https://doi.org/10.31925/farmacia.2026.4.16 **ORIGINAL ARTICLE**

COMPARATIVE EVALUATION OF EXTRACTION TECHNIQUES FOR THE PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF *VIOLA TRICOLOR* L. EXTRACTS

PATRICIA MONICA BOZGA [1], ANA-MARIA VLASE [2], RADU COSMIN BOZGA [1]*, IONUȚ VENTER [1], BOBU ADRIAN [4], LAURIAN VLASE [3], FLORIN BĂNICĂ [5], SEBASTIAN NEMETH [1,5]

[1]*Doctoral School of Pharmaceutical Sciences, University of Oradea, 410087, Oradea, Romania*
[2]*Department of Pharmaceutical Botany, Faculty of Pharmacy, Iuliu Haţieganu University of Medicine and Pharmacy, 8 Victor Babeş Street, 400012, Cluj-Napoca, Romania*
[3]*Department of Pharmaceutical Technology and Biopharmacy, Faculty of Pharmacy, Iuliu Haţieganu University of Medicine and Pharmacy, 8 Victor Babeş Street, 400012, Cluj-Napoca, Romania*
[4]*Faculty of Automatic Control and Computers, National University of Science and Technology Politehnica, 060042, Bucharest, Romania*
[5]*Department of Pharmacy, Faculty of Medicine and Pharmacy, University of Oradea, 410073, Oradea, Romania*

**corresponding author: bozga.raducosmin@student.uoradea.ro*

Manuscript received: May 2026

Abstract

Viola tricolor L. is a medicinal plant with a long history of traditional use and is recognized as a valuable source of antioxidant phenolic compounds. This study investigated the influence of the extraction technique on the phenolic composition and antioxidant capacity of extracts obtained from the aerial parts of the plant. Five extraction techniques (maceration, microwave, Soxhlet, extraction assisted by ultrasound, and turbo-extraction) were comparatively evaluated by HPLC-MS, determination of total phenolic and flavonoid content, and antioxidant assessment using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and differential pulse voltammetry. The phenolic profile was dominated by rutin (up to 170 µg/mL), together with quercetin glycosides and phenolic acids. Maceration yielded the highest total phenolic content (1.17 mg GAE/mL) and flavonoid (0.29 mg RE/mL) contents, as well as the greatest antioxidant capacity (4.03 mg and 5.61 mg AAE/mL by DPPH assay and differential pulse voltammetry, respectively), followed by Soxhlet extraction. The strong correlation between the antioxidant assays confirmed the reliability of the analytical evaluation, identifying maceration as the most efficient extraction technique for recovering antioxidant compounds from *Viola tricolor* L.

Rezumat

Viola tricolor L. este o plantă medicinală cu utilizare îndelungată în medicina populară, recunoscută ca sursă valoroasă de compuși fenolici cu proprietăți antioxidante. Acest studiu a investigat influența tehnicii de extracție asupra conținutului de compuși fenolici și a capacității antioxidante a extractelor obținute din părțile aeriene ale plantei. Au fost evaluate comparativ cinci tehnici de extracție (macerare, extracție Soxhlet, extracție asistată de ultrasunete, microunde și turbo-extracție) utilizând analiza HPLC-MS, determinarea conținutului total de polifenoli și flavonoide și evaluarea capacității antioxidante prin metoda 2,2-difenil-1-picrilhidrazil (DPPH) și voltametrie puls diferențială. Profilul fenolic a fost dominat de rutozidă (până la 170 µg/mL), alături de glicozide ale quercetinei și acizi fenolici. Macerarea a înregistrat cele mai mari valori: 1,17 mg GAE/mL polifenoli, 0,29 mg RE/mL flavonoide și o capacitate antioxidantă de 4,03 mg AAE/mL prin metoda DPPH, respectiv 5,61 mg AAE/mL prin voltametrie puls diferențială, fiind urmată de extracția Soxhlet. Rezultatele celor două metode au prezentat o corelație puternică, confirmând fiabilitatea determinării. Aceste rezultate evidențiază macerarea drept cea mai eficientă pentru extragerea compușilor antioxidanți din *Viola tricolor* L.

Keywords: *Viola tricolor* L., rutin, polyphenols, differential pulse voltammetry, HPLC-MS

Introduction

Viola tricolor L. (*Violaceae*), commonly known as wild pansy, is a herbaceous species widely distributed throughout the temperate regions of Europe and Asia, with a long history of use in traditional medicine (1). Its aerial parts have been used to treat respiratory disorders and dermatological conditions; effects largely attributed to its high content of phenolic compounds (2). The high phenolic content, together with the significant antioxidant activity, has generated increasing scientific interest over recent decades, establishing *Viola tricolor* L. as a valuable natural source of bioactive compounds with potential pharmaceutical and nutraceutical applications (3).

Phenolic compounds, mainly flavonoids and phenolic acids, constitute one of the most important classes of plant secondary metabolites with antioxidant

properties, due to their ability to neutralize reactive oxygen species and limit the oxidative processes involved in numerous chronic diseases (4).

The phenolic profile of *Viola tricolor* L. is predominantly composed of flavonol glycosides, particularly rutin, together with quercetin derivatives and phenolic acids, compounds that are recognized for their significant contribution to the antioxidant activity of the extracts (5, 6). The recovery of phenolic compounds depends directly on the extraction method, which determines both the yield and composition of the resulting phenolic fraction. Extraction parameters, including temperature, solvent-matrix contact time, and the mode of energy transfer to the plant material, influence both the release of phenolic compounds and the preservation of their structural integrity (7). Classical methods, such as maceration and Soxhlet extraction, are used in parallel with techniques based on ultrasound, microwaves, or mechanical homogenisation, each providing a different balance between extraction efficiency, processing time, and preservation of labile compounds (8). The choice of extraction technique is therefore a crucial step in obtaining extracts rich in active ingredients.

A comprehensive characterization of extracts requires the combination of several complementary analytical methods. The identification and quantification of individual compounds are achieved by high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS), an advanced technique that combines the efficient separation of complex mixtures with structural confirmation based on the mass-to-charge ratio, providing the most detailed information on sample composition (9). This analysis is complemented by the determination of total polyphenol and flavonoid content, which reflects the overall phenolic load, and by the assessment of antioxidant activity, which estimates the functional capacity of the extract (10).

Although the phytochemical profile of *Viola tricolor* L. is well documented, a comparative evaluation of multiple extraction techniques using the same set of determinations provides additional insight into how the choice of method influences the phenolic content and antioxidant capacity of the extracts. Such an approach, applied to plant material collected from a specific region, may contribute to the identification of the most suitable technique for recovering the active constituents of the species.

This study aimed to compare five extraction techniques (maceration, microwaves, Soxhlet, extraction assisted by ultrasound and turbo-extraction) applied to the aerial parts of *Viola tricolor* L. The resulting extracts were characterized by HPLC-MS, total phenolic and flavonoid content determination, and antioxidant capacity assessment using the DPPH assay and differential pulse voltammetry, in order to identify the most efficient extraction method.

Materials and Methods

Plant material

The plant material was collected from the spontaneous flora of Bihor County, Husasău de Tinca commune, Sititelec village, Romania, 46.886105, 21.900203 (46°53'10.0"N 21°54'00.7"E), an unpolluted area, in July, 2025, during the flowering stage. The aerial parts, consisting of flowering stems with leaves and flowers, were collected and cleaned of foreign matter. One specimen was pressed and is preserved in the herbarium of the Faculty of Medicine and Pharmacy, Department of Pharmaceutical Botany, UOP code 05.709. The aerial parts were dried naturally at room temperature (20 - 25°C), in a well-ventilated room protected from direct sunlight, for 10 days, until a constant mass was achieved, defined as a difference of less than 0.1% between two successive weighings performed at 24 h intervals. The residual moisture content of the dried material was 8.7%, determined by drying at 105°C to constant mass. After drying, the plant material was finely ground using a mortar to increase the surface area and passed through sieve V (Romanian Pharmacopoeia, 10^{th} edition, nominal aperture 0.315 mm, wire diameter 0.2 mm, 362 openings/cm^2). The powdered material was stored in hermetically sealed amber glass containers, protected from light, at room temperature, until extraction.

