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Effect of Nutmeg on Collagen Remodeling and Epithelialization Using Picrosirius Red and Gold-Orange Staining

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Abstract
Burn injuries, especially second-degree burns, remain a global health concern. Their complex healing mechanisms and socioeconomic burden make them challenging to treat. This study evaluates the effects of 3% nutmeg (*Myristica fragrans*) cream on collagen remodeling and epithelial regeneration in grade II burns, using advanced histological techniques. A post-test-only experimental design was used. Five groups were included: normal control, untreated burn, burn treated with nutmeg cream, burn treated with silver sulfadiazine (SSD), and burn treated with both nutmeg cream and SSD. Collagen subtype distribution was examined using Picrosirius Red–Fast Green staining under polarized light. Epithelialization was assessed using Gold-Orange staining. Results showed that nutmeg cream significantly enhanced the transition from collagen type III to type I. It improved epithelial thickness and promoted keratohyalin granule formation compared to untreated burns. The combination therapy improved some histological features but did not consistently outperform single therapy by day 18. These findings indicate that nutmeg cream supports extracellular matrix remodeling and epithelial maturation. This validates its traditional use and suggests its potential as an accessible adjunctive therapy. Overall, this study underscores the value of integrating plant-based bioactives with standard treatments to improve burn wound healing.

Keywords: Collagen remodeling, epithelialization, nutmeg, Picrosirius Red staining, Gold-Orange staining

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Efek Pala terhadap Remodeling Kolagen dan Proses Epitelisasi Luka Bakar Menggunakan Pewarnaan Picrosirius-Red dan Gold-Orange

Abstrak
Luka bakar, khususnya luka bakar derajat II, masih menjadi masalah kesehatan global. Mekanisme penyembuhan yang kompleks serta beban sosial ekonomi yang ditimbulkan menjadikannya sulit untuk ditangani. Penelitian ini bertujuan untuk mengevaluasi pengaruh krim pala (*Myristica fragrans)* 3% terhadap remodeling kolagen dan regenerasi epitel pada luka bakar derajat II menggunakan teknik histologi lanjut. Metode penelitian ini adalah desain eksperimental *post-test only*. Terdapat lima kelompok, yaitu kontrol normal, luka bakar tanpa perlakuan, luka bakar yang diberi krim pala, luka bakar yang diberi silver sulfadiazine (SSD), serta luka bakar yang diberi kombinasi krim pala dan SSD. Distribusi subtipe kolagen dianalisis menggunakan pewarnaan *Picrosirius Red–Fast Green* di bawah cahaya terpolarisasi. Proses epitelisasi dinilai menggunakan pewarnaan Gold-Orange. Hasil penelitian menunjukkan bahwa krim pala secara signifikan meningkatkan transisi kolagen tipe III menjadi kolagen tipe I. Krim pala meningkatkan ketebalan epitel dan mendorong pembentukan granula keratohialin dibandingkan dengan luka bakar tanpa perlakuan. Perlakuan kombinasi tidak selalu melampaui terapi tunggal di hari ke-18. Hasil menunjukkan bahwa krim pala mendukung remodeling matriks ekstraseluler dan pematangan epitel. Hasil ini memvalidasi penggunaan tradisional krim pala dan menunjukkan potensinya sebagai terapi tambahan yang mudah diakses. Penelitian ini menegaskan pentingnya integrasi senyawa bioaktif berbasis tanaman dengan terapi standar untuk meningkatkan penyembuhan luka bakar.

Kata Kunci: Epitelisasi, pala, pewarnaan Picrosirius Red, pewarnaan Gold-Orange, remodeling kolagen.

1. Introduction

Burn injuries represent a major global health challenge, particularly in low- and middle-income regions where limited healthcare access and inadequate safety regulations often lead to delayed treatment and prolonged recovery.[1] Second-degree burns, with damage to the epidermis and partial dermis, cause prolonged healing and increased risk of infection. Most burns occur in developing regions, often exacerbated by delayed treatment and inadequate care. Burn survivors also face productivity loss and long-term rehabilitation needs.[2]

Healing of second-degree burns involves tightly coordinated inflammatory, proliferative, and remodeling phases, characterized by dynamic collagen deposition and remodelling.[3] Type III collagen dominates early, providing a scaffold for regeneration, and is gradually replaced by type I during remodeling to enhance tissue strength. Proper transition between these subtypes is critical for recovery, as disruptions may lead to hypertrophic scarring and deformities. Understanding these changes is essential for improving burn wound therapies.[4,5]

Silver sulfadiazine (SSD), a conventional topical therapy, has long been used to treat wound infection and promote healing. However, evidence indicates that SSD has limitations, including delayed epithelialization, impaired collagen remodeling, and systemic toxicity.6 While antimicrobial, SSD may impede key biological processes needed for matrix formation and epithelial regeneration, potentially extending recovery.[7] These drawbacks have driven interest in alternatives that support wound repair without adverse effects.

