RESEARCH PAPER

Phytochemical Analysis and Determination of the Antioxidant Activity of Pomegranate (Punica Granatum) Peel Growing in Armenia

Tonoyan Inga[1]*, Topchyan Hakob[1], Mkhitaryan Seda[1], Zhamharyan Arusyak[2]

[1]Faculty of Pharmacy, Yerevan State Medical University,
[2]Department of Drug Technology, Yerevan State Medical University,
[3]Department of Pharmacy, Yerevan State Medical University

Received: 18th Sep, 2025; Revised: 22th Oct 2025; Accepted: 4th Nov, 2025; Available Online: 1st December, 2025

ABSTRACT
Pomegranate peel is a promising source due to its rich content of biologically active compounds and proven medicinal properties. This study aimed to obtain extracts from pomegranate peels grown in Armenia using different technological methods and to perform a phytochemical analysis of the compounds responsible for the main biological activity in these extracts: flavonoids and organic acids. The raw material was standardized by determining the content of flavonoids, organic acids, and water content using appropriate methods. Liquid extracts from pomegranate peel were obtained through maceration for three days and ultrasonic extraction using 80% ethyl alcohol, with varying degrees of raw material grinding. The optimal extraction method, which ensures the maximum content of active substances, was ultrasonic extraction using 80% ethyl alcohol with a particle size of 0.5-1 mm of the raw materials. The results demonstrate that the extracts show high antioxidant properties in in vitro tests, comparable to those of ascorbic acid.

Keywords: pomegranate peel, flavonoids, organic acids, ultrasonic extraction, maceration, antioxidant activity
How to cite this article: Tonoyan I, Topchyan H, Mkhitaryan S, Zhamharyan A, Phytochemical Analysis and Determination of the Antioxidant Activity of Pomegranate (Punica Granatum) Peel Growing in Armenia. Int J Drug Deliv Technol. 2026;16(1): 361-366. DOI: 10.25258/ijddt.16.1.39
Source of support: Nil.
Conflict of interest: None

INTRODUCTION

The pomegranate (Punica granatum L.) is a deciduous fruit-bearing shrub or tree belonging to the Lythraceae family. The pomegranate tree is ornamental and long-lived; some trees in France are known to be over 200 years old [1]. It has been used in the Middle East as a healing food and traditional medicine for thousands of years. The pomegranate is an ancient shrub that is still popular today.

Pomegranate peel makes up about 30-40% of the total fruit, but it was previously considered waste. Recent research has shown that pomegranate peel is a rich source of biologically active compounds, such as phenolic substances and polysaccharides [2]. It exhibits antioxidant, anti-inflammatory, and antimicrobial effects, due to the presence of its polyphenolic compounds: tannins (punicalgin, punicalin, granatin A, B), flavonoids (catechin, epicatechin, quercetin, etc.), and organic acids (gallic acid, ellagic acid, vanillic and other acids) [3].

Different dosage forms obtained from the peel are used in the treatment of various inflammatory pathologies: colitis, arthritis, hepatitis, and contact dermatitis, as well as anti-osteoporosis, anti-hyperglycemic, anti-hypertensive, vasoprotective, hepatoprotective, neuroprotective, and immunomodulatory agents [4].

Considering the above, our study aimed to obtain extracts from pomegranate peels by using raw materials with varying degrees of grinding and different extraction methods. The objective was to identify the optimal technological condition for obtaining the extract with the highest content of biologically active compounds and study their antioxidant activity.

MATERIALS AND METHODS

Herbal Material

The pomegranate was harvested from the Armavir region of Armenia in September, during the fruit ripening period. The peels were manually separated from the fruit, thoroughly washed with distilled water, and dried in the shade until complete dryness was achieved [5]. The dried peels were divided into two parts and ground into a powder with a grain grinder, resulting in particles of 0.5-1 mm and larger particles of 2 mm.

Standardization of herbal raw material

The raw material was standardized by determining the content of flavonoids, organic acids, and residual moisture.

