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Synergistic Interaction between Topical Application of Allicin and Quercetin Enhances Diabetic Wound Healing via Inflammatory in Wistar Rats

(Interaksi Sinergi antara Aplikasi Topikal Alisin dan Kuersetin Meningkatkan Penyembuhan Luka Diabetis melalui Radang pada Tikus Wistar)

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ABSTRACT

Prolonged and excessive inflammatory processes at the wound site result in delayed healing of diabetic wounds. Allicin and quercetin have significantly inhibited tumor necrosis factor alpha (TNF-α) and increased levels of transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) in rats. However, there is no known investigation on the combination of both compounds on TNF-α, TGF-β, and VEGF as a topical healing agent via inflammatory mechanisms. This study aims to determine the synergistic interaction between topical application of allicin and quercetin in enhancing diabetic wound healing via inflammatory in Wistar rats. A total of 45 male Wistar rats weighing 180-250 g were induced diabetes by a single intraperitoneal injection of streptozotocin (45 mg/kg bw). Diabetic rats underwent wound creation under anaesthesia and a square-shaped open excision wound was made with a full-thickness measurement of 1 cm × 1 cm on the right side of the rat's back. Rats were randomly allocated into group namely, vehicle control, allicin, quercetin, and a combination of allicin and quercetin which is applied topically once a day at a dose of 10 mg/mL for 7 days. Our finding showed that once daily topical application of allicin and quercetin, both independently and synergistically, increased levels of TGF-β and VEGF, as well as decreased levels of TNF-α of the rat diabetic wound model compared with the vehicle control group over 7th day. The synergistic effects of allicin and quercetin significantly enhanced diabetic wound healing via inflammation in rats, by suppressing inflammatory cytokines and stimulating growth factors hence offering a new window of an experimental study.

Keyword: Allicin; inflammation; quercetin; rats; wound healing

ABSTRAK

Proses keradangan yang berpanjangan dan berlebihan di tapak luka mengakibatkan kelewatan penyembuhan luka diabetes. Alisin dan kuersetin telah menghalang faktor nekrosis tumor alfa (TNF-α) dengan ketara dan meningkatkan faktor pertumbuhan mengubah beta (TGF-β) dan faktor pertumbuhan endotelial vaskular (VEGF) pada tikus. Walau bagaimanapun, terdapat kajian yang tidak diketahui mengenai gabungan kedua-dua sebatian pada TNF-α, TGF-β, dan VEGF sebagai agen penyembuhan topikal melalui mekanisme keradangan. Kajian ini bertujuan untuk menentukan interaksi sinergi antara penggunaan topikal alisin dan kuersetin dalam meningkatkan penyembuhan luka diabetes melalui keradangan pada tikus Wistar. Sebanyak 45 ekor tikus Wistar jantan seberat 180-250 g telah diakibatkan kencing manis oleh satu suntikan intraperitoneal streptozotocin (45 mg/kg bb). Tikus diabetes menjalani pemodelan luka di bawah bius dan luka eksisi terbuka berbentuk segi empat sama dibuat dengan ukuran ketebalan penuh 1 cm × 1 cm di sebelah kanan belakang tikus. Tikus telah diperuntukkan secara rawak kepada kumpulan keempat iaitu, plasebo, alisin, kuersetin serta gabungan alisin dan kuersetin yang diberikan sekali sehari pada dos 10 mg/mL selama 7 hari. Penemuan kami mendedahkan bahawa satu aplikasi topikal harian alisin dan kuersetin, secara bebas dan sinergistik, meningkatkan TGF-β dan VEGF, serta menurunkan TNF-α model Tikus dengan diabetes luka berbanding dengan kumpulan plasebo sepanjang hari ke-7. Kesan sinergistik alisin dan kuersetin dengan ketara meningkatkan penyembuhan luka diabetes melalui keradangan pada tikus, justeru menawarkan ruang baharu untuk kajian penyelidikan.

Kata kunci: Alisin; keradangan; kuersetin; penyembuhan luka; tikus

Introduction

Diabetic wounds have an exceptionally complex pathology due to prolonged hyperglycemia with the destruction of dermal tissue (Aldana & Khachemoune 2020; Everett & Mathioudakis 2018). An epidemiology study showed that diabetic wounds have emerged as a primary influence of amputations, which leads to poor quality of life and elevated mortality rate (Burgess et al. 2021; Sothornwit et al. 2018). Approximately 19% to 34% of diabetic patients had wounds in the lower extremity, and a staggeringly high mortality rate of 50% to 59% worldwide (Okonkwo et al. 2020). In Indonesia, it is forecast that 7.3% to 24% of individuals had diabetic wounds (Soewondo, Ferrario & Tahapary 2013). Diabetic wounds were closely linked with both a prolonged inflammatory phase, which inhibited the formation of angiogenesis, leading to chronic wounds, and easy infection (Patel et al. 2019).

The inability to transition from the inflammatory to the angiogenesis phase could be caused by the activation of overexpression of tumor necrosis factor-alpha (TNF-α) and the downregulation of transforming growth factor beta (TGF-β). Notably, TNF-α is primarily responsible for the early wound healing response, and its persistently high level causes hampering of normal healing (Ban et al. 2020). Low levels of TGF-β can decrease collagen formation and decline re-epithelialization (Burgess et al. 2021; Mi et al. 2007). TNF-α also helps vascular endothelial growth factor (VEGF) control vascular processes throughout the inflammatory phase via hypoxia-inducible factor-α, thereby may be a possible mechanism for accelerating the healing of wounds (Kim, Lee & Kim 2017).

