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Effective Combination of *Nigella sativa* and *Trigonella foenum-graecum* Seed Extract on Wound Healing in Diabetic Mice

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Abstract. *High levels of free radicals in diabetic wounds often cause chronic inflammation. Nigella sativa and Trigonella foenum-graecum seed extracts are rich in antioxidants and have anti-inflammatory effects. Trigonella foenum-graecum seed extracts can also accelerate the reepithelialization process in wound healing. This study aimed to determine the combination of ethanol extract of Nigella sativa and Trigonella foenum-graecum seeds on wound length and the number of neutrophils, macrophages, fibroblasts, and collagen density in the incision wound of diabetic mice induced by streptozotocin. T. This study used three types of controls, namely Normal (Normal mice, HPMC 3%), DM (Diabetic mice, HPMC 3%), and Iodine (Diabetic mice, Povidone Iodine 10%). Topical treatment with 70% ethanol extract ointment combined with Nigella sativa and Trigonella foenum-graecum in various variations, namely N (20%:0%), T (0%:10%), NT (10%:5%), Observations of the wound healing process were carried out on 3rd, 7th and 14th day. Data were analyzed using the One Way ANOVA and Duncan Multiple Range Test. The study's results by observing the morphology of the wound length on day 7th day showed that the combination treatment of extracts of Nigella sativa and Trigonella foenum-graecum resulted in the shortest wound, which was significantly different from the wound in untreated diabetic mice. In addition, diabetic wounds without treatment showed infection, and on the contrary, the infection did not occur in wounds treated with a combination of extracts of Nigella sativa and Trigonella foenum-graecum. The results of wound histology observations showed that the combination treatment of extracts of Nigella sativa and Trigonella foenum-graecum showed a decrease in inflammation which was indicated by a significant decrease in the number of neutrophil cells, macrophages, and accelerated reepithelialization of wound healing as indicated by a significant increase in the number of fibroblasts and collagen density since the third day of treatment.*

Keywords: *Diabetes Mellitus (DM), Nigella sativa, Trigonella foenum-graecum, wound healing*

Citation
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INTRODUCTION

Diabetes Mellitus (DM) is one of the

metabolic disorders with hyperglycemia characteristics, usually due to abnormalities in the insulin secretion process, insulin performance, or both. High blood glucose levels due to diabetes mellitus in the long term can cause organ damage and dysfunction of various organs, especially the heart, kidneys, eyes, blood vessels, and nerves (American Diabetes Association, 2013).

Based on the International Diabetes Federation (2017), the prevalence of diabetes mellitus globally is 387 million people. In 2035, it is predicted that the prevalence of DM worldwide will increase by 529 million people, or a 53% increase at the time. An estimated 9.1 million people worldwide suffer from DM (International Diabetes Federation, 2017). Complications of foot ulcers in DM patients start from minor wounds but can lead to amputation, about 85% of DM patients experience amputation within five years (Jupiter et al., 2016).

Wounds are conditions of body tissue damage, especially skin epithelial tissue, connective tissue, and muscle tissue, to cause damage to nerve tissue and blood vessels that cause bleeding (Amalia, 2015). Diabetic wounds are characterized by delayed healing of acute wounds so that they become chronic wounds (Raziyeva et al., 2021). An incision wound is slicing a particular body part at a certain depth (Payne-James, 2016).

The wound healing phase generally starts from the inflammatory phase, the proliferative or granulation phase, and finally, the maturation or remodeling phase (Gonzalez & Andrade, 2016). The normal wound healing process starts from the hemostasis phase, where the system initiates blood clotting factors-followed by the inflammatory phase that aims to clean the damaged tissue through phagocytosis of microorganisms by neutrophils and macrophages. This stage occurs approximately 24 hours after the injury and can end within three days or longer if there is interference in the healing process by infection, trauma, or other causes. The second phase of the healing process is the proliferative phase characterized by decreasing in inflammatory cells to form granulosa tissue and lasts from day 3 to day 14. The maturation phase, the last phase of the wound healing process, is marked by the formation of new tissue on the 14th day to 3-6 months (Primadina et al., 2019).

