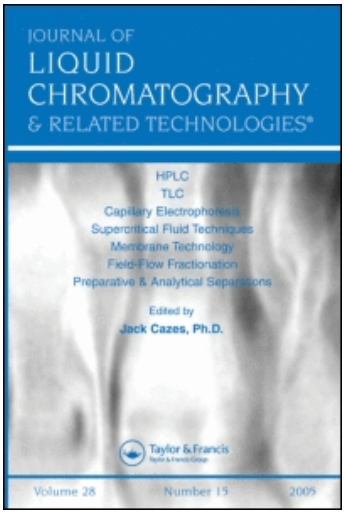

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Abstract: Phenolic compounds in winter savory (*Satureja montana* L.) extracts were analysed by HPLC with diode array detection on reversed phase, C_8 columns. Gradient elution with acetonitrile-acetic acid-water mixture gave complete separation of all phenolic acids and flavanols of interest. The identified phenolic acids and flavanols, as well as tentatively identified phenolic acids in ethyl acetate and *n*-butanol extracts, were quantified. Total content of phenolic acids in ethyl acetate and *n*-butanol extracts was 47.59 and 96.70 μg/g, respectively. Flavanols, (±)-catechin and (−)-epicatechin were present in ethyl acetate extract in quantities of 622.47 and 16.29 μg/g, respectively, while *n*-butanol extract contained these flavanols in extent of 239.08 and 199.82 μg/g.

Keywords: Winter savory, Phenolic acids, Flavanols, HPLC

INTRODUCTION

Phenolic compounds, important constituents in many plants, have received considerable attention as potentially protective factors against cancer and heart diseases because of their potent antioxidant properties and their ubiquity in a wide range of commonly consumed foods of plant origin.[1–4] Plant phenolics are a very diversified group of phytochemicals, which

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includes simple phenolic, phenolic acids (benzoic and cinnamic acid derivatives), lignans, lignins, coumarins, styrylpyrones, flavonoids, stilbenes, flavonolignans and tannins.[5] Identification and quantification of phenolic compounds can give vital information relating to antioxidant function, food quality, and potential health benefits. Many separation techniques such as thin-layer chromatography (TLC), gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE) have been proposed to separate and determine phenolic compounds in various plant materials.[6–10]

Herbal and especially herbal extracts, are very attractive not only in the modern phytotherapy but also in the food industry, as spices and additives.[11] Winter savory (*Satureja montana* L.) is native to the Mediterranean region, but is now found all over Europe, Russia, and Turkey. In the Mediterranean region, it has been used as a culinary herb since antiquity. In traditional and homeopathic medicine it has been used for various ailments, especially for digestive complaints, such as colic, and diarrhoea. Also, winter savory has been traditionally used as stimulants, stomachic, carminative, expectorant, anticatarrhal, astringent, and aphrodisiac.[12] Essential oils, as well as ethanolic extracts of winter savory possesses antioxidant[13] and antimicrobial properties (antibacterial and antifungal).[14,15] This plant contains various biologically active constituents such as essential oils,[16,17] triterpenes,[18] and phenolic compounds.[19]

According to the fact that winter savory extracts, obtained by successive extraction, exhibited antioxidative and antiproliferative activities,[20] the goal of this study was to examine phenolic compounds in these extracts. Winter savory (*Satureja montana* L.) extracts were obtained by successive extraction with solvents of different polarities such as petroleum ether, chloroform, ethyl acetate, and *n*-butanol. The total phenolic content in winter savory extracts was determined by the Folin-Ciocalteau method. Phenolic compounds, phenolic acids, and flavanols, were analysed by HPLC with diode array detection on a reversed phase, C_8 column.

EXPERIMENTAL

Chemicals and Plant Material

HPLC grade acetonitrile and acetic acid (Merck, Darmstadt, Germany), and filtered bidistilled water were used for HPLC analysis. The solvents (methanol, ethyl acetate, petroleum ether, chloroform, and *n*-butanol) used for extraction were from "Zorka" (Šabac, Serbia and Montenegro). Standards of phenolic acids (gallic, protocatechuic, vanillic, caffeic, syringic, coumaric, ferulic, and 3,5-dimethoxy-4-hydroxycinnamic acid), and flavanols ((±)-catechin and (−)-epicatechin) were obtained from Sigma Chemicals Co., USA. Solvents and standards of phenolic acids and flavanols were of analytical grade.