Extraction methods

All five extraction methods used were applied to identical quantities of plant material and solvent, maintaining a plant material: solvent ratio of 1:10 (w/v), with 70% (v/v) ethanol used as the extraction solvent. At the end of each extraction, the solutions were filtered under vacuum through filter paper with a pore size of 15 - 17 µm, and the volume was adjusted back to its initial value with the same solvent, to strictly maintain the 1:10 (w/v) plant material to solvent ratio and ensure comparability among the extracts. A single extraction was performed for each technique, and the resulting extract was analysed in triplicate in all subsequent determinations, and the reported values therefore represent analytical replicates. The extracts were stored at 4°C, protected from light. The spectrophotometric and electrochemical determinations were performed within 2 - 3 days of preparation, while the HPLC-MS analysis was carried out within 7 days.

Maceration. Maceration was carried out according to the protocol described in the Romanian Pharmacopoeia, 10^{th} edition. The ground plant material was placed in a sealed amber glass container, to which the extraction solvent was added, and the system was kept at room temperature (20 - 25°C), protected from light, for 10 days, with periodic manual agitation (3 - 4 times a day, approximately 2 minutes each time) to enhance mass transfer (11, 12).

Microwave-assisted extraction. Microwave-assisted extraction was performed in a microwave oven (Samsung, model MS23K351AW/OL, Dublin, Ireland), using a two-stage irradiation regimen: 5 minutes at high power (750 W), followed by 10 minutes at medium power (450 W) (13). The extraction was carried out in a 250 mL borosilicate glass flask containing 5 g of powdered plant material and 50 mL of solvent. The flask was connected to a reflux condenser, and the temperature of the solvent was monitored with a digital thermometer, reaching a maximum of 70°C during irradiation.

Soxhlet extraction. The extraction was performed in a standard Soxhlet apparatus; the powdered plant material was placed in a cellulose cartridge (28 × 80 mm, Filter-Lab). The round-bottom flask contained 70 mL of solvent. The process was carried out over 12 successive cycles, each lasting around 40 minutes, for a total extraction time of approximately 8 hours (14, 15).

Ultrasound-assisted extraction. Ultrasound-assisted extraction was carried out by sonication for 10 minutes in an ultrasonic bath (Polsonic, Sonic 3 model, series 136443, frequency 40 kHz, power 310W, Warsaw, Poland) (16, 17). Sonication was performed without external heating or cooling, and the bath temperature increased from 24.2°C to 29.5°C during the 10 min treatment.

Turbo-extraction. High-speed homogenization was performed for 3 minutes at 9000 rpm using a turbo-extraction device (Biobase, model BK-HG160, serial number DA213AC000283, Shandong, China), in a 50 mL glass beaker. No cooling was applied during homogenization (18, 19).

HPLC-MS Analysis of Phenolic Compounds

The phenolic compounds were determined by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), using a Nexera X3 HPLC system (Shimadzu Corporation, Kyoto, Japan) coupled to a QTOF 9030 mass spectrometer (Shimadzu). Reference standards of phenolic compounds used in quantitative analysis were purchased from Sigma Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Supelco (Bellefonte PA, USA), and other commercial suppliers. Stock solutions of the reference standards were prepared in methanol, from which seven calibration levels were obtained by serial dilution, at concentrations of 0.05, 0.2, 0.8, 3.2, 12.8, 25.6, and 50 µg/mL. LC-MS-grade acetic acid and methanol were obtained from Merck KGaA (Darmstadt, Germany).

The separation was achieved using a Zorbax SB-C18 column (100 mm x 3.0 *i.d*, particle size 3.5 µm, Agilent Technologies), thermostated at 48°C. The mobile phase consisted of solvent A, 0.2% (v/v) acetic acid in water, and solvent B, 0.2% (v/v) acetic acid in methanol. Gradient elution was performed as follows: 0 - 30 minutes, a linear gradient from 5% to 70% B, followed by a re-equilibration step at 5% B for 4 minutes. The mobile phase flow rate was 1 mL/min, and the injection volume was 1 µL.

Mass spectrometry detection was carried out in negative ionization mode, using high resolution (HR-MS) and tandem fragmentation (HR-MS/MS) model, with a mass accuracy tolerance of 15 ppm. The electrospray ionization (ESI) source parameters were set as follows: source voltage, -2000 V; interface temperature, 300°C; spray gas flow rate, 3 L/min; heating gas flow rate, 15 L/min (air), drying gas flow rate, 15 L/min (nitrogen); desolvation line temperature, 300°C, heating block temperature, 400°C. The methodology was adapted from previously published analytical protocols for the determination of phenolic compounds (20-23).

Quantitative analysis was performed using a time-scheduled event specific to each available standard (beginning 0.8 min before and ending 0.8 min after its retention time), in HR-MS or HR-MS/MS mode. A compound was quantified only after its positive qualitative identification, based on Data Dependent Acquisition (DDA) spectral data. The results were expressed in micrograms of phenolic compound *per* milliliter of plant extract (µg/mL). Compounds for which an authentic standard was available were quantified against the corresponding calibration curve, whereas compounds lacking a specific standard were expressed as equivalents of the most structurally related reference compound. Data acquisition and processing were carried out using LabSolutions 5.137 software (Shimadzu Corporation, Kyoto, Japan). The analytical method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision. Linearity was evaluated over the concentration range of 0.05 - 50 µg/mL, and the LOD and LOQ were established at signal-to-noise (S/N) ratios of ≥ 10 and ≥ 20, respectively.

Total phenolic content

The total polyphenol content was evaluated using the Folin-Ciocâlteu spectrophotometric method (24, 25), and the results were expressed as acid equivalents. For each reference compound: gallic acid (Fluka Chemicals, Buchs, Switzerland) and caffeic acid (Sigma-Aldrich, Hamburg, Germany), a 0.5% (m/v) stock solution was prepared, dissolved in a 1:10 (v/v) ethanol (Chemical Company, Iasi, Romania) water mixture. The solutions were prepared on the day of analysis or stored at 4°C. Calibration standards corresponding to concentrations of 50, 100, 150, 200, 250 and 300 µg/mL were obtained by diluting the stock solutions.

A volume of 0.04 mL of each calibration standard or extract, diluted 1:5 (v/v), was mixed under vigorous agitation with 0.2 mL of Folin-Ciocâlteu reagent (Merck, Darmstadt, Germany) and 3.16 mL of distilled water. After 5 minutes of homogenization, 0.6 mL of 20% sodium carbonate (Reagent Bucharest, Bucharest,

Romania) solution was added. The reaction mixture was incubated at 20°C for 2 hours, after which the absorbance was measured at 765 nm against a reagent blank (26-28). For each reference standard, calibration curves were constructed by plotting absorbance against concentration. The total polyphenol content of each extract was calculated by interpolating the measured absorbance values onto the corresponding calibration curves, applying the appropriate dilution factor, and expressed as mg gallic acid/caffeic acid equivalents *per* millilitre of extract (mg/mL).

Total flavonoid content

The total flavonoid content was determined using the aluminium chloride colorimetric method (29, 30), with the result expressed in both rutin and quercetin equivalents, reference substances which were used. For rutin (Sigma-Aldrich, Hamburg, Germany), a stock solution of 1 mg/mL was prepared, from which, through successive dilutions, six calibration standards were obtained at 100, 200, 300, 400, 500 and 600 µg/mL. For quercetin (Sigma-Aldrich, Hamburg, Germany), a 1 mg/mL stock solution was prepared in ethanol, from which six standards were prepared at 50, 100, 150, 200, 250 and 300 µg/mL. Both sets of standards were subjected to the same reaction procedure.

