Original Article

The Effect of Zataria multiflora Nanoemulsion Gel on Rat Surgical Wound Healing

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ABSTRACT

Background: We aimed to determine the effect of *Zataria multiflora* Nanoemulsion gel on surgical wound healing.

Methods: This experimental study was conducted in the years 2021-2022 at the Animal Research Center of Mazandaran University of Medical Sciences, northern Iran. Forty two male Westar rats were randomly divided into six groups (n=7). After a surgical incision of full thickness with a 3 cm diameter, they were treated for 21 days with diltiazem 2% (positive control), placebo, and *Z. multiflora* emulsions and nanoemulsions at 2% and 4%, respectively. Macroscopic parameters of wound area and contraction, as well as pathological factors such as granulation, angiogenesis, epithelialization, collagen organization, bacterial colony, inflammation, creatine and epidermal thickness, hair follicles, and lymphatic ducts, were examined.

Results: The mean wound size and contraction of the placebo group differed significantly from the other groups on all days, and on some days, the results indicated more favorable effects of nanoemulsions than Diltiazem. Based on the microscopic findings, the average scores on the seventh day were nearly different (P= 0.051); however, all groups scored higher than the placebo group. On the 21st day, the best results were related to the 4% Nanoemulsion (242.5), 2% Nanoemulsion (159.4), and 4% emulsion (159.3), followed by diltiazem (154.60), 2% emulsion (146.5), and placebo (70.7).

Conclusion: *Z. multiflora* emulsions and nanoemulsions at 2% and 4% could be effective in healing surgical wounds, and the use of 4% Nanoemulsion yields the best results. This is recommended for use in clinical trial studies.

KEYWORDS
Medicinal herbs; Nanoemulsion; Wounds; Gel; *Zataria multiflora*

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INTRODUCTION

The skin serves as the body's largest organ, playing a crucial role in protecting internal structures from environmental hazards, pathogens, and dehydration. Additionally, it is essential for thermoregulation, sensory perception, and the synthesis of vitamin D, all of which contribute to overall health and well-being Skin injuries include surgical

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and traumatic wounds, pressure, diabetes, vascular ulcers, and burns [1-3].

Surgical wounds are common, and Surgical Site Infection (SSI) is expected due to reasons such as aseptic failure and bacteria like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Antibiotic resistance and side effects also pose problems, and antiseptics have limited efficacy in reducing infection burden [4,5]. Wound healing is a physiological response to skin and subcutaneous tissue damage. It helps restore skin structure and function in stages of homeostasis, inflammation, proliferation, and remodeling [6]. Initially, vasoconstriction, decreased blood flow, and platelet aggregation stabilize the wound. During the inflammatory phase, increased vascular permeability leads to neutrophil, macrophage, and lymphocyte migration, phagocytosis of pathogens, damaged proteins, and cell debris [7,8]. In the proliferative phase, fibroblasts, macrophages, growth factors, collagen, blood vessels, and myofibroblasts cause granulation, epithelialization, and wound contraction. The remodeling phase usually starts 2-3 weeks post-injury, with collagen deposition, scar maturation, wound contraction, pigmentation, and epidermal formation [9,10].

Factors like surgical wound size, microorganism presence, age, and comorbidities (e.g., diabetes, cancer, malnutrition, pain) affect healing [11]. Delay in this process can lead to chronic wounds, infections, scars, and hinder adjuvant therapy initiation, impacting economic and psychological aspects. Thus, accelerating the process in all surgery stages is crucial [12].

In Iranian traditional medicine, various herbal remedies have been suggested for wound healing, including *Z. multiflora*. This plant, belonging to the Lamiaceae family, is found in Iran, Afghanistan, and Pakistan. Its phenolic and non-phenolic compounds, such as thymol and carvacrol, have anti-cancer, anti-spasm, anti-fever, anti-thrombosis, and anti-diabetes properties [14,15], along with antimicrobial, anti-apoptotic, analgesic, anti-inflammatory, and antioxidant effects that contribute to its healing potential [16].

