RESEARCH ON THE EXTRACTION AND PROCESSING OF CHICKEN EGGSHELL MEMBRANE TOWARD POTENTIAL BIOMEDICAL APPLICATIONS

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Abstract: *Eggshell membrane (ESM) is a promising waste by-product from the food processing industry, easily collected at no material cost. Rich in collagen, glycosaminoglycans, and a natural fibrous structure, ESM represents a valuable low-cost biomaterial with inherent biological functionality. Moreover, utilizing ESM aligns with the waste-to-value concept, contributing to both biomedical innovation and environmental sustainability. This study investigated the extraction and treatment of ESM by using different ratios of organic acids (acetic acid and citric acid) to improve its physical properties and evaluate its potential biomedical applications. The processing method does not require advanced fabrication technologies such as electrospinning or 3D printing, making it suitable for laboratories with limited technical resources. ESM samples were treated with acid ratios ranging from 1:1 to 1:10 and analyzed thickness, porosity, fluid absorption capacity, and surface morphology using scanning electron microscopy (SEM). Cytotoxicity tests indicated that the treated ESM is potentially suitable as a scaffold for cell culture. Based on these findings, ESM treated with a 1:8 organic acid ratio shows promising potential for applications in biomedical and pharmaceutical fields, particularly in wound dressing materials and in vitro tissue models.*

Keywords: *biomedical potential, cell culture scaffold, cytotoxicity, eggshell membrane, in vitro tissue model, low-cost biomaterial, organic acid treatment*

1. INTRODUCTION

The eggshell membrane (ESM) is a semi-permeable fibrous layer located between the eggshell and the egg white. As a waste-to-value material, ESM represents a sustainable and low-cost source of functional biopolymers, reducing environmental burden while offering potential biomedical utility. Biologically, it is naturally rich in type I collagen, glycosaminoglycans, glycoproteins, and other filamentous proteins, forming a fibrous architecture that resembles the extracellular matrix and supports cellular adhesion and regeneration. Several studies have suggested that ESM may be suitable for applications such as wound dressings, drug delivery systems, and tissue engineering scaffolds (Chen et al., 2022; Kalluri et al., 2024). However, untreated ESM typically exhibits a dense and compact structure with relatively low porosity (often below 50–55%) and limited fluid interaction (Aggarwal et al., 2024; Esmaeili et al., 2024; Torres-Mansilla et al., 2023), which restrict nutrient exchange, water uptake, and cellular infiltration. Addressing these structural gaps is therefore critical to unlock the full biomedical potential of ESM.(Aggarwal et al., 2024; Esmaeili et al., 2024)

In recent years, natural-origin biomaterials have attracted growing interest

due to their renewability, accessibility, and biocompatibility. ESM has been highlighted for its structural similarity to the extracellular matrix (ECM), which allows it to function as a biological scaffold for cell adhesion and tissue regeneration (Malahayati & Wardhani Widowati, 2024; Mensah et al., 2023; Torres-Mansilla et al., 2023). However, to fully realize these potential applications, appropriate processing methods are needed to improve its microstructure and surface properties.

One practical approach involves chemical modification using organic acids. Acetic acid and citric acid are widely used agents due to their mild reactivity, availability, and biosafety. These acids disrupt hydrogen bonding within the protein network, resulting in partial denaturation of collagen fibers and subsequently improving surface area, porosity, and hydrophilicity (Choi et al., 2021). Most existing studies, however, focus on single-acid treatments or a limited range of ratios, lacking comprehensive evaluations of how acid combinations affect the physical and biological performance of ESM.

Unlike many current tissue engineering approaches that rely on advanced fabrication technologies such as electrospinning, 3D bioprinting, cold plasma treatment, or laser ablation, the method proposed in this study does not require such complex techniques. Instead, it embraces the principle of utilizing what nature provides by developing a simple and accessible approach for processing ESM without reliance on high-tech methods.

This research investigates the effects of different acetic–citric acid ratios on the morphology, porosity, thickness, fluid absorption capacity, and cytotoxicity of ESM. It is hypothesized that the organic acid ratio plays a critical role in determining the structural and functional properties of the material. The outcomes of this study are expected to contribute to the development of a low-cost, scalable method for preparing ESM-based biomaterials for potential applications in biomedical and pharmaceutical contexts, particularly in wound dressings and *in vitro* tissue models.

