

Inhibitory effect of *Streptomyces fradiae* isolates against microbial pathogens (*in vitro*) and study its compatibility with biosynthesized zinc oxide nanoparticles

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ABSTRACT

Background: *Streptomyces* comprises Gram-positive, filamentous, and spore-forming bacteria. *Streptomyces fradiae* has the capability to synthesize the antibiotics neomycin, tylosin, and fosfomycin. **Aim of the study:** Detection of the inhibitory effect of *S. fradiae* isolates against microbial pathogens (*in vitro*) and study its compatibility with biosynthesized zinc oxide nanoparticles. **Methodology:** Fifty soil samples were gathered between December 2023 and January 2024. Specimens were gathered from several locations inside the city of Baghdad. Following the dilution of the sample, it was plated onto ISP2 agar to isolate *Streptomyces*. Multiple biochemical assays were conducted on the isolates. Zinc oxide nanoparticles were produced using *Eucalyptus* extract and evaluated against harmful microorganisms. **Results:** A total of 33 *Streptomyces* isolates were obtained, then identified as *Streptomyces fradiae* , 12 isolates did not exhibit any antibacterial effects against pathogenic bacteria. On the other hand, 21 isolates (63.6%) demonstrated strong antibacterial activity. Of the total isolates, 48.5% (16) exhibited antibacterial activity against both *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*Staph. aureus*). However, only 15% (5 isolates) demonstrated activity, particularly against *Staph. aureus*. The zone of inhibition of the nanoparticles against the tested strains was 9mm, while for the *Eucalyptus* extract was 2.5mm against *Staph. aureus*. The inhibition zones against *E. coli*, on the other hand, were 11mm for nanoparticles and 3mm for the *Eucalyptus* extract. The effective concentration was 12.5% for the plant's extract and 20% for nanoparticles. **Conclusion:** The *Streptomyces*'s bioactive molecule exhibited antibacterial properties. It was more effective against Gram-positive bacteria (*Staph. aureus*) than Gram-negative bacteria (*E. coli*). This study presents an environmentally friendly method for producing zinc oxide nanoparticles (ZnO NPs) using a leaf extract from *Eucalyptus globulus* as a reducing agent. The ZnNPs have antibacterial efficacy against both *E. coli* and *Staph. aureus*. The antibiotic of *Streptomyces* was more effective than Zn NPs and *Eucalyptus* plants against pathogenic bacteria

Keywords: *Streptomyces*, *Eucalyptus*, Nanoparticles.

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INTRODUCTION

The Actinomycetales order includes actinomycetes. The phylum Actinobacteria is one of the largest bacterial taxonomic groups. Gram-positive bacteria with high DNA guanine-cytosine (G + C) concentration comprise the phylum. From 51% in certain *Corynebacterium* to over 70% in *Streptomyces* and *Frankia* (1). They are bacteria because their cell walls contain peptidoglycan, they lack a nuclear membrane, are vulnerable to lysozyme and antibiotics, and lack chitin and cellulose (2). *Streptomyces* are Gram-positive, aerobic, and non-acid-fast. Their global existence influences soil ecology. When nutrients are scarce, *Streptomyces* species self-synthesize antimicrobials during sporulation. These natural chemicals

prevent other organisms from competing with *Streptomyces* for nutrients. (3) *Actinomycetes* produce various bioactive substances, including anthracyclines, glycopeptides, aminoglycosides, macrolides, polyenes, β -lactams, peptides, nucleosides, terpenes, polyethers, and tetracyclines. These chemicals have many biological functions. Some are microbicidal; others are inhibitors (4). Antimicrobials—including antibiotics—include antiviral, antifungal, and antiparasitic drugs (5). Nanotechnology is increasingly important in modern society. With this technology, scientists can understand and regulate material composition and properties at the atomic and molecular level. Nanoparticles spearhead nanotechnology's rapid growth. In the last decade, nanostructured materials, particularly metallic nanoparticles, have received interest due to their unique properties, making them lucrative and crucial in many fields. Nanoparticles differ from bulk materials due to their small size and large specific surface area (6).

