The investigation for eradication and inhibition of Gram-positive and Gram-negative bacteria by metal zinc oxide nanoparticles

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ABSTRACT

The purpose of the current research was to assess ZnO nanoparticles (ZnO NPs) effectiveness *versus* the bulk one as antibacterial agent towards Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) as well as Gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*). ZnO NPs were prepared through the interaction between Zn (CH_3COO) $2.2H_2O$ and NaOH. The titration reaction was performed by the addition of ethanol for obtaining a gel-like product. Then the gel was dried. On the other hand, the antibacterial activity assay was carried out using the agar well diffusion method. Overnight cultures of the test organisms (100 µl) were spread on tryptone soya agar in plates. Wells with diameters of about 6 mm were created aseptically and 20 µl of normal ZnO or ZnO NPs (whether Lab made or produced by Sigma) was performed at 25 or 50 µg/ml in wells against both tested Gram-negative and Gram-positive bacteria. The plates were incubated at 37 °C ± 1 °C for 24 and 48 h. Any resulting clear zone in mm (zone of inhibition) around the wells was measured. Antibacterial activity was recorded if the zone of inhibition was greater than 8 mm. The results showed that both transmission and scanning electron microscopies confirmed the nanoparticles being formed in Nano scale particles. The particle size ranged between 38.45 and 39.95 nm, with an average size 38.93 or ranged between 83.14 and 88.21 nm, with an average size 87.24 nm for Sigma or Lab made ZnO NPs, respectively. The particle size of normal was ranged between 551.25 and 553.20 nm, with an average size 552.2 nm. Z-potential for ZnO NPs showed negatively charged (-11.7 mV for Lab made and -11,6 for Sigma, respectively),while normal ZnO possessed ζ-potential value of +23.0 mV. Highest inhibition zone was observed at the concentration of 50 µg/ml ZnO NPs against all pathogens compared to lower one (25 µg/ml) of the ZnO NPs. Gram-negative bacteria, namely *Escherichia coli* or *Pseudomonas aeruginosa* exhibited relatively lower sensitivity towards ZnO, whether in the normal or rather in the NPs form (Sigma or Lab made), compared to the two studied Gram-positive bacterial strains (*Bacillus cereus* or *Staphylococcus aureus)*. It could be concluded that besides ZnO, especially in the NPs form, there is a promising inorganic substance with numerous applicable benefits in a wide range of industries. It is has a harmful impact against considerable pathogenic strains.

Keywords: Preparation of zinc oxide nanoparticles; Sigma ZnO NPs; transmission; scanning electron microscopies and ζ-potential properties of ZnO forms.

INTRODUCTION

Antibiotic-resistant bacteria are now a major global concern (Moravej *et al.*, 2018). In recent years, when looking at potential solutions to this issue, nanomaterials like metal oxide nanoparticles have emerged as intriguing contenders. The science of nanotechnology has substantially advanced due to its widespread use (Suresh *et al.*, 2016).

The health of people and animals is put at risk by the unchecked abuse of antibiotics, which encourages multidrug resistance in microbes. Therefore, it is imperative to create classic antimicrobial substitutes that are more potent and have novel action mechanisms (Li *et al*., 2010 and Nie *et al*., 2020). Nano biotics, sometimes referred to as nanoparticles (NPs) having antibacterial capabilities, is one of the current approaches to treat diseases that are resistant to several drugs. Due to their unique physicochemical and biological characteristics compared to their bulk phase, inorganic NPs, such as gold (Au NPs), silver (Ag NPs), zinc oxide (ZnO NPs), and selenium NPs (Se NPs), have extensive applications in the medical and biological fields, including medical diagnosis, biosensor, and personal care products (Li *et al*., 2010 and Nie *et al*., 2020). Among the NPs, ZnO NPs are particularly important as effective metal oxide NPs and showed a variety of antibacterial characteristics against a wide range of microorganisms, including major foodborne pathogens and Gram-positive and Gram-negative bacteria. ZnO NPs have a wide range of applications due to its characteristics like high surface area, biocompatibility, biodegradability, semiconductor behavior and UV Light barrier render their vast application (Ahmadi *et al.*, 2020).

Due to their potent bactericidal action linked to their small particles and higher surface energy, ZnO NPs have also been used as antimicrobial agents in animal feed (Ahmadi *et al*., 2020), topical lotions, and antibiotic agents in animal feed (Ye *et al*., 2020).

ZnO is an inorganic substance with many common uses. The Food and Drug Administration now recognizes ZnO as a substance that is generally regarded as safe (GRAS) and uses it as a food additive (Espitia *et al*., 2012). In vitro tests with *Staphylococcus aureus* and *Salmonella typhi* showed that ZnO NPs have antibacterial properties. ZnO NPs demonstrated the highest toxicity against microbes of all the metal oxide nanoparticles that have been examined to date (Hu *et al*., 2009). ZnO NPs have been shown to be a potential replacement for antibiotics in the production of poultry by Hidayat *et al*. (2021), and they also open up new opportunities for the control of harmful microorganisms.

The purpose of the current research was to assess ZnO NPs effectiveness *versus* the bulk one as antibacterial agent towards Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) as well as Gram-positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*).

MATERIALS AND METHODS

Materials

Bacterial strains

Cultures of *Escherichia coli ATCC8739*, *Pseudomonas aeruginosa ATCC9207*, *Bacillus cereus ATCC6633* and *Staphylococcus aureus ATCC7484* were obtained from the Sigma–Aldrich Co., St. Louis, USA.

Normal Zinc Oxide

Normal zinc oxide "Food grade" was purchased from Brighten Technology Pacific Co. LTD, Egypt.

Sigma Zinc Oxide nanoparticles

Nano zinc oxide "Food grade" made in Sigma–Aldrich Co., St. Louis, USA was purchased from Brighten Technology Pacific Co. LTD, Egypt.

