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The Effect Of Giving Ethanol Extract Of African Leaves (Vernonia Amygdalina) On Wound Closure And Pmn Cell Infiltration In Wistar Rats With Streptozotocin Induced Diabetes

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Abstract

Purpose: *This study aims to prove the role of the ethanol extract of African leaves (Vernoniaamygdalina) in improving wound healing in streptozotocin-induced diabetic ulcers in rats.*

Methods: *In this study four groups of rats were induced with diabetes using 55 mg/kgBWStreptozotocin (STZ) intraperitoneally on the first day. All rats with serum glucose levels above 250 mg / dL were then excised on the back. For 16 days, 3 groups of rats were given African leaf extract (Vernonia amygdalina) at doses of 10, 30, and 90 mg/kgBW. One group was only given aquadest after excision and the other group was excised without STZ injection. On the 17th day surgery was performed, blood and skin tissue samples were taken for histopathological examination.*

Results: *The ethanol extract of African leaves (Vernonia amygdalina) has been shown tosignificantly reduce blood glucose level positive control 480.5 mg/dl, negative control 166.75 mg/dl, extract dose 10 mg/kgBW 275 mg/dl, extract dose 30 mg/kgBW 285,75 mg/dl, extract dose 90 mg/kgBW 325,5 mg/dl, increase the speed of wound closure (p= 0.000, p <0.05), reduce PMN cell infiltration (p= 0,000, p <0.05), and reduce TNF-α level but statistically not significant (p = 0.075, p> 0.05).*

Conclusions: *The administration of ethanol extract of African leaves (Vernonia amygdalina)has been shown to reduce blood glucose levels, increase the speed of wound closure, reduce PMN cell infiltration, and reduce TNF-α in Wistar rat with diabetic ulcers with the induction of Streptozotocin.*

Keywords: *Vernonia amygdalina, diabetic ulcer, blood glucose level, wound closure rate,PMN cell infiltration, serum TNF-α.*

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I. Introduction

A diabetic ulcer is a complication of macroangiopathy in Diabetes mellitus (DM) due to vascular insufficiency and neuropathy. Ulcers can carry bacterial infections, causing difficult sores to heal and lead to amputations.[1] The limitations of therapy cause the need for the development of companion therapy to treat diabetic ulcers. African leaf plants (*Vernoniaamygdalina*) with its active ingredient luteolin have been widely consumed as herbs to reduceglucose levels in diabetic patients. *Vernonia amygdalina* is known to have hypoglycemia, vasodilator, antibacterial, and reepithelization effects and induce collagen formation in topical administration.[2,3] This study aims to prove the role of the ethanol extract of African leaves (Vernonia amygdalina) in improving wound healing in streptozotocin-induced diabetic ulcers in rats.

II. Methods

Research Design and Animal Models

In this study, four groups of rats (16 rats) were induced with diabetes using 55 mg/kgBW Streptozotocin (STZ) intraperitoneally on the first day. All rats with serum glucose levels above 250 mg / dL were then excised on the back. For 16 days, 3 groups (12 rats) of rats were given African leaf extract (*Vernonia amygdalina*) at doses of 10, 30, and 90 mg/kgBW. One group (4 rats) was only given aquadest after excision and the other group was excised without STZ injection. On the 17th day surgery was performed, blood samples were taken for glucose serum examination and skin tissue samples were taken for analysis of CT-50, PMN infiltration, TNF-α on histopathological examination.

Blood Glucose Examination
Blood glucose examination is using Glucose Stick Easy Touch from peripheral blood samples three days after injection and on the day of surgery.

TNF-α Examination
On the 17th day before surgery, the TNF-α serum level was examined using the ELISA method with a wavelength of 450nm.

Histopathologic Examination
Each group was fixed with a paraffin block, then stained with hematoxylin and eosin (HE), and the amount of PMN infiltration that infiltrates the wart and subcutis layer was counted.

The number of field of view is determined by counting the minimum number of field of view (magnification 1000) for each preparation. The amount of PMN associated with injury was assessed quantitatively with a 100x objective lens (magnification of 1000) using emersion oil.

Wound Closure Examination
Measurement of the wound area is done by taking photos with a digital camera every 2 days. On the edge of the wound is placed a grid/ruler. The wound area was measured by manual tracing using ImageJ software (64-bit) with the measurement results in mm2. Half closure time (CT-50) is the time required for the wound to cover 50%, calculated using a formula that includes the area of the wound and the day of measurement. The results then performed linear regression analysis using GraphPad Prism V software.

PMN Infiltration Examination
On observation under a microscope, quantitatively counted the number of PMN cells that experience infiltration in 10 fields of view. The total number of PMN cells found in the epithelial layer is data that will be processed by statistical analysis.

Statistical Analysis
Data were collected and processed using SPSS 11.0 for windows computers, and the data were expressed as mean ± standard deviation (mean ± SD). Then the histological score difference test between groups was carried out using the One-way ANOVA test, with a limit of the degree of significance of $p < 0.05$ with a 95% confidence interval and the presentation in tables and graphs.

