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

Aulanni’am Aulanni’am1, Ricadonna Raissa1, Wibi Riawan1, Dyah Kinashi Wuragil1, Fajar Shodiq Permata2, Ma Asuncion Guiang Beltran3

1Biochemistry Laboratory, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, Indonesia; 2Department of Molecular and Biochemistry, Faculty of Medicine, Brawijaya University, Malang, Indonesia; 3Laboratory of Veterinary Biochemistry, Faculty of Veterinary Medicine, Brawijaya University, Malang, Indonesia; 4Laboratory of Veterinary Histology, Faculty of Veterinary Medicine, Brawijaya University, Malang, Indonesia; 5Department of Microbiology and Veterinary Public Health, College of Veterinary Medicine, Tarlac Agricultural University, Tarlac, Republic of the Philippines

**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, and 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, and LGR-6 was observed by immunohistochemistry staining.  

**RESULTS:** *G. sepium* leaves significantly (p < 0.05) upregulated the expression of TGF-β1, Smad-3, and LGR-6 in the group treated with Indonesian *G. sepium* leaves significantly (p < 0.05) upregulated the expression of TGF-β1, Smad-3, 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 Varian leaves 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-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. A7BS-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].
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.
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 *Gliricidia 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 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 *G. sepium* from the Philippines.](image-url)
Measurement of LGR-6, beta-catenin, transforming growth factor-beta1, smad-3 expression by immunohistochemistry

Skin samples were processed in the standard protocol of fixation, embedding, deparaffinization, labeling primary antibody (TGF-beta1, Smad3, beta-catenin, Lgr-6) and secondary antibody, counterstaining. An immunohistochemistry technique was performed to analyze TGF-beta1, Smad3, beta-catenin, and 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-beta1, Smad3, beta-catenin, LGR-6 expression in immunohistochemistry evaluations, the positive cells show brown color. Immunostaining intensity for TGF-beta1, Smad3, beta-catenin, and LGR-6 was moderate to strong for both extracts in the treatment group. As shown in Figures 1-4, TGF-beta1, Smad3, beta-catenin, and LGR-6 immunoreactivity was higher in both extracts.
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 (TGF-β1), Smad-3, Beta-catenin and LGR-6

| Group | TGF-β1 | Smad-3 | β-catenin | LGR-6 |
|-------|--------|--------|-----------|-------|
| T1    | 8.33 ± 2.18^a | 7.33 ± 2.59^a | 8.17 ± 1.83^b | 5.17 ± 2.56^a |
| T2    | 2.83 ± 1.72^b | 3.50 ± 1.87^a | 3.17 ± 1.94^b | 4.96 ± 2.49^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^a | 10.00 ± 2.83^a | 10.83 ± 2.48^b | 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.

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].
We report here 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.

**References**

1. Gonzalez AC, Costa TF, Andrade ZA, Medrado AR. Wound healing – A literature review. An Bras Dermatol. 2016;91(5):614-20. https://doi.org/10.1590/abd1806-4841.20164741
PMid:27828635

2. Coalson E, Bishop E, Liu W, Feng Y, Spezia M, Liu B, et al. Stem cell therapy for chronic skin wounds in the era of personalized medicine: From bench to bedside. Genes Dis. 2019;6(4):342-58. https://doi.org/10.1016/j.gendis.2019.09.008
PMid:31832514

3. Cañedo-Dorantes L, Cañedo-Ayala M. Skin Acute wound healing: A comprehensive review. Int J Inflamm. 2019;2019:3706315. https://doi.org/10.1155/2019/3706315
PMid:31275545

4. Rodrigues M, Kosaric N, Bonham CA, Gurtner GC. Wound healing: A cellular perspective. Physiol Rev. 2019;99(1):665-706. https://doi.org/10.1152/physrev.00067.2017
PMid:30475656

5. Sorg H, Tilkorn DJ, Hager S, Hauser J, Mirastchijski U. Skin wound healing: An update on the current knowledge and concepts. Eur Surg Res. 2017;58(1-2):81-94. https://doi.org/10.1101/sqb.2008.73.011
PMid:27974711

