POLYPHENOLIC DETERMINATION FROM MEDICINAL PLANTS USED IN VETERINARY MEDICINE BY AN UHPLC-LC-MS/MS METHOD

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Abstract

Flavonoids and polyphenols are known for positive health effects and are used for their medicinal properties. They are considered functional foods and are widely distributed in plants having an important role in health benefits. In this study, several phenolic compounds have been determined quantitatively from eighteen medicinal plants. Chromatographic separation of analytes was performed using a NUCLEODUR C18 Gravity, 3 μ m, 150 x 3 mm column using a mobile phase consisting of 0.2% formic acid in water and methanol with gradient elution and a flow rate of 0.6 mL/min. Detection was performed in multiple reaction monitoring (SRM) mode for chlorogenic acid, caffeic acid, quercitrin, luteolin, quercetin, apigenin, and kaempferol. Plant extracts with methanol and ethanol (70%) were prepared. The studied medicinal plants revealed richness in flavonoids and polyphenols. The present study serves as a contribution to the characterization of phenols and flavonoids from medicinal plants traditionally used in human and veterinary medicine.

Rezumat

Flavonoidele și polifenolii sunt compuși cu efecte pozitive asupra sănătății, motiv pentru care se folosesc în medicina naturistă. Acești compuși sunt considerați alimente funcționale și se găsesc într-o varietate mare de plante. Extractele a 18 plante medicinale au fost analizate, folosind tehnica LC-MS, în scopul identificării și determinării cantitative a unor compuși reprezentativi din clasa fenolilor. Separarea cromatografică s-a realizat folosind o coloană NUCLEODUR C18 Gravity (3 μ m, 150 x 3 mm) și o fază mobilă formată din apă acidulată cu 0,2% acid formic - metanol, în gradient de concentrație. Debitul fazei mobile a fost 0,6 mL/minut. Detecția compușilor de interes (acid clorogenic, acid cafeic, quercitrină, luteolină, quercetină, apigenină și campferol) s-a făcut în modul SRM. Din plantele studiate s-au preparat extracte metanolice și extracte alcoolice (70%). Rezultatele au arătat prezența în cantitate mare a compușilor flavonoidici și polifenolici în plantele studiate. Lucrarea de față servește ca o contribuție la caracterizarea profilului flavonoidic și polifenolic al plantelor medicinale folosite în mod tradițional în medicina umană și cea veterinară.

Keywords: medicinal plants, polyphenols, flavonoids, LC-MS/MS

Introduction

Flavonoids and phenols are widely distributed in plants and play an important role in health-promoting effects, such as: anti-inflammatory effect, antiproliferative activity, reducing the risk of cancer and cardiovascular diseases, skin conditions, mainly based on their spin trapping properties, not only in phytotherapy, but also in veterinary medicine as well [12, 18, 28, 36]. There are many assumptions and a general belief regarding the dietary benefits of these compounds [6]. However, the epidemiological evidence shows that a diet rich in polyphenols reduces the risk of several illnesses. Poly-phenol administration decreases bowel inflammation, gastrointestinal inflammation in animal

models [8, 11]. Some medicinal plants rich in polyphenols and with their commercial formulas were experimented successfully in chicken coccidiosis [30]. Many medicinal plants are known to be particularly diverse in their contents of chlorogenic acids [7, 9], kaempferol, and other flavonoids or their glycosides forms [5, 15, 20, 21]. All plants contain significant levels of flavonoids and phenolic acids. Several phenolic compounds have been identified and quantified in medicinal plants [3, 19, 23, 34].

The following plants, such as *Achillea millefolium* L. (Asteraceae) (yarrow), *Agrimonia eupatoria* L. (Rosaceae) (agrimony), *Allium sativum* L. (Alliaceae) (garlic), *Arctium lappa* L. (Asteraceae) (greater burdock), *Artemisia absinthium* L. (Asteraceae) (wormwood), *Betula pendula*

