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RESEARCH ARTICLE

Effect of Moringa Leave Ethanol Extract on Accelerating Wound Healing Process

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Abstract

TGF-β and VEGF are vital in cell proliferation and regeneration, as evidenced in processes like wound healing. The leaves of *Moringa oleifera* Lam exhibit anti-inflammatory and cell regenerative properties that may facilitate the transition from the inflammatory to the proliferative phase, enhancing wound repair. This research sought to discern the potential of orally administered moringa leaf extract in augmenting systemic wound healing, focusing on TGF-β and VEGF serum as *in vivo* molecular markers. This research was conducted at the Animal Laboratory of the Faculty of Medicine, Universitas Padjadjaran, and the Laboratory of Molecular Genetics, Universitas Padjadjaran, from January to March 2022. We divided thirty Swiss Webster mice into two categories: healthy and wound-treated. Wounded mice received 100 mg/kgBW Na CMC as a negative control, 500 mg/kgBW zinc syrup as a positive control, and *M. oleifera* leaves ethanol extract (MOLE) in doses of 280 and 560 mg/kgBW orally from day 3 to day 6. Wound progression was documented and measured on days 0, -1, -3, and -6. Day-6 blood samples were obtained, and TGF-β and VEGF serum levels were gauged using ELISA. Results from day 6 revealed that wound coverage in the 280 and 560 mg/kgBW MOLE groups was 13.76±5% and 13.38±4%, respectively. These percentages notably surpassed that of the negative control group (p=0.005). However, the TGF-β and VEGF serum levels in the MOLE-treated groups did not differ significantly from the negative control (p=0.081 and p=0.149, respectively). Thus, the study concludes that while MOLE expedites wound closure, it does so without the systemic involvement of TGF-β and VEGF *in vivo*.

Keywords: *Moringa oleifera* leaves extract, TGF-β, VEGF, wound healing

Introduction

Wounds are defined as a loss or damage of body tissue due to a sharp or blunt trauma, temperature changes, chemical exposure, or electric shock.[1] They cause damage to the skin due to loss of epithelial tissue continuity, with or without loss of other underlying tissues, such as muscles, bones, blood vessels, and nerves.[2] According to the National Health Service (NHS), the annual prevalence of injuries in the United Kingdom increased by 71% between 2012/2013 and 2017/2018.[3] There is no epidemiology data on wounds in Indonesia. Still, it can be estimated that they are almost the same in number. The wounds that are difficult to heal affect the health costs incurred.[4] Wound healing treatments optimize controllable healing factors, such as infection clearance, mechanical protection, and nutritional support.[5]

Wound healing is a dynamic and complex process in response to injury. The skin cells are activated to promote healing by attracting other cells and substances from different body parts. The healing process has four overlapping phases: hemostasis, inflammatory proliferative, and remodeling. The inflammatory phase begins in 24 hours, lasting three days after wound onset.[5] In the proliferative phase, granulation tissue is formed by transforming growth factor β (TGF-β) and vascular endothelial growth factor (VEGF).[6] Some wound healing processes involve these factors, including inflammation, stimulation of angiogenesis, the proliferation of fibroblast, synthesis, and deposition of collagen, and remodeling of new extracellular matrix (ECM). Additionally, an increase in these factors on day 6 of onset accelerates wound closure, seven, and the disruption of this signaling causes impaired healing. Wound healing disorders are abnormal scarring or chronic injuries characterized by itching and pain. They may cause functional impairment, aesthetic, and psychological problems, leading to decreased quality of life.[8]

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Synthetic and natural medicines accelerate wound healing, and tropical regions rich in biological resources can be used as herbal medicines, including *Moringa oleifera* Lam, which has a high economic value and spread in many tropical and subtropical regions. Moringa has a range of medicinal uses with high nutritional value.[9] The leaves contain beta-carotene, vitamin C, vitamin E, flavonoids including quercetin, kaempferol, vicenin-2, and natural antioxidants. Therefore, they are reported to improve various biological functions, including anti-inflammatory, antimicrobial, anti-cancer, and wound healing properties.[10] The flavonoids increase the growth factor required for wound healing, such as epidermal growth factor (EGF), transforming growth factor α (TGF-α), TGF-β, platelet-derived growth factor (PDGF), VEGF, and fibroblast growth factor (FGF), making it faster. These benefits accelerate the inflammatory phase, resulting in an earlier proliferative phase and faster healing.[6,11] Histologically, the wound tissue of rats given Moringa leaves showed increased growth, proliferation, and migration of fibroblasts in palatal wounds.[11] The diabetic rat group given Moringa leave aqueous extract orally demonstrated faster wound healing by increasing the inflammatory substance.[13] However, the proliferative effect of Moringa leaves on the levels of TGF-β and VEGF has not been elucidated well before. This study aimed to investigate the ability of oral Moringa leave extract to improve the wound healing process systemically using TGF-β and VEGF serum as molecular targets.

