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The utilization of oral and topical rosella calyx extract (*Hibiscus sabdariffa* L.) to enhance re-epithelialization in Wistar rats (*Rattus novergicus*)

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Background: The management of third-degree burns primarily aims to enhance the re-epithelialization process. Any impairment in this process may cause delayed wound healing. Rosella calyx (*Hibiscus sabdariffa* L.) can act as an immunomodulator, possibly affecting the re-epithelialization process.

Methods: Rats were randomized after adaptation for a week. Rats (n = 30) were induced with third-degree burns, and it was ensured that they met the criteria for third-degree burns. We divided them into six groups and administered treatment according to each group: 1) negative control group using cold cream only; 2) positive control using silver sulfadiazine (SSD) only; 3) treatment group using rosella calyx extract orally (250 mg/kg) + cold cream; 4) treatment group using rosella calyx extract topically only (250 mg/kg); 5) treatment group using rosella calyx extract orally (250 mg/kg) + SSD; and 6) treatment group using rosella calyx extract orally and topically (each 250 mg/kg). They were sacrificed on day 17 and then stained with hematoxylin and eosin. The variable studied was the re-epithelialization length (mm). Statistical analysis involved analysis of variance (ANOVA) and post hoc correction with $P < 0.05$ taken as significant.

Results: Despite treatment using rosella calyx extract orally + topically showing the longest re-epithelialization, this group showed no significant difference compared with SSD treatment, indicating they may show a similar effect in the re-epithelialization process.

Conclusion: Rosella calyx extract applied orally and topically may show a therapeutic effect in the re-epithelialization process. Further research needs to be conducted.

Keywords: plant extract, re-epithelialization, burns

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INTRODUCTION

In one report from Indonesia, 275 were admitted to Cipto Mangunkusumo Hospital due to burn-related injuries [1]. According to the report, a third-degree burn was one of the absolute causes of mortality alongside the second-degree burn. A third-degree burn is a wound showing epidermis and dermis destruction with no epidermis left for regrowth. Infection occurs easily because of inadequate blood flow and formation of necrotic tissues [2].

Wound management aims to accelerate re-epithelialization and remove the debris [3]. A non-

epithelialized wound is not considered "healed" no matter how perfectly restored the underlying dermal structures may be [4].

Re-epithelialization is the restoration of an intact epidermal barrier through wound epithelialization [4]. Re-epithelialization of the wound can be conceptually viewed as the result of three overlapping keratinocyte functions: migration, proliferation, and differentiation [5]. The directed migration of keratinocytes is critical for wound epithelialization, and defects in this function are associated with the clinical phenotype of chronic non-healing wounds [6].

Rosella calyx (*Hibiscus sabdariffa* Linn) contains quite a high amount of anthocyanin, evident from its red color [7]. Rosella also contains citric acid, natural malate, protein, and vitamin C [8]. These agents may contribute to the process of burn healing. Currently, the use of oral rosella calyx is common; it has proven therapeutic effects in lowering cholesterol, blood pressure, and creatinine levels while also being good for liver function [9]. Rosella calyx acts as an anti-inflammatory agent that inhibits the production of reactive oxygen species (ROS) and tumor necrosis factor (TNF)-α [7]. Thus, fibroblast proliferation, keratinocyte production, and migration for the remodeling phase can occur more quickly. This study examines the effect of third-degree burn treatments using oral and topical rosella calyx extracts (*Hibiscus sabdariffa* L.) in the re-epithelialization process in Wistar rats (*Rattus norvegicus*).

PARTICIPANTS AND METHODS

We used a pure experimental research method with a post-test control group design. We used 30 Wistar rats with the following inclusion criteria: female, aged 2.5–3 months, weight 150–250 g, in a healthy condition, and not received prior treatment. The exclusion criteria were having a wound infection, excessive exudate, or being dead. We obtained rosella calyx extract from Balai Medika, Batu, using a maceration extraction procedure with methanol and ethanol as soluble agents. This research was performed in the pharmacology laboratory of the Universitas Brawijaya, Malang, Indonesia. From 100 g dried rosella calyx, we extracted 30 g of rosella calyx.

