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Determining the Applicability of Milkfish (*Chanos chanos*) for Skin Grafting through Microbiological and Histological Evaluations

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Abstract. Tilapia fish skin, rich in Type I collagen, has been shown to accelerate wound healing, reduce infection rates, and create an optimal environment for wound care. However, the potential application of other fish species, like milkfish, for skin grafting remains underexplored. Milkfish (*C. chanos*), an economically significant species in the Philippines, could expand the donor pool for fish skin grafting, addressing the accessibility issues in resource-limited regions where traditional graft materials may be costly or scarce. The current study investigates the feasibility of applying tilapia fish skin sterilization techniques to milkfish skin. Collected and prepared milkfish skin was subjected to a sterilization process using an established protocol for silver nanoparticle synthesis and characterization, to ensure sterility without compromising collagen integrity. Microbiological and histological evaluations were conducted to determine the collagen quality and sterility of milkfish skin. The results indicate that milkfish skin, after undergoing the same sterilization procedures as tilapia skin, maintained high collagen integrity and was free from microbial contamination. By demonstrating that milkfish skin can be effectively sterilized and used similarly to tilapia skin, this research provides innovative, cost-effective, and sustainable wound-healing solutions. This finding has the potential to transform wound care in underserved areas, improving patient outcomes in regions with limited access to advanced medical facilities.

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1 Introduction

Wound healing is a complex physiological process that restores skin integrity after injury. The body's natural healing mechanisms often suffice for minor injuries, but more severe conditions such as extensive burns, chronic ulcers, and traumatic injuries pose significant challenges to the body's ability to repair itself fully. In such cases, advanced interventions, including skin grafts and collagen-based therapies, have emerged as crucial solutions to facilitate wound healing and promote tissue regeneration [1-2].

Collagen, the most abundant protein in the human body, is a fundamental component of the extracellular matrix that provides structural support and scaffolding for cells involved in tissue repair. It plays a vital role in maintaining tissue integrity and facilitating cellular processes essential for wound healing, such as cell migration, differentiation, and proliferation [3-4]. The use of collagen in wound care has been transformative, providing a framework for new tissue growth and enhancing the body's natural healing capacity.

Skin grafting, a surgical procedure that involves transplanting skin from a donor site to a recipient site, is a well-established technique in regenerative medicine. It is used to treat various wounds, including burns, traumatic injuries, and chronic ulcers, by providing a protective covering that prevents infection, promotes tissue regeneration, and facilitates the formation of new blood vessels [5-6]. However, traditional sources of graft materials, such as autografts and allografts, are often limited by availability, high costs, and risks of immune rejection or infection.

Recently, the use of fish skin, particularly from *Oreochromis niloticus* (Nile tilapia), has gained attention in wound care due to its high collagen content and biocompatibility. Tilapia skin, rich in Type I collagen, has demonstrated the ability to accelerate wound healing, reduce infection rates, and provide an optimal environment for tissue regeneration. Its low immunogenicity further reduces the risk of immune rejection, making it a suitable alternative to traditional graft materials [7-8]. The successful application of tilapia skin in wound healing has sparked interest in exploring the potential of other fish species for similar uses [9-10].

Despite the promising outcomes with tilapia skin, the application of fish skin grafting techniques to other species remains underexplored. Among the economically significant fish species in the Philippines, *Chanos chanos* (milkfish), locally known as *bangus*, is one of the most widely cultivated. However, limited research has been conducted on the suitability of milkfish skin for wound care. Given its abundance and potential collagen content, milkfish skin presents a valuable opportunity to expand the donor pool for fish skin grafting [11]. Investigating its properties and comparing them with tilapia skin could provide new insights into alternative graft materials.