Nigella sativa or black cumin is a plant from the Ranunculaceae family that has been used traditionally for the treatment of various diseases. *Nigella sativa* contains 36-38% fixed oil, alkaloids, proteins, saponins, and 0.4-2.5% essential oils (Ramadan, 2016). *Nigella sativa* seeds at concentrations of 20% and 40% can accelerate wound healing in streptozotocin-induced diabetic rats within 15-18 days (Nourbar et al., 2019). Fenugreek seeds (*Trigonella foenum-graecum)* are rich in polysaccharides and saponins (Jyothi et al., 2014). Hydroalcoholic extract of fenugreek seeds with a concentration of 5% and 10% given topically can accelerate the proliferative process in wound healing in diabetic rats (Muralidharan et al., 2016). Thymoquinone contained in black cumin seeds can work as an antioxidant in breaking down free radicals and increasing intracellular antioxidant capacity (Alexander et al., 2019). It is also optimized by the synergistic effect of the flavonoid compounds present in the fenugreek seed extract which are responsible for producing anti-inflammatory activity (Jyothi et al., 2016).

The effectiveness of topical drug preparations is strongly influenced by the duration of skin surface contact with the drug, this requires the adhesive properties of the preparation. In addition, the effectiveness of topical preparations must also be able to release the drug well. HPMC (Hydroxyl Propyl Methyl Cellulose) is a gelling agent that acts as an emulsifier, that is stable at pH 3 to 7 (Li et al., 2020), the resulting gel is clear, neutral, and its viscosity is stable even though it is stored for a long time (Binder et al., 2019). The higher concentration of HPMC in the preparation has higher adhesive properties, HPMC concentration of 3% has an adhesiveness of 0.8 N and a viscosity of 29 Pa.s. Sulphadiazine sodium release test from gel preparations showed that the use of HPMC at a concentration of 1-3% increased the ability to penetrate deeper layers of the skin in greater amounts than without the gelling agent (Binder et al., 2019). This study aimed to obtain a synergistic effect on the combination of 70% ethanol extract of black cumin seeds (*Nigella sativa* L.) and fenugreek seeds (*Trigonella foenum-graecum*) using HPMC 3% as gelling agent on incisional wound healing in mice induced by strept zotocin (STZ).

MATERIALS AND METHODS

This research was conducted in April-June 2021 at the Laboratory of Animal Physiology and Experimental Animal, Biology Study Program, Faculty of Science and Technology, UIN Maulana Malik Ibrahim Malang, using a Completely Randomized Design (CRD) method with six treatments, namely normal control (Normal), negative control (DM), Diabetic mice with 10% povidone-iodine as positive control (Iodine), topical treatment on diabetic mice with various combinations extract of black cumin: fenugreek in a ratio of 20%:0% (N), 10%:5% (NT), and 0%:10% (T) in HPMC 3%. The wound healing parameters observed included wound morphology, namely wound-length and wound assessment, and histological observations included the number of neutrophils, macrophages, fibroblasts, and collagen density.

The tools used were a shaver (Gilette), scalpel (OneMed), 1 ml syringe (OneMed), analytical scale, blood-sugar level measuring device (EasyTouch), beaker glass (80 ml and 100 ml), stirrer, spatula, vortex, oven, rotary evaporator, shaker, blender, section board, ruler, camera, paraffin bath, paraffin oven, microtome, object glass, and cover.

The materials used were male mice (*Mus musculus*) strain Balb/C, 3-4 months old and weighing about 30-40 grams, husks, feed for BR-1 and BR-2 mice, drinking water for mice, label paper, plastic, aluminum foil, streptozotocin (Santa Cruz Biotech), NaCl, citrate buffer 0.1 M pH 4.5, 70% ethanol, povidone-iodine (Betadine) ointment, HPMC (Hydroxypropyl Methylcellulose), alcohol swabs, blood sugar measuring strip (EasyTouch), black cumin seeds (*Nigella sativa* L.) and fenugreek seeds (*Trigonella foenum-graecum*).

