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Research Article

Formulation and Clinical Evaluation of A Topical Dosage Form of *Alkanna orientalis* Root Extract for Management of Pressure Lesions: A Pilot Cross-Sectional Clinical Trial

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Abstract

Background: Pressure lesions are chronic wounds causing the development of infection and inflammation into deeper structures and finally necrosis. In Persian medicine, *Alkanna orientalis* (Boraginaceae) has been used for centuries as a naturally derived remedy for managing lesions. A cross-sectional pilot clinical trial was conducted to assess the wound healing effect of an ointment made of chloroform extract of roots of *A. orientalis* (CERAO).

Methods: Sixty patients (36 men and 24 women) diagnosed with bedsore staging 1-2 entered the study for one year. They were divided into two groups of control and treatment with equal proportions. The control group received conventional treatment from the hospital, including irrigation serum, mupirocin, phenytoin ointments, and gauze dressing. After rinsing and cleansing with normal saline, in the intervention group, patients received a thin layer of CERAO once daily for four weeks. Clinical outcomes were measured at weeks 2 and 4.

Results: Recovery assessment was carried out by measuring wound area, days of epithelia formation, and complete wound closure. The difference between the two groups was statistically significant (P-value <0.05) in terms of the mentioned criteria. The recovery percentage was 26.7% and 60% for the control and treatment groups, respectively. In the control group, 16.7% of the study population experienced the development of wounds, while in the intervention group, wound progression was not observed.

Conclusion: The results of this pilot study indicated that the clinical efficacy of CERAO could be promising and a replacement for conventional treatment of pressure ulcers.

Introduction

Wounds fall in the category of acute and chronic ones. A bedsore is a painful and chronic lesion that develops in disabled and hospitalized patients who receive long-term care owing to continuous pressure. It is also called pressure sores, injuries, or decubitus ulcers.[1] The leading cause of this chronic ulcer is the constant lying position that negatively affects the capillary beds. It decreases blood supply or ischemia, contributing to local tissue hypoxia, production of excess moisture, the disintegration of skin and subcutaneous tissue, and finally, necrosis occurs.[2] A chronic ulcer needs a prolonged treatment duration, and sometimes treatment failure occurs.[3] The global prevalence of pressure injuries was reported to be 6.3%.[4] According to previous studies, pressure lesions were the most high-cost wounds that impose more than 11 billion dollars on healthcare systems.[2] Thus, proceeding with cost-effective approaches could lighten the financial burden of the medical system. The standard therapeutic approach for decubitus ulcers is similar to treating open wounds. First and foremost, debridement is carried out to prevent the progression of ulcers. The second contemporary strategy is healing moisture with some dressings, which are prepared based on traditional knowledge. Moreover, antiseptics are used; however, they delay the progression of wound healing.[5] There are controversial concepts regarding

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the management and treatment of these ulcers. In this regard, herbs and herbal preparations have shown positive effects to manage wounds.[6-8] Among suggested wound healing herbs, species belonging to the *Alkanna* genus (Boraginaceae), known as "Shengar" in Persian, includes 50 species traditionally used to heal ulcers. They wildly grow in the Mediterranean and Eastern Asia regions. Usage of this herb for skin ulcers dates back to ancient times. In ancient manuscripts and folklore, it is claimed to treat burns, anal ulcers and hemorrhoids, infected and oozing wounds and crusts, bedsores, and dermatitis.[9-10] Plants of this genus contain phytochemicals responsible for anti-ulcerogenic effects, including phenolic compounds such as tannins, flavonoids, and naphthoquinones. The mentioned components have anti-inflammatory, antibacterial, anti-fungal, antioxidant, and wound healing activities. Although these components act collaboratively to indicate an anti-ulcerogenic effect, naphthoquinones of *Alkanna*, alkannin, and shikonin (hydroxyl naphthoquinones) are known as significant and potent phytoconstituents of the root of the plant.[10] *Alkanna orientalis* (L) Boiss. is a perennial herb endemic to Iran with the Persian name of "Shengar sharghi". In Iranian folk medicine, it was used to prepare ointments and pastes for ulcer dressings. Even though some studies were conducted to elucidate the pharmacological properties of *A. orientalis.*[11-12] To our knowledge, no clinical trial was conducted to evaluate its effect on pressure ulcers. Thus, this study aimed to prepare an ointment from chloroform extract of roots of *A.orientalis* and assess its wound healing effect on patients suffering from pressure ulcers.

