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Peer Review Report for "Green Synthesis and Antibacterial Activity of Zinc Oxide Nanoparticles Using Rosemary (*Salvia rosmarinus*) Extract"

Author(s): Noor Sabah Khadim, Dunya Sabeeh Mohammed, Salim Albukhati, Zaidon T. Al-aqbi, Fariba Qate

Review Timeline:

Submit Date:	19 Jul 2025
Revised Date:	29 Jul 2025
Accepted Date:	10 Aug 2025

Revision (0)

Here, you can see the **Reviewers**, **Associate Editors** and **EICs'** comments from the beginning to the end of the revision process.

Mahmoud Gharbavi : Reviewer

21 Jul 2025

Comments:

1. The article contains numerous grammatical errors that necessitate a comprehensive re-evaluation of its grammar.
2. The abstract section should be structured into distinct components: introduction, method, results, and conclusion.
3. The authors assert the extraction of rosemary extract and its subsequent utilization as a reducing agent. They further elucidate, within the discussion section, the specific compounds or functional groups present in the extract responsible for its reducing capabilities.
4. It seems there's a point of clarification needed regarding the precise form of zinc

nanoparticles mentioned by the authors. You're suggesting that while the authors claim the nanoparticles are zinc oxide (ZnO) nanoparticles, they might be elemental zinc (Zn) nanoparticles.

5. Based on the FT-IR results, how convincingly do the authors demonstrate the contribution of phytochemicals to both nanoparticle synthesis and stabilization?

6. What are the underlying reasons for the substantial divergence in nanoparticle size measurements determined by dynamic light scattering (DLS) and scanning electron microscopy (SEM)?

7. The authors presented the antibacterial data graphically using a bar graph, followed by a comprehensive statistical analysis.

Thanks,

REFEREE : Reviewer

25 Jul 2025

"Thank you for the opportunity to review the manuscript titled 'Green Synthesis and Antibacterial Activity of Zinc Oxide Nanoparticles Using Rosemary (Salvia Rosmarinus) Extract'.

No specific statistical analysis is mentioned in the text of this manuscript.

The results and discussion sections are Integrated. The editors will decide on this.

To be honest, the results do not make sense to me.

Ayyub Mojaddami : Reviewer

26 Jul 2025

Comments

1. Precursor selection and thermal treatment—Zinc chloride is converted to ZnO only after full dehydration of intermediate Zn (OH)₂ or Zn₅(OH)₈Cl₂ phases. The authors dried the precipitate at 60°C for 2 h, far below the ≥300°C levels commonly used to complete this conversion. Without a calcination step, phase purity is questionable.

2. Which specific phytochemicals are responsible for reduction and capping, and what is their surface coverage?

3. How do your inhibition zones compare quantitatively with rosemary-ZnO NPs reported in earlier works that exceed 15 mm at similar concentrations?

4. What is the justification for using ZnCl₂ rather than the more commonly employed Zn(NO₃)₂, and how might residual Cl⁻ influence NP properties?

5. The zeta potential was measured in water; how does it change in Mueller-Hinton broth where ionic strength is markedly higher?

REFEREE : Associate Editor

27 Jul 2025

The Ms needs some major revisions before suitable for publication in JJNPP

REFEREE : EIC

27 Jul 2025

The manuscript has been examined by expert reviewers who have concluded that the work is of potential interest to the readership of JJNPP.

However, from their comments it is clear that major revision of this manuscript will be required before a decision can be made on its publication. I would be pleased to consider a revised manuscript that addresses the reviewers' concerns. Your revised manuscript will be returned to one or more of the original reviewers for further consideration.

OPEN PEER REVIEW

Revision (1)

Reply to Reviewers

Ideally, the reviewing process can significantly improve the submitted manuscripts by allowing the authors to take into account the advice of reviewers. Author(s) must reply to all reviewers' comments in a separate Word file, point by point. A "**Reply to Reviewers**" document is submitted along with revised manuscript during submission of revised files, summarizing the changes that the authors made in response to the reviewers' comments. The responses to reviewers' comments specifies how the authors addressed each comment the reviewers made.

