Original Article

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Therapeutic Effect of *Zataria Multiflora* Essential Oil on Burn Wound Infected by *Pseudomonas aeruginosa*

Maryam Zare[1,2];MSc, Elahe Sattarrinezhad[3];Ph.D, Ali Karimi Akhormeh[1,4];MSc, Maryam Motevasel[5*];Ph.D, Marzieh Ashrafmansouri[5];Ph.D, Maral Mokhtari[6];Ph.D, Omid Koohi Hosseinabadi[7];MSc, Aida Iraji[7];Ph.D, Azar Purkhosrow[3];MSc

[1]Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Canada
[3]Department of Pharmacology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[4]Cardiovascular Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[5]Department of Medical Laboratory Sciences, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
[6]Department of Pathology, Shiraz University of Medical Sciences, Shiraz, Iran
[7]Central Research Laboratory, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the most important microorganisms causing burn wound infection. Due to the rapid increase in resistance to the currently used antimicrobial agents, finding new antibiotics is one of the research priorities. This study was designed to investigate antibacterial and wound healing effects of *Zataria multiflora* essential oil (ZMEO) on burn wounds infected with *P. aeruginosa* in Sprague-Dawley rats. Experimental burn wounds were created on the back of the animals and infected with *P. aeruginosa*. The animals were randomly divided into 4 groups of 7 to 10 as follow: negative control (no treatment), carrier gel group treated with carboxymethyl cellulose (CMC) gel, ZMEO group that received CMC gel loaded with ZMEO, and positive control group that received silver sulfadiazine (SSD). All medications were applied topically once daily for 28 days. On the days 7th, 14th, 21st, and 28th after the start of treatments, the surfaces of the wounds were measured and some samples were collected for histopathological evaluations. The tensile strengths of rats' skins were also measured on the 28th day. The results showed that on the 7th day, while a significant healing of the wounds was observed in the ZMEO group, the other groups did not show remarkable wound healing ($P<0.05$). Therefore, ZMEO showed an accelerating effect on the healing process of burn wounds and could be considered for further evaluations in order to develop new medications for the treatment of burn wounds infected with *P. aeruginosa.*

Keywords: *Pseudomonas aeruginosa*, burn wound, *Zataria multiflora*

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1. Introduction

Wound infection is a constant concern in hospital burn care units. Burn wounds provide favorable environments for the growth of various types of pathogenic bacteria and may lead to sepsis which is associated with high mortality (1). Among the pathogenic microorganisms in this

Corresponding Author: Maryam Motevasel, Department of Medical Laboratory Sciences, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran
Email address: motevaselm10@gmail.com

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field, *P. aeruginosa* is one of the most common hospital infectious bacteria that accounts for about 50% of burn wound infections (2, 3).

Despite the development of numerous and diverse anti-pseudomonas antibiotics, unfortunately, the rate of drug resistance is increasing rapidly. Recent studies show that approximately 80% of clinical isolates of *P. aeruginosa* demonstrate resistance to various antibiotics (4). In this regard, the use of traditional herbal medicines that have antimicrobial properties can be a promising solution. *Zataria multiflora* (*Z. multiflora*), a thyme-like plant from the Lamiaceae family, could be considered as a potential wound dressing (5). *Z. multiflora* has shown antibacterial properties in both lab and animal studies (6). Various components of *Z. multiflora*, such as phenolic compounds, carvacrol, and thymol have been shown to disrupt the bacterial cell wall and inhibit the growth of pathogenic bacteria through different mechanisms (7). Additionally, *Z. multiflora* stimulates innate immune system responses (8) which can expedite the wound healing process by reducing the microbial load. Although many studies have been conducted on the antibacterial effects of this vegetable oil, few researches have evaluated its therapeutic effects on infected burn wounds caused by *P. aeruginosa*. Therefore, we designed this study with the aim of investigating the antimicrobial and wound healing properties of ZMEO in a rat model of burn wound infected with *P. aeruginosa*.

2. Materials and Methods

2.1. Animals

Male Sprague Dawley rats weighing 180±10 g were housed in standard cages under a 12-hour light/dark cycle, at a temperature of 20-25 °C and humidity of 25-35%. Food and water were provided ad libitum. All experimental procedures adhered to the guidelines set forth by the Institutional Ethics Committee for Animal Care and Use at Shiraz University of Medical Sciences (Ethical code number: IR.SUMS.REC.1392.6106).

