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The Role of Mount Lawu Propolis in Modulating Oxidative Stress and Angiogenic Factors During the Proliferation Phase of Wound Healing

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A B S T R A C T

Background: The skin, the body's largest and first-line defense organ, is crucial for protection against environmental aggressors, thermoregulation, fluid balance, immune surveillance, sensory perception, and the intricate process of wound healing. This study investigated the modulatory role of Mount Lawu propolis extract on Malondialdehyde (MDA), a marker of oxidative stress, and Vascular Endothelial Growth Factor (VEGF), a key mediator of angiogenesis, during the critical proliferation phase of wound healing. **Methods:** A post-test-only control group design experimental study was conducted using twenty-four male rats (*Rattus norvegicus*). Following the creation of standardized full-thickness skin excision wounds, the rats were divided into four groups (n=6 per group): a control group receiving no propolis, and three treatment groups receiving topical applications of 200mg/kgBB (Group 1), 400mg/kgBB (Group 2), and 800mg/kgBB (Group 3) ethanol propolis extract daily for seven days. MDA and VEGF expression in granulated tissue samples were evaluated using immunohistochemistry and semi-quantitatively scored. Statistical analysis involved the Kruskal-Wallis non-parametric test, followed by the Mann-Whitney test due to non-normal data distribution. **Results:** MDA levels showed a significant reduction ($p<0.05$) in Group 2 (400mg/kgBB propolis) compared to the control, indicating a dose-specific antioxidant effect. VEGF expression demonstrated a significant increase ($p<0.05$) in all propolis-treated groups (Groups 1, 2, and 3) compared to the control, confirming the pro-angiogenic potential of propolis. Notably, the highest VEGF expression was observed in Group 3 (800mg/kgBB propolis). **Conclusion:** Mount Lawu ethanol propolis extract effectively modulated both oxidative stress and angiogenesis during the proliferation phase of wound healing in this rat model. A 400mg/kgBB/day dose of propolis optimally reduced MDA expression, while propolis at all doses, particularly 800mg/kgBB/day, significantly enhanced VEGF expression. These findings suggest propolis as a promising therapeutic agent to enhance wound healing by mitigating oxidative stress and promoting angiogenesis.

1. Introduction

The skin, as the largest and most exposed organ of the human body, is critical in maintaining overall health and well-being. Its functions are diverse and essential, ranging from providing a physical barrier against the external environment to participating in complex physiological processes. As a primary defense, the skin acts as a shield, protecting the body from mechanical injury, harmful chemicals, and the invasion of pathogenic microorganisms. This barrier function is vital for preventing infection and maintaining the body's internal milieu. Beyond its protective role, the skin is actively involved in thermoregulation. It regulates body temperature through mechanisms such as perspiration, which cools the body through evaporation, and the modulation of blood flow to the skin's surface, which can either conserve or dissipate heat depending on the body's needs. This ability to regulate temperature is crucial for maintaining optimal physiological function

in varying environmental conditions. The skin also plays a vital role in maintaining fluid balance. By preventing excessive water loss, it helps to ensure that the body remains adequately hydrated, which is essential for numerous bodily functions. This function is particularly important in dry environments or during periods of increased physical activity when the risk of dehydration is elevated. Furthermore, the skin is an integral component of the body's immune system. It functions as a site of immune surveillance, housing various immune cells that detect and respond to potential threats. These cells play a critical role in recognizing foreign substances and initiating an immune response to protect the body from infection and disease. The skin also functions as a sensory organ. It is richly innervated with a variety of sensory receptors that allow us to perceive a wide range of sensations, including touch, pressure, pain, and temperature. These sensory inputs provide crucial information about our surroundings, enabling us to interact effectively with our environment and to detect and respond to potential dangers.[1-3]

One of the most remarkable and complex functions of the skin is its ability to repair itself following injury. This process, known as wound healing, is fundamental to the maintenance of skin integrity and the body's overall homeostasis. Wound healing is not a single event but rather a dynamic and intricate sequence of cellular and molecular events that occur in a series of distinct yet overlapping phases. The process of wound healing is classically divided into four main phases: hemostasis, inflammation, proliferation, and tissue remodeling. Each of these phases is characterized by specific cellular activities, the release of various signaling molecules, and changes in the extracellular matrix that ultimately lead to the restoration of tissue integrity. Hemostasis, the initial response to skin injury, is a rapid and critical process aimed at minimizing blood loss. This phase is triggered by damage to blood vessels, which leads to an immediate vasoconstriction. Vasoconstriction, the narrowing of blood vessels, reduces blood flow to the injured area, helping to control bleeding. Following vasoconstriction, platelets, small cellular fragments in the blood, adhere to the site of injury and aggregate, forming a temporary plug that helps to stem the bleeding. Simultaneously, the coagulation cascade, a complex series of enzymatic reactions, is activated. This cascade culminates in the formation of fibrin, a protein that strengthens the platelet plug and forms a more stable clot. This intricate process not only halts bleeding but also provides a provisional matrix that serves as a scaffold for subsequent cellular events in the wound healing process. The inflammatory phase, which follows hemostasis, is essential for clearing debris and pathogens from the wound site and for initiating the subsequent repair processes. This phase is characterized by the recruitment of various immune cells to the wound, including neutrophils, macrophages, and lymphocytes. These cells are drawn to the site by a variety of signaling molecules, including pro-inflammatory cytokines, which are released in response to tissue damage and the presence of bacteria. Neutrophils, the first responders, play a crucial role in phagocytosing bacteria and cellular debris, thus preventing infection. They also contribute to the inflammatory environment by releasing additional cytokines. Macrophages, arriving later, take over the phagocytic role, further clean the wound, and secrete growth factors that are essential for the proliferation of cells involved in tissue repair. The inflammatory response, while crucial for wound healing, must be tightly regulated. Excessive or prolonged inflammation can hinder the healing process and may lead to the development of chronic wounds. The proliferative phase is characterized by the actual rebuilding of tissue. This phase involves several key events, including angiogenesis, fibroplasia, and re-epithelialization. Angiogenesis is the formation of new blood vessels, a process that is critical for providing the developing tissue with the necessary oxygen and nutrients. This process involves the sprouting of new capillaries from existing blood vessels and is stimulated by growth factors such as Vascular Endothelial Growth Factor (VEGF). Fibroplasia

