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Effects of *Stelechocarpus burahol* [Blume] Leaf Ethanol Extract Ointment on Burns Healing

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ABSTRACT

Stelechocarpus burahol [Blume] Hook.f & Thomson is a plant native to Indonesia with antibacterial, antioxidant, antifungal and antiseptic properties. This study aimed to determine the action on burns healing of a 70 % ethanol extract of *Stelechocarpus burahol* leaf, which was formulated as an ointment. Testing was conducted by modelling burns on rats with four parameters, namely the number of macrophages, the density of fibroblasts, the rate of re-epithelialisation and measurement of the decrease in the burn wound surface area. A total of 30 rats of the Sprague Dawley strain were used in this study, divided into five groups: *Stelechocarpus burahol* leaf extract ointment with concentrations of 3.25%, 6.5% and 13%, vaselin flavum as a negative control and silver sulfadiazine as a positive control. Observations were made on days 3, 7 and 14 histologically. The histological observations revealed a significant decrease in the number of macrophages, increased fibroblast density and re-epithelialisation compared to the negative control group, and at a concentration of 13 %, the extract's effects were comparable to those of silver sulfadiazine. It can be concluded that *Stelechocarpus burahol* leaf ointment extract is capable of accelerating the healing of burn wounds, with the best results obtained at a concentration of 13%.

Keywords: *Stelechocarpus burahol*, Macrophages, Fibroblast, Re-epithelialisation.

Introduction

Indonesia is a country with rich biological resources. Its biodiverse forests are a national asset that provides human beings with priceless benefits. One such benefit is the use of the *Stelechocarpus burahol* plant as medicine. *Kepel* (*Stelechocarpus Burahol* [Blume] Hook.f. & Th) is a native Indonesian plant; it is the symbol of the Special Region of Yogyakarta and can be found in palaces in the region. A burn wound is a form of tissue damage or tissue loss caused by exposure to heat sources, i.e. fire, hot water, chemical substances, electricity or radiation.[1,2] The severity of the wound is determined by two factors. The first is the width of the surface area exposed, and the second is the depth of the burn, which is categorised as a first-degree burn, second-degree burn or third-degree burn.[2] A third-degree burn is a full-depth burn involving the epidermis, dermis and appendix parts of the skin. Burns typically have a very complex healing process; thus, stabilising the general condition, providing healing care and offering prevention and treatment for complications are considered costly, especially because any such complications from burns can lead to morbidity and mortality. There is a need to resolve these problems successfully, safely and reasonably cost-effectively. One solution involves the use of traditional medicine. Based on the findings of Sunarni *et al.*,[3] *Stelechocarpus burahol* leaves have antioxidant properties. Another study found that the juice of *Kepel* leaves at a concentration of 60 % showed healing activity in open wounds in rats, with 59.84 % healing.[4] The antioxidant and antibacterial activity of *Stelechocarpus burahol* leaves, as well as the role of its juice in the healing process of open wounds, suggest that 70 % ethanol extract of *Kepel* leaves could boost the healing process of burn wounds. This study therefore aimed to evaluate the potential of *Stelechocarpus burahol* leaves in burn wound healing. The study was conducted using rats that were induced with third-degree burns. The rats were observed by measuring four parameters, namely the number of macrophages, the density of fibroblasts, the speed of re-epithelialisation and the decrease in the surface area of the burn wound. Measurement was carried out using the *Image Raster 3.0* application. This parameter measurement supported the data for evaluating the potential of *Stelechocarpus burahol* leaves as a burn wound medication.

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Materials and Methods

Chemicals
The following chemicals were used in this study: $HgCl_2$ (Merck), KI (Merck), $CHCl_3$ (Merck), $FeCl_3$ (Merck), metal Mg (Merck), methanol (Merck), xylol (Merck), paracetamol (Indofarma), ketamin HCl injections (Guardian Pharmatama Indonesia), vaselin flavum (Pharma Laboratoria Bandung Indonesia) and silver sulfadiazine (SSD)/Burnazin® (Darya Varia Laboratoria Tbk). The following equipment was used: a microscope (Leica, Germany), a rotary vacuum evaporator (Eyela), a microtome (Thermo, USA), an analytical scale (Ohaus) and an oven (Memmert). All chemicals used in this study were of analytical grade.