Herbal-based therapies have emerged as accessible, affordable options for burn management.[8–10] *Myristica fragrans* (nutmeg), traditionally used in Aceh, Indonesia, has attracted attention for its anti-inflammatory, antioxidant, and antibacterial properties.[11–14] Studies identify myristicin, eugenol, and elemicin as active constituents that reduce oxidative stress, modulate inflammation, and promote fibroblast activity, thereby supporting healing processes such as collagen synthesis and epithelialization.[14] These mechanisms suggest nutmeg may offer a beneficial alternative to conventional treatments for burn wounds. Despite these promising indications for nutmeg, previous investigations assessing nutmeg-based formulations have been limited by the use of Hematoxylin–Eosin (HE) staining, which provides insufficient detail to distinguish collagen subtypes or to evaluate epithelial maturation. As collagen dynamics are critical indicators of healing progression, more specific histological techniques are needed to provide robust evidence of nutmeg's therapeutic effectiveness.[15] Picrosirius Red staining, viewed under polarized light, precisely differentiates between collagen types I and III by their birefringence patterns. Gold-Orange staining enhances visualization of keratinization and granule formation within the stratum granulosum, providing a more accurate assessment of epithelial regeneration.[16] Integrating these techniques offers a comprehensive evaluation of burn wound healing at the microscopic level, using natural, multifunctional agents that support wound-healing pathways while minimizing toxic side effects.[15]

Therefore, this study analyzes grade II burn wound healing. The study introduces a novel combination of histological techniques to quantify collagen subtypes and assess epithelial maturation. By comparing nutmeg cream alone, SSD alone, and their combination, the research aims to clarify efficacy, identify potential synergistic effects, and inform the development of safe, affordable, and scientifically validated interventions for burn injuries. This study bridges an essential need in providing specific histological data about collagen remodeling and epithelial maturation with nutmeg-based treatment.

2. Materials and Methods

2.1. Tools

This study utilized several tools and equipment, including light microscopeDM 1000 (Leica®), rotary microtome HistoCore Nanocut (Leica®), paraffin embedding station (Leica®), HistoCore water bath (Leica®), oven (Memmert ®), slide box, mask, handscoons (gloves), slide glass, cover slip, and Image J Software.

2.2. Materials

Skin tissue samples were obtained from a prior study by Angilia et al.[14]Paraffin-embedded skin tissue blocks were used for histological analysis. Collagen remodeling was evaluated using Picrosirius Red Direct Red 80 (dye content 25 %) staining, combined with Fast Green, while epithelialization was assessed using Gold Orange staining combined with Mallory trichrome. All reagents, including Picrosirius Red (Direct Red 80), Fast Green, Gold Orange, hematoxylin, and eosin, were of analytical grade and were supplied by Sigma-Aldrich® (St. Louis, MO, USA). Materials for staining included xylene, absolute ethanol, 90% ethanol, 80% ethanol, and 70% ethanol, Hematoxylin, acid alcohol, ammonia solution, distilled water, picric, entellan, acid fuchsin, and aniline blue–orange G.

2.3. Methods

2.3.1. Study Design

This study used a post-test-only experimental design to evaluate the effects of topical treatments on grade II burn healing. The study was approved by the institutional ethical committee of the Faculty of Veterinary Medicine, Universitas Syiah Kuala (No. 25/KEPH/5/2023). Skin tissue samples were obtained from a prior study by Angilia et al..14 There were five experimental groups: normal control (N), burn without treatment (LB), burn treated with 3% nutmeg cream (LB+P), burn treated with SSD (LB+SSD), and burn treated with both 3% nutmeg cream and SSD (LB+P+SSD). These groups enabled comparison of single- and combination-therapy approaches. Sample size was determined using the degrees of freedom (DF) method for ANOVA, indicating that a minimum of four rats per group was required. Skin tissues from four rats per experimental group were evaluated, and histological analysis was performed across five representative fields of view for each sample. Histological evaluation was performed at two time points: day 6 (proliferative phase) and day 18 (remodeling phase) after injury. A 3% cream of nutmeg seed (*Myristica fragrans* Houtt.) was formulated specifically for the study. The cream was applied to the designated treatment group. Silver sulfadiazine (SSD), an established topical antimicrobial agent, served as the comparator treatment. For the combination group, nutmeg cream and SSD were administered concurrently (ratio 1:1) to evaluate potential synergistic effects. All treatments began immediately after burn induction and continued until tissue harvesting on day 6 or day 18.

