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Evaluation of histopathological and healing potentials of the full-thickness cutaneous wound for a topical ointment formulation containing extract of bark *Quercus aegilops* in mice

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Abstract

Cutaneous wounds are significant problems that can be treated with traditional herbal supplements. This study evaluated the wound healing potential of *Quercus aegilops* barks extract formulations on excision wounds. BQAE extractions were acquired and employed to make two distinct formulas, namely, 10 and 20% barks of *Quercus aegilops* extract. These formulations were applied topically once daily for 12 days to check out their capacity to heal wounds in a mice model of excision wound repair. At 0,3,6,9 and 12 days, wound sizes and healing areas were observed. Hematoxylin and Eosin staining were used to skin tissue samples for histopathological evaluation. The formulations of the ointments were found to be stable and skin-safe. Comparing the wound contraction and healing area to the positive (standard reference Povidone-iodine) and negative controls, the 10 and 20% BQAE formulations both caused a substantial ($P<0.05$) reduction in these two parameters (wound contraction and healing area). A significant ($P<0.05$) increase in the levels of Reduced glutathione, Superoxide dismutase, and Catalase, as a decrease in the levels of Malondialdehyde was observed in 10 and 20% BQAE groups when compared with positive and negative groups. The histopathological studies of excision biopsy on day 12 observed 10 and 20% BQAE groups increased collagen formation, increased number of neovascularization, and reformation of sebaceous glands, with full thickness epithelization of the epidermal layer as soon as compared with the standard reference Povidone- iodine 10% (positive control) and negative control groups. Thus, the study in vitro (physical properties of the ointment) and in vivo scientifically validated the wound-healing activity for barks of extract (BQAE), which explained the increased collagen production and potential antioxidant activity, thereby supporting the traditional claims.

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Introduction

Wound healing is a complex biological process, including tissue regeneration (1). When such tissues are damaged, the complicated and multi-stage healing development, which includes a complex sequence of cellular responses, is triggered, leading to repair, resurfacing, and the recovery of the wounded skin's tensile strength (2). Soon after the injury, the inflammatory stage begins, with vasodilatation that maintains homeostasis while producing an inflammation mediator. The angiogenesis process and the proliferation of granulation tissue, which is mainly produced by fibroblasts, define the proliferative phase. The remodeling stage is characterized by collagen fiber component

reformulations and enhancements that increase tensile strength (3). Multiple factors, including skin injury, hinder the healing of wounds, and an imbalance between the body's antioxidants and free radicals leads to the over-production of reactive oxygen species (ROS), causing cell and tissue damage and delayed wound healing. As a result, decreasing levels of ROS via anti-oxidative processes may aid in healing by minimizing the damage caused by oxidative stress (4). In Iraq, the oak tree (*Quercus aegilops*) accounts for 70% of the oak woodlands (KRI) (5). According to current literature, *Quercus aegilops* is used as a hemostatic, anti-diarrheic, anti-bacterial, wound healing, and antiseptic agent, in addition to for the treatment of burns, anal, and oral mucosa irritation (6), also cosmetic applications (7). The bark of these trees has been used to make infusions for medical or nutritional purposes for humans and animals (8). This genus comprises phytochemical compounds such as phenolic acids, flavonoids, tannins, stilbenoids, lignans, coumarins, monoterpenes, steroids, and triterpenes (9); also has a variety of phenolic acids and simple phenols, including ellagic acids, gallic, ellagitannins, and Gallo, in addition, their conjugates with catechin/epicatechin, was extracted or identified (6,10). By performance quantitative high-performance liquid chromatography (HPLC) analysis containing gallic acid and Quercetin (11). Recently, it described the wound-healing properties of this kermes oak (*Quercus coccifera L.*), which was performed *in-vitro* as an alternative wound-healing agent (12).

The current study carefully examines the wound-healing effects of bark *Quercus aegilops* in the formula of a topical ointment preparation, which has still not been scientifically studied *in vivo*. Model excision in mice was used to study wound healing activities.

Materials and methods

Chemicals solvent

The UAE's AMEYA FZC Co. Ltd. supplied the ethanol with a 70% content. The diluents employed were of the highest purity and grade HPLC. The inquiry also used a variety of additional substances, all of which were analytical grades.

