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Anti-Inflammatory Activity Of Neem Leaves (Azadirachta Indica L.) Extract In Wound Healing Of Diabetic Rat Model

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ABSTRACT

The use of commercially available anti-inflammatory drugs in the treatment of diabetic ulcers is relatively expensive and has side effects. However, addressing both inflammation and epithelialization during the treatment of diabetic foot ulcers is an important step, but current treatment options are limited. Neem leaves extract in combination with VCO has potential as an alternative therapy, but further research is needed regarding the correct dose. The aim was to evaluate the effect of Neem leaves extract in combination with VCO on IL-6 levels on wound healing of diabetic rats model. This research is true experimental with a post-test only control group design. Conducted on 25 white mice divided into 5 groups: P1, P2, and P3 contain 5%, 10%, and 15% of Neem leaf extract, respectively. Povidone Iodine was utilized as positive control and physiologic saline NaCl 0.9% as negative control. The grouping of subjects was carried out randomly. Data were normally distributed by using Shapiro Wilk which shows the p value in each group > 0.05. The average wound diameter in the P1 is 3.79 mm, P2 3.01 mm and P3 3.80 mm. The highest reduction of IL-6 from day 4 to day 14 was showed in P2 group (43.80%). There was significant difference in the effectiveness of treatment groups compared to negative and positive control. Significant values were obtained in IL-6 levels on day 4 with p value 0.003 (p > 0.05) and day 14 with p value 0,046 (p > 0.05). It concluded that Neem leaves extract in combination with VCO at 10% concentration has an effect on wound healing through a decrease in IL-6 levels on day 14.

Keywords: diabetes mellitus, VCO, Indian lilac leaf extract, wound healing, Interleukin-6

Introduction

Diabetes mellitus (DM) is the most common metabolic disorder for all age groups, this condition is associated with abnormal carbohydrate, fat, and protein metabolism in the body. Diabetic ulcers are one of the most serious complications that occur in 5-10% of the diabetic population. The five-year mortality prevalence for diabetic neuropathic ulcers, diabetic neuroischemic ulcers, and diabetic ischemic ulcers are 15%, 18% and 55%, respectively (Zulkarnain, dkk., 2023).

The wound healing process involves a complex relationship between cellular, humoral and connective tissue elements. The phases of wound healing consist of inflammation, proliferation and maturation, each of which is interrelated. Fibroblasts are the most important cells in the re-modeling process of healing damaged tissue. Fibroblasts are the primary cellular component of connective tissue and the main synthetic source of protein matrix (Sihotang, dkk. 2019).

The inflammatory phase occurs immediately after trauma and continues until day 5 post trauma. In the inflammatory phase there will be a buildup of neutrophils and an increase in the secretion of pro-inflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-6), and Interleukin-6 (IL-6). IL-6 plays a role in regulating the inflammatory immune response and hematopoiesis. Increased levels of IL-6 are associated with inflammation and tissue damage (Salsabila, dkk., 2022; Sarihati, dkk., 2020).

The use of anti-inflammatory commercial drugs such as steroids and nonsteroids in healing diabetic ulcers is relatively more expensive and has side effects such as growth suppression, osteoporosis, aggravating DM disease, susceptible to infection, and muscle weakness (Salsabila, dkk., 2022). However, until now the available topical treatments are still limited, therefore other alternative therapies are needed to accelerate the healing of diabetic ulcers from natural ingredients which are expected to minimize the unwanted side effects of drug use.

VCO has known to increase the formation of new blood vessels in wound healing process. The anti-inflammatory content in VCO has the ability to accelerate the wound healing process through the mechanism of reducing wound surface area. VCO also plays a role in improving circulation in diabetic ulcers so that there is an increase in the angiogenesis process, so that the need for nutrients and oxygen in the wound healing process is well met (Suarni, dkk, 2019); Dafrani, dkk., 2020). The inhibitory power of VCO can be increased by making VCO as hydrolyzed virgin coconut oil or by adding other active ingredients that can dissolve in VCO (Karta, dkk., 2022).