Determination of water content in raw materials

The moisture content of the crushed raw materials, weighed to the nearest 0.01g, was determined in an oven at 100-105^{0}C according to Quality Control for Herbal Materials (**World Health Organization**)[6] and using a KERN MLB 50-3 moisture meter. Weighings were performed at 30-minute intervals until a constant mass was obtained; the mass differences between weighings did not exceed 0.01 grams.

Quantitative determination of flavonoid content in raw materials

*Author for Correspondence: ingatonoyan@yahoo.com

1 g±0.01g of pomegranate peel powder was weighed and replaced in a conical cylinder, and 50 *ml* of 96% ethyl alcohol was added. The graduate was connected to a reflux condenser, heated in a boiling water bath for 45 minutes, shaking periodically to remove raw material particles from the walls. Then, the mixture was cooled to room temperature and, if necessary, made up to the original volume with 96% ethyl alcohol.

2 ml of alcoholic extract was transferred to a 25 ml volumetric flask, 4 drops of dilute acetic acid solution and 4 ml of 2% aluminum chloride solution were added, and the volume was brought to the mark with 96% ethanol and left for 20 minutes.

The optical density of the resulting solution was measured using a UV spectrophotometer Unicam 8625 at a wavelength of 410±2 nm in a 10 mm layer thickness cuvette.

The total flavonoid content was calculated based on rutin and absolute dry matter using the following formula:

$$X,\% = \frac{A * 0{,}025 * 5000}{A_0 * m * (100 - W)}$$

A- is the optical density of the test solution

A_0- is the optical density of the rutin solution

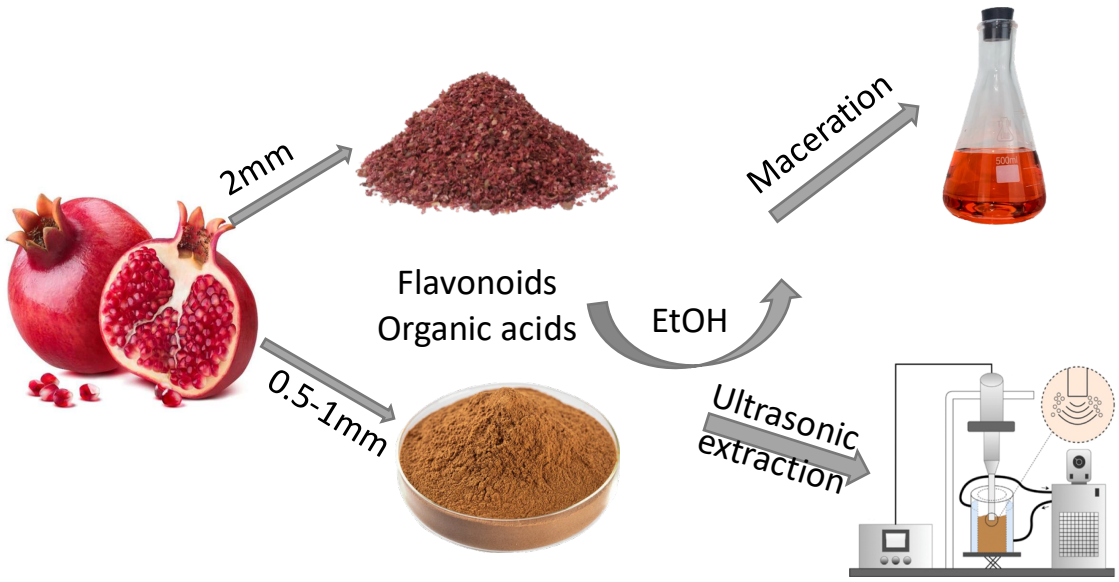
m- the mass of the raw material is g,

W- raw material moisture (%).

Preparation of rutin standard solution: 0.025g of weighed rutin standard was transferred to a 50ml volumetric flask, 35ml of 96% ethyl alcohol was added and heated in a water bath until dissolved. Then it was cooled, and the volume was brought to the mark with the same alcohol and mixed. 1 *ml* of this solution was transferred to a 25 *ml* volumetric flask, 1 drop of dilute acetic acid was added, 2 *ml* of a 2% solution of aluminum chloride was added, and the volume was brought to the mark with 96% ethyl alcohol. It was mixed and left for 30 minutes. The optical density of the resulting solution was measured spectrophotometrically at a wavelength of 410±2 *nm* in a 10 *mm* layer thickness cuvette [7].