Collection and authentication of plant materials

The fresh barks of *Quercus aegilops*, were collected from healthy, fully-grown plants in November 2021 from the oak tree (*Quercus aegilops*) located in Erbil city (Figure 1).

Topical formulations preparation

BQAE topical ointment preparations were created to compare their wound healing performance to povidone iodine ointment. According to British Pharmacopoeia, a simple ointment base was made (13). In a beaker, 5 g wool fat, 5 g hard paraffin, 5 g ceto-stearyl alcohol, and 85 g soft white paraffin were combined and heated in the apparatus of a water bath at 65 °C up to all of the materials were melted. After cooling, the mixture was homogenized with a homogenizer at 1500 rpm for 10-15 minutes. By adding the needed quantity of BQAE into 100 g of simple ointment base, different ointment preparations, BQAE 10% w/w, and BQAE 20% w/w were created.

Figure 1: Showed fresh barks of *Quercus aegilops.*

Evaluation of physiochemical formulation of ointment

All the ready ointments were characterized for parameters such as physical appearance, spreadability, color, texture, pH (digital pH meter, China), phase separation, and immediate skin feel (including greasiness, stiffness, and grittiness) (14).

Preparation of extracts

Fresh barks were softly washed in tap water to eliminate dust before being shade-dried in the research laboratory at room temperature at 24±2°C for 2-4 weeks. After the drying, the dehydrated plant materials were crushed with an automatic grinder and sieved to produce a soft powder. Using the cold extract, the milled plant material was extracted through ethanol 70%. After the crude extract solution was filtered with Whatman No. 42 filter paper, the surplus solvents were evaporated using lyophilized water (Freeze Dryer, Bioevopeak Co., Ltd., China). Before being analyzed, the crude ethanol extract was kept at 4 °C with the abbreviation name barks of *Quercus aegilops* extract (15).

Gas chromatography-mass spectrometry evaluation

BQAE was analyzed in (Scientific Center for Chemical Analysis (SCCA), No:54) for the presence of various compound active groups. These compounds have peaks initiated from 5.5 (0.33%) to 29.24 (0.46%).

Experimental animals and housing conditions

The wound healing activity was investigated using adult healthy male mice (age 6-8 weeks; body weight, 25-30g). Before testing, the animals were kept in clean poly-propylene cages with stainless steel top grills for a minimum of one week in the research laboratory animal room under

normal circumstances (temperature, 24±2°C; relative humidity, 55 percent; 12/12 h light/dark cycle). For the excision model (10), the animal was randomly divided into four groups of mice; five animals per group were used. Animals in groups 1 and 2 were treated with BQAE (10 and 20% w/w respectively) from experimental plant extracts and positive control (Povidone-Iodine 10% (PI)), also negative control with simple ointment (SO). The treatment process was initiated 24 hours after the wound was created, with the day of the wound's genesis taken as zero. Every 12 hours for a total of 12 days, formulations are applied topically onto the wound area. On Day 12 After anesthetizing the laboratory animals with ketamine hydrochloride (100 mg/kg, i.m.), then taking the piece of tissue from the place of the surgical cut and observing the histopathological changes under a microscope. Finally, The animal sacrifices according to approved ethics.

Ethical approval

The College of Veterinary Medicine, University of Tikrit, Salah Al-Deen, Tikrit, Iraq, approved all experimental protocols. Experiments were carried out following the laboratory animal care and use guide in college.

Test of acute irritation of the skin

The BQAE ointment formulation was examined for any potential acute irritation of skin tests (16) on mice. A 3*3 cm region was defined on the dorsal side, and the hairs of the dorsal area were clean-shaven and cleaned. The BQAE sample was applied to each skin compartment on the cleansed region (3 grams/site) and secured with tape to avoid contamination and dehydration. (17). The shaved portions of various animal groups were subsequently treated with the ointment formulations (10 and 20%). The animals were observed for 24 hours after applying the ointment formulations to look for any indications of inflammation or other abnormal changes on their skin (18,19).

