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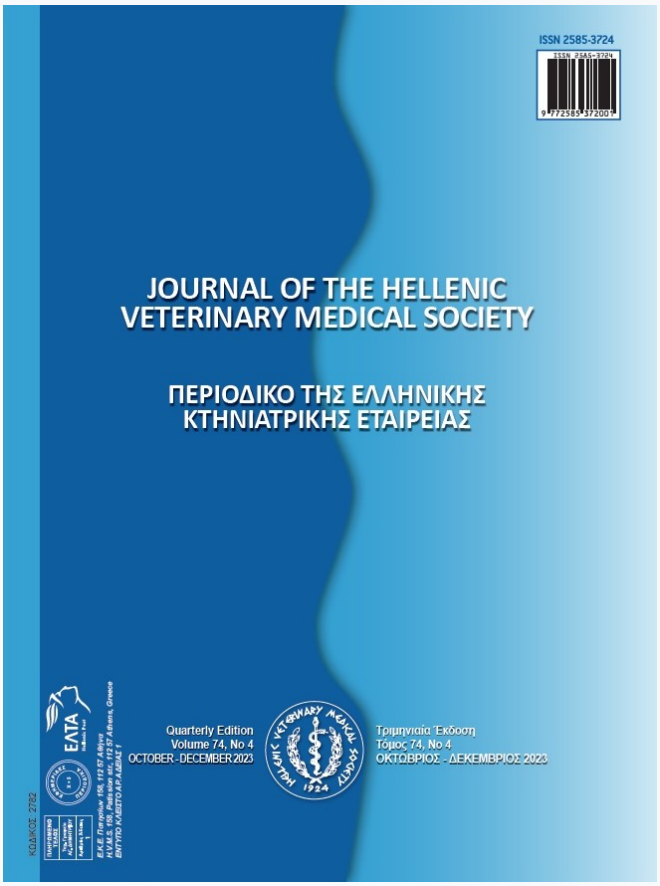

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MA Bozkurt, E Keyvan, S Donmez, E Sen

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Research article
Ερευνητικό άρθρο

Eco-friendly preparation of zinc oxide nanoparticles and effects on *Staphylococcus aureus* isolated from subclinical mastitis cow milk

M.A. Bozkurt[1], E. Keyvan[1*], S. Donmez[2], E. Sen[1]

[1]*Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Burdur, Turkey*

[2]*Burdur Mehmet Akif Ersoy University, Bucak School of Health, Turkey*

ABSTRACT: The study aimed to investigate the eco-friendly preparation of zinc oxide nanoparticles (ZnO NPs) using *Origanum minutiflorum* extract and the inactivation effect of the ZnO NPs to *Staphylococcus aureus*isolates from subclinical mastitis cowmilk. In dairy farms with capacities of 30 or more, a total of 350 cows were evaluated. In a consequence of the evaluation for subclinical mastitis, 144 (41.14%) of the dairy animals were positive. Thirty-four isolates (14.70%) were identified as *Staphylococcus aureus* by PCR, and 29 (20.13%) of the dairy animals with subclinical mastitis were also found to be *S. aureus* positive. The isolates were found to be resistant to various antibiotics, including ampicillin (85.29%), penicillin (82.35%), oxacillin (61.76%), cefoxitin (58.82%), erythromycin (44.11%), vancomycin (35.29%), rifampin (38.23%), tetracycline (26.47%), ciprofloxacin (17.64%), and gentamicin (17.64%). *Origanum minutiflorum*-mediated ZnO NPs have been found to have antibacterial activity against all isolates, with minimum inhibitory concentration (MIC) values ranging from 2.5 to 2000 µg/mL. The ZnO NPs were found to peak at around 350 nm in the UV-Visible analysis. Scanning electron microscopy (SEM) images confirmed the nanoparticle (NP) size and distribution of ZnO. The typical crystallite measured at 15.86 nm. Green produced ZnO NPs exhibited antioxidant activity by scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. In conclusion, an alternative antibacterial product synthesis has been provided for antibiotic substances. Because of the rising incidence of antibiotic resistance in isolates, more research on antibiotic alternatives needs to be considered.

