Monometallic 'zinc oxide and copper oxide' nanoparticles by ecofriendly synthesis for suppression of mastitis-causing bacteria via ξ potential

Sara M. Mahmoud[a], Laila E. Kortam[b], Olfat S. Barakat[c], Rasha H. Eid[d], Noha E. Aref[d]

[a]Biotechnology Department, Faculty of Graduate Studies and Environmental Researches, Ain Shams University, Cairo, 11311,Egypt, [b]Immunity Department, Animal Reproduction Research Institute, Agriculture Research Center (ARC), Giza, [c]Agricultural Microbiology Department, Faculty of Agriculture, Cairo University, [d]Mastitis and Neonatal Disease Department, Animal Reproduction Research Institute, Agriculture Research Center (ARC)

Correspondence to Sara M. Mahmoud, PhD Biotechnology Department, Faculty of Graduate Studies and Environmental Researches, Ain Shams University, Cairo, Tel: +01277330637 Egypt. E-mail: Sara.attaya@iesr.asu.edu.eg

Received: 10 June 2023
Revised: 1 July 2023
Accepted: 5 July 2023
Published: 2 February 2024

Egyptian Pharmaceutical Journal 2024, 23:129–141

Background
Metallic nanoparticles (MNPs) are extensively employed in biology and medicine because they may freely pass through organisms' barriers. Monometallic zinc oxide (MM ZnO) and copper oxide nanoparticles (CuO NPs) by ecofriendly synthesis are safe, economical, and promising future alternatives as antimicrobial agents.
Objective
This study focused on the functioning zeta (ξ) potential (ZP) of (MM ZnO) and (CuO NPs) for suppression of mastitis-causing bacteria.
Materials and methods
Monometallic nanoparticles (MMNPs) were biosynthesized by Bacillus megaterium and characterized by UV-Vis spectra, SEM-EDS, TEM, particle size distribution, ZP, and particle concentrations calculated by ICP-AES. Then, the biocidal activity of MM ZnO and CuO NPs against some mastitis causing bacteria isolates was studied.
Results and conclusion
The obtained data reveal that the resulting cationic ZnO and anionic CuO are zero-dimensional (0-D) oval and spherical NPs with 5–17 and 10–34 nm in size, respectively. The IC_{50} of the biosynthesized ZnO and CuO NPs was 1717 ±33.7 μg/ml and 1493±42.52 μg/ml, respectively. The obtained results showed no cytotoxic effect of the MMNPs on somatic cells. Data suggested that a high dose of 100 μg/ml of cationic ZnO represents a highly significant effect ($P<0.05$) over anionic CuO for suppressing mastitis bacteria. The terminus point was in evaluating the toxicity of MMNPs by comet assay; the effects of the variation were based on the ZP and interactivity of layers carrying opposite charges. These findings elucidate that cationic ZnO NPs have advantages in targeting pathogenic bacteria because of enhanced delivery to the cells, which causes water dehydration and decreases the moisture required for bacterial viability and plasmolysis via ionic interactions.

Egypt Pharmaceut J 23:129–141

1687-4315

Introduction

Bovine mastitis, characterized as mammary gland inflammation, poses significant risks to the general public's health of animal because the main bacterial species that cause mastitis are toxin producers which can contaminate milk, resulting in significant financial losses for the dairy industry [1]. One of the most significant infectious microorganisms affecting sick cows' mammary glands is *Staphylococcus aureus*; the main environmental pathogens that cause mastitis are *Escherichia coli*, *Bacillus cereus*, and *Klebsiella* spp. [2]. Antibiotics have been used often to treat cow mastitis up until now [3]. This method has many drawbacks, including an increase in antibiotic resistance, a low cure rate, and antibiotic residues in milk that pose a risk to one's health if one consumes raw milk [4]. So, confirming the critical need for innovative, efficient, safe, and affordable methods to stop the spread of the disease [5], nanoparticles have improved distinctive physical, chemical, and biological properties [6]. These nanomineral particles minimize the required amount because they have better potential than their conventional sources. In this context, metal oxide nanoparticles have proven powerful antibacterial agents. Metallic nanoparticles (MNPs) are extensively employed in biology and medicine because they may freely pass through organisms' barriers [7]. MNPs are attractive candidates for risk-free antimicrobial therapies due to their nonspecific mode of action [8]. MNPs disrupt cell layers and produce reactive oxygen species that harm interior structures as one approach to interacting with bacterial cells [9,10]. Therefore, it has been suggested that metallic

nanoparticles may have applications in the treatment of infections caused by cow mastitis [4].

The safety of Monometallic zinc oxide nanoparticles (MM ZnO NPs) for both humans and animals has been established, as zinc is required for the body's healthy physiological processes, including appropriate growth, reproduction, DNA synthesis, cell division, and gene expression, as well as boosting the immune system [11]. Monometallic copper oxide nanoparticles (MM CuO NPs) are advancing in importance, effectiveness, speed, simplicity, affordability, and environmental friendliness due to their unique electrical, magnetic, thermal, and antimicrobial properties [12]. Only one copper atom forms a bond with an oxygen molecule in copper (II) oxide (CuO), which has an oxidation number of +2. This compound is known as CuO; refer to it as 'fully oxidized'.

The aim of this study was to overcome the resistant mastitis-causing bacteria (gram positive and gram negative) in the dairy industry by using different concentrations of MM ZnO and CuO NPs, which have been evidenced to be safe, economical, and promising future alternatives as antimicrobial agents against several bacteria.

Materials and methods

Biosynthesis of MM ZnO and CuO NPs

Bacillus megaterium used in this study for synthesizing MM ZnO and CuO NPs was grown in nutrient broth, pH 7, for 24 h at 30°C on a shaker incubator at 200 rpm (Sartorius Stedim Biotech, Aubagne, France) [13]. The cell-free broth culture was recovered by centrifugation at 10 000 rpm using a high-speed laboratory centrifuge (YING TAI Instrument, Shenzhen, China). Aqueous $ZnSO_4$ and $CuSO_4$ solutions were added independently to the 50 mL medium at a final concentration of 5 mM to create MM ZnO and CuO NPs. The developed MMNPs suspensions were characterized by the following characteristics.

Characterization of MM ZnO and CuO NPs

The colored supernatant containing independently the MM ZnO and CuO NPs was used for various physical characterizations.

UV–*vis* spectra analysis ranging from 200 to 800 nm was carried out using 'Thermo Scientific HERYIOS ϒ' Instrument. Ultraviolet-visible (UV–*vis*) detects the capability of the NPs products to absorb electromagnetic radiation and appear at specific spectrum.

Scanning electron microscopy (SEM) using 'JEOL MODEL JSM 6360 SEM-EDS' operated at voltage 25 kV and Transmission Electron Microscopy (TEM) 'Jeol Hrten-2100 TEM' worked with a total magnification of 80.00 kx at 200 kV were used to determine the particles size and shape.

Particles size distribution (PSD) and Zeta (ξ) Potential (ZP) index were estimated by the strategy of Dynamic light scattering by using (Zetasizer Nano ZS Ver. 7.13, Malvern, UK).

The concentration of Zn and Cu in the different samples was determined by ICP (Inductively Coupled Plasma, ICP-AES, Thermo Sci, model: iCAP6000 series, USA), with easy control software HPR1000/10 S.

Antioxidant activity of MM ZnO and CuO NPs

Antioxidant activity was measured for each sample MM zinc oxide and copper oxide NPs using the stable DPPH (Diphenyl Picrylhydrazyl) free Radical Scavenging Activity (RSA) method with a laboratory spectrophotometer 'Thermo Scientific HERYIOS ϒ'. RSA of samples concentration at (10, 50, and 100 μg/ml) was expressed as percent inhibition [14].

Antimicrobial effect of MM ZnO and CuO NPs against seven different pathogenic strains

The antimicrobial activity of green synthesized MM ZnO and CuO NPs was evaluated against seven different pathogens gram negative (G^{-ve}) bacteria *i.e., Pseudomonas aeroginosa* ATCC 9027, *Escherichia coli* O157 H:7 93111, *Salmonella typhimureum* ATCC 14028 and gram positive (G^{+ve}) bacteria *i.e., Bacillus cereus* ATCC 33018, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19115, *Enterococcus faecalis* ATCC 19433 adopting the disc diffusion method [15]. The previous control strains were used for monitoring the accuracy of susceptibility tests, according to Clinical and Laboratory Standards Institute [16]. All experiments were designed in triplicates at four different concentrations being 5, 25, 50, and 100 μg/ml.