Plant material was purchased from a local herbal drugstore. Aerial parts of winter savory (*Satureja montana* L.) were collected in the period May–June, 2004, in the region of the mountains of Zlatibor, Serbia, as labelled. Voucher specimens of the collected plants were confirmed and deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Medicine, University of Novi Sad.

Successive Extraction

The dried aerial parts of winter savory were ground in a grinder (DLFU Bühler Miag Laboratory Disc Mill, Germany) with a 2 mm diameter mesh. This material (10 g) was macerated with 70% methanol in water (2 × 250 mL) at room temperature for 2 × 24 h. The obtained extract was concentrated under reduced pressure to remove methanol, and then consecutively extracted with petroleum ether (2 × 20 mL), chloroform (2 × 20 mL), ethyl acetate (2 × 20 mL), and *n*-butanol (2 × 20 mL) as shown in Figure 1. The solvent extractions were performed in separatory funnels, shaking for 10 min. The petroleum ether, chloroform, ethyl acetate, *n*-butanol and remained water extract were evaporated to dryness under reduced pressure at 40°C with a water bath.

The yields, average of triplicate analysis, of extracts were: petroleum ether, $m = 0.0604 \pm 0.0030$ g; chloroform, $m = 0.0462 \pm 0.0022$ g; ethyl acetate, $m = 0.0554 \pm 0.0027$ g; *n*-butanol, $m = 0.2376 \pm 0.0118$ g and water, $m = 0.6126 \pm 0.0306$ g.

Total Phenolic Content

The amount of total soluble phenolics in extracts was determined spectrophotometrically according to the Folin-Ciocalteau method.[21] The reaction mixture was composed by mixing 0.1 mL of methanolic solution (concentration of 1 mg/mL) of extract, 7.9 mL of distilled water, 0.5 mL of the Folin-Ciocalteau's reagent, and 1.5 mL of 20% sodium carbonate. After 2 h, the absorbance was measured at 750 nm in a spectrophotometer. The total phenolic content is expressed as chlorogenic acid equivalents per g dry weight of plant.

HPLC Analysis

Instrumentation and Chromatographic Conditions

HPLC was performed with a Hewlett-Packard Liquid Chromatograph HP1090 equipped with a Diode Array Detector (79880A DAD). Data collection and

