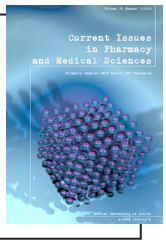
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In vivo healing potential of *Vitis Vinifera* L. and *Punica Granatum* L. fruit extracts in excision and burn models in rabbits

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ABSTRACT

Aim. The present study was carried out to evaluate the wound healing potential of crude Hydroalcoholic fruit extracts of Grape (*Vitis vinifera* L.) and Pomegranate (*Punica granatum* L.) in experimental animals.

Material and methods. Extracts were formulated as ointments (250 mg), and their wound healing properties were determined by using two models: i.e. excision and burn models, in rabbits. Wound healing potential was assessed by measuring rate of wound contraction (percentage wound contraction) and the epithelialization period. *Tukey test* (HSD) test was used to analyze the obtained results. The value of $p < 0.05$ was considered as a level of significance. The reference standard was wadded Cicatryl® ointment.

Results. The result showed that crude fruit extracts of *Vitis vinifera* L. and *Punica granatum* L. exhibited a significant ($p < 0.0001$) rate of wound contraction and significant ($p<0.0001$) decrease in the period of epithelialization compared to the control (untreated) group in both the models studied.

Conclusion. Our results suggest that topical treatment of fruit extracts of *Vitis vinifera* L. and *Punica granatum* L. accelerate wound healing activity, thus our study supports their traditional use. Both extracts can be used as a potential source of wound healing therapeutics.

INTRODUCTION

A wound is characterized as condition known by the loss of epithelial integrity, as well as the disruption of the skin's and underlying tissues' normal structure and function. The wound healing process is usually divided into: inflammation (0-3 days), cellular proliferation (3-12 days) and tissue remodeling (3-6 months), that are the three overlapping phases. Any substance which stimulates wound healing is considered as an enhancer of the wound healing process [1].

Many ointments are used to treat and to reduce the risks in wounds. However, these topical ointments are usually only partially successful in the healing mechanism. Hence, the need of new wound healing drugs is imperative. Plant-based chemicals are considered as potential wound healing agents due to their extensive homeopathic use, low toxicity on dermal cells, effectiveness and simplicity in administration – even as a raw preparations [2,3].

Recently, plant-based wound healing preparations have received enormous attention from the scientific community. Currently, about one-third of the treatments used for wound healing and skin diseases are achieved from plant sources, only 1-3% of the total were synthetically produced [4,5]. Still, a review of current literature has revealed that little or no recent studies on the wound healing activity of plant fruit extracts have been conducted, despite a number of investigations demonstrating a considerable beneficial relationship between human health and fresh fruit consumption due to their bioactive constituents, which have strong biological effects [6,7].

Vitis vinifera L. (locally known as "Dalya" or "black grape") of the family Vitaceae is a vine widely distributed in central Europe, southwestern Asia and the Mediterranean

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regions, and is cultivated today in all temperate regions of the world [8,9]. Traditionally, *V. vinifera* is used in conditions like hemorrhages, anemia, anti-inflammatory, leprosy, skin diseases, syphilis, anti-carcinogenic, asthma, platelet aggregation inhibiting, jaundice, bronchitis and metal chelating properties [10]. Studies have reported that black grape seed extract possess a broad range of therapeutic effects including antioxidant, antibacterial, and anti-inflammatory activities, as well as neuro-protective, hepato-protective and cardio-protective effects [11]. Research indicates the presence of phenolic acids (malic acid, citric acid, oxalic acid, succinic acid, tartaric acid etc.), flavonoids (quercetin and kempferol glucosides), phenylacrylic acid derivatives (caffeoyl acid, p-coumaroyl acid and feruloylsuccinic acid), tannins (procyanidolic oligomers) and Stilbenes (viniferins and resveratrol) in the *V. vinifera* leaves and fruits [12].

