

Original Research Article

In vivo analgesic activity and safety assessment of *Vitis vinifera* L and *Punica granatum* L fruits extracts

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Abstract

Purpose: To investigate the analgesic properties of fruit extracts of *Vitis vinifera* (grape) and *Punica granatum* (pomegranate) in Albino mal mice.

Methods: The analgesic activity of fruit extracts of *V. vinifera* and *P. granatum* were examined in vivo using thermal stimulus assays (i.e., tail immersion and hot plate) and acetic acid-induced writhing test using acetylsalicylic acid (0.1 g/kg, per os) as standard. The extracts were administered orally in doses of 1.0, 2.0 and 3.0 g/kg.

Results: In acetic acid writhes test, both fruit extract pretreatments (1.0, 2.0 and 3.0 g/kg, per os) significantly decreased the number of writhes ($p < 0.0001$) in a dose-dependent manner compared to control. The Index of Pain Inhibition (IPI) values following *V. vinifera* extract treatments were 36.52 % (1.0 g/kg), 66.67 % (2.0 g/kg) and 89.71 % (3.0 g/kg) which were significantly different from those for *P. granatum* extracts (45.39 %, 1.0 g/kg), 70.93 %, 2.0 g/kg) and 86.88 %, 3.0 g/kg) at equivalent doses of 2.0 and 3.0 g/kg of the extracts. The fruit extracts of both species increased the reaction latency time. In tail-immersion assay, only the fruit extract of *P. granatum* significantly increased the response to heat stimulus at doses of 2.0 g/kg ($p < 0.05$).

Conclusion: The hydroalcohol fruit extracts of *P. granatum* and *V. vinifera* have potential analgesic effects. Further studies are needed to determine the active component responsible for this effect.

Keywords: *Vitis vinifera*, *Punica granatum*, Analgesic activity, Tail immersion test, Hot plate test, mouse writhings inhibition assay

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INTRODUCTION

Most drugs currently used for the management of pain and inflammatory conditions are either NSAIDs or opiates [1]. Most of these medicines can carry a risk of adverse side effects (like gastric lesions caused by NSAIDs and tolerance and dependence induced by opiates). They also do not produce effective pain relief in all the cases [2]. According to World Health Organization, traditional herbal remedies are still extensively used by population, particularly

throughout all over the rural regions with restricted access to modern medicines [3]. Investigation of pain retrieve potential of plant-based remedies used in the traditional medicine is a viable option to discover new analgesic agents which could be beneficial in the management scheme of pain [4].

An ethno-pharmacological survey of plant-based remedies commonly used to relieve pain, conducted as part of our research program on traditional medicinal plants of the Maghreb

region, has allowed establishing a priority list of a dozen of plants, among which figure prominently two well-known species, *Vitis vinifera* (grape) and *Punica granatum*, (pomegranate). These plants are extensively cultivated from ancient time from Tunisia to Morocco for their socio-economical values. Both species are source of juicy and sweet fruits. Traditional therapeutic uses of both species are well documented in the literature of North Africa traditional medicine through several comprehensive reviews [5-8].

V. vinifera L. (Vitaceae), known locally as "Dalya", is a climbing shrub native to the Mediterranean region, central Europe and south-western Asia [9]. Leaves, grapes and seeds are described for their astringent, homeostatic and anti-inflammatory properties [9,10]. Plant parts are traditionally utilized to stop bleeding, inflammation, and also remedies for painful conditions, such as the kind brought on by hemorrhoids and headaches [7]. Flavonoids (including kampferol-3-O-glucosides, quercetin-3-O-glucosides), tannins (procyanidolic oligomers), Stilbenes (resveratrol and viniferins), phenolic acids (tartaric acid, malic acid, succinic acid, citric acid, oxalic acid) and phenylacrylic acid derivatives (p-coumaroyl acid, caffeoyl acid, feruloylsuccinic acid) have been identified in the leaves and fruits of *V. vinifera* [11,12]. From a pharmacological point of view, *in vitro* and *in vivo* studies have reported a wide range of biological effects, including anti-inflammatory, anti-oedematous effect, hepatoprotective activity, antimicrobial activity, antioxidative activity, vasorelaxant effect, spasmolytic effects [13]. The most recent review of the literature conducted on *V. vinifera* revealed no study on the analgesic activity of the edible part of the plant's fruit orbits extracts.

