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IN VITRO AND ANIMAL STUDIES

Antinociceptive and anti-inflammatory activities of a pomegranate (*Punica granatum* L.) extract rich in ellagitannins

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Abstract

Pomegranate (*Punica granatum* L.) has been used for centuries for the treatment of inflammatory diseases. However, there is a lack of comprehensive information focused on the properties of a certain pomegranate (poly)phenolic profile to cure pain and gastric injury induced by anti-inflammatory drugs. This study investigated the systemic effects of different doses of a HPLC-characterized pomegranate extract on the formalin-induced nociceptive behavior in mice. The effect of the extract against gastric injury caused by non-steroidal anti-inflammatory drugs and ethanol was also assessed. Pomegranate reduced nociception in both phases of the formalin test, suggesting central and peripheral activities to inhibit nociception. Indomethacin-induced gastric injury was not produced in the presence of pomegranate, which also protected against ethanol-induced gastric lesions. The present results reinforce the benefits of pomegranate (poly)phenolics in the treatment of pain as well as their anti-inflammatory properties.

Keywords

Anti-inflammatory, antinociception, anthocyanin, ellagitannin, formalin test, gastroprotection

History

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Introduction

Pomegranate (*Punica granatum* L.) is a tropical and subtropical fruit. It is native to Southwestern Asia, and spread from there to North Africa and South Europe, and then to America (Sheets et al., 2013). It is well known that pomegranate is a dietary source of diverse bioactive phytochemicals. Pomegranate fruit contains mainly anthocyanins and ellagitannins, but it is also rich in gallotannins, non-coloured flavonoids, and lignans, among other (poly)phenolic compounds (Fischer et al., 2011; Mena et al., 2012a). This particular phytochemical profile has been related to the wide range of biological properties of pomegranate products (Mena et al., 2011b), moving pomegranate under the spotlight of nutritional and pharmacological research. Several plants are known to be good sources of health-promoting phytochemicals, but their potency for biological activity may vary depending on their genetic background and the agronomical conditions (Halvorsen et al., 2002).

Pomegranate is considered among the plants with the highest values of antioxidants (Abdel Moneim, 2012). It has become popular due to its nutraceutical and therapeutic effects such as reducing the blood pressure by preventing angiotensin-converting enzyme activity, reversing the damage on vessels, preventing prostate cancer and arthritis, stopping the diarrhoea, protecting phagocyte cells against auto-oxidative damages through β-carotene, maintaining blood glucose level in normal range (Aviram & Dornfeld, 2001), stimulating T cell functions, supporting formation of cytokines, and increasing the capacity of cells which naturally inhibit the tumors (Malik & Mukhtar, 2006). They have also been proven to be effective against inflammation (Lansky & Newman, 2007). The neuroprotective properties of pomegranate polyphenols have been reported in an animal model of Alzheimer's disease (Choi et al., 2011). Transgenic mice with Alzheimer's-like pathology treated with it had 50% less accumulation of soluble amyloid-beta and less hippocampal amyloid deposition than mice consuming sugar water, suggesting it may be neuroprotective. Animals also exhibited improved learning of water maze tasks and swam faster than control animals (Choi et al., 2011; Hartman et al., 2006). Nevertheless, the effects of pomegranate on the nociceptive behavior and adverse effects have been poorly explored. In the present study, the main constituents and the antinociceptive and anti-inflammatory activities of a pomegranate extract obtained from whole fruits was examined using high-performance liquid chromatography coupled to diode array detector (HPLC-DAD) and tested after systemic administration in the formalin-induced-neurogenic and inflammatory behavior in rats.

Materials and methods

Reactives and drugs

Ellagic acid was purchased from Sigma-Aldrich (Steinheim, Germany); cyanidin 3-glucoside from Polyphenols (Sandnes, Norway); formic acid, and methanol, all of analytical grade, from Panreac Química S.A (Barcelona, Spain). Diclofenac,

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pharmaceutical grade (98%), was acquired from Novartis Farmacéutica, S.A. de C.V. (Mexico, D.F.). Indomethacin was from Sigma (St. Louis, MO) and absolute ethanol was from Química Meyer (Mexico, D.F.). Milli-Q water used was produced using an Elix®3 Millipore water purification system coupled to a Milli-Q module (model Adventage10) (Molsheim, France). The pomegranate extract, obtained from whole fruits, was provided by Nutracitrus S.L. (Elche, Alicante, Spain).