involves the proliferation of fibroblasts and the deposition of extracellular matrix components. Fibroblasts, which migrate into the wound, proliferate and synthesize collagen and other components of the extracellular matrix, providing structural support and strength to the healing tissue. Re-epithelialization is the process by which keratinocytes migrate and proliferate to restore the epithelial layer of the skin. This process involves the migration of keratinocytes from the wound edges to cover the wound surface, effectively restoring the protective barrier function of the skin.[4-7]

The final phase of wound healing, tissue remodeling, is a long-term process that can continue for many months or even years after the initial injury. During this phase, the newly formed tissue is reorganized and strengthened. Collagen fibers are remodeled, cross-linked, and aligned, resulting in increased tensile strength of the scar tissue. Cellular activity decreases, and the tissue gradually matures. While the healed tissue may regain much of its functionality, it rarely achieves the full strength and elasticity of undamaged skin. Within the complex cascade of wound healing, the regulation of oxidative stress and angiogenesis plays a pivotal role. Oxidative stress, an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defenses, can significantly impact the healing process. ROS are produced as a byproduct of cellular metabolism and in response to injury. While they serve important functions in wound healing, such as signaling and defense against pathogens, an excess of ROS can overwhelm the antioxidant systems, leading to cellular damage and impaired healing. Malondialdehyde (MDA) is a commonly used biomarker to assess the level of oxidative stress in biological systems, reflecting lipid peroxidation, a process where ROS damage cell membranes. Angiogenesis, the formation of new blood vessels, is equally critical, particularly during the proliferative phase of wound healing. This process ensures that the healing tissue receives an adequate supply of oxygen and nutrients, which are essential for cellular survival, proliferation, and differentiation. VEGF is one of the most potent pro-angiogenic factors, stimulating the proliferation and migration of endothelial cells, the cells that line blood vessels. The intricate balance between pro-angiogenic and anti-angiogenic factors determines the extent and quality of new blood vessel formation, which in turn influences the efficiency of wound healing. Given the complexity of wound healing and the potential for disruptions to this process, there is a continued need for therapeutic strategies that can enhance and accelerate tissue repair. Traditional medicine has long utilized natural products to promote wound healing, and propolis, a resinous substance collected by honeybees, is one such example. Propolis has been used for centuries due to its diverse biological properties, which include antimicrobial, anti-inflammatory, and antioxidant effects. Its therapeutic potential in wound healing is attributed to its complex composition, which includes flavonoids, phenolic acids, essential oils, and other compounds that can modulate various aspects of the healing process. Flavonoids, a major class of compounds in propolis, are known for their potent antioxidant activity. They can neutralize free radicals, reduce oxidative stress, and protect cells from damage. Phenolic acids, such as caffeic acid, contribute to propolis's anti-inflammatory properties by modulating key signaling pathways involved in inflammation. Essential oils present in propolis provide antimicrobial activity, which can help to prevent wound infections.[8-10] This study focuses on the potential role of Mount Lawu propolis in modulating oxidative stress and angiogenesis, two critical factors in the proliferation phase of wound healing.

2. Methods

The study employed a post-test-only control group design. In this experimental design, the primary outcome measures were the expression levels of Malondialdehyde (MDA) and Vascular Endothelial Growth Factor (VEGF) in granulated wound tissue. The experimental animals used in this study were rats, specifically *Rattus norvegicus*. A total of 24 rats were

used in the study. These animals were divided into four groups, with 6 rats in each group. This approach to sample size calculation and allocation is consistent with established best practices in experimental animal research, aiming to provide a sufficient number of replicates to enhance the reliability and validity of the study findings.

The study utilized male rats that were approximately 2 months of age, with a body weight ranging from 150 to 200 grams. The use of animals with a relatively uniform age and weight helps to minimize variability in the results, as these factors can influence physiological responses and wound healing processes. The animals were housed under standard laboratory conditions to minimize any potential environmental influences on the wound healing process. Standard laboratory conditions typically include; Controlled temperature and humidity: Maintaining a stable temperature and humidity level is crucial for ensuring animal well-being and for achieving consistent physiological responses. Fluctuations in these environmental parameters can introduce variability and potentially confound the results of the study; 12-hour light/dark cycle: This controlled lighting schedule helps to regulate the animals' circadian rhythms. Circadian rhythms are endogenous biological processes that oscillate with a period of approximately 24 hours and can influence various physiological processes, including metabolism, immune function, and wound healing; Standard rat chow and water ad libitum: The animals were provided with unrestricted access to standard rat feed and water, ensuring that their nutritional needs were met. The amount of feed was adjusted based on the average body weight of the rats to maintain appropriate nutrition. Providing ad libitum access to food and water ensures that the animals are not under any nutritional stress, which could affect the wound healing process.

All experimental procedures involving animals were conducted in accordance with ethical guidelines and were approved by the appropriate institutional animal care and use committee. Ethical approval is a fundamental requirement for any research involving animal subjects, ensuring that the animals are treated humanely and that the research is conducted in a responsible and ethical manner. These guidelines are designed to minimize pain and distress to the animals and to ensure that the research contributes to scientific knowledge in a way that justifies the use of animal subjects.

