TOPICAL *HELICHRYSUM ITALICUM* ESSENTIAL OIL-BASED OINTMENT HASTENS EXCISIONAL WOUND HEALING BY ALLEVIATING INFLAMMATION AND OXIDATIVE STRESS IN DIABETIC RATS

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ABSTRACT

This study aimed to develop novel topical semi-solid formulation based on Helichrysum italicum (H. italicum) essential oil and to assess its wound-healing capacity through macroscopic and biochemical examination of markers of inflammation and oxidative stress. Methods: The wound-healing effect was evaluated on an excision wound model. The study was carried out on male Wistar albino rats with induced diabetes type 1. Diabetic rats were randomly divided into four groups: control (CTRL), 1% silver sulfadiazine (SSD), ointment base (OINT), and HIEO OINT (H. italicum essential oil-based ointment). The investigated formulations were applied once daily, for three weeks. The formulation containing H. italicum essential oil has shown no changes in color, smell, consistency, or homogeneity during the storage period at room temperature for six months. Three-week administration of HIEO ointment led to a significant reduction in wound size and the percentage of wound contraction was the highest in HIOE point group. A significant increase in wound contraction percentage was observed from day 7. The treatment with HIEO ointment significantly reduced tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), and enhanced IL-10 levels. Additionally, a three-week administration of HIEO ointment elevated antioxidative enzymes detected in wound tissue. Our findings showed that topical application of HIEO ointment showed immense potential in augmenting skin wound regeneration in diabetes rats by upregulating the antioxidant status as well as modulating cytokines.

Keywords: *Helichrysum italicum, essential oil, wound, inflammation, oxidative stress.*

INTRODUCTION

Wound healing is the body's natural response to tissue injury. It represents a complex and dynamic biological process consisting of integrated cellular and biochemical cascades, which lead to the re-establishment of the structural and functional integrity of the damaged tissue (1). The healing process consists of four temporally and spatially overlapping phases: hemostasis, inflammation, proliferation, and remodeling (2, 3). Different factors are associated with a compromised capacity to promote the regeneration of damaged tissue and, consequently delayed wound healing. Factors affecting the healing process can be categorized into local and systemic. Local factors are factors that directly of the affect the characteristics wound, while systemic factors represent the individual's health condition, which affects the ability to heal. There is an association of several factors and systemic factors act through local effects that affect wound healing (4).

One of the major contributors to delayed and consequently chronic wound healing problems is diabetes mellitus (DM). Prolonged wound healing in patients with DM occurs due to a delayed, incomplete and uncoordinated healing process, which permeates all phases, from inflammation to remodeling (5). As a result, people with diabetes can develop a variety of complications, including chronic wounds that do not heal, such as foot ulcers (6).

Considering the huge impact of chronic diabetic wounds on the economy and society, there is an urgent need to develop new therapeutic strategies that can contribute to proper wound healing and exhibit as few side effects as possible. Although various therapeutic protocols are available for wound healing, including antibiotics, antiseptics, and anti-inflammatory agents, medicinal herbs and natural products have been the main source of wound healing agents in recent years. The significant increase in the use of medicinal plants is the result of greater safety compared to synthetic drugs, but also more affordable prices (7).

Helichrysum italicum (H. italicum) is a perennial, aromatic plant belonging to the *Asteraceae* family. *H. italicum*, commonly known as "immortelle," is renowned for its therapeutic properties and has been traditionally used in folk medicine for its wound-healing abilities (8, 9). The essential oil derived from *H. italicum* contains a complex mixture of bioactive molecules, including monoterpenes and sesquiterpenes, which have demonstrated potent anti-inflammatory, antioxidant, and antimicrobial actions (10, 11). These properties make *H. italicum* essential oil a promising ingredient for topical preparations developed to enhance wound healing efficacy, particularly in diabetic conditions where inflammation and oxidative stress are heightened.

Therefore, this investigation was designed to develop and characterize *H. italicum* essential oil-based topical semi-solid dosage form and evaluate wound-healing properties by performing a biochemical analysis in diabetic animal model.

MATERIALS AND METHODS

H. italicum essential oil obtaining

H. italicum essential oil was purchased from "A-lekpharm", Belgrade, Serbia. This commercial essential oil was obtained by hydrodistillation from flowering parts of *H. italicum*.