Methods

Collection of herb and extraction

The roots of *Alkana orientalis* (L.) Boiss. (Boraginaceae) were collected from Arasbaran, East Azarbaijan, Iran. The voucher specimen (TbzMed-FPh 4003) was deposited in the herbarium of the faculty of pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran. The powdered roots were soxhlet extracted by chloroform within 14 hours. Afterward, the extract was filtered, and a rotary evaporator evaporated the solvent at 45°C.

Keratinolytic activity and feather degradation test of CERAO

For assessment of keratolytic effect of the extract, firstly, keratin of chicken feather soluble in DMSO was prepared using the method of Wawrzkiewicz *et al.*[13] One g of chicken feathers were suspended in 50 mL of DMSO. Afterward, the mixture was heated at 100°C for 1 hour. For precipitation of keratin in DMSO, cold acetone (-20°C) was gradually added. The following step was a 10 min centrifugation of the mixture at 12000 rpm. The residue was twice washed with distilled water and suspended in phosphate buffer (0.1M, pH=7) to acquire keratin suspension. This suspension was applied for further assessments.[14-15]

Assessment of keratinolytic activity of the extract in culture medium containing keratin

0.25 g peptone, 0.15 g yeast extract, and 0.6 g agar were mixed and solved in 50 mL phosphate buffer (0.1M), and then the mixture was heated. Before completely cooling the solution, 600 mg keratin was solved in a 50 cc phosphate buffer and 50 µl DMSO was added. The solution was added to cultivation plates. Afterward, 20mg of extract was solved in 20 mL acetone, resulting in a solution with a concentration of 1mg/ml. Disks were classified into three categories. Disks were immersed in extract solution, phosphate buffer 0.1M, and TGA (thioglycolic acid) solution resulting in test, negative control, and positive control disks. Cultivation plates were divided into three regions, each region contained one category of disks. Finally, plates were incubated in an incubator of 37°C for 24 h. After incubation, clearing zones produced by three categories of disks were measured.[14-15]

Different concentrations of extract were solved in 1 mL DMSO. Then, phosphate buffer of 0.1 M was added to containers up to 100 mL. In each container, a feather was immersed, and the keratolytic effect was assessed after 24 hours. The positive control solution was 5 mL TGA solved in a 45 mL buffer. Two feathers were suspended in the positive control solution and incubated for 24h.[14-15]

Formulation of the ointment, microbiological and physical stability testing of CERAO

For the preparation of an ointment, 6 g of CERAO (20%) was solved in 3 g (10%) of transcutol. Afterward, the mixture was heated to 30°C till a transparent solution was prepared. 18 g (60%) of white petroleum and 3 g (10%) of stearyl alcohol were added to the solvent and mixed till the 20% CERAO ointment was prepared. Ultimately, the ointments were packaged in 30 g aluminum tubes.

The method for detection of microbial contamination of CERAO was carried out according to USP to determine TAMC (Total Aerobic Microbial Count) and TYMC (Total Yeast and Mold Count). To assess TAMC, the ointment was solved in polysorbate 80 to obtain a well-dispersed emulsion. 1 mL of the prepared sample was mixed with soybean casein digest agar on a plate. It was incubated in an incubator at 35°C temperature for five days. Then, the number of colonies were counted (CFU mL^{-1}). To obtain TYMC, one cc of the sample was mixed with Sabouraud Dextrose Agar (SDA) in another plate. It was incubated in an incubator at 25°C temperature for five days. Finally, the colonies were counted (CFU mL^{-1}).[16] The experiments were carried out in duplicate. Microbial stability testing was carried out to describe the product characteristics in terms of bio burden numbers over time. The plates were incubated in an incubator for one month, and the number of colonies was counted again.