Propolis extract was prepared from Mount Lawu propolis. The preparation of the extract is a crucial step in the study, as the extraction process can significantly influence the composition and concentration of the active compounds present in the final product. These active compounds are responsible for the biological activities of propolis, including its antioxidant and pro-angiogenic properties. In this study, ethanol was used as the solvent for the extraction process. Ethanol extraction is a commonly employed method for propolis, and it is known for its efficiency in extracting a broad range of bioactive compounds, including flavonoids and phenolic acids. The choice of ethanol as a solvent is based on its ability to solubilize a wide variety of compounds with different polarities, ensuring a comprehensive extraction of the potentially therapeutic components of propolis. The specific details of the propolis extraction procedure, such as the precise ratio of propolis to ethanol, the extraction time, and the temperature at which the extraction was performed, were not provided in the source text. However, these parameters are critical for the standardization and reproducibility of the extraction process. Variations in these parameters can lead to differences in the composition and concentration of the extract, which can in turn affect the results of the study. After the extraction process, the ethanol would typically be removed from the extract. This is often achieved through evaporation techniques, which concentrate the propolis extract. The resulting extract would then be diluted to the desired concentrations for topical application to the animals' wounds. The process of concentrating and diluting the extract allows for precise control over the dosage of propolis administered to the animals.

To simulate the proliferative phase of wound healing in the experimental animals, a granulated wound tissue model was created on the rats' skin. This model is widely used in preclinical wound healing studies as it allows for the controlled study of the tissue repair processes, specifically the formation of granulation tissue. Granulation tissue is a hallmark of the proliferative phase of wound healing and is characterized by the formation of new connective tissue and blood vessels. The use of this model enables researchers to investigate the effects of therapeutic interventions on the key processes involved in tissue regeneration. Prior to the wound creation procedure, the rats were anesthetized to minimize pain and distress. Anesthesia is essential for ensuring the humane treatment of animals in research and for preventing any unnecessary suffering during experimental procedures. In this study, anesthesia was induced using a combination of ketamine and xylazine. The specific dosages used were 87 mg ketamine per kg of body weight and 13 mg xylazine per kg of body weight. This combination is commonly used in rodent anesthesia, as ketamine provides immobilization and analgesia, while xylazine provides sedation and muscle relaxation. The dorsal area of each rat was carefully prepared before the surgical procedure. This preparation involved shaving the hair from the dorsal region and then cleaning the skin with an antiseptic solution. Shaving the hair is necessary to provide a clear surgical field and to prevent hair from contaminating the wound. Cleaning the skin with an antiseptic solution, such as iodine or chlorhexidine, is crucial for reducing the risk of infection at the surgical site. Following the preparation of the surgical site, a standardized full-thickness skin excision was created. The size of the excision was 1.5 x 1.5 cm. A full-thickness excision involves cutting through both the epidermis and the dermis layers of the skin, creating a wound that mimics a deep tissue injury. The use of a standardized wound size is essential for ensuring consistency between the experimental groups and for allowing for accurate comparisons of the wound healing process. Following the creation of the wounds, the rats were randomly assigned to one of four groups. Random assignment is a critical aspect of experimental design as it helps to minimize bias and ensures that the characteristics of the animals are evenly distributed across the different treatment groups. The four groups were; Control group: This group served as the negative control and received no propolis treatment. The control group is essential for providing a baseline against which the effects of the propolis treatments can be compared; Group 1: This group was topically treated with 200mg/kgBB ethanol propolis extract; Group 2: This group was topically treated with 400mg/kgBB ethanol propolis extract; Group 3: This group was topically treated with 800mg/kgBB ethanol propolis extract. The propolis extract was applied topically to the wound area once daily for a period of seven days. The duration of the treatment was specifically chosen to focus on the proliferation phase of wound healing, which typically occurs within this timeframe. The proliferation phase is a critical stage in the healing process, characterized by the formation of new tissue and blood vessels. The method of application of the propolis extract was not specified in the source text. However, it is likely that the extract was applied using a pipette or a swab to carefully and precisely deliver the treatment to the wound bed. The specific method of application can influence the distribution and absorption of the treatment, so consistency in the application method is important.

On day seven post-wounding, the rats were euthanized, and the granulated tissue from the wound site was harvested. Euthanasia was performed using a combination of Ketamine 300-360 mg/kg + xylazine 30-40 mg/kg administered Intraperitoneal. Euthanasia methods are carefully selected to ensure a humane and painless death for the animals. The specific method used in this study is a commonly accepted procedure for rodents. The granulated tissue, which had formed in the wound bed during the proliferative phase of healing, was then carefully collected. This tissue is the focus of the study as it contains the cells and extracellular matrix

components that are actively involved in the repair process.

