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Article

HPLC Determination of Phenolic Acids, Flavonoids and Juglone in Walnut Leaves

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A high-performance liquid chromatographic method with gradient elution and diode-array detection was developed to quantify free phenolic acids (gallic, vanillic, chlorogenic, caffeic, syringic, *p*-coumaric, ferulic, sinapic, salycilic, elagic and *trans*-cinnamic), flavonoids (catechin, epicatechin, rutin, myricetin and quercetin) and juglone in walnut leaves. Chromatographic separation was performed on a Hypersil Gold C18 column (5 μm particle size, 250 × 4.6 mm) and detection was conducted at three different wavelengths (254, 278 and 300 nm) according to the absorption maxima of the analyzed compounds. Validation procedures were conducted and the method was proven to be precise, accurate and sensitive. The developed method has been applied to analyze walnut leaves samples from nine different cultivars, with the same agricultural, geographical and climatic conditions. The experimental results revealed high concentrations of myricetin, catechin hydrate and rutin, and low concentrations of quercetin and epicatechin aglycones. Ellagic acid was established as the dominating phenolic acid of walnut leaves, followed by *trans*-cinnamic, chlorogenic and caffeic acids. Juglone content varied between 44.55 and 205.12 mg/100 g fresh weight. Significant differences were detected among cultivars for the concentration levels of phenolics.

Introduction

Phenolic compounds are secondary metabolites of plants and are generally involved in the defense against ultraviolet radiation or aggression by pathogens (1, 2). They have been associated with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis, structural components and allelopathy (3).

Several thousands of natural polyphenols have been identified in plants, many of them in plant foods, although a more limited number are at significant levels in most human diets. Dietary intake of phenolics is estimated to be approximately one gram per day. This is significantly higher than that of all other dietary antioxidants, including vitamin C, vitamin E and carotenoids (4). Several studies showed that phenolic compounds are the major bioactive phytochemicals with human health benefits.

The term "plant phenolics" encompasses simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans and lignins (3).

Phenolic acids comprise two primary groups, both of which are hydroxy derivatives of aromatic carboxylic acids: benzoic acid and cinnamic acid. They differ according to the number and position of hydroxylations and methoxylations of the aromatic ring (5). The most common hydroxycinnamic acids are caffeic, *p*-coumaric and ferulic acids, which frequently occur in foods as simple esters with quinic acid or glucose. Probably the most well-known bound hydroxycinnamic acid is the chlorogenic acid, which is a combination of caffeic and quinic acids. Hydroxybenzoic acid derivatives are primarily present as glucosides; *p*-hydroxybenzoic, vanillic and protocatechuic acids are most common (2). Phenolic acids occur in plants at different concentrations, and each plant sample could be specific enough for the presence of different phenolic acids and their derivates in combination with the other groups of phenolics (6).

Flavonoids are a large family of compounds (over 4,000 flavonoids have been identified) synthesized by plants that are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, anthocyanidins and flavanols (catechins and proanthocyanidins) (1). Apart from their physiological roles in plants, flavonoids are considered to be important components in the human diet, although they are generally considered non-nutrients. Flavonoid intake can range between 50 and 800 mg a day, depending on the diet: consumption of vegetables, fruits, red wine, tea and unfiltered beer. Another significant source of flavonoids includes different medicinal plants and related phytomedicines (3).

Epidemiological data suggest that the consumption of plant-derived antioxidants such as flavonoids and phenolic acids prevents various diseases. Phenolic acids show a broad spectrum of pharmacological activity; they have antioxidant, antimutagenic, antitumor and anticarcinogenic properties (5, 7). They have also been found to possess antiviral and antibacterial properties (6). Also, several pharmacological effects have been ascribed to flavonoids, such as central vascular effects and anti-inflammatory, antihepatotoxic, antitumor, antimicrobial, antiviral and enzyme-inhibiting activities (8).

Walnut leaves have widely been used in folk medicine for treatment of venous insufficiency and hemorrhoidal symptomatology, and for their antidiarrheic, antihelmintic, depurative and astringent properties. Keratolytic, antifungal, hypoglycemic, hypotensive, anti-scrofulous and sedative activities have also been described (9–11). Several of these traditionally attributed actions may be due to tannins known to occur in these leaves, but also to several phenolic compounds, namely flavonoids and phenolic acids (8). Different works have investigated the phenolic composition and the antimicrobial and antioxidant properties of walnut leaves (8, 9, 12–15).