This study aims to explore the feasibility of using *C. chanos* skin for wound care by evaluating its collagen quality and sterility post-sterilization. By developing optimized, easily replicable sterilization methods and assessing the microbiological and histological properties of milkfish skin, the research seeks to determine whether it can serve as an effective alternative to tilapia skin. The findings could have significant implications for expanding wound care options, particularly in resource-limited settings where access to advanced medical treatments is constrained. This research not only contributes to the field of regenerative medicine but also promotes the sustainable use of underutilized resources in the Philippine aquatic industry, potentially transforming waste products into valuable medical materials.

2 Materials and methods

2.1 Sample collection

Tilapia skin was collected from fresh tilapia (*O. niloticus*) fish, and milkfish skin was collected from fresh *bangus* (*C. chanos*). Both were obtained from Cubao Farmers Market, Cubao, Metro Manila, Philippines, skinned and descaled. The skin samples were rinsed with a 0.85% normal saline solution to remove any trace of blood and other impurities. These were then placed into a normal saline solution and transected into 3 x 2 cm strips. A total of 24 fish strips were prepared.

2.2 Preparation of silver nanoparticles for sterilization

The established protocol for AgNP synthesis and characterization provided a basis for this study's subsequent experimentation and analysis [12-13]. To initiate the synthesis, 1.0 g of soluble starch was added to 100 mL of distilled water and heated until complete dissolution. Subsequently, 1 mL of 100 mM aqueous silver nitrate (AgNO3) crystals was added to the starch solution and stirred thoroughly. This reaction mixture was then subjected to autoclaving. The resulting solution exhibited a clear yellow colour, indicating successful silver nanoparticle formation.

The stock solution of AgNPs was stored in dark glass bottles and kept from direct exposure to sunlight. These bottles were maintained at room temperature (27 °C) to prevent temperature-induced alterations in the nanoparticle properties.

2.3 Sterilization procedures

The tilapia and milkfish fish strips were randomly allocated into four main groups with three replicates each: tilapia treated, tilapia untreated, milkfish treated, and milkfish untreated. Samples in the treated group were subjected to the sterilization procedure of immersion in silver nanoparticles for 5 min [13].

2.4 Microbiological evaluation

All twelve samples were individually swabbed with sterile cotton tips, followed by submersion in 10 mL nutrient broth and vortexed for 1 min. For microbial counting, the pour plate method was employed, where a diluted bacterial sample was mixed with melted agar, and this mixture was poured into a petri dish. Specifically, one mL of the sample swab solution was transferred into sterilized petri dishes, and a sterilized isolation medium was poured into the dishes. Nutrient agar was used for total aerobic bacteria, and potato dextrose agar was used for filamentous fungi. The plates were then left to incubate for 48 h at 35 C. Subsequently, the colonies were counted, and the viable cell count was calculated.

2.5 Histological evaluation

The remaining 12 treated and untreated fish skin samples were sent to the College of Veterinary Medicine-Veterinary Paraclinical Sciences Veterinary Histopathology Laboratory, University of the Philippines Los Baños, Laguna, Philippines for slide preparation and staining. The specimens were processed, embedded, and stained. After staining with hematoxylin and eosin, the slides were randomly observed under a compound microscope.

Histological evaluation to assess the integrity and organization of collagen fibres was based on a 0 to 3 score [13]. Integrity of collagen fibres refers to the structural soundness and continuity of collagen fibres within the tissue. This aspect is crucial for determining the tissue's ability to maintain its structural roles in the body. Organization of collagen fibres relates to the arrangement and alignment of collagen fibres within the tissue. Proper organization is essential for the functional biomechanics of tissue, influencing its strength and elasticity. The scoring evaluation is shown in Table 1.