Plant Materials
The plant material used was the leaf part of the *Stelechocarpus burahol* plant, which was obtained from the Indonesian Research Institute for Spices and Medicines (BALITTRO). The *Stelechocarpus burahol* leaves were deposited in the 'Herbarium Bogoriense', Botanical Field, Biology Research Centre, Indonesian Institute of Sciences (LIPI), with register no. 1592 / IPH.1.01 / If.07 / VI / 2017.

Preparation of the Extract
The *Stelechocarpus burahol* leaves (7 kg) were washed with running water and dried in the sun. The sample was ground and sieved with a

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40-mesh sieve. Then, 1.2 kg of the sample was macerated with 8 L of 70 % ethanol. The maceration process was repeated twice for residue for the same duration (48 hours). The macerated was filtered and concentrated using a rotary evaporator and the obtained extracts were concentrated. This extract was labelled *Kepel* leaves' ethanol extract (KLEE).

Preparation of the Test Animals
Thirty Sprague Dawley male rats weighing 150–200 g was used. Test animals were acclimatised for a week, and fed, along with water every day. The Health Research Ethics Commission of the University of Muhammadiyah Prof. Dr. Hamka approved the research procedure, with ethical approval letter number 02/17.10/017.

Determination of the Extract's Characteristics
The organoleptic observations of the KLEE included its shape, colour, odour and taste. Loss on drying was determined using gravimetric analysis, where 2 g of extract was weighed and dried at 105°C in an oven for 30 minutes until it attained a constant weight.[5] Preliminary phytochemical screening was then conducted on the KLEE by testing several secondary metabolites. Following this, the extract was tested for its alkaloid content using the three reagents of Dragendorff's, Mayer and Bouchardat, a flavonoid test (Shinoda and ammonia test), a tannin test (a test with gelatine and $FeCl_3$), Glycoside test (Fehling A and B), saponin test (a foam test), steroids and terpenoid test (Liebermann Burchard test).[6,31]

Preparation of the Stelechocarpus burahol Leaves' Ethanol Extract Ointment
KLEE ointment was created with concentrations of 3.25 %, 6.5 % and 13 % (w/w) by weighing 0.325 g, 0.65 g and 1.3 g of KLEE, then adding vaselin flavum until the ointment reached 10 g and crushing all ingredients until they were homogeneous.

Generating Third-Degree Burns and Treating the Test Animals
The rats were anaesthetised using ketamine HCl injection at a dose of 40.08 mg/kg BW intramuscularly. A special metal plate measuring 1.5 cm × 1.5 cm in diameter was then heated until it reached 100°C and placed for 5 seconds on the back part of the rat, which had already been shaved. After the wound had been generated, the rat was given analgetic medication orally (paracetamol tablet 51.37 mg/kg BW, a single dose). There were five test groups, namely the three KLEE groups with KLEE concentrations of 3.25 %, 6.5 % and 13%, a positive (silver sulfadiazine) control group and a negative control group. Silver sulfadiazine was used as the positive control because it is the drug of choice in the topical treatment of burns and vaselin flavum as the negative control. All group spread evenly over the wound surface twice daily (morning and afternoon) for each treatment for 14 days.[7] Wound tissue was taken on days three, seven and 14 after burns were induced for histological observations.

Histology Sample Preparations
Skin tissue samples were taken from the biopsy of the burn wound and the subcutaneous fat tissue. The samples were taken on the third, seventh and 14th days after giving the test sample. Before the samples were taken, the test animals were anaesthetised using a ketamine injection. The specimens were then fixated using a Buffer Neutral Formalin 10 % solution.

