

Development of gel containing Psidium glaziovianum essential oil has in vitro antimicrobial activity and improves healing of excisional wounds in mice

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Abstract

Wounds are physical, chemical, biological or even medical procedure-related damage to the skin or mucous membranes. One of the major problems in the treatment of wounds is the development of infections, as they cause systemic damage to the patient, delaying recovery from the injury. In order to accelerate the healing process, there is currently an interest in finding drugs that can interact with the injured tissue and fight the associated infections. Previously *P. glaziovianum* essential oil (PgEO) has been shown to have antinociceptive and anti-inflammatory properties, as well as minimal oral toxicity. Therefore, our objective was to evaluate the impact of topical application of a gel formulation containing PgEO in excisional wounds in mice. Additionally, *in vitro* antimicrobial evaluation was also performed. The results of this research demonstrate that the gel containing essential oil of *P. glaziovianum* has action against gram-positive and gram-negative bacteria, as well as fungi. Furthermore, the results show that PgEO gel therapy improves skin wound healing, presumably because of its ability to stimulate antioxidant enzymes and inhibit pro-inflammatory cytokines. The gel also demonstrated minimal erythrocyte toxicity and skin irritation, which may hold promise for future preclinical and clinical studies in various types of wounds.

1. Introduction

Wounds are physical, chemical, biological, or even medical procedure-related damages to the skin or mucous membranes. Wound healing is a natural reaction of the body formed as a tissue response to injury. Inflammatory phase, proliferation or granulation phase, and remodeling or maturation phase are the three interactive and overlapping phases of wound healing (Saporito et al., 2018; Salas-Oropeza et al., 2020; Andji et al., 2022).

One of the biggest issues when treating wounds is the development of infections because they cause the patient's systemic harm by delaying the lesion's healing. Additionally, it is important to note that the immune system's response is exacerbated by the growth of the microbial load, which can result in sepsis and even critical colonization (Wang et al., 2021; Alanazi et al., 2022).

The cost of treating wounds rises annually, placing a significant financial strain on the global health system. As a result, there is a growing interest in finding drugs that can interact with the injured tissue to hasten the healing process (Andji et al., 2022). In order to treat cutaneous wounds, some studies using medicinal plants have been conducted with an emphasis on the healing process and with the goal of bringing new therapeutic alternatives (Farahpour et al., 2020; Andji et al., 2022).

Psidium glaziovianum Kiaersk it is a small to medium-sized tree that is found in the Caatinga and Atlantic Forests of Brazil and is thought to be an endemic species there (Landrum et al., 2017). *P. glaziovianum* essential oil (PgEO) was previously reported to have antinociceptive and anti-inflammatory activity as well as low oral toxicity (Costa et al., 2022, 2023).

We therefore examined the effects of topical application of a gel formulation containing PgEO on excisional wounds in mice, taking into account that PgEO has an anti-inflammatory effect, and that inflammation is a crucial stage of the healing process. An in vitro antimicrobial assessment was also carried out.

2. Materials and Methods

2.1 Vegetable material and essential oil extraction

Psidium glaziovianum Kiaersk (Myrtaceae) leaves were collected in December 2019 (drought period) in the town of Exu in the Brazilian state of Pernambuco, Caatinga region. Exsicata was deposited in the 93,728 specimen collection at the IPA Herbário Dárdano de Andrade Lima. The plant material was registered under the number A08E18B in the Sistema Nacional de Gesto do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen).

The essential oil was extracted from *P. glaziovianum* (PgEO) leaves by hidrodestilation and identified by gas chromatography mass spectrometry (GC/MS); the results were previously published by Costa et al. (2022). It contained 48 constituents and made up 98.22% of the composition, which was dominated by 1,8-cineole (24.29%), α -pinene (19.73%), and β -pinene (17.31%).