Table 1. Scoring evaluation of the integrity and organization of collagen fibres

Score	Integrity	Organization
0	Continuous, long fibers, indicating excellent structural integrity	Fibers that are compact and parallel, exemplifying ideal alignment for optimal function
1	Slight fragmentation, suggesting minimal damage	Fibers that are slightly loose and wavy, indications some disarray but generally organized
2	Moderate fragmentation, showing more significant damage but still functional	Fibers that are moderately loose, wavy, and cross each other, reflecting a more disorganized structure that could affect tissue function
3	Severe fragmentation, where the structural integrity is heavily compromised	Fibers with no identifiable pattern, representing a highly disorganized structure that likely compromises tissue functionality

2.6 Statistical analysis

The statistical analysis employed to evaluate the significance of collagen integrity and organization scores in histopathological assessments involved the application of a t-test, utilizing Microsoft Excel (Microsoft 365) as the software for data processing. The objective was to discern whether there existed a statistically significant difference between the mean scores of two distinct groups, specifically a treatment group and a control group.

The collagen integrity and organization scores were gathered from the histopathological evaluations. The subsequent data preparation involved organizing the scores into their respective groups and calculating mean and standard deviation values. Assumption checks were conducted to ensure the normal distribution of scores within each group and verify the variances' homogeneity. Depending on the nature of the data, an independent samples t-test or a paired samples t-test was chosen. The t-test statistics were computed, and significance was determined by comparing the calculated t-statistic with critical values from the t-distribution table or obtaining p-values using statistical software.

3 Results

3.1 Microbiological evaluation

The findings reveal a remarkable outcome, indicating that applying AgNP to fish skin significantly reduced microbial growth, yielding a sterile bandage. The effectiveness of silver nanoparticles on microbial growth is summarized in Figures 1 and 2.

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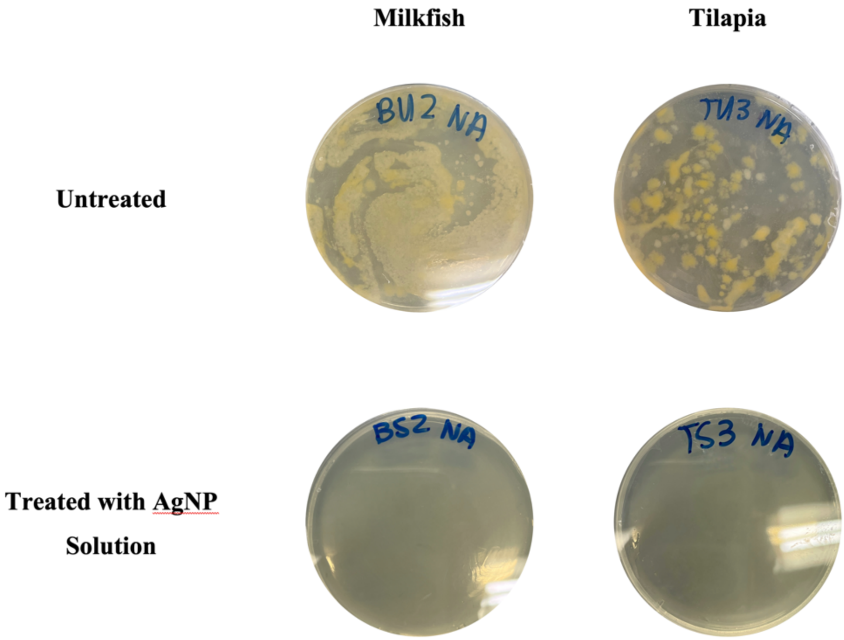

Fig. 1. Nutrient agar plates from both treated and untreated milkfish and tilapia skin. Results show complete suppression of microbial growth in AgNP-treated fish skin.

