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2. **Mokhnache, M.**, Belhadj, H., Alien Mohamed Bachir, A., Douadi, N., Harzallah, D. Physicochemical, phytochemical, and microbiological analyses of some Algerian bee-collected pollen samples. *Acta Periodica Technologica*. **56**: 1-13. https://doi.org/10.2298/APT250403015M

Communications

1. **Mokhnache, M.**, Belhadj, H. Evaluation Of Antibacterial Activity Of Silver Nanoparticles Green-synthesized Using Algerian Bee Pollen Extract. 2nd Online International Conference on Applied Microbiology and Beneficial Microbes. August 26 - 27, 2021. Coalesce Research Group, USA. (Oral communication).

2. **Mokhnache, M.**, Belhadj, H., Alien Mohamed Bachir, A., Harzallah, D. Biological activities of pigment produced by Actinomycetes associated with bee pollen. National Seminar on Phytotherapy and Pharmacognosy (NSPP-2023). March 14-15, 2023. Setif, Algeria. (Oral communication).

3. **Mokhnache, M.**, Belhadj, H., Alien Mohamed Bachir, A., Harzallah, D. Actinomycetes Isolated from Algerian Bee pollen As a Source of Antimicrobial Compounds: Preliminary Study. The First International Congress on Food and Environmental Security (FES-2023). May 15-17, 2023. Bordj Bou Arreridj, Algeria. (Oral communication).

4. **Mokhnache, M.**, Belhadj, H., Koca, F. D., Nasrat, A., Basma, A. Antimicrobial activity of silver nanoparticles (AgNPs) green synthesized using a pigment of Actinobacteria isolated from bee pollen. The Second International Conference on Materials, Energy and Environment (MEE'2023). October 23-24, 2023. El-oued, Algeria. (Poster).

5. **Mokhnache, M.**, Belhadj, H., Alien Mohamed Bachir, A., Harzallah, D. Microbiological analysis of Algerian bee bread: a preliminary study. 2nd National Seminar on Therapeutic and Agro-Food Applications of Natural Substances (ATASN, 2023). November 11, 2023. Mila, Algeria. (Poster).

6. **Mokhnache, M.**, Belhadj, H., Alien Mohamed Bachir, A., Douadi, N., Harzallah, D. Microbiological quality control of bee products: Bee pollen. The 1st International Seminar on Ecology and Biotechnology in Mediterranean Atmosphere Climate (ISEBMCE-2023). November 14-16, 2023. Setif, Algeria. (Poster).

7. **<u>Mokhnache, M.</u>**, Belhadj, H., Alien Mohamed Bachir, A., Harzallah, D. Actinomycetes isolated from local bee pollen as a source of enzymes: a qualitative study. The 1st International Seminar on Ecology and Biotechnology in Mediterranean Atmosphere Climate (ISEBMCE-2023). November 14-16, 2023. Setif, Algeria. (Poster).

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الملخص

تهدف هذه الدراسة إلى عزل بكتيريا الأكتينوميسات من بعض عينات طلع النحل، والتي تم جمعها من مناطق مختلفة من الجزائر، مع تقييم تطبيقاتها وأنشطتها المختلفة. أولاً، تمت مراقبة الجودة الميكروبيولوجية لعينات طلع النحل، حيث أظهرت التحاليل أن الحمل الميكروبي تراوح بين 7.70×10^2 و 1.93×10^6 و م م/غ للفلورا الكلية الهوائية، و بين 1.10×10^3 و 5.30×10^5 و م م/غ للفلورا الكلية اللاهوائية. كما تراوح محتوى الأعفان والخمائر بين 2.70×10^3 و 3.70×10^3 و م م/غ. قدرت الحمولة القصوى لبكتيريا القولون والمكورات العنقودية الذهبية بـ 1.73×10^4 و م م /غ و2.33×10^2 و م م/غ على التوالي. كما تم العثور على sulfite-reducing *Clostridium* في عينة واحدة فقط، بينما لم يتم العثور على *Salmonella* sp. في كل العينات. علاوة على ذلك، تم عزل 33 سلالة من الأكتينوميسات حيث تم توصيفها وفحصها لإنتاج إنزيمات خارج خلوية وكذلك للنشاط المضاد للميكروبات، ثم تم تصنيفها بطريقة UPGMA. أظهرت السلالات المعزولة ثمانية مجاميع متنوعة، حيث كان معظمها قادرًا على إنتاج إنزيمات خارج خلوية، بما في ذلك البروتياز، الأميلاز والليباز، بينما أظهرت خمس سلالات فقط نشاطية ضد ميكروبية. تم اختيار مستخلص أسيتات الإيثيل الخام للسلالة *Streptomyces* sp. BPA-6 والتي أظهرت نشاطية ضد ميكروبية معتبرة في الفحص الأولي لتقييم قدرتها على قتل أو تثبيط نمو وتكاثر بعض مسببات الأمراض الميكروبية، وكذلك تحليل وتحديد مركباتها النشطة. أظهرت طريقة الآبار أن المستخلص كان له فعالية فقط ضد *S. aureus* ATCC 6538P، و*B. subtilis* ATCC 6633، و*C. albicans* ATCC 10231، مع مناطق تثبيط قدرت بـ 42.33 ± 3.79 ملم، و41.66 ± 3.51 ملم، و48.67 ± 2.08 ملم، على التوالي. كانت قيمة MIC المسجلة هي 62.5 مكغ/مل ضد *S. aureus* ATCC 6538P و*B. subtilis* ATCC 6633، بينما كانت 250 مكغ/مل ضد *C. albicans* ATCC 10231. كما لوحظت أفضل قيمة MMC ضد *B. subtilis* ATCC 6633، بقيمة 1000 مكغ/مل. كشف تحليل GC-MS عن العديد من المركبات المضادة للميكروبات، بما في ذلك hexadecanoic acid، 9, 12-octadecadienoic acid و 9-octadecenoic acid. من جهة أخرى، تم استخدام الصبغة الحمراء للسلالة *Streptomyces* sp. A23 للتصنيع الأخضر لجزيئات الفضة النانوية (AgNPs)، وتقييم أنشطتها الضد ميكروبية وضد سرطانية ووقاية الخلايا العصبية، حيث لوحظ نشاطا مضادا للميكروبات جيدا ضد *E. faecalis* ATCC 19433 و *C. albicans* ATCC 10231 و *S. aureus* ATCC 6538P و *P. aeruginosa* ATCC 27853 و *B. subtilis* ATCC 6633 و *K. pneumoniae* ATCC 13883 و *E. coli* ATCC 7839، مع مناطق تثبيط 32 ملم و30 ملم و30 ملم و27 ملم و25 ملم و20 ملم و19 ملم على التوالي. كما تم تسجيل أقل قيم لـ MIC و MBC ضد *B. subtilis* ATCC 6633 بقيمة 62.5 مكغ/مل. ومن المثير للاهتمام أن معظم جزيئات الفضة النانوية المصنعة والمحضرة بتراكيز 2 مكغ/مل و4 مكغ/مل و8 مكغ/مل كان لها تأثيرات معتبرة على خلايا الورم العصبي SH-SY5Y. بالإضافة إلى ذلك، أظهرت جزيئات الفضة النانوية (1 ملي مول، درجة حموضة 7) نشاطًا وقائيًا للخلايا العصبية عند أقل تركيز تم اختباره.

الكلمات المفتاحية: بكتيريا الأكتينوميسات، طلع النحل، الجودة الميكروبيولوجية، *Streptomyces*، الصبغات الميكروبية، جزيئات الفضة النانوية، الإنزيمات خارج خلوية، النشاطية ضد ميكروبية.

Abstract

This study aimed to isolate actinomycetes from some bee pollen samples collected from different Algerian places and evaluate their applications. First, the microbiological quality control of bee pollen samples was investigated, where the analyses showed a microbial load ranged from 7.70 x 10^2 to 1.93 x 10^6 CFU/g for the aerobic mesophilic bacteria and from 1.10 x 10^3 to 5.30 x 10^5 CFU/g for the anaerobic mesophilic bacteria. Molds and yeasts content varied from 2.70 x 10^3 to 3.70 x 10^3 CFU/g. The maximum concentrations for total coliforms and *S. aureus* were 1.73 x 10^4 CFU/g and 2.33 x 10^2 CFU/g, respectively. Sulfite-reducing *Clostridium* was detected only in one sample. In addition, *Salmonella* sp. was not detected in all samples. Furthermore, 33 strains of actinomycetes were isolated, characterized, screened to produce extracellular enzymes as well as for antimicrobial activity, and then classified by the UPGMA method. The isolated strains showed eight diversified clusters, where most of them were able to produce extracellular hydrolytic enzymes, including proteases, amylases, and lipases, while only five strains demonstrated significant antimicrobial activity. The crude ethyl acetate extract of the *Streptomyces* sp. BPA-6 strain was selected to assess its ability to kill or inhibit the growth and reproduction of some pathogens and to analyze and identify its bioactive compounds. The agar-well diffusion method showed that it was only effective against *Staphylococcus aureus* ATCC 6538P, *Bacillus subtilis* ATCC 6633, and *Candida albicans* ATCC 10231, with inhibition zones of 42.33 ± 3.79 mm, 41.66 ± 3.51 mm, and 48.67 ± 2.08 mm, respectively. The recorded MIC value was 62.5 µg/mL against *S. aureus* ATCC 6538P and *B. subtilis* ATCC 6633, while it was 250 µg/mL against *C. albicans* ATCC 10231. Also, the best MMC value was observed against *B. subtilis* ATCC 6633, with a value of 1000 µg/mL. The GC-MS analyses revealed many antimicrobial compounds, including hexadecanoic acid, 9,12-octadecadienoic acid, and 9-octadecenoic acid. On the other hand, the red pigment of the *Streptomyces* sp. A23 strain was used for the green synthesis of silver nanoparticles (AgNPs), evaluating their antimicrobial, anticancer, and neuroprotective activities. Good antimicrobial activity was observed against *Enterococcus faecalis* ATCC 19433, *C. albicans* ATCC 10231, *S. aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 27853, *B. subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 13883, and *Escherichia coli* ATCC 7839, with inhibition zones of 32 mm, 30 mm, 30 mm, 27 mm, 25 mm, 20 mm, and 19 mm, respectively. The lowest MIC and MBC were recorded against *B. subtilis* ATCC 6633 with a value of 62.5 µg/mL. Intriguingly, the most of synthesized AgNPs at concentrations of 2 µg/mL, 4 µg/mL, and 8 µg/mL had cytotoxic effects on SH-SY5Y neuroblastoma cell lines. In addition, AgNPs (1 mM, pH 7) exhibited significant neuroprotective activity at the lowest tested concentration.

Keywords: Actinomycetes, bee pollen, microbiological quality, *Streptomyces*, microbial pigments, silver nanoparticles, extracellular enzymes, antimicrobial activity.

Résumé

Cette étude avait pour objectif d'isoler les actinomycètes à partir de quelques échantillons de pollen d'abeille collectés dans différentes localités algériennes et d'évaluer leurs applications. Dans un premier temps, le contrôle de la qualité microbiologique des échantillons de pollen d'abeille a été effectué, où les analyses ont une charge microbienne allant de 7,70 x 10^2 à 1,93 x 10^6 UFC/g pour les bactéries mésophiles aérobies et de 1,10 x 10^3 à 5,30 x 10^5 UFC/g pour les bactéries mésophiles anaérobies. La teneur en moisissures et en levures variait de 2,70 x 10^3 à 3,70 x 10^3 UFC/g. Les concentrations maximales pour les coliformes totaux et les *Staphylococcus aureus* étaient respectivement de 1,73 x 10^4 UFC/g et 2,33 x 10^2 UFC/g. Les *Clostridium* sulfito-réducteurs n'ont été détectés que dans un seul échantillon, et les *Salmonella* sp. étaient absentes dans tous les échantillons. De plus, 33 souches d'actinomycètes ont été isolées, caractérisées, et criblées pour la production d'enzymes extracellulaires ainsi que pour une activité antimicrobienne, puis classées par la méthode UPGMA. Les souches isolées ont montré huit groupes diversifiés, où la plupart d'entre elles étaient capables de produire des enzymes hydrolytiques extracellulaires, y compris des protéases, des amylases et des lipases, tandis que seulement cinq souches ont démontré une activité antimicrobienne significative. L'extrait brut d'acétate d'éthyle de la souche *Streptomyces* sp. BPA-6 a été sélectionné pour évaluer sa capacité à tuer ou à inhiber la croissance et la reproduction de certains agents pathogènes et pour analyser et identifier ses composés bioactifs. La méthode de diffusion en puits d'agar, a montré qu'elle n'était efficace que contre *Staphylococcus aureus* ATCC 6538P, *Bacillus subtilis* ATCC 6633 et *Candida albicans* ATCC 10231, avec des zones d'inhibition de 42,33 ± 3,79, 41,66 ± 3,51 et 48,67 ± 2,08 mm, respectivement. Les valeurs des CMI enregistrées étaient de 62,5 µg/mL contre *S. aureus* ATCC 6538P et *B. subtilis* ATCC 6633, tandis qu'elle était de 250 µg/mL contre *C. albicans* ATCC 10231. Aussi, la meilleure valeur de CMM a été observée contre *B. subtilis* ATCC 6633, avec une valeur de 1000 µg/mL. Les analyses GC-MS ont révélé de nombreux composés antimicrobiens, dont l'acide hexadécanoïque, l'acide 9,12-octadécadiénoïque et l'acide 9-octadécénoïque. D'autre part, le pigment rouge de la souche *Streptomyces* sp. A23 a été utilisé pour la synthèse verte des nanoparticules d'argent (AgNPs), évaluant leurs activités antimicrobiennes, anticancéreuses et neuroprotectrices. Une bonne activité antimicrobienne a été observée contre *Enterococcus faecalis* ATCC 19433, *C. albicans* ATCC 10231, *S. aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 27853, *B. subtilis* ATCC 6633, *Klebsiela pneumoniae* ATCC 13883 et *Escherichia coli* ATCC 7839, avec des zones d'inhibition de 32, 30, 30, 27, 25, 20 et 19 mm, respectivement. Les valeurs les plus faibles de CMI et de CMB ont été enregistrées contre *B. subtilis* ATCC 6633 avec une valeur de 62,5 µg/mL. De manière intrigante, la plupart des nanoparticles d'argent synthétisés à des concentrations de 2, 4 et 8 µg/mL ont eu des effets cytotoxiques sur les lignées cellulaires de neuroblastome (SH-SY5Y). De plus, les AgNPs (1 mM, pH 7) ont montré une activité neuroprotectrice significative à la concentration la plus faible testée.

Mots clés : Actinomycètes, pollen d'abeille, qualité microbiologique, *Streptomyces*, pigments microbiens, nanoparticules d'argent, enzymes extracellulaires, activité antimicrobienne.

List of Figures

List of Tables

List of Abbreviations

AIA : Actinomycete Isolation Agar

Ag^0 : Elemental silver

$AgNO_3$: Silver nitrate

AgNPs : Silver nanoparticles

ANOVA : Analysis of variance

ATCC : American Type Culture Collection

AuNPs : Gold nanoparticles

BLAST : Basic Local Alignment Search Tool

BPA : Baird-Parker Agar

BPS : Bee pollen sample

CZA : Czapek Agar

$CaCO_3$: Calcium carbonate

CFU : Colony-forming unit

CuONPs : Copper oxide nanoparticles

DLS : Dynamic light scattering analysis

DMSO : Dimethyl sulfoxide

DNA : Deoxyribonucleic acid

DPPH : 2,2-Diphenyl-1-picrylhydrazyl

EDX : Energy dispersive X-Ray analysis

FI-SEM : Field Emission Scanning Electron Microscopy

FT-IR : Fourier transform infrared spectroscopy

GAA : Glycerol asparagine agar

GC-MS : Gas chromatography-mass spectrometry

H_2O_2 : Hydrogen peroxide

HCN : Hydrogen Cyanide

HEA : Hektoen Enteric Agar

HPLC : High-Performance Liquid Chromatography

HPLC-MS : High-Performance Liquid Chromatography-Mass spectrometry

HPLC-UV-vis : High-Performance Liquid Chromatography- Ultraviolet–visible spectroscopy

IAA : Indole Acetic Acid

IC_{50} : Half maximal inhibitory concentration

ISP-2 : International Streptomyces Project-2 Medium

LC-MS : Liquid Chromatography-Mass spectrometry

LC-MS-MS : Liquid Chromatography-Mass spectrometry- Mass spectrometry

MBC : Minimum Bactericidal Concentration

MEGA : Molecular Evolutionary Genetics Analysis

MIC : Minimum Inhibitory Concentration

MMC : Minimum Microbicidal Concentration

MS : Mass spectrometry

MTT : 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide

NA : Nutrient Agar

NCBI : National Center for Biotechnology Information

nm : Nanometer unit

NPs : Nanoparticles

PCA : Plate Count Agar

PCR : Polymerase chain reaction

PGPR : Plant growth-promoting rhizobacteria

ROS : Reactive oxygen species

rpm : Revolutions per minute

rRNA 16S : 16S ribosomal ribonucleic acid

RVB : Rappaport-Vassiliadis Broth

SA : Streptomyces Agar

SCA : Starch Casein Agar

SEM : Scanning Electron Microscopy

SSA : Salmonella shigella Agar

STM : Scanning Tunneling Microscopy

TEM : Transmission Electron Microscopy

TTC : Triphenyl tetrazolium chloride

UPGMA : Unweighted Pair Group Method with Arithmetic mean

UPLC-MS : Ultra-Performance Liquid Chromatography-Mass spectrometry

UV-vis : Ultraviolet-visible spectrophotometry

VRBLA : Violet Red Bile Lactose Agar

XLDA : Xylose Lysine Deoxycholate Agar

XRD : X-Ray diffraction analysis

YMA : Yeast extract Malt extract Agar

ZnONPs : Zinc oxide nanoparticles

Content

General Introduction

General Introduction

Bee pollen, as one of the beehive products, known for its rich composition of carbohydrates, proteins, lipids, vitamins, and minerals, has gained attention as a natural source for the isolation of beneficial microorganisms (Pełka et al., 2021). In recent years, there has been a growing interest in exploring the potential of bee pollen for isolating actinomycetes as a promising source for the discovery of new bioactive compounds with pharmaceutical and biotechnological applications (Ecem Bayram et al., 2023).

Actinomycetes are gram-positive and filamentous bacteria that belong to the phylum Actinobacteria, which is one of the largest bacterial phyla within the Bacteria Domain (Barka et al., 2016). They are considered a remarkable group of microorganisms due to their ability to produce a variety of valuable bioactive secondary metabolites as natural products, including antibiotics, pigments, and enzymes, which are extremely important and potentially useful for diverse fields, including medical, pharmaceutical, agricultural, environmental, and industrial applications (Rai & Bai, 2022).

The discovery of novel actinomycetes strains producing unique bioactive compounds is essential for addressing the rising concerns of antibiotic resistance (De Simeis & Serra, 2021). According to Mast and Stegmann (2019), the known world's antibiotics come from actinomycetes, with *Streptomyces* species recognized as significant producers of a major proportion of these antibiotics.

Also, actinomycetes are well known as great producers of extracellular enzymes, such as proteases, lipases, cellulases, amylases, chitinases, L-asparaginase, and xylanases, which have an increased commercial value due to their many applications, especially in the pharmaceutical, food, and detergent industries (Selim et al., 2021).

On the other hand, the increasing interest in nanotechnology has led to the exploration of nanomaterials, such as silver nanoparticles (AgNPs), for their various biological activities (Xu et al., 2020). AgNPs are known for their antimicrobial properties, but recent studies have expanded their potential applications, including anticancer and neuroprotective activities (Khorrami et al., 2023; Rangavitala & Taranath, 2024). While conventional methods for synthesizing AgNPs often involve toxic chemicals and energy-intensive processes, green synthesis offers eco-friendly alternative approaches to nanoparticle production. One of these promising approaches for green synthesis involves the use of bioactive secondary metabolites produced by actinomycetes (Dhanasekaran & Jiang, 2016).

Therefore, to evaluate the potential applications of secondary metabolites produced from microorganisms as valuable sources of bioactive compounds, we have investigated the actinomycetes associated with some local bee pollen samples that were collected from different Algerian sites.

Our study is divided into two main sections, a literature review and an experimental part, as follows:

Section one: Literature review about actinomycetes and their applications.

Section two: Experimental part, which contains four chapters:

1. Physicochemical, phytochemical, and microbiological analyses of some Algerian bee pollen samples.

2. Isolation, characterization, and screening of actinomycetes from Algerian bee pollen for extracellular enzyme production and antimicrobial activity.

3. Molecular identification, antimicrobial activity, and GC-MS analyses of bioactive compounds produced by the *Streptomyces* sp. BPA-6 strain isolated from bee pollen collected from Algeria.

4. Evaluation of antimicrobial, anticancer, and neuroprotective activities of silver nanoparticles (AgNPs) green-synthesized using a red pigment produced by the *Streptomyces* sp. A23 strain isolated from Algerian bee pollen.

Literature Review

Actinomycetes and their applications

1. Biology and taxonomy of actinomycetes

1.1. General characteristics

Actinomycetes are aerobic, gram-positive, and filamentous bacteria that belong to the phylum Actinobacteria, which is one of the largest bacterial phyla within the Bacteria Domain. They are distinguished by their complex life cycle, and distinctive morphological features, including a high content of guanine and cytosine (G + C), which differentiates them from other bacteria (Shrestha et al., 2021; Zahr et al., 2022). The name "Actinomycetes" is derived from the Greek words Actini (ray) and mycetes (fungus), referring to their radiating, fungal-like morphology (Shrestha et al., 2021). They are widely distributed in both terrestrial and aquatic habitats, mainly in soil, where they play a crucial role in the decomposition of organic materials, dead plants, animals, and fungi (Sharma et al., 2014). Actinomycetes are also considered a remarkable group of microorganisms for their ability to produce a variety of valuable bioactive secondary metabolites, including antibiotics, pigments, and enzymes (Selim et al., 2021).

1.2. Morphology and life cycle

Actinomycetes display significant morphological varieties, particularly concerning the presence or absence of aerial and substrate mycelia, their pigmentation, the secretion of melanoid pigments, and the characteristics of their spores. The distinction between substrate and aerial mycelia is based on their functional and morphological differences. Substrate mycelium primarily serves in nutrient absorption, facilitating the growth of the organism, and can exhibit a wide range of colors. In contrast, aerial mycelium is produced by vegetative mycelium and is mainly involved in reproductive processes, with spores developing (Zahr et al., 2022). As presented in Figure 1, actinomycetes always initiate their life cycle with the formation of spores, which then undergo germination. This process results in the spreading of filaments, leading to the formation of vegetative mycelium in the solid medium. This vegetative mycelium develops sporophores, which then grow and extend vertically toward the surface to form aerial mycelium, leading to the formation of further aerial filaments. These filaments then divide to form sheaths, which contribute the formation of new spores, initiating a new cycle (Barka et al., 2016).

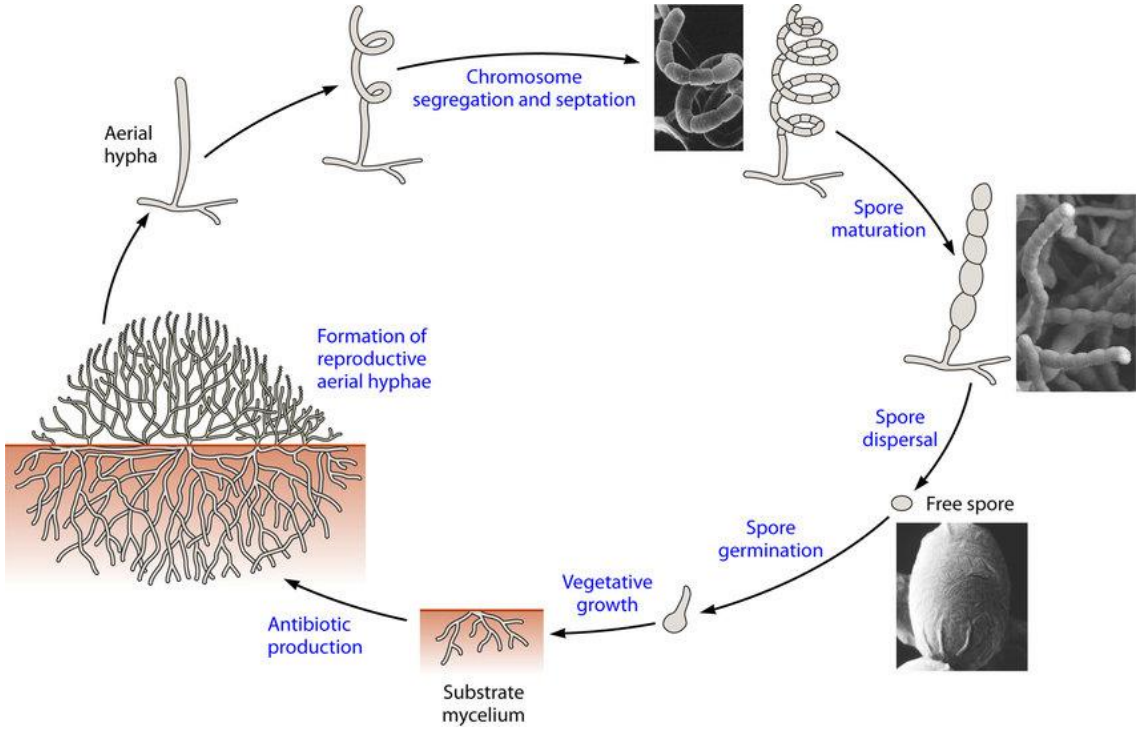


Figure 1. Life cycle of actinomycetes (Barka et al., 2016).

1. 3. Taxonomy and classification

The progress in the polyphasic approach has emerged as a crucial tool in the classification of actinomycetes, based on information obtained from the phenotypic and chemotaxonomic characteristics and also from the molecular methods, including the sequencing of the 16S rRNA gene (Meenakshi et al., 2024). Firstly, actinomycetes are identified and classified according to their morphological and cultural characteristics, including aerial and substrate mycelia, their pigmentation, the secretion of melanoid pigments, and the characteristics of their spores. Subsequently, the physiological and biochemical characteristics will be determined (Sharma et al., 2014; Shirling & Gottlieb, 1966). Actinomycetes are now primarily classified based on nucleotide sequence analysis, with 16S rRNA genes being the ideal target sequence. This analysis provides a phylogenetic tree that facilitates the investigation of their evolution (Sharma et al., 2014). Currently, the most recent data about the taxonomic classification of the actinomycetes are present in Bergey's Manual of Systematic Bacteriology (2nd Edition, Volume 5), which categorizes actinomycetes as a group of bacteria within the phylum Actinobacteria, divided into six classes, namely Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria, and Thermoleophilia. The first class, Actinobacteria, was further divided into sixteen orders that were Actinomycetales, Actinopolysporales, Bifidobacteriales, Catenulisporales,

Corynebacteriales, Frankiales, Glycomycetales, Jiangellales, Kineosporiales, Micrococcales, Micromonosporales, Propionibacteriales, Pseudonocardiales, Streptomycetales, Streptosporangiales, and Incertae sedis (Goodfellow et al., 2012). Most of the well-known genera of actinomycetes are *Streptomyces*, *Nocardia*, *Micromonospora*, *Actinoplanes*, and *Streptosporangium* (Dhanasekaran & Jiang, 2016).

2. Ecology and habitats of actinomycetes

According to Meenakshi et al., (2024), the distribution of actinomycetes is influenced by several ecological factors such as pH, temperature, moisture, aeration, salinity, and organic matter content. Actinomycetes are an ecologically significant group of bacteria that are primarily found in soil and other terrestrial habitats, but they are also widespread in aquatic environments, extreme habitats, and as part of the microbiota in plants and animals (Figure .2) (Meenakshi et al., 2024; Van Der Meij et al., 2017).

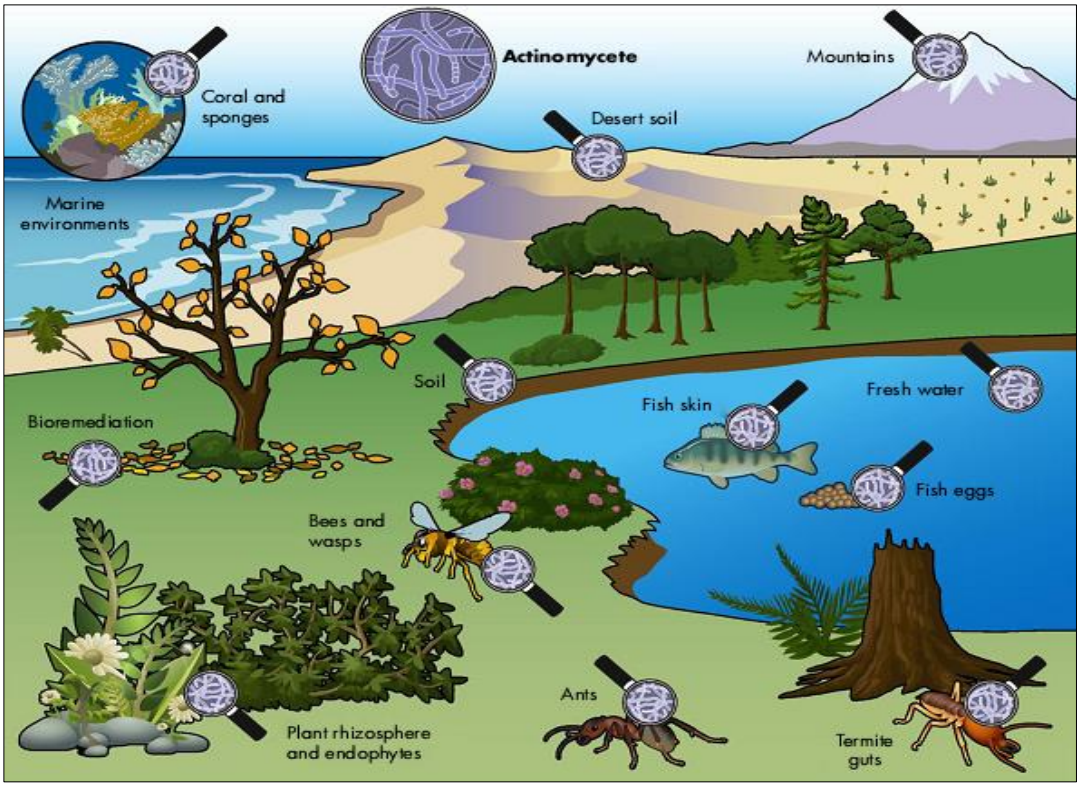


Figure 2. Habitats and distribution of actinomycetes (Van Der Meij et al., 2017).

2.1. Actinomycetes from terrestrial habitats

Terrestrial habitats, particularly soil, serve as the principal reservoir for actinomycetes, playing an important role in organic matter degradation, nutrient cycling, symbiotic relationships, and the production of bioactive compounds (Barka et al., 2016; Bhatti et al.,

2017). They are also found as constituents of humus, dung, litter, and surfaces of rocks. They have also been related to the rhizospheric soil associated with plant roots, which harbors different populations of actinomycetes, particularly *Streptomyces*, *Nocardia*, and *Micromonospora*, which are considered valuable and significant sources of antimicrobials, antitumors, pigments, and extracellular enzymes (Aly et al., 2019; Wink et al., 2017).

2.2. Actinomycetes from aquatic habitats

Actinomycetes and their significance in aquatic habitats, including freshwater and marine environments, have been well investigated by many studies. They were distributed in the water and sediments of lakes, streams, rivers, and marine habitats, playing a role in recycling organic materials and minerals, phosphate solubilization, nitrogen fixation, environmental protection, and improving physical parameters (Alharbi, 2016; Dhanasekaran & Jiang, 2016). Recently, aquatic actinomycetes are ecologically as well as industrially significant due to their ability to produce excellent bioactive secondary metabolites, including antibiotics, enzymes, and other useful metabolites (Jagannathan et al., 2021). The principal genera of actinomycetes that have been found in aquatic ecosystems include *Streptomyces*, *Micromonospora*, and *Actinoplanes* (Das et al., 2022).

2.3. Actinomycetes from extreme environments

Actinomycetes are known for their adaptation to extreme environments, where they exhibit a remarkable diversity and potential capabilities. They were isolated from different extreme habitats, such as Antarctic soils, volcanic areas, hot springs, hyper-alkalinity and hyper-salinity regions, acidic streams, hyper-arid soils, subsurface rock, caves, frigid, and hypogean environments. Due to their characteristics, such as osmoregulation, residue degradation, nitrogen fixation, and the production of bioactive metabolites, they hold promise for the discovery of novel compounds with potential biotechnological applications, including extracellular enzyme production and biological properties such as antioxidant, anticancer, and antimicrobial activities (Ezeobiora et al., 2022; Wink et al., 2017).

2.4. Actinomycetes from honeybees and their products

Several recent studies reported and confirmed the presence of actinomycetes as one of the major microbial communities associated with honeybees and their products, including bee pollen, beebread, propolis, and honey, which may serve as valuable sources of new bioactive compounds, including antibiotics and others (Ghosh et al., 2022; Grubbs et al., 2021; Khan et al., 2020; Mokhnache et al., 2025; Santos-Beneit et al., 2022).

2.4.1. From honeybees

Due to their potential advantages, numerous studies have been focused on the isolation and screening of novel actinomycetes strains producing bioactive compounds from different parts of honeybees, including digestive tracts that support the transitory and symbiotic microbial communities (Cui et al., 2022; Patil et al., 2010). Previous studies have reported that honeybees harbor actinomycetes, including *Streptomyces aurantiogriseus*, *Nonomuraea roseoviolacea*, *Nocardiopsis alba*, and *Actinomadura apis*, which were isolated from three different honeybee species (*Apis mellifera*, *Apis florea*, and *Apis cerana*) collected in Thailand (Promnuan et al., 2009, 2011). In the study of Cui et al., (2022), a total of 61 actinomycetes strains were isolated from different parts of collected honeybee samples, including larvae, gut, head, cuticle, and abdomen, belonging to the genus *Streptomyces*, and displaying significant antibacterial activities against some Gram-positive and Gram-negative pathogenic bacteria, including *Staphylococcus aureus*, *Micrococcus tetragenus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Also, a recent study found that out of 93 isolates of actinomycetes, 19 were from honeybees and included three strains of *Streptomyces* that demonstrated antifungal activity against *Beauveria bassiana* and *Fusarium oxysporum*, as well as antibacterial activity against *Paenibacillus larvae* (Grubbs et al., 2021).

2.4.2. From beehive products

In addition to their presence in honeybees, actinomycetes have been found in honey, bee pollen, and bee bread, including the *Streptomyces* species, indicating a potential role in preserving and protecting these beehive products from spoiling microorganisms (Erdem, 2024; Khan et al., 2020; Wang et al., 2023). The study of Santos-Beneit et al., (2022) allowed the isolation of two *Streptomyces* strains from bee pollen and honey, which were collected from beehives located in southeast England and showed antibacterial activity against some Gram-positive pathogens, including *S. aureus*, *E. faecalis*, *B. cereus*, and *M. luteus*, as well as antifungal activity against *C. albicans*. Also, another study allowed the isolation of about 22 *Streptomyces* strains from bee pollen stores, and some of them were demonstrated to have the ability to produce piceamycin (a macrocyclic polyene lactam), which exhibited a potent antibacterial effect against *Paenibacillus larvae*, the causative agent of severe disease affecting honey bee larvae (American foulbrood). In addition to the antibacterial activity, some of these isolated strains displayed antifungal activity against *Beauveria bassiana* (entomopathogenic fungus) and *Fusarium oxysporum* (a common plant pathogen) (Grubbs et al., 2021).

3. Applications of actinomycetes

Current studies indicate that actinomycetes represent a valuable resource of different secondary metabolites as natural products, which are extremely important and potentially useful for diverse fields, including medical, pharmaceutical, agricultural, environmental, and industrial applications (Mebrat, 2024; Raut et al., 2023).

3.1. Medical and pharmaceutical applications

3.1.1. Antibiotics

Actinomycetes are well known as an inexhaustible source for antibiotics, which are bioactive compounds with various structures and action mechanisms (De Simeis & Serra, 2021; Zahr et al., 2022). According to Mebrat (2024), about 80 % of the known world's antibiotics come from actinomycetes, with *Streptomyces* species recognized as significant producers of a major proportion of these antibiotics. The important antibiotics produced from actinomycetes are differentiated into several major structural classes, such as β-lactams, tetracyclines, macrolides, aminoglycosides, chloramphenicol, and peptides (Table 1) (De Simeis & Serra, 2021; Zahr et al., 2022).

Table 1. The most natural antibiotics produced by actinomycetes (De Simeis & Serra, 2021).

Structural classes	Antibiotics		Some producer strains
β-lactams	Penicillin (Penicillin N)		*Streptomyces lipmanii* *Streptomyces microflavus* *Streptomyces griseus* *Streptomyces cattleya*
	Cephalosporines	Cephamycins	*Streptomyces griseus* *Streptomyces chartreusis* *Streptomyces lipmanii* *Streptomyces cattleya* *Amycolatopsis lactamdurans*
		Cephalosporines	*Streptomyces microflavus* *Streptomyces lipmanii* *Streptomyces hygroscopicus* *Streptomyces clavuligerus*
	Carbapenems (Thienamycins)		*Streptomyces cattleya* *Streptomyces penemifaciens* *Streptomyces flavogriseus* *Streptomyces olivaceus* *Streptomyces flavoviridis*
	Monobactam		*Nocardia uniformis* *Streptomyces alcalophilus* *Actinosynnema mirum*

	(Nocardicins)		*Microtetraspora caesia*
	PBP inhibitors	Clavulanic acid	*Streptomyces clavuligerus* *Streptomyces jumonjinensis* *Streptomyces katsuharamanus*
		Olivanic acid	*Streptomyces olivaceus* *Streptomyces griseus*
Macrolides	Oleandomycin Spiramycin Josamycin Midecamycin Rosaramycin Erythromycin		*Streptomyces antibioticus* *Streptomyces ambofaciens* *Streptomyces narbonensis* *Streptomyces mycarofaciens* *Micromonospora rosaria* *Streptomyces erythreus* *Aeromicrobium erythreum*
Lincosamides	Lincomycin		*Streptomyces lincolnensis* *Streptomyces spinosus* *Actinomyces roseolus* *Micromonospora halophytica*
Tetracyclines	Tetracycline		*Streptomyces aureofaciens* *Streptomyces avellaneus* *Streptomyces lusitanus* *Streptomyces viridifaciens*
	Chlortetracycline		*Streptomyces lusitanus* *Streptomyces aureofaciens* *Streptomyces lividans*
	Oxytetracycline		*Streptomyces alboflavus* *Streptomyces albofaciens* *Streptomyces erumpens* *Streptomyces griseus*
	6-demethyltetracycline		*Streptomyces platensis* *Streptomyces aureofaciens*
Aminoglycosides	Streptomycin		*Streptomyces bikiniensis* *Streptomyces streptomycinii* *Streptomyces ornatus* *Streptomyces glaucescens* *Streptomyces griseocarneus*
	Neomycin		*Streptomyces fradiae* *Streptomyces catenulae* *Streptomyces chrestomyceticus* *Streptomyces albogriseolus*
	Tobramycin		*Streptoalloteichus hindustanus* *Streptomyces tenebrarius*
	Kanamycin		*Streptomyces cremeus* *Streptomyces kanamyceticus*
	Paromomycin		*Streptomyces rimosus* *Streptomyces catenulae* *Streptomyces chrestomyceticus* *Streptomyces fradiae*
	Gentamycin		*Micromonospora purpurea* *Micromonospora pallida* *Micromonospora echinospora*

Peptides	Glycopeptides (Vancomycin) Lipopeptides (Daptomycin) Streptogramins (Streptogramins A and B)	*Amycolatopsis orientalis* *Streptomyces roseosporus* *Streptomyces lividans* *Streptomyces halstedii* *Streptomyces pristinaespiralis* *Streptomyces virginiae*
Aminocumarines	Novobiocin	*Streptomyces spheroides* *Streptomyces caeruleus* *Streptomyces niveus*
Epoxides	Fosfomycin	*Streptomyces fradiae* *S. viridochromogenes* *Streptomyces wedmorensis*

3.1.2. Antifungals

Actinomycetes are also a valuable source of antifungal agents with different mechanisms of action, including influences on nucleic acid, disruption of cell membrane integrity, inhibition of cell wall synthesis, virulence factors, spore germination, and the flow of electrons in the mitochondrial respiratory chain (Setiawati & Yusan, 2022). Due to the high degree of phylogenetic relationship between fungi and humans, many antifungals are toxic to eukaryotic host cells, which makes the number of antifungal compounds approved for use in human therapy very limited compared with antibiotics (Huang et al., 2023; Mohamadtalebi et al., 2022). Among these approved therapeutic antifungals are nystatin, amphotericin B, and natamycin, which are produced by *Streptomyces noursei*, *Streptomyces nodosus*, and *Streptomyces natalensis*, respectively (Mohamadtalebi et al., 2022).

3.1.3. Antivirals

The bioactive secondary metabolites of actinomycetes have demonstrated their ability to act against viruses. Antimycin A showed high activity against Western Encephalitis Virus and against a variety of RNA viruses, including members of the families Togaviridae, Flaviviridae, Bunyaviridae, Picornaviridae, and Paramyxoviridae. In addition, panosialins showed significant effects against HIV, influenza, and HSV (Meenakshi et al., 2024). Also, complestatins, which are known as peptides that are produced by *Streptomyces lavendulae*, act by inhibiting the adsorption of HIV-1 to the cells (Selim et al., 2021). Unfortunately, due to insufficient interest, none of these compounds exhibit enough potency to be developed as novel commercial antivirals compared to existing antiviral medicines, such as peramivir, zanamivir, baloxavir, and acyclovir (Rai & Bai, 2022).

