OPTIMAL CONDITIONS FOR THE EXTRACTION OF POLYPHENOLIC COMPOUNDS FROM THE PEEL OF PUNICA GRANATUM FRUITS

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https://doi.org/10.5281/zenodo.20694567

Abstract. *Optimal conditions for the extraction of polyphenolic compounds from the peel of Punica granatum L. fruits grown in the Tashkent region of the Republic of Uzbekistan were selected. The peel of the Uzbek pomegranate cultivar "Kai-anor," popular in Central Asia, was dried in late October – early November 2025 to study the total polyphenol content. A comprehensive study was conducted to examine the influence of various factors on the total polyphenol yield, including extractant composition, aqueous acetone concentration, extraction rate, raw material-to-extractant ratio, temperature, extract concentration conditions, treatment of the aqueous residue with organic solvents, and precipitation and drying conditions for the obtained polyphenolic compounds. To isolate biologically active substances, 1 kg of dried and crushed peel was pre-treated with chloroform and then extracted three times with aqueous acetone at concentrations ranging from 25 to 70%. After removing the acetone, the aqueous residue was treated with ethyl acetate. The ethyl acetate fraction was concentrated and precipitated with chloroform, yielding the total polyphenolic compounds. Optimal extraction conditions were found to yield 3.9% of the total polyphenolic compounds based on the air-dried weight of the raw material. Column chromatography on bald powder was used for preliminary separation of the polyphenol complex. The composition of the isolated fractions was studied using modern physicochemical methods, including high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS). As a result of the research, citric acid ($C_7H_{12}O_6$), gallic acid ($C_7H_6O_5$), gallacatechin ($C_{15}H_{14}O_7$), di-galloyl-hexahydroxydiphenyl-D-glucose ($C_{27}H_{22}O_{18}$), garnetin A ($C_{34}H_{24}O_{22}$), procyanidin B1 ($C_{30}H_{26}O_{12}$), punigluconin ($C_{34}H_{26}O_{23}$), garnetin A (form B) ($C_{34}H_{24}O_{23}$), myricetin-3-O-xyloside ($C_{20}H_{18}O_{12}$), garnetin B ($C_{41}H_{28}O_{27}$), chebulagic acid ($C_{41}H_{30}O_{27}$), isorhamnetin-3-galactoside ($C_{22}H_{22}O_{12}$), kaemferol-3-O-rutinoside were identified in the peel of Punica L. fruits. ($C_{27}H_{30}O_{15}$), ellagic acid-rhamnoside, rutin ($C_{26}H_{26}O_{17}$), and*

myricetin ($C_{15}H_{10}O_8$). The obtained results demonstrate the rich composition of polyphenolic compounds in pomegranate peel and its potential for use as a source of natural biologically active substances.

Keywords: *punica granatum L., extract, high-performance liquid chromatography mass spectrometry (HPLC-MS).*

