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ORIGINAL ARTICLE

Biochemical, histopathological, and pharmacological evaluations of cutaneous wound healing properties of *Quercus brantii* ethanolic extract ointment in male rats

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Abstract

The recent experiments have revealed the property of ethnomedicinal plants on the treatment of the cutaneous wound. The purpose of the experiment was to investigate the wound healing and antioxidant potentials of the internal layer of *Quercus brantii* ethanolic extract ointment. DPPH free radical scavenging test was carried out to examine the antioxidant effect of the internal layer of *Q. brantii* ethanolic extract, which indicated similar antioxidant activity with butylated hydroxy toluene (BHT) as a positive control. In vivo design, 120 Sprague Dawley male rats were used. After creating the cutaneous wound, the animals were randomly divided into four groups: untreated control, treatment with Eucerin ointment, treatment with 3% tetracycline ointment, and treatment with 3% *Q. brantii* ethanolic extract ointment (3 g of *Q. brantii* ethanolic extract + 97 g base ointment). At days 10, 20, and 30 after creating the wound, for histopathological and biochemical analysis of the cutaneous wound healing trend, a section was prepared from all dermal thicknesses. At days 10, 20, and 30, *Q. brantii* ethanolic extract ointment significantly ($p > 0.05$) enhanced wound contraction, fibrocyte count, hexuronic acid, and hydroxyproline levels and decreased the wound area, all cell, macrophage, lymphocyte, and neutrophil counts as compared with the basal ointment and control groups. In conclusion, the acquired findings showed the cutaneous wound healing potential of *Q. brantii* ethanolic extract ointment.

Keywords *Quercus brantii* · Ethanolic extract · Leaf · Cutaneous · Wound healing · Rats

Introduction

Wound healing involves different stages, including inflammation, proliferation, and regeneration. Each of these stages includes other stages, some of which interfere with each other and are not easily differentiated. Therefore, qualitative and quantitative improvement of each stage can lead to the acceleration of wound healing and reduction of the associated side effects (Khorasgni et al. 2010). Many studies have made an attempt to discover compounds that heal the wounds with minimum side effects. Iranian medicinal plants are of a special significance in this regard (Malekzadeh 1986; Dastmalchi et al. 2008; Sayyedrostami et al. 2018). A long list of medicinal plants was used for the treatment of cutaneous wound healing including *Rhus coriaria* L, *Punica granatum* L, and *Ziziphus jujuba* Mill among others (Hosseinkhani et al. 2016). One of the most important herbal medicines which are widely used for the treatment of cutaneous wounds is *Quercus brantii* (Persian oak). It is a species of oak native to Western Asia, including Iran, Iraq, Syria, and Turkey (Pithayanukul et al. 2005; Taleshi and Maasoumi Babarabi 2013). In traditional medicine, *Q. brantii* tea is recommended for topical baths, lotion, and washing in different ways to treat diseases. This tea is traditionally used as local bath for conditions such as ductal obstruction of mammary glands, hematomas melancholy, chronic skin diseases such as eczema, and varicose veins (Bahmani et al. 2015). In addition, the plant has been alleged to treat diarrhea, indigestion, stomach pain, and many other conditions (Bahmani et al. 2015).

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Considering the therapeutic potential of *Q. brantii*, we made an attempt to study the antioxidant and cutaneous wound healing potentials of the internal layer of *Q. brantii* ethanolic extract ointment in rats.

Materials and methods

Extract preparation method

Q. brantii was collected from Kermanshah city in the west of Iran. After complete drying of *Q. brantii* internal layer in the dark without humidity for 1 week, the obtained material was powdered. Of the powder, 200 g was weighed, mixed with 2000 ml (1/10 weight/volume ratio) ethanol, heated at 40 °C, and stirred for 2 h. It was then kept at ambient temperature for 24 h. Next, the extract was filtered with Whatman paper #2. The primary extract was fed into a vacuum distillation apparatus (a rotary machine with a vacuum pump), and the solvent was evaporated at 80 °C for 1 h, yielding the condensed extract. To prepare the powder of the extract, the condensed solution was put in the oven for 48 h at 40 °C, and the obtained substance was lyophilized (Hagh-Nazari et al. 2017; Hamelian et al. 2018; Zangeneh et al. 2018b, c). To prepare the *Q. brantii* ethanolic extract ointment, 3 g extract was mixed with 97 g Eucerin.

Measurement of antioxidant activity of *Q. brantii* ethanolic extract by DPPH

In this study, 11 dilutions of *Q. brantii* ethanolic extract (1000, 500, 250, 125, 62, 31, 15, 7, 3, 2, and 1 μg/mL) were analyzed. Two hundred microliter 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in ethanol, and the equal volume of DPPH was added to each concentration. After mixing, the solution was incubated in darkness for 30 min, and its absorption was read at the wavelength of 517 nm. BHT was used as a positive control, and trapping activity of free radicals was calculated by the following formula (Hosseinimehr et al. 2011). The experiment was repeated three times.