2.2. Materials

Zataria multiflora essential oil was purchased from Barij Essence Company (Iran). Silver sulfadiazine cream (1%) was purchased from Sobhan Darou Company (Iran). Carboxymethyl cellulose was purchased from Sigma Company (USA).

2.3. Methods

2.3.1. Preparation of infectious bacterial inoculum

Three young colony of *P. aeruginosa* (ATCC 27853) were cultured in Mueller Hinton Broth and aerobically incubated at 35 ºC for 18 hours for reaching to logarithmic growth phase and preparing 1.5×10^8 CFU/mL (9).

2.3.2. GC-MS analysis of ZMEO

The essential oil was analyzed using an Agilent 7890A GC instrument coupled with a Triple Quad MS detector (Agilent Technologies, CA, USA). A DB-1ms capillary column (30 m×0.25 mm ID, 0.25 µm film thickness) was used with high-purity helium (99.999%) as the carrier gas at a flow rate of 1.2 mL/min. The injection volume was 0.1 µL, with a split ratio of 1:30. The oven temperature was initially set at 70 °C which was increased to 280 °C at a rate of 1.2 °C/min and held at 280 °C for 4 minutes. The MS system operated in electron ionization mode with a quadrupole detector at 70 eV ionization energy. The chemical composition of the ZMEO was identified by calculating Kovats retention indices using the retention times of an n-alkane ladder, injected after the injection of ZMEO sample. Mass spectra were compared with those in the computer library for component identification.

2.3.3. Determination of in vitro antibacterial activity of ZMEO

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests were conducted to assess *in vitro* antibacterial activity of ZMEO. The MIC was determined using micro-broth dilution method (10). Briefly, 0.5 to 256 µL/mL serial dilutions of the ZMEO were prepared and exposed to 1.5×10^8 CFU/mL inoculum size of *P. aeruginosa* (ATCC 27853). The experiments were performed in duplicate and the tested micro-plates were incubated at 35 °C for 20 hours. To interpret the results, the MIC was defined as the concentration at which the well showed complete clarity and the MBC was determined as the lowest concentration at which no subculture bacterial

colonies were visible. In this study, the MBC of ZMEO was utilized for contaminating the experimental burn wounds in the animals.

2.3.4. Preparation of ZMEO carboxymethyl cellulose gel

The gel-forming agent was prepared based on the previously reported procedure (11). Briefly, 1% (W/V) sodium carboxymethyl cellulose was prepared in deionized water and continuously stirred with a mixer for 15 minutes. Next, ZMEO (1% V/V) which was dried with sodium sulfate was gradually added to the prepared gel. Finally, the prepared gel was homogenized for 30 minutes and the prepared gel was collected in an aluminum tube in the refrigerator.

2.3.5. Creating experimental burn wounds

To induce burn wounds, animals were anesthetized by intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The hair on the back of the rats was shaved and a standard third-degree burn wound of 2×2 cm was created using a hot metal plate. The resulting burn was confirmed by a pathologist and was determined to be equivalent to twelve percent of total body surface area (TBSA) calculated using equation 1 (Equation 1) (12):

$$\text{Total body surface area } (cm^2) = k \times \left[\textit{Animal weight } (g)^{2/3} \right] \quad \text{(Eq 1.)}$$

Where k is the Meeh's constant (9.46 for Sprague Dawley rats (13).

2.3.6. Microbial inoculation of burn wounds with P. aeruginosa

15 minutes after burn wound induction, 1 ml of *P. aeruginosa* inoculum (1.5 x 10^8 CFU/ml) was applied to the burned areas. After leaving the wounds overnight, samples were collected from the surrounding areas using wet sterile swabs. These swabs were then sent to the microbiology laboratory and analyzed by an expert bacteriologist to confirm the microbial infection of the burn wounds.

2.3.7. Treatments

24 hours after creating experimental burn wounds and infecting them with *P. aeruginosa*, the animals were randomly divided into 4 groups of 7 to 10 rats as follow: group 1: the negative control group that received no treatment; group 2: the carrier gel group which was treated with CMC gel topically; group 3: ZMEO group that received CMC gel loaded with ZMEO (1%); and group 4: the positive control group that treated with silver sulfadiazine (SSD) ointment (1%). All treatments were applied topically every day for 28 days.

