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Original Research Article

Effect of ethanolic propolis extract on hypoxia-induced skin flap survival in white rats (*Rattus norvegicus*) – an experimental study

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ABSTRACT

Background: Hypoxia in skin flaps promotes oxidative stress and inflammation, which may impair angiogenesis and tissue survival. Propolis contains flavonoids and polyphenols with antioxidant, anti-inflammatory, and angiogenic properties. This study evaluated the effect of ethanolic propolis extract on malondialdehyde (MDA), vascular endothelial growth factor (VEGF), necrosis, and acute inflammation in a rat skin-flap model.
Methods: This experimental pre-test and post-test control-group study included 24 male white rats (Rattus norvegicus) allocated to four groups: control (K) and three treatment groups receiving ethanolic propolis extract 800 mg/kg body weight/day with different flap proportions (P1, P2, and P3). Blood MDA and VEGF levels were measured before and after treatment, and skin-flap specimens were assessed histopathologically. MDA and VEGF were analyzed using one-way ANOVA with post hoc testing, while necrosis and acute inflammation were analyzed using the Kruskal-Wallis test.
Results: MDA decreased least in K (-3.89±0.47) and most in P3 (-6.81±0.53), with a significant between-group difference ($p<0.001$). VEGF increased least in K (6.30±1.10) and most in P3 (11.55±1.19), also with a significant between-group difference ($p<0.001$). Between-group differences were not significant for necrosis ($p=0.256$) or acute inflammation ($p=0.513$).
Conclusions: Propolis administration significantly increased skin flap survival, as indicated by a decrease in MDA and an increase in VEGF levels. At the histopathological level, propolis administration reduced the degree of necrosis and acute inflammation in the skin flap, although this reduction was not statistically significant.

Keywords: Propolis, Malondialdehyde, Vascular endothelial growth factor, Necrosis, Acute inflammation

INTRODUCTION

Complex wound closure requires a technical approach tailored to the specific characteristics of each defect, including anatomical location, defect dimensions, post-intervention functional potential, patient comorbidities, skin tissue architecture, and primary etiology, whether neoplastic or traumatic.

The principal goal of modern wound management is to achieve an optimal aesthetic outcome together with maximal patient satisfaction.[1]

The biochemical process of wound healing involves several important mediators, including reactive oxygen species (ROS), malondialdehyde (MDA), caspase-9, nuclear factor kappa beta (NF-κB), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF). ROS play a physiological role in activating expression of hypoxia-inducible factor (HIF)-1α while also modulating the cellular inflammatory response to signaling factors such as VEGF. The success of skin flap techniques depends heavily on the quality of vascularization achieved through angiogenesis, which is significantly influenced by ROS activity.[2] Excessive ROS

activity can trigger lipid peroxidation of cell membranes and cause cellular damage, whereas limited ROS activity can produce optimal angiogenesis through regulation of inflammation and vascular signaling. Strategies to improve skin flap success therefore include optimizing angiogenesis quality by reducing ROS activity through exogenous antioxidant supplementation.[2]

Propolis is a natural substance believed to be a source of exogenous antioxidants. Produced by Apis mellifera, propolis has been identified as a potential source of exogenous antioxidants with antioxidant, anti-inflammatory, and angiogenic effects owing to its comprehensive polyphenol and flavonoid content. The term propolis refers to a complex mixture of resinous substances collected by honeybees from various plant parts, including buds and exudates, in temperate regions extending from the tropics to the Arctic Circle. The main botanical sources of propolis include poplar, willow, birch, elm, alder, beech, conifer, and horse-chestnut trees.[3]

The bioactive molecular composition of propolis varies substantially depending on geographic, botanical, seasonal, and environmental factors, as well as bee genetics. The quality and quantity of propolis collected depend on floral diversity, availability of plant sources, duration of the collection period, beekeeping practices, and local ecosystem health. The chemical profile of propolis extract is also strongly influenced by the solvent used for extraction, the solvent-to-material ratio, and the extraction procedure applied. More than 500 bioactive molecules have been identified in propolis, most of which are plant secondary metabolites with significant pharmacological activity, including antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, antitumor, antiulcer, and wound-healing-promoting properties. Extraction with solvents such as ethanol, ether, or water is required to isolate the active polyphenol and flavonoid compounds. Ethanol extraction of propolis has been reported to yield the highest concentration of polyphenols and flavonoids, thereby providing optimal anti-inflammatory and antioxidant activity.[4]

This study was designed to investigate the effect of propolis administration on flap hypoxia using a skin flap model in white rats, in order to evaluate its influence on flap survival.

