization and identification of the most active actinobacterial strains

Aerial hyphae and spore chain morphology

The aerial hyphae and spore chains of the active 16 actinobacteria strains were investigated on ISP2 agar medium using the coverslip technique (Kawato and Shinobu, 1959). To create a trough, a strip of agar was taken from the poured plates. Sterile coverslips were carefully placed onto the surface of the solid medium. This creates a space or angle between the medium and the coverslip, which is crucial for the inoculation the selected isolates separately. The inoculated plates were then incubated at 28°C for specific time intervals (7, 14, and 21 days). The coverslips were gently removed along with the adherent growth and then examined microscopically and photographed using a light microscope at the appropriate magnification. A JEOL (JSM-6060 LV) scanning electron microscope was also employed to examine gold-coated dehydrated hyphal culture for spore shape, surface, and spore structure.

Cultural characterization

The cultural characteristics of the most active 5 actinobacteria strains, particularly the aerial spore mass color, substrate mycelial pigmentation, and color of

any diffusible pigment, were observed after two weeks of incubation at 28°C using the methods and media recommended by (Shirling and Gottlieb, 1966). The most active 5 actinobacteria strains were selected based on their highest antibacterial activity as determined by the diameter of the inhibition zones in the paper disc diffusion method. The following ISP media were used to study the cultural properties of active actinobacteria strains: Tryptone-yeast extract medium (ISP 1) (Pridham and Gottlieb, 1948); Yeast extract-malt extract agar (ISP2); Oatmeal agar medium (ISP 3) (Küster and Williams, 1964); Inorganic salts-starch agar medium (ISP 4) (Küster and Williams, 1964); and Glycerol-asparagine agar medium (ISP 5) (Pridham and Lyons Jr, 1961). The sterilized agar medium was placed onto sterilized Petri plates and allowed to solidify. The plates were then inoculated with 100 ml of actinobacteria spore suspension and incubated at 28°C for 21 days. The growth rate, the color of the mature sporulating aerial surface, substrate mycelium, and diffusible soluble pigments were all recorded.

Chemotaxonomic characteristics of cell wall

For the DAP analysis, the approach of Hasegawa *et al*. (1983) was used. The polar lipid pattern of the actinobacteria strains was determined using thin-layer chromatography (TLC) following the method outlined by Lechevalier *et al.* (1977). This method involves extracting the polar lipids from the cell material and separating them on a silica gel plate using a suitable solvent system. The separated lipids were then visualized and identified based on their migration patterns compared to known standards. The polar lipid pattern of strain Am 90 consisted of phosphatidyl-choline (PC) as the diagnostic polar lipid, along with phosphatidylmethylethanolamine (PME), phosphatidyl-ethanolamine, and diphosphatidyl glycerol.

Phylogenetic analysis of the 16S rRNA gene sequence

The strains Am 139, Am 152, and Am 183 were chosen for the phylogenetic study due to their potential antibacterial and anti-cancer activity. The phylogenetic analysis of the 16S rRNA gene sequence was carried out in the manner previously reported in detail (Hozzein and Goodfellow, 2007a). Gel electrophoresis was performed to visualize the PCR products, and sequence chromatograms were generated to confirm the quality and accuracy of the amplified DNA. The standard genomic DNA extraction kit was used to extract genomic DNA from the organism's biomass. The primers 8-27 f (5′-AGAGTTTGATCC-TGGCTCAG-3′) and 1523-1504 r (5′-AAGGAGGT-GATCCAGCCGCA-3′) were used for PCR-mediated amplification of 16S rRNA. The PCR products were purified using a PCR purification kit according to the manufacturer's instructions, and the sequencing was done at a commercial company (Macrogen, South Korea).

BLAST (Basic local alignment search program, available online at www.ncbi.nlm.nih.gov) was used to compare the resulting 16S rRNA gene sequences to the corresponding bacterial sequences available from the public DDBJ, EMBL, and GenBank databases. The most comparable sequences were chosen and manually aligned using the MEGA X software tool (Kumar *et al.*, 2004). The neighbor-joining tree-making technique was used to infer phylogenetic trees (Saitou and Nei, 1987). To produce evolutionary distance matrices, the Jukes and Cantor distance model was employed (Jukes and Cantor, 1969). Bootstrap studies of the neighbor-joining approach based on 1000 resampling were performed on the resulting tree topologies using the SEQBOOT and CONSENSE programs from the PHYLIP package (Felsenstein, 1981, 1993). Finally, the results were interpreted, as well as the strains' taxonomic affiliation.

Statistical analysis

Statistical analysis was conducted to assess the significance of the extracts' anti-cancer properties. The SPSS statistical tool (SPSS for Windows, Version 16.0, SPSS Inc., Chicago, USA) was employed to determine the effects at a significance level of 0.05%. This involved calculating the means of three microplate readings along with their standard deviations. Mean separations were performed using Duncan's Multiple Range tests (at $p \leq 0.05$).

RESULTS

Evaluation of Antimicrobial activities of the metabolic extraction

The antibacterial properties of the 37 extracts were assessed against representative pathogenic Gram-positive bacteria, Gram-negative bacteria, a yeast strain, and a filamentous fungus. According to Table (1), it was discovered that 16 strains were biologically active against the tested strains. The data clearly show that yeast and Gram-negative bacteria were less inhibited than Gram-positive bacteria, with stronger inhibition observed against the latter. The action-bacteria strains isolated from the cave system of Beni-Suef, Egypt, showed great promise and potential in the antimicrobial results, with 43.2% of the chosen strains exhibiting antimicrobial activities against at least some of the tested pathogenic organisms. Five actinobacteria extracts, namely Am 43, Am 90, Am 139, Am 152, and Am 183, exhibited particularly potent antibacterial properties.