Pomegranate extract and quantification of major (poly)phenolic compounds by HPLC-DAD

In order to assess the (poly)phenolic composition of the extract used, 0.2 g of powdered pomegranate extract were extracted with 2 mL of MeOH 70% (v/v) as fully described by Mena et al. (2014a). The methanol extract was filtered through 0.45 µm PVDF filters (Membrane Solutions, Spring View Lane Plano, TX) and analyzed using a Merck-Hitachi D-7000 HSM PC-based chromatography data system (Tokyo, Japan), according to a previous report (Mena et al., 2013). Compounds were quantified by the absorbance of their corresponding peaks and using calibration curves. Anthocyanins were quantified as cyanidin-3-*O*-glucoside (detected at 520 nm) and ellagitannins as ellagic acid (at 360 nm).

Animals and extract administration

Male Swiss mice weighing 25–30 g and male Wistar rats (180–200 g) (both aged 7–8 weeks) obtained from the vivarium of the ''Instituto Nacional de Psiquiatría, Ramón de la Fuente Muñiz'' were used in this study. Animals were housed in a temperature- and light-controlled room under a 12-h light/12-h dark cycle (lights on at 7:00 a.m.) with water and food available *ad libitum*. Twelve hours before the experiments, food was withdrawn, though animals had only free access to tap drinking water. All experimental procedures followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals (Zimmermann, 1983), and were carried out according to a protocol approved by the local Animal Ethics Committee.

The pomegranate extract was resuspended in 0.9% saline solution (s.s.) and tested at 10, 30 and 100 mg/kg. Control animals received the same volume of vehicle (s.s.). All treatments were administered by intraperitoneal route (i.p.) in a volume of 1 mL/kg. Thirty minutes after administration, the nociception was induced using formalin-test in mice. As diclofenac is one of the most commonly used analgesics from the pharmacological group of the non-steroidal anti-inflammatory drugs (NSAIDs), it was chosen as a positive control by using 100 mg/kg, i.p. at the same route and volume of administration. Indomethacin (a NSAID too) was selected as a positive drug of reference for gastric injury since it is associated with greater gastrointestinal toxicity compared to other NSAIDs (Seo et al., 2012).

All experiments were performed during the light phase. The number of experimental animals was kept to a minimum and they were used only once. For each experimental procedure, animal groups consisted of six mice and at least three doses of the extract were used to create a dose–response curve.

The formalin-test

The formalin assay was performed according to Hunskaar and Hole (1987). In brief, mice were individually acclimated to the observation chamber for 30 min before injection of 20 µL of diluted formalin (1%) subcutaneously in the dorsal surface of the right hind paw by using a 30 gauge needle. The number of shakings observed in the injected paw of mice was taken as a nociceptive response. Two periods of high shaking activity were considered: the first one comprises the time elapsing immediately after injection to the 10th min known as the early or neurogenic phase. A second period was observed 10–60 min after formalin injection; it is named late or inflammatory phase.

Gastric lesions analysis

In order to analyze if the antinociceptive and anti-inflammatory effects of pomegranate are associated to the presence of hemorrhagic lesions commonly produced by NSAIDs (Seo et al., 2012); rats receiving 30 mg/kg, i.p. of pomegranate or 20 mg/kg, i.p. of indomethacin (as positive control from NSAIDs group) were sacrificed and then their stomachs qualitatively observed for gastric injury. Other groups of rats were used to examine gastroprotection, and then rats that received 30 mg/kg, i.p. and 30 min after they received 1 mL of absolute ethanol, p.o. to induce gastric ulcers. One hour after ethanol, the animals were sacrificed to get the stomachs. Each stomach was dissected out from esophagi to pyloric portion and inflated with 10 mL of 4% formalin and placed in 4% formalin for at least 15 min to fix both the inner and outer gastric layers. Stomachs were incised along the greater curvature and photographed to observe the presence of ulcers. The hemorrhagic lesions were observed under a dissection microscope (10×) with an ocular micrometer (Zeige XSP-313K, Mexico City, Mexico). The ulcer index was defined as hemorrhagic lesions present in the corpus of the stomach for each animal. Gastric injury was compared to that produced by indomethacin.