3.1.4. Antiparasitics

Actinomycetes are also known as producers of effective antiparasitic compounds with high effects against parasitic infections, particularly those caused by *Leishmania* sp., *Plasmodium* sp., *T. brucei*, and some helminths. Cyclomarin A, an antimalarial agent, has been isolated from *Streptomyces* sp. and demonstrated activity against *P. falciparum*. Valinomycin and indolocarbazole, which were also produced by *Streptomyces* sp., exhibited antiparasitic effects against *T. brucei* and *L. major*. Also, milbemycins and avermectins obtained from *Streptomyces avermectinius* are now utilized as antihelminthics in veterinary medicine (Rai & Bai, 2022).

3.1.5. Antitumor compounds

Actinomycetes are considered the main source of different potent cancer chemotherapeutics, which belong to a wide range of classes, including macrolides, anthracyclines, enediynes, isoprenoids, indolocarbazoles, and non-ribosomal peptides. Their antitumor activity is exhibited by triggering apoptosis through different mechanisms (Table 2) (Zahr et al., 2022).

Table 2. Some antitumor compounds from different actinomycetes strains (Zahr et al., 2022).

Compounds	Actinomycetes strain	Activity
Actinomycin D	*Streptomyces antibioticus*	Treat Wilms tumors in kids
Doxorubicin	*Streptomyces peucetius*	Treating different cancers including lung, thyroid, breast, ovarian, gastric, sarcoma, Hodgkin's and non-Hodgkin's lymphoma, multiple myeloma, and pediatric cancers
Bleomycin	*Streptomyces verticillus*	It is used to treat testicular tumors, squamous cell carcinoma of the neck and head, and Hodgkin's lymphoma
Mitomycin C	*Streptomyces caespitosus*	It is potent against different solid tumors, including bladder, breast, lung, and gastrointestinal tumors
Calicheamicin	*Micromonospora echinospora*	It is used to treat acute myeloid leukemia

3.2. Industrial applications

3.2.1. Enzymes

The values of the commercial enzymes have recently increased substantially due to their many applications in the pharmaceutical, food, and detergent industries (Muazi Alenazi

et al., 2023). According to the BRENDA database, around 66,900 industrial enzymes applied in biotechnological industries and biomedical fields were produced by numerous actinomycetes genera, including L-asparaginase, proteases, lipases, cellulases, amylases, chitinases, xylanases, pectinases, keratinases, phytases, and glucose oxidases (Table 3) (Rai & Bai, 2022).

Table 3. Significant enzymes produced by actinomycetes (Bawazir et al., 2019).

Enzymes	Producing strains	Industrial applications
Protease	*Streptomyces pactum* *Streptomyces thermoviolaceus*	Pharmaceutical field Leather Food Detergents
Cellulase	*Streptomyces ruber* *Thermobifida halotolerans*	Detergents Paper and pulp
Lipase	*Nocardia alba* *Streptomyces exfoliates*	Detergent industries Foodstuff Oleochemical Diagnostic settings Pharmaceutical field
Xylanase	*Streptomyces* spp. *Actinomadura* sp.	Paper and pulp Animal feed
Pectinase	*Streptomyces lydicus*	Beverage Textile
Amylase	*Thermobifida fusca* *Streptomyces avermitilis*	Paper and pulp Textile Food Brewing Distilling industries
L-asparaginase	*Streptomyces griseus* *Streptomyces karnatakensis* *Streptomyces albidoflavus* *Nocardia* sp.	Therapeutic agent in the cure of certain human cancers, mostly in acute lymphoblastic leukemia
Keratinase	*Actinomadura keratinilytica* *Streptomyces erumpens*	Leather Detergents
Chitinase	*Streptomyces thermoviolaceus* *Nocardiopsis prasina* *Streptomyces hygroscopicus* *Streptomyces aureofaciens*	Textile Leather

3.2.2. Pigments and dyes

Due to the negative impacts caused by synthetic pigments on the environment, microbial pigments are offered as an alternative. They are eco-friendly and non-toxic, serving as a valuable natural alternative to synthetic pigments in foods, medicines, textiles, and

cosmetics (Abidin et al., 2023). Actinomycetes can produce several pigments with different colors, including blue, purple, red, rose, yellow, green, brown, and black (Muazi Alenazi et al., 2023). Also, actinomycetes are hyper producers of dark-brown-colored melanin or melanoid pigments that are widely used in medicine, pharmacology, and cosmetics (Kumar et al., 2023; Selim et al., 2021). *Streptomyces* is a major industrially important genus that has the ability to produce various pigments. Actinorhodin is a blue pigment produced by *Streptomyces coelicolor*, *Streptomyces violaceusruber*, and *Streptomyces lividans*. It may be used in the food industry during the making of beverages and desserts and might even be in the cosmetics (Selim et al., 2021). Although actinomycetes produced many pigments, industrial extraction is not much practiced due to the unavailability of protocols for large-scale fermentation and downstream processing (Abidin et al., 2023).

3.3. Nanotechnology applications

Nanobiotechnology has now emerged as an integration between nanotechnology and biotechnology, comprising novel approaches for the synthesis, design, and manipulation of microscopic particles that have sizes ranging from 1 to 100 nm, known as nanoparticles (Manivasagan et al., 2014; Rahman et al., 2019; Sharma & Sharma, 2018). Nanoparticles are in high demand by the scientific community due to their potential properties and technological applications (Rajoriya et al., 2021). They exhibit various physicochemical properties, including size, distribution, shape, charge, optical, electronic, and magnetic properties, as well as catalytic and biological activities, making them unique and suitable for various applications (Khan et al., 2019; Khan et al., 2018). Given the numerous disadvantages associated with physical and chemical methods for nanoparticle synthesis, there was a pressing need to find alternative, more advantageous methods. The green and biological methods have recently gained popularity as alternatives to chemical and physical methods due to their proven simplicity, rapid synthesis, cost-effectiveness, high safety, non-toxicity, solvent-free, and eco-friendly (Leila et al., 2018). These methods are usually considered a novel area of nanotechnology, known as nanobiotechnology (Rajoriya et al., 2021). Many biological systems, including plants, algae, bacteria, fungi, viruses, biopolymers, and agricultural and food wastes, were examined for their capacity to produce nanoparticles (Kanchi & Ahmed, 2018). Microbial synthesis of nanoparticles, which includes bacteria, molds, yeasts, and actinomycetes, has shown enormous potential in recent times due to their ability to secrete large amounts of enzymes and emit many reducing agents (Kanchi & Ahmed, 2018). Due to their high abundance and ability to adapt to extreme environments, bacteria are a wonderful

tool for the synthesis of various nanoparticles. As well as the substantial abundance, other advantages include rapid growth rates, cost-effective cultivation, and easily controllable growth parameters such as incubation time, temperature, oxygenation, and pH (Panpatte & Jhala, 2019). Furthermore, actinomycetes are helpful in the green synthesis of nanoparticles with excellent surface and size characteristics, showing a wide range of promising properties that have applications in medical, agricultural, and industrial fields. This biogenic approach uses an eco-friendly, easier, and rapid synthesis methodology, utilizing bioactive metabolites that can reduce the metal compounds, such as gold, silver, copper, and zinc, into corresponding nanoparticles (Dhanasekaran & Jiang, 2016). Numerous reports have demonstrated the effectiveness of actinomycetes isolated from various ecosystems as potential synthesizers of nanoparticles. These nanoparticles possess attractive physicochemical properties with various biological activities, including anticancer, antioxidant, antibacterial, antifungal, antiparasitic, and antibiofouling activities. Furthermore, with cytotoxicity being a major concern, researchers have examined and discussed the toxicity of these biogenic nanoparticles to humans and the environment (Golinska et al., 2014; Manivasagan et al., 2014). According to Rai and Bai (2022), *Arthrobacter*, *Thermomonospora*, *Streptomyces*, and *Nocardiopsis* are among the most common genera used in the green synthesis of nanoparticles, which can show antimicrobial, anticancer, antiparasitic, and antioxidant activities. The study of Pallavi et al. (2022) indicated that the synthesized AgNPs through the cell-free supernatant of the *Streptomyces hirsutus* strain SNPGA-8, which was isolated from sediment samples, exhibit considerable potential for biomedical applications, demonstrating significant antimicrobial activity against bacterial and fungal pathogens, as well as anticancer activity against the human lung carcinoma cell line A549. In another study, the mycelial biomass of the *Streptomyces* KBR3 strain isolated from a rhizospheric soil was utilized to synthesize AgNPs, where the in vitro evaluation of their antioxidant, antimicrobial, and antiproliferative activities indicated that they are promising alternative materials for various biomedical applications (Samuel et al., 2023).

3.4. Agricultural applications

3.4.1. Soil fertility

Actinomycetes, due to their remarkable characteristics compared to other microorganisms, are beneficial for soil quality improvement. They participate in all processes that enhance soil's fertility, such as nutrient cycling, decomposition of organic materials, and the formation of beneficial soil humus (Mebrat, 2024).

3.4.2. Plant growth-promoting and biological control

Actinomycetes, due to their adaptability to desiccation and resistance to both dry and wet heat, are utilized as plant growth-promoting rhizobacteria (PGPR). They exhibit direct biofertilizer activities, including nitrogen fixation, phosphate solubilization, and potassium solubilization, as well as the production of phytohormones such as indole-3-acetic acid (IAA), gibberellic acid, and cytokinins. Additionally, they act indirectly as biocontrol agents through the production of antimicrobials, siderophores, hydrogen cyanide (HCN), lytic enzymes, and volatile organic compounds (Figure .3) (Mebrat, 2024). Also, the ability of actinomycetes to produce organic acids, antimicrobials, volatile oils, and enzymes and to create a competitive microbial microenvironment through siderophore production and pathogen elimination constitutes additional benefits (Rai & Bai, 2022). *Streptomyces* species, already being the largest part of the soil microbiome, have the ability to enhance nutrient mobility within the soil. Also, other species of *Streptomyces*, *Micromonospora*, *Frankia*, *Nocardia*, and *Streptosporangium* play a beneficial role and act as potential plant growth-promoting bacteria, providing the plants with necessary nitrogen. Furthermore, *Streptomyces griseus*, *Micromonospora endolithica*, and *Micromonospora aurantiaca* are currently used as phosphate-solubilizing bacteria in bean and wheat cultivations. Additionally, many species of *Arthrobacter*, *Streptomyces*, and *Nocardia* help with iron absorption in plants by producing siderophores (Rai & Bai, 2022). *Streptomyces diastaticus*, *Streptomyces fradiae*, and *Streptomyces collinus* can produce active biomolecules used to control some plant pathogens, such as *Alternaria solani*, *Sclerotium rolfsii*, and *Fusarium oxysporum*. *Actinoplanes* spp. and *Microbispora* spp. have also demonstrated biocontrol effects against *Pythium* and *Gaeumannomyces graminis* in wheat, respectively (Rai & Bai, 2022).

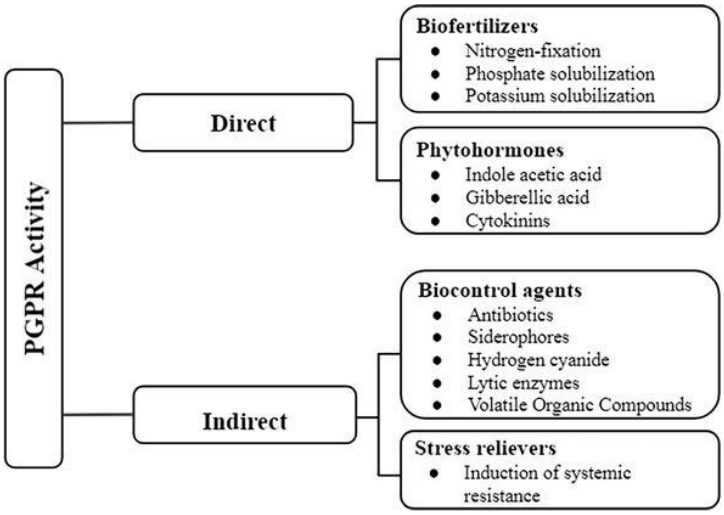


Figure 3. Plant Growth-Promoting Rhizobacteria activities of actinomycetes (Mebrat, 2024).

3.5. Environmental applications

3.5.1. Bioremediation

Actinomycetes play an important role in the bioremediation of soils contaminated with organic pollutants, including hydrocarbons, pesticides, aliphatic and aromatic compounds, and inorganic pollutants like heavy metals. The ability of various actinomycetes species to live in an oily environment is an opportunity to use these microorganisms to reduce oil pollutants. Many reports indicated that *Streptomyces* species could play a significant role in the degradation of hydrocarbons (Sharma et al., 2014). Also, actinomycetes are used in the bioremediation of different heavy metals (Table 4) (Rai & Bai, 2022).

Table 4. Bioremediation of heavy metals using some actinomycetes (Rai & Bai, 2022).

Heavy metals	Actinobacterial strains
Cadmium	*Actinomyces turicensis* AL36Cd, *Nocardia* spp., *Frankia* spp., *Arthrobacter rhomb* RE, *S. rimosus*, *S. lunalinharesii*, *S. zinciresistens*, *Streptomyces* spp. F4, *Promicromonospora* sp. UTMC 2243.
Chromium	*S. griseus*, *S. thermocarboxydus* NH50, *S. rimosus*, *S. flavovirens* ON3, *S. flavovirens* M4, *S. lunalinharesii*, *S. acrimycini* NGP, *S. albogriseolus* NGP, *S. variabilis* NGP, *Streptomyces* sp., *S. zinciresistens*
Lead	*S. viridochromogenes*, *S. srimosus*, *S. plumbiresistens*
Nickel	*S. rimosus*, *S. acidiscabies* E13
Mercury	*S. coelicolor* M130, *S. lividans* 1326, *S. lividansstrain* 8, *S. espinosusstrain* 5

3.5.2. Waste management

Members of many actinomycetes species represent a promising tool for use in the bioconversion of rural, urban, and agricultural wastes into valuable chemical products. Amylolytic actinomycetes, such as *Streptomyces* and *Thermoactinomyces*, are observed to produce amylase and may be utilized for the bioconversion of wastes from food containing huge amounts of starch to maltose. Also, actinomycetes incorporate an extensive variety of strains that decompose lignocellulosic plant sediment. Furthermore, *Streptomyces lavendulae* was observed as a cyanide degrader. It has a rhodanase enzyme that transfers sulfur from sodium thiosulfate to cyanide, forming thiocyanate (Bawazir et al., 2019).

Experimental Section

Chapter 1

Physicochemical, phytochemical, and microbiological analyses of some Algerian bee pollen samples

Introduction

As one of the important beehive products, bee pollen comes to the attention of the scientific community due to the nutritional and healthy properties demonstrated in ancient times (Anjum et al., 2024). It is considered the only fully complete food due to its high nutritional content and beneficial effects on human and animal health (Su et al., 2021). Accumulative research reports demonstrated clearly its biological and therapeutic properties, such as antioxidant, antitumor, antiviral, antimicrobial, antimutagenic, antiallergenic, hypolipidemic, hypoglycemic, anti-inflammatory, immune stimulant, and hepato-renal protection (Vargas-Abella et al., 2020). Bee pollen exhibits good antimicrobial effects against a range of pathogenic bacterial strains such as *Staphylococcus aureus*, *Clostridium perfringens*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and fungal strains such as *Candida albicans*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Rhizopus oryzae*, making it a candidate for use as a natural alternative antimicrobial agent (Anjum et al., 2024; Denisow & Denisow-Pietrzyk, 2016). Significant antioxidant activity has been shown in bee pollen, which helps and contributes to protecting and preventing the human body from diseases caused by oxidative stress (Su et al., 2021). Advanced analytical techniques, including chromatography and spectrophotometry, allowed identifying approximately 250 chemical compounds in samples of bee pollen collected from various regions in the world according to the botanical and geographical origin (Denisow & Denisow-Pietrzyk, 2016). It contains carbohydrates as the major content, which ranged between 13 and 55%, followed by proteins and amino acids (10 - 40%), water (20 - 30%), lipids (1 - 13%), and dietary fiber (0.3 - 20%) (El Ghouizi et al., 2023). For the minor components, bee pollen is an important source of minerals with about 25 elements, such as Fe, Mg, Zn, Cu, Ca, K, and N, which amounted to 2 - 6% of its total content. Additionally, almost all of the vitamins are present in a range of 0.02 to 0.7%. Furthermore, polyphenols and flavonoids, mainly gallic acid, coumaric acid, quercetin, kaempferol, and catechin, were determined with an average concentration of 3 - 8 % (Alvarez-Suarez, 2017; Denisow & Denisow-Pietrzyk, 2016; El Ghouizi et al., 2023). Due to its nutritional composition, bee pollen can contain different species of microorganisms, including bacteria, molds, and yeasts, which come from beekeepers' manipulation and natural habitats during collection, processing, and storage. Some of these microbial contaminants can be beneficial, such as lactic acid bacteria species, while others, such as *Staphylococcus aureus*, *Salmonella*, *Clostridium*, *Penicillium*, *Mucor*, and *Aspergillus*, can pose risks for consumer safety (Fernández et al., 2021; Hani et al., 2012;

Mauriello et al., 2017). Therefore, due to only some countries worldwide having regulatory specifications for bee pollen, standardization and legislation for microbiological quality are critical to reducing the risks associated with microbial contamination (Hani et al., 2012).

This study aims to analyze the microbiological quality, determine the physicochemical characteristics, and evaluate the polyphenol and flavonoid contents and their antioxidant activity in six local bee pollen samples collected from various Algerian regions.

Material and Methods

1. Chemicals and reagents

All chemicals used in this study were of analytical grade. Folin-Ciocalteu, gallic acid, quercetin, ascorbic acid, sodium carbonate, and ethanol were purchased from Sigma-Aldrich. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) reagent was purchased from SpeciLab. Aluminium chloride was obtained from Biochem Chemopharma. Culture media were provided from INSEP national institute and Applied Microbiology Laboratory, Setif, Algeria.

2. Samples collection

Four samples of bee pollen were collected by the International Api-Phyto Therapy Center -Setif- using pollen traps from different Algerian geographic sites. Bee pollen sample 1 (BPS 1) was collected from Guelma (36° 28′ 00″ N, 7° 26′ 00″ E), bee pollen sample 2 (BPS 2) was obtained from Medea (36° 16′ 03″ N, 2° 45′ 00″ E), and bee pollen sample 4 (BPS 4) was from Setif (36° 09′ 00″ N, 5° 26′ 00″ E), whereas the bee pollen sample 5 (BPS 5) was from Bejaia (36° 45′ 00″ N, 5° 04′ 00″ E). Two samples (BPS 3 and BPS 6) were purchased from a local market in Setif (36° 09′ 00″ N, 5° 26′ 00″ E). All samples were transported to the laboratory and conserved at 4 °C to analyze.

3. Physicochemical analyses

3.1. Determination of pH

To determine the pH values of bee pollen samples, one gram of each sample was mixed with 7.5 mL of distilled water for 24 hours at room temperature. Thereafter, the prepared solutions were centrifuged at 6000 rpm and filtered through filter paper. The pH values were measured using a pH meter (HANNA pH 211, USA) (Adaškevičiūtė et al., 2019).

3.2. Determination of moisture content

Three grams of each sample were dried at 105 °C for 2 hours (Dias et al., 2012; Fuenmayor et al., 2014). The moisture was calculated using the mathematical equation (A):

$$\text{Moisture (\%)} = \frac{[\,A1 - A2\,]}{[A1]} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \text{(A)}$$

Where, A1: weigh of fresh bee pollen sample, A2: weigh of dried bee pollen sample.

3.3. Determination of ash content

The ash content was determined after the complete burning of 3g of each tested bee pollen sample using a muffle furnace at 550°C (De Arruda et al., 2017).

4. Phytochemical analyses

4.1. Preparation of bee pollen extracts

Five grams from each bee pollen sample was extracted in 75 mL of 70 % ethanol. The extraction was performed by agitation using a hot plate magnetic stirrer set to 1500 rpm and 40 °C for 2 hours. This experiment was carried out in duplicate with two repetitions. After extraction, the mixture solution for each sample was filtered through a filter paper. Finally, ethanol was evaporated using a rotary evaporator (BUCHI R-215, Switzerland), and the obtained extracts were kept at 4°C (Lawag et al., 2021).

4.2. Determination of total polyphenols

Total polyphenol content was evaluated by the Folin-Ciocalteu method as reported elsewhere (Benmerzoug et al., 2020). 100 µL of each bee pollen extract was mixed with 500 µL of a 10 % Folin-Ciocalteu reagent for 4 minutes. Subsequently, 400 µL of 7.5 % sodium carbonate was added. After incubation in dark conditions for 90 min at room temperature, the absorbances were recorded at 765 nm using a UV-visible spectrophotometer (Shimadzu BioSpec-mini, Japan). Gallic acid was used as a standard at various concentrations ranging from 10 to 90 µg/mL. The total content of polyphenols was determined in milligrams of gallic acid equivalents per gram of bee pollen extract.

4.3. Determination of flavonoids content

Total flavonoid content was determined by the aluminum chloride method (Barbieri et al., 2020). Briefly, 500 µL of each extract was mixed with 500 µL of 2% aluminum chloride. After 10 minutes, the absorbances were measured at 430 nm. Different concentrations of quercetin ranging from 5 to 40 µg/mL were used as a standard. Flavonoid content was expressed as milligrams of quercetin equivalent per gram of bee pollen extract.

4.4. Antioxidant activity

The antioxidant activity of each bee pollen extract was studied using DPPH free radical scavenging assay, as reported by Kohole Foffe et al. (2023) with little modification. 0.5 mL of different concentrations (0.2 to 6 mg/mL) of each bee pollen extract was added to 0.3 mL of 0.5 mM 2,2-diphenyl-1-picryl-hydrazyl solution and 3 mL of methanol. The mixtures were incubated for 30 minutes at room temperature in dark conditions. The absorbances were recorded at 517 nm. Different concentrations of ascorbic acid from 5 to 40 µg/mL were used as a standard. The IC_{50} values of extracts were determined directly from the graph of scavenging effect percentage against the extract concentrations. They were expressed in mg/mL and compared with the IC_{50} of ascorbic acid.

5. Microbiological analyses

Before the microbiological analyses, 5 g of each bee pollen sample were diluted and homogenized in 45 mL of sterile peptone water, followed by incubation for 2 hours at 30 °C. For each sample, serial dilutions were performed as required in sterile saline solution. Adequate dilutions were plated on a suitable medium. The obtained colonies were counted, and expressed as colony-forming units per gram of bee pollen (CFU/g) using the formula (B):

$$\mathrm{CFU/g} = \frac{\mathrm{N}}{\mathrm{V \times D}} \quad \text{..... (B)}$$

Where, N: number of counted colonies, V: volume of plated inoculum, D: strength of the dilution.

5.1. Determination of total aerobic and anaerobic mesophilic bacteria

The aerobic and anaerobic mesophilic bacteria were enumerated by spreading 100 µL of the adequate dilutions of each bee pollen sample onto plate count agar (PCA). Then, all plates were incubated for 3 days at 30 °C under aerobic and anaerobic conditions (Jaya et al., 2020).

5.2. Determination of molds and yeasts

Molds and yeasts were counted by plating 100 µL of the dilutions of each bee pollen sample onto sabouraud chloramphenicol agar (SCA) and incubating at 28 °C for 3 to 5 days (Vargas-Abella et al., 2020).

5.3. Determination of total coliforms

The total coliforms were counted by spreading 100 µL of the appropriate dilution of each bee pollen sample onto violet red bile lactose agar (VRBLA) and incubating it at 35 °C. After 24 to 48 hours of incubation, the typical black-reddish colonies were counted (Fernández et al., 2017).

5.4. Determination of *Staphylococcus aureus*

S. aureus was enumerated by plating 100 µL of the dilutions of each bee pollen sample on Baird-Parker agar (BPA), enriched with egg yolk emulsion, and supplemented with a 3 % potassium tellurite solution. After incubation at 37 °C for 48 hours, all colonies that were circular, smooth, convex, moist, and gray to jet-black, surrounded by a clear zone, were counted (Kouamé et al., 2021; Nuvoloni et al., 2021).

5.5. Detection of *Sulfite-reducing clostridium*

For sulfite-reducing *Clostridium*, 1 mL of the initial suspension of each bee pollen sample was put into an empty screw-capped glass tube, treated at 80°C for 15 minutes, and covered with iron sulfite agar (ISA) followed by a layer of paraffin oil. Then, all tubes were incubated for 5 days at 37°C. The presence of black colonies was noted as a positive result (Estevinho et al., 2012).

5.6. Detection of *Salmonella* sp.

The presence of *Salmonella* sp. was detected by three steps. The first step involved pre-enrichment of 25 g of each sample in 225 mL of sterile buffered peptone water, followed by 20 hours of incubation at 37 °C. Secondary selective enrichment was performed in Rappaport-Vassiliadis broth (RVB) with 24 hours of incubation at 42 °C. Finally, the growth was done on three different selective media, including xylose-lysine-deoxycholate agar (XLDA), *Salmonella-Shigella* agar (SSA), and Hektoen enteric agar (HEA). After 24 hours of incubation at 37 °C, the presence of black and green to blue-green colonies was recorded as a positive result (Azonwade et al., 2018; Fernández et al., 2017; Mauriello et al., 2017).

6. Statistical analysis

In this study, unless otherwise stated, all experiments were performed in triplicate. Statistical analyses were assessed by one factor ANOVA and Pearson's correlation tests using IBM SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, USA). The results were expressed as means ± standard deviations. The variance percentage was calculated with 95% confidence ($\alpha = 0.05$).

Results and discussion

1. Physicochemical analyses

In this study, some physicochemical parameters, including pH, moisture, and ash content of six collected bee pollen samples, have been tested. The obtained results are shown in Table 1.1.

Table 1.1. Physicochemical analyses of collected bee pollen samples.

Samples	pH	Moisture (%)	Ash (%)
BPS1	3.90 ± 0.52 (a)	23.26 ± 0.07 (d)	1.93 ± 0.26 (a)
BPS2	4.41 ± 0.05 (b)	23.87 ± 0.22 (e)	2.65 ± 0.25 (b)
BPS3	5.33 ± 0.13 (d)	11.13 ± 0.03 (a)	2.00 ± 0.08 (a)
BPS4	4.74 ± 0.04 (c)	27.80 ± 0.18 (f)	2.78 ± 0.08 (b)
BPS5	3.99 ± 0.06 (a)	16.25 ± 0.40 (c)	2.52 ± 0.28 (a b)
BPS6	3.87 ± 0.02 (a)	14.62 ± 0.16 (b)	3.73 ± 0.31 (c)
Mean	4.37 ± 0.55	19.49 ± 6.04	2.60 ± 0.64
Maximum	5.33 ± 0.13	27.80 ± 0.18	3.73 ± 0.31
Minimum	3.87 ± 0.02	11.13 ± 0.03	1.93 ± 0.26
p value	0.0001	0.0001	0.0001
Significance level	***	***	***

*: $p < 0.05$, **: $p < 0.01$, ***: $p<0.001$, (a, b, c, d, e, f): subgroups.

1.1. pH

According to the obtained results, a highly significant difference between the pH values of all tested bee pollen samples ($p<0.001$) was noticed (Table 1.1). The pH values were recorded in the acidic range with variation from one sample to another and with an estimated mean of 4.37 ± 0.55. BPS 3 collected from Setif showed the highest value of pH (5.33 ± 0.13), while BPS 6, which was also collected from Setif, showed the lowest value (3.87 ± 0.02).

1.2. Moisture

By the same way, for the moisture or water content, there is also a highly significant difference between the values of the samples ($p<0.001$) (Table 1.1), which varied from 11.13 ± 0.03 to 27.80 ± 0.18%, with an average value of 19.49 ± 6.04 %. The BPS 4, which was collected from Setif, presented the maximum value, while the BPS 3, which was also collected from Setif, had the minimum value. Eventually, the fresh samples, BPS 1 (Guelma), BPS 2 (Medea), and BPS 4 (Setif), showed elevated values when compared to the dried ones.

1.3. Ash

The ash content values vary significantly between samples ($p<0.001$) (Table 1.1). The results ranged from 3.73 ± 0.31 % in BPS 6, which was collected from Setif, to 1.93 ± 0.26 % in BPS 1, which was collected from Guelma, with a mean value estimated at 2.60 ± 0.64 %.

Regarding the physicochemical analyses, the results indicate the presence of highly significant differences in pH, moisture, and ash content of all tested bee pollen samples due to the variety of their geographical region, botanical origin, and soil type (De Arruda et al., 2017; Nogueira et al., 2012). The pH values were recorded in the acidic range with an average of 4.37 ± 0.55, which is within the range of pH suggested by the regulatory specifications of Argentina, Brazil, and Mexico (Fuenmayor et al., 2014). The obtained results for moisture vary from 11.13 ± 0.03 to 27.80 ± 0.18 % with an average value of 19.49 ± 6.04% and are similar to that reported by Barajas et al. (2012) in their study on the effect of the drying process of bee pollen. Furthermore, the obtained results of ash correspond with the range of ash content between 2 and 6% proposed in 2008 by Campos et al. in their standardization of bee pollen.

2. Phytochemical analyses

Table 1.2 presents the total polyphenols, flavonoids, and antioxidant activity analyses of the collected bee pollen samples.

Table 1.2. Phytochemical content and antioxidant activity of collected bee pollen samples.

Samples	Polyphenols (mg/g)	Flavonoids (mg/g)	IC_{50} (mg/mL)
BPS1	17.21 ± 0.44 (d)	6.80 ± 0.09 (d)	1.21 ± 0.02(a)
BPS2	12.02 ± 0.51 (b)	4.19 ± 0.34 (b)	2.42 ± 0.05(b)
BPS3	22.46 ± 0.05 (e)	11.07 ± 0.29 (e)	1.08 ± 0.05(a)
BPS4	14.30 ± 0.27 (c)	4.78 ± 0.01 (c)	4.77 ± 0.09(c)
BPS5	15.94 ± 0.96 (d)	3.76 ± 0.13 (b)	2.38 ± 0.06(b)
BPS6	9.76 ± 0.22 (a)	2.67 ± 0.06 (a)	2.23 ± 0.11(b)
Mean	15.28 ± 4.17	5.54 ± 2.85	2.35 ± 1.25
Maximum	22.46 ± 0.05	11.07 ± 0.29	4.77 ± 0.09
Minimum	9.76 ± 0.22	2.67 ± 0.06	1.08 ± 0.05
p value	0.0001	0.0001	0.0001
Significance level	***	***	***

IC_{50}: amount of extract required to reduce DPPH radical by 50%. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, (a, b, c, d, e): subgroups.

2.1. Total phenolic content

As presented in Table 1.2, a highly statistically significant difference was recorded between the phenolic content of all studied samples of bee pollen ($p<0.001$). According to the

results, BPS 3 (Setif) has the higher content of polyphenols, followed by BPS 1 (Guelma), BPS 5 (Bejaia), BPS 4 (Setif), BPS 2 (Medea), and BPS 6 (Setif), with obtained amounts estimated by 22.46 ± 0.05, 17.21 ± 0.44, 15.94 ± 0.96, 14.30 ± 0.27, 12.02 ± 0.51, and 9.76 ± 0.22 mg/g, respectively. Figure 1.1 shows the total polyphenol content in the extracts of the collected samples of bee pollen.

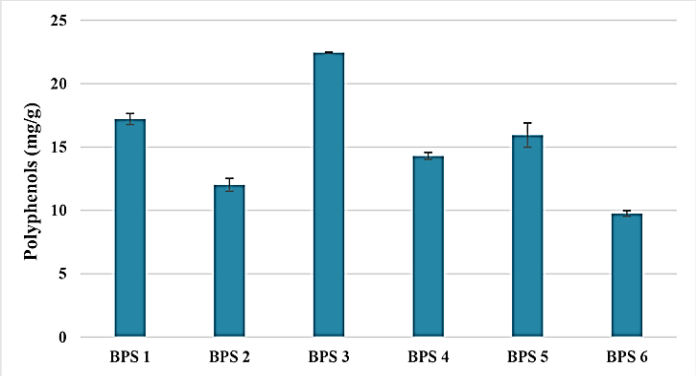


Figure 1.1. Total polyphenols of bee pollen extracts

2.2. Flavonoids content

Concerning the flavonoid content, there is also a highly significant difference between the values of the collected samples of bee pollen ($p<0.001$) (Table 1.2). Figure 1.2 displays the results, showcasing the variation and differences in the obtained flavonoid content concentrations. A considerable concentration was observed in BPS 3 (11.07 ± 0.29 mg/g). The sample BPS 6 showed the lowest concentration of flavonoids (2.67 ± 0.06 mg/g).

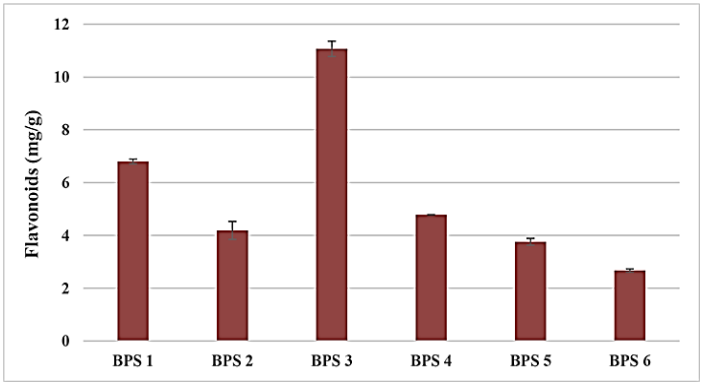


Figure 1.2. Flavonoids content of bee pollen extracts

2.3. Antioxidant activity

As presented also in Table 1.2, a highly significant difference also was recorded between the IC_{50} values of the examined samples ($p<0.001$). Figure 1.3 shows that the BPS 3,

which was collected from Setif, had the best antioxidant activity, with an IC_{50} value of 1.08 ± 0.05 mg/mL. It was followed by BPS 1 (Guelma), BPS 6 (Setif), BPS 5 (Bejaia), BPS 2 (Medea), and BPS 4 (Setif), which had IC50 values of 1.21 ± 0.02, 2.23 ± 0.11, 2.38 ± 0.06, 2.42 ± 0.05, and 4.77 ± 0.09 mg/mL, respectively. These results are hardly comparable with ascorbic acid, which is used as a standard, presenting an IC_{50} value of 0.035 mg/mL.

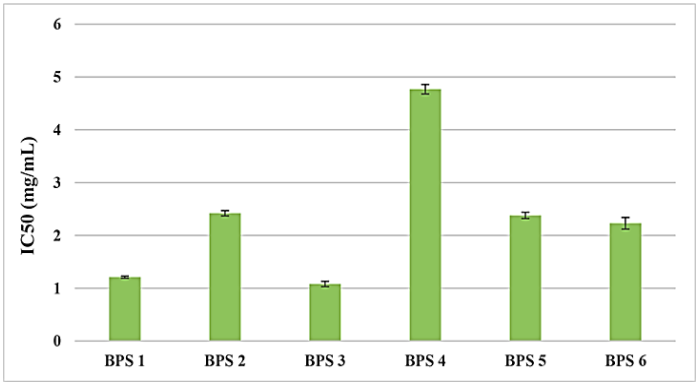


Figure 1.3. IC_{50} values of bee pollen extracts

2.4. Correlation between polyphenols, flavonoids, and antioxidant activity

According to the statistical analyses illustrated in Table 1.3, a strong positive significant correlation occurred between flavonoids and total polyphenols ($r = 0.917$; $p < 0.01$). As expected, this result indicates that the total polyphenols have a positive effect on flavonoid content in collected bee pollen samples. Furthermore, a negative significant correlation was observed between IC_{50} and flavonoids ($r = - 0.501$; $p < 0.05$).

Table 1.3. Correlations between polyphenols, flavonoids, and IC_{50}.

	Polyphenols	**Flavonoids**	**IC_{50}**
Polyphenols	1		
Flavonoids	**0.917****	1	
IC_{50}	- 0.449	**- 0.501***	1

(**): the correlation is significant at the 0.01. (*): the correlation is significant at the 0.05.

As shown in the results of phytochemical analyses, the obtained polyphenol and flavonoid contents of tested bee pollen samples are in agreement with some previous studies and comparable with others (Adaškevičiūtė et al., 2019; Anjos et al., 2019; Hemmami et al., 2020; Morais et al., 2011). The study of Carpes et al. (2007) indicated that bee pollen has significant polyphenol and flavonoid contents with some variations due to its botanical origin and the type of solvent used during the extraction. In this study, the antioxidant activity was

evaluated by the DPPH test. When the extracts of bee pollen were mixed with the DPPH reagent, the color changed from purple to yellow, indicating the reduction of the DPPH radical by the antioxidant compounds present in the extracts (Su et al., 2021). The average of IC_{50} values of bee pollen extracts was 2.35 ± 1.25 mg/mL. Pascoal et al. (2014) studied the biological activities of eight samples of bee pollen from Portugal and Spain and obtained IC_{50} values ranging from 2.98 ± 0.47 to 6.69 ± 0.75 mg/mL using DPPH assay. According to El Ghouizi et al. (2023)**,** there is a significant variation in the antioxidant activity of bee pollen samples from one country to another. The study of Su et al. (2021) reported that the DPPH radical scavenging assay is the common method for assessing antioxidant activity. However, it is infeasible to assess the antioxidant activity of any natural product thoroughly and accurately through just one evaluation method. In addition, some studies have reported that the antioxidant activity of bee pollen extracts is not only related to the polyphenol content but may also be related to the types of polyphenols (Hemmami et al., 2020; Su et al., 2021). In addition, the results revealed a positive significant correlation between flavonoids and total polyphenols ($r = 0.917$; $p < 0.01$), and a negative significant correlation between IC_{50} and flavonoids ($r = - 0.501$; $p < 0.05$). Due to flavonoids being a group of polyphenols, an increase in polyphenol content leads to a corresponding increase in flavonoid concentration, and vice versa (Tamuly et al., 2013). Furthermore, previous studies have reported that the higher flavonoid concentration generally corresponds to decreased IC_{50}, where the antioxidant activity was increased (Hmamou et al., 2023; Kocabaş Oğuz, 2023). It is noteworthy that the antioxidant activity of bee pollen is not just dependent on flavonoids, because the presence of other phenolic compounds can also contribute to an increase in antioxidant potential (Rojo et al., 2023).

3. Microbiological analyses

In the microbiological analyses, various microorganisms were found in the tested bee pollen samples, such as aerobic and anaerobic mesophilic bacteria, molds and yeasts, coliforms, *S. aureus*, and sulfite-reducing *Clostridium*, with the exception of *Salmonella* sp., which was not detected. According to the obtained results in Table 1.4, significant differences in microbial loads were found between all studied samples of bee pollen. They ranged from 7.70×10^2 to 1.93×10^6 CFU/g for the aerobic mesophilic bacteria, 1.10×10^3 to 5.30×10^5 CFU/g for the anaerobic mesophilic bacteria, 2.70×10^3 to 3.70×10^3 CFU/g for molds and yeasts, 0.00 to 1.73×10^4 CFU/g for total coliforms, and from 0.00 to 2.33×10^2 CFU/g for *S. aureus*. Sulfite-reducing *Clostridium* was detected only in the BPS 1 sample, which was

collected from Guelma. Furthermore, *Salmonella* sp. was not detected in all studied samples. Vargas-Abella et al. (2020) evaluated the microbiological analyses of some fresh and dried bee pollen from Colombia. They found that there were between 2.00 x 10^3 and 12.00 x 10^3 CFU/g for aerobic mesophilic bacteria, 0.00 to 6.00 x 10^3 CFU/g for molds and yeasts, 0.00 to 3.00 x 10^3 CFU/g for total coliforms, and between 0.00 and 2.00 x 10^3 CFU/g for *S. aureus*. However, none of the tested samples contain sulfite-reducing *Clostridium*. The European Union Standard for microbiological quality proposed the following microbial load allowed in bee pollen: total mesophilic bacteria (<100,000/g), molds and yeasts (<50,000/g), *Enterobacteriaceae* (Max. 100/g), *S. aureus* (absent/1 g), *Salmonella* sp. (absent/10 g), and *E. coli* (absent/1g) (Campos et al., 2008). The comparison of these recommendations with our results reveals that the bee pollen samples collected from Guelma (BPS 1), Medea (BPS 2), and Setif (BPS 4) have a poor microbiological quality. Due to its nutritional composition, different species of microorganisms, including bacteria, molds, and yeasts, can be present in bee pollen during collection, processing, and storage, depending strongly on its geographic region and beekeepers activities, necessitating the following hygienic and quality standards to minimize the microbial load that can pose risks to consumer safety (Altintas et al., 2022; De Arruda et al., 2017; Straumite et al., 2022).

In Algeria, people continue to consume bee pollen as food and medicine, even though there are no regulatory specifications or legislation for its microbiological quality (Hani et al., 2012). Therefore, hygienic standards should be adopted, as the high number of mesophilic bacteria reflects the microbiological quality of bee pollen (Vargas-Abella et al., 2020). The presence of molds and yeasts is responsible for changes in the organoleptic characteristics of bee pollen and the production of mycotoxins such as aflatoxins and ochratoxins, which are thermostable and carcinogenic substances (Ecem Bayram et al., 2023; Hani et al., 2012; Nuvoloni et al., 2021). Also, the presence of coliforms, including *Salmonella* sp., is an epidemiological risk due to their ability to cause food-borne diseases in humans (Altintas et al., 2022; Vargas-Abella et al., 2020). In addition, the presence of *S. aureus* in bee pollen can cause food poisoning by producing thermostable enterotoxins. Furthermore, the presence of sulfite-reducing *Clostridium* can lead to neuroparalytic illness through the production of neurotoxins (Altintas et al., 2022).