Composition and preparation of semi-solid product: ointment

For the formulation of the ointment, as a semi-solid pharmaceutical dosage form, cholesterol, lanolin, paraffinum liquidum, and vaselinum album were used to form an ointment base. The *H. italicum* essential oil-based ointment was prepared by incorporating 0.5% (w/w) essential oil *H. italicum* into the ointment base by levigation method (12). The formulation is presented in Table 1.

Table 1. Ointment base and *H. italicum* essential oil –based ointment formulations.

Ingredient	Ointment Base (%)	Ointment HIEO (%)
HIEO	/	0.50
Cholesterol	5.00	5.00
Lanolin	15.00	15.00
Parafinum liquidum	15.00	15.00
Vaselinum album	65.00	ad 65.00

HIEO – *H. italicum* essential oil

Assessment of physical properties of ointment containing *H. italicum* essential oil

Organoleptic properties

Organoleptic properties, which include examination of the appearance, odour, color, consistency, homogeneity and spreadability of the prepared ointment with incorporated *H. italicum* essential oil, were assessed by visual observation. Homogeneity was tested by pressing a small amount of the formulation between the thumb and forefinger (13). Spreadability was assessed by applying preparation in circular motions on the skin and visually monitoring the trace that remains on the skin.

Assessment of long-term stability of the H. italicum essential oil-based ointment

The long-term stability of the *H. italicum* essential oil-based ointment was assessed by determination of organoleptic characteristics and spreadability during the storage period. The ointment was stored at room temperature (22° ± 2°C) for 6 months and the sampling was conducted after 6 months of ointment storage.

Wound healing effects of ointment with *H. italicum* essential oil in diabetic rats

Ethical statement

This investigation was conducted in the Laboratory for pharmaceutical technology and Center for experimental and preclinical investigations of the Faculty of Medical Sciences, University of Kragujevac, Serbia. The part of study involving animals was performed in accordance with the the European Directive for Protection of the Vertebrate Animals used for Experimental and Other Scientific Purposes 86/609/EES and the principles of Good Laboratory Practice. Ethical committee for the welfare of laboratory animals of the Faculty of medical sciences, University of Kragujevac was approved the experimental protocol (Number 01-6292).

Animals

Male *Wistar albino* rats (280–350 g) were obtained from the Military Medical Academy, Belgrade, Serbia and housed in clean cages under adjusted conditions (12:12 h light–dark cycle, at a temperature of 22 ± 2 °C). The rats had ad libitum access to water and food.

Diabetes mellitus induction

Diabetes mellitus type 1 was induced with a single injection of streptozotocin in a dose of 50 mg/kg. Streptozotocin was dissolved in 1 ml of 0.05 M freshly prepared citrate buffer solution (pH 4.5) and administrated by intraperitoneal injection after a 12 h starvation (14). Seventy-two hours after streptozotocin administration diabetes was confirmed by measuring tail-veinous blood glucose level using a portable glucometer. Rats with blood glucose levels >11.1 mmol/l were included in the investigation.

Excision wound model

One week after confirmation of diabetes type 1, excision wounds were created (15). First of all, the rats were anesthetized by intraperitoneal injection of the mixture of ketamine and xylazine in a dose of 5 mg/kg and 10 mg/kg respectively. The dorsal of animals were shaved, disinfected with 70% ethanol and wounds were created by scissors and a scalpel. The dimension of the wound was 2x2 cm.

Groups and treatment

Immediately after the creation of excision wound, animals were photographed and placed in individual cages. Animals were randomly divided into the following groups (n = 8 per group):

1. Negative control group - animals without treatment (CTRL);
2. Positive control group - animals treated with a commercially available ointment 1% silver sulfadiazine (SSD);
3. Animals treated with an ointment base (OINT);
4. Animals treated with a 0.5% *H. italicum* essential oil based ointment (HIOINT).

The formulations were applied once daily in an amount of 0.5 g by sterile cotton for three weeks (16). At the end of the experimental protocol, the rats were anesthetized with ketamine and xylazine mixture and sacrificed by decapitation.