Punica granatum L. (locally known as "Rouman" and 'Pomegranate") belongs to the family Lythraceae. It is a Middle Eastern native fruit bearing shrub that is now commonly grown in temperate and tropical climates around the world, including the Mediterranean region [13,14]. *P. granatum* has been traditionally used by local cultures as an important medicinal plant. Pomegranate is said to have antibacterial, antioxidant, anticancer and anti-proliferative activities. Pomegranate peels are used in homeopathy as a popular therapy for dysentery, diarrhea and stomatitis. Pomegranate juice is known to possess anti-carcinogenic properties and beneficial effects against platelet aggregation and oxidative stress. Various phytochemical studies have reported that pomegranate fruits are rich sources of polyphenolic compounds [9,15], among others, punicic acid, ellagic acid, punicalagin, ellagitannin, anthocyanidins, oestrogenic flavones and flavanoles [16,17].

A thorough investigation of literature data suggests that the eating of grape (*Vitis vinifera* L.) and pomegranate (*Punica granatum* L.) reduces the risks of infections and oxidative stress [18]. However, the healing activity of the selected fruit extracts and their mechanisms of action still remain unknown. Therefore, the goal of current study is to determine the cicatrizing activity of Algerian grape and pomegranate fruit extracts.

To the best of our knowledge, there is no published evidence in form of literature on the therapeutic properties of fruit extracts from *V. vinifera* and *P. granatum*. Therefore, the present work was aimed to assess the wound healing activity of *V. vinifera* and *P. granatum* fruits extracts. Our study is the first to report the increasing of wound contraction rate and the lowering of the healing time in rabbits upon treatment with the selected fruit extracts of *V. vinifera* and *P. granatum*. We also observed that treatment with fruits extracts of *P. granatum* significantly enhanced the rate of wound contraction when compared with *V. vinifera.* Moreover, fruit extracts of *V. vinifera* and *P. granatum* showed higher healing activity than that of the commercial cream Cicatryl Bio ®.

MATERIAL AND METHODS

Plant Material

Plant samples were obtained from the local market of Constantine, eastern Algeria, in the 2020 harvest season. An expert taxonomist undertook authentication of the samples of both the black grape (*Vitis vinifera* L.) and the pomegranate (*Punica granatum* L.). Samples consisted of mature fresh fruits that have been thoroughly cleaned with tap water to eliminate any sticking contaminants. The samples were freeze-dried, powdered, and stored at -25°C until they were extracted.

Drugs and Chemicals

For the extraction, methanol used were purchased from Sigma Aldrich, while ketamine hydrochloride®, diazepam® and Cicatryl Bio® were purchased from a private pharmacy.

Extraction process

The lyophilized edible fruits were subjected to extraction by maceration assisted by ultrasound [9,19] in an ultrasonic bath (Fisher scientific fb 15046, Leicestershire, England) (>20 kHz in frequency) for 30 min at room temperature using methanol/water (70/30) as solvent in a solid/liquid ratio of 25 g/500 ml, followed by maceration in a magnetic stirrer for 24h. The remaining vegetal material was extracted again twice under identical conditions. Combined extracts were filtered and concentrated in vacuum at 45°C to obtain a crude extract which was immediately subjected to lyophilization (Christ Alpha 2e4 LD plus, Osterode am Harz, Germany) and kept at -25°C until further analysis.

Experiment animals

Healthy Albino rabbits (male sex) weighing 2.72±0.3 kg were selected at the beginning of the experiment for this study. We used male animals as females demonstrate more individual variability due to cyclical reproductive hormones. Animals were housed individually, and allowed free access to standard pellet diet and water *ad libitum* in a standard environment [20,21]. The animals' dorsal fur was shaved using an electrical clipper. An intramuscular injection of ketamine hydrochloride® 15 mg/kg was used to anesthetize the rabbits. The anesthesia was supplemented with 5 mg/kg of diazepam® locally at the place of excision or burns prior the experimental wounds [20,21].

