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RESEARCH PAPER

Antibacterial Activity of Reduced Graphene Oxide Nanoparticles Synthesized by *Klebsiella Oxytoca*

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ABSTRACT

The biological activity was studied using biogenic reduced graphene oxide nanocomposites (RGO) that combine some unique properties such as high surface area, conductivity, with biocompatibility, making them ideal for many medical and biological applications. The RGO nanocomposite were prepared from *Klebsiella oxytoca (K. oxytoca*) against the commonly occurring pathogenic bacterium *Staphylococcus aureus* (*S. aureus*) isolated from diabetic foot infections. The antibacterial activity of reduced graphene oxide nanocomposites against *S. aureus* was investigated at different concentrations. The synthesized nanoparticles were examined for antibacterial activity with 125, 250, 500, and 1000 µg/ml concentrations against pathogenic *S. aureus*. Reduced graphene oxide nanocomposites with these concentrations showed antioxidant activity by scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. The inhibition percentage in the DPPH-reduced graphene oxide mixture was 29.42% at 125 µg/ml, 37.27% at 250 µg/ml, 40.76% at 500 µg/ml, and 54.06% at 1000 µg/ml. Reduced graphene oxide nanocomposites did not show any hemolysis for the tested blood. Additionally, biogenic reduced graphene oxide nanocomposites did not cause degradation of human DNA at the concentrations used in the current study.

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INTRODUCTION

Klebsiella oxytoca, a Gram-negative bacterium, has gained substantial consideration in recent years for its role in the fabrication of nanoparticles. As a facultative anaerobe with varied metabolic competencies, *K. oxytoca* can reduce metal ions and yield nanoparticles using their enzymatic action and secreted biomolecules. This green fabrication method offers a sustainable and eco-friendly substitute for traditional chemical approaches [1]. Notably, molecular research has highlighted the genetic versatility of *K. oxytoca*, counting the recognition of resistance genes [2]. Graphene is a two-dimensional allotrope of carbon composed of layers formed by single carbon atoms. In graphene, these carbon atoms demonstrate sp^2 hybridization within a two-dimensional hexagonal crystal lattice, connected by σ and π bonds. Research into the theoretical aspects of graphene commenced well before the material itself was physically produced. In 1947, Wallace, a Canadian theoretical physicist, was the pioneer in exploring

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graphene theory. Subsequently, in 2004, Jim, a Dutch-British physicist, and Novoselov, a Russian-British physicist, successfully synthesized the first graphene samples, leading to their receipt of the Nobel Prize in 2010[3]. Graphene exhibits several outstanding characteristics, such as excellent conductivity, a vast surface area, and strong mechanical and thermal stability. These attributes render graphene a valuable material for numerous uses, including fuel cell catalysis, supercapacitors, photocatalysis, heterogeneous catalysis, water purification, and applications in pharmaceuticals and biosensing [4]. Graphene oxide (GO) is a material that holds significant potential for various graphene-based applications across fields such as electronics, optics, chemistry, energy storage, and biology [5].

Nanotechnology studies the design of nanometer-sized materials with chemical and physical properties different from macroscopic materials [6]. Nanoparticles have been employed in many biological and medical applications, especially in addressing challenges of bacterial growth [7] and biofilm-related infections [8]. Developments in nanoparticle fabrication, driven by artificial intelligence (AI), have improved their properties and antimicrobial capabilities, allowing for precise targeting and minimized toxicity [9]. Magnetite (Fe_3O_4) is one of the most studied magnetic nanoparticles (NPs) due to its low cost, easy surface modification, and high magnetization levels that enable these particles to be used in many diverse applications [10,11]. Graphene and its derivatives, particularly graphene oxide (GO), have demonstrated significant effectiveness in stabilizing Fe_3O_4 nanoparticles (NPs) due to their layered structure, which provides a substantial surface area and enhances the reactivity of their functional groups. NPs that are immobilized by GO not only prevent aggregation but also preserve their inherent properties through a synergistic interaction between the two components. Furthermore, the creation of graphene-based antimicrobial nanocomposites has emerged as a key research focus, which can be classified into three categories: graphene–metal nanocomposites, graphene–metal oxide nanocomposites, and graphene–polymer nanocomposites. Recently, there has been increased interest in the development of graphene–metal nanocomposites because metal cations can bind directly to the oxygen groups on the surface of GO via electrostatic forces. Nanocomposites of stable metal-decorated graphene, such as GO-Ag, with antibacterial characteristics are formed when this interaction causes surface deoxygenation of the GO nanosheets. In order to achieve pure, biocompatible materials with antibacterial activity, a large financial investment is necessary for the extra processing of GO, which includes reduction, surface activation, and composite synthesis. If we compare GO to reduced graphene oxide (RGO), we see that GO is more stable in water and has better bactericidal action [12-17]. Different approaches were available for the synthesis of graphene oxide nanoparticles. Graphene is generally produced by Chemical Vapor Deposition (CVD) [18], chemical reduction of graphene oxide or thermal treatment of graphene oxide [19], and mechanical exfoliation of graphite with sonication in the presence of solvent or chemical /biological surfactant [20].

