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EVALUATION OF THE ANTINOCICEPTIVE EFFECT OF THE ETHANOLIC EXTRACT OF *PUNICA GRANATUM*

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Abstract

Background: There are severe adverse effects of analgesic drugs on human body. Extraction of analgesic drugs from natural products has therefore become the prime objective of the study. In this study, we aimed to evaluate the antinociceptive activity of the pomegranate fruit.
Materials and Methods: Antinociceptive activity of ethanol pomegranate extract was examined using three models of pain: the writhing test, the hot tail flick test and the plantar test. The ethanolic extract of pomegranate was administered by oral gavages in doses of (100,150 and 200mg/kg, p.o (orally)), for all the tests and compared with aspirin (100mg/kg, p.o.) which was considered as the standard drug. Phytochemical screening and HPLC analysis of the plant species was carried out.
Results: In the writhing test, the index of pain inhibition (IPI) was 37% for ethanolic extract of pomegranate (200mg/kg, p.o.), and 59% for aspirin. In the hot tail flick test, the ethanolic extract of pomegranate (200mg/kg, p.o.), has shown significant analgesia reaching its peak at 60 min maximum possible analgesia (MPA), was 24.1% as compared with aspirin 37.5%. Hyperalgesia was successfully induced by the plantar test and the ethanol extract of pomegranate (100,150,200mg/kg, p.o.), reduced the hyperalgesia in a dose dependent manner comparable to aspirin at (100mg/kg, p.o.). HPLC analysis revealed the presence of gallic acid, ellagic acid and Punicalagins A&B.
Conclusion: The results demonstrated that ethanol pomegranate extract has an antinociceptive effect that may be related to the presence of identified phytochemicals.

Key words: Pain, ethanolic extract of pomegranate, analgesia, phytochemicals

Introduction

Pomegranate (*Punica granatum* L.), a species of Punicaceae, has been widely researched for its nutritional and medicinal values in recent years given its active phytochemical compounds with pharmacological effect and lack of toxicity (Wang et al., 2010).
The major class of phytochemical agents present in pomegranate are the polyphenols that are predominant in the fruit (Seeram et al., 2006), which includes flavonoids (flavonols and anthocyanins), (Hernandes et al., 1999; Santgati et al., 1984), condensed tannins (proanthocyanidins), and hydrolysable tannins (ellagitannins and gallotannins), (Gil et al., 2000). Other phytochemicals identified from different parts of the pomegranate tree include organic and phenolic acids sterols and triterpenoids, fatty acids and triglycerides and alkaloids such as pelletierine and hygrine (Santgati et al., 1984).

Anthocyanins are the major constituents of the pomegranate arils. Pomegranate juice is an important source of anthrocyanins and phenolic tannins (puicalin, pedunculagin, punicalagin and ellagic acid), which adds red color to the fruit and aril (Gil et al., 2000; Kulkarni and Aradhya 2005).

Pain is the most common symptom encountered in clinical practice. It is well known that current analgesic drugs such as opiates and non-steroidal anti-inflammatory drugs (NSAID), possess some severe side effects like kidney damage, hepatic damage etc. This provoked the need for a search for natural alternative analgesics which derives from sources of plant origin.
A few studies did evaluate antinociceptive effect of different species and parts of pomegranate peels (Quarshi et al., 2012; Olapour et al, 2010), seeds (Kumar et al., 2008). However, there is no published data which did prove the antinociceptive effect of Libyan variety of pomegranate. Thus, this would be the first antinociceptive study showcasing the Libyan pomegranate fruit.

Materials and methods

Plant material

Fresh pomegranate fruits were collected from an orchard in the region of Tajoura, Tripoli, Libya in the fall of 2010. The sample was identified by Professor Alsherif, a taxonomist from the Faculty of Sciences, University of Tripoli. A Voucher specimen with the number 01563 was deposited at the Herbarium of the same University.

Drugs and treatment

Aspirin (Bayer®100mg/kg, 10ml/kg b.w.), procured from Kuala Lumpur, Malaysia. It was used as the reference agent in all tests.

