

THE EFFECT OF BINJAI LEAVES EXTRACT (*Mangifera caesia*) ON INCISIONAL BACK WOUND OF MICE (*Mus musculus*) TO THE NUMBER OF NEUTROPHIL CELLS

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**THE EFFECT OF BINJAI LEAVES EXTRACT (*Mangifera caesia*) ON INCISIONAL
BACK WOUND OF MICE (*Mus musculus*) TO THE NUMBER OF
NEUTROPHIL CELLS
(Preliminary Study of Drug Preparations for Wound Healing)**

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Wound is a tissue damage that causes functional and structural disorder of the body. When there was a tissue damage, the healing process will immediately occur to restore its integrity.¹ The wound healing process have 3 phases, namely inflammation, proliferation or granulation, and remodeling.² Clinical symptoms caused by inflammatory reactions are in the form of reddish color (*rubor*), warmth (heat), pain (*dolor*), swelling (*tumor*), and decreased function (*functio laesa*).³

The inflammatory phase is characterized by the emigration of neutrophil cells.⁴ Neutrophils act as the first defense which is come out to phagocytes the debris and bacteria. Phagocytosis will cause

neutrophils death and releasing of enzymes around the matrix which going to destroy the tissue.⁵ Neutrophil cells began to infiltrate 6 hours after the injury and increased on day 1 then gradually dropped on the 3rd day.⁴ Macrophage cells will enter the wound area and continue the role of neutrophils to phagocyte debris and bacteria and also produce the growth factors. Lymphocyte cells and mast cells will be the last one that entered the injury area on inflammatory phase.^{6,7}

One of the wound healing treatments that can be done is by consuming chemical drugs and herbal medicines.^{8,9} Herbal medicine is a medicine that made from plant which has a secondary metabolite with its function to support the healing process, such

as flavonoid. Herbal medicines can be utilized as an additional drug after the main treatment (gold standard therapy). This therapy is called as adjuvant therapy which can help to enhance the effectiveness of the treatment process.^{9,10,11} One of the herbal plants that have the potential to be an alternative treatment for wound healing is binjai plant.

Binjai (*Mangifera caesia*) is a plant which is widely used by Indonesian, especially in South Kalimantan. One of its use is as the mixture of chili sauce.^{12,13} Based on research by Rosita et al, 2017, the results of the phytochemical test of binjai leaves are known to have bioactive contents in the form of flavonoid and saponin.¹⁴

Flavonoids are known to be an anti-inflammatory and antimicrobial which is beneficial in the process of wound healing.¹⁵ The anti-inflammatory activity of flavonoid on its molecular mechanism involves the inhibition of pro-inflammatory enzymes, such as cyclooxygenase-2 and lipoxigenase.¹⁶ This study is done to determine whether there is an effect of binjai leaves extract on increasing the number of neutrophil cells on day 1 and decreasing the number of neutrophil cells on day 3 and 5 after topically administered on the incisional back wound of mice (*Mus musculus*).

METHODS

The research process began with a request for the necessary research permit and ethical clearance to be issued the Ethics Committee of the Faculty of Dentistry, University of Lambung Mangkurat NO.114/KEPKG-FKGULM/EC/1/2019. The research methodology was true experimental with a posttest-only control group design. The sample used thirty six mice which was divided into four groups; three groups administered with binjai leaves ethanol extract at varying concentrations (1 mg/mL, 2 mg/mL, and 3 mg/mL) and one control group without extract administering on day 1, 3, and 5.

The making process of binjai leaves extract used maceration method. Leaves were subsequently washed in water, dried in the open air, and mashed it up to obtain the simplicia powder. The dried simplicia was soaked for 3x24 hours in ethanol 70%. The result of maceration was filtered and subsequently concentrated using a rotary evaporator at 37°C. The filtrate being evaporated using waterbath until it form a thick ethanol extract of binjai leaves and then it was diluted using aquadest to get varying concentrations (1 mg/mL, 2 mg/mL, and 3 mg/mL).

