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Antinociceptive, anti-gastric ulcerogenic and anti-inflammatory activities of standardized egyptian pomegranate peel extract

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ABSTRACT

The utilization of the peels of the widely popular pomegranate fruit is the subject of this study. This bio waste product, which has been under study for some time as a source of potential bioactive constituents is investigated for its biological activity. A method for the extract standardization was developed using HPLC and ellagic acid as a reference standard. Results revealed that the pomegranate methanolic extract exhibited potent analgesic and anti-inflammatory activity comparable to indomethacin, used as a reference, and furthermore, caused no gastric ulcer formation.

INTRODUCTION

Punica granatum L. Puniaceae, commonly known as pomegranate produces an edible fruit that is popularly consumed worldwide. A native to Afghanistan, Iran, China and the Indian sub-continent, this fruit was widely used as part of folk medicine in many cultures which was verified by extensive reports of various biological activities (Lansky and Newman 2007, Jurenka 2008, Ismail, et al. 2012). Nowadays, pomegranates are cultivated around the world in subtropical and tropical regions with different microclimatic zones including Egypt. The world production of this fruit amounts to approximately 1,500,000 tons, where the peels amounts to approximately 60% of the pomegranate fruit weight (Lansky and Newman 2007).

The pomegranate husk, or peels, are characterized by an interior network of membranes comprising almost 26–30% of total fruit weight and constitute a higher amount of phenolic compounds than in the fruit pulp (Li et al. 2006). These compounds include flavonoids (anthocyanins, catechins and other complex flavonoids) and hydrolyzable tannins (punicalin, pedunculagin, punicalagin, gallic and ellagic acid) (Ismail, et al. 2012). The therapeutic potential of pomegranate peels has been widely recognized by different cultures. It was used for treating diarrhea and dysentery in traditional Chinese herbal medicine. In Egyptian culture, several common ailments such as inflammation, diarrhea, intestinal worms, cough and infertility were treated using the peel extract. Punicalagin and ellagic acid, the main bioactive constituents in the pomegranate husk, have shown antioxidant, antiproliferative and apoptotic activities (Seeram et al. 2005a, Seeram et al. 2005b; Lu et al. 2007; Madrigal-Carballo et al. 2009). It was also reported that punicalagin in addition to punicalin, strictinin A and granatin B significantly reduced production of nitric oxide and PGE2 by inhibiting the expression of pro-inflammatory proteins (Lee et al. 2008; Romier et al. 2008). Successful *in vitro* and *in vivo* assays indicated that pomegranate peel extract and hydrolysable tannins, in the form of standardized active components, are very effective treatment measure against various inflammatory disorders (Ismail et al. 2012). The use of the pomegranate fruit peel as a biowaste product to be used in pharmaceutical preparations has been recently presented (Abdel Motaal and Shaker, 2011) and in the work at hand, we accentuate the idea by assessing the analgesic and anti-inflammatory activities of the very same model.

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MATERIALS AND METHODS

Plant material and extract preparation

The fruits of *Punica granatum* L. were purchased from the local Egyptian market. A voucher specimen was kept at the Pharmacognosy Department, Faculty of Pharmacy, Ain Shams University under number (P-PG-8).

The seeds were removed from a kilogram of picked fruits and the peel was left to dry then grinded to yield a weight of 400g. Ten grams of the dried grinded husk were extracted with 70% methanol then concentrated under vacuum.

HPLC standardization of pomegranate peel extract

Samples were dissolved in MeOH (HPLC grade) and filtered then injected into the column. Gradient elution with two solvents was used solvent A (Methanol) and solvent B (Acetic acid in water, 1:25).

The gradient program was begun with 100 % B and was held at this concentration for the first 4 minutes. This was followed by 50 % eluent A for the next 6 minutes after which concentration of A was increased to 80 % for the next 10 minutes and then reduced to 50 % again for the following 2 minutes. Total run time was 22 minutes. The sample was repeated in triplicates (Gupta, et al. 2012).