Animal ethical approval

The animals during the experiment were handled in accordance with the Institutional Animal Care and Use Committee (IACUC), of Animal Reproduction Research Institute (ARRI), Egypt [17]. Samples collected from lactating cows at two different areas, El-Menoufia Governorate and El-Beheira Governorate, Egypt.

Cytotoxic effect of MM ZnO and CuO NPs on Somatic Cells (SCs)

Somatic cells (SCs) (mainly leukocytes) are an important component in milk of the dairy animal. They are used as an indicator of udder health and milk quality. SCs are essential part of the innate immune system that involved in protecting the mammary gland against infection [18].

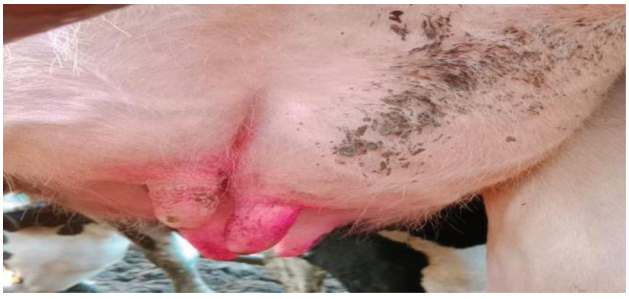
SCs were isolated to study the DNA integrity of cells as cytotoxicity assay of MM ZnO and CuO NPs after exposure. From healthy cow cells, ten Quarter Milk Samples (QMS) were obtained at the morning milking. Milk samples were firstly filtered with 70 µm cell strainers to discard any clots. Then, 1 ml of each milk sample was centrifuged (300×g) for 15 min at 4°C until obtained clear SC pellets [19].

To determine the effect of different concentrations of MM ZnO and CuO NPs on SCs, Griess reagent was utilized for colorimetric measurement of nitric oxide creation; at 570 nm using an ELISA plate reader (Epoch, BioTek, Germany). The color intensity formed was considered proportional to the number of viable cells.

Animals and Milk samples

A number of 120 milk samples was inspected from lactating cows and the udders were examined for subclinical mastitis by CMT (California mastitis test) of the milk samples and clinical mastitis were identified through observation, palpation of the udders, clots in milk and mammary gland inflammation (Fig. 1). Animals were not treated with any antibiotic for at least 30 days before sample collection. Then, after examination only 50 milk samples were collected from lactating cows affected with mastitis and were used in this study. The udder teats were sterilized with 70% ethyl alcohol before collection of milk samples. A 10 ml of milk was milked into sterilized McCartney and samples were kept on ice during transportation to the laboratory. Microbiological examination was performed after 24 h incubation [20].

Figure 1



Observation the mastitis infected udders.

In order to determine whether tissue damage had occurred, a biopsy sample from the afflicted mammary quarter was fixed in 10% buffered formalin, paraffin embedded, sectioned at 4 mm, and stained with hematoxylin and eosin and Ziehl-Neelsen/Fite (EasyPath) for acid-fast rods [21].

Isolation and identification of some mastitis causing bacteria isolates

Bacteria were isolated on specific agar media and identified using biochemical test, 10 µl of each milk sample were cultured on MacConkey agar and Levine's Eosin-Methylene Blue (EMB) agar for the isolation of gram-negative bacteria, as well as cultured on selective agar media (HiMedia) for isolation of *B. cereus* and mannitol salt agar for isolation of *S. aureus*. CHROM agar was used for identification as/the methodology, the cultures were incubated at 37°C for 24 h. followed by Gram staining and biochemical tests for confirmation (catalase, DNase, coagulase tests and IMViC tests) [22,23].

Biocidal activity of MM ZnO and CuO NPs against some mastitis causing bacteria isolates

Biocidal activity of MM ZnO and CuO NPs was determined using Mueller-Hinton agar (MH) culture medium (Oxoid) as described for the diffusion disc method that is commonly used for antibiotic susceptibility tests [24]. Bacteria isolates were instilled with MM ZnO and CuO NPs at different concentrations (5, 25, 50, and 100 µg/ml); were assessed for their antimicrobial activities *in vitro* against *Escherichia coli*, *Klebsiella pneumonia, Bacillus cereus,* and *Staphylococcus aureus*. The Petri dishes were incubated at 37°C for 24 h. after incubation the inhibition zones were measured (mm) [25].

Evaluation of apoptosis using comet assay

Cell's DNA damage of both somatic and mastitis causing bacteria isolates were determined according to comet assay, or Single-cell gel electrophoresis [26]. Out of 100 randomly selected nuclei were photographed and observed at 40x in an Optica Axioscope fluorescence microscope (OPTIKA-ZEISS).

Statistical analysis

All data were subjected to statistical analysis according to Ref. [27]. Results were expressed as standard error of the mean (±SEM). The outcomes were expressed by

one-way analysis of variance (ANOVA) employing a completely randomized design at *P*-value <0.05 was considered statistically significant.

Results

Biosynthesis of MM ZnO and CuO NPs

The color change in the *B. megaterium* culture suspension to brownish yellow and dark green colored is considered as an indication for formation of MM ZnO and CuO NPs, respectively. The obtained NPs were further subjected to physical characterization for the particle size and shape determination as following.

The UV-*vis* spectrum of solution MM ZnO and CuO NPs shows a characteristic surface plasmon resonance (SPR) with strong peak at 283 nm and 317 nm for MM ZnO and CuO NPs, respectively (Fig. 2). Moreover, there is not any peaks at *vis* range at 400–800 nm.

SEM-EDS micrographs indicated that MM ZnO and CuO NPs were aggregated NPs and a perfect example of zero-dimensional (0-D) NPs with oval and spherical particles in shape, respectively (Fig. 3a). TEM micrographs of MMNPs revealed the morphological discrimination with numerous shape and size at the bar beneath scale 100 nm. Particles were 5–17 and 10–34 nm in size for MM ZnO and CuO NPs, respectively (Fig. 3b).

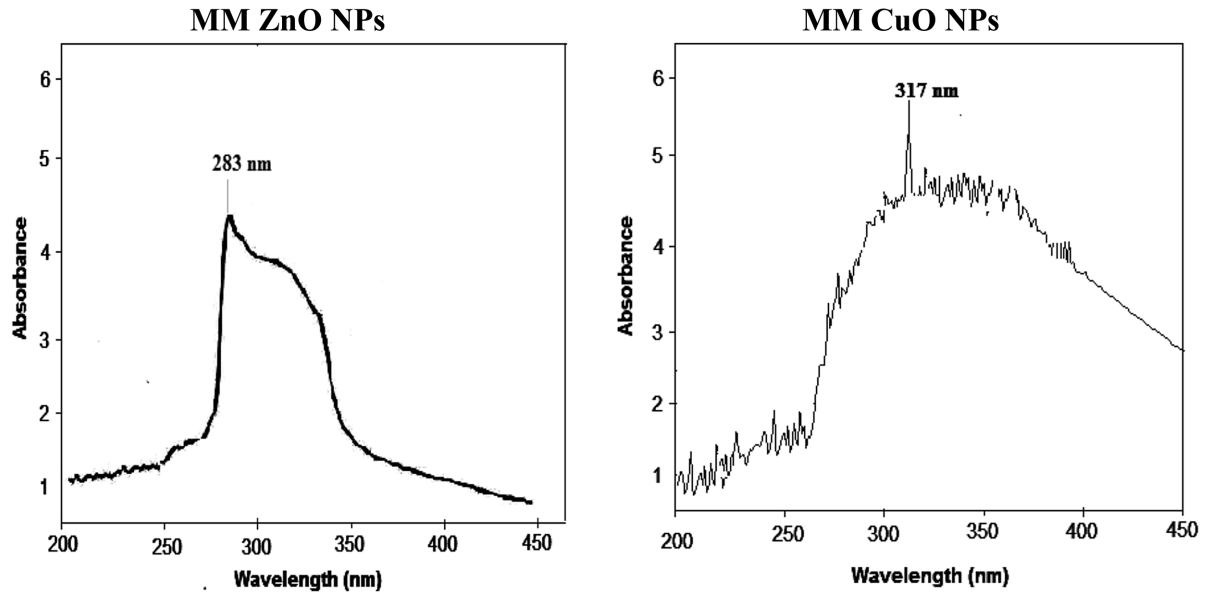
By counting 100 NPs, the particle size distribution pattern of MM ZnO and CuO NPs was created, revealing that the majority of the particles (above 60%) had narrow and homogeneous size distributions and were 6–9 nm and 11–30 nm in size, respectively (Fig. 4a). ZP was cationic charge +10 ±1.82 mV and anionic charge −14±1.32 mV for MM ZnO and CuO NPs, respectively (Fig. 4b). The ZP, which gauges the potency of the attraction, was used to determine the electro kinetic potential of MM ZnO and CuO NPs.

