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Antimicrobial and Free Radical Scavenging Activity of *Syzygium aromaticum* Mediated Zinc Oxide Nanoparticles against Oral Pathogens

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Abstract: The aim of this study was to evaluate the antimicrobial and free-radical elimination activity of ZnO nanoparticles mediated by *Syzygium aromaticum* (*S. aromaticum*) (clove) against oral pathogens. The synthesized *S. aromaticum* mediated ZnO NPs (*S. aromaticum* ZnO NPs) were studied by UV-visible spectroscopy. The antimicrobial activity of ZnO NPs was determined by the diffusion method of agar wells and by time-killing kinetic assay of *Streptococcus mutans*, *Lactobacillus*, *Staphylococcus aureus*, and *Candida albicans*. The results show that ZnO NP has a significant antimicrobial activity against all tested microorganisms, and *S. mutans* and *Lactobacillus* are the most prone. Additionally, the ZnO NPs exhibited significant free radical scavenging activity. The study suggests that *S. aromaticum* ZnO NPs have the potential to be used as a natural alternative to synthetic antimicrobial agents in the prevention and treatment of oral infections.

Keywords: Antioxidant activity, *Syzygium aromaticum*, Zinc oxide nanoparticles, Antimicrobial activity

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Introduction

In recent years, the emergence of antibiotic-resistant strains of bacteria has become a significant concern in healthcare. This has led to a growing interest in the use of alternative antimicrobial agents such as nanoparticles (Tarin-Pello *et al*., 2022, Junaid *et al*., 2022). Among these, zinc oxide nanoparticles (ZnO NPs) have shown promising potential as effective antimicrobial agents due to their unique physicochemical properties (Sana *et al*., 2023). Additionally, ZnO NPs have also been found to possess antioxidant properties, making them useful in scavenging free radicals (Yousof *et al*., 2022). Cloves, the flower buds of the *S. aromaticum* tree, have been

traditionally used as a spice and a medicinal herb in various cultures. Cloves are rich in phenolic compounds such as eugenol, which have been reported to have antimicrobial properties against several pathogenic microorganisms (Benmakhlouf *et al*., 2022). The combination of the inherent antimicrobial properties of cloves and the unique properties of ZnO NPs make them an attractive candidate for developing novel antimicrobial agents (Elisha *et al*., 2022, Kiki *et al*., 2023). Oral health is crucial for overall health, and the oral cavity is a site for colonization of several microorganisms, including *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Candida albicans*, among others (Baty *et al*., 2022). These micro-organisms are responsible for several diseases, such as periodontal diseases, dental caries, and oral candidiasis. Current antimicrobial agents used to treat these infections have several limitations, including resistance development, toxicity, and limited efficacy. Therefore, there is a need to develop novel antimicrobial agents that can effectively target these pathogens (Kumar *et al*., 2022; Jardon-Romero *et al*., 2022). The objective of this study was to use the aqueous extract of *S. aromaticum* to synthesise ZnO NPs and evaluate their antimicrobial and free radical scavenging activity against oral pathogens such as *S. mutans*, *Lactobacillus sp*, *S. aureus*, and *C. albicans*. The use of green synthesis, i.e., synthesis using plant extracts, has gained significant attention in recent years due to its eco-friendly nature and low cost. Additionally, using plant extracts to synthesize NPs can impart the antimicrobial properties of the plant to the NPs, making them more effective. The study will use the Brine shrimp lethality assay to assess the cytotoxic nature of ZnO NPs. The findings of this study can provide valuable insights into the antioxidant potential of ZnO NPs synthesized using the *S. aromaticum* extract and as an effective antimicrobial agent against oral pathogens. Additionally, the study can also contribute to the development of safe and effective alternative therapies for oral infections that are affordable and sustainable.

Materials and Methods

Preparation of S. aromaticum extract:

The study utilises *S. aromaticum* extract as a reducing and capping agent to synthesize zinc oxide nanoparticles. To prepare *S. aromaticum* extract, 1 g of *S. aromaticum* was measured and added to 100 ml distilled water. Then it was heated using a heating mantle at 60-70 ^{o}C for 20 min. Whatman N. 1 filter paper was used to filter the extract after heating. The filtered extract was kept for silver nanoparticle synthesis.

Green synthesis of Zinc Oxide nanoparticles:

The 20 mM zinc nitrate was measured and added to a conical flask containing 60 ml distilled water. *S. aromaticum* solution extract was first filtered and 40 ml of this filtrate was added to the previous reaction mixture. The reaction mixture was then stirred on a magnetic stirrer at 700 rpm for 48 h. The synthesized zinc oxide nanoparticle extract was then put in a centrifuge for 10 min at 8000 rpm to separate the pellet from the supernatant. The resultant pellet from the tube was collected and stored in an airtight Eppendorf tube for further characterization and biomedical activities.