P. granatum L. (Lythraceae), locally called "Rouman", is a fruit-bearing shrub native to the Middle East and now widely cultivated in warm regions of the world, particularly throughout the Mediterranean region [14-17]. The entire fruit is used in folklore medicine as remedy of various diseases, such as dyspepsia, ulcer, hepatic damage, to treat jaundice and diarrhea, and to relieve pain due to sore throat and menstruation in women [7,18]. *P. granatum* is characterized by the presence of polyphenols, including ellagic acid, punicalic acid, ellagitannin, punicalagin, anthocyanidins, oestrogenic flavonols and flavones [19,20]. Pharmacology studies proved its antimicrobial, antioxidant, anti-inflammatory, anthelmintic and molluscicidal activities [18,19]. Its other benefits include the chemopreventive potential in case of prostate cancer [21].

The current study aims to investigate the possible protective role of alcoholic fruits extracts of *V. vinifera* and *P. granatum* against thermal and chemically induced pain.

EXPERIMENTAL

Drugs and chemicals

Acetic acid, acetylsalicylic acid and other chemicals used for extraction purpose and phytochemical screening were purchased from Sigma Aldrich (Poole, UK).

Plant materials

Mature fresh fruits of the black grape (*Vitis vinifera* L.) and the pomegranate (*Punica granatum* L), purchased from local markets from the 2012 harvest season, were authenticated by qualified taxonomist. Voucher specimen was stored in the herbarium as appropriate. The fruits were collected in bulk, and washed under running tap water to remove adhering material and the edible portion of each was freeze-dried, powdered using a dry grinder and stored at low temperature (-25 °C) until extracted.

The extraction process was carried out according to Babero *et al* and Ma *et al* [22,23]. Samples (25g) were extracted by maceration for 24 h using 500 ml of methanol/water (70:30) with an automatic shaker, assisted by ultrasound for 30 min at room temperature, and the remaining material was extracted twice under identical conditions. The extracts were combined and filtered; the combined filtrates were concentrated in a rotary evaporator under vacuum at low temperature (<40 °C) to yield the crude extracts which were subjected immediately to lyophilization. Freeze dried samples were kept at low temperature (-25 °C) until required for further experiments. Extracts of both species were reconstituted in distilled water for the evaluation of analgesic activity.

Presence of flavonoids, tannins, alkaloids, saponins, steroids, terpenoids, and coumarins in the extracts was determined as previously described [24,25]:.

Animals

Adult albino male mice, weighing between 20 - 32 g and aged 4 - 5 weeks, were used for the studies. The animals were kept in standard polypropylene cages at room temperature (24 ± 2 °C) in a 12 h light/dark cycle. They were allowed free access to standard pellet diet and water *ad libitum*, and acclimatize to the laboratory conditions for seven days before the

experiment. The study was carried out following the guidelines of the principles of Laboratory Animal Care "Guide for the Care and Use of Laboratory Animals" [26].

Acute toxicity study

After an overnight fast, healthy animals were weighed and randomly distributed into 5 groups of six animals each (one control group and four treated groups). The control and herbal groups received per os distilled water and serial doses (0.5, 2.5, 5.0 and 10.0 g/kg) of extracts reconstituted in distilled water respectively. Each animal was fed by oral gavage using a specially designed mice needle. Animal observation was carried within the first 30 min, then periodically during the first 24 h and once daily for two weeks. Death or changes in general behavior and other physiological activities of each animal were noted [27,28]. Mice of all groups were weighted on days 7 and 14. At the end of the experiment, the animals were sacrificed and their internal organs including heart, liver, kidneys, lungs, and spleen were examined [29,30].

Analgesic activity

The peripheral analgesic effect of the extracts was evaluated by chemical-induced writhing test [32-34], while the involvement of central mechanisms was studied using the hot-plate and tail-immersion tests as previously reported [31]. These latter assays are known to activate supra spinal and spinal nociceptive pathways, respectively [35]. Tests were conducted after 24 h fast and healthy animals were then weighed and randomly assigned into groups of six animals each (n = 6).

Acetic acid-induced writhing test

The method described by Collier *et al* [36], was used. Writhing was elicited by an intraperitoneal (i.p) injection of 1 % acetic acid aqueous solution. Animals were pretreated with fruits extracts (1.0, 2.0 and 3.0 g/kg per os, single dose) reconstituted in distilled water, and acetylsalicylic acid (standard drug, 0.1 g/kg, per os). Then they were allowed to adapt for 60 min before intraperitoneal (i.p) injection of acetic acid aqueous solution. The number of writhes in 20 min was recorded for each mouse.

Index of Pain Inhibition (IPI) was expressed as in Eq 1.

$$IPI = [(Nc - Nt) / (Nc)] \times 100 \dots\dots\dots (1)$$

where Nc represents the number of writhes observed for control group, and Nt :is number of writhes in tested groups (fruit extracts or acetylsalicylic acid).

Central nervous system analgesic activity

Central analgesic activity was monitored using both the hot plate and tail immersion tests.

