The effect of Abelmoschus esculentus Ethanol Extract on TNF-α expression in wound healing of diabetic: implications for physical recovery

El efecto del extracto de etanol de Abelmoschus esculentus sobre la expresión de TNF-α en la cicatrización de heridas de diabéticos: implicaciones para la física

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Abstract

Introduction: For the wound healing process, the role of pro-inflammatory mediators such as Tumor Necrotic factor (TNF-α) and Interleukin-1. TNF-α is a pro-inflammatory mediator produced by macrophages that stimulates inflammatory cells, fibroblasts, and epithelial cells. One of the herbal plants suitable for treatment is okra fruit (*A. esculentus*) because it has beneficial properties that include antidiabetes, antioxidants, and antibacterial and anti-inflammatory properties.
Objective: The research aims is to know the effect of *A. esculentus* ethanol extract on tnf-α expression in wound healing of diabetic Wistar rats.
Methodology: Laboratory experimental research design with post-test-only control design using male white Wistar rats (*Rattus norvegicus*) as a sample of 30. The treatment consisted of negative control (K-), positive control (K+), experiments with various doses of P1 dose of 300 mg/kgBB, P2 dose of 600 mg/kgBB, and P3 dose of 900 mg/kgBB extra ethanol okra fruit. On the 21st day, blood was taken through the retroorbital vein to examine TNF-alpha levels using the enzyme-linked immunosorbent assay (ELISA) method using a manual ELISA tool from Bioeureaux.
Result and Discussion: The results of measuring TNF-α expression showed that TNF-α expression in the positive control group had an average expression of 6.95 ± 0.33 and decreased by 174.35% against the negative control (K-), with an average expression of 19.06 ± 0.29.
Conclusion: There is a statistically significant difference in Okra groups P1, P2, and P3. The administration of ethanol okra fruit can significantly reduce the expression of tumor necrosis factor α (TNF-α) in the wound tissue of Wistar rats model of Diabetes Mellitus.

Keywords

ELISA; diabetes mellitus; okra; tumor necrosis factor; wistar rats.

Resumen

Introducción: En el proceso de cicatrización de heridas, el papel de los mediadores proinflamatorios como el factor necrótico tumoral (TNF-α) y la interleucina-1. El TNF-α es un mediador proinflamatorio producido por los macrófagos que estimula las células inflamatorias, los fibroblastos y las células epiteliales. Una de las plantas herbáceas adecuadas para el tratamiento es el fruto del quimbombó (*A. esculentus*) porque tiene propiedades beneficiosas que incluyen antidiabetes, antioxidantes y propiedades antibacterianas y antiinflamatorias.
Objetivo: El objetivo de la investigación es conocer el efecto del extracto etanólico de A. esculentus sobre la expresión de tnf-α en la cicatrización de heridas de ratas Wistar diabéticas.
Metodología: Diseño de investigación experimental de laboratorio con diseño de sólo control post-test utilizando ratas Wistar blancas macho (*Rattus norvegicus*) como muestra de 30. El tratamiento consistió en control negativo (K-), control positivo (K+), experimentos con varias dosis de P1 dosis de 300 mg/kgBB, P2 dosis de 600 mg/kgBB, y P3 dosis de 900 mg/kgBB extra etanol fruto de okra. El día 21, se extrajo sangre a través de la vena retroorbital para examinar los niveles de TNF-alfa mediante el método de ensayo inmunoenzimático (ELISA) utilizando una herramienta ELISA manual de Bioeureaux.
Resultados y discusión: Los resultados de la medición de la expresión de TNF-α mostraron que la expresión de TNF-α en el grupo de control positivo tuvo una expresión media de 6,95 ± 0,33 y disminuyó un 174,35% frente al control negativo (K-), con una expresión media de 19,06 ± 0,29.
Conclusiones: Existe una diferencia estadísticamente significativa en los grupos Okra P1, P2 y P3. La administración del fruto de okra en etanol puede reducir significativamente la expresión del factor de necrosis tumoral α (TNF-α) en el tejido de la herida de ratas Wistar modelo de Diabetes Mellitus.

Palabras clave

ELISA; diabetes mellitus; okra; factor de necrosis tumoral; ratas wistar.