2.3.2. Histological Staining and Outcome Measures

This study employed several histological staining procedures for tissue analysis, namely Hematoxylin–Eosin (H&E), Picrosirius Red-Fast Green, and Gold Orange-Mallory stains. Based on H&E staining, a semi-quantitative analysis was performed to assess collagen fiber density and epithelial thickness (Table 1). Collagen remodeling was assessed using Picrosirius Red combined with Fast Green staining, which offers improved specificity for distinguishing collagen subtypes. Under polarized light microscopy, type III collagen appears greenish-yellow, whereas type I collagen appears bright red or orange, enabling detailed visualization of extracellular matrix organization. Epithelialization was evaluated using Gold-Orange staining, which enhances visualization of keratinocyte maturation and keratohyalin granule formation in the stratum granulosum.

Table 2 was used for a quantitative analysis to assess the percentage of type I and type III collagen fibers, whereas epithelization in Gold Orange + Mallory–stained sections was evaluated by counting keratohyalin granules in the stratum granulosum and categorizing them into five scores: 0–20 (very few/absent granules), 21–40 (initial, unevenly distributed granules), 41–60 (moderate granules with more homogeneous distribution), 61–80 (numerous granules clearly visible throughout the layer), and 81–100 (abundant, large granules resembling mature epithelial tissue).

2.3.3. Data Analysis

All measurements were conducted throughout multiple microscopic fields (five fields per slide) to minimize sampling bias. Semi-quantitative scores were treated as ordinal data, and both percentage-area measurements and granule counts/scores were treated as quantitative outcomes. Data are expressed as mean ± SD, with statistical significance assumed at $p < 0.05$.

3. Results

3.1. Collagen Fiber Density and Distribution

Histopathological examination revealed distinct differences in collagen fiber density and organization among the experimental groups. Figure 1 illustrates representative histopathological features on day 18 with HE staining. In the untreated burn group (LB),

Table 1. Scoring criteria for HE staining

	Parameters and description	Score
1.	Collagen fiber density (compared with the normal group)	
	Very sparse collagen fibers	0
	Sparse collagen fibers	1
	Moderate collagen fiber density (normal/ reference)	2
	Dense collagen fibers	3
2.	Epithelial thickness (relative to the normal group)	
	Very thin epithelium	0
	Thin epithelium	1
	Normal/moderate thickness (normal/ reference)	2
	Thick epithelium	3

Table 2. Scoring criteria for the percentage of type I and type III collagen fibers

Collagen Type	Percentage of Stained Collagen Area	Color Under the Microscope (Polarized Light)	Fiber Description
Type I	0 - 100%	Bright red to orange	Thick, dense fibers; tightly packed; parallel arrangement
Type III	0 - 100%	Green to yellowish-green	Fine, thin fibers; irregular/ disorganized arrangement

collagen fibers appeared sparse, fragmented, and irregularly distributed, indicating impaired extracellular matrix remodeling following second-degree burn induction. In contrast, animals treated with 3% nutmeg cream (LB+P), silver sulfadiazine (LB+SSD), or the combination therapy (LB+P+SSD) showed markedly improved collagen deposition. The LB+SSD and LB+P groups exhibited thicker, more cohesive collagen bundles, suggesting enhanced fibroblast activity and matrix formation.

Figure 2a semi-quantitatively supports the increased collagen density observed across treatment groups. The combination therapy group (LB+P+SSD) demonstrated the highest collagen fiber density and most organized arrangement, closely resembling the normal control group (N), particularly by day 18. These findings align with expected collagen remodeling patterns in which treatment accelerates the transition from immature, loosely arranged fibers to denser, aligned collagen structures.

