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Original Research Article

Antimicrobial Efficacy of Biogenic Manganese Oxide Nanoparticles Against Klebsiella pneumonia: A Novel Approach to Combat Antibiotic Resistance

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Abstract: *The rise of multidrug-resistant (MDR) bacteria has emerged as a critical global health concern, significantly impeding effective infection management. Conventional antibiotics are increasingly failing against pathogens such as Klebsiella pneumoniae, which is notorious for its resistance and prevalence in nosocomial infections. This has necessitated the exploration of alternative antimicrobial strategies.* ***Objective:*** *To evaluate the antibacterial, antibiofilm, and cytotoxic effects of biogenically synthesized manganese oxide nanoparticles (MnO NPs) against multidrug-resistant Klebsiella pneumoniae.* ***Methods:*** *This experimental study involved the biosynthesis of manganese oxide nanoparticles followed by their evaluation against Klebsiella pneumoniae. Antibacterial activity was assessed using the agar well diffusion assay. Minimum inhibitory concentration (MIC) was determined via the broth dilution method. Antibiofilm activity was quantified using the crystal violet microtiter plate assay. The effect of sub-inhibitory concentrations of MnO NPs on extracellular polymeric substances (EPS) was also studied. Cytotoxicity was evaluated using the neutral red uptake assay and morphological analysis of HepG2 human liver cell lines.* ***Results:*** *Manganese oxide nanoparticles demonstrated notable antibacterial activity with clear zones of inhibition in agar diffusion assays. MIC testing revealed significant growth inhibition of K. pneumoniae at defined nanoparticle concentrations. MnO NPs also significantly reduced biofilm formation and disrupted EPS production at sub-MIC levels. Cytotoxicity assays indicated that MnO NPs exhibited minimal toxic effects on HepG2 cells at effective antimicrobial concentrations, suggesting good biocompatibility.* ***Conclusion:*** *Biogenically synthesized manganese oxide nanoparticles possess potent antibacterial and antibiofilm activity against Klebsiella pneumoniae, with minimal cytotoxicity to human liver cells. These findings suggest that MnO NPs may offer a promising alternative therapeutic approach to combat multidrug-resistant bacterial infections.*

Keywords: Drug Resistance, Bacterial Nanoparticles, Manganese Compounds, Klebsiella pneumoniae Biofilms

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Introduction

The emergence of bacterial resistance to antibiotics has become a significant global health concern (1). The overuse and misuse of antibiotics have accelerated the development of resistance, making it challenging to treat bacterial infections (1). Klebsiella pneumoniae, a Gram-negative bacterium, is a major nosocomial pathogen that has gained public interest due to its alarming multidrug resistance worldwide (2) (3). This bacterium is known for its biofilm-forming ability and various virulence factors, which contribute to its evasion of the immune system and antibiotic resistance (4).

The increasing prevalence of antibiotic resistance has led to a search for alternative therapeutic options (5). Nanotechnology has emerged as a promising solution, with nanoparticles showing potential as antimicrobial agents. Metal-based nanoparticles, in particular, have demonstrated effectiveness against antibiotic-resistant bacteria (6). The mechanism of action of these nanoparticles differs from traditional antibiotics, and they can target various biomolecules, reducing the likelihood of resistance development (7). Manganese oxide nanoparticles, with their unique physical and chemical properties, have shown promise as antimicrobial agents.

Manganese oxide nanoparticles have been found to exhibit antimicrobial activity against a range of bacteria, including Klebsiella pneumoniae. Their mechanism of action involves the generation of highly reactive species that damage bacterial cell membranes and walls (8) (9) (10). Additionally, manganese oxide nanoparticles have been shown to have antioxidant properties, which could be beneficial in reducing oxidative stress in various diseases (11) (12). The use of manganese oxide nanoparticles as an alternative therapeutic option for treating bacterial infections is an area of ongoing research (13).

This study aims to evaluate the antimicrobial and antibiofilm activities of manganese oxide nanoparticles against clinical isolates of Klebsiella pneumoniae (14, 15). The study will also assess the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of manganese oxide nanoparticles, as well as their antioxidant potential and cytotoxic effects against HepG2 cell lines (16). The findings of this study may provide valuable insights into the potential use of manganese oxide nanoparticles as an alternative therapeutic option for treating Klebsiella pneumoniae infections (17).

Methodology

Sub-Culturing and Biochemical Characterization of Bacterial Isolates

Three clinical isolates of MDR Klebsiella pneumoniae obtained from the Department of Microbiology, Government College University, Faisalabad, were used in the present study. These isolates were categorized as Klebsiella pneumoniae A, Klebsiella pneumoniae B, and Klebsiella pneumoniae C. The bacterial culture was stabilized in LB (Luria-Bertani) broth and agar (Merck, Germany). Gram staining of

bacterial isolates was performed using the method of Becerra et al. (2016). When a primary stain is fixed with a mordant, decolorized, and then counterstained, bacteria do not retain the primary stain. Instead, they are counterstained, indicating that they are Gram-negative bacteria. First of all, a drop of water was added to the clean glass slide. A loopful of colony from the agar plate was mixed in a drop of water. The inoculating loop was moved towards the outer edges of the slide in a circular motion to create a thin smear. The smear was air-dried. Then the smear was heat fixed by gently moving the slide over the flame. The crystal violet dye was used on the smear for one minute before the slide was washed with tap water. After rinsing, the Gram's iodine was applied for one minute. The slide was again rinsed with tap water. The smear was decolorized by covering the smear with acetone for ten seconds. At last, the slide was washed and the smear was covered with a counterstain called safranin for one minute. The slide was washed, dried, and observed under an oil immersion lens of a light microscope by adding a drop of cedar wood oil on the smear. All three isolates were swabbed on MacConkey agar plates. Biochemical tests were performed to confirm the identification of different bacterial isolates.

Table 1. Biochemical tests for the identification of bacteria.

Catalase	Positive
Coagulase	Negative
Oxidase	Negative
Citarate	Positive
Indole	Negative
Methyl Red	Negative
Voges Proskauer	Positive

Set up of MacFarland Turbidity Standards

1% H_2SO_4 (sulphuric acid) and 1.175% $BaCl_2.2H_2O$ (barium chloride dihydrate) were mixed in two different volumes to establish McFarland turbidity standards (Chapin & Lauderdale, 2003).

Table 2. Set up of McFarland Turbidity Standards

McFarland Standard No.	Volume (ml)		No. of bacteria/ml (10^8) represented
	$BaCl_2.2H_2O$ (1.175%)	H_2SO_4 (1%)	
0.5	0.05	9.95	1.5
1	0.1	9.90	3
2	0.2	9.80	6
3	0.3	9.70	9
4	0.4	9.60	12
5	0.5	9.50	15

Preparation of Bacterial Culture

For preparing the bacterial inoculum, bacteria were freshly grown on nutrient agar. The isolated colonies were added to the sterile Muller-Hinton broth to make a uniform suspension. Then these broth tubes were kept in an incubator at 37°C for 24 hours. After 24 hours of incubation, the turbidity of bacteria was adjusted according to 0.5 McFarland standards (Unnisa et al., 2012). After preparation of the suspensions, they were instantly used.

Synthesis of Manganese Oxide Nanoparticles (MnO NPs)

MnO NPs were prepared by a biogenic method (Green synthesis), adopting the method of (Chatterjee et al., 2017) with some alterations. Leaf extracts of Brassica were used to synthesize the MnO NPs.

Protocol

Leafs of Brassica were rinsed in distilled water and dried until all of the water molecules were evaporated. In 50 mL of distilled water, 8.0 g of finely chopped Brassica was added. Then the mixture was boiled in a microwave oven at 100°C for 5 minutes. The solution was filtered and diluted to 10 percent for future usage. After adjusting the pH of Brassica to 6.0, $KMnO_4$ crystals (0.2 M concentration) were added to it. This solution was mixed for four hours, yielding a brown suspension that was then sonicated to generate a homogeneous solution. The amorphous microparticles were obtained after centrifuging and drying the resulting solution at 100°C overnight. Finally, to acquire fine powder, the product was ground and later used for characterization.

Characterization of Manganese Oxide Nanoparticles

Metallic nanoparticles were characterized by EDX (Energy dispersive X-ray spectroscopy) and SEM (Scanning electron microscopy).

