Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2022 Apr 27; 10(A):1143-1150.
https://doi.org/10.3889/oamjms.2022.8637
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Immunology*

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Epidermal Stem Cell in Wound Healing of *Gliricidia sepium* Leaves from Indonesia and the Philippines in Rats (*Rattus norvegicus*)

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Edited by: Slavica Hristomanova-Mitkovska
Citation: Aulanni'am A, Raissa R, Riawan W, Wuragil DK, Permata FS, Beltran MA. Epidermal Stem Cell in Wound Healing of *Gliricidia sepium* Leaves from Indonesia and the Philippines in Rats (*Rattus norvegicus*). Open Access Maced J Med Sci. 2022 Apr 27; 10(A):1143-1150. https://doi.org/10.3889/oamjms.2022.8637
Keywords: *Gliricidia sepium* leaves; Wound healing; Epidermal; Stem cell; Herbal plant
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Received: 14-Jan-2022
Revised: 14-Apr-2022
Accepted: 17-Apr-2022

Funding: This study was supported by the Professor Research Funding Program 2020 (Grant No: 01/UN10.F09/PN/2020)
Competing Interests: The authors have declared that no competing interests exist

Abstract

AIM: This study intended to investigate the regenerate wound, due to the ointment therapy containing *Gliricidia sepium* leaves that has potential-induced epidermal stem cells producing. It determined its effect on the expression of transforming growth factor-β1 (TGF-β1), Smad-3, β-catenin, LGR-6.

MATERIALS AND METHODS: About 16 Wistar male rats aged approximately 2 months (150–200g) were used and were divided into four treatment groups (T1, positive control; T2, negative control; T3, wounds treated with *G. sepium* from Indonesia; and T4, wounds treated with *G. sepium* from the Philippines). The treatment of ointment was applied to the wound for 3 days. The expression of TGF-β1, Smad-3, β-catenin, and LGR-6 was observed by immunohistochemistry staining.

RESULTS: *G. sepium* leaves significantly ($p < 0.05$) upregulated the expression of TGF-β1, Smad-3, β-catenin, and LGR-6 in the group treated with Indonesian *G. sepium* leaves were higher than that in the group treated with *G. sepium* leaves from the Philippines.

CONCLUSIONS: Both leaves Varian contain flavonoids, saponins, and tannins, which act as producing epidermal stem cell agents to enhance the wound healing process. It can be concluded that both *Gl. sepium* Varian Indonesia and the Philippines have a potential effect on wound healing.

Introduction

Wounds are the destruction of body tissue [1]. Wounds occur in the cutaneous that cause damage to the skin epithelium or the disruption of the normal anatomical structure of the tissue due to trauma [2]. After the injury, cutaneous integrity must be promptly restored to maintain its functions. In this process, cutaneous wound healing is an important step for survival, completing in wound closure [3].

Cutaneous wound healing is a complex process of devitalizing missing cellular structures [4] [5]. The process of tissue repair occurs due to the repair and regenerative abilities of cutaneous tissue. It is related to epidermal stem cells [6]. Epidermal stem cells are multipotent cell types, where the amounts of LGR-6, β-catenin, transforming growth factor-β1 (TGF-β1), and Smad3 protein. These proteins are produced in response to optimally wound healing of tissue damage [7], [8], [9], [10], [11], [12], [13].

A balance of cellular processes is necessary to maintain tissue homeostasis. TGF-β is a cytokine that plays an important role in regulating several cellular processes, including self-renewal and cell differentiation [14]. Smad2 and Smad3 are transcription factors in the TGF- branch through binding between the ligands and the TGF-β1 receptor [15]. TGF-β ligands activate the Smad2/3 intracellular pathway and promote wound contraction resulting in a reduction wound's size area [16], [17].

β-Catenin/Wnt could enhance the healing process. A7B5-Catenin regulates fibroblast behavior during the proliferative phase of dermal wound repair [18]. Lgr6 belongs to the type B family of LGR proteins, which have been intensively studied as markers and regulators of adult stem cells [19].