Determination of organic acids content in raw materials

1 g of pomegranate peel powder was taken in a conical graduate, and 50 *ml* of 96% ethyl alcohol was added. The test tube was connected to a reflux condenser, heated in a boiling water bath for 45 minutes, shaking periodically to remove particles of the raw material from the wall. Then, the mixture was cooled to room temperature and, if necessary, made up to the original volume with 96% ethyl alcohol. To prepare the titration solution, 10 *ml* of the test sample was taken and mixed with 200 ml of freshly boiled water. Then, 1 ml of 1% phenolphthalein and 2 *ml* of 0.1% methylene blue solutions were added. The resulting solution was titrated until a blue-green color was obtained. A 0.1M solution of NaOH was used as the titrant. 1 *ml* of 0.1M NAOH solution corresponds to 0.0067 g of maleic acid [8]



Picture 1. The scheme of the extraction procedure from the Pomegranate peel

Extraction procedure

Maceration and ultrasonic extraction methods were used to obtain extracts (pic. 1).

Maceration: To prepare pomegranate peel extracts, the peels were separated, washed, and then cut into small pieces. The pomegranate peels were dried in the shade. The dried peels were ground in a grain grinder until a powder with a diameter of 0.5-1 *mm* was obtained. To prepare the extracts, 20 g of crushed peels was mixed with 200 *ml* of 80% ethanol solution in a ratio of 1:10. The mixture was left for 72 hours, away from light, at room temperature [9]. Then, alcohol was removed using a rotary evaporator at 50°C. The resulting extract was stored under refrigerated conditions for further analysis.

Using the same preparation technology, an extract was also obtained from coarsely ground (2mm diameter) pomegranate peel raw material.

Ultrasonic extraction: 20g of raw material was weighed and transferred to a 250 *ml* beaker, 200 *ml* of 80% ethyl

alcohol was added, and the solution was subjected to ultrasound at a frequency of 22kHz for 1h and filtered through a gauze. The resulting extract was centrifuged at 3500 rpm for 15 minutes. The extract was obtained from both powdered and coarsely ground raw materials [10].
Thick extracts were obtained from liquid extracts using ultrasonication, maceration, and subsequent evaporation. The evaporation was performed under vacuum conditions at a temperature of 40-50°C using a "Unipam" vacuum evaporator type 350, after which the obtained thick extract was dried in a drying cabinet at a temperature of 60°C until a constant mass was obtained [11].

Phytochemical analysis of extracts
0.1 g of the dry extract was weighed and transferred to a 50 ml volumetric flask, and the volume was made up to the mark with distilled water. The organic acid content and the total flavonoid content, recalculated on rutin and absolute dry matter, were determined by the above-described specified methods. All experiments of analysis were performed in triplicate, and the data are represented as an average of measurements.

Determination of the antioxidant activity
The *in vitro* method of determination of the antioxidant activity of extracts was based on the use of a relatively stable free radical, 2,2-diphenyl-1-picrylhydrazyl(DPPH), which was performed spectrophotometrically by measuring the kinetics of reduction of the DPPH molecule [12].
50 µl of the test extract and 2 ml of DPPH methanol solution were placed in a 1 cm cuvette. Since the extracts showed high antioxidant activity, a 1:100 dilution with an appropriate solvent was performed. A methanol solution of ascorbic acid was also tested as a control: 2.29 mg of ascorbic acid was taken and dissolved in 10 ml of 80% methanol. 50 µl of ascorbic acid solution, 1 ml of methanol, and 1 ml of DPPH methanol solution were placed in a 1 cm^3 cuvette.
Absorbance measurements were started immediately. The kinetics of the change in optical density were recorded with a Unicam 8625 spectrophotometer at a wavelength of 515 nm for 16 min until the absorbance stabilized. The percentage of DPPH radical inhibition was calculated using the following formula.

