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Original article

A novel source of biologically active compounds – The leaves of Serbian herbaceous peonies

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ABSTRACT

In order to gain further insight into how various extraction techniques (maceration, microwave-, and ultrasound-assisted extractions) affect the chemical profile and biological activities of leaf extracts from *Paeonia tenuifolia* L., *Paeonia peregrina* Mill., and *Paeonia officinalis* L., this research was performed. The targeted chemical characterization of the extracts was achieved using the Ultra-High-Performance-Liquid-Chromatography-Linear-Trap-Mass-Spectrometry OrbiTrap instrumental technique, while Fourier Transform Infrared Spectroscopy was conducted to investigate the structural properties of the examined leaf extracts. According to the results, the species *P. officinalis,* Božurna locality as the origin of the plant material, and microwave-assisted extraction produced the maximum polyphenol yield, (491.9 ± 2.7 mg gallic acid equivalent (GAE)/mL).

The ethanolic extracts exhibited moderate antioxidant activity as evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) and phosphomolybdenum tests. With MIC values of 0.125 mg/mL, the leaf extracts produced by ultrasound-assisted extraction and maceration (Deliblato sands and Bogovo gumno) had the best antibacterial activity against *Pseudomonas aeruginosa* and *Salmonella* Typhimurium. Ultrasound-assisted extraction has proven to produce the most effective antimicrobial agents. Inhibitory potential towards glucosidase, amylase, cholinesterases, and tyrosinase was evaluated in enzyme inhibition assays and molecular docking simulations. Results show that leaves of *P. tenuifolia* L. obtained by ultrasound-assisted extraction had the highest acetylcholinesterase and butyrylcholinesterase inhibitory activity. Namely, the complexity of the polyphenol structures, the extraction method, the used locality, and the different mechanisms of the reactions between bioactives from leaf extracts and other components (free radicals, microorganisms, and enzymes) are the main factors that influence the results of the antioxidant tests, as well as the antibacterial and enzyme-inhibitory activities of the extracts. Hydroxymethyl-phenyl pentosyl-hexoside and acetyl-hydroxyphenyl-hexoside were the first time identified in the leaf extract of the *Paeonia* species. Due to their proven biological activities and the confirmed existence of bioactive compounds, leaf extracts may find use in foodstuffs, functional foods, and pharmaceutical products.

1. Introduction

Out of all peonies, only herbaceous ones spontaneously grow on the territory of the Republic of Serbia. Five native taxa were recently confirmed after assessments of their populations: *Paeonia tenuifolia* L., *Paeonia peregrina* Mill., *Paeonia officinalis* L., *Paeonia daurica* Andrews, and *Paeonia banatica* Rochel (Marković et al., 2022). All of them are protected by the Law on Nature Protection (Official Gazette of RS), and their collection from nature requires the permission of the Ministry of Environmental Protection of the Republic of Serbia. With the exception

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of *P. banatica* (which is relict and endemic in Serbia) and *P. daurica* (which is very scarce in Serbia), the remaining three taxa have a wider geographic distribution (Lazarević et al., 2012).

P. tenuifolia, also known as the steppe peony or fern-leaf peony, is native along the entire region of the Carpathian-Balkan massif, including the territories of all countries in Eastern Europe (Suleymanova et al., 2019). In Serbia, it spontaneously grows in steppes, rocky fields, dry meadows, and pastures, most abundantly in Deliblato sands, Southern Banat (the south-western slopes of the Carpathian Mountains), but could also be found on the slopes of Stara Planina Mountain and Devica Mountain (Lazarević et al., 2012; Čutović et al., 2022). Also, the extracts of *P. tenuifolia* proved to be rich in polyphenol compounds (phenolic acids, flavonoids, anthocyanins, tannins, and terpene derivatives), which possess different biological activities such as antimicrobial, antioxidant, and wound healing properties (Čutović et al., 2022).

P. peregrina, also known as the Balkan peony, is considered the most widespread in Serbia (Marković et al., 2023), particularly in Kosovo and Metohija, and eastern rural parts of Serbia (Krivi vir, Skrobnica, Golina, and Pirot). It prefers deciduous broad-leaved woods, pine, or mixed forests. So far, its flowers and roots have been proven to have many beneficial effects, including anticonvulsant, anxiolytic, hypoglycaemic, anti-inflammatory, antiosteoporosis (Nikolova and Ivanovska, 2000; Orhan et al., 2010), anticoagulant, analgesic, and sedative activities (Ivancheva et al., 2006; Li et al., 2021).

P. officinalis is also known as European, garden, or common peony and is native to mainly mountainous areas of southern Europe. In Serbia, it can be found on Tara Mountain, where it spontaneously grows along forest edges and in meadows (Marković et al., 2023). In addition to its ornamental value, this plant has been primarily used for medicinal purposes, as an antiepileptic and antispasmodic agent, while its flowers have been used to prepare cough syrup (Dienaitė et al., 2019; Lieutaghi, 2009). As the leaves have been proven to be a rich source of antioxidants with great potential for disease prevention, they could be used as a valuable ingredient in many medicine, cosmetic, and food products (Dienaite et al., 2019). They are also used in traditional Chinese medicine to detoxify and promote blood circulation (Lieutaghi, 2009).

Since ancient times, herbaceous peonies have been used for ornamental purposes (Kamenetsky-Goldstein and Yu, 2022; Zhang et al., 2023), although studies are focusing on their edible (Arya et al., 2014; Wang et al., 2023) and medicinal potential (Fernandes et al., 2017). Apart from several biologically active molecules found in flowers, roots, and stems (Ahmad and Tabassum, 2013; Li et al., 2021; Wang et al., 2020), the leaves have not received much attention as a potential source of bioactives, although they make a significant contribution to the total biomass (Dienaitė et al., 2019).

Recent studies related to the wild herbaceous peonies identified polyphenols and their derivatives (flavonoids, anthocyanins, and tannins) as the main constituents responsible for the different biological activities, referring to antioxidant, antimicrobial, and anti-inflammatory potential (Čutović et al., 2022; Dienaitė et al., 2019; Marković et al., 2023). The petals of *P. tenuifolia* show skin-beneficial qualities, as well as the possibility of inhibiting the growth of some skin-surface microflora (*Staphylococcus lugdensis*, *Candida albicans*, etc.) (Čutović et al., 2022). Also, the petals of *P. peregrina* possess strong antimicrobial activity against *S. lugdensis, Pseudomonas vulgaris*, and *Staphylococcus aureus* (Marković et al., 2023). Earlier studies show that the roots of *P. peregrina* modulated the antibody response against T-dependent antigen and augmented host resistance against *Klebsiella pneumoniae* infection (Nikolova and Ivanovska, 2000). On the other hand, Dienaitė et al. (2019) show that leaf extracts of *P. officinalis* are stronger antioxidants than root extracts, while methyl alcohol was more effective than water in the extraction of bioactive compounds. A long time ago, it was discovered that the leaves of *P. tenuifolia* are the sources of flavonoid derivatives, concretely quercetin 3-*O*-β-D-galactopyranoside and quercetin 3-*O*-β-D-glucopyranoside (Stošić and Gorunović, 1989). To the best of the authors' knowledge, no recent study has been done on the chemical makeup and content of secondary plant metabolites identified in the leaves of any wild species of herbaceous peonies found in Serbia.

Extracts are one of the forms that make the use of herbal drugs more convenient in the modern world. To produce them, an efficient extraction technique should be employed that captures and preserves all the important ingredients while retaining all their beneficial properties (Batinić et al., 2022; Oancea et al., 2021).

Since the chemical profile and biological activity of the leaf extract are dependent on the extraction method applied, starting alcoholic extracts in this investigation are obtained by using maceration, ultrasound-, and microwave-assisted extractions under conditions defined elsewhere (Batinić et al., 2023). The final goal of this research was to determine the biological activities (antioxidant, antimicrobial, and anti-enzymatic potential) and chemical fingerprint of the leaves of wild *P. tenuifolia, P. peregrina*, and *P. officinalis* from different localities in Serbia, using various analytical techniques (ultraviolet–visible spectroscopy – UV–Vis, Ultra-High-Performance-Liquid-Chromatography-Linear-Trap-Mass-Spectrometry OrbiTrap – UHPLC-LTQ-OrbiTrap MS, and Fourier Transform Infrared Spectroscopy – FTIR), and to estimate the possibility of their application in the food and pharmaceutical industries. Due to the extensive research, this study also aimed to identify the best plant resources for further cultivation, which would allow interested companies to get a steady supply of standardized-quality herbal raw material, the leaves of herbaceous peonies. In addition, previous investigations show that photosynthetic organs – leaves of herbaceous peonies are stronger antioxidants than other vegetative organs – roots, and have stronger potential against α-amylase inhibitors (Dienaitė et al., 2019).

Based on the above-stated background and the past utilization of peony leaves in folk medicine, this study aimed to assess the various biological activities of leaf extract from three different wild peonies. Also, it is generally known that leaves represent a big part of waste biomass, so their processing would contribute to more complete waste management and biodiversity preservation. Lastly, due to the influence of different habitats on the secondary metabolism of the plant, *i.e.*, the production of bioactive compounds, it is necessary to examine whether there are differences between the same species in different localities/habitats.

2. Materials and methods

2.1. Plant material

The fresh leaves of *P. tenuifolia* (LPT), *P. peregrina* (LPP), and *P. officinalis* (LPO) were collected from eight different localities in Serbia (Table 1). The plants were collected in their full flowering phenophase, in May 2022. As all herbaceous peonies in Serbia are protected by law, their collection was carried out with the permission of the Ministry of Environmental Protection of the Republic of Serbia (353–01-1467/2021–04, issued on May 26, 2021, and 353–01-162/2022–04, issued on February 24, 2022). Following collection, voucher specimens of these

Table 1
Information about plant material (leaves, L).

Acronym	Species	Locality
LPT	*Paeonia tenuifolia* L.	Gulenovci Deliblato sands Pančevo
LPP	*Paeonia peregrina* Mill.	Bogovo gumno Krivi vir Pirot
LPO	*Paeonia officinalis* L.	Pančevo Bogovo gumno Rujevica Božurna

protected plant species were confirmed and deposited in the Herbarium BUNS at the Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Serbia. Prior to extraction, the harvested plant material was left to dry on air at 21 °C for three weeks. According to European Pharmacopoeia, dried leaves were ground in the laboratory mill (M−20, IKA Universal mill, IKA®-Werke GmbH & Co. KG, Staufen, Germany), and they were further separated by analytical sieving technique to obtain a particle size of 0.75 mm for further extraction procedures (Ćujić et al., 2016).

2.2. Standards and reagents

Ethyl alcohol (Zorka Pharma, Serbia, 96 %, v/v) and deionized water, as a mixture, were used as the extraction solvent. Distilled water was purified through a Simplicity® UV water purification system (Merck Millipore, Germany). Folin-Ciocalteu's phenol reagent (2 N, ACS), potassium(III) hexacyanoferrate (≥99 %, ACS), gallic acid (97.5–102.5, p.a.), catechin monohydrate (96.0 %, p.a.), aluminum(III) chloride (98 %, RG), sodium hydroxide (98 %, AG), sodium nitrite (97 %, ACS), and sodium hydrogen carbonate (≥99.7 %, ACS) were commercially purchased from Sigma Aldrich, Germany. Tannic acid (99 %, ACS), sulfuric acid (99 %, AG), hydrochloric acid (38 %, w/w, AG), D-(+)-glucose (99.5 %, GC), phenol (99.0–100.5 %, p.a.), copper(II) sulfate (98 %, p.a.), potassium sodium tartrate (≥99.5 %, p.a.), DPPH (2,2-Diphenyl-1-picrylhydrazyl, ACS), sodium hydrogen phosphate monohydrate (98 %, p.a.), and ammonium molybdite tetrahydrate (81.0–83.0 %, AG, ACS) were purchased from Fisher Scientific, United States. Bovine serum albumin (≥98 %, p.a.) was obtained from Merck, Germany. Acetonitrile (99.9+%, HPLC GG) grade was purchased from Chem-Lab NV, Belgium. L(+)-ascorbic acid (>99 %, ACS) was purchased from Carlo Erba Reagents GmbH, Germany. Butyl alcohol (>99 %, GC) was obtained from Fluka AG, Switzerland. l-3,4-dihydroxyphenylalanine (>98 % TLC), 5,5′-dithiobis(2-nitrobenzoic acid); acetylcholinesterase from *Electrophorus electricus* (electric eel), Type-VI-S, EC 3.1.1.7, butyrylcholinesterase from equine serum, EC 3.1.1.8, acetylthiocholine iodide (AS, >99 %), butyrylthiocholine chloride, kojic acid (AS, >99 %), alpha-amylase solution (ex-porcine pancreas, EC 3.2.1.1), acarbose (>95 %), *p*-iodonitrotetrazoliumviolet (>95 %), alpha-glucosidase solution (from *Saccharomyces cerevisiae*, EC 3.2.1.20), Lugol reagents (diluted iodine-potassium iodide solution), formic acid (98–100 %, HPLC grade), and Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, 97 %, a.g.) were obtained by Sigma Aldrich, USA.

2.3. Extraction methods

2.3.1. Microwave-assisted extraction (MAE)

The leaf extracts of all three herbaceous peony species were obtained using microwave-assisted extraction equipment (Milestone ETHOS X, Milestone, Italy), equipped with a 2.45 GHz reactor with an infrared temperature sensor that monitors the process temperature and 2 magnetrons achieving a maximum operative power of 1.8 kW (0.9 kW x 2). The method was previously described by Batinić et al. (2023). All the experimental tests were conducted at the normal atmospheric pressure in the SR-15 rotor segment containing a high-density polypropylene mold with a modified poly(tetrafluoroethylene) Teflon vessel (0.1 l), cover, and stirrer bar (Ø12x30 mm). The irradiation stages were separated by the time needed to reach a predetermined process temperature, and experimental runs were carried out in three steps (cycles). Ethyl alcohol concentration of 50 %, time of extraction of 2 min, solid-to-solvent ratio of 1:10, and temperature of 100 °C were employed in the MAE operation. The samples were filtered using a laboratory glass funnel and 200 nm quantitative filter paper. After the filtration, the permeate was collected in a dark glass bottle and stored at 0–4 °C until analysis. The schematic illustration of the experiment is presented in Fig. 1.

2.3.2. Ultrasound-assisted extraction (UAE)

UAE was performed using the concentration of ethyl alcohol of 50 %, extraction time of 30 min, and solid-to-solvent ratio of 1:10. The method was previously described by Batinić et al. (2023). The extraction process was performed in the ultrasound bath (Digital ultrasound bath, DU-32, ARGO LAB, Italy) with a frequency of 35 kHz at a temperature of 21 °C, which was maintained by adding ice in a small portion during the extraction process. The extracts were filtered through the 200 nm quantitative filter paper and stored at 0–4 °C until analysis.

2.3.3. Maceration (MAC)

MAC was carried out using a Tube Roller Mixer (Stuart SRT6,

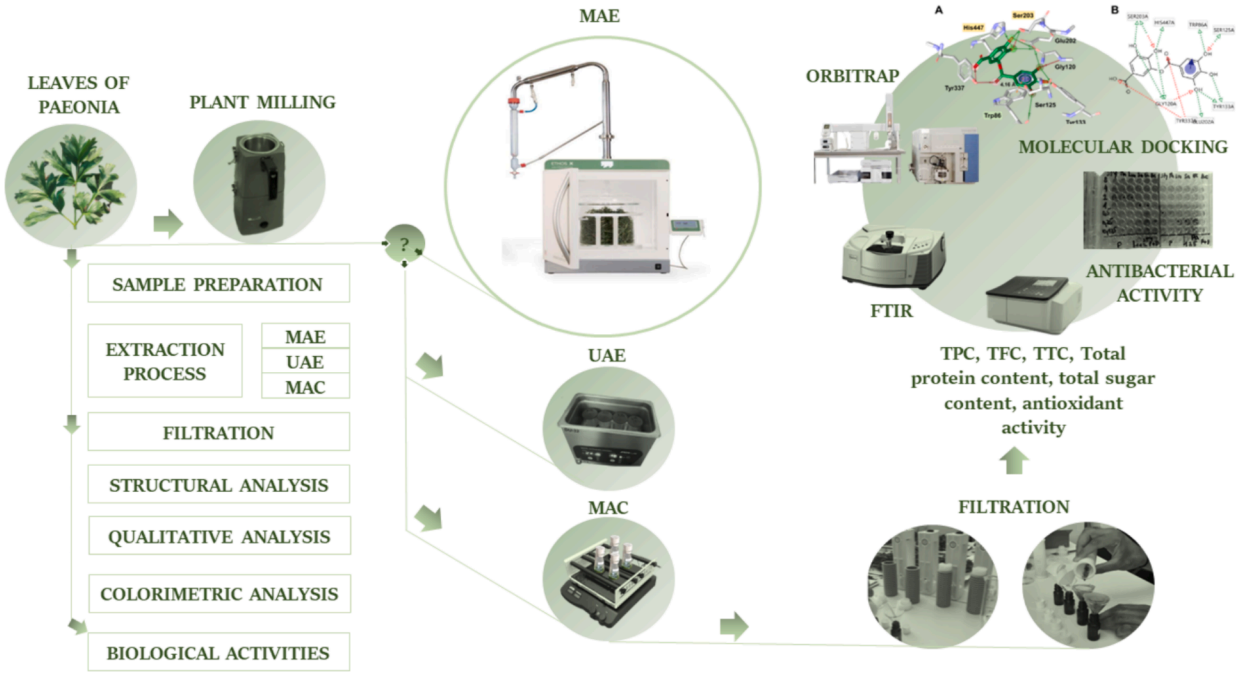

Fig. 1. The schematic illustration of the experimental procedure.