2. RESEARCH METHODS

2.1. Materials

Eggshell membranes (ESM) were collected from the inner lining of commercial chicken eggs sourced in Vietnam. The eggs were not traceable to any specific breed or supplier, and the source was not subject to controlled conditions.

All chemicals used in this study were of analytical reagent (AR) grade and applied without further purification. The L-929 mouse fibroblast cell line was obtained from the American Type Culture Collection (ATCC). Cell culture experiments were conducted using DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. All procedures were performed followed laboratory biosafety protocols.

2.2. Preparation and acid treatment

The inner membranes were manually separated from commercial chicken eggshells and thoroughly rinsed with distilled water to remove residual albumen. Clean membranes were then divided into six experimental groups and immersed in mixed organic acid solutions at different volume ratios of acetic acid (Xilong Scientific, PRC) to citric acid (Xilong Scientific, PRC): 1:1, 1:2, 1:4, 1:6, 1:8, and 1:10. The treatment was conducted at room temperature (25 ± 2°C) for 24 hours.

After treatment, all samples were rinsed with distilled water until neutral pH was reached, then freeze-dried to preserve the structural integrity and biological activity of the material. The drying process was performed using a freeze dryer (BenchTop Pro, Virtis – SP Scientific, USA), simulating

the method described by (Merivaara et al., 2021). The dried samples were stored at room temperature (25 ± 2°C) for subsequent analyses.

2.3. Thickness

The membrane thickness was determined using a digital micrometer (293-240-30, Mitutoyo, Japan) with a resolution of 0.001 mm. The device was regularly calibrated and verified at a metrology laboratory accredited under ISO 17025 standards. To ensure measurement reliability and eliminate local variation, each membrane was measured at five fixed positions: top, bottom, left, right, and center. The results were used to assess material uniformity and served as a basis for analyzing correlations between thickness and other physicochemical parameters.

2.4. Fluid absorption capacity

The fluid absorption capacity of the ESM was determined by immersing dry samples in laboratory-prepared PBS solution for 24 hours. Each sample was first weighed (W0) using a analytical balance (AX224N Adventurer, Ohaus, USA), calibrated and certified by an ISO 17025-accredited calibration laboratory. After immersion, the samples were briefly rinsed several times with distilled water to remove excess salts, then soaked again in distilled water to stabilize. The samples were then removed and reweighed (W_{24}). Absorption capacity was calculated using the formula which following a gravimetric method commonly applied in ESM studies (Esmaeili et al., 2024; Torres-Mansilla et al., 2023):

$$Absorption\ (\%) = \frac{W_{24} - W_0}{W_0} \times 100$$

2.5. Porosity

The porosity of the ESM samples was estimated using a gravimetric method. Each dry sample was weighed (W_0) and then fully immersed in distilled water until saturation. After soaking, surface water was gently blotted using filter paper, and the sample was weighed again (W_1) using the same calibrated analytical balance as described in the previous section. The geometric volume (V) of each sample was calculated based on its measured thickness and surface area. The volume of water retained within the porous structure was calculated as the absorbed water mass divided by the density of water (ρ = 1 g/cm³). Porosity was then expressed using the following equation which following a gravimetric method commonly applied in ESM studies (Esmaeili et al., 2024; Torres-Mansilla et al., 2023):

$$Porosity\ (\%) = \frac{W_1 - W_0}{\rho \times V} \times 100$$

2.6. Surface morphology

The surface morphology of the treated ESM samples was analyzed using a scanning electron microscope (SEM, Zeiss, Germany) at a magnification of 2000×. SEM analysis enabled detailed observation of the microstructure, including fiber organization, pore distribution, fiber-fiber connectivity, and surface uniformity. These characteristics are crucial in assessing the material's potential for biomedical applications, particularly as scaffolds for cell culture or as bioactive wound coverings. The acquired micrographs served as a basis for comparing the effects of different organic acid ratios on the structural integrity and surface features of the ESM.

2.7. Cytotoxicity

The indirect cytotoxicity of the treated ESM materials was evaluated based on the effect of extraction media on cell viability and proliferation. Prior to testing, the ESM samples were sterilized using gamma irradiation at a dose of 25 kGy and subjected to sterility testing in accordance with the Vietnamese Pharmacopoeia V to ensure no microbial contamination during *in vitro* assessment.

