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The effect of Indonesian propolis dosage on vascularization of skin graft in skin wound of white rat's skin graft model: molecular studies of malondialdehyde (MDA), nuclear factor-kappa beta (NF-kB), interleukin-6, vascular endothelial growth factor (VEGF), caspase-3, and microvessels density (MVD)

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ABSTRACT

Background: Extensive wound closure due to trauma, infection and post tumor excision often requires a skin graft procedure. One of the successful factors of skin graft is the good quality of recipient tissue vascularization. Reactive oxygen species (ROS) is a factor that plays a role in vascularization, an excessive accumulation of ROS can interfere with the process of vascularization. Administration of the antioxidant can keep the ROS levels from being excessive thus producing the recipient vascularization for a successful skin graft. Propolis has an effect of antioxidant, inflammation and immunomodulator, anti-ROS and anti-inflammatory effects due to the large polyphenol content. This study aims to examine the antioxidant effect of propolis ethanol extract Lawu mountain (EEP) in a rats skin graft model by examining its effect on oxidative stress levels (Malondialdehyde blood levels/MDA), effect on inflammation (IL-6 blood levels and NF-kB expression), effect on angiogenesis (VEGF blood levels and Caspase 3 expression) and skin graft vascularization (Micro Vessels Density /MVD).

Methods: The study used 24 white male rats (*Rattus norvegicus*) aged 3-4 months, body weight 150-300 grams, and divided into a control group with skin graft without treatment and three groups of skin graft treated with a dosage, 200 mg/kg-BW EEP, a dosage, 400 mg/kg-BW EEP, dan a dosage, 800 mg/kgBW EEP) for 7 days. Wound tissue granulation is made according to previous research. Skin graft procedure uses allograft donors for 7 days. Blood draw from retro orbita veins and skin graft with a small amount of recipient tissue done after the seventh day. The propolis ethanol extract of mount Lawu (EEP) is made by maceration technique 3,75 liters of ethanol 70% as a solvent. Using the SPSS 22 for windows program, blood MDA levels, blood IL-6 levels, and blood VEGF levels were analyzed by One Way ANOVA and post hoc. Examination of Nuclear Factor-Kappa Beta (NF-kB) expression, Caspase-3 expression, and MVD was carried out by using Immunohistochemistry (IHC) tested with non-parametric Mann-Whitney test.

Results: There was a significant decrease in MDA blood levels ($p<0.05$), with an effective dosage, 400 mg/kgBW EEP and a dosage, 800 mg/kg-BW EEP. There was a significant decrease of IL-6 blood levels ($p<0.05$) at administration dosage, 200 mg/kg-BW EEP and a dosage, 400 mg/kg-BW EEP, and statistically significant ($p<0.05$). Vascular Endothelial Growth Factor (VEGF) blood levels increased with a dosage from 200 mg/kg-BW EEP until a dosage, 800 mg/kgBW EEP and significant ($p<0.05$), the post hoc test showed significant results in all group comparisons. There was no significant increase of skin graft vascularization (MVD) ($p=0.254$). Caspase-3 and NF-kB expressions decreased at certain dosages, although the decrease of Caspase-3 ($p=0.557$) and NF-kB ($p=0.916$) were not significant in statistic analysis test.

Conclusion: Propolis ethanol extract of mount Lawu (EEP) has anti-ROS, anti-inflammatory, and angiogenic effect through decreased MDA blood levels, decreased IL-6 blood levels and increased VEGF blood levels, no significant reduction in NF-kB and caspase-3 expression and there was no increase in skin graft vascularization (MVD).

Keywords: reactive oxygen species, inflammation, angiogenesis, propolis, vascularisation, skin graft.
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INTRODUCTION

Skin graft is still the golden standard in the management of extensive skin defects. The successful skin graft in extensive wounds is still a management challenge by a plastic surgeon specialist. Skin grafting has been a part of management for more than 2 millennia and is the number 2 most frequently used to close the wound after primary wound closure. The incidence of skin grafting increased in 1996 by 98.000 cases compared to 2002 by 240.000 cases, according to the Healthcare Cost and Utilization Project more than 160.000 skin graft procedures were performed annually among 1 patient from 3 burn patients.[1-3]

Skin grafting is a surgical technique for reconstructive wound closure by moving the epidermis and parts or all the dermis from place to another place in order to live in new place, either a permanent or temporary procedure. The indication of Split Thickness Skin Graft (STSG) is to cover the extensive defect such as the reconstruction of extensive and deep burn, extensive defect post excision or contracture release, congenital skin deficiencies, post soft tissue trauma, post extensive tumor excision and infection because of Diabetes Mellitus (Diabetic Foot Ulcers/DFU).[2,4,5]

The success of Split Thickness Skin Graft (STSG) depends on the quality of the recipient bed's vascularization, affected by hematoma, infection, foreign body, chronic wounds, adequate immobilization and the type or thickness of the skingraft. The successful of skin-graft was assessed by the take percentage that is the occurrence of revascularization of the skin graft which clinically characterized by pink STSG and histopathological examination using light microscopy, will see an image of new blood vessels that can be measured in diameter and the number of new vessels per mm at the fibrin interface as vascular density metric.[6-10]

The failure rate of skin graft at lower extremities was described from 70 skin graft procedures in 50 patients, there were 1/3 skin graft failures of all procedures during 6 weeks follow up after operation. The cause of this failure is associated with an increase in Body Mass Index (BMI), peripheral vascular disease, and giving of immunosuppression drugs and infection. On a meta-analysis of successful study of STSG in Diabetic Foot Ulcer (DFU) cases reported the highest reach 85% and skin graft failure was 16%.[11,12]

Granulation tissue as a product of the angiogenesis process has vascularity that depends on the quality of angiogenesis. ROS is one of the factors that play a role in the angiogenesis process, either directly or indirectly through VEGF. The quantitative assessment of angiogenesis can be in vitro or in vivo by manually calculating the density of the blood vessels, the length of the blood vessels and the diameter of the blood vessels.[13,14]

The angiogenesis process have several stages involving activate endothelial cell (EC) by angiogenic factor (EC proliferation, degradation of basal membrane, formation of the structure of the blood vessel tube, and vascular stabilization.[15] Blood VEGF levels expression the tissue VEGF (keratinocytes and macrophages) associated with proangiogenic activity. High VEGF levels were found in the normal wound healing process, a low VEGF levels were found in patients with chronic wounds (DM). Adequate and sufficient blood supply necessary to maintain healthy tissue where it requires oxygen and nutrients from the blood.[16,17]

Propolis is a natural product from various types of trees, which vary in material content depending on demographics, seasons, tree species and seasons. Propolis has a fairly broad spectrum of antioxidant effects. The mechanisms of the antioxidative activity of propolis such as the ability to inhibit the ROS formation, inactivate various compounds in the ROS formation, and the clearance of ROS thereby disrupting the flow of reactions leading to lipid peroxidation and increasing synergy with other types of antioxidants. Propolis shows as a good anti-inflammatory in chronic inflammatory processes, and this is mainly due to its large polyphenol content and angiogenic effects both as proangiogenic and as antiangiogenic (pinocembrin) and the effect of inducing apoptotic pathways by stimulating Bax, p53, p21 protein, cytochrome c release and activate the caspase cascade.[18,19]