Phenolic acids, flavonoids and naphthoquinones are the primary phenolic compounds in walnut leaves. One constituent

in particular that is unique to walnuts, juglone (5-hydroxy-1, 4-napthoquinone), is a chemical compound released by walnut trees, which can be toxic at various levels to several plant species. Juglone is present in considerable amounts in all green and growing parts of the tree and unripe hulls of the nuts, whereas the juglone level in kernels is either low or absent (16).

The determination of phenolic compounds in walnut leaves is important both for their characterization and to facilitate more efficient uses of this important plant resource.

There are several methods for the determination of phenolic acids and flavonoids in plants and foods. These include thin-layer chromatography (TLC) (17), gas chromatography (GS) (18), high-performance liquid chromatography (HPLC) alone or coupled with mass spectrometry (MS) (5–8, 12, 19–25), capillary electrophoresis (CE) (2, 26–29) and micellar electrokinetic chromatography (MEKC) (30, 31). In the last 20 years, HPLC has been the analytical technique that has dominated the separation and characterization of phenolic compounds (32, 33).

In the present work, a new reversed-phase HPLC method with diode array detection was developed for the identification and quantification of 17 major phenolic compounds; namely, phenolic acids (gallic, vanillic, chlorogenic, caffeic, syringic, *p*-coumaric, ferulic, sinapic, salycilic, elagic and *trans*-cinnamic acids), flavonoids (catechin hydrate, epicatechin, rutin, myricetin and quercetin) and juglone that are present in walnut leaves. The method was applied to walnut leaves of nine different cultivars growing under the same agricultural, geographical and climatic conditions.

Materials and Methods

Chemicals

Standards of phenolic acids (gallic, vanillic, chlorogenic, caffeic, syringic, *p*-coumaric, ferulic, sinapic, salycilic, elagic and *trans*-cinnamic), flavonoids (catechin, epicatechin, rutin, myricetin and quercetin) and juglone were purchased from Sigma-Aldrich (Germany).

Methanol (HPLC gradient grade) was purchased from Baker (Netherlands), acetic acid (HPLC grade) was purchased from Merck (Germany) and 2,6-di-*tert*-butyl-4-methylphenol, butylhydroxytoluene (BHT) was from Sigma-Aldrich (Germany). Water used throughout the experiments was obtained from an SG water purification system (Germany).

Samples

Studies were conducted on walnut leaves from nine cultivars (Fernette, Vina, Muscelean, Fernor, Pedro, Valrex, Orastie, Hartley and Valcor). Fresh leaves of the different cultivars were collected on July 15, from an experimental plantation at Ramnicu Valcea (Romania) research station (located at 45° 07′ N / 24° 22′ 21″ E), and preserved by freezing at −40°C.

All samples were extracted and analyzed in triplicate. Intergenotype significance of differences was calculated according to the LSD test. Data were reported as means ± standard error of the mean. Differences at $P \leq 0.05$ were considered to be statistically significant.

Sample preparation

Walnut leaves were finely chopped and phenolics were extracted from 1,000 mg samples in a DK 102p Bandelin ultrasonic bath with 20 mL methanol and 1% BHT at 25°C for 40 min. Extracts were centrifuged at 1,200 × *g* and the supernatants were filtered through a 0.2 μm polyamide membrane and stored at −20°C.

HPLC equipment

HPLC analyses were performed on a Finningan Surveyor Plus system (Thermo Electron Corporation, San Jose, CA) including a vacuum degasser, a Surveyor Plus LCPMPP pump, a Surveyor Plus ASP autosampler and a PDA5P diode array detector with 5 cm flow cell and with Chrom Quest 4.2 system manager as data processor. Separation was achieved by a reversed-phase Hypersil Gold C18 column (5 μm particle size, 250 × 4.6 mm) provided by Thermo Electron Corporation.

Chromatographic analysis of phenolic compounds

The mobile phase consisted of 1% aqueous acetic acid solution (A) and methanol (B). Samples were eluted with the following gradient: 90% A from 0 to 27 min, from 90 to 60% A in 28 min, 60% A for 5 min, from 60 to 56% A in 2 min, 56% A for 8 min, from 56 to 90% A in 1 min and 4 min 90% A to re-establish the initial conditions, before the injection of another sample. All gradients were linear. The flow rate was 1 mL/min and the injection volume was 5 μL. Column temperature was maintained at 20°C.