A volume of 1.5 mL of each calibration standard or undiluted extract was mixed with 1.5 mL ethanol, 0.1 mL of 10% aluminium chloride (Merck, Darmstadt, Germany) solution, 0.1 mL of 1 M sodium acetate (Merck, Darmstadt, Germany), and 1.25 mL distilled water. The reaction mixture was maintained at room temperature for 60 minutes, after which the absorbance was measured at 420 nm (rutin)/415 nm (quercetin) against a reagent blank (31). The blank sample was prepared under identical conditions, replacing the standard solution with an equivalent volume of ethanol. A calibration curve (absorbance *versus* concentration) was constructed for each reference substance. The total flavonoid content of each extract was calculated by interpolating the measured absorbance on two curves, and expressed as mg rutin equivalents or mg quercetin equivalents *per* millilitre of extract (mg/mL).

Antioxidant activity

DPPH method

The antioxidant capacity of the extracts was evaluated using a spectrophotometric method based on the scavenging of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, measured in the visible spectrum at 517 nm (32, 33). Three reference substances were used to plot the calibration curves: ascorbic acid, gallic acid and caffeic acid. For each standard, an accurately weighed amount of the pure compound was dissolved in ethanol to obtain a primary stock solution (1 mg/mL); this was subsequently diluted with ethanol to the concentration of the working stock solution, corresponding to the highest concentration on the calibration curves. From each working stock solution, five calibration standards were prepared by successive dilutions in ethanol, as follows: ascorbic acid (Fluka Chemicals, Buchs, Switzerland) 20, 10, 5, 2.5 and 1.25 µg/mL, gallic acid 12, 6, 3, 1.5 and 0.75 µg/mL and caffeic acid 25, 12.5, 6.25, 3.125, and 1.5625 µg/mL. A freshly prepared DPPH (Cayman Chemical, Ann Arbor, MI, USA) solution (0.1 mM in ethanol) was used throughout the study. The solution was protected from light during all experimental procedures.

For each determination, 1 mL of sample, either a calibration standard solution or extract diluted between 1:50 and 1:400 (v/v) with ethanol, depending on the extract, was mixed with 3 mL DPPH solution, and the mixture was kept in the dark at room temperature for 30 minutes. The absorbance was then measured at 517 nm in quartz cuvettes with a 1 cm path length. The control sample was prepared by mixing 1 mL of ethanol with 3 mL DPPH solution, and ethanol was used as the blank (12, 34, 35). All determinations were performed in triplicate.

Calibration curves were constructed by plotting absorbance against the concentration of each reference standard. Sample dilutions were selected to ensure that the measured absorbance values fell within the linear range of the respective calibration curves. The antioxidant capacity of each extract was determined by interpolation of the measured absorbance on the calibration curves, followed by correction for the appropriate dilution factor and expressed as milligrams of standard equivalents (ascorbic acid, gallic acid and caffeic acid) *per* millilitre of extract (mg/mL).

Differential Pulse Voltammetry

The voltammetric measurements were performed using an Autolab PGSTAT128N potentiostat (Metrohm Autolab, Utrecht, the Netherlands), controlled by Nova 2.1.8 software. All electrochemical experiments were carried out in a 20 mL electrochemical cell, equipped with a conventional three-electrode system: an Ag/AgCl reference electrode, a platinum (Pt) auxiliary electrode, and a glassy carbon working electrode (GCE, diameter 3 mm, Metrohm Autolab, Switzerland) (36, 37). All measurements were conducted at room temperature (25 ± 2°C).

A Britton-Robinson (B-R) buffer solution was used as the supporting electrolyte over the pH range of 2 - 10. The buffer was prepared from a mixture of phosphoric acid (H_3PO_4), boric acid (H_3BO_3) and acetic acid (CH_3COOH), each at a concentration of 0.04 mol/L. The pH was adjusted using 0.01 mol/L NaOH and measured with a Metrohm 632 pH meter (Metrohm AG, Herisau, Switzerland) equipped with a combined glass pH electrode. All electrochemical measurements, including calibration standards and sample analyses, were performed at pH 3.0 (38).

Differential pulse voltammetry (DPV) measurements were performed over the potential range of 0.0 - 1.2 V *vs.* Ag/AgCl using a potential step of 10 mV, a pulse amplitude of 50 mV, and a pulse duration of 50 ms.

Calibration curves were established using three reference compounds: ascorbic acid (AA), gallic acid (GA), and caffeic acid (CA) (39, 40). Six standard solutions of each analyte were prepared from 10^{-3} M stock solutions in Britton-Robinson buffer. The concentration range was 0.047 - 0.166 mM for AA, 0.011 - 0.031 mM for GA, and 0.009 - 0.029 mM for CA. Differential pulse voltammograms were recorded under the optimized experimental conditions. Peak currents were measured relative to the corresponding baseline of each oxidation peak, and all measurements were carried out in triplicate. Before each measurement, the glassy carbon electrode surface was mechanically polished with 0.05 µm alumina slurry on a polishing pad and subsequently rinsed with ultrapure water to minimize electrode surface passivation resulting from the adsorption of phenolic compounds (41). The electrode was then immersed in the supporting electrolyte and allowed to equilibrate for 1 min before recording each voltammogram. For each extract, an appropriate sample volume was added to 10 mL B-R buffer in the electrochemical cell. The aliquot volume (50 - 120 µL, depending on the sample) was selected so that the resulting analyte concentration in the electrochemical cell fell within the calibration range of the corresponding acid, and therefore within the linear range of the calibration curves of the acids. Peak current measurements were performed in triplicate for each sample. The equivalent concentration was determined by interpolation from the calibration curves of the acids, corrected using the appropriate dilution factor for each aliquot, and converted based on the molar mass of acids. The antioxidant capacity of the extracts was expressed as milligrams of acid equivalents *per* millilitre of extract.

Statistical analysis

The results are expressed as the mean ± standard deviation (n = 3). Prior to analysis of variance, the normality of the data was verified using the Shapiro-Wilk test and the homogeneity of variances using Levene's test; both assumptions were met for all determinations ($p > 0.05$). For each analytical method, the differences among the five extraction techniques were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons, with a p-value < 0.05 considered statistically significant. Groups not significantly different are denoted by the same letter. Calibration curves were constructed using linear regression, with linearity evaluated based on the coefficient of determination (R^2). Data processing and statistical analysis were performed using OriginPro version 9.4 (OriginLab Corporation, Northampton, MA, USA).

Results and Discussion

HPLC-MS Analysis of Phenolic Compounds

The polyphenolic composition of the extracts was characterized using an advanced analytical technique, high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS), which enabled the identification and quantification of 36 compounds belonging to the classes of flavonoids, phenolic acids and related derivatives. The results are presented in Table I.