Evaluation of Anticancer activities of the metabolic extraction

The potential anticancer activities of five action-bacteria extracts were evaluated using the MTT assay against the MCF-7 cancer cell line. The data presented in Table (2) revealed that only two extracts, Am 139 and Am 152, exhibited significant cytotoxic effects against the tested cancer cell line when compared to the methanol-negative control. Consequently, these two extracts were selected for further investigation, where their anticancer effects were assessed against three cancer cell lines: MCF7, HepG2, and JURKAT, at various concentrations. The results indicated that, compared to the methanol control, the Am 139 extract of exhibited anticancer activity against the cancer cell

Table (1): The antimicrobial activities of the selected actinobacteria isolates, isolated from cave soil sample locations surrounding the Meidum Pyramid in El Wasta, Beni-suef governorate, Egypt, expressed as inhibition zones of growth in mm against the used test organisms.

Designated Code for actinobacteria extract	**Test human pathogens (Inhibition zone measured as diameter in mm)**								
	Bacillus cereus ATCC 10876	*Bacillus subtilis* ATCC 6633	*S.aureus ATCC 6538*	*E. coli* ATCC 25922	*K. pneumoni a* ATCC 10031	*P. aeruginosa* ATCC 25668	*Salmonell a typhi* ATCC 9992	*Candida albicans* ATCC 90028	*Aspergillus niger* ATCC 16404.
Am 18	-	-	-	-	-	-	-	-	-
Am 22	-	-	-	-	-	-	-	-	-
Am 23	7.0	8.0	7.0	-	-	-	-	-	-
Am 27	-	-	-	-	-	-	-	-	-
Am 31	15.0	16.0	18.0	-	-	-	-	-	-
Am 43	20.0	23.0	19.0	18.0	16.0	20.0	14.0	35.0	11.0
Am 49	-	-	-	-	-	-	-	-	-
Am 54	-	-	-	-	-	-	-	-	-
Am 57	12.0	11.0	11.0	-	-	-	10.0	-	-
Am 59	-	-	-	7.0	7.0	-	7.0	-	-
Am 66	-	-	-	-	-	-	-	-	-
Am 72	-	-	-	-	-	-	-	-	-
Am 73	17.0	16.0	15.0	-	-	-	-	-	-
Am 90	23.0	23.0	26.0	-	-	-	-	20.0	-
Am 99	9.0	8.0	7.0	16.0	-	-	9.0	-	-
Am 103	-	-	-	-	-	-	-	-	-
Am 117	-	-	-	-	-	-	-	-	-
Am 118	-	-	-	-	-	-	-	-	-
Am 120	7.0	7.0	9.0	-	-	-	-	-	-
Am 125	-	-	-	-	-	-	-	-	-
Am 131	-	-	-	-	-	-	-	-	-
Am 135	-	-	-	13.0	14.0	9.0	9.0	-	-
Am 137	-	-	-	-	-	-	-	-	-
Am 139	28.0	23.0	26.0	8.0	9.0	13.0	18.0	-	-
Am 143	12.0	13.0	12.0	-	-	-	-	15.0	-
Am 144	13.0	14.0	14.0	-	-	-	-	11.0	-
Am 146	-	-	-	-	-	-	-	-	-
Am 150	13.0	15.0	16.0	-	-	-	-	11.0	-
Am 152	20.0	21.0	18.0	14.0	11.0	11.0	16.0	17.0	-
Am 157	-	-	-	-	-	-	-	-	-
Am 159	-	-	-	-	-	-	-	-	-
Am 163	-	-	-	-	-	-	-	-	-
Am 164	-	-	-	-	-	-	-	-	-
Am 171	-	-	-	-	-	-	-	-	-
Am 183	17.0	19.0	18.0	7.0	7.0	-	8.0	12.0	-
Am 186	-	-	-	-	-	-	-	-	-
Am 188	-	-	-	-	-	-	-	-	-
Standard antibiotics									
Erythromycin (25µg)	32.0	35.0	35.0	13.0	10.0	11.0	34.0	-	-
Tetracycline (30µg)	24.0	25.0	24.0	23.0	7.0	7.0	22.0	-	-

-, no inhibition detected.

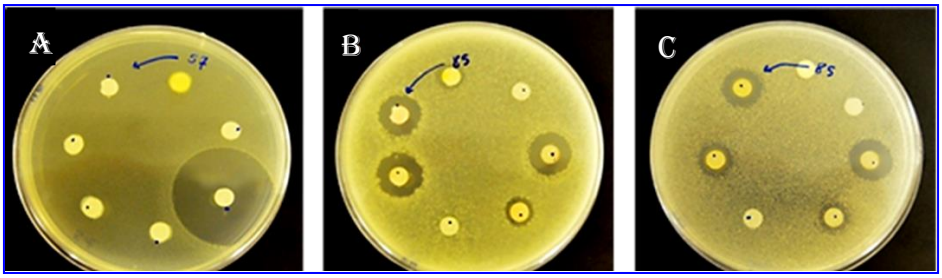

Figure (2): Representative plates showing the antimicrobial activities of the actinobacteria extracts against the used test organisms. (A) *Salmonella typhi* ATCC 9992, (B) *Bacillus subtilis* ATCC 6633 and (C) *Candida albicans* ATCC 90028.