Figure 1. Leaves of Brassica

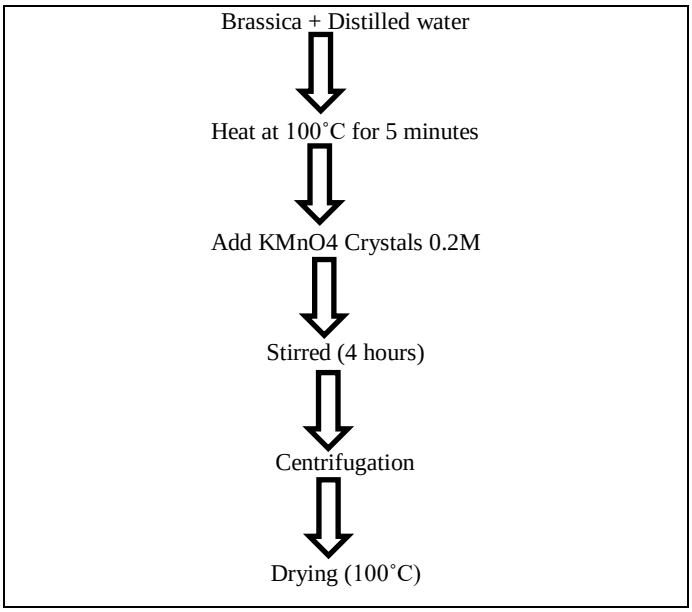

Figure 2. Steps involved in the synthesis of MnO NPs.

Assessment of Antibacterial Activity of Manganese Oxide Nanoparticles by Agar Well Diffusion Assay

Antibacterial potential was checked by the use of agar well diffusion assay following the protocol of Manyasree et al. (2018). Bacterial suspensions of all three isolates were made whose turbidity was equal to 0.5 McFarland standard. The bacterial suspensions were then swabbed onto Muller-Hinton agar plates. Then, on swabbed agar plates, 5 mm wells were made. In 100% dimethyl sulfoxide, different quantities of MnO nanoparticles (25, 50, 75, and 100µg/ml) were prepared (DMSO). DMSO was used as a control. 100µl of nanoparticle concentration was distributed into the wells. The plates were incubated for 24 hours at 37°C. The widths of zones of inhibition against all microorganisms were measured to assess antibacterial efficacy—determination of Minimum Inhibitory Concentration (MIC) of Manganese Oxide Nanoparticles. The Minimum Inhibitory Concentration (MIC) is defined as the minimum concentration of an antibacterial agent that decreases the growth rate of bacteria. MIC of MnO nanoparticles was calculated by following the broth microdilution method by Alekish et al. (2018). The overnight cultures of bacterial isolates were prepared in normal saline, whose turbidity was compared with a 0.5 MacFarland standard. A 1500 µg/ml stock solution of manganese oxide nanoparticles was prepared in DMSO.

100 µl of Muller Hinton broth was added to all 12 wells of 96 96-well microtitration plate. 100 µl of the stock solution of nanoparticles was added to the first well. The nanoparticles were serially diluted in 2-fold increments up to the 10th well. Then 100 µl of bacterial suspension was dispensed into the 1st to 10th wells and then into the 12th well. The 11th acted as a positive control as it contained only broth. The 12th well acted as a negative control as it contained broth and bacterial suspension. The plate was covered with parafilm and kept in an incubator at 37ºC for 24 hours. After incubation for 24 hours, the viability of bacterial cells was evaluated by using a 0.02 % solution of redox nitro-blue tetrazolium chloride (NBT) dye in methanol. The presence of viable cells was determined by the change of yellow-colored dye to blue.

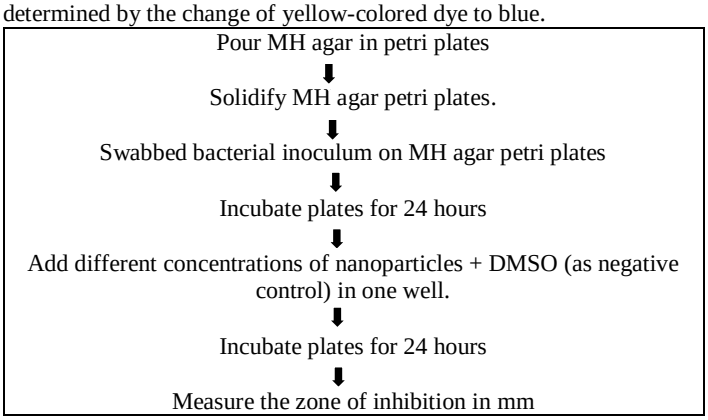

Figure 3. Flow Chart of steps involved in the evaluation of antibacterial activity of MnO nanoparticles

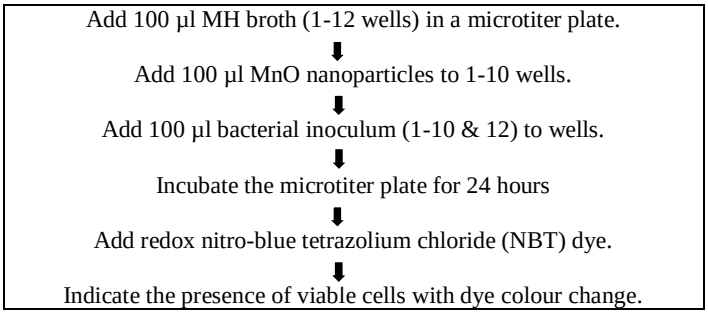

Figure 4. Flow Chart of steps involved in the Minimum inhibitory concentration of manganese oxide nanoparticles

Determination of Minimum Bactericidal Concentration (MBC) of Manganese Oxide Nanoparticles

Minimum bactericidal concentration is the concentration of an antibacterial that completely stops bacterial growth by killing them. MBC of Manganese oxide nanoparticles was evaluated following the method of Alekish et al. (2018) by sub-culturing the broth dilutions from the MIC well and the wells with higher concentrations of nanoparticles than MIC. With a glass spreader, 50 µl of broth from the wells of microtitration plates was distributed on Muller Hinton agar plates. These plates were kept in an incubator for 24 hours at 37°C. MBC was the minimum concentration of nanoparticles that demonstrated no observable growth on plates.

Evaluation of Antioxidant Activity of Manganese Oxide Nanoparticles

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method was used to evaluate the antioxidant activity of MnO NPs following the method of Jacob & Rajiv (2019). The dark violet colour of DPPH solution changed to light yellow in the presence of ascorbic acid and manganese oxide nanoparticles, showing the antioxidant activities of manganese oxide nanoparticles. Manganese oxide nanoparticles and ascorbic acid were synthesized at various concentrations (50-1000 µg/ml). Dissolving 39.4 milligrams of DPPH in 1000 ml of methanol yielded a 0.1 mM DPPH solution. 3 ml of each concentration of nanoparticles and ascorbic acid were added to 2.96 ml of 0.1 mM DPPH solution. For incubation, the mixtures were continually mixed and placed in a dark room for 20 minutes. A spectrophotometer read the absorbance of mixtures at 517 nm. 0.1 mM DPPH acted as a control, and ascorbic acid was used as a standard solution. The scavenging ability of nanoparticles was evaluated by following the formula of Otunola & Afolayan (2018). DPPH scavenging activity (%) = $[(Abs_{control} - Abs_{sample}) / (Abs_{control})] \times 100$

$Abs_{control}$ = (Absorbance of DPPH + ascorbic acid) Abs_{sample} = (Absorbance of DPPH + sample/nanoparticles)

Antibiofilm activity of Manganese Nanoparticles Microtiter plate assays were used to assess the effects of two different sub-inhibitory concentrations (0.5 x MIC) and (1 x MIC) of manganese oxide nanoparticles on biofilm development adopting the method of Shi et al. (2016) with some modification. Biofilm was quantified after staining of attached cells with crystal violet dye. Cells grown in Muller Hinton broth were diluted with fresh broth media to match the turbidity to 0.5 McFarland for preparing a standard inoculum of each isolated bacterium. In a 96-well microtitration plate, 100 µl of bacterial inoculum and 100 µl Muller Hinton broth with manganese oxide nanoparticles were dispensed. After sealing the microtitration plates with parafilm, they were put in an incubator for time intervals of 24 hours at 37°C. The non-adherent cells were removed by discarding the broth and then washing them twice with normal saline. The plates were left to dry for 30 minutes. The adhering cells were fixed with ethanol before being stained with 200 µl of 0.1 percent (v/v) crystal violet dye for 20 minutes. Following staining, the excess dye was washed out of each well three times with 200 µl of sterile normal saline. To quantify the attached cells, 200µl of glacial acetic acid (33% (v/v) was added to each well. The absorbance was measured at 594 nm using a microplate reader. Muller Hinton broth, having only bacteria, was used as positive control, while sterile Muller Hinton broth, having nanoparticles, acted as negative control. The %age of inhibition of biofilm was calculated by the formula: %age inhibition = Control- OD594 of cells treated with MnO NPs/OD594 of non-treated control x 100.

