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Indonesian Propolis Extract Acts as Antioxidant in Angiogenesis Based on Microvascular Density and Vascular Endothelial Growth Factor Assay: An Experimental Study in Skin Graft Murine Model

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Authors' contributions

This work was carried out in collaboration among all authors. Author BN designed the study, performed the statistical analysis, literature searches and wrote the first draft of the manuscript. Authors AS and KYY managed the analyses of the study. Author BW provided assistance with statistical analysis and manuscript writing. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Skin plays an important part as the foremost protector of human body and is prone to injuries. Wound healing process involves pro-inflammatory cytokines which trigger angiogenesis. Propolis is a byproduct that has been proven to play a role in angiogenesis through its antioxidant effect and modulating angiogenesis and inflammatory substances.
Study design: Experimental trial with post-test-only control.
Place and Duration of Study: This experiment is conducted from September to October 2020 in Gadjah Mada University, Yogyakarta.

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Methodology: This study used white male rat (*Rattus Norvegicus*) aged 8-10 weeks and weighing 150–200 grams with skin graft model and divided into four groups. Skin grafting was done four days after wound incision. Propolis was administered right afterwards for a week. Blood sample was taken at the last day of propolis intervention while the VEGF and MVD assessment were done the next day.
Results: We found differences in VEGF expression between four groups. Slowest angiogenesis was observed in control group (25.13 ± 1.36 pg/ml; $P < .001$). Group 1 had the lowest microvascular density (MVD) (84,48 ± 20,53; $P > .05$). We found very weak and insignificant correlation between VEGF and MVD (r = .002; $P = .993$).
Conclusion: Propolis as antioxidant affects MVD and VEGF expression. Propolis enhances angiogenesis, marked by elevation of VEGF but not MVD.

Keywords: Propolis; wound healing; microvascular density; vascular endhothelial growth factor.

1. INTRODUCTION

Skin is the first and outermost layer of human protection that shields the body from environmental exposures [1-2]. The skin is very fragile and prone to wound or scarring. Destruction of an epithelial layer or its surrounding soft tissue due to excessive tissue destruction, underlying pathological process, and decreasing perfusion and oxygenation creates a wound [3]. Wound healing, therefore, is an important mechanism to prevent further damage or open-wound contamination [2].

Wound healing involves biochemical substances such as Reactive oxygen species (ROS), Malondialdehyde (MDA), caspase-9, Nuclear factor kappa beta (NF-κβ), Tumor growth factor beta (TGFβ) and Vascular endothelial growth factor (VEGF) [4]. ROS in its normal, physiological level activates the expression of Hypoxia inducible factor (HIF)-1α and stabilizes or increases inflammatory cells response to signaling factors such as VEGF [5]. ROS induces VEGF expression during wound healing process to stimulate angiogenesis through VEGF receptor-2 (VEGFR-2) inside endothelial cells. Angiogenesis can be quantitatively measured as microvascular density (MVD) through the use of several biomarkers such as VEGF, Cluster of differentiation (CD) 31, CD34, CD105, and factor VIII. This assessment method is done by immunohistochemistry staining. An increase in certain biomarkers signifies higher MVD, implying an ongoing angiogenesis [6-7].

Propolis has several positive or synergic effect in wound healing process [8]. Juanes et al. [9]. have proved that propolis works as an antioxidant which inhibits angiogenesis through modulation of angiogenesis and inflammatory-inducing factors. Kakehashi et al. [10] have stated that administration of propolis suppresses tissue inflammation and cell proliferation. The anti-inflammatory property of propolis was marked by a decrease in neutrophils, increased macrophages activation, B- and T-lymphocyte modulation, and proliferation of antibodies and inflammatory cytokines such as Interleukin (IL)-2, IL-10, dan Interferon (IFN)-γ. This study aimed to determine antioxidant and angiogenesis effect of propolis in wound healing.

2. MATERIALS AND METHODS

This is an experimental, post-test-only control design using male white murine (*Rattus norvegicus*) model with skin graft, aged around 8 – 10 weeks, and weighted around 150–200 grams. Study subjects were divided into 4 different groups consisted of one control group with no propolis administration and three intervention groups, each with different dosage of propolis. Blood sample was taken at the last/ 7th day of propolis intervention post skin grafting while the VEGF and MVD assessment was carried the next day. This study was done in Gadjah Mada University, Yogyakarta from September 2020 until October 2020.