The harvested granulated tissue samples underwent a series of processing steps to prepare them for immunohistochemical analysis. These steps are essential for preserving the tissue structure and enabling the visualization of specific proteins of interest, namely MDA and VEGF. The tissue samples were immediately fixed in 10% formalin. Formalin fixation is a widely used method for preserving biological tissues. The formalin acts by cross-linking proteins, which prevents tissue degradation and maintains cellular morphology. This process effectively stabilizes the tissue, preventing autolysis, which is the self-destruction of cells by their own enzymes. The rapid fixation of tissue samples is crucial to preserve the integrity of the tissue and to ensure that the immunohistochemical staining accurately reflects the in vivo expression of the target proteins. Following fixation, the tissue samples were embedded in paraffin wax. This process involves several sequential steps; Dehydration: The fixed tissues were dehydrated through a graded series of ethanol solutions. This process gradually removes water from the tissue, which is necessary because paraffin wax is hydrophobic and will not infiltrate water-containing tissue. The use of a graded series of ethanol solutions, starting with lower concentrations and gradually increasing to 100% ethanol, minimizes tissue damage during the dehydration process; Clearing: After dehydration, the tissues were cleared using xylene. Xylene is a solvent that is miscible with both ethanol and paraffin wax. It replaces the ethanol in the tissue, making it receptive to infiltration by the wax. This step is essential for preparing the tissue for paraffin infiltration, as it removes the dehydrating agent and replaces it with a substance that is compatible with the embedding medium; Infiltration: The tissues were then infiltrated with molten paraffin wax. This is typically done in a series of steps with increasing concentrations of paraffin in a vacuum oven to ensure complete penetration of the wax into the tissue. The use of a vacuum oven facilitates the infiltration process by removing air from the tissue, allowing for better penetration of the paraffin wax. Complete infiltration is essential for providing adequate support for the tissue during sectioning; Embedding: Finally, the paraffin-infiltrated tissues were embedded in blocks of paraffin wax. This process creates a solid matrix that allows for thin sectioning of the tissue. The embedded tissue blocks provide the necessary structural support for the microtome, which is used to cut thin sections of the tissue for microscopic examination. The paraffin blocks containing the tissue samples were sectioned into thin slices using a microtome. The sections were cut to a thickness of 4 µm. A microtome is a precision instrument that uses a sharp blade to cut very thin sections of tissue. The thickness of the sections is critical for optimal visualization of cellular structures and for ensuring that the immunohistochemical staining is consistent and reproducible. The tissue sections were then mounted onto glass slides. This involves carefully placing the sections onto the slides and allowing them to adhere. The slides were then prepared for the subsequent steps of deparaffinization and rehydration. Mounting the sections onto glass slides provides a stable platform for the staining and microscopic examination of the tissue.

Immunohistochemistry (IHC) is a technique that uses antibodies to detect specific proteins in tissue sections. In this study, IHC was used to determine the expression of MDA and VEGF, the proteins of interest, in the granulated tissue. The paraffin wax was removed from the tissue sections by placing the slides in xylene. This step reverses the embedding process and prepares the tissue for the aqueous solutions used in IHC. Following deparaffinization, the sections were rehydrated through a graded series of ethanol solutions, gradually increasing the water content of the tissue. This step is essential for allowing the aqueous reagents used in the IHC procedure to penetrate the tissue effectively. Antigen retrieval is a crucial step in IHC, as formalin fixation can cause protein cross-linking. This cross-linking can mask the epitopes, which are the specific parts of the protein

that antibodies recognize, and thus prevent antibody binding. In this study, antigen retrieval was performed by heating the sections in citrate buffer. Heating in citrate buffer is a commonly used method for breaking these protein cross-links and exposing the epitopes, thus improving antibody binding and enhancing the sensitivity of the IHC staining. Endogenous peroxidase enzymes are present in some tissues and can cause background staining in IHC, which can interfere with the interpretation of the results. To prevent this, endogenous peroxidase activity was blocked by treating the sections with 3% hydrogen peroxide. Hydrogen peroxide inactivates peroxidase enzymes, preventing them from reacting with the chromogen used in the staining procedure and thus reducing background staining. The tissue sections were then incubated with primary antibodies against MDA and VEGF. Primary antibodies are specific to the target proteins and bind directly to them. The primary antibodies used in this study were; MDA antibody (Abcam); VEGF antibody (Santa Cruz Biotechnology). The sections were incubated with the primary antibodies overnight at 4°C. This prolonged incubation time at a low temperature allows for optimal antibody binding to the target proteins, increasing the specificity and sensitivity of the staining. After washing to remove any unbound primary antibodies, the sections were incubated with appropriate secondary antibodies. Secondary antibodies are antibodies that bind to the primary antibodies. These secondary antibodies are typically conjugated to a label, which is usually an enzyme, that allows for the visualization of the antibody-protein complex. The choice of secondary antibody depends on the species in which the primary antibody was raised. The immunoreaction was visualized using diaminobenzidine (DAB) as the chromogen. DAB reacts with the enzyme conjugated to the secondary antibody to produce a brown precipitate at the site of the target protein. This brown staining indicates the location and distribution of the protein of interest within the tissue section. The sections were counterstained with hematoxylin. Hematoxylin is a basic dye that stains cell nuclei blue. Counterstaining provides contrast to the brown DAB staining, making it easier to visualize the tissue morphology and to identify the location of the target proteins within the cells. This step aids in the interpretation of the staining pattern and provides a clearer picture of the tissue architecture. After staining, the slides were dehydrated through a graded series of ethanol solutions and cleared with xylene. These steps remove water from the tissue and replace it with a hydrophobic solvent that is miscible with the mounting medium. Finally, a coverslip was mounted onto the slide using a mounting medium. The mounting medium protects the tissue section and provides a clear and permanent image for microscopic examination.

The evaluation of immunohistochemical staining is a critical step in the analysis of IHC results. It involves a detailed assessment of the intensity and distribution of the staining to determine the relative levels of protein expression in the tissue samples. The expression of MDA and VEGF was evaluated by a pathologist who was blinded to the experimental groups. This means that the pathologist was unaware of which tissue sections came from which treatment group. Blinded evaluation is essential to minimize bias in the interpretation of the staining results. By preventing the pathologist from knowing the origin of the samples, the evaluation becomes more objective and less susceptible to preconceived notions or expectations. The intensity of staining was graded semi-quantitatively. This involves assigning scores based on the visual intensity of the staining. The scoring system used in this study was as follows; 0: No staining; 1: Weak staining; 2: Moderate staining; 3: Strong staining. This semi-quantitative approach allows for a systematic and consistent assessment of the staining intensity, providing a relative measure of protein expression. In addition to the intensity of staining, the percentage of positive cells was also recorded. This involves estimating the proportion of cells in the tissue section that show positive staining for the target protein. This assessment provides

information on the extent of protein expression within the tissue. The immunoreactivity score (IRS) was calculated by multiplying the intensity score by the percentage of positive cells. The IRS provides a semi-quantitative measure of protein expression that takes into account both the intensity and the extent of staining. This method provides a more comprehensive assessment of protein expression compared to simply evaluating staining intensity alone, as it incorporates both the intensity of the staining and the proportion of cells that are expressing the protein.