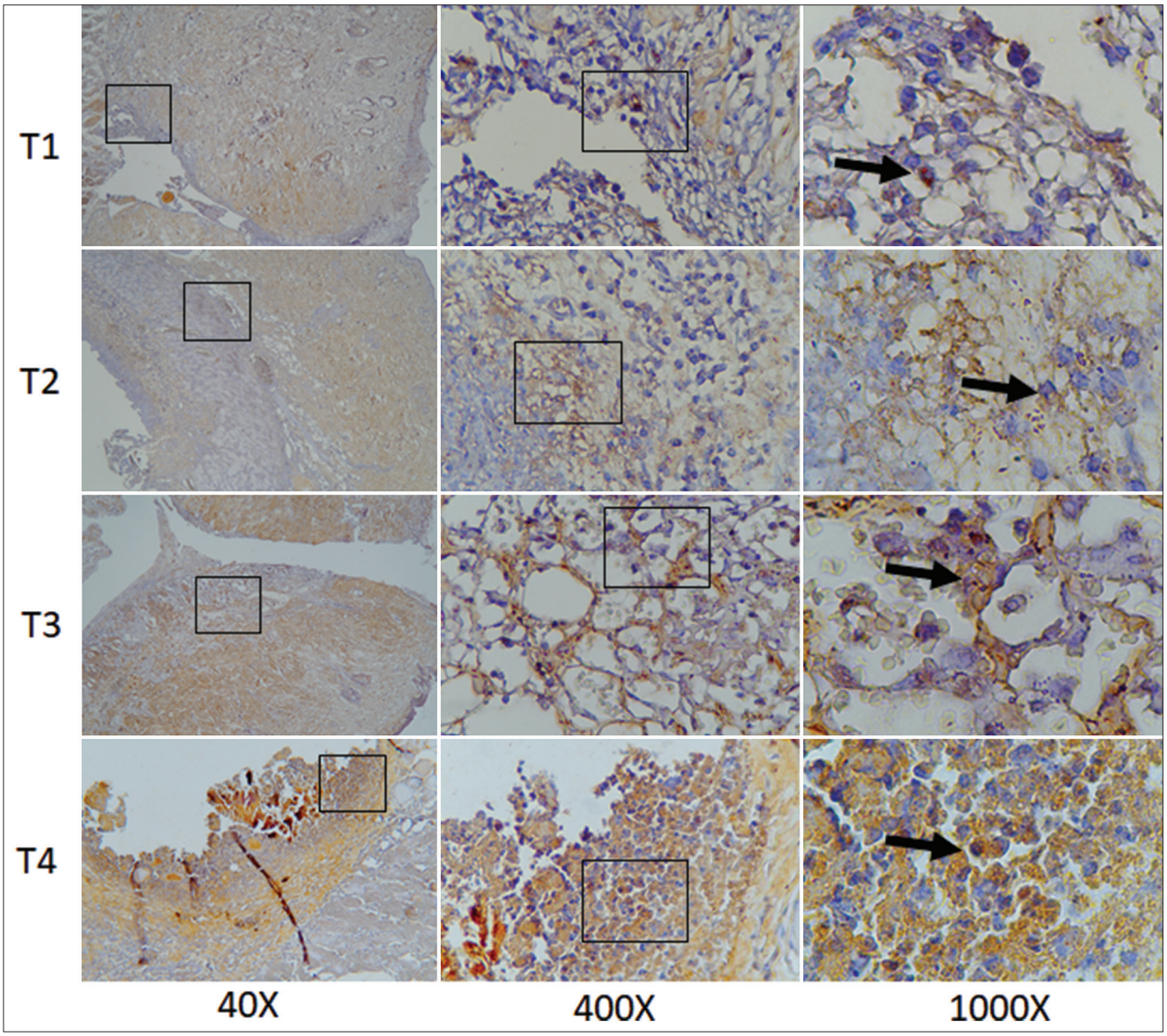

Figure 1: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against transforming growth factor-β1. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with Gliricidia sepium from Indonesia; (T4) treated with Gliricidia sepium from the Philippines

Enhancing β-Catenin results to strengthen the β-Catenin/Wnt signaling pathway [20].

