

## Original Research Article

# Difference of Propolis Administration as an Antioxidant against Oxidative Stress (Malondialdehyde Level (MDA)) and Apoptosis (Caspase-3 Expression) in Skin Graft Model White Rats (*Rattus Norvegicus*)

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### ABSTRACT

**Background:** The wound healing process is influenced by various substances with Reactive Oxygen Species having a fundamental role in destroying microorganisms and degrading damaged tissue structures. However, excessive production of ROS can lead to oxidative stress which is the basis of the pathogenesis of chronic, non-healing wounds. Therefore, an antioxidant that can fight oxidative stress is needed by administering propolis extract as an antioxidant to the granulation tissue of male white rats with skin graft model.

**Aims:** Here clearly write the aims of this study. Sample: To correlate platelet count, splenic index (SI), platelet count/spleen diameter ratio and portal-systemic venous collaterals with the presence of esophageal varices in advanced liver disease to validate other screening parameters.

**Study design:** Experimental post-test only control.

**Methodology:** This study was an experimental post-test only control group design using 24 male white rats (*Rattus Norvegicus*) as experimental animals which were divided into 4 groups consisting of 6 rats in each group. Wounds of granulation tissue were made on the sample by excising the skin on the back with a size of 1x1 cm which was treated for 7 days then was given Skin Graft of 2x2 cm from the back of the rat's fat layer. Propolis extract was given at a dose of 200 mg / kg, 400 mg / kg, and 800 mg / kg for 7 days after giving the skin graft. MDA serum and caspase-3 expression were examined after giving the propolis extract. The values of malondialdehyde (MDA) and Caspase-3 expression were analyzed by comparative test using 95% confidence interval and  $p < 0.05$  were considered significant.

**Results:** The comparative test of the mean MDA level was  $p < 0.05$  with the post hoc test showed  $p < 0.05$  between the control group and the 3 doses group of propolis extract. Meanwhile, the comparative test mean of Caspase-3 histoscore showed  $p > 0.05$ , indicating that there was no significant difference between the control group and the 3 doses group of propolis extract.

**Conclusion:** There was a significant difference in levels of Malondialdehyde (MDA) although there was

no significant difference in the expression of Caspase-3 by giving three different doses of propolis extract to the granulation tissue of male rats (*Rattus Norvegicus*) skin graft model.

*Keywords: Skin graft, White Mouse, MDA, Caspase-3*

## 1. INTRODUCTION

The prevalence of wounds continues to increase every year. Data from the 2013 Riskesdas reports that the prevalence of wounds in Indonesia is 8.2%, with the highest prevalence in South Sulawesi at 12.8% and the lowest prevalence in the Jambi area of 4.5% (1). The high incidence of injuries can become a health problem that requires treatment and a large amount of funds allocated.

Theoretically, the wound will undergo a physiological healing process. The wound healing process occurs through the role of various substances such as ROS, MDA, caspase 9, NF $\kappa$ B, TGF $\beta$  and VEGF. Gorlach *et al.*, (2015) stated that Reactive oxygen species (ROS) are associated with the inflammatory process (2).

Reactive oxygen species represent the principal agents of oxidative stress. Oxidative stress is an imbalance between oxidative and antioxidant events, which causes oxidative reactions; ROS is involved in the production of free radicals, and is a factor responsible for skin aging and disease progression (3). The fundamental purpose of the formation of high ROS during skin inflammation is to remove and destroy microorganisms that attack and / or degrade the damaged tissue structure. The ROS system is ubiquitous in the aging process, photoaging, inflammation, wound healing, tumorigenesis, and other processes in the skin. (4).

Excessive production of ROS can lead to oxidative stress, which has been identified as an important feature in the pathogenesis of chronic non-healing wounds. Excessive levels of ROS lead to oxidative modification and biomolecular damage, altering lipid / protein / DNA structure and function, inducing irreversible oxidation of reactive thiol protein groups, which are a feature of oxidative stress, and dysregulation of cell signaling pathways, triggering a downstream signaling cascade leading to impaired release of cytokines and exacerbation of skin diseases (5,6).

