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# Molecular identification of two *Actinomycetes* (*Streptomyces* sp. and *Norcardia* sp.) with the evaluation of cytotoxicity and antioxidant properties of *Streptomyces* sp. Extract

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**Abstract:** The current study was carried out to isolate *Actinomycetes* bacteria from soil samples from Al-Diwaniyah City, Iraq, and to evaluate the toxicity and antioxidant characteristics of the extract of the isolated species. For that aim, 500gm of soil sample was collected and subjected to series of bacterial cultivation that ended up with bacterial identification using morphological, biochemical, and *16S rRNA*-gene-polymerase chain reaction (PCR) tools. The final identification revealed the isolation of two *Actinomycetes*; *Streptomyces* sp. (named as QZS11) and *Nocardia* sp. (named as QZS9), and since many members of *Streptomyces* are beneficial bacteria, the toxicity and antioxidant characteristics of the extract of the isolated QZS11 were evaluated using the Vero cell viability assay and the diphenyl picryl hydrazine (DPPH) reduction assay, respectively. The results showed that the viability rates of the Vero cells were 100% at 0.125mg/ml and 90% at 0.25mg/ml. The findings regarding the DPPH assay revealed an important antioxidant level (20%) at 0.25mg/ml. The study reports the isolation of two *Actinomycetes*; *Streptomyces* sp. (QZS11) and *Nocardia* sp. (QZS9), with low toxicity and important antioxidant level at a very low concentration for the QZS11 isolate.

**Keywords**: *Actinomycetes*, antibiotics, *Nocardia*, soil bacteria, *Streptomyces*.

## Introduction

As far back as recorded history, people have relied on chemicals to preserve or enhance their health. Chemicals that can kill or defeat germs and pathogens are considered one of the greatest medical discoveries of the 20th century, and millions of people have been saved as a consequence, in latest years. Medical therapies have always relied on natural ingredients. Nature-derived chemicals or their derivatives made up 65% of the 1211 small molecule medications that the FDA authorized during a 34-year period, from 1981 to 2014 (1,2).

As soon as it was introduced to humans in 1942, the antibiotic penicillin changed the way bacteria were treated. As a result, it has been essential in the hunt for other antibacterial and antimicrobial compounds, saving the lives of hundreds of millions of people worldwide. Scientific and economic considerations are likely to postpone the development of new antibiotics. Human usage takes 10–15 years after preclinical testing has been completed (3–5). International response on antimicrobial resistance was adopted by the World Health Assembly in 2015. Nations must build comprehensive strategy and take aggressive efforts in order to support the development and effective utilization of new antibiotics and those with revolutionary mechanisms of action (6,7).

The Actinomycetes are a diverse community of Gram-positive bacteria having a high guanine (G) and cytosine (C) composition in their DNA. At a minimum of 350 genera have been identified so far for this group of bacteria. They make up a significant portion of the bacterial kingdom and may be found in both aquatic and terrestrial environments. Soil-dwelling microorganisms such as the Streptomycetes (in particular) may also be found in fresh and salt water, as well as in the air. Streptomycetes make up more than 95% of all Actinomycete isolates found in the soil, and they
may be found at concentrations of 106 to 109 cells per gram of soil. Many species are safe to animals and upper plants, whereas a few are major pathogens that pose a serious threat to human health (8).High-impact compounds have been produced by Actinomycetes, notably species from the group *Streptomyces*. Streptomycin, actinomycin, and streptothricin are just a few of the antimicrobials they've produced (9).The current study was carried out to isolate *Actinomycetes* bacteria from soil samples from Al- Diwaniyah City,

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Iraq, and to evaluate the toxicity and antioxidant characteristics of the extract of the isolated species.

**Materials and methods Soil samples**

The study included taking of 500gm of soil sample from Al-Diwaniyah City, Iraq, in a sterile plastic bags. An air-drying based step was done on the samples for one week. Later, these samples were crushed and sieved. The isolation of Actinomycetes was performed on the final product of the sieving step.