Nowadays, wound therapies are limited, therefore finding to develop better therapeutic strategies is occurring. According to the World Health Organization, 80% of Asian and African populations use traditional medicine or herbal medicine in their healthcare needs, due to easy and low side effects [21]. Leaves are parts that are often used as herbal medicines, one of which is *Gliricidia sepium (G. sepium)* leaves. *G. sepium* is a legume plant belonging to the family *Fabaceae* and is found widely in subtropical and tropical areas, such as in Indonesia and the Philippines [22]. Molina-Botero *et al.* studied its active substances, including flavonoids, saponins, tannins, alkaloids, polyphenols, hydroxyl acid, and coumarin [23]. Aulanni'am *et al.* use *G. sepium* leaves can heal excision wounds with their anti-inflammatory effect because it contains bioactive compounds to enhance the wound healing process [24]. According to research by Carandang *et al.* wound treated with 7.5% gel *G. sepium* on excision wound is safe, effective, and stable [25].

Hence, this study was performed to further determine the efficacy of *G. sepium* leaves as a wound-healing agent based on the evidence of increased potential of the epidermal stem cells as well as increased expression of TGF-β1, Smad3, β-catenin, and LGR-6 protein.

Materials and Methods

Animals and ethical approval

Inbred male *Rattus norvegicus* used in this study were obtained from Institut Biosains Laboratory.