DPPH free radical scavenging (%) = (control − test/control) × 100

In vivo design

A total of 120 male rats of the same race with a weight of 220 ± 50 g were used in this study. The rats were kept in individual cages at 22 ± 2 °C, in 12:12-h dark-light cycle, and with free access to water and food. The rats were anesthetized by intramuscular administration of 5 mg/kg xylazine. To induce anesthesia, 40 mg/kg ketamine was administered according to the previous solution. After induction of anesthesia, the hair between the two scapulae was shaven, and 3 × 3 cm of the area was disinfected with 70% ethanol. A wound (2 × 2 cm) was made by a scalpel, which involved the removal of all cutaneous layers. The depth of the wound included dermis and hypodermis. Then, the animals were randomly divided into four groups, 30 animals in each group:

- Group I: untreated control
- Group II: treatment with Eucerin ointment
- Group III: treatment with 3% tetracycline ointment
- Group IV: treatment with 3% *Q. brantii* ethanolic extract ointment (3 g of *Q. brantii* ethanolic extract + 97 g base ointment) (Ghashghaii et al. 2017)

The ointment was applied to the wound bed for 30 consequent days.

The wound area and shrinkage percentage were accurately calculated as follows:

Percentage of the remaining wound = area of the remaining wound in the sampling day/ area of the remaining wound in day zero × 100

Percentage of wound contractures = 100−percentage of the remaining wound

On days 10, 20, and 30 after complete anesthesia by inhalation of chloroform in a desiccator, a sample was taken from the wound in each group ($n = 10$). Histological sections were equally divided into half, half of which was sent to the laboratory in 10% formalin. After staining the samples by hematoxylin-eosin staining technique, they were analyzed by an optic microscope. In a histopathological study, the levels of all cells, vessel, fibrocyte, fibroblast, ratio of fibrocyte to fibroblast, neutrophil, lymphocyte, and macrophage were measured.

Biochemical studies by determining of hydroxyl proline and hexuronic acid concentrations were performed on another half of the samples:

Hydroxyproline measurement

First, the cutaneous wound sample was transferred to 5 ml Eppendorf. Then, 0.3 ml hydrolysate, 2.5 ml N NaOH, 0.01 M $CuSO_4$, and 6% H_2O_2 were added to each Eppendorf. Next, the Eppendorf tubes were transferred to 80 °C bain-marie. After 15 min, the Eppendorf tubes were cooled in cold water for 5 min. After that, 0.6 ml 5% paradimethyl amino-benzaldehyde and 1.2 ml 3 NH_2SO_4 were added to the Eppendorf. Following this, the Eppendorf tubes were put in 75 °C bain-marie for 15 min and transferred then to cold water for 5 min. Finally, hydroxyproline level was measured by a spectrophotometer at 540 nm wavelength (Caetano et al. 2016).

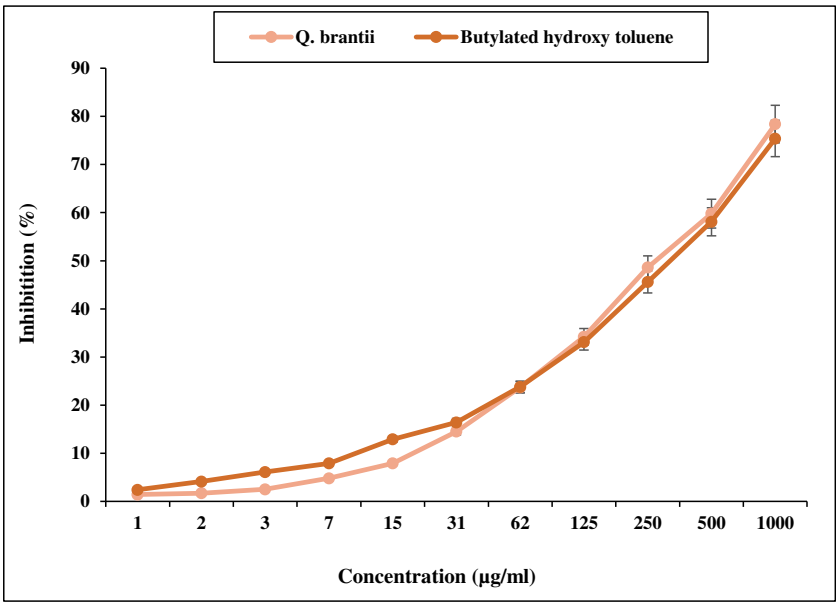

Fig. 1 Antioxidant activity of *Q. brantii*

Hexuronic acid measurement

2.5 mL of 0.025 M Borax in concentrated sulfuric acid is placed in stoppered tubes fixed in a rack and cooled to 4 °C. 0.125 mL of hydrolysate was diluted 0.5 mL by adding distilled water. Now, this 0.5 mL of hydrolysate is layered carefully on Borax-sulfuric acid mixture kept in a rack at 4 °C. The tubes were closed with glass stoppers and then shaken, first slowly then vigorously, with constant cooling by placing tubes in an ice container. The tubes were then heated for 10 min in a vigorously boiling water bath and cooled to room temperature. Thereafter, 0.1 mL of 0.125% carbazole reagent in absolute alcohol was then added to each tube, shaken, again heated in the boiling water bath for further 15 min, and then cooled to room temperature. Color intensity was measured at 530 nm against the blank. Hexuronic acid content of the samples was determined from the standard curve prepared with D (+) Glucurono-6, 3-lactone (Bitter and Muir 1962).