Due to low solubility, rapid oxidation, and low stability of essential oils, they are not typically used directly but in the form of emulsions and nano-emulsions [17]. However, nano-systems can solve these issues and increase drug permeability rates [18]. *Z. multiflora* has shown increased antibacterial and healing properties using nanotechnology [19].

We aimed to determine the effect of *Z. multiflora* Nanoemulsion gel on surgical wound healing in Wistar rat animal models.

METHODS

Ethical approval

This research was conducted after obtaining research ethics code number IR.MAZUMS.REC.1400.457 from the Research Committee of Mazandaran University of Medical Sciences, adhering to ethical principles for working with laboratory animals, and obtaining the required training in this field.

Study design

This experimental study was conducted from 2021 to 2022 at the Animal Research Center of Mazandaran University of Medical Sciences, Northern Iran. The study utilized the ARRIVE (Animal Research: Reporting in Vivo Experiments) checklist for reporting animal research. The groups studied were divided into comparative and control groups based on the study's purpose and intervention design. The groups included: P (negative control consisting of placebo or gel base), D (positive control consisting of 2% Diltiazem gel), Z2% (2% Zataria multiflora emulsion), Z4% (4% Zataria multiflora emulsion), NZ2% (2% Zataria multiflora Nanoemulsion), and NZ4% (4% Zataria multiflora Nanoemulsion).

Formulation and Characterization of nanoemulsions of essential oil and Preparation of nanoemulgel

The coarse emulsion was prepared by dispersing essential oil into the deionized water and Tween 80, Tween 20, or a mixture of Tween 80/Span 80 as emulsifiers. The concentration of surfactant(s) was kept constant at 3 wt%. A range of HLB values from 9.65 to 16.7 was utilized to prepare essential oil nanoemulsion, and the influence of HLB value was investigated (Table 1). The mixture was then homogenized by a high-speed homogenizer (Silent Crusher M, Heidolph, Germany) at 8000 rpm for 10 min. The obtained coarse emulsions were then subjected to homogenization by utilizing an FBF laboratory high-pressure homogenizer (Italy) at 500 bars for five cycles. The device was covered with

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Table 1: Composition (wt%), mean droplet size (nm), polydispersity index (PDI), and zeta potential (mV) of nanoemulsion

Formulation	Essential oil[a] (%)	Tween 80[b] (%)	Span 80 (%)	Tween 20 (%)	HLB[c]	Water up to (ml)	Particle size (nm)	PDI[d]	Zeta potential (mv)
F1	2			3	16.7	97	77.06±3.70	0.326±0.030	-2.51±0.12
F2	2	3			15	97	52.5±2.75	0.196±0.015	-2.77±0.11
F3	4	1.5	1.5		9.65	97	343.36±12.15	0.350±0.015	-4.75±0.21
F4	2	1.5		1.5	9.65	97	343.06±12.05	0.320±0.10	-6.52±0.25
F5	2	1	2	1.5	7.27	97	366.23±13.26	0.936±0.056	-0.76±0.09

ice bags to prevent an increase in the temperature. Optimum conditions have been selected for further investigation based on droplet size and particle size distribution.

The dynamic light scattering (DLS) with Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK) equipped with disposable capillary cuvette (DTS 1060) was used to determine the size, zeta potential, and polydispersity index (PDI) of nanoemulsion formulations. The DLS technology provides a PDI and an intensity weighted mean diameter (Z-average) [20]. The measurements were conducted at a fixed scattering angle of 90° at 25 °C. The reported values are the mean± standard deviation (SD) of at least three determinations. The Malvern Zeta sizer was also employed to measure the zeta potential through electrophoretic mobility measurements. The zeta potential was measured using the same cuvette. The average value was calculated immediately after the measurement of particle size.

In order to prepare a plain gel base, first, Carbopol 940 polymer (0.75 wt%) was dispersed in distilled water and was allowed to swell in the dark at 25±2 °C for 24 h to remove air bubbles and allow to reach the equilibrium condition. Finally, the pH value of the polymeric solution was adjusted to 6 using triethanolamine after the swelling of the polymer and result in a transparent and clear gel [21].