UV-visible spectrophotometer analysis:

The zinc oxide nanoparticles produced by the green synthesis were studied using a Double beam-UV-Visible spectrophotometer

Antimicrobial activity:

Agar well diffusion technique was utilised to evaluate the antimicrobial nature of zinc oxide nanoparticles produced by green synthesis. Mueller-Hinton agar plates were created and subjected to sterilization in an autoclave, where they were exposed to temperatures of 121°C for a duration of 15 to 20 min. Following the sterilization process, the medium was poured onto the surfaces of sterile Petri plates and allowed to cool down to reach room temperature. The bacterial suspension containing *S. mutans*, *Lactobacillus*, *S. aureus*, and *C. albicans* was uniformly distributed across the agar plates by

means of sterile cotton swabs. Using a sterile polystyrene tip, wells measuring 9 mm in diameter were generated within the agar plates. These wells were subsequently loaded with varying concentrations (25 µg, 50 µg, 100 µg) of ZnO NPs, while an antibiotic (such as Amoxyrite for bacteria or Flucanazole for fungi) served as the reference standard. The plates were placed in an incubator set at 37°C for 24 h, while fungal cultures were incubated for 48 h. The assessment of antimicrobial effectiveness involved determining the diameter of the zone where growth inhibition occurred around the wells. This measurement was carried out using a ruler and recorded in millimeters (mm), after which the size of the inhibition zone was calculated.

Time kill curve assay:

1 ml aliquot of the bacterial and fungal suspension (*S. mutans*, *Lactobacillus*, *S. aureus*, and *C. albicans*) was added to 9 ml of Mueller Hinton broth containing the ZnO NPs at a concentration of 25 µg, 50 µg, and 100 µg. The final microbial concentration was approximately 10^6 CFU/ml. The mixture was then incubated at 37°C with shaking at 200 rpm for varied time intervals (0, 4, 6, 8, 10, 12, and 24 h). Subsequently, the percentage of deceased cells was determined at specific time intervals by measuring the absorbance at a wavelength of 600 nm.

Antioxidant activity:

DPPH assay: A stock solution containing of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was initially prepared using methanol. The concentration of this solution was 0.1 mM. For each individual assay, a new working solution was created by diluting the stock solution to a final concentration of 20 µM in methanol. In a 96-well plate, several concentrations ranging in multiples of 10, such as 20, 30, 40, and 50 µg/ml of zinc oxide nano-particles synthesized with the assistance of *S. aromaticum* were introduced into 200 µl of the DPPH working solution. The plate was kept under incubation in the dark at room temperature for about half an hour. With the help of a microplate reader the optical density was measured at a wavelength of 517 nm. The blank solution was that of methanol. Utilising the given formula the DPPH Scavenging Activity was calculated in percentage:

$$\% \text{ DPPH Scavenging Activity} = [(\text{Control} - \text{Sample})/\text{Control}] \times 100$$

Where, the value of Control is the measurement of absorbance of the control (DPPH solution which does not have the sample), and the value of Sample is the measurement of absorbance of the sample (DPPH solution which contains the *S. aromaticum* mediated zinc oxide nanoparticles). The positive control group consisted of ascorbic acid (1 mg/ml).

H_2O_2 assay: Hydroxyl radical scavenging assay was utilised in this study to determine the degree of antioxidant activity using the method proposed by Halliwell *et al*. (1989). 100 µl of 28 mM of 2-deoxy-2-ribose was mixed with 1 ml of reaction mixture for preparation. To those various concentrations of *S. aromaticum* ZnO NPs (10-50 µg/ml) was added. Along with that, 200 µl of 200 µm ferric chloride, 200 µl of EDTA, 100 µl ascorbic acid was added. Subsequently, it was left to incubate at 37°C for one hour, and the optical density was recorded at 532 nm compared to the blank solution. Vitamin E acted as the positive control in this regard.

$$\text{Hydroxyl radical scavenging activity (\%)} = [(\text{Blank} - \text{Sample})/\text{Blank}] \times 100$$

Where, the value of Blank is the measurement of absorbance of Control (which does not have the sample), and the value of Sample is the absorbance of the reaction with the sample.

Cytotoxic impact – Brine shrimp lethality assay:

The zinc oxide nanoparticles produced by the green synthesis method were subjected to the Brine shrip lethality assay to determine their cytotoxic impact. For the preparation of saline water, 2 g of salt which is devoid of any iodine was measured and dissolved in 200 ml distilled water. Six well ELISA plates were then taken and 10 – 12 ml of saline water was pipetted in it. In each well,

