Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2023;48(2):472-479
DOI: 10.17826/cumj.1242762

RESEARCH

Effects of herbal mixture on burned rat skin infected with *Pseudomonas aeruginosa*

Pseudomonas aeruginosa ile enfekte olmuş ratlarda oluşan yanıklar üzerine bitkisel karışımların etkileri

Serdar Yiğit[1], Arzu Gezer[2], Çiğdem Eda Balkan Bozlak[3], Seyit Ali Bingöl[4], Seda Çelik[5]

[1]Kafkas University, Faculty of Medicine, Histology and Embryology Department, Kars, Turkey [2]Ataturk University, Vocational School of Health Services, Erzurum, Turkey [3]Kafkas University, Departments of Microbiology, Kars, Turkey [4]Kafkas University, Faculty of Medicine, Histology and Embryology Department, Kars, Turkey [5]Kafkas University, Department of Biochemistry, Kars, Turkey

Abstract

Purpose: The purpose of this study is to investigate the healing impact of an ointment derived from the *Alkanna tinctoria* plant upon *Pseudomonas aeruginosa*-induced infections.

Material and Methods: In this study, 18 male adolescent rats (mean age 6 weeks) weighed an average of 180 g were used. Animals were divided into 3 groups. Group 1: Control group (consisting of burns, no treatment was done), Group 2 *(P. aeruginosa*): Burn was created and infected with *P. aeruginosa*, Group 3 (Cream): *P. aeruginosa* was used to infect the burns area and the herbal mixture was administered twice a day, once in the morning and once in the afternoon. Under anesthesia, the backs of the rats were shaved, and a specially produced steel bar with a diameter of 1*1 cm was immersed in boiling water for 15 seconds before being applied to their backs for 20 seconds. The burned area was subsequently infected with the ATCC *Pseudomonas aeruginosa* strain, and samples were collected 24 hours later. To detect bacterial growth in this area, the samples were inoculated on blood and EMB (Eosin Methylene Blue) media in a microbiology laboratory. After inoculation, the animals were placed in separate sterile cages and randomly divided into three groups. Once the growth was observed, the tissue and blood samples were harvested from the rats on the 2nd, 7th, 14th, and 21st days.

Results: Epithelial regeneration in this group was more prominent. Vascularization was remarkable on the 2nd day, especially in the group in where we induced a burn and applied the ointment. VEGF levels increased more in the ointment group than in that of others. On the 2nd day of the study, the average bacterial count was 10^5 in sample of both 2nd and 3rd groups. At the end of the study, while the

Öz

Amaç: *Alkanna tinctoria* bitkisinden elde edilen bir merhemin *Pseudomonas aeruginosa* kaynaklı enfeksiyonlar üzerindeki iyileştirici etkisini araştırmaktır.

Gereç ve Yöntem: Bu çalışmada ortalama 180 gr ağırlığında 18 adet erkek ergen sıçan (ortalama yaş 6 hafta) kullanıldı. Hayvanlar 3 gruba ayrıldı. Grup 1: Kontrol grubu (sadece yanık oluşturuldu, herhangi bir tedavi yapılmadı), Grup 2 (*P. aeruginosa*): Yanık oluşturuldu ve *P. aeruginosa* ile enfekte edildi, Grup 3 (Krem): Yanık oluşturularak *P. aeruginosa* ile enfekte edilerek bitki karışımı sabah akşam uygulandı. Anestezi altında sıçanların sırtları tıraş edildi ve özel olarak üretilmiş 1*1 cm çapında çelik çubuk 15 saniye kaynar suda bekletildikten sonra 20 saniye sırtlarına uygulandı. Yanan alan daha sonra ATCC *Pseudomonas aeruginosa* suşu ile enfekte edildi ve örnekler 24 saat sonra toplandı. Bu alanda bakteri üremesini saptamak için, numuneler bir mikrobiyoloji laboratuvarında kan ve EMB (Eosin Metilen Mavisi) besiyerine inoküle edildi. Aşılamadan sonra hayvanlar ayrı steril kafeslere yerleştirildi ve rastgele üç gruba ayrıldı. Üreme görüldükten sonra 2., 7., 14. ve 21. günlerde sıçanlardan doku ve kan örnekleri alındı.