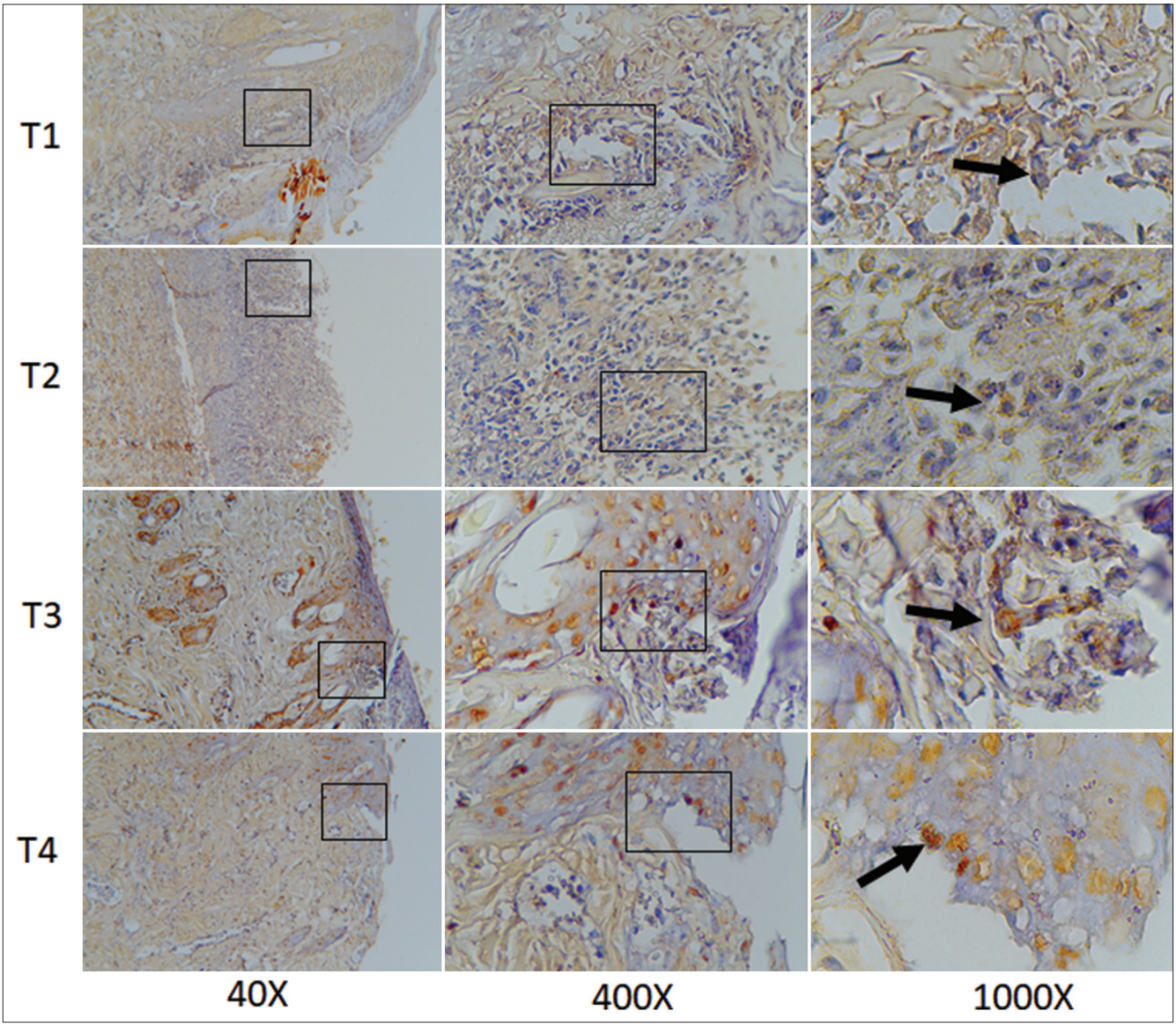

Figure 2: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against Smad3. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with Gliricidia sepium from Indonesia; (T4) treated with Gliricidia sepium from the Philippines

Rats are approximately 2 months old and weigh 150–200 g. The experimental procedures applied in this study were approved by the Brawijaya University Research Ethics Committee (No. 1004-KEP-UB).

Study period

The research was conducted at the Animal Disease and Diagnostic Laboratory, Faculty of Veterinary Medicine, Brawijaya University, Malang, Indonesia, from May to October 2020.

Experimental design

This experiment used a completely randomized experimental design. Rats were divided into four treatment groups comprising four rats per group as follows: T1, positive control, treated with a commercial wound healing agent; T2, negative control; T3, wounds treated with *G. sepium* from Indonesia; and T4, wounds treated with *G. sepium* from the Philippines. The rats were anesthetized with an intramuscular injection of ketamine (10 mg/kg body weight).

Gliricidia sepium preparation and wound treatment

G. sepium leaves from Indonesia and the Philippines were identified in the Plant Taxonomy Laboratory of the Biology Department, Brawijaya University. All leaves were dry-aired and grounded into a powder. After that, powder the ointment by adding petroleum jelly. The ointment was put into the wounds for 3 days.