Determine the sample size

Fewer samples may lead to uncertain results while more samples can conflict with ethical issues due to unnecessary use of animals. Therefore, after designing the study, the sample size should be determined by considering the level of significance (=∝0.05) and test power (1-β=0.80) in a one-way ANOVA test. G Power software version 3.1.9.2 can assist in estimating the appropriate sample size. For this specific study, a sample size of 42 male Wistar rats (n = 7 in each group) was estimated and a 21-day treatment period was considered.

Characteristics of the studied community

Studied rats weighed 200-250 g, were 14-15 weeks old, clinically healthy, and kept in optimal conditions (25 ± 0.5 °C temperature, 12-hour light/dark cycle, 40-60% relative humidity, good air quality, low noise, free water access, wood pellets and adequate physical space). Inclusion criteria: weight within the desired range; exclusion criteria: loss due to anesthesia or ethical issues such as unacceptable conditions predicted before the study.

Creating a surgical wound

Depending on the animal's age, species, and surgical intervention type, rats received intraperitoneal injection of 100 mg/kg ketamine hydrochloride and 20 mg/kg xylazine hydrochloride for analgesia, and pain prevention during surgery. The animal was placed prone on a surgical table, and after shaving its dorsal part with an electric caliper, the area was prepped with 70% alcohol. A circular full-thickness wound (including epidermis, dermis, hypodermis, and panniculus) with a diameter of 3 cm was created using Blade No.15 and sterile Iris scissors [22] in aseptic conditions, after marking the area with a pattern and marker.

Determination of study groups

The rats were monitored after being wounded until they fully recovered before being randomly assigned to six groups of seven based on weightlifting. To perform randomization, an observer outside the intervention group numbered the rats from 01 to 42 using a Random Number Generator and Table, then placed them in groups accordingly.

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The rats were renumbered for macroscopic and microscopic examinations, and the table destroyed by the observer. Each treatment group was placed in separate laboratory units (animal cages at least 800 cm^2 in size) to reduce stress and prevent interactions. Labels recorded information about each group for easy identification, and daily control measures ensured clean cages, water access, and food. The daily topical application of the compound in each group was 1-2 mg.

Macroscopic and microscopic examinations

To examine macroscopic parameters (wound size and contraction percentage), five rats were photographed from each group on days 0, 3, 7, 10, 14, 17, and 21 under the same conditions. Image J software accurately calculated wound area [23] and percentage of contraction [24].

$$N^{th} = \frac{Wound\ area\ 0\ day - Wound\ area\ on\ Nth\ day}{Wound\ area\ on\ 0\ day} \times 100$$

For Histopathological examinations, two samples were taken from each group on day 7 and another set on day 21 after humane euthanasia with chloroform 99% composition, following scientific and ethical principles. Skin samples were obtained using a blade from the restored area and 1-2 mm of healthy tissue around the wound to the depth of the hypodermis and sent to the pathology unit.

After specimen fixation and standard histological techniques, tissue sections of 3 microns thickness were prepared using paraffin. H/E staining was used to prepare a slide for each section, and the tissue parenchyma examined for morphological changes. A pathologist evaluated tissue sections blindly, recording an average of ten microscopic fields of view for each sample. Based on modified scoring criteria from previous studies [25-27]. histopathological criteria and morphological parameters were graded as (-), (±), (+), (++), or (+++), indicating mild, moderate, or severe foci in HPF microscopic fields (x400). Parameters included macrophage infiltration, matrix mycopolysaccharide deposition, fibroblast proliferation, epithelial thickness and epithelialization, bacterial colonization, neovascularization, granular cell layers, degree of orthokeratosis, hair follicle and lymphatic duct number, keratin layer thickness, squamous cell maturation organization, scar formation, and collagen orientation and organization. Total scores were compared between groups.

Statistical analysis

The data were analyzed using SPSS software version 21 (IBM Corp., Armonk, NY, USA), and normality was determined by the Kolmogorov-Smirnov test. For normally distributed data, one-way ANOVA and LSD tests were used, while Kruskal-Wallis and Mann-Whitney tests were applied for non-normal distributions. Statistical significance was considered at *P*-value <0.05 in this study.