2.2 Gel preparation with PgEO

Initial additions included 7.6 grams of distillate water and 0.05 grams of Carbopol® 940. The mixture was then homogenized using an Ultra-Turrax (Modelo T-10, IKA®, German) at 13.000 revolutions per minute for five minutes. Propilenoglicol (0,2 g) and *P. glaziovianum* essential oil (0,1 g) were combined, and stirring was maintained at 13,000 rpm for five minutes. Finally, the addition of the preservatives propilparaben (Nipazol, 0.025 g) and metilparaben (Nipagin, 0.025 g) was made while continuing the agitation for another minute. The obtained gel was known as PgEO-gel 1%.

2.3 PgEO-gel Characterization

The PgEO-gel was evaluated to find visible changes or instability like color and phase separation. The analysis was completed 24 hours after preparation. The test of resistance to centrifugation was carried out by subjecting the sample to increasing rotations of 980, 1800, and 3000 rpm for the same duration of time during each rotation (5 min), at room temperature (25 °C). The pH value was measured using a pH meter (pH 21, Hanna instruments) and properly calibrated to room temperature. The Rotavisc hi-vi II, IKA® viscosimeter was used for viscosity measurement with spindle 1 and appropriate rotation (5 rpm), at stabilized ambient temperature.

2.4 In vivo experiments

For the tests, Camundongos Swiss males weighing between 30 and 35g and aged between 8 and 10 weeks were used. The animals were kept in temperature-controlled rooms (22–25°C) with a clear-and-dark cycle of 12–12 hours, and they were given unlimited access to food and water. The Comission on

Ethics in Animal Use of the Federal University of Pernambuco has deemed the study to be favorable (nº. 122/2019) and all execution procedures are following Brazilian laws governing animal experimentation.

2.5 Evaluation of PgEO-gel hematologic capacity

According to Costa et al. (2020), the hemolytic activity test evaluated the PgEO-gel's toxicological profile. Ultimately, a cardiac puncture was performed in sedated mice and then transferred to a tub containing EDTA acid, which was centrifuged at 3,000 rpm for 10 minutes. Later, the cell pellet was homogenized with saline solution at a concentration of 2% (v/v). Different concentrations of PgEO-gel (0,250–10,0 mg/mL) were added to 96-well plates, and they were then incubated for 60 minutes at 28°C with a solution of erythrocytes in triplicate. After the incubation period, a new centrifugation was carried out under the same conditions as before, and the supernatant was collected to determine the amount of hemoglobin released in the 540 nm spectrophotometer. The Triton X was used as a positive control.

2.6 Dermal irritability

The irritability test was carried out on Mice swiss males. The experimental protocol was created in accordance with Directives 404 for Economic Cooperation and Development (OCDE, 2002). The mice were grouped into a single group (n = 3), and 500 mg of PgEO gel was topically applied to the rasped areas. The animals were monitored for mortality as well as some toxicology symptoms (tremors, salivation, convulsions, and diarrhoea) or degenerative skin effects (irritation, edema, itching, and erythema), paying particular attention to the first four hours and then once daily for a period of 14 days.

2.7 Microbial strains

For the evaluation of antimicrobial activity, four strains of gram-positive bacteria (*Staphylococcus aureus* ATCC 29213; *S. aureus* UFPEDA 670; *S. epidermidis* UFPEDA 183 and *Enterococcus faecalis* UFPEDA 138), seven strains of gram-negative bacteria (*Escherichia coli* ATCC 25922; *E. coli* UFPEDA 224; *Klebsiella pneumoniae* ATCC 700603; *K. pneumoniae* UFPEDA 396; *Pseudomonas aeruginosa* ATCC 27853; *P. aeruginosa* UFPEDA 416 and *Proteus mirabilis* UFPEDA 737) and five *Candida* (*Candida albicans* URM 5852; URM 6391; *C. glabrata* URM 6343; *C. tropicalis* URM 6557 and *C. parapsilosis* URM 6711). The ATCC strains were obtained from the American Type Culture Collection, the UFPEDA strains were obtained from the microorganisms collection of the Antibiotics Department of the Federal University of Pernambuco and the fungi were obtained from the URM culture collection (Universidade Recife Mycologia) of the Mycology Department of the University Federal of Pernambuco.