The ICP-AES analysis carried out on the SD inhibitor samples, displayed considerable elemental concentrations of MM zinc and copper elements were 1063 and 1049 ppm, respectively. It is stressed that the elemental composition is (ideally) the same as what is predicted by the stoichiometry of the synthesis process and that ICP-AES provides adequate data for MMNPs.

Antimicrobial activity of MM ZnO and CuO NPs

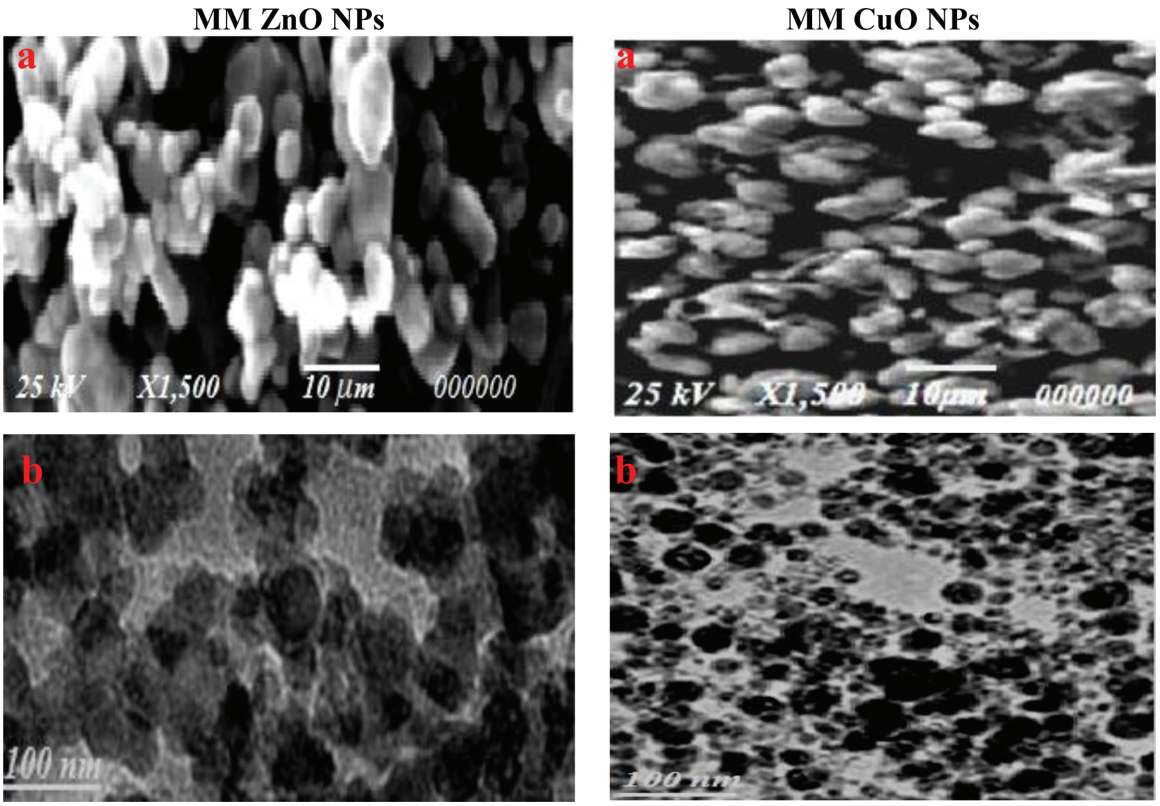
Data reveal that increasing concentrations of MM ZnO NPs have significant effect on all tested G^{-ve} bacteria (Table 1), however, the lowest dose (5 μg/ml) did not effect of *Salmonella typhimureum* ATCC 14028. The largest inhibition zone diameter (29 mm) of MM ZnO NPs at 100 μg/ml was recorded against the most susceptible bacteria, *Pseudomonas aeroginosa* ATCC 9027, followed by *Escherichia coli* H:7 O157 93111 (27 mm), while *Salmonella typhimureum* ATCC 14028 was less affected (10 mm). A similar trend was observed with *Bacillus cereus* ATCC 33018 and *Staphylococcus aureus* ATCC 25923 were 23 mm and 22 mm at 100 μg/ml,

Figure 2



Spectrum curves of monometallic zinc oxide and copper oxide nanoparticles.

Figure 3



Electron microscope micrographs, Monometallic zinc oxide and copper oxide nanoparticles (a) SEM, (b) TEM.

respectively. Therefore, cationic MM ZnO NPs have positive effect on only two tested G^{+ve} strain.

Data in Table 1 show that 5 and 25 µg/ml of anionic MM CuO NPs had negative effect on growth of *Pseudomonas aeroginosa* ATCC 9027 and *Escherichia coli* O157 H:7 93111, while 50 and 100 µg/ml were less efficient growth inhibitors compare with reference standard. In addition, raising the concentration of copper oxide NPs led to increase the growth inhibition of *Salmonella typhimurium* ATCC 14028 record the largest inhibition zone diameter (18 mm) at 100 µg/ml. Therefore, anionic MM CuO NPs have high positive effect on only one tested G^{-ve} strain.

Antioxidant activities of biosynthesized nanoparticle

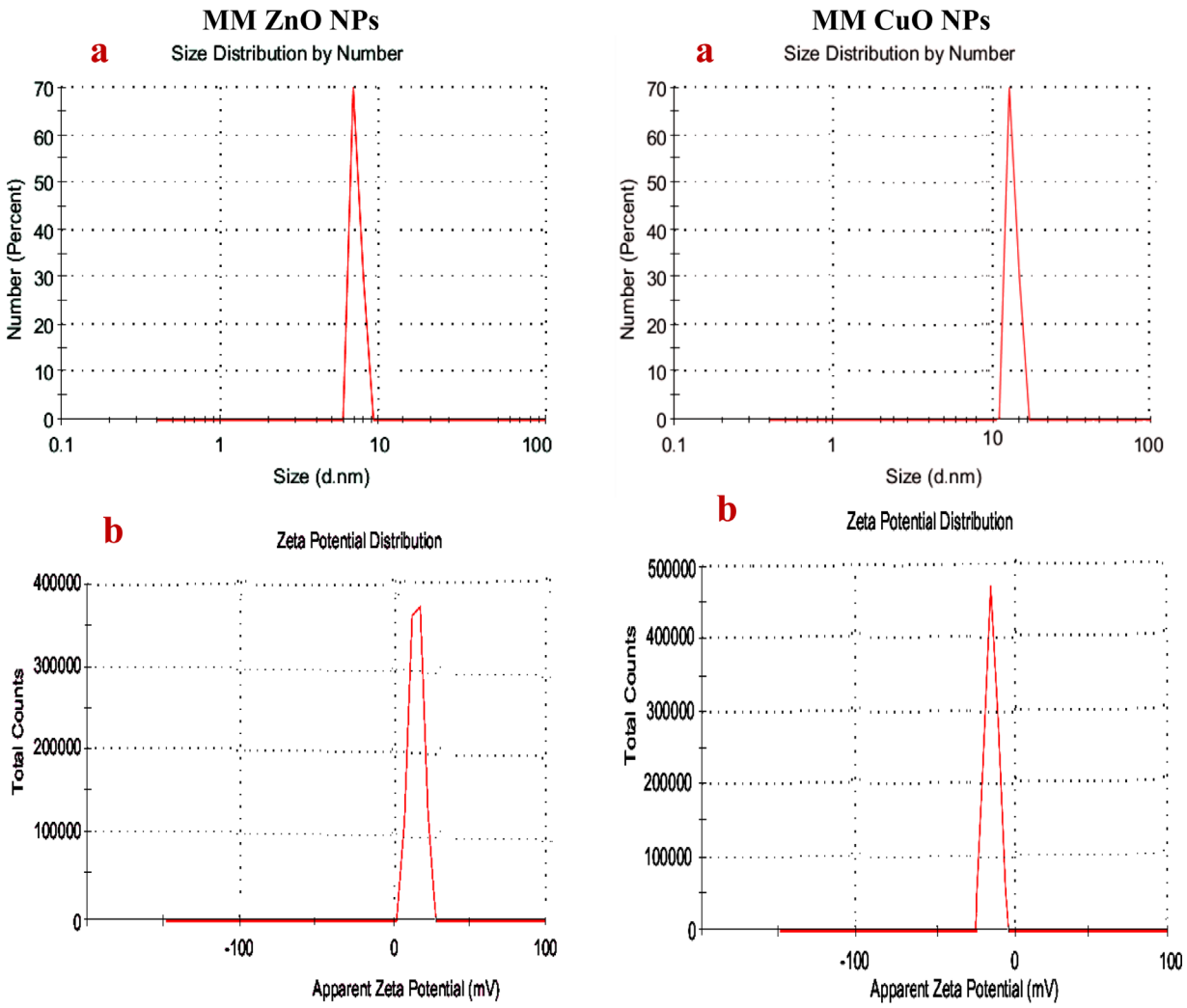
With rising MMNP concentrations, free radical scavenging activity rises [6]. The best MMNPs, were positively charged MM ZnO NPs at a high concentration of 100 µg/ml, which showed strong inhibition of 67.97%***; and activity 35.32%, 51.84%** for dosages of 10 and 50 µg/ml, respectively. While at three dosages of 10, 50, and 100 µg/ml, the inhibition of negatively charged MM CuO NPs was 28.8%, 40.91%*, and 49.33%**, respectively. At this concern, the activity increases ascend in order to doses, IC_{50} were 1717±33.7 µg/ml and 1493±42.52 µg/ml for MM ZnO and CuO NPs, respectively. Utilizing MM cationic ZnO and anionic CuO NPs antioxidant activity was assessed to predict reactive oxygen species (ROS), MMNPs effective at boosting antioxidant activity and serve as carriers or antioxidant delivery vehicles.