Methods

A laboratory experiment with a case-control design was conducted at the Animal Laboratory of the Faculty of Medicine, Universitas Padjadjaran, and the Laboratory of Molecular Genetics, Universitas Padjadjaran, from January to March 2022. This study was approved by the Research Ethics Committee, Faculty of Medicine, Universitas Padjadjaran, with Protocol No. 1064/ UN6.KEP/ EC/2021.

Swiss Webster (*Mus musculus*) male mice from the Institut Teknologi Bandung, aged 5–10 weeks and weighing 20–25 grams, were used and adapted for at least seven days before the study. The exclusion criteria include weight loss >10% during the adaptation period, death, or illness. Mice were housed in polypropylene cages at 22°C with a 12-hour light and dark cycle and ad libitum access to water and pallets.

The Moringa leaves powder (PT Moringa Organik Indonesia, Blora, Indonesia) is a fine powder (500 mesh) that contains 100% organic Moringa leaves without additives, which, based on laboratory tests, contains minerals, beta carotene, thiamine, riboflavin, niacin, pyridoxine, biotin, ascorbic acid, cholecalciferol, tocopherol, vitamin K, amino acids, polyphenols, oleic acid, zeatin, folic acid, and chlorophyll. The Moringa leaves powder was extracted using the maceration method with 96% ethanol (Merck, Rahway, NJ, USA) and water in a 1:3 ratio for 24 h and then filtered. This process was repeated three times. The entire filtrate was homogenized and evaporated in a rotatory evaporator until it was one-third of the initial volume. The filtrate was frozen at −20°C for 24 hours and dried further using the freeze-drying method until it turned into a greenish concentrate. The extract was kept in the refrigerator until treatment use. The ethanol extract was dissolved in 1% Na CMC for oral administration.[14]

The mouse on the left side was wounded after the fur was shaved and the skin was disinfected using 70% alcohol. Then, an intraperitoneal injection of 10 µl/gBW ketamine/xylazine solution (1:1:8) was used to anesthetize the mice. Furthermore, a punch biopsy with a diameter of 5 mm and a depth of 2 mm was used to make a wound, which was treated according to the procedure.[15]

The mice were divided into five groups, containing six mice each. Group 1 was unwounded and received Na CMC (PT Anggana Catur Prima, Jakarta, Indonesia) at 100 mg/kg BW. Groups 2 and 3 were wounded and treated with 100 mg/kgBW Na CMC and 500 mg/kgBW zinc syrup (PT Darya-Varia Laboratoria Tbk, Jakarta, Indonesia), representing the normal, positive and negative controls, respectively.[16,17] Meanwhile, groups 4 and 5 were wounded and treated with *M. oleifera* leaves ethanol extract (MOLE) with a dose of 280 and 560 mg/kgBW mixed with 1% Na CMC, respectively, given orally each day from day-3 to -6.[14] The wound area was photographed on day-0, -1, -3, and -6 using an iPhone 11 Pro camera (Apple, Cupertino, CA, USA), then analyzed with ImageJ version 1.53k (National Institute of Health, Bethesda, MD, USA) was used to quantify the wound area. The wound area percentage was calculated using the

formula described as the initial wound area minus the current, divided by the initial in percent, and then the wound area percentage between groups on day six was compared.

The mice were anesthetized and sacrificed on day 6, and the blood was collected using cardiac puncture. Then, the blood was centrifuged at 1,000 × g for 15 minutes to assemble the serum. Furthermore, the enzyme-linked immunosorbent assay (ELISA) kit was used to measure the TGF-β and VEGF serum levels based on the manufacturer's instructions (EliKineTM kits, Abbkine Scientific Co., Ltd, Wuhan, China, cat. no. KET7014 and KET7016, respectively).

The data were analyzed for the normality distribution using the Shapiro-Wilk normality test. A normally distributed data set was analyzed using one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. Meanwhile, Kruskal-Wallis analysis was used when the data was not normally distributed, followed by the Mann-Whitney test. The differences were statistically significant at $p<0.05$. Statistical analyses were performed using SPSS for Windows software version 20 (IBM, Armonk, NY, USA).