Introduction

Multisystem disorder that causes physiological changes in tissues and cells that disrupt the normal healing process. Diabetic wounds are trapped in an inflammatory phase characterized by a continuous influx of neutrophils that release cytotoxic enzymes, free radicals, and inflammatory mediators, causing significant damage to the surrounding tissue (Kant *et al.*, 2014). There is much evidence that inflammation is a contributing factor to delayed healing and indicates that there is an infectious response. There are several phases to restore the integrity of damaged skin, including the inflammatory, proliferative, and remodeling phases (Raina *et al.*, 2023; Khorsandi and Fekrazad, 2024).

Changes in these phases lead to delayed healing or inability to heal wounds. Wound healing after damage to the skin involves complex interactions between many cellular components of the skin, especially keratinocytes, fibroblasts, vessel endothelial cells, recruited immune cells, and their associated extracellular matrix (Martin and Nunan, 2015). Macrophages are contributors to wound healing such as neutrophils, keratinocytes, fibroblasts, mast cells, and endothelial cells significantly produce cytokines such as TNF-α and IL-10. Sustained production of pro-inflammatory cytokines can lead to impaired angiogenic response and microvascular complications (Izzatunnisa *et al.*, 2024), impair macrophage function, interfere with the migration and proliferation of keratinocytes and fibroblasts, and interfere with the production of growth factors (Okizaki *et al.*, 2015).

The wound healing process requires the role of pro-inflammatory mediators such as Tumor Necrotic factor (TNF-α) and Interleukin-1. TNF-α, as part of the inflammatory cascade, plays a crucial role in the development of atherosclerotic lesions (Popa *et al.*, 2007). The higher level of TNF-α in the wound indicates an ongoing inflammatory process (Brizeno *et al.*, 2016). Although the wound-healing process is the same for every patient, the results achieved depend on the biological conditions of each individual (Zabihi, Pashapour and Mahmoodi, 2023; Liu *et al.*, 2024). When a wound occurs, inflammation will occur, which then removes nonvital tissue and prevents invasive bacterial infection. When inflammation begins, a few minutes later, macrophages will secrete important factors in inflammation, such as Tumor Necrosis Factor (TNF) and Interleukin-1 (IL-1), which stimulate granulocyte-monocyte colonies.

In the wound process, TNF-α levels rise. The increase in TNF-α levels can induce the release of endothelial adhesion molecule, intercellular adhesion molecule 1 (ICAM-1), which will increase the attachment of neutrophils to endothelial cells before entering the extravascular space or intracellular space. Therefore, reducing or smoothing persistent inflammation and eliminating free radicals by introducing anti-inflammatory agents and antioxidants into wound treatment could be an essential strategy to improve diabetic wound healing (Kant *et al.*, 2014). Alternative treatments using natural ingredients are widely practised because they are more economical and have limited side effects (Luthfi *et al.*, 2021). One suitable treatment plant is okra fruit (*A. esculentus*).

A. esculentus has many beneficial properties, including anti-diabetic, antioxidant, antibacterial, and anti-inflammatory. Okra pods and polyphenols and flavonoids were active constituents, which are crucial in managing diabetic wounds. (Xia *et al.*, 2015). Okra extract contains flavonoids, which act as antioxidants and have anti-inflammatory and antidiabetic properties. These properties help in reducing oxidative stress and inflammation, which are critical factors in the impaired wound healing observed in diabetic conditions (Ahmed and Kumar, 2016; Luthfi *et al.*, 2021). The ethanol extract of okra was shown to reduce the mRNA expression of TNF-α in diabetic mice, indicating its potential to control inflammation (Mahdavi, Javadivala and Ahmadian, 2022).

Okra fruit inhibits the alpha-glucosidase enzyme, inhibiting glucose absorption in the intestine and lowering glucose levels (Trisnadi, 2023). Active ingredients contained in okra fruit include saponins, tannins, flavonoids, and alkaloids (Bello *et al.*, 2015). Flavanoids can reduce ROS, thus accelerating wound healing (Ciz *et al.*, 2012). Based on the above statement, the research aims to know the effect of *A. esculentus* ethanol extract on TNF-α expression in wound healing of diabetic Wistar rats.

