

Topical Binahong (*Anredera cordifolia*) Leaf Extract Increases Interleukin-6 and VEGF (Vascular Endothelial Growth Factor) during Burn Wound Healing in Wistar Rats Infected with *Pseudomonas aeruginosa*

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Abstract

Burn injuries still remain a major problem. It leads to nosocomial infection by *Pseudomonas aeruginosa*, the most common bacteria that infects burn injuries, due to its long duration of hospitalization. Previous research found that the extract of *Anredera cordifolia*, locally known as "binahong" in Indonesia, is effective in burns healing. Interleukin-6 (IL-6) and VEGF are substances that are important in healing process. This study aims to determine how topical *Anredera cordifolia* leaf extract accelerates burn wound healing, increases IL-6 level, and increases VEGF production in rats with *Pseudomonas aeruginosa*-infected burn injuries. This is an experimental study with the *Posttest Only Control Group Design*, involving a total of 32 samples of wistar rats with infected burn wounds. The treatment group received 2 ml of the topical extract of *Anredera Cordifolia* leaf while the control group received 2 ml of topical tetracycline 3%. The analysis of plasma IL-6 level was done on day 3, the VEGF analysis was done on day 5, and the observation of wound closure was done on days 3, 5, and 7. Independent t-Test was performed to obtain the mean difference of IL-6 and VEGF in control and treatment groups. The treatment group was found to have faster wound healing. Plasma IL-6 and VEGF in the treatment group was significantly higher than those in the control group (IL-6 $p = 0.001$, and VEGF $p = 0.001$). The application of topical *Anredera cordifolia* leaf extract accelerates burn wound healing, increases IL-6 level, and increases VEGF production in burns infected by *Pseudomonas aeruginosa*.

Keywords: *Anredera cordifolia*, IL-6; VEGF; burn wound healing; *Pseudomonas aeruginosa*

Introduction

Burn injuries, especially above first-degree burn injuries, still remain a major problem: high treatment defrayment, mortality, and morbidity, as well as a long duration of hospitalization, resulting in patients becoming prone to nosocomial infection.

Pseudomonas aeruginosa, which is resistant to extreme temperatures, is the most common bacteria that infects burn injuries and the major cause of nosocomial infections in hospitals, leading to sepsis and death. The infection that occurs in burn injuries is often accompanied by antibacterial drug resistance, which makes the treatment become more complex [1]. *Pseudomonas aeruginosa* is resistant to many antibacterial drugs and multiplies rapidly if normal flora is suppressed [2,3].

Allergic reaction and skin irritation often occur in commonly used topical, antibacterial, disinfectant drugs that are used to treat burn injuries with infection, leading to delay skin regeneration and prolonging the duration of hospitalization. Silver sulfadiazine (SSD) is a common topical treatment used in burns. In addition to delayed wound healing process, it also evokes kidney toxicity and leukopenia, which increase the cost of treatment, so SSD is not recommended for the long-term treatment of burns.

Burn injury treatments with short hospitalization duration is still a challenge. Reports from the use of herbal medicine showed that herbal medicinal plants have a promising therapeutic effect with low toxicity. It is also less expensive compared with synthetic drugs [4,5].

Natural ingredients, particularly plants that have medicinal properties, become more dependable, mainly for its pharmacological effects as an antibacterial and cell regeneration.

Anredera cordifolia, locally known as "binahong" in Indonesia, is a traditional plant that has medicinal properties. Amertha, 2007, found that the extract of *Anredera cordifolia* leaf proves to be more effective in healing burn injuries in chicks compared with the drug that is commonly used to treat burns [6]. Based on this result, it is estimated that the mechanism involved in the process of burn wound healing is through faster cell regeneration.