Statistical analysis was performed to determine whether there were significant differences in MDA and VEGF expression between the different treatment groups. The choice of appropriate statistical tests depends on the distribution of the data. Data were tested for normality using the Shapiro-Wilk test. This test is commonly used to determine whether a dataset follows a normal distribution. Normality is an important assumption for many parametric statistical tests, and violating this assumption can lead to inaccurate results. If the data were normally distributed, one-way ANOVA (Analysis of Variance) followed by Tukey's post hoc test was used to compare the groups. ANOVA is a parametric test used to compare the means of three or more groups. Tukey's post hoc test is used to make pairwise comparisons between the groups after a significant ANOVA result, allowing for the identification of specific differences between the groups. If the data were not normally distributed, the Kruskal-Wallis test followed by the Mann-Whitney U test was used. The Kruskal-Wallis test is a non-parametric test used to compare the medians of three or more groups when the data are not normally distributed. The Mann-Whitney U test is a non-parametric test used to make pairwise comparisons between groups after a significant Kruskal-Wallis test, enabling the determination of which specific groups differ significantly from each other.

Statistical analysis was performed using SPSS version 26.0. SPSS is a widely used statistical software package for data analysis in scientific research. It provides a comprehensive suite of statistical tools for data management, analysis, and visualization. A p-value of <0.05 was considered to indicate statistical significance. The p-value is a measure of the probability of obtaining the observed results (or more extreme results) if there were actually no effect of the treatment. A p-value of less than 0.05 is a commonly accepted threshold for rejecting the null hypothesis, which is the hypothesis that there is no effect. In this context, if the p-value is less than 0.05, it suggests that the observed differences in MDA and VEGF expression between the treatment groups are unlikely to be due to chance and are likely to reflect a real effect of the propolis treatment.

3. Results

Figure 1 presents a visual comparison of MDA expression levels across four groups: A (Control), B (Group 1), C (Group 2), and D (Group 3). The red arrows in each image indicate the location of MDA expression, which is represented by the brown staining. In image A, the Control group, there is a relatively high degree of brown staining distributed throughout the tissue. This suggests a substantial level of MDA expression in the control group, indicating a baseline level of oxidative stress in the wound tissue without propolis treatment. Image B, representing Group 1 (200mg/kgBB propolis), shows a noticeable decrease in the intensity and distribution of brown staining compared to the control group. This suggests that the lowest dose of propolis resulted in a reduction of MDA expression. Image C, representing Group 2 (400mg/kgBB propolis), exhibits the least intense brown staining among all four groups. The staining is sparse and faint, indicating the lowest level of MDA expression. This suggests that the 400mg/kgBB dose of propolis was most effective in reducing oxidative stress, as measured by MDA levels. Image D, representing Group 3 (800mg/kgBB propolis), shows an increase in the intensity of brown staining compared to Group 2, but it is still less intense than the staining observed in the Control group. This indicates that while the highest dose of

propolis (800mg/kgBB) did reduce MDA expression compared to the control, it was not as effective as the 400mg/kgBB dose.

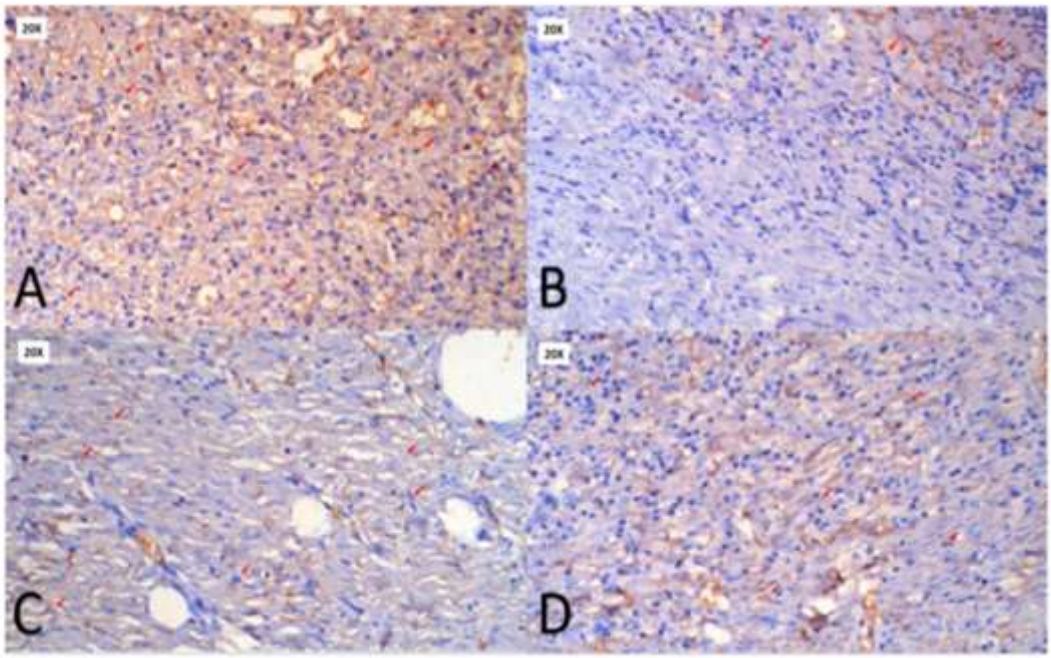

Figure 1. Immunohistochemistry for MDA expression. A. Control; B. Group 1; C. Group 2; D. Group 3. Red arrow: MDA expression.