***Keywords*:** *Origanum minutiflorum*; *Staphylococcus aureus*; Subclinical mastitis; Milk;ZnO NPs.

Corresponding Author:
Erhan Keyvan, Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology,Burdur, Turkey.
E-mail address: erhankeyvan@mehmetakif.edu.tr

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INTRODUCTION

Milk is essential in human nutrition due to its high protein content (Guha et al., 2021). Mastitis is an economically significant disease due to the treatment costs in dairy animals (Zigo et al., 2018). Because of dysfunction of the udder, mastitis changes the milk components (Paixão et al., 2017). In subclinical mastitis, contamination of raw milk may occur in the mammary glands without clinical signs. Major pathogens such as *Staphylococcus aureus, Escherichia coli, Streptococcus* spp. are important subclinical mastitis agents (Ariffin et al., 2019). *S. aureus* is one of the most common causes of subclinical mastitis in dairy cows, and it is also one of the most common reasons for antibiotic treatment(Zigo et al., 2022). Antibiotic resistance has been developed due to the widespread use of antibiotics in agriculture, animal husbandry, and medicine (Normanno et al., 2007). As a result of the widespread use of β-lactam antibiotics in dairy cows, methicillin-resistant *S. aureus* (MRSA) may increase in milk. New strategies are needed to combat bacteria due to resistance to antibiotics(Silva et al., 2021).

The higher potential is due to the nanoparticles' small size, high surface area to volume ratio, quantum confinement, and other special physicochemical features (Prasad et al., 2021). Nanoparticle metal oxides are developing research areas for health-related applications and antibacterial activities (Jones et al., 2008).Nanoparticles indicate an alternative approach for focusing on the problem of antibiotic resistance (Thakral et al., 2021). The Food and Drug Administration (FDA) classifies zinc oxide (ZnO) as Generally Recognized As Safe (GRAS) (FDA, 2019).ZnO NPs have antibacterial effects against a wide variety of pathogenic bacteria for humans and animals with low toxicity to mammalian cells (Beyth et al., 2015). It was concluded that ZnO NPs produce reactive oxygen species that cause destructive oxidative stress, resulting in the inactivation of bacterial cells (Beyth et al., 2015).

Origanum minutiflorum O. Schwarz et. H. Davis known as "Sütçüler thyme" is an endemic species that grows wild only in Sütçüler region of Isparta province and is collected(Baydar, 2005). *Origanum minutiflorum* O. Schwarz et. H. Davis has the highest carvacrol content than other *Origanum* species. The carvacrol ratio in its essential oil was over 90 percent(Baser, 2008). Besides carvacrol, p-cymene (7.7%) and γ-terpinene (2.2%) were determined as the main components in Sütçüler thyme(Özkum et al., 2010). Phenolic compounds such as carvacrol, rutin, rosmarinic acid, eriodyctiol, and luteolin were found in Sütçüler thyme. In addition, quercetin, naringenin, vitexin, and apigenin have been identified (Askun et al., 2009). The effective antimicrobial effect of carvacrol in *Origanum minutiflorum* is attributed to its hydrophobic properties, which enable it to disrupt the structural integrity of bacterial membranes (Baser et al., 2008). The primary objectives of this research were to (1) isolate *S. aureus* from farms in the Burdur region, which is the most common cause of subclinical mastitis; (2) create an eco-friendly synthesis of ZnO nanoparticles by using *Origanum minutiflorum* extract; and (3) investigate the inactivation activity of ZnO nanoparticles.

MATERIALS AND METHODS

Collection of milk samples with subclinical mastitis

Burdur is one of the significant milk-producing provinces in Turkey. A total of 350 dairy cows were monitored in farms with a capacity of 30 or more, representing the entire province ofBurdur. Teats were disinfected with an antiseptic solution, and the first drops of milk were discarded. California mastitis test (CMT) was applied to the milk obtained from teats(Zigo et al., 2019).The samples were collected in sterile plastic collection tubes, transferred to the laboratory under refrigeration (4-8°C), and processed immediately for additional analyses.

S. aureus isolation from milk samples

Milk samples obtained from subclinical mastitis were plated on Columbia blood (5% (v/v)) agar (CBA,Oxoid, Italy) and incubated at 37 °C for 24-48 hours. Cream-colored colonies on the CBA were analyzed with some additional tests (Gram stain test, catalase activity, hemolysis test, deoxyribonuclease test, and coagulase test) (National Mastitis Council, 1999; ISO, 2003; Wang et al., 2015). The isolates were kept at -80°C in a 20% glycerol stock.