Method

Design

The research design was laboratory experimental with a post-test-only control design using male white Wistar rats (R. norvegicus). The treatment consisted of negative control (K-), positive control (K+), experiments with various doses of P1 dose of 300 mg/Kg BB, P2 dose of 600 mg/Kg BB, and P3 dose of 900 mg/Kg BB of okra fruit ethanol extract. Ethanol extract from okra fruit was made using the maceration technique using glass maceration container (Pyrex®, USA) with a capacity of 1000 ml for 24 hours, ensuring chemical resistance and preventing contamination. Okra fruit was dried in an oven at 60 °C and turned over every 4 hours (Husen et al., 2020). The research sample consisted of 30 male white Wistar rats aged 8-10 weeks, weighing 200-300 grams. Animals for each homogeneous treatment were randomized to maintain internal validity.

Data Collection

This study was conducted in two stages on 30 Wistar rats. The first stage of Wistar rats was made into diabetes mellitus rats by injecting NA (110 mg/kg BB) after 15 minutes in single dose STZ induction (45 mg/Kg BB). Three days after injection, retroorbital venous blood was taken using a microcapillary tube on all white rats. Blood collection is done when the rat is fasting. Rats are declared DM if, three days after STZ and NA injection, GDP levels ≥ 250 mg/d (El-Gohary and Said, 2016). At the stage of preparing to make wounds on the back of rats previously anaesthetized with a dose of 10 mg/kg BB based intramuscularly (Siqueira *et al.*, 2010).

After anesthetizing the rats, they were prepared to make wounds on the back area and then shave the hair on the back. The back was incised with a size of 1 cm x 1 cm and then cleaned with 70% alcohol (Candra, Laksmitawati and Rahmat, 2022). Wistar rats that have been made wounds are grouped into five groups, each consisting of 6 rats. Group 1 was a negative control that was not given treatment; group II gave metformin 45 mg/kg BB; groups III, IV, and V gave ethanol extract of okra fruit at 300 mg/Kg BB, 600 mg/kg, 900 mg/Kg BB. The treatment of ethanol extract of okra fruit was given orally using a sonde. This treatment was carried out for 21 days. On the 21st day, blood was taken through the retroorbital vein to examine TNF-alpha levels. Examination of TNF-alpha levels of blood taken from the retroorbital vein using the enzyme-linked immunosorbent assay (ELISA) method using a manual ELISA tool from Bioeureaux.

Data analysis

Data were analyzed using a parametric test, the One-Way ANOVA test. This test is used to determine whether there are differences in mean values between treatment groups. If there is a significant difference between the one-way ANOVA tests, the post-test using the LSD and Duncan tests to determine which groups have significant differences should continue. Data were previously tested for normality with Shapiro-Wilk and Levene's homogeneity.

Results

The normality and homogeneity test analysis showed that TNF-α expression was normally distributed and homogeneous. Therefore, the One-way Analysis of Variance (ANOVA) parametric test was used. Then, if the results of the One-way ANOVA test show significant differences, proceed with the Post Hoc test using LSD and Duncan's test. The results of the research can be seen in Table 1.

Table 1. Post-hoc Test of TNF-α Expression

Treatment	Average±SD	Increasing	Decreasing	P Value
K (-)	19.06±0.29^{e}	-	-	0.001
K (+)	6.95±0.33^{a}	-	174.35%	-
P 1	13.96±0.37^{d}	100.98%	-	0.001
P 2	9.97±0.60^{c}	43.52%	-	0.001
P 3	7.91±0.34^{b}	13.89%	-	0.001