Excision wound model

According to the excision wound model, the wound site was created via anesthetizing the experimental animals. The surgical procedures were performed under antiseptic settings using ketamine 10% (80 mg/kg) + xylazine 2% (10 mg/kg), and a 6.5 mm full-thickness circular excision incision was made on the shaved dorsal back area. The wound closure mice were evaluated by tracing the wound on the 0^{th}, 3^{rd}, 6^{th}, 9^{th}, and 12^{th} post-wounding days using a Vernier caliper. The percentage of wound closure was calculated using the formulary (20-22). % of wound contraction= [(wound area on day 0 - wound area on day n)/initial wound are on day 0]*100. where n=3^{rd}, 6^{th}, 9^{th}, and 12^{th} post-wounding days. The epithelialization period was also determined as the days necessary for the dead tissue to fall without any leftover raw wound. Healed area = original wound area − present wound area.

Estimation of free radicals and antioxidants in granulation tissue

The samples of wet granulation tissue were taken from the animals' excised wound patches on the tenth day after wounding to evaluate the levels of free radicals and antioxidants in the granulation tissue. Phosphate-buffer-saline (PBS, 10% homogenate) was used to homogenize the tissues at 4 °C, following which the mixture was centrifuged at 40,000 g for 30 minutes. The resulting supernatant underwent tests for Malondialdehyde (MDA) (ELISA Kit, E0048Ge), reduced glutathione (GSH) (ELISA kit, AK0479), catalase (CAT) (ELISA Kit, SL0747Mo), and superoxide dismutase (SOD) (ELISA Kit, CSB-E08556m).

Histopathological evaluation

On day 12, skin tissue samples were taken from the wound sites and stored in 10% formalin to evaluate the histopathological changes. Tissue slices underwent hematoxylin/eosin (H&E) staining followed by a microscopic imaging scale bar (μm) (23).

Statistical analysis

The investigational result was expressed as mean ± SEM (standard error of the mean). The results were statistically analyzed using a one-way analysis of variance (ANOVA). GraphPad InStat Version 8 finished the statistical analysis and data presentation (Software GraphPad, Inc. La Jolla, CA, USA).

Results

Physiochemical ointment formulation

The results exhibited that the ointments had a good, appealing appearance, which remained unchanged through the 30 days *in vivo*, besides smooth texture, and they were all homogenous with no marks of phase separation; in addition, immediate skin feel showed no grittiness, greasiness, and stick. Also, no severe offensive odor was advanced during the study to characterize the physiochemical of the ointment formulation. The results showed no difference significantly in pH between BQAE 10% and BQAE 20%. All formulas have pH levels within the skin's typical pH range.

GC-MAS test

The ethanol extract bark *Quercus aegilops* revealed the presence of many phytoconstituents have peaks (Table 1), and these peaks contribute to many acids such as Oleic acid, Octadecenoic acid, 9-octadecenoic acid, n-hexadecanoic acid, and Pentadecanoic acid (Figure 2).

Acute skin irritation test

No redness, irritation, edema, inflammation, or other unexpected modifications were visible on the exposed skin of mice using BQAE ointment formulation at 10 % or 20 %.

Table 1: Showed phytoconstituents in extract bark *Quercus aegilops* analysis by GC-MS

No.	Peaks	Contents	Total (%)
1.	23.5	Oleic Acid	36.0
2.	25.4	Cyclohexanecarboxylic acid	4.6
3.	18.9	9-Octadecenoic acid	9.4
4.	17.5	6-Octadecenoic acid	3.2
5.	28.9	3-hydroxypropyl ester	2.1
6.	22.8	12-Octadecenoic acid	1.9
7.	26.8	Hexanedioic acid	1.2
8.	28.5	Benzeneacetic acid	0.6
9.	22.3	Glutamic acid	0.4
10.	20.7	7-Hexadecenoic acid	0.3
11.	5.5	2-amino-6-methyl benzoic acid	0.3
		Total	60

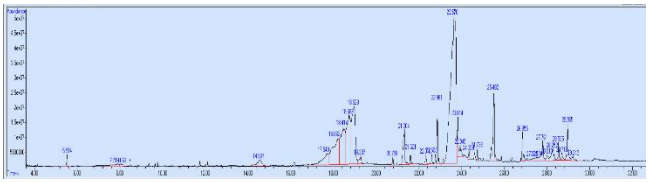

Figure 2: Showed peak of the phytoconstituents compound in the extract of bark *Quercus aegilops.*