Statistical analysis

The obtained results were fed into SPSS-22 software and analyzed by one-way ANOVA, followed by the Duncan post hoc test. Values of $p < 0.05$ were considered significant.

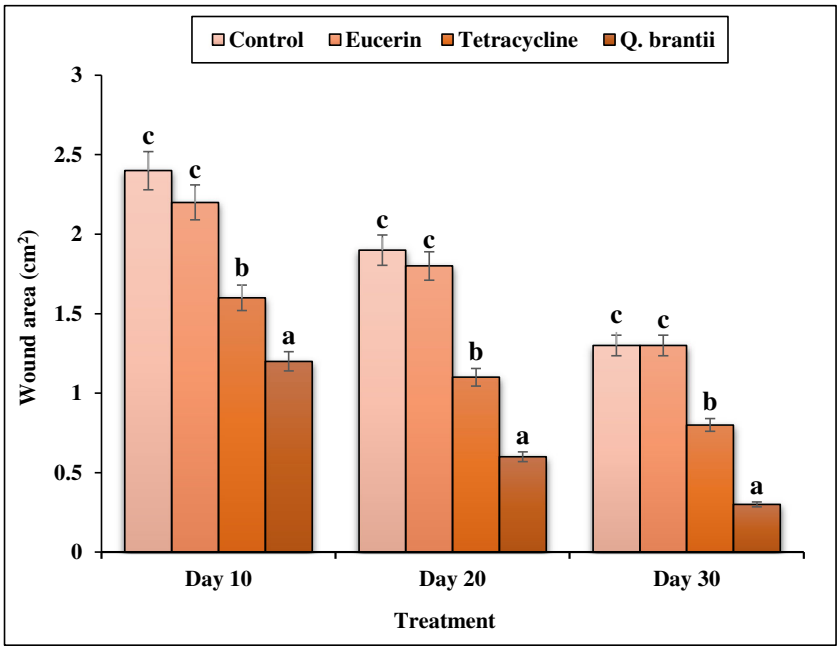

Fig. 2 The level of wound area in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

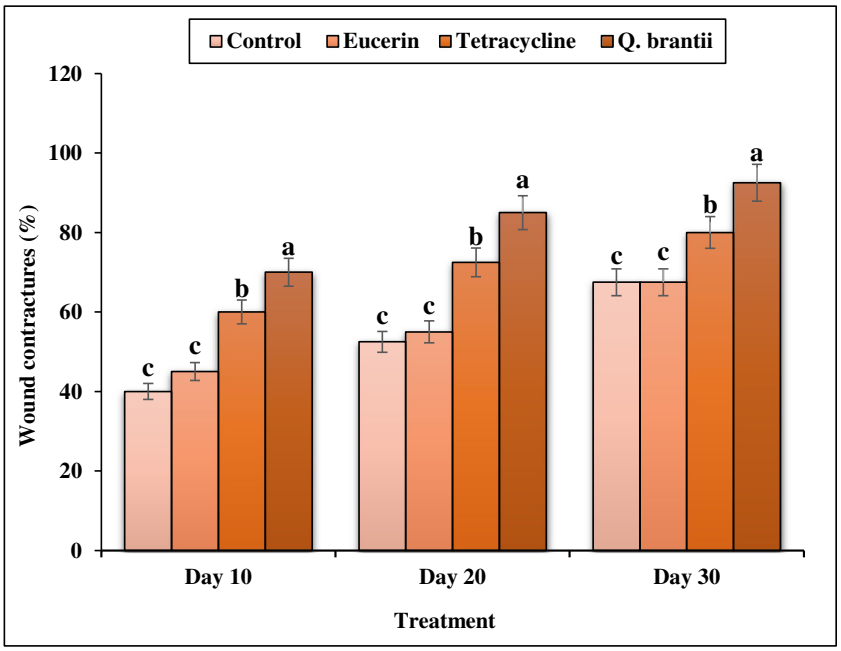

Fig. 3 The percent of wound contractures in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

Results

Antioxidant activity of *Q. brantii* ethanolic extract

DPPH free radical scavenging effect of the *Q. brantii* ethanolic extract indicated impressive prevention in similar with BHT as a positive control. As shown, the free radical scavenging effect of *Q. brantii* increased as a result of an increase in a dose-depended manner. The findings demonstrated antioxidant activity of *Q. brantii* ethanolic extract (Fig. 1).

Effect of *Q. brantii* ethanolic extract ointment on the macroscopic parameters

As demonstrated in Figs. 2 and 3, tetracycline and *Q. brantii* ethanolic extract ointments decreased significantly ($p > 0.05$) the level of the wound area and increased significantly ($p > 0.05$) the percent of wound contracture as compared with the basal ointment and control groups. *Q. brantii* ointment ameliorated significantly ($p > 0.05$) the level of the wound area and wound contracture in comparison of the tetracycline ointment. No significant changes ($p > 0.05$) were observed in

