

## Ethanol extract of Neem (*azadirachta indica* a. juss) twigs peel gel increased neovascularization, fibroblast and epithelialization in wound healing of male albino Wistar rats (*Rattus norvegicus*)

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**Introduction:** Proper wound healing is characterized by increased neovascularization, fibroblast cell number and epithelialization. This process can be accelerated using several natural substances and one of them is Neem (*Azadirachta indica* A. Juss) twigs extract. However, its wound healing effect is still poorly investigated. Therefore, the aim of this study was to assess the efficacy of ethanol extract of Neem twigs peel gel toward wound healing in male albino Wistar rats (*Rattus norvegicus*).

**Methods:** A randomized posttest only control group study was conducted using 28 male albino Wistar rats as a subject. Subjects were divided into four groups; the first and second group was the control group which was treated using placebo gel for 4 and 12 days while the third and fourth groups were

treated with 12.5% extract gel for 4 and 12 days. On day 4 and 12, rats were euthanized then examined histologically.

**Results:** The results showed a significant increase of neovascularization in the P1.4 compared to P0.4. However, it decreased significantly at day 12. Similarly, the number of fibroblast cells was also increased in the treatment group until day 4 but decreased significantly and even lower than the control group at day 12. On contrary, epithelialization was continuously increased in both group and the treatment groups consistently demonstrated higher epithelialization compared to control.

**Conclusion:** It can be concluded that ethanol extract of Neem twigs peel gel enhanced wound healing process in male albino Wistar rats.

**Keywords:** epithelialization, fibroblast, neem twigs peel, neovascularization, wound healing

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### INTRODUCTION

Aged skin is characterized by various structural changes and disrupted functions that disturb the integrity of the skin. Aging alter many physiological functions including cell renewal and regenerative capacity of tissues and organs. Therefore, it is undeniable that aging also hampers wound healing process.<sup>1,2</sup>

Wound healing is a complex process that restores the integrity and function of the damaged skin tissue. The wound healing process consists of the coagulation phase, inflammatory phase, the proliferation phase - migration and the remodeling phase. A proper wound healing process is characterized by an increase in new blood vessels (neovascularization) which peaks on the third to the fifth day, increase in the number of fibroblasts, and epithelialization which reaches its peak on the 10th day onwards until that the wound is repaired.<sup>3,4</sup>

Advancing age resulted in deterioration of body

response toward free radicals, which make it more susceptible to oxidative stress. During oxidative stress, the levels of Reactive Oxygen Species (ROS) increased and exceed the endogenous antioxidant capacity to neutralize these free radicals that used to kill bacteria in wounds and prevent infection, but excessive levels of ROS will also slows wound healing process and damage of the surrounding tissue. These free radicals will increase with advancing age due to a decrease in endogenous antioxidant responses.<sup>5</sup>

In clinical setting, a lot of efforts have been made to improve wound healing, most commonly by applying antiseptics. However, some traditional medicines have been known to have potential to accelerate wound healing. One of the herb that has potential to improve wound healing is Neem (*Azadirachta indica* A. Juss).<sup>4</sup>

Neem contains flavonoids that able to neutralize free radicals and modulate the inflammatory process, accelerating wound healing process. High reactivity of the hydroxyl component of flavonoids

plays a key role in free radicals neutralization, thus preventing the activation of inflammatory mediators by free radicals.<sup>6</sup> Additionally, tannin triggers wound contractions, increasing neovascularization and fibroblasts proliferation. Tannin functions as an astringent which constricts the skin pores, stopping exudation and minimizes bleeding.<sup>7</sup> Meanwhile, saponin acts as antiseptic to prevent severe wound infections. Saponin also accelerates epithelialization which improved wound healing and epithelial cover.<sup>7</sup> Therefore, this study was aimed to assess the efficacy of ethanol extract of Neem twigs peel gel toward wound healing in male albino Wistar rats (*Rattus norvegicus*).

## METHODS

### Study design, samples and grouping

This study was an experimental study using a randomized posttest only control group design. *In vivo* study on animal was carried out using 28 male albino Wistar rats (*Rattus norvegicus*). Subjects were injured using punch biopsy, and were divided into four groups. The first group was the control group with placebo gel for 4 days (P0.4), the second group was treated with 12.5% extract gel for 4 days (P1.4), the third group was the control group with placebo gel for 12 days (P0.12) and the fourth group was the treatment group with 12.5% gel extract for 12 days (P1.12). On day 4 and 12, rats were in euthanized then histological preparations were made to examine the neovascularization, fibroblast, and epithelialization.

