MULTIDISCIPLINARY APPLIED RESEARCH AND INNOVATION
e-ISSN: 2773-4773

MARI

Vol. 6 No. 2 (2025) 133-140
https://publisher.uthm.edu.my/periodicals/index.php/mari

Synthesis of Green Zinc Oxide-Citrus Hystrix Nanoparticles and Study of its Antimicrobial Properties

Aida Muhamad*, Raja Nur Dalilah Batrisyia Raja Zainal, Nurzaty Iman Shariff, Nurul Syazwani Enche Zaiton

Department of Science and Mathematics, Centre for Diploma Studies,
Universiti Tun Hussein Onn Malaysia, Pagoh Higher Education Hub, 84600 Pagoh, Johor, MALAYSIA

*Corresponding Author: aidamohd@uthm.edu.my
DOI: https://doi.org/10.30880/mari.2025.06.02.016

Article Info

Received: 31 August 2024
Accepted: 31 December 2024
Available online: 20 February 2025

Keywords

Citrus hystrix, green synthesis,
Agar Well Diffusion Method,
zinc oxide nanoparticles

Abstract

Green synthesis nanoparticles using plant extract have recently become one of the most preferred methods. This is due to its environmentally friendly and economical procedure and synthesis of more stable nanoparticles. This study synthesized green Zinc oxide (ZnO-CH) nanoparticles using a *Citrus hystrix* (CH) leaf extract solution. The ZnO-CH nanoparticles were prepared using the precipitation method at different concentrations of CH extract solution (1%, 4%, and 8%). The ZnO-CH nanoparticles were characterized for surface morphology, functional groups, and crystalline structure. The results showed that the particle size of ZnO-CH using 8% CH extract and zinc acetate with an oxalic acid solution is in the range of 30.50 nm to 74.29 nm, which is lower than ZnO-CH with 1% and 4% CH extract. The FTIR analysis of all ZnO-CH samples shows multiple peaks between 660 cm^{-1} and 400 cm^{-1}, indicating the ZnO's presence. The XRD patterns of ZnO-CH synthesized show all the 2θ values corresponding to the confirmation of ZnO NPs. The ZnO-CH*'s* antimicrobial properties were assessed using an agar well diffusion method against gram-positive and gram-negative bacteria. The concentrations of ZnO-CH NPs for antimicrobial properties are from 0.5 mg/ml to 4 mg/ml. The results from the susceptibility test of ZnO-CH against *Escherichia coli* (*E. Coli*) and *Staphylococcus aureus* (*S. aureus*) show that the inhibition zone diameter does not yield any results around the sample; this indicates no antimicrobial activity.

1. Introduction

Antimicrobials, including antibiotics, antivirals, antifungals, antiseptics, and antiparasitics, are pharmaceutical substances that treat or prevent infections. On non-living surfaces, antimicrobial chemicals are utilized as disinfectants. Multidrug-resistant bacteria have emerged due to the overuse of conventional antibiotics, posing a danger to public health worldwide. By concentrating on critical phases in cellular metabolism, such as synthesizing biological macromolecules, antimicrobials can destroy bacteria and prevent them from increasing the activity of cellular enzymes or cellular structures like the cell wall and cell membranes [1]. 50–70% ethanol, 50–70% isopropanol, formaldehyde, silver salts, hypochlorite, ethylene oxide gas, and metal oxides are among the antimicrobial agents. Metal oxide nanoparticles' diverse physicochemical properties enable them to act as antibacterial agents in several ways. Metal oxide nanoparticles have antibacterial qualities and act as medication transporters, making it difficult for microorganisms to become resistant. Bacterial antibiotic resistance is one of global healthcare's most urgent issues. Applying metal nanoparticles and their oxides is one of the best

strategies to combat microbial resistance to antibiotics. Consequently, applying medical dressing materials containing ZnO nanoparticles helps promote early wound healing by preventing microbial contamination. Multidrug-resistant bacteria have emerged due to the overuse of conventional antibiotics, posing a danger to public health worldwide. Zinc oxide nanoparticles have exhibited remarkable antimicrobial properties, while *Citrus hystrix* is known for its rich bioactive compounds with potential medicinal applications.

