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Original Research Article

Effect of ethanolic propolis extract on TNF-α, caspase-3 expression and histopathological findings in hypoxic skin flaps: an experimental study in rats

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ABSTRACT

Background: Hypoxia remains a major cause of skin flap failure due to excessive production of reactive oxygen species (ROS), which triggers inflammation and apoptosis through increased expression of tumor necrosis factor-alpha (TNF-α) and caspase-3. Propolis contains bioactive compounds with antioxidant and anti-inflammatory properties that may improve skin flap survival under hypoxic conditions. This study aimed to evaluate the effect of ethanolic propolis extract on TNF-α expression, caspase-3 expression, and histopathological changes in a hypoxic skin flap model.
Methods: An experimental post-test only control group study was conducted using 24 male *Rattus norvegicus* divided into four groups (n=6/group): control, P1, P2, and P3. Different flap dimensions (1/3, 1/2, and 2/3) were used to induce varying degrees of hypoxia. Ethanolic extract of Gunung Lawu propolis was administered orally at a dose of 800 mg/kg bw/day for seven days. TNF-α and caspase-3 levels were measured using enzyme-linked immunosorbent assay (ELISA). Histopathological evaluation included necrosis, acute inflammation, and re-epithelialization. Statistical analysis was performed using one-way ANOVA followed by post hoc tests and Kruskal–Wallis analysis.
Results: Propolis administration significantly reduced TNF-α levels in all treatment groups compared with controls ($p<0.001$), with the greatest reduction observed in the P3 group (55.5%). Caspase-3 expression was also significantly decreased ($p<0.001$), with the highest reduction found in the P3 group (79.5%). Histopathological assessment demonstrated lower necrosis and acute inflammation scores and higher re-epithelialization scores in treatment groups; however, these differences were not statistically significant ($p>0.05$).
Conclusions: Ethanolic propolis extract effectively attenuated inflammatory and apoptotic responses in hypoxic skin flaps by reducing TNF-α and caspase-3 expression. Although histopathological improvements were observed, they did not reach statistical significance. Propolis may serve as a promising adjunctive therapy to enhance skin flap viability.

Keywords: Propolis, Skin flap, Hypoxia, TNF-α, Caspase-3, Apoptosis

INTRODUCTION

Skin flap reconstruction remains one of the most important techniques in reconstructive surgery for the management of soft tissue defects caused by trauma, tumor resection, vascular ulcers, and diabetic wounds. The success of a skin flap largely depends on adequate vascular perfusion and tissue oxygenation. However, flap ischemia and hypoxia remain major causes of partial or complete flap necrosis, resulting in prolonged hospitalization, increased healthcare costs, and unsatisfactory functional and aesthetic outcomes.[1-3]

Hypoxic conditions trigger excessive production of reactive oxygen species (ROS), leading to oxidative stress, endothelial dysfunction, inflammation, and apoptosis. Although physiological levels of ROS are required for angiogenesis and wound healing, excessive ROS accumulation disrupts vascular integrity and impairs tissue regeneration. Furthermore, ROS-induced activation of inflammatory pathways stimulates the production of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), which contributes to tissue injury and prolongation of the inflammatory phase of wound healing.[4-7]

Apoptosis is another critical mechanism involved in skin flap failure. Oxidative stress and inflammatory mediators activate both intrinsic and extrinsic apoptotic pathways, resulting in increased expression of caspase-3, the principal executioner caspase responsible for programmed cell death. Elevated caspase-3 expression has been associated with increased cellular apoptosis, impaired angiogenesis, and reduced flap viability. Therefore, interventions capable of suppressing inflammation and apoptosis may improve skin flap survival under hypoxic conditions.[8-10]

Propolis is a natural resinous substance collected by honeybees from various plant sources and contains more than 300 biologically active compounds, including flavonoids, phenolic acids, terpenoids, and caffeic acid phenethyl ester (CAPE). Numerous studies have demonstrated that propolis possesses antioxidant, anti-inflammatory, antimicrobial, and wound-healing properties. CAPE, one of the major active components of propolis, has been shown to inhibit nuclear factor-kappa B (NF-κB) activation, reduce TNF-α production, suppress oxidative stress, and attenuate apoptosis in various experimental models.[11-14]

Although the beneficial effects of propolis on wound healing have been reported, evidence regarding its role in improving survival of hypoxic skin flaps remains limited, particularly using Indonesian Gunung Lawu ethanolic propolis extract. Therefore, this study aimed to evaluate the effects of ethanolic propolis extract on TNF-α expression, caspase-3 expression, and histopathological findings in a hypoxic skin flap model in *Rattus norvegicus*. We hypothesized that propolis administration would reduce inflammatory and apoptotic responses and subsequently improve tissue viability in hypoxic skin flaps.