This research process was certified with Ethical Clearance certification No. 020/EC/KEP-FST/2020. The research procedure included acclimatization of test animals for ±14 days, preparation of 70% ethanol extract of black cumin (*Nigella sativa* L.) and fenugreek seeds (*Trigonella foenum-graecum*) by maceration until a concentrated extract was obtained. Furthermore, HPMC 3% was used as a gelling agent for the preparation of the ointment. The treatment consisted of the induction of experimental mice with streptozotocin (STZ) at a dose of 45 mg/kgBW for three consecutive days and 60 mg/kgBW for two intermittent days. Mice with blood sugar levels of 200 mg/dL are referred to as hyperglycemia.

The incision wound was made by shaving the hair on the right back of the mouse with an area of 1 cm x 2.5 cm. Mice were anesthetized using a combination of 0.025 ml (10% w/v) ketamine and 0.025 ml (2% w/v) xylazine (Nourbar et al., 2019) intramuscularly. The incision was subcutaneously made ±2 cm long using a razor blade. The experimental animal therapy was administered by applying the extract to the wound evenly once a day for 14 days; the positive control group was treated with 10% povidone-iodine ointment daily. Measurement of wound length and observation of wound healing was carried out on the 3rd, 7th, and 14th after the injury. The histological observation was prepared on the same day of the wound healing process.

Statistical data analysis was carried out using the SPSS ver. 21.0. Data that qualify the parametric rules were statistically tested using the parametric ANOVA test (Analysis of Variances) and continued with Duncan's Multiple Range Test (DMRT) for data that had significant ANOVA test results ($p < 0.05$). The data from the wound healing assessment were tested using the non-parametric Kruskal-Wallis statistical test; when there was a significant difference ($p < 0.05$), then the data analysis was continued with the Mann-Whitney Test (Sugiyono, 2014).

RESULTS AND DISCUSSION

The results of the incision wound length on day three (3) and day seven (7) each showed a significant effect after treatment ($p<0.05$). The combination therapy of *N. sativa* and *T. foenum-graecum* extracts (NT) gave the best result by accelerating the wound closure process observed on the 7th day of treatment (Table 1). All treatments using extracts of *N. sativa* and *T. foenum-graecum* either in single or combination extract showed the ability to improve the healing process without causing infection and allergic reactions significantly to diabetic and normal mice (Table 1). Infectious reactions can be found in the preliminary inflammatory phase. This stage usually occurs approximately 24 hours after the wound appears and can end within three days, or it can be longer if there is interference in the healing process by infection, trauma, or other causes (Eming et al., 2014).

Table 1. Average percentage of wound length and incision wound assessment score in Mice (*Mus musculus*)

Treatment Groups	Average Wound Length ±SD (%)			Wound Assessment Score		
	Day 3	Day 7	Day 14	Infection	Allergy	Wound Healing Time
KN (normal control)	55.6 ± 16.2[a]	36.6 ± 9.0[bc]	5.6±6.6[a]	1.0	1.6	3.6
K- (negative control)	82.0 ± 12.1[b]	41.6 ± 5.7[c]	8.2±7.7[a]	2.8	1.0	3.6
K+ (positive control)	53.4 ± 11.3[a]	24.6 ± 16.2[ab]	6.4±9.5[a]	1.0	1.0	3.2
N (*N. sativa* 20%)	69.0 ± 15.4[ab]	33.6 ± 10.5[bc]	0[a]	1.0	1.0	3.0
T (*T. foenum-graecum* 10%)	62.2 ± 13.6[a]	29.2 ± 12.1[abc]	3.8±5.8[a]	1.0	1.0	3.4
NT (*N. sativa* 10% and *T. foenum-graecum* 5%)	66.2 ± 6.8[ab]	17.0 ± 10.8[a]	5.0±11.2[a]	1.0	1.0	3.2

The results in the number of neutrophil cells in the incision wound on on the 3rd, 7th, and 14th days showed a significant difference in the number of neutrophils in the wound between treatments (p<0.01). The NT extract treatment gave the best effect significantly compared to the control treatment (Normal, Iodine, and DM) on the 3rd, 7th, and 14th days (Figure 1).

