

The possibility of using zinc oxide nano particles for treatment of bovine-subclinical mastitis, *in vitro* antibacterial activity.

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Abstract

Bovine mastitis is an inflammation of mammary gland parenchyma in cows. It is caused by multiple pathogens that result in economic losses because of the reduction in milk production and poor quality of the milk. It is commonly caused by bacteria that multiply in the mammary gland and regional lymph nodes, damaging the mammary parenchyma. The present study focuses on the bovine mastitis treating capacity of zinc oxide nanoparticles by examining *in vitro* antibacterial activity, MIC and MBC against pathogens isolated and identified from sub-clinical mastitis in dairy cows. Zinc oxide nanoparticles were synthesized both chemically and green methods from a plant extract of *Coriandrum sativum* and characterized for phase and microstructural analysis. The milk sample from subclinical mastitis cows was collected from the veterinary hospital. The bacteriological examination revealed the presence of *S. aureus* and *E. coli*. The antibacterial activity of the zinc oxide nanoparticle was evaluated at 650, 850, 1300, 1700, 1950 and 2500 µg/ml concentrations and the minimum inhibitory concentration was found to be 650 µg/ml and 850 µg/ml for *S. aureus* and *E. coli* respectively, and minimum bactericidal concentration was 650 µg/ml and 1700 µg/ml for *S. aureus* and *E. coli* respectively.

Keywords: Antibacterial activity, Bovine subclinical mastitis, *Coriandrum sativum*, Chemical synthesis, Green synthesis, Minimum inhibitory concentration, Minimum bactericidal concentration, Zinc oxide nanoparticles.

Introduction

The world human population is expected to reach 9.4 billion. Food from animal sources is an important aspect of the human diet globally and it contributed to the development and the eradication of poverty in human society. To meet the increasing food demand of the world population animal health plays a crucial role. Animal health has a major role in food security for human consumption especially in developing countries.

Bovine mastitis is mammary gland parenchyma inflammation and it is one of the important diseases in the dairy industry. It is the most complex dairy cow disease which is negatively affecting milk production [1]. It is commonly caused by bacteria in dairy cattle and characterized by the chemical, physical and bacteriological changes occurring in the milk. In the dairy industry, it is a disease with an important economic impact which results in huge losses. The majority of antibiotics used in these industries are applied for the prevention and control of mastitis. However, drugs used for control, prevention and treatment of mastitis are not effective currently, this is mainly due to the development of resistance to wide range antibiotics used, as these drugs have been used for a long period of time and other possible causes.

Nanotechnology is being applied in a variety of fields like medicine, food, and agriculture. It is used to produce nano-sized particles that have different physical, optical and mechanical properties from their bulk. In the field of medicine, nanotechnology is being implemented for drug delivery, diagnostics and disease treatment [2]. Zinc Oxide (ZnO) nanoparticle is one of the recent outcomes of nanotechnology.

Zinc Oxide (ZnO) as Generally Recognized As Safe" (GRAS). ZnO has been incorporated in materials used for packaging of fish, meat and other foods to prevent spoilage and enhancement of preservation. Nanosized ZnO particles have antibacterial activities against a wide range of bacteria than their bulk counterparts. This is due to its small size and high surface to volume ratio of nanoparticles which improves interaction with bacteria. Zinc nanoparticle also has selective toxicity against bacteria.

The most common pathogens responsible for subclinical bovine mastitis are *S. aureus*, *S. agalactiae*, and other *Streptococcus* species and *Coliforms*. Antibiotic resistance in bacterial and fungal species is significantly increasing which causes diseases like mastitis incurable using antibiotics. This condition triggered for other options, one of which is the use of metals as antibiotics against bacterial and fungal agents [3].