METHODS

Study design and ethical considerations

This experimental study employed a post-test only control group design using male *Rattus norvegicus* as the animal model. All experimental procedures were conducted in accordance with institutional guidelines for animal research and were approved by the appropriate Research Ethics Committee (approval number to be inserted).

Study setting and period

Animal treatment and sample collection were conducted at the Inter-University Center Laboratory, Universitas Gadjah Mada, Yogyakarta, Indonesia, between May and June 2025. Histopathological and immunohistochemical analyses were performed at the Department of Anatomical Pathology, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia, in November 2025.

Animals

Twenty-four healthy male *Rattus norvegicus* aged 3–4 months and weighing 150–300 g were included in the study. Animals were obtained from the Faculty of Veterinary Medicine, Universitas Gadjah Mada, and were maintained under standardized laboratory conditions with free access to water and standard rodent chow.

Animals presenting physical abnormalities or signs of illness were excluded from the study.

Sample size and grouping

Sample size was determined according to the World Health Organization recommendation for experimental animal studies, requiring a minimum of five animals per group. One additional animal was included in each group to compensate for possible attrition, resulting in six animals per group and a total sample size of 24 animals.

The animals were randomly allocated into four groups (n=6 per group): control group (K+), P1 group (1/3 flap hypoxia model), P2 group (1/2 flap hypoxia model), and P3 group (2/3 flap hypoxia model).

Skin flap model and intervention

A modified dorsal skin flap model based on the technique described by Karypidis et al was used. Full-thickness dorsal skin flaps including the epidermis, dermis, and part of the panniculus carnosus were elevated under sterile conditions. Hemostasis was achieved using adrenaline 1:100,000 solution or 6-0 monofilament ligation when necessary.

Different flap dimensions (1/3, 1/2, and 2/3 of the wound area) were used to induce varying degrees of tissue hypoxia. The skin flaps were repositioned and secured using 6-0 monofilament sutures. The operative area was covered with sterile saline-moistened gauze and maintained using a tie-over dressing for seven days.

All treatment groups received ethanolic extract of Gunung Lawu propolis at a dose of 800 mg/kg body weight/day administered orally through a gastric gavage once daily for seven consecutive days.

Outcome measurements

Primary outcomes

The primary outcomes were serum TNF-α and caspase-3 levels measured on day 7 following flap creation.

TNF-α analysis

TNF-α expression was evaluated using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol.

Caspase-3 analysis

Caspase-3 expression was evaluated using ELISA according to the manufacturer's protocol.

Histopathological evaluation

Skin flap specimens were harvested on postoperative day 7 and processed for histopathological examination. Hematoxylin-eosin staining was performed to evaluate tissue necrosis, acute inflammatory cell infiltration, and re-epithelialization. Histopathological assessment was conducted by an experienced pathologist blinded to group allocation.

Statistical analysis

Data analysis was performed using statistical package for the social sciences (SPSS) software. Quantitative variables were expressed as mean ± standard deviation. Normality was assessed using the Shapiro-Wilk test, while homogeneity of variance was evaluated using Levene's test.

Differences in TNF-α and caspase-3 levels among groups were analyzed using one-way analysis of variance (ANOVA), followed by post hoc multiple comparison testing when appropriate. Histopathological variables were analyzed using the Kruskal-Wallis test. A p value <0.05 was considered statistically significant.

Ethical approval

This study was conducted in accordance with the principles of replacement, reduction, and refinement (3Rs) established by the National Centre for the replacement, refinement and reduction of animals in research (NC3Rs). Ethical approval was obtained from the Health Research Ethics Committee of Universitas Gadjah Mada, Yogyakarta, Indonesia.

RESULTS

A total of 24 male *Rattus norvegicus* completed the study and were included in the final analysis. The animals were randomly allocated into four groups consisting of six rats each: control (K+), P1 (one-third flap coverage), P2 (one-half flap coverage), and P3 (two-thirds flap coverage). No mortality or major complications occurred during the experimental period.

Effect of propolis on TNF-α expression

Baseline TNF-α levels were comparable among all groups, with no statistically significant difference observed before treatment (p=0.877). Following seven days of intervention, TNF-α levels decreased significantly in all groups.