Chromatograms were acquired at three different wavelengths (254, 278 and 300 nm) according to absorption maxima of analyzed compounds.

Each compound was identified by its retention time and by spiking with standards under the same conditions. The identities of constituents were also confirmed with a photodiode array (PDA) detector by comparison with ultraviolet (UV) spectra of standards in the wavelength range of 220–450 nm. Each compound was quantified according to the peak area measurements, which were reported in calibration curves of the corresponding standards. Data are reported as means ± standard deviations of three independent analyses.

Validation of the HPLC method

The following requirements related to linearity, limits of detection (LODs) and quantification (LOQs), accuracy and precision were taken into account in the validation process.

Linearity

The linearity of the detector response (peak area versus concentration) was evaluated by preparing five mixed calibration solutions (S1–S5). Each standard solution was injected in triplicate into the HPLC system and the calibration curves were established by plotting peak areas against the corresponding concentrations for each standard compound. The correlation coefficients were used as the measure of linearity.

LOD and LOQ

The LOD and LOQ of the individual compounds were calculated based on a signal-to-noise (S/N) ratio > 3, using the following formulas:

$$\mathrm{LOD} = 3 \times c/(S/N), \mathrm{LOQ} = 10 \times c/(S/N)$$

where c = concentration.

The S/N ratio was calculated by the Chrom Quest 4.2 software.

Precision

The repeatability of the retention times was determined from all injections of the five mixed calibration solutions made for linearity and were expressed by relative standard deviation (RSD).

Peak areas were checked for repeatability at two concentration levels by injecting the mixed standard solutions S1 and S4 into the HPLC system and calculating the RSD for 10 replicate determinations.

The precision (repeatability) of the method was evaluated based on the results for walnut leaves samples, calculating the average RSDs of peak areas obtained at three different times with six repetitions.

Accuracy

The accuracy, which was described as the percentage recovery of all analytes, was evaluated at two concentration levels by spiking the investigated walnut leaves extracts with mixed calibration solutions (S2 and S5). Three samples were prepared for each level. The contents of investigated compounds were previously determined in the extract of non-spiked walnut leaves. For each phenolic compound, the recovery was calculated based on the ratio of the obtained amount in the spiked extract to the calculated amount (sum of the amounts originating from extract and standard).

Results and Discussion

Validation of the HPLC method

A chromatogram of the external standard mixture recorded at 278 nm is presented in Figure 1. As shown in the chromatogram, all investigated compounds had responses at 278 nm, where they were successfully separated. The constituents under investigation were also identified by the recorded absorption spectra, which were comparable both for extracts of walnut leaves and standard substances.

The regression equation and linearity range for each compound, together with LOD and LOQ values, are shown in Table I. Good response linearity was obtained for all compounds ($R^2 > 0.9987$) in the ranges of standard concentrations analyzed by using the wavelengths of maximum absorption (Table I).

The repeatability of the retention times for all standards was below 1% and the repeatability of peak areas for S1 and S4 standards was below 2%. Also, the RSDs of the results for samples of walnut leaves were all less than 2%, proving the precision of the method, while the recoveries were very high (more than 89.8%) for all analytes, proving the method's accuracy.

By analyzing the results presented in Tables I and II, it can be concluded that the developed method is precise, accurate and sensitive enough for the simultaneous quantitative evaluation of the investigated phenolic compounds in walnut leaf extracts.

Under the described chromatographic conditions, this method enabled accurate resolution of the mixture of 17 phenolics commonly encountered in plant material, including those that are most abundant. This method was applied in routine measurement of the phenolic contents of walnut leaves from nine cultivars: Fernette, Vina, Muscelean, Fernor, Pedro, Valrex, Orastie, Hartley and Valcor.

Analysis of walnut leaves samples

A chromatogram of walnut leaf extract is presented in Figure 2. As shown in the figure, this method enabled very good resolution of all phenolic compounds of interest in these samples.

The contents of investigated phenolic compounds was expressed in mg/100 g fresh weight (FW) as a mean value ± standard deviation (Table III).

The results for phenolic acid contents revealed that ellagic acid was the most abundant phenolic acid in all samples, followed by *trans*-cinnamic and chlorogenic acids.

