Synthesis of Titanium Nanoparticles and Loaded With Chitosan as Membranes for Soft Tissue Regeneration Using Zebrafish Model

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**Abstract:** Soft tissue regeneration is crucial in biomedical research, addressing injuries from trauma, surgery, or disease. Traditional treatments have limitations, leading to the exploration of novel biomaterials. Titanium nanoparticles (TiNPs) exhibit excellent biocompatibility, mechanical strength, and the ability to enhance cell adhesion and proliferation. Chitosan, a natural polysaccharide, is biodegradable, antimicrobial, and supports tissue growth. This study investigates a TiNPs-loaded chitosan membrane for soft tissue regeneration using zebrafish as an in vivo model.TiNPs were synthesized via the sol-gel method and incorporated into a chitosan matrix. SEM analyzed surface morphology, FTIR confirmed chemical composition, and tensile testing assessed mechanical properties. Cytotoxicity was evaluated using DPPH and H₂O₂ assays, while zebrafish models were used to monitor wound healing and tissue regeneration.SEM revealed a porous structure facilitating cell interaction. FTIR confirmed key functional groups, and mechanical testing demonstrated improved strength. Cytotoxicity assays showed low toxicity and enhanced antioxidant activity. Zebrafish studies indicated accelerated wound closure, improved tissue organization, and reduced inflammation in the test group.The TiNP-chitosan membrane enhances soft tissue regeneration, collagen synthesis, and wound healing, making it a promising biomaterial for future clinical applications.

**Keywords:** Titanium nanoparticles (TiNPs), Tissue regeneration, Zebra fish, chitosan, Tissue repair, membrane, healing, biocompatibility.

# INTRODUCTION

Tissue regeneration is a rapidly evolving field in biomedical science, addressing the growing demand for advanced biomaterials capable of promoting wound healing and repairing damaged tissues[(Kreller et al., 2025)](https://paperpile.com/c/ZxNbMh/sKZy). Traditional treatment methods often lack efficiency, necessitating the development of novel materials that enhance biological responses and accelerate tissue integration[(Ramsundar et al., 2023; Rieshy et al., 2023; Singh et al., 2023)](https://paperpile.com/c/ZxNbMh/e0beE+4Npik+ACBCB). Nanotechnology has emerged as a powerful tool, offering innovative solutions to improve the regenerative potential of biomaterials. Among these, titanium dioxide nanoparticles (TiO₂ NPs) have garnered significant attention due to their biocompatibility, chemical stability, and antimicrobial properties[(Sieniawski & Ziaja, 2013)](https://paperpile.com/c/ZxNbMh/ElsD).Titanium nanoparticles (TiNPs) play a crucial role in soft tissue regeneration by promoting cell adhesion, growth, and differentiation. Their oxidative properties help control infections in wound environments, thereby accelerating the healing process[(Nosrati & Heydari, 2025)](https://paperpile.com/c/ZxNbMh/vPQn). However, TiNPs alone may not provide optimal regenerative outcomes. To overcome this, chitosan, a natural polysaccharide derived from chitin, is incorporated due to its biocompatibility, biodegradability, and antimicrobial activity [(Pavithra et al., 2023; Shenoy et al., 2023; Thomas & Jain, 2023)](https://paperpile.com/c/ZxNbMh/sLyW2+leIeX+PlyuU). Despite its advantages, chitosan has poor solubility in neutral and alkaline solutions, which can limit its applications[(Halawa et al., 2022)](https://paperpile.com/c/ZxNbMh/tZKz).Combining TiO₂ NPs with chitosan enhances the scaffold’s mechanical stability, wound healing potential, and biocompatibility. TiO₂-based materials serve as platforms for cell adhesion and proliferation, while also aiding in haemorrhage control through improved blood clotting[(Doshi et al., 2023; Lampl et al., 2023; Pandiyan et al., 2023)](https://paperpile.com/c/ZxNbMh/IU7rv+i9Qi9+F68pd). Chitosan further supports tissue growth, drug delivery, and antimicrobial defense, making it a promising material for biomedical applications[(Cordoba et al., 2023)](https://paperpile.com/c/ZxNbMh/CbKt).To evaluate the regenerative potential of TiNP-chitosan membranes, zebrafish (Danio rerio) were used as an in vivo model [(Gandhi et al., 2021; Katyal et al., 2023; Priyadharshini et al., 2023)](https://paperpile.com/c/ZxNbMh/svQdk+LhPSm+vp4dD). Zebrafish possess remarkable regenerative capabilities, making them ideal for studying the biological effects of biomaterials[(*The Influence of Organic Coatings on the Toxicity of Titanium Dioxide Nanoparticles to Developing Zebrafish (Danio Rerio)*, 2013)](https://paperpile.com/c/ZxNbMh/QozW). Several analytical techniques were employed, including scanning electron microscopy (SEM) to assess surface morphology, Fourier-transform infrared spectroscopy (FTIR) to examine chemical composition, and tensile strength testing to evaluate mechanical properties. Histological analyses, including hematoxylin and eosin (H&E) staining and Masson’s trichrome staining, were performed to study tissue organization, collagen deposition, and extracellular matrix (ECM) formation at different time points[(Janani et al., 2021; Kachhara et al., 2021; Subramanian et al., 2023)](https://paperpile.com/c/ZxNbMh/t8wAP+ZKcjO+RkeBm).By analyzing the structural, mechanical, and biological properties of TiNP-chitosan membranes, this study aims to determine their potential for enhancing ECM formation, reducing inflammation, and accelerating soft tissue regeneration. The results will contribute to the development of effective biomaterials for clinical applications in tissue engineering and wound healing.

