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The Effect of *Hypericum* perforatum (St. John's Wort) and *Nigella* sativa (Black Cumin) Oils on Wound Healing in Type-1 Diabetic Mice

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Abstract: Wound healing is a well-known therapeutic challenge in animal and human medicine. This study aimed to investigate the effect of topical application of *Nigella* sativa (black cumin, NS) and *Hypericum* perforatum (St. John's Wort, HP) on wound healing in streptozotocin (STZ) induced diabetic mice. For this aim, 25 male BALB/c mice were divided into five groups: i. *Hypericum* perforatum (HP), ii. *Nigella* sativa (NS), iii. Standard saline solution 0.9% NaCl (NSS), iv. Natural extra virgin olive oil (OL), and v. Fusidic acid-*Centella* asiatica cream (FM). A single dose (200 mg/kg) of STZ was intraperitoneally administered to induce type-1 diabetes. After diabetes was induced, six symmetrical excision wounds were performed on the dorsal areas of mice using a dermal biopsy punch. Wound areas were photographed every three days for 21 days, and the images were analyzed using software to achieve the daily wound closure rate in pixel values. On day one and day 21, wound tissues were dissected, total protein and hydroxyproline levels were measured by ELISA. Statistically significant differences were found in hydroxyproline change rates between the NS group with HP, NSS, and FM groups ($P<0.05$). The only significant difference was found between NS with OL groups ($P<0.05$) on wound closure rate. This study illustrated that topically administered HP and NS may not have wound-healing effects in type-1 diabetic conditions in mice.

Keywords: *Black Cumin, Mouse, Nigella sativa, Type-1 Diabetes, Wound Healing.*

Hypericum perforatum (Sarı Kantaron) ve *Nigella* sativa (Çörek Otu) Yağlarının Tip-1 Diyabetik Farelerde Yara İyileşmesi Üzerine Etkisi

Özet: Yara iyileşmesi, hayvan ve insan sağlığında iyi tanınan terapötik bir durumdur. Sunulan çalışma STZ (streptozotosin) ile indüklenen diyabetik farelerde *Nigella* sativa (çörek otu, NS) ve *Hypericum* perforatum (sarı kantaron, HP) yağının deri yarası iyileşmesi üzerine etkilerinin araştırılmasını amaçladı. Bu amaçla, 25 adet erkek BALB/c ırkı fare gruba ayrıldı: i. *Hypericum* perforatum (HP), ii. *Nigella* sativa (NS), iii. Fizyolojik tuzlu su (%0,9 NaCl, NSS), iv. Natürel sızma zeytinyağı (OL) ve v. Fusidik asit-*Centella* asiatica kremi (FM) olacak şekilde beş gruba ayrıldı. Tip-1 diyabeti indüklemek için intraperitoneal olarak tek doz (200 mg/kg) STZ uygulandı. Diyabet indüksiyonunundan sonra farelerin sırt bölgelerinde dermal biyopsi punch kullanılarak altı simetrik eksizyon yarası oluşturuldu. Yaralar 21 gün boyunca her üç günde bir fotoğraflandı ve günlük yara küçülme oranlarını piksel değeri olarak elde etmek için bu fotoğraflar yazılım aracılığı ile analiz edildi. Çalışmanın 1. ve 21. günlerinde yara dokuları diseke edildi ve ELISA ile hidroksiprolin ve total protein düzeyleri ölçüldü. HP, NSS ve FM grupları ile NS grubu fareler arasında hidroksiprolin değişim oranlarında istatistiksel olarak anlamlı farklılıklar bulundu ($P<0,05$). Yara kapanma oranında ise sadece NS ile OL grupları arasında istatistiksel olarak anlamlı bir farklılık tespit edildi ($P<0,05$). Bu çalışmanın sonuçları, topikal olarak uygulanan HP ve NS'nin farelerde tip-1 diyabetik koşullarda yara iyileştirici etkileri olmayabileceğini göstermiştir.