Wound healing process consists of four phases: hemostasis, inflammation, proliferation, and maturation. Interleukin 6 (IL-6), secreted by T-cell and macrophage, is one of the foremost inflammatory markers at the inflammation phase. In this phase, macrophage stimulates the secretion of *vascular endothelial growth factor* (VEGF), which triggers angiogenesis and plays an important role in the proliferation phase by increasing the amount of capillary vessels under the site of injuries [7,8]. This study aims to determine the effect of topical *Anredera cordifolia* leaf extract on the acceleration of burn wound healing observed by the rate of wound closure, the increase of IL-6, and the increase of VEGF level in rats with *Pseudomonas aeruginosa*-infected burn injuries.

Materials and Methods

Research design

This is an experimental study using the posttest only control group design to test binahong (*Anredera cordifolia*) leaf extract to increase IL-6

and VEGF and accelerate the closure of burn wound in rats infected with *Pseudomonas aeruginosa*.

Place and time

The research was conducted at the Analytical Laboratory of the University of Udayana to make binahong leaf extract, rats breeding, burn wound making, and examination of IL-6, VEGF, and burn wound closure. Also, the Laboratory of Microbiology, Faculty of Medicine, Udayana University, was used for the *Pseudomonas aeruginosa* growth. This research study was carried out over a short duration, starting from January 2016 through March 2016.

Population and sample

Thirty-two male wistar rats were used in this study, which were divided into two groups. Qualified samples were the ones who fitted the inclusion criteria: wistar, male, age of two months, 200-250 g, and suffering from burn injuries infected with *Pseudomonas aeruginosa*. In the control group, rats were treated with 2 ml of topical tetracycline 3%. In the treatment group, rats treated with 2 ml of the topical extract of *Anredera Cordifolia* leaf. The analysis of plasma IL-6 level was done on day 3, the VEGF analysis was done on day 5, and the observation of wound closure was done on days 3, 5, and 7.

Procedure

Preparation of binahong leaf extract

750 g binahong fresh leaves were washed and wind dried for 4 days. The dried leaves were blended to make powder. The formulation of *Anredera Cordifolia* leaf extract was prepared by the maceration of the dried powdered *Anredera Cordifolia* leaf with one liter of methanol 95% for 24 h. Filtration was done after 24 h, and vaporization of crude extract was conducted using a rotary evaporator to obtain concentrated extract.

Burn wound on the rat model

The use of rats for this study meets the ethical worthiness in accordance with the letter no: 216/KE-PH-Lit-2/II/2016 issued by the Commission on Ethics Use of Animals in Research and Education, Faculty of Veterinary Medicine, Udayana University. Samples were anesthetized with 3 mg/kg of ketamine before inflicting burn wounds. Their back hair was shaved and sterilized. Burn inflictor was heated for 1 min before introduced to the skin for 1 s with a diameter of 1 cm and a depth of 2 mm. All burn injuries were inoculated with *Pseudomonas aeruginosa* bacterial suspension. The samples were then divided into two groups.

Preparation of *Pseudomonas aeruginosa* suspension

Pseudomonas aeruginosa bacterial suspension was obtained through agar preparation and isolate cultivation. *Mueller Hinton* (MH) agar was chosen as *pseudomonas* isolation agar. A few colonies of *Pseudomonas aeruginosa* were inoculated to MH agar and incubated for 24 h, 37°C. Two growth colonies was mixed with 0.9% NaCl and concentrated into 0.5 Mc Farland to gain the suspension. The amount of 0.2 ml of suspension was taken with syringe and dripped evenly on burn wounds.

Observation of wound healing

Wound closure rate

The rate of wound closure was analyzed macroscopically on days 1, 3, and 5. The identification of wound healing process was done with

push score (length × width, tissue type, exudates amount). In this study, only length × width and the amount of exudates were documented.

Interleukin 6 (IL-6) in rat plasma

Plasma IL-6 was assayed using *BioSains* ELISA kit on day 3. Plasma IL-6 bound with an anti-IL-6 monoclonal antibody in the ELISA wells. The wells were washed and the biotinylated anti-IL6 antibody was added. The unbound biotinylated anti-IL6 antibody was removed by washing the wells, and streptavidin-horse radish peroxidase (HRP) was added. The wells were washed again and 3,3',5,5'-Tetramethylbenzidine (TMB) solution was added, which produced a blue color, equivalent with the plasma IL-6 level. Stop solution changed the color into yellow, and its intensity was measured at 450 nm wavelength to quantitatively measure the plasma IL-6 level.