Cytotoxicity assay on milk Somatic Cells (SCs)

Geimsa-stained SCs slides under light microscope showed impacts of MM ZnO and CuO NPs on SCs, based on SC phagocytosis to the intensity of zymosan particles, which reveal that SCs were not affected by MMNPs (Fig. 5). This was determined through microscopic examination depending on how many zymosan particles were ingested.

Cytotoxicity assay is the primary biological supposing toxicity of all MMNPs, (Table 2 and Fig. 6). The evaluation of the impact different concentrations of MM ZnO and CuO NPs on isolated SCs revealed there is not any significant impact compared with control sample SCs expressed by comet% and DNA damage expressed by Tail Moment, which showed non-significant change of different concentration of ZnO and CuO nanoparticles on DNA integrity of

Figure 4



Distribution of monometallic zinc oxide and copper oxide nanoparticles, (a) Size by numbers (b) Zeta potential.

somatic cells. Indeed, there are slightly cytotoxic effects of the ZP of anionic CuO NPs greater than cationic ZnO NPs, after exposure being with the SCs at low dose (5 µg/ml).

Effect of different doses of MM ZnO and CuO NPs on nitric oxide (NO) level of SCs

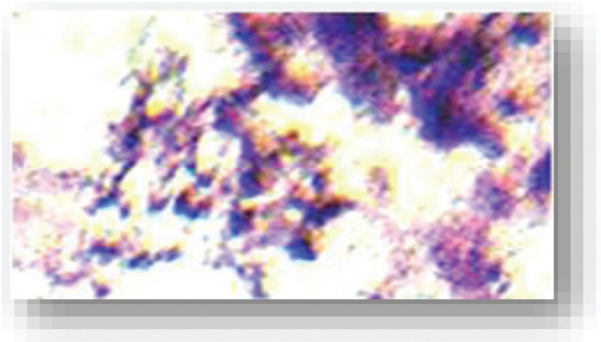
Results manifested that nitric oxide levels in somatic cells that were treated with MM cationic ZnO and

Table 1 Antimicrobial activity (growth inhibition zone diameter mm) of monometallic cationic zinc oxide and anionic copper oxide nanoparticles against some pathogenic bacteria

		Zone of inhibition (mm)								
		Cationic MM ZnO NPs Concentration (µg/ml)				Anionic MM CuO NPs Concentration (µg/mL)				
Pathogenic microorganisms		5	25	50	100	5	25	50	100	PRS
(G^{-ve})	*Pseudomonas aeroginosa* ATCC 9027	8	17**	21**	29****	0	0	7	14	15
	Escherichia coli O157 H:7 93111	5	15**	20**	27***	0	0	9	12*	10
	Salmonella typhimurium ATCC 14028	0	6	8	10	4	10	15*	18**	10
(G^{+ve})	*Bacillus cereus* ATCC 33018	0	10.5	17	23**	0	6	10	20*	20
	Staphylococcus aureus ATCC 25923	0	8.6	15*	22**	0	4.2	8	14*	13
	Listeria monocytogenes ATCC 19115	0	0	3	4	4	9	14*	17**	10
	Enterococcus faecalis ATCC 19433	0	0	0	0	4	9	11*	18**	10

*Superscript star means significantly different ($P<0.05$). * Positive Reference Standard (PRS).

Figure 5



Light microscope micrograph for the effects monometallic zinc oxide and copper nanoparticles on somatic cells via zymozan particles.

anionic CuO NPs did not substantially increase with any dose when compared with the control group. For cationic ZnO and anionic CuO NPs, respectively, high concentrations of 37.1±0.34 and 38.3±2.2 were found in (Table 3). Control cells were taken without any MMNPs treatment. Ditto, the NO levels increases ascend with MM cationic ZnO and anionic CuO NPs at concentrations of 5, 25, 50, and 100 µg/ml, but anionic CuO NPs at low dose 5 µg/ml appeared significant effect, which has high nitric oxide level compare with the control.

Isolation and identification of some mastitis-causing bacteria

Examination of mastitis infected cells under microscope (Fig. 7) reveals that numerous areas of severe and widespread inflammatory infiltration were present.

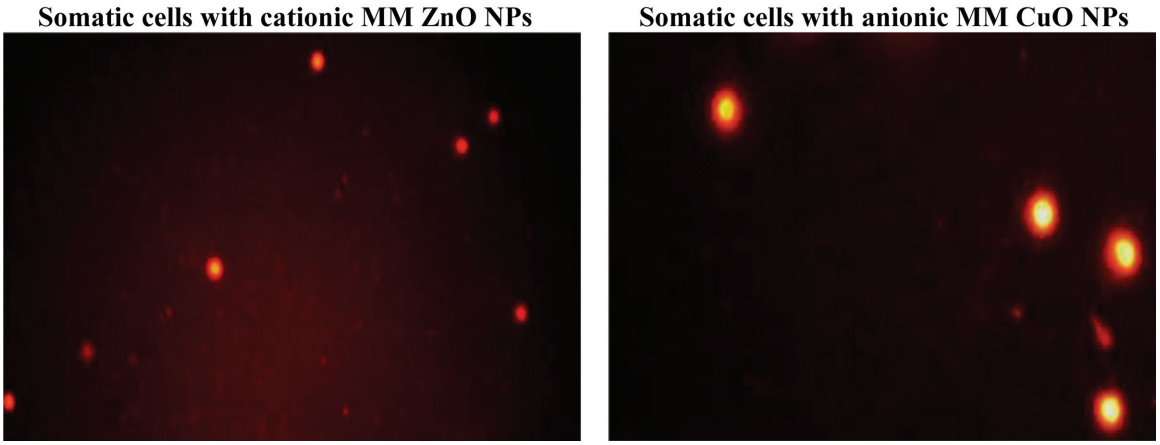
Microbiological analysis showed that, 18/50 samples golden yellow colonies on mannitol salt agar that was initially as *S. aureus*, which was confirmed by Gram staining, biochemical tests; catalase, DNase and coagulase activity assays and 9/50 *B. cereus* confirmed on CHROM agar plates the typical Light blue large flat colonies with blue center observed. On the other hand, 15/50 samples showed a metallic sheen on EMB agar, characteristic of *E. coli* spp., which was confirmed