Table I

Phenolic composition of *Viola tricolor* L. extracts determined by HPLC-MS

No.	Compound	Extraction technique				
		Maceration	**Microwave**	**Soxhlet**	**Ultrasound**	**Turbo-extraction**
1	Protocatechuic acid-4-O-glucoside	1.536 ± 0.143	1.227 ± 0.069	1.587 ± 0.063	1.359 ± 0.077	1.252 ± 0.080
2	Vanillic acid-4-O-glucoside	0.310 ± 0.014	0.253 ± 0.017	0.321 ± 0.018	0.275 ± 0.020	0.264 ± 0.020
3	Salicylic acid O-glucoside	1.635 ± 0.139	1.453 ± 0.093	0.522 ± 0.030	1.560 ± 0.055	1.473 ± 0.068
4	Quercetin-3-O-2,6-dirhamnosylglucoside	8.324 ± 0.508	6.096 ± 0.402	10.855 ± 0.423	6.411 ± 0.192	6.015 ± 0.229
5	Luteolin-7-O-glucoside	0.326 ± 0.015	0.325 ± 0.025	0.411 ± 0.040	0.319 ± 0.021	0.305 ± 0.017
6	Kaempferol-3-O-(2,6-di-O-rhamnosyl-glucoside)	0.251 ± 0.010	0.184 ± 0.013	0.330 ± 0.012	0.185 ± 0.012	0.197 ± 0.014
7	Quercetin-3-O-robinobioside	65.801 ± 2.566	57.287 ± 3.953	65.826 ± 5.069	60.564 ± 4.906	58.139 ± 3.663
8	Isorhamnetin-3-O-dirhamno-glucoside	0.398 ± 0.020	0.306 ± 0.009	0.505 ± 0.025	0.300 ± 0.021	0.304 ± 0.027
9	Kaempferol-3-O-rutinoside	1.712 ± 0.169	1.363 ± 0.059	1.576 ± 0.099	1.491 ± 0.054	1.415 ± 0.075
10	Isorhamnetin-3-O-rutinoside	3.096 ± 0.121	2.453 ± 0.164	2.930 ± 0.217	2.682 ± 0.118	2.511 ± 0.090
11	Gallic acid	0.413 ± 0.041	0.361 ± 0.036	0.616 ± 0.040	0.363 ± 0.017	0.302 ± 0.026
12	Protocatechuic acid	0.156 ± 0.013	0.886 ± 0.038	0.692 ± 0.055	0.629 ± 0.040	0.453 ± 0.040
13	Procyanidin B3	0.285 ± 0.010	0.388 ± 0.037	0.143 ± 0.006	0.384 ± 0.034	0.375 ± 0.030
14	Procyanidin B1	0.044 ± 0.003	0.130 ± 0.012	0.026 ± 0.001	0.115 ± 0.008	0.113 ± 0.010
15	3-Hydroxybenzoic acid	0.330 ± 0.020	0.299 ± 0.027	0.112 ± 0.009	0.321 ± 0.024	0.314 ± 0.013
16	Esculin	0.610 ± 0.028	0.631 ± 0.043	0.776 ± 0.047	0.653 ± 0.052	0.607 ± 0.021

No.	Compound	Extraction technique				
		Maceration	Microwave	Soxhlet	Ultrasound	Turbo-extraction
17	Catechin	0.608 ± 0.059	0.657 ± 0.065	0.632 ± 0.057	0.636 ± 0.032	0.562 ± 0.042
18	Vanillic acid	1.371 ± 0.080	1.015 ± 0.046	1.433 ± 0.066	1.057 ± 0.069	1.020 ± 0.042
19	Chlorogenic acid	1.294 ± 0.040	1.103 ± 0.072	1.458 ± 0.120	1.127 ± 0.073	1.049 ± 0.042
20	Caffeic acid	0.116 ± 0.006	0.079 ± 0.007	0.118 ± 0.011	0.076 ± 0.004	0.077 ± 0.006
21	Esculetin	0.989 ± 0.096	0.665 ± 0.052	0.853 ± 0.078	0.721 ± 0.050	0.662 ± 0.032
22	Syringic acid	0.234 ± 0.018	0.164 ± 0.006	0.234 ± 0.018	0.191 ± 0.006	0.175 ± 0.016
23	p-Coumaric acid	0.810 ± 0.024	0.611 ± 0.035	0.816 ± 0.047	0.689 ± 0.052	0.627 ± 0.020
24	Syringaldehyde	0.101 ± 0.004	0.056 ± 0.005	0.119 ± 0.011	0.083 ± 0.008	0.060 ± 0.003
25	Salicylic acid	49.970 ± 3.748	42.329 ± 1.482	53.240 ± 3.194	45.084 ± 2.119	42.944 ± 1.331
26	Scopoletin	1.121 ± 0.035	0.805 ± 0.056	1.075 ± 0.058	0.880 ± 0.063	0.820 ± 0.074
27	Ferulic acid	0.403 ± 0.026	0.306 ± 0.026	0.381 ± 0.019	0.342 ± 0.011	0.320 ± 0.028
28	Coniferyl aldehyde	0.083 ± 0.005	0.093 ± 0.006	0.111 ± 0.007	0.071 ± 0.006	0.082 ± 0.006
29	Sinapaldehyde	0.078 ± 0.007	0.069 ± 0.005	0.094 ± 0.008	0.067 ± 0.002	0.067 ± 0.005
30	Vitexin	0.536 ± 0.048	0.423 ± 0.022	0.493 ± 0.031	0.461 ± 0.036	0.427 ± 0.038
31	Isoquercitrin	7.151 ± 0.515	5.431 ± 0.456	6.963 ± 0.613	6.056 ± 0.218	5.547 ± 0.338
32	Rutin	170.231 ± 12.086	157.034 ± 6.909	169.185 ± 16.411	162.633 ± 5.204	158.396 ± 13.622
33	Rosmarinic acid	0.280 ± 0.011	0.095 ± 0.007	0.324 ± 0.032	0.268 ± 0.008	0.145 ± 0.010
34	Apigenin 7-glucoside	0.035 ± 0.001	0.030 ± 0.001	0.030 ± 0.002	0.030 ± 0.001	0.030 ± 0.003
35	Quercetin	3.249 ± 0.136	0.147 ± 0.004	0.408 ± 0.016	0.591 ± 0.057	0.564 ± 0.047
36	Hispidulin	0.061 ± 0.002	0.044 ± 0.004	0.050 ± 0.003	0.045 ± 0.004	0.041 ± 0.004

The results were expressed as mean ± standard deviation (n = 3), in µg/mL of extract.

Compounds for which a specific reference standard was available were quantified accordingly, and the others were expressed in terms of a structurally related compound: phenolic acids as gallic acid equivalents (compounds no. 1 - 3), and flavonoids as quercetin equivalents (compounds no. 4 - 10). The phenolic profiles of all extracts were dominated by a limited number of major constituents: rutin, quercetin-3-O-robinobiose and salicylic acid, which together accounted for the majority of the total phenolic content, with rutin representing the main constituent.

In addition to these, numerous minor flavonoids were identified, particularly quercetin, kaempferol and isorhamnetin glycosides, as well as a diverse range of phenolic acids, including caffeic, ferulic, p-coumaric and chlorogenic acids, together with glycosylated derivatives of protocatechuic and vanillic acids.

The quantified total phenolic content varied within a narrow range among the five extracts, due to the high proportion of major compounds, whose concentrations remained relatively constant. The Soxhlet and maceration extracts exhibited the highest values, consistent with the results obtained by spectrophotometric and electrochemical methods.

The predominance of quercetin-type flavonoids, together with the presence of salicylic acid, is characteristic of the species and supports the correspondence between the identified chemical profile and the observed antioxidant activity.

Total phenolic content

The total polyphenol content in the five extracts was determined using the Folin-Ciocâlteu method, with gallic acid and caffeic acid as reference standards. Calibration curves were plotted, yielding the equations $y = 0.00307x + 0.03245$ ($R^2 = 0.9967$) for gallic acid and $y = 0.00294x + 0.01274$ ($R^2 = 0.9987$) for caffeic acid, both showing very good linearity. For each extract, the measured absorbance was interpolated against calibration curves, and the polyphenolic content was calculated after applying the dilution factor correction and expressed as milligrams equivalent *per* millilitre of extract. The results are presented in Table II.

Table II

Total polyphenol content of *Viola tricolor* L. extract

Extraction method	Total polyphenol content (mg/mL)	
	GAE	CAE
Maceration	1.171 ± 0.033[a]	1.257 ± 0.029[a]
Microwave	0.375 ± 0.010[d]	0.425 ± 0.012[d]
Soxhlet	0.873 ± 0.019[b]	0.946 ± 0.020[b]
Ultrasound	0.569 ± 0.015[c]	0.627 ± 0.017[c]
Turbo-extraction	0.342 ± 0.012[d]	0.391 ± 0.009[d]

The results are expressed as mean ± standard deviation (n = 3). GAE – gallic acid equivalents, CAE – caffeic acid equivalents. Within the same column, values followed by different letters differ statistically significantly (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

The highest values were recorded for the maceration extract, followed by the Soxhlet extract, indicating a superior efficiency of techniques based on prolonged contact between the solvent and the plant material, during which the diffusion of phenolic compounds into the liquid phase occurs more completely. Extracts obtained using rapid techniques showed lower values, a result that can be attributed to the significantly shorter duration of the extraction process. The values obtained using both standards followed the same trend, supporting the consistency of the determination.