Table (2): The anticancer activities of the selected five actinobacterial extracts on the MCF7 cancer cell line.

Cytotoxicity	Selected actinobacteria extracts					Control
	Am 43	Am 90	Am 139	Am 152	Am 183	
	0.497	0.522	0.942	0.944	0.376	0.739
Viable cell at 595 nm	0.554	0.592	0.923	0.932	0.615	0.725
	0.599	0.595	0.983	0.916	0.520	0.712
Average	0.556^{c}	0.569^{c}	0.949^{a}	0.930^{a}	0.503^{c}	0.725^{b}
Standard deviation	0.051	0.041	0.030	0.014	0.120	0.013

Data are represented in mean (n=3) ± SD. Data with different superscript small letters, per row, are significantly different at $p \leq 0.05$ according to Duncan's Multiple Range Tests.

Table (3): The cytotoxic effect of Am 139 extract on MCF7 cancer cell line.

Cytotoxicity	Am 139 concentration (µg/ml)				
	25	50	100	200	Control
	0.869	0.869	0.721	0.587	0.708
Viable cell at 595 nm	0.750	0.772	0.816	0.567	0.719
	0.814	0.783	0.776	0.582	0.712
Average reading	0.809^{a}	0.807^{bc}	0.769^{c}	0.578^{c}	0.712^{b}
Standard deviation	0.059	0.053	0.047	0.010	0.005

Data showed represent mean values (n=3) ± SD. Data with different superscript small letters, per row, are significantly different at $p \leq 0.05$ according to Duncan's Multiple Range Tests.

Table (4): The cytotoxic effect of Am 139 extract on HepG2 and JURKAT cancer cell lines.

Cell line	Am 139 concentration (µg/ml)				
	25	50	100	200	Control
HepG2	0.57 ± 0.02^{a}	0.67 ± 0.05^{b}	0.78 ± 0.008^{c}	0.81 ± 0.02^{c}	0.7 ± 0.01^{b}
JURKAT	0.52 ± 0.004^{a}	0.53 ± 0.04^{ab}	0.55 ± 0.004^{b}	0.60 ± 0.04^{c}	0.56 ± 0.02^{ab}

Data are represented in mean (n=3) ± SD. Data with different superscript small letters, per row, are significantly different at $p \leq 0.05$ according to Duncan's Multiple Range Tests.

lines MCF7 and JURKAT at concentrations ranging from 25 to 100 µg/ml, and against the cancer cell line HepG2 at concentrations between 25 and 50 µg/ml (Tables 3 and 4). The extract of Am 152, on the other hand, demonstrated anticancer activity against the MCF7 cancer cell line at concentrations ranging from 25 to 100 g/ml when compared to the methanol control, and the results were significant (Table 5). The extract of Am 152, on the other hand, did not affect the cancer cell lines HepG2 and JURKAT.

Our findings showed that the extracts of Am 139 and Am 152 have good and mostly substantial cytotoxic effects on the cancer cell lines evaluated. As a result, the results demonstrated that the two extracts could be viable anti-cancer medications, although more research is needed.

Identification of the most potent actinobacteria strains

Morphological characteristics

The morphological characteristics of the 16 biologically active Actinobacteria strains were systematically examined using the coverslip culture technique, which enabled detailed observation of cellular structures. Spore arrangement and surface ornamentation were further analyzed with scanning electron microscopy (SEM), providing high-resolution images that reveal intricate details of spore morphology. Representative micrographs, presented in Figure (3), display the diverse morphological features observed across these strains.

Based on these morphological assessments, the strains were tentatively classified into six distinct genera: *Streptomyces*, *Nocardiopsis*, *Amycolatopsis*, *Nocardia*, *Actinomadura*, and *Microbispora*. This classification is summarized in detail in Table (6), which outlines the specific characteristics associated with each genus. The results indicate a rich diversity of actinobacterial genera, highlighting the ecological significance of the sampled locations in Beni-Suef, Egypt.

Cultural characteristic

The five most active actinobacteria strains, namely Am 183, Am 152, Am 139, Am 90, and Am 43, were characterized by examining their cultural characteristics on various International Streptomyces Project (ISP) media, with the results summarized in Table (7). Following the guidelines established by Shirling and Gottlieb (1966), the cultural characteristics of these desert actinobacteria were analyzed based on their growth abundance and coloration across ISP media types ISP1 to ISP5. The evaluation revealed diverse growth patterns among the strains. Some strains exhibited abundant growth, characterized by dense colony formation, while others showed moderate to weak growth. The coloration of the colonies varied significantly, ranging from grayish white to reddish brown. This variation indicates a range of pigment production among the isolates, suggesting that different strains possess unique metabolic capabilities that contribute to their pigmentation.

Table (5): The cytotoxic effect of Am 152 extract on MCF7, HepG2, and JURKAT cancer cell lines.

Cell line tested	Am 152 concentration (µg/ml)				
	25	50	100	200	Control
MCF7	0.50±0.02a	0.52±0.08[a]	0.54±0.04[b]	0.63±0.003[c]	0.56±0.02[a]
HepG2	0.73±0.02b	0.75±0.03[b]	0.81±0.008[a]	0.87±0.009[a]	0.70±0.01[b]
JURKAT	0.73±0.14b	0.74±0.02[b]	0.76±0.03[b]	0.78±0.003[a]	0.70±0.006[b]

Data are represented in mean (n=3) ± SD. Data with different superscript small letters, per row, are significantly different at $p \leq 0.05$ according to Duncan's Multiple Range Tests.