Roth (*Betulaceae*) (silver birch), *Calendula officinalis* L. (*Asteraceae*) (marigold), *Convolvulus arvensis* L. (*Convolvulaceae*) (bindweed), *Equisetum arvense* L. (*Equisetaceae*) (horsetail), *Hypericum perforatum* L. (*Hypericaceae*) (St John's wort), *Lythrum salicaria* L. (*Lythraceae*) (purple loosestrife), *Origanum vulgare* L. (*Lamiaceae*) (oregano), *Plantaginis lanceolata* L. (*Plantaginaceae*) (narrowleaf plantain), *Polygonum aviculare* L. (*Polygonaceae*) (common knotgrass), *Potentilla anserina* (L.) Rydb. (*Rosaceae*) (silverweed), *Symphitum officinale* L. (*Boraginaceae*) (comfrey), *Thymus serpyllum* L. (*Lamiaceae*) (wild thyme), *Urtica dioica* L. (*Urticaceae*) (common nettle) are widely used in human and veterinary medicine with similar indications in both fields.

In our study, we identified and quantified flavonoid aglycons (kaempferol, luteolin, quercetin, apigenin), flavonoid glycoside (quercitrin), and phenolic acids (chlorogenic acid, caffeic acid) in the above listed plants using ultra performance liquid chromatography with diode array detector and quadrupole time of flight mass spectrometer (UHPLC-DAD-QTOF-MS).

The aim of this paper is to highlight the polyphenol content of the many medicinal plants used in veterinary medicine with a large effect in many veterinary illnesses.

Materials and Methods

Reagents and apparatus

HPLC grade methanol (Merck, Darmstadt, Germany) and formic acid 98 - 100% (analytical grade, Scharlau, Barcelona, Spain) were used. A Millipore Direct-Q 3 Water purification system (Millipore - Milford, USA) was used to obtain ultrapure water. Chlorogenic acid, caffeic acid, quercitrin, luteolin, quercetin, apigenin and kaempferol analytical standards were purchased from Extrasynthese-SAS (France).

Digital ultrasonic bath (Nahita, China) was used for extracts and filtered using nylon micropore 0.45 µm filters (Roth, Germany). A Perkin-Elmer (Waltham, USA) Flexar FX-10 HPLC system composed of two single pumps with inline degasser, auto-sampler and column thermostat coupled with an AB Sciex (Framingham, USA) QTOF 4600 mass spectrometer was used for analytical separation and detection.

Plant material

Several plants, such as *Achillea millefolium* L. (aerial parts), *Agrimonia eupatoria* L. (aerial parts), *Allium sativum* L. (bulbs), *Arctium lappa* L. (roots), *Artemisia absinthium* L. (aerial parts), *Betula pendula* Roth (leaf), *Calendula officinalis* L. (flower), *Convolvulus arvensis* L. (aerial parts), *Equisetum arvense* L. (aerial parts), *Hypericum perforatum* L. (aerial parts), *Lythrum salicaria* L. (aerial parts), *Origanum vulgare* L. (aerial parts), *Plantaginis lanceolata* L. (leaf), *Polygonum bistorta* (L.) Samp. (aerial parts), *Potentilla anserina* (L.) Rydb. (aerial parts), *Symphitum officinale* L. (roots), *Thymus serpyllum* L. (aerial parts), *Urtica dioica* L. (aerial

parts) were sampled from the spontaneous flora in Mureş County, Romania, during the summer of 2015. The plants were harvested in July and August and were stored at Larix Com Impex SRL manufacturing company Sovata, Mureş County, Romania.

Extraction procedure

Plant extracts with methanol or ethanol (70^o) were prepared. The harvested medicinal plants were ground and 5 g were extracted with 50 mL solvent for 30 minutes in an ultrasonic bath at a temperature of 25°C. Extracts were filtered through filter and diluted to 50 mL solvent in volumetric flasks.