### Neem twigs peel extraction

The extraction and phytochemical analysis of Neem twigs peel was carried out in the Laboratory of Agricultural Technology, Udayana University. *In vivo* experiment and histological examinations were performed in Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University.

### End-points assessment

The number of neovascularization, fibroblast, and epithelialization were carried out on the

HE-stained skin tissue, and observed under 100 and 400 x magnification using Olympus light microscope. The number of neovascularization and fibroblast were calculated from the average of 3 visual fields. Epithelialization was measured using histomorphometry method.

## RESULTS

Phytochemical test analysis of the Neem (*Azadirachta indica A. Juss*) twigs peel was carried out in the Laboratory of Agricultural Technology, Udayana University. The phytochemical analysis showed that the extract contains several active compounds as shown in Table 1.

Histological examination to assess the number of neovascularization, fibroblast cell and epithelial thickness were conducted using routine HE staining (Figure 1). Results showed that on day 4, the average neovascularization in the P1.4 group was significantly higher than the P0.4 group ( $p=0.002$ ; Fig.1). However, on day 12, neovascularization of the P1.12 group was significantly lower than the P0.12 group ( $p=0.002$ ; Figure 1). This is suggested that the topical administration of 12.5% extracts of the Neem (*Azadirachta indica A. Juss*) twigs peel improves neovascularization in the wound healing process on day four (Figure 1). Neovascularization is a new blood vessel that develop as a new branches of the already exist vascular in the wound tissue. The formation of neovascularization reaches its peak on days 3-5 after the injury and slowly the number will decrease around the 7th day.<sup>3</sup>

Fibroblast cells count also has similar trend as neovascularization. Initially, the number of fibroblast in the P1.4 group was higher than the P0.4 group ( $p<0.001$ ; Figure 1) but it was reversed on the day 12, where the number of fibroblast in control group was significantly higher ( $p<0.001$ ; Figure 1). Thus, this finding supports the vascularization patterns in the treatment group which indicates that the extract accelerate wound healing process. On contrary, epithelial thickness was continuously increase in both group but it was more significant in treatment group both at day 4 and day 12 (Figure 2).

## DISCUSSION

Antioxidants have been extensively studied to accelerate the process of wound healing. In this study, we provide evidences that topical administration 12.5% extracts of the Neem twigs peel accelerated wound healing process which was marked by increasing neovascularization, fibroblast cells, and

**Table 1. Phytochemical Analysis of Neem (*Azadirachta indica A. Juss*) twigs**

	Compound	Concentration	Units
1.	Flavonoid	2000.00	mg/100gr QE
2.	Tanin	10313.12	mg/100gr TAE
3.	Polyphenols	3806.27	mg/100gr GAE
4.	Vitamin C	1644.09	mg/100g
5.	Saponin	+	none

epithelial thickness. These effects were thought to be the result of bioactive compounds in the Neem twigs peel extract. According to phytochemical test, it contains flavonoid, tannin, polyphenols, vitamin C and saponins.

Flavonoids have been widely known to inhibit lipid peroxidation and neutralized free radicals. It also prevents and slows apoptosis as well as enhancing vascularity in injured areas.<sup>8</sup> In addition, the flavonoid content in herbal plants has been proved to accelerate wound healing by increasing the epithelialization process, which is a process of epithelial renewal after injury, which involves proliferation and migration of epithelial cells to the center of the wound followed by wound contraction by myofibroblasts.<sup>9</sup>

Ethanol extract of neem twigs peel also contains saponins and tannins which play an important role in tissue regeneration in the wound healing process and have the ability as an antiseptic. Saponin stimulates collagen synthesis which plays an important role in wound closure and increase tissue epithelialization.<sup>8</sup> Saponins also accelerates epithelial cell migration and induce vascular endothelial growth factor (VEGF) production.<sup>10,11</sup>

On the other hand, tannin acts as an antioxidant, antimicrobial, and vasoconstrictor which help in stopping mild bleeding.<sup>10,12</sup> Tannin also accelerates wound healing with a number of cellular mechanisms, such as antioxidant activity, increasing wound closure and increasing neovascularization as well as triggering fibroblasts proliferation.<sup>13</sup> Overall, high antioxidant activity can accelerate wound healing because it can stimulate antioxidant production in wounds and provide a conducive environment for wound healing.<sup>14</sup> Neem twigs peel extract gel also has antioxidant activity that can increase contraction of the wound and increase the speed of epithelialization which positively affects wound healing.