Wound contraction

The evolution of the wounds was followed every one week by measuring the areas in the treated animals (in cm^2). The percentage of wound contraction was calculated for each animal on various days by using following formula (17):

% Wound contraction = [(Initial WA – Specific day WA) /Initial WA] * 100;
where is: WA – wound area

Tissue collection

After sacrifice, samples of the rat's skin were isolated and stored at -80°C for further biochemical analysis. Wound tissues were taken to estimate the oxidative status and markers of inflammation.

Markers of inflammation

A sample of skin tissue (100 mg) was homogenized with 0.5 mL of cell lysis buffer. The obtained homogenates were centrifuged at 5000 rpm, for 10 minutes, at a temperature of 4°C. The obtained supernatants were stored at a temperature of -70°C.

Determination of the concentration of inflammation markers TNF-α, IL-6, and IL-10 in the supernatant obtained from skin tissue was carried out via ELISA kit according to the instructions.

Markers of oxidative stress

The wound tissue sample was homogenized in cold PBS (phosphate-buffered saline) (1:10). The obtained homogenates were centrifuged at 1200 × g for 10 minutes at a temperature of 4°C and the obtained supernatants were stored at a temperature of -70°C.

The parameters of the tissue redox state were measured from obtained supernatants according to previously described protocol (18). The supernatant was used for the determination of the pro-oxidative parameter index of lipid peroxidation (measured as TBARS). Additionally, we determined the parameters of the antioxidative defense system such as level of reduced glutathione (GSH), and the activity of antioxidant enzymes: catalase (CAT), and superoxide dismutase (SOD).

Statistical analysis

IBM SPSS Statistics 20.0 Desktop for Windows was used for statistical analysis. All data were expressed as mean ±

standard deviation. The distribution of data was assessed by the Shapiro–Wilk test. For the normal distribution, the data were assessed via a one-way ANOVA followed by Tukey's multiple comparison post hoc tests. When the distribution was different from than normal comparison between groups was assessed by the Kruskal–Wallis test. A p-value < 0.05 was considered statistically significant.

RESULTS

Organoleptic characteristics

Physico-chemical characterization of the ointment with incorporated *H. italicum* essential oil was carried out by organoleptic testing and spreadability testing. Organoleptic examination revealed that the formulation with *H. italicum* essential oil has a characteristic appearance of homogeneous ointment, without lumps, yellow color and intense odor. The preparation has a fine, uniform, semi-solid consistency (Figure 1). In terms of spreadability, it was observed that the examined ointment spreads well on the skin. During the storage period at room temperature for six months, no changes in color, smell, consistency, or homogeneity of the ointment were observed (Table 2).

Table 2. Organoleptic characteristics and spreadability of ointment with *H. italicum* essential oil after 3 and 180 days of preparation

Parameters	***3 days***	***180 days***
Colour	Yellow	Yellow
Odour	Characteristic odor of the essential oil	Characteristic odor of the essential oil
Consistency	Semi-solid	Semi-solid
Homogenity	Homogeneous ointment	Homogeneous ointment
Spreadability	Easily	Easily

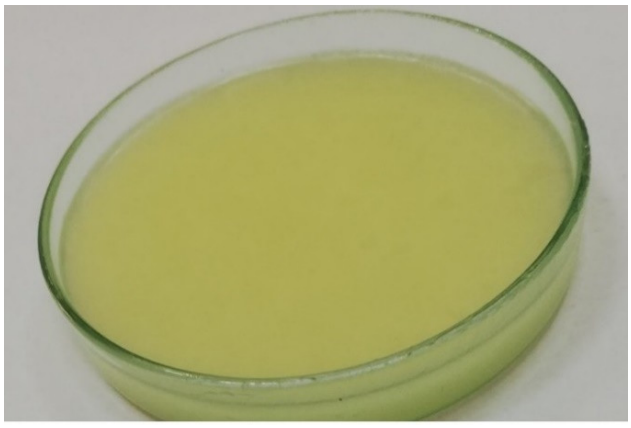


Figure 1. Photograph of *H. italicum* essential oil – based ointment

Wound healing effects

Wound contraction

The progress of wound-healing contraction, examined over the three weeks is shown in Figure 2. The results have shown significant differences in the wound closure rate between the group treated with *H. italicum* ointment and the other examined groups. Therefore, these data indicated that *H. italicum* essential oil formulated in the form of ointment possesses wound healing activity. The results of wound contraction, represented in Figure 2 point out that topical application of *H. italicum* ointment resulted in a significant increase in the percent of wound contraction compared to the control group, on all key days of the duration of the experiment. Additionally, three-week administration of *H. italicum* ointment resulted in a significant increase in wound contraction compared to the group treated with an ointment base. On the other side, the wound contraction in the group was slightly higher compared to the SSD group, but not significantly.

