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Determination of The Content of Phenolic Compounds in The Septum of Walnut Fruit Using the HPLC Method

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Abstract: This article presents comprehensive data regarding the determination of the quantitative and qualitative composition of phenolic compounds within a 96% ethanolic extract derived from the walnut fruit septum (Juglans regia L.), utilizing the method of High-Performance Liquid Chromatography (HPLC). The experimental findings reveal the presence of several key bioactive substances, specifically gallic acid, rutin, salicylic acid, quercetin, and apigenin, within the investigated extract. Conversely, the presence of kaempferol was not detected under the established chromatographic conditions. Among the identified phenolic constituents, gallic acid and rutin were found to be present in relatively higher concentrations compared to other components. The analytical results obtained throughout this study demonstrate that the walnut fruit septum represents a highly promising source of natural antioxidants, possessing significant potential for application as a raw material within the pharmaceutical and food industries.

Keywords: Walnut fruit septum, Gallic acid, Rutin, Salicylic acid, HPLC, Natural antioxidants.

Introduction: The walnut fruit septum (diaphragm) is a highly valuable product containing compounds beneficial to the human organism; however, in many instances, these components are discarded as waste. Its composition includes essential compounds required for the proper functioning of the human body, such as flavonoids, vitamins, macro- and microelements, saponins, and carbohydrates. The specific substances present within these septa, as well as their respective concentrations, can vary significantly depending on the variety of the walnut plant, its cultivation conditions, the type of soil, and the regional climate [1].

Extensive research has been conducted by European and Asian scientists to determine the chemical composition of the walnut fruit septum. These researchers have tested the identified substances and their biological activities through both in vivo and in vitro experiments.

Scientists from Russia and Poland, including D. Chernikova, J. Bazarnova, U. Gašić, and S. Durović, have successfully isolated a complex of phenolic compounds from the walnut fruit septum, subsequently determining their phytochemical profiles and antioxidant properties [2]. Daria Chernikova, a professor at the Peter the Great St. Petersburg Polytechnic University, conducted direct experiments on the walnut fruit diaphragm, during which she developed specific methods for extraction and sample preparation [3]. Within the same university, Professor Julia Bazarnova acted as the scientific supervisor for projects focused on developing technological regulations for the processing and standardisation of walnut fruit septa. For these experiments, the researchers utilised walnut septa obtained from the "Valentine's Gift", "Burlyuk", and "Alminskiy" varieties harvested between 2021 and 2023 [4].

The phenolic compounds were isolated via ultrasonic extraction (20–25 kHz) using a water–ethanol mixture (at a weight ratio of 1:30). The resulting extracts were

concentrated under vacuum and prepared in the form of a "total extract of phenolic compounds (TEPC)". Through the application of HPLC–MS/MS, twelve primary phenolic compounds were identified, including:

− Gallic acid;

− Chlorogenic acid;

− Catechin;

− Quercetin;

− Kaempferol-3-O-glucoside;

− Rutin, amongst others.

The concentration of flavonoids and their glycosides was recorded at 119.75 mg/g, while phenolic acids were present at a level of 69.43 mg/g. The moisture content was determined to be 6.2%. The total amount of extractive substances reached 285 mg/g, and the antioxidant activity, as determined by the DPPH assay, was found to be 94% [5].

Experimental Part

Determination of the content of phenolic compounds in walnut septum extract using the HPLC method.

Reagents and Equipment. The following reagents and reference standards were utilised: Gallic acid from "Macklin" (China), Salicylic acid from "Rhydburg Pharmaceuticals" (Germany), and quercetin, apigenin, and kaempferol from "Regal" (China). Rutin was isolated from natural sources using extraction and column chromatography methods. For the mobile phase and sample preparation, HPLC-grade water and acetonitrile were employed, along with chemically pure grade acetic acid and sodium hydroxide.

The quantification of polyphenols in the plant material was performed using an LC-40 Nexera Lite High-Performance Liquid Chromatograph manufactured by the Shimadzu Corporation (Japan).

Preparation of Standard Solutions. Standard solutions were prepared by dissolving gallic acid (5.2 mg), salicylic acid (5.2 mg), rutin (5 mg), quercetin (5 mg), apigenin (5 mg), and kaempferol (5 mg) in 96% ethanol. The mixture was placed in an ultrasonic bath for 20 minutes to ensure complete dissolution, transferred to a 50 ml volumetric flask, and diluted to the mark with ethanol. A 200 µl aliquot was taken from each individual solution and mixed. Subsequently, four different concentrations were prepared through serial dilution. Each solution was transferred into a vial for chromatographic analysis.

Preparation of the Plant Extract. To extract the phenolic compounds, a 1 g sample of the test material was weighed with an accuracy of 0.01 g using an OHAUS NV222 analytical balance (USA). The sample was placed in a 50 ml conical flask, and 25 ml of 96% ethanol was added. The extraction process was conducted in a GT SONIC-D3 ultrasonic bath (China) at a temperature of 60 °C for a duration of 20 minutes.