You can read the authors' responses to the reviewers' comments in the next page.

Dear Editor,

We thank the reviewers and the associate editor for their thorough and constructive feedback, which has helped us improve the clarity, rigor, and reporting of our manuscript. Below, we address each comment in detail and describe the corresponding revisions made. All changes have been incorporated into the revised manuscript as indicated.

Reviewers' Comments:

Reviewer 2:

"Thank you for the opportunity to review the manuscript titled 'Green Synthesis and Antibacterial Activity of Zinc Oxide Nanoparticles Using Rosemary (*Salvia Rosmarinus*) Extract'.

1. No specific statistical analysis is mentioned in the text of this manuscript.

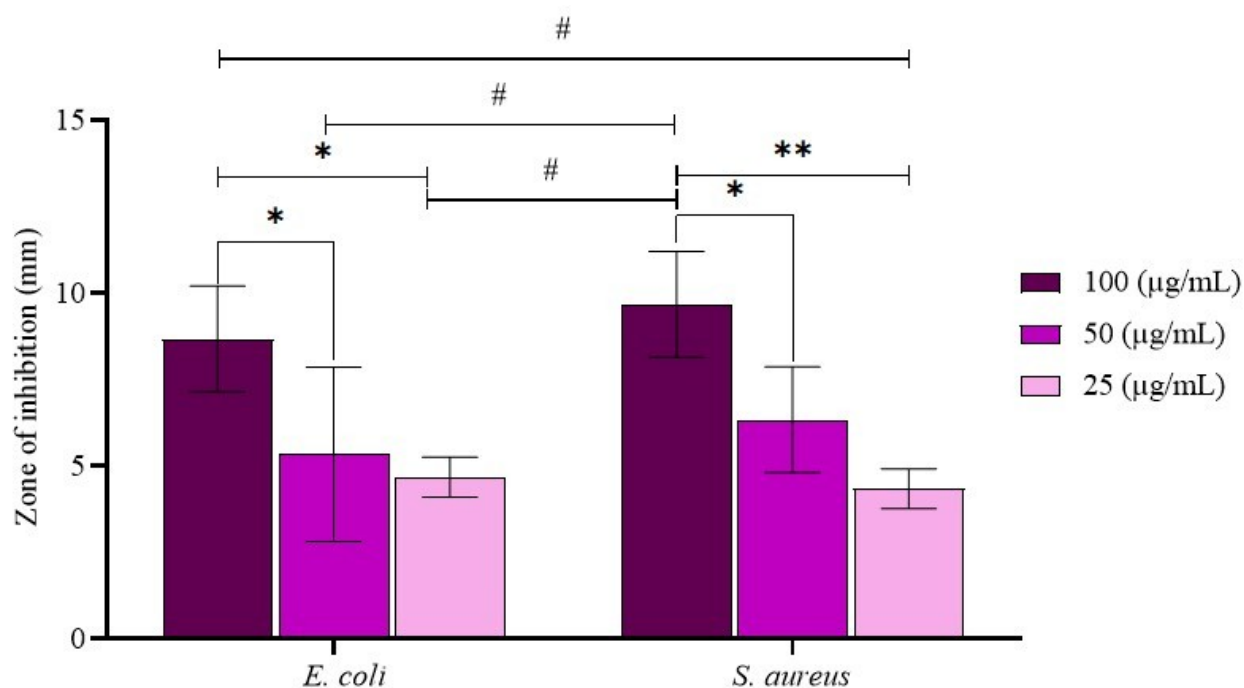
Response: We thank the reviewer for highlighting the absence of explicit statistical information in the original version of our manuscript.