INTRODUCTION

Pomegranates are processed for juice, with the peel and seeds serving as secondary raw materials. The seeds are dried and transferred to the oil and fat industry to extract valuable pharmacopoeial oil. Although pomegranate peel contains natural compounds such as polyphenols, flavonoids, carotenoids, anthocyanins, catechins, tannins, etc., it is not processed; furthermore, peel removal and disposal is problematic [1-2]. The development of resource-saving, waste-free technologies may find application in the food, light industry, and pharmaceutical industries. An important property of natural dyes is their absolute safety. The collection includes 69 varieties of pomegranate. Bitter Kazakh, Tuyatish, Shakhrisabz, May, Belozerny, Red Pomegranate, Ulfi, and other varieties are grown in Uzbekistan. Since the peel of these varieties is red, a red dye is produced [3-4]. Punica granatum L. is a famous fruit tree. Pomegranate is grown in gardens and vegetable patches throughout Central Asia. It also grows wild in mountain river valleys. [5-6]. The fruits, flowers, and bark of the pomegranate have long been used in folk medicine as a tonic and astringent [7]. They are included in popular herbal teas [8] and are described in many historical medical treatises [9, 10]. The chemical composition of the plant: the juice and pulp of the pomegranate fruit contain up to 20% sugars, organic acids, and up to 6% citric and malic acids [11]. Tricosane, heptacosanyl n-hexanoate, oleic acid, β-sitosterol laurate, and β-sitosterol myristate have been identified in pomegranate flowers [12; 16]. In addition, pomegranate, ellagic acid, O-methyl-ellagic acid, ethyl brevicarboxylate, ursolic and butyric acids and daucasterol [17], flavone tricetin 4 '-O-β -glucopyranoside, flavones tricetin, luteolin, pomegranate B [18; 9] were identified. The leaves and bark of the fruits contain ursolic acid; alkaloids - pseudopeltierine, isopeltierine; triterpenoids; steroids; resins and a large amount of tannins (up to 25%). All parts of the plant are rich in polysaccharides [20]. Pomegranate peel contains a significant amount of phenolic compounds, such as hydrolyzable tannins (punicalin, punicalagin, ellagic acid and gallic acid), flavonoids (anthocyanins and catechins) and nutrients [21,22]. The seeds contain up to 20% fatty oil, consisting mainly (40%) of linoleic, palmitic (16%), oleic acids, and also contain fatty acids, triglycerides, steroids, lignins, phenolic acids, phytosterols such as β-sitosterol, campesterol, stigmasterol, and α-, β-, γ-, δ-tocopherols, and proteins [23]. Pomegranate peel makes up about 50% of the total fruit content and, due to its polysaccharides and phenolic substances, has antioxidant, anti-inflammatory, antimicrobial, antityrosinase, anti-osteoporotic, anti-hyperlipidemic, and anti-cancer properties [24]. Pomegranate seeds have antioxidant, cardioprotective, anti-osteoporotic, anti-diabetic, anti-inflammatory, and anti-cancer properties [25]. In this regard, the aim of our work is to determine the optimal conditions for the extraction of polyphenolic compounds from the peel of the fruits of the Punica granatum L plant using physicochemical research methods.

EXPERIMENTAL SECTION

Isolation of Total Polyphenols.[26,27] The subject of the study was dried peels of the Uzbek pomegranate variety "Kai-anor" (Punica granatum L), prepared in late October - early November 2025. Extraction of the raw material with organic solvents was used to extract the total

polyphenols. For this purpose, several factors influencing the yield of total polyphenols were studied: the degree of raw material grinding, the composition of the extractant, the extraction modulus, the extraction frequency, the raw material-extractant ratio, the extraction temperature, the thickening conditions, the treatment of the aqueous residue with organic solvents, the conditions for the precipitation of the total polyphenols and their drying. Based on the obtained results, an optimal method for the isolation of total polyphenols was developed. It consists of the following: air-dried, crushed (to a size of 5-7 mm) peel of P. granatum L (1 kg) is pre-extracted with chloroform to remove lipophilic substances. For this purpose, the raw material was placed in a flask equipped with a reflux condenser and extracted with chloroform in a water bath in ratios from 1:2 to 1:10 (raw material:chloroform), at a temperature of 20 to 60 °C for 2 h, from a single to fivefold extraction of the raw material. After treating the raw material with chloroform, extraction was continued with aqueous acetone at concentrations from 25 to 70%. Extraction was repeated from one to three times. Then, the obtained aqueous-ethyl extracts were evaporated under vacuum on a rotary evaporator until the aqueous portion remained. The purified aqueous extract was treated with ethyl acetate in a separatory funnel, in ratios from 1:1 to 1:6 (aqueous residue: ethyl acetate). The ethyl acetate fractions were concentrated and precipitated with chloroform in ratios from 1:1 to 1:5 (concentrate:precipitant), resulting in the formation of a flocculent precipitate. The total polyphenols were preliminarily separated on gallium powder. 5 g of the total polyphenols, soluble in ethyl acetate, were fractionated on gallium powder according to the method [28]. The column was eluted with diethyl ether, distilled water, pure acetone and 60% acetone. The fraction washed with ether contained 1 substance, which, based on the chromatographic behavior in the solvent systems n.butanol-acetic acid-water (40:12:28, Rf 0.72) and 15% aqueous acetic acid solution (Rf 0.55), was identified as gallic acid. The fraction washed with water was treated with ethyl acetate five times. After drying the ethyl acetate extract with anhydrous Na2SO4, the mixture was concentrated in vacuo, and the concentrate was precipitated with chloroform. The precipitate was filtered and dried in a vacuum oven. 1.2 g of the total product was obtained. Paper chromatography in a n-butanol-acetic acid-water (40:12:28) system revealed the presence of two compounds with Rf 0.68 and Rf 0.60. The product was dissolved in ethanol and chromatographed on a column.

