Vera Ladeska-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. The number of macrophages and fibroblast density were measured by observing the cells in 10 fields of view under a 400x microscope magnification. The thickness of the re-epithelialization was measured using the Image Raster 3.0 application. The measurement of the burn area used the Macbiophotonic Image J program. 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 %.

INTRODUCTION

Indonesia is a country with rich biological resources. Indonesia's forest biodiversity is a national asset which have priceless benefits to human beings. One of those benefits is its use as medicine, i.e. kepel plant. Kepel (*Stelechocarpus burahol* [Blume] Hook.f. & Th) is a native Indonesian plant which is the symbol of Special Region of Yogyakarta and can be found in the palaces in the region. 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, and radiation.[1,2] The severity of the wound is determined by two factors, first is the width of the surface area exposed, and second is the depth of the burn, which categorized into first-degree burn, second-degree burn, and third-degree burn.[2] Third-degree burn is a full depth burn involving the epidermis, dermis, and appendix part of the skin. The healing process of burn is very complex, thus stabilizing the general condition, the healing care, and also preventing and treating the complications is considered costly, especially since the complications could lead to morbidity and mortality. There's a need to resolve the problems effectively, safely, and reasonably affordable. One of the alternatives is by using traditional medicine.

Based on a research by Sunarni et al.,[3] kepel leaves have antioxidant ability. Another research also stated that kepel leaves's juice with 60% concentration showed healing process activity in open wound on rats with 59.84% of healing percentage.[4] Based on the kepel leaves' antioxidant and antibacterial activity, and also including the kepel juice's role in the healing process of open wound, there's a possibility that the 70% kepel leaves' ethanol extract could have the ability to boost the healing process of burn wound. This research aim is to proof the efficacy of kepel leaves in healing burn wounds.

This research is carried out on rats which have been induced with third-degree burn and will be observed by measuring four parameters, the number of macrophages, fibroblast density, reepithelialization speed, and the decrease of burn wound surface area. The measurement will be done by using Image Raster 3.0 application. This parameters measurement will support the data that will prove the efficacy of kepel leaves on burn wound medication. It is hoped that the

result of this study could increase the knowledge on the efficacy of native Indonesian plants and useful in the burn wound healing process.

MATERIAL 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

Materials used in this research are kepel leaves obtained from The Research Center for Spices and Medicinal Plants (BALITTRO), Vaseline, Silver Sulfadiazine (Burnazin®), Ketamine injection. All chemical used in this study were analytical grade.

Preparation of Extract

The kepel leaves could be determine in "Herbarium Bogoriense", Botanical field, Biology Research Center, Indonesian Institute of Sciences (LIPI), Cibinong with register No. 1592/IPH.1.01/If.07/VI/2017 . Kepel leaves (7 kg) was washed with running water and dried under the sun. The sample was grinded and sieved with a 40 mesh sieve. Then extracted (1.2 kg) with 8 L ethanol 70% by maceration. The maceration process was repeated twice for residue at same duration (48 hours). The macerat was evaporated in vacuum rotary evaporator and continued with 40°C waterbath until it attains the form of thick extract. This extract labelled as KLEE (Kepel Leaves' Ethanol Extract)

Preparation of Test Animals

Rats that was used as test animal was acclimatized and given food and drink daily. There were 30 Sprague Dawley male white rats (*Rattus norvegicus*) weighed 150-200 g. The research procedure has been approved by the Health Research Ethics Commission University of Muhammadiyah Prof.DR.Hamka with ethical approval letter number : 02/17.10/017

Determination of Extract Characteristics

Organoleptic observations of KLEE include of shape, color, odor, and taste. Determination of Loss on Drying is done by using gravimetric, where 2g of thick extract is weighed in a calibrated and dried it at 105 °C in an oven for 30 minutes until constant weight.[5] Preliminary phytochemical screening was carried out for KLEE by testing several secondary metabolites. This extract were being tested for its alkaloids content using Dragendorff, Mayer and Bouchardat reagents, flavonoid test (Shinoda and ammonia test), tannin test (test with gelatin and FeCl3), saponin test (foam test) and steroid and terpenoid test (Liebermann Burchard test).[6,31]

Preparation of *Stelechocarpus burahol* Leaves' Ethanol Extract Ointment

KLEE ointment with a concentration of 3.25% ; 6.5% ; 13% (w/w) is made by weighing 0.325; 0.65; 1.3 g of KLEE then add vaselin flavum until 10 g and crushed until homogeneous.