Experimental procedures

Preparation of zinc oxide Nano particles (ZnO NPs)

ZnO NPs were prepared according to Chung, *et al.* (2015) as follow: At first, 20 g Zn (CH_3COO) $2.2H_2O$ b was mixed into 150 ml distilled water and stirred for 20 min at 35 °C to produce a zinc acetate solution. Again, 80 g NaOH powder was weighed, mixed into 80 ml water and stirred for around 20 min at 35°C for producing NaOH solution. After mixing both solutions, the titration reaction was performed by the addition of 100 ml ethanol into the drop-wise manner accompanied by vigorous stirring. The stirring was continued for around 90 min to complete the reaction for obtaining a gel-like product. Then the gel was dried at 80 °C overnight and calcined in an oven at 250 °C for 4 h. Finally, ZnO NPs were prepared. However, the overall chemical reaction for the preparation of ZnO NPs by using NaOH can be expressed as:

$$Zn(CH_3COO)2.2H_2O + 2NaOH \longrightarrow ZnO + 2NaCH_3COO + H_2O$$

Analytical methods

Determination of ZnO NPs characterization

Scanning electron microscopy (SEM)

The structural morphology of ZnO NPs was examined and measured using SEM, TM-1000 (Hitachi, Japan) as described by Nabila *et al.* (2018). An aliquot of each sample was fixed on a carbon-coated copper grid, and the film on the SEM grid was then dried by fixing it under a mercury lamp for 5 min. The instrument was equipped with an energy dispersive spectrum (EDS) to ensure the presence of nanoparticles.

Transmission electron microscopy (TEM)

The structural characterization and particle size examination of ZnO NPs were carried out using TEM, JEM-1230, JEOL (Akishima, Japan) as mentioned by Nabila *et al.,* (2018).The samples were put on the carbon-coated copper grid for 1 min to make a small film of the sample. The excess liquid was removed with a filter paper, and it was then set in a grid box sequentially.

Z-potential measurement for nano emulsion

The mean z-potential of ZnO NPs were determined as described by Hamed *et al.* (2019) using a Zeta sizer (Nano-ZS, Malvern Instruments, UK). Before measurement, samples were diluted with phosphate buffer solution (10mM, pH 7).

Determination of antibacterial activity of ZnO NPs *versus* normal ZnO

Tryptone soya agar (TSA) was used for the antimicrobial activity of ZnO NPs *versus* bulk ZnO against the Gram-negative bacteria (*E. coli* and *P. aeruginosa*) as well as Gram-positive

bacteria (*B. cereus* and *Staph. aureus*). Bacterial strains were firstly grown in tryptone soya broth followed by further activation on agar broth, the systems were preserved at 37 °C ± 1 °C for 24 h. to allow successive incubation. A concentration of colony-forming units (CFU) of 10^6 /ml was used according to Klink *et al.* (2022).

The antibacterial activity assay was carried out as in Balouiri (2016) using the agar well diffusion method with some modifications. Overnight cultures of the test organisms (100 µl) were spread on TSA in plates. Wells with diameters of about 6 mm were created aseptically using sterile cork poorer on the agar plates. Twenty µl of normal ZnO or ZnO NPs were performed at 25 or 50 µg/ml in wells against all tested Gram-negative and Gram-positive bacteria. The plates were incubated at 37 °C ± 1 °C for 24 and 48 h. Any resulting clear zone in mm (zone of inhibition) around the wells was measured. Antibacterial activity was recorded if the zone of inhibition was greater than 8 mm. The antibacterial activity results were expressed in term of the diameter of zone of inhibition and < 9 mm the zone was considered as inactive; 9-12 mm as partially active; while 13-18 mm as active and > 18 mm as very active. The diameter of inhibition zones was calculated. The agar well diffusion experiment was performed in duplicate.

RESULTS AND DISCUSSION

Characterization of the ZnO NPs *versus* normal ZnO

Morphology surface and particle characteristics

The ZnO NPs structure can be viewed in a TEM and SEM without further preparation and certain conclusions can be drawn from the images (Mason *et al.*, 2006).

Morphology and particle size characteristic of normal ZnO, ZnO NPs Sigma and ZnO NPs Lab made powder was examined using TEM with direct magnifications (40000 ×), the results are shown in Figure(1) [A(a and b), B(a and b) and C(a and b)] and SEM, the results are shown in Figure (2) [A, B and C]. Lab made ZnO NPs powder mixtures are depicted in Table (1), and Figure (1)[C(a and b] as opaque solutions with a smooth spherical Form Table (1), and Figure, (3C), and average mean size 87.24 nm, while the given size of that obtained from Sigma was 38.93 nm in Table (1), and Figure, (3B), *versus* that of normal ZnO, which was 552.2 nm in Table (1), and Figure (3A). Currently, materials made of structures with dimensions of around 1 to 100 nm are considered nanoscale materials (Ravichandran, 2009). The obtained results are similar to those of Hegazy and El-Agamy (2021) and Chang *et al.* (2021), who reported that the particle size of synthesized ZnO powder is about 84.98, 85.3 nm, respectively.

Furthermore, SEM image showed the interior structure of ZnO NPs showed dark granular shape with an irregular surface and aggregations (Figure, 2). In comparison with the previous mixtures description, the mixture of ZnO NPs was an opalescent solution, indicating the formation ZnO nanoparticles. The interior structure of ZnO NPs powder nanoparticles demonstrated a circular shape consisting of dark core.

Surface electrical charge of ZnO NPs

Zeta potential is a physical property which used to measure of the electrical charge of particles that are suspended in liquid. Colloidal system least stable at zero ζ-potential which called isoelectric point, ζ-potential value will be positive at low pH and negative at high pH, According to Orevi *et al.* (2014), a nano emulsion's physical stability can be ensured with a ζ-potential value of ±30 mV, ζ-potential values of ZnO NPs are displayed. Because the surrounding aqueous phase's pH (pH 7) was significantly higher than the isoelectric point of the materials used to coat the lipid droplets, the ζ-potential of Lab made ZnO NPs was -11.7 mV (Table, 2 and Figure, 4) indicating that, they were negatively charged like that of Sigma product (-11.6 mV). Nevertheless, ζ-potential value of normal ZnO was +23.0 mV (Table, 2 and Figure, 4).

Antibacterial activity

Many foods deteriorate primarily through microbial activity, which frequently results in a loss of quality and safety (Tauxe, 1997). Antibacterial compounds are used in food for two major purposes: to prevent or regulate the growth of bacteria for food safety and to control natural spoiling processes (Naidu, 2000). Antibacterial properties of ZnO NPs were measured using inhibition zone method against *B. cereus, E. coli*, *Staph. aureus* and *P. aeruginosa*. The results are shown in Figures (5, 6,7 and 8 as well as A, B, C and D) and Table (3).