Study setting and population

This clinical trial was a case-control, single-center, and pilot study that enrolled 60 patients (36 men and 24

women) randomly assigned to treatment (30 control and 30 treatment) in Sina hospital, Tabriz, Iran within one year from Aug 2017 to Aug 2018. Patients' history was obtained, and their general health was assessed as a starting point. The pressure lesions of patients were graded according to the scoring system of NAUAP. National Pressure Ulcer Advisory Panel (NPUAP) introduced a four-stage approach to scale pressure injuries. The patients with pressure ulcer staging 1 and 2 were classified into two groups; control and intervention. 8 and 22 patients in the control group were diagnosed with stage 1 and 2 pressure ulcers, respectively. In the intervention group, 7 and 23 patients were diagnosed with pressure lesions of stage 1 and 2, successively.

The control group received the current treatment protocol of Sina hospital, including rinsing the wound surface with normal saline, applying mupirocin, phenytoin ointments, and dressing the wound surface with gauze. The intervention group received CERAO in conjunction with the conventional treatment protocol of Sina hospital. First, the wound surface was rinsed with normal saline and dried. Second, a thin layer of CERAO was placed on gauze, and the wound was packed with it. This process was carried out once daily. Treatment continued for one month, and the efficacy outcomes were assessed in weeks 2 and 4 (Figure 1). Assessment of the ulcers and efficacy endpoint were based on the closure of the lesions. The following criteria evaluated efficacy assessment; first, measurement of the area of ulcer surface; second, days of generation of a new epithelium layer, and third, days of wound closure. Finally, the recovery percentage was documented according to the number of patients who experienced the closure of the wound.

The ethics committee of Tabriz University of Medical Sciences approved the protocol of this study (ethical code: IR.TBZMED.REC.1395.1053). Procedures followed were according to the ethical standards of the responsible committee on human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2008. The study protocol was declared to the study population, and the volunteers obtained a written consent form.

The subjects included in this study were males and females, 18 years of age and older, who presented pressure ulcer staging 1 and 2. The exclusion criteria were pregnancy and lactation, organ failure, patients using immunosuppressant, corticosteroids, antibiotics, anticonvulsants, angiogenesis inhibitors, and nicorandil, patients with the weakened or suppressed immune system, and those aged below 18.

Statistical analysis

Values of this study were expressed as Mean±SD, frequency, and percentage. Fishers' chi-square test expressed nominal non-parametric data. The variables were analyzed using t-test and Mann–Whitney test. P-values less than 0.05 were considered statistically significant. SPSS version 20 software was used for statistical analysis.

Results

Keratinolytic activity and feather degradation test

Observing feathers under a microscope revealed that feathers immersed in extract solutions were not decomposed, while the feather in positive control solution (TGA) was completely disintegrated. A clear zone was not observed around the extract and negative control disks in the keratin-containing culture medium. This result demonstrated that the extract did not decompose keratin.