Markers of inflammation

The concentrations of pro-inflammatory markers TNF-α and IL-6, as well as anti-inflammatory IL-10, in wound tissue homogenate are shown in **Figure 3**. The highest concentration of the pro-inflammatory mediator TNF-α was detected in the control group. Topical administration of ointments has caused a significant reduction in the concentration of this parameter in all observed groups compared to the control group (**Figure 3A**).

At the same time, the three-week application of H. italicum essential oil-based ointment significantly reduced the concentration of IL-6 compared to the control and ointment groups. On the other side, the concentration of IL-6 was almost identical compared to the SSD group (**Figure 3B**).

Additionally, the statistically highest concentration of the anti-inflammatory marker, IL-10, was observed in the group of animals treated with the H. italicum essential oil-based ointment (**Figure 3C**).

Markers of oxidative stress

The results of the oxidative status in the wound tissue homogenate created by the excision method are shown in **Figure 4**. Topical treatment with H. italicum essential oil-based ointment significantly increased the activity of SOD relative to all examined groups. On the other hand, the activity of CAT was remarkably increased in the SSD group, comparing the values of this enzyme in groups treated with vehicle or H. italicum ointment. Neither treatment made a difference in the level of GSH and TBARS.

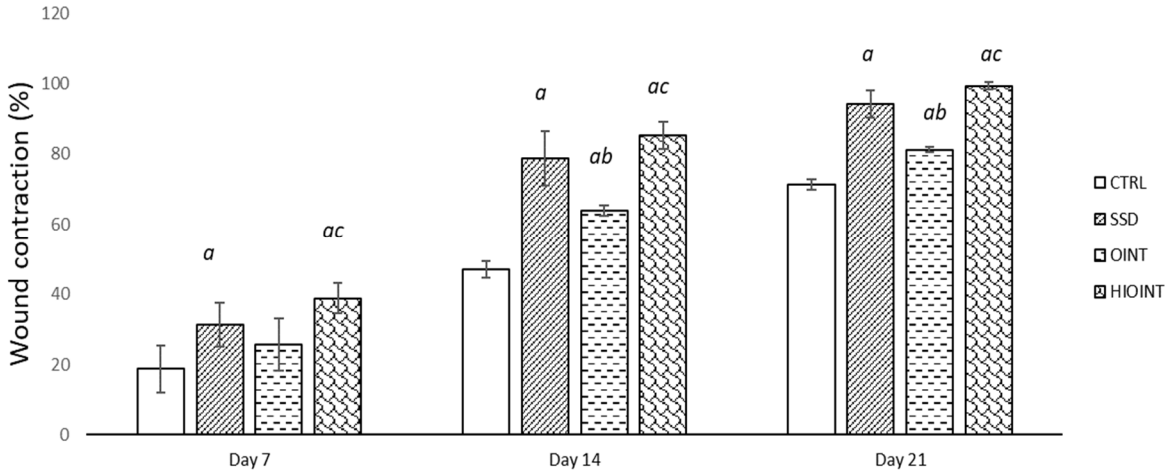


Figure 2. Effects of applied ointments on the wound contraction.
Values are expressed as mean ± standard deviation (n = 8). [a] $p < 0.05$ compared to CTRL group;
[b] $p < 0.05$ compared to SSD group; [c] $p < 0.05$ compared to OINT group;
[d] $p < 0.05$ compared to HIOINT group. CTRL –control; SSD – silver sulfadiazine;
OINT – ointment base;
HIOINT – *H. italicum* ointment;

