Research Article

In vivo Antibacterial Activity of Biosynthesized Selenium Nanoparticles using a Zebrafish Model

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ABSTRACT

Background: *Staphylococcus aureus* is a leading cause of both healthcare-associated and community-acquired infections, contributing to an extensive array of illnesses, ranging from skin infections to life-threatening conditions like pneumonia and bloodstream infections. Despite extensive efforts, its resistance to multiple antibiotics poses a significant public health threat. This paper employed an experimental design to explore the potential of nanotechnology, specifically selenium nanoparticles, in combating *in vivo* antibacterial resistance of *Staphylococcus aureus* (*S. aureus*) infections using a zebrafish model. **Materials and Methods:** Selenium Nanoparticles (SeNPs) were synthesized from orange peel waste extract and sodium selenite. 93 zebrafish were infected with *S. aureus* via intramuscular injection and then exposed to various concentrations of SeNPs via medicated bath using Ciprofloxacin as positive control. Following the IACUC guidelines for euthanasia, fish muscle was cultured using MSA and then antimicrobial activity was noted in CFUs, or colony-forming units. **Results:** Results indicated significant antibacterial activity at all selenium nanoparticle concentrations, with 20 µg/mL showing the most pronounced effect, notably reducing colony counts compared to the negative control. Statistical analysis demonstrated differences between selenium nanoparticle concentrations, particularly with 15 µg/mL and 20 µg/mL having shown similar effectiveness. **Conclusion:** Overall, selenium nanoparticles exhibited a high level of antimicrobial activity against *S. aureus*, with an effectiveness rate of 95.9%.

Keywords: Antibacterial, Selenium Nanoparticles, Zebrafish.

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INTRODUCTION

Antimicrobial resistance of bacteria has become one of the significant threats faced in the healthcare setting today. One is the gram-positive opportunistic pathogen, *Staphylococcus aureus* (*S. aureus*), which is found to be a substantial causative agent of healthcare-associated and community-acquired infections.[1] Despite steady research to improve the effect of antibacterial agents against *S. aureus*, the bacteria leaves a constricted number of therapeutic options against the increasing infections as it continues to demonstrate resistance against multiple forms of antibiotics, including vancomycin, penicillin and methicillin.[2]

Medicine and technology have combined different methods to counter significant threats in human healthcare provision, leading to the discovery of nanotechnology, which has molded the contemporary world to be more prosperous and productive. Nanotechnology shows potential in being a solution to drug resistance exhibited by bacteria, which is regarded as an emergent threat to public health worldwide. Selenium Nanoparticles (SeNPs), in particular, exhibit function as an antioxidant, potential chemo-preventive agent, anti-inflammatory and anti-diabetic.[3-5]

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More importantly, SeNPs attracted attention in the medical field through findings about their anticancer and antimicrobial activity, with their minuscule size enabling them to gain easier access and increased contact with bacterial cells, resulting in a more potent response.[6]

Meanwhile, animal models have been employed to investigate the pathogenesis of various conditions and to assess the potential medicinal applications of different substances. Zebrafish (*Danio rerio*) has been recognized in biomedical studies as an ideal animal model due to its optical transparency, low maintenance expenses, fully sequenced genome and susceptibility to bacterial infection.[7,8] Thus, this paper will focus on the *in vivo* antibacterial activity of different concentrations of biosynthesized selenium nanoparticles against the infection and growth of *Staphylococcus aureus* using a Zebrafish model.

MATERIALS AND METHODS

Preparation of Biosynthesized Selenium Nanoparticles (SeNPs)

Selenium nanoparticles were synthesized using orange peels bought from the Trabajo public market located at J. Marzan St., Sampaloc, Manila. First, the orange peels were washed with distilled water twice. Then, the peels were cut into smaller pieces (~1 cm) and weighed at precisely 200 g. The peels were then blended until only small particles were visible and the water turned into a yellow-orange color. Consequently, the combination was strained using Whatman No. 1 filter paper and stored in a sterile bottle at 4ºC until use. One liter (1000 mL) of the prepared orange peel waste extract was used for the biosynthesis of selenium nanoparticles by adding it to 100 mL of 100 mM Sodium Selenite (Na_2SeO_3) purchased from 186 Seohaean-ro, Siheung-si, Gyeonggi-do, Korea's Daejung Chemicals.[9] This concoction was heated at 40ºC with a magnetic stirrer for at least 5 hr or until the solution achieved a dark yellow-brown hue.[10] The Se-NPs were separated and purified via washing with distilled water and centrifugation technique.[9]

Characterization of Biosynthesized Selenium Nanoparticles

An aliquot of biosynthesized SeNPs was submitted to the Department of Science and Technology (DOST) for characterization via Scanning Electron Microscopy (SEM). The results of SEM produced various sizes of NP ranging from 114.7 nm (s) to 227.1 nm (s), as can be pictured in Figure 1.