Results

The wound healing process for all groups was followed by visual monitoring of the wound size at day 0, -1, -3, and -6 (Figure 1), which was then measured and analyzed under ImageJ. On day 0, -1, and -3, the mean wound area of the group treated with MOLE at doses of 280 and 560 mg/kg was not significantly different from the negative control group ($p>0.05$). The mean area of wounds on day 6 in the positive control group had a lower percentage ($p=0.018$) than the opposing group, constituting 13.62±4% vs 21.93±5%, respectively. Similarly, in groups treated with 280 and 560 mg/kgBW MOLE, there was a lower percentage of wound area compared to the negative control group, accounting for 13.76±5% and 13.38±4%

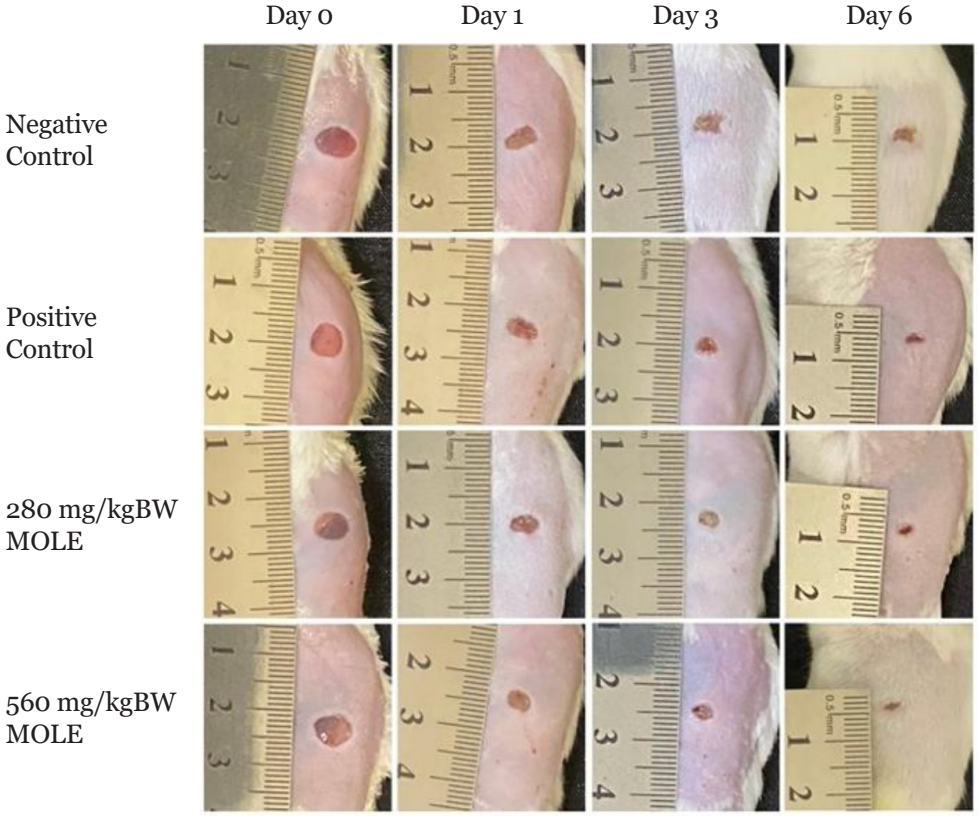

Figure 1 Photographically Recorded the Wound Area on Days 0, 1, 3, and 6 after Wounding

Note: The wound areas in 280 mg/kgBW MOLE and 560 mg/kgBW MOLE on days 1 and 3 were slightly smaller than in the negative control and showed significantly smaller wound areas than in the negative control on day 6

vs 21.93±5% (p=0.021, p=0.015, respectively). However, the groups treated with MOLE were insignificantly statistical compared to the positive control group, 13.76±5% and 13.38±4% vs 13.62±4%, respectively (p=1.000), indicating that both doses had a similar effect to the positive group, as shown in Figure 2.

In the positive control group, the mean TGF-β serum level was higher than the negative and normal control group, 178.2 pg/ml vs 121.4 pg/ml and 98.2 pg/ml (p=0.674, p=0.124, respectively). Meanwhile, in groups treated with 280 mg/kgBW MOLE, it was lower than the positive and negative control, generating 101.2 pg/ml versus 178.2 pg/ml and 121.4 pg/ml (p=0.157, p=1.000, respectively), though higher than the normal control group, 101.2 pg/ml vs 98.2 pg/ml, (p=1.000). A similar result was obtained at a dose of 560 mg/kgBW, generating 118.2 pg/ml vs 178.2 pg/ml and 121.4 pg/ml, when compared to the positive and negative control (p=0.541, p=1.000 respectively), but higher than the normal control group, 118.2 pg/ml vs 98.2 pg/ml (p=1.000) as indicated in Figure 3.