Table 1.4. Microbiological analyses of tested samples of bee pollen.

Microbiological analyses *	Bee pollen samples						p value	Significance level
	BPS 1	BPS 2	BPS 3	BPS 4	BPS 5	BPS 6		
Aerobic mesophilic bacteria	$1.17 \times 10^6 \pm 2.00 \times 10^4$	$1.93 \times 10^6 \pm 7.20 \times 10^5$	$7.70 \times 10^2 \pm 5.80 \times 10$	$1.11 \times 10^6 \pm 3.50 \times 10^4$	$4.06 \times 10^5 \pm 3.50 \times 10^4$	$8.66 \times 10^3 \pm 1.53 \times 10^3$	0.0001	***
Anaerobic mesophilic bacteria	$5.30 \times 10^5 \pm 8.00 \times 10^4$	$4.30 \times 10^5 \pm 5.57 \times 10^4$	$4.67 \times 10^2 \pm 5.80 \times 10^1$	$1.23 \times 10^5 \pm 1.39 \times 10^4$	$2.53 \times 10^4 \pm 8.73 \times 10^3$	$1.10 \times 10^3 \pm 1.00 \times 10^2$	0.0001	***
Molds and yeasts	$3.03 \times 10^3 \pm 1.07 \times 10^3$	$2.90 \times 10^3 \pm 3.00 \times 10^2$	$2.80 \times 10^3 \pm 5.57 \times 10^2$	$3.70 \times 10^3 \pm 5.29 \times 10^2$	$2.70 \times 10^3 \pm 0.00 \times 10^1$	$2.87 \times 10^3 \pm 4.51 \times 10^2$	0.386	
Total coliforms	-	$3.67 \times 10^2 \pm 1.53 \times 10^2$	-	$1.73 \times 10^4 \pm 4.93 \times 10^3$	-	-	0.0001	***
Staphylococcus aureus	$2.33 \times 10^2 \pm 1.53 \times 10^2$	$1.33 \times 10^2 \pm 1.53 \times 10^2$	-	-	-	-	0.026	*
Sulfite-reducing Clostridium	+	-	-	-	-	-		
***Salmonella* sp.**	-	-	-	-	-	-		

(*) The microbiological analyses were expressed in CFU/g. *: $p < 0.05$. **: $p < 0.01$. ***: $p<0.001$.

Conclusion

In conclusion, the comprehensive analyses of the physicochemical, phytochemical, and microbiological features of some Algerian bee pollen samples provided us with additional information about their nutritional and therapeutic benefits. Physicochemical analyses revealed significant differences in pH, moisture, and ash content, which indicated the influence of geographical location and botanical origin on bee pollen quality. The phytochemical analyses quantified the bioactive compound content, including total polyphenols and flavonoids, and indicated that the bee pollen is a substantial source of potent antioxidant compounds. In addition, the microbiological study demonstrated the presence of different microorganisms, including some pathogenic species, and underscored the necessity of following hygienic and quality standards to reduce the microbial contamination, which can pose microbiological risks to consumer safety. All things considered, these results affirm the potential of bee pollen as a functional food and its importance in alternative medicine, warranting more studies into its health benefits and applications.

Chapter 2

Isolation, characterization, and screening of actinomycetes from Algerian bee pollen for extracellular enzyme production and antimicrobial activity

Introduction

Bee pollen is one of the beehive products collected by worker honey bees, which is composed of natural flower pollen mixed with nectar and bee secretions (Su et al., 2021). Due to the nutritive composition, bee pollen provides a unique source for microbial communities, including bacteria, and offers a great chance for the isolation of one dominant group of bacteria, namely actinomycetes (López et al., 2020; Mahajan & Balachandran, 2012). As one of the dominant groups of microorganisms, actinomycetes are being widely distributed in various terrestrial and aquatic habitats. Actinomycetes are a group of Gram-positive bacteria that have DNA with a high guanine and cytosine content (GC %). Their growth is characterized by the formation of mycelium during the early stages of its life cycle (Mahajan & Balachandran, 2012; Wink et al., 2017). They are capable of producing a wide range of bioactive secondary metabolites, including antibiotics, pigments, and enzymes, which makes them invaluable in diverse sectors, such as pharmaceuticals, agriculture, biotechnology, and environmental sustainability (Dhanasekaran & Jiang, 2016). Due to the increasing phenomenon of antibiotic resistance and the emergence of new infectious diseases, it's necessary to seriously enhance the therapeutic strategies against antibiotic-resistant pathogens by discovering and exploiting novel bioactive compounds from actinomycetes, which are known for their potential antimicrobial activities (Bernardi et al., 2019). Many studies reported that these bioactive compounds exhibited antibacterial activities and are effective against a range of Gram-positive bacteria, such as *S. aureus*, *B. subtilis*, and *E. faecalis*, as well as against Gram-negative bacteria, such as *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *S. paratyphi* (Al-Dhabi et al., 2020; Ayoubi et al., 2018; Naligama et al., 2022; Sapkota et al., 2020). Furthermore, the bioactive metabolites produced by actinomycetes showed significant antifungal activity against different pathogenic fungal strains, such as *A. flavus*, *A. niger*, *C. albicans*, *C. glabrata*, and *S. cerevisiae* (Aku et al., 2022; Dharmaveera et al., 2018; Sripreechasak & Athipornchai, 2019). In addition to antimicrobials, actinomycetes are also known for their ability to produce an extensive number of valuable extracellular enzymes, such as proteases, cellulases, amylases, chitinases, xylanases, and lipases, making them promising candidates for industrial and biotechnological applications, including the production of pharmaceuticals, bioremediation, and waste management (Adeoyo, 2023; Rai & Bai, 2022).

Due to the actinomycetes associated with bee pollen being poorly investigated, the aim of this preliminary study is to assess the presence of them in some bee pollen samples collected from different Algerian places, as well as the screening of their extracellular enzyme production and antimicrobial properties.

Material and methods

1. Collection of bee pollen samples

A total of twelve bee pollen samples were collected in this study, eleven samples from different areas in Algeria that included, Medea (36° 16′ 03″ N, 2° 45′ 00″ E), Guelma (36° 28′ 00″ N, 7° 26′ 00″ E), Setif 1, 2 and 3 (36° 09′ 00″ N, 5° 26′ 00″ E), Skikda (36° 52′ 00″ N, 6° 54′ 00″ E), Mila (36° 27′ 00″ N, 6° 16′ 00″ E), Bejaia (36° 45′ 00″ N, 5° 04′ 00″ E), Tipaza 1 and 2 (36° 35′ 31″ N, 2° 26′ 58″ E) and Blida (36° 29′ 00″ N, 2° 50′ 00″ E). One sample was purchased from a local market in Setif (36° 09′ 00″ N, 5° 26′ 00″ E). All the obtained samples were transported to the laboratory and kept at 4°C until pretreatment. Figure 2.1 shows the origins of collected bee pollen samples.

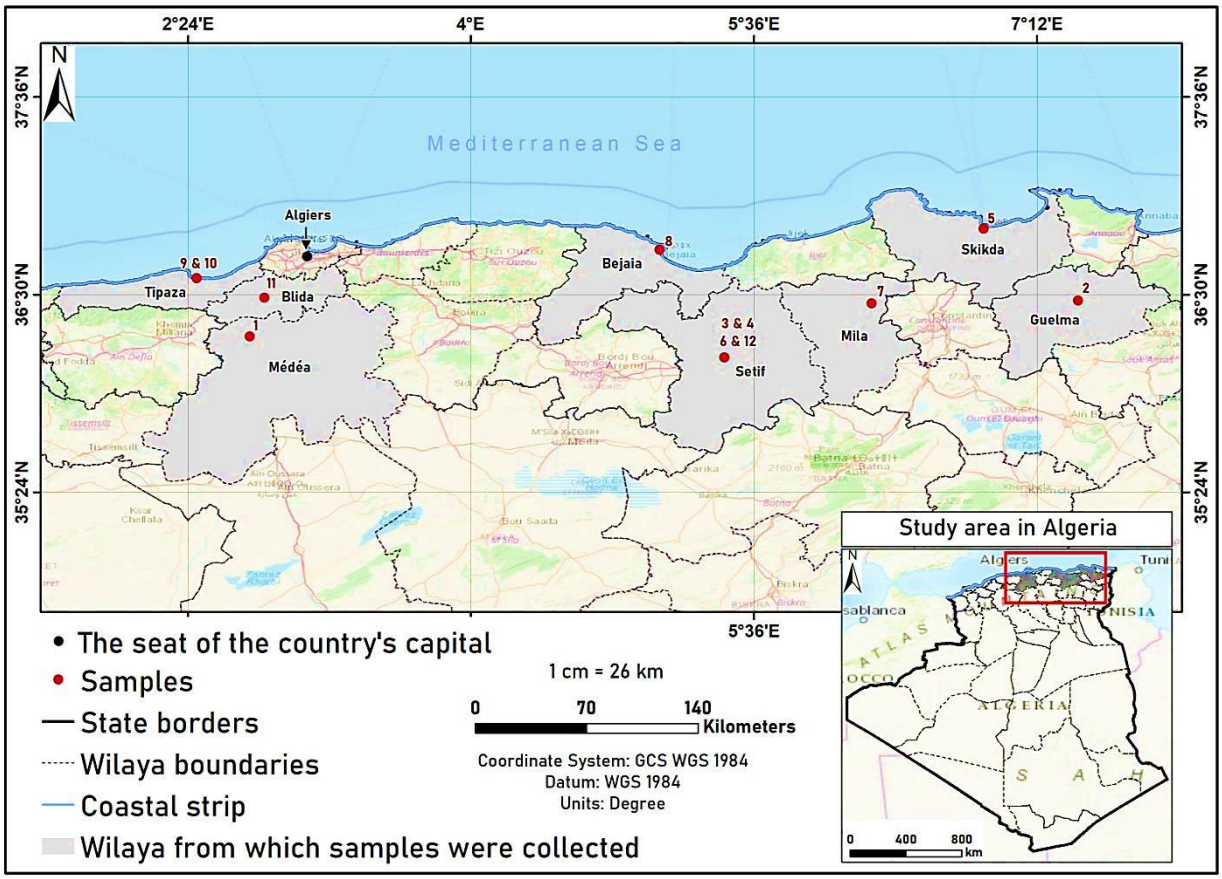


Figure 2.1. Geographic locations of bee pollen sampling (Created by ArcGIS maps).

2. Pretreatment of collected samples

The collected bee pollen samples were crushed and exposed to three different pretreatments. First, air drying at room temperature for 4 days was performed (Figure 2.2). Then, they were heated in a water bath at 70 °C for 60 minutes. Finally, 1 g of each bee pollen sample was mixed with 0.1 g of calcium carbonate ($CaCO_3$) and incubated in an atmosphere saturated with humidity at 28 °C for 6 to 7 days (Boussaber, et al., 2012.; Juby & Usha, 2018).

Figure 2.2. Air drying of collected bee pollen samples.

3. Isolation, purification, and conservation of actinomycetes strains

Actinomycetes strains were isolated from collected bee pollen samples using serial dilution. 1 g of each pretreated bee pollen sample was suspended in 9 mL of sterile saline, vortexed for a few minutes, and diluted up to 10^{-2} in a series of test tubes (Juby & Usha, 2018; Kumar & Jadeja, 2018). Then, 100 µL of diluted samples were plated by the spread plate technique on different media, including starch casein agar (SCA), yeast extract-malt extract agar (YMA), streptomyces agar (SA), glycerol asparagine agar (GAA), actinomycetes isolation agar (AIA), Czapeck agar (CZA), and nutrient agar (NA) that were prepared with or

without cycloheximide (75 µg/ml) and fluconazole (25 µg/ml). All plates were incubated at 28°C for about 21 days. During incubation, powdery colonies were selected and purified using yeast extract-malt extract agar (ISP-2 medium). Finally, all purified strains were conserved with some previously isolated strains in distilled water at 4 °C (Ahmed et al., 2020; Ayoubi et al., 2018; Bawazir et al., 2018).

4. Actinomycetes strains characteristics

4. 1. Macroscopic description

Using ISP-2 agar medium, morphological and cultural properties of all isolated actinomycetes strains were described based on colony size, colony color, colony shape, colony texture, growth intensity, and pigmentation (Kavitha et al., 2020).

4. 2. Gram staining

The standard Gram stain procedure was employed to identify the isolated actinomycetes strains (Balachandar et al., 2018). Firstly, the smear was prepared by spreading the pure colony of each actinomycetes strain onto a glass slide, followed by heat-drying. Next, the smear was covered with crystal violet for 1 minute and rinsed with sterile distilled water. After that, the smear was subsequently covered with Lugol for 1 minute and rinsed with sterile distilled water. The next step involved decolorizing the smear with drops of alcohol for 10 to 15 seconds, then rinsing it with sterile distilled water. Finally, the smear was stained with fuchsin for 1 minute. After washing and drying using filter paper, the slides were observed at 100× under a light microscope with the help of immersion oil.

4.3. Physiological and biochemical properties

4. 3.1. Catalase test

Using a sterile inoculating loop, a young bacterial colony of each isolated actinomycetes strain was transferred and placed on glass slides. A 3% hydrogen peroxide (H_2O_2) drop was added to the colonies. A positive test is the production of effervescence or bubbles in 5-10 seconds, and the absence of effervescence or bubbles is considered a negative test (Reiner, 2010).

4. 3.2. Carbon source utilization

According to Shirling and Gottlieb (1966) carbon source utilization was determined using the Pridham and Gottlieb carbon utilization medium. After autoclaving and cooling the basal agar medium, sterile carbon sources, including glucose, fructose, lactose, maltose,

sorbitol, arabinose, galactose, mannitol, and saccharose, were added separately and aseptically to give a concentration of 1% under a magnetic stirrer. The mixtures were distributed into sterile petri dishes to be ready for use. Positive and negative controls were used to compare the growth.

4.3.3. Assimilation of nitrogen source

The ability to utilize different nitrogen sources was studied using the Pridham and Gottlieb medium added with 0.5 % amino acid solutions, including asparagine, arginine, glycine, and proline (Chávez-Hernández et al., 2023).

5. Screening of extracellular hydrolytic enzymes production

5.1. Protease production

Protease production was tested by growing all isolated actinomycetes strains on glucose yeast extract peptone agar medium [g/mL: glucose 1 g, yeast extract 0.1 g, peptone 0.5 g, agar 15 g, and distilled water 1000 mL, pH 7.3] amended with 5% sterile skim milk. After incubation for 5 to 7 days at 28 °C, a clear zone around colonies indicate the production and secretion of proteases (Mahfooz et al., 2017).

5.2. Amylase production

Amylase production was also tested using glucose yeast extract peptone agar medium [g/mL: glucose 1 g, yeast extract 0.1 g, peptone 0.5 g, agar 15 g, and distilled water 1000 mL, pH 7.3] containing 1% soluble starch. After incubation for 5 to 7 days at 28°C, all plates were flooded with Lugol to observe a clear zone around the actinomycetes colonies (Sadar et al., 2017).

5.3. Lipase production

The production of lipase from isolated actinomycetes strains was evaluated on peptone agar [g/mL: peptone 10 g, NaCl 5 g, agar 17 g, and distilled water 900 mL, pH 7.3] supplemented with 10 g of Tween 80 suspended in 100 mL of distilled water and sterilized separately. After incubation for 5 to 7 days at 28 °C, a white precipitate surrounding the colonies was recorded as a positive result for the production of lipases (Naligama et al., 2022).

6. Diversity analysis of isolated actinomycetes strains

The physiological and biochemical properties of all the isolated actinomycetes strains

and the production of extracellular enzymes were analyzed using cluster analysis with the PAST software and a binary matrix (1: positive test and 0: negative test). A dendrogram was constructed using the classical clustering with a Jaccard similarity and the unweighted pair group method with arithmetic mean (UPGMA) (Khirennas et al., 2023; Sharma et al., 2021).

7. Screening of antimicrobial activity

7.1. First screening

The first screening of all the isolated actinomycetes strains was performed using the perpendicular streak method against *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *C. albicans* ATCC 10231. Using Mueller-Hinton agar plates, the isolated strains were streaked along the diameter of the plates (one strain in one plate) and incubated for 5 to 7 days at 28 °C. Then, the microbial suspensions of test microorganisms were adjusted to 10^7 CFU/mL and streaked perpendicular to isolated strains, and subsequently all plates were incubated at 37°C for 24 h. The inhibition zones revealed antimicrobial activity (Sapkota et al., 2020).

7.2. Second screening

Based on the first screening results, the actinomycetes strains that demonstrated good activity were selected for the second screening using the double-layer agar method. The selected strains were spotted on ISP-2 agar plates and incubated at 28°C for 5 to 7 days. Then, assay tubes containing 9 mL of semi-solid Mueller-Hinton agar were seeded with 1 mL of microbial suspensions of test microorganisms used in the first screening and poured on spotted plates. After incubation at 37 °C for 24 h, the antimicrobial activity was determined by measuring the inhibition zones in millimeters (mm) (Ahmad et al., 2017).

8. Identification of selected actinomycetes strains

8.1. Scanning electron microscopy (SEM)

After growing for 14 days at 28°C on ISP-2 agar, the morphology of aerial mycelium of selected actinomycetes strains was observed directly through environmental scanning electron microscopy at 10,000x and 20,000x magnification with an accelerating voltage of 10 kV (Thermo Fisher Scientific, USA) (Messaoudi et al., 2020).

8.2. Molecular identification

The molecular identification of the selected actinomycetes strains was carried out as a paid service. The strains A6, A24, and A28 were identified by the Gene Life Sciences

Laboratory (Sidi Bel Abbes, Algeria), while the other two strains, A1 and A27 were identified by the AGRIMAQ laboratory (University of L'Aquila, Italy). Initially, the strains were subcultured in the ISP-2 agar and incubated for 7 days at 28°C. Then, the extraction of bacterial genomic DNA was performed using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia) according to the manufacturer's instructions. After that, and following the PCR conditions, 16S rRNA gene amplification was achieved using universal primers, including 27F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1429R (5'-GGTTACCTTGTTACGACTT-3'), 27f-Bif (5'-AGGGTTCGATTCTGGCTCAG-3'), and Bif662-r (5'-CCACCGTTACACCGG GAA-3'). After the PCR reaction, the products were separated into a 1.5% agarose gel (Sigma-Aldrich, USA), purified using a clean-up kit (Vivantis Technologies, Malaysia), and sequenced using a 3130 Genetic Analyzer Capillary (Applied Biosystems, USA). Subsequently, 16S rRNA gene sequences of strains were compared using the BLAST program available at the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the neighbor-joining tree method and Clustal W algorithm, a phylogenetic tree was constructed using MEGA 11 software (Khirennas et al., 2023; Mokhnache et al., 2025).

Results and discussion

1. Isolation, purification, and conservation of actinomycetes

In this study, 33 actinomycetes strains were isolated using different media, purified, and conserved from 12 bee pollen samples collected from many places in Algeria. As presented in Figure 2.3, out of all used culture media, starch casein agar (SCA) showed the highest number of isolated actinomycetes strains, followed by yeast extract-malt extract agar (YMA), glycerol asparagine agar (GAA), actinomycetes isolation agar (AIA), streptomyces agar (SA), Czapeck agar (CZA), and nutrient agar (NA), with growth percentages amounting to 24.24, 21.21, 15.15, 12.12, 12.12, 9.10, and 6.06 %, respectively. These results are in agreement with other previous studies that reported that the starch casein agar (SCA) is the most suitable medium for the isolation of actinomycetes (Bawazir et al., 2018; Lee et al., 2014; Rahman et al., 2011).

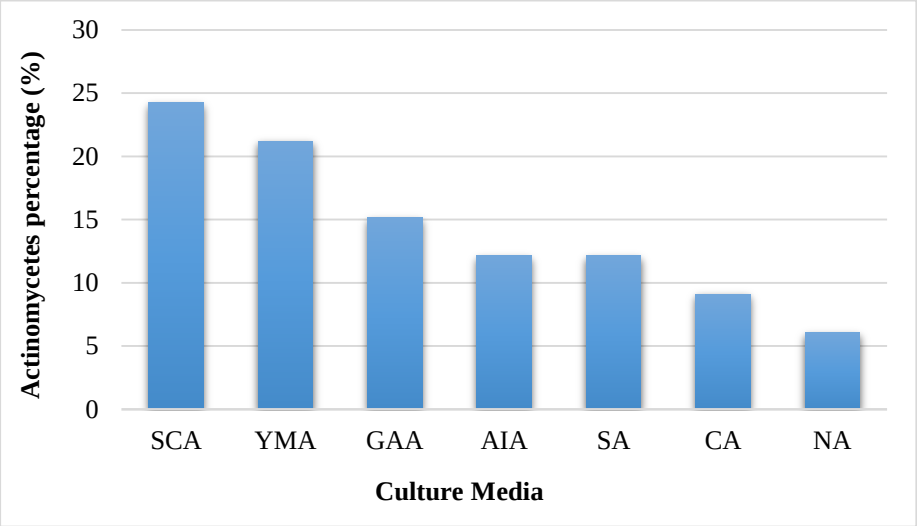


Figure 2.3. The distribution of isolated actinomycetes strains using different culture media.

According to our results shown in Table 2.1, from 12 samples of bee pollen, only eight samples contained actinomycetes were found. Of the 33 strains that were isolated, eight strains were found in the sample obtained from the local market in Setif, followed by Bejaia (7 strains), Tipaza 2 (7 strains), Mila (4 strains), Tipaza 1 (3 strains), Setif 1 (2 strains), Setif 2 (1 strain), and Skikda (1 strain). The combination of the pretreatment applied on collected bee pollen samples with the use of different culture media allowed for isolating a maximum of actinomycetes strains. The air drying and heat treatment used as a physical pretreatment stimulated the isolation of actinomycetes by eliminating unwanted microorganisms and reducing the growth of Gram-negative bacteria (Goodfellow, 2014; Vijayakumar et al., 2007). The enrichment treatment with calcium carbonate, which was used as a chemical pretreatment, helped actinomycetes grow as an additional carbon source, and it also changed the acidic pH of the bee pollen samples to a basic pH, which promoted the growth of mycelium and the formation of spores (Hernández-Bolaños et al., 2020). Furthermore, the addition of cycloheximide and fluconazole into the culture media as antifungal agents strongly suppressed the growth of fungi (Belyagoubi et al., 2018; Fernandes et al., 2021; Subhashini, 2018).

Table 2.1. Number of isolated strains of actinomycetes in the collected bee pollen samples.

Samples	Origin	Number of isolated strains
1	Medea	00
2	Guelma	00
3	Setif 1	02
4	Setif 2	01

5	Skikda	01
6	Setif 3	00
7	Mila	04
8	Bejaia	07
9	Tipaza 1	03
10	Tipaza 2	07
11	Blida	00
12	Local market	08
Total : 33 strains		

2. Characteristics of isolated actinomycetes strains

2.1. Morphological and cultural properties

The morphological and cultural properties of actinomycetes strains isolated from the collected bee pollen samples were observed using ISP-2 agar. All isolated strains were tabulated in Table 2.2 according to their colony size, colony color, colony shape, colony texture, growth intensity, pigmentation, and Gram staining.

Table 2.2. Morphological and cultural properties of isolated actinomycetes strains.

Strains	Growth intensity	Colony size	Colony color	Colony shape	Colony texture	Pigmentation	Gram staining
A1	Excellent	3 mm	Grey	Irregular	Powdery	-	+
A2	Good	4 mm	Dark brown	Circular	Cottony	-	+
A3	Excellent	6 mm	Grey	Irregular	Powdery	-	+
A4	Excellent	1 mm	White cream	Circular	Powdery	-	+
A5	Good	5 mm	Pale white	Irregular	Powdery	-	+
A6	Excellent	3 mm	White	Circular	Powdery	-	+
A7	Good	2 mm	Pale white	Circular	Powdery	-	+
A8	Excellent	5 mm	Dim grey	Irregular	Powdery	-	+
A9	Good	6 mm	White	Circular	Cottony	-	+
A10	Excellent	4 mm	Dark grey	Circular	Powdery	-	+
A11	Good	6 mm	Brown grey	Circular	Powdery	-	+
A12	Excellent	5 mm	Dark grey	Irregular	Powdery	Dark green	+
A13	Excellent	4 mm	Light grey	Irregular	Powdery	-	+
A14	Medium	3 mm	White	Circular	Powdery	-	+
A15	Excellent	5 mm	Grey black	Circular	Powdery	-	+
A16	Good	3 mm	Light grey	Irregular	Powdery	-	+
A17	Good	4 mm	White smoke	Circular	Powdery	-	+
A18	Good	5 mm	Yellow	Circular	Sticky	-	+
A19	Excellent	4 mm	Grey	Circular	Powdery	-	+
A20	Excellent	4 mm	Light grey	Circular	Powdery	-	+
A21	Excellent	4 mm	Dim grey	Circular	Powdery	-	+
A22	Good	1 mm	Black	Circular	Hard	-	+
A23	Excellent	3 mm	Light grey	Circular	Powdery	Dark red	+

A24	Excellent	2 mm	White	Circular	Powdery	-	+
A25	Excellent	3 mm	White smoke	Circular	Powdery	-	+
A26	Excellent	3 mm	Light grey	Circular	Powdery	-	+
A27	Good	3 mm	Light pink	Circular	Sticky	-	+
A28	Good	6 mm	Off-white	Irregular	Cottony	-	+
A29	Medium	3 mm	Light brown	Circular	Powdery	-	+
A30	Excellent	5 mm	Grey	Irregular	Powdery	-	+
A31	Excellent	3 mm	Pale white	Irregular	Powdery	-	+
A32	Excellent	4 mm	Grey	Irregular	Powdery	-	+
A33	Excellent	3 mm	Dark grey	Irregular	Powdery	-	+

Generally, the observation of morphological and cultural properties of isolated strains using ISP-2 agar showed powdery colonies, an earthy odor, excellent growth, and abundant mycelial formation with a significant diversity of color that varied from white to grey, brown, yellow, pink, and black (Figure 2.4). Furthermore, among all isolated strains, only two produced dark green and dark red diffusible pigments, namely strain A12 and strain A23, respectively. On the other hand, all isolated strains subjected to Gram staining showed a Gram-positive reaction with filamentous structures. Based on Bergey's manual of systematic bacteriology, actinomycetes are Gram-positive bacteria that are characterized by the mycelial and spore formation, variation in their colors, and can present an earthy odor due to their ability to produce distinctive odorous substances, including pigments (Goodfellow et al., 2012). Numerous studies have demonstrated that the diffusible pigments produced by actinomycetes can exhibit various biological activities, including antioxidant, antimicrobial, antitumor, and others (Fernandes et al., 2021; Parmar & Singh, 2018).

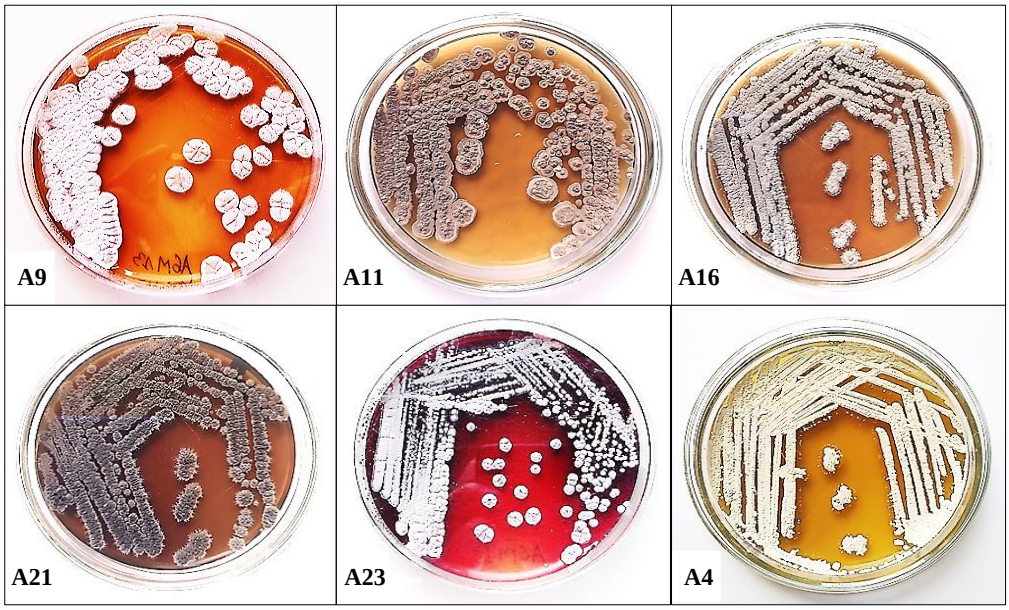


Figure 2.4. Some actinomycetes strains isolated from collected bee pollen.

2.2. Physiological and biochemical properties

The catalase test and the ability to use different sources of carbon and nitrogen are important biochemical and physiological properties for the study of actinomycetes (Goodfellow et al., 2012; Shirling & Gottlieb, 1966). As illustrated in Figure 2.5, our actinomycetes strains isolated from the collected bee pollen samples revealed physiological and biochemical variations. Regarding the catalase test, the majority of isolated strains showed a positive catalase test (84.85 %), while 15.15 % of strains were catalase negative. Different sugars were used as carbon sources, including glucose, fructose, lactose, maltose, sorbitol, arabinose, galactose, mannitol, and sucrose. Out of all isolated actinomycetes, most of the strains had the ability to utilize all sugars (19 strains), and 13 strains utilized at least one sugar, while only one strain didn't utilize any sugar as a carbon source. Amino acids, including asparagine, arginine, proline, and glycine, were used as nitrogen sources. The majority of isolated actinomycetes (23 strains) utilized all amino acids, and 9 strains utilized at least one amino acid, while only one strain didn't utilize any amino acid as a nitrogen source. In previous studies, some strains of actinomycetes didn’t have the ability to utilize some sugars and amino acids as carbon and nitrogen sources (Khirennas et al., 2023; Sharma et al., 2021).

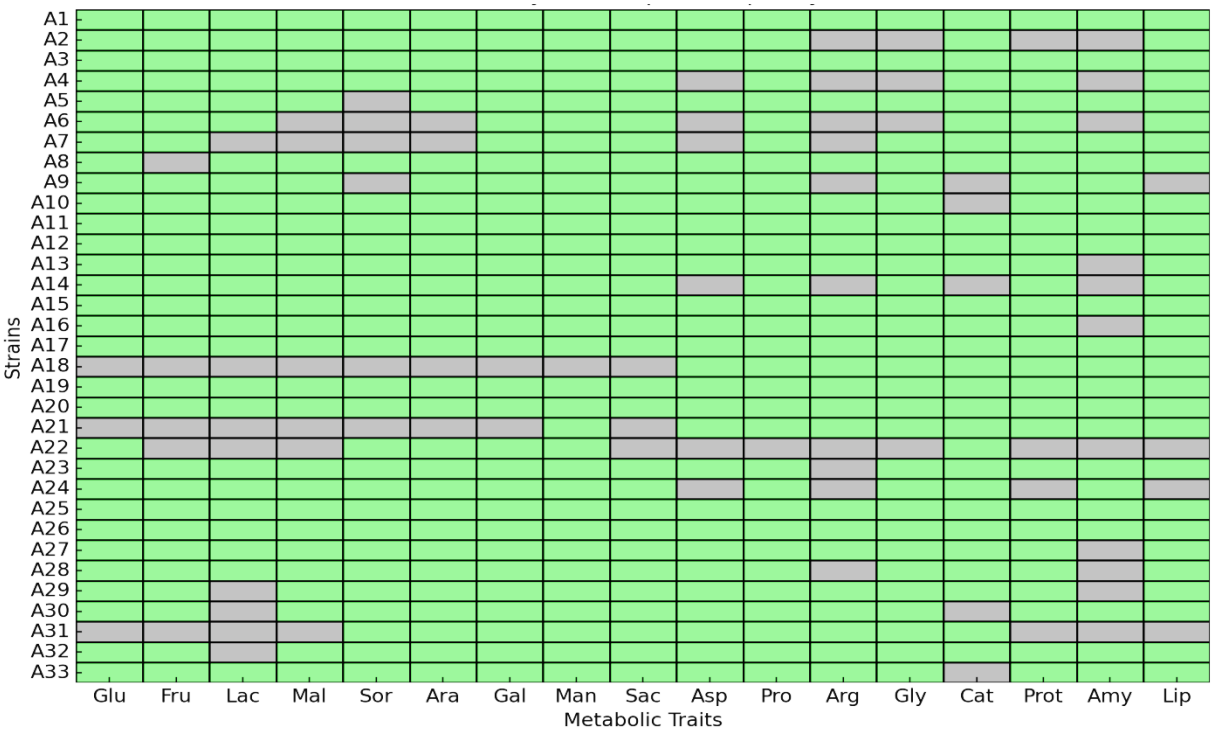


Figure 2.5. Physiological and biochemical properties of isolated actinomycetes. Glu: Glucose, Fru: Fructose, Lac: Lactose, Mal: Maltose, Sor: Sorbitol, Ara: Arabinose, Gal: Galactose, Man: Mannitol, Sac: Saccharose, Asp: Asparagine, Pro: Proline, Arg: Arginine, Gly: Glycine, Cat: Catalase, Prot: Protease, Amy: Amylase, Lip: Lipase. Green: Positive result, Grey: Negative result.

3. Screening of extracellular enzymes production

All isolated actinomycetes strains were subjected to three different enzymatic activity screenings, including protease, amylase, and lipase. Figure 2.6 presents a qualitative screening for extracellular enzyme production in some isolated strains. As shown in Figure 2.5, the majority of isolated strains have the ability to produce extracellular hydrolytic enzymes. However, four strains (A2, A22, A24, and A31) don't show a proteolytic activity. Also, eleven strains (A2, A4, A6, A13, A14, A16, A22, A27, A28, A29, and A31) don't display an amylolytic activity. And also four strains (A9, A22, A24, and A31) don't demonstrate a lipolytic activity. On the other hand, only the strain A31 was the one that didn't have the ability to produce any enzyme. Various previous studies have reported and confirmed the production of extracellular enzymes by actinomycetes, including proteases, amylases, and lipases (Naligama et al., 2022; Singh et al., 2019). In addition, actinomycetes are important bacteria that have the ability to produce a variety of extracellular hydrolytic enzymes used in various industrial applications (Jassim & Jarallah, 2023; Khirennas et al., 2023).

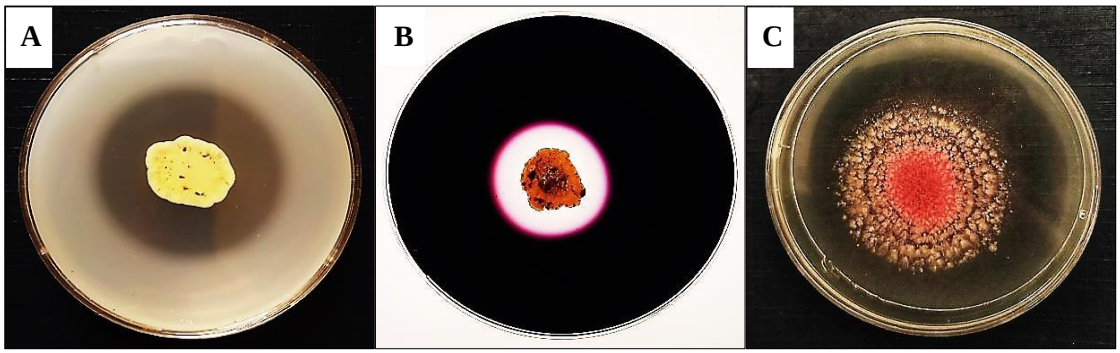


Figure 2.6. Qualitative screening for extracellular enzyme production. **(A)** Protease of A11, **(B)** Amylase of A32, **(C)** Lipase of A27.

4. Diversity and numerical analysis of isolated actinomycetes strains

This study evaluated the diversity of isolated actinomycetes strains by comparing their different physiological and biochemical properties, including carbon source utilization, nitrogen source assimilation, catalase test, and extracellular hydrolytic enzyme production. According to the dendrogram shown in Figure 2.7, eight diversified clusters were obtained, each containing two strains or more. Among the isolated actinomycetes, cluster III is the major cluster with ten strains (A1, A3, A11, A12, A15, A17, A19, A20, A25, and A26), accounting for 30.30 % of the strains. The diversity study of the phenotypic characteristics of actinomycetes through numerical analysis is critical to knowing and understanding the

relationships, similarities, and variations among them (Khirennas et al., 2023; Sharma et al., 2021).

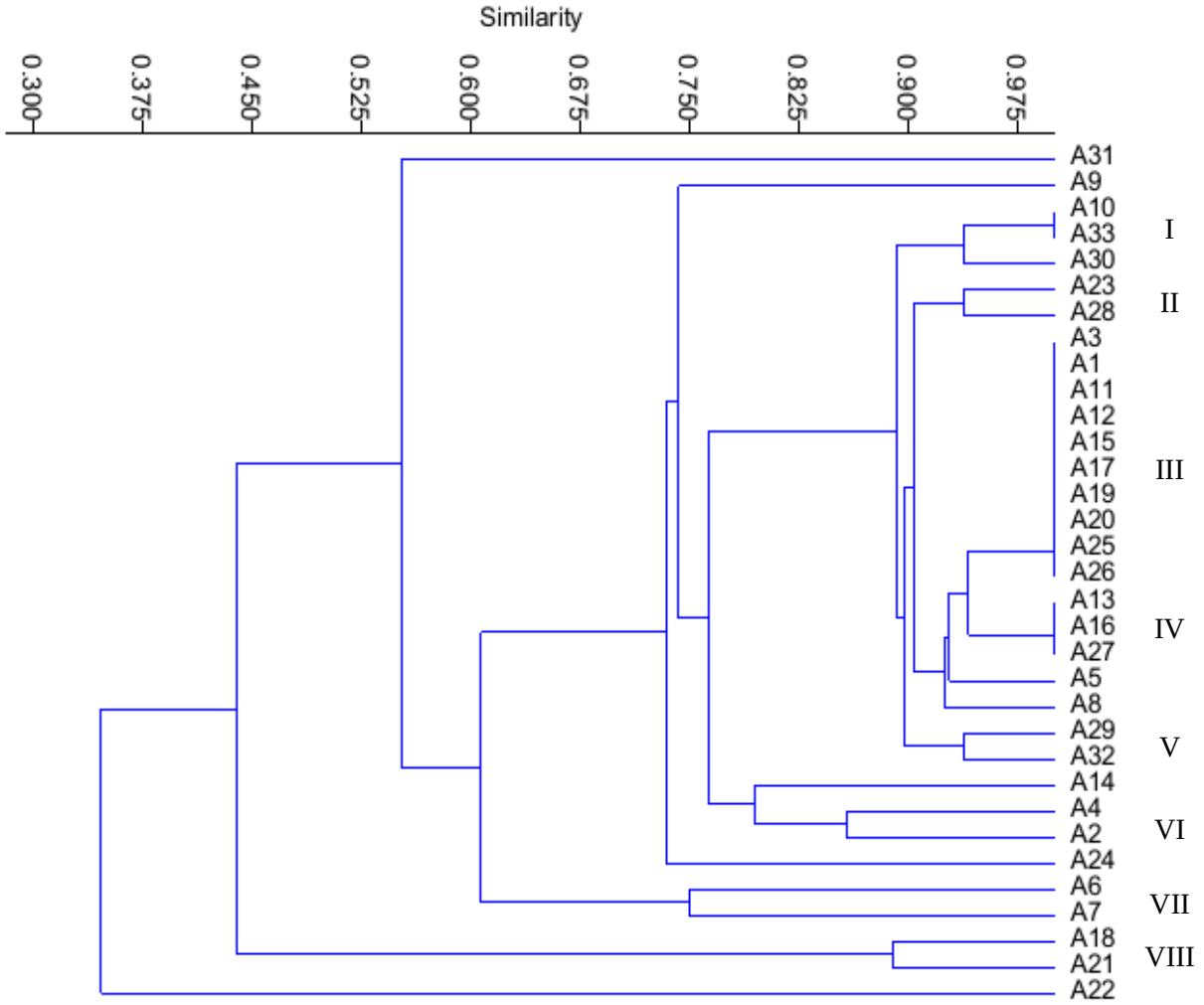


Figure 2.7. Dendrogram based on comparing of physiological and biochemical properties of isolated actinomycetes.

5. Screening of antimicrobial activity

5.1. First screening

The first screening of isolated actinomycetes for antimicrobial activity was evaluated by the perpendicular streak method (Figure 2.8). Only five of the isolated strains, namely A1, A6, A24, A27, and A28, demonstrated antibacterial activity against two or more of the tested bacterial strains, including Gram-positive bacteria (*S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633) and Gram-negative bacteria (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 13883). Additionally, only three isolated strains, namely A6, A24, and A28, exhibited antifungal activity against *C. albicans* ATCC 10231 (Table 2.3). The study of Aku et al., (2022) reported that the results of the first screening for antimicrobial

activity using the perpendicular streak method showed that only 27 of the 113 actinomycetes strains that were isolated from 21 samples collected from different sources in Nigeria exhibited antibacterial activity against at least one of the tested bacterial strains, which included *S. aureus* ATCC 25923, *B. cereus* IFO 13804, *E. coli* JCM 20135, *P. aeruginosa* ATCC 25783, and *K. pneumonia*. Furthermore, just three strains showed antifungal activity against one or more of the fungal strains, including *C. albicans* ATCC 10231.