Intaran leaves or also known as Neem leaves have active ingredients that can act as anti-inflammatory, antitumor, diuretic effect, antifungal, insecticide, antibacterial, antimalarial, and mosquito larvicide (Dewi, dkk., 2017). Phytochemically, Neem leaves extract contains active ingredients such as alkaloids, terpenoids and steroids, saponins, tannins, and flavonoids that can work together with VCO as carrier oil (Karta, dkk., 2022). Neem leaves extract has no fat content so it requires a carrier oil that does not cause irritation when applied to the skin. Neem plants contain several toxic compounds and have side effects that can cause damage to liver and kidney structures so that the use of plants as drugs needs to be done by giving the right dose (Karta, dkk., 2022). Based on those phytochemical screening that has been carried out on Neem leaves which can accelerate the wound healing

process, it is necessary to test the effectiveness of Neem leaves extract in combination with VCO to accelerate the healing process of wound healing in diabetic rats model.

Research Method

This experimental study using the Posttest Only Control Group Design which consists of two groups. ``This research was conducted in the immunology laboratory of the Poltekkes Kemenkes Denpasar Jl. Sanitation No. 1, Sidakarya, South Denpasar, Denpasar City and the Laboratory of Maintenance and Breeding of Bio Mice and Rat Experimental Animals. Ethical approval for all procedures involving animals was granted by the Ethics Committee of Poltekkes Kemenkes Denpasar (No.DP.04.02/F.XXXII.25/0093/2024). The samples used in this study were utilized white rat population with male sex. Data collection was carried out by measuring immunologically through quantitative analysis of antigen-antibody reactions. The Shapiro-Wilk test was used to determine whether the data were normally distributed; otherwise, the Kolmogorov Smirnov test was used. After the data were normally distributed, One Way ANOVA test was conducted to determine the difference in IL-6 levels in mice between the control and treatment groups, followed by Least Significantly Difference (LSD) test to confirm the difference.

The materials used in this study include male white rats aged 2-3 months weighing 100-120 gr, standard feed and drinking water, NaCl 0.9%, Streptozocin (STZ), sterile bandages, wound closure plasters, and supplementation materials such as Neem leaves, ethanol, and Virgin Coconut Oil (VCO).

The preparation of Neem leaves simplicial and extracts began with washing the leaves, drying them in the open air, baking for 24 hours, crushing, and extracting with ethanol for seven days. Preparation of VCO supplementation was carried out by making 5%, 10%, and 15% concentrations of neem leaf extract using the formula %b/b to produce a total mass of mixture per concentration of 5 g. Grouping of experimental animals involved 25 rats divided into five groups:

negative control (NaCl 0.9%), positive control (povidone iodine), and three treatment groups with concentrations of 5%, 10%, and 15%. Body weight weighing and STZ administration were performed by weighing the body weight of the rats and injecting STZ 40 mg/kg to induce diabetes. Fasting blood sugar measurement was done by taking blood from the lateral vein after the rats were fasted for 10 hours. The excision wound was made by shaving, cleaning, and sterilizing the rat's back, then making a circular wound with a diameter of 5 mm. The treatment and wound diameter measurements were carried out every day by measuring the wound diameter vertically, horizontally, and diagonally. Blood sampling was performed on the 4th and 14th days through the retro orbital sinus, then centrifuged. Sandwich ELISA test was performed for further analysis.

Results and Discussions

1. Wound diameter analysis on VCO supplementation with Neem leaves extract (Azadirachta indica L.)

The average results of wound diameter measurements that have been carried out in this study are shown in table 1 and figure 1.

Table I. Average wound diameter in each group.

Group	n	Wound Diameter Measurement Day (mm)			*Asymp. Sig One Way Anova*
		Day 1	Day 7	Day 14	
K (-)	4	5,00	4,07	1,58	
K (+)	4	5,00	2,55	0,83	
P1	4	5,00	4,57	1,21	0.008
P2	4	5,00	3,04	0,40	
P3	4	5,00	4,36	1,98	