Histopathology Sample Preparations
The tissue was fixated using a Buffer Neutral Formalin (BNF) 10 % solution and left at room temperature for 24 hours. The tissue was then cut into pieces and placed in a specimen container made from plastic. Next, it was subjected to a dehydration process using a graded alcohol concentration of 70 %, 80 % and 90 % for 2 hours each. Later, the clearing process was conducted using xylol to eliminate alcohol traces. After this, the moulding process was conducted using paraffin blocks, and the moulds were stored in the fridge. These paraffin blocks were then sliced thinly, around 6–8 µm, using a microtome (Thermo, USA). Afterwards, the pieces were floated on 60 °C warm water (a water bath) to stretch the tissue and prevent creasing. The specimens were then lifted and placed on object glass to perform the haematoxylin and eosin (HE) staining and were later observed under a microscope (Leica, Germany).[8]

Data analysis
The data were analysed statistically using one-way Analysis of Variance (ANOVA), followed by the Tukey–Kramer Post Hoc test for multiple comparisons. $p < 0.05$ was considered to represent statistical significance.

Results and Discussion

Stelechocarpus burahol ethanol leaf extract was obtained using the maceration method, with 70% ethanol as the solvent. In terms of characteristics, the 70% KLEE ointments were semi-solid and they had a unique smell, bitter taste and blackish-green colour. The phytochemical screening results showed that the extract contained flavonoids, saponin, glycoside, tannin, steroids/terpenoid but the alkaloids was not detected. This extract yielded 11.25%, and the loss on drying was 8.92%. Organoleptic and homogeneity observations of the 70 % KLEE ointment showed homogenous consistency, with the colour of the ointment darkening as the extract's concentration increased.
The parameters observed from the histology samples were the number of macrophages, the density of fibroblasts and the thickness of re-epithelialisation, which were obtained by observing 10 field views. The width of the burn wound was measured by processing the image using the *Macbiophotonic Image J* program. The burn wound model was made by inducing third-degree burns on the rats, which damaged the tissue through to the dermis. The prepared samples used were 70% KLEE ointments at concentrations of 3.25%, 6.5 % and 13%, silver sulfadiazine for the positive control group and vaselin flavum for the negative control group. Silver sulfadiazine was chosen for the positive control group because it is the drug of choice for topical burn wound treatment.[9] Silver sulfadiazine inhibits bacterial DNA replication and damages the bacterial cell wall and also has antibacterial functions that help cleanse the wound, thus preventing tissue regeneration from being compromised.[10,11,12] The sample preparation in the form of ointment with a vaselin flavum base had hydrocarbon characteristics. These rendered it difficult to dissolve in water and thus prolonged contact between the medical ingredients and the skin.[13] A burn wound is a form of tissue damage or loss caused by exposure to heat sources, i.e. fire, hot water, chemical substances, electricity or radiation.[1] Generally, the healing process is divided into three phases.[14] The early phase of inflammation begins immediately after the injury is incurred and involves the elimination of dead tissue to prevent infection. The second phase is the proliferation phase during which a balance occurs between scar tissue formation and tissue regeneration. The third phase is the maturation phase, which is aimed at maximising the structural strength and integrity of the wound.[14] While the healing process of a burn wound has similarities with other wound healing processes, the duration of each phase differs.[15] The macrophage cell calculation process was conducted by taking an image with a light microscope and then observing it and counting the macrophages using the *Image Raster 3.0* application. The number of macrophages found on the third-, seventh- and 14th-day observations showed significant differences in every group ($p < 0.05$).