DNA isolation

The isolates identified phenotypically as *S. aureus* were confirmed by PCR technique. GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA) was used to extract DNA according to the manufacturer's procedure.

PCR analysis

In this study, *S. aureus* isolates were confirmed by primer pairs of species-specific *nuc* gene and *coa* gene (Goh et al., 1992; Lem et al., 2001). The PCR analysis was carried out using 5x FIREPol® Master Mix (Solis Biodyne, Tartu, Estonia) in a total volume of 20 µL, followed by pre-denaturing at 95°C for 4 min, followed by 30 cycles; it was applied at 95°C for 40 s, at 55°C for 30 s, at 72°C for 40 s and 72°C for 10 min. The amplified PCR products were visualized on 1.5% agarose (Keyvan et al., 2020).

Determination of antibiotic resistance

The determination of antibiotic susceptibility in isolates was analyzed by the Kirby-Bauer disc diffusion method using eleven different antibiotic discs: ampicillin, penicillin, oxacillin, cefoxitin, erythromycin, vancomycin, rifampin, tetracycline, ciprofloxacin, gentamicin and chloramphenicol (Hudzicki, 2009; CLSI, 2018).

Synthesis of ZnO NPs from thyme extract

Thyme extract was cleaned from dust particles by washing it three times with ultrapure water, and was weighed 2.5 grams and diluted with 200 mL of ultrapure water.The mixture was heated to boiling for 30 min and cooled to 25°C.The prepared extract was filtered with Whatman filter paper.1.0 gram of zinc acetate dihydrate ($Zn(CH_3CO_2)_2 \cdot 2H_2O$) was taken, dissolved in 50 mL of ultrapure water, and mixed with 50 mL of thyme extract. Afterwards, 1.0 M NaOH was added to the mixture dropwise until pH 10 was achieved.Then, it was kept in a magnetic stirrer (500 rpm) at 80°C until whitish ZnO NPs were formed. After the pellets were collected by centrifugation at 10,000 rpm for 10 min, ZnO NPs were washed several times with ultrapure water and ethanol dried at room temperature. The resulting ZnO NPswere calcined for 1 hour in an oven set at 400°C (Janaki et al., 2015).The obtained ZnO NPs were kept in an amber-colored sample bottle at room temperature until further use.

Characterization of ZnO NPs

Scanning electron microscopy (SEM-EDS) (JEOL JSM-7100F), UV-Visible spectrophotometer (Pg instrument, T60), and X-ray Diffractometry (XRD) (PANalytical Empyrean) were used for the characterization of ZnO NPs (Ramesh et al., 2015).

Detection of ZnO NPs antibacterial effect on *S. aureus* isolates by MIC method

To determine ZnONPs's minimum inhibitory concentration (MIC) value in *S. aureus* isolates identified from subclinical mastitis cow milk, the Clinical and Laboratory Standards Institute (CLSI) performed a tube microdilution method (CLSI, 2018). ZnO NPs were dissolved in dimethyl sulfoxide (DMSO, Sigma, 472301). The ZnO NPs concentration was diluted twice in Mueller-Hinton broth (MHB, Oxoid, CM0405B) starting from 2000 µg/µL, and eight different dilutions were obtained. The prepared solutions were transferred into sterile microplates with 200 µL dilution in each well.0.5 McFarland turbidity standard of the bacterial suspension was adjusted, and 20 µL was added to each well.The control wells included eight dilutions of ZnO NPs without the bacterial suspension. The microbial growth rate was determined using a 600 nm microplate reader (Epoch, BioTek, USA) after incubating microplates at 37±2°C for 24±2 hours (Keyvan and Tutun, 2019). The lowest substance concentration with an OD600 value of ≤0.1 and no microbial growth was accepted as the MIC value (Kang et al., 2008). In this study, the MIC values of isolates were determined in triplicate.

Determination of antioxidant activity

As previously described, the antioxidant activity of ZnO NPs was determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Safawo et al., 2018). Briefly, 1 mL of 0.1 mM DPPH was dissolved in methanol and mixed with 2 mL of ZnO NPs solution with various concentrations between 0.03125-4.0 mg/mL. Then, the mixture was incubated at 25°C for 30 min. The absorbance was recorded at 517 nm against a methanol blank. DPPH scavenging activity was estimated using the following Equation 1:

$$\text{Scavenging Activity (\%)} = \frac{(Ac-As)}{Ac} \text{ x100 (1)}$$

As and *Ac* are referred to as the sample's absorbance and controls at 517 nm, respectively.