In a previous review study, it was presented that allicin is an organosulfur chemical compound found in garlic which contains high concentrations of the alliinase enzyme. The alliinase enzyme is responsible for the formation of the bioactive compound allicin from the stable alliin precursor (Liu et al. 2013). Interestingly, only one study evaluated the effectiveness of allicin on fibroblast, collagen, and neutrophils as indicators of diabetic wound healing, but did not investigate the TNF-α, TGF-β, and VEGF for detecting the inflammatory biomarkers mechanism (Toygar et al. 2020). Allicin at 10 mg/mL inhibited VEGF expression in neuroblastoma-bearing mice treated with cyclophosphamide demonstrating the positive synergistic effect (Gao et al. 2015). In addition, prior research showed that a combination treatment of allicin and other antimicrobials may represent a highly feasible approach for preventing drug resistance and increasing the potential effect of antimicrobials (Choo et al. 2020).

Remarkably, quercetin is a potent antimicrobial, anti-inflammatory, and antioxidant agent against ameliorating infectious (Nguyen & Bhattacharya 2022). A prior study reported that 10 mg/mL of quercetin demonstrated a wound-healing benefit in a rat model with diabetic wounds via inhibition of inflammation and escalate the fibroblast distribution as well as the angiogenesis rate (Fu et al. 2020). Quercetin affects cytokines, growth factors, and protease, optimizing diabetic wound repair in rats (Kant et al. 2021).

To the best of our knowledge, there has been no experimental investigation of the synergistic effect of allicin and quercetin enhancing diabetic wound healing by overcoming prolonged inflammation in the Wistar rats model involving TNF-α, TGF-β, and VEGF. This study aimed to explore the potential mechanisms underlying the wound-healing process effect of these two compounds in diabetic Wistar rats rather than of a single compound.

Materials and Methods

Preparation of allicin and quercetin emulsion

Making allicin and quercetin emulsion preparations as wound medicine can maintain the stability of the contents of these two substances, especially allicin, so that they are not degraded.

The emulsion base consisting of allicin from ChemFaces (Wuhan, China), quercetin from Sigma-Aldrich (Saint Louis, MO), 80% sucrose, PEG 400, ethanol, Tween 80, peppermint oil, and nipagin with aquadest was mixted under stirring at 500 rpm using a magnetic stirrer for 30 min. Further, the temperature of the allicin or quercetin and the combination of these two compounds must be maintained at 2-8 °C due to stability issues (Fujisawa et al. 2008; Niazi 2019). To maintain the stability of allicin, quercetin, and a combination of these two compounds, the finished preparations are placed in the primary packaging and then stored at a temperature approximately 4 °C, with the primary packaging coated with aluminum foil. For the vehicle control formulation, the emulsion was prepared using a pure emulsion procedure without the presence of allicin or quercetin compounds. All groups received a formula according to the ingredients specified in each group in the form of an emulsion that was administered topically.

EXPERIMENTAL ANIMALS

The design used in this research was a Randomized Post-test only with a control group design. The experimental unit in this study used Wistar rats which were divided into four groups, namely group 1 (vehicle control), group 2 (allicin), group 3 (quercetin), and group 4 (combination of allicin and quercetin). Group 1 (vehicle control) was the control group and groups 2, 3, and 4 as the treatment group. Determining the sample size using the formula from Lemeshow with a correction factor using the Higgin Formula of 20%. A total of 45 Wistar rats were used in this study which met the inclusion criteria, namely males with a body weight of 180-250 g, and according to the exclusion criteria, namely rats that had been used as experimental animals. Wistar rats were obtained from the animal research center of the Faculty of Veterinary Medicine, Airlangga University, Indonesia. All Wistar rats were allowed to acclimatize for one-week. All rats were maintained on a 12:12-h light/dark cycle with a climate-controlled environment temperature of 24 ± 1 °C and had *ad-lib* access to food and water in the Biochemical laboratory, Universitas Airlangga. The ethical approval number (2.KEH.005.02.2022) were conferred by the Animal Care and Use Ethic Committee at the Faculty of Veterinary Medicine, Universitas Airlangga.

DIABETIC MODEL

In induction of diabetes, a single intraperitoneal injection of streptozotocin (45 mg/kg b.wt., Sigma S0130, USA) was dissolved in citrate buffer solution (0.1 mol/L, pH 4.5, stored at 4 °C). Before the injection of streptozotocin, rats also fasted overnight (12 h), and then were orally administrated glucose 10% in the next day (Ahmed et al. 2018). Their fasting blood glucose levels were measured using a glucometer (ACCU-CHEK ® Active, Roche Diagnostics, Germany). Rats whose fasting blood glucose levels remained stable at ≥300 mg/dl over seven days were diagnosed with diabetes and eligible for our study.

WOUND MODEL

Wistar rats that had blood sugar levels of ≥ 300 mg/dl were then anesthetized by intraperitoneal administration of ketamine at a dose of 50 mg/kg, i.p., plus xylazine at a dose of 5 mg/kg, i.p. (Kant et al. 2021). After the rats were anesthetized, the right side of the rat's back was shaved and the size of the wound was determined using a square mold with a size of 1 cm × 1 cm and then outlined using a marker. The backs of rats that had been marked were disinfected with 70% ethanol. A square-shaped open excision wound was made with a full-thickness and extended to the subcutaneous tissue using a scalpel and scissors (Kandhasamy et al. 2021; Thawer & Houghton 2001). The wound was left open without dressing and the animal was placed in a clean polycarbonate cage with bedding material using fine wood shavings.