METHODS

This was an experimental study using a pre-test and post-test control group design with male white rats (Rattus norvegicus) as experimental animals. The animal treatment and blood sample examinations were carried out at the PAU UGM animal facility, Yogyakarta, from May to June 2025.

Study subjects were male white rats (Rattus norvegicus) aged 3-4 months with a body weight of 150-300 g, obtained from the Faculty of Veterinary Medicine, Universitas Gadjah Mada. The animals were fed standard BR I rat feed.

A total of 24 animals were used, with 5 animals plus 1 reserve animal per treatment group. The treatment groups were as follows: positive control group (wound tissue + skin flap) given aquadest (K); treatment group 1, wound tissue + 1/3 skin flap + propolis 800 mg/kgBW-1 (P1); treatment group 2, wound tissue + 1/2 skin flap + propolis 800 mg/kgBW-1 (P2); and treatment group 3, wound tissue + 2/3 skin flap + propolis 800 mg/kgBW-1 (P3).

The skin flap animal model was created by full-thickness skin excision (epidermis, dermis, and part of the subcutaneous tissue/panniculus carnosus) over a 1×1 cm area on the dorsum using a no. 10 scalpel, leaving the underlying muscle tissue as the wound base (Figure 1).

The skin flap was harvested from the dorsal skin of the rat using a random advancement flap design. As much of the donor panniculus (fat layer) as possible was harvested from the donor rat's dorsum, with hemostasis achieved using 1: 100,000 adrenaline or, for bleeding from larger vessels, ligation with 6-0 monofilament suture. The donor skin flap was raised with a no. 10 blade and sutured to the wound bed using 6-0 Lene® monofilament suture, then fixed with moist gauze (0.9% NaCl). A tie-over dressing was used to cover the skin flap wound for 7 days, until the vascularization phase was reached.

The ethanolic extract of Mount Lawu propolis used in this study was prepared by ethanol extraction at the Inter-University Center Laboratory, Faculty of Animal Husbandry, Universitas Gadjah Mada. The dose of ethanolic propolis extract administered was 800 mg/kgBW/day, given orally by gastric gavage once daily for 7 consecutive days.

Blood VEGF and MDA levels were measured from retro-orbital venous blood using the enzyme-linked immunosorbent assay (ELISA) method, with a manual ELISA reader from Bioeureaux and VEGF and MDA reagent kits from Quantikine (catalog number D6050), expressed in pg/ml. High VEGF and MDA levels were defined as blood levels ≥ the median level across all groups.

Skin flap tissue, together with a small amount of surrounding healthy tissue, was subsequently collected after the rats were euthanized with chloroform. Specimens were fixed in formalin solution for immunohistochemical examination (VEGF and MDA expression) at the Department of Anatomical Pathology, Faculty of Medicine, Universitas Sebelas Maret. Flap tissue was then biopsied for histological assessment of cellular-level necrosis and inflammation.

Data on blood VEGF and MDA levels were analyzed using one-way analysis of variance (ANOVA) to determine whether mean VEGF and MDA levels differed among

treatment groups. Data not meeting the assumptions for one-way ANOVA were analyzed with the non-parametric Kruskal-Wallis test. When one-way ANOVA showed a significant difference, this was followed by post hoc testing, using statistical package for the social sciences (SPSS) for Windows Release 26. The significance level was set at α=0.05, with statistical significance defined as p<0.05.

All animal procedures were carried out in accordance with institutional ethical standards for the use of laboratory animals in research.