2.3.8. Measuring the wound area and wound contraction percentage

The wound areas were measured using a standard ruler. The percentage of wound contraction was calculated using equation 2 (Equation 2) (14و 15):

$$\text{Wound contraction percentage} = \left(\frac{(X - Y) \times 100}{X} \right) \quad \text{(Eq 2.)}$$

X=the area of the wound on day 0
Y = the area of the wound on the day of the test

2.3.9. Histopathological evaluations

Tissue samples that were collected from the edges of the infected wounds were immediately stored in 10% neutral buffered formalin before being sent to the histopathology laboratory. The tissue sections were stained with hematoxylin and eosin and were evaluated for histological indices of wound healing including epithelialization, granulation tissue formation, collagen organization, fibroblast proliferation, and vessel density (14, 16). The scoring system used for wound healing evaluation is shown in Table 1.

2.3.10. Tensometric evaluations

Tensile strength test was performed on all skin tissue samples 28 days after starting the treatments as follows. Four rats from each group were killed on the last day of the experiment, and the skin samples were collected to evaluate skin elasticity. Skin samples were prepared by removing any fat and muscle. Then, the samples were fixed in the chambers of the tensometer for testing under opposing forces (at a speed of 30 mm/min). The resulting data were recorded, and the tensile strength was calculated using equation 3 (Equation 3) (17):

Table 1. Histological scoring of wound healing.

Indices	Scores				
	0	1	2	3	4
Epithelialization	None	Partial	Moderate	Complete, immature	Complete, mature
Granulation tissue formation	None	Immature	Moderate	Mature	-
Collagen organization	None	Thin bundle of collagen	Thick bundle of collagen	Completely organized	-
Fibroblast proliferation rate	None	Mild	Moderate	Marked	-
Density of vessels	None	Mild	Moderate	Abundant	-

$$TS = T/A \quad \text{(Eq 3.)}$$

TS: Tensile strength (g/cm^2)
T: Maximal tensometer reading (g)
A: Cross - sectional area (cm^2)

2.3.11. Statistical analyses

Data were expressed as mean±SEM and analyzed statistically using Kruskal-Wallis followed by Bonferroni tests. The data were analyzed using SPSS software version 23 and $P \leq 0.05$ were considered statistically significant.

3. Results

3.1. GC-Mass analysis of the ZMEO components

The GC-Mass analysis results of ZMEO showed that cymene (ρ), terpinene (γ), and thymol were the most abundant molecules found in ZMEO (Table 2).

3.2. Inhibitory and bactericidal effects of ZMEO on P. aeruginosa

ZMEO exhibited inhibitory and bactericidal effects on *P. aeruginosa* with an MIC of 4 µL/mL and an MBC of 256 µL/mL.

3.3. Effect of ZMEO on wound area

Calculation of wound contraction percentage at 7 days after the start of the treatments showed that ZMEO caused the highest wound contraction percentages ($P<0.05$). At this time, no wound contraction effect was observed in other groups instead, the wound extent was appeared to be increased in these groups. At days 14, 21, and 28, an increasing trend of wound contraction was observed in all groups so that the differences between most of the groups were not statistically significant. The SSD-treated group was appeared to have less wound healing effect compared to other groups and its difference with ZMEO was significant at all time intervals ($P<0.05$) (Table 3, Figure 1).

3.4. Effect of ZMEO on total histological scores of

Table 2. GC-Mass analysis of the *Z. multiflora* essential oil.

Peak	Area=100	Calculated Kovats index	Name	Reference Kovats index
1	1.32	917	Thujene (α)	924
2	0.39	925	Pinene (α-)	932
3	1.79	965	Pinene (β-)	974
4	1.45	975	Myrcene	988
5	36.64	1011	Cymene (ρ)	1020
6	1.75	1017	Phellandrene (β)	1025
7	35.99	1047	Terpinene (γ)	1054
8	0.46	1078	Bicyclo[3.1.0]hexan-2-ol	1080
9	0.52	1157	Terpinen-4-ol	1174
10	1.03	1211	Carvone	1239
11	18.14	1273	Thymol	1289
12	0.49	1284	Carvacrol	1298

Table 3. The percentage of wound contraction in the studied groups at different times after the start of treatments.