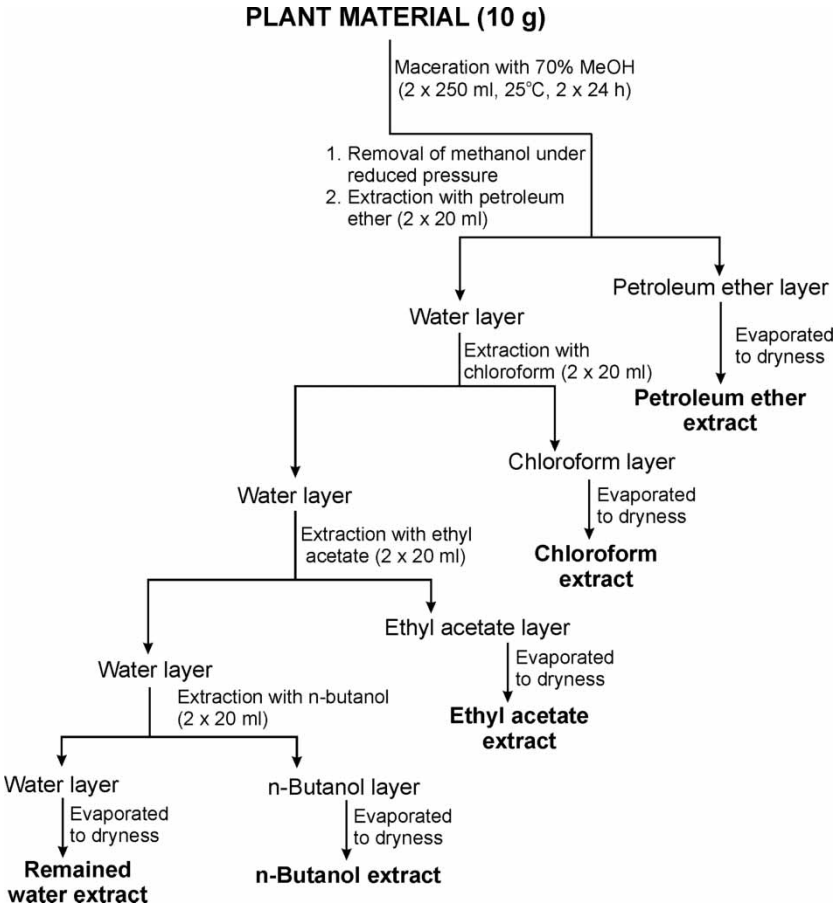

Figure 1. Scheme for preparation of extracts.

calculations were accomplished with use of a Hewlett Packard Chemstation equipped with Hewlett Packard Software (Pascal). A reversed-phase column, Hypersil MOS (250 × 2.1 mm) with a 5 μm particle size (Hewlett-Packard, USA), was used at the flow rate of 0.200 mL/min. The solvent system used was a linear gradient of solvent A (water-acetic acid pH 2.8) and solvent B (acetonitrile): from 1% to 32.2% B in 40 minutes, 32.2% to 40% B in 5 minutes, 40% to 1% B in 5 minutes, hold at 1% B for 10 minutes.

The injected volume was 10 μL of a water solution of extracts (10 mg/mL) and standards (1 mg/mL). The injection was performed manually. All solutions were filtered prior to the injection through 0.45 μm membrane filters (Millipore, Bedford, MA, USA). The column temperature was 26°C. The spectra were acquired in the range 210–400 nm and chromatograms plotted at 290/4 nm with reference wavelength 550/100 nm.

Identification and Quantification

Phenolic acids and flavanols in extracts were identified by matching the retention time and their spectral characteristics against those of standards. The purity of the peaks was determined to ensure the identification, and because of that, for each peak, spectra taken from five points were compared. The external standard method was the technique used for quantification. For each compound, the stock solution (concentration of 1 mg/mL) was made by accurately weighing out commercial standards of phenolic acids and flavanols, followed by dissolution in water. Solutions used for calibration were prepared by dilution of the stock solutions. Peak areas from chromatograms were plotted against the known concentrations of standards. Equations generated via linear regression were used to establish concentrations of phenolic acids and flavanols in extracts.

Statistical Analysis

All measurements were carried out in triplicate, and presented as mean $\pm$ SD. Regression analyses and significance of differences were carried out using a SPSS Statistical Software package (SPSS for Windows, 8.0, 1997, SPSS Inc., Chicago, IL, USA). Statistical significance level was fixed at $p < 0.05$, unless indicated otherwise.

RESULTS AND DISCUSSION

Total Phenolic Content

The phenolic compounds are generally distributed in the herbal extracts as a function of polarity. Phenolic compounds were not generally found in the petroleum ether fractions, while only very small quantities could be found in the chloroform fractions. The ethyl acetate and *n*-butanol fractions contain phenolic acids and their glycosides, as well as flavonoids and their glycosides. The most polar compounds remain in the water fraction.[22]

Table 1 lists the total phenolic content expressed as μg per g dry weight of plant, of petroleum ether, chloroform, ethyl acetate, *n*-butanol, and water extracts of winter savory. The obtained results showed that the quantity of phenolic compounds in ethyl acetate (969.43 μg/g) and *n*-butanol (1358.14 μg/g) extracts were significantly higher ($p < 0.001$) than in water extract (96.36 μg/g). Petroleum ether did not extract any of the phenolic compounds, while phenolic compounds are present in chloroform extract in a very small quantity (8.36 μg/g).

Table 1. Total phenolic content in savory extracts

Extract	Total phenolic content (μg/g)[a]
Petroleum ether	0
Chloroform	8.36 ± 0.43
Ethyl acetate	969.43 ± 48.47
n-Butanol	1358.14 ± 67.91
Water	96.36 ± 4.82

[a]Expressed as μg per g dry weight of plant.