Total flavonoid content

The total flavonoid content was determined using the aluminium chloride method, with the results expressed as both rutin and quercetin equivalents, based on two calibration curves: y = 0.00159x - 0.0972 (R^2 = 0.9962) for rutin and y = 0.00261x + 0.02526 (R^2 = 0.9978) for quercetin. Following the same procedure, the absorbance of each extract was recorded at both wavelengths corresponding to the two standards and plotted on the respective calibration curves, and the values were expressed as milligrams equivalent *per* millilitre of extract. The obtained values are presented in Table III.

Table III

Total flavonoid content of *Viola tricolor* L. extract

Extraction method	Total flavonoid content (mg/mL)	
	RE	QE
Maceration	0.287 ± 0.007[a]	0.174 ± 0.004[a]
Microwave	0.243 ± 0.006[cd]	0.143 ± 0.003[bc]
Soxhlet	0.268 ± 0.005[b]	0.166 ± 0.004[a]
Ultrasound	0.256 ± 0.006[bc]	0.151 ± 0.005[b]
Turbo-extraction	0.237 ± 0.004[d]	0.139 ± 0.003[c]

The results are expressed as mean ± standard deviation (n = 3). RE – rutin equivalents, QE – quercetin equivalents. Within the same column, values followed by different letters differ statistically significantly (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$)

The total flavonoid content varied depending on the extraction technique, with the maceration extract again recording the highest values, consistent with the trend observed for total polyphenol content and with the results of the other determinations. Compared with total polyphenol, the values were distributed over a narrower range, reflecting consistent extractability of flavonoids and in good agreement with the chromatographic profile, in which the flavonoid fraction is predominantly represented by rutin. The values expressed using both standards followed the same trend, confirming the reproducibility of the results.

Antioxidant activity

The antioxidant activity of the extracts was evaluated using two complementary methods: DPPH radical scavenging, a spectrophotometric method, and differential pulse voltammetry (DPV), an electrochemical technique. In both cases, three reference substances (ascorbic acid, gallic acid, and caffeic acid) were used, and calibration curves were plotted for DPPH absorbance at 517 nm against concentration (µg/mL) and for DPV, the peak current against concentration (mM), as presented in Figure 1.

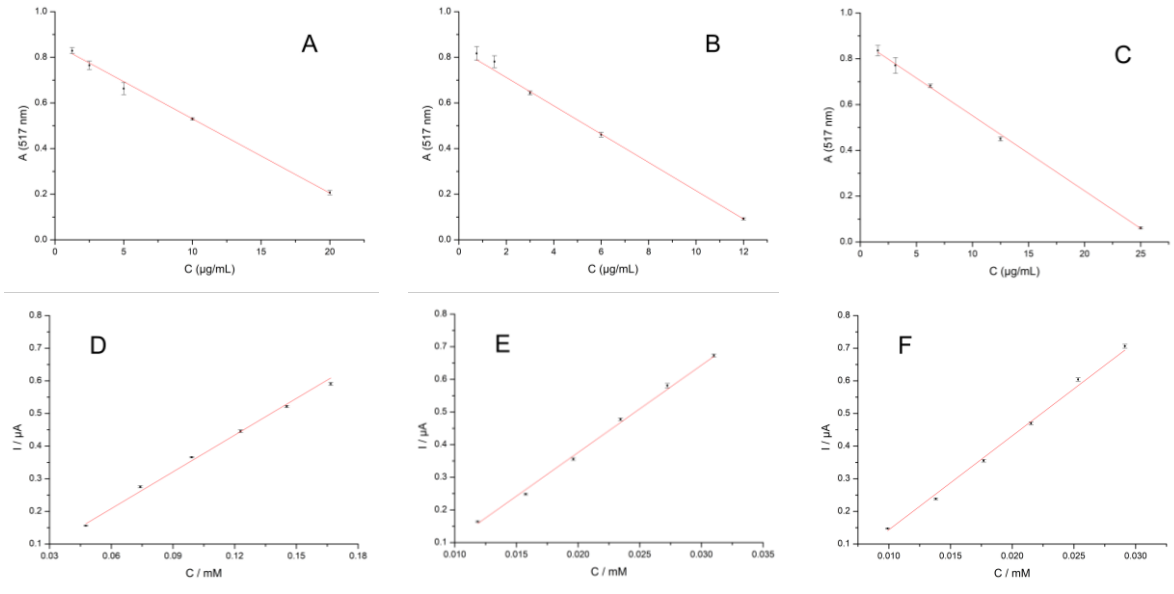

Figure 1.

Calibration curves of the antioxidant standards used for DPPH (**A-C**) and DPV (**D-F**) analyses: (**A, D**) ascorbic acid, (**B, E**) gallic acid, (**C, F**) caffeic acid. Data are presented as mean ± standard deviation (n = 3)

The calibration curves showed a linear relationship between the concentration of the standards and the absorbance values, as confirmed by the high coefficients of determination (R^2), indicating the reliability of the method within the investigated concentration range. The regression equations and R^2 values are presented in Table IV.

Table IV

Calibration curve parameters for the DPPH and DPV method

	Standard substance	Equation of the standard curve	Regression Coefficients (R^2)
DPPH	AA	y = - 0.03256x + 0.85631	0.9981
	GA	y = - 0.06203x + 0.83571	0.9989
	CA	y = - 0.03284x + 0.88003	0.9987
Differential Pulse Voltammetry	AA	y = 3.75362x - 0.01674	0.99486
	GA	y = 26.7415x - 0.15889	0.99558
	CA	y = 28.78503x - 0.14417	0.99490

The validated calibration curves were subsequently used to quantify the antioxidant capacity of the *Viola tricolor* L. extract. Based on the regression equations obtained, the antioxidant activity of each extract was determined and expressed in equivalents of ascorbic acid (AA), gallic acid (GA), and caffeic acid (CA). The values obtained by the DPPH method and DPV method are comparatively presented in Table V.

Table V

Antioxidant activity of *Viola tricolor* L. extract (DPPH and DPV)

Extraction method	Antioxidant activity (mg/mL)					
	DPPH			DPV		
	AAE	GAE	CAE	AAE	GAE	CAE
Maceration	4.033 ± 0.11^{a}	1.984 ± 0.050^{a}	4.287 ± 0.10^{a}	5.604 ± 0.15^{a}	0.941 ± 0.020^{a}	0.907 ± 0.019^{a}
Microwave	0.579 ± 0.02^{d}	0.287 ± 0.009^{d}	0.610 ± 0.02^{d}	0.911 ± 0.02^{d}	0.214 ± 0.006^{d}	0.202 ± 0.005^{d}
Soxhlet	2.551 ± 0.07^{b}	1.256 ± 0.035^{b}	2.710 ± 0.06^{b}	3.496 ± 0.09^{b}	0.604 ± 0.015^{b}	0.581 ± 0.014^{b}
Ultrasound	1.029 ± 0.03^{c}	0.507 ± 0.013^{c}	1.093 ± 0.03^{c}	1.363 ± 0.04^{c}	0.336 ± 0.009^{c}	0.315 ± 0.008^{c}
Turbo-extraction	0.633 ± 0.02^{d}	0.315 ± 0.010^{d}	0.663 ± 0.01^{d}	0.814 ± 0.02^{d}	0.186 ± 0.005^{d}	0.175 ± 0.004^{d}

The results are expressed as mean ± standard deviation (n = 3). AAE – ascorbic acid equivalents, GAE – gallic acid equivalents, CAE – caffeic acid equivalents. Within the same column, values followed by different letters differ statistically significantly (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$)

Both methods yielded the same ranking of extracts, with the macerate exhibiting the strongest antioxidant activity, followed by the Soxhlet extract, while the extracts obtained using turbo-extraction and microwaves showed the lowest values. The agreement between an optical and an electrochemical method, evaluated independently, was reflected in a very strong correlation between the two datasets, which confirms the reliability of the estimations and supports voltammetry as a valuable complementary approach. The two assays nevertheless rely on distinct chemical principles. The DPPH assay measures the ability of the extract constituents to scavenge a stable free radical, mainly through hydrogen atom transfer or single electron transfer, whereas differential pulse voltammetry quantifies their direct electrochemical oxidation at the electrode surface. Since these mechanisms are not equivalent, the relative ranking of samples may differ between assays; the concordance observed in the present study therefore provides additional support for the reliability of the results (36).