Germany) at a temperature of 21 °C. MAC was performed using a 50 % concentration of ethyl alcohol, an extraction time of 30 min, and a solid-to-solvent ratio of 1:10. Batinić et al. (2023) provided an earlier description of the experimental procedure. The extracts were filtered through the 200 nm quantitative filter paper and stored at 0–4 °C until analysis.

2.4. Chemical analysis of the extracts performed by UV–Vis spectrophotometry

2.4.1. Total polyphenol content (TPC)

TPC in the leaf extracts of all herbaceous peonies was performed by the Folin-Ciocalteu method with some modifications described by Čutović et al. (2022). In short, 0.02 mL of the diluted plant extract (extract: extraction medium = 1:1) was mixed with 0.1 mL of previously diluted Folin-Ciocalteu phenol reagent with deionized water (1:2), after which 0.3 mL of sodium hydrogen carbonate (20 %, w/v) and 1.5 mL of deionized water were added to the working solution. After 120 min of incubation at room temperature (21 °C), the absorbance (λ_{max} = 765 nm) was measured using a UV–Vis spectrophotometer (UV/Vis 1800, Shimadzu, Japan). An analytical standard of gallic acid (GA) was used for the construction of a calibration curve ranging from 100–1600 mg/L ($A = 8{\cdot}10^{-4}c–0.0317$; $r^2 = 0.9845$). The results of the experiment were expressed as milligramms of gallic acid equivalents per mL of the extract (mg GAE/mL).

2.4.2. Total flavonoid content (TFC)

The modified aluminum(III) chloride colorimetric method of Shraim et al. (2021) was used to estimate the TFC in all studied extracts. In brief, 0.25 mL of the properly diluted extract and 0.75 mL of sodium nitrite (5 %, w/v) were combined with 1.25 mL of deionized water. The working solution was incubated in the dark for 6 min at 21 °C. The mixture was then treated with 0.15 mL of aluminum(III) chloride (10 %, w/v), and 0.5 mL of sodium hydroxide solution (1 mol/L) before being topped off with deionized water to a final volume of 3 mL. Then, the working solution was vortexed (Vortex 1, IKA®, Germany), and the tube containing the sample was kept in the dark for 30 min before the UV–Vis examination. The absorbance of the samples was evaluated at λ_{max} = 510 nm. Catechin monohydrate, CA (0.037–0.3 mg/L), was used as a standard for making a calibration curve ($A = 6{\cdot}10^{-4}c + 0.0544$; $r^2 = 0.9917$). The results are given as mg of catechin monohydrate equivalents per mL of the extract (mg CAE/mL).

2.4.3. Total tannin content (TTC)

TTC in all studied extracts was determined using the colorimetric method described by Balaky et al. (2021). The extraction solution was prepared by mixing 50 mg of iron(III) sulfate, 95 mL of butyl alcohol, and 5 mL of hydrochloric acid (38 %, w/w). For determining the TTC (in the form of a condensed derivative), 10 mg of dry plant material in a glass test tube and 10 mL of the extraction solution were added and placed in a water bath for heating for 60 min at 80 °C. The extraction solution was filtered through cellulose acetate filter paper (0.22 mm) and processed spectrophotometrically. The absorbance was measured at λ_{max} = 580 nm. The results of the analysis were expressed as milligrams of tannic acid equivalents per millilitre of the extract (mg TAE/mL). The calibration curve was plotted by dissolving the standard of tannic acid in deionized water in five different concentrations (1, 2, 4, 8, and 16 mg/mL). Equation of the calibration curve: $A = 0.0414c–0.0148$; $r^2 = 0.9987$.

2.4.4. Total protein content

This experiment is based on the measurement of protein content in the extracts with the Folin-Ciocalteu phenol reagent after alkali copper treatment, first described by Waterborg (2009). In short, the experimental protocol was as follows: 1 mL of the appropriately diluted extract (1 mg/mL) was mixed with 2 mL of sodium hydroxide (0.1 mol/L) containing sodium carbonate (2 %, w/v), copper(II) sulphate (0.01 %, w/v), and potassium sodium tartrate (0.02 %, w/v). After 10 min, 0.2 mL of previously diluted Folin-Ciocalteu phenol reagent with deionized water (1:3) was added and homogenized using a vortex mixer for 30 s. The working solution was kept in the dark for 30 min at 21 °C after which the absorbance was measured at λ_{max} = 750 nm. Bovine serum albumin (BSA) was used as a standard for the construction of the standard curve ($A=(c + 0.072)/0.5573$), $r^2 = 0.9997$) in the range of concentration between o.o6 and 1 mg/mL. The results are given as total protein content in the extract, expressed in milligrams of protein per mL of the extract (mg/mL).

2.4.5. Total sugar content

The rapid and sensitive method of colorimetric determination was used for the content of soluble sugars in all studied leaf extracts (Abidi et al., 2011). In brief, the experimental test was performed as follows: 0.2 mL of the extracts (0.2 mg/mL) were diluted with deionized water (1:1), and the samples were further enriched with 0.4 mL of phenol (5 %, w/v), and 2 mL of concentrated sulphuric acid (96 %, w/w). Then, the extracts were degraded by slowly adding sulphuric acid along the vessel's walls while simultaneously cooling them in the ice bath. The aqueous solution of D-(+)-glucose (0.01–0.12 mg/mL) was used for the creation of a calibration curve ($A = 4.9923c-0.0028$; $r^2 = 0.9991$). The results were expressed as milligrams of soluble sugars per mL of the extract (mg/mL).

2.5. UHPLC-LTQ-OrbiTrap MS

An LTQ OrbiTrap XL mass spectrometer connected to an Accela 600 UHPLC system operating in positive and negative ionization mode (heated electrospray ionization or HESI) was utilized for LC/MS analysis (Thermo Fisher Scientific, Bremen, Germany). A Syncronis C18 analytical column (50 2.1 mm, 1.7 m particle size) was utilized for the separation process. Previous reports on MS parameters and UHPLC settings are reported in Čutović et al. (2022). ChemDraw (Version 12.0, CambridgeSoft, USA) was used for structure drawing. With the use of the information in the published literature, the deprotonated molecule mass $[M-H]^-$, and the MS^2, MS^3, and MS^4 fragmentation behaviour were used to identify the chemical compounds in the extracts.

For instrument control and data processing, Xcalibur software (version 2.1, Thermo Fisher Scientific, Waltham, MA, USA) was utilized. A sophisticated LC/MS technique (UHPLC-LTQ-OrbiTrap-MS) was used to evaluate the chemical composition of the ethanolic extracts of the leaves. Some of the compounds for which no standards were available were tentatively identified using previously reported MS fragmentation data (Waterhouse, 2002).

2.6. Fourier transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR)

FTIR spectra of the extracts were performed to identify the present characteristic functional groups. The spectra were recorded using a Nicolet™ iS™ IR-spectrometer equipped with a smart iTX™ accessory (Thermo Fischer Scientific, USA) in the wavenumber range between 4000–400 cm^{-1}, and a resolution of 4 cm^{-1} at 25 ± 5 °C. The analysis was conducted on dry leaf extracts (Figure S1, Supplementary material), previously prepared using a Rotary vacuum evaporator (Rotavapor Heidolph 4001-efficient, Heidolph Instruments, Germany) under pressure of 0.25 bar at 25 ± 5 °C. Instrumental analysis was performed by fixing a small amount of the extract (20–30 mg) to the metal chassis of the IR-spectrometer equipped with diamond crystal. FTIR spectra were obtained by OMNIC™ series software (Thermo Fischer Scientific, USA). The graphical view of results was generated by ORIGIN™ (Version 9.0, OriginLab Corporation, USA).

2.7. Antioxidant activity

2.7.1. DPPH radical scavenging activity (DPPH)

The radical scavenging capacity measured by the DPPH method (RSC_{DPPH}) used in this experiment was previously described by Čutović et al. (2022). The test solution is based on mixing 2.8 mL of ethanolic DPPH radical working solution (0.028 mg/mL) with 0.2 mL of extract. All examined aliquots of test solutions were kept in the dark for 13 min and processed spectrophotometrically at $\lambda_{max} = 517$ nm. The control probe consists of 2.8 mL of the DPPH test solution and 0.2 mL of solvent, which was used for the extraction (ethyl alcohol, 50 % (v/v)). The RSC_{DPPH} was calculated using the formula presented in Equation (1):

$$RSC_{DPPH}, \% = [(A_C - A_S)/A_C] \times 100 \quad (1)$$

where A_C was the absorbance of the control solution, while A_S was the absorbance of the test solution treated with the ethanolic DPPH radical working solution. The standard curve was constructed using a Trolox solution (0.031–1.0 mg/mL), and the analysis results are presented as millimoles of Trolox equivalents per mL of the extract (mmol TE/mL).

2.7.2. Total antioxidant capacity assay (TAC)

Aliquots of 0.1 mL of the studied leaf extracts were mixed in an Eppendorf plastic tube with 1 mL of the main reagent solution containing the mixture of sulfuric acid (0.6 mol/L), sodium phosphate (0.028 mol/L), and ammonium molybdate ($4{\cdot}10^{-3}$ mol/L) (Milošević et al., 2020). The tubes were capped, wrapped up with parafilm, and incubated in a digital shaker (Thermo Fischer Scientific, China) at 95 °C for 90 min. The samples were cooled to 25 °C, and the absorbance was measured at $\lambda_{max} = 695$ nm. The calibration curve was plotted using a solution of L-ascorbic acid (0.2–5.7 mmol/L). The equation of the calibration curve was A = 0.0414c-0.0148; $r^2 = 0.9987$. The results are expressed as millimole of L-ascorbic acid equivalents per milliliter of the extract (mmol LAE/mL).

2.8. Antibacterial activity

Antibacterial activity was performed using dry leaf extracts dissolved in ethyl alcohol (30 %, v/v) before analysis. For the evaluation of their antibacterial activities, six bacterial strains gained from the Collection of the Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, were tested: three Gram-positive (*Listeria monocytogenes* NCTC 7973, *Staphylococcus aureus* ATCC 11633, and *Bacillus cereus* human isolate), and three Gram-negative bacteria (*Salmonella* Typhimurium ATC 13311, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922). Fresh overnight cultures of all bacteria were adjusted with sterile saline to a concentration of 1.0×10^6 CFU (colony-forming unit) per well.

The experimental procedure used in this study was the microdilution method in 96-well microtiter polystyrene plates (Čutović et al., 2022), and the obtained minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were expressed in mg/mL. The MIC and MBC values were determined by serial subcultivations of 0.01 mL of extract into microtiter plates containing 0.1 mL of broth per well and further incubation for 24 h at 37 °C; the values were detected following the addition of 0.04 mL of *p*-iodonitrotetrazolium violet (0.2 mg/mL) and incubation at 37 °C for 30 min, as previously described by Nikolić et al. (2014).

2.9. Enzyme-inhibitory activity

Dry leaf extracts, dissolved in ethyl alcohol (30 %, v/v), were used for testing enzyme-inhibitory activity.

2.9.1. Assay of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity

The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity of the extracts was accessed following instructions given by Uysal et al. (2017). In short, 0.025 mL of working solution of the leaf extract (1 mg/mL) was mixed with 0.125 mL of 5,5′-dithiobis(2-nitrobenzoic acid) (3×10^{-3} mol/L) and 0.025 mL of AChE (0.265 u/mL) or BchE (0.026 u/mL) in Tris-Hydrochloride buffer solution (1 mol/L, pH 8.0) in a 96-well microplate and incubated for 30 min at 25 °C. The enzymatic reaction was initiated with the addition of 0.025 mL of acetylthiocholine iodide or butyrylthiocholine chloride substrate. Similarly, a control probe was prepared by adding a sample solution to all reagents without the enzyme. The sample and control absorbances were recorded at $\lambda_{max} = 405$ nm after 10 min of incubation at 25 °C. The cholinesterase inhibitory activity was quantified as milligrams of galanthamine equivalents per gram of dried extract (mg GALAE/g) by subtracting the absorbance of the control probe from that of the sample.

2.9.2. Alpha-amylase inhibitory activity assay

The α-amylase inhibitory activity assay was carried out as follows: 0.05 mL of working solution of the leaf extract (1 mg/mL) was mixed with 0.05 mL of α-amylase solution (*ex*-porcine pancreas, 10 u/mL) in phosphate buffer solution ($6{\cdot}10^{-3}$ mol/L) in a 96-well microplate and incubated for 10 min at 37 °C (Savran et al., 2016). The enzymatic reaction was initiated with the addition of 0.05 mL of the starch solution (0.05 %, w/v). A control probe was prepared by adding the working solution to all reagents without α-amylase. Then, the reaction mixture was thermostated for 10 min at 37 °C. After that, 0.025 mL of hydrochloric acid (1 mol/L) and 0.1 mL of Lugol reagents (diluted iodine-potassium iodide solution) were added to entire the process. The absorbances of the sample and control probe were read at $\lambda_{max} = 630$ nm; the absorbance of the control was subtracted from that of the working solution, and the α-amylase inhibitory activity was expressed as millimoles of acarbose equivalents per gram of dried extract (mmol ACAE/g).

2.9.3. Alpha-glucosidase inhibitory activity assay

The α-glucosidase inhibitory activity assay was conducted as follows: 0.05 mL of the working solution of the extract (1 mg/mL) was mixed with a mixture containing 0.05 mL of glutathione (0.5 mg/mL) and 0.05 mL of α-glucosidase solution (0.2 u/mL) in phosphate buffer solution (1 mol/L, pH 6.8) in a 96-well microplate and incubated for 15 min at 37 °C (Llorent-Martínez et al., 2016). A control probe solution was prepared by adding a working solution of the extract to all reaction reagents without adding α-glucosidase. The enzymatic reaction was then ended with the addition of 0.05 mL of disodium carbonate (0.2 mol/L). The absorbances of the sample and control probe were read at $\lambda_{max} = 400$ nm. The alpha-glucosidase inhibitory activity was expressed as millimoles of acarbose equivalents per gram of dried extract (mmol ACAE/g).

2.9.4. Tyrosinase inhibitory activity assay

The tyrosinase inhibitory activity assay was carried out according to Mocan et al. (2017). In short, 0.025 mL of the working solution of the extract (1 mg/mL) was mixed with 0.04 mL of tyrosinase solution (200 u/mL) and 0.1 mL of phosphate buffer (0.04 mol/L, pH 6.8) in a 96-well microplate and incubated for 15 min at 25 °C. The enzymatic reaction was initiated with the addition of 0.04 mL of L-DOPA (L-3,4-dihydroxyphenylalanine). A control probe was prepared by adding the tested solution to all reagents without tyrosinase. The absorbances of the sample and control probe were read at $\lambda_{max} = 492$ nm after 10 min of incubation at 25 °C. Tyrosinase inhibitory activity was determined as milligrams of kojic acid equivalents per gram of dried extract (mg KAE/g) by subtracting the absorbance of the control from that of the sample.

2.10. Molecular docking

The initial 3D structures of proteins were retrieved from the Protein

Databank (PDB). For BChE, its complex with the nanomolar and selective inhibitor N-((1-(2,3-dihydro-1H-inden-2-yl)piperidine-3-yl) methyl)-N-(2-methoxyethyl)-2-naphthamide was used (ID: 4TPK) (Brus et al., 2014). The complex of human AChE with hyperzine was employed for this enzyme (ID: 4EY5) (Cheung et al., 2012). In the case of amylase and glucosidase, their complexes with acarbose (IDs: 1OSE and 5NN8, respectively) were utilized (Gilles et al., 1996). Finally, for tyrosinase, its complex with kojic acid (ID: 3NQ1) was used (Sendovski et al., 2011). The ionization state of the protein was set to resemble pH 7.40 using PROPKA (Olsson et al., 2011). Prior to docking, water, and co-crystallized ligands were removed to create binding space for the investigated natural products. Vega ZZ 3.2.2. was used as a graphical user interface (GUI) (Pedretti et al., 2021).

The 2D structures of natural products identified by HRMS/MS[4] were downloaded from PubChem, and their starting conformations were generated using the MMFF94s force field (Halgren, 1999). Subsequently, their final geometries were optimized using the semiempirical PM7 method (Stewart, 2013) implemented in MOPAC2016 (Stewart, 1990), along with the COSMO solvation model of water. The final structures of all ligands were stored in the database in sdf format.

For molecular docking, all residues within 10 Å of the co-crystallized ligand were selected for active site definition. AutoDock Vina 1.1 was employed for molecular docking (Trott and Olson, 2010). The virtual screening module within Vega ZZ 3.2.2 was used to screen the binding affinities of the entire natural product database against each of the five enzymes. The exhaustiveness was set to 25, and five binding modes were saved for each ligand.