**Isolation of Actinomycetes**

Suspension of 5gm of the sieved sample/50ml NaCl0·85g/L was prepared, incubated at 28˚C with in a shaker at 200 rpm for 3mins. Spread plate techniques were used for the isolation after a serial dilution. Starch casein agar medium, Actinomycetes Isolation Agar media, and Water-yeast extract-agar (WYE) were used and incubated at 28°C for 7-15 days.

**Morphological and biochemical identification**

Colony shape, color, and type were used. Gram straining based microscopic examination was performed, using structure of mycelium, color, and the appearance of conidiophores and Arthrospore at (1000x). For the biochemical features, IMVC (indole methyl red voges-proskauer citrate), TSI (triple sugar- iron agar), urease, oxidase, and catalase tests were used.

**Polymerase chain reaction**

A modified tryptone-yeast glucose extract broth was used to grow maintained microorganisms, incubating the organism culture for 6 to 8 days at 28°C in a shaker. The “guanidine thiocyanate DNA isolation method” followed from Pitcher et al (10).

The primers (*16S rRNA* gene) adopted from Lane (11) 27f: “AGA GTT TGA TCM TGG CTC AG” and 1525r: “AAG GAG GTG WTC CAR CC”. The volume for the PCR reaction was (50 µl), which included 20µM for each primer (Invitrogen, USA), 25µM dNTPs (Promega), and 2.5U Taq polymerase buffer (HotStarTaq®, Germany), and 50-300ng DNA. For the PCR thermal cycler (ThermoHybaid, UK), initial denaturation (95°C for 5min), 35 cycles (denaturation (95°C for 60s), annealing (55°C for 120s), and extension (72°C for 180s)), and a final extension (72°C for 600s). To visualize the PCR products, 1% agarose gel (Merck, Germany) with ethidium bromide was used in an electrophoresis, which finally was visualized using a UV-light-based viewer and imager. PCR-product-based sequencing was performed to identify the bacterial isolates.

**Evaluation of *Streptomyces* sp. QZS11 cytotoxicity**

The cytotoxicity of the *Streptomyces* sp. QZS11 ethyl acetate based crude extract was evaluated using the Vero cell (Kidney cells of African green monkey) viability assay was performed via the utilization of the MTT assay, relying on a method described by Raheel et al (12). The cells line was obtained from the Laboratory of Virology, School of Biosciences and Biotechnology, Universiti Kebangsaan Malaysia. Cell culture in 96-well microplates ($2^{x}10^{4}$ cells /ml density/100µl/well) were subjected to a range of 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 mg/ml of the *Streptomyces* sp. QZS11 extract. An ELISA reader (BioRad, Chapan) was used to study the optical density (OD). The cell viability percentage was calculated using the following equation:

V(%)=[OD treated cell (Mean )/OD untreated cell (Mean])×100. V: Viability

**Evaluation of *Streptomyces* sp. QZS11 antioxidant properties**

The reduction of DPPH, a stable free radical, was tested to evaluate the antioxidant properties of the *Streptomyces* sp. QZS11 ethyl-acetate extract, in which the test was performed based on a method by Patel and Patel (13). The procedure was started by mixing 3ml (0.06mM) DPPH with (77µL) 0.125, 0.25, 0.5, 1, 2, 4, 8, or 16 mg/ml of the Streptomyces sp. QZS11 extract. Vitamin C (Vit C) was the positive control. OD (at 517nm) was measured using a spectrophotometer (Shimadzu,UV, Germany). The radical scavenging activity was calculated using the following equation: Inhibition (%) = [(OD control – OD sample /OD control)] × 100

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**Results**

The final identification revealed the isolation of two *Actinomycetes*; *Streptomyces* sp. (named as QZS11) and *Nocardia* sp. (named as QZS9) (Table 1 and 2).