# MATERIALS AND METHODS

## Synthesis of Titanium Nanoparticle-Loaded Chitosan Membranes

Titanium nanoparticles (TiNPs) were synthesized following a standard green synthesis approach. The synthesized TiNPs were characterized using scanning electron microscopy (SEM) to assess their morphological properties. To prepare the composite membrane, chitosan (CS) was dissolved in acetic acid and mixed with different concentrations of TiNPs (20 µL, 40 µL, and 80 µL). The solution was stirred continuously to ensure homogeneity before being cast into molds and allowed to dry at room temperature, forming a flexible membrane structure. The resulting TiNP-CS membranes were then stored in a desiccator until further analysis.

## Evaluation of Soft Tissue Regeneration Using the Zebrafish Model

To assess the regenerative potential of TiNP-CS membranes, a controlled artificial injury was introduced in the caudal region of zebrafish (Danio rerio). The injured zebrafish were treated with TiNP-loaded chitosan at three concentrations (20 µL, 40 µL, and 80 µL). The healing process was monitored over time, and at designated intervals, the affected tissue was excised, fixed in 10% formalin, and dehydrated using a graded ethanol series. The samples were embedded in paraffin, sectioned at 5 µm thickness, and mounted on glass slides for histological analysis.

## Histological Analysis: Haematoxylin and Eosin (H&E) Staining

## Antioxidant Activity: DPPH Assay

The antioxidant properties of TiNPs were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. A 0.1 mM DPPH solution was prepared in ethanol, and various TiNP concentrations (5, 10, 15, 20, and 25 µg/mL) were tested. In a 96-well microplate, 100 µL of each TiNP solution was mixed with 100 µL of DPPH solution and incubated at room temperature for 30 minutes. The absorbance was measured at 570 nm using a microplate reader. Antioxidant activity was calculated by comparing the absorbance values of TiNPs against a standard antioxidant reference.

## Antimicrobial Activity: Well Diffusion Method

The antimicrobial properties of TiNPs were assessed using the well diffusion method against clinical bacterial strains, including Enterococcus faecalis, Streptococcus mutans, Pseudomonas aeruginosa, and Staphylococcus aureus. Pure cultures of these bacteria were grown on nutrient agar plates, and wells were created for TiNP solutions at concentrations of 25, 50, and 100 µg/mL. Ampicillin (1 µg/mL) served as the positive control. The plates were incubated at 37°C for 12–24 hours, after which the zones of inhibition around each well were measured to determine bacterial susceptibility to TiNPs.