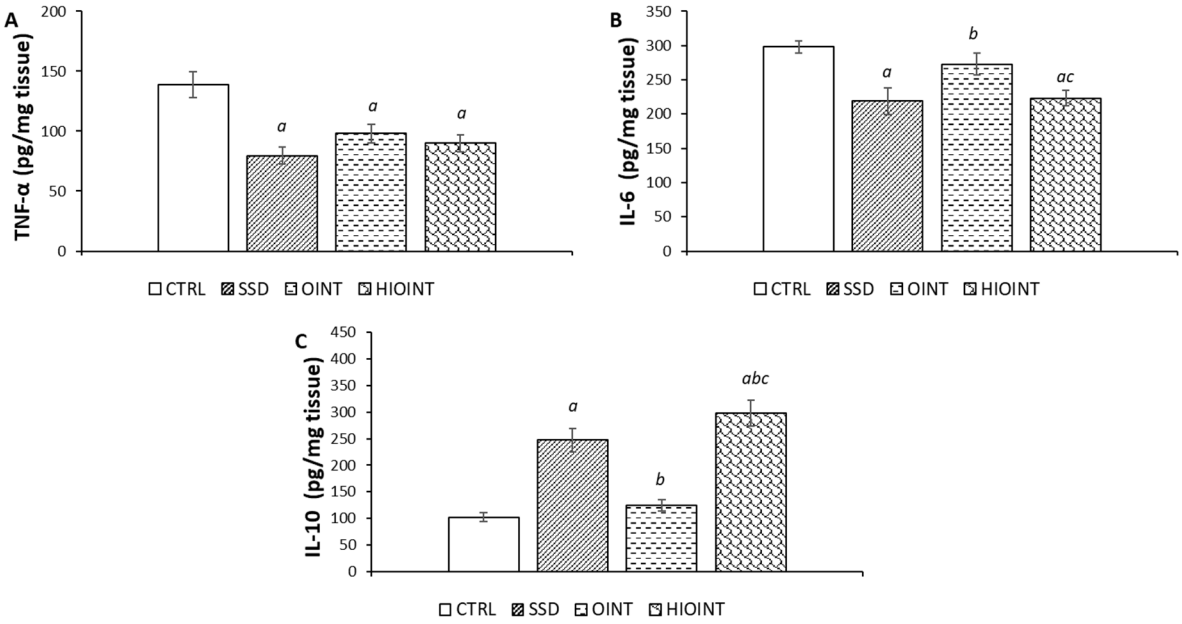


Figure 3. Effects of applied ointments on the markers inflammation:
A) TNF-α; B) IL-6; C) IL-10. Values are expressed as mean ± standard deviation (n = 8).
[a] $p < 0.05$ compared to CTRL group; [b] $p < 0.05$ compared to SSD group; [c] $p < 0.05$ compared to OINT group;
[d] $p < 0.05$ compared to HIOINT group. CTRL –control; SSD – silver sulfadiazine;
OINT – ointment base; HIOINT – *H. italicum* ointment;

