ORIGINAL ARTICLE

HPLC/MS ANALYSIS OF CAFFEIC AND CHLOROGENIC ACIDS FROM THREE ROMANIAN *VERONICA* SPECIES AND THEIR ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES

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Abstract

Aerial parts of *Veronica* species are used in Romanian traditional medicine for the treatment of various conditions like kidney diseases, cough, and catarrh, and are known for their wound-healing properties. In the present study three relevant *Veronica* species (*Plantaginaceae*), namely *V. officinalis* L., *V. teucrium* L. and *V. orchidea* Crantz from the Romanian spontaneous flora were investigated considering their content in caffeic and chlorogenic acids, antioxidant and antibacterial properties against anaerobic bacteria. The identification and quantification of the phenolic compounds performed by LC/MS revealed the presence of the two phenolic acids in all samples. The antioxidant potential investigated by several assays showed *V. officinalis* and *V. orchidea* as superior sources of natural antioxidants. The antibacterial screening against two anaerobic bacterial strains was assessed by a micro-dilution technique; the results indicating *V. teucrium* and *V. orchidea* extracts being more active on *Peptostreptococcus anaerobius*.

Rezumat

Părțile aeriene ale speciilor genului *Veronica* sunt folosite în medicina tradițională românească pentru tratarea diverselor afecțiuni cum ar fi bolile de rinichi, tusea, catarul, fiind cunoscute și pentru proprietățile lor cicatrizante. În studiul de față, trei specii reprezentative ale genului *Veronica* (*Plantaginaceae*) au fost investigate privind conținutul acestora în acizii cafeic și clorogenic precum și proprietăților lor antioxidante și antimicrobiene asupra a două tulpini bacteriene anaerobe. Identificarea și cuantificarea celor doi compuși polifenolici s-a realizat prin HPLC/MS, fiind pusă în evidență prezența celor doi analiți în toate speciile studiate. Potențialul antioxidant al celor două specii a fost studiat prin diverse metode, speciile *V. officinalis* și *V. orchidea* fiind indicate ca surse importante de antioxidanți naturali. Potențialul antimicrobian a fost evaluat prin tehnica microdiluțiilor, rezultatele indicând o activitate superioară pentru *V. teucrium* și *V. orchidea* asupra *Peptostreptococcus anaerobius*.

Keywords: *Veronica officinalis* L., *V. teucrium* L., *V. orchidea* Crantz., antioxidant, antimicrobial

Introduction

Veronica L. genus is the largest genus of *Plantaginaceae* family, with about 500 species widespread over most of the northern hemisphere and in many parts of the southern hemisphere; it presents a high ecological diversity with species growing in aquatic to dry steppe habitats from sea level to high alpine regions. This diversity and the fact that many species have beautiful blue flowers may explain the interest *Veronica* has drawn for a long time. In the Romanian spontaneous flora, the *Veronica* genus gathers 41 species and 3 hybrids grouped in 5 subgenus [1, 6, 10].

Aerial parts of *Veronica* species are used as traditional remedies for treatment of various inflammatory conditions, including rheumatism [3]. Some of the *Veronica* species, like *Veronica officinalis* L. have a long history of medicinal use as diuretic and diaphoretic agents. In the Romanian folk medicine, it has been used for kidney diseases, cough, and catarrh, and was known for its wound healing properties and its

indication in lung diseases and hypercholesterolemia [4-6, 19].

The chemical composition of *Veronica* species has been previously investigated regarding the iridoid profile [6, 10], but no other data were found regarding the composition of indigenous *V. officinalis* L., *V. teucrium* L. and *V. orchidea* Crantz. Recently, however few studies confirm that certain *Veronica* species exhibit noticeable bioactivity such as antibacterial [8, 22], antioxidant [11, 23], anti-inflammatory [3, 9] and citotoxic [9, 18].