A huge Myrtaceae genus, *Eucalyptus* has over 900 species and subspecies. *Eucalyptus* leaf extracts are approved as food additives and cosmetics despite their widespread cultivation. Recently, these extracts' functional properties have been emphasized. The extracts are antibacterial, antihyperglycemic, and antioxidant, according to studies (7). Biocompatible, cost-effective, and regenerative phytochemicals from medicinal plants can be used to produce nanoparticles in an environmentally friendly way. Nanoparticles (NP) of different shapes, sizes, and optical properties can be made from phytochemical plant extracts. The green production of Metallic Nanoparticles (MNPs) has revolutionized nanobiotechnology. Thus, producing MNPs from plants, microorganisms, and algae has great potential, particularly because metal ion reduction is faster with plant extracts(8). The use of reducing agents, stabilizing chemicals, and environmentally friendly solvents makes green chemistry advantageous. Plant extracts contain amines, amides, alkaloids, flavonoids, phenols, terpenoids, proteins, and pigments. The phytochemical components stabilize and reduce metal ions during the formation of green nanoparticles(9).

Study objective: *Streptomyces fradiae* isolates' in vitro inhibition of microbial pathogens and compatibility with biosynthesized zinc oxide nanoparticles.

METHODOLOGY

Sample collection

50 soil samples were taken from several locations in Baghdad.

Isolating and identifying *Streptomyces* spp. from soil samples

One gram of dried soil samples was added to 99 mL of sterile distilled water (stock suspension) and agitated at 120 rpm for 30 minutes at room temperature. For 10 minutes, stock solution serial dilutions from 10^1 to 10^9 were left. After shaking, 0.1 mL of each dilution was pipetted onto ISP2 Yeast Extract-malt Extract Agar containing 50 μ g/L Tetracycline and 50 μ g/L Nystatin, evenly smeared with a sterile brush. Inoculated plates were incubated at 28°C for 7–10 days. To ensure pure actinomycete growth on ISP2 agar, colonies were transferred from the initial screening plates to separate plates and cultured at $28 \pm 1^\circ\text{C}$ for 7 days. The process was repeated often. Biochemical tests were used for further identification. The pure culture was kept at 4°C for investigation. (10).

Antibacterial Activity of *Streptomyces* spp.

Primary screening (Giant Colony/ cross-streak technique). Nutrient agar plates were inoculated with the *Streptomyces* isolate by a single streak of inoculum in the middle of the plates. After 7 days of incubation at 28°C, the test bacterial isolates were streaked perpendicular to the *Streptomyces* (a single streak at a 90° angle to the *Streptomyces* isolates), and the plates were re-incubated at 37°C for 24 hours. The antibacterial activity was observed by the naked eye, in which the tested bacteria failed to grow near the *Streptomyces* line. The antibacterial effectiveness of all *Streptomyces* spp. isolates were assessed on Yeast extract-malt extract agar medium ISP2 using the cross-streak method against two pathogenic bacteria, *Escherichia coli* as a representative of Gram-negative, and *Staphylococcus aureus* as a representative of Gram-positive. The standard inoculum is prepared by cultivating the required bacteria in Nutrient Broth at $37 \pm 0.1^\circ\text{C}$ for 24 hours, utilizing 4-5 colonies. The inoculum was calibrated using a turbidity standard known as the McFarland standard. As an illustration, a turbidity level of 0.5 McFarland corresponds to a concentration of 1.5×10^8 CFU/mL, as determined by visual inspection (10).

Preparation of Plant Aqueous Extracts

The aquatic extracts of *Eucalyptus* leaves were obtained using a traditional method: washing the fresh leaves with well-run water to remove surface contaminants, then soaking them for 30 minutes. After that, the leaves' moisture was removed, and they dried thoroughly with dry air. 10 g of dried plant leaves was placed in a 250 mL glass flask containing 100 mL of deionized water and heated in a 45 °C water bath for about 30 min, after which the extracts were filtered through Whatman filter paper No .1 and stored at 4 °C for later use. (11). The inhibitory effect of alcoholic *Eucalyptus* extract on *E.coli* & *Staph. aureus* isolates were tested by the well -diffusion method. Inhibition Zone Diameter (IZD) values were measured in millimeters (mm).

Biosynthesis of Zinc Oxide Nanoparticles

Synthesis of zinc oxide nanoparticles

Zinc oxide nanoparticles (ZnO NPs) were synthesized using a green approach known as Eucalyptus extract-mediated synthesis. Following the previously described plant extract procedure, 30 mL of the extract was transferred to a beaker and heated gradually. At 60 °C, precisely 3 grams of zinc nitrate hexahydrate were introduced into this extract. Subsequently, the mixture was continuously churned at 60 °C until it formed a yellowish paste within 1 hour. The reaction temperature had a significant impact on nanoparticle production. The highest yield of nanoparticles was obtained at 60 °C. Subsequently, the paste was subjected to intense heat in a furnace at 400 °C for approximately 2 hours. Following this, the remaining residue was often cleansed using ethanol and distilled water. The powder was dried at 100 °C and meticulously prepared for subsequent characterization and treatment (12).as illustrated in Figure1.