Table 1 presents the data for MDA expression in granulated tissue across the four experimental groups. The table includes the Mean Immunoreactivity Score (IRS) ± Standard Deviation (SD) for each group and the corresponding p-value. The control group, which received no propolis treatment, exhibited a mean IRS of 6.50 ± 1.29. This value represents the baseline level of MDA expression in the wound tissue without any propolis intervention. Group 1, treated with 200mg/kgBB propolis extract, showed a mean IRS of 5.25 ± 1.04. While the mean IRS is lower than the control group, the p-value is 0.062, which is greater than 0.05. This indicates that the reduction in MDA expression in Group 1 compared to the control group is not statistically significant. Group 2, treated with 400mg/kgBB propolis extract, demonstrated a mean IRS of 3.00 ± 0.82. This is the lowest mean IRS among all four groups. The p-value is 0.046, which is less than 0.05. This result is statistically significant, indicating that the 400mg/kgBB dose of propolis extract significantly reduced MDA expression compared to the control group. Group 3, treated with 800mg/kgBB propolis extract, had a mean IRS of 3.50 ± 0.58. The mean IRS is lower than the control group but higher than Group 2. The p-value is 0.071, which is greater than 0.05. This suggests that the reduction in MDA expression in Group 3 compared to the control group is not statistically significant.

Table 1. MDA expression in granulated tissue.

Group	Mean IRS ± SD	p-value
Control	6.50 ± 1.29	
Group 1	5.25 ± 1.04	0.062
Group 2	3.00 ± 0.82	0.046
Group 3	3.50 ± 0.58	0.071

*Significant difference compared to the control group ($p<0.05$).

Figure 2 visually compares VEGF expression levels across four groups: A (Control), B (Group 1), C (Group 2), and D (Group 3). The red arrows indicate the location of VEGF expression, represented by the brown staining. Image A, the Control group, shows a relatively low level of brown staining. The staining is generally light and less intense compared to the other groups. This suggests a baseline level of VEGF expression in the wound tissue without propolis treatment. Image B, representing Group 1 (200mg/kgBB propolis), displays a noticeable increase in the intensity and distribution of brown staining compared to the Control group. The staining appears more pronounced and widespread, indicating an elevated level of VEGF expression with the application of the lowest dose of propolis. Image C, representing Group 2 (400mg/kgBB propolis), also shows increased brown staining compared to the Control group. The staining intensity is comparable to, or perhaps slightly greater than, Group 1, suggesting a further enhancement of VEGF expression with the 400mg/kgBB propolis treatment. Image D, representing Group 3 (800mg/kgBB propolis), exhibits the most intense and extensive brown staining among all four groups. The staining is very dark and widespread throughout the tissue, indicating the highest level of VEGF expression. This suggests that the highest dose of propolis (800mg/kgBB) resulted in the greatest increase in VEGF expression.

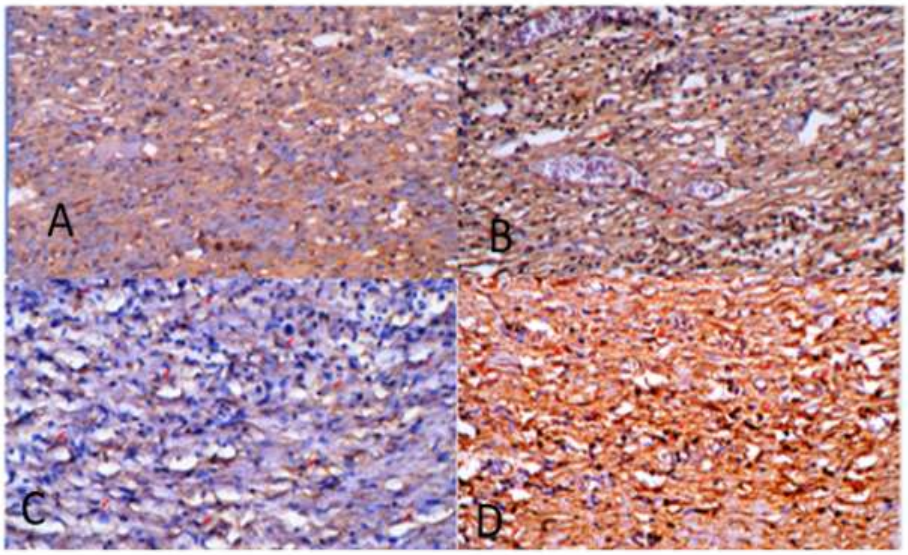

Figure 2. Immunohistochemistry for VEGF expression. A. Control; B. Group 1; C. Group 2; D. Group 3. Red arrow: VEGF expression.

Table 2 presents the data for VEGF expression in granulated tissue across the four experimental groups. The table includes the Mean Immunoreactivity Score (IRS) ± Standard Deviation (SD) for each group and the corresponding p-value. The control group, which received no propolis treatment, exhibited a mean IRS of 4.75 ± 0.96. This value represents the baseline level of VEGF expression in the wound tissue without any propolis intervention. Group 1, treated with 200mg/kgBB propolis extract, showed a mean IRS of 8.50 ± 1.04. The p-value is 0.000, which is less than 0.05. This indicates that the increase in VEGF expression in Group 1 compared to the control group is statistically significant. Group 2, treated with 400mg/kgBB propolis extract, demonstrated a mean IRS of 9.25 ± 0.96. The p-value is 0.000, which is less than 0.05. This result is statistically significant, indicating that the 400mg/kgBB dose of propolis

extract significantly increased VEGF expression compared to the control group. Group 3, treated with 800mg/kgBB propolis extract, had a mean IRS of 11.00 ± 1.41. The p-value is 0.000, which is less than 0.05. This suggests that the increase in VEGF expression in Group 3 compared to the control group is statistically significant.

