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IN VIVO EVALUATION OF COMBRETUM INDICUM FLOWER METHANOLIC EXTRACT AS OINTMENT FOR BURN WOUND ON MALE MICE

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Abstract

Burns that are not treated properly can be dangerous, even can lead to death. Combretum indicum is reported to have anti-inflammatory, antibacterial, and high antioxidant activities, making it effective in healing dermal wounds. This study aimed to evaluate the wound healing effect of C. indicum flower methanolic extract on burn wound model in male mice. A total of 36 adult male mice were randomly assigned to 6 groups namely Negative control (I) without treatment; Positive Control I (II) with Burnazin treatment; Positive control II (III) with Vaseline treatment; Treatment group I (IV) with 25% concentration of C. indicum flower methanolic extract ointment; Treatment group II (V) with 50% concentration of C. indicum flower methanolic extract ointment; Treatment group III (VI) with 75% concentration of C. indicum flower methanolic extract ointment. Mice were treated daily for 20 days, and wound areas were observed every 2 days. Hydroxyproline level and total DNA content were tested on day-11. Skin tissue was analysed histologically. The results indicate that C. indicum flower extract accelerate wound healing process and repair the damaged skin tissue by accelerate the Proliferative phase.

Keywords: *Combretum Indicum; Burn Wound; Hydroxyproline; Wound Healing; Total DNA Content*

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INTRODUCTION

Burn wounds result from contact with heat sources such as fire, chemicals, and electricity. Untreated burns can lead to complications like infection, dehydration, organ failure, and even death (Anggraeni & Bratadiredja, 2018). Plants like *Combretum indicum* are reported to have anti-inflammatory, antibacterial effects, high antioxidant activity, and are excellent in healing dermal wounds (Arundhati *et al*., 2020).

C. indicum, known as melati belanda in East Borneo, belongs to the Combretaceae family and is classified as a weed by the Global Compendium of Weeds. It is widespread across Asia, Africa, North America, and Australia, and is cultivated as a houseplant for its medicinal benefits (Ningrum, 2019). *C. indicum* has numerous pharmacological benefits, including antidiabetic, anticancer, antiviral, anti-inflammatory, and anthelmintic properties (Kulshreshtha *et al*., 2018). *C. indicum* has so many benefits such as antidiabetic, anticancer, antiviral, antiinflammation and anthelmintic. *C. indicum* can be used for malaria, filariasis and diarrhea. Besides, this plant has analgesic activity that can reduce pain.

C. indicum flower extract reported has antibacterial activity for pathogen bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* dan *Bacillus subtilis* (Mukherjee & Chandra, 2017). *C. indicum* extract reported has good antioxidant activity (Kumar *et al*., 2019). Based on research by Bhangale & Qureshi, (2021) noted that *C. indicum* extract given orally for mice proven can accelerate wound healing in skin. This is because *C. indicum* contain such as alkaloid, saponin, tannin, phenol, steroid, triterpenoid, dan flavonoid that potentially for accelerate wound healing process (Bharti *et al*., 2018; Barik *et al*., 2020; Jasiem *et al*., 2018; Shamili & Santhi, 2017; Singh *et al*., 2017. Currently, there is limited evidence using ointment from *C. indicum* flower methanolic extract as solitary treatment to improve wound healing. This study aims to evaluate the wound healing effect of *C. indicum* flower methanolic extract on burn wounds in a male mice model.

RESEARCH METHODS

Materials

This study used *C. indicum* flower, 36 male mice, Hydroxyproline kit, total DNA kit, 70% methanol, 70% alcohol, vaseline, ketamine, Burnazin ointment, distilled water, phosphate-buffered saline (PBS), 6 NHCl, Chloramine T-Oxidan, Perchloric acid, citrate buffer, Erlich's reagent, filter paper, 1 mL syringe, and aluminum foil. The study was conducted out at January-March 2022 in the Animal

Physiology Development and Molecular Laboratory, Faculty of Mathematics and Science, Mulawarman University. The study was approved by the Health Research Ethics Committee of Medical College of Mulawarman University (Approval no.22/KEPK-FK/III2022).

Flower extract and ointment preparation

500 grams of dried *C. indicum* flower were macerated in 1.5 L of 70% methanol for 5 days. The macerate was filtered and evaporated using a rotary evaporator at 34-40°C. The extract paste was mixed with vaseline as an ointment base in concentrations of 25%, 50%, and 75% (Sentat & Permatasari, 2015).