Table 2 Effect different concentrations of monometallic zinc oxide and copper oxide nanoparticles on DNA integrity of somatic cells

Concentration (µg/mL)	Comet %	Tail Length (px)	Tail Moment
Cationic ZnO NPs			
5	5.1±0.5*	5.6±0.6*	0.661±0.01*
25	4.41±0.78	5.23±0.64	0.52±0.045
50	4.39±0.38	4.58±0.18	0.594±0.02
100	4.27±0.22	3.93±0.33	0.516±0.44
Anionic CuO NPs			
5	5.3±0.66***	5.9±0.42***	0.697±0.13***
25	4.86±0.53*	5.4±0.55*	0.587±0.008
50	4.3±0.5	5.3±0.89	0.57±0.053
100	4.8±0.59	4.7±0.55	0.631±0.08

*Superscript star means significantly different ($P<0.05$). *Values are shown as means of 3 replicates per experiment±SEM. *Control of comet % 4.9±0.27; tail length, 5.5±0.29; tail moment, 0.646±0.07.

Figure 6



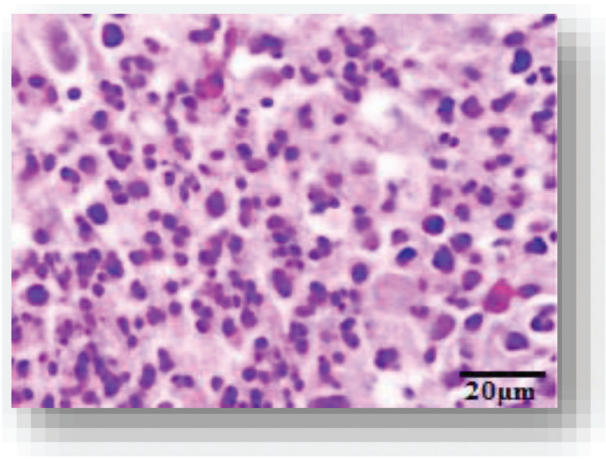
Effect different concentration of monometallic zinc oxideand copper oxide nanoparticles on DNA integrity of somatic cells.

Table 3 Effect of different doses of monometallic zinc oxide and copper oxide nanoparticles on nitric oxide level of somatic cells

	Concentration (μg/mL)			
MMNPs	5	25	50	100
Cationic ZnO	33.3±0.98	34.0 ±1	36.3±1.3	37.1±0.34
Anionic CuO	38.9±3.6[s]	36.8±2.0	37.91±1.8	38.3±2.2

[*]Superscript star means significantly different ($P<0.05$). [*]Values are shown as means of 3 replicates per experiment±SEM. [*]Control of nitric oxide, 38.8±1.65.

Figure 7



Microscopic examination micrograph of mastitis infected cells; magnification, x40.

by biochemical identification using IMViC tests that showed positive results with both methyl red and indole. The remaining 8/50 samples showed opaque, mucoid colonies that were pink in color on MacConkey agar and exhibited urease activity, which is characteristic of *K. pneumoniae*, and were biochemically positive for citrate.

Bactericidal activity of MM ZnO and CuO NPs against some mastitis causing bacteria isolates

The maximum zone of inhibition was reached by cationic MM ZnO NPs against *Escherichia coli* and *K. pneumoniae* at a concentration of 100 μg/ml, reaching 36 mm and 35 mm, respectively. *Bacillus cereus* and *Staphylococcus aureus* were next, with high significant inhibition reaching 34 mm and 30 mm, respectively. On the other hand, MM ZnO NPs were unable to inhibit the growth of *Bacillus cereus* and *Staphylococcus aureus* at the lowest dose (5 μg/ml), as shown in Table 4.

Anionic MM CuO NPs showed biocidal activity against *Escherichia coli* and *K. pneumoniae* with the maximum zone of inhibition at high concentration 100 μg/ml reaching 30 mm and 28 mm, respectively, followed by *Bacillus cereus* and *Staphylococcus aureus* with a maximum zone inhibition 25 mm and 22 mm, respectively. Moreover, MM CuO NPs were unable to inhibit all isolates at lowest dose, 5 μg/ml and *Staphylococcus aureus* at 25 μg/ml shown in Table 4. In addition, raising the concentration of MM cationic ZnO and anionic CuO NPs led to increase the growth inhibition of all G^{-ve} and G^{+ve} tested strains to record the largest inhibition zone diameter at 100 μg/ml. Obviously, cationic MM ZnO NPs appear highly significant inhibition of clear growth, great effect against G^{-ve} and G^{+ve} isolates at all doses than anionic MM CuO NPs.

Comet assay for studying the effect of MM ZnO and CuO NPs on the isolated mastitis bacteria

In comparison, MM cationic ZnO NPs had a highly significant effect against four investigated mastitis-causing bacteria isolates at comet%, tail length, and tail moment (Table 5 and Fig. 8). Results show that anionic MM CuO NPs at low dose 5 μg/ml has no effect on all bacteria isolates compared with the control as appeared in results of comet%, Tail length and Tail Moment. The apoptosis of mastitis causing bacteria isolates increased ascend with increased the doses of MM cationic ZnO and anionic CuO NPs. On the other side, MM cationic ZnO NPs at high dose 100 μg/ml caused strong significant effective ($P<0.05$), comet% which 27.6±0.66, 27.42±1.5, 23.1

Table 4 Bactericidal of different doses monometallic cationic zinc oxide and anionic copper oxide nanoparticles on some Mastitis isolates

		Zone of inhibition (mm)							
		Concentration (μg/mL)							
		MM ZnO NPs				MM CuO NPs			
Mastitis Isolates		5	25	50	100	5	25	50	100
(G^{-ve})	*Escherichia coli*	13**	27****	33****	36****	0	24***	27**	30*
	Klebsiella pneumoniae	12*	24***	31***	35***	0	21*	25*	28*
(G^{+ve})	*Bacillus cereus*	0	21**	29**	34**	0	12*	20***	25*
	Staphylococcus aureus	0	19*	27*	30*	0	0	15**	22**

[*]Superscript star means significantly different ($P<0.05$). [*]Values are shown as means of 3 replicates per experiment±SEM.

Table 5 Comet study the effect of monometallic cationic zinc oxide and anionic copper oxide nanoparticles on isolates causing mastitis

	Comet %				Tail Length (px)				Tail Moment			
Concentration (µg/mL)	5	25	50	100	5	25	50	100	5	25	50	100
E.coli												
MM ZnO NPs	16.63±0.78*	20.2±0.85**	24.3±1.17***	27.6±0.66****	6.43±0.44*	6.92±0.72**	7.3±0.4***	8±0.52****	0.633±0.08	0.75±0.06**	0.81±0.094***	0.97±0.086****
MM CuO NPs	5.96±0.588	9.3±0.67**	12.8±0.89**	13.2±1.1***	4.83±0.45	5.2±0.33**	5.6±0.5**	5.84±0.4***	0.42±0.05	0.45±0.027**	0.49±0.094**	0.53±0.03***
K.pneumoniae												
MM ZnO NPs	16.3±0.63*	18.82±0.91**	23.42±0.53***	27.42±1.5****	6.12±0.7*	6.41±0.73**	7.14±0.8***	7.4±0.33****	0.61±0.052*	0.7±0.09**	0.76±0.065***	0.92±0.06****
MM CuO NPs	6.89±1.2	11.6±0.72*	13.8±1.33**	16.1±1***	4±0.35	5.75±0.6*	6±0.34**	6.36±0.44***	0.32±0.047	0.48±0.066*	0.51±0.04**	0.62±0.08***
B. cereus												
MM ZnO NPs	8.4±0.44	14.8±0.43**	20.3±0.64***	23.1±0.58****	5.1±0.31	6±0.97**	6.56±0.6***	7.1±0.38****	0.55±0.022	0.62±0.03**	0.7±0.05***	0.89±0.076****
MM CuO NPs	6.2±0.63	12.7±0.4*	15.6±0.56**	16.79±1.2***	4.3±0.42	5.92±0.5**	6.14±0.53**	6.82±0.6***	0.45±0.076	0.51±0.035*	0.59±0.04**	0.7±0.056***
S. aureus												
MM ZnO NPs	8.91±0.55	13.62±0.5*	17.5±. 1.5**	19±0.98 ***	5.3±0.44	5.7±0.2*	6.11±0.3**	6.94±0.7***	0.49±0.021	0.57±0.06*	0.68±0.08**	0.81±0.05***
MM CuO NPs	5.87±0.76	5.87±0.76	15.82±1.23*	17.1±1.1**	4.2±0.62	4.2±0.62	6±0.57*	6.92±0.93**	0.35±0.035	0.35±0.035	0.61±0.058*	0.71±0.08**

