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The Inhibitory Effect of Manganese Oxide Nanoparticles Synthesized by Prodigiosin against Pathogenic *P. aeruginosa*

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Abstract

The present work aims to biosynthesis of manganese oxide nanoparticles (MnO NPs) using Prodigiosin pigment, produced by *Serratia marcescens*, as a stabilizing and reducing agent. In addition to the characterizing of the biosynthesized MnO NPs were characterized utilizing several optical methods, including FT-IR, XRD, AFM and UV-Vis. A cut-off wavelength of the biologically synthesized MnO NPs was established at around 440 nm using UV-Vis. Furthermore, the analysis of AFM revealed that MnO NPs have an average diameter size of 74.51 nm. The results showed concentration-dependent antibacterial effect (25, 50, 100 and 200 mg/ml) of MnO nanoparticles on *Pseudomonas aeruginosa*. Overall, it is concluded that the biosynthesized MnO NPs retain the potential to serve as an alternative antibacterial agents.

Keywords: *Serratia marcescens*, nanoparticles against bacteria, Nanoparticles Synthesized.

التأثير التثبيطي لجزيئات أكسيد المنغنيز النانوية المحضرة بالبروديجيوسين ضد بكتيريا *P. aeruginosa*

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الخلاصة

الهدف من العمل الحالي هو الكشف عن طريقة التخليق الحيوي لجسيمات أكسيد المنغنيز النانوية (MnO NPs)باستخدام صبغة تنتجها*Serratia marcescens* ، تسمىProdigiosin ، والتي تستخدم كعامل تثبيت واختزال. بالإضافة إلى ذلك، تم تحديد الظروف المثلى للتخليق الحيوي لجسيمات أكسيد المنغنيز النانوية من خلال عدة طرق، بما في ذلك FT-IR و XRDو AFMو UV-Vis. P بالإضافة الى ظاهرة قطع MnO المركبة بيولوجيًا عند حوالي 440 نانومتر باستخدام الاشعة فوق البنفسجية. في حين كشف تحليل AFMأن متوسط قطر جسيمات اوكسيد المنغنيز المحضرة هو 74.51 نانومتر. وتم اثبات تأثير التراكيز المختلفة (25، 50، 100 و 200 ملجم/مل) من الجسيمات النانوية اوكسيد المنغنيز على البكتيريا *Pseudomonas aeruginosa*. بشكل عام، تم التوصل إلى أن MnO NPs المُصنّعة حيويًا تحتفظ بالقدرة على العمل كعوامل بديلة مضادة للبكتيريا..

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1. Introduction

Pseudomonas aeruginosa is a motile, aerobic, non-spore forming, rod-shaped Gram-negative bacterium belongs to the *Pseudomonadaceae* family which includes eight classes [1, 2]. *P. aeruginosa* are ubiquitous microorganisms found in both natural and human-made environments, affecting humans, animals and plants [3]. *Pseudomonads* are opportunistic pathogens that can cause infections in the lungs, skin, and eyes of people with cystic fibrosis (CF), urinary tract, and burns and abrasions [4-5].

Serratia marcescens is a Gram-negative rod-shaped bacillus that belongs to the family Enterobacteriaceae [6]. Prodigiosin (PG) is one of the alkaloid secondary metabolites released from numerous microorganisms, including *Nocardia* spp., *Streptomyces lividans*, *Vibrio gazogenes*, *Pseudomonas magneslorubra*, *Serratia rubidaea*, *Serratia marcescens*, and *Pseudoalteromonas rubra*. The chemical structure of this pigment includes three pyrrole rings. This natural pigment has achieved significant biological roles via displaying a wide range of useful properties, including immunosuppressive, antifungal, antimalarial, anticancer, antibacterial potentials [7].

Particles with a mean diameter of less than or equal to 100 nm and a high surface-to-volume ratio are known as nanoparticles (NPs) [8]. The visual features, catalytic activity, and antimicrobial capabilities of nanoparticles have garnered a lot of attention in recent years. There is a greater possibility of their use in fields like medicine, communications, and electronics due to their special qualities. Nanoparticles, endowed with these characteristics, have proven useful in a wide variety of industries including biomedical applications [9]. Manganese is considered as a high-performance metal in numerous applications such as catalysis, photoelectronics, electrochemistry, electronics, purification, water treatment, biosensors, biomedicine, medicine etc. [10].
Manganese nanoparticles are a viable new antibacterial therapeutic option. Therefore, the current work aims to synthesize manganese nanoparticles utilizing Prodigiosin derived from *Serratia marcescens* and evaluate their anti-*P. aeruginosa* potential.