The preparation of extraction media

followed the guidelines outlined in ISO 10993-12. Each ESM sample was incubated in cell culture medium under sterile conditions at 37°C for 24 hours. The resulting extracts were then applied to mouse fibroblast L-929 cells and incubated under standard culture conditions (37°C, 5% CO_2) for another 24 hours. Cell viability was assessed using the MTT assay, and absorbance was measured at 570 nm. The relative growth rate (RGR) was calculated using the following equation:

$$RGR\ (\%) = \frac{OD_{ESM}}{OD_{negative\ control}} \times 100$$

According to ISO 10993-5, a material is considered non-cytotoxic if RGR ≥ 70%; otherwise, it is classified as cytotoxic.

2.8. Statistical analysis

Data are expressed as mean ± standard deviation (SD), and the coefficient of variation (CV%) was calculated as (SD/ mean × 100) to indicate data stability among replicates.

3. RESULTS AND DISCUSSION

3.1. Thickness

The thickness of ESM samples exhibited clear variation depending on the acid treatment ratio (Figure 1). Among the tested groups, the 1:8 ratio (1 part acetic acid to 8 parts citric acid) resulted in the highest average thickness (0.072 ± 0.004 mm), with statistically significant differences compared to all other ratios ($p < 0.05$). This group also demonstrated consistent values across replicates, indicating processing stability. The lowest thickness was observed in the 1:1 group (0.051 ± 0.002 mm), while intermediate values were recorded for the other ratios (ranging from 0.054 to 0.068 mm).

Thickness is a critical physical parameter for ESM intended for biomedical use, particularly in wound dressing and soft tissue scaffolding. Membranes with insufficient thickness may lose mechanical integrity in wet conditions, whereas excessively thick membranes may hinder flexibility and permeability. The 1:8 group presented the highest average thickness (0.072 ± 0.004 mm), with a coefficient of variation (CV%) of 5.6%, indicating consistent measurement across replicates. This group therefore represents a desirable balance between robustness and flexibility, making it a promising candidate for further development.

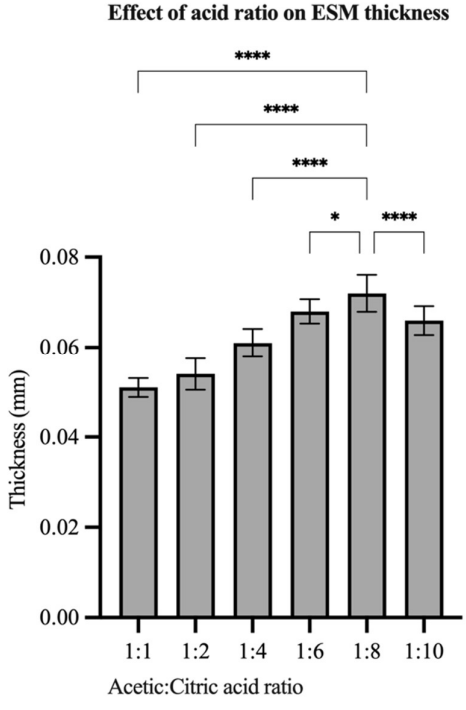

Figure 1. Thickness of ESM after acid treatment

*Data are presented as mean ± SD and CV% (n = 3). Asterisks indicate statistically significant differences: *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001.*

These findings are consistent with previous reports. Carboxylic acid treatment has been shown to enhance the biological performance of ESM in wound healing applications (Choi et al., 2021). Although that study did not evaluate different acid ratios, it supports the potential of acid-modified ESM for regenerative medicine. Our identification of the 1:8 ratio as the optimal

condition underscores the importance of fine-tuning treatment parameters to obtain membranes with favorable structural and functional properties through a simple and accessible protocol.

3.2. Fluid absorption capacity

The fluid absorption capacity of ESM samples showed a progressive increase with higher proportions of citric acid in the treatment solution (Figure 2). The 1:8 acetic:citric acid group demonstrated the highest absorption value (252.8 ± 3.9%, CV = 1.54%), followed by the 1:10 group (228.5 ± 4.4%, CV = 1.93%). These values were significantly higher than those recorded in the 1:1 and 1:2 groups (188.4 ± 4.1%, CV = 2.18% and 194.2 ± 3.5%, CV = 1.80%, respectively), suggesting enhanced hydrophilicity and swelling behavior associated with increased citric acid content.