The current study emphasizes the biosynthesis of reduced graphene oxide (RGO) nanoparticles via the bacterium *Klebsiella oxytoca*. The fabricated RGO nanoparticle properties were characterized by employing advanced analytical techniques such as Field Emission Scanning Electron Microscopy (FESEM), Energy Dispersive X-ray Spectroscopy (EDX), Atomic Force Microscopy (AFM), X-ray Diffraction (XRD), and Fourier Transform Infrared Spectroscopy (FT-IR) to approve their structural and chemical properties. The antibacterial activity of RGO nanoparticles was assessed in contradiction of *Staphylococcus aureus* isolates from diabetic foot infections using the agar well diffusion method. Additionally, the antioxidant potential of RGO nanoparticles was assessed using the DPPH radical scavenging assay, and their biocompatibility was tested through hemolysis analysis. This method not only highlights the potential of *K. oxytoca* as a sustainable and eco-friendly alternative for nanoparticle synthesis but also explores the multifunctional applications of RGO nanoparticles in antimicrobial and biomedical fields.

MATERIALS AND METHODS

Isolation and Identification of pathogenic bacteria from diabetic foot

Isolation and diagnosis of *Staphylococcus aureus* bacteria were performed by collecting 100 samples from diabetic patients for this study. The identification of the bacteria was performed using their morphological features, specific

characteristics, biochemical assays, and the VITEK2 system.

Preparation of Reduced graphene oxide nanoparticle by K. oxytoca

Different types of bacteria were screened to test their ability to synthesize reduced graphene oxide nanoparticles. The efficient bacterium, *Klebsiella oxytoca*, was chosen for nanoparticle synthesis. *K. oxytoca* was cultivated in brain heart infusion broth for 24 hours at 37°C in a shaking incubator at 150 rpm. The colloidal suspension was centrifuged for 15 minutes at 10,000 rpm to separate the precipitate. Graphene oxide was then added to the supernatant in a ratio of 1:1 of *K. oxytoca* at a concentration of 2.4 mg/ml to prepare graphene oxide nanoparticles. The mixture was incubated at 37°C for 48 hours at 150 rpm and centrifuged for 15 minutes at 10,000 rpm.

Characterization of reduced graphene oxide

The RGO NPs were characterized using several analyses, including FESEM, EDX, AFM, XRD, and FT-IR.

Antibacterial activity of RGO against S.aureus isolated from diabetic foot

The antibacterial activities of RGO NPs were tested using Agar well diffusion method against *S aureus* bacteria isolated from diabetic foot. A dipping cotton swab was used to streak the entire surface of a Mueller Hinton agar tray. Then, using a sterile cork borer, pores (7 mm diameter) were created and filled with RGO NPs (100ul) in four concentrations (1000, 500, 250 and 125 µg/ ml), and the four wells were filled with distal water as a control. The Petri dishes were then incubated at 37°C for 24 hours. The diameter of the growth inhibition zones in millimeters was measured to determine antimicrobial activity [21,31].

The antioxidant activity of RGO against S. aureus isolated from diabetic foot

Antioxidant activity evaluation used an offline (DPPH) assay. The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical cation technique was adapted to assess the ability of one hundred pure chemical compounds to scavenge free radicals. The DPPH reagent was DPPH 200 µl as a control in the first well of the microplate. In a 96-well microplate, 100 µL DPPH reagent was mixed with 100 µL of the sample (RGO NPs at different concentrations of 1000, 500, 250, and 125 µg/ml) and incubated in the dark at room temperature for 30 min to measure scavenging activity. The absorbance was determined at 514 nm using an ELISA reader (TECAN, Grading, Austria). After incubation, 100 percent methanol was used as a blank [22]. The following formula was used to calculate the DPPH scavenging effect:

Radical scavenging (%) = [(A)control − (A) sample/ (A)control] × 100.

