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Moringa oleifera Mediated Green Synthesis of Zinc Oxide Nanoparticles and their Characterization and Evaluation of Biological Activities

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The green synthesis of nanoparticles has attracted the attention of scientific communities due to their simple, economical, and environment-friendly properties. This study focuses on the biological synthesis of zinc oxide nanoparticles (ZnONPs) using an aqueous extract of *Moringa Oleifera* as a reducing and stabilizing agent. The formation, structure, and other physical and chemical properties of ZnONPs have been extensively studied using various microscopy and spectroscopic techniques. The biogenic synthesis of ZnONPs was confirmed by UV-visible (UV-Vis) spectrophotometer analysis and further characterized by Fourier transform infrared (FTIR), X-ray diffraction (XRD) analysis, and Scanning electron microscopy (SEM). Potential antibacterial and antioxidant activity for ZnONPs were also studied. A sharp peak at 450 nm was observed by UV-Vis analysis, while FTIR analysis showed the presence of -O-H-, -C=C-and -C-H- stretching. SEM analysis revealed that ZnONPs were cubic and hexagonal with500 nmto 1µmsize. The results of antimicrobial activity presented that the zone of inhibition of ZnONPs against *Pseudomonas aeruginosa* ATCC25923,

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Bacillus subtilis ATCC6633, *Klebsiella pneumoniae* ATCC4617, and *Escherichia coli* ATCC15224 were in the range of 16-36 mm. The antioxidant potential was evaluated by DPPH assay and the IC50 value was 144.59 µg/mL. This study provides an ecofriendly green approach for ZnONPs synthesis with less time and energy consumption.

Keywords: Moringa oleifera; ZnONPs; green synthesis; characterization; antibacterial; antioxidant.

1. INTRODUCTION

Nanotechnology has emergedas a revolutionary approachfor several fields of sciencesespecially health and medicine. Inorganic materials, like metal and their oxides, have gotten a lot of popularity in the last ten years because of their ability to sustain extreme processing conditions. Moreover, they are safe for humans as well as animals due to their unique nature. Such abilities of these metals have drawn the attention of scientists [1]. The inorganic nanoparticles like silver (Ag), gold (Au), copper (Cu), copper oxide (CuO), titanium dioxide (TiO_2), and zinc oxide (ZnO) have shown significant antibacterial activities against different bacterial strains. Among these, ZnONPs are of great interest because they can be prepared easily without much cost and they are quite safe for humans and animals and they possess several medicinal properties [2]. They are extensively used in the formulation of healthcare products [3]. Other reasons that ZnONPs have drawn the attention of scientists are their semiconductor properties along with the unique antibacterial, anti-fungal, wound healing, and UV filtering properties, high catalytic and photochemical activities [4].Several methods have been used for the synthesis of ZnONPs such as Wet chemical [5], chemical Microemulsion [6], Hydrothermal method [7], Solvothermal method [8], Sono-chemical [9], Solar photocatalysis [10]. But increasing awareness towards green chemistry and other biological processes led to the development of an eco-friendly approach for the synthesis of nanoparticles which is more facile, sophisticated, and requires less expensive and toxic chemical substances. Such biological approaches appear to be a promising, cost-effective alternative to conventional physical and chemical methods of nanoparticles synthesis [11,12].