The median VEGF serum level in the normal group was higher than the negative and positive control group, producing 171.6 pg/ml vs 96.7 pg/ml and 67.9 pg/ml (p=0.262, p=0.078, respectively). In groups treated with 280 mg/kgBW MOLE, it was lower than the normal, negative, and positive control, generating 59.5 pg/ml vs 171.6 pg/ml, 96.7 pg/ml, and 67.9 pg/ml (p=0.016, p=0.200, p=0.522, respectively). Also, at a dose of 560 mg/kgBW, the serum level decreased compared to the normal and negative control, yielding 72.2 pg/ml vs 171.6 pg/ml and 96.7 pg/ml (p=0.150, p=0.749, respectively), but higher than the positive control group, 72.2 pg/ml vs 67.9 pg/ml (p=1.000). Hence, there was no significant difference in VEGF expression in groups treated with 280 and 560 mg/kgBW MOLE, 59.5 pg/ml, and 72.2 pg/ml (p=0.262), as indicated in Figure 4.

Discussion

Skin trauma is shared globally, and studies have shown that more than one million people experience skin injuries yearly. Therefore, effective drugs with low side effects are required urgently.[18] The *M. oleifera* leaves ethanol extract (MOLE), with its flavonoid compounds, is a novel candidate for skin wound healing with minimum side effects.[19]

The gross wound size (Figure 1) significantly reduced after oral MOLE administration and was accompanied by a dose-dependent wound area reduction (Figure 2). This was in line with a previous study, where topical MOLE accelerated excisional wound healing in diabetic rats by reducing wound size, increasing wound contraction and tissue regeneration, down-regulating inflammatory mediators, antibacterial activity, and regulating VEGF angiogenic

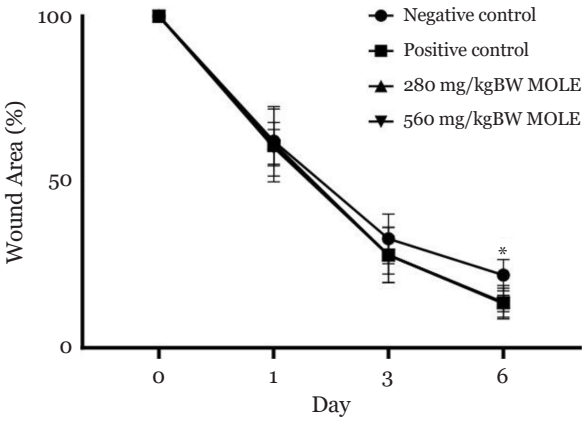

Figure 2 The Average Wound Area Percentage in Mice Treated with *Moringa oleifera* Leaves Extract

Note: *p<0.05 negative control vs positive control and MOLE groups. Data were presented as mean±SD, n=6 for each group

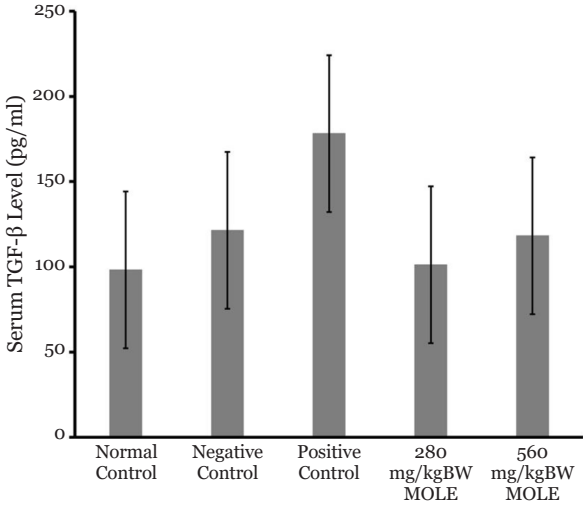

Figure 3 Serum TGF-β Levels

Note: serum TGF-β levels were measured using the ELISA method from serum samples obtained on day 6 in mice treated with *Moringa oleifera* leaf extract. Data were presented as mean+SD (n=6)