Liquid chromatography tandem mass spectrometry conditions

Chromatographic separation of analytes was performed using a NUCLEODUR C18 Gravity, 3 µm, 150 x 3 mm (Macherey-Nagel) column with a mobile phase consisting of 0.2% formic acid in water and methanol with gradient elution and a flow rate of 0.6 mL/min. Detection was done in multiple reaction monitoring (SRM) mode for chlorogenic acid, caffeic acid, quercitrin, luteolin, quercetin, apigenin and kaempferol, monitoring the following transitions: chlorogenic acid: ion 191.10 *m/z* derived from ion 353.08 *m/z* at a collision energy of 20 V; caffeic acid: ion 135.08 *m/z* derived from ion 179.00 *m/z* at a collision energy of 20 V; quercitrin: sum of ions 300.10 *m/z* and 301.10 *m/z* derived from ion 447.03 *m/z* at a collision energy of 30 V; luteolin: ion 133.06 *m/z* derived from ion 285.00 *m/z* at a collision energy of 40 V; quercetin: ion 151.04 *m/z* derived from ion 300.99 *m/z* at a collision energy of 30 V; apigenin: ion 117.07 *m/z* derived from ion 269.01 *m/z* at a collision energy of 40 V; kaempferol: ion 93.06 *m/z* derived from ion 285.03 *m/z* at a collision energy of 41 V.

The proposed structures and negative ion MS data of the studied components are presented in Figure 1.

Ionization of analytes was performed using negative electrospray ionization mode. Ionization parameters used for the ionization source were: Spray voltage: 2500 V, vaporizer temperature: 350°C, Ion Gas Source 1: 25, Ion Gas Source 2: 25, Curtain Gas: 10, Declustering Potential: 100, Ion Release Delay: 30, Ion Release Width: 15. Sample run-time was 30 minutes.

Standard solutions

Stock solutions of each analyte having concentrations of 100 µg/mL each were prepared in methanol. These stock solutions were used to obtain a mixture containing a concentration of 10 µg/mL of each analyte, also in methanol. Furthermore, stock solutions with concentrations of 0.1 (lower limit of quantification - LLOQ), 0.25, 0.5, 1 and 10 µg/mL (the mixture itself) of each analyte were prepared using this mix. Calibration curve standard solutions were prepared diluting 100 µL of each stock solution (concentrations 0.1 - 10 µg/mL of each analyte) with 900 µL purified water.

Samples preparation

A volume of 100 μL of each sample and 900 μL purified water in Eppendorf tubes. The mixtures were centrifuged at 10000 rpm for 10 minutes, filtered using 0.45 μm nylon syringe filters. Solutions were

transferred to HPLC vials and 5 μL of the samples were injected into the LC-MS system.

Each sample from the medicinal plants extracts with methanol or ethanol (70%) used as solvents were diluted as the standards.

Figure 1.

The proposed structures and negative ion MS data of the studied components

Results and Discussion

Method validation

Specificity. Due to the lack of a bank sample, the specificity of the method is assured by the MS/MS technique, which allows the analysis of complex

mixtures, since we used a specific fragment derived from the parent compounds as identification method. A total ion chromatogram of a standard solution (5 $\mu\text{g/mL}$) is shown in Figure 2.

Figure 2.

A total ion chromatogram of a 5 $\mu\text{g/mL}$ standard solution (1-chlorogenic acid, 2-caffeic acid, 3-quercitrin, 4-quercetin, 5-luteolin, 6-kaempferol, 7-apigenin)

Table I

Parameters of the calibration curves and lower limit of quantifications

Compound	Regression equation ($y = ax + b$) slope (a) and intercept (b)		Correlation coefficient	Range of linearity ($\mu\text{g/mL}$)	LLOQ ($\mu\text{g/mL}$)
Apigenin	a = 22943	b = 4840	0.9996	0.11 - 11.0	0.11
Kaempferol	a = 1229	b = -168	0.9998	0.10 - 10.2	0.10
Luteolin	a = 19984	b = 350	0.9995	0.10 - 10.8	0.08
Quercetin	a = 17893	b = -406	0.9999	0.08 - 8.73	0.08
Quercitrin	a = 39529	b = 1726	0.9999	0.10 - 10.2	0.10
Chlorogenic acid	a = 29061	b = 1467	0.9999	0.10 - 10.8	0.10
Caffeic acid	a = 41724	b = 5217	0.9999	0.11 - 11.0	0.11

Linearity and LLOQ. Table I shows the parameters of the calibration curves and the LLOQs for all studied compounds. The LLOQ is set at signal to noise ratio of 10:1. The precision of the LLOQ was acceptable with a CV% < 15%.

Precision. The precision of the method was tested using the spiked sample technique at three concentration levels and was expressed in CV%. The results are presented in Table II.