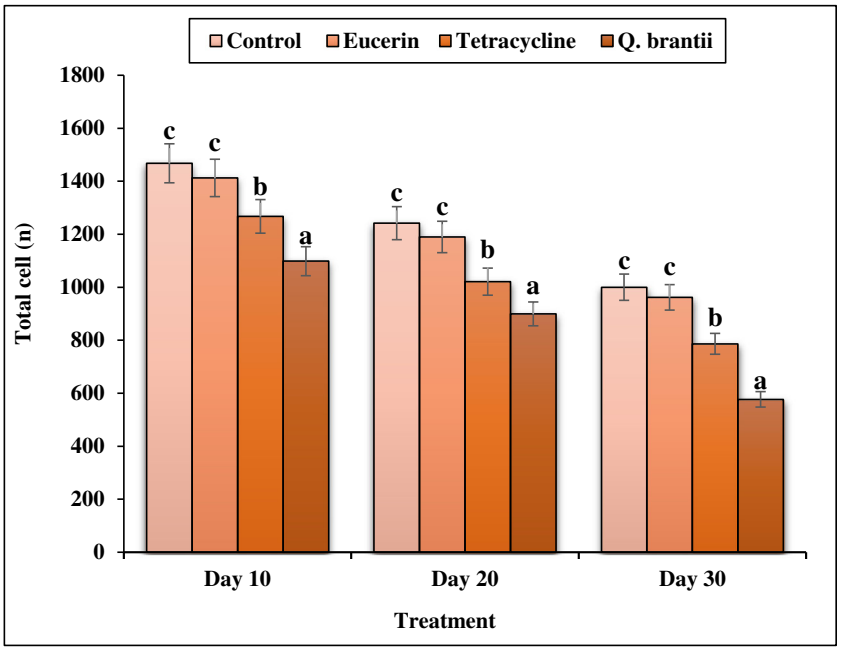

Fig. 4 The number of total cell in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

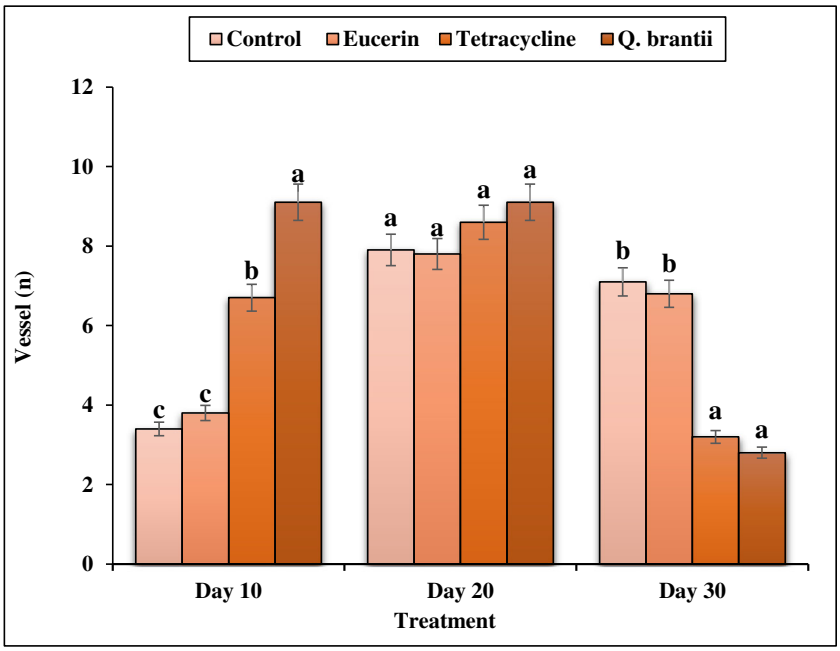

Fig. 5 The number of vessel in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

the above parameters between basal ointment and control groups. There was no sign of pus and infection in the wound area in tetracycline and *Q. brantii* ethanolic extract ointments, but it observed in basal ointment and control groups.

Effect of *Q. brantii* ethanolic extract ointment on the histopathological parameters

Q. brantii ethanolic extract and tetracycline ointments significantly ($p > 0.05$) increased the number of fibrocyte and reduced the number of all cell, lymphocyte, macrophage, and neutrophil per field as compared with the control and basal ointment groups. Also, there were no significant changes ($p > 0.05$) in the above parameters between basal ointment and control groups (Figs. 4, 5, 6, 7, 8, 9, 10, and 11).

Effect of *Q. brantii* ethanolic extract ointment on the biochemical parameters

The level of the hexuronic acid and hydroxyproline increased significantly ($p > 0.05$) in tetracycline and *Q. brantii* ethanolic extract groups as compared with the basal ointment and