2.8 Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Microbicidal Concentration (MMC)

The CLSI M100 and M60 protocols were used to determine the minimum inhibitory concentrations (MIC) and minimum microbicidal concentrations (MMC) using the broth microdilution method. Using Mueller-Hinton broth for bacteria and RPMI-1640 for yeast, PgEO and PgEO-gel (2048–1 µg/ml) were serially diluted. Standardized microorganisms were then added to the wells at a concentration of about 10⁸ CFU/

mL for bacteria and 10^7 CFU/mL for yeast, and the plates were incubated at 37°C for bacteria for 24 hours or 25°C for 48 hours for yeast. As a colorimetric indicator of cell viability, 10 µL of 0.01% resazurin was added following this time frame. The lowest concentration that prevented microbial growth was then recorded as the MIC after the microplates were incubated once more for 4 hours.

Aliquots of 10 µL from the wells before application of resazurin were transferred to Petri dishes containing Mueller-Hinton-Agar (MHA) for bacteria or Sabouraud Dextrose Agar (SDA) for yeast and incubated at 37°C for 24 h for bacteria or 25°C for 48 h for yeast. CMM was considered the lowest concentration from which no microbial growth was observed after cultivation. Assays were performed in triplicate and Ampicillin and Fluconazole were used as positive controls.

2.9 Wound model

On day 0, the animals were anesthetized by intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (5 mg/kg) and the back shaved and cleaned with 70% ethyl alcohol and size 1 open excision wounds $\times$ 1 cm and 2 mm deep with the aid of a punch. After wound induction, the mice were housed in cages (Boudjelal et al., 2020).

2.10 Experimental design

After wound induction, mice were randomly divided into three groups ($n = 8$). 24 h after wound induction, the following treatment groups were performed; group I, negative control where the wounds were not treated; group II, positive control where the animals were treated with FitoScar® ointment; and group III, treated with a 1% PgEO-gel formulation. All treatments were applied topically once a day for 14 days, using a sterile Swab, in the amount of 0.5 g. On the 15th day after starting treatment, the animals were anesthetized by intraperitoneal injection of a mixture of ketamine (10 mg/kg) and xylazine (5 mg/kg). Blood samples were collected.

2.11 Wound contraction

The wound of each mouse was photographed immediately after formation and on the 8th and 15th day after injury induction and the wound area was measured using Image J software. The percentage of wound contraction was calculated following the model described by Boudjelal et al., (2020) for each animal on multiple days using the following formula: % Wound Contraction = $[(\text{Initial Wound Area} - \text{Day Specific Wound Area}) / \text{Initial Wound Area}] \times 100$.

2.12 Measurement of serum cytokine levels

Serum TNF- α and IL-1 β levels were measured by ELISA kits according to the instructions provided by the manufacturer. Results are expressed as pictograms of cytokines per milliliter of serum (pg/ml).

2.13 Determination of antioxidant system defense parameters

The evaluations of the oxidative stress parameters were carried out on blood samples collected by cardiac puncture at the time of the animals' euthanasia. After centrifuging the blood samples, Bradford (1976)'s method was used to obtain the soro and determine the protein content. The peroxidation of lipids was evaluated by estimating the level of substances reactive to tiobarbitric acid (TBARS), with the results expressed in nmol of malondialdehyde (MDA) per mg of protein (Ohkawa et al., 1979). Superoxide dismutase (SOD) activity was assessed using the cinétique of preventing the oxidation of epinephrine in accordance with 480 nm absorbance, with the enzyme activity of the treated material expressed in U/mg of protein. The activity of catalase (CAT) was measured at 240 nm to observe the change in absorbance between the first and sixth minutes; the enzyme activity of the CAT-containing material is expressed in mU/mg of protein (Beers and Sizer, 1952).

2.15 Statistical analyzes

Statistical analysis was performed using Graph Pad Prism software version 8.0. One-way ANOVA followed by Tukey's post-test was used when analyzing data for an independent variable. Data were expressed as mean $\pm$ SEM, and $p < 0.05$ was considered statistically significant.