Vascular endothelial growth factor (VEGF) in rat plasma

Plasma VEGF was assayed using *BioSains* VEGF ELISA kit on day 5. Plasma VEGF was bound with a monoclonal antibody in ELISA wells and was then covered with aluminum and incubated for 2.5 h. The solution was removed and 300 µl of wash solution was used to wash the wells for four times. Then 100 µl of biotinylated VEGF detection antibody was added and incubated for 1 h at room temperature. The solution was removed and washed four times using wash buffer. As much as 100 µl of HRP-streptavidin solution was added to the wells and incubated for 45 min at room temperature. The solution was removed again and washed four times using wash buffer and then 100 µl of TMB 106 substrate was added to each well and incubated for 30 min. 50 µl of stop solution was added to each well after incubation and measured at 450 nm wavelength.

All data were analyzed statistically. Normality test was performed with Shapiro Wilk. Independent t-Test was performed to obtain the mean difference of IL-6 and VEGF in control and treatment groups.

Results

All subjects in the groups ($n = 32$) were divided in a manner that each group would contain 16 subjects. The rate of wound closure was observed macroscopically every day (Figure 1). On day 3, the entire surface of burn wound in the treatment group has been covered by scar tissue, but not in the control group. On day 5, the burn wound of the treatment group has perfectly closed, but in the control group, the scar tissue only began to appear.

All plasma IL-6 level and VEGF data obtained were normally distributed ($p > 0.05$), and their variances were also homogeneous ($p > 0.05$). Based on independent t-test analysis, it was observed that the plasma IL-6 level on day 3 of the treatment group was significantly higher than that of the control group ($p = 0.001$). A similar trend was also observed for VEGF data (Table 1). This indicates a significant difference between the treatment group, which received topical *Anredera Cordifolia* leaf extract, compared with the control group, which received topical tetracycline 3%.

Discussion

This result showed that burn wounds on the treatment group heal faster with the use of the topical extract of *Anredera cordifolia* leaf compared with the control group with the use of tetracycline 3% (Figure 1). It is also seen from several wound healing indicators, such as interleukin-6 and VEGF. Previous research done by Amertha, 2007, showed that *Anredera cordifolia* leaf extract proves more effective

