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ANALGESIC EFFECTS OF LIBYAN FRESH POMEGRANATE FRUIT OF PUNICA GRANATUM

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Context: The severe side effects of analgesic drugs require the search of new analgesics from natural products and nutritional resources known as nutraceutical.

Objective: This work aimed to evaluate the analgesic effect of fresh pomegranate *Punica granatum* fruit juice (fpj) been consumed by Libyans for a century. In this study we investigated the fresh fruit juice given to mice for three subsequent days versus the effect of (fpj) given instantly and those given the ethanol extract of pomegranate

Materials and Methods: Antinociceptive activity of fresh pomegranate fruit juice (fpj) was examined using two models of pain. In the writhing test the (fpj) was administered for three subsequent days where no food was given,the (fpj) was also given instantly i.p.(0.15ml/kg) and orally p.o.(0.15ml/kg) for hot tail one group of animals were prefed (fpj) the ethanol pomegranate extract was administered by intraperitoneal route in

doses of (100,150 and 200mg/kg) for writhing , hot tail flick test and compared to aspirin 100mg/kg in all tests

Results:

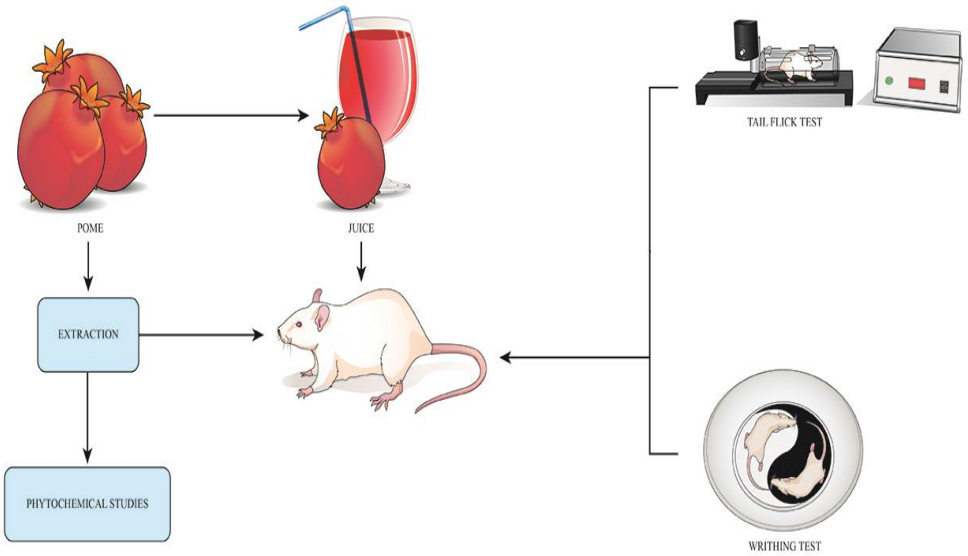
In the writhing test for mice the index of pain inhibition (IPI) was 47.8% for mice prefed with (fpj) and 37% for ethanol pomegranate extract (200mg/kg i.p.) and 59% for aspirin whereas, the groups treated instantly (0.15ml/10g) i.p.and orally failed to inhibit acetic acid induced writhings.

In the hot-tail flick test fresh pomegranate fruit juice (fpj) antinociceptive effect was evident within 15min the maximum possible analgesia (MPA) was 18% and remained elevated throughout the observation period ethanol pomegranate extract (200mg/kg) showed significant analgesia reaching its peak at 60 min MPA was 24.1% as compared to aspirin 37.5%

Conclusion: The results demonstrated that consuming pomegranate fresh fruit leads to significant analgesic activity which may be both peripheral and central.