Excision wound model contraction and healing area

The excision wound model studies have shown that the rate of wound contraction in control mice was 3.07%-49.53% from day 3 to day 6 and 4.07%- 5.62% from day 9 to day 12. The 10% treated mice showed an increase in wound contraction from 4.06% on day 3 - to 92.61% on day 12 (Table 2). The percentage rate of wound contraction in mice treated topically with BQAE 20% was from 15.84% (P<0.0001) to 86.24% (P<0.0001) on day 3 to day 9 and 100% wound contraction on day 12. Similarly, the BQAE 10% treated animals' group had wound contraction of 10.36% (P<0.0001) on day 3 day and 78.64% (P<0.0001) on day 9, and complete epithelization was observed on day 12. The percent rate of healing area of 10 and 20% BQAE on day 3 to day 12 showed different significant (P<0.0001) from 0.67% to 3.18%, 1.03% to 0.0%, respectively, when compared with positive and negative groups from 0.26% to 6.02, 0.2% to 5.62% at the same time. Studies on excision wound models show that both groups treated with the individual extracts experienced an improvement in the re-establishment of normal skin constructions, with complete epithelization occurring at day 10. The same findings were obtained using standard povidone-iodine, demonstrating the plant extract's potent wound healing efficiency at day 11 (Figure 3).

Table 2: Wound contraction and healed area percentage of bark Quercus aegilops BQAE 10 and 20%

Group		Day 0	Day 3	Day 6	Day 9	Day 12	EIN
G1	HA	0.0±0.0	0.2±0.04	3.22±0.15	4.07±0.26	5.62±0.12	12
	WC	0.0±0.0	3.07±0.68	49.53±2.45	62.67±4.02	86.46±1.97	
G2	HA	0.0±0.0	0.26±0.07	3.41±0.16	4.51±0.31	6.02±0.15	11
	WC	0.0±0.0	4.06±1.11	52.55±2.49	69.50±4.88	92.61±2.45	
G3	HA	0.0±0.0	0.67±0.08	4.35±0.16	5.11±0.21	3.81±1.55	11
	WC	0.0±0.0	10.36±1.37	67.04±2.60	78.64±3.24	98.73±0.58	
G4	HA	0.0±0.0	1.03±0.24	4.53±0.30	5.60±0.42	0.0±0.0	10
	WC	0.0±0.0	15.84±3.75	69.72±4.63	86.24±6.60	100±0	

G1: Control, G2: PI; Povidone-Iodine 10% w/w. G3: BQAE 10%. G4: BQAE 20%. WC: % of wound contraction, HA: % healed area, EIN: Epithelialization in days. Values are expressed as mean ± SEM (n = 5) and analyzed by one-way ANOVA. ****P<0.0001 compared to the control group.

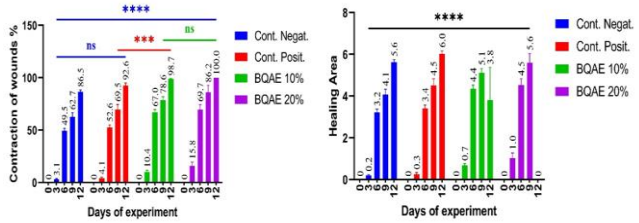

Figure 3: Showed *P* value of wound contraction and healed area between experiment groups. ****P<0.0001; ***P<0.0008; ns: Non-significant. Note: The small number above each column represents the group's mean at days.

Effect of BQAE ointment preparation of antioxidants and free radicals in wet granulation tissue

The results of the anti-oxidant and free radicals are obtainable in figure 4. In comparison to the control (simple ointment) group, both the BQAE (10 and 20%) and standard reference Povidone-iodine (10%) treated groups exhibited a substantial (P<0.05) rise in antioxidant marker enzymes, for instance, SOD, GSH, and CAT. The tissue of wet granulation collected from BQAE 20% ointment-treated mice presented maximum SOD, GSH, and CAT activity. In comparison, free radicals, for example, MDA, reduced significantly in BQAE at 10 and 20%, respectively (P<0.05) compared with the control group.