Bulgular: Merhem uygulanan grupta epitelyal rejenerasyon daha belirgindi. Özellikle yanık oluşturup pomad uyguladığımız grupta 2. gün damarlanma dikkat çekiciydi. VEGF seviyeleri merhem grubunda diğerlerine göre daha fazla arttı. Çalışmanın 2. gününde, hem 2. hem de 3. grup örneklerinde ortalama bakteri sayısı 105'tir. Çalışmanın sonunda, 2. gruptaki bakteri sayısı ortalaması artarken, 3. gruptaki bakteri sayısı ortalaması azalmıştır.

Sonuç: *A. tinctorial*'dan elde edilen merhemin epitel dokuyu başarılı bir şekilde onardığı ve kanda artan

Address for Correspondence: Serdar Yiğit, Kafkas University, Faculty of Medicine, Histology and Embryology Department, Kars, Turkey Email: yigitserdar85@gmail.com
Received: 26.01.2023 Accepted: 31.05.2023

average of bacterial count was increased in the 2nd group, the average of bacterial count was decreased in the 3rd group.
Conclusion: It was concluded that the ointment obtained from A. tinctorial successfully repaired the epithelial tissue and contributed to the healing of wounds by modifying increasing VEGF in the blood. However, further research is needed before this ointment can be highly recommended for therapeutic usage.
Keywords:. Burn, *Alkanna tinctoria*, VEGF, rat, *pseudomonas aeruginosa*

VEGF'yi modifiye ederek yaraların iyileşmesine katkı sağladığı sonucuna varıldı. Bununla birlikte, bu merhem terapötik kullanım için tavsiye edilmeden önce daha fazla araştırmaya ihtiyaç vardır.

Anahtar kelimeler: Yanık, *alkanna tinctoria*, VEGF, sıçan, *pseudomonas aeruginosa*

INTRODUCTION

As far as burn infections are concerned, the burned wound surfaces become vulnerable to infections particularly post second-degree burns. For this reason, eradicating resistant microorganisms detected on the wound surface in burn patients may entail complicated and long processes for both the doctor and the patient. Throughout this emotionally tough process, additional antibiotics may be required to be given to the patient as part of the treatments launched upon the diagnosis of a secondary infection at the wound site. Besides being abundant in nature, the bacteria of Pseudomonas spp. also cause serious infections not only for burn victims but also for immunosuppressive patients as well. These bacteria are particularly dangerous in the case of hospital-acquired infections. Herbal and pharmacological treatments have remained popular today due to the benefits of antimicrobials applied to the wound surface. Vascular Endothelial Growth Factors (VEGFs) are a family of polypeptides with a highly conserved receptor-binding cysteine-knot structure like that of platelet-derived growth factors[1].

While inflammatory cell infiltration ceases a few days after injury, sustained pro-inflammatory and decreased anti-inflammatory cytokine levels have been reported in infected wounds during the acute healing process. Chronic inflammation reduces the mechanical integrity of the temporary matrix, which limits the formation of new blood vessels[2]. The use of *A. tinctoria* to treat wounds in traditional medicine can be traced back to the times of Hippocrates and Theophrastus. Due to such properties, these active compounds have received increasing attention in the pharmaceutical, cosmetic, and food sectors in recent years[3]. However, most of the research on *A. tinctoria* has focused either on its chemical composition or the function of the active components.

It is utilized as a dye in the cosmetic and textile industries. *A. tinctoria* roots have been used to cure wounds since ancient times. Numerous researchers from all around the globe have investigated plants' antibacterial properties[4]. According to the World Health Organization (WHO), nearly 2000 therapeutic plants are found in 91 countries. Because of their expanding popularity in treating or preventing numerous diseases, medicinal herbs are still used by rural communities[5]. While data on the therapeutic applications of medicinal plants in Pakistan is scant, *Alkanna tinctoria* is being explored for its numerous therapeutic purposes. However, it remains to be documented in terms of multidrug-resistant bacteria activity and phytochemical content[6]. Plants act as reservoirs for a wide variety of secondary metabolites with medicinal qualities, such as alkaloids, flavonoids, tannins, and terpenoids[7].