III. Results

Blood Glucose Level

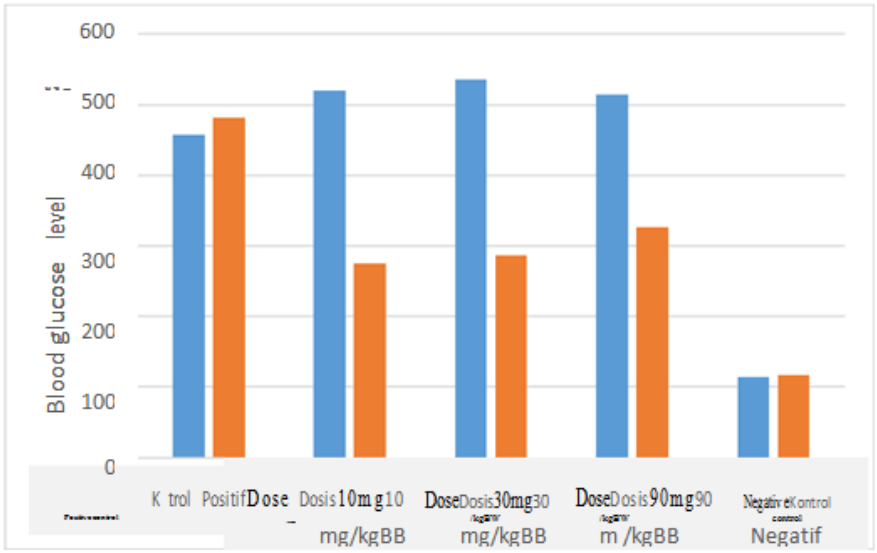

Figure 1. Mean Blood Glucose Levels in Rat. Description: Blue (blood glucose level on day 0); Red (blood glucose level 17th day)

In the group that received the STZ injection on the third day after induction, the mean blood glucose level of the positive control group was 457 mg/dl, the extract dose was 10 mg / kgBW 518.75 mg/dl, the extract dose was 30 mg / kgBW 535.25 mg/dl. , the extract dose is 90 mg / kgBW 513.50 mg / dl. There was a significant increase in blood glucose levels compared to the negative control group (114.5 mg/dl). This indicates that there has been a hyperglycemia (diabetes mellitus) condition in the group of rats that were given STZ induction. On the 17th day before the surgery, the blood glucose level was again checked. Obtained mean blood glucose levels random positive control group 480.5 mg / dl, extract dose 1 10 mg / kg body weight 275 mg / dl, extract dose 2 30 mg / kgBW 285,75 mg / dl, extract dose 3 90 mg / kgBW 325 , 5 mg / dl and the negative control group 166.75 mg / dl. There was a significant decrease in mean blood glucose levels after giving African leaf extract (Vernonia amygdalina) compared to the positive control group.

