

THE EFFECTIVENESS OF EXTRACT THE SEED OF POMEGRANATE IN HEALING THE WOUND INDUCED IN RABBITS SKIN

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ABSTRACT

The present study was conducted to evaluate wound induced in rabbit skin by using alcohols and aqueous extracts of pomegranate seeds. Total flavonoids concentration were estimated using high performance liquid chromatography (HPLC). The highest flavonoids concentration were recorded in ethanol extract. Acute inflammation was induced in laboratory animals (rabbits) by injecting 0.1mL of 2.5% formalin inside the wound incision. Aqueous and ethanol extracts of pomegranate seeds an anti-inflammatory and healing effect compared with the Vaseline on rabbit skin. A histopathological study was conducted to observe healing development of the induced wound which proved that healing of the skin was obvious by vanishing of odema and decrease in scar size, enhancement of fibroblast proliferation, collagen regeneration, Keratinization and Epithelialization. Extracts have the ability to reduce the inflammation that induced by formalin and appeared to be efficient in healing wounded rabbits skin.

Keywords: pomegranate, phytochemical analysis, wound healing activity.

سالم وآخرون

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فعالية مستخلص بذور الرمان في شفاء الجروح المستحثة في جلد الأرنب

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المستخلص

أجريت هذه الدراسة لتقييم إمكانية شفاء الجروح المستحثة في جلد الأرنب باستخدام مستخلص بذور الرمان الكحولي والمائي. حضر المستخلصان وجمعت المواد الخام الفعالة بجهاز السكسليت (الكحولي) والنقع (المائي). تم تقييم المحتوى الكلي للفلافونويدات بتقنية HPLC ولوحظ أن التركيز الأعلى للفلافونويدات كانت في المستخلص الكحولي مقارنة مع المائي. تم استحداث الجروح في جلد حيوانات التجربة (الأرانب) عن طريق الحقن بالفورمالين (2.5 %) تحت الجلد. وتم تقييم التأثير المضاد للالتهابات في شفاء الجروح الجلدية بالمقارنة مع الفازلين. أجريت دراسة نسيجية لملاحظة تطور شفاء الجروح، وأوضحت هذه الدراسة أن شفاء الجلد تم من خلال اختفاء الخبز (edema) وانخفاض في حجم ندبة الجرح، وتعزيز انتشار الخلايا الليفية، والأوعية الدموية والتقرن والاندمال بتشكل النسيج الظهري بالمقارنة مع مجموعات السيطرة كذلك بينت النتائج قابلية المستخلصين في خفض الالتهابات الحادة التي استحدثت بالفورمالين كما كانت أكثر فعالية في شفاء الجروح.

كلمات المفتاحية: الرمان، التحليلات الكيميائية النباتية، فعالية شفاء الجروح.

INTRODUCTION

Wound healing process involves many steps including coagulation, inflammation, formation of granulation tissue, matrix formation, remodeling of connective tissue, collagenization and acquisition of wound strength (11). According to Mirele *et al.* (24), four phases of normal wound healing are involved hemostasis, inflammation, proliferation, and remodeling. Healing occur after the disrupted surface is firmly knit by collagen. Kumarasamyraja *et al.*, (22) and Barwary *et al.* (8) showed that nearly six million people suffer from chronic wounds in many countries as a result of the synthetic modern drugs that might have side effects. This issue became a focus to researchers who try to solve the wound infection by using plant extracts as alternative medicine. Bioproducts may have the potential to heal wound since they promote the wound repair mechanisms in natural way. Dhinesh and Ramasamy (13), Jumaa and Ali (18), stated that pomegranate is a shrub or small tree, the fruit is spherical, leathery skin, and contains many seeds embedded in the pulp, green when unripe and turns pink or yellow when ripe. Studies on pomegranate confirmed the presence of several secondary metabolites in different plant parts, mainly ellagic acid (ellagitannins including punicalagins), puniceic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones (9, 30). Traditionally, pomegranate has been used to treat numerous diseases, such as dysentery, diarrhea, cough, diabetic, bleeding disorders, bronchitis, cancers, AIDS, malaria, hyperlipidemia, and obesity (1, 20, 23, 33). Clinical assays *in vivo* and *in vitro* have shown that juice, flower and fruit extracts have antioxidant and anti-inflammatory activities (10, 27). An *in vitro* assay using four separate testing methods demonstrated that pomegranate juice and seed extracts are rich with antioxidants than red wine or black and green tea (4, 32). Thus, to confirm the role of pomegranate seed extracts in wound healing, this study was aimed to use pomegranate seed extract on induced wound healing in rabbits as alternative medicine.