Healing activity

Hydroalcoholic fruit extracts of *Vitis vinifera* and *Punica granatum* were tested for their therapeutic properties using burn wound and excision models according to the procedures described previously [22,23]. Six excisions or burns wounds were performed on the same animal; each rabbit received one of the following treatments:

- cream of hydroalcoholic fruit extract of *Vitis vinifera* L. or *Punica granatum* L. at the dose of 250 mg;
- a healing cream Cicatryl Bio® at the dose of 250 mg; A rabbit with wounds or burns, without treatment is used as a control.

Excision test

In the standard excision test, prior to the experiment, the test animals were anesthetized. A full thickness skin of circular area of about 17 mm was then incised [22]. Daily wound therapy with a precise amount of tested extracts

or ointment was subsequently given until the wound healed completely.

The percentage of healing of the excision wounds was calculated every three days, according to the following equation:

$$Cw\ (\%) = \frac{\text{wound area on day}_0 - \text{wound area on day}_n}{\text{wound area on day}_0} \times 100$$

where: Cw – Contraction of the wound.

Burn test

For this test we adopted the technique of burns by hot plate. The protocol followed is described by Farnood Shokouhi *et al.* [23]. Six wounds were made on the back of each rabbit on either side of the thoracolumbar spine, by holding a metal cylinder heated at 170°C on the skin of the anesthetized animals for 10 sec. without exercising any strength. Just after the induction of burns, the cream of the tested hydroalcoholic fruit extracts or commercial cream control are applied topically on the burned area.

The frequency of application of creams on wounds was fixed at one application per day in a daily way until complete healing. A macroscopic observation was performed before each application. The percentage of wound healing was calculated every three days relative to the initial wound size in accordance with the given equation:

$$Cw\ (\%) = \frac{\text{wound area on day}_0 - \text{wound area on day}_n}{\text{wound area on day}_0} \times 100$$

where: Cw – Contraction of the wound.

Statistical analysis

The wound contraction percentage means are presented as mean values ± standard deviation (n=6). The *Tukey HSD* test was used to examine the differences between the groups. Statistical significance is defined as a value of $p < 0.05$. The results were comparable to those of the control group. *XL Stat* version was used to perform the statistical analysis.

RESULTS

Preparation of extracts

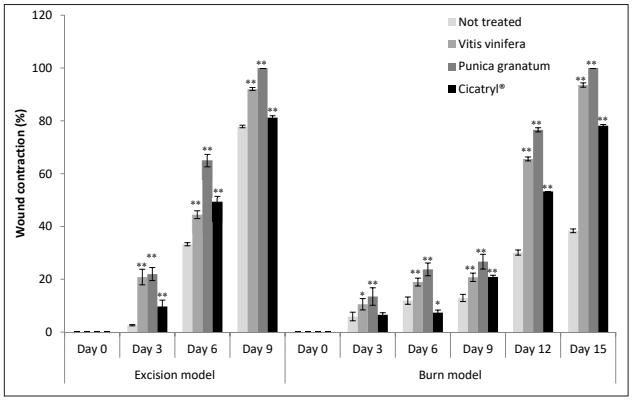
The maceration process assisted with ultrasound that was employed to prepare ointments from grape and pomegranate edible fruit, yielded a sufficient amount of the extracts. The physicochemical properties of the ointments are shown in Table 1.

Excision test

The measurement of the progress of the wound healing in excision wound model is presented in Figures 1, 2 and Table 2. The present study revealed that the skin wounds treated with fruits extracts of *V. vinifera*, *P. grantum* and the commercial cream Cicatryl Bio® showed a faster ($p<0.0001$) contraction of the wounds at 3rd, 6th and 9th days when compared to the control (untreated) group. With the exception of group II at 6th day, the degree of wound contraction of the groups treated with the fruits extracts of *V. vinifera* and *P. granatum* was faster ($p <0.0001$) than that observed for the group treated with Cicatryl Bio®. From the 6th day onwards, the contraction of wounds was faster in the group treated with *P. granatum* fruit extract compared to that of *V. vinifera.* We observed complete wound healing on the 9th day for the group treated with fruit extract of *P. granatum*.