Wound Closure

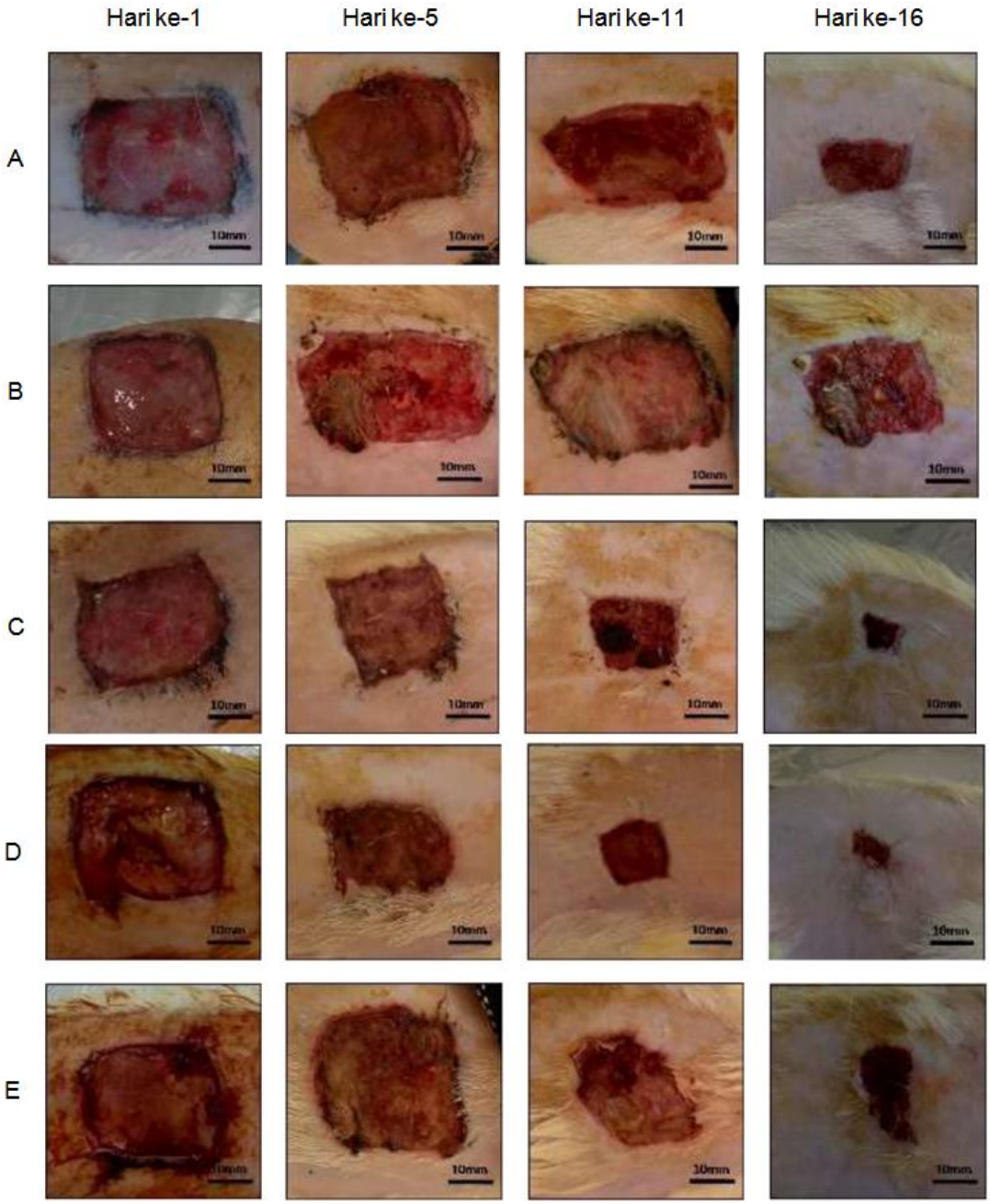

Figure 2. Wound Closure Description: Line A (Negative Control); B (Positive Control); C (Extract 10mg / kgBB); D (Extract 30mg / kgBB); E (Extract 90mg / kgBB)

The figure in line A shows wound closure in rats without diabetes mellitus. It was seen that the wound area appeared to widen at the start of the treatment, then it became more and more closed on the 17th day. In rat with the induction of diabetes mellitus, it appears that the wound tends to get wet, it becomes deeper and the wound bed is dirty (containing pus). The images in rows C, D, and E show wounds in rats with diabetes mellitus by administering the extract at a dose of 10 mg/kg, 30 mg/kg, and 90 mg/kg. On the 17th day, the wound area was getting smaller, with a cleaner wound bed, except in group E.

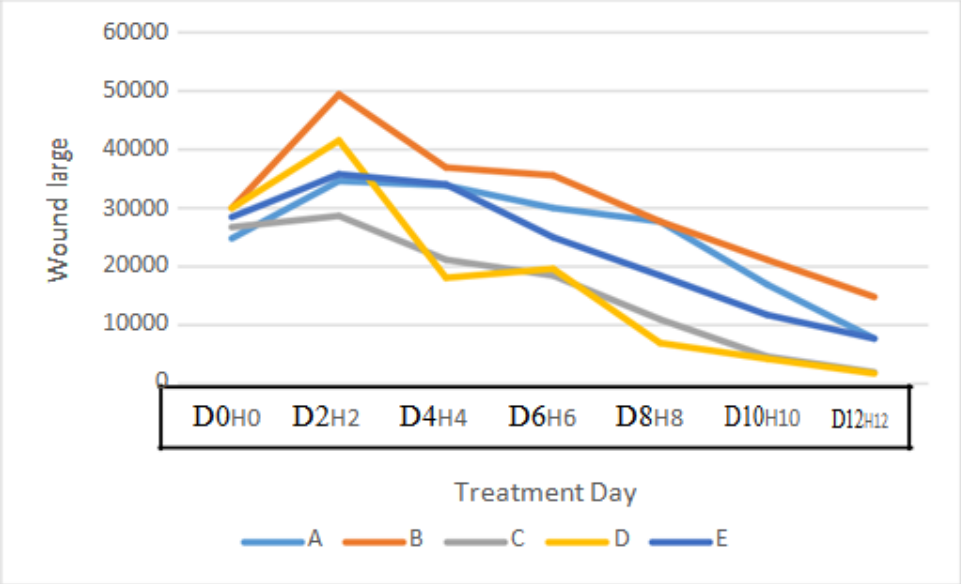

Figure 3. Average Wound Area in Rat Description: A (Negative control); B (Positive control); C (dose 10 mg / kgBW); D (Dose 30 mg / kgBW); E (Dose 90 mg / kgBW)

Table 1. Wound Closure Linear Regression Analysis

Group	Equation	CT-50	CT-100
A	Y = -1070*x + 33806	11.63	19.98
B	Y = -1643*X + 42566	16.80	25.90
C	Y = -4683*X + 55676	7.44	11.17
D	Y = -2369.5*X + 32653	7.72	13.78
E	Y = -2264*X + 38832	9.22	17.51

Description: A (Negative control); B (Positive control); C (dose 10 mg / kgBW); D (Dose 30 mg / kgBW); E (Dose 90 mg / kgBW); CT-50 (half closure time); CT-100 (closure time)

Simple linear regression analysis produces equations that can be used to estimate the CT-10 and CT-100 of each treatment group (table 1). The longest CT-50 obtained was the negative control group, with the fastest from the Dose 1 and 2 groups. While the fastest wound closure was from the Dose 1 group with 11 days, followed by the Dose 2 group on the 13th day, then the Dose 3 group. on the 17th day. The wound closure in STZ-induced rats with African leaf supplementation was faster than in the non-supplemented group.

Table 2. ANOVA Test of Wound Closure Speed

Parameter		Sum of Squares	Df	Mean Square	F	Sig.
CT-50	Between Groups	170.587	4	42.647	1625.669	.000
	Within Groups	.394	15	.026		
	Total	170.980	19			
CT-100	Between Groups	238.761	4	59.690	222.657	.000
	Within Groups	4.021	15	.268		
	Total	242.782	190			

The homogeneity test results show that the significance value of p = 0.098 on CT-50 and 0.106 on CT-100, is greater than the specified α = 0.05 value. It means that the five treatment groups on wound closure speed are identical or have the same variance. The data obtained were normal and homogeneous, then performed a parametric test, namely one way ANOVA to determine differences in treatment of the speed of wound closure on the rats' backs. The summary of the results of the one way ANOVA is listed in table 2.