**Table 1**: Biochemical properties of *Actinomycetes* isolates

| Tests | Results |
|---|---|
| **Hydrolysis of starch** | Variable |
| **Casein** | Variable |
| **Glucose** | + |
| **Utilization of sucrose** | Variable |
| **Hydrolysis of lipid** | + |
| **Utilization of citrate** | Variable |
| **Utilization of Rhamnose** | + |
| **Reduction of nitrate** | – |
| **Production of $H_2S$** | – |
| **Ability to grow in 8%** | + |

Two genera of *Actinomycetes* (*Streptomyces* sp. QZS11 and *Nocardia* sp. QZS9) were detected by performing different cultivation and biochemical based tests (Table 2 and figure 1)

**Table 2**: The growth and biochemical characteristics of *Streptomyces* sp. QZS11 and *Nocardia* sp. QZS9.

| Characteristics | Results | |
|---|---|---|
| | ***Streptomyces sp.*QZS11** | ***Nocardia sp.*QZS9** |
| **Gram stain** | Positive | Positive |
| **Microscopic appearance** | Long filamentous | Long filamentous |
| **Optimum growth temperature (˚C)** | 28 | 30 |
| **Range of growth temperature (˚C)** | 25- 45 | 25- 45 |

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| | | |
|---|---|---|
| **pH optimum-growth** | 7 | |
| **pH Range for growth** | 4 – 9 | 4-10 |
| **NaCl range for growth (%)** | 1-6 | 1-7 |
| **Anaerobic growth** | - | - |
| **Motility** | Non-motile | Non-motile |
| **Activity of oxidase** | + | + |
| **Hydrolysis of starch** | + | + |
| **Hydrolysis of casein** | + | + |
| **Hydrolysis of urea** | + | + |

| | | |
|---|---|---|
| **Skim milk agar** | + | + |
| **Hydrolysis of gelatin** | + | + |
| **Xanthine Agar** | - | - |
| **Mannitol** | + | + |
| **Indole** | - | - |
| **Activity of catalase** | + | + |
| **Xylose** | + | + |
| **Rhamnose** | - | - |
| **D-Galactose** | + | + |
| **D-Fructose** | + | + |
| **L-Arabinose** | + | + |
| **VP** | - | - |
| **Utilization of citrate** | + | + |
| **TSI** | Alk/Alk | Alk/acid |

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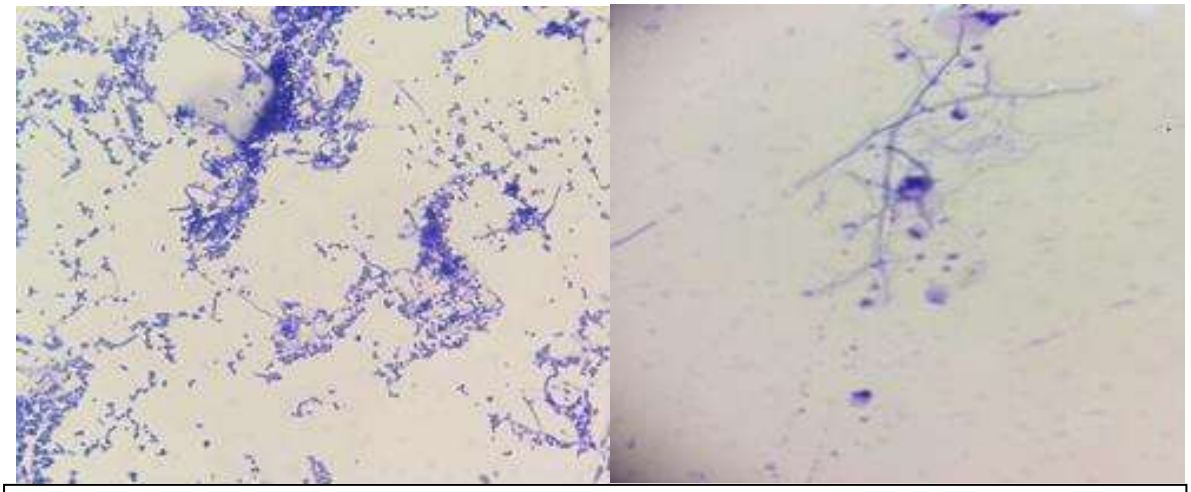

Figure 1: Gram staining of *Streptomyces* sp. QZS11 and *Nocardia* sp. QZS9

The amplification of *16S rRNA* gene revealed the identity of *Streptomyces* sp. QZS11 and *Nocardia* sp. QZS9 (Figure 2).