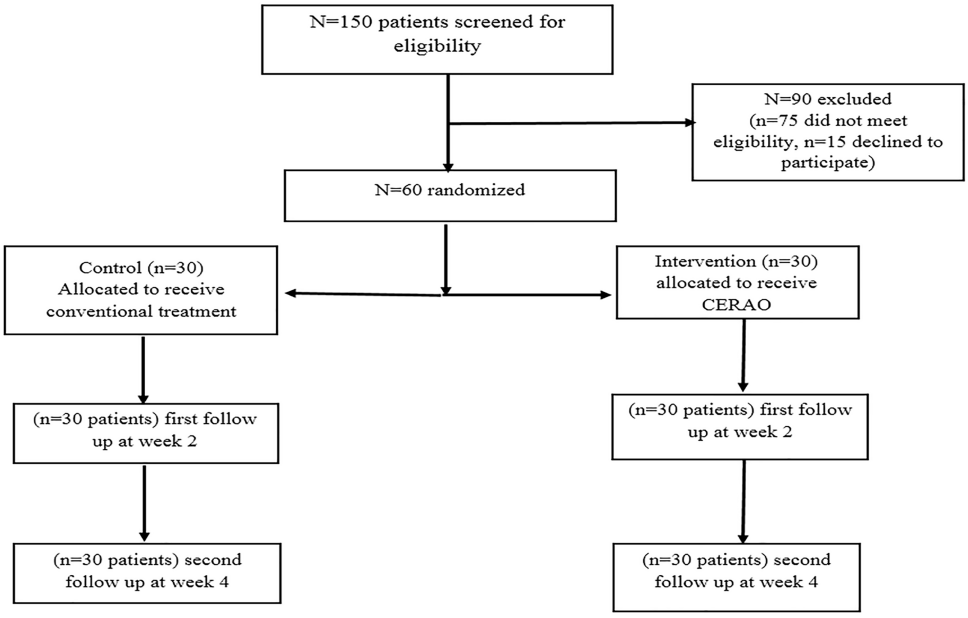

Figure 2. Consort flow chart of the study.

Microbiological stability tests

Maximum acceptable count of TAMC and TYMC were 250 and 50 (CFU mL^{-1}), respectively, which were within a reasonable range according to USP guidelines.

Profile of patients in control and intervention groups

This research was conducted on 60 patients of both sexes, presenting pressure ulcers staging 1-2. Patients were randomly divided into intervention (30 patients) and control (30 patients) groups. There was no significant difference between patients diagnosed with pressure ulcers in control and intervention groups regarding ulcer staging, number, and distribution in hospital wards (P-value >0.05). In control and intervention groups, the interval between onset of pressure ulcer and beginning of treatment was 15±15.2 and 12.9± 12.4 days, respectively, and the P-value was 0.077 (P-value >0.05), indicating that this difference was not statistically significant (Table 1).

Comparison of control and intervention groups

Pearson chi-square and fisher's exact test were used to compare qualitative values (gender, staging of ulcer, etc.) between two groups of control and intervention. Mann Whitney test was used for quantitative values, such as duration of treatment, area of wounds, and duration of epithelium formation. Table 2 summarizes two groups regarding surface area of ulcers, days of re-epithelialization, and wound closure and recovery percentage.

Discussion

The healing progress of chronic wounds, like pressure ulcers, requires an extended period. These ulcers mainly turn into pathologic inflammation or prolonged inflammatory response. Extended inflammation generates a perfect environment for bacteria and fungi proliferation and infection which delays process of wound healing. Also, excessive production of free radicals hinders wound healing in that environment.[17] Wound care consists of orchestrated activities to assist in the healing of wounds in the shortest period leading to wound closure and tissue regeneration.[18] Medicinal plants, as natural resources, bring about rapid tissue regeneration by several mechanisms. Especially, herbs used by folklore are valuable agents to be assessed for wound healing effects. From ancient civilizations to modern times, different species of *Alkanna* have been used as an alternative medicine to treat ailments and promote health. The most medically effective part of *Alkanna* is its roots. Due to producing loud red color, alkanna root is mainly used to prepare cosmetics. Moreover, according to ancient records, it was used for the treatment of skin injuries, such as burns, anal ulcers, hemorrhoids, infected crusts, oozing dermatitis, and bedsores.[12] *A. orientalis* contains a broad spectrum of phytochemicals, for instance, flavonoids, pyrrolizidine alkaloids, and naphthoquinones responsible for pharmacological effects, such as antibacterial, antifungal, wound healing, and antioxidant activities.[19-20] Among mentioned phytoconstituents, naphthoquinones, alkannin, shikonin, and their derivatives have been reported

Table 1. Profile of patients in study and intervention groups.