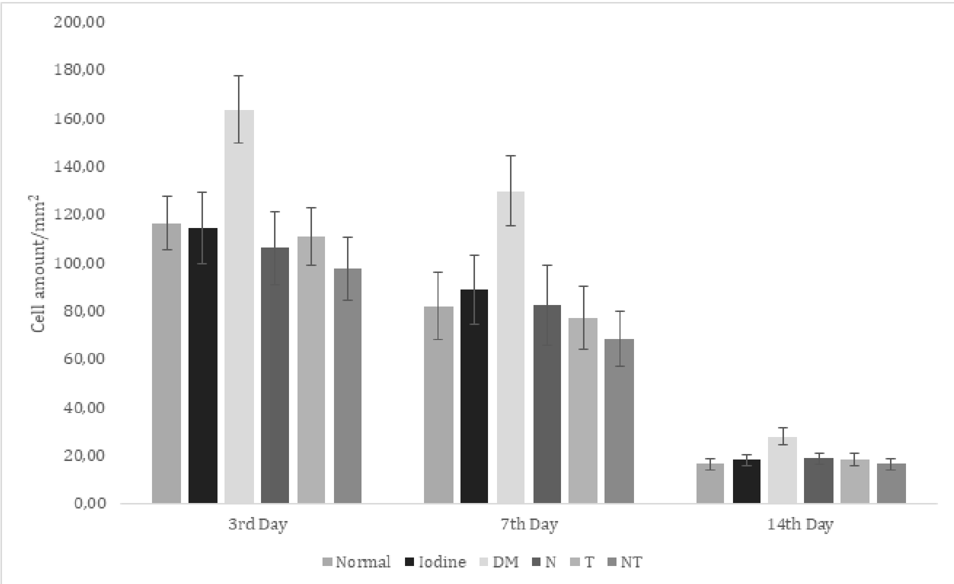

Figure 1. The average number of neutrophils on the 3rd, 7th, and 14th days after treatment with a combination of black cumin seed extract and fenugreek. Note: N (treatment of *Nigella sativa* 20%), T (treatment of *Trigonella foenum-graecum* 10%), NT (treatment of a combination of *Nigella sativa* 10% and *Trigonella foenum-graecum* 5%), Iodine (positive control of povidone-iodine 10%), DM (negative control), Normal (normal control). Differences in letter notation indicate significant differences between treatments.

Neutrophils are the first leukocytes present at the wound site in the first 24 hours after the injury (Portou et al., 2015). The number of neutrophils indicates the stage of inflammation in the open wound healing process. The neutrophil count will be high when the wound becomes inflamed, and it will decrease as the wound heals. Neutrophils play a crucial role in the inflammatory phase of wound repair by secreting cytokines and various growth factors (Nguyen et al., 2013). Thymoquinone contained in black cumin can accelerate wound healing in the inflammatory phase, which is associated with its anti-inflammatory properties. Thymoquinone can significantly reduce inflammation in diabetic ulcer models (Chen et al., 2016).

The results in the number of macrophage cells in incision wounds on on the 3rd, 7th, and 14th days showed a significant difference in the number of macrophages in the wound between treatments (p<0.01). The NT extract treatment gave the best effect significantly compared to the control treatment (Normal, Iodine, and DM) on 3rd day and gave the best effect compared to other treatments, which were equivalent to Normal on the 7th day (Figure 2).

