Continental Veterinary Journal
ISSN: 3079-0212 (ONLINE)
www.cvetj.com; editor@cvetj.com

RESEARCH ARTICLE
DOI: 10.71081/cvj/2024.030

Evaluation of Anti-Bacterial Efficacy of Nanoparticles Against Major Mastitis Associated Pathogens

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ARTICLE INFO

ARTICLE HISTORY: CVJ-24-924

Received: 24 September 2024
Revised: 15 November 2024
Accepted: 18 November 2024
Published online: 21 November 2024

Key words:
Bovine mastitis
E. coli
Staphylococcus aureus
Zinc oxide nanoparticles (ZnO-NPs)

ABSTRACT

The present study reports the efficacy of zinc oxide nanoparticles (ZnO-NPs) and traditional antibiotics against *E. coli* and *Staphylococcus aureus* (*S. aureus*). The results showed that ZnO-NPs, regardless of concentration, did not demonstrate considerable antibacterial action against *E. coli* on their own but ZnO-NPs had a strong antibacterial action against *S. aureus* at different doses, suggesting that they may be employed to treat illnesses brought on by these bacteria. The study assessed the efficacy of traditional antibiotics, such as ciprofloxacin, and demonstrates that they had a strong antibacterial impact on *S. aureus* and *E. coli*. There were no appreciable variations in the antibacterial effectiveness of the nanoparticles at concentrations of 30 µL, 60 µL, and 120 µL, according to statistical analysis performed with Tukey's test through SPSS software. The concentration of ciprofloxacin alone and the combination of ciprofloxacin and ZnO-NPs, on the other hand, showed substantial differences, suggesting that the latter combination was not more effective. Furthermore, as compared to employing ZnO-NPs or antibiotics alone, the study showed that combining ZnO-NPs with conventional antibiotics has a decreased but still substantial antibacterial impact against both *E. coli* and *S. aureus*. In particular, the antibacterial action was much decreased when ciprofloxacin and ZnO-NPs were combined, especially against *E. coli*. The study's overall findings highlighted the possible advantages of ZnO-NPs antibacterial activity having more alone against *S. aureus* but in combination with traditional antibiotics were somehow reduced against bacteria. These results implied that enhancing the formulations of nanoparticles may be a viable tactic to fight bacterial infections and deal with the expanding problem of antibiotic resistance bacteria.

To Cite This Article: Ahmed B and Nawaz MA, 2024. Evaluation of anti-bacterial efficacy of nanoparticles against major mastitis associated pathogens. Continental Vet J, 4(2): 222-230. http://dx.doi.org/10.71081/cvj/2024.030

INTRODUCTION

Mastitis is an inflammatory response of the mammary tissue carried on by somatic damage to the mammary glands or a microbiological infection in cattle. It is believed to be the utmost predominant disorder that roots commercial victims for the dairy industry due to lower milk production and worth. Depending on the level of inflammation, cow mastitis can be categorized as clinical, sub-clinical, or chronic. In dairy cows, external abnormalities including fever and a red, swollen udder are indicative of clinical bovine mastitis. The clots and flakes in the cow's milk give the impression that it is thin. Clinical mastitis can be characterized as acute, sub-acute, or per-acute by condition on the level of inflammation. Furthermore, fatal cases of severe clinical mastitis are possible. Unlike clinical mastitis, sub-clinical mastitis does not indicate any obvious symptoms in the mammary tissue or fluid yet, when the somatic cell count (SCC) rises, milk production declines. Specialists agree that sub-clinical mastitis causes the herd to lose more money monetarily than clinical cases do, despite the fact it is difficult to pinpoint the precise amount of loss it causes (Elshaer and Shaaban 2023). The most serious issue of dairy sector is mastitis which is an estimated 533 billion dollars worth of economic damages were attributed to mastitis globally (El-Sayed and Kamel 2021). It is reported that financial loss of milk per cow per lactation cycle due to mastitis is 70%, premature culling results in 14%, the segregation of the mastitis milk causes 7% losses; and the rate of the vet medicine accounts for 8% losses globally (Mubeen et al. 2021). Numerous risk variables, such as microbes, host, and ecological aspects, are known to be significantly linked to the occurrence of bovine mastitis. The mastitis rheostat

plains took all these issues into account. The course of mastitis is influenced by numerous microbial, host, and environmental variables. Certain traits are intrinsic to every animal species and shared by all of them (Mohammad et al. 2019). Drugs are now the most effective way to treat mastitis, however, infections cannot always be completely cured, which promotes microbial resistance, and the accumulation of drug remains in milk. Dairy farm's cows with infections were primarily found to have *S aureus* and coagulase-negative staphylococci (CNS) as their primary microbes (Luan et al. 2018). The persistence of intramammary infections has been linked to bacterial biofilms, which have been suggested as a significant virulence component (Lange et al. 2021). The most prevalent of these bacteria, *S aureus*, causes the worst instances of bovine mastitis and represents the utmost danger to the global dairy sector (Krishnamoorthy et al. 2021). Significant financial losses are brought on by this bacterium which also causes reproductive issues, a sharp drop in milk revenue, and additional costs for veterinarian care, culling diseased animals, and tainted milk replacement and a variety of poisons and enzymes that *S aureus* produces in milk can cause serious food-borne illnesses. Moreover, they are connected to cow mastitis in its preclinical, recurrent, and clinical forms. Their continuous existence within the cells may serve as a cause of recurrent infection (Krishnamoorthy et al. 2021). *S. aureus* is one of the primary causes of clinical mastitis in cows. This study planned to confine and portray *S. aureus* from cow clinical mastitis cases utilizing phenotypic location of MRSA and VRSA (Varghese et al. 2024). Moreover, *S aureus* can transform into a multi-drug-resistant strain known globally as vancomycin and methicillin-resistant *S. aurous* (VRSA) and methicillin-resistant *S aurous* (MRSA). Although MRSA is widely recognized as a hospital infection with developed multi-drug resistance, recent findings have linked the bacteria to incidences of cow mastitis (Algammal et al. 2020). Their continued intracellular presence in various forms shielded them from host defense mechanisms and medications, but they can deteriorate to additional infectious wild-type phenotypes, which is likely what causes recurrent infection. Additionally, prolonged use of large doses of antibiotics causes *S. aureus* to become progressively resistant to them. Antibiotic resistance in living organisms has been shown to be halted by employing nanoparticles as a secure and effective substitute treatment against common infectious agents (Jin and Jin 2021).