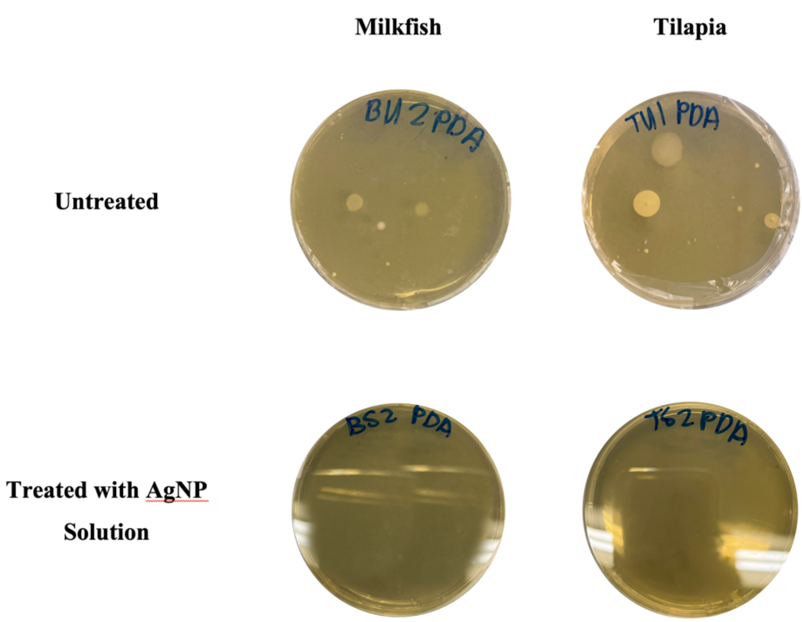

Fig. 2. Potato dextrose agar plates from both treated and untreated milkfish and tilapia skin. Results show complete suppression of microbial growth in AgNP-treated fish skin.

For untreated *bangus* fish skin strips, the average colony-forming unit (CFU) counts on nutrient agar were too many to count (TMTC) CFU/g. Meanwhile, AgNP-treated *bangus* fish skin strips consistently exhibited an absolute inhibition of microbial growth across all three replicates, with no discernible CFUs observed on the nutrient agar plates. A similar trend was observed in the case of untreated tilapia fish skin strips, where the average CFU counts on nutrient agar were TMTC CFU/gram. Similarly, AgNP-treated tilapia fish skin strips exhibited a complete suppression of microbial growth in all three replicates, with no observable CFUs.

In investigating filamentous fungi on potato dextrose agar, untreated *bangus* fish skin strips revealed an average CFU count of 16.33±4.04 CFU/g. Contrasting this, AgNP-treated *bangus* fish skin strips consistently displayed a complete inhibition of filamentous fungi growth across all replicates, with no visible CFUs on the potato dextrose agar plates. Untreated tilapia fish skin strips showed an average CFU count of 14.67±3.51 CFU/g. AgNP-treated tilapia fish skin strips mirrored the results of *bangus* fish skin strips, displaying complete inhibition of filamentous fungi growth in all replicates, with no detectable CFUs.

3.2 Histological evaluation

The histological evaluations were conducted to compare the structural integrity and organization of collagen fibres in fish skin samples from milkfish (*C. chanos*) and tilapia (*O. niloticus*) post treatment with silver nanoparticles, using hematoxylin and eosin staining techniques. These evaluations were crucial to ascertain the effects of treatments on tissue structure, particularly in terms of collagen preservation and organization.

In the histological sections of the skin, the main basis for comparing *bangus* and tilapia was the collagen fibre integrity and organization, scored from 0 to 3. The integrity of the collagen fibres was assessed based on their continuity and structural soundness, while their organization was evaluated based on the arrangement and alignment of the fibres. These criteria are essential for determining the functional biomechanics of the skin, which influences its strength and elasticity.

The histological sections of the skin revealed distinct characteristics between treated and untreated groups (Figure 3). In untreated samples, collagen fibres displayed slight to moderate disorganization, indicating potential degradation. In contrast, treated samples generally showed better preservation and alignment of collagen fibres, suggesting the effectiveness of the treatments in maintaining structural integrity.