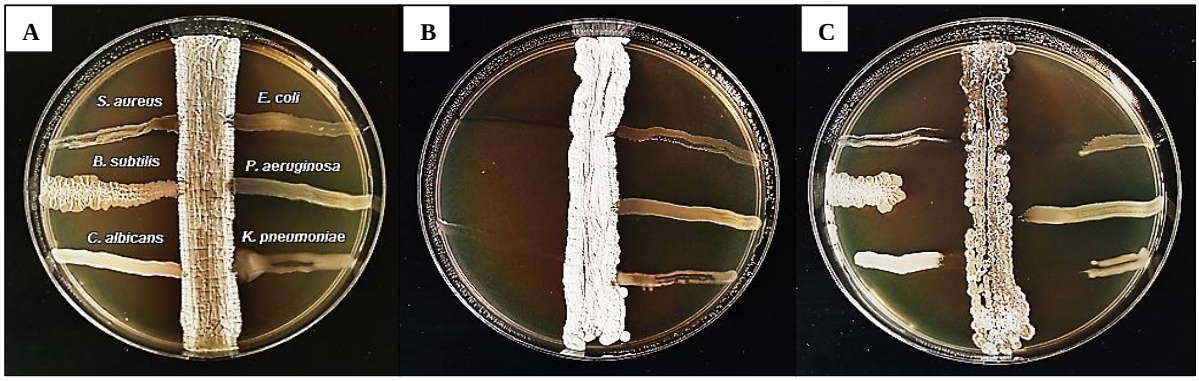


Figure 2.8. First screening of antimicrobial activity by perpendicular streak method. **(A)** A25 strain, **(B)** A6 strain, **(C)** A28 strain.

Table 2.3. Results of the first screening of antimicrobial activity.

Strains	First screening of antimicrobial activity					
	S. aureus	***B. subtilis***	***E. coli***	***P. aeruginosa***	***K. pneumoniae***	***C. albicans***
A 1	+	+	-	-	-	-
A 6	+	+	-	-	-	+
A 24	+	+	-	-	-	+
A 27	+	+	+	-	+	-
A 28	+	+	+	+	+	+
Other strains	-	-	-	-	-	-

(+) : positive activity, (-) : negative activity.

5.2. Second screening

The second screening of the selected strains for antimicrobial activity was evaluated using the double-layer agar method (Figure 2.9). As presented in Table 2.4, the second screening showed that the selected actinomycetes strains were effective against at least two of test microorganisms. These microorganisms include *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *C. albicans* ATCC 10231. The strains A6 and A24 demonstrated significant

antibacterial and antifungal activities against *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, and *C. albicans* ATCC 10231, with inhibition zones of 70, 49, 64, 48, 36, and 31 mm, respectively. However, the strains A1, A27, and A28 displayed only antibacterial activity. Actinomycetes grown on agar plates can produce diffusible bioactive secondary metabolites with a strong growth inhibition of the test microorganisms (Charousová et al., 2019; El Karkouri et al., 2019). These bioactive metabolites can target different parts of the microbial metabolism with both antibacterial or antifungal effects, such as cell wall biosynthesis, DNA wrapping, transcription or translation, and some specific features (De Simeis & Serra, 2021). In our study, Gram-positive test bacteria and fungi are more susceptible to the bioactive secondary metabolites produced by the selected strains. In contrast, the Gram-negative test bacteria are more resistant. Similarly, a previous study determined a qualitative antimicrobial activity of actinomycetes isolated from Moroccan soils against *S. aureus* ATCC 29213, *B. subtilis* ATCC 6633, *E. coli* K12, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 using a double-layer method, revealing that the strongest inhibitory effects were noted against Gram-positive bacteria (*S. aureus* and *B. subtilis*) and fungi (*C. albicans*) as compared to those tested against Gram-negative bacteria (Ait Assou et al., 2023). The structural and functional differences in their cell walls mainly cause of this differential susceptibility (Breijyeh et al., 2020). Gram-negative bacteria have an outer membrane composed of lipopolysaccharides, proteins, and phospholipids, an effective permeability barrier. This outer membrane makes it more difficult for antibiotics and other antimicrobial agents to penetrate and reach their targets (Lehman & Grabowicz, 2019). While Gram-negative bacteria have this important outer membrane layer, Gram-positive bacteria don't. Instead, Gram-positive bacteria have a more straightforward cell wall structure with a thick peptidoglycan layer, which is not as effective as an outer membrane layer. This makes Gram-positive bacteria more susceptible to antimicrobial agents than Gram-negative bacteria (Abou-Dobara et al., 2018; Riu et al., 2022).

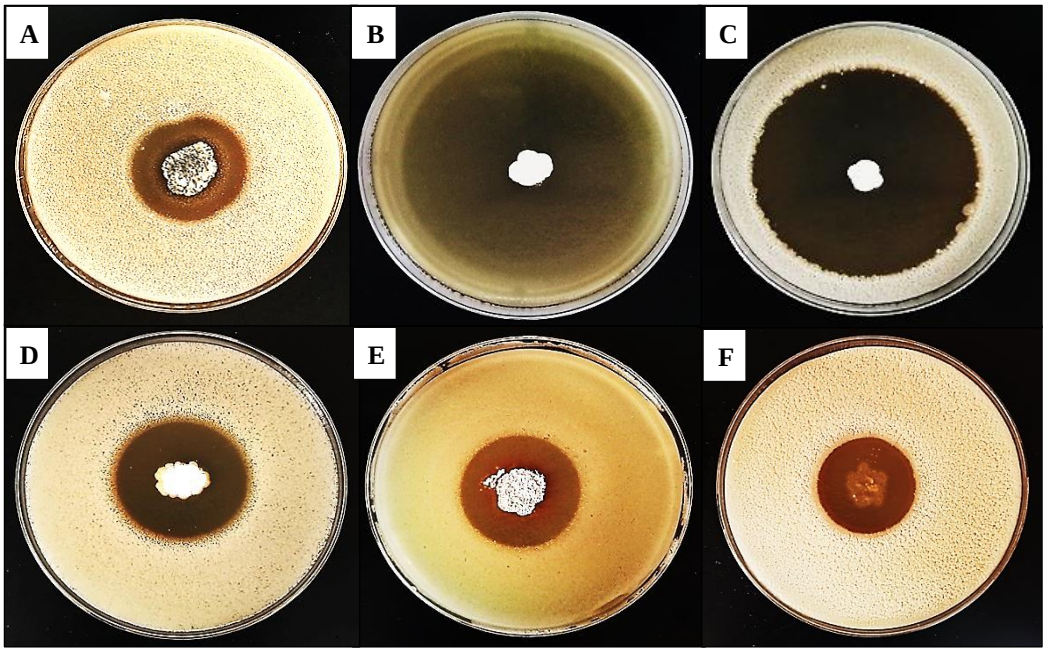


Figure 2.9. Second screening of antimicrobial activity by double layer agar method. **(A)** A1 against *B. subtilis*, **(B)** A6 against *S. aureus*, **(C)** A6 against *B. subtilis*, **(D)** A24 against *C. albicans*, **(E)** A27 against *S. aureus*, **(F)** A28 against *B. subtilis*.

Table 2.4. Results of the second screening of antimicrobial activity.

Strains	Second screening of antimicrobial activity					
	S. aureus	***B. subtilis***	***E. coli***	***P. aeruginosa***	***K. pneumoniae***	***C. albicans***
A 1	27 mm	25 mm	00 mm	00 mm	00 mm	00 mm
A 6	70 mm	64 mm	00 mm	00 mm	00 mm	36 mm
A 24	49 mm	48 mm	00 mm	00 mm	00 mm	31 mm
A 27	34 mm	26 mm	00 mm	00 mm	00 mm	00 mm
A 28	00 mm	29 mm	22 mm	00 mm	16 mm	00 mm

6. Identification of selected actinomycetes strains

6.1. Scanning electron microscopy (SEM)

The detailed structural characteristics of the selected actinomycetes strains' surface morphology were examined by direct scanning electron microscopy (SEM). They were shown to have a filamentous morphology that forms a complex network and a mycelium with many branches that hold mass and mature chains of cylindrical spores with smooth surfaces (Figure 2.10). The scanning electron microscope (SEM) analysis of actinomycetes typically can provide a view of the structural features, particularly their filamentous morphology, as well as a detail of the shape, size, and arrangement of their spores. Actinomycetes appear as long and

branching filaments known as mycelia for their resemblance to fungal mycelium, organizing their spores in chains or clusters. Many actinomycetes species, such as those in the genus *Streptomyces*, produce spores exhibiting surface textures that can be either rough or smooth, depending on the species (Sailaja Rani J. et al., 2024; Uasoontornnop et al., 2022).

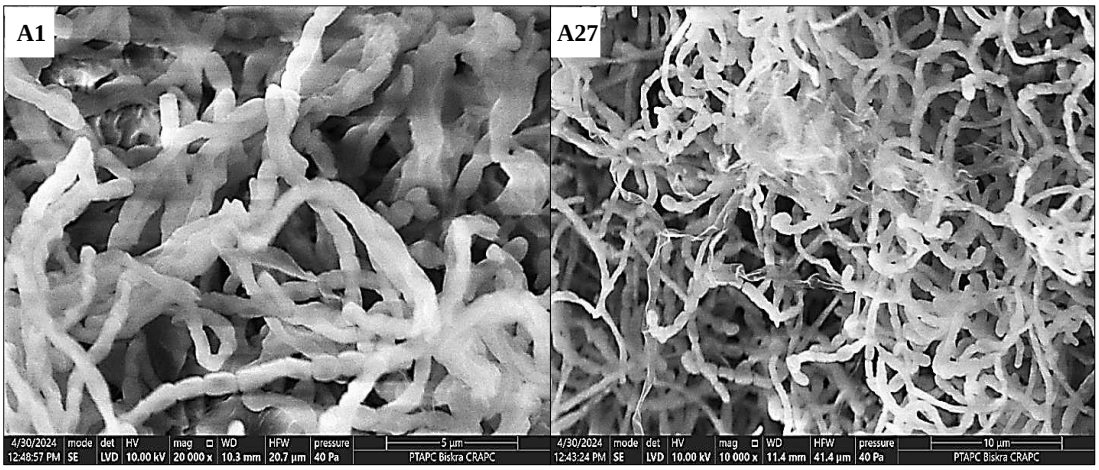


Figure 2.10. SEM microscopic morphology of A1 and A27 strains.

6.2. Molecular identification

The obtained 16S rRNA sequences identify strains A1, A24, A27, and A28 as belonging to the *Streptomyces* genus. They were registered in GenBank with the accession numbers PP577932.1, PQ821170.1, PV131599.1, and PP577933.1, respectively. The phylogenetic tree, shown in Figure 2.11, constructed with the help of BLAST and MEGA 11 for the selected strains A1, A6, A24, A27, and A28, illustrates the evolutionary relationships among the various strains based on sequence analysis. *Nocardia* sp. (OQ235041.1) was selected as an outgroup to root the tree. All strains were placed within the *Streptomyces* genus. Strains A1 and A24 were grouped with *Streptomyces globosus* and *S. atroolivaceus*, respectively, and closely related to *S. amulatus*. Strain A28 grouped with *S. olivoreticillatus*, closely related to *S. caelestis* and *S. luteus*. Strains A6 and A27 closely grouped with *Streptomyces tuirus.*

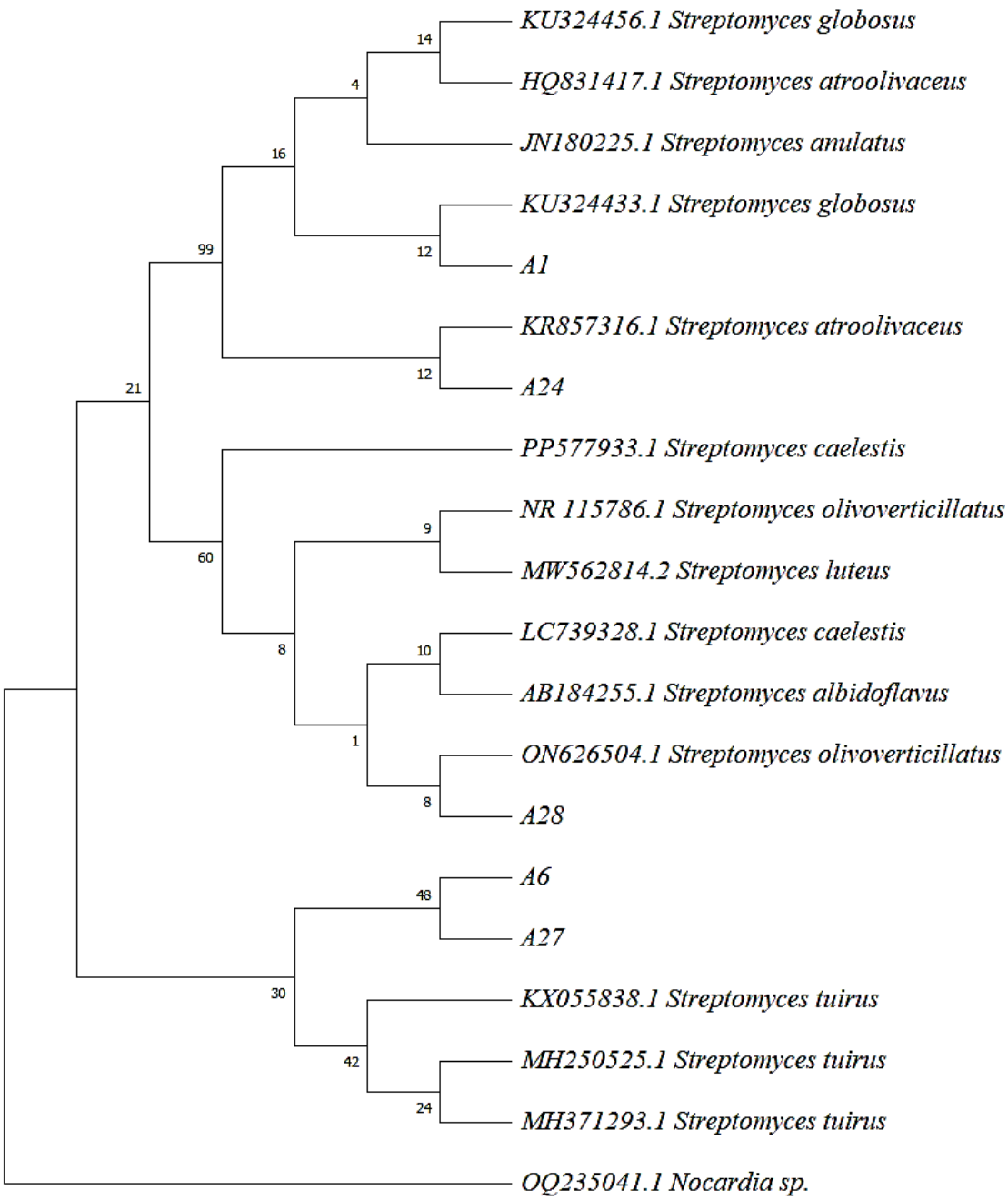


Figure 2.11. Phylogenetic tree using the Maximum Likelihood method and Kimura 2-parameter model of A1, A6, A24, A27, and A28 strains and NCBI sequences that obtained a percent identity above 99%.

Conclusion

The isolation, characterization, and screening of actinomycetes from bee pollen samples collected from different Algerian places have been successfully studied, revealing a diverse array of *Streptomyces* strains that have a significant potential for the production of extracellular enzymes and antimicrobial compounds. Through the screening for extracellular

enzyme production, the majority of isolated strains demonstrated considerable production of proteases, amylases, and lipases, which are highly valuable and necessary in various industrial applications. Furthermore, the antimicrobial screening revealed that several strains exhibited significant inhibitory activity against *S. aureus*, *B. subtilis*, and *C. albicans*, suggesting them as a promising source for novel and natural alternative antimicrobials. Further studies are required to optimize the production, extraction, purification, and identification of produced bioactive compounds and evaluation of their applications.

Chapter 3

Molecular identification, antimicrobial activity, and GC-MS analyses of bioactive compounds produced by the *Streptomyces* sp. BPA-6 strain isolated from bee pollen collected from Algeria

Introduction

Antimicrobial resistance is currently an important health problem that has emerged as a global crisis, affecting the health of humans as well as animals. This phenomenon led to the ever-increasing appearance and spreading of novel diseases and infections caused by microorganisms, including bacteria, fungi, viruses, and parasites, which developed different mechanisms to resist the available antimicrobial agents (Reygaert, 2018; Collignon et al., 2018; Prestinaci et al., 2015). In addition to the misuse and overuse of antimicrobials, numerous factors contribute to antimicrobial resistance, such as spontaneous gene mutation, poor hygiene and sanitation, food processing, migration, and the use of biocides as disinfectants (Aslam et al., 2018; Oniciuc et al., 2019; World Health Organization, 2014). This situation resulted in a reduction in the efficacy of the available antimicrobials, leading the researchers to discover and develop novel alternative antimicrobial agents with enhanced effectiveness to fight the problem of antimicrobial resistance (Ghosh et al., 2019; Ong et al., 2020). The most important of these alternatives are the secondary metabolites, which are bioactive compounds with significant potential to be used as pigments, growth hormones, antitumor agents, and antimicrobial agents. These bioactive natural products can be secreted by microorganisms such as fungi and bacteria, including actinomycetes (Singh et al., 2019). Actinomycetes are considered the major producers of secondary metabolites, and they represent one of the largest natural microbial sources, as about 61% of the discovered bioactive microbial metabolites have been isolated from them (Djinni et al., 2019; Hassan et al., 2019). One of the most important genera of actinomycetes, *Streptomyces* is known for producing a wide range of antimicrobial compounds, including terpenes, tetracyclines, macrolides, glycopeptides, aminoglycosides, and ansamycins, showing different antibacterial and antifungal activities (Alam et al., 2022). Even though the studies on the *Streptomyces* associated with beehive products are limited, the 16S rRNA high-throughput sequencing revealed that it is one of the dominant bacterial genera present in bee pollen and bee bread (Ghosh et al., 2022). The presence of *Streptomyces* in bee pollen confirms that this bee product could be a source of new bioactive compounds that have the capability to function as antimicrobials in the face of antibiotic-resistant diseases (Grubbs et al., 2021).

In the present study, the aim is to produce, extract, and identify bioactive secondary metabolites of the *Streptomyces* sp. BPA-6 strain isolated from bee pollen collected from Algeria, as well as to demonstrate their potential as antimicrobials against some pathogenic microorganisms, including bacteria and fungi.

Materials and methods

1. Reagents, media, and microorganisms

Dimethyl sulfoxide (DMSO) and yeast extract were purchased from Sigma. Glycerol and ethyl acetate were purchased from Merck. Tetrazolium salt (0.25 %) was from MicroBiotech. Muller-Hinton broth was obtained from Conda and malt extract was obtained from Liofilchem. The GF-1 Nucleic Acid Extraction Kit and universal primers (27F and 1492R) were from Vivantis Technologies. Gentamycin discs were provided from Oxoid™. *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, *E. coli* ATCC 7839, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *C. albicans* ATCC 10231 were obtained from the applied microbiology laboratory, Ferhat Abbas University, Algeria.

2. Characterization of the actinobacterial strain (BPA-6)

The actinobacterial strain (BPA-6) used in this study was obtained from the isolate collection of the applied microbiology laboratory, University of Ferhat Abbas Setif 1, Algeria, that were isolated from bee pollen samples in 2020. The strain was subcultured, purified, and maintained in slant culture on 15 % glycerol yeast extract-malt extract agar (ISP-2) and stored at 4°C as stock culture.

2.1. Morphological, cultural, and biochemical characteristics

The BPA-6 strain was initially characterized using the ISP-2 agar based on macroscopic and microscopic properties, including the growth abundance, texture, spore color, aerial and substrate mycelium color, soluble pigment, biochemical characters, Gram staining, and scanning electron microscopy (Ayoubi et al., 2018; Dharmaveera et al., 2018; Wadetwar & Patil, 2013).

2.2. Molecular identification

The BPA-6 strain was molecularly identified by the Gene Life Sciences Laboratory (Sidi Bel Abbes, Algeria) as a paid service. Briefly, the DNA was extracted using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia), following the manufacturer's instructions. Then, 27F and 1492R primers were used to amplify the 16S rRNA under the following PCR conditions: initial denaturation at 94 °C for 12 minutes, denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute and 40 seconds. The amplification process was repeated for 30 cycles, followed by a final extension at 72 °C for 7 minutes. After that, the PCR products were subjected to electrophoresis,

purified, and subsequently sequenced with the help of an automated DNA sequencer (Applied Biosystems®, USA) (Lakhdar et al., 2023). After sequencing, The Basic Local Alignment Tool (BLAST) program was used to compare the obtained sequence of the 16S rRNA gene of our strain (BPA-6) with eight homologous sequences available in the NCBI GenBank database (Table 3.1), and subsequently the phylogenetic analysis was performed using MEGA 10 software (Balachandran et al., 2021).

Table 3.1. Homologous sequences to the BPA-6 strain available in the GenBank database.

No.	Description	Query Cover	E value	Pairwise Identity	Accession Number
1	*Streptomyces* sp. strain A4	100%	0.0	90.24%	OR098551.1
2	*Streptomyces thermolilacinus* strain G31	100%	0.0	89.11%	ON810411.1
3	*Streptomyces* sp. NIIST A23	100%	0.0	89.09%	KM873340.1
4	*Streptomyces luteoverticillatus* strain T0907-107	99%	0.0	89.14%	KM657645.1
5	*Streptomyces cinnamoneus* strain JCM 4633	99%	0.0	88.98%	MT760587.1
6	*Streptomyces buecherae* strain act3	99%	0.0	88.98%	OM491327.1
7	*Streptomyces fradiae* strain Fim09-0041	99%	0.0	88.83%	JQ819743.1
8	*Streptomyces* sp. strain Kukup BR1-19	99%	0.0	88.54%	OL635598.1

3. Production and extraction of crude extract

Firstly, the actinobacterial isolate (BPA-6) was cultured on ISP-2 agar for 7 days at 28 °C. Then, with the help of a sterile cork borer, a 2 cm^2 agar disc was obtained from pure culture and inoculated into an Erlenmeyer flask containing 100 mL of sterile ISP-2 broth, which was subsequently incubated at 28 °C ± 2 °C in an orbital shaker (200 rpm) for 7 days. After that, the mycelia were removed from the resulting culture broth by filtration through a filter paper, followed by centrifugation at 4000 rpm and 4 °C for 10 minutes. The cell-free clear filtrate (supernatant) was divided into two parts: the first part was filtered through a 0.45 µm syringe filter and kept directly at 4°C for the antimicrobial activity screening, while the second part was extracted by an equal volume of pure ethyl acetate, evaporated, dissolved in 20% DMSO, filtered through a 0.45 µm syringe filter, and conserved at 4°C for GC-MS analyses and antimicrobial activity (Al-Dhabi et al., 2020; Mohammed-Amin & Al-Jaf, 2024; Salim et al., 2017).

4. Antimicrobial activity

4.1. Perpendicular streak method

The perpendicular streak method was used to evaluate the primary screening for antimicrobial activity of the actinobacterial strain (BPA-6) against some pathogenic microorganisms, including *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, *E. coli* ATCC 7839, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *C. albicans* ATCC 10231 (Hossain, 2024; Sapkota et al., 2020). At first, a sterile inoculation loop was used to streak the BPA-6 strain in a straight line across the surface of a Mueller-Hinton agar plate at the center. The plate was then incubated for 5 to 7 days at 28 °C, allowing any produced antimicrobial compounds to diffuse into the agar medium. After the incubation period, all pathogenic microorganisms were adjusted to 10^7 CFU/mL and streaked in separate straight lines, intersecting the central streak at a 90-degree angle. The plate was once again incubated for 18 to 24 hours at 37 °C, and observations were recorded to evaluate the presence of growth or inhibition at the intersection of straight lines and the central streak. As a result, if the actinobacterial strain (BPA-6) produces antimicrobial compounds, they will diffuse into the surrounding agar medium, inhibiting the growth of the test microorganisms. As a result, the formation of clear inhibition zones at the intersections is a positive outcome.

4.2. Agar well diffusion method

As a secondary screening, the antimicrobial activities of the cell-free clear filtrate and the crude ethyl acetate extract obtained from the resulting culture broth after submerged fermentation of the BPA-6 strain were evaluated on Muller-Hinton agar plates using the agar well diffusion method against the previously tested pathogenic microorganisms (Balagurunathan et al., 2020; Balouiri et al., 2016). Prepared suspensions of 10^7 CFU/mL from the young cultures of tested pathogenic microorganisms were swabbed on Muller-Hinton agar plates. Then, 100 mL from the cell-free clear filtrate and the crude ethyl acetate extract were poured into wells that were made using sterilized micropipette tips. All plates were incubated for 18 to 24 hours at 37 °C. Gentamycin was used as a positive control. The inhibition zone diameter observed around each well was measured and is expressed in millimeters (mm). All experiments were carried out in triplicate. The results were expressed as means ± standard deviations.

4.3. Microbroth dilution method

4.3.1. MIC assay

With little modification, the microbroth dilution method in a 96-well microplate was used to determine the minimum inhibitory concentration (MIC) of the crude ethyl acetate extract of the actinobacterial strain (BPA-6) (Balagurunathan et al., 2020; Balouiri et al., 2016; Sadrati et al., 2020). 160 µL of sterile Mueller-Hinton broth was transferred to each well of the microplate, followed by the addition of 20 µL of 10^7 CFU/mL from young culture suspensions of *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, and *C. albicans* ATCC 10231. Then, 20 µL of different concentrations of the crude ethyl acetate extract from 7.81 to 1000 µg/mL were added to the required wells. Gentamycin and sterile Mueller-Hinton broth were used as positive and negative controls, respectively. After then, all microplates were incubated at 37 °C for about 24 to 48 hours. Finally, 20 µL of 0.25 % tetrazolium salts (TTC) was added to wells of microplates as an effective growth indicator and incubated in dark conditions for 30 minutes. As a result, the first concentration at which it gave no color change was considered the MIC value. All experiments were performed in triplicate.

4.3.2. MMC assay

The minimum microbicidal concentration (MMC) was determined directly from the MIC results prior to the addition of 0.25 % tetrazolium salts (TTC) (Balouiri et al., 2016; Sadrati et al., 2020). With the help of a bacteriological platinum handle, a loop from each well of 96-well microplates was streaked on Mueller-Hinton agar plates and incubated for 24 hours at 37 °C. The lowest concentration of the crude ethyl acetate extract that showed no visible microbial growth was recorded as the MMC value. All experiments were performed in triplicate.

5. GC-MS analysis

The crude ethyl acetate extract was dissolved in DMSO, filtered through a 0.22 µm syringe filter, and injected for direct analysis by the GC-MS Shimadzu QP-2030 system equipped with an Rxi-5ms capillary column (length 30 m, diameter 0.25 mm, and film thickness 0.25 µm). The obtained MS spectrum was interpreted by comparing it with the National Institute of Standards and Technology database to determine the bioactive compounds produced by the strain (BPA-6) with names, retention times, amounts, and suggested chemical structures (Balachandar et al., 2018; Chemmam et al., 2024).

Results and discussion

1. Morphological, cultural, and physiological characteristics of the isolate (BPA-6)

Morphological, cultural, and physiological characteristics of the actinobacterial isolate (BPA-6), including the growth abundance, texture, spore mass color, aerial and substrate mycelium color, diffusible pigment, Gram staining, shape, and biochemical properties, were well studied on ISP-2 medium and tabulated in Table 3.2.

Table 3.2. Morphological, cultural, and biochemical properties of the BPA-6 strain.

Properties	**The isolate (BPA-6)**
Morphological and cultural characteristics	
Growth abundance	Abundant growth
Growth texture	Powdery
Spore mass color	Light yellow
Aerial mycelium color	White
Substrate mass color	Dark yellow
Gram staining	Positive
Shape	Filamentous
Diffusible pigment	Negative
Carbon and nitrogen sources assimilation	
Glucose	+
Fructose	+
Maltose	-
Mannose	+
Arabinose	-
Saccharose	+
Galactose	+
Glycine	-
Proline	+
Arginine	-
Enzymes production	
Catalase	+
Oxidase	-
Protease	+
Lipase	+
Amylase	-

The BPA-6 strain was grown well on ISP-2 agar and produced powdery colonies with light yellow, white, and dark yellow spores and aerial and substrate mycelia, respectively. Also, the strain exhibited a filamentous shape and a positive Gram reaction (Figure 3.1). And also, the strain assimilated various carbon sources such as glucose, fructose, mannose, and saccharose, but it did not use maltose or arabinose. In addition, the isolate (BPA-6) utilized only proline as a source of nitrogen. Regarding the biochemical characteristics, the isolate (BPA-6) was positive for catalase and negative for oxidase, and it can produce proteases and lipases as extracellular enzymes. Actinomycetes are filamentous bacteria, exhibiting a wide

range of colors such as white, yellow, pink, red, brown, and orange (Rahman et al., 2011; Sripreechasak & Athipornchai, 2019). They have the ability to produce extracellular enzymes, including proteases, amylases, and lipases, and they may not be able to utilize some sugars and amino acids as carbon and nitrogen sources. (Khirennas et al., 2023; Singh et al., 2019). Based on the morphological, cultural, and biochemical properties and the comparison with Bergey's manual of systematic bacteriology (Goodfellow et al., 2012), the BPA-6 strain was initially identified as *Streptomyces*.

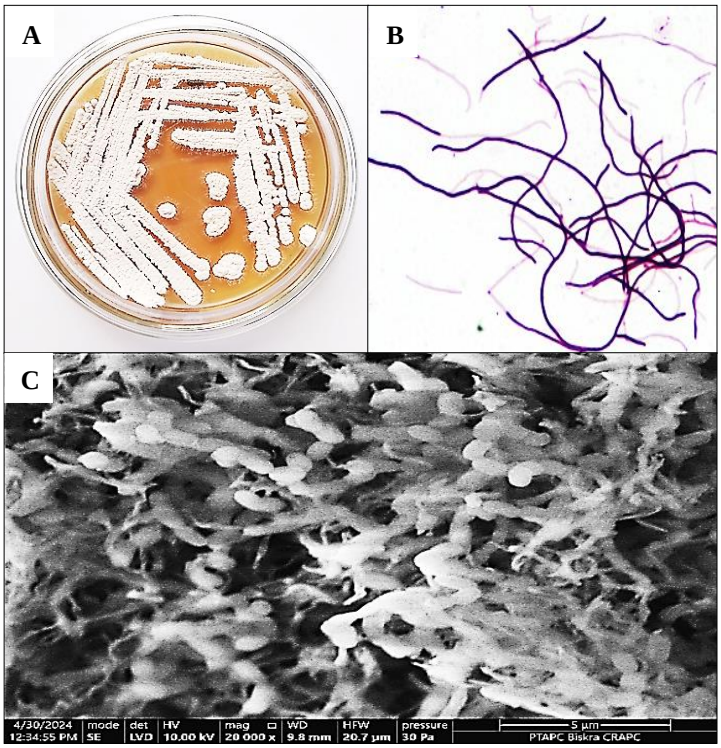


Figure 3.1. Morphological and cultural properties of the BPA-6 strain. **A)** BPA-6 strain on ISP-2, **B)** Gram staining, **C)** SEM image.

2. Molecular identification of the isolate BPA-6

With the help of the Basic Local Alignment Tool (BLAST) program, the obtained 16S rRNA gene sequence of our isolate (BPA-6) was compared and analyzed with eight homologous sequences that are available in the NCBI GenBank database. Figure 3.2 illustrates the phylogenetic tree, which was constructed based on the neighbor-joining tree method. As a result, the BPA-6 strain was identified as *Streptomyces* sp., demonstrating a similarity of 89.11% with the relative sequence of *Streptomyces thermolilacinus* strain G31 (ON810411.1).

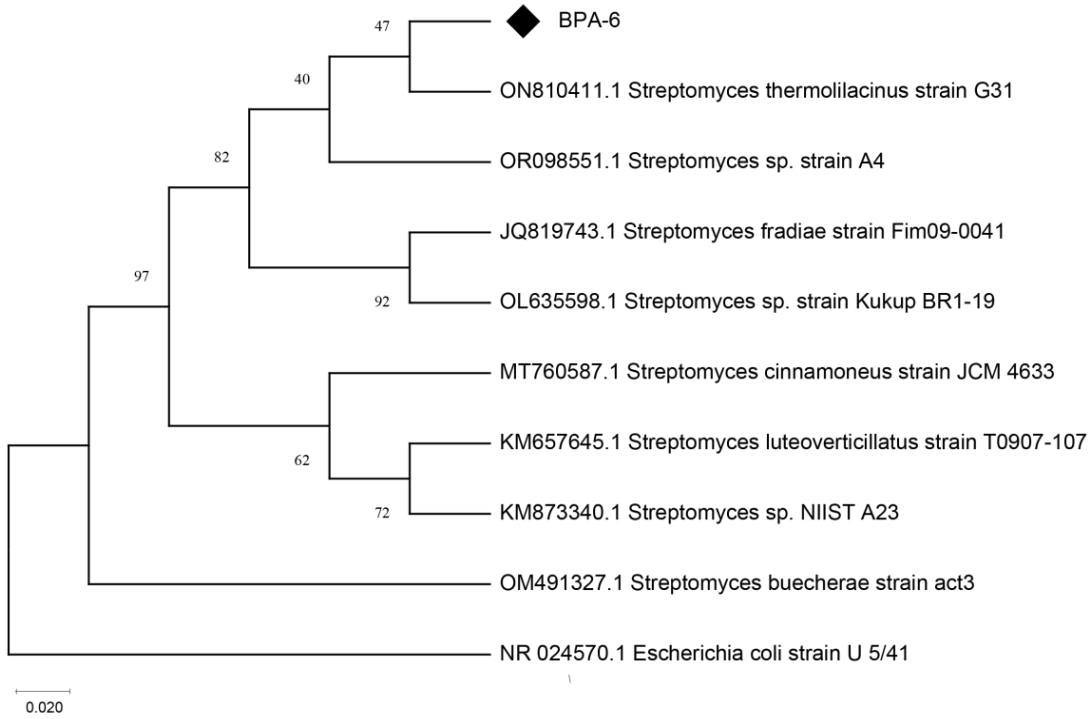


Figure 3.2. Neighbor-joining phylogenetic tree using the Maximum Likelihood method based on 16S rRNA gene sequences of the BPA-6 strain.

3. Antimicrobial activity

3.1. Perpendicular streak method

Based on the perpendicular streak method, the *Streptomyces* sp. BPA-6 strain was subjected to primary screening for antimicrobial activity to determine its ability to secrete antimicrobial secondary metabolites against several pathogenic microorganisms, including *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, *E. coli* ATCC 7839, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *C. albicans* ATCC 10231 (Figure 3.3). According to the results, the BPA-6 strain displayed only antagonistic activity against *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, and *C. albicans* ATCC 10231. In a similar study, the majority of isolated actinomycetes subjected to primary screening using the perpendicular streak method showed antagonistic activity only against Gram-positive bacteria, including *S. aureus* ATCC 25925 and *B. subtilis* ATCC 6633, and only one isolate demonstrated activity against *C. albicans* ATCC 10231 (Sripreechasak & Athipornchai, 2019). On the other hand, the *Streptomyces* sp. ERINLG-201 strain that was isolated from the Indian soil sample of Kodanad forest showed promising antagonistic activity in the primary screening against all tested microorganisms, including *S. aureus* MTCC 96, *B. subtilis* MTCC 441, *E. coli* (ESBL-3904), *P. aeruginosa* MTCC 741, *K. pneumoniae* MTCC 109, and *C. albicans* MTCC 227 (Balachandran et al., 2021).

Figure 3.3. Primary screening of the *Streptomyces* sp. BPA-6 strain against **1)** *S. aureus* ATCC 6538P, **2)** *B. subtilis* ATCC 6633, **3)** *C. albicans* ATCC 10231, **4)** *E. coli* ATCC 7839, **5)** *P. aeruginosa* ATCC 27853, **6)** *K. pneumoniae* ATCC 13883.

3.2. Agar well diffusion method

In this study, the culture supernatant (clear filtrate) and the crude ethyl acetate extract of the *Streptomyces* sp. BPA-6 strain were also tested against *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, *E. coli* ATCC 7839, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *C. albicans* ATCC 10231 using the agar well diffusion method as a secondary screening for antimicrobial activity (Figure 3.4).

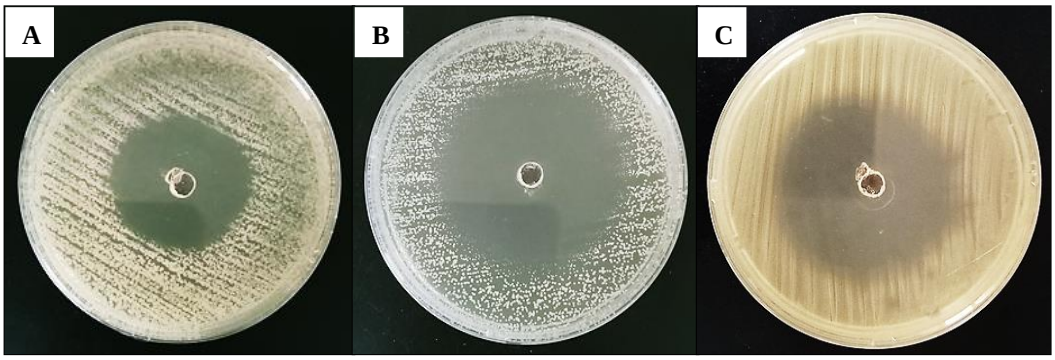


Figure 3.4. Secondary screening for antimicrobial activity of the crude ethyl acetate extract of the *Streptomyces* sp. BPA-6 strain against: **(A)** *B. subtilis* ATCC 6633, **(B)** *C. albicans* ATCC 10231, **(C)** *S. aureus* ATCC 6538P.

As shown in Table 3.3, the culture supernatant of the *Streptomyces* sp. BPA-6 strain showed a positive antimicrobial activity that corresponds to the results of the primary screening against *S. aureus* ATCC 6538P (30.33 ± 0.94 mm), *B. subtilis* ATCC 6633 (33.33 ± 1.53 mm), and *C. albicans* ATCC 10231 (33.00 ± 1.41 mm). And as expected, the crude ethyl acetate extract was stronger than the standard antibiotic against *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, and *C. albicans* ATCC 10231, with inhibition zones of 42.33 ± 3.79,

41.66 ± 3.51, and 48.67 ± 2.08 mm, respectively. Sripreechasak and Athipornchai (2019) evaluated the supernatant of the culture broth from the *Streptomyces* sp. SM4-HV7 strain, isolated from mountain soil in Thailand, for its antimicrobial activity using agar against *S. aureus* ATCC 25925, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 1023. The results showed a significant effect only against Gram-positive bacteria, including *S. aureus* ATCC 25925 and *B. subtilis* ATCC 6633, with inhibition zones of 43.0 ± 12.12 and 37.3 ± 6.66 mm, respectively. In the study of Mohammed-Amin and Al-Jaf (2024), the supernatant of the *Streptomyces* sp. 26 strain, which was isolated from Iraqi soil, displayed antimicrobial activity against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *C. albicans* ATCC 10231, and the zones of inhibition were 32.3 ± 2.5, 23.3 ± 1.2, and 17.3 ± 1.2 mm, respectively. In another study, the ethyl acetate extract of the *Streptomyces albus* AN1 strain, which was isolated from bee pollen obtained from beehives in Southeast England, revealed only antifungal activity against *C. albicans* ATCC 32077, while it did not demonstrate any antibacterial effects against all tested Gram-positive bacteria, such as *S. aureus* BAA-1747 and *B. cereus* ATCC 14579, or Gram-negative bacteria, such as *E. coli* ESS and *P. aeruginosa* ATCC 10145 (Santos-Beneit et al., 2022). Furthermore, the secondary screening for antimicrobial activity of the ethyl acetate extract of the *Streptomyces* sp. ERINLG-201 strain demonstrated a moderate activity against *S. aureus* MTCC 96, *B. subtilis* MTCC 441, *P. aeruginosa* MTCC 741, *K. pneumoniae* MTCC 109, and *C. albicans* MTCC 227, with inhibition zones of 18 ± 0.7, 15 ± 1.4, 15 ± 0.7, 16 ± 1.4, and 12 ± 1.4 mm, respectively (Balachandran et al., 2021).

According to our results of the perpendicular streak method as primary screening and the agar well diffusion method as secondary screening, the *Streptomyces* sp. BPA-6 strain was effective against the tested Gram-positive bacteria and non-filamentous fungi, while it did not show any activity against Gram-negative bacteria. Due to the thick peptidoglycan layer, Gram-positive bacteria are more susceptible to some antimicrobial compounds that can deteriorate cell wall synthesis or function. In contrast, Gram-negative bacteria have an outer membrane that acts as an additional permeability barrier, protecting them from their external environment by limiting or reducing the penetration of antimicrobial compounds, as observed in our results (Gauba & Rahman, 2023; Halawa et al., 2024).

Table 3.3. Agar well diffusion method results of the crude ethyl acetate extract of the *Streptomyces* sp. BPA-6 strain.