2.Materials and methods

Collection, isolation and identification of bacteria

A total of one hundred-eighty clinical samples were obtained from burn and wound infections of different patients with different ages and sexes, who were attending Kadhimiya Hospital, Karkh General Hospital, and Yarmouk Hospital during the period from September 2022 to December 2022. All isolates were subjected to various examinations, including microscopic examination (G staining), cultural characteristics (MacConkey agar and Cetrimide agar) and biochemical tests (catalase, oxidase, indole, Simmons citrate and urease tests). Also, VITEK-2 system was employed in order to isolate and identify isolates of *Pseudomonas aeruginosa* [11– 13].

Furthermore, a ready isolate of *Serratia marcescens* was obtained after confirming its species using VITEK-2 system. The isolates were obtained from Biotechnology Department, Collage of Science, University of Baghdad and were utilized for the production of prodigiosin after performing VITEK-2 system to confirm its identity.

Antibiotic susceptibility test

Ten antibiotics were utilized in order to estimate the multi-drug resistance of the isolated *P. aeruginosa*. These antibiotics were *as* follows: Tobramycin (TOB, 10 µg), Piperacillin-tazobactam (PIT, 100/10 µg), Meropenem (MEM, 10 µg), Azithromycin (AT, 30

µg), Ceftazidime (CAZ, 30 µg), Piperacillin (PRL, 100 µg), Ofloxacin (OF, 5 µg), Levofloxacin (LE, 5 µg), Gentamicin (CN, 10 µg) and Imipenem (IPM, 10 µg). The antibiotic susceptibility test was performed according to CLSI [14].

Prodigiosin pigment production

A ready medium of nutrient agar (Himedia Company / India) was utilized for the production of prodigiosin according to the manufacturer's recommendations. Medium was autoclaved for 15 minutes at 121 ° C thereafter its pH was adjusted to 7.0. The medium was cooled after sterilization, then 2% of the chosen bacterial isolate was added (using a 0.5 McFarland standard, which equates to 1.5×10^8 CFU/ml), and the mixture was incubated in a shaker incubator for 48 hours at 28 °C with 120 rpm.

Extraction and purification of prodigiosin

After 48 hours of incubation at 28 °C, the cell-free broth culture of *S. marcescens* was used to harvest the prodigiosin. The culture medium was placed in a centrifuge for 15 minutes at 8000 rpm to remove any debris. The collected supernatant was then systematically removed, and methanol (250ml) was added to the collected cells before being well mixed for three hours at temperature of room. After combining methanol with culture medium, the resulting mixture was placed in a centrifuge at 8000 rpm for twenty minutes, after which the supernatant was obtained and filtered through a fine mesh filter (0.2 µm, Millipore filter). Hence, a rotary evaporator set at 70°C was utilized for the concentration of the methanol filtrate, and two as much chloroform was added to the pigment extraction mixture. The methanol and chloroform were thoroughly combined in a reparatory funnel, and then the organic chloroform phase was separated and left to dry at 45 °C. Ultimately, methanol was added to dissolve the extracted pigment and then it is stored refrigerated in an opaque bottle [15].

Syntheses of manganese oxide nanoparticles (MnO NPs)

Manganese oxide nanoparticles (MnO NPs) were synthesized utilizing the biological method by mixing prodigiosin and manganese (II) sulphate monohydrate ($MnSO_4.H_2O$, 98.0%) [16]. This was done by adding of 5 gm of manganese (II) sulphate monohydrate to deionized distilled water (DDW) and the mixture then subjected sonication technique for thirty minutes for dissolving (solution A). Furthermore, 10 mg/ml of prodigiosin was dissolved solution A to create solution B. The two solutions (A and B) were then fully combined in an ultra-sonication bath for 30 mins at a temperature of 50 ^oC and a pH of 7.0 before being stored in the dark for 12 hours. Then, the mixture was centrifuged and rinsed with DDW multiple times. The resulting weight residuals were then left to dry at 60°C and stored in the darkness until needed [17].

Characterization of manganese oxide nanoparticles (MnO NPs)

Different optical techniques were utilized to characterize the biosynthesized MnO NPs, including ultra-violate visible light (UV-Vis), atomic force microscopy (AFM), X-ray diffraction (XDR) analysis, and Fourier transforms infrared (FTIR) spectroscopy.

