Effects of the *Hydnophytum formicarum* plant extract on collagen density, angiogenesis, wound length, and re-epithelialization in wound healing: Experimental study on rats

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Abstract

Background. The formation of scar tissue in the wound healing process is associated with fibroblasts that are produced during the proliferation phase (3–14 days after surgery/injury). One of the strategies to suppress the formation of excessive scar tissue is to use wound care material. The use of herbal extracts is currently being investigated by researchers, as it allows avoiding the side effects of synthetic drugs. The *Hydnophytum formicarum* extract has antioxidant and anti-inflammatory potential.

Objectives. The aim of the study was to analyze the effects of the *Hydnophytum formicarum* plant extract on collagen density, angiogenesis, wound length, and re-epithelialization in wound healing.

Material and methods. Twenty-four Sprague–Dawley rats were divided into 2 groups: the control group; and the treatment group. Skin wounds were made on the dorsum of the rats, using the biopsy punch technique. Four rats from each group were sacrificed on days 4, 7 and 14 after injury. Collagen density, angiogenesis, wound length, and re-epithelialization were analyzed using hematoxylin and eosin (H&E) staining and Masson's trichrome staining.

Results. There were significant differences in the results of the angiogenesis analysis, wound length and re-epithelialization between the treatment and control groups. When considering angiogenesis, there were fewer vessels in the treatment group, but they were more mature as compared to the control group. There was also a meaningful interaction between the application of the *Hydnophytum formicarum* extract and the necropsy day with regard to collagen density and the re-epithelialization rate. No secondary infection was found in either group.

Conclusions. The topical use of the *Hydnophytum formicarum* extract affected the formation of scar tissue, as indicated by the positive area of collagen, the extent of angiogenesis, wound length, and the re-epithelialization rate in the early, middle and final granulation phases. The inhibition of angiogenesis through the application of *Hydnophytum formicarum* was probably related to the formation of scar tissue in the wound.

Keywords: angiogenesis, wound healing, scar formation, *Hydnophytum formicarum*

Introduction

Oral and maxillofacial surgeons often treat patients with skin wounds; the occurrence of these wounds activates the wound healing process. Wound healing is a process of restoration of the anatomical and physiological integrity of the injured tissues that consists of several phases (hemostasis, inflammation, proliferation, and remodeling).[1,2] Scar tissue that forms during the wound healing process is indeed a common complication. Hypertrophic scar tissue results in skin deformity, malfunctioning and psychological distress.[3]

During the inflammation phase, macrophages release cytokines and growth factors that attract fibroblasts to the wounded area. The proliferation phase starts on the 3rd day after injury and is marked by the presence of fibroblasts.[1] Fibroblast activities include the production of collagen, the induction of angiogenesis and the contraction of wounds, as well as the facilitation of re-epithelialization, which leads to the formation of hypertrophic scar tissue if it happens excessively.[4]

Many strategies have been used to reduce excessive scar tissue, including the use of herbal agents.[5] People tend to use herbal medicines to avoid the side effects of synthetic drugs, chiefly in developing countries.[6] *Hydnophytum formicarum* is one of many medicinal plants known to Southeast Asian people. The tuber part of this plant is believed to cure various diseases. In addition, it is generally accepted that *Hydnophytum formicarum* has a significant anti-inflammatory effect that inhibits scar formation.[7,8] Thus, this study aimed to collect baseline data and analyze the effects of *Hydnophytum formicarum* on collagen density, angiogenesis, wound length, and the re-epithelialization rate as an indicator of scar formation in the early, middle and late proliferation phases.

Material and methods

This research was conducted at the Faculty of Medicine of the University of Indonesia, Jakarta, and the Faculty of Veterinary Medicine of the IPB University (Institut Pertanian Bogor), Bogor, Indonesia, from August until November 2020.

Hydnophytum formicarum extract

The *Hydnophytum formicarum* plants were cleaned, dried and macerated using ethanol. The separation of ethanol and the active compound of the extract was accomplished by applying the rotary evaporator technique. The density and percentage of the active compound were determined.