1.1 Green Synthesis of Nanoparticles

One approach for creating nanoparticles with plants is called "green synthesis of nanoparticles". This new method's growth and popularity are mostly because biological synthesis uses less energy and harmful chemicals than previous methods, making it a more economical and environmentally benign process. Furthermore, this method's primary benefit is that the raw materials are naturally abundant in amino, carboxyl, and hydroxyl groups, frequently employed as capping or stabilizing agents in aqueous media to promote the production of nanoparticles. Plant material is the most popular biological substrate for creating green nanoparticles containing metallic ions. Because they generate unique phytochemicals, plant parts such as leaves, stems, roots, fruits, and seeds have been employed to manufacture ZnO nanoparticles. This might have to do with the fact that vegetable substrates are often seen as less hazardous, simpler, and more affordable than microbes. Plants are the most recommended source for NP synthesis because they can produce NPs on a vast scale and in various shapes and sizes [2].

1.2 Definition of Antimicrobial and Its Significance

Antimicrobials are chemicals or agents that can prevent microorganisms' growth, development, or survival. Microorganisms are microscopic living creatures, including bacteria, viruses, fungi, and parasites. Antimicrobial agents are chemicals or compounds that have antimicrobial activity. Antibiotics, antivirals, antifungals, and antiparasitic medicines are natural or manufactured agents. Antimicrobial agents exert their effects through various modes of action—for example, cell wall inhibition, protein synthesis inhibition, nucleic acid inhibition, and enzyme inhibition. Antimicrobials can have a wide range of effects or a limited range. A narrow-spectrum antimicrobial only targets germs, whereas a broad-spectrum antimicrobial is effective against various microorganisms. The importance of antimicrobial agents highlights medical significance, which is the treatment of infections in treating bacterial, fungal, viral, and parasitic diseases in humans, animals, and plants using antimicrobial agents, including antibiotics, antivirals, antifungals, and antiparasitic. Furthermore, various products such as toiletries, cosmetics, skincare, and household items use antimicrobial agents to prevent the growth of microorganisms that could spoil the product, thus posing health risks. Lastly, studying antimicrobials and their mechanisms of action is essential for developing new drugs and therapies. The ongoing research helps combat the emergence of antimicrobial resistance.

1.3 Green Zinc Oxide Mechanisms of Antimicrobial Action

Zinc oxide nanoparticles (ZnO-NPs) are among the most promising inorganic materials with antibacterial characteristics. They are used in food packaging, cosmetics, sanitizers, and pharmaceutical products [3]. Green zinc oxide exhibits antibacterial properties through a variety of mechanisms. Plant parts such as leaves, stems, bark, roots, rhizomes, fruits, flowers, and seeds have been extensively used for the synthesis of zinc oxide nanoparticles in the recent past and are found to be stable, highly pure, cost-effective, and possess greater biomedical properties [4]. Phytochemicals are plant-based bioactive compounds produced by plants for their protection. The plant extract was used as a bio-reducing agent in C*itrus hystrix* leaf extract-assisted green synthesis. Alkaloids, terpenoids, polyphenolic chemicals, amino acids, tannins, polysaccharides, saponins, steroids, and other bioactive substances comprise *Citrus hystrix* leaf extract. The concentration of green zinc oxide nanoparticles can affect their antibacterial activity. The lowest antimicrobial concentrations that, following an overnight incubation period, will prevent the visible growth of bacteria are known as minimum inhibitory concentrations (MIC). The bacterial cell wall becomes damaged when ZnO NPs are introduced. Direct solid contact between the antibacterial agent ZnO NPs and the surface of bacteria may clarify this effect. The bacteria's membrane retains tiny pores that allow the ZnO-NPs to enter the cells. Reactive oxygen species (ROS) generation is the other mechanism that activates zinc oxide nanoparticles' antibacterial qualities. Reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radicals, can be produced by ZnO nanoparticles [5]. These ROS can cause oxidative stress in microbiological cells, harming cellular constituents and ultimately resulting in cell death. ROS's high reactivity and oxidizing potential are the leading causes of their toxicity to microorganisms. The peptidoglycan layer that envelops gram-positive bacteria is thick yet porous, and it contains a single lipid bilayer with a negative charge provided by lipoteichoic and teichuronic acids. The peptidoglycan layer in the cell walls of gram-positive bacteria comprises an amino acid and sugar mesh structure. This peptidoglycan layer may interact with zinc ions, changing its structure and weakening the bacterial cell wall.