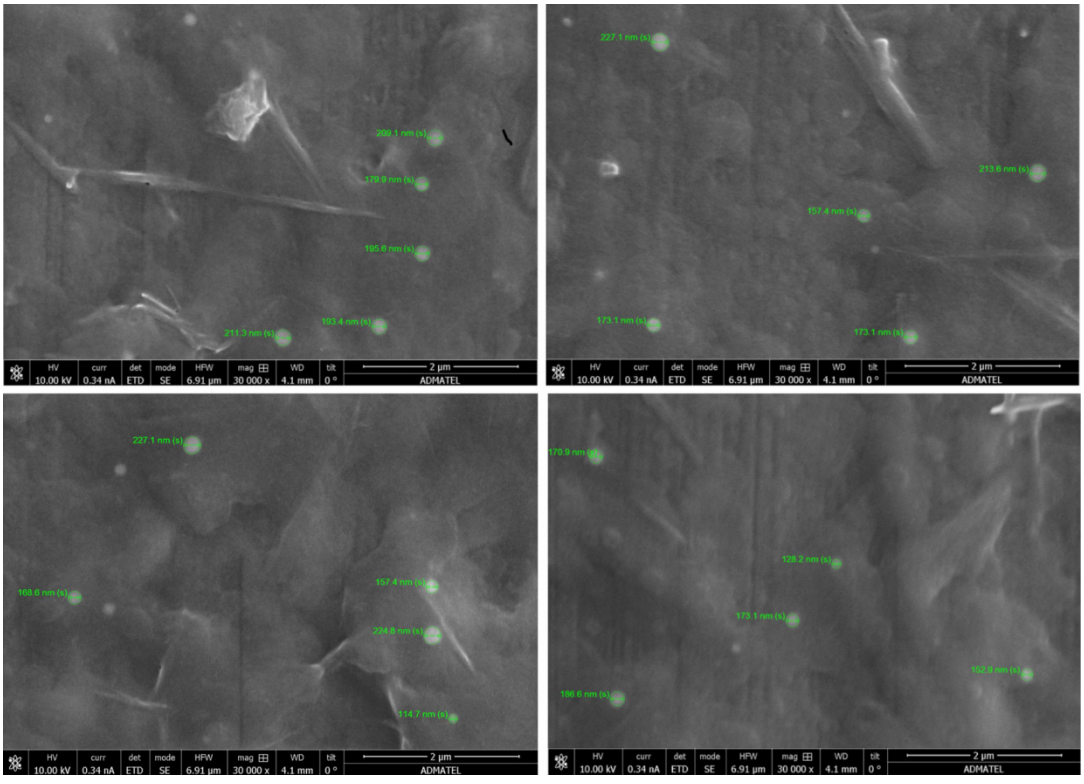

Figure 1: SEM images with particle size measurements.

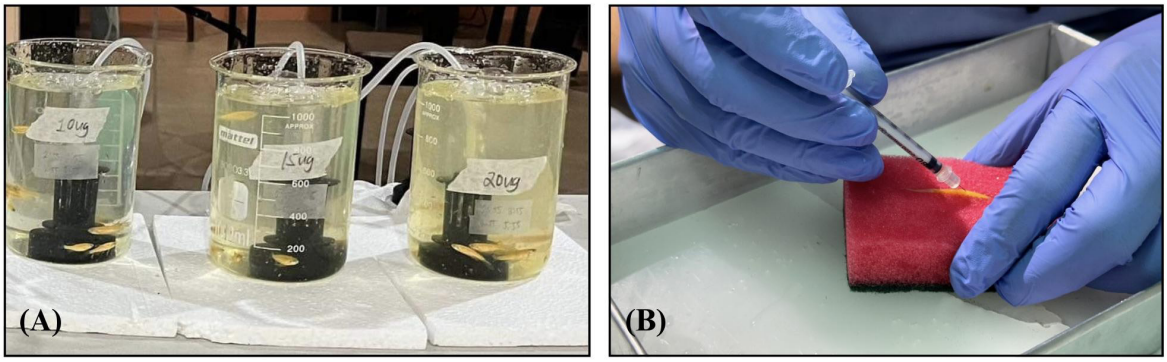

Figure 2: (A) Preparation of medicated baths containing 5 µg/mL, 10 µg/mL, 15 µg/mL and 20µg/mL concentrations of SeNP. Figure 2. (B) The zebrafish was infected via the intramuscular injection method.