Anahtar Kelimeler: *Çörek Otu, Fare, Hypericum perforatum, Nigella sativa, Sarı Kantaron, Tip-1 Diyabet, Yara İyileşmesi.*

Introduction

It is known that individuals with diabetes suffer from chronic unhealable wounds. Wound healing is the unification process in a specific pattern and order of epithelial, endothelial, inflammatory cells, platelets, and fibroblasts (Lerman et al., 2013; Loomans et al., 2004). This process is influenced by several systemic and local variables such as oxygenation, infections, age, sex hormones in aged individuals, stress, diabetes, obesity, nutrition, and medications (Guo and Dipietro, 2010). Approaches for wound healing have significant advantages for the patients. However, they may also have drawbacks, such as the relative cost of products or the undesirable side effects of the chemicals used.

Therefore, studies have focused on alternative remedies of natural wound healing products such as natural herbs (Sharma et al., 2021).

Hypericum perforatum is considered as a valuable herbal medicine. It contains hyperforin, flavonoids, and hypericin (Fu et al., 2006) which may be used as antiseptic, anti-inflammatory, and wound healer (Sharma et al., 2021; Suntar et al., 2010). *Nigella* sativa, known as black cumin in English literature, is an herb that originated mainly from South Asia and the Middle East (Ghedira, 2006). *Nigella* sativa and its components have many pharmacological effects; antioxidant, anti-inflammatory, and anticancer are only a few examples (Khader and Eckl, 2014). The activation of angiogenesis enhanced fibroblast proliferation, and subsequent collagen synthesis is the fundamental mechanism by which *Nigella* sativa promotes wound healing (Shahani et al., 2013).

Type-1 diabetes is a chronic metabolic disease characterized by autoimmune degradation of pancreatic β-cells. Hyperglycemia and dyslipidemia lead to the failure of the wound healing processes which causes an increase in mortality of type-1 diabetes (American Diabetes Association, 2014; Petrie et al., 2018). Treatments of diabetic wounds are challenging as their healing processes are extremely slow and can last for weeks. Although there has been a significant effort with the enhanced technology, the specific pathogenesis and treatment of the impaired wound healing in diabetes remains unclear (Bagdas et al., 2014; Spampinato et al., 2020). The present study aimed to investigate the wound healing effect of *Hypericum* perforatum and *Nigella* sativa in type-1 diabetic mice.

Materials and Methods

Animal Preparation: This study, including animal experiments and protocols, was approved by the Institutional Animal Care and Ethical Committee of Aydin Adnan Menderes University (ADU-HADYEK, approval number #64583101.2017.051). Twenty-five male BALB/c mice, six weeks of age, were used. Mice were housed individually in the appropriate environment (12 hours light/12 hours dark) and room temperature (22 ± 2°C). Tap water and chow diet (Mice chow feed, Radon Medical, Dikmen, Ankara, Turkey) were given ad libitum. All mice were housed in individual cages. After an initial environmental adaptation period (10 days), mice were randomly divided into five groups (n=5/each); group 1 *Hypericum* perforatum (HP, Zade Vital®, Konya, Turkey), group 2 *Nigella* sativa (NS, Zade Vital®, Konya, Turkey), group 3 standard saline solution (NSS, 0.9% NaCl, Polifarma®, Tekirdag, Turkey), group 4 natural extra virgin olive oil (OL, Zade Vital®, Konya, Turkey), and group 5 fusidic acid (Furacin Cream®, Zentiva, Luleburgaz, Turkey)-*Centella* asiatica (Madecassol Cream®, Bayer, Umraniye, Istanbul, Turkey) cream group (FM). Since *Hypericum* perforatum oil from a licensed company was macerated in natural extra virgin olive oil like other products on the market, one group (natural extra virgin olive oil treatment) (OL) was added to distinguish the effectiveness of the olive oil used in it.

