MALAYSIAN JOURNAL OF MICROSCOPY

Journal homepage: https://malaysianjournalofmicroscopy.org

RESEARCH ARTICLE

QUALITATIVE METHYLENE BLUE MICROSCOPIC COMPARISON OF TRITON X-100 AND SDS DECELLULARIZATION PROTOCOLS FOR *CHANNA STRIATA* SKIN

Terry Previo Avianto[1,2,*], Nooraini Ahmad[3], Refan Maulana Putra[4], Afrisya Idzni Nurashiddiqy[4]

[1]*Department of Marine, Faculty of Fisheries and Marine, Universitas Airlangga, 60115, Surabaya, Indonesia.*
[2]*Post harvest, Fish Processing, and Fish Bio-Products Research Group, Faculty of Fisheries and Marine, Universitas Airlangga, 60115, Surabaya, Indonesia.*
[3]*Department of Chemistry, Faculty of Science, Universiti Malaya, 50603, Kuala Lumpur Malaysia.*
[4]*Fisheries Product Technology Study Program, Faculty of Fisheries and Marine, Universitas Airlangga, 60115, Surabaya, Indonesia.*

Abstract: Fish skin-derived extracellular matrix (ECM) has emerged as a promising biomaterial for chronic wound management and commercial grafts. Compared to mammalian xenografts, acellular fish skin demonstrates favourable clinical outcomes alongside a reduced risk of disease transmission and fewer cultural limitations. *Channa striata* (snakehead fish) skin, naturally rich in type I collagen, is an excellent candidate for developing these biopolymer dressings. Effective decellularization is critical to remove cellular material while preserving the underlying ECM architecture; however, common detergents like sodium dodecyl sulphate (SDS) and Triton X-100 impact ECM composition and mechanics differently. This exploratory study qualitatively compared three detergent-based decellularization protocols for *C. striata* skin: 1 % Triton X-100, 1 % SDS, and a combined 0.5 % Triton X-100/0.5 % SDS solution. Fresh skin was divided into matched pairs of treated and untreated groups, processed accordingly, and evaluated via methylene blue (MB) staining and bright-field microscopy. Untreated controls exhibited abundant MB-positive nuclei and dense background staining. Treatment with 1 % Triton X-100 reduced nuclear staining while largely preserving the fibrillar collagen network. Conversely, 1 % SDS produced the greatest reduction in visible nuclei but caused apparent swelling and structural disruption of the collagen architecture. The combined 0.5 % Triton/0.5 % SDS protocol achieved intermediate nuclear clearance while better maintaining collagen organization than SDS alone. These findings support MB microscopy as a rapid, qualitative screening tool for decellularization strategies. They also suggest that mixed, low-concentration Triton/SDS protocols best balance cell removal with ECM preservation in *C. striata* skin. Future quantitative DNA, glycosaminoglycan, and mechanical testing will be required to optimize this protocol for scaffold development in diabetic ulcer applications.

Keywords: Decellularization, *channa striata* skin, methylene blue microscopy, responsible consumption and production.

Article Info

Received 12 December 2025
Accepted 12 May 2026
Published 8 June 2026

***Corresponding author: terry_avianto@fpk.unair.ac.id**
Copyright Malaysian Journal of Microscopy (2026). All rights reserved.
ISSN: 1823-7010, eISSN: 2600-7444

1. INTRODUCTION

Chronic wounds, including diabetic foot ulcers, venous ulcers and pressure ulcers, remain a major global health burden and are difficult to manage with standard care alone [1, 2]. Acellular fish-skin grafts have gained increasing attention as xenogeneic skin substitutes that can accelerate wound closure, reduce scarring and improve patient quality of life [1–6]. Several systematic reviews and pre-clinical studies report that acellular fish skin enhances healing in chronic ulcers and burns, often outperforming standard dressings [2, 4, 5, 7].

Fish skin has multiple advantages as a scaffold source: its collagen-rich ECM resembles human dermis, the risk of viral and prion transmission is lower than for mammalian tissues, and there are fewer cultural or religious concerns [1, 3, 6, 7]. Cod-derived acellular dermal matrices such as Kerecis are now in routine clinical use and contain both structural ECM proteins and bioactive lipids that contribute to angiogenesis and modulation of inflammation [7-9].