GROUPING

The rats with wounds were randomly divided into four groups (8 rats per group) which were given the same intervention once a day for seven days: group 1 (vehicle control containing pure emulsion), group 2 (allicin emulsion at a dose of 10 mg/mL), group 3 (10 mg/mL quercetin emulsion), and 4 groups (combination emulsion containing allicin 10 mg/mL and quercetin 10 mg/mL). The dose of 10 mg/mL was chosen based on the median effective dose of allicin and quercetin in the wound healing process (Fu et al. 2020; Kant et al. 2017; Salehi et al. 2019). A volume of 100 µL of emulsion with different contents was applied to the wound according to the group of experimental animals until completely absorbed by the wound.

TISSUE HARVESTING AND IMMUNOHISTOCHEMICAL STUDY

Five rats from each group were euthanized on days 3 and 7 post-wound treatment to collect the rats' healing tissue. The obtained tissue was further processed based on the results of a series of analyses. All tissue was preserved in 10% neutral buffer formalin, embedding the sample tissue with a paraffin block for immunohistochemical analysis. The paraffin sections are preheated at 60.0 ± 0.50 °C in the microwave for 12 min before being blocked with endogenous peroxide for 15 min and incubated with the primary rat monoclonal antibodies for TNF-α (52B83; sc-52746, Santa Cruz, USA), TGF-β (3C11; sc-130348, Santa Cruz, USA), and VEGF (NB100-664, Novusbio, United Kinddom) for 60 min. Secondary antibodies were added for 30 min. Finally, slides were taken with glycerine and examined under a Nikon Eclipse microscope.

The expression of immunohistochemical performance was measured using an Immunoreactivity Remmele Scale (IRS) index (Rammele & Stegner 1987). The multiplication result of the scores for staining

intensity and number of stained cells is defined as the immunoreactivity score. The intensity of staining was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (intensive). Furthermore, the extent of staining was scored as 0 (0%), 1 (<10%), 2 (10-50%), 3 (51-80%), and 4 (81-100%), indicating the percentage of positive staining in the anastomosis. Multiplication of intensity score (0-3) and extended score (0-4) resulted in the IRS, which ranges between 0 and 12, on a numerical scale.

STATISTICAL ANALYSIS

The experimental value was analyzed using one or two-way analysis of variance (ANOVA) test method using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Statistical significance was determined by a p-value of <0.05 and expressed as mean ± S.E.M with n equal to the number of replicates.

RESULTS AND DISCUSSION

WOUND SIZE AND WOUND CONTRACTION IN DIFFERENT GROUPS TREATED

Examination of wound size and contraction in diabetic rats was measured on days 0, 3, and 7 after injury and is depicted in Figure 1. Examination on the 3rd day after injury, the size of the wound and wound contraction in the four groups had a mean wound contraction score of 20% and there were no significant differences. On the 7th day after injury, wound contraction increased rapidly as a result of the treatment of diabetic rats with allicin, quercetin, and allicin+quercetin; treating with both is the most potent. The diabetes control (vehicle control) group healing tissue was thin and pale in color. The mean ± SEM values of wound contraction in the vehicle control, allicin, quercetin, and allicin+quercetin groups on the 7th day was 44.50±2.90; 69.25±6.10; 68.75±5.06; and 76.25±4.48, respectively. Wound contraction in the allicin, quercetin, and allicin+quercetin group increased and was significantly different compared to the vehicle control group (control group).

THE EFFECT OF TOPICAL APPLICATION ON BIOMARKER EXPRESSION

Immunoreactivity Remmele Score Index of TNF-α, TGF-β, and VEGF of different groups on days 3 and 7 post-wounding treatment was presented in Figure 2. On the 3rd day, our findings showed that TNF-α was statistically lower in the topical allicin-treated group than in the vehicle control group (p-value < 0.01). Moreover, the allicin-treated group did not have a significant effect on the TGF-β and VEGF expression. Conversely, after the 7th day of treatment, the TNF-α, TGF-β, and VEGF expression was significantly higher than the control group (p-value < 0.01). The topical quercetin-treated group did not have a significant effect on the TGF-β, and VEGF expression, but significantly in TNF-α after 3rd days than compared to the control group. The topical quercetin-treated group was significantly different on TNF-α, TGF-β, and VEGF after the 7th day (p-value < 0.01) of treatment.