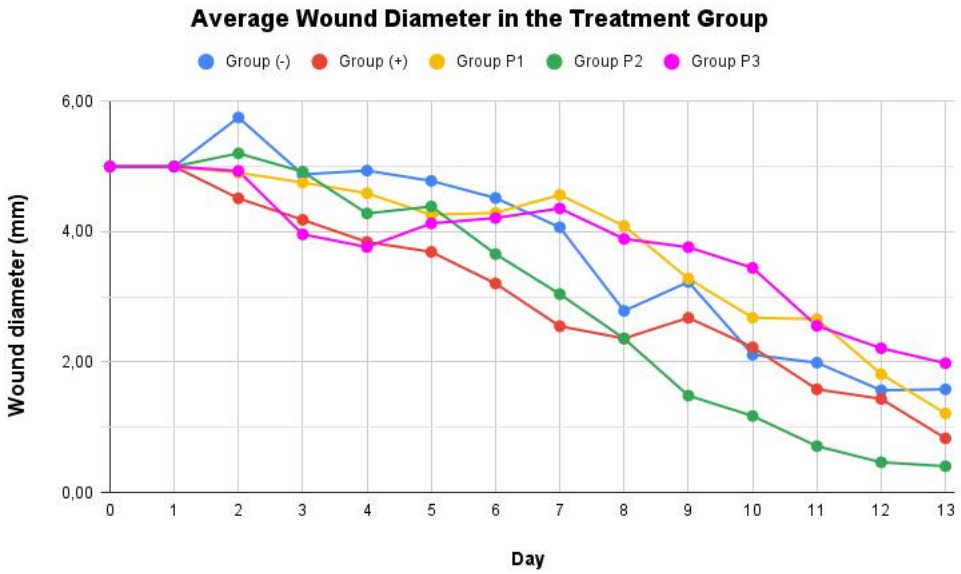

Figure 1. Average wound diameter of the groups. In group P2, the green line with the treatment of VCO supplementation with 10% Neem leaves extract showed the fastest wound closure with an average wound diameter of 0.40 mm.

Based on the results of the study, the diagram above shows that the K (-) and P3 groups have the

highest average wound diameter compared to other groups. At P2 group has the average wound diameter smallest, so the P2 group with group P2 with the treatment of VCO supplementation with Neem leaves extract with a concentration of 10% has macroscopically significant activity in healing type 2 diabetes mellitus wounds. VCO supplementation with Neem leaves extract plays a role in shortening the inflammatory phase compared to the K (-) and K (+) groups.

2. **Effect of VCO supplementation with Neem leaves extract (*Azadirachta indica* L.) on IL-6 levels**

Table 2. Mean difference in IL-6 levels at day 4 and day 14.

Group	N	IL-6 day 4 (mg/dL)	IL-6 day 14 (mg/dL)	% Decrease in IL-6 Levels
K (-)	4	15,310	10,602	30,75
K (+)	4	13,512	10,509	22,23
P1	4	15,042	9,184	38,94
P2	4	16,087	9,041	43,80
P3	4	17,443	9,812	43,75
Asymp. Sig. One Way Anova		0.003	0.046	

Table 3. Post Hoc LSD (Least Significant Difference) test results of the average measurement of IL-6 levels on day 4 and day 14.

Group Result of IL-6 Levels on Day 4	K (-)	K (+)	P1	P2	P3
K (-)	-	0.036	0.851	0.037	0.099
K (+)	0.036	-	0.025	0.001	0.000
P1	0.851	0.025	-	0.053	0.138
P2	0.037	0.001	0.053	-	0.603
P3	0.099	0.000	0.138	0.603	-
IL-6 Level Results Group 14th Day	**K (-)**	**K (+)**	**P1**	**P2**	**P3**
K (-)	-	0.097	0.010	0.007	0.108
K (+)	0.097	-	0.251	0.196	0.953
P1	0.010	0.251	-	0.876	0.229
P2	0.007	0.196	0.876	-	0.178
P3	0.108	0.953	0.229	0.178	-

Based on table 3, it is known that the difference in mean IL-6 levels on day 4 is most significant in groups K (+) and P3 with a difference in mean IL-6 levels Sig. 0.000. It is known that the average difference in IL-6 levels on day 14 is most significant in group K (-) and P2 with an average difference in IL-6 levels Sig. 0.007.

One of the factors in the wound healing process is the immune system that plays a role

in recognizing and fighting antigens from the wound, high blood sugar levels can cause nutrients to not be able to enter the cells so that protein and calories decrease, rehydration and wound washing, lack of nutrients, low blood albumin levels, oxygen supply and vascularization, pain that can produce glucocorticoid hormones which play a role in inhibiting the wound healing process, and corticosteroids (Kartika, 2015).

Wounds cause inflammation which results in the production of free radicals by phagocytic cells. Increased free radical production can delay the wound healing process, so strategies are needed that can inhibit free radical production with the aim that the therapy can function in action. wound healing. Macrophages are the main cells that factor into wound healing. These phagocytic cells actively remove foreign bodies in the wound area such as bacteria. After that, fibroblasts emerge from the wound tissue and endothelial cells that migrate towards the wound, these fibroblasts play a role in increasing tissue permeability and collagen fiber production (Asrul, dkk., 2023).