The antihemolytic activity of RGO against S. aureus isolated from diabetic foot

On the blood of one healthy donor, haemolysis experiments were performed. The haemolysis was identified using Triton X-100 as a positive control indicator. A sterilized phosphate-buffered saline solution was employed as a negative control, allowing the stock solution to be stored at room temperature on a shaking plate for 2-4

Table 1. Bacteria isolated types from diabetic foot.

	Bacteria isolated	Number of isolates
1	*P. aeruginosa*	15
2	*E. coli*	18
3	*Klebsiella spp*	12
4	*S. aureus*	21
5	*Protus spp*	10
6	*Klebsiella pneumonia*	9
7	*Protus mirabilus*	7
8	*No growth*	8
	Total	100

hours. After the incubation period, the sample was centrifuged at 10,000 rpm. In a 96-well plate, the supernatant was read using a microplate scanning spectrophotometer at 550 nm. RGO NPs throughout all the concentrations (1000, 500, 250, and 125 µg/ ml) did not cause haemolysis in the entire examined blood samples [33].

RESULTS AND DISCUSSION

Isolation and Identification of bacterial pathogen from diabetic foot

Several pathogenic bacterial species were isolated and diagnosed from the feet of diabetic patients, and the diagnosis results were as shown in Table 1. The isolation findings specify that *S. aureus* is the most commonly isolated bacterium from the infections of diabetic foot, accounting for 21% of the isolates. This prevalence indicates the necessity for targeted antibacterial strategies, particularly in diabetic patients who are extremely vulnerable to infections due to weakened immunity [24].

Biosynthesis of RGO NPs by K. oxytoca

K. oxytoca demonstrated the capacity for the extracellular production of GO nanosheets. The change in color of the reaction mixture from a transparent brown to black, accompanied by the formation of a precipitate, serves as an indicator of successful GO biosynthesis [23], as illustrated in the Fig. 1. This visual validation, joint with characterization findings, proves the ability of *K. oxytoca* to perform as a green and eco-friendly biosynthesis agent for RGO.

Fig. 1. Change color of supernatant.