Each murine underwent a one-week adaptation in which they properly fed and caged before divided into four different study groups. A two times two centimeters (cm) wound was done in the back of each murine using number 10 scalpel as per full-thickness skin graft donor procedure. Murine scaring procedure is shown in Fig. 1. No treatment except application of talcum powder to induce granulation process and inhibition of secondary wound closure was given for 4 days. All murine in control group was only given two milliliters (ml) solution of .5% carboxymethyl

cellulose sodium (Na CMC)/ murine per oral every morning while murines in intervention group 2 to 4 received 50, 100, and 200 milligrams (mg)/ kilograms (kg) of body weight (BW)/ murine of propolis solution orally every morning accordingly for a week after skin grafting procedure. Skin grafting was done together with propolis application and wound dressing after the wound was left open for 4 days as shown in Fig. 2. Each murines in every group were weighted around 185-200 grams in average right before skin grafting and gained 3-4 grams after the procedure. Blood sample was taken from every murine for angiogenesis assessment at the 7th day of propolis administration and the VEGF and MVD assessment were done the next day. During the experiment, all murines received analgesics to ensure freedom from pain and received proper burial afterwards. This experiment has been done in accordance to 1964 Declaration of Helsinki.

Skin sample from each murines was also taken for histopathological analysis which used Hematoxylin-Eosin (HE) staining. Each study group consisted of five murines, which is in accordance to World health organization (WHO) standard for animal experiment [11].

Results of this study were analyzed using IBM SPSS Statistics version 25.0. Normality test was conducted using Kolmogorov-Smirnov to determine data distribution. Comparative analysis of VEGF and MVD in each study group was done using one-way Analysis of variance (ANOVA) for normal data distribution. Relationship between VEGF and MVD was determined using Pearson's correlation test for normal data distribution. All results of this study were deemed significant for P = .05.

3. RESULTS AND DISCUSSION

We demonstrated different VEGF and MVD in each study groups. Murines which received propolis had relatively higher amount of VEGF and MVD compared to control group. The lowest VEGF level was found in the control group (25.13) and steadily increasing along with the increasing propolis dosage. The highest VEGF was observed in the third intervention group (34.64) which received the most amount of

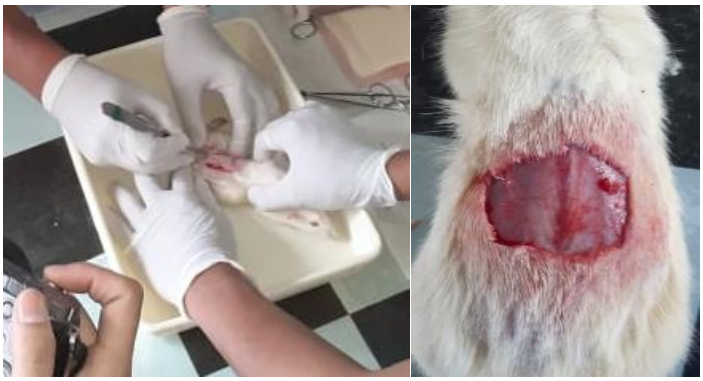

Fig. 1. Murine scaring procedure. Incision was made on using number 10 scalpel (Left). A 2x2 cms full thickness wound was made on the back of each murine (Right)

Fig. 2. Each murine received skin graft after the wound was left open for 4 days (Left). This process was also done together with wound dressing and propolis application (Right)

propolis. However, we observed an abnormal pattern in MVD distribution among all study groups as shown by the histograms in Fig. 2 and Fig. 3. MVD assessment using CD34 as the biomarker revealed that the control group had a higher MVD (166.58) compared to other groups. This uneven distribution of MVD shows a discrepancy between the results of this study with angiogenesis theory. Overall results of VEGF and MVD assessment across all murine models are shown in Table 1, Fig. 3, and Fig. 4.

Kolmogorov-Smirnov test showed a normal data distribution for VEGF dan MVD in every study group. This was shown by asymptotic significance value of .200 in every study group, which is higher than *P* value. Normality test results for VEGF and MVD is shown in Table 2.