RESULTS

The mean reduction in MDA level was -3.89±0.47 in group K, -5.87±0.70 in group P1, -6.72±0.56 in group P2, and -6.81±0.53 in group P3 (Table 1). These data were further analyzed with the ANOVA difference test, which showed p<0.05 for the delta-MDA comparison.

Post hoc testing showed that group K differed significantly from groups P1, P2, and P3 (p<0.05), indicating that treatment with Mount Lawu ethanolic propolis extract reduced MDA more than in the control group. The reduction in MDA differed significantly between treatment groups, except between P2 and P3, which showed no significant difference (p=0.777).

The mean increase in VEGF level was 6.30±1.10 in group K, 6.51±1.33 in group P1, 8.12±0.87 in group P2, and 11.55±1.19 in group P3 (Table 2). These data were further analyzed with the ANOVA difference test, which showed p<0.05 for the delta-VEGF comparison.

Post hoc testing showed that group K differed significantly from groups P2 and P3 (p<0.05) but not from P1 (p=0.752), indicating that treatment with Mount Lawu ethanolic propolis extract increased VEGF more than the control group, except in the group with a 1/3-area skin flap. Post hoc comparisons between the treatment groups themselves showed significant differences in VEGF increase (p<0.05).

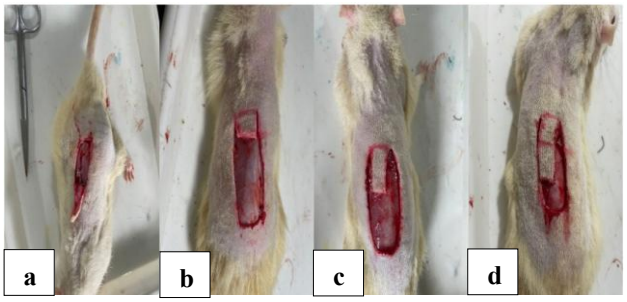

Figure 1: Experimental animal design in each treatment group (a) control flap; (b) one-third flap; (c) one-half flap; and (d) two-thirds flap.

Histopathological examination showed severe necrosis in most specimens across all groups. The group with the highest degree of necrosis was K, and the lowest was P2 (Table 3).

Between-group comparison showed no significant difference in the degree of necrosis (p=0.256).

In addition to the degree of tissue necrosis, the degree of acute inflammation was also assessed on histopathology. Most of the observed tissue showed severe acute inflammation. The group with the highest proportion of severe acute inflammation was K, and the lowest was P3 (Table 4 and Figure 2).

Between-group comparison showed no significant difference in the degree of acute inflammation (p=0.513).

Table 1: ANOVA difference test of MDA among treatment groups.

Groups	MDA pre (mean±SD)	MDA post (mean±SD)	MDA delta (mean±SD)	% Decrease	P value
K	11.74±0.53	7.85±0.39	-3.89 ±0.47	-33.1	<0.001*
P1	11.80±0.67	5.92±0.20	-5.87 ±0.70	-49.8	<0.001*
P2	11.73±0.61	5.02±0.16	-6.72 ±0.56	-57.2	<0.001*
P3	11.75±0.57	4.94±0.28	-6.81 ±0.53	-58.0	<0.001*
P value	0.998	<0.001*	<0.001*	-	-

ANOVA test (normally distributed, homogeneous data); *statistically significant at p<0.05

Table 2: ANOVA difference test of VEGF among treatment groups.

Groups	VEGF pretest (mean±SD)	VEGF posttest (mean±SD)	VEGF delta (mean±SD)	% Increase	P value
K	18.8+1.11	25.10+0.45	6.30 ±1.10	33.5	<0.001*
P1	19.33+1.26	25.84+0.55	6.51 ±1.33	33.7	<0.001*
P2	18.84+0.67	26.96+0.95	8.12 ±0.87	43.1	<0.001*
P3	19.01+0.98	30.56+1.06	11.55 ±1.19	60.8	<0.001*
P value	0.805	<0.001*	<0.001*	-	-

ANOVA test (normally distributed, homogeneous data); (*) statistically significant at p<0.05

Table 3: Necrosis findings.

