EVALUATION OF THE BIOLOGICAL EFFECT SYNTHESIZED ZINC OXIDE NANOPARTICLES ON *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

This study was aimed to demonstrate the biosynthesis procedure of Zinc oxide nanoparticles (ZnO NPs) using Prodigiosin pigment which in turn produced from particular environmental bacteria isolates *Serratiamarcescens*as a stabilizing and reducing agent. Additionally, the synthesis conditions were precisely taken into consideration as a pH of 7 and a temperature of 50°C alongside a concentration of prodigiosin of 12 mg/ml with 5mg precursor of zinc acetate in deionized distilled water (DDW) 50ml. Biosynthesized ZnO nanoparticles have presented many applications such as catalysis, biosensing, anticancer, and biomedical, etc. The optimum condition for ZnO biosynthesis was characterized through several techniques such as UV-Vis, AFM, XRD, FT-IR, and FE-SEM. In particular, a cut-off phenomenon of the biological synthesized ZnO was found at around 280 nmusing UV-Vis, while spherical shape particles were noticed using FE-SEM techniques. Also, the AFM analysis revealed that ZnO NPs an average diameter size of 45.02 nm.And the effect of ZnO NPs on bacteria*Pseudomonas aeruginosa*on an inhibition zone 29mm.

Keywords: ZnO nanoparticles, antimicrobial activity, prodigiosin.

تقييم التأثيرالبيولوجي لجسيمات اوكسيد الزنك المصنعة النانويه على بكتريا *pseudomonas aeruginosa*

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المستخلص :

هدفت هذه الدراسة الى توضيح إجراء التخليق الحيوي لجسيمات أوكسيد الزنك النانوية (ZnO NPs) باستخدام صبغة Prodigiosin التي تنتج بدورها من بكتيريا بيئيه معينة *Serratia marcescensas*كعاملا ستقرارواختزال. بالإضافةإلى ذلك،تم أخذ ظروف التوليف في الاعتبار بدقة على أنها درجةحموضة 7 ودرجةحرارة 50 درجة مئوية جنبًا الى جنب مع تركيزبروديجيوسين 12 مجم / ملمع 5 ملغ من خلات الزنك في الماء المقطر منزوع الأيونات(DDW) 50مل. قدمت جسيمات ZnOالنانويةالمصنعة حيوياً العديد من التطبيقات مثلا لتحفيز،والاستشعارالحيوي،ومضادالسرطان،والطب الحيوي،وما إلى ذلك. تميزت الحالة المثلى لتخليقZnO NPsالحيوي من خلال العديد من التقنيات مثل UV-Vis و AFM و XRD و FT-IRو FE-SEM على وجه الخصوص ،تم العثورعلى ظاهرةالانقطاع لـ ZnO للمركب البيولوجي عند حوالي 280 نانومتر باستخدام تقنياتUV–Vis ،بينما لوحظت جزيئات الشكل الكروية باستخدام FE-SEM . وكذلك ،كشف فحص AFMأن معدل حجم الجسيمات ZnO NPs يبلغ 45.02 نانومتر.وان تاثير الزنك النانوي على بكتريا *Pseudomonas aeruginosa* بتثبيط نمو بكتيري هو29ملم.

الكلمات المفتاحية: جزيئات أكسيد الزنك النانوية،نشاط مضاد للميكروبات،بروديجيوسين.

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INTRODUCTION

Pseudomonas aeruginosa is a widely known Gram-negative bacteriathat is isolated using several clinical sources. This type of bacterium is characterized using the Vitek-2 system, polymerase chain reaction (PCR), and biochemical tests. Specifically, the antibiotic susceptibility revealed that *P. aeruginosa* possesses some resistance to Methicillin and Nitrofurantoin as well as a noticeable sensitivity tocolistin and Ciprofloxacin. Among Gram-negative bacteria Pseudomonas*aeruginosa*is a rod-shaped, motile bacterium that is capable of growing in both anaerobic and aerobic manners, Gram-negative bacteria are known as a major cause of nosocomial infections, leading to an extended period of hospitalization, mortality rate, and higher hospitalization charges(1). The *P. aeruginosa* can be located in several moist environments and able to grow in many others. This particular adaptable pathogen is frequently linked to clinical infections, specifically in immunocompromised patients. Furthermore, this type of bacteria is world-wide considered as one of the most commonly recovered pathogens within the intensive care unit (ICU) patients. Moreover, wide-ranging reports of nosocomial infection due to this particular bacterium are common, typically caused by the hospital environment and cross-contamination which is related to the improper utilization of medical devices/equipment(2, 3, 4, 5).Additionally, the majority of infections that occur in immunodeficiency patients result from mucous membrane loss. The latter is attributed to the germination mechanism existence which in turn is caused by antibiotic resistance (6). Antibiotic is a natural substance that inhibits many microbes' activity, such as bacteriostatic (7, 8). Nanoparticles and nano-biomedicine provide an alternative pathway to overcoming the addressed typical antibiotic limitations due to excellent biocompatibility, high antimicrobial activity, and good thermal stability of the nanoparticles(9, 10). Several nanoparticles, ZnO in particular, delivers an outstanding antimicrobial performance in a wide range of bacteria such as *Escherichia coli* and *Staphylococcus aureus*(9, 11). Furthermore, ZnO has demonstrated some attractive applications such as solar cells, catalysts, photodetectors, and biomedical/antibacterial activity(12, 13, 14, 15).Herein, the interaction between the bacteria and ZnO nanoparticles are highly effective if the ZnO is prepared using a biological method due to the absence of undesired/toxic chemical compounds. Also, ZnO nanoparticles are biocompatible and non-toxic (16, 17, 18). The aforementioned advantages provide great potential in the utilization of ZnO nanoparticles as an antibacterial agent. Therefore, this study aims to evaluate the antibacterial activity of ZnO nanoparticles against Gram-negative *P. aeruginosa* through a biosynthesis approach using Prodigiosin as a reducing agent.