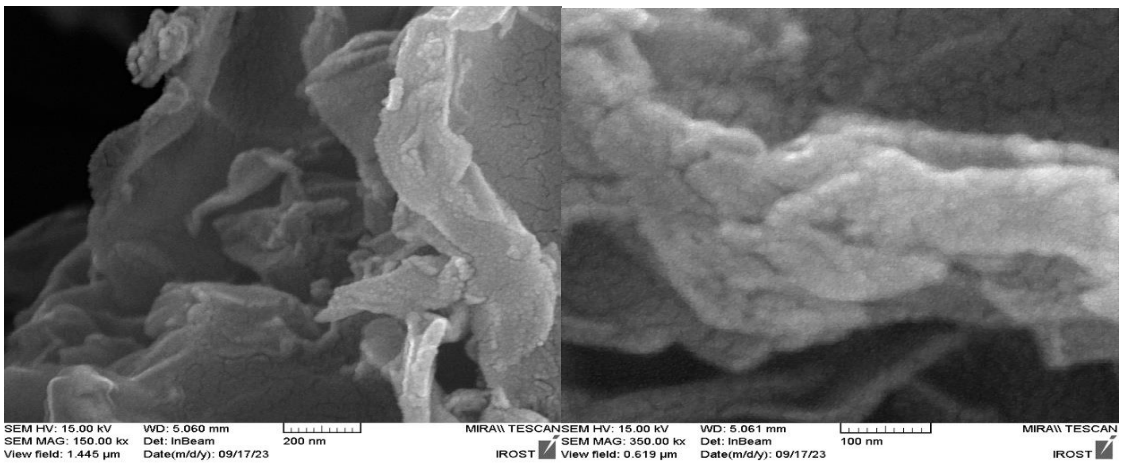

Fig. 2. FESEM analysis of RGO NPs biosynthesis by *K. oxytoca.*

Characterization of RGO nanoparticles

The characteristics of reduced Graphene Oxide (RGO) nanosheets produced by *K. oxytoca* were assessed using FESEM, EDS, AFM, FTIR, and XRD techniques. The RGO derived from *K. oxytoca* is regarded as exceptional and environmentally friendly. The employed analytical methods confirm the successful reduction of graphene oxide to RGO. A noticeable decrease in oxygen functional groups was observed (FTIR), and the nanosheets exhibited a thin, wrinkled appearance (FESEM), aligning with findings from earlier research [19, 25]. These findings confirm the eco-friendly method of using *K. oxytoca* for nanoparticle synthesis. In RGO NPs the nanomaterial displayed a thin and wrinkled texture which was caused by the stacking of individual sheets by various self-assembly techniques. Nanoscale surface modification allows accurate engineering of materials through tailored properties, enhancing biomedical applications such as drug delivery and tissue engineering [26]. The images revealed that the RGO material consists of individual sheets closely associated with each other, with the RGO size range between (35-85 nm) and 49.31 nm as the average diameter as shown in Fig. 2. The RGO sample's chemical composition was examined through energy dispersive X-ray spectroscopy (EDX). The analysis involved evaluating the optical absorption peaks related to carbon and oxygen to determine their concentrations on the surface of the sample. The reduction of graphene oxide (GO) was indicated by the weight percentages of carbon and oxygen, as illustrated in Fig. 3. Analysis using atomic force microscopy (AFM) revealed information about the exterior morphology, surface roughness, and average diameter of RGO nanosheets synthesized by *K. oxytoca*. So, the size and form of the final structures can be affected by the engraving duration and current density. The average diameter of the GO nanosheets synthesized by *K. oxytoca* was found to be 45.26 nm. Additionally, three-dimensional images and distribution charts of the granularity accumulation

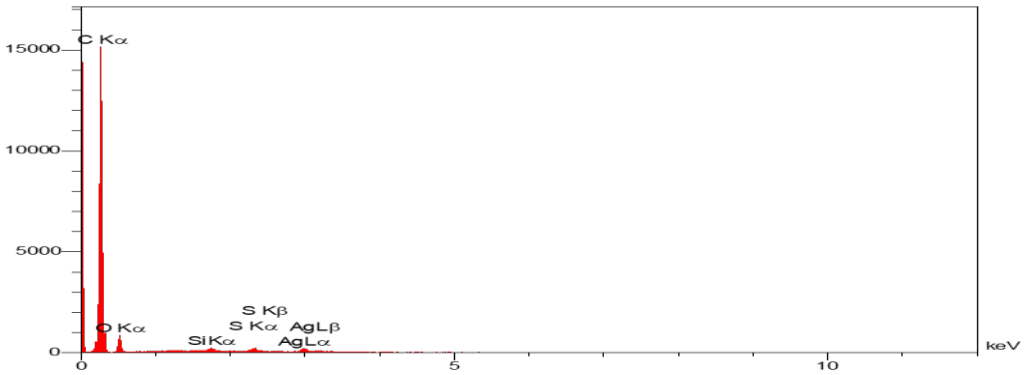

Fig. 3. EDX analysis of RGO NPs biosynthesis by *K. oxytoca.*

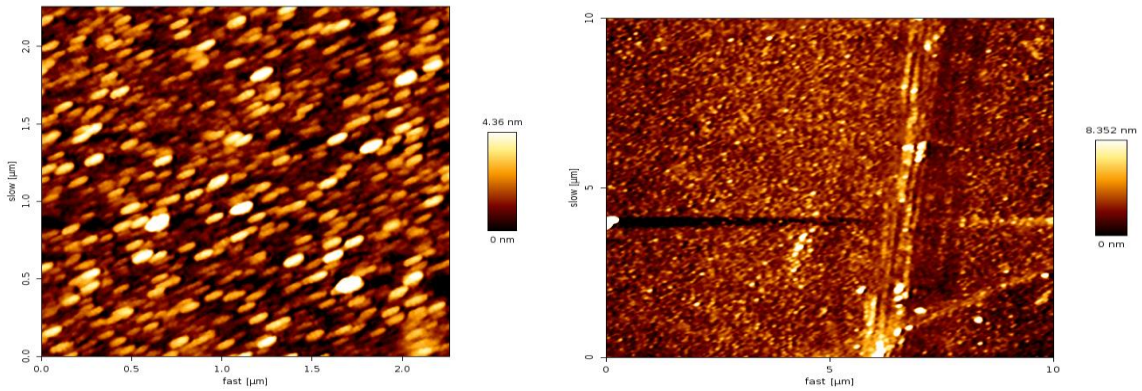

Fig. 4. AFM analysis of RGO NPs biosynthesis by *K. oxytoca.*

of RGO nanosheets are presented in Fig. 4.