Table (3) and Figures (5, 6,7 and 8) show that the existence of an inhibitory zone made the antibacterial activity of ZnO NPs very evident. Highest inhibition zone was observed at 50 µg/ml of the ZnO NPs concentration

against all pathogens for ZnO NPs compared to 25 μg/ml of the ZnO NPs. Low antimicrobial effects on tested Gram-negative bacteria, *E. coli* [Fig. (6,7 and 8A)] and *P. aeruginosa* [Fig. (6,7 and 8B)], were observed compared to Gram-positive bacteria, *B. cereus* [Fig. (6,7 and 8C)]) and *Staph. aureus* [Fig. (6,7 and 8D)]. These results agreed with those of Rana *et al*. (2014). Moreover, Mirhosseini and Firouz Abad (2015) found that whether in milk or in the media, ZnO NPs exhibit antibacterial action against *L. monocytogenes* and *B. cereus*. Furthermore, Manna (2012) declared that, ZnO NPs' precise antibacterial mechanism is still a mystery. However, a number of putative ZnO NPs antibacterial processes have been put forth, including the generation of reactive oxygen species, the contact of the nanoparticles with the bacterium and consequent harm to the bacterial cell, and the release of Zn^{2+}. Results also indicated that ZnO NPs had highest an antimicrobial activity as a compared to the bulk zinc ZnO powder. The antibacterial activity of engineered ZnO NPs was examined by Yamamoto *et al.* (1998) against gram-negative and gram-positive pathogens, namely *E. coli* and *S. aureus* and compared with commercial zinc oxide powder. They observed that polymer coated spherical ZnO NPs showed maximum bacterial cell destruction compared to normal ZnO powder.

CONCLUSION

ZnO is a promising inorganic substance with numerous applicable benefits in a wide range of industries. This current study sought to evaluate and discuss research that addressed the possible use of NPs for their antibacterial activity. Highest inhibition zone was observed at the concentration of 50 μg/ml ZnO NPs against all pathogens compared to lower one (25 μg/ml) of the ZnO NPs. Gram-negative bacteria, namely *Escherichia coli* or *Pseudomonas aeruginosa* exhibited relatively lower sensitivity towards ZnO, whether in the normal or nanoparticles form compared to the two studied Gram-positive bacterial strains (*Bacillus cereus* or *Staphylococcus aureus*).

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Table 1: The mean dimeter of particles of normal ZnO, Sigma ZnO NPs and Lab made ZnO NPs

	dimeter average (nm)		
Particle No.	Normal ZnO	Sigma ZnO NPs	Lab made ZnO NPs
1	553.20	39.95	88.21
2	552.75	39.13	88.13
3	552.45	39.12	87.95
4	552.40	39.11	87.93
5	552.37	39.10	87.92
6	552.35	38.94	87.75
7	552.34	38.93	87.54
8	552.33	38.90	87.49
9	552.25	38.89	87.36
10	551.95	38.84	87.23
11	551.91	38.81	87.22
12	551.85	38.72	86.95
13	551.82	38.65	86.91
14	551.78	38.46	86.87
15	551.25	38.45	83.14
Mean dimeter	552.2 nm	38.93 nm	87.24 nm

Table 2: Zeta potential value of normal ZnO, Sigma ZnO NPs and Lab made ZnO NPs

Normal ZnO	Sigma ZnO NPs	Lab made ZnO NPs
+23 mV	-11.6 mV	- 11.7 mV

Table 3: Antibacterial activity of normal ZnO, Sigma ZnO NPs and Lab made ZnO NPs on some strains grown in Tryptone Soya Agar

Organism	Gram dye response	Zone inhibition (mm)					
		ZnO Normal		ZnO NPs Sigma		ZnO NPs Lab made	
		25 µg/ml	50 µg/ml	25 µg/ml	50 µg/ml	25 µg/ml	50 µg/ml
Escherichia coli	negative	8	12	12	18	11	17
Pseudomonas aeruginosa	negative	9	13	13	20	12	19
Bacillus cereus	positive	10	18	16	23	15	23
Staphylococcus aureus	positive	12	19	17	26	16	25