Preparation of Positive Control and Various Concentrations of SeNP Medicated Baths

As seen in Figure 2A, the preparation of medicated baths containing various concentrations of SeNP is as follows: To prepare the various concentrations of biosynthesized SeNP medicated baths, predetermined volumes of SeNP suspension were dissolved in distilled water. To prepare the 5 µg/mL concentration of medicated bath, 5 mL of SeNP suspension was incorporated into 1 L of distilled water. Accordingly, 10 mL of suspension was transferred to 1 L of distilled water to create a 10 µg/mL bath. Then, 15 mL of SeNP suspension was combined with 1 L of distilled water to produce a concentration of 15 µg/mL. Finally, 20 mL of biosynthesized SeNPs was diluted with 1 L of distilled water to create a medicated bath of 20 µg/mL. For the positive control, 0.02 g of a pulverized Ciprofloxacin tablet was dissolved in 1 L of distilled water to yield a concentration of 20 µg/mL.

Preparation of Mannitol Salt Agar Media

Mannitol Salt Agar was prepared by dissolving 111 g of MSA powder in water, heating until fully dissolved, sterilizing in an autoclave at 15 psi at 121ºC for 15 min and cooling.[11]

Zebrafish Maintenance

The study used adult zebrafish from A and R Aquarium Fish in Cavite, Philippines. An aerated glass tank was utilized as a temporary habitat for the ninety-three fish and the water was regularly cleaned and sterilized. The water parameters were pH-7.45 and 25±2ºC temperature.[12] The fish were fed as desired with a commercial fish diet and allowed to acclimate a week before being treated with SeNPs medicated bath.[12]

Preparation of *Staphylococcus aureus* Suspension

At least 3-5 well-isolated colonies of *Staphylococcus aureus* ATCC 25923 were selected from a Blood Agar Plate (BAP) that was incubated for at least 18 hr. The colony surfaces were gently tapped using a sterile inoculating loop and the resulting growth was then transferred to a tube with 2 mL of Tryptic Soy Broth (TSB). The turbidity of the broth culture was modified with TSB in order to achieve an optical density similar to that of the 0.5 McFarland standards. The VITEK DENSICHEK was used to perform this step.[13]

Zebrafish Infection

As seen in Figure 2B, the zebrafish was infected via the intramuscular injection method.[14] A BD Ultra Fine insulin syringe (0.3 mL; 31-gauge; 6 mm) was utilized to infect ninety-three adult zebrafish with *Staphylococcus aureus* bacterial suspension. The fish were settled in the slit of a damp sponge in a prostate condition and then the bevel was inserted anterior to the dorsal fin at a 45º angle, delivering 10 uL of the bacterial suspension. Three fish were euthanized via rapid chilling at 4ºC to extract muscle tissue to confirm the infection.[15] Then, sterilized tubes were filled with 1 mL of sterile PBS, where the muscle tissue was added. Then, the tube was vigorously agitated for 1 min using a vortex mixer until homogenization was achieved.[16] After the procedure, 100-200 µL of each suspension were plated on Mannitol Salt Agar (MSA) and incubated for 18-24 hr at 37ºC. Colony-Forming Unit (CFU) counting was then performed to confirm the *S. aureus* infection.

Zebrafish Treatment with Biosynthesized Selenium Nanoparticles

The antimicrobial activity of biosynthesized selenium nanoparticles was determined by treating *Staphylococcus aureus*-infected zebrafish with SeNPs.[12] The *Staphylococcus aureus*-infected zebrafish were divided into three groups: the treatment group, the positive control and the negative control. The treatment group was exposed to a medicated bath containing different concentrations of SeNPs; the positive control group received 20 µg/mL Ciprofloxacin, while the negative control group was left