2. Materials and Methods

The Citrus Hystrix (CH) was collected from a garden near the Batu Pahat Johor area. The chemicals used to synthesize ZnO-CH NPs in this study were zinc acetate and oxalic acid. Zinc oxide was semi-soluble. Therefore, the recommended solutions from the previous study were used to find the most suitable solution to disperse the ZnO-CH. The solutions tested to disperse the ZnO-CH antimicrobial properties were Mueller Hinton Broth, Normal Saline, and distilled water.

2.1 Preparation of the Citrus Hystrix Leaves Extract

To prepare the aqueous C*itrus hystrix* leaf extract, the fresh green C*itrus hystrix* leaves were collected, washed, and dried in an oven at 60℃ for 24 hours. The dried leaves are then ground using a grinder and weighed for 10g for 1%, 40g for 4%, and 80g for 8%. Then, the leaves were boiled at 80℃ under stirring in 1 litre of deionised water for 2 hours. After 2 hours of boiling, the light brown extract was filtered using a Whatman filter paper to remove the residues, and the leaves extract was then stored in the refrigerator for further use.

2.2 Green Synthesis of Zinc Oxide Nanoparticles

ZnO-CH was prepared by using the precipitation method. The chemical was prepared by weighing 43.9g of Zinc Acetate, and 37.82g of Oxalic Acid was dissolved in 2 litre of deionised water. Then, 400 mL of CH 1%, 4%, and 8% extract was poured into three separate beakers and mixed with 600 mL of Zinc Acetate under stirring for 30 minutes. After 30 minutes, 600 mL of Oxalic Acid was poured into each beaker slowly under stirring, and the solution was left overnight under stirring while covered with aluminium foil. The next day, the solution is filtered using Whatman filter paper and left in an oven below 100℃ for 1 hour. Then, the resultant white powder was calcined at 550℃ for 3 hours in a furnace. The white powder is crushed using a mortar and pestle to produce a fine white powder.

2.2.1 Characterisation of Zinc Oxide-Citrus Hystrix

The characterization of the synthesized ZnO-CH nanoparticles was performed using field-emission scanning electron microscopy (FESEM) to determine the surface morphology of the ZnO-CH. A Fourier-transform infrared spectrophotometer (FTIR) was employed to identify the functional groups present in the ZnO-CH. Lastly, X-ray diffraction analysis (XRD) was used to analyze the crystalline structure and confirm the presence of ZnO.

2.3 Preparation of Antimicrobial Properties in Mueller Hinton Agar

The antimicrobial tests were conducted using Mueller Hinton Agar as the medium. The Mueller Hinton Agar was weighed and dissolved in 1 litre of distilled water. Then, the Mueller Hinton Agar was autoclaved for sterilization at 121℃ for 2 hours. After that, the agar was poured into 22 petri dishes and left to harden before swabbing the culture bacteria onto the media. Once the agar had hardened, the swabbing method was applied by inoculating the culture using a microbial swab. Then, a well with a 6 mm diameter was formed using the back of the pipette tips. After that, the bottom of the well in the petri dish was coated again with 50 µL of Mueller Hinton Agar to prevent the sample from dispersing at the bottom of the petri dish. Subsequently, the four concentrations of the ZnO-CH nanoparticles suspension (4, 2, 1, and 0.5 mg/mL) for each ZnO-CH 1%, 4%, and 8% were prepared by dispersing the required amount in 20 mL of normal saline. Following this, 100 µL of each concentration of ZnO-CH 1%, 4%, and 8% were added into the wells using a micropipette. The *Amoxicillin* antibiotic was used as the positive control, and sterilized distilled water was used as the negative control. Finally, the petri dishes were incubated at 37℃ for 16-18 hours, and the inhibition zones were measured.

2.3.1 Preparation of Bacteria in Mueller Hinton Broth

In this experiment, the cultures used were *S. aureus*, a gram-positive bacterium, and *E. coli*, a gram-negative bacterium. They were prepared using Mueller Hinton Broth before being used to streak the antimicrobial properties. The Mueller Hinton Broth had been dissolved in 1 liter of distilled water, and the broth was autoclaved to ensure its sterility. Then, the broth was poured into two conical flasks of 250 mL and mixed with five colonies of each bacterium. The cultures were then incubated at 37℃ for 20 hours.