Antibacterial test

In order to estimate the antibacterial activity of the biosynthesized MnO NPs on Gram-negative *P. aeruginosa*, the agar well diffusion technique was used [18]. In this case, 25 ml of sterile from Muller Hinton agar medium was poured into pre-cleaned petri dishes and let to settle overnight in the lab. The agar medium with the grown test species was expanded using the sterile cotton swab method. As a result, solutions of varying concentrations of MnO NPs (25, 50, 100, 200 mg/ml) were introduced into the previously drilled wells. At 37 ℃, the

obtained plates were infected for a period of 24 hours. After that, the size of the inhibition zone surrounding each of the prepared wells was determined [17].

3. Results and Discussion

Collection, isolation and identification of bacterial samples

Different examinations techniques were utilized for the identification of 180 samples that were collected from burn and wound infections of different patients. Firstly, cells of *P. aeruginosa* displayed negative Gram reaction and appeared as single bacterial cell or arranged in small pairs or rods. The cultural characteristics of *P. aeruginosa* were determined on MacConkey agar and Cetrimide agar. On MacConkey, colonies of this bacterium appeared in pale shape because *P. aeruginosa* is a non-lactose fermenting bacterium, while greenish-yellow color colonies of this bacterium appeared on cetrimide agar medium, due to the ability of *P. aeruginosa* to survive with the toxic cetrimide material, as shown in Figure 1.

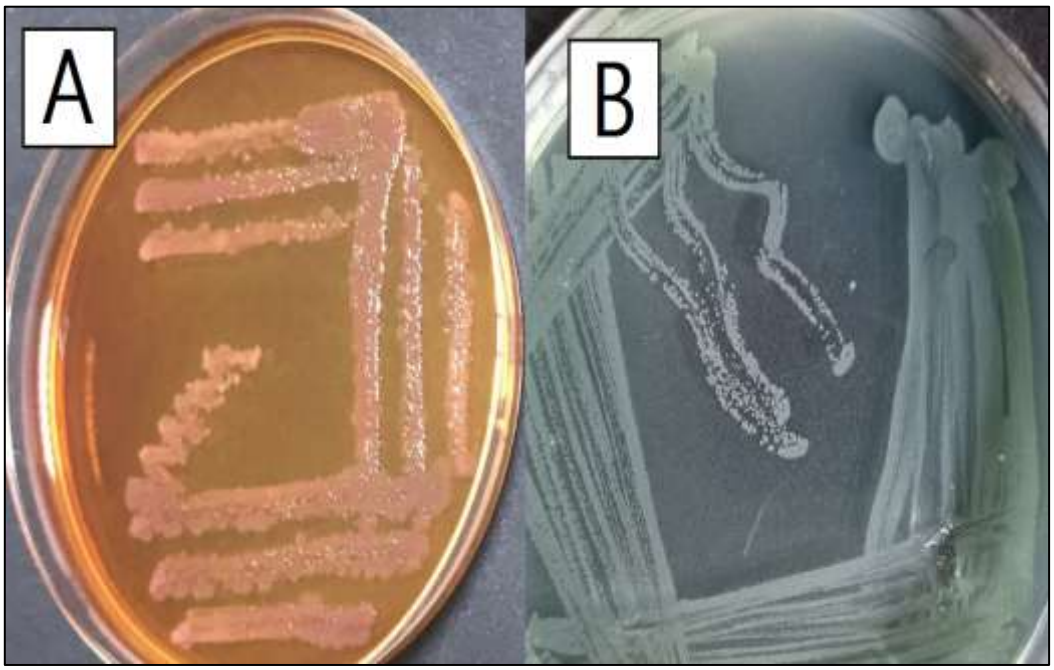


Figure 1: Colonies of *P. aeruginosa* on: A) MacConkey agar, and B) Cetrimide agar.

Moreover, several biochemical tests were performed to confirm identity *P. aeroginosa* isolates. The findings of these tests are listed in Table 1.

Table 1: The results of the biochemical tests of *P. aeruginosa*

Test	*Result*
Oxidase	+
Catalase	+
Indole test	-
Methyl red	-
Vogues-Proskauer	-
Simmons Citrate test	+

Furthermore, the ready used isolate of *S. marcescens* was utilized for the production of prodigiosin after performing VITEK-2 system to confirm its identity.

