

Citrus limon based synthesis of zinc oxide nanoparticles: Antibacterial applications with improved outcomes

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ABSTRACT

Background: Biosynthesis of nanoparticles by biological means, especially plant extract plays a major role in the field of nanotechnology. Zinc Oxide nanoparticles (ZnO-NPs) along with natural resources have demonstrated several advantages over traditional synthetic processes, including ease of use, low cost, eco-friendliness, nontoxic by products, and the absence of critical temperature and pressure conditions.

Methods: In the present study, the aqueous extract of lemon (*Citrus limon*) peel, pulp and leaves was used for the green synthesis of ZnO-NPs using 0.1 M Zinc nitrate dihydrate ($Zn(NO_3)_2$) as a precursor in alkaline condition using NaOH. UV-visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR) analysis and Scanning Electron Microscopy (SEM) were used to characterize the structures. Disc diffusion method was used to evaluate the antibacterial effects of the synthesized nanoparticles.

Results: ZnO-NPs obtained were characterized by UV-visible spectroscopy in which the surface plasmon resonance was 280 nm. FTIR analysis showed appropriate peaks for various chemical groups present in ZnO-NPs and stretching vibrations at 432 cm^{-1} indicating the presence of Zinc oxide bond in nanoparticles. SEM results showed spherical and well dispersed structures. Antibacterial property of ZnO-NPs, evaluated by the well diffusion assay, showed zone of inhibition in various pathogenic strains of Gram positive as well as Gram negative bacteria. It was observed that pulp-based nanoparticles had highest activity amongst all strains.

Conclusion: Use of aqueous lemon extract as a capping agent would improve the antibacterial property of ZnO-NPs and can be developed as antibacterial agents against a wide range of microorganisms to control and prevent bacterial infections.

Key Words: Nanoparticles, Green synthesis, Citrus limon, Zinc Oxide, Antibacterial

INTRODUCTION

Infections caused by various bacterial pathogens are treated with antibiotics. Antibiotics are agents that inhibit bacterial growth either by disrupting its membrane or protoplasmic content. Recent studies have shown that various bacterial pathogens are developing resistance against these agents and their mode of actions. Antimicrobial resistance (AMR) is a growing global health concern whereby bacteria develop resistance to the drugs that were once effective in treating infections caused by these microorganisms. This resistance is primarily a result of the overuse and misuse of antimicrobial agents, leading to proliferation of resistant strains [1].

The consequences of antimicrobial resistance are alarming, as it makes infections harder to treat, increases the risk of severe illnesses, and can lead to higher mortality rates. Development of resistance not only affects effectiveness of common antibiotics but also impacts on other critical medical procedures such as organ transplants, cancer treatments, and surgeries, which heavily rely on the availability of effective antimicrobial agents to prevent and control infections. The emergence and spread of antibiotic-resistant bacteria have become a global health concern, necessitating the development of alternative and effective antibacterial agents. To address the antimicrobial resistance, several approaches are being pursued including nanotechnology [2].

Nanotechnology is an emerging field of science and engineering that deals with the manipulation of matter on an atomic and molecular scale, typically involving structures with sizes ranging from 1-100 nanometers.