3. Results and discussion

Wound treatment can be done with different pharmacological agents, which can be administered locally or systemically. However, the benefits of topical formulations over systemically administered drugs include deeper skin penetration, greater bioavailability, and immediate action at the application site (Dantas et al., 2016; Kazemi et al., 2020). Additionally, topical administration using gels can prolong the duration of cutaneous drug resistance and increase drug delivery and release by prolonging drug residence at the injection site (Karadzovska et al., 2013). Therefore, in our study, a gel containing the essential oil obtained from the leaves of *P. glaziovianum* was produced and treated in excisional wounds in mice.

As the physicochemical behavior and stability of the preparation during manufacture, storage and application can be anticipated in physicochemical assays, we performed the characterization of PgEO-gel in the first part of our study. The PgEO-gel was evaluated 24 hours after it was obtained, and showed a translucent white color, with the characteristic odor of the essential oil of *P. glaziovianum*, with no signs of macroscopic instability even after the centrifugation resistance test. When evaluating the pH of the formulation we had a value of 5.03 ± 0.0156 (0.32) and the viscosity was equal to $134,810 \pm 3505$ mPa.s (2.06%).

After the characterization, the PgEO gel was submitted to safety evaluation tests. Initially, the ability of the gel to cause hemolysis of erythrocytes was evaluated and the results showed that the hemolysis rate ranged from 0.12–0.17%, indicating a low ability to cause hemolysis. Additionally, the evaluation of dermal irritation was carried out, the animals submitted to topical application of PgEO-gel did not show clinical signs of dermal toxicity and other adverse reactions such as pruritus, erythema, inflammation,

and edema during 14 days of observation. These results demonstrate that PgEO gel has low toxicity or is non-toxic for the development of topical formulations.

Previously PgEO was evaluated for safety in use, the rate of hemolysis showed that PgEO has a limited ability to lyse cells (0.33–1.78%). Oral administration with up to 5,000 mg/kg did not show mortality or physiological abnormalities during the investigation of acute toxicity (Costa et al., 2023).

Although several therapeutic products present in their formulation antibiotics, antiseptics and anti-inflammatory agents for the treatment of wounds, infection is a problem that delays healing because it can prolong the inflammatory stage by destroying the tissue, slowing the formation of collagen and delaying the components of epithelialization (Farahpour et al., 2020; Joughi et al., 2022). Therefore, the antimicrobial activity of PgEO and PgEO-gel were evaluated against gram positive and gram negative bacteria, as well as yeast.

PgEO showed activity against gram-positive and gram-negative bacteria, exhibiting minimum inhibitory concentrations (MICs) of 256–1024 µg/mL. The MIC against yeast varied between 512 and 1024 µg/mL. The gel containing PgEO showed a MIC that varied between 512 and 1024 µg/mL against gram-positive and negative bacteria, however it did not show results against *Pseudomonas aeruginosa*. Regarding the yeasts, the PgEO gel presented MIC that varied from 1024 to 2048 µg/mL for the different species of *Candida* (Table 1).

Table 1
Determination of the Minimum Inhibitory Concentration (MIC) and the Minimum Microbicidal Concentration (MMC) of PgEO and the gel containing PgEO.