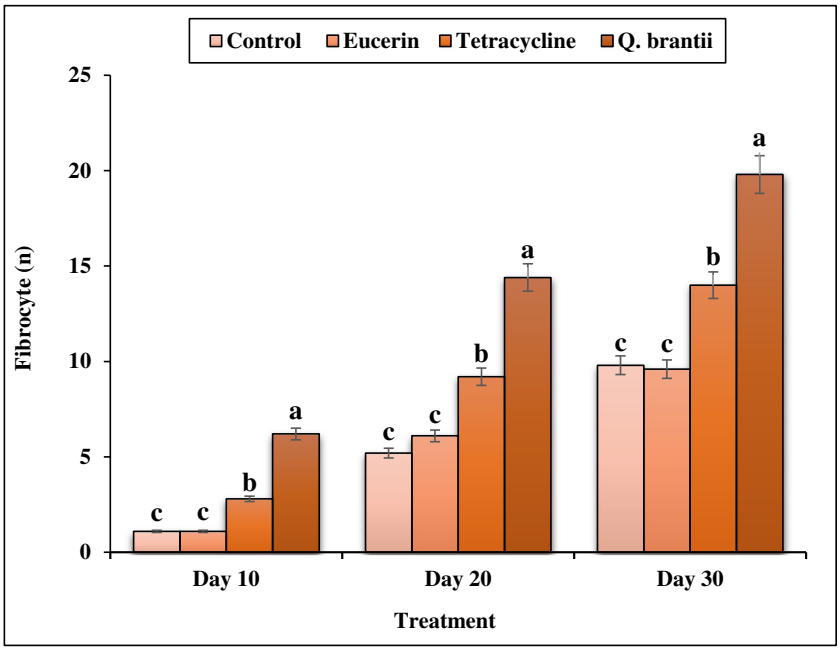

Fig. 6 The number of fibrocyte in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

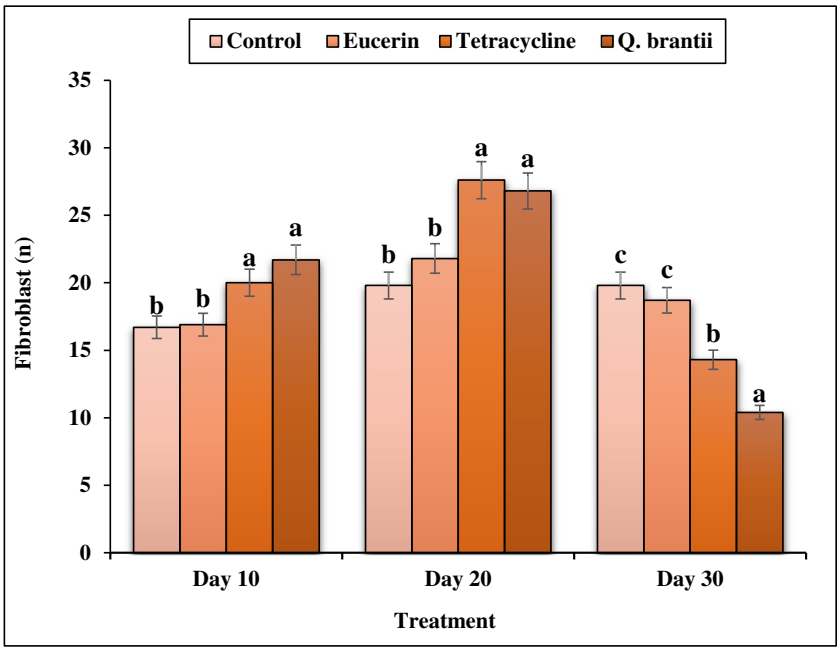

Fig. 7 The number of fibroblast in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

control groups. *Q. brantii* ointment increased significantly ($p > 0.05$) the level of hexuronic acid and hydroxyproline in comparison with the tetracycline ointment. Also, no significant changes ($p > 0.05$) were found between basal ointment and control groups in the above parameters (Figs. 12 and 13).

Discussion

From past to now, many articles indicated that medicinal plants had therapeutical properties (Zhaleh et al. 2018; Goorani et al. 2018; Moradi et al. 2018; Zangeneh et al. 2018a). These studies have revealed that natural drugs are the only treatment modality in some cases, and the compounds available in them have been used in pharmaceutical industries (Rezvanipour et al. 2007; Sherkatolabbasieh et al. 2017; Goorani et al. 2019; Hemmati et al. 2019; Jalalvand et al. 2019). Since no definitive drug has been introduced for wound healing, it is necessary to perform studies on the effects of herbal drugs on wound healing (Rezvanipour et al. 2007).

Wound healing is a dynamic, complex, and regular response to impairment that requires the interaction of different

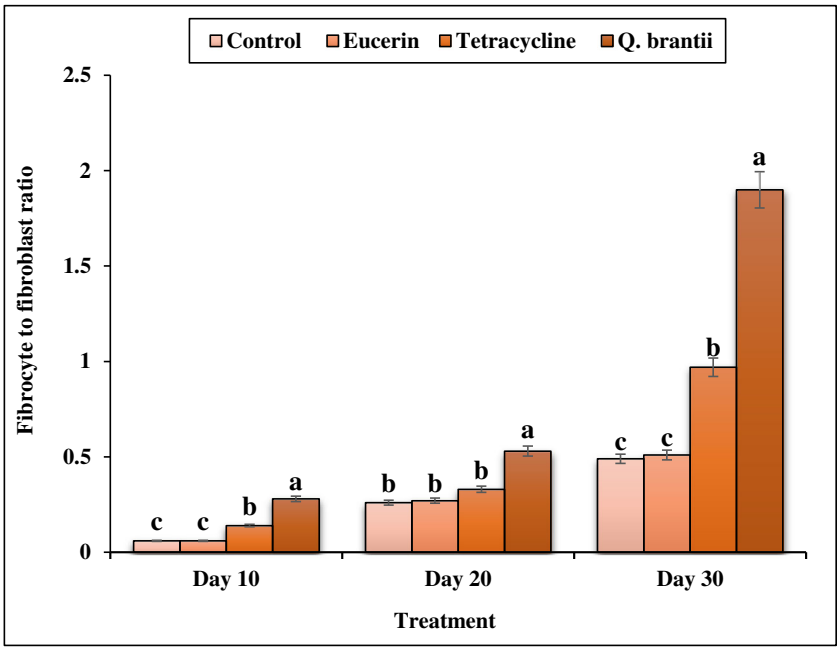

Fig. 8 The ratio of fibrocyte to fibroblast in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

