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Original Article

COMBINED EFFECT OF TOPICAL APPLICATION OF VIRGIN COCONUT OIL (VCO) AND BLACK CUMIN OIL (*NIGELLA SATIVA*) ON THE UPREGULATION OF VEGF GENE EXPRESSION AND WOUND HEALING IN DIABETIC ULCERATED RATS.

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ABSTRACT

Objective: Traditional therapies are increasingly explored as alternative methods for the management of diabetic ulcer. VCO and black cumin oil has attracted attention for its potential therapeutic benefits in promoting skin wound healing.

Methods: The rats were induced with one dose diabetes mellitus through the of intraperitoneal injection of streptozotocin 55 mg/kg body weight. Furthermore, fasting blood glucose (FBG) levels were monitored weekly for assessment. The wound was created using a 10-mm diameter punch biopsy. An experimental methodology was used, comprising the division of 30 rats into six groups, namely control, VCO, black cumin oil, and combinations of VCO and black cumin oil labeled as C1, C2, and C3. The formulated treatments were topically applied to wound for 7 and 14 d. At the end of the treatment, the samples were sacrificed and wound was excised, followed by molecular biological analysis and histopathological examination.

Results: On day 7, VEGF gene expression showed the highest increase in the C3 group, with an average of 1.85±0.10. Meanwhile, the highest increase on day 14 was observed in the C3 group, with an average of 1.69±0.11. C3 group treated wounds healed much faster, as indicated by a decreased time of complete epithelization and higher levels of various skin components.

Conclusion: The combination of VCO and black cumin oil could be used as an agent to accelerate wound healing in diabetic conditions, as indicated by the increased expression of VEGF gene.

Keywords: Black cumin oil, Diabetic ulcer, VCO, VEGF, Wound

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INTRODUCTION

Diabetes mellitus is a progressively prevalent chronic metabolic disease characterized by prolonged hyperglycemia, leading to long-term health complications [1, 2]. Among these complications, diabetic ulcer stand out as a persistent clinical challenge necessitating further treatment development [3, 4]. Furthermore, approximately 25% of Diabetes mellitus patients experience impaired wound healing, culminating in lower limb amputation. This condition incurs significant economic and psychosocial burdens [5, 6, 1]. Despite the advancements in standard clinical care, such as local wound management and repeated debridement of necrotic tissue, approximately 14-20% of patients suffering from diabetic ulcer often require amputation. These statistics underscore the limitations of current therapeutic methods and emphasize the need to develop new and more effective treatment modalities [6, 1].

Conventional wound care therapies offer significant potential for expediting healing process by acting as an anti-inflammatory and accelerating growth factors and cell migration [7]. In this context, Virgin Coconut Oil (VCO) has been reported to be a potential alternative therapeutic agent. This can largely be attributed to its high concentration of medium-chain saturated fatty acids, including lauric and capric acids [8, 9]. These specific fatty acids facilitate penetration through the skin barrier, thereby enhancing fibroblast proliferation, neovascularization, and accelerated epithelialization processes [10]. Furthermore, VCO has been found to significantly improve the migratory capabilities of CCD-18 and RGC-5 cells [10]. VCO also elevates collagen levels, substantiating its integral role in promoting wound healing [11]. Several studies have shown that both VCO and its hydrogenated variant enhance expression of wound healing-related proteins, such as MMP-9, PDGF-BB, and TGF-beta [11, 12]. Recent *in vivo* studies have provided insights into the therapeutic benefits of black cumin oil (*nigella sativa*), including anti-inflammatory, antioxidant, antibacterial, and anticancer properties, as well as identify its crucial component, thymoquinone (TQ) [13]. Oil from *nigella sativa* has been found to expedite wound healing by promoting angiogenesis, fibroblast proliferation, and collagen synthesis while also positively impacting the formation of granulation tissue and epithelialization, as well as reducing vascularization and inflammation [14].

Wound-healing process is initiated at the inflammation stage, characterized by vascular injury and extracellular matrix degradation, leading to the activation of platelet aggregation, degranulation, and the coagulation cascade [4]. During degranulation, platelets release alpha granules that secrete growth factors, such as Epidermal Growth Factor (EGF), Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor-beta (TGF-beta), and Platelet Activating Factor (PAF) [2]. Among these factors, VEGF has been reported to be a potent positive regulator of angiogenesis and stimulates crucial endothelial cell functions for new blood vessel formation, including proliferation, migration, and differentiation [5]. VEGF also influences the rate of wound closure and wound strength during the proliferative phase, as well as promotes scar tissue formation during the remodeling phase [16-19]. Therefore, the innovative method of combining black cumin oil (*nigella sativa*) and VCO offers a promising unexplored therapeutic pathway, particularly concerning its impact on expression of VEGF gene. VEGF plays a critical role in the complex biological mechanisms of diabetic wound healing.

