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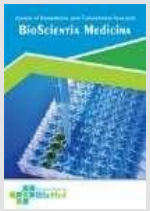

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The Effect of Ethanolic Extract of *Moringa oleifera* Leaves on the Macrophage Count and VEGF Expression on Wistar Rats with Burn Wound

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A B S T R A C T

Background: One modality type of burn wound therapy is topical silver sulfadiazine. Recently, research about topical herb medicine has grown. Moringa oleifera (MO) is a kind of herb plant that has an anti-inflammation effect and has an influence on angiogenesis, so it tends to faster burn wound healing. This study aimed to prove the effect of ethanolic extract of Moringa oleifera leaves on the macrophage count and VEGF expression in Wistar rats with burn wounds. **Methods:** The research design that was used is "Randomized post-test with a control group." The study population used 24 male Wistar rats that were induced burn wounds and randomly divided into 4 groups which were given medicine topically once daily for 7 days. Treatment groups included: I (MO leaves extract 10%), II (SSD + MO leaves extract 10%), III (SSD), dan IV (pure vehiculum). Exudation was assessed macroscopically, while PMN and macrophage amount were assessed microscopically with Hematoxylin-Eosin. Data were analyzed and processed using hypothesis test One Way ANOVA – Post Hoc Bonferroni, Kruskal Wallis – Mann Whitney, and correlation test Spearman and Pearson with SPSS 25.0 program. **Results:** Significant difference obtained in macrophage amount between II group and IV group (p 0,000). There was no significant difference in VEGF expression. There was also no correlation between macrophage amount and VEGF expression. **Conclusion:** Ethanolic extract of Moringa oleifera leaves extract proved to be effective in decreasing macrophage cell count on Wistar rats with burn wounds.

1. Introduction

World Health Organization (WHO) estimates about 180.00 mortalities each year are caused by burn injury. Burn injury is the main cause of morbidity. Indonesian Ministry of Health in 2015 noted that burn injury was in the 6th order for accidental injury with a total case of 7,7%.[1-4]

Generally, burn is categorized into three degrees based on injury depth and skin layer involvement. Superficial and partial-thickness burns can heal spontaneously, while full-thickness burns can't heal spontaneously. There are three phases of burn wound healing; inflammation, proliferation, and remodeling.[5]

Body defense to burn includes innate and adaptive immune responses. Innate immunity response is dominated by cellular components. Neutrophils and macrophages will increase in the early days of a wound and decrease on the 5th day. Polymorphonuclear/ neutrophile (PMN) dysfunction, also macrophage decrease, and natural killer cell activation will decrease the level of proinflammatory cytokine.[6]

The proliferative phase in burn wound healing is marked with angiogenesis, starting with vascular endothelial growth factor (VEGF) response, capillary formation, collagen deposition, granulation formation, wound contracture, and epithelisation. Clinically, this phase can be seen from light red colorization on wound tissue, so the existence of VEGF expression, new

capillary, and granulation show that the wound is in the proliferative phase.[6,7]

Burn wound management consists of different aspects. One of them is with topically silver sulfadiazine because of its effect as broad-spectrum antimicrobials and ease to use. But, silver sulfadiazine can't penetrate eschar and has toxicities against pregnant women and babies.[8]

Recently, there has been a lot of research about the effectiveness of herb topical drug for burn wounds. *Moringa oleifera* is an herb plant that contains many efficacies, e.g., anti-proliferative, hepatoprotective, anti-inflammation, anti-atherosclerotic, anti-oxidant, antimicrobe, etc.[9]

Hossain et al. and Masurekar et al., in their research, concluded that extract of *Moringa oleifera* leaves anti-inflammation and antimicrobe effects that are effective enough in burn wound healing.[10,11]

The general objective of this research is to prove that ethanolic extract of *Moringa oleifera* leaves effective toward macrophage count and VEGF expression on Wistar rat's burn wound, with specific objectives to prove that ethanolic extract of *Moringa oleifera* leaves effective in decreasing macrophage count and increasing VEGF expression on Wistar rat's burn wound, also prove that there is a negative correlation between macrophage count and VEGF expression on Wistar rat's burn wound.

The hypothesis in this research is that ethanolic extract of *Moringa oleifera* leaves is effective toward macrophage count and VEGF expression in Wistar rats' burn wounds. Also, there is a negative correlation between macrophage count and VEGF expression on Wistar rats' burn wounds.