Channa striata (snakehead fish) represents a valuable tropical species whose skin is rich in type-I collagen which being the part for 90 % of human body and being used extensively particularly in tissue engineering and regenerative medicine [10]. Chemical and enzymatic extraction studies show that *C. striata* skin yields high amounts of acid-soluble collagen with preserved triple-helical structure, and that cross-linking this collagen with alginate produces biopolymers with enhanced thermal stability suited for wound dressings [5]. These findings support the exploration of *C. striata* skin not only as a collagen source but also as a whole-tissue ECM scaffold for wound repair.

Decellularization is a critical step in transforming native skin into a safe and functional scaffold. The goal is to remove immunogenic cellular components while preserving key ECM constituents such as collagen, elastin, glycosaminoglycans (GAGs) and growth factors, as well as mechanical integrity [7, 11]. Reviews by previous research emphasizing that decellularization protocols must be tailored to tissue type and that detergents, particularly ionic agents, can profoundly alter ECM composition and biomechanics [7, 11].

Sodium dodecyl sulphate (SDS), a strong ionic detergent, is highly effective at solubilizing cell membranes and nuclear material but tends to denature proteins, strip GAGs and weaken tissue mechanics [6, 7]. Triton X-100, a non-ionic detergent, is milder and better preserves ECM ultrastructure but may be less efficient at removing tightly bound nuclear remnants [7,11,12]. Comparative studies in various tissues, including cornea and vascular grafts, show that SDS often achieve greater DNA reduction at the cost of GAG loss and reduced cytocompatibility, whereas Triton or low-concentration SDS/Triton combinations better preserve ECM biochemistry and structure [6,12].

Methylene blue (MB) is a cationic dye that binds nucleic acids and acidic macromolecules, making it a simple tool for visualizing residual cellular material in decellularized tissues. In addition, MB is the basis of the “methylene blue active substances” (MBAS) assay, which detects anionic surfactants such as SDS by forming an ion pair that partitions into an organic phase and can be measured spectrophotometrically [9,13,14].While MB is not a quantitative DNA assay, it provides a rapid qualitative screen for decellularization efficiency and, in MBAS format, for residual detergent.

The objective of this study was therefore to qualitatively compare three detergent-based decellularization protocols for *C. striata* skin 1 % Triton X-100, 1 % SDS, and a combined 0.5 % Triton X-100/0.5 % SDS using MB staining and light microscopy. We focused on two key outcomes relevant for scaffold design: (i) qualitative removal of MB-positive cellular material and (ii) preservation of collagen network morphology. The intent was not to provide a definitive validation of the decellularization. Instead, it was to find the protocol that best maintains ECM integrity while removing cells, setting the stage for future quantitative improvements.

2. MATERIALS AND METHODS

2.1 Sample Collection And Preparation

Fresh *Channa striata* skin was obtained from a local farmer and from the same pond and we select the same weight as possible we bring the fish alive to laboratory and being processed at the same time to ensure the same freshness between each other. The whole skin is extracted then Scales and adherent muscle were carefully removed, and the skin was rinsed with physiological saline to eliminate surface blood and debris. extracted skin to be cleaned thoroughly and washed using NaCl 0.9 % and moved to sterile container and soaked in chlorhexidine gluconate 2 % for 1 hour and be washed again NaCl 0.9 % then be soaked using glycerol 75 % and salt 25 % the placed in waterbath shaker in 37 ºC and 15 rpm and placed in vacuum plastic or sterile container and placed in 4 ºC temperature and being kept in the same temperature for 7 days process corresponding to groups. Each group contained an untreated control sample and a corresponding treated sample.

There is no need for Ethical Clearance for the use of fish as research subjects as per regulations in Indonesia.