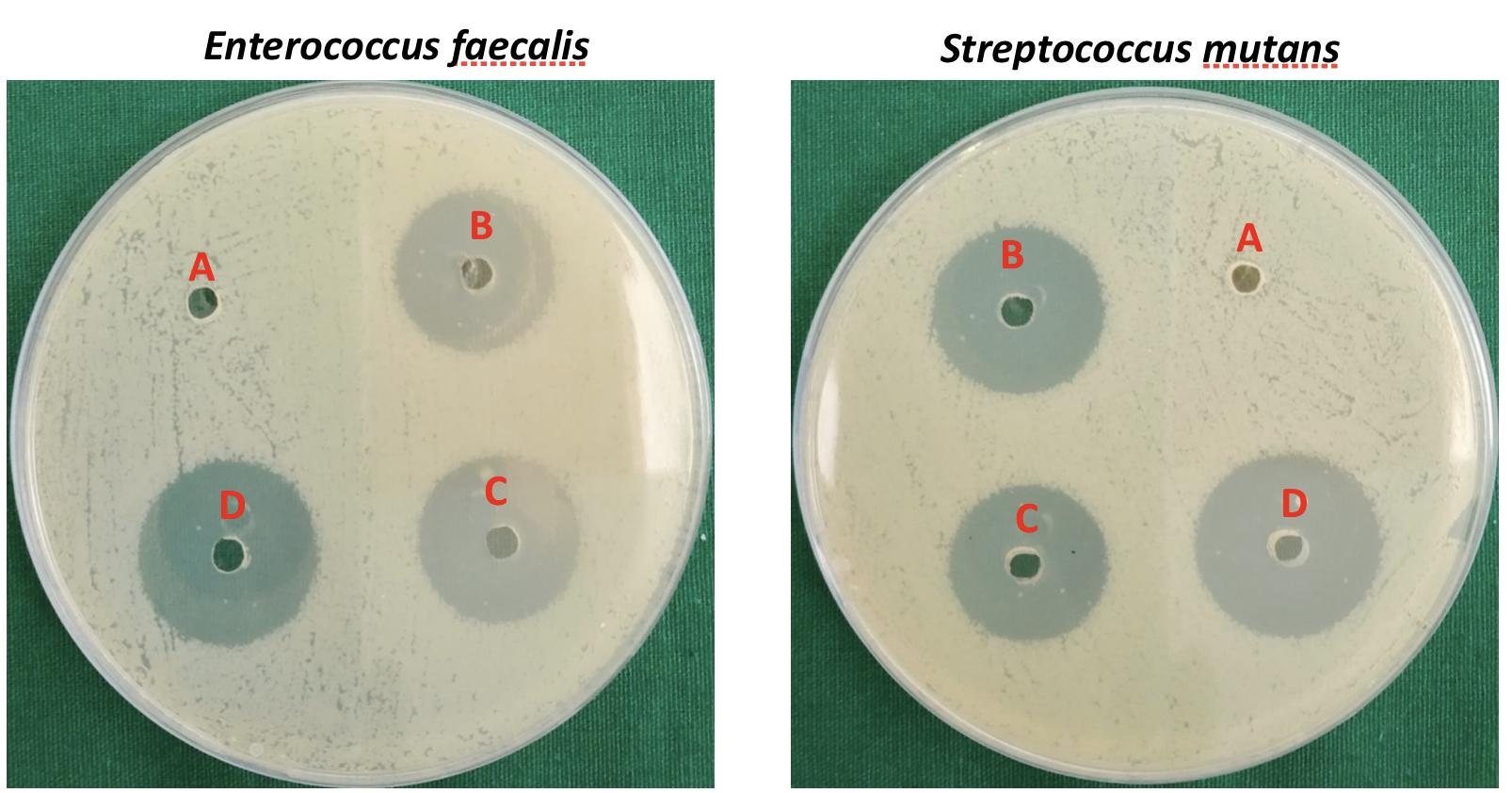


Fig 1: Zone

# Statistical Analysis

All experimental data were analyzed using IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA). A one-way ANOVA was conducted to compare the antimicrobial effectiveness of TiNPs with the antibiotic control, with p < 0.05 considered statistically significant. Additionally, an unpaired t-test was used to assess the antioxidant activity of TiNPs relative to a standard reference, with a significance threshold of p ≤ 0.05.