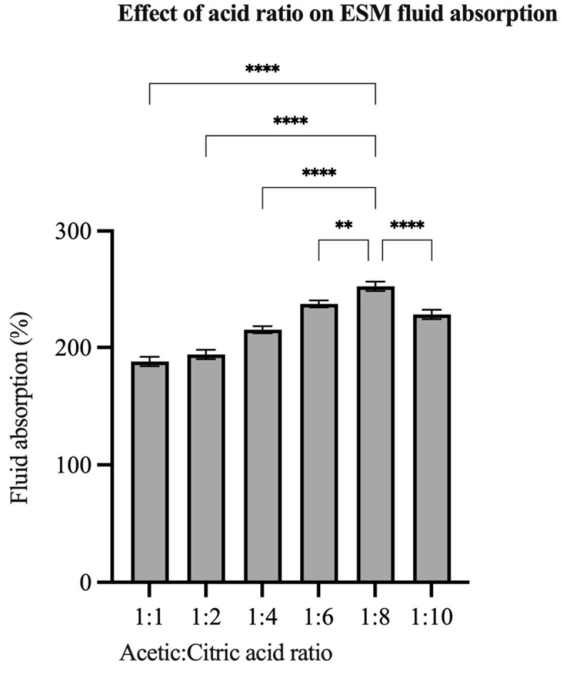

Figure 2. Fluid absorption capacity of ESM after acid treatment

*Data are presented as mean ± SD and CV% (n = 3). Asterisks indicate statistically significant differences: *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001.*

Although citric acid plays a dominant role in improving fluid retention, further increase beyond the 1:8 ratio (i.e., in the 1:10 group) resulted in slightly reduced absorption, likely due to excessive structural softening or collapse. Therefore, 1:8 appears to offer the best balance between porosity, fluid retention, and material integrity.

Statistical analysis confirmed that the 1:8 group exhibited a significantly higher fluid absorption capacity compared to all other experimental groups ($p < 0.05$). This highlights the effectiveness of the 1:8 ratio in modifying ESM for moisture-sensitive biomedical applications such as wound dressings or hydrated scaffolds.

3.3. Porosity

Porosity is a key determinant in evaluating the biomedical applicability of membrane-based biomaterials, especially in contexts requiring moisture retention, nutrient exchange, and cellular infiltration. In this study, the porosity values of ESM samples exhibited a clear upward trend with increasing citric acid content (Figure 3). The 1:8 acetic:citric acid group exhibited the highest porosity (72 ± 2%, CV = 2.78%), followed by the 1:6 group (65 ± 2%, CV = 3.08%). In contrast, lower-ratio groups such as 1:1 and 1:2 recorded markedly reduced porosity values (49 ± 2%, CV = 4.08% and 51 ± 3%, CV = 5.88%, respectively). These results highlight that increased citric acid treatment enhances porosity and structural openness, which are desirable features for biomedical applications.

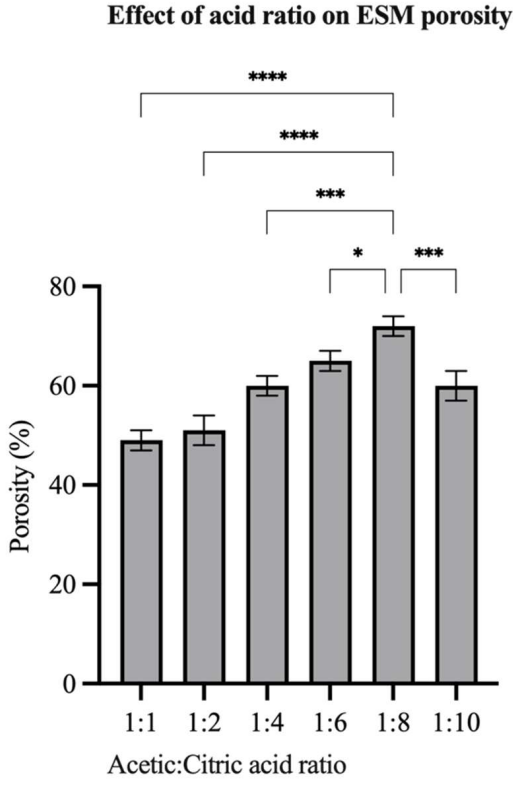

Figure 3. Porosity of ESM after acid treatment

*Data are presented as mean ± SD and CV% (n = 3). Asterisks indicate statistically significant differences: *p < 0.05, **p < 0.01, ***p < 0.001, ***p < 0.0001.*

This progressive increase is likely due to enhanced disruption of inter-fiber hydrogen bonding and partial denaturation of collagenous proteins under mildly acidic conditions, particularly when citric acid concentration is sufficiently high. The resulting structural loosening facilitates the formation of an open, sponge-like architecture — a favorable characteristic for applications in wound dressings or scaffold matrices.