Additionally, the growth and coloration differences observed across various media types underscore the influence of nutrient composition on the morphological characteristics of actinobacteria. Each ISP medium likely provided distinct nutrients and environmental conditions that affected the growth rate and pigment production of the strains.

Analysis of diaminopimelic acid (DAP)

Amino acid analysis of cell wall hydrolysates revealed that only strain Am 43 contained LL-diaminopimelic acid, a characteristic diamino acid of the peptidoglycan, while the other four strains (Am 90, Am 139, Am 152, and Am 183) contained meso-diaminopimelic acid as their characteristic diamino acid. Morphologically, strain Am 43 exhibited extensively branched mycelia with long spore chains, aligning with characteristics typical of the genus *Streptomyces*. This was supported by the chemotaxonomical markers, which confirmed its classification within the *Streptomyces* genus as it possessed the LL-isomer of diaminopimelic acid and lacked major characteristic sugars, fitting the criteria for wall chemotype 1.

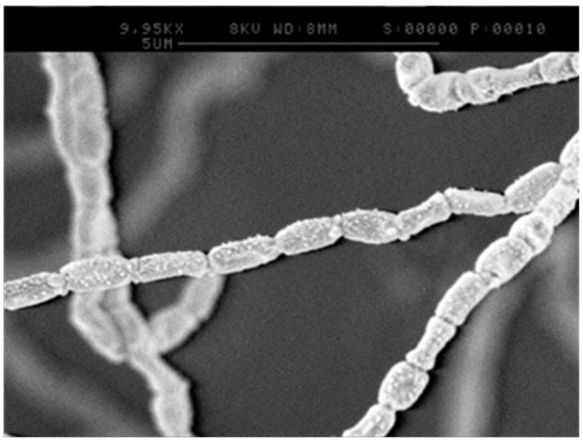

Figure (3): SEM of Actinobacterial isolate as a representative micrograph of the *Streptomyces* isolate obtained in this study.

Additionally, strain Am 90 displayed morphological traits consistent with species of *Nocardiopsis*, featuring aerial mycelium fragments that formed branched and straight spore chains, as well as elongated and smooth spores. Its cell wall composition included meso-diaminopimelic acid and specific sugars, while its polar lipid profile was dominated by phosphatidylcholine and phosphatidylmethylethanolamine, typical of the *Nocardiopsis* genus. Strain Am 139 exhibited fragmented substrate mycelia and long chains of smooth spores, characteristic of the genus *Amycolatopsis*, supported by chemotaxonomic analysis indicating the presence of meso-diaminopimelic acid and specific polar lipids. In contrast, both strains Am 152 and Am 183 exhibited fragmented substrate mycelia and short spore chains, leading to their classification within the *Nocardia* genus, as evidenced by their chemotaxonomical markers indicating meso-diaminopimelic acid and specific polar lipids.

Phylogenetic analysis of the 16S rRNA gene sequence

The actinobacteria isolates Am 139, Am 152, and Am 183 were meticulously selected for phylogenetic analysis due to their significant antimicrobial and anticancer activities observed in previous studies. To elucidate their taxonomic relationships, the 16S rRNA gene sequences of these selected isolates were amplified and analyzed. The phylogenetic analysis revealed that strains Am 152 and Am 183 were firmly placed within the genus *Nocardia* (Figures 4, and 5). Strain Am 152 exhibited a particularly strong genetic affinity, clustering closely with two distinct strains of *Nocardia carnea*, which suggests a very high level of genetic similarity with a remarkable similarity value of 99.8%. This close relationship indicates that strain Am 152 may share similar genetic and metabolic characteristics with *Nocardia carnea*, which could have implications for understanding its ecological roles and potential applications in biotechnology and pharmacology.

Table (6): Classification of biologically active actinobacteria isolates according to their morphological characterization.

Isolate code	Characteristic features	Proposed classification [*]
Am 43, Am 57, Am 59, Am 120	Extensively branched aerial mycelium with short to long chains of coiled spores	*Streptomyces*
Am 31, Am 73, Am 90, Am 150	The aerial mycelium fragments into branched and straight spore chains with elongated, irregular, and smooth spores.	*Nocardiopsis*
Am 135, Am 143, Am 144	Short chains of conidia on the aerial mycelium.	*Actinomadura*
Am 99, Am 139	Fragmented substrate mycelia. Aerial hyphae may be formed; they may also segment into long chains of smooth spore-like structures.	*Amycolatopsis*
Am 152, Am 183	Fragmenting substrate mycelium and short chains of spores.	*Nocardia*
Am 23	Chains of conidia mainly of two spores formed on conidiophore	*Microbispora*

[*]Proposed classification based on the practical guide to generic identification of actinobacteria introduced by Lechevalier and Lechevalier in Bergey's Manual for Systematic Bacteriology (1989).

Table (7): The cultural characteristics of the most active desert actinobacteria on ISP media.