An antioxidant is needed that will fight oxidative stress so that the wound healing process is not interrupted. Therefore, this study aims to determine the effect of propolis extract as an antioxidant and to correlate it by assessing the quality of angiogenesis in granulation tissue in male white rats with skin graft model.

## 2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY (ARIAL, BOLD, 11 FONT, LEFT ALIGNED, CAPS)

This research is an experimental post-test only control group design with 24 male white rats (*Rattus Norvegicus*) as experimental animals. Measurements on test animals are carried out at a certain time after giving the treatment. The entire sample was divided into four groups consisting of:

- a. Control group
- b. Group I: Skin Graft model rats given 200 mg / kgBW propolis extract dose
- c. Group II: Skin Graft model rats given 400 mg / kgBW propolis extract dose
- d. Group III: Skin Graft model rats given 800 mg / kgBW propolis extract dose

Each group consisted of 6 male white rats (*Rattus Norvegicus*) treated with Skin Graft and then the levels of malondialdehyde (MDA) and the expression of Caspase-3 granular tissue were measured after treatment.

### 2.1. Provision of Propolis Extract

Dry propolis is cleaned and blended until smooth, then weighed as much as 500 grams, then put into a beaker glass. The maceration process was carried out by adding 3.75 liters of 70% ethanol as a solvent. The mixture of ingredients is stored for seven days in a room that is not exposed to sunlight with strong shaking or stirring with a stirring spatula twice a day (7).

The next step is filtering with a Buchner funnel and filter paper to separate the filtrate from the dregs into the Erlenmeyer flask to obtain the filtrate. The filtration results obtained are evaporated by a rotary evaporator at a temperature of 45oC with vacuum pressure (<1 atm) for approximately 4 hours so that a thick concentration of propolis extract is obtained ( $\pm$  100 g). Furthermore, the propolis extract is evaporated for 24 hours in a beaker glass so that the ethanol content evaporates (8).

Provision of propolis extract in different doses to male white rats (*Rattus Norvegicus*) skin graft model was given orally using a round. The dose of propolis extract used is a dose of 200 mg / kg BW, a dose of 400 mg / kgBW and 800 mg / kgBW which is given orally every day for 7 days.

## 2.2. Skin Graft Model Mouse Formation

The experimental animal model of granulation tissue wound was formed through the skin excision method (epidermis, dermis and part of the sub-cutan / panniculus carnosus) in the back area with a size of 2 x 2 cm using scalpel No. 10 such as the procedure for taking the Skin Graft donor with hemostasis using adrenaline 1: 100,000 or using 6-0 monofilament thread ligation. The edges of the wound are anchored with continuous sutures to prevent contraction of the wound edges. The wound was treated using Na Cl 0.9%. The wound was left for 7 days without treatment. This model is useful for creating inflammatory wounds of granulation tissue - Extra Cellular Matrix. This model can be induced by either mice or rat and is independent of a particular strain. This model within 2 weeks can cause inflammation in the proliferation phase (granulation tissue) so that inflammatory factors can be investigated.

Skin graft donor is taken in the area around the back with a size of 1 x 1 cm<sup>2</sup>. The skin graft was sutured to the wound with 6-0 mono filament thread and fixed with moist gauze (0.9% NaCl) and cotton for a bolster covered with Tegaderm (3M Company, St. Paul, Minn.). The donor skin graft wound was sutured with 6-0 monofilament suture and the wound was treated with gentamycin skin ointment and covered with Tegaderm (3M Company, St. Paul, Minn.)

