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Biosynthesis of silver nanoparticles using curcumin against the bovine mastitis bacteria

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ABSTRACT

Bovine mastitis is the most common disease and has greatly affected economies around the world. This study aimed to determine the antibacterial ability of bovine mastitis by curcumin - silver nanoparticles (Cur-AgNPs). The study included experiments on presenting a new process for synthesizing silver nanoparticles using curcumin from fresh turmeric as a reducing agent and stabilizer. UV-visible spectroscopy of the samples revealed the localized surface plasmon resonance absorbance of the dispersion of silver nanoparticles at 430 nm. The prepared Cur-AgNPs has a spherical shape with an average size of 30 nm and a size distribution of 15–47 nm. FT-IR (Fourier transform infrared spectroscopy) measurements of the samples showed that silver nanopartilces has been encapsulated well by curcumin. Cur-AgNPs with a concentration of 50-200 µg/mL has very effect to *Staphylococcus aureus*, *Pseudomonas aeruginosa* causing bovine mastitis *in vitro*. The maximum inhibition zone formed was 15 ± 0.85 mm for *Staphylococcus aureus* and 14 ± 0.56 mm for *Pseudomonas aeruginosa*. Plant materials mediating for the synthesis of silver nanoparticles have relatively rapid, less expensive, and widespread applications for antimicrobial therapy in the livestock sector.

Keywords: antibacterial activity, curcumin, mastitis causing bacteria, silver nanoparticles

INTRODUCTION

Bovine mastitis is the most widespread and economically important disease worldwide (Kovacevic et al., 2021; Reshi et al., 2015). Mastitis in dairy cows caused by infection, trauma or poisoning is one of the diseases that cause serious damage leading to health deterioration, loss of milk-producing ability, and death if not treated promptly, which results mainly from bacterial colonization of the mammary gland (Orellano et al., 2021). Previous studies show thatthe most common bacteria causing intramammary inflammation are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli* and *Streptococcus uberis* (Nguyen et al., 2015; Dufour et al., 2019; Ashraf and Imran, 2022).

Antibiotics are the most widely used strategy for its prevention and treatment, but overuse has led to growing antimicrobial resistance (Zigo et al., 2018). Treat cows with mastitis with antibiotics either intramuscularly or directly into the udder. The antibiotics used are Kanamycin, Gentamicin, Amoxicillin, Tylosin. This method of treatment is highly effective, but after injecting antibiotics, in the first time, cow's milk must be abandoned, due to the content of antibiotics in milk, which does not meet the specified criteria for cow's milk. On the other hand, the phenomenon of antibiotic resistance occurs more and more rapidly and strongly, requiring new antibacterial drugs and more effective treatment methods (Zigo et al., 2022).

Today, nanotechnology is developing rapidly and being widely applied in many different fields. Silver nanoparticles have a very strong ability to kill fungi and bacteria at low concentrations, making them a promising option for reducing bacterial growth (Woodrow Wilson, 2006; Vo-Van et al., 2021). Silver nanoparticles have been shown to have broad-spectrum antimicrobial potential

(Humberto et al., 2011; Bao et al., 2018). The release of silver ions and the combination of antibiotics and silver nanoparticles have been proven effective in killing bacteria, even those with antibiotic resistance (Birla et al., 2009). Many studies on the bactericidal effect of silver nanoparticles have been carried out and proven effective at the farm scale (Perugini et al., 2015). Along with the high ability to kill bacteria and fungi because only at very low concentrations, silver nanoparticles is not harmful to humans and animals. At the same time, silver that does not accumulate in the body can be eliminated through different routes. Nanomaterials have been used in the human health field for diagnostic and therapeutic purposes, and they also have applications in veterinary medicine, such as drug delivery, cell diagnostics and classification, and antibiotics (Hill and Li, 2017; Youssef et al., 2019; El-Sayed and Kamel, 2020; Cerbu et al., 2021) and animal production, especially animal nutrition (Huang et al., 2015; Gopi et al., 2017; Konkol and Wojnarowski, 2018; Fesseha et al., 2020), is still relatively new. However, nanotechnology is a rapidly growing field that offers the possibility of producing new materials at the nanoscale, with great potential to revolutionize the agricultural sector by offering new options. New treatment options for common and costly diseases, such as bovine mastitis, are urgently needed to reduce the use of antibiotics. Nanotechnology is increasingly being implemented in the design of new therapies for bovine mastitis. Silver nanoparticles, which have been extensively tested on bovine mastitis isolates, have shown a significant bactericidal effect at the nanoscale (Berni et al., 2013; Kazemi et al., 2014; Cardozo et al., 2014). Meanwhile, curcumin, a natural phenolic, extract from turmeric (*Curcuma longa* L.), has displayed natural anti-biofilm properties through anti-inflammatory effects, anti-oxidative effects, and wound healing effects (Tejada et al., 2016; Peng et al., 2021). Therefore, the biological activity of the combination of AgNPs and curcumin can enhance (Loo et al., 2016). In Vietnam, there has been little research on the use of silver nanoparticles for dairy farming, including the prevention and treatment of mastitis. This paper presents the results of a study on the biosynthesis of silver nanoparticles from curcumin (Cur-AgNPs) an evaluation of their antimicrobial activity against bacteria, causing bovine mastitis. This research contributes to the search for alternative materials to antibiotics for treating mastitis in dairy cows.

MATERIALS AND METHODS

Synthesis of curcumin

Fresh turmeric (*Curcuma longa* L.) was collected from the A Luoi mountainous district in Thua Thien Hue province, Vietnam, and washed to remove impurities. For mountain people, turmeric was an excellent natural antiseptic, disinfectant, anti-inflammatory, and analgesic, while at the same time, the plant treats skin irritations. The cleaned material was then cut into small pieces and ground finely with a liter of distilled water for every two kilograms of the sample. The cleaned material was then cut into small pieces and ground finely with a liter of distilled water for every two kilograms of sample. The extract was obtained after thorough filtration with Whatman No.1 filter paper, stored at 4 °C and used within 24 h. To synthesize curcumin, 500 mL of fresh turmeric extract was placed in an Erlenmeyer flask (1000 mL) containing 50 mL of acetone and 250 mL of ethyl acetate. The mixture was gently stirred at 50 °C for an hour. The suspension was then ultrasonicated (27 kHz) for 30 min and irradiated in a 150 W microwave oven for 15 s. The solution was allowed to settle, filtered, and vacuum evaporated to obtain the crude curcuminoid product. The crude product was dissolved in 200 mL of diethyl ether, magnetically stirred at 500 rpm for an hour, and filtered through filter paper to remove insoluble impurities. The resulting solution was then ultracentrifuged at 10 000 rpm for 20 min and the supernatant was lyophilized to obtain pure curcumin.

The synthesis of Cur-AgNPs using curcumin

Cur-AgNPs were synthesized using curcumin solution as both reducing and stabilizing agent. To a 250 mL beaker containing 150 mL of $AgNO_3$ solution (0.02 M), 5 to 20 µg/mL of curcumin solution was added. The

contents were homogenized using an ultrasonicator at 30 °C for 20 min. The color of the $AgNO_3$ solution gradually changed from colorless to pale yellow, and finally to dark red-brown, indicating the formation of Cur-AgNPs.

Characterization of Cur-Ag NPs

The Cur-AgNPs were characterized using various techniques. Ultraviolet-visible spectroscopy (UV-vis) was performed using a UV-2600 spectrophotometer (SHIMADZU Corp., Kyoto, Japan) in the wavelength range of 280 nm to 600 nm. The topographic images were obtained using scanning electron microscopy (SEM), and transmission electron microscopy (TEM) analysis was performed using a JEM 1010 instrument (JEOL, Ltd., Tokyo, Japan) (Inkson, 2016). Fourier transform infrared spectroscopy (FTIR) analyses were carried out at room temperature using a Nicolet 380 spectrophotometer in the spectral range of 150–4500 1/cm, with a resolution of 1.91/cm.

Antibacterial activity by Cur-AgNPs

Preparation of two strains of bacteria causing bovine mastitis: *Staphylococcus aureus* and *Pseudomonas aeruginosa* used in the antibacterial research, isolated, identified and provided by the Faculty of Animal Science and Veterinary Medicine, Hue University of Agriculture and Forestry.

The antibacterial activity of Cur-AgNPs was determined based on colony formation by performing *in vitro* Petri dish assays (Balouiri et al., 2016). Method to evaluate the bactericidal ability of Cur-AgNPs: Using agar well diffusion method. Plate count agar (PCA) medium is poured into sterilized Petri dishes and left overnight. Put in the PCA medium 100 mL of the prepared bacterial fluid and spread the bacteria on the surface of the Petri dishes until dry. The turbidity of the suspension was adjusted with a spectrophotometer at 530 nm to obtain a final concentration to match that of a 0.5 McFarland standard (~1.5 × 10^8 CFU/mL). Punch 5 mm diameter wells and 20 µl of the Cur-AgNPs solution at concentrations of 50, 75, 100, 125, 150, and 200 ppm were placed into each well. Incubate at 37 °C and measure antibacterial ring diameter after 36 hours of culture. The experiment was carried out in triplicate.

Statistical Analysis

The data was statistically analyzed by SPSS software version V.20.0.

RESULTS AND DISCUSSION

Biosynthesis of silver nanoparticles

For the synthesis of curcumin, fresh turmeric from the A Luoi district of the Thua Thien-Hue province in Vietnam was collected, and this high-quality turmeric was used to synthesize curcumin solution after heating the samples at 80 °C for two hours. The nanoparticle synthesis reaction was started after the curcumin extract was introduced into $AgNO_3$ solution and stirred for 36 hours at room temperature; the emulsion turned dark brown corroborating the silver nanoparticle synthesis (Figure 1). The color changes in solutions are due to the surface-plasmon resonance (SPR) phenomenon. This color change indicates the formation of Cur-AgNPs via reducing Ag^+ ions to Ag°. Cur-AgNPs are synthesized in the presence of curcumin act as a stabilizer and a reducing agent (Hemlata et al., 2020). Ag^+ ions are reduced by curcumin solution to form $[Ag\ (curcumin)]^+$ complex (Equation 1), which reacted with aldehyde groups in the molecular structure of the methanolic extract to form [Ag (Curcumin)], due to the reduction of silver ions through the oxidation of aldehyde to carboxylic acid groups (Equation 2). The explained mechanism of [Ag (Curcumin)] was similar to the study of Shameli et al. (2012). The Ag^+ ions were reduced into Ag° nanoparticles by curcumin through the following reactions (Equations (1) and (2)) (Khan et al., 2019):

$$Ag^+_{(aq)} + \text{curcumin} \xrightarrow{\text{Stirring at 25 °C}} [Ag\ (\text{curcumin})]^+ \quad (1)$$

$$[Ag\ (\text{curcumin})]^+ + \text{R-CHO} \xrightarrow{\text{Stirring at 25 °C}} [Ag\ (\text{curcumin})]^+ \text{R-CHOOH} \quad (2)$$

In this experiment, Cur-AgNPs were synthesized and stored at room temperature.

Figure 1. Synthesized silver nanoparticles using curcumin as a reducing agent

Characterization of Cur-AgNPs

UV-Visible Spectroscopy of Cur-AgNPs

UV–vis spectra were recorded for the various curcumin concentrations intervals (5, 10, 15, and 20 µg/mL) was introduced with 1 mM $AgNO_3$ solution. After 36 h of incubation in the dark room condition yellow colour reaction mixture was turned into dark brown (Figure 1). The synthesized silver nanopaticles in features a strong localized absorption peak at 430 nm when the concentration of curcumin solution increased from 5 to 15 µg/mL. Figure 2 also demonstrates that increasing the concentration of the curcumin solution steadily increases the absorbance intensity, and this is in correspondence with the continuous reduction of silver ion and subsequent production of Cur-AgNPs. However, the decrease in intensity at 20 µg/mL curcumin concentration might be caused by the excess reducing agent. The obtained absorption spectrum is similar to that reported by Moghaddasi et al. (2018). The results are in agreement with those reported by Venugopal and Mitra (2013), and Hemlata et al. (2020), where the silver nanoparticles exhibited a UV–Vis spectral range of 400–500 nm, which clearly indicates the formation of Cur-AgNPs in the solution. According to Soto-Quintero et al. (2019), the maximum surface plasmon resonance of spherical silver nanoparticles occurs at 400–500 nm and depends on their size, shape, and surrounding dielectric medium. The achieved suspension of Cur-AgNPs displayed an intense dark brownish-red color due to the plasmon resonance absorption phenomenon (Figure 2). The optimized curcumin concentration for the synthesis of Cur-AgNPs was found to be 15µg/mL, and this is consistent with the results of Verma et al. (2016).

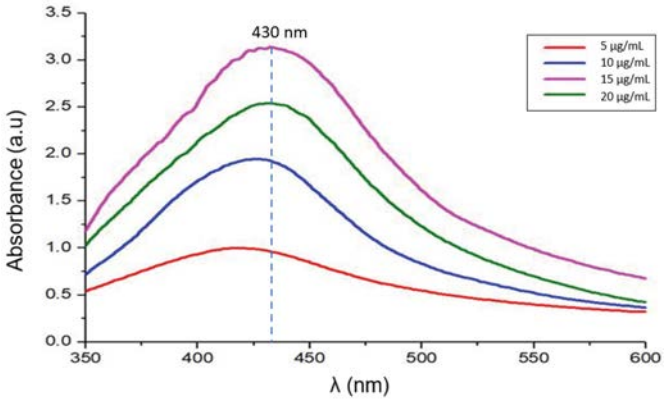


Figure 2. UV–Vis absorption spectra of silver nanoparticles with different contents of the curcumin solution

Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR spectrum of the curcumin showed transmission peaks at 3511; 1628; 1510 and 1282 cm^{-1} corresponding to the oscillate ν (OH), ν (C = C), ν (C = O), [ν (C = O), δ CCC), và δ (CC = O)], và δ (C-O-C), respectively (Kolev et al., 2005; Maliket al., 2016). With the FTIR spectrum of nanoparticles (Figure 3), the peaks for the oscillations of the O–H, C–O–C, C=O shifted to the frequency region 3441,0; 1635,6; 1383,0 cm^{-1}. From that, the characteristic peaks of silver nanoparticles have a slight shift compared to that of curcumin. This showed that the surface of silver nanoparticles has been coated by a curcumin, which protected the silver nanoparticles from agglomeration and this is consistent with the results of Kasthuri et al (2009).

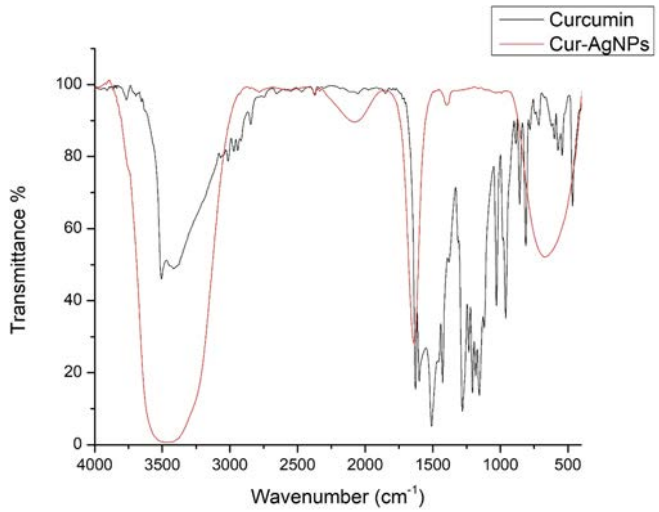


Figure 3. FTIR spectra of Curcumin, and Cur-AgNPs stabilized by curcumin

SEM (Scanning electron microscopic) and TEM (Transmission electron microscopy) analysis

SEM analysis to gather information related to the surface morphology, and particle size of as-synthesized silver nanoparticles (Cur-AgNPs) were assessed after heating the samples at 80 °C for two hours. The SEM image of as-synthesized Cur-AgNPs obtained by using curcumin solutions is given in Figure 4. Accordingly, the Cur-AgNPs showed relatively spherical and uniform with an average size of 5–90 nm. The images suggest the presence of organic moieties on the surface of nanoparticles as stabilizing agents.

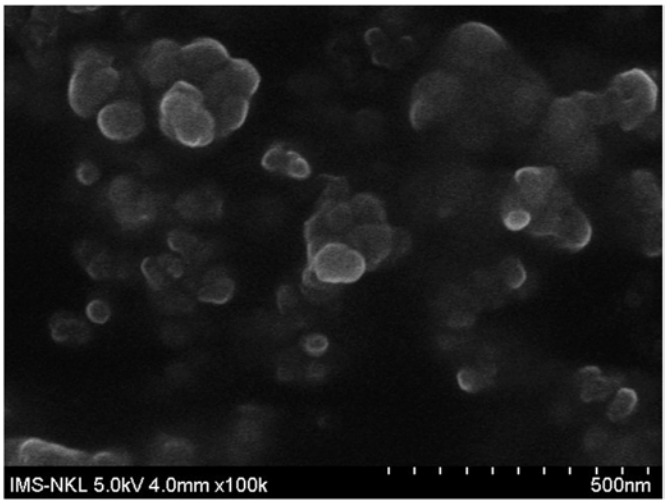


Figure 4. SEM image of Cur-AgNPs with a magnification of 100000x

The shape, size and dispersion of silver nanoparticles were determined by TEM as shown in Figure 5. Thus, it shows that the silver nanoparticles have spherical shape, the particle size ranges from (15 – 47) nm. It observed similar results in SEM image of Cur-AgNPs (Figure 4). According to the research results of Dong et al. (2019), the silver nanoscale size determines the antibacterial activity, the smaller the silver nanoparticle size, the higher the bacteriostatic activity. So, the prepared silver nanoparticles are promising bactericidal ability as the research direction.

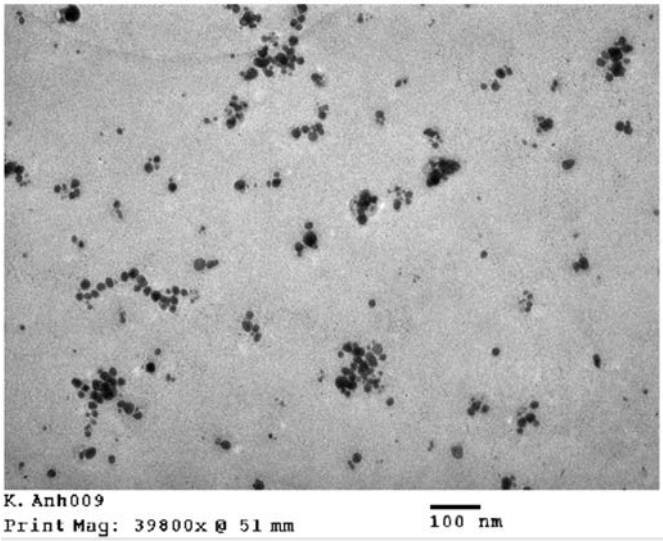
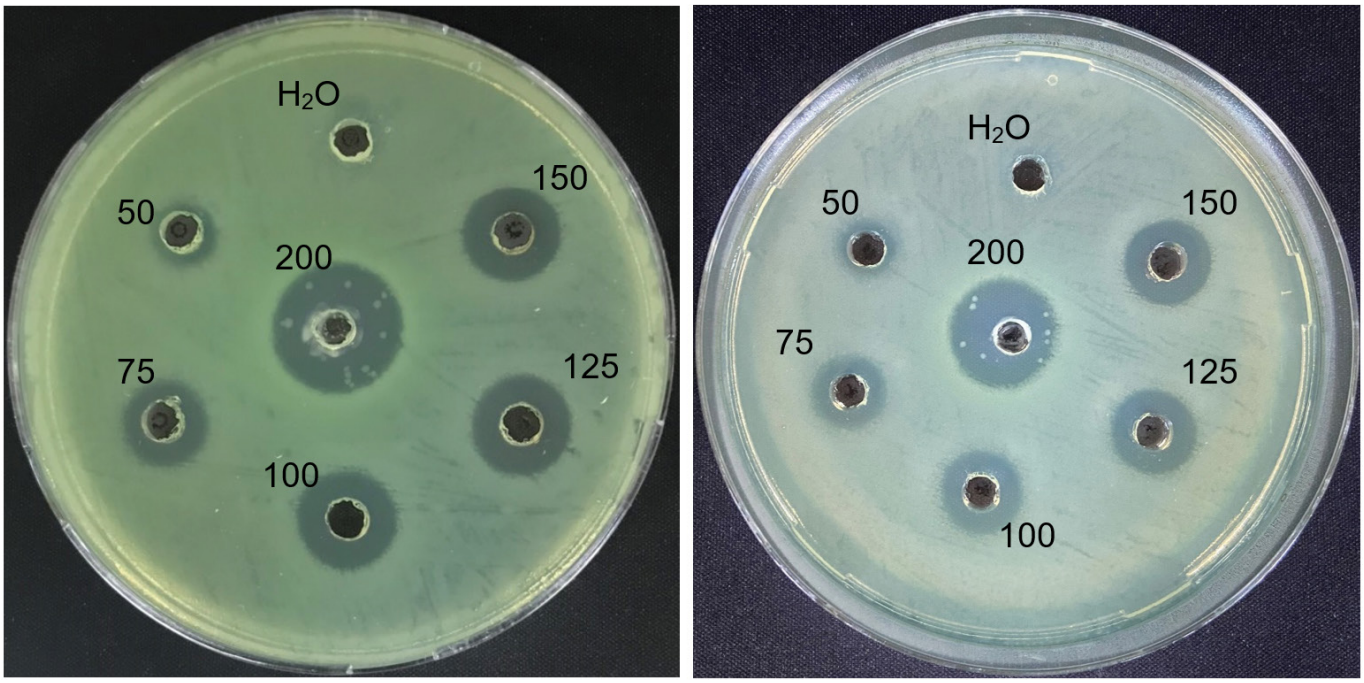


Figure 5. TEM image of Cur-AgNPs with a magnification of 39800x

Antimicrobial activity of Cur-Ag NPs against bovine mastitis in vitro

The antibacterial activity of Cur-AgNPs against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are pathogens that cause bovine mastitis, was determined using the diffusion method, as shown in Figure 6a. The antibacterial activity was tested at concentrations of 50, 75, 100, 125, 150, and 200 µg/mL of Cur-AgNPs (Figure 6b), and the bacterial inhibition zone was found to be proportional to the concentration of Cur-AgNPs. As the concentration of Cur-AgNPs increased, the bactericidal ability also increased, resulting in a larger diameter of the antibacterial ring. The maximum inhibition zone observed was 15 ± 0.85 mm for *Staphylococcus aureus* and 14 ± 0.56 mm for *Pseudomonas aeruginosa* when the Cur-AgNPs concentration was 200 µg/mL (Table 1).



a) *Staphylococcus aureus* b) *Pseudomonas aeruginosa*

Figure 6. Antibacterial effect of *Staphylococcus aureus* and *Pseudomonas aeruginosa* of synthesized Cur-AgNPs

Table 1. Inhibition zone of Cur-AgNPs against some bacteria causing bovine mastitis

Zone of inhibition (M ± SD, mm)	Contents of Cur-AgNPs						
	0 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL	125 µg/mL	150 µg/mL	200 µg/mL
Staphylococcus aureus	0^{a}±0.00	4^{b}±0.17	6^{c}±0,54	7^{cd}±0.36	8^{d}±0.28	9^{d}±0.73	15^{e}±0.85
Pseudomonas aeruginosa	0^{a}±0.00	3^{b}±0.84	5^{c}±0,72	6^{cd}±0.41	7^{d}±0.66	9^{e}±0.08	14^{f}±0.56

M ± SD: Sample means ± standard deviation; The values with different superscript letters in a row are significantly different (P<0.05)

The results of this study show that Cur-AgNPs synthesized using curcumin have excellent antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro*. This is significant because bacterial mastitis is difficult to treat (Zigo et al. 2019). Cur-AgNPs synthesized using curcumin as a reducing and stabilizing agent demonstrated excellent antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida auris* (Gupta et al., 2020). The results of this study correspond to the conclusions of Marslin et al. (2015) also showed that nanosilver strongly inhibited the growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*. Thus, the obtained results show that using Cur-AgNPs has a good premise for further research into this difficult to treat bacterial mastitis. It may become the compound replacing future antibiotic therapy.

CONCLUSION

A new process for synthesizing silver nanoparticles from $AgNO_3$ has been presented in this study. The resulting silver nanoparticles were spherical in shape with a size range of 15 to 47 nm and a good distribution. Additionally, the FT-IR spectrum showed that the curcumin effectively encapsulated the nanoparticles. Furthermore, the Cur-AgNPs exhibited antimicrobial activity against two pathogenic bacteria known to cause bovine mastitis.

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