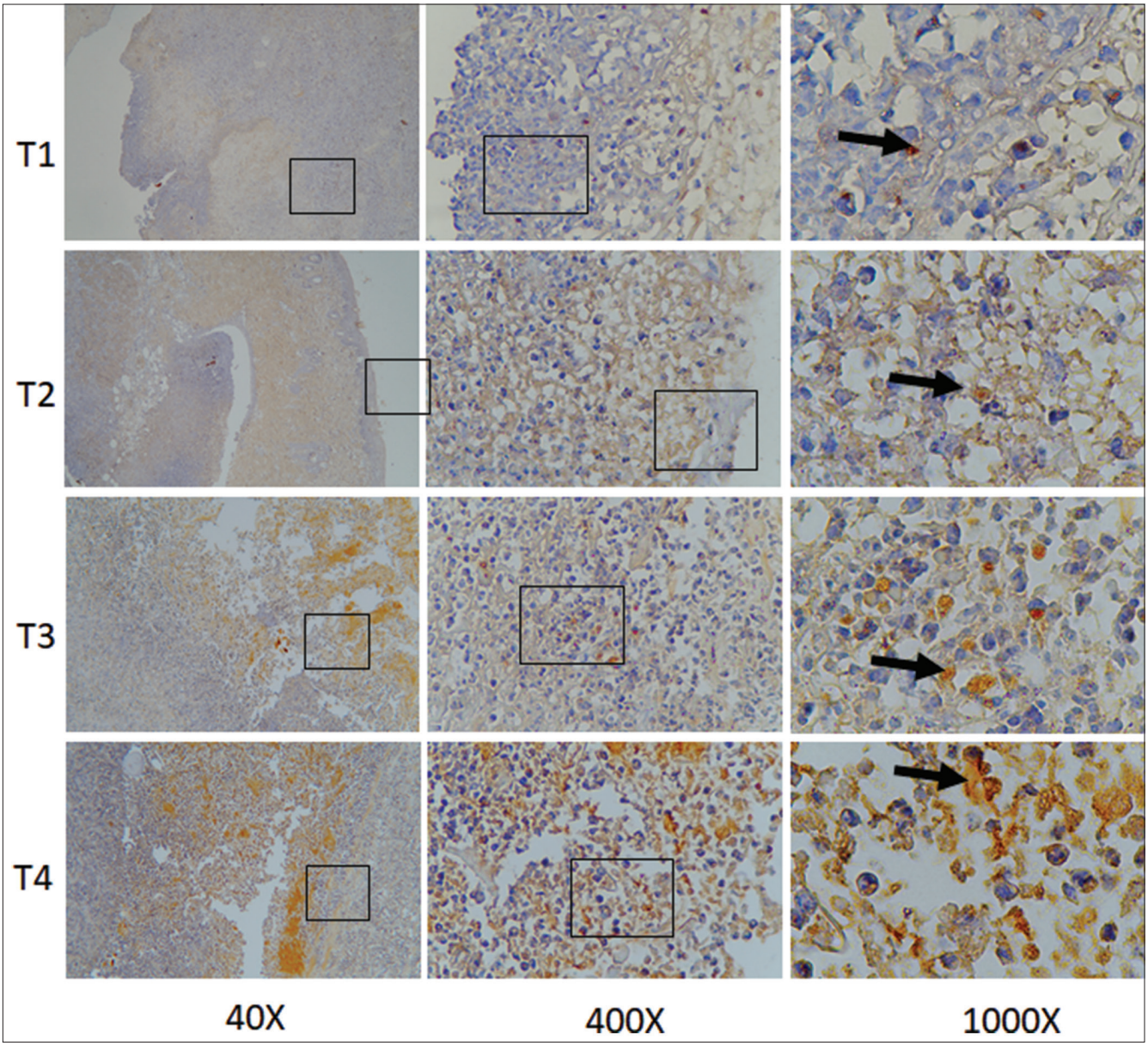

Figure 3: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against β-catenin. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with Gliricidia sepium from Indonesia; (T4) treated with Gliricidia sepium from the Philippines

Measurement of LGR-6, beta-catenin, transforming growth factor-β1, smad-3 expression by immunohistochemistry

Skin samples were processed in the standard protocol of fixation, embedding, deparaffinization, labeling primary antibody (TGF-β1, Smad-3. β-catenin, Lgr-6) and secondary antibody, counterstaining. An immunohistochemistry technique was performed to analyze TGF-β1, Smad3. β-catenin, LGR-6 expression based on the previous methods [26].

Statistical analysis

Statistical analyses were using SPSS software version 14.0 (IBM, USA). The data were analyzed with a one-way analysis of variance and a Tukey test with $\alpha = 0.05$ to determine differences between the treatment groups.

Results and Discussion

Effect of an ointment containing *G. sepium* leaves on TGF-β1, Smad3, β-catenin, LGR-6 expression in immunohistochemistry evaluations, the positive cells show brown color. Immunostaining intensity for TGF-β1, Smad3, β-catenin, and LGR-6 was moderate to strong for both extracts in the treatment group. As shown in Figures 1-4, TGF-β1, Smad3, β-catenin, and LGR-6 immunoreactivity was higher in both extracts