MATERIALS AND METHODS

Bacterial isolation and culture media: In this study, the isolated bacteria (*p. aeruginosa*) were collected from December 2019 to February 2020 from three different hospitals namely, Sheikh Zayed, Al-Yarmouk, and Baghdad/Medical city; this includes 210 clinical specimens' comparison concerning urine and wounds. The collected specimens were directly streaked on macconkey agar and subsequently incubated at 37 °C for 24 hr. Using macconkey agar, the corresponded specimens were found to be black colonies, while another test such as the biological and morphological analysis was also performed (19, 20). Furthermore, identification of the collected specimens was demonstrated via automated and/or manual techniques (Vitek II, bioMe ´rieux, Marcy l'Etoile, France); the attained VitekIIoutcomesare presented in Figure1.

bioMérieux Customer: Microbiology Chart Report Printed Sep 3, 2020 21:58 CDT

Patient Name: Patient ID:
Location: Physician:
Lab ID: 03 Isolate Number: 1

Organism Quantity:
Selected Organism : Pseudomonas aeruginosa

Source: **Collected:**

Comments:	

Susceptibility Information	**Analysis Time:** 15.58 hours			**Status:** Final	
Antimicrobial	**MIC**	**Interpretation**	**Antimicrobial**	**MIC**	**Interpretation**
Ticarcillin	>= 128	R	Amikacin	<= 2	S
Ticarcillin/Clavulanic Acid	>= 128	R	Gentamicin	<= 1	S
Piperacillin	16	*R	Tobramycin	<= 1	S
Piperacillin/Tazobactam	32	S	Ciprofloxacin	<= 0.25	S
Ceftazidime	4	S	+Levofloxacin		S
+Ceftriaxone		R	Pefloxacin		
Cefepime	2	S	Minocycline		
Aztreonam			Colistin	<= 0.5	S
Imipenem	2	S	Rifampicin		
Meropenem	2	S	Trimethoprim/Sulfamethoxazole		

+= Deduced drug *= AES modified **= User modified

AES Findings	
Confidence:	Consistent

Figure 1.Vitek II test for *Pseudomonas aeruginosais*

Prodigiosin pigmentproduction: In a typical procedure, the fermentation media was prepared by (21). In detail, the corresponded media was attained by mixing peptone (5g/L) and source sucrose (10g/L) as the nitrogen and carbon sources, respectively. Additionally, other compounds such as $MgSO_4.7H_2O$ (0.61g/L), $MnSO_4.4H_2O_2$ (2g/L), $CaCl_2.2H_2O$ (8.82g/L), and $FeSO_4.4H_2O$ (0.33g/L). The PH was set to 7.0 and then sterilized at 121 °C for 15 min by autoclaving. After sterilization, the medium left to cool and inoculated 2% of the selected bacteria isolate a 0.5 McFarland standard corresponds to 1.5×10^8 CFU/ml. And cultured in a shaker incubator at 28 °C for 48 hrs at 120 revolutions per minute (rpm) (22).

Extraction and purification of prodigiosin: The prodigiosin was produced by using cell-free broth culture of *Serratiamarcescens* which in turn was acquired after an incubation period of 48 hrs. at 28 °C. The culture media was subject to the centrifuging process at 8000 rpm for 15 min. Continuously, the acquired supernatant was then discarded, while a particular amount of methanol (250 ml) was poured into the obtained cell; the mixture was systematically mixed for 3 hrs. at room temperature. The attained mixture (methanol and culture media) was, hereinafter, centrifuged at 8000 rpm. for 20 min where the supernatant was collected and filtered using specific filtering paper (0.2 µm, millipore filter). Consequently, the methanol filtrate was concentrated using a rotary evaporator at a temperature of 70 °C, while double the amount of chloroform was supplemented for the red pigment extraction. Both methanol and chloroform were mixed thoroughly using a reparatory funnel whereby the organic chloroform phase was separated and later dried at 45 °C. Finally, the obtained pigment was liquefied using a particular amount of methanol and deposited in a fridge using an opaque bottle for further use (7).