The greatest reduction in TNF-α expression was observed in the P3 group, followed by the P2 and P1 groups. The control group demonstrated the smallest reduction. Mean TNF-α reductions were -9.08±0.66 pg/ml in the control group, -11.31±0.63 pg/ml in the P1 group, -11.73±0.38 pg/ml in the P2 group, and -12.38±0.51 pg/ml in the P3 group.

Post-treatment analysis demonstrated significant differences among groups (p<0.001). Percentage reductions in TNF-α were 41.1%, 50.7%, 52.8%, and 55.5% in the K+, P1, P2, and P3 groups, respectively. Post hoc analysis revealed significant differences between the control group and all treatment groups (all p<0.001).

A significant difference was also observed between the P1 and P3 groups (p=0.003), whereas no significant differences were found between P1 and P2 (p=0.209) or between P2 and P3 (p=0.058).

Effect of propolis on caspase-3 expression

Baseline caspase-3 levels did not differ significantly among groups (p=0.899). After treatment, caspase-3 expression decreased significantly in all groups.

The largest reduction was observed in the P3 group (-10.33±0.41 pg/ml), followed by the P2 (-9.75±0.66 pg/ml) and P1 groups (-9.14±0.87 pg/ml). The control group demonstrated the smallest reduction (-6.39±0.92 pg/ml).

Percentage reductions in caspase-3 expression were 49.1%, 69.9%, 76.0%, and 79.5% in the K+, P1, P2, and P3 groups, respectively. Significant differences among groups were observed for both post-treatment values and delta changes (p<0.001).

Post hoc analysis demonstrated significant differences between the control group and all treatment groups (all p<0.001). The P3 group also showed a significantly greater reduction compared with the P1 group (p=0.011). No significant differences were identified between P1 and P2 (p=0.170) or between P2 and P3 (p=0.188).

Table 1: Comparison of TNF-α levels among study groups.

Group	Pre-treatment (mean±SD)	Post-treatment (mean±SD)	Δ TNF-α (mean±SD)	Percentage reduction
K+	22.09±0.58	13.00±0.75	-9.08±0.66	41.1
P1	22.30±0.67	10.98±0.47	-11.31±0.63	50.7
P2	22.21±0.30	10.48±0.33	-11.73±0.38	52.8
P3	22.30±0.40	9.92±0.40	-12.38±0.51	55.5

Overall ANOVA p value: pre-treatment=0.877, post-treatment <0.001, and Δ TNF-α <0.001

Table 2: Comparison of caspase-3 levels among study groups.

Group	Pre-treatment (mean±SD)	Post-treatment (mean±SD)	Δ caspase-3 (mean±SD)	Percentage reduction
K+	13.02±0.60	6.62±1.05	-6.39±0.92	49.1
P1	13.07±0.71	3.93±0.71	-9.14±0.87	69.9
P2	12.83±0.42	3.09±0.53	-9.75±0.66	76.0
P3	12.99±0.51	2.66±0.38	-10.33±0.41	79.5

Overall ANOVA p value: pre-treatment=0.899, post-treatment <0.001, and Δ caspase-3 <0.001

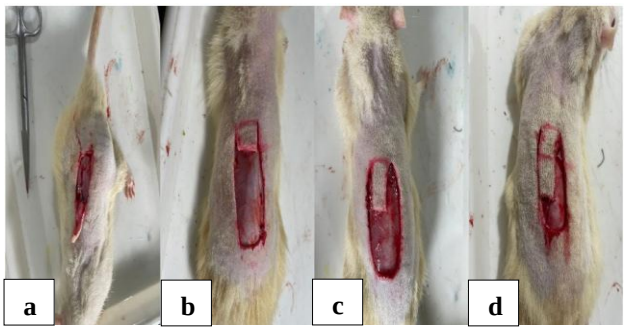

Figure 1: Experimental design of the hypoxic skin flap model. Representative schematic of the skin flap models used in this study (a) control flap, (b) one-third flap (P1), (c) one-half flap (P2), and (d) two-thirds flap (P3). Increasing flap dimensions were used to induce progressively greater degrees of tissue hypoxia.

Figure 2: Representative macroscopic appearance of skin flaps on postoperative day 7. Representative photographs of skin flaps following treatment with ethanolic extract of Gunung Lawu propolis. Flap viability, skin color, and areas of partial necrosis were evaluated macroscopically among the control and treatment groups.