Strains	PgEO (µg/mL)		PgEO Gel (µg/mL)		Amp (µg/mL)	Flu (µg/mL)
	MIC	MMC	MIC	MMC	MIC	MIC
<i>Gram-positive</i>						
<i>S. aureus</i> ATCC 29213	256	256	512	512	0,5	-
<i>S. aureus</i> UFPEDA 670	256	256	512	1024	4	-
<i>S. epidermidis</i> UFPEDA 183	256	512	512	1024	4	-
<i>E. faecalis</i> UFPEDA 138	256	512	512	1024	1	-
<i>Gram-negative</i>						
<i>E. coli</i> ATCC 25922	256	1024	1024	1024	4	-
<i>E. coli</i> UFPEDA 224	256	1024	1024	1024	4	-
<i>K. pneumoniae</i> ATCC 700603	512	1024	1024	2048	4	-
<i>K. pneumoniae</i> UFPEDA 396	1024	1024	1024	2048	8	-
<i>P. aeruginosa</i> ATCC 27853	512	1024	> 2048	> 2048	> 128	-
<i>P. aeruginosa</i> UFPEDA 416	1024	2048	> 2048	> 2048	> 128	-
<i>P. mirabilis</i> UFPEDA 737	512	1024	1024	2048	4	-
<i>Yeast</i>						
<i>C. albicans</i> URM 5852	512	1024	1024	2048	-	0,5
<i>C. krusei</i> URM 6391	512	1024	1024	2048	-	0,25
<i>C. glabrata</i> URM 6343	1024	1024	2048	> 2048	-	1
<i>C. tropicalis</i> URM 6557	1024	1024	2048	> 2048	-	1
<i>C. parapsilosis</i> URM 6711	1024	1024	2048	> 2048	-	2
MIC: minimum inhibitory concentration; MMC: minimum microbicidal concentration. Amp: Ampicillin; Flu: Fluconazole.						

This bactericidal potential of PgEO and PgEO-gel may be due to the presence of the major compounds 1,8-cineole, α -pinene and β -pinene present in the essential oil. Studies performed by Li et al. (2014) demonstrated that 1,8-cineole alters the shape and size of the bacterial cell of both gram-negative and gram-positive bacteria, as strains of *S. aureus* showed strong condensation of nuclear chromatin,

characteristic of apoptosis. On the other hand, strains of *E. coli* showed a reduction in nucleoplasm and nuclear chromatin, characteristic of necrosis. Li et al. (2022) also showed that 1,8 cineole had a significant inhibitory effect on dermatophytes.

Studies have shown that α -pinene has antimicrobial activity against gram positive and gram negative (Sousa Eduardo et al., 2018; Freitas et al., 2021). Additionally, this compound is capable of inhibiting *S. aureus* efflux pumps, an important mechanism of resistance and virulence (Freitas et al., 2021), in addition to exhibiting strong antifungal activity against non-albicans *Candida* yeasts (Mandras et al., 2021). β -pinene, on the other hand, showed antimicrobial and synergistic activity against MRSA (Silva Rivas et al., 2012). In addition, this substance also inhibits the adhesion of *Candida* species and the development of the biofilm, in addition to eliminating mature *Candida* biofilm populations (Alves-Silva et al., 2019).

PgEO gel effectively enhanced the healing of excision wounds without causing any noticeable skin irritation. Wound contraction calculated as a percentage reduction in wound area was monitored in all groups examined every seven days. The results shown in Fig. 1 show that the topical application of PgEO gel increases the wound contraction rate by $90.62\% \pm 7.89$ after 14 days of treatment. The positive control group, treated with the reference drug FitoScar®, presented contracting of $78\% \pm 4.07$. The negative control group, on the other hand, presented a wound closure contraction of $32.75\% \pm 3.53$.

Previously, PgEO demonstrated the ability to modulate the inflammatory response (Costa et al., 2022), the composition of the essential oil and its major components, such as 1,8-cineole, α -pinene and β -pinene, which played a significant role in driving the anti-inflammatory activity, are probably the cause of the faster healing efficiency of PgEO-gel.

Mohammed et al. (2022) produced an ointment containing 1,8-cineol that accelerated the healing of burn wounds, in addition to exerting antioxidant and anti-inflammatory effects. Wound repair may have been stimulated by regulation of the inflammatory response, this phase of healing is known to control collagen and fibroblast proliferation and synthesis, production of wound granulation tissue, and differentiation of fibroblasts into myofibroblasts in the granulation tissue. (Rezvani Ghomi et al., 2019). Furthermore, the major components present in PgEO have been identified as anti-inflammatory agents that inhibit several factors involved in inflammation (Khoshnazar et al., 2019; Zhang et al., 2020; Santos et al., 2022).

Cytokines IL-1, IL-6 and TNF- α are released in large amounts in response to tissue damage, which allows them to play a significant role in the inflammatory response and gradually decrease with the progress of the healing process (Landén, Li, Ståhle 2016; Arabska et al., 2020). Therefore, we evaluated the influence of PgEO-gel on the levels of pro-inflammatory cytokines.