LSD and Duncan post-hoc tests

KN: Negative control

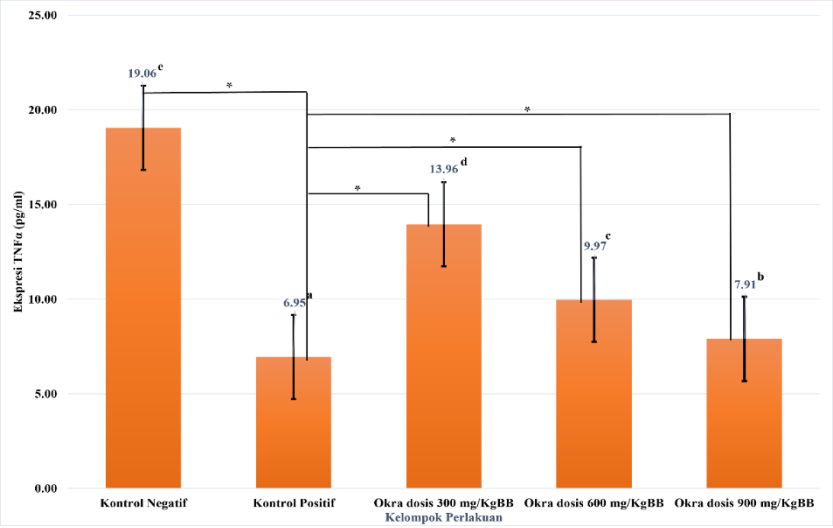
KP: Positive control by administering metformin 45 mg/Kg BB

P1: Treatment with ethanol extract at a dose of 300 mg/Kg BB

P2: Treatment with ethanol extract at a dose of 600 mg/Kg BB

P3: Treatment with ethanol extract at a dose of 900 mg/Kg BB

Figure 1. TNF-α Expression of the Treatment Group



Discussion

The average measurement results of TNF-α expression tend to be higher in the Okra treatment group at a dose of 300 mg/Kg BB, 600 mg/Kg BB, and 900 mg/KgBB compared to the positive control group. Furthermore, the sign (*) in Figure 1 shows the significance of P value <0.05 using the Post-hoc One-way Anova test, namely the LSD test, which indicates that there are statistically significant differences in the dose of 300 mg/Kg BB, 600 mg/Kg BB, and 900 mg/Kg BB. Then, from Table 1, the Duncan test shows that the Positive Control group is in subgroup "a", then the treatment groups of Okra dose 300 mg/kg BB, Okra dose 600 mg/kg BB, and Okra dose 900 mg/kg BB are in subgroups "d", "c", and "b", while the Negative Control group is in subgroup "e". This means that the average value of the positive control group is significantly different from the treatment groups of the dose 300 mg/Kg BB, 600 mg/Kg BB, and 900 mg/Kg BB because they have different notations. This condition shows that the highest increase in TNF-α expression occurred in the 300 mg/Kg BB okra treatment group by 100.98% or once. Additionally, okra extract demonstrates antioxidant and antimicrobial activities, protecting against oxidative stress and promoting tissue repair (Sipahi *et al.*, 2022).

The results of measuring the average expression of TNF-α in Table 1 show that the TNF-α expression of the positive control group has an average expression of 6.95 ± 0.33 and decreased by 174.35% against the negative control (K-) with an average expression of 19.06 ± 0.29. The post-hoc test results showed a significant difference between the expression of TNF-α in the group (K+) and the group (K-), which was characterized by a difference in notation. This is due to tissue inflammation due to excision wounds without therapy. The negative control group was used as a standard indicator of TNF-α expression and compared with the positive control group given metformin and the treatment group with the administration of ethanol okra fruit. The results of the study are in line with research by Siqueira et al., (2010) an increase in TNF-α due to the condition of the injured tissue will give the body a response in the form of inflammation so that it will activate phospholipase which will free arachidonic acid and cells phospholipids so that it will activate macrophages to secrete proinflammatory cytokines.

TNF-α expression in groups (P1, P2, P3) treated with ethanol extract of *A. esculatus* with different concentrations, namely 300 mg/Kg BB (P1), 600 mg/Kg BB (P2), 900 mg/Kg BB (P3) showed different results. Expression of TNF-α in the P1 group giving 300 mg/Kg BB dose of okra fruit ethanol decreased against the negative control with an average expression of 13.96±0.37. Based on the post hoc test showed a significant difference with the group (K-) and (K +). Group P2, administered with 600mg/Kg

BB dose of okra fruit ethanol, decreased against the negative control with an average expression of 9.97±0.60. The P3 group administered 900 mg/Kg BB dose of fruit ethanol reduced the average expression of 7.91±0.34 against the negative control. Post hoc test results showed a significant difference between the control (K-) and control (K+).