Considering these matters, this study aimed to investigate the composition in caffeic and chlorogenic acids of aerial parts of relevant *Veronica* species (*V. officinalis*, *V. teucrium* and *V. orchidea*) harvested from the Romanian spontaneous flora. The quantitative content of the two phenolic acids was estimated by using a newly developed, short and rapid LC/MS method. This study was also intended to explore the antioxidant capacities of the three species by using 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox Equivalent Antioxidant Capacity (TEAC) and two more physiologically relevant methods, one based on the interaction of the antioxidant molecules with the ferryl haemoglobin species - HAPX (haemoglobin ascorbate peroxidase activity) and the other on cytochrome *c*-induced oxidation of lecithin liposomes, as well as EPR spectroscopy. Furthermore, the antibacterial properties of the three species were assessed by using the microdilution method [24, 25].

Materials and Methods

Plant material and extraction procedure

The aerial parts of *V. officinalis* L., *V. teucrium* L. and *V. orchidea* Crantz were collected in the summer of 2014, from Cluj and Alba counties. Voucher specimens were deposited in the Herbarium of the Pharmaceutical Botany Department, Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania. The vegetal material was air dried at room temperature in shade, separated and grinded to a fine powder (< 300 μm) before the extraction procedure. One gram of each sample was extracted with 10 mL of 70% ethanol, twice for 30 min in a sonication bath at room temperature. The samples were then centrifuged at 4.500 rpm for 15 min, and the supernatant was recovered.

Chromatographic conditions for the analysis of caffeic and chlorogenic acids

The two compounds were separated using a Zorbax SB-C18 (Agilent, Santa Clara, CA, SUA) reversed-phase analytical column (100 × 3.0 mm i.d., 3.5 μm particle) operated at 42°C. The separation was achieved under isocratic conditions using a mobile phase consisting of 0.1% acetic acid and acetonitrile (V/V). The flow rate was 0.8 mL/min with an injection volume of 5 μL. Mass spectrometry analysis was performed on an Agilent Ion Trap 1100 VL mass spectrometer with electrospray ionization (ESI) interface in the negative mode. The operating conditions were optimized in order to achieve maximum sensitivity values: gas (nitrogen) temperature 360°C at a flow rate of 12 L/min, nebulizer pressure 60 psi and capillary voltage +3500 V. The full identification of compounds was performed by comparing the retention times and mass spectra with those of standards in the same chromatographic conditions. To avoid or limit the interference from background, the multiple reactions monitoring analysis mode was used instead of single ion monitoring (e.g., MS/MS instead of MS).

Antioxidant activity assays

DPPH assay

An ethanolic solution of 100 μM DPPH was prepared. To measure the antioxidant capacity, 5 μL of *V. officinalis* or *V. teucrium* sample from 5 times diluted extracts were added, while for the *V. orchidea* sample, 3 μL extract were added. The process was monitored spectrophotometrically at 517 nm, for 30 minutes, at room temperature. The same parameters, as in the TEAC assay, were calculated (ΔA) and expressed as quercetin equivalents using a calibration curve (R = 0.99) with quercetin standard solutions of 0-12 μM [7, 13, 14, 21].

Trolox equivalent antioxidant capacities (TEAC) assay

100 μM ABTS solution was enzymatically prepared to obtain the ABTS radical ($ABTS^{\bullet+}$). After that, kinetic measurements were performed at 735 nm for 30 minutes, in the presence of 3 μL extract from 5 times diluted extracts. Typical decay curves were obtained for every sample (see in allowing one to calculate the total change in absorbance (ΔA), which correlates directly to the antioxidant capacities of the samples. The results are expressed as trolox equivalents (TE) using a calibration curve (R = 0.98) with trolox standard solutions of 0-16 μM [12, 14, 20].

Inhibition of haemoglobin ascorbate peroxidase activity (HAPX) assay

The HAPX assay was performed as in previous published studies [7, 14]. Thus, in a quartz cuvette, 6 μL ascorbate of 20 mM, 9.23 μL H_2O_2 of 75 mM and 3 μM extracts, from the five times diluted extracts, were added to 979 μL sodium acetate buffer, pH 5.5. Absorbance changes were monitored at 405 nm, at room temperature. After absorbance stabilization, the reaction was started by the addition of 3 μL met-Hb of 2 mM.