To investigate the crystalline structure and interlayer spacing of reduced graphene oxide (RGO), X-ray diffraction (XRD) was used, confirming the conversion from graphene oxide (GO) to RGO by *K. oxytoca*. The analysis showed a broad diffraction peak for RGO at 2θ = 24.5°, indicating interlayer distances of 0.30 nm. This peak signifies RGO's well-ordered crystalline structure and suggests effective removal of oxygen functional groups and water molecules during the reduction of GO nanosheets, illustrated in Fig. 5.

FTIR is a method employed to analyze the bonding characteristics of various types of oxygen. The FTIR spectroscopy examination of RGO synthesized using *K. oxytoca* isolates, conducted within a wavelength range of 400 to 3800 cm^{-1}, revealed a significant reduction in the distinctive peaks related to oxygen functional groups. Notably, the peaks corresponding to hydroxyl and alkoxy groups were entirely absent, as illustrated in Fig. 6. The absorption peaks detected were 2918.30 cm−1; C–H , 1627.92 cm−1; C=O, 1469.76 cm−1; C=C, 1053.13 cm−1; C–O, These results indicate a significant reduction of oxygen functional groups from GO nanosheets [17].

Antibacterial of ORG NPs

RGO exhibited clear inhibitory activity against *S. aureus* isolated from the feet of diabetic patients at all studied concentrations. The inhibition increased with increasing concentration as shown in Fig. 7. The high-diameter inhibition zone was 36 mm at a concentration of 1000 µg/ml, while the low-diameter inhibition zone was 15 mm at a concentration of 125 µg/ml as shown in Table 2. The antibacterial activity findings reveal that RGO inhibits *S. aureus* growth successfully, with inhibition growing proportionally to concentration. This proposes that RGO is probably an effective

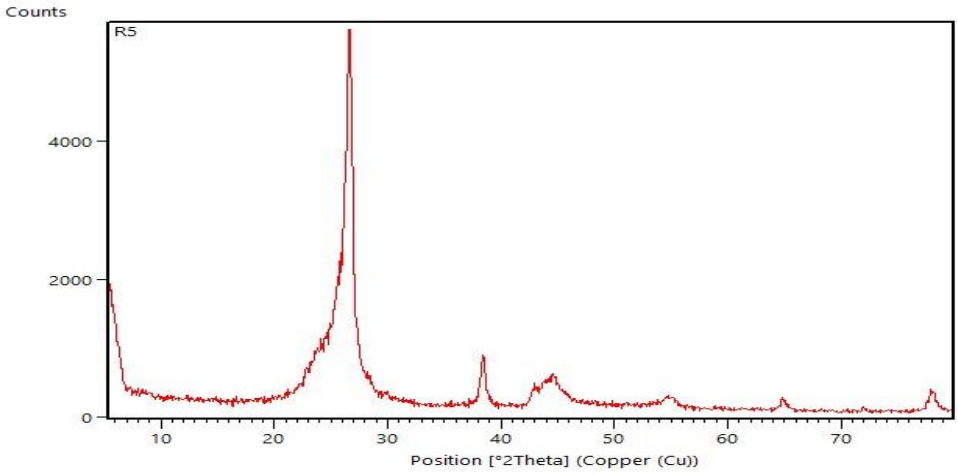

Fig. 5. XRD analysis of RGO NPs biosynthesis by *K. oxytoca.*

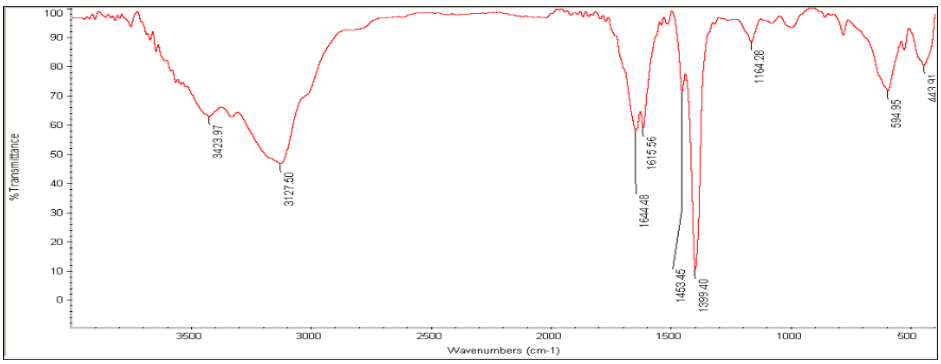

Fig. 6. FTIR analysis of RGO NPs biosynthesis by *K. oxytoca*.

antibacterial agent [27], predominantly for treating infections related to diabetic foot ulcers.