RESULTS AND DISCUSSION

As a result of screening 350 dairy cows for subclinical mastitis, 144 (41.14%) dairy cows were detected as positive by CMT. A total of 230 samples were collected from these animals, which were positive for subclinical mastitis. 119 (51.74%) of these samples were classified as presumptive *S. aureus*. Thirty-four (14.70%) isolates were performed as *S. aureus* with

PCR. According to these results, 29 (20.13%) dairy cows positive for subclinical mastitis were also positive for *S. aureus*. Among the studies on mastitis in Turkey, 106 of 480 milk samples with subclinical mastitis at the location of Van Province in Turkey were reported as positive for *S. aureus*. It was determined that 25.5% of the isolates were enterotoxigenic (Boynukara et al., 2008). As a result of screening 400 dairy cows for subclinical mastitis in Kars province, 96 (24%) dairy animals were evaluated as positive. By examining 235 milk samples from positive animals for the presence of *S. aureus*, 76 (64.95%) were confirmed as *S. aureus* (Sağlam et al., 2017).In Aydın province, 85 (28.3%) of 300 milk samples with mastitis were isolated as *S. aureus* (Kirkan et al., 2005). Studies have been conducted in various countries regarding the presence of *S. aureus* in subclinical and clinical mastitis. By screening 224 dairy animals for subclinical mastitis in Thailand, 132 (52%) dairy animals were positive. Twenty-four of the 229 isolates were confirmed as *S. aureus* (Pumipuntu et al., 2019). In a study conducted in China, 773 (67.4%) samples of 1,153 milk samples were evaluated as subclinical mastitis positive. *S. aureus* was determined as 6.24% (Song et al., 2020). Olivares-Pérez et al. (2015) determined the rate of subclinical mastitis as 20.5% in 259 dairy animals in a study they conducted in Mexico. In a study conducted over eight years in Uruguay, the annual rate of *S. aureus* was reported as 78% in subclinical mastitis and 22% in clinical mastitis (Santos et al., 2017). In comparison to other research from Egypt, China, and Iran, the prevalence of *S. aureus* in mastitis was shown to be greater in this study by 95.83%, 77.3%, and 20%, respectively(Jamali et al., 2014; Ewida and Al-Hosary, 2020; Ren et al., 2020). The connection between milking methods and subclinical mastitis, it was shown that the incidence of mastitis was lower in establishments employing automatic milking systems. It is believed that the variations in washing and disinfection procedures utilized throughout the milking process may be the root of this issue.This study and results from various countries show that *S. aureus* is an important factor in subclinical mastitis.To combat this significant subclinical mastitis agent, effective measures should be taken.

Antibiotic resistance of isolates

Thirty-four isolates identified as *S. aureus* were found to have high levels of resistance to the tested antibiotics in the current study (Table 1). The bacterial strains were identified to be susceptible to chloramphenicol. Eighty-three *S. aureus*-positive isolates were obtained from the analysis of 463 milk samples with subclinical mastitis from Central West Anatolia. Eighty-two (98.8%) isolates were determined to be resistant to at least one tested antibiotic (Kenar et al., 2017).In analyzing *S. aureus* isolated from mastitis in Aydin province, resistance to penicillin and oxacillin was determined (Kirkan et al., 2005). In a previous study from Iran, 111 *S. aureus* isolates were obtained from milk with subclinical mastitis. *S. aureus* strains were susceptible to antibiotics, including ciprofloxacin, gentamicin, imipenem, and minocycline. (Sahebekhtiari et al., 2011). According to the results of 107 *S. aureus* antimicrobial susceptibility tests collected from milk with mastitis in Brazil, 59 (55.1%) isolates were resistant to at least one antibiotic group. Resistance to ampicillin (55.1%), erythromycin (2.8%), penicillin (55.1%) and tetracycline (7.4%) was determined in isolates (Rabello et al., 2005). High levels of antibiotic-resistant isolates have been reported in dairy animals. The development of antibiotic alternatives is an important approach in treating mastitis.

Table 1. Levels of resistance to tested antibiotics in the isolates.