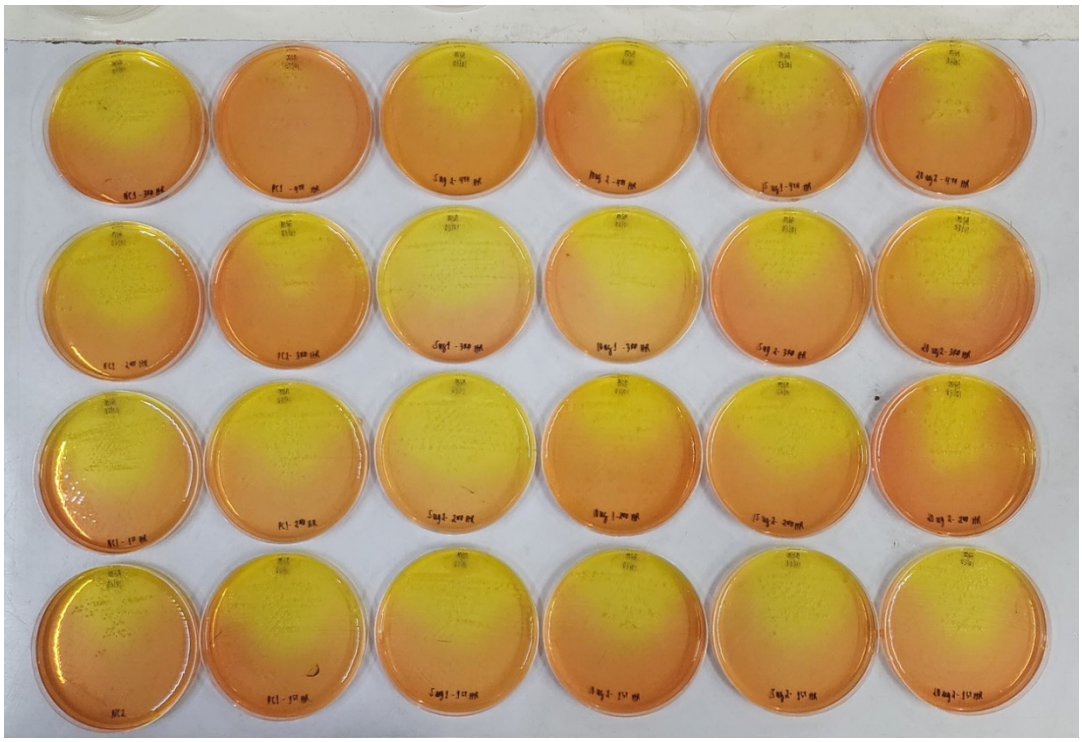

Figure 3: Colony count of *Staphylococcus aureus* infection from extracted zebrafish muscle.

untreated. The experiments were conducted in triplicate. After infection and treatment, the bacterial load was determined through euthanasia, homogenization and CFU counting repeatedly for each group after 1, 2, 3 and 4 hr. The total colony count forming units in MSA culture plates, as seen in Figure 3, were then recorded and collated by multiplying the number of colonies in each group by 100 as a 0.01 mL calibrated loop was used. All groups of fish were also observed for mortality in the succeeding 48 hr.

Statistical Analysis

The study used a zebrafish model to analyze the antibacterial activity of SeNPs against *Staphylococcus aureus*. The mean ($\bar{x}$) was used to calculate the mean antibacterial activity for each concentration, while the standard deviation (σ) was used to calculate the amount of variation from the mean. Furthermore, significant differences across variables were identified using SPSS and one-way Analysis of Variance (ANOVA). The F-statistic formula was used to quantify differences among means and an F-distribution table was used to assess data. A significance value of 0.05 (5%) was used to control values and obtain valid data.

Ethical Consideration

The researchers obtained authorization to conduct the scientific procedures on the zebrafish model from the Bureau of Animal Industry (BAI), with BAI-Registration No. LAF-0007 and reference no. AR-2024-0094, following the provisions of Republic Act 8485 as amended by Republic Act 10631 and DA-Administrative Order (AO) No. 40, series of 1999.

RESULTS

The mean CFUs of the control and treatment groups are presented in Figure 4. When described against the negative control, all concentrations of Selenium Nanoparticles (SeNPs) showed *in vivo* antibacterial activity against *Staphylococcus aureus* (*S. aureus*) using a zebrafish model. Within the 1st hr post-treatment, 20 µg/mL of SeNPs elicited the most antibacterial activity with 254.33 CFU/mL, as compared to the Negative Control (NC) of 372.67 CFU/mL. For the 2nd hr, the steepest drop in CFU was similarly observed at a concentration of 20 µg/mL SeNPs, with 197.33 CFU/mL versus 450.67 CFU/mL of the negative control. The same concentration (20 µg/mL SeNPs) likewise exhibited the most inhibition contrary to the negative control at the 3rd and 4th hr, with 144.33 CFU/mL (525.33 CFU/mL NC) and 138.00 CFU/mL (149.33 CFU/mL NC), respectively.