2.2 Experimental Groups and Decellularization Protocols

Three detergent conditions with 3 replications were investigated:

- Groups 1–3: 1 % (v/v) Triton X-100 in phosphate-buffered saline (PBS), pH 7.4
- Groups 4–6: 1 % (w/v) SDS in PBS
- Groups 7–9: 0.5 % Triton X-100 + 0.5 % SDS in PBS

For each treated sample, skin pieces were fully immersed in the respective detergent solution and incubated under gentle agitation at room temperature (24 ºC). The samples were immersed in the solution for 7 days and being controlled every two days to check the changes in solution color or the skin color. After detergent treatment, samples were rinsed repeatedly into large volumes of PBS or distilled water under agitation until the rinse solutions were clear and free from visible foam. Untreated control samples were handled in parallel but remained in buffer without detergent.

2.3 Methylene Blue Staining

Following washing, all samples were stained with methylene blue. A working MB solution (for example, 0.05–0.1 % w/v in distilled water) was freshly prepared skin pieces (treated and untreated) were immersed in MB solution at room temperature, then briefly rinsed in distilled water or PBS to remove unbound dye. Stained samples were cut in 2 cm x 2 cm size taken from the middle part of the whole skin samples to avoid tears and unevenness. and were gently flattened on glass slides or placed in a transparent holder and imaged in a hydrated state. No counterstains were applied.

2.4 Light Microscopy

Bright-field images were acquired using a compound light microscope equipped with Dinolite eyepiece wit 30 mm lens. Olympus CX-23 Microscope equipped with Low to medium power objectives were used to capture both the collagen network and cell-associated staining in this research the magnification used is 40x. Fields were chosen to avoid folds and tears and to represent typical regions of each sample.Representative images from each group were assembled into composite panels showing paired untreated and treated samples to allow direct visual comparison of decellularization protocols.

2.5 Qualitative Image Evaluation

Images were evaluated qualitatively by visual inspection for:

1. Residual MB-positive nuclei – discrete, dark-blue dots or clusters embedded within the collagen matrix. [15]
2. Diffuse MB background staining – a general blue haze indicative of residual RNA or other acidic components. [15,16]
3. Collagen network morphology – continuity and organization of collagen bundles, presence of gaps or swelling, and any obvious disruption of the fibrillar pattern. [17,18]

No numerical scoring or statistical analysis was performed; the study was designed as a qualitative comparative assessment of the three protocols.

3. RESULTS AND DISCUSSION

3.1 Native Channa striata Skin (Untreated Controls)

As shown in Figure 1, untreated control samples exhibited intense MB staining. Numerous discrete, dark-blue structures, morphologically consistent with nuclei, were distributed throughout the tissue and frequently aligned along the collagen bundles. The background matrix displayed strong, diffuse blue staining that partially obscured the underlying fibrillar architecture. Additionally, the collagen network appeared dense and continuous, characterized by intersecting bundles and minimal interstitial spacing.

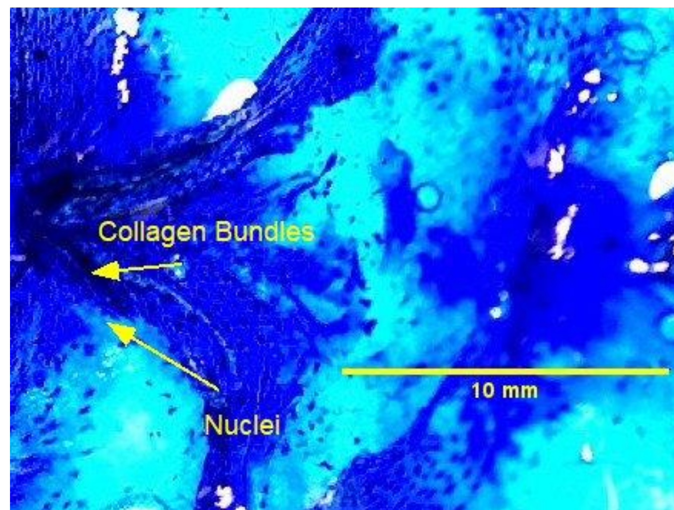

Figure 1: Light microscopy image of stained *Channa striata* ECM from an untreated control

3.2 Triton X-100 (1 %) Group

Triton X-100–treated samples exhibited a clear reduction in MB-positive nuclei compared with corresponding controls. Many fields contained only occasional small blue dots, suggesting partial removal of cellular material. However, scattered nuclear-like foci persisted, indicating incomplete decellularization at the tested conditions.

