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EFFECT OF IRONWOOD BARK EXTRACT GEL ON LYMPHOCYTE COUNT IN BUCCAL MUCOSA WOUND HEALING RATS

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ABSTRACT

Background: *Wounds are disruptions caused by chemical or physical trauma, resulting in tissue damage. During the healing process of oral mucosal wounds, the body initiates defensive and protective immune responses against injury. The acute inflammatory phase is the initial stage of wound healing, marked by the body's response to injury within a few hours. Lymphocyte cells play a crucial role in the inflammatory phase as part of the adaptive immune system in wound healing. Secondary metabolites in the ironwood bark extract gel, such as proanthocyanidins, phenolics, and flavonoids, function as immunomodulators to enhance immune activity and act as anti-inflammatory and antioxidant agents to accelerate wound healing.* ***Purpose****: To prove the effect of ironwood bark extract gel at concentrations 5%, 12.5%, and 20% on lymphocyte cell number in the buccal mucosa incision wounds of male Wistar rats on days 3, 5, and 7.* ***Methods****: This study employed a pure experimental design with a post-test only with a control group design, using 36 male Wistar rats divided into treatment and negative control groups. Results: Two-way ANOVA tests revealed significant effects based on treatment and observation days ($p < 0.05$).* ***Conclusion****: Ironwood bark extract gel at concentrations of 5%, 12.5%, and 20% increased lymphocyte cell number on day 3, peaked on day 5 and decreased on day 7 compared to the control group (gel base).*

Keywords: *eusideroxylon zwageri gel, lymphocyte cells, oral mucosal wound healing,*

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INTRODUCTION

Surgical procedures in dentistry, especially in oral surgery, involve an incisional process that traumatizes the oral mucosa.[1,2] The prevalence of wounds caused by trauma in the oral cavity is quite high, namely 3-24%.[2] The presence of trauma to the oral mucosa causes open wounds on the mucosa causes inflammation and provides discomfort and pain in the mouth.[3]

Treatment of wounds to the oral mucosa used in general by the community is the administration of povidone iodine 1% which is a local antiseptic therapy and as an anti-inflammatory in the wound healing process in the oral cavity.[4,5] The use of 1% povidone iodine solution over time has disadvantages, namely that it can cause redness, pain, sensitivity, mucosal erosion, and disruption of the thyroid gland.[5] Alternative treatments with herbs are known to have minimal side effects and can be used as wound healing agents, have the ability to fight infection and support faster healing.[2] One of the typical Kalimantan plants used by the community for herbal medicine is ironwood bark (*Eusideroxylon zwageri*) which can be used for wound healing.[6]

Based on the results of the phytochemical test by Kusuma (2018), it is stated that the ironwood bark (*Eusideroxylon zwageri*) contains secondary metabolite compounds including proanthocyanidins with levels of 183.30 mg PE/g, phenolics with levels of 31.28 mg GAE/g, and flavonoids with levels of 30,48 mg CE/g.[7] Secondary metabolite compounds in ironwood bark can function as antibacterials, antioxidants, and anti-inflammatories that can accelerate wound healing.[6,8]

Based on Mariam's (2020) research on *Aggregatibacter actinomycetemcomitans* bacteria and Noor's (2021) on *Porphyromonas gingivalis* bacteria at concentrations of 5%, 10%, 20%, 40%, 60%, 80%, 100%, it states that ironwood bark extract (*Eusideroxylon zwageri*) has an effect that can inhibit bacteria with a minimum inhibitory value (KHM) at a concentration of 5% and a killer drink value (KBM) at a concentration of 20%.[9,10] Based on Salwa's research (2021)

on in vitro toxicity tests on BHK-21 cells at concentrations of 5%, 15%, 25%, 35%, 45%, 55%, 65%, 75%, 85%, 95%, and Arum's research (2023) on in vivo toxicity tests at concentrations of 25%, 55%, 95% on the kidneys of Wistar rats at ureum and creatinine levels, it is known that ironwood bark extract (*Eusideroxylon* zwageri) has no toxic effects so it is safe to use as a herbal ingredient in supporting the wound healing process.[6,11]