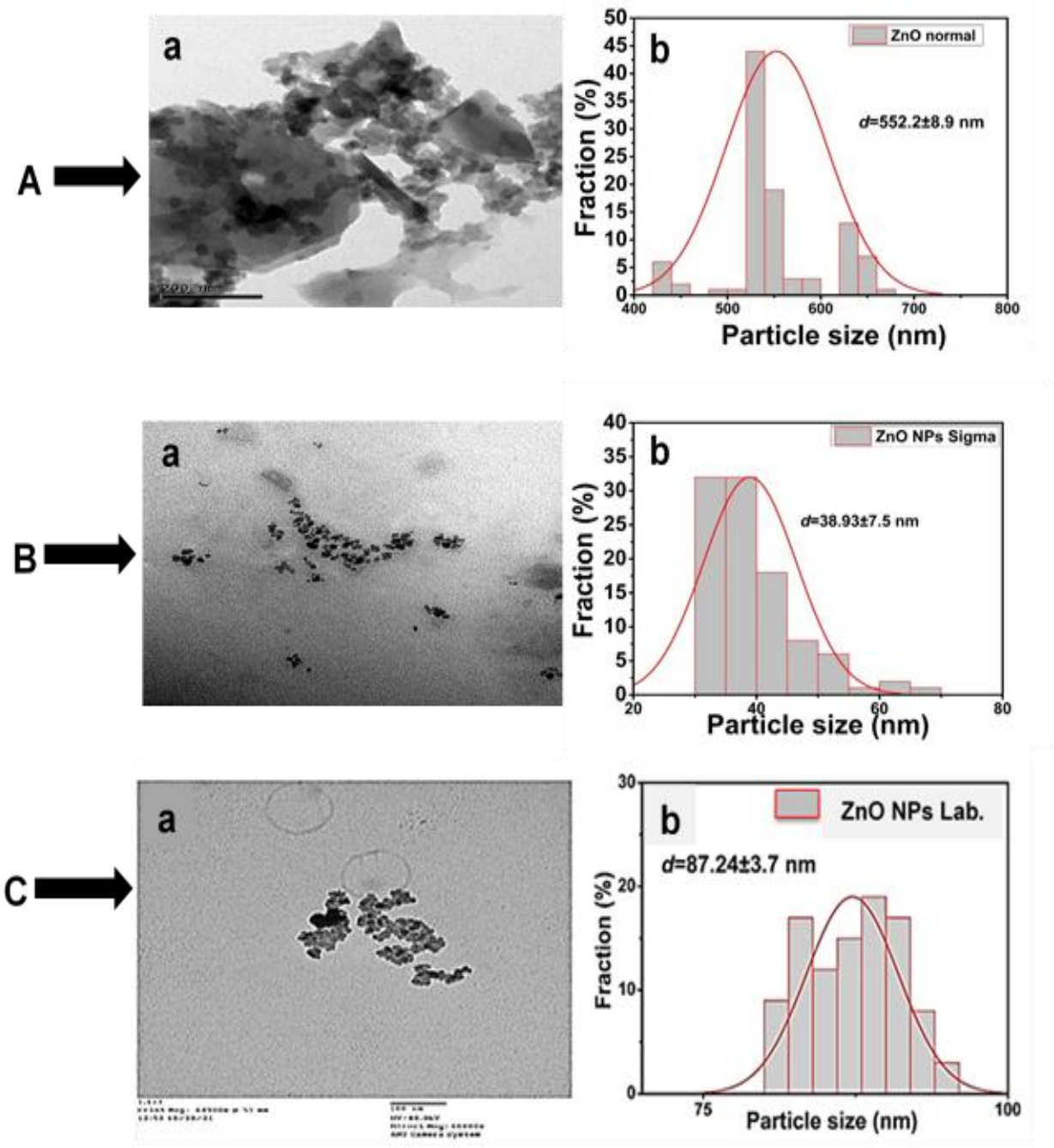

Figure 1: TEM photographs of (A) normal ZnO, (B) Sigma ZnO NPs and (C) Lab made ZnO NPs, respectively using 40000 x magnifications showing nanoparticles shapes and size

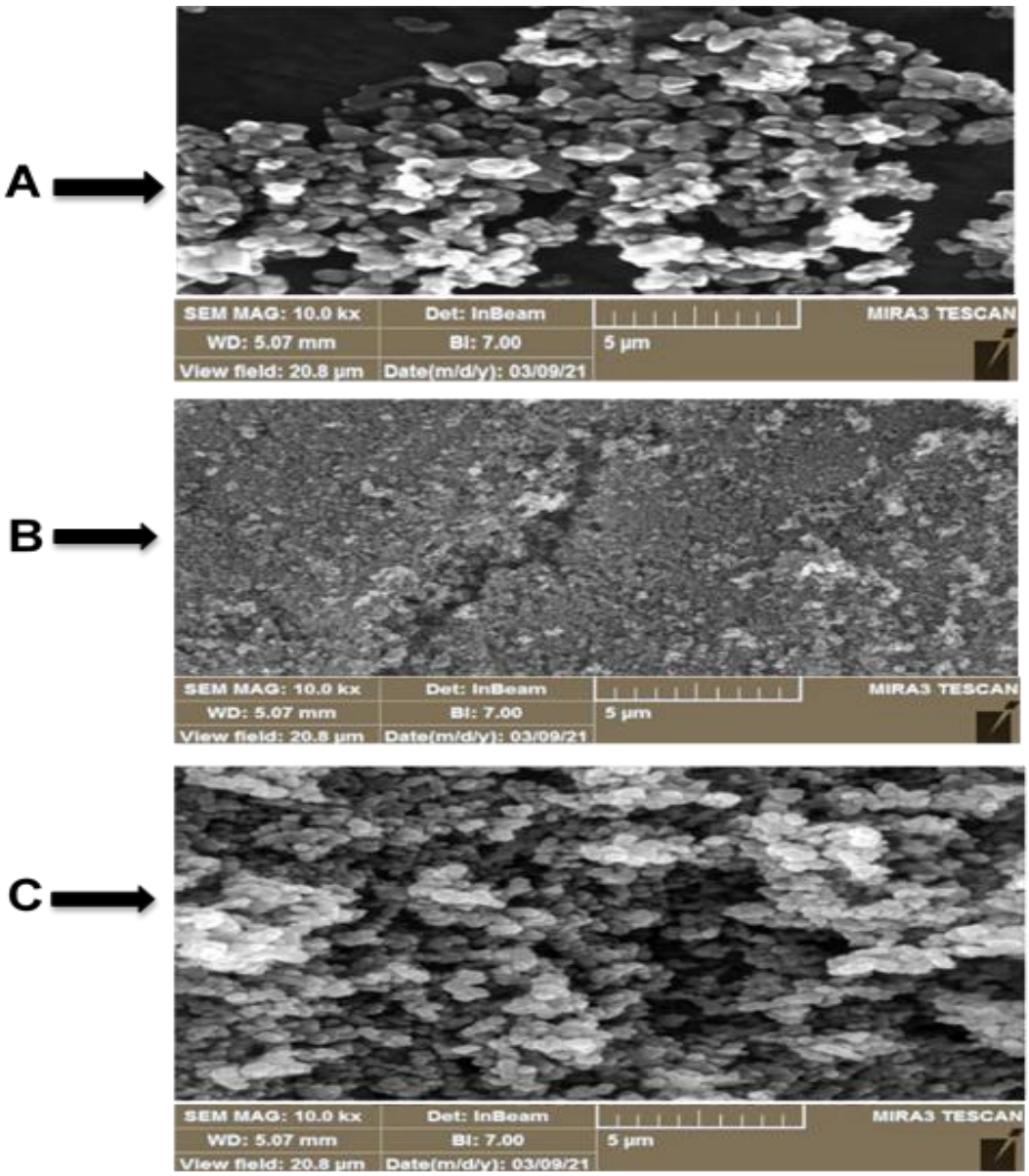

Figure 2: SEM image of (A) normal ZnO, (B) Sigma ZnO NPs and (C) Lab made ZnO NPs, respectively using 10.0 kx magnifications