Table II

Precision of the quality control samples (n = 5) in the case of methanolic extraction

Compound	Spiked concentration ($\mu\text{g/mL}$)	CV%
Apigenin	0.550	8.8
	0.275	10.2
	0.110	17.8
Kaempferol	0.514	9.6
	0.257	18.1
	0.102	10.3
Luteolin	0.436	17.7
	0.218	9.0
	0.087	19.0
Quercetin	0.540	17.6
	0.270	16.3
	0.108	14.8
Quercitrin	0.514	2.6
	0.257	1.9
	0.102	5.0
Chlorogenic acid	0.540	5.0
	0.271	19.1
	0.108	17.4
Caffeic acid	0.550	3.4
	0.275	10.3
	0.110	18.9

An extracted ion chromatogram of the *Millefoli herba* methanolic extract is shown in Figure 3.

Figure 3.

An extracted ion chromatogram of the *Millefoli herba* methanolic extract

The amount of polyphenols and flavonoids found in the studied plant materials are presented in Table III and Table IV.

Table IIIThe amount ($\mu\text{g/g}$) of the studied compounds in the tested plants (using methanol as extraction solvent)

Plant	Apigenin	Kaempferol	Quercetin	Luteolin	Quercitrin	Chlorogenic acid	Caffeic acid
<i>Achillea millefolium</i> (a.p.)	104.24 $\pm$ 50.44	16.13 $\pm$ 1.57	BLQ*	77.40 $\pm$ 35.78	18.69 $\pm$ 11.30	6512.60 $\pm$ 1454.21	365.56 $\pm$ 82.96
<i>Agrimonia eupatoria</i> (a.p.)	28.84 $\pm$ 9.26	146.09 $\pm$ 48.46	492.26 $\pm$ 51.43	196.34 $\pm$ 59.24	932.37 $\pm$ 37.31	37.01 $\pm$ 10.70	BLQ
<i>Allium sativum</i> (bulbs)	23.69 $\pm$ 0.81	18.71 $\pm$ 3.82	BLQ	BLQ	BLQ	BLD**	BLD
<i>Arctium lappa</i> (root)	BLD	16.81 $\pm$ 1.42	26.21 $\pm$ 5.53	BLD	10.51 $\pm$ 3.31	7677.89 $\pm$ 1901.79	234.88 $\pm$ 48.13
<i>Artemisia absinthium</i> (a.p.)	BLD	22.93 $\pm$ 3.40	9.63 $\pm$ 2.16	BLQ	BLQ	740.20 $\pm$ 65.79	42.66 $\pm$ 12.24

Plant	Apigenin	Kaempferol	Quercetin	Luteolin	Quercitrin	Chlorogenic acid	Caffeic acid
<i>Betula pendula</i> (leaf)	207.11 ± 71.78	27.35 ± 3.14	124.02 ± 22.58	BLQ	131.12 ± 38.28	1443.64 ± 424.36	91.14 ± 22.25
<i>Calendula officinalis</i> (flower)	BLQ	24.99 ± 6.51	50.70 ± 5.38	25.86 ± 7.03	23.20 ± 4.58	2638.51 ± 326.81	325.56 ± 86.59
<i>Convolvulus arvensis</i> (a.p.)	16.83 ± 1.27	18.89 ± 4.18	BLQ	BLD	BLQ	2377.70 ± 605.50	309.66 ± 49.46
<i>Equisetum arvense</i> (a.p.)	15.32 ± 0.24	16.35 ± 1.45	BLQ	BLQ	BLQ	145.07 ± 18.05	395.91 ± 7.92
<i>Hypericum perforatum</i> (a.p.)	47.03 ± 5.96	386.76 ± 35.80	5771.86 ± 1075.22	221.58 ± 44.74	3717.98 ± 600.88	1210.32 ± 99.02	40.65 ± 1.27
<i>Lythrum salicaria</i> (a.p.)	BLD	16.64 ± 1.17	BLQ	BLD	12.79 ± 4.83	535.89 ± 182.29	35.39 ± 2.63
<i>Origanum vulgare</i> (a.p.)	70.67 ± 22.46	24.43 ± 0.75	BLQ	256.02 ± 46.46	92.55 ± 30.56	348.22 ± 22.81	402.52 ± 31.17
<i>Plantaginis lanceolata</i> (leaf)	26.14 ± 6.84	32.17 ± 1.43	BLQ	BLQ	BLQ	237.99 ± 22.56	BLQ
<i>Polygonum aviculare</i> (a.p.)	13.74 ± 2.80	19.13 ± 1.85	BLQ	BLD	BLQ	147.35 ± 23.07	BLQ
<i>Potentilla anserina</i> (a.p.)	15.94 ± 1.09	45.10 ± 2.40	71.06 ± 16.40	13.11 ± 2.47	229.83 ± 40.79	68.29 ± 11.10	114.21 ± 12.88
<i>Symphitum officinale</i> (roots)	13.78 ± 3.94	19.78 ± 0.98	BLQ	BLQ	BLQ	BLQ	90.53 ± 4.84
<i>Thymus serpyllum</i> (a.p.)	28.45 ± 0.52	17.29 ± 0.90	BLQ	13.86 ± 2.73	BLQ	160.29 ± 5.68	230.64 ± 35.74
<i>Urtica dioica</i> (leaf)	14.96 ± 0.83	29.20 ± 2.31	BLQ	BLQ	16.83 ± 1.10	3569.69 ± 106.13	200.32 ± 29.16