Meanwhile, the optimum concentrations of SeNPs that exhibited *in vivo* antibacterial activity against *S. aureus* were visualized in Figure 4. Under the 1st hr,

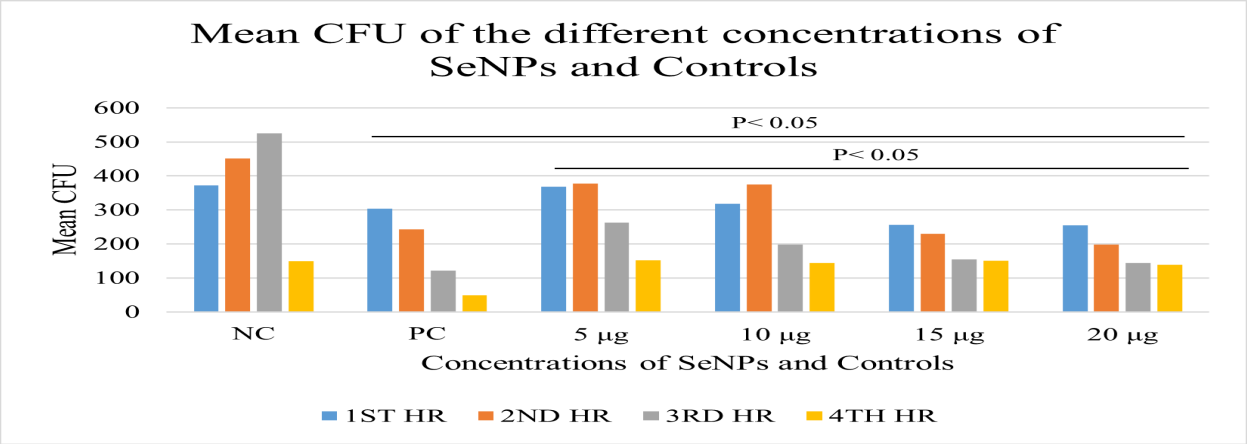

Figure 4: Mean CFU of the different concentrations of SeNPs and Controls.

the optimal concentration of SeNPs was 10 µg/mL with a colony count of 318.33 CFU/mL, the closest to the positive control value of 303.00 CFU/mL. At the 2nd hr, 15 µg/mL with 229.67 CFU/mL showed the most similar antibacterial activity with the Positive Control (PC) of 242.67 CFU/mL. The concentration of 20 µg/mL SeNPs then generated a colony count of 144.33 CFU/mL as opposed to a PC of 122 CFU/mL at the 3rd hr. Finally, at the 4th hr, 20 µg/mL SeNPs produced 138 CFU/mL, while the PC produced 49 CFU/mL.

Moreover, the different concentrations of SeNPs against *S. aureus* using a zebrafish model were also showcased in Figure 4. The findings of the analysis showed that the *p*-value is 0.000 (*p<0.05*), which indicated that there was a significant difference in the *in vivo* antibacterial activity of the varying concentrations of SeNPs against *S. aureus*. Following this, post-hoc analysis using Tuckey revealed that 15 µg/mL and 20 µg/mL SeNPs had the same effectivity as an *in vivo* antibacterial agent against *S. aureus*. The rest of the concentrations, on the other hand, exhibited a significant difference in inhibiting the *in vivo* growth of *S. aureus*. Figure 4 also revealed that all concentrations of selenium nanoparticles showed 95.9% (*r2=0.959*) *in vivo* antibacterial activity against *S. aureus*.

Moreover, Figure 4 displays the different concentrations of SeNPs against the control variable. According to this Figure 4, there was a significant difference between the different concentrations of selenium nanoparticles and the positive control, as shown by a p-value of 0.000 *(p<0.05)*. The *in vivo* antibacterial activity of biosynthesized selenium nanoparticles was further accounted for by an r^2=0.977, which signified a 97.7% effectiveness against *S. aureus* when compared to the control. Post-hoc analysis using LSD also relayed that while there was a notable distinction between the treatment groups of selenium nanoparticles and the positive control, the same effect was produced at the 1st and 2nd hr with *p=0.988 (p>0.05)*.

DISCUSSION

Staphylococcus aureus is a common clinical isolate that causes a myriad of clinical manifestations, from mild boils and impetigo to potentially fatal bacteremia, sepsis and lung infection.[16-18] As a consequence of the rise in antimicrobial resistance, various biomedical studies have focused on exploring nanoparticles, which possess antiviral, anticancer and antibacterial properties proven through the use of *in vitro* and *in vivo* protocols. [5,12]

In vitro research found that Selenium Nanoparticles (SeNPs) synthesized from orange peel waste effectively inhibited the growth of *S. aureus* even at low concentrations such as 10 µg/mL and 20 µg/mL and that SeNPs synthesized from potato extract exhibited notable stability and antibacterial properties when used within the concentration range of 10 to 20 µg/mL.[19,20] Furthermore, investigations on Silver Nanoparticles (AgNPs) discovered that antibacterial activity relied on both time and dose, while analyses of biogenic Silver Nanoparticles (bAgNPs) indicated that such nanostructures expressed dose-dependent antibacterial activity against pathogenic and nonpathogenic isolates at specific time intervals.[21,22]