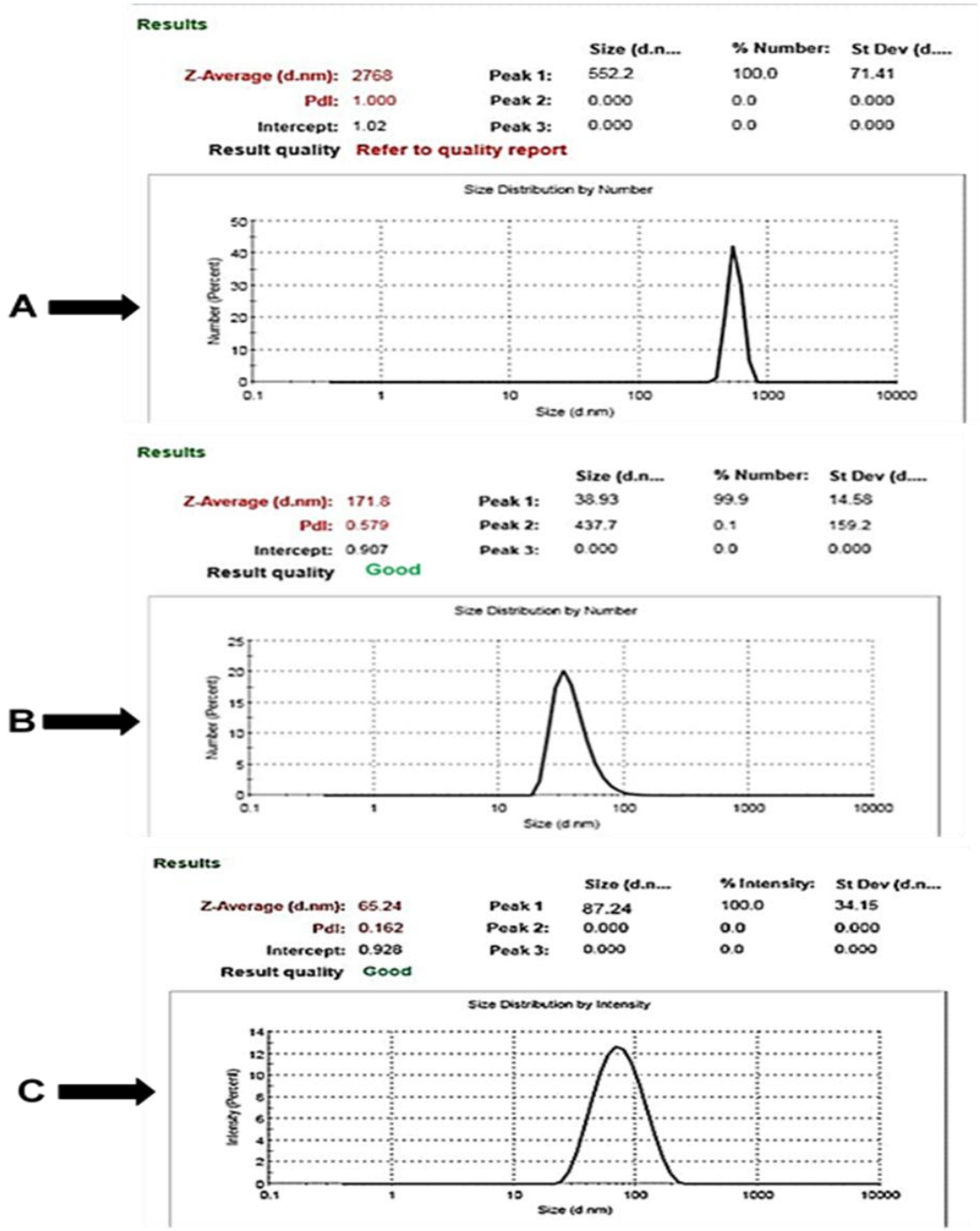

Figure 3: The mean dimeter of Average Particles Size of (A) normal ZnO, (B) Sigma ZnO NPs and (C) Lab made ZnO NPs

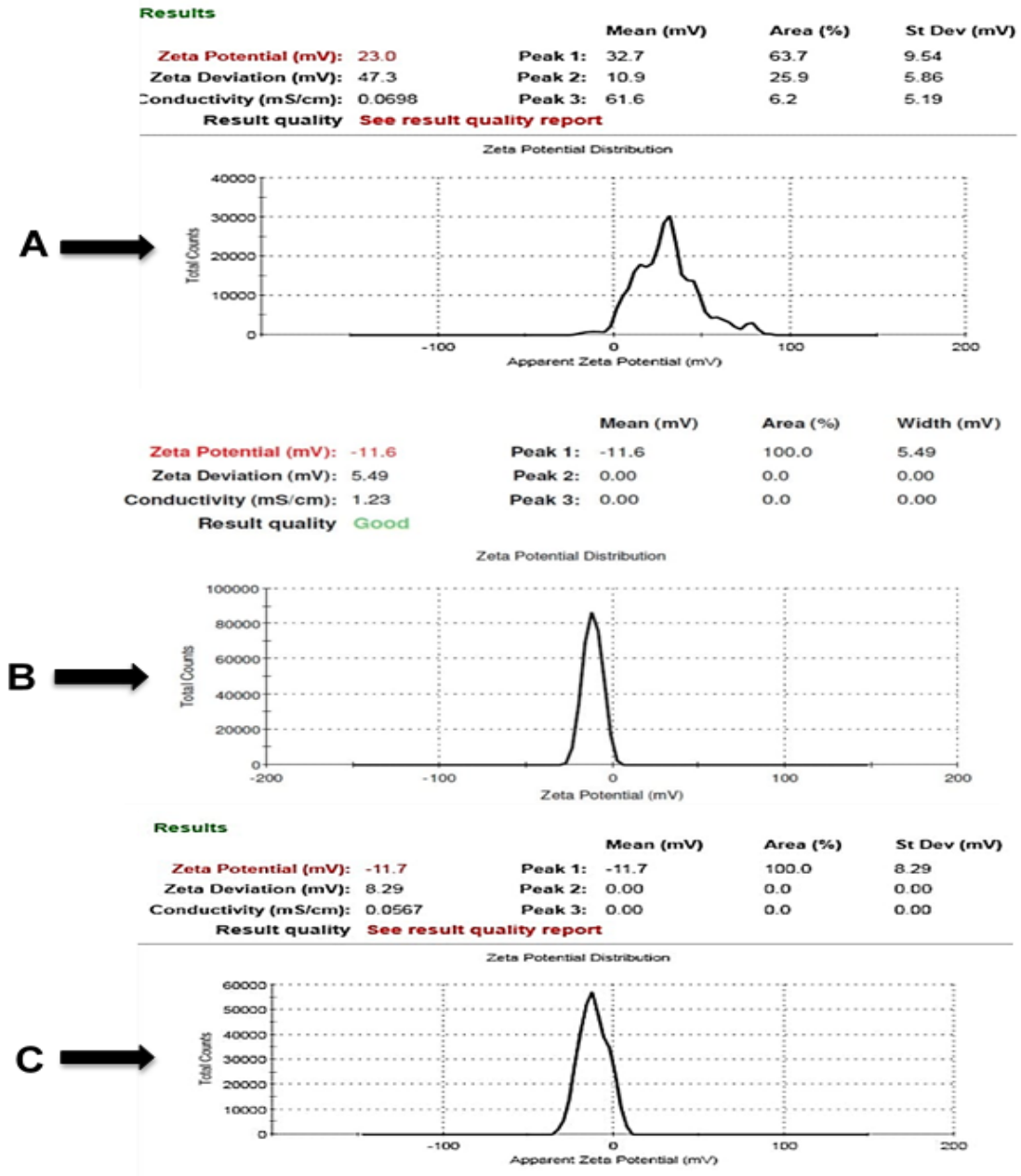

Figure 4: Zeta potential value of (A) normal ZnO, (B) Sigma ZnO NPs and (C) Lab made ZnO NPs, respectively