Induction of Type-1 Diabetes: Type-1 diabetes was induced by administering a single (200 mg/kg) intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich, St. Louis, Missouri, United States) dissolved in sterile saline after overnight fasting. Blood glucose levels from the tail vein were measured one week after STZ injection to confirm diabetes (Guz et al., 2002). A commercial glucometer (Contour Plus®, Bayer, Leverkusen, Germany) measured blood glucose levels. A mouse with 300 mg/dL or above blood glucose levels was considered type-1 diabetic (Guz et al., 2002). Mice that are resistant to streptozocin-induced type-1 diabetes were excluded from the study.

Forming Full-Thickness Excision Wounds and Biochemical Analyses: Mice were anesthetized with an intraperitoneal injection of ketamine HCl (50 mg/kg) (Ketasol® 10%, Interhas, Ankara, Turkey) and 10 mg/kg xylazine HCl (Xylazinbio® 2%, Bioveta, Ankara, Turkey). The dorsal areas of mice were fully shaved by using an electric shaver and disinfected with 70% ethanol. Six symmetrical excision wounds (5 mm diameter/each) were punched from cranial to caudal on the right and left of the medial line on each animal with a dermal punch (Moreira et al., 2015). With such a simple procedure, the wound model was constructed faster. At the end of the study, mice were sacrificed with isoflurane (Isoflurane USP®, Adeka Ilac, Istanbul, Turkey). Tissues from the healed wound areas were collected following the euthanasia procedure (Liu et al., 2013) and stored at -70°C for further biochemical analysis. Tissues were dissected 1 mm around the healing scar tissue. Hydroxyproline and total protein levels were measured using the ELISA (SunRed® Mouse (Hyp), Shanghai, China, cat no: 201-02-0543) and BCA Protein Assay Kit (Thermo Scientific®, Rockford, United States) from collected tissues. Tissues were weighed before and then homogenized by a mechanical homogenizer (Lab-Blender 400 Stomacher®, Interscience, Saint Nom la Brétèche, France) in chilled water 10 minutes at 2000 rpm. Homogenates were transferred to Eppendorf tubes and centrifuged at 3000 rpm (4 °C). Supernatants were transferred to new Eppendorf tubes for total protein, and hydroxyproline

measurements were performed according to the manufacturer's instructions.

Photographic Follow-up of Excision Wounds: Image processing techniques have been used to assess wound healing, and methodologies that have been well addressed in the literature have been performed in the present study (Marotte et al., 2010; Saranya et al., 2016). The methodology of using images of wound areas to monitor the daily closure amount of the wound area was preferred in the research as it is a non-invasive method that reduces measurement-related errors. Wound areas were photographed regularly, and these photos were stored on a computer. For this purpose, photographs of wound areas were taken every 3 experiment days following the treatments and forming excisional wounds, using a Canon® EOS 550D brand and model camera by the primary investigator (IA). Photographs were taken while the mice were anesthetized with isoflurane (Isoflurane USP®, Adeka Ilac, Istanbul, Turkey) to ensure the best picture quality. The photographs were analyzed in a computer with an interface (software) developed/written in the R2015a version using the MATLAB® (The Mathworks Inc., Natick, Massachusetts, USA) program, and the size of the wound area for each mouse was calculated. Steps involved in wound area processing are presented in Figure 1.

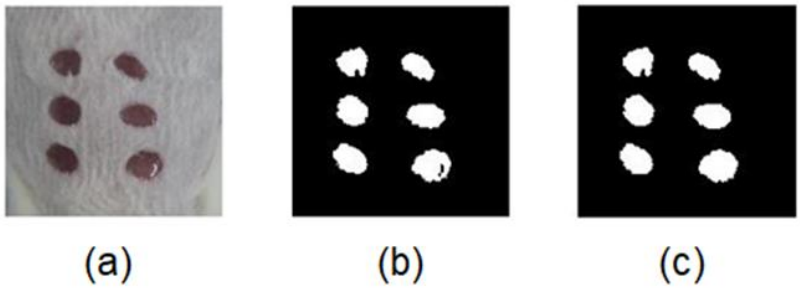

Figure 1. Excision wounds and image processing with the computer software.
(a) The image of excision wound areas, (b) the resolution of wound margins with image processing software, and (c) improving the quality of the image with MATLAB® software.

