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Research Article

QUANTITATIVE DETERMINATION OF PHENOL CARBOXYLIC ACIDS IN POMEGRANATE FRUIT PULP BY THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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ABSTRACT

Objective: Pomegranate (*Punica granatum* L.) is a broadly used plant possessing a wide range of medicinal properties. In this research, we have mainly focused on the investigation of phenolic compounds of pomegranate fruit pulp (PFP).

Methods: Fresh fruits of "Çəhrayı Gülöyşə," "Kizil-anor," and pomegranate varietal mixture were used as samples. High-performance liquid chromatography-ultraviolet (HPLC-UV) analysis of phenol carboxylic acids was performed with metal column Kromasil® C_{18} (4.6×250 mm, particle size 5 µm) and the acetonitrile-water-concentrated acid phosphoric system (400:600:5) under isocratic elution conditions (flow rate of 0.5 ml/min). Detection was carried out using a UV detector "GILSTON" UV/Visible model 151 at a wavelength of 280 nm.

Results and Discussion: As a result of our research, we proposed chromatographic conditions for the separation of phenolic compounds, the conditions for sample preparation of PFP. Procedure for determination of phenolic carboxylic acids total content in terms of gallic acid by HPLC-UV method was developed. According to the obtained data, the content of phenolic carboxylic acids should be at least 0.7%.

Conclusion: Procedure for the quantitative determination of gallic acid using the HPLC-UV method was developed. This method which can be used in the standardization of new medicinal plant raw materials - PFP, as well as extract preparations based on it in the future.

Keywords: Pomegranate fruit pulp, *Punica granatum L.*, Phenol carboxylic acids, Gallic acid, high-performance liquid chromatography with ultraviolet detection.

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INTRODUCTION

Pomegranate (*Punica granatum* L.) - a low evergreen tree or deciduous shrub, is widespread in the wild form on the Balkan Peninsula, West Asia, reaching in the east to the borders of Northwest India. Wild pomegranate grows on dry gravelly and stony slopes, up to above 1000 m above sea level. In the Russian Federation, pomegranate is cultivated in Transcaucasia and the Crimea. Fruits, flowers, and bark of pomegranate have long been used in traditional medicine as a general strengthening and astringent remedy [1], also they are the part of the herbal species used in phytotherapy [2]. Information about this raw material found in the prescriptions of the famous ancient Roman physician-encyclopaedist - Aulus Cornelius Celsus [3]. In the "Canon of Medicine" Avicenna included a separate article "Rumman" ("Pomegranate"), which was used in therapy of many diseases. Moreover, the great doctor of the middle ages was the first to suggest the use of crushed pomegranate fruit pulp (PFP), which remains after the production of juice, called "pomegranate oatmeal," as an independent remedy [4]. Pomegranate medicinal plant raw material (MPRM) is included in the list of nomenclature of single-component homeopathic medicines homeopathic mother tinctures produced from the trunks and branches bark of *P. granatum* [5]. In the plural microbiological studies, it was established that different pomegranate varieties fruits possess antibacterial properties against a wide range of microorganisms [6]. Moreover, this effect is directly related to the content of polyphenols in MPRM [7]. Taking into account the high content of polyphenol complex in PFP after the juice production, there were attempts to develop a non-waste technology for the processing of pomegranate fruits. Unfortunately, they did not find industrial application [8].

Analysis of modern scientific literature shows the growing interest of researchers, both in the study of biologically active compounds (BAC) of pomegranate fruits and in the pharmacological action of MPRM extracts, as well as in solving the problem of creating a non-waste processing technology [6,7]. *P. granatum* leaf extracts contain phenols, tannins, flavonoids, and triterpenoids [9]. Thus, in studies, the high hypoglycemic activity of MPRM extracts was experimentally proved [10]. Membrane-stabilizing effect of *P. granatum* polyphenolic complex in MPRM was established using an experimental model of hepatitis [11]. According to the data obtained [12], the pomegranate peel can be used as a natural food preservative, due to the high antioxidant activity. The pomegranate antitumor potential for the treatment of different cancer localizations was described by researchers from Iran in detail [13]. Extracts that can be obtained from different parts of *P. granatum* possess various activities: Antioxidant [14], hypolipidemic, anti-fatty liver effects [15], and antimicrobial activity [16]. However, until now, the pomegranate fruits and PFP are not official MPRMs.

In view of the foregoing, the aim of our study was to study phenolic compounds in PFP extracts, as well as to develop a methodology for quantitative determination of gallic acid by high-performance liquid chromatography-ultraviolet (HPLC-UV) for further MPRM standardization.