The ellagic acid content ranged from 38.56 to 134.54 mg/100 g FW, which represents a 3.5-fold difference between Pedro and Hartley cultivars. Previous experimental results have indicated that ellagic acid is a major component of walnuts (34–36), and it has been proposed to exert anti-atherogenic, anti-carcinogenic and antioxidative properties (37–41). Daniel *et al.* (42) found 59 mg/100 g ellagic acid in walnuts, and Stampar *et al.* (35), after analyzing walnut husks collected at four different sampling dates, found that the content of ellagic acid ranged from 3.9 to 98.3 mg/100 g dry weight (DW). Likewise, Colaric *et al.* (34) found ellagic acid to be predominant in walnut kernel and pellicle (average values of 5.90 and 128.98 mg/100 g, respectively).

The amount of chlorogenic acid ranged between 3.9 and 7.14 mg/100 g FW, and there were also significant differences due to cultivars. Chrzanowski *et al.* (43) found 15.3 mg/100 g DW chlorogenic acid in walnut leaves and 30.67 mg/100 g DW in green husks, both collected in July, and Stampar *et al.* (35) found 7.56 mg/100 g DW chlorogenic acid in walnut husks collected in July. Regarding *trans*-cinnamic acid, this study found an average content of 7.92 mg/100 g FW, while Chrzanowski *et al.* (43) found 8.75 mg/100 g DW *trans*-cinnamic acid in walnut leaves collected in July.

Among the investigated cultivars, significantly high contents of these phenolic acids located in the top three (ellagic, *trans*-cinnamic and chlorogenic) were found in leaves of the cultivars Pedro, Valrex and Fernor. Cosmulescu *et al.* (44), in a study on 12 different walnut cultivars, found the highest content of total phenolics in the leaves of Valrex, followed closely by Fernor. Additionally, in this study, walnut leaves of the cultivar Fernor showed significantly higher contents of catechin-hydrate and epicatechin, while Valrex was distinguished for having the highest contents of caffeic, *p*-coumaric, vanillic acids and rutin.

At lower concentrations, phenolic acids found in walnut leaves were caffeic, ferulic, sinapic, salycilic, syringic, *p*-coumaric, gallic

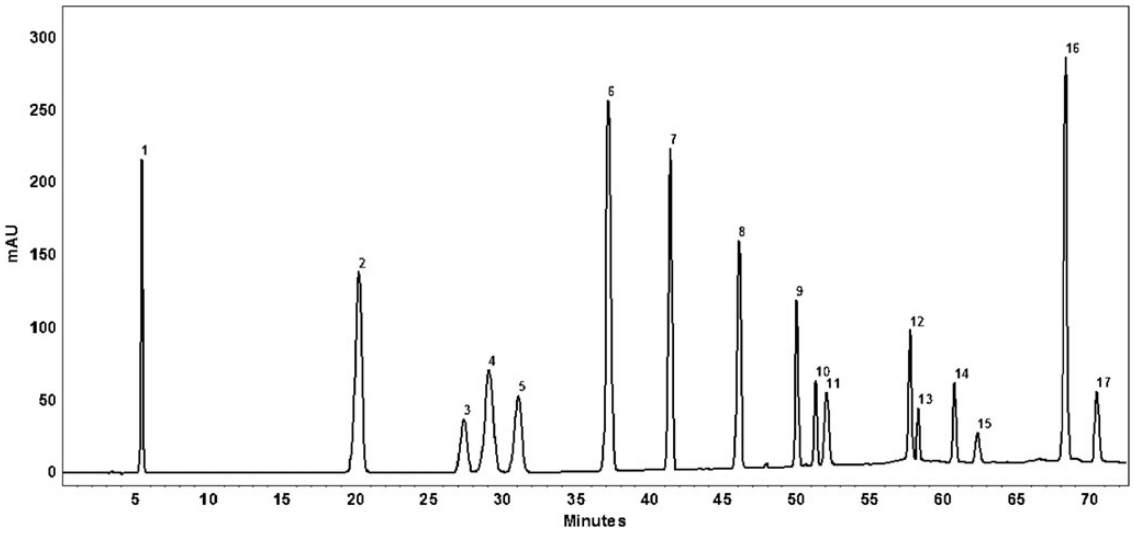

Figure 1. Chromatogram of an external standard mixture at λ = 278 nm. Peaks 1, gallic acid; 2, catechin hydrate; 3, vanillic acid; 4, chlorogenic acid; 5, caffeic acid; 6, syringic acid; 7, epicatechin; 8, *p*-coumaric acid; 9, ferulic acid; 10, sinapic acid; 11, salicylic acid; 12, rutin; 13, ellagic acid; 14, myricetin; 15, juglone; 16, *trans*-cinnamic acid; 17, quercetin.