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Received: 27.07.2025

Revised: 27.12.2025

Accepted: 28.12.2025

Published: 08.01.2026

At this scale, materials exhibit unique properties and behaviors that are different from their bulk counterparts. Nanotechnology has numerous applications in various industries, including electronics, medicine, energy, and materials science. Nanoparticles have emerged as promising candidates due to their unique physicochemical properties and potential for antimicrobial applications [3]. Materials at nano size show more effective properties such as small size, high adsorption, high catalytic activity, large surface area, large number of reactive sites, and chemical stability. For this reason, nano-sized materials are widely being used in different disciplines such as water purification, drug delivery, medicine, agriculture, solar cells, food textiles, cosmetics, and electronics catalysis [4]. Nanoparticles are one of the key components in nanotechnology. These particles can be engineered and tailored to have specific properties, making them valuable for a wide range of applications. Nanoparticles can be made from different materials, such as metals, semiconductors, polymers, ceramics, and natural resources [4]. The choice of nanoparticle formation method depends on factors such as the desired material, size, shape, and intended application. Some nanoparticles can be synthesized using biological entities, such as bacteria, fungi, or plants. These biological methods are often referred to "green synthesis" and are environmentally friendly [5]. Nanotechnology and use of nanoparticles also raise important questions about safety, regulation, and potential environmental impacts and in previous studies on Nanoparticle formation it was observed that green synthesis of nanoparticles from plants is cheap, ecofriendly and forms more stable nanoparticles [6]. Among various green synthesis approaches, the utilization of natural sources, such as plants, has gained significant attention for producing nanoparticles with enhanced antibacterial activity. Phytochemical and pharmacological studies suggest that plants and plant products contain important phytochemicals that possess anti-oxidative and antibacterial effects, hence biosynthesis of nanoparticles can be based on utilizing plant extracts [7]. Many biomolecules in plants such as proteins, polysaccharides, amino acids, organic acids, and vitamins, and phytochemicals such as polyphenols, flavonoids, terpenoids, alkaloids, tannins, and alcoholic compounds are readily available in plant extracts and can act as reducing and stabilizing agents in green synthesis of nanoparticles. Nanoparticles production using ecofriendly, cost-effective, reducing and stabilizing materials from plants, yielding negligible toxic chemicals reduces health and environmental risks at source level [8].

Citrus limon, a nature's gift with therapeutic properties, commonly known as lemon, is a popular citrus fruit widely recognized for its distinctive flavor

and numerous health benefits. Lemons are typically available year-round in many regions due to their wide range of cultivation. It is rich in bioactive compounds, including flavonoids, polyphenols, and terpenoids, which endow lemon with potent antioxidant, anti-inflammatory, and antimicrobial properties [9]. In recent studies metal and metal oxide nanoparticles, especially those synthesized from plants, have been explored as alternative biocontrol agents [10]. ZnO nanoparticles (ZnO-NPs) are center of focus among metal oxide nanoparticles in their anti-bacterial activity and recently an antiviral agent against COVID-19. One unique feature of ZnO-NPs is their biocompatibility that makes it ideal candidate to employ in health care. Based on the previous literature reports, ZnO-NPs have been synthesized from various plant extracts [11, 12]. ZnO potent antibacterial action has found widespread application in industry, and it also has the potential to serve as a safe and effective antibiotic substitute. Noble metals like gold (Au) and silver (Ag) have been extensively used in biosynthesis of NPs and medically evaluated. The distinct antibacterial activities of metal and metal oxide NPs were achieved by their smaller sizes and larger surface-area-to-mass ratios and generation of oxidative stress on bacterial cells [13]. Because of their smaller size and larger surface area, nanoparticles may easily adsorb bacterial cells and a higher proportion of atoms on their surface, as well as improve their capacity to pass through membranes and interfacial reactivity to directly interfere with cell membrane functions. The nanoparticles get in direct contact with the cell wall and as a result, destroy the integrity of bacterial cells. ZnO-NPs, like other metal oxides, exhibit significant antibacterial activities against a broad spectrum of bacterial strains [14]. In this study, ZnO-NPs were synthesized by using lemon structures and were evaluated for their antibacterial efficiency. Outcomes will attract scientific community to synthesize ZnO-NPs from green resources like citrus limon for potential use as antibacterial agents.

METHODS

Preparation of Extractions

Fresh lemons and leaves samples from household plant of *Citrus limon* and were collected and authenticated by Botany Department, Govt. College University, Lahore, Pakistan. Lemons and leaves were washed thoroughly four-to-five times with tap water, then by distilled water to remove the dust particles. Clean lemons were peeled off and afterwards peel, and leaves were dried under the shade for 5 days at room temperature. Lemon pulp juice was extracted using reamer and collected in sterile bottle. Dried peel and leaves of lemon were grinded using mortar and pestle into fine powder. Peel and leaves extract was prepared by heating 10 gm dried leaves and peel

powder separately in 100 mL of deionized distilled water at 80°C stirred continuously with the help of magnetic stirrer for 2 hours. Mixture was cooled down and filtered using Whatman No. 1 Filter paper. Extracts were collected in sterile bottles. All three extracts were stored at 2-8°C for further use.