Rats

Twenty-four 8-week-old Sprague–Dawley rats with a body weight of 200–300 g were used in this study. The rats were randomly divided into 2 groups: the control group; and the treatment group. The rats were kept in accordance with the applicable standards in animal cages in the animal research facilities of the Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, University of Indonesia. The experiment was approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia (protocol No. 20-07-0783), as well as Dr. Cipto Mangunkusumo National Central Public Hospital, Jakarta, Indonesia.

Wound model

Anesthesia was performed with an intraperitoneal injection of a ketamine/xylazine solution. Circular, full-thickness skin wounds of a diameter of 8 mm were made in the dorsum of the rats with punch biopsy tools. Wound dressing changes were carried out every day and in the treatment group, the *Hydnophytum formicarum* extract was applied topically on the wounds. Four rats from each group were anesthetized and sacrificed on days 4, 7 and 14, using the exsanguination technique. No antibiotics or analgesics were used in this study.

Wound healing observation

The wound areas were resected and fixated in 10% formalin. Histopathological observations were carried out using hematoxylin and eosin (H&E) staining and Masson's trichrome staining. Quantitative analyses of collagen density, angiogenesis, wound length, and re-epithelialization were performed using the ImageJ software (https://imagej.nih.gov/ij/index.html).

The appearance of collagen density areas in Masson's trichrome staining is characterized by a bluish color in thick, wavy and having transverse fibers cytoplasm without the nucleus. The collagen density area [mm^2] was measured as the total area of collagen in relation to the total wound area in 6 regions. The image of angiogenesis which emerges from H&E staining is in the form of purple endothelial cells and red-colored erythrocytes. The total number of blood vessels found in the 6 regions was used as a measurement of angiogenesis. Wound length [mm] was determined by measuring the distance between both edges of the wound, based on the H&E staining examination. The re-epithelialization rate was calculated using the following equation (Equation 1):

$$\text{re-epithelialization rate} = \frac{S_t}{S_0} \times 100\% \qquad (1)$$

where:
S_t – residual wound area at the indicated time; and
S_0 – initial wound area.

Statistical analysis

The data was statistically analyzed using the IBM SPSS Statistics for Windows software, v. 26.0 (IBM Corp., Armonk, USA). The normality of data distribution was tested using the Shapiro–Wilk test, and the parametric two-way analysis of variance (ANOVA) was performed to compare differences between the groups and the necropsy days.

Results

The values for collagen density, angiogenesis, wound length, and the re-epithelialization rate are presented in Table 1. Generally, the average values of collagen density and the mean re-epithelialization rates in both the control and treatment groups increased from the early to late proliferation phases, while wound length decreased. The average angiogenesis values in both the control and treatment groups peaked in the middle proliferation phase (Fig. 1).

The Shapiro–Wilk test showed normally distributed data ($p > 0.05$). The two-way ANOVA with Tukey's post hoc honestly significant difference (HSD) test was used to compare the wound healing variables (collagen density, angiogenesis, wound length, and the re-epithelialization rate) between the groups and the necropsy days, as presented in Table 2 and Table 3.

Table 1. Collagen density, angiogenesis, wound length, and the re-epithelialization rate in the study groups on different necropsy days

Variable	Control group			Treatment group		
	day 4	day 7	day 14	day 4	day 7	day 14
Collagen density area [mm²]	0.04 ±0.03	0.10 ±0.05	0.29 ±0.33	0.04 ±0.01	0.13 ±0.07	0.19 ±0.02
Angiogenesis (*n*)	46.75 ±12.97	70.50 ±15.02	41.00 ±23.65	11.75 ±7.13	40.25 ±16.21	19.00 ±7.43
Wound length [mm]	9.66 ±2.62	6.24 ±3.02	2.18 ±4.23	12.73 ±4.96	9.62 ±2.36	7.27 ±2.83
Re-epithelization rate [%]	0.38 ±0.49	18.61 ±11.35	84.84 ±30.31	2.27 ±1.63	14.59 ±10.29	36.25 ±16.09