Figure 1: Green synthesis and characterization of zinc oxide nanoparticles using *Eucalyptus*. Leaf extract and zinc nitrate hexahydrate salt (12).

Preparation of different concentrations of plant extract and nanoparticles

Four different concentrations (50, 25, 12.5, and 6.25)% of each plant extract were prepared by adding 5% Dimethyl sulfoxide (DMSO) as a polar solvent. Serial dilutions of ZnO Nps (20,10,5,2.5) µg/mL were prepared according to (13). The MIC for ZnO NPs was determined against clinical source isolates of *Staph. aureus* and *E. coli* .

Statistical analysis

Prog. SPSS Version (13) Experimental results are reported as mean ± SD. Differences in ZnO-NPs concentrations were evaluated for significance using one-way ANOVA. A statistical significance was indicated by a p-value of ≤0.05.

RESULTS

Out of the 50 soil samples analyzed, 35 (70%) were found to potentially contain *Streptomyces*. Among these samples, 33 (94.3%) isolates were successfully isolated, each exhibiting distinct morphological traits. The colonies presumed to be Actinomycetes were meticulously transplanted onto agar medium containing ISP2 to obtain a pure isolate. The isolate was then defined by its colorful aerial and substrate mycelium, dry and smooth or rough texture, and irregular or regular edge. The colony was generally of a convex shape. The majority of solitary colonies exhibit earthy aromas. Figure 2 displays small powdery colonies ranging from white to grey, likely representing Actinomycetes isolates.



Figure 2: *Actinomycetes* powdery colonies

Biochemical test

Streptomyces spp. possesses the capacity to synthesize enzymes such as catalase. Simmon's citrate usage was confirmed as positive, but indole synthesis was not detected, indicating a negative outcome. The use of sugar was investigated by cultivating *Streptomyces* in media containing Dextrose, Starch, or Glycerol as carbon sources (14).

Table (1): Biochemical results of *Streptomyces fradiae*

No	Test	Reaction	Result
1.	Melanin	Black to brown	-ve
2.	Catalase	Bubbles	+ve
3.	Citrate Utilization	Deep blue color	+ve
4.	Indole production	No color zone	-ve
5.	Sugar utilization	Growth	+ve
6.	Hydrogen sulfide production	No reaction	-ve
7.	Nitrate reduction	Red color	+ve
8.	Oxidase production	No reaction	-ve
9.	Casein hydrolysis	Clear transparent zone	+ve
10.	Melanine reaction	No reaction	-ve
11.	Starch hydrolysis	Clear halo	+ve

Screening of Antibiotic-Producing *Streptomyces fradiae*

Of the 33 *Streptomyces fradiae* isolates collected from Baghdad, 12 exhibited no antibacterial activity against pathogenic bacteria, including Gram-negative and Gram-positive bacteria. In contrast, 21 isolates (63.6%) showed intense antibacterial activity. Among all isolates, 48.5% (16) exhibited antibacterial properties against both *Staph. aureus* and *E. coli*. However, only 15% (5 isolates) showed activity, mainly targeting *Staph. aureus*. The Agar-Well Diffusion technique was utilized to assess the *Streptomyces* isolates, and the growth inhibition zones were quantified in millimeters. Figure 3.



Figure 3: Antimicrobial activity of *Streptomyces fradiae* isolates against *Staph. aureus* and *E. coli* on Muller Hinton agar media at 28°C for 7-10 days, for *Streptomyces* grown, then re-incubated at 37°C for 24 hours using cross streaking. A-Negative results. B-Positive results

Determine the antibacterial effect of *Eucalyptus* extract against Pathogenic bacteria

Four different concentrations (6.25, 12.5, 25, 59)% of each plant extract were prepared by adding 5% Dimethyl sulfoxide (DMSO) as a polar solvent. The ethanolic crude extract showed remarkable results with a clear inhibition zone around each well, whereas DMSO, as a negative control, showed no inhibition zone. The results showed an increase in the size of the inhibition zone with increasing concentration. The results in Figure 4 showed that the highest bacterial inhibition was recorded at a concentration of 12.5% as the inhibitory diameter reached 17mm, and at concentrations of 50%, 15mm was recorded, at a concentration of 25%, and 6.25%, inhibitory diameters reached 12 mm and 10 mm, respectively.