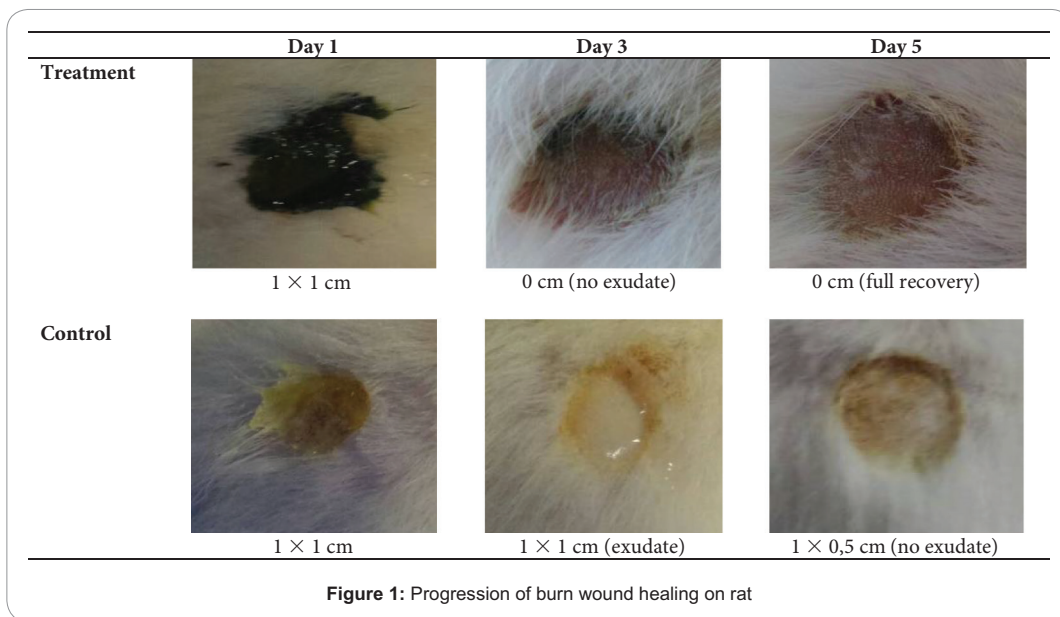


Figure 1: Progression of burn wound healing on rat

Variable	Groups		Independent t-test
	Treatment (<i>Anredera Cordifolia</i> leaf extract, n = 16)	Controlled (tetracycline 3%, n = 16)	p
IL-6 (pg/ml)	1.40 ± 0.22	0.42 ± 0.12	0.001*
VEGF	27.99 ± 2.15	14.44 ± 0.56	0.001*

*Significantly independent t-test $p < 0.05$.

Table 1: Comparison of IL-6 and VEGF among groups

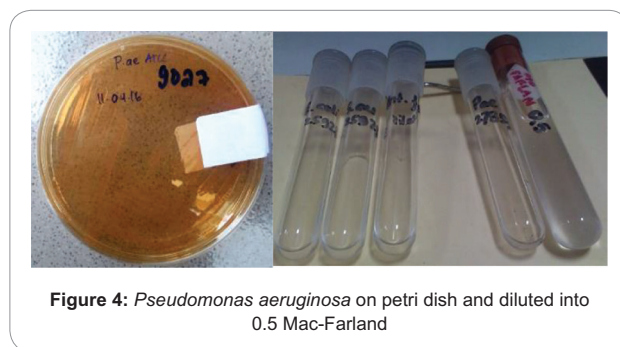


Figure 4: *Pseudomonas aeruginosa* on petri dish and diluted into 0.5 Mac-Farland

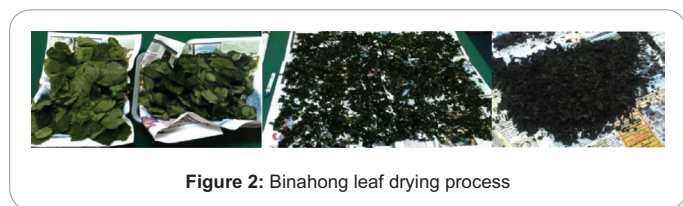


Figure 2: Binahong leaf drying process



Figure 3: Vaporization of crude extract was conducted with a rotary evaporator to obtain concentrated extract

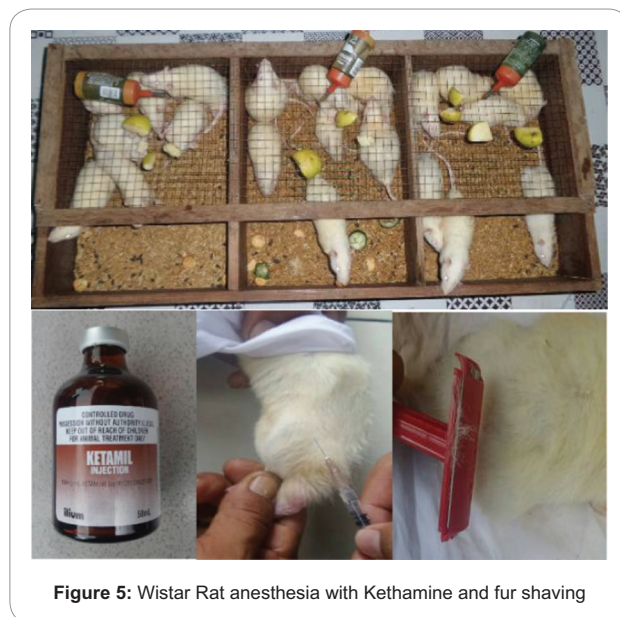


Figure 5: Wistar Rat anesthesia with Kethamine and fur shaving

in healing burns on chicks compared with one of the clinically used burn treatments [6]. The phytochemical analysis of *Anredera cordifolia* leaf extract found its anti-hyperlipidemic, analgesic,