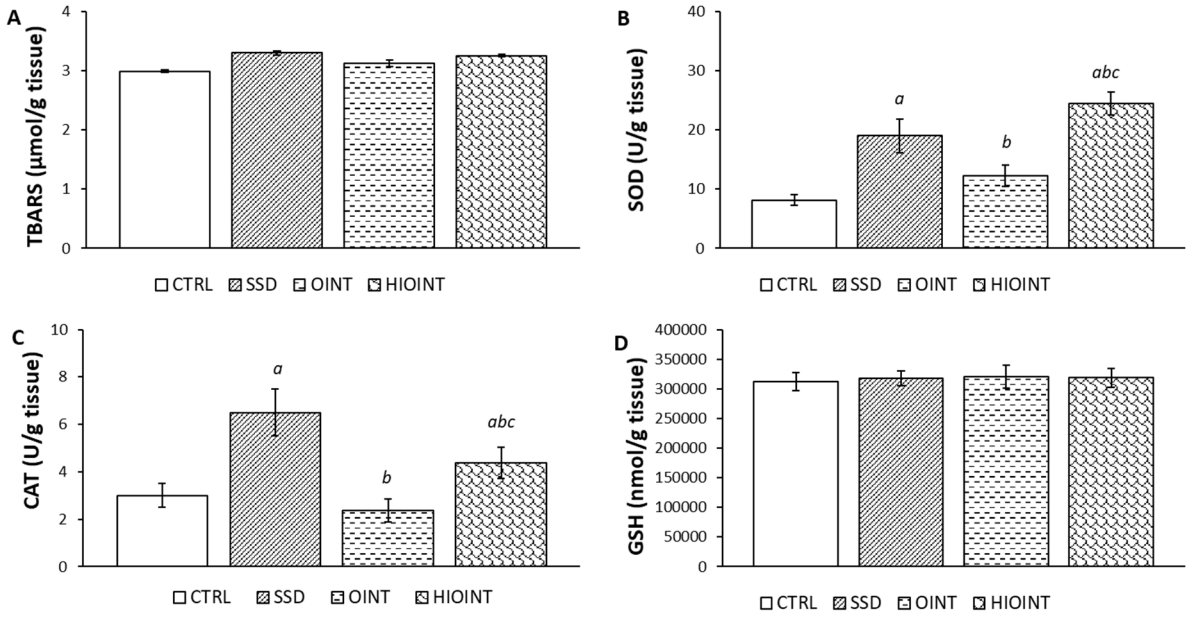


Figure 4. Effects of applied ointments on the oxidative stress markers in wound tissue:
A) TBARS; B) SOD; C) CAT; D) GSH. Values are expressed as mean ± standard deviation (n = 8).
[a] $p < 0.05$ compared to CTRL group; [b] $p < 0.05$ compared to SSD group; [c] $p < 0.05$ compared to OINT group;
[d] $p < 0.05$ compared to HIOINT group. CTRL –control; SSD – silver sulfadiazine;
OINT – ointment base; HIOINT – *H. italicum* ointment;

DISCUSSION

Over the years, the use of synthetic preparations has been in drastic decline, primarily due to their close association with the occurrence of serious side effects. As a result, their unsafe use has enabled preparations based on natural products to regain their popularity and gain great interest from the scientific public in the prevention and treatment of various conditions. Although the wound-healing activity of immortelle extracts and their ingredients is well known, there is almost no data on the effects of different topical preparations based on immortelle essential oil. Accordingly, the first part of this investigation was to formulate semi-solid topical preparation containing *H. italicum* essential oil and to determine its stability during six months of storage on the room conditions.

Literature data suggest that a concentration of immortelle essential oil of 0.5% is optimal and suitable for topical application and that its increase is not recommended due to the possibility of skin irritation (19). Accordingly, immortelle essential oil was incorporated in a concentration of 0.5% into the ointment base to obtain immortelle-based ointment. In order to assess the physical stability of the prepared semi-solid formulation, the ointment sample was stored for six months at a room temperature of 22 ± 0.1ºC, the parameters of interest were monitored in the following time points: at the beginning and end of the storage period.

Within the organoleptic characteristics, changes in color, smell, consistency and homogeneity were monitored over a period of six months. The results of the six-month follow-up indicate that there were no changes in the organoleptic properties of the tested formulation. From the aspect of physical stability, the absence of change in color, smell, consistency and homogeneity is an indicator of the preliminary stability of the investigated immortelle ointment.

Determining spreadability, as an index of ease of application, is one of the necessary methods before placing the preparation on the market, considering that it is necessary for semi-solid products for local application to be easily applied to the surface of the skin. Spreadability greatly affects the effectiveness of topical therapy and is an important characteristic responsible for the delivery of the active substance to the target site, ease of application and most importantly, the needs of the user (20). So, we monitored the spreadability of our preparations over time, which remained unchanged, which is an additional confirmation of the stability of semi-solid preparation based on immortelle. Bearing in mind the percentage of immortelle essential oil in the mentioned formulation, it can be concluded that the ability to spread is mainly influenced by the substrates used for the production of ointments.

After confirmation of the stability of H. *italicum* essential oil-based ointment, we investigated the effects of developed ointment on the wound-healing process in diabetic rats with special emphasis on the markers of inflammation and oxidative stress in wound tissue.

The most important factors in the wound-healing process are the contraction and epithelization of the wound (21), therefore monitoring these parameters is of key importance in assessing the wound-healing potential of a different formulation. The results of our research showed that the percentage of wound contraction was significantly higher in the group of animals treated with preparation based on immortelle essential oil. The impressive effect of the immortale ointment was observed from the first moment, more precisely after seven days of application and was recorded until the last day of the experimental period. Therefore, the given results indicate a shorter re-epithelialization time in the groups treated with essential oil compared to the other groups. The potential for wound healing may be attributed to the presence of terpenes known for their astringent and antimicrobial properties, which contribute to enhanced wound contraction and promote epithelialization.