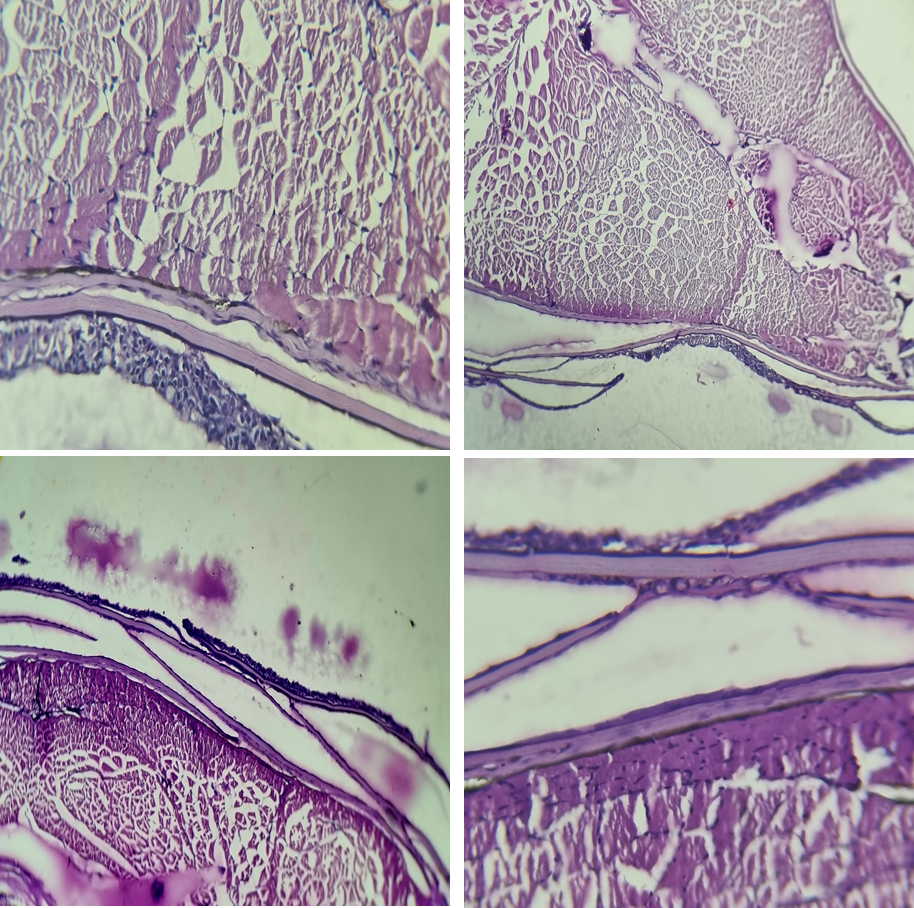
# RESULT

## Scanning Electron Microscopy of the Membrane

The morphological characteristics of the experimental (chitosan-titanium nanoparticle membrane) and control groups were examined using scanning electron microscopy (SEM). The SEM images revealed noticeable differences in surface structure between the two membranes. The control membrane displayed a relatively smooth and uniform surface, whereas the chitosan-titanium nanoparticle-infused membrane exhibited a rougher and more porous texture. This increased surface roughness is advantageous for tissue integration, as it can promote better cell attachment and proliferation, potentially enhancing tissue regeneration outcomes. Additionally, SEM imaging confirmed the presence of nanoparticle incorporation in the test membrane, indicating successful integration of bioactive components into the material matrix.

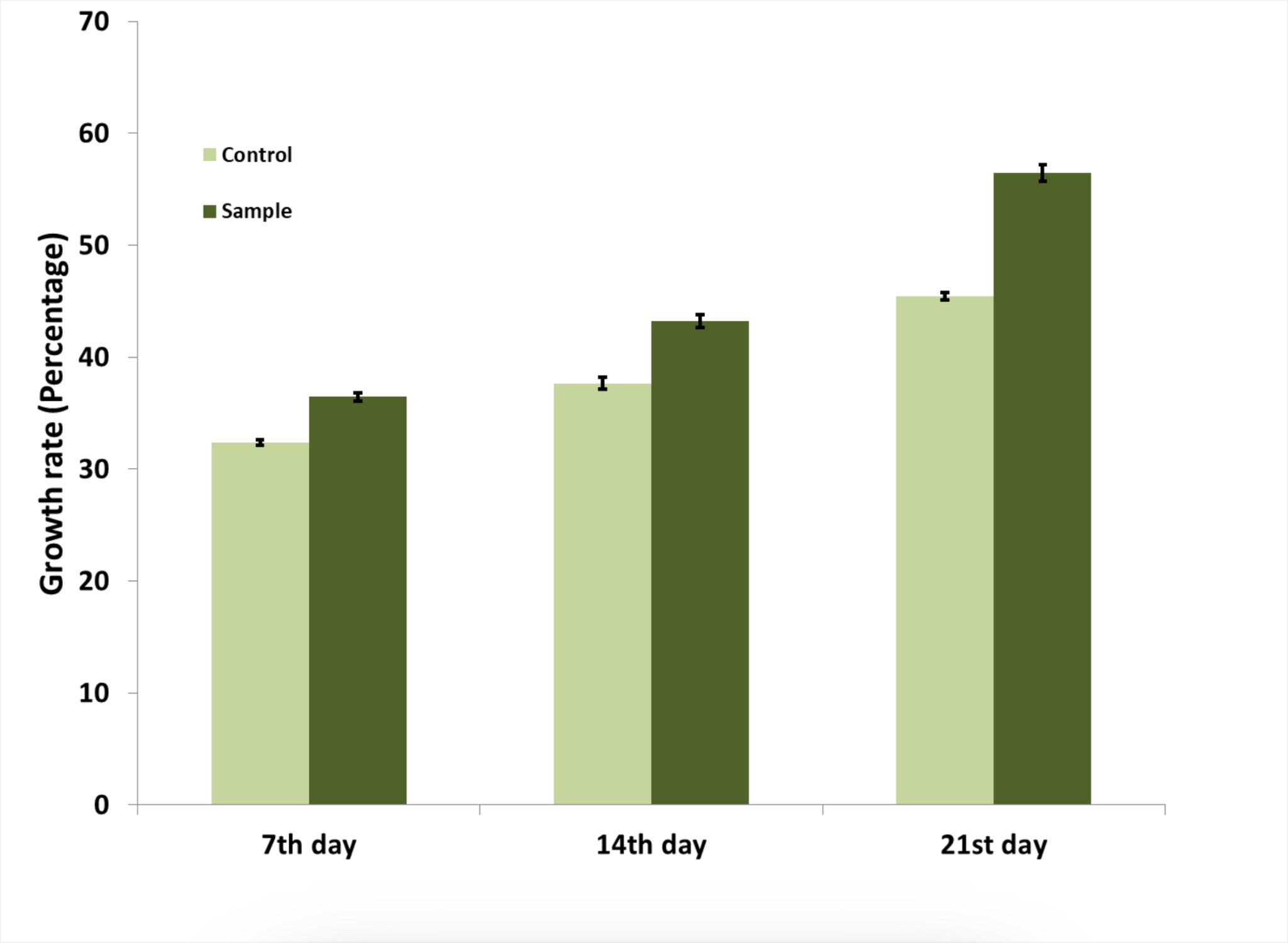
**Figure:1** Histological Analysis of Tissue Regeneration Using Hematoxylin and Eosin (H&E) Staining