Animals

Albino mice (20-30g) and Sprague dawley rats (180-200g), of both sexes were used. They were supplied by the animal unit, faculty of medicine with ethics approval from the Animal Ethics Committee of University of Malaya ethic number FIS/27/01/2012/LAB(R).
The experiments were performed according to the guidelines set by the National institute of health regarding the treatment of experimental animals

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Extraction method

3 kg of Fresh fruits were cleaned freeze-dried and grounded into fine powder using electric blender. The powder was dried in an oven at 40°C for 24 hrs. The fine powder was sieved through 24 mesh stainless steel filter. The fine powdered sample of 1 kg was extracted with 2500ml 80% ethanol in water at room temperature for 24 hr in a shaking water bath and was finally filtered. The extraction was repeated twice. The solvent was removed by using RV10 rotary evaporator (IKA, Guangzhou) and the resultant residue was crude ethanolic extract (110g), which was used for the experiment:

Phytochemical analysis

Chemical tests for the screening and identification of bioactive chemical constituents of the extract was carried out using standard procedures (Trease and Evans 1989; Raman 2006).

HPLC conditions

All samples and standards were dissolved in methanol at a concentration of 500ppm. Solutions were injected into an Agilent SB-C18 RRHD 1.8um 2.1×150mm column. The mobile phase for elution were mobile phase A 0.1% H3PO4 mobile phase B 100% methanol and separated with the following gradient: gradient 0-1min :0% mobile phase B 6.7-7.7 min :50% mobile phase B 7.9-9 : min 0% mobile phase B . The flow rate was maintained at 2 ml min-1, UV detection was maintained at 280nm, 246nm and 366nm. The identification of each compound was achieved by comparing the retention times and UV spectra with those corresponding to standards.

Acetic acid writhing test

Mice were inducted by oral gavages with vehicle saline (10ml/kg, p.o.), ethanol pomegranate extract (100,150,200mg/kg, p.o.), and aspirin (100mg/kg, p.o). Acetic acid solution (0.6%, 10ml/kg), was injected intra-peritoneally 30 min after the groups were treated. Five groups of six mice each were used (n=6). The number of writhes response consisting of contraction of abdominal wall and pelvic rotation followed by hind limb extension produced in each animal by the acetic acid injection was counted for a period of 15 minutes immediately after the acetic acid administration (Collier et al., 1968)

The index of pain inhibition was calculated as follows:

$$IPI = \left(\frac{X0 - Xi}{X0}\right) \times 100$$

X0 is the number of writhes observed in control group. Xi the number of writhes in the tested groups

Hot tail flick test

Albino mice weighing between 25-30g were fasted for 24 hrs with water *ad libitum* maintained at room temperature and were divided into five groups (n=6). Mice were treated by oral gavages with normal saline (10ml/kg, p.o.), aspirin (100mg/kg, p.o.), and ethanol pomegranate extract (100,150 and 200mg/kg, p.o.). Nearly 1 or 2 cm of the tail was immersed in warm water kept constant at 50 ºC. The reaction time was the time taken by the mice to deflect their tails. The first reading was discarded and the reaction time was taken as the mean of the next two readings. The latent period of tail flick response was taken as the index of antinociception and was determined before and at 15, 30, 45 and 60 min after the administration of drugs. The maximum reaction time was fixed at 15 sec (Sewel and Spencer., 1976).
The maximum possible analgesia was calculated as (MPA).

$$\text{Therefore; MPA} = \frac{\text{Test reaction time-Saline reaction time}}{\text{15-Saline reaction time.}}$$

Plantar test

The nociceptive stimulus was applied using the Hargreaves apparatus (Ugo Basile Italy). Each rat was individually situated in a compartment (Plexiglas boxes 20 cm high and 69 cm in diameter), on a glass surface for a previous training 30 min then (0.1ml of 1% carrageenan), was injected subcutaneously into the right hind paws of the rats weighing 180-200g. Then the same amount of saline was injected onto the plantar surface of the left hind paw. Ethanol pomegranate extract (100,150, 200mg/kg, p. o.), and aspirin (100mg/kg, p.o.) was administered by gavages 30 minutes before the subcutaneous injection of both rat hind paws. A thermocouple was placed under the heel of the hind paws. The withdrawal latencies were recorded every 30 min for the duration of 5 hours. Analgesic effect was detected when carrageenan induced hyperalgesia on the right hind paw prolong the reaction time (withdrawal latencies), compared to base line values. The baseline value is the recorded withdrawal latencies before the hind paw was injected with the carrageenan (Hargreaves et al., 1988).

Statistical analysis

Data were represented as mean± standard error of the mean (SEM). The results were analyzed using one way analysis of variance (ANOVA) for comparison between groups followed by students't-test.