Importantly, statistical analysis confirmed that the porosity of the 1:8 group was significantly higher than that of all other treatment groups ($p < 0.05$). This indicates that the 1:8 acid ratio not only improves porosity but does so in a reproducible and statistically robust manner, positioning it as the optimal treatment condition for enhancing material permeability and biological responsiveness.

3.4. Surface morphology

The surface morphology of the ESM samples before and after acid treatment was visualized using SEM at 2000× magnification (Figure 4). The untreated sample exhibited a dense and compact fibrous network with minimal porosity and poor surface roughness (Figure 4A). In contrast, the 1:8 acetic: citric acid-treated sample displayed a clearly loosened structure with increased fiber separation, well-distributed pores, and an open mesh-like appearance (Figure 4B).

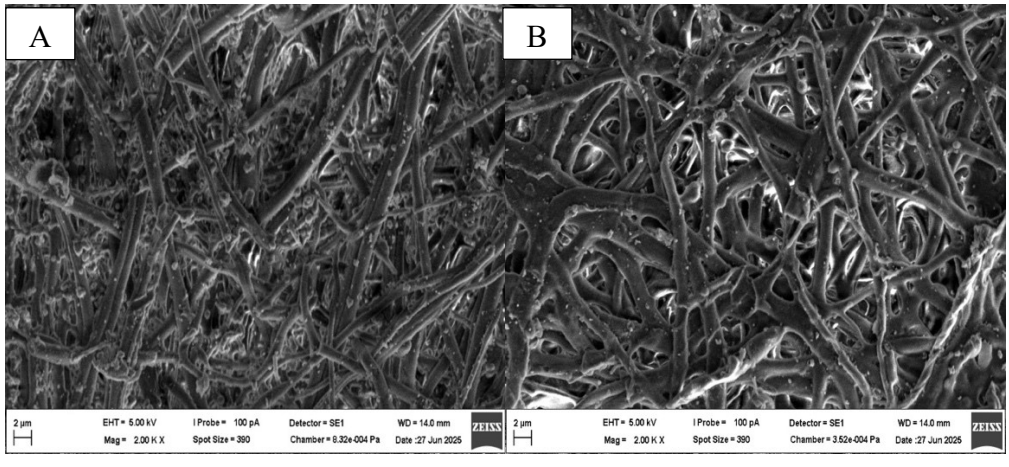

Figure 4. SEM images of ESM surface morphology before and after acid treatment

(A) Untreated ESM: Dense and compact fiber structure with limited visible porosity
(B) Treated ESM (1:8 acetic:citric): Loosely arranged fibers with expanded gaps and increased pore density, forming an open porous network

This morphological transformation can be attributed to the acid blend's ability to disrupt cross-linking within protein matrices, especially collagen and glycoproteins, resulting in increased pore formation (Choi et al., 2021). In addition, the use of sublimation drying preserved the delicate porous architecture without collapsing the matrix (Merivaara et al., 2021).

This finding is consistent with multiple prior studies. Previous reports demonstrated that carboxylic acid-treated ESM promoted wound healing and fibroblast attachment (Choi et al., 2021), and emphasized the versatility of ESM as a sustainable scaffold for biomedical applications (Mensah et al., 2023). Processed ESM has also been successfully applied in wound healing models (Ahmed et al., 2019), while its porous and biocompatible characteristics have been reported to support tissue regeneration (Torres-Mansilla et al., 2023). The structural changes observed in our 1:8 treatment group reflect these principles, showing improved microstructure suitable for cell interaction and fluid transport.

In conclusion, the 1:8 acid treatment provides an effective, low-cost approach to enhance ESM microarchitecture, aligning with existing literature and supporting its use as a bioactive scaffold.

3.5. Cytotoxicity

The cytotoxicity of the ESM sample treated with a 1:8 acetic:citric acid ratio was evaluated using an extract-based method with L-929 fibroblast cells, in accordance with ISO 10993-5 and ISO 10993-12 standards. The membrane was sterilized by gamma irradiation at 25 kGy and confirmed sterile following the Vietnamese Pharmacopoeia V. For extraction, samples were immersed at a ratio of approximately 3 cm^2/mL of surface area of culture medium, as recommended by ISO 10993-12.