METHODS

For identification and quantitative determination of phenol carboxylic acids, fresh fruits of "Çəhrayı Gülöyşə," "Kizil-anor," and pomegranate varietal mixture, collected in October in Azerbaijan, were used. Samples were subjected to grinding and mechanical juice quenching after they were dried in isothermal mode at 100°C and crushed to the size of particles passing through the sieve with a hole diameter of 2 mm.

PFP extract, obtained by the infusion method (1 h in a boiling water bath with 70% ethanol, MPRM-extractant ratio – 1:10), was used for selection of the conditions in chromatographic analysis.

Analysis of literature data shows the advisability of using the C_{18} reverse phase as a sorbent, and mixture of acetonitrile-water-concentrated phosphoric acid in a ratio of 20:80:0.05 as a mobile phase. BAC detection was carried out at a wavelength of 280 nm. In reversed-phase chromatography optimum separation efficiency and the shape of the peaks are typical for the substances that are in molecular form. Gallic acid contains carboxyl and phenolic groups, it has acidic properties, which determines the choice of an inorganic eluent with pH <7 to suppress ionization. In the case of acidification, the retention volumes of acid nature compounds increase, and the volumes of basic compounds decrease.

It should be remembered that the use of eluents with a pH <2 is highly undesirable for maintaining the stability of the stationary phase since at pH <2 hydrolysis of the silanol groups of the sorbent is possible. Taking into account the foregoing, to create the system pH value in the acidic region, we selected a concentrated phosphoric acid [17-20].

Taking into account the described conditions, we used a metal column Kromasil® C_{18} (4.6×250 mm, particle size 5 μm), the acetonitrile-water-concentrated acid, phosphoric system (400:600:5) under isocratic elution conditions. The analysis was carried out at room temperature at a flow rate of 0.5 ml/min. The duration of the analysis was from 20 to 40 min. Detection was carried out using a UV detector "GILSTON" UV/ VIS model 151 at a wavelength of 280 nm.

Sample preparation

The analytical sample of PFP was ground to the size of particles passing through a sieve with a hole diameter of 2 mm. Fresh MPRM was ground to the size of particles passing through a sieve with a hole diameter of 7 mm. About 10.00 g (precise test portion) of crushed PFP was placed in a 250 ml flask, 70 ml of 70% ethanol was added, and the flask was attached to a reflux condenser and heated in a boiling water bath for 1 h from the boiling point. At the end of the extraction, the mixture was filtered through a membrane filter with a pore diameter of 0.25–0.45 μm into a 100 ml volumetric flask (the first 7 ml of the filtrate was discarded), then the volume was adjusted to the flask's calibration mark by 70% ethanol. In parallel, 0.05% solutions of phenolic compounds working standard (gallic acid, coffeic acid, catechin, epigallocatechin gallate (EGCG), and ellagic acid) were prepared in 70% ethanol by the procedure described in Skurkhin and Tutelyan [17]. 20 μl of the test solutions and the reference solution were introduced into a chromatograph using a 25 μl Hamilton Microliter™ Syringe and chromatography was performed.

RESULTS AND DISCUSSION

The fruits of the pomegranate of "Çəhrayı Gülöyşə," "Kizil-anor," and pomegranate varietal mixture met the GOST 27573-87 "Fresh pomegranate fruits" quality requirements (Table 1).

As a result of the analysis, we proposed a methodology for quantitative determination, described in the section "Materials and methods."

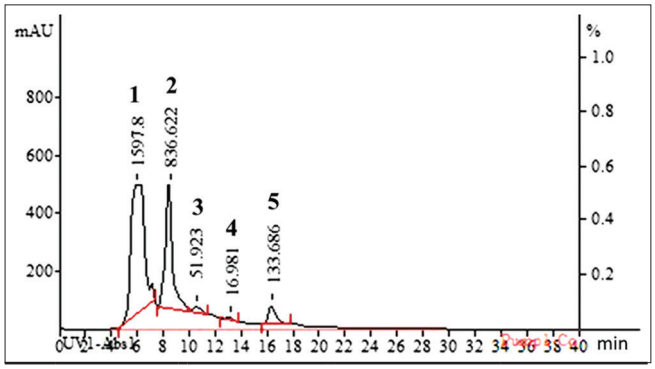
Table 1: Quality indicators of pomegranate fruits used for PFP production according to GOST 27573-87 "Fresh pomegranate fruits"