Preparation of Nanoparticles

Synthesis of ZnO-NPs involved the use of Zinc Acetate dehydrate Zn (CH₃COO)₂·2H₂O, Sodium hydroxide (NaOH) and distilled water. Both the chemicals were purchased from Sigma-Aldrich while distilled water was obtained from the laboratory of the university. Zinc acetate dehydrate solution (0.2 M) was freshly prepared by adding 43.9 g of it in 1 liter of distilled water and stirred continuously until clear solution was prepared. 230 mL freshly prepared 0.2 M Zn (CH₃COO)₂·2H₂O was added to 100 mL of each extract separately, previously prepared. The content was covered with aluminum foil. It was placed on hotplate temperature was set to 60°C and was stirred continuously with the help of magnetic stirrer for roughly 2 hours. After 1 hour and 30 minutes, 1M NaOH (2 g/ 50 ml) was added dropwise as it was observed that nanoparticles were best synthesized in alkaline medium. Formation of white, pale yellow and pale green precipitates of ZnO-NPs based on lemon pulp, peel and leaves were obtained respectively. The mixture containing ZnO-NPs based on lemon pulp was filtered using Whatman filter paper and other containing peel and pulp based were centrifuged at 10,000 rpm for 30 min. The filtrate and supernatant were discarded, and the pellets were washed with deionized water three to four times. Finally, pellets were placed in hot air oven at 60 °C for 24 h to obtain crystals of ZnO-NPs. These crystals were further dried for 1 h at 100°C, cooled, grinded in mortar and pestle and stored in 5 mL sterile airtight tubes covered with aluminum foil for future studies.

Characterization of Nanoparticles

Qualitative tests were performed for confirmation of ZnO-NPs formation. Lemon peel, pulp and leaves mediated ZnO-NPs were scanned for UV-visible spectroscopy to determine light absorbance. UV-visible spectrum of synthesized ZnO-NPs were obtained using NanoDrop 2000 spectrophotometer by Thermo Scientific. The different functional groups in ZnO-NPs were identified by FTIR. FTIR spectroscopy detects functional groups such as vibrational bonds like N-H, O-H, C-H, C = O (ester, ether, amine, ketone, aldehyde), C = C, C = N (vibrational modes of a tetrapyrrole ring) and simply C = N. SEM analysis was achieved to determine surface topography. SEM analysis was performed on TESCAN Vega LMU – Variable pressure Scanning Electron Microscope. ZnO-NPs were well-dispersed, less aggregated, and spherical in shape and were in nm range.

Antibacterial Activity

Antibacterial activity of synthesized ZnO-NPs was checked against different strains of pathogenic bacteria by agar well diffusion assay. This method involved preparing bacterial cultures and agar plates, followed by introducing ZnO-NPs solutions into wells on the agar plates. The zone of inhibition around the wells indicates antibacterial activity of nanoparticles. Briefly, aqueous solution of ZnO-NPs, synthesized from lemon leaves, pulp and peel were prepared by dissolving 100 mg powder of each in 1 mL of distilled water. Gram positive (*Staphylococcus aureus*, MRSA) and gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacteria were spread uniformly on Muller Hinton agar plates having wells of 4 mm that were prepared by sterile filter tips of 1uL. Aqueous solution of pulp, peel and leaves based nanoparticles were introduced in the wells under sterilized conditions. Aqueous extract (leaves, pulp and peel) and antibiotic Ciprofloxacin were used as standard in each plate. Aseptic conditions were maintained to prevent contamination. Plates were left for 30 min in safety hood so that nanoparticles could diffuse and then plates were incubated at 37°C for 24 h. The zone of inhibition (mm) around well was measured.

