

Effect of Beluntas Leaf Extract (*Pluchea indica*) on Oral Mucosal Wound Healing in Terms of Density of Inflammatory Cells and Collagen

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ABSTRACT

Traumatic injury is one of the acute injuries that often occur in the oral cavity. The wound healing process consists of the phases of hemostasis, inflammation, proliferation, and tissue remodeling. Some of the active components of herbal plants can help speed up the wound healing process and have anti-inflammatory effects. Beluntas leaves which contain flavonoids, tannins, and saponins have the potential to be used as herbal medicine because they have anti-inflammatory effects, but standardization is needed first. The purpose of this study was to determine the effect of beluntas leaf extract (*Pluchea indica*) on wound healing in the oral mucosa in terms of the number of inflammatory cells and collagen in tissue preparations stained with Hematoxylin Eosin. The method in this research is a descriptive laboratory experimental research design with the post-test only control group design. The number of samples of 30 Wistar rats, divided into 6 groups that had been treated and terminated on the 3rd and 6th days were then continued to the microscopic stage by making paraffin blocks, making preparations, and staining using Hematoxylin Eosin (HE). The assessment of HE streaks was semiquantitative, all data from the research results were statistically tested using the Kruskal-Wallis Test. Based on the test results, it is known that there are significant differences in the effect of beluntas leaf extract on the density of inflammation and collagen cells compared to the negative control and the positive control (povidone iodine). The conclusion from this study is that it can be seen that beluntas leaf extract can help accelerate the process of wound healing in the oral mucosa as indicated by a significant decrease in the density of inflammatory cells and an increase in collagen density.

Keywords: Anti-inflammatory, beluntas leaves, collagen, wound healing

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INTRODUCTION

Traumatic injuries or injuries caused by injuries can range from abrasions and small skin incisions or lacerations to wounds with extensive tissue damage or loss. The degree of tissue damage is influenced by the mechanism of injury, traumatic injuries can be caused by blunt trauma, penetrating trauma (such as stabbing and gunfire), crush injuries, explosion injuries, burns, and animal bites.^{1,2}

Traumatic injury is one of the most common acute injuries to the oral mucosa, which can be caused by thermal, physical, and chemical trauma. Sores that form can occur as a result of being bitten, eating hot and hard food, sharp teeth edges, or excessive brushing. Some injuries can also be caused by iatrogenic damage during dental work or other procedures related to the oral cavity such as intubation during general anesthesia. Likewise, chemical or physical injuries can result from improper or careless handling of chemicals and dental instruments during dental procedures. This condition causes the patient to feel uncomfortable, therefore medicine is needed to help accelerate wound healing.³⁻⁵

Acute or chronic wound healing is a challenge and there are various ways to heal wounds using synthetic polymers (iodine, ethyl alcohol, ether), growth factors, stem cells, and medicinal plants.^{6,7} Wound healing is a natural physiological reaction to the injury network. After the injury, rapid wound closure and rapid skin regeneration are essential to restore barrier function. Effective repair requires communication and interaction between many cells and this process is precisely regulated at any level. Wound healing has stages as in the following phases:

hemostasis or fibrin clot formation (0-hours after injury), inflammation/granulation (1-3 days), proliferation (4-21 days), and tissue remodeling (21 days-1 year).⁸

These phases can occur due to the integration of biological and molecular activities, such as cellular proliferation, differentiation, migration, secretion of cytokines and growth factors, as well as differentiation and production of the extracellular matrix. However, wound healing can be delayed if there is a failure to move from one phase to the next resulting in an imbalance in the normal sequence of acute wound healing.⁹

The main key to the wound healing process is the transition from the inflammatory phase to the proliferation phase. The inflammatory phase is very important because it leads to hemostasis and recruitment of the innate immune system, which protects us from invading pathogens and helps eliminate dead tissue.⁸ This inflammatory phase is then followed by the formation of granulation tissue, re-epithelialization, and the formation of the connective tissue matrix (the proliferation phase).¹⁰ One of the herbal plants that can be used to help accelerate wound healing is beluntas leaves (*Pluchea folium*). This can occur because this plant has astringent, antipyretic, and anti-inflammatory activities.¹¹ Beluntas (*Pluchea folium*) is one of the most widespread native Indonesian plants. Plants that are included in the Asteraceae family grow wild and are planted as hedges, as well as a traditional medicine because one of them has anti-inflammatory activity.¹²