We used cold cream (produced following the procedure described by Yaman *et al.*) as a negative control, and silver sulfadiazine (SSD) as a positive control [10]. Thirty rats were induced with third-degree burns. We shaved their back area and then injected lidocaine as a local anesthetic. We prepared a 100-g cylindrical stainless-steel rod for the induction and heated it for 13 min; we then taped the heated rod onto the shaved back. The injury had to meet the criteria for third-degree burns: no bullae; gray and pale burnt skin; dryness; wound laying lower than surrounding skin due to protein coagulation in the epidermal layer and dermis. Following the induction of a third-degree burn, we treated the rats with normal saline 0.9% and then proceeded according to the treatment group. In this study, we divided the rats into six groups (n = 5 in each group): group 1, negative control group using cold cream only; group 2, positive control using SSD only; group 3, treatment group using rosella calyx extract orally (250 mg/kg) + cold cream; group 4, treatment group using rosella calyx extract topically only (250 mg/kg); group 5, treatment group using rosella calyx extract orally (250 mg/kg) + SSD; and group 6, treatment group using rosella calyx extract orally and topically (each 250 mg/kg).

Thirty rats were treated every day over a 16-day period; on day 17, all rats were sacrificed using ether and prepared for hematoxylin and eosin (H&E) staining. We used microscopy techniques and Olivia software for observation. For the variables, we observed the re-epithelialization length measured in millimeters. In determining the length of re-epithelialization, we drew a line from the closest hair follicle to the wound tissue. The longer the re-epithelialization length, the better is the wound closure process since the wound site is filled by the epithelial cells. The process for measuring re-epithelialization was conducted following the method of Engel *et al.* [11] We used one-way analysis of variance (ANOVA) with 95% confidence intervals for statistical analysis and used the post hoc least significant difference (LSD) test to show any significant differences between the groups. Significance was taken at $P < 0.05$ by ANOVA and the post hoc test.

RESULTS

Before we viewed the results of the length of re-epithelialization, we needed to observe the

histology images. Figure 1 shows the identified differences among the groups. These images were taken from third-degree burns of rats with treatments according to the groups mentioned earlier. Extensive inflammation (indicated by a bluish-purple color) persisted in group 1 (cold cream) and group 3 (rosella calyx extract orally + cold cream). Data indicated a re-epithelialization process, yet inflammation was still evident with treatment using topical rosella calyx extract (group 4). Interestingly, the wound showed almost complete closure and almost no inflammatory cell colonies in group 6 (rosella calyx extract orally + topically). Group 5 (rosella calyx extract orally + SSD) looked less inflamed than group 2. Despite group 5 appearing incompletely closed, no inflammatory cell colonies were seen compared with group 2 (Figure 1). We concluded that group 6 showed the best re-epithelialization process with the least inflammation. Third-degree burn treatments using the combination of rosella calyx extract orally + topically or the combination of rosella calyx extract orally + SSD showed better therapeutic effects on the re-epithelialization compared with treatment using SSD only. Meanwhile, the use of rosella calyx extract orally combined with cold cream showed no difference to treatment using cold cream only, with extensive inflammation present (massive bluish-purple color seen in groups 1 and 3).

As stated earlier, we used the method of Engel *et al.* to measure re-epithelialization length [11]. Data were tabulated and statistically analyzed (Table 1). The