Histopathological findings

Histopathological examination revealed a trend toward reduced tissue necrosis and acute inflammatory infiltration and increased re-epithelialization in the propolis-treated groups. However, these differences did not reach statistical significance.

For tissue necrosis, severe necrosis was observed in 100% of control specimens, compared with 83.3%, 50.0%, and 66.7% of specimens in the P1, P2, and P3 groups, respectively (p=0.256).

Similarly, acute inflammation scores tended to be lower in treatment groups, with severe inflammation observed in 100% of control specimens compared with 83.3%, 83.3%, and 66.7% in the P1, P2, and P3 groups, respectively (p=0.513).

Re-epithelialization tended to be more prominent in the treatment groups than in controls. Nevertheless, no statistically significant difference was observed among groups (p=0.485).

Table 3: Histopathological findings among study groups.

Variable	P value
Necrosis	0.256
Acute inflammation	0.513
Re-epithelialization	0.485

Macroscopic flap assessment

Macroscopic examination demonstrated generally favorable flap survival in all groups. Most flaps retained a color comparable to the surrounding skin, while partial necrosis was observed in a limited number of specimens. The proportion of viable flaps appeared higher in the propolis-treated groups compared with controls.

Representative histopathological sections demonstrated areas of tissue necrosis and neutrophilic inflammatory infiltration, with relatively improved tissue preservation observed in the treatment groups.

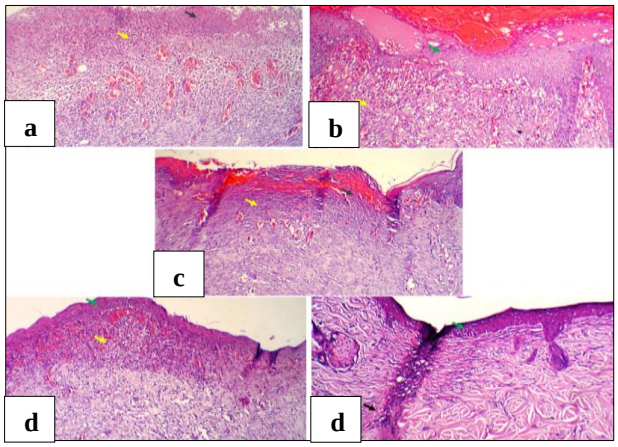

Figure 3: Histopathological findings of hypoxic skin flap tissue. Representative hematoxylin and eosin-stained sections from the experimental groups (a) control group, (b and c) one-third flap group (P1), (d) one-half flap group (P2), and (e) two-thirds flap group (P3). Black arrows indicate areas of tissue necrosis, whereas yellow arrows indicate acute inflammatory cell infiltration (neutrophils). Histopathological evaluation included assessment of necrosis, acute inflammation, and re-epithelialization.

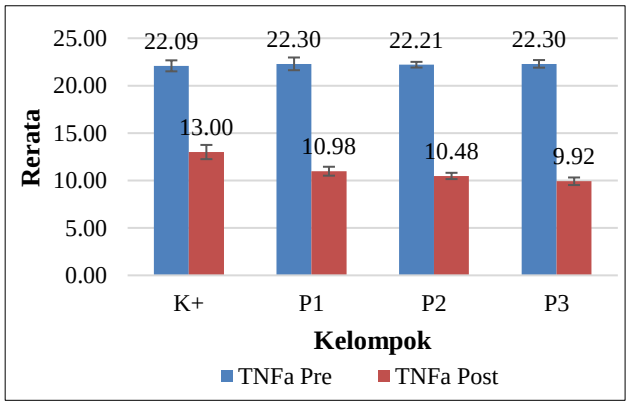

Figure 4: Comparison of serum TNF-α levels among study groups. Mean serum TNF-α levels in the control group and treatment groups (P1, P2, and P3). Administration of ethanolic extract of Gunung Lawu propolis significantly reduced TNF-α levels compared with the control group, with the greatest reduction observed in the P3 group.

DISCUSSION

The present study demonstrated that administration of ethanolic extract of Gunung Lawu propolis significantly reduced TNF-α and caspase-3 levels in hypoxic skin flap models. The greatest reductions were observed in the P3 group, which represented the highest degree of induced hypoxia. These findings suggest that propolis exerts substantial anti-inflammatory and anti-apoptotic effects under conditions of tissue ischemia and oxygen deprivation. Although histopathological parameters showed favorable trends toward reduced necrosis and inflammation and enhanced re-epithelialization, these changes did not reach statistical significance.