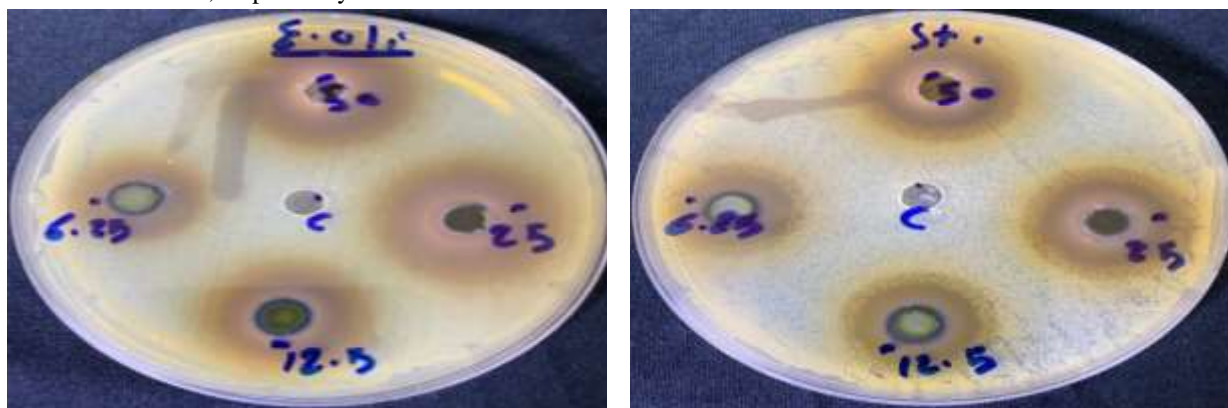


Figure 4: Antibacterial effect of *Eucalyptus* extract against *E.coli* and *Staph. aureus*

Determine the minimum inhibitory concentration (MIC) of the ZnO nanoparticles against Pathogenic bacteria

The results of prepared serial dilutions of ZnO NPs (2.5, 5, 10, 20) $\mu\text{g/mL}$ showed that the MIC of ZnO NPs was against clinical-source isolates of *Staph. aureus* and *E. coli*, which were (2.5, 5) $\mu\text{g/mL}$, respectively.

Table 2: The minimum inhibitory concentration (MIC) of ZnO nanoparticles (NPs) against harmful bacteria

Isolated bacteria	MIC of ZnO NP ($\mu\text{g/mL}$)
<i>Staph. aureus</i>	2.5
<i>E. coli</i>	5

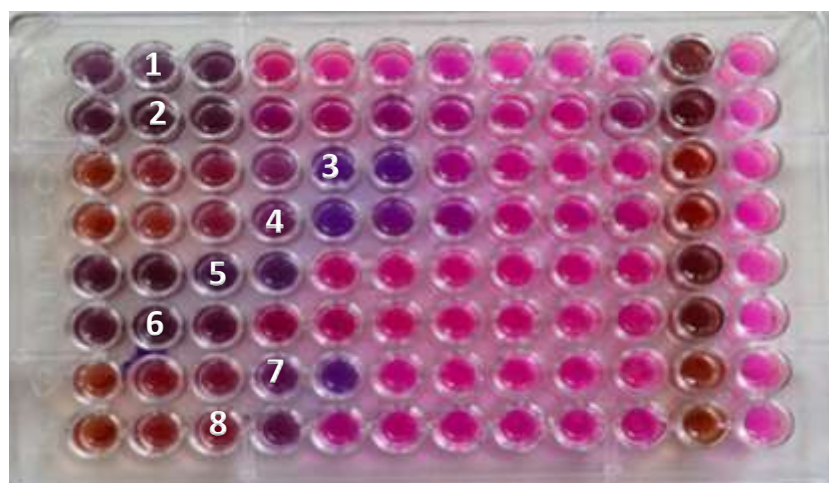


Figure 5: Flat-bottom microtiter plates for determining MIC of ZnO nanoparticles against the *E.coli*(1,2,3&4) & *Staph. aureus*(5,6,7&8).