RESULTS

Droplet size, polydispersity index and Particle surface charge (zeta potential)

The essential oil nanoemulsion droplet size distribution is illustrated in Table 1. Having a suitable HLB value used in the formulation was one of the major driving forces for stable nanoemulsions. The range of droplet size was from 52.5±2.75 to 366.23±13.26 nm. This size range with a narrow distribution caused by the high-pressure homogenization, leading to nanoemulsions with small droplet size that met the nanoemulsion criteria (less than 400 nm). Nanoemulsions with different concentrations of essential oil (2%, and 4% w/w%) showed droplet sizes of 343.06±12.05 and 343.36±12.15 nm, respectively. A mixture of surfactants with the fnal HLB 7.27 formed the biggest droplet nanoemulsion size (366.23±13.26 nm). The narrow distribution with the smallest droplets can be obtained if the HLB value of the emulsifer is closer to the HLB of the oil. A mixture of surfactants produces the stable and suitable nanoemulsion droplet size.

PDI results are represented in Table 1 showed that all formulations exhibited PDI values in the range of 0.196±0.015 and 0.936±0.056. A similar trend with particle size was found when the PDI was compared to various HLB values (Table 1). The larger PDI (0.936) was obtained at an HLB value equal to 7.27.

The zeta potential of the nanoemulsion droplets varied from -0.76±0.09 mV to -6.52±0.25mV (Table 1).

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Findings of Macroscopic assessments

Macroscopic examinations were conducted on days 0, 3, 7, 10, 14, 17, and 21 to determine mean wound area and contraction (Figure 1) using One Way-ANOVA and LSD tests. Results showed no significant difference in mean weight of rats or wound area among treatment groups on the surgery day. However, Table 2 revealed a significant difference in wound area parameter between *Z. multiflora* and positive control groups with the placebo group on days 3, 7, 10, 14, 17, and 21. On days 7, 14, and 17, *Z. multiflora* groups, particularly nanoemulsions of 2% and 4%, showed better wound reduction compared to the placebo group ($P<0.05$). While on the 21st day, all *Z. multiflora* and diltiazem groups had significantly different mean wound areas from the placebo group. Similarly,