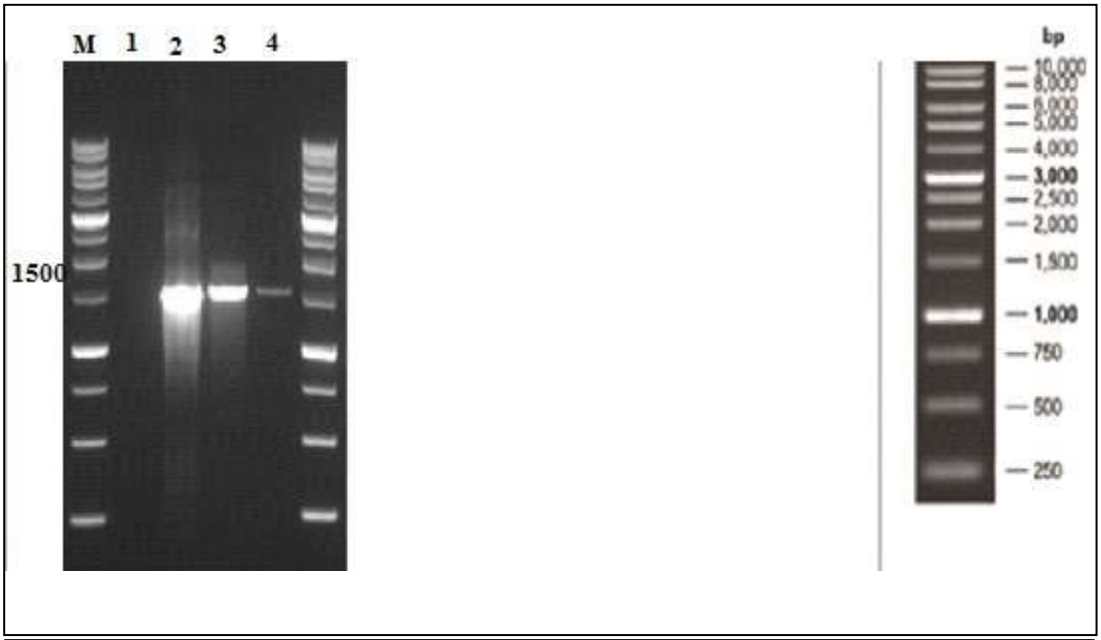


**Figure 2**: Image of agarose-gel electrophoresis of the *16S rRNA* gene of *Actinomycetes*. M: 1kb ladder; lane 1: No DNA control ($H_2O$); lane 2: *E. coli* positive control; lane 3: *Streptomyces* sp. QZS11; lane 4: *Nocardia* sp. QZS9.

Since many members of *Streptomyces* are beneficial bacteria, the toxicity and antioxidant characteristics of the extract of the isolated QZS11 were evaluated using the Vero cell viability assay and the diphenyl picryl hydrazine (DPPH) reduction assay, respectively. The results showed that the viability rates of the Vero cells were 100% at 0.125mg/ml and 90% at 0.25mg/ml (Figure 3).