2.3.2 Types of Solvent Used to Disperse Zinc Oxide

In this experiment, the zinc oxide was a semi-soluble powder. Table 1 shows the solvent used to disperse ZnO in a previous study. It was found that Mueller Hinton broth, normal saline, and distilled water were used to disperse the ZnO-CH. In this study, normal saline was used to disperse the ZnO-CH sample.

Table 1 *The solvent to dispersed ZnO*

Solvent Used	References
Each well-received 100uL from stock (5 mg of Zno NPs dissolved in 1 mL of 10% dimethyl sulfoxide). 10% DMSO as an adverse control.	[6]
The necessary quantity of ZnO NPs was dispersed in deionized water while being stirred to create our concentrations of the ZnO NP suspension (4, 2, 1, and 0.5 mg/mL).	[7]
On average, 10 mg/mL of all the samples were dissolved using 0.9% NaCl. Saline had no antibacterial effect against any of the pathogenic strains examined.	[8]
To produce a powder concentration of 1000 mg/mL, ZnO nanoparticles were suspended in sterile normal saline and continuously agitated until a homogenous colloidal suspension was created.	[9]
Before every test, ZnO NP suspensions at varying concentrations were made using an ultrasonic bath to disperse the particles in Milli-Q water for half an hour, then vortex mixing for 5 seconds.	[10]

3. Results and Discussion

3.1 FTIR Analysis of ZnO-CH NPs

Fig. 1 shows the comparison of FTIR spectra of ZnO-CH 1%, 4%, 8% and ZnO-Commercial.