Inhibition of lipid peroxidation

In the liposomes experiment the oxidation of liposomes was monitored at 235 nm and at room temperature, in the presence of cytochrome *c*

(2 μM) and the extracts (5 μL from 2000 times diluted extracts). Liposomes were obtained from soybean lecithin dissolved in phosphate buffer (20 mM, pH 7), followed by sonication for 15 minutes in an ultrasonic bath (using a Power Sonic 410 device) [7, 14, 16].

Free radical generation experiment

EPR spectra were collected with a Bruker ELEXSYS E-580 spectrometer as previously described [17]. The extracts were diluted 10 times in 90% ethanol and mixed with 5 mM NaOH in a 100/1 ratio, yielding a pH of 11.7 [17]. The spectra of the reference compounds were obtained from 2 mM pure polyphenol (luteolin, quercetin, caffeic acid, chlorogenic acid, etc.) prepared in the same manner.

Antibacterial activity

Microorganisms and culture conditions

For the bioassay two anaerobic bacterial strains were used: *Peptostreptococcus anaerobius* (ATCC 27337) and *Fusobacterium nucleatum* (ATCC 25586). All of the tested microorganisms were obtained from Food Biotechnology Laboratory, Life Sciences Institute, University of Agricultural Sciences and Veterinary Medicine Cluj Napoca, Romania. The bacteria were cultured on Muller-Hinton agar and cultures were stored at 4°C and subcultured once a month [15].

Microdilution method

The modified microdilution technique was used to evaluate the antimicrobial activity. Anaerobic bacteria were cultured overnight at 37°C on Thioglycollate broth with resazurin media at 37°C. The bacterial cell suspensions were adjusted with sterile saline to a concentration of approximately $3*10^5$ CFU/mL in a final volume of 100 μL per well. The inoculum was stored at 4°C for further use. Dilutions of the inoculum were cultured on solid Muller–Hinton (MH) for bacteria to verify the absence of contamination and to check the validity of the inoculum. Determinations of minimum inhibitory concentrations (MICs) were performed by a serial dilution technique using 96-well microtiter plates. Different dilutions of the ethanolic extracts were carried out over the wells containing 100 μL of Thioglycollate broth with resazurin and afterwards, 10 μL of inoculum was added to all the wells. The microplates were incubated for 24-48 h at 37°C. The MIC of the samples was detected following the addition of 20 μL (0.2 mg/mL) of resazurin solution to each well, and the plates were incubated 2 h at 37°C. A change from blue to pink indicates reduction of resazurin and therefore bacterial growth. The MIC was defined as the lowest drug concentration that prevented this colour change. The minimum bactericidal concentrations (MBCs) were determined by serial sub-cultivation of a 2 μL into microtiter plates containing 100 μL of broth per well and further incubation for 48 h at 37°C. The lowest concentration with no visible growth was defined as MBC, indicating 99.5% killing of the original inoculum. Amoxicillin and metronidazole were used as positive control for the anaerobic bacteria. A 10% solution of ethanol in water was used as negative control [15].

Statistical analysis

The average of multiple measurements (triplicates) was listed in the tables together with the standard deviations. Statistical analysis was performed using Excel software package.

Results and Discussion

HPLC/MS analysis of caffeic and chlorogenic acids

Under the described chromatographic conditions, the retention times of the two phenolic acids were 2.2 min for chlorogenic acid and 3.3 for caffeic acid, as seen in Figure 1.