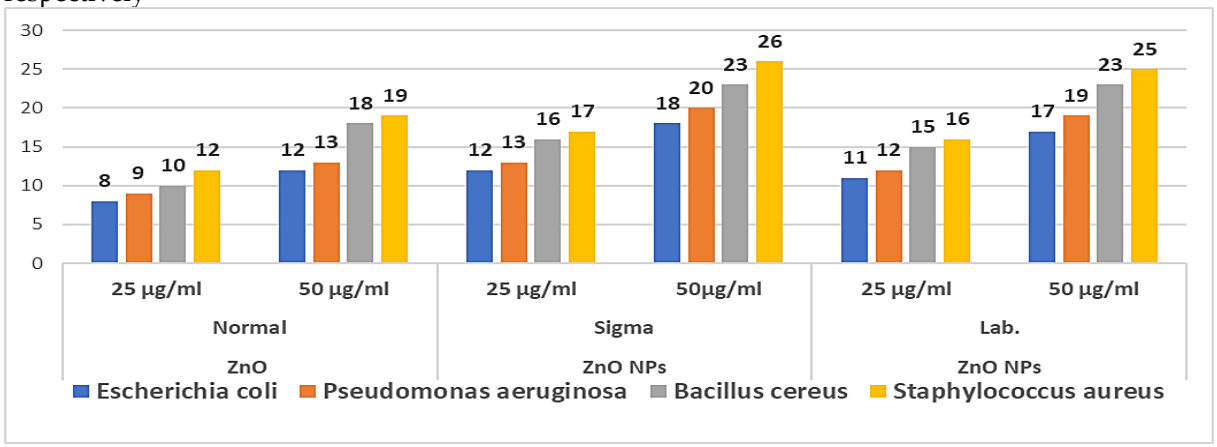

Figure 5: Antibacterial activity of normal ZnO, Sigma ZnO NPs and Lab made ZnO NPs on some strains grown in Tryptone Soya Agar

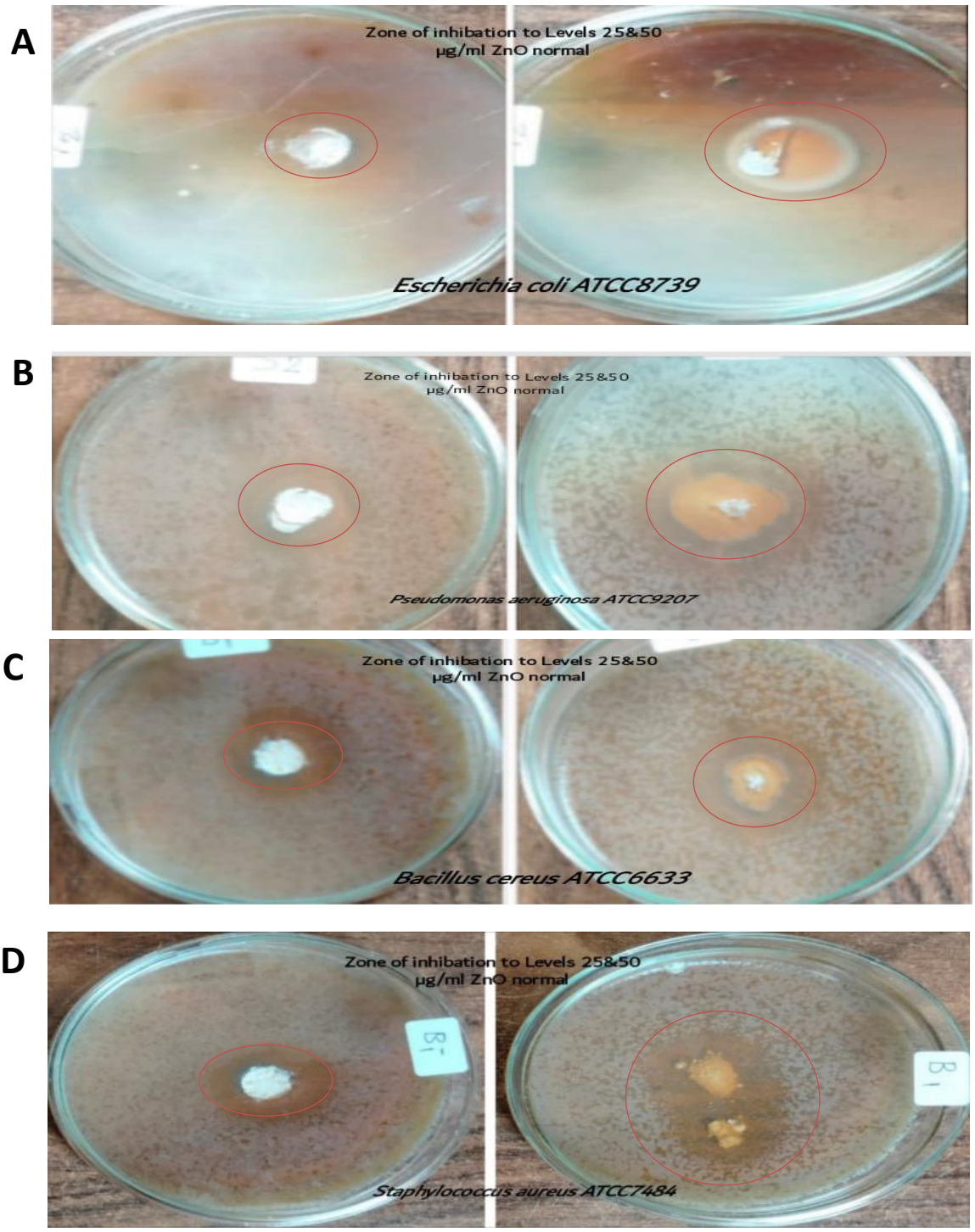

Figure 6: Inhibition zone due to the presence of 25 or 50 µg/ml of normal ZnO of (A) *Escherichia coli*, (B) *Pseudomonas aeruginosa*, (C) *Bacillus cereus and (D) Staphylococcus aureus* grown in Tryptone Soya Agar