The structures of the isolated compounds were established using physicochemical methods—high-performance liquid chromatography and mass spectrometry. Individual phenolic compounds were identified by high-performance liquid chromatography–mass spectrometry (HPLC-MS/MS).Chromatography conditions: chromatograph—HPLC Ultimate 3000, Thermo Fisher Scientific (USA), equipped with an automatic sampler; Hypersil GOLDaQ column 100 mm × 2.1 mm, particle size 1.9 μm; mobile phase—bidistilled water acidified with 0.1% formic acid (A) and acetonitrile (B), flow rate 0.1 ml/min, 15:85 isocratic mode. Instrument name and chromatography–mass spectrometry conditions: mass spectrometry with electron impact ionization (HESI-II); Spray chamber voltage – 4000 V; evaporator temperature – 25 °C. Solution concentration gradient in minutes: 0–3 min – 2%, 3–35 min – 55%, 35–38 min – 2%. Solutions were degassed using an Agilent Technologies 1260 μ-degasser.Mass spectrometric analysis of isolated polyphenols. Samples were loaded onto the column using an Agilent Technologies Micro WPS instrument (1 μL at a time) from a polyphenol solution with a concentration of 0.1 mg/mL. Mass spectrometric studies of the isolated polyphenols were performed on an Agilent Technologies Q-TOF LC-MS 6520B series instrument under the following conditions: ionization source – ESI, drying gas flow – 5 μ/min, drying gas temperature – 300 °C, voltage on: kimmer

cone – 20 V, fragmentor 125 V, mass range: in MS mode 100 – 2000 m/z, and in Auto MS/MS50 mode – 2000 m/z, collision energy – 35, 50 eV. Ionization method: negative. was introduced into the mass spectrometer using an Agilent Technologies 1200 series chromatograph. Mobile phase: A – 0.1% formic acid solution, B – acetonitrile + 0.1% formic acid. Elution was performed on an Agilent Technologies 1260 Cap Pump at a flow rate of 15 µL/min. The solution concentration gradient was as follows: 0–3 min – 2%, 3–35 min – 55%, 35–38 min – 2%. Solutions were degassed on an Agilent Technologies 1260 µ-degasser.