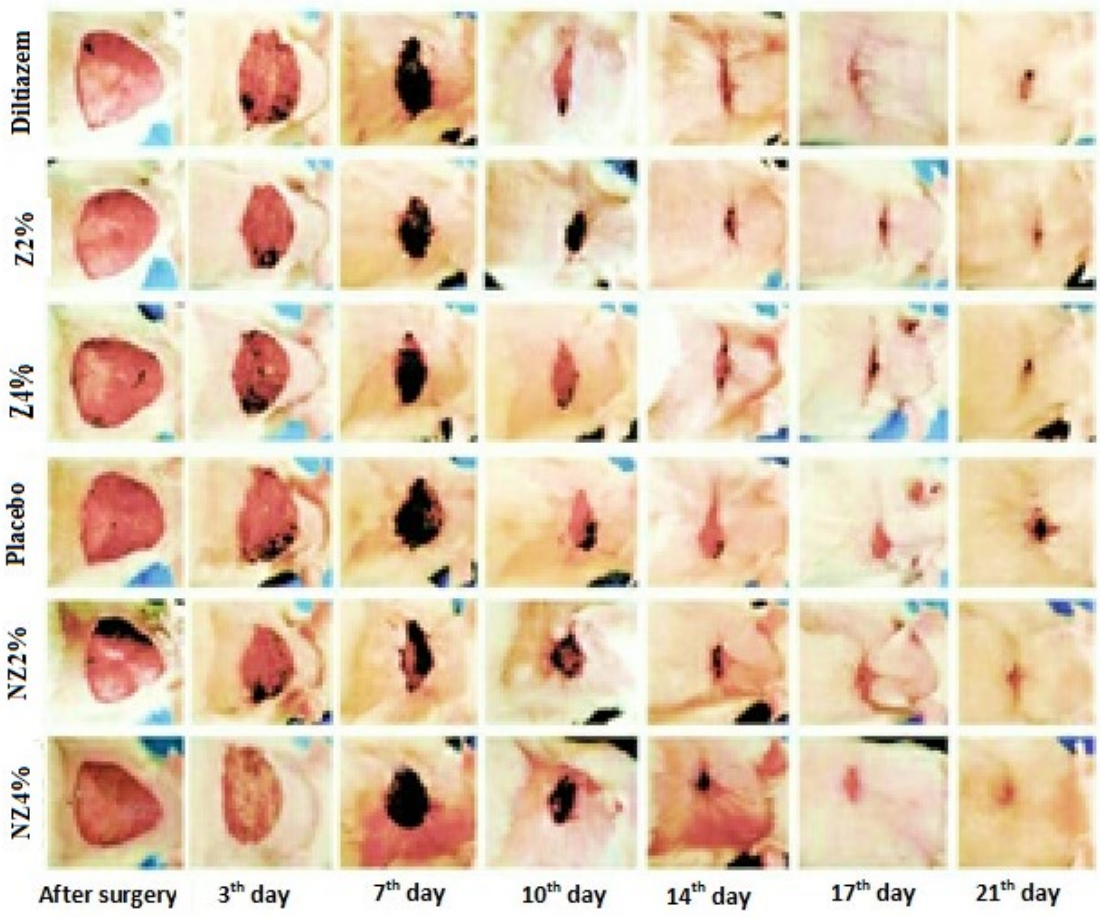

Figure 1: General macroscopic condition of surgery wounds healing of various treatment groups (D: Diltiazem- Z2%: Zataria multiflora 2% - Z4%: Zatarai multiflora 4%- NZ2%: Nanoemulsion Zataria multiflora 2%- NZ4%: Nanoemulsion Zataria multiflora 4%- *P*: Placebo) during 21 days after skin surgery

Table 2: Comparison of wound area (Mean ± SD) in different treatment groups (mm)

Groups	Surgery Day	3 Day	7 Day	10 Day	14 Day	17 Day	21 Day
D	7.29±0.14	5.89±0.58	2.84±0.32	1.27±0.30	0.62±0.33	0.52±0.28	0.12±0.06
Z2%	7.23±0.15	5.71±1.36	2.36±0.18	1.19±0.10	0.48±0.10	0.30±0.09	0.08±0.07
Z4%	7.27±0.08	5.20±0.42	2.45±0.48	1.36±0.21	0.46±0.09	0.42±0.05	0.09±0.05
NZ2%	7.27±0.17	5.33±0.76	2.40±0.59	1.21±0.20	0.37±0.06	0.31±0.05	0.07±0.04
NZ4%	7.34±0.16	5.59±0.41	2.40±0.31	1.11±0.11	0.32±0.05	0.28±0.06	0.06±0.05
P	7.25±0.14	6.89±0.42	2.23±0.56	1.69±0.15	0.91±0.11	0.85±0.16	0.33±0.07
***P* Value**	0.869	0.021<*	*0.023	*0.001	0.001<*	0.001<*	0.001<*

D: Diltiazem- Z2%: Zataria multiflora 2% - Z4%: Zatarai multiflora 4%- NZ2%: Nanoemulsion Zataria multiflora 2%- NZ4%: Nanoemulsion Zataria multiflora 4%- *P*: Placebo- *statistically significant

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Table 3 showed a significant difference in wound contraction for *Z. multiflora* and positive control groups compared to the placebo group on all days. On days 3, 10, 14, and 17, the *Z. multiflora* groups, especially nanoemulsions of 2% and 4%, had higher average percentage wound shrinkage compared to the diltiazem group. Only the placebo group had a statistical difference on the 21st day from other treatment groups.