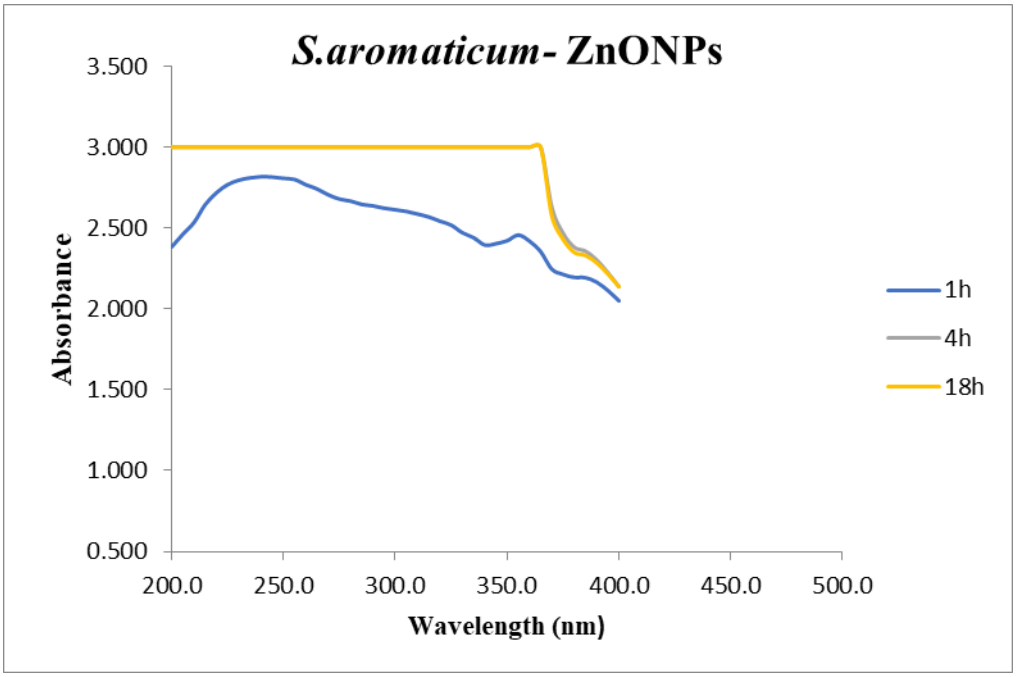

Fig. 1: Production of zinc oxide nanoparticles by the method of Green synthesis and its spectroscopic analysis by UV visible rays.

10 nauplii were gently introduced. Varying amounts of zinc oxide nanoparticles (5, 10, 20, 40 and 80 µl) were then introduced based on their respective concentrations. Subsequently, the plates with the introduced nauplii were incubated for a day at room temperature. After this incubation period, the ELISA well plates were checked for the surviving Nauplii and the following formula was utilised:

Percentage of live nauplii = Number of live nauplii/Total number of nauplii) × 100

Results and Discussion

UV-vis spectroscopy:

The analysis of *S. aromaticum* ZnO NPs showed a specific peak at 360 nm, which is indicative of the presence of zinc oxide nanoparticles (Fig. 1). This peak signifies the occurrence of surface plasmon resonance (SPR) associated with the nano-particles, confirming their generation. Additionally, visual inspection corroborated the formation of silver nanoparticles, evident by the transformation in the color of the reaction mixture from a pale yellow hue to a deep brown shade. The change in color suggests that *S. aromaticum* extract has reducing and stabilizing power, which has facilitated the formation of the zinc oxide nanoparticles and also contributes to it's stabilility. *Limonium pruinosum* aqueous extract-mediated zinc oxide nanoparticles are known to exhibit a characteristic peak at 370 nm (Naiel *et al*., 2022). *Thymbra spicata* plant extract-mediated ZnO NPs showed a surface plasmon resonance peak at 360 nm which seems to be in concordant with the current study results (Gur *et al*., 2022).

Antimicrobial activity:

Zinc oxide nanoparticles have a broad-spectrum antimicrobial activity and have been shown to be effective against bacteria, fungi, and viruses. They work by disrupting the cell membranes and DNA of microbes, which causes lethality (Imade *et al*., 2022; Archana *et al*., 2022). Figure 2 shows the results of the antimicrobial properties of Zinc oxide nanoparticles against four different pathogens, namely *S. aureus*, *S. mutans*, *Lactobacillus*, and *C. albicans*. The zone of

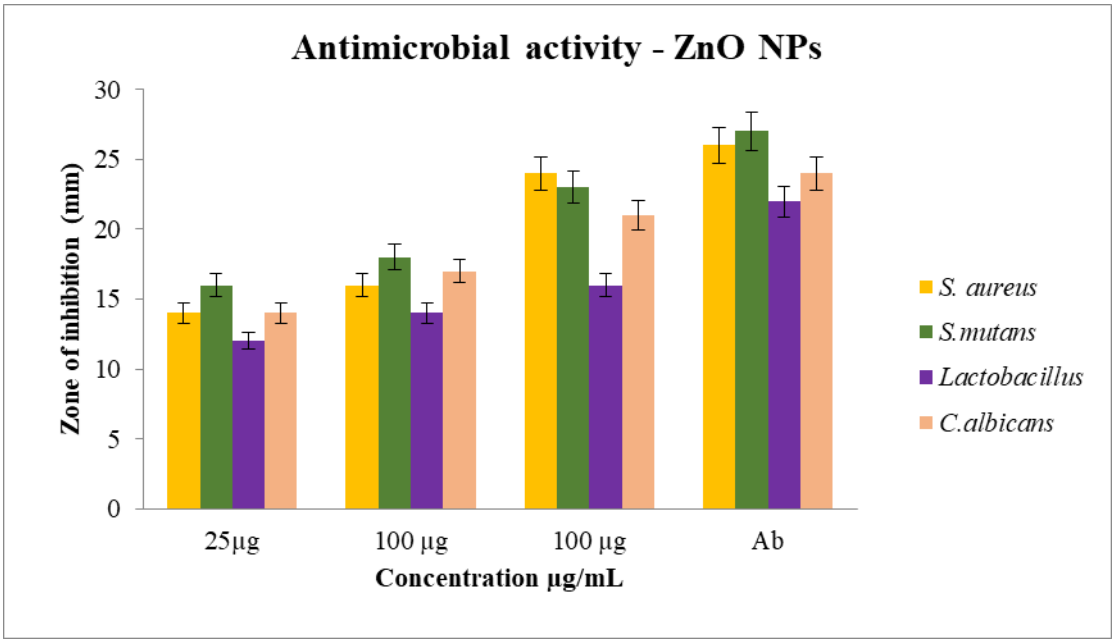

Fig. 2: Antimicrobial nature of zinc oxide nanoparticles using agar well diffusion method.