The wound healing process in the oral mucosa consists of 4 phases, namely hemostasis, inflammation, proliferation, and remodeling.[2] In the initial inflammatory phase, there are leukocyte cells that play an important role, one of which is neutrophil cells that start secreting cytokines to call monocytes that will migrate to the wound and then differentiate into macrophages. Then, lymphocyte cells begin to increase on day 3 after the appearance of pro-inflammatory M1 macrophage cells on day 2 which will further increase the synthesis of inflammatory mediators in the form of *interleukin-1* (IL-1) and *tumor necrosis factor- α* (TNF- α) which will increase lymphocyte cell proliferation on day 3.[12–14]

Lymphocytes are activated by cytokines in the form of IL-1 and TNF produced by macrophages. Activated lymphocytes release lymphokines, *interferon* (IFN) and *interleukin* (IL) to stimulate macrophage aggregation for enhanced phagocytosis.[12,15] Lymphocytes migrate to the wound area on day 1. On days 3 and 5 there was an increase in lymphocyte cells and a decrease in lymphocyte cells on day 7. The decrease in the number of lymphocyte cells indicates that the inflammatory phase ends and continues to the proliferation phase so that wound healing takes place quickly.[12]

There has been no research on the effect of ironwood bark extract gel (*Eusideroxylon zwageri*) on the number of lymphocyte cells in buccal mucosal wound healing, so it is necessary to conduct research on the effect of ironwood bark extract gel (*Eusideroxylon zwageri*) concentrations of 5%, 12.5%, 20% on the number of lymphocyte cells in buccal mucosal wound healing of male Wistar rats on days 3, 5, and 7.

MATERIALS AND METHODS

This study was conducted using a pure experimental method (*true experimental*), namely using a *posttest-only* design *with control group design.* This study has been approved by the Ethics Commission for Health Research of FKG ULM with *Ethical Clearance* Letter No.013/KEPKG FKGULM/EC/VII/202. A total of 36 male white rats of the Wistar strain (*Rattus norvegicus*) with a body weight of 200-250 g and 2-3 months old in a healthy condition characterized by normal eating, drinking, and movement patterns. The experimental animals were divided into 12 groups consisting of 9 treatment groups and 3 control groups with different euthanasia on days 3, 5, and 7

Ironwood Bark Extraction (*Eusideroxylon zwageri*)

Ironwood bark (*Eusideroxylon zwageri*) was taken using a knife on the inside of the red-brown color weighing ± 5 kg, then cleaned and dried in an oven at 40°C for 4 hours. The dried bark was ground into powder using a *hammer mill* and sieved with a *mesh screen*, resulting in a dry simplisia weighing 1.004g. Extraction was carried out by maceration method, namely mixing ironwood bark powder and 96% ethanol in a ratio of 1:6, then stirring in a *shaker* for 24 hours and repeating 4 times. The results of the extraction process of ironwood bark that has been homogenized are filtered using WH04 filter paper and the filtrate is put into an *erlenmeyer*. While the residue was re-macerated until a clear brown liquid was obtained. The evaporation process using a *vacuum rotary evaporator* with a temperature of 60°C for 4-6 hours and heated with a water bath until the entire solution evaporated and obtained a brownish residue of 24,24 g. The thick extract was then tested for ethanol-free by adding distilled water and a few drops of *potassium dichromate* ($K_2Cr_2O_7$) and heated for 20 minutes. A total of 5 ml of solution was mixed with 2,5 ml of distilled water, then the absorbance was measured using a spectrophotometer

Preparation of Gel Base and Variation of Ironwood Bark Extract Gel Concentration (*Eusideroxylon zwageri*)

Preparation of the gel base for each formulation begins with mixing 1g sodium carboxymethyl cellulose (Na-CMC), 2g glycerin, and 1g propylene glycol into a beaker glass. The mixture was homogenized using a stirring rod on a hot plate at 60-70°C. Aquadest were then added up to a volume of 20ml and stirred into the gel base mixture until it coalesced to form a homogeneous and stable gel base. Gel preparations were made in tifa concentration variations, namely 5%, 12.5%, and 20%.

Treatment of Male Wistar *Rats* (*Rattus norvegicus*)

Male Wistar rats (*Rattus norvegicus*) as experimental animals were adapted for 1 week in a cage with a temperature of 25-28°C with a good ventilation system. During the adaptation period, rats were fed BR2 *Comfeed* 2 times a day and drinking aquadest. Before the wounding procedure, all experimental animals were first anesthetized by *intramuscular* injection using a combination of *xylazine hydrochloride* 2mg/kg BW and *ketamine hydrochloride* 20mg/kg BW. An incision wound was made on the lower left buccal mucosa with a length of about ±10mm and a wound depth of ±1mm using a *scalpel* and *blade* no.11. Blood that came out during the opening process was cleaned using sterile cotton and given aquadest.