Statistical analysis

The results are presented as mean ± SEM from at least 6 rats or mice per group. In the behavioral assays, statistically significant differences among the treatments were tested by one way analysis of variance (ANOVA) followed by Dunnett's or Tukey's test. p Values < 0.05 were considered to be significant. All statistical analyses involved use of SPSS v 21.0 (SPSS Inc., Chicago, IL).

Results and discussion

(Poly)phenolic profile of the pomegranate extract

The phytochemical fingerprint of the pomegranate extract used revealed that hydrolysable tannins and anthocyanins were the most relevant (poly)phenolic compounds identified in the extract (Table 1).

Other phenolic compounds (phenolic acids and flavan-3-ols) were also detected only as traces. Ellagic acid derivatives (both the glucoside and the free form) were the main phenolics in the extract, followed by punicalagin isomers. The content in ellagitannins was 8-fold higher than the content in anthocyanins, the natural pigments of pomegranate products. Among these coloured substances, cyanidin glycosides accounted for the 71% of the total anthocyanins in the extract (Table 1). This composition matched the common profile of pomegranate extracts and products (Madrigal-Carballo et al., 2009; Mena et al., 2011a, 2012b, 2014a).

Antinociceptive and anti-inflammatory activities

The formalin test in rodents employs a tonic stimulus to elicit nociceptive behavior (shakings) at both early (0–10 min) and late (10–60 min) phases (Abbott et al., 1995). In the present study, formalin injection produced the expected nociceptive response in mice receiving vehicle (0.9% saline solution) (Figure 1). It was observed an initial number of 32 ± 2 shakings that progressively decreased to 5 ± 1 shakings in the 10th min, described as the neurogenic phase. It was followed by an increase in the inflammatory nociception to reach a maximal response of

Table 1. Concentration (mg of compound/g of dry extract) of main (poly)phenolic compounds in the pomegranate extract.

Compound	Retention time (min)	Absorption maxima (nm)	Conc. (mg/g dry extr)
Ellagitannins			
Punicalagin α	11.06	377 258	2.08 ± 0.04
Punicalagin β	16.80	377 258	3.38 ± 0.07
Total Punicalagins			5.46 ± 0.04
Ellagic acid glucoside	31.95	358 253	3.97 ± 0.15
Ellagic acid	36.24	363253	6.33 ± 0.04
Total Ellagic acid			10.30 ± 0.11
Total ellagitannins			15.77 ± 0.15
Anthocyanins			
Delphinidin-3,5-di-*O*-diglucoside	19.02	522 276	0.28 ± 0.01
Cyanidin-3,5-di-*O*-glucoside	21.73	514 277	0.64 ± 0.20
Pelargonidin-3,5-di-*O*-diglucoside	24.05	502 274	0.18 ± 0.01
Delphinidin-3-*O*-glucoside	26.07	525 277	0.02 ± 0.01
Cyanidin-3-*O*-glucoside	26.87	517 279	0.85 ± 0.02
Pelargonidin-3-*O*-glucoside	29.85	505 276	0.12 ± 0.01
Total anthocyanins			2.09 ± 0.23