Necrosis grades	K, N (%)	P1, N (%)	P2, N (%)	P3, N (%)
Mild	0 (0.0)	1 (16.7)	2 (33.3)	1 (16.7)
Moderate	0 (0.0)	0 (0.0)	1 (16.7)	1 (16.7)
Severe	6 (100.0)	5 (83.3)	3 (50.0)	4 (66.7)
P value	0.256			

Kruskal-Wallis test (ordinal categorical data)

Table 4: Acute inflammation findings.

Acute inflammation grades	K, N (%)	P1, N (%)	P2, N (%)	P3, N (%)
Mild	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Moderate	0 (0.0)	1 (16.7)	1 (16.7)	2 (33.3)
Severe	6 (100.0)	5 (83.3)	5 (83.3)	4 (66.7)
P value	0.513			

Kruskal-Wallis test (ordinal categorical data)

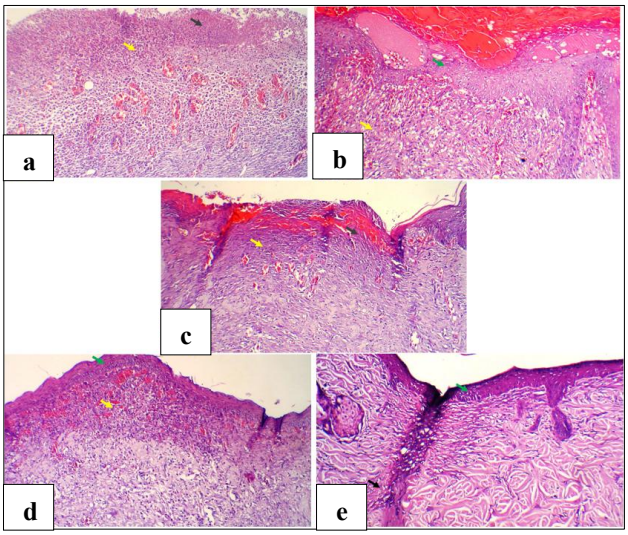

Figure 2: Histopathological appearance of the control group (a) control; (b and c) one-third flap group; (d) one-half flap group; and (e) two-thirds flap group.
Black arrow: necrosis; yellow arrow: acute inflammation (neutrophils)

DISCUSSION

In this study, the smallest reduction in MDA level was found in group K (33.1% decrease), with an increasing trend across treatment groups and the largest reduction in group P3 (58.0% decrease); the reduction in MDA in every group was statistically significant ($p<0.05$).

MDA is widely recognized as a central biomarker for evaluating oxidative stress, particularly that involving lipid peroxidation. When redox balance is disrupted, free radicals such as ROS attack polyunsaturated fatty acids (PUFA), producing toxic end products including MDA, 4-HNE, and F2-isoprostanes. Although all three are relevant, MDA is most frequently measured because of its strong correlation with pathological and physiological conditions involving oxidative damage. Its formation can occur through enzymatic (physiological) or non-enzymatic (pathological) pathways, making it a dynamic yet complex indicator. In group K, the smallest reduction in MDA occurred, indicating that without an exogenous antioxidant such as propolis, antioxidant defense was weak, resulting in largely uncontrolled lipid peroxidation. This finding reinforces the role of MDA as a key marker of oxidative stress. Previous studies confirm that MDA not only reflects the degree of oxidative damage but can also serve as a parameter for evaluating interventions aimed at restoring antioxidant balance, particularly in the context of tissue perfusion such as skin flap viability.[5,6]

MDA is formed as an end product of lipid peroxidation triggered by oxidative stress. When ROS exceed the capacity of the antioxidant system, they attack PUFA in cell membranes, generating lipid radicals. This triggers a chain reaction with oxygen to form peroxyl radicals, which subsequently form lipid hydroperoxides. These intermediates readily degrade into secondary products, including MDA. Because of its close relationship with oxidative damage, MDA is widely used as a biomarker of oxidative stress.[5-7]