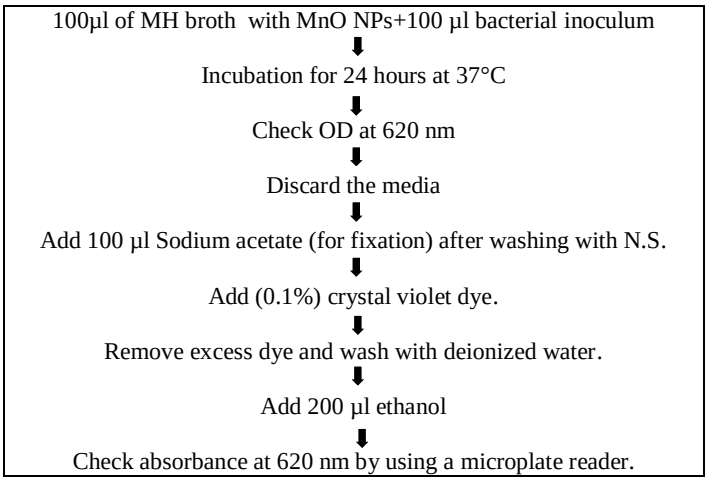

Figure 5. Flow Chart of steps involved in the evaluation of anti-biofilm activity of MnO nanoparticles

Effect of Manganese Oxide Nanoparticles on Established Biofilms

The effect of manganese oxide nanoparticles on preformed biofilms was investigated using the method of Ahmed et al. (2016) with some modifications. Biofilms were created and treated with sub-inhibitory concentrations (0.5 x MIC) and (1 x MIC) of manganese oxide nanoparticles. In a 96-well microtitration plate, 100 µl bacterial inoculum and 100 µl Muller Hinton broth were added according to the method. After sealing the microtitration plates with parafilm, they were kept in an incubator at 37°C for 24 hours. After each interval, unbound cells were removed by washing the wells twice with 200 µL of normal saline. The wells were then treated with manganese oxide nanoparticles at sub-inhibitory concentrations, with untreated wells acting as a control. Microtitration plates were incubated at 37°C for 24 hours. Washing the

cells in normal saline yielded the number of adherent cells. The cells were then stained for 20 minutes with 200 µL of 0.1 percent (v/v) crystal violet dye.

Excess dye was washed out of the wells three times with 200 µL of sterile normal saline after staining. The absorbance was measured at 594 nm after eluting the adherent cells with 200 µL of 33 percent (v/v) glacial acetic acid. A sterile Muller Hinton broth containing zinc oxide nanoparticles served as a positive control, whereas a sterile Muller Hinton broth containing solely bacterial isolates served as a negative control.

Effect of Manganese Oxide Nanoparticles on Extracellular Polymeric Substance (EPS) Production

Extraction of EPS

To check the production of EPS by Klebsiella pneumonae, Muller-Hinton broth medium was used. Overnight cultures of tested bacterial isolates were prepared and diluted to make a final concentration of around 0.5 McFarland (1.5×10^{8}CFU/ml). The standardised inoculum was distributed into flasks containing 100ml of media with sub-inhibitory doses of metallic nanoparticles in relation to each isolate. The medium without nanoparticles was used as a control. For 24 hours, the flasks were put in a shaking incubator at 37°C. After incubation, flasks were removed and EPS was obtained according to the procedure of De Vuyst et al. (1998). To extract EPS, at 4°C, bacterial cultures were centrifuged at 10,000 rpm for 15 minutes. After centrifugation, the supernatant was removed in a separate flask and kept in a refrigerator overnight. The precipitation of EPS was done by dispensing two volumes of acetone in each flask, followed by overnight refrigeration. To obtain the EPS product, the mixture was centrifuged (at 10,000 rpm at 4°C/15 min). The supernatant was removed, and the pellet obtained was oven dried at 40°C for 24 h. The dry and wet weight of extracted EPS was measured.

Evaluation of anticancer activity of Manganese oxide nanoparticles

Anticancer activity of manganes oxide nanoparticles was checked using 3-(4, 5 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay by adopting the method of Jeevanandam et al. (2019) with some modifications. We used HepG2 human liver cell lines; the cells were maintained in a suitable medium.

The cell density was 2×10^{4} cells/mL for anticancer potential assays. A 96-well plate was used for experimentation, 100 µl cells were dispensed into each well and treated with different amounts (500, 400, and 300 µg/ml) of nanoparticles, a negative control was also used in this assay (without nanoparticles treatment), and incubated for 24 hours under suitable conditions. The sample with the medium was taken from the wells after the incubation period, and the cells were rinsed with PBS (pH 7.4). Following that, 10 µM MTT dye (5 mg/ml) was applied to each well and incubated for 4 hours before adding 150 µL DMSO to each well to determine optical density (OD) at 590 nm using a reader plate. Each experiment was carried out in three, and the findings were reported as a mean. The proportion of cells that died was determined using the calculation below.

$$\%\text{age cell death} = \frac{\text{Control - Treated}}{\text{Control.}} \times 100$$

Results

Biochemical Characterization of Bacterial Isolates. The biochemical characterization of bacterial isolates was done by Gram staining and biochemical tests. **Gram Staining.** The stained slides were washed, dried, and observed under an oil immersion lens of a light microscope after the addition of cedar wood oil on the smear. Gram-negative rods (Klebsiella pneumoniae) were identified through Gram staining. **Biochemical Tests:** Klebsiella pneumoniae colonies on MacConkey agar plates were pinkish, mucoid, and lactose positive. The citrate utilisation test and the Voges–Proskauer test were used to validate the findings. A bacterial colony was injected onto a citrate agar slant and incubated for 24 hours. Because bacteria used citrate, the colour of the slant changed from green to blue during incubation. This indicated a positive citrate utilisation test. A cherry red hue emerged on the top of the broth when a few drops of 40 percent KOH and alpha naphthol were added to a tube of MR-VP broth with overnight bacterial growth, indicating a positive result of the VP test. Positive results of both citrate utilization test and Voges-Proskauer test confirmed that the test bacterium was Klebsiella pneumoniae. Evaluation of Antibacterial Activity of Manganese Oxide Nanoparticles by Agar Well Diffusion Assay. Antibacterial efficacy of Manganese oxide nanoparticles was evaluated against three bacterial isolates of Klebsiella pneumoniae by agar well diffusion assay. The inhibitory zones around each agar well were measured in millimeters. Zones of inhibition of bacterial growth represented bactericidal activity of manganese oxide nanoparticles (Table 4.1).

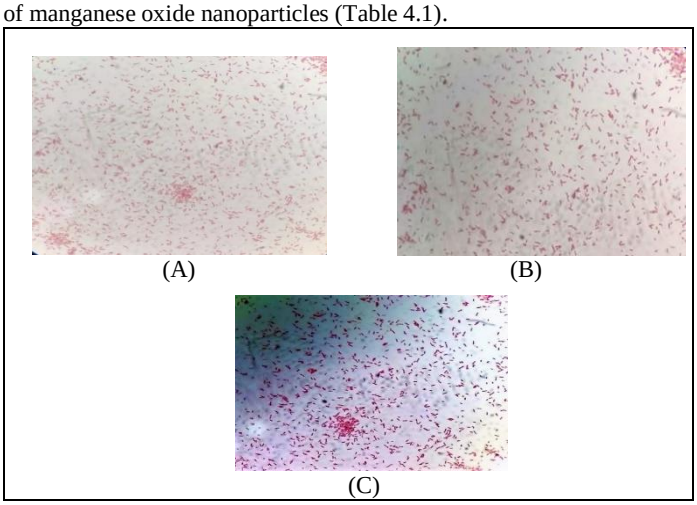

Figure 4.1. Microscopic images of Gram staining of three different isolates of Klebsiella pneumoniae