inhibition (diameter in mm) was measured for each pathogen at different concentrations of Zinc oxide nanoparticles (25 µg, 50 µg and 100 µg). The findings suggest that Zinc oxide nanoparticles possess antimicrobial attributes against all four examined pathogens. As the concentration of Zinc oxide nanoparticles escalated from 25 µg to 100 µg, the inhibition zone also expanded for all the pathogens. For *S. aureus*, the zone of inhibition was 14 mm at 25 µg and increased to 16 mm and 24 mm at 50 µg and 100 µg, respectively. Similarly, for *S. mutans*, it was observed that the zone of inhibition was 16 mm at 25 µg and increased to 18 mm and 23 mm at 50 µg and 100 µg, respectively. For *Lactobacillus*, the zone of inhibition was 12 mm at 25 µg and increased to 14 mm and 16 mm at 50 µg and 100 µg, respectively. Finally, for *C. albicans*, it was observed that the zone of inhibition was 14 mm at 25 µg and increased to 17 mm and 21 mm at 50 µg and 100 µg, respectively.

Time kill curve assay:

The time kill curve assay of zinc oxide nanoparticles was performed against four different microorganisms, namely *S. mutans*, *Lactobacillus sp.*, *S. aureus* and *C. albicans*. The assay was conducted for up to 4 h, and the bacterial/fungal growth was measured at several time intervals such as 0 h, 1 h, 2 h, 3 h, and 4 h using optical density. It appears that the standard group also showed a notable inhibitory effect on bacterial and fungal growth (Figs. 3a, b, c, d), reducing it to a great extent. By the end of the assay, the optical density measurements showed that the standard group was able to reduce the bacterial/fungal growth to a great extent, with the final value being only 70-80% of the initial value (Donga and Chanda, 2022; Velumani *et al*., 2022). In the case of *S. mutans*, all three concentrations of zinc oxide nanoparticles (25 µg, 50 µg, and 100 µg) showed a gradual decrease in bacterial growth over time, with the highest concentration (100 µg) showing the most significant effect. By the end of the assay, the 100 µg concentration of nanoparticles was able to reduce the bacterial growth to 29.4% of the initial value. For *Lactobacillus sp*, the highest concentration of nanoparticles (100 µg) showed a significant reduction in bacterial growth, with a decrease of

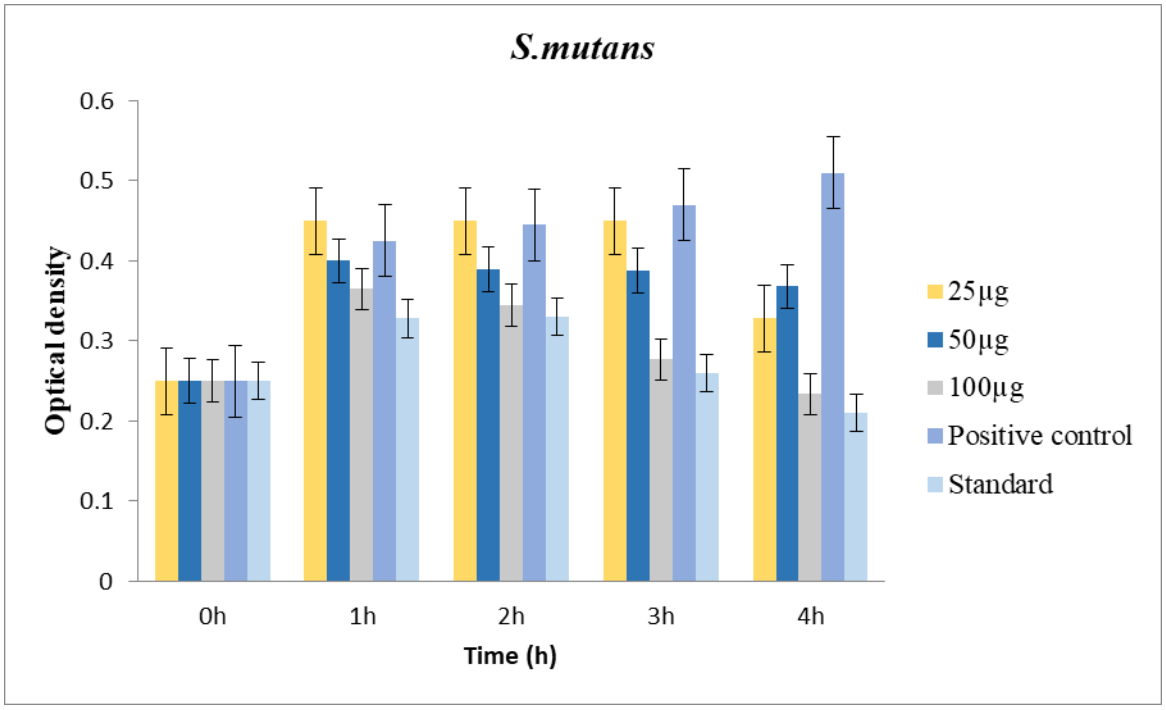

Fig. 3a: Zinc oxide nanoparticles when assessed through a time kill curve assay against *Streptococcus mutans.*