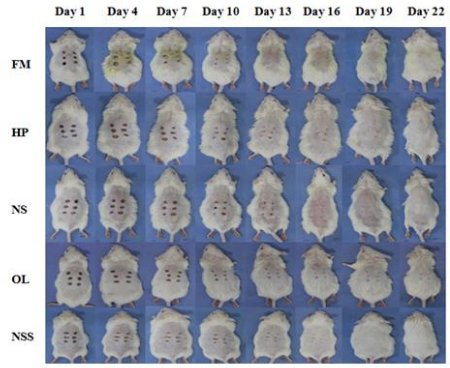

Figure 2. Photographic observations of excision wounds.
HP: *Hypericum* perforatum (St. John's Wort), NS: *Nigella* sativa (Black cumin oil), NSS: Normal saline solution (0.9% NaCl), OL: Natural extra virgin olive oil, FM: 2% fusidic acid+*Centella* asiatica cream.

The wound images were cropped in a particular frame, and the image's unwanted parts were deleted. Images were transformed into black and white images to quantify the area of the wound in pixels. The number of pixels belonging to the wound area was computed after counting the pixels corresponding to the white regions in contact with each image. Thus, the average daily closure rates of scar tissues were calculated as pixels. The wound closure rate was calculated according to the following equation:

The daily rate of wound closure = Cumulative pixel value / Day of wound closure

Wound Management: *Hypericum* perforatum oil (macerated in natural extra virgin olive oil), natural extra virgin olive oil (cold-pressed), and *Nigella* sativa oil (cold-pressed) were purchased from a commercial company (Zade Vital, Konya, Turkey) which holds product certificates and has an organic production permit. Wound creams *Centella* asiatica (titrated extract, 1% Madecassol Cream®, Bayer, Umraniye, Istanbul, Turkey) and fusidic acid (2% Furacin Cream®, Zentiva, Luleburgaz, Turkey) were obtained from companies that hold a license for medical use. Physiological saline (NSS) 0.9% isotonic sodium chloride solution was purchased from a company (Polifarma, Tekirdag, Turkey).

Treatments according to treatment groups were carried out every day until each mouse's wound healing was completed. The tip of the cotton swab was first dipped in the active ingredient and then applied to the wounds on the skin surface of the mice at one time until coating the wound surface, and cotton swabs were discarded in every mouse and application.

Statistical Analyses: A p-value equal to or less than 0.05 was considered as a statistically significant difference to reject the null hypothesis. Statistical analyses were performed using the SPSS®

22.0 (Armonk, NY, USA) program. The Shapiro-Wilk test was used to determine the distribution of the obtained data, and the Levene test was used to evaluate the homogeneity of the variances. Two-way repeated-measures ANOVA was first conducted to determine whether interventions were significant on hydroxyproline concentration, wound closure rate, wound pixel values, body weights, and blood glucose levels. General linear model (GLM) procedures were conducted for *post hoc* Duncan multiple comparisons when the treatments were significant. The results are presented as mean±standard error of the mean.

Results

No mortality or infection was observed in treatment groups. According to treatment groups (mean±standard error of the mean), wound healing days are given in Table 1. Body weights and blood glucose levels of mice (mean±standard error of the mean) at the beginning and end of the experiment are given in Table 2.

Wound Closure: Pictures from the wound healing period and the rate of wound closures are given in Figure 2, Figure 3, and Table 1, respectively. Based on the photographic follow-up, the daily rate of wound closure between NS (707.74±82.28 pixels) with OL (904.78±111.38 pixels) groups was statistically significant (P<0.05). There was a statistically significant difference between NS and OL groups on the first day of the experiment (P<0.05, Figure 3). However, all other comparisons on the rest of the experiment days among treatment groups were not statistically significant (P>0.05, Figure 3). The closure time of wound healing was not significantly different among other treatment groups (P>0.05, Table 1, Figure 3).