Mice were adapted for 7 days in Laboratory. The mice were taken and measured on its back to make an incisional wound. First of all the anesthesia was performed by inhalation technique to the mice

using 1 mL of diethyl ether until its breathing was changed and the movement was simmer down, after that the mice back was shaved with a size of 4 cm in diameter and alcohol was administered on it to remove the hair. After being adapted for 2 days, the incisional wound was made after anesthesia given to the mice using diethyl ether. The wound was made on its back for about 1 cm in length and in depth till sub cutis by scalpel and disposable blade no.15. The overflowing blood was cleaned then.

The application of binjai leaves ethanol extract on injured area was done using a cotton bud once a day for 5 days. After that, the wound was wrapped with a sterile gauze which has been given NaCl and layered with a dry sterile gauze to maintain the moisture of wound. The mice on each groups were sacrificed in 1st, 3rd, and 5th day using 5 mL of diethyl ether by inhalation until it dead. Furthermore, biopsy was taken for tissue extraction in 2.5 cm length, 3 mm width, and 3 mm depth. The specimens were made into histology preparations and stained using the *Haematoxyllin Eosin* (HE) method. The preparations were observed and counted using light microscope with 40x10 magnification in 5 observing fields. The data obtained was quantitative of neutrophil cells number.

Data was processed using SPSS with One Way ANOVA as the analytical statistic with a confidence level of 95% ($\alpha = 0.05$), followed by Post Hoc Least Significant Difference (LSD) analysis to determine the value of significance.

RESULTS

Statistic result shows all the data was normally and homogenously distributed ($p > 0.05$). The mean value of neutrophil cells number can be seen on table 1. The mean value of neutrophil cells number in incisional wound healing of mice back in day 1, 3, and 5 is illustrated in Figure 1.

Table 1. Mean \pm SD value of Neutrophil Cells Number in Incisional Back Wound Healing of Mice

Day Groups	Mean \pm SD Cells Number			
	Control	Binjai 1 mg/mL	Binjai 2 mg/mL	Binjai 3 mg/mL
Day 1	23.0 \pm 2.0 (21-25 cells)	15.3 \pm 1.5 (14-17 cells)	17.0 \pm 2.0 (15-19 cells)	32.0 \pm 2.0 (30-34 cells)
Day 3	16.0 \pm 2.0 (14-18 cells)	9.0 \pm 2.0 (7-11 cells)	10.7 \pm 1.5 (9-12 cells)	7.0 \pm 1.0 (6-8 cells)
Day 5	10.3 \pm 1.5 (9-12 cells)	5.0 \pm 1.0 (4-6 cells)	3.0 \pm 1.0 (2-4 cells)	4.0 \pm 1.0 (3-5 cells)

Figure 1. A Diagram Represent the Average Number of Neutrophil Cells On The Incisional Back Wound of Mice for 5 Days in Each Groups.

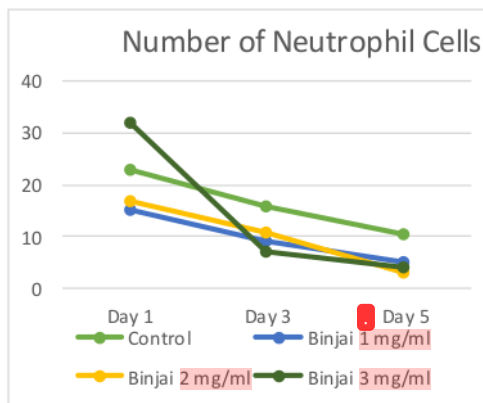


Figure 1 shows the difference in average number of neutrophil cells in incisional back wound of mice for 5 days in each group. On the day 1 there is an increase in the average number of neutrophil cells, whereas on day 3 and 5 there is a decrease in the number of neutrophil cells. The highest average on the 1st day can be found in 3 mg/mL of binjai leaves ethanol extract group (32.0 \pm 2.0 cells). The lowest average on the 3rd day can be found in 3 mg/mL of binjai leaves ethanol extract group (7.0 \pm 1.0 cells), while the 2 mg/mL of binjai leaves ethanol extract group has the lowest average on the 5th day (3.0 \pm 1.0 cells).