Male Wistar rats were divided into 12 groups consisting of 9 treatment groups and 3 control groups (gel base). Groups 1, 2, 3 were applied with a 5% concentration of ironwood bark extract gel, groups 4, 5, 6 were applied with a 12.5% concentration of ironwood bark extract gel, groups 7, 8, 9 were applied with 20% concentration of ironwood bark extract gel, and group 10, 11, 12 were applied with gel base. Each group that was given (*Eusideroxylon zwageri*) ironwood bark extract gel and gel base was given as much as 0.05g using a sterile *cotton bud* and waited for 1 minute

after application. The application was carried out twice a day, namely in the morning at 08.00 WITA and in the afternoon at 16.00 WITA. The application was done in the direction of the entire wound area. On days 3, 5, and 7, rats in all study groups were euthanized with 3x doses of anesthesia intraperitoneally using *ketamine* 60mg/kg and *xylazine* 6mg/kg in a ratio of 10:1. The injection technique is done intraperitoneally until the Wistar rats die. *Euthanasia* on day 3 in groups 1, 4, 7, 10, post-treatment, then *euthanasia* on day 5 in groups 2, 5, 8, 11 post-treatment, and *euthanasia* on day 7 in groups 3, 6, 9, and 12 post-treatment..

The buccal mucosal incision wound was removed using a sterile *scalpel* and *blade* No.15, *surgical scissors*, and *tweezers*. Tissue from the incision wound was cut and then separated with anatomical *tweezers* and *surgical scissors*. The cut tissues were immersed in 10% buffer neutral formaldehyde (BNF) fixation solution for 1x24 hours.

Animal Handling

Male Wistar rats (*Rattus norvegicus*) that have been sacrificed will be cleaned first wrapped in cloth and then buried at a depth of ± 25-75cm.

Preparation Preparation and Histopathology Observation

Tissue fixation by inserting normal tissue and incision wound area into 10% *Buffer Neutral Formalin* (BNF) solution for approximately 24 hours. The results of tissue fixation were then cut with a *scalpel* of ± 10mm. The tissue that has been cut is inserted into the *cassette embedding* tool. The tissue is placed in a *cassette embedding* basket and rotated with a *tissue processing* tool for a span of ± 18 hours. The tissue is then *blocked* by moving the tissue into a *base mold* containing liquid paraffin to be attached to the *embedding cassette* until it cools, the tissue is stored in a refrigerator. *Cutting* or cutting tissue blocks with a thickness of 5 microns using a microtome is placed on a microtome. The results of cutting the tissue are entered into a *water bath* at a temperature in the range of 37-47 ° C, the shape is smoothed then placed on a glass object and a good cut. Preparations that have been made are then dried. Observation of histopathological preparations was carried out using a *Leica DM 1000* light microscope at 400x magnification with 5 fields of view. Lymphocyte cells on light microscope observation have round and oval shapes and narrow cytoplasm that do not contain purplish blue granules. The number of lymphocyte cells per field of view was averaged by 4 observers and the average number of lymphocyte cells in each group was taken.

Data Processing and Analysis

The data obtained will first be tested using normality and homogeneity tests. *Shapiro-Wilk* test is a normality test used on the number of samples <50, and *Levene's Test* is a homogeneity test to see normally distributed and homogeneous data (p>0.05). Data with normally distributed and homogeneous results can be continued with the *Two-Way ANOVA* test to see significant differences between treatments. *Bonferroni Post Hoc* test is used to see groups that have significant differences. The research data were processed and processed with the SPSS computer *software* program

RESULTS

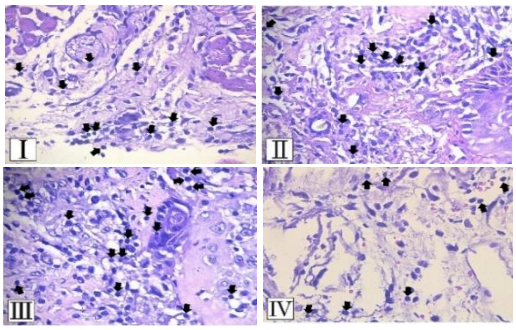

Figure 1. Histopathology of Lymphocytes Cells (Black arrows), I (5%), II (12,5%), III 20%) and IV (Gel base) on day 3 with 400x magnification using a Leica DM 1000 microscope**.**