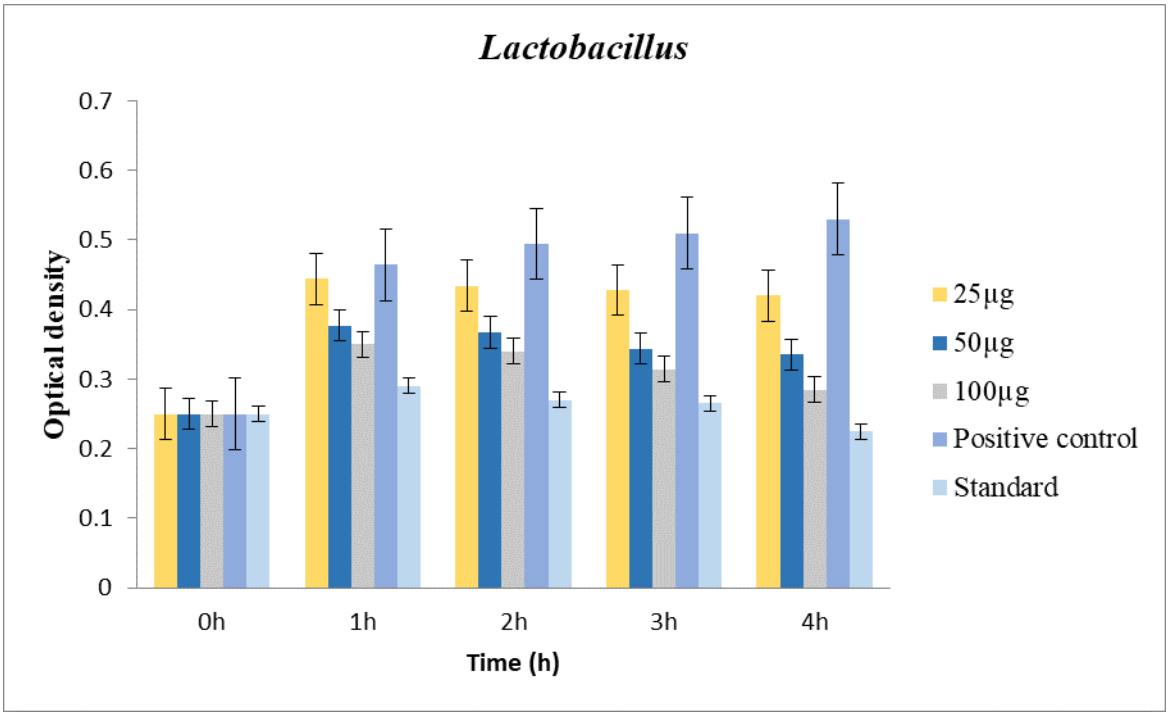

Fig. 3b: Zinc oxide nanoparticles when assessed through a time kill curve assay against *Lactobacillus sp.*

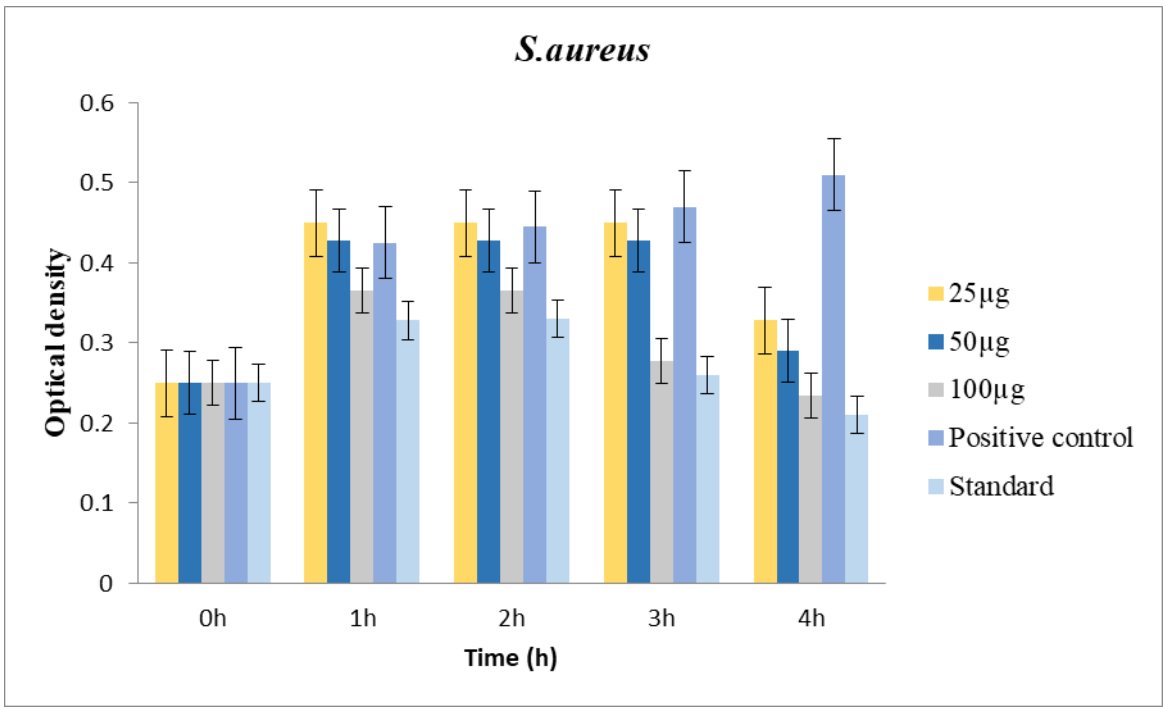

Fig. 3c: Zinc oxide nanoparticles when assessed through a time kill curve assay against *Staphylococcus aureus.*