Test microorganisms	Inhibition zones (mm)		
	Supernatant	Ethyl acetate extract	Gentamycin
S. aureus ATCC 6538P	30.33 ± 0.94	42.33 ± 3.79	28
B. subtilis ATCC 6633	33.33 ± 1.53	41.66 ± 3.51	36
E. coli ATCC 7839	00	00	21
K. pneumoniae ATCC 13883	00	00	27
P. aeruginosa ATCC 27853	00	00	21
C. albicans ATCC 10231	33.00 ± 1.41	48.67 ± 2.08	00

3.3. Determination of MICs and MMCs

The minimum inhibitory concentrations (MICs) and the minimum microbicidal concentrations (MMCs) of the crude ethyl acetate extract of the *Streptomyces* sp. BPA-6 strain against *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, and *C. albicans* ATCC 10231 were evaluated using the microbroth dilution method in a 96-well microplate with the help of tetrazolium salts (TTC) as an effective growth indicator and subculture technique (Figure 3.5).

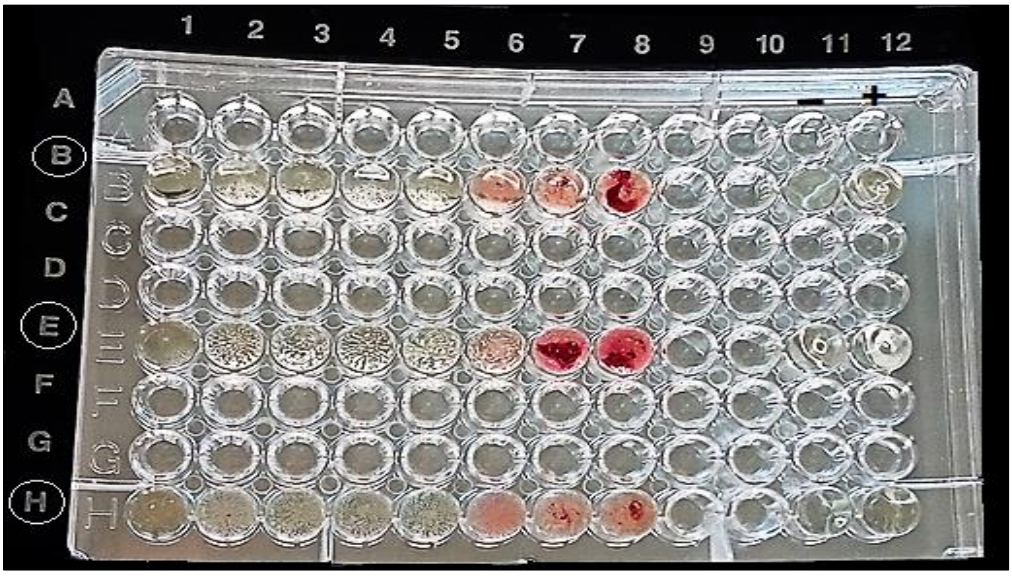


Figure 3.5. A 96-well microplate showing MIC of crude ethyl acetate extract against *B. subtilis* ATCC 6633.

The MICs and MMCs of the crude ethyl acetate extract of *Streptomyces* BPA-6 were determined successfully and were presented in Table 3.4. The recorded MIC value was 62.5 µg/mL against *S. aureus* ATCC 6538P and *B. subtilis* ATCC 6633, while it was 250 µg/mL against *C. albicans* ATCC 10231. These results indicate that the crude ethyl acetate extract of the *Streptomyces* BPA-6 strain contains bioactive secondary metabolites that can be used as

antibiotics to inhibit the growth of pathogenic microorganisms, particularly against *B. subtilis* ATCC 6633, with an MMC value of 1000 µg/mL. The MIC value of the crude ethyl acetate extract of our isolate (BPA-6) against *S. aureus* ATCC 6538P was significantly lower than what was reported by Aku et al. (2022) (250 µg/mL), while it was relatively higher than what was reported by Rajaram et al. (2020) (25 µg/mL). The MIC value of the ethyl acetate extract from *Streptomyces achromogenes* TCH4 against *B. subtilis* ATCC 6633 was found to be 125 µg/mL, with the MBC value being more than 4,000 µg/mL (Tangjitjaroenkun et al., 2021). In another study, the MICs of different organic solvent extracts of the *Streptomyces* Al-Dhabi-97 isolated from the marine region of Saudi Arabia, including hexane, chloroform, and ethyl acetate extracts, were tested against *B. subtilis* and indicated that the ethyl acetate extract was the most effective with an MIC value of 500 µg/mL (Al-Dhabi et al., 2020). Also, the MIC value of our isolate against *C. albicans* ATCC 10231 was relatively higher than what was found in previous studies (125 µg/mL, 100 µg/mL) (Aku et al., 2022; Rajaram et al., 2020). These results showed distinct MIC and MBC values against the same test microorganism, which were probably related to the variation in the strains of actinomycetes isolated from different geographical regions (Aku et al., 2022; Rai, 2018).

Table 3.4. MICs and MMCs results of the crude ethyl acetate extract of the BPA-6 strain.

Test microorganisms	MIC (µg/mL)	MMC (µg/mL)
S. aureus ATCC 6538P	62.5	> 1000
B. subtilis ATCC 6633	62.5	1000
C. albicans ATCC 10231	250	> 1000

4. GC-MS analysis

According to the obtained GC-MS analysis, different bioactive compounds that have antimicrobial activity were detected and identified in the crude ethyl acetate extract of the *Streptomyces* sp. BPA-6 strain (Figure 3.6).

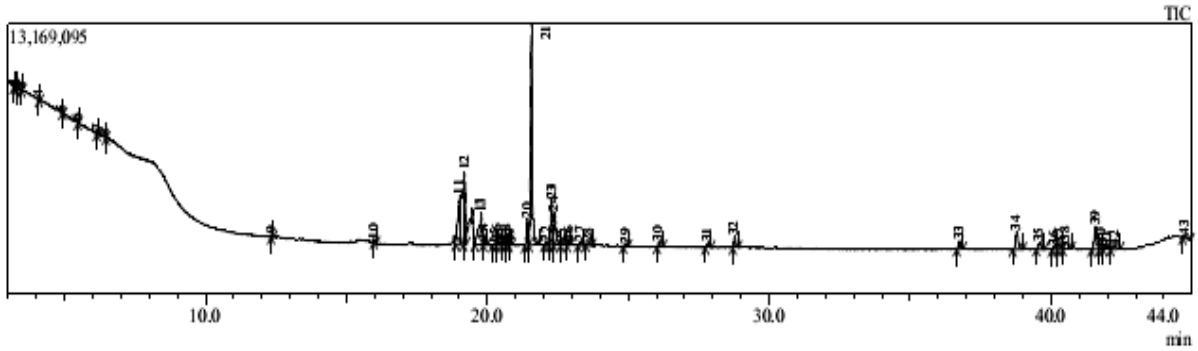
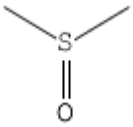
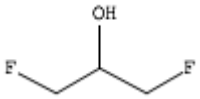
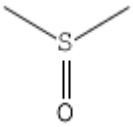
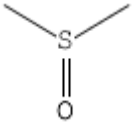
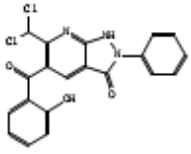
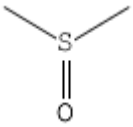
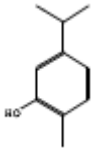
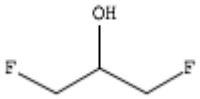
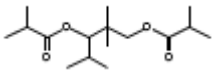
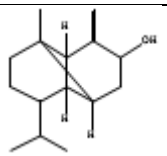
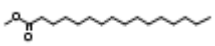
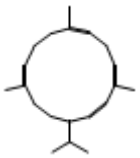
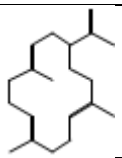
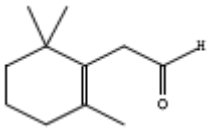
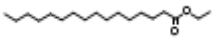
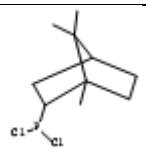
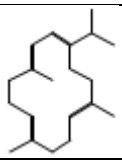
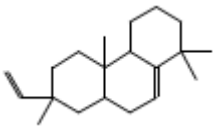


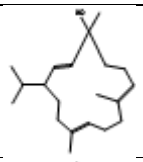
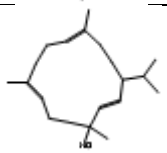
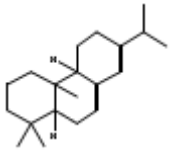
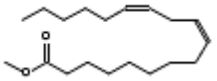
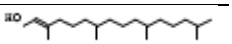
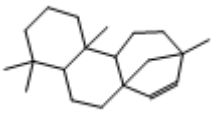
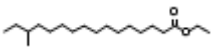
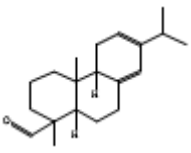
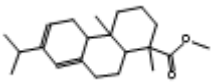
Figure 3.6. GC-MS chromatogram of the crude ethyl acetate extract of the BPA-6 strain.

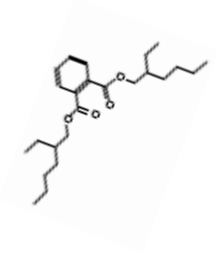
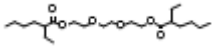
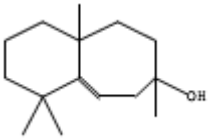
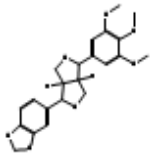
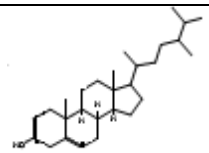
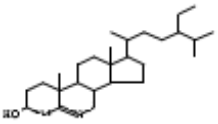
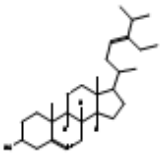
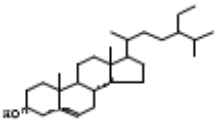
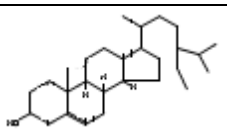
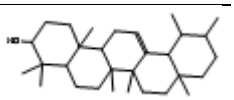
The *Streptomyces* sp. BPA-6 strain produced a wide range of bioactive compounds, as illustrated in Table 3.5. The main abundant compounds were 4-isopropyl-1,7,11-trimethyl-2,7,11-cyclotetradecatrien-1-ol (28.90%), hexadecanoic acid, methyl ester (11.33%), 1,3,6,10-cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]- (8.90%), (R,1E,5E,9E)-1,5,9-trimethyl-12-(prop-1-en-2-yl)cyclotetradeca-1,5,9-triene (8.13%), 9,12-octadecadienoic acid (Z,Z)-, methyl ester (6.00%), .gamma.-sitosterol (5.36%), and 9-octadecenoic acid, methyl ester, (E)- (4.92%). These compounds exhibit a variety of biological properties, including antibacterial, antifungal, insecticidal, antioxidant, anticancer, anti-inflammatory, and neuroprotective activities (Fajdek-Bieda et al., 2020; Khan & Javaid, 2021; Narwade et al., 2024; Taiyeb et al., 2024; Youssef et al., 2023). As reported in previous studies, other *Streptomyces* strains, such as *Streptomyces* sp. MMM2, *Streptomyces* sp. strain GLD25, and *Streptomyces* sp. 1S1, also can produce hexadecanoic acid, 9,12-octadecadienoic acid, and 9-octadecenoic acid as secondary metabolites, demonstrating antimicrobial activities against several pathogenic microorganisms, such as *S. aureus*, *B. subtilis*, and *C. albicans* (Djebbah et al., 2022; Malash et al., 2022; Mothana et al., 2022).

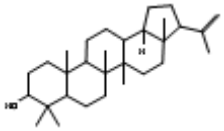
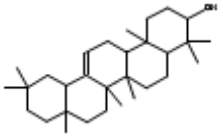
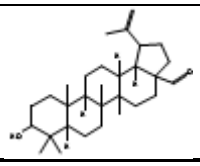
Table 3.5. The bioactive compounds detected by GC-MS analysis in the crude ethyl acetate extract of the *Streptomyces* sp. BPA-6 strain.

No	Compound name	Retention time (min)	Area (%)	Formula	Chemical structure
1	Dimethyl sulfoxide	3.203	0.05	C2H6OS	
2	1,3-Difluoro-2-propanol	3.313	0.06	C3H6F2O	
3	Dimethyl sulfoxide	3.487	0.04	C2H6OS	
4	Dimethyl sulfoxide	4.096	0.10	C2H6OS	
5	6-(Dichloromethyl)-5-salicyloyl-2-phenyl-1H-pyrazolo[3,4-b]pyridin-3-one	4.924	0.04	C20H13Cl2N3O3	

6	Dimethyl sulfoxide	5.472	0.09	C2H6OS	
7	Phenol, 2-methyl-5-(1-methylethyl)-	6.142	0.17	C10H14O	
8	1,3-Difluoro-2-propanol	6.461	0.05	C3H6F2O	
9	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	12.365	0.23	C16H30O4	
10	(1R,2R,4S,6S,7S,8S)-8-Isopropyl-1-methyl-3-methylenetricyclo[4.4.0.02,7]decan-4-ol	15.998	0.18	C15H24O	
11	Hexadecanoic acid, methyl ester	19.027	**11.33**	C17H34O2	
12	1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl-14-(1-methylethyl)-, [S-(E,Z,E,E)]-	19.187	**8.90**	C20H32	
13	(R,1E,5E,9E)-1,5,9-Trimethyl-12-(prop-1-en-2-yl)cyclotetradeca-1,5,9-triene	19.781	**8.13**	C20H32	
14	1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl-	19.905	0.83	C11H18O	
15	Bisabolene <beta->	20.265	0.25	C15H24	ND
16	Hexadecanoic acid, ethyl ester	20.391	0.74	C18H36O2	
17	1,7,7-Trimethylbicyclo[2.2.1]hept-2-ylphosphonous dichloride	20.578	0.33	C10H17Cl2P	
18	(1E,3E,7E,11E)-4-Isopropyl-1,7,11-trimethylcyclotetradeca-1,3,7,11-tetraene	20.700	0.65	C20H32	
19	Phenanthrene, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,8a,9-dodecahydro-1,1,4b,7-tetramethyl-,	20.815	0.25	C20H32	

	[4aS-(4a.alpha.,4b.beta.,7.alpha.,8a.alpha.)]-				
20	Thunbergol	21.416	2.96	C20H34O	
21	4-Isopropyl-1,7,11-trimethyl-2,7,11-cyclotetradecatrien-1-ol	21.563	**28.90**	C20H34O	
22	(4aS,4bR,10aS)-7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,4b,5,6,10,10a-decahydrophenanthrene	22.104	0.33	C20H32	
23	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	22.278	**6.00**	C19H34O2	
24	9-Octadecenoic acid, methyl ester, (E)-	22.393	**4.92**	C19H36O2	
25	Phytol	22.655	0.35	C20H40O	
26	Methyl stearate	22.878	0.45	C19H38O2	
27	17-Norkaur-15-ene, 13-methyl-, (8.beta.,13.beta.)	23.311	0.68	C20H32	
28	Ethyl 14-methyl-hexadecanoate	23.607	0.50	C19H38O2	
29	(1R,4aR,4bS,10aR)-7-Isopropyl-1,4a-dimethyl-1,2,3,4,4a,4b,5,9,10,10a-decahydrophenanthrene-1-carbaldehyde	24.904	0.46	C20H30O	
30	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,4b,5,9,10,10a-decahydro-1,4a-dimethyl-7-(1-methylethyl)-, methyl ester, [1R-(1.alpha.,4a.beta.,4b.alpha.,10a.alpha.)]-	26.084	0.67	C21H32O2	

31	1,2-Benzenedicarboxylic acid, 1,2-bis(2-ethylhexyl) ester	27.826	0.65	C24H38O4	
32	Triethylene glycol di(2-ethylhexoate)	28.776	1.22	C22H42O6	
33	1H-Benzocyclohepten-7-ol, 2,3,4,4a,5,6,7,8-octahydro-1,1,4a,7-tetramethyl-, cis-	36.748	1.36	C15H26O	
34	5-((1S,3aR,4S,6aR)-4-(3,4,5-Trimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)benzo[d][1,3]dioxole	38.786	3.55	C22H24O7	
35	Campesterol	39.587	1.37	C28H48O	
36	(24R)-Stigmast-5-en-3.beta.-ol	40.135	0.77	C29H50O	
37	(3S,8S,9S,10R,13R,14S,17R)-17-[(E,1R)-4-ethyl-1,5-dimethyl-hex-3-enyl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol	40.285	1.26	C29H48O	
38	(24R)-Stigmast-5-en-3.beta.-ol	40.512	2.02	C29H50O	
39	.gamma.-Sitosterol	41.579	**5.36**	C29H50O	
40	Urs-12-en-3-ol, (3.beta.)-	41.785	1.22	C30H50O	

41	(3.beta.,21.beta.)-A'-neogammacer-22(29)-en-3-ol	41.895	1.04	C30H50O	
42	.beta.-Amyrin	42.253	0.82	C30H50O	
43	Betulinaldehyde	44.757	0.70	C30H48O2	

ND: not determined.

Conclusion

The study successfully highlights the molecular identification, antimicrobial activity, and GC-MS analyses of bioactive compounds produced by the *Streptomyces* sp. BPA-6 strain isolated from bee pollen. Molecular identification confirmed its classification within the *Streptomyces* genus, known for producing secondary metabolites used in different applications. GC-MS analysis revealed a variety of bioactive compounds, such as hexadecanoic, 9,12-octadecadienoic, and 9-octadecenoic acids, suggesting that the *Streptomyces* sp. BPA-6 is a valuable source for compounds that exhibited significant inhibitory effects against pathogenic microorganisms, including *S. aureus*, *B. subtilis*, and *C. albicans*, underlining its potential as an alternative and natural antimicrobial agents. Future research should focus on the purification and structural elucidation of the specific compounds responsible for the antimicrobial activity, as well as their potential applications in medicine, agriculture, and food safety.

Chapter 4

Evaluation of antimicrobial, anticancer, and neuroprotective activities of silver nanoparticles (AgNPs) green-synthesized using a red pigment produced by the *Streptomyces* sp. A23 strain isolated from Algerian bee pollen

Introduction

Recently, the utilization of nanomaterials in the medical field has attracted increased amounts of attention, especially as novel and alternative therapeutic agents with multifaceted applications (Malik et al., 2023).One of these nanomaterials is silver nanoparticles (AgNPs), which are emerging as possible hopeful alternatives due to their beneficial features (Xu et al., 2020). Two conventional methods were applied to synthesize AgNPs: chemical and physical methods. Due to their undesirable disadvantages, suitable and green alternative methods must be found. Among these methods, the biological methods that use bacteria, fungi, algae, plants, and others; have gained substantial prominence due to their high safety (Dhaka et al., 2023). Many researchers have used actinomycetes, including *Streptomyces* bacteria, to synthesize AgNPs (Barbuto Ferraiuolo et al., 2021; Rosyidah et al., 2022). The genus *Streptomyces* is known as a source of valuable and varied secondary metabolites, which are excellent tools for the green synthesis of nanoparticles (Al-Dhabi et al., 2018; Pallavi et al., 2022).One such secondary metabolite is pigments, which not only serve as an identifier product for *Streptomyces* but also demonstrate a prominent potential for synthesizing AgNPs (Singh et al., 2021). This process of synthesis provides a fast, safe, and eco-friendly approach and imparts significant biological properties, such as antioxidant, antimicrobial, and antiproliferative activities, to the resulting nanoparticles, which hold promise as multifaceted therapeutic agents capable of treating both communicable and noncommunicable diseases (Datkhile et al., 2023; Mechouche et al., 2022). Regarding communicable diseases, the growth of antibiotic-resistant microorganisms is one of the most important public health problems, which has emerged as a global crisis, underscoring the urgent need to discover novel and alternative antimicrobial agents to the conventional antibiotics (Ghosh et al., 2019). It has been shown that AgNPs synthesized using the secondary metabolites produced by the *Streptomyces* species exhibit good antimicrobial effects against a wide range of pathogenic microorganisms, including bacteria and fungi (Abd-Elhady et al., 2021). Moreover, when AgNPs are combined with conventional antibiotics, they can have synergistic effects that may overcome antibiotic resistance (Aabed and Mohammed, 2021). For noncommunicable diseases, cancer and neurodegenerative disorders remain major health concerns, necessitating the discovery of new effective therapeutic agents (Kopeć et al., 2023; Xu et al., 2023). AgNPs synthesized using the microbial pigments have shown promising anticancer and neuroprotective effects (Abd-Elhady et al., 2021; Salem et al., 2022). These nanoparticles are promising agents in cancer therapy because they can target the cancer cells while avoiding the

cells that are normal (Baranwal et al., 2023). Furthermore, the distinctive physicochemical properties of these nanoparticles allow them to exhibit antioxidant activities, reducing the oxidative stress that might lead to neurodegenerative disorders. Additionally, these nanoparticles can modulate inflammatory responses, further contributing to their neuroprotective effect (Zhu et al., 2021).

Therefore, this study aims to synthesize AgNPs using a red pigment produced by the *Streptomyces* sp. A23 strain isolated from Algerian bee pollen as a new biological reducing agent to evaluate their antimicrobial, anticancer, and neuroprotective activities

Material and methods

1. Materials

Silver nitrate ($AgNO_3$), hydrogen peroxide (H_2O_2), and dimethyl sulfoxide (DMSO) were purchased from Sigma. Muller-Hinton broth was obtained from Conda, and carbonate calcium ($CaCO_3$) was obtained from Merck. The GF-1 Nucleic Acid Extraction Kit, universal primers (27F and 1492R), and Clean-Up Kit were from Vivantis Technologies. Gentamycin discs were provided from Oxoid™. *S. aureus* ATCC 6538P, *B.* ATCC 6633, *E. faecalis* ATCC 19433, *E. coli* ATCC 7839, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *C. albicans* ATCC 10231 were obtained from the applied microbiology laboratory, Ferhat Abbas University, Algeria. SHSY-5Y human neuroblastoma cells (ATCC®, CRL-2266) were obtained from ERFARMA Center, Erciyes University, Turkey.

2. Bee pollen sampling

Bee pollen samples were provided by the Api-Phyto Therapy Center - Setif - Algeria, from different Algerian clean lands during the spring of 2019, in sterile plastic containers. The samples were subsequently transported to the laboratory and stored at 4°C until use.

3. Isolation, purification, and conservation of the A23 strain

All the samples were crushed, air dried at room temperature and heated at 50°C for 30 minutes in a water bath (Memmert, Germany). One gram from all bee pollen samples was mixed with 0.1g of calcium carbonate ($CaCO_3$) and incubated at 28 °C for one week (Hernández-Bolaños et al., 2020). Then, 1g of each pre-treated sample was suspended in 9 mL of sterile saline water and diluted to 10^{-2}. After then, 100 µL of the diluted sample was plated on different media for Actinomycetes isolation, and incubated at 28 °C for approximately 10 days. During the period of incubation, the selected strain was purified using

yeast extract-malt extract agar (ISP-2 medium), labeled by the A23 strain and conserved at 4°C (Mohamed et al., 2017).

4. Phenotypic identification of the A23 strain

The primary phenotypic characteristics of the A23 strain were determined via Gram staining, catalase and oxidase tests (Lakhdar et al., 2023; Smith and Hussey, 2005). In addition, the culture characteristics were described based on the growth intensity, aerial and substrate mycelia, and pigment production on ISP-2 agar medium (Tandale et al., 2018). Also, carbohydrate assimilation was assessed on minimal growth medium supplemented with the carbon source at a final concentration of 1 %, as described by Shirling and Gottlieb (1966). The production of extracellular hydrolytic enzymes (starch hydrolysis, protease and lipolysis) was studied on minimum agar medium added to the substrates at a concentration of 1 % (Mahfooz et al., 2017). Assignments of the A23 strain at least to the genus level were performed by comparing the results of the phenotypic characterization to those reported in Bergey's Manual of Systematic Bacteriology (Goodfellow et al., 2012).

5. Genotypic identification of the A23 strain

5.1. DNA extraction, 16S rRNA amplification, and electrophoresis

The DNA extraction of the selected strain was carried out by using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia). The 16S rRNA amplification was performed by PCR using thermocycler (iCycler Bio-Rad, USA) with the help of two universal primers, 27F and 1492R. This process was repeated for 30 cycles (Djebbah et al., 2022). After, the products of PCR were subjected to electrophoresis and subsequently purified by a Clean-Up Kit (Vivantis Technologies, Malaysia) (Lakhdar et al., 2023).

5.2. DNA sequencing and bioinformatics analysis

The PCR products were purified and subsequently sequenced by using 3130 Genetic Analyzer (Applied Biosystems®, USA) (Lakhdar et al., 2023). BLAST was used to compare the obtained sequence of the studied strain with 16S rRNA gene sequences of other Actinomycetes in GenBank. MEGA 11 software was used to construct the phylogenetic tree (Almuhayawi et al., 2021).

6. Green synthesis, purification and characterization of silver nanoparticles (AgNPs)

6.1. Production and purification of red pigment

Red pigment was produced from the A23 strain by submerged fermentation on ISP-2 broth using an orbital shaker at 180 rpm and 28°C. A week later, the culture broth was centrifuged at 4000 rpm and 4 °C for 10 minutes to remove the mycelia. The supernatant that contained the produced pigment was collected and stored at 4°C (Nuanjohn et al., 2023).

6.2. Synthesis and purification of AgNPs

Two milliliters of the obtained pigment were filtered through a 0.45 µm syringe filter and mixed with 18 mL of 1 mM and 5 mM silver nitrate solutions at pH 5, pH 7, and pH 9 using a magnetic hotplate stirrer at 1500 rpm and room temperature for 24 hours in dark conditions (Ahila et al., 2016; Mechouche et al., 2022). The first part of the nanoparticles suspension was obtained directly and kept at 4 °C for UV-Vis spectrophotometer, Zeta potential, DLS analyses, and further experiments. The second part was centrifuged and washed two times with distilled water at 15000 rpm for 10 minutes, after which the precipitates were dried at 70 °C for 2 days and subjected to characterization using XRD, FT-IR, FE-SEM, and EDX analyses (Koca et al., 2022).

6.3. Characterization of synthesized AgNPs

Several techniques have been used to characterize the synthesized AgNPs. The formation of AgNPs was confirmed by visual observation and Ultraviolet visible spectrophotometry (UV-Vis) (Perkin Elmer, USA) (Mechouche et al., 2022). The hydrodynamic diameter was determined via Dynamic light scattering (DLS) (Malvern, UK). The surface charge was analyzed by Zeta potential (Malvern, UK) analyzer. The presence of functional groups playing a role in the synthesis was determined by FT-IR (Perkin Elmer Spectrum 400, USA) analysis. Crystallization was determined by X-ray diffraction (XRD) (BRUKER AXS D8, Germany) analysis. Morphology and elemental presence determined by field emission scanning electron microscopy (FE-SEM) and Energy-dispersive (EDX) analyses (ZEISS GeminiSEM 500, Germany) (Shukla and Iravani, 2018).

7. Biological activities of synthesized AgNPs

7.1. Antimicrobial activity

The first test of antimicrobial activity of the synthesized AgNPs was evaluated using the agar well diffusion method as described by Balouiri *et al.* (2016) against *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, *E. faecalis* ATCC 19433, *E. coli* ATCC 7839, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, and *C. albicans* ATCC 10231. Fresh microbial suspensions (10^7 CFU/mL) were prepared and swabbed using sterile cotton swabs on Muller-

Hinton agar plates. After that, the wells were made using sterilized micropipette tips, and 100 µL of synthesized AgNPs were poured into each well. Finally, all plates were kept at room temperature for 30 minutes for diffusion and subsequently incubated at 37 °C for 24 hours. After incubation, the inhibition zone diameter observed around each well was measured and is expressed in millimeters (mm). Gentamycin discs (Oxoid™) and silver nitrate solutions were used as positive controls; pigment alone and distilled water were used as negative controls. After, minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of AgNPs (5 mM, pH 9) were determined by the microbroth dilution method using tetrazolium salts (TTC) in 96-well microplates in triplicate and on Mueller-Hinton agar plates, respectively (Mechouche et al., 2022).

7.2. Anticancer and neuroprotective activities

Final concentrations of 2, 4, 8, 12, and 16 µg/mL of synthesized AgNPs were prepared to use in the evaluation of cytotoxicity and neuroprotective effects on the SHSY-5Y neuroblastoma cells by using the MTT test.

7.2.1. Cytotoxicity assay

After the preparation of SHSY-5Y cells, they were exposed to all prepared concentrations of AgNPs for 24 hours in 96-well plates containing approximately 10^4 cells per well. Then, 100 µL of prepared MTT solution was added to each well and incubated at 37 °C for 4 hours. Afterwards, the supernatant portion was subsequently removed from the wells, followed by the addition of 100 µL of DMSO to each well in order to dissolve the MTT salts. Finally, the microplate reader (BioTek, Synergy HT, USA) was used to measure the absorbances at a wavelength of 560 nm (Koca et al., 2022).

7.2.2. Neuroprotective activity

To evaluate the potential neuroprotective effects of the synthesized nanoparticles on neuroblastoma cells, the prepared concentrations of AgNPs compounds were added 1 hour after the treatment of cells by H_2O_2 (300 µM) in 96-well plates. Cell viability was measured by the MTT test after 24 hours of incubation. The same volume of medium without AgNPs or H_2O_2 was used as a control (Koca et al., 2022).

8. Statistical analysis

Data analysis was performed using GraphPad Prism 8.0 software by using one-way ANOVA and Dunnett's post-hoc tests. All differences were considered significant when $P < 0.05$.The *n* value was four for the cell culture studies.

Results and discussion

1. Phenotypic and genotypic identification of the isolated strain (A23)

As shown in Figure 4.1, the isolated strain in our study was characterized by earthy odor and powdery colonies with a grey-white color, and it was a filamentous, branching, and Gram-positive bacterium. Morphological and cultural properties have shown abundant growth with variations in the colour of aerial and substrate mycelia. Additionally, it has the potential to produce a diffusible red pigment. Actinomycetes can produce visible powdery colonies with different colors from the 3rd to the 7th day, with an earthy odor as a result of these bacteria's ability to produce various secondary metabolites, including pigments (Abou-Dobara et al., 2018). Furthermore, the strain can utilize different carbon sources for growth, and it is also positive for catalase and negative for the oxidase reactions. Protease, lipase, and amylase are produced by this strain (Table 4.1). With the help of Bergey's manual of systematic bacteriology, these phenotypic properties are consistent with the typical characteristics described for the genus *Streptomyces* (Goodfellow et al., 2012).

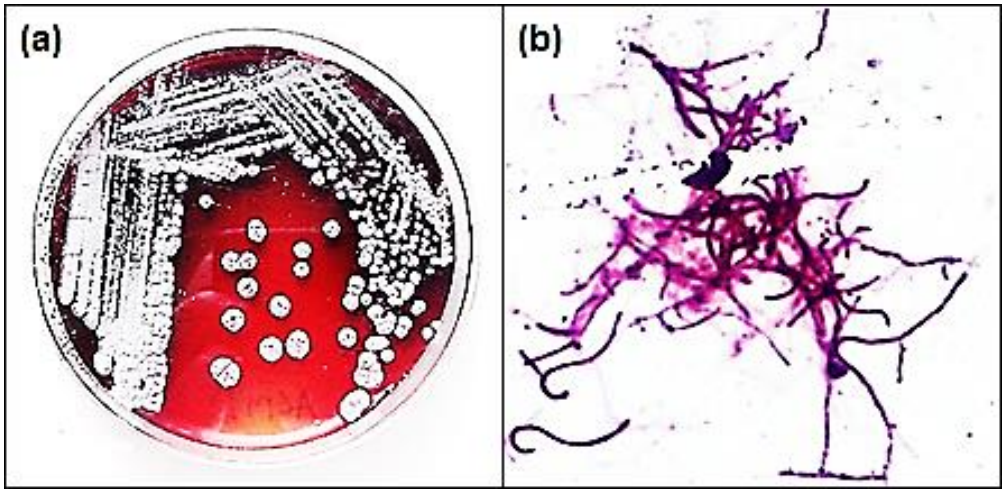


Figure 4.1. The *Streptomyces* sp. A23 strain: **a)** Colony morphology, **b)** Gram staining.

Table 4.1. The phenotypic characteristics of the isolated strain (*Streptomyces* sp. A23).

Phenotypic characteristics of the *Streptomyces* sp. A23 strain	
Morphological and cultural characteristics	
Growth intensity on ISP-2 agar	+++
Arial mycelium colour on ISP-2 agar	Grey white
Substrate mycelium colour on ISP-2 agar	Dark red
Diffusible pigment	+++
Gram staining	Positive
Carbon sources assimilation	
Glucose	+
Fructose	+
Lactose	+
Maltose	+
Galactose	+
Mannitol	+
Enzymes production	
Catalase	+
Oxidase	-
Starch hydrolysis	+
Casein hydrolysis	+
Lipolysis	+

(+): positive reaction, (-): negative reaction, (+++): abundant growth and production.

According to the obtained 16S rRNA sequence (1096 bp) and the phylogenetic tree analyses (Figure 4.2), the A23 strain belongs to the genus *Streptomyces*. It was labeled *Streptomyces* sp. A23 strain and registered in GenBank under the accession number OR236137.1.

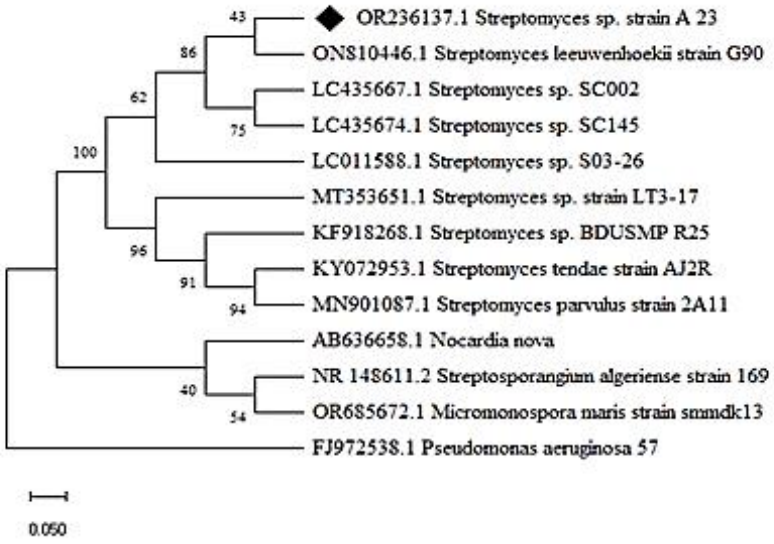


Figure 4.2. Phylogenetic tree of the *Streptomyces* sp. A23 strain.

2. Green synthesis and characterization of AgNPs

AgNPs were successfully synthesized via a rapid, easy, and eco-friendly procedure using the red pigment of the *Streptomyces* sp. A23 strain that was isolated from bee pollen as a bio-reducing agent source. Silver nitrate ($AgNO_3$) is a colorless solution, and the pigment is red color. When the pigment was mixed with silver nitrate solution, the mixture slowly turned to the brown color after 24 hours in the dark (Figure 4.3). The color change was due to silver nitrate interacting with the pigment compounds and being bio-reduced from silver nitrate ($AgNO_3$) to elemental silver (Ag°). These elementals aggregate to form stable AgNPs (Jabeen et al., 2021). A previous study indicated the formation of AgNPs using a pink pigment produced from *Streptomyces* sp. NS-05 isolated from a rhizospheric soil by observing the change in color to brown (Singh et al., 2021).

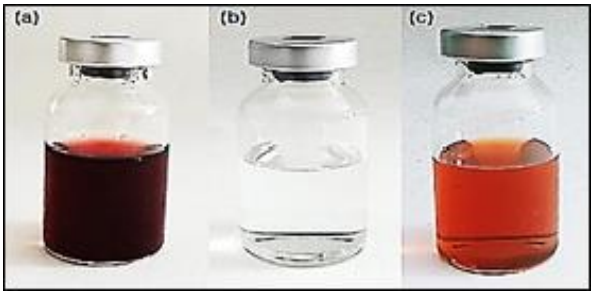


Figure 4.3. Visual observation of the synthesized AgNPs: **a)** Pigment, **b)** $AgNO_3$ solution, **c)** AgNPs suspension.

In this study, the light absorption points, hydrodynamic diameter, surface charge, crystallization, phytochemical component, morphology, and elemental presence of synthesized AgNPs (5 mM, pH 9) were determined by UV-vis, DLS, Zeta potential, XRD, FT-IR, FE-SEM, and EDX analyses, respectively. The optical and electrical properties of nanoparticles are interrelated, and unlike those of their bulk forms, they have a UV-Vis absorption band called surface plasmon resonance (SPR).

UV-vis spectroscopy was used for characterizing the nanoparticles synthesized by various methods. Diffractions observed by analysis can be controlled by many factors, such as reducing agents and solvents (Khan et al., 2019). According to our results, the characteristic light absorption bands detected at 278 nm and 433 nm by UV-vis analysis were associated with the presence of biomolecules in the pigment and AgNPs, respectively (Figure 4.4). The characteristic light absorption bands of AgNPs synthesized by using the secondary metabolites of *Streptomyces rochei* and marine *Streptomyces* sp. were measured at 413 and 434 nm, respectively (Bano et al., 2023; Kamala et al., 2023). The structural properties of

nanoparticles, which depend on the synthesis conditions, also affect the UV-vis characterization bands (Asif et al., 2022).

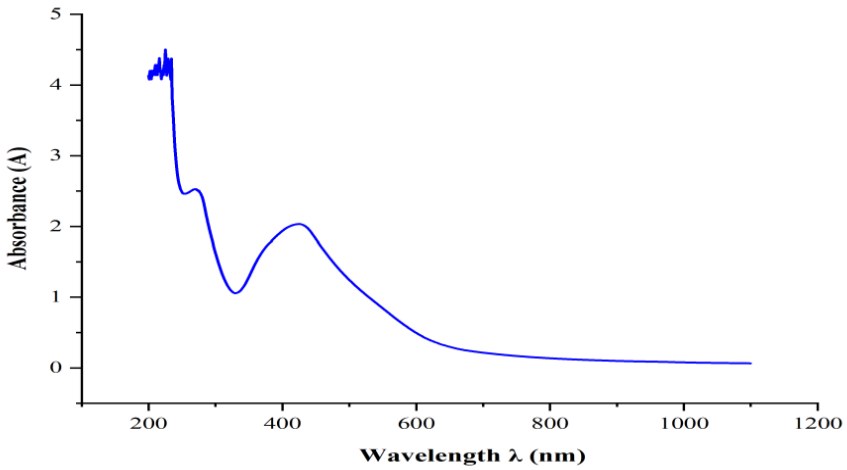


Figure 4.4. UV-vis spectrum of AgNPs (5 mM, pH 9).

The surface charge of nanoparticles is an important parameter for the characterization of nanomaterials, which determines the distribution properties of particles, adsorption properties of ions, and molecules (Mourdikoudis et al., 2018). The surface charge of the AgNPs synthesized in our study was determined to be -33 mV (Figure 4.5). A relatively low zeta potential increases the electrostatic repulsion force between nanoparticles, which prevents their aggregation and ensures their stabilization (Varadavenkatesan et al., 2020). The surface charge (negative, positive, or neutral) of nanoparticles is related to the functional groups on the surface structure (Awashra and Młynarz, 2023).

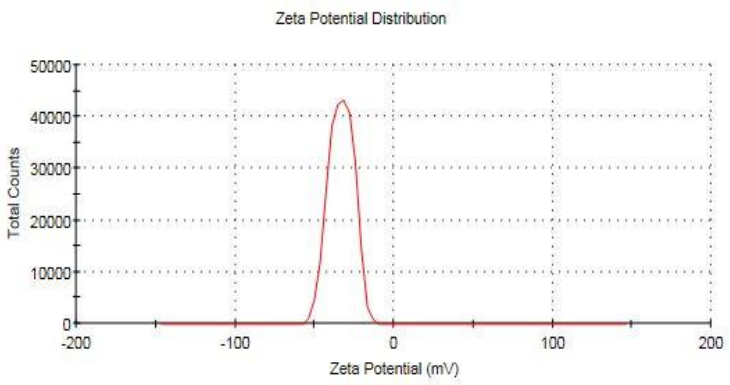


Figure 4.5. Zeta potential analysis of AgNPs (5 mM, pH 9).

Dynamic light scattering (DLS) is a spectroscopic analysis technique used to determine the size of particles suspended in a liquid from 1 to 1000 nm (Jia et al., 2023). The average hydrodynamic size of our synthesized AgNPs was 112 nm (Figure 4.6). DLS

measurements are expected to yield more intense results than microscopic analyses, which can be explained by the presence of ions or molecules bound and the increase in the thickness of the hydration shell around nanoparticles in solution (Gharari et al., 2023; Moosavy et al., 2023).

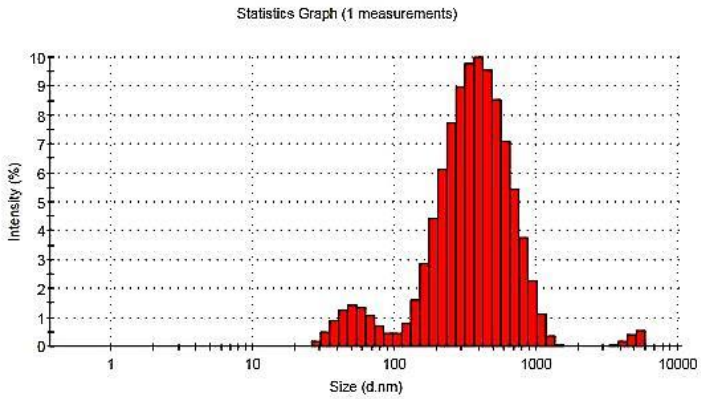


Figure 4.6. DLS analysis of AgNPs (5 mM, pH 9).