In biological methods, different organisms, microbes, fungi, algae, and plants are used to reduce the large metallic particles into nanoscale particles [13,14]. The use of plants for the synthesis of nanoparticles is most common [15]. Plant-based synthesis methodsoffer safe, labor-intensive protocols which do not require equipment, are easy to perform, cost-effective, and reproducible [16]. Nanoparticles are also prepared using microorganisms but the rate of conversion is much slower as compared to the plant-mediated methods [17,18,16,17]. Nanoparticles are prepared using different plant extracts which contain the reducing agents which reduce the metal into metal and metal oxide nanoparticles [19]. ZnONPs prepared by the green synthesis methods are safe and non-toxic so, they are desirable to be used in the biomedical field [20]. ZnONPs are important in the health industry as they have antibacterial, antidiabetic, and anticancer activities [21]. The properties of ZnONPs depend on their size, shape, and morphology. The antibacterial activity of ZnONPs increases with a decrease in the size of nanoparticles and an increase in concentration [22]. The morphology and surface area of the ZnONPs also plays an important role. Conventional methods of preparation of ZnONPs involve the use of hazardous chemicals or higher pressure and temperature which is not safe and not cost-effective [23]. Previously ZnONPs have been prepared using the extracts of *Aloe barbadensis*, *Sargassum muticum*, *Parthenium hysterophorusAspergillus niger*, and *Abrus precatorius* which exhibit antibacterial and antifungal activities [24,25]. Green synthesis of ZnONPs can be done in a variety of ways. Such approaches have the following advantages: they take relatively little time and do not require any intermediate substances, they use a low-cost precursor, the product is of high purity and quantity. Moreover, the processing is easy, and they do not need expensive machinery or a setting [26].

Moringa oleifera tree is a part of the flowering plant family Moringaceae. The *moringa oleifera* species has many nutritional and medicinal properties. Mostly the tree is native in the tropical and subtropical areas of Pakistan, Bangladesh, India, Afghanistan, and Sri Lanka. It is known as one of the most valuable trees in the world because every single part of the tree has multiple properties [27,28]. *Moringa oleifera* contains large amounts of phytochemicals such as gallic acid, carotenoids, kaempferol, rutin, vicenin-2,

quercetin, chlorogenic acid, fatty acids, niazirin I and II, niazinin, and niazimicin [29,30]. This plant has several medicinal applications such as antidiabetic, anticancer, anti ulcerative, anti-hypertensive, antipyretic, and many more [29,31-33].Therefore, *Moringa oleifera* is used to produce nanoparticles.

This study involved the eco-friendly synthesis of ZnONPs by leaf extract of *Moringa oleifera.*Synthesized nanoparticles were characterized by different characterization techniques. Their antibacterial potential was determined against both gram-negative and gram-positive bacterial strains through the agar well diffusion method. Antioxidant potential was evaluated by DPPH radical scavenging activity.

2. MATERIALS AND METHODS

2.1 Material Collection

Disease-free, fresh leaves of *Moringa oleifera*were collected from the botanical garden of Bahauddin Zakariya University Multan, Pakistan. Zn $(NO_3)_2.6H_2O$ and all other chemicals of standard analytical grade were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2 Preparation of *Moringa oleifera* powder

2.2.1 Washing and drying

The plant fresh leaves were properly rinsed with tap water to remove dust particles. After rinsing the leaves with tap water, the leaves were washed with distilled water to remove any impurities. Washed leaves were kept in shade for three days for drying purposes. Dried leaves were crushed to obtain a fine powder. The powdered leaves were passed through a mesh sieve to remove dust particles [34].

2.2.2 Preparation of *Moringa oleifera* leaf extract

The extract of the leaves powder was prepared according to the method described previously with minor modifications. 25 g of leaf powder was added into 250 mL of distilled water in a beaker and heated on a hot plate at 70 °C for 3 hours with constant stirring. A brown-colored extract was obtained which was cooled at room temperature. After cooling the extract, it was filtered Wattman (No.1) to obtain pure plant extract. The pH of the extract was maintained at 5.9. The resulting plant extract was stored in a refrigerator at 4 °C for later use and prevented from direct light exposure [34,35].

2.2.3 *Moringa oleifera* mediated ZnONPs synthesis

To prepare the ZnONPs using plant leaves extract a modified method was used [12], 80 mL of plant extract was poured into a flask and 5.0 g of Zn $(NO_3)_2.6H_2O$salt was added to it; according to a method described earlier [35] with slight modification. The pH of the reaction mixture was recorded 4.62 at room temperature and the reaction mixture was heated at 60 °C for 2.5 hours. The reaction mixture was cooled at room temperature and subjected to a centrifuge at 12,000 rpm for 10 minutes. As a result, precipitates were obtained which again centrifuged three times with distilled water at 6000 rpm for 20 minutes for washing purposes. After that, the precipitates were dried in an oven at 100 °C for 2 hours and then annealed at 500 °C in an open-air furnace to get crystalline ZnONPs. Green synthesized nanoparticles were subjected to further characterization processes and biological activities.