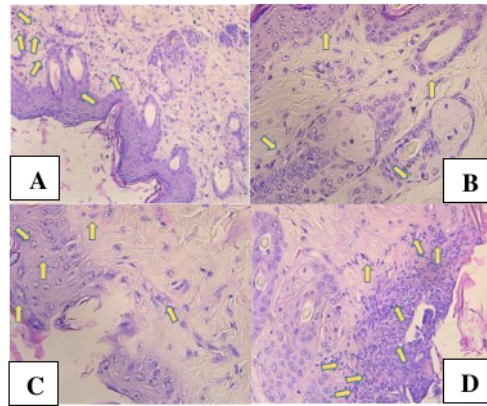


Figure 2. Histopathology of Neutrophil Cells on Incisional Back Wound Healing of Mice Using Light Microscope with 400x Magnification in Control Group (A), Binjai Leaves Extract 1 mg/mL (B), Binjai Leaves Extract 2 mg/mL (C), Binjai Leaves Extract 3 mg/mL (D) at 1st Day.

Figure 2 shows the histopathology of neutrophil cells number in incisional wound of mice given feed only with a total of (21-25 cells), the 1 mg/mL of binjai leaves ethanol extract group with a total of (14-17 cells), the 2 mg/mL of binjai leaves ethanol extract group with a total of (15-19 cells), and the 3 mg/mL of binjai leaves ethanol extract group with a total of (30-34 cells) on the 1st day.

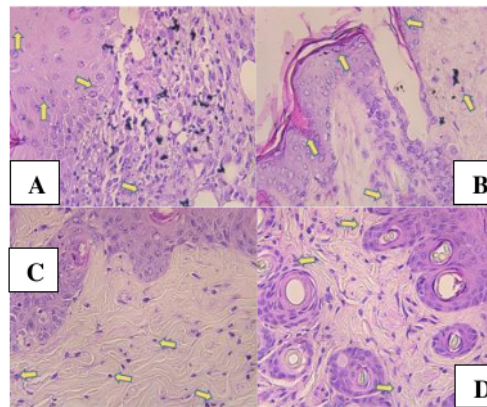


Figure 3. Histopathology of Neutrophil Cells on Incisional Back Wound Healing of Mice Using Light Microscope with 400x Magnification in Control Group (A), Binjai Leaves Extract 1 mg/mL (B), Binjai Leaves Extract 2 mg/mL (C), Binjai Leaves Extract 3 mg/mL (D) at 3rd Day.

Figure 3 shows the histopathology of neutrophil cells number in incisional wound of mice given feed only with a total of (14-18 cells), the 1 mg/mL of binjai leaves ethanol extract group with a total of (7-11 cells), the 2 mg/mL of binjai leaves ethanol extract group with a total of (9-12 cells), and the 3 mg/mL of binjai leaves ethanol extract group with a total of (6-8 cells) on the 3rd day.

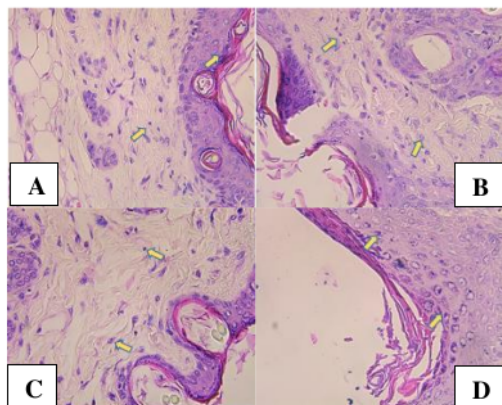


Figure 4. Histopathology of Neutrophil Cells on Incisional Back Wound Healing of Mice Using Light Microscope with 400x Magnification in Control Group (A), Binjai Leaves Extract 1 mg/mL (B), Binjai Leaves Extract 2 mg/mL (C), Binjai Leaves Extract 3 mg/mL (D) at 5th Day.

Figure 4 shows the histopathology of neutrophil cells number in incisional wound of mice given feed only with a total of (9-12 cells), the 1 mg/mL of binjai leaves ethanol extract group with a total of (4-6 cells), the 2 mg/mL of binjai leaves ethanol extract group with a total of (2-4 cells), and the 3 mg/mL of binjai leaves ethanol extract group with a total of (3-5 cells) on the 5th day.