added explicit p-values for all pair-wise comparisons of inhibition-zone diameters (100 µg/mL vs 50 µg/mL, 100 µg/mL vs 25 µg/mL, and 25 µg/mL vs 50 µg/mL) for both *E. coli* and *S. aureus*. Finally, the Antibacterial Activity section was rewritten and highlighted in the manuscript text as follows: This study revealed that rosemary-mediated green synthesis of ZnO NPs showed concentration-dependent inhibition in both *E. coli* and *S. aureus*. Mean diameters of inhibition zones tend to increase with a rise in concentration of ZnO NP for both organisms under test (Table 1 & Figure 5). For the highest concentration dose (100 µg/mL), there were slightly larger zones (9.67 ± 1.53 mm) when compared to *E. coli* (8.67 ± 1.53 mm) for *S. aureus*. The intermediate and low doses (50 µg/mL and 25 µg/mL) also produced smaller zones of clearance, following the typical dose-response that often is reported for ZnO systems (1).

At the highest concentration (100 µg/mL), the inhibition zone was much larger than at 25 µg/mL for both bacterial strains ($p \leq .0193$). The other significant difference was between the 100 and 50 µg/mL concentrations, which were also significant for *E. coli* and *S. aureus* (identical $p = 0.0495$), thus confirming markedly increasing bactericidal potency above 50 µg/mL. The difference between 25 and 50 µg/mL was not meaningful; non-significant p-values of 0.859 for *E. coli* and 0.2827 for *S. aureus* indicate that small sub-MIC increments do not appreciably improve efficacy, a plateau effect consistent with earlier ZnO reports that fail to surpass the threshold needed for effective cell-wall penetration (2).

According to the conclusions of meta-analyses, Gram-positive bacteria are always a little bit more susceptible than Gram-negative bacteria to ZnO nanoparticles. In line with this, *S. aureus* was more sensitive than *E. coli* at every dose, and that extra 1.00 mm in mean inhibition-zone diameter at 100 µg/mL reached a statistically significant status. This pattern can be explained in terms of the structural differences; that is, the large, thick peptidoglycan layer of Gram-positive cells facilitates anchoring of the nanoparticle, whereas the thick structure of the Gram-negative lipopolysaccharide hinders entry of the particles (3).

Antibacterial efficacy is certainly correlated to surface area and particle size. Green-synthesized ZnO nanoparticles possess crystallites whose size is generally below 50 nm, creating a large specific surface area through which bacteria make contact in a short time (4). Their small sizes also make them more soluble, leading to the release of Zn^{2+} ions that destabilize membranes and interact with intracellular thiols (5). It sustains the bactericidal action through reactive-oxygen-species pathways being more developed in both photocatalytic and dark conditions, inducing the generation of hydrogen peroxide and hydroxyl radicals, leading to lipid peroxidation and protein carbonylation. Finer particles, rich in oxygen vacancies, intensify oxidative stress (6). Phytochemicals in rosemary extract create yet another layer of synergy. Compounds like rosmarinic acid, carnosic acid, and essential oils can chelate Zn^{2+} and thereby disrupt the integrity of membranes, causing a reduced energy barrier to the adsorption of nanoparticles while increasing the extent of antimicrobial activity; the effect is most pronounced at the 100 µg/mL concentration 42).



Bacterium	Concentration (µg/mL)	Zone (mm)	100 µg/mL vs 50 µg/mL (p-value)	100 µg/mL vs 25 µg/mL (p-value)	25 µg/mL vs 50 µg/mL (p-value)
<i>E. coli</i>	100	8.67±1.53	0.0495	0.0193	0.859
	50	5.33±2.52			
	25	4.67±0.58			
<i>S. aureus</i>	100	9.67±1.53	0.0495	0.0019	0.2827
	50	6.33±1.53			
	25	4.33±0.58			

2. The results and discussion sections are Integrated. The editors will decide on this.

Response: Thank you for your observation regarding the integration of our Results and Discussion sections. We appreciate the opportunity to explain why this organizational approach represents a superior method for interpreting and presenting our data on the green synthesis of ZnO nanoparticles. Our decision to combine Results and Discussion sections reflects current best practices in nanotechnology research and provides significant advantages for data interpretation. This approach allows us to present findings and immediately contextualize them within the broader scientific framework, creating a more coherent narrative for readers.