3.2. Percentage of Collagen Type III and Type I

Picrosirius Red–Fast Green staining under polarized light microscopy enabled clear differentiation between collagen type III (green–yellow) and type I (red–orange). On day 6, the early wound-healing phase revealed heterogeneous responses in collagen type III expression across the treatment groups. The LB+SSD group exhibited the highest percentage of collagen type III (41.46 ± 33.36%), followed by the LB group (27.84 ± 23.04%). In contrast, LB+P had the lowest value (6.57 ± 7.31%), which was close to the normal group (7.26 ± 1.53%). The combination group (LB+P+SSD) showed an intermediate expression level (12.57 ± 16.67%). The large standard deviations in several groups, particularly LB and LB+SSD, indicate that collagen type III deposition during this early phase was still highly variable and unstable across individuals.

The expression of collagen type I on day 6 was relatively similar across treatment groups. The percentage

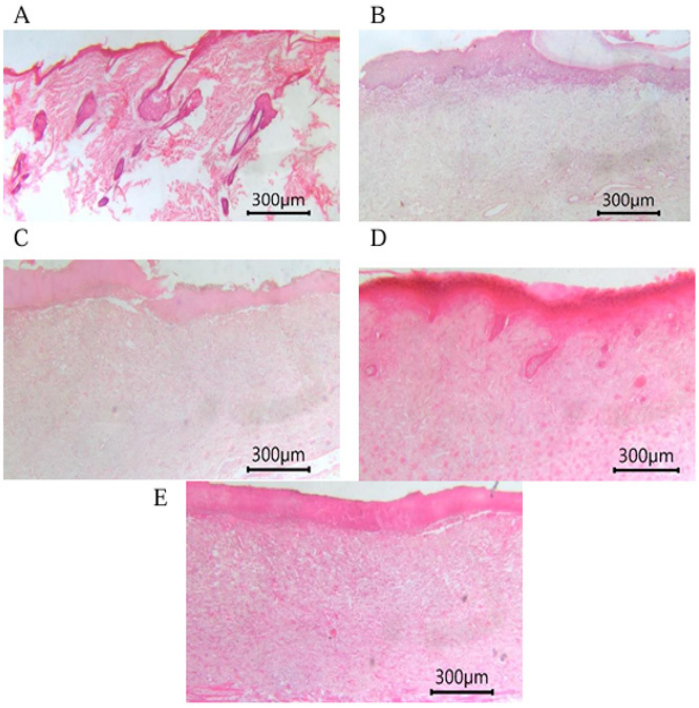

Figure 1. Histopathological features at day 18. A: Normal Control, B: Untreated Burn, C: Burn + Silver Sulfadiazine, D: Burn + 3% Nutmeg Cream, E: Burn + Silver Sulfadiazine + 3% Nutmeg Cream

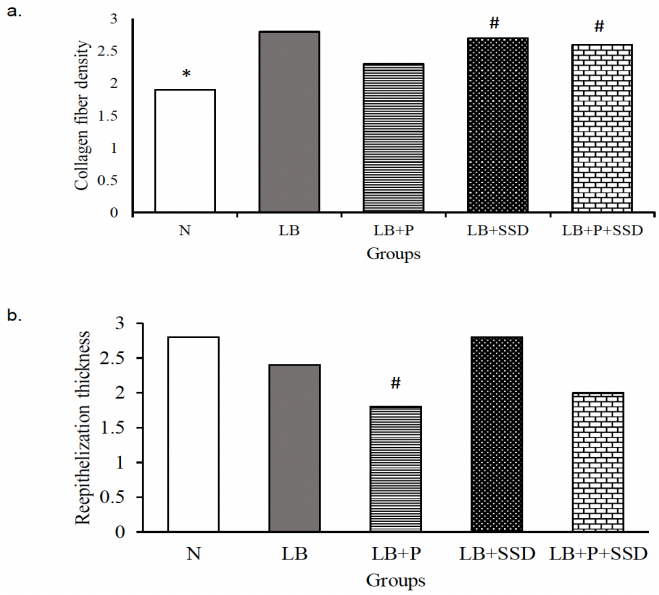

Figure 2. Collagen Fiber Density (a) and Re-epithelialization thickness (b) in rat skin tissue across all experimental groups. The data are categorical and represent the mean values across five fields. HE staining. N: Normal Control; LB: Untreated Burn; LB+P: Burn + 3% Nutmeg Cream; LB+SSD: Burn + Silver Sulfadiazine;LB+SSD+P: Burn + Silver Sulfadiazine + 3% Nutmeg Cream. *p<0.05 vs LB, # p<0.05 vs N.