Indonesian propolis (Sulawesi) contains a mixture of ingredients that cannot be separated from 4 elements including alk (en) ylresorcinols (5-pentadecyl resorcinol), 5-(8'Z, 11'Z-heptadecadienyl)-resorcinol, 5-(11'Z-heptadecenyl)-resorcinol, and 5-heptadecyl resorcinol), and also contains 4 prenylflavonoids, propolis D, C, F, and G and 3 types of cycloartane that is triterpenes, mangiferolic acid, isomangiferolic acid, and 27-hydroxysomangiferolic acid have been identified. Propolis ethanol extract of Lawu mountain contains Caffeic Acid Phenethyl Ester (CAPE) with levels of $30.24 \pm 3.53 \times 10^{-6}$gr and quercetin $4.42 \pm 0.50 \times 10^{-6}$gr. The flavonoid content of *Trigona sp* bee propolis was higher than *Apis mellifera* bees, from the origin of propolis the highest antioxidant activity was from Pandeglang and the highest total flavonoids came from Kendal.[20]

The administration of antioxidants can control the levels of ROS in the normal angiogenesis process, which is necessary for successful skin graft vascularization.

This study aims to analyze the effect of the dose of Lawu mountain propolis ethanol extract (EEP) on skin wounds of white rats with skin grafts on the effect of ROS (MDA blood levels), inflammation (IL-6 blood levels, NF-kB expression) and angiogenesis (VEGF blood levels and expression of caspace-3) on the vascular skin graft (Microvessels density / MVD). It is hoped that a certain dosage of EEP will be used as an additional therapy to increase the successful of skin grafts on skin wounds.

METHODS

Study design and experimental animals

This study used a post-test only control group design in a group of white male rats (*Rattus novergicus*) as a sample. The number of white male rats used in the study was 24 aged 3-4 months and body weight 150-300 grams. Treatment and blood sampling was carried out at PAU UGM Yogyakarta, while immunohistochemical examinations were carried out at the Anatomical Pathology Laboratory, Faculty of Medicine, Sebelas Maret University Surakarta by a pathologist.

Procedure and injury observation

The rats were subjected to skin excision

(full-thickness/epidermis, dermis, and partially subcutaneous or panniculus carnosus) in the back area with a size of 1 x 1 cm using scalpel no. 10. The wound was given sterile talc to stimulate the growth of granulation tissue for 7 days. Furthermore, all rats were subjected to an allograft type skin graft. Rats that had skin grafts were divided into four groups, namely group 1 (K) which was given 2 ml of Na CMC 0.5% for 7 days, group 2 (P1) which was administered ethanol propolis extract (EEP) at a dosage of 200 mg/kg body weight/day orally for 7 days, group 3 (P2) were given EEP at a dosage, 400 mg/kg body weight/day orally for 7 days, group 4 (P3) were given EEP at a dosage, 800 mg/kg body weight/day orally for 7 days (Figure 1).

Assessment of Malondialdehyde (MDA), IL-6, NF-kB, VEGF, Caspase-3, MVD

After 7 days of post skin graft, MDA blood levels were measured using the thiobarbituric acid reacting substances (TBARS) method and expressed in units of nmol / ml. Blood IL-6 levels were measured by the Enzyme-Linked Immunosorbent Assay (ELISA) method, expressed in units of pg/ml. VEGF levels will be measured by means of Enzyme-Linked Immunosorbent Assay (ELISA) with the VEGF Human ELISA kit, expressed in units of pg/ml. Blood draws are carried out through retroorbital veins. NF-kB expression was assessed based on the expression of inactive NF-kB in the cytoplasm of macrophage cells and appeared as brown on IHC preparations, and was calculated by histologist score.

Assessment of the positivity of caspase-3 expression used immunohistochemical examination with monoclonal antibody to caspase-3. Assessments were performed quantitatively visually using a light microscope with 400x magnification of caspase-3 expression cells. Then count the number of immunoreactive cells stained silver brown on the cell membrane. Then, the percentage of all existing immunoreactive cells was carried out by scoring: a score of 1 = 1-30%, a score of 2 = 31-70% and a score of 3> 70%.[21]

MVD histological examination used a CD34 marker, for staining new vascular endothelial cells by counting the number of blood vessels per mm^2.[22] The vascular number was calculated by manual marking using image pro plus software, then the data summed and entered into an Excel table for automatic calculation of vascular density. The calculation results are also carried out by taking a microscope image with a 40X magnification equipped with a 5 MP CCD color sensor. Images are captured, stored, and analyzed using Image-Pro Plus software.[23]

Statistical analysis

The data about MDA blood levels, IL-6 blood levels and VEGF blood levels were analyzed using the One Way Analysis of Variance (ANOVA) parametric test. The One Way Anova test was used to determine whether there were differences in the mean values of MDA, IL-6 and VEGF blood levels between the treatment groups. To find out whether there were differences in the mean values of MDA, IL-6 and VEGF blood levels between the treatment groups, the One Way Anova test was used. Data that did not meet the requirements to be analyzed using the One Way Anova test were analyzed using the non-parametric Mann-Whitney test. If the One Way Anova test results show a significant difference, then proceed with the post hoc test.

The data about NF-kB expression, Caspase-3 expression, and MVD will be tested using the Mann-Whitney non-parametric test. The level of significance used was $\alpha = 0.005$. Thus, the statistical value was declared significant if $p<0.05$.

RESULTS

Assessment of MDA Blood Levels

The administration of EEP decreased MDA blood levels. There was a significant difference in the MDA results between the normal group and the treatment group. The MDA blood levels in each control group and administering of EEP dosage

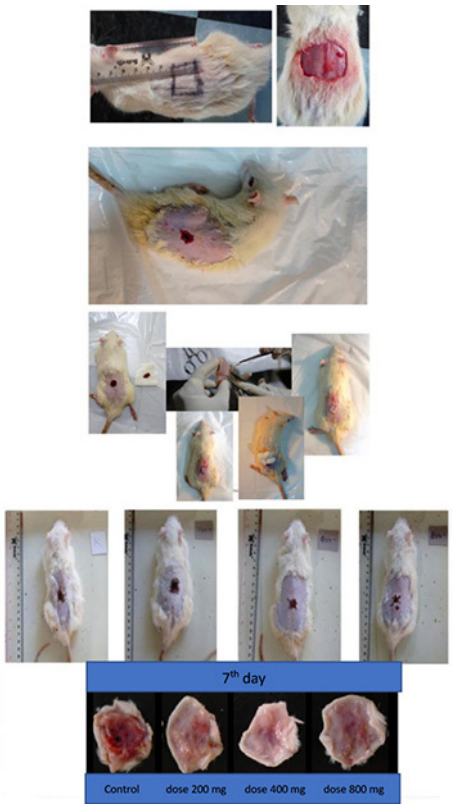

Figure 1. Rat model treatment and observation of skin graft injuries

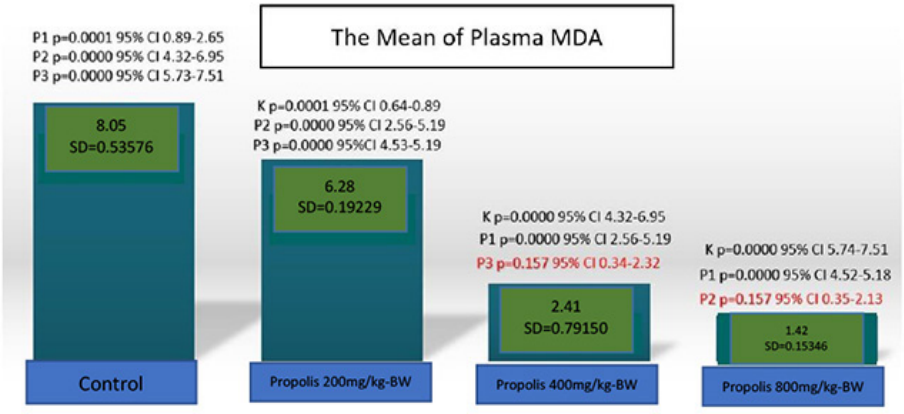

Figure 2. Hologram of the mean MDA blood levels of each control group and administration of EEP dosages, it appears that at K, P1, P2 and P3 MDA blood levels decreased gradually.