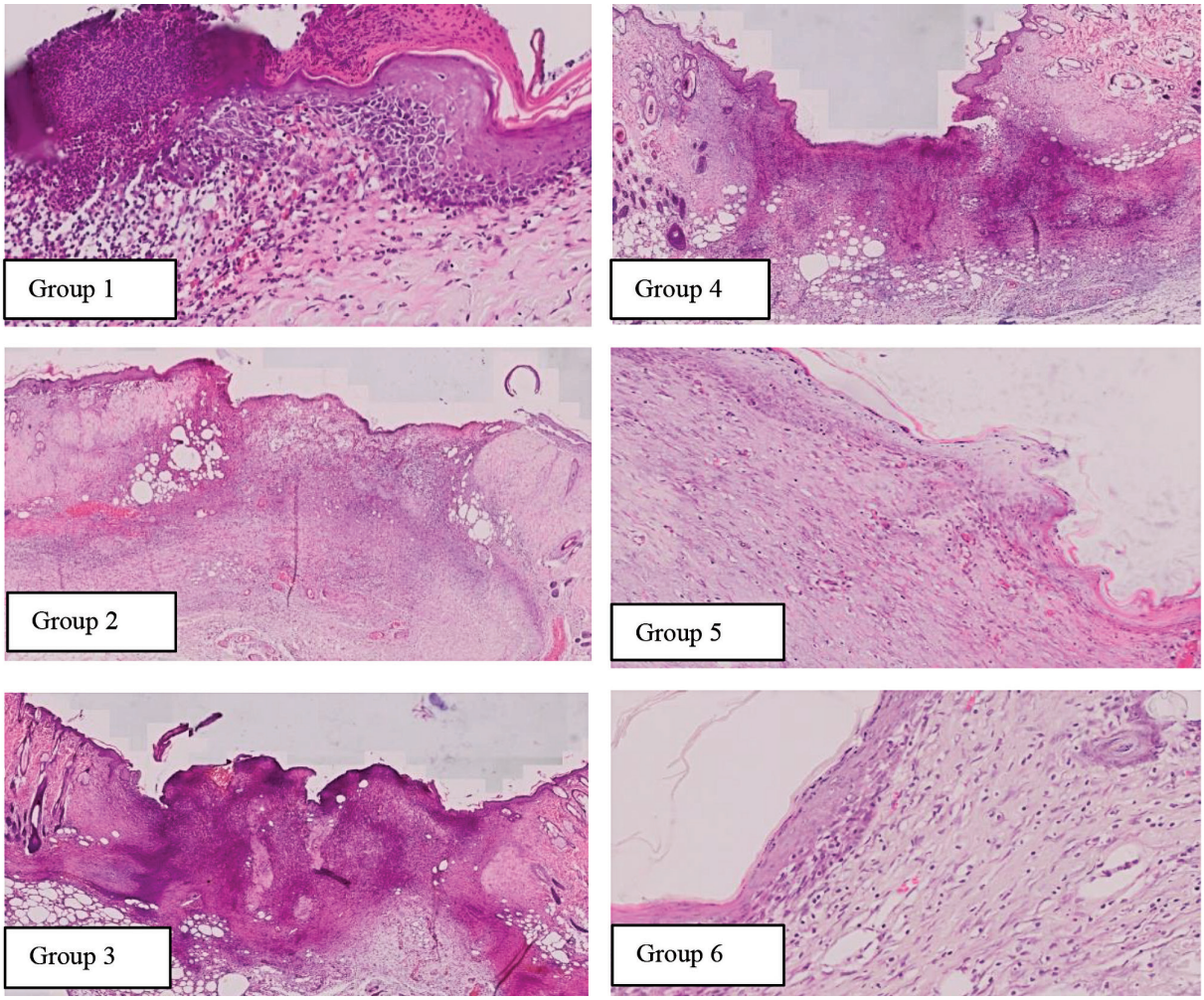

Figure 1. Representative image from all groups. We used H&E staining at 400× magnification over 20 µm to measure the re-epithelialization length. Inflammation could be seen from the bluish-purple color (white blood cells); collagen has a pink color. It is seen that groups 1 and 3 show the most inflammation, while group 6 shows the least inflammation.

Table 1. Re-epithelialization length (mm)

Group	Re-epithelialization length (mm)
Group 1 (cold cream)	0.6728 ± 0.33085 [a]
Group 2 (SSD)	2.0740 ± 1.09576 [b]
Group 3 (rosella calyx extract orally + cold cream)	0.9660 ± 0.33916 [a]
Group 4 (rosella calyx extract topically)	2.0020 ± 0.54316 [b]
Group 5 (rosella calyx extract orally + SSD)	2.2720 ± 0.59910 [b]
Group 6 (rosella calyx extract orally + topically)	2.8040 ± 0.68178 [b]