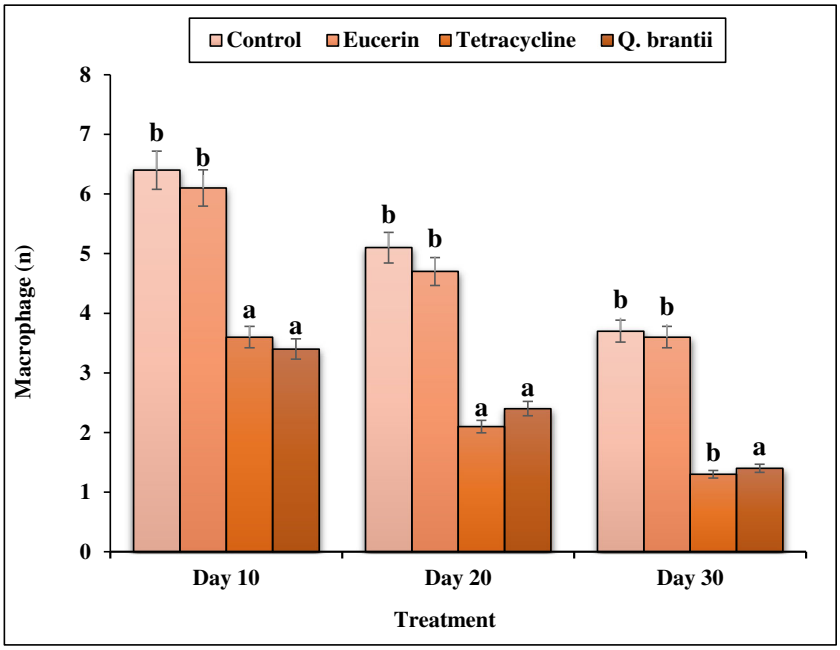

Fig. 9 The number of macrophage in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

types of cells, structural proteins, growth factors, and proteinases (Mussel et al. 2003). The basic principles of wound healing are minimization of tissue damage, adequate blood supply, oxygenation, proper diet, humid environment to create anatomic integrity, and function of the affected site (Kumar et al. 2008). The wound healing process includes accumulation of platelets, coagulation, inflammatory response to damage, changing the underlying materials, angiogenesis, and re-epithelization (Kumar et al. 2008). Restoration is not completed unless the disturbed surfaces of collagen are completely connected to each other (Kumar et al. 2008). Inflammation is a normal phenomenon in the wound healing process and is important for the elimination of the contaminating microorganisms. Prolonged inflammation occurs in the absence of effective decontamination. When microbial cleaning is incomplete, bacteria and endotoxins can prolong the pro-inflammatory cytokines as well as inflammatory phase (Guo and Dipietro 2010). Subcutaneous cells begin to make collagen following injury and regenerate the epithelial cells. Hence, it is therapeutically important to discover medicines to accelerate the regeneration of dermis and epidermis against skin injuries.

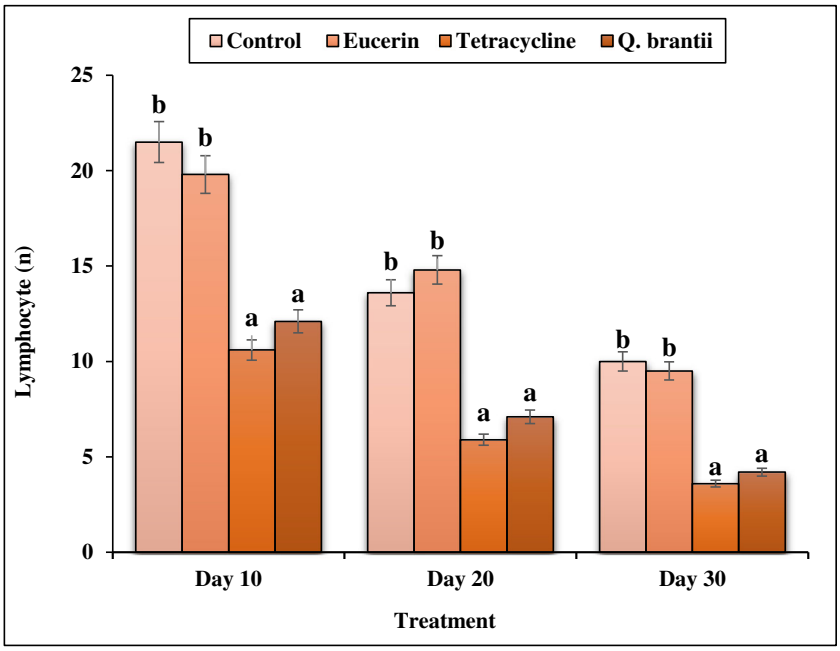

Fig. 10 The number of lymphocyte in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