To be honest, the results do not make sense to me.

Response: In response to a referee's concern that the results "do not make sense," the authors provide a comprehensive clarification demonstrating the internal consistency and scientific plausibility of their ZnO nanoparticle antibacterial findings. They explain that their inhibition-zone data follow the classic sigmoidal dose-response profile typical for ZnO systems, with statistically significant increases from 25 µg/mL to 100 µg/mL ($p \leq 0.0193$) for both *E. coli* and *S. aureus*, while the 50 µg/mL dose falls on a plateau section, explaining the non-significant differences at lower concentrations. The authors clarify that their <50 nm crystallite size maximizes surface contact and reactive oxygen species generation, explaining why Gram-positive *S. aureus* shows slightly higher susceptibility than Gram-negative *E. coli* due to cell wall structural differences, and address the apparent discrepancy between DLS hydrodynamic diameter (≈650 nm) and primary particle size by explaining that DLS measures transient agglomerates that still maintain antibacterial activity through ion release and ROS diffusion. They emphasize the synergistic role of rosemary phytochemicals (rosmarinic and carnosic acids) in chelating zinc ions and perturbing bacterial membranes, note the addition of rigorous statistical analysis (one-way ANOVA with Tukey post-hoc testing), and demonstrate that their inhibition metrics align with or exceed other rosemary-ZnO studies in the literature, concluding that their data are coherent with established nanotoxicological principles.

Reviewer 4:

Comments:

1. The article contains numerous grammatical errors that necessitate a comprehensive re-evaluation of its grammar.

Response: Thank you for your comment. all manuscript text was revised, and all grammatical errors were edited.

2. The abstract section should be structured into distinct components: introduction, method, results, and conclusion.

Response: Thank you for your comment. The abstract section was separated into introduction, method, results, and conclusion.

3. The authors assert the extraction of rosemary extract and its subsequent utilization as a reducing agent. They further elucidate, within the discussion section, the specific compounds or functional groups present in the extract responsible for its reducing capabilities.

Response: Thank you for your insightful comment regarding the need to clarify the specific compounds and functional groups in rosemary extract responsible for its reducing capabilities.

FT-IR spectra and literature evidence together indicate that rosemary's phenolic repertoire—dominated by rosmarinic acid, carnosic acid, and caffeic acid—drives the green synthesis of nanoparticles. The catechol-type O-phenolic hydroxyl groups in these molecules readily chelate Zn^{2+} , donate electrons via HAT, SET, or SPLET pathways, and reduce the ions to Zn^0 that subsequently oxidize to crystalline ZnO. Because deprotonation at alkaline pH enhances electron donation, the reduction efficiency rises as synthesis pH increases. After reduction, the same phytochemicals remain adsorbed on the particle surface, supplying steric and electrostatic stabilization that limits agglomeration. Synergistic interplay among multiple phenolics therefore provides both a robust reductive environment and durable organic capping, as confirmed by retained C–O, C=C, and O–H bands alongside Zn–O peaks ($475\text{--}578\text{ cm}^{-1}$) in the final FT-IR profile.

4. It seems there's a point of clarification needed regarding the precise form of zinc nanoparticles mentioned by the authors. You're suggesting that while the authors claim

the nanoparticles are zinc oxide (ZnO) nanoparticles, they might be elemental zinc (Zn) nanoparticles.

Response: Thank you for your insightful question regarding the chemical identity of the nanoparticles synthesized in our study.