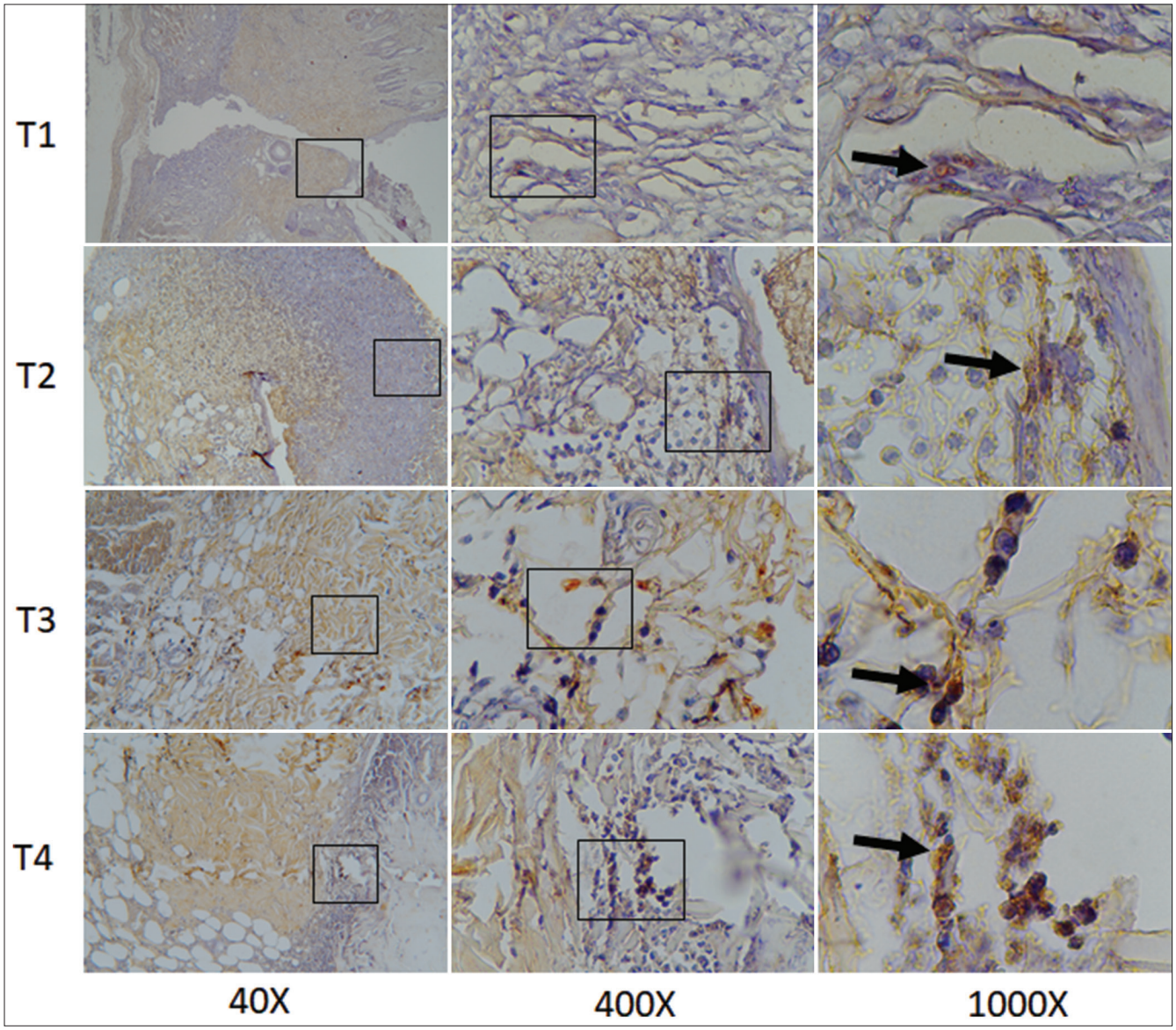

Figure 4: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against LGR-6. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with Gliricidia sepium from Indonesia; (T4) treated with Gliricidia sepium from the Philippines

treated than in the control group. The treatment group had shown a significant increase in H-SCORE than the control group ($p < 0.05$, Table 1).