HPLC Separation of the Standard Mixture of Phenolic Compounds

To identify and quantify individual phenolic compounds in winter savory extracts, a standard mixture was prepared containing phenolic acids and flavanols, which were commercially available. After multiple preliminary assays, a gradient elution program using acetonitrile-acetic acid-water as mobile phase was chosen (as given under Instrumentation and chromatographic conditions). Linear gradient elution was set using a standard mixture of phenolic acids and flavanols for achieving good peak shapes and resolution (Figure 2). As shown in Figure 2, a good separation of phenolic acids and flavanols, with sharp symmetrical peaks, can be performed within 25 min.

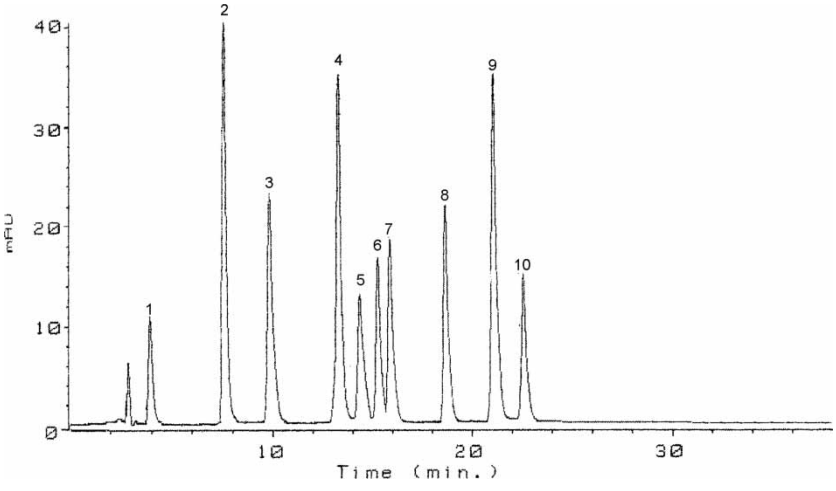

Figure 2. HPLC chromatogram of the mixture of standards at 290 nm. Peaks: 1, gallic acid; 2, protocatechuic acid; 3, (±)-catechin; 4, vanillic acid; 5, caffeic acid; 6, syringic acid; 7, (−)-epicatechin; 8, coumaric acid; 9, ferulic acid; 10, 3,5-dimethoxy-4-hydroxycinnamic acid.

Table 2. Calibration curves of the phenolic acids and flavanols

		Calibration curves			
Peak	Phenolic acid	Regression equation	Linear range (μg/mL)	r	Detection limit (μg/mL)
1	Gallic acid	$y = (8.22x - 0.0150)10^4$	1–20	0.9950	0.5
2	Protocatechuic acid	$y = (6.14x - 0.0017)10^4$	1–20	0.9988	0.5
3	(±)-Catechin	$y = (0.45x + 0.0101)10^4$	50–1500	0.9986	10
4	Vanillic acid	$y = (5.24x + 0.0040)10^4$	1–20	0.9966	0.5
5	Caffeic acid	$y = (18.06x - 0.0137)10^4$	10–60	0.9993	1.0
6	Syringic acid	$y = (7.3x - 0.0019)10^4$	10–60	0.9951	0.8
7	(−)-Epicatechin	$y = (2.07x + 0.0003)10^4$	20–100	0.9999	2.3
8	*p*-Coumaric acid	$y = (19.45x + 0.0002)10^4$	1–20	0.9986	0.2
9	Ferulic acid	$y = (10.61x + 0.0366)10^4$	1–20	0.9995	0.3
10	3,5-Dimethoxy-4-hydroxycinnamic acid	$y = (52.68x + 0.0054)10^4$	1–80	0.9999	0.8

y – peak area at 290 nm; x – concentration (mg/mL); r – correlation coefficient.

A calibration curve was established for each phenolic acid and flavanol at concentration ranges that were expected in extracts. Validation results are summarized in Table 2.

All investigated phenolic acids and flavanols showed excellent linearity with correlation coefficients between peak areas and concentrations greater than 0.995 in the range studied. The detection limit values were calculated as the concentration corresponding to three times the standard deviation of the background noise. The detection limits ranged from 10 μg/mL for (±)-catechin to 0.2 μg/mL for *p*-coumaric acid. These results suggest that the presented HPLC screening method is sufficiently sensitive for determination of the phenolic compounds in this type of sample. As could be seen from the chromatogram (Figure 2) and Table 2, target compounds are separated well on a C_8 column (Hypersil MOS) in a short analysis time (25 min). Good separation of these phenolic compounds could be achieved on C_{18} columns in a longer time, within 30 min,[23] 45 min,[24] 55 min,[25] or 60 min.[26,27]

HPLC Analysis of Phenolic Compounds in Winter Savory Extracts

Winter savory extracts were analyzed by the HPLC assay described above. Chromatograms of petroleum ether, chloroform, and water extracts showed that these extracts were poor in phenolic compounds (Figure 3). This fact is in agreement with the previously determined low content of phenolic compounds in these three extracts (Table 1).