As shown in Figure 2 (A–C), the collagen architecture in the Triton-treated tissues closely resembled that of native skin. The collagen bundles remained well-organized and continuous, with no obvious swelling or large gaps. Overall, the 1% Triton X-100 treatment produced moderate nuclear clearance while maintaining good preservation of ECM morphology.

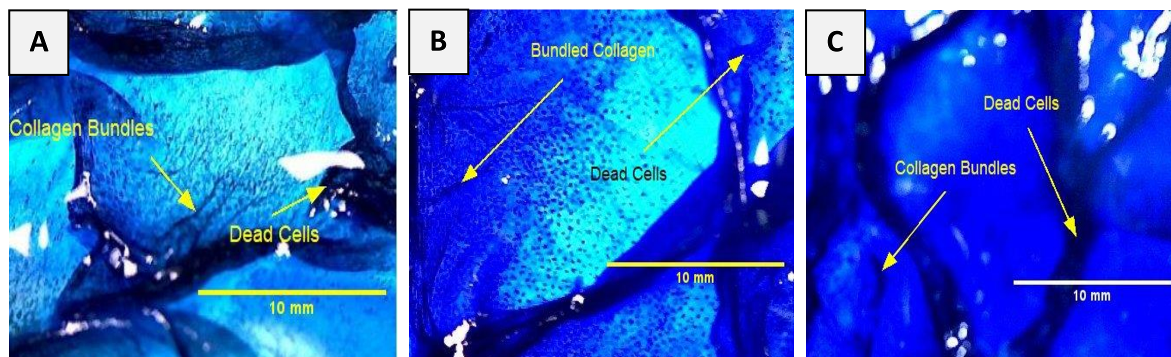

Figure 2: Light microscopy images (A–C) of stained *Channa striata* ECM treated with 1% Triton X-100.

3.3 SDS (1 %) Group

As shown in Figure 3 (A–C), samples treated with 1% SDS demonstrated a pronounced reduction in visible MB-positive nuclei. In some fields, discrete nuclear structures were nearly absent, and the overall staining appeared lighter and more uniform, indicating the extensive removal of nuclear material. However, these SDS-treated tissues also exhibited noticeable alterations in collagen morphology. The collagen network appeared loosened, characterized by widened spaces between bundles and less sharply defined fibers. In certain regions, the matrix appeared swollen or partially delaminated, which is consistent with the known tendency of SDS to denature proteins and disrupt ECM architecture. [7,11,12].

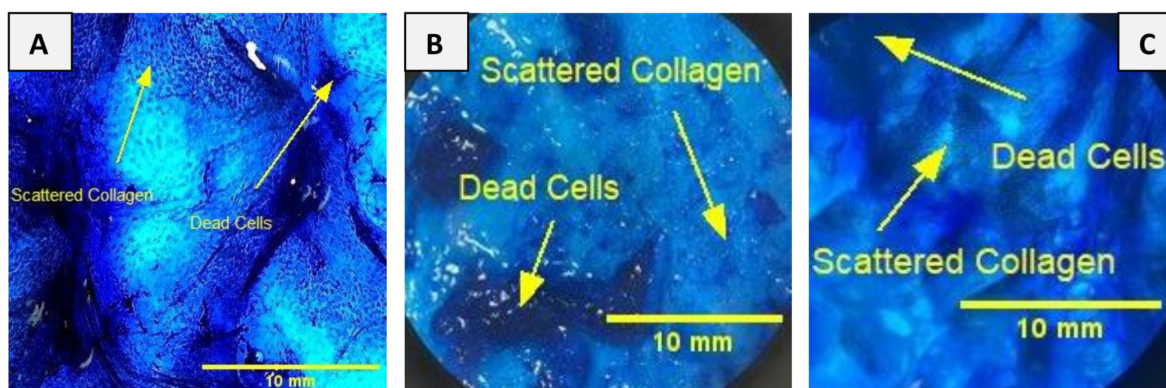

Figure 3: Light microscopy images (A–C) of stained *Channa striata* ECM treated with 1% SDS.