Data presented as mean ± standard deviation (*M* ±*SD*). *n* – total number of blood vessels.
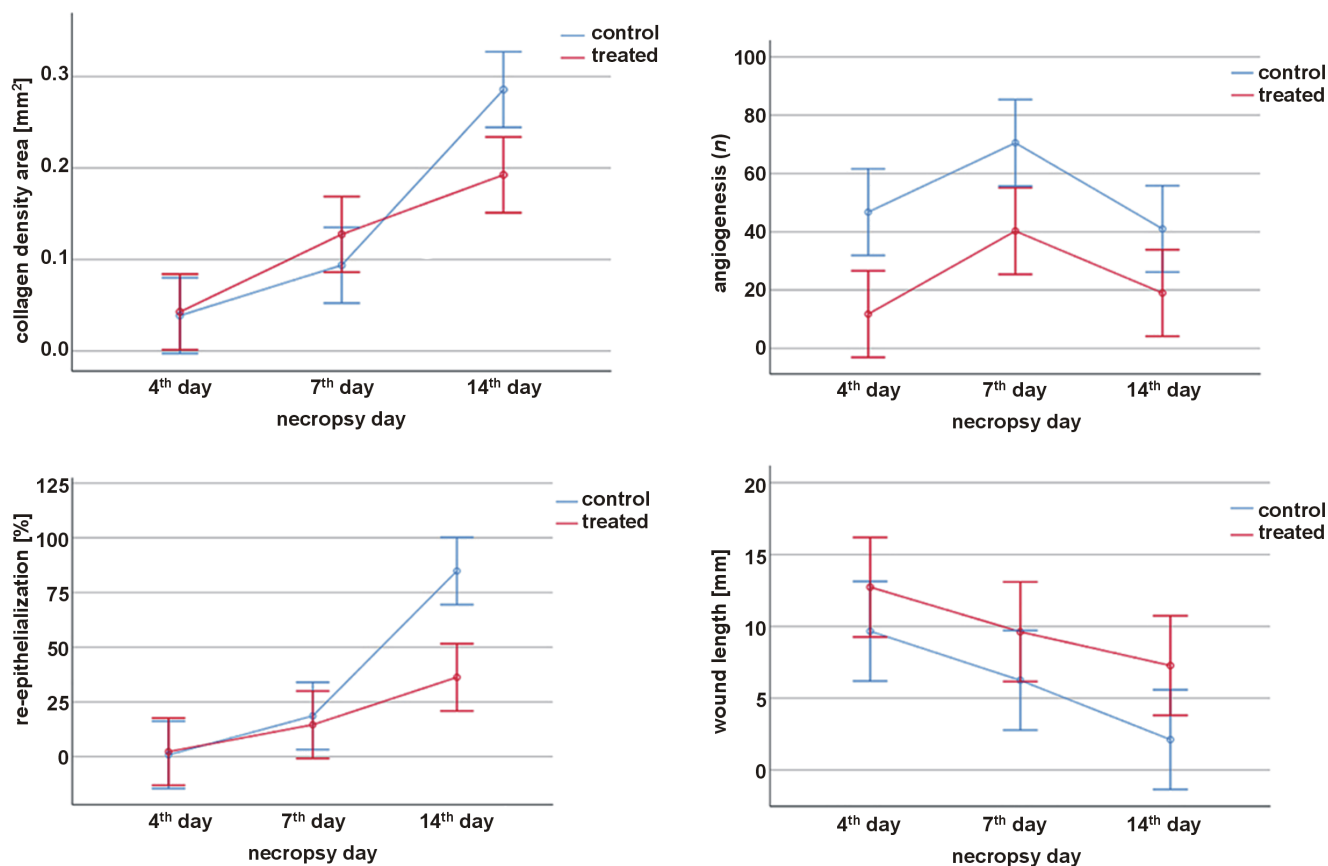

Fig. 1. Line graphs showing collagen density, angiogenesis, wound length, and the re-epithelialization rate in the study groups on different necropsy days

Table 2. Results of the two-way ANOVA with regard to differences in collagen density, angiogenesis, wound length, and the re-epithelialization rate between the groups and the associated necropsy days

