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# The Effectiveness of Cream of Centella asiatica Ethanol Extract in Chitosan Nanoparticles Compared with Centella asiatica Ethanol Extract Cream, Silver Sulfadiazine Cream, and Control on Superficial Dermal Burn Healing in Rats

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## ABSTRACT

**Introduction:** Centella asiatica extract is known to promote wound healing. Nowadays nanotechnology, especially nanoparticle is used to increase the bioavailability of active ingredients into the skin, one of which is chitosan nanoparticle. However, the effectiveness of Centella asiatica ethanol extract in chitosan nanoparticle (CAEECN) towards superficial dermal burn healing is not fully understood.

**Aim:** To determine the effectiveness of CAEECN cream compared to Centella asiatica ethanol extract (CAEE) cream, silver sulfadiazine (SSD) cream and control in superficial dermis burn healing in Sprague-Dawley rats. **Materials and Methods:** A total of 20 male Sprague-Dawley rats were divided into 4 groups. After anesthetizing, 4 superficial dermis burns were made on the back of each rat and was given 4 treatments (CAEECN, CAEE, SSD, and control) afterward. Each group was sacrificed separately (respectively on day 3rd, 7th, 14th, and 21st post-burn induction). Several evaluations were macroscopic (wound size), and microscopic parameters (reepithelialization ratio and distance, wound contraction index, wound size, and angiogenesis). **Results:** There was no significant difference in wound size between treatments on day 0, 3, 7, 14, and 21. But three active agents (CAEECN, CAEE, and SSD) treated group showed early decrease of wound size compare to control group on day-3. Microscopically, there were no significant differences in reepithelialization ratio and distance, wound contraction index, wound size, angiogenesis. Nevertheless, CAEECN treated group showed the smallest wound size on day-3 and highest angiogenesis on day 21. **Conclusion:** Effectiveness of CAEECN in burn wound healing in rats showed no significant difference compared to CAEE, SSD, and control. But in three active agents (CAEECN, CAEE, and SSD) treated group, showed an early decrease of wound size compared to control group on day-3. Microscopically, CAEECN treated group showed the smallest wound size on day-3 and highest angiogenesis on day 21.

**Keywords:** Wound healing, re-epithelialization, fibroblasts, angiogenesis, nanotechnology

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## INTRODUCTION

Burns are injuries to the skin or other tissues of organs directly caused by heat or other causes, such as radiation, radioactivity, electricity, or chemicals. Burns are a global public health problem, World Health Organization (WHO) in 2014 estimated 265,000 deaths that mostly occur in developing and underdeveloped countries (1). Burns cause high morbidity including longer hospital stay duration, deformity, and disability, resulting in patients experiencing stigma and rejection of the social environment (2). One of the principle management of superficial dermal burns one is the prevention of infection with topical antibiotic treatment (3). One of the most widely used topical antibiotic treatment is silver sulfadiazine (SSD) (3). SSD has antimicrobial effects, including against gram-positive bacteria, gram-negative bacteria and yeasts. SSD also has a wound healing effect by reducing matrix metalloproteinase (MMP) and inhibiting Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) (4,5). *Centella asiatica* (CA) or Pegagan leaf has been used as a traditional medicine for wound healing. CA plants are commonly found in numerous countries in Asia. CA contains numerous active components in burn healing activities such as asiaticoside and madecassoside. Although it has potentially high biologic activity, the clinical use of CA extract is limited due to its hygroscopic nature. The use of nanoparticle (e.g chitosan) may protect the CA extract against external humidity (6,7). Nanoparticle is one of the active material delivery systems that is widely used due to its stability and compatibility with other cosmetic formulations (7,8). Chitosan is a biopolymer of crustacean's shell that acts as natural antibacterial and wound healing agent. The use of chitosan in the food and pharmaceutical industries is increasing, due to its biodegradability and biocompatibility, relatively low-cost manufacturing, and approved by the Food and Drug Administration (FDA) (7,9,10). Thus, the aim of this study was to study the

effectiveness of CAEECN in superficial dermal burn healing in rats.

## MATERIALS AND METHODS

This experimental study was conducted in the Department of Histology, Faculty of Medicine, Universitas Indonesia, Indonesia, in December 2016. The experiment was started after obtaining the approval from The Ethics Committee of the Faculty of Medicine, Universitas Indonesia, with the number of ethical approval letter is 1037/UN2.F1/ETIK/2016.