While general preventative measures like improved milking cleanliness, post-milking teat cleaning, and milking gear maintenance are important, antibiotics are the principal therapy for current mastitis infections. However, the dairy industry reduced its use of antibiotics due to worries about germs that are resistant to antibiotics as an outcome of the extensive usage of antibiotics. As a result, researchers have looked for alternative medicines, especially natural compounds derived from plants and animals, to prevent and treat bovine mastitis. We identified the nanoparticles physicochemical characteristics, minimal inhibitory concentration, and interactions with the cell membrane in addition to measuring the degree of biofilm reduction. According to the findings, out of all the nanomaterial examined, the silver–copper compound was the utmost dynamic (biofilm was reduced by almost 100% at a concentration of 200 ppm for each tested germ class). But individual silver nanoparticles were equally effective (biofilm was reduced by about 100% at 200 ppm, but the level of decrease was less at 50 and 100 ppm than for the complex). New alternative treatments for cow mastitis may employ nanoparticles (Lange et al. 2021).

The area focuses on using Nano sciences and nanostructure for enhanced biotechnological applications. Green techniques that make usage of different plant components, bacteria, fungus, and algae have largely superseded the usage of physical and chemical means for the creation of nanostructures. The biosynthesis of Nano-based structures is very helpful for medical applications since it produces inexpensive, non-toxic nanomaterial (Ahmed et al. 2022).

Toxic chemicals are not needed as reducing agents since organic sources are used in the biosynthesis of nanomaterial. Because they include a variety of metabolites that serve as both stabilizing and reducing agents, plants are thought to be one of the most beneficial sources for the green production of nanoparticles. Depicts the environmentally friendly process used to create ZnO nanoparticles and the many instruments used to characterize them. In addition to the creation of several pharmaceutical molecules, bacterial contaminations remain to be a foremost cause of fatality (Hamad et al. 2020).

The dimensions, form, and synthetic chemistry of the nanoparticles are among the physicochemical characteristics that influence their intracellular delivery. Numerous studies have demonstrated the benefits of submicron versus micron size, meaning that small particles are more efficient than large ones at delivering drugs to infected loci (Algharib et al. 2020).

For instance, it was shown that silver nanoparticles, which series in dimensions from 10nanometer to 50nM, were useful in treating *S aureus* related infections. The manners of the nanoparticles in the biological fluid and their intracellular uptake are significantly influenced by their size. Moreover, it measures the constancy, drug stuffing and discharge, toxicity, and in vivo bio distribution of the nanoparticles (Hamad et al. 2020).

A recent investigation against bacterial spp. *E. coli, S. aureus, B. subtillis* and *K. pneumonia* revealed a greater zone of inhibition achieved by biologically manufactured silver nanoparticles (AgNPs) than chemically produced ones. Tri-sodium and lemon-synthesized silver nanoparticles showed comparable outcomes, with the former exhibiting superior antibacterial action against both gram-positive and gram-negative bacteria (Agarwal et al. 2018).

In the past ten years, nanoparticles have become a popular antibacterial agent. The nanoparticle's little toxicity and precise targeting make it suitable for use as a customized medication. In particular, they have shown efficacy in suppressing microorganisms that are resistant to antibiotics. Through the distraction of their cell membrane or the restriction of their food source, nanoparticles demonstrate their bacteriostatic or bactericidal activity. Because of its huge surface area to volume ratio, the nanoparticle may hold a huge number of ligands for improved directing of harmful bacteria (Ghodake et al. 2020).

There have already been several reports of metal and metal oxide nanoparticles with anti-microbial characteristics, including titanium oxide, copper oxide, iron oxide, gold, copper, and silver. The distinct electrical, optical, and therapeutic capabilities of ZnO NP have drawn the most attention of any other nanoparticle. Zinc oxide nanoparticles are suited for use as a biological membrane or in other biological uses due to their great biocompatibility and rapid electron transport kinetics. The antimicrobial activity of ZnO NPs in contradiction of a wide range of pathogenic bacteria (Akbar et al. 2019).

A numeral appliances are employed by nanoparticles to function as antibacterial agents. Loss of cell membrane integrity brought on by phospholipid bilayer breakdown is regarded as single of the utmost significant mechanisms. Another important mechanism that nanoparticles exploit is oxidative stress that is caused by reactive oxygen species (ROS). This ROS molecule impedes or modifies the processes of respiration, protein synthesis, food metabolism, and DNA replication, which ultimately leads to more cell death. Zinc oxide nanoparticles have superior anti-microbial action than soluble zinc compounds like zinc chloride because of their dynamic directing prospective, ability to yield ROS in the cell membrane and subsequently disturb cell membrane integrity, and capacity to further aid in protein, lipid, and DNA denaturation. As a result, Zinc chloride's antibacterial movement is mostly related to its capacity to oxidize thiol groups on glycolytic enzymes, hence suppressing the glycolytic process due to the Zn+2 ion's unique attraction for the S group (Jin and Jin 2021).