These secondary metabolites are enantiomeric naphthoquinones that are produced by the roots. In the phospholipid layer, they split and gather to form granules. Thus, they can be found in the fungal layer of mature roots. Besides, their accumulation leads to the red or purple color of the root[8]. Furthermore, compared to other Alkanna species, *A. tinctoria* generates a large quantity of alkannin/shikonin (A/S) and its derivatives. A/S derivatives play an important role in the plant's antibacterial activity and establish a chemical barrier against soil-borne microbes. They are also recognized to have wound healing, anticancer, and anti-inflammatory effects[9]. They include the active pharmaceutical components of effective wound-healing medications licensed by Greece's National Medicines Organization[10]. These taken into account, the present study aims to determine the healing effect of *A. tinctorial* plant upon burn wounds to generate A/S for medical applications considering its therapeutic properties. The hypothesis of this study is to determine whether *A. tinctoria* can accelerate infected wound healing in

burns due to its anti-inflammatory effect. We think that our finding on the effect of *A. tinctoria* on infected burns will contribute to the literature.

MATERIALS AND METHODS

Sample

This study was approved by the Kafkas University's Faculty of Veterinary Animal Experiments Local Ethics Committee with the decision number 2020-92, in which 18 male adolescent rats (average age 6 weeks) weighing approximately 180 grams were used. Animals were divided into 3 groups. Group 1: Control group (no treatment was done), Group 2 (*P. aeruginosa*):

Procedure

Burn was created and infected with *P. aeruginosa*, Group 3 (Cream): *P. aeruginosa* was used to infect the burns area. The herbal mixture was administered twice a day, once in the morning and once in the afternoon. (Table 1) The animals were subjected to standard feeding (ad libitum), housing, and maintenance conditions in a laboratory setting with 12 hours of light and 12 hours of darkness at constant temperature and humidity. The rats were divided into three groups, for which sterile microsurgical sets were prepared during the surgical procedure and whose tissues were collected on the 21st day using the punch biopsy method[11]. Then, they were anesthetized via an aseptic intraperitoneal infusion of 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride under aseptic conditions. Next, the backs of the rats were shaved, and the specially prepared metal with a diameter of Steel 1*1 cm was immersed in boiling water for 15 seconds before being applied to their backs for 20 seconds. Finally, the rats with burns were administered 0.02 micg/kg subcutaneous 2x1 injections of fentanyl citrate and analgesics for three days. Our study was launched once the histological evidence showed that second-degree burns had been acquired on the skin at the temperature and time achieved in the pilot study. Once the burn area had been infected with the ATCC *Pseudomonas aeruginosa* strain, culture samples were harvested 24 hours later to determine whether there was bacterial growth. The samples were then inoculated in blood and EMB (Eosin Methylene Blue) media in a microbiology laboratory. The animals were placed in sterile cages separated after cultivation before being randomly divided into three groups. Following the detection of growth, sample tissues were harvested from the rats using a punch biopsy on the 2nd, 7th, 14th, and 21st days under anesthesia. As for the blood samples, they were collected from the tail vein.

Table 1. Experimental groups and procedures.

Group Number	Number of rats	Groups
1	6	Control Group (consisting of burns)
2	6	*P. aeruginosa* Group (*P. aeruginosa* was used to infect the burns area)
3	6	Cream Group (*P. aeruginosa* was used to infect the burns area. The herbal mixture was administered twice a day, once in the morning and once in the afternoon)

Histological analysis

The tissue samples were taken from each animal group (n = 6)[12]. Under anesthesia through intraperitoneal administration of 50% ketamine and 10mg/kg of xylazine hydrochloride. The skin area to be infected was prepared aseptically, and the rats were then fixed on the operating table in ventral posture before the designated area could be marked. The tissue samples were put in formaldehyde and prepared for histopathological processes after collection in such a way that the other burn areas would not be harmed. During 48 hours, the tissue samples were quickly fixed in 3.7% solution formaldehyde. All samples were routinely processed for histology tissue after fixation. All tissues were passed to increasing alcohol (50, 60, 70, 80, 96, 99-1 h) series for histological tissue processing. The tissues were kept in xylene for 15 min three times. Ultimately, they were held in molten liquid paraffin that was both soft and firm. Then, each tissue sample was blocked in paraffin. After that, 5 μm thick slices were taken from each paraffin block. This treatment was performed on the 2nd, 7th, 14th, and 21st days. The sections were stained with hematoxylin and eosin, Masson trichrome, and Periodic acid sift. The percentage of epithelialization, scar formation,

inflammation, and angiogenesis were evaluated in all the samples[13].