Organism Quantity:
Selected Organism : Serratia marcescens

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	3.97 hours	**Status:**	Final
Selected Organism	99% Probability **Bionumber:**	**Serratia marcescens** 6125711455006210		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	+	4	PyrA	+	5	lARL	+	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	+	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	-	39	5KG	+
40	lLATk	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	lLATa	-			

Figure 2: Identification of *S. marcescens* using VITEK 2 system

Antibiotic susceptibility test of *P. aeruginosa*

Ten antibiotic discs were utilized for estimating the multi-drug resistant *P. aeruginosa* isolate in order to use this isolate for further steps. The results showed that the majority of *Pseudomonas aeruginosa* isolates were sensitive to antibiotics, including Meropenem, Tobramycin, Piperacillin-tazobactam, Azithromycin and Gentamicin with sensitivity values of 82%, 76%, 82%, 86% and 68%, respectively, as shown in Figure 3. In addition, the majority of isolates showed intermediate resistance to Ofloxacin, Piperacillin and Levofloxacin with values of 74.6%, 60.3% and 86.3%, respectively.

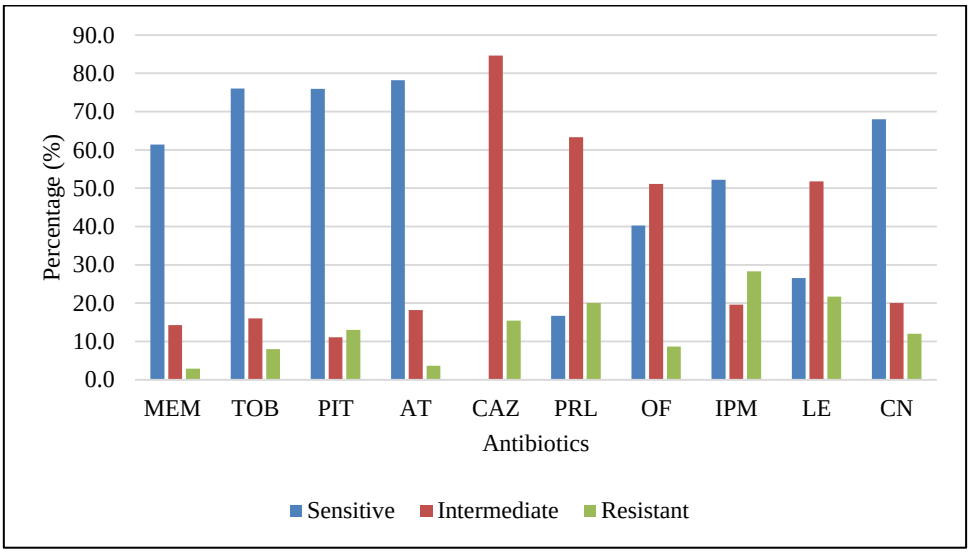
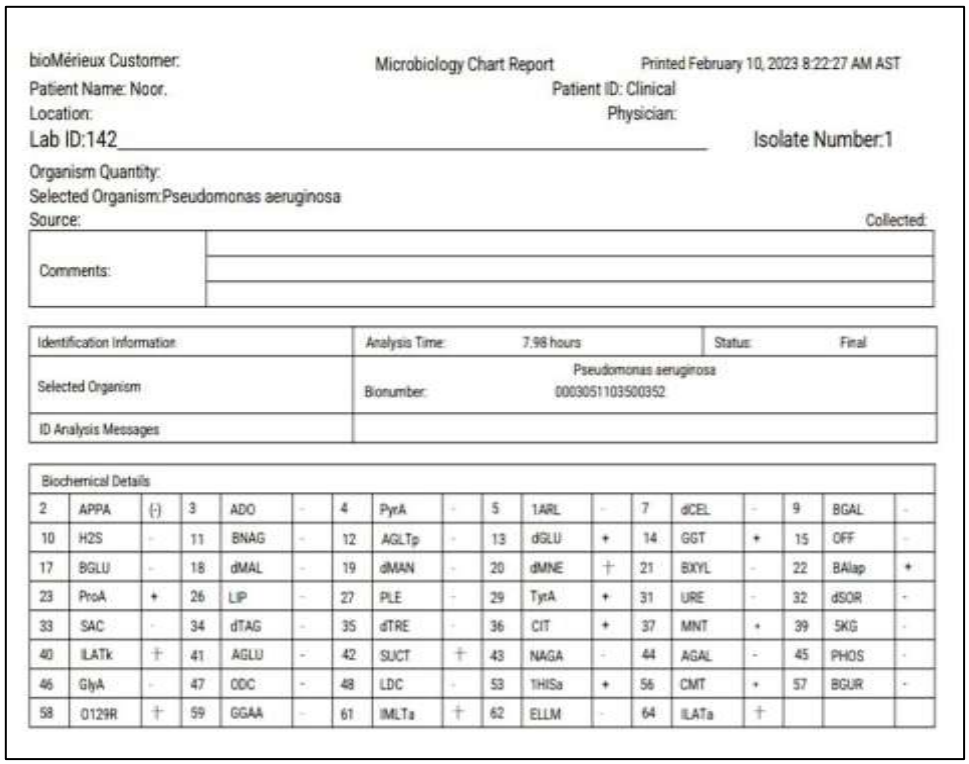