*Superscript star means significantly different ($P<0.05$). *Values are shown as means of 3 replicates per experiment±SEM. *Control of *E.coli:* Comet %, 5.96±0.588; Tail Length (px), 4.83±0.45; Tail Moment, 0.42 ±0.05. *Control of *K.pneumoniae:* Comet % 6.89±1.2; Tail Length (px), 4±0.35; Tail Moment, 0.32±0.047. *Control of *B.cereus:* Comet % 6.2±0.63; Tail Length (px), 4.3±0.42; Tail Moment, 0.45±0.076. *Control of *S. aureus:* Comet %5.87±0.76; Tail Length (px), 4.2±0.62; Tail Moment, 0.35±0.035.

±0.58, and 19±0.98, while the anionic MM CuO NPs showed effect at high dose but less than positively charged zinc oxide was 13.2±1.1, 16.1±1, 16.79±1.2, and 17.1±1.1, on *E.coli*, *K. pneumoniae*, *B. cereus* and *S. aureus*, respectively.

Discussion

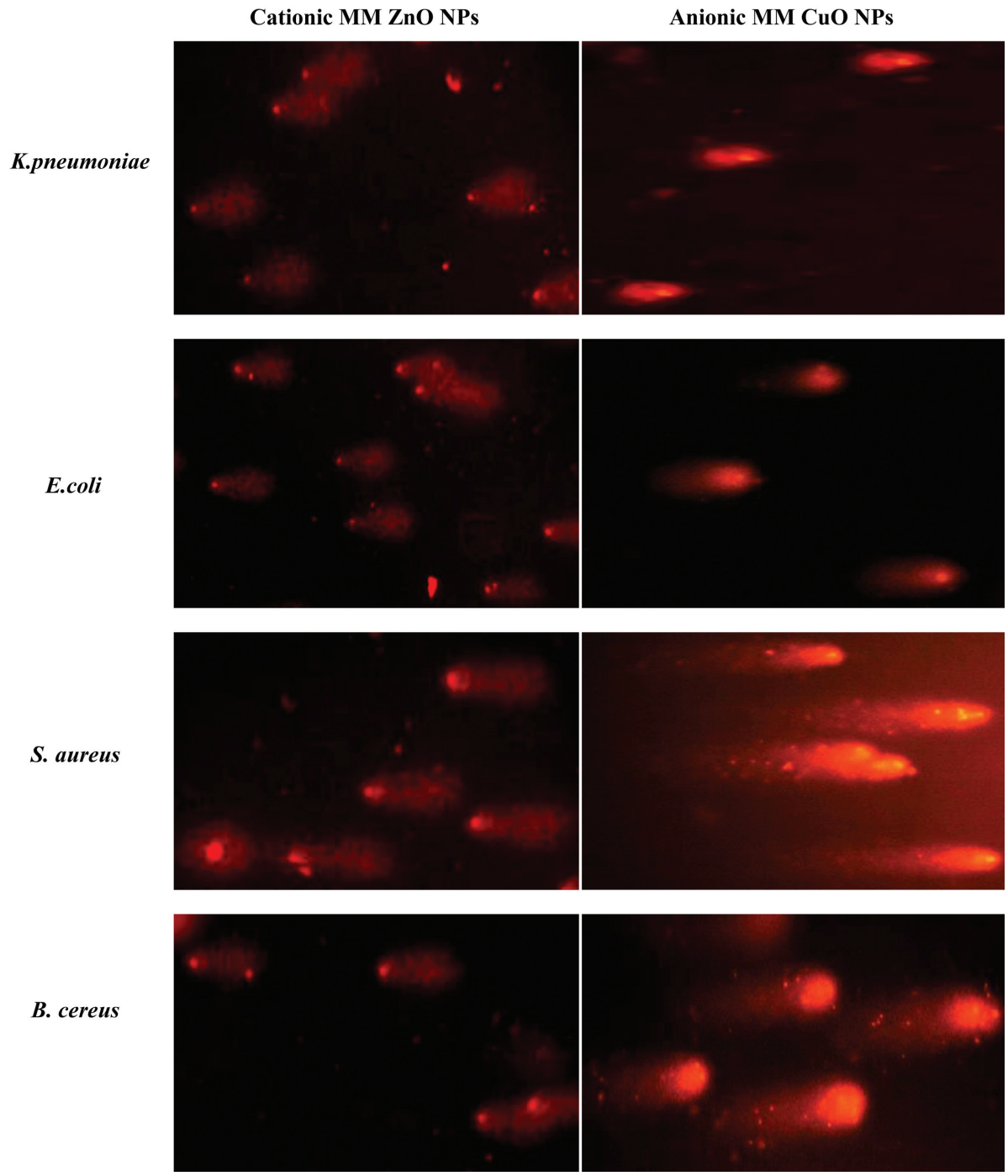
This essay contributes to current debates about the effects of biosynthesis of MM ZnO and CuO NPs on the suppression of the growth of mastitis-causing bacteria. The added advantage is that MMNPs can efficiently be synthesized by using biological methods, which are cheap and easy [28]. Regarding the perceived color and absorption peak at UV spectra as LSPR, this is a common occurrence that is a hint of the feedback of MM ZnO and CuO NPs formation. Additionally, a bigger band gap is associated with smaller particle sizes because fewer molecular orbitals contribute to the particle's potential energy states [29,30].

TEM analysis and SEM-EDS impacted by this, whose outcomes are in accordance with previous authors studies, confirmed that the ZnO and CuO NPs were synthesized with a width typically between 5 and 34 nm in the nanometer scale [13,31].

ZP is a crucial metric for understanding the charge, stability, and dispersion of synthetic MNPs since the charge exerts influence over the immunological response. The increased surface charge suggests increased stability for MNPs. These findings are consistent with the antioxidant activities of MM ZnO and CuO NPs as well as the greater radical scavenging activity of low-molecular-weight DPPH radicals compared with higher-molecular-weight ones [32]. Cationic ZnO NPs have a higher IC_{50} than anionic CuO NPs was 1717±33.7 µg/ml, so positively charged MM ZnO NPs have been anticipated to be powerful antioxidants.

Cytotoxic activity results on SCs showed a nonsignificant effect of cationic ZnO and anionic CuO NPs on DNA integrity, in addition to no increase in nitric oxide concentration. This result indicates the use of MM ZnO and CuO NPs as antibacterial agents save for the innate immune system. This result agreed with Machado *et al.* [33], who showed that injection of a multimineral preparation of ZnO and CuO NPs has a positive impact on udder health and the incidence of subclinical and clinical mastitis, with no effect of MMNPs supplementation on SC count or the impermeable mammary epithelium intact. The

Figure 8



Comet micrograph of the effect monometallic zinc oxideand copper oxidenanoparticles on isolates of mastitis causing bacteria.

present fruitful results of the comet are in harmony with studies that confirm ZnO NPs are crucial for the development and proper function of cells mediating innate immunity, such as SCs, which are considered an innate part of the udder immune system [34,35].