Table I
Parameters of Calibration Curves, LOD and LOQ for HPLC Method Validation

Analyte	λ (nm)	Concentrations (mg/L)					Retention time (min)	Calibration curve			LOD (mg/L)	LOQ (mg/L)
		S1	S2	S3	S4	S5		intercept	slope	r^2		
Vanillic acid	254	5	10	15	20	25	27.35	0.6042	1.16663 *e*−005	0.9994	0.133	0.443
Rutin	254	10	20	30	40	50	57.74	0.8447	4.74197 *e*−006	0.9989	0.053	0.176
Ellagic acid	254	10	20	30	40	50	60.81	−0.3113	2.21081 *e*−005	0.9988	0.162	0.539
Myricetin	254	10	20	30	40	50	61.40	−0.4929	1.38462 *e*−005	0.9993	0.122	0.406
Juglone	254	10	20	30	40	50	62.37	−0.0487	1.02449 *e*−005	0.9991	0.117	0.390
Quercetin	254	5	10	15	20	25	70.50	0.2500	1.01167 *e*−005	0.9992	0.126	0.420
Gallic acid	278	5	10	15	20	25	5.42	0.3109	1.26622 *e*−005	0.9992	0.179	0.599
Catechin hydrate	278	40	80	120	160	200	20.14	2.7311	5.68028 *e*−005	0.9999	0.905	3.019
Syringic acid	278	10	20	30	40	50	37.10	4.8145	7.80232 *e*−006	0.9997	0.227	0.759
Epicatechin	278	40	80	120	160	200	41.37	1.3485	5.73111 *e*−005	0.9987	0.348	1.162
Trans-cinnamic acid	278	5	10	15	20	25	68.32	−0.2451	4.99316 *e*−006	0.9993	0.029	0.095
Chlorogenic acid	300	10	20	30	40	50	29.07	1.4673	1.58729 *e*−005	0.9995	0.243	0.812
Caffeic acid	300	5	10	15	20	25	31.06	0.2171	8.52071 *e*−006	0.9998	0.095	0.319
Coumaric acid	300	5	10	15	20	25	46.13	0.1255	5.60511 *e*−006	0.9989	0.033	0.109
Ferulic acid	300	5	10	15	20	25	50.02	−0.0627	9.21424 *e*−006	0.9997	0.045	0.130
Sinapic acid	300	5	10	15	20	25	51.28	−0.2589	1.22805 *e*−005	0.9994	0.050	0.167
Salicylic acid	300	10	20	30	40	50	52.12	0.6164	2.71310 *e*−005	0.9996	0.212	0.707

and vanillic. Significant differences were also found for the contents of these compounds among various cultivars.

A recent study on naturally occurring phenolic acid mixtures extracted from the leaves of black currant, sour cherry and walnut, and from the green husks of walnut, revealed that ferulic acid was found only in *J. regia* (45). This study found ferulic acid contents between 0.45 and 1.26 mg/100 g FW, values much lower than those reported by Chrzanowski *et al.* (43) (9 mg/100 g DW and 29.25 mg/100 g DW for walnut leaves collected in May and July, respectively). Instead, Stampar *et al.* (35) reported a ferulic acid content of 0.91 mg/100 g DW for green husks of walnut sampled in July.

Gallic acid was found in low amounts (0.14–0.50 mg/100 g FW), which compared reasonably well with the content found by Chrzanowski *et al.* (43) in July walnut leaves (1.26 mg/100 g DW). At the same time, Chrzanowski *et al.* found no syringic acid in leaves collected in July, but only in those collected in May and in green husks, while this study found low contents of syringic acid in July leaves, in the range of 0.018–1.42 mg/100 g FW.

Vanillic acid was found in the lowest concentrations and, moreover, it was not detected in leaf samples of Orastie and Hartley.