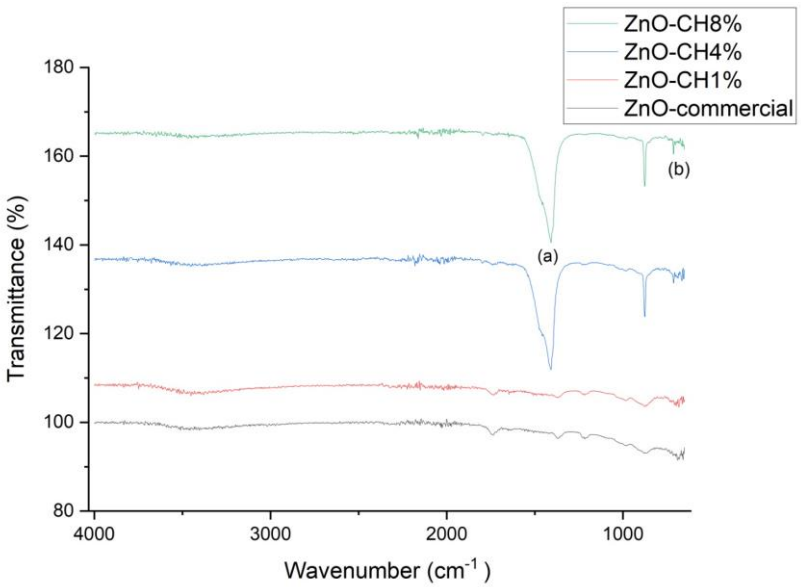


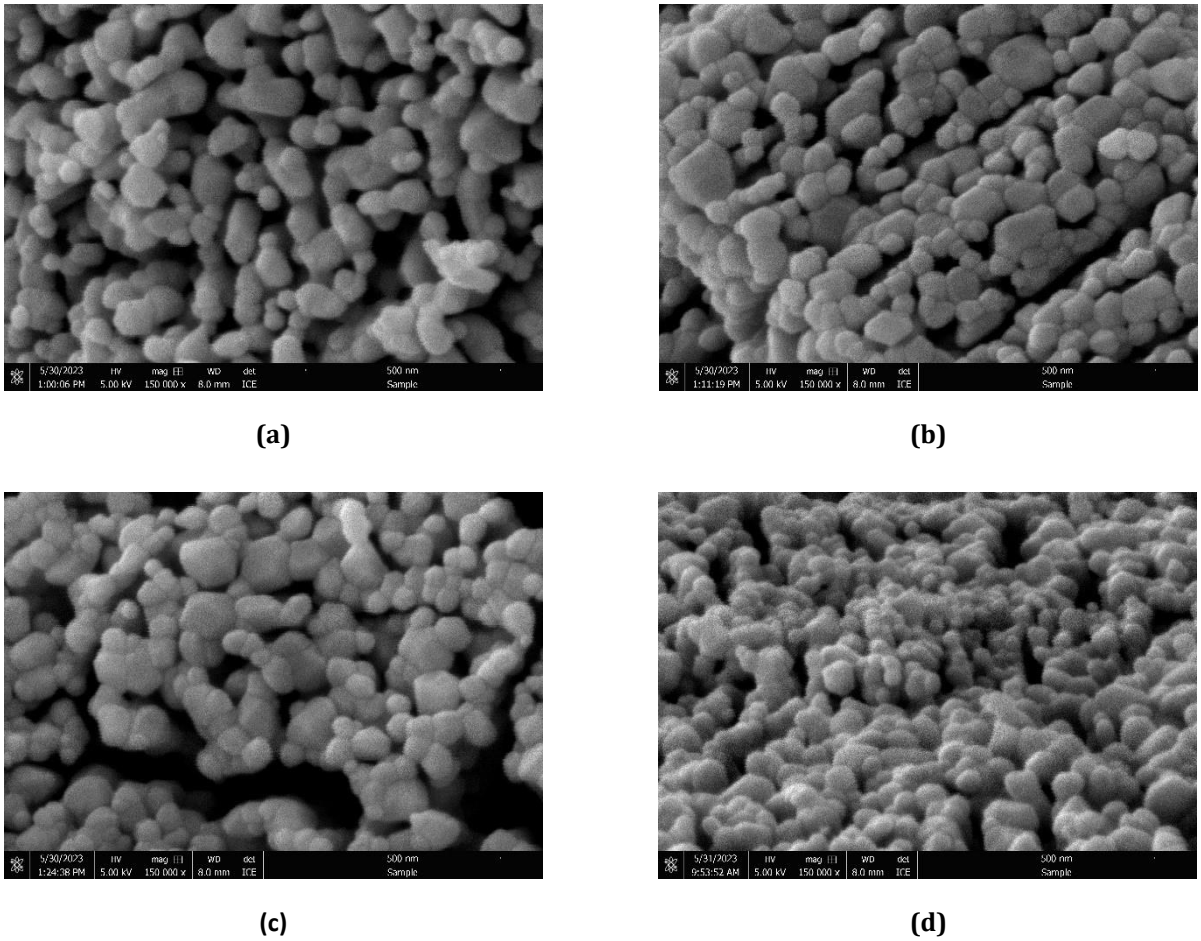
Fig. 1 *FTIR spectrum of ZnO-CH NPs and ZnO-CH*

The functional groups present in CH leaf extract, ZnO-Commercial, and ZnO-CH at concentrations of 1%, 4%, and 8% were identified based on Fig. 1. The figure's functional group corresponding to peak (a) is attributed to the amide bond formed by the carbonyl (C-O) group at the peaks between 1400 cm^{-1} and 1460 cm^{-1}, which acts

as a capping and reducing agent. The figure's next peak (b) is ZnO at the 400 cm^{-1} to 600 cm^{-1} stretching vibration peak. The highest peak that could be seen is around a range of 1400nm, where the functional group of C-O is found.

3.2 FESEM Analysis of ZnO-CH NPs

In Fig. 2, ZnO-CH NPs were subjected to a FESEM study to determine their morphologies.



(a) **(b)**

(c) **(d)**

Fig. 2 *FESEM images of ZnO NPs surface morphology (a) ZnO commercial; (b) ZnO-CH 1%; (c) ZnO-CH 4%; (d) ZnO-CH 8%*

This FESEM analysis aims to examine the microstructure of synthesised Citrus Hystrix to understand its surface morphology and grain size distribution. Fig. 2 (a) to (d) were all captured at a magnification of 150,000x with a resolution of 500nm, using an accelerating voltage of 5.00kV and a working distance of 8.0mm. The images show that the surface morphology across all figures remains consistent, appearing smooth, slightly rounded, and interconnected. As the concentration of ZnO-CH increases, the figures show a higher number of particles and reduced distances between them, indicating the presence of the expected elements. However, the nanoparticles may not exhibit high-quality properties, as the grain sizes are not uniform.