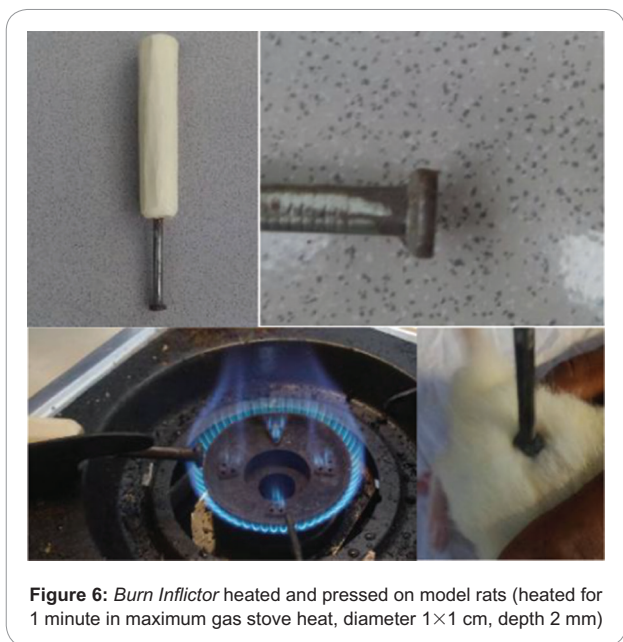


Figure 6: Burn Inflictor heated and pressed on model rats (heated for 1 minute in maximum gas stove heat, diameter 1×1 cm, depth 2 mm)

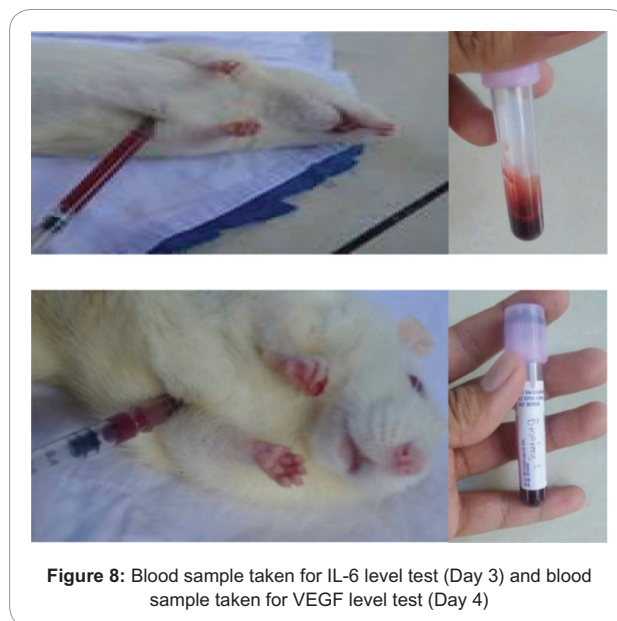


Figure 8: Blood sample taken for IL-6 level test (Day 3) and blood sample taken for VEGF level test (Day 4)