Hematoxylin and eosin (H&E) staining protocol

After the deparaffinization process, the slides were kept in xylene for 30 min. Then, they were put in three different series of 96%, 80% and 70% alcohol respectively for per 10 min. Subsequently, the slides were retained in hematoxylin dye for 1 min, in Acid-Alcohol mixture for 1 min, in eosin solution for 1 min, wash in water for 1 min, in 70% alcohol for 10 min, in 80% alcohol 10 min, in 96% alcohol 10 min, in three separate xylene series for 10 min each, respectively. At the end this process, and they were covered with entellan.

Masson trichrome staining protocol

After deparaffinization process, they were kept in xylene for 30 min, in absolute alcohol for 5 min, in 96% alcohol for 5 min, in 80% alcohol for 5 min, in 70% alcohol for 5 min, in distilled water for 5 min, in iron hematoxylin + ferric choloride for 10 min, in distilled water for 5 min, in Ponceu-acid fuchsin solution for 15 min, in distilled water for 5 min, in Phosphotungustc acid for 15 min, in aniline blue for 5 min, in distilled water for 5 min, in 70% alcohol for 5 min, in 80% alcohol for 5 min, in 96% alcohol in 80% alcohol for 5 min, absolute alcohol in 80% alcohol for 5 min, in three separate xylene series for 10 min each, and covered with entellan, respectively.

Periodic Acid-Schiff (PAS staining) protocol

After the deparaffinization process, the slides were held in xylene for 30 min, in Absolute alcohol for 5 min, in 96% alcohol for 5 min, in 70% alcohol for 5 min, in 80% alcohol for 5 min, in 70% alcohol in 80% alcohol for 5 min, distilled water for 5 min, Periodic acid for 5 min, distilled water for 5 min, Sciff's reagent for 15 min, in running tap water for 5 min, in Hemotoxylene for 5 min, in 70% alcohol for 5 min, in 80% alcohol for 5 min, in 96% alcohol for 5 min, in absolute alcohol for 5 min, in three separate xylene series for 10 min each, and covered with entellan, respectively.

Biochemical analysis

The plasma levels of VEGF from the blood samples having been harvested from the tail vein of the rats on the 2nd, 7th, 14th, and 21st days were used randomly by the enzyme-linked immunosorbent assay method (ELISA) (Biosource International, Nivelles, Belgium). VEGF levels were measured in the blood of animals based on the small amount of plasma sample required for the analysis kit, which has a high degree of sensitivity and specificity specific to rat studies.

Statistical analysis

SPSS 20.0 (SPSS 20.0 Software Package Program) was used to perform the statistical analysis. One-way ANOVA, Tukey's test of Post hoc-tests, was used for comparisons between groups. The threshold for statistical significance between groups was set at $p<0.05$

RESULTS

ELISA findings

When the VEGF levels of groups are concerned, the cream group had the highest level of VEGF and P. aeroginose group had the lowest level of VEGF. There was a statistically significant difference between groups in terms of VEGF levels ($p<0.05$) (Table 2).

Table 2. Comparison of VEGF level between groups.

Groups	Mean +SD	P
2.day		
Cream	0.05+0.05	0.0081*
P. aeruginosa	0.24+0.01	0.2431
Control	0.37+0.09	0.0001*
7.day		
Cream	0.50+0.04	0.0001*
P. aeruginosa	0.27+0.03	0.7051
Control	0.52+0.02	0.0001*
14.day		
Cream	0.61+0.048	0.0001*
P. aeruginosa	0.27+0.04	0.672
Control	0.59+0.007	0.0001*
21.day		
Cream	0.72+0.00	0.0001*
P. aeruginosa	0.31+0.00	0.0007*
Control	0.62+0.00	0.0001*

Mean +SD (The mean ± standard deviation), cream group (*P. aeruginosa* was used to infect the burns area. The herbal mixture was administered twice a day), *P. aeruginosa* (*P. aeruginosa* was used to infect the burns area), Control group (consisting only of burns). *$p<0.05$.