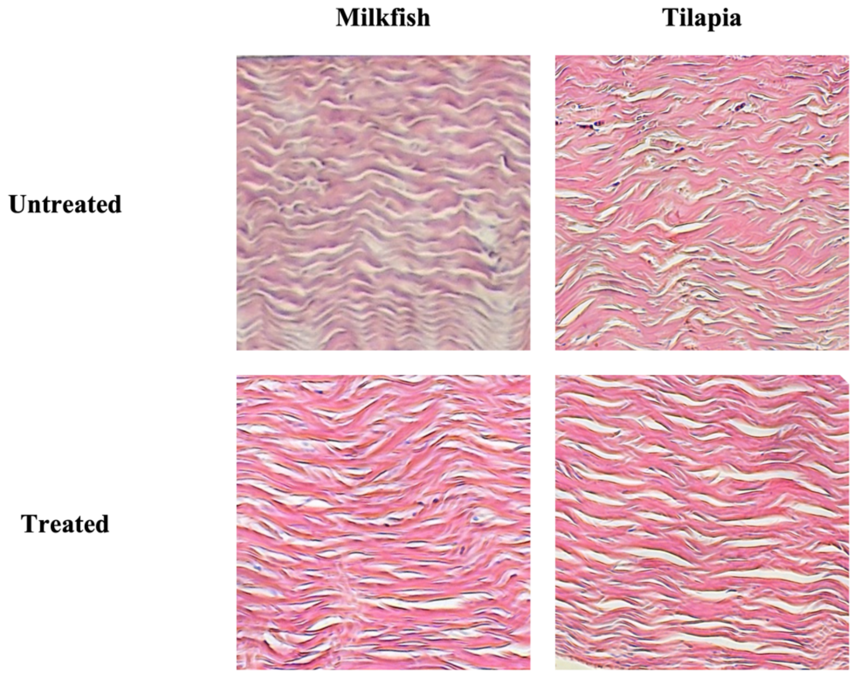

Fig. 3. Comparative histological results of milkfish and tilapia fish skins demonstrating the collagen fiber integrity and organization in treated versus untreated groups.

An indication of collagen preservation in the histological section can be observed through the appearance of continuous, long fibres (scored as 0), which signify excellent structural integrity. This is contrasted with the presence of fragmented fibres, which suggest various degrees of damage and degradation.

Statistical analysis conducted on the scores of collagen integrity and organization (as detailed in Table 2) revealed no significant difference between treated and untreated groups of both *bangus* and tilapia, indicating that the treatment was effective in maintaining the structural integrity and organization of the collagen across both species.

Table 2. Mean scoring evaluation scores of the integrity and organization of collagen fibres

Samples	Untreated	Treated
Integrity Scores		
Tilapia	1.00±0.00	0.00±0.00
Milkfish	1.00±1.00	0.00±0.00
Organization Scores		
Tilapia	1.33±0.58	0.33±0.58
Milkfish	0.67±1.15	0.33±0.58

For the integrity scores, both untreated tilapia and *bangus* exhibit comparable mean scores of 1.00±0.00. Following treatment, both species demonstrate a remarkable reduction in mean integrity scores to 0.00±0.00, indicating a potential positive impact of the sterilization process on collagen integrity.

While the organization scores, untreated tilapia shows a mean score of 1.33±0.58, whereas untreated *bangus* scores slightly lower at 0.67±1.15. Following treatment, both species exhibit a decrease in mean organization scores, both obtaining a comparable score of 0.33±0.58. The t-test results conducted indicate no statistically significant difference between the sterilized tilapia and *bangus* in terms of collagen integrity and organizational scores.

4 Discussion

The in-depth analysis of microbial and histological outcomes provides substantial support for the viability of *bangus* treated with silver nanoparticles as a viable candidate alongside tilapia bandages in wound care. The discussion delves into microbial inhibition, histological improvements, and collagen organization.

Firstly, the microbial growth inhibition demonstrated the remarkable efficacy of the one-step sterilization procedure with silver nanoparticles both in tilapia and milkfish. The untreated tilapia skin showed a significant microbial load, necessitating effective sterilization. The AgNP-treated tilapia skin exhibited complete inhibition of microbial growth, showcasing the potency of silver nanoparticles in providing a sterile environment. This trend was also mirrored in the untreated *bangus* skin, emphasizing the comparable antimicrobial efficacy of AgNP in both fish species.