At 14 days post-treatment, H&E staining of zebrafish tissue sections revealed distinct histological differences between the control and test groups. In the control group, the regenerated tissue displayed an irregular arrangement with dispersed cellular structures and minimal vascularization, suggesting a slower healing response. Conversely, the chitosan-titanium nanoparticle-treated group exhibited more organized tissue layers, increased cell proliferation, and well-defined vascular structures. These findings indicate that the experimental membrane facilitated a more effective regenerative response by promoting cellular organization and neovascularization, essential for tissue repair and functional recovery.



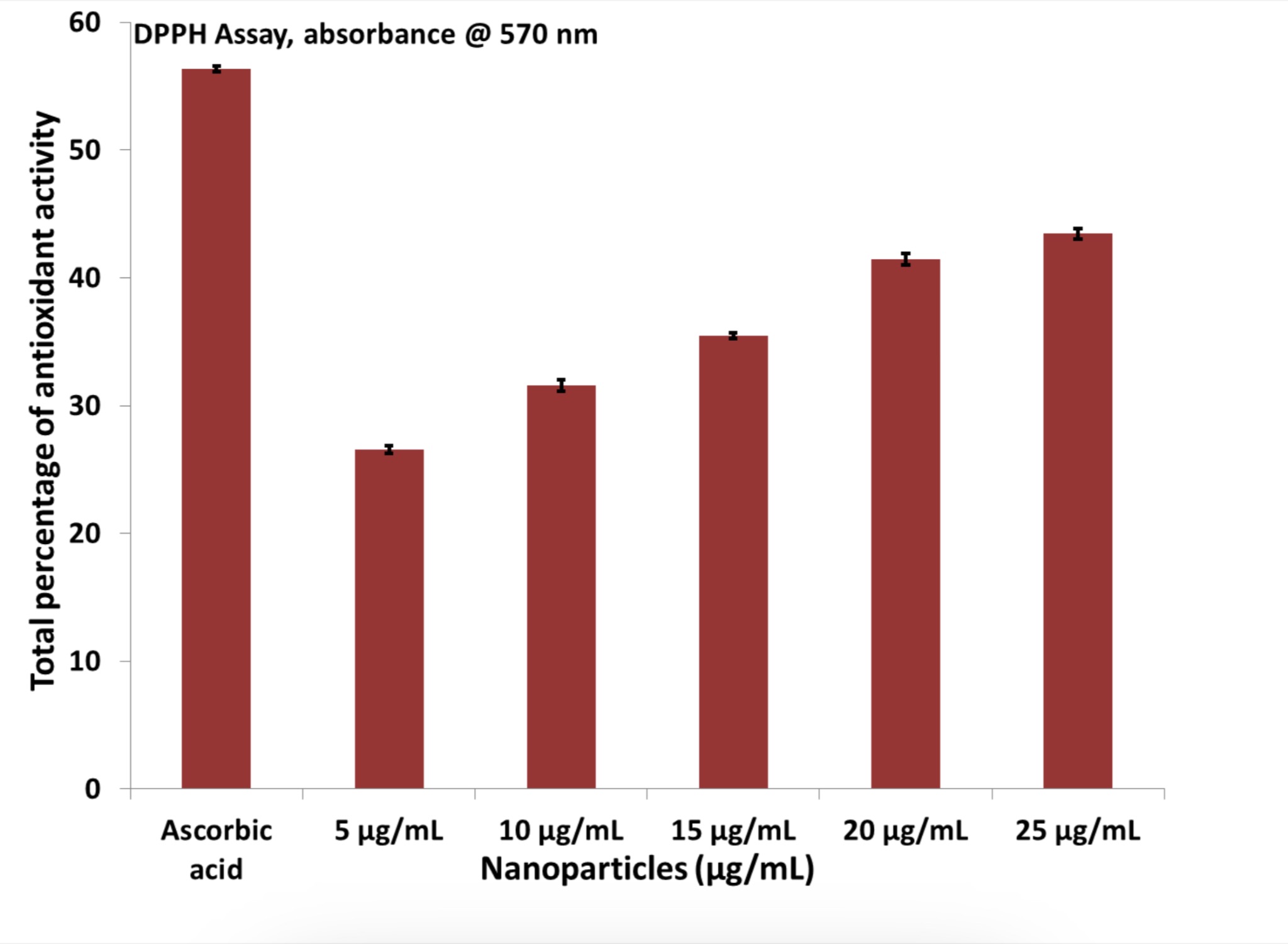
**Figure 2:** Growth Rate Analysis of Zebrafish Model