MATERIALS AND METHODS

Plant Collection: Fresh pomegranate seeds were collected from a local Iraqi market, dried in air at room temperature, and grinding into powder by using a coffee grinder.

Aqueous extract

Fifty gram of pomegranate seeds powder was heated in distilled water for 15-20 min at 80 °C, and filtered, then concentrated at 40 °C by a rotary evaporator there after kept in a deep freeze until use (28). Samples of crude extract were prepared at (100, 50, 25)mg/ml.

Alcohol extract

Fifty gram of pomegranate seeds powder was mixed with 250 ml of 96% ethanol and the extract was obtained by soxhlet apparatus for 6 hrs. at 70 °C, then the solvent was removed under reduced pressure by a rotary evaporator at 40 °C. The obtained powder of crude extract were kept in a deep freeze until use (28). Samples of crude extracts were prepared at concentration 100, 50, and 25 mg.ml⁻¹ and mixed well with Vaseline in a ratio of (3:1 v/v) applied topically daily for 8 days (14).

Phytochemical analysis

Identification of total flavonoids

The ethanol and aqueous extracts were subjected for HPLC analysis (21, 29). Standard compounds included rutin, quercetin, apigenin, leuteolin, kaempferol, pinobanksin, pinocembrin, pinobanksin-3-acetate, chrysin, galangen, tetrochrysin, genistein, curcumin, and merietin, were prepared at 10 mg.ml⁻¹ using distilled water. Optimum conditions: Column: DB-C18 (50×2.0 mm, 3 μm particle size), Flow rate : 1.2 ml.min⁻¹; Mobile phase:- phosphate buffer: methanol (60: 40 v/v).

Experimental Animals

Nine healthy mature rabbits 2.5-3 Kg weight were used in this study. They were maintained at a temperature of 20- 25 °C, with free access to food (standard pellets) and water throughout the experimental work. The animals were divided into three groups, first group (A): the animal skin was wounded at the left side of the dorsal surface with no treatment (negative control), whereas the right side of the dorsal surface was treated with Vaseline (positive control), second group (B): Animal skin was wounded and treated with ethanol extract at three concentrations (100, 50, and 25 mg.ml⁻¹), third group (C): Animal skin was wounded

and treated with the aqueous extract at 100, 50, and 25 mg.mL⁻¹.

Animal treatment

Both sides of the animal dorsal surfaces were cleaned with sterile distilled water and then shaved with sharp blade. After removal of hair from both sides, all animals were wounded by making incisions at both sides of dorsal surface (1 cm) below the scapula, which was 3 cm long and 1cm deep according to (26, 3). Three incisions were made on each side and treated with the extracts. After half an hour, a syringe was used to inject 0.1 ml of 2.5% formalin into the wound incision daily for two days to induce an acute inflammation (6).

Wound healing activity

Wounded area was measured by digital photographs using the image analysis program. The evaluated surface area was used to calculate the percentage of wound contraction (15, 12, 3):

$$\% \text{ of wound contraction} = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100$$

Histopathological studies

Samples of skin from healing wound of rabbits were treated with 2.5% formalin fixative for 24 hours. Sections of skin were embedded in paraffin wax and made at a thickness of 4-7 microns, and stained with hematoxylin and eosin. They were

viewed under microscope and photographed to examine histopathological changes.

Statistical analysis

Analysis of variance (ANOVA) was performed, to calculate the differences in whether group variance was significant or not, according to Al-Samurai, (5). The level of significance was considerable at $p \leq 0.05$.

RESULTS AND DISCUSSION

Phytochemical analysis by HPLC

The ethanol and aqueous extracts of pomegranate seeds showed different peak areas representing the presence of different flavonoids (Table 1). The concentration of these compounds in the aqueous extract was 90.460, 170.230, 168.415, and 314.640 $\mu\text{g.mL}^{-1}$ for quercetin, apegenin, leuteolin, and kaempferol, respectively. While, the ethanol extract recorded 132.321, 224.531, 63.52, 776.887, and 167.82 $\mu\text{g.mL}^{-1}$ for quercetin, apegenin, leuteolin, kaempferol, and merietin, respectively. Ali *et al.*, (2) stated similar content of phytochemicals in alcohol extract of pomegranate fruit.