Results are expressed as the mean ± standard deviation
SSD silver sulfadiazine
Note that [a] and [b] denote significant differences ($P < 0.05$) for a versus b

findings suggested an effect of rosella calyx extract in the re-epithelialization process as a treatment for the third-degree burns of rats ($P < 0.05$). We continued to a post hoc test, which confirmed that the longest re-epithelialization was seen in group 6 (2.8040 ± 0.68178 mm), significantly greater than group 1 (0.6728 ± 0.33085 mm; $P < 0.001$). However, the re-epithelialization length in group 6 was not significantly different from groups 2 (2.0740 ± 1.09576 mm), 4 (2.0020 ± 0.54316 mm), or 5 (2.2720 ± 0.59910 mm). Data were also graphically represented to see if the re-epithelialization length in third-degree burn rats was significantly different between groups 2, 4, 5, and 6. Although the rosella calyx extract orally + topically group showed the longest re-epithelialization (2.8040 ± 0.68178 mm), no significant difference was found (Figure 2).

Topical rosella calyx extract showed a therapeutic effect on re-epithelialization, but not oral rosella calyx extract + cold cream. Treatment using rosella calyx extract orally + cold cream (0.9660 ± 0.33916 mm) showed no significant difference compared with negative controls treated only with cold cream (0.6728 ± 0.33085 mm), and both had the shortest re-epithelialization lengths compared with the other groups.

DISCUSSION

Despite treatment using oral and topical rosella calyx extract showing similar effectiveness as SSD in the re-epithelialization length for third-degree burns, further studies need to be conducted due to the marked difference between treatment using rosella calyx extract orally + topically compared with SSD only. We observed that the re-epithelialization process in rosella calyx extract orally + topically was better than the other groups (Figure 1). Though no significant difference was identified (Figure 2), we found the treatment using rosella calyx extract orally + topically may have a therapeutic effect on re-epithelialization in third-degree burns. We were not surprised by the effect of SSD since it is already used as standard therapy for third-degree burn management in our hospital. However, we expected the combination management for third-degree burns to accelerate the re-epithelialization process. Re-epithelialization is a crucial step for wound healing because the deceleration of the re-epithelialization process is associated with a high risk of infection, skin contracture onset, and sepsis.

As commonly known, SSD is the standard

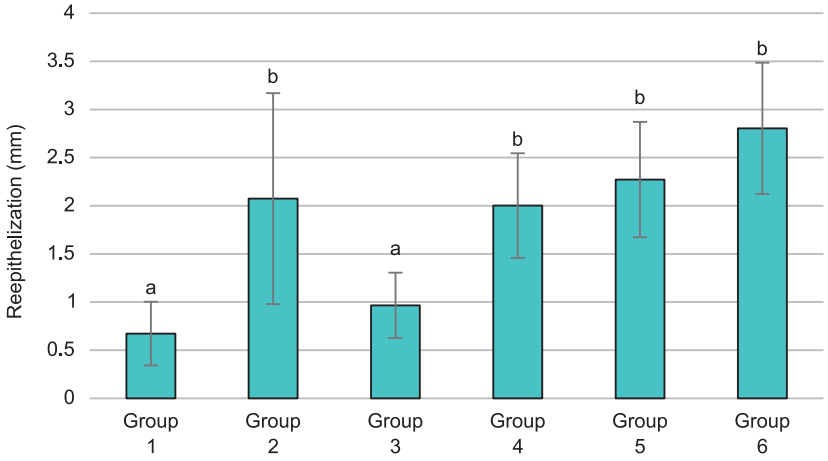

Figure 2. Re-epithelialization length (mm). Results are expressed as the mean ± standard deviation. Note that [a] and [b] denote significant differences ($P < 0.05$) for a versus b.

management for third-degree burns [12]. Our reasoning for this study was assembled from the findings of studies conducted by Atiyeh *et al.* and Poon and Burd, where SSD was not only toxic and lethal to bacteria but also to keratinocytes and fibroblasts [13,14]. Despite SSD having antibacterial properties, this therapy has a weakness that slows the re-epithelialization process, leading to a longer inflammation process. This is because keratinocytes and fibroblasts are needed to accelerate the main proliferation process, especially for re-epithelialization. With SSD having a lethal effect on these two components, this will lead to delayed re-epithelialization [13].