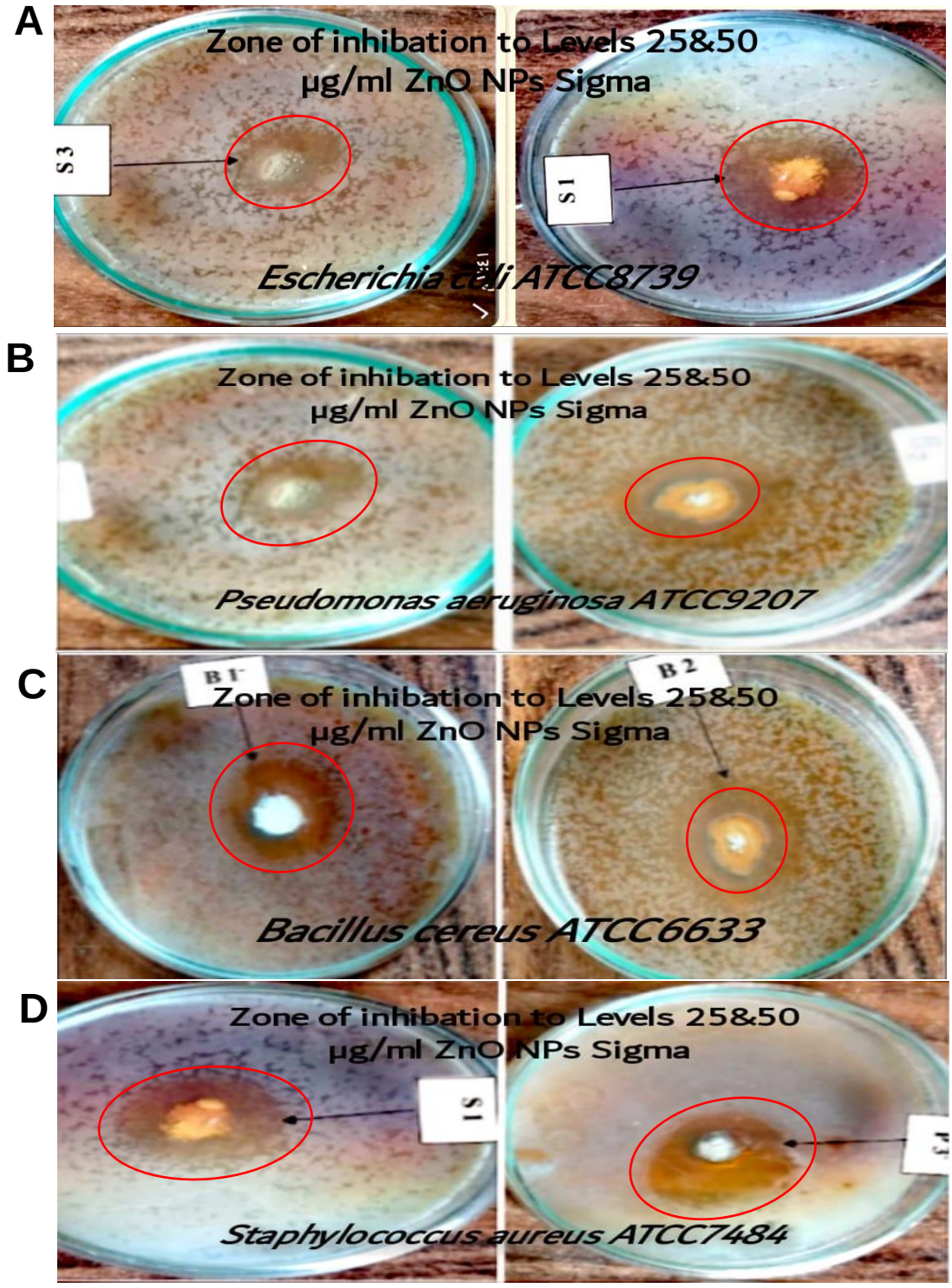

Figure 7: Inhibition zone due to the presence of 25 or 50 µg/ml of Sigma ZnO NPs of (A) *Escherichia coli,* (B) *Pseudomonas aeruginosa,* (C) *Bacillus cereus and (D) Staphylococcus aureus* grown in Tryptone Soya Agar