Groups	Wounds(n)	Wound Contraction Percentage (%)			
		Day 7	Day 14	Day 21	Day 28
ZMEO	4	1.35±1.54*	63.74±5.11	87.32±1.3	99.85±0.08
Negative control	4	-14.19±1.45	58.17±4.56	87.00±3.43	98.50±0.55
SSD	4	-60.99±9.81	52.14±3.31	74.89±5.92	95.38±0.11
Carrier gel	3	-25.41±5.54	60.36±1.58	77.84±1.4	99.65±0.06

Values show mean±SEM. Kruskal-Wallis followed by Bonferroni test was used for data analysis.

*P≤0.05 as compared with the negative control group

ZMEO: *Zataria multiflora* essential oil; SSD: Silver sulfadiazine

wound healing

The total histological scores of wound healing in the four groups of this study at different times are demonstrated in Figure 3. Data analyses revealed significant differences in the mean total histological scores between the ZMEO-treated group and the other groups particularly at 7th day after the start of the treatments ($p < 0.05$).

As shown in figure 2, at 7th day after the start of the treatments, ZMEO induced better epithelialization and granulation tissue formation, higher fibroblast proliferation, and vessel length density compared to the other groups.

3.5. Effect of ZMEO on skin tensile strength

Figure 4 shows the results of skin tensometry in four studied groups at 28 days after the start of the treatments. The skin samples of ZMEO group exhibited significantly higher strength properties compared to the other groups ($P<0.05$).

4. Discussion

Burn wounds provide an ideal environment for the proliferation of pathogenic bacteria. *P. aeruginosa* is an opportunistic pathogen that causes potentially life-threatening nosocomial infections in burn patients. Rapidly developing resistance to existing antibiotics has made the wounds infected with this microorganism really hard to manage (18). Herbal essential oils have been considered in wound healing due to their valuable bioactive compounds. *Zataria multiflora* essential oil (ZMEO) has been shown to possesses numerous antimicrobial and immunomodulatory properties that greatly enhances wound repair mechanisms (19). In the present study, we evalu-

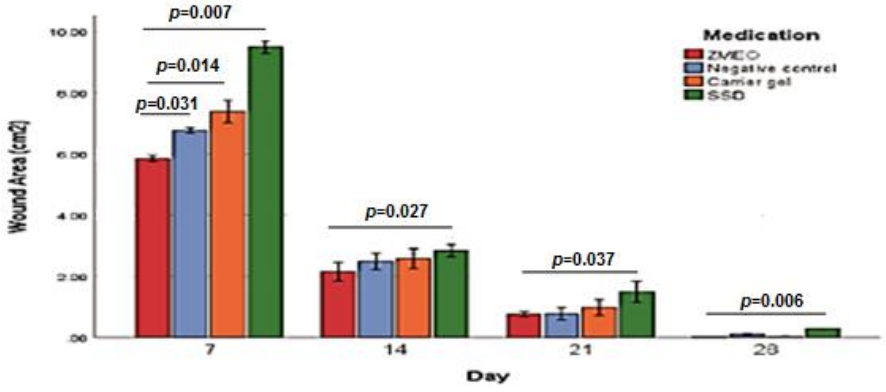

Figure 1. Wound area on days 7, 14, 21 and 28 after the start of the treatments in the ZMEO, negative control, carrier gel, and SSD groups. Values show mean ± SEM. Kruskal-Wallis followed by Bonferroni test was used for data analysis. ZMEO: *Zataria multiflora* essential oil; SSD: Silver sulfadiazine