Histopathological findings

Table 2 presents the burn histology scoring. On the 2nd day,all the groups showed acute inflammatory cells. It was remarkable to see vascularization on the 2nd day, especially in the group in which we had induced a burn and applied the ointment. On the 7th, the ointment group showed considerable improvement (Table 3). Hair follicles were more prominent in this group than in the others. Also, epithelial regeneration in this group was more prominent (Figure 1). The areas of inflammation observed in the control group were noticeable on the 14th day. On the 21st day, not only the dermis but also the epidermis was normal in the ointment group. In addition, collagen was normal in Masson trichrome staining on the 21st day, just as the basement membrane was normal in PAS staining. (Figure 2).

Table 3. Histopathological scores in burn and treatment groups.

Histopathologic parameters	Control				*P. aeruginosa*				Cream			
	2. day	7. day	14. day	21. day	2. day	7. day	14. day	21. day	2. day	7. day	14. day	21. day
Epithelial regeneration	-	-	+	+	-	-	-	+	+	++	+++	+++++
Inflammatory cell	+	+	++	+++	+	+	++	++	++	+++	++++	++++
Vascularization	-	-	+	++	-	-	-	+	+	++	+++	++++
Keratinization	-	-	+	++	-	-	+	+	-	+	++	+++

negative, + very mild, ++ mild, +++ mild to moderate, ++++ moderate, +++++ severe.

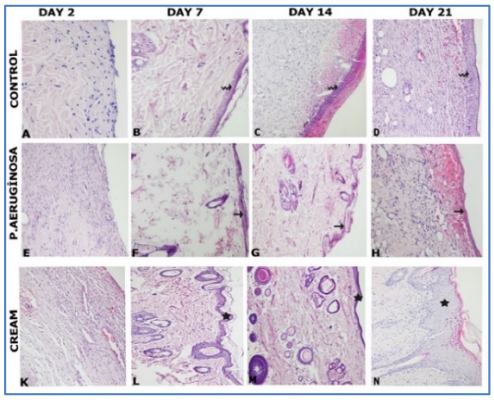

Figure 1. A) Control Group, 2nd day, Hematoxylin and eosin staining (H&E), 20x magnification. B) 7th day, H&E staining, 20x magnification, convoluted arrow; epithelia. C) 14th day, H&E staining, 20x magnification, convoluted arrow; epithelia. D) 21st day, H&E staining, 20x magnification, convoluted arrow; epithelia. E) *P. aeruginosa* Group, 2nd day, H&E staining, 20x magnification. F) 7th day, H&E staining, 20x magnification, normal arrow; epithelia. G) 14th day, H&E staining, 20x magnification, normal arrow; epithelia. H) 21st day, H&E staining, 20x magnification, normal arrow; epithelia. K) Ointment Group (Burn+ *P. aeruginosa* +Ointment), 2nd day, H&E staining, 20x magnification. L) 2nd day, H&E staining, 20x magnification, star; epithelia. M) 21st day, H&E staining, 20x magnification, star; epithelia. N) 21st day, H&E staining, 20x magnification, star; epithelia.

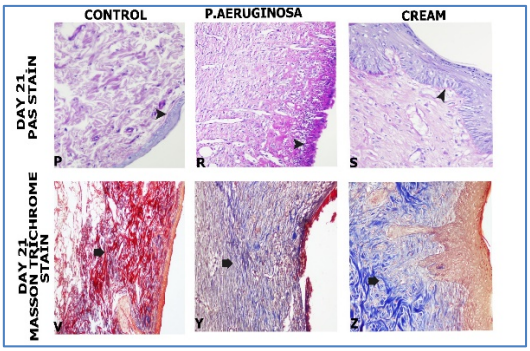

Figure 2. P) Control Group, 21st day, Periodic Acid Schiff (PAS) staining, 20x magnification R) *P. aeruginosa* Group. 21st day, PAS staining, 20x magnification, arrowhead; basal membrane. S) Ointment Group (Burn+ *P. aeruginosa* +Ointment). 21st day, PAS Staining, 20x magnification, arrowhead; basal membrane V) Control Group. 21st day. Masson Trichrome staining, 20x magnification, thick arrow; ligament Y) *P. aeruginosa* Group. 21st day, Masson Trichrome staining, 20x magnification, thick arrow; ligament. Z) Ointment Group (Burn+ *P. aeruginosa* +Ointment). 21st day. Masson Trichrome staining, 20x magnification, thick arrow; ligament.