MATERIALS AND METHODS

Materials

Streptozotocin (STZ) and RNase Free water were purchased from Sigma (USA). Triazole was acquired from Thermo Fisher Scientific (CA, USA), while the cDNA Kit, the GAPDH, and VEGF genes were obtained from Thermo Fisher Scientific (Vilnius, Lithuania). VCO

was purchased from PT. Indo Fureco pratman (Indonesia), while black cumin oil was obtained from PT. Habbatusauda (Indonesia).

Study design

The study rats were confirmed to have diabetes when FBG levels were>300 mg/dl three days (3) after STZ injection. Furthermore, diabetic samples were randomly divided into six (6) groups (n = 5 per group). VCO, black cumin oil, and combination were applied topically with a volume of 1 ml [11, 20] over wound area for 7 and 14 consecutive days. All procedures were approved by the animal ethics committee Andalas University, Indonesia. The groupings were as follows:

Control: Diabetic control (DC) with no intervention

VCO: 100% VCO

Black cumin oil: 100% black cumin oil (*nigella sativa*).

C1: received 50% VCO and 50% black cumin oil (*nigella sativa*).

C2: received 70% VCO and 30% black cumin oil (*nigella sativa*).

C3: received 30% VCO and 70% black cumin oil (*nigella sativa*).

Preparation of rats with diabetic ulcer

The investigation was conducted on 30 male Wistar rats weighing 220 and 250 grams. All rodents were housed at a controlled temperature (23±2 °C) and 12 h dark/12 h light cycle. Furthermore, the samples had unrestricted access to food and water. Intraperitoneal STZ 55 mg/kg of body weight was used to induce Diabetes in rats housed for one week. The rats were subjected to fasting blood glucose and random blood glucose assays from tail vein blood once a week for four weeks to ensure their diabetic status [21]. Samples with diabetes were anesthetized with xylazine, followed by the removal of their fur. The ten mm-diameter punch biopsies were used to create the incision, and each rat was placed in its separate cage [21, 22].

Wound tissue collection

Skin tissue samples were extracted on days 7 and 14 [21, 22]. In the first stage, the mouse was positioned dexter-lying, and then surgery was performed to isolate the skin by excision to the subcutis depth. The epidermis was then dissected with tweezers before being cut with scissors. After the collection of tissue samples, the rats were euthanized by neck dislocation [21, 22].

VEGF gene expression analysis

RNA isolation

All tissues from experimental groups were isolated using reagents TRIzol® (Thermo Fisher Scientific, CA, USA) and homogenized into a sample using a homogenizer 1 ml Reagen TRIzol™. The process was then continued with the addition of 200 µl of chloroform, incubation at room temperature, and centrifugation at 12,000 x g and 4 °C for 15 min. Subsequently, the top layer was added with 2x isopropanol, set for 10 min at room temperature, and centrifuged for 10 min until a white pallet formed. The pellets were then washed with 350 µl of 70% ethanol, vortexed, and centrifuged again for 5 min at 7500 x g at 4 °C. The supernatant was removed and rested for 10 min. The pellets were resuspended in 25–40 µl of RNase-free water (depending on the number of shells). The RNA was calculated and adjusted to a concentration of 1000 ng [23-25].

cDNA synthesis

The synthesis of cDNA was carried out using a synthesis kit (Thermo Fisher Scientific, Vilnius, Lithuania). The composition of total cDNA synthesis was 5 µg total RNA, 1x RT buffer, 20 pmol oligodT, 4 mmol dNTP, 10 mmol DTT, 40 U enzyme SuperScript TMII RTase, and Nuclease Free Water with a reaction volume of 20 µl. Total cDNA synthesis was performed at 52 °C for 50 min with a working protocol according to the kit manual (iScript cDNA synthesis, Biorad) [23-25].

PCR gradient amplification

The PCR process was carried out in the amplification range for 40 amplification cycles, consisting of predenaturation at 95.0 °C for 3 min, initial denaturation at 94 °C for 5 min, core cycle consisting of 94 °C for 45 seconds, 55 °C for 30 seconds, 72 °C for 45 seconds, and then extension at 72 °C for 7 min [23, 25].