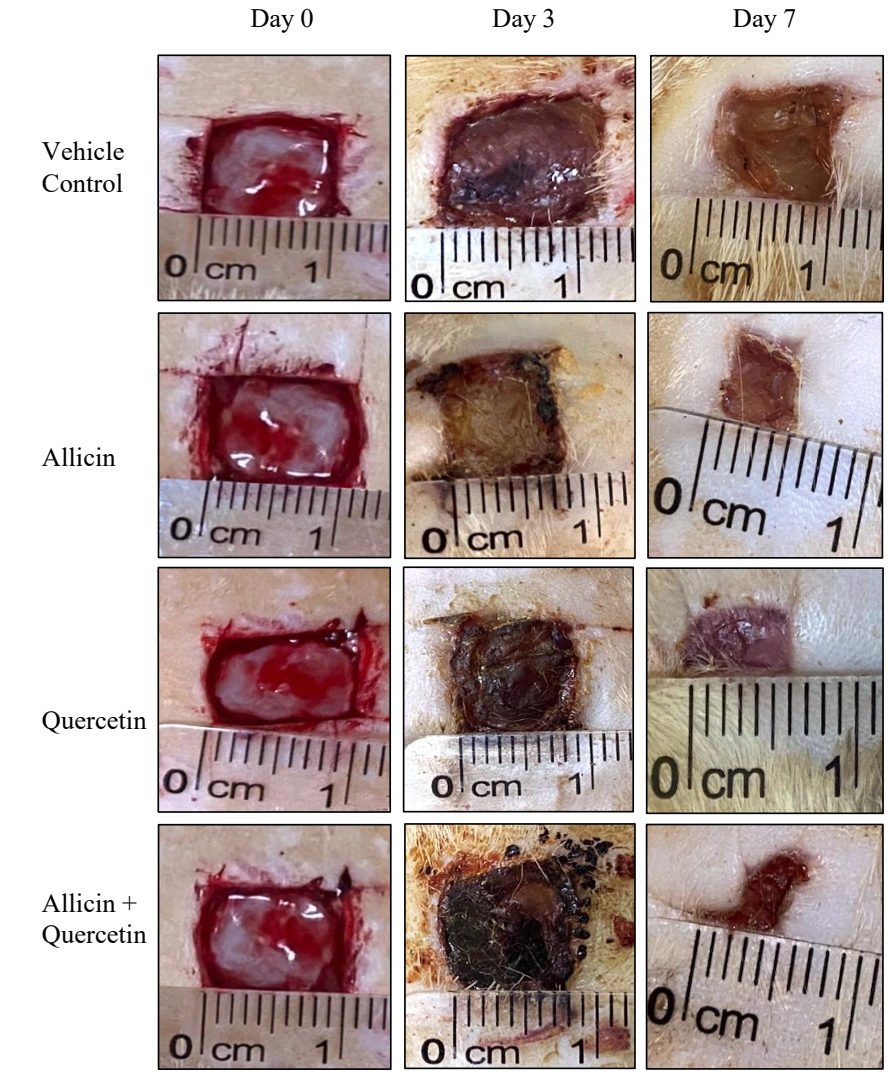
The combination of allicin and quercetin-treated group indicated a significant effect on lowering TNF-α and increasing TGF-β, and VEGF. In every timeframe of evaluation, which 3rd and 7th day, the combination of topical allicin and quercetin had a significant result (p-value <0.05). This finding indicates that the combination treatment was more impactful than compared to a single treatment (Figure 2). Immunohistochemical examination of TNF, and VEGF on days 3 and 7 in the four treatment groups after wound care is presented in Figures 3 to 5 (all images 20x).

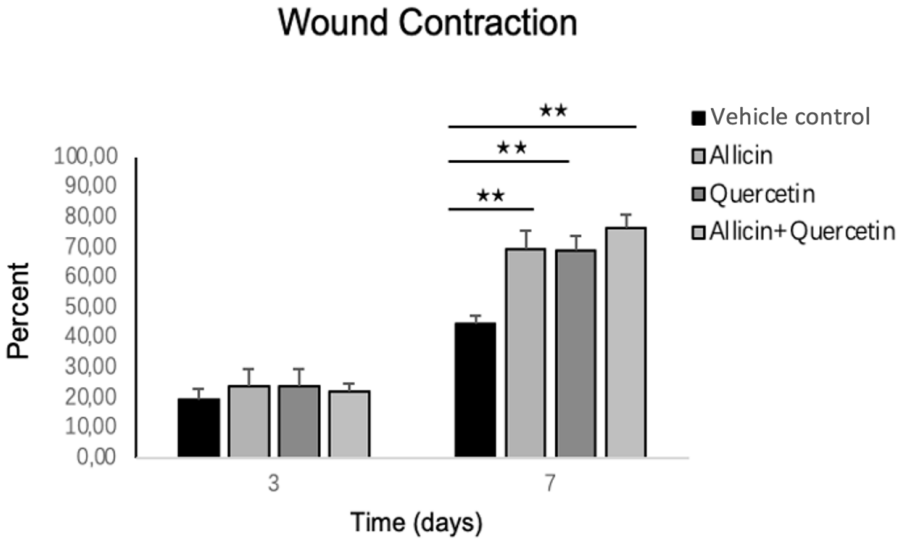
DISCUSSIONS

This investigation was the first time to explore the synergistic effect of topical allicin and quercetin enhancing diabetic wound healing via inflammatory in the Wistar rats model involving TNF-α, TGF-β, and VEGF. The current finding highlighted that the combination of allicin and the quercetin-treated group significantly increased TGF-β, and VEGF, as well as declined TNF-α in 3rd and 7th day.