The presence of alkaloid, terpenoid and steroid compounds, saponins, tannins, and flavonoids in VCO supplementation with Neem leaves extract. The active ingredients in the extract were able to collaborate with VCO. Semi-quantitatively, this mixture contains a lot of tannins and flavonoids that can be used as a supplement to helps the wound healing process of diabetes mellitus rats through antioxidant, antimicrobial, astringents, and anti-inflammatory activities (Karta, 2022). The anti-inflammatory content of VCO can improve the wound healing process by reducing the surface area of the wound (Dafriani, dkk., 2020). The study shows that group P2 has the fastest average wound healing and is in line with the research of Karta & Burhannuddin (2022) which states that VCO supplementation with Neem leaves extract with a concentration of 10% has the highest inhibition zone diameter with a strong category inhibiting the growth of P. acnes. This study also mentioned that the higher the extract of Neem leaves in VCO does not necessarily have an impact on increasing the strength of inhibition due to the solubility factor and saturation of extracts in the mixture. The higher the concentration, the more saturated the mixture and the absorption is not optimally distributed.

This study showed that the mean ratio of IL-6 levels on day 4, which is the peak of inflammation, was higher in the VCO supplementation group with 15% neem leaf extract at 17.443 mg/dL compared to the mean ratio in the positive control group at 13.512 mg/dL. VCO supplementation with neem leaf extract has an effect on IL-6 levels in type 2 diabetes mellitus model rats. The effect can be seen from the IL-6 levels on day 4 significantly which is addressed by the p value of $p < 0.05$, namely $p < 0.003$. In group K (+) with povidone iodine treatment showed the lowest IL-6 levels due to povidone iodine only having an antimicrobial effect [14]. In line with research by Ferdina (2022) povidone iodine is a complex iodine that acts as an antiseptic, which is able to kill microorganisms. The ability of povidone iodine in the inflammatory process is to inhibit IL-1 beta and IL-8 (Ferdina, dkk., 2022).

In the P2 group with the treatment of VCO supplementation with 10% Neem leaves extract, the average IL-6 levels decreased faster. The mean IL-6 level in the positive control group was 9.041 mg/dL compared to the 14th day, which was 10.509 mg/dL. The table shows that the P2 group experienced a decrease of 43.80% faster than the K (-) group which experienced a decrease of 22.23%.

The role of IL-6 as a pleiotropic cytokine that carries out pro- and anti-inflammatory activities. In the research of Masfufatun et al

(2018) mentioned IL-6 is considered a pro-inflammatory cytokine and IL-6 also has regenerative and anti-inflammatory activities. This study also explains that IL-6 levels will increase at the peak of inflammation. In the inflammatory phase, pro- and anti-inflammatory levels must be balanced because they are important for immune system activity . Neem leaves extract contains flavonoid compounds that have the ability to inhibit nitric oxide (NO) activity, which inhibits IL-6 expression. NO is a free radical with odd electrons that can bind to other molecules such as oxygen, superoxide, and transition metals.

As a result, excessive production of NO can lead to increased expression of IL-6 which is associated with various cardiovascular pathogenesis and complications [18]. The presence of IL-6 on day 14 in the wound healing process because on day 7 to day 14 is a proliferation phase that still requires IL-6 as a receptor in stimulating keratinocytes that play a role in the wound healing process. The proliferation phase occurs around day 4 to day 14 after trauma or injury which is characterized by the formation of granulation tissue. IL-6 is needed in the wound healing process in the proliferation phase because IL-6 can stimulate epithelial proliferation. A decrease in IL-6 levels indicates that IL-6 is successful in controlling systemic inflammation and will return to normal because the presence of IL-6 is no longer needed. IL-6 concentration must decrease because the remodeling phase does not require IL-6 and is a sign that there is no infection or chronic inflammation in the wound (Hafizsha, dkk., 2021).

The main obstacle in this study to putting topical therapy into practice is its cost, as it is more expensive than commercial drugs due to the preparation of the extract. More research is required to assess the efficacy and potency of topical interleukin therapy in DM wounds as well as other cost-effective options. Further research is required to study the available treatment modalities for a diabetic wound with respect to possible drug therapies, the cytokine environment of the wound and the side effects of such treatment

It can be concluded that there is significant difference in effectiveness of Neem leaves extract (*Azadirachta*

indica L.) 5%, 10%, and 15% in combination with VCO compared to negative control group and the positive control group on wound healing in diabetic rat models. Neem leaf extract, in combination with VCO at 10% concentration, has accelerated wound healing of diabetic rats and inhibited the inflammation of diabetic wounds in the healing process through a decrease in IL-6 levels on day 14.

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Conflic of Interest

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