The inflammation phase plays an important role in the healing process, which is necessary for proper wound healing. The pro-inflammatory cytokines IL-6 and TNF-α are involved in cell differentiation and proliferation, coordinating granulation tissue synthesis, angiogenesis, reepithelialization, and collagen remodeling mechanisms (22). Moreover, these cytokines enhance the migration and proliferation of leukocytes in the wound, enhancing the removal of necrotic tissue and the phagocytosis of antigens (23). IL-10 is another interleukin involved in the inflammatory response in wounds, which acts as an anti-inflammatory mediator, by inhibiting the synthesis of pro-inflammatory cytokines and plays a role in angiogenesis (24). However, an imbalance between inflammatory cytokines can lead to a chronic inflammatory process, which results in the disruption and delay of the healing process (25). The results of cytokine quantification in the tissue homogenate isolated from the wound area, previously caused by the excision method, showed that the anti-inflammatory activity of topical formulation based on immortelle essential oil in skin wounds is a consequence of the reduction of IL-6 and TNF-α levels. In addition, treatment with preparation based on immortelle increased the activity of IL-10, which reduces the expression of pro-inflammatory cytokines, as well as scar formation. Therefore, immortelle ointment achieved anti-inflammatory potential similar to the standard, silver sulfadiazine. Considering the concentration of inflammation markers detected in the tissue sampled at the end of the experimental period, it can be noticed that immortelle essential oil achieved the given anti-inflammatory activity in the remodeling phase. Our results are consistent with literature data, which shows the anti-inflammatory activity of immortelle essential oil. Previous research has shown that the inflammatory phase restores homeostasis, and that in the proliferative phase, fibroblasts and other cells from the connective tissue infiltrate the wound site and secrete cytokines, attract keratinocytes, and lead to re-epithelialization (26). Preventing prolonged inflammation by suppressing the production of inflammatory cytokines is a desirable target for wound healing products, whereas excessive inflammation leads to the development of chronic wounds and scarring (27). Modulation of cytokine production by topical formulations based on immortelle essential oil indicates anti-inflammatory activity, which supports the previously described results of the essential oil itself and its dominant ingredients.

Disruption of the wound healing process is also associated with oxidative stress, which occurs due to an imbalance in the generation of reactive oxygen species (ROS) and the endogenous antioxidant defense mechanism. Essential oxidants, such as ROS, are generated in various physiological processes. Their effects on the wound healing process are concentration dependent, generally showing positive effects as secondary transporters at low concentrations. However, at high concentrations they damage DNA, proteins and lipids in cells causing toxicity. In diabetic patients, oxidative stress caused by hyperglycemia adversely affects the wound healing process and accordingly, herbal products with antioxidant activity may be useful agents for re-establishing anatomical and cellular tissue continuity (28). Numerous studies have shown that at the site of skin wounds, the concentration of ROS increases and the production of antioxidants decreases, which leads to delayed wound healing (29). In the inflammatory phase, neutrophils and macrophages produce large amounts of ROS, which directly attack pathogens, but also damage healthy surrounding tissues. Inhibition of ROS production triggers angiogenesis and fibroblast proliferation, stimulating skin wound closure. The results of a three-weeks administration of topical formulation indicate that rats treated with immortelle essential oil formulation showed a significant increase in CAT and SOD activity. The increase in the activity of antioxidant protection enzymes, achieved by immortelle formulation, indicate that the reduction of oxidative stress is one of the mechanisms responsible for the achieved effects in the wound healing process.

The limitations of our investigation include the fact that biochemical analysis of the measurement of oxidative stress and inflammation parameters was conducted only on the last day of the treatment protocol. To provide comprehensive and accurate findings regarding the formulation's impact on specific phases of the healing process, these examinations should be performed at multiple time points throughout the 21-day protocol. Evaluating different time points would illuminate the varying effects of H. italicum ointment on each stage of wound healing.

CONCLUSION

The results of the six-month follow-up indicate that there were no changes in the organoleptic properties of the tested formulation, what demonstrated the stability of immortelle ointment during six months storage under room temperature conditions. Additionally, the our investigation reveal that

immortelle ointment treatment accelerated the wound healing by modulating the expression of cytokines TNF-α, IL-6 and IL-10 as well as elevating the secretion of antioxidative markers.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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