Our experimental study demonstrated that topical allicin treatment significantly increased TGF-β and VEGF expression, but decreased TNF-α on 7th day. The allicin, garlic's active component has anti-inflammatory, antioxidant, and anti-bacterial characteristics that can effectively treat diabetic wounds (Salehi et al. 2019). In agreement, a previous study showed that allicin promoted diabetic wound healing by reducing inflammation through decreasing neutrophils and increasing angiogenesis (Toygar et al. 2020). Allicin can effectively exert antioxidant, anti-inflammatory, and antibacterial activities (Remmele & Stegner 1987). Beyond that, allicin may decline the expression of TNF-α, IL-6, and IL-8 (Li et al. 2020; Liu et al. 2019). Previous research has demonstrated the effects of topical allicin on wound healing in dogs. Histopathological investigations showed that the density of fibrocytes and fibroblasts in the

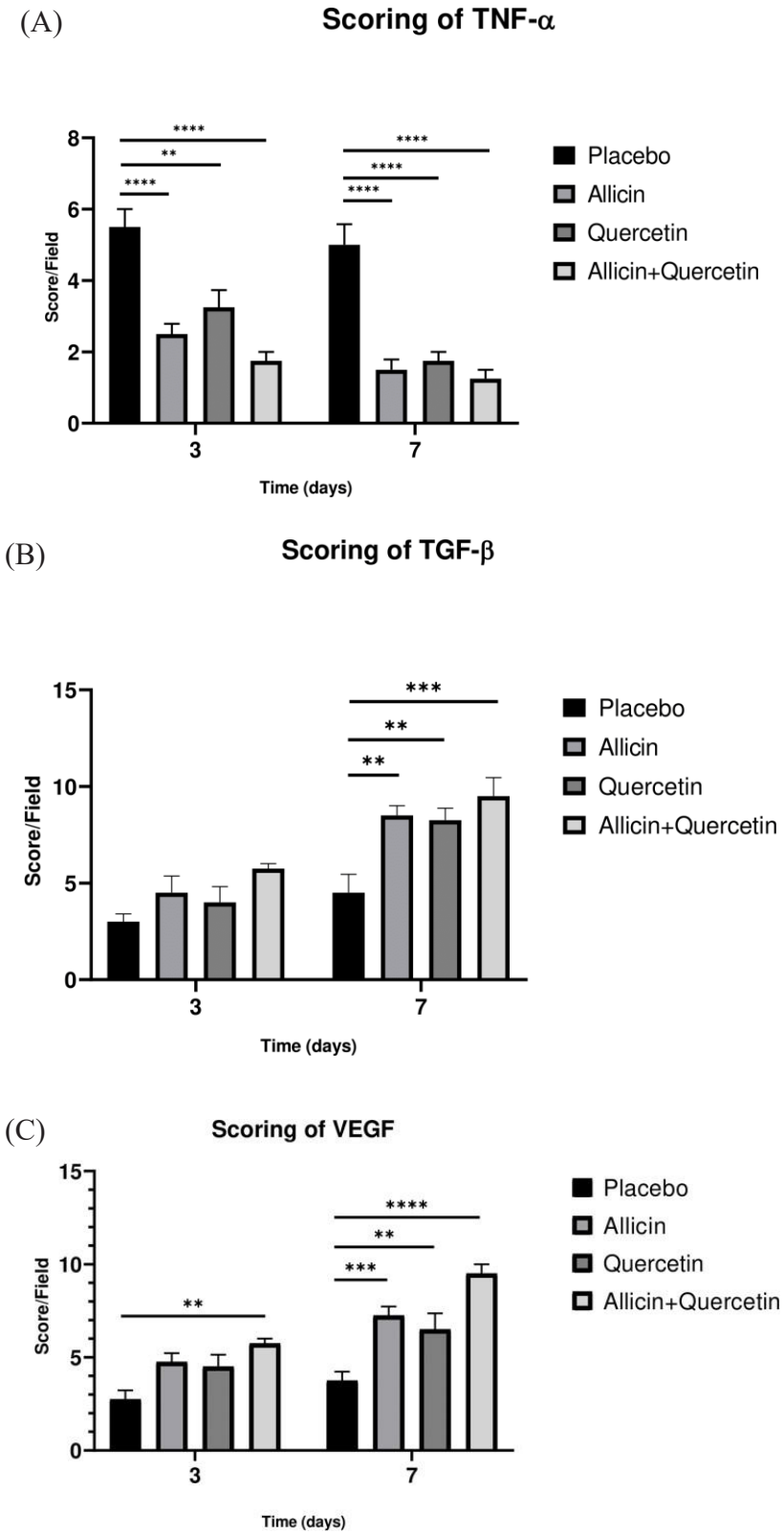
Figures





Model = placebo group, allicin group, quercetin group, and allicin+quercetin group. Data are expressed as mean ± SEM. **P < 0.01

FIGURE 1. Wound size and wound contractions on different days as an illustration of the wound healing process in diabetic rats. Model = vehicle control group, allicin group, quercetin group, and allicin+quercetin group. Data are expressed as mean ± SEM. **P < 0.01.



Data are expressed as means ± SEM, (n = 4). *p< .05; ** p< .01; ***p< .001vs. control group

FIGURE 2. Immunoreactivity Remmele Score Index of (A) TNF-α; (B)TGF-β; and (C) VEGF of different groups on days 3, and 7 post-wounding treatment