These findings align with studies that elucidate the antibacterial mechanism of AgNPs. AgNPs exhibit efficacy by producing high levels of reactive oxygen species (ROS) and free radical species, including hydrogen peroxide, superoxide anion, hydroxyl radical, hypochlorous acid, and singlet oxygen [14-16]. Moreover, there was a size-dependent antibacterial effect, where smaller nanoparticles possess a larger surface area in contact with bacterial cells, facilitating their penetration into the bacteria [14].

Importantly, the safety of silver nanoparticles for animal cells is highlighted, positioning AgNPs as a safe and promising antibacterial agent against highly infectious drug-resistant bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [17-19]. Three proposed mechanisms further elucidate AgNP actions [20-22]. The first postulates their action at a membrane level, destabilizing the bacterial cell membrane and inducing leakage of cellular content. The second mechanism suggests that AgNPs not only break and cross the cell membrane and enter the cell, altering DNA and protein structures, affecting the respiratory chain, and inducing reactive oxygen species. The third mechanism proposes the release of silver ions from the nanoparticles, interacting with cellular components and altering metabolic pathways, membranes, and genetic material.

The demonstrated efficacy of AgNP in inhibiting microbial growth in tilapia and *bangus* skin aligns with the multifaceted antibacterial mechanisms proposed in the referenced studies. These mechanisms, encompassing ROS production, size-dependent effects, and safe interactions with animal cells, collectively support the potential of AgNP-treated *bangus* skin as a promising alternative to tilapia skin for sterile bandage development.

Secondly, the histological improvements observed in AgNP-treated tilapia skin were similarly reflected in treated *bangus* skin, affirming the positive impact of AgNP treatment on tissue structure. These enhancements are crucial for wound healing, indicating that AgNP-treated *bangus* skin could offer comparable benefits to the previously established AgNP-treated tilapia skin in promoting tissue regeneration. Collagen organization, a key aspect of skin integrity, demonstrated no significant differences between AgNP-treated and untreated skin for both tilapia and *bangus*. This finding is crucial, as it suggests that the AgNP treatment did not compromise the structural integrity of collagen, an essential component for effective wound dressings.

Supporting this observation, silver nanoparticles were established to represent an identical preservative of collagen integrity, particularly at a concentration of 250 µg/mL, the same concentration used to treat the tilapia and *bangus* samples [23]. Notably, this concentration was associated with higher collagen deposition than other concentrations.

Corroborating evidence from two studies indicated that increasing AgNP concentration promotes macrophage migration and higher collagen deposition, contributing to discriminatory remodelling [24-25].

Furthermore, previous studies have also emphasized the benefits of silver usage in wound healing, including reducing inflammation and scarring, inhibiting bacterial outgrowth, and enhancing the healthy healing process [26-27]. This is attributed to the direct expression of growth factors induced by silver nanoparticles, leading to vasculogenesis, re-epithelialization, and collagen fibre deposition. The transformation of fibroblasts to myofibroblasts, responsible for wound contraction and accelerated healing, is also stimulated by silver nanoparticles, along with the proliferation of keratinocytes in the injured area.

Additional studies have shown that silver-containing materials increase fibroblast proliferation within the skin [28]. Prolonged exposure of epithelial cells to silver nanoparticles has been proven to stimulate the secretion of TGF-β, further promoting the increased production and deposition of extracellular matrix proteins, including collagen [29-30].

The preservation of collagen organization in AgNP-treated tilapia and *bangus* skin was demonstrated through meticulous histological evaluations. These evaluations involved staining the skin samples with hematoxylin and eosin, followed by detailed microscopic examination. The treated samples showed a noticeable improvement in the arrangement and continuity of collagen fibers compared to untreated samples. Specifically, the collagen fibres in the treated groups exhibited greater alignment and less fragmentation, which are indicative of preserved structural integrity. These observations were quantitatively supported by lower scores in collagen integrity and organization assessments, suggesting a more compact and orderly collagen matrix. Such findings provide clear evidence of how silver nanoparticles not only counteract microbial infection but also enhance and preserve the structural properties of collagen, vital for effective wound healing. This dual functionality of AgNP reinforces its utility as a promising component in advanced wound dressing materials, aimed at both preventing infection and supporting tissue repair processes.