This study is an experimental test. The subjects were 20 rats (*Rattus norvegicus*) of Sprague Dawley strain in accordance with acceptance criteria and rejection criteria. Mouse rats were adapted in the laboratory for 1 week (1st to 7th day of research) with separate rods. Meals and drinks are given on ad libitum. Rats were divided into four groups, based on the day sacrificed ie on day 3, 7, 14, 21 after treatment.

Burn optimization is done to determine the desired depth of the superficial dermis. In this study used 100° C for 5 seconds. The process of making the injury using Eryani *et al*<sup>11</sup> modification method that is rat is anesthetized with combination of ketamine (85 mg/kg BB) and xylazine (10 mg/kg BB) intraperitoneally. Rats Injected Penicillin Procaine-G 100 mg / kg BW intramuscularly. The treatment area on the back measuring 5 cm x 5 cm was shaved using an electric shaver with a 45° angle to the skin. The wound is made parallel to the mouse vertebral line with a distance of 1 cm on the right and left side of the vertebral bone; 0.5 cm below the bone of the scapula and 2 cm distance between the vertical wound. Skin is disinfected with 10% povidone-iodine solution. Plates 1 cm in diameter, heated to 100° C, measured using a thermometer. Prior to the injury, sedation is assessed by pinching the skin on two different limbs. The hot plate is placed perpendicular to the skin of the mouse for 5 seconds. Before starting the injury, the plate is reheated and measured to the desired temperature.

The same step was performed until 4 models of burns were created on each mouse and each wound was applied with different treatments ie CAEENPK, CAEE, SSD (positive control) creams, and not applied anything (negative control). A macroscopic examination was performed to measure the wound area of Visitrak® on days 0, 3, 7, 14, and 21. At the time of sampling, the mice were sacrificed by means of decapitation, the skin tissue around the wound was cut and inserted into a spot containing a formalin buffer solution 10% were then prepared histologically with Hematoxylin and Eosin (HE) staining. Microscopic examination was performed to assess the reepithelialization ratio, reepithelialization distance, WCI, wound area, and angiogenesis. Parameters are measured quantitatively by measurement through Image Raster® 3 software. The data were presented in tables, drawings, and graphs.

**Statistical Analysis**

Data were analyzed using Statistical Product and Service Solution (SPSS) program version 17.0. The data obtained were tested for normality first, then analyzed with appropriate statistical tests (Repeat measures or Friedman for wound and microscopic parameters, unpaired T-test and Mann Whitney for comparison of wound range inter-day). Statistical conclusions use a 5% significance limit with meaningful criteria if  $p < 0.05$ .

**RESULTS**

**3.1 Macroscopic Evaluation**

Based on observation of 0, 3, 7, 14, and 21-day wounds, it was found that the median and the mean of the wounded area of all groups showed the same relative and uninfected area. An example of the process of wound closure between treatments can be seen in [Table/Fig-1].

[Table/Fig-1]: The result of observation of macroscopic wound closure process based on the passage of time. (CAEENPK = Centella asiatica ethanol extract with chitosan nanoparticles, CAEE = Centella asiatica ethanol extract, SSD = silver sulfadiazine)

Group	Day	
	0	3
CAEENPK		
CAEE		
SSD		
Control		

Based on observation of the 0th, 3rd, 7th, 14th, and 21st day wounds, it was found that the median and the mean widespread of all groups showed relatively similar area and the result of statistical analysis did not differ significantly. However, on the 3rd day the group has given CAEENPK and CAEE showed the smallest wound area compared to the controls can be seen in [Table/Fig-2].

Comparison of wound area between days in each treatment group can be seen in [Table/Fig-3] and illustrated in [Table/Fig-4]. In the control treatment between day 0 and 3 groups, there

was a statistically significant difference in wound sizes ( $p > 0.05$ ). In the Kimura *et al*<sup>6</sup> study, the extent of second degree deep burns without treatment will increase until day 6. However, in

treatment with CAEENPK, CAEE, and SSD there was no significant increase in the extent of the wound. This may be due to an asiaticoside content that can modulate inflammation by