Keywords: Antinocieptive, pomegranate fruit juice, maximum possible analgesia, *Punicagranatum*

Graphical abstract



Introduction

The pomegranate (Punica granatum L.Punicaceae) ,is one of the oldest known fruit species. It has a long history of herbal use dating back more than 3000 years. In Libya pomegranate is considered as a health giving fruit and home remedy and is consumed widely as a fruit juice, pomegranate fruit peels are used for gastric ulcers, visceral pain, and as a natural dye. It grows in the middle east extending throughout the Mediterranean, eastward to China and India, and on to the American Southwest, California and Mexico in the New world. The pomegranate plant is either a small tree or a large shrub, its fruit often considered to be a large berry (Levin,1994). Pomegranate is used traditionally for treatment of many illnesses these include ulcers diarrehea, aphthe and diabetes mellitus (Lansky and Newman,2007). It has been reported to possess anti-inflammatory effect (Lansky and Newman,2007;Shukla et al 2008 ;

Quachrif et al, 2012),antioxidant effect(Mertens-Talcott et al., 2006) anticancer and antiatherosclerotic effect (Perze-Vicente et al., 2002).

The major class of phytochemical present in pomegranate is the polyphenols and includes flavoinoids, condensed tannins and hydrolysable tannins(Gil et al., 2000). In addition pomegranate fruit husk is rich in ellagitannins and gallotannins (Mayer, 1977). Pomegranate juice is an important source of anthrocyanins and phenolic tannins (puicalin,pedunculagin,puicalagin and ellagic acid) which give the fruit and aril its red colour (Gil et al,2000;Kulkarnit Aradhya,2005). It has been reported that pomegranate juice has an antioxidant capacity three times of that known beverages such as red wine and green tea, presumably due to the presence of hydrolysable tannins in the rind along with anthrocyanins and ellagic acid derivatives (Gil et al., 2000). In a comparative study, anthocyanins from pomegranate fruit were also shown to possess higher antioxidant activity than vitamin E(alpha tochopherol),ascorbic acid and B carotene (Seeram and Nair,2002).

NSAIDs remain among the most widely used drugs for management of pain and inflammation despite their serious side effects. This highlights a need for safe and alternative treatments. The prevention and alternative treatments could come from nutrition. By nature nutrition is better positioned to have long term rather than short term health effect (Ameye and Chee, 2006).

NSAIDs inhibit both COX1which has a protective effect and COX2 enzyme which is responsible for inflammation and pain which lead to discovery of selective COX2 inhibitors which are known to be very expensive (Peterson and Cryer ,1999) .This gave a rise to research for natural COX2 inhibitors with no side effects and less expenses. Pomegranate is rich in flavonoids and phenolics which are plant based chemicals that hold great promise as COX2 inhibitors. Despite the wide consumption of pomegranate fruit and juice in Libya no scientific studies

have been done regarding the Libyan variety of *Punica granatum*. In this study we tested the fruit juice and fruit extract which are consumed by Libyans in order to verify their ethno pharmacological use and to join other colleagues in the search of natural analgesics.

Materials and methods

Plant material

Fresh pomegranate were collected from an orchard in the region of Tajoura,in the fall of 2010

Animals

For the writhing test and hot tail flick test albino mice (20-30g) of both sexes were used. The animals were supplied by the animal house of faculty of medicine University of Tripoli. Two groups of mice was fed pomegranate fruit juice for 3 subsequent days and no other food was given.

The experiments were performed according to the guidelines set by the National institute of health regarding the treatment of experimental animals

Preparation of juice

The fresh pomegranate fruits were washed and manually peeled without separating the seeds. Juice was obtained using commercial blender filtered and diluted with distilled water.

Extraction method

Fresh fruits were cleaned freeze-dried and grounded into fine powder using electric blender. The powder was dried in an oven at 40 ℃ for 24hrs then the fine powder was sieved through 24 mesh .The fine powdered sample was extracted with 250ml 80%ethanol in water at room temperature

for 24hr in a shaking water bath. The extract was filtered by a Millipore filter with a 0.45um nylon membrane under vaccum at 25℃

Phytochemical analysis

Preliminary phytochemical analysis of pomegranate extract implicated determination of the following compounds alkaloids, flavoinoids, tannins, anthocyanes, sterols ,terpenes and saponins (Trease and Evans 1989).