Table 1. Physiochemical properties of *Vitis vinifera* L. and *Punica granatum* L. fruit extracts formulations

Parameter	Grape extract	Pomegranate extract
Species	*Vitis vinifera* L.	*Punica granatum* L.
Vegetative part	Berry of grapes	Berry of Pomegranate
Vegetative stage	Mature fruit (September 2020)	Mature fruit (September 2020)
Used parts	Whole fruit with seeds and envelope	Fruit without bark
Extraction yield (%)	50.29±0.06	68.09±0.05
Color	Purplish-red	Pink
Odor	Characteristic	Characteristic
Taste	Sour sweet	Sour sweet
Spreadability (seconds)	Good	Good
pH	4.0-5.0	3.0-4.0
Stability Skin irritation study	No skin irritation was observed	No skin irritation was observed
Washability	Satisfactory	Satisfactory
Homogeneity	Good	Good
Solubility	Water	Water



mean value ± SD; *p <0.001, **p <0.0001 *vs* control

Figure 1. Healing wound contraction in both models (excision and burn) in different group treatments at different time intervals

Table 2. Percentage of excision wound contraction in different group treatments at different time intervals

		Excision wound contraction (%) Δ		
Group	Treatment	Day 3	Day 6	Day 9
Group I	Not treated	2.59 ±0.24	33.26 ±0.59	77.86 ±0.48
Group II	*Vitis vinifera* L	20.84 ±2.95**■	44.52 ±1.36**■#	92.03 ±0.58**■#
Group III	*Punica granatum* L	21.96 ±2.48**■	65.01 ±2.77**■	100 ±0.00**■
Group IV	Cicatryl Bio®	9.67 ±2.42**	49.32 ±2.07**	81.18 ±0.74**

Δ mean value± SD (n=6); ** p<0.0001 *vs* control; ■p<0.0001 *vs* Cicatryl®; # p <0.0001 *vs* P. granatum

Burn test

The results of the wound healing effect fruits extracts *V. vinifera* and *P. granatum* and commercial cream Cicatryl bio® in the burn wound model is presented in Figures 1, 3 and Table 3. Both fruit extracts (from the 3rd day) and Cicatryl bio® (from the 6th day) showed significant effects of wound

Table 3. Percentage of burn wound contraction in different group treatments at different time intervals

		Burn wound contraction (%) Δ				
Group	Treatment	Day 3	Day 6	Day 9	Day 12	Day 15
Group I	Control	5.89 ±1.64	11.91 ±1.39	12.92 ±1.37	30.12 ±0.99	38.35 ±0.72
Group II	*Vitis vinifera* L	10.53 ±2.15*▪	18.94 ±1.50**▪▪#	20.76 ±1.60**▪▪#	65.56 ±0.76**▪▪#	93.55 ±0.83**▪▪#
Group III	*Punica granatum* L	13.46 ±3.31**▪▪	23.73 ±2.47**▪▪	26.66 ±2.81**▪▪	76.59 ±0.80**▪▪	100.00 ±0.00**▪▪
Group IV	Cicatryl Bio®	6.53 ±0.80	7.35 ±0.99*	20.88 ±0.64**	53.23 ±0.00**	78.12 ±0.47**

Δ Mean value±SD; *p<0.001, **p<0.0001 *vs* control; ▪p<0.05; ▪▪p<0.0001 *vs* Cicatryl Bio®; # p<0.0001 *vs P. granatum*