The rate of zebrafish growth was evaluated at three different time intervals—7, 14, and 21 days—comparing the control group with the test group treated with chitosan-titanium nanoparticle membranes. The results demonstrated a consistently higher growth rate in the test group across all time points, with the most significant difference observed at 21 days. This suggests that the nanoparticles positively influenced tissue development, possibly by enhancing cellular metabolism and proliferation within the regenerating tissue.



**FIGURE 3:** DPPH Assay (Total Percentage of Antioxidant activity at 570 nm) and

The antioxidant capacity of the titanium nanoparticles was assessed using the DPPH assay, which measures free radical scavenging activity at 570 nm. The results showed a concentration-dependent increase in antioxidant activity, although the values remained lower than those observed for the positive control, ascorbic acid. These findings indicate that while the nanoparticles exhibit measurable antioxidant properties, their efficacy remains inferior to conventional antioxidants.



**Figure 4:** H202 Assay (Total Percentage of Antioxidant Activity at 480 nm)

The antioxidant capacity of the titanium nanoparticles was assessed using the H₂O₂ assay, conducted at 480 nm using Vitamin E as the standard, revealed an increase in antioxidant potential with higher nanoparticle concentrations. However, the maximum antioxidant activity recorded was still lower than that of Vitamin E. These findings indicate that while the nanoparticles exhibit measurable antioxidant properties, their efficacy remains inferior to conventional antioxidants.