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Different researches have been done to develop new antimicrobial agents in order to combat antimicrobial resistance microorganisms as an alternative to existing drugs. Current studies are showing that different nanoparticles have antibacterial and antifungal activities, but having minimal effects on human and animal cells. When metal oxides are synthesized in the nano range, they exhibit unique properties which are size, chemical composition and surface chemistry dependent. The antimicrobial property of Zinc Oxide (ZnO) Nanoparticles (NPs) is extensively studied among metal oxide nanoparticles. Activities of a wide range of bacteria have been reduced by zinc oxide. The antimicrobial properties of zinc oxide are significantly increased at the nanoparticle level. Zinc Oxide (ZnO) Nanoparticles (NPs) possess antibacterial, antifungal and anti-inflammatory properties and facilitate the healing of both acute and chronic wounds. Therefore, the current work was undertaken to synthesize ZnO NPs using both green and chemical methods, characterize and investigate the subclinical mastitis treating capacity of the nanoparticle by examining its *in vitro* antibacterial activities against pathogens isolated and identified from mastitis dairy cow [4].

Methods and Materials

Collection of milk samples

Dairy cows with clinical signs of mastitis were diagnosed for subclinical mastitis by a certified veterinarian from a veterinary hospital in Vijayawada city, Andhra Pradesh, India. A total of ten milk samples from ten confirmed subclinical mastitis dairy cows were aseptically collected. In brief, each teat was cleaned, disinfected using a 0.5% iodine solution and dried with a sterile paper towel. The milk sample of about 15 ml- 20 ml was stripped from teats to the sterile test tube. Then each tube was labelled and transported in an ice box to the microbiology laboratory at KL University [5].

Bacteriological examination

Standard bacteriological analytical methods were used to recover bacterial isolates from subclinical bovine mastitis in the milk sample. Isolation of bacterial strain was conducted according to the National Mastitis Council procedures. Blood agar in which sheep red blood cell (5%) was added and MacConkey agar were prepared and loop full of the milk sample was streaked on it. The plates were incubated at 37 °C for 24-48 h aerobically. The plates were inspected for colony morphology and characteristics of hemolysis after 24 and 48 h. The morphological characteristic of the bacteria was identified by Gram's Method of staining and colony morphology. The catalase test and coagulase test were used to identify the strain in addition to, the pattern of hemolysis and colony morphology.

Zinc nanoparticles preparations

The synthesis of ZnO NPs was carried both chemically and by green synthesis methods using a zinc precursor in an alkaline medium. All chemicals used throughout this work were purchased from Merck and, are of analytical grade. The input parameters of the synthesis process were identified and optimized. Then the synthesis and characterization studies were performed at optimum input conditions.

Preparation of plant extract

The *Coriandrum sativum* plant leaf was used as a reducing and capping agent to synthesize ZnO NPs as indicated. The fresh plants were collected from a local market in Vijayawada city, Andhra Pradesh, India. The leaves were surface cleaned using distilled water for about three times to remove the minor organic contents from the surface of the leaves [6]. Then 10 g of the leaf was mixed with 200 ml of distilled water and heated at a temperature of 70°C for about 30 min to get the plant extract.

After boiling, the liquid extract is separated through filtering and then the extract was cooled down for 4 h at room temperature.

Green synthesis of ZnO-NPs

0.2 M of Zinc acetate dihydrate solution ($Zn(CH_3COO)_2 \cdot 2H_2O$) was prepared and the *Coriandrum sativum* plant extract was added to the solution in 1:1 (v/v) ratio. This mixture was stirred and heated at 70 °C and sodium hydroxide (NaOH) is added drop by drop to it till light yellow colour precipitate was formed. The yellowish precipitate was centrifuged at 5000 rpm for 20 min and washed three times with distilled water followed ethanol. Then the precipitate was dried in an oven and finally calcinated at 500 °C for 2 h.