Cationic MM ZnO NPs at a high dose 100 µg/ml appear to have a higher significant inhibition of clear growth on G^{-ve} (*Pseudomonas aeroginosa* ATCC 9027; *Escherichia coli*; *K. pneumoniae*) and G^{+ve} (*Bacillus cereus*; *Staphylococcus aureus*) in Tables 1 and 4. This may be because these bacteria have a high negative charge on their surfaces compared with the Positive Reference Standard (PRS), but strains G^{-ve} (*Salmonella typhimureum* ATCC 14028) and G^{+ve} (*Listeria monocytogenes* ATCC 19115; *Enterococcus faecalis* ATCC 19433) were less significantly affected as these bacteria have a slightly positive or neutral charge on their surfaces [36]. High concentrations of cationic MM ZnO NPs were effective at locating,

interacting with, and disrupting the cell wall, cell membrane, or active enzymes of these bacteria, resulting in a positive antimicrobial impact. Additionally, the depolarization of the contents of the cell wall membrane as a result of positively charged MM ZnO NPs binding to the anionic cell wall causes the effect of bacterial killing, which inhibits growth as a blocker inhibitor, the loss of cellular contents, protein denaturation, and cell death. This process then led to water dehydration, which lowered the moisture needed for bacterial survival, thereby inducing plasmolysis via ionic interactions [29,30]. Since bacteria have high negative surface charges, cationic ZnO NPs appear to suppress bacteria the most, and it is easy for cationic MNPs to adhere to the bacterial cell wall, causing destruction and death of bacteria. The interaction of the layers with the opposite charge resulted in the surface generating a fluffy layer of charged particles known as the Stern layer, which contributed to the formation of the electrical double layer force, which is composed of the diffusive layer. This mobility causes a barrier to build between these ions and the other ions in the bulk dispersant [29,30,37].

Anionic MM CuO NPs are less effective than cationic MM ZnO NPs at a high dose 100 μg/ml, for inhibition of G^{-ve} (*Escherichia coli*; *K. pneumoniae*) and G^{+ve} (*Bacillus cereus*; *Staphylococcus aureus*) in Tables 1 and 4. This may be because copper nanoparticles, characterized by a high surface-to-volume ratio, have a great potential effect as a good catalytic agent, so that CuO NPs demonstrate significant inhibitory activity against several bacteria species. Also, it is due to the fact that, due to its small size in comparison to the holes, it may easily enter porous cells when combined with the proper surface-tunable features cells [38]. According to certain research, the MNPs' surface softness had the primary influence on the contact. According to the theory, soft and hydrophilic MNPs can reduce the hemolytic potential because biological cells do not perceive them as alien objects [39]. Moreover, anionic CuO NPs were more effective in strains G^{-ve} (*Salmonella typhimureum* ATCC 14028) and G^{+ve} (*Listeria monocytogenes* ATCC 19115; *Enterococcus faecalis* ATCC 19433) in Table 1, was a significant effect as these bacteria have a neutral or slightly positive charge on their surface, and the opposite charge makes MNPs easy to adhere to cells.

MM ZnO and CuO NPs have a slightly higher effectiveness against G^{-ve} than G^{+ve}, *which is* due to the differing cell wall structures of both gram-positive and gram-negative cells. The cell wall of gram-negative bacteria is covered by a thin peptidoglycan layer with an additional outer lipopolysaccharide membrane. This arrangement may facilitate the entry of released ions from NPs into the cell. But gram-positive bacteria are covered with very thick peptidoglycan with covalently attached teichoic and teichuronic acids, which restricts the entry of NPs and acts as a protective layer [20,40].

The comet assay is one of the most crucial and widely used in vitro techniques in genotoxicology and DNA damage investigations. Some MNPs damage cells by producing ROS, lipid peroxidation, and protein peroxidation [39]. Also, the accumulation and dissolution of NPs in the bacterial membrane change its permeability, with subsequent release of lipopolysaccharides, membrane proteins, and intracellular biomolecules. The dissipation of the proton motive force across the plasma membrane inactivates the proteins, decreasing the membrane permeability and eventually causing cellular death [38,41].

Cationic ZnO NPs were more effective against G^{-ve} and G^{+ve} (Table 5 and Fig. 8) than anionic CuO NPs due to the fact that the small nanoparticles have high surface reactivity, can be easily internalized by cells, and release Zn+, which could be toxic to the biomolecules in bacterial cells [12,20]. Additionally, positively charged MNPs increase cytotoxicity by having a contact that simultaneously destabilizes the membrane and has a vandalizing effect [42]. The uptake can be thought of as a two-step process, with internalization coming after binding to the plasma membrane. The ZP rises as a result of cationic ZnONPs adhering to the cell plasma membrane. Similar to how vesicular transport-based cell endocytosis causes changes in the ZP, internalization also causes these changes [29,30].

The anionic CuO NPs, in contrast, had a lower ZP than ZnO in the comet assay, which may be because the negatively charged particles were unable to adhere to the negatively charged bacterial cell membrane, resulting in less contact membrane fusion and a low electro kinetic potential strength of the attraction [43].

Hence, MM cationic ZnO NPs at high doses are more effective than anionic CuO NPs because of their medical potential and ability to treat many diseases and play a crucial role in the immune system. MM ZnO enhances resistance to udder stress in dairy goats with supplementation [44]. In general, our findings were consistent with their explanation of cytotoxicity as an impediment to ion reciprocity, resulting in a

deficiency in exocytosis activity due to the buildup of extra MNPs on the cell surface [42]. The sudden rise in demand for zinc oxide nanoparticles is due to their unique properties compared with conventional ZnO [45,46].

Overall, it was found that cationic ZnO NPs inhibited microbial growth at the best dose of 100 µg/ml, which showed strong significant antibacterial activity ($P<0.05$), and whose mode of action varied between bacteria due to various cell surface charges and structures, as demonstrated in the conducted study.

Conclusion

The current results demonstrated that the positive surface charge MM ZnO NPs at a high dose 100 µg/mL, exhibit strong antibacterial properties against *E.coli*, *K. pneumoniae*, *B. cereus*, and *S. aureus*, which were isolated from the milk of cows with clinical mastitis locally and killed or slowed down their growth. Also, because they have a positive impact on udder health without being toxic to surrounding tissues, MMNPs can be considered promising multifunctional antibacterial agents. As stated in previous sections, cationic MM ZnO NPs had a cytotoxic effect on bacterial cells, as 'complexes' is the term by which particle formation is elicited by the tropism between DNA molecules and MNPs with positive surface charge [29,30]. According to numerous studies, ZP is a function of MMNP in various media due to the interaction between particle surface molecules and medium molecules, which affects surface ionization and, in turn, the stability of particle dispersion. The cationic charge sometimes increases cytotoxicity by disrupting membranes and having an associated vandal effect as a result of interaction. Interestingly, diminutive size with positive charge of ZnO NPs reflects the significant effective role of ZnO NPs in suppressing mastitis and indicates a powerful relation with the ZP and the cytotoxicity strength. Finally, concluding remarks indicate that the cationic MM ZnO NPs were recommended for suppression of mastitis-causing bacteria as a cheap, effective, and ecologically safe solution.

Financial support and sponsorship

Nil.

Conflicts of interest

The authors declare that they have no competing interests.

References

1 Tomanić D, Božin B, Kladar N, Stanojević J, Ivana Č, Nebojša S, *et al.* Environmental Bovine Mastitis Pathogens: Prevalence, Antimicrobial Susceptibility, and Sensitivity to *Thymus vulgaris L., Thymus serpyllum L., and Origanum vulgare L.* Essential Oils. Antibiotics (Basel) 2022; 11:1077.

2 Cheng WN, Han SG. Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments-A review. Asian-Australas J Anim Sci 2020; 33:1699–1713.

3 Krömker V, Leimbach S. Mastitis treatment-reduction in antibiotic usage in dairy cows. Reprod Domest Anim 2017; 52(S3):21–29.

4 Gomes F, Henrique M. Control of bovine mastitis: Old and recent therapeutic approaches. Curr Microbiol 2016; 72:377–382.

5 Hossain M. Bovine Mastitis and Its Therapeutic Strategy Doing Antibiotic Sensitivity Test. Austin J Vet Sci Anim Husb 2017; 4:1030.

6 La-Beck M, Rakibul M, Maciej M. Nanoparticle-induced complement activation: implications for cancer nanomedicine. Front Immunol 2020; 11:603039.

7 Lavor UL, Guimarães FF, Salina A, Mioni MS, Langoni H. Bacterial identification, somatic cell count, antimicrobial profile and toxigenic Staphylococcus strains search from mastitic cow milk samples on small farms properties. Pesqui Veterinária Bras 2019; 39:715–722.