Following extraction, the mixture was cooled and filtered, then diluted to a final volume of 25 ml with ethanol in a volumetric flask. A 1.5 ml portion of the extract was centrifuged using a Mini-7 centrifuge (BIOBASE, China) at a speed of 7,000 rpm. Finally, the supernatant was filtered through a 0.45 µm syringe filter before being used for analysis.

Chromatographic Conditions

Determination of Phenolic Compounds. To determine the phenolic compounds, a Shim-pack GIST C18 reverse-phase column (150 × 4.6 mm; 5 µm, Shimadzu, Japan) was employed. The mobile phase consisted of a gradient system (Table 1) comprising acetonitrile (A) and a 0.5% aqueous solution of acetic acid (B). The experimental parameters were set as follows:

− Injection volume: 10 µl;

− Flow rate: 0.5 ml/min;

− Column thermostat temperature: 40 °C.

The analytical signals (peak areas) for the phenolic compounds were recorded at a wavelength of 300 nm (Figure 1).

Table 1. Gradient programme for the mobile phase.

Time (min)	Solvent A (Acetonitrile, %)	Solvent B (0.5% Acetic Acid, %)
0	5	95
5	5	95
17	40	60
22	40	60
22,1	5	95
40	Completion	

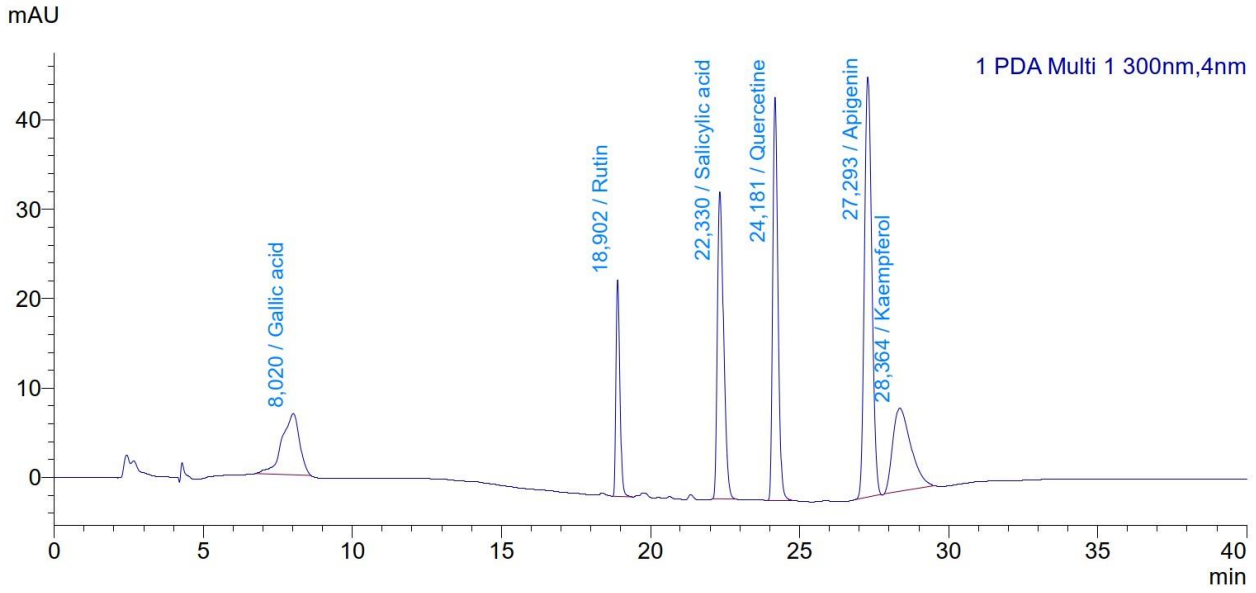

Figure 1. Chromatogram of standards at 300 nm.

RESULTS AND DISCUSSION

Professor Uros Gasic from the University of Belgrade, Serbia, performed the identification and quantitative analysis of phenolic compounds in walnut fruit septum extracts using HPLC–MS/MS methods [6]. Additionally, Professor Sasa Durovic from the Institute of General and Physical Chemistry in Serbia determined the optimal conditions for chromatography and physicochemical analyses of walnut fruit septum extracts [7].

Determination of the content of phenolic compounds in the sample extract. We successfully determined the concentration of polyphenols in the fruit septum extract of walnut plants grown under the environmental conditions of the Andijan region using the HPLC method. For this purpose, a chromatogram of an extract from a 1 g sample of the walnut fruit septum was obtained (Figure 2). Based on these results, the amounts of phenolic compounds present in 100 g of the sample were calculated using the following formula and are presented in Table 2:

$$X = \frac{C_{phen} \cdot V_{extract}}{m_{sample}} \cdot 100\,g \qquad (1)$$

Where:

X – the total quantity of phenolic compounds per 100 grams of the sample, measured in mg;

C_{phen} – the concentration of the phenolic compound within the extract as determined by the HPLC method, expressed in mg/l;

$V_{ekstract}$ – the total volume of the sample extract, measured in l;

m_{sample} – the mass of the sample taken for the preparation of the extract, expressed in g..