It is known that certain nanoparticles have efflux pump-inhibiting properties. Bacterial cell membranes include efflux pumps, which are involved in expelling waste products and hazardous substances from the cell. These pumps are also known to carry antibiotics out of the cell, extending the life of the cell. When excess Zn^{+2} ions inside the cell reach dangerous amounts, the Zn^{+2} efflux transporter protein (cation diffusion facilitator and P-type ATPase) exports the excess ions out of the cell. Because of their distinct membrane structures, G^+ and G^- microbes use diverse mechanisms for the addition of nanoparticles to their surfaces and for the movement of those particles inside the cell (El-Sayed and Kamel 2021).

Both teichoic acid and lipoteichoic acid exist in G^+ bacteria, along with a dense covering of peptidoglycan. Teichoic acid and lipoteichoic acid give the cell stiffness while acting as chelating agents. From the ZnO NP complex, they chelate the Zn^{+2} ions and move it throughout the cell. The outer layer of the peptidoglycan triple layer that makes up the cell walls of gram-positive bacteria contains porins. Ion channels called porins originated in the external layer of peptidoglycan and help passive passage of nanoparticles throughout the cell (Chonwerawong and Ferrero, 2021).

Depending on their size and shape, ZnO NPs can also be absorbed by common uptake, membrane diffusion, or endocytosis through membrane-based pores. Surface functionalization improves a nanoparticle's absorption by the cell. LamB Maltodextrin, a significant cause of glucose, is diffused into the periplasmic region via porins. Maltodextrin enters the intracellular matrix by attaching itself to a protein called maltodextrin binding. Nanoparticle uptake in *E. coli* cells was enhanced many times when maltodextrin and nanoparticles were conjugated on the surface (Bains et al. 2020).

Zinc oxide nanoparticles tend to liquefy in liquid solutions and discharge Zn+2 ions when a surface's free energy changes. The electrostatic attraction of Zn^{+2} ions to negatively charged membranes is also measured to be a typical mechanism of nanoparticle addition to the cell surface. The concentration of Zn^{+2} rises in the bacterial cytoplasm due to the local breakdown of the linked ZnO NP, which results in membrane outflow and the damage of the proton motive force. It has been discovered that smaller nanoparticles relate with cell membranes more strongly and are more hazardous (Ajdary et al. 2018).

G- Bacteria are extra vulnerable to the harmful effects of nanoparticles than G+ bacteria because the former have a thinner peptidoglycan layer and are less resistant to the attachment of nanoparticles with their cell membranes. Consequently, the first contact between nanoparticles and bacteria is significantly influenced by the thickness, content, and structure of the cell wall (Luan et al. 2018).

MATERIALS AND METHODS

Preparation of leaf extract

The leaves of the *Cassia fistula* plant were collected from UAF agriculture land Faisalabad, Pakistan. Leaves were placed under the sun for 4 weeks to dry properly. After drying, the leaves were meshed in the grinder completely. A 20g of cleaned mesh powder was mixed with 100mL of deionized water in 500 mL flask, placed at 80^oC in magnetic hot stirrer for 30 minutes, the solution turned bright yellow. Then the solution was filtrated through watt man's filter paper number 01, this process was repeated thrice **(**Bandeira et al. 2020).

Preparation of zinc oxide nanoparticles

Preparation of zinc oxide nanoparticles through green synthesis was done and followed by many steps (Bandeira et al. 2020). The molar mass of zinc acetate is 183.48g. so, 1 molar solution of $Zn(C_2H_3O_2)_2$ (zinc acetate) was prepared by dissolving 183.48g zinc acetate in 1000mL of distilled water. As 0.02M solution of zinc acetate was prepared, by dissolving 3.6696g of $Zn(C_2H_3O_2)_2$ (zinc acetate) in 1000mL of distilled water (Lange et al. 2021). The molar mass of sodium hydroxide is 40g. So, 1 molar solution of sodium hydroxide (NaOH) was prepared by dissolving 40g. Sodium hydroxide (NaOH) in 1000mL of distilled water. 0.02M solution of sodium hydroxide (NaOH) was prepared by dissolving 0.8g. of sodium hydroxide (NaOH) in 1000mL of distilled water (Elshaer et al. 2023).

Procedure of zinc oxide nanoparticles

- 25mL of 0.02M solution zinc acetate was mixed with 1mL of leaves extract; it was placed on magnetic stirrer for 2hrs. And then 0.02M solution of sodium hydroxide was added drop wise till pH reached to 12 and then further it was placed on magnetic stirrer for one hrs. and white precipitates were formed and the whole process was repeated thrice to obtain the required number of nanoparticles.

- Supernatant was discarded, the sedimentation was washed with distilled water thrice through centrifugation at 3000 rpm for 20 min.
- After that, this sediment was placed on Petri plate and distilled water was added and placed in Hot Air Oven at 150°C till dried powder was formed.
- After that dried powder was collected gently and stored it at 4°C (Varghese et al. 2024).

Isolation of pathogen of mastitis milk

Sample collection

Sample was collected from different animals, had clinical singe of mastitis and examined by visual inspection and palpation. In addition, the milk sample was checked for change in tint and steadiness. A sterile 50mL falcon tube was used to collect the third stream of milk (10 mL), which was then transported in an insulated ice box to the microbiology department of the University of Agriculture Faisalabad (UAF) within four hours of the samples were being collected aseptically (Bains et al. 2020).