2.3 Characterization of ZnO Nanoparticles

2.3.1 UV–vis spectrophotometry

The synthesis of the ZnO nanoparticles was confirmed by using a T80 UV-Vis spectrophotometer. The absorption was measured in the 300-700 nm wavelength range [36].

2.3.2 Fourier-transform infrared (FTIR) spectroscopy

FTIR is an analytical method for determining the structures and functional groups involved in the formation of ZnONPs. The infrared frequencies applied to the radiation absorbed by the sample correspond to the oscillations of the functional groups and the natural frequencies of the atomic bonds. The frequency of natural oscillations is related to each part of the molecule, such as the mass of the atom, the bond and bond strength, or the bond ends between the atom and each functional group. ZnONPs were scanned and monitored using Alpha Bruker FTIR with an infrared spectrum of 500-4500 cm^{-1} [36,37].

2.3.3 Scanning electron microscopy (SEM)

The particle size distribution, size, and surface morphology of the ZnONPs were investigated using a scanning electron microscope (Philips XL-30S FEG) [38].

2.3.4 nX-ray diffraction (XRD)

X-ray diffraction is a new non-destructive, non-contact method used to determine the average distance between rows of atoms, the orientation of the crystal, the structure of the crystal, and the size and shape of the crystal region. The particle size and crystal purity of the ZnONPs were investigated by an X-ray diffractometer (SIEMENS D5005) [39].

2.4 Biological Activities of Green Synthesized ZnONPs

2.4.1 Antibacterial activity of ZnO nanoparticles

The antimicrobial activity of *Moringa oleifera* mediated ZnONPs against different bacterial strains was evaluated. For this purpose, cultures of *Bacillus subtilis* (ATCC6633TM), *Escherichia coli* (ATCC25922TM), *Pseudomonas aeruginosa* (ATCC25923), and *Klebsiella pneumonia* (ATCC4617) were prepared in nutrient broth. Agar well method was performed to evaluate the antibacterial potential of ZnONPs [40]. Nutrient agar plates were prepared, and bacterial cultures were spread on these plates with a sterile glass spreader. After this, wells were made with the help of a sterile borer of 6mm size. Solution of ZnONPs at different concentration i.e., 100µg/ml, 150 µg/ml, 200 µg/ml and 250 µg/ml were added into respective wells. Ciprofloxacin was used as standard. Plates were kept at 4°C for 5 minutes, and then in an incubator for 24 hours at 37 °C. Next day zones for measured.

2.4.2 Antioxidant activity of ZnO nanoparticles

The antioxidant potential of ZnONPs was evaluated by DPPH radical scavenging assay. 0.1 M solution of DPPH (1,1 Diphenyl 2- picryl hydrazyl) was prepared in methanol. 150 µl methanolic solution of DPPH was further mixed with an equal volume of different ZnONPs dilutions (i.e., 100 µg/ml, 200 µg/ml, 400 µg/ml, and 500 µg/ml) [41]. The reaction mixture was kept in dark for 30 minutes at 37°C. After incubation, a color change was observed and absorbance was measured at 517 nm wavelength. Ascorbic acid was used as a positive control. The following formula was used to calculate the %age inhibition.

%age inhibition= (Abs Control – Abs Sample/Abs Control) × 100

3. RESULTS

3.1 Biological Synthesis of ZnONPs

The change in color from reddish-brown to reddish-black was observed after incubation. This color change indicated that the$Zn(NO_3)_2.6H_2O$ has been reduced by leave the extract of *Moringa oleifera*into ZnONPs.

3.2 Biophysical Characterization of ZnO Nanoparticles

A variety of techniques were used to characterize the biosynthesized ZnONPs.