The consistently robust microbial inhibition observed in both tilapia and *bangus* skin, following treatment with silver nanoparticles, validates the sterilization method's effectiveness. The preservation of collagen integrity by AgNP not only emphasizes its suitability for wound care applications but directly addresses the challenges associated with traditional sterilization methods. The incorporation of AgNP emerges as a promising alternative, ensuring microbial sterility and collagen preservation. This innovation becomes particularly significant in this study's objective to determine whether *C. chanos* fish skin shares similar properties with tilapia fish skin, presenting a potential alternative for wound care applications.

Ultimately, the comparison between sterilized and unsterilized conditions for both *bangus* and tilapia skin samples yielded valuable insights into their microbiological characteristics. The results indicate that sterilization effectively reduced microbial contamination on both types of fish skin. This is crucial for potential applications in wound care, as a lower microbial load enhances the safety and efficacy of the materials used. Furthermore, the comparable performance of sterilized *bangus* and tilapia skin, measured in terms of mean collagen integrity and organization scores, suggests that both could be viable candidates for use as potential bandages. The study supports the hypothesis that *bangus* skin is on par with tilapia skin in terms of structural characteristics, affirming its potential as a wound dressing material. This finding may have implications for the development of alternative, sustainable materials for medical applications, particularly in regions where these fishes are readily available.

Moreover, this research aimed to establish optimized and easily replicable sterilization methods for fish skin grafts, specifically enhancing accessibility and promoting adoption in resource-limited healthcare settings. The study employed a streamlined approach to AgNP synthesis, utilizing a one-step method with an autoclave. This efficient and simplified process attests to the practicality of AgNP production. It ensures the stability of the resulting solution, allowing it to be preserved in the dark for up to six months [31]. This method's simplicity holds promise for easy replication and adoption in various healthcare settings, especially those with limited resources. Leveraging the accessibility of *bangus* as the national fish of the Philippines, which are readily available in wet markets nationwide, this research established concise methodologies for obtaining fresh, sterilized fish skin for wound bandages.

The straightforward AgNP synthesis method and the ubiquity of *bangus* in local markets provide a unique opportunity for enhancing wound care accessibility, potentially revolutionizing the availability of beneficial, sterilized bandages across diverse healthcare landscapes in the Philippines and beyond. This innovative approach aligns seamlessly with this study's objectives of optimizing sterilization methods for fish skin grafts, ensuring their accessibility, and fostering practical solutions for wound care.

5 Conclusions

This study effectively developed and validated a one-step sterilization process using silver nanoparticles for *C. chanos* (milkfish) skin to optimize its use as a wound dressing material. The AgNP sterilization method demonstrated complete microbial growth inhibition, confirming its effectiveness in maintaining sterility, which is crucial for wound care applications. Moreover, histological analyses showed that the collagen structure in treated milkfish skin remained intact, preserving its essential mechanical properties and scaffold function for tissue regeneration. These results indicate that AgNP-treated milkfish skin could serve as a viable, sterile, and effective material for wound dressings.

Furthermore, the study found that the collagen quality of milkfish skin post-treatment was comparable to that of tilapia skin, a well-established material in wound care. This finding highlights the versatility and potential of AgNP-treated milkfish skin as an alternative wound dressing material. The streamlined one-step process for AgNP synthesis and application enhances the accessibility of fish skin grafts, particularly in resource-limited settings. Overall, this research offers a promising, sustainable solution for wound care, expanding the availability of practical and effective medical materials in diverse healthcare environments.

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