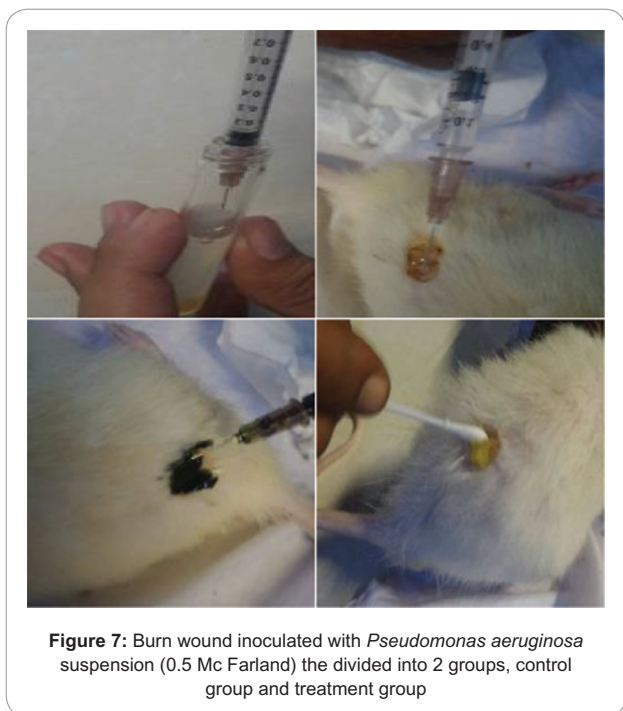


Figure 7: Burn wound inoculated with *Pseudomonas aeruginosa* suspension (0.5 Mc Farland) the divided into 2 groups, control group and treatment group

anticonvulsant, and cytotoxic activities. The leaf of *Anredera cordifolia* contains compounds such as phytol, alpha-pinene, 6,10,14-trimethyl pentadecanone, neophytadiene, methyl hexadecanoate, methyl-9,12,15-octadecatrienoate, methyl-9,12-octadeca dienoate, and C-flavone-glycosides [9]. Wound healing consists of four phases: hemostasis phase, inflammation phase, proliferative phase, and maturation or differentiation phase. Phytol, contained in the extract of *Anredera cordifolia* leaf, is a precursor of vitamin E and vitamin K, which are important for body hemostatic in the first phase of wound healing and to promote this phase. Phytol also has a role in wound contraction and promotes epithelialization [10,11].

Independent samples test (pg/ml)	Mean	T	p	Confidence interval 95%	
				Lower	Upper
IL-6 level control group vs. treatment group	0.99	15.86	0.001	-1.11	-0.86

p significant <0.05.

Table 2: Resume t-test IL-6

Parameter	Group		p**
	Control	Binahong treatment	
VEGF (pg/ml)	14.44 ± 0.56	27.99 ± 2.15	0.062
p*	0.627	0.136	

p* normal distribution > 0.05.
p** homogeny variant > 0.05.

Table 3: Average VEGF level in control group and treatment group

Independent samples test (pg/ml)	Mean	T	p	Confidence interval 95%	
				Lower	Upper
VEGF level control group vs. treatment group	13.54	24.35	0.001	-14.68	-12.41

p significant <0.05.

Table 4: Resume t-test VEGF

In this research, macroscopic analysis of wound healing found shorter and faster inflammatory phase in the treatment group. The proliferative phase generally takes place on day 5 until day 20. In this phase, fibroblast produces collagen and connective tissue. The formation of new capillaries also took place at this phase, which began when there was inflammation [12,13]. It indicates ongoing healing process, starting from capillary growth and granulation tissue growth. Granulation process runs concurrently with re-epithelialization that took place on the surface of the wound.

The wound will develop into scar tissue that consists of plasma mixed with dead cells. In this phase, the wound appears like a yellow line, indicating that epithelialization takes place. The next phase is the maturation phase, which takes place for 21 days or 2 months until several years after the wound occur. In this phase, collagen preserves scar tissue. Epithelialization also covers the skin. Jar scar (keloid), that is, an inelastic and strong connective tissue, sometimes occur. In this research, we found that topical *Anredera cordifolia* leaf extract shortened these four phases of wound healing. Wound repair in the treatment group was marked by the occurrence of scar tissue on day 3.

This indicates a good sign of wound healing, since wound closure signifies the pass of the inflammation phase and the proliferative phase has already taken place without any microorganism infection or other disturbance. As for the control group, scar tissue has not been identified until day 3. Yellow color found on the surface of the control group's wound on day 3 is a sign of drainage disorders or plasma proteins that results from microorganism infection.