Table1. Total flavonoids concentration in ethanol and aqueous extracts of pomegranate seeds by HPLC

Compound	Retention time minute	Area μ volt	Standard %	Aqueous extract $\mu\text{g.mL}^{-1}$	Alcohol extract $\mu\text{g.mL}^{-1}$
quercetin	2.67	180334	19.3316	90.460	132.321
apegenin	3.77	275845	16.5504	170.230	224.531
leuteolin	4.67	295773	18.3409	168.415	63.52
kaempferol	5.52	316581	16.4409	314.640	776.887
merietin	1.46	334426	18.6571	0.000	167.82
Total flavonoids concentration				743.745	1365.070

Effect of ethanol and aqueous extracts of pomegranate seeds on wounds healing

All treated groups exhibited reduced wound area from day to another. Wound healing activity was observed after eight days of treatment compared to control one (Table 2). Treated wounds showed closure with small scar, smaller incision skin breaking stretch, and inflammation of edema was disappeared. The results of the present study revealed that the highest wound healing activity was observed after using in ethanol extract at 100

mg.mL⁻¹, followed by aqueous extract at 100 mg.mL⁻¹. Measurement of wound healing at 8th day showed that the means percentage of wound closure areas were 50.24 of negative control group, 69.03 of positive control group, and 69.54, 75.49, and 79.64 for the concentrations 25, 50, 100 mg.mL⁻¹, respectively in aqueous extract; and 69.13, 78.68, 84.24 for the concentrations 25, 50, 100 mg.mL⁻¹, respectively in alcoholic extract (Table 2).

Table 2. Effect of different treatments on wounds contraction in induced skin rabbits.

Compound	Retention time minute	Area μ volt	Standard %	Aqueous extract $\mu\text{g.mL}^{-1}$	Alcohol extract $\mu\text{g.mL}^{-1}$
quercetin	2.67	180334	19.3316	90.460	132.321
apegenin	3.77	275845	16.5504	170.230	224.531
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Total flavonoids concentration				743.745	1365.070

* Values are means of three replicates \pm S.D

Histological features of incision in animals skin are illustrated in Figure 1, (Group A). The histopathological changes in the negative control (N) are characterized by the presence of wound injury, and congestion of blood vessels and neutrophils aggregation which led to form pus of dead neutrophils surrounded by capsule consisted of fibrous connective tissue. There was epithelialization of epidermis and also a granulation tissue characterized by fibroblast and collagen fiber. While the histopathological changes of the positive control (Vaseline treated) involved the presence of granulation tissue covered by few layers of epithelialization cells which represented the epidermis. Vaseline (petroleum jelly) kept wounds clean and moist, and also provided an occlusive layer. It kept germs out, thus decreasing the risk of infection. Apart from that it hydrated the wound and stimulated the healing process (17). The groups B in figure 2 section (d, e, f), shows wound healing after treatment with ethanol extract of pomegranate seeds at (100, 50, 25) mg.mL^{-1} respectively. At 100 mg.mL^{-1} the epithelialization started with more than one layer covering the dermis layer and the healing area consisted of granulation tissue resulted mostly from fibrous connective tissue, and collagen. While at 50 mg.mL^{-1} the epithelialization these consisted from small area while most of wound area filled with granulation was less than the treatment with 100 mg.mL^{-1} . At 25 mg.mL^{-1} , the healing area composed of just granulation tissue as area of wound without epithelialization. Group C (d, e, f) from the recovery aqueous extract of pomegranate, at (100, 50, 25) mg.mL^{-1} . At 100 mg.mL^{-1} the wound healing was less than the wound treated with alcohol extract, the epithelialization appeared more clear with one

layer covered the granulation tissue with congestion of blood vessels. While the healing area at 50 mg.mL^{-1} , is characterized by granulation tissue covered by necrotic area forming scab separated inflammatory cells mostly mononuclear cells but 25 mg.mL^{-1} , just necrotic area was obvious forming scab covered by granulation tissue which appeared wider than 0.5 mg.mL^{-1} . The effect of extracts was clear and rapid after eight days of treatment, through reduced scar formation, exhibited increased fibroblast proliferation, collagen regeneration, angiogenesis, keratinization and epithelialization as compared to treatment groups. Park *et al.*, (25) observed that a decrease of open skin wounds, leaves an extraordinarily little scar as the circumference of skin moves centripetally to close the wound. Enhanced healing was ascribed to generate collagen fibers, and angiogenesis. During the process of wound healing, various inflammatory cells (neutrophils, macrophages, and fibroblasts) produce several types of reactive oxygen species (ROS) and increase proteases, which led to kill fibroblasts, other cells and less flexible for skin lipids, so oxidative stress occupies a central role in wound healing against microorganisms preventing host cells damage by inhibiting ROS (7). The antioxidants such as flavonoids, tannins, vitamin C and vitamin E are identified to accelerate the wound healing action by mainly eliminating the oxidants, the alteration in homeostasis leading to oxidative stress (31). The results demonstrated that extracts affected differently in wound healing activity, but ethanol extract of pomegranate seeds was the best in this regard. Such potent activity seemed to be correlated with the total polyphenolic compounds, because the strong wound healing activity occurred in extracts that are rich in

phenolic and flavonoid compounds. It may due to the constituents possess very potent antioxidant and antimicrobial activities. Furthermore, the results are in agreement with Kandermir *et al.* (19) who studied the wound healing activity of methanol extract for pomegranate seed on rabbits skin by oral administrate, animals treated with 5% of the extract showed complete healing after 10 days. Flavonoids are mostly have pharmacological active constituents of various samples and they

are well known that these products are powerful anti-oxidants. For this reason pomegranate seeds are considered as natural source of wound healing compounds.