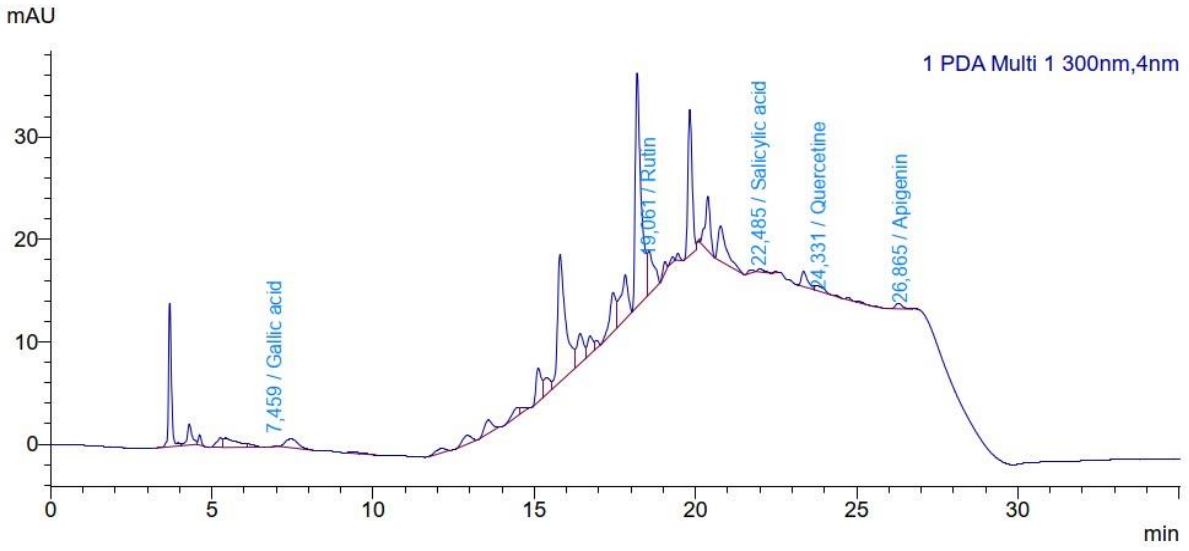

Figure 2. Chromatogram for the determination of polyphenols in walnut fruit plate extract.

Table 2. Amount of polyphenols in the extract and retention times.

Name of Phenolic Compound	Retention Time (s)	Concentration (mg/l)	Amount in 100 g of Sample (mg)
Gallic acid	7.459	1.141	2.853
Rutin	19.061	0.632	1.580
Salicylic acid	22.485	0.029	0.073
Quercetin	24.331	0.024	0.060
Apigenin	26.865	0.006	0.015
Kaempferol	Not detected	0	0.000

An analysis of the chromatogram obtained from the 96% ethanolic extract of the walnut fruit septum (Figure 2) reveals distinct characteristics regarding the identified polyphenols. Among the peaks corresponding to the five different flavonoids detected in the polyphenolic chromatogram, those for gallic acid and rutin are prominently manifested. Conversely, the chromatogram confirms the absence of a peak for kaempferol within the investigated extract.

The experimental analysis conducted on the chemical composition of the walnut fruit septum (Table 2) demonstrates that among the six types of polyphenols analysed in the ethanolic extracts prepared from a 100 g sample, the concentrations of gallic acid (2.853 mg), rutin (1.580 mg), and salicylic acid (0.073 mg) were found to be the most significant. Given that even low doses of polyphenols have been shown to protect melanocytes from lipid peroxidation, these findings suggest that the walnut fruit septum serves as a viable raw material for the production of therapeutic food supplements.

CONCLUSION

As a result of the comprehensive investigations conducted throughout this study, it has been established that the chemical composition of the walnut fruit septum is exceptionally rich in phenolic compounds, specifically flavonoids. The analytical data obtained through the application of the HPLC method demonstrated the presence of relatively high concentrations of:

– Gallic acid;

– Rutin;

– Salicylic acid.

These identified bioactive substances possess potent antioxidant properties, which play a fundamental role in safeguarding cellular structures against oxidative stress and lipid peroxidation. Consequently, the findings of this research significantly expand the possibilities for utilizing the walnut fruit septum as an efficient and highly promising secondary raw material. Its integration into the industrial production of:

– Pharmaceutical formulations;

– Dietary supplements;

– Therapeutic food additives.

The utilization of this biomass, which is frequently discarded as waste, could lead to the development of novel health-promoting products with substantial bioactive potential.

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