3.2.1 UV-Vis analysis of ZnONPs

The stability and formationof the green synthesized ZnONPswere measured by UV-Vis spectroscopy.The UV-Vis spectrum, recorded in the 300-700 nm range of wavelength, showed a sharp peak at 450 nm (Fig. 1). This peak indicated that the phenomenon of surface plasmon resonance occurred due to the reduction of the Zn into ZnO nanoparticles.

3.2.2 FTIR analysis of ZnONPs

The FTIR analysis revealed the functional groups present on the surface of ZnONPs as stabilizing agents. The presence of -O-H- bond due to sharp peaks at 1390 $cm^{-1}$and 3500$cm^{-1}$was observed [42]. The other peak at879 cm^{-1}represented the presence of-C-H- stretching [43]. The peak at515cm^{-1} indicated-C=C-stretching [44] and the presence of metal oxides i.e., ZnO (Fig. 2). Metal oxides are characterised by intrinsic absorption bands below 1000 cm^{-1} (the "fingerprint area"), which are generated by inter-atomic vibrations [17,44,45].

3.2.3 SEM analysis

The size andsurface morphology of biosynthesized ZnONPs were determined by SEM. The surface morphology plays an important role to control the physicochemical properties of nanoparticles.The size of ZnONPs was in the range of 500 nmto 1µm with cubic hexagonalshape and uniformly distributed Fig. 3.