Preparing the sample for identification and separation

The milk samples were centrifuged for 20 minutes at 3000 rpm. The supernatant was discarded, and the pellet was lifted and inoculated 05mL of Brain-Heart Infusion broth (BHI) and incubated for 24 hrs. at 37°C (Chonwerawong and Ferrero 2021).

Bacterial identification

For bacterial identification were passed from different steps of identification like microscopic examination and chemical examination.

Microscopic examination

Gram staining

A smear of suspension was prepared on glass slide with loopful of sample, heat fixed and air dried, crystal violet was poured on, left for 1 mint almost, washed with distilled water, gram iodine (as mode rent) was applied then washed with distilled water, applied acid alcohol of (95%) (As decolorizer) for 30sec, then washed with water, added safranin (as counter stain) almost left for 1mint, air dried and observed under microscope (Ghodake et al. 2020).

Biochemical examination

Biochemical tests were performed for identification of *E. coli* and *S. aureus.*

Methyl Red (MR) and Vogues–Proskauer (VP)

Methyl red test

MRVP broth was taken, inoculation was done in the broth, placed in incubator for 24hrs, after that broth was taken, isolated 2mL of inoculated broth and added 2 to 3 drop of methyl red indicator, color was observed which indicated *E coli* and color remained same indicated salmonella (Mohammad et al. 2019).

Vogues–Proskauer (VP)

MRVP broth was taken, inoculation was done in the broth, placed in incubator for 24hrs, after that broth taken, isolated 2mL of inoculated broth and added 4 drop Beret's reagent then shacked well up to 30mint and color was observed.

Indole test procedure

A sterilized test-tube was taken, 4mL tryptophan broth was poured in it, inoculated the sample, incubated it at 37°C for 24hour, after that it was taken, added 0.5mL of Kovac's reagent and observed ring on the surface of cultured broth which indicated positive.

Citrate test procedure

Citrate broth was taken, slant was made through cooling, it was inoculated from pure colony and after left for 2 days the colored was changed which indicated that was positive for salmonella, *E. coli* was negative for citrate test because no growth had occurred in media.

Coagulase test procedure

Clean slide was taken and placed drop of normal saline on each end of slide emulsified two thick colony of isolated sample, then added rabbit plasma in one sample, mixed properly and clump was observed in second indicate coagulase positive for *S. aureus*, but other *S. epidermis* and *S. saprophyticus* are negative.

Catalase test procedure

The cleaned glass slide was taken, placed small colony of growth sample in slide, and 3% H_2O_2 was poured on the slide and observed evolution of oxygen bubbles. Which indicated catalase positive of *Staph species*.

RESULTS

Biosynthesis of zinc oxide nanoparticle

Preparation of leaf extract

Dried leaves were meshed in the grinder completely. A 500mL beaker was taken, 20g of powder of leaves mixed with 100mL of deionized water, placed at 80°C of magnetic hot stirrer for 30minutes. Then the solution was filtrated through Wattman,s filter paper number 01 having size of (0.45 µm). This extract was used for reduction of zinc acetate into zinc oxide nanoparticles (Bains et al. 2020).

Preparation of 0.02M solution of zinc acetate

For the preparation 0.02M solution of zinc acetate, 3.6696g of $Zn(C_2H_3O_2)2$ (zinc acetate) was dissolved in 1000mL of distilled water in the glass after completely mixed the zinc acetate and then added extract of the leaves and placed on magnetic stirrer at 60°C for 2hrs.

For the preparation 0.02M solution of sodium hydroxide (NaOH), 0.8gram of sodium hydroxide (NaOH) was dissolved in 1000 ml of distilled water in the glass and after completed the formation of 0.02M solution of sodium hydroxide (NaOH), and then added drop wise sodium hydroxide (NaOH) to the solution of zinc acetate and leaves extract and a white precipitates were found at pH reached 12.

Synthesis of zinc oxide nanoparticles

After complete mixing of solution zinc acetate and leaves extract, placed on magnetic stirrer for 2hrs and then added drop wise of 0.02M solution of sodium hydroxide till pH was reached to 12 and then further placed on magnetic

stirrer for one hour and white precipitate was formed. Now discarded supernatant and sedimentation was washed with distilled water through centrifugation at 3000rpm for 20min. After that, this sediment was poured on Petri plate and added distilled water, and it was placed in hot air oven at 150ºC till dried powder was formed.

Determination of MIC and Antibacterial Activity

Agar well diffusion assay of *E. coli*

For performing antibacterial activity though agar well diffusion assay by using zinc oxide nanoparticles (ZnO-NPs) against the *E. coli*. picked a single colony from the pure isolated colony of *E. coli* plate, mixed in the normal saline and then made lawn on Muller Hilton plate and the 4 mM deep and 2 mM width of three wells were made with the help of steel borer, and then three concentration of zinc oxide nanoparticles (ZnO-NPs) were added and control well in the center having antibiotics disc of metronidazole (Fig. 1).