6. Teng M, Huang Y, Zhang H. Application of stem cells in wound healing – An update. Wound Repair Regen. 2014;22(2):151-60. https://doi.org/10.1111/wrr.12152
PMid:24635168

7. Watt FM, Collins CA. Role of beta-catenin in epidermal stem cell expansion, lineage selection, and cancer. Cold Spring Harb Symp Quant Biol. 2008;73:503-12. https://doi.org/10.1101/sqb.2008.73.011
PMid:19022747

8. Sakaki-Yumoto M, Katsuno Y, Derynck R. TGF-β family signaling in stem cells. Biochim Biophys Acta. 2013;1830(2):2280-96. https://doi.org/10.1016/j.bba.gen.2012.08.008
PMid:22959078

9. Shi X, DiRenzo D, Guo LW, Franco SR, Wang B, Seedial S, et al. TGF-β/Smad3 stimulates stem cell developmental gene expression and vascular smooth muscle cell de-differentiation. PLoS One. 2014;9(4):e93995. https://doi.org/10.1371/journal.pone.0093995
PMid:24718260

10. Ojeh N, Pastar I, Tomic-Canic M, Stojadinovic O. Stem cells in skin regeneration, wound healing, and their clinical applications. Int J Mol Sci. 2015;16(10):25476-501. https://doi.org/10.3390/ijms161025476
PMid:26512657

11. Lichtenberger B, Mastrogiannaki M, Watt F. Epidermal β-catenin activation remodels the dermis via paracrine signalling to distinct fibroblast lineages. Nat Commun 2016;7:10537. https://
Aulanni'am et al. Epidermal Stem Cell in Wound Healing of Gliricidia sepium Leaves from Indonesia and the Philippines in Rats

PMid:26837596

12. Wang PH, Huang BS, Horng HC, Yeh CC, Chen YJ. Wound healing. J Chin Med Assoc. 2018;81(2):94-101. https://doi.org/10.1016/J.JCMA.2017.11.002

13. Yang R, Liu F, Wang J, Chen X, Xie J, Xiong K. Epidermal stem cells in wound healing and their clinical applications. Stem Cell Res Ther. 2019;10(1):229. https://doi.org/10.1186/s13287-019-1312-z

14. Park SR, Lee JH, Kim PH. Smad3 and Smad4 mediate transforming growth factor-beta1-induced IgA expression in murine B lymphocytes. Eur J Immunol. 2001;31(6):1706-15. https://doi.org/10.1002/1521-4141(200106)31:6<1706::AID-ejim1706>3.0.co;2-z

15. Jian H, Shen X, Liu J, Semenov M, He X, Wang XF. Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-mediated proliferation of bone marrow-derived adult human mesenchymal stem cells. Genes Dev. 2006;20(6):666-74. https://doi.org/10.1101/gad.1388806

16. Penn JW, Grobbelaar AO, Rolfe KJ. The role of the TGF-beta1 family in wound healing, burns and scarring: A review. Int J Burns Trauma. 2012;2(1):18-28. PMid:3415964

17. Walton KL, Johnson KE, Harrison CA. Targeting TGF-beta-mediated SMAD signaling for the prevention of fibrosis. Front Pharmacol. 2017;8:461. https://doi.org/10.3389/fphar.2017.00461

18. Cheon SS, Wei Q, Gurung A, Youn A, Bright T, Poon R, et al. Beta-catenin regulates wound size and mediates the effect of TGF-beta in cutaneous fibrosis. FASEB J. 2006;20(6):692-701. https://doi.org/10.1096/fj.05-4759com

19. Liao XH, Nguyen H. Epidermal expression of Lgr6 is dependent on nerve endings and Schwann cells. Exp Dermatol. 2014;23(3):195-8. https://doi.org/10.1111/exd.12340