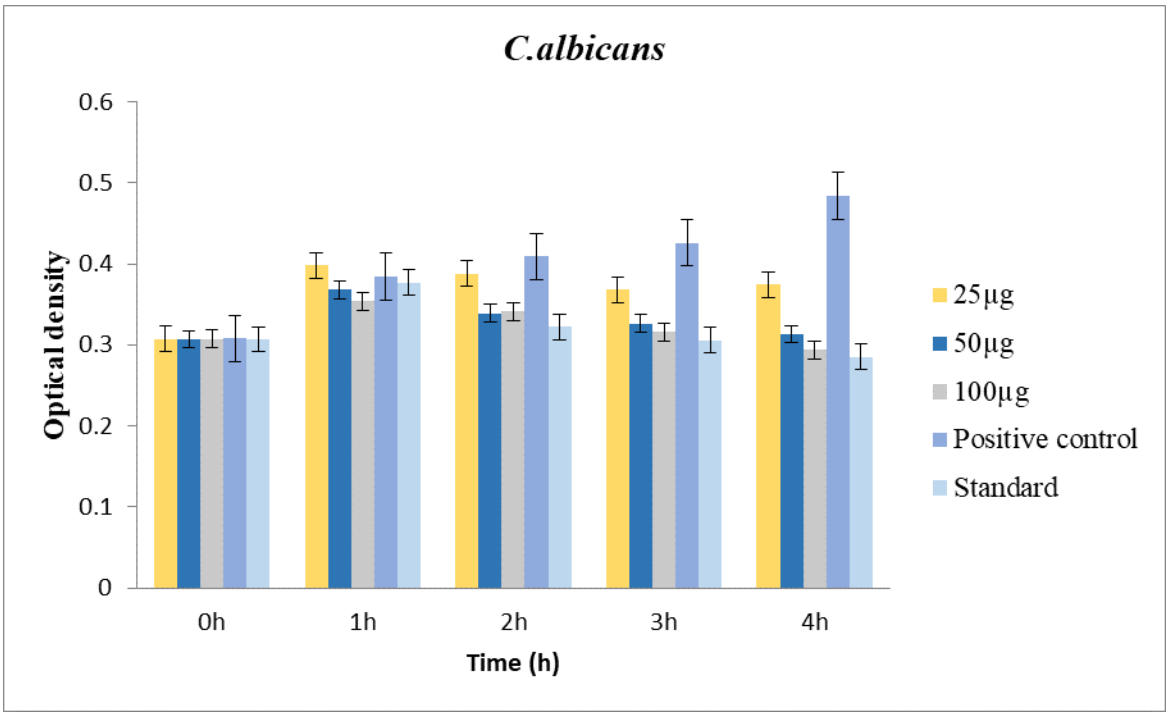

Fig. 3d: Zinc oxide nanoparticles when assessed through a time kill curve assay against *Candida albicans.*

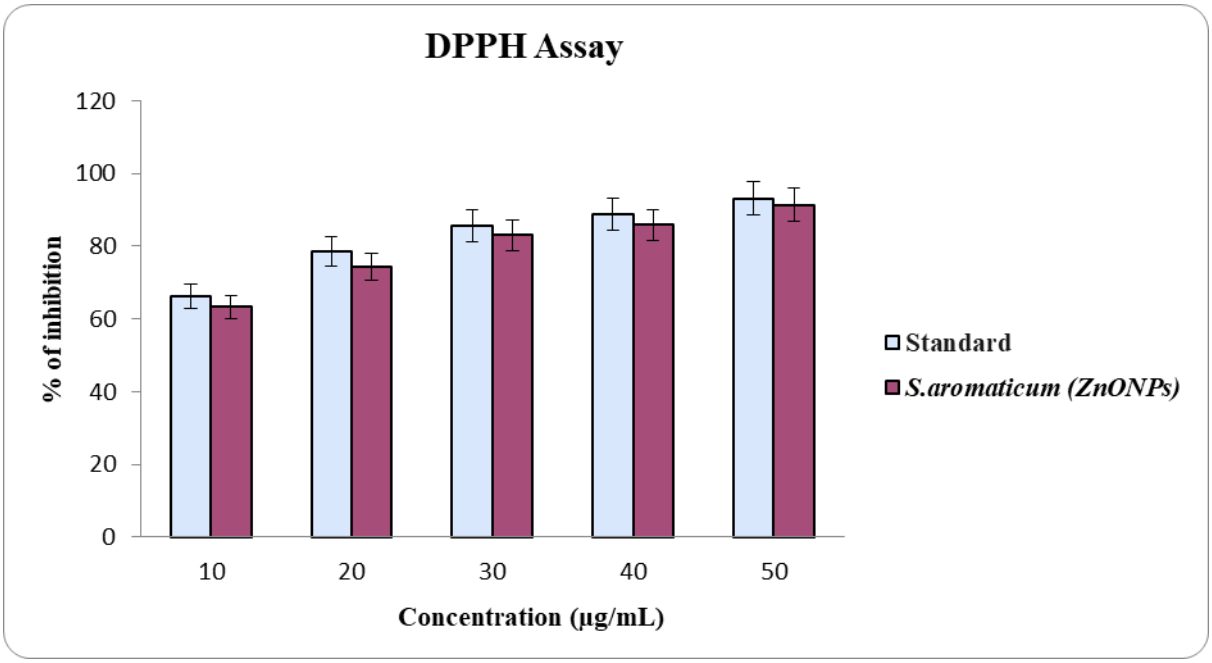

Fig. 4: Antioxidant activity exhibited by the synthesised zinc oxide nanoparticles after utilization of DPPH assay.