Animal Preparation

Mice aged 8-12 weeks, weighing 20-30g, were randomly divided into 6 groups (6 mice each) and acclimatized for 3 weeks prior to the experiment. The mice were kept at room temperature with ad libitum access to water and chow.

Experimental Protocol

Burn wounds were created on the back of 36 mice. The animals were anesthetized with ketamine, and the back was shaved and cleaned with 70% alcohol. A hot flat-round bottom stainless steel (10g) was applied to the shaved area for 3 seconds to create burn wounds (Karina *et al*., 2021). Mice were divided into six groups: group I (negative control), group II (Burnazin treatment), group III (Vaseline treatment), group IV (25% *C. indicum* extract ointment), group V (50% *C. indicum* extract ointment), and group VI (75% *C. indicum* extract ointment). Treatments were administered once daily for 20 days, and burn areas were observed and measured every 2 days. Skin tissue was collected on day 11.

Percentage Value of Burn Wound Closure Measurement

Burn areas were measured using ImageJ software. The percentage of burn wound closure was calculated using the following equation:

$$Px = \frac{(L^2) - (Lx^2)}{(L^2)} \times 100\%$$

Where:

Px : Percentage value of burn wound closure on day-x

L2 : Wound area day-0

Lx2: Wound area day-x

Hydroxyproline Levels Evaluation

Skin tissue (0.05 g) was dried, hydrolyzed with 6 NHCl, homogenized, and analyzed for hydroxyproline content using a spectrophotometer at 557 nm (Afifah, 2016).

Total DNA Content Evaluation

Skin tissue was homogenized in PBS, and total DNA was measured using a Qubit DNA assay (Nugroho *et al*., 2019).

Histological Process

Skin tissue was fixed, processed, and stained with hematoxylin-eosin for histological analysis. Tissue regeneration was assessed based on re-epithelization and connective tissue presence.

Statistical Analysis

Data were analyzed using SPSS version 22.0. One-way ANOVA and Kruskal-Wallis tests were used to analyze differences in wound area, hydroxyproline levels, and total DNA content among groups, with post-hoc analysis performed as needed.

RESULTS AND DISCUSSION

Effect of *C. indicum* Flower on Burn wound area

Burn wound improvement was observed from day-0 to day-20 was observed from day-0 to day-20 which was done visually by observing the condition of the burn wound, the size of the burn area, edema and erythema. The results obtained in Figure 1. are as follows:

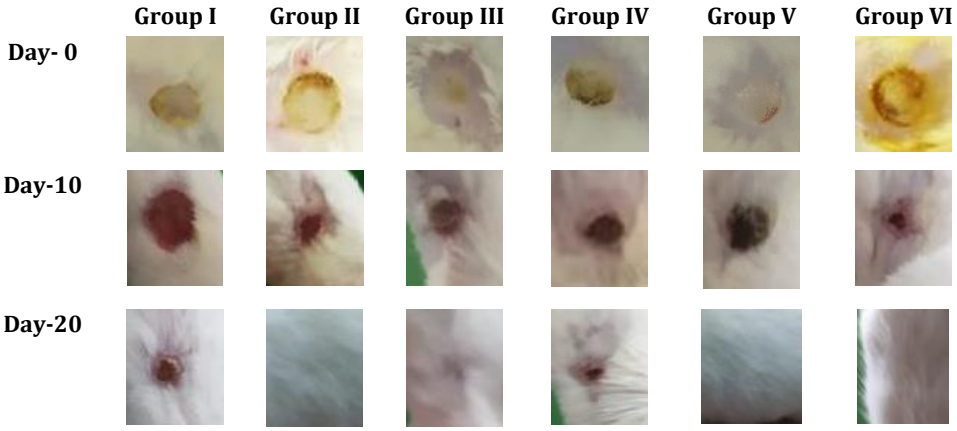

Figure 1. Burn Wound Improvement of 20 Days *C. indicum* Flower Methanol Extract Treatment to Burn Wound on Mice Skin from Day-0 to Day-20.

Burn wound improvement was observed from day 0 to day 20. Macroscopic observations showed that by day 20, burn wounds in groups II, III, V, and VI had completely closed and were covered in hair, whereas groups I and IV still had visible wounds. The treatment groups showed faster wound healing compared to the control group, indicating the efficacy of *C. indicum* extract.