Specification of the HPLC instrument: Analysis of all the standard samples was performed using quaternary pump G1311A, vacuum degasser G1322A, standard preparative autosampler G1329A with an injection volume 20 µl and Diode Array and multiple wavelength detector of Agilent technologies, having reverse phase column ZOBRADEX Eclipse XDB- C18 (4.6x150mm, 5µm), guard column ZOBRADEX Eclipse XDB-C18 (4.6x12.5 mm, 5µm)and The data analysis was done using Chemstation 4.02 software.

Validation of the HPLC method

Validation of the analytical method was performed according to the International Conference on Harmonization guideline (ICH, 2005). The method was validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantitation (LOQ).

Linearity

Linearity was determined by using ellagic acid as a standard solution of 0.00279g/ ml methanol. Dilutions of the standard solution were prepared. The calibration graphs were obtained by plotting the peak area versus the concentration of the standard solutions.

Precision

The precision was determined by analyzing the concentration of ellagic acid in the extract on the same day for intraday precision and on 3 different days for inter-day precision by the proposed method. The precision was expressed as relative standard deviation (RSD).

Accuracy

The accuracy of the method was tested by performing a recovery study of ellagic acid reference standard by adding 0.008g of ellagic acid to the extract sample solution (0.00423g/ml) and analyzed by the proposed HPLC method. The recovery and average recovery were calculated. Three replicates were performed.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

According to the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use recommendations, the approach based on SD of the response and the slope were used for determining the detection and quantitation limits

Biological Study

The rats (male wister,120-170g) were kept at the laboratory animal home, National Research Center (NRC), Dokki, Egypt. The animal were maintained under environmental conditions and had free access to standard diet and water

Antinociceptive activity of pomegranate peel extract

The animals were divided into 4 groups, each composed of 6 rats. The first group served as negative control where saline was administered in one group of animals subcutaneously, while the second group served as positive control receiving standard Indomethacin (20 mg/Kg b.wt.) and the third and fourth groups received the pomegranate peel extract at doses 100 and 200 mg/ Kg b. wt., respectively.

Each animal was placed gently on the tail flick such that the tail is subjected to the IR beam. Latency to exhibit nociceptive responses, such as removing the tail was determined 30, 60, 90 minutes after administration of the test substances or saline. All drugs were taken orally 30 minutes before placing the animal on the hot plate. The data represents the mean ± standard error of the mean (n=6). Statistical analysis was carried out using repeated measures one way ANOVA followed by Tuky test.

Antigastric–ulcerogenic effect of pomegranate peel extract

After the employment of antinociceptive activity experiment, rats were sacrificed under deep ether anesthesia and the stomachs of each mouse were removed. Then the abdomen of each mouse was opened through the greater curvature and examined under dissecting microscope for lesions or bleedings. Statistical analysis was carried out using Kruskall-Wallis non parametric one way ANOVA.

Acute Anti-inflammatory effect (Carrageenan induced paw edema) of pomegranate peel extract

Paw edema was induced by receiving a 100 µl sub plantar injection of 1% carrageenan solution in saline, in the right hind paw of the rats. One hour before induction of edema, saline was administered orally to a group of animals serving as a negative control.

Table 1: Antinociceptive (Tail flick) and Antigastric–ulcerogenic activity of *Punica granatum* L. peel extract.

Groups	Tolerance time (% change from baseline)				Ulcer number (mean ± SE)	Ulcer severity (mean ± SE)
	0 min.	30 min.	60 min.	90 min		
Control (saline)	2.583 ± 0.2810	3.650 ± 0.123 *	3.467 ± 0.1764 *	4.367 ± 0.2512	-	-
Indomethacin (20 mg/Kg b. wt)	2.433 ± 0.4240	4.777 ± 0.3790	7.717 ± 0.4159	7.100 ± 0.5704	3.33 ± 0.614 *	4.66 ± 0.71 *
P. granatum L. peel extract (100 mg/Kg b. wt)	2.540 ± 0.1075	4.580 ± 0.2835	6.00 ± 0.5762	6.920 ± 0.4705	-	-
P. granatum L. peel extract (200 mg/Kg b wt)	2.633 ± 0.1464	4.650 ± 0.3797	6.725 ± 0.4608	7.000 ± 0.6069	-	-

*statistically significant from the control $p < 0.05$

Table 2: Acute Anti-inflammatory effect (Carrageenan induced paw edema) of *Punica granatum* L. peel extract.