According to the FE-SEM image, our synthesized spherical AgNPs tend to form agglomerations and appear to be monodispersed with a mean particle size of 54.5 nm (Figure 4.7). The average size of the spherical AgNPs synthesized by using the pink pigment of *Streptomyces* sp. NS-05 was 42.5 nm (Singh et al., 2021). The size, shape, and structural properties of NPs synthesized by biological methods can vary depending on the biological agent used and the synthesis conditions, such as the concentration and type of metal salt used and the bio-reducing agent, temperature, and reaction time (Kizildag et al., 2019).

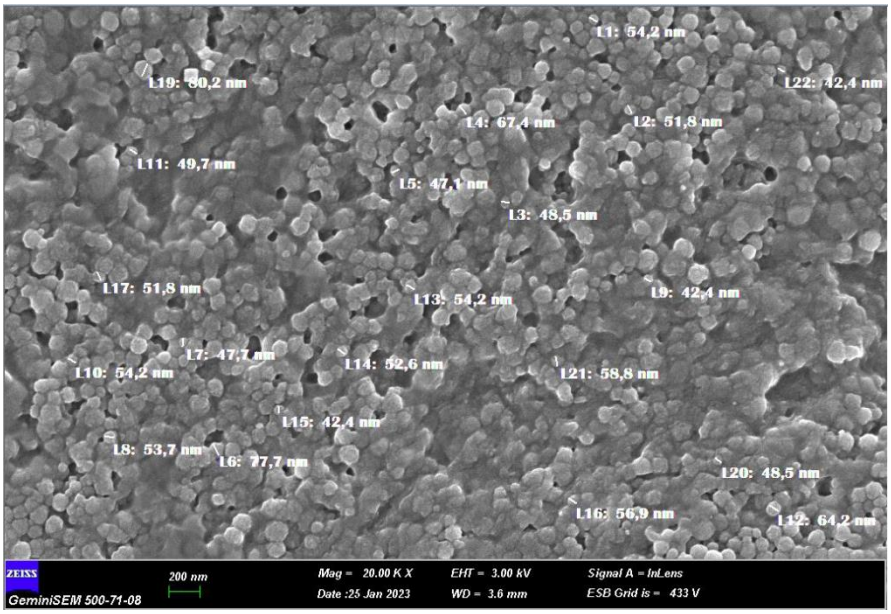


Figure 4.7. FE-SEM image of AgNPs (5 mM, pH 9).

EDX and XRD were performed to determine the elemental composition and crystal structure of the AgNPs. The presence of Ag was revealed by EDX analysis (Figure 4.8).

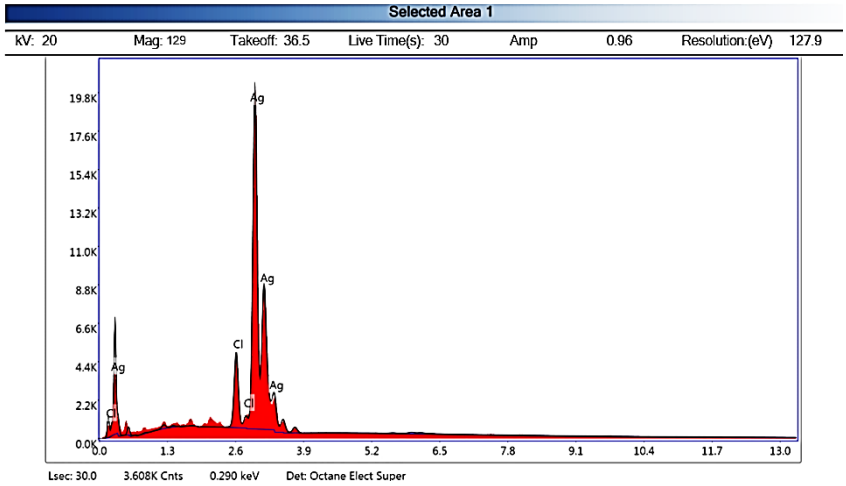


Figure 4.8. EDX analysis of AgNPs (5 mM, pH 9).

According to the XRD analysis of the Ag/Ag^0 NPs, the 27.7°, 32.3°, 38.4°, 44.1°, 46.2°, 54.5°, 57.6°, 64.3° and 77.2° peaks observed at 2 theta corresponded to the (1, 0, 0), (1, 2, 2), (1, 1, 1), (2, 0, 0), (2, 0, 0), (2, 2, 0), (2, 2, 0), (2, 2, 0), and (3, 1, 1) planes, respectively (Figure 4.9). The obtained peaks at 27.7°, 32.3°, and 54.5° indicate Ag^0 NPs (JCPDS (01–076-1489)). The peaks at 32.3°, 38.4°, 46.2°, 54.5°, 57.6°, 64.3° and 77.2° reflect the face-centered cubic Ag phase (JCPDS (00-001-1167)) (Ali et al., 2023; Fouad et al., 2021; Korpayev et al., 2024).

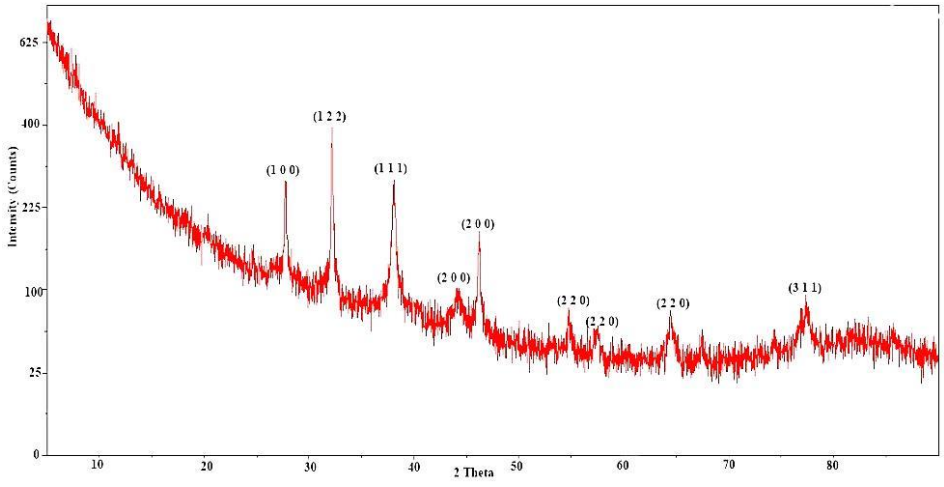


Figure 4.9. XRD pattern of AgNPs (5 mM, pH 9).

FT-IR can provide information about the bioactive molecules that may be responsible for reducing silver ions into AgNPs (Abd-Elhady et al., 2021). The functional groups that play

a role in the green synthesis of our AgNPs were revealed by the peaks observed in the FT-IR spectrum with diffractions at 3471, 2918, 1620, 1409, 1321, and 478 cm^{-1}, which indicate the presence of alcohol (O-H), alkane (C-H), amine (N-H), fluoro compound (C-F), aromatic amine (C-N), and metal (Ag), respectively (Figure 4.10). Similar to our findings, previous studies reported that alcohol, alkane, and amine groups were responsible for the reduction and capping of Ag ions for the synthesis of NPs (Ghosh et al., 2019; Kamala et al., 2023; Varadavenkatesan et al., 2020).

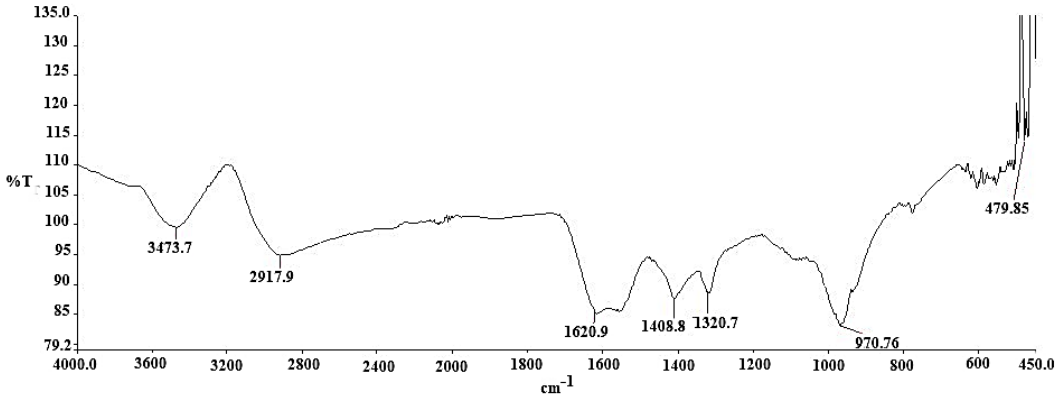


Figure 4.10. FT-IR spectrum of AgNPs (5 mM, pH 9).

3. Antimicrobial activity

Firstly, the antimicrobial activity of synthesized nanoparticles (AgNPs) using the red pigment produced by the *Streptomyces* sp. A23 strain was evaluated using an agar well diffusion assay as illustrated in Figure 4.11.

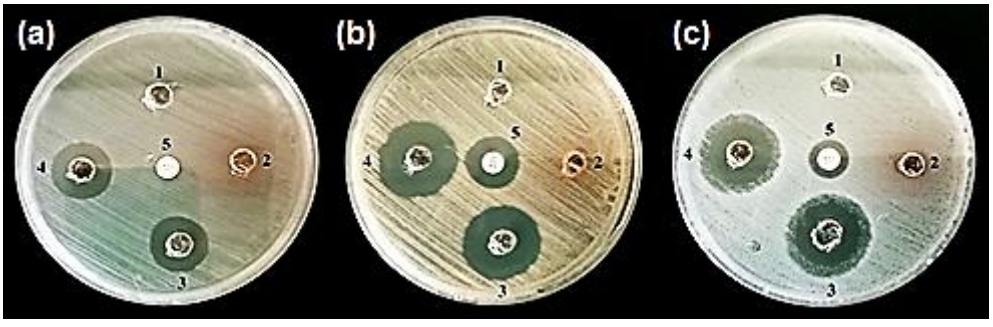


Figure 4.11. Antimicrobial activity of AgNPs (1 mM, pH 7) against: **a)** *E. faecalis*, **b)** *S. aureus*, **c)** *P. aeruginosa.* **1)** H_2O, **2)** Pigment, **3)** $AgNO_3$, **4)** AgNPs, **5)** Gentamycin.

The obtained results showed that AgNPs (5 mM, pH 9) demonstrated the best antimicrobial effect against the tested pathogenic microorganisms (Table 4.2). The zone with the greatest diameter of inhibition was observed for *E. faecalis* ATCC 19433 (32 mm),

followed by *S. aureus* ATCC 6538P (30 mm), *C. albicans* ATCC 10231 (30 mm), *P. aeruginosa* ATCC 27853 (27 mm), *B. subtilis* ATCC 6633 (25 mm), *K. pneumoniae* ATCC 13883 (20 mm), and *E. coli* ATCC 7839 (19 mm). The AgNPs synthesized using the secondary metabolites of the *Streptomyces hirsutus* isolated from Indian sediment samples exhibited significant effects against *E. faecalis*, *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans* with inhibition zones of 21, 19, 17, 12 and 12 mm, respectively (Pallavi et al., 2022).

Table 4.2. Antimicrobial activity of synthesized AgNPs.

Pathogenic microorganisms	**Inhibition zones diameter (mm)**					
	AgNPs			**Controls**		
	(1 mM, pH5)	**(1 mM, pH7)**	**(5 mM, pH9)**	**$AgNO_3$ (1 mM)**	**Pigment**	**Gentamycin (10 µg)**
S. aureus ATCC 6538P	21	24	30	22	00	17
B. subtilis ATCC 6633	22	16	25	15	00	14
E. faecalis ATCC 19433	25	18	32	16	00	00
E. coli ATCC 7839	18	21	19	20	00	14
P. aeruginosa ATCC 27853	23	23	27	23	00	12
K. pneumoniae ATCC 13883	20	26	20	24	00	14
C. albicans ATCC 10231	27	28	30	33	11	00

(ATCC): American Type Culture Collection.

AgNPs (5 mM, pH 9) are more effective because of their spherical shape and smaller size (54.5 nm), and their contact with microbial surfaces is good and easy due to their high negative surface charge (-33 mV). According to different reports, physical properties such as size, shape, and surface charge can influence the antimicrobial activity (Kalwar and Shan, 2018; More et al., 2023; Rasmussen et al., 2020). In addition, many previous studies have shown that one of the critical characteristics of nanoparticles is size, and a smaller size has a higher effect on the ability of nanoparticles to penetrate into microbial cells (More et al., 2023). Additionally, the shape of AgNPs plays an important role in the antimicrobial activity, and it was decided that a spherical shape would result in excellent activity (Menichetti et al., 2023). Furthermore, the surface charge plays a crucial role in the initial adsorption of nanoparticles to cell membranes (Rasmussen et al., 2020).

For the MICs and MBCs of AgNPs (5 mM, pH 9), we recorded the lowest MIC against *S. aureus* ATCC 6538P and *B. subtilis* ATCC 6633 with a value of 62.5 µg/mL, while

the highest MIC was against *P. aeruginosa* ATCC 27853 (250 µg/mL). The lowest MBC (62.5 µg/mL) was recorded against *B. subtilis* ATCC 6633, while the highest MBC (>1000 µg/mL) was against *P.aeruginosa* ATCC 27853 (Table 4.3). The minimum inhibitory concentration (MIC) value is the lowest concentration of the tested substance as an antibiotic that inhibits the growth of the test microorganisms after an overnight incubation, while the minimum bactericidal concentration (MBC) value is the lowest concentration required to kill 99.9% of the test microorganisms (Mechouche et al., 2022; Pallavi et al., 2022).

Table 4.3. MICs and MBCs results of AgNPs (5 mM, pH 9).

Pathogenic microorganisms	MIC (µg/mL)	MBC (µg/mL)
S. aureus ATCC 6538P	62.5	125
B. subtilis ATCC 6633	62.5	62.5
E. coli ATCC 7839	125	125
P. aeruginosa ATCC 27853	250	> 1000
K. pneumoniae ATCC 13883	125	125

(ATTC): American Type Culture Collection.

There is no clear or exact mechanism for the antimicrobial effect of AgNPs, but many existing studies have reported that the most common and prominent mechanisms are as follows: a) AgNPs can adhere to the cell wall and membrane of microbial cells, causing cell rupture and leading to cell lysis. b) AgNPs can penetrate the inside of microbial cells and damage biomolecules (DNA and enzymes) and intracellular structures (mitochondria, vacuoles, and ribosomes). c) AgNPs have the potential to increase ROS production, which can cause oxidative stress and damage to microbial cells (Dakal et al., 2016; Roy et al., 2019).

4. Anticancer and neuroprotective activities

In the MTT test, SH-SY5Y cells were treated with various concentrations of AgNPs to determine their cytotoxic effects (Figure 4.12). Compound-1 (AgNPs, 1 mM, pH 5) did not alter cell viability at concentrations of 2 or 4 µg/mL, but at concentrations of 8, 12, and 16 µg/mL ($p<0.001$), it significantly reduced cell viability compared to that of the control group. For compound-2 (AgNPs, 1 mM, pH 7), a concentration of 2 µg/mL tended to increase cell viability, but this increase was not statistically significant. Additionally, there was no change in the cell viability at 4 µg/mL. Also, at concentrations of 8, 12, and 16 µg/mL ($p<0.001$), the cell viability was markedly lower than that in the control group. Compound-3 (AgNPs, 5 mM, pH 9) significantly decreased ($p<0.001$) cell viability at concentrations of 2, 4, 8, 12, and 16 µg/mL compared to that of the control.

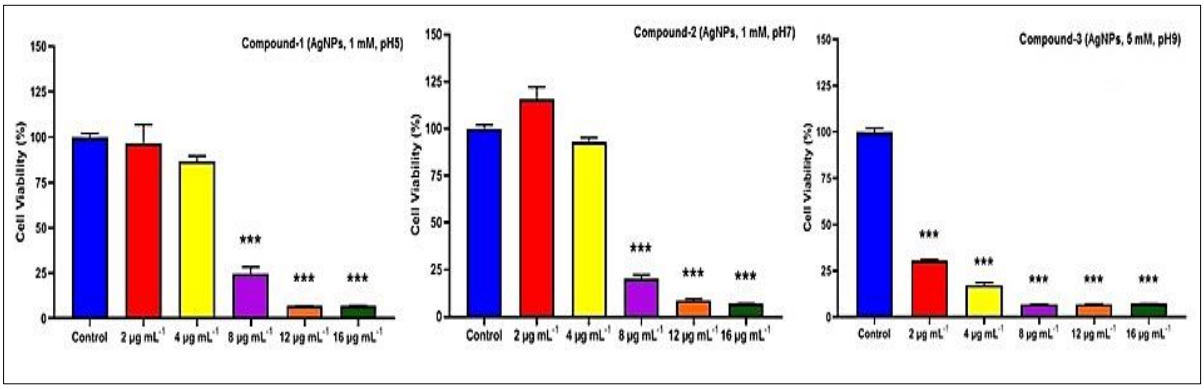


Figure 4.12. Effects of synthesized AgNPs on the viability of SH-SY5Y cells. ***: $p < 0.001$.

For the neuroprotective activity, the MTT assay was performed to determine the neurotoxic dose of H_2O_2 as shown in Figure 4.13. Treatment with 100, 150, 200, 300, 400, and 600 µM of H_2O_2 reduced ($p<0.001$) cell viability compared to that of the control group. To evaluate the neuroprotective effect of synthesized nanoparticles, 300 µM H_2O_2 was added one hour before the AgNPs were added, and cell viability was investigated via the MTT test at the 24th hour of AgNPs treatment. When the neuroprotective effects of the AgNPs were examined, compound-1 (AgNPs, 1 mM, pH 5) at concentrations of 2, 4, 8, 12, and 16 µg/mL ($p<0.001$) significantly reduced the viability of the SH-SY5Y cells compared to that of the H_2O_2 group. Compound-2 (AgNPs, 1 mM, pH 7) at a concentration of 2 µg/mL ($p<0.01$) significantly increased the cell viability with the presence of H_2O_2. In addition, at concentrations of 8, 12, and 16 µg/mL ($p<0.001$), the cell viability was markedly lower than that in the H_2O_2 group. Treatment with compound-3 (AgNPs, 5 mM, pH 9) at concentrations of 2, 4, 8, 12, and 16 µg/mL ($p<0.001$) significantly reduced the viability of the SH-SY5Y cells compared to that of the H_2O_2 group.

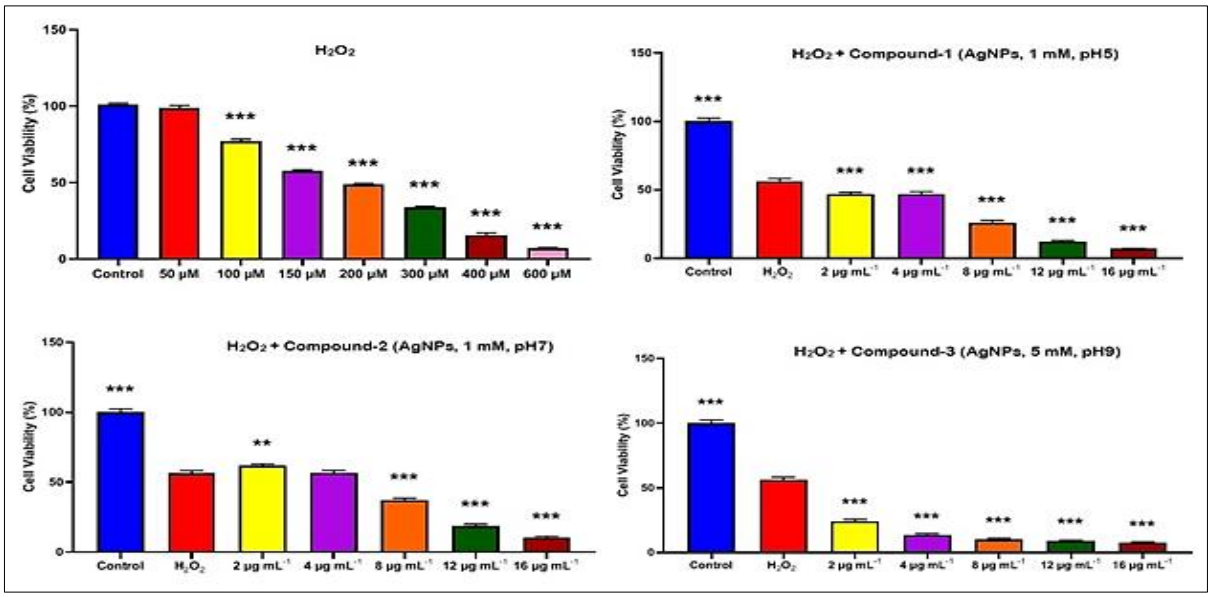


Figure 4.13. Neuroprotective activity of synthesized AgNPs. **: $p < 0.01$ ***: $p<0.001$.

In this study, cell culture experiments were conducted to address two primary objectives concerning SH-SY5Y neuroblastoma cells. First, the anticancer activity of synthesized AgNPs was investigated by assessing their cytotoxic effects on neuroblastoma cells. Determination of the optimal cytotoxic dose was crucial for evaluating the anticancer potency of the synthesized materials. Notably, Compound-1, Compound-2, and Compound-3 exhibited cytotoxic effects at concentrations of 2, 4, and 8 µg/mL. Comparative analysis revealed that compound-3 had superior anticancer activity against neuroblastoma cells. This observation aligns with recent studies, confirming the anticancer efficacy of silver nanoparticles synthesized by using bioactive molecules produced by some *Streptomyces* strains on different cancer cell lines (Asif et al., 2022; Pallavi et al., 2022). Additionally, our study was the first to show that AgNPs synthesized using a red pigment of *Streptomyces* sp. had anticancer activities on human neuroblastoma cells, although there are several studies showing anticancer and cytotoxic effects of different source-based AgNPs (Shkryl et al., 2021; Zhai et al., 2022). The second objective focused on elucidating the potential neuroprotective effects of AgNPs against hydrogen peroxide-induced cytotoxicity, a common mechanism underlying neurodegenerative diseases. Hydrogen peroxide (H_2O_2) at a concentration of 100 µM demonstrated cytotoxic effects, and a concentration of 300 µM was selected for its moderate cytotoxic effects, as suggested by prior study (Koca et al., 2019). Intriguingly, compound-2 exhibited neuroprotective effects at the lowest concentration tested, distinguishing it from the other compounds. The observed neuroprotective effects of compound-2 were hypothesized to be associated with its potential antioxidant properties,

drawing parallels with findings from Alkhalaf et al. in 2020, who demonstrated the antioxidant and anti-inflammatory effects of AgNPs synthesized by using *Nigella sativa* extract in rodents. Controversially, Zhai et al. (2022) showed the cytotoxic effects of AgNPs via oxidative stress mechanisms in SH-SY5Y cells. It has been seen that AgNPs induce oxidative stress at very high doses (40 and 60 µg/mL), whereas we observed the neuroprotective effect of low doses of AgNPs (2 and 4 µg/mL) against a well-known oxidative stressor, H_2O_2, in our study. These differences suggest a dose-dependent antioxidant or oxidant effect of AgNPs synthesized using the red pigment produced by the *Streptomyces* sp. A23 strain. These findings suggested a nuanced interplay between antioxidant effects at lower concentrations and antiproliferative effects at higher concentrations for the compound-2.

Conclusion

In conclusion, the green synthesis of silver nanoparticles (AgNPs) using the red pigment produced by the *Streptomyces* sp. A23 strain isolated from Algerian bee pollen represents a new approach with remarkable neuroprotective, anticancer, and antimicrobial activities. This synthesis method not only harnesses the inherent capabilities of the *Streptomyces* sp. A23 strain but also produces silver nanoparticles with unique biological properties, paving the way for their application in diverse therapeutic avenues. Future studies should address the molecular mechanisms underlying the therapeutic properties of these nanoparticles, including the molecular interactions between the AgNPs and biological targets, which will also provide deeper insight into their mode of action. Additionally, the exploration of synergistic effects with other therapeutic agents could open new possibilities for enhanced treatment strategies, especially in multidrug-resistant infections and complex diseases like cancer and neurodegeneration.

General Discussion and Conclusion

General Discussion and Conclusion

Bee pollen, a natural product collected by honeybees from plant flowers, is recognized for its rich nutritional and bioactive compounds (Straumite et al., 2022). Therefore, the comprehensive evaluations of its physicochemical, phytochemical, and microbiological analyses are critical for assessing its quality, safety, and any potential health benefits. In this study, conventional techniques were used to obtain preliminary qualitative and quantitative data on the microbiological and phytochemical quality of some local bee pollen samples. The results revealed the presence of various microorganisms, including bacteria, yeasts, and fungi, as well as important concentrations of polyphenols and flavonoids. Furthermore, the ethanolic extracts of these samples demonstrated notable antioxidant activities.

Among the important physicochemical analyses are the pH, moisture, and ash content. In this study, the obtained pH values were in the acidic range between 5.33 ± 0.13 and 3.87 ± 0.02. The pH is one of bee pollen's quality indicators, influencing its nutritional properties, microbial safety, and storage stability. It has been reported that it should be between 4.3 and 7 because a pH below 4 can lead to a number of unwanted microbial activities in unsafe storage conditions (Fuenmayor et al., 2014; Straumite et al., 2022). The moisture of bee pollen greatly affects its nutritional composition, shelf life, and susceptibility to microbial contamination (El Ghouizi et al., 2023). The obtained moisture content in this study, which ranged from 11.13 ± 0.03 to 27.80 ± 0.18%, is approximately in agreement with several previous studies that reported that the moisture content of fresh bee pollen ranges from 10 to 30%, while dried bee pollen contains less than 8%, which is influenced by some factors, such as botanical origin and storage conditions (Castagna et al., 2020; El Ghouizi et al., 2023; Prđun et al., 2021). Furthermore, ash content, representing the total mineral content, is important for the nutritional value of bee pollen, reflecting variations due to plant species, soil composition, climate, and geographical origin (De Arruda et al., 2017; Prđun et al., 2021). The values obtained for ash in this study, which ranged between 3.73 ± 0.31 and 1.93 ± 0.26%, are similar to those suggested by Campos et al. (2008) in their standardization of bee pollen.

Concerning the phytochemical study, the total polyphenols and flavonoid content of bee pollen contribute to various health benefits, including antioxidant activity. The obtained averages of total polyphenols, flavonoids, and IC_{50} values of the studied bee pollen samples were 15.28 ± 4.17 mg/g, 5.54 ± 2.85 mg/g, and 2.35 ± 1.25 mg/mL, respectively. As reported in the study of Rojo et al. (2023), which indicated the relationship between polyphenols,

flavonoids, and antioxidant potential, these results revealed a positive significant correlation between total polyphenols and flavonoids ($r = 0.917$; $p < 0.01$) and a negative significant correlation between IC_{50} and flavonoids ($r = -0.501$; $p < 0.05$).

Due to its nutritional composition, different species of microorganisms, including bacteria, molds, and yeasts, can be present in bee pollen during collection, processing, and storage, depending strongly on its geographic region and beekeepers activities, necessitating the following hygienic and quality standards to minimize the microbial load that can pose risks to consumer safety (Altintas et al., 2022; De Arruda et al., 2017; Straumite et al., 2022). The obtained results of the microbiological analyses in the present study showed significant differences between all studied samples, with the presence of different microorganisms, including some pathogenic species, such as *S. aureus* and sulfite-reducing *Clostridium*. In (2008), Campos et al. reported that the microbial load in bee pollen should correspond to the European Union's standards for microbiological quality, which are as follows: total mesophilic bacteria (<100,000/g), molds and yeasts (<50,000/g), Enterobacteriaceae (maximum 100/g), *S. aureus* (absent/1 g), *Salmonella* sp. (absent/10 g), and *E. coli* (absent/1g).

Unfortunately, unlike previous studies, some other important physicochemical parameters, including carbohydrates, proteins, and lipids, were not determined, which would have revealed and provided additional information about the microbiological and phytochemical quality of the collected pollen samples (De Arruda et al., 2017; Fuenmayor et al., 2014; Spulber et al., 2018).

In addition, the present study has revealed some potential applications of actinomycetes, particularly *Streptomyces* species, isolated from the collected samples of bee pollen. The study not only enhanced the isolation and identification of this kind of microorganisms but also highlighted their significant medical and biotechnological applications in the fields of antibiotherapy, enzyme production, and nanomedicine.

The increasing problem of antimicrobial resistance and the growing demand for industrial enzymes and pigments have pushed the search to discover new valuable bioactive compounds from natural origins (Álvarez-Martínez et al., 2020; Celedón & Díaz, 2021; Mesbah, 2022). Actinomycetes species, especially those from the genus *Streptomyces*, are known for their wide production of antibiotics, pigments, and enzymes (Alam et al., 2022; Janaki, 2017; Sarmiento-Tovar et al., 2022).

To explore the presence of actinomycetes producing antibiotics, pigments, and enzymes in bee pollen and their diversity, the collected samples were subjected to different pretreatments and cultured in various media, including starch casein agar, which favors the actinomycetes' growth to obtain a maximum number of strains. As a result, 33 strains were isolated, characterized, and then classified, revealing eight diverse clusters within the *Streptomyces* genus, exhibiting varied morphological, cultural, physiological, and biochemical properties.

First, the enzymatic potential of the isolated actinomycetes strains was evaluated qualitatively by testing their capacity to produce extracellular enzymes, such as proteases, amylases, and lipases. It was found that most of the strains were able to produce these enzymes, which are essential for biotechnological and industrial uses. Several studies have demonstrated the potential of actinomycetes, including *Streptomyces* sp., in producing proteases, amylases, and lipases (Hasan et al., 2024; Pham et al., 2024; Singh et al., 2019). The ability of actinomycetes to produce stable and robust proteases, which represent an important class of industrial enzymes, makes them highly attractive for biotechnological exploitation, such as pharmaceuticals, food processing, detergents, and leather processing (Bawazir et al., 2019; Rai & Bai, 2022). Also, actinomycetes represent a valuable source of amylases, which are hydrolytic enzymes that facilitate the degradation of starch into simpler sugars, including maltose, glucose, and dextrin. These enzymes are one of the most important commercial enzymes, which being used in various industrial processes, including food, paper, and textiles (Bawazir et al., 2019; Singh et al., 2019). In addition to proteases and amylases, actinomycetes also have the capability to secrete lipases, which can hydrolyze fats and triglycerides to form diglycerides, monoglycerides, fatty acids, and glycerol. Lipases have the potential to be used in various industrial applications, including the processing of oils and fats, detergents, food, and cosmetics (Janaki, 2017; Mukhtar et al., 2017).

Furthermore, the ability of the isolated actinomycetes to produce antimicrobials against some pathogenic microorganisms was evaluated using two tests: the perpendicular streak method for the first screening and the double-layer agar method for the second screening. The results revealed that only five potent strains, namely, A1, A6, A24, A27, and A28, exhibited considerable antibacterial and antifungal activities against *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883, and *C. albicans* ATCC 1023, demonstrating their potential as sources of alternative natural antimicrobials. These potent strains were subjected to characterization by SEM analysis and

16S rRNA gene sequencing, confirming their classification belonged within the *Streptomyces* genus. Prior studies indicated that honeybees and their products, such as honey, bee pollen, and bee bread, provide a unique niche for the isolation of actinomycetes, including *Streptomyces* sp., with potential antimicrobial properties (Grubbs et al., 2021; Santos-Beneit et al., 2022).

The crude ethyl acetate extract from the *Streptomyces* BPA-6 strain, which was one of the five potent strains, showed excellent antimicrobial activity against *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, and *C. albicans* ATCC 10231. The GC-MS analysis of this extract revealed a variety of bioactive secondary metabolites, including hexadecanoic acid, 9,12-octadecadienoic acid, and 9-octadecenoic acid. Several recent studies have indicated that *Streptomyces* species have the ability to produce a wide range of bioactive compounds, such as hexadecanoic acid, 9,12-octadecadienoic acid, and 9-octadecenoic acid, exhibiting antibacterial and antifungal activities against various disease-causing microorganisms, including *S. aureus*, *B. subtilis*, and *C. albicans* (Djebbah et al., 2022; Malash et al., 2022; Mothana et al., 2022). Many previous studies used the GC-MS method to analyze and determine the bioactive compounds produced by the *Streptomyces* species, which exhibited various biological activities, including the antibacterial, antifungal, antioxidant, and anticancer activities (Adeyemo et al., 2024; Nofiani et al., 2025; Tangjitjaroenkun et al., 2021). The GC-MS is a powerful tool for identifying and characterizing bioactive compounds, especially the volatile ones, but unfortunately, it's not enough to find all of them, including the non-volatile compounds (Al-Rubaye et al., 2017; Peguy Flora Djuidje et al., 2024; Ranjan Maji et al., 2023). Therefore, more advanced analytical techniques are necessary and required to purify and identify the bioactive secondary metabolites produced by actinomycetes and elucidate their chemical structures, including HPLC, UPLC-MS, LC-MS, LC-MS-MS, FT-IR, and NMR. In addition to GC-MS analyses, some studies also used HPLC, UPLC-MS, or LC-MS-MS to identify non-volatile constituents of some *Streptomyces* species, which demonstrated antimicrobial, antioxidant, and anticancer effects (Elghwas et al., 2024; Nazeeh et al., 2024; Peguy Flora Djuidje et al., 2024). With the help of AntiSMASH software, another study combined LC-MS with FT-IR for the prediction of antimicrobial substances in the crude extracts of the *Streptomyces* sp. CRB46 and their chemical structures, which showed antibacterial and antifungal activities (Ambarwati et al., 2020). Furthermore, NMR spectroscopy, HPLC-UV-vis, and HPLC-MS analyses were used together to identify

antimicrobial and cytotoxic metabolites that were produced by the *Streptomyces* sp. PU-KB10-4 and to elucidate their chemical structures (Saleem et al., 2023).

In the last part of this study, the red pigment produced by the *Streptomyces* sp. A23 strain was used as a new biological reducing agent for the green synthesis of silver nanoparticles (AgNPs), which demonstrated significant antibacterial and antifungal activities. In addition, these synthesized AgNPs exhibited potent anticancer effects on SHSY-5Y human neuroblastoma cell lines with significant neuroprotective activity at the lowest tested concentrations. In reality, this synthetic approach and the mentioned activities of AgNPs are not new, but the use of the red pigment produced by the *Streptomyces* sp. A23 strain, which was isolated from bee pollen as a new reducing agent, may influence the AgNPs properties and be more effective than others reported in the literature. Additionally, this study was the first to show that AgNPs synthesized using a microbial pigment had antimicrobial activity and anticancer and neuroprotective effects on human neuroblastoma cell lines (SH-SY5Y). Other studies reported that the AgNPs, which were synthesized using various sources, including plant extracts, demonstrated the same studied effects (Bayav et al., 2024; Rangavitala & Taranath, 2024; Zhai et al., 2022).

Based on the obtained results in this study, our perspectives can be summarized as follows:

1. Isolation of other actinomycetes strains from other bee pollen samples and other beehive products.

2. Optimization of bioactive secondary metabolite production using the best fermentation conditions including the effects of carbon and nitrogen sources, mineral elements, agitation, and pH.

3. Continuation of study by purifying and identifying the produced bioactive secondary metabolites using the advanced analysis methods, as well as determining their chemical structures.

4. Exploring the enzymatic potential applications and other functional properties of the isolated actinomycetes strains.

5. The *in vivo* evaluation of the produced secondary metabolites and AgNPs is required.

6. Valorization of the red pigment in different applications, particularly as a color and medicine.

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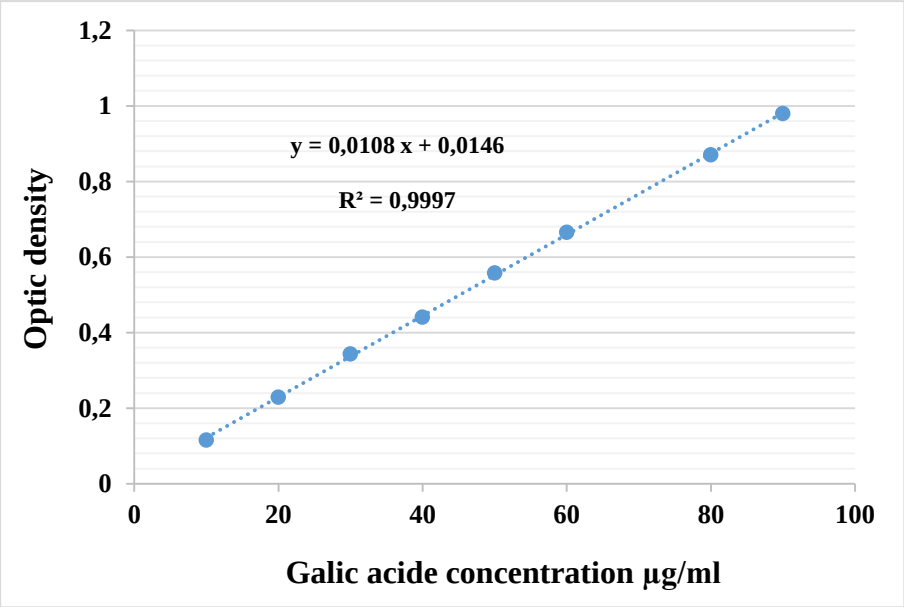
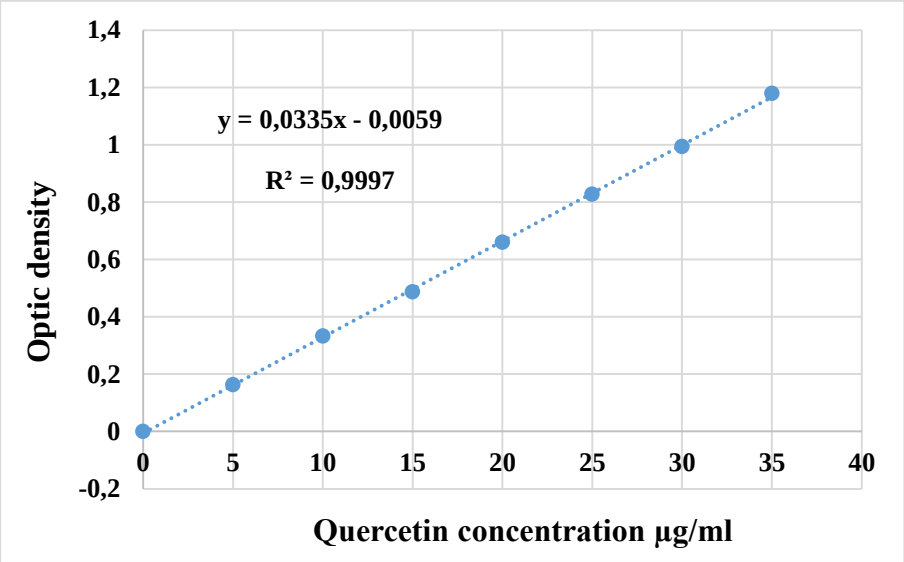
Appendices

Appendix A. Standard curve of gallic acid.



Appendix B. Standard curve of quercetin.



Appendix C. The culture media used in the isolation of actinomycetes.

1. Yeast extract-malt extract agar (YMA):

- Yeast Extract ……..……..…. 4 g
- Malt Extract …….……...…. 10 g
- Dextrose ……...…… 4 g
- H_2O D ……..…….... 1000 mL

- pH …… 7,3
- Agar ……...… 20 g

2. Starch casein agar (SCA):

- Starch ……...….. 10 g
- Casein ……...…..... 1 g
- $CaCO_3$ ……..... 0.02 g
- $FeSO_4$ ……..... 0.01 g
- KNO_3 ……...….. . 2 g
- H_2O D ……...….. 1000 mL
- pH: ……...….. 7 - 7,4
- Agar ……...……. 20 g

3. Streptomyces agar (SA):

- Glucose …… 4 g
- Yeast Extract …….. 4 g
- Malt Extract ……... 10 g
- $CaCO_3$ ……..… 2 g
- H_2O D …… 1000 mL
- pH ……. 7,2
- Agar ……... 15 g

4. Glycerol - asparagine agar (GAA):

- L-asparagine …… 1 g
- Glycerol …… 10 g
- K_2HPO_4 …….. 1 g
- H_2O D …… 1000 mL
- Trace salts solution ……. 1 mL
- pH ……..... 7 - 7,4
- Agar ……... 20 g

Trace salts solution:

- H_2O D ……..…….. 100 mL
- $FeSO_4 \cdot 7H_2O$ ……..... 0.1 g
- $MnCl_2 \cdot 4H_2O$ ……..... 0.1 g
- $ZnSO_4 \cdot 7H_2O$ ……..... 0.1 g

5. Actinomycetes isolation agar (AIA):

- Sodium propionate ……..…..….. 4 g
- Sodium caseinate ……..... 2 g
- L-asparagine …….…… 0.1 g
- K_2HPO_4 …..……... 0.5 g
- MgSO4 ……..... 0.1 g
- $FeSO_4$ ……..….... 0.001 g
- Agar ……..…….…. 15 g
- H_2O D ……..…… 1000 mL
- Glycerol ……..…….……. 5 g
- pH ……..……... 8.2

6. Nurient agar (NA):

- Gelatine peptone …..…… 5 g
- Beef extract …..……..... 3 g
- H_2O D …… 1000 mL
- Agar ……... 17 g

7. Czapek agar (CZA):

- Sucrose ..... 30 g
- $NaNO_3$ …..... 3 g
- KCl ……..... 0.5 g
- $MgSO_4 \cdot 7H_2O$ ..... 0.5 g
- $FeSO_4 \cdot 7H_2O$ ..... 0.01 g
- K_2HPO_4 ..... 1 g
- Agar ..... 15 g
- H_2O D ..... 1000 mL

Appendix D. 16S rRNA gene sequences of the selected actinomycetes strains.