The results shown in Fig. 2 demonstrate that treatment with PgEO gel was able to reduce TNF- α levels by 48.17%, while IL-1 β showed a reduction of 43.79%. FitoScar® reduced TNF- α and IL-1 β by 48.29% and 35.00%, respectively.

The level of IL-1 β and TNF- α was significantly restored by treating the animals for 14 days with PgEO gel, demonstrating remarkable improvement in inflammation with topical application. These results are consistent with previously published research using PgEO, in which IL-1 β and TNF- α values decreased with oral treatment, attenuating the inflammatory condition (Costa et al., 2022). This decrease may be an effect of the presence of 1,8-cineol, as in a study carried out by Ehrnhofer-Ressler et al. (2013), 1,8-cineol was applied on monocytes stimulated with LPS at a concentration of 1.5 $\mu\text{g/mL}$, the results revealed a considerable drop in TNF- α and IL-1 β levels, with a reduction of 99 and 84%, respectively.

To assess the involvement of PgEO-gel treatment in modulating the oxidative profile, plasma levels of superoxide dismutase (SOD), catalase (CAT) and Malondialdehyde (MDA) were evaluated. The results of the measured oxidative stress parameters are summarized in Table 2. After 14 days of treatment with PgEO-gel a significant increase in CAT (294.11%) and SOD (220.86%) levels and a reduction in MDA levels (27.90%). Similar results were observed in the group treated with FitoScar® (CAT: 370.58%; SOD: 211.73% and MDA (26.13%). However, the lowest antioxidant activity was observed in the negative control group.

Table 2
Effect of PgEO gel treatment on malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) levels in mice.

Parameter	Negative Control	PgEO gel	FitoScar®
MDA	6.81 $\pm$ 0.66	4.91 $\pm$ 0.51*	5.03 $\pm$ 0.30*
SOD	2.30 $\pm$ 0.35	5.08 $\pm$ 0.65*	4.87 $\pm$ 0.43*
CAT	0.17 $\pm$ 0.09	0.51 $\pm$ 0.08*	0.63 $\pm$ 0.09*
Malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT). Values represent mean $\pm$ SEM (n = 8/group). (*) indicates significant difference (p < 0.05) compared to the control.			

Oxidative stress occurs due to an imbalance between levels of antioxidants and reactive oxygen species, playing a negative role in the wound repair process. Thus, measuring the activity of antioxidant enzymes such as SOD and CAT is important, as it promotes the reduction of free radicals (Andjić et al., 2022; Mohammed et al., 2022). The results of the present study reported that PgEO gel has antioxidant effects, possibly due to the presence of active components such as terpenes, which exert antioxidant activity and wound repair capacity.

In a previous study, 1,8-cineole and α -pinene protected PC12 cells (rat pheochromocytoma) against oxidative damage induced by hydrogen peroxide, reducing the generation of intracellular reactive oxygen species while significantly increasing the expression of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Porres-Martínez et al., 2016).

Conclusion

The findings of this study show that the gel containing *P. glaziovianum* essential oil has antimicrobial activity against gram-positive and gram-negative bacteria and fungi. In addition, the results indicate that treatment with PgEO-gel improves the repair of skin wounds possibly due to its anti-inflammatory and antioxidant enzyme-stimulating action. Additionally, the gel showed low erythrocyte toxicity and dermal irritation, which may be encouraging for new preclinical and clinical trials in different types of wounds.