Groups	Edema volume (% change from baseline)			
	1 h	2 h	3 h	4 h
Control (saline)	67.04 ± 5.338	67.04 ± 5.338	108.9 ± 9.658	109.1 ± 6.273
Indomethacin (20 mg/Kg b. wt)	44.86 ± 2.894	44.86 ± 2.894	53.86 ± 6.945*	47.29 ± 3.669*
P. granatum L. peel extract (100 mg/Kg b. wt)	39.32 ± 2.863	39.32 ± 6.008*	39.32 ± 2.658*	31.48 ± 2.658*
P. granatum L. peel extract (200 mg/Kg b. wt)	42.61 ± 3.256	53.30 ± 5.879*	44.41 ± 3.658*	32.29 ± 3.265*

*statistically significant from the control normal inflamed group at the corresponding time $p < 0.05$

Indomethacin (20 mg/Kg b. wt.) was administered to a group of rats that served as a positive control. The extract was administered at dose levels 100 and 200 mg/Kg b.wt. All the drugs were orally administered one hour before induction of inflammation. The right hind paw volume was measured immediately before carrageenan injection and at selected times (1, 2, 3 and 4 hours) thereafter by water plethysmometer (Winter, et al. 1962). Statistical analysis was carried out using repeated measures one way ANOVA followed by Least test for multiple comparison.

$$\% \text{ edema} = \frac{T-C}{C} \times 100$$

where T; test and C: control.

RESULTS AND DISCUSSION

HPLC standardization of pomegranate peel extract

The method of standardization used showed a linear relationship between peak areas and concentrations over the range for ellagic acid. Standard solutions of ellagic acid were prepared and analyzed in a concentration range of 0.0027 to 0.0108 g/ml. The regression equation of the curve is Y=11040x+111.4 and the coefficient of regression (R2) was 0.984 confirming the linearity of the method. The LOD and LOQ values for ellagic acid ranged from 1.974 and 5.983 µg/ml, respectively. The quantitative repeatability of the injection was determined by analyzing the quantity of the marker in the extract. A high repeatability was observed with RSD values lower than 0.11843 % and 0.0753 % for inter-day and intraday assay, respectively. Accuracy (expressed as recovery) of the method was determined by analyzing the percentage recovery of the marker. The high recovery value (98.2 %) obtained indicated satisfactory accuracy.

Antinociceptive, Antigastric–ulcerogenic and Antiinflammtory activities of pomegranate peel extract

The results of antinociceptive test are summarized in Table (1 & 2). The Pomegranate peel extract significantly ($p<0.05$) increased the tolerance of the rats towards the IR beam. Tolerance time was elevated from 2.54 to 4.58 min. after 30 minutes then increased to 6 min. after 60 minutes then to 6.92 min. after 90 minutes at a dose level 100 mg/Kg b. wt. of the pomegranate peel extract. Similarly, the tolerance time for dose 200mg/Kg b. wt. of the extract showed the same pattern but with a minor change after 60 minutes (6.7 min).

The rats were sacrificed to examine the effect of pomegranate peel extract on the gastric ulcer number and severity after 5 hours of oral drug administration. It was found that the pomegranate peel extract showed no ulcer formation compared to the reference indomethacin which formed 3.33 ± 0.614 and severity 4.66 ± 0.71.

Concerning the anti-inflammatory effect of the pomegranate peel extract, the edema volume, in the group receiving the extract (200 mg/Kg.b.wt.) had decreased similarly to the group receiving indomethacin (20 mg/Kg.b.wt.) reference standard after 1 h. After 2 hours, the test group showed a high decrease in edema volume by 53.3% which was considerably more than that resulting from indomethacin administration (44.86%).

CONCLUSION

Punica peel extract exhibited potent antinociceptive, anti-gastric ulcerogenic and anti-inflammatory activities which is very promising in regards to the use of biowaste that is usually discarded. A validated HPLC method for the analysis of *Punica* peel extract had been developed to test for the concentration of the marker ellagic acid which proved to be reliable and efficient for future standardization experiments.

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