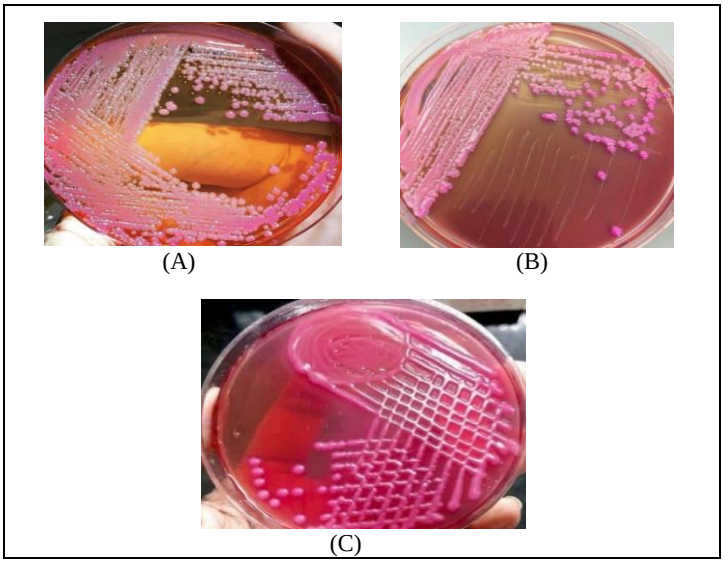

Figure 4.2. Pinkish mucoid colonies of Klebsiella pneumonia isolates on MacConkey agar

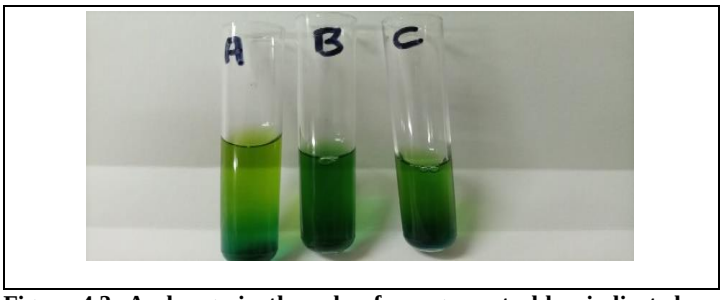

Figure 4.3. A change in the color from green to blue indicated a positive citrate utilization test for Klebsiella pneumoniae

Table 4.1. Antibacterial activity of MnO nanoparticles.

Bacterial isolates	Zone of inhibition in mm			
	25 µg/ml	50 µg/ml	75 µg/ml	100 µg/ml
A	20	19	23	30
B	13	24	21	28
C	18	22	25	36

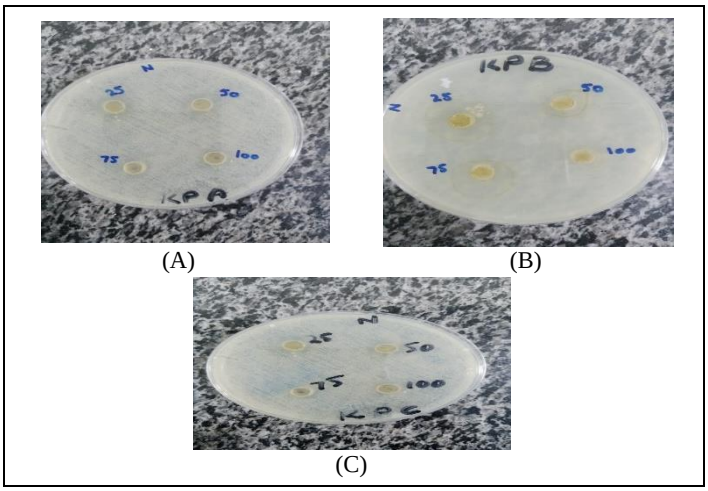

Figure 4.4. Zones of inhibition for bacterial isolates due to antibacterial activity of manganese oxide nanoparticles by agar well diffusion assay

Determination of Minimum Inhibitory Concentration (MIC) of Manganese Oxide Nanoparticles

Using 96-well microtitration plates, the minimum inhibitory concentration of manganese oxide nanoparticles against K. pneumoniae was demonstrated. Using the redox nitro blue tetrazolium chloride (NBT) dye, the MIC values of manganese oxide nanoparticles were obtained. In the presence of living bacterial cells, the colour of the dye changed from yellow to blue, whereas in the absence of viable bacterial cells, the dye remained yellow. The highest MIC was obtained against isolate C, as shown in Figure 4.3. MICs of different isolates are given in Table 4.2.

Table 4.2. Minimum inhibitory concentrations in µg/ml of manganese oxide nanoparticles against selected bacterial isolates

Bacterial isolates	MIC (µg/ml) of manganese oxide nanoparticles
Isolate A	375
Isolate B	375
Isolate C	750

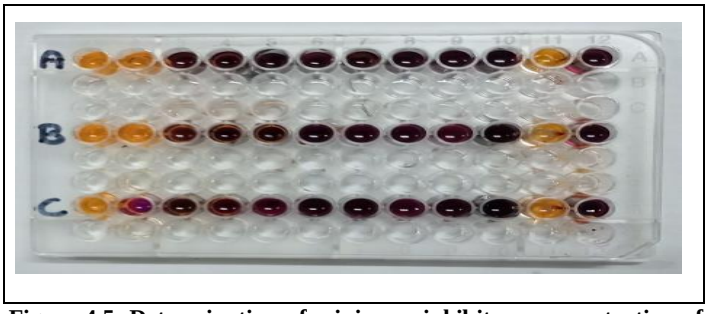

Figure 4.5. Determination of minimum inhibitory concentration of manganese oxide nanoparticles by broth microdilution technique against Klebsiella pneumoniae using Nitro-blue tetrazolium chloride (NBT) dye. Blue color denoted the presence of metabolically active cells (growth), and yellow color indicated the inhibition of growth of the isolate.

Determination of Minimum Bactericidal Concentration (MBC) of Manganese Oxide Nanoparticles

Minimum bactericidal concentration was evaluated by sub-culturing the broth dilutions from the MIC well and wells with higher concentrations of nanoparticles than the MIC. The absence of bacterial growth on agar plates was used to determine MBC values. Bacterial growth was seen on agar plates using broth containing nanoparticles at the lowest inhibitory concentration. However, no bacterial growth was seen on agar plates containing nanoparticle quantities greater than the minimal inhibitory concentration. MBCs of different isolates are given in Table 4.3

Table 4.3. Minimum bactericidal concentrations (µg/ml) of manganese oxide nanoparticles against selected bacterial isolates.

Bacterial isolates	MBC (µg/ml) of manganese oxide nanoparticles
Isolate A	750
Isolate B	750
Isolate C	1500

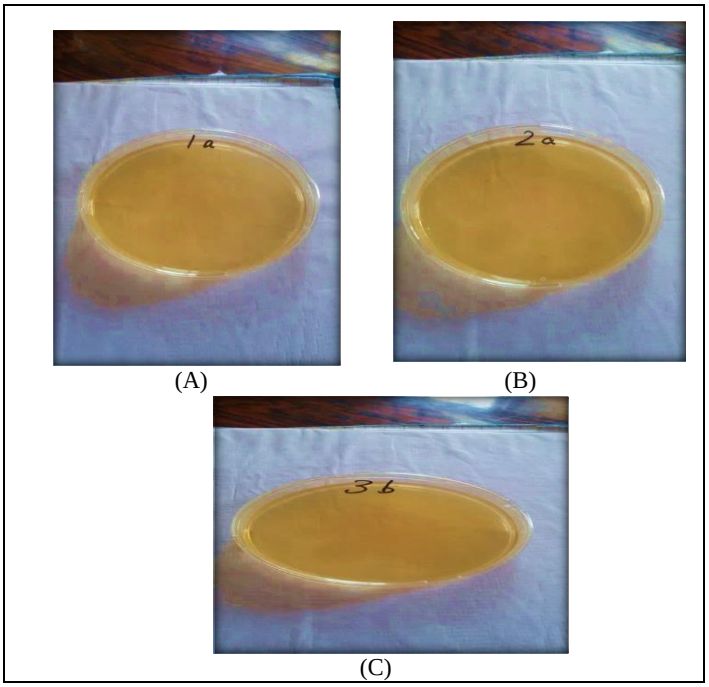

Figure 4.6. Minimum bactericidal concentrations (MBC) of manganese oxide nanoparticles against Klebsiella pneumoniae A, B, and C. 750µg/ml was the least concentration at which MBC was observed

Evaluation of Antioxidant Activity of Manganese Oxide Nanoparticles

DPPH radical scavenging assay is a quick and easy method to estimate the antioxidant potential of nanoparticles with the use of a spectrophotometer. It was observed that the radical scavenging potential of manganese oxide nanoparticles increased with the increase in the quantity of nanoparticles. A noteworthy increase was observed at the concentration of 200µg/ml, but this was a little less than that of ascorbic acid. The % scavenging activity of manganese oxide nanoparticles ranged from 15 to 65 %. Effect of Manganese Oxide Nanoparticles on Preformed Biofilms

Antibiofilm potential of manganese oxide nanoparticles against preformed biofilms was evaluated by the use of the microtiter plate method. Antibiofilm potential was evaluated against pre-established biofilms. Absorbance results showed that the biofilm inhibition was dependent upon the concentration of nanoparticles used. The percentage inhibition of biofilm was increased with a greater concentration of nanoparticles.