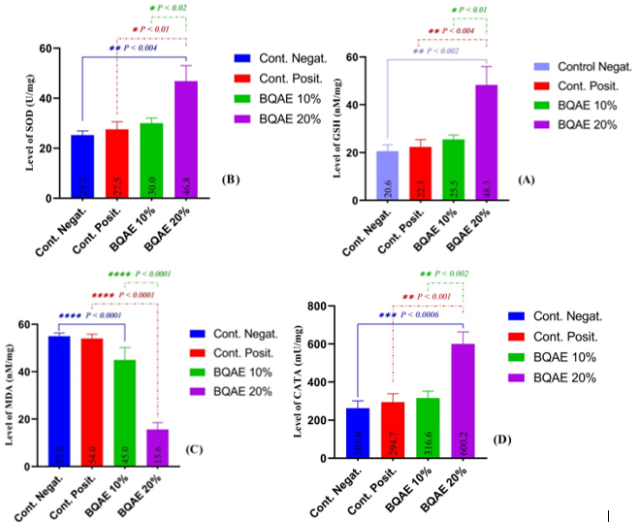

Figure 4: Effects of topical BQAE ointment application on various antioxidants and free radicals (A) Reduced glutathione (GSH), (B) Superoxide dismutase (SOD), (C) Malondialdehyde (MDA), (D) Catalase. Values are expressed as mean ± S.E. (n=5) between groups. Statistical analysis was done by one-way ANOVA at P<0.05.

Histopathological analysis

The histopathological excision biopsy section of the skin wound on day 12 showed healed skin structures without any inflammatory reactions, full-thickness epithelization of the epidermal layer with the restoration of a thin layer of keratin, increased number of neovascularization, improved reformation of collagen fibers as horizontal orientation, and sebaceous gland of the adnexa in BQAE 10% and BQAE 20% treated groups. The reference standard Povidone-iodine 10% and control (simple ointment) group lagged behind the treated group, with no complete sebaceous gland and collagen fibers reformation. At the same time, the newly formed hair follicles can be seen in all groups (Figures 5 and 6).

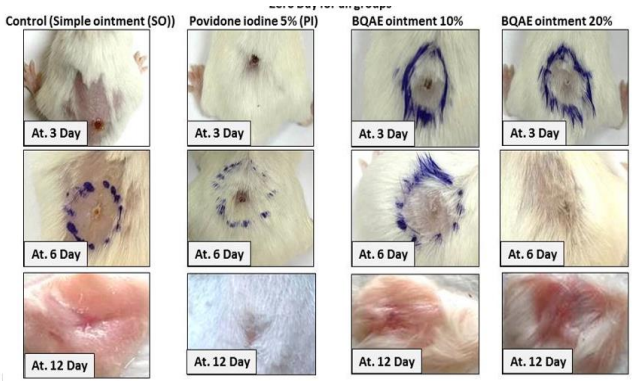

Figure 5: The gross appearance of excisional wound healing at three-day intervals over the 12-day research period.