Synthesis of ZnO NPs by precipitation method

ZnO NPs were synthesized by direct precipitation method using zinc acetate dihydrate solution and potassium hydroxide as precursors and a precipitating agent respectively as described elsewhere. In brief, an aqueous solution of 0.2 M Zn ($CH_3COO)_2 \cdot 2H_2O$ and 0.4 M KOH solutions were prepared with deionized water. The KOH solution was slowly added into Zinc acetate dihydrate solution at room temperature under vigorous stirring [7]. This resulted in the formation of a white suspension. The white product was centrifuged at 5000 rpm for 20 min and washed three times with distilled water followed by absolute alcohol. The obtained product was calcinated at 500 °C for 3 h after drying in an oven.

Characterization of ZnO NPs

Phase analysis: Phase analysis by X-ray diffraction is done to confirm the phases and impurities present in the calcined chemically and green synthesized ZnO powders. It involves the irradiation of a monochromatic X-ray beam generated from an X-ray tube on the sample and recording the intensity of the diffracted beam as a function of the goniometer position (2θ). It follows and works on the principle based on Bragg's law.

Where λ =wavelength of the incident X-ray beam, d =interplanar spacing or d -spacing, θ =position of the diffraction angle. Phase analysis is done in the X-Ray powder diffractometer fitted with a Ni filter of 0.154 nm. The diffraction patterns were measured using $CuK\alpha$ ($\lambda=1.5418 \text{ \AA}$) radiation generated at a voltage of 40 kV and a current of 40 mA. The samples were scanned in the interval $10^\circ < \theta < 50^\circ$ at a scanning rate $20^\circ/\text{min}$ with a step size of 0.05° in a continuous mode. XRD analysis of the samples was performed using X'pert High Score software in

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comparison with the reference powder diffraction data given by the Joint Committee on Powder Diffraction Standards (JCPDS).

Microstructural analysis: Field Emission Scanning Electron Microscope (FESEM) along with EDX (Energy-Dispersive X-ray) analysis attachment was used to analyze the morphology of the powders [8]. FESEM is a typical electron microscope, which uses a high energy focused beam of electron produced by Field Emission Gun (FEG) for generating an image by just scanning with the beam of electrons that interacts with the surface atoms of the samples to develop relevant information. As the wavelength, of the electron, is very small, a very high magnification can be achieved in field emission electron microscopy. Prior to that, the samples were gold coated for 240 s in a sputter coater in an Ar atmosphere. The electrons at 15 kV sources were used to develop relevant information about the samples.

Determination of antibiotic activity

Different concentrations of synthesized ZnONPs using both green and chemical methods were evaluated for their antimicrobial activity against the pathogens isolated from milk samples of subclinical mastitis cow. ZnONPs were dissolved in distilled water using a sonicator. The nanoparticles antibiotic activity was evaluated at 650, 850, 1300, 1700, 1950 and 2500 µg/ml concentrations of ZnO for each of pathogen isolated from the sample, with both green and chemically synthesized NPs.

The antibiotic activity against each isolate was determined by agar well diffusion method using Muller Hilton agar medium for both types of the nanoparticle synthesized according to CLSI procedure. After the agar was solidified the plates were inoculated and spread with a bacterial suspension of approximately $1-2 \times 10^8$ CFU/ml. The wells having a diameter of 6 mm were prepared. Each of these wells was filled up with 50 µL of the ZnO NPs solution to be tested [9]. Then the inhibition zones around the wells were observed after 24 and 48 h of incubation at 37°C.

Determination of MIC and MBC

The MIC of prepared ZnO NPs against bacteria isolated from subclinical mastitis was determined using a standard broth dilution method. The MIC value of the nanoparticle was evaluated at 650,850,1300,1700,1950 and 2500 µg/ml concentrations of ZnO for each of pathogen isolated from the sample. Tryptone Soya Broth (TSB) was used to determine MIC and MBC, as antibacterial assay media. The MIC was identified by observation of turbidity in each tube before and after incubation, the test was done in triplicate. The inhibitory concentration was taken as the lowest concentration that inhibited the growth of each isolated bacteria. The lowest concentration that killed the bacteria was the Minimum Bactericidal Concentration (MBC) and it was determined by sub-culturing 100 µL of each sample remaining clear, with no turbidity, in tubes containing 1 mL of fresh medium, were incubated for 24 h at 37 °C, and these were compared between the green and chemical method of synthesis of the Nanoparticles [10].