MTT assay was used to determine cell viability under aseptic conditions. The negative control consisted of untreated L-929 cells (cell-only), while the positive control was 20% DMSO. The treated ESM group exhibited Relative Growth Rate (RGR) values ranging from 92% to 97%, significantly exceeding the 70% threshold for cytotoxicity classification. In contrast, the positive control group showed RGR values between 20% and 23%, confirming the assay's sensitivity and reliability.

These findings strongly indicate that the processed ESM is non-cytotoxic and possesses excellent in vitro biocompatibility. The high RGR suggests that the acid treatment and freeze-drying processes did not produce harmful residues. Overall, the result supports the biosafety and potential biomedical applicability of the treated ESM, particularly as a scaffold for cell culture and tissue engineering.

4. CONCLUSION AND IMPLICATIONS

In summary, this study demonstrated that controlled acid treatment and freeze-drying enhanced the physicochemical and biological properties of chicken eggshell membrane (ESM), improving its hydrophilicity, porosity, and structural performance. The treated ESM exhibited excellent cytocompatibility with fibroblast cells, supporting its potential as a low-cost, waste-to-value biomaterial for applications such as wound dressings and soft tissue scaffolds.

Nevertheless, some limitations should be acknowledged. The scope of the present work was restricted to a limited set of acid ratios, and no systematic optimization strategy was employed. In addition, the study did not incorporate in silico modeling approaches that could help reduce unnecessary experimental conditions and provide predictive insights for comparison with in vitro results. Furthermore, mechanical properties such as

tensile strength, elasticity, and degradation rate were not investigated, which are crucial parameters for biomedical translation.

Despite these limitations, the current findings provide a strong foundation for further research. Future work should integrate computational modeling with experimental validation, expand the evaluation of physicochemical and mechanical properties, and explore scalable processing strategies to accelerate the translation of ESM into biomedical applications.

Author contributions and authors' declaration

Thi Thanh Lan Le and Viet Hoang Le: Conceptualization, methodology design, experimental supervision, final manuscript revision

Minh Tien Nguyen: Data analysis, writing – original draft, visualization

Thi Hong Lien Nguyen, Huynh Tan Tran, and Kieu My Nguyen: Material preparation, experimental procedures, data collection

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All authors have read and approved the final version of the manuscript. The authors declare that this manuscript was reviewed with the assistance of an artificial intelligence (AI) tool, limited to proofreading and grammatical correction. All scientific content, analyses, and interpretations are entirely the work and responsibility of the authors.

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NGHIÊN CỨU THU NHẬN VÀ BIẾN TÍNH MÀNG VỎ TRỨNG GÀ ĐỊNH HƯỚNG ỨNG DỤNG TRONG Y SINH HỌC

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Tóm tắt: *Màng vỏ trứng (ESM) là phế phẩm tiềm năng từ ngành công nghiệp chế biến thực phẩm, có thể được thu gom dễ dàng với số lượng không hạn chế. Nghiên cứu này khảo sát quy trình thu nhận và biến tính ESM bằng hỗn hợp acid acetic : acid citric nhằm cải thiện các đặc tính vật lý và đánh giá tiềm năng ứng dụng trong y sinh học. Phương pháp biến tính này cũng không đòi hỏi trang thiết bị hiện đại, phù hợp với mọi phòng thí nghiệm. Các mẫu ESM được biến tính với hỗn hợp acid acetic : acid citric tỷ lệ từ 1:1 đến 1:10 và được xác định độ dày, độ trương, độ xốp và hình thái bề mặt. Kết quả thử nghiệm độc tính tế bào cho thấy ESM sau biến tính có tiềm năng sử dụng làm giá thể nuôi cấy tế bào. Dựa trên các kết quả thu được, ESM đã được biến tính với hỗn hợp acid acetic : acid citric tỷ lệ 1:8 hoàn toàn có tiềm năng ứng dụng đáng kể trong các lĩnh vực y sinh và dược học, đặc biệt là trong vật liệu phủ vết thương và kỹ nghệ mô in vitro.*

Từ khóa: *độc tính tế bào, khung nâng đỡ tế bào, kỹ nghệ mô in vitro, màng vỏ trứng, vật liệu sinh học, vật liệu y sinh*

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Ghi chú

Các tác giả xác nhận không có tranh chấp về lợi ích đối với bài báo này.