Characteristics	Control	Treatment	P-value[3]
Gender[1]			
Male	17	19	-
Femele	13	11	-
Age[4]	70.33±16.9	66.17±19.6	0.43
Hospital ward[2]			
Internist	53.3%	53.3%	0.89
ICU	13.3%	16.7%	0.89
Infectious	20%	20%	0.89
poisoning	3.3%	6.7%	0.89
general	10%	3.3%	0.89
Ulcer staging			
Grade 1	(8) 26.7%	(7) 23.3%	1
Grade 2	(22) 73.3%	(23) 76.7%	1
Underlying conditions			
Diabetes	30%	30%	1
Hypertension	40%	40%	1
Alzheimer's disease	20%	10%	0.47
Cardiovascular disease	6.7%	10%	1
Arthritis rheumatoid	3.3%	3.3%	1
Hepatitis history	0%	3.3%	1
CVA	0%	3.3%	1
Days between the onset of pressure ulcer before treatment[4]	15±15.2	12.9±12.4	0.77

[1] Number of patients belonging to each gender
[2] Proportion of patients who entered to study
[3] P-value greater than 0.05 means that the difference is not significant.
[4] Mean ± SD

Table 2. Comparison of control and intervention groups in terms of area of ulcers, days of epithelia formation and wound closure, as well as recovery percentage.

Assessment of recovery	Control		Intervention		P-value	
Surface area (cm^2)[1]	Before	After	Before	After	Before	After
	23.9±43.9	21.1±41.9	33.2±54.1	6.2±15.4	0.67	0.005
Days of re-epithelization[1]	10.4±7.4		3.2±2.1		<0.001	
Days of wound closure[1]	16.1±10.9		7.1±4.7		<0.001	
Recovery percentage[2]	26.7%			60%	0.009	

[1] Mean ± SD

[2] Number of patients experiencing wound closure in both control and intervention groups.

to be the main pharmacologically active compounds of *A. orientalis*.[21-22] Interestingly, alkannin and shikonin are enantiomers. The S enantiomer is alkannin used as a dying agent owing to its highly colored nature. Shikonin, the R enantiomer, is the other main red-colored component of *A.orientalis* root extract.[8] These chiral ingredients and their derivatives have indicated free radical scavenging, anti-bacterial, proliferative, anti-thrombotic, wound healing, and anti-inflammatory effects.[11] Previous reports indicated that these phytochemicals and their derivatives could bring about regeneration and proliferation due to upregulation of biosynthetic processes. Hence, in prior clinical studies, they were known as broad-spectrum antibacterial agents. They mainly acted against *Pseudomonas aeruginosa*, an important pathogen which inhibits wound healing by making biofilms. This pathogen utilizes the host's iron for growth and biofilm, which changes wounds into the chronic forms. Shikonin could chelate iron which gives rise to inhibition of bacterial growth and biofilm formation. According to previous manuscripts, alkannin and shikonin are potent ROS scavengers and inhibitors of oxidase enzymes that augment wound healing activity. Prior studies demonstrated that shikonin could prevent the biosynthesis of inflammatory mediators related to leukotrienes and cytokines, namely leukotriene B4, 5-hydroxy eicosatetranoic acid, and TNF-alpha. Thus, inhibition of these mediators takes center stage in the anti-inflammatory effect of shikonin. Hence, shikonin could prevent the expression of Cox-2 gene, and it is a selective antagonist of CC chemokine receptor 1. Therefore, shikonin could block the inflammatory process from multiple pathways.[12]