>Seq A1 strain (PP577932.1)

AAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACA
CGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACC
GGATAACACTCTGTCCCTCATGGGACGGGGTTAAAAGCTCCGGCGGTGAAGGAT
GAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACG
GGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC

AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGG
AAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAG
AGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCTCGGGGCTTAACCCCGAGT
CTGCATTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATT

>Seq BPA-6 strain

GCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGA
TGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGT
TCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGG
AGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCG
AAAGCGTGGGGAGCGAACAGGATTATATACCCTGGTAGTCCACGCCGTAAAACG
TTGGAGAACTAGGTTGTGGGCGACATTCCACGTTCGTCCGGTGCCGCAGTCTAAC
GCATTAAGTTCCCCCGCCTTGGGGAGTACGGCCCGCCAAGGCTAAAAACTTCAAA
GGAAATTGAACGGGGGGCCCCGCACAAAGCGGCCGGAAGCATTGTGGGCTTTAA
TTTCGGACGCCAACCGCGGAAAGAACCCTTACCCAAGGGCTTGGACATTACACCC
GGGAAAAACGGGCCCAGAAGAATGGGTCGGCCCCCCCTTTGTGGGTTCGGGTGG
TAACAGGGTGGGTGGCATGGGCCTTGTCCGTTCAAGCTTCGTGGTCGGTGGAGGA
TGGTTGGGGGTTAA

>Seq A23 strain (OR236137.1)

GCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAA
CACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATA
CCGGATACGAGCCTCACGGGCATCTGTGAGGTTGGAAAGCTCCGGCGGTGCAGG
ATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGA
CGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGAT
GCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAG
GGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAA
AGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGG
GTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTG
GTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGAT
CTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCGACATTCC
ACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCG
CAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGT
GGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAA
ACGTTCAGAGATGGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGT
CCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGG
TCAACTC

>Seq A24 strain (PQ821170.1)

GTCGAACGATGAAGCCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACA
CGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACC
GGATATGACTACCGATCGCATGGTTGGTGGTGGAAAGCTCCGGCGGTGCAGGAT
GAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACG
GGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGG
AAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAG
AGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGT
CTGCATTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTG
TAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTC
TGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCCACG
TCGTCCGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAA
GGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGC
TTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACG
GCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCT
GTGTTGCCAGCATGCCTTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTC
A

>Seq A27 strain (PV131599.1)

GCGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAA
AGTGACGGTACCTGCAGAAGATGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGG
CGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGA
TACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGA
AATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGAT
ACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTA
GTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTG
CCGCTGCTAACGCATTAAGTGCCGGGCCTGGGGAGTACGGCCGCAAGGCTAAAA
CTCAAAGGAATTGACGGGGGCCCGCACATGCGGCGGAGCATGTGGCTTAATTCG
ACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAACCCTGGAGA
CAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTC
TGGTGAGATGTTGGGTTAAGTCCCGCAACGA

>Seq A28 strain (PP577933.1)

AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGA
TGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCA
GGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTA

AAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCG
GGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCT
GGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGA
TCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTC
CACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCC
GCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATG
TGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAA
AACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGT
CCCGTGTTGCCAGCAAGCCCCTTCGGGGGTGTTGGG

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Evaluation of antimicrobial, anticancer and neuroprotective activities of silver nanoparticles (AgNPs) green-synthesized using a red pigment produced by *Streptomyces* sp. A23 strain isolated from Algerian bee pollen

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Abstract: In this work, the red pigment of *Streptomyces* sp. A23 strain isolated from Algerian bee pollen was used for the green synthesis of silver nanoparticles (AgNPs) as well as for evaluating their antimicrobial, anticancer and neuroprotective activities. AgNPs were synthesized as a result of the reduction of 1 and 5 mM silver nitrate solutions at various pH values (5, 7 and 9) and were subsequently characterized. AgNPs (5 mM, pH 9) exhibited a maximum UV–Vis absorbance at 433 nm. Dynamic light scattering revealed that the average diameter was 112 nm. A zeta potential peak was found at –33 mV corresponding to the increased stability. XRD analysis confirmed the crystallization nature of the material. Furthermore, FT-IR analysis revealed the specific functional groups at 3471 to 478 cm^{-1}. In addition, FE-SEM showed that the mean size of the spherical AgNPs was 54.5 nm in diameter. The presence of Ag was revealed by EDX analysis. Additionally, good antimicrobial activity was observed against *Enterococcus faecalis* ATCC 19433, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 13883 and *Escherichia coli* ATCC 7839, with inhibition zones of 32, 30, 30, 27, 25, 20 and 19 mm, respectively. The lowest minimum inhibitory concentration and minimum bactericidal concentration were recorded against *B. subtilis* ATCC 6633, with a value of 62.5 μg mL^{-1}. Intriguingly, all the synthesized

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AgNPs at concentrations of 2, 4 and 8 μg mL^{-1} had cytotoxic effects on SH-SY5Y neuroblastoma cell lines. In addition, AgNPs (1 mM, pH 7) exhibited the significant neuroprotective activity at the lowest tested concentration. Finally, the AgNPs synthesized using the red pigment of *Streptomyces* sp. strain A23 can be considered as promising therapeutic agents.

Keywords: actinomycetes; microbial pigment; green synthesis; antimicrobial activity; cancer; neuroprotective activity.

INTRODUCTION

Recently, the use of nanomaterials in the medical field has attracted increased attention, especially as novel and alternative therapeutic agents with multifaceted applications.[1] One of these nanomaterials are silver nanoparticles (AgNPs), which are emerging as possible hopeful alternatives due to their beneficial features.[2] Two conventional methods were applied in order to synthesize AgNPs and they are chemical and physical methods. Due to their undesirable disadvantages, some suitable and green alternative methods must be found. Among these methods, the biological methods which use bacteria, fungi, algae, plants, and others have gained the substantial prominence due to their high safety.[3] Many researchers have used Actinomycetes, including *Streptomyces* bacteria to synthesize AgNPs.[4,5] The genus *Streptomyces* is known as a source of valuable and variety of secondary metabolites, which are excellent tools for the green synthesis of nanoparticles.[6,7] One such secondary metabolite is pigments which not only serve as an identifier product for *Streptomyces*, but also demonstrate a prominent potential for synthesizing AgNPs.[8] This process of synthesis provides a fast, safe and eco-friendly approach, and imparts significant biological properties like antioxidant, antimicrobial, and antiproliferative activities, to the resulting nanoparticles, which hold promise as multifaceted therapeutic agents capable of treating both communicable and noncommunicable diseases.[9,10] Regarding communicable diseases, the growth of antibiotic-resistant microorganisms is one of the most important public health problems, which have emerged as a global crisis, underscoring the urgent need to discover novel and alternative antimicrobial agents to the conventional antibiotics.[11] AgNPs synthesized using secondary metabolites produced by *Streptomyces* exhibit good antimicrobial effects against a wide range of pathogenic microorganisms, including bacteria and fungi.[12] Moreover, when AgNPs are combined with conventional antibiotics, they can have synergistic effects that may overcome antibiotic resistance.[13] For noncommunicable diseases, cancer and neurodegenerative disorders remain major health concerns, necessitating the discovery of new effective therapeutic agents.[14,15] AgNPs synthesized using the microbial pigments have shown promising anticancer and neuroprotective effects.[12,16] These nanoparticles are promising agents in cancer therapy because they can target the cancer cells, while

avoiding the cells that are normal.[17] Furthermore, the distinctive physicochemical properties of these nanoparticles allow them to exhibit antioxidant activities, reducing the oxidative stress that might lead to neurodegenerative disorders. Additionally, these nanoparticles can modulate inflammatory responses, further contributing to their neuroprotective effect.[18]

Therefore, this study aims to synthesize AgNPs using a red pigment produced by the *Streptomyces* sp. A23 strain isolated from Algerian bee pollen as a new biological reducing agent to evaluate their antimicrobial, anticancer and neuroprotective activities.

EXPERIMENTAL

Materials

Silver nitrate ($AgNO_3$), hydrogen peroxide (H_2O_2) and dimethyl sulfoxide (DMSO) were purchased from Sigma. Muller-Hinton broth was obtained from Conda and calcium carbonate ($CaCO_3$) was obtained from Merck. The GF-1 nucleic acid extraction kit, universal primers (27F and 1492R) and clean-up kit were from Vivantis Technologies. Gentamicin discs were provided from Oxoid™. *Staphylococcus aureus* ATCC 6538P, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 19433, *Escherichia coli* ATCC 7839, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883 and *Candida albicans* ATCC 10231 were obtained from the applied microbiology laboratory, Ferhat Abbas University, Algeria. SHSY-5Y human neuroblastoma cells (ATCC®, CRL-2266) were obtained from Erfarma Center, Erciyes University, Turkey.

Bee pollen sampling

The bee pollen samples were provided by the International Api-Phyto Therapy Center, Setif, Algeria, from different Algerian clean lands during the spring of 2019, in sterile plastic containers. The samples were subsequently transported to the laboratory and stored at 4 °C until use.

Isolation, purification and conservation of the A23 strain

All the samples were crushed, air dried at room temperature and heated at 50 °C for 30 min in a water bath (Memmert, Germany). One gram from all bee pollen samples was mixed with 0.1 g of calcium carbonate and incubated at 28 °C for one week.[19] Then, 1 g of each pre-treated sample was suspended in 9 mL of sterile saline water and diluted to 10^{-2}, after which 100 µL of the diluted sample was plated on different media for Actinomycetes isolation and incubated at 28 °C for approximately 10 days. During the period of incubation, the selected strain was purified using yeast extract-malt extract agar (ISP 2 medium), labelled by the A23 strain and conserved at 4 °C.[20]

Phenotypic identification of the A23 strain

The primary phenotypic characteristics of the A23 strain were determined *via* Gram staining, catalase and oxidase tests.[21,22] In addition, the culture characteristics were described based on the growth intensity, aerial and substrate mycelia and the pigment production on the ISP 2 agar medium.[23] Also, the carbohydrate assimilation was assessed on minimal growth medium supplemented with the carbon source at a final concentration of 1 %, as described by Shirling and Gottlieb (1966).[24] The production of extracellular hydrolytic enzymes (starch hydrolysis, protease and lipolysis) was studied on the minimum agar medium added to the

substrates at a concentration of 1 %.[25] The assignments of the A23 strain at least to the genus level were performed by comparing the results of the phenotypic characterization to those reported in Bergey's Manual of Systematic Bacteriology.[26]

Genotypic identification of the A23 strain

DNA extraction, 16S rRNA amplification and electrophoresis. The DNA extraction of the selected strain was carried out by using the GF-1 Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia). The 16S rRNA amplification was performed by PCR using thermocycler (iCycler Bio-Rad, USA) with the help of two universal primers, 27F and 1492R. This process was repeated for 30 cycles,[27] after which the products of PCR were subjected to electrophoresis and subsequently purified by a Clean-Up Kit (Vivantis Technologies, Malaysia).[22]

DNA sequencing and bioinformatics analysis. The PCR products were purified and subsequently sequenced using 3130 Genetic Analyzer (Applied Biosystems®, Waltham, MA, USA).[22] BLAST was used to compare the obtained sequence of the studied strain with 16S rRNA gene sequences of other Actinomycetes in GenBank. MEGA 11 software was used to construct the phylogenetic tree.[28]

Green synthesis, purification and characterization of silver nanoparticles (AgNPs)

Production and purification of red pigment. Red pigment was produced from the A23 strain by the submerged fermentation on ISP 2 broth using an orbital shaker at 180 rpm and 28 °C. A week later, the culture broth was centrifuged at 4000 rpm and 4 °C for 10 min to remove the mycelia. The supernatant that contained the produced pigment was collected and stored at 4 °C.[29]

Synthesis and purification of AgNPs. Two mL of the obtained pigment were filtered through a 0.45 μm syringe filter and mixed with 18 mL of 1 or 5 mM silver nitrate solutions at pH 5, 7 and 9 using a magnetic hotplate stirrer at 1500 rpm and room temperature for 24 h in dark conditions.[9,30] The first part of the nanoparticles suspension was obtained directly and kept at 4 °C for UV–Vis spectrophotometry, zeta potential and DLS analyses and further experiments. The second part was centrifuged and washed two times with distilled water at 15000 rpm for 10 min, after which the precipitates were dried at 70 °C for 2 days and subjected to the characterization using XRD, FT-IR, FE-SEM and EDX analyses.[31]

Characterization of synthesized AgNPs. Several techniques have been used to characterize the synthesized AgNPs. The formation of AgNPs was confirmed by the visual observation and UV–Vis (Perkin Elmer).[9] The hydrodynamic diameter was determined *via* dynamic light scattering (DLS, Malvern Panalytical, Malvern, UK). The surface charge was examined by zeta potential analyser. The presence of functional groups, playing a role in the synthesis, was determined by FT-IR (Perkin Elmer Spectrum 400) analysis. The crystallization was determined by XRD (Bruker AXS D8, Ettlingen, Germany) analysis. The morphology and the elemental presence was determined by the field emission scanning electron microscopy (FE-SEM) and energy-dispersive (EDX) analyses (Zeiss Gemini SEM 500, Aalen, Germany).[32]

Biological activities of synthesized AgNPs

Antimicrobial activity. The first test of antimicrobial activity of the synthesized AgNPs was evaluated using the agar well diffusion method, as described by Balouiri *et al.* (2016)[33] against *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, *E. faecalis* ATCC 19433, *E. coli* ATCC 7839, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883 and *C. albicans* ATCC 10231. The fresh microbial suspensions (10^7 cfu mL^{-1}) were prepared and swabbed

using sterile cotton swabs on Muller–Hinton agar plates. After that, the wells were made using sterilized micropipette tips and 100 µL of synthesized AgNPs were poured into each well. Finally, all plates were kept at room temperature for 30 min to diffusion and subsequently incubated at 37 °C for 24 h. After incubation, the inhibition zone diameter observed around each well was measured and is expressed in mm. The gentamicin discs (Oxoid™) and silver nitrate solutions were used as positive controls; pigment alone and distilled water were used as negative controls. After that the minimum inhibitory concentrations (*MIC*s) and the minimum bactericidal concentrations (*MBC*s) of AgNPs (5 mM, pH 9) were determined by the micro-broth dilution method using tetrazolium salts (TTC) in 96 well microplates in triplicate and on Mueller–Hinton agar plates, respectively.[9]

Anticancer and neuroprotective activities. The final concentrations of 2, 4, 8, 12 and 16 µg mL^{-1} of the synthesized AgNPs were prepared to be used in the evaluation of the cytotoxicity and the neuroprotective effects on the SHSY-5Y neuroblastoma cells by using the MTT test.

Cytotoxicity assay. After the preparation of SHSY-5Y cells, they were exposed to all prepared concentrations of AgNPs for 24 h in 96 well plates contains approximately 10^4 cells per well. Then, 100 µL of the prepared MTT solution was added to each well and incubated at 37 °C for 4 h. Afterwards, the supernatant portion was subsequently removed from the wells, followed by the addition of 100 µL of DMSO to each well in order to dissolve the MTT salts. Finally, the microplate reader (BioTek, Synergy HT, Winooski, Vt, USA) was used to measure the absorbances at a wavelength of 560 nm.[31]

Neuroprotective activity. To evaluate the potential neuroprotective effects of the synthesized nanoparticles on neuroblastoma cells, the prepared concentrations of AgNPs compounds were added 1 h after the treatment of cells by H_2O_2 (300 µM) in 96 well plates. Cell viability was measured by the MTT test after 24 h of incubation. The same volume of medium without AgNPs or H_2O_2 was used as a control.[31]

Statistical analysis. Data analysis was performed using the GraphPad Prism 8.0 software by using ANOVA one-way and Dunnett's post-hoc tests. All differences were considered significant when $P < 0.05$. The n value was four for the cell culture studies

RESULTS AND DISCUSSION

Phenotypic and genotypic identification of the isolated strain (A23)

As shown in Fig. 1, the isolated strain in our study was characterized by earthy odour and powdery colonies with a grey white colour, and it was a filamentous, branching and gram-positive bacterium. The morphological and the cultural properties have shown abundant growth with the variations in the colour of aerial and substrate mycelia. Additionally, it has the potential to produce a diffusible red pigment. Actinomycetes can produce visible powdery colonies with different colours from the 3rd to 7th day, with an earthy odour as a result of these bacteria's ability to produce various secondary metabolites, including pigments.[34] Furthermore, the strain can use different carbon sources for growth, and it is also positive for catalase and negative for the oxidase reactions. Protease, lipase, and amylase are produced by the described strain (Table I). With the help of Bergey's manual of systematic bacteriology, these phenotypic properties are consistent with the typical characteristics described for the genus *Streptomyces*.[26]

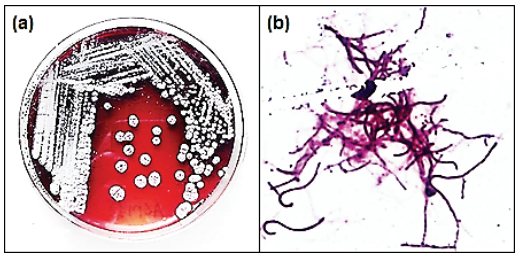

Fig. 1. *Streptomyces* sp. A23 strain a) Colony morphology b) Gram staining.

TABLE I. The phenotypic characteristics of the isolated strain (*Streptomyces* sp. A23); +) positive reaction; –) negative reaction; +++) elevated production

Growth intensity on ISP2 agar	+++
Arial mycelium colour on ISP2 agar	Grey white
Substrate mycelium colour on ISP2 agar	Dark red
Diffusible pigment	+++
Gram staining	Positive
Carbon sources assimilation	
Glucose	+
Fructose	+
Lactose	+
Maltose	+
Galactose	+
Mannitol	+
Enzymes production	
Catalase	+
Oxidase	–
Starch hydrolysis	+
Casein hydrolysis	+
Lipolysis	+

According to the obtained 16S rRNA sequence (1096 bp) and the phylogenetic tree analyses (Fig. 2), the A23 strain belongs to the genus *Streptomyces*. It was labelled *Streptomyces* sp. A23 strain and registered in GenBank under the accession number OR236137.1.

Green synthesis and characterization of AgNPs

AgNPs were successfully synthesized via a rapid, easy, and eco-friendly procedure, using the red pigment of the *Streptomyces* sp. A23 strain that isolated from bee pollen as a bio-reducing agent source. Silver nitrate ($AgNO_3$) is a colourless solution, and the pigment is red colour. When the pigment was mixed with silver nitrate solution, the mixture slowly turned to the brown colour after 24 hours in the dark (Fig. 3). The colour change was due to silver nitrate interacting with the pigment compounds and being bio-reduced from silver nitrate ($AgNO_3$) to elemental silver. These elementals aggregate to form stable AgNPs.[35] A previous study indicated the formation of AgNPs using a pink pigment

produced from *Streptomyces* sp. NS-05 isolated from a rhizospheric soil by observing the change in colour to brown.[8]

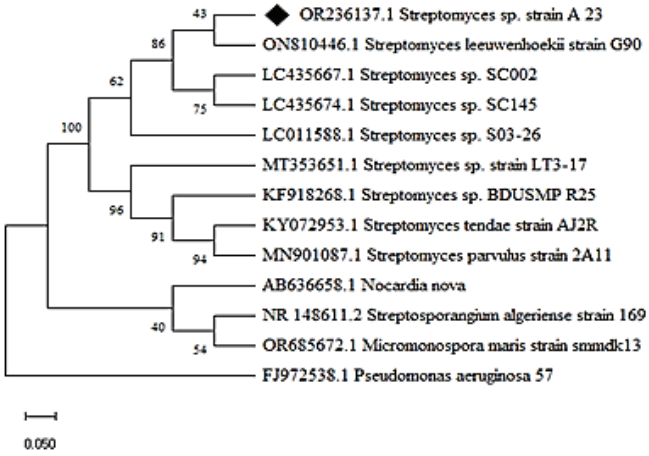

Fig. 2. Phylogenetic tree of the *Streptomyces* sp. A23 strain.

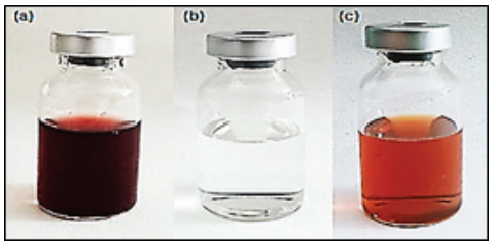

Fig. 3. Visual observation of synthesized AgNPs: a) pigment, b) $AgNO_3$ solution and c) AgNPs suspension.

In this study, the light absorption points, the hydrodynamic diameter, the surface charge, the crystallization, the phytochemical composition, the morphology and the elemental presence of synthesized AgNPs (5 mM, pH 9) were determined by UV–Vis, DLS, zeta potential, XRD, FT-IR, FE-SEM and EDX analyses, respectively. The optical and electrical properties of nanoparticles are interrelated, and unlike those of their bulk forms, they have a UV–Vis absorption band called surface plasmon resonance (SPR).

UV–Vis spectroscopy was used for characterizing the nanoparticles synthesized by various methods. The diffractions observed by analysis can be controlled by many factors, such as reducing agents and solvents.[36] According to the obtained results, the characteristic light absorption bands detected at 278 and 433 nm by UV–Vis analysis were associated with the presence of biomolecules in the pigment and AgNPs, respectively (Fig. 4). The characteristic light absorption bands of AgNPs synthesized by the secondary metabolites of *Streptomyces rochei* and marine *Streptomyces* sp. were measured at 413 and 434 nm, respectively.[37,38]

The structural properties of nanoparticles which depend on the synthesis conditions, also affected the UV–Vis characterization bands.[39]

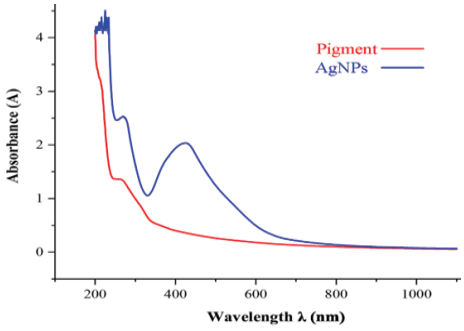

Fig. 4. UV–Vis spectrum of AgNPs (5 mM, pH 9).

The surface charge of nanoparticles determines the distribution properties of particles, the adsorption properties of ions, and molecules, and it is an important parameter for the characterization of nanomaterials.[40] The surface charge of the AgNPs synthesized in our study was determined to be –33 mV (Fig. 5). A relatively low zeta potential increases the electrostatic repulsion force between nanoparticles, which prevents their aggregation and ensures their stabilization.[41] The surface charge (negative, positive or neutral) of nanoparticles is related to the functional groups on the surface structure.[42]

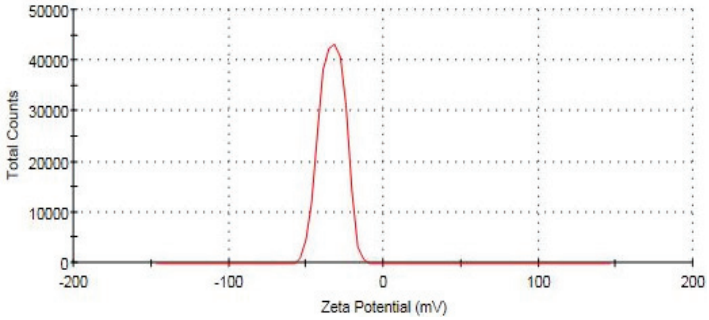

Fig. 5. Zeta potential analysis of AgNPs (5 mM, pH 9).

The dynamic light scattering (DLS) is a spectroscopic analysis technique used to determine the size of particles suspended in a liquid from 1 to 1000 nm.[43] The average hydrodynamic size of our synthesized AgNPs was 112 nm (Fig. 6). The DLS measurements are expected to yield more intense results than microscopic analyses, which can be explained by the presence of ions or molecules bound, and the increase in the thickness of the hydration shell around nanoparticles in solution.[44,45]

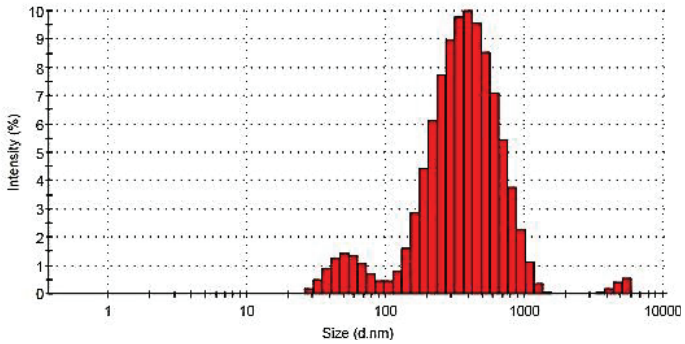

Fig. 6. DLS analysis of AgNPs (5 mM, pH 9).

According to the FE-SEM image, our synthesized spherical AgNPs tend to form agglomerations and appear to be monodispersed with a mean particle size of 54.5 nm (Fig. 7). The average size of the spherical AgNPs synthesized, using the pink pigment of *Streptomyces* sp. NS-05 was 42.5 nm.[8] The size, shape, and structural properties of NPs synthesized by biological methods can vary depending on the biological agent used and the synthesis conditions, such as the concentration and type of metal salt used and the bio-reducing agent, temperature and reaction time.[46]

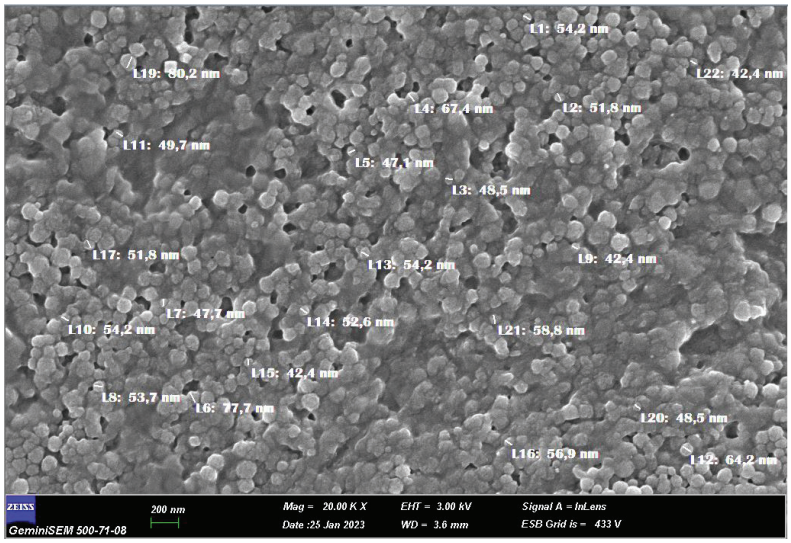

Fig. 7. FE-SEM image of AgNPs (5 mM, pH 9).

EDX and XRD were performed to determine the elemental composition and crystal structure of the AgNPs. The presence of Ag was revealed by EDX analysis (Fig. 8).

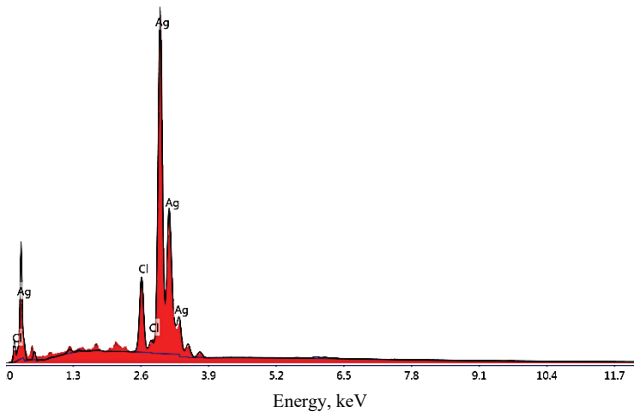

Fig. 8. EDX analysis of AgNPs (5 mM, pH 9).

According to the XRD analysis of the AgNPs, the 27.7, 32.3, 38.4, 44.1, 46.2, 54.5, 57.6, 64.3 and 77.2° peaks observed at 2 theta corresponded to the (1,0,0), (1,2,2), (1,1,1), (2,0,0), (2,0,0), (2,2,0), (2,2,0), (2,2,0) and (3,1,1) planes, respectively (Fig. 9). The obtained peaks at 27.7, 32.3 and 54.5° indicate AgNPs (JCPDS (01–076-1489)). The peaks at 32.3, 38.4, 46.2, 54.5, 57.6, 64.3 and 77.2° reflect the face-centered cubic Ag phase (JCPDS (00-001-1167).[47–49]

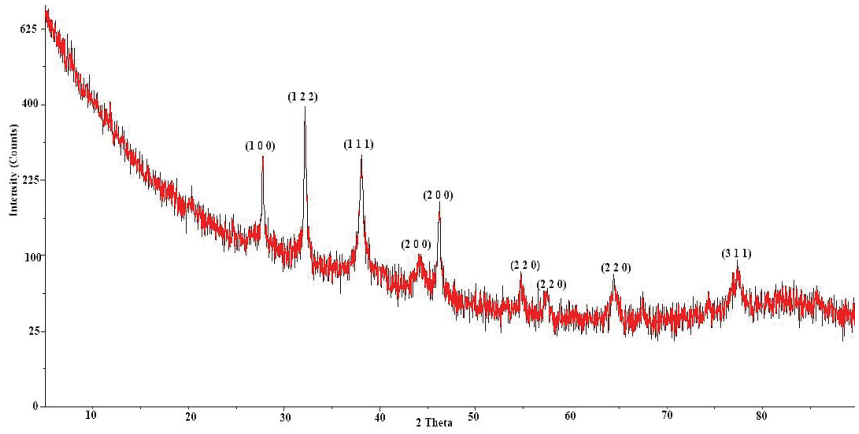

Fig. 9. XRD pattern of AgNPs (5 mM, pH 9).

FT-IR can provides the information about the bioactive molecules that may be responsible for reducing silver ions into AgNPs.[12] The functional groups that play a role in the green synthesis of our AgNPs were revealed by the peaks observed in the FT-IR spectrum with diffractions at 3471, 2918, 1620, 1409, 1321 and 478 cm^{-1}, which indicate the presence of alcohol (O–H), alkane (C–H), amine (N–H), fluoro compound (C–F), aromatic amine (C–N) and metal (Ag),

respectively (Fig. 10). Similar to our findings, previous studies reported that alcohol, alkane, and amine groups were responsible for the reduction and capping of Ag ions for the synthesis of NPs.[11,38,41]

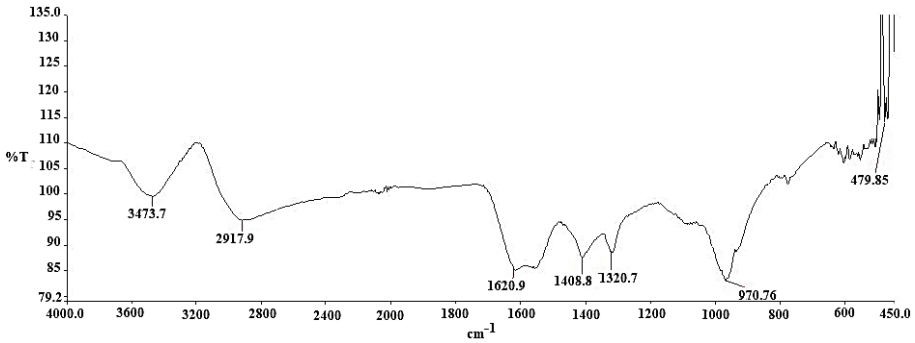

Fig. 10. FT-IR spectrum of AgNPs (5 mM, pH 9).

Antimicrobial activity

Firstly, the antimicrobial activity of synthesized nanoparticles (AgNPs) by using the red pigment produced from *Streptomyces* sp. A23 strain was evaluated using an agar well diffusion assay as illustrated in Fig. 11.

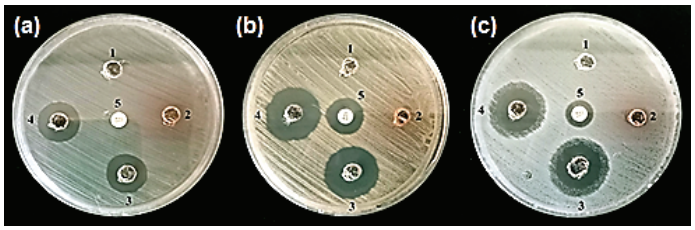

Fig. 11. Antimicrobial activity of AgNPs (1 mM, pH 7) against: a) *E. faecalis*, b) *S. aureus* and c) *P. aeruginosa*; 1) H_2O, 2) pigment, 3) $AgNO_3$, 4) AgNPs and 5) gentamicin.

AgNPs (5 mM, pH 9) are more effective because of their spherical shape and smaller size (54.5 nm), and their contact with microbial surfaces is good and easy due to their high negative surface charge (–33 mV). According to several reports, physical properties such as size, shape, and surface charge can influence the antimicrobial activity.[50–52] In addition, many previous studies have shown that one of the critical characteristics of nanoparticles is size, and a smaller size has a higher effect on the ability of nanoparticles to penetrate into microbial cells.[52] Additionally, the shape of AgNPs plays an important role in the antimicrobial activity, and it was decided that a spherical shape would result in excellent activity.[53] Furthermore, the surface charge plays a crucial role in the initial adsorption of nanoparticles to cell membranes.[51]

For the *MIC*s and *MBC*s of AgNPs (5 mM, pH 9), we recorded the lowest *MIC* against *S. aureus* ATCC 6538P and *B. subtilis* ATCC 6633 with a value amounted by 62.5 μg mL^{-1}, while the highest *MIC* was against *P. aeruginosa* ATCC 27853 (250 μg mL^{-1}). The lowest *MBC* (62.5 μg mL^{-1}) was recorded against *B. subtilis* ATCC 6633, while the highest *MBC* (> 1000 μg mL^{-1}) was against *P.aeruginosa* ATCC 27853 (Table II). *MIC* value is the lowest concentration of the tested substance as an antibiotic that inhibits the growth of the test microorganims after an overnight of incubation, while *MBC* value is the lowest concentration required to kill 99.9 % of the test microorganisms.[7,9]

TABLE II. *MIC*s and *MBC*s results of AgNPs (5 mM, pH 9)

Pathogenic microorganism	*MIC* / μg mL^{-1}	*MBC* / μg mL^{-1}
S. aureus ATCC 6538P	62.5	125
B. subtilis ATCC 6633	62.5	62.5
E. coli ATCC 7839	125	125
P. aeruginosa ATCC 27853	250	> 1000
K. pneumoniae ATCC 13883	125	125

There is no clear or exact mechanism for the antimicrobial effect of AgNPs, but many existing studies have reported that the most common and prominent mechanisms are as follows. a) AgNPs can adhere to the cell wall and membrane of microbial cells, causing cell rupture and leading to cell lysis. b) AgNPs can penetrate the inside of microbial cells and damage biomolecules (DNA and enzymes) and intracellular structures (mitochondria, vacuoles and ribosomes). c) AgNPs have the potential to increase ROS production, which can cause oxidative stress and damage to microbial cells.[54,55]

Anticancer and neuroprotective activities

In the MTT test, SH-SY5Y cells were treated with various concentrations of AgNPs to determine their cytotoxic effects (Fig. 12). The compound-1 (AgNPs, 1 mM, pH 5) did not alter cell viability at concentrations of 2 or 4 μg mL^{-1}, but at concentrations of 8, 12 and 16 μg mL^{-1} ($p < 0.001$), it significantly the reduced cell viability compared to that of the control group. For the compound-2 (AgNPs, 1 mM, pH 7), the concentration of 2 μg mL^{-1} tended to increase the cell viability, but this increase was not statistically significant. Additionally, there was no change in the cell viability at 4 μg mL^{-1}. Also, at the concentrations of 8, 12 and 16 μg mL^{-1} ($p < 0.001$), the cell viability was markedly lower than that in the control group. Compound-3 (AgNPs, 5 mM, pH 9) significantly decreased ($p < 0.001$) cell viability at concentrations of 2, 4, 8, 12 and 16 μg mL^{-1} compared to that of the control.

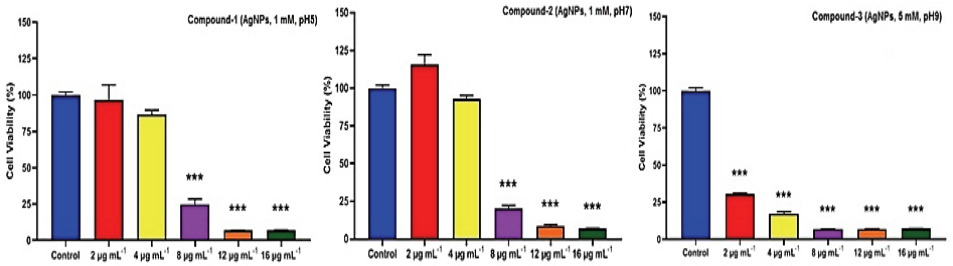

Fig. 12. Effects of synthesized AgNPs on the viability of SH-SY5Y cells; ***: $p < 0.001$ compared to the control.

For the neuroprotective activity, MTT assay was performed to determine the neurotoxic dose of H_2O_2 as shown in Fig. 13. The treatment with 100, 150, 200, 300, 400 and 600 μM of H_2O_2 reduced ($p < 0.001$) the cell viability compared to that of the control group. To evaluate the neuroprotective effect of synthesized nanoparticles, 300 μM H_2O_2 was added one hour before the AgNPs were added, and the cell viability was investigated via the MTT test at the 24th hour of AgNPs treatment. When the neuroprotective effects of the AgNPs were examined, the compound-1 (AgNPs, 1 mM, pH 5) at concentrations of 2, 4, 8, 12 and 16 μg mL^{-1} ($p < 0.001$) significantly reduced the viability of the SH-SY5Y cells, when compared to that of the H_2O_2 group. The compound-2 (AgNPs, 1 mM, pH 7) at a concentration of 2 μg mL^{-1} ($p < 0.001$) significantly increased the cell viability with the presence of H_2O_2. In addition, at the concentrations of 8, 12 and 16 μg mL^{-1} ($p < 0.001$), the cell viability was markedly lower than that in the H_2O_2 group. The treatment with the compound-3 (AgNPs, 5 mM, pH 9) at the concentrations of 2, 4, 8, 12 and 16 μg mL^{-1} ($p < 0.001$) significantly reduced the viability of the SH-SY5Y cells compared to that of the H_2O_2 group.

In this study, the cell culture experiments were conducted to address two primary objectives concerning SH-SY5Y neuroblastoma cells. First, the anticancer activity of the synthesized AgNPs was investigated by assessing their cytotoxic effects on neuroblastoma cells. The determination of the optimal cytotoxic dose was crucial for the evaluation of the anticancer potency of the synthesized materials. Notably, Compound-1, Compound-2, and Compound-3 exhibited cytotoxic effects at concentrations of 2, 4 and 8 μg mL^{-1}, respectively. Comparative analysis revealed the superior anticancer activity of compound-3 against neuroblastoma cells. This observation aligns with the recent studies, confirming the anticancer efficacy of silver nanoparticles synthesized using the bioactive molecules produced by some *Streptomyces* strains on different cancer cell lines.[7,39] Additionally, our study was the first to show that AgNPs synthesized using a red pigment of *Streptomyces* sp. had anticancer activities on human

neuroblastoma cells, although there are several studies showing anticancer and cytotoxic effects of different source-based AgNPs.[56,57]

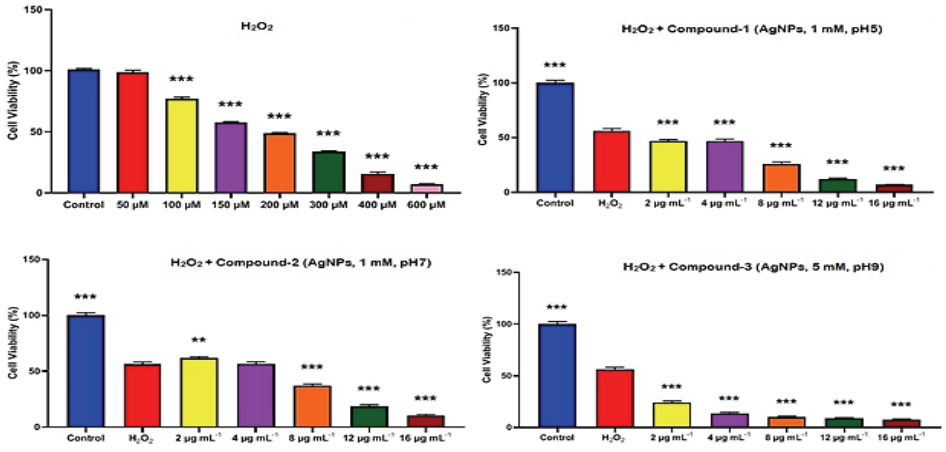

Fig. 13. Neuroprotective activity of synthesized AgNPs; **: $p < 0.01$; ***: $p < 0.001$ compared to the control group.