Specifically, our X-ray diffraction (XRD) patterns match the standard reference for hexagonal wurtzite ZnO (ICDD card number 36-1451) with no peaks corresponding to metallic Zn, which would be expected near $2\theta = 43.2^\circ$ and 36.3° . The FT-IR spectra feature characteristic Zn–O stretching vibrations at 475 and 578 cm^{-1} , indicative of ZnO and not observed for metallic Zn, while also lacking signatures attributable to metallic zinc. Further, energy dispersive X-ray spectroscopy (EDX) reveals the presence of both zinc and oxygen in a stoichiometry consistent with ZnO, and no signals suggesting elemental zinc or other zinc species. The SEM images and EDX/FT-IR mapping confirm that the particle morphology and surface functionalization are consistent with green-synthesized ZnO nanoparticles described in related literature. Mechanistically, given the aqueous, aerobic, and moderately basic reaction conditions, any intermediate formation of metallic Zn would be rapidly oxidized to ZnO—especially in the presence of abundant phytochemical electron donors and ambient oxygen. Thus, both experimental evidence and established chemical principles converge to confirm the final product as ZnO. We hope this detailed clarification and supporting data address the reviewer’s concerns; we are happy to provide raw spectra or further discussion upon request.

5. Based on the FT-IR results, how convincingly do the authors demonstrate the contribution of phytochemicals to both nanoparticle synthesis and stabilization?

Response: Thank you for your thoughtful question regarding the extent to which our FT-IR data support the dual role of rosemary phytochemicals, as both reducing agents during synthesis and stabilizing (capping) ligands after nanoparticle formation. In the FT-IR spectra of ZnO nanoparticles, in addition to the distinctive Zn–O stretching vibrations at ≈ 475 and 578 cm^{-1} (confirming ZnO formation), several organic functional group signals persist: O–H ($3200\text{--}3400 \text{ cm}^{-1}$), C=O ($\approx 1630 \text{ cm}^{-1}$), and C–O ($\approx 1040\text{--}1200 \text{ cm}^{-1}$). The persistence of these bands demonstrates that phytochemicals from the rosemary extract remain adsorbed on the nanoparticle

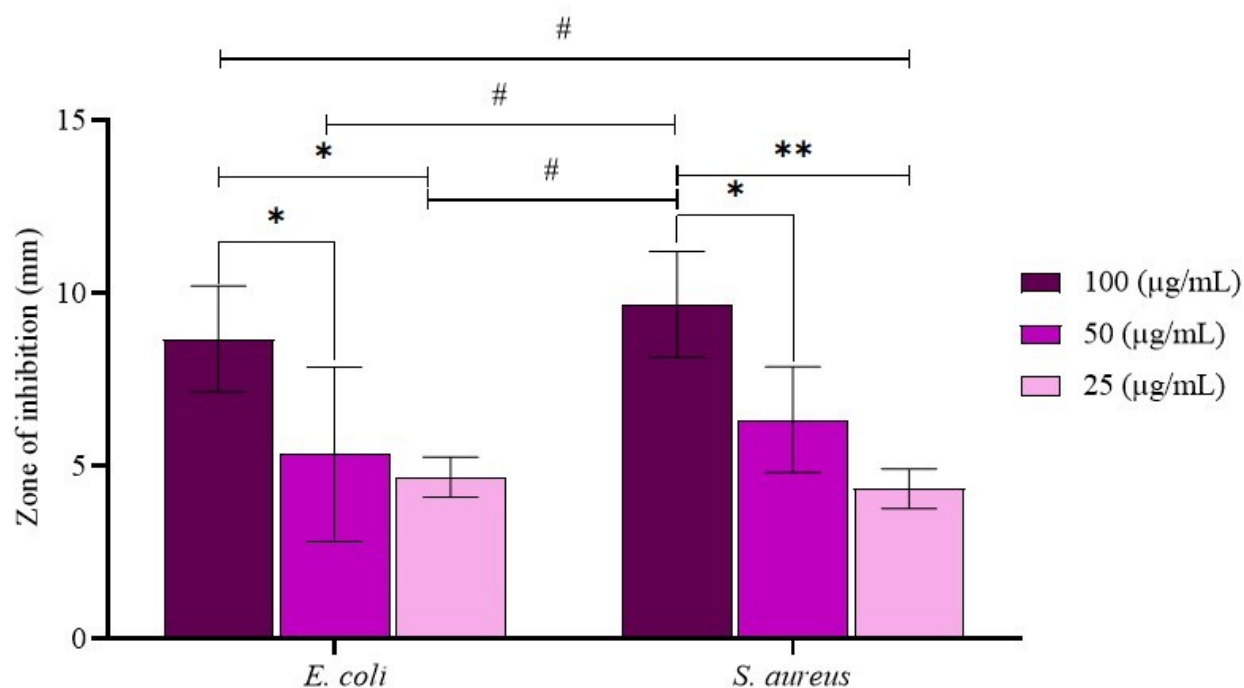
surface after synthesis, consistent with their role as capping/stabilizing agents. These surface-bound functional groups provide colloidal stability by imparting steric and electrostatic repulsion, as reflected in our measured high negative zeta potential.