Antibiotics	Resistant (%)	Intermediate (%)	Susceptible (%)
Penicillin	28 (82.35%)	ND	6 (17.65%)
Oxacillin	21(61.76%)	ND	13 (38.24%)
Cefoxitin	20 (58.82%)	ND	14 (41.18%)
Gentamicin	6 (17.65%)	9 (26.47%)	19 (55.88%)
Erythromycin	15 (44.12%)	12 (35.29%)	7 (20.59%)
Tetracycline	9 (26.47%)	4 (11.76%)	21(61.76%)
Ciprofloxacin	6 (17.65%)	13(38.24%)	15 (44.12%)
Ampicillin	29 (85.29%)	ND	5 (14.71%)
Rifampin	13(38.24%)	1 (2.94%)	20 (58.82%)
Chloramphenicol	ND	ND	34 (100%)
Vancomycin	12(35.29%)	ND	22 (64.71%)

ND: Non detected

UV-Vis analysis

According to the UV-vis spectra in Figure 1, an absorbance peak at 356 nm, which can be characterized as the intrinsic band-gap absorption of ZnONPs was revealed. In the previous literature, it has been reported that the absorbance peaks obtained by performing UV spectroscopy on ZnONPs are at 355 and 356. The same peak level supporting our results was obtained in similar studies (Rashidian et al., 2021; Al-Mohaimeed et al., 2022).

SEM-EDX analysis

The SEM images of ZnO NPs by using *Origanum minutiflorum* extract is presented in figure (2a). Each spherical aggregate is composed of several individual small nanoparticles with a diameter range about 13 to 27 nm. The EDX spectra of ZnO NPs are shown in figure (2b). The EDX analysis confirmed the presence of zinc and oxygen ions in ZnO NPs where the atomic percentages were Zn = 48.3% and O = 51.7%. No other element was found, indicating the high purity of ZnO NPs. Our result is in close agreement with the previously reported data (Yashni et al., 2019).

XRD analysis

In the XRD pattern (Figure 3), various significant peaks were located at the 31.7, 34.4, 36.3, 47.6, 56.6, 62.9, 66.4, 68.0, 69.3, 72.6, and 77.0º, for crys-

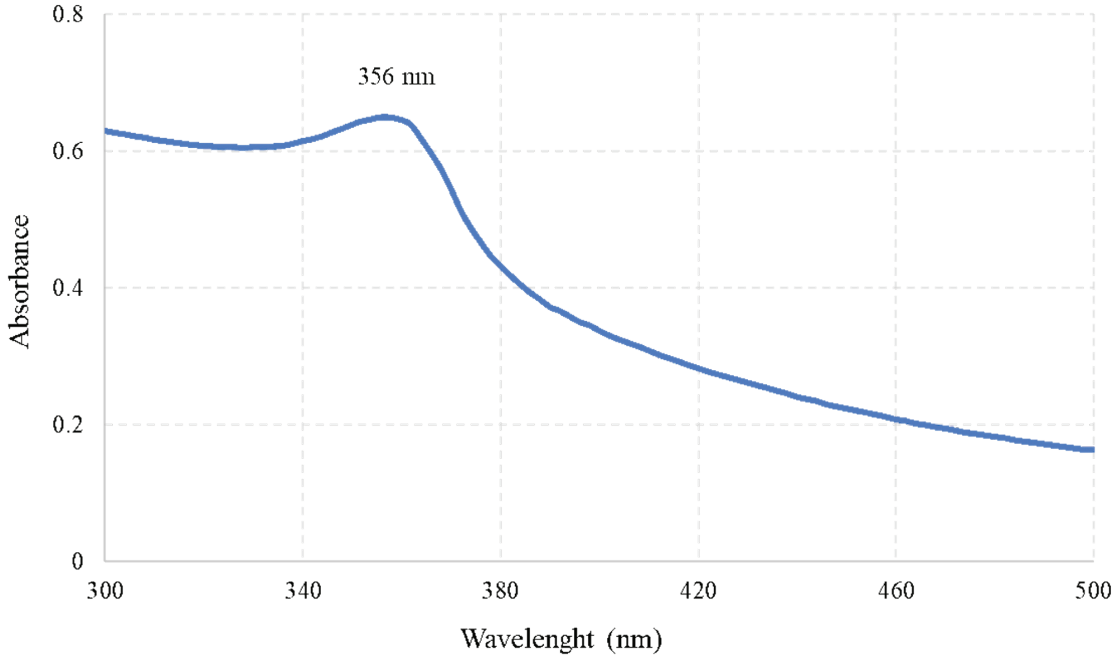

Figure 1. UV-vis spectraof ZnO-NPs.