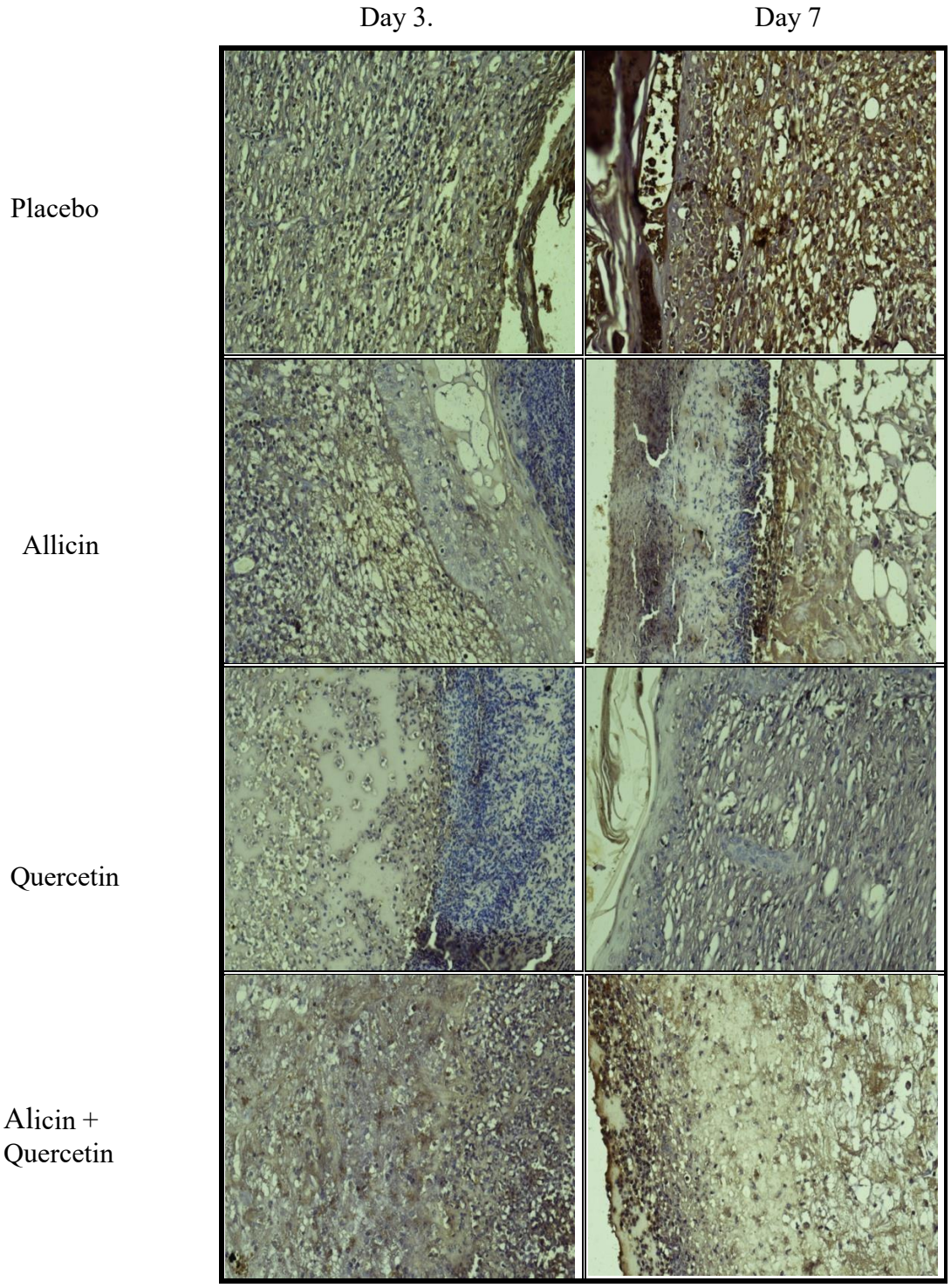


FIGURE 3. Immunohistochemistry of TNF-α (images 20x) of different groups on days 3, and 7 post-wounding treatment