Variable	Comparison		F	p-value
Collagen density	groups	control treatment	1.201	0.288
	necropsy days	4 7 14	47.359	0.000*
	interaction test group–necropsy day		5.159	0.017*
Angiogenesis	groups	control treatment	23.026	0.000*
	necropsy days	4 7 14	8.028	0.003*
	interaction test group–necropsy day		0.393	0.681
Wound length	groups	control treatment	7.473	0.014*
	necropsy days	4 7 14	7.035	0.006*
	interaction test group–necropsy day		0.211	0.812
Re-epithelization rate	groups	control treatment	23.026	0.014*
	necropsy days	4 7 14	8.028	0.000*
	interaction test group–necropsy day		0.393	0.008*

* statistically significant.

Table 3. Results of Tukey's post hoc HSD test with regard to differences in collagen density, angiogenesis, wound length, and the re-epithelialization rate between different necropsy days

Variable	Necropsy days	p-value
Collagen density	4–7	0.009*
	4–14	0.000*
	7–14	0.000*
Angiogenesis	4–7	0.007*
	4–14	0.994
	7–14	0.008*
Wound length	4–7	0.151
	4–14	0.000*
	7–14	0.000*
Re-epithelization rate	4–7	0.173
	4–14	0.004*
	7–14	0.176

* statistically significant.

Collagen density

The smallest average area of collagen density was in the control group on necropsy day 4 (0.04 ±0.03 mm²), while the largest average area was in the control group on necropsy day 14 (0.29 ±0.33 mm²). The two-way ANOVA revealed a statistically significant difference between all necropsy days. In addition, there was a statistically significant interaction between the study group and the necropsy day, influencing the average area of collagen density.

Angiogenesis

In terms of angiogenesis, it was found that the average number of blood vessels in the treatment group was lower than in the control group on all necropsy days. The peak in the control group was on necropsy day 7 (40.25 ±16.21). Furthermore, the multivariate analysis showed that there were significant differences in the average number of blood vessels between the treatment group and the control group, and between the necropsy days.

Wound length

The shortest average wound length was found in the control group on necropsy day 14 (2.18 ±4.23 mm), while the longest was found in the treatment group on necropsy day 4 (12.73 ±4.96 mm). The multivariate analysis revealed that there were significant differences in the average wound length between the treatment group and the control group, and between the necropsy days.

Re-epithelialization rate

The lowest average re-epithelialization rate was found in the control group on necropsy day 4 (0.38 ±0.49%), while the highest average re-epithelialization rate was found in the control group on necropsy day 14 (84.84 ±30.31%). The two-way ANOVA showed that there was a statistically significant interaction between the study group and the necropsy day, influencing the average value of the re-epithelialization rate.

Discussion

This study found that the collagen density area, angiogenesis (the number of blood vessels), wound length, and the level of re-epithelialization were significantly different on different necropsy days. The values for collagen density and the re-epithelialization rate increased with time, which was influenced by the increased fibroblast activity required for wound healing in the granulation phase. Meanwhile, the length of the wound tended to decrease, reflecting that the longer a wound undergoes the wound healing process, the shorter the wound length is because of the wound contraction process. The angiogenesis value was at its highest on necropsy day 7, indicating the commencement of angiogenesis in the initial proliferation phase and its continuous rise in the middle proliferation phase until it attained its peak and started to decrease in order to prevent excessive scarring.

A study conducted by Ismardianita et al. found that the oral administration of the *Hydnophytum formicarum* plant extract had an inversely proportional relationship with the number of macrophages in the inflammatory phase of wound healing after tooth extraction.[9] Macrophages play an important role in the secretion of cytokines to attract fibroblasts that become an essential component in the proliferation phase.[10] As already known, fibroblasts are responsible for important actions that support wound healing, such as the production of collagen, angiogenesis, wound contraction, and re-epithelialization. However, these activities can cause the formation of scar tissue if they occur excessively.[4] Previous studies determined that decreasing the number of macrophages in the inflammatory phase could be correlated with the suppressed fibroblast activities.[9,10]