3. Results and discussion

3.1. UV–Vis chemical characterization

3.1.1. Determination of TPC and TFC values

The results of TPC and TFC for all prepared *Paeonia* leaf extracts are presented in Table 2. Both the extraction process and the origin of the plant material had a significant impact on the TPC values, which were in the range of 205.56 to 491.90 mg GAE/mL. When MAC was employed, the highest TPC was for the LPO (Rujevica) extracts (480.00 mg GAE/mL), whereas for MAE, it was LPO (Božurna), with a TPC value of 491.90 mg GAE/mL, and the TPC of the mentioned extracts was significantly higher in comparison to the remaining ones from both mentioned methods. The lowest TPC value was obtained by using the UAE method (205.6 mg GAE/mL, LPP from Pirot). Regarding the TFC values, they were in the range of 51.85 to 224.35 mg CAE/mL. The highest TFC value was obtained with MAC in the LPP (Pančevo) extract (224.35 ± 3.35 CAE/mL), but it can also be seen that the LPP extracts had the highest TFC values, compared to the remaining two *Paeonia* species leaves, in all employed extraction methods.

The statistical analysis using one-way ANOVA, Duncan's *post hoc* test, and experimental design (2^3 full factorial design) was carried out to study the statistical significance of the factors' influence, the interactions between the factors, as well as the combination of the factors for achieving the highest TPC.

Preliminary screening of factor levels (one-way ANOVA and Duncan's post hoc test). The selection of the levels of each factor (plant species, locality, and extraction procedure) that have a significant influence on the polyphenol content was carried out, and the results are presented in the supplementary material (Figures S2–S4, Table S1). Statistical significance between factor levels has been estimated on triplicate samples through a one-way analysis of variance, followed by Duncan's *post hoc* test at $p < 0.05$ level. Values followed by different letters in the graphs differ significantly, based on Duncan's test at $p < 0.05$ level. Selected two levels of each factor with the highest TPC were included in future experimental design, *i.e.*, 2^3 full factorial design.

Plant species is an important factor that significantly influenced the polyphenol yield in all tested extracts. As can be seen, *P. officinalis*

Table 2
Total polyphenol (TPC) and total flavonoid contents (TFC) of the leaf extracts of *Paeonia tenuifolia* (LPT), *Paeonia peregrina* (LPP), and *Paeonia officinalis* (LPO) from different localities, obtained by different extraction methods.

Plant species (leaf extract)	Locality	Extraction method	*TPC [mg GAE/mL]	**TFC [mg CAE/mL]
Paeonia tenuifolia (LPT)	Gulenovci	UAE	259.40 ±1.46 ^{f}	165.20 ±5.80 ^{fg}
	Deliblato sands		231.90 ±2.85 ^{i}	97.70±3.30 ^{mn}
	Pančevo		242.55 ±5.30 ^{gh}	126.05 ±6.65 ^{jk}
	Bogovo gumno		237.50 ±3.61 ^{hi}	91.90±7.50 ^{no}
	Gulenovci	MAC	238.15 ±4.46 ^{hi}	73.55±4.15 ^{p}
	Deliblato sands		221.90 ±2.06 ^{j}	102.70 ±1.70 ^{lm}
	Pančevo		234.40 ±7.92 ^{hi}	171.05 ±3.35 ^{f}
	Bogovo gumno		247.55 ±5.05 ^{g}	160.20 ±2.50 ^{g}
	Gulenovci	MAE	230.65 ±3.62 ^{i}	86.85±4.15 ^{o}
	Deliblato sands		245.65 ±9.69 ^{g}	77.70±3.30 ^{p}
	Pančevo		240.65 ±3.22 ^{gh}	51.85±5.85 ^{q}
	Bogovo gumno		246.90 ±6.22 ^{g}	129.35 ±3.35 ^{jk}
Paeonia peregrina (LPP)	Krivi vir	UAE	206.30 ±3.54 ^{k}	134.35 ±3.35 ^{j}
	Pirot		205.65 ±2.62 ^{k}	96.05±3.35 ^{n}
	Pančevo		230.00 ±3.54 ^{i}	155.20 ±4.20 ^{gh}
	Bogovo gumno		236.90±3.9 ^{hi}	127.70 ±5.00 ^{j}
	Krivi vir	MAC	231.90 ±2.69 ^{i}	101.00 ±5.00 ^{lm}
	Pirot		225.05 ±1.77 ^{j}	131.85 ±4.15 ^{jk}
	Pančevo		448.15 ±6.76 ^{d}	224.35 ±3.35 ^{a}
	Bogovo gumno		471.90 ±6.22 ^{bc}	216.05 ±3.35 ^{b}
	Krivi vir	MAE	449.40 ±2.69 ^{d}	122.70 ±5.00 ^{k}
	Pirot		469.40 ±4.38 ^{c}	203.50 ±2.50 ^{c}
	Pančevo		471.25 ±9.45 ^{bc}	172.70 ±6.70 ^{f}
	Bogovo gumno		470.00 ±7.11 ^{b}	186.05 ±1.65 ^{c}
Paeonia officinalis (LPO)	Rujevica	UAE	266.25 ±1.77 ^{c}	131.90 ±2.50 ^{j}
	Božurna		244.40 ±4.38 ^{g}	104.35 ±3.35 ^{lm}
	Rujevica	MAC	480.00 ±7.07 ^{b}	154.35 ±1.65 ^{h}
	Božurna		466.30 ±5.07 ^{c}	149.40 ±5.00 ^{h}
	Rujevica	MAE	475.05 ±1.77 ^{b}	195.20 ±5.80 ^{d}
	Božurna		491.90 ±2.69 ^{a}	141.90 ±1.85 ^{i}

GAE: gallic acid equivalent; CAE: catechin equivalent; values with the same letter ($^{a-p}$) in each column showed no statistically significant difference ($p > 0.05$, $n = 3$, the p-value is defined as the probability under the assumption of no effect or no difference (null hypothesis) of obtaining a result equal to or more extreme than what was observed; the n value is defined as the number of repetitions, one-way ANOVA, analysis of variance, Duncan's *post hoc* test); UAE: ultrasound-assisted extraction; MAC: maceration; MAE: microwave-assisted extraction.

provided the extracts with the highest TPC, followed by *P. peregrina* and *P. tenuifolia*. Since *P. tenuifolia* gave the extracts with the lowest polyphenol yield, this species was excluded for the future 2^3 full factorial design (Figure S2, Supplementary material). The impact of locality follows the trend: Rujevica and Božurna ≥ Bogovo gumno and Pančevo ≥ Krivi vir and Pirot ≥ Gulenovci and Deliblato (Figure S3, Supplementary material). Thus, Krivi vir and Pirot, as well as Gulenovci and Deliblato sands, were not included in future experimental design, due to lower TPC (applied to all mentioned localities) and belonging to a species that was previously excluded from the experimental design, *i.e.*, *P. tenuifolia* (applied to Gulenovci and Deliblato sands). Regarding the results of the influence of employed extraction techniques on the TP yield (Figure S4, Supplementary material), MAE gave the samples with significantly higher TPC, followed by MAC and UAE. Thus, the UAE was excluded for further full factorial design. Therefore, regarding the results of one-way ANOVA and Duncan's *post hoc* test and the highest polyphenol yield, the following two levels of each of three parameters were included in future experimental design: *P. peregrina* and *P. officinalis* (among the species), Pančevo and Bogovo gumno for *P. peregrina* and Rujevica and Božurna for *P. officinalis* (among the localities), and MAC and MAE (among the extraction procedures).

3.1.2. 2^3 full factorial design

The influence of three factors (species, locality, and extraction procedure) with two levels on the TPC was observed through absolute values of standardized estimated effects and presented on Pareto charts with the level of significance set at $p < 0.05$ for the factorial design (Fig. 2).

The effects and corresponding regression coefficients of factors and factor interactions are presented in Table S1, while observed and predicted values for the polyphenol yield, as a dependent variable, are presented in Table 3.

Selected plant species (variable number 1), extraction procedures (variable number 3), and localities (variable number 2), as well as all interactions, did not have a significant impact on the polyphenol yield (Fig. 2 and Table S1).

As can be seen in Table 3, the highest polyphenol yield was achieved under the following parameters: *P. officinalis* (species), Božurna (locality), and MAE (extraction method), 491.9 ± 2.7 mg GAE/mL (observed value). The model predicted the maximum polyphenol yield under the same conditions, 487.9 ± 5.4 mg GAE/mL (predicted value). The lowest polyphenol yield was measured under the following factors: *P. peregrina* (species), Pančevo (locality), and maceration (extraction technique), 448.1 ± 6.8 mg GAE/mL (observed value). The model predicted the minimal polyphenol concentration under the same conditions, 455.6 ± 4.2 mg GAE/mL (predicted value).

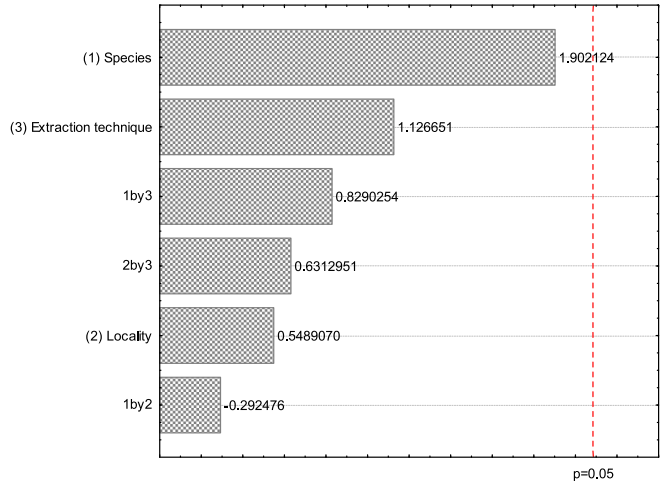

Fig. 2. Pareto charts of selected factors' and their interactions' influence (three factors on two levels) on the total polyphenol content (TPC) in leaf extracts of *Paeonia* species.

3.1.3. Total flavonoid content (TFC)

The results of TFC for the samples of LPT from Gulenovci, Deliblato sands, and Pančevo showed a significant failure of TFC values when MAE was used as the extraction method (Table 2, 86.85 ± 4.15 mg CAE/mL, 77.70 ± 3.30 CAE/mL, and 51.85 ± 5.85 mg CAE/mL, respectively). On the other hand, in the case of LPP and LPO, the values of TFC for the extracts obtained using the UAE and MAC revealed significantly lower values than those obtained using the MAE. Also, the samples of LPO obtained using the MAC method and a sample of LPP obtained using the MAE method show a low degree of correlation compared to the TPC counterparts. Considering the values of TFC for the samples of LPP and LPO obtained from the MAE method, it can be seen that the values of TFC are significantly higher due to the diversity of the main analyte and other external/internal influences, such as the mechanism of the extraction process, pressure, temperature, sensitivity of the extracted components, etc.

3.1.4. Total tannin content (TTC)

Tannins in condensed forms are derivatives of flavanols and are commonly used to stabilize emulsions to protect unsaturated lipids from oxidation (Figueroa-Espinoza et al., 2015). Also, tannins improve the stability of anthocyanin and make its anthocyanin solution darker (Zhu et al., 2006). The study by Bao et al. (2020) revealed that the accumulation of tannins in plants is crucial to the formation of a purplish red color in herbaceous peonies, which tannins delegate to the organic molecules that contribute to the ornamental potential.

In this study, TTC ranged from 0.018 to 0.026 mg TAE/mL; the highest value was observed in the extract of the LPT from Gulenovci (Table 4), however, there was no significant difference between all prepared extracts (except in the case of Deliblato sands). Namely, leaves from Deliblato sands provided the extract with the lowest TTP. To the best of the authors' knowledge, there are no contemporary publications on TTC in herbaceous peonies.

3.1.5. Total protein and total sugar content

The determination of the amount of total proteins in foods is important because it evaluates the economic value of the food product and can impact the economic feasibility of new industries for alternative protein production. Therefore, the type and quality of protein consumed by humans are important for health (Hayes, 2020). Colorimetric protein quantitation assays use UV or visible spectroscopy to rapidly determine the concentration of protein in the examined analyte, relative to a conventional standard (Shen, 2023). These methods are fast, reliable, and do not require the use of expensive equipment.

To the best of our knowledge, there are no previous data on total protein content in leaves of herbaceous peonies, only in petals (Weixing et al., 2017) and seeds of *P. lactiflora* (Ren et al., 2020). In this study, total protein content ranged from 3.13 ± 0.45 to 6.14 ± 0.51 mg/mL of leaf extract (Table 5). Therefore, the highest total protein contents were observed in the MAC extract of LPP from Pančevo, approximately 5.85 mg/mL, and the UAE extract of LPO from Rujevica, approximately 6.14 mg/mL. Significantly lower total protein content was observed in the LPP extract obtained by MAE (Krivi vir).

Total sugar content in this study ranged from 0.10 ± 0.02 to 0.69 ± 0.05 mg/mL (Table 5). The highest values, which did not differ between themselves, were observed in two LPT extracts by MAC (Pančevo and Bogovo gumno), LPP extract by MAC (Pančevo) and LPO extract by MAE (Rujevica). Thus, it can be seen that the MAC was the most efficient technique for protein extraction.

3.2. Qualitative analyses of the extracts

The chemical assembly of the leaves of the wild peonies that grow in

Table 3
Full factorial design (2^3 experimental design) for the screening of the parameters' impact on total polyphenol content (TPC) in *Paeonia peregrina* Mill. and *Paeonia officinalis* L. leaf extracts, with the observed and predicted values.

Plant species	Locality	Extraction technique	Plant species	Locality	Extraction technique	TPC [mg GAE*/mL]	
Design			*Factor levels*			*Observed*	*Predicted*
-1	-1	-1	*P. peregrina*	Pančevo	MAC	448.0±6.8	455.6±4.2
-1	1	-1	*P. peregrina*	Pančevo	MAE	471.1±9.5	464.8±5.3
1	-1	1	*P. peregrina*	Bogovo gumno	MAC	474.9±6.2	467.7±4.7
1	1	1	*P. peregrina*	Bogovo gumno	MAE	470.0±7.1	472.7±5.4
-1	-1	1	*P. officinalis*	Rujevica	MAC	480.0±7.1	473.9±6.6
-1	1	1	*P. officinalis*	Rujevica	MAE	475.0±1.8	480.1±5.2
1	-1	-1	*P. officinalis*	Božurna	MAC	466.3±5.1	471.9±5.4
1	1	-1	*P. officinalis*	Božurna	MAE	491.9±2.7	487.9±5.4

* GAE: gallic acid equivalent; MAC: maceration; MAE: microwave-assisted extraction.

Table 4
The total tannin content (TTC) of the leaf extracts of *Paeonia tenuifolia* L. (LPT), *Paeonia peregrina* Mill. (LPP), and *Paeonia officinalis* L. (LPO).

Plant species (leaf extract)	Locality	Extraction method/Solvent	TTC [mg TAE/mL]
Paeonia tenuifolia (LPT)	Gulenovci	Heat-assisted extraction/Solution of iron (III) sulfate in a mixture of butanol/HCl (95:5, w/v)	0.026 ±0.02 [a]
	Deliblato sands		0.018 ±0.01 [b]
	Pančevo		0.025 ±0.02 [a]
	Bogovo gumno		0.026 ±0.02 [a]
Paeonia peregrina (LPP)	Krivi vir		0.024 ±0.04 [a]
	Pirot		0.025 ±0.01 [a]
	Pančevo		0.026 ±0.02 [a]
	Bogovo gumno		0.023 ±0.02 [a]
Paeonia officinalis (LPO)	Rujevica		0.026 ±0.01 [a]
	Božurna		0.022 ±0.02 [a]

TAE: tannic acid; values with the same letter ([a-b]) in the column showed no statistically significant difference ($p > 0.05$, $n = 3$, the p-value is defined as the probability under the assumption of no effect or no difference (null hypothesis) of obtaining a result equal to or more extreme than what was observed; the n value is defined as the number of repetitions, one-way ANOVA, analysis of variance, Duncan's *post hoc* test).

the territory of the Republic of Serbia is summarised in Table 6. In the analyzed leaf extracts originating from different regions, 84 different organic molecules were identified. They were classified into four major groups, as follows: 1) Gallic acid derivates (compounds **1–44**) representing 75,3 to 75,8% of the total content of the examined extracts; 2) Flavonoids (compounds **45–66**) representing 11,71 to 13,2% of the total content of the examined extracts; 3) *Paeonia*-specific monoterpenoids (compounds **67–78**) representing 3,41 to 5,57 % of the total content of the examined extracts; and 4) Other compounds (compounds **79–84**) representing 4,28 to 7,24 % of the total content of the examined extracts.

Gallic acid derivates. The most varied organic molecules can be found in this class. Some of the identified compounds have the appropriate chromatographic distinctions and are adequately matched with the analytic standards of gallic acid **(3)**, ellagic acid **(20)**, cinnamic acid **(42)**, and *p*-coumaric acid **(43)**. Phenolic acids **2, 6, 9, 20, 42,** and **43** were identified in the different *Paeonia* varieties, while derivatives numbered as **1, 2, 5, 7, 8, 11, 12, 15, 17, 18, 21, 23, 25, 29,** and **31** were identified as galloyl-hexosides and their isomers (Čutović et al., 2022). Gallic acid **(3),** ellagic acid **(20),** and *p*-coumaric acid **(43)** were previously identified in the roots of *Paeonia ostii* and *Paeonia lactiflora* (Bai et al., 2021; Yang et al., 2021), while cinnamic acid **(42)** was found in *Paeonia radix* (Gao et al., 2020). Also, it should be emphasized that the chemical quantification of some gallic acid derivates has been undertaken so that the amount of gallic acid is in a wide range (7.33 to 9.19 %) concerning the plant varieties. On the other hand, ellagic acid and *p*-coumaric acid were found in all examined extracts, and their mass content was in the range of 0.11 to 0.22 %. All galloyl-hexoside derivatives showed very similar fragmentation patterns, being the MS^2 fragment ion at *m/z* 313, MS^3 abundant ion at *m/z* 169 (corresponding to the mass of deprotonated gallic acid), and the MS^4 base fragment being mainly formed by further loss of CO_2 (44 Da). Compound 14 with a molecular ion at *m/z* 183.02832 was identified as methyl gallate 1 ($C_8H_7O_5^-$). The MS^2 and MS^3- fragment ions at *m/z* 168 (side chain) and *m/z* 124 (pyrogallol group $[M-H-CH_3-CO_2]^-$) were formed by neutral losses of 15 Da (methyl radical, $CH_3\bullet$) and 44 Da (CO_2).