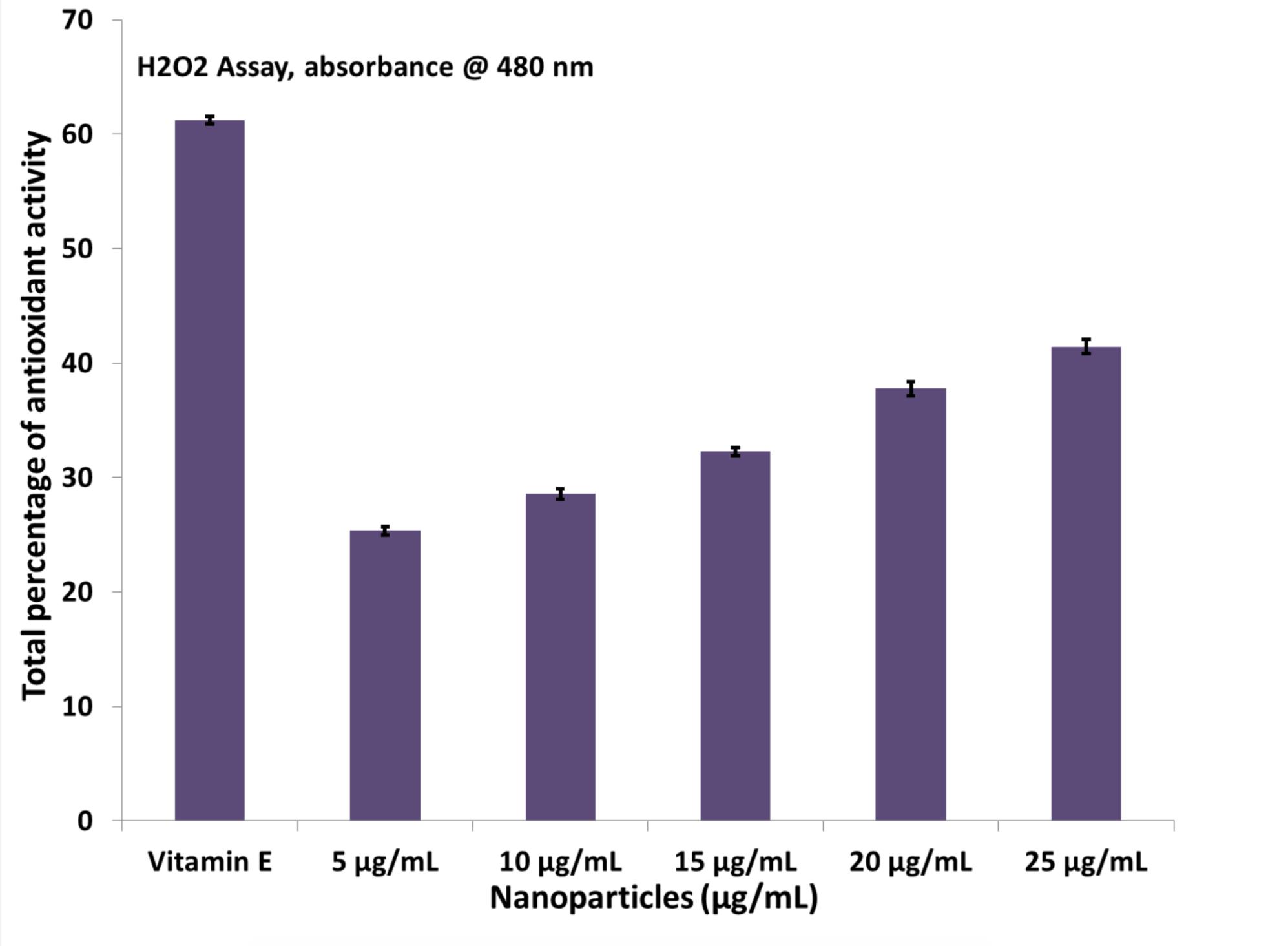


Fig 4: Antioxidant activity vs. nanoparticles

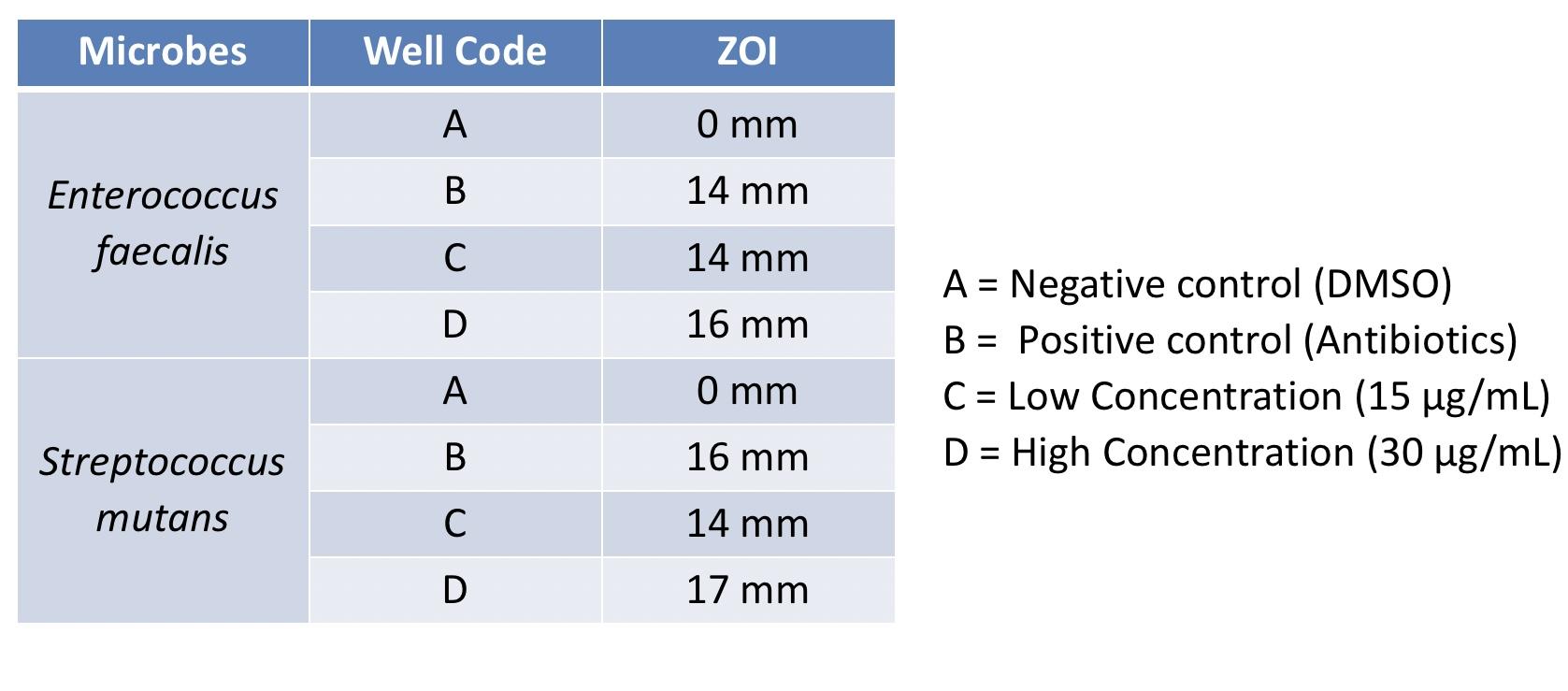


Fig 5: Microbes

# DISCUSSION

The synthesis of titanium nanoparticles (TiNPs) has gained significant attention in biomedical research due to their unique properties, including high biocompatibility, antimicrobial activity, and mechanical durability[(Li et al., 2016)](https://paperpile.com/c/ZxNbMh/iYZz). Studies have demonstrated that TiNPs improve osteoconductivity, support cellular proliferation, and enhance bone tissue regeneration, making them valuable for applications in tissue engineering[(Bharadwaz & Jayasuriya, 2020)](https://paperpile.com/c/ZxNbMh/evaF). Additionally, TiNPs have been investigated for their potential in drug delivery systems, where they not only promote tissue repair but also help prevent infections at injury sites[(Chokkattu et al., 2023; Dharman et al., 2023; Govindaraj & Shanmugam, 2023)](https://paperpile.com/c/ZxNbMh/meDU6+gZ8Oe+7obCz).Chitosan, a naturally derived biopolymer, is widely recognized for its beneficial role in tissue regeneration. Its biodegradability, biocompatibility, and ability to support cell adhesion and migration make it an ideal material for regenerative medicine.[(van den Broek & Boeriu, 2020)](https://paperpile.com/c/ZxNbMh/V1cV) Research has shown that chitosan-based scaffolds significantly accelerate wound healing, particularly in complex healing environments such as diabetic wounds, by creating a supportive microenvironment for cellular activity[(Adetunji et al., 2025)](https://paperpile.com/c/ZxNbMh/GJWo). Furthermore, chitosan exhibits antioxidant properties, which can mitigate oxidative stress at injury sites, thus contributing to a more efficient healing process[(Rajeshkumar & Lakshmi, 2021; Sivakumar et al., 2021)](https://paperpile.com/c/ZxNbMh/9lExA+7fF2d).Previous studies have reported that chitosan has been employed as an antimicrobial gel-forming agent to enhance the distribution of nanoparticles such as magnesium nanoparticles (MNPs) in wound-healing applications[(Loo et al., 2022)](https://paperpile.com/c/ZxNbMh/fiLu). In this study, we explored the potential of incorporating TiNPs into a chitosan-based composite to develop a material that not only promotes antimicrobial activity but also supports tissue regeneration, with a particular focus on soft tissue repair. The integration of TiNPs and chitosan within this composite material leverages the complementary characteristics of both components, enhancing both mechanical reinforcement and biological activity[(Wu & Ren, 2020)](https://paperpile.com/c/ZxNbMh/3tTr). TiNPs contribute to the structural integrity of the membrane while also providing antibacterial benefits, reducing the likelihood of infection—a common concern in wound