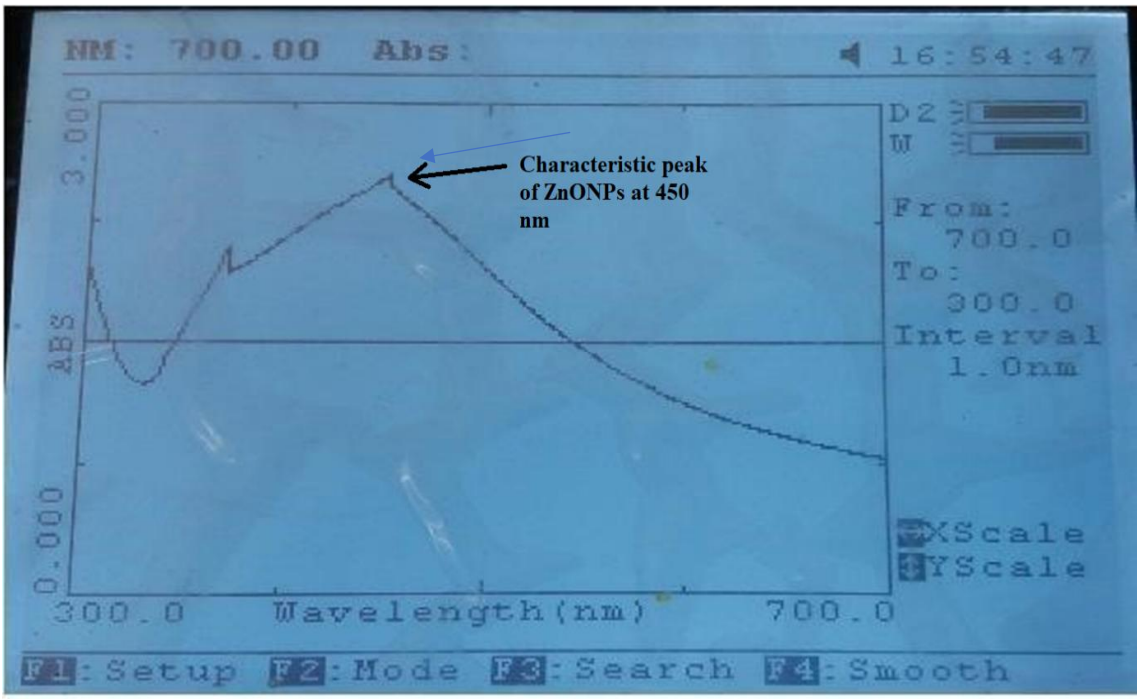

Fig. 1. UV-Vis spectroscopy analysis confirmed the synthesis of ZnONPs

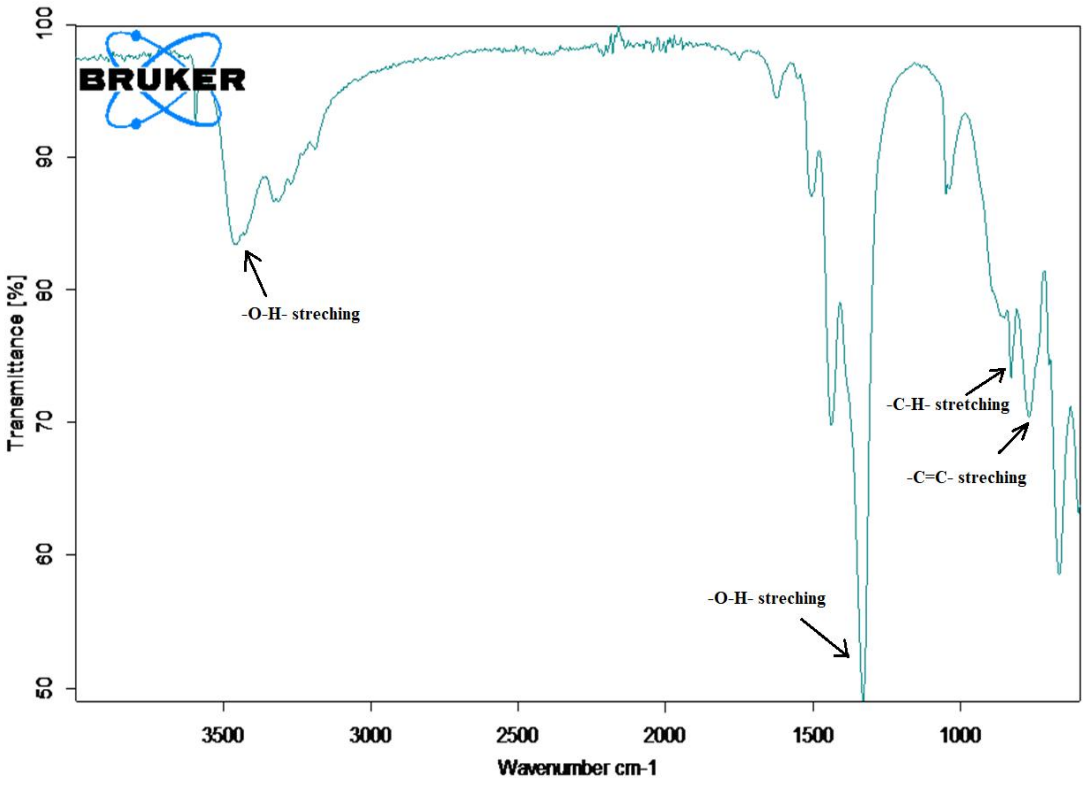

Fig. 2. FTIR spectrum of ZnONPs shows the presence of functional groups that act as stabilizing agents