Used Medium	Character†	Actinobacterial isolates				
		Am 43	**Am 90**	**Am 139**	**Am 152**	**Am 183**
ISP1	G	Abundant	Abundant	Weak	Moderate	Abundant
	AM	Dark gray	Moderate grey	Reddish white	Brownish white	Moderate grey
	SM	Reddish brown	Yellowish brown	Brown	Brownish white	Grayish white
	SP	None	None	None	None	None
ISP2	G	Good	Abundant	Abundant	Moderate	Abundant
	AM	Reddish white	Grayish white	Brownish white	Yellowish white	Grayish white
	SM	Brownish white	Maroon brown	Brown	Yellowish white	Reddish brown
	SP	None	None	Yellowish brown	None	None
ISP3	G	Abundant	Abundant	Abundant	Abundant	Abundant
	AM	Dark gray	Moderate grey	Yellowish white	Yellowish green	Grayish white
	SM	Reddish brown	Maroon brown	Brownish white	Brownish white	Brown
	SP	None	None	Yellowish brown	None	None
ISP4	G	Moderate	Abundant	Weak	Weak	Abundant
	AM	Yellowish white	Grayish white	Yellowish white	Off white	Grayish white
	SM	Reddish white	Maroon brown	Yellowish brown	Yellowish white	Brown
	SP	None	None	None	None	None
ISP5	G	Moderate	Abundant	Moderate	Moderate	Abundant
	AM	Yellowish white	Moderate grey	Yellowish white	Greenish white	Grayish white
	SM	Yellowish white	Marron brown	Brownish white	Brownish white	Brownish white
	SP	None	None	None	None	None

†G, Growth feature (Abundant, Moderate, Weak); AM, Aerial mycelium color; SM; Substrate mycelium color; SP, Soluble pigment color. Growth characteristics assessed on ISP media (ISP1 to ISP5 as described by Shirling and Gottlieb (1966).

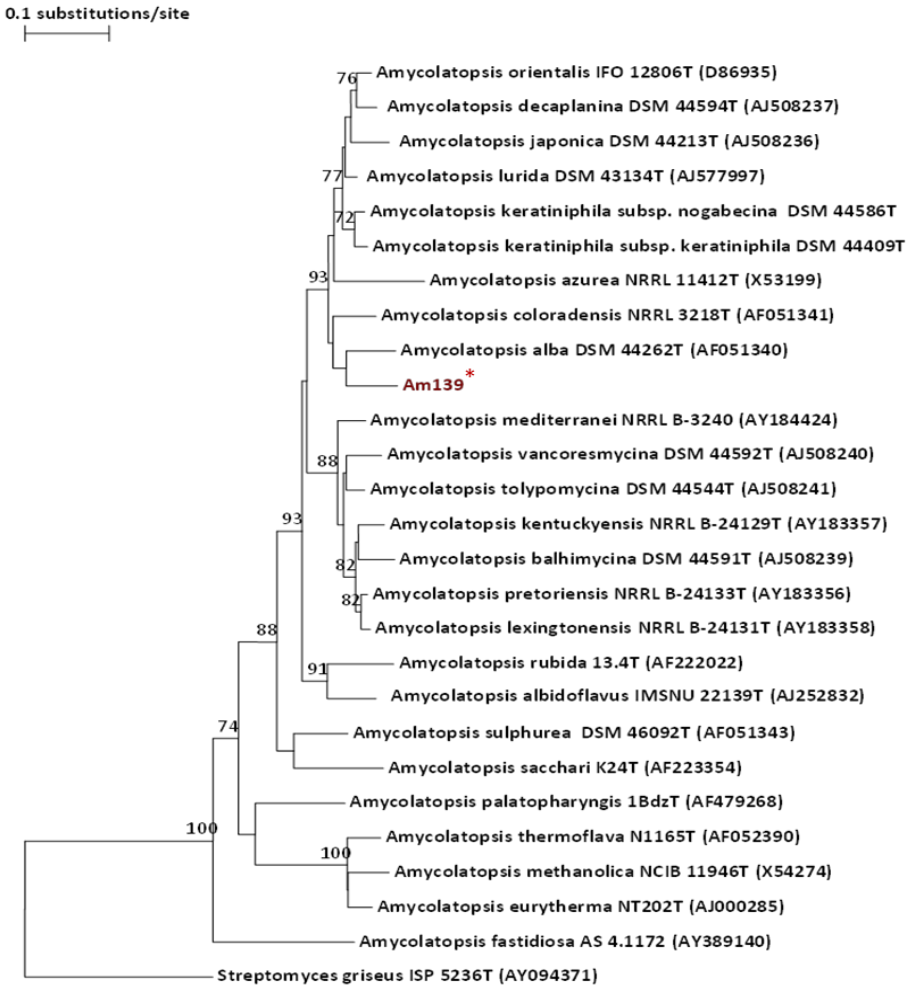

Figure (4): A neighbor joining tree showing the relationships between strain Am 139 and its closest relatives of the genus *Amycolatopsis* based on 16S rRNA gene sequence.