Results

Phytochemical screening

Preliminary phytochemical analysis revealed the presence of alkaloids, saponins, phenolic compounds, tannins, flavonoids, sterols and terpenes. HPLC chromatograms have emphasized four marker components present in ethanol extract of pomegranate as shown in **figure 1.** These

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phenolic components have been identified as gallic acid (Rt:1.11 min), ellagic acid (Rt:1.11min), and punicalagin A, B (Rt:7.62min) by their retention time and UV absorbance of purified standards.

Acetic acid –induced writhing test

The results of the writhing test showed that the ethanol pomegranate extract at doses (100,150,200mg/kg, p.o.), induced a significant reduction in pain response in a dose dependent manner when compared to control group. Furthermore, aspirin (100mg/kg, p.o.), significantly reduced the number of writhing as a reference drug. The index of pain inhibition (IPI), induced by the ethanol pomegranate extract at dose (100,150,200mg/kg, p.o.) *p<0.001 was 21%, 28% and 37% respectively, whereas aspirin produced IPI 37%.

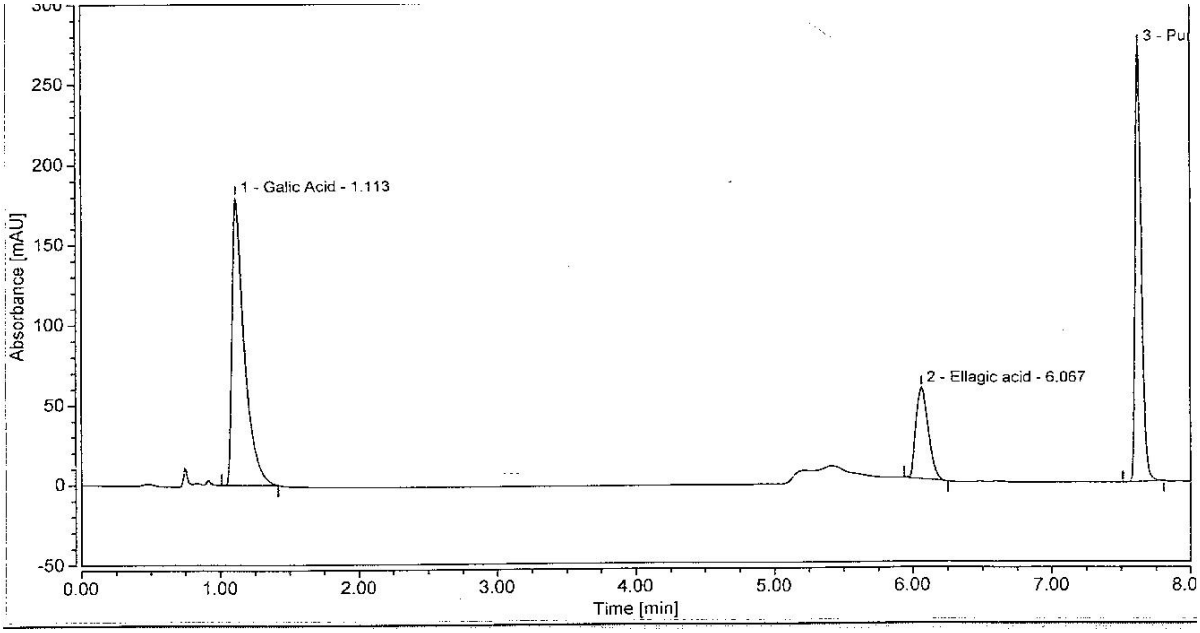

Figure 1. HPLC chromatogram of ethanol pomegranate extract. Peaks indicate the following 1.Gallic acid 2. Ellagic acid 3. Punicalagin A&B