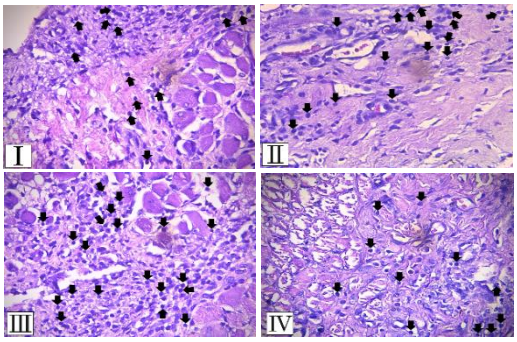

Figure 2. Histopathology of Lymphocytes Cells (Black arrows), I (5%), II (12,5%), III (20%) and IV (Gel base) on day 5 with 400x magnification using a Leica DM 1000 microscope**.**

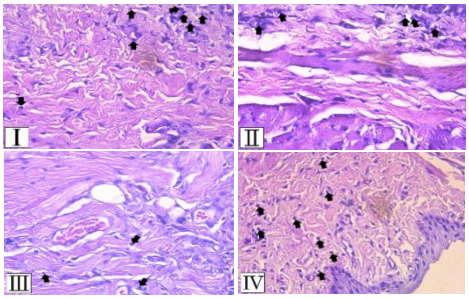

Figure 3. Histopathology of Lymphocytes Cells (Black arrows), I (5%), II (12,5%), III (20%) and IV (Gel base) on day 7 with 400x magnification using a Leica DM 1000 microscope**.**

To see the average lymphocytes cells on day 3, 5, and can be seen in table 1.

Table 1. Mean and Standard Deviation of lymphocytes Cell Counts

Kelompok	Mean ± SD		
	Hari ke-3	Hari ke-5	Hari ke-7
GEKBU 5%	11,2 ± 1,83	16,06 ± 0,46	9,53 ± 0,7
GEKBU 12,5%	13,67 ± 0,5	18,13 ± 1,3	7,13 ± 0,46
GEKBU 20%	17,8 ± 1,2	22,2 ± 1,21	4,4 ± 0,87
Kontrol (Basis gel)	6,8 ± 1,44	13,33 ± 1,62	11,86 ± 1,22

The average number of lymphocyte cells in the group treated with a 20% concentration of ironwood bark extract gel (*Eusideroxylon zwageri*) showed the highest average number of lymphocyte cells on day 3 and on day 5 lymphocyte cells reached the highest peak and on day 7 lymphocyte cells experienced the largest decrease compared to other treatment groups and the control group (gel base). On day 5, the 20% concentration treatment group showed that the average number of lymphocyte cells was higher than the treatment group on day 3 and the control group (gel base). On day 7, the 20% concentration treatment group showed that the average number of lymphocyte cells decreased the most compared to the average number of lymphocyte cells on day 3 and day 5 and the control group (gel base).

The results of data analysis of the average number of lymphocyte cells have gone through the *Shapiro-Wilk* normality test and *Levene* homogeneity. The *Shapiro-Wilk* normality test shows statistically significant results with a sig value of $p>0.05$ ($p=0.987$), which means that the data distribution is normally distributed. The homogeneity test using *Levene's test* showed meaningful results with a sig value of $p>0.05$ ($p=0.275$), indicating that the data varied and was homogeneous. Data analysis continued with parametric analysis using *Two-way ANOVA*.

Two-way ANOVA statistical analysis has shown the results that the treatment group and control group have a significant effect on the number of lymphocyte cells and gel base as evidenced by $p<0.05$ ($p=0.001$). The results of statistical analysis showed a significant effect on days 3, 5 and 7 with a value of $p<0.05$ ($p=0.001$). The values for the treatment and control groups on days 3, 5, and 7 were all less than $p<0.05$ ($p=0.001$). It can be concluded that there is a significant difference or interaction between all groups.