Results showed that the percentage inhibition produced by manganese oxide nanoparticles was found to be 30.14-54.89% when a sub-inhibitory concentration of nanoparticles (0.5 x MIC) was used. On the other hand, the percentage inhibition was 40-59.78%, with (1 x MIC) concentration of nanoparticles. The percentage inhibition of biofilm formation ranged from 30 to 59.78%.

Effect of Manganese Oxide Nanoparticles on the Production of Extracellular Polymeric Substance (EPS)

From cultures of Klebsiella pneumoniae isolates (A, B, and C), EPS was extracted using acetone after treatment of bacterial cultures with sub-inhibitory concentrations of manganese oxide nanoparticles. Cultures growing in the absence of sub-inhibitory concentrations of manganese oxide nanoparticles were used as controls. Wet and dry weights of EPS were measured after extraction, and the influence of manganese oxide nanoparticles on EPS production was found. Table 4.4 shows that in cultures developing in the presence of sub-inhibitory doses of manganese oxide nanoparticles, there was a reduction in the quantity of extracted EPS compared to control cells.

The findings revealed that the percentage decrease in the wet weight (mg/100ml) of EPS was 46.6-61.5 percent, while the percentage reduction in the dry weight of EPS was 41.1-61.3 percent. After treatment with manganese oxide nanoparticles, isolate A showed the greatest reduction in wet and dry weight of EPS, whereas isolate A showed the least reduction in wet and dry weight of extracted EPS (Table 4.4).

Evaluation of anti-cancer activity of manganese oxide nanoparticles

Anticancer activity of manganese oxide nanoparticles was evaluated following the MTT method. Different concentrations (500µg/ml, 4000µg/ml, and 300µg/ml) of manganese oxide nanoparticles were used. Results showed that the anti-cancer activity of manganese oxide nanoparticles was dose-dependent (Figure 4.7.B). Percentage % viability as determined by measuring absorbance was found to be 44.48-51.56%, 51.67-60%, 56.82-60.54% at the concentrations of 300µg/ml, 400µg/ml, and 500µg/ml receptively. Maximum anticancer activity was observed at the concentration of 500µg/ml (Figure 4.7.A-4.7.C)

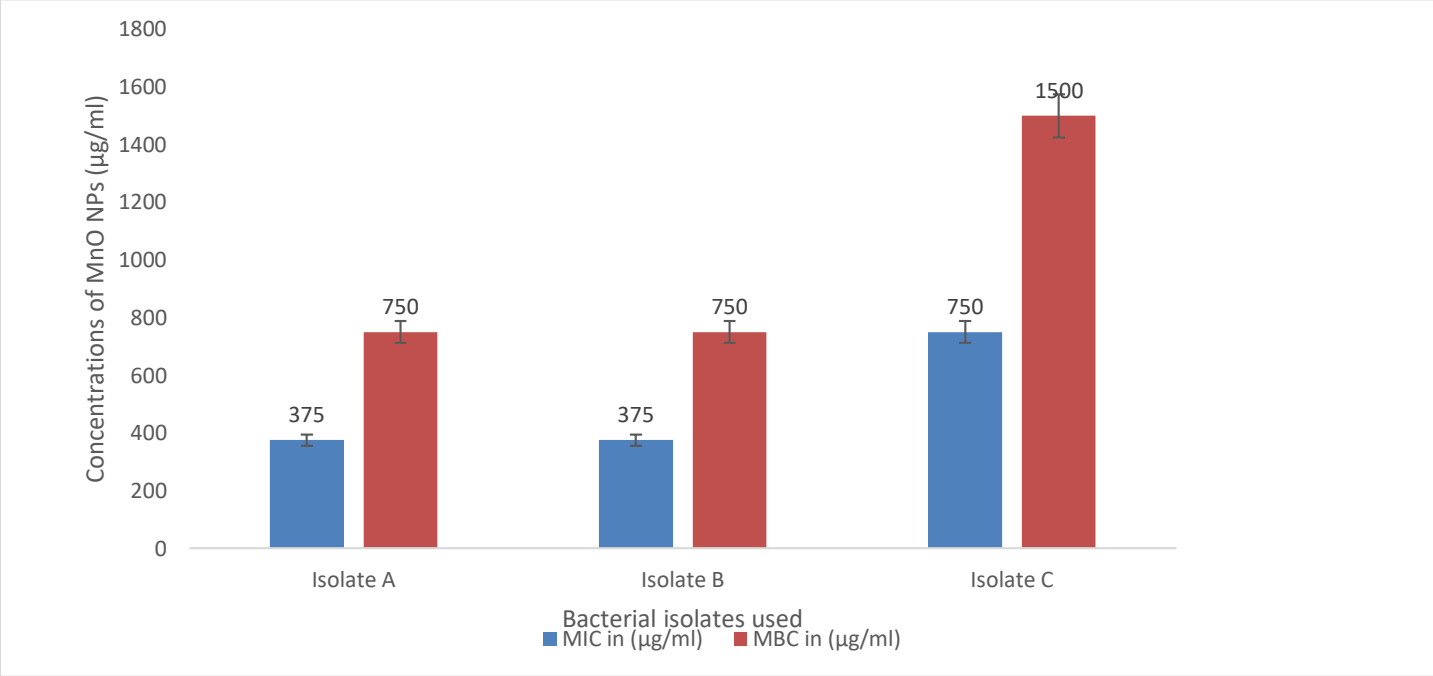

Figure 4.7. MICs and MBCs of manganese oxide nanoparticles against K. pneumoniae

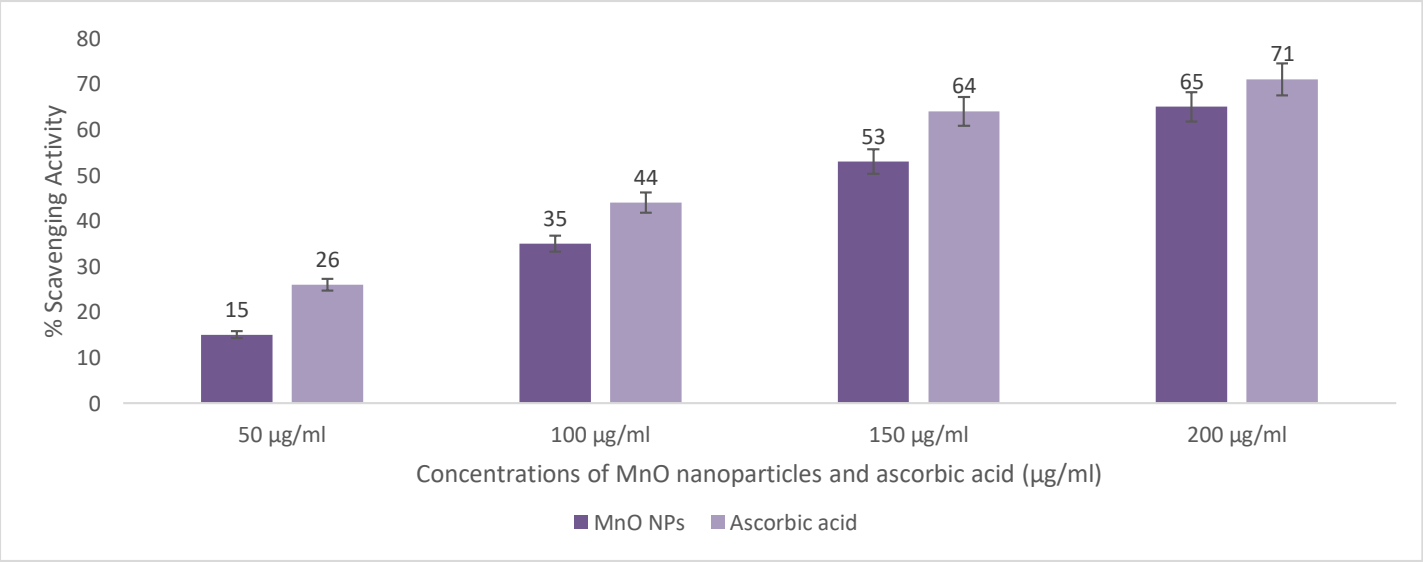

Figure 4.8. % scavenging activity of manganese oxide nanoparticles. Ascorbic acid was used as a standard compound