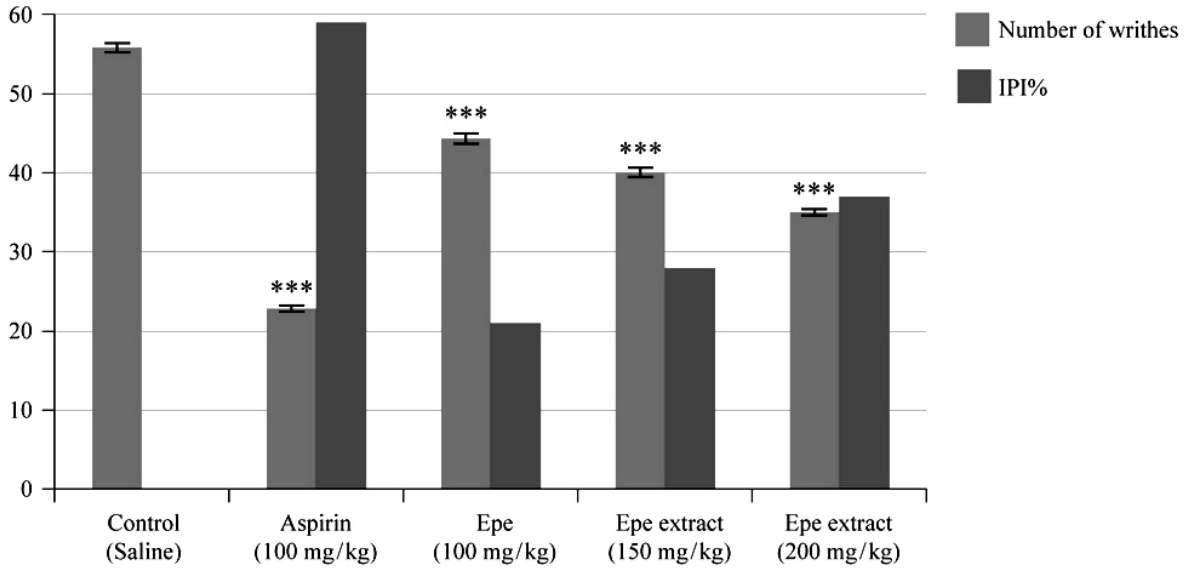

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Figure 2.The effect of the ethanol pomegranate extract (100,150 and 200mg/kg, p.o.), and aspirin (100mg/kg,p.o.), on acetic acid induced visceral pain in mice. Values are expressed as mean ± SEM (n=6). Differences between groups were statistically analyzed by ANOVA followed by t- test***p<0.001 versus control (saline). Epe:ethanol extract of pomegranate IPI: index of pain inhibition

Hot tail flick test

The hot tail flick test results showed that the mean reaction time increased in a dose related manner reaching a peak at 60 minutes for doses 150 and 200mg/kg 5.2±0.04, 6.5±0.04 ***p<0.001 respectively compared to aspirin 100mg/kg increased the mean reaction time to 8±0.03sec***p<0.001 whereas for 100mg/kg the ethanol pomegranate extract produced significant effect at 30min**p<0.05 .The MPA similarly

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increased in a dose related manner reaching its peak at 60 minutes for all doses 100,150 and 200mg/kg 3.6%,12.5%,24% respectively and 37.5% for aspirin Table 1, Figure 3

Plantar test

Peripheral antinociceptive action on right hind paw injected with carrageenan

Hyperalgesia was successfully induced and withdrawal latencies were shortened from10.0±0.1 to 5.7±0.2 for the control group (carrageenan), and for the ethanol pomegranate extract (100,150 and 200mg/kg). Carrageenan induced hyperalgesia was reduced and antinociceptive effect was exhibited in a dose dependent manner the 200mg/kg showed the highest effect withdrawal latency prolonged to 13.2±0.2 ***p<0.001compared to aspirin 13.7±0.2 ***p< 0.001 and remained elevated throughout the observation period of 5 hours Figure 4.

Table 1: The effect of the ethanol pomegranate extract (100,150,200mg/kg, p.o.), and aspirin (100mg/kg, p.o.), on hot tail tail flick test

Treatment	0 min	15 min	30 min	45 min	60 min
Saline	2.5 ± 0.0	2.8 ± 0.03	3 ± 0.02	3.5 ± 0.05	3.8 ± 0.06
Aspirin (100 mg/kg)	3 ± 0.02	4 ± 0.04***	5 ± 0.04***	6 ± 0.04***	8 ± 0.03***
Epe (100 mg/kg)	2.6 ± 0.03	3 ± 0.02	3.3 ± 0.04*	4 ± 0.03**	4.2 ± 0.03
Epe (150 mg/kg)	2.5 ± 0.04	3.1 ± 0.04*	3.9 ± 0.05***	4.4 ± 0.06**	5.2 ± 0.04***
Epe (200 mg/kg)	2.7 ± 0.02	3.5 ± 0.04***	4.5 ± 0.04***	5.5 ± 0.06***	6.5 ± 0.04***