Acetic acid writhing test

Writhing was induced by the intra peritoneal injection of 0.6% acetic acid at a dose of 0.1ml/kg (Collier et al, 1968). Five groups of 6 mice were used. Saline, ethanol pomegranate extract 100,150and 200mg/kg, aspirin 100mg/kg were given to animals 30min before the injection of acetic acid and the number of writhes were counted for a period of 15min

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 24hrs with water given ad libitium maintained at room temperature and were divided into five groups of six mice. Mice were treated with normal saline 0.15ml/10g, aspirin100mg/kg and ethanol pomegranate extract(Epe)100,150 and 200mg/kg. One or two cm of the tail was immersed in warm water kept constant at 50℃.The reaction time was the time taken by the mice to deflect their tails. The first reading is 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 (Sewell and Spencer ,1976).

The maximum possible analgesia was calculated as (MPA)

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

Results

Acetic acid –induced writhing test

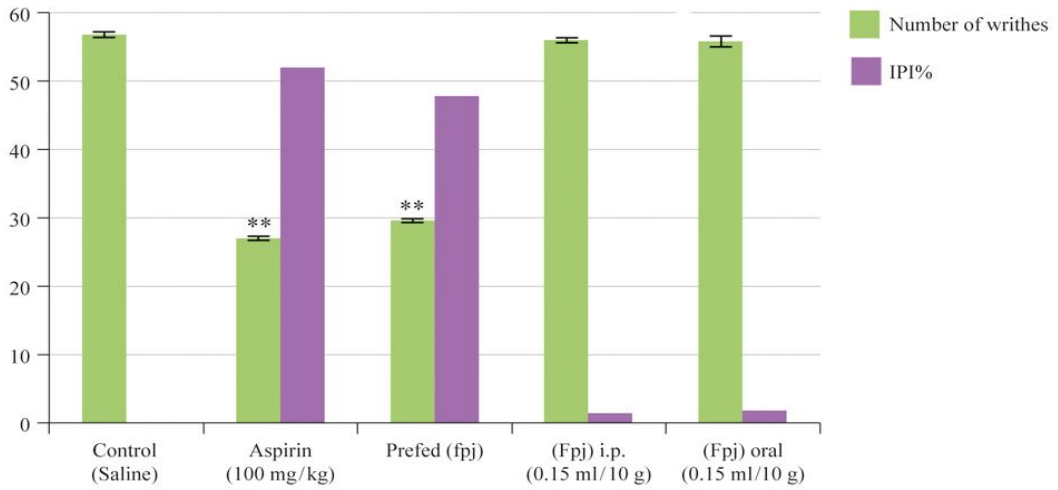
The results in Table 1 showed that pomegranate fruit juice given for a period of three subsequent days reduced the number of writhes induced by acetic acid IPI was 47.8%

Whereas, pomegranate fruit juice given instantly i.p or orally failed to produce significant effect. Aspirin (100mg/kg,i.p.) exhibited 52%of pain inhibition.

Table 1: The antinociceptive effect of administration of *P.granatum* juice and aspirin on acetic –acid induced visceral pain in mice.