Indicator name	Indicator value according to GOST		Objects of research		
	I sort	II sort	Çəhrayı Gülöyşə	Kizil-anor	Pomegranate varietal mixture
1. Appearance	The fruits are fresh, whole, mature, healthy, clean, without excessive external moisture, without damage from diseases and pests, fully developed, typical of a pomologic variety of form and color, with or without a flower cup, and with a peduncle smoothly cut at the base of the fruit		Meet	Meet	Meet
2. Taste and smell	Characteristic of this pomologic variety, without foreign smell and taste		Meet	Meet	Meet
3. Size by the largest transverse diameter, mm, not less than:					
For fresh consumption	75.0	60.0	75.0	70.0	70.0
For industrial processing	75.0	50.0	75.0	60.0	70.0
4. Degree of maturity	Consumer		Consumer		
5. Fruit peeling from sunburn, not more	1/8 of the fruit surface	1/4 of the fruit surface	1/6 of the fruit surface	1/8 of the fruit surface	1/4 of the fruit surface
6. Caustic fungus, no more than	Not allowed	1/4 of the fruit surface	Not allowed	Not allowed	Not allowed
7. Peel rubbing, not more:					
In places (shopping centers, shops)	1/8 of the fruit surface	1/4 of the fruit surface	1/8 of the fruit surface	1/8 of the fruit surface	1/6 of the fruit surface
8. Contents of rotten, crushed fruits, immature, damaged by agricultural pests, with unhealed cracks, punctures at the places of shipment and at the time of release to the buyer in the retail trade network	Not allowed		Not allowed		

Table 2: Metrological characteristics of the procedure for the quantitative determination of gallic acid in PFP alcohol extracts

n	f	X_{av}	S^2	S_x	p, %	t (p, f)	ΔX	ε, %
Fresh PFP								
5	4	1.107	0.0017	0.0412	95	2.78	0.0512	4.6
Dry PFP								
5	4	0.964	0.0011	0.0332	95	2.78	0.0413	4.3

n - number of repeat tests, f - number of degrees of freedom, p% - confidence figure, t (p, f) – Student's coefficient, X_{av} $\bar{x}$ - mean value, S^2 - dispersion, S - standard deviation, $S_{\bar{x}}$ - the standard deviation of the mean value, ΔX - confidence interval, ε,% - relative error. PFP: Pomegranate fruit pulp



No.	Time, min	Height, mAU	Area, mAU* sec	Name
1	5.832	451.74	31,957.31	gallic acid
2	8.418	430.32	16,732.44	catechin
3	10.54	19.26	1038.46	not identified
4	12.97	10.82	339.61	EGCG
5	16.18	62.79	2673.72	ellagic acid

Fig. 1: High-performance liquid chromatography-ultraviolet chromatogram of 70% ethanol fresh PFP extract at 280 nm

Gallic acid, catechin, EGCG, and ellagic acid were determined in PFP alcohol extracts with the use of the internal normalization method. It should be noted that the chromatograms of dry and fresh PFP alcohol extracts are identical (Fig. 1).

Calculation of the content of gallic acid was carried out by absolute calibration using the computer program Multichrome for Windows according to the formula:

$$C\,\% = \frac{S_{test} \times C_{st} \times 100 \times 100 \times 100}{S_{st} \times 25 \times \alpha \times (100 - W)}$$

Where
S_{test} - peak area of gallic acid in the test solution;
S_{st} - peak area of gallic acid in standard sample solution;
C (%) - content of gallic acid, %;
C_{st} – weight of the sample of gallic acid standard sample, g;
α - mass of the raw material taken for analysis, g;
W - loss in mass when dried, %;

Metrological characteristics of the procedure for quantitative determination of gallic acid in PFP alcohol extracts in five independent replicates are presented in Table 2.

The relative error in the procedure for determining the gallic acid content with a 95% probability is±4.6% for fresh PFP and ±4.3% for dried PFP.

CONCLUSION

Thus, we developed a procedure for the quantitative determination of gallic acid using the HPLC-UV method, which can be used in the standardization of new MPRMs - PFP, as well as extract preparations based on it in the future.

AUTHORS' CONTRIBUTIONS

We declare that this study was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Pogosyan R.A. collected the data, analyzed the data, all the laboratory work performed, and wrote the introduction, discussion, the material, and method part. Nesterova O.V., Samylina I.A. proofread the whole manuscript, and Bokov D.O. helps in designing and conducting the study.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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