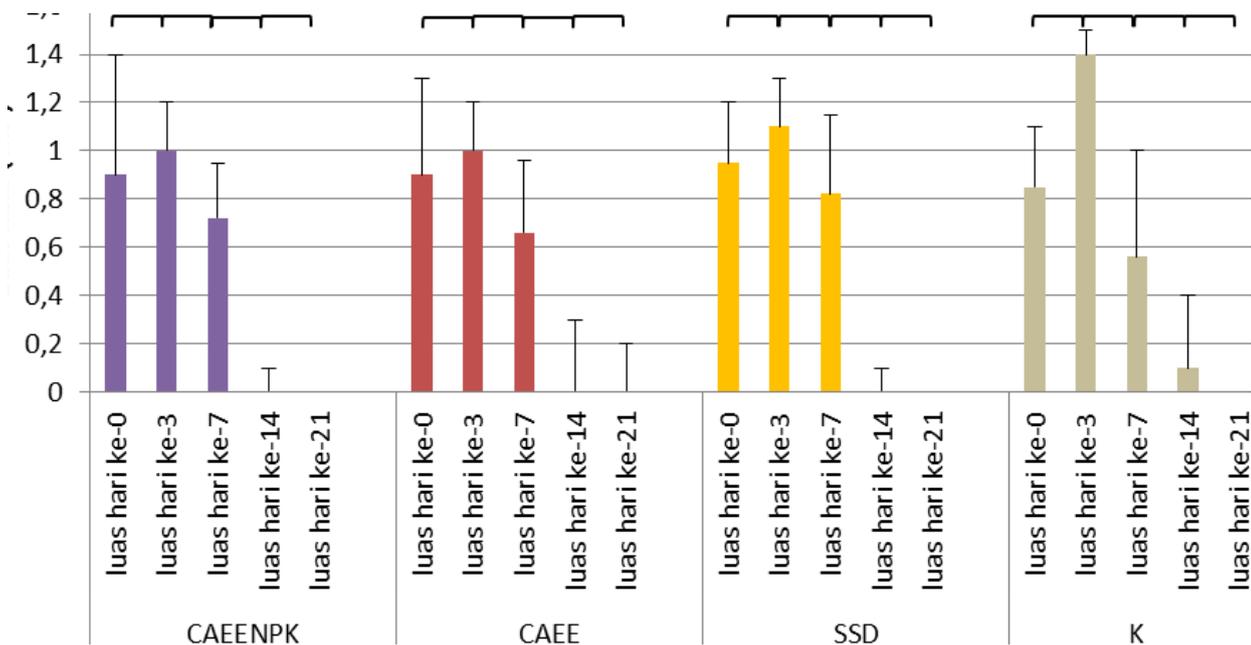
increasing the accumulation of macrophages and decreasing the number of neutrophils.<sup>17</sup> While SSDs can help lower MMP levels and are suspected to have anti-inflammatory effects.

[Table/Fig-3]: Comparison of wound range between days in each treatment group

Wound Area	Day	Description (cm <sup>2</sup> )	p-value	Wound Area	Day	Description (cm <sup>2</sup> )	p-value
CAEENPK	Day 0	0.90 (0.70-1.40)	0.620	SSD	Day 0	0.95 (0.60- 1.20)	0.110
	Day 3	1.00 (0.90-1.20)	0.034*		Day 3	1.10 (0.50- 1.20)	0.399
	Day 7	0.72 ± 0.23	0.008*		Day 7	0.82 ± 0.33	0.007*
	Day 14	0.00 (0.00-0.10)	0.134		Day 14	0.00 (0.00-0.10)	0.317
	Day 21	0.00 (0.00-0.00)			Day 21	0.00 (0.00-0.00)	
CAEE	Day 0	0.90 (0.60- 1.30)	0.740	Control	Day 0	0.85 (0.60- 1.10)	0.034*
	Day 3	1.00 (0.80- 1.20)	0.071		Day 3	1.40 (1.00- 1.50)	0.027*
	Day 7	0.66 ± 0.30	0.009*		Day 7	0.56 ± 0.44	0.054
	Day 14	0.00 (0.00-0.30)	0.881		Day 14	0.10 (0.00-0.30)	0.151
	Day 21	0.00 (0.00-0.20)			Day 21	0.00 (0.00-0.00)	

In [Table/Fig-4] it can be seen also the CAEENPK treatment and control between the 3rd and 7th day groups, there was a statistically significant decrease in wound area (p <0.05). This was caused by day 3, the extent of wound