Treatment	Number of writhes	IPI%
Control (Saline)	56.8 ± 0.4	0
Aspirin (100 mg/kg)	27 ± 0.3***	52%
Prefed (fpj)	29.6 ± 0.3***	47.8%
(Fpj)i.p. (0.15 ml/10 g)	56.0 ± 0.4	1.4%
(Fpj)oral (0.15 ml/10 g)	55.8 ± 0.8	1.8%

Values are represented as the mean ± SEM (n=6).Differences between groups were statistically analysed by ANOVA followed by t-test ***p<0.001vs control (saline).Fpj: pomegranate fruit juice; IPI index of pain inhibition



In Table 2 the ethanol pomegranate extract reduced writhes in a dose dependent manner IPI was 37% for (200mg/kgi.p.) and 28% for (150mg/kg.i.p.) and 21% for (100mg/kg i.p.) aspirin 100mg/kg exhibited 59% of pain inhibition

Table 2: The antinociceptive effect (i.p.) of P.*granatum* extracts and aspirin on acetic acid induced visceral pain in mice.

Treatment	Number of writhes	IPI%
Control (Saline)	55.8 ± 0.6	0%
Aspirin (100 mg/kg)	22.8 ± 0.4***	59%
Epe extract (100 mg/kg)	44.3 ± 0.7***	21%
Epe extract (150 mg/kg)	40 ± 0.6***	28%
Epe extract (200 mg/kg)	35 ± 0.4***	37%

Values are represented as the mean ±SEM(n=6).Differences between groups were statistically analysed by ANOVA followed by t test.***p<0.001vs control (saline).Epe: ethanol extract of pomegranate; IPI:index of pain inhibition

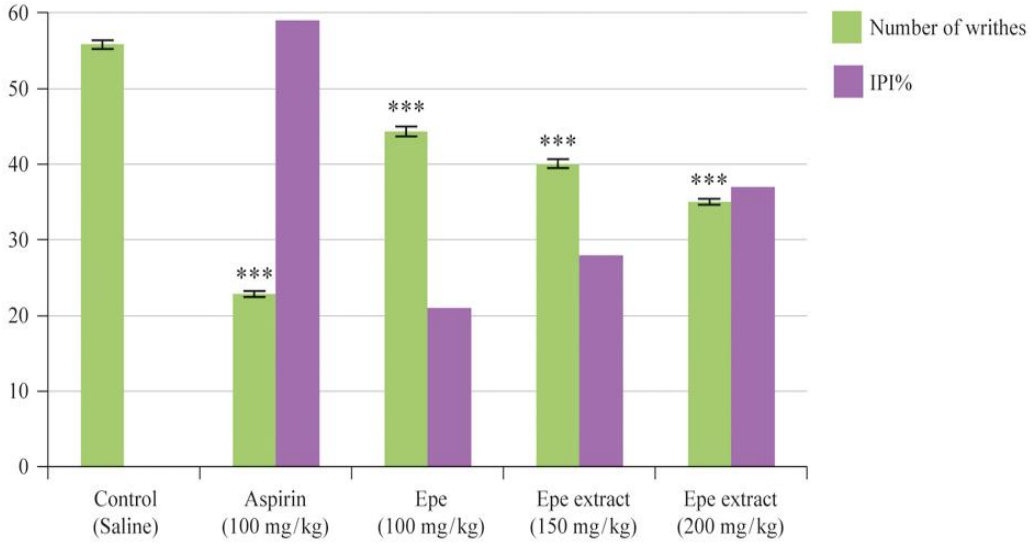


Table 3 : The effect of administration of *P.granatum* extract,juice and aspirin(100mg/kg) on hot tail tail flick test