Compounds **6** and **9** with identical exact masses (*m/z* 321) were identified as digallic acid derivatives (Sun et al., 2021; Xiong et al., 2021). Additionally, compounds **24** and **28,** as well as compounds **30** and **36** with the exact masses at *m/z* 197.04 and 349.06, respectively, were marked as ethyl gallate and ethyl digallate isomers, and their fragmentation ions at *m/z* 168.01 and *m/z* 125 were identified as $[M-H-CH_3-CO_2]^-$ and $[M-H-C_2H_5-CO_2]^-$, respectively. Concerning the remaining compounds shown in Table 6 **(13, 16, 37,** and **38)**, all of them were classified based on their specific MS spectra and fragmentation patterns. Nevertheless, compounds **16** and **37** were present in the traces (0.01 %).

Flavonoids. These organic molecules, with a C_6-C_3-C_6 structure containing two (A + B) condensed benzene rings connected by a heterocycle pyrene ring (C) that contains oxygen, are one of the most heterogenic classes of polyphenols, and in this study, 22 compounds were identified. Compound **46** was recently identified in the seed of *P. lactiflora* (Nie et al., 2021). Compounds **47** and **48** were identified in the *Paeonia kesrouanensis* (Sut et al., 2019), while compound **49** was identified in the *Paeonia rockii* (Wang et al., 2004). Compounds **55, 60, 64, 65,** and **66** were previously reported only in tree peony species (Wang et al., 2004), with the latter two compounds being detected for the first time in the leaves of herbaceous species *P. tenuifolia* and *P. officinalis* in this study.

Paeonia-specific monoterpenoids. It is the third class of polyphenolic compounds observed in this study; 10 complex organic molecules from this category were found, and most of them were already established as dominant for the *Paeonia* taxa. Compounds **74** and **75** were previously detected in the flowers, and **68** and **73** in the petals of *P. tenuifolia* (Stošić and Gorunović, 1989), while compound **71,** with the fragment ion *m/z* 525.16, was detected in the roots of *P. lactiflora* and *Paeonia veitchii* (Wang et al., 2006; Yang et al., 2021). In addition, mudanpiozide isomers (compounds **77** and **78**) with molecular ions at *m/z* 629.19 exhibit highly comparable fragmentation patterns that are consistent with their chemical structure and reactivity.

Other compounds. In this research, some molecules were characterized for the first time, and to the best of the authors' knowledge, there is

Table 5
The total protein and total sugar contents of the leaf extracts of *Paeonia tenuifolia* L. (LPT), *Paeonia peregrina* Mill. (LPP), and *Paeonia officinalis* L. (LPO).

Plant species (leaf extract)	Locality	Extraction method	Total protein content	Total sugar content
			[mg/mL]	
Paeonia tenuifolia (LPT)	Gulenovci	UAE	3.94±0.65 ^{cd}	0.16±0.02 ^{g}
	Deliblato sands		4.92±0.92 ^{bc}	0.14±0.02 ^{g}
	Pančevo		3.75±0.85 ^{cd}	0.21±0.03 ^{f}
	Bogovo gumno		3.72±0.14 ^{cd}	0.21±0.03 ^{f}
	Gulenovci	MAC	3.81±0.46 ^{cd}	0.31±0.03 ^{e}
	Deliblato sands		3.82±0.65 ^{cd}	0.37±0.03 ^{de}
	Pančevo		3.68±0.49 ^{cd}	0.69±0.05 ^{a}
	Bogovo gumno		3.95±0.35 ^{cd}	0.62±0.03 ^{a}
	Gulenovci	MAE	3.48±0.21 ^{d}	0.32±0.02 ^{e}
	Deliblato sands		3.71±0.61 ^{cd}	0.12±0.02 ^{gh}
	Pančevo		3.54±0.17 ^{d}	0.34±0.04 ^{de}
	Bogovo gumno		3.58±0.77 ^{cd}	0.46±0.05 ^{c}
Paeonia peregrina (LPP)	Krivi vir	UAE	3.52±0.98 ^{cd}	0.34±0.03 ^{de}
	Pirot		4.92±0.75 ^{bc}	0.31±0.02 ^{e}
	Pančevo		5.23±0.22 ^{b}	0.10±0.02 ^{h}
	Bogovo gumno		3.68±0.56 ^{cd}	0.31±0.03 ^{e}
	Krivi vir	MAC	3.66±0.15 ^{d}	0.37±0.04 ^{de}
	Pirot		5.62±0.90 ^{ab}	0.67±0.05 ^{a}
	Pančevo		5.85±0.19 ^{a}	0.42±0.06 ^{cd}
	Bogovo gumno		4.60±0.44 ^{bc}	0.48±0.02 ^{c}
	Krivi vir	MAE	5.21±0.55 ^{ab}	0.25±0.02 ^{f}
	Pirot		3.42±0.74 ^{cd}	0.43±0.04 ^{c}
	Pančevo		3.38±0.56 ^{d}	0.35±0.02 ^{de}
	Bogovo gumno		3.56±0.19 ^{d}	0.49±0.06 ^{c}
Paeonia officinalis (LPO)	Rujevica	UAE	6.14±0.51 ^{a}	0.31±0.03 ^{e}
	Božurna		3.68±0.49 ^{cd}	0.34±0.05 ^{de}
	Rujevica	MAC	3.13±0.45 ^{d}	0.52±0.01 ^{b}
	Božurna		3.18±0.13 ^{d}	0.54±0.02 ^{b}
	Rujevica	MAE	3.68±0.54 ^{cd}	0.67±0.04 ^{a}
	Božurna		3.57±0.89 ^{cd}	0.31±0.03 ^{e}

UAE: ultrasound-assisted extraction; MAC: maceration; MAE: microwave-assisted extraction; values with the same letter ($^{a-h}$) in each column showed no statistically significant difference ($p > 0.05$, $n = 3$, the p-value is defined as the probability under the assumption of no effect or no difference (null hypothesis) of obtaining a result equal to or more extreme than what was observed; the n value is defined as the number of repetitions, one-way ANOVA, analysis of variance, Duncan's *post hoc* test).

no publication on this topic. Therefore, compounds **80** and **82** with the molecular ions at *m/z* 417.14 and *m/z* 313.09 were first published in this study. Compounds **79** and **84** were earlier found in the roots and flowers of *P. officinalis* (Ahmad and Tabassum, 2013; Dienaitė et al., 2019), while compounds **81** and **83** were detected in the flowers of *Paeonia suffruticosa* (Pan et al., 2020). Also, compound **81** in the analyzed extracts was presented in traces (0.01 %).

3.3. Structural properties of the extracts

The ATR-FTIR analysis was performed to verify the specific functional groups of LPT, LPP, and LPO extracts obtained by the different extraction methods (Fig. 3 and Figures S5–S10).

In the presented figure, it can be seen that the most absorption bands were observed in the range between 1708 and 1024 cm^{-1}. The vibration at 3335 cm^{-1} originates from the –OH stretching modes of both phenolic and hydroxyl groups (Lee et al., 2009). The broad peaks centred at 2970 and 2932 cm^{-1} represent different modes of the –CH stretching vibrations originating from = CH_2/methoxy/–CH_3 groups (Lee et al., 2009; Oancea et al., 2021). The vibration at 1708 cm^{-1} originates from the C = O stretching vibration present in the carboxyl and ester forms (Lazzari et al., 2018). The broad band at 1612 cm^{-1} is mainly attributed to the C = C stretching vibrations of the aromatic structure (Geng et al., 2016; Krysa et al., 2022; Robb et al., 2002). The vibration at 1446 cm^{-1} is due to the C–H stretching vibration and O-C–H *in-plane* bending and has been associated with the phenyl core of phenolic acids (Lee et al., 2009; Oancea et al., 2021). The band at 1321 cm^{-1} is close to the O–H bending modes in the gallic acid derivates and flavonoids. Vibration at 1197 cm^{-1} results from the C-O stretching modes of aromatic alcohols (Lazzari et al., 2018). Also, the band at 1024 cm^{-1} is due to the C-O-C stretching asymmetric results to the primary alcohols in gallic acid derivates. It also seen little bands in the region of 900–757 cm^{-1} which can be assigned to the O–H wagging vibrations and C–H deformation vibrations (Geng et al., 2016; Krysa et al., 2022; Robb et al., 2002).

3.4. Antioxidant potential of the extracts

In the case when the amount of oxidants, such as reactive oxygen species, exceeds the amount of antioxidant compounds, oxidative stress causes irreversible damage to the cell components, including DNA, lipids, and proteins, resulting in tissue injury and diseases (Sbieh et al., 2022). The influence of the extraction methods (UAE, MAC, and MAE) on the antioxidant potential of the extracts was examined using $DPPH^{\bullet}$ radical scavenging and total antioxidant capacity (TAC) assays. The results of the $DPPH^{\bullet}$ scavenging and TAC tests of LPT, LPP, and LPO extracts are shown in Table 7. According to the results, the extract of LPT collected from Gulenovci had strong $DPPH^{\bullet}$ scavenging potential. The extracts of LPP collected from Pančevo and Bogovo gumno showed the highest $DPPH^{\bullet}$ scavenging activity when the UAE was used, followed by MAE and MAC. The sample prepared using leaves from Bogovo gumno and the UAE showed the lowest antioxidant potential. In LPO extracts, MAE provided the extracts with the highest antioxidant activity determined in the DPPH test. Also, these findings are consistent with those of a study by Ćutović et al. (2022), who investigated the effect of different extraction techniques on the efficacy of polyphenol extraction from *P. tenuifolia* L. petal extract. On the other hand, the results of the TAC test indicate that the highest antioxidant activity of extracts of LPT was obtained in the samples of Gulenovci (UAE and MAC). The results of the antioxidant activity of LPP extracts demonstrate that the extract from Krivi vir has a higher ability to chelate molybdenum ions. Therefore, in all three extraction techniques, this trend is observed. According to the results of the TAC assay, the extract prepared using Božurna and UAE showed the highest antioxidant effect among LPO samples.

3.5. Antimicrobial activity of the extracts

The results of the antimicrobial activity evaluated by the microdilution test are summarized in Table 8. The LPT, LPP, and LPO extracts originating from different localities were all tested for their antibacterial activity, as plant materials may differ in chemical profile, *i.e.*, in the content of the bioactive compounds responsible for the achieved antimicrobial effect. Namely, the leaf extracts of all three *Paonia* species were assessed as a potential source of antimicrobial agents intended for use in the treatment of disorders in the human gastrointestinal tract. The

Table 6
HRMS and MS[4] data for secondary plant metabolites identified in the leaf extracts of *Paeonia tenuifolia* L. (LPT), *Paeonia peregrina* Mill. (LPP), and *Paeonia officinalis* L. (LPO).

No	Compound name	tR, min	Molecular formula [M–H]–	Calculated mass [M–H]–	Exact mass [M–H]–	Δ mDa	MS2 fragments	MS3 fragments	MS4 fragments	Content [%]		
							(% Base Peak)			LPO	LPP	LPT
Galic acid derivatives										75.8	76.1	75.3
1	Galloyl-hexoside 1	0,57	C13H15O10–	331,06707	331,06438	2,69	125(8), 151(4), 169 (100), 170(3), 193(9), 211(20), 271(38)	125(100)	69(55), 76(8), 79(17), 81(100), 97(56), 107 (49)	0.79	0.63	0.32
2	Galloyl-hexoside 2	0,87	C13H15O10–	331,06707	331,06398	3,09	125(14), 169(100), 170(7), 193(12), 211 (28), 271(59), 272(7)	125(100)	79(19), 81(100), 97 (66), 107(26)	1.11	1.00	0.57
3	Gallic acid	1,00	C7H5O5–	169,01425	169,01286	1,39	124(3), 125(100)	51(5), 53(5), 69(17), 79(10), 81(100), 97 (91), 107(14)	NA	9.19	8.18	7.33
4	Dihydroxybenzoic acid 1	1,84	C7H5O4–	153,01933	153,01847	0,86	109(100), 110(7), 123 (3)	81(93), 85(94), 92 (100)	NA	0.99	2.36	1.43
5	Digalloyl-hexoside 1	2,19	C20H19O14–	483,07803	483,07383	4,20	168(9), 169(100), 170 (6), 193(4), 271(7), 313(14), 331(25)	125(100)	53(30), 81(100), 97 (59)	0.06	0.00	0.00
6	Digallic acid 1	3,00	C14H9O9–	321,02521	321,02246	2,75	125(4), 169(100), 170 (7)	125(100)	67(13), 69(16), 81 (100), 81(20), 82(12), 97(72), 107(24)	0.67	0.57	0.76
7	Digalloyl-hexoside 2	3,05	C20H19O14–	483,07803	483,07458	3,45	169(3), 271(12), 313 (3), 330(5), 331(100), 332(14), 423(12)	125(11), 151(4), 169 (82), 193(11), 211 (27), 241(19), 271 (100)	169(23), 211(100)	0.06	0.07	0.03
8	Digalloyl-hexoside 3	3,49	C20H19O14–	483,07803	483,07378	4,25	169(11), 193(16), 211 (16), 271(100), 272 (12), 313(22), 331 (26)	169(12), 211(100)	124(26), 125(8), 139 (5), 165(11), 167(46), 168(100), 183(9)	0.14	0.26	0.03
9	Digallic acid 2	3,49	C14H9O9–	321,02521	321,02212	3,08	125(3), 169(100), 170 (4)	125(100)	69(28), 79(16), 81 (100), 95(10), 96(9), 97(57), 107(15)	0.57	1.29	0.99
10	Dihydroxybenzoic acid 2	3,61	C7H5O4–	153,01933	153,01801	1,32	97(55), 109(100), 110 (9), 123(6), 125(9), 135(8), 138(7)	58(38), 62(36), 81 (100)	NA	0.34	0.19	0.20
11	Trigalloyl-hexoside 1	3,62	C27H23O18–	635,08841	635,08409	4,32	313(6), 421(6), 465 (100), 483(52)	161(7), 169(62), 193 (7), 235(10), 295(32), 313(100), 421(50)	125(13), 137(4), 151 (9), 169(100), 179(5), 193(4), 295(13)	0.14	0.04	0.06
12	Digalloyl-hexoside 4	3,70	C20H19O14–	483,07803	483,07411	3,92	169(20), 211(18), 271 (100), 272(13), 313 (52), 331(42), 439 (13)	169(12), 211(100)	124(16), 125(9), 165 (12), 167(67), 168 (100), 183(7), 193(9)	0.04	0.24	0.11
13	Digalloyl-rhamnoside	3,99	C20H19O13–	467,08311	467,07949	3,63	313(9), 315(22), 421 (8), 423(100), 424 (18), 425(3), 449(4)	125(6), 151(4), 168 (5), 169(49), 211(4), 313(100), 314(11)	125(17), 151(5), 169 (100), 211(5), 223(8), 241(4), 253(5)	0.05	0.28	0.31
14	Methyl gallate 1	4,06	C8H7O5–	183,02990	183,02832	1,58	124(83), 125(5), 140 (8), 153(14), 167(7), 168(100), 169(7)	111(6), 124(100), 137 (5), 139(4), 140(9)	78(100), 96(44)	12.54	8.71	8.80
15	Trigalloyl-hexoside 2	4,07	C27H23O18–	635,08841	635,08481	3,61	465(100)	169(36), 193(4), 211 (6), 235(8), 295(10), 313(100), 447(4)	125(16), 151(7), 169 (100), 193(29), 241 (17), 253(16), 295 (15)	1.46	0.09	0.11

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Table 6 (*continued*)