Correspondingly, the current study revealed similar results, such that all four concentrations of selenium nanoparticles showed *in vivo* antibacterial activity against *S. aureus*. However, 20 µg/mL in particular, produced the most notable decline in bacterial growth within 1, 2, 3 and 4 hr post-treatment. Additionally, while

the optimal concentrations of SeNPs varied in the 1st and 2nd hr, the concentration of 20 µg/mL SeNPs possessed the closest activity with the positive control (Ciprofloxacin) and exhibited an ideal antibacterial effect against *S. aureus* in the 3rd and 4th hr. The survival of the un-euthanized pre-treated zebrafish was also achieved post-experimentation. The summation of these outcomes suggests that *in vivo* inhibition of bacteria is concentration dependent. Thus, the potency of biosynthesized SeNPs against *S. aureus* may be evaluated as a function of time in subsequent investigations, with longer time intervals being adopted to examine the relationship between antibacterial activity and length of exposure.

Nanoparticles of various sizes and shapes interact with various biological macromolecules, including DNA, lipids, proteins, flavonoids and polysaccharides, to form the bio-nano interface.[23] When nanoparticles with photocatalytic activity interact with biological entities, they are capable of generating Reactive Oxygen Species (ROS) that result in the death of cells.[24] This particular process was shown to be among the key mechanisms by which SeNPs generated antibacterial activity against Antibiotic-Resistant Strains (MRSA), as ascending concentrations of SeNPs equated to descending colony counts.[25]

Related studies have also reported that higher SeNP concentrations inhibited and decreased the *in vitro* viability of both gram-negative and gram-positive bacteria.[23,24] Specifically, *B. subtilis* viability dropped from 70% to 30% when exposed to 25 µg/mL to 250 µg/mL of SeNPs. For *E. coli,* viability decreased from 60% to 10% at the same concentrations. [23,24]

In parallel, this study established that 15 µg/mL SeNPs and 20 µg/mL SeNPs possessed the same effectivity *in vivo* against *S. aureus* and the concentrations lower than this (5 µg/mL and 10 µg/mL) displayed noticeable differences. Nevertheless, while this is the case, all concentrations of selenium nanoparticles still elicited a marked 95.9% antibacterial activity against *S. aureus.*

Compared to compounds containing Se, SeNPs have distinct properties, such as selective cytotoxicity, which kills cancer cells without harming healthy cells. Another mechanism by which nanoparticles inhibit bacterial growth is biomolecule degradation. The antimicrobial activity of AgNPs was found to occur intracellularly, where cell death was induced due to the damage sustained by bacterial biomolecules.[26] On a related note, the antimicrobial action of SeNPs also exhibited DNA damage, protein degradation and cell destruction.[26]

Specific cellular processes including those previously mentioned have been further examined using *in vivo* paradigms. A particular study employing a Golden Syrian hamster model explored the utilization of various concentrations of selenium and discovered that macrophage activation, phagocytosis and bacterial killing displayed dosage reliance.[27] This likewise illustrates that different concentrations of selenium nanoparticles significantly affect the antibacterial activity against *S. aureus*. Meanwhile, other *in vivo* research implemented on zebrafish have shown important phagocytic activity on foreign pathogens, increased lysosomal activity by 3-fold and significantly higher intracellular and extracellular activity response after SeNP stimulation. This attested that the treatment was able to trigger *in vivo* responses to bacterial pathogens like *S. aureus* due to the immunostimulant effect of SeNPs.[28] Notably, the results of the present study closely align with those regarding rodent or murine subjects, signifying that zebrafish can serve as a comparable animal model.[27]

Ciprofloxacin, a fluoroquinolone antibiotic, demonstrates significant potential in combating bacterial infections through its specific targeting of bacterial DNA gyrase and topoisomerase IV. These enzymes are crucial for DNA replication and cell division in bacteria. By inhibiting their function, ciprofloxacin causes disruption of bacterial DNA, resulting in fragmentation and the eventual prevention of cell division and growth. This mechanism is comparable to the action of SeNPs, which create oxidative stress that consequently disturb the cell membrane of bacteria and lead to the death of the entire bacterial cell.[29,30] As such, the high efficacy of biosynthesized SeNPs as an antibacterial agent is elucidated.

This study discovered a notable distinction among the treatment groups and Ciprofloxacin (positive control) and that biosynthesized SeNPs presented 97.7% of *in vivo* antibacterial activity against *S. aureus.* The parallel action of ciprofloxacin and SeNPs, through different pathways, highlights diverse and effective ways to combat bacterial pathogens. In sum, the aforementioned biological activities contribute to the vast arsenal of antibacterial properties possessed by various nanoparticles. These modes of action thereby scaffold the collective and concentration-dependent antibacterial activity demonstrated by the biosynthesized SeNPs discussed in this paper.