Hypoxia is one of the most important factors contributing to skin flap failure. Adequate oxygen supply is essential for maintaining cellular metabolism, angiogenesis, fibroblast proliferation, collagen synthesis, and epithelial regeneration. When tissue perfusion becomes compromised, oxygen deprivation results in metabolic imbalance and accumulation of reactive oxygen species (ROS), particularly during ischemia-reperfusion injury.[4-6] Excessive ROS production subsequently activates multiple inflammatory pathways, resulting in endothelial dysfunction, vascular leakage, leukocyte recruitment, and tissue damage. These pathological processes eventually increase the risk of flap necrosis and compromise reconstructive outcomes.[1-3]

One of the most important mediators involved in hypoxia-induced inflammation is TNF-α. This cytokine plays a dual role in wound healing. At physiological concentrations, TNF-α contributes to host defense and tissue repair by promoting inflammatory cell recruitment and activation. However, excessive or prolonged TNF-α expression may become detrimental by perpetuating inflammation, impairing angiogenesis, and delaying progression toward the proliferative phase of healing.[7-9] Previous studies have demonstrated that elevated TNF-α levels are associated with poor wound healing outcomes and increased tissue necrosis.[7]

In the present study, TNF-α levels decreased significantly in all treatment groups, with the greatest reduction observed in the P3 group. This finding suggests that propolis effectively attenuated the inflammatory response induced by severe tissue hypoxia. Similar findings have been reported by Ma et al, who demonstrated that apigenin significantly improved random skin flap survival by reducing inflammatory cytokine production and oxidative stress.[13] Likewise, Li et al reported that enhancement of antioxidant defense mechanisms improved flap viability through suppression of inflammatory injury.[14] These observations support the hypothesis that modulation of inflammatory pathways is an important mechanism underlying flap preservation.

The anti-inflammatory effect of propolis can be explained by its rich content of flavonoids, phenolic acids, terpenoids, and caffeic acid phenethyl ester (CAPE). CAPE is recognized as one of the most biologically active components of propolis and has been shown to inhibit NF-κB activation, a key transcription factor regulating TNF-α production.[15] By suppressing NF-κB signaling, CAPE reduces the expression of TNF-α, interleukin-1β, interleukin-6, and other pro-inflammatory mediators.[15-17]

Furthermore, flavonoids contained in propolis act as free-radical scavengers, thereby reducing oxidative stress and limiting the downstream inflammatory cascade.[16,17] These mechanisms likely contributed to the marked reduction in TNF-α observed in this study.

Besides inflammation, apoptosis plays a critical role in the pathogenesis of skin flap injury. Apoptosis is a tightly regulated process that eliminates damaged cells without provoking excessive inflammation. However, excessive apoptosis may significantly impair tissue viability by reducing the number of cells available for angiogenesis, extracellular matrix production, and tissue regeneration.[10-12] Hypoxia and oxidative stress activate both intrinsic and extrinsic apoptotic pathways, resulting in mitochondrial dysfunction, cytochrome c release, and activation of executioner caspases, particularly caspase-3.[11,12]

The significant reduction in caspase-3 observed in the present study indicates that propolis effectively suppressed apoptotic activity in hypoxic flap tissue. Interestingly, the percentage reduction in caspase-3 was even greater than the reduction observed for TNF-α. This finding may suggest that the anti-apoptotic effects of propolis extend beyond its anti-inflammatory activity and involve direct modulation of intracellular survival pathways. Polyphenols and flavonoids have been reported to stabilize mitochondrial membranes, reduce oxidative DNA damage, inhibit activation of pro-apoptotic proteins such as Bax, and preserve cellular antioxidant defenses.[16,17] Consequently, fewer cells undergo programmed cell death despite exposure to hypoxic stress.

The present findings are supported by previous studies. Swamy et al demonstrated that propolis significantly reduced caspase-3 activation in an experimental neurotoxicity model while simultaneously improving inflammatory biomarkers.[18] Tri et al reported similar findings, showing that Indonesian propolis decreased apoptotic activity and inflammatory cytokine expression in experimental animals.[19] Moreover, protective effects of propolis have also been demonstrated in models of renal and testicular ischemia-reperfusion injury, where treatment reduced apoptosis and improved tissue preservation.[20-22] Collectively, these studies support the notion that propolis possesses broad cytoprotective properties across multiple organ systems.