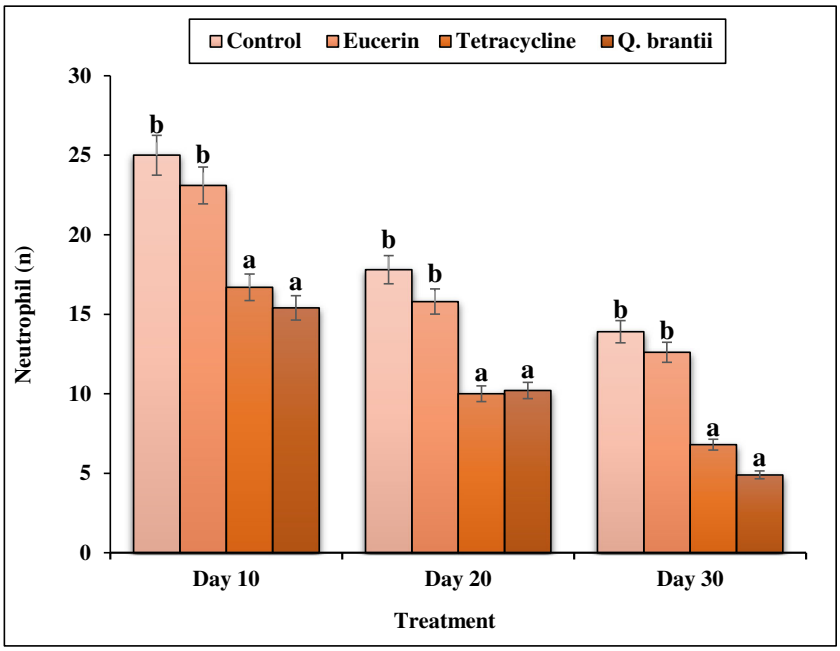

Fig. 11 The number of neutrophil in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

In our study, the findings of wound area and contractures, all cell and vessel levels revealed that ointment of *Q. brantii* significantly ($p > 0.05$) ameliorated the above parameters at 10, 20, and 30 days as compared with the basal ointment and control groups. Angiogenesis is defined as formation of new capillaries from previous vessels. Angiogenesis is a controlled process that is rarely seen in adults except in instances of wound healing and menstrual cycle in women (Lazarus et al. 1994). It is also a phenomenon that mostly occurs in the impaired areas, which is aimed at secreting cytokines in the vessels to repair tissues. Angiogenesis is higher in the early days, reaching its maximum level from days 15 to 20. This level is then reduced with complete withdrawal of cytokines and other tissue repair factors (Phillips et al. 1991).

In a recent study, ointment of *Q. brantii* increased the level of fibrocyte, fibroblast, hydroxyproline, and hexuronic acid and the ratio fibrocyte to fibroblast at 10, 20, and 30 days as compared with the basal ointment and control groups. Fibroblasts are removed through the blood vessels formed at the wound site and are developed into fibrocytes after some time. The amount of fibroblast is usually high until day ten. The main role of fibroblasts is making collagen (Caetano et al.

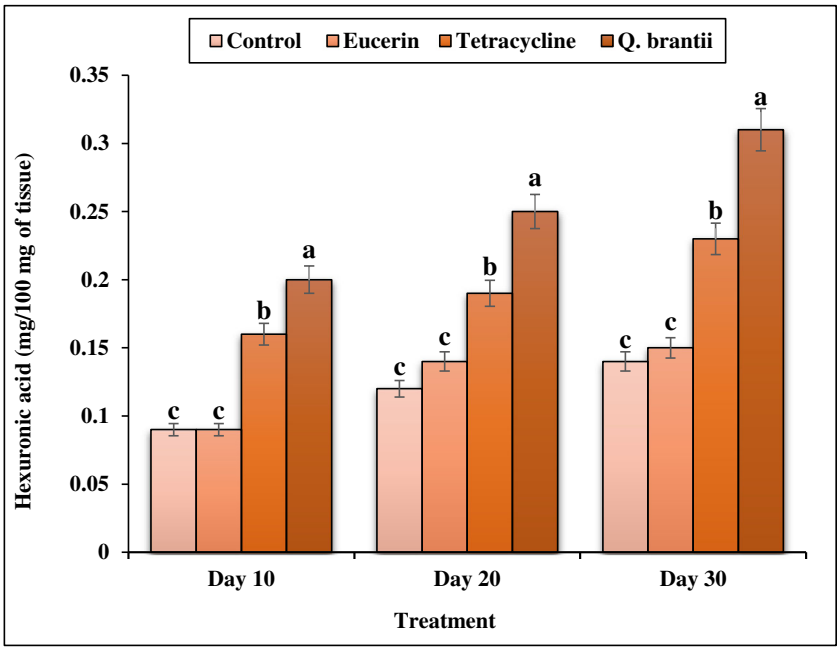

Fig. 12 The level of hexuronic acid in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