appeared on K, P1, P2, and P3 MDA blood levels decreased gradually. There was a significant difference between the P1 and P2 and P3 dosages, but the data for the ratio of dosages of P2 to P3 were not significant. The use of the cut-off points method that has a value below the cut-off point will have no difference [Natural Cut-off Points = (Maximum Score + Minimum Score)/4=800/4=200]. The dosage of EEP administration of 200 mg/kg-BW is the cut-off point value. The use of EEP with a dosage of P2 and P3 is the most effective dosage to decrease MDA blood levels. The comparison of the use of P2 and P3 dosages did not significantly affect the results of decreasing MDA (Figure 2).

Assessment of IL-6 blood levels

The administration of EEP decreases IL-6 blood levels. It appears that in K, P1, P2, and P3 IL-6 blood levels decreased gradually. There was a significant difference between the control group and the P1 group, the control group with the P2 group, the control group with the P3 group, the P1 group with the P2 group, the P1 group with P3, and the P2 group with P3. The dosage in the P3 group gave the largest reduction when compared to the control group, and it was statistically significant (p<0.05) (Figure 3).

Assessment of VEGF Blood Levels

Administration of EEP increased VEGF blood levels. It appears that on K, P1, P2, and P3, VEGF blood levels increased gradually. There was a significant difference between the control group and the P1 group, the control group with the P2 group, the control group with the P3 group, the P1 group with the P2 group, the P1 group with P3, and the P2 group with P3. The dosage in the P3 group gave the largest reduction when compared to the control group, and it was significant (p<0.05) (Figure 4).

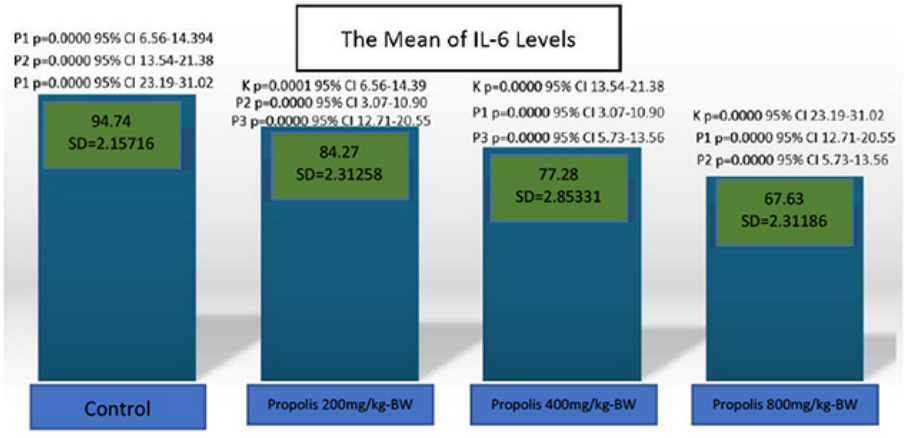

Figure 3. Hologram of the mean IL-6 data. Decrease in IL-6 blood levels in each control group and administering of EEP dosages, it appears that at K, P1, P2, and P3 IL-6 blood levels decreased gradually with increasing the dosage of EEP.

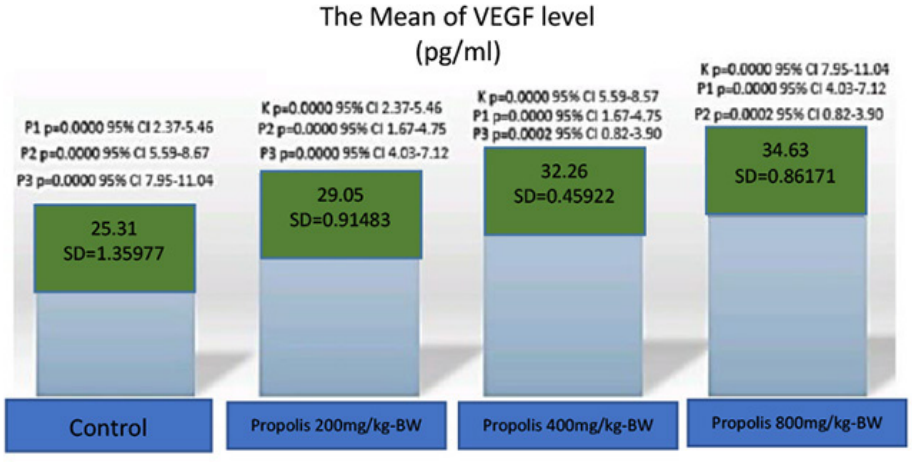

Figure 4. Histogram of the mean increase in VEGF blood levels of each control group and administering of EEP dosages, it appears that at K, P1, P2, and P3 VEGF blood levels increased gradually according to the increasing the dosage of EEP.

Assessment of NF-kB Expression

Administration of EEP decreases NF-kB expression. The median histological score of inactive NF-kB expression was high in the group of normal white rats, white rats were administered a dosage, 200 mg/kg-BW EEP and a dosage, 400 mg/kg-BW EEP, whereas in the group of white rats with a dosage, 800 mg/kg-BW EEP the median histological score was low. The median histological score of the P1 group (administration a dosage, 200 mg/kgBW EEP) and P2 group (administration a dosage, 400 mg/kg-BW EEP) were the same. Thus, the lower the EEP dosage administered, the higher the median histological score and the more inactive NF-kB expression that appears in the cell cytoplasm or the less activated NF-kB enters the cell nucleus. It can also be said that the lower the EEP dosage administered, the smaller the resistance to NF-kB expression. The median histological score of inactive NF-kB expression among the four white rats (Figure 5 & 6).

Assessment of Caspase-3 Expression

Administration of EEP decreases the expression of caspase-3. It shows administration EEP in K, P1, P2, and P3 groups reveal the expression of caspase 3 gradually decreased. At the dosage, 200 mg/kgBW EEP, there was no decrease in the activity of caspase-3 expression. At a dosage, 400 mg/kgBW EEP, there was no significant decrease in the activity of caspase -3 expression. In statistical test, there was no group that had a median caspase-3 intensity which was statistically significant (p=0.557) (Figure 7 & 8).