Comparison between the inhibitory effect of ZnO nanoparticles and the antibiotic of *Streptomyces fradiae* on pathogenic bacteria

The results revealed that ZnO NPs and the antibiotic obtained from *Streptomyces fradiae* showed similar zones of inhibition for both agents against *Staph. aureus* and *E.coli* bacteria on separate plates. The zone of inhibition for nanoparticles, plant drug, antibiotic of *Streptomyces* S1, and antibiotic of *Streptomyces* S2 was (9, 2.5, 20, 18) mm, respectively, against *Staph. aureus*, while against *E. coli* were (11, 3, 15, 17) mm, respectively, as shown in Table 3.

Table 3: inhibitory effect of ZnO nanoparticles and the antibiotic of *Streptomyces fradiae* on pathogenic bacteria

Zone of inhibition	Nanoparticles	plant drug	<i>Streptomyces</i> S1	<i>Streptomyces</i> S2
<i>Staph. aureus</i>	B 9 ± 0.70	C 2.5 ± 0.35	A 20 ± 1.74	A 18 ± 2.36
<i>E.coli</i>	B 11 ± 1.96	C 3 ± 0.47	A 15 ± 2.88	A 17 ± 2.33

Different latter mean significant differences at level 0.05 ($p \leq 0.05$)

DISCUSSION

The *Streptomyces* isolates were classified by analyzing the differences in their microscopic characteristics and colony morphology, including the presence of aerial and substrate mycelium, soluble pigment, and the arrangement of spore chains. Multiple strains of *Streptomyces fradiae* demonstrated the capacity to produce a colored material that diffused into the surrounding medium, matching the color of the aerial mycelium (15). Out of the 50 soil samples analyzed, 35 (70%) were found to potentially contain *Streptomyces*. Among these samples, 33 (94.3%) isolates were successfully isolated, each exhibiting distinct morphological traits. In another study by (16), 154 *Streptomyces* isolates were isolated from 46 soil samples collected from various locations in Nineveh, while according to (17), approximately 44 (88%) of the soil samples were suspected of containing *Streptomyces*, which agrees with our result. Out of the approximately 42 isolated *Streptomyces* species, 84% have displayed distinct physical characteristics. The results of the present investigation were consistent with the findings of Parungao *et al.* (18), who showed that the antibacterial effect of *Streptomyces* secondary metabolites on Gram-positive bacteria is stronger than their effect on Gram-negative bacteria, while Al-Ansari *et al.* showed that the crude extract of *Streptomyces* spp. has high activity against both Gram-positive and Gram-negative bacteria (19), implying that environmental conditions also impact the synthesis of secondary metabolites and should be considered. A number of *Streptomyces* species isolated from different settings have recently demonstrated potent anti-Methicillin-Resistant-Staphylococci (MRSA) activity. These organisms have the potential to produce a high molecular weight glycopeptide with novel anti-MRSA activity. The investigation conducted by Sharma *et al.* (20) found that among the *Streptomyces* isolates, 51 (38%) showed antibiotic activity against one or more test organisms (*Staph. aureus* and *E. coli*), and six of these isolates had promising broad-spectrum activity against all of the species that were considered for testing. An investigation that was done out in Iraq demonstrated that methanol extracts showed differing degrees of inhibitory zones against the microorganisms that were examined at various concentrations, beginning with 12.5 mg/mL and going all the way up to 10 mg/mL. Furthermore, the methanol extracts demonstrated a greater level of activity against *Staph. aureus* in comparison to *E. coli* (21).

The study examined the potential of ZnO NPs as a substitute for antibiotics to enhance their effectiveness against pathogenic strains. Their distinct physicochemical properties may influence the biological and toxicological reactions of microorganisms. The primary processes responsible for their antibacterial activity involve releasing metal ions, adsorbing particles, and generating reactive oxygen species (22). Emami-Karvani *et al.* (23) demonstrated that ZnO's minimum inhibitory concentration (MIC) against *E. coli* and *Staph. aureus* was 1 mg/mL and 0.5 mg/mL, respectively. The findings indicated that ZnO NPs exhibited an antibacterial inhibition zone measuring 29 mm and 19 mm at a dosage of 10 mg/mL against *E. coli* and *Staph. aureus*. Gram-negative bacteria exhibited greater resistance to ZnO NPs compared to Gram-positive bacteria. The

study concluded that manipulating the particle size and concentration of ZnO NPs led to enhanced antibacterial efficacy. The antibacterial efficacy of ZnO NPs exhibited a time-dependent trend, gradually increasing in effectiveness. El-Masry *et al.* (24) did a study that found that the utilization of ZnO NPs with a diameter of 20 nm and a concentration of 10 µg/mL resulted in the most substantial reduction in growth. The population of *Staph. aureus* was decreased by 98.99%. In their study, Mwafy *et al.* (25) discovered that the minimum inhibitory concentration (MIC) of zinc oxide ZnO NPs was 31.3 mg/mL for *E. coli* and 7.8 mg/mL for *Staph. aureus*.