Hot plate test: Hot plate test was performed according to the method previously described [33]. Five groups of mice (6 mice per group) each received, 1 h before testing, fruit extracts at different doses (1.0, 2.0 and 3.0 g/kg, single dose, per os), distilled water (control), and acetylsalicylic acid (0.1g/kg, per os). The animals were placed on a heated surface of a hot plate maintained at 55.0 ± 0.5 °C. The pain threshold was considered to be reached when the animals licked their hind paws or jumped out [37].

Tail immersion test: Tail immersion test was performed using an adapted method described previously [34]. Each of five groups of mice (6 mice per group) received different doses (1.0, 2.0 and 3.0 g/kg, single dose, per os) of the extract, distilled water (control), and acetylsalicylic acid (0.1g/kg, per os). The lower portion of each animal tail was immersed gently in a hot water bath maintained at 55.0 ± 0.5 °C for some seconds and withdrawn as soon as the mice reacted. The time it took each animal to withdraw its tail was recorded (using a chronometer) [38].

Statistical analysis

The results of pharmacological testing were expressed as mean \pm SD and analyzed using Tukey test (HSD) to determine the level of significance. A 2-tailed *p* value less than 0.05 was considered to be significant.

RESULTS

Acute toxicity

Fruit extracts did not produce mortality of any animal within 24 h at the concentrations used. Moreover, there was no visible signs of acute toxicity up to 10.0 g/kg within 14 days of observation. Macroscopic examination performed later on the main organs (heart, liver, kidneys, lungs, spleen) also revealed no abnormality.

Analgesic activity

Results of acetic acid induced abdominal writhing test are provided in Table 1.

Table 1: Anti-nociceptive effect of fruits extracts and acetylsalicylic acid on acetic acid-induced pain in mice

| Drug/Plant extract | Dose (g/kg) | Number of writhings* (IPI, %) |
|---------------------------|-------------|-------------------------------------|
| Control (distilled water) | - | 94.00 ± 3.03 (-) |
| Acetylsalicylic acid | 0.1 | 22.50 ± 1.00 [#] (76.06%) |
| <i>Vitis vinifera</i> | 1.0 | 59.67 ± 4.76 ^{#■} (36.52%) |
| | 2.0 | 31.33 ± 5.95 ^{#■} (66.67%) |
| | 3.0 | 09.67 ± 1.97 ^{#■} (89.71%) |
| <i>Punica granatum</i> | 1.0 | 51.33 ± 0.82 ^{#■} (45.39%) |
| | 2.0 | 27.33 ± 5.43 [#] (70.93%) |
| | 3.0 | 12.33 ± 4.41 ^{#■} (86.88%) |

* Values are expressed as mean ± SD (Tukey HSD-test, n =6); [#]p<0.0001:vs. control group; [■]p<0.0001, [■]p<0.01vs. acetylsalicylic acid (standard drug)treated group; IPI: Index of Pain Inhibition (%)

Table 2: Central analgesic activities of the fruit extracts, measured by hot plate and tail immersion tests

| Drug/Plant extract | Dose (g/kg) | Reaction time (s) * | |
|------------------------|-------------|------------------------------------|-----------------------------|
| | | Hot plate test | Tail immersion test |
| Control | - | 02.98 ± 0.47 | 02.12 ± 0.51 |
| Acetylsalicylic acid | 0.1 | 01.92 ± 0.61 ^{ΔΔ} | 02.80 ± 1.17 |
| <i>Vitis vinifera</i> | 1.0 | 03.16 ± 0.82 | 02.38 ± 0.61 |
| | 2.0 | 04.17 ± 1.09 | 02.43 ± 0.60 |
| | 3.0 | 12.77 ± 2.79 ^{ΔΔΔΔ####} | 03.06 ± 1.32 |
| <i>Punica granatum</i> | 1.0 | 04.13 ± 0.6 ^{##} | 02.39 ± 0.49 |
| | 2.0 | 10.32 ± 1.27 ^{ΔΔΔΔ####§§} | 03.31 ± 0.96 ^Δ |
| | 3.0 | 16.18 ± 1.02 ^{ΔΔΔΔ####§§} | 03.90 ± 0.51 ^{ΔΔΔ} |

* RT in seconds expressed as mean ± SD, Tukey (HSD)-test, n = 6; ^Δp < 0.05, ^{ΔΔ}p < 0.01, ^{ΔΔΔ}p < 0.001, ^{ΔΔΔΔ}p < 0.0001: compared to control group; [#] p < 0.05, ^{##} p < 0.01, ^{###} p < 0.001, ^{####} p < 0.0001: compared to acetylsalicylic acid treated group. [§]P < 0.01, ^{§§}P < 0.0001: compared to *V. vinifera* treated group