***Realtime PCR* (RT-PCR)**

RT-PCR used gene primers following the design and temperature optimization. The primary sequence of the alpha VEGF gene was as follows [23]:

F: 5′-GCTCCGTAGTAGCCGTGGTCT-3′,

R: 5′-GGAACCCGGCGGGACACGGAC-3′

Histopathological analysis on animal skin tissue

The tissue was firstly fixed using a 4% phosphate-buffered formalin solution. Subsequently, the tissue was processed into paraffin blocks, which were sectioned using a microtome to a thickness of 4 mm. These sections were then stained with Hematoxylin and Eosin to facilitate observation. Microscopic examination was performed using a CX 33 light microscope, and photomicrographs were captured using a 3.1MP Sony Exmor CMOS camera, followed by analysis using the Betaview software. Quantitative measurements were carried out to determine the thickness of the epidermis and dermis at a 40x magnification. The epidermal thickness was measured by drawing a straight line from the basal epidermis to the upper limit of the stratum granulosum beneath the stratum corneum at ten different points. Furthermore, dermal thickness was measured by drawing a straight line from the basal epidermis to the lower limit of the dermis at ten different points. The two measurements were presented as mean values in micrometers (µm). Other histological parameters, such as edema, leucocytes, granulation, fibroblasts, collagen, and epithelization, were semi-quantitatively evaluated based on criteria outlined by McMinn [26].

Measurement of gene concentration

This study's gene concentration measurement used the relative quantification method [27]. ΔCT experiment = CT experiment target–CT experiment housekeeping. ΔCT control = CT control target–CT control housekeeping. ΔΔCT experiment = ΔCT experiment–ΔCT control. The comparison of gene expression levels = 2^ΔΔCT.

Data analysis

Data were analyzed using SPSS version 21.0 with One-way analysis of variance (ANOVA) and Tukey's tests presented as mean±standard deviation with a confidence interval of 95%. A p-value less than 0.05 was recognized as significant.

Ethics of study

All experimental procedures comprising animals complied with ethical guidelines and were approved by the appropriate ethics committee with number 42/UN.16.10. D. KEPK-FF/2023.

RESULTS

This study was carried out to explain and prove effect of VCO, black cumin oil, C1, C2, and C3 on wound healing in diabetic ulcer model rats. Each treatment's average expression of VEGF gene was calculated using the RT PCR method with GAPDH as a housekeeping gene and quantified using the Livak method. Expression of VEGF gene on day 7 in the skin of the sample was presented in table 1. Furthermore, the results showed that there were differences in VEGF gene expression on day 7 in all groups with a P value of 0.00. The highest mean value was in group C3, which used a 70% black cumin oil ratio and 30% VCO (1.85±0.10). To determine the pairs of groups that were different from others, the analysis was continued with the Tukey HSD test. Fig. 1 showed that the C3 combination group (70% black cumin oil and 30% VCO) had a significant difference from other groups. The results also showed that groups C1(500% VCO and 50% black cumin oil) and C2 (70% VCO and 30% black cumin oil were not different from the group given 100% VCO and 100% black cumin oil only. VCO 100% and 100% black cumin oil groups were not significantly different from the controls.

Expression of VEGF gene on day 14 in the skin of diabetic ulcer model ratas is presented in table 2. The results showed that there were differences in VEGF gene expression on day 14 in all groups, with a P value of 0.00. The highest mean value was obtained in group C3, which was treated with 70% black cumin oil and 30% VCO (1.69±0.11). To determine the pair of groups that were different from others, the analysis was continued with the Turkey HSD test. Based on fig. 2, C2 (70% VCO and 30% black cumin oil) and C3 (70% black cumin oil and 30% VCO had a significant difference from the controls, but was not different from the group with a single treatment of 100% VCO and 100% black cumin oil, as well as C1 (50% VCO and 50% black cumin oil).