A study conducted by Velanita et al. found that the administration of the *Hydnophytum formicarum* plant extract orally at a dose of 4.65 mg was able to significantly increase the production of collagen fibers in wound healing in the oral cavity after tooth extraction.[11] This is in contrast to the results of the present study, which indicated that collagen density in the control and treatment groups did not present significantly different values, although there was a statistically significant interaction between the use of the extract and the necropsy day, affecting collagen density. This interaction indicates that the *Hydnophytum formicarum* plant extract may change the value of collagen density when used for 14 days. Differences between the results of the present collagen density analysis and previous studies are probably related to the choice of tissue morphometric analysis. In this study, a quantitative method was employed, using the image processing software, whereas previous research used qualitative analyses, which tend to be more subjective.[12] Another potential cause of differences between the present study and previous research is the concentration of the administered extract; in the current study, pure extract without dilution was used. Moreover, the extract was applied topically, whereas in previous studies, it was administered orally.[9,11]

With regard to angiogenesis, this study indicates that the topically applied *Hydnophytum formicarum* plant extract can significantly reduce angiogenesis that occurs during the early, middle and late proliferation phases. This result is consistent with research by Ismardianita et al., who concluded that the oral administration of the *Hydnophytum formicarum* plant extract at a dose of 6.2 mg was able to significantly reduce angiogenesis in the proliferation phase.[9] By contrast, research conducted by Putri and Ismardianita found that the orally administered *Hydnophytum formicarum* plant extract at a dose of 4.65 mg was able to significantly increase angiogenesis.[13] Wound healing with minimal to no scarring occurs in the oral mucosa and the fetal skin in the uterus, presenting less angiogenesis as compared to wound healing in the skin; however, the capillaries become mature more rapidly. Many new capillaries in the healing wounds in the skin do not function effectively.[14] Histopathologically, less angiogenesis was observed in the treatment group in this study, but the blood vessels were more mature (Fig. 2).

Several clinical studies have concluded that excessive angiogenesis is associated with the formation of keloids. In addition, previous animal studies suggested that the partial inhibition of angiogenesis resulted in less hypertrophic scarring.[14] This effect occurs because of the presence of pericytes that stabilize the newly formed blood vessels. Therefore, the extent of angiogenesis parallels the number of pericytes. Pericytes play a role in myofibroblast transition, which affects the fibrosis process; thus, reducing angiogenesis leads to a decrease in the number of pericytes and suppresses the formation of excessive scar tissue.[14,15]

In the initial proliferation phase in this study, the re-epithelialization rate in the treatment group was higher than in the control group. However, in the middle and late proliferation phases, the re-epithelialization rate in the treatment group was lower than in the control group. Statistical analysis determined that the difference in the re-epithelialization rate between the control and treatment groups was significant, and that there was a significant interaction between the study group and the necropsy
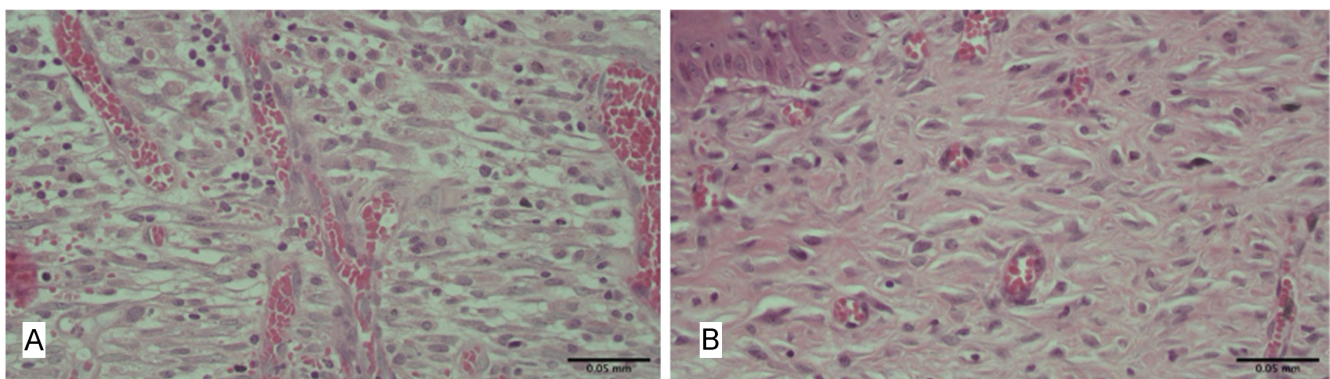