The results of the study are in line with research conducted by Luthfi et al. (2019), showing that there is a significant difference between the control group and the treatment group. This is because okra extract is given to the treatment group at a dose of 250 mg/Kg BB, which contains flavonoids that act as antidiabetics, antioxidants, and anti-inflammatory agents. The content of o *A. esculentus* extract as an antidiabetic is isoquercetin and quercetin-3-O-beta-glucopyranosyl-(1"- 6")-glucoside which is an ÿ-glucoside inhibitor works by inhibiting the absorption of glucose complexes caused by oligosaccharides and (1 "ÿ 6")-glucoside which is an inhibitor of ÿ-glucoside. The efficacy of okra extract in wound healing is attributed to its rich phenolic content, particularly isoquercitrin, which contributes to its antioxidant and anti-inflammatory effects (Sipahi *et al.*, 2022). Glucosides, which are ÿglucoside inhibitors, work by inhibiting glucose uptake, which causes oligosaccharides and polysaccharides not to be converted into monosaccharides so that blood glucose levels can decrease (Li *et al.*, 2015). By lowering blood glucose levels and improving glucose tolerance, okra extract can help manage diabetes more effectively, which in turn supports better wound healing outcomes (Yang *et al.*, 2018; Mahdavi, Javadivala and Ahmadian, 2022).

The flavonoid content as an antioxidant in *A. esculentus* has a high reactive hydroxyl group that can react with reactive components of free radicals so that the formation of ROS can be stable and controlled, which is characterized by a balance between oxidants and antioxidants. Flavonoids also act as anti-inflammatory agents through immunomodulatory mechanisms. Flavonoids can increase Th-1 and Th-2 production and IFN production, which can induce the formation of M1 and M2. The formed M1 can play a role in the phagocytic apoptosis of neutrophils. This causes a switch from M1 to M2 phenotype macrophages that produce growth factors such as VEGF. The anti-inflammatory mechanism in flavonoids can also reduce cyclooxygenase activity, which causes diminished prostaglandin synthesis, making the inflammatory process faster. The anti-inflammatory and antioxidant properties of okra extract can accelerate the wound healing process by reducing pro-inflammatory cytokines like TNF-α, thereby mitigating prolonged inflammation and promoting faster tissue repair (Husen *et al.*, 2020; Mahdavi, Javadivala and Ahmadian, 2022).

In diabetic foot ulcers, increased levels of TNF-ÿ increased fibroblast apoptosis and decreased fibroblast proliferation, followed by impaired ulcer healing. TNF-ÿ is an inflammatory marker in the tissue healing process (Rosyid, 2016). Previous studies have shown the relationship of TNF-ÿ to the wound healing process. Decreased levels of TNF-ÿ indicate that inflammation control and healing progress are inadequate as TNF-ÿ stimulates MMP synthesis. High levels of proteases in the wound lead to the degradation of matrix proteins and growth factors, essential factors in wound healing. As a result, the recovery process becomes disconnected and uncoordinated (Rosyid, 2016).

Diabetic wounds had increased TNF-α, fibroblast apoptosis, caspase-3/7 activity, pro-apoptotic transcription factor FOXO1 activation, and decreased proliferating cell nucleus antigen-positive fibroblasts ($p < 0.05$). TNF-α inhibition improved healing in diabetic rats and increased fibroblast density. This can be explained by decreased fibroblast apoptosis and increased proliferation when TNF-α was blocked ($p<0.05$) (Ravanti *et al.*, 1999). Results Diabetic wounds had increased TNF-α, fibroblast apoptosis, caspase-3/7 activity, and activation of the pro-apoptotic transcription factor FOXO1, and decreased proliferating cell core antigen positive fibroblasts ($p<0.05$). TNF-α inhibition improved healing in diabetic rats and increased fibroblast density (Siqueira *et al.*, 2010).

Conclusions

Avoid presenting conclusions that are not a consequence of what is stated in the results or repeating those previously presented. Based on data analysis ethanol extract of okra fruit (A. esculentus) can significantly reduce the expression of tumor necrosis factor α (TNF-α) in wound tissue of Wistar rats with

Diabetes Mellitus model. The findings suggest that okra extract could be a valuable natural therapeutic agent for enhancing wound healing in diabetic patients, potentially leading to improved physical recovery and reduced complications associated with diabetic wounds. Further research is needed to determine the effect of doses and duration of wound healing.

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