No	Compound name	tR, min	Molecular formula [M–H]–	Calculated mass [M–H]–	Exact mass [M–H]–	Δ mDa	MS2 fragments	MS3 fragments	MS4 fragments	Content [%]		
							(% Base Peak)			LPO	LPP	LPT
16	Galloyl-vanilloyl-rhamoside	4,15	C21H21O12–	465,10385	465,09999	3,86	313(10), 421(100), 422(21), 447(26), 448 (5)	125(5), 151(3), 169 (45), 313(100), 314 (4)	125(18), 151(5), 169 (100), 211(4), 223(4), 241(5), 253(3)	0.01	0.57	0.01
17	Tetragalloyl-hexoside 1	4,32	C34H27O22–	787,09947	787,09506	4,41	465(5), 617(15), 617 (100), 618(25), 635 (8)	277(10), 295(23), 313 (8), 447(25), 449(7), 465(100), 573(6)	169(21), 193(4), 271 (6), 295(14), 313 (100)	0.25	0.12	0.10
18	Trigalloyl-hexoside 3	4,34	C27H23O18–	635,08841	635,08459	3,82	295(5), 313(19), 423 (7), 465(100), 483 (94)	169(29), 295(14), 313 (100), 314(9)	125(18), 151(4), 169 (100), 193(4), 241 (11), 253(9), 295(3)	0.12	0.30	0.09
19	Methyl digallate 1	4,64	C15H11O9–	335,04086	335,03802	2,84	182(4), 183(100), 184 (5)	111(3), 124(77), 137 (3), 139(4), 140(7), 168(100)	111(4), 124(100), 137 (5), 139(4), 140(10)	2.35	3.42	4.30
20	Ellagic acid	4,69	C14H5O8–	300,99899	300,99682	2,17	185(53), 229(87), 257 (100), 271(66), 272 (24), 284(51)	157(4), 185(82), 201 (13), 213(22), 229 (100), 230(4), 240(9)	145(11), 147(12), 157 (46), 173(35), 185 (100), 201(92)	0.28	0.11	0.23
21	Tetragalloyl-hexoside 2	4,73	C34H27O22–	787,09947	787,09491	4,56	465(17), 617(98), 618 (22), 619(6), 635 (100), 636(27), 637 (7)	465(100), 483(8)	169(31), 193(3), 211 (4), 235(6), 295(8), 313(100), 447(4)	1.22	0.67	0.74
22	Methyl gallate 2	4,89	C8H7O5–	183,02990	183,02856	1,34	111(4), 124(72), 137 (3), 139(4), 140(7), 168(100)	111(6), 124(100), 127 (4), 137(7), 139(6), 140(14)	NA	0.09	0.01	10.20
23	Pentagalloyl-hexoside 1	5,02	C41H31O26–	939,11043	939,10421	6,22	617(7), 769(100), 770 (24), 771(7), 787(7), 788(3)	429(13), 431(13), 447 (25), 599(24), 601 (30), 617(100), 725 (9)	271(7), 277(6), 295 (5), 313(8), 423(12), 447(22), 465(100)	3.01	2.03	2.01
24	Ethyl gallate 1	5,04	C9H9O5–	197,04555	197,04412	1,43	124(5), 125(8), 167 (3), 168(7), 169(100), 170(4)	125(100)	69(19), 79(13), 81 (100), 96(5), 97(52), 107(17)	2.12	5.94	0.04
25	Pentagalloyl-hexoside 2	5,19	C41H31O26–	939,11043	939,10522	5,22	769(4), 787(100), 788 (22)	403(4), 447(6), 465 (11), 573(7), 617(10), 617(100), 635(20)	295(15), 403(33), 421 (14), 447(41), 449 (11), 465(100), 573 (55)	0.18	0.15	0.07
26	Methyl digallate 2	5,25	C15H11O9–	335,04086	335,03822	2,63	183(100), 184(4)	111(4), 124(72), 137 (3), 139(4), 140(7), 168(100)	111(6), 124(100), 127 (4), 137(7), 139(6), 140(14)	9.27	10.71	9.48
27	Galloyl-benzoyl-hexoside 1	5,28	C20H19O11–	435,09329	435,09033	2,96	151(100), 169(89), 175(42), 193(79), 223 (24), 300(18), 313 (31)	65(8), 83(5), 107 (100)	65(100)	0.36	0.08	0.00
28	Ethyl gallate 2	5,33	C9H9O5–	197,04555	197,04420	1,34	124(6), 125(9), 168 (9), 169(100)	125(100)	NA	8.15	6.57	8.90
29	Hexagalloyl-hexoside	5,37	C48H35O30–	1091,12139	1091,11446	6,93	939(100), 940(36)	599(3), 617(9), 769 (100), 787(11)	429(12), 431(12), 447 (23), 599(30), 601 (39), 617(100), 725 (9)	3.51	3.70	3.12
30	Ethyl digallate 1	5,56	C16H13O9–	349,05651	349,05390	2,60	197(100), 198(6)	124(4), 125(7), 168 (9), 169(100)	125(100)	2.64	2.10	2.59
31	Heptagalloy-hexoside	5,59	C55H39O34–	1243,13234	1243,12545	6,89	939(26), 940(16), 1090(22), 1091(100), 1092(36)	939(100)	599(3), 617(9), 769 (100), 787(10)	2.96	4.43	2.41
32	Galloyl-benzoyl-hexoside 2	5,75	C20H19O11–	435,09329	435,09060	2,69	150(13), 168(80), 169 (74), 313(100), 314	125(32), 137(87), 151 (21), 161(15), 168	108(4), 123(4), 125 (62), 151(100)	1.11	1.31	0.58

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Table 6 (*continued*)

No	Compound name	tR, min	Molecular formula [M–H]–	Calculated mass [M–H]–	Exact mass [M–H]–	Δ mDa	MS2 fragments	MS3 fragments	MS4 fragments	Content [%]		
							(% Base Peak)			LPO	LPP	LPT
							(12), 417(90), 418 (20)	(75), 169(100), 269 (47)				
33	Octagalloy-hexoside	5,80	C62H43O38–	1395,14327	1395,13672	6,55	939(19), 1091(92), 1092(32), 1243(100), 1244(19)	938(25), 939(90), 1090(38), 1091(100)	768(3), 769(4), 788 (4), 938(7), 939(100)	0.59	1.22	0.28
34	Trigalloyl-methyl ester	5,94	C22H15O13–	487,05181	487,04859	3,22	183(9), 334(16), 335 (100), 336(8)	183(100)	111(5), 124(74), 137 (3), 139(3), 140(7), 168(100)	0.09	0.52	0.18
35	Galloyl-hydroxybenzoyl-hexoside	5,97	C20H19O12–	451,08820	451,08493	3,27	137(8), 169(7), 313 (92), 314(13), 331 (100), 332(12), 349 (7)	125(34), 150(12), 167 (19), 168(95), 169 (29), 313(100), 314 (13)	108(48), 117(42), 125 (90), 135(48), 137 (31), 150(100), 151 (44)	0.07	0.11	0.20
36	Ethyl digallate 2	6,02	C16H13O9–	349,05651	349,05366	2,85	197(100), 198(6)	124(4), 125(7), 169 (100)	125(100)	8.41	7.86	8.37
37	Methyl galloyl-dihydroxybenzoate	6,07	C15H11O8–	319,04594	319,04355	2,39	183(100), 184(7)	111(3), 124(72), 137 (5), 139(4), 140(8), 168(100)	82(3), 111(8), 124 (100), 137(6), 139(7), 140(6)	0.01	0.01	0.01
38	Galloyl-di-dihydroxybenzoyl-rhamnoside	6,16	C27H23O15–	587,10370	587,10041	3,29	169(100), 170(5), 417 (38), 435(41), 465(4)	125(100)	51(48), 55(50), 81 (63), 97(47), 107 (100)	0.84	0.26	0.09
39	Galloyl-benzoyl-hexoside 3	6,49	C20H19O11–	435,09329	435,09002	3,26	137(7), 152(5), 153 (9), 297(100), 298 (14), 315(26), 316(3)	107(8), 108(38), 109 (14), 135(8), 152 (100), 153(45), 179 (15)	108(100)	0.00	0.00	0.24
Other phenolic acid derivatives										1,04	1,68	1,78
40	Benzoyl-hexosyl-hexoside	3,61	C19H25O12–	445,13515	445,13137	3,78	161(59), 162(6), 179 (24), 221(7), 321(10), 323(100), 324(26)	113(73), 125(78), 143 (57), 179(45), 221 (100), 245(41), 263 (90)	NA	0.11	0.88	0.20
41	Coumaroyl-hexoside	3,68	C15H17O8–	325,09289	325,09011	2,78	119(9), 145(5), 163 (100), 164(7)	119(100)	101(100)	0.03	0.03	0.22
42	Cinnamic acid	4,56	C9H7O2–	147,04515	147,04445	0,70	53(3), 92(3), 103 (100), 119(4), 121(5)	NA	NA	0.01	0.02	0.02
43	p-coumaric acid	5,82	C9H7O3–	163,04007	163,03925	0,81	91(3), 119(100), 120 (10)	91(100), 101(74)	NA	0.77	0.05	1.32
44	Dihydroxybenzoyl-pentosyl-pentoside	6,27	C21H21O9–	417,11911	417,11605	3,06	151(17), 152(67), 153 (100), 163(11), 165 (7), 297(15), 373(6)	108(7), 109(100)	81(100)	0.12	0.70	0.02
Flavonoids										12,48	11,71	13,2
45	Quercetin 3-O-(2“-rhamnosyl)-hexoside-7-O-hexoside	3,52	C33H39O21–	771,19846	771,19315	5,30	299(10), 301(8), 462 (28), 463(18), 609 (100), 610(57), 611 (9)	255(17), 271(32), 300 (100), 301(41), 445 (18), 463(11), 489 (11)	151(5), 227(4), 254 (11), 255(33), 256 (11), 271(100), 272 (31)	0.10	0.06	0.02
46	Quercetin 3-O-hexoside-7-O-hexoside	3,74	C27H29O17–	625,14055	625,13602	4,53	301(39), 302(7), 462 (3), 462(24), 463 (100), 464(19), 505 (4)	271(5), 299(3), 300 (47), 301(100), 343 (8)	107(9), 151(100), 179 (52), 229(13), 255 (16), 272(14), 273 (10)	0.40	0.58	0.59
47	Quercetin 3-O-pentoside-7-O-hexoside	3,80	C26H27O16–	595,12999	595,12574	4,25	301(36), 302(5), 433 (100), 434(21), 462 (71), 463(61), 464 (11)	179(3), 271(6), 300 (100), 301(25), 343 (4)	151(5), 179(3), 254 (11), 255(25), 271 (100), 272(14)	0.64	0.33	0.32

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Table 6 (*continued*)

No	Compound name	tR, min	Molecular formula [M–H]–	Calculated mass [M–H]–	Exact mass [M–H]–	Δ mDa	MS2 fragments	MS3 fragments	MS4 fragments	Content [%]		
							(% Base Peak)			LPO	LPP	LPT
48	Kaempferol 3-O-pentoside-7-O-hexoside	3,99	C26H27O15–	579,13554	579,13192	3,62	285(7), 417(100), 418 (20), 446(12), 447(5), 459(13)	255(9), 284(100), 285 (22), 327(11)	227(14), 255(100), 256(21)	0.08	0.05	0.12
49	Kaempferol 3-O-(2“-hexosyl)-hexoside	4,02	C27H29O16–	609,14611	609,14187	4,24	285(24), 286(4), 327 (4), 447(100), 489 (12)	151(4), 227(4), 255 (18), 284(100), 285 (39), 327(16)	227(16), 255(100), 256(20)	0.07	0.16	0.17
50	Isorhamnetin 3-O-hexoside-7-O-hexoside	4,13	C28H31O17–	639,15610	639,15208	4,02	315(16), 357(3), 477 (100), 519(10)	271(10), 285(8), 299 (5), 314(100), 315 (45), 357(18)	243(33), 257(10), 271 (84), 285(100), 286 (48), 299(12), 300 (79)	0.42	0.14	0.53
51	Isorhamnetin 3-O-(2“-pentosyl)-hexoside	4,19	C27H29O16–	609,14611	609,14232	3,79	315(7), 447(100), 476 (8), 489(13)	271(6), 285(4), 299 (9), 314(100), 315 (26), 357(10), 432(5)	243(30), 257(10), 271 (89), 285(100), 286 (38), 299(30), 300 (19)	0.15	0.00	0.11
52	Quercetin 3-O-hexoside-7-O-rhamnoside	4,51	C27H29O16–	609,14611	609,14187	4,24	301(51), 302(8), 446 (50), 447(100), 448 (17), 463(72), 464 (12)	300(6), 301(100)	107(14), 151(100), 179(58), 211(9), 229 (13), 255(22), 273 (12)	0.00	2.88	0.00
53	Quercetin 3-O-galloyl-hexoside	4,71	C28H23O16–	615,09860	615,09548	3,12	300(5), 301(16), 302 (3), 463(100), 464 (17)	300(31), 301(100)	151(80), 179(100), 193(6), 229(7), 257 (12), 273(18), 283(7)	3.36	0.99	0.16
54	Quercetin 3-O-(2“-rhamnosyl)-hexoside	4,73	C27H29O16–	609,14611	609,14246	3,65	255(10), 271(23), 299 (14), 300(100), 301 (30), 445(11), 489(8)	243(4), 254(8), 255 (47), 256(3), 271 (100), 272(10)	199(23), 203(9), 215 (34), 227(76), 229 (10), 243(100), 271 (15)	1.58	0.38	0.03
55	Kaempferol 3-O-hexoside-7-O-rhamnoside	4,76	C27H29O15–	593,15119	593,14688	4,31	285(28), 286(4), 431 (50), 432(9), 447 (100), 448(17)	151(3), 227(5), 255 (17), 256(4), 284 (100), 285(28), 327 (16)	227(15), 255(100), 256(18)	0.00	1.70	0.00
56	Isorhamnetin 3-O-hexoside-7-O-rhamnoside	4,84	C28H31O16–	623,16176	623,15762	4,14	315(15), 316(3), 461 (44), 462(7), 477 (100), 478(17)	271(7), 285(10), 286 (4), 299(5), 314(100), 315(23), 357(16)	243(31), 257(13), 271 (81), 285(100), 286 (44), 299(13), 300 (11)	0.00	0.92	0.00
57	Kaempferol 3-O-rhamnoside-7-O-pentoside	4,85	C26H27O14–	563,14016	563,13695	3,20	285(55), 286(9), 417 (57), 418(8), 430(41), 431(100), 432(16)	284(6), 285(100)	169(62), 185(52), 213 (100), 229(68), 239 (51), 243(91), 257 (65)	0.00	1.26	0.00
58	Quercetin 3-O-hexoside Quercetin 3-O-hexoside	4,95	C21H19O12–	463,08820	463,08534	2,86	300(31), 301(100), 302(9)	107(7), 151(81), 179 (100), 256(10), 257 (11), 272(14), 273 (19)	151(100), 169(3)	1.03	0.66	4.60
59	Quercetin 3-O-(2“-rhamnosyl)-pentoside	5,08	C26H27O15–	579,13554	579,13212	3,42	255(7), 271(15), 300 (100), 301(28), 415 (5), 433(3), 489(3)	151(3), 243(3), 254 (10), 255(48), 256(4), 271(100), 272(13)	199(25), 215(28), 227 (72), 229(16), 242 (13), 243(100), 271 (16)	0.56	1.24	0.00
60	Quercetin 3-O-pentoside	5,16	C20H17O11–	433,07764	433,07458	3,06	299(5), 300(100), 301 (81), 302(8)	151(10), 179(8), 254 (6), 255(54), 256(5), 271(100), 272(15)	199(25), 203(11), 215 (28), 227(68), 229 (12), 243(100), 271 (14)	3.14	0.30	2.43
61	Kaempferol 3-O-hexoside Kaempferol 3-O-hexoside	5,31	C21H19O11–	447,09329	447,09067	2,61	255(15), 256(5), 284 (100), 285(57), 286 (8), 316(7), 327(12)	227(14), 255(100), 256(19), 257(4)	183(5), 187(5), 210 (7), 211(62), 213(5), 227(100), 255(8)	0.02	0.01	0.41

(*continued on next page*)

Table 6 (*continued*)

No	Compound name	tR, min	Molecular formula [M–H]–	Calculated mass [M–H]–	Exact mass [M–H]–	Δ mDa	MS2 fragments	MS3 fragments	MS4 fragments	Content [%]		
							(% Base Peak)			LPO	LPP	LPT
62	Isorhamnetin 3-O-hexoside	5,37	C22H21O12–	477,10385	477,10104	2,81	271(6), 285(8), 300 (6), 314(100), 315 (61), 316(7), 357(12)	243(24), 257(9), 271 (77), 285(100), 286 (30), 299(41), 300 (25)	270(100), 271(4)	0.11	0.03	1.26
63	Kaempferol 3-O-pentoside	5,45	C20H17O10–	417,08272	417,07999	2,73	255(10), 256(3), 284 (100), 285(33), 327 (6)	227(13), 255(100), 256(18)	167(6), 183(5), 210 (6), 211(64), 213(6), 227(100), 255(10)	0.00	0.00	0.87
64	Isorhamnetin 3-O-pentoside	5,64	C21H19O11–	447,09329	447,09051	2,78	271(4), 285(5), 286 (3), 314(100), 315 (25), 357(9)	243(30), 257(12), 271 (77), 285(100), 286 (44), 299(12), 300 (19)	270(100), 271(4)	0.34	0.00	0.44
65	Quercetin	6,36	C15H9O7–	301,03538	301,03305	2,32	107(6), 151(86), 179 (100), 180(8), 257 (11), 271(32), 273 (17)	151(100)	63(7), 65(3), 83(17), 107(100)	0.30	0.01	0.67
66	Isorhamnetin	7,30	C16H11O7–	315,05103	315,04865	2,38	300(100), 301(9)	151(100), 227(40), 228(22), 255(31), 271 (88), 272(66), 283 (33)	83(7), 107(100)	0.18	0.01	0.39
Paeonia specific monoterpenoids										3.41	3.47	5.57
67	Galloyl desbenzoylpaeoniflorin 1	3,16	C23H27O14–	527,14016	527,13701	3,14	169(19), 271(25), 313 (100), 345(37), 375 (22), 491(31), 497 (48)	125(21), 151(5), 167 (3), 169(100), 211(6), 241(5), 253(5)	107(4), 125(100)	0.22	0.13	0.09
68	Oxypaeoniflorin	3,61	C23H27O12–	495,15033	495,14670	3,63	245(18), 333(24), 447 (92), 448(22), 465 (100), 466(22), 477 (14)	137(31), 165(11), 179 (12), 217(17), 281 (27), 299(100), 447 (18)	89(68), 143(70), 206 (68), 209(100), 219 (68), 226(68)	0.00	0.03	0.16
69	Galloyl desbenzoylpaeoniflorin 2	3,72	C23H27O14–	527,14016	527,13640	3,76	345(19), 347(100), 348(16), 365(18), 375 (22), 479(13), 481 (12)	125(25), 151(9) 169 (100), 195(10), 285 (5), 303(3)	97(5), 125(100)	0.18	0.14	0.24
70	Desbenzoyl paeonin B	3,79	C16H21O9–	357,11911	357,11626	2,85	191(3), 195(100), 196 (9)	119(7), 123(15), 134 (9), 135(100), 136 (89), 151(66), 177 (20)	91(32), 91(41), 107 (100), 113(25)	0.03	0.01	0.02
71	Mudanpioside E	4,75	C24H29O13–	525,16137	525,15795	3,41	449(100), 450(3), 479 (34)	165(27), 309(6), 327 (100)	113(5), 123(14), 137 (3), 165(100), 309 (10)	0.06	0.71	1.05
72	Galloylpaeoniflorin	5,11	C30H31O15–	631,16684	631,16273	4,12	271(21), 313(12), 479 (13), 491(23), 509(8), 613(100), 614(22)	211(23), 241(8), 271 (100), 313(38), 375 (13), 399(17), 491 (81)	169(11), 211(100)	0.12	0.64	1.27
73	Paeoniflorin	5,53	C23H27O11–	479,15589	479,15250	3,39	183(5), 195(4), 213 (6), 449(100), 450 (36)	135(5), 139(5), 165 (4), 183(16), 327 (100), 328(6), 431 (13)	101(37), 121(50), 124 (44), 139(100), 183 (60), 225(45), 309 (44)	2.11	0.13	0.02
74	Albiflorin	5,76	C23H27O11–	479,15589	479,15289	2,99	151(4), 183(3), 195 (13), 196(3), 213(7), 449(100), 450(63)	137(4), 139(18), 140 (6), 183(17), 184(4), 327(100), 328(63)	139(100), 143(16), 163(24), 165(25), 183 (74), 235(19), 237 (21)	0.12	0.80	0.51