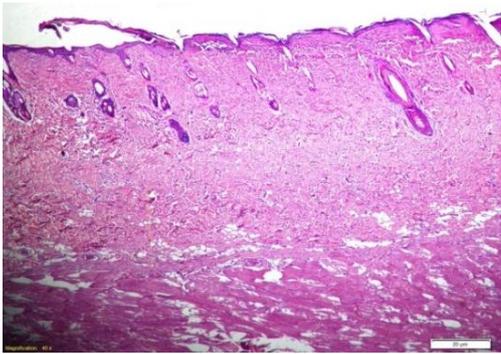
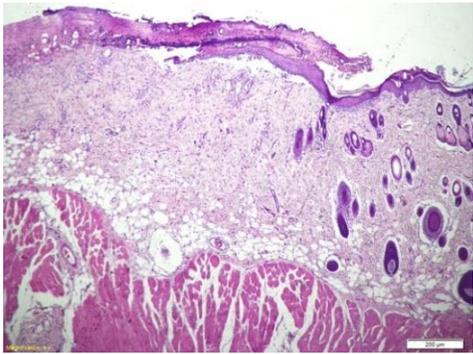
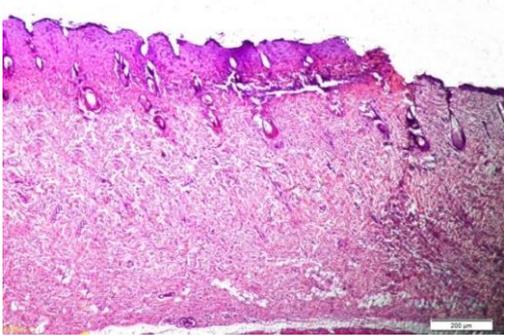
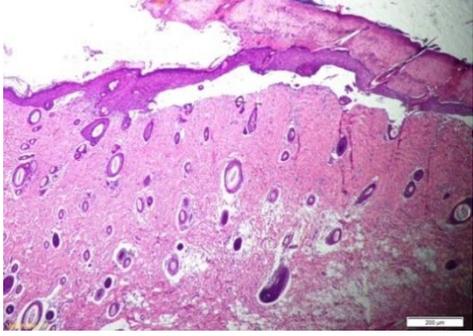
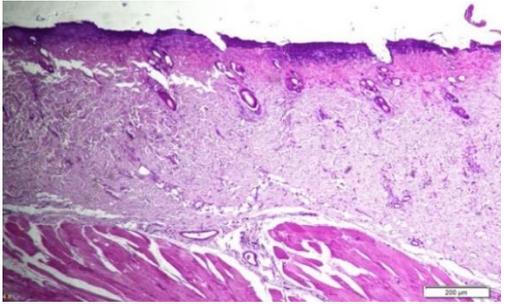
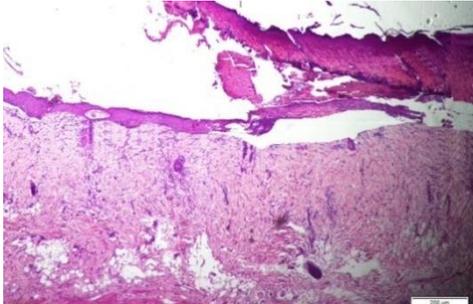
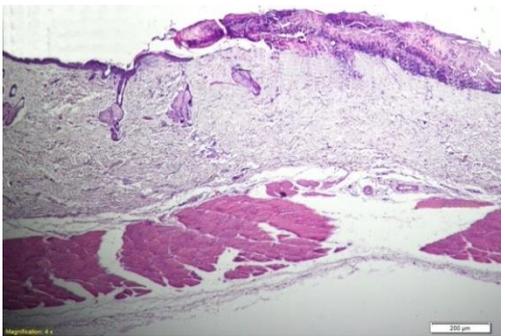
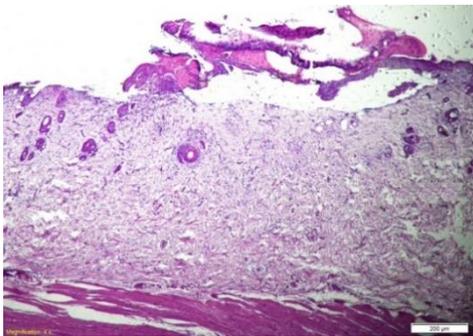
with CAEENPK did decrease significantly, while in control there was a significant increase on the 3rd day and then the wound decreased on the 7th day. Treatment with CAEE and SSDs was not significantly decreased.