The primary focus of this research was to assess the antibacterial activity of biosynthesized Selenium Nanoparticles (SeNPs) through the exploration of an *in vivo* process using a zebrafish model. With that, it must be noted that *in vivo* procedures may introduce certain limitations that affect their reproducibility. Since the experimentation requires live subjects, ethical clearance

must be attained, which entails a rigorous review process and additional documentation. Furthermore, methods in this study such as intramuscular injection and muscle dissection rely on the skills of an expert, which may be subject to human errors. The use of an *in vivo* design, however, allowed for the application and confirmation of findings from previous research conducted *in vitro,* thereby increasing the validity of consequently generated results. Thus, the data reported in this study could be utilized for pharmacological applications and in-depth inspections regarding the effectivity of biosynthesized selenium nanoparticles against more potent pathogens.

CONCLUSION

The *in vivo* antibacterial activity of biosynthesized selenium nanoparticles, as demonstrated in this study using a Zebrafish model, exhibits their promising potential as effective antimicrobial agents. The significant reduction in bacterial load observed post-treatment shows the efficacy of these nanoparticles in combating bacterial infections. This study contributes to the growing body of evidence supporting the utility of nanotechnology in diagnostic medicine and suggests a novel approach for fighting antibiotic-resistant bacteria. Given the findings of this study, future research endeavors may explore a more extensive inspection of the antibacterial activity of SeNPs using different animal models targeting other biological pathogens, with emphasis on the duration of exposure to ascertain their optimum antibacterial activity and potential for toxicity.

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FUNDING

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

***S. aureus*:** *Staphylococcus aureus*; **SeNPs:** Selenium nanoparticles; **CFU:** Colony forming unit; **IACUC:** Institutional Animal Care and Use Committee; **MSA:** Mannitol salt agar; **DOST:** Department of Science and Technology; **SEM:** Scanning electron microscopy; **NP:** Nanoparticles; **BAP:** Blood Agar Plate; **TSB:** Tryptic soy broth; **PBS:** Phosphate buffered saline; **ANOVA:** Analysis of variance; **SPSS:** Statistical software suite; **BAI:** Bureau of Animal Industry; **AO:** Administrative Order; **DA:** Department of Agriculture; **HR:** Hour; **NC:** Negative control; **PC:** Positive control; **LSD:** Least significant difference; **AgNPs:** Silver nanoparticles; **bAgNPs:** Biogenic silver nanoparticles; ***B. subtilis*:** *Bacillus subtilis;* **DNA:** Deoxyribonucleic acid; **ROS:** Reactive oxygen species; **MRSA:** Methicillin-resistant *Staphylococcus aureus.*