RESULTS

Formation of Nanoparticles

Biosynthesis of ZnO-NPs was carried out using lemon leaves, peel and pulp extracts and zinc acetate dihydrate as a precursor. On addition of the plant extracts (leaf, peel and pulp) with a greenish, yellowish and brown color, respectively, to the colorless zinc acetate dihydrate solution, green, yellowish and white precipitate occurred, indicating the presence of zinc oxide nanoparticles as shown in Figure 1.

Confirmation of Nanoparticles

The confirmation of formation of ZnO-NPs was performed by analysis with UV-Vis spectroscopic technique with a wavelength range of 200-800nm. ZnO-NPs formed showed absorbance at 280nm (Figure 2). FTIR analysis was done to detect the various characteristics functional groups associated with the synthesized nanoparticles. FTIR spectrum of pulp based nanoparticles showing stretching vibrations at wavelength 3344.57cm⁻¹(alcohol/phenol group), 2978.09cm⁻¹ (carboxylic acid group), 1562.34 cm⁻¹ (aromatic group), 1409.96 cm⁻¹ (ethyl alcohol group), 1018.41 cm⁻¹ (ethyl group), 966.04cm⁻¹, and 651.94cm⁻¹ (C-H aromatic group) and 412.77 cm⁻¹(metallic oxide group) (Figure 3). SEM was done to do surface morphology analysis of the sample. It showed that nanoparticles were in nanometer range. It detected scattered electrons from the particle's

surface and revealed that nanoparticles formed were spherical in shape, well dispersed and less aggregated as shown in Figure 4.

Antibacterial Activity

Antibacterial activity was checked by agar well diffusion assay and Zone of inhibitions were measured



Figure 1: ZnO Nanoparticles of (A) Lemon leaves (B) Peel and (C) Pulp

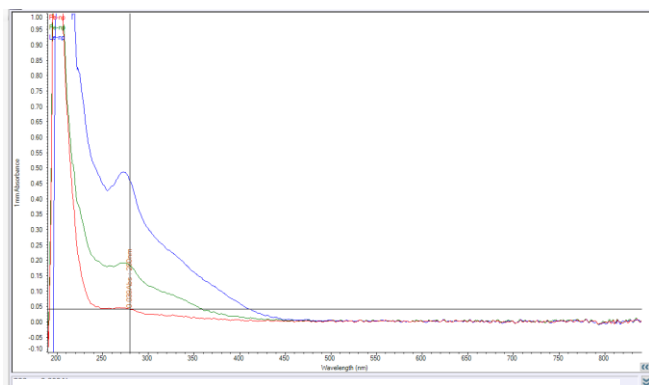


Figure 2: UV Vis absorption spectra for ZnO-NPs synthesized from lemon showing absorption peak at 280nm

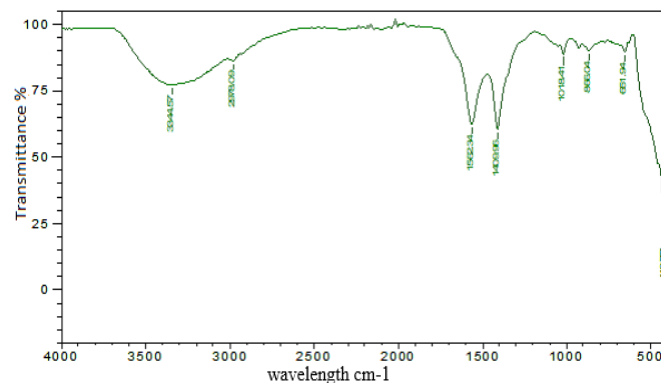


Figure 3: FTIR spectrum of Pulp based nanoparticles

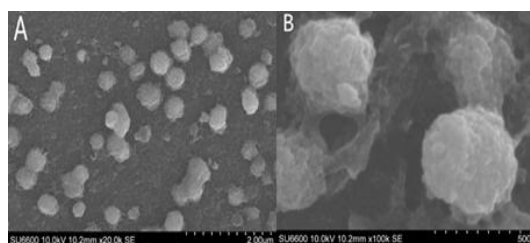


Figure 4: Micrographs of SEM of pulp-based NPs. (A) 2.00 um and (B) 500 nm.