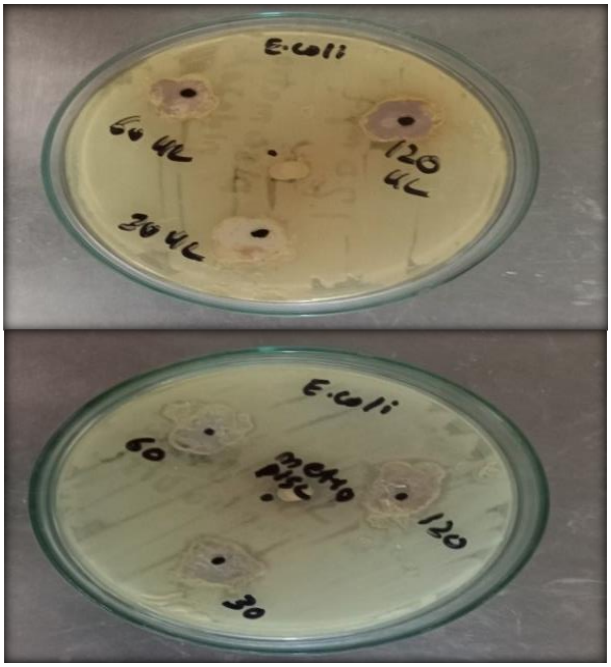

Fig. 1: The result of zinc oxide nanoparticles (ZnO-NPs) against *E coli.* Agar well diffusion assay of *S. aureus*

For performing antibacterial activity though agar well diffusion assay by using zinc oxide nanoparticles (ZnO-NPs) against the *S. aureus*. picked a single colony from the pure isolated colony of *S. aureus* plate, mixed in the normal saline and then made lawn on Muller Hilton plate and the 4mM deep and 2mM width of three wells were made with the help of steel borer, and then three concentration of zinc oxide nanoparticles (ZnO-NPs) were added and control well in the center having antibiotics disc of metronidazole (Fig. 2).

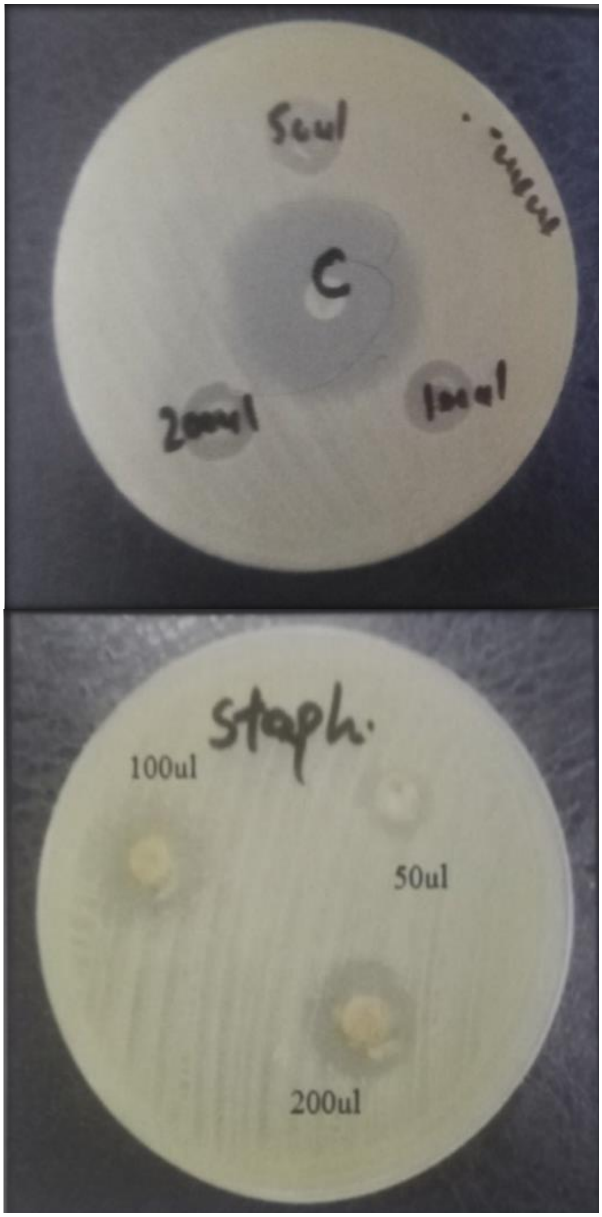

Fig. 2: Result of ZnO-NPs against *S. aureus*

Disc diffusion assay of *E. coli*

For performing antibacterial activity though agar disc diffusion assay by using zinc oxide nanoparticles (ZnO-NPs) against the *E. coli*. Picked a single colony from the pure isolated colony of *E. coli* plate, it was mixed in the normal saline and then made lawn on Muller Hilton plate and discs of zinc oxide nanoparticles (ZnO-NPs) were placed in the MHA agar and control group in the center had antibiotics disc of ciprofloxacin (Fig. 3).

For performing antibacterial activity though agar disc diffusion assay by using zinc oxide nanoparticles (ZnO-NPs) against the *S. aureus*. Picked a single colony from the pure isolated colony of *S. aureus* plate, it was mixed in the normal saline and then made lawn on Muller Hilton plate and discs of zinc oxide nanoparticles (ZnO-NPs)