In this research, we found that plasma IL-6 level on the treatment group was significantly higher than that of the control group. Interleukin-6 (IL-6) is a pro-inflammatory cytokine that is found in small number, except for certain conditions such as infection, trauma, and stress. Blood flow to the site of the wound increases when inflammation occurs, conveying fibrins to shut off the injured blood vessel and protecting it from bacterial infection. A good inflammation phase is an important step in wound healing process. IL-6 has important roles to support that phase, for it prevents infection, monocytes, and macrophage chemoattractant, as well as cell signaling to start the proliferation phase. It was proved by Zi-Qing *et al.* [14] They compared wound healing process between wild-type (WT) mice and IL-6-deficient BALB/c mice. The wound on WT mice on day 6 closed for 50% from initial wound size and leukocytes infiltration was found microscopically. It was also found that re-epithelialization reached 80% on day 6, with the increase of angiogenesis and hydroxyproline. IL-1 gene expression, chemokines, adhesion molecules, transforming growth factor- β 1, and vascular endothelial growth factor also significantly increased compared with IL-6-deficient BALB/c mice [14]. Slow wound healing, marked by lack of leukocytes infiltration, re-epithelialization, angiogenesis, and collagen accumulation, was found in IL-6-deficient BALB/c mice. The study that was done through injection of neutralizing anti-IL-6 monoclonal antibody to WT mice resulted in the decrease of wound closure rate. These results proved that IL-6 has a crucial role in wound healing process [14].

C-flavone-glycosides, contained in *Anredera cordifolia* leaves, are flavonoids thought to have crucial part as a metabolic stimulant through the increase of cell signaling pathways. It maximizes IL-6 function as chemoattractant to stimulate monocytes, macrophages, and other cells in inflammatory and proliferative phases of wound healing [15].

Tanin is a polyphenol and part of complex phenolic compound of *Anredera cordifolia* leaf. It acts as a bowel astringent, decreasing fluid turn-over in gut and supporting body metabolic process, an antidote (alkaloid toxicity), a reagent for the detection of gelatin, alkaloid, and protein, and a wound healing stimulant through the enhancement of body metabolism [16].

This research found a significant difference in VEGF level between treatment and control groups ($p < 0.05$). The result showed that topical *Anredera cordifolia* leaf extract increases the expression of VEGF significantly. VEGF accelerates wound healing through

angiogenesis. In the angiogenesis process, VEGF acts through the stimulation of endothelial cells to proliferate. Angiogenesis in wound healing includes vasodilatation, basement membrane degradation, and migration and proliferation of endothelial cells, subsequently forming blood capillaries. VEGF also enhances tissue granulation. In areas where granulation occurs, fibroblasts and endothelial slowly undergo apoptosis and the tissue is filled by collagen.

VEGF is produced by various cells that involve in wound healing, for instance, endothelial cells [17-19], fibroblasts [20], smooth muscle cells [21,22], platelets [23], neutrophils [24] and macrophage [25]. The result from this research found that VEGF production could accelerate wound healing and could be viewed as a mechanism involved in wound healing [26]. They also found that VEGF administration gives a very good outcome on rats with skin graft.

A similar result was reported by Anom, 2013, that involved 32 male wistar rats [27]. Incision was made on the back, and then *Catharanthus roseus* extract was administered. VEGF in the treatment group was significantly higher than the group with no treatment. VEGF affects angiogenesis, leading to acceleration of wound healing process [27].

In molecular process, oxygen is required by cells and is obtained from blood flow. This indicates that blood capillaries are very important for oxygen supply, since oxygen is needed for molecular process [28,29]. VEGF serves as important signal that is used by oxygen-hungry cells to trigger blood vessel formation [30-32]. VEGF is required for endothelial cell growth and division [19,32].

Conclusion

The application of topical *Anredera cordifolia* leaf extract accelerates burn wound healing, increases IL-6 level, and increases VEGF production in burns infected by *Pseudomonas aeruginosa*.

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