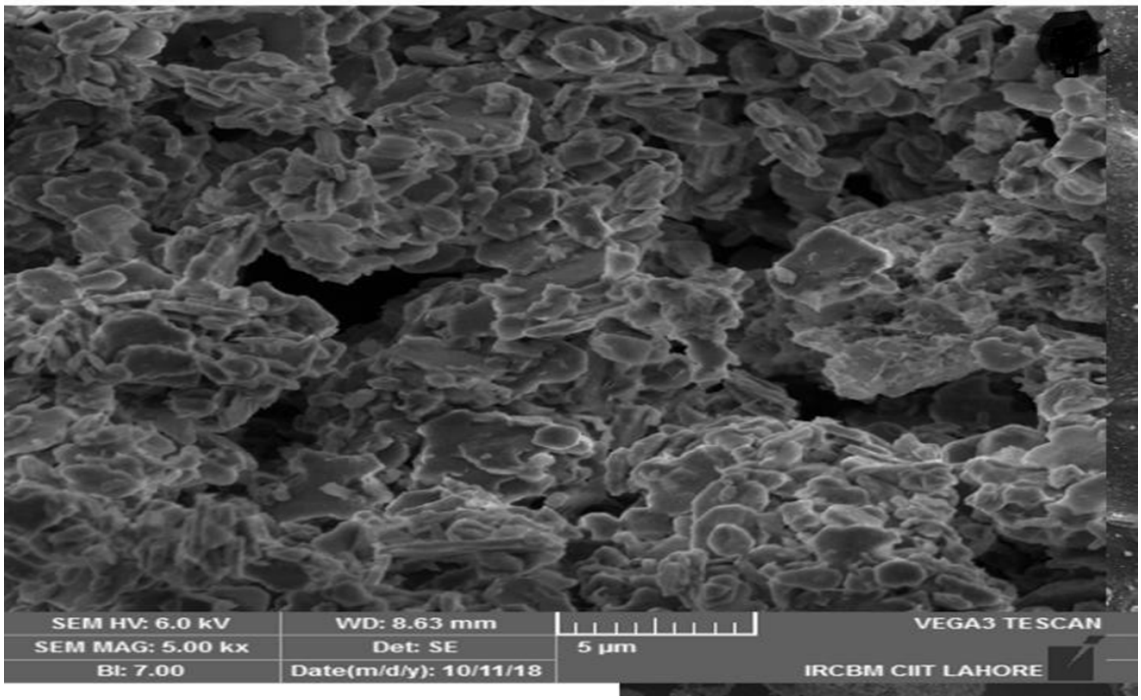

Fig. 3. SEM analysis revealed the morphology and size of green synthesized ZnONPs

3.2.4 XRD Analysis

In this study, XRD spectroscopy was used to determine the crystallinity and size of nanoparticles. The XRD pattern in Fig. 4 shows strong, sharp, and intense Bragg reflections indications 17.5º, 23.45°, 31.72°, 34.5°, 36.3°, 43.1° 47, 47.76° and 59°, these peaks indicate the presence of ZnO nanoparticles (Fig. 4). On the other hand, these peaks were compared to Miller indices (100), (002), (101), (102), (103), (110), and (201) similar to the standard wurtzite structure of ZnO. Consequently, X-ray diffraction evaluation confirms the crystallization of ZnONPs. Some extra peaks were appeared due to impurities.

3.3 Biological Activities of ZnONPs

3.3.1 Antibacterial activity

The antimicrobial activity of ZnONPs has been evaluated against Gram-positive bacteria *Bacillus subtilis* ATCC6633 and Gram-negative *Escherichia coli* ATCC15224, *Pseudomonas aeruginosa* ATCC25923, and *Klebsiella pneumonia* ATCC4617. Ciprofloxacin was used as positive control and leaves extract of *Moringa oleifera*as a negative control. The antibacterial potential of different concentrations of nanoparticles was evaluated by measuring the zone of inhibitions (ZOIs) against test samples. Table 1 demonstrated the ZOIs of ZnONPs in the range of 16 mm to 36 mm. Results indicated that as the concentration of nanoparticles increased their antibacterial potential also increased.

3.3.2 Antioxidant activity

The results of the DPPH assay revealed that ZnONPs were potential antioxidant agents. The %age inhibition was increased by increasing the concentration of nanoparticles as shown in Table 2. The IC50 value of ZnONPs was 144.59 ± 0.12 µg/mL which was comparable with the IC50 value of ascorbic acid i.e., 20.88 ± 0.21 µg/mL.

4. DISCUSSION

Nanoparticles can be synthesized by special methods, but the green approach for the synthesis of nanoparticles is gaining importance as compared to other chemical and physical methods. The synthesis of nanoparticles by physical and chemical methods is often affected by many negative aspects such as cost, time, energy consumption, environmental difficulties or damage, and the excessive pressure and heat required can leave harmful chemicals on the surface of nanoparticles. Such nanoparticles cannot be used for medical purposes [46,47].