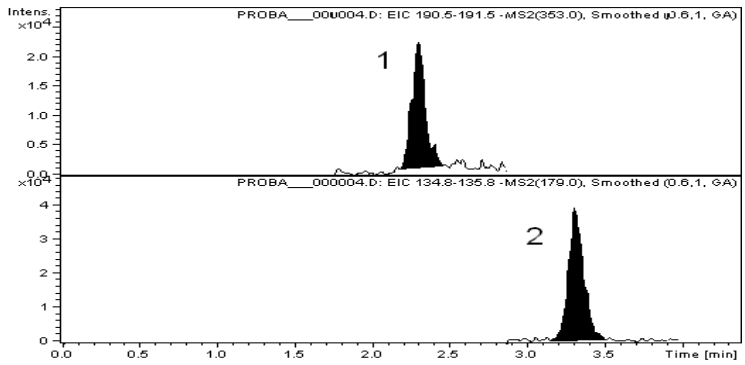

Figure 1.
MS chromatograms of chlorogenic (1) and caffeic (2) acids

Due to the fact that in the ionization conditions both chlorogenic and caffeic acids loose a proton, the ions monitored in the mass spectrometry process are in the form $[M-H]^-$. Thus, the ions recorded

have m/z = 353 for chlorogenic acid and m/z = 179 for caffeic acid. However, in order to increase the selectivity and sensitivity of the analytical method, for each of the analytes a daughter ion was monitored from the MS/MS spectrum, as seen in Table I.

Table I

Quantitative determination of chlorogenic and caffeic acids

Sample	Caffeic acid (µg/g)	Chlorogenic acid (µg/g)	Specific ions for identification (m/z)
V. officinalis	230.1 ± 6.21	9.1 ± 0.32	m/z 179 > m/z 135 (caffeic acid); m/z 353 > m/z 191 (chlorogenic acid).
V. teucrium	11.3 ± 1.12	70.6 ± 2.15	
V. orchidea	149.5 ± 3.52	16.6 ± 0.02	

The method can also be applied for quantitative determinations because the intensity of ions in the mass spectrum is proportional to the concentration of the substance in the sample. The ions with m/z = 191 and m/z = 135 were further used for that purpose. Previous data regarding the presence of these two phenolic acids in *Veronica* species were reported by Beara *et al*. who found these compounds in *V. teucrium*, *V. jaquinii* and *V. urticifolia* harvested in Serbia [3]. However the results are not comparable due to the fact the authors expressed their results per dry weight (d.w.) extract and not per d.w. vegetal material. In another study conducted by Živković *et al*. on *V. urticifolia* the presence of 3-*O*-caffeoylquinic acid and caffeic acid was indicated [22]. Nevertheless, Barreira *et al*. identified 3-*O*-caffeoylquinic acid in *V. montana*, 5-*O*-caffeoylquinic acid in *V. montana* and *V. polita* alongside with caffeic acid in *V. montana* and *V. spuria* [2].

Antioxidant activity assays

The antioxidant activity of the ethanolic extracts of *V. officinalis*, *V. teucrium* and *V. orchidea* was evaluated using the DPPH assay, Trolox equivalent antioxidant capacity (TEAC) method, haemoglobin ascorbate peroxidase activity inhibition (HAPX) assay and by testing the inhibition of lipid peroxidation catalysed by cytochrome *c*, as shown in Table II and Figure 2.

Table II

Antioxidant capacity assays and EPR measurements

Sample	DPPH (mg QE) / g plant material	ABTS (mg TE) / g plant material	HAPX %	EPR area (t0)
V. officinalis	12.54 ± 0.15	4.18 ± 0.21	110.04 ± 25.34	5.55
V. teucrium	5.03 ± 0.04	2.06 ± 0	45.41 ± 5.90	3.48
V. orchidea	20.90 ± 1.33	6.27 ± 0.25	120.81 ± 35.91	2.53

DPPH assay

The antioxidant activity of the three samples was assessed by the DPPH radical bleaching method and the results were expressed as quercetin equivalents (QE) (Table II). The highest radical scavenging activity was shown by *V. orchidea* (20.90 ± 1.33 mg QE/g d.w. plant material); followed by *V. officinalis* (12.54 ± 0.15 mg QE/g d.w. plant material); the lowest results being obtained for *V. teucrium* (5.03 ± 0.04 QE/g d.w. plant material). In this case, the percentage of DPPH consumption was converted to quercetin equivalents by using a calibration curve (R^2 = 0.99) with quercetin standard solutions of 0 - 12 µM [7, 14].