Generating Third-Degree Burn and Treating of Test Animals

The rats are anesthetized by using ketamine injection at a dose of 40.08 mg/kgBB intramuscular. A special metal plate with 1.5 cm x 1.5 cm in diameter is heated until it reached 100°C, which then would be paste for 30 seconds on the back part of the rat that was shaved previously. After the wound was generated, the rat is given analgetic medication orally. There are 6 test groups namely KLEE with concentration of 3.25%, 6.5%, and 13%, Burnazin® (positive control), and vaselin flavum (negative control) is then spread evenly on the wound surface twice daily (morning and afternoon) for each treatment for 14 days.[7] Dosing and treatment of test animals can be seen in table 1.

Histology Sample Preparations

Skin tissue samples are taken from the biopsy of the burn wound and the subcutaneous fat tissue. The sample is taken on the third, seventh, and 14th day after giving the test sample. Before samples are taken, the testing animal is anesthetized by using ketamine injection. The specimen is then fixated by using Buffer Neutral Formalin 10% solution.

Histopathology Sample Preparations

The tissue is fixated by using Buffer Neutral Formalin (BNF) 10% solution and left at room temperature for 24 hours. The tissue is then cut to pieces and placed in a specimen container made from plastic. Subsequently, it will go through dehydration process done with a graded alcohol concentration, which is 70%, 80%, 90%, for 2 hours each. Later, the clearing process is done by using xylol to eliminate alcohol traces. After that, the molding process is done by using paraffin blocks and stored in the fridge. These paraffin blocks are then sliced thinly around 6-8 μm by using microtom. Afterwards, these pieces are floated on 60°C warm water (waterbath) to stretch the tissue and avoid creasing. These specimens are then lifted and placed on object glass to do the Hematoxyllin and Eosin (HE) staining and later observed under the microscope.[8]

Data Analysis

Obtained data in form of numbers of macrophages, numbers of fibroblast, and collagen density is statistically analyzed by using the one-way ANOVA test with 95% confidence (α = 0,05). Tukey test is then used to observe whether there is significant difference.

RESULTS (AND DISCUSSION)

Kepel leaves' ethanol extract (KLEE) is obtained through maceration method by using ethanol 70% as the solvent. The maserat is then evaporated by using vacuum rotary evaporator at 50°C until the thick extract is obtained. This 70% kepel leaves' ethanol extract characteristics are semi solid, has a unique smell, bitter taste, and blackish green color. The phytochemical screening results showed that this extract contain flavonoid, saponin, and tannin. This extract yields 11.25% and the loss on drying is 8.92%. Organoleptic and homogeneity observations of the 70% kepel leaves' ethanol extract ointment showed homogenous consistency with the color of the ointment darkens as the extract concentration increases.

The parameters observed from the histology samples are the numbers of macrophages, fibroblast density, and reepithelialization thickness by observing 10 field views. The width of

the burn wound is measured by processing the image using the *Macbiophotonic Image J* program.

The burn wound model on rats as test animal is made by inducing third-degree burn which damage the tissue until the dermis. The prepared samples used are 70% kepel leaves' ethanol extract at the concentration of 3.25%, 6.5%, 13%; Burnazin® as the positive control group and vaseline flavum as the negative control group. The reason Burnazin® was chosen as the positive control group is because it is the gold standard for burn wound topical treatment due to the Silver Sulfadiazine (SSD) as its active agent. SSD inhibits bacterial DNA replication and damages bacterial cell wall. The silver content in SSD also has antibacterial functions that help cleanse the wound thus avoids compromising the tissue regeneration. [10,11,12] The sample preparation in the form of ointment with vaseline flavum base has a hydrocarbon characeristics which is not easily disolved in water, thus prolonged the contact between the medical ingredients and skin.[13]

Burn wound is a form of tissue damage or loss caused by exposure to heat sources, i.e. fire, hot water, chemical substances, electricity, and radiation.[1] Generally, the healing process is divided into 3 phases.[14] The early phase or the inflammation phase is started immediately after the injury happened where it eliminates dead tissues and avoid infection. The second phase is the proliferation phase where the balance between the scar tissue formation and tissue regeneration occur. The third phase is the maturation phase that aimed at maximizing the structural strength and integrity of the wound.[14] The healing process of burn wound has similarities with other wound healing process in general, yet had a different duration for each phase.[15]

Macrophage cells calculation process is done by taking the image from a light microscope which then observed and counted by using Image Raster 3.0 application. The result of the ANOVA table on the number of macrophage on the third, seventh, and 14th day showed significant difference on every group ($p<0.05$). On the third day of observation, the number of macrophage cells on the 13% concentration group is higher than the 6.5%, 3.25%, and negative control group, with the lattest has the lowest number (Table 2). This is due to the macrophages became the predominant cell at the third day after the wound occured. Macrophage is an effective cell for the phagocytosis process where the macrophage phagocytoses pathogens, foreign bodies, and other unnecessary cells. Macrophages in the tissue originates from the monocyte cells in the blood that migrated to the connective tissue. In cases that inflammation happened, the number of monocytes that migrated to the connective tissue will increase, thus the macrophages is activated.[17,18]