The one way ANOVA test results obtained a significance value of p = 0.000 (p <0.05) between the control group and the test group so that it can be concluded that there is a significant difference in wound closure speed in 2 or more treatment groups. The analysis was continued with a post hoc analysis, which aims to determine which group was significantly different from the one way ANOVA test results. From the posthoc test the following results were obtained (Table 2).

Table 3 Post-Hoc Test of Wound Closure Speed

Parameter	Mean Difference	A	B	C	D	E
CT-50	A	-	-3.3500*	3.5425*	4.9050*	2.5550*
	B	3.3500*	-	6.8925*	8.2550*	5.9050*
	C	3.5425*	-6.8925*	-	1.3625	-.9875
	D	-4.9050*	-8.2550*	-1.3625*	-	-2.3500*
	E	-2.5550*	-5.9050*	.9878*	23500*	-
CT-100	A	-	-4.3200*	4.3175*	5.4125*	2.0675*

B	4.3200*	-	8.6375*	9.7325*	6.3875*
C	-4.3175*	-8.6375*	-	1.0950	-2.2500*
D	-5.4125*	-9.7325*	-1.0950*	-	-3.3450*
E	-2.0675*	-6.3875*	2.2500*	3.3450*	-

From the post hoc test, the significance value of the difference in wound closure speed was p

= 0.000 in each group. This value means that the difference in each group is significant (p <0.05). To determine the magnitude of the relationship and the concentration of African leaf extract on the speed of wound closure, the Pearson correlation test was used. Pearson's parametric correlation test showed a significance value of p = 0.000 (p <0.05) and a correlation coefficient of -.0646 which means that there is a significant correlation between extract concentration and wound closure speed. The negative correlation direction means that the correlation is inversely related, which indicates that the higher the dose or concentration of the extract, the faster the wound closure will be, and shows the strength of the strong correlation.

TNF-α serum levels

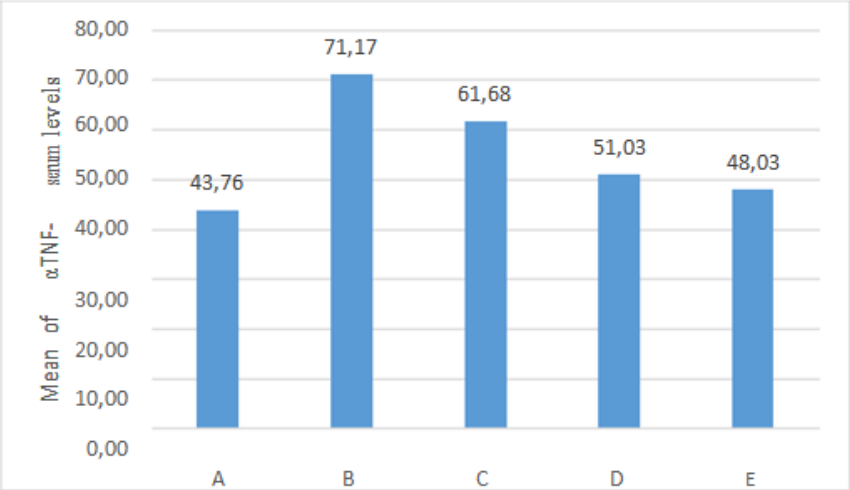

Figure 4. Average TNF-α Serum Levels in Rat Description: A (negative control); B (positive control); C (extract dose 10mg / kgBW); D (extract dose of 30 mg / kgBW); E (extract dosage 90 mg / kgBW)

On the 17th day before surgery, the TNF-α serum level was examined. The average serum TNF-α level in the positive control group was 71.17 pg / dl, the extract dose was 10 mg / kgBW 61.68 pg / ml, the extract dose was 30 mg / kgBW 51.03 pg / ml, the extract dose was 90 mg / kgBW 48.03 pg / ml and negative control group 43.76 pg / ml. There was an increase in the mean serum TNF-α level after diabetes mellitus injection compared to the negative control group. This suggests that diabetes mellitus causes systemic inflammatory conditions. There was a decrease in the mean serum TNF-α level after giving African leaf extract (Vernonia amygdalina) compared to the positive control group.

Table 4 ANOVA Test of TNF-α Serum Levels

Parameter	Sum of Squares	Df	Mean Square	F	Sig.
serum TNF-α	Between Groups	1987.411	4	496.853	2.648	.075
	Within Groups	2814.069	15	187.605		
	Total	4801.479	19			

TNF-α serum data were tested for normality using the Kolmogorov-Smirnov. The test results showed that the probability for all treatment groups was 0.200, greater than 0.05 (p> 0.05). The homogeneity test results show that the significance value is p = 0.138, greater than the specified α = 0.05. The one way ANOVA test results obtained a significance value of p = 0.075 (p <0.05) between the control group and the test group so that it can be concluded that there is no significant difference in TNF-α serum in 2 or more treatment groups.