Low MDA levels correlate strongly with better skin flap viability, since they reflect controlled oxidative stress. Interventions that reduce MDA, such as antioxidant administration, have been shown to improve flap tissue survival by protecting against lipid peroxidation and cellular damage.[8-12]

Our study found that propolis administration at 800 mg/kg BW produced a significant reduction in MDA values after treatment ($p<0.001$) in all groups. This is consistent with previous studies reporting that propolis administration reduces MDA values.[13,14]

Propolis reduces MDA levels through several multi-target mechanisms directed at oxidative stress. Rich in polyphenols, flavonoids, and phenolic acids, propolis can directly scavenge ROS and interrupt the lipid peroxidation chain.[13,15,16] Its phenolic compounds not only suppress

ROS production but also increase total antioxidant capacity (TAC) and stimulate endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which act synergistically to minimize oxidative damage.[14,16,17] Propolis also increases non-enzymatic antioxidants such as glutathione.[15] In addition, propolis suppresses inflammation by inhibiting pro-inflammatory cytokines (TNF-α, IL-1β) and NF-κB activation, thereby reducing secondary ROS production arising from inflammation.[18,19] Caffeic acid phenethyl ester (CAPE), an important propolis constituent, acts as a key scavenger of oxidative stress; propolis also regulates gene expression by activating Nrf2 and suppressing pro-oxidative genes, which can reduce MDA accumulation.[19]

CAPE reduces MDA by enhancing the activity of antioxidant-related enzymes such as SOD and GSH, strengthening cellular defenses against oxidative stress. CAPE also inhibits pro-inflammatory cytokines (TNF-α, IL-1β), interrupting the chain of ROS production and lipid peroxidation. Through this dual action, CAPE effectively suppresses MDA accumulation.[20,21]

The post hoc LSD test showed that nearly all between-group comparisons were significantly different ($p<0.001$), except between P2 and P3 ($p=0.965$), indicating that the MDA-lowering effect is directly proportional to wound area and inversely proportional to skin flap area, consistent with previous studies reporting that MDA values are inversely proportional to wound area.[8]

The absence of a significant difference between P2 and P3 in our study could potentially be addressed by leveraging the dose-dependent property of propolis, whereby higher doses produce greater therapeutic effects.[22-24] Exploiting this property, it is expected that an increase in wound area could be offset by an enhanced therapeutic effect achieved through a higher dose.

The lowest increase in VEGF level was found in group K (33.5% increase), while the highest was in group P3 (60.8% increase); the increase in VEGF in every group was statistically significant ($p<0.05$). This finding that propolis administration produces a significant increase in VEGF is consistent with previous studies.[23]

VEGF plays a central role in vascular development, from vasculogenesis and angiogenesis to lymphatic maturation. VEGF maintains vascular stability, regulates blood pressure, and preserves fluid balance between cells; it also regulates organ function in the kidneys, lungs, and central nervous system. Imbalanced VEGF production can trigger pathological conditions such as hypertension, proteinuria, impaired wound healing, and cognitive or motor impairment from neural damage.[25]

VEGF, particularly VEGF-A, is regarded as the principal biomarker of angiogenesis given its role as the central regulator of new blood vessel formation. It is expressed under hypoxic conditions and during wound healing, and it induces endothelial cell proliferation and migration; deletion of VEGF-A disrupts vascularization, underscoring its crucial role in both physiological and pathological angiogenesis.[25,26]

The role of VEGF in angiogenesis has also been linked to improved skin flap survival: two meta-analyses reported that increased VEGF is associated with a 15.7% increase in flap survival area, an effect attributed to the relationship between increased VEGF and increased vascular density.[27]

The post hoc LSD test in our study showed significant differences between groups ($p<0.05$), except between K and P1 ($p=0.401$), indicating that the VEGF increase is directly proportional to flap area and inversely proportional to wound area, consistent with previous studies.[10,28]