Table 1: The expression of transforming growth factor-β1, Smad-3, Beta-catenin and LGR-6

Group	TGF-β1	Smad-3	β-catenin	LGR-6
T1	8.33 ± 2.16^{b}	7.33 ± 2.58^{a}	8.17 ± 1.83^{b}	5.17 ± 2.56^{a}
T2	2.83 ± 1.72^{a}	$3.50 \pm 1.87a^{b}$	3.17 ± 1.94^{a}	4.86 ± 2.48^{a}
T3	9.17 ± 1.60^{b}	13.33 ± 2.42^{c}	12.33 ± 2.58^{c}	12.00 ± 2.10^{b}
T4	8.17 ± 2.32^{b}	$10.00 \pm 2.83^{b,c}$	$10.83 \pm 2.48^{b,c}$	10.33 ± 2.16^{b}

T1: Positive control, T2: Negative control, T3: Wounds treated with *G. sepium* from Indonesia, T4: Wounds treated with *G. sepium* from the Philippines. TGF-β1: Transforming growth factor-β1, *G. sepium: Gliricidia sepium*

The result of Smad3, β-catenin, LGR-6, and TGF-β1 expression in this study are shown in Table 1. The Smad3, β-catenin, LGR-6, and TGF-β1 expression level in the negative control group (T2) were obtained below the expression level in the positive group (T1) and the treated group (T3 and T4). Normally, epidermal stem cells in normal conditions act to maintain the skin homeostasis that displaces the lost keratinocyte through normal differentiation and tissue turnover [27]. After treatment, the treated group with *G.sepium* var. Indonesia and Philippine extract ointment increase TGF-β1 protein expression [28]. The release of TGF-β1 happens at an early stage of the healing process to the recruitment of inflammatory cells into the injury area. TGF-β1 encourages the expression of vascular endothelial growth factor that improves the angiogenic process in the injured area and stimulates the fibroblast to contract for closing the wound [29], [30].

The Smad3 expression of the treatment group (T3 and T4) has significantly increased in this study. Smad family proteins are phosphorylated by TGF-β receptors and will activate Smad 3 pathways [31], [32],

[33]. TGF-β/Smad3 plays a role in the development of vascular reconstruction. It is important in the wound healing process [9].

Epidermal stem cells acquire the re-epithelialization process [34]. The treated group (T3 and T4) showed an increase of β-catenin expression that indicates active Wnt signaling through β-catenin. Wnt signaling through β-catenin plays a crucial role in skin regenerating [35]. Wnt/β-catenin signaling is the first molecular signal that is required to instruct epithelial cells [27].

Protein expression of LGR-6 also enhances after both treatments. LGR-6 is responsible as marker adult stem cells for fueling the renewal of the sebaceous gland and skin [36]. LGR-6 is also a Wnt downstream target gene. LGR-6 cells give rise during homeostatic growth [37], [38], [39]. In this study, the LGR-6 protein significantly increases both the treated group; it indicates that there is enhancement of epidermal stem cells to regenerate wounds.

The wound treated with *G. sepium* leaves Varian Indonesia showed increasing the protein expressions of epidermal stem cells, while wounds treated with *G. sepium* Varian the Philippines (T4). Both therapies showed a significant difference ($p < 0.05$) compared with the positive control (T1). *G. sepium* leaves Varian Indonesia and the Philippines contain active ingredients, such as flavonoids, saponins, tannins, and alkaloids that could enhance the epidermal stem cell function and stimulate healing the wound. Cutaneous wound healing is a vital physiological process that involves the cooperation of a variety of cell strains and their products [40], [41], [42], [43], [44], [45], [46].

We report here that *G. sepium* leaves extract ointment enhances the acquisition of epidermal stem cells in wound healing *in vivo* in a rat model. We demonstrated that *G. sepium* treatment significantly improved the expression of LGR-6, β-catenin, TGF-β1, and Smad3 protein in rat skin cells. These findings imply that *G. sepium* leaves extract to improve reprogramming efficiency and tissue regeneration.

Conclusions

These studies suggest that natural plant products from *G. sepium* leaf exhibit positive histopathological effects on *in vivo* wound healing in a rat model. Based on these findings, we suggest that *G. sepium* extracts potentially represent useful supplements for the regeneration of wound healing direct treatment, but this needs to be studied on tissue before animal models.

Authors' Contribution

AA designed the experiment. RR and WR helped statistically. The study was supervised by DKW, FSP, and MAGB.

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