Re-epithelialization cannot completely occur when the inflammation phase is overstimulated [15]. This inflammation is affected by the inflammation-inducing activation of ROS, TNF-α, interleukin (IL)-1, IL-6, and other pro-inflammatory mediators to the wound site. All of these would delay the wound healing process [16]. In third-degree burns, overstimulation of inflammation results in destruction occurring in the hypodermis; meanwhile, the immunomodulatory mediators are located in the epidermis and dermis as well [17,18]. Additionally, delays to the process occur when colonization of microorganisms exists at a wound site, yet the body's response to activating an immune response is lacking due to disruption of the immunomodulatory function. The delayed re-epithelialization process with mass inflammation can be clearly seen in group 1 and group 3 (Figure 1). This research proved that mass inflammation would delay the wound healing process, as shown by a short length of re-epithelialization.

The therapeutic effect of rosella calyx extract orally + topically in this study supports other studies on the effect of rosella in accelerating the wound healing process. Nizamutdinova *et al.* reported that anthocyanin therapy for 48 h significantly stimulated fibroblast and keratinocyte migration. Also, anthocyanins inhibited the excessive accumulation of ROS and TNF-α. The inflammation activator NF-kB was also blocked. By inhibiting the inflammation process, anthocyanins will affect the healing process more quickly [19]. The study by Dahiru *et al.* reported that administration doses of as much as 250 mg/kg would accelerate immunomodulator activity [8].

Immunomodulation is the ability to stimulate or inhibit the cell response. For wound healing, it is linked to IL-10 and TNF-α. The function of TNF-α is to modulate wound healing by stimulating fibroblasts and angiogenesis, and the main function of IL-10 is inhibiting or terminating the inflammation process [20]. IL-10 regulates immune cell growth or the differentiation process [21,22]. Besides anthocyanin, rosella also contains vitamin C (for collagen formation), malate acid (anti-aging), protein (immune development), and citrate acid (decreases pH and as an antibacterial) [9]. Based on the above statements, we conclude that oral and topical rosella calyx may affect the wound healing process in third-degree burns by accelerating the re-epithelialization process. The study findings showed that rosella calyx treatment orally and topically has a considerable therapeutic effect. The combination of treatment using rosella calyx extract orally + SSD is an interesting finding as, using H&E staining, it showed a better re-epithelialization process than isolated SSD.

This study has some limitations. Our euthanasia method used ether and should use cervical dislocation for best results. However, several similar studies used ether, and the relevant ethics committee approved this study. Also, we still used crude rosella calyx extract. This is due to the absence of any study on the effect of rosella calyx in third-degree burns; we thus conducted a preliminary study on the anthocyanin/rosella effect on the re-epithelialization process in third-degree burn treatment, and we conclude that this study is feasible and has no major errors.

The development of this study is still far from perfect considering that this is a novel therapy. We need to study whether it is necessary to combine the anthocyanins from rosella as a topical wound dressing with SSD (also supported by the oral consumption of anthocyanins of rosella) or with other substances, or merely use the anthocyanins of rosella as an oral and topical wound treatment since it already showed the longest re-epithelialization length in group 6 of this study. Additionally, dosage manipulation was not used in this study and should be included in any future study. The administration of anthocyanin of rosella is also unsuitable for certain conditions; it is, for example, not suitable for the treatment of electric, radiation, or chemical burns. Therefore, this research still needs to be investigated further regarding its effectiveness and efficiency.

CONCLUSION

Rosella calyx extract applied topically shows a therapeutic effect in the re-epithelialization process, with no significant difference compared with SSD, the standard for third-degree burn treatment. Despite the lack of significant differences, it may still be too early to draw a conclusion. As the H&E staining images showed a difference between treatment using rosella orally + topically compared with rosella extract orally + SSD, topical rosella extract, and isolated SSD, treatment using rosella orally + topically may lead to a better healing process, with no white blood cell colonies seen and re-epithelialization occurring better than with the other treatments. Therefore, we suggest further studies to investigate this issue. We also suggest further research using immunocytochemicals to count the inflammatory cells or immunohistochemistry staining for inflammation marker staining, using the specific anthocyanin extract from rosella calyx, adding various parameters (such as granulation level, wound contraction, and hyperpigmentation), and using different re-epithelialization measurement techniques.