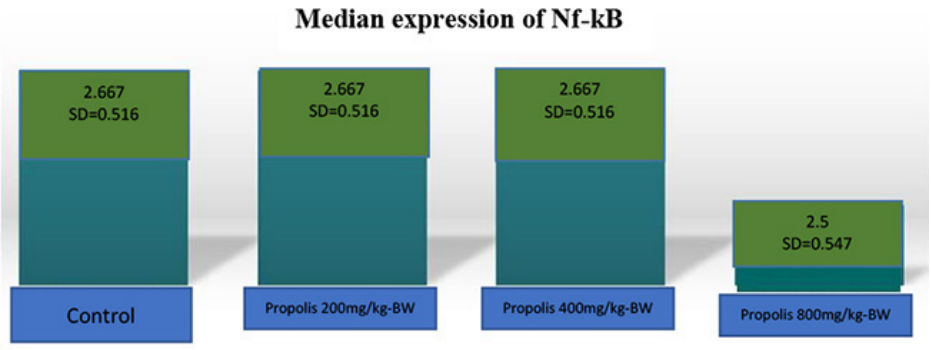

Figure 5. The median decrease in NF-kB expression in each control group and administration of EEP dosages. From the figure, it can be seen that the mean histological score of inactive NF-kB expression was high in the control white rat group, administration at dosage 200 mg/kg-BW EEP and a dosage of 400 mg/kg-BW EEP did not show decreasing of expression NF-kB, while in the group of white rats with a dosage, 800 mg/kg-BW EEP, the median histological score was low.

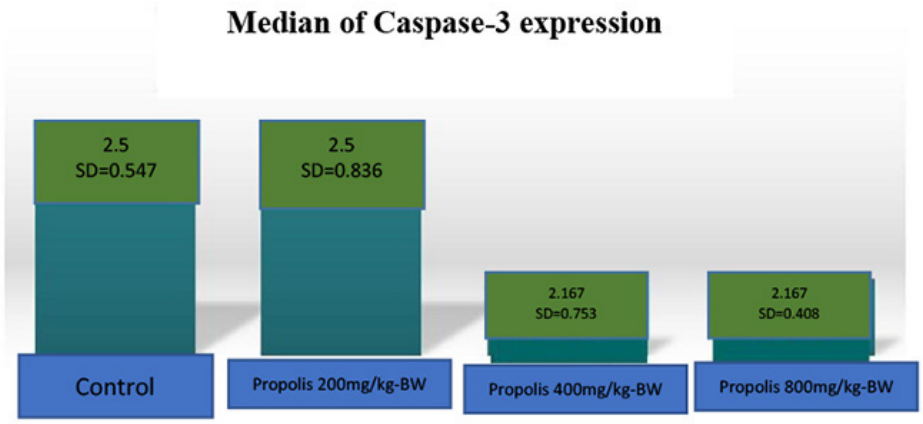

Figure 7. The median of caspace-3 expression for each control group and administering of EEP dosages showed that in K, P1, P2, and P3 the expression of caspase-3 decreased gradually. Administration of both dosages, 400 and 800 mg/kg-BW of EEP, shows there were decreases in the activity of caspase 3 expressions. But statistic analysis did not significant (p>0.005).

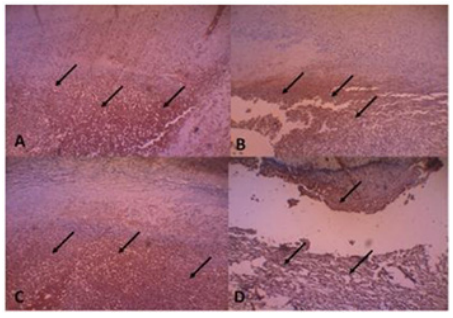

Figure 6. The expression of NF-kB protein showed positive staining (arrow) on immunohistochemistry staining, A. Group K score 3; B. Group P1 scores 3; C. Group P2 score 3; D. Group P3 score 2 (Magnification 100x)

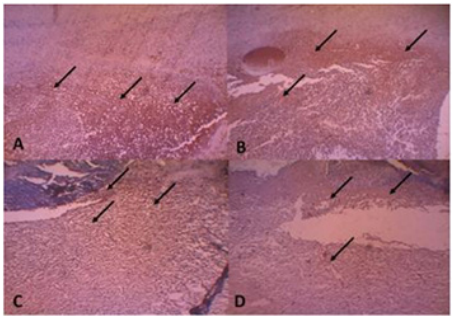

Figure 8. The expression of caspase 3 protein showed positive staining (arrow) on immunohistochemistry staining, A. Group K score 3; B. Group P1 scores 3; C. Group P2 scores 2; D. Group P3 score 2 (Magnification 100x)

Assessment of Micro Vessel Density (MVD)

Administration of EEP did not significantly increase the amount of MVD (p> 0.05). Mann-Whitney test was not carried out because there was no significant difference in the median data between different groups (Figure 9 & 10).

DISCUSSION

One of the therapeutic approaches for chronic wounds is to cover the wound with a skin graft which has become the most widely used option. One of the causes of skin graft failure is the quality of angiogenesis which is initiated by inflammation involving ROS, cytokines and apoptotic activity. Therapeutic approaches that target by reducing oxidative stress and inflammation are expected to improve the quality of angiogenesis indirectly by suppressing the effects of excessive ROS through the mechanism of reducing inflammatory activity so it will increase the vascularity of the skin graft donors. Propolis as a natural product derived from plant resins collected by honey bees has a number of biological activities such as antioxidants and anti-inflammatory.[24] This study describes ethanol extract propolis of Lawu mountain (EEP) in preventing oxidative and inflammatory reactions in the male white rat skin graft model.

One of the causes of the disturbance of the normal healing process is imbalance of redox homeostasis. This imbalance occurs because of the excessive production of ROS. Reactve oxygen species is a form of free radical which has high reactivity because it has tendencies to attract electrons and can turn a molecule into another molecule. ROS will activate the transcription factor (NF-kB) through

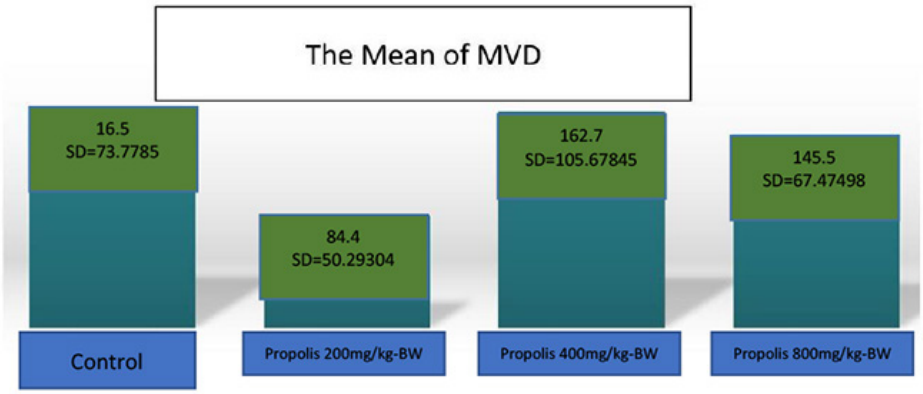

Figure 9. The median of administration at dosage 200 mg/kg-BW, 400 mg/kg-BW and 800 mg/kg-BW did not show increasing amount vessels (MVD) for each control group comparing EEPs administration groups.