DISCUSSION

This study was conducted to prove the effect of ironwood bark extract gel (*Eusideroxylon zwageri*) concentrations of 5%, 12.5%, and 20% on the number of lymphocyte cells on days 3, 5, and 7 on wound healing of male Wistar rat buccal mucosa incision (Rattus norvegicus). The results showed that the treatment group of ironwood bark extract gel (*Eusideroxylon zwageri*) concentrations of 5%, 12.5%, and 20% had an effect as indicated by an increase in the number of lymphocyte cells on day 3 and on day 5 lymphocyte cells reached the highest peak and the number of lymphocyte cells decreased the most on day 7 compared to the control group (gel base). The increase in the number of lymphocyte cells is due to the influence of the content of secondary metabolite compounds contained in the ironwood bark extract gel, namely proanthocyanidins with levels of 183.30 mg PE/g, phenolics with levels of 31.28 mg GAE/g, flavonoids 30.48 mgCE/g.[7,8,10] Secondary metabolite compounds contained in ironwood bark can function as immunomodulators that can improve the immune system through increased lymphocyte cell proliferation and antioxidants and anti-inflammatory to reduce lymphocyte cell infiltration into the wound area.

Based on the research that has been done, it is found that on day 3, lymphocyte cells began to increase after being given ironwood bark extract gel (*Eusideroxylon zwageri*) and the control group (gel base). This research is in line with that conducted by Zayyan's (2016) and Fatimatuzzahro (2021) which states that lymphocyte cells increased on day 3.[12,16]

In the early phase of inflammation, neutrophils initiate cytokine secretion to call monocytes that migrate to the wound and differentiate into macrophages on day 2 to day 3. The increase in lymphocyte cell numbers on day 3 is in accordance with the theory that lymphocyte cells migrate to the wound area on day 3 after the emergence of pro-inflammatory M1 macrophage cells on day 2 which play a role in increasing the synthesis of inflammatory mediators including *interleukin-1* (IL-1) and *tumor necrosis factor-α* (TNF-α) which causes lymphocyte proliferation on day 3 to increase. Secondary metabolite compounds such as proanthocyanidins, phenolics, and flavonoids of ironwood bark extract gel can act as immunomodulators by increasing lymphocyte cell proliferation on day 3. This is supported by research by Rauf (2019) which states that proanthocyanidin is abundant in the stem bark and can play a role in stimulating lymphocyte cells increasing the phagocytic activity of macrophages and increasing TNF-α (*tumor necrosis factor-alpha*). On the 3rd day, macrophage cells will reach their peak by increasing the release of inflammatory mediators such as *interleukin-1* (IL-1), *interleukin-6* (IL-6) and *tumor necrosis factor* (TNF) which will cause the proliferation of B lymphocytes and T lymphocytes as the formation of antibodies and cell-mediated immunity to increase.[13,14,17]

The average number of lymphocyte cells reached the highest peak in the treatment group and control group on day 5 compared to day 3. This is in line with Zayyan's research (2016) which states that on day 5 lymphocyte cells reach their peak. On day 5 lymphocyte cells are activated by forming lymphokines, interferons, and interleukins.

Lymphokines and interleukins are used by lymphocyte cells to increase the phagocytic power of macrophage cells and *interferon-gamma* (IFN-y) to stimulate monocytes in the wound area. Increased phagocytosis of macrophages will help the release of proinflammatory cytokines, namely IL-1 and TNF, which will increase the proliferation of lymphocyte cells.[12,13] Other secondary metabolite compounds in the ironwood bark extract gel, namely phenolics and flavonoids, are known to function as immunomodulators by increasing the number of lymphocyte cells. This is supported by Nitbani's (2019) research on an ethanol extract of faloak bark (*Ipomea carnea jaco*) which states that many active phenolic compounds can increase lymphocyte cell proliferation and increase *interferon-gamma* (IFN-y) and phenolics from phenolics which act as immunomodulators by increasing the production of CD4+ T cells or helper T cells and CD8+ cytotoxic T cells as body immunity.[18,19] T helper cells activate lymphocyte cells and macrophages resulting in the proliferation of a higher number of lymphocyte cells.[13] The role of flavonoids as immunomodulators is supported by Siregar (2021) on lakum stem extract (*Cayratia trifolia*) which states that the flavonoid mechanism is to act as an antigen with recognized B cell and T cell receptors. Flavonoids react with hydrogen bonds on the surface of T cell receptors and on the surface receptors of IgM bind to B cells. The two resulting bonds will cause the activation of G-proteins to produce phospholipase. The reaction that occurs results in the hydrolysis of *Phosphatidyl Inositol Biophosphate* (PIP2) to produce the compounds *Inositol Triphosphate* (IP3) and *Diacylglycerol* (DAG). The cytoplasm receives Ca^{2+} release from the stimulation of Inositol Triphosphate which causes the Ca^{2+} concentration to increase, protein kinase C and 5-lipoxygenase resulting in the formation of IL-2 to activate the proliferation of B cells and T cells.[20]