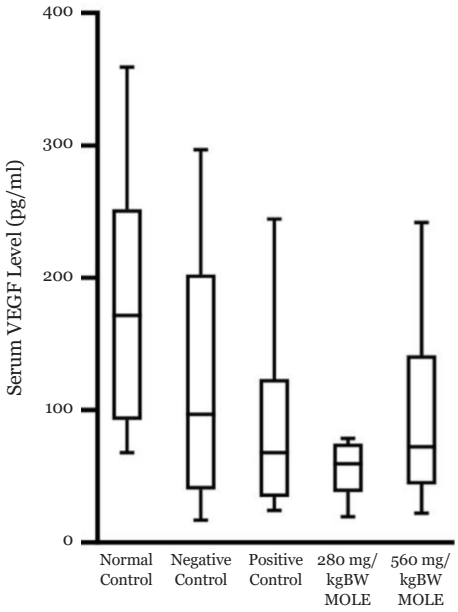

Figure 4 Serum VEGF Levels

Note: serum VEGF levels were measured using the ELISA method from serum samples obtained on day 6 in mice treated with *Moringa oleifera* leaves extract. Data were presented as median (n=6)

markers.[20] There was no significant difference in the percentage of wound contraction between the positive and MOLE groups. Hence, MOLE can be an alternative to wound healing therapy.

Skin re-epithelialization begins after injury, as growth factors are released by cells to induce keratinocytes, macrophages, and fibroblasts proliferation and migration into the wound space. TGF-β plays a critical role by directing inflammatory cells into the wound, accelerating ECM deposition and granulation tissue formation, and increasing the expedition of collagen type 1 to replace type 3 during the inflammation, proliferation, and remodeling phase, respectively. Meanwhile, VEGF is the angiogenetic factor regulated to promote angiogenesis and revascularization. Therefore, MOLE accelerates wound healing through TGF-β and VEGF signaling pathways.[21]

The presence of vicenin-2 and quercetin in MOLE increases the expression of TGF-β and VEGF by activating the critical transcription factor of the VEGF gene, hypoxia-inducible factor 1α (HIF-1α), reducing the expression of MMP-2, MMP-9, cathepsin. B, and cathepsin K, and increasing tissue inhibitory MMP-1 gene expression.[21,22] In this study, the increase in TGF-β levels in the dosage groups of MOLE was insignificant compared to the negative and the positive control groups (Figure 3) because MOLE acts locally in the wound.[23,24] A previous study showed that the expression of TGF-β in injuries treated with topical lavender oil increased significantly compared to the control.[25]

The VEGF levels decreased in the wounded group compared to the average (Figure 4). When a wound occurs on the skin of a healthy human, there is a spike in the local VEGF expression in response to hypoxia and local inflammation conditions.[26] The results were inconsistent with the previous study, where topical administration of *Callicarpa nudiflora* showed increased VEGF serum levels in rats with scald wounds.[27] In another study, using curcumin ointment for skin ulcers in a diabetic rat showed higher VEGF expression compared to the control group.[28] However, other studies demonstrated that oral MOLE reduces VEGF expression in Wistar rats because the isothiocyanates in the leaves lower the amount of VEGF through inhibitory pathways in the transcription of hypoxia-inducible factor (HIF).[29] Flavonoid-based compounds in Moringa leaves and seed residue extracts, such as isoquercetin, zeatin, rutin, quercetin, β-sitosterol, caffeoylquinic acid, and kaempferol, have potential antiangiogenic activity. These compounds act as inhibitors of CaN, blocking the CaN/NFAT pathway, which promotes cancer growth, migration, as well as angiogenesis, and suppresses angiogenesis.[30]

Other active substances play a role in the wound-healing process. Further studies should be conducted using flavonoids from Moringa leaf extract (quercetin, kaempferol, and vicenin-2) for wound healing. This study also did not perform histological research to confirm the fibroproliferative effect of Moringa leaf extract. Therefore, a histological examination is necessary to determine the number of fibroblasts and the level of granulation tissue formation in the wound. It is also suggested to measure VEGF and TGF-β protein levels in wound tissue to confirm the effect of Moringa leaf extract in the local wound area.

Conclusions

MOLE improves wound closure macroscopically, and the results showed that it is a promising alternative for therapy. However, MOLE may not

affect systemic TGF-β and VEGF levels. Further studies are required to examine the histology of fibroblast cells and VEGF and TGF-β protein levels in wound tissue.

Conflict of Interest

None declared.

Acknowledgments

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