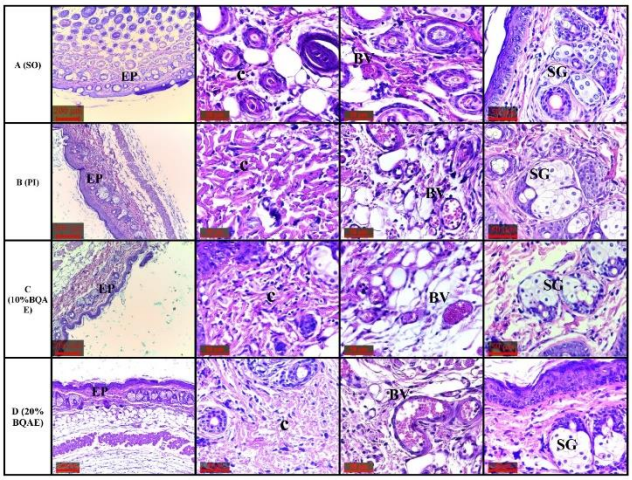

Figure 6: Histopathological investigation of an excised healed wound treated through (A) Control (Simple ointment (SO) (B) Povidone-iodine 5% (PI) (C) BQAE ointment 10% (D) BQAE ointment 20%. In (SO) and (PI) groups, the re-epithelization developed through a thin epidermal layer, with the thick irregular orientation of collagen fibers and tiny blood vessels with slightly developed sebaceous glands. In (C) and (D) groups, a thick epidermal layer set with thin regular orientation collagen fibers and the wall of blood vessels appeared well developed and increased in luminal diameter, while the sebaceous gland became more spherical and lobulated. Abbreviation: SG: Sebaceous glands; C: Collagen; EP: Epidermis; BV: Blood vessels.

Discussion

The multistage cutaneous wound healing process involves several cellular and molecular interactions that control cell activity and dynamic remodeling of the extracellular matrix to promote regeneration and recovery (2). Numerous phytochemicals are known to have potential wound-healing effects, and their application in the treatment of cutaneous wounds has been investigated using a variety of traditional medicines (24). The Topical ointment of medicinal plants used as complementary medicine with varying mechanisms of action can diminish scar formation due to their antioxidant, anti-inflammatory, and antimicrobial properties (25,26). It is crucial to choose the proper components for our study's formulations and to use the appropriate proportions of each ingredient (27-30). As well as the various factors such as physiochemical appearance, color, phase separation, texture, homogeneity, and immediate skin feel, in addition to pH, spreadability in the study attuned with Bharti *et al.* (31) who described no significant modification over the research period which increased the potential of wound healing. As a result, the BQAE ointment formulation at 10 and 20 % may be applied to mice's skin without having an adverse impact (32,33).

Reactive oxygen species (ROS) overproduction and a rise in free radical activity are the two main contributors to oxidative stress, which slows the healing of wounds. Determining antioxidants (SOD, CAT, and GSH) and free radicals (MDA) in granulation tissue becomes essential because they aid in accelerating the progression of wound healing, and eliminating ROS and reducing free radicles could therefore be crucial in the strategy of the healing of wounds. The excessive ROS level is associated with sustained inflammatory response, which limits efficient wound repair and stimulates antioxidant capacity in accelerating wound closure (34,35). In our study, BQAE ointment-treated groups exhibited potent in-vivo antioxidant activity by decreasing free radical's stress. Thus, BQAE could help stop oxidative damage and encourage healing.

In an excision wound, the wound contraction is the progression of mobilizing the healthy skin around the wound to overlay the denuded area. In our study, the BQAE 20% and standard reference Povidone- iodine 10% showed faster healing than the control (simple ointment) group. In addition, an excision biopsy of a skin wound on day 12 revealed that the BQAE 20 % and PI-treated groups had healed skin constructions with normal epithelization, re-establishment of the adnexa, and fibrosis in the dermis, whereas the control group delays behind the treated group in terms of information about the aggregate of ground substance in the tissue granulation. The more rapid wound contraction via BQAE may be because of phytoconstituents that are present in bark *Quercus aegilops* can help support fibroblast function by transforming growth factor-beta 1 (TGF-β1), accelerating wound healing (36-38). In addition, the phytoconstituents may be induced by myofibroblasts situated in skin granulation tissue to diminish the boundaries of the wound to decrease the surface to be repaired by yielding large amounts of matrix components, and they encourage angiogenesis and can recruit immune cells. These activities occur via the secretion of particles into their environment or indirectly by manufacturing micro-vesicles holding pro-fibrotic and pro-angiogenic molecules (39,40). The ointment prepared from bark *Quercus aegilops* extract efficacy of BQAE is characterized by improved fibroblast emigration, collagen production, and collagen deposition, with rapid re-epithelialization. This is primarily because of the augmented discharge of TNF-α through glucan receptors by encouraged fibroblasts and macrophages (41,42).

From the histopathological investigations, the facilitated thorough re-epithelialization, re-structuring of the wound area through blood capillaries, fibrosis, and very mild infiltration of inflammatory cells in the BQAE-treated group. The range of healing looks to be moderate when compared to the control group with PI. The newly formed sebaceous glands and hair follicles at the area of the wound were also seen. The range of healing seems to be better in the BQAE-treated group than in other groups. Using the BQAE in damages progresses and decreases the healing area in the primary phases of usual wound healing. This might mainly be because of immuno-modulatory effects on wound healing and collagen deposition (43-45).