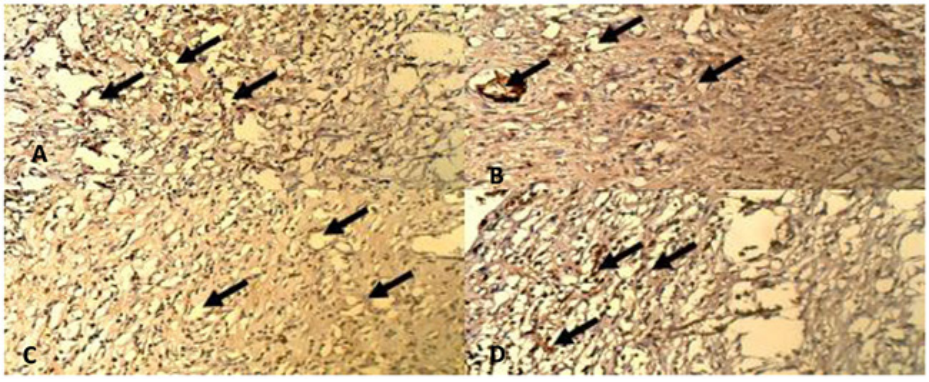

Figure 10. Microvessel density (MVD) was calculated using CD34 immunohistochemistry, blood vessel endothelial cells were stained with CD34 staining and showed the presence of blood vessels (arrows), A. Group K; B. Group P1; C. Group P2; D. Group P3 (200x Magnification)

the destruction of Inhibitor Kappa Beta (IKB) to produce inflammatory cytokines, lipid peroxidation by ROS produces various aldehydes such as propanal, hexanal, 4-hidroxynenal (4HNE) and Malondialdehyde (MDA). MDA is the most mutagenic and used as biomarkers for the peroxidation of cell wall fat.[25,26]

Administration a dosage, 200 mg/kg-BW EEP compared with the administration of a dosage, 400 mg/kg-BW EEP ($p = 0.0000$) or a dosage, 800 mg/kg-BW EEP ($p = 0.0000$) there is a significant difference. Giving a dosage, 400 mg compared to giving a dosage, 800 mg/kg-BW EEP is not significant ($p = 0.517$). So the use of EEP at a dosage, 400 mg to a dosage, 800 mg is the most effective dose to reduce MDA, so the "cut-off point" for dosing is effective in reducing MDA blood levels is the administration of dosage, 200 mg/kg-BW EEP.

Caffeic Acid Phenyl Esther (CAPE) is an active biological material anti-inflammatory propolis which has an important role in reducing the inflammatory process by inhibiting the production of cytokines, chemokines, lymphokines and T cell proliferation. Russo has proven that propolis containing 10.44% CAPE and 9.04% galangin is capable of inhibits the formation of superoxide anions, the higher the CAPE level, the higher the ability to inhibit the effects of lipid peroxidation which is indicated by a decrease MDA levels in plasma.[27,28]

Administration of propolis from Indian ethanol extract at a dosage, 100, 200, 300 mg /kg/BW for 14-21 days per oral decreases ROS, MDA and SOD with an increasing effect according to the increase in dose in Alzheimer mice.[44-47] The effect of propolis increases the activity of antioxidant enzymes SOD and GPx which decrease MDA levels in the administration of propolis ethanol extract in rat kidney disorders model Type 1 diabetes mellitus, testicular malignancy, vascular endothelial cell disorders and exposure to pesticide drugs.[29,30]

In this study, the content of CAPE EEP from Mount Lawu propolis which has an anti-oxidant effect that causes a decrease in MDA, the pathway of decrease mechanism is proven by measuring antioxidant levels enzymes (SOD, GPx, CAT). A decrease in MDA blood levels and SOD levels in the administration of Lawu mountain's EEP in infection and sepsis model rats given a dosage, 200 mg/kgBW/day for 7-14 days showed significant results compared to control.[31-33]

The decrease of the IL-6 blood levels, showed the number of $p=0.000$ ($p<0.05$). Post Hoc test, there were two groups that had significantly different mean of IL-6 blood levels. The post hoc test results showed that the differences between groups were statistically significant ($p<0.05$). There was a statistical difference between the control group and the P1 group, the control group with the P2 group, the control group with the P3 group, the P1 group and the P2 group, the P1 group with P3, and the P2 group with P3. The dose in the P3 group gave the largest reduction when compared to the dose in the control group, and it was statistically significant.

Propolis contains anti-inflammatory materials including CAPE, quercetin, naringenin, apigenin, galangin, vestitol and others which can suppress the formation of prostaglandins, leukotriene and suppress the activity of myeloperoxidase, NADPH-oxidase, ornithine decarboxylase and tyrosine-protein kinases. Wang's molecular analysis study evaluating the effect of propolis ethanol extract from Chinese to the production and expression of inflammatory mediators of mRNA showed that the suppression of IL-1β, IL-6 and nitric oxide production was dose dependent.[33]

The study using Iranian propolis ethanol extract doses at a dosage, 50 mg/kg, 100 mg/kg and 200 mg/kg-BW in rats experiencing postoperative peritoneal adhesion showed anti-inflammatory

effects of cytokines (IL-6, IL-1β and TNF-α) which decreased significantly with the best dose of 200 mg/kgBW the effect on MDA and NO as well as the effect of propolis on angiogenesis biomarkers (VEGF). Giving of Brazilian red propolis containing phenol in wound model rat showed that the wound area was reduced due to suppression of inflammation with decreased IL-6 levels.[33,34]

The expression of NF-kB in this study show decreasing expression in a dosage 800 mg/kg-BW but not significant, p = 0.927 (p>0.05). It can be concluded that there was no group that had a significantly different median of NFKB intensity statistically. Mann-Whitney test was not carried out because there was no difference in the median data between different groups.

Funakoshi-Tago's et al.[35] studies on the anti-inflammatory effect of Nepal's propolis isolate, that is 3,4 dihydroxy-4-methoxydalbergione, 4-methoxydalbergion, cearoin and chrysin inhibits the expression of inflammatory genes including IL-6, TNF-α and IL-13 in bone marrow-derived mast cells (BMMC) and also inhibits NF-kB activation through IKK inhibition and IkBα degradation. Inhibition of LPS production due to oxidative stress (NO) as an anti-inflammatory effect of brazilian caffeic acid propolis which was mediated by decreased NF-kB activity, p38 MAP Kinase and JNK1/2. CAPE is known to be the most active ingredient as an anti-inflammatory modulator compared to caffeic acid, quercetin and naringenin which work to inhibit NF-kB activation.

The transcription signaling pathway of NF-kB becomes irregular in several types of human malignancies. Evidences show that accumulation of ROS is an important signal in regulating the function of various cells, high levels of ROS induce oxidative stress that causes a number of disease effects including malignancy. In most types of malignant cells found that NF-kB is very active, inhibition of NF-kB has been shown to stop tumor cell proliferation with tumor cell death or to become more sensitive to the action of anti-tumor agents, especially antioxidants. Ethanol extract of Chinese propolis decreased the activity of NF-kB p65 (a subunit of NF-kB) and resisted translocation from the cytoplasm to the nucleus to activate invitro breast tumor cells.[36]

In the inflammatory phase, tissue response to trauma activates the macrophage cells. Macrophage also produces Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF) which will trigger the angiogenesis process for formation of Extra Cellular Matrix (ECM) materials, as results of decrease in pH, oxygen tension, and increase of lactate in the area around the wound will trigger the process that encourage the formation of new blood vessels or what can be known as angiogenesis or neovascularization, which VEGF, bFGF, and TGF-β mainly influenced. This process is vital in continuity of the next process, that is the formation of granulation on days 4-7.[37,38]

In this study, the administration of EEP shows the increase of VEGF blood levels statistically significant, p=0.000 (p<0.05). It can be concluded that there were at least two groups that had significantly different mean of VEGF.