Juglone is known as the characteristic compound of *Juglans spp.* and is reported to occur in fresh walnut leaves (46, 47). Lately, the interest in juglone has increased because several studies revealed that juglone exerts cytotoxic and genotoxic effects against cultured melanoma tumor cells, thus significantly decreasing the proliferation of tumor cells (48). Also, juglone is reported to be a potential anticancer drug based on its strong inhibitory properties of the peptidyl-prolyl isomerase Pin1, a therapeutic target for anticancer research (49).

Gîrzu *et al.* (50), by using HPLC, determined that fresh walnut leaves contain significant amounts of juglone (up to 500 mg/100 g DW), while Thakur and Cahallan (51) found that juglone content in fresh leaves collected during the first week of August was in the range of 13.1 to 1556.0 mg/100 g DW on the basis of 1,121 samples of tree leaves, with an overall mean juglone content of 357 mg/100 g DW. This study found a juglone content between 44.55 and 205.12 mg/100 g FW. The obtained results were comparable with those reported by Babula *et al.* (52), who determined in Persian walnut leaves a juglone content of 200–400 mg/100 g FW by using electrochemical methods. In healthy walnut husks, Stampar *et al.* (35) found higher juglone contents, ranging between 218 and 1404 mg/100 g DW, while Cheniany *et al.* (53) found between 21.18 and 38.47 mg juglone/100 g DW in microshoots of *J. regia* cultivars.

Most of the flavonoids present in plants are attached to sugars (glycosides), although they are occasionally found as aglycones (54). Therefore, previous studies have identified and quantified the following flavonoids in walnut leaves: quercetin 3-galactoside, quercetin 3-arabinoside, quercetin 3-xyloside, quercetin 3-rhamnoside and two other partially identified quercetin 3-pentoside and kaempferol 3-pentoside derivatives (8, 9). In this study, some flavonoids were identified and quantified as aglycones. Thus, myricetin, one of the most common flavonols, was found in concentrations between 81.01 and 230.99 mg/100 g FW. Myricetin contents in walnut leaves have not been reported in other papers, but Lugasi *et al.* (55) found an extremely high level of myricetin in walnut (456.5 mg/100 g), and Stampar *et al.* (35) found myricetin between 2.88 and 25 mg/100 g DW in walnut husks. These high amounts of myricetin can contribute to the health benefits of walnut leaves, because, apart from the potent antioxidant and free-radical scavenging activities of myricetin (56), several

Table II
Parameters Related to Precision and Accuracy for HPLC Method Validation

Analyte	Precision, RSD (%)			Precision of the method, RSD (%)	Accuracy	
	Retention times	Peak areas			Recovery (%)	
		Level 1 (S1)	Level 2 (S4)		Level 1	Level 2
Vanillic acid	0.373	0.892	0.956	1.442	95.2 ± 1.2	99.3 ± 1.1
Rutin	0.667	0.682	0.786	1.266	100.6 ± 0.8	101.1 ± 0.7
Ellagic acid	0.539	0.556	0.892	1.543	96.3 ± 1.6	98.7 ± 0.9
Myricetin	0.331	0.721	0.543	1.108	89.8 ± 1.1	91.9 ± 1.9
Juglone	0.278	0.443	0.684	1.782	93.3 ± 0.9	96.3 ± 1.2
Quercetin	0.442	0.583	0.621	1.765	91.8 ± 1.5	93.2 ± 1.5
Gallic acid	0.122	0.908	1.083	1.619	98.9 ± 1.6	100.2 ± 0.6
Catechin hydrate	0.366	0.484	0.425	1.166	92.5 ± 1.2	92.9 ± 2.1
Syringic acid	0.309	1.082	0.967	1.722	93.4 ± 1.8	94.5 ± 1.8
Epicatechin	0.482	0.644	0.782	1.464	98.7 ± 1.1	99.6 ± 0.9
Trans-cinnamic acid	0.188	0.828	0.872	1.885	96.3 ± 0.6	97.8 ± 1.2
Chlorogenic acid	0.166	0.891	0.663	1.028	97.4 ± 0.9	99.6 ± 1.1
Caffeic acid	0.628	0.423	0.547	1.556	91.1 ± 1.6	91.8 ± 2.0
p-Coumaric acid	0.541	0.565	0.782	1.191	95.9 ± 1.4	97.0 ± 1.3
Ferulic acid	0.384	0.965	1.121	1.891	92.2 ± 1.8	96.1 ± 1.4
Sinapic acid	0.223	1.161	0.882	1.337	97.3 ± 1.0	99.2 ± 1.1
Salicylic acid	0.243	0.551	0.722	1.667	97.7 ± 0.8	100.5 ± 0.8