A similar correspondence was observed at the level of the individual compounds. Rutin, the predominant constituent, together with quercetin, reached the highest concentrations in the maceration and Soxhlet extracts and the lowest in the microwave and turbo-extraction ones, following the same order as the antioxidant activity determined by both methods. The difference was most pronounced for quercetin, a flavonoid with strong antioxidant properties, whose concentration in the maceration extract clearly exceeded that of the other extracts. This agreement between the recovery of the main constituents and the measured antioxidant capacity supports a direct relationship between the phenolic profile and the functional properties of the extracts.

Across all five analytical methods, the maceration and Soxhlet extracts consistently showed the highest values, followed by the ultrasound extract, while the microwave and turbo-extraction techniques yielded the lowest. This ranking reflects the efficiency of the extraction techniques in recovering phenolic compounds and should be considered in relation to the duration of each process, as the highest values were obtained with the most time-consuming techniques, while the rapid methods provided lower contents in a considerably shorter time. The rapid techniques therefore present an advantage in terms of processing time and solvent use, consistent with the current interest in green extraction (42), which may compensate for their lower yields depending on the intended application.

The relative performance of conventional and assisted extraction techniques has been the subject of numerous comparative studies, covering a wide range of plant matrices. Ultrasound-assisted extraction yielded 3.12 g GAE/100 g from black locust flowers against 2.54 g GAE/100 g for maceration, with correspondingly better radical-scavenging activity, IC_{50} values of 120.5 and 150.6 μg/cm³ respectively (43), 7.01 mg/g against 5.18 mg/g for fresh olives (44), and 418.01 mg GAE/100 g against 357.34

mg GAE/100 g for baobab seeds (45). Larger differences have been reported for by-product matrices, with 34.87 against 21.45 mg GAE/g for kinnow mandarin peel, 7.85 against 4.62 mg GAE/g for rice bran and 22.80 against 15.40 mg GAE/g for orange peel, corresponding to increases of approximately 63%, 70% and 48% (46). Microwave-assisted extraction has shown a comparable advantage for broccoli, increasing the phenolic yield by between 45.7% and 133.6% depending on the plant part, relative to a 24-hour maceration (47). For *Cassia alata*, the same technique provided the highest phenolic content among maceration, ultrasound, and microwave-assisted extraction, reaching 37.92 mg GAE/g, followed by ultrasound-assisted extraction with 26.91 mg GAE/g (48). In these designs, the operating parameters of the assisted techniques were individually optimized, generally by response surface methodology, and applied at temperatures between 40 and 59°C, while the maceration procedures against which they were compared lasted from 105 minutes to 24 hours.

The outcome of such comparisons depends closely on the experimental conditions adopted, and the design of the present study differed in three respects from those described above. The five techniques were applied without individual optimization, so that their performance could be compared on a common basis; the ultrasound-assisted extraction was carried out without external heating, the bath temperature rising only from 24.2 to 29.5°C, and the maceration was extended over ten days, a contact time approximately one order of magnitude longer than the longest procedure reported in the studies cited above. Under these conditions, the conventional techniques provided the highest values, an outcome that is consistent with observations reported where the experimental settings were similarly configured. For nettle, maceration provided 25.67 mg GAE/g, comparable to or slightly above the 23.86 mg GAE/g obtained by ultrasound-assisted extraction under optimized conditions of 30 minutes at 80% power and the 24.64 mg GAE/g obtained by microwave-assisted extraction in 10 minutes. The authors attributed the limited performance of sonication to the possible formation of hydrogen peroxide and oxidative radicals during the process (49). A comparable effect has been described for microalgal biomass, where sonication in methanol reduced the phenolic content relative to maceration, an outcome ascribed to the localized thermal spikes exceeding 80°C generated during the collapse of cavitation bubbles (50). Taken together, these observations suggest that the energy input of the assisted techniques may act on phenolic compounds in two opposing ways, promoting their release from the matrix while also exposing them to conditions that favour degradation.

Reports comparing maceration with Soxhlet extraction likewise vary according to the plant matrix. Soxhlet extraction proved more efficient for black locust flowers, where it gave the highest phenolic content of the three techniques compared, 3.22 g GAE/100 g (43), and for *Lavandula mairei*, with 35.12 - 99.37 mg GAE/g against 27.63 - 58.99 mg GAE/g for maceration, and 119 - 224.6 mg QE/g against 111.8 - 148.51 mg QE/g for total flavonoids, using an eight hour extraction (51). Cold maceration, in turn, was more efficient for the aerial parts of *Thymelaea hirsuta*, both in phenolic content and in radical-scavenging activity, with IC_{50} values of 4.75 - 15.86 mg/L against 12.46 - 17.82 mg/L for the Soxhlet extracts, a difference attributed by the authors to the degradation of thermolabile constituents at the temperature required by the latter technique (52). Similar observations have been reported for microalgal biomass, where Soxhlet extraction gave the lowest phenolic content of the three techniques compared, 5.05 mg GAE/g against 20.15 mg GAE/g for maceration (50). A similar outcome was reported for *Syzygium cumini* seed kernels, where a six-hour Soxhlet extraction yielded 30.05 mg GAE/g, compared with 79.89 mg GAE/g obtained by stirred batch extraction in 105 minutes at 50°C, the corresponding IC_{50} values being 35.89 and 12.97 µg/mL. The authors attributed the lower recovery to the prolonged exposure of the solvent to 100°C in the distillation flask, which affects thermosensitive constituents, and to the fact that extraction within the thimble proceeds essentially by diffusion (53). A recent systematic review of olive leaf extraction reconciles these observations quantitatively, reporting a mean phenolic recovery of approximately 52 mg GAE/g for maceration and stirring procedures, not significantly different from that of Soxhlet extraction in the pooled analysis, and concluding that simple solvent extraction can approach the recovery achieved by Soxhlet provided that sufficient contact time or repeated solvent renewal is allowed (54).

The values obtained in the present study for the maceration and Soxhlet extracts correspond to this last pattern. The prolonged contact of the ten-day maceration and the repeated percolation with freshly condensed solvent in the Soxhlet apparatus appear to represent two distinct routes towards a comparably exhaustive recovery, the elevated temperature of the latter being offset by the continuous renewal of the extraction medium.

The efficiency of maceration observed for *Viola tricolor* L. can also be related to the nature of its phenolic constituents. As the phenolic fraction of this species is dominated by rutin and other flavonol glycosides, the extended contact between solvent and plant material favours their gradual release, rutin reaching comparably high concentrations in the maceration and Soxhlet extracts. Free quercetin, by contrast, was distinctly higher in the maceration extract

than in all the others, which is consistent with an extraction carried out at room temperature.

The values obtained in the present study are in agreement with previously published data on *Viola tricolor* L. Expressed on a plant material basis, taking into account the 1:10 (w/v) ratio used, the total phenolic content of the extracts corresponds to 3.4 - 11.7 mg GAE/g of dried plant material, the maceration extract being close to the contents of up to 12.84 mg/g described for this species in flower fractions (5). The phenolic profile is likewise consistent with earlier reports, rutin being identified as the main constituent both in the present work and in previous studies on the species (3, 5).