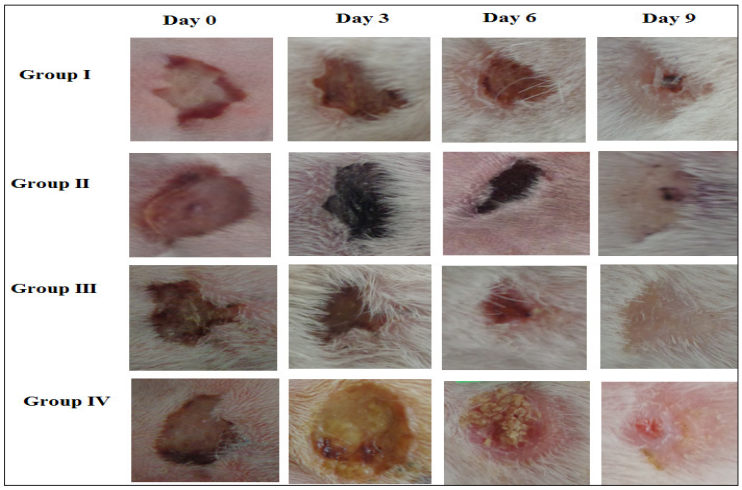


Figure 2. Chronology of healing of treated and untreated (control) excisional wounds in rabbits (Group I: not treated, Group II: treated by *Vitis vinifera* L. extract, Group III: treated by *Punica granatum* L. extract, Group IV: treated by Cicatryl Bio®)

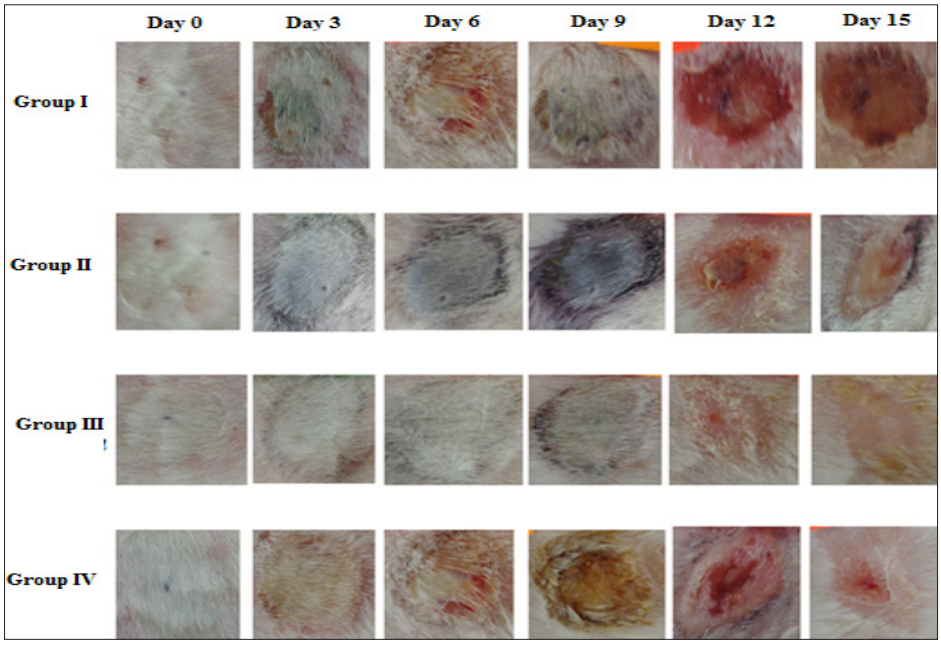


Figure 3. Chronology of healing of treated and untreated (control) burns in rabbits (Group I: not treated, Group II: treated by *Vitis vinifera* L. extract, Group III: treated by *Punica granatum* L. extract, Group IV: treated by Cicatryl Bio®)

healing potential. Over the whole period (3rd day → 15th day), the groups treated with fruits extracts of *V. vinifera* and *P. granatum* demonstrated significant reduction in wound area as compared to that treated with Cicatryl Bio®. The group treated with the fruit extract of *P. granatum* showed significantly (p <0.0001) higher wound repair activity as compared to the group treated with the fruit extract of *V. vinifera*. On the 15th day, we noted that the group treated with *P. granatum* fruit extract had completely healed wounds.