**Reepithelialization**

Microscopic reepithelialization observations were performed on days 3 and 7 in each treatment group. On the 3rd day most of the

wounds have not been reepithelialized, while the seventh day most of the wounds have been reepithelialized. The process of reepithelialization as an example is summarized in [Table/Fig-5]

Group	Histopathology	
	3	7
CAEENPK		
CAEE		
SSD		
Control		

The highest ratio and lowest reepithelialization distance indicate the furthest epithelial migration.

The results of the reepithelialization ratio test on days 3 and 7 showed no statistically significant

difference ( $p > 0.05$ ) between treatments. The highest ratio was obtained in wound with SSD. The results of reepithelialization distance analysis on days 3 and 7 between treatments showed no statistically significant difference ( $p > 0.05$ ) between treatments. The

### Wound Area

The analysis of the wound healing process of the burn area was carried out on the 3rd, 7th, 14th and 21st-day openings. The analysis results are

### Contraction of the wound

Analysis of wound contractions on days 3, 7, and 14, reflected by WCI in [Table/Fig-9], showed

### Angiogenesis

This angiogenesis data is assessed for compatibility between two assessors with the Bland-Altman method. Because of the discrepancy between the two assessors, the statistically analyzed angiogenesis data is the principal assessor data (histopathologist) as the gold standard. The result of angiogenesis analysis showed that on day 7 there was no statistically significant difference between treatments ( $p > 0.05$ ). On day 21 the injury with CAEENPK treatment had the highest density of blood vessels, although there was no statistically significant difference between treatments ( $p > 0.05$ ). The mean density of blood vessels on days 7 and 21 can be seen in [Table/Fig-10]. Observation of angiogenesis can be seen in [Table/Fig-11].

### DISCUSSION

CAEENPK and CAEE base creams used contain propylene glycol (4.0%) and cetyl alcohol (1.5%). In addition, the SSD base cream also contains propylene glycol.<sup>12</sup> Propylene glycol is an irritating ingredient, which can affect wound healing.<sup>13</sup> Another superficial dermal burn is a self-healing type of burn and the healing of burns in mice is generally faster than humans.<sup>14</sup> The concentration of CAEENPK or CAEE 1.5% active ingredient should have an effect on wound healing.<sup>15</sup> Kimura *et al.*<sup>16</sup> studied burns in mice with topical ethanol extract of 70%

treatment on day 3 and CAEE on day 7 can be seen in [Table/Fig-6].

reepithelialization distance in [Table/Fig-7] shows that wound with SSD treatment on day 3 and CAEE on day 7 had the lowest distance with slight differences between treatments.

illustrated in [Table/Fig-8]. The wound area of CAEENPK treatment on day 3 was smaller than other wounds, but in CAEE treatment the area of the wound was larger than the others, then decreased on the 7th day.

that there was no statistically significant difference between treatments ( $p > 0.05$ ).

CA in 2 and 5 × 10<sup>-4</sup>% vaseline doses (w/w), wound size decreased on days 8 to 19 compared with vehicle (vaseline) treatment.