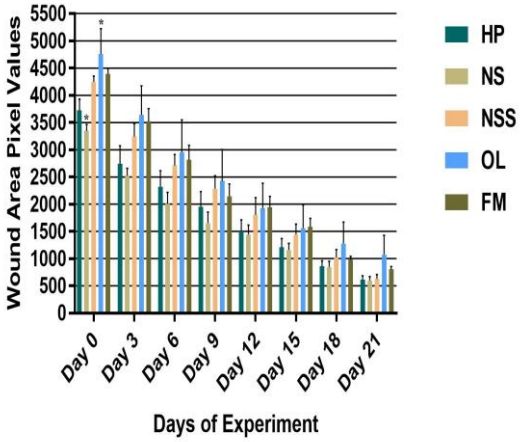

Figure 3. Treatment groups' mean wound area pixel values by days of treatment with standard error of the mean (SEM) bars.
* Indicates a statistically significant difference between groups. HP: *Hypericum* perforatum (St. John's Wort), NS: *Nigella* sativa (Black cumin oil), NSS: Normal saline solution (0.9% NaCl), OL: Natural extra virgin olive oil, FM: 2% fusidic acid+*Centella* asiatica cream.

Hydroxyproline Biochemistry Assays: Percentage changes in hydroxyproline levels are shown in Table 1. Changes in hydroxyproline levels were significantly higher in groups HP and NSS than in other treatment groups (P<0.05).

Table 1. The effect of topical application of treatments on wound size in pixels and hydroxyproline change rate per day throughout the experiment.

Groups	The daily rate of wound closure (pixel)	Hydroxyproline change rate throughout the experiment (%)	Day of wound healing
	Mean±SEM	Mean±SEM	Mean±SEM
HP	676.88±64.3$^{a, b}$	108.57±6.14^{a}	21±0
NS	707.74±82.28^{a}	67.10±9.10^{b}	19.2±0.73
NSS	908.37±182.63$^{a, b}$	127.56±1.36^{a}	18±1.34
OL	904.78±111.38^{b}	63.00±0.98^{b}	20.4±0.60
FM	826.07±61.88$^{a, b}$	83.59±7.41^{b}	21±0

a,b,c: Different superscripts are indicating statistical differences (P<0.05), SEM: Standard error of the mean, HP: *Hypericum* perforatum (St. John's Wort), NS: *Nigella* sativa (Black cumin oil), NSS: Normal saline solution (0.9% NaCl), OL: Natural extra virgin olive oil, FM: 2% fusidic acid+*Centella* asiatica cream.

Discussion

The OL group was included in the study to differentiate the effect of Hypericum *perforatum* clearer and more distinct. FM was also chosen as a control group since it represents common wound creams. Unlike acute wounds, diabetic wounds exhibit a variety of molecular abnormalities in the healing process, including fibroblast and keratinocyte dysfunction (Lerman et al., 2013), angiogenesis deficiency (Loomans et al., 2004), and phagocytic activity failure (Khanna et al., 2010). Since ancient times, herbal medicines have been used to treat various ailments (Sharma et al., 2021).

NS extracts have been shown to have various therapeutic effects, including anti-inflammatory, antibacterial, antidiabetic, and antitumor effects (Khader and Eckl, 2014; Yaman et al., 2010). Wound healing has also been aided by HP's antispasmodic and antiseptic properties (Sharma et al., 2021; Suntar et al., 2010). In the present study, there was a statistically significant difference between NS and OL groups on the first day of the treatment ($P<0.05$). In contrast, there was no statistically significant difference in wound area pixel values between treatment groups on the rest of the experiment ($P>0.05$). However, a statistically significant difference was found in the wound closure rate between the NS and OL groups ($P<0.05$). Whereas hydroxyproline changes through the study were significantly higher in groups HP and NSS compared to other groups ($P<0.05$).