TEAC assay

The TEAC results are in agreement with the DPPH values being also correlated with HAPX results. Both, DPPH and TEAC assays are based on free radical scavenging by electron transfer mechanism [14].

HAPX assay

The recently described enzymatic assay (HAPX) measures the ability of antioxidants to protect against peroxide-induced free radicals in a physiologically-relevant system, involving the protein haemoglobin [7, 14, 17]. For the particular set of samples examined in the present work, the trend in HAPX values is very similar to the one seen in the DPPH and ABTS measurements, suggesting that the antioxidant capacity in these *Veronica* species is dominated by compounds for which the reactivity can be safely approximated as involving non-specific redox reactions, rather than any particular affinities towards protein matrices.

Inhibition of lipid per-oxidation

The cytochrome *c* assay monitors the ability of an antioxidant to inhibit the cytochrome (heme)-catalysed formation of lipid conjugated dienes at 235 nm. The antioxidant capacity is reflected in the delay of the onset of lipid oxidation. The mechanism whereby this may occur, may involve interaction with lipids and especially with lipid hydroperoxides, as well as interaction with the reactive Fe (IV) form of cytochrome *c*, or with protein-located free radicals generated by the Fe (IV) [7, 14, 17]. Thus, in this experiment, the *V. teucrium* extract delayed the oxidation of lipids about 100 minutes, *V. orchidea* about 200 minutes while the *V. officinalis* extract completely blocked the oxidation during the time of the experiment (1000 min). The order of reactivity is thus identical to that seen in

the other assays – except that the magnitude of the difference between the two most active extracts is distinctly larger in the cytochrome *c* assay than in any of the other ones.

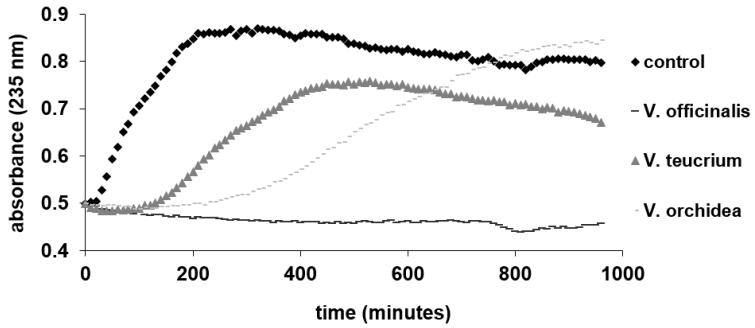

Figure 2.
Liposome oxidation by cytochrome *c*, in the presence of the tested samples

EPR direct detection of polyphenolic radicals

Alkali-treated polyphenols generate semiquinone radicals, whose EPR spectra are easily observable even in matrices as complex as natural extracts/products [17]. The hyperfine structure and the intensity of the signal of such spectra have been shown to offer information regarding the antioxidant capacity (Table II) as well as the chemical composition of the samples. The spectra collected for the three *Veronica* extracts immediately after adding NaOH are relatively similar to each other; they then slowly decrease in the intensity, and change shape (Figure 3. A). The initial spectra, bear no resemblance to those of pure polyphenols such as luteolin, quercetin, rutin or the spectra of the poliphenolic acids as caffeic and chlorogenic acids. Attempts to simulate the *V. officinalis* spectrum at t = 0 min as a sum of spectra of known compounds, led to a mixture of 50% quercetin and 50% luteolin free radical signals; this match is not perfect, suggesting that either compounds beyond our library of EPR spectra are present, or there is a synergy effect which affects the line-shapes of the spectra. By contrast, the spectrum at t = 30 minutes is very similar to the spectrum of chlorogenic acid (Figure 3 B).

Antibacterial activity

The results of antibacterial activity from *V. officinalis*, *V. teucrium* and *V. orchidea* ethanolic extracts and standard antibiotics amoxicillin and metronidazole, tested by the microdilution assay, are presented in Table III.