DISCUSSION

Wound healing is a process by which damaged tissue is restored as closely as possible to its normal state, while wound contraction is the process of shrinkage of the area of the wound [24]. Therefore, results obtained in the present study (excision and burn) demonstrate that the treatment of excision and burn wounds with crude hydroalcoholic fruit extracts of grape and pomegranate accelerated the wound healing process. The increased rate of wound contraction, lesser epithelialization phase, and lesser scar area led to faster healing as confirmed by the increased healed area compared to (untreated) control. The wound healing results of the present study are in agreement with those reported in literature; topical application of grape seed extract [25,26] or pomegranate seed extract [27,28] on excision wounds in animals exhibited a significant (p <0.001) diminishing in wound area compared to untreated wounds, in addition to accelerated wound closure rate in *Vitis vinifera* and *Punica granatum* seed extracts-treated wounds.

Moreover, the preliminary phytochemical investigation on the fruit extracts of *V. vinifera* and *P. granatum* demonstrated that *V. vinifera* and *P. granatum* are a rich source of phenolic compounds, including gallic acid, catechins and resveratrol, as well as a wide range of procyanidins [9,19] which can accelerate wound healing. The presence of these metabolites, which are reported to play a critical role in the wound healing process by increasing the rate of wound contraction, lowering the period of epithelialization and preventing secondary bacterial infections that would induce complex and delayed wound healing, could explain the wound healing activity [29,30]. Other studies have shown that phenolic compounds are effective in the treatment of skin diseases, aging skin and skin lesions – including wounds and burns. What is more, they were found to reduce the wound healing time and, by creating constriction at the wound site, facilitated reconstruction of the epithelial cells [31].

Considering the results of recent studies, fruit extracts has been shown to contain antioxidant and anti-inflammatory

properties and to strengthen the immune system [32]. Many studies have proposed that wound healing activities of plants extracts might be related to their antioxidant activity. According to Casado-Diaz *et al*. and Pitz *et al.* [33,34], bioactive phyto-constituents that reduce free radicals are able to offer protection against skin damage by preventing depreciation at the cellular level through inhibiting the inflammation that leads to collagen reduction. Overall, it could be concluded that the extract of grape and pomegranate fruits accelerates wound healing by enhancing the number of immune cells, accelerating the second stage of the healing, and accelerating the migration of fibroblast to the wounded tissue. Herein, fibroblasts produce collagens, elastin, and proteoglycans, while collagens increase the elasticity of the wound, thereby enabling its contraction [35]. Treatment by these extracts was associated with a more well-defined epithelial hypo-proliferative region, greater cell density, greater connective tissue storage and better tissue structure. Topical treatment with these extracts also resulted in a higher production of vessel enclosure growth factor (VEGF) at the edge of the wound. Altogether, the topical application of this extract facilitated skin wound healing [28,32].

However, further studies are needed for better assessment of the potential of *Vitis vinifera* and *Punica granatum* fruits extracts as wound healing agents. To isolate the active compound(s) responsible for its pharmacological effect, more phytochemical research is required and histological examinations are recommended.

CONCLUSION

The present study revealed that topical treatments of fruit extracts of *Vitis vinifera* L. and *Punica granatum* L are able to aid in the healing of wounds and that such extracts can efficiently be used as wound-healing agents. The outcome might be attributable to the phyto-constituents included in them, which increased wound healing activity either independently or cumulatively. Further studies are highly warranted to fractionate and identify the pharmacologically active compounds. The exact molecular mechanism of action and the active principles remain to be investigated.

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CONFLICT OF INTERESTS

There are no conflicts of interests as declared by the authors.

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