a.p.= aerial parts; * – BLQ = below quantification limit; ** – BLD = below detection limit

Table IV

Flavonoid and polyphenol content (µg/g) of the samples using ethanol as extraction solvent

Plant	Apigenin	Kaempferol	Quercetin	Luteolin	Quercitrin	Chlorogenic acid	Caffeic acid
<i>Achillea millefolium</i> (a.p.)	256.58	17.37	16.68	318.47	BLQ	2004.64	2566.27
<i>Agrimonia eupatoria</i> (a.p.)	BLQ	113.08	240.60	102.62	1340.92	106.92	39.79
<i>Allium sativum</i> (bulbs)	22.43	27.11	BLQ	42.93	11.82	32.90	41.64
<i>Arctium lappa</i> (root)	BLD	19.06	114.97	BLD	40.71	10115.85	271.73
<i>Artemisia absinthium</i> (a.p.)	BLD	24.76	BLD	BLQ	BLQ	1167.70	43.21
<i>Betula pendula</i> (leaf)	196.50	45.74	307.53	BLQ	21.13	2387.66	31.75
<i>Calendula officinalis</i> (flower)	24.19	92.14	268.62	103.51	151.51	2795.41	286.91
<i>Convolvulus arvensis</i> (a.p.)	12.59	29.13	BLQ	BLQ	BLQ	2389.99	186.75
<i>Equisetum arvense</i> (a.p.)	BLQ	27.89	5.38	BLQ	BLQ	264.83	420.22
<i>Hypericum perforatum</i> (a.p.)	98.35	236.60	4564.57	287.20	3900.44	1426.26	42.47
<i>Lythrum salicaria</i> (a.p.)	BLD	14.95	BLQ	BLD	BLQ	986.37	BLD
<i>Origanum vulgare</i> (a.p.)	38.62	29.51	BLQ	68.42	32.42	61.48	58.00
<i>Plantaginis lanceolata</i> (leaf)	24.25	18.68	BLQ	BLQ	BLQ	82.03	BLD
<i>Polygonum aviculare</i> (a.p.)	14.19	16.50	BLQ	BLQ	BLQ	204.60	BLD
<i>Potentilla anserina</i> (a.p.)	42.82	33.65	111.76	25.55	9.40	390.68	629.87
<i>Symphitum officinale</i> (roots)	16.60	20.51	BLQ	BLQ	BLQ	13.26	314.55
<i>Thymus serpyllum</i> (a.p.)	24.03	27.88	BLQ	BLQ	BLQ	24.03	108.39
<i>Urtica dioica</i> (leaf)	15.44	25.24	19.97	BLQ	6.99	5534.36	310.69

a.p.= aerial parts; * – BLQ = below quantification limit; ** – BLD = below detection limit

In rather few studies involving some medicinal plants, such as aerial parts of *Achillea millefolium* for caffeic acid, luteolin and apigenin [14, 35], bulb of *Allium sativum* for chlorogenic acid, caffeic acid, luteolin, quercetin and apigenin [11, 12], *Betula pendula* leaves for chlorogenic acid [13], and aerial parts of lyophilized *Polygonum aviculare* for quercetin, quercitrin and kaempferol [17, 25] from methanolic extracts had higher concentrations of polyphenols and flavonoids than our Romanian methanolic extract samples.