$$\%\text{inhibition} = \frac{\text{Ac}(0) - A_A(t)}{\text{Ac}(0)} 100\%$$

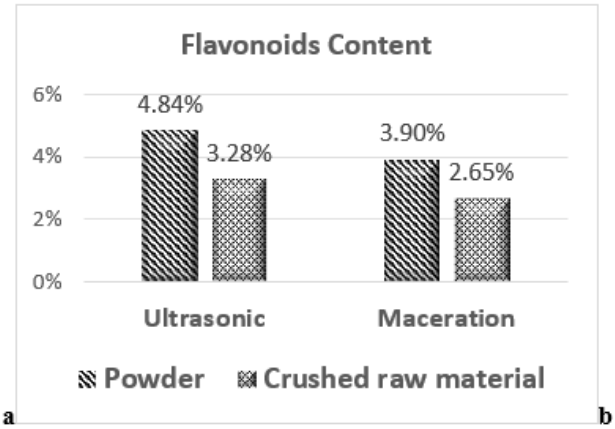
where $A_C(0)$ is the absorbance of the control at t=0 min, $A_A(t)$ is the absorbance of the reaction solution over a given time interval.

RESULTS AND DISCUSSION
The collected raw herbal materials were pre-processed, dried, and then standardized. The residual moisture content of the raw materials was determined; as a result, it does not exceed 7-8% and complies with the pharmacopoeial requirements for herbal raw materials.

	Water content (%)	
	Drying oven	KERN MLB 50-3 moisture meter
Powder (0,5-1mm)	8%	6.21%
Coarsely ground (2mm)	7%	6.8%

Table 1. Results of water content obtained through evaporation in a drying oven and moisture meter for raw material with varying grinding sizes.

The main pharmacological effects of pomegranate peel are due to flavonoids and organic acids. Consequently, the standardization of the plant's raw material was carried out according to the quantitative content of organic acids and flavonoids contained in it.
According to the results obtained, pomegranate peel contained 3.2% flavonoids calculated on rutin and 3.214% organic acids calculated on dry raw material. According to literature data [13], the total content of flavonoids in different parts of the Punica granatum plant ranges from 0.1-2%, which indicates that the peel of pomegranates grown in Armenia contains significant flavonoid content and is suitable as a raw material for obtaining extracts. Considering the rich composition of dried pomegranate peel with biological compounds and its compliance with the requirements for USP (United States Pharmacopeia) herbal raw materials, standardization of extracts obtained by ultrasound and maceration was also performed. The content of flavonoids and free organic acids, the predominant active substances in the phytochemical composition of pomegranate peel, was determined in extracts obtained from both the powder (0.5-1mm) and crushed (2mm) herbal raw material.



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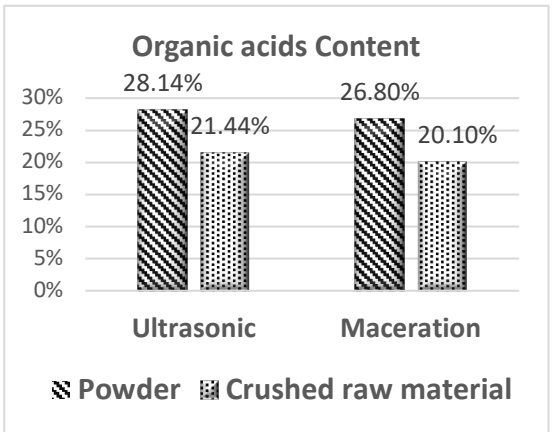
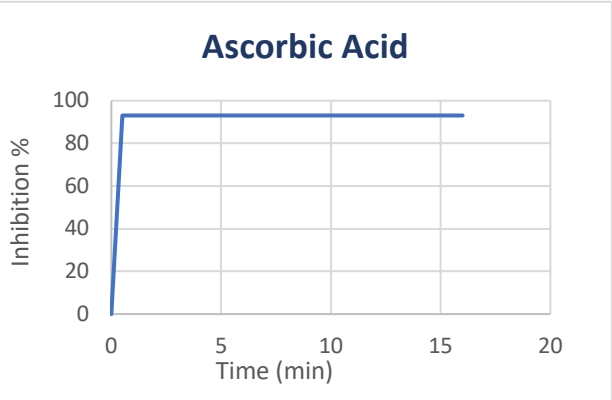