Figure 3: Antibiotic susceptibility test of *P. aeruginosa*.

MEM**:** meropenem; TOB: Tobramycine; PIT: Piperacillin-tazobactam; AT: Azithromycin; CAZ: Ceftazidime; PRL: Piperacillin; OF: Ofloxacin; IPM: Imipenem; LE: Levofloxacin; CN: Gentamicin.

Alsaimary *et al*. (2010) found that 47.5% of *P. aeruginosa* isolates exhibited resistance to meropenem [19]. The growth and spread of multidrug-resistant (MDR) strains of *P. aeruginosa* have lately become a health problem for many reasons. In the first place, *P. aeruginosa* is a leading cause of death from infection, especially in hospitals and among those with impaired immune systems. Secondly, it can be selectively favored and disseminate antimicrobial resistance *in vivo* to an extraordinary degree [20].

The multi-drug resistant isolate of *P. aeruginosa* was selected for further experiments, after performing VITEK2-system, which ensures the diagnosis of *P. aeruginosa*, as shown in figure 4.



bioMérieux Customer: Microbiology Chart Report Printed February 10, 2023 8:22:27 AM AST
Patient Name: Noor. Patient ID: Clinical
Location: Physician:
Lab ID:142 Isolate Number:1
Organism Quantity:
Selected Organism:Pseudomonas aeruginosa
Source: Collected:

Comments:	

Identification Information	Analysis Time: 7.98 hours	Status: Final
Selected Organism	Bionumber:	Pseudomonas aeruginosa 0003051103500352
ID Analysis Messages		

Biochemical Details

2	APPA	(-)	3	ADO	-	4	PyrA	-	5	1ARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	±	21	BXYL	-	22	BAlap	+
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	±	41	AGLU	-	42	SUCT	±	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	1HISa	+	56	CMT	+	57	BGUR	-
58	O129R	±	59	GGAA	-	61	IMLTa	±	62	ELLM	-	64	ILATa	±			

Figure 4: Identification of *P. aeruginosa* NJ23 using VITEK 2 system

Production of prodigiosin pigment

Serratia marcescens were incubated for 12 hours before their prodigiosin synthesis was initiated. Changes in medium hue, especially which is predominantly observed during the stationary phase, may be attributable to prodigiosin accumulation [21].

Ultra-violate visible light (UV-Vis) investigation of the bio-synthesized MnO NPs

The optical characteristics of the biosynthesized MnO NPs were studied utilizing UV-Vis spectroscopy method. As demonstrated in Figure 5, the attained MnO NPs showed a pronounced UV absorption at around 440 nm.