Beluntas (*Pluchea folium*) is commonly used for traditional medicine in Asia in the form of stew, poultices,

and infusions. In Thailand, beluntas sticks are used as a treatment for kidney stones or work as a diuretic agent. The fresh leaves are used as a treatment for gangrenous ulcers. In India, boiled beluntas root has astringent and antipyretic action. Meanwhile, in Indonesia, boiled beluntas leaves can stimulate appetite, work as an antipyretic, aid in digestion, as a deodorant, antibacterial, and anti-diarrhea.¹¹

Beluntas leaves (*Pluchea folium*) contain phytochemical compounds such as essential oils, flavonoids, phenolics, tannins, saponins, phenol hydroquinones, and glycoside compounds.^{12,13} Flavonoids have biological functions such as anti-oxidant, anti-inflammatory, anti-mutagenic, anti-virus, and anti-allergies.¹⁴ The anti-inflammatory effect occurs by inhibiting a series of enzymes that are activated during the inflammatory process.¹⁵ The anti-inflammatory action of flavonoids involves inhibition of the synthesis and activity of various pro-inflammatory mediators such as eicosanoids, cytokines, adhesion molecules, and protein C-reactive.¹⁶ Tannins are antioxidants that induce TGF- β so that the proliferation of fibroblasts can occur. The more fibroblasts that are formed, the collagen will be formed which then accelerates wound contraction and will also accelerate wound healing.^{17,18}

The cytotoxicity test of beluntas leaf extract (*Pluchea folium*) against fibroblasts has been carried out and has shown non-toxic results, with an IC₅₀ value of 265.388 μ g / mL.¹⁹ Therefore, beluntas leaves are safe if used as herbal medicine. Besides, beluntas leaves (*Pluchea folium*) have also been used traditionally to increase appetite, smooth muscle relaxants, dysentery, eliminate body odor and disorders that cause cachexia.²⁰

MATERIALS AND METHODS

Animal preparation

This research has obtained ethical approval issued by the ethical committee of the Faculty of Medicine, Maranatha Christian University-Immanuel Hospital Bandung. The main goal is that in this study the minimum possible use of experimental animals, by treating them humanely. The experimental animals are kept in the Pharmacology Laboratory of the Faculty of Medicine, Padjadjaran University, Bandung. This study used thirty male Wistar

rats (weight 200-250 grams) aged 40-60 days. experimental animals were divided into six groups (n = 5 / group). Groups one and four were given wounds without topical treatment of Beluntas as negative controls. Group two and five were given the injuries and povidone iodine topical treatment as a positive control. Groups three and six were treated with topical beluntas leaf extract. All treatments were given to rats anesthetized with ketamine (10 mg/kg body weight) intramuscularly. Then a 5 mm long wound was made on the labial gingiva of the mandible and the treatment was applied. At the end of the experimental period, groups one, two, and three on the three days were terminated and groups four, five, and six on the sixth day were terminated and decapitated.

Beluntas leaf ethanol extract

The leaves of beluntas to be used in this study are \pm 10 years old, obtained from an experimental garden at the Indonesian Medicinal and Spice Crops Research Institute (Balitro), Agricultural Research and Development Agency (IAARD), Ministry of Agriculture in Bandung, West Java, Indonesia. This leaf was later determined by Dr. Iriawati at the Identification and Assignment Laboratory of the Faculty of Life Sciences, Bandung Institute of Technology (ITB). After the determination was carried out, then the crude extract of beluntas leaves was made by maceration method using 70% ethanol which was carried out at Aretha Medika Utama, the Center for Biomolecular and Biomedical Research.

Beluntas extract is applied to the wound that has been made twice a day at a dose of 5 μ g / gbw in the morning and evening. After the third day, the tissue cutting was done in groups one, two, and three which were then made preparations and stained with hematoxylin-eosin staining. The same procedure was performed on the sixth day for groups of four, five, and six.

Tissue preparations and microscopic examination

Make a paraffin block from the tissue to be examined and then cut the tissue 4-5 μ m thick using a microtome. Dehydrate using a multilevel ethanol solution, then color with Hematoxylin Eosin (HE). Inflammatory cells and collagen density were quantitatively calculated by an anatomical pathologist, unaware of the experimental data, using a light microscope (Olympus CX21FS1, Tokyo, Japan) at 100x magnification.²¹

Table 1: Scoring of Inflammatory Cell Density and Collagen Density

Score	Inflammatory Cell Density	Collagen Density
0	There are no inflammatory cells	There is no collagen
1	<20%	Slightly on the granulation tissue
2	20-50%	Enough on the granulation tissue
3	>50%	Lots and clear in granulation tissue

Statistical Analysis

Calculation of data based on measurement results according to each group with statistical testing of data analysis using the Kruskal-Wallis Test. All analyzes used Minitab version 17.0 software. The p-value of less than 0.05 (P<0.05) is considered significant.