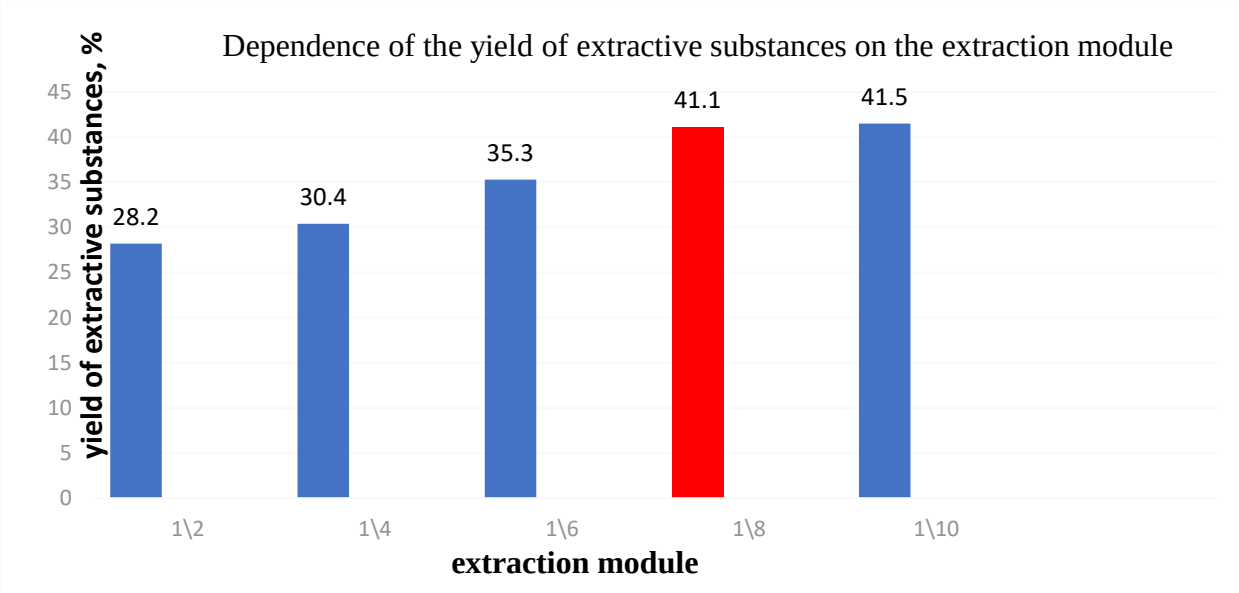
The following public databases were used for identification: Chemical Entities of Biological Interest (ChEBI, https://www.ebi.ac.uk/chebi/), Chemical Compounds Deep Data Source (https://www.molinstincts.com/), ChemSpider (www.chemspider.com), and Phenol_Explorer (www.phenol-explorer.eu).

RESULTS DISCUSSION

Results Discussion. The process of isolating polyphenols from plant materials involves several stages: raw material extraction, processing the extracted material with organic solvents, evaporation, precipitation of total polyphenols, purification, etc. Increased efficiency in raw material use is achieved primarily at the first stage – extraction. The yield of total polyphenols was studied as a function of the following parameters: extractant composition, extraction modulus, extraction rate, raw material-to-extractant ratio, extraction temperature, thickening conditions, processing the aqueous residue with organic solvents, and the conditions for precipitation of total polyphenols and their drying [11, 12]. To determine the optimal extraction modulus, raw material-to-extractant ratios of 1:2, 1:4, 1:6, 1:8, and 1:10 were used. Extraction efficiency was assessed based on the yield of extractive substances, calculated as a percentage.

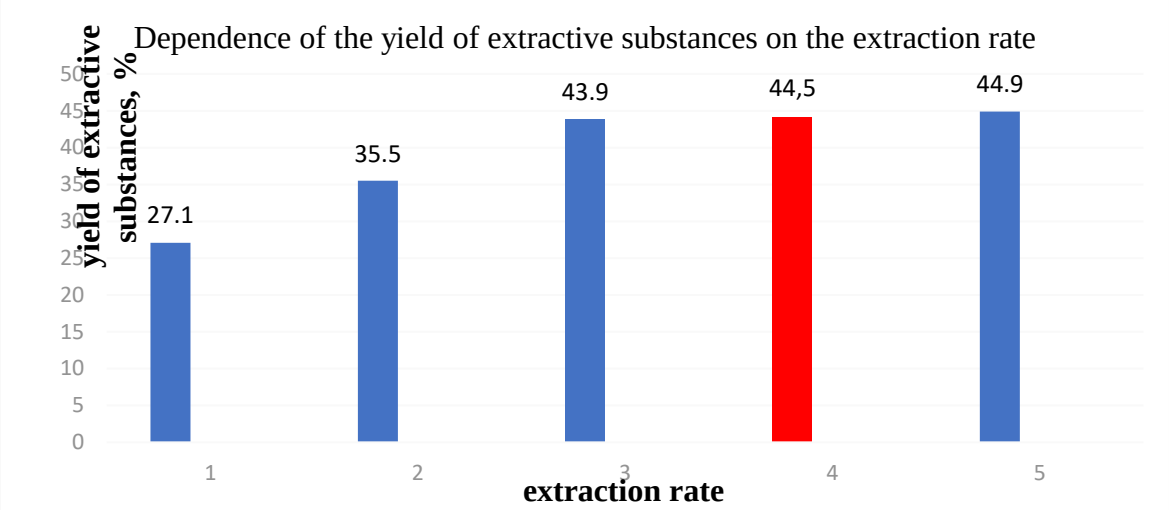
The results are shown in Figure 1.

