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ANTIBACTERIAL ACTIVITY OF METALLIC-CORE GOLD AND SILVER NANOPARTICLES AGAINST SOME ANIMAL PATHOGENS

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Abstract

The current work aimed to find substitutes for antibiotics because of the side effects of antibacterial agents and the expansion of bacterial resistance to these agents. The scope of this study was to evaluate the antibacterial activity of gold and silver nanoparticles (AuNPs and AgNPs) against selected animal pathogens (*Staphylococcus aureus*, *Klebsiella pneumonia*, *Streptococcus pneumoniae*, *Escherichia coli*, *Bacillus abortus* and *Mycobacterium bovis*). The synthesized nanoparticles were distinguished by scanning electron microscopy (SEM) analysis and tested for antibacterial activity with the broth microdilution method, well diffusion assay, and minimum bactericidal concentration procedure. Results showed that both AuNPs and AgNPs displayed good antibacterial activity against all tested bacteria. The strongest antibacterial action of AgNPS (18 mm) was against *E. coli*. AuNPs displayed good antibacterial activity against *S. aureus* and *B. bovis* with a suppression area of 14 mm. Therefore, it is suggested that AgNPs and AuNPs could be effectively used against animal pathogens and may contribute to reducing antibiotic resistance. However, there is a need for further research on the *in vivo* toxicity and mechanisms of action of AuNPs and AgNPs.

Key words: animal pathogenic microorganisms, antibacterial activity, green synthesis, silver and gold nanoparticles

The extensive use of antibiotics in animal diets for preventing or treating diseases could produce multidrug-resistant (MDR) bacteria (Abdalhamed et al., 2021 a, b; Abo Ghanima et al., 2023; Al-Otaibi et al., 2023). Thus, MDR is one of the major global menaces to the health and welfare of animals in the era of the 21st century, which implies the capability of bacteria to resist multi antibiotics drugs (Franci et al., 2015; Alagawany et al., 2016; Saeed et al., 2017; Talapko et al., 2020; Okkeh et al., 2021). The presence of MDR bacteria strains in animal products such as milk, meat and others can comprise the principal reservoir of zoonotic pathogens (Kotzamanidis et al., 2021).

Consequently, those pathogenesis bacteria can be moved from animals to humans directly or indirectly by consuming contaminated animal products or contact with animals or their waste in the environment (Abd El-Hack et al., 2016, 2021; Abdel-Moneim et al., 2020; Zalewska et al., 2021). Some bacteria stains such as *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Mycobacterium bovis*, *Staphylococcus aureus* isolated from animals are showing resistance to many common antibiotics (Trott et al., 2018; Guo et al., 2020; Sofiana et al., 2020), which attain resistance via genetic manipulations or gene mutations. According to the above reports, the occurrence and drug resistance outline of most antibiotics utilized against those pathogenic bacteria isolated from animals have been documented (Trott et al., 2018; Guo et al., 2020; Abdalhamed et al., 2021 a, b). For mitigating MDR bacteria, studies have shown that the superior levels of antibiotics will be controlled and could produce insupportable toxic and adversative impacts, which will boost the progress of alternative approaches (Masimen et al., 2022).

Nanotechnology is an emergent approach as a fast upward multidisciplinary scientific scope and has drained global thoughtfulness in various areas viz., biology, chemistry, medicine, and physics (Farouk et al., 2020; Adegbeye et al., 2021).

Additionally, using NPs (nanoparticles) offers a solid policy to cope with infections and diseases triggered by MDR bacteria in animals (Farouk et al., 2020;

Salah et al., 2020; Abdalhamed et al., 2021 a; Masimen et al., 2022). Salah et al. (2020) reported AgNPs exhibited a robust bactericidal influence against *S. aureus* isolated from mastitic ewes and goats. In investigations on small ruminants, Farouk et al. (2020) clarified the influential capacity of AgNPs to combat MDR *Salmonella* spp., according to the *in vivo* and *in vitro* trials without adverse impacts.

Recently, using the green production of metallic NPs such gold nanoparticles (AuNPs) exhibited significant effects on some pathogens bacteria viz. *K. pneumonia* and *S. aureus* (Barani et al., 2022; Dhanker et al., 2022; Shah et al., 2022). However, there is a lack of data regarding using AgNPs and AuNPs as antimicrobial agents against several animal pathogen resistance bacteria. For targeting this scope, metal nanoparticles (AgNPs and AuNPs) are synthesized using an eco-friendly or green approach.

The current work has provided biogenic metallic nanoparticles (AgNPs and AuNPs) through metal recycling. These particles are not only environmentally friendly and efficient but also quickly created. Based on *in vitro* results, this study hypothesizes that AgNPs and AuNPs nanoparticles can control some animal-resistant pathogenic bacteria, including *Staphylococcus aureus*, *Klebsiella pneumonia*, *Streptococcus pneumoniae*, *Escherichia coli*, *Bacillus abortus*, and *Mycobacterium bovis*. The current work aimed to find substitutes for antibiotics because of the side effects of antibacterial agents and the expansion of bacterial resistance to these agents.

Material and methods

Synthesis of green metal nanoparticles (GMNPs) by recycling minerals

The green synthesis of metallic nanoparticles is a novel nanotechnology that is less costly and ecologically sound compared to conventional physical and chemical synthesis methods. Green methods synthesize metal nanoparticles of specific shapes and sizes by enhancing their properties more safely. Recycling metals synthesize GMNPs without chemicals within a short period of fewer than 33 hours. These minerals are converted to powder and liquid at different concentrations to produce nano-gold and nano-silver.

Structural characterization techniques

The optical structure and morphology of deposited GMNPs were characterized using topographical analysis by SEM (scanning electron microscopy). The external morphology and distribution of the GMNPs were evaluated, corresponding to Jakinala et al. (2021). All samples were analyzed at the Egyptian Nanotechnology Center (ENC), Cairo University, Egypt.

Preparation of the tested microbial strains

Microorganisms

The bacteria tested for susceptibility included *E. coli* ATCC BAA-196, *K. pneumoniae* ATCC 700603, *S. pneumoniae* ATCC 49619, *S. aureus* ATCC 25923, *M. bovis* PG45, and *B. abortus* strain 544 (ATCC 23448). All bacteria strains were acquired from the ATCC (American Type Culture Collection).

Preparation of the bacterial suspensions

The bacterial suspensions were prepared by taking a single colony from a 24-hold stock bacterial culture to inoculate 10 ml of sterile Muller Hinton broth (MHB) medium and Mycoplasma broth medium. The broth culture was then kept in a shaking incubator overnight at 37°C and 110 rpm. After incubation, 1 ml culture was serially diluted in 10 ml of sterile normal saline, and the optical density of the diluted culture was then estimated at 660 nm. The culture was adjusted to an initial optical density between 0.10 and 0.15 and an initial bacterial concentration of approximately 107–108 CFU/ml (Alizadeh et al., 2014; Gouyau et al., 2021; Jakinala et al., 2021).

Evaluation of gold and silver nanoparticles for activity against bacteria

Minimum inhibitory concentrations (MICs)

Minimum inhibitory concentrations were evaluated by performing the broth microdilution technique in 96-well plates to detect the action of the plant extracts against the selected bacteria. The extracts were extended multiple times with MHB (sterile diluent) and Mycoplasma broth medium to obtain a concentration range of 10–0.3 mM. Excluding the positive control, each well was added 10 µl of the bacterial suspension. Subsequently, the dishes were preserved in the incubator at 37°C for 24 hours, then after 18 h, a few amounts (50 µl) of TTC solution (2,3,5-triphenyl tetrazolium chloride) were supplemented.

Following the incubation of the plates for 1 h, either a red color develops in the wells due to the reduction of TCC by active bacteria or the color remains the same, indicating the inhibition of bacterial growth (Theivasanthi et al., 2011; Alizadeh et al., 2014; Gouyau et al., 2021).

Minimum bactericidal concentrations (*MBCs*)

The minimum bactericidal concentrations (MBCs) of AuNPs and AgNPs were determined by sub-culturing 0.1 ml of the inoculum from each tube onto Mueller Hinton agar plates. The MBCs were identified as the smallest levels of AuNPs and AgNPs can kill the bacteria. After an incubation period of 72 h, the number of colonies grown on the agar was calculated and compared to the number of colony-forming units (CFUs) per ml in the original inoculum. These tests were repeated three times (Alizadeh et al., 2014).

Well-diffusion method

A good diffusion technique was applied to ascertain the antimicrobial responses of the nanoparticles. Accordingly, a suspension of bacterial pathogens was introduced to Mueller Hinton agar medium and Mycoplasma agar medium and swirled gently to mix well. After the solidi-

fication of the medium, the 6-mm wells were added 10 mM of silver and gold nanoparticles. After incubation at 37°C for 24 h, the microbial inhibition zone surrounding the disc was assessed to evaluate the antimicrobial activity of the GMNPs (Theivasanthi et al., 2011; Senthilkumar et al., 2017; Gouyau et al., 2021).

Results

SEM analysis of the GMNP

The SEM screening demonstrated the existence of a spherical Ag shape (Figure 1) and a size extending between 60 to 160 nm and Au nanoparticles (Figure 2) have a rod shape and a size extending between 300 to 500 nm. Overall, the results revealed that modifying the pH value during the reaction mixture could alter the metallic nanoparticle size.

Evaluation of AuNPs and AgNPs against selected bacterial strains

Minimum inhibitory concentration (MIC)

The antibacterial response of the AuNPs and AgNPs against the picked bacterial strains are presented in Table 1. The results obtained for the AuNPs showed strong antibacterial activity against the bacterial strains at different levels. AuNPs showed the strongest inhibitory activity with a MIC value of 0.3125 mM against *S. aureus* and *P. aeruginosa.* The lowest MIC values (0.625–2.5 Mm) of the AuNPs were observed against *K. pneumoniae*, *M. bovis*, *B. abortus*, and *E. coli.* The AgNPs displayed their strongest inhibitory activity with a MIC value of 0.15625 mM against *P. aeruginosa* and *S. aureus*. The AgNPs also showed their lowest MIC values of 0.3125–2.5 mM against *K. pneumoniae*, *M. bovis*, *B. abortus* and *E. coli* (Table 1).

Minimum bactericidal concentrations (MBCs)

AuNPs and AgNPs were able to kill the tested bacteria at various concentrations. AuNPs showed strong antibacterial activity with MBCs of 2.5 mM against all tested bacteria. AgNPs exhibited bactericidal activity with a MBC of 2.5 mM against *P. aeruginosa*, *K. pneumoniae*, *M. bovis*, and *B. abortus*. AgNPs also showed strong bactericidal activity against *E. coli* with an MBC of 1.25 mM. AgNPs showed no bactericidal activity against *S. aureus* (Table 2).

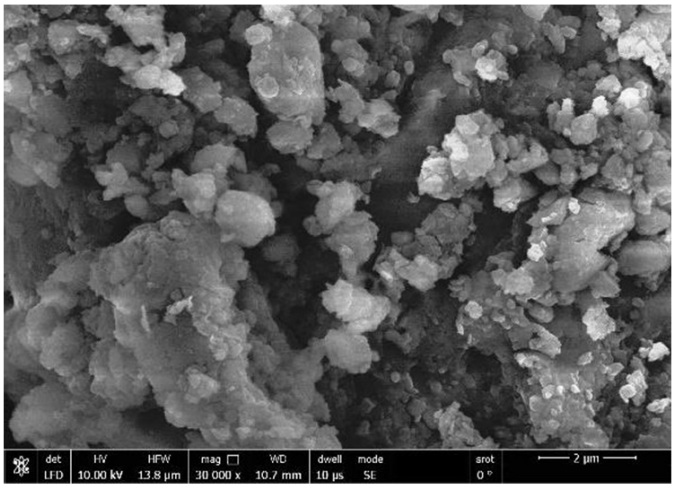


Figure 1. SEM image of spherical Ag nanoparticles at 10 kV operating voltage

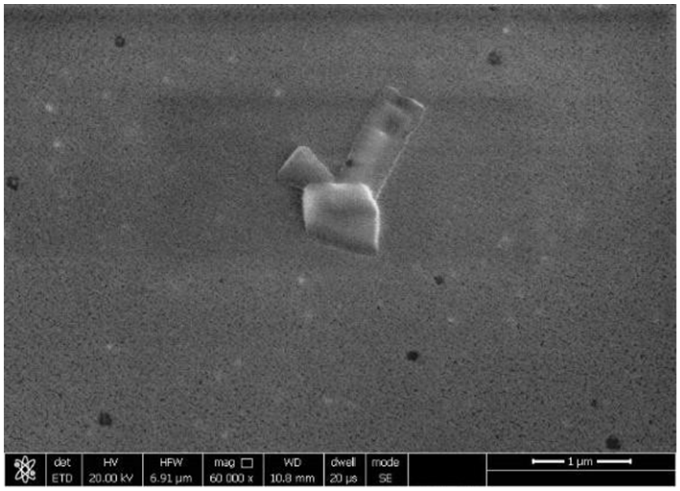


Figure 2. SEM image of rod-shaped Au nanoparticles at 20 kV operating voltage

Table 1. Minimum inhibitory concentrations (MICs) against the selected bacteria

MIC (mM)	Animal pathogenic bacteria					
	E. coli	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*	*M. bovis*	*B. abortus*
AuNPs	2.5	0.3125	0.625	0.3125	2.5	2.5
AgNPs	1.25	0.15625	0.3125	0.15625	2.5	1.25

Table 2. Minimum bactericidal concentrations (MBCs) against the selected bacteria

MBC (mM)	Animal pathogenic bacteria					
	E. coli	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*	*M. bovis*	*B. abortus*
AuNPs	2.5	2.5	2.5	2.5	2.5	2.5
AgNPs	1.25	–	2.5	2.5	2.5	2.5

Table 3. Well-diffusion method against the selected bacteria

Well-diffusion method (mm)	Animal pathogenic bacteria					
	E. coli	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*	*M. bovis*	*B. abortus*
AuNPs	13	14	13	12	14	13
AgNPs	18	14	14	14	15	14

Well-diffusion assay

AuNPs and AgNPs showed great antibacterial activity against all checked bacterial types with diameters extending between 12–18 mm. The strongest antibacterial action of AgNPS (18 mm) was against *E. coli*. AuNPs displayed good antibacterial activity against *S. aureus* and *B. bovis* with a suppression area of 14 mm. It was 13 mm for three pathogenic bacteria including *E. coli*, *B. abortus* and *K. pneumoniae*. AgNPs showed strong antibacterial activity against *P. aeruginosa*, *K. pneumoniae* and *S. aureus* with a growth suppression zone of 14 mm diameter (Table 3).

Discussion

For many years, antibiotics have been extensively utilized in animals at sub-therapeutic dosages for enhancing and promoting productive efficiency, as well as controlling many diseases, which increases the resistance of bacteria against these agents (Farouk et al., 2020; Sofianae et al., 2020). In this regard, developing a new approach using an eco-friendly nanotechnology approach for combating the pathogenic resistance bacteria in veterinary science (Abdalhamed et al., 2021 a, b; Barani et al., 2022) is very critical for the animal as well as the environmental ecosystem.

Today, "green technologies" are highly popular owing to their non-toxicity, efficiency, and environmental friendliness. This approach aims to produce elemental AgNPs of AuNPs for various medical and scientific fields (Mikhailova, 2020, 2021; Swolana and Wojtyczka, 2022). In this work, the formation of AuNPs or AgNPs was confirmed by SEM and they have average sizes of 60–160 and 300–500 nm (Figure 2). Moreover, the SEM displayed that the AgNPs have a spherical shape, while the AuNPs are rod-shaped, as reported in this research.

Abdalhamed et al. (2021 a) examined AuNPs produced using phytochemicals for the green production of metallic NPs and found similar results to ours. According to Nagar et al. (2016), while bio-surfactants used to synthesize AgNPs alter the pH value and decrease their size (60 nm), nanoparticles are uniform at pH 9. Our findings are in mark with the results of Anbu et al. (2020), who stated that AuNPs have a size of 500 nm.

The differences in the size of metallic nanoparticles syntheses in this work compared with other previous research could be due to abundant factors affecting the size viz., pH, methods of synthesis, oxidized agent, and type of metallic, which is evidenced by several researchers (Narayan and Dipak 2015; Ahmed et al., 2016), who used herbs and seaweed extracts. Novel approaches and medicinal tools are being established to enhance fighting bacteria-antibiotic resistance. In this sense, combining metallic nanomaterials and naturally revolving components is highly appreciated (Murei et al., 2021). Most metallic nanomaterial is adjusted using functionalizing mediators to various conjugate drugs that can be successfully applied for treatment (Singh et al., 2013). Our results showed strong antibacterial responses to both AuNPs and AgNPs against the Gram-positive (GPB) and Gram-negative bacteria (GNB). The mechanisms of action of AuNPs and AgNPs differ and depend on the conformation and structure of the bacterial membrane.

The antibacterial activity of different metallic nanoparticles, such as AgNPs and zinc nanoparticles, was evidenced and effectively used as promising options instead of traditional antibiotics in the fish farm (Shaalan et al., 2017). According to studies on small ruminants, AgNPs have verified a substantial capability to fight resist-

ant *Salmonella* spp. *in vivo* and *in vitro* without adverse impacts (Farouk et al., 2020). This antimicrobial activity was reported through its ability to reduce inflammation indices in mice. Our results demonstrated that both AuNPs and AgNPs exhibited antibacterial action against all tested bacteria based on MIC values (0.15625–2.5 mM) ascertained by the broth microdilution technique. As identical with the current data, Farouk et al. (2020) found that the MIC was ≤0.02–0.313 µg/mL for AgNPs. The study by Abdalhamed et al. (2021 a) exhibited that the biogenic AuNPs produced by plant extracts have the highest MIC for *E. coli* and *Salmonella* spp., and isolated from ruminants. The same results were reported on the antimicrobial AuNPs and AgNPs against *E. coli* and *Salmonella* spp. isolated from the animal origin by many previous works implemented by Hegazi et al. (2014), Athreya et al. (2020) and El-Gohary et al. (2020).

Authors suggested that this action might be associated with the thin wall of the peptidoglycan layer in GPB, which permit AuNPs to pass in the cytoplasm of bacteria, preventing bacterial growth or development. Another study by Masimen et al. (2022) showed that AgNPs induced a significant reduction in the growth of *S. aureus*.

In this study, the antimicrobial properties of AuNPs and AgNPs against the tested bacteria were demonstrated by inhibition zones ranging from 12 mm to 18 mm in diameter with a good diffusion assay. These results agree with those reported by Zawrah and El-Moez (2011), suggesting strong antimicrobial activity for AuNPs with the largest inhibition zones against *P. aeruginosa* (17 mm) and *S. aureus* (13 mm). The strong antimicrobial activity of AgNPs and AuNPs could be accredited by their small size, which enables them to readily attach to the cell wall surface and impede bacterial functions such as respiration and permeability (Zawrah and El-Moez, 2011; Swolana and Wojtyczka, 2022). Vazquez-Muñoz et al. (2019) stated that AgNPs displayed strong antibacterial action against both GNB and GPB.

The potential effects of AgNPs on pathogenic bacteria via different pathways include adhering to the bacterial membrane and cell wall, penetrating the cellular membrane and disrupting intracellular biomolecules and organelles, inducing oxidative stress, and modulating singling transduction paths (Chauhan et al., 2016; Singh et al., 2018).

K. pneumoniae is the bacteria most affecting the lungs, triggering clinical and subclinical mastitis, increasing the mortality in newborn calves and decreasing milk yield and quality (Langoni et al., 2015). This species has shown antibiotic resistance in farm animals, causing severe mastitis and ultimate animal death (Munoz et al., 2007; Langoni et al., 2015). This research showed that the MIC for *K. pneumoniae* was 0.625 and 0.3125 for AuNPs and AgNPs, respectively. Our results are partially in consequence of some previous studies (Hegazi et al., 2014; Athreya et al., 2020), which described that the AgNPs created from camel or cow milk exhibited a significant effect on the growth of *K. pneumoniae* via increasing the inhibition zone and MIC.

The same results were detected for *P. aeruginosa*, and Athreya et al. (2020) showed that AgNPs demonstrated influential inhibition zones extending between 11 and 15 mm against *P. aeruginosa* . The pathogenic bacteria *P. aeruginosa* is associated with many diseases in dairy cows and has wide host ranges (El-Gohary et al., 2020). As the previous authors mentioned, the resistance pattern of *P. aeruginosa* against MDR was 85.7%. It is critical to discover alternative antimicrobial agents for this species to achieve this target, and further studies are required. While AuNPs and AgNPs showed a strong bactericidal effect against all tested bacteria with an MBC of 2.5 Mm, AgNPs demonstrated no bactericidal activity against *S. aureus*. Based on their study on the impacts of AgNPs on streptomycin-resistant *Brucella abortus*, Mirnejad et al. (2013) determined that dilutions greater than 25 mM could impede the development of this species.

The investigation of the synergistic impacts of AgNPs with streptomycin revealed the development of inhibition zones in the agar plates containing streptomycin discs at a dilution of 12.50 mM. Alizadeh et al. (2014) reported that AgNPs illustrate antimicrobial action against the intramacrophagic *B. abortus* 544. AgNP concentrations of 1 and 2 ppm have been determined to be highly effective in the intramacrophagic clearance of *B. abortus* 544 within 24 hours. Thus, AgNPs are capable of penetrating macrophages and killing *B. abortus* 544. In another study by Selim et al. (2018), the antimicrobial activity of synthetic AgNPs of the mean size of 50 nm was tested against two picked bacteria species (*M. bovis* and *M. tuberculosis*), the MDR *M. tuberculosis* strain and clinical separates (*M. bovis* and *M. tuberculosis*). The synthetic nanoparticles enabled a substantial inhibition in the growth of all the samples examined. The MIC values of the *M. bovis* and *M. tuberculosis* reference strains were 1 and 4 µg/ml, respectively.

Brucella abortus GNB is the main reason for domestic animals' morbidity. Using *in vitro* evaluation, Alizadeh et al. (2014) illustrated that the values of MBC and MIC were 8 and 6 ppm for AuNPs against *B. abortus* 544. In a recent study on rats infected by *B. abortus*, Elbehiry et al. (2022) showed that MIC values were 12.45 and 18.77 g/ml for AuNPs and AgNPs, respectively. The capability of those nanoparticles, such as AgNPs and AuNPs, to increase the Ca^{+2} levels in the cytoplasm and inhibit the bacterial cell membrane suggests their strong antibacterial activity (El-Gohary et al., 2020; Elbehiry et al., 2022). Moreover, some investigators suggested that the AgNPs and AuNPs could be attached to the bacterial DNA, inducing damage and causing apoptosis-like cell death.

Few studies are available regarding utilizing AuNPs (gold nanoparticles) against *M. bovis* and *B. abortus*. Thus, additional exploration is necessary to shed additional light on the mechanism of AuNPs against these bacteria. AgNPs penetrate bacteria via water-filled channels (porins) found in the outer membrane of GNB. In

bacteria, porins are for the passive transport of hydrophilic molecules of different charges and sizes across the membrane. Given that GPB has a thicker cell wall, silver ions penetrate the cytoplasm and show a greater impact against GNB than GPB (Chauhan et al., 2016). Furthermore, the negative charge of the cell membrane of GNB and GPB makes them highly affinitive to positively charged AuNPs. While GNBs are easily and highly exposed to AuNPs owing to their thinner cell wall, GPBs are spared from exposure to AuNPs due to the rigid peptidoglycan layers on their cell surface.

Conclusions

The present study has shown that AgNPs and AuNPs could be ecologically synthesized at low costs and within short periods by recycling minerals. The synthesized nanoparticles were distinguished by SEM analysis and their antibacterial activity was confirmed by broth micro-dilution system and good diffusion assay. Referring to our results, it is suggested that AgNPs and AuNPs could be used as an effective antibacterial agent against selected animal pathogens. However, further studies are required to better understand metallic-core nanoparticles' *in vivo* toxicity and mechanisms of action.

Author Contributions

Conceptualization, A.A. and R.A.; methodology, A.A., A.Y.M.A., R.Al. and S.E.; software, validation, investigation, M.A.; resources, M.E.; data curation, visualization, writing – original draft preparation, A.Y.M.A., A.A. and R.A.; funding acquisition, A.A. and R.A. All writers have read and approved the published version of the manuscript.

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Institutional Review Board Statement

This work was conducted in acquiescence with the ARRIVE procedures at the Department of Veterinary Science, Eskil Vocational School, Aksaray University, Aksaray, Turkey.

Conflicts of Interest

The authors declare no conflict of interest.

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