DISCUSSION

The result of this study demonstrates that binjai leaves extract was able to increase the number of neutrophil cells on day 1 and decrease the number of neutrophil cells on day 3 and 5. On the 1st day, binjai leaves extract with a concentration of 3 mg/mL had the highest average number of neutrophil cells. This is in accordance with the theory mentioned by Kumar et al, 2013 that neutrophil cells are inflammatory cells which has a dominant role in the first 6-24 hours and act as the main cellular defense that works by phagocytes the foreign debris in damaged tissue when acute inflammation occurs, so the number increases in the early time post-injury.^{4,16,17}

Inactive neutrophils in the body cell will be active if there's a signal from damaged cells by producing the inflammatory mediators, such as cytokines (TNF, IL-1, and IL-6), chemokines, and growth factors. TNF and IL-1 will stimulate the release of adhesion mediators in endothelial cells, thus triggering neutrophil cell attachment and accumulation.⁴ An active neutrophil cells will immediately clean the microbes that invade the wound tissue. Neutrophils have various antimicrobial, substance, and protease contents which are mostly stored in cytoplasmic granules before they are released.¹⁸ Hypochlorous acid (HOCl) is one of the most powerful antimicrobials and is produced by the myeloperoxidase enzyme (MPO) which can be found in neutrophil lysosomes.⁴

Neutrophils that were previously in the bloodstream will go along the side of blood vessels' wall, the process is helped by vascular permeability increased during the initial phase of inflammation because the blood flow will be slowing down and neutrophil cells will accumulate more easily and also attach to capillary endothelial cells, after that neutrophils will enter the tissue assisted by adhesion molecules, namely ICAM-1 and VCAM-1. ICAM-1 will bind to LFA-1 and Mac-1 integrins, while VCAM-1 binds to VLA-4.⁴ The chemotaxic factors also help neutrophils to enter the tissue, namely complement fragments C5a, small peptides from bacteria, and a number of leukotrienes which has a role in attracting these neutrophils.¹⁹

Emigration of neutrophil cells from capillary blood vessels to damaged tissue is one of the important components that occurs in the acute inflammatory phase that lasts 1-4 days.⁴ However, the high number of neutrophil cells in the treatment group of the 3 mg/mL binjai leaves extract on day 1 allowed a toxic effect on the extract. This is in accordance with the results of the study by Syahdana et al, 2017 which states that the higher the concentration of binjai leaves extract, the higher the toxicity level of the extract is, moreover the research by Santi, 2015 proved that at the highest dose of methanol and n-hexane extract of papaya leaves administrated which contain flavonoid had a toxic effect on rats because they caused diarrhea from the 3rd to the 8th day, But the toxicity effect did not cause death in wistar rats.^{12,20}

Binjai leaves extract with concentration of 1 mg/mL has the lowest number of neutrophil cells below the control group on day 1. This is due to the content of flavonoid and saponin in binjai leaves which have an anti-inflammatory effect, so they can control the wound healing process. The anti-inflammatory activity of flavonoid on its molecular mechanism involves the inhibition of pro-

inflammatory enzymes, such as cyclooxygenase-2 and lipoxygenase, besides that flavonoid can also regulate up and down the transcription factors in inflammatory and antioxidant pathway, such as NF- κ B and Nrf-2.^{21,22}

In the LOX pathway there is lipoxygenase 5-Lipoxygenase (5-LOX), an enzyme which is metabolize the production of arachidonic acid (AA). Hydroperoksi AA 5-HPETE derivatives (eikosatetraenoic 5-hydroxy acid) are chemotactic to neutrophil cells or converted to leukotriene. The leukotriene A₄ will turn into leukotriene B₄ (LTB₄) which is a potent chemotaxic agent and causes neutrophil cell aggregation. If 5-LOX is inhibited, then neutrophil chemotaxis is inhibited as well, so the number of neutrophils will decrease in inflamed tissue.^{4,23}