Fig. 1



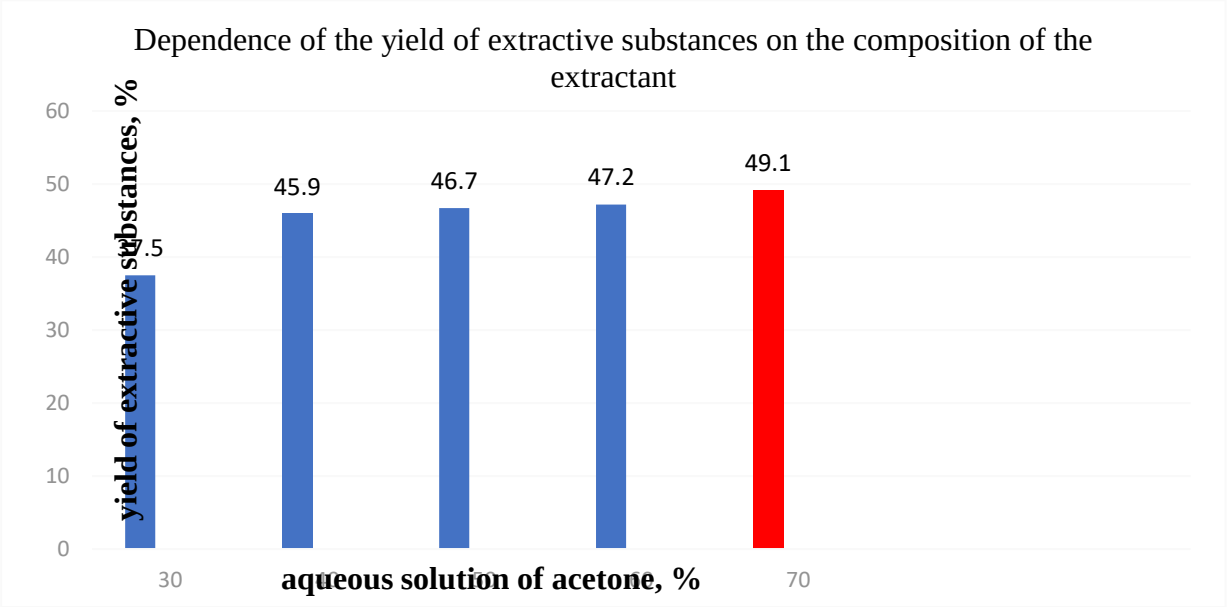
The data presented show that the optimal raw material:extractant ratio is 1:8. The average yield of extractive substances was 41.1%. With a lower extraction module (1:2; 1:4; 1:6), the yield of extractive substances is incomplete, and the use of a ratio of 1:10 leads to an increase in the extraction volume, which in turn leads to an overconsumption of organic solvents. We similarly conducted a study of the optimal extraction multiplicity. In this case, 1-, 2-, 3-, 4-, 5-fold extraction methods were used. The results obtained are shown in Figure 2.