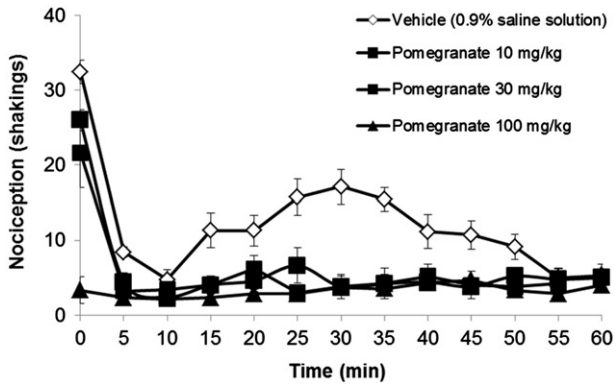

Figure 1. Temporal course curve of the antinociceptive effect of pomegranate 10 (●), 30 (■) and 100 mg/kg (▲), i.p. dosage in comparison to the vehicle group (0.9% saline solution) in the 1% formalin-induced nociception in a period of 60 min. Response is expressed as the number of shakings produced by injection of 20 μL of 1% formalin into the dorsal surface skin of the right hind paw in mice.

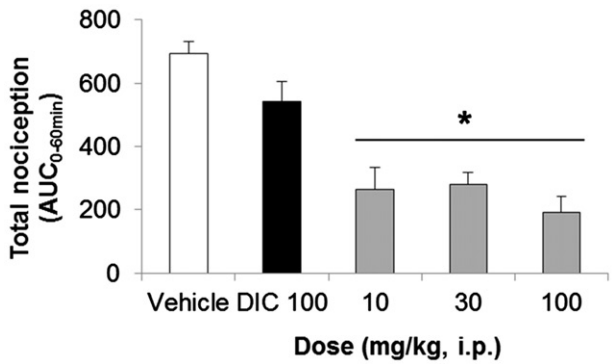

Figure 2. Effect of pomegranate (10, 30 and 100 mg/kg, i.p.) and diclofenac (DIC, 100 mg/kg, i.p.) in the nociception expressed as the total area under the curve (AUC) ($F_{4,25} = 20.25$, $p < 0.001$) induced with 20 μL of 1% formalin into the dorsal surface skin of the right hind paw in mice. *$p < 0.001$ ANOVA followed by Tukey's test of at least six repetitions per group.

17 ± 2 shakings in the 30th min which decreased to 5 ± 1 shaking at the end of the observation period of 60 min (Figure 1). Nociceptive response was decreased significantly in a dose-dependent manner by pomegranate extract administration in the first 5 min of induction. It was evident an inhibition of the inflammatory phase in the presence of 10, 30, and 100 mg/kg of pomegranate extract (Figure 1). This inhibition of the inflammatory phase was already observed from a dosage of 10 mg/kg of pomegranate extract, with almost a total inhibition in all the nociceptive behavior at a dosage of 100 mg/kg, as observed in the entire temporal course (Figure 1).

Mice receiving different systemic administrations of pomegranate extract demonstrated a significant ($F_{4,25} = 30.29$, $p < 0.001$) decrease in the nociceptive response with a better effectiveness than diclofenac 100 mg/kg, i.p. (Figure 2). First, total nociceptive response was calculated as the area under curve (AUC) with 694 ± 29 units of area (ua) in the control group that was reduced to 265.42 ± 62.24 ua, 281 ± 67.59 ua and 192.50 ± 36.02 ua to the dosage of 10 mg/kg, 30 mg/kg, and 100 mg/kg of pomegranate extract, respectively, and in comparison to 543.33 ± 50.47 ua for diclofenac (Figure 2). This total response was divided into the neurogenic phase (0–10 min) with 135.0 ± 4.23 ua and in the inflammatory phase (10–60 min) with 559.29 ± 39.73 ua (Figure 3) of the formalin test in the control group. The early phase is thought to be produced by the direct activation of nociceptive neurons, whereas the late phase reflects nociception generated by the acutely inflamed tissue (Hunskaar & Hole, 1987). Nociceptive responses were significant ($F_{4,25} = 30.29$, $p < 0.001$) and dose-dependently reduced in the neurogenic phase in the presence of pomegranate extract as follows: 10 mg/kg, 77.50 ± 16.57 ua; 30 mg/kg, 78.50 ± 10.54 ua and 100 mg/kg, 25.42 ± 5.93 ua, in comparison to diclofenac 108.08 ± 6.75 ua (Figure 3A). Whereas in the inflammatory phase, pomegranate extract demonstrated a significant ($F_{4,25} = 20.13$, $p < 0.001$) ceiling effect in the following diminution: 10 mg/kg, 187.92 ± 48.30 ua; 30 mg/kg, 202.50 ± 58.05 ua and 100 mg/kg, 167.08 ± 30.80 ua (Figure 3B) in comparison to diclofenac 408.08 ± 37.51 ua (Figure 3B).