RESULTS

HE staining showed the density of inflammatory and

collagen cells in the wound healing process of the mandibular labial gingival under a light microscope, as shown in Figure 1. Preparation of preparations was repeated 2 times for each subject so that the total number was 60 preparations. Semi-quantitative analysis was performed based on the percentage of inflammatory cells and collagen density in 5 visual fields for each preparation.

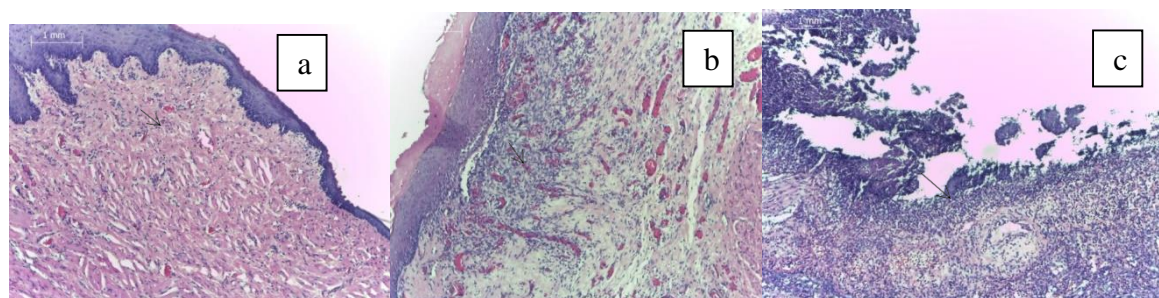


Figure 1: HE Staining for Inflammatory Cell and Collagen Density (100x enlargement)
 (a) number of inflammatory cells >50% and a little collagen;
 (b) The number of inflammatory cells is 20-50% and sufficient collagen;
 (c) The number of inflammatory cells <20% and a lot of collagen

Table 2: Score Inflammatory Cell Density

Group	1	2	3	4	5	6
	3	2	2	1	2	1
	2	2	1	1	2	1
	2	2	1	1	1	2
	2	2	1	1	1	2
	3	2	2	2	3	1
	3	2	2	3	3	2
	2	2	2	3	2	1
	2	2	1	3	1	1
	2	2	2	2	1	1
	2	2	1	3	1	1
Average	2,3	2	1,5	2,0	1,7	1,3

Information:

- Group-1: Group as a negative control was terminated on day 3
- Group-2: Group was given povidone iodine which was terminated on day 3
- Group-3: The group was given beluntas leaf extract which was terminated on day 3
- Group-4: The group as a negative control was terminated on day 6
- Group-5: Groups were given povidone iodine which was terminated on day 6
- Group-6: The group was given beluntas leaf extract which was terminated on day 6

Table 3: Collagen Density Score

Group	1	2	3	4	5	6
	1	3	2	3	2	3
	1	3	3	3	2	3
	2	3	3	3	3	3
	2	3	3	3	3	3
	1	3	2	2	2	3
	2	3	2	2	2	3
	2	3	3	2	3	3
	2	3	3	2	3	3
	3	3	3	3	3	3
	3	3	3	3	3	3
Average	1,9	3	2,7	2,6	2,6	3

Information:

- Group-1: Group as a negative control was terminated on day 3
- Group-2: Group was given povidone iodine which was terminated on day 3
- Group-3: The group was given beluntas leaf extract which was terminated on day 3
- Group-4: The group as a negative control was terminated on day 6
- Group-5: Groups were given povidone iodine which was terminated on day 6
- Group-6: The group was given beluntas leaf extract which was terminated on day 6

Based on the data obtained and tested by the Kruskal-Wallis Test, it was found that there was a significant decrease in the density of inflammatory cells ($P < 0.05$) with a value of $P = 0.028$. Therefore, it can be concluded that there is a significant difference in the effect of beluntas leaf extract on decreasing the density of inflammatory cells compared to negative controls (without treatment) and positive controls (povidone iodine) both on day 3 and day 6. Based on the data obtained and tested by the Kruskal-Wallis Test, it was found that there was a significant increase in collagen density ($P < 0.05$) with a value of $P = 0.012$. Therefore it can be concluded that there is a significant difference in the effect of beluntas leaf extract on the increased collagen density compared to negative controls (without treatment) and positive controls (povidone iodine) on both day 3 and day 6.