Table 1: Effect of *Stelechocarpus Burahol* ethanol extract ointment on Macrophages Density

Groups	Third day (cells ± SD)	Seventh day (cells ± SD)	14th day (cells ± SD)
Positive control	139.3 ± 8.944	104.725 ± 5.998	94.975 ± 6.602
Negative control	109.875 ± 7.221	119.95 ± 7.083	112.3 ± 4.231
3.25 % KLEE oint.	116.421 ± 5.549^{b}	114.900 ± 5.780^{b}	106.37 ± 3.398^{b}
6.5 % KLEE oint.	124.175 ± 7.322^{b}	109,925 ± 4.332^{b}	99.875 ± 4.678^{b}
13 % KLEE oint.	134.3 ± 6.710^{a,b}	107.025 ± 4.0343^{a,b}	97.025 ± 3.927^{a,b}

Note: ^{a} not significantly different from the positive controls ($p > 0.05$)
^{b} significantly different from the negative controls ($p < 0.05$)

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Table 2: Effect of *Stelechocarpus Burahol* ethanol extract ointment on fibroblast density

Groups	Third day (cells ± SD)	Seventh day (cells ± SD)	14th day (cells ± SD)
Positive control	58.65 ± 4.577[a]	73.15 ± 4.577[a]	34.87 ± 3.4674[a]
Negative control	29.5 ± 2.8191	49.55 ± 3.1030	50.9 ± 4.9139
3.25 % KLEE oint.	45.95 ± 3.2602[a]	63.2 ± 4.1688[a]	43 ± 3.9603[a]
6.5 % KLEE oint.	49.75 ± 4.2534[a]	67.35 ± 4.3922[a]	38.95 ± 3.5388[a]
13 % KLEE oint.	51.2 ± 3.6685[a]	70.18 ± 4.1372[a,b]	37.02 ± 3.0842[a,b]

Note: [a] significantly different from the negative controls ($p < 0.05$)
[b] not significantly different from the positive controls ($p > 0.05$)

Table 3: Effect of *Stelechocarpus Burahol* ethanol extract ointment re-epithelialisation thickness

Groups	Third day (cells ± SD)	Seventh day (cells ± SD)	14th day (cells ± SD)
Positive control	15.86 ± 0.63	21.67 ± 0.67	30.67 ± 0.90
Negative control	7.72 ± 0.55	10.84 ± 0.60	25.06 ± 0.94
3.25 % KLEE oint.	10.78 ± 0.58[b]	15.79 ± 0.69[b]	26.10 ± 0.74[b]
6.5 % KLEE oint.	12.72 ± 0.59[b]	18.22 ± 0.65[b]	28.95 ± 0.73[b]
13 % KLEE oint	13.65 ± 0.77[b]	21.66 ± 0.73[a]	55 ± 0.90[a,b]

Note: [a] = not significantly different from the positive controls ($p > 0.05$) [b] = significantly different from the negative controls ($p < 0.05$)