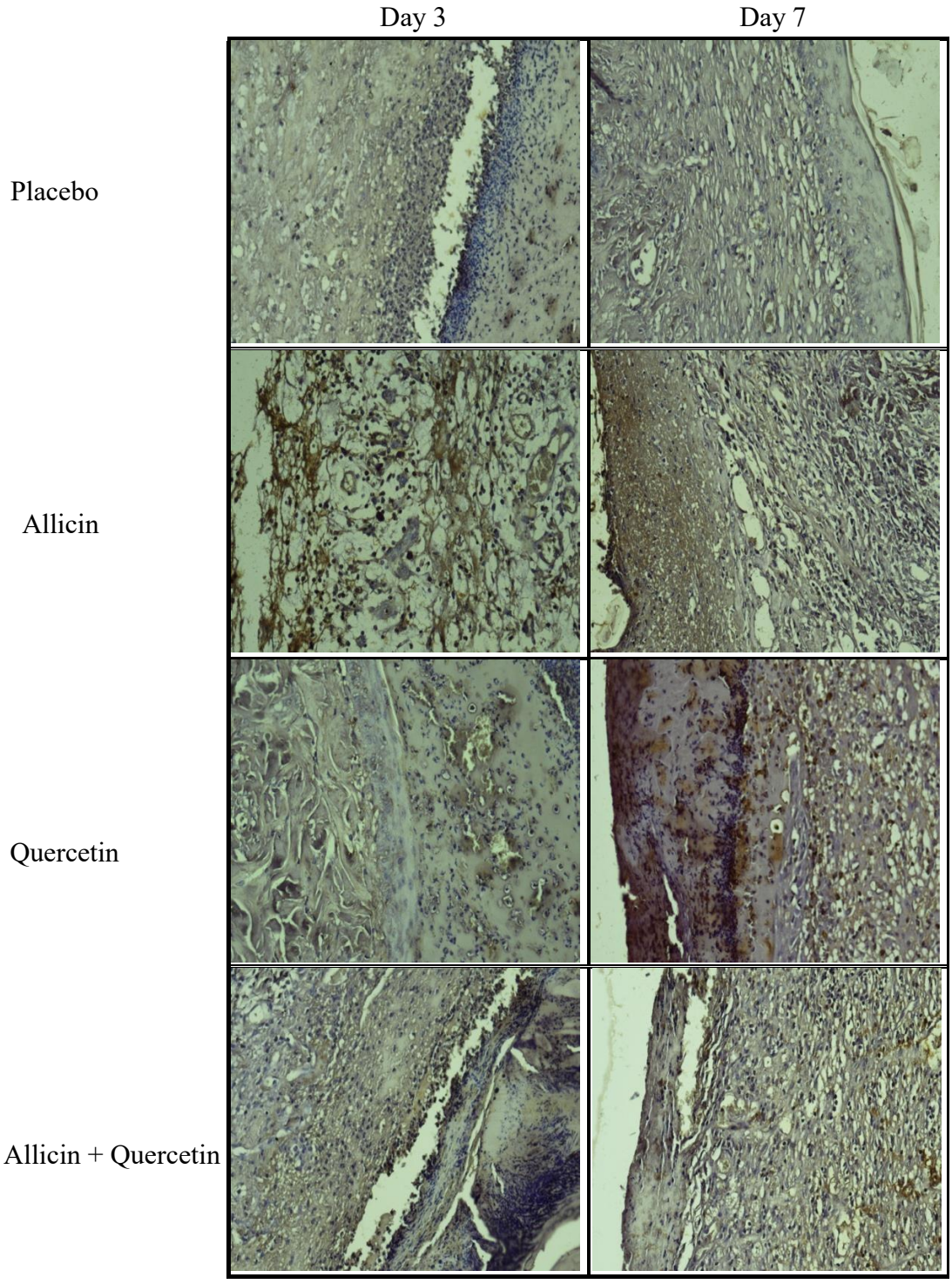


FIGURE 4. Immunohistochemistry of TGF-β (images 20x) of different groups on days 3, and 7 post-wounding treatment

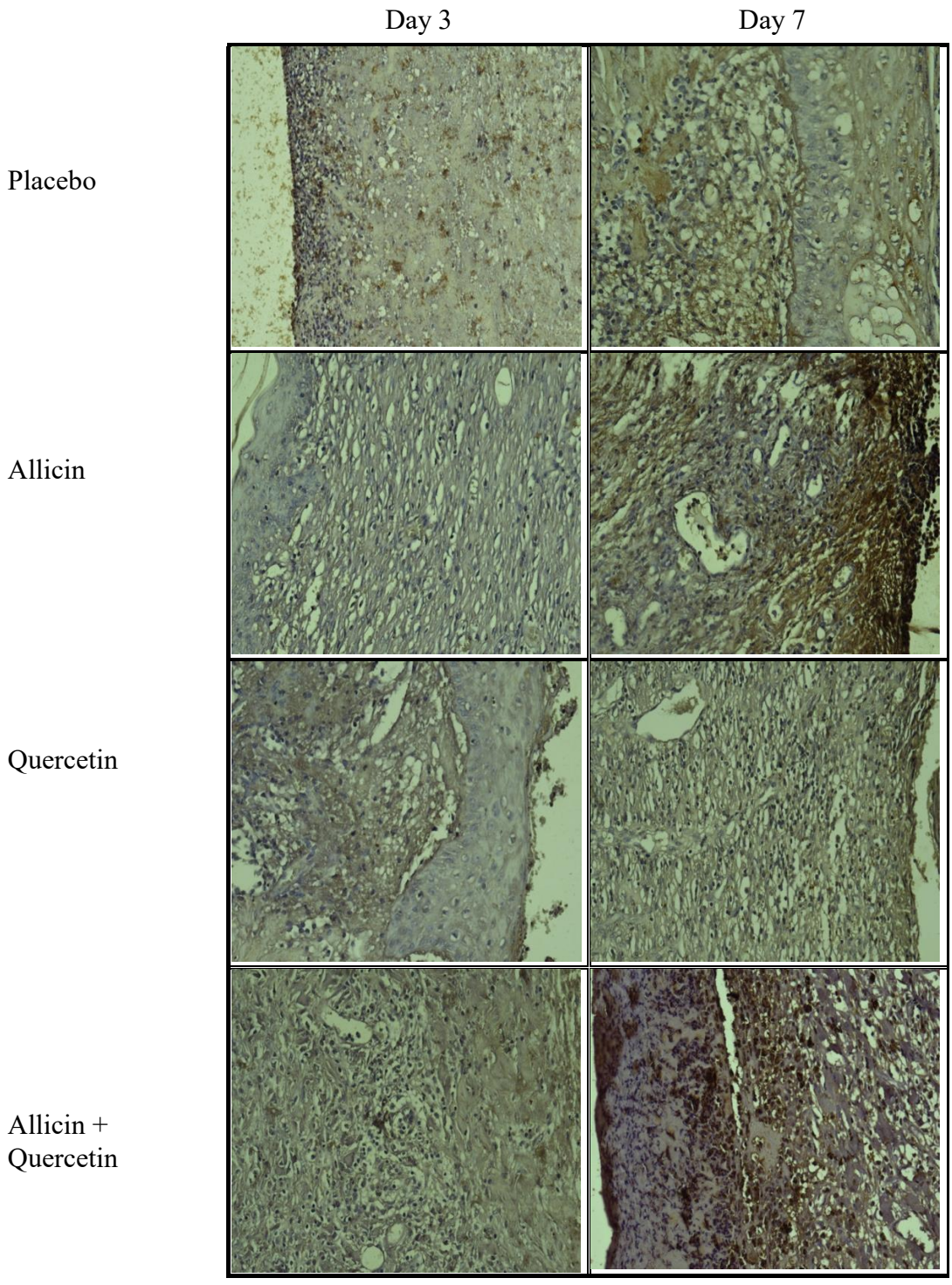


FIGURE 5. Immunohistochemistry of VEGF (images 20x) of different groups on days 3, and 7 post-wounding treatment