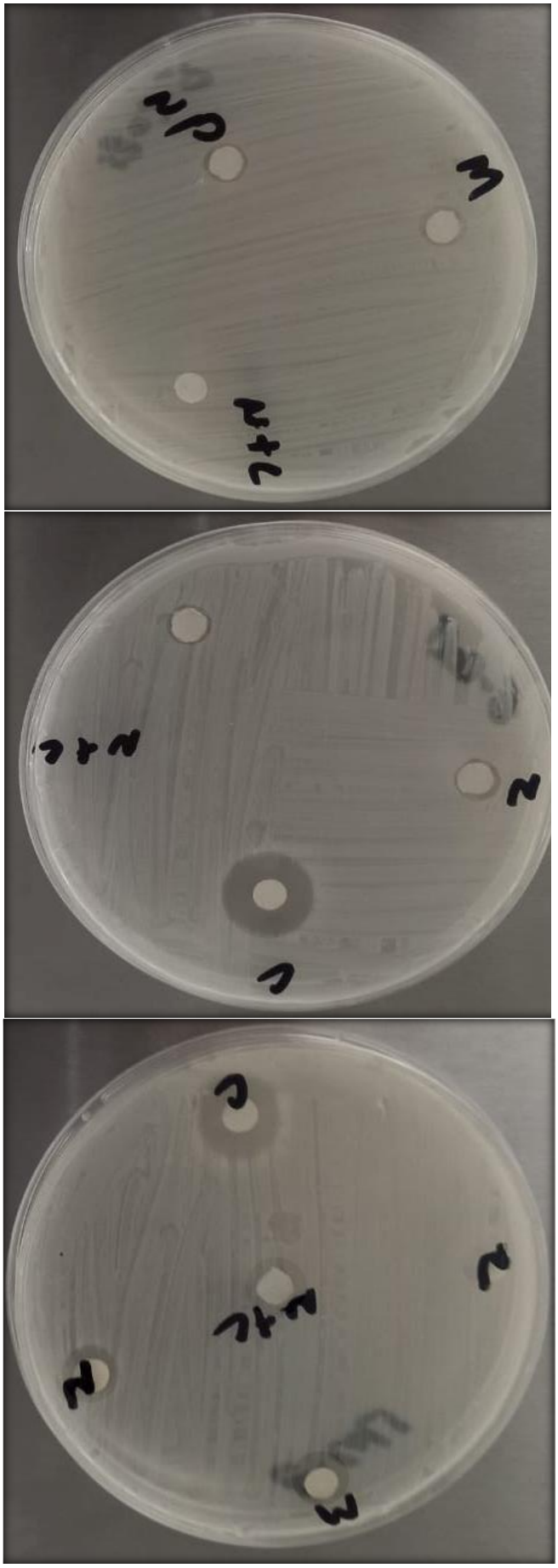

Fig. 3: Result of zinc oxide nanoparticles (ZnO-NPs) against *coli* bacteria by using disc. Disc diffusion assay of *S. aureus*

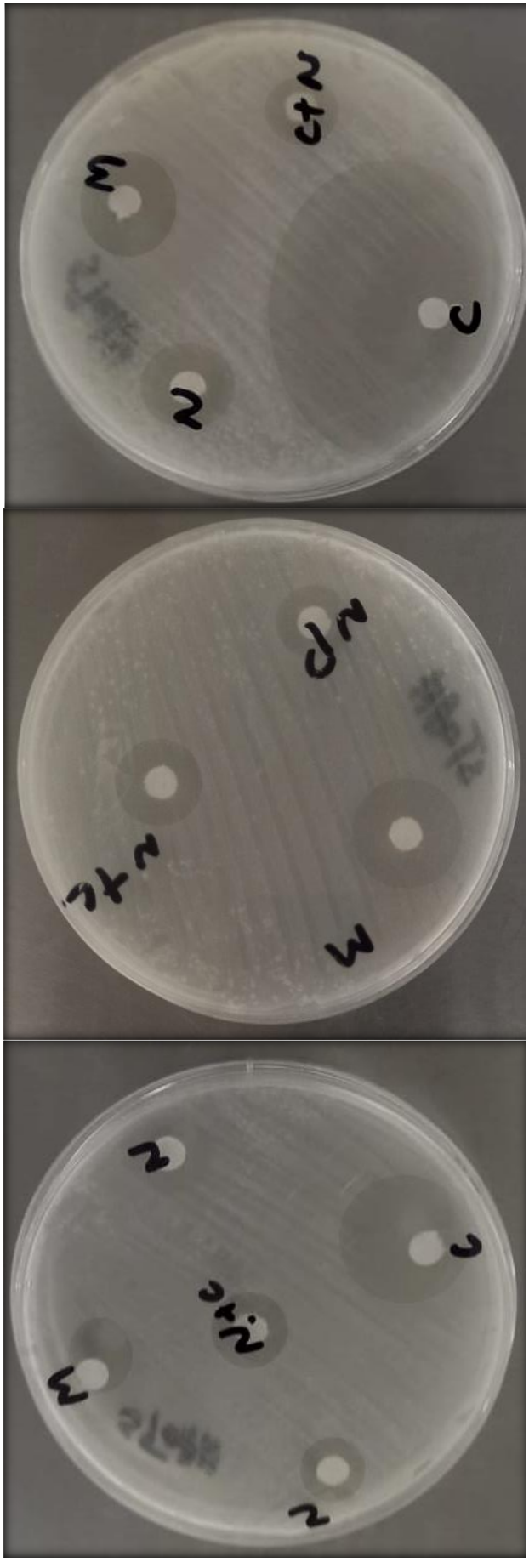

Fig. 4: Result of zinc oxide nanoparticles (ZnO-NPs) against *S. aureus* bacteria using disc

were placed in the MHA agar and control group in the center had antibiotics disc of ciprofloxacin (Fig. 4).

Combined effect of zinc oxide nanoparticles (ZnO-NPs) and antibiotics

For performing antibacterial activity through agar disc diffusion assay by using zinc oxide nanoparticles (ZnO-NPs) against the *S. aureus* and *E. coli* picked a single colony from the each pure isolated colony of *S. aureus* and *E. coli* plate, mixed in the normal saline and then made lawn on Muller Hilton plate and discs of zinc oxide nanoparticles (ZnO-NPs) and antibiotics combination were placed, in the MHA agar and control group in the center had antibiotics disc of ciprofloxacin (Fig. 5).