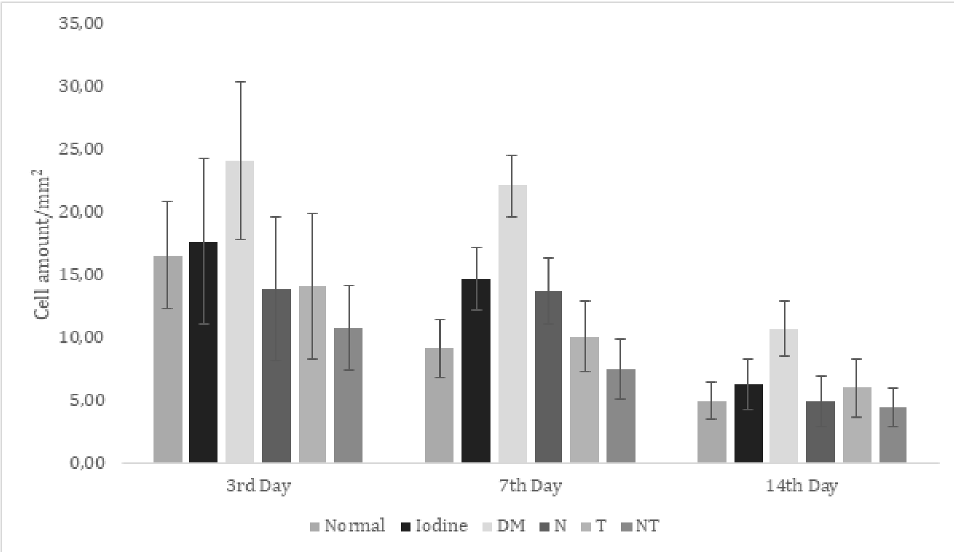

Figure 2. The average value of the number of macrophages on the 3rd, 7th, and 14th days after treatment with a combination of black cumin seed extract and fenugreek. Note: N (treatment of *Nigella sativa* 20%), T (treatment of *Trigonella foenum-graecum* 10%), NT (treatment of a combination of *Nigella sativa* 10% and *Trigonella foenum-graecum* 5%), Iodine (positive control of povidone-iodine 10%), DM (negative control), Normal (normal control). Differences in letter notation indicate significant differences between treatments.

Macrophages are cells that play a role in the inflammatory stage in addition to neutrophils. Macrophages infiltrate the wound area after receiving signals from neutrophils. Macrophages act as immunocompetent cells whose duty is to continue the phagocytosis of neutrophil cells. Macrophages begin to be present in the wound area 48-72 hours after the wound (Gonzalez & Andrade, 2016). Fenugreek seed extracts rich in polyphenols significantly reduced H_2O_2-induced oxidative modification in normal and diabetic human erythrocytes, indicating strong antioxidant properties of fenugreek seeds (Kilambi & Shah, 2021).

In this study, the highest number of neutrophils and macrophages was observed in the negative control mice compared to other treatments until the observation's end, indicating a persistent inflammatory phase. The prolonged inflammatory phase becomes an obstacle in the formation of mature granulation tissue and a decrease in the tensile strength of the wound (Alavi et al., 2014) so that it can cause a tendency for infection and the healing process to run slowly, as shown in table 1 and the low number of fibroblasts and collagen cells (Figure 3 & 4).

The prolonged inflammatory phase in diabetic mice may be due to increased levels of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (Baltzis et al., 2014). This factor causes the inflammatory pathway to be interrupted (Patel et al., 2019). In addition, diabetic conditions impact high ROS production and inhibition of endogenous antioxidant action; this oxidative stress condition can worsen the wound condition (Matough et al., 2012).