VEGF production under hypoxic conditions increases with the duration of hypoxia. VEGF is a major factor in the angiogenesis process. Giving of CAPE at different doses showed decreased production by setting HIF-1α although it was not statistically significant. Giving CAPE to Choroidal Neovascularization (CNV) disorders which can cause blindness can reduce VEGF production through ROS inhibition mechanisms, phosphorylation of Posphoinositide3-kinase (PI3K) and inhibition of HIF 1-α activation by preventing translocation of transcription factors in the nucleus during hypoxic conditions.[39]

In hypokinesia and hypodynamic, rats showed decreased muscle activity which led to capillary regression, especially in muscle fibers with low oxidative levels. This condition causes oxidative stress which increases the regulation of negative angiogenic factors such as thrombospondin-1 (TSP-1). TSP-1 has anti-angiogenic activity through anti-proliferation and pro-apoptotic effects, while pro-angiogenic factor of VEGF plays a role in angiogenesis by stimulating the formation of new blood vessel tissue by attracting and stimulating endothelial cell differentiation. Giving of Brazilian propolis at a dose of 1000 mg/kg-BW showed a significant improvement in capillary lumen volume and diameter compared to the control group, as well as an increase in VEGF expression and a decrease in endothelial cell apoptosis.[40]

In this study, the effect of VEGF on tissue has not been proven so that it has a pro-angiogenic effect on the angiogenesis process which will further increase skin graft vascularization on MVD examination, it is necessary to examine tissue VEGF expression to prove the proangiogenic effect of EEP administration.

A preliminary study about the potential of propolis from Sulawesi which has anti-angiogenic activity shows that Sulawesi propolis does not affect increasing VEGF levels but has a protective effect of pericyte against hypoxic conditions. Pericyte is one of the main factors in angiogenesis which supports the stability of blood vessels by protecting the endothelium and producing chemical signals to maintain the attachment of endothelial cells. The vascular stabilizing effect of pericyte was also associated with decreased HIF-1α activation and reduced ROS of the antioxidant propolis.[41]

The quality of angiogenesis is influenced by many factors such as hypoxic effect, growth factors (especially VEGF), inflammatory cytokines (especially IL-6), apoptosis of endothelial cells. The apoptosis process is regulated by cysteine aspartate-specific proteases (caspases) through selective cleavage of cell substrates with cytoskeleton rearrangement and nuclear degradation characterized by cell shrinkage, chromatin condensation, membrane swelling and finally DNA fragmentation. Apoptosis can be triggered by inflammatory cytokines, ROS and cytotoxic (hypoxic) ROS These factors play a role in the angiogenesis process either directly or indirectly through various pathways starting from the inflammatory and immune processes. Caspase-3 as the executor of apoptosis's apoptosis, apoptosis of macrophages and endothelial (Vascular Smooth Muscle Cells/VSMC) causes vascular instability by forming atherosclerotic plaques which

reduce oxygen supply and nutrients to donor blood vessels.[42]

In this study, there was no significant decrease in the expression of Caspase-3 (p = 0.057), it is possible that in the skin graft process with allograft donors there was a tissue rejection reaction that led to the emergence of oxidative stress conditions, where there was an increase in excessive ROS production due to cell damage. ROS itself plays an important role in the signaling pathway for apoptosis activation.[43,44]

Increased Calcium (Ca) in the mitochondrial wall caused by cell damage will cause Bad defosforilation (proapoptotic of Bcl-2 family) and cause dissociation and translocation mitochondria which blocks the anti-apoptotic action of Bcl-XL resulting in mitochondrial apoptosis. Bcl-2 is an anti-apoptotic marker showed an increase with a significant decrease in caspase-3 on the giving of flavonoid propolis (chrisin) to rat testes induced with paracetamol which caused decreased sperm motility, sperm abnormalities and sperm death. Apoptosis that occurred in this study can occur not through the caspases pathway but through the apoptosis-inducing factor (AIF) pathway which binds directly to DNA and activates DNAase which causes condensation and fragmentation of DNA and activates Endonuclease G (endo G) through an unclear mechanism.[44]

Revascularization is required to ensure the survival of the transplanted skin. Skin graft vascularization is precisely adjusted for endothelial cell regression and replacement via angiogenesis and vasculogenesis. The skin graft requires sufficient vascularization to live before it is in good contact with the recipient. The process occurs due to passive displacement of red blood cells into the capillary graft. The capillary effect occurs within 12 hours after the procedure. The nutrition of the skin graft is given through the plasmatic circulation process and the process of plasma or serum imbibition and oxygen into the graft will occur. The graft will experience gradual edema and increase in weight up to 40%.[45]

In this study, the administration of EEP did not increase skin graft vascularization p = 0.254 (p>0.05). It can be concluded that there was no group that had a significantly different mean of MVD. Mann-Whitney test was not carried out because there was no difference in the mean data between different groups.

MVD examination is used as a prognostic marker of human malignancy, increasing MVD is associated with aggressive tumor behavior and provides a worse prognosis although it is still debated regarding factors that can influence, such as counting method, immunohistochemical staining techniques and specimen storage method. Immunohistochemical staining uses the CD34 antigen which will show a brown stain on endothelial cells or a collection of cells that are clearly separated from blood vessels, normal blood vessels, tumor cells, and surrounding connective tissue. Vascular counts, at 200 magnification (10 x ocular, 20 x objective) 4 fields of view, with 200 magnification were calculated and averaged to produce an average score.[46]

The process of skin graft vascularity depends on the thickness of the donor skin graft, STSG is faster than FTSG. The vascularization of donor skin grafts is not the same throughout the donor area, with the highest density in the peripheral area adjacent to normal skin and the least in the central area.[45,46]

In skin graft with allograft donors will undergo a tissue rejection process, cellular immunity (Langerhans epidermal cells) plays an important role in initiating the reaction by activating T cells (CD4 and CD8 T cells) and triggering the release of cytokines IL-2, IFN- γ, TNF, IL-6, IL-10 and several cytokines that lasted on days 7 to 14. The most dominant inflammatory cytokines played a role is IL-6 as a regulator of the balance of activation and suppression of T cells. Rejection reactions vary from the occurrence of local inflammation, bulla formation, bleeding, necrosis and degeneration of skin appendages.[47,48]

CONCLUSION

We concluded that the propolis ethanol extract of mount Lawu (EEP) had anti-ROS effect, anti-inflammatory effect and angiogenic effect in skin graft model rats which have been assessed from its ability: significantly decrease MDA blood levels, significantly decrease IL-6 blood levels and significant in increasing VEGF blood levels, but not significant in decreasing NF-kB expression and decreasing caspase-3 expression. Skin graft vascularization did not increase in numbers significantly (MVD).