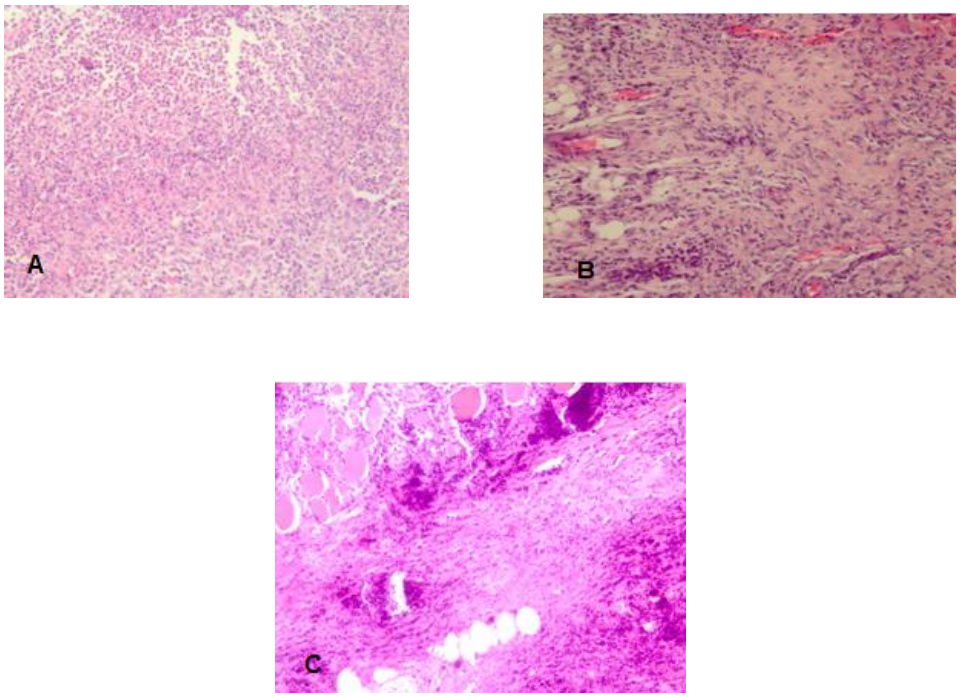

Figure 2. Stained samples of rats' wounds 7 days after the start of treatments: (A) carrier gel group shows ulceration with many acute inflammation, focal necrosis, and minimal fibroblast infiltration; (B) ZMEO group shows fewer inflammatory cells, many fibroblast and vessel proliferation with collagen deposition and maturing granulation tissue; (C) SSD group shows necrotic tissue with acute inflammation and fat necrosis. Hematoxylin and eosin staining, 200×
ZMEO: *Z. multiflora* essential oil; SSD: Silver sulfadiazine.

ated the antibacterial effect of ZMEO against *P. aeruginusa*. We also examined the wound healing properties of ZMEO at different time intervals over a 28-day period. ZMEO showed a remarkable bactericidal effect on this pathogen with an MIC of 4µL/mL and an MBC of 256 µL/mL. Our find-

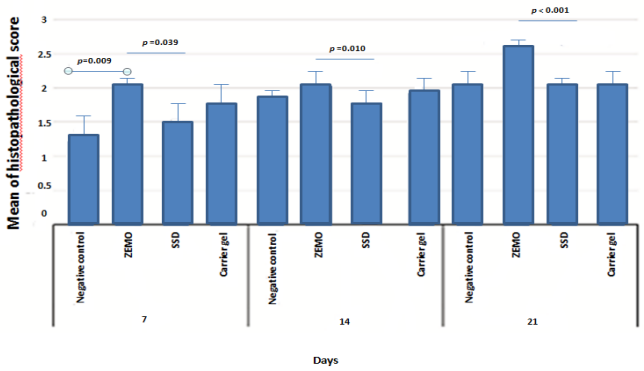

Figure 3. Histopathological scores of wound healing on days 7, 14 and 21 after the start of the treatments in the ZMEO, negative control, carrier gel, and SSD groups
Values are expressed as mean±SEM. Kruskal-Wallis followed by Bonferroni tests was used for data analysis $p \leq 0.05$ were considered significant.
ZMEO: *Z. multiflora* essential oil; SSD: Silver sulfadiazine.

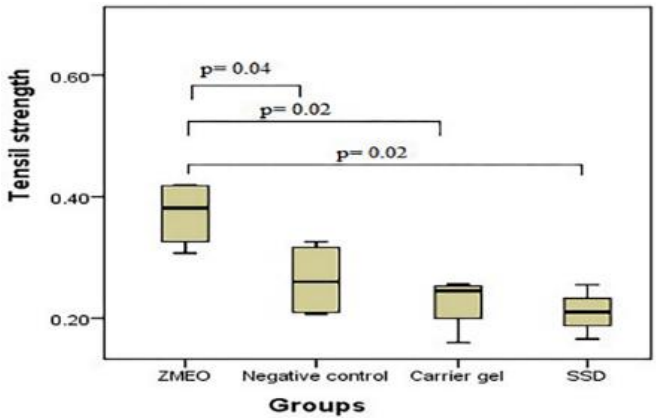

Figure 4. Tensile strength of the skin samples of rats 28 days after the start of the treatments in the ZMEO, negative control, carrier gel, and SSD groups
Values show mean±SEM. Kruskal-Wallis followed by Bonferroni test was used for data analysis. $p \leq 0.05$ were considered significant.
ZMEO: *Zataria multiflora* essential oil, SSD: Silver sulfadiazine.