Values are represented as mean reaction time ± SEM (n=6). Differences between groups were statistically analyzed by ANOVA followed by the test *p<0.05,**p<0.01,***p<0.001vs control(saline), Epe: Ethanol extract of pomegranate

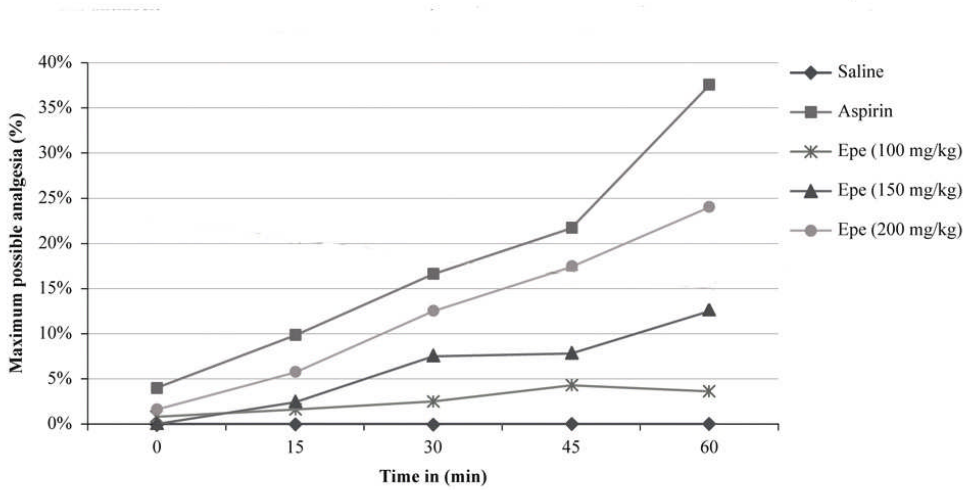

Figure 3: The effect of the ethanol pomegranate extract (100,150,200mg/kg, p.o.), and aspirin (100mg/kg, p.o.) on hot tail flick test. Values are represented as MPA MPA: maximum possible analgesia.

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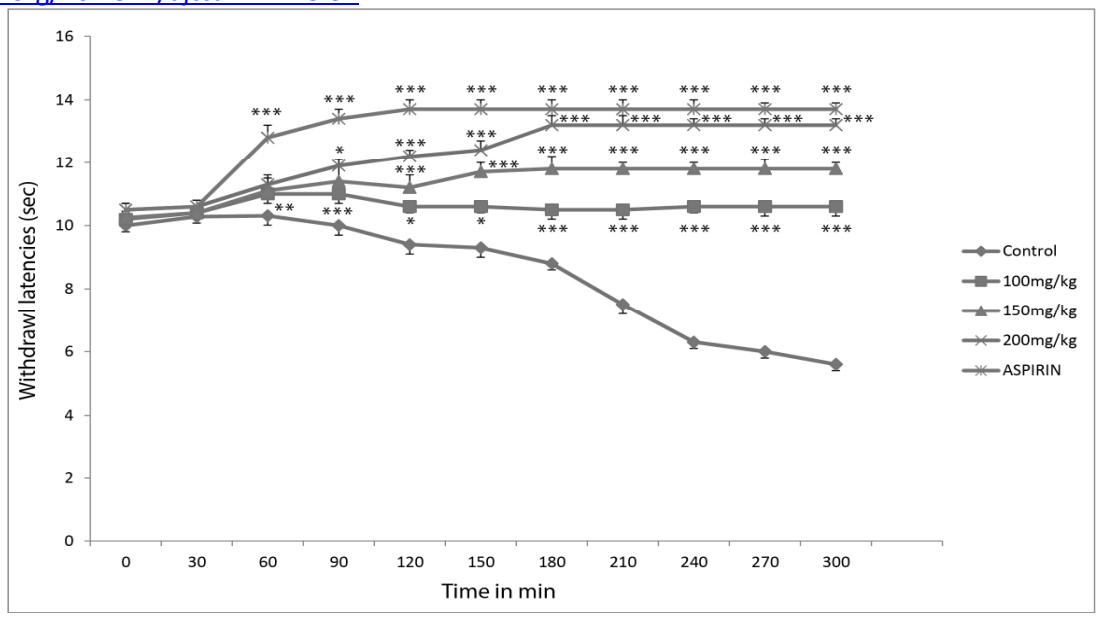

Figure 4. The effect of withdrawal latencies of ethanol pomegranate extract (100,150,200mg/kg, p.o.), and aspirin (100mg/kg, p.o.), versus control (carrageenan), on right hind paw in plantar test. Values are expressed as mean ±SEM. Differences between groups were statistically analyzed by ANOVA followed by t-test *p<0.05,**p<0.01,***p<0.001vs control.