of collagen type I in all groups was markedly lower than the normal group (63.07 ± 4.04%). This result indicates that collagen type I deposition was still in its early phase and had not yet dominated the wound matrix. The significant variation in collagen type III at day 6 may be related to biological heterogeneity during the initial proliferative phase of wound healing, a period marked by dynamic and transitional collagen synthesis. This variation occurs frequently in the early stages of burn healing, before stabilization during the remodelling phase. On day 18, a clear increase in collagen type I was observed in all burn groups, indicating progression into the remodeling phase of wound healing. The highest increase was noted in the LB+SSD group, followed by LB+P and LB. Interestingly, the combination group (LB+P+SSD) showed the lowest expression (53.85 ± 24.33%) compared with the other treatment groups. These findings suggest that collagen type I maturation was more pronounced in groups treated with SSD alone, and that combination therapy did not necessarily enhance it.

In contrast, collagen type III expression on day 18 tended to decrease, especially in the LB+SSD group, which dropped significantly to 8.66 ± 3.61%, approaching the normal level. The LB+P+SSD group retained a relatively higher level of collagen type III, while LB remained elevated and LB+P decreased moderately. These results indicate that the remodeling phase, characterized by the replacement of collagen type III by collagen type I, occurred at different rates across treatment regimens. These quantitative findings are represented in Table 3.

3.3. Epithelial Thickness and Epithelialization

The degree of epithelialization evaluated by HE staining on day 18 is shown in Figure 2b. The highest epithelialization score was observed in the burn group treated with SSD, while the lowest epithelialization occurred in the burn group treated with nutmeg. These findings are different from the results obtained with Gold-Orange staining.

Gold–Orange staining demonstrated differences in

Table 3. Collagen Type III and I (Mean ± SD) at Day 6 and Day 18

Group	Day 6		Day 18	
	Collagen Type III	Collagen Type I	Collagen Type III	Collagen Type I
N	7.26 ± 1.53	63.07 ± 4.04	7.26 ± 1.13	64.05 ± 1.92
LB	27.84 ± 23.04	35.62 ± 11.54	20.34 ± 22.30	60.72 ± 22.15
LB+P	6.57 ± 7.31	33.78 ± 8.91	11.51 ± 7.99	65.26 ± 14.46
LB+SSD	41.46 ± 33.36	26.04 ± 16.94	8.66 ± 3.61	68.82 ± 9.32
LB+P+SSD	12.57 ± 16.67	35.41 ± 18.22	21.24 ± 14.01	53.85 ± 24.33

Table 4. Epithelial cell number

Group	Day 6	Day 18
N	32.88 ± 9.17	38.63 ± 1.17
LB	14.31 ± 16.55	20.13 ± 18.45
LB+P	24.69 ± 9.52	27.5 ± 15.63
LB+SSD	9.44 ± 11.24	18.31 ± 16.91
LB+P+SSD	18.13 ± 6.96	17.25 ± 14.44

epithelial growth between the experimental groups, as shown in Table 4. On day 6, the LB+P group showed higher levels of epithelialization than the LB group, when the LB+SSD group presented reduced numbers, and the combination group showed an intermediate response. By day 18, epithelialization improved in both the LB and LB+P groups, with LB+P indicating the greatest level of epithelialization among the treated groups. The combination of treatments produced no greater improvement than any of the therapies. Furthermore, the variations in this parameter were not statistically significant.

4. Discussion

The findings of this study demonstrate that the healing of second-degree burns is profoundly influenced by the modulation of collagen remodeling and epithelial regeneration, which collectively determine tissue strength, functional restoration, and overall wound recovery. Second-degree burn wounds that were untreated had delayed healing characteristics, illustrated by weak and unorganized collagen fibers and reduced epithelial replacement by day 18. These findings are consistent with the persistence of a transient extracellular matrix composed mainly of collagen type III and an inadequate transition to collagen type I. This feature indicates a delayed transition into the remodeling phase, throughout which collagen maturation and epidermal restoration are required to restore tissue integrity.