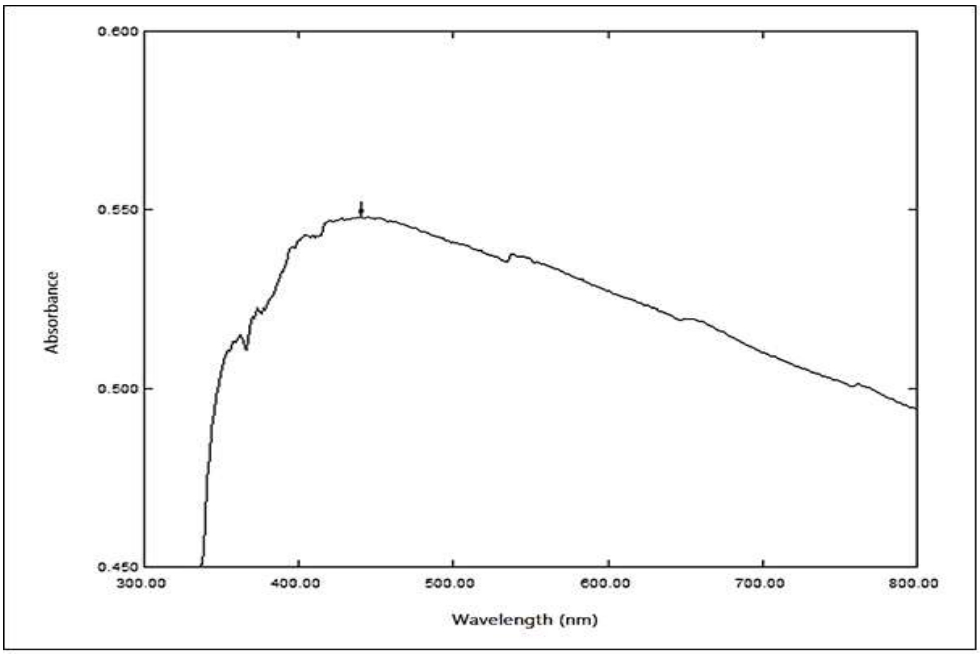


Figure 5: Spectrum of UV-Vis of the bio-synthesized MnO NPs.

Atomic force microscopy (AFM) investigation of the bio-synthesized MnO NPs

This technique was used to examine the surface properties of MnO nanoparticles in 2D and 3D. The results showed that the MnO nanoparticles are round and having an average diameter of 74.51 nm (Figure 6).

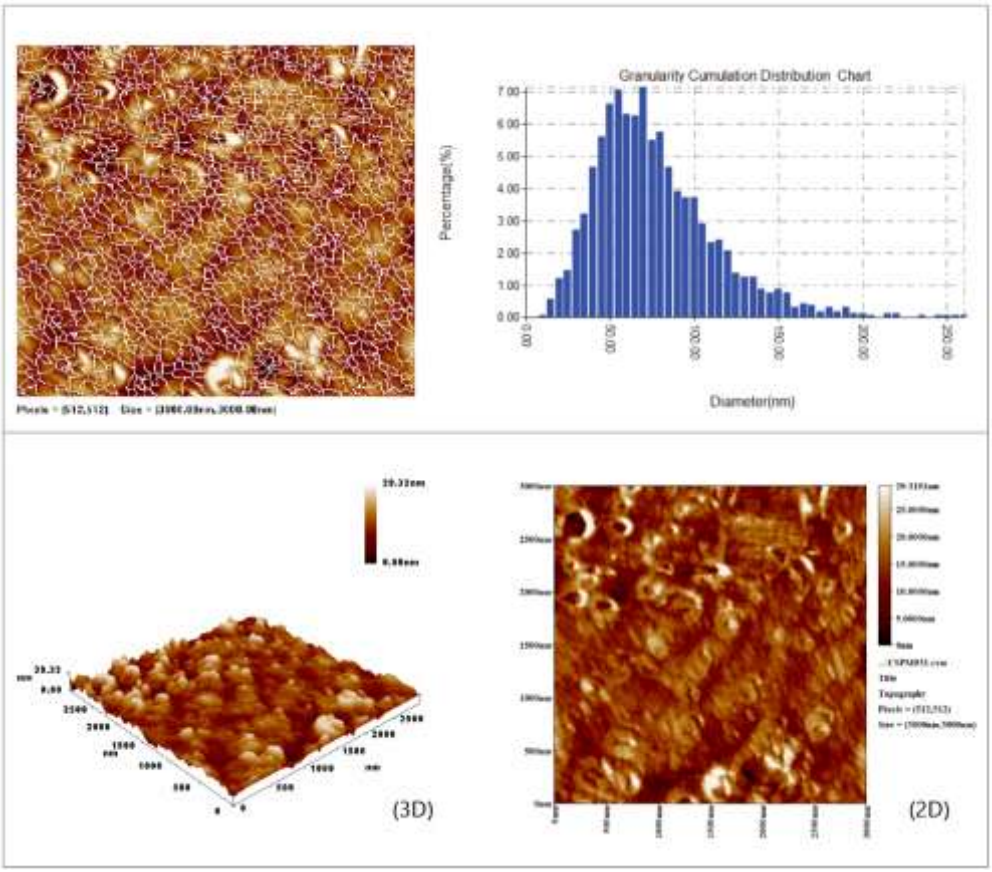


Figure 6: Atomic Force Microscopic examination of the synthesized MnO NPs.