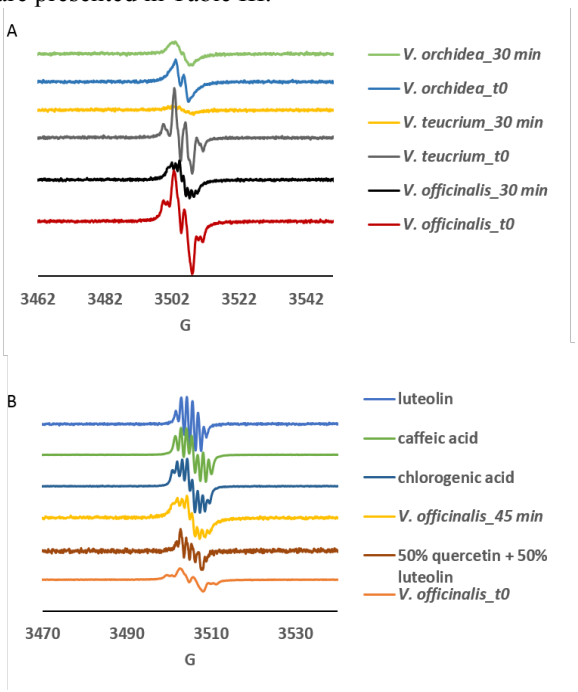

Figure 3.
EPR spectra of *Veronica* sp. extracts (A) and for reference compounds (B), treated with NaOH, in ethanol 90%

Table III
Antibacterial activity of *Veronica* extracts

Samples	MIC (mg/mL)		MBC (mg/mL)	
	Peptostreptococcus anaerobius (ATCC 27337)	*Fusobacterium nucleatum (ATCC 25586)*	*Peptostreptococcus anaerobius (ATCC 27337)*	*Fusobacterium nucleatum (ATCC 25586)*
V. officinalis	62.5	62.5	125	125
V.teucrium	31.25	62.5	62.5	125
V. orchidea	31.25	62.5	62.5	125
Amoxicillin (µg/mL)	3.9	-	7.81	-
Metronidazole (µg/mL)	-	7.81	-	15.62

In the case of *V. officinalis*, both anaerobic strains presented low sensitivity, with equal values of MIC and MBC of 62.5 mg/mL and respectively, 125 mg/mL. Regarding *V. teucrium* and *V. orchidea* antibacterial activity, the strain of *Peptostreptococcus anaerobius* showed the same values for MIC (31.25 mg/mL) and MBC (62.5 mg/mL), being more sensitive. Nevertheless,
Fusobacterium nucleatum exhibited the lowest sensitivity to *Veronica* extracts. The antibacterial activity of Romanian *Veronica* species was already documented regarding aerobic bacterial strains in our previous study [15] and it is mainly related to their content in phenolic compounds and phyto-sterols. Thus the results of the present study contribute with further data regarding the antibacterial potential of *Veronica* species by extending their applications as antimicrobial agents against anaerobic bacteria strains.

Conclusions

In the present study, *V. officinalis*, *V. teucrium* and *V. orchidea* from Romanian spontaneous flora were investigated in relation to their content in caffeic and chlorogenic acids content, antioxidant and antimicrobial activity against anaerobic bacterial strains. Both, caffeic and chlorogenic acids were found in all species, the richest source of caffeic acid being *V. officinalis* while the highest amount for chlorogenic acid was present in *V. teucrium*. The antioxidant assays indicate *V. orchidea* as a superior source of natural antioxidants, however the best ability of lipid peroxidation inhibition was presented by *V. officinalis*. Concerning the antimicrobial potential of the investigated species, *V. teucrium* and *V. orchidea* presented a higher activity than *V. officinalis*, the most sensitive strain being *Peptostreptococcus anaerobius*. The results of this study bring new perspectives for further pharmaceutical and nutraceutical development of *Veronica* species as natural sources of bioactive compounds with antioxidant and antimicrobial potential. However, further studies are necessary in order to elucidate the mechanisms of *in vivo* antioxidant action, bioavailability and the involved metabolic pathways.

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