Table 2. Glucose levels and body weights of treatment groups at the beginning and end of the experiment.

Groups	Glucose levels (mg/dL) (Day 0)	Glucose levels (mg/dL) (Day 21)	Body weights (g) (Day 0)	Body weights (g) (Day 21)
	(Mean±SEM)	(Mean±SEM)	(Mean±SEM)	(Mean±SEM)
HP	402.4±17.1	424±40.49	39.6±1.21	37±1.48
NS	469.2±31.35	515.6±36.53	42±0.99	40±1.31
NSS	401.4±16.83	432.8±46.38	42.2±1.02	41.6±0.98
OL	385±26.73	431.2±24.17	41.2±0.77	39±1.92
FM	463.4±24.2	505±25.6	43.4±0.51	41.2±0.58

SEM: Standard error of the mean, HP: *Hypericum* perforatum (St. John's Wort), NS: *Nigella* sativa (Black cumin oil), NSS: Normal saline solution (0.9% NaCl), OL: Natural extra virgin olive oil, FM: 2% fusidic acid+*Centella* asiatica cream.

Hydroxyproline is an amino acid almost exclusively confined to collagen. The hydroxyproline content of wound tissues is often measured to estimate the amount of collagen contained in the wound bed (Gao et al., 2006). Paheerathan et al. (2017) found that *Nigella* sativa (NS) seed powder has a significant accelerating wound healing activity in an incised wound model (Paheerthan et al., 2017). Sari et al. (2018) reported that *Nigella* sativa oil gel reduced inflammation and enhanced reepithelialization and granulation tissue development in diabetic wounds. In the present study, the increase in hydroxyproline of groups NSS and HP suggests that the collagen synthesis in mice that received HP and NSS was higher than NS and the other groups (Table 1). Wounds and burns should be kept moist during the recovery and treatment for optimum healing to ensure faster epithelialization, increased angiogenesis, and collagen synthesis compared to a dry environment. A moist wound condition often promotes the degradation of dead tissue and fibrin, resulting in less scarring (Junker et al., 2013). In the present study, topical application of standard saline solution seems to be the superior treatment for hydroxyproline change and wound closure rate (Table 1). Our findings might be interpreted as the pH, and hydrostatic pressure of the applied treatments higher or lower than healthy skin condition was speculated to influence the treatment's outcome (Lim et al., 2000; Percival et al., 2014). As a limitation in our study, osmolalities, pH, and hydrostatic pressure of the present topical treatments were not evaluated, which might be considered confounding. In future studies, osmolality, pH, and hydrostatic pressure of topical agents should be investigated to determine their effects on wound treatments.

Conclusion

Topical application of *Nigella* sativa and *Hypericum* perforatum oils did not accelerate wound healing in type-1 diabetic wound conditions in mice. Also, the present findings illustrated that the collagen synthesis and daily wound closure rate of *Nigella* sativa oil were higher than *Hypericum* perforatum oil. However, topical applied standard saline solution was the superior treatment in the present results. Further research considering osmolality, pH, and hydrostatic pressure in wound healing would be beneficial to reveal the effect of alternative topical wound healing agents.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval

This study including animal experiments and protocols was approved by the Institutional Animal Care and Ethical Committee of Aydin Adnan Menderes University (ADU-HADYEK, approval number #64583101.2017.051). In addition, the authors declared that Research and Publication Ethical rules were followed.

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Author Contributions

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Design: IA, CA
Control/Audit: IA, YK, OC, AGU
Data Collection and/or Processing: IA, YAO, CA
Analysis and/or Interpretation: YAO, CA
Literature Review: IA, YAO, CA, YK, AGU
Critical Review: IA, YAO, CA, YK, OC, AGU

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