20. Whyte JL, Smith AA, Liu B, Manzano WR, Evans ND, Dhamdhere GR, et al. Augmenting endogenous Wnt signaling improves the function of mesenchymal stem cells in the wound bed. Front Dev Biol. 2017;5:28. https://doi.org/10.3389/fcell.2017.00028

21. Liao XH, Nguyen H. Epidermal expression of Lgr6 is dependent on nerve endings and Schwann cells. Exp Dermatol. 2014;23(3):195-8. https://doi.org/10.1111/exd.12340

22. Oyebode O, Kandala NB, Chilton PJ, Lilford RJ. Use of traditional medicine in middle-income countries: A WHO-SAGE study. Health Policy Plan. 2016;31(8):984-91. https://doi.org/10.1093/heapol/czw022

23. Molina-Botero IC, Montoya-Flores MD, Zavala-Escalante LM, Barahona-Rosales R, Arango J, Ku-Vera JC. Effects of long-term diet supplementation with Gliricidia sepium foliage mixed with Enterolobium cyclocarpum pods on enteric methane, apparent digestibility, and rumen microbial population in crossbred heifers. J Anim Sci. 2019;97(4):1619-33. https://doi.org/10.1093/jas/sky067

24. Aulanni'am A, Ora KM, Ariandini NA, Wuragil DK, Permata FS, Riawan W, et al. Wound healing properties of Gliricidia sepium leaves from Indonesia and the Philippines in rats (Rattus norvegicus). Vet World. 2021;14(3):820-4. https://doi.org/10.14202/vetworld.2021.820-824

25. Carandang RR, Buemio KC, Lopez A. The wound healing action of kakawati gel from Gliricidia sepium (Jacques) Steudel (Family Fabaceae). JIPTP. 2015;6(4):2642-9.

26. Kim SW, Roh J, Park CS. Immunohistochimistry for pathologists: Protocols, pitfalls, and tips. J Pathol Transl Med. 2016;50(6):411-8. https://doi.org/10.4132/jptm.2016.08.08

27. Blanpain C, Fuchs E. Epidermal homeostasis: A balancing act of stem cells in the skin. Nat Rev Mol Cell Biol. 2009;10(3):207-17. https://doi.org/10.1038/nrm2636

28. Han G, Li F, Singh TP, Wolf P, Wang XJ. The pro-inflammatory role of TGF(R)-beta1: A paradox? Int J Biol Sci. 2012;8(2):228-35. https://doi.org/10.1006/excr.2000.4930

29. Ferrari G, Cook BD, Terushkin V, Pintucci G, Mignatti P. Transforming growth factor-beta1 induces angiogenesis through vascular endothelial growth factor (VEGF)-mediated apoptosis. J Cell Physiol. 2009;219(2):449-58. https://doi.org/10.1002/jcp.21706

30. Ghosh D, McGrail DJ, Dawson MR. TGF-beta1 Pretreatment improves the function of mesenchymal stem cells in the wound bed. Front Dev Biol. 2017;5:28. https://doi.org/10.3389/fcell.2017.00028

31. Mori Y, Chen SJ, Varga J. Modulation of endogenous Smad expression in normal skin fibroblasts by transforming growth factor-beta. Exp Cell Res. 2000;258(2):374-83. https://doi.org/10.1006/excr.2000.4930

32. Owens P, Han G, Li AG, Wang XJ. The role of Smads in skin development. J Invest Dermatol. 2008;128(4):783-90. https://doi.org/10.1038/sj.jid.5700969

33. Nakerakanti S, Trojanowska M. The role of TGF-beta receptors in fibrosis. Open Rheumatol J. 2012;6:156-62. https://doi.org/10.2174/1874312901206010156

34. Li Y, Zhang J, Yue J, Guo X, Wu X. Epidermal stem cells in skin wound healing. Adv Wound Care (New Rochelle). 2016;7(9):297-307. https://doi.org/10.1089/wound.2017.0728