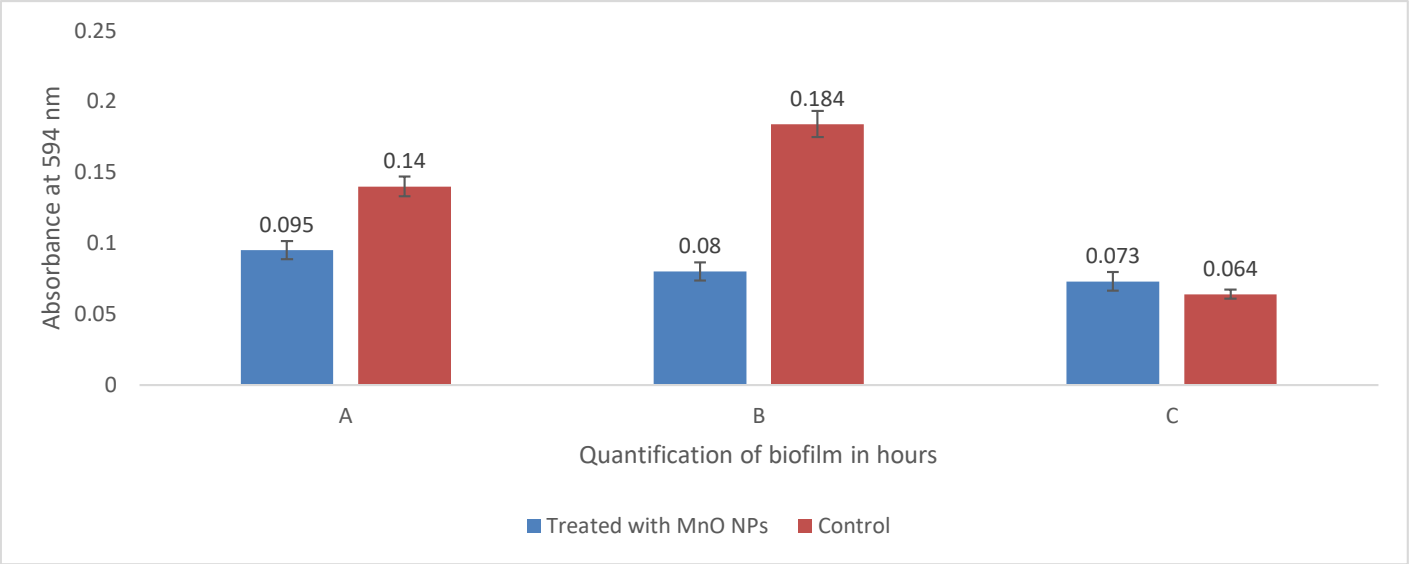

Figure 4.9. Effect of MnO nanoparticles on biofilm formation of K. pneumoniae with (0.5x MIC) concentration of nanoparticles

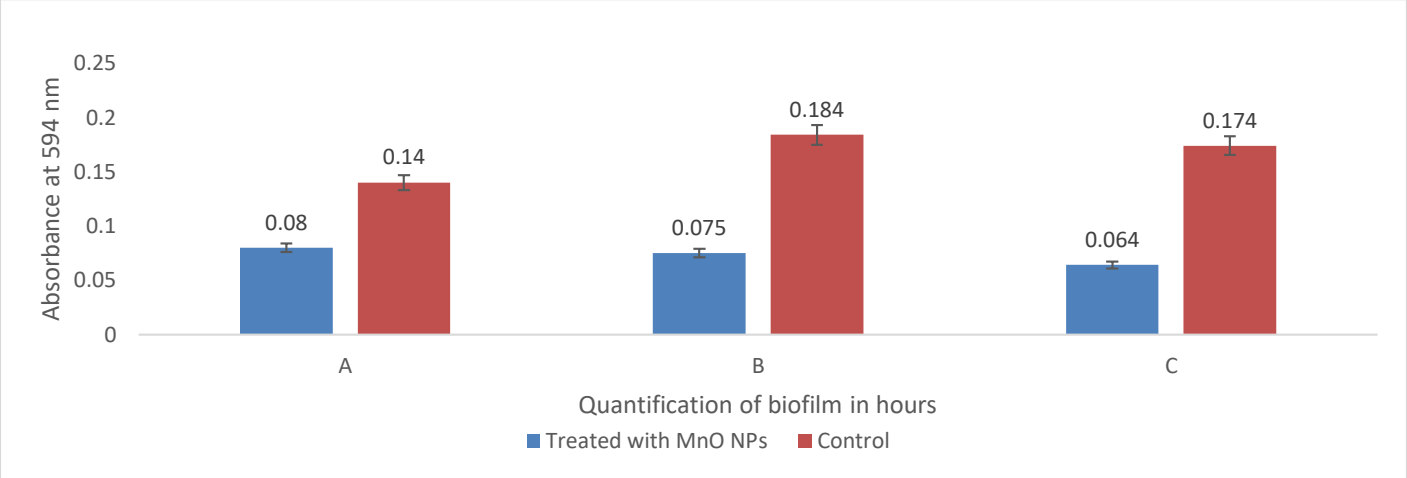

Figure 4.10. Effect of MnO nanoparticles on biofilm formation of K. pneumoniae with (1x MIC) concentration of nanoparticles

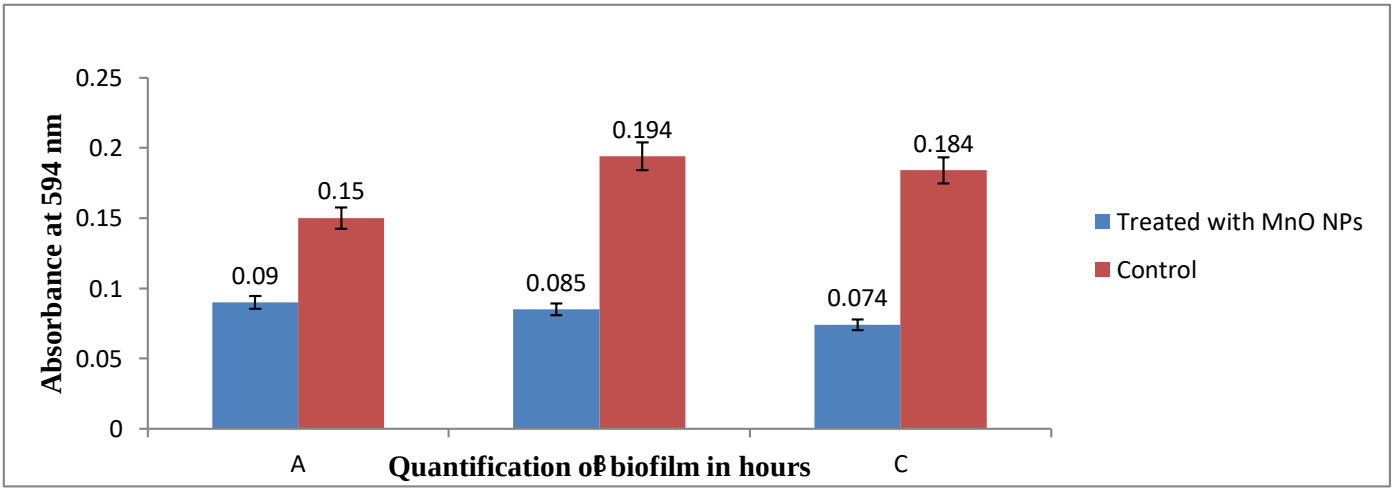

Figure 4.11. Effect of sub-inhibitory concentrations (0.5xMIC) of MnO nanoparticles on established biofilm of K. pneumonia