Table 2. VEGF expression in granulated tissue.

Group	Mean IRS ± SD	p-value
Control	4.75 ± 0.96	
Group 1	8.50 ± 1.04	0.000
Group 2	9.25 ± 0.96	0.000
Group 3	11.00 ± 1.41	0.000

*Significant difference compared to the control group ($p<0.05$).

4. Discussion

This study investigated the effects of Mount Lawu propolis extract on oxidative stress and angiogenesis during the proliferation phase of wound healing in a rat model. The key findings of this research indicate that propolis extract modulates the expression of Malondialdehyde (MDA), a marker of oxidative stress, and Vascular Endothelial Growth Factor (VEGF), a crucial mediator of angiogenesis, thereby influencing the wound healing process. Specifically, the results demonstrate that propolis extract, particularly at a dose of 400mg/kgBB, effectively reduces oxidative stress, as evidenced by the decreased MDA levels observed in Group 2. Furthermore, propolis treatment significantly increased VEGF expression in the granulated tissue, indicating its pro-angiogenic effect. These findings suggest that propolis has the potential to enhance wound healing by mitigating oxidative stress and promoting the formation of new blood vessels.[11-13]

The results of this study demonstrate a dose-specific antioxidant effect of propolis extract. The most significant reduction in MDA levels was observed in Group 2, which received a propolis dose of 400mg/kgBB. MDA is a well-established biomarker of lipid peroxidation, a process initiated by reactive oxygen species (ROS) that can cause cellular damage. The decrease in MDA levels in Group 2 suggests that propolis possesses antioxidant properties that can effectively mitigate oxidative damage in the wound tissue. This observation aligns with the understanding that oxidative stress, characterized by an imbalance between ROS production and antioxidant defenses, can impair the healing process. Propolis is known to be rich in flavonoids and phenolic compounds, both of which are recognized for their potent antioxidant activities. These compounds can act as scavengers of free radicals, neutralizing them and inhibiting the production of ROS. By doing so, they protect cells from oxidative damage, preserving cellular function and promoting tissue repair. The reduction in MDA levels observed in this study supports the concept that propolis can enhance the antioxidant defense mechanisms within the wound environment, contributing to a more favorable healing milieu. The study revealed a dose-dependent effect of propolis on MDA expression. While the 400mg/kgBB dose was most effective in reducing MDA levels, the 800mg/kgBB dose also showed a reduction compared to the control group, although it was not as pronounced. This suggests that the antioxidant components of propolis may exert their effects optimally at specific concentrations, with higher concentrations potentially not conferring additional benefits in terms of MDA reduction. It is possible that at higher concentrations, other compounds in propolis might interfere with the antioxidant mechanisms or that saturation of the antioxidant capacity is reached.[14-17]

A key finding of this study is the significant increase in VEGF expression in the granulated tissue of propolis-treated groups, indicating a pro-angiogenic effect. VEGF is a crucial signaling protein that plays a vital role in angiogenesis, the formation of new blood vessels. This process is essential for wound healing as it ensures the delivery of oxygen and nutrients to the healing tissue, supporting cellular survival, proliferation, and differentiation. The enhanced VEGF expression observed in this study strongly suggests that propolis can promote the formation of new blood vessels in the wound bed. The pro-angiogenic effect of propolis may be attributed to its ability to stimulate the release of various growth factors and cytokines that are known to promote angiogenesis. Propolis contains a complex mixture of compounds that can interact with cellular signaling pathways involved in the regulation of angiogenic factors. These compounds may influence the expression of genes encoding pro-angiogenic molecules or modulate the activity of proteins that control their production and release. Similar to the findings on MDA expression, the study also revealed a dose-dependent effect of propolis on VEGF expression. However, in contrast to MDA, higher doses of propolis were associated with greater VEGF expression. The highest VEGF expression was observed in Group 3, which received the 800mg/kgBB dose of propolis. This suggests that different components or mechanisms within propolis may be responsible for its pro-angiogenic effects, and these components or mechanisms may be more effectively activated or enhanced at higher concentrations. It is plausible that the complex composition of propolis allows for a range of biological activities that are elicited at different dosage levels.[18-20]

5. Conclusion

In conclusion, this study provides compelling evidence that Mount Lawu ethanol propolis extract effectively modulates oxidative stress and angiogenesis during the proliferation phase of wound healing in a rat model. The findings highlight a dose-specific antioxidant effect, with a 400mg/kgBB/day dose of propolis demonstrating the most significant reduction in MDA expression, a key marker of oxidative stress. This suggests that propolis can mitigate oxidative damage, potentially creating a more favorable environment for tissue repair. Furthermore, the study demonstrates a significant increase in VEGF expression across all propolis-treated groups, confirming the pro-angiogenic potential of propolis. Notably, the highest dose of propolis (800mg/kgBB/day) resulted in the greatest enhancement of VEGF expression, indicating a dose-dependent effect on angiogenesis. This pro-angiogenic activity is crucial for the formation of new blood vessels, which are essential for delivering oxygen and nutrients to the healing tissue. The ability of propolis to simultaneously reduce oxidative stress and promote angiogenesis underscores its therapeutic potential in enhancing wound healing. By modulating these critical processes, propolis may facilitate the repair and regeneration of damaged tissue, offering a promising avenue for the development of novel wound healing strategies.