Findings of histological assessments

Microscopic findings were examined on days 7 and 21 using Kruskal-Wallis and Mann-Whitney tests. On the seventh day, a near-significant difference was observed in the studied factors (P=0.051). In fact, the intervention group and positive control group had higher scores in macrophage infiltration, fibroblast proliferation, inflammation, angiogenesis, and deposition of Mucoplis acarid matrix (7.50) compared to the negative control group or placebo group (1.50). The drug intervention groups (8.50) scored better in epithelial sufficiency parameter than the positive control and negative control groups (2.50). Additionally, emulsion 4%, nanoemulsions 2%, and 4% groups (3.50%) had lower scores in bacterial colonization compared to other groups (9.50), indicating more antimicrobial effects of *Z. multiflora* groups. Figure 2 shows the Histopathological picture of different treatment groups on day 7.

On day 21, there was no significant difference between the groups in new vascular formation (P=1.00), but the diltiazem and nanoemulsion 4%

Table 3: Comparison of wound contraction percentage in different treatment groups

Groups	Wound Contraction % (Mean ± SD)					
	3 Day	7 Day	10 Day	14 Day	17 Day	21 Day
D	19.28±6.88	61.05±4.67	82.46±4.17	91.44±4.60	92.81±3.85	98.35±0.86
Z2%	25.77±10.40	67.36±2.77	83.53±1.33	93.23±1.50	95.75±1.42	98.77±1.06
Z4%	28.49±5.56	66.15±7.11	81.18±2.95	93.68±1.32	94.17±0.78	98.71±0.75
NZ2%	26.62±11.23	66.95±8.48	83.37±2.25	94.92±0.85	95.63±0.65	98.91±0.62
NZ4%	23.87±4.13	67.25±4.27	84.81±1.76	95.65±0.64	96.17±0.86	99.12±0.81
P	7.58±2.87	55.34±7.99	76.61±2.06	87.36±1.61	88.21±2.26	95.43±0.98
P VALUE	*0.021	*0.029	*0.001	0.001<*	0.001<*	0.001<*

D: Diltiazem- Z2%: Zataria multiflora 2% - Z4%: Zatarai multiflora 4%- NZ2%: Nanoemulsion Zataria multiflora 2%- NZ4%: Nanoemulsion Zataria multiflora 4%- *P*: Placebo- *statistically significant

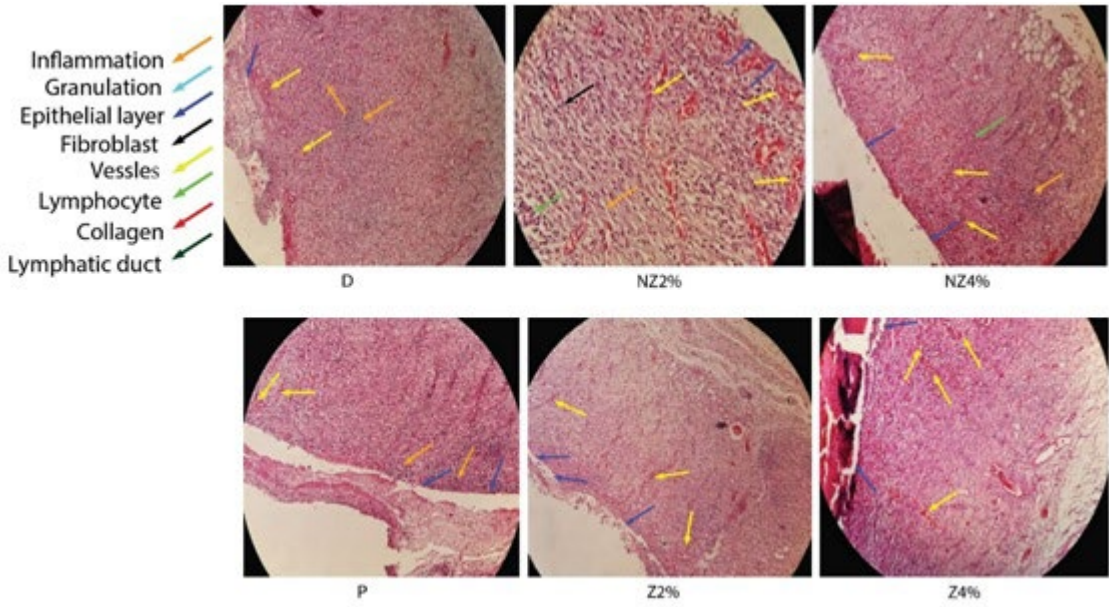

Figure 2: Histopathological properties of cutaneous specimens of various treatment groups (D: Diltiazem- Z2%: Zataria multiflora 2% - Z4%: Zatarai multiflora 4%- NZ2%: Nanoemulsion Zataria multiflora 2%- NZ4%: Nanoemulsion Zataria multiflora 4%- P: Placebo) on 7 days after skin surgery