3.3 XRD Analysis of ZnO-CH NPs

Fig. 3 shows the XRD pattern of ZnO-CH 8% compared to ZnO commercial.

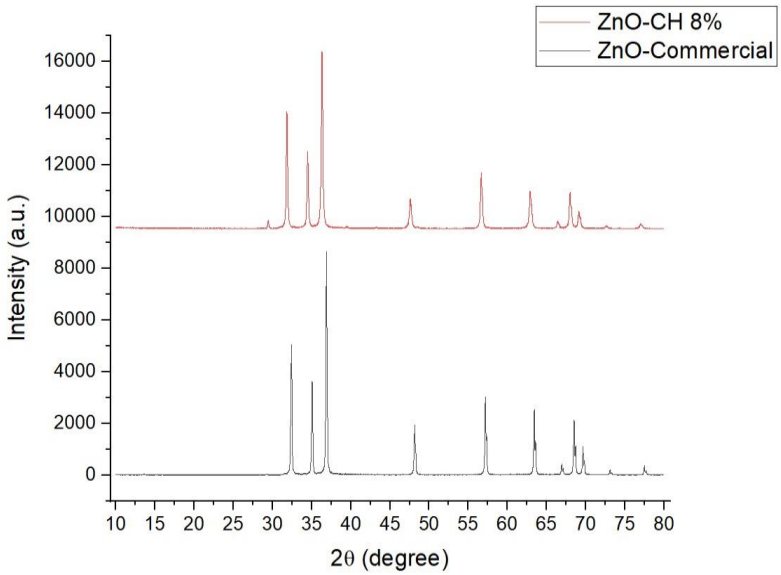
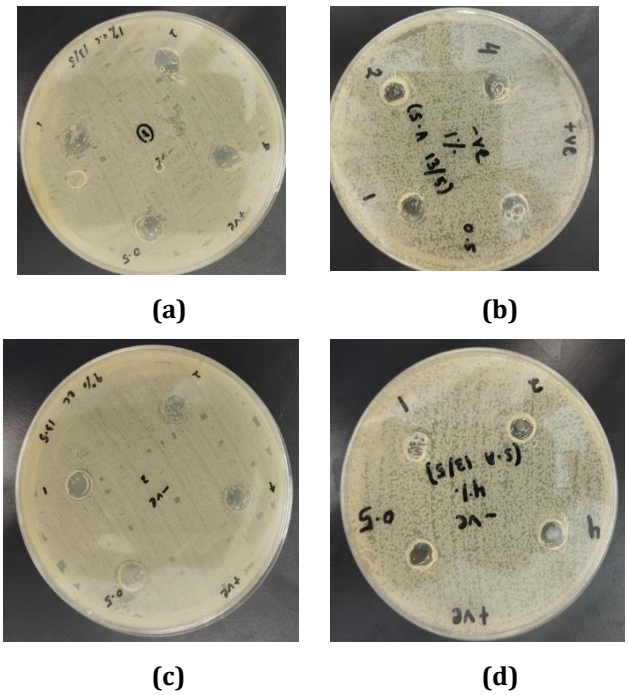


Fig. 3 *XRD patterns of the ZnO commercial and ZnO-CH 8%*

In Fig. 3, the synthesized ZnO-CH 8% and ZnO-Commercial crystalline structure was characterized by XRD. The XRD patterns display vast reflection peaks, indicating the formation of the hexagonal phase of zinc oxide. The characteristic diffraction peaks of the zinc oxide nanoparticles were observed at 2Θ values of 31.77°, 34.40°, 36.22°, 47.61°, 56.58°, 62.85°, 66.41°, 67.93°, 69.08°, 72.54°, and 76.85°, corresponding to the planes (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202) respectively. To compare, there is a slight difference at 47.61° value; the structure for ZnO-Commercial is slightly sharper than ZnO-CH 8%, as seen in the figure. Besides that, both structures show the same characteristics and values.

3.4 Antimicrobial Activity

Fig. 4 shows the antimicrobial properties of ZnO-CH samples against *E. coli* and *S. aureus*.