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Table 6 (*continued*)

No	Compound name	tR, min	Molecular formula [M–H]–	Calculated mass [M–H]–	Exact mass [M–H]–	Δ mDa	MS2 fragments	MS3 fragments	MS4 fragments	Content [%]		
							(% Base Peak)			LPO	LPP	LPT
75	Galloylalbiflorin	5,88	C30H31O15–	631,16684	631,16286	3,98	417(5), 509(100), 510 (21), 511(3), 553(3), 556(10)	169(100), 179(7), 195 (7), 295(44), 407(18), 465(7), 479(8)	107(4), 125(100)	0.29	0.00	0.00
76	6′-Hemisuccinyl paeoniflorin	6,63	C27H31O15–	595,16637	595,16209	4,28	427(11), 549(100), 550(3)	163(5), 223(7), 283 (9), 325(15), 367(9), 426(20), 427(100)	135(92), 221(40), 227 (100), 305(58), 324 (41)	0.03	0.34	0.05
77	Mudanpioside B	7,18	C31H33O14–	629,18758	629,18334	4,24	431(3), 552(6), 553 (100), 582(4), 583 (67)	165(22), 265(6), 309 (4), 413(8), 430(25), 431(100), 525(4)	147(10), 162(9), 165 (100), 217(20), 243 (8), 249(7), 413(38)	0.25	0.50	1.87
78	Mudanpioside J	7,91	C31H33O14–	629,18758	629,18372	3,86	431(3), 535(4), 552 (26), 553(100), 554 (4), 583(98), 584(3)	163(3), 165(30), 245 (6), 291(3), 309(3), 413(9), 431(100)	165(100), 171(29), 205(26), 217(45), 309 (23), 413(60), 469 (17)	0.00	0.04	0.29
Other compounds										7.24	7.01	4.28
79	Quinic acid	0,59	C7H11O6–	191,05560	191,05453	1,07	85(51), 93(28), 111 (100), 127(37), 129 (13), 171(14), 173 (36)	67(100), 81(32), 83 (7)	NA	7.15	6.06	3.17
80	Hydroxymethyl-phenyl pentosyl-hexoside	3,99	C18H25O11–	417,14024	417,13703	3,21	181(34), 209(26), 284 (29), 285(13), 293(8), 295(100), 343(17)	101(23), 127(25), 133 (100), 175(62), 205 (17), 215(15), 217 (27)	NA	0.03	0.31	0.02
81	Apiopaeonoside	4,22	C20H27O12–	459,15080	459,14748	3,32	164(10), 269(9), 296 (100), 297(60), 310 (21), 326(9), 327(17)	176(37), 180(35), 239 (57), 240(100), 251 (35), 267(71), 268 (69)	NA	0.00	0.01	0.01
82	Acetyl-hydroxyphenyl-hexoside	4,47	C14H17O8–	313,09289	313,08936	3,53	169(100), 170(8), 187 (28), 197(11), 212(8), 213(34), 241(18)	121(3), 133(3), 135 (3), 137(100), 139(7), 149(27)	57(9), 60(3), 83(100), 109(4)	0.01	0.03	0.62
83	Paeonoside	4,57	C15H19O8–	327,10854	327,10636	2,18	113(4), 123(12), 137 (3), 165(100), 166(6), 179(3), 309(15)	95(4), 121(12), 122 (5), 123(100), 137(8), 147(3), 150(5)	80(14), 81(100), 95 (30), 105(11), 108 (25)	0.04	0.46	0.39
84	Paeonol	5,20	C9H9O3–	165,05572	165,05504	0,68	121(94), 122(39), 123 (75), 129(100), 136 (42), 137(70), 139 (51)	56(12), 60(12), 67 (13), 85(74), 96(13), 101(100), 130(12)	NA	0.01	0.14	0.07
									Total	100.0	100.0	100.0

NA: not available; the peaks that were further fragmented in the MS^3 and MS^4 tests are shown by **bold** numbers and show 100% of the base peak.

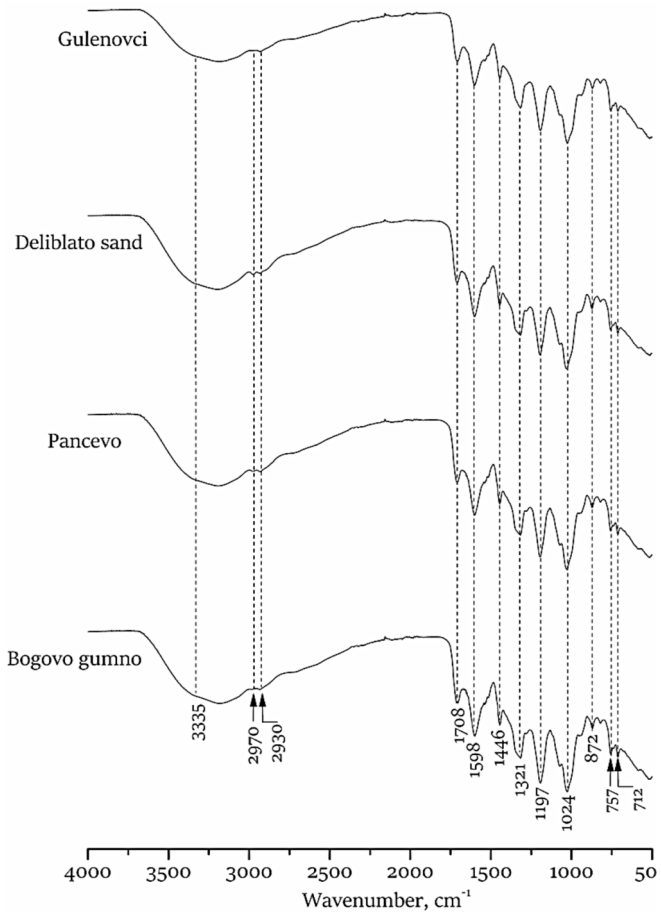

Fig. 3. The ATR-FTIR spectra of the dry extract of *Paeonia tenuifolia* L. leaves from different localities by ultrasound-assisted extraction.

LPT (Deliblato sands) extract had the highest antibacterial activity towards *S.* Typhimurium and *P. aeruginosa* (Gram-negative), followed by *L. monocytogenes* (Gram-positive), and was the least effective against *E. coli* (Gram-negative), thus requiring a higher extract concentration for the inhibition of bacterial growth. The LPP and LPO extracts showed a lower ability to inhibit the degree of bacterial growth, both being the most effective against *S. aureus, S.* Typhimurium, *E. coli,* and *L. monocytogenes.*

3.6. Enzyme inhibitory activity

3.6.1. The AChE and BChE inhibitory activities

The most widely employed therapeutically active substances for treating the symptoms of Alzheimer's and Parkinson's diseases are cholinesterase inhibitors. Since then, peonies have been used in traditional and conventional therapies as protective agents related to autoimmune neurodegenerative diseases (Kayani et al., 2015; Lev and Amar, 2002). Therefore, the potential of the leaf extracts of the studied herbaceous peonies to act as inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was investigated.

As shown in Table 9, the extracts of LPT obtained by the UAE showed similar effects on both cholinesterases, ranging from 1.65 to 1.78 mg GALAE/g. The MAE extract of LPT collected from Gulenovci was the most potent (1.47 mg GALAE/g). On the other hand, the extract of LPP obtained by the UAE shows significantly better inhibition of AChE (1.99–2.05 mg GALAE/g) compared to the other two groups of extracts.

3.6.2. The alpha-glucosidase and alpha-amylase inhibitory activities

Type 2 diabetes mellitus has become a serious global problem in the

Table 7
Antioxidant potential (DPPH and TAC assays) of the leaf extracts of *Paeonia tenuifolia* L. (LPT), *Paeonia peregrina* Mill. (LPP), and *Paeonia officinalis* L. (LPO).

Plan species (leaf extract)	Locality	Extraction method	Antioxidant potential	
			DPPH [mmol TE/mL]	**TAC** [mmol LAE/mL]
Paeonia tenuifolia (LPT)	Gulenovci	UAE	28.86±1.17 ef	34.08±1.74 g
	Deliblato sands		8.24±3.51 k	31.98±1.12 gh
	Pančevo		18.90±0.14 g	28.11±0.95 i
	Bogovo gumno		3.99±0.89 m	31.83±2.12 gh
	Gulenovci	MAC	23.35±2.02 de	24.37±1.50 j
	Deliblato sands		22.68±0.86 de	26.54±1.86 ij
	Pančevo		18.83±1.33 fg	26.88±1.29 ij
	Bogovo gumno		22.88±1.96 de	19.92±2.20 k
	Gulenovci	MAE	33.40±0.47 a	18.84±0.95 k
	Deliblato sands		30.23±1.12 b	16.69±1.01 kl
	Pančevo		31.19±0.55 b	15.21±1.43 l
	Bogovo gumno		27.19±3212 bc	17.77±2.30 kl
Paeonia peregrina (LPP)	Krivi vir	UAE	13.88±1.80 i	63.64±1.25 a
	Pirot		12.86±0.99 i	34.03±0.76 g
	Pančevo		29.69±2.04 bc	38.26±0.88 ef
	Bogovo gumno		20.22±0.35 f	39.54±1.43 ef
	Krivi vir	MAC	10.60±0.51 j	64.22±1.02 a
	Pirot		12.94±0.92 i	30.77±0.29 h
	Pančevo		5.12±1.11 lm	43.86±0.55 d
	Bogovo gumno		7.47±1.63 kl	38.64±2.10 ef
	Krivi vir	MAE	19.36±1.26 fg	63.69±1.86 a
	Pirot		27.42±1.91 bc	58.81±1.23 b
	Pančevo		26.08±1.46 cd	48.62±2.14 c
	Bogovo gumno		22.96±2.33 d	43.26±0.93 d
Paeonia officinalis (LPO)	Rujevica	UAE	6.14±1.55 lm	40.14±0.66 e
	Božurna		16.70±0.40 h	47.29±1.22 c
	Rujevica	MAC	9.19±0.89 jk	28.93±1.88 i
	Božurna		9.66±1.33 jk	38.96±2.50 ef
	Božurna	MAE	24.44±1.51 d	36.64±1.86 fg
	Rujevica		23.43±1.25 d	40.99±1.08 e

TE: Trolox equivalent; LAE: L-ascorbic acid equivalent; DPPH: 2,2-diphenyl-1-picrylhydrazyl; TAC: Total antioxidant capacity assay; UAE: ultrasound-assisted extraction; MAC: maceration; MAE: microwave-assisted extraction; values with the same letter ($^{a-m}$) in each column showed no statistically significant difference ($p > 0.05$, $n = 3$, the p-value is defined as the probability under the assumption of no effect or no difference (null hypothesis) of obtaining a

result equal to or more extreme than what was observed; the n value is defined as the number of repetitions, one-way ANOVA, analysis of variance, Duncan's *post hoc* test).

past twenty years. In addition, it is predicted that the prevalence of diabetes mellitus will rise dramatically in the next two decades, primarily due to inadequate and unbalanced nutrition and genetic predispositions (Ginter and Simko, 2013). Therefore, many therapeutic strategies have been developed for the mentioned health disorder, and the key enzyme inhibitory theory is one of the most accepted approaches.

Dietary carbohydrates, such as starch and its hydrolysates, digested by pancreatic alpha-amylase and alpha-glucosidases and then absorbed by the small intestine, are the main source of blood–sugar–glucose. A vital enzyme required for the hydrolysis of carbohydrates in the gastrointestinal tract is alpha-glucosidase (Uysal et al., 2017). Hence, alpha-amylase and alpha-glucosidase inhibitors have been recognized as therapeutic targets for the control of postprandial hyperglycaemia, the earliest metabolic disorder manifesting in type 2 diabetes mellitus (Sut et al., 2019; Uysal et al., 2017). Therefore, the anti-enzymatic capability of LPT, LPP, and LPO extracts needs to be investigated due to their extensive use in food supplements, pharmacy, and health protection.

The results of the inhibitory effects of LPT, LPP, and LPO extracts on alpha-amylase and alpha-glucosidase are presented in Table 9. Analyzing the effects of LPT, LPP, and LPO extracts on the inhibition of alpha-amylase, all tested analytes had a mostly similar effect on the observed enzyme. In the case of extracts of LPT, the most effective was the group of samples obtained by the MAC, with the concentration in the range of 0.19 ± 0.01 to 0.22 ± 0.01 mmol ACAE/g. The anti-enzymatic potential of the other two parallels (the extracts obtained by the UAE and MAE) ranged from 0.22 ± 0.00 to 0.27 ± 0.01 mmol ACAE/g. The extracts of LPP have uniform activity against the tested alpha-amylase, with a concentration higher than the extract of LPT (0.27 ± 0.01 to 0.33 ± 0.01 mmol ACAE/g).

The extracts of LPT show a moderate inhibitory effect on alpha-glucosidase (1.02 ± 0.01–1.14 ± 0.00 mmol ACAE/g), presented in the following order: LPT UAE > LPT MAE > LPT MAC. Our results related to *Paeonia* species inhibitory effects on glucosidase are not in agreement with the previous studies on this topic. Further, a similar pattern was observed for the extracts of LPP and LPO. Namely, the most effective inhibition was found in the extracts of LPP obtained by the UAE (the values are in the range of 1.08 ± 0.01 to 1.21 ± 0.00 mmol ACAE/g) compared to the other two tested groups of extracts.

Samples LPP MAC (Pančevo), LPP MAE (Krivi vir), and LPP MAE (Bogovo gumno) have the greatest impact against alpha-glucosidases and alpha-amylases. Therefore, it is recommended to utilize them to reduce the undesirable symptoms of diabetes mellitus type 2. Nonetheless, additional studies on the model of the mentioned disease are needed to confirm the claim.

3.6.3. The tyrosinase inhibitory activity of the extracts

Tyrosinase, the rate-limiting enzyme of the melanogenic pathway, is crucial for the production of melanin, the substance in the human body that affects the pigmentation of skin, hair, and eyes (Cavalieri et al.,

Table 8
Antibacterial activity of the leaf extracts of *Paeonia tenuifolia* L. (LTP), *Paeonia peregrina* Mill. (LPP), and *Paeonia officinalis* L. (LPO) (MIC and MBC, mg/mL).