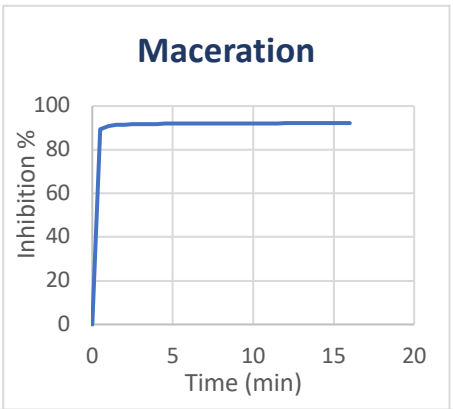
Figure 1. Quantitative content of flavonoids (a) and organic acids (b) in pomegranate peel extracts (%).

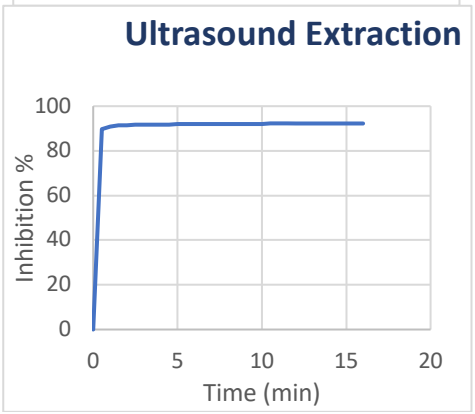
As shown in Figure 1, the highest quantitative content of organic acids and flavonoids was found in the ultrasonic extract obtained from pomegranate peel powder: 28.14% and 4.84%, respectively. In extracts obtained by the ultrasonic extraction method in all samples, the content of organic acids and flavonoids is higher than in extracts prepared by the maceration method. In addition to the low yield, the maceration method is time-consuming, so obtaining an extract from pomegranate peel is more expedient using an ultrasonic method. Thus, the ultrasonic method of extracting bioactive compounds from pomegranate peel is several times faster, more efficient, and more economically beneficial.

Results of the antioxidant activity of the extracts

Antioxidant activity was determined by measuring the kinetics of the reactions of the extracts obtained by ultrasonication and maceration with the DPPH reagent. Picture 2 shows the change in the percentage of DPPH inhibition over time in the conditions of the extracts and the addition of ascorbic acid solution. Experimental data show that the extracts have a very high rate of neutralization of free radicals, as well as the control preparation. Thus, already at the 2nd minute, this indicator is 89.6% and 89.4% for extracts obtained by ultrasound and maceration methods, respectively. Already at the 2nd minute, this indicator for the methanol solution of ascorbic acid was 93%.







Picture 2. Kinetic profile of the reactions of Punica granatum L. ethanolic extracts (a,b) and ascorbic acid with DPPH.

According to the test data, the extracts obtained by both methods showed high antioxidant properties comparable to the antioxidant activity of ascorbic acid, which is most likely due to the high content of flavonoids. This corresponds to previous investigations, which found that pomegranate peel is a good source of natural antioxidants, mainly attributed to ellagitannins [14, 15, 16, 17]. Pomegranate peel grown in Armenia had a rich total phenolic content, and it has the potential to become the source of raw material for pharmaceutical formulations. Future studies are necessary to investigate the most active compounds responsible for the antioxidant activity of pomegranate peel.

CONCLUSION

Pomegranate peel collected from the Armavir region of Armenia contains high levels of flavonoids and organic acids and can be used as raw material for obtaining preparations.

It is most appropriate to use raw materials ground to a diameter of 0.5-1 mm and an ultrasonic method to ensure a high content of substances responsible for the pharmacological effect in the extract.

Extracts of pomegranate peels grown in Armenia, exhibiting high antioxidant activity, can serve as the basis for the development of various dosage forms

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