Microbiology findings

The samples were taken from the wounds with sterile swab which was inoculated into blood and EMB medium. On the 2nd day of the study, the average bacterial count was 10^5 in sample of both 2nd and 3rd groups. At the end of the study, while the average of bacterial count was increased in the 2nd group, the average of bacterial count was decreased in the 3rd group (Table 4).

Tablo 4. Results of the *P. aeruginosa* numbers by days.

	Control	*P. aeruginosa*	Cream
DAYS	Non infected	Infected not threated with mixture	Infected and threated with mixture
DAY 2	-	10^5 microorganism / ml	10^5 microorganism / ml
DAY 7	-	10^5 microorganism / ml	10^3 m microorganism / ml
DAY 14	-	10^7 microorganism / ml	10^3 microorganism / ml
DAY 21	-	10^8 microorganism / ml	10^2 microorganism / ml

DISCUSSION

A. tinctoria is known for having antimicrobial effects upon the epidermis, thus being very effective in treating injuries. Also, it exhibits anticancer and antithrombotic properties in addition to not having toxic effects[14]. The purpose of the present study is to investigate the effectiveness of *A. tinctoria* upon experimentally burned wounds that were infected by *P. aeruginosa*. Burned wound treatments aim to regenerate the vascularization, re-epithelialization, and collagen production of the burned skin. Scientific studies conducted thus far have aimed to demonstrate the therapeutic effects of the direct use of herbal cures, particularly those based on plant mixtures and extracts. Many studies in the literature have revealed that the epidermis and dermis layers are severely damaged in burned cases[15]. Medicinal herbs, which are increasingly gaining popularity, particularly in rural communities, are used not only in curing various infections but also in preventing them as well. *A. tinctoria* is one of these therapeutic plants that is widely utilized for its medicinal properties across the Mediterranean countries. *A. tinctoria* generates quinone and phenolic chemicals, as well as antioxidants, in addition to antibacterial and anti-inflammatory effects[16]. The present study used the antibacterial and anti-inflammatory properties of this plant to add to the literature on the efficacy of herbal remedies. In experimentally induced lesions, the epithelialization values of the group given a cream derived from the *A. tinctoria* were statistically more useful than those in the other groups[17]. Another study into the wound-healing properties of *A. tinctoria* has found that this herb boosts the production of fibroblasts, which is of the most crucial phases of healing that stimulate angiogenesis[18]. Studies have also shown that elevated fibroblast activity and angiogenesis help epithelialization of the wound more rapidly[19]. Besides, scientific studies argue that alkannin and shikonin found in *A. tinctoria* have a strong effect on wound healing[20]. In line with the literature, the present study demonstrated that the *A. tinctoria* mixture significantly boosted fibroblasts and angiogenesis compared to the other groups. For this study, we used Masson's trichrome, a special staining technique used in wound healing research. Masson's trichrome staining is widely used in pathology laboratories to distinguish between collagen and smooth muscle in tumors and to determine collagen growth in such diseases as cirrhosis. It is also a type of staining commonly used for liver and kidney tissues[21]. Given that the staining process in question shows the migration and restructuring of collagen fibers in the skin following a burn, it is crucial in detecting wound healing[22]. This staining is used to identify collagen fibers as well as other skin components such as dark hair follicles and adipose tissues[23]. In this study, the relevant data were used to determine the wound healing score once the structures were detected thanks to this staining. The basement membrane, which plays a crucial role in burn healing, is responsible for fusing the epidermis and dermis. Therefore, the recovery takes the form of a scar if this membrane has been damaged during the burn[24].

The present study performed the Periodic Acid Schiff staining to highlight the burns-induced basal membrane damage. The ointment group had the most regular basement membrane of all study groups. Traditional Chinese medicine is widely used in the treatment of wounds in clinical practice due to its