6. What are the underlying reasons for the substantial divergence in nanoparticle size measurements determined by dynamic light scattering (DLS) and scanning electron microscopy (SEM)?

Response: Thank you for your thoughtful inquiry about the substantial difference observed between nanoparticle sizes determined by dynamic light scattering (DLS) and scanning electron microscopy (SEM) in our study. Green-synthesized ZnO nanoparticles are coated with phytochemicals from the plant extract. DLS captures the full hydrodynamic radius, encompassing these organic layers and any hydration shell. SEM, however, primarily detects the dense inorganic core, and organic capping layers—being light and often amorphous—may not be well resolved or may appear thinner due to electron beam penetration and sample dehydration.

7. The authors presented the antibacterial data graphically using a bar graph, followed by a comprehensive statistical analysis.

Response: Thank you for your comment. The reviewer's request was done.



Thanks,

Reviewer 5:

Comments

1. Precursor selection and thermal treatment—Zinc chloride is converted to ZnO only after full dehydration of intermediate $\text{Zn}(\text{OH})_2$ or $\text{Zn}_5(\text{OH})_8\text{Cl}_2$ phases. The authors dried the precipitate at 60°C for 2 h, far below the $\geq 300^\circ\text{C}$ levels commonly used to complete this conversion. Without a calcination step, phase purity is questionable.

Response: Thank you for your insightful comment on the crucial issue of zinc precursor conversion and the adequacy of the thermal treatment applied in our green synthesis of ZnO nanoparticles.

The current study was designed to prioritize “green” and energy-efficient processing, and therefore employed low-temperature drying at 60°C for 2 hours. This choice was aimed at demonstrating the feasibility of biosynthesizing ZnO nanoparticles under mild conditions using rosemary extract, as has been reported in some prior green synthesis literature. However, we acknowledge that drying at this low temperature without a high-temperature calcination step does introduce the risk that intermediate hydroxide or oxychloride phases remain partially unconverted in the final product. To address this, we conducted X-ray diffraction (XRD) analysis of the dried sample. Our XRD patterns display well-defined peaks matching the standard reference for hexagonal wurtzite ZnO (ICDD card no. 36-1451), and we did not observe any distinct reflections attributable to intermediate phases such as $\text{Zn}(\text{OH})_2$ or $\text{Zn}_5(\text{OH})_8\text{Cl}_2$. This suggests that, under our synthesis conditions and in the presence of excess plant phytochemicals, rapid conversion to ZnO was favored even at relatively mild temperatures.