Syntheses of zinc oxide nanoparticles: ZnO nanoparticles were synthesized via the biological synthesis approach using zinc acetate [$Zn(CH_3CO_2)_2$](15). In a typical procedure, 5 gm of zinc acetate was dissolved in deionized distilled water (DDW) using the sonication technique for 30 min (solution A). Concurrently, solution (B) was obtained by dissolving a certain amount of prodigiosin (10 mg/ml) using the aforementioned technique. Subsequently, both solutions (A and B) were mixed thoroughly using an ultra-sonication bath for 60 min; with a pH level of 7.0 and the temperature at 50 ˚C and later kept in a dark condition overnight. The resultant solution was then centrifuged and washed using deionized water several times. Hereinafter, the obtained weight residuals were dried at 60 °C and then kept in a dark condition for further use.

Antibacterial test (in vitro): The antibacterial activities of the biologically synthesized ZnO nanoparticles against Gram-negative *p. aeruginosa* were tested using the agar well diffusion technique in which the minimal inhibition concentration (MIC) of ZnO nanoparticles was estimated (23). Herein, Müller Hinton agar sterilized medium (25 ml) was added into the sterilized Petri dishes and allowed to solidify at laboratory conditions overnight. The grown test species were extended on the agar medium through the sterile cotton swab technique. Consequently, variety of ZnO concentrations (5,10, 20, 40, 80, 160, and 320) µg/mlwere poured into the pre-made wells. The attained plates were then inoculated for 24 hrs. at a temperature of 37 °C. Hereinafter, the zone of inhibitions was measured around the pre-made wells(24).

RESULTS AND DISCUSSION

Production of prodigiosin pigment: The identification of the collected bacterial isolates for the production of prodigiosin was accomplished through a biological test so-called Vitek-2 compact system where the regarded outcomes are illustrated in Figure 1. The prodigiosin production was started after the incubation period (12 hrs.). Herein, the prodigiosin concentration (at the end of the exponential phase) was found to be 0.29 g/L at 48 hrs. of incubation and 0.4145 g/L(during the stationary phase) at 35 hrs. The alteration of the medium to red color noticed could be attributed to the prodigiosin production which was accumulated primarily throughout the stationary phase (25).

Characterization of prodigiosin pigment: The absorbance characteristics of the extracted prodigiosin using *S. marcescens* was achieved through UV-Vis spectrophotometer (Shimadzu, Japan) with a range of 400-600 nm (Figure 2). In particular, the maximum absorption was noticed at around 525 nm which is in good agreement with previous studies(25).

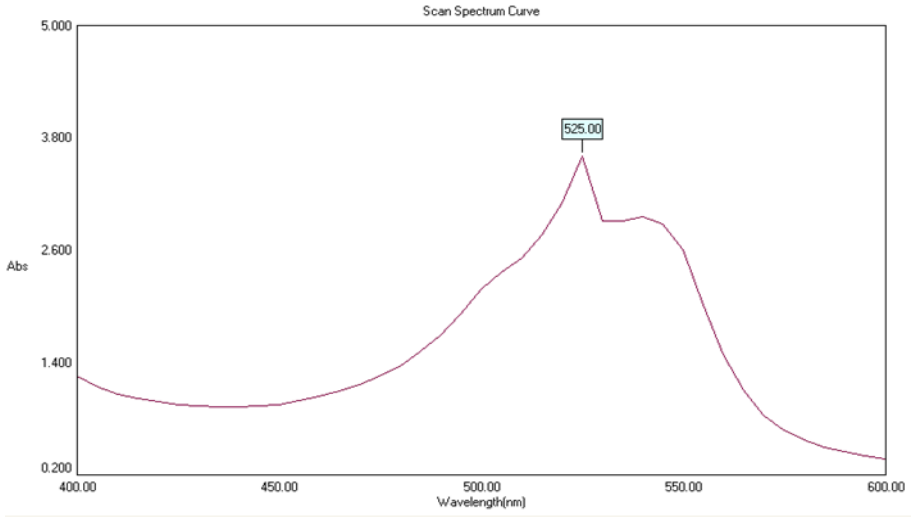

Figure 2.Absorption spectra of the purified pigment, extracted from *Serratia* spp

Ultra-violate visible light (UV-Vis) analysis: The optical properties of the biosynthesized ZnO NPs was examined using UV-Vis spectroscopy technique. As demonstrated in Figure 3, the attained ZnO NPs exhibited a pronounced UV absorption at around 287 nm. The stated absorption could be mainly attributed to the biosynthesized ZnO's direct band emission (12, 18).