This can be explained by the direct relationship between skin flap area and the degree of tissue hypoxia: the more hypoxic the tissue, the greater the VEGF production triggered by that hypoxic state.[10,28] The larger the skin flap, the greater the degree of hypoxia, as indicated by increased HIF-1α levels.[10,28] Hypoxic conditions inhibit HIF-1α degradation, causing accumulation of the molecule, which then dimerizes with HIF-1β. This complex translocates to the nucleus, where it binds the hypoxia response element (HRE) on the VEGF gene promoter and triggers VEGF transcription; the resulting VEGF then stimulates angiogenesis through activation of VEGFR2 on endothelial cells.[29,30]

Our findings also show that propolis enhances angiogenesis by increasing VEGF levels, as demonstrated by the fact that all propolis-treated groups showed a greater increase in VEGF than the control group. This is consistent with previous studies showing a positive correlation between propolis administration and increased VEGF.[31] The lack of a significant difference between K and P1 in our study is presumed to be addressable by increasing the propolis dose, exploiting its dose-dependent property.[22,23]

Propolis can increase VEGF production because of its flavonoid content. Flavonoids stimulate macrophages and polymorphonuclear cells (PMNs) to release VEGF and fibroblast growth factor-2 (FGF-2), both key drivers of angiogenesis; the resulting VEGF increase subsequently triggers endothelial cell migration, proliferation, and differentiation, which ultimately facilitates new blood vessel formation and supports tissue repair. Propolis also inhibits the cyclooxygenase (COX) and lipoxygenase pathways, reducing leukotriene production and excessive inflammation; this anti-inflammatory activity creates conditions favorable for VEGF release by macrophages and for angiogenesis.[32]

Beyond its flavonoid content, other propolis compounds also strongly influence VEGF expression. CAPE increases VEGF through activation of the p38 MAPK, ERK, and

JNK signaling pathways and the NF-κB transcription factor, which are important for VEGF production, while also suppressing pro-inflammatory cytokines such as CXCL10, creating an environment that supports tissue repair and sustains angiogenesis.[33]

Our study showed that propolis administration did not produce a statistically significant difference in the degree of necrosis (p=0.256), although a trend toward reduction compared with control was observed; most specimens still showed severe necrosis, indicating a limited effect within the context of the flap model used. This finding differs from several previous studies that reported significant effects of propolis on controlling necrosis, both macroscopically and histopathologically.[34] This discrepancy is likely influenced by dose variation, given the dose-dependent nature of propolis, and by the absence of a standardized experimental formulation.[22-24,35] In addition, the composition of active compounds in propolis varies considerably according to geographic and botanical source, which contributes to inconsistency of results across studies.[35]

Experimental model factors also play a role. The choice of ligated artery, flap type (pedicled versus free), and incision technique are all known to affect the degree of necrosis.[36] In this study, these factors may have contributed more strongly to necrosis outcomes than the propolis intervention itself.

On histopathology, propolis also did not significantly reduce acute inflammation (p=0.513), with all groups showing a severe degree of inflammation. This appears to contradict the general evidence that propolis is anti-inflammatory.[37,38] However, several studies have reported an increase in early inflammation as part of the normal healing mechanism, with increased neutrophils and cytokines such as TNF-α and TGF-β during the early phase, followed by rapid resolution by day 7 to 14.[39]

This suggests that propolis may not suppress inflammation from the outset, but instead modulates its dynamics, enhancing the early phase to accelerate regeneration before promoting faster resolution. In our study, tissue was possibly sampled too early or too late to capture this transitional phase.

Overall, although propolis did not show a statistically significant effect in reducing necrosis or acute inflammation in this study, this does not negate its potential benefit. Variables such as dose, timing of administration, flap model, and healing phase should be re-evaluated in future studies to reveal its optimal effect.

CONCLUSION

Administration of ethanolic propolis extract significantly increased skin flap viability, as indicated by a decrease in blood MDA and an increase in blood VEGF levels. However, propolis administration did not show a statistically significant effect in preventing tissue necrosis or acute inflammation on histopathological examination. Further studies evaluating dose, timing of administration, and flap model are warranted to clarify the optimal therapeutic effect of propolis on flap survival.

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