In contrast, topical application of 3% nutmeg seed cream accelerated collagen maturation by enhancing the transition from collagen type III to collagen type I. This transition is essential for improving tensile strength and restoring normal extracellular matrix (ECM) architecture.[17] Bioactive constituents of nutmeg, including myristicin, eugenol, terpinene, safrole, and terpinen-4-ol, possess notable antioxidant and anti-inflammatory properties that are likely to have contributed to the improved remodeling observed in this study. These compounds are known to modulate fibroblast activity, reduce oxidative stress, and support collagen synthesis, thereby fostering a favorable biochemical environment for tissue repair.[11,12]

Silver sulfadiazine (SSD), a standard antimicrobial therapy for burns, improved epithelialization and collagen organization. Silver sulfadiazine treatment (LB+SSD) demonstrated a different pattern. During the early phase, this group exhibited the highest percentage of collagen type III, reflecting active but unstable matrix deposition.[7] SSD's antimicrobial action reduces infection-related inflammation, which indirectly aids healing; however, its potential to delay epithelial proliferation may have limited its overall effect on collagen remodeling. By day 18, however, collagen type I levels were the highest among all burn groups, while collagen type III decreased substantially toward normal values. This suggests that SSD effectively promoted collagen maturation during the remodeling phase, likely through infection control and reduction of prolonged inflammation. Nevertheless, the early accumulation of collagen type III and the variability observed indicate that SSD does not uniformly regulate early matrix formation.[18]

The combination therapy followed the same general healing sequence but did not enhance collagen maturation beyond that achieved with single treatments. Although HE staining showed high collagen density in this group, polarized light analysis revealed that collagen type I expression on day 18 was lower than in both LB+SSD and LB+P groups, while collagen type III remained relatively elevated. This indicates that increased collagen density did not correspond to improved collagen quality or maturity. Therefore, the combination therapy increased matrix quantity but did not optimize collagen remodeling. These findings are different from some literature, which reported that combining herbal extracts with SSD enhances therapeutic outcomes through antioxidative, anti-inflammatory, and antimicrobial mechanisms.[5,19,20]

The outcomes of epithelialization also emphasize treatment-specific effects. HE staining indicated increased epithelial thickness in SSD-treated wounds by day 18; however, Gold-Orange staining offered more comprehensive insights into epithelial maturation. According to keratohyalin granule counts, nutmeg-treated wounds demonstrated the highest epithelial cell counts at day 18, suggesting enhanced keratinocyte differentiation and maturation within the stratum granulosum. These histological features were associated with enhanced keratinocyte migration and

differentiation, followed by epithelial maturation that contributes to epidermal barrier restoration. Increased granule density further suggests enhanced structural and functional recovery of the epidermis.[5] These findings support and extend previous reports on the beneficial effects of plant-derived anti-inflammatory and antioxidant agents in improving wound healing outcomes. Furthermore, the results provide scientific validation for the traditional Acehnese practice of using nutmeg for burn treatment and demonstrate its potential as an affordable, accessible adjunctive therapy for second-degree burns.[14,21]

In line with the results, nutmeg cream alone provided the most balanced healing response, characterized by regulated collagen remodeling and improved epithelial maturation. SSD mostly facilitated collagen type I deposition during the remodeling phase, although it had a diminished impact on epithelial quality. The combined therapy enhanced collagen density but did not improve collagen maturation or epithelial regeneration. These findings underscore the need to examine both collagen subtype composition and epithelial maturity, rather than relying solely on basic histological density or thickness, when evaluating burn wound healing outcomes.

Several limitations must be acknowledged. The use of an animal model, although advantageous for controlled experimental investigation, inherently limits direct translation to human clinical settings due to interspecies differences in skin structure, healing mechanisms, and immune responses. Additionally, the controlled environment does not account for clinical variables such as comorbidities, age variability, or secondary infections that can affect human burn outcomes. These limitations necessitate a cautious interpretation of the findings and underscore the need for follow-up studies, including clinical trials or advanced human skin models.

5. Conclusion

This study provides comprehensive histological evidence that 3% nutmeg (*Myristica fragrans*) cream accelerates the healing of grade II burns by enhancing collagen remodeling and epithelial regeneration. Treatment with nutmeg cream accelerated the transition from collagen type III to type I, indicating enhanced extracellular matrix maturation and increased tissue tensile strength. Improvements in epithelial thickness and keratohyalin granule formation further confirmed its positive impact on re-epithelialization, contributing to the restoration of the skin barrier. While the histological analyses adequately support the study aims, future investigations may incorporate additional mechanistic assessments, such as inflammatory or angiogenic markers, to further clarify the underlying healing mechanisms.

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Conflict of Interest

The authors declare no conflicts of interest.

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