Discussion

The present study has emphasized that the ethanol pomegranate extract administered orally produced a significant analgesic effect according to three models of nociception i.e. the writhing test, hot tail flick test and the plantar test.

The results elucidated that ethanolic extract of pomegranate has produced significant dose related analgesic effect in the writhing test similar findings have also been reported by Qurashy and Olapour. The inhibition of writhing in mice by the extract suggests a peripheral mechanism of action possibly mediated by inhibition of PGE among several possibilities.

Tail flick test is used to determine both centrally acting analgesics (Rambadrane et al., 1989), like morphine (Domer.,1990), and peripherally acting analgesics like NSAIDs' which inhibit cyclooxygenase in peripheral tissues thereby interfering with the mechanism of transduction of primary afferent nociceptors (Fields et al.,1987).

The results observed by tail flick test clearly stated that the ethanolic extract of pomegranate did possess a dose dependent antinociceptive activity. Our results are close with that of (Kumar et al., 2008), who reported that pomegranate seed extract possesses antinociceptive effect when investigated by hot tail flick test.

Administration of aspirin, (100mg/kg, p.o), which was used as a reference drug produced significant antinociception in both tests which agrees with (Miranda et al., 2001), who reported that NSAIDs elicited antinociception in writhing test and hot tail flick test.

Although acetic acid and hot tail flick tests are useful tests for studying analgesic drugs, they are not selective pain tests and may not be conclusive enough to determine the mechanism of action of antinociceptive effects of the extract. Therefore we studied the antinociceptive effect using a more selective method the plantar test (Hargreaves method). We are not aware of previous evaluation of pomegranate antinociceptive effect by the plantar test.

The Hargreaves model (Hargreaves et al., 1988), was used to evaluate the effect of pomegranate extract on thermal hyperalgesia. In the present study we have confirmed the findings of previous researchers which have shown that thermal hyperalgesia develops in hind paw injected with carrageenan and saline (Hargreaves et al., 1988). Carrageenan –induced inflammatory pain is well known to involve inflammatory mediators like cyclooxygenase products (PGE2), leukotrienes histamine, 5-HT, and cytokines (Morris, 2003; Yaksh et al., 2001), which are released as a result of tissue injury. Our results demonstrated increase in withdrawal latency in (right paw) post carrageenan administration which suggests that the pomegranate extract analgesic effect may be acting against some of these mediators.

Phytochemicals are plant molecules that enter into our bodies through diet. The relationship between phytochemicals and the reduced possibility of adverse effects has attracted many scientists (Rasheed et al., 2009). In addition, more people are consuming herbal {drug}, mixture preparations which offer natural therapies with fewer side effects. As the chemical constituents of pomegranate and their pharmacological effect is less known, current research seems to indicate that most therapeutically beneficial pomegranate constituents are ellagic acid ellagitannins (including punicalagin) punic acid , flavonoids , anthocyanidins, anthocyanins, estrogenic flavonols and flavones (Gil et al., 2000; Kulkarni and Aradhya 2005). Several studies on ellagic acid and its related antinociceptive effect have been conducted (Rogerio et al., 2006; Beltz et al., 2008), and was proved that the ellagic acid's antinociceptive effect could be due to COX inhibition. Other studies on flavonoids revealed that, they play a role on analgesic activity and work by targeting prostaglandins and inhibiting prostaglandin synthetase and tannins. Furthermore, flavoinoids were also known to suppress COX2 transcription (Oleary et al., 2004). The presence of ellagic acid and flavonoids in our extract unravels its analgesic activity and may give an indication that it acts by COX inhibition.

Conclusion

Based on the results of this study, it may be concluded that ethanolic extract of Libyan variety of pomegranate has an analgesic effect which may be related to the phytochemicals identified in the extract. Currently, cell culture studies are on-going to determine the effect of the extract on inflammation and analgesic activity mediators COX, NO, PGE and IL-6.

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Declaration of interest: The authors report no declaration of interest.

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