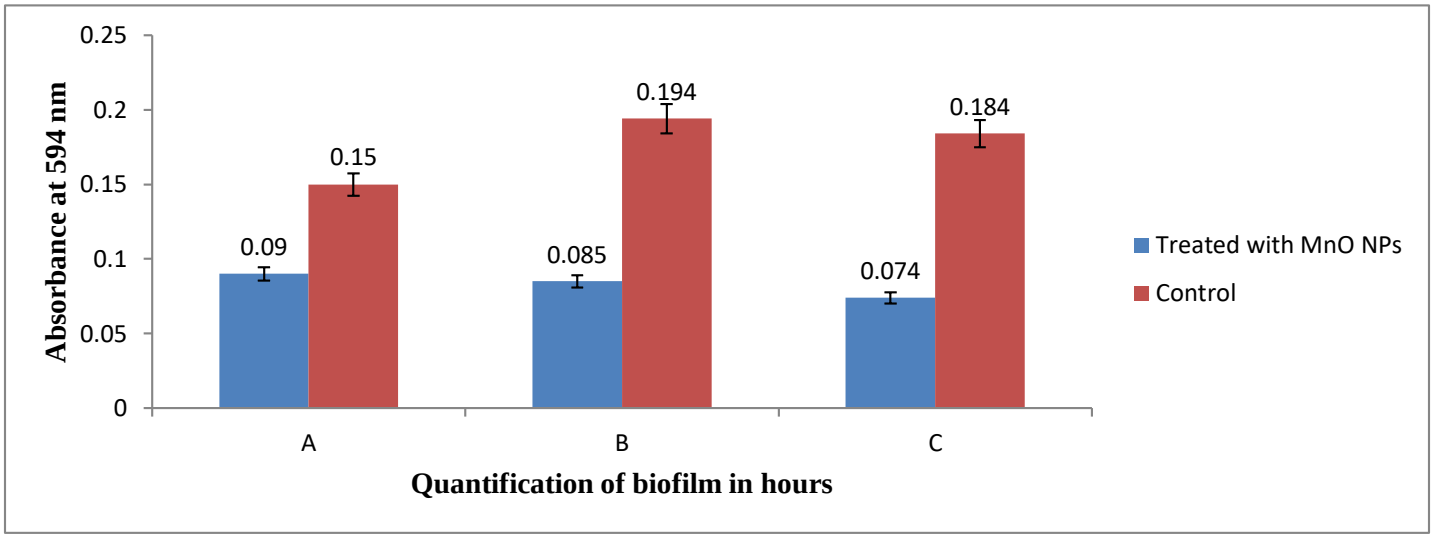

Figure 4.12. Effect of sub-inhibitory concentrations (1xMIC) of MnO nanoparticles on established biofilm of K. pneumonia

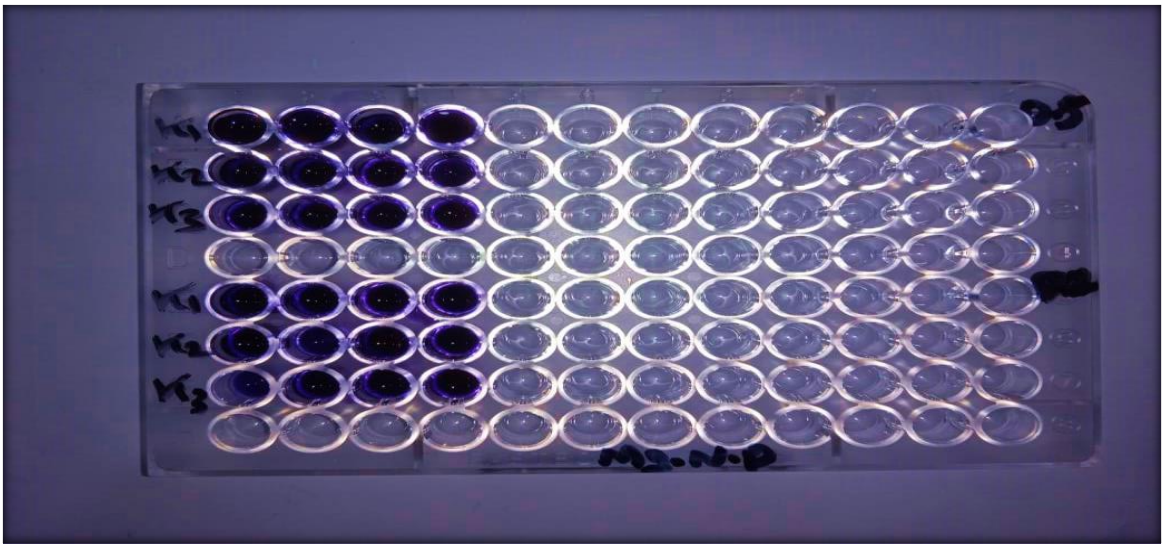

Figure: 4.13. Determination of anti-biofilm activity of manganese oxide nanoparticles against Klebsiella pneumoniae isolates K1 (Klebseilla pneumoniae A), K2 (Klebseilla pneumonia B), K3 (Klebseilla pneumoniae C) by microtiter plate assay

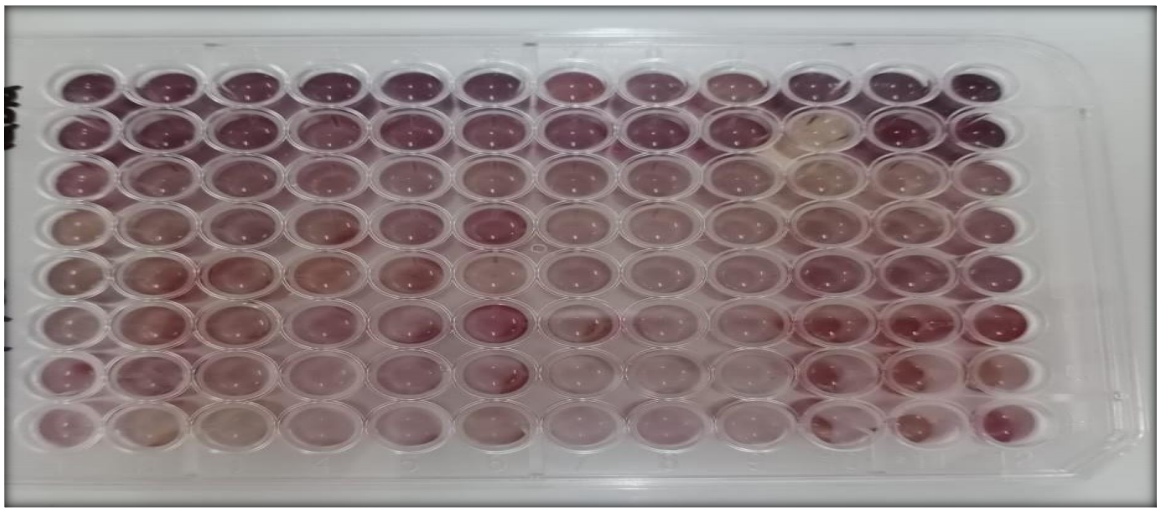

Figure: 4.14. Anti-cancer activity of magnesium oxide nanoparticles against liver cancerous cell line cells

Table 4.4. Wet and dry weight of EPS recovered from Klebsiella pneumoniae isolates treated with and without MnO NPs at sub-inhibitory doses (mg/ 100 ml of culture).

Bacterial Isolates	Control (Without Nanoparticles)		Treated cells (MnO)	
	Wet weight	Dry weight	Wet weight	Dry weight
A	39 mg	59 mg	24 mg	36.2 mg
B	42 mg	36 mg	22 mg	24.7 mg
C	45 mg	43.7 mg	21 mg	18 mg

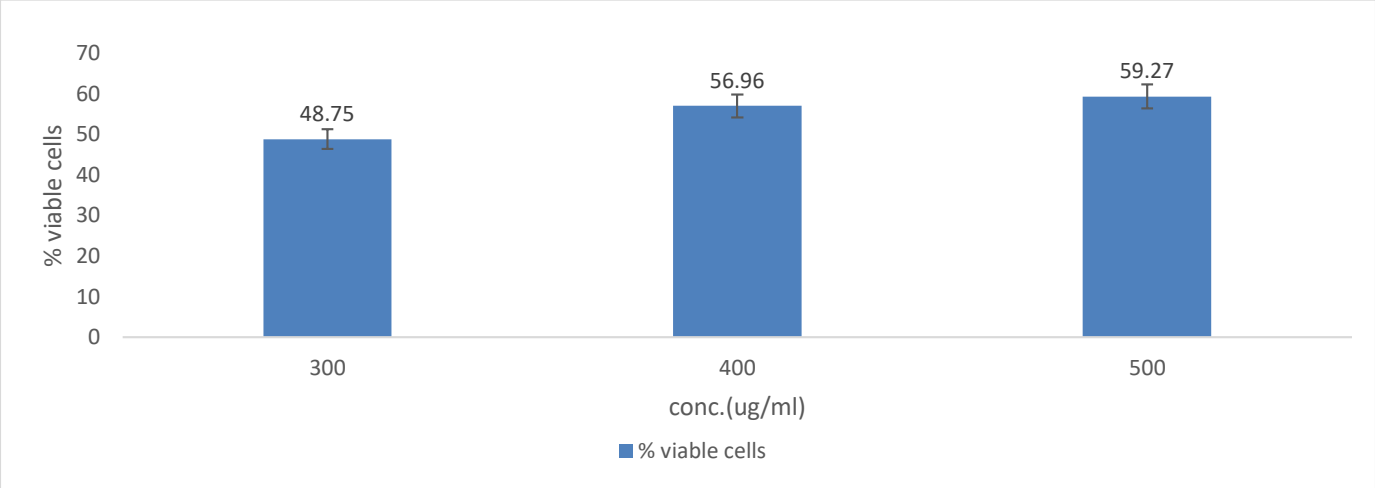

Figure 4.15. Kinetics of viability of cells treated with different concentrations of manganese oxide nanoparticles