The histopathological (fig. 3 and fig. 4) features of wound healing process in animal skin models showed distinct differences between the controls and those treated with VCO and black cumin oil. The control group, induced with STZ, exhibited incision wound with ulceration, scabs, necrotic tissue, and inflammatory cell infiltration on day 7. Loose granulation tissue with numerous capillaries, sparse collagen, and dense inflammatory cell infiltration were also observed. On day 14, wound still showed incomplete epithelization, with the surface still covered by scabs and ulceration. This was consistent with diabetic ulcer, where wound epithelization and granulation were impaired. VCO treatment exhibited complete epithelization by day 7, with wound surfaces covered by epithelium. The dermis used dense granulation and less inflammatory infiltration compared to the positive control. On day 14, the granulation tissue was more compact, with a higher density of fibroblasts and collagen and reduced edema and inflammatory cells. Black cumin treatment showed complete epithelization on day 7. Furthermore, its epidermal thickness was more significant than VCO treatment on the same day. On day 14, the epidermal thickness was the highest among all groups. Granulation tissue with fibroblast and collagen density was higher than the control but lower than VCO group. Inflammatory cell distribution was higher than the control and VCO group on the same day. Combined treatment of VCO and black cumin oil led to complete epithelization by day 7, with epidermal thickness being more significant than VCO group and granulation tissue greater than black cumin oil group. From day 14, collagen and fibroblast density was higher than day seven within the same dosage group, and inflammatory cell infiltration was lower.

Table 1: VEGF gene expression on Day 7 in diabetic ulcer rat models

Group	N	mean±SD	P value
Control	5	1.02±0.25	0.000
VCO	5	1.28±0.16	
Black cumin oil	5	1.42±0.13	
C 1	5	1.69±0.29	
C 2	5	1.68±0.14	
C 3	5	1.85±0.10	

Table 2: VEGF gene expression on day 14 in diabetic ulcer rat models

No.	Group	N	mean±SD	P value
1	Control	5	1.01±0.17	0.001
2	VCO	5	1.18±0.25	
3	Black cumin oil	5	1.35±0,27	
4	C 1	5	1.44±0.20	
5	C 2	5	1.48±0.22	
6	C 3	5	1.69±0.11	

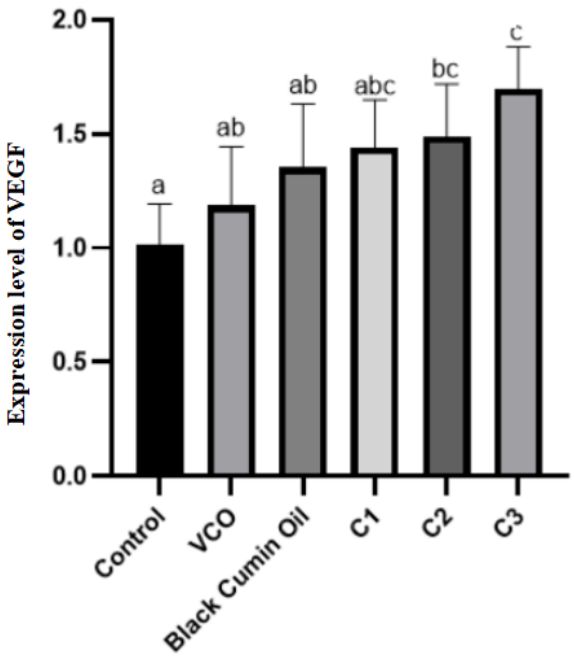

Fig. 1: VEGF gane expression on day 7 across groups using ANOVA and Tukey HSD Post Hoc Analysis. Different letters show significant differences among group with P<0.05

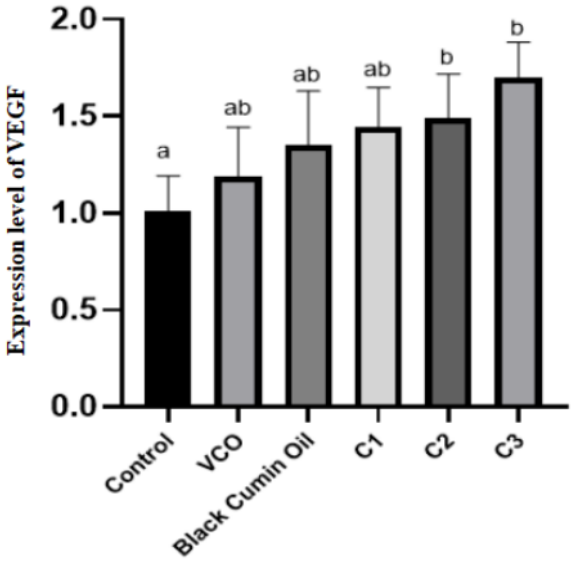

Fig. 2: VEGF gane expression on day 14 across groups using ANOVA and Tukey HSD Post Hoc Analysis. Different letters show significant differences among group with P<0.05