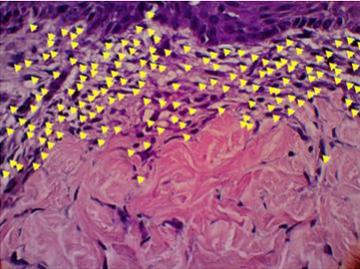
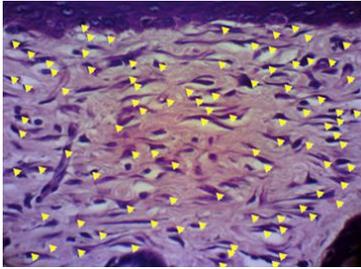
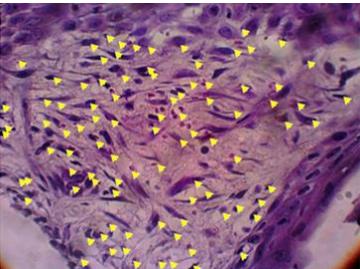
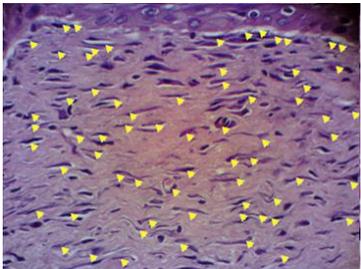
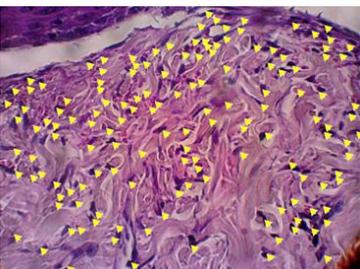
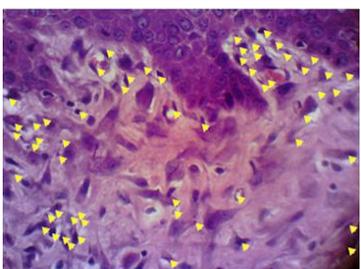
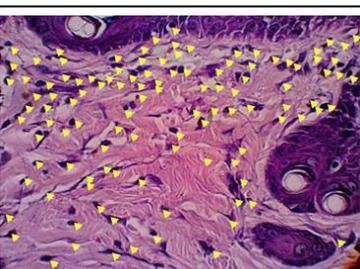
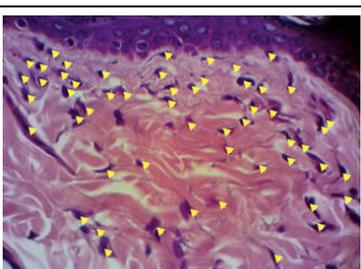
In vitro studies by Yulianti *et al.*<sup>18</sup> show that CAEENPK may increase the proliferation of fibroblasts and keratinocytes. CAEE can improve the process of reepithelialization and increase the synthesis of collagen.<sup>19</sup> Avsar *et al.*<sup>20</sup> shows on days 3 to 7, SSDs can increase fibroblast activity and epithelial regeneration.

The significant decrease in wound area ( $p < 0.05$ ) in the treatment of CAEENPK, CAEE, and SSD between day 7 and 14. In control, wound area decreased but not significant ( $p > 0.05$ ). The Kimura *et al.*<sup>16</sup> study showed that topical asiaticoside administration increased the accumulation of macrophages and IL-1 $\beta$  by the ninth day to accelerate the healing of burns. In addition, antioxidant levels after 7 days of topical asiaticoside were also increased.<sup>21</sup> In CAEENPK and CAEE also contained madecoside which increased the wound coverage between the 7th and 14th.<sup>22</sup> Yuksel *et al.*<sup>23</sup> examined SSDs against second-degree burns in rat skin, 7 began to form proliferation of fibroblasts, the formation of collagen and increased by day 14.

On day 14, the median widescreen control treatment was highest compared to other treatments although not statistically significant. The Somboonwong *et al.*<sup>17</sup> study showed that

administration of methanol, aqueous, ethyl acetate and hexane OTAs in the topical Tween 20® (polyoxyethylene (20) sorbitan monolaurate)

vehicle on burns, there was a significantly different reduction in the 14th day wound than control and vehicle solutions.

Group	Day	
	7	21
CAEENPK		
CAEE		
SSD		
Control		

On day 3 SSDs have the highest ratio caused by silver inducing metal-binding-metallothioneins thus increasing the levels of zinc and copper. Both of these are important micronutrients for epithelial proliferation.<sup>4</sup> However, SSDs have decreased ratios by day 7 which may be due to in vitro SSD has a cytotoxic effect on keratinocytes, but this is still not proven in vivo.<sup>4</sup> CAEE contains asiaticocic acid which can accelerate the process of reepithelialisation.<sup>19</sup> In

vitro studies by Yulianti *et al*<sup>18</sup>, CAEENPK with a concentration of 6.25 mg/ml after 24 h had an effect on fibroblast proliferation of 160% compared to control, and CAEENPK with concentrations of 3.125 mg/ml after 24 hours increased keratinocyte proliferation by 145% compared to control. But in vitro studies are an incomplete method of studying wound dynamics, as there is a complex in vivo interaction between

tissue stress, effector cells, extracellular matrix, humoral factors and growth factors.<sup>24</sup>