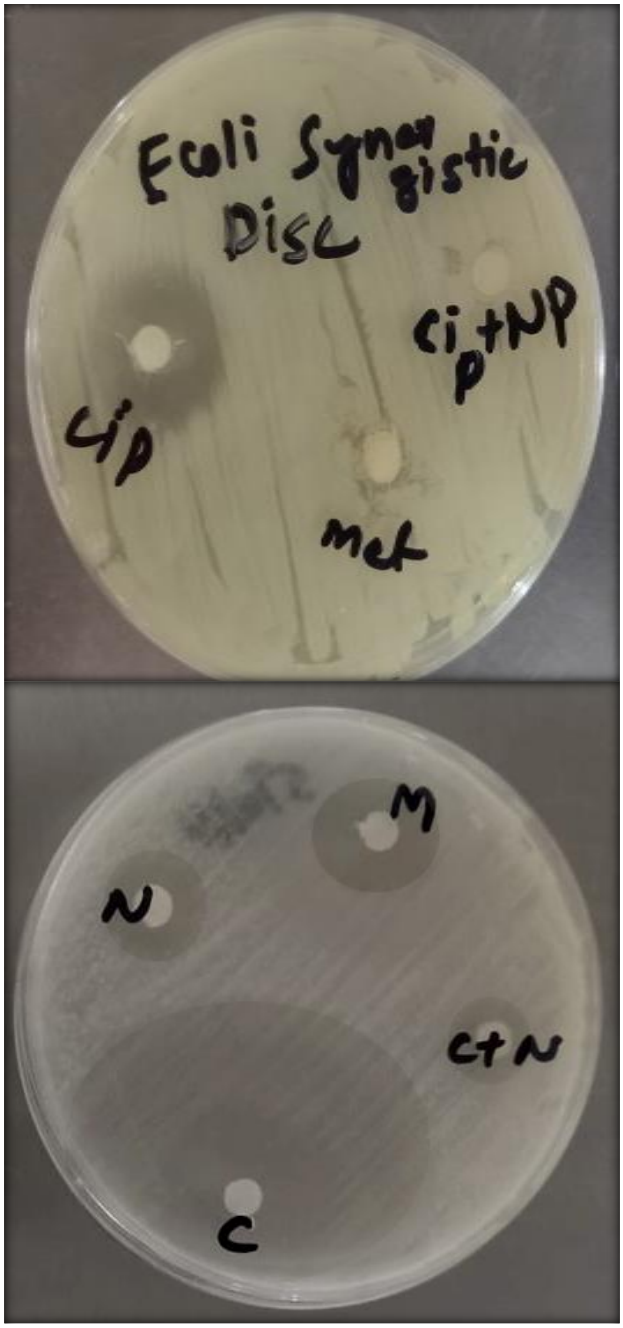

Fig. 5: Result of zinc oxide nanoparticles (ZnO-NPs) against Ajdary and *Staph aureus*. Histogram and statistical analysis

The result shown that, the drugs were used against *S. aureus* were highly significant. The statistical analysis shown the drugs that were used against *S. aureus* were highly significant. Tukey's test specifically identified that drugs that were used in the research that, there were no significant difference between 30uL with 60uL and 120uL but there were significant differences between 30uL with ciprofloxacin and Cipro plus NPs combination (Fig. 6; Table 1).

Statistical Analysis

The results were tested through Tukey's (HSD) of SPSS software and Excel were used to draw the results through Histogram by using the mean zone of inhibition against two test cultures.

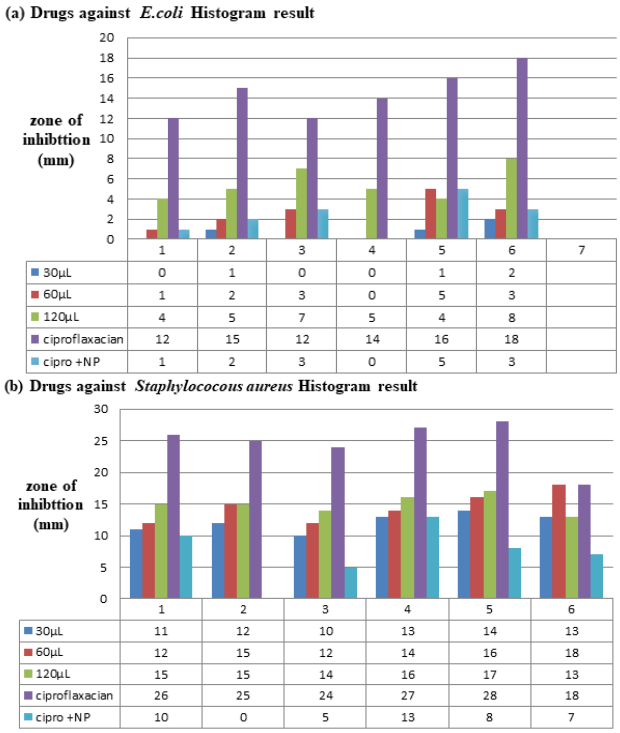

Fig. 6: (a) Antibacterial efficacy of different products against *E. coli* (b) Antibacterial efficacy of different products against *S. aureus*

DISCUSSION

The antibacterial effectiveness of conventional medications and ZnO-NPs against *S. aureus* and *E. coli*. Regardless of the concentration used, ZnO-NPs by themselves did not significantly hinder the development of *E. coli* bacteria, suggesting that ZnO-NPs are ineffective against this kind of bacterium. On the other hand, ZnO-NPs had a noteworthy antimicrobial activity against *S. aureus* over a range of doses, indicating their possible application in the management of infections resulting from this bacterium.