In another studies on aerial part of *Agrimonia eupatoria* with chlorogenic acid [32], roots of *Arctium lappa* with chlorogenic acid, caffeic acid, quercitrin, quercetin and luteolin [10, 22], flower of *Calendula officinalis*

for chlorogenic acid content [32], aerial parts of *Convolvulus arvensis* for chlorogenic acid and caffeic acid [16], aerial parts of *Hypericum perforatum* for quercetin and quercitrin [1, 24], chlorogenic acid from aerial parts of *Lythrum salicaria* [2], aerial parts of *Origanum vulgare* with caffeic acid, chlorogenic acid, luteolin, apigenin, quercetin and kaempferol [31], aerial parts of *Potentilla anserina* with kaempferol, quercetin and caffeic acid [33] and the roots of *Symphitum officinale* with chlorogenic acid, caffeic acid, luteolin, quercetin, kaempferol and apigenin [29] had lower concentrations of polyphenols and flavonoids than in our Romanian samples.

Finally, some papers revealed similar or appropriate concentration values to our results, like aerial parts of *Artemisia absinthium* for caffeic acid [22], aerial parts of *Equisetum arvense* for chlorogenic acid (except for caffeic acid with lower concentrations than in Romanian samples) [26], aerial parts of *Thymus serpyllum* [4] for caffeic-chlorogenic acid, apigenin and quercetin from methanolic extracts.

Caffeic acid from leaves of *Urtica dioica* showed similar concentration with the Romanian *Urticae* leaf concentrations, whereas flavonoids such as kaempferol and quercitrin were not detectable. However, in our samples with methanolic extracts these compounds were present [27]. The caffeic acid from leaves of *Plantago major* in ethanolic extracts 26% was not detectable according to Meinhart *et al.* similarly to our Romanian methanolic extracts from leaves of *Plantaginis lanceolata* [22].

Regarding the differences between extraction efficiency of the two solvents used, a high variability of the extracted polyphenol and flavonoid amounts was observed: neither the type of the drug nor the used solvent explains the highest extracted polyphenol and/or flavonoid. Statistically, no significant differences were observed between the two sets of data using the Wilcoxon matched paired, two-tailed test at 95% confidence interval and $p = 0.05$ significance level. The observed differences in flavonoid concentration are determined by the solvents used as well as by the matrix.

The high variability in flavonoid content in the studied plants can be explained by the different extractability for these active ingredients dependent on the solvent used and the complexity of the sample matrix.

Conclusions

The results of our study show that the developed UHPLC-DAD-QTOF-MS/MS method is a fast and suitable method for the determination of polyphenolic profiles of medicinal plants. Using methanolic extracts, we determined high concentrations of chlorogenic acid from aerial parts of *Achillea millefolium*, flower of *Calendula officinalis*, aerial parts of *Convolvulus arvensis*, aerial parts of *Hypericum perforatum*, aerial parts of *Origanum vulgare*, leaf of *Plantaginis lanceolata* and aerial parts of *Thymus serpyllum*. Caffeic acid was in higher concentration in methanolic extracts from leaves of *Betula pendula*, flower of *Calendula officinalis*, aerial parts of *Lythrum salicaria*, aerial parts of *Origanum vulgare*, aerial parts of *Thymus serpyllum*. Flavonoid aglycon concentrations were in higher in methanolic extracts of aerial parts of *Agrimonia eupatoria*, aerial parts of *Origanum vulgare* and aerial parts of *Potentilla anserina*. In some cases, ethanolic extraction is more effective for flavonoid aglycons compared with methanol, like in cases of aerial parts of *Achillea millefolium*, bulbs of *Allium*

sativum, roots of *Arctium lappa*, flower of *Calendula officinalis*, aerial parts of *Hypericum perforatum*. Therefore, these constituents explain the richness of medicinal plants in flavonoids and polyphenolics. The present study serves as a contribution to the characterization of phenolics and flavonoids from medicinal plants traditionally used in veterinary medicine.

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Conflict of interest

The authors declare no conflict of interest.

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