Fig. 2



The extraction efficiency was assessed based on the yield of extractive substances. As can be seen from the data presented, a 4-fold extraction was the most optimal. The average yield of extractive substances was 44.5%. With low extraction rates (1-, 2-fold), the yield of extractive substances was incomplete, and with increasing extraction rates (4-, 5-fold), the yield of extractive substances changed insignificantly. However, the extraction process is time-consuming and increases the volume of the extract, which also further leads to an increase in the consumption of organic solvents. To determine the optimal extractant composition, we used an aqueous acetone solution of varying concentrations. 30%, 40%, 50%, 60%, and 70% aqueous acetone solutions were used as the extractant. The results are shown in Figure 3.

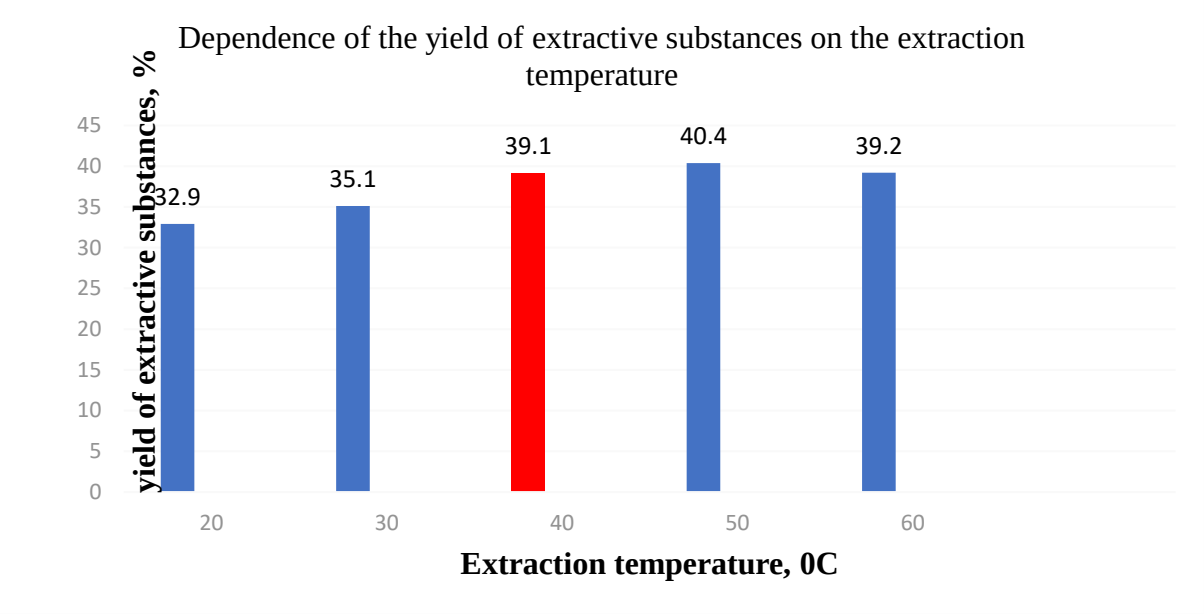
Fig. 3



As can be seen from the data presented, 70% aqueous acetone is the most optimal extractant. The average extractive yield was 49.1%. Using low-concentration aqueous acetone results in incomplete extraction of extractives from the raw material, while high acetone concentrations also do not increase the extractive yield.

Similarly, we searched for the optimal extraction temperature. Extraction was carried out at the following temperatures: 200°C, 300°C, 400°C, 500°C, and 600°C. The obtained data are shown in Figure 4.