Discussion

Nanotechnology has revolutionised aerospace engineering, biology, and electronic sectors based on electrical and physiochemical characteristics (18). Nanoparticles have also acquired popularity in medical sectors, such as the pharmaceutical business, where they are used to transport monoclonal antibodies, DNA, proteins, and medicines (19). Present study evaluated antibacterial, antioxidant, antibiofilm and anticancer activity of manganese oxide (MnO) nanoparticles against the three isolates of Klebsiella pneumoniae which were chemically characterized by performing biochemical tests. These bacteria were obtained from Deparment of Microbiology, Government College University Faisalabad. Similar to the current study, Rinita et al., (2021) also prepared MnONPs by biogenic method and obtained NPs were further characterized by scanning electron microscopy (20). Results revealed that regular geometric shapes and coryndrum spheres of NPs with diameter of 10 to 20 µm were obtained and it was also observed that shape and morphology of these NPs could be monitored via controlling the pressure and temperature conditions (21). In another study by Medina-Cruz, (2021) an isolated aerobic bacteria from Persian Gulf water was used to make MnO NPs (22). This isolate belonged to a gram-negative Acinetobacter that generated nano Mn-oxide crystal particles, according to 16SRNA sequencing. TEM, SEM, EDAX, XRD, and FTIR were used to characterise the complement morphology, size, and chemical structure of these particles.

In current research anticancer activity of manganese oxide nanoparticles was evaluated against HepG2 human liver cell lines. Results showed that manganese oxide nanoparticles have ability of anticancer activity in dose dependant manners (11). Percentage of cancer cells death was 44.48-51.56%, 51.67-60% and 56.82-60.54% at the concentrations of 300µg/ml, 400µg/ml and 500µg/ml receptively. In order to study the cytotoxicity and safety of metallic nanoparticles (MnO) for human beings, cancerous liver cells were used (23). Cell viability assay using neutral red dye revealed 90-96 % viability cells treated with manganese oxide nanoparticles as compared to control at a concentration of above 100 µg/ml. Cytotoxicity experiments revealed that the metallic oxide nanoparticles were safer to use for human beings upto that concentration (24). In another study by Uski et al., (2017) cytotoxic assay (MTT) was performed for copper, zinc, titania-zirconia based NPs, ceria, alumina and tungsten carbide NPs against human macrophage cell lines and alveolar epithelium cells (25). The results revealed that Cu and Zn nanoparticles appeared to be more toxic for human cells while alumina, zirconia and ceria based NPs showed mild to moderate toxicity against human cells while no toxicity was observed for tungusten carbide NPs.

Agar well diffusion technique was performed to evaluate the antibacterial potential of manganese oxide nanoparticles. Different concentrations (50, 40, 30, 20, 10, 5, 2.5, 1.25 µg/ml) of MnO nanoparticles were used against isolates. Antibacterial activity increased by increase in concentration of nanoparticles (26). Naumenko et al. (2015) conducted study on antibacterial activity of Manganese Oxide nano particles using concentrations of 39, 78, 156, 312, 625 and 1250 µg ml-1 (27). Using the Broth Microdilution Method, the antibacterial activity of different concentrations of Mn3O4-NPs against human pathogenic bacteria, namely Escherichia coli and Staphylococcus aureus, were investigated.

Similarly, antibacterial activity of magnesium oxide nanoparticles was performed by Hayat et al. (2018) against different strains of Klebsiella pneumoniae and E.coli. Different concentrations (500 µg/mL, 1000 µg/mL and 2000 µg/mL) of nanoparticles were used and maximum zone of inhibition was observed at the concentration of 2000 µg/mL (28). In another study Khan et al. (2020) antibacterial activity of magnesium oxide nanoparticles was evaluated against gram-positive (Streptococcus pyogenes, Staphylococcus aureus, Staphylococcus epidermidis) and gram-negative (Klebsiella pneumonia, Serratia mercescens Pseudomonas aeruginosa, Escherichia coli) (29). Maximum zone of inhibition was measured at the concentration of 25µg/ml that was the highest.

In present study antioxidant potential of manganese oxide nanoparticles was evaluated by using DPPH radical scavenging assay which explained that the antioxidant potential of nanoparticles increased by increase in the

concentration of nanoparticles (30). It was calculated by formula that % scavenging activity of four different concentrations (50, 100, 150 and 200 µg/ml) of nanoparticles was 15, 35, 53 and 65 % respectively. The % scavenging potential of same concentrations of ascorbic acid was higher (26, 44, 64 and 71 %) than % scavenging potential of nanoparticles. Nazir et al., (2020) did a similar investigation on the antioxidant and antibacterial properties of silver and manganese dioxide nanoparticles .Using the conventional well diffusion technique, AgNPs and MnO2NPs were evaluated for antibacterial activity against human pathogenic Enterococcus faecalis ATCC 29212, Escherichia coli ATCC 35218, ATCC 25922, Klebsiella pneumonia ATCC 700603, and Staphylococcus aureus ATCC 43300 and ATCC 29213 (31). MnO2NPs showed higher antioxidant activity than AgNPs, indicating that the capping agent affects the size and shape of nanoparticles, which affects antioxidant activity (32). Current study determined antibiofilm activity of manganese oxide nanoparticles by microtiter plate assay. It was observed that the sub inhibitory concentrations of manganese oxide nanoparticles exhibited good antibiofilm activity and also treated the mature biofilms efficiently by targeting biofilm viability, biomass and matrix (33). The results of current studies indicated that the antibiofilm potential of manganese oxide nanoparticles depended upon time of nanoparticles-bacterial surface interactions i.e. biofilm inhibition was greater at 24 hours and 48 and 72 hours. Study by Ogunyemi, et al., (2023) demonstrated that manganese oxide nanoparticles exhibited antibiofilm activity against Xoo strain GZ 0006. It was stated that the nanoparticles can inhibit the formation of biofilm but have lower efficacy of inhibition on the preformed biofilms (34).

Effect of sub-inhibitory concentrations of metallic nanoparticles on extracellular polymeric substance (EPS) extracted from various isolates was determined (35). The results indicated a considerable reduction in wet and dry weight of EPS. Similarly, there was a decrease in protein and carbohydrate contents of extracted EPS from cells growing in the presence of manganese oxide nanoparticles. In a study by Gangadhar et al., (2022), it was observed that NPs caused reduction in EPS (36).

The entire findings of the current research work specified that manganese oxide (MnO) nanoparticles have wonderful potentials such as antibacterial, antioxidant and antibiofilm activities against multidrug resistant Klebsiella pneumonia (14). Manganese oxide nanoparticles can be used in combination to different antibiotics for enhanced antibacterial effect against the multidrug resistant bacteria (9).

Overall findings of the current research work illustrated that manganese oxide (MgO) nanoparticles have marvelous potential of anti-biofilm, antibacterial and antioxidant activities against multidrug resistance three strains of Klebsiella pneumonia (37). Cytotoxicity experiment revealed that manganese oxide nanoparticles were safer to use for human beings. More research work is needed to determine anti-biofilm and antibacterial potential of manganese oxide nanoparticles against other human pathogens (38). Moreover, cytotoxicity assays should be extended to other human cell lines to confirm the safety of these nanoparticles for human beings (39).

Conclusion

Biogenically synthesized manganese oxide nanoparticles possess potent antibacterial and antibiofilm activity against Klebsiella pneumoniae, with minimal cytotoxicity to human liver cells. These findings suggest that MnO NPs may offer a promising alternative therapeutic approach to combat multidrug-resistant bacterial infections.

Declarations

Data Availability statement

All data generated or analysed during the study are included in the manuscript.

Ethics approval and consent to participate

Approved by the department concerned. (IRBEC-MMNCS-0331d-24)

Consent for publication

Approved

Funding

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Conflict of interest

The authors declared the absence of a conflict of interest.

Author Contribution

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All authors reviewed the results and approved the final version of the manuscript. They are also accountable for the integrity of the study.

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