8 Nasr A, Sara Mohamed Mahmoud, Badawi M, Barakat O. Sugarcane Bagasse Wastes Represent Untraditional Pillars For Antifungal Silica-Based Nanoparticle And ß-Glucosidase Production. Egyptian Journal of Chemistry 2022; 65:199–213.

9 Fulaz S, Vitale S, Quinn L, Casey E. Nanoparticle-Biofilm Interactions: The Role of the EPS Matrix. Trends Microbiol 2019; 27:915–926.

10 Aboelmaaty AM, Sayed MA, Elgabry MA, Kotp MS, Fouad GI, El-Shamarka ME, *et al.* Therapeutic effects of silver nanoparticles on Escherichia coli-induced endometritis in rats. Egypt Pharmaceut J 2022; 21:456–71.

11 Atalla SM, El Gamal NG, Khalil MSA. Evaluation of enzyme production and biocontrol agent of zinc nanoparticles from Gonatorrhodiella parasitica. Egypt Pharmaceut J 2020; 19:252–9.

12 Chatterjee AK, Chakraborty R, Basu T. Mechanism of antibacterial activity of copper nanoparticles. Nanotechnology 2014; 25:135101.

13 Fawzy MH, Mahmoud SM, Hanafy MA, Bakr MH, Mahmoud AEM, Ali MAA, *et al.* Production of Zinc and Copper as Nanoparticles by Green Synthesis Using *Pseudomonas fluorescens*. Pakistan Journal of Biological Sciences 2021; 24:445–453.

14 Ali SK, Mahmoud SM, El-Masry SS, Dalal Hussien M, Wael N, Aboel-Ainin MA. Phytochemical screening and characterization of the antioxidant, anti-proliferative and antibacterial effects of different extracts of *Opuntia ficus-indica* peel. Journal of King Saud University – Science 2022; 34:7.

15 Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. Pharm Anal 2016; 6:71–9.

16 CLSI. VET01- A4: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. Approved Standard. 4th edn. Wayne, PA: Clinical and Laboratory Standards Institute; 2013.

17 Romero J, Benavides E, Meza C. Assessing Financial Impacts of Subclinical Mastitis on Colombian Dairy Farms. Vet Sci 2018; 5:273.

18 Abeer M Anwer, Hanaa AE, Inas M. Apoptosis in Somatic Cells and Immunological Bioactive Parameters of Cow's Milk and Their Relation to Subclinical Mastitis. Alexandria Journal of Veterinary Sciences 2016; 49:31–41.

19 Boutet P, Boulanger D, Gillet L, *et al.* Delayed neutrophil apoptosis in bovine subclinical mastitis. J. Dairy Sci 2004; 87:4104–4114.

20 Hozyen HF, Ibrahim ES, Khairy EA, El-Dek SI. Enhanced antibacterial activity of capped zinc oxide nanoparticles: A step towards the control of clinical bovine mastitis. Veterinary World 2019; 12:1225–1232.

21 Berg JW. Acid-fastness as a histochemical test. J Histochem Cytochem 1953; 1:436–41.

22 Rhodehamel EJ, Harmon SM. Bacteriological analytical manual online. 1998; 8th ed. Revision. Chapter 14 [monograph on the internet]. U.S. Food and Drug Administration; 2001.

23 Kateete DP, Kimani CN, Katabazi FA, Okeng A, Okee MS, Nanteza A, *et al.* Identification of Staphylococcus aureus: DNase and mannitol salt agar improve the efficiency of the tube coagulase test. Ann Clin Microbiol Antimicrob 2010; 9:23.

24 Case CL, Johnson TR. Laboratory Experiments in Microbiology. 5th ed. San Francisco, CA: Benjamin Cummings Pub., Inc; p 126–129; 1984.

25 Pérez MJ, Falqué E, Domínguez H. Antimicrobial Action of Compounds from Marine Seaweed. Mar Drugs 2016; 14:52.

26 Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988; 175:184–191.

27 Armonk. IBM SPSS Statistics for Windows, Version 20.0. NY: IBM Corp; 2011.

28 Swain PS, Rajendran D, Rao SBN, Dominic G. Preparation and effects of nano mineral particle feeding in livestock: a review. Vet World 2015; 8:888–891.

29 Mahmoud SM, Barakat OS, Kotram LE. Stimulation the immune response through ξ potential on core-shell 'calcium oxide/magnetite iron oxides' nanoparticles. Animal Biotechnology 2022; 34:2657–2673.

30 Mahmoud SM, Ali SH, Omar MMA. Cationic Cellulose Nanocrystals as Sustainable green material for Multi Biological applications via ξ Potential. Journal of Biomaterials Science Polymer Edition 2023; 34:1618–1642.

31 Elshamy AA, Kotram LE, Barakat OS, Mahmoud SM. The effects of green synthesized anionic cupric oxide nanoparticles on Zaraibi goat spermatozoa during cryopreservation with and without removal of seminal plasma. Animal Biotechnology 2022; 34:2582–2595.

32 Ibrahim K, Wageeh A, Alaitz E, *et al.* Nanoantioxidants: Recent Trends in Antioxidant. Antioxidants (Basel) 2020; 9:24.

33 Machado VS, Bicalho MLS, Pereira RV, *et al.* Effect of an Injectable Trace Mineral Supplement Containing Selenium, Copper, Zinc, and Manganese on the Health and Production of Lactating Holstein Cows. Vet J 2013; 197:451–456.

34 Libera K, Konieczny K, Witkowska K, ⊠urek K, Szumacher-Strabel M, Cieslak A, Smulsk S, *et al.* The Association between Selected Dietary Minerals and Mastitis in Dairy Cows-A Review. Animals (Basel) 2021; 7:11:2330.

35 Chang YN, Zhang M, Xia L, Zhang J, Xing G. The toxic effects and mechanisms of CuO and ZnO nanoparticles. Materials 2012; 5:2850–2871.

36 Syed B, Nagendra Prasad MN, Dhananjaya BL, Mohan Kumar K, Yallappa S, Satish S. Synthesis of silver nanoparticles by endosymbiont Pseudomonas fluorescens CA 417 and their bactericidal activity. Enzyme Microb Technol. 2016; 95:128–136.

37 Rasmussen MK, Pedersen JN, Marie R. Size and surface charge characterization of nanoparticles with a salt gradient. Nat Commun 2020; 11:2337.

38 Ganesh Kumar N, Pandey SD, Mallick S, Ghosh SK, Pramanik P, Ghosh AS. Thiol stabilized copper nanoparticles exert antimicrobial properties by preventing cell division in *Escherichia coli*. Indian Journal of Biochemistry and Biophysics 2020; 57:151–157.

39 Li Y, Zhang W, Niu J, Chen Y. Mechanism of Photogenerated Reactive Oxygen Species and Correlation with the Antibacterial Properties of Engineered Metal-Oxide Nanoparticles. ACS Nano 2012; 6:5164–5173.

40 Slavin YN, Asnis J, Häfeli UO, Bach H. Metal nanoparticles: Understanding the mechanisms behind antibacterial activity. J Nanobiotechnol 2017; 15:65.

41 Bakhshizadeh S, Mirzaei AF, Taghizadeh A, Seifdavati J, Navidshad B. Effect of zinc sources on milk yield, milk composition and plasma concentration of metabolites in dairy cows. South African J Animal Science 2019; 49:5.

42 Hilchie AL, Wuerth K, Hancock RE. Immune modu-lation by multifaceted cationic host defense (anti-microbial) peptides. Nat Chem Biol 2013; 9:761–768.

43 Xu R, Wu C, Xu H. Particle size and zeta potential of carbon black in liquid media. Carbon 2007; 45:2806–2809.

44 Salama AAK, Caja G, Albanell E, Such X, Casals R, Plaixats J. Effects of dietary supplements of zinc-methionine on milk production, udder health and zinc metabolism in dairy goats. J Dairy Res 2003; 70:9–17.

45 Padmavathy N, Vijayaraghavan R. Enhanced bioactivity of ZnO nanoparticles- an antimicrobial study. Sci Technol Adv Mater 2008; 9:3.

46 Aboelmaaty AM, Omara ST, Aly MS, Kotp MS, Ali AH. The antibacterial and anti-inflammatory effects of zinc oxide nanoparticles synthesized by Thymus vulgaris medicinal plant against Escherichia coli and Escherichia coli lipopolysaccharides.Egypt Pharmaceut J 2022; 21: 153–66.