## 2.3. MDA level examination

Blood MDA levels in this study were serum MDA levels of white rats with skin graft treatment after fasting for 12 hours, which was measured by the Thiobarbituric Acid Reacting Substances (TBARS) method and expressed in units of nmol / mL. Malondialdehyde levels were measured using the KitDiaSys measuring instrument which was done by an expert from the PAU UGM Yogyakarta Animal Laboratory. Blood is drawn from the retroorbital vein. Measurement of blood MDA levels was carried out after being given the propolis extract, after 7 days of giving the skin graft.

## 2.4. Immunohistochemical examination of Caspase-3 expression

The caspase-3 examination used standard immunohistochemical staining procedures. Immunohistochemical staining used polyclonal primary antibody, anti-rabbit / Ig G, anti caspase-3 (Thermo Scientific), 1: 100 dilution, retrieval antigen using citrate buffer with Mayer hematoxylin counterstaining. Calculation of caspase-3 expression is done by observing cells with no less than 500 cells and more than 5 high power fields. Interpretation criteria are based on the intensity of staining and the percentage of positive cells. Determination of the number of cells was used the Olympus CX 21 microscope with a small magnification of 40X first then proceed with a medium magnification of 100X and a large of 400 X and read the preparations in sequence from left to right, top to bottom.

## 2.5. Statistical Analysis

The statistical test used is the comparative test by comparing the average levels of MDA and caspase-3 expression using the one-way ANOVA test if the data is normal, and the Kruskal-Wallis comparative test if the data is not normal. All analysis of the research results will be analyzed using the statistical application tool IBM-SPSS version 25. A P value <0.05 was considered statistically significant with 95% as the confidence interval value.

## 3. RESULTS AND DISCUSSION

Post test MDA level data collection was carried out on August 21, 2020 after being given treatment in the form of propolis extract for 7 days. The mean MDA levels after being given skin graft treatment is shown in Table 1..

**Table 1. Results of the analysis of MDA levels using the one-way ANOVA test**

| Group | N | Mean ± Standard Deviation (nmol/ml) | P value |
|-------|---|-------------------------------------|---------|
| K     | 6 | 8,050 ± 0,536                       | 0,000   |
| P1    | 6 | 8,050 ± 0,536                       |         |
| P2    | 6 | 8,050 ± 0,536                       |         |
| P3    | 6 | 8,050 ± 0,536                       |         |

The results in table 1 show that there are differences in MDA levels given skin graft treatment when compared to MDA levels given skin grafts with propolis extract. The mean MDA levels treated with skin graft treatment was 8.050 nmol / ml, while the mean MDA levels treated with skin graft and 200 mg / kgBW of propolis extract showed a difference of 6.282 nmol / ml. Giving skin graft and propolis extract treatment with a larger dose of 400 mg / kgBW made the average MDA

level to 2.407 nmol / ml, and giving the propolis extract with the largest dose, 800 mg / kgBW showed a much greater difference in MDA levels. compared to the control group that is to be 1.425 nmol / ml. From table 1, it is obtained a p value <0.05, which indicates that at least two groups have significantly different mean MDA levels. Post hoc test analysis was conducted to find out which between groups had differences. The results of the post hoc tests can be seen in table 2.

**Table 2. Results of analysis of MDA levels using the one-way ANOVA test**

| Group      |                      | P value |
|------------|----------------------|---------|
| MDA levels | Group K vs Group P1  | 0,001   |
|            | Group K vs Group P2  | 0,000   |
|            | Group K vs Group P3  | 0,000   |
|            | Group P1 vs Group P2 | 0,000   |
|            | Group P1 vs Group P3 | 0,000   |
|            | Group P2 vs Group P3 | 0,157   |

\*Group K: Skin graft control

Group P1: Skin graft with 200 mg / kgBW propolis extract

Group P2: Skin graft with 400 mg / kgBW of propolis extract

Group P3: Skin graft with 800 mg / kgBW of propolis extract

From the results of the post hoc analysis, it was found that there were significant differences in the mean levels of MDA between groups K and groups P1, P2, and P3 (p value <0.05) and between groups P1 with groups P2 and P3. These results indicate that the differences in the results of existing MDA levels were significant between group K and groups P1, P2, and P3 and group P1 with groups P2 and P3. On the other hand, there was no significant difference in the results of MDA levels between the P2 group and the P3 group (p> 0.05). These results indicate that the difference in the results of MDA levels between the P2 and P3 groups was not statistically significant.