up to 61.8% of the initial value after 4 h. The lower concentrations of nanoparticles (25 µg and 50 µg) also showed some inhibitory effect, but it was less pronounced compared to the higher concentration. Against *S. aureus*, the highest concentration (100 µg) of nanoparticles showed a more significant effect compared to the lower concentrations, with a reduction in bacterial growth of up to 71.5% after 4 h. In the case of *C. albicans*, all three concentrations of nanoparticles showed a gradual decrease in fungal growth over time, with the highest concentration (100 µg) showing the most significant effect. By the end of the assay, the 100 µg concentration of nanoparticles was able to reduce the fungal growth to 32.2% of the initial value. Overall, the results of the time kill curve assay suggest that the zinc oxide nanoparticles have a concentration-dependent inhibitory effect on the growth of *S. mutans*, *Lactobacillus sp.*, *S.aureus*, and *C. albicans*. The highest concentration (100 µg) showed the most significant effect against all microorganisms tested, while the lower concentrations also showed some inhibitory effect, albeit to a lesser extent. The positive control maintained stable growth throughout the assay.

Antioxidant activity:

The activity of antioxidance of *S. aromaticum* ZnO NPs was determined by using the DPPH assay to check their ability to scavenge free radicals (Fig. 4) and H_2O_2 assays (Fig. 5). At 10 µg/ml concentration, *S. aromaticum* ZnO NPs showed 63.38% antioxidant activity, while the standard ascorbic acid exhibited 66.25% antioxidant activity. At 20 µg/ml concentration, *S. aromaticum* ZnO NPs showed 74.27% antioxidant activity, while the standard ascorbic acid exhibited 78.52% antioxidant activity. At 30 µg/ml concentration, *S. aromaticum* ZnO NPs showed 82.97% antioxidant activity, while the standard ascorbic acid exhibited 85.63% antioxidant activity. At 40 µg/ml concentration, *S. aromaticum* ZnO NPs showed 85.81% antioxidant activity, while the standard ascorbic acid exhibited 88.68% antioxidant activity. The DPPH assay results indicated a rise in the antioxidant activity of *S. aromaticum* ZnO NPs as the concentration increased, culminating at its

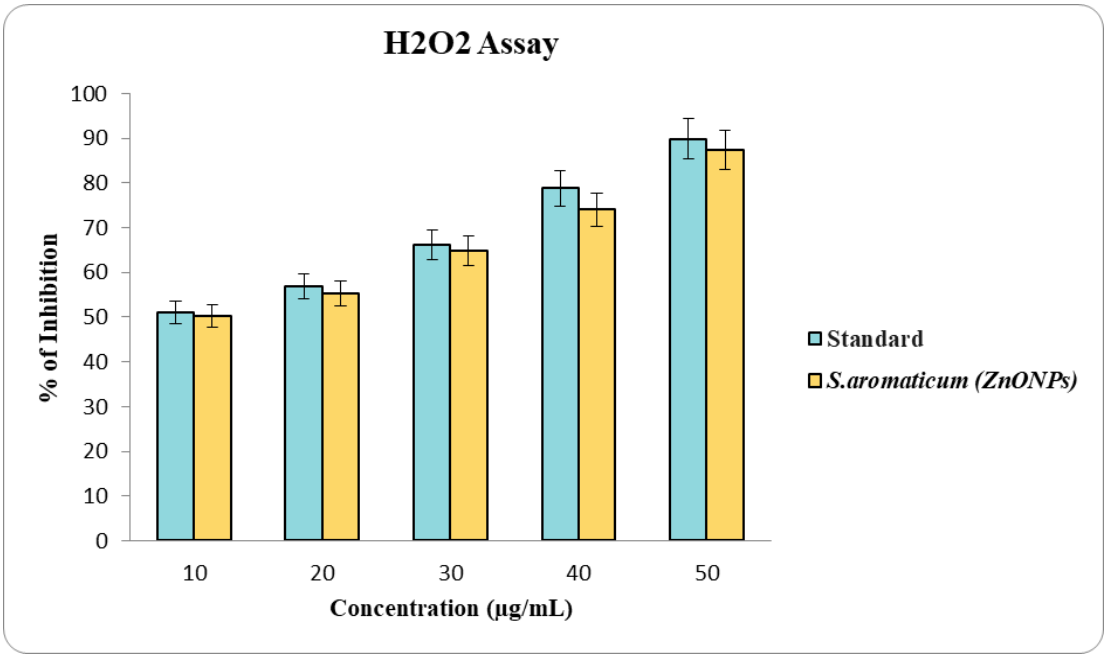

Fig. 5: Antioxidant activity exhibited by the synthesized zinc oxide nanoparticles after utilization of H_2O_2 assay.