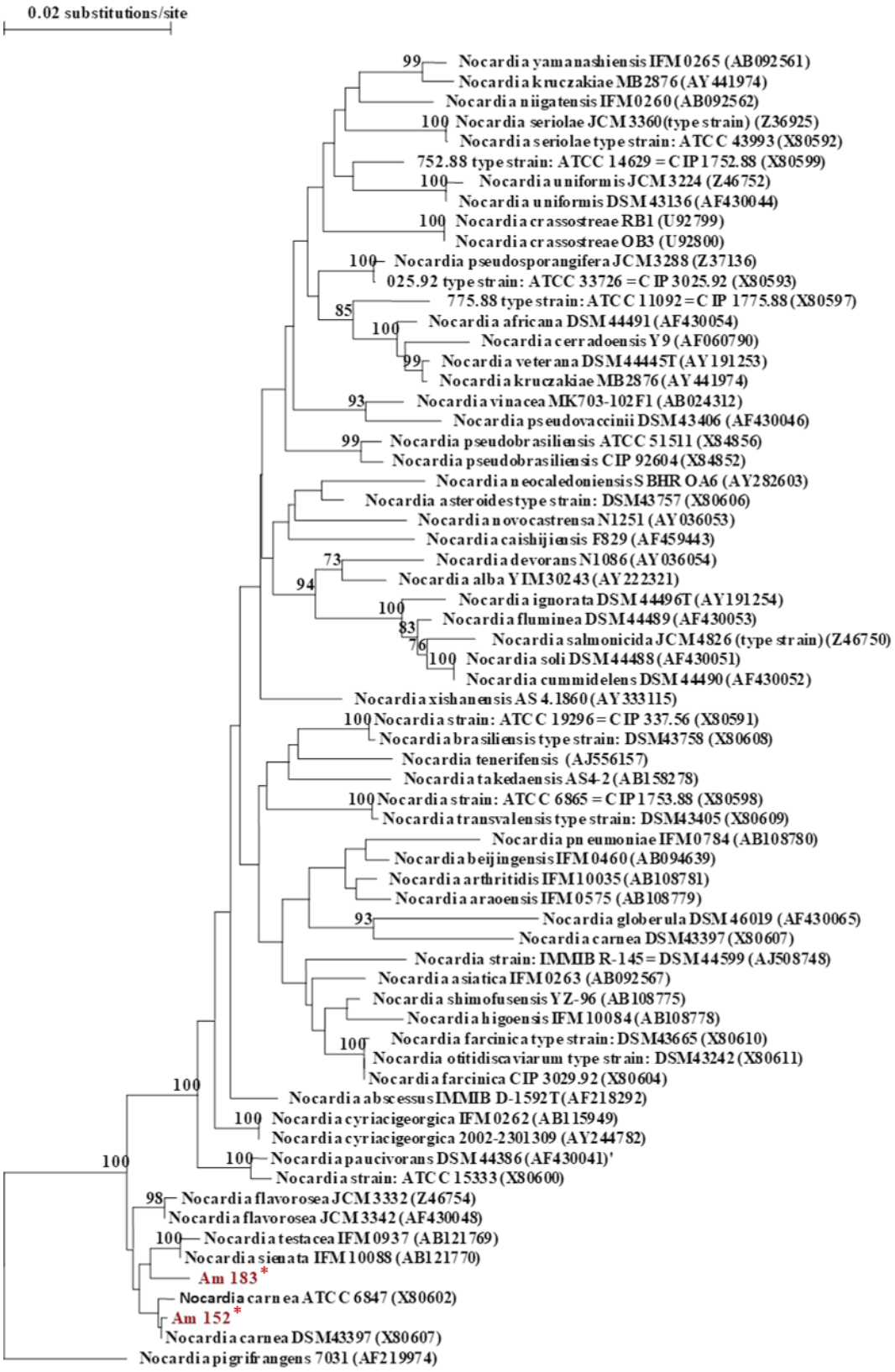

Figure (5): A neighbor joining tree showing the relationships between strains Am 152 and Am 183 with their closest relatives of genus Nocardia based on 16S rRNA gene sequences.

In contrast, strain Am 183 was found to associate with *Nocardia sienata* and *Nocardia testacea*, with a sequence similarity value of 98.2% to *Nocardia sienata*. This notable degree of genetic similarity suggests that strain Am 183 is closely related to *Nocardia sienata*, yet it also indicates that there are distinct differences that may deserve further investigation. The phylogenetic positioning of Am 183 raises the possibility that it could represent a unique strain within the *Nocardia* genus, potentially characterized by specific metabolic pathways or bioactive compound production that distinguishes it from its closely related counterparts.

Generally, the phylogenetic analysis not only confirms the placement of these actinobacteria isolates within the *Nocardia* genus but also emphasizes the importance of molecular techniques in resolving taxonomic relationships and exploring the evolutionary significance of these strains. The findings pave the way for further studies aimed at investigating the biochemical properties and potential biotechnological applications of these promising actinobacteria isolates.

DISCUSSION

The discovery of new actinobacteria is increasingly important for sourcing commercially significant bioactive compounds, particularly from extremophilic environments. This study aligns with previous reports on the antimicrobial properties of actinobacteria isolated from similar extreme habitats, notably in Saudi Arabia, where studies by Al-Dhabi *et al*. (2016) and Atta et al. (2010) documented significant antimicrobial potential. Similarly, Toukam *et al*. (2019) reported the antimicrobial activity of actinobacteria isolated for the first time from oil-contaminated sites in Cameroon. Meanwhile, Nithya *et al*. (2015) also reported that only 16 out of 134 actinobacterial isolates from Saudi Arabian desert soils exhibited antibacterial activity, reinforcing the need for exploration in unique environments like the cave system of Beni-Suef, Egypt. Our findings demonstrate the antimicrobial activities of actinobacteria extracts against test organisms such as *Salmonella typhi*, *Bacillus subtilis*, and *Candida albicans*, as illustrated in Figure (3). These visual representations substantiate the inhibitory effects detailed in Table (5), showing the extracts' efficacy as potential antimicrobial agents.

The study revealed that extracts from strains Am 139 and Am 152 have significant cytotoxic effects on various cancer cell lines, including MCF7, HepG2, and JURKAT. The broad-spectrum anticancer activity of Am 139, alongside Am 152's selective efficacy against MCF7, emphasizes the need to further investigate their mechanisms of action and therapeutic applications. While promising, additional research is required to explore their potential side effects and efficacy in vivo, paving the way for novel anticancer therapies derived from these actinobacteria. Identification of six distinct genera among the 16 biologically active actinobacteria strains highlights the remarkable diversity within the cave soil ecosystem surrounding the Meidum Pyramid. The ecological conditions of this area provide a suitable habitat for various actinobacteria species that have adapted to its unique environmental factors. Morphological characteristics, particularly spore arrangement and surface ornamentation, are crucial for taxonomic classification. For instance, *Streptomyces* species are recognized for their spiral spores and filamentous growth, while *Nocardiopsis* displays distinctive branched hyphae and spore chains.