wounds in the allicin group was much higher than in the vehicle control group, indicating that allicin promotes the fibroblasts (Negrini et al. 2017). Collagen deposition was significantly correlated with escalating VEGF expression, which indicated the allicin will increase the VEGF expression. Interestingly, a previous study reported that the escalation of VEGF ameliorated inflammation in diabetic rats (Bao et al. 2009; Lin et al. 2019). Mechanistically, the up-regulated expression of VEGF may activate both the endogenous defence and repair mechanism and effectively promote neovascularization (Lv, Ge & Zhao 2017). Allicin, as an antioxidant, could decrease inflammatory cytokines inhibit the expression of TNF-α and up-regulate TGF-β.

Moreover, allicin may alleviate inflammatory infections and enhance vascular endothelial function (Liu et al. 2013). A previous experimental study investigated the anti-inflammatory effects of allicin in a model of diabetic rats, and their findings significantly reduced the expression of TNF-α in an allicin-treated group. Furthermore, the expression of inflammation-related proteins, such as vascular cell adhesion molecule-1 and inducible nitric oxide synthase (iNOS) was elevated in diabetic rats and was significantly decreased by allicin treatment (Li et al. 2020). In diabetic wounds, iNOS contributes to oxidative stress-mediated cellular differentiation and wound remodeling (Badr et al. 2016). Thus, allicin indicates that minimizes protracted inflammation, and these findings imply that lower inflammation may be the mechanism behind the improved antioxidant. Consequently, it highlighted the potential wound-healing mechanism in diabetic mice treated with allicin.

To provide light on the potential anti-inflammatory mechanism of quercetin on diabetic wound healing, we studied the expression of TNF-α, TGF-β, and VEGF. In line with a previous study, quercetin declined the expression of TNF-α and escalated the expression of TGF-β, and VEGF (Fu et al. 2020; Salehi et al. 2019). Another study presented that quercetin significantly improved wound healing by escalating the proliferation and declining fibrosis (Jangde et al. 2018). Moreover, a clinical study showed that quercetin can up-regulate the expressions of VEGF and TGF-β in healing tissue (Gopalakrishnan et al. 2016). Delay of granulation tissue development and failure of wound closure in diabetes are caused by a persistently high level of TNF-α. Thus, the present investigation indicated that the inflammation long-process formed due to persistently elevated TNF-α expression in the control group. However, using topical quercetin on wound diabetics significantly reduced these negative alterations. In diabetic wound, counts of VEGF and TGF-β was significantly decreased after receiving topical quercetin (Kant et al. 2021). In the present investigation, we also observed the decline of VEGF and TGF-β in the quercetin group than in the vehicle control group. Remarkably, VEGF plays a critical role in angiogenesis by increasing endothelial cell processes required for new blood vessel development, including proliferation and migration; hence, the amount of VEGF in the healing tissue substantially affects the healing process (Wilgus 2019). The association between VEGF expression patterns and permeable blood vessels in wounded skin demonstrates that VEGF promotes vascular permeability during the early wound-healing process (DiPietro 2016). Along with VEGF, TGF-β is another factor in the promotion of angiogenesis, and it also acts as a modulator of the proliferation and differentiation of endothelial cells (Bertolino et al. 2005; Öztürk & Ermertcan 2011). Thus, it is possible that the therapeutic impact that topical quercetin plays in our model is perhaps partially due to this specific targeting mechanism that increased the angiogenesis in the wound area to accelerate the process of healing.

A combination of quercetin and allicin had an additive synergistic effect on angiogenesis induced by TGF-β and VEGF. In contrast, a combination of quercetin and allicin inhibits the expression of TNF-α. A previous study suggested that when combined with antibiotics or antifungals, allicin improved the antibacterial efficacy and enhanced the antimicrobial activity (Choo et al. 2020). In fact, quercetin has been demonstrated to inhibit the growth of different Gram bacteria and as viruses and fungi agents (Nguyen & Bhattacharya 2022). Moreover, combining quercetin with another antioxidant, anti-inflammatory, and antimicrobial agent could promote wound healing compared the single compounds (Chittasupho et al. 2021). Consequently, this highlights that a combination of allicin with quercetin as an additional antimicrobial treatment may serve as a reasonable alternative for trying to counter the progression of TNF-α and improving the TGF-β and VEGF expression in the diabetic wound healing process. Furthermore, the management of diabetic wounds can prevent delay the of inflammation and escalated angiogenesis process (Okonkwo & DiPietro 2017). This mechanism may recognize the potential insights into the pathways that regulate the wound healing process via inflammation action in Wistar rats.

Conclusions

In summary, it might be concluded that one daily topical application of allicin and quercetin, both independently and synergistically, escalated TGF-β and VEGF, as well as declined TNF- α of rat model with diabetic wound over 7 days, so it is a feasible treatment in advanced wound diabetic process. Taken together, these biological mechanisms may be attributed to the synergistic effect of both compounds. Therefore, further research is warranted to explore the deep mechanism and synergistic effects of the maturation phase with the longer time required to create possibility.

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