Number of Infiltrated PMN Cells

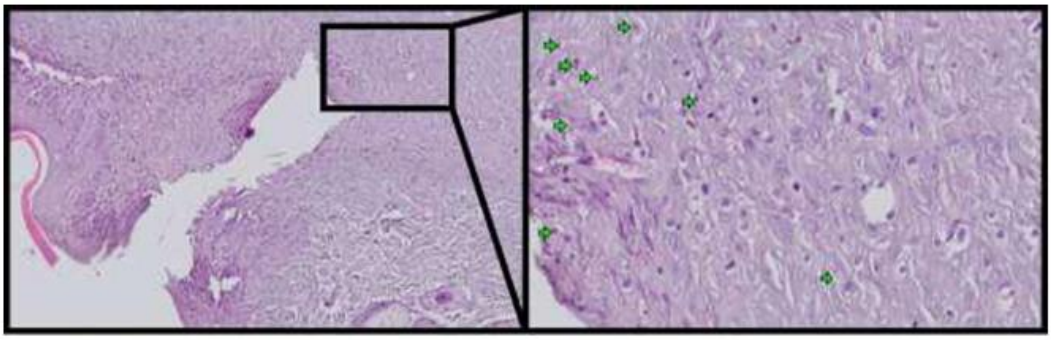

Figure 5. Back Wound of Rats in Negative Control Group. Description: Left (Full view, transverse cut, HE stain, 10x magnification); Right (epidermis, transverse cut, HE stain, 40x magnification) green arrow showing PMN cell infiltration

Figure 5. shows normal skin tissue after wound creation. It can be seen that the connective tissue (brush) is neatly arranged under the epithelium with minimal PMN cell infiltration.

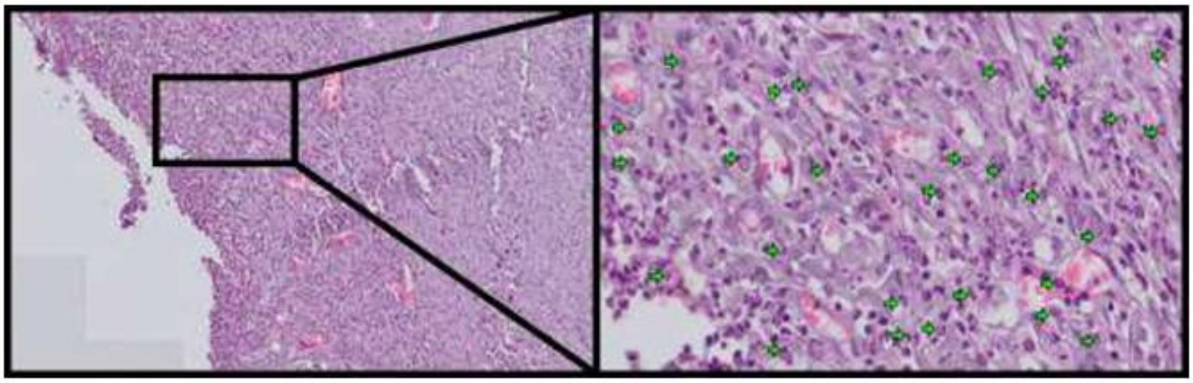

Figure 6. Back Wound of Rats in Positive Control Group (Induced by STZ). Description: Left (Full view, transverse cut, HE stain, 10x magnification); Right (epidermis, transverse cut, HE stain, 40x magnification) green arrow showing PMN cell infiltration.

Figure 6. shows the skin tissue in STZ-induced rats after wound making. Visible connective tissue (cicatrix) under the epithelium with massive PMN cell infiltration.

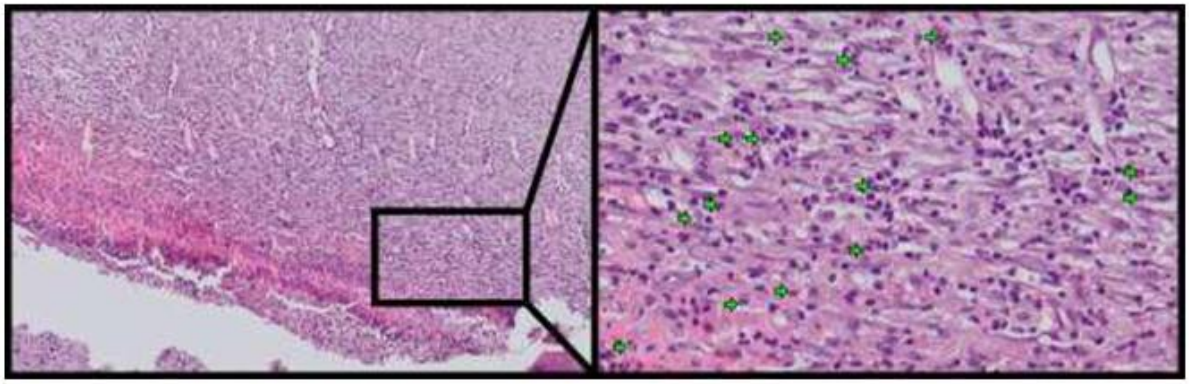

Figure 7. Back Wound of Dose Group 1 Rats (Induced by STZ; Dose 10 mg / kgBW). Description: Left (Full view, transverse cut, HE stain, 10x magnification); Right (epidermis, transverse cut, HE stain, 40x magnification) green arrow showing PMN cell infiltration.

Figure 7. shows the skin tissue in STZ-induced rats after wound making with a therapeutic dose of 10 mg / kgBW. It can be seen that the PMN cell population infiltrating the epithelium but not as much as the positive control group and the cyclic tissue looks tidier.