CONCLUSION

The *Streptomyces* bioactive molecule exhibited antibacterial properties. It was more effective against Gram-positive bacteria (*Staph. aureus*) than Gram-negative bacteria (*E. coli*). This study presents the environmentally friendly production of zinc oxide nanoparticles (ZnO NPs) using a leaf extract from *Eucalyptus globulus* as a reducing agent. The ZnO NPs have antibacterial efficacy against both *E. coli* and *Staph. aureus*. The antibiotic of *Streptomyces* was more effective than Zn NPs and *Eucalyptus* plants against pathogenic bacteria.

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التأثير التثبيطي لعزلات *Streptomyces fradiae* ضد مسببات الأمراض الميكروبية (في المختبر) ودراسة توافقها مع جزيئات أكسيد الزنك النانوية المخلفة حيويًا

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كلية التقنيات الاحيائية ، جامعة النهرين ، العراق

الخلاصة

خلفية البحث : تشتمل الستربتوميسيس على بكتيريا موجبة الجرام، وخطيئة، ومكونة للأبواغ. تمتاز بكتيريا *S. fradiae* بالقدرة على تصنيع المضادات الحيوية نيومايسين وتيلوسين وفوسفوميسين. **الهدف من الدراسة:** الكشف عن التأثير التثبيطي لعزلات *Streptomyces fradiae* ضد مسببات الأمراض الميكروبية (في المختبر) ودراسة مدى توافقها مع جزيئات أكسيد الزنك النانوية المخلفة حيويًا. **طرق العمل:** تم جمع خمسين عينة من التربة في الفترة ما بين كانون الأول 2023 وكانون الثاني 2024. وتم جمع العينات من عدة مواقع داخل مدينة بغداد. بعد تخفيف العينة، تم زرعها على أجار ISP2 لعزل الستربتوميسيس. تم إجراء فحوصات كيميائية حيوية متعددة على العزلات. تم إنتاج جزيئات أكسيد الزنك النانوية باستخدام مستخلص الأوكالبتوس وتم تقييمها ضد الكائنات الحية الدقيقة الضارة. **النتائج:** تم الحصول على 33 عزلة من العزلات. *Streptomyces* شخصت لاحقًا بانها عزلة *Streptomyces fradiae* ، لم تظهر اثنتا عشرة عزلة أي تأثير مضاد للجراثيم ضد البكتيريا المسببة للأمراض. من ناحية أخرى أظهرت إحدى وعشرون عزلة (63.6%) نشاطاً قوياً ضد البكتيريا. من إجمالي العزلات أظهرت 48.5% (16 عزلة) نشاطاً مضاداً للبكتيريا ضد كلا من الإشريكية القولونية. و المكورات العنقودية الذهبية. ومع ذلك، أظهرت 15% فقط (5 عزلات) نشاطاً خاصاً ضد المكورات العنقودية الذهبية. تم قياس منطقة التثبيط للجسيمات النانوية 9 ملم، بينما للعقار النباتي 2.5 ملم. في حالة المكورات العنقودية الذهبية. بالنسبة للإشريكية القولونية، كانت منطقة التثبيط 11 ملم للجسيمات النانوية و 3 ملم للدواء النباتي. **الاستنتاج:** أظهرت جزيئات *Streptomyces* النشطة بيولوجياً خصائص مضادة للبكتيريا. كانت أكثر فعالية ضد البكتيريا إيجابية الجرام (*Staph. aureus*) من البكتيريا سلبية الجرام (*E. coli*). تقدم هذه الدراسة الإنتاج الصديق للبيئة لجسيمات أكسيد الزنك النانوية (ZnO NPs) باستخدام مستخلص أوراق *Eucalyptus globulus* كعامل اختزال. تتمتع جزيئات أكسيد الزنك النانوية بفعالية مضادة للبكتيريا ضد كل من *Staph. aureus* و *E. coli*. كان المضاد الحيوي *Streptomyces* أكثر فعالية من جزيئات أكسيد الزنك النانوية ونباتات الأوكالبتوس ضد البكتيريا المسببة للأمراض.

الكلمات المفتاحية: بكتيريا *Streptomyces* ، الأوكالبتوس، الجسيمات النانوية.