The second objective focused on elucidating the potential neuroprotective effects of AgNPs against hydrogen peroxide-induced cytotoxicity, a common mechanism underlying neurodegenerative diseases. Hydrogen peroxide (H_2O_2) at a concentration of 100 µM demonstrated cytotoxic effects, and a concentration of 300 µM was selected for its moderate cytotoxic effects, as suggested by prior study.[58] Intriguingly, compound-2 exhibited some neuroprotective effects at the lowest concentration tested, distinguishing it from the other compounds. The observed neuroprotective effects of compound-2 were hypothesized to be associated with its potential antioxidant properties, drawing parallels with findings of Alkhalaf *et al.* in 2020,[59] who demonstrated that the antioxidant and anti-inflammatory effects of AgNPs synthesized using *Nigella sativa* extract in rodents. Controversially, Zhai *et al.* (2022) showed the cytotoxic effects of AgNPs *via* oxidative stress mechanism in SH-SY5Y cells. It has been seen that AgNPs causes oxidative stress at very high doses (40 and 60 µg mL^{-1}), whereas we noticed the neuroprotective effect of low doses of AgNPs (2 and 4 µg mL^{-1}), against a well-known oxidative stressor, H_2O_2, in our study. These differences suggest a dose-dependent antioxidant or oxidant effect of AgNPs synthesized, using the red pigment produced by the *Streptomyces* sp. A23 strain.[57] These findings suggested a nuanced interplay between the antioxidant effects at lower concentrations and antiproliferative effects at higher concentrations for the compound-2.

CONCLUSION

In conclusion, the green synthesis of silver nanoparticles (AgNPs) using the red pigment produced by the *Streptomyces* sp. A23 strain isolated from Algerian bee pollen represents a new approach with the remarkable neuroprotective, anticancer and antimicrobial activities. This synthesis method not only harnesses the inherent capabilities of the *Streptomyces* sp. A23 strain, but also produces the silver nanoparticles with unique biological properties, paving the way for their application in diverse therapeutic avenues. The future studies should address the molecular mechanisms underlying the therapeutic properties of these nanoparticles, including the molecular interactions between the AgNPs and biological targets, which will also provide deeper insight into their mode of action. Additionally, the exploration of synergistic effects with other therapeutic agents could open new possibilities for the enhanced treatment strategies, especially in the multidrug-resistant infections and the complex diseases like cancer and neurodegeneration.

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ИЗВОД

ОДРЕЂИВАЊЕ АНТИМИКРОБНЕ, АНТИКАНЦЕРОГЕНЕ И НЕУРОПРОТЕКТИВНЕ АКТИВНОСТИ НАНОЧЕСТИЦА СРЕБРА (AgNP), СИНТЕТИСАНИХ ПРИМЕНОМ ЗЕЛЕНЕ ХЕМИЈЕ, КОРИШЋЕЊЕМ ЦРВЕНОГ ПИГМЕНТА СОЈА *Streptomyces* sp. A23 ИЗОЛОВАНОГ ИЗ АЛЖИРСКОГ ПЧЕЛИЊЕГ ПОЛЕНА

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Црвени пигмент из соја *Streptomyces* sp. A23 изолован из алжирског пчелињег полена је коришћен за синтезу наночестица сребра (AgNP) применом зелене хемије, а затим је одређивана њихова антимикробна, антиканцерска и неуропротективна активност. AgNP су синтетисане редукцијом 1 mM и 5 mM раствора сребро нитрата на различитим pH (5, 7 и 9), а затим окарактерисане. AgNP (5 mM, pH 9) су испољиле максимум UV–Vis апсорбанције на 433 nm. Методом DLS је утврђено да је просечни пречник честица 112 nm. Максимум зета потенцијала је утврђен на –33 mV, потврђујући повећану стабилност. XRD анализом је констатована кристална природа материјала. FT-IR анализом су утврђене специфичне функционалне групе на положају од 3471 до 478 cm^{-1}. FE-SEM методом је показано да је средња вредност сферних пречника AgNP 54,5 nm. Присуство Ag је потврђено EDX анализом. Наночестице су испољиле значајну антимикробну активност спрам *Enterococcus faecalis* ATCC 19433, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 13883 и *Escherichia coli* ATCC 7839, уз зону инхибиције од 32, 30, 30, 27,

25, 20, односно 19 mm. Најниже вредности *MIC* и *MBC* су измерене спрам *B. subtilis* ATCC 6633 – 62,5 μg mL^{-1}. Занимљиво је да су све синтетисане AgNP, у концентрацијама 2, 4 и 8 μg mL^{-1}, испољиле цитотоксични ефекат према SH-SY5Y ћелијској линији неуробластома. Додатно, AgNP (1 mM, pH 7) су испољиле значајну неуропротективну активност при најнижим тестираним концентрацијама. Из свега наведеног произилази да се AgNP синтетисане применом црвеног пигмента *Streptomyces* sp. сој A23 могу сматрати за обећавајуће терапеутске агенсе.

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PHYSICOCHEMICAL, PHYTOCHEMICAL, AND MICROBIOLOGICAL ANALYSES OF SOME ALGERIAN BEE-COLLECTED POLLEN SAMPLES

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Bee-collected pollen is one of the most beehive products. It is considered a functional food due to its nutritional value and therapeutic benefits. Due to its nutritional composition, bee pollen may contain pathogenic and spoilage microorganisms, including bacteria, molds, and yeasts. This study aims to determine the total phenolic and flavonoid contents, antioxidant activity using DPPH radical scavenging assay, and microbiological quality of some bee-collected pollen samples from different locations in Algeria. The results indicate that the mean pH, moisture, and ash content were 4.37 ± 0.55, 19.49 ± 6.04 %, and 2.60 ± 0.64 %, respectively. The total phenolic content varied from 9.76 ± 0.22 to 22.46 ± 0.05 mg GAE/g of dried bee-collected pollen extract, while the flavonoid content ranged from 2.68 ± 0.06 to 11.07 ± 0.29 mg QE/g of dried bee-collected pollen extract. The DPPH radical assay also showed that the IC_{50} values were ranging from 1.08 ± 0.05 to 4.77 ± 0.09 mg/ml. Furthermore, the microbiological analyses showed a microbial load ranging from 7.70 x 102 to 1.93 x 106 cfu/g for the aerobic mesophilic bacteria and from 1.10 x 103 to 5.30 x 105 cfu/g for the anaerobic mesophilic bacteria. Mold and yeast content varied from 2.70 x 103 to 3.70 x 103 cfu/g. The maximum concentrations for total coliforms and Staphylococcus aureus were 1.73 x 104 cfu/g and 2.33 x 102 cfu/g, respectively. In addition, sulfite-reducing Clostridium was detected only in one sample, which was collected from Guelma (BCPS 1), while Salmonella sp. was not detected in all samples. In conclusion, given its nutritional and therapeutic benefits, the local authorities should adopt regulatory legislation that includes microbiological and mycotoxicological standards for the Algerian bee-collected pollen to protect consumer health.

Keywords: Bee-collected pollen, Microbiological quality, Phenolic content, Flavonoids, Antioxidant activity.

INTRODUCTION

As one of the most beehive products, bee-collected pollen comes to the attention of the scientific community due to the nutritional and healthy properties demonstrated in ancient times (1). It is considered a functional food and feed ingredient due to its rich composition of nutrients and bioactive compounds. In addition, it is recognized for its potential to enhance human health and animal well-being, offering various benefits such as antioxidant, antitumor, antiviral, antimicrobial, antimutagenic, antiallergenic, hypolipidemic, hypoglycemic, anti-inflammatory, immune stimulant, and hepato-renal protection (1-4). Bee pollen exhibits good antimicrobial effects against a range of pathogenic bacterial strains such as *Staphylococcus aureus*, *Clostridium perfringens*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and fungal strains such as *Candida albicans*, *Aspergillus flavus*, *Aspergillus fumi-*

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gatus, *Aspergillus niger*, and *Rhizopus oryzae*, making it a candidate for use as a natural alternative antimicrobial agent (1, 5). Significant antioxidant activity has been shown in bee pollen, which helps and contributes to protecting and preventing the human body from diseases caused by oxidative stress (2, 6, 7).

Advanced analytical techniques, including chromatography and spectrophotometry, allowed identifying approximately 250 chemical compounds in samples of bee-collected pollen collected from various regions in the world according to the botanical and geographical origin (6). It contains carbohydrates as the major component, which ranged between 13 and 55%, followed by proteins and amino acids (10 - 40%), water (20 - 30%), lipids (1 - 13%), and dietary fiber (0.3 - 20%) (7). As the minor components, bee-collected pollen is an important source of minerals with about 25 elements, such as Fe, Mg, Zn, Cu, Ca, K, and N, which amounted to 2 - 6% of its total content. Additionally, almost all of the vitamins are present in a range of 0.02 to 0.7%. Furthermore, polyphenols and flavonoids, mainly gallic acid, coumaric acid, quercetin, kaempferol, and catechin, were determined with an average concentration of 3 - 8 % (5, 7, 8).

Due to its nutritional composition, bee-collected pollen can contain different species of microorganisms, including bacteria, molds, and yeasts, which come from beekeepers' manipulation and natural habitats during collection, processing, and storage. Some of these microbial contaminants can be beneficial, such as lactic acid bacteria species, while others, such as *Staphylococcus aureus*, *Salmonella* spp., *Clostridium* spp., *Penicillium* spp., *Mucor* spp., and *Aspergillus* spp., can pose risks for consumer safety (9-11). Therefore, due to only some countries worldwide, including Argentina, Brazil, Mexico, Spain, Poland, Bulgaria, and Switzerland, having regulatory specifications for bee-collected pollen, standardization and legislation for microbiological quality are critical to reduce the risks associated with microbial contamination (12, 13).

This study aims to analyze the microbiological quality, determine the physicochemical characteristics, and evaluate the contents of phenolics and flavonoids, along with a preliminary examination of their radical quenching ability in six local bee-collected pollen samples obtained from various Algerian regions.

EXPERIMENTAL

COLLECTION OF SAMPLES

Four samples of bee-collected pollen were provided by the International Api-Phyto Therapy Center -Setif- using pollen traps from different Algerian geographic sites. Bee-collected pollen sample 1 (BPS 1) was obtained from Guelma (36° 28′ 00″ N, 7° 26′ 00″ E), bee-collected pollen sample 2 (BPS 2) was from Medea (36° 16′ 03″ N, 2° 45′ 00″ E), and bee-collected pollen sample 4 (BPS 4) was from Setif (36° 09′ 00″ N, 5° 26′ 00″ E), whereas the bee-collected pollen sample 5 (BPS 5) was obtained from Bejaia (36° 45′ 00″ N, 5° 04′ 00″ E). Two samples (BPS 3 and BPS 6) were purchased from a local market in Setif (36° 09′ 00″ N, 5° 26′ 00″ E). All samples were transported to the laboratory and conserved at 4 °C until analysis.

PHYSICOCHEMICAL ANALYSES

Determination of pH

To determine the pH values of bee-collected pollen samples, one gram of each sample was mixed with 7.5 ml of distilled water for 24 h at room temperature. Thereafter, the prepared solutions were centrifuged at 6000 rpm and filtered through filter paper. The pH values were measured using a pH meter (HANNA pH 211, USA) (14).

Determination of moisture content

The moisture was determined by drying of 3 g of each sample at 105 °C for 2 h (15, 16). The moisture was calculated using the mathematical equation [1]:

$$\text{Moisture (\%)} = \frac{[\,A1 - A2\,]}{[A1]} \times 100 \quad \ldots\ldots\ldots\ldots \quad \ldots\ldots\ldots\ldots [1]$$

where: A_1 - weigh of fresh bee pollen sample, A_2 - weigh of dried bee pollen sample.

Determination of ash content

The ash content was determined after the complete burning of 3 g of each sample using a muffle furnace at 550 °C (17). The results were expressed as a percentage per gram of bee-collected pollen sample.

PHYTOCHEMICAL ANALYSES

Preparation of bee-collected pollen extracts

5 g of each bee-collected pollen sample was extracted in 75 ml of 70% ethanol. The extraction was performed by agitation using a hot plate magnetic stirrer set to 1500 rpm and 40 °C for 2 h. This experiment was carried out in duplicate with two repetitions. After extraction, the mixture solution for each sample was filtered through a filter paper. Finally, ethanol was evaporated using a rotary evaporator (BUCHI R-215, Switzerland). The obtained extracts were kept at 4 °C (18).

Determination of total phenolic content

Total phenolic content (TPC) was evaluated by the Folin-Ciocalteu method as reported elsewhere (19). 100 µl of each bee-collected pollen extract was mixed with 500 µl of a 10% Folin-Ciocalteu reagent for 4 min. Subsequently, 400 µl of 7.5% sodium carbonate (Na_2CO_3) was added. After incubation in dark conditions for 90 min at room temperature, the absorbances were recorded at 765 nm using a UV-visible spectrophotometer (Shimadzu BioSpec-mini, Japan). Gallic acid was used as a standard at various concentrations ranging from 10 to 90 µg/ml. The total phenolic content was determined in milligrams of gallic acid equivalents per gram of dried bee-collected pollen extract.

Determination of total flavonoid content

Total flavonoid content (TFC) was determined by the aluminum chloride method (20). Briefly, 500 µl of each extract was mixed with 500 µl of 2% aluminum chloride solution ($AlCl_3$). After 10 min, the absorbances were measured at 430 nm. Different concentrations of quercetin ranging from 5 to 40 µg/ml were used as a standard. Total flavonoid content was expressed as milligrams of quercetin equivalent per gram of dried bee-collected pollen extract.

Evaluation of antioxidant activity

The antioxidant activity of each bee-collected pollen extract was studied using DPPH radical scavenging assay, as reported by Kohole Foffe *et al.* (21) with little modification. 0.5 ml of different concentrations (0.2 to 6 mg/ml) of each bee-collected pollen extract was added to 0.3 ml of 0.5 mM 2,2-diphenyl-1-picryl-hydrazyl solution and 3 ml of methanol. The mixtures were incubated for 30 min at room temperature in dark conditions. The absorbances were recorded at 517 nm. Different concentrations of ascorbic acid from 5 to 40 µg/ml were used as a standard. The IC_{50} values of extracts were determined directly from the graph of scavenging effect percentage against the extract concentrations. They were expressed in mg/ml and compared with the IC_{50} of ascorbic acid.

MICROBIOLOGICAL ANALYSES

Before the microbiological analyses, 5 g of each bee-collected pollen sample were diluted and homogenized in 45 ml of sterile peptone water, followed by incubation for 2 hours at 30 °C. For each sample, serial dilutions were performed as required in sterile saline solution. Adequate dilutions were plated on a suitable medium. The obtained colonies were counted, and expressed as colony-forming units per gram of bee-collected pollen (cfu/g) using the formula [2]:

$$\text{cfu/g} = \frac{N}{V \times D} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad \ldots\ldots. [2]$$

where: N - number of counted colonies, V - volume of plated inoculum, D - strength of the dilution.

Determination of total aerobic and anaerobic mesophilic bacteria

The aerobic and anaerobic mesophilic bacteria were enumerated by spreading 100 µl of the adequate dilutions of each sample onto plate count agar (PCA). Then, all plates were incubated for 3 days at 30 °C under aerobic and anaerobic conditions (22).

Determination of molds and yeasts

Molds and yeasts were counted by plating 100 µl of the dilutions of each sample onto sabouraud chloramphenicol agar (SCA) and incubating at 28 °C for 3 to 5 days (4).

Determination of total coliforms

The total coliforms were counted by spreading 100 µl of the appropriate dilution of each sample onto violet red bile lactose agar (VRBLA) and incubating it at 35 °C. After 24 to 48 h of incubation, the typical black-reddish colonies were counted (23).

Determination of *Staphylococcus aureus*

S. aureus was enumerated by plating 100 µl of the dilutions of each bee-collected pollen sample on Baird-Parker agar (BPA), enriched with egg yolk emulsion, and supplemented with a 3% potassium tellurite solution. After incubation at 37 °C for 48 h, all colonies that were circular, smooth, convex, moist, and gray to jet-black, surrounded by a clear zone, were counted (24, 25).

Detection of sulfite-reducing *Clostridium*

For sulfite-reducing *Clostridium*, one ml of the initial suspension of each sample of bee-collected pollen was put into an empty screw-capped glass tube, treated at 80 °C for 15 min, and covered with iron sulfite agar (ISA) followed by a layer of paraffin oil. Then, all tubes were incubated for 5 days at 37 °C. The presence of black colonies was noted as a positive result (26).

Detection of *Salmonella* spp.

The presence of *Salmonella* spp. was tested by three steps. The first step involved pre-enrichment of 25 g of each sample of bee-collected pollen in 225 ml of sterile buffered peptone water, followed by 20 h of incubation at 37 °C. Secondary selective enrichment was performed in Rappaport-Vassiliadis broth (RVB) with 24 h of incubation at 42 °C. Finally, the growth was done on three different selective media, including xylose-lysine-deoxycholate agar (XLDA), *Salmonella-Shigella* agar (SSA), and Hektoen enteric agar (HEA). After 24 h of incubation at 37 °C, the presence of black and green to blue-green colonies was recorded as a positive result (11, 23, 27).

STATISTICAL ANALYSIS

In this study, unless otherwise stated, all experiments were performed in triplicate. Statistical analyses were assessed by ANOVA and Pearson's correlation tests using IBM SPSS Statistics 21 software (IBM Corp., Armonk, NY, USA). The results were expressed as means ± standard deviations. The variance percentage was calculated with 95% confidence ($\alpha = 0.05$).

RESULTS AND DISCUSSION

PHYSICOCHEMICAL ANALYSES

In this study, some physicochemical parameters, including pH, moisture, and ash content of six bee-collected pollen samples, have been tested. The obtained results are shown in Table 1.

Table 1. Physicochemical analyses of bee-collected pollen samples.

Samples	pH	Moisture (%)	Ash (%)
BPS 1	3.90 ± 0.52^{a}	23.26 ± 0.07^{d}	1.93 ± 0.26^{a}
BPS 2	4.41 ± 0.05^{b}	23.87 ± 0.22^{e}	2.65 ± 0.25^{b}
BPS 3	5.33 ± 0.13^{d}	11.13 ± 0.03^{a}	2.00 ± 0.08^{a}
BPS 4	4.74 ± 0.04^{c}	27.80 ± 0.18^{f}	2.78 ± 0.08^{b}
BPS 5	3.99 ± 0.06^{a}	16.25 ± 0.40^{c}	$2.52 \pm 0.28^{a, b}$
BPS 6	3.87 ± 0.02^{a}	14.62 ± 0.16^{b}	3.73 ± 0.31^{c}
Mean	4.37 ± 0.55	19.49 ± 6.04	2.60 ± 0.64
Maximum	5.33 ± 0.13	27.80 ± 0.18	3.73 ± 0.31
Minimum	3.87 ± 0.02	11.13 ± 0.03	1.93 ± 0.26
p value	0.0001	0.0001	0.0001
Significance level	***	***	***

*** $p<0.001$. a-f: subgroups

According to the obtained results, a highly significant difference between the pH values of all tested bee-collected pollen samples ($p<0.001$) was noticed (Table 1). The pH values were recorded in the acidic range with variation from one sample to another and with an estimated mean of 4.37 ± 0.55. BPS 3 collected from Setif showed the highest value of pH (5.33 ± 0.13), while BPS 6, which was also collected from Setif, showed the lowest value (3.87 ± 0.02). By the same way, for the moisture or water content, there is also a highly significant difference between the values of the samples ($p<0.001$) (Table 1), which varied from 11.13 ± 0.03 to 27.80 ± 0.18%, with an average value of 19.49 ± 6.04 %. The BPS 4, which was collected from Setif, presented the maximum value, while the BPS 3, which was also collected from Setif, had the minimum value. Eventually, the fresh samples, BPS 1 (Guelma), BPS 2 (Medea), and BPS 4 (Setif), showed elevated values when compared to the dried ones (BPS 3, BPS 5, and BPS 6). The ash content values vary significantly between samples ($p<0.001$) (Table 1). The results ranged from 3.73 ± 0.31 % in BPS 6, which was collected from Setif, to 1.93 ± 0.26 % in BPS 1, which was collected from Guelma, with a mean value estimated at 2.60 ± 0.64 %.

Among the important physicochemical analyses are the pH, moisture, and ash content. The results indicate the presence of highly significant differences in pH, moisture, and ash content of all tested bee-collected pollen samples due to the variety of their geographical region, botanical origin, and soil type (17, 28). In this study, the obtained pH values were in the acidic range between 5.33 ± 0.13 and 3.87 ± 0.02. The pH is one of bee-collected pollen's quality indicators, influencing its nutritional properties, microbial safety, and storage stability. It has been reported that it should be between 4.3 and 7 because a pH below 4 can lead to a number of unwanted microbial activities in unsafe storage conditions (16, 29). The moisture of bee-collected pollen greatly affects its nutritional composition, shelf life, and susceptibility to microbial contamination (7). The obtained moisture in this study, which ranged from 11.13 ± 0.03 to 27.80 ± 0.18 %, is approximately in agreement with several previous studies that reported that the water content of fresh bee-collected pollen ranges from 10 to 30%, while dried bee-collected pollen contains less than 8%, which is influenced by some factors, such as botanical origin and storage conditions (7, 30-32). Furthermore, ash content, representing the total mineral content, is crucial for the nutritional value of bee-collected pollen, reflecting variations due to plant species, soil composition, climate, and geographical origin (17, 31). The values obtained for ash in this study,

which ranged between 3.73 ± 0.31 and 1.93 ± 0.26 %, are similar to those suggested by Campos *et al.* (32) in their standardization of bee-collected pollen.

PHYTOCHEMICAL ANALYSES

Table 2 presents the total phenolics, flavonoids, and antioxidant activity analyses of the bee-collected pollen samples.

Table 2. Phytochemical content and antioxidant activity of bee-collected pollen samples.

Samples	TPC (mg GAE/g)	TFC (mg QE/g)	IC_{50} (mg/ml)
BPS1	17.21 ± 0.44 (d)	6.80 ± 0.09 (d)	1.21 ± 0.02(a)
BPS2	12.02 ± 0.51 (b)	4.19 ± 0.34 (b)	2.42 ± 0.05(b)
BPS3	22.46 ± 0.05 (e)	11.07 ± 0.29 (e)	1.08 ± 0.05(a)
BPS4	14.30 ± 0.27 (c)	4.78 ± 0.01 (c)	4.77 ± 0.09(c)
BPS5	15.94 ± 0.96 (d)	3.76 ± 0.13 (b)	2.38 ± 0.06(b)
BPS6	9.76 ± 0.22 (a)	2.67 ± 0.06 (a)	2.23 ± .011(b)
Mean	15.28 ± 4.17	5.54 ± 2.85	2.35 ± 1.25
Maximum	22.46 ± 0.05	11.07 ± 0.29	4.77 ± 0.09
Minimum	9.76 ± 0.22	2.67 ± 0.06	1.08 ± 0.05
p value	0.0001	0.0001	0.0001
Significance level	***	***	***

*** $p<0.001$; a-e: subgroups.

As presented in Table 2, a highly statistically significant difference was recorded between the total phenolic content (TPC) of all studied samples of bee-collected pollen ($p<0.001$). According to the results, BPS 3 (Setif) has the higher content of phenolics, followed by BPS 1 (Guelma), BPS 5 (Bejaia), BPS 4 (Setif), BPS 2 (Medea), and BPS 6 (Setif), with obtained amounts estimated by 22.46 ± 0.05, 17.21 ± 0.44, 15.94 ± 0.96, 14.30 ± 0.27, 12.02 ± 0.51, and 9.76 ± 0.22 mg GAE/g of extract, respectively.

Concerning the total flavonoid content (TFC), there is also a highly significant difference between the values of the tested samples of bee-collected pollen ($p<0.001$) (Table 2). The results showcase the variation and differences in the obtained flavonoid content concentrations. A considerable concentration was observed in BPS 3 (11.07 ± 0.29 mg QE/g of extract). The sample BPS 6 showed the lowest concentration of flavonoids (2.67 ± 0.06 mg QE/g of extract).

As presented in Table 2, a highly significant difference also was recorded between the IC_{50} values of the examined samples ($p<0.001$). The results show that the BPS 3, which was collected from Setif, had the best radical quenching ability, with an IC_{50} value of 1.08 ± 0.05 mg/ml. It was followed by BPS 1 (Guelma), BPS 6 (Setif), BPS 5 (Bejaia), BPS 2 (Medea), and BPS 4 (Setif), which had IC_{50} values of 1.21 ± 0.02, 2.23 ± 0.11, 2.38 ± 0.06, 2.42 ± 0.05, and 4.77 ± 0.09 mg/ml, respectively. These results are hardly comparable with ascorbic acid, which is used as a standard, presenting an IC_{50} value of 0.035 mg/ml.

Concerning the phytochemical analyses, the total phenolic and flavonoid contents of bee-collected pollen contribute to various health benefits, including antioxidant activity. The obtained averages of total phenolics and flavonoids of the studied bee pollen samples were 15.28 ± 4.17 mg GAE/g and 5.54 ± 2.85 mg QE/g, respectively. As shown in the results, the phenolic and flavonoid contents of the tested bee-collected pollen samples are in

correspondence with some previous studies and comparable to others (14, 33-35). The study of Carpes *et al.* (36) indicated that bee pollen has a significant phenolic and flavonoid contents with some variations due to its botanical origin, and the type of solvent used during the extraction.

In this study, the antioxidant activity was evaluated by the DPPH test. When the extracts of bee-collected pollen were mixed with the DPPH reagent, the color changed from purple to yellow, indicating the reduction of the DPPH radical by the antioxidant compounds present in the extracts (2). The average of IC_{50} values of bee-collected pollen extracts was 2.35 ± 1.25 mg/ml. Pascoal *et al.* (37) studied the biological activities of eight samples of bee-collected pollen from Portugal and Spain and obtained IC_{50} values ranging from 2.98 ± 0.47 to 6.69 ± 0.75 mg/ml using DPPH assay. According to El Ghouizi *et al.* (7), there is a significant variation in the antioxidant activity of bee-collected pollen samples from one country to another. The study of Su *et al.* (2) reported that the DPPH radical scavenging assay is the common method for assessing antioxidant activity. However, it is infeasible to assess the antioxidant activity of any natural product thoroughly and accurately through just one evaluation method. In addition, some studies have reported that the antioxidant activity of bee-collected pollen extracts is not only related to the phenolic content but may also be related to the types of phenolics (2, 34).

According to the statistical analyses illustrated in Table 3, a strong positive significant correlation occurred between TFC and total TPC ($r = 0.917$; $p = 0.0001$; $p<0.01$). As expected, this result indicates that the total phenolics have a positive effect on flavonoid content in bee-collected pollen samples. Due to flavonoids are a group of phenolics, an increase in the phenolic content leads to a corresponding increase in flavonoid concentration, and vice versa (38). Furthermore, a negative moderately significant correlation was observed between IC_{50} and TFC ($r = - 0.501$; $p = 0.034$; $p<0.05$). Previous studies have reported that the higher flavonoid concentration generally corresponds to decreased IC_{50}, where the antioxidant activity was increased (39, 40). It is noteworthy that the antioxidant activity of bee-collected pollen is not just dependent on flavonoids, because the presence of other phenolic compounds can also contribute to an increase in antioxidant potential (41). In addition, the antioxidant capacity of bee-collected pollen is not always limited to phenolic compounds because some others, including carotenoids, glutathione, and vitamins, also play a role in its radical quenching ability (42, 43).

Table 3. Correlations between TPC, TFC, and IC_{50}.

	TPC	**TFC**	**IC_{50}**
TPC	1		
TFC	**0.917****	1	
IC_{50}	- 0.449	**- 0.501***	1

* The correlation is significant at the 0.05 level. ** The correlation is significant at the 0.01 level.

MICROBIOLOGICAL ANALYSES

According to the obtained results (Table 4), significant differences in microbial loads were found between all studied samples of bee-collected pollen, with the presence of different microorganisms, including some pathogenic species, such as *S. aureus* and sulfite-reducing *Clostridium*.

Table 4. Microbiological analyses of tested samples of bee-collected pollen.

Microbiological analyses	Bee-collected pollen samples						p value	Significance level
	BPS 1	BPS 2	BPS 3	BPS 4	BPS 5	BPS 6		
Aerobic mesophilic bacteria	$1.17 \times 10^6 \pm 2.00 \times 10^4$	$1.93 \times 10^6 \pm 7.20 \times 10^5$	$7.70 \times 10^2 \pm 5.80 \times 10$	$1.11 \times 10^6 \pm 3.50 \times 10^4$	$4.06 \times 10^5 \pm 3.50 \times 10^4$	$8.66 \times 10^3 \pm 1.53 \times 10^3$	0.0001	***
Anaerobic mesophilic bacteria	$5.30 \times 10^5 \pm 8.00 \times 10^4$	$4.30 \times 10^5 \pm 5.57 \times 10^4$	$4.67 \times 10^2 \pm 5.80 \times 10^1$	$1.23 \times 10^5 \pm 1.39 \times 10^4$	$2.53 \times 10^4 \pm 8.73 \times 10^3$	$1.10 \times 10^3 \pm 1.00 \times 10^2$	0.0001	***
Molds and yeasts	$3.03 \times 10^3 \pm 1.07 \times 10^3$	$2.90 \times 10^3 \pm 3.00 \times 10^2$	$2.80 \times 10^3 \pm 5.57 \times 10^2$	$3.70 \times 10^3 \pm 5.29 \times 10^2$	$2.70 \times 10^3 \pm 0.00 \times 10^1$	$2.87 \times 10^3 \pm 4.51 \times 10^2$	0.386	
Total coliforms	-	$3.67 \times 10^2 \pm 1.53 \times 10^2$	-	$1.73 \times 10^4 \pm 4.93 \times 10^3$	-	-	0.0001	***
Staphylococcus aureus	$2.33 \times 10^2 \pm 1.53 \times 10^2$	$1.33 \times 10^2 \pm 1.53 \times 10^2$	-	-	-	-	0.026	*
Sulfite-reducing Clostridium	+	-	-	-	-	-		
***Salmonella* spp.**	-	-	-	-	-	-		

The microbiological analyses were expressed in cfu/g. *$p < 0.05$; ***$p<0.001$.

They ranged from 7.70 x 10^2 to 1.93 x 10^6 cfu/g for the aerobic mesophilic bacteria, 1.10 x 10^3 to 5.30 x 10^5 cfu/g for the anaerobic mesophilic bacteria, 2.70 x 10^3 to 3.70 x 10^3 cfu/g for molds and yeasts, 0.00 to 1.73 x 10^4 cfu/g for total coliforms, and from 0.00 to 2.33 x 10^2 cfu/g for *S. aureus*. Sulfite-reducing *Clostridium* was detected only in the BPS 1 sample, which was collected from Guelma. Furthermore, *Salmonella* spp. was not detected in all studied samples. Vargas-Abella et al. (4) evaluated the microbiological analyses of some fresh and dried bee-collected pollen from Colombia. They found that there were between 2.00 x 10^3 and 12.00 x 10^3 cfu/g for aerobic mesophilic bacteria, 0.00 x 10^3 to 6.00 x 10^3 cfu/g for molds and yeasts, 0.00 x 10^3 to 3.00 x 10^3 cfu/g for total coliforms, and between 0.00 to 2.00 x 10^3 cfu/g for *S. aureus*. However, none of the tested samples contain sulfite-reducing *Clostridium*.

Campos *et al.* (32) reported that the microbial load in bee-collected pollen should correspond to the European Union's standards for microbiological quality, which are as follows: total mesophilic bacteria (<100,000/g), molds and yeasts (<50,000/g), Enterobacteriaceae (maximum 100/g), *S. aureus* (absent/1 g), *Salmonella* spp. (absent/10 g), and *E. coli* (absent/1g). The comparison of these recommendations with our results reveals that the samples collected from Guelma (BPS 1), Medea (BPS 2), and Setif (BPS 4) have a poor microbiological quality. Due to its nutritional composition, different species of microorganisms, including bacteria, molds, and yeasts, can be present in bee-collected pollen during collection, processing, and storage, depending strongly on its geographic region and beekeepers activities, necessitating the following hygienic and quality standards to minimize the microbial load that can pose risks to consumer safety (17, 29, 44). Furthermore, high moisture or water content in bee-collected pollen creates a favorable environment for microbial growth, including bacteria, molds, and yeasts, which can lead to spoilage, degradation of nutrients, and even safety concerns for human consumption. Therefore, drying and reducing water activity are crucial for preserving bee-collected pollen and preventing spoilage (10, 45, 46).

In Algeria, people continue to consume bee-collected pollen as food and medicine, even though there are no regulatory specifications or legislation for its microbiological quality (9). Therefore, hygienic standards should be adopted because the high number of mesophilic bacteria reflects the microbiological quality of bee pollen (4). The presence of molds and yeasts is responsible for changes in the organoleptic characteristics of bee-collected pollen and the production of mycotoxins such as aflatoxins and ochratoxins, which are thermostable and carcinogenic substances (9, 25, 45). Also, the presence of coliforms, including *Salmonella* spp., is an epidemiological risk due to their ability to cause food-borne diseases in humans (4, 44). In addition, the presence of *S. aureus* in bee-collected pollen can cause food poisoning by producing thermostable enterotoxins. Furthermore, the presence of sulfite-reducing *Clostridium* can lead to neuroparalytic illness through the production of neurotoxins (44).

CONCLUSION

In conclusion, the comprehensive analyses of the physicochemical, phytochemical, and microbiological features of some Algerian bee-collected pollen samples provided us with additional information about their nutritional and therapeutic benefits. Physicochemical analyses revealed significant differences in pH, moisture, and ash content, which indicated the influence of geographical location and botanical origin on bee-collected pollen quality. The phytochemical analyses quantified the bioactive compound content, including total

phenolics and flavonoids, and indicated that the bee-collected pollen is a substantial source of potent antioxidant compounds. In addition, the microbiological study demonstrated the presence of different microorganisms, including some pathogenic species, and underscored the necessity of following hygienic and quality standards to reduce the microbial contamination, which can pose microbiological risks to consumer safety. All things considered, these results affirm the potential of bee-collected pollen as a functional food and its importance in alternative medicine, warranting more studies into its health benefits and applications.

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الملخص

تهدف هذه الدراسة إلى عزل بكتيريا الأكتينوميسات من بعض عينات طلع النحل، والتي تم جمعها من مناطق مختلفة من الجزائر، مع تقييم تطبيقاتها وأنشطتها المختلفة. أولاً، تمت مراقبة الجودة الميكروبيولوجية لعينات طلع النحل، حيث أظهرت التحاليل أن الحمل الميكروبي تراوح بين 7.70 × 10^2 و 1.93 × 10^6 و م م/غ للفلورا الكلية الهوائية، و بين 1.10 × 10^3 و 5.30 × 10^5 و م م/غ للفلورا الكلية اللاهوائية. كما تراوح محتوى الأعفان والخمائر بين 2.70 × 10^3 و 3.70 ×10^3 و م م/غ. قدرت الحمولة القصوى لبكتيريا القولون والمكورات العنقودية الذهبية بـ 1.73 × 10^4 و م م /غ و2.33 × 10^2 و م م/غ على التوالي. كما تم العثور على sulfite-reducing *Clostridium* في عينة واحدة فقط، بينما لم يتم العثور على *Salmonella* sp. في كل العينات. علاوة على ذلك، تم عزل 33 سلالة من بكتيريا الأكتينوميسات حيث تم توصيفها وفحصها لإنتاج إنزيمات خارج خلوية وكذلك للنشاط المضاد للميكروبات، ثم تم تصنيفها بطريقة UPGMA. أظهرت السلالات المعزولة ثمانية مجاميع متنوعة، حيث كان معظمها قادرًا على إنتاج إنزيمات خارج خلوية، بما في ذلك البروتياز، الأميلاز والليباز، بينما أظهرت خمس سلالات فقط نشاطية ضد ميكروبية. تم اختيار مستخلص أسيتات الإيثيل الخام للسلالة *Streptomyces* sp. BPA-6 والتي أظهرت نشاطية ضد ميكروبية معتبرة في الفحص الأولي لتقييم قدرتها على قتل أو تثبيط نمو وتكاثر بعض مسببات الأمراض الميكروبية، وكذلك تحليل وتحديد مركباتها النشطة. أظهرت طريقة الآبار أن المستخلص كان له فعالية فقط ضد *S. aureus* ATCC 6538P، و*B. subtilis* ATCC 6633، و*C. albicans* ATCC 10231، مع مناطق تثبيط قدرت بـ 42.33 ± 3.79 ملم ، و41.66 ± 3.51 ملم ، و48.67 ± 2.08 ملم، على التوالي. كانت قيمة MIC المسجلة هي 62.5 مكغ/مل ضد *S. aureus* ATCC 6538P و*B. subtilis* ATCC 6633، بينما كانت 250 مكغ/مل ضد *C. albicans* ATCC 10231. كما لوحظت أفضل قيمة MMC ضد *B. subtilis* ATCC 6633، بقيمة 1000 مكغ/مل. كشف تحليل GC-MS عن العديد من المركبات المضادة للميكروبات، بما في ذلكhexadecanoic acid ، 9, 12-octadecadienoic acid و 9-octadecenoic acid. من جهة أخرى، تم استخدام الصبغة الحمراء للسلالة *Streptomyces* sp. A23 للتصنيع الأخضر لجزيئات الفضة النانوية (AgNPs)، وتقييم أنشطتها الضد ميكروبية وضد سرطانية ووقاية الخلايا العصبية، حيث لوحظ نشاطا مضادا للميكروبات جيدا ضد *E. faecalis* ATCC 19433 و *C. albicans* ATCC 10231 و *S. aureus* ATCC 6538P و *P. aeruginosa* ATCC 27853 و *B. subtilis* ATCC 6633 و *K. pneumoniae* ATCC 13883 و *E. coli* ATCC 7839، مع مناطق تثبيط 32 ملم و 30 ملم و 30 ملم و 27 ملم و 25 ملم و 20 ملم و 19 ملم على التوالي. كما تم تسجيل أقل قيم لـ MIC و MBC ضد *B. subtilis* ATCC 6633 بقيمة 62.5 مكغ/مل. ومن المثير للاهتمام أن معظم جزيئات الفضة النانوية المصنعة والمحضرة بتراكيز 2 مكغ/مل و 4 مكغ/مل و 8 مكغ/مل كان لها تأثيرات معتبرة على خلايا الورم العصبي SH-SY5Y. بالإضافة إلى ذلك، أظهرت جزيئات الفضة النانوية (1 ملي مول، درجة حموضة 7) نشاطًا وقائيًا للخلايا العصبية عند أقل تركيز تم اختباره.

الكلمات المفتاحية: بكتيريا الأكتينوميسات، طلع النحل، الجودة الميكروبيولوجية، *Streptomyces*، الصبغات الميكروبية، جزيئات الفضة النانوية، الإنزيمات خارج خلوية، النشاطية ضد ميكروبية.

Abstract

This study aimed to isolate actinomycetes from some bee pollen samples collected from different Algerian places and evaluate their applications. First, the microbiological quality control of bee pollen samples was investigated, where the analyses showed a microbial load ranged from 7.70 x 10^2 to 1.93 x 10^6 CFU/g for the aerobic mesophilic bacteria and from 1.10 x 10^3 to 5.30 x 10^5 CFU/g for the anaerobic mesophilic bacteria. Molds and yeasts content varied from 2.70 x 10^3 to 3.70 x 10^3 CFU/g. The maximum concentrations for total coliforms and *S. aureus* were 1.73 x 10^4 cfu/g and 2.33 x 10^2 CFU/g, respectively. Sulfite-reducing *Clostridium* was detected only in one sample. In addition, *Salmonella* sp. was not detected in all samples. Furthermore, 33 strains of actinomycetes were isolated, characterized, screened to produce extracellular enzymes as well as for antimicrobial activity, and then classified by the UPGMA method. The isolated strains showed eight diversified clusters, where most of them were able to produce extracellular hydrolytic enzymes, including proteases, amylases, and lipases, while only five strains demonstrated significant antimicrobial activity. The crude ethyl acetate extract of the *Streptomyces* sp. BPA-6 strain was selected to evaluate its ability to kill or inhibit the growth and reproduction of some pathogens and to analyze and identify its bioactive compounds. The agar-well diffusion method showed that it was only effective against *Staphylococcus aureus* ATCC 6538P, *Bacillus subtilis* ATCC 6633, and *Candida albicans* ATCC 10231, with inhibition zones of 42.33 ± 3.79 mm, 41.66 ± 3.51 mm, and 48.67 ± 2.08 mm, respectively. The recorded MIC value was 62.5 µg/mL against *S. aureus* ATCC 6538P and *B. subtilis* ATCC 6633, while it was 250 µg/mL against *C. albicans* ATCC 10231. Also, the best MMC value was observed against *B. subtilis* ATCC 6633, with a value of 1000 µg/mL. The GC-MS analyses revealed many antimicrobial compounds, including hexadecanoic acid, 9,12-octadecadienoic acid, and 9-octadecenoic acid. On the other hand, the red pigment of the *Streptomyces* sp. A23 strain was used for the green synthesis of silver nanoparticles (AgNPs), evaluating their antimicrobial, anticancer, and neuroprotective activities. Good antimicrobial activity was observed against *Enterococcus faecalis* ATCC 19433, *C. albicans* ATCC 10231, *S. aureus* ATCC 6538P, *Pseudomonas aeruginosa* ATCC 27853, *B. subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 13883, and *Escherichia coli* ATCC 7839, with inhibition zones of 32 mm, 30 mm, 30 mm, 27 mm, 25 mm, 20 mm, and 19 mm, respectively. The lowest MIC and MBC were recorded against *B. subtilis* ATCC 6633 with a value of 62.5 µg/mL. Intriguingly, the most of synthesized AgNPs at concentrations of 2 8 µg/mL, 4 8 µg/mL, and 8 µg/mL had cytotoxic effects on SH-SY5Y neuroblastoma cell lines. In addition, AgNPs (1 mM, pH 7) exhibited significant neuroprotective activity at the lowest tested concentration.

Keywords: Actinomycetes, bee pollen, microbiological quality, *Streptomyces*, microbial pigments, silver nanoparticles, extracellular enzymes, antimicrobial activity.