healing(Rafi et al., 2024). Meanwhile, chitosan facilitates cell attachment and nutrient exchange, thereby improving biocompatibility and supporting tissue development(13).Additionally, the ability of TiNPs to act as carriers for bioactive molecules offers another layer of therapeutic potential. Studies suggest that TiNPs can facilitate the controlled release of growth factors, which play a crucial role in cellular signaling and tissue regeneration. This sustained release mechanism may further accelerate healing and improve overall tissue functionality[(Vo et al., 2012)](https://paperpile.com/c/ZxNbMh/gAwc).Titanium dioxide (TiO₂) nanoparticles, known for their antimicrobial efficacy, generate reactive oxygen species (ROS) under UV exposure, disrupting bacterial membranes and contributing to antibacterial action (Tuluwengjiang et al., 2024). Research has shown that the anatase form of TiO₂—alone or in combination with rutile or brookite—enhances antibacterial and photocatalytic properties[(Younis et al., 2023)](https://paperpile.com/c/ZxNbMh/uOx3). Our study expands on this by incorporating TiNPs into a chitosan-based composite, shifting the focus from antibacterial properties alone to also investigating its effectiveness in soft tissue regeneration. While prior studies have established that chitosan-TiO₂ composites promote wound healing through biocompatible scaffolding, facilitating cell proliferation and adhesion, our research specifically evaluates the material’s potential for soft tissue repair using a zebrafish model Unlike earlier research that primarily emphasized bacterial inhibition[(de Sousa Victor et al., 2020)](https://paperpile.com/c/ZxNbMh/LSuN), our study provides insight into the regenerative capabilities of TiNP-chitosan composites. By assessing both antimicrobial and regenerative outcomes, we highlight the potential for these materials to be utilized in clinical applications requiring both structural support and biological functionality.This study contributes to the growing body of knowledge on biomaterials by demonstrating the synergistic effects of TiNPs and chitosan in tissue engineering. The findings emphasize the importance of multifunctional biomaterials in advancing regenerative medicine, offering a promising strategy for developing safe, effective, and innovative solutions for soft tissue repair.

# CONCLUSION

The titanium nanoparticle (TiNP)-chitosan composite exhibits strong antimicrobial properties and enhances soft tissue regeneration. Its dual functionality in bacterial inhibition and tissue repair highlights its potential for biomedical applications, particularly in advanced wound healing. Further studies are needed to optimize its formulation and evaluate its effectiveness in more complex models.

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