On the third day of observation, the number of macrophage cells in the 13 % concentration group was higher than that in the 6.5 %, 3.25 % and negative control groups (Table 1). This is because the macrophages became the predominant cell type on the third day after the wound occurred. A macrophage is an effective cell in the phagocytosis process, as it phagocytoses pathogens, foreign bodies and other unnecessary cells.[16] Macrophages in the tissue originate from monocyte cells in the blood that migrate to connective tissue. In the case of inflammation, the number of monocytes that migrate to the connective tissue will increase; thus, the macrophages are activated.[17,18]
On the seventh and 14th days of observation, the number of macrophages in the 13 % concentration group was comparable to that of the positive control group and the 6.5 % concentration group, yet it differed significantly from that of the negative control group and the 3.25 % concentration group (Table 1). This result shows that the inflammation process in the negative control group was still ongoing.
The high number of macrophages in the negative control group indicated prolonged inflammation due to the growth of more microorganisms in the burn wound. The absence of active ingredients in the negative control group may have accounted for the presence of microbes and the amount of tissue damage that the macrophages were required to phagocytose in the wound area.[19,20] Thus, the wound healing process in the negative control group was prolonged and led to the proliferation phase being delayed. In the 13 % concentration group, as well as in the other concentration groups, the number of macrophages was lower, indicating the end of the inflammation phase and the beginning of the proliferation phase. During the proliferation phase, macrophages are needed to produce growth factors such as the fibroblastic growth factor and the transforming growth factor-Beta (TGF-β). Macrophages also activate fibroblasts and increase their migration, which plays a role in the tissue formation process and produces collagens.[21,22] Administration of the KLEE ointment can hasten the inflammation phase of burn wounds. This effect is related to the presence of secondary metabolite compounds in the *Stelechocarpus burahol* ethanol leaf extract, such as flavonoids, saponin and tannin, that aid the healing process by functioning as antioxidant and antimicrobial agents that affect wound healing.[23,24] Tannin and saponin also have antiseptic properties. For example, saponin can trigger the vascular endothelial growth factor (VEGF) and increase the number of macrophages that migrate toward the wound area, thus increasing the production of cytokines, which activate fibroblasts in the wound tissue.[25] Glycosides are known to accelerate the wound healing process and our study revealed the presence of such phytochemical groups in the extract.[26] The third-day observation showed that the mean densities of fibroblasts in all of the concentration groups were significantly different from those of the negative control group. Figure 1 shows that the fibroblast densities in all of the concentration groups remained low as the fibroblasts were yet to play a role in the inflammation phase. Fibroblasts begin to play their part only in the proliferation and maturation phases.[27] The seventh-day observation showed that the mean density in the 13% concentration group was not significantly different from that of the positive control and 6.5 % concentration groups. A significant difference was only found between the negative control and 3.25% concentration groups (Table 2). This is because the increased number of fibroblast cells triggers an increase in the number of collagen fibres, which accelerates the wound healing process. The 14th-day observation showed that the mean density of fibroblasts in the 13% concentration group was not significantly different from that of the positive control and 6.5% concentration groups. This shows that the proliferation of fibroblasts determines the result of wound healing. Fibroblasts produce an extracellular matrix, which is replaced by collagen. Fibroblasts disappear immediately as the collagen matrix fills the wound cavity.[14]

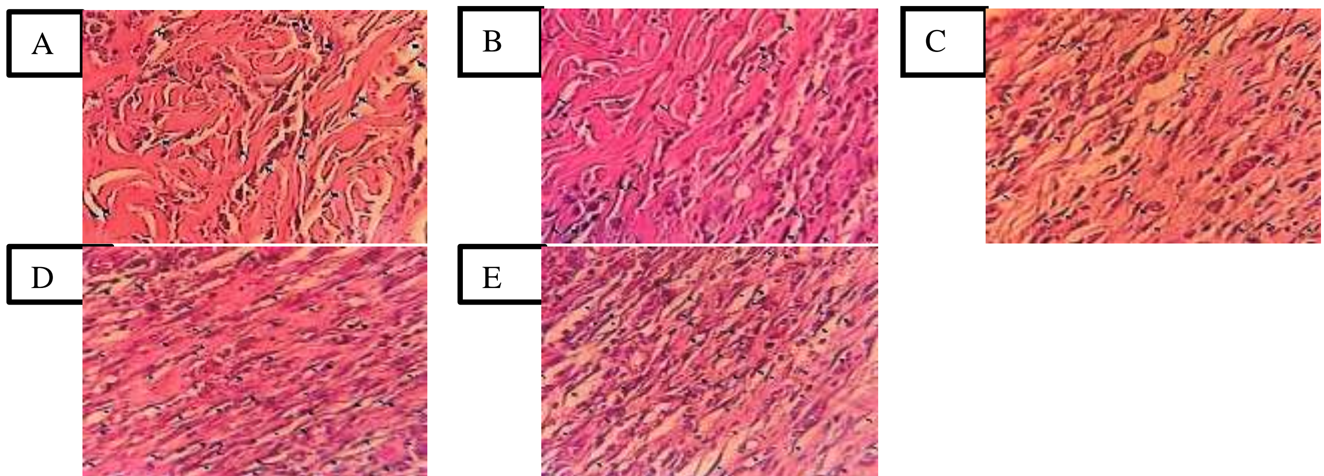

Figure 1: The histological picture on day 7 at 400x magnification under a light microscope (*Olympus*). Arrows indicate fibroblasts: A) negative control group; (B) 3.25 % KLEE ointment; (C) 6.5 % KLEE ointment; (D) 13 % KLEE ointment; E) positive control group. KLEE: *Kepel* leaves' ethanol extract.