Table 1. MVD and VEGF results across all models

Group	Component	Minimum	Maximum	Mean	Standard deviation
Control	MVD	41.38	272.25	166.58	30.12
	VEGF	23.43	26.82	25.13	1.36
1	MVD	34.00	161.38	84.48	20.53
	VEGF	27.67	30.21	29.05	.91
2	MVD	62.88	303.13	162.71	43.14
	VEGF	31.69	32.97	32.26	.46
3	MVD	38.63	213.25	145.58	27.55
	VEGF	33.60	35.93	34.63	.86

MVD = Microvascular density; VEGF = Vascular endothelial growth factor

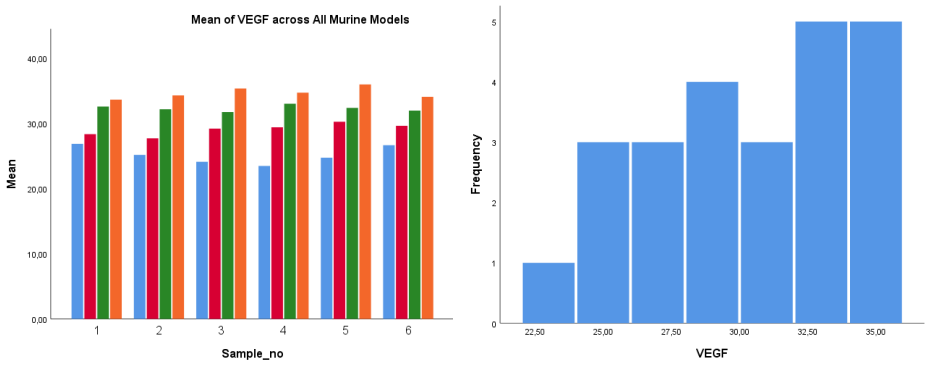

Fig. 3. Mean expression of VEGF shown in clustered bar graphs (left) and histogram (right). Blue = control group; Red = group 1; Green = group 2, Orange = group 3

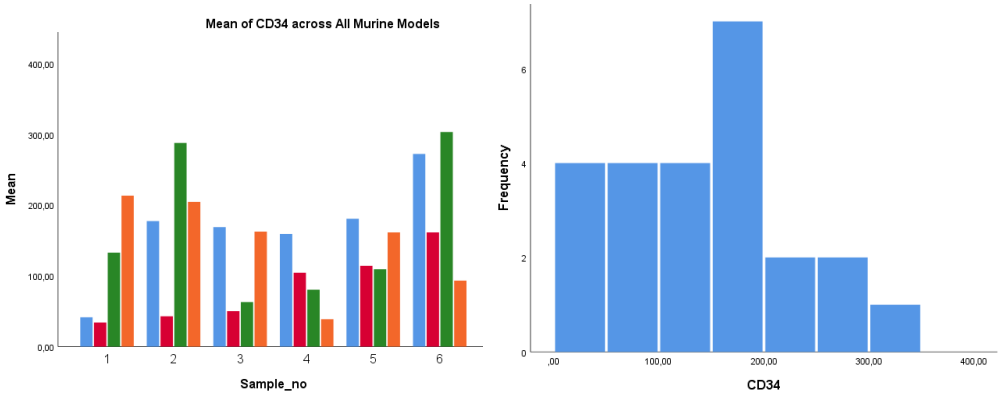

Fig. 4. Mean expression of CD34 in clustered bar graphs (left) and histogram (right). Blue = control group; Red = group 1; Green = group 2, Orange = group 3

Comparative analysis showed a significant difference with relatively small gaps in VEGF value among every study groups (F = 111.87; *P* <.001). Tukey's post-hoc analysis showed that angiogenesis process in third intervention group (34.63 ± .86 pg/ml; *P* < .001) was higher compared to control, first (29.05 ± .91 pg/ml; *P* < .001), and second (32.26 ± 0.46; *P* < .001) intervention group. The slowest angiogenesis rate was observed in control group (25.13 ± 1,36 pg/ml; *P* < .001). Result of one-way ANOVA analysis for VEGF in every study group is shown in Table 3.

Compared to our VEGF findings, we did not manage to find a significant difference in MVD among our study groups (F = 1.464; *P* = .254). Tukey's post-hoc analysis showed a higher, but insignificant, number of MVD in control group compared to the intervention groups (155.68 ± 30.12; *P* > .05). The lowest MVD was observed in first intervention group (84.48 ± 20.53; *P* > .05). This result shows that MVD expression is not always accompanied by increasing VEGF. One-way ANOVA result for MVD is shown in Table 4. Immunohistochemistry results using CD34 are shown in Fig. 5.

Relationship between VEGF and MVD was determined using Pearson's correlation test. We obtained a weak, insignificant, but positive correlation between VEGF and MVD (r = .002; *P* = .993). This finding proved that angiogenesis is not always followed by an increase in MVD. Results of Pearson's correlation test for VEGF and MVD are shown in Table 5.