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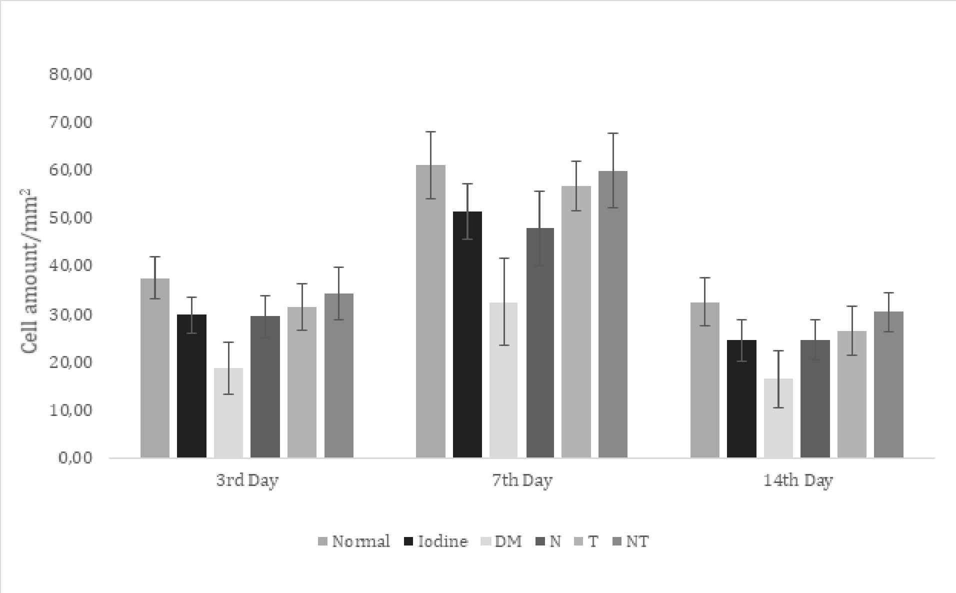

Figure 3. The average value of the number of fibroblasts on the 3rd, 7th, and 14th days after treatment with a combination of black cumin seed extract and fenugreek. Note: N (treatment of *Nigella sativa* 20%), T (treatment of *Trigonella foenum-graecum* 10%), NT (treatment of a combination of Nigella sativa 10% and *Trigonella foenum-graecum* 5%), Iodine (positive control of povidone-iodine 10%), DM (negative control), Normal (normal control). Differences in letter notation indicate significant differences between treatments.

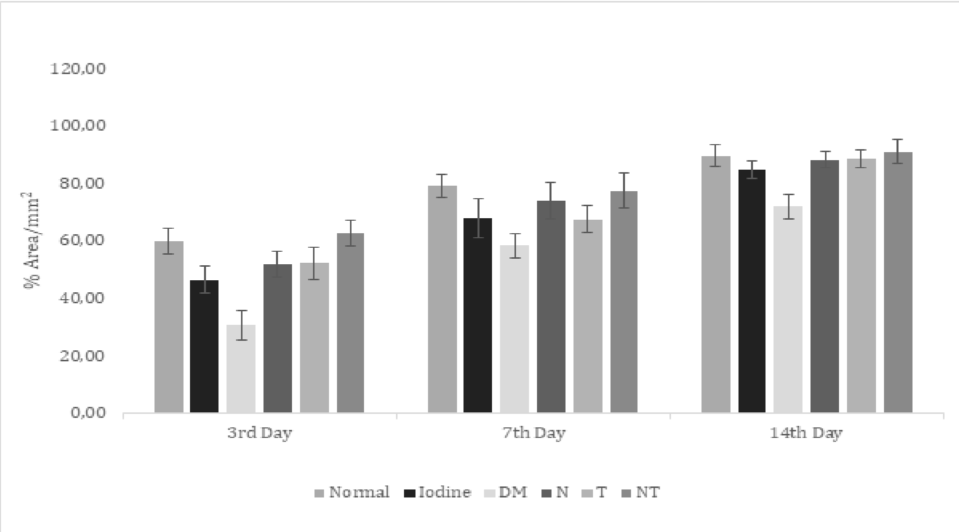

Figure 4. The average density of collagens on the 3rd, 7th, and 14th days after treatment with a combination of black cumin seed extract and fenugreek. Note: N (treatment of *Nigella sativa* 20%), T (treatment of *Trigonella foenum-graecum* 10%), NT (treatment of a combination of *Nigella sativa* 10% and *Trigonella foenum-graecum* 5%), K+ (positive control of povidone-iodine 10%), K- (negative control), KN (normal control). Differences in letter notation indicate significant differences between treatments.