2. Methods

This research is experimental research with a randomized design post-test with a control group. Research subjects are 24 Wistar rats that were randomly divided into 4 intervention groups that were given topical therapy once daily for 10 days. Intervention groups include: Group I got a topically ethanolic extract of *Moringa oleifera* leaves 10%, group II got a combination of ethanolic extract of *Moringa oleifera* topically leaves 10% + silver sulfadiazine (SSD), group III got SSD topically, and group IV got vehiculum topically. Inclusion criteria in this research include 2 months old male rats, Wistar strain that induced partial thickness burn wound, weight ± 150 – 200 gram after acclimatization for one week in the individual cage, and no anatomical abnormalities visible. Exclusion criteria in this research are if there is no partial thickness burn wound, whereas drop-out criteria are if, during induction and intervention, rats look ill (no active movement) or dead.

The Independent variable in this research is an ethanolic extract of *Moringa oleifera* leaves 10% topically. The dependent variable in this research is an examination of macrophage and VEGF expression by 2 observers.

Data were analyzed using SPSS 25.0 for windows. Data was shown in table and graphic, then processed with normality test of Shapiro-Wilk and homogeneity test. The hypothesis test that was used on the macrophage variable was One Way ANOVA, continued with Post-Hoc Test Bonferroni. The hypothesis test that was used on VEGF expression was Kruskal-Wallis. The result was said to be significant if $p \leq 0,05$.

This research was agreed by the Ethical Commission of Research, Faculty of Medicine, Universitas Diponegoro, Semarang.

3. Results

The normality test was done with the Shapiro-Wilk test because the sample count was less than 50. The normality test result on macrophage count showed normal distribution ($p>0,05$), and from the homogeneity test, there was homogenous data in all groups (p 0,083). A normality test result of VEGF expression showed that data distribution was abnormal (p 0,006).

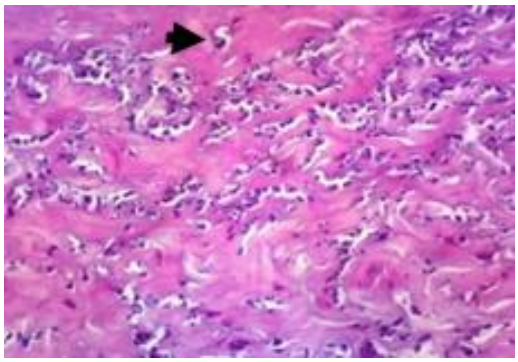

Figure 1. Macrophage cell (HE, 400x)

Shapiro-Wilk and homogeneity test on macrophage variable resulted in normal and homogeny data distribution and continued with One Way ANOVA. One Way ANOVA test concluded that there was a significant difference in macrophage count in all groups (p 0,000). From the Post-Hoc Bonferroni test, there was a significant difference between group II-IV (p 0,000).

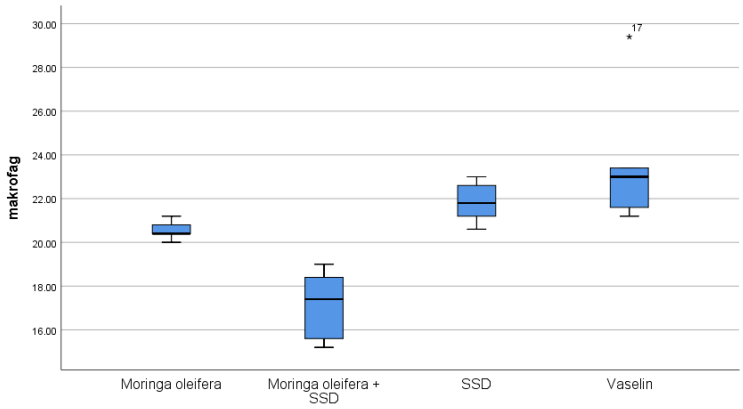

Figure 2. Box plot graphic of macrophage count in burn wound

Table 1. One Way ANOVA test about macrophage count

Group	Macrophage count (Mean ± SD)	P
I	20,56 ± 0,45	0,000*
II	17,12 ± 1,68	
III	21,84 ± 0,98	
IV	23,72 ± 3,30	

Shapiro-Wilk test of VEGF expression showed abnormal data distribution and continued with the Kruskal-Wallis test. The result of the Kruskal-Wallis test showed that there is no significant difference in VEGF expression in all groups (p 0,550).