X-ray diffraction (XRD) investigation of the bio- synthesized MnO NPs

The XRD investigation of the synthesized MnO NPs was demonstrated as shown in Figure 7. Over the course of observation, three distinct peaks at 2θ = 11.9867^o, 19.9940^o, and 22.5561^o were seen. The Debye-Scherrer equation was used to determine the crystalline particles' sizes in the meantime, range about 10.42 - 28.19nm as follows:

$$D = \left[\frac{K\lambda}{\beta\cos\theta}\right] A^o$$

In this context, the symbol D stands for the crystallite size, K represents the form factor, which is a constant (0.9), and λ stands for the x-ray wavelength (1.5406 A^o). Nanoparticles' Bragg angles and corrected line broadening are denoted by θ and β, respectively.

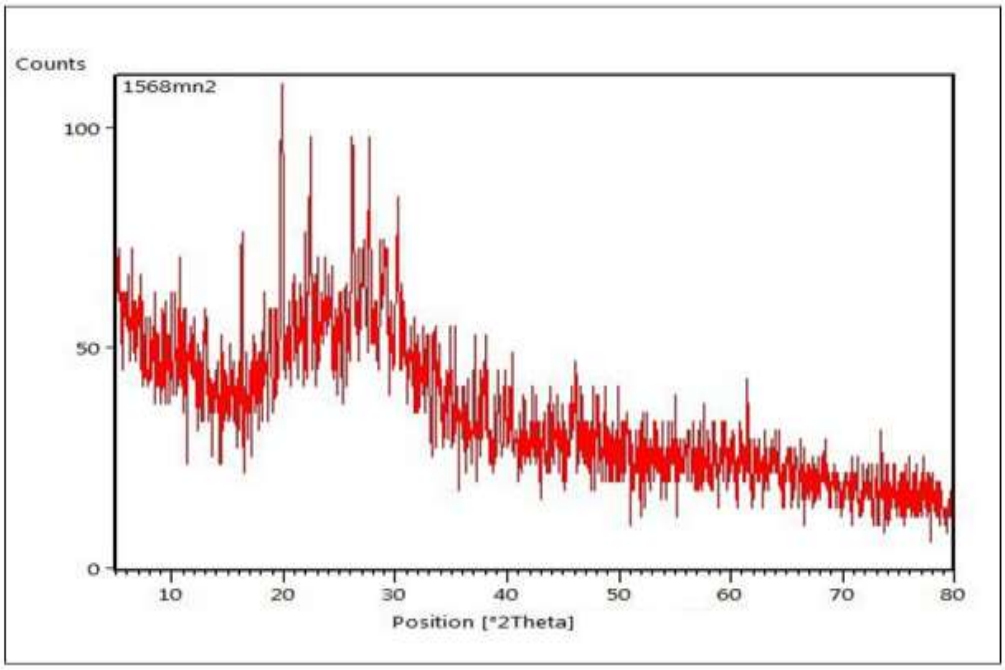


Figure 7: XRD patterns of the bio-synthesized MnO NPs.

Fourier transforms infrared (FTIR) spectroscopy analysis of the bio-synthesized MnO NPs

Figure 8 displays the FT-IR data for the MnO NPs produced via biosynthesis. It is common to see a succession of absorption peaks between 400 and 4000 cm-1, which corresponds to alcohol in the O-H bonds stretching mode for a broad frequency of 3433.06 cm^{-1}. As for the N-O bonds stretching vibration is responsible for the peak 1562.23 cm^{-1} which represents Nitro compound, and absorption from 524.60 cm^{-1} is due to the metal oxygen representation of the MnO.