2. Which specific phytochemicals are responsible for reduction and capping, and what is their surface coverage?

Response: Thank you for your insightful comment regarding the need to clarify the specific compounds and functional groups in rosemary extract responsible for its reducing capabilities. FT-IR spectra and literature evidence together indicate that rosemary’s phenolic repertoire—dominated by rosmarinic acid, carnosic acid, and caffeic acid—drives the green synthesis of nanoparticles. The catechol-type O-phenolic hydroxyl groups in these molecules readily chelate Zn^{2+} , donate electrons via HAT, SET, or SPLET pathways, and reduce the ions to Zn^0 that subsequently oxidize to crystalline ZnO. Because deprotonation at alkaline pH enhances electron donation, the reduction efficiency rises as synthesis pH increases. After reduction, the same

phytochemicals remain adsorbed on the particle surface, supplying steric and electrostatic stabilization that limits agglomeration. Synergistic interplay among multiple phenolics therefore provides both a robust reductive environment and durable organic capping, as confirmed by retained C–O, C=C, and O–H bands alongside Zn–O peaks ($475\text{--}578\text{ cm}^{-1}$) in the final FT-IR profile.

3. How do your inhibition zones compare quantitatively with rosemary-ZnO NPs reported in earlier works that exceed 15 mm at similar concentrations?

Response: Thank you for your pertinent question regarding how our inhibition zones for rosemary-mediated ZnO nanoparticles (ZnO NPs) quantitatively compare to those reported in earlier publications, particularly those exceeding 15 mm at similar concentrations.

In our study, the maximum inhibition zones observed at $100\text{ }\mu\text{g/mL}$ were 9.67 mm for *S. aureus* and 8.67 mm for *E. coli*. We recognize that several reports in the literature describe larger inhibition zones—often in the range of 17–23 mm for ZnO NPs at comparable concentrations and under seemingly similar testing conditions. Notably, some rosemary-extract-mediated ZnO NPs have been reported to yield inhibition zones as high as 17.97 mm (*S. aureus*) or 22.27 mm (*E. coli*) at doses equivalent to or just above $100\text{ }\mu\text{g/mL}$ (1). In other green ZnO NP studies, inhibition zones of 22–23 mm have been observed against both Gram-positive and Gram-negative strains at $100\text{ }\mu\text{g/mL}$, though methodological details such as NP size, culture medium, and bacterial inoculum density can vary substantially (7). Our results, therefore, fall on the lower spectrum of reported antibacterial efficacy.

Several factors may account for these differences. The average hydrodynamic size of our ZnO NPs was larger ($\approx 652\text{ nm}$, DLS) than those reported in many studies with higher inhibition zones, where mean sizes can be below 100 nm. Larger or more aggregated particles often show reduced bioavailability and diffusion into agar media, which directly impacts the measured zone of inhibition. In addition, we did not employ a high-temperature calcination step or post-synthesis size refinement, both of which tend to improve crystallinity and reduce aggregation, key parameters influencing nanoparticle-bacteria interactions. The presence and availability of active phytochemical capping agents, differences in bacterial strain sensitivities, and even slight protocol variations (such as agar composition or disc size) also influence inhibition zone outcomes.

4. What is the justification for using ZnCl_2 rather than the more commonly employed $\text{Zn}(\text{NO}_3)_2$, and how might residual Cl^- influence NP properties?

Response: Thank you for highlighting the important question of precursor selection—specifically, our use of zinc chloride (ZnCl_2) rather than the more commonly applied zinc nitrate ($\text{Zn}(\text{NO}_3)_2$)—and for raising the issue of potential effects from residual chloride ions (Cl^-) on nanoparticle (NP) properties.