Plant species (leaf extract)	**Locality**	**Extraction method**	***S.* Typhimurium**		***L. monocytogenes***		***B. cereus***		***P. aeruginosa***		***S. aureus***		***E. coli***	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Paeonia tenuifolia (LPT)	Gulenovci	UAE	2	4	0.25	0.5	0.5	1	1	2	1	2	2	4
	Deliblato sands		0.125	0.25	0.25	0.5	0.5	1	0.125	0.25	0.5	1	1	2
	Pančevo		0.25	0.5	0.25	0.5	0.25	0.5	0.125	0.25	0.25	0.5	1	2
	Bogovo gumno		0.25	0.5	0.25	0.5	0.5	1	0.125	0.25	0.25	0.5	0.5	1
	Gulenovci	MAC	0.5	1	0.5	1	0.5	1	1	2	0.25	0.5	1	2
	Deliblato sands		0.25	0.5	0.25	0.5	0.5	1	0.125	0.25	0.25	0.5	0.25	0.5
	Pančevo		2	4	2	4	0.5	1	2	4	2	4	0.5	1
	Bogovo gumno		0.25	0.5	0.25	0.5	1	2	0.125	0.25	0.25	0.5	0.25	0.5
	Gulenovci	MAE	1	2	1	2	1	2	1	2	1	2	1	2
	Deliblato sands		1	2	1	2	1	2	1	2	1	2	1	2
	Pančevo		2	4	2	4	2	4	2	4	2	4	2	4
	Bogovo gumno		2	4	2	4	0.25	0.5	2	4	1	2	1	2
Paeonia peregrina (LPP)	Krivi vir	UAE	1	2	1	2	1	2	1	2	1	2	1	2
	Pirot		0.5	1	1	2	0.5	1	0.5	1	1	2	1	2
	Pančevo		0.5	1	0.5	1	1	2	0.25	0.5	0.5	1	0.5	1
	Bogovo gumno		0.5	1	1	2	0.5	1	0.5	1	0.5	1	1	2
	Krivi vir	MAC	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	0.25	0.5
	Pirot		1	2	1	2	0.5	1	1	2	1	2	1	2
	Pančevo		0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	1	2
	Bogovo gumno		0.5	1	1	2	1	2	0.5	1	1	2	1	2
	Krivi vir	MAE	1	2	1	2	1	2	1	2	1	2	1	2
	Pirot		0.5	1	1	2	0.5	1	0.5	1	1	2	1	2
	Pančevo		0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1
	Bogovo gumno		0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.25	0.5	0.5	1
Paeonia officinalis (LPO)	Rujevica	UAE	0.5	1	0.25	0.5	0.5	1	0.5	1	0.25	0.5	0.25	0.5
	Božurna		0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	1	2
	Rujevica	MAC	1	2	1	2	0.5	1	1	2	1	2	1	2
	Božurna		0.5	1	1	2	0.5	1	1	2	1	2	1	2
	Rujevica	MAE	0.25	0.5	0.5	1	0.5	1	0.5	1	0.5	1	0.5	1
	Božurna		0.5	1	0.5	1	0.5	1	0.5	1	0.5	1	1	2

UAE: ultrasound-assisted extraction; MAC: maceration; MAE: microwave-assisted extraction; MIC: minimal inhibitory concentration; MBC: minimal bactericidal concentration; *S.* Typhimurium: *Salmonella* Typhimurium; *L. monocytogenes*: *Listeria monocytogenes*; *B. cereus*: *Bacillus cereus; P. aeruginosa: Pseudomonas aeruginosa; S. aureus: Staphylococcus aureus; E. coli: Escerichia coli.*

Table 9
The anti-enzymatic activity of the leaf extracts of *Paeonia tenuifolia* L (LPT), *Paeonia peregrina* Mill. (LPP), and *Paeonia officinalis* L. (LPO).

Plant species (leaf extract)	**Locality**	**Extraction method**	**AchE inhibition**	**BChE inhibition**	**Amylase inhibition**	**Glucosidase inhibition**	**Tyrosinase inhibition**
			[mg GALAE/g]		[mmol ACAE/g]		[mg KAE/g]
Paeonia tenuifolia (LPT)	Gulenovci	UAE	1.65±0.02 ^{bc}	0.31±0.01 ^{a}	0.27±0.01 ^{d}	1.14±0.00 ^{j}	46.85±0.30 ^{c}
	Deliblato sands		1.62±0.03 ^{b}	0.35±0.01 ^{b}	0.24±0.01 ^{bc}	1.02±0.00 ^{b}	49.48±0.66 ^{de}
	Pančevo		1.71±0.05 ^{cd}	0.29±0.05 ^{ab}	0.27±0.01 ^{d}	1.02±0.01 ^{bc}	53.78±0.08 ^{g}
	Bogovo gumno		1.78±0.03 ^{d}	0.55±0.03 ^{c}	0.22±0.00 ^{ab}	1.13±0.00 ^{i}	46.99±1.08 ^{c}
	Gulenovci	MAC	1.75±0.07 ^{cd}	0.71±0.06 ^{e}	0.22±0.01 ^{ab}	1.03±0.01 ^{c}	51.28±0.23 ^{f}
	Deliblato sands		1.65±0.04 ^{bc}	1.30±0.09 ^{jk}	0.19±0.01 ^{a}	1.03±0.00 ^{c}	50.37±0.45 ^{e}
	Pančevo		1.81±0.08 ^{de}	0.78±0.03 ^{ef}	0.20±0.02 ^{a}	1.09±0.00 ^{ef}	48.75±0.33 ^{d}
	Bogovo gumno		1.79±0.06 ^{d}	0.86±0.05 ^{fg}	0.21±0.00 ^{a}	1.03±0.00 ^{c}	50.66±0.56 ^{ef}
	Gulenovci	MAE	1.47±0.01 ^{a}	0.03±0.05 ^{ab}	0.22±0.00 ^{a}	1.06±0.00 ^{d}	47.36±0.19 ^{c}
	Deliblato sands		1.99±0.04 ^{fg}	0.89±0.12 ^{gh}	0.24±0.00 ^{c}	1.07±0.00 ^{d}	43.42±0.65 ^{a}
	Pančevo		1.77±0.03 ^{d}	1.17±0.14 ^{ij}	0.24±0.00 ^{c}	1.07±0.01 ^{d}	44.66±0.20 ^{b}
	Bogovo gumno		1.88±0.04 ^{e}	1.68±0.03 ^{l}	0.22±0.01 ^{ab}	1.08±0.01 ^{de}	41.83±1.14 ^{a}
Paeonia peregrina (LPP)	Krivi vir	UAE	1.99±0.03 ^{fg}	0.76±0.07 ^{ef}	0.30±0.01 ^{ef}	1.16±0.00 ^{k}	50.50±0.35 ^{e}
	Pirot		2.04±0.04 ^{g}	1.08±0.01 ^{i}	0.32±0.01 ^{fg}	1.13±0.00 ^{i}	54.33±0.43 ^{i}
	Pančevo		2.05±0.04 ^{g}	1.14±0.12 ^{ij}	0.27±0.01 ^{d}	1.21±0.00 ^{l}	53.11±0.64 ^{gh}
	Bogovo gumno		2.03±0.02 ^{g}	1.30±0.05 ^{k}	0.25±0.01 ^{cd}	1.08±0.01 ^{de}	55.85±0.18 ^{j}
	Krivi vir	MAC	1.91±0.04 ^{ef}	1.19±0.13 ^{ij}	0.27±0.01 ^{d}	1.21±0.00 ^{l}	52.58±0.55 ^{g}
	Pirot		1.94±0.04 ^{ef}	1.09±0.11 ^{ij}	0.31±0.01 ^{f}	1.15±0.01 ^{jk}	55.99±0.67 ^{j}
	Pančevo		1.95±0.03 ^{f}	1.03±0.06 ^{i}	0.27±0.00 ^{d}	1.10±0.00 ^{g}	57.36±0.09 ^{k}
	Bogovo gumno		1.97±0.07 ^{fg}	1.14±0.15 ^{ij}	0.30±0.00 ^{ef}	1.07±0.00 ^{d}	57.67±0.13 ^{k}
	Krivi vir	MAE	2.05±0.02 ^{g}	0.61±0.02 ^{d}	0.29±0.00 ^{e}	1.06±0.00 ^{d}	51.77±0.84 ^{fg}
	Pirot		1.92±0.04 ^{ef}	0.89±0.04 ^{g}	0.30±0.00 ^{ef}	1.10±0.00 ^{g}	57.71±0.54 ^{k}
	Pančevo		1.99±0.09 ^{fg}	0.80±0.02 ^{fg}	0.31±0.01 ^{f}	1.15±0.00 ^{jk}	59.18±1.13 ^{l}
	Bogovo gumno		1.89±0.02 ^{e}	1.01±0.08 ^{i}	0.33±0.01 ^{g}	1.10±0.00 ^{g}	58.72±0.31 ^{l}
Paeonia officinalis (LPO)	Rujevica	UAE	1.99±0.04 ^{fg}	1.53±0.11 ^{l}	0.32±0.01 ^{fg}	1.09±0.00 ^{ef}	54.00±0.60 ^{hi}
	Božurna		1.86±0.02 ^{de}	1.18±0.04	0.31±0.00 ^{f}	1.09±0.00 ^{ef}	54.28±1.62 ^{hi}
	Rujevica	MAC	1.95±0.03 ^{f}	1.49±0.18 ^{kl}	0.32±0.01 ^{fg}	1.12±0.00 ^{h}	54.50±0.36 ^{i}
	Božurna		1.89±0.01 ^{e}	1.22±0.04 ^{j}	0.31±0.01 ^{f}	0.98±0.00 ^{a}	54.69±0.29 ^{i}
	Rujevica	MAE	1.95±0.02 ^{f}	0.93±0.03 ^{h}	0.31±0.00 ^{f}	1.08±0.00 ^{d}	54.62±0.66 ^{i}
	Božurna		1.82±0.02 ^{d}	1.11±0.14 ^{ij}	0.32±0.01 ^{fg}	1.09±0.00 ^{ef}	54.14±0.55 ^{hi}

UAE: ultrasound-assisted extraction; MAC: maceration; MAE: microwave-assisted extraction; GALAE: galanthamine equivalents; ACAE: acarbose equivalents; KAE: kojic acid equivalents; AChE: acetylcholinesterase; BChE: butyrylcholinesterase; values with the same letter ($^{a\text{-}l}$) in each column showed no statistically significant difference ($p > 0.05$, $n = 3$, where the p-value is defined as the probability under the assumption of no effect or no difference (null hypothesis) of obtaining a result equal to or more extreme than what was observed; the n value is defined as the number of repetitions, one-way ANOVA, analysis of variance, Duncan's *post hoc* test).

2002; Da Silva and Ming, 2005). Thus, the influence of various compounds on tyrosinase activity is important in treating cutaneous hyperpigmentation. Also, the inhibitors of tyrosinase have a significant impact on some neurological disorders, such as Parkinson's disease (Pan et al., 2011).

The tested extracts of LPT were slightly potent against tyrosinase (Table 9). Namely, the extracts of LPT obtained by the MAE had the weakest effect, with the highest concentration of 41.83 ± 1.14 mg KAE/g (the extract of LPT collected from Bogovo gumno) and 47.36 ± 0.19 mg KAE/g (the extract of LPT collected from Gulenovci). The other two groups of the tested extract generate values in the range from 48.75 ± 0.33 to 51.28 ± 0.23 mg KAE/g, and 46.85 ± 1.08 to 53.78 ± 0.08 mg KAE/g. Among these, the highest inhibitory effect on tyrosinase was achieved by the LPT extract collected from Pančevo (obtained by the UAE), with a concentration of 53.78 ± 0.08 mg KAE/g. Further, the tyrosinase inhibitory activity of the studied extracts of LPP (for all three extraction methods) ranged from 50.50 ± 0.35 to 59.18 ± 1.13 mg KAE/g. Therefore, the highest tyrosinase inhibitory activity was found in the extract of LPP collected from Pančevo (obtained by the MAE). The extracts of LPO show a similar pattern to the previous group of the tested analytes; the strongest inhibitory activity was found at the concentration of 54.69 ± 0.29 mg KAE/g, which can be assigned to the extract of LPO collected from Božurna (obtained by the MAC).

3.7. Molecular docking

To gain insights into the compounds identified in the leaf extracts of wild herbaceous peony species that contribute most to the overall potency of the extracts, molecular docking simulations were performed for the compounds listed previously in Table 6 into the active sites of AChE, BChE, glucosidase, amylase, and tyrosinase. The heatmap presented in Fig. 4 revealed that these compounds express high binding affinities across all five enzymes, with the most pronounced affinity observed for BChE. Galloyl paeoniflorin (–11.7 kcal/mol) showed the highest affinity for BChE, which is primarily attributed to its rich pattern of non-covalent interactions with the macromolecular target. These interactions include hydrophobic contacts with Leu286, Trp231, Phe398, Phe329, Ile 442, and Trp82 and hydrogen bonding interactions with Gly115, Gly 121, Tyr 332, Asn68, Asn83, Tyr440, and Trp82 (Fig. 5). Notably, interactions with Trp82 and Phe329 from the anionic site, as well as Tyr332 from the peripheral anionic site (PAS) of BChE, emerged as particularly prominent.

Compounds that exhibited high affinities towards other macromolecular targets examined in this study include digallic acid, with a binding affinity of –10.0 kcal/mol for AChE; mudanpioside J, with a binding affinity of –9.4 kcal/mol for amylase; mudanpioside B, with a binding affinity of –8.5 kcal/mol for glucosidase; and galloyl

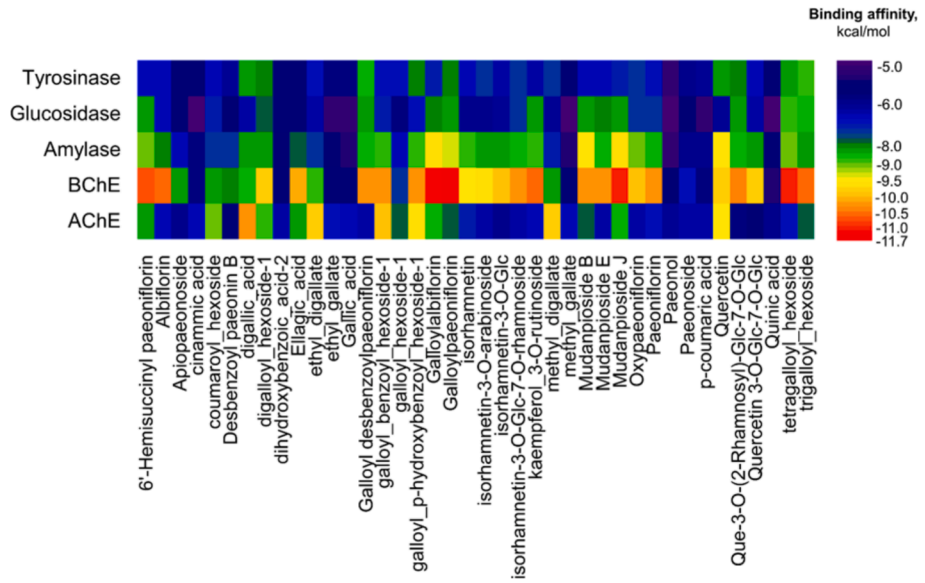

Fig. 4. AutoDock Vina binding affinities of individual compounds from the leaf extracts of herbaceous peonies (*Paeonia tenuifolia* L., *Paeonia peregrina* Mill., and *Paeonia officinalis* L.) to experimentally evaluated enzymes, tyrosinase, glucosidase, amylase, butyrylcholinesterase (BChE), and acetylcholinesterase (AChE). More negative values indicate a higher affinity of the ligands for the respective enzyme.

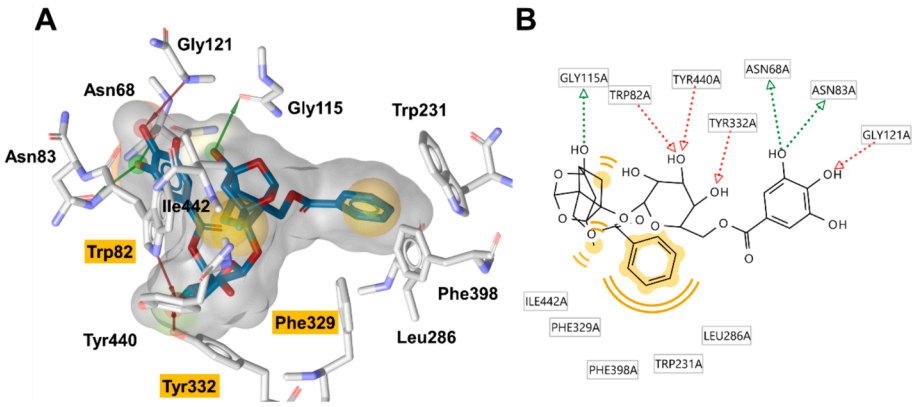

Fig. 5. **(A)** The binding mode of galloyl paeoniflorin into the BChE (butyrylcholinesterase) active site (PDB ID: 4TPK); **(B)** 2D ligand interaction diagram for this protein–ligand complex. Hydrogen bond donor, hydrogen bond acceptor, and hydrophobic interactions are depicted as green arrows, red arrows, and yellow spheres, respectively.

desbenzoylpaeoniflorin, with a binding affinity of –8.6 kcal/mol for tyrosinase. Detailed information on their respective binding modes is provided in Figures S11-S14.

4. Discussion

Medicinal plants and their formulations (extracts, decoctions, infusions, etc.) have been primary sources of antioxidants, such as gallic acid, quercetin, and isorhamnetin, which are among the most effective bioactives currently available (Bajpai et al., 2005). Nevertheless, peonies have been the main ingredient in most Chinese medical formulations throughout history and are still an important source of biotherapeutics that are being tested in clinical trials, specifically as antibacterial and anti-inflammatory agents (Yan et al., 2021). The herbaceous species that are grown in the Central Balkan region include *P. tenuifolia, P. peregrina*, and *P. officinalis* (Marković et al., 2023). They have potential uses that are similar to those of their Chinese herbaceous counterparts, including food and medical applications. However, there is little evidence that supports their traditional use in Serbia (Lazarević and Stojanović, 2012). It is commonly known that a variety of factors, such as the origin of plant material, harvest time, climatic/environmental conditions, and investigated plant parts, as well as experimental setup, can affect a plant chemical profile (AL-Hmadi et al., 2021; Sbieh et al., 2022). Therefore, in the current research, the leaves of *P. tenuifolia, P. peregrina*, and *P. officinalis* that were collected from wild localities in Serbia were screened for their content of polyphenol derivatives, proteins, and sugars, as well as antioxidant, antibacterial, and enzyme-inhibitory activities.