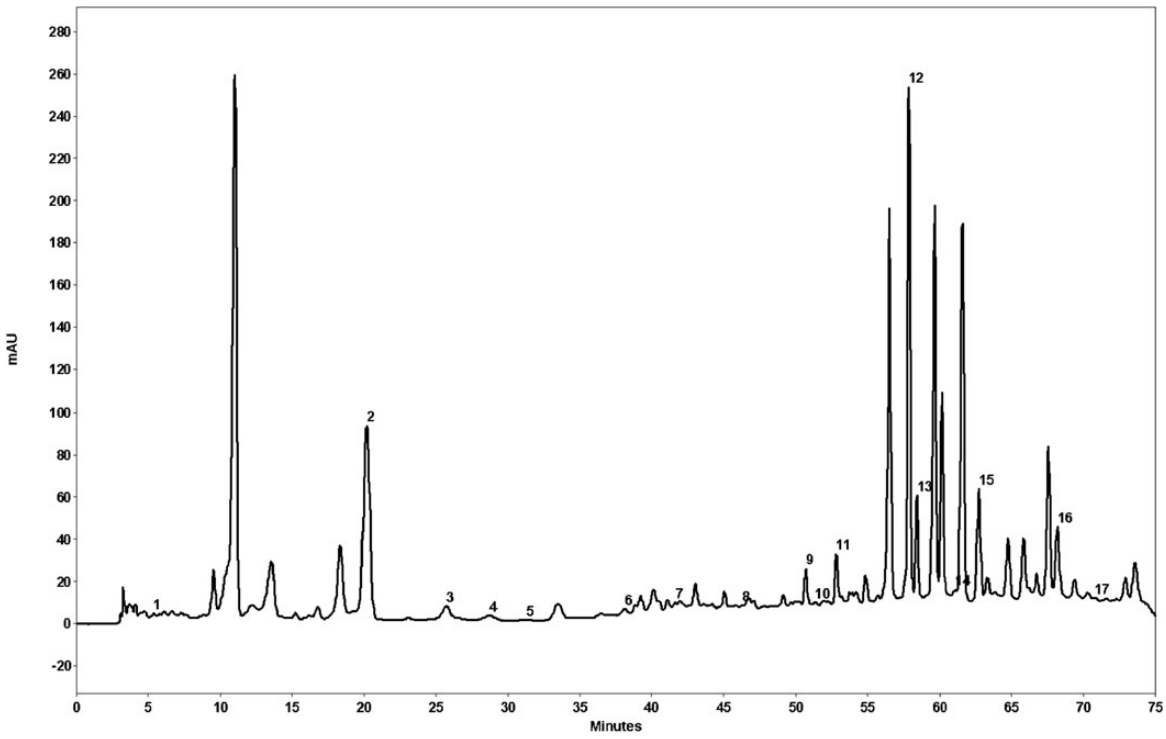

Figure 2. Chromatogram at λ = 278 nm of the extract of walnut leaves. Peaks: 1, gallic acid; 2, catechin hydrate; 3, vanillic acid; 4, chlorogenic acid; 5, caffeic acid; 6, syringic acid; 7, epicatechin; 8, *p*-coumaric acid; 9, ferulic acid; 10, sinapic acid; 11, salicylic acid; 12, rutin; 13, ellagic acid; 14, myricetin; 15, juglone; 16, *trans*-cinnamic acid; 17, quercetin.