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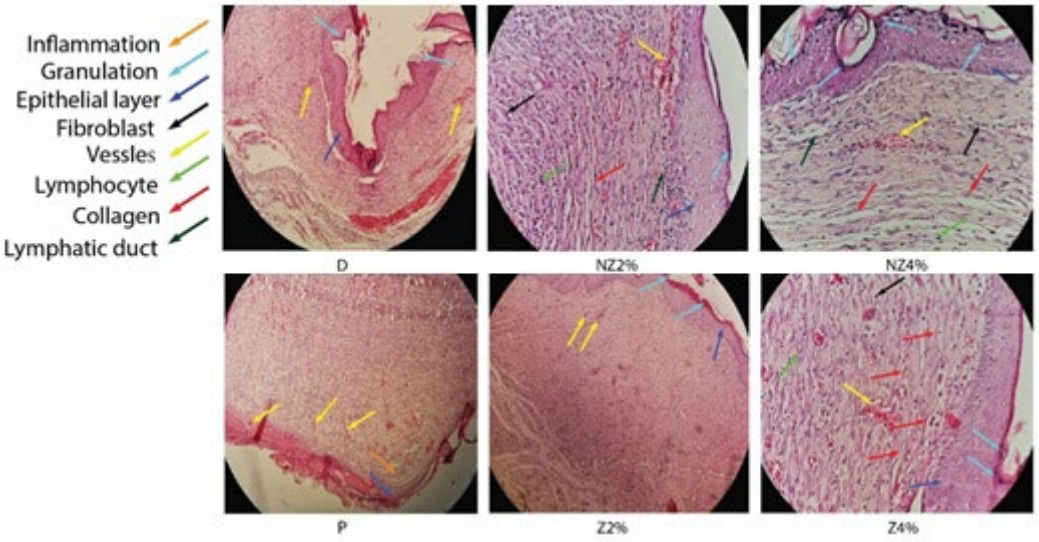

Figure 3: Histopathological properties of cutaneous specimens of various treatment groups (D: Diltiazem- Z2%: Zataria multiflora 2% - Z4%: Zatarai multiflora 4%- NZ2%: Nanoemulsion Zataria multiflora 2%- NZ4%: Nanoemulsion Zataria multiflora 4%- P: Placebo) on 21 days after skin surgery

groups received the highest score in hair follicle formation (P=0.082).

The 4% nanoemulsion group had the highest mean score and statistical difference in scar formation (P=0.001) and collagen organization (P <0.001) compared to other treatments (P=0.008). Diltiazem, emulsion 4%, and Nanoemulsion 2% also received good scores. Diltiazem, emulsion 4%, nanoemulsions 2%, and 4% groups had the highest scores in lymphatic duct formation (P=0.031). Nanoemulsions 2% and 4% showed significant differences in epidermal thickness (P=0.001) and granular layer formation (P <0.001) compared to placebo groups (P=0.008). The 4% nanoemulsion group had the highest scores in squamous cell maturity (P <0.001) and keratin layer thickness (P <0.001) parameters. Intervention groups had higher mean scores than control groups for orthokeratosis and epidermal layer development (P <0.001), with the highest rates in the 4% and 2% nanoemulsion groups. Figure 3 shows the histopathological picture of different treatment groups at 21 days.