It has been demonstrated that the formalin's test late phase depends mainly on an inflammatory reaction of the peripheral tissue. In line, diclofenac and drugs with preferential peripheral actions like NSAIDs, such as acetylsalicylic acid, indomethacine, naproxen, oxyphenbutazone and dexamethasone, which block prostaglandin synthesis, reduce nociception by exclusively affecting this phase (Hunskaar & Hole, 1987; Rosland et al., 1990); while other drugs, such as opioids, equally inhibit both phases

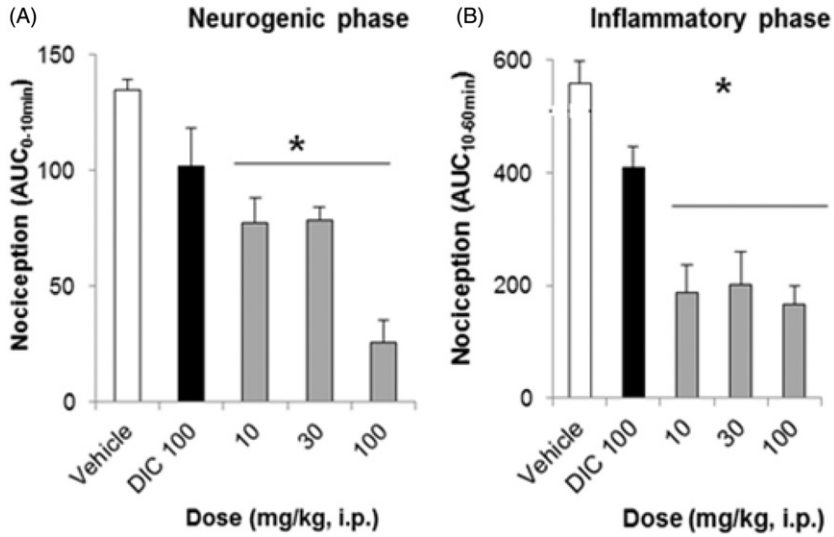

Figure 3. Effect of pomegranate (10, 30 and 100 mg/kg, i.p.) and diclofenac (DIC, 100 mg/kg, i.p.) in the nociception expressed as the neurogenic ($F_{4,25} = 30.29$, $p < 0.001$) (A) and inflammatory phases ($F_{4,25} = 20.13$, $p < 0.001$) (B) induced with 20 µL of 1% formalin into the dorsal surface skin of the right hind paw in mice. *$p < 0.001$ ANOVA followed by Tukey's test of at least 6 repetitions per group.

(Shibata et al., 1989). Our data reinforce the recent report by Mo et al. (2013) indicating peripheral anti-inflammatory activity by local effect of a pomegranate rind extract in the formalin test in mice. It is important to mention that the frequent occurrence of gastrointestinal disturbances including gastrointestinal bleeding, perforation and peptic ulceration observed in the presence of NSAIDs has limited the chronic use of this kind of analgesics. The gastric damage induced by diclofenac or aspirin is reported to be similar to that caused by indomethacin, but the depth of ulcers is shallow and the damage extent is less severe than that caused by indomethacin (Seo et al., 2012). In our study, no gastric damage was observed with systemic administration of pomegranate alone (Figure 4A) in comparison to the indomethacin administration. In addition, the pomegranate extract produced protection against the ethanol-induced gastric lesions (Figure 4B), reinforcing preliminary reports suggesting that pomegranate polyphenols can likely strengthen the gastric mucosal barrier (Ajaikumar et al., 2005; Gharzouli et al., 1999).