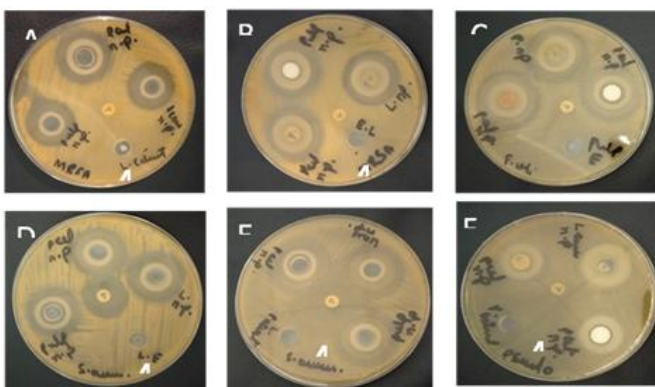


Figure 5: Comparison of antibacterial activity of ZnO-NPs with Ciprofloxacin against MRSA (A & B), *E. coli* (C), *S. aureus* (D&E), and *P. aeruginosa*. All three extracts-NPs were used (leaf, pulp, and peel). Well 4 of all plates contain simple extract as control.

Table 1. Zone of inhibition measured by well-diffusion method

ZnO-NPs and Standard	Zone of Inhibition	Bacteria Tested
ZnO-NPs Pulp	30mm	Staphylococcus aureus
ZnO-NPs leaves	28mm	
ZnO-NPs peel	25mm	
Ciprofloxacin	27mm	
Extract	No Zone	
ZnO-NPs Pulp	28mm	Methicillin resistant Staphylococcus aureus
ZnO-NPs leaves	26mm	
ZnO-NPs peel	25mm	
Ciprofloxacin	No Zone	
Extract	No Zone	
ZnO-NPs Pulp	24mm	Escherichia coli
ZnO-NPs leaves	22mm	
ZnO-NPs peel	23mm	
Ciprofloxacin	28mm / No Zone	
Extract	No Zone	

DISCUSSION

Antibiotic resistance is a critical global health issue that arises when bacteria develop the ability to withstand the effects of antibiotics. Over time, bacteria can mutate and acquire resistance genes, making the antibiotics ineffective against them. This phenomenon is fueled by the overuse and misuse of antibiotics in both humans and animals, as well as the lack of development of new antibiotics. The consequences of antibiotic resistance are severe, leading to prolonged illnesses, increased healthcare costs, and even deaths that could have been preventable with effective antibiotics. It poses a significant challenge for healthcare providers and researchers worldwide, as many common bacterial infections are becoming increasingly difficult to treat.

In the face of this problem, the search for alternate agents to combat bacterial infections has become a top priority. Several approaches are being adopted, and researchers are exploring use of nanotechnology to deliver antimicrobial agents directly to infected areas. Nanoparticles can be designed to target bacteria selectively and disrupt their cellular processes. Plants have been explored in recent years for their potential as a green and eco-friendly source for the synthesis of nanoparticles. Nanoparticles are particles that have at least one dimension in the nanometer range and possess unique properties compared to their bulk counterparts. Their small size and increased surface area make them promising candidates for various applications, including antibacterial applications.

Present study was aimed at synthesizing nanoparticles using *Citrus limon* various parts such as the peel, juice, and leaves. These parts contain bioactive compounds like flavonoids, terpenoids, and phenolic

compounds, which act as reducing and stabilizing agents during the nanoparticle synthesis process.

The synthesized nanoparticles were characterized using UV-visible spectroscopy, FTIR and SEM that confirmed the presence of ZnO-NPs in a colloidal solution. The absorbance range of ZnO (zinc oxide) nanoparticles by UV-visible spectroscopy typically fall within the wavelength range of 200 to 400 nm. However, it is important to note that the exact absorbance range can vary depending on factors such as the size, shape, and concentration of the nanoparticles. ZnO-NPs of present study displayed characteristic peak at 280 nm.