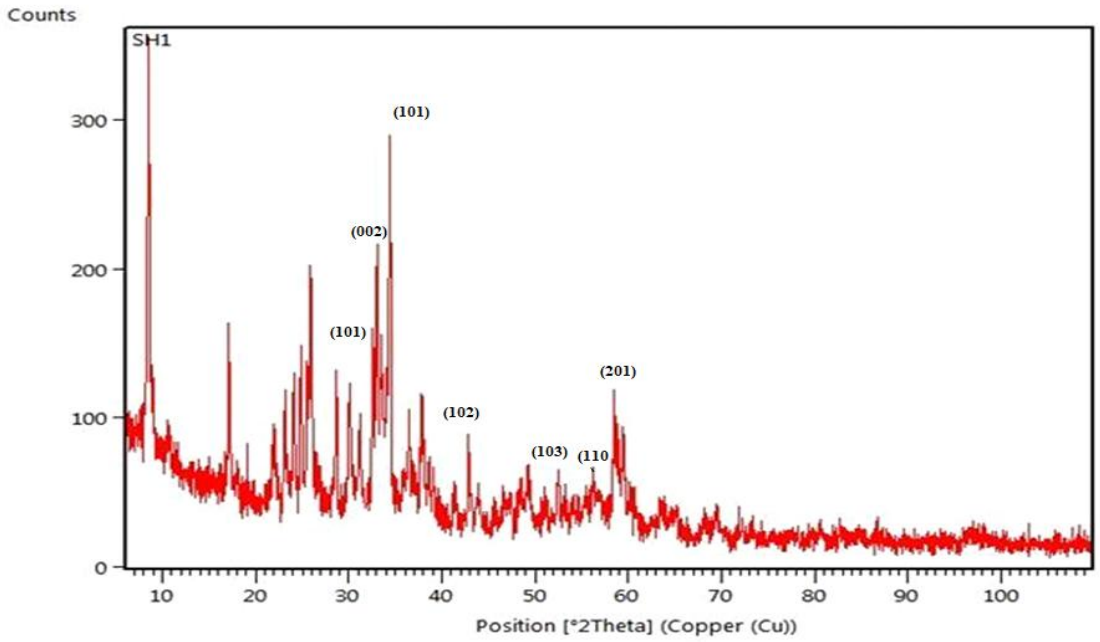

Fig. 4. XRD analysis confirmed the crystalline nature of ZnONPs

Table 1. The zone of inhibition (mm) of ZnONPs against bacterial strains

	ZOI (mm)				
Bacterial strains	**Ciprofloxacin (1mg/ml)**	**ZnONPs (100µg/ml)**	**ZnONPs (150µg/ml)**	**ZnONPs (200µg/ml)**	***Moringa oleifera* Leaf extract (200mg/ml)**
Bacillus subtilis ATCC6633	42	20	29	36	0
Escherichia coli ATCC15224	28	16	23	29	0
Pseudomonas aeruginosa ATCC25923	38	19	26	33	0
Klebsiella pneumonia ATCC4617	40	18	24	30	0

Table 2. Percentage of inhibition and IC50 values of ZnONPs and ascorbic acid from DPPH assay

Sample	Concentration (µg/mL)	%age inhibition	IC50 (µg/mL)
ZnONPs	100	49.71± 0.07	144.59± 0.12
	200	51.4± 0.23	
	400	60.61± 0.33	
	500	72.29± 0.19	
Ascorbic acid	100	54.71 ± 0.09	20.88± 0.21
	200	61.75 ± 0.26	
	400	73.61 ± 0.37	
	500	80.29 ± 0.17	