Results and Discussion

Isolation and Identification of pathogens from bovine mastitis milk

From the milk samples, the two most prominent strains *S. aureus* and *E. coli* isolate were identified. On MacConkey agar *E. coli* appeared smooth, circular pink colonies; it was also Catalase positive and Gram-negative. *S.aureus* showed hemolysis on blood agar, morphologically it was a Gram-positive and biochemical test that indicates catalase and coagulase positive. Growth isolates were sub cultured on nutrient agar, plates at 37°C for 24 h to obtain pure cultures. The bacteria isolated in this investigation are, the more frequently isolated bacterial causes of subclinical mastitis in bovine, this was in agreement with the findings from other researches. Because of long-time application of antibiotic for the treatment of mastitis in dairy industries, now days almost all of commonly used drugs are not effective against these pathogens isolated from subclinical mastitis due to antibiotic resistance as described in findings [11].

Phase analysis

The XRD diffractogram of the green and chemically synthesized calcined ZnO powders are shown in (Figure 1). The XRD patterns showed diffraction lines characteristic of ZnO, both present in standards and in the literatures. The peaks well matched with the JCPDS card no 75-0576. It had the hexagonal crystal system with a space group of P63 mc. Single and pure phase ZnO powders were observed with the absence of any other impurity phase even in minor quantity. Therefore the calcination temperature was optimized at 500°C (Figure 1).

Microstructural analysis

The micrograph of the chemically and green synthesized calcined ZnO powders is shown in (Figure 2). The microstructure as observed under the microscope showed the shape and particle size of the powders. A comparative analysis of both is discussed in this section. The ZnO nanosized particles were found to be agglomerated. It is observed that the shape of both particles was more or less spherical. Though very few elongated grains were noticed for the chemically synthesized ZnO, the particle size of the chemically synthesized powder was found to be larger than that of the green synthesized powder [12]. The range for the particle size of the synthesized powder was found to be within the nano range of 100 nm (Figure 2).

The corresponding elemental distribution mapping and EDX spectra of the ZnO powder microstructure chemically and green synthesized is shown in (Figures 3 and Figure 4) respectively. It shows the presence and distribution of the different elements such as O and Zn. The presence of Au is due to the gold coating done on the powder surface (Figures 3 and Figure 4).

Antibiotic activity

The antibiotic activity of the nanoparticle was investigated by measuring the size of the inhibition zone. The nanoparticle, at

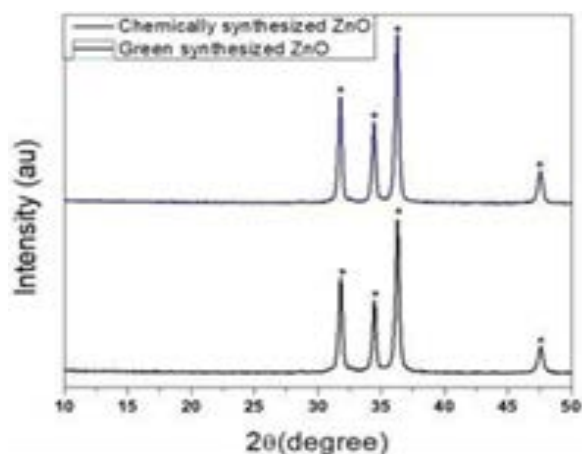


Figure 1. 1A) A very cryptative area black leg sicked heifer typical sign of cryptation and 1B) Lameness and Emphysematous of fore left limb on the last day of the treatment.