beneficial effects in China and other Asian countries. Traditional Chinese medicine is commonly used in wound healing due to its established advantages not just in China but also in other Asian countries as well. However, compelling evidence for safe application is lacking and its mechanisms remain to be clarified. Excisional wounds have long been associated with disturbed tissue integrity, resulting in vascular injury. Fibrin fibronectin promotes clot formation and platelet uptake. As a result, it regulates growth factors and cytokines, including VEGF and bFGF[25]. Granulation tissue formation is known to promote wound healing, which is also aided by vascular endothelial and fibroblast cells. It is commonly believed that VEGF stimulates endothelial cell proliferation and migration, promotes vascular permeability and angiogenesis, and increases collagen deposition[26]. Repair of scar tissue occurs through the proliferation of many extracellular matrix proteins and fibroblast cells, which are growth factors[27]. Angiogenesis, which is required for wound healing, feeds new tissue with both oxygen and metabolites. Furthermore, it eliminates metabolic waste products[28]. VEGF is crucial to facilitate oxygen and nutrient delivery by enhancing angiogenesis during wound healing and tissue regeneration[29]. Furthermore, VEGF also mediates vascular hyperpermeability and promotes the secretion of both active growth factors and cytokines that are essential for wound repair[30]. In our study, in which a burn model was created, the VEGF growth factors were significantly higher in the ointment group than in the normal wound group ($p<0.05$). Our histopathological results also demonstrated that re-epithelialization was higher in the ointment group than the others ($p<0.05$), which also confirmed our ELISA results. As a result, we suggest that this cream should be used in clinical settings. However, further research is needed before this ointment can be highly recommended for therapeutic usage. An important of the limitations of our study is not to check the VEGF levels by using Real Time PCR. In order to apply the cream, we studied in this study, especially against the hospital infection that occurs on burns area, we need to evaluate more advanced techniques.

Author Contributions: Concept/Design : SY, SAB; Data acquisition: SY, ÇEBB, Data analysis and interpretation: SY,AG; Drafting manuscript: SY,AG; Critical revision of manuscript: SY, SAB; Final approval and accountability: SY, SAB, AG, ÇEBB, SÇ; Technical or material support: SY; Supervision: SAB, SY; Securing funding (if available): n/a.
Ethical Approval: Ethical approval was obtained for this study from the Ethics Committee of the Faculty of Veterinary Medicine, Kafkas University with the decision dated 26.03.2020 and numbered 2020/092.
Peer-review: Externally peer-reviewed.
Conflict of Interest: The authors declared that there is no conflict of interest.
Financial Disclosure: The authors declare no financial support.

REFERENCES

1. Holmes, D. I.; Zachary, I., The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease. Genome Biol. 2005;6:209.
2. Arroyo, A. G.; Iruela-Arispe, M. L., Extracellular matrix, inflammation, and the angiogenic response. Cardiovasc. Res. 2010;86:226-35.
3. Malik S., Bhushan S., Sharma M., Ahuja P. S., Biotechnological approaches to the production of shikonins: a critical review with recent updates. Crit. Rev. Biotechnol. 2016;36:327-40.
4. Gümüş K., Özlü Z. K., The effect of a beeswax, olive oil and alkanna tinctoria (l.) tausch mixture on burn injuries: An experimental study with a control group. Complement. Ther. Clin. Pract. 2017;34:66-73.
5. WHO. WHO traditional medicine strategy: 2014-2023. World Health Organization. 2013.
6. Elsharkawy E., Elshathely M., Jaleel G. A., Al-Johar H. I. Anti-inflammatory effects of medicinal plants mixture used by Bedouin people in Saudi Arabia. Herba Pol. 2013:59.
7. Talib W. H., Mahasneh A. M., Antimicrobial, cytotoxicity and phytochemical screening of Jordanian plants used in traditional medicine. Molecules (Basel, Switzerland). 2010;15:1811-24.
8. Brigham L. A, Michaels P. J, Flores H. E. Cell-specific production and antimicrobial activity of naphthoquinones in roots of lithospermum erythrorhizon. Plant Physiol. 1999;119:417-28.
9. Papageorgiou V. P, Assimopoulou A. N, Couladouros E. A, Hepworth D, Nicolaou K. C. The chemistry and biology of alkannin, shikonin, and related naphthazarin natural products. Angew Chem. Int. Ed. Engl. 1999;38:270-301.
10. Papageorgiou V. P, Assimopoulou A. N, Ballis A. C. Alkannins and shikonins: a new class of wound healing agents. Curr. Med. Chem. 2008;15:3248-67.
11. Desai M. H, Rutan R. L, Herndon D. N. Conservative treatment of scald burns is superior to early excision. J Burn Care Res J. 1991;12:482-4.
12. Salhi N, El Guourrami O., Rouas L., Moussaid S., Moutawalli A., Benkhouili F. Z et al. Evaluation of the Wound healing potential of cynara humilis extracts in the treatment of skin burns. Evid. Based Complementary Altern. Med. eCAM. 2023;2023:5855948.
13. Kumar V, Abbas AK, Fausto N. Cellular adaptations, cell injury, and cell death. In: Kumar V, Abbas AK, Fausto N, eds. Robbins and cotran pathologic basis of disease. 7th ed. Philadelphia, PA: Saunders Elsevier. 2005;3-46.
14. Esfahani H. M., Esfahani Z. N., Dehaghi N. K., Hosseini-Sharifabad A., Tabrizian K., Parsa M et al.