**Table 3. Results of Caspase-3 histoscore analysis using the Kruskal-Wallis test**

| Group | N | Mean ± Standard Deviation | P value |
|-------|---|---------------------------|---------|
| K     | 6 | 10,00 ± 2,19              | 0.557   |
| P1    | 6 | 9,84 ± 3,71               |         |
| P2    | 6 | 8,50 ± 3,33               |         |
| P3    | 6 | 8,67 ± 1,63               |         |

The results in table 3 show that there is a difference between Caspase-3 histoscore given skin graft treatment when compared to Caspase-3 histoscore given skin graft with propolis extract. The mean of Caspase-3 histoscore treated with skin graft was 10.0 while the mean Caspase-3 histoscore treated with skin graft and 200 mg / kgBW of propolis extract had a mean of 9.83. Giving skin graft and propolis extract treatment with a larger dose of 400 mg / kgBW made the average Caspase-3 histoscore to be 8.50. Finally, the administration of propolis extract with the largest dose, 800 mg / kgBW showed a significant difference between Caspase-3 histoscore compared to the control group.

The distribution of Caspase-3 histoscore data was checked using the Shapiro-Wilk test. The results of the normality test show that the data are not normally distributed. Because the data were not normally distributed, the comparative test of mean histoscore score in each trial group was carried out using the Kruskal-Wallis test. The results of the Kruskal-Wallis mean comparative test can be seen in table 3.

Based on this study, it was found that the difference between the MDA levels in the control group (K) and the group giving 200 mg / kgBB of propolis extract, 400 mg / kgBW, and 800 mg / kgBB. The results also showed that the administration of 400 mg / kgBB of propolis extract was the dose that gave the most statistically significant difference in MDA levels because there was no statistically significant difference between the administration of 400 mg / kgBB of propolis extract and 800 mg / kgBB of doses.

In a study conducted by Damayanti *et al* (2016) which compared MDA levels between the administration of propolis doses to the brain of a rat brain injury model due to trauma, it was found that there was a difference between the control group mice and the mice given propolis although there was no significant difference between the 50 mg / kgBB dose , 100 mg / kg, and 200 mg / kg. The researcher explained that the higher the dose of propolis extract had the tendency to reduce MDA levels in the brain tissue of mice in the traumatic brain injury model, but the insignificant difference was probably due to the still too low dose range of propolis administration. Meanwhile, based on the correlation test, it was found that there was a weak negative correlation between each dose, indicating that the higher the dose of propolis extract, there was a tendency that MDA levels would decrease. The results obtained by this study indicate that between the dosages of

propolis extract 50 mg / kg, 100 mg / kg, and 200 mg / kg, there is no significant difference in MDA levels, but Damayanti *et al* Suggest using even higher doses of MDA for future research. (9).

The results obtained are in line with other studies regarding the effect of reducing MDA levels by propolis extract conducted by Hoesada *et al* (2016) in their research on propolis extract with a total of 14.5 mg / kg, 27 mg / kg, and 41.5 mg / kgBB against MDA levels in male Wistar rats treated with cigarette smoke for 21 days. The results obtained indicate that the propolis extract can significantly reduce MDA levels in male Wistar rats treated with cigarette smoke (10). In addition, in a study conducted by Prasetyo *et al* (2013) which examined the effects of ethanol extract of propolis on levels of inflammation and serum MDA in sepsis model mice, MDA levels of mice treated with propolis extract decreased drastically from 0.39 micromol / L in the group of mice with a sepsis model was made to be 0.09 micromol / L in a group of mice made a sepsis model and then given propolis extract. The results showed that the ethanol extract of propolis could reduce serum MDA levels in sepsis model experimental animals (11).