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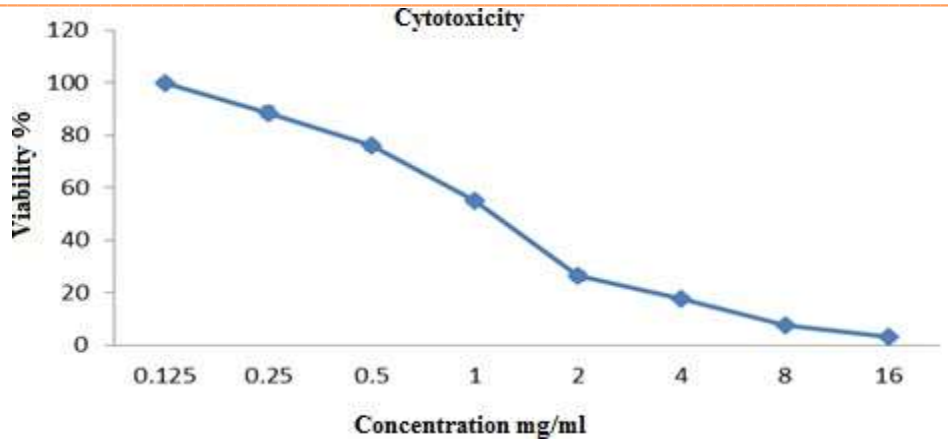


**Figure 3**: Vero cell based cytotoxicity of the ethyl acetate crude extract of *Streptomyces* sp. QZS11. Each value represents triplicate (Mean±SD).

The findings regarding the DPPH assay revealed an important antioxidant level (20%) at 0.25mg/ml (Figure 4).