A component of alkannin named β-acetoxy isovaleryl alkannin indicated the healing of ulcers in rabbits by suppressing the inflammatory process. In crude terms, reducing the levels of pro-inflammatory and surging the levels of anti-inflammatory cytokines brought about a wound healing effect. Furthermore, the mentioned compound led to the proliferation of dermal fibroblasts which regulate, support, and accelerates wound healing process. According to the results of this study, there was connection between β-acetoxy isovaleryl alkannin and TGF-β/smad3 signaling. The mentioned signaling could regulate immune response, angiogenesis, cell growth, and finally, ulcer healing.[23] These naphthoquinones and their derivatives could accelerate and promote wound healing by induction of proliferation. Prior studies indicated that shikonin derivatives brought about re-epithelialization, angiogenesis, and granulomatous tissue formation. All in all, alkannin and shikonin, as multifaceted compounds, offer simultaneous and unparalleled pharmacological effects.[12] Owing to mentioned broad-spectrum activities of these enantiomers, chloroform extract of *A.orientalis* roots was selected to contain a maximum level of alkannin, shikonin, and their derivatives.[24]

In this study, a keratolytic test on chloroform extract of the roots of *A.orientalis* revealed that high concentrations of this extract have fewer keratolytic effects. Consequently, it could dissolve and remove skin flakes and scales, leading to better penetration of topical medicaments. Since the keratolytic effect is delicate enough, it neither injures the wounds nor delays the process of wound healing.

One of the significant difficulties in formulating pharmaceuticals for the treatment of pressure ulcers is water in topical medications formulations that could trigger bacterial growth. Thus, the ointment was decided as a suitable topical formulation for consistency, skin spread, and the minimum bacterial load.

The results of this study indicated promising results at the end of the first week using CERAO. Similarly, the reduction of surface area, acceleration of re- epithelialization, wound closure, and increase of recovery percentage was revealed in the intervention group. Before the intervention, the difference in surface area of control and study groups was not statistically significant (P-value=0.67), while after the intervention, that was statistically significant (P-value=0.005). Epithelial layer formation occurred in 3.2±2.1 days in the study group, while that occurred within 10.4±7.4 days in the control group (P-value=0.000). These results showed the acceleration of epithelialization in the population using CERAO.

Moreover, wound closure was observed within 16.1±10.9 days in the control group, while that of the intervention group was 7.1±4.7 days (P-value<0.001). These results illustrated that the rate of wound closure doubled after using CERAO. Recovery percentage or in crude terms, the number of patients experiencing wound closure in control and intervention groups was 26.7% and 60%, respectively (P-value =0.009), illustrating that the number of improved patients tripled in the intervention group. Furthermore, in the control group, 16.7% of patients experienced the development of pressure ulcers to higher stages, while in the intervention group, the development of ulcers was not observed. Therefore, applying CERAO ointment to

wounds resulted in much better wound healing effects than the current approach of in the studied hospital.

Conclusion

From this pilot study, it can be concluded that CERAO ointment induced wound healing. Also, it decreased days of recovery. According to the results of this study, CERAO had better clinical efficacy than the current treatment protocol of pressure ulcers. Due to encouraging results, CERAO can be applied with conventional treatment to manage pressure ulcers. For further confirmation, double-blind, randomized clinical trial studies are required.

Ethical Issues

The ethics committee of Tabriz University of Medical Sciences approved the protocol of this study (ethical code: IR.TBZMED.REC.1395.1053). Procedures followed were according to the ethical standards of the responsible committee on human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2008. The study protocol was declared to the study population, and the volunteers obtained a written consent form.

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Data Sharing

The data that support the findings of this study are available from the corresponding author.

Author Contributions

Elham Lazarzareh: Investigation, Formal Analysis. Babak Davami: Methodology, Resources. Hadi Valizadeh: Methodology. Kavous Shahsavarinia: Methodology, Resources. Hossein Nazemiyeh: Conceptualization, Supervision. Laleh Khodaie: Formal Analysis, Writing - Original Draft. Afshin Gharekhani: Methodology, Formal Analysis, Project Administration, Writing - Review & Editing.

Conflict of Interest

The authors report no conflicts of interest.

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