The average number of lymphocyte cells decreased on day 7 compared to day 3 and day 5. This is in line with Riyani's research (2021) which states that lymphocyte cells decreased on day 7. The decrease in the number of lymphocyte cells on day 7 was due to lymphocyte cells undergoing apoptosis which caused their duties as phagocytic agents to be replaced by fibroblast cells to form new tissues or regeneration processes. The decrease in the number of lymphocyte cells indicates that the wound healing process has entered the proliferation phase. Secondary metabolite compounds proanthocyanidin, flavonoids, phenolics in ironwood bark extract gel have functioned as anti-inflammatory and antioxidant by reducing the number of lymphocyte cells.[7,12,21] Dwitiyanti's research (2022) on the extract of kecapi bark (*Sandoricum koetjape (Burm.f.) Mer*r.) states that flavonoids can act as anti-inflammatory by inhibiting the *cyclooxygenase* (COX) and *lipooxygenase* (LOX) pathways, accumulation of leukocytes in the trauma area, inhibiting histamine in the form of prostaglandins, leukotrins, and IL-1 and TNF mediators so that lymphocyte cell infiltration decreases.[22,23] Flavonoids belong to a large group of phenolics and can act as antioxidants in reducing lymphocyte cells. This is supported by research by Azminida (2022) on ulin bark which states that flavonoids can act as antioxidants by preventing the formation of oxidation reactions and capturing free radicals and increasing the activity of the enzyme *superoxide dismutase* (SOD) so that lymphocyte cell infiltration into the wound area decreases.[22,24] Safaruddin's research (2022) states that Java wood bark contains condensed tannins or proanthocyanidins which act as wound healing agents and are supported by Savitri's research (2020) which states that proanthocyanidins function as anti-inflammatory by inhibiting IL-2 and IL-1β cytokines which cause T cells to decrease lymphocyte cells.[25,26]

Oral mucosal wound healing is supported by biological substances in saliva which have important functions in wound healing, namely as antioxidants, and anti-inflammatory in response to wound healing. The oral mucosa intrinsically produces a lower infiltration of T lymphocytes, macrophages, and neutrophils. The oral cavity in particular has saliva and a buffer system at pH 5.5-7 providing a wet environment and has been shown to accelerate the wound reepithelialization process.[27] A study conducted by Azizah (2021) stated that saliva contains B lymphocytes consisting of IgA, IgG, and IgM antibodies which are subsets of B lymphocytes for oral mucosal protection and mucin as an antimicrobial to support oral mucosal wound healing.[28] According to Waasdorp (2021), on day 3 after the wound, T cells or T lymphocytes are in the oral mucosa and begin to disappear on day 6. T cell chemokines CCL5 (*C-C chemokine ligand 5*) and CXCL 10 or IP-10 were found more in the oral mucosa, indicating that Th1 is abundant in the oral mucosa so that the proliferation of T lymphocytes and B lymphocytes is abundant to accelerate the wound healing process.[29]

The results of this study showed that 20% concentration had the highest average number of lymphocyte cells in the gel treatment group of ironwood bark extract (*Eusideroxylon zwageri*) on day 3 and day 5 and on day 7 experienced the largest decrease in the number of lymphocyte cells compared to the gel treatment group of ironwood bark extract concentrations of 5%, 12.5%, and the control group (gel base). The difference in the average number of lymphocyte cells is influenced by different concentration levels. The higher the concentration used, the stronger the activity and effectiveness of the secondary metabolite compound content. This is supported by Mariam's research (2020) on the concentration of ironwood bark extract (*Eusideroxylon zwageri*) which states that the higher the concentration of extract used, the greater the activity of secondary metabolite compounds contained in the extract.[9]

Based on the results of the research that has been done, it can be concluded that the ironwood bark extract gel group (*Eusideroxylon zwageri*) is effective in increasing the number of lymphocyte cells on days 3 and 5 and reducing the number of lymphocyte cells on day 7 in the wound healing process.

Ironwood bark extract gel (*Eusideroxylon zwagery*) at concentrations of 5%, 12.5%, and 20% had a more significant effect when compared with the control in the form of a gel base on the number of lymphocyte cells on days 3, 5, and 7.

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