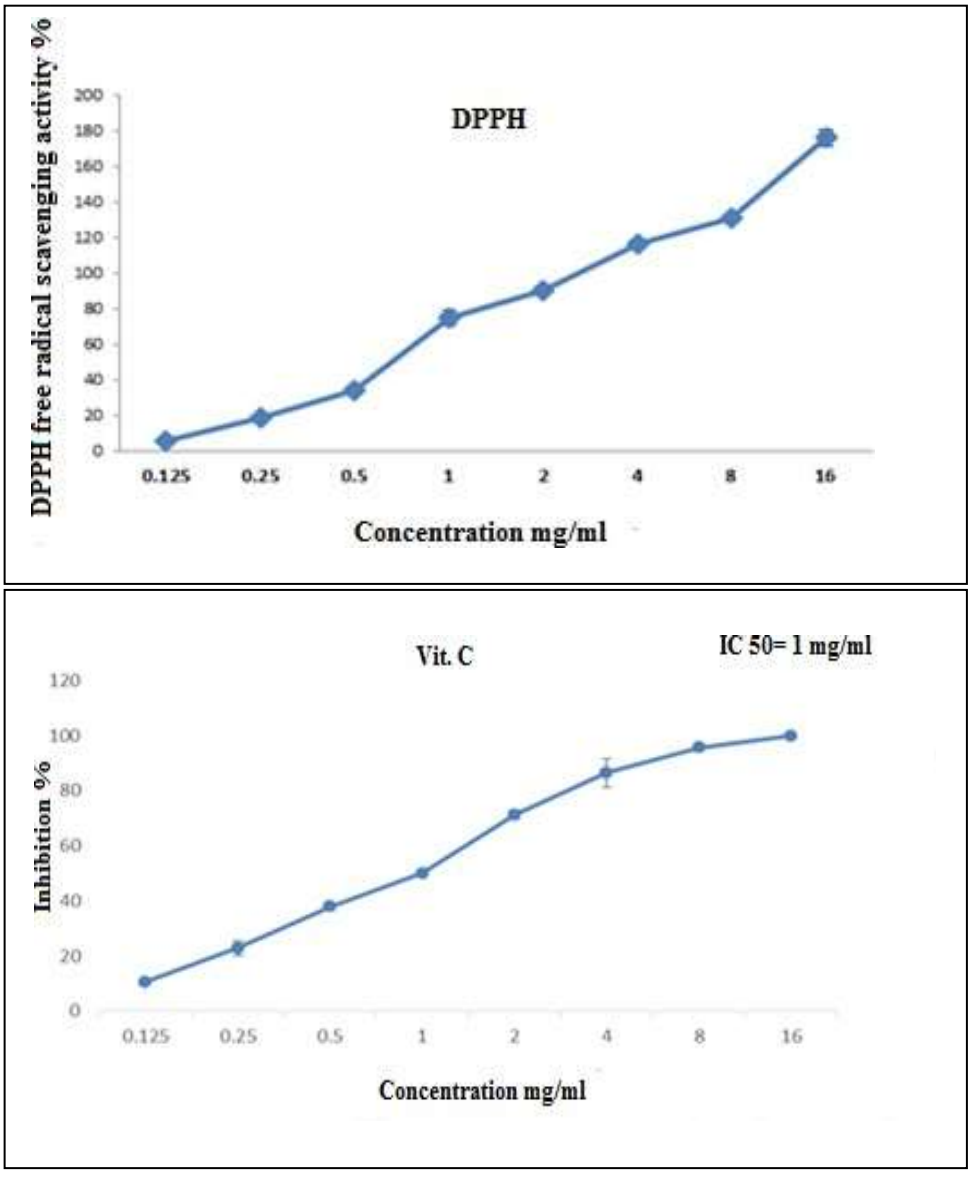


**Figure 4**: DPPH-based scavenging activity of the ethyl acetate crude extract of *Streptomyces* sp. QZS11. Each value represents triplicate (Mean±SD).

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**Discussion**

The current study revealed the presence of two genera of *Actinomycetes* in the soil samples collected from Al-Diwaniyah City, Iraq. These isolates are *Streptomyces* sp. QZS11 and *Nocardia* sp. QZS9. The findings agree with those by Sapkota et al (14), who reported that the soil samples they collected showed that about 71% of their soil samples had *Streptomyces* spp. and 19.5% had *Nocardia* spp. Moreover, the authors (14) revealed that 43.34% of the isolates had antimicrobial activity against different species of bacteria, *Staphylococcus aureus*, *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, which was demonstrated by using the ethyl-acetate based extract of their *Actinomycete* isolates via the use of an agar well diffusion method.

Due to the problem of antibiotic resistance by bacterial and fungal pathogens, researchers around the world work continuously to find novel natural compounds that can overcome this resistance issue, and that *Actinomycete* isolates represent the best suppliers for such compounds. Singh et al (15) found that soil samples from some areas in India showed the presence of different species that belong to *Actinomycetes*, in which they used growth and biochemical tests to identify these isolates and deeply identified these isolates by the use of the *16S rRNA* gene sequencing. Their (15) samples included the presence of *Streptomyces xanthophaeus*, *S. variabilis*, *S. xanthochromogenes*, and *S. levis*. They isolated some antibacterial and antifungal substances from the soil isolates of *Actinomycetes* as were effective in inhibiting the growth of some bacterial and fungal pathogens, such as *S. aureus*, *E. coli*, *Candida albicans*, *C. tropicalis*, and *Trichophyton rubrum*. *Shrestha* et al (16) isolated 9 (22) *Nocardia* and 7 (22) *Streptomyces* from soil samples from some regions in Nepal and tested their crude-extract ability to inhibit the growth of some bacterial pathogens.

Sudha and Masilamani (17) reported that marine sediment isolates of *Streptomyces* crude extract revealed 64.5µg and 250µg of IC50 against Hep-2 cell line and VERO cell line, respectively, which were, as they declared, very close to the crude-extract cytotoxicity criteria, IC50<30 µg/ml, of the American National Cancer Institute (NCI). The present study showed antioxidant activity of the crude extract of the *Streptomycete* isolate as revealed by the DPPH reduction assay. The finding agrees with those by Tan et al (18), who demonstrated that soil samples from some areas in Malaysia showed the presence of *Streptomyces* sp., which revealed strong antioxidant actions via the utilization of the *Streptomyces* crude extract in a DPPH reduction assay.

**Conclusion**

The study reports the isolation of two *Actinomycetes*; *Streptomyces* sp. (QZS11) and *Nocardia* sp. (QZS9), with low toxicity and important antioxidant level at a very low concentration for the QZS11 isolate.

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