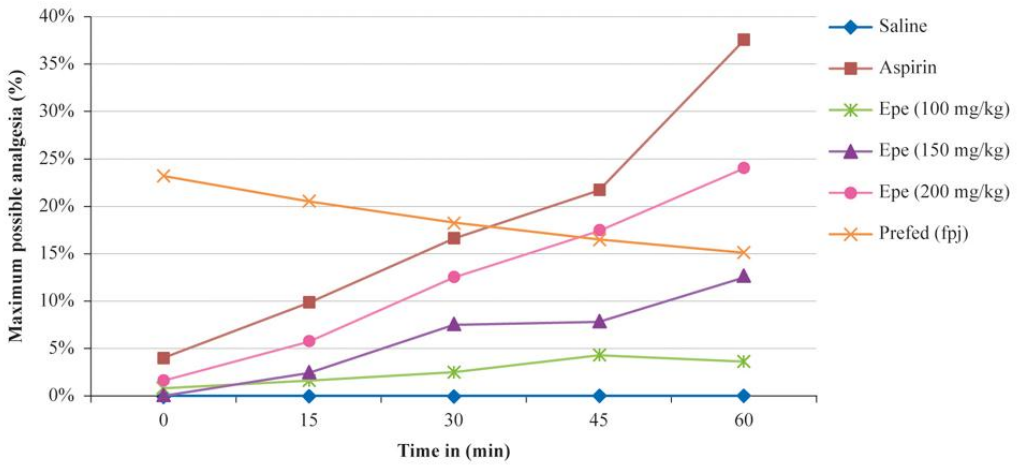
Treatment	0 min	15 min	30 min	45 min	60 min
Saline	2.5 ± 0.0	2.8 ± 0.03	3 ± 0.02	3.5 ± 0.05	3.8 ± 0.06
Aspirin (100 mg/kg)	3 ± 0.02	4 ± 0.04***	5 ± 0.04***	6 ± 0.04***	8 ± 0.03***
Epe (100 mg/kg)	2.6 ± 0.03	3 ± 0.02	3.3 ± 0.04*	4 ± 0.03**	4.2 ± 0.03
Epe (150 mg/kg)	2.5 ± 0.04	3.1 ± 0.04*	3.9 ± 0.05***	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***
Prefed (fpj)	4.7 ± 0.03	5 ± 0.06***	5.2 ± 0.08	5.4 ± 0.06***	5.5 ± 0.06***

Values are represented as mean+SEM(n=6).Differences between groups were statistically analysed byANOVA followed by t test
*p<0.05,*p<0.01,***p<0.001vs control(saline)

Epe:Ethanol extract of pomegranate IPI:index of pain inhibition

Table 4: The percentage of maximum possible analgesia (MPA%) caused by *P.granatum* extract (100,150,200mg/kg) *P.granatum* juice and aspirin100mg/kg

Treatment	0 min	15 min	30 min	45 min	60 min
Saline	0%	0%	0%	0%	0%
Aspirin	4%	9.8%	16.6%	21.7%	37.5%
Epe (100 mg/kg)	0.8%	1.6%	2.5%	4.3%	3.6%
Epe (150 mg/kg)	0%	2.4%	7.5%	7.8%	12.5%
Epe (200 mg/kg)	1.6%	5.7%	12.5%	17.4%	24%
Prefed (fpj)	18%	18%	18.3%	16.5%	15.1%



Discussion

The aim of this study was to evaluate the analgesic effects of *P.granatum* fruit juice and ethanolic extract which is commonly used by Libyan traditional medicine and to try to find alternative for analgesics which are widely used and known for their common side effects. We also focused on making a preliminary comparison between the juice and fruit extract.

To investigate the analgesic effect of pomegranate fruit juice and ethanolic extract three different models were conducted. The peripheral analgesic effect of the extract was tested by using the writhing test. This test is widely accepted as a model for visceral pain (Kozak et al 1998;Vogel andVogel1997).Writhing induced by chemical substances eg acetic acid injected i.p. are due to sensitization of nociceptors by prostaglandins and this test is useful for the evaluation of mild analgesic nonsteroidal anti-inflammatory compounds which act peripherally.

The group of mice given pomegranate ethanolic extract produced a significant dose related effect in writhing test , the prefed group with pomegranate fruit juice also produced a significant effect whereas those given (fpj) instantly i.p or p.o.failed to produce response.

This could be to the fact that biologically active dietary constituent has only limited effects on its relevant target and significant differences are only reached over time through a cumulative effect where daily benefits add up day after day (Ameye and Chee ,2006).