35. Ku AT, Shaver TM, Rao AS, Howard JM, Rodriguez CN, Miao Q, et al. TCF7L1 promotes skin tumorigenesis independently of beta-catenin through induction of LCN2. Elife. 2017;6:e23242. https://doi.org/10.7554/eLife.23242

36. Kretzschmar K, Weber C, Driskell RR, Calonje E, Watt FM. The role of TGF-beta1 in epithelial-mesenchymal transition. Exp Dermatol. 2017;26(9):781-9. https://doi.org/10.1111/exd.13749

37. Romero N, Areche C, Cubides-Cárdenas J, Escobar N, García-Beltrán O, Simirgiotis MJ, et al. In vitro anthelmintic evaluation of Gliricidia sepium, Leucaena leucocephala, and Pithecellobium dulce: FingerPrint analysis of extracts by UHPLC-orbitrap mass spectrometry. Molecules. 2020;25(13):3002. https://doi.org/10.3390/molecules25133002
38. Zhang Y, Guo L, Lu X, Cheng C, Sun S, Li W, et al. Characterization of Lgr6+ cells as an enriched population of hair cell progenitors compared to Lgr5+ cells for hair cell generation in the neonatal mouse cochlea. Front Mol Neurosci. 2018;11:147. https://doi.org/10.3389/fnmol.2018.00147
PMid:29867341

39. Kim JY, Suh W. Stem cell therapy for dermal wound healing. Int J Stem Cells. 2010;3(1):29-31. https://doi.org/10.15283/ijsc.2010.3.1.29
PMid:24855538

40. Saputro ID, Rizaliyana S, Noverta DA. The effect of allogenic freeze-dried platelet-rich plasma in increasing the number of fibroblasts and neovascularization in wound healing. Ann Med Surg. 2022;73:103217. https://doi.org/10.1016/j.jamsu.2021.103217
PMid:35079361

41. Husen SA, Syadzha MF, Setyawan MF, Pudjiastuti P, Ansori AN, Susilo RJ, et al. Evaluation of the combination of Sargassum duplicatum, Sargassum ilicifolium, Abelmoschus esculentus, and Garcinia mangostana extracts for open wound healing in diabetic mice. Syst Rev Pharm. 2020;11(9):888-92. https://doi.org/10.31838/srp.2020.9.129

42. Husen SA, Syadzha MF, Susilo MF, Hayaza S, Ansori AN, et al. A novel therapeutic effects of Sargassum ilicifolium alginate and okra (Abelmoschus esculentus) pods extracts on open wound healing process in diabetic mice. Res J Pharm Technol. 2020;13(6):2764-70. https://doi.org/10.5958/0974-360X.2020.00491.6

43. Puspitaningrum MS, Rahmadhani D, Rizqianti Y, Ridwan RD, Ansori AN, Fadholly A, et al. Freeze-dried epigallocatechin-3-gallate and stem-cells from human exfoliated deciduous-teeth scaffold as the biocompatible anti-relapse material post-orthodontic treatment: A review. Biochem Cell Arch. 2020:2935-42.

44. Fadholly A, Ansori AN, Proboningrat A, Kusala MK, Putri N, Pertwri VR, et al. An investigation on the Euphoria longan (Lour.) Steud Seeds in Wound Healing in Rattus norvegicus. Indian Vet J. 2020;97(2):26-9.

45. Budi AC, Hamid IS, Legowo D. Tekelan leaves (Chromolaena odorata) infusion and 10% Povidone-Iodine on incision wound healing process of mice (Mus musculus) infected with Staphylococcus aureus. World Vet J. 2021;11(1):60-5. https://dx.doi.org/10.54203/scil.2021.wvj8

46. Wulandari PA, Ilmi ZN, Husen SA, Winarni D, Alamsjah MA, Awang K, et al. Wound healing and antioxidant evaluations of alginate from Sargassum ilicifolium and Mangosteen rind combination extracts on diabetic mice model. Appl Sci. 2021;11(10):4651. https://doi.org/10.3390/app11104651