3.4 Combined Triton X-100 0.5 % + SDS 0.5 % Group

The mixed-detergent protocol yielded an intermediate pattern between Triton alone and SDS alone. Residual MB-positive nuclei were less frequent than in Triton-only samples but generally more evident than in 1 % SDS-treated samples. Background staining was reduced compared with controls, indicating substantial removal of cellular and soluble components.

Importantly, the collagen network appeared better preserved than in the 1% SDS groups. As shown in Figure 4 (A–C), the collagen bundles remained relatively organized, and widespread swelling

or separation was less apparent. Overall visual inspection suggested that the combined 0.5% Triton/0.5% SDS protocol achieved substantial nuclear clearance with comparatively limited ECM damage.

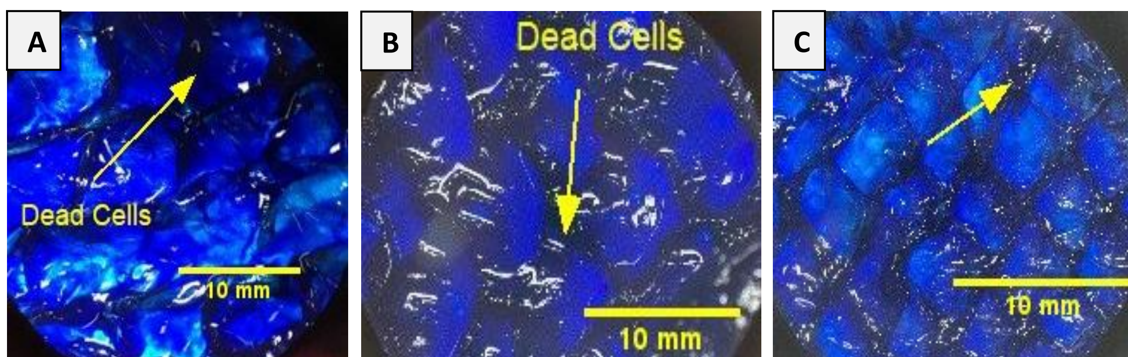

Figure 4: Light microscopy images (A–C) of stained *Channa striata* ECM treated with a combination of 0.5% Triton X-100 and 0.5% SDS.

3.5 Comparison of Detergent Protocols

The 1 % SDS protocol showed the strongest qualitative decellularization effect, with near-complete disappearance of visible nuclear staining in many fields. This is consistent with SDS's role as a powerful ionic detergent capable of solubilizing cell membranes and denaturing nuclear proteins [7]. However, SDS-treated samples also showed clear disruption of collagen architecture, manifested as swelling and loosening of the matrix. Similar SDS-associated GAG loss and mechanical weakening have been reported in ECM hydrogels and decellularized tissues [6,7,12]. For wound dressings that must withstand handling and in vivo forces, such structural compromise is a significant concern.

In contrast, the 1 % Triton X-100 protocol preserved the global collagen network with minimal visible damage, mirroring findings in other tissues where Triton maintains ECM ultrastructure and tensile properties [7,11,12]. Yet, residual MB-positive nuclei were commonly observed, indicating incomplete decellularization under the chosen conditions. Triton alone may therefore require longer exposure, repeated cycles, or combination with enzymatic treatments to meet quantitative DNA criteria such as <50 ng dsDNA/mg dry tissue [11].

The combined 0.5% Triton/0.5% SDS protocol displayed intermediate characteristics: nuclear staining was reduced more than with Triton alone, while collagen organization was better preserved than with 1 % SDS. These observations align with work in vascular and cartilage tissues where low-concentration SDS/Triton combinations effectively remove cells while limiting ECM damage [6]. For *C. striata* skin, the mixed protocol appears to offer the most favorable qualitative balance between cell removal and ECM preservation and is therefore a logical candidate for further optimization.

3.6 Role of Methylene Blue Microscopy

Methylene blue light microscopy proved useful as a rapid, low-cost means of comparing decellularization methods. The presence or absence of distinct blue nuclei provided an intuitive indicator of residual cellular material, and the collagen network could be assessed simultaneously under MB counter-stain. This is particularly valuable in early-stage protocol development, where multiple conditions must be screened before investing in more complex assays.