DISCUSSION

The wound healing process occurs in four interrelated phases. In the second phase, namely, the inflammatory phase, which lasts for 2-5 days and stops once the harmful stimuli have been removed, the cells that play a role in this phase are leukocyte cells, namely neutrophils, macrophages, and lymphocytes.⁸ The parameters used in this observation are the number of inflammatory cells (leukocytes and PMN) with the preparations used were preparations that had been stained with HE staining.

The data in Table 2 and Table 3 show that the results of statistical tests on day 3 and 6, showed a significant difference ($P < 0.05$) in the number of inflammatory cells and also collagen for the three groups. The difference between the untreated group, the group given povidone iodine, and the group was given beluntas leaf extract was influenced by the active ingredients contained in beluntas leaves. In the event of trauma, the circulating protein will recognize the microbes that enter the blood. The increased permeability of blood vessels will allow plasma proteins and leukocytes, as well as body defense mediators, to enter the site of infection or areas of damaged tissue. The wound healing process occurs because of the interaction between inflammatory cells, fibroblasts, and keratinocytes which causes changes in the microenvironment in the wound area.^{7,10}

Beluntas leaf extract contains several biologically active components such as saponins, flavonoids, and tannins, which can help accelerate wound healing through various mechanisms. Saponins have anti-inflammatory, antioxidant, antibacterial, and antiviral activity. The antibacterial activity of saponins occurs through denaturation of proteins present in bacteria so that cell membranes in bacteria become damaged and lysis, this causes the function of leukocytes for phagocytosis to be reduced, therefore from the 3rd and 6th day there is a decrease in the number of inflammatory cells namely leukocytes and PMN. With a series of reactions from the active ingredient of beluntas leaves, the inflammatory process occurs faster. In the wound healing process, saponins can also inhibit inflammatory reactions in the initial phase of wound healing and increase the synthesis of the collagen matrix through the phosphorylation of the SMAD-2 protein.^{7,22}

Flavonoids are one of the bioactive components of beluntas leaves which have biological and pharmacological activities, including activities as anti-inflammatory, antimicrobial, increase angiogenesis, and act as antioxidants that work together in accelerating

wound healing by controlling prostaglandins, leukotrienes, and cyclooxygenase cycles in the inflammatory phase of wound healing.^{6,23} Flavonoids contained in beluntas leaves can also inhibit cyclooxygenase and accumulation of leukocytes.²⁴ Inhibition of the cyclooxygenase enzyme can control pain and edema so that the wound healing process takes place better because there is an increase in the immune response during wound healing. In the cell signaling process, flavonoids also act as modulators and inhibit procollagen type I mRNA, so that they directly affect collagen synthesis which will help accelerate the wound healing process.^{23,25}

Tannins will protect the body's cell membranes by reducing inflammation by releasing hydrogen atoms to bind free radicals so that the lipid autoxidation reaction can be controlled and reduced.²⁶ Besides, tannins are also antioxidants that induce TGF- β so that they can cause increased fibroblast proliferation. The more fibroblasts that are formed, the more collagen will be formed to support other cells involved in the wound healing process, so that it will speed up wound contraction which will affect the acceleration of wound healing. In addition, TGF- β also directly plays a role in stimulating collagen production.^{17,18} In the process of wound healing, fibroblasts contribute to the formation of extracellular matrix (ECM) components consisting of collagen by breaking down fibrin clots through the expression of matrix metalloproteinases (MMPs). This collagen-containing extracellular matrix will support and regulate the migration and activity of fibroblasts which will then help speed up the wound healing process by signaling angiogenesis, granulation tissue formation, and reepithelization.^{17,18}

The low number of inflammatory cells on the 3rd day in the beluntas leaf extract group showed a faster healing process compared to the positive control group and the negative control group. The number of inflammatory cells in the negative control group was the highest compared to the positive control group and with beluntas leaf extract, so there may be still tissue damage and infection due to the absence of treatment which causes the inflammatory process to be long. The comparison between the positive control group (povidone iodine) and beluntas leaf extract showed different results, both on day 3 and 6, the number of inflammatory cells in beluntas leaf extract was lower than that in the positive control, so beluntas leaf extract had a significant effect. Better for wound healing as seen from the number of inflammatory cells compared with positive controls. Povidone iodine is an antimicrobial agent that contains iodine.²⁷ The above comparison shows that both the beluntas leaf extract group and the povidone iodine group experienced a decrease in the density of inflammatory cells. However, beluntas leaves have a lower amount because of the high content in beluntas and have astringent, antipyretic, and anti-inflammatory activity so that they have complementary work and help accelerate the wound healing process.

CONCLUSION

Beluntas leaf extract can help accelerate the process of wound healing in the oral mucosa as indicated by a decrease in the density of inflammatory cells and a significant increase in collagen density.

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