Asiaticoside, madecoside, madecassic acid and asiatic acid contained within CA, can induce reepithelialization and play a role in wound healing process.<sup>25-27</sup> While SSD has antimicrobial and anti-inflammatory effect by decreasing MMP, inhibiting TNF- $\alpha$ , and modulate local inflammatory response.<sup>4,28</sup> CAEENPK has an important antimicrobial effect for wound healing since in general the first 48-72 hours of bacterial colonization begin to develop.<sup>29</sup> However, this study uses Penicillin-procaine G as a secondary infection prophylaxis therapy that may affect the effect. CAEENPK also promotes the proliferation of fibroblasts and keratinocytes so that it is expected to accelerate wound healing.<sup>18</sup> In vitro study by Yulianti *et al* showed that CAEENPK with concentrations of 3.125 mg/ml in 24 hours and 12.5 mg/ml within 48 hours increased keratinocyte proliferation by 145% and 115%.<sup>30</sup>

Burns until day 6 can still be widespread, indicating that CAEENPK prevents worsening injury by modulating inflammation.<sup>17</sup> In vitro study by Yulianti *et al*<sup>18</sup> showed that within the first 48 hours of CAEENPK exposure to fibroblasts would increase the synthesis of collagen III required to begin the remodeling process. The CAEE treatment showed the widest area of the wound on day 3 of the other treatments. In vitro studies Yulianti *et al*<sup>30</sup> showed results that in the first 24-72 hours of CAEE extract inhibited collagen 3 synthesis compared with CAEENPK and control. While SSDs have anti-inflammatory effects by inhibiting TNF- $\alpha$ .<sup>4</sup> On day 7 the median area of the wound area of all treatments decreases and does not differ greatly from one another.

On the 14th day, all treatments have the perfect WCI. Studies by Schencke *et al*<sup>31</sup> showed that postpartum wounds on guinea pig on day 10 had a WCI of 0.9 in the positive control group (tulle hydrogel) and topical Ulmo and WCI 1 in Ulmo honey group and ascorbic acid supplementation. In mice, wound healing is mostly played by

wound contraction, which is reflected by WCI. The wound contraction in rats was largely played by carnosus panniculus, the striated muscle in the hypodermic layer.<sup>14,32</sup> Venter *et al* studied burns as deep as the superficial dermis in mice generally experienced relatively faster wound healing than humans. However, according to Vidinsky *et al*<sup>34</sup> mice can still be used as a burn model because the time difference is not too far (comparable). Another advantage of mice is that the body size is relatively small so it is easier to maintain and obtain, also economical.<sup>32,35</sup>

The content of asiaticosida is suspected to play a role in this process. Kimura *et al*<sup>16</sup> studies show that angiatikoside stimulates angiogenesis and VEGF in burns allegedly through the mechanism of enhancing IL-1 $\beta$  expression by macrophages or by enhancing MCP-1 expression by keratinocytes and / or macrophages. In addition, Hou *et al*<sup>36</sup> investigated that 100 ng/ml of madecasocide in vitro significantly increased VEGF levels at 6, 12, 24, 36, 48 hours post-application. The role of angiogenesis in wound healing is to provide oxygen supply, metabolites for newly formed tissues, exclude useless metabolites, and maintain normoxic conditions.<sup>26</sup> The study by Nasiri *et al*<sup>36</sup> shows that the 8th day found moderate angiogenesis in second degree burns in mice that are smeared SSD. But the mechanism is still unknown.

The maturation of angiogenesis is characterized by the incidence of smooth muscle, pericitation, and regression of new branches of blood vessels.<sup>37</sup> In the 21st day injury preparation, blood vessel regression is characterized by decreased blood vessel density. In the elongated truncated blood vessels can be found layers of smooth muscle cells and pericites. In the angiogenesis process formed many new small blood vessels or buds. So the calculation of blood vessel density with HE routine staining in this study is quite difficult and it takes high flying hours, especially to determine small blood vessels or buds.

## CONCLUSION

Overall, from the macroscopic and microscopic evaluation results although no treatment with the three active ingredients ie CAENPK, CAEE, and SSD showed a decrease in macroscopic injury area earlier than control on day 3, treatment with CAENPK showed the smallest microscopic lesion area on day 3, and treatment with CAENPK showed the highest angiogenesis on day 21. We recommend follow-up studies in the form of additional analytical methods for the assessment of angiogenesis (eg, specific coloring of CPI CD 31 or CD 34), different CAENPK concentration and vehicular studies, as well as types of internal or complicated burns of the dermis.

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