However, MB has important limitations. It binds not only DNA and RNA but also other acidic components, so diffuse background staining cannot be interpreted as specific for nucleic acids. Likewise, the absence of visible nuclei does not guarantee that residual DNA levels meet accepted

decellularization thresholds [7,11]. Fluorescent nuclear stains (e.g. DAPI) and quantitative assays such as Pico Green remain the gold standard for DNA assessment [7,12].

MB is also the basis of the MBAS assay for detecting anionic surfactants such as SDS, in which MB–surfactant ion pairs are extracted into chloroform or another organic solvent and measured at ~650 nm [9,13]. Applying a MBAS-type assay to wash effluents from SDS-containing protocols would enable quantification of residual detergent and help ensure that concentrations fall below cytotoxic thresholds before any recellularization or in vivo use [9,13,14]. Incorporating such detergent monitoring would be an important next step in validating SDS-containing protocols for clinical translation.

3.7 Implications for Channa striata–derived Dressings

Collagen from *C. striata* skin has already been shown to form Type-I collagen biopolymers that can be cross-linked with alginate to produce thermally stable wound dressings [5]. Combining those extraction-based approaches with whole-tissue decellularized *C. striata* skin could yield a family of related products ranging from collagen hydrogels to acellular dermal matrices. Decellularized fish-skin ECM from other species has demonstrated favorable biocompatibility and accelerated wound healing compared with porcine matrices and untreated controls [4,8,10]. An optimized *C. striata* skin scaffold could provide a locally sourced, culturally acceptable alternative for chronic wound care in regions where this species is abundant.

Based on the present qualitative findings, mixed low-concentration Triton/SDS protocols are particularly attractive: they appear to achieve meaningful nuclear clearance while retaining an organized collagen framework that should support mechanical integrity and cellular repopulation. Triton-only protocols might be further refined (e.g. extended treatment time or combination with nucleases) for applications where maximal ECM preservation is critical, whereas high-concentration SDS protocols may need substantial modification or extensive post-washing, combined with detergent assays, before being considered safe for clinical use.

4. CONCLUSIONS

Using methylene blue staining and brightfield microscopy as a rapid qualitative screen, this study compared three detergent-based protocols for decellularizing Channa striata skin. Untreated controls consistently showed abundant dark-blue nuclear staining and strong diffuse background staining, confirming the high cellular content of native skin. Among the tested methods, 1 % Triton X100 reduced nuclear staining while largely preserving the organized collagen bundle architecture. In contrast, 1 % of SDS produced the strongest apparent clearance of nuclear material but was associated with visible loosening, swelling and disruption of the collagen network. The combined 0.5 % Triton X100 + 0.5 % SDS protocol achieved substantial reduction of nuclear staining with better maintenance of collagen organization than SDS alone, suggesting a more favorable qualitative balance between cell removal and extracellular matrix preservation for scaffold development. These findings support methylene blue microscopy as an inexpensive and accessible approach for preliminary screening of decellularization conditions in fish-skin ECM. Nevertheless, qualitative loss of visible nuclei does not confirm compliance with decellularization benchmarks. Future work should quantify residual dsDNA, evaluate glycosaminoglycan retention and mechanical properties, and assess cytocompatibility, recellularization and in vivo wound-healing performance. For SDS-containing protocols in particular, detergent-removal monitoring and standardized post-wash and sterilization steps will be important to advance Channa striata acellular skin toward safe, locally sourced biomaterials for chronic wound applications such as diabetic ulcers.

Acknowledgements

The Authors Sincerely Thank the Faculty of Fisheries and Marine, Universitas Airlangga, for providing the laboratory facilities and equipment which is essential to this study.

Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

Not Applicable.

References

[1] Esmaeili, A., Biazar, E., Ebrahimi, M., Heidari, K.S., Kheilnezhad, B. & Saeedi L.F. (2023). Acellular fish skin for wound healing. *International Wound Journal*, 20(7), 2924-2941

[2] Gao, J., Ge, L. X., Gao, Q. Y., Zhang, A.M. & Hu, L. J. (2024). Efficacy of acellular fish skin graft in the management of chronic ulcer: a systematic review and meta-analysis. *Langenbeck's Archives of Surgery*, 409, 64.