Further analyses were performed only on the ethyl acetate and *n*-butanol extracts, rich in phenolic compounds. The chromatograms of these extracts are presented in Figure 4.

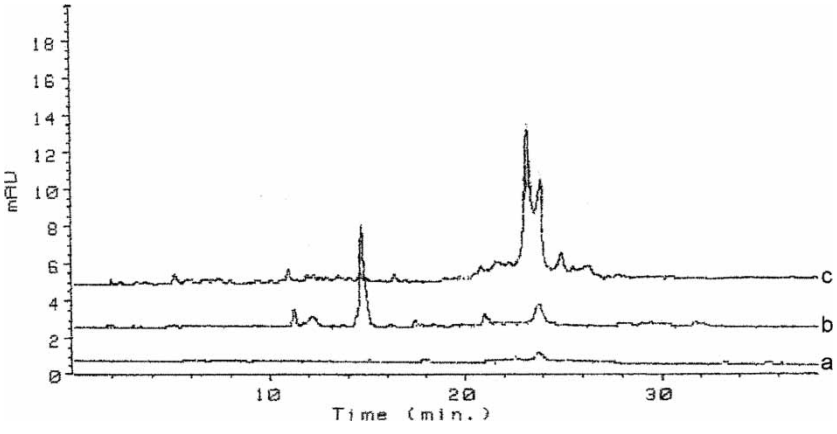

Figure 3. HPLC chromatogram of (a) 10 mg/mL petroleum ether, (b) 10 mg/mL chloroform, and (c) 10 mg/mL water extracts at 290 nm.

Protocatechuic, syringic, and vanillic acids (hydroxybenzoic acid derivatives), caffeic, *p*-coumaric, and ferulic acids (hydroxycinnamic acid derivatives), and (±)-catechin and (−)-epicatechin (flavanols), were identified in both extracts (Figure 4). In addition, peaks IV and VI–X named unidentified compounds, that had similar spectra to ferulic (Figure 5a) and/or isoferulic acids[28] and because of that, UC4 and UC6 to UC10 were

