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In vitro and *in vivo* Antibacterial and Antibiofilm Efficacy of Selenium Nanoparticles against *Staphylococcus aureus* Supported with Toxicopathological and Behavioral Studies in Rats

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ABSTRACT

The present study aimed to investigate the antibacterial and antibiofilm effects of selenium nanoparticles (SeNPs) synthesized by two different methods against *Staphylococcus aureus* forming biofilm *in vivo* and *in vitro*, with a focus on the toxicological and behavioral changes of various SeNPs concentrations in rats. SeNPs were prepared by green and chemical methods and characterized by X-ray diffraction (XRD), Transimission electron microscope TEM. Their antibacterial efficacy was evaluated by agar well diffusion test, also minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) values were detected. Experimental design sixty female lactating rats were divided into 6 groups: G1 control negative, G2 control positive, G3-G6 infected animals received SeNPs with different concentrations. A bacteriological and histopathological examination of the mammary gland was carried out, an antioxidants assay was evaluated and finally a toxicological study was done. Green synthesized SeNPs showed antibacterial effects at different concentrations (600 µg/mL to 10µg/mL) with MIC and MBC values of 10µg/mL and 25µg/mL. Time kill kinetics showed that SeNPs inhibited the growth of *S. aureus* completely after 4 hours. The treated group showed pronounced improvement in the main lactiferous duct with normal histological structure compared with the infected group without treatment; by increasing dose the main lactiferous duct structure and acini became nearly identical to the control negative group. Bacteriological examination showed a complete absence of bacterial colonies in all tested concentrations in female rats. The highest doses of SeNPs showed mild cytoplasmic vacuolization of some hepatocytes with activation of kupffer cells with normal hepatocytes structure compared to the control group, a decrease in catalase (CAT) and total antioxidant capacity (TAC) in most examined doses compared to the control group, and an increase in superoxide dismutase (SOD) level in all treated animal. SeNP supplementation could be a safe and helpful treatment for *Staphylococcus aureus* infection. It prevents biofilms formation both in vitro and in vivo, and it had no notable toxicological or behavioral effects on rats.

Key words: Selenium nanoparticle, *Staphylococcus aureus*, *In vivo*, Toxicity, Antioxidant.

INTRODUCTION

One of the most destructive illnesses in dairy herds worldwide is bovine mastitis, which is typically brought on by a variety of bacteria (Demontier et al. 2021). *Staphylococcus aureus* (*S. aureus*) is the most prevalent bacteria among these bacteria that causes the most severe cases of bovine mastitis and poses the biggest threat to the production of dairy products in the majority of nations (Campos et al. 2022). According to (Gomes and Henriques 2016), this bacterium results in significant economic losses, including a sharp fall in milk sales, problems during

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reproduction, costs associated with culling affected animals, higher veterinary medicine expenditures, and the need to replace tainted milk. Additionally, *S. aureus*-produced milk contains a variety of toxins and enzymes that can cause serious food-borne illnesses (Freitas et al. 2023). They are also linked to the clinical, subclinical, and recurring infections of bovine mastitis, and their persistence in cells can create a reservoir for relapsing infection (Zhou et al. 2018).

When *S. aureus* adheres to a surface, biofilm formation is uncomplicated. Due to the polymeric exopolysaccharide matrix that these biofilms produce, they are simple to create yet challenging to eradicate. This polymeric matrix functions as a barrier to stop medications from accessing the biofilm. Therefore, it would be ideal to create active molecules that eliminate *S. aureus* at the earliest stages of an infection, avoiding the development of difficult-to-treat biofilms (Tuon et al. 2023).

Researchers may now create nanoparticles and employ them in a variety of applications (Malik et al. 2023). Compared to traditional, micron-sized particles, nanoparticles (NPs) have a larger surface area, which results in more interactions with biological targets (such as bacteria). Selenium (Se) is a halogen element that occurs naturally and has both metallic and nonmetallic characteristics (Hunter 2014). At low concentrations, it is a crucial micronutrient for both prokaryotes and eukaryotes, but at greater amounts, it can be hazardous to living things (Kessi et al. 1999). Se, for instance, can function as an antioxidant and defend against cellular harm from oxygen radicals.

Although the application of NPs in animal production is still in its early stages and has not been fully tested *in vivo*, encouraging *in vitro* study results are encouraging future research. To develop safe NP antibacterial drugs for use in animal systems, it is necessary to examine the toxicological characteristics and pharmacokinetics of metal-based NPs. It is also important to determine their intracellular fate, biological interaction, and function (Nel et al. 2009). Frameworks and varied methods are needed to ensure safety and to pinpoint the knowledge gaps and uses of NPs in cattle that still need to be filled.

The major objective of this research is to assess the antibacterial and antibiofilm efficiency of both naturally occurring and chemically produced SeNPs as a helpful and effective antibiotic alternative. *In vitro* and *in vivo* studies on the antibacterial capabilities of chemically and environmentally friendly SeNPs were conducted on *S. aureus* generating biofilms isolated from bovine mastitis. Laboratory animals were also employed to assess the NPs' toxicological effects.

MATERIALS AND METHODS

Ethical Approval

The experimental design was approved by the Animal Ethics Committee of the National research center (approval number: #19-155#).

Preparation of SeNPs by Green Method

SeNPs were formulated using a modified version of (Chen et al. 2011) approach. In brief, 100mL of ethylene glycol (Merck Schuchardt - Hohenbrunn, Germany) and 100ml of water were combined with 4g of Na2SeO3 (LobaChemie, India; MW=173.01); 4g of glucose (ADWIC, Egypt); and the beaker containing the reactants was sealed and placed in an oven that had been preheated to 80°C. The beaker was removed and the NPs were cleaned multiple times with distilled water after the reaction had been running for an hour.

Preparation of SeNPs by Chemical Method

With slight adjustments (Gao et al. 2002), chemical precipitation approach was used to create SeNPs. In summary, 0.5mmol of sodium selenite was dissolved in 97mL of distilled water, and 2mmol of mercaptoethanol was added. The sodium selenite and mercaptoethanol solution (Carl Roth GmbH, Cat # 4227) was then stirred magnetically at 1000rpm for 30min at room temperature with 3ml of 0.9mM aqueous NaOH (Merck). Centrifugation at 5000rpm for 15min was used to separate SeNPs, and the settling NPs underwent three successive washes in deionized water. To obtain the powder nano-selenium, the purified SeNPs were then dried for an overnight period at 80°C.

Characterization of SeNPs

Cu K1 radiation (wavelength 1.5406) was used in powder X-ray diffraction (XRD) studies on a PANalytical (Empyrean) Xray diffraction at a voltage of acceleration of 40kV, an applied current of 30mA, a scanning angle range of 5-80°, and a scanning step of 0.02°. The morphology and microstructure of samples were examined by HRTEM (JEOLJEM2100, Japan).

In vitro Assessment for the Antibacterial and Antibiofilm Activity of SeNPs

Determination of SeNPs for Antibacterial Activity by the Agar Well Diffusion Test

According to Jin et al. (2009), the synthesized SeNPs were examined for their antibacterial efficacy against *S. aureus* that was recovered from the mastitic milk samples using the agar well diffusion method. The media used in this test was TSA, which was added to plates containing one-mL of bacterial cell suspension (10^5 cells/ml), shaken rotating over the table, and left until solidification. A sterile cork borer or the tip of a pasture pipette was used to aseptically punch a hole with a diameter of 6 to 8mm after solidification. 50µl of SeNPs concentrations (100, 200, 300, 400, 500, and 600 µg/mL) were pipetted into various plates. The varied sizes of the inhibitory zone were measured following incubation under appropriate circumstances (for 24 hours at 37°C).

Determination of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

Using the micro dilution approach according to (Parvekar et al. 2020), the MIC and MBC values were found. Briefly, the strain of *S. aureus* was cultured in TSB at 37°C and shaken overnight. Preparation of various concentrations (100-600µg/ml) of SeNPs in Muller Hinton broth and added (1×10^5 CFU/mL) to a 96-well plate. As a control, bacteria without SeNPs were employed. Incubation at 37°C for 24h. The lowest concentration of SeNPs completely inhibit the growth of *S. aureus* were

considered as the MIC values. The lowest concentration that prevented colony formation was determined to be the minimum bactericidal concentration (MBC) value by applying 20 ml of the suspensions without bacterial turbidity to the surface of agar.

Determination of Minimum Biofilm Inhibitory Concentration (MBIC)

The selected *S. aureus* strain was cultured in TSB-g (TSB with 0.5% glucose added) at 37°C overnight. The bacteria were then diluted with medium until they were $1*10^6$ CFU/mL after being diluted with TSB-g until their OD600 was 0.4 ($5*10^8$ CFU/mL). The suspension was added to a 96-well plate and cultured there for 48 hours at 37°C to allow for biofilm formation. After then, PBS was used to wash the biofilm three times. The biofilm was then exposed to various SeNPs concentrations for 24 hours. The MBIC was considered the lowest SeNPs concentration that, after 24-hour incubation, visibly inhibited the development of *S. aureus* (Raffaella et al. 2017).

Time-killing Kinetics

SeNps' time-kill kinetics test was conducted with a few changes as described by Eleftheriadou et al. (2021). *S. aureus* strain colonies that had grown over night were resuspended and incubated for two hours at 37°C and 180rpm. Each strain was inoculated into sterilized Muller Hinton broth media at a density of $1*10^6$ CFU/mL, and then various concentrations of SeNps were added, followed by an incubation period at 37°C and 180rpm. Additionally, there was also a growth control with no SeNPs. At intervals of 1, 2, 3 and 4 hours, aliquots of 1ml of the cultures were obtained, placed on Muller Hinton agar plates, and incubated at 37°C for 24 hours. CFU/mL measurements were made of the live cells.

***In vivo* Assessment for the Antibiofilm Activity of SeNPs**

Animals and Experimental Design

The ability of SeNPs formulized using the green technique to lessen *S. aureus* colonization of rat mammary glands was assessed using a rat mastitis model (Zhu et al. 2007). A total of 60 female Sprague dewalley rats, weighing, and 250-280 g were allocated in 6 groups of 10 rats. Pups were removed from the lactating rat 72h post parturition, approximately 1h before infection of the mammary glands of rats. The pups were euthanized and not allowed to suckle after inoculation of the mammary gland with the *S. aureus* strains or normal PBS.

Preparation of Bacterial Inoculum

On Mueller Hinton (MH) agar plates, the *S. aureus* strains were cultivated for 18h at 37°C. To obtain a final viable bacterial count of $4 \times 10^{11}.mL^{-1}$, the organisms were suspended in isotonic saline after being rinsed off the plates with 20 ml of isotonic saline (Anderson and Chandler 1975).

Induction of Mastitis

In order to infect the mammary glands with *S. aureus* (biofilm hyperproducing strain), the female nursing rats were isolated from their young, put under ketamine and xylazine anesthesia at doses of 87 and 13, respectively, per kilogram of body weight. First, 70% ethanol was used to disinfect the fourth pair of glands (L4 and R4 glands) found from the head to the tail. The lactiferous duct was then gradually infused with 100µl of PBS containing live bacteria. Two doses of SeNPs formulated by using the green method (in PBS) were delivered to the infected mammary glands with concentrations 25, 100, 300, 500µg/ml. The first dose was given four hours after the bacteria were inoculated and the second dose was administrated twelve hours later.

Post-inoculation Macroscopic Examination

Rat mammary glands were checked for the presence of mastitis. The clinical symptoms of mastitis include redness, swelling, mammary gland discolouration and exudate extrusion. Rats were observed for morbidity and mortality 6 hours after infection with *S. aureus*. Based on the observed clinical indicators, the level of symptoms were categorized as 0 (no macroscopic alterations), + (low), ++ (medium), and +++ (severe) (Gogoi-Tiwari et al. 2016).

Bacteriological and Histological Procedures for Mammary Glands (Gogoi-Tiwari et al. 2015)

After 12h, rats were euthanized using ketamine-xylazine anesthesia. The L5 mammary glands from all rats were harvested and individually ground in sterile Griffith tubes containing 2ml of sterile normal saline. Tenfold serial dilution was prepared from the homogenates of the mammary glands and cultured on Baird Parker (BP) agar plates using spread plate method and incubated at 37°C for 48h. Colony counts of *S. aureus* per mammary gland were then determined. The R5 mammary glands were gathered for histological analysis after the surrounding hair was aseptically shaved. After that, they were processed using an automatic tissue processor, fixed for 24h in 10% neutral buffered formalin, and then embedded in paraffin wax. Sections were cut at 4µm thicknesses and stained by the Haematoxylin and Eosin stain (H and E) (Bancroft and Gamble 2008).

Toxicological Effect of different SeNPs Concentrations in Rats

Animals and Experimental Design

In this investigation, 60 mature female albino rats (8–12 weeks old, 150–180 20g body weight) were employed. Five rats per plastic cage were used to house the rodents. Six groups of ten rats each were established: G1 to G6, G1 got 0.9% NaCl solution as the experiment's control group. The five extra groups, designated as G21 to G65, were used as the experimental groups. The rats received SeNPs for two weeks at doses of 0.5, 1, 2, 4, and 8mg/kg. The rats were examined behaviorally after two weeks, after that they were euthanized by cervical dislocation under ketamine-xylazine anesthesia according to a previous study (Elbehiry et al. 2018).

Behavioral Assessment

Anxiety-based tests were adapted to evaluate the intactness of the brain using open field test and Dark light activity box according to previous research (Hamdan et al. 2020; Khalil et al. 2020).

Body Weight and Relative Organ Weight Percentage

Body weights of rats were measured weekly and body weight change was calculated as follows (Final body weight (g) – initial body weight (g)). After euthanasia, vital organs, including the brain, liver, kidney, and heart were gently removed and weighed using a sensitive balance then the following equation was adopted to calculate the relative organ weight percentage (organ weight (g)/ Final body weight of the rats (g) *100).

Antioxidant Assessments

According to Koracevic et al. (2001), the total antioxidant status of rat serum was determined spectrophotometrically with a commercial kit (Biodiagnostic, Egypt). For the manufacture of 10% stock hemolysate, a portion of the RBC pellet was diluted in cold distilled water at a ratio of 1:10, which was then used to estimate the activities of SOD (Madesh and Balasubramanian 1998), CAT (Bergmayer 1983), and total antioxidant (Placer et al. 1966).

Histopathological Examination

The tissue organs were separated, cleaned with 0.9% sodium chloride, weighed, and examined for morphology. In order to preserve them for histological analysis, they were first stabilized in 10% buffered formalin. Prior to creating paraffin blocks, all tissue samples were processed using a tissue processing apparatus (Sakura Tissue Tek VIP E300). In order to conduct a histological analysis, 5µm sections were cut after paraffin embedding and stained with H and E.

RESULTS

Characterization of Prepared Nanoparticles

The development of Nano selenium structures prepared by green method was confirmed by the acquired XRD patterns in Fig. 1. The X-ray diffractogram's peaks were all indexed in accordance with the literature (Joint Committee on Powder Diffraction Standards, No. 00-042-1425). The X-ray diffraction patterns of chemically produced SeNPs are shown in Fig. 2. The standard JCPDS data (JCPDS card No. 01-086-2246) and all of the diffraction peaks in the 2 range are in excellent agreement. The preparation samples appear to be well crystallized based on the sharpness of the diffraction peaks.

HRTEM of the SeNPs prepared by green and chemical methods is shown in Fig. 3 and 4, respectively. The micrographs (Fig. 3) present SeNPs of hexagonal shape with a wide particle size distribution from 60 to 200nm. Moreover, Fig. 4 confirms that SeNPs, with an average diameter of 67nm, have a spherical shape.

***In vitro* Assessment**

Antibacterial Activity, MIC and MBC of Green Synthesized SeNPs against *S. aureus*

The average diameters for zones of inhibition (in mm) for green synthesized SeNPs (Fig. 5) showed strong antibacterial activity against *S. aureus* with a zone of inhibition ranging from 19 to 10mm at concentrations between 600 to 10µg/mL. Conversely, chemically synthesized SeNPs were unable to stop *S. aureus* growth at any of the tested concentrations (600, 500, 400, 300, 200, and 100 µg/ml). The MIC and MBC values were detected as 10 and 25 µg/ml respectively (Table 1).

Table 1: MIC and MBC of green synthesized SeNPs against *S. aureus* isolated from milk of cows with clinical mastitis.

S. aureus	MIC	MBC
Green synthezed SeNPs	10µg/mL	25µg/mL
Chemical synthezed SeNPs	----------	----------

Se-NPs=Selenium nanoparticles, MIC=Minimum inhibitory concentration, MBC=Minimum bactericidal concentration, *S. aureus =Staphylococcus aureus.*

Time Killing Kinetics

Results showed a reduction in the bacterial growth pattern in the presence of SeNPs formulated by the green method compared with control one after 1 hour and showed a gradual decrease in the number of CFU after the second and third hour till nearly complete inhibition of growth after 4h.

***In vivo* Assessment for the Antibiofilm Activity of Green Synthesized SeNPs**

Macroscopic and Bacteriological Examination for Mammary Gland

Rat's mammary glands exhibited a little bit of redness and swelling, but no discharge. Neither morbidity nor death was noted throughout the experiment. Total colony counts used to measure bacterial load revealed that no bacteria existed in concentrations of 500 to 25µg /ml, whereas the control group revealed 284x104CFU.

Histological Findings of the Mammary Gland

Fig. 6 showing the main lactiferous duct of mammary gland of rats of the following groups: (a, b) normal control rats showing normal duct (a) lined by cuboidal to columnar epithelium (b), (c, d) *S. aureus* infected rats showing necrosis of the epithelial lining (c) with massive infiltration of neutrophils (d), (e, f) G3 (25µg/m) treated rats showing normal epithelial lining (e) with no inflammatory cellular infiltrates (f), (g, h) G4 (100µg/mL) treated rats showing normal epithelial lining (g) without inflammatory cellular infiltrates (h), (I, j) G5 (300µg/mL) treated rats showing normal active epithelial lining, (k, l) G6 (500µg/mL) treated rats showing normal histological structure (Stain:H&E, 20X for a, c, e, g, I, k and 40X for b, d, f, h, j, l).

Toxicological Study

The Survival Rate, Body Weight and Organ Body Weight of Female Rats during the Experiment

Results showed that from a total number of 10 rats in each group,100% of rats received 0.5mg/kg SeNPs survived, 8 rats survived in the 1mg/kg treated group,7 rats survived in the 2mg/kg treated group, 5 rats survived in 4mg/kg treated group, and 2 rats survived in 8mg/kg treated group (Fig. 7). The final body weight after the second week of the experiment for groups receiving 0.5, 1, 4, and 8mg/kg body weight was nearly the same as the initial weight except for the group that received 2mg/kg body weight showed a slight reduction in body weight (Fig. 8).

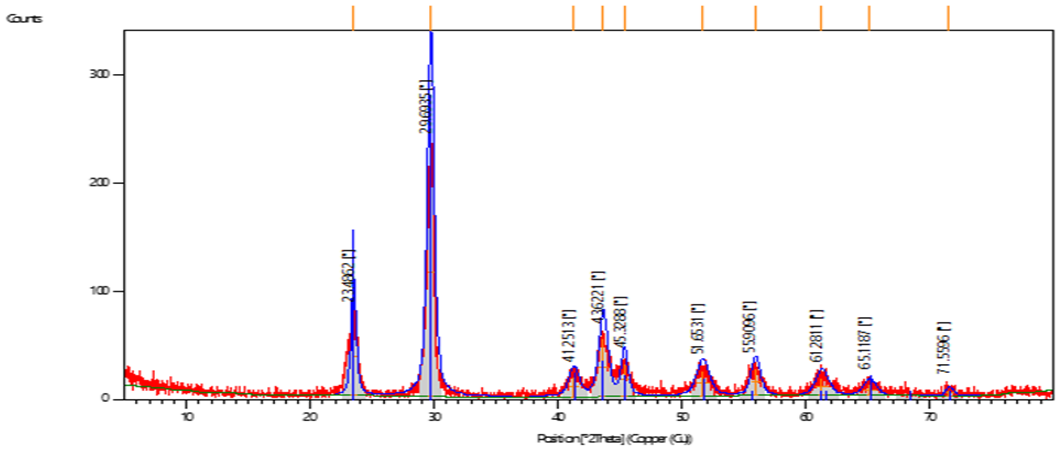

Fig. 1: X-ray diffraction patterns of selenium nanoparticles prepared by green method.

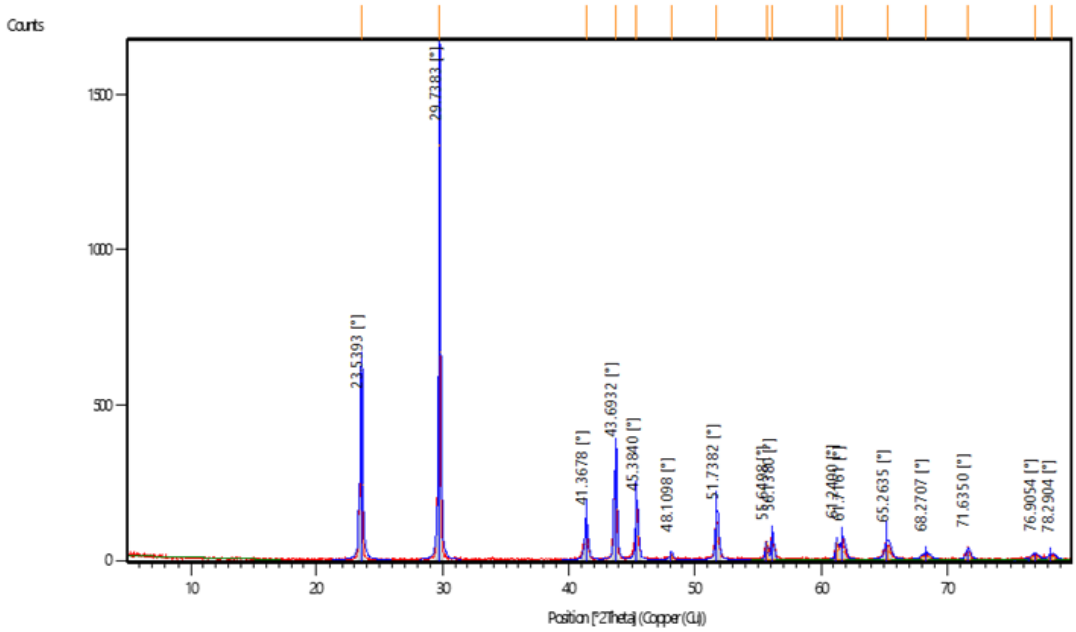

Fig. 2: X-ray diffraction patterns of selenium nanoparticles prepared by chemical method.

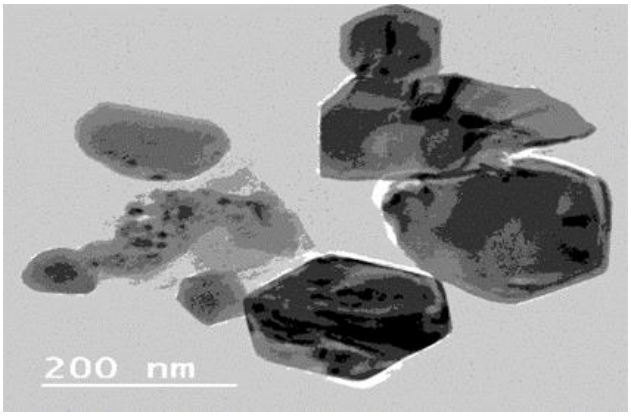

Fig. 3: High resolution transmission electron microscope of green synthesized selenium nanoparticles.

Results showed that relative brain, heart, and kidney weight showed a significant increase in all administrated doses of SeNPs as well as that observed for relative liver weight except for the group that received 2mg/kg BW showed a slight decrease in weight (Fig. 9).

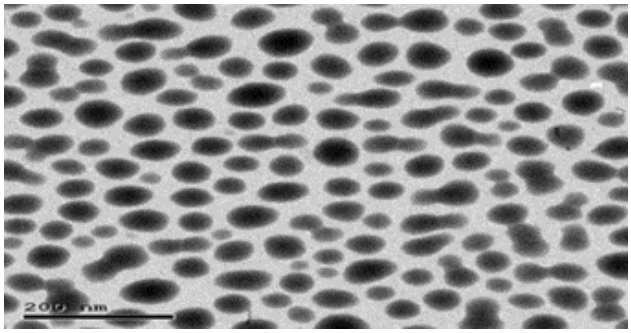

Fig. 4: High resolution transmission electron microscope of chemically synthesized selenium nanoparticles.

Behavioral Measures

As displayed in Fig. 8 (a, b), administration of SeNPs at a dose of 2, 4, and 8mg/kg significantly decreased the number of crossing squares in a dose dependent manner with the highest reduction in the number of crossing at the highest deses. Similarly, the rearing frequency markedly decreased in the high doses of SeNPs treated rats (4 and 8mg/kg). Concerning the light activity box, administration

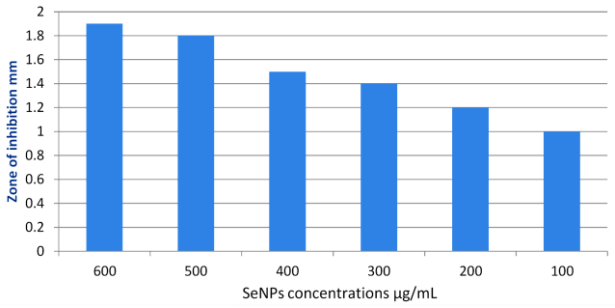

Fig. 5: The size of the zones of inhibition of green synthesized selenium nanoparticles (SeNPs) against *S. aureus.*

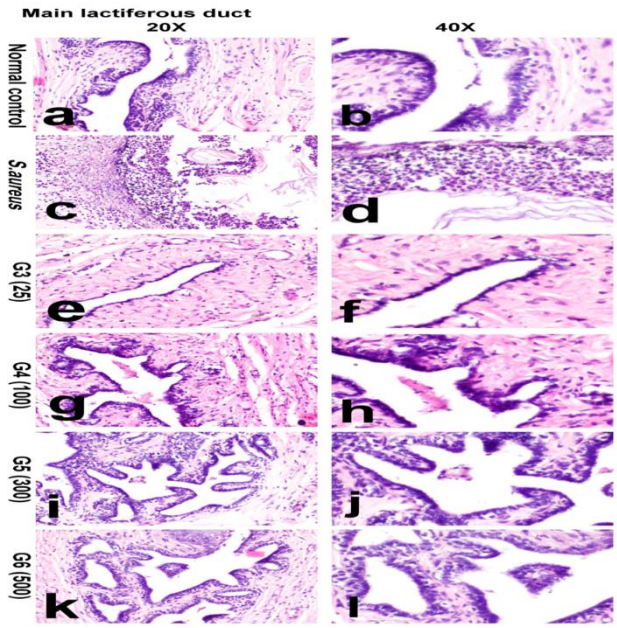

Fig. 6: Histological findings of mammary gland.

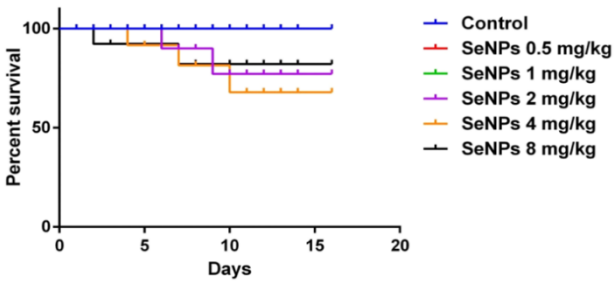

Fig. 7: Survival curve of mal rat treated with different concentrations of green synthesized selenium nanoparticle.

of SeNPs at 8mg/kg significantly reduced the frequency of light chamber entries associated with statistically non-significant reduction in the dark chamber's entries. However, rats treated with SeNPs at a dose of 4 and 8mg/kg displayed a marked decrease in the duration inside the light chamber associated with an increase in the dark chamber duration at all doses of SeNPs compared to control rats (Fig. 8 c-f).

Chemical Biomarkers

SeNPs administered at a dose of 0.5mg Se/kg and 4mg Se/kg BW increased the levels of TAC and SOD and decreased CAT, Groups administrated SeNPs at a dose of 1, 2, and 8mg Se/kg showed increase in SOD and decrease in CAT and TAC, with the exception of dose 8mg se/kg, where TAC remains unchanged. (Table 2).

Table 2: Serum antioxidant capacity in Rat

Group number	Superoxide dismutase (µ/mL)	Catalase (µ/L)	Total antioxidant (mM/L)
Control group	5.91	354.45	0.64
0.5mg/kg Treated group	27.91	319.62	0.79
1mg/kg Treated group	31.85	338.02	0.53
2mg/kg Treated group	30.99	220.92	0.54
4mg/kg Treated group	26.20	246.68	0.71
8mg/kg Treated group	45.55	272.18	0.64

Histopathological Investigation

Normal hepatic parenchyma with normal hepatocytes was demonstrated in normal control negative group (Fig. 10a). Similarly, normal hepatocytes with scant leucocytes in the sinusoids were demonstrated in the liver of G2 (0.5mg/kg) treated group (Fig. 10b). On the other hand, sparse apoptosis and activation of kupffer cells were demonstrated in G3 (1mg/kg) treated group (Fig. 10c). Normal histological structure with normal hepatocytes was demonstrated in G4 (2mg/kg) treated group (Fig. 10d). But mild cytoplasmic vacuolization of some hepatocytes with activation of kupffer cells were demonstrated in G5 (4mg/kg) treated group (Fig. 10e). Normal hepatocytes with mild activation of kupffer cells were demonstrated in G6 (8mg/kg) treated group (Fig. 10f).

DISCUSSION

Antibiotic misuse has contributed to the recent rise in multidrug-resistant microorganisms (Salam et al. 2023; Mwafy et al. 2023). Nanotechnology methods offer an alternative to antibiotics for the possible killing of mastitis bacteria, overcoming the limitations of conventional synthetic antimicrobial drugs (Dehkordi et al. 2011; Azam et al. 2023; Alorainy et al. 2023). The tested *S. aureus* isolates were shown to be very susceptible to the green synthesized SeNPs. The presence of an inhibition zone ranging from 19 to 10mm at concentrations ranging from 600 to 10 µg/ml indicated the potent antibacterial effect of the green synthesized SeNPs as presented in Fig. 5 which is similar to that reported by **(**Alagesan and Venugopal 2019) who observed a zone of inhibition (19.66mm) against *S. aureus* and stated that the application of green manufacturing metal nanoparticles will be important in the medical field. This result could be attributed to the bacterial wall composition, including abundant pores and a thin layer of peptidoglycan that allow the interaction between SeNPs and bacterial cells. In contrast, SeNPs prepared by the chemical method were unable to inhibit *S. aureus* growth at all the tested concentrations (600, 500, 400, 300, 200, and 100µg/mL) this result may be due to that the isolated *S. aureus* carries many virulence genes related to biofilm formation and *agr* group which make it resistant to the effect of SeNPs prepared by this method. Another study compared the antimicrobial activity of biogenic and chemically synthesized SeNPs and obtained significantly better results for the first one (Cremonini et al. 2016).

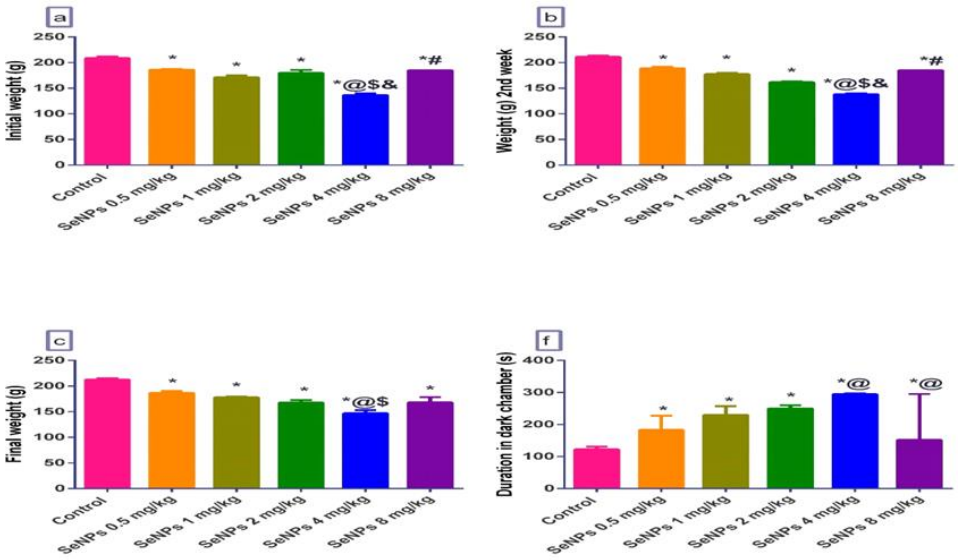

Fig. 8: Effect of administration of SeNPs on body weight.

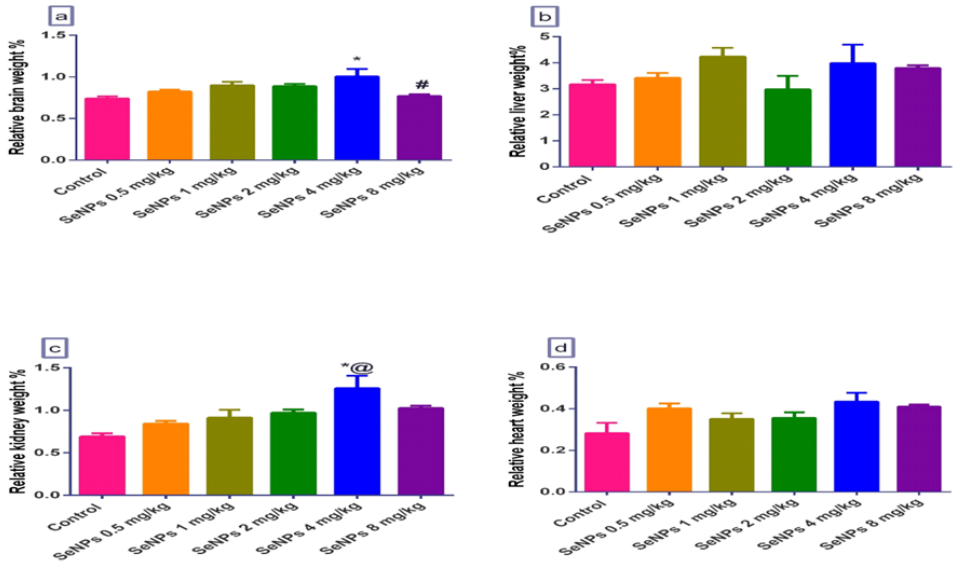

Fig. 9: Effect of administration of SeNPs on different organ weight.

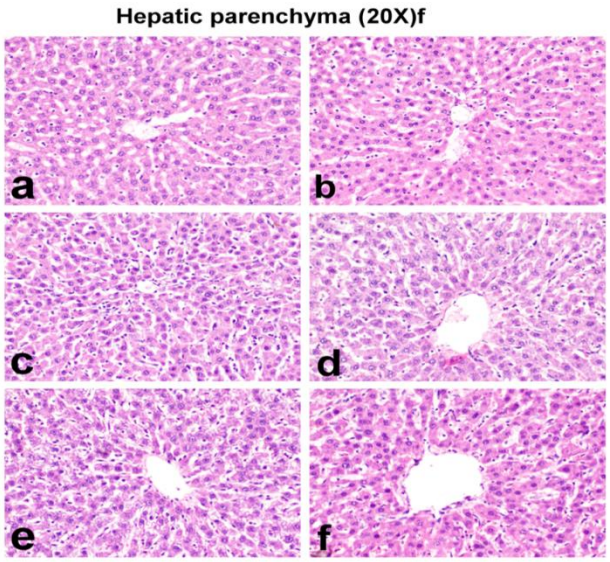

Fig. 10: Histopathological alterations in liver.

The MIC and MBC values were detected as 10 and 25µg/mL respectively as shown in Table 1. Other authors reported higher MIC 72µg/mL for SeNPs-Gluc (Filipović et al. 2021) conversely to our results. Recently, Rangrazi et al. (2020) tested the antimicrobial activity of chitosan-stabilized SeNPs with a diameter range of 50–105nm and they documented MICs of 137µg/mL against *S. Aureus*.

There is not much research showing how well NPs may kill bacteria, particularly *S. aureus*. According to several studies, SeNPs can be created and have biological effects on human cells *in vitro* (Zhang et al. 2004). Others have noted that Se compounds (used as coatings) inhibit the growth of specific types of bacteria (Tran et al. 2009). However, nothing is known about how SeNPs affect bacterial development. In this study, our results presented clearly the profile of bacterial growth in the presence of SeNPs. After one-hour, SeNPs formulated by the green method inhibited the bacterial growth profile in comparison to the control group, after the second and third hours, the number of CFUs gradually decreased until nearly

all growth was inhibited after four hours. This finding supports SeNPs' capacity to stop the onset of biofilm in the early stage after four hours. Additionally, it completely inhibited growth beginning with the first hour at high concentrations (600-500 µg/ml). In early time points (up to 5 hours), Tran and Webster (2011) demonstrated that the growth of *S. aureus* was greatly slowed down by the novel SeNPs created using the simple colloidal synthesis technique by up to 60 times as compared to no treatment. After 3, 4, and 5h, according to live/dead assays, the SeNPs appeared to have killed roughly 40% of *S. aureus*.

Assessment of SeNPs' antibacterial and antibiofilm effects in *in vivo* research shown that all tested concentrations of 500, 300, 100 and 25µg/mL considerably decreased the bacterial load in mammary gland analyzed homogenate Fig. 6. SeNPs lowers inflammation by reducing the production of inflammatory cytokines, as shown by several studies (Gao et al. 2016) modulating the immune response (Bi et al. 2016). In this study, we showed that SeNPs prevented mammary epithelial cells from becoming inactive and decreased inflammation caused by infection with *S. aureus* in the rat mastitis model.

In comparison to groups treated with SeNPs, which displayed normal lactiferous ducts with normal epithelial lining free of inflammatory cellular infiltrates, *S. aureus* infection resulted in mammary tissue damage, acinar structural organization disruption, infiltration of inflammatory cells, and necrosis of tissue cell. This outcome is consistent with research by (Sun et al. 2017), who showed that selenium and miR-146a jointly modulate the anti-inflammatory response in mammary epithelial cells of mouse mastitis model infected with *S. aureus*. They also hypothesized that Se reduces inflammation by up regulating the expression of miR-146a. Additionally, shows the therapeutic value of Se supplementation for lowering and preventing *S. aureus* mastitis.

Se is commonly brought up when discussing the role of the organism's health. The proper and acceptable form of Se is being discussed in light of the development of SeNPs. This potential choice needs to be thoroughly investigated in terms of toxicity. The emotional status of rats, antioxidant parameters, changes to the liver's histology have been evaluated in the present study. Throughout the trial, the weight of the test animals has been tracked. Se supplementation has often been linked to higher weight gain in mammals because it improves nutritional availability (Reed et al. 2007). In this study, the body weight largely remained unchanged except for those receiving SeNPs in dose 2mg/kgBW (Fig. 8). Similar results were reported by (Urbankova et al. 2021) who determined that there were no variations in the rat weight gain among the tested groups. A dissected liver's weight, heart, brain, kidney in relation to body weight was also examined. Many researchers recognized alterations in both liver and intestinal weight as a result of its damage as its role as a detoxifying organ (Strubelt et al. 1996). In our study, there was a significant increase in the tested four organs (Fig. 9).

In the current study, the emotional status of SeNPs exposed rats at different concentrations was investigated using open field test and dark light activity box. The open field test measures the tendency of rats to fear from open areas (Kraeuter et al. 2019) while dark light activity box depends on the natural fear of rats from light areas (Bourin and Hascoët 2003). Herein, administration of SeNPs at high doses (4 and 8 mg/kg) increase the anxious state of rats as portrayed by a reduction in the horizontal (number of crossing squares) and vertical activities (rearing activity) as well as a reduction in the light and dark chamber entries (Fig. 8). These findings were in agreement with a previous study (He et al. 2014) who demonstrated the toxic effects of SeNPs at doses higher than 4mg/kg on the reproductive organs of rats. On the other hand, administration of low doses of SeNPs ranging from 0.4 to 2mg/kg has a reported beneficial activity on different organs, including brain (Bashir et al. 2021; Shalaby et al. 2023) and reproductive organs (Hozyen et al. 2020).

These behavioral data were confirmed by measuring the serum oxidative stress. Administration of high doses of SeNPs have an impact on the TAC and levels of antioxidant enzymes. The findings showed an elevation in SOD and decrease in CAT across all administrated doses (Table. 2). In terms of TAC, there was an increase for groups that received 0.5 and 4mg/kg BW, a decrease for that received 1 mg/kg, 2 mg/kg, and a constant value for 8 mg/kg BW receiving groups. In contrast, the TAC and GPx concentrations in relation to the control and treatment groups have not differed noticeably from those reported by Eid et al. **(**2019). Our findings are supported by Wang et al. (2021) who found Se activated TAC and SOD in a manner dependent on dose at both 0.1 and 0.2mg Se/kg. In addition, Hamza and Diab (2020) discovered that the rats used in the experiment that received 5mg Se/kg had significantly less SOD activity. However, lower dosages of SeNPs (0.1mg Se/kg) significantly enhance SOD, which has a positive overall effect on the antioxidant system (Hozyen et al. 2020).

Histopathological examination of the liver showed normal histological structure with normal hepatocytes in all tested doses (Fig. 10), mild cytoplasmic vacuolization of some hepatocytes with activation of kupffer cells was demonstrated in G5 (4mg/kg) treated group (Fig. 10e), mild activation of kupffer cell in G6 (8mg/kg). Mild sinusoidal expansions were discovered, according to Deniz et al. (2021), when Se first-day groups were compared to the control group which is in line with our results. The spherical, central nuclei of hepatocytes were found to be present. Sinusoids showed little expansion on days 6 and 28. On days 1, 6, and 28, it was observed that the intensity of activated Kupffer cells was constant. Parallel to this, it was discovered that there were no substantial harmful effects, with the exception of slight sinusoidal expansions, in rat livers after light microscopy investigation. An investigation by Messarah et al. (2012) demonstrated that Se has no harmful effects on the Westar albino, according to research done on rats.

Conclusion

Our study found that green synthesized selenium nanoparticles (SeNPs) had a strong antibacterial effect against *Staphylococcus aureus* (*S. aureus*) biofilms in both *in vitro* and *in vivo* tests. *In vitro*, SeNPs completely inhibited the growth of *S. aureus* at the early stages of infection (after 4 hours). *In vivo*, SeNPs treatment in female rats resulted in significant improvement of the major lactiferous duct with normal histological structure, and

complete absence of bacterial colonies in all tested concentrations. SeNPs did not show significant toxicity in the mature female rat model. The highest doses of SeNPs only mildly vacuolated some hepatocytes with activation of Kupffer cells, and had no significant effect on the liver function. These results suggest that SeNP supplementation could be both safe and beneficial for the specific treatment of *S. aureus*.

Author's Contribution

Eman Shafeek designed the plan of work , performed *in vitro* and *in vivo* assessment for the antibacterial and antibiofilm activity of SeNPs, review and drafting the manuscript.Abeer Mostafa shared in design of experiment, shared *in vivo* and *in vitro* laboratory work, review and drafting the manuscript, Rasha Hamdy *invivo* assessment of antibacterial activity of SeNPs and time killing kinetics.Riham Hassan shared in Antioxidant assessments. Amany Ahmed performed antibacterial activity of SeNPs *in vitro*. Sohad Mohamed performed preparation of bacterial inculum and bacteriological examination of mammary gland. Heba Fawzy prepared nanoparticles and characterized it. Heba Mohamed performed toxological study and behavioral study. All authors read and approved the final manuscript.

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