These findings verify previous research by Hozzein and Goodfellow (2008); Hozzein *et al.* (2011); Nithya *et al.* (2015) which identified *Nocardiopsis* and *Streptomyces* as frequent isolates from various soil habitats with potent antimicrobial activities. The diverse actinobacteria genera identified not only reflect the ecological complexity of the environment but also highlight their potential as sources of bioactive compounds, including antibiotics and other pharmacologically relevant metabolites. This study ultimately reinforces the critical role of environmental exploration in discovering novel actinobacteria with therapeutic potential.

Further investigation into the biochemical and genetic characteristics of actinobacteria strains from the Beni-Suef cave soil is essential for exploring their potential to produce novel bioactive compounds. Understanding the metabolic capabilities and biosynthetic pathways of these microorganisms could lead to the discovery of new antimicrobial agents, emphasizing the critical role of environmental biodiversity in sourcing therapeutic resources. Additionally, examining the ecological interactions among these strains could provide valuable insights into their roles within microbial communities, enhancing our understanding of soil microbiology and its implications for health and disease management. The cultural characteristics of the five most active strains Am 183, Am 152, Am 139, Am 90, and Am 43 reveal significant insights into their ecological adaptations and metabolic diversity. The observed growth patterns, categorized as abundant, moderate, and weak, suggest varying capacities for environmental adaptation, which is crucial for survival in the extreme conditions of desert ecosystems, where nutrient availability and environmental factors fluctuate significantly.

Variations in colony color, ranging from grayish white to reddish brown, indicate the potential of actinobacteria to produce diverse secondary metabolites, including pigments (Kazi *et a*l., 2021; Wink *et al*., 2017). These pigments serve ecological functions such as protecting against UV radiation, acting as antioxidants, and contributing to antimicrobial properties (Agarwal et al., 2023). Pigmentation may also attract beneficial microorganisms or deter predators, enhancing survival (Chatragadda and Dufossé, 2021). Differences in growth and coloration across various ISP media highlight the influence of nutrient composition on the morphology and metabolic expression of Actinobacteria (Bahamdain, 2020). Each medium provides distinct nutrient profiles that can

promote or inhibit specific growth characteristics and metabolite production, allowing for the optimization of culture conditions to enhance the yield of bioactive compounds. The correlation between cultural characteristics and bioactivity is significant (Kazi et al., 2021); the abundance and pigmentation of strains may relate to their production of antimicrobial and anticancer agents, necessitating further analysis to establish these connections. Investigating the biosynthetic pathways responsible for metabolite production could lead to the identification of novel compounds with therapeutic potential.

To fully understand the capabilities of these desert actinobacteria, additional research should include metabolic profiling, molecular characterization, and bioactivity assays (Xie and Pathom-Aree, 2021). Techniques such as high-performance liquid chromatography (HPLC) and mass spectrometry (MS) can isolate and identify specific bioactive metabolites produced by these strains, while genetic studies can elucidate the biosynthetic genes involved (Mukherjee *et al.*, 2024).

Given the rising antibiotic resistance and the need for effective anticancer drugs, the exploration of microbial diversity in under-researched environments like desert ecosystems is of paramount importance. By leveraging the unique biosynthetic capability of these actinobacteria, there exists a significant opportunity to discover novel bioactive compounds that could address critical health challenges and contribute to advancements in pharmaceuticals and agriculture.

The analysis of diaminopimelic acid (DAP) was pivotal in the taxonomic classification of actinobacteria isolates from the Beni-Suef cave system. The presence of LL-diaminopimelic acid in strain Am 43 confirmed its affiliation with the genus *Streptomyces*, supported by its morphological traits, such as branched mycelia and long spore chains. This aligns with existing literature that emphasizes these features as reliable taxonomic indicators (Kämpfer, 2012; Williams and Cross, 1971).

Conversely, isolates Am 90, Am 139, Am 152, and Am 183 were associated with different genera based on the detection of meso-diaminopimelic acid. Strain Am 90 exhibited characteristics typical of the genus *Nocardiopsis*, while strain Am 139 was classified under *Amycolatopsis* due to its unique sugar composition and lipid profile. In addition, isolate Am 152 and Am 183 were linked to the genus *Nocardia*, based on their morphological characteristics and polar lipid presence. Previous studies have documented the prevalence of *Nocardiopsis* in desert environments, confirming that strains from such habitats often exhibit similar biochemical and morphological characteristics (Hozzein *et al.*, 2004; Zitouni *et al.*, 2005).

Phylogenetic analysis of the 16S rRNA gene sequences for strains Am 139, Am 152, and Am 183 revealed significant insights into their taxonomic affiliations. Strains Am 152 and Am 183 were confirmed as *Nocardia*, with Am 152 closely related to *Nocardia carnea* at 99.8% similarity, indicating potential similarities in biochemical pathways and ecological roles. Strain Am 183 showed a close relationship with *Nocardia sienata*, suggesting it may represent a new species, warranting further investigation into its unique characteristics.