Antioxidant of ORG NPs

RGO showed the removal of free radicals (DPPH) at all concentrations studied, and the inhibition increased with increasing concentration (Table 3). The inhibition titer in the mixture of DPPH with reduced graphene oxide was 29.42% at 125 µg/ml, 37.27% at 250 µg/ml, 40.76% at 500 µg/ml, and 54.06% at 1000 µg/ml. RGO showed the removal of free radicals (DPPH) at all concentrations studied, and the inhibition increased with the increasing concentration. The antioxidant assay revealed a substantial growth in scavenging activity with higher RGO concentrations. This is credited to the plentiful functional groups capable of neutralizing free radicals [28, 29]. These results highlight the potential application of RGO in mitigating oxidative stress-related conditions. Nanotechnology integration amplifies their functionality, driving innovation in biomedical research, including studying bacterial and cell antioxidants and chemotaxis. [34, 35].

Antihemolytic of ORG NPs

The ability of RGO to degrade blood was tested, and no hemolysis appeared at all concentrations studied (Table 4). The antihemolytic activity findings reveal that RGO is biocompatible at all

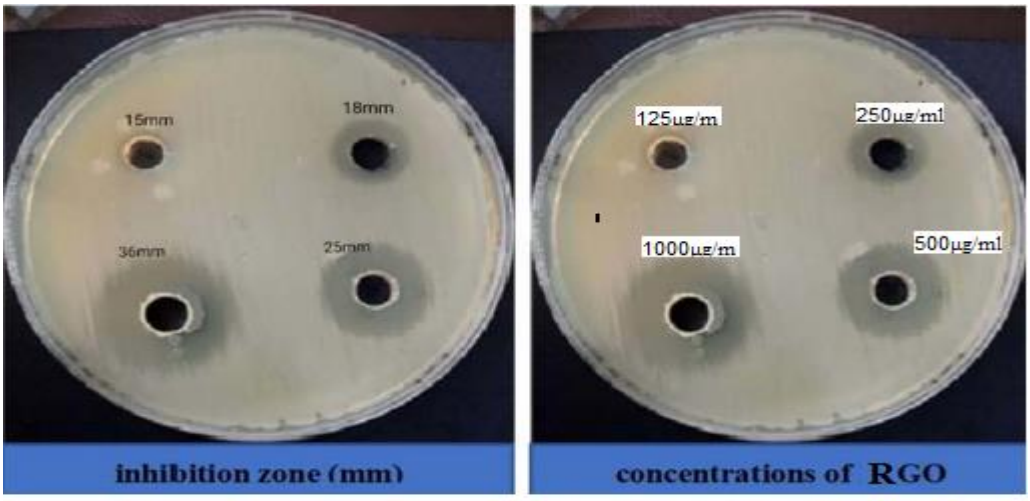

Fig. 7. Antibacterial of RGO NPs biosynthesis by *K. oxytoca.*

Table 2. Antibacterial Efficacy of RGO NPs Against S. aureus bacteria.

RGO nano µg/ml	*S.aureus*
Con.1000	36mm
Con.500	25 mm
Con.250	18 mm
Con.125	15 mm

Table 3. Antioxidant of ORG NPs.

no	concentration	scavenging %
1	125 µg/ml	29%
2	250 µg/m	37%
3	500 µg/m	41%
4	1000 µg/m	54%

Table 4. Antihemolytic of ORG NPs.

concentration (µg/ ml)	test result	hemolysis %
1000	0.751	0
500	0.675	0
250	0.55	0
POSITIVE	3.385	100
NEGATIVE	0	0

verified concentrations, and display no hemolysis. This result is critical for biomedical applications, demonstrating that RGO can be securely used in therapeutic contexts without causing hostile effects on red blood cells [30, 31].

CONCLUSION

This report effectively validates the biosynthesis of reduced graphene oxide nanoparticles (RGO) using *Klebsiella oxytoca*, showcasing an eco-friendly and sustainable method. The RGO nanoparticles displayed effective antibacterial activity against *S. aureus* isolates from diabetic foot infections, ROG nanocomposites showed strong and clear activity against *S. aureus* in different concentrations (125, 250, 500, 1000 µg/ ml). In addition to their potential role, they have substantial antioxidant properties and outstanding biocompatibility. These results highlight the potential applications of RGO in antimicrobial and biomedical fields, paving the way for further investigation in clinical and therapeutic settings.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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