Fig. 2. Histopathological observation of angiogenesis on necropsy day 7
A – treated group; B – control group.

day, influencing the re-epithelialization rate. The re-epithelialization process is closely related to wound length. In this study, the wound length results were inversely proportional to the rate of re-epithelialization. Research on the effect of the *Hydnophytum formicarum* plant extract on re-epithelialization and wound length has not been conducted yet, but based on the prior knowledge of the wound healing process, it can be suggested that the re-epithelialization rate should be directly proportional to fibroblast activities. On the other hand, wound length is inversely proportional to the level of re-epithelialization and fibroblast activities.[10] This study concluded that the use of the *Hydnophytum formicarum* plant extract could significantly reduce the re-epithelialization rate if used for a sufficient period of time. Meanwhile, the observation of wound length indicated that the treatment group had significantly greater wound length values throughout the granulation phase. This led to the conclusion that the *Hydnophytum formicarum* plant extract has a significant effect on reducing wound length.

The production of collagen by fibroblasts in a balanced condition plays an important role in wound healing. Collagen deficiency can lead to chronic wounds, resulting in prolonged re-epithelialization, whereas excessive collagen production can lead to hypertrophic scarring.[4] The use of hypertrophic scar-preventing agents can have side effects if it is not properly timed. This initial study succeeded in confirming the ability of the *Hydnophytum formicarum* plant extract to suppress factors that cause scar tissue, but it was accompanied by a decrease in the re-epithelialization rate, which affected wound length. Therefore, further research is needed on this plant extract with different application times in the wound healing phase. One example of a wound care agent that is used topically to prevent scarring is silicone gel. The application of silicone gel is carried out when the wound has undergone full epithelialization, since it is considered that the material has an unfavorable effect if it is applied on an open wound.[16]

The systemic use of active ingredients administered via the oral route or with an intravenous injection has several drawbacks, such as toxicity on other organs, and requires higher doses to achieve a therapeutic effect. On the contrary, the advantage of the topical use of active ingredients consists in their continuous application while preventing them from being metabolized hepatically and in the gastrointestinal tract.[17] Based on these reasons, this study was conducted using active ingredients administered topically.

As reported in previous studies, the use of antibiotics and analgesics can influence the wound healing process. Antibiotics can suppress interleukin 1 beta (IL-1β), C-C motif chemokine ligand 2 (CCL2) and interferon alpha/beta (IFN-α/β), thereby leading to slower wound healing. Studies on the effect of non-steroidal anti-inflammatory drugs (NSAIDs) on wound healing provided conflicting results, yet the role of NSAIDs in inhibiting the COX pathway, which is related to the proliferation phase,

is undeniable. Antibiotics and analgesics were not used in this study to prevent bias. In general, this study demonstrated normal morphology in the wound healing process in both groups. Also, there were no signs of secondary infection in any of the rats.

Conclusions

The topical use of the *Hydnophytum formicarum* plant extract affected the formation of scar tissue, as demonstrated by the positive area of collagen, the extent of angiogenesis, wound length, and the re-epithelialization rate in the early, middle and final granulation phases. The inhibition of angiogenesis through the application of *Hydnophytum formicarum* was probably related to the formation of scar tissue in the wound. However, the plant extract showed the ability to reduce the re-epithelialization rate, which affected wound length. Accordingly, further research is needed on this plant extract with different application times in the wound healing phase to achieve an optimal anti-scar tissue effect with minimal unwanted complications.

Ethics approval and consent to participate

The experiment was approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia (protocol No. 20-07-0783), as well as Dr. Cipto Mangunkusumo National Central Public Hospital, Jakarta, Indonesia.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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