Table III
Phenolic Compounds in Samples of Walnut Leaves (mg/100 g FW)*

Compound	Cultivar									Mean
	Fernette	Vina	Muscelean	Fernor	Pedro	Valrex	Orastie	Hartley	Valcor	
Vanillic acid	0.04 ± 0.002	0.02 ± 0.001	0.03 ± 0.001	0.10 ± 0.004	0.01 ± 0.001	0.48 ± 0.056†	ND	ND	0.22 ± 0.028†	0.13 ± 0.17
Rutin	57.83 ± 3.17	47.39 ± 1.89	78.04 ± 3.61	150.24 ± 4.59	153.95 ± 6.06†	186.76 ± 5.66†	96.62 ± 4.05	33.97 ± 1.06	86.98 ± 2.02	99.08 ± 53.06
Ellagic acid	55.12 ± 2.16	44.88 ± 1.34	67.34 ± 2.37	130.00 ± 3.33†	134.54 ± 2.89†	128.61 ± 2.08†	70.96 ± 1.90	38.57 ± 0.97	91.64 ± 2.18	84.62 ± 38.06
Myricetin	117.66 ± 2.07	111.32 ± 1.89	91.71 ± 1.67	81.02 ± 1.14	98.67 ± 1.70	230.99 ± 4.46†	218.40 ± 4.89†	114.96 ± 1.88	204.68 ± 4.23†	141.05 ± 59.23
Juglone	44.55 ± 2.27	45.35 ± 1.89	48.23 ± 2.45	80.62 ± 3.19	108.08 ± 2.87	205.13 ± 5.11†	86.21 ± 2.33	51.90 ± 1.67	128.26 ± 3.06†	88.73 ± 52.90
Quercetin	1.79 ± 0.06	1.53 ± 0.05	3.11 ± 0.07	2.27 ± 0.02	4.78 ± 0.05†	3.39 ± 0.04	5.17 ± 0.05†	2.64 ± 0.02	3.30 ± 0.03	3.20 ± 1.24
Gallic acid	0.18 ± 0.01	0.50 ± 0.02†	0.39 ± 0.03	0.32 ± 0.03	0.21 ± 0.02	0.15 ± 0.01	0.32 ± 0.02	0.48 ± 0.04†	0.15 ± 0.02	0.30 ± 0.13
Catechin hydrate	145.35 ± 5.55	381.37 ± 6.79†	304.93 ± 4.58	457.11 ± 6.67†	224.89 ± 2.28	59.03 ± 1.88	50.78 ± 1.25	213.87 ± 2.66	77.91 ± 1.89	212.81 ± 145.50
Syringic acid	0.74 ± 0.01	1.29 ± 0.02†	0.76 ± 0.02	1.42 ± 0.02†	0.62 ± 0.01	0.02 ± 0.002	0.22 ± 0.01	0.65 ± 0.02	0.21 ± 0.02	0.66 ± 0.47
Epicatechin	0.22 ± 0.01	5.04 ± 0.07	2.17 ± 0.03	20.41 ± 0.27†	1.20 ± 0.09	6.43 ± 0.16	0.63 ± 0.02	7.28 ± 0.20	6.35 ± 0.16	5.53 ± 6.21
Trans-cinnamic acid	6.92 ± 0.19	2.55 ± 0.12	9.58 ± 0.33	11.75 ± 0.33†	15.08 ± 0.51†	9.22 ± 0.23	3.13 ± 0.11	3.21 ± 0.05	9.71 ± 0.23	7.92 ± 4.30
Chlorogenic acid	4.36 ± 0.11	4.78 ± 0.27	4.51 ± 0.19	6.96 ± 0.30†	7.14 ± 0.28†	5.74 ± 0.17	3.90 ± 0.10	4.15 ± 0.09	5.27 ± 0.12	5.21 ± 1.18
Caffeic acid	0.57 ± 0.03	0.14 ± 0.01	0.15 ± 0.01	1.28 ± 0.06	1.84 ± 0.09	5.04 ± 0.19†	3.06 ± 0.16†	0.80 ± 0.10	2.46 ± 0.05	1.73 ± 1.60
p-Coumaric acid	0.67 ± 0.03†	0.28 ± 0.01	0.42 ± 0.02	0.27 ± 0.01	0.42 ± 0.03	0.69 ± 0.04†	0.17 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.35 ± 0.21
Ferulic acid	0.46 ± 0.02	0.86 ± 0.02	1.56 ± 0.07†	0.69 ± 0.02	1.26 ± 0.04†	1.04 ± 0.02	0.59 ± 0.02	0.62 ± 0.02	0.97 ± 0.02	0.90 ± 0.35
Sinapic acid	0.78 ± 0.03	1.20 ± 0.03†	1.18 ± 0.04†	1.30 ± 0.04†	0.80 ± 0.02	0.61 ± 0.01	0.35 ± 0.02	0.80 ± 0.03	0.63 ± 0.03	0.85 ± 0.31
Salicylic acid	1.10 ± 0.05	