In this study, ZnONPs were synthesized using the biological method. Leaf extract of*Moringa oleifera* was used to synthesize ZnONPs as plants based synthesis methods have many benefits such as they are easy to deal with, inexpensive, and achievable without the use of natural solvents or harmful substances [48]. Several researchers have reported the green synthesis method of ZnONPs such as Siripireddy and colleagues(41)used the *Eucalyptus globulus*plant to synthesize ZnONPs and evaluated their photocatalytic properties.Moreover, the *Moringa oleifera*plant has been extensively studied for the synthesis of nanoparticles. Prasad and coworker [49] used *Moringa oleifera*leaf extract as a reducing agent to synthesize silver nanoparticles (AgNPs) and evaluated the antimicrobial potential of AgNPs. *Moringa oleifera*leaf extractmediated AgNPs synthesis was also reported by [50] and they used AgNPs for optical limiting purposes. In another study by [51] reported the AgNPs synthesis and evaluated the antifungal potential of AgNPs against *Candida albicans*. Synthesis of nickel oxide nanoparticles (NiONPs) via *Moringa oleifera* was reported byEzhilarasi et al. [52]. Anand et al. [53] fabricated flower extract of *Moringa oleifera*plant and synthesized palladium nanoparticles (PdNPs). Ngom et al. [44] synthesized ZnONPs by using extracts of different parts of the *Moringa oleifera*plant. But they only focused onthe structural and optical properties of ZNONPs. In the current study, we have studied the biological activities including the antibacterial and antioxidant potential of ZnONPs.

To evaluate the stability and formation of nanoparticles UV-Vis spectroscopic analysis was performed inthe range of 300nm to 700nm wavelength. This analysis has confirmed the reduction of Zn into ZnONPs.A sharp absorption peak at 450nm confirmed the nanoparticle synthesis. Our results are in accordance with the study of [54]. FTIR analysis revealed the presence of -C-H-, -C=C- and -O-H- functional groups supported by the Vijayakumar et al. [55] and Anand et al. [56]. SEM analysis was carried out to find the surface morphology and size of nanoparticles which revealed that ZnONPs were spherical and have 500nm size. Muhammad and colleguesalso carried out SEM analysis of ZnONPs. Our results are in accordance with this study [57].

Further ZnONPs were evaluated for their antibacterial and antioxidant potential. The results of antibacterial activity have revealed that bio-reduced ZnONPs are potent antibacterial agents against gram-positive bacterial strain*Bacillus subtilis*with 36mm ZOI. Since ZnO may form free radicals, which can cause cell damage and ultimately cell death, by damaging the cell membrane [58]. The nanosized ZnONPs can easily penetrate the cell membrane of bacterial strains and cause cell damage. Thus, bio-reduced ZnONPs can act as potent antimicrobial agents against infectious diseases. Previous studies have also reported similar results [26,55,56] % age inhibition and IC50 value obtained from DPPH assay of ZnONPs demonstrated the antioxidant potential of these nanoparticles. Das et al. reported thatZnONPs exhibited greater free radical scavenging potential as compared to their bulk counterpart due to the high surface-to-volume ratio [59]. In current study ZnONPs of different concentration showed significant %age inhibition i.e., 49.71 µg/mL, 51.4µg/mL, 60.61µg/mL, and 72.29 µg/mL with 144.59 IC50 value. These results revealed that our synthesized ZnONPs can be used as potent antioxidant agents.This study was only limited to evaluating the antibacterial and in vitro antioxidant activities of ZnONPs. Further studies should be carried out to evaluate more biomedical applications such as the anti-cancerous and anti-diabetic potential of ZnONPs at laboratory as well as clinical level.

5. CONCLUSION

In this study, ZnONPs have been successfully synthesized by green route using *Moringa oleifera* leaf extract as a reducing and stabilizing agent which appeared as an ecofriendly, cheaper, and suitable approach. The UV-Vis analysis showed the formation of NPs and FTIR and XRD revealed the bioactive phytochemicals of *Moringa oleifera* leaves extract on the surface of ZnONPs. Besides, ZnONPs have been tested for pathogenic strains of various bacterialstrains which indicated the antibacterial potential of ZnONPs. Such nanoparticles could be used as drug carriers. ZnONPs also showed potent antioxidant properties. Thus, further studies should be carried out to evaluatebiomedical applicationsof ZnONPs.

ETHICAL APPROVAL

We conducted our research after obtaining proper IEC approval.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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