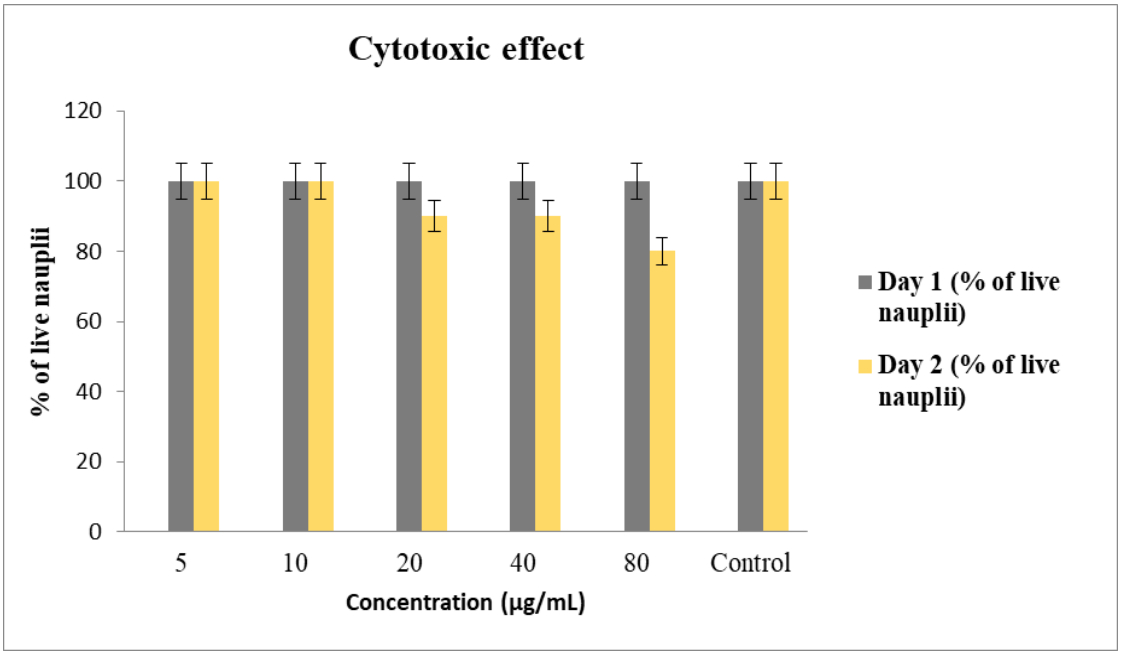

Fig. 6: Cytotoxic effect of zinc oxide nanoparticles using BSLA.

highest point of 91.38% at a 50 µg/ml concentration. The standard ascorbic acid showed an antioxidant activity of 93.15% at the same concentration. Similarly, the results of the H_2O_2 assay exhibited that the antioxidant activity of *S. aromaticum* ZnO NPs increased proportionally with concentration, reaching a maximum value of 87.5% at a concentration of 50 µg/ml. The standard ascorbic acid exhibited an antioxidant activity of 89.9% at the same concentration. Overall, antioxidant activity of *S. aromaticum* ZnO NPs was assessed to be matching that of the ascorbic acid. The findings suggest that *S. aromaticum* ZnO NPs have significant antioxidant

potential and can be used as a natural antioxidant agent in various pharmaceutical applications (Abbes *et al*., 2022; Chemingui *et al*., 2024).

Cytotoxic effect:

The cytotoxic effect of *S. aromaticum* ZnO NPs was evaluated by exposing nauplii to different concentrations (5 µl, 10 µl, 20 µl, 40 µl, and 80 µl) of the nanoparticles for two consecutive days. The viability of nauplii was measured and expressed as a percentage of live Nauplii in each group. On day 1, all concentrations showed 100% viability, which was similar to the control group. However, on day 2, the viability of Nauplii exposed to 20 µl, 40 µl, and 80 µl of nanoparticles decreased to 90%, 80%, and 80%, respectively. The control group, on the other hand, maintained 100% viability on day 2. These results indicate that the cytotoxic effect of *S. aromaticum* ZnO NPs is concentration-dependent (Fig. 6) and can cause a decrease in the viability of Nauplii at higher concentrations. The highest concentration (80 µl) caused a similar decrease in viability as the 40 µl concentration. Recently, Saleemi *et al*. (2022) synthesized Zinc oxide nanoparticles using bioflavonoid rutin and reported no toxicity on *Artemia* Nauplii.

Conclusion

In conclusion, the present study highlights the ability of free radical scavenging and the degree of antioxidant character of *S. aromaticum* ZnO NPs against oral pathogens. The results demonstrate that the synthesized ZnO NPs have significant antimicrobial activity against *S. mutans*, *Lactobacillus*, *S. aureus*, and *C. albicans*, with *S. mutans* followed by *Lactobacillus* being the most susceptible. Furthermore, the ZnO NPs exhibited significant free radical scavenging activity, indicating their potential as a natural antioxidant. These findings suggest that *S. aromaticum* ZnO NPs can be a promising substitues to synthetic antimicrobial agents in the prevention and treatment of oral infections. The use of natural compounds such as *S. aromaticum* may offer a safer and more sustainable approach to combating oral pathogens.

Ethical Statement

The authors confirm that all ethical issues have been dealt with the research ethics guidelines provided by the Research Department of Biotechnology, Marudhar Kesari Jain College for Women, Vaniyambadi, Tamil Nadu, India.

Author Contributions

Gomathi M: Supervision, critical review of the manuscript, and final editing**;** *Ayesha Naaz M*: Data analysis, preparation of tables and figures, and manuscript refinement; *Moganapriya S:* Literature collection, data interpretation, and manuscript writing; *Malarkodi R*: Assisted in manuscript writing, proofreading, and technical validation; *Sharma Bhoomika*: literature survey, data analysis. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

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