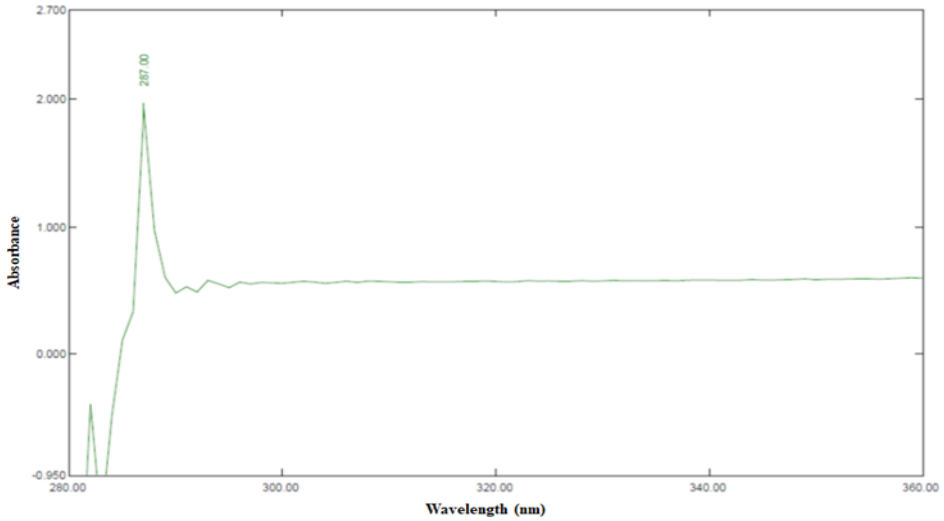

Figure 3.UV-Vis spectrum of the biosynthesized ZnO

Atomic force microscopy (AFM) analysis: The AFM was introduced to investigate the ZnO nanoparticles' surface features using a 2D and 3D imaging approach (Figure 4). Specifically, the AFM outcomes revealed that the ZnO nanoparticles exhibit a spherical shape with an average diameter size of 45.02 nm.