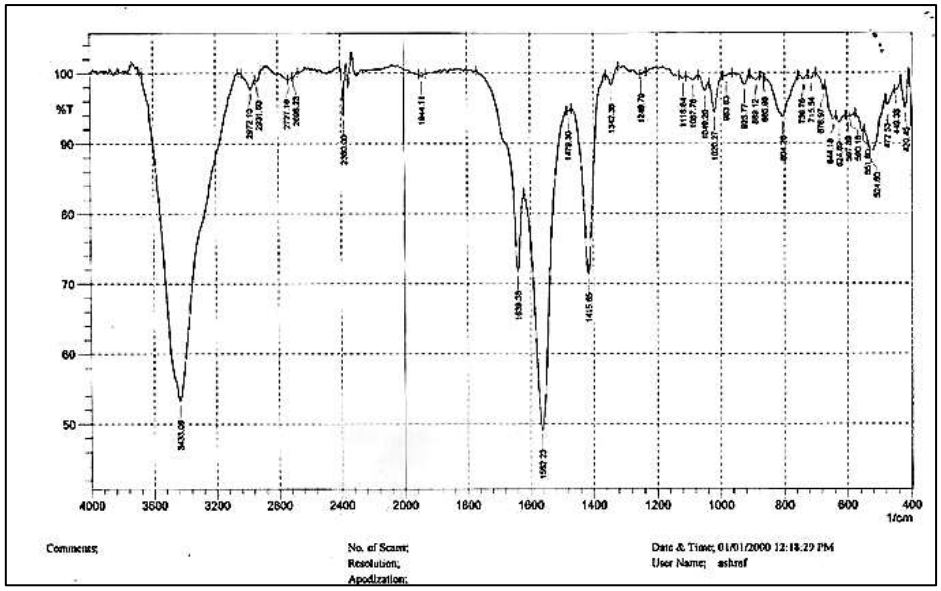


Figure 8: Fourier transforms infrared (FTIR) spectroscopy analysis of the bio-synthesized MnO NPs

Antibacterial susceptibility test of the bio-synthesized MnO NPs

The antibacterial activity of the biosynthesized MnO NPs at various concentrations (25, 50, 100, 200 mg/ml) is depicted in Figure 10. It is clear to be noticed that the MnO NPs antibacterial activity is immediately dependent on the utilized concentrations. The MnO NPs exhibited strong antibacterial activity against pathogenic bacteria, including *Pseudomonas aeruginosa* [22, 23]. Nanoparticles may penetrate bacterial membranes and disrupt DNA replication, and they can also function as a catalyst to inactivate enzymes that microorganisms need for their metabolism. Antibacterial efficacy was maximized in metal nanoparticles with a higher surface-to-volume ratio [24]. Generally, the present study findings clearly showed that the biosynthesized MnO2 NPs have antibacterial activity that is immediately dependent on the utilized concentrations.

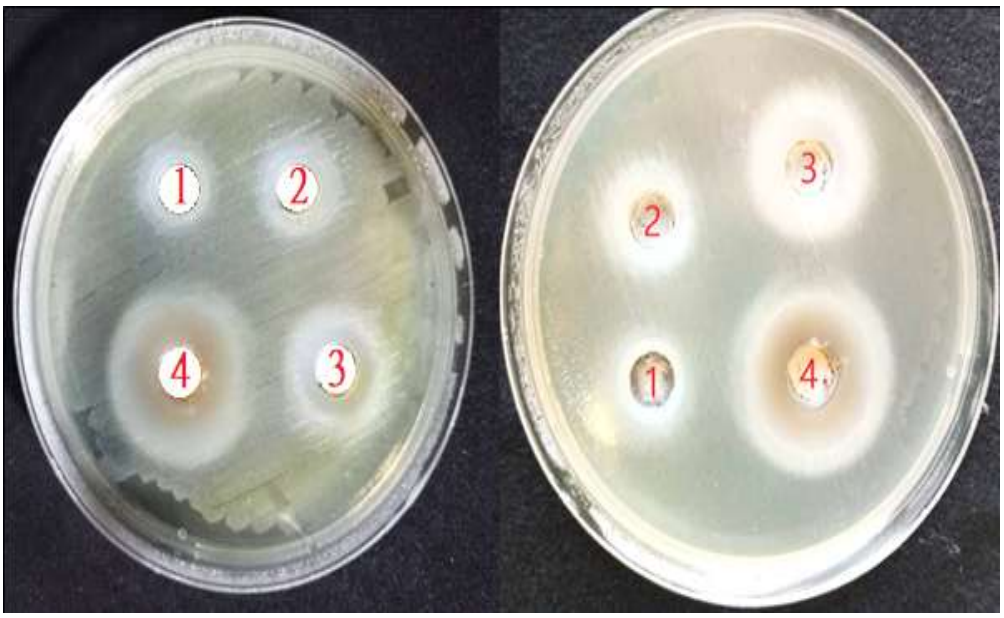


Figure 10: Antibacterial activities of the biosynthesized MnO NPs against *P. aeruginosa* at concentrations of: 1) 25 mg/ml, 2) 50 mg/ml, 3) 100 mg/ml and 4) 200 mg/ml

Conclusion

The results indicated that the biosynthesized MnO NPs retain considerable antibacterial activity against MDR *P. aeruginosa*. Based on these findings, it is suggested that MnO NPs could be used as a promising treatment against *P. aeruginosa* that correlated with burn and wound infections.

Conflict of interest

There are no conflicts of the interest to be declared.

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