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Figures

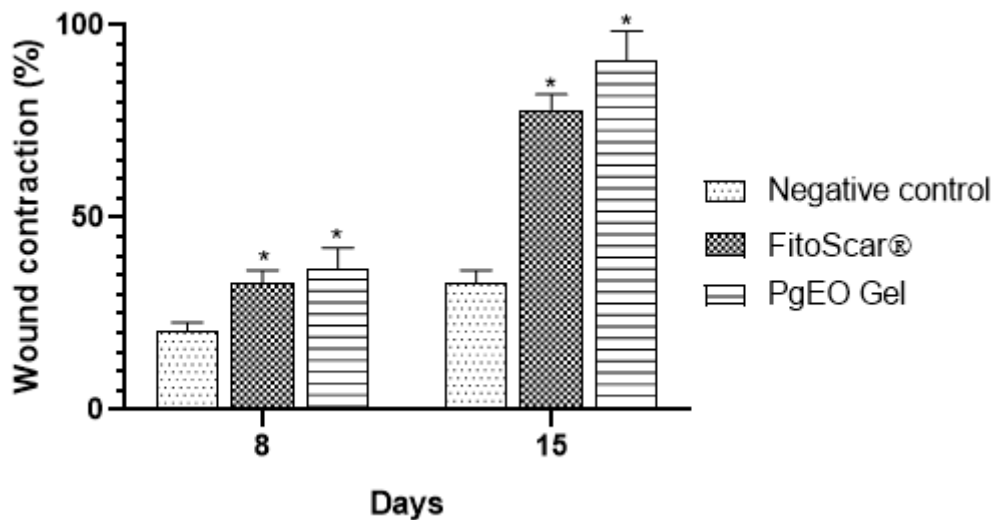


Figure 1

Effects of PgEO gel treatment on wound contraction in mice.

Values represent mean $\pm$ SEM (n = 8/group). (*) indicates significant difference (p < 0.05) compared to the control.

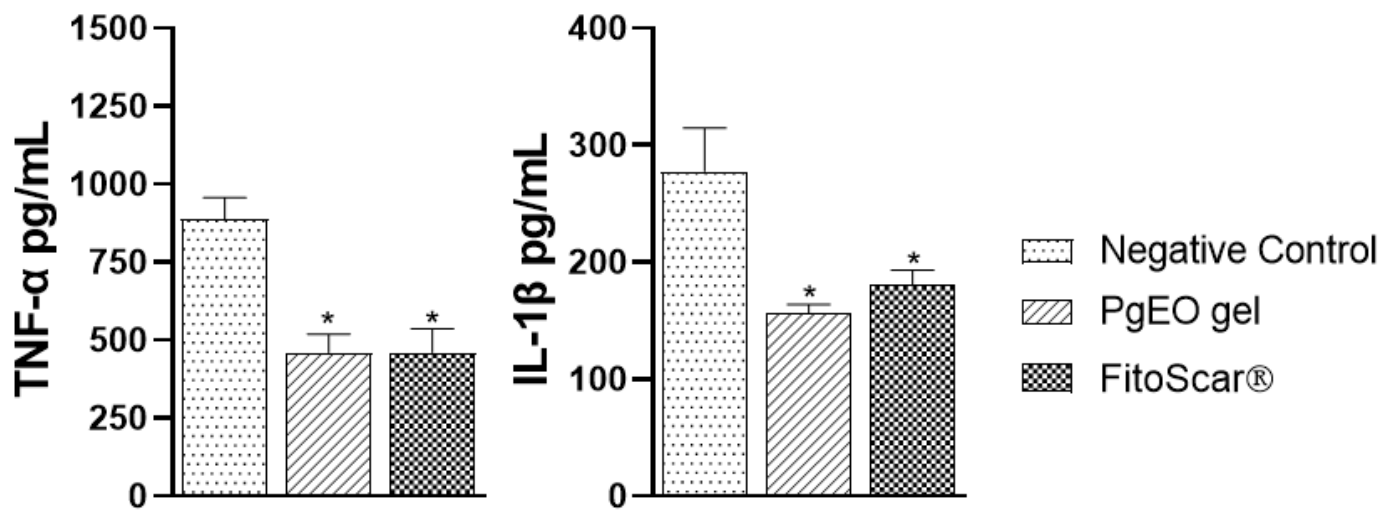


Figure 2

Effect of PgEO gel treatment on TNF- α and IL-1 β levels.

Values represent mean $\pm$ SEM. *p<0.05 compared to control, one-way analysis of variance (ANOVA) followed by Dunnett's test.