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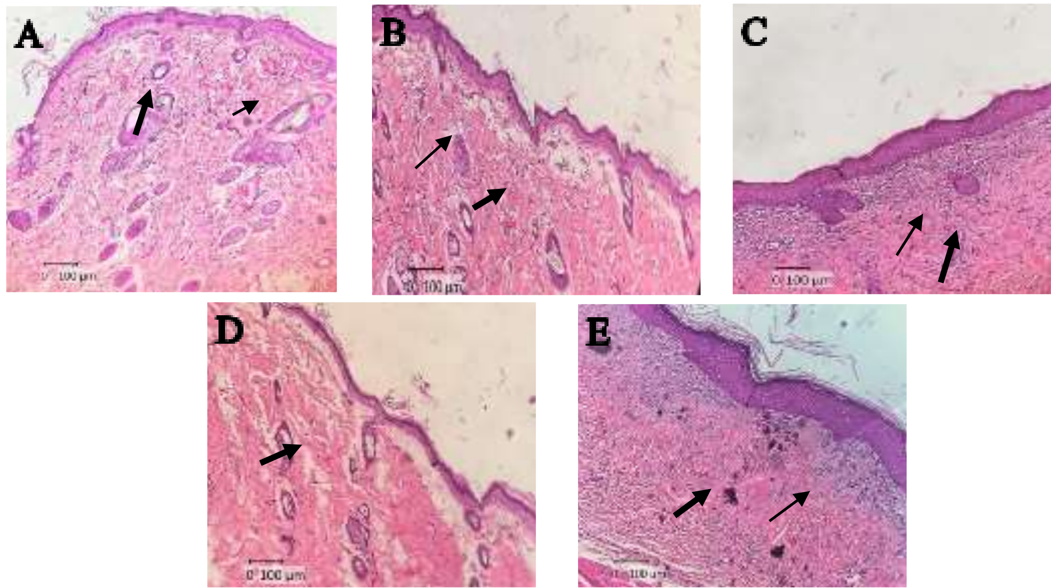

Figure 2: The histological picture on day 14 at 100x magnification, observed under a light microscope (*Olympus*). The arrows show re-epithelialisation: (A) 3.25 % KLEE ointment; (B) 6.5 % KLEE ointment; (C) 13 % KLEE ointment; (D) negative control group; (E) positive control group. KLEE: *Kepel* leaves' ethanol extract.

Table 4: Effect of *Stelechocarpus Burahol* ethanol extract on percentages of burn healing

Day	Negative control	Positive control	3.25 % KLEE oint.	6.5 % KLEE oint.	13 % KLEE oint.
1	1.22 ± 0.45	1.23 ± 0.07	1.55 ± 0.31	1.60 ± 0.33	1.83 ± 0.35
2	1.35 ± 0.55	3.43 ± 0.74*	2.32 ± 0.69	3.57 ± 1.02	3.18 ± 0.55*
3	1.84 ± 1.00	7.08 ± 2.16*	3.62 ± 1.46	50 ± 1.60	6.52 ± 2.93*
4	3.55 ± 2.23	10.87 ± 2.24*	7.40 ± 4.07	8.50 ± 1.77	10.11 ± 3.12*
5	6.44 ± 3.20	16.72 ± 2.80*	11.16 ± 6.24	12.44 ± 3.86	15.50 ± 5.93*
6	8.84 ± 4.04	22.98 ± 3.04*	13.52 ± 6.41	15.89 ± 3.58	20.33 ± 6.02*
7	11.02 ± 3.30	26.92 ± 2.42*	17.50 ± 5.50	21.09 ± 5.81*	27.13 ± 3.54*
8	13.06 ± 3.40	35.84 ± 8.54*	21.92 ± 7.91	24.71 ± 4.92*	31.03 ± 3.10*
9	15.81 ± 2.65	46.77 ± 10.71*	26.27 ± 7.36	29.75 ± 5.80*	37.93 ± 3.71*
10	18.25 ± 2.54	57.46 ± 7.06*	32.54 ± 7.94*	40.33 ± 3.58*	48.94 ± 6.57*
11	20.04 ± 2.20	67.41 ± 6.85*	39.72 ± 4.09*	46.64 ± 6.48*	57.04 ± 3.3*
12	22.67 ± 1.85	84.00 ± 5.70*	44.25 ± 2.74*	51.70 ± 5.47*	76.55 ± 6.56*
13	25.09 ± 1.51	92.25 ± 3.00*	48 ± 54 ± 1.44*	56.30 ± 4.12*	84.34 ± 4.67*
14	26.72 ± 1.77	95.31 ± 2.72*	51.64 ± 2.49*	61.70 ± 4.34*	92.32 ± 2.58*

Note: * = significantly different from the negative controls ($p < 0.05$)

Re-epithelialisation thickness was another of the wound-healing parameters that did not show any significant results during the third day of observation. On the seventh and 14th days of observation, however, the 13% concentration group had a value equivalent to that of the positive control group: The mean value of re-epithelialisation thickness was 13.65μm ± 0.77 (Table 3). It could thus be interpreted that wound proliferation started on the fourth day and continued until the 14th day, when epithelial cell proliferation closed the wound affected by the mitosis activity of the epithelial cells around the wound's edges. Subsequently, the mature epithelial cells moved from the edges of the wound to the dermis, migrating to and attaching to the centre part of the wound. Figure 2 demonstrates that the positive control group and 13% *Stelechocarpus burahol* ethanol leaf extract ointment concentration group had thicker epithelial formation compared to the other test groups. During the proliferation phase, the thickness of the epithelial layer continues to increase until the wound area closes completely. The epithelium layered in the epidermis is composed of multiple layers of cells called keratinocytes. These cells are constantly renewed through the mitosis of cells in the basal layer, which are gradually shifted to the epithelial surface.[27] Observation of the wound surface area was carried out using the *Macbiophotonic Image J* program. Based on the microscopic observation from the first to the 14th day, there was a decrease in the wound surface area. On the first day, the wound appeared pale white and was still wet. On the third day, the wounds in all test groups appeared large and swollen, which indicated that the inflammation process was ongoing. This reflects the role of the inflammation phase in preventing the entry of bacteria, eliminating dirt particles from the wound tissue and preparing the wound for the advanced healing process.[14] On the seventh day, the wound appeared reddish-brown in the positive control group, while in the 6.5 % and 13 % concentration groups, the wounds showed the formation of scabs and shrinkage. On the 14th day, the wound had dried out, and the scabs

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began to fall off. The shedding of scabs indicates the growth of new cells, which help to speed up the process and attach the edges of the wound.[28,29,30] On the 14th day of observation, the wound constriction percentage in the 13 % concentration group was 92.32 %; in the positive control group, it was 95.31 % (Table 4). This proves that the 13 % KLEE ointment had the fastest rate of healing burn wounds, with a percentage that was proportional to that of the positive control group (Silver sulfadiazine).

Conclusion

The 70% *Stelechocarpus burahol* ethanol leaf extract KLEE ointment with 13 % concentration showed burn wound healing acceleration activity, a decreased number of macrophages, a decrease in the wound surface area and an increase in fibroblast density and re-epithelialisation thickness.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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