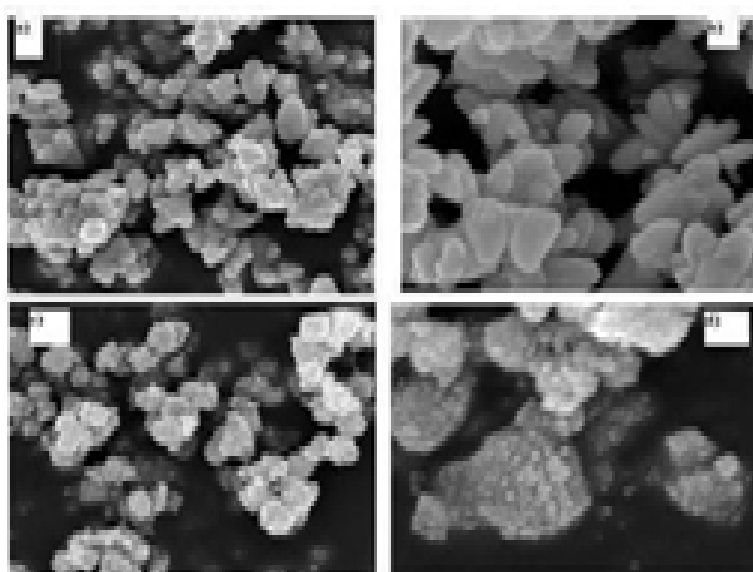


Figure 2. 2a) Micrograph of chemically synthesized ZnO particles at lower magnification; 2b) Higher magnification green synthesized ZnO particles at; 2c: Lower magnification; 2d: Higher magnification.

the tested concentrations, showed different levels of antibiotic activity against each bacterial strain [13]. Antimicrobial activity of ZnO NPs against broad spectrum microorganism was reported and it has been used in biomedical and therapeutic research in human medicine, its application in the field of veterinary medicine would be effective in treating diseases like mastitis as suggested in this work. Both green and chemically synthesized ZnO NPs have shown almost the same level of antibiotic activity (Figures 5 and Figure 6), showing that, no significant difference in antibiotic activity value between them against the pathogens isolated from subclinical mastitis (Figures 7 and Figure 8). The results indicated that the diameter of the zone of inhibition increases with the concentration of the nanoparticle (Figures 5- 8).

Determination of MIC and MBC

The test tubes used for MIC were examined for turbidity after 24 hours of incubation at 37 °C. The turbidity was different between both bacterial species. All the tested concentrations of ZnONPs, including the minimum concentration at

650 µg/ml did not show any turbidity for *S. aureus*, that it prevented the growth of the bacteria. The concentration of ZnONPs starting from 850 µg/ml did not show turbidity for *E. coli* bacteria. From these outcomes we concluded, that the MIC is 650 µg/ml and 850 µg/ml for *S. aureus* and *E. coli* respectively for both green and chemical methods of nanoparticle synthesis methods (Table 1). The MBC value is found to be 650 µg/ml and 1700 µg/ml for both *S. aureus* and *E.coli* respectively for both synthesis methods. *S. aureus* was susceptible for lower concentration of ZnO NPs than *E. coli* which supported the reports that Gram-positive bacteria were more sensitive to the ZnO Nanoparticle than Gram-negative bacteria. The inhibitory efficacy of ZnO-NPs is dependent on its concentration and size [14]. The wound healing activities of ZnO-NPs were shown by the work, this means that ZnO NPs have anti- inflammatory activity, since mastitis is inflammation the combined anti-inflammatory and antibiotic activity of the nanoparticle would be effective in the treatment of mastitis (Table 1).

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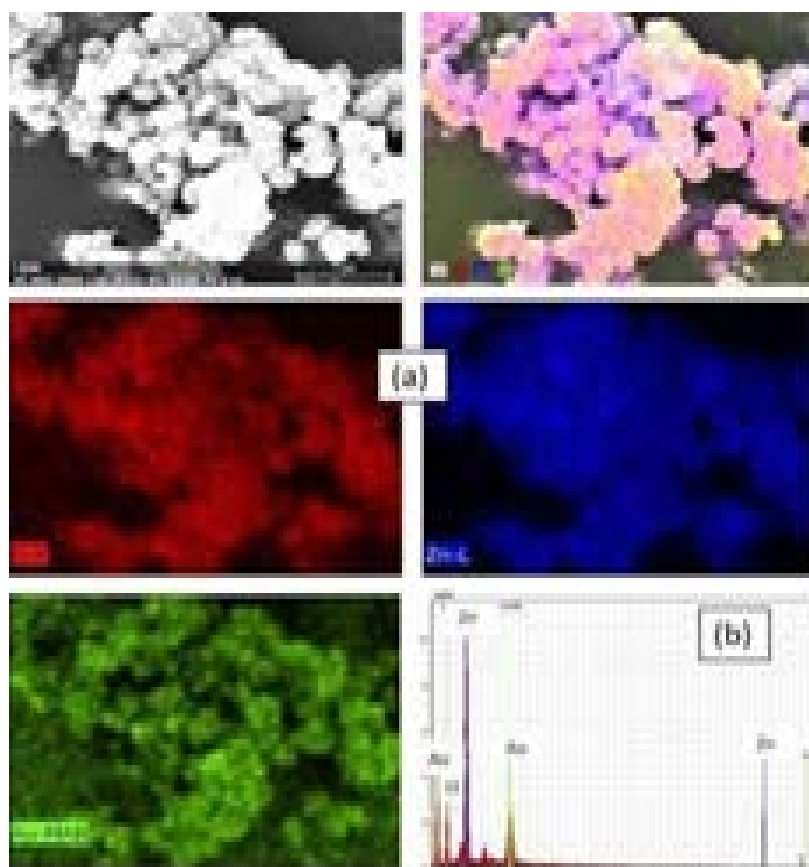


Figure 3. 3a) Elementary mapping of 500°C calcined chemically synthesized ZnO powder showing the distribution of elements such as O and Zn; 3b) EDX spectra of green synthesized ZnO powder.

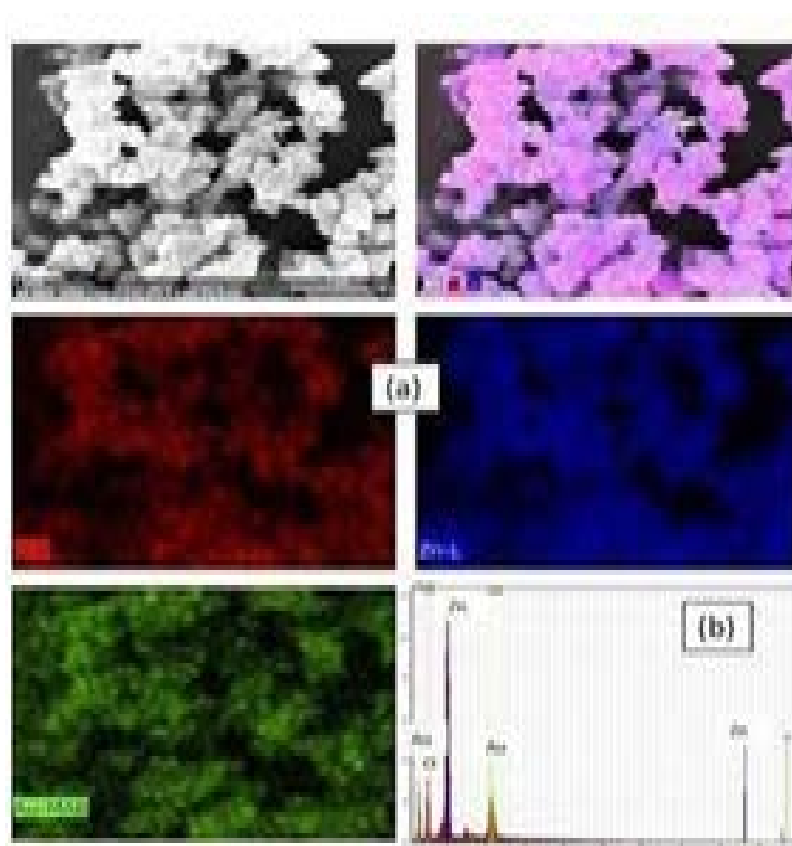


Figure 4. 4a) Elementary mapping of 500°C calcined green synthesized ZnO powder showing distribution of elements such as O and Zn; 4b) EDX spectra of green synthesized ZnO powder.

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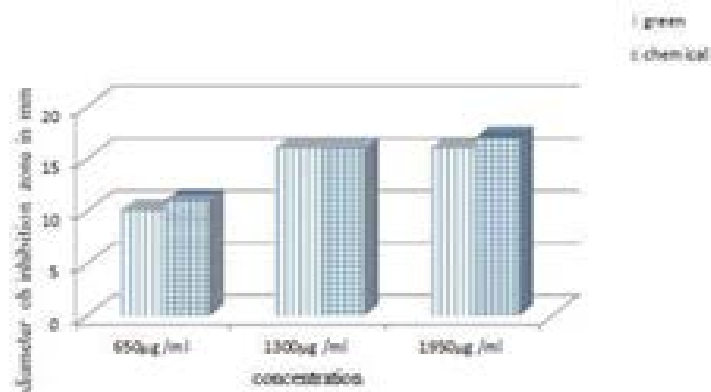


Figure 5. Comparison of zone of inhibition of green and chemical methods in diameter (mm) against *S. aureus* at 650, 1300 and 1950 µg/ml.

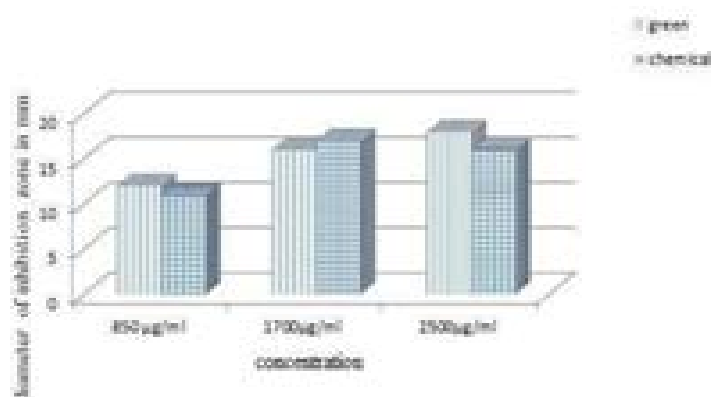


Figure 6. Comparison of zone of inhibition of green and chemical methods in diameter (mm) against *E. coli* at 850, 1700 and 2500 µg/ml.

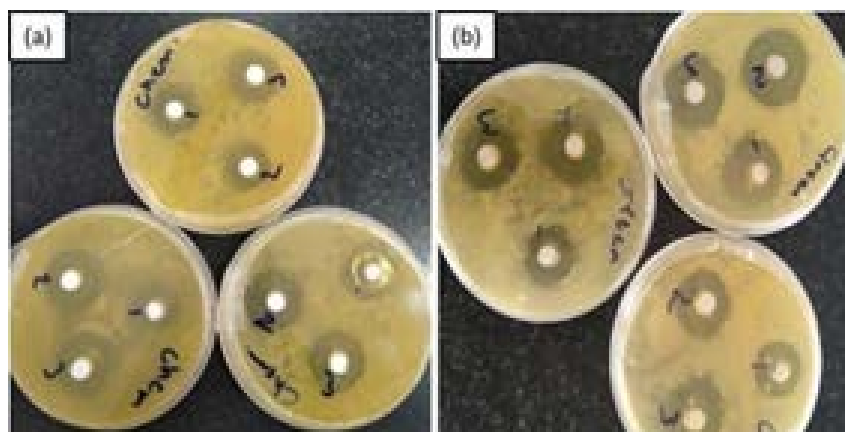


Figure 7. 7a: Comparison of chemically; 7b: green synthesized ZnO NPs against *S. aureus* at 650, 1300 and 1950 µg/ml.

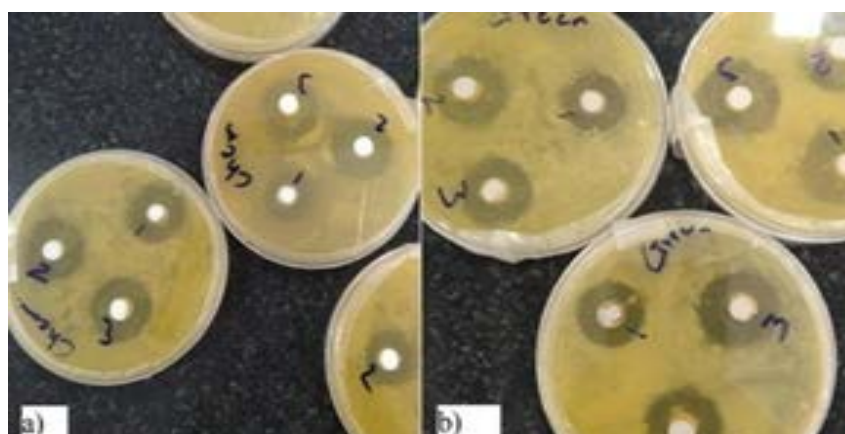


Figure 8. 8a: Comparison of chemically; 8b: green synthesized ZnO NPs against *E. coli* at 850, 1700 and 2500 µg/ml.

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Table 1. The MIC and MBC values ($\mu\text{g/ml}$) of ZnO-NPs against *S. aureus* and *E. coli* strains isolated from bovine affected with subclinical mastitis.

Method of zinc oxide nano particle synthesis			
Bacterial isolates		MIC	MBC
Green synthesis	<i>S. aureus</i>	650 $\mu\text{g/ml}$	650 $\mu\text{g/ml}$
	<i>E. coli</i>	850 $\mu\text{g/ml}$	1700 $\mu\text{g/ml}$
Chemical synthesis	<i>S. aureus</i>	650 $\mu\text{g/ml}$	650 $\mu\text{g/ml}$
	<i>E. coli</i>	850 $\mu\text{g/ml}$	1700 $\mu\text{g/ml}$

Note: MIC=Minimum inhibitory concentration, ZnO NPs=Zinc oxide nanoparticles, *S. aureus*=*Staphylococcus aureus*, *E. coli*=*Escherichia coli*

Conclusion

In conclusion, ZnONPs was synthesized using green and chemical methods of synthesis and characterized by FTIR, powder XRD. The antibiotic activity of the nanoparticle against common causes of subclinical bovine mastitis was investigated as an alternative treatment approach for mastitis in the dairy cow. The results of this study indicate the potential anti-bacterial effects of ZnO NPs against *S. aureus* and *E. coli* isolated from bovine subclinical mastitis at concentrations of 650 $\mu\text{g/ml}$ and 850 $\mu\text{g/ml}$, respectively. Isolated pathogens which are *S. aureus* and *E. coli* strains were susceptible to ZnO NPs and there is no significant difference between the efficacy of green and chemically synthesized ZnO NPs. As subclinical Mastitis is inflammation caused commonly by bacteria, the antibiotic and wound healing activities of ZnO NPs can facilitate effective treatment of mastitis in dairy. This study is limited to the effect of the nanoparticles on common bacterial causes of subclinical bovine mastitis and it is conducted *in vitro*; further studies are required to know the effect on other causes of mastitis as well as clinical trials are required.

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Conflict of Interest

The authors declare no conflict of interest in this work.

Authors' Contributions

Rupita Ghosh corresponding author, involved in the study design. Zerihun Beyene involved in the project implementation, editing, and major revisions of manuscript.

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