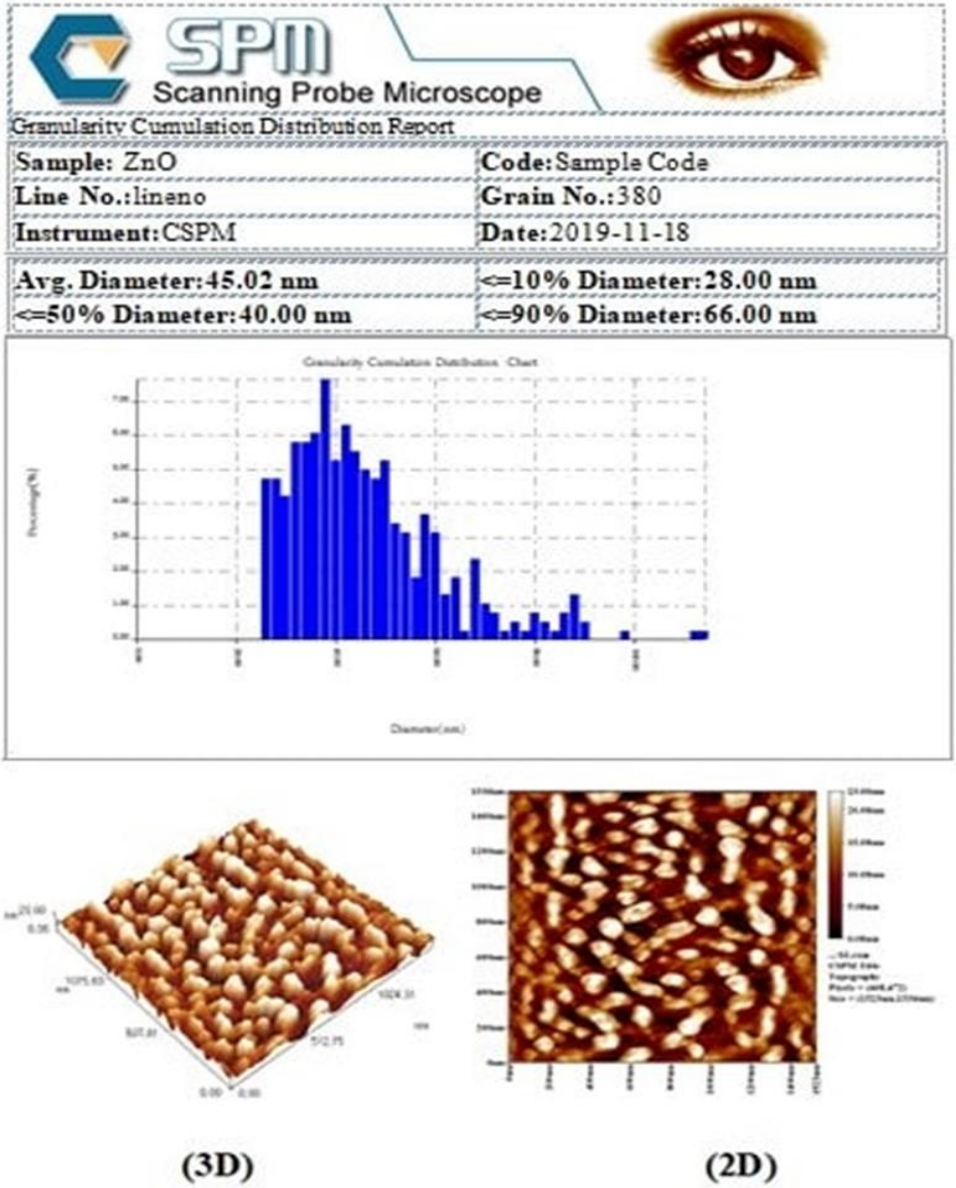

Figure 4. Atomic Force Microscopy of the bio-synthesized ZnO

X-ray diffraction (XRD) analysis: The XRD patterns obtained from the bio-synthesized ZnO nanoparticles is elucidated in Figure 5. Continuously, three pronounced peaks were observed between 2θ = 30 to 40 degrees (JCPDS No. 89-1397) which can be indexed to the occurrence of metal oxide ZnO nanoparticles hexagonal phase wurtzitestructure (26). In the meanwhile, the crystalline particles were calculated using the well-known Debye-Scherrer equation:

$$D = \left[\frac{K\lambda}{\beta cos\theta}\right] \text{Å}$$

Herein, the crystallite size is represented by the symbol (D), while K denotes the shape factor which is a constant (0.9) and λ is the x-ray wavelength (1.5406 Å). The Bragg angle and corrected line broadening of the nanoparticles are represented by the symbols $\theta$and β, respectively.

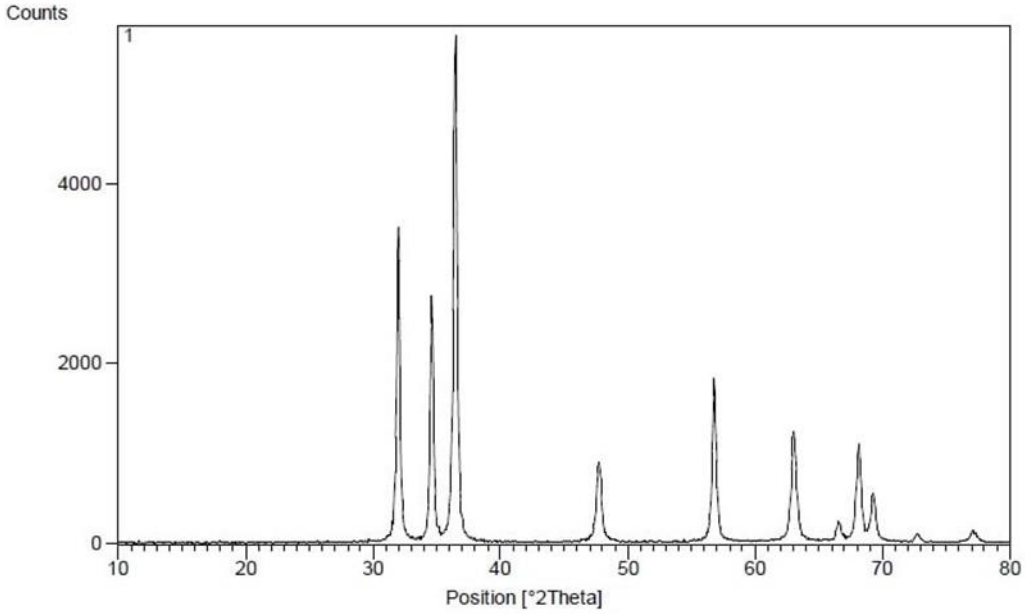

Figure 5. XRD patterns of the bio-synthesized ZnO nanoparticles

Fourier transforms infrared (FTIR) spectroscopy analysis: The FT-IR results for the bio-synthesized ZnO nanoparticles are demonstrated in Figure 5. Generally, absorption peaks series ranging between 400 to 4000 cm^{-1} could be noticed which are corresponded to the hydroxyl and carboxylate in materials. Particularly, a broadband around3120.61 cm^{-1} is attributed to the stretching mode of the C-Haromatics group. In the meanwhile, other peaks located within the 1560.30 $cm^{-1}$are mainly caused by the stretching vibration of N-O (Nitrocompounds). Furthermore, peak perceived at 1446.51 cm^{-1} is attributed to the stretching mode of C-C(in-ring) aromatics, and 694.33 cm^{-1}aremetal oxygen attributed to symmetrical as well as asymmetrical zinc carboxylate stretching (27).