The ability of the EEP as antioxidative stress activation reveals MDA blood level decrease with a cut-off point of giving at a dose of 200 mg/kg BW / day. We did not conclude the cut off point for EEP dosages either to decrease IL-6 blood level or increase VEGF blood level. The cut-off point administration dosage of EEP for decreasing IL-6 blood level and increasing VEGF blood level were not yet established. Further research could be recommended based on the amount and duration of EEP

CONFLICT OF INTEREST

The author declares there is no conflict of interest regarding publication of this study.

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ETHICAL STATEMENT

Ethical Committee Faculty have approved this study of Medicine/Dr. Moerwadi Hospital, Solo, Indonesia with ethical clearance references number: 720/VI/HREC/2020.

AUTHOR CONTRIBUTION

All author has contributed for writing the original draft and agree for the final version of the manuscript for publication.

REFERENCES

1. McDermott KW, Weiss AJ, Elixhauser A. Burn-Related Hospital Inpatient Stays and Emergency Department Visits, 2013: Statistical Brief #217. 2016 Dec. In: Healthcare Cost and Utilization Project (HCUP) Statistical Briefs. Rockville (MD): Agency for Healthcare Research and Quality (US); 2006 Feb–. PMID: 28121115.
2. Kanapathy M, Smith OJ, Hachach-Haram N, Bystrzonowski N, Mosahebi A, Richards T. Protocol for a systematic review of the efficacy of epidermal grafting for wound healing. Syst Rev. 2016;5:92. doi: 10.1186/s13643-016-0268-7.
3. Serebrakian AT, Pickrell BB, Varon DE, Mohamadi A, Grinstaff MW, Rodriguez EK,

Nazarian A, Halvorson EG, Sinha I. Meta-analysis and Systematic Review of Skin Graft Donor-site Dressings with Future Guidelines. Plast Reconstr Surg Glob Open. 2018;6(9):e1928. doi: 10.1097/GOX.0000000000001928.
4. Santema TB, Poyck PP, Ubbink DT. Skin grafting and tissue replacement for treating foot ulcers in people with diabetes. Cochrane Database Syst Rev. 2016;2(2):CD011255. doi: 10.1002/14651858.CD011255.pub2.
5. Hallock GG, Morris SF. Skin grafts and local flaps. Plast Reconstr Surg. 2011;127(1):5e-22e. doi: 10.1097/PRS.0b013e3181fad46c.
6. Wollina U, Meseg A, Weber A. Use of a collagen-elastin matrix for hard to treat soft tissue defects. Int Wound J. 2011;8(3):291-6. doi: 10.1111/j.1742-481X.2011.00785.x.
7. Thorne CH, et al. Grabb and Smith's plastic surgery: Seventh edition. Wolters Kluwer Health Adis (ESP). 2013; pp: 68.
8. Frueh FS, Sanchez-Macedo N, Calcagni M, Giovanoli P, Lindenblatt N. The Crucial Role of Vascularization and Lymphangiogenesis in Skin Reconstruction. Eur Surg Res. 2018;59(3-4):242-254. doi: 10.1159/000492413.
9. Wu X, Kathuria N, Patrick CW, Reece GP. Quantitative analysis of the microvasculature growing in the fibrin interface between a skin graft and the recipient site. Microvasc Res. 2008;75(1):119-29. doi: 10.1016/j.mvr.2007.04.012.
10. Gould DJ, Reece GP. Skin graft vascular maturation and remodeling: a multifractal approach to morphological quantification. Microcirculation. 2012;19(7):652-63. doi: 10.1111/j.1549-8719.2012.00200.x.
11. Reddy S, El-Haddawi F, Fancourt M, Farrant G, Gilkison W, Henderson N, Kyle S, Mosquera D. The incidence and risk factors for lower limb skin graft failure. Dermatol Res Pract. 2014;2014:582080. doi: 10.1155/2014/582080.
12. Yammine K, Assi C. A Meta-Analysis of the Outcomes of Split-Thickness Skin Graft on Diabetic Leg and Foot Ulcers. Int J Low Extrem Wounds. 2019;18(1):23-30. doi: 10.1177/1534734619832123.
13. Kim YW, Byzova TV. Oxidative stress in angiogenesis and vascular disease. Blood. 2014;123(5):625-31. doi: 10.1182/blood-2013-09-512749.
14. Honnegowda TM, Kumar P, Udupa EGP, Kumar S, Kumar U, Rao P. Role of angiogenesis and angiogenic factors in acute and chronic wound healing. Plast Aesthet Res. 2015;2:243-249. http://dx.doi.org/10.4103/2347-9264.165438
15. Frisca S, Sardjono CT, Sandra F. Angiogenesis: pathophysiology and clinical application. Public Health J. 2009;8(2):174-187.
16. Bösmüller H, Pfefferle V, Bittar Z, Scheble V, Horger M, Sipos B, Fend F. Microvessel density and angiogenesis in primary hepatic malignancies: Differential expression of CD31 and VEGFR-2 in hepatocellular carcinoma and intrahepatic cholangiocarcinoma. Pathol Res Pract. 2018;214(8):1136-1141. doi: 10.1016/j.prp.2018.06.011.
17. Apte RS, Chen DS, Ferrara N. VEGF in Signaling and Disease: Beyond Discovery and Development. Cell. 2019;176(6):1248-1264. doi: 10.1016/j.cell.2019.01.021.
18. Sawicka D, Car H, Borawska MH, Nikliński J. The anticancer activity of propolis. Folia Histochem Cytobiol. 2012;50(1):25-37. doi: 10.2478/18693.
19. Pasupuleti VR, Sammugam L, Ramesh N, Gan SH. Honey, Propolis, and Royal Jelly: A Comprehensive Review of Their Biological Actions and Health Benefits. Oxid Med Cell Longev. 2017;2017:1259510. doi: 10.1155/2017/1259510.
20. Sarsono S. Identification of caffeic acid phenyl esther in the ethanol extract of propolis isolate Mount Lawu. JBAI. 2012;8(2):132-6
21. Ummanni R, Lehnigk U, Zimmermann U, Woenckhaus C, Walther R, Giebel J. Immunohistochemical expression of caspase-1 and-9, uncleaved caspase-3 and -6, cleaved caspase-3 and -6 as well as bcl-2 in benign epithelium and cancer of the prostate. Exp Ther Med. 2010;1:47–52.
22. Park S, Tepper OM, Galiano RD, Capla JM, Baharestani S, Kleinman ME, Pelo CR, Levine JP, Gurtner GC. Selective recruitment of endothelial progenitor cells to ischemic tissues with increased neovascularization. Plast Reconstr Surg. 2004;113(1):284-93. doi: 10.1097/01.PRS.0000091169.51035.A5.
23. Mogoșanu GD, Popescu FC, Busuioc CJ, Lascăr I, Mogoantă L. Comparative study of microvascular density in experimental third-degree skin burns treated with topical preparations containing herbal extracts. Rom J Morphol Embryol. 2013;54(1):107-13.
24. Cano Sanchez M, Lancel S, Boulanger E, Neviere R. Targeting Oxidative Stress and Mitochondrial Dysfunction in the Treatment of Impaired Wound Healing: A Systematic Review. Antioxidants (Basel). 2018;7(8):98. doi: 10.3390/antiox7080098.
25. Broughton G 2nd, Janis JE, Attinger CE. The basic science of wound healing. Plast Reconstr Surg. 2006;117(7 Suppl):12S-34S. doi: 10.1097/01.prs.0000225430.42531.c2.
26. Kurahashi T, Fujii J. Roles of Antioxidative Enzymes in Wound Healing. Journal of Developmental Biology. 2015;3(2):57-70. https://doi.org/10.3390/jdb3020057
27. Mahmoud AM, Abd El-Twab SM. Caffeic acid phenethyl ester protects the brain against hexavalent chromium toxicity by enhancing endogenous antioxidants and modulating the JAK/STAT signaling pathway. Biomed Pharmacother. 2017;91:303-311. doi: 10.1016/j.biopha.2017.04.073.
28. Barros Silva R, Santos NA, Martins NM, Ferreira DA, Barbosa F Jr, Oliveira Souza VC, Kinoshita A, Baffa O, Del-Bel E, Santos AC. Caffeic acid phenethyl ester protects against the dopaminergic neuronal loss induced by 6-hydroxydopamine in rats. Neuroscience. 2013;233:86-94. doi: 10.1016/j.neuroscience.2012.12.041.
29. Nanaware S, Shelar M, Sinnathambi A, Mahadik KR, Lohidasan S. Neuroprotective effect of Indian propolis in β-amyloid induced memory deficit: Impact on behavioral and biochemical parameters in rats. Biomed Pharmacother. 2017;93:543-553. doi: 10.1016/j.biopha.2017.06.072.
30. Alyane M, Kebsa LB, Boussenane H, Rouibah H, Lahouel M. Cardioprotective effects and mechanism of action of polyphenols extracted from propolis against doxorubicin toxicity. Pak J Pharm Sci. 2008;21(3):201-9.
31. Prasetyo AP, Hestianah E, Handijatno D. Protective Effect of propolis extract on Histyopathological Features of Mice's (Mus Musculus) Liver induced by Lead Acetate. Journal of Basic Medicine Veterenary. 2017;6(2):91-99.
32. Redhono D, Purwanto B, Wasita B, Indarto D, Adji RS, Kusumawardani A, Cilmiaty R. The Antioxidant Effect of Indonesian Propolis In Rats Induced by Anthrax Spores. SRP. 2021;12(1):557-562. doi:10.31838/srp.2021.1.80
33. Askari VR, Rahimi VB, Zamani P, Fereydouni N, Rahmanian-Devin P, Sahebkar AH, Rakhshandeh H. Evaluation of the effects of Iranian propolis on the severity of post operational-induced peritoneal adhesion in rats. Biomed Pharmacother. 2018;99:346-353. doi: 10.1016/j.biopha.2018.01.068.
34. Corrêa FR, Schanuel FS, Moura-Nunes N, Monte-Alto-Costa A, Daleprane JB. Brazilian red propolis improves cutaneous wound healing suppressing inflammation-associated transcription factor NFκB. Biomed Pharmacother. 2017;86:162-171. doi: 10.1016/j.biopha.2016.12.018.
35. Funakoshi-Tago M, Ohsawa K, Ishikawa T, Nakamura F, Ueda F, Narukawa Y, Kiuchi F, Tamura H, Tago K, Kasahara T. Inhibitory effects of flavonoids extracted from Nepalese propolis on the LPS signaling pathway. Int Immunopharmacol. 2016;40:550-560. doi: 10.1016/j.intimp.2016.10.008.
36. Xuan H, Li Z, Yan H, Sang Q, Wang K, He Q, Wang Y, Hu F. Antitumor Activity of Chinese Propolis in Human Breast Cancer MCF-7 and MDA-MB-231 Cells. Evid Based Complement Alternat Med. 2014;2014:280120. doi: 10.1155/2014/280120.
37. Kondo T, Ishida Y. Molecular pathology of wound healing. Forensic Sci Int. 2010;203(1-3):93-8. doi: 10.1016/j.forsciint.2010.07.004.
38. Bao P, Kodra A, Tomic-Canic M, Golinko MS, Ehrlich HP, Brem H. The role of vascular endothelial growth factor in wound healing. J Surg Res. 2009;153(2):347-58. doi: 10.1016/j.jss.2008.04.023.
39. Paeng SH, Jung WK, Park WS, Lee DS, Kim GY, Choi YH, Seo SK, Jang WH, Choi JS, Lee YM, Park S, Choi IW. Caffeic acid phenethyl ester reduces the secretion of vascular endothelial growth factor through the inhibition of the ROS, PI3K and HIF-1α signaling pathways in human retinal pigment epithelial cells under hypoxic conditions. Int J Mol Med. 2015;35(5):1419-26. doi: 10.3892/ijmm.2015.2116.
40. Tanaka M, Kanazashi M, Maeshige N, Kondo H, Ishihara A, Fujino H. Protective effects of Brazilian propolis supplementation on capillary regression in the soleus muscle of hindlimb-unloaded rats. J Physiol Sci. 2019;69(2):223-233. doi: 10.1007/s12576-018-0639-z.
41. Iqbal M, Fan TP, Watson D, Alenezi S, Saleh K, Sahlan M. Preliminary studies: the potential