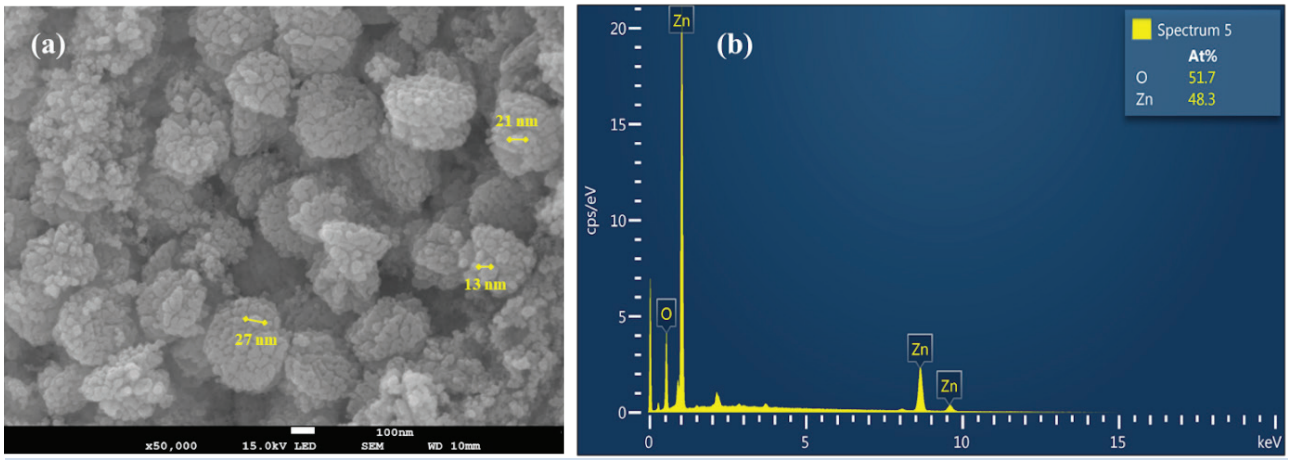

Figure 2. (a)The SEM images and (b) the EDX spectrum of ZnO NPs.

tal planes of (100), (002), (101), (012), (110), (013), (200), (112), (201), (004), and (202), respectively. Compared with the results of the Crystallography Open Database (COD; card No. 96-230-0114, the XRD pattern reflected a well-matching with the hexagonal phase (wurtzite structure). No other peaks are associated with impurities, showing the high purity of the produced ZnO NPs. Scherer's equation was used to estimate the mean crystallite size of the ZnO NPs (Equation (2)):

$$D = \frac{K\lambda}{\beta \cos\theta} \quad (2)$$

λ is the X-Ray wavelength (0.154178 nm), *K* is Scherer's constant (0.9), D is the crystal particle size, θ is the Bragg angle, and β is the width of the XRD peak at half height. The average crystallite size of the green synthesized ZnO NPs was about 9.99 nm.

Antioxidant activity

The antioxidant potential of green synthesized ZnO NPs was evaluated by DPPH method, mainly used to study the radical scavenging activity of the nanoparticles (Safawo et al., 2018). DPPH is a free radical as DPPH, and colour of it is violet in solution. When DPPH mixed with an antioxidant by either accepting hydrogen atom or electron, the DPPH radical is reduced to DPPH-H (yellow). When the concentration of the green-synthesized ZnO NPs increased from 0.03125 mg/mL to 4.000 mg/mL, the dark purple color of the DPPH solution gradually changed to pale yellow (Figure 4) (Das et al., 2013). The half maximal inhibitory concentration (IC_{50}) was estimated as 0.5663 mg/mL (Figure 5). Kavya et al. (2020)