The results should be interpreted considering a few limitations that also highlight directions for future research. All determinations were performed in triplicate, which was sufficient to detect the marked differences observed among the extraction techniques. However, a higher number of replicates would strengthen comparisons among extracts with similar values. In addition, the extraction methods were applied under standardized conditions without individual optimization of the operating parameters for each technique. Consequently, their relative performance may differ following method-specific optimization. It should also be noted that the absolute values reported here may not be directly comparable across laboratories, as antioxidant results are known to depend on the extraction procedure and on the conventions adopted for expressing the data, which differ between studies. Comparisons are therefore likely to be more informative when based on relative trends among samples than on absolute values (9, 30). Furthermore, the phenolic composition and antioxidant activity within the genus *Viola* may vary considerably with the species, the plant tissue analysed and the geographical origin of the material (55). The present results therefore reflect the profile of the aerial parts of *Viola tricolor* L. from a specific region and should be interpreted accordingly, particularly as the plant material came from a single site and a single harvest year. Antioxidant capacity was assessed using chemical assays, and the results provide a basis for further investigation of effects in biological systems. Overall, the marked antioxidant capacity of the *Viola tricolor* extracts, together with their content in flavonoids and phenolic acids, supports the traditional use of the species and its potential as a source of natural antioxidants for pharmaceutical and nutraceutical applications.

Conclusions

The study compared five extraction techniques applied to the aerial parts of *Viola tricolor* L., and the extracts were evaluated using a combination of chromatographic, spectrophotometric and electrochemical methods. Maceration yielded the highest values of phenolic content and antioxidant capacity, followed by Soxhlet extraction, indicating the superior efficiency of techniques based on prolonged contact between the solvent and the plant material. The results obtained using the DPPH method and voltammetry were closely correlated, and the assessment confirms the usefulness of voltammetry as a complementary method. The phenolic profile determined by HPLC-MS was dominated by rutin and other flavonol glycosides, along with phenolic acids, which supports the connection between chemical composition and the antioxidant activity of the extracts. The results provide a useful reference for selecting the most appropriate extraction technique for *Viola tricolor* L. and further support its potential as a natural source of antioxidant compounds.

Acknowledgement

Not applicable.

Funding

Not applicable.

Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

Not applicable.

AI use disclosure

During the preparation of this manuscript, the author(s) used an AI-based language tool for adaptation from Romanian to English and for editing assistance and language improvement, specifically to improve spelling, grammar, word choice, readability, and clarity. The author(s) reviewed and revised all outputs from these tools and take(s) full responsibility for the final content of the work.

Conflict of interest

The authors declare no conflict of interest.

References

1. Tobyn G, Denham A, Whitelegg M. Viola odorata, sweet violet; Viola tricolor, heartsease. In: The Western herbal tradition: 2000 years of medicinal plant knowledge. Edinburgh: Churchill Livingstone; 2011. p. 337-348.
2. Batiha GES, Lukman HY, Shaheen HM, Wasef L, Hafiz AA, Conte-Junior CA, et al. A systematic review of phytochemistry, nutritional composition, and pharmacologic application of species of the genus Viola in noncommunicable diseases (NCDs).

Evid Based Complement Alternat Med. 2023;2023(1):5406039.
3. Supty IZ, Choudhury T, Parvin M. Viola tricolor L.: a systematic review on ethnopharmacology, phytochemistry and pharmacology of an important traditional medicinal plant. J Pharmacogn Phytochem. 2025;14(4):501-508.
4. Coman OA, Smeu A, Muntean D, Vlase AM, Vlase L, Toth S, et al. Comparative analysis of Ribes nigrum leaf extracts in different solvents: antioxidant, antimicrobial and cytotoxic activity. Farmacia. 2025;73(5):1164-1174.
5. Gonçalves AFK, Friedrich RB, Boligon AA, Piana M, Beck RCR, Athayde ML. Antioxidant capacity, total phenolic contents and HPLC determination of rutin in Viola tricolor (L.) flowers. Free Radic Antioxid. 2012;2(4):32-37.
6. Hellinger R, Koehbach J, Fedchuk H, Sauer B, Huber R, Gruber CW, et al. Immunosuppressive activity of an aqueous Viola tricolor herbal extract. J Ethnopharmacol. 2014;151(1):299-306.
7. Naczk M, Shahidi F. Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. J Pharm Biomed Anal. 2006;41(5):1523-1542.
8. Saad AM, Mohammed DM, Alkafaas SS, Ghosh S, Negm SH, Salem HM, et al. Dietary polyphenols and human health: sources, biological activities, nutritional and immunological aspects, and bioavailability - a comprehensive review. Front Immunol. 2025;16:1653378.
9. Losseva I, Tulebayev Y, Ishmuratova M, Kukula-Koch W, Akhmetova S, Karilkhan I, et al. Evaluation of anti-inflammatory activity and chemical composition of Filipendula vulgaris Moench and Filipendula ulmaria (L.) Maxim extracts obtained by ultrasonic method. Farmacia. 2025;73(1):103-111.
10. Zhang Y, Cai P, Cheng G, Zhang Y. A brief review of phenolic compounds identified from plants: their extraction, analysis, and biological activity. Nat Prod Commun. 2022;17(1):1934578X2110697.
11. Romanian Pharmacopoeia. 10th ed. Bucharest: Editura Medicală; 1993. [Available in Romanian]
12. Irfan S, Ranjha M, Nadeem M, Safdar M, Jabbar S, Mahmood S, et al. Antioxidant activity and phenolic content of sonication and maceration-assisted ethanol and acetone extracts of Cymbopogon citratus leaves. Separations. 2022;9(9):244.
13. Levaya YK, Badekova KZh, Zholdasbayev ME, Kurmantayeva GK, Atazhanova GA, Sadyrbekov DT, et al. Efficient microwave-assisted extraction of polyphenol-rich extract from Salvia dumetorum leaves. Compounds. 2025;5(4):58.
14. Lama-Muñoz A, Contreras MDM, Espínola F, Moya M, Romero I, Castro E. Content of phenolic compounds and mannitol in olive leaves extracts from six Spanish cultivars: extraction with the Soxhlet method and pressurized liquids. Food Chem. 2020;320:126626.
15. M'hiri N, Ioannou I, Ghoul M, Boudhrioua NM. Extraction methods of citrus peel phenolic compounds. Food Rev Int. 2014;30(4):265-290.
16. Mobasheri F, Khajeh M, Ghaffari-Moghaddam M, Piri J, Bohlooli M. Machine learning optimization of microwave-assisted extraction of phenolics and tannins from pomegranate peel. Sci Rep. 2025;15(1):19439.
17. Bouaoudia-Madi N, Boulekbache-Makhlouf L, Madani K, Silva AMS, Dairi S, Oukhmanou-Bensidhoum S, et al. Optimization of ultrasound-assisted extraction of polyphenols from Myrtus communis L. pericarp. Antioxidants (Basel). 2019;8(7):205.
18. Rusu ME, Gheldiu AM, Mocan A, Moldovan C, Popa DS, Tomuta I, et al. Process optimization for improved phenolic compounds recovery from walnut (Juglans regia L.) septum: phytochemical profile and biological activities. Molecules. 2018;23(11):2814.
19. Vázquez-Vázquez J, Barajas-Salazar M, Casas-Godoy L, Alcázar-Valle M, Arellano-García L, Barrera-Martínez I. Enhanced extraction of antioxidant phenolics: quercetin, kaempferol, gallic acid, syringic acid, and epicatechin from fresh berries and their waste using Ultra-Turrax and ultrasonication. Appl Food Res. 2024;4(2):100539.
20. Brudiu MD, Nicolescu A, Moroșan AG, Urcan AC, Vlase L, Vlase AM, et al. Optimized ultrasound-assisted extraction reveals Salvia transsylvanica as a rosmarinic acid-rich endemic sage with antioxidant potential. Antioxidants (Basel). 2026;15(5):561.
21. Jojić AA, Pașcalau NA, Antal AG, Uțu D, Muntean D, Vlase L, et al. Fraction-dependent polyphenolic profile and biological activities of Juniperus communis pseudo-fruit extracts: antioxidant, antimicrobial and selective antimelanoma effects. Antioxidants (Basel). 2026;15(6):738.
22. Vlase AM, Toiu A, Tomuță I, Vlase L, Muntean D, Casian T, et al. Epilobium species: from optimization of the extraction process to evaluation of biological properties. Antioxidants (Basel). 2022;12(1):91.
23. Safta DA, Vlase AM, Pop A, Cherfan J, Carpa R, Iurian S, et al. Optimized Sambucus nigra L., Epilobium hirsutum L., and Lythrum salicaria L. extracts: biological effects supporting their potential in wound care. Antioxidants (Basel). 2025;14(5):521.
24. Waterman PG, Mole S. Analysis of phenolic plant metabolites. Oxford; 1994.
25. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965;16(3):144-158.
26. Termentzi A, Kefalas P, Kokkalou E. Antioxidant activities of various extracts and fractions of Sorbus domestica fruits at different maturity stages. Food Chem. 2006;98(4):599-608.
27. Poljak I, Vahčić N, Liber Z, Tumpa K, Pintar V, Zegnal I, et al. Morphological and chemical diversity and antioxidant capacity of the service tree (Sorbus domestica L.) fruits from two eco-geographical regions. Plants (Basel). 2021;10(8):1691.
28. Islamčević Razboršek M, Ivanović M, Krajnc P, Kolar M. Choline chloride based natural deep eutectic solvents as extraction media for extracting phenolic compounds from chokeberry (Aronia melanocarpa). Molecules. 2020;25(7):1619.
29. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two