On the seventh and 14th day of observation, the number of macrophages in the 13% concentration group is equivalent with the positive control group and the 6.5% concentration group, yet showed significant difference with the negative control group and the 3.25% concentration group (Table 2). This result shows that inflammation process in the negative control group is still in progress. The high number of macrophages in the negative control group indicates a prolonged inflammation due to the growth of more microorganism in the burn wound. The absence of active ingredients in the negative control group might be the reason of the presence of microbes and the number of tissue damage that needs to be phagocytosed by the macrophage in the wound area.[19,20] Thus, the wound healing process in the negative control group will be prolonged and lead to the delay of proliferation phase. In the 13% concentration group and also other consentration group, the number of macrophages is lower which indicates the end of inflammation process and mark the beginning of the proliferation process.

During the proliferation phase, macrophage is also needed to produce growth factors such as fibroplatic growth factor, transforming growth factor-beta (TGF-β), and DGF in which they stimulate the migration of fibroblast towards the wound area. Macrophages also activated

fibroblasts and increase their migration which play a role in tissue formation process and produce collagens.[21,22]

Administration of KLEE ointment could speed the inflammation phase on burn wound. It is supposed to be related to the presence of secondary metabolite compounds in the kepel leaves' extract that helps the healing process such as flavonoids, saponin, dan tannin which functions as antioxidant and antimicrobial agent that affects the wound healing.[23,24] Tannin and saponin also have the ability as an antiseptic agent. Saponin could trigger the vascular endothelial growth factor (VEGF) and increase the number of macrophages that migrate towards the wound area thus increase the production of cytokine that activates fibroblast in the wound tissue.[25,26]

Another wound healing parameter which is fibroblast density could be seen in Table 3. The third day observation showed the mean density of the fibroblast in all of the concentration groups have significant difference with the negative control group. Fig.1 showed that fibroblast density in all of the concentration groups still have a small number due to the fibroblast is yet to have a role in the inflammation process.[27] Fibroblast start to have a part in both proliferation and maturation phase.[27] The seventh day of observation showed the mean density in the 13% concentration group does not have significant difference with the positive control and 6.5% concentration groups. Significant difference is only found between the negative control and 3.25% concentration groups. The increase number of fibroblast cell would trigger the increase number of collagen fibers which speed the process of wound healing. The 14th day observation showed the mean density of fibroblast in the 13% concentration group does not show significant difference with the positive control and 6.5% concentration groups. This shows that the proliferation of fibroblast determines the end result of wound healing. Fibroblast produces extracellular matrix which then will be replaced by collagen. Fibroblast will disappear immediately as the collagen matrix fill the wound cavity and the formation of neovascular will decrease through the apoptosis process.[14]

Reepithelialization thickness as another wound healing parameter does not show any significant results during the third day of observation. On the seventh and 14th day of observation, the 13% concentration group has an equivalent value with the positive control group, where the mean value of the reepithelialization thickness is 13.65 μm ± 0.77 (Table.4). It could be interpreted that wound proliferation started on the fourth day until the 14th day, where the epithelial cell proliferation closed the wound that was affected by the epithelial cells' mytosis activity in the wound edges, subsequently the mature epithelial cells will move from the wound edges to the dermis, thus epithelial cells migrated and attached together at the center part of the wound.

Figure 2 it can be seen that the positive control group and KLEE ointment concentration of 13% showed thicker epithelial formation compared to all test groups. In the proliferation phase, the thickness of the epithelial layer continues to increase until the wound area closes completely. Epithelium layered in the epidermis which is composed of many 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]

The observation of the wound surface area is done by using *Macbiophotonic Image J* program. Based on the microscopic observation from the first day until the 14th day, it is showed that there's a decrease in wound surface area. On the first day, the wound appears pale white and is still wet. On the third day, the wound in all test groups appears large and swollen, which indicates that the inflammation process is still in progress. The inflammation process role is to prevent the entry of bacteria, eliminate dirts from the wound tissue and prepare the advanced healing process.[14] On the seventh day, the wound appears reddish brown on the positive control group while on the 6.5% and 13% concentration group showed the formation of scab and the wound began to shrink in size. On the 14th day, the wound has dried out and the scabs started

to come off.. The removal of scab indicates the growth of new cells thus speeds the process and help attaching the wound edges.[28,29,30] Wound constriction percentage in the 13% concentration group is 92.32% and in positive control group is 95.31% on the 14th day of observation. This proves that the 13% kepel leaves' ethanol extract oinment is the fastest in healing burn wound with a percentage that is proportional to the positive control group (Silver sulfadiazine®).