FTIR analysis was done and stretching vibrations at 502 and 432 cm⁻¹ detected in the FTIR spectrum generally stand for the tetrahedral coordination of the Zn and O molecules in the ZnO-NPs. The peaks at 3344.57 cm⁻¹ corresponds to the alcohol/phenol and assigned as stretching vibration of O-H group, absorbance at 2978.09 cm⁻¹ belongs to the group of carboxylic acid, 1562.34 cm⁻¹ corresponds to the vibration and bending modes of C=C aromatic group, the peaks at 1409.96 and 1018.41 cm⁻¹ were designate to the O-H bond of ethyl alcohol group and the peaks at 866.04 and 651.94 belongs to the bending modes of C-H aromatic group. The results indicated the existence of organic biomolecules such as proteins and enzymes on surface of nanoparticles that are accountable for reduction and stabilization of ZnO nanoparticles. Presence of hexagonal phase ZnO nanoparticles was indicated by a weak band at 866.04 cm⁻¹. Adnan Alnehia *et al.*, 2022 also reported similar results of the FTIR study of ZnO-NPs, with the presence of signified O-H bond stretching, metal-oxide, primary amines, nitriles and alkyls [12].

SEM analysis of ZnO-NPs revealed the size and shape of sample. The current study showed that ZnO-NPs have spherical shape. The maximum and minimum particle size of ZnO-NPs was in nanometer range. Present results were in good agreement with Shagufta Saeed *et al.*, 2020 that documented green synthesis of ZnO-NPs from *Achyranthes aspera* leaf extract [15]. Shagufta Irshad *et al.*, 2018 and Meron Girma Demissie *et al.*, 2020 stated that zinc oxide nanoparticles have a significant antimicrobial effect on several Gram-positive as well as on Gram-negative bacteria [14, 16]. The antibacterial activity of biologically synthesized ZnO-NPs was studied against *Staphylococcus aureus*, MRSA, *Escherichia coli* and *Pseudomonas aeruginosa* in current research. ZnO-NPs showed comparable antibacterial activity as compared to antibiotic and plant extract. The diameter of zone of inhibition produced by ZnO-NPs was almost equal to the standard antibiotic. The results indicated that use of nanoparticles is a promising option to control the problem of antibiotics resistance. The results of antibacterial activity of ZnO-NPs against different bacterial strains are reported in literature.

In conclusion, the use of citrus limon based synthesis of nanoparticles for antibacterial applications is an exciting area of research that holds potential for developing effective and eco-friendly antimicrobial agents. Broad-spectrum activity of Citrus limon-based nanoparticles have shown promise in inhibiting the growth of various bacteria, including both Gram-positive and Gram-negative strains. This broad-spectrum activity is essential in combating antibiotic-resistant bacteria, which pose a significant global health challenge. The synthesis of ZnO-NPs using citrus limon lemon as a reducing and stabilizing agent for anti-bacterial applications revealed promising results. The study demonstrated the successful green synthesis of ZnO nanoparticles, which offers several advantages over conventional chemical methods. Citrus limon extract as a reducing agent is a green and sustainable alternative to traditional chemical methods, which often involve hazardous chemicals and generate toxic waste.

Overall, the study suggests that citrus limon-based synthesis of ZnO nanoparticles could be a promising approach for producing anti-bacterial agents with reduced environmental impact and potential medical applications. However, further research and testing are necessary to fully understand the nanoparticles' antibacterial properties, toxicity levels, and their effectiveness against various bacterial strains.

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Author Contributions: MU performed experiments, SF helped in data analysis. RA and NA supervised work and writing of the article.

Competing Interests: There are no competing interests among the authors.

Ethical Statement: Not Applicable

Financial Support: No specific grant was availed for this research.

Data Availability: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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