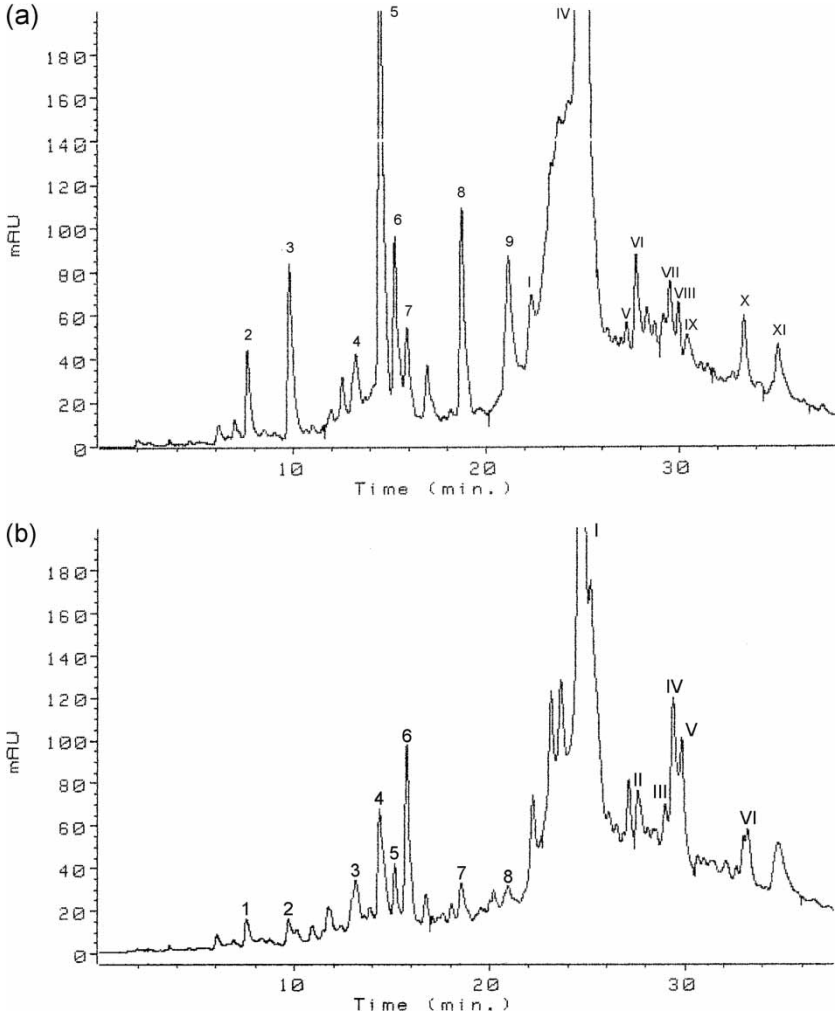

Figure 4. HPLC chromatogram of (a) 10 mg/mL ethyl acetate and (b) 10 mg/mL *n*-butanol extracts at 290 nm. Peaks: 2, protocatechuic acid; 3, (±)-catechin; 4, vanillic acid; 5, caffeic acid; 6, syringic acid; 7, (−)- epicatechin; 8, *p*-coumaric acid; 9, ferulic acid I–XI, unidentified compounds UC1–UC11.

tentatively identified as isomeric forms of ferulic acid. Spectra of unidentified compounds UC1 to UC3, UC5, and UC11 are shown on Figure 5b.

The retention times including the standard deviation for three replicates of ethyl acetate and *n*-butanol extracts were studied, UV absorption maxima of each peak, as well as content of phenolic acids and flavanols in these extracts are listed in Table 3. The content of phenolic acids and flavanols were calculated by using the obtained concentrations of these compounds from calibration curves (Table 2), expressed as μg per gram dry weight of plant.