CONCLUSION

Based on the results mentioned above, the following deductions can be addressed as follows: The study evaluated antimicrobial activities of metabolic extracts from actinobacteria strains isolated from the cave of Beni-Suef, Egypt. Sixteen out of 37 extracts exhibited significant biological activity against representative pathogenic microorganisms. Gram-positive bacteria were more strongly inhibited compared to yeast and Gram-negative bacteria. Actinobacteria strains from the cave system showed promise, with 43.2% exhibiting antimicrobial activities against tested pathogens. Five extracts (Am 43, Am 90, Am 139, Am 152, and Am 183) displayed potential antibacterial properties, emphasizing the importance of extremophilic actinobacteria in bioactive compound discovery. MTT test was used to assess anticancer properties against the MCF-7 cancer cell line. Only extracts from Am 139 and Am 152 showed substantial cytotoxic effects, leading to further evaluation against multiple cancer cell lines (MCF7, HepG2, and JURKAT). Extracts of Am 139 and Am 152 demonstrated significant anticancer activity, particularly against MCF7 and JURKAT cells. These findings suggest the potential of these extracts as viable anti-cancer medications. Morphological and chemotaxonomic analyses identified the most potent strains, tentatively assigned to six genera: *Streptomyces*, *Nocardiopsis*, *Amycolatopsis*, *Nocardia*, *Actinomadura*, and *Microbispora*. Phylogenetic analysis of the 16S rRNA gene sequences confirmed membership of Am 152 and Am 183 in the genus *Nocardia*, with Am 183 possibly representing a new species within the genus. Further research is necessary to isolate, characterize, and evaluate these compounds for pharmaceutical development to address challenges in antimicrobial resistance and cancer therapy. Future research on the promising actinobacteria extracts identified in this study should focus on several key areas to enhance their therapeutic potential. Detailed genomic and proteomic analyses are essential for elucidating the biosynthetic pathways of the active compounds, while metabolomic profiling can aid in identifying novel metabolites. *In vivo* efficacy testing in animal models, along with dose-response and combination therapy studies, will provide insights into the pharmacokinetics, pharmacodynamics, and potential toxicity of the extracts. Investigating the cellular and molecular mechanisms of action, as well as resistance mechanisms, will enhance our understanding of their anticancer and antimicrobial properties. Developing advanced formulation and delivery systems, such as nanotechnology-based and controlled-release formulations, can improve the bioavailability

and targeted delivery of the active compounds. Structure-activity relationship (SAR) studies and chemical modification efforts will help optimize the potency and stability of these compounds. Additionally, screening actinobacteria strains from diverse and extreme environments using high-throughput methods can expand the repertoire of potentially useful bioactive compounds. Pursuing these research directions will significantly advance the development of actinobacteria extracts as effective therapeutic agents.

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مضادات الميكروبات والسرطان المحتملة للبكتيريا الأكتينية الجديدة المعزولة من كهوف بني سويف، مصر

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[1] قسم الميكروبيولوجيا الصيدلية والمناعة، كلية الصيدلة، جامعة بني سويف، بني سويف، مصر.

[2] قسم الأحياء، كلية العلوم، جامعة الأميرة نورة بنت عبدالرحمن، ص.ب. ص.ب 84428، الرياض 11671، المملكة العربية السعودية

[3] قسم النبات والأحياء الدقيقة، كلية العلوم، جامعة بني سويف، بني سويف، مصر

الملخص العربي

في السنوات الأخيرة، ظهرت البكتيريا الأكتينية كمرشح واعد لعلاج السرطان، وحظيت باهتمام كبير في البحوث الطبية الحيوية. تركز هذه الدراسة على عزل ووصف البكتيريا الأكتينية من أنظمة الكهوف المتنوعة داخل محافظة بني سويف في مصر. هدفت هذه الدراسة الي تقييم النشاط البيولوجي، وخاصة الخصائص المضادة للبكتيريا ومضادات السرطان، للمركبات النشطة بيولوجيًا التي تنتجها هذه البكتيريا الأكتينية. من خلال الوسائط والتقنيات الانتقائية، تم عزل 112 سلالة من عينات تربة الكهوف، ثم تنقيتها لمزيد من التحليل. كشف تقييم المستقلبات الثانوية المستخرجة من سلالات ممثلة عن نشاط مضاد للميكروبات كبير ضد البكتيريا المسببة للأمراض المختلفة والخميرة والفطريات، مع تفضيل ملحوظ للبكتيريا إيجابية الجرام. بالإضافة إلى ذلك، أظهرت خمسة مستخلصات من البكتيريا الأكتينية خصائص مضادة للبكتيريا، في حين أظهرت مستخلصات من سلالتين، Am 139 وAm 152، تأثيرات سامة للخلايا بشكل كبير على العديد من سلالات الخلايا السرطانية. وأكدت تجارب أخرى إمكاناتها الواعدة في مكافحة السرطان ضد سلالات الخلايا MCF7 و HepG2 و JURKATعبر تركيزات مختلفة. وتؤكد نتائجنا على الإمكانات غير المستغلة لمستقلبات البكتيريا الأكتينية المشتقة من كهوف بني سويف، مما يلقي الضوء على قدراتها الجديدة المضادة للميكروبات ومضادة للسرطان في البحث الطبي الحيوي. وعلاوة على ذلك، يشير عزل البكتيريا الأكتينية من مواقع لم يتم استكشافها من قبل مثل الكهوف إلى مصدر غني للمركبات النشطة بيولوجيًا ذات الأنشطة المضادة للميكروبات ومضادة للسرطان، مما يعد بآفاق جديدة لاكتشاف الأدوية وتطويرها.