In this study, systemic administration of pomegranate showed that its antinociceptive action is possible at central level, decreasing neurogenic response in a dose-dependent manner. In fact, some pomegranate products that include not only juice (Malek et al., 2014), but also extracts from flowers (Sarker et al., 2012), leaves, and fruit peel (Salwe & Sachdev, 2014) or seeds (Malek et al., 2014; Saad et al., 2014) have recently shown to exert antinociceptive and anti-inflammatory effects (Salwe & Sachdev, 2014) at different levels (local or systemic), and by using different experimental models (Ismail et al., 2012; Larrosa et al., 2010; Ouachrif et al., 2012). These effects could be related to their content in ellagitannins and/or ellagic acid (Gainok et al., 2011; Rosillo et al., 2012; Saad et al., 2014) and anthocyanins (de Pascual-Teresa et al., 2010; Mena et al., 2014b). Although this cannot be the case since the pomegranate extract was supplied via i.p., urolithins, metabolites produced after the consumption of pomegranate ellagitannins as a consequence of gut microbiota transformations, have also displayed notable anti-inflammatory effects (Giménez-Bastida et al., 2012; Larrosa et al., 2010). All the same, despite the proven effects of pomegranate products and their bioactive phytochemicals, the real responsible compounds have not been fully described and the mechanisms of action remain still unknown. Therefore, further studies should be performed in order to shed light on these critical points related to the biological activities of pomegranate and their unique composition. Moreover, the role of the pomegranate-derived circulating metabolites, those generated not only by gut microbiota but also phase II conjugates, should be taken into account in future *in vivo* studies.

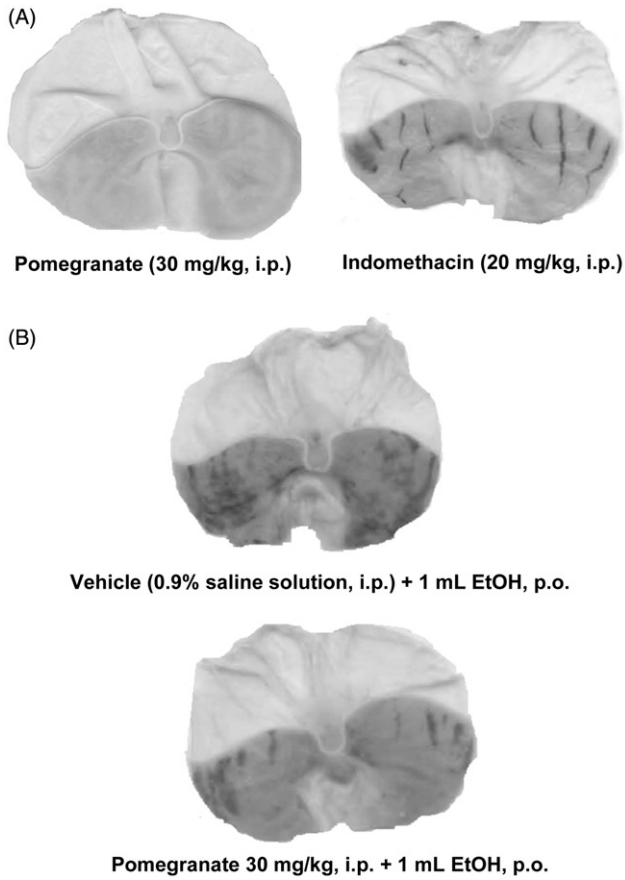

Figure 4. Representative-image of the rat stomachs to observe gastric damage induced by indomethacin (20 mg/kg, i.p.) alone, as positive control (A), and after the administration of pomegranate extract (30 mg/kg, i.p.) alone (A) and in the presence of absolute ethanol (EtOH 1 mL, p.o.) in animals that received vehicle (B).

Conclusions

Systemic administration of pomegranate extract produced anti-nociceptive and anti-inflammatory activities without producing

gastric damage and even exerting gastroprotection, possibly by modulation at central and peripheral levels, suggesting its utility in the therapy of pain. Intervention trials are needed to further examine the impact of phenolic-rich pomegranate extracts on the treatment of pain and gastric inflammation.

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Declaration of interest

The authors have no potential conflicts of interest to disclose.

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