DISCUSSION

The study found that *Z. multiflora* and diltiazem 2% treatments were more effective in reducing wound area than the placebo group. Emulsion and nanoemulsions of *Z. multiflora* groups showed significantly better results than diltiazem on some days. *Z. multiflora* treatment groups had a more favorable effect than the negative control group (placebo) on average wound shrinkage percentage, especially nanoemulsions on some days. Zataria multiflora, particularly in nano-formulation, had favorable effects on reducing the area and increasing wound contraction from a macroscopic point of view. Histopathological scoring on days 7 and 21 indicated acceptable effects of *Z. multiflora* compared to placebo. Nanoemulsion at a concentration of 4% had a more favorable effect on surgical wound healing than other groups from a microscopic point of view. In the present study, the range of droplet size was from 52.5±2.75 to 366.23±13.26 nm. A blend of two non-ionic surfactants with high HLB differences could produce the suitable droplet size with acceptable distribution.

Some studies have reported the beneficial effects of *Z. multiflora* in reducing the size of various wounds, such as oral mucositis, gastric ulcers, burns, infected skin wounds, and leishmaniasis. These effects are due to the presence of phenolic compounds like thymol and carvacrol in *Z. multiflora* essential oil [28-30].

Some clinical studies and research have pointed to the analgesic and anti-inflammatory effects of thyme extract and essential oil, as well as their effects on oxidative stress and harmful free radicals that are effective in wound healing [4,31,32]. In all of these studies, the reduction of wound size due to the effect of *Z. multiflora* has been reported. This is consistent with the macroscopic results obtained

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in the present study, where both the *Z. multiflora* emulsion and nanoemulsion groups exhibited statistically significant improvements compared to the placebo group.

The microscopic and histopathological results obtained in the present study indicate that *Z. multiflora* emulsions and nanoemulsions can affect factors that promote wound healing such as increased epithelialization and granulation, thicker creatine layers, reduced bacterial colonies, adequate horny layer, and accelerated inflammatory phase. These effects could potentially accelerate the healing process of surgical wounds.

Other studies have shown that *Z. multiflora* can reduce bacterial colonization. Nano-fiber pads infused with *Z. multiflora* herbal nanogels have also demonstrated the herb's antibacterial effects against common human pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*, which cause nosocomial infections [33-35]. Furthermore, another study has evaluated *Z. multiflora's* antimicrobial effectiveness against gram-positive and gram-negative pathogens, including *Helicobacter pylori*, the causative agent of ulcers and gastric cancer, *Trichomonas vaginalis*, the pathogen responsible for vaginitis, and *Echinococcus granulosus*, the hydatid cysts causative agent [36]

Microscopic findings from Farahpour et al study (4) indicated that *Z. multiflora* could increase angiogenesis, fibroblast count, lymph duct formation, creatine and hair follicle thickness, as well as epithelialization. These studies have reported significant reduction in bacterial count, growth factor-secreting gene expression, angiogenesis, fibroblast and fibrocyte count, collagen activity, antioxidant activity, inflammatory factors, and proliferative phase in the presence of *Z. multiflora* against pathogens such as *P. aeruginosa* and *staphylococcus aureus*[4]. Although the nano-formulation was not used to prepare *Z. multiflora* in Farahpour et al study[4], our findings show that the use of nano-formulation is more effective than emulsion. Farahani et al study also demonstrated the accelerated healing of burn wounds through a combination of acetate-gel cellulose nanofibers Ethnic and *Z. multiflora* nanoemulsions [37,38].

Several studies have reported increased antimicrobial and antioxidant effects of *Z. multiflora* through the use of nano-systems [39]. The primary mechanism of nanoparticles is the increase in skin permeability rate and speed, modulation of intracellular calcium concentration, activation of transcription factors, and changes in cytokines during the inflammatory phase of the healing process, which can accelerate the process and accompany wound healing.

LIMITATIONS

Blinding was not possible at all stages of the present study, and due to the COVID pandemic, attendance at the research site and interventions were delayed.

CONCLUSION

On both macroscopic and histopathological scales, the use of 2% and 4% *Z. multiflora* ra emulsions and nanoemulsions were found to be effective in promoting surgical wound healing. The superior results obtained from the nanoemulsion groups suggest that the use of nano-formulations can increase effectiveness. Therefore, we recommend the use of *Z. multiflora* nanoemulsion products in clinical trials for surgical wounds.

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DECLARATION OF COMPETING INTEREST

The authors declare that there is no conflict of interests.

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