(a) **(b)**

(c) **(d)**

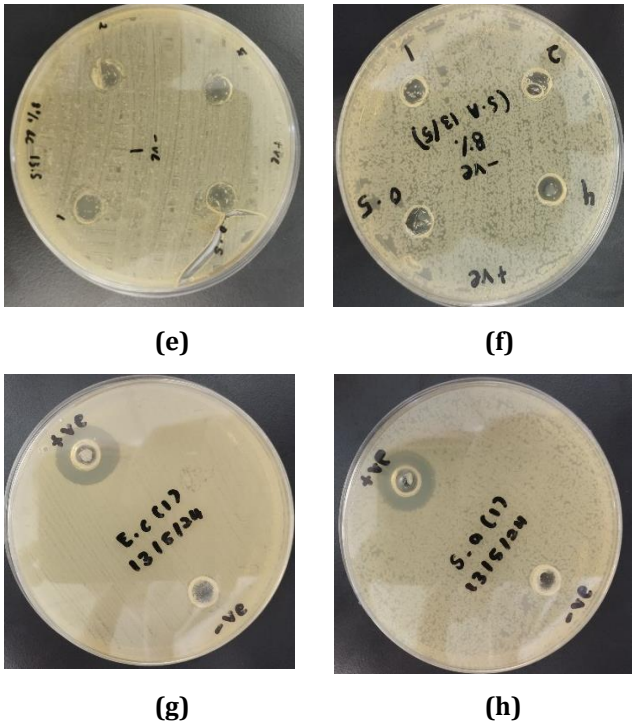


Fig. 4 *ZnO-CH antimicrobial properties (a) ZnO-CH 1% with E. coli; (b) ZnO-CH 1% with S. aureus; (c) ZnO-CH 4% with E. coli; (d) ZnO-CH 4% with S. aureus; (e) ZnO-CH 8% with E. coli; (f) ZnO-CH 8% with S. aureus; (g) Positive and Negative Control with E. coli; (h) Positive and Negative Control with S. aureus*

To evaluate the antimicrobial properties of the green synthesized ZnO NPs, different concentrations of the sample (0.5, 1, 2, and 4 mg/mL) of each ZnO-CH 1%, 4%, and 8% were tested against two bacterial gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria using an agar well diffusion method. The inhibition zone was used to evaluate the antibacterial activity. As can be seen from the results in Fig. 4(a) to Fig. 4(f,) the inhibition zone diameter does not show up around the sample; this shows no antimicrobial activity for each ZnO-CH sample. Fig. 4(g) and Fig. 4(h) show that the positive control has a significant antimicrobial activity with maximum inhibition zones of 1.9 cm for *E. coli* and 1.7 cm for *S. aureus*. In this study, *Amoxicillin* antibiotic was used as the positive control, meanwhile distilled water was used as a negative control. This shows that the *Amoxicillin* antibiotic has antimicrobial properties on both *E. coli* and *S. aureus*. The green ZnO-CH samples did not produce any inhibition zones, showing a lack of antimicrobial action under the investigated conditions. The results show that the well-known antibiotic *amoxicillin* efficiently suppresses microbial growth, as seen by the visible inhibition zones.

4. Conclusion

In this study, the successful synthesis of zinc oxide nanoparticles using an environmentally friendly method involving an aqueous extract of *Citrus Hystrix* leaves represents a significant advancement in green chemistry. In addition to minimizing the environmental effect usually associated with conventional ways of synthesizing nanoparticles, this strategy uses plant extract's inherent natural reducing and stabilizing properties. Previously, ZnO–CH was synthesized using leaf extracts obtained from *Citrus Hystrix*. The ZnO – CH were characterized using XRD, FESEM, and FTIR. ZnO – CH exhibits two distinct biological properties: antibacterial and antioxidant. The antimicrobial efficacy of the NPs was demonstrated against two bacterial strains, *S. aureus* and *E. coli*. The ZnO–CH concentration (0.5, 1, 2, and 4 mg/mL) for each ZnO–CH 1%, 4%, and 8% were tested on each bacteria strain. *Amoxicillin* antibiotic was used as a positive control, and distilled water was used as a negative control. The attempt to prove antimicrobial agent using the ZnO–CH agar well diffusion method did not yield the expected results. Despite thorough experimentation and rigorous testing, the synthesized compound did not demonstrate significant antimicrobial activity against the tested bacterial strains. Thus, objectives 1 and 2 of this study were achieved. Further investigation on the antimicrobial properties of ZnO-CH could be carried out.