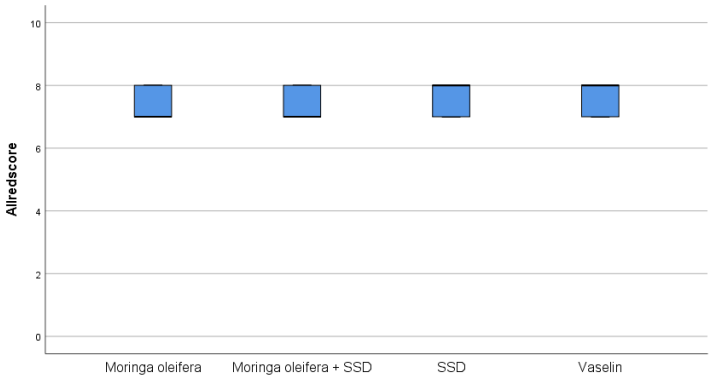

Figure 3. Box plot graphic of VEGF expression

Table 2. Kruskal-Wallis test for VEGF expression

Group	VEGF expression (Mean ± SD)	P
I	7,40±0,548	0,550*
II	7,40±0,548	
III	7,80±0,447	
IV	7,60±0,548	

The correlation test that was used on macrophage and VEGF expression was the Spearman correlation test. Spearman test concluded that there wasn't a significant correlation between macrophage count and VEGF expression (p 0,370).

4. Discussion

This research showed that ethanolic extract of *Moringa oleifera* leaves has a positive effect on a decrease in macrophage count in the burn wound.

The lowest mean of macrophage count was obtained from a group that was given a combination of ethanolic extract of *Moringa oleifera* leaves and SSD topically, whereas the highest was in the control group. *One Way ANOVA* test showed that there was a significant difference in macrophage count in all groups, so the test continued with the Post-Hoc Bonferroni test. From the Post-Hoc Bonferroni test, we conclude that there was a significant difference in macrophage count among a group that was given a combination of ethanolic extract of *Moringa oleifera* leaves + SSD with a control group.

Some previous research stated that extract of *Moringa oleifera* leaves and roots had an anti-inflammation effect on the animal trial wound, topically or orally. This was seen by decreasing parameters: edema, erythema, proinflammatory cytokine (IL-1β, IL-6, TNF-α), and inflammatory cell.[12,13]

Ethanolic extract of *Moringa oleifera* leaves in this research was proved to decrease inflammatory cells (macrophages) in animal burn wounds. This result correlates with previous research that showed that *Moringa oleifera* extract had a sufficient anti-inflammatory effect. 4-[(2'-O-acetyl-alpha-l-rhamnosyloxy) benzyl isothiocyanate, glukosinolat, flavonoid, and dibenzil-urea have inhibition potency against NO and pro-inflammatory cytokine, so it decreased burn inflammation. This decrease in inflammation had a positive correlation with inflammation cell decrease, including neutrophile and macrophage. Macrophage existence was triggered by

neutrophiles and the amount will decrease in the 4th day. It corresponded with the research result, which on the 5th day, ther was lower macrophage count in a group that was given an ethanolic extract of *Moringa oleifera* leaves.[11,14]

The hypothesis test for VEGF expression used *Kruskal-Wallis* showed that there is no significant difference (p=0,550) between group that used *Moringa oleifera* topically or not. It might be caused the examination of VEGF expression to be done on the 7th day where all groups (intervention or control) had an increase of VEGF expression, so there was no significant difference among groups.

Wound healing process includes 3 phases:[4] coagulation, proliferation and remodeling phases. Coagulation, hemostasis, and inflammation phases start shortly after wound occurs. There is formation of pro-inflammatory chemokines followed by neutrophil production 24-36 hours after wound occurs. The late inflammation phase, 48-72 hours after wound occurs, was signed with the release of macrophages to begin phagocytosis. After debris is cleared, macrophages, together with other chemoattractant will trigger growth factors like VEGF, and fibroblast growth factor (FGF).

The proliferation phase starts on the 3rd-day for 2 weeks. VEGF plays an important role in angiogenesis in this phase.[4,15] Remodelling phase starts after granulation is formed and the synthesis of the extracellular matrix. This phase can last for 1-2 years.

Spearman correlation test was done to investigate the correlation between macrophage count and VEGF expression on the provision of ethanolic extract of *Moringa oleifera* leaves (single or combination). This test showed that there was no correlation between macrophage count and VEGF expression on ethanolic extract of *Moringa oleifera* leaves provision.

The macrophage is an important cell in angiogenesis and wound healing. The macrophage can produce VEGF. This research resulted that there was no significant correlation between macrophages and VEGF. This can be caused by the existence of other factors that stimulate VEGF formation, including hypoxic conditions.[5]

5. Conclusion

The ethanolic extract of *Moringa oleifera* leaves proved to be effective in decreasing macrophage cell count on Wistar rats with burn wounds but not effective in increasing VEGF expression. There is also no correlation between macrophage count and VEGF expression on Wistar rats with burn wounds.

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