## CONCLUSION

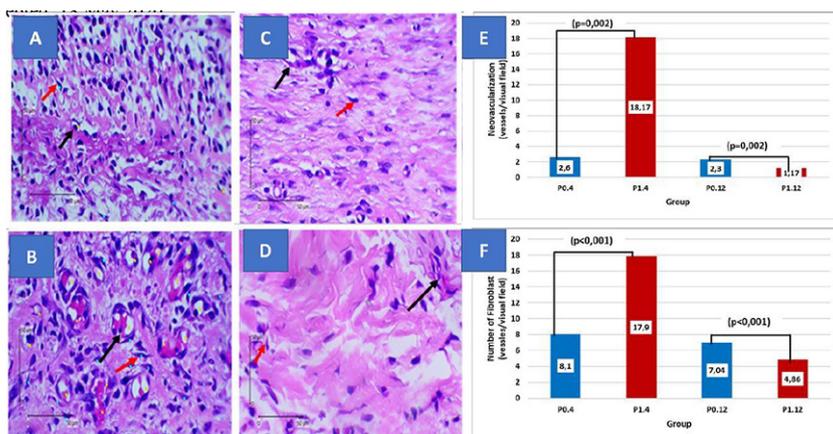
According to the results of this study, it can be concluded that ethanol extract of Neem (*Azadirachta indica* A. Juss) twigs peel gel improved wound healing process by increasing neovascularization, fibroblast and epithelialization in male albino Wistar rats (*Rattus norvegicus*). Further study on long-term effect, optimum dose and clinical trial are required to validate these findings and to assess its clinical applicability.

## CONFLICT OF INTEREST

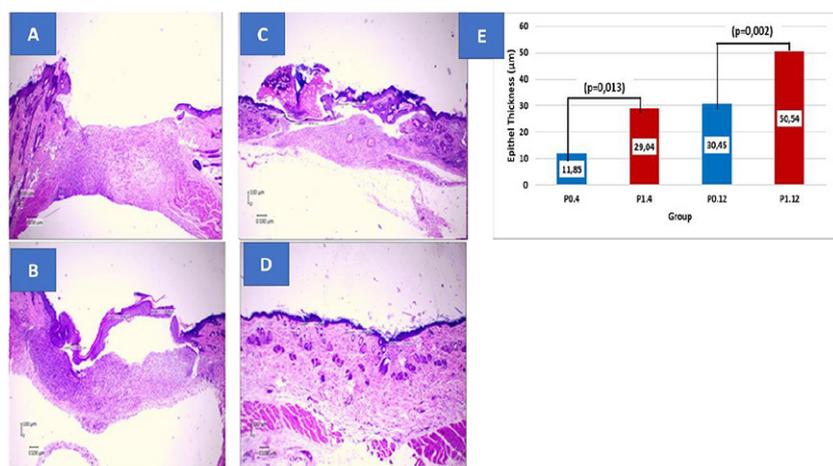
All authors declared that there is no conflict of interest regarding this publication

## AUTHOR CONTRIBUTION

All authors contributed equally in the writing of this article



**Figure 1.** Histological evaluation of neovascularization and fibroblast between sample's groups. (A) Histopathological examination of neovascularization and fibroblast in the P0.4 group. (B) Histopathological examination of neovascularization and fibroblast in the P1.4 group. (C) Histopathological examination of neovascularization and fibroblast in the P0.12 group. (D) Histopathological examination of neovascularization and fibroblast in the P1.12 group. (Black Arrow indicates neovascularization, red arrow indicates fibroblast cell; HE staining, 400x magnification). (E) Graphical comparison of neovascularization among group, (F) Graphical comparison of fibroblast cell among group. P0.4= Control group day 4, P1.1= Treatment group day 4, P0.12= Control group day 12, P1.12= Treatment group day 12.



**Figure 2.** Histological assessment of epithelialization between study groups. (A) Histopathological examination of epithelial thickness in the P0.4 group. (B) Histopathological examination of epithelial thickness in the P1.4 group. (C) Histopathological examination of epithelial thickness in the P0.12 group. (D) Histopathological examination of epithelial thickness in the P1.12 group. (HE staining, 100x magnification). (E) Graphical comparison of epithelial thickness among group, P0.4= Control group day 4, P1.1= Treatment group day 4, P0.12= Control group day 12, P1.12= Treatment group day 12.

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**ETHIC APPROVAL**

This study had been ethically approved by ethical commission of Faculty of Medicine Udayana University with approval letter number 339/KE-PH-Lit-2/VII/2019

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