Acknowledgement

This research was supported by Universiti Tun Hussein Onn Malaysia (UTHM) through Tier 1 (vot Q519). The authors would also like to thank the Centre for Diploma Studies, Universiti Tun Hussein Onn Malaysia, for its support.

Conflict of Interest

Authors declare that there is no conflict of interests regarding the publication of the paper.

Author Contribution

The authors confirm their contribution to the paper as follows: ***Problem Statement, Literature Review Introduction, Definition of Antimicrobial and its Significance, Green Synthesis of Nanoparticles, Classes of Antimicrobials, Preparation of Antimicrobials, Gantt Chart, Conclusion:*** *Raja Nur Dalilah Batrisyia Raja Zainal;* ***Introduction, Objectives of Study, Scope of Study, Cost Estimation Project, Zinc Oxide Antimicrobials Properties Against Bacteria, Methodology Introduction, Materials:*** *Nurzaty Iman Shariff;* ***Problem Statement, Physical and Chemical Parameters/Properties of Antimicrobial, Methods of Antimicrobial Properties, Preparation of Bacteria, Cost Estimation, Results and Discussion:*** *Nurul Syazwani Enche Zaiton. All authors reviewed the results and approved the final version of the manuscript.*

References

[1] P. Di Martino, "Antimicrobial agents and microbial ecology," Microbiology, vol. 8, pp. 1–4, Jan. 2022. [Online]. Available: https://doi.org/10.3934/microbiol.2022001

[2] M. Thakur, S. Poojary, and N. Swain, "Green synthesis of iron oxide nanoparticles and its biomedical applications," Nanotechnology in the Life Sciences, vol. 1, no. 1, pp. 83–109, 2021. [Online]. Available: https://doi.org/10.1007/978-3-030-64410-9_5

[3] C. R. Mendes et al., "Antibacterial action and target mechanisms of zinc oxide nanoparticles against bacterial pathogens," Scientific Reports, vol. 12, p. 2658, 2022. [Online]. Available: https://www.nature.com/articles/s41598-022-06657-y

[4] M. Murali et al., "Plant-mediated zinc oxide nanoparticles: Advances in the new millennium towards understanding their therapeutic role in biomedical applications," Pharmaceutics, vol. 13, p. 1662, 2021.

[5] A. Sirelkhatim et al., "Review on zinc oxide nanoparticles: Antibacterial activity and toxicity mechanism," Nano-Micro Letters, vol. 7, no. 3, pp. 219–242, 2015. [Online]. Available: https://doi.org/10.1007/s40820-015-0040-x

[6] S. Lal et al., "Antioxidant, antimicrobial, and photocatalytic activity of green synthesised ZnO-NPs from Myrica esculenta fruits extract," Inorganic Chemistry Communications, vol. 141, p. 109518, Apr. 2022. [Online]. Available: https://doi.org/10.1016/j.inoche.2022.109518

[7] T. S. Aldeen, H. E. Ahmed Mohamed, and M. Maaza, "ZnO nanoparticles prepared via a green synthesis approach: Physical properties, photocatalytic and antibacterial activity," Journal of Physics and Chemistry of Solids, vol. 160, p. 110313, Aug. 2021. [Online]. Available: https://doi.org/10.1016/j.jpcs.2021.110313

[8] B. F. Naiel, M. Fawzy, and M. W. A. H. Halmy, "Green synthesis of zinc oxide nanoparticles using sea lavender (Limonium pruinose L. Chaz.) extract: Characterization, evaluation of anti-skin cancer, antimicrobial and antioxidant potentials," Scientific Reports, vol. 12, no. 1, p. 20370, 2023. [Online]. Available: https://doi.org/10.1038/s41598-022-24805-2

[9] E. K. Zarrindokht and P. Chehraz, "Antibacterial activity of ZnO nanoparticle on gram-positive and gram-negative bacteria," African Journal of Microbiology Research, vol. 5, no. 12, pp. 1368–1373, 2012. [Online]. Available: https://doi.org/10.5897/AJMR10.159

[10] C. S. Roberta, U. H. Leticia, G. R. Humberto, D. H. B. Roberta, and B. A. M. Chehraz, "Antibacterial activity of zinc oxide nanoparticles synthesized by solochemical process," Brazilian Journal of Chemical Engineering, vol. 36, no. 2, pp. 885–893, 2019.