[3] Heo, S. Y., Kim, T. H. Kim, S. C., Oh, G. W., Heo, S. J. & Jung, W. K. (2024). Therapeutic effect of decellularized extracellular matrix from fish skin for accelerating skin regeneration. *Marine Drugs*, 22(10), 437.

[4] Lee, V. C. (2023). Use of acellular fish skin grafts in wound healing: a literature review. *Wounds* UK, 19(4), 56–64.

[5] Issains, F. B., Trinanda, A. F. & Basyir, A. M. (2019). Extraction of collagen Type I from snakehead fish skin (*Channa striata*) and synthesis of biopolymer for wound dressing. In Proceedings of the International Symposium of Biomedical Engineering (ISBE 2019), Padang, 22-24 July 2019.

[6] Cai, Z., Gu, Y. & Xiao, Y. (2021). Porcine carotid arteries decellularized with a suitable concentration combination of Triton X-100 and sodium dodecyl sulfate for tissue engineering vascular grafts. *Cell and Tissue Banking*, 22(2), 277–286.

[7] Mendibil, U., Ruiz, H. R. & Retegi, C. S. (2020). Tissue-specific decellularization methods: rationale and strategies to achieve regenerative compounds. *International Journal of Molecular Science*, 21(15), 5447.

[8] Kim, T.H., Park, J. H., Jeong, J. W., Wee, S.Y. (2021). The utility of novel fish-skin derived acellular dermal matrix (Kerecis) as a wound dressing material. *Journal of Wound Management Research*, 17(1), 39-47.

[9] American Public Health Association (APHA). (1992). 5540 C: Anionic surfactants as methylene blue active substances (MBAS). In: *Standard Methods for the Examination of Water and Wastewater*, 18th Edition, American Public Health Association , pp. 6-8.

[10] Acharya, P. P., Kupendra, M. H., Fasim, A., More, S. S. & Murthy, V. K. (2022). A comparative assessment of collagen type 1 from silver carp (fresh water) and milk shark(marine) fish waste. 3 *Biotech*, 12, 82.

[11] Crapo, P. M., Gilbert, T. W. & Badylak, S. F. (2011). An overview of tissue and whole organ decellularization processes. Biomaterials, 32(12), 3233–3243.

[12] Fernández-Pérez, J. & Ahearne, M. (2019). The impact of decellularization methods on extracellular matrix-derived hydrogels. *Scientific Reports*, 9, 14933.

[13] Bell, J., Mottet, G., Halliday, H. & Barsh, R. (2013). Methylene Blue Active Substances (MBAS) assay for surfactants in environmental samples. (Report 081408, Kwiaht center for the historical ecology of the Salish Sea)

[14] Yeerum, C., Wongwilai, W., Grudpan, K., & Vongboot, M. (2018). Green assay of anionic surfactant via ion-association with methylene blue sorbed on polyurethane foam monolithic rod and using a smartphone. *Talanta*, *190*, 85–88.

[15] Nedu, M. E., Tertis, M., Cristea, C. & Georgescu, A. V. (2020). Comparative study regarding the properties of methylene blue and proflavine and their optimal concentrations for in vitro and in vivo applications. *Diagnostics*, 10(4), 223.

[16] Braden, A. W. H., & Austin, C. R. (1953). The distribution of nucleic acids in rat eggs in fertilization and early segmentation II. Histochemical studies. *Australian Journal of Biological Sciences*, 6(4), 665-673.

[17] Cheng, C. W., Solorio, L. D., & Alsberg, E. (2014). Decellularized tissue and cell-derived extracellular matrices as scaffolds for orthopaedic tissue engineering. *Biotechnology Advances*, 32(2), 462–484.

[18] Consolo, F., Brizzola, S., Tremolada, G., Grieco, V., Riva, F., Acocella, F., Fiore, G. B. & Soncini, M. (2013). A dynamic distention protocol for whole-organ bladder decellularization: histological and biomechanical characterization of the acellular matrix. *Journal of Tissue Engineering and Regenerative Medicine*, 10(2), E101–E112.