CONCLUSION

Ethanol extracts of pomegranate have a therapeutic effect on induced wounds in rabbits skin during eight days. Further studies are required for isolating the compounds responsible for the wound healing activity and evaluate the mechanism of wound healing

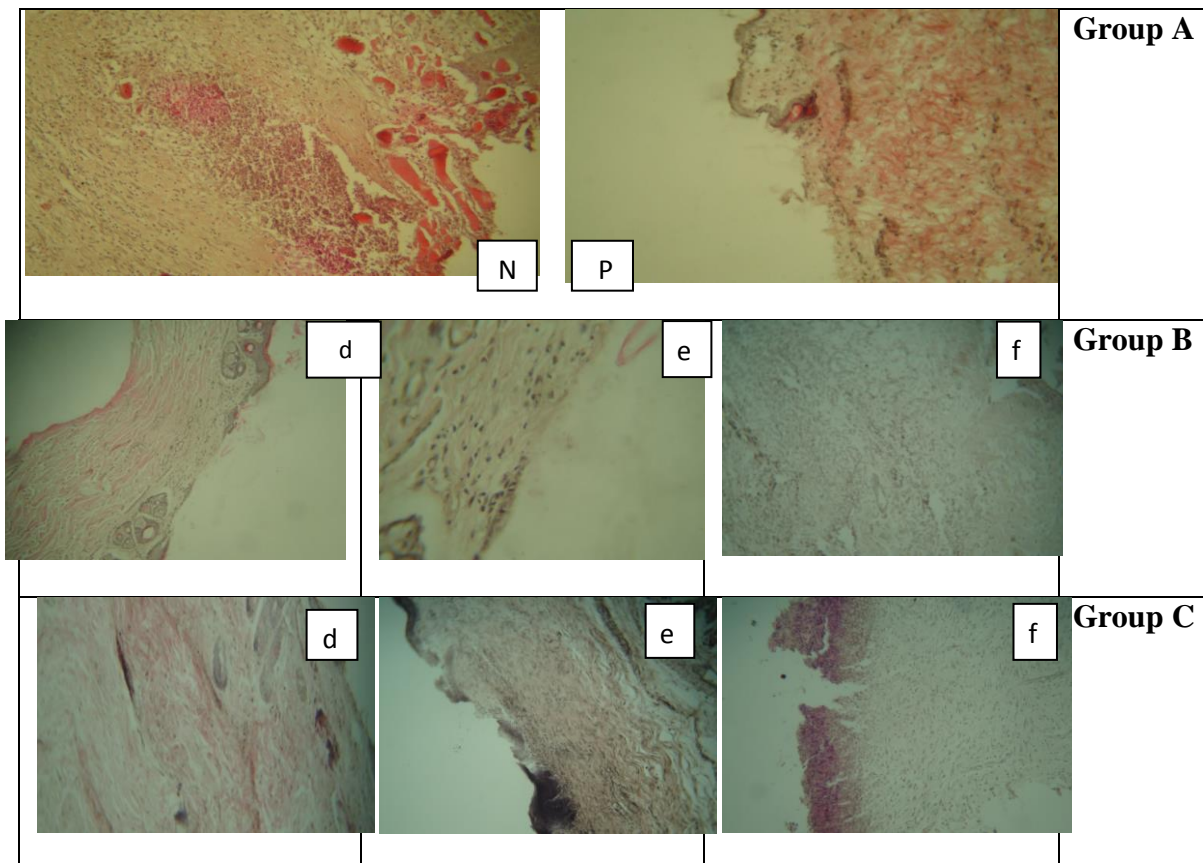


Figure 1: Longitudinal section in incision of rabbit skin.

Group A: (N)Negative control, (P) positive control (treated with Vaseline only).

Group B: treated with ethanolic extract of pomegranate seed(d: 100 mg/ml⁻¹, e: 50 mg/ml⁻¹, f: 25 mg/ml⁻¹)

Group C: treated with aqueous extract of pomegranate seed(d: 100 mg/ml⁻¹, e: 50 mg/ml⁻¹, f: 25 mg/ml⁻¹).

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