An important observation in this study is the simultaneous reduction of TNF-α and caspase-3. These findings are biologically plausible because inflammation and apoptosis are closely interconnected processes. Excessive TNF-α signaling can activate apoptotic pathways through TNF receptor-mediated mechanisms, resulting in caspase-3 activation and programmed cell death.[7,10] Therefore, suppression of TNF-α may indirectly reduce apoptosis. Conversely, reduced apoptosis may limit the release of damage-associated molecular patterns (DAMPs), thereby attenuating secondary inflammatory responses. This reciprocal relationship may explain why both biomarkers decreased significantly following propolis administration.

Despite significant molecular improvements, histopathological outcomes did not differ significantly among groups. Tissue necrosis tended to be less severe in treatment groups, while inflammatory infiltration appeared reduced and re-epithelialization appeared enhanced. However, these changes failed to achieve statistical significance. Similar discrepancies between molecular and histological findings have been reported in previous wound-healing studies, where changes in cytokine expression preceded visible tissue remodeling.[23-25]

Several factors may explain the absence of significant histopathological differences. First, histological scoring systems are inherently less sensitive than quantitative biochemical measurements. Small biological changes may therefore remain undetected when categorized into broad ordinal grades. Second, the sample size was relatively small, with only six animals per group. Although sufficient for detecting differences in molecular markers, this sample size may not provide adequate statistical power to identify subtle histological changes. Third, evaluation was performed only on postoperative day seven. At this time point, inflammatory resolution and tissue remodeling may still be ongoing, particularly in severely hypoxic tissues. Earlier and serial observations might reveal temporal changes that were not captured in the present study.

The tendency toward improved re-epithelialization observed in the treatment groups remains clinically meaningful. Re-epithelialization is essential for restoration of skin integrity and serves as an important indicator of wound healing progression.[23] Previous studies have shown that propolis enhances keratinocyte migration, fibroblast proliferation, collagen deposition, and extracellular matrix remodeling.[15,16] Therefore, the favorable trend observed in this study may indicate genuine biological improvement despite the absence of statistical significance.

From a clinical perspective, the findings of this study have potential implications for reconstructive surgery. Partial flap necrosis remains a major complication that can compromise both functional and aesthetic outcomes. Current strategies to improve flap survival include surgical delay procedures, pharmacological agents, hyperbaric oxygen therapy, and optimization of vascular supply. The present findings suggest that propolis may serve as a complementary therapeutic option because it targets two critical mechanisms of flap failure, namely inflammation and apoptosis. Furthermore, propolis is relatively inexpensive, widely available, and generally considered safe, making it an attractive candidate for future translational applications.

Several limitations should be acknowledged. Tissue oxygenation and perfusion were not measured directly, preventing objective quantification of the degree of hypoxia. Only a single dose of propolis was evaluated,

precluding assessment of dose-response relationships. The chemical composition of the propolis extract was not standardized through quantitative analysis of active compounds such as CAPE or total flavonoid content. In addition, molecular and histological assessments were performed at a single endpoint, limiting evaluation of dynamic changes throughout the healing process.

Despite these limitations, this study provides evidence that ethanolic extract of Gunung Lawu propolis significantly attenuates inflammatory and apoptotic responses in hypoxic skin flaps. The reductions in TNF-α and caspase-3 observed across treatment groups support the potential role of propolis as a biologically active adjunct for improving flap viability. Future studies involving larger sample sizes, longer observation periods, standardized characterization of propolis constituents, and direct measurements of tissue perfusion are warranted to further elucidate its therapeutic potential in reconstructive surgery.

CONCLUSION

The present study demonstrated that administration of ethanolic extract of Gunung Lawu propolis significantly reduced serum TNF-α and caspase-3 levels in hypoxic skin flap models in *Rattus norvegicus*, indicating its anti-inflammatory and anti-apoptotic effects. The greatest reductions were observed in the group with the largest hypoxic flap area (P3), with TNF-α and caspase-3 levels decreasing by 55.5% and 79.5%, respectively. These findings suggest that propolis may modulate key molecular pathways involved in tissue injury and flap survival under hypoxic conditions. Although histopathological evaluation showed trends toward reduced necrosis and acute inflammation and improved re-epithelialization, these differences were not statistically significant. Overall, ethanolic extract of Gunung Lawu propolis shows potential as a promising adjunctive therapeutic agent for improving skin flap viability by attenuating inflammatory and apoptotic responses associated with tissue hypoxia.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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