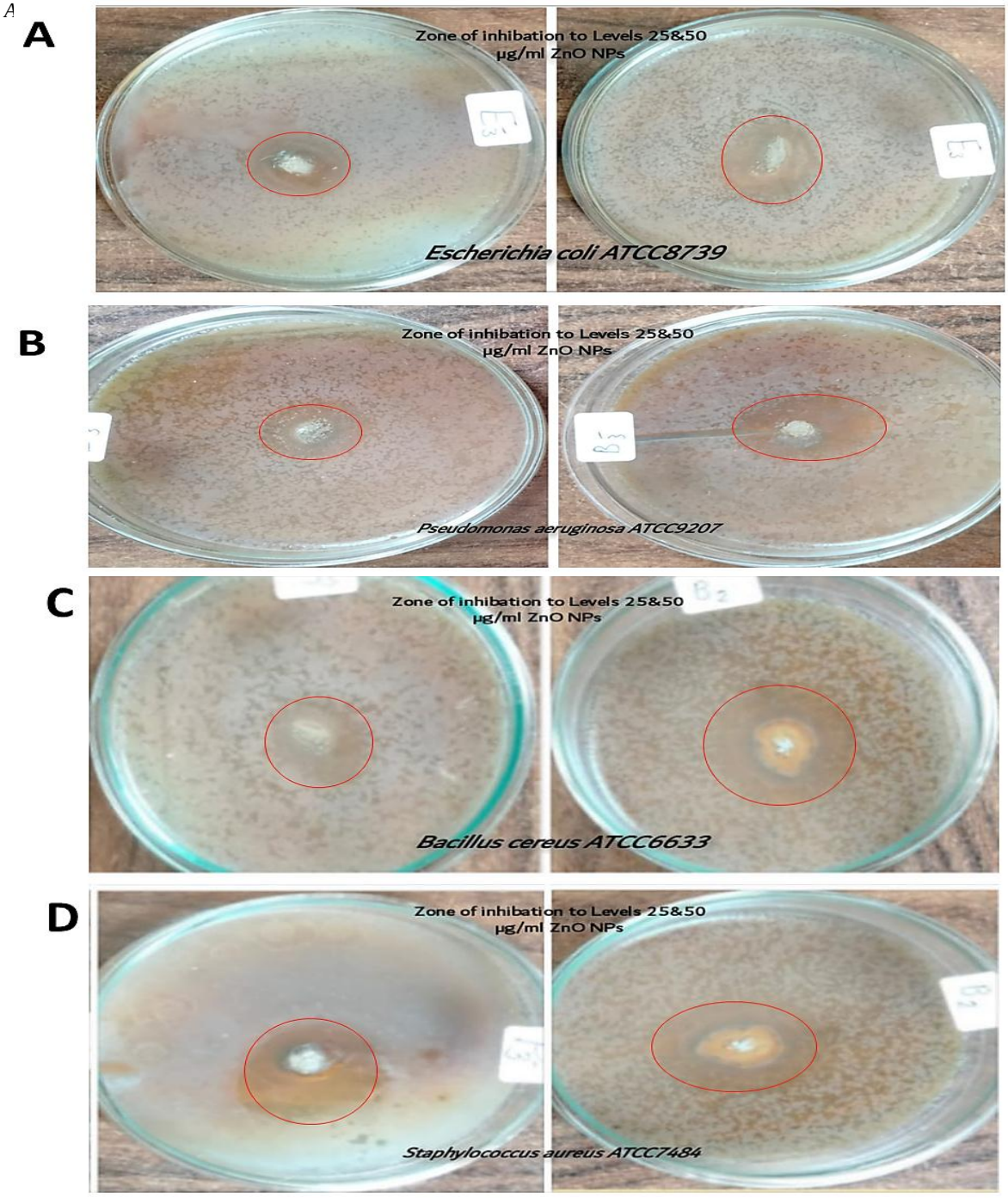

Figure 8: Inhibition zone due to the presence of 25 or 50 µg/ml of Lab made ZnO NPs of (A) *Escherichia coli*, (B) *Pseudomonas aeruginosa*, (C) *Bacillus cereus and (D) Staphylococcus aureus* grown in Tryptone Soya Agar

التحقيق في استئصال وتثبيط البكتيريا موجبة الجرام وسالبة الجرام بواسطة جزيئات أكسيد الزنك المعدنية النانوية

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الملخص العربى

الغرض من البحث الحالي هو تقييم فعالية الجزيئات النانوية لأكسيد الزنك (ZnO NPs) مقابل الجسيمات العادية لأكسيد الزنك كمواد مضادة للبكتيريا تجاه البكتيريا سالبة الجرام (*Escherichia coli* و*Pseudomonas aeruginosa*) وكذلك البكتيريا موجبة الجرام (*Bacillus cereus* و*Staphylococcus aureus*). تم تحضير ZnO NPs من خلال التفاعل بين $Zn(CH_3COO)_2.2H_2O$ و NaOH. ثم تم إجراء تفاعل المعايرة بإضافة الإيثانول للحصول على منتج يشبه الهلام (الجل) . كما تم تجفيف الجل. من ناحية أخرى ، تم إجراء اختبار النشاط المضاد للبكتيريا باستخدام طريقة الإنتشار على الآجار. ولقد تم نشر المزارع البكتيرية المنماة مسبقا لمدة ليلة (100 ميكرولتر) على أجار تربتون الصويا في أطباق. تم إنشاء آبار بأقطار حوالي 6 مم بمعدات معقمة وضع 20 ميكرولتر من ZnO الطبيعي أو ZnO NPs ، سواء المشترى من سيجما أو المحضر معمليا ، بتركيز 25 أو 50 ميكروغرام / مل في الآبار ضد كل من البكتيريا سالبة الجرام والموجبة الجرام. حيث تم تحضين الأطباق على 37 درجة مئوية ± 1 درجة مئوية لمدة 24 و 48 ساعة. تم قياس قطر أي منطقة ناتجة خالية من النموات بالملليمتر (منطقة التثبيط) حول الآبار. على أن تم تسجيل النشاط المضاد للبكتيريا إذا كانت منطقة التثبيط أكبر من 8 مم. أظهرت النتائج أن كلا من الميكروسكوب الإلكتروني النافذ والمسحى transmission and scanning electron microscopies أكدا أن الجسيمات النانوية تتشكل في جسيمات مقياس النانو حيث يتراوح حجم الجسيمات بين 38.45 و 39.95 نانومتر ، بمتوسط حجم 38.93 نانومنر لـ ZnO NPs المشترى من سيجما بالمقارنة بالمركب النانو المحضر معمليا حيث يتراوح حجم الجسيمات بين 83.14 و88.21 نانومتر ، بمتوسط حجم 87.24 نانومتر فى حين أن حجم الجسيمات فى المركب العادى كانت تتراوح بين 551.25 و 553.20 نانومتر ، بمتوسط حجم 552.2 نانومتر. أظهرت النتائج أيضا أن قيم زيتا الوضعية 0 -potential لـ ZnO NPs (سيجما) كانت سالبة الشحنة بقيمة -11.6 مللي فولت بالمقارنة بالمحضرة معمليا والتي كانت سالبة الشحنة أيضا بقيمة -11.7 مللي فولت بالمقارنة بالمركب العادى الذى كان له شحنة موجبة بقيمة +23.0 مللى فولت. لوحظ أكبر منطقة تثبيط عند تركيز 50 ميكروغرام / مل من ZnO NPs ، سواء المحضر معمليا أو إنتاج سيجما ، ضد جميع البكتيريا الممرضة مقارنة بالتركيز الأقل (25 ميكروغرام / مل) من ZnO NPs. كما أظهرت البكتيريا سالبة الجرام ، وهي *E. coli* أو *P. aeruginosa* ، حساسية أقل نسبيًا تجاه ZnO وهذا يعني أنها أكثر مقاومة تجاه ZnO ، سواء كانت في الصورة الطبيعية أو الجسيمات النانوية ، مقارنة بالسلالتين البكتيريتين الموجبة الجرام محل الدراسة (*B. cereus* أو *B. cereus*) و *Staph. aureus*). ويمكن الإستنتاج أنه إلى جانب كون ZnO ، وخاصة في الصورة النانوية NPs ، فإنه مادة غير عضوية واعدة لها العديد من الفوائد القابلة للتطبيق في مجموعة واسعة من الصناعات ، فإن لها أيضا تأثير مثبط للسلالات المسببة للأمراض سواء السالبة أو الموجبة لجرام إلا أن تأثيرها تجاه الموجبة لجرام أعلى من السالبة مما يدل على أن السالبة أكثر مقاومة نسبيا من الموجبة .

الكلمات الإسترشادية: تحضير الجزيئات النانوية لأكسيد الزنك، الميكروسكوب الإلكتروني النافذ والمسحى، خصائص زيتا الوضعية للجزيئات النانونية لأكسيد الزنك.