Table 2. Normality test for VEGF and MVD

		Control	Group 1	Group 2	Group 3
VEGF	Asymp. Sig. (2-tailed)	.200	.200	.200	.200
MVD		.117	.200	.161	.200

Table 3. VEGF differences between groups using ANOVA

		F	Mean Diff.	*P* value
Between Groups		**111.869**		**.000**
Control	1		-3.92000	.000
	2		-7.13333	.000
	3		-9.50000	.000
1	Control		3.92000	.000
	2		-3.21333	.000
	3		-5.58000	.000
2	Control		7.13333	.000
	1		3.21333	.000
	3		-2.36667	.002
3	Control		9.50000	.000
	1		5.58000	.000
	2		2.36667	.000

Table 4. MVD differences between groups using ANOVA

		F	Mean Diff.	*P* value
Between Groups		**1.464**		**.254**
Control	1		82.10417	.281
	2		3.87500	1.000
	3		21.00000	.964
1	Control		-82.10417	.281
	2		-78.22917	.321
	3		-61.10417	.529
2	Control		-3.87500	1.000
	1		78.22917	.321
	3		17.12500	.980
3	Control		-21.00000	.964
	1		61.10417	.529
	2		-17.12500	.980

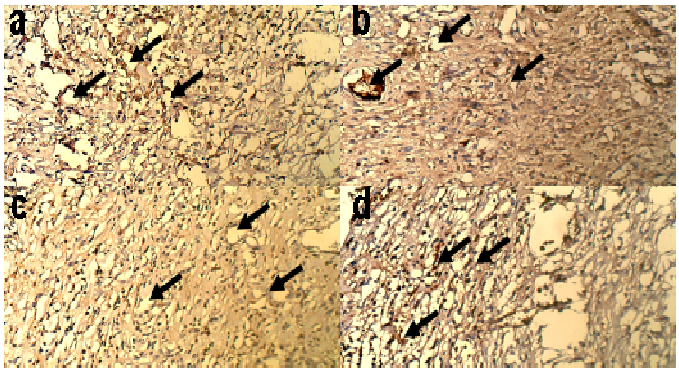

Fig. 5. Immunohistochemistry assessment using CD34 showed endothelial formation in all study groups as shown by the black arrows. Control group (a) showed the highest MVD compared to group 1 (b), group 2 (c), and group 3 (d). Group 1 had the least MVD

Table 5. Correlation between VEGF and MVD

Groups	Correlation coefficient (r)	*P* value
Control	-.101	.848
1	.750	.086
2	-.331	.522
3	-.112	.832

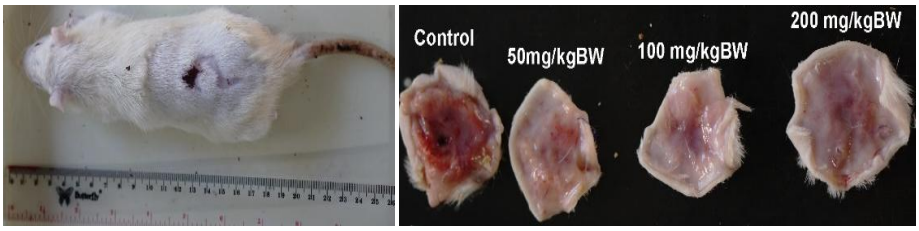

Fig. 6. Administration of propolis induces wound healing in a murine which also had received skin graft (Left). Administration of 200 mg/kgBW propolis results in a better wound healing quality compared to other groups (Right)

We also obtained a weak, inversed, and insignificant correlation for VEGF in every study group except the first intervention group (r=.750, *P* = .086). This particular group showed a strong but insignificant correlation between VEGF and MVD. We hereby concluded that VEGF expression is not always followed by an increase in MVD. Visual comparison of wound healing process at seventh day after intervention between every study group is shown in Fig 4. Compared to other groups, group 3 which received 200 mg/kgBW dose of propolis presented better wound healing as shown by the formation of mature granulated tissue from the skin graft.