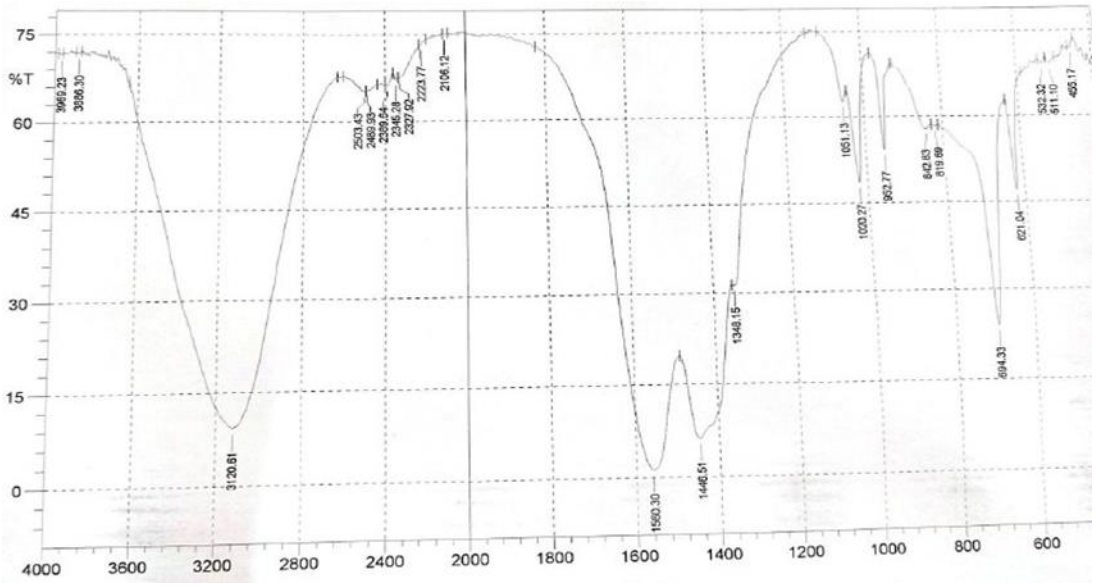

Figure 5. FTIR spectrum of the biosynthesized ZnO

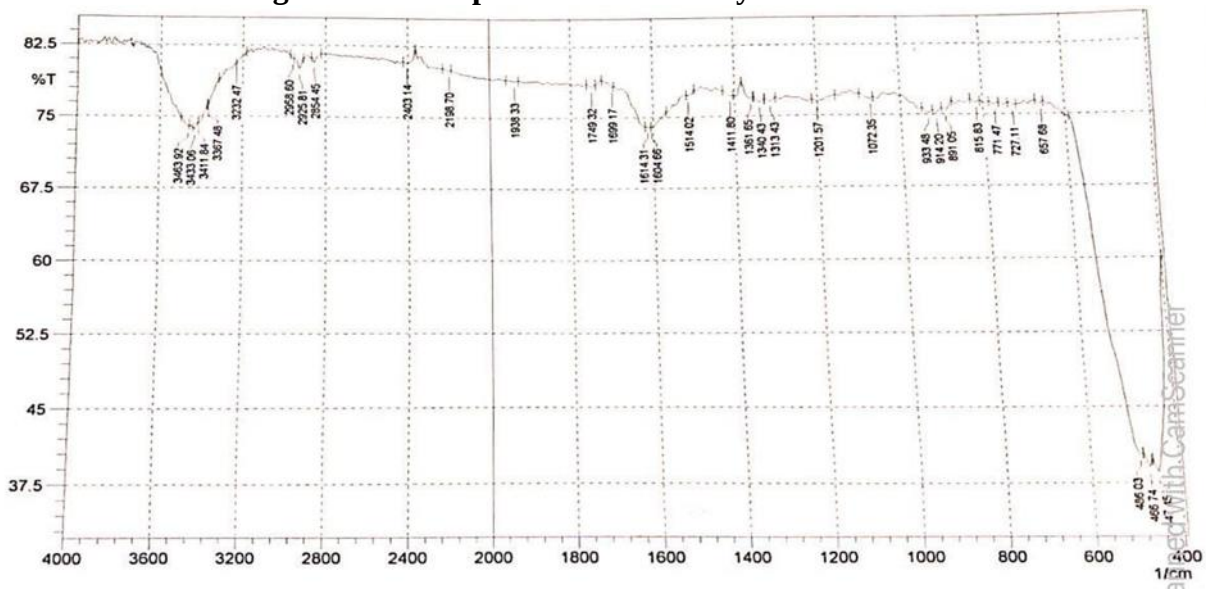

Figure 6.FTIR spectrum of the biosynthesized nano

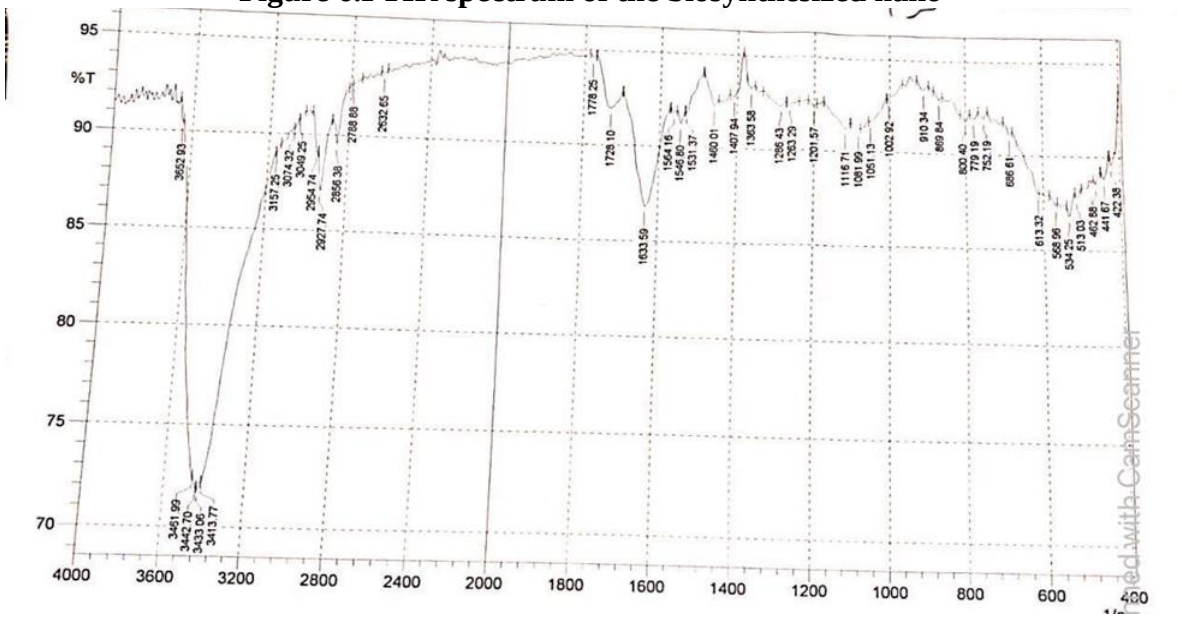

Figure 7. FTIR spectrum of the biosynthesized prodigiosin pigment

Field emission scanning electron microscopy (FE-SEM) analysis: The morphological properties of the bio-synthesized ZnO NPs were examined using the FE-SEM technique. As illustrated in Figure 6, the prepared ZnO NPs sample exhibited spherical particles as well as plate-like structures. It is worth mentioning that the average nanoparticles diameter was found to be around 40 nm using ImageJ software.

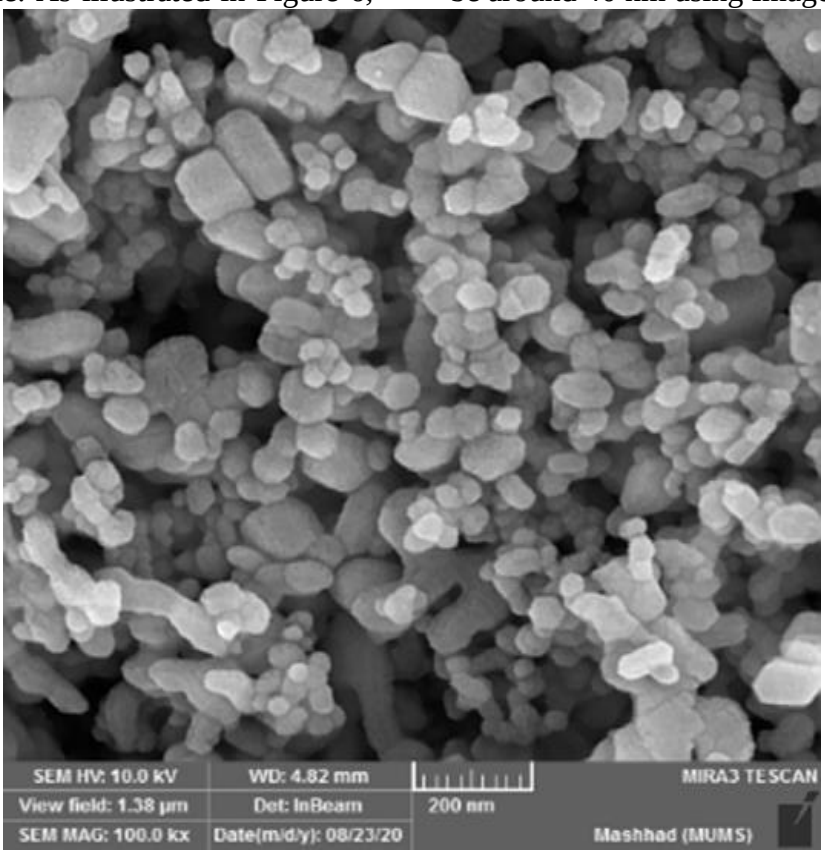

Figure 8.FE-SEM image of the bio-synthesized ZnO nanoparticles

Antibacterial susceptibility test: The antibacterial activity of the bio-synthesized ZnO nanoparticles at different concentrations (5,10, 20, 40, 80, 160, and 320) µg/ml is depicted in Figure 7 and Table 1. It is clear to be noticed that the ZnO NPs antibacterial activity is directly dependent on the utilized concentrations. Furthermore, Table 1 reveals that the concentration as low as 5 µg/ml of ZnO did not show any zone of inhibition, while an inhibition zone of 29mm was acquired at a ZnO concentration of 320µg/ml. The range in the inhibition zone, demonstrated in Table 1, could be attributed to different ZnO interaction mechanism with the microorganism utilized as well as the bacteria susceptibility. The ZnO NPs toxicity on any bacteria is mainly due to the reactive oxygen species (ROS)generation. Specifically, the ROS toxicity to the cell's wall is attributed to the cellular constituent damage like proteins, lipids, and DNA. The generation of ROS is widelyconsidered as the major factor of antibacterial activity associated with the ZnO phototoxicity(28, 29). This in turn leads to oxidation which in turn kills/inhibits the microorganisms. Using the serial dilution method, the MIC was evaluated with a range of 5-320µg/mL as pre-described CLSI (30, 31).The antibacterial activity of ZnO NPs is of great importance because of the pathogenetic bacteria's ability in joining the ecosystem food chain(32).