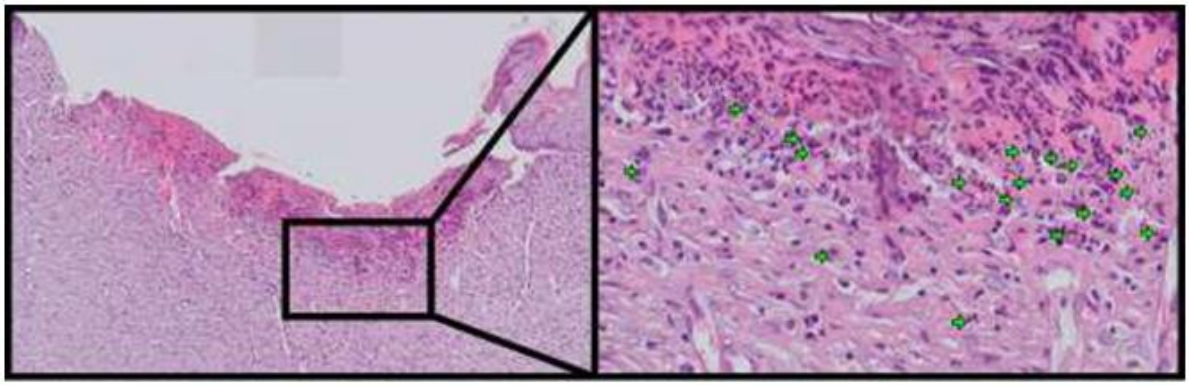

Figure 8. Back Wound of Dose Group 2 Rats (Induced by STZ; Dose 30 mg / kgBW). Description: Left (Full view, transverse cut, HE stain, 10x magnification); Right (epidermis, transverse cut, HE stain, 40x magnification) green arrow showing PMN cell infiltration

Figure 8. shows skin tissue in STZ-induced rats after wound making with a therapeutic dose of 30 mg / kgBW. The PMN cell population that infiltrated the epithelium was seen but not as much as the Dose 1 group and tissue.

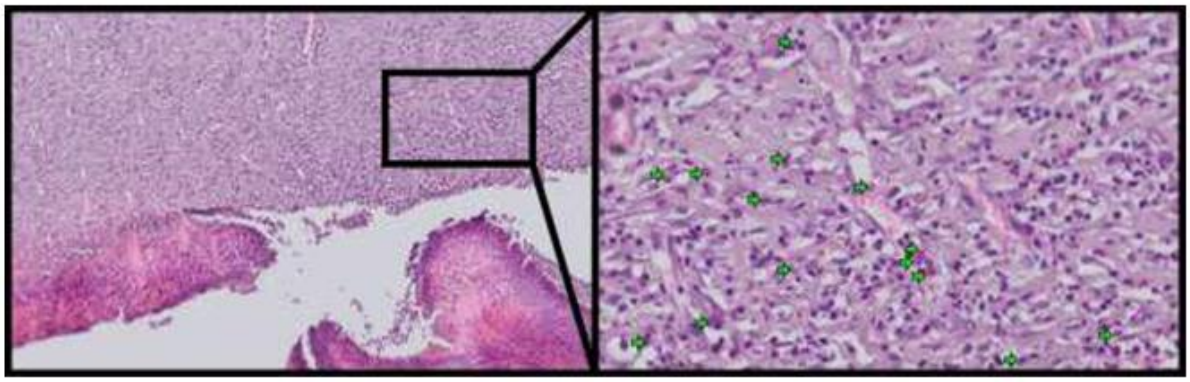

Figure 9. Back Wound of Dose Group 1 Rats (Induced by STZ; Dose 90 mg / kgBW). Description: Left (Full view, transverse cut, HE stain, 10x magnification); Right (epidermis, transverse cut, HE stain, 40x magnification) green arrow showing PMN cell infiltration.

Figure 9. shows the skin tissue in STZ-induced rats after wound making with a therapeutic dose of 90 mg / kgBW. The PMN cell population that infiltrated the epithelium was seen but not as much as the dose group 2.

Observation under a microscope quantitatively counted the number of PMN cells that experience infiltration in 10 fields of view. The total number of PMN cells found in the epithelial layer is data that will be processed by statistical analysis. Obtained data such as table 5.2.

Table 5. Number of PMN Cells Experiencing Infiltration in the Overall Field of View Replication

Group	1	2	3	4	Mean	SD
A	56	48	52	47	50,75	3,56
B	425	423	440	429	429,25	6,57
C	226	252	267	248	248,25	14,67
D	161	168	152	160	160,25	5,67
E	92	146	133	153	131,00	23,63

Description: A (Negative Control); B (Positive Control); C (STZ + Extract 10mg / kgBW); D (STZ + Extract 30mg / kgBW); E (STZ + Extract 90mg / kgBW)