Anti-inflammatory and anti-nociceptive effects of the ethanolic extracts of Alkanna frigida and Alkanna orientalis. J. Nat. Med. 2012;66:447-52.
15. Avsar U., Halici Z., Akpinar E., Yayla M., Avsar U., Harun U et al. The Effects of Argan Oil in Second-degree Burn wound healing in rats. Ostomy Wound Manag. 2016;62:26-34.
16. Takci H. A. M., Turkmen F. U., Anlas, F. C., Alkan F. U., Bakirhan P., Demir C et al. Antimicrobial activity and cytotoxicity of alkanna tinctoria (l.) tausch root extracts. Karadeniz Fen Bilimleri Dergisi. 2019;9:176-85.
17. Moustafa A., Atiba A. The effectiveness of a mixture of honey, beeswax and olive oil in treatment of canine deep second-degree burn. Glob. Vet. 2015;14:244-50.
18. Yazdinezhad A., Monsef-Esfahani, H., Ghahremani M. H. Effect of alkanna frigida extracts on 3t3 fibroblast cell proliferation. Int. J. Pharm. Biol. Sci 2013;3:212-15.
19. Abdulrhman M., Elbarbary N. S, Ahmed Amin D., Saeid Ebrahim R., Honey and a mixture of honey, beeswax, and olive oil-propolis extract in treatment of chemotherapy-induced oral mucositis: a randomized controlled pilot study. Pediatr. Hematol. Oncol. 2012;29:285-92.
20. Sengul M., Yildiz H., Gungor N., Cetin, B., Eser Z., Ercisli S. Total phenolic content, antioxidant and antimicrobial activities of some medicinal plants. Pak. J. Pharm. Sci. 2009;22:102-6.
21. Zhang L., Liu C., Yin L., Huang C., Fan S. Mangiferin relieves CCl4-induced liver fibrosis in mice. Sci. Rep. 2023;13:4172.
22. Enoch, S., Leaper D. J. Basic science of wound healing. Surgery (Oxford). 2008;26:31-37.
23. Noorlander M. L., Melis P., Jonker A., Van Noorden C. J. A quantitative method to determine the orientation of collagen fibers in the dermis. J Histochem Cytochem. 2002;50:1469-74.
24. Shanmugasundaram N., Uma T. S., Ramyaa Lakshmi T. S., Babu M. Efficiency of controlled topical delivery of silver sulfadiazine in infected burn wounds. J. Biomed. Mater. Res. Part A. 2009;89:472-82.
25. Furie B., Furie B. C. Molecular and cellular biology of blood coagulation. NEJM. 1992;326:800-6.
26. Nissen N. N., Polverini P. J., Koch A. E., Volin M. V., Gamelli R. L., DiPietro L. A. Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. Am. J. Clin. Pathol. 1998;152:1445-52.
27. Ma L., Gan C., Huang Y., Wang Y., Luo G., Wu J. Comparative proteomic analysis of extracellular matrix proteins secreted by hypertrophic scar with normal skin fibroblasts. Burns & trauma. 2014;2:76-83.
28. Inan, A., Şen M., Koca C., Ergin M., Dener C. Effects of Aloe vera on colonic anastomoses of rats. Surg. Pract. 2007;11:60-5.
29. Yu Y., Chen R., Sun Y., Pan Y., Tang W., Zhang S et al. Manipulation of VEGF-induced angiogenesis by 2-N, 6-O-sulfated chitosan. Acta Biomater. 2018;71:510-21.
30. Johnson K. E., Wilgus T. A., Vascular endothelial growth factor and angiogenesis in the regulation of cutaneous wound repair. Adv. Wound Care. 2014;3:647-61.