Flavonoids can act as a radical scavenger against ROS by releasing hydrogen atoms (H) from their hydroxyl groups, so the free radicals will be more stable. Free radicals will stop doing extreme movement which can damage the lipid membranes, proteins, and DNA that are targeted by cellular damage.²⁴ Based on the result of the study by Ambriz et al, 2016, the types of flavonoids such as apigenin, genistein, and luteolin are known to be able to inhibit the excessive formation of ROS, so the tissue damage can be minimized.²²

Another secondary metabolites in binjai leaves are saponin compounds. Saponin has anti-inflammatory properties by reducing prostaglandin production through inhibition of the cyclooxygenase (COX) pathway. Suppression of the COX pathway results in reduced pain, redness, and vasodilation of blood vessels. Decreased ability of blood vessel vasodilation will result the depression in the number of neutrophil cells coming out of the bloodstream to the endothelial surface, so the number of neutrophils that do diapedesis is reduced.⁴ In addition of being able to inhibit the release of COX-2 according to Sumaddar and Koner, 2019, saponin can also inhibit the release of iNOS, PGE₂, IL-6, IL-1 β , TNF- α , and NO. Inhibition of cytokines such as IL-6, IL-1 β , and TNF- α is the key of success in controlling the inflammatory process.²⁶

On the 3rd day, the highest concentration of binjai leaves extract with 3 mg/mL has the lowest number of neutrophil cells compared to other groups. On day 3 the number of neutrophil cells gradually decrease because the wound healing process in the inflammatory phase is almost complete, marked by the presence of randomly scattered neutrophil cells around the edge of the wound.⁴ Flavonoid compound in binjai leaves extract also has a role to activate macrophage cells and do the phagocytosis together with neutrophil cells. Macrophage cells will release

the inflammatory mediators which help attachment of neutrophil cells and macrophage cells to the endothelial wall, then these cells will do diapedesis and move to the inflamed tissue. Increased number of macrophage cells will replace the role of neutrophils to clean debris and phagocytes the remnants of neutrophils, so the number of neutrophil cells will decrease.²⁷ The types of flavonoid such as quercetin, apigenin, and epigallocatechin gallate (EGCG) are known to work as modulators of macrophage cells in the inflammatory phase.²⁸

The decrease of neutrophil cells number on day 3 was proven by Canesso et al, 2014 which was seen through MPO enzyme activity. On day 3 MPO activity is lower than day 1. The phagocytosis activity of neutrophils could be calculated through MPO activity.^{19,29} It proves that the number of neutrophil cells gradually decreases because the wound healing process in the inflammatory phase is almost complete.²⁹ The reduced number of neutrophil cells is a marker of the continuous of healing process to the next step, so that the healing process of the inflammatory phase can be accelerated.³⁰

On the 5th day, binjai leaves extract with a concentration of 2 mg/mL has the lowest number of neutrophil cells compared to the other groups. Based on the research by Suharto et al, 2017, on the 5th day there is a significant decrease in the number of neutrophil cells compared to the number of neutrophil cells on day 1 after being given ginger extract which contained flavonoid compound.³¹ This is because the remaining neutrophils in the tissue will be phagocytes by macrophage cells. Macrophages will enter the wound site and continue the role of neutrophils to phagocyte debris and bacteria, produce growth factors, and become the main cell to repair the wound in proliferation phase.³⁰ Proliferation is characterized by the presence of a new blood vessel formation process (angiogenesis), granulated tissue formation with a reddish protruding surface, and re-epithelialization.⁴

The most influential group of binjai leaves extract on day 1 was at the concentration of 1 mg/mL because the number of neutrophil cells did not exceed the control group and it counted as the lowest. The most influential group of binjai leaves extract on the 3rd day in reducing neutrophil cells is at the concentration of 3 mg/mL because it has the lowest amount of neutrophil and the most influential group of binjai leaves extract on the 5th day in reducing neutrophil cells is found at the concentration of 2 mg/mL. The results showed that the ethanol extract of binjai leaves has an anti-inflammatory function, so it can be given after the administration of the main treatment as an additional drug, this therapy is called adjuvant therapy which can help increase the

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