All tested extracts exhibited a notable change in polyphenol yield depending on the plant species. *P. officinalis* provided the extracts with the highest TPC, followed by *P. peregrina* and *P. tenuifolia*. The impact of locality on the TPC was also significant. A possible explanation of the significant differences is that the content of complex polyphenol structures (gallic acid derivates and their esters forms) can vary, and it depends on plant material, the region, and specific geomorphological features. Among the extraction procedures, the MAE gave the samples with significantly higher TPC, followed by the MAC and UAE. Numerous scientific studies give advantages to the MAE method due to its benefits, such as a short extraction time, followed by a reduced amount of extraction agent, and higher extraction efficiency (Asofiei et al., 2016;

Dandena et al., 2014; Milutinović et al., 2015; Simić et al., 2016; L. Wang and Weller, 2006). Chakma et al. (2023) have also shown that a higher extraction yield of bioactives from *Stevia* leaves was achieved using elevated temperatures in comparison to the conventional maceration technique. Additionally, when a solid substance and extraction agent heat up, the high vapor pressure of free water molecules in the plant substrate breaks the cell wall and accelerates the release of the matter into the extraction medium (Jovanović et al., 2016; Wang and Weller, 2006). Although literature data showed the advantages of UAE, including increased extraction yield, improved extract quality, fast kinetics, lower price, simplicity, and employment of a wide range of mediums (Jovanović et al., 2017; Khan et al., 2010; Lee et al., 2013), the results from this study suggest that the UAE procedure (performed in an ultrasound bath) provided significantly lower values of TPC compared to the MAE and MAC methods. The cause of this phenomenon is well recognized and reinforced by the scientific literature (Horžić et al., 2012; Jovanović et al., 2017). Namely, the disadvantages of UAE include the degradation of polyphenol compounds by ultrasound waves and the production of free radicals, dependence on the characteristics of plant material and consequently, contribution to the ultrasound wave attenuation by the presence of a larger quantity of plant particles that causes the restriction of the active part of ultrasound inside the zone located in the vicinity of the ultrasonic emitter (Horžić et al., 2012; Jovanović et al., 2021; Wang and Weller, 2006). Additionally, the impact of all used extraction parameters and the extraction time, the temperature regime, and the uniformity of the process temperature is not negligible and significantly affects the extraction efficiency, resulting in different extraction yields between various extraction procedures (Jovanović et al., 2022).

These results are more or less similar to the findings of Chakma et al. (2023), who found that binary water–ethanol solvent had the highest efficiency for extracting TPC from *Stevia* leaf sample when extraction on a shaker was used.

The LPP and LPO extracts showed higher values of total flavonoids after the MAE, while in LPT samples, the mentioned method provided the extracts with lower TFC. A possible explanation for this phenomenon is that the MAE method influences the release of the polar molecules in the polar extraction medium by simultaneously increasing the internal pressure of the solid and producing high extraction efficiency. More specifically, the polar flavonoids in the extract that are exposed to high temperatures during the extraction process have a stronger affinity for the solvent, resulting in easier degradation than those with a lower affinity for the solvent. Moreover, in some literature reports, it can be found that high temperatures (≥100 °C) during MAE favor the degradation of flavonoids, especially catechin (Burns et al., 2000; Pulido et al., 2000) and epicatechin (Piñeiro et al., 2004). In contrast, Liazid et al. (2007) showed that some flavonoids, such as kaempferol, are stable above 100 °C. It is generally accepted that the power of applied microwaves and the time of microwave application affect the MAE of flavonoids (Routray and Orsat, 2012). The extraction of flavonoids has been found to increase as microwave power is increased (Routray and Orsat, 2014). Similarly, the temperatures of the solvent-analyte mixture and the cellular disintegration are increased as the time of microwave application is increased. The dielectric properties of solvents can also significantly influence the extraction of flavonoids (Routray and Orsat, 2012). However, these results and data related to the impact of different extraction procedures on the flavonoid yield represent a promising step towards the utilization of natural flavonoid derivatives from peony leaf extracts rather than chemical synthetic substances in food and pharmaceutical applications.

In the present study, there was no significant difference in the TTC values between all prepared extracts (except in the case of Deliblato sands, which gave the lowest tannin yield). There is an assumption that the lower content of tannins could be influenced by the reduced extractability of presented tannins (caused by changes in tannins' chemical reactivity) (Udensi et al., 2007). To the best of the authors' knowledge, there are no contemporary publications on TTC in herbaceous peonies. Apart from some biological and environmental factors (plant age, genotype, seasonal changes, etc.) that may influence the TTC and their accumulation in plant material, the temperature during plant growth (in controlled conditions) may also affect them. The chemical structure and degree of polymerization of tannins (condensed forms) can affect their solubility in organic solvents (Besharati et al., 2022). For instance, in the crude fractions of *Paeonia emodi* Wall. ex-Royle, the TTC varied from 0.002 to 0.005 mg/mL, depending on the nature and polarity of the used solvent (Uddin et al., 2013).

The highest protein contents were detected in the LPO extract by the UAE (Rujevica) and the LPP extract by the MAC (Pančevo), which did not differ. By reviewing the literature, it can be found that microwave irradiation can be well used to break up plant cells and may be applied to extract cellular substances, such as simple carbohydrates and proteins. This phenomenon can be explained by the fact that during the MAE process, the intracellular temperature of plant material rises sharply, which causes the intracellular pressure to rise above what the cell wall can withstand. As a consequence, plasmatorrehexis occurs, and the intracellular components diffuse and dissolve in the extraction agent, enabling great extraction efficiency (Zhang et al., 2014). However, this kind of action failed to happen in our experiments. Probably, boiling caused by microwave irradiation (MAE) can intensify the denaturation of peony cellular proteins and unstable complexes of cell pigments bound to proteins. Some investigations show that these complexes are unstable and tend to be converted to stable forms, such as pheophytin (Komolafe and Obayanju, 2003; Lola, 2009), which are unable to react with the Folin reagent, so the lower protein concentration can sometimes be underrated.

In the case of *Peaonia* leaf extracts, the MAC was the most efficient extraction procedure to obtain higher yields of sugars. Due to its long extraction time, it promotes the solubilization of other poorly soluble sugar derivates, which, together with lower temperatures, reduces the possibility of a Maillard reaction between sugars and proteins, resulting in the extraction of more carbohydrates. There were only two studies estimating the total sugar content in the leaves of herbaceous peonies, both were conducted on *P. lactiflora* using the colorimetric method (Walton et al., 2010; Li et al., 2019); the detected secondary sugars were monosaccharides (fructose and glucose) and disaccharide (saccharose).

Summarising the results of antioxidant activity, it can be seen that the highest DPPH$^{\bullet}$ scavenging activity showed LPT collected from Gulenovci and LPP collected from Pančevo and Bogovo gumno. The primary factors influencing the antioxidant test results are the complex structures of polyphenol molecules, the extraction method used, and the possible interaction between the sample's antioxidants and free radicals (Piluzza and Bullitta, 2011). Comparing the LPO extracts, it is clear that the UAE and MAE were the most successful approaches considering the results of the DPPH scavenging potential. According to the literature data, microwave extraction was shown to be an effective procedure to achieve the highest polyphenol yield and antioxidant potential, due to the reduced activity of enzymes such as polyphenol oxidases by microwave treatment (Riguene et al., 2023). It should be noted that the paper of Dienaitė et al. (2019) is the only one that describes the DPPH$^{\bullet}$ scavenging capacity of leaf extract of herbaceous peonies. Al Qaisi et al. (2024) have reported that the ability of wild medicinal plant extracts to scavenge free radicals may be attributed to their high amounts of polyphenol and flavonoid compounds. On the other hand, the TAC assay is a very common and significant indicator of the health benefits of medicinal plants and other natural compounds related to food (mushrooms, beverages, fruit juices, etc.). Still, it is crucial to identify and measure the antioxidant potential of plants from several perspectives (Tütem et al., 2014). The phosphomolybdenum test describes the reduction of Mo(VI) to Mo(V) and observes a corresponding change in the absorbance, which was used to determine the total antioxidant capacity. Concretely, the results of the antioxidant activity of the extracts of LPO, evaluated by TAC, show that the UAE and MAE were more

effective in extracting strong antioxidants than MAC. Probably, the MAE and UAE extracts were more potent in antioxidant activity than MAC because the microwave and ultrasonic irradiation, and the higher temperatures during the extraction process, promoted higher diffusion rates and better solubility of antioxidant compounds in the solvent (Dorta et al., 2012). The study of Elakremi et al. (2023) also showed that the use of different extraction procedures resulted in statistically different antioxidant activities. To the best of the authors' knowledge, no articles have yet been published that analyze the antioxidant activity of leaf extracts of herbaceous paeonies evaluated by the phosphomolybdenum test.

In low- and high-income countries, food intoxications continue to be the primary and secondary causes of death, even though the use of antibiotics has reduced the severity and spread of many infectious diseases. The recent increase in our knowledge of the human gastrointestinal tract and microbiota has changed our view of antibiotics and synthetic anti-inflammatory drugs. Antibiotic resistance is the result of uncontrolled and widespread antibiotic usage (Ianiro et al., 2016). Therefore, it is not surprising that the World Health Organization estimates that by half of the 21st century, the number of deaths worldwide from antibiotic resistance will rise from 700,000 to 10,000,000 annually (de Kraker et al., 2016). Examples of known pathogens that develop antibiotic resistance are *S. aureus*, *P. aeruginosa*, and *E. coli* (de Kraker et al., 2016). For that reason, the leaf extracts of three *Paonia* species were assessed as a potential source of antibacterial agents intended for use in the treatment of disorders of the human gastrointestinal tract, as a natural alternative to synthetic antibiotics. The results show that LPT (Deliblato sands) extract had the highest antibacterial activity against *S.* Typhimurium and *P. aeruginosa*, while the LPP and LPO extracts showed a lower ability to inhibit the degree of bacterial growth, both being the most effective against *S. aureus, S. typhimurium, E. coli,* and *L. monocytogenes*. Further, analyzing the results of antimicrobial activity, it is evident that the extraction method proven to produce the most effective antimicrobial agents is UAE. It could be due to the fact that the cavitation process during ultrasonication causes swelling and rupture of plant cell walls, allowing either higher rates of diffusion through the cells or washing out of the content, thus making various bioactive components available, in this case, the ones with antimicrobial potential (Khan et al., 2010). Previous studies have shown that the leaves of the herbaceous peony *P. lactiflora* have strong antibacterial effects (Liu et al., 2022; Zhou et al., 2019). It can also be found that the ethanolic extract of *P. officinalis* (aerial parts) has strong antimicrobial potential against *P. aeruginosa* compared to the standard antimicrobial agent, meropem (Samy et al., 2022). On the other hand, there has been only one study that evaluated the antimicrobial activity of extracts of *Paeonia japonica* against *B. subtilus, E. coli,* and *S. aureus* using the agar diffusion method. Furthermore, these results are consistent with those of Sbieh et al. (2022), who found that an alcoholic extract of the aerial parts of some endemic species native to the countries of the Arabian Peninsula, *i.e.*, *Chiliadenus iphinoides,* had a broad spectrum of antibacterial activity (strongly potent against *E. coli, P. aeruginosa,* and *S. aureus*). Also, earlier reports have indicated that plant-dominant structures, such as polyphenols, are more susceptible to disruption of the bacterial cell wall of both Gram-positive and Gram-negative bacteria since polyphenol compounds contain the hydroxyl functional group and the benzene ring, which readily penetrate the bacterial cell and crosslink with the enzymes resulting in cell death (Chakma et al., 2023).

In a multitude of results, it is evident that the UAE extracts of LPT collected from Gulenovci and Pančevo might be emphasized as the most successful candidate that could mitigate or slow neurodegeneration in Alzheimer's disease. Other tested extracts do not show statistically significant results. In addition, some literature reports suggest that some active ingredients contained in peonies, such as paeoniflorin and its derivatives, may be responsible for noncovalent interactions with the macromolecular receptor and may increase this effectiveness against AChE and BChE (Montanari et al., 2019). Also, it can be found that the aerial parts of *Paeonia kesrounansis* can strongly inhibit AChE and BChE activity, and act as excellent neuroprotectants (Sut et al., 2019).

The capacity of LPT, LPP, and LPO extracts to inhibit of alpha-amylase was similar. Sut et al. (2019) report that the non-direct relationship between phenolic quantity and activity may be due to the unique activities of select molecules that are responsible for the observed effects, or to the synergistic effects of the various extract constituents. Additionally, Zengin et al. (2017) reported that there is a direct correlation between the nature/polarity of the solvent and anti-enzymatic activity (it is preferred that the organic compounds in the extract that modulate enzymatic activity be highly extractable). Recent literature inducate that the alpha-amylase inhibitory property of *P. mascula* can also be attributed to the presence of gallic acid, the most abundant active compound in *Paeonia* species (Abdel-Moneim et al., 2022; Kurt-Celep et al., 2023). Additionally, gallic acid has been early studied and shown to decrease alpha-amylase activity, increase glucose-induced insulin secretion and glucose uptake in peripheral blood *in vitro*, as well as serum insulin levels and liver glycogen *in vivo* (Abdel-Moneim et al., 2022). On the other hand, Zhang et al. (2023) reported the synergistic and complementary effects of paeoniflorin and berberine in the treatment of diabetes mellitus type 2.

The results related to the glucosidase inhibitory activity of the prepared *Paeonia* extracts are not in agreement with the previous studies on this topic. Namely, the findings from Sut et al. (2019) show that ethyl acetate extracts of the aerial parts of *P. arietina* and *P. kesrouanensis* had about nine times higher glucosidase inhibitory effects compared to our results. One of the possible explanations for this non-correlation is that some of the phenolic molecules that cause the observed effects are more soluble in ethyl acetate than in their alcohol counterparts (in this study, ethyl alcohol was used as a solvent). Additionally, the main analyte nature (herbaceous *Paeonia* species) and its chemical composition (concentration and ratio between secondary metabolites) can affect the outcomes of the anti-enzymatic activity.

Regarding the results of the *Paeonia* extracts' effect on tyrosinase, some literature reports indicate that the methanolic extract of aerial parts of *P. kesrouanensis* generated the highest inhibition of tyrosinase, about 145.43 mg KAE/g (Sut et al., 2019). Also, by reviewing the literature, it can be found that extracts of aerial parts of some specific herbaceous varieties, such as Greek *Paeonia mascula* ssp. *hellenica*, generate high anti-enzymatic potential towards tyrosinase (Chaita et al., 2017). Probably, the complex polyphenol structures presented in plant extract block hydroxylation of L-tyrosine and oxidation of L-DOPA, which are the factors that intensify the production of reactive oxygen species (Sturm et al., 2001). However, some research suggests that certain flavonoid glycosides (precisely some derivates of kaempferol) extracted from the generative organs of *P. lactiflora* can inhibit fungal tyrosinase more potently than conventional inhibitors like kojic acid (Magid et al., 2017).

5. Conclusion

In the current study, the extracts have been obtained from *P. tenuifolia, P. peregrina*, and *P. officinalis* leaves using three extraction methods (UAE, MAC, and MAE). This work succeeded in identifying different classes of organic molecules that constitute the different leaf extracts. Quercetin-3-*O*-pentoside and quercetin-3-*O*-hexoside-7-*O*-rhamnoside were the primary constituents of the LPT and LPO extracts, while the dominant molecule in the LPP extract is quercetin-3-*O*-hexoside-7-*O*-rhamnoside. The highest polyphenol yield was achieved in LPO extract from Božurna, using the MAE method. The results of this study confirm the biological properties of LPT, LPP, and LPO extracts. In terms of antioxidant activity, although the observed differences were smaller, better antioxidant capacities were also determined in the LPP from Pančevo and Bogovo gumno when the UAE was implemented. The antibacterial assay shows that LPO extracts obtained from Rujevica were the most effective analytes against the pathogen strains *L. monocytogenes*

and *S. aureus*. The highest enzyme-inhibitory activity towards cholinesterases was found in the extracts of LPT from Gulenovci and Pančevo, obtained by the MAC method. Molecular docking simulation identifies the peony compounds with the highest affinities for the five investigated enzymes, accentuating the high binding affinity of galloyl-paeoniflorin against BChE. Overall, this research provides endless opportunities for the use of leaf extracts in different fields, such as medicine, food, or pharmacy. Nevertheless, before their use for edible/nutritional and medicinal purposes, it will be necessary to perform more in-depth studies on the properties of these plant entities, especially on their side effects and toxicity.

CRediT authorship contribution statement

Petar Batinić: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Aleksandra Jovanović:** Writing – review & editing, Writing – original draft, Software, Conceptualization. **Dejan Stojković:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Natalija Čutović:** Writing – original draft, Methodology, Investigation, Formal analysis. **Ilija Cvijetić:** Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Uroš Gašić:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis. **Tamara Carević:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Gökhan Zengin:** Software, Resources, Methodology, Investigation, Formal analysis. **Aleksandar Marinković:** Writing – review & editing, Supervision, Resources. **Tatjana Marković:** Writing – original draft, Supervision, Resources, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2024.102090.

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