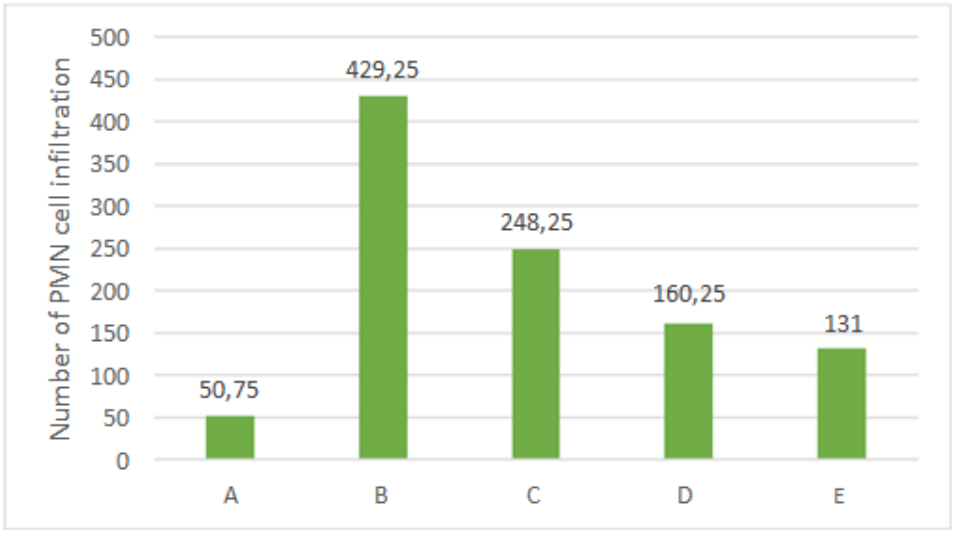

Figure 10. Average PMN Cell Infiltration. Description: A (negative control); B (positive control); C (extract dose 10mg / kgBW); D (extract dose of 30 mg / kgBW); E (extract dosage 90 mg / kgBW)

Table 5. shows that the lowest number of PMN cells infiltration was in group A of 50.75 cells, then group E was 131.00 cells, while group D was 160.25 cells, group C had 248.25 cells and group B had total cells. The highest number of PMN was 429.25 cells. For details, see graph 10.

Table 10. ANOVA Test of PMN Infiltration

Parameter	Sum of	Df	Mean Square	F	Sig.
	Squares				
Infiltrate PMN	Between Groups	306777.77	4	76694.425	1485.367 .000
	Within Groups	774.500	15	51.633	
	Total	307552.200	19		

The PMN cell infiltration data were tested for normality using the Kolmogorov-Smirnov. The test results showed that the probability for all treatment groups was 0.101, greater than 0.05 (p> 0.05). The results of the homogeneity test showed that the significance value of p = 0.443 on PMN cells was greater than the specified α = 0.05. The one way ANOVA test results obtained a significance value of p = 0.000 (p <0.05) between the control group and the test group so that it can be concluded that there is a significant difference in PMN cell infiltration in 2 or more treatment groups.

Table 11 Post-Hoc Test of PMN Infiltration

Parameter	Mean Difference	A	B	C	D	E
PMN	A	-	-340.25*	-232.75*	-117.25*	-49.50
Infiltration	B	340.25*	-	107.50*	223.00*	290.75*
	C	232.75*	-107.50*	-	115.50*	183.25*
	D	117.25*	-223.00*	-115.50*	-	67.75*
	E	49.50*	-290.75*	-183.25*	-183.25*	-

The analysis was continued with a post hoc analysis, which aims to determine which group was significantly different from the one way ANOVA test results. From the posthoc test, the following results were obtained (Table 10). From the post hoc test, it was found that the significant difference in PMN cell infiltration was p = 0.000 in each group. This value means that the difference in each group is significant (p <0.05). To determine the magnitude of the relationship and the concentration of African leaf extract on PMN cell infiltration, the Pearson correlation test was used. Pearson's parametric correlation test shows a significance value of p = 0.552 (p <0.05) and a correlation coefficient of -.141 which means that there is no significant correlation between extract concentrations and PMN cell infiltration.

IV. Discussion

The Effect of Giving African Leaf Extract (Vernonia amygdalina) on the Blood Glucose Level The alcohol extract of Vernonia amygdalina was reported to significantly improve glucose tolerance in STZ-induced mice, lower fasting blood glucose levels, and show a protective effect on pancreatic beta cells, thereby increasing insulin levels. The same researchers reported administering Vernonia amygdalina increased GLUT-4 expression in muscle and its translocation on the plasma membrane.[4] The combination of vernonia amygdalina extract and metformin with a ratio of 1: 2 caused a significant reduction in glucose levels compared to the control group.[5]

*The effect of giving African leaf extract (Vernonia amygdalina) on TNF-α seru*m
The anti-inflammatory effect of vernonia amygdalina extract has been compared with acetylsalicylic acid. Inflammation induction is done by applying a mixture of pyridine, water, diethyl-ether, and croton to the ears of rats. The inflammatory response is measured and compared, it is known that vernonia amygdalina extract can significantly reduce the inflammatory response.[6]

In this study, giving African leaf extract (Vernonia amygdalina) was proven to reduce the mean serum TNF-α level (Figure 4) at a dose of 10 mg / kgBW (61.68 pg/dl), 30 mg / kgBW (51.03 pg/dl), or 90. mg / kgBW (48.03 pg / dl). The one way ANOVA parametric test results showed a significance value of $p = 0.075$ ($p < 0.05$). It can be concluded that there is no significant difference in TNF-α serum after administration of vernonia amygdalina extract.