6. References

1. Kresnoadi U, Lunardhi LC, Agustono B. Propolis extract and bovine bone graft combination in the expression of VEGF and FGF2 on the preservation of post extraction socket. J Indian Prosthodont Soc. 2020; 20(4): 417–23.
2. Hadjab W, Zellagui A, Mokrani M, Öztürk M, Ceylan Ö, Gherraf N, et al. Pharmacological potential effects of Algerian Propolis against oxidative stress, multidrug-resistant pathogens biofilm and quorum-sensing. Turk J Pharm Sci. 2024; 21(1): 71–80.
3. Adham EKE, Hassan AI, Dawoud MMA. Retraction Note: Evaluating the role of propolis and bee venom on the oxidative stress induced by gamma rays in rats. Sci Rep. 2024; 14(1): 8849.
4. Wang S, Yang C, Luo Y, Chen Q, Xu M, Ji Y, et al. Poplar bud (Populus) extraction and

Chinese Propolis counteract oxidative stress in Caenorhabditis elegans via insulin/IGF-1 signaling pathway. Antioxidants (Basel). 2024; 13(7): 860.

5. Wang S, Yang C, Luo Y, Chen Q, Xu M, Ji Y, et al. Poplar bud (Populus) extraction and Chinese Propolis counteract oxidative stress in Caenorhabditis elegans via insulin/IGF-1 signaling pathway. Antioxidants (Basel). 2024; 13(7): 860.
6. Cikman O, Bulut A, Taysi S. Protective effect of propolis in protecting against radiation-induced oxidative stress in the liver as a distant organ. Sci Rep. 2024; 14(1): 21766.
7. Laaroussi H, Bakour M, Ousaaid D, Ferreira-Santos P, Genisheva Z, El Ghouizi A, et al. Protective effect of honey and Propolis against gentamicin-induced oxidative stress and hepatorenal damages. Oxid Med Cell Longev. 2021; 2021(1): 9719906.
8. Cury BJ, Jerônimo DT, da Silva LM, Farias de Queiroz E Silva T, França TCS, Dos Santos AC, et al. Hydroalcoholic extract of Araucaria sp. brown propolis alleviates ulcerative colitis induced by TNBS in rats by reducing inflammatory cell infiltration and oxidative damage. J Pharm Pharmacol. 2024; 76(10): 1379–92.
9. Zhong-Yong L, ZHi-Qing D, Li-Qiong X, Poorasadollah E, Shirvani S. The impact of propolis supplementation on inflammatory biomarkers: a meta-analysis and systematic review of randomized controlled clinical trials. Prostaglandins Other Lipid Mediat. 2024; 175(106915): 106915.
10. Ribeiro IS, Muniz IPR, Galantini MPL, Gonçalves CV, Lima PHB, Silva NR, et al. Antimicrobial photodynamic therapy with Brazilian green propolis controls intradermal infection induced by methicillin-resistant Staphylococcus aureus and modulates the inflammatory response in a murine model. Photochem Photobiol Sci. 2024; 23(3): 561–73.
11. Li Z, Liu Z, Guo Y, Gao S, Tang Y, Li T, et al. Propolis alleviates acute lung injury induced by heat-inactivated methicillin-resistant Staphylococcus aureus via regulating inflammatory mediators, gut Microbiota and serum metabolites. Nutrients. 2024; 16(11): 1598.
12. Mautone Gomes H, Dalló GC, Martins Silva A, Kleber Silveira A, Ferreira dos Santos J, dos Santos da Silva L, et al. Propolis of Tubuna bee (*Scaptotrigona bipunctata*) from the Meliponini tribe alleviates glycaemic, oxidative, and inflammatory conditions during STZ induced diabetes in Wistar rats also aiding in injury recovery. Pharmacological Research - Natural Products. 2024; 3(100025): 100025.
13. Lesmana R, Tandean S, Christoper A, Suwantika AA, Wathoni N, Abdulah R, et al. Propolis as an autophagy modulator in relation to its roles in redox balance and inflammation regulation. Biomed Pharmacother. 2024; 175(116745): 116745.
14. Yigit E, Deger O, Korkmaz K, Huner Yigit M, Uydu HA, Mercantepe T, et al. Propolis reduces inflammation and dyslipidemia caused by high-cholesterol diet in mice by lowering ADAM10/17 activities. Nutrients. 2024; 16(12): 1861.
15. Kang E-S, Jeong S-Y, Cho B-O, Shin J-Y, Kim M-Y, Hur Y-K, et al. Propolis and Petasites japonicus leaf extract mixture attenuates PM2.5-induced inflammation in NCI-H292 lung epithelial cells. Korean J Food Sci Technol. 2024; 56(4): 458–64.
16. Garbis DVO, Fortes TS, Brito JM, Silva LDM, Trovão L de O, Oliveira AS, et al. Prophylactic use of standardized extract of propolis of Apis mellifera (EPP-AF®) reduces lung inflammation and improves survival in experimental lethal sepsis. J

Ethnopharmacol. 2024; 331(118294): 118294.

17. Khabazian A, Mirhashemi FS, Sadeghi F. Investigating the effect of propolis-containing chewing gum in comparison with propolis-containing mouthwash on reducing gingival inflammation in patients with gingivitis. BMC Oral Health. 2025; 25(1): 231.
18. Wang K, Jin X-L, Shen X-G, Sun L-P, Wu L-M, Wei J-Q, et al. Effects of Chinese Propolis in protecting bovine mammary epithelial cells against mastitis pathogens-induced cell damage. Mediators Inflamm. 2016; 2016: 8028291.
19. Xool-Tamayo J, Chan-Zapata I, Arana-Argaez VE, Villa-de la Torre F, Torres-Romero JC, Araujo-Leon JA, et al. In vitro and in vivo anti-inflammatory properties of Mayan propolis. Eur J Inflamm. 2020; 18: 205873922093528.
20. Ripari N, Lopes EC, Francisco ÁF, de Almeida JFS, Honorio M da S, Júnior AF, et al. Guttiferone E from Brazilian red propolis inhibited wound-isolated methicillin-resistant Staphylococcus aureus and enhanced the bactericidal action of suppressed macrophages. Phytomedicine. 2025; 140(156615): 156615.