We managed to determine the wound-healing property of propolis in white murine. This study revealed that oral administration of propolis significantly increases the expression of VEGF. Murines in group 3 which received 200mg/kgBW dose of propolis showed a higher amount of VEGF compared to control and other study groups (*P* < .001). Our study has managed to prove that propolis-induced angiogenesis plays an important role in wound healing process. In a latest study by Kresnoadi et al. [12], it was also shown that propolis administration increases VEGF which assists with wound closure. This study combined propolis extract with bovine bone graft and polyethylene glycol. These angiogenesis stimulatory components inhibit attachment of Receptor activator of NF-κβ ligands (RANKL) to its receptors by increasing osteoprotegerin. This process increases Fibroblast growth factor (FGF)2 and VEGF which eventually promotes vascular endothelial cells proliferation. Increased FGF2 and VEGF also

promotes osteoblasts production and promoting bone growth. Another study in 2013 has also shown that propolis promotes dermal connective tissue remodeling process through formation of mature granulation tissue [13]. Propolis is also known for its acute anti-inflammatory effect as proven through a study by Jacob et al. [14]. The antimicrobial effect of propolis indirectly lowers the expression of neutrophiles which in turn decreases free radicals and tissue damage due to inflammation process.

Iqbal et al. [15] stated a different result, in which Indonesian propolis does not have the ability to induce VEGF and FGF expression. This study implied that pro-apoptotic factors such as p53, ROS, and caspase-3 may inhibit VEGF and FGF production, a mechanism that is believed to inhibit tumor growth. Direct inhibition of VEGF genes is also another proposed mechanism. Apoptosis targets pericyte, a component that stabilizes blood vasculature. VEGF and pericytes work hand-in-hand to support angiogenesis. Inhibition of VEGF increases pericytes, a mechanism that is believed to act as an anti-angiogenic factor in tumors [16] However, VEGF induction also promotes angiogenesis and increases MVD in both normal and pathologic condition, as shown in neoplastic growth [17].

Yang et al. [18] showed that pinocembrin, a key flavonoid component in propolis, triggers expression of CD34. Pinocembrin stimulates expression of Phosphorylated endothelial nitric-oxide synthase (p-eNOS) and nitric oxide (NO) through phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/eNOS pathway. Phosphorylation of eNOS is mediated by VEGF, which together with CD34 induces angiogenesis through marcophages differentiation. Pinocembrin, through this mechanism, increases endothelial progenitor cells proliferation and migration [13,18]. Propolis' ability in increasing CD34 is not followed by a decrease in number of cells or cell death, proving that propolis administration is relatively safe to be administered [19].

An increase in CD34 marks a higher MVD. A study by Mogoşanu et al. [13] found that application of flavonoid extract for burns increase MVD, which signifies an increase in microvasculature. Our finding showed that VEGF increase is not always accompanied by rising MVD. This is in contrast with basic concepts of angiogenesis which implies that a high VEGF is also followed by a high MVD as shown in hematologic malignancies and cancers [20]. Another study by Suga et al. [21] using human adipose stem cells found that that cell differentiation disorders, expression of telomerase, an increase in CD133, and hypoxia are factors that influence CD34 expression dan MVD. Changes in intracellular environments due to trauma and inflammatory processes can also lower the value of CD34. Certain types of fixative reagents such as FACS™ Lysing Solution and sample washing techniques can also affect the expression of CD34 in a decreasing manner [22]. Endothelial cell culture and cell cycle initiation can also lower the value of CD34 [23]. Age is also a contributing factor in CD34 expression, in which elderly population tends to have a smaller expression of CD34 [24].

This study is not without limitations. First, we did not obtain VEGF and MVD data from the murines before administration of propolis. This means that we were not able to find any differences in VEGF or MVD before and after administration of propolis. We also used CD34, a not well-known biomarker for MVD assessment unlike CD105. Using CD105 as biomarker for MVD may yield a better result due to its sensitivity and specificity [25]. This study only utilized one type of propolis which originates from Central Java, Indonesia. Different propolis from another geographical area may have different wound healing effect, as shown by a study by Iqbal et al. [15]. This study assesses the wound healing property of propolis as a whole, so we did not find the exact flavonoid component which plays the biggest role as an antioxidant.

4. CONCLUSION

We have managed to prove that propolis is an antioxidant that affect MVD and VEGF expression in granulation tissue of male murine. Our findings have shown that propolis stimulates angiogenesis through increase in VEGF but not MVD. We concluded that propolis has a potential as wound healing agent. Further study using other types of propolis and analysis/identification of the exact flavonoid component may facilitate researches in wound treatment and care.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study has received Animal ethic committee from Dr. Moewardi General Hospital Health

Ethics Committee and in accordance to 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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