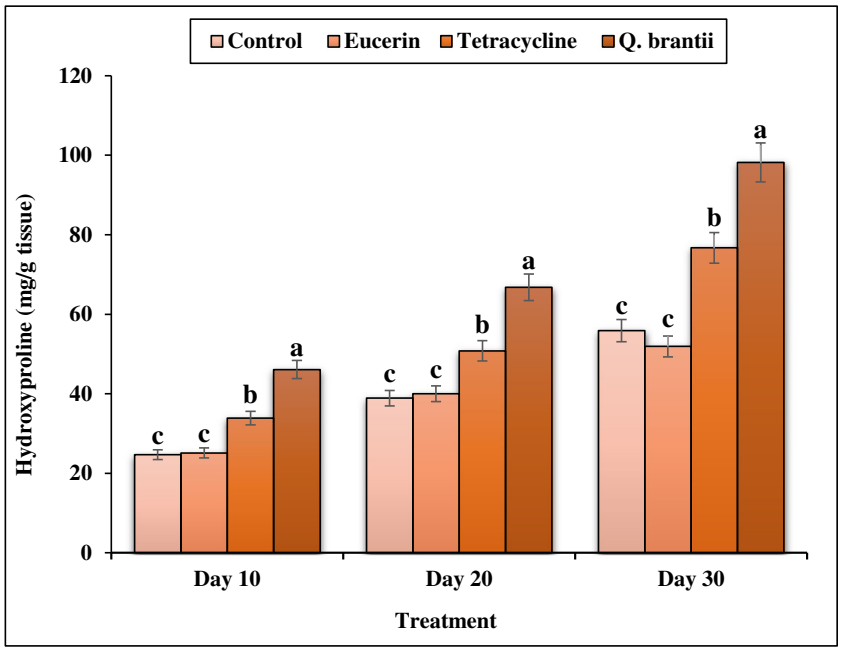

Fig. 13 The level of hydroxyproline in several groups. Non-identical letters reveal a significant difference between the experimental groups ($p < 0.05$)

2016). Fibroblasts synthesize some components of primary extracellular matrix of the wound bed such as fibronectin, hexosamine, and hexuronic acid, which provides a favorable ground for cell migration and proliferation. Fibroblasts then synthesize collagen, which provides a tensile strength in the wound bed (Dwivedi et al. 2017). Fibrocytes are developed fibroblasts that have a higher ability in making collagen than fibroblasts. The more the number of fibroblasts, the better is the wound healing (Caetano et al. 2016). Collagens are protein strains that are made of glycine, praline, and hydroxy proline amino acids. The amount of collagen is very low in the early days but abundantly found in the final days due to an increased number of fibroblasts. The tensile strength of wound is dependent not only on the content of tissue collagen but also on the organization and arrangement of collagen fibers and maturity of fibers (Caetano et al. 2016; Dwivedi et al. 2017).

Also, other results indicated that ointment of *Q. brantii* regulated the number of inflammatory cells (Lymphocyte, macrophage, and neutrophil) at 10, 20, and 30 days as compared with the basal ointment and control groups. Neutrophils prepare the wound area for tissue regeneration by cleaning the wound site from infections and microorganisms. These cells contribute to the acceleration of inflammatory response by releasing some chemotoxic factors to absorb other leucocytes (Guo and DiPietro 2010). Macrophages are the most important cells in the inflammatory stage that contribute to the elimination of necrotic tissues and bacteria (Oryan et al. 2012). These cells also contribute to the localization of inflammation process and absorption of fibroblasts to initiate proliferation by releasing some chemotoxic factors. Therefore, any factor that absorbs or activates the macrophages may have a positive impact on the repair process. In the absence of macrophages, the number of fibroblasts migrating to the wound is also reduced (Oryan et al. 2012). Stimulation of receptors on the surface of cutaneous macrophages stimulates these cells to produce cytokines and advance some stages of wound healing (Nayak et al. 2007; Dong et al. 1993). Lymphocytes, existing in the human peripheral blood mononuclear cells, are an important source of immunoregulatory cytokines in the blood circulation and inflammatory parts of the body. Lymphocytes are increased in the early days (Nayak et al. 2007). Accumulation and overactivation of lymphocytes, macrophages, and neutrophils in the wound site and their extreme secretion produce pus in the infection site reduce the wound healing process and may lead to complete loss of the impaired tissue and even amputation. Further, the presence of free radicals in the wound site may increase the amount of pus (Koh and DiPietro 2011; Foschi et al. 1988). Antioxidant compounds reduce the free radicals and pus in the wound area, thereby healing the wound completely. Other papers reported that medicinal plants rich in anti-inflammatory and antioxidant compounds significantly decreased the production of pus and enhanced wound healing process (Geethalakshmi et al. 2013; Robards et al. 1999). Our study determined that *Q. brantii* ethanolic extract had strong antioxidant property. Therefore, it was usual to observe that *Q. brantii* had a notable wound healing activity.

Conclusion

To conclude, our study demonstrated the antioxidant and wound healing activities of the internal layer of *Q. brantii* ethanolic extract ointment, suggesting its possible use as a

therapeutic supplement or drug. Additional clinical trials are needed to further evaluate the clinical potential of this plant in humans.

Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

Ethic approval All institutional and national guidelines for the care and use of laboratory animals were followed.

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