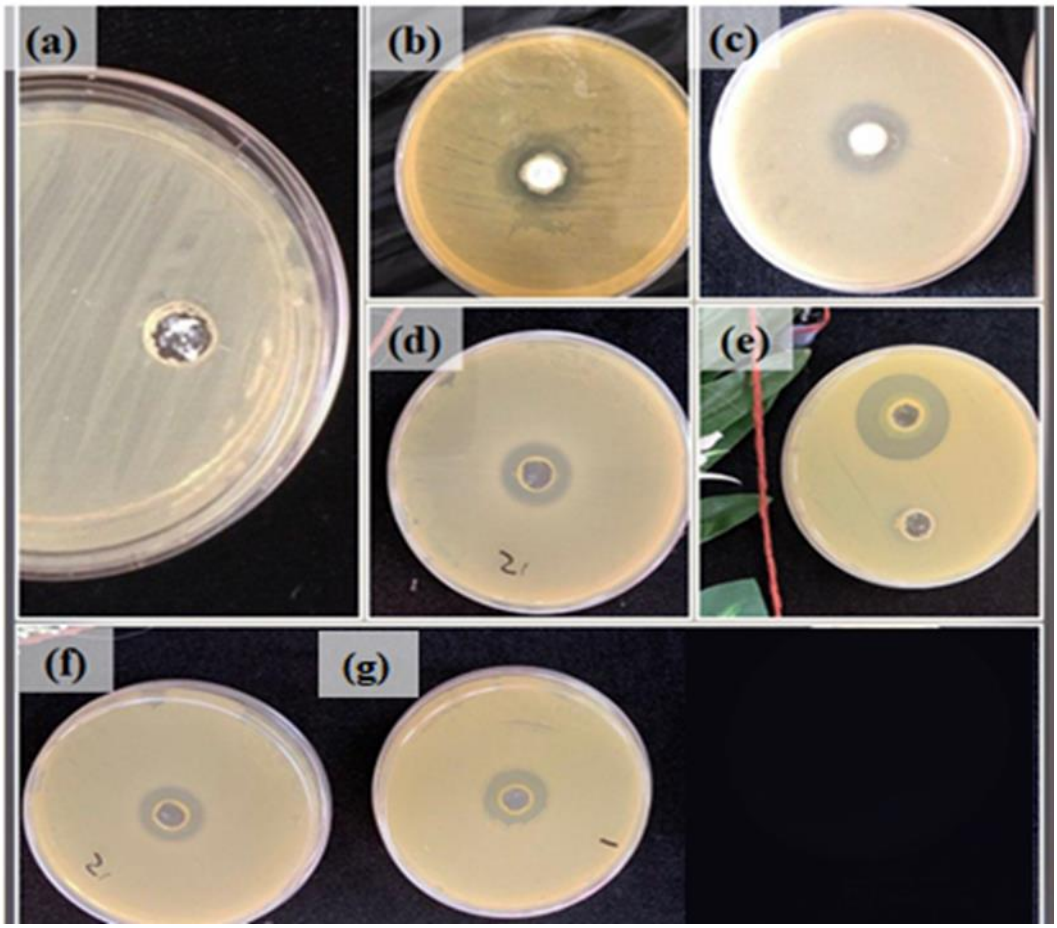

Figure 9. Antibacterial activity of the bio-synthesized ZnO nanoparticles against *ps*eudomonas aeruginosa at concentration of (a) 5, (b) 10, (c) 20 (d) 40, (f) 80, (g) 160 and (e) 320µg/ml

Table 1. Inhibition zone of ZnO nanoparticles

ZnO concentration (µg/mL)	Inhibition zone (mm)
5	Nill
10	7
20	15
40	19
80	21
160	25
320	29

CONCLUSION

In this study, the biosynthesis of ZnO nanoparticles using prodigiosin as a reducing agent was demonstrated successfully. Additionally, the attained ZnO NPs were characterized using UV-Vis, AFM, XRD, FT-IR, and FE-SEM. Techniques. In particular, The XRD patterns showed the successful ZnO NPs phase formation, while the FE-SEM demonstrated that the prepared ZnO NPsexhibited spherical particles as well as plate-like structures with an average diameter size ranging between 30-50 nm. While the AFM revealed an average diameter of 45.02 nm. In the antibacterial activity test, it was found that the bio-synthesized has a strong antibacterial activity against the introduced bacteria. The maximum inhibition zone was found to be 29 mm at a concentration of 320 µg/mL.

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