The effect of this difference in MDA levels occurs due to the presence of antioxidants from propolis such as terpenoids, flavonoids, and cinnamic acid. The antioxidant property of flavonoids is that they can take free radicals (ROS) directly by donating hydrogen atoms or transferring electrons. In addition, flavonoids can also act as intracellular antioxidants through inhibition of free radical-forming enzymes such as xanthine oxidase (XO), lipoxygenase, protein kinase C, cyclooxygenase (COX), monooxygenase, and NADPH oxidase (12). This is also in line with the research conducted by Hardiningtyas *et al* (2014) which examined the antioxidant activity of the white apiary mangrove leaves, which found the phytochemical content of ethyl acetate extract from the leaves of the white fire mangrove which was rich in flavonoids, steroids, and triterpenoids. The extract was then given to Sprague Dawley rats which were hepatotoxic induced with carbon tetrachloride CCL4 which caused high levels of liver MDA then administered with ethyl acetate extract of white fire mangrove leaves and there was a statistically significant difference indicating a protective effect and inhibition of lipid peroxidation from Ethyl acetate extract of the white flame mangrove leaves which is rich in these flavonoids (13).

Based on the measurement of Caspase-3 levels after 7 days the experimental animals were made skin graft and given propolis extract, the Kruskal-Wallis mean comparative analysis test showed  $p > 0.05$ . This shows that there are no two groups that have different Caspase-3 means which are statistically significant (14).

The Caspase-3 protein has an important influence on apoptosis. Caspase-3 was expressed in normal tissue and significantly lower in gastric cancer. Another study also showed that Caspase-3 expression decreased in tongue cancer with overexpression of survivin which had an effect on inhibition of caspase-3 expression (15). In the process of angiogenesis, VEGF is expressed and increases the expression of survivin which can inhibit apoptosis through the apoptotic signaling pathway, thereby protecting the newly arising immature tumor vascular endothelial cells, enabling them to avoid apoptosis and subsequently producing more VEGF (16).

The results obtained in this study are not in line with research conducted by Tandean *et al* (2019) on Sprague Dawley rats that were treated with closed head injury and then given 200 mg / kgBW of propolis extract for 7 days, showing that administration of propolis extract reduced caspase 3 expression which is statistically significant through the caspase-dependent pathway and caspase-independent pathway inhibition mechanisms (17). The results obtained are also different from other studies conducted by Sun *et al* (2016) which examined the effects of propolis extract on a Neuro-2a neuroblastoma (N2a) cell model that was given hypoxia and hypoglycemia treatment, showing that propolis extract can downregulate Caspase-3 (18).

The results of the comparative test between the control group and the group given propolis extract, which was not statistically significant, still showed a decrease in Caspase-3 expression in the group given propolis extract compared to the control group. This is due to the content possessed by the propolis extract, namely Chrysin, pinocembrin, pinobanksin, apigenin, and galangin which apparently show an inhibitory effect on the apoptotic pathway such as reducing the expression of Caspase-3 and Bax, and increasing the expression of Bcl-2 mRNA (18).

#### 4. CONCLUSION

There was a significant difference in malondialdehyde (MDA) levels between the administration of three different doses of propolis extract and there was an insignificant difference in the expression of Caspase-3 in the male mouse granulation tissue propolis extract (*Rattus Novvergicus*) given the Skin Graft model. The most statistically significant difference in MDA levels occurred when the sample was given propolis extract at a dose of 400 mg / kgBW.

## CONSENT

As per university standard guidelines, participant consent have been collected and preserved by the authors.

## ETHICAL APPROVAL

This research obtained ethical approval from the Research Ethics Committee of RSUD Dr. Moewardi Surakarta with number 721/VI/HREC/2020. All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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