Fig. 4

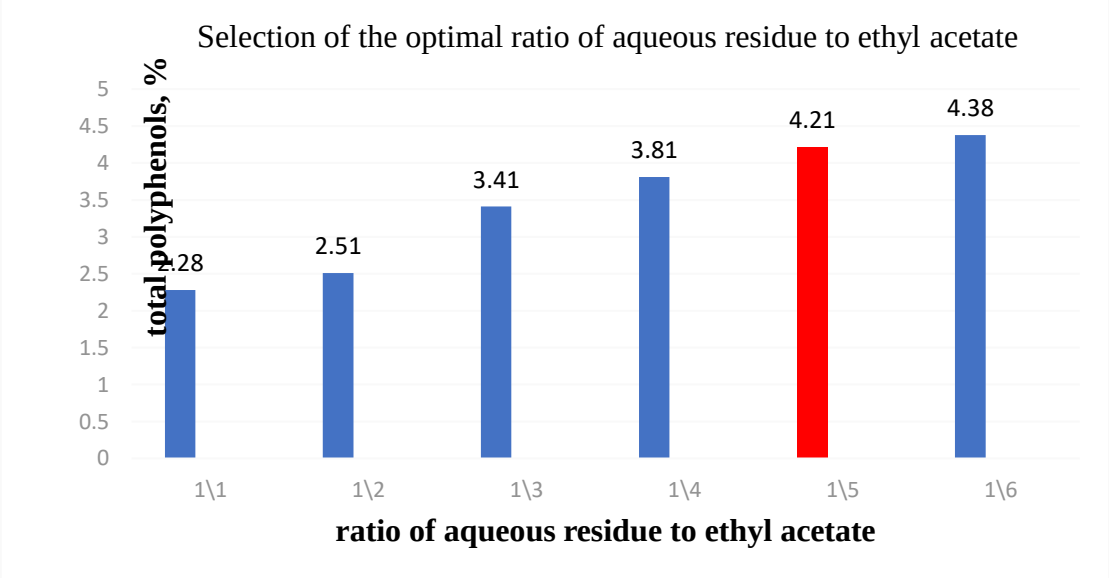


From the data presented it is clear that the most optimal extraction temperature is 40 ^{0}C and under this regime the average value of extractives was 39.1%. At lower temperatures, the yield of extractive substances is incomplete, and when the extraction temperature increases to 600C, polyphenolic substances are hydrolyzed, which is confirmed by paper chromatography data.

In a similar way, the dependence of the yield of extractives on the extraction time was studied, which varied from 1 to 3 hours. It was found that the most optimal is a 2-hour extraction.

The resulting extracts were concentrated under vacuum to an aqueous residue. The extraction of polyphenols from the aqueous residue was carried out with ethyl acetate in a ratio of 1:1; 1:2; 1:3; 1:4; 1:5; 1:6. Ethyl acetate extracts were combined, dried over anhydrous Na2SO4 and concentrated. The results obtained are shown in the figure. 5.