Conclusion

The current investigation showed that the BQAE formulation, which contains bark *Quercus aegilops* extract, improves angiogenesis at wound sites, wound closure, and epithelial wound healing. This potency may be related to the phytoconstituents in the whole section and may support the claim that the bark of *Quercus aegilops* has some ethnomedicinal characteristics. Before starting the wound healing research, stability experiments on the ointment formulations were carried out, and the findings showed that they were stable under various situations. A high dosage of BQAE ointment formulation demonstrated a substantial wound contraction rate in the excision wound model. This may be attributed to an increase in *in-vivo* antioxidant activity, a decrease in myeloperoxidase, and a rise in collagen deposition. The histopathological results of the healed excised skin after 12 days, which presented almost recovered skin constructions with usual epithelization, further corroborate the outcome of the excision wound. The findings of this research suggest that one investigate the effectiveness of a BQAE formulation that combines several plant extracts for wound healing.

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Conflict of interest

The author attests that they did not get any financial support for this work and do not see any potential conflicts of interest.

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تقييم الفعالية النسيجية المرضية والالتئام للجرح الجلدي كامل الطبقات لتركيبة مرهم موضعي تحتوي على مستخلص لحاء السنديان الطابوري في الفئران

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الخلاصة

تعد الجروح الجلدية من المشاكل الكبيرة التي يمكن علاجها بالمكملات العشبية التقليدية. قيمت هذه الدراسة إمكانات التئام الجروح لتركيبات مستخلصات لحاء السنديان الطابوري على جروح الاستئصال. تم الحصول على مستخلص السنديان الطابوري واستخدامه في صنع صيغتين متميزتين، وهما ١٠ و٢٠ ٪ من مستخلص اللحاء السنديان الطابوري. تم تطبيق هذه التركيبات موضعيًا مرة واحدة يوميًا لمدة ١٢ يومًا للتحقق من قدرتها على التئام الجروح في نموذج الفئران لإصلاح الجروح الاستئصالية. في ٠ و ٣ و ٦ و ٩ و ١٢ يوم، لوحظت أحجام الجرح ومنطقة الالتئام. تم استخدام صبغة هيماتوكسلين/الايوسين لعينات أنسجة الجلد لتقييم الأنسجة المرضية. وجد ان تركيبات المراهم مستقرة وآمنة على الجلد. وبمقارنة تقلص الجرح ومنطقة الالتئام بالسيطرة الإيجابية والسلبية، تسببت تركيبات ١٠ و٢٠٪ السنديان الطابوري في انخفاض كبير في هاتين المعاملتين (تقلص الجرح ومنطقة الالتئام). لوحظ زيادة معنوية في مستويات الكلوتاثيون المختزل، سوبر أكسيد ديسميوتيز، والكتاليز، وكذلك انخفاض في مستويات المالوندالديهايد في مجموعات ١٠ و٢٠٪ السنديان الطابوري عند مقارنتها بالمجموعات الإيجابية والسلبية. لاحظت الدراسات النسيجية المرضية لخزعة الاستئصال في اليوم الثاني عشر أن ١٠ و٢٠٪ من مجموعات السنديان الطابوري زادت من تكوين الكولاجين، وزيادة عدد الأوعية الدموية الجديدة، وإعادة تشكيل الغدد الدهنية، مع ظهور النسيج الطلائي للسمك الكامل لطبقة البشرة بمجرد مقارنتها مع المرجع القياسي من ١٠% ايودين بوفيدين (السيطرة الموجبة) ومجموعة السيطرة السالبة. وبالتالي، أثبتت الدراسة في المختبر (الخصائص الفيزيائية للمرهم) وداخل الجسم علميًا صحة نشاط التئام الجروح لحاء مستخلص السنديان الطابوري الذي أوضح زيادة إنتاج الكولاجين والنشاط المضاد للأكسدة، وبالتالي دعم الادعاءات التقليدية.