CONCLUSION

Based on this research, it could be concluded that the 70% kepel leaves' ethanol extract with 13% concentration shows burn wound healing acceleration activity with decreased number of macrophages and wound surface area and also the increase of fibroblast density and reepithelialization thickness.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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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 ($p < 0.05$)

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 ($p < 0.05$)

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 ($p < 0.05$)

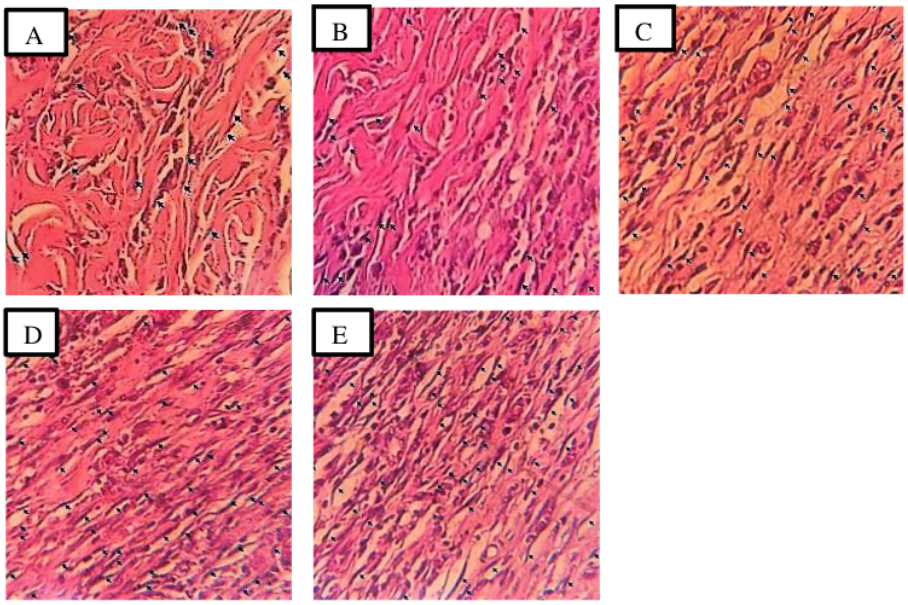

Figure 1. The histological picture on day 7 at 400x magnification under a light microscope (*Olympus*). Arrows indicate fibroblasts: A) negative control group; (B) 3.25 % KLEE ointment; (C) 6.5 % KLEE ointment; (D) 13 % KLEE ointment; E) positive control group. KLEE: *Kepel* leaves' ethanol extract.

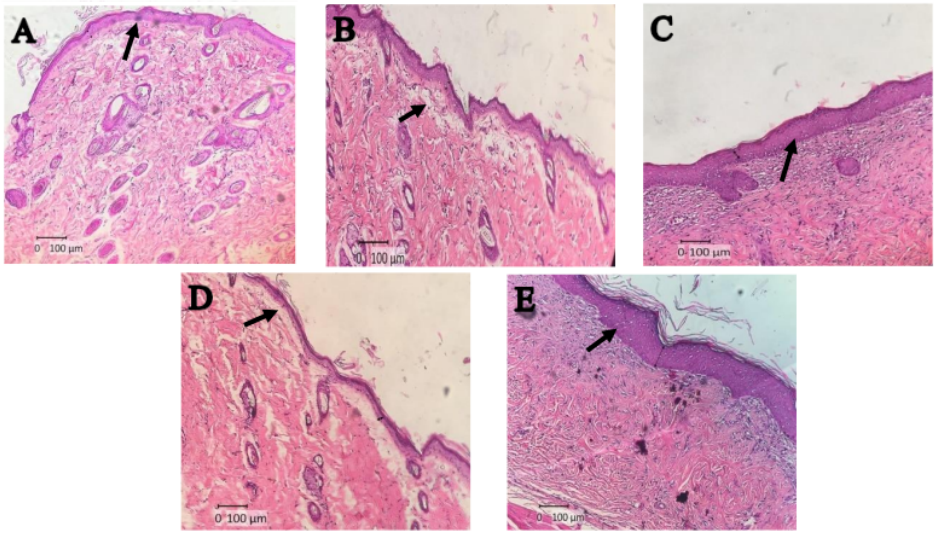

Figure 2. The histological picture on day 14 at 100x magnification, observed under a light microscope (*Olympus*). The arrows show re-epithelialisation: (A) 3.25 % KLEE ointment; (B) 6.5 % KLEE ointment; (C) 13 % KLEE ointment; (D) negative control group; (E) positive control group. KLEE: *Kepel* leaves'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

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