The results on the number of fibroblasts in the incision wound on the 3rd day showed a significant difference in the number of fibroblasts in the wound between treatments ($p<0.01$). The NT extract treatment gave the best effect significantly compared to the control treatment (Normal, Iodine, and DM control) on the 3rd day and gave a better effect than the N, Iodine, and DM treatment on the 7th day. Meanwhile, on the14th day, the NT treatment was better than the N treatment (Figure 3).

Fibroblasts are cells that play a role in the proliferative stage. Fibroblasts start in the wound area 3rd days after injury and continue to increase up to 7th day after injury. After fibroblasts peaked on 7th day, their numbers decreased over time. These fibroblasts migrate into the wound, stimulated by factors such as TGF-β and PDGF released by inflammatory cells and platelets. Fibroblasts then synthesize the extracellular matrix (ECM), which serves as a medium for cell migration (Velnar et al., 2009). In addition to fibroblasts, neutrophil cells and macrophages also produce PDGF, TGF-β, FGF, and VEGF, which function as the central cytokines capable of stimulating the formation of granulation tissue prior to the work of fibroblast cells.

Fenugreek seed extract can accelerate the proliferative process in wounds which can be found in the previous study by Muhammed (2012) on rabbits, where the topical application of 10% fenugreek seed ointment accelerates the formation of cellular fibrous connective tissue, granulation tissue, and early maturation of fibrous connective tissue and thereby increases wound healing. Goorani et al (2019) showed that the administration of flavonoid antioxidants was able to increase the number of fibroblasts and the rate of formation of fibrocytes into fibroblasts, thereby increasing the wound healing process.

The results on 3rd, 7th, and 14th days of incision wound collagen density showed a significant difference in wound collagen density between treatments ($p<0.01$). The NT extract treatment gave the best effect, equivalent to Normal on the 3rd day and better than the T, Iodine, and DM treatments on the 7th day. While on 14th day, NT treatment gave the best effect compared to other treatments, which were not significantly different from Normal (Figure 4).

Collagen is an essential component in all phases of wound healing and is synthesized by collagen fibroblasts (Chattopadhyay & Raines, 2014). Collagen will continue to increase daily, with a peak on 14th day (Zomer & Trentin, 2017). A previous study by Nourbar et al (2019) showed that collagen fibers in excision wounds of diabetic mice treated with black cumin seed extract with a concentration of 40% were thicker than normal mice that were not treated. Belaïd-Nouira et al (2013) added that the fatty acids present in fenugreek seeds help in the reconstruction of collagen thereby promoting wound healing and maintenance of skin elasticity.

This study found the highest number of fibroblast cells and collagen matrix in diabetic mice treated with the combination of *N. sativa* and *T. foenum-graecum* extracts (Figure 2). This condition is supported by several reports of other researchers related to the discovery of thymoquinone in the combination therapy of treatment extracts. *Nigella sativa* seed extract is the quinone group's main component, especially thymoquinone (Kumar & Venkatachallam, 2010) (Ahmad et al., 2013).

CONCLUSION

Based on the study's results, it can be concluded that the NT treatment can accelerate the wound healing process by a decrease in the number of neutrophils and macrophages, which showed by the normalization of the inflammatory process, the proliferative phase characterized by a high number of fibroblast cells that become collagen producers, which showed the imminent occurrence of a critical granulation process in the final stages of the wound healing process.

AUTHOR CONTRIBUTION

R.S. made study conception design, supervised all the process, proofread and edited manuscript, U.M.R. carried out research, collected and analyzed the data, as well as wrote the manuscript.

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CONFLICT OF INTEREST

All authors have no conflict of interest.

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