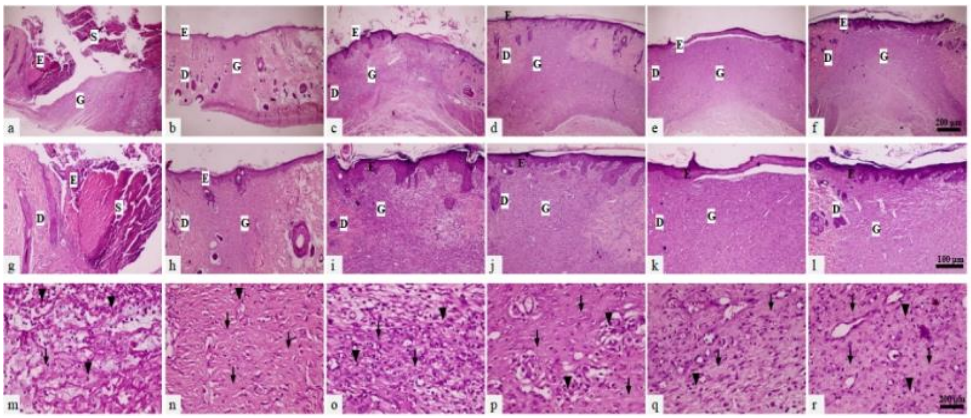

Fig. 3: Histology of animal skin tissue on day 7. Control group for Diabetes (a, g, m), treatment with VCO (b, h, n), treatment with black cumin oil (*nigella sativa*) (c, i, o), treatment with a combination of VCO+black cumin oil *(nigella sativa)*, 1;1 (d, j, p), treatment with a variety of VCO+black cumin oil (*nigella sativa)*, 2;1 (e, k, q), treatment with a combination of VCO+black cumin oil, 1;2 (f, l, r). They were showing the epidermis (E) and dermis (D). Post-wound granulation tissue (G) in the dermis contains collagen matrix (↓) with inflammatory cells (▼)

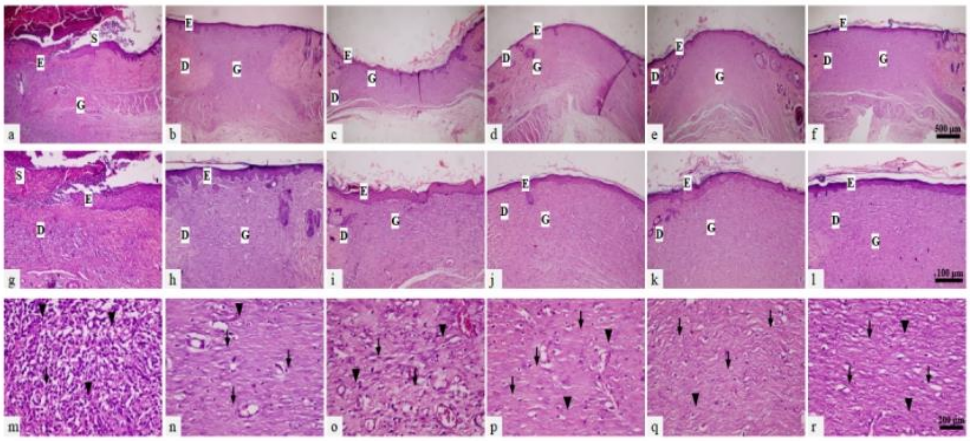

Fig. 4: Histology of animal skin tissue on day 14. Control group for Diabetes (a, g, m), treatment with VCO (b, h, n), treatment with black cumin oil (*nigella sativa)* (c, i, o), treatment with a combination of VCO+black cumin oil (*nigella sativa)*, 1;1 (d, j, p), treatment with a combination of VCO+black cumin oil (*nigella sativa*) 2;1 (e, k, q), treatment with a combination of VCO+black cumin oil, 1;2 (f, l, r), showing the epidermis (E) and dermis (D). Post-wound granulation tissue (G) in the dermis contains a collagen matrix (↓) with inflammatory cells (▼)

DISCUSSION

This study provided compelling evidence for the synergistic impact of VCO and black cumin oil on wound healing. The results showed a significant positive effect of combined VCO and black cumin oil on intracellular and extracellular matrix components and VEGF gen expression wound healing in rats model. VEGF gene expression a key regulatory element in multiple biological processes, including angiogenesis and tissue regeneration. A statistically significant enhancement was observed in VEGF expression when these two oil were combined. Furthermore, they had collaborative potential in activating specific biological pathways [28]. VEGF was acknowledged as an essential growth factor that promoted angiogenesis, forming new blood vessels [29]. The enhanced VEGF expression could potentiate angiogenesis, facilitating more rapid tissue regeneration and optimizing wound healing through improved blood supply to the affected area [18, 16]. Previous studies corroborated these results, indicating that VCO alone had a beneficial effect on fibroblast proliferation and neovascularization in burn wound [30]. Black cumin oil (*nigella sativa*) had also been shown to accelerate wound healing in STZ-induced diabetic rats, specifically when combined with honey [29, 16].