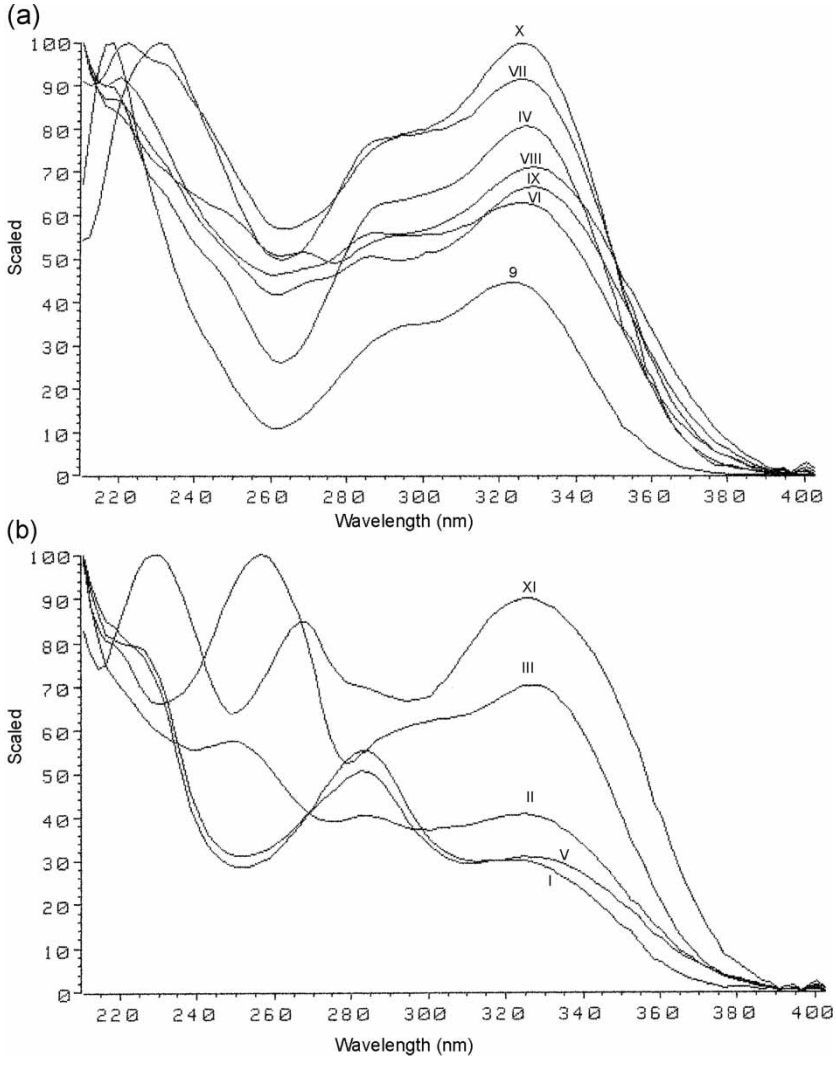

Figure 5. Photodiode array UV spectra of (a) ferulic acid (9) and peaks IV, VI-X and (b) peaks I–III, V, and XI.

Table 3. Retention times, UV absorbtion maxima and content of phenolic acids and flavanols in ethyl acetate and *n*-butanol extracts

Peak	Phenolic acid	Retention time (min)	UV_{max} (nm)	Content in ethyl acetate extract (μg/g)[a]	Content in *n*-butanol extract (μg/g)[a]
2	Protocatechuic acid	7.56 ± 0.06	258, 292	5.48 ± 0.27	7.60 ± 0.37
3	(+)-Catechin	9.73 ± 0.06	242, 279	622.47 ± 28.03	238.08 ± 11.64
4	Vanillic acid	13.16 ± 0.06	260, 290	4.99 ± 0.23	20.43 ± 0.95
5	Caffeic acid	14.42 ± 0.07	238, 296, 322	13.19 ± 0.65	15.44 ± 0.73
6	Syringic acid	15.19 ± 0.06	274	14.07 ± 0.70	45.86 ± 2.18
7	(−)-Epicatechin	15.80 ± 0.07	240, 278	16.29 ± 0.77	199.82 ± 9.74
8	*p*-Coumaric acid	18.63 ± 0.09	294, 308	4.82 ± 0.22	4.04 ± 0.20
9	Ferulic acid	21.05 ± 0.09	236, 294, 322	5.04 ± 0.24	3.33 ± 0.16

[a]Expressed as μg per g dry weight of plant.

All identified phenolic aids and flavanols were present in both of the investigated extracts in different extents. Total content of phenolic acids in ethyl acetate extracts was 47.59 μg/g, while (±)-catechin and (−)-epicatechin were present in quantities of 622.47 and 16.29 μg/g, respectively. *n*-Butanol extract contained these compounds in the extent of 96.70 μg/g for phenolic acids; 239.08 μg/g for (±)-catechin; 199.82 μg/g for (−)-epicatechin.

Compounds UC4 and UC6 to UC10 were present in ethyl acetate and *n*-butanol extracts in quantities of 108.25 and 294.86 μg/g, respectively, and expressed as ferulic acid equivalents.

The total phenolic content of ethyl acetate and *n*-butanol extracts (Table 1) are higher than the sum of the individual phenolics in these extracts identified by HPLC. The sum of the individual phenolics was 794.60 μg/g in ethyl acetate extract and 830.46 μg/g in *n*-butanol extract. This difference can be explained by fact that the Folin-Ciocalteau method is not an absolute measurement of the amount of phenolics, and various phenolic compounds have different responses in this assay.[21] The statistical analysis showed a good relationship between total phenolic content obtained by the Folin-Ciocalteau method and HPLC ($r = 0.9819$).

CONCLUSION

The ethyl acetate and *n*-butanol winter savory (*Satureja montana* L.) extracts, obtained by successive extraction, possessed the highest content of phenolics compared to petroleum ether, chloroform, and water extracts. Hydroxybenzoic acid (protocatechuic, syringic, and vanillic acids), hydroxycinnamic acid (caffeic, *p*-coumaric and ferulic acids), derivatives, and flavanols ((±)-catechin and (−)-epicatechin), as well as some tentatively identified phenolic acids were identified and quantified in ethyl acetate and *n*-butanol extracts of winter savory, using the HPLC screening method. These compounds were separated well on C_8 columns (Hypersil MOS), in standard mixtures as well as in extracts.

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