Our choice of ZnCl_2 as a precursor was informed by practical, economic, and mechanistic considerations. ZnCl_2 is highly water-soluble, readily available, and often less expensive than $\text{Zn}(\text{NO}_3)_2$, which enables more accessible and scalable green synthesis—an important aim of our study. In preliminary experiments and in a number of prior green synthesis reports, ZnCl_2 was also found to facilitate rapid precipitation under mild conditions, particularly in the presence of plant extracts. Its high solubility permitted effective interaction with the reducing phytochemicals in rosemary extract at moderate pH and ambient temperature, supporting a greener synthesis route without the need for vigorous heating or harsh chemicals. Additionally, the chloride ion has been shown in some biosynthetic contexts to improve the nucleation rate, which can aid in controlling particle uniformity.

5. The zeta potential was measured in water; how does it change in Mueller-Hinton broth where ionic strength is markedly higher?

Response: Thank you for your insightful question regarding the measurement conditions of zeta potential and its implications for nanoparticle stability under biological assay conditions.

We acknowledge that zeta potential values are highly sensitive to the ionic strength and composition of the dispersion medium. In our study, zeta potential was measured in distilled water, yielding a value of -45.4 mV, which reflects strong electrostatic repulsion and excellent colloidal stability under low-ionic-strength conditions. However, we recognize that antibacterial assays were conducted in Mueller-Hinton broth, a complex medium with much higher ionic strength (due to added salts, peptides, and organic matter) than pure water.

Sincerely,

1. Uysal Y, Görkem Doğaroğlu Z, Çaylali Z, Karakulak DS. Rosemary-Mediated Green Synthesis of ZnO Nanoparticles and their Integration into Hydrogel Matrices: Evaluating Effects on Wheat Growth and Antibacterial Properties. *Global Challenges*. 2024;8(11):2400120.
2. Pitjamit S, Vichiansan N, Leksakul K, Boonyawan D. Optimization of Ultrasonic and Microbubble Disinfection for *Escherichia coli* and *Staphylococcus aureus*: Experimental Design and Effectiveness Evaluation. *Journal of Engineering and Technological Sciences*. 2024;56(6):742-55.
3. Al-Hamad KA, Asiri A, Alqahtani AM, Alotaibi S, Almalki A. Monitoring the Antibacterial Activity of the Green Synthesized ZnO Nanoparticles on the Negative and Positive Gram Bacteria Mimicking Oral Environment by Using a Quartz Tuning Fork (QTF) Micromechanical Sensor. *International Journal of Nanomedicine*. 2025;7975-85.
4. Babayevska N, Przysiecka Ł, Iatsunskyi I, Nowaczyk G, Jarek M, Janiszewska E, et al. ZnO size and shape effect on antibacterial activity and cytotoxicity profile. *Scientific Reports*. 2022;12(1):8148.
5. Albarakaty FM, Alzaban MI, Alharbi NK, Bagrwan FS, Abd El-Aziz AR, Mahmoud MA. Zinc oxide nanoparticles, biosynthesis, characterization and their potent photocatalytic degradation, and antioxidant activities. *Journal of King Saud University-Science*. 2023;35(1):102434.
6. Slman AA. Antibacterial activity of ZnO nanoparticle on some gram-positive and gram-negative bacteria. *Iraqi Journal of physics*. 2012;10(18):5-10.
7. Mohanasundaram P, Saral A M. Binding properties and biological applications of green synthesized ZnO nanoparticles from neem flower. *Scientific Reports*. 2025;15(1):17727.

OPEN PEER REVIEW

Revision (1)

Mahmoud Gharbavi : Reviewer

29 Jul 2025

All of the questions have been thoroughly answered, and I have no further comments.

REFEREE : Reviewer

9 Aug 2025

Dear Author(s),

Thank you for your revised manuscript. I have reviewed the changes you've made and am pleased to confirm that all my previous comments have been successfully addressed. The manuscript is now in a strong position for publication.

Best regards

REFEREE : Associate Editor

10 Aug 2025

The Ms is now suitable for publication in JJNPP.

REFEREE : EIC

10 Aug 2025

We are pleased to inform you that your manuscript is now acceptable for publication in JJNPP.