The wound healing process is influenced by several factors. Slow wound healing can be caused by secondary inflammation in burns. One of the appropriate burn therapy efforts is treating wounds with active ingredients to

prevent secondary inflammation (Balqis *et al*., 2014). These factors are found in the secondary metabolites of the *C. indicum* (Kulshreshtha *et al*., 2018; Mukherjee & Chandra, 2017; Barik *et al*., 2020; Kumar *et al*., 2019; Singh *et al*., 2017).

The burn wound area on the documentations each day was measure by using ImageJ. Burn area on mice skin surface data were analyzed using SPSS Statistics 22. Based on the results of the analysis, the results obtained in Table 1. are as follows.

Table 1. Average Burn Area on the Mice Skin Surface

Day	Burn Wound Area on Mice Skin (Cm^2)					
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
0	1.062 ± 0^{a}	1.062 ± 0^{a}	1.064 ± 0^{a}	1.063 ± 0^{a}	1.063 ± 0^{a}	1.062 ± 0^{a}
2	1.045 ± 0.004^{e}	0.814 ± 0.003^{a}	0.964 ± 0.009^{c}	1.018 ± 0.004^{d}	0.914 ± 0.003^{b}	0.817 ± 0.002^{a}
4	1.018 ± 0.009^{c}	0.632 ± 0^{a}	0.800 ± 0.013^{b}	0.865 ± 0.045^{b}	0.769 ± 0.010^{b}	0.643 ± 0.006^{a}
6	0.970 ± 0.003^{d}	0.546 ± 0.028^{ab}	0.671 ± 0.017^{c}	0.856 ± 0.078^{d}	0.613 ± 0.011^{b}	0.521 ± 0.005^{a}
8	0.885 ± 0.062^{c}	0.405 ± 0.005^{a}	0.429 ± 0.031^{a}	0.613 ± 0.064^{b}	0.492 ± 0.015^{ab}	0.413 ± 0.012^{a}
10	0.826 ± 0.078^{d}	0.296 ± 0.002^{a}	0.376 ± 0.009^{b}	0.507 ± 0.018^{c}	0.364 ± 0.023^{b}	0.287 ± 0.014^{a}
12	0.726 ± 0.088^{d}	0.193 ± 0.004^{a}	0.305 ± 0.002^{b}	0.416 ± 0.027^{c}	0.256 ± 0.058^{b}	0.180 ± 0.009^{a}
14	0.685 ± 0.067^{d}	0.083 ± 0.013^{a}	0.230 ± 0.022^{b}	0.288 ± 0.044^{c}	0.143 ± 0.005^{b}	0.087 ± 0.006^{a}
16	0.464 ± 0.037^{e}	0 ± 0^{a}	0.125 ± 0.003^{c}	0.216 ± 0.005^{d}	0.060 ± 0.008^{b}	0 ± 0^{a}
18	0.317 ± 0.067^{d}	0 ± 0^{a}	0.074 ± 0.006^{c}	0.197 ± 0.003^{b}	0.005 ± 0.002^{a}	0 ± 0^{a}
20	0.314 ± 0.053^{c}	0 ± 0^{a}	0.005 ± 0.002^{a}	0.083 ± 0.007^{b}	0 ± 0^{a}	0 ± 0^{a}

All values are represented as average ± SE of 3 mice (n = 3); Values marked with the different superscript (a, b, c, d, e) in the same line shows a differ significantly with ($P<0.05$). Group I was the negative control group, group II was treated with Burnazin and group III was treated with vaseline as the positive control groups, groups IV, V, and VI were treated with 25, 50 and 75% concentrations of *C. indicum*flower methanol extract.

Based on the results of statistical analysis (Table 1.), the average burn area on the skin surface of the mice began to show a significant difference on day-2. The II and VI group did not have a significant difference from day 0 to day 20. The average value of burn area in the group I decreased relatively much more slowly than the II and VI groups. This proves that there is a significant effect of *C. indicum* methanol extract ointment treatment compared to the control group without treatment. The VI treatment group which was given *C. indicum* flower extract ointment with a concentration of 75% had the same effect as the treatment group treated with Burnazin ointment as a positive control. This statement is supported by the results of Handayani *et al*., (2015) regarding the effect of gambier on healing burns, which stated that the positive control group (treated with branded ointment) healed faster than other groups. The positive control group used a commercial ointment specifically for burn wounds. The ointment used has gone through a pre-clinical and clinical testing process and is marketed legally

because it has been tested and proven to speed up wound healing.