Treatment with each of the fruit extracts (1.0, 2.0 and 3.0 g/kg, per os) significantly ($p < 0.0001$) reduced, in a dose-dependent manner, the number of writhes induced by acetic acid when compared to control (Table 1). *V. vinifera* extract treatments produced IPI values of 36.52 % (1.0 g/kg), 66.67 % (2.0 g/kg) and 89.71 % (3.0 g/kg). However, the corresponding values of IPI for *P. granatum* extract treatments were 45.39 % (1.0 g/kg), 70.93 % (2.0 g/kg) and 86.88 % (3.0 g/kg). At each of the extract doses of 2.0 and 3.0 g/kg, the writhes were not significantly different for the extracts of the different species. It is noteworthy that fruit extracts administered at a dose of 3.0 g/kg, the extracts produced writhing inhibition effect significantly ($p < 0.0001$) significantly different from that observed in the acetylsalicylic acid (0.1 g/kg) pre-treated group (Table 1).

The tail immersion assay revealed no significant difference in reaction time observed between the control group and *V. vinifera* pretreated groups over the range of tested doses (1.0, 2.0 and 3.0 g/kg), or between the different fruit extracts (Table 2). Similarly, no significant difference was noted between the acetylsalicylic acid group and control group. However, mice groups receiving doses of 2.0 and 3.0 g/kg of *P. granatum* extract showed a significant increase in reaction time in comparison to the control group. These values of

reaction time measured for *P. granatum* extract groups at doses of (2.0, 3.0 g/kg) were even superior to those for the acetylsalicylic acid group.

In the hot plate assay, both groups of mice treated with *V. vinifera* extract (at doses of 3.0 g/kg) and *P. granatum* extract (at doses of 2.0 and 3.0 g/kg) showed significant increase in reaction time when compared with the control group. Compared to *V. vinifera* treatment, *P. granatum* extract was significantly more potent when administrated at the same doses (1.0, 2.0 and 3.0 g/kg). At all treated doses (1.0, 2.0 and 3.0 g/kg), fruit extract protection against pain was dose-dependent and significantly greater than that obtained for acetylsalicylic acid at a dose of 0.1 g/kg.

DISCUSSION

To investigate the analgesic potential of fruit extracts of *Punica granatum* and *Vitis vinifera*, three different experiments were conducted. The peripheral analgesic effect of the extracts was tested by using the chemical (acetic acid) induced writhing test which is widely accepted as a model for visceral pain [39,40]. The involvement of central mechanisms was studied by using the hot-plate and tail-immersion tests,

known to activate supra-spinal nociceptive and spinal nociceptive pathways, respectively [39,40]. In toxicity studies, substances that presents LD₅₀ higher than 5.0 g/kg administered by oral route is considered practically non-toxic [41].

Although no significant difference was seen between the alcoholic fruit extracts of *P. granatum* and *V. vinifera* at doses of 2.0 g/kg and 3.0 g/kg in acetic acid writhing test, these species produced a significant ($p < 0.0001$) dose-related peripheral analgesic effect (1.0, 2.0 and 3.0 g/kg) compared to control. These results are in agreement with previous reports on antinociceptive effect using the entire fruit, peel or seed extracts of the plants [45,46].

Furthermore, it is established that the abdominal constriction response is induced by activation of local peritoneal receptors by mediators of pain [42]. Thus, the peripheral analgesic effect of the fruit extracts could be mediated by inhibition of the release of these endogenous nociceptive mediators [43].

The results from the hot-plate test and tail-immersion assay clearly show that the fruit extract of *P. granatum* possesses analgesic potential which suggests the existence of both peripherally and centrally mediated mechanisms as demonstrated by acetic acid writhing, tail immersion and hot plate assays. This assumption is in agreement with previous reports on extracts from different parts of *P. granatum* [47,55]. Similarly, results obtained for *V. vinifera*, in the different experiments favors analgesic effect due to peripheral and central-based mechanisms. This is thought to involve only supraspinal nociceptive pathway as earlier reported [46,49,56].

The chemistry of these plants (pomegranate and grape) is characterized by the presence of high amount of phenolic compounds, including tannins and flavonoïds. These constituents are known to possess analgesic and anti-inflammatory effects in experimental animals [47,48,51,54,56,57].

CONCLUSION

This study demonstrates that the fruits extracts of *V. vinifera* and *P. granatum* exhibit significant analgesic activities using acetic acid induced writhing, hot-plate and tail-immersion assays. These plants are thus good candidates for development of herbal-based drugs as alternatives for management of pain and inflammatory conditions.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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Trop J Pharm Res, September 2016; 15(9): 1920

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