The results suggested a likely synergistic mechanism underlying the observed elevation in VEGF expression. The bioactive constituents in VCO, particularly medium-chain fatty acids, influenced angiogenesis positively [31]. Black cumin oil, rich in thymoquinone, had been implicated in gene regulation and activating angiogenesis-related biological pathways [32]. These results were in line with existing literature underscoring the independent merits of these oil in wound healing processes [33]. Topical application of VCO to rats, which was used as a model for diabetic ulcer, where the fatty acid content in VCO matched the characteristics of the skin, acted as a bioactive molecule encouraging and increasing expression of VEGF. This correlated with the angiogenesis process during the inflammatory period [16].

In this study (fig. 3 and fig. 4), treatment with VCO showed a significant ability to facilitate wound healing, as evidenced by reduced granulation tissue and diminished inflammation levels. VCO could accelerate healing through its anti-inflammatory effect and expedited collagen formation. Black cumin oil manifested a unique healing trajectory characterized by thicker epithelization and a broader distribution of inflammatory cells. Given wound healing challenges faced by type II diabetes patients; this method could provide therapeutic benefits. Furthermore, the combination of VCO and black cumin oil offered promising outcomes, seemingly amalgamating the advantages of both agents and the potential of combined therapies for patients with wound healing disorders, such as diabetic ulcer. VCO had been found to have similar properties.

A histopathological study on young rats showed an increase in fibroblast proliferation and neovascularization in VCO-treated wound compared to controls [11]. Another study found that wound treated with VCO healed faster, increased collagen tissue and fibroblast proliferation [34]. Studies had shown that VCO-treated wound healed faster, as indicated by a decreased time of complete epithelization and higher levels of various skin components [10]. Oil had also been found to promote wound healing due to its anti-inflammatory, analgesic, antipyretic, and antioxidant properties [10]. Black cumin oil (*nigella sativa*) was shown to have a therapeutic effect on skin wound healing through its anti-inflammatory, tissue growth stimulation, and antioxidative properties [33]. Studies had shown that black cumin oil (*nigella sativa*) could heal burn-related skin wound in rat models and topical application of oil prepared from its seeds accelerated wound healing [35].

The histopathological results suggested that both individual and combined treatments with VCO and black cumin oil positively accelerated wound healing in diabetic animal models. Healing patterns differed between VCO and black cumin oil treatments, while combined treatment exhibited an intermediate pattern. Further studies were needed to provide a full understanding of these effect. Although this study elucidated the synergistic potential of VCO and black cumin oil (*nigella sativa)*, it also necessitated further investigation to explore the underlying mechanisms responsible for the observed upregulation of VEGF gene expression. Future *in vitro* studies using molecular methods could offer insights into this response's specific interactions and biological pathways. Furthermore, comprehensive preclinical trials using animal models were warranted to validate these results in a more nuanced physical context. The clinical implications of this study were particularly promising for diabetic ulcer patients, who frequently faced complications in wound healing. Using topical treatment comprising a combination of VCO and black cumin oil (*nigella sativa*) could revolutionize therapeutic strategies for these patients by enhancing VEGF gene expression. This strategy could accelerate tissue and blood vessel formation, thereby mitigating the risk of complications and significantly improving patient prognosis.

CONCLUSION

In conclusion, the results showed the synergistic potential of VCO and black cumin oil (*nigella sativa*) in upregulating the expression of VEGF gene, an essential mediator of angiogenesis and tissue regeneration. This study served as a foundational step toward the development of alternative therapeutic regimens that could yield significant improvements in the clinical management of diabetic ulcer. These results were expected to catalyze further investigations clinical translation, and the implementation of natural substance-based therapies for optimized wound healing management.

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AUTHORS CONTRIBUTIONS

All authors were involved in the completion of this research.

CONFLICT OF INTERESTS

There is no conflict of interest in this study

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