anti-angiogenic activities of two Sulawesi Island (Indonesia) propolis and their chemical characterization. Heliyon. 2019;5(7):e01978. doi: 10.1016/j.heliyon.2019.e01978.
42. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol. 2013 Apr 1;5(4):a008656. doi: 10.1101/cshperspect.a008656. Erratum in: Cold Spring Harb Perspect Biol. 2015;7(4). pii: a026716. doi: 10.1101/cshperspect.a026716.
43. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta. 2016;1863(12):2977-2992. doi: 10.1016/j.bbamcr.2016.09.012.
44. Aksu EH, Özkaraca M, Kandemir FM, Ömür AD, Eldutar E, Küçükler S, Çomaklı S. Mitigation of paracetamol-induced reproductive damage by chrysin in male rats via reducing oxidative stress. Andrologia. 2016;48(10):1145-1154. doi: 10.1111/and.12553.
45. Capla JM, Ceradini DJ, Tepper OM, Callaghan MJ, Bhatt KA, Galiano RD, Levine JP, Gurtner GC. Skin graft vascularization involves precisely regulated regression and replacement of endothelial cells through both angiogenesis and vasculogenesis. Plast Reconstr Surg. 2006;117(3):836-44. doi: 10.1097/01.prs.0000201459.91559.7f.
46. Duff SE, Jeziorska M, Kumar S, Haboubi N, Sherlock D, O'Dwyer ST, Jayson GC. Lymphatic vessel density, microvessel density and lymphangiogenic growth factor expression in colorectal cancer. Colorectal Dis. 2007;9(9):793-800. doi: 10.1111/j.1463-1318.2006.01199.x.
47. Uehara M, Li X, Sheikhi A, et al. Anti-IL-6 eluting immunomodulatory biomaterials prolong skin allograft survival. Sci Rep. 2019;**9:**6535. https://doi.org/10.1038/s41598-019-42349-w
48. Liang Y, Christopher K, Finn PW, Colson YL, Perkins DL. Graft produced interleukin-6 functions as a danger signal and promotes rejection after transplantation. Transplantation. 2007;84(6):771-7. doi: 10.1097/01.tp.0000281384.24333.0b.