ings are consistent with the results of Zomorodian K, et al. and Mahboubi M, et al. who explored the antimicrobial property of ZMEO against several strains of *P. aeruginosa in vitro* (20). In addition to evaluating the *in vitro* antibacterial effects, we examined the wound healing properties of ZMEO. Our results showed that ZMEO is excellent in improving wound healing indices, including reducing the burn wound area, improving histological indices of wound healing, and increasing skin tension. The wound healing effects of ZMEO started as soon as 7 days after the start of the treatments, while the other groups showed no effect on wound area and even increased the wound surface during this period. After this time, an increasing pattern of wound healing was observed in all groups which may be related to the natural mechanisms of wound healing in the body. By days 5 through 7, the fibroblasts start to produce new collagen and glycosaminoglycans which help stabilize the wound (21). Therefore, it appears that the advantage of ZMEO lies in its accelerating effect on wound healing. Other researchers have reported similar results. Farahpour et al. have reported the therapeutic effects of ZMEO ointment on wounds infected with P. aeruginosa (22). Also, a study by Stupin V. et al. showed that the topical application of ZMEO increases the histological indices of wound healing (23). The mechanisms by which the ZMEO exerts its antimicrobial effects are not fully understood, but it has been proposed that the main components of ZMEO such as ρ-cymene, terpinene (γ-), and thymol have antimibacterial and immunomodulatory properties (24, 25) via stimulating the innate immune responses (26) as well as their direct enhancing effects on skin cell proliferation (27). As we showed in the current study, ZMEO positively affected the proliferative phase of wound healing that leaded to significant improvements in histological indices such as collagen organization, fibroblast proliferation rate, vessel density, epithelialization, and granulation tissue formation. Moreover, it has been proposed that these compounds may enhance the formation of granulation tissue and elevate the level of transforming growth factor beta (24, 28, 29). ZMEO also has shown the ability of increasing the levels of endothelial growth factors, promoting fibroblast proliferation and differentiation, improving collagen organization, and enhancing vessel density (22, 30, 31).

At last, we evaluated the effects of ZMEO on the skin mechanical properties. According to our results, ZMEO enhanced skin tensile strength. The skin samples derived from ZMEO-treated rats were able to tolerate more force and exhibited higher tensile strength than the other groups. This property is possibly due to the enhancing effect of ZMEO on the thick bundles of collagen and fibroblast proliferation rate, as highlighted by Nair's report on the relationship between the structural integrity of the skin and quality of the extracellular

matrix (30, 32).

Another observation of this study was that silver sulfadiazine, a common antimicrobial treatment for burn wounds, initially delayed wound healing. This effect has been reported in several studies by other authors (33, 34). Intoxication of fibroblasts and keratinocytes with silver as a heavy metal has been proposed as the mechanism of effect of SSD in delayed wound healing (35).

5. Conclusion

Considering the accelerating effects of ZMEO on wound healing and skin tensile strength as well as its strong antibacterial properties, we suggest this plant essential oil as a suitable candidate for further evaluations in order to develop new topical drugs for the treatment of wounds infected with *P. aeruginosa.*

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Authors contributions

Maryam Motevasel, Maryam Zare, and Ali Karimi Akhormeh conceived the presented idea, developed the theory of the study, verified the analytical methods, and supervised the findings of this work. Aida Iraji contributed in GC-MS analysis of compounds and their interpretation. Maryam Zare, Ali Karimi Akhormeh, Maryam Motevasel, Marzieh Ashrafmansouri, and Omid Koohi Hosseinabadi, contributed to sample preparation, treatments, data collection, and data visualization. Maral Mokhtari contributed to histopathological data analysis and visualization. Maryam Motevasel, Elahe Sattarrinezhad, Maryam Zare, Ali Karimi Akhormeh and Azar Purkhosrow took the lead in writing the manuscript. Maryam Motevasel prepared the primary draft of the manuscript. All authors discussed the results and contributed to the final version of the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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