Another explanation is that the effect given by pomegranate fruit juice may be due to one or more metabolites. A study by (Seeram et al., 2006) confirmed that after pomegranate juice injestion ellagic acid metabolites which were not present in juice consumed as dimethylellagic acid glucoronide were detected in plasma and urine while urolithins formed

by intestinal bacteria were detected in urine samples and urolithin metabolites excreted in urine can persist for 48 hours thereby suggesting an explanation of long term administration.

In another study three pomegranate juice metabolites were detected in plasma urolithinA, urolithinB and a third unidentified metabolite ,maximum excretion rate occurred 3-4days after juice ingestion (Cerda et al., 2004). The same author also reported that ellagitannins bioaccumulate in small animals (Cerda et al.,2003).

The inhibition of writhing in mice by the extract suggest a peripheral mechanism of action possibly mediated by inhibition of PGE among several possibilities. Pomegranate extract revealed the presence of phytochemicals including flavoinoids which have a role in analgesic activity and work by targeting prostaglandins and inhibiting prostaglandin synthetase and tannins. Flavoinoids are also known to suppress COX2 transcription (Oleary et al., 2004). Cold pressed pomegranate seed oil has been shown to inhibit both cyclooxygenase and lipooxygenase enzymes invitro (Schubert et al., 1999).

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,1987). In this study aspirin 100mg/kg given intraperitonealy 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.

The pomegranate ethanolic extract produced significant dose related analgesia with the hot tail flick test, at the dose of100mg/kg the onset of action started at 30min whereas at dose150,200mg/kg the onset was at 15min which reached its peak at 60 min similar to aspirin This suggests that

it is possible that the extract produces analgesia by both a central and peripheral component on the other hand, the juice showed significant analgesia at 15min MPA18% which then declined to MPA15.1% at 60 min this either could be due to low concentration of bioavailable compounds such as ellagic acid or it may be due to a conditioning effect that develops due to repeated testing (Adovokat and McInnis 1992)Pomegranate extracts 100,150 and 200mg/kg showed significant suppreesion on pain induced by the radiant heat applied to the plantar surface of the heel of the right hind paw injected with carrageenan and left hind paw injected with saline.These results indicate that pomegranate action was via peripheral(right paw) and via central nervous system mechanisms (left paw)

The dose 200mg/kg of pomegranate produced an analgesic effect comparable to aspirin 100mg/kg in both right and left hind paw.This confirms our previous finding that pomegranate extract acts by peripheral and central mechanism of action.

While several researches have reported the health promoting effects of pomegranate juice (eg.,protection against cancer,diabetes ,cardiovascular disease, inflammation ,dental conditions erectile functions,bacterial functions antibiotic resistance ,UV-induced skin damage, infant brain ischemia, male infertility, Alzeheimers disease and arthritis (Lansky and Newman,2007;Aviram et al2000;Aviram et al 2004;Jurenka,2008 ; Baso and Penugonda 2009) no studies on pomegranate juice analgesic effect have been reported .The most striking observation in this study is the ability of pomegranate juice to possess an analgesic effect, it has been reported that regular consumption of pomegranate juice provide significant amounts of water soluble hydrolysable ellagitannins (Gil et al 2000,Seeram et al 2004). Ellagitannins are hydrolysable tannins releasing ellagic acid on hydrolysis it was also reported that after a single administration of pomegranate juice 250 ml urolithins A and urolithin B are formed and conjugated in the liver prior excrection in the urine over12-56 hrs (Heber 2011). Furthermore a

study by Adams et al. (2006) reported that pomegranate juice inhibits COX2 enzyme. This evidence gives rise to use pomegranate as an alternative to NSAIDs

Conclusion

Based on the results of this study, it can be concluded that pomegranate fruit juice has an analgesic effect which may be peripheral and pomegranate ethanol extract has both central and peripheral analgesic effects .These findings may justify the use of this plant in medicine to manage pain.

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