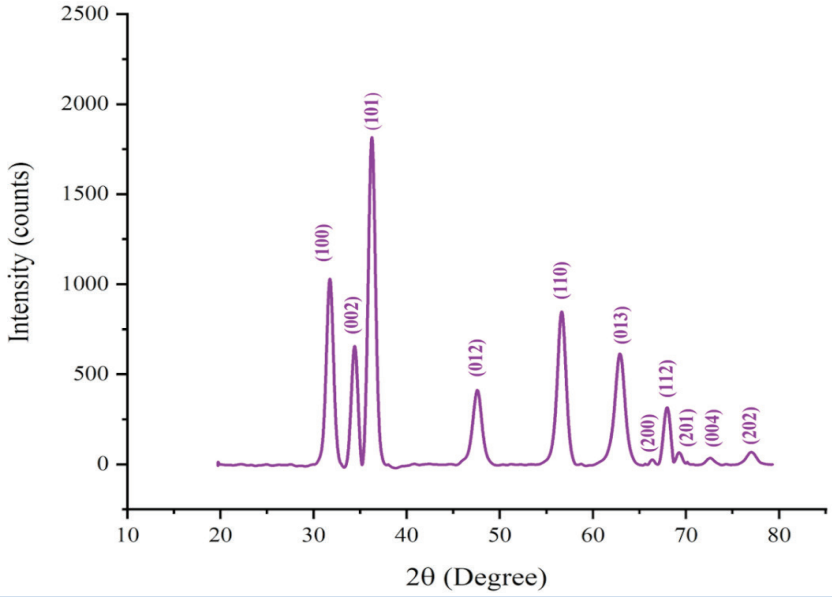

Figure 3. The XRD pattern of ZnO NPs.

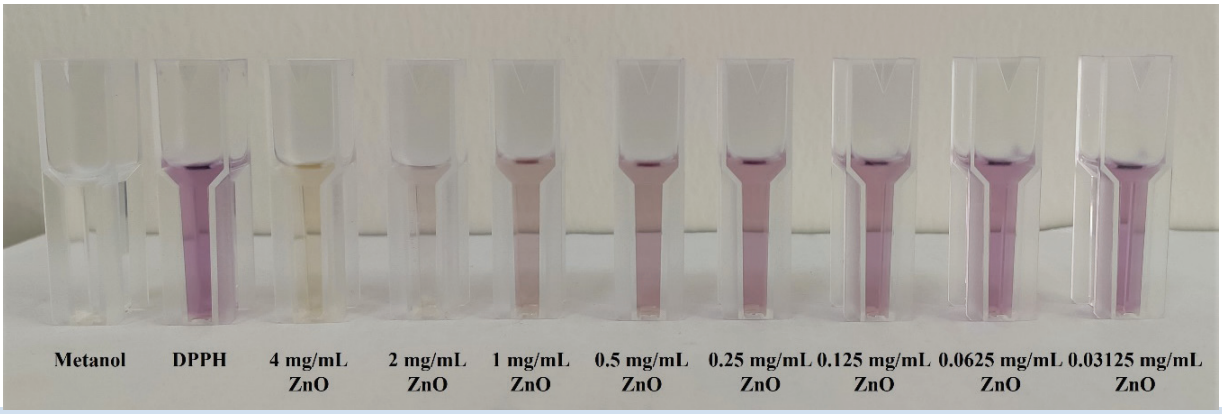

Figure 4. DPPH assay at various concentration of green synthesized ZnO NPs.

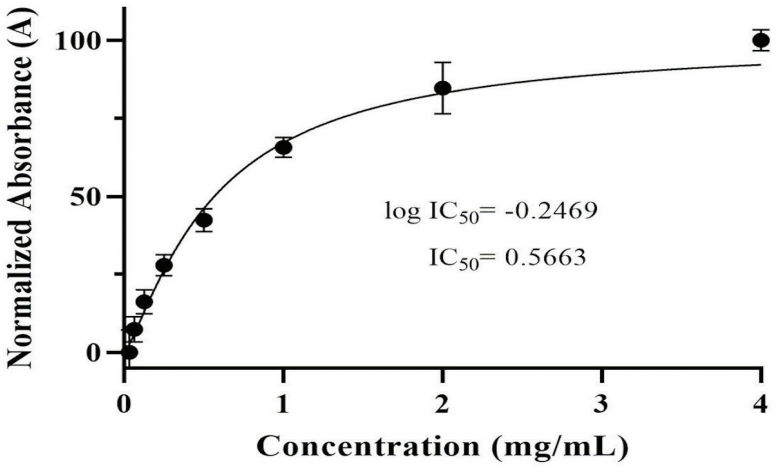

Figure 5. IC_{50} values of green-synthesized ZnO NPs.

found the IC_{50} value of the ZnO NPs synthesized by *Sidarhombifolia* to be 0.9745 mg/mL. The antioxidant activity of the ZnO NPs may be due to the electron donation of oxygen atom in the ZnO NPs (Das et al., 2013).

Antibacterial activity of ZnO NPs

The whole antibacterial mechanism of ZnO NPs is under investigation. However, various antibacterial mechanisms of ZnO NPs have been studied. It has been reported that ZnO NPs produce reactive oxygen species that cause destructive oxidative stress to bacterial cells (Dutta et al., 2012; Gold et al., 2018). This present study determined the antibacterial effect on all isolates of thyme-derived ZnO NPs. The value at which the antimicrobial effect occurred was determined to be between 62.5 µg/mL and 2000 µg/mL (Figure 6). Zhang et al. (2007) reported that the occurrence of ZnO NPs disrupts the membrane wall of the bacteria. Another study reported that the attachment of metal oxide NPs to the bacterial surface is due to electrostatic forces that directly kill the bacteria (Stoimenov et al., 2002). The antibacterial activity of ZnO NPs is related to the nanoparticle size, and releasing antimicrobial ions such as Zn^{+2} may damage bacterial cell integrity (Raghupathi et al., 2011).ZnO was obtained using *Nephelium lappaceum L*. extract with an innovative green synthesis method.It has been determined that the obtained ZnO has an antibacterial effect on *E. coli* and *S. aureus* (Yuvakkumar et al., 2014). The antimicrobial activity of ZnO NPs, which was synthesized by the green method using *Solanum-nigrum* leaf extract, was characterized by UV spectrum and FE-SEM method. It has been reported that the substance synthesized by Uv-Vis DRS analysis is effective in the light spectrum ranges of 402 nm, 447 nm, 469 nm, 483 nm,and 529 nm. It has been found that ZnO NPs has an antibacterial effect on *S. aureus* (Ramesh et al., 2015).Janaki et al. (2015) reported the effect of ZnO NPs, which they carried out using a ginger root extract, on *S. aureus* as 10 mm at 1000 µg, 9 mm at 500 µg, 9 mm at 250 µg, 9 mm at 125 µg, and 9 mm at 62.5 µg concentration. In another study by Nagajyothi et al. (2014), it was observed that ZnO NPs synthesized using *Coptidisrhizoma* had an antibacterial effect on Gram positive (*Bacillus megaterium, Bacillus pumilus* and *Bacillus cereus*) and Gram negative (*E. coli*) bacteria. Dehkordi et al. (2011) found the MIC value of silver nanoparticles on *S. aureus* to be 1.25-10 µg/mL. Ansari et al. (2020) using cinnamon flower, determined that ZnO NPs were effective on *E. coli* and *S. aureus* at the level of 125 µg/mL and 62.5 µg/mL, respectively. Compared with the data obtained in this study, similar results were obtained. The zone diameter of ZnO NPs synthesized using *Azadirachtaindica* (L.) plant, measured on *S. aureus* at doses of 200 µg/mL, 100 µg/mL and 50 µg/mL, was determined as 23, 19 and 14.4, respectively. MIC values were found between 6.25µg/mL and 50 µg/mL (Elumalai and Velmurugan, 2015). The MIC value of ZnO NPs obtained using Spirulina (*Arthrospira platensis*) on *S. aureus* was determined as 50 ppm (El-Belely et al., 2021). The difference between the results obtained in this study and other studies may be due to the isolate types and the various types of plant extracts used for the synthesis.

CONCLUSIONS

This study shows that subclinical mastitis is still a problem for the dairy industry. *S. aureus* is a signifi-

cant pathogen in the development of subclinical mastitis, according to research on milk with the infection. Antibiotic resistance was discovered to be common among these isolates. The synthesized ZnO nanoparticles were evaluated for their antibacterial properties by determining the inactivation of all isolates using a range of MIC values (62.5 µg/mL and 2000 µg/mL). The synthesis of a product for antibiotic alternatives has been successfully achieved. At the levels determined in this study, further research on animals is required.

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CONFLICT OF INTEREST

None declared.

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