Conflict of Interest: None declared.

REFERENCES

1. Moenadjat Y. Luka bakar: masalah dan tatalaksana. Jakarta: Balai Penerbit FKUI. 2009.
2. Cobb N, Maxwell G, Silverstein P. Patient perception of quality of life after burn injury: results of an eleven-year survey. J Burn Care Rehab. 1990;11:330-3.
3. Sullivan TP, Eaglstein WH, Davis SC, et al. The pig as a model for human wound healing. Wound Repair Regen. 2001;9:66.
4. Pastar I, Stojadinovic O, Yin NC, et al. Epithelialization in wound healing: a comprehensive review. Adv Wound Care. 2014;3:7.
5. Chen D, Hao H, Fu X, et al. Insight into reepithelialization: how do mesenchymal stem cells perform? Stem Cell Int. 2016;2016:6120173.
6. Rosińczuk J, Taradaj J, Dymarek R, et al. Mechanoregulation of wound healing and skin homeostasis. BioMed Res Int. 2016;2016 .
7. Shen CY, Zhang TT, Zhang WL, et al. Anti-inflammatory activities of essential oil isolated from the calyx of Hibiscus sabdariffa L. Food Funct. 2016;10:4451–9.
8. Dahiru D, Obi OJ, Umaru H. Effect of Hibiscus sabdariffa calyx extract on carbon tetrachloride induced liver damage. Biokemistri. 2003;1:27–33.
9. Da-Costa-Rocha I, Bonnlaender B, Sievers H, et al. Hibiscus sabdariffa L. A phytochemical and pharmacological review. Food Chem. 2014;165:424-43.
10. Yaman I, Durmus AS, Ceribasi S, et al. Effects of Nigella sativa and silver sulfadiazine on burn wound healing in rats. Vet Med. 2010;12:619–24.
11. Engel H, Kao SW, Larson J, et al. Investigation of dermisderived hydrogels for wound healing applications. Biomed J. 2015;38:58–64.
12. Lloyd ECO, Rodgers BC, Michener M, et al. Outpatient burns: prevention and care. Am Fam Physician. 2012;1:25–32.
13. Atiyeh BS, Costagliola M, Hayek SN, et al. Effect of silver on burn wound infection control and healing: review of the literature. Burns. 2007;2:139-48.
14. Poon VK, Burd A. In vitro cytotoxicity of silver: implication for clinical wound care. Burns. 2004;30:140–7.
15. Khanna S, Biswas S, Shang Y, et al. Macrophage dysfunction impairs resolution of inflammation in the wounds of diabetic mice. PLoS One. 2010;5:e9539.
16. Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. Expert Rev Mol Med. 2013;13:e23.
17. Children's Hospital of Wisconsin. Third-degree burns: what is a third-degree burn? (full thickness), http://www.chw.org/medical-care/burn-program/burn-treatments/classification-and-treatment-of-burns/third-degree-burns; 2016. [accessed 12 December 2017].
18. Seneschal J, Clark RA, Gehad A, et al. Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells. Immunity. 2012;36:873–84.
19. Nizamutdinova IT, Kim YM, Chung JI, et al. Anthocyanins from black soybean seed coats stimulate wound healing in fibroblasts and keratinocytes and prevent inflammation in endothelial cells. Food Chem Toxicol. 2009;47:2806–12.
20. Lech M, Anders HJ. Macrophages and fibrosis: how resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair. Biochim Biophys Acta. 2013;1832:989–97.
21. Moore KW, de Waal Malefyt R, Coffman RL, et al. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683–765.
22. Couper KN, Blount DG, Riley EM. IL-10: The master regulator of immunity to infection. J Immun. 2008;180(9):5771–5777.