The average value of burn area on mice skin was substituted to the equation (1) to get the Burn Wound Closure Percentage value. The comparison was made with the positive and negative control groups to know the comparative burn wound closure percentage changes over a period of 20 days. The result obtained in Table 2. are as follows:

Table 2. Burn Wound Closure percentage

Day-	Burn Wound Closure Percentage (%)					
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**	**Group VI**
2	3	41	17	8	26	40
4	9	64	43	33	43	63
6	16	73	60	35	72	75
8	30	85	83	66	78	84
10	41	92	91	77	88	92
12	53	96	95	84	93	97
14	58	99	98	92	98	99
16	81	100	99	95	99	100
18	91	100	100	96	100	100
20	91	100	100	99	100	100

Group I was the negative control group, group II was treated with Burnazin and group III was treated with vaseline as the positive control groups, groups IV, V, and VI were treated with 25, 50 and 75% concentrations of *C. indicum*flower methanol extract.

Based on the results, the group that reached 100% burn wound closure percentage on day-16 while the other group (III and V) reached 100% at day-18. The lowest percentage are from group I that reached 91% at day-20. This proved that *C. indicum* can accelerate the healing process of burns.

Flavonoids as anti-inflammatory and antioxidants that can counteract free radicals during wound healing. Triterpenoids has astringent effect that can clean dirt. Tannins are antiseptic and antibacterial so it can reduce and accelerate inflammation. Saponins can stimulate the formation of new collagen. Steroids are active substances that can reduce pain in wounds by reducing oxidative stress on damaged tissue. The contents of the *C. indicum* greatly affect to accelerate healing burn wound.

Hydroxyproline Level Evaluation and Total DNA Content

The test results have various values. Hydroxyproline level and total DNA content were examined on day-11. The comparison was made with the positive and negative control groups to know the comparative hydroxyproline level and total DNA content in mice's skin tissue. The result obtained in Table 3. are as follows:

Table 3. Hydroxyproline Level and Total DNA Content

Group Name	Hydroxyproline Level (µg/mL)	Total DNA Content (µg/mL)
I	54.259 ± 3.638[d]	44.467 ±1.964[c]
II	3.981 ± 0.244[a]	13.433 ± 1.855[a]
III	31.759 ± 1.058[c]	30.467 ± 0.581[b]
IV	44.491 ± 6.670[d]	31.067 ± 1.047[b]
V	17.034 ± 0.467[b]	27.033 ± 0.643[b]
VI	4.120 ± 0.483[a]	15.400 ± 1.501[a]

Group I was the negative control group, group II was treated with Burnazin and group III was treated with vaseline as the positive control groups, groups IV, V, and VI were treated with 25, 50 and 75% concentrations of *C. indicum*flower methanol extract.

Hydroxyproline and total DNA content were measured on day 11. Group I showed higher hydroxyproline levels due to being in the proliferative phase, while groups II and VI showed lower levels as they had moved past this phase.

Based on the results, the highest average value of hydroxyproline and total DNA levels is from the I treatment group, while the lowest is from II and VI. The levels of hydroxyproline in the I group were higher than in the II and VI groups because on the 11th day, the I group was still in the proliferative phase while the II and VI groups had passed the proliferative phase. Hydroxyproline levels will decrease after the proliferative period as happened in the II and VI treatment groups. During the proliferative period, there is an increase of hydroxyproline level which is known to be a constituent of immature collagen. Immature collagen (gelatinous collagen) formed during the proliferation period will condense into collagen fibers that are more mature and stronger. This process is known as the remodeling process. The remodeling process plays an important role in the reconstruction of damaged dermal tissue (Nasution, 2016). Collagen synthesis reaches the highest activity in the lag phase, which is between one to two weeks after the tissue is injured, increasing the tensile strength of the wound tissue (Maulana, 2019). In the proliferative phase there is an increase in DNA synthesis. The increase in DNA synthesis that occurs is due to the increased need for protein to repair the condition of injured or damaged tissues. Protein and DNA multiplication occurs during the wound healing process. This is what reduces the length of the inflammatory phase (Ahmad *et al*., 2017). Reduced inflammation occurs due to increased levels of fibroblasts, collagen and proteoglycans so that they can regenerate damaged tissue and wound traction. Tissue regeneration is also influenced by blood vessels which play a role in delivering new cells from the bone marrow to damaged tissues.

Regeneration can be accomplished by the formation of new vessels in the injured tissue area due to mitotic activity on vascular endothelial cells. The process of mitosis that occurs can increase the total DNA content in skin tissue during the proliferative period (Balqis *et al*., 2014)

Histological Analysis

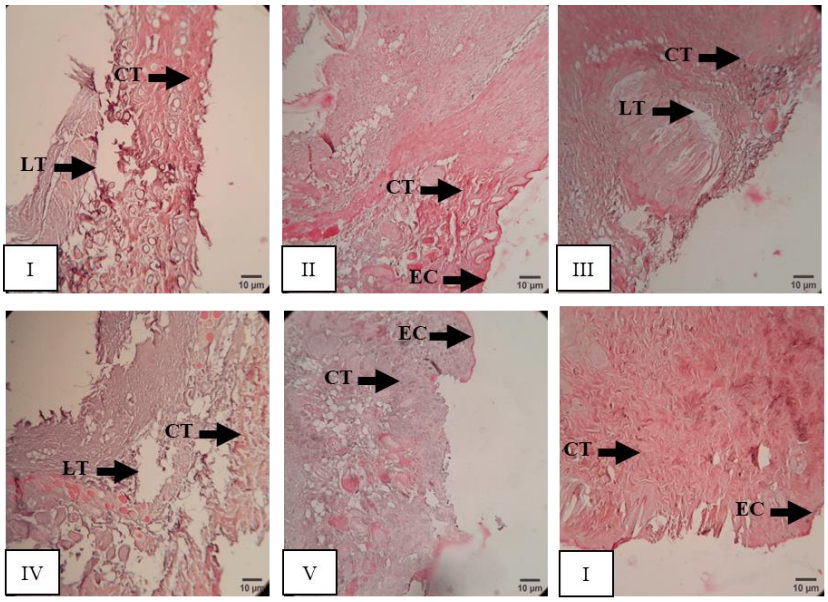

Figure 2. Skin Tissue of Burn Wound on Mice at Day-11 treated with *C. indicum* Flower Methanol Extract with Hematoxylin and Eosin stain photomicrographs (100×). EC: Epithelial Cells; CT: Connective Tissue and LT: Loss Tissue

Histological observations showed that groups II and IV had the best outcomes, with visible epithelial lining and reduced inflammatory cells, indicating effective wound healing and tissue regeneration. Epithelial lining is a sign of re-epithelialization and regeneration process. A sign of good dermal tissue regeneration is when the new tissue can have the same functionality as the previous tissue that was damaged. Also, have the high collagen density and organization, full and mature epithelium, low number of inflammatory cells and angiogenesis (Salhi *et al*., 2023). In other words, samples that were treated with Burnazin and *C. indicum* 75% ointment had the best effect for burn wound healing process. Meanwhile, mature epithelial cell that form the epithelial lining were not found in the I, IV and III. Rather, I, IV, III had tissue loss and lot of inflammation cell that indicate the groups still in inflammation phase.

The wound healing process goes through 3 phases, the inflammatory phase, the proliferative phase and the

maturation phase (Suharto & Etika, 2019). When the tissue was injured, capillary and arterial in vasoconstriction phase to stop bleeding in the injured area. In addition, vasodilation occurs so that blood flow increases towards the wound area and can cause erythema and edema (Nugraha & Patimah, 2016).

The inflammatory phase is characterized by the occurrence of constriction of blood vessels accompanied by a hemostatic reaction due to the aggregation of platelets and fibrin to stop bleeding. Platelet aggregates will synthesize Transforming Growth Factor-Beta 1 (TGF-β1) which will activate fibroblasts to synthesize collagen (Nasution, 2016). The wound healing process then enters the proliferative phase which is characterized by the formation of granulation tissue in the wound and the migration of epithelial, fibroblast and endothelial cells. Wounds in the proliferative stage are filled with fibroblasts and collagen which form a reddish tissue with an uneven surface. This newly formed tissue is known as granulation tissue.

The process of repairing skin tissue will continue until the wound is completely closed (Fitri, 2015). Maturation aims to perfect the formation of new skin tissue into stronger skin tissue. Fibroblasts begin to leave the granulation tissue, the erythema decreases as the vessels begin to regress and there is an accumulation of fibrin fibers turn into collagen to strengthen the scar tissue (Nasution, 2016).

CONCLUSION

The methanol extract of *C. indicum* flower significantly accelerates ($P<0.05$) the wound healing process in mice with burn wounds. The extract improves burn wound closure, hydroxyproline levels, total DNA content, and skin tissue regeneration. These findings support the potential therapeutic effect of *C. indicum* flower in burn wound healing, with the 75% extract ointment showing the best results.

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