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 (CH₃COO)₂.2H₂O 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:COO) 2.2H2O 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:

\[
\text{Zn(CH}_3\text{COO) } 2.2\text{H}_2\text{O}+2\text{NaOH} \rightarrow \text{ZnO} + 2\text{NaCH}_3\text{COO}+\text{H}_2\text{O}
\]

**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 mm 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 Zn²⁺. 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*).

**REFERENCES**


Mirhosseini, M., Firouz, B.F. 2015: Reduction of *Listeria monocytogenes* and *Bacillus cereus* in...


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

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<th>Particle No.</th>
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<th>Lab made ZnO NPs</th>
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Table 2: Zeta potential value of normal ZnO, Sigma ZnO NPs and Lab made ZnO NPs

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<th>Sigma ZnO NPs</th>
<th>Lab made ZnO NPs</th>
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<td>+23 mV</td>
<td>-11.6 mV</td>
<td>-11.7 mV</td>
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Table 3: Antibacterial activity of normal ZnO, Sigma ZnO NPs and Lab made ZnO NPs on some strains grown in Tryptone Soya Agar

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<th>Organism</th>
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<th>Zone inhibition (mm)</th>
<th>Zone inhibition (mm)</th>
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<td></td>
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<td>25 µg/ml</td>
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<td>Escherichia coli</td>
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<td>8</td>
<td>12</td>
<td>12</td>
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</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>positive</td>
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<td>19</td>
<td>17</td>
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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 diameter 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 النانوية

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الخلاصة:
الغرض من البحث الحالية هو تقييم فعالية الجزيئات النانوية لكسيد الزنك (ZnO NPs) مقابل الجسيمات العادية لكسيد الزنك كمواد مضادة للكبكتيريا تجاه البكتيريا سالبة الجرام (Escherichia coli وPseudomonas aeruginosa) وكذالك البكتيريا موجبة الجرام (Bacillus cereus وStaphylococcus aureus).

تم تحضير ZnO NPs من خلال التفاعل بين NaOH وZn (CH₃COO)₂.2H₂O و Что NaOH، و بضافة الكحول للحصول على منتج يشبه الهلام (الجل). ثم تم إجراء اختبار التسرب الكيميائي باستخدام طريقة الاستشارة و منتج الجل مع الماء الساخن (100 ميكروتيرل). و احتواء البكتيريا في اطراف الأبر. و إضافة ال ZnO NPs الطبيعية أو ZnO NPs الصناعية بعد 6 ساعات من اضافة البكتيريا و إضافة الجل (الجل). ثم تم إجراء Tests الفيزيائيات واستشعار البكتيريا في الإبر بأقطار حوالي 6 مم بمعدات معقمة وضع 21 ميكروغرام من ZnO NPs في الأبر، سواء المشتري من سوسنة أو المحضر محليًا، بترك 21 أو 48 ساعة. ثم قياس قطر منطقة ناتجة خالية من النموات بلملل (منطقة التثبيط) حول الأبر. على أن تم تسجيل النشاط المضاد للبكتيريا إذا كانت منطقة التثبيط بقدر 21 مكروغرام / مل من ZnO NPs (من المشتري أو المحضر محليًا) كانت أكبر من منطقة التثبيط عند تركيز 48 مكروغرام / مل من ZnO NPs (من المشتري أو المحضر محليًا) ضد جميع البكتيريا الممرضة مقارنة بلتركيز ال 48 مكروغرام / مل من ZnO NPs (من المشتري أو المحضر محليًا).

النتائج:
نتيجة تتماشى مع كشفة التعرض النانوي للجزيئات، حيث زادت نسبة نبات البكتيريا بين 38 و 95 نانوغرام / مل، متوسط حجم ZnO NPs حوالي 38.93 نانوغرام / مل. ZnO NPs من سوسنة بين 83.14 و 88.21 نانوغرام / مل، متوسط حجم ZnO NPs 87.24 نانوغرام / مل. ZnO NPs من المشتري بين 55.52 و 55.20 نانوغرام / مل، متوسط حجم ZnO NPs 55.52 نانوغرام / مل. حمضية كانت سالبة الشحنة 0.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 11.6 مللي فولت، بحيث كانت سالبة الشحنة 23.0 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 1.6 مللي فولت، بحيث كانت سالبة الشحنة 4.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 7.6 مللي فولت، بحيث كانت سالبة الشحنة 10.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 13.6 مللي فولت، بحيث كانت سالبة الشحنة 16.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 19.6 مللي فولت، بحيث كانت سالبة الشحنة 22.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 25.6 مللي فولت، بحيث كانت سالبة الشحنة 28.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 38.6 مللي فولت، بحيث كانت سالبة الشحنة 41.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 44.6 مللي فولت، بحيث كانت سالبة الشحنة 47.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 50.6 مللي فولت، بحيث كانت سالبة الشحنة 53.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 56.6 مللي فولت، بحيث كانت سالبة الشحنة 59.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 62.6 مللي فولت، بحيث كانت سالبة الشحنة 65.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 68.6 مللي فولت، بحيث كانت سالبة الشحنة 71.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 74.6 مللي فولت، بحيث كانت سالبة الشحنة 77.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 80.6 مللي فولت، بحيث كانت سالبة الشحنة 83.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 86.6 مللي فولت، بحيث كانت سالبة الشحنة 89.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 92.6 مللي فولت، بحيث كانت سالبة الشحنة 95.6 مللي فولت. ZnO NPs من المشتري كان له شحنة موجبة 98.6 مللي فولت، بحيث كانت سالبة الشحنة 101.6 مللي فولت.

الكلمات المشتركة:
تحضير الجزيئات النانوية لكسيد الزنك، الميكروسكوب الإلكتروني النافذ والمسحى، خصائص زيتا الوضعية لـZnO NPs.