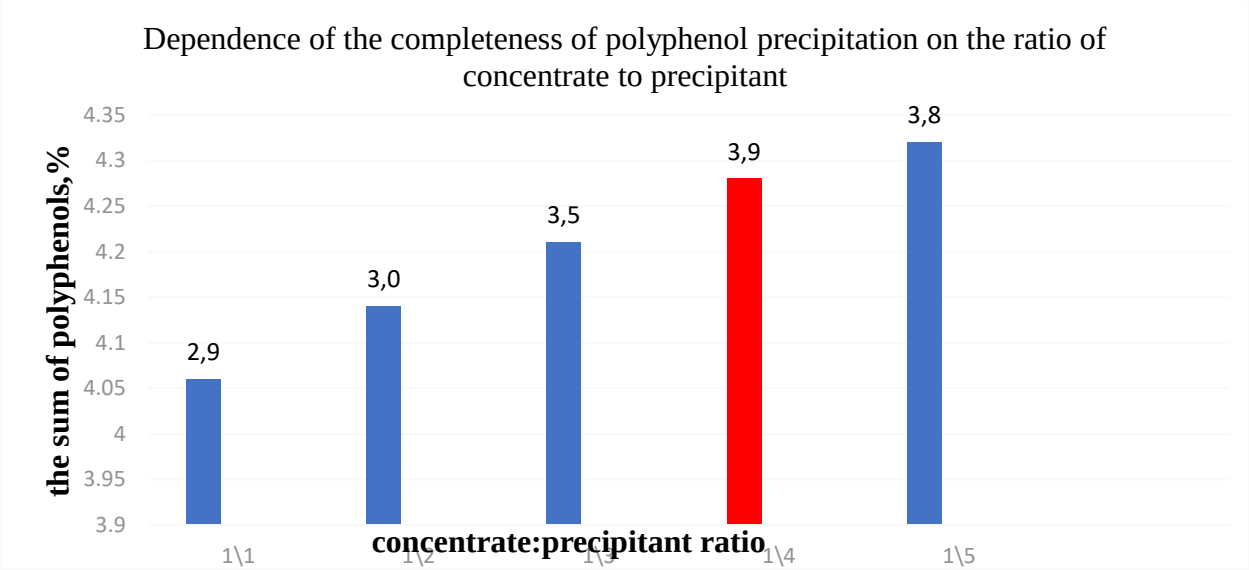
Fig.5



The data show that the optimal ratio of aqueous residue to ethyl acetate is 1:4. At lower aqueous residue to solvent ratios, polyphenols are incompletely extracted from the aqueous residue. The polyphenol yield increases slightly at a ratio of 1:6, but this also increases solvent consumption.

Chloroform was used to precipitate polyphenols from the ethyl acetate concentrate. The concentrate to precipitant ratios were 1:1; 1:2; 1:3; 1:4; 1:5. The obtained data are shown in Figure 6.

Fig.6



The resulting polyphenolic mixture from the ethyl acetate fraction of Punica L. fruit rind is an amorphous, light-yellow powder with an astringent taste. With ferric chloride, the mixture yields a blue color (developed with an iodine chamber), and with a 1% vanillin solution in concentrated hydrochloric acid, it yields a bright red color (developed with aqueous ammonia).

Elution was performed with diethyl ether, water, acetone, and 60% acetone. The phenolic compounds of the ether fraction are phenoloxyacids, the phenolic compounds of the aqueous fraction are flavonols, and the phenolic compounds of the 60% aqueous acetone fraction are tannins. In the fraction washed with ether, gallic acid (R_f 0.72 and 0.55), citric acid ($C_7H_{12}O_6$), and gallacatechin ($C_{15}H_{14}O_7$) were identified by paper chromatography.

The water-soluble fraction was rechromatographed on polyamide using a mixture of chloroform and methanol in various ratios as an eluent, yielding individual compounds: myricetin-3-O-xyloside ($C_{20}H_{18}O_{12}$), isorhamnetin-3-galactoside ($C_{22}H_{22}O_{12}$), kaemferol-3-O-rutinoside ($C_{27}H_{30}O_{15}$), ellagic acid-rhamnoside, rutin ($C_{26}H_{26}O_{17}$), and myricetin ($C_{15}H_{10}O_8$).

The fraction soluble in aqueous acetone was chromatographed on silica gel columns using a solvent system of diethyl ether - ethyl acetate (1:1) and pure ethyl acetate. As a result, several compounds were isolated and identified by their physicochemical properties using modern physicochemical methods, including high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS). As a result of the studies, di-galloyl-hexahydroxy-diphenyl-D-glucose ($C_{27}H_{22}O_{18}$), garnetin A ($C_{34}H_{24}O_{22}$), procyanidin B1 ($C_{30}H_{26}O_{12}$), punigluconin ($C_{34}H_{26}O_{23}$), garnetin A (form B) ($C_{34}H_{24}O_{23}$), garnetin B ($C_{41}H_{28}O_{27}$), and chebulagic acid ($C_{41}H_{30}O_{27}$) were identified in the Punica L. fruit peel. The obtained results indicate a rich composition of polyphenolic compounds in the pomegranate peel and the potential for its use as a source of natural biologically active substances.

CONCLUSIONS

Optimal conditions for the extraction and isolation of polyphenolic compounds from the peel of the Kai Anor pomegranate (Punica granatum L.) variety were determined. High-

performance liquid chromatography coupled with mass spectrometry (HPLC-MS) was used to identify the main polyphenolic components of the studied raw material.

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