complementary colorimetric methods. J Food Drug Anal. 2002;10(3):178-182.
30. Shraim AM, Ahmed TA, Rahman MM, Hijji YM. Determination of total flavonoid content by aluminum chloride assay: a critical evaluation. LWT. 2021;150(2):111932.
31. Noviyanti, Martiani I, Perdana F, Rahmaniah. Determination of total phenol and total flavonoid, and antioxidant activities of chocolate leaves (Zephyranthes candida (Lindl.) Herb.). Bali Med J. 2022;11(2):927-933.
32. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT. 1995;28(1):25-30.
33. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181(4617):1199-1200.
34. Islam T, Hasan MR, Roy A, Islam MS, Uddin MA, Islam MA, et al. Screening of in-vitro antioxidant, brine shrimp lethality bioassay and antimicrobial activities of extracts of Bridelia retusa (L.) Spreng. fruit. 2015;5(4):1058-1067.
35. Gulcin İ, Alwasel SH. DPPH radical scavenging assay. Processes. 2023;11(8):2248.
36. Osorio-Valencia AI, De Jesús Franco-Mejía J, Hoyos-Arbeláez JA, Blandón-Naranjo L, Vega-Castro OA, Del Carmen Contreras-Calderón J, et al. Evaluation of antioxidant capacity in different food matrices through differential pulse voltammetry and its correlation with spectrophotometric methods. J Appl Electrochem. 2023;53(12):2495-2505.
37. Alam MW, Najeeb J, Naeem S, Usman SM, Nahvi I, Alismail F, et al. Electrochemical methodologies for investigating the antioxidant potential of plant and fruit extracts: a review. Antioxidants (Basel). 2022;11(6):1205.
38. Banica F, Bungau S, Tit DM, Behl T, Otrisal P, Nechifor AC, et al. Determination of the total polyphenols content and antioxidant activity of Echinacea purpurea extracts using newly manufactured glassy carbon electrodes modified with carbon nanotubes. Processes. 2020;8(7):833.
39. Montemerlo AE, Azcarate SM, Camiña JM, Messina G. Chemometrically assisted differential pulse voltammetry for simultaneous and interference-free quantification of gallic and caffeic acids. Anal Methods. 2024;16(23):3774-3783.
40. Pisoschi AM, Pop A, Iordache F, Stanca L, Bilteanu L, Serban AI. Antioxidant determination with the use of carbon-based electrodes. Chemosensors. 2021;9(4):72.
41. Lima AP, Dos Santos WTP, Nossol E, Richter EM, Munoz RAA. Critical evaluation of voltammetric techniques for antioxidant capacity and activity: presence of alumina on glassy-carbon electrodes alters the results. Electrochim Acta. 2020;358(1):136925.
42. Martins R, Barbosa A, Advinha B, Sales H, Pontes R, Nunes J. Green extraction techniques of bioactive compounds: a state-of-the-art review. Processes. 2023;11(8):2255.
43. Savic Gajic I, Savic I, Boskov I, Žerajić S, Markovic I, Gajic D. Optimization of ultrasound-assisted extraction of phenolic compounds from black locust (Robiniae pseudoacaciae) flowers and comparison with conventional methods. Antioxidants (Basel). 2019;8(8):248.
44. Deng J, Xu Z, Xiang C, Liu J, Zhou L, Li T, et al. Comparative evaluation of maceration and ultrasonic-assisted extraction of phenolic compounds from fresh olives. Ultrason Sonochem. 2017;37:328-334.
45. Ismail BB, Guo M, Pu Y, Wang W, Ye X, Liu D. Valorisation of baobab (Adansonia digitata) seeds by ultrasound assisted extraction of polyphenolics. Optimisation and comparison with conventional methods. Ultrason Sonochem. 2019;52:257-267.
46. Nayak A, Shah A, Bhatt S, Bhushan B, Kumar A, Gaur R, et al. Ultrasound and microwave assisted extraction of bioactives from food wastes: an overview on their comparative analysis towards commercialization. Ultrason Sonochem. 2026;124:107712.
47. Rodríguez García SL, Raghavan V. Microwave-assisted extraction of phenolic compounds from broccoli (Brassica oleracea) stems, leaves, and florets: optimization, characterization, and comparison with maceration extraction. Recent Prog Nutr. 2022;2(2):011.
48. Ling YY, Fun PS, Yeop A, Yusoff MM, Gimbun J. Assessment of maceration, ultrasonic and microwave assisted extraction for total phenolic content, total flavonoid content and kaempferol yield from Cassia alata via microstructures analysis. Mater Today Proc. 2019;19:1273-1279.
49. Ince AE, Sahin S, Sumnu G. Comparison of microwave and ultrasound-assisted extraction techniques for leaching of phenolic compounds from nettle. J Food Sci Technol. 2014;51(10):2776-2782.
50. Ruzi II, Mohd Shahpudin SN, Mohamad S, Kassim MA. Impact of extraction techniques and solvent systems on the antioxidant activity and fatty acid composition of Scenedesmus parvus extracts. Food Anal Methods. 2025;18(10):2220-2229.
51. Ghanimi R, Ouhammou A, El Atki Y, Bouchari MEH, Cherkaoui M. The antioxidant activities of ethanolic, methanolic, ethyl acetate, and aqueous extracts of the endemic species, Lavandula mairei Humbert (a comparative study between cold and hot extraction). Ethiop J Health Sci. 2022;32(6):1231-1236.
52. Yahyaoui M, Ghazouani N, Sifaoui I, Abderrabba M. Comparison of the effect of various extraction methods on the phytochemical composition and antioxidant activity of Thymelaea hirsuta L. aerial parts in Tunisia. Biosci Biotechnol Res Asia. 2017;14(3):997-1007.
53. Sridhar A, Ponnuchamy M, Kumar PS, Kapoor A, Vo DVN, Prabhakar S. Techniques and modeling of polyphenol extraction from food: a review. Environ Chem Lett. 2021;19(4):3409-3443.
54. Rosenhech E, Lobiuc A, Boz I, Zamfirache MM. The phytochemical potential of Viola species, Melanium subgenus, subsection Bracteolatae. Int J Mol Sci. 2025;26(23):11614.