Olaniyan et al., Compared the levels of TNF-α and IL-10 in plasma of 33 diabetes mellitus patients with insulin therapy compared to administration of Vernonia amygdalina orally. There was a significant decrease in TNF-α levels after administration of Vernonia amygdalina extract ($p < 0.05$).[7]

Vernonia amygdalina is suspected to have anti-inflammatory activity through the bioactive content of flavonoids and saponins.[3] Ngatu, et al., Conducted a study assessing cytokine levels in rats with atopic dermatitis after administration of vernonia amygdalina extract. There was a significant decrease in TNF-α, IFN-Ɣ, and IL-5 in serum and skin lesions.[8]

The effect of giving African leaf extract (Vernonia amygdalina) on the speed of wound closure

In this study, giving African leaf extract (Vernonia amygdalina) was proven to reduce the mean CT-50 and CT-100 at doses of 10 mg / kgBW (7.44 and 11.17), 30 mg / kgBW (7.72 and 13.78), or 90 mg / kgBW (9.22 and 17.51). The mean reduction of CT-50 and CT-100 compared to the positive control group (STZ-induced rats) and in the post-hoc analysis (Table 3), there was a significant difference ($p < 0.05$) between the treatment groups ($p = 0.000$). Pearson's correlation test found that the correlation direction is inversely proportional, meaning that increasing the dose causes a decrease in CT-50 and CT-100. This suggests that the administration of vernonia amygdalina extract can significantly accelerate wound closure of diabetic rats.

Nafiu et al., Compared wound healing in healthy rats with topical administration of Vernonia amygdalina and honey. It was found that the administration of Vernonia amygdalina significantly accelerated wound closure, as well as minimal exudation compared to the negative control group.[9] Eyo et al., Modeled incision in rat and compared several extracts topically. Vernonia amygdalina has been shown to accelerate wound contraction of Zingiber officinalis extract, and Ocimum gratissimum in every phase of wound healing.[2]

In phytochemical studies, Vernonia amygdalina extract was known to compete with various bioactive components of the saponin, flavonoid, alkanoid, and steroid groups.[10] The dominant saponin content is vernoniosides, while flavonoids include luteolin, luteolin 7-O-β-glucosidase.[11] In vitro, luteolin can induce the proliferation and migration of fibroblast cells so that it can play a role in wound healing.[12] In an in vivo study, Ozay et al. applying luteolin topically to the excised wound of the rat with diabetes. Luteolin increases the wound healing ratio and tensile strength significantly. Tensile strength is an important parameter because it reflects the organization of collagen fibers in subdermal tissue.[13] This healing effect is caused by the hydroxyl group and the 2-3 carbon double bond in the structure of Luteolin so that it can act as an anti-inflammatory, antioxidant, and antimicrobial.[14]

The effect of giving African leaf extract (Vernonia amygdalina) on PMN cell infiltration

In this study, giving African leaf extract (Vernonia amygdalina) was proven to reduce the mean PMN cell infiltration (Table 5) at a dose of 10 mg / kgBW (248.25), 30 mg / kgBW (160.25), or 90 mg / kgBW (131.00). The decrease in mean PMN cell infiltration was supported by posthoc analysis (Table 11). There was a significant difference ($p < 0.05$) between the treatment groups ($p = 0.000$). Pearson's correlation test found that the correlation direction was inversely proportional, meaning that increasing the dose caused a decrease in PMN cell infiltration. This shows that the administration of Vernonia amygdalina extract can significantly reduce PMN cell infiltration in diabetic rat's wounds.

In the study of Nafiu et al., A semi-quantitative calculation was carried out on wound histopathological preparations with one of the infiltration parameters. There was a significant reduction in PMN infiltration after topical administration of Vernonia amygdalina extract compared to the negative control group. Histomorphological, Nafiu also has increased fibroblast recruitment, endothelial cell formation, and

epithelialization. It can be concluded, Vernonia amygdalina has a role at the cellular level in wound healing through activation, migration, proliferation, and differentiation of cells in the skin.[9]

Asante et al. conducted a trial of administering vernonia amygdalina extract topically to inflamed rats' feet. There was a significant reduction in leukocyte infiltration compared to the negative control group with doses of 100 and 200 mg / kgBW. Administration of Vernonia amygdalina extract is also known to reduce mast cell degranulation. Asante stated that the ethanol extract of young Vernonia amygdalina leaves can reduce or inhibit the transmigration of pro-inflammatory cells such as PMN through stabilization of resident mast cells in the tissue.[3]

Onasanwo in 2017 conducted a similar study aimed at anti-inflammatory effects on rat feet using topical methanol extract of Vernonia amygdalina. There was a significant reduction in leukocyte infiltration in edematous tissues, as well as protein concentrations and MDA levels.[15]

V. Conclusion

1. The administration of ethanol extract of African leaves (Vernonia amygdalina) is proven to reduce serum glucose levels in rats with diabetic ulcers with the induction of Streptozotocin

2. The administration of ethanol extract of African leaves (Vernonia amygdalina) is proven to reduce CT-50 in rats with diabetic ulcers with the induction of Streptozotocin with an effective dose of 10 mg / kgBW

3. Administration of African leaf ethanol extract (Vernonia amygdalina) has been shown to reduce PMN cell infiltration in rat with diabetic ulcers with the induction of Streptozotocin

4. The administration of ethanol extract of African leaves (Vernonia amygdalina) was not proven to reduce TNF-α serum levels in rats with diabetic ulcers by induction of Streptozotocin

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DISCLOSURE

All author reports no conflicts of interest in this work.

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PENELITI UTAMA	:	**dr. Andreas Nicolaus Ola**
UNIT / LEMBAGA	:	**PPDS I Bedah - Fakultas Kedokteran - Universitas Brawijaya Malang.**
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