The medications under examination, such as ciprofloxacin, have a strong antimicrobial deed contrary to *S. aureus* and *E. coli*. The 30µL, 60µL and 120µL concentrations of the medications did not significantly differ from one another in terms of their antibacterial activity, according to statistical analysis performed using

Table 1: Multiple Comparisons of drugs against *S. aureus* analysis adopted from Tuckey's HSD test

(I) drug use against bacteria	(J) drug use against bacteria	Mean Difference (I-J)	Std. Error	Sig.P<0.05	95% Confidence Interval	
					Lower Bound	Upper Bound
30uL	60uL	-2.3333	1.67531	.638	-7.2535	2.5868
	120uL	-2.8333	1.67531	.457	-7.7535	2.0868
	Ciprofloxacin	-12.5000*	1.67531	.001	-17.4202	-7.5798
	Cipro+NPs	5.0000*	1.67531	.045	.0798	9.9202
60uL	30uL	2.3333	1.67531	.638	-2.5868	7.2535
	120uL	-.5000	1.67531	.998	-5.4202	4.4202
	Ciprofloxacin	-10.1667*	1.67531	.001	-15.0868	-5.2465
	Cipro+NPs	7.3333*	1.67531	.002	2.4132	12.2535
120uL	30uL	2.8333	1.67531	.457	-2.0868	7.7535
	60uL	.5000	1.67531	.998	-4.4202	5.4202
	Ciprofloxacin	-9.6667*	1.67531	.001	-14.5868	-4.7465
	Cipro+NPs	7.8333*	1.67531	.001	2.9132	12.7535
Ciprofloxacin	30uL	12.5000*	1.67531	.001	7.5798	17.4202
	60uL	10.1667*	1.67531	.001	5.2465	15.0868
	120uL	9.6667*	1.67531	.000	4.7465	14.5868
	Cipro+NPs	17.5000*	1.67531	.000	12.5798	22.4202
Cipro+NPs	30uL	-5.0000*	1.67531	.045	-9.9202	-.0798
	60uL	-7.3333*	1.67531	.002	-12.2535	-2.4132
	120uL	-7.8333*	1.67531	.001	-12.7535	-2.9132
	Ciprofloxacin	-17.5000*	1.67531	.000	-22.4202	-12.5798

Tukey's test. The concentration of ciprofloxacin alone and the combination of ciprofloxacin and ZnO-NPs, on the other hand, show substantial differences, suggesting that the latter combination is not more effective.

The study also demonstrates that, in comparison to using ZnO-NPs or the medications alone, the combined treatment decreased the overall antibacterial activity. This was true even though the combination of ZnO-NPs and conventional antibiotics had a diminished but still significant antibacterial effect against both *E. coli* and *S. aureus*. In particular, the antibacterial action of ciprofloxacin with ZnO-NPs is much not increased, particularly against *S. aureus*.

Overall, the results highlighted the high performance of ZnO-NPs alone against *E. coli* and *S. aureus* and highlighted the potential disadvantages of combining them with other antibiotics almost decreased antibacterial activity, particularly against resistant organisms like *E. coli*.

One important finding of the Jordanian study on antibacterial sensitivity is that isolates of *S. aureus* and *E. coli* exhibit widespread antimicrobial resistance (AMR). The *E. coli* strains exhibited resistance to six antibacterial drugs, whereas all examined strains of *S. aureus* showed resistance to five. This result is in line with recent research showing an increasing prevalence of AMR in Jordanian sheep and dairy cows. The multidrug resistance patterns for *S. aureus* and *E. coli* that have been identified are consistent with findings from other parts of the world, suggesting that controlling bacterial infections globally is becoming more difficult owing to rising resistance levels (Al-Tamimi et al. 2022).

This study, which focuses on *S. aureus* and *E. coli* strains sequestered from subclinical mastitis patients, is the primary to examine the antimicrobial properties of ZnO-NPs contrary to multidrug-resistant mastitis pathogens in sheep. ZnO-NPs were shown to have Minimum Inhibitory Concentration (MIC) of 3.9µg/mL and Minimum Bactericidal Concentration (MBC) of 7.81µg/mL for *S. aureus*, according to the study. The MBC was 62.5µg/mL and the MIC for *E. coli* was 31.25µg/mL. These findings support earlier studies that found ZnO-NPs to be more efficient against Gram-positive bacteria (such as *S. aureus*) than Gram-negative bacteria (such as *E. coli*) (Alekish et al. 2018).

The results also support previous research indicating that ZnO-NPs' antibacterial movement is mostly prejudiced by their concentration and particle size. This reliance emphasizes how crucial it is to enhance the antibacterial capabilities of nanoparticle compositions through optimization. In view of the rising incidence of AMR in both human and veterinary medicine, the study stresses the potential of ZnO-NPs as an alternative or supplementary treatment in the fight against microbial disorders. The study lays the groundwork for future research and possible clinical uses of ZnO-NPs in treating resistant bacterial infections in cattle by determining the MIC and MBC values (Mubeen et al. 2021).

Conclusion

The current work provides a thorough analysis of the zinc oxide nanoparticle-adopted antibacterial action mechanism. Zinc oxide nanoparticles could block or inhibit the zinc ion efflux pump, which helps to raise the concentration of zinc ions locally when conditions are susceptible. The tabular column has a summary of the literature that highlights the many green sources for nanoparticle production, the various nanoparticle morphologies, and the mechanisms used to demonstrate antibacterial action. The physiological characteristics of nanoparticles, such as their size, shape, concentration, and surface flaws, influence their potential for toxicity. Zinc oxide nanoparticles ' antibacterial properties can be utilized in various segments, including food, cosmetics, and agriculture. They may also be able to cure germs that are resistant to many drugs in the pharmaceutical sector. The investigation of nanoparticles presents a viable path toward the creation of novel bovine mastitis therapies, tackling the drawbacks of conventional antibiotics and offering efficient substitutes for the management of this illness that affects the cattle industry greatly.

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