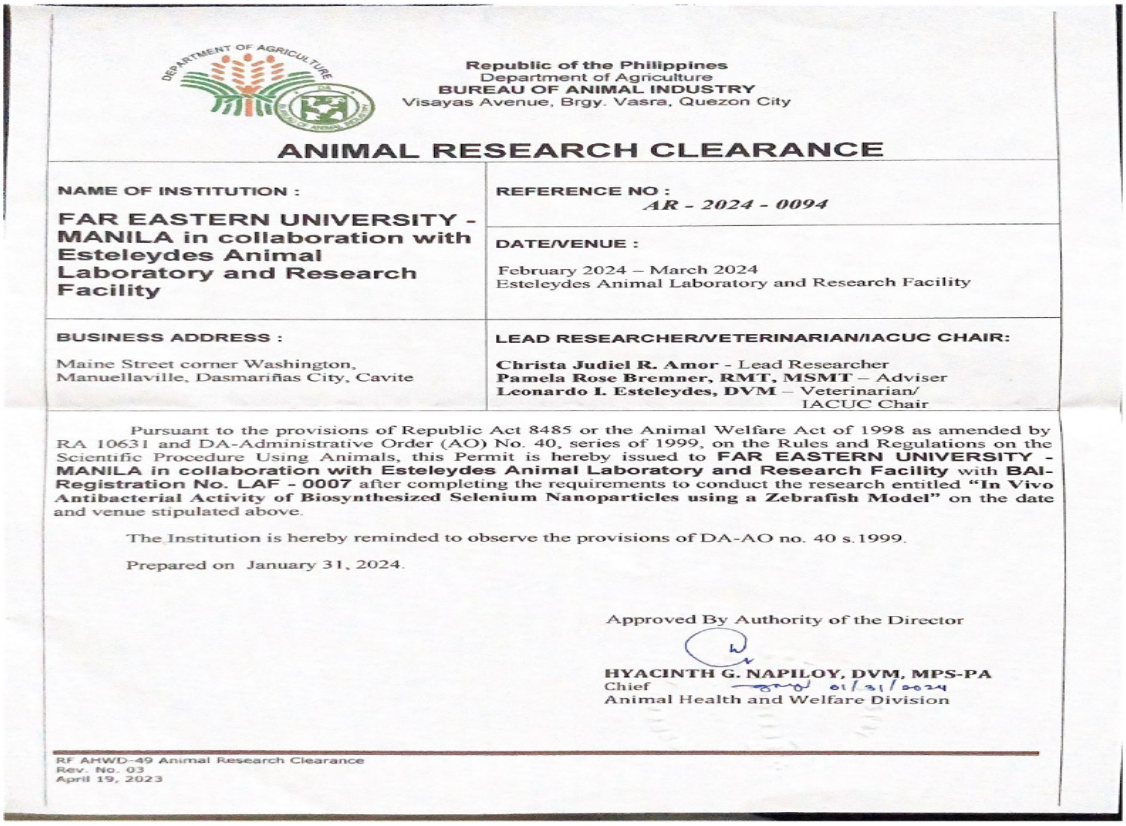
SUMMARY

This research focuses on the antibacterial activity of selenium nanoparticles against *S. aureus*. Selenium nanoparticles were synthesized using orange peels and sodium selenite. Ninety-three zebrafish were infected with *S. aureus* via intramuscular injection. Subsequently, solutions with varying concentrations (5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL) of the selenium nanoparticles were prepared for water bath treatment. The infected zebrafish were divided into three groups: the positive control, the negative control and the treatment group. The treatment group was treated in the different concentrations of the prepared medicated water bath. The positive group was treated with 20 µg/mL of ciprofloxacin and the negative group remained untreated. After the treatment, the zebrafish were euthanized, dissected, homogenized and plated in the MSA after 1, 2, 3 and 4 hr repeatedly to determine its antibacterial load.

The findings revealed that all SeNPs concentrations exhibited significant antibacterial activity, with 20 µg/mL being the most effective, leading to a notable decline in bacterial growth within 1 to 4 hr. Furthermore, the 20 µg/mL concentration displayed efficacy comparable to the positive control, Ciprofloxacin, showing 97.7% effectiveness against *S. aureus*. These results suggest that SeNPs could be a potential alternative for combating bacterial infections, with further exploration warranted to elucidate their mechanism of action. Moreover, in a zebrafish model, the varying concentrations of SeNPs exhibited different levels of inhibition against *S. aureus,* showing a significant difference in the *in vivo* growth of the bacteria. The study revealed that concentrations of 15 µg/mL and 20 µg/mL of SeNPs were equally effective in inhibiting *S. aureus,* whereas lower concentrations (5 µg/mL and 10 µg/mL) showed

measurable variations. Overall, SeNPs demonstrated a remarkable 95.9% antibacterial activity against *S. aureus* using a zebrafish model.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE



Republic of the Philippines
Department of Agriculture
BUREAU OF ANIMAL INDUSTRY
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ANIMAL RESEARCH CLEARANCE

NAME OF INSTITUTION : FAR EASTERN UNIVERSITY - MANILA in collaboration with Esteleydes Animal Laboratory and Research Facility	**REFERENCE NO :** AR - 2024 - 0094
	DATE/VENUE : February 2024 – March 2024, Esteleydes Animal Laboratory and Research Facility
BUSINESS ADDRESS : Maine Street corner Washington, Manuellaville, Dasmariñas City, Cavite	**LEAD RESEARCHER/VETERINARIAN/IACUC CHAIR:** **Christa Judiel R. Amor** - Lead Researcher; **Pamela Rose Bremner, RMT, MSMT** – Adviser; **Leonardo I. Esteleydes, DVM** – Veterinarian/IACUC Chair

Pursuant to the provisions of Republic Act 8485 or the Animal Welfare Act of 1998 as amended by RA 10631 and DA-Administrative Order (AO) No. 40, series of 1999, on the Rules and Regulations on the Scientific Procedure Using Animals, this Permit is hereby issued to **FAR EASTERN UNIVERSITY - MANILA in collaboration with Esteleydes Animal Laboratory and Research Facility** with **BAI-Registration No. LAF - 0007** after completing the requirements to conduct the research entitled **"In Vivo Antibacterial Activity of Biosynthesized Selenium Nanoparticles using a Zebrafish Model"** on the date and venue stipulated above.

The Institution is hereby reminded to observe the provisions of DA-AO no. 40 s.1999.

Prepared on January 31, 2024.

Approved By Authority of the Director

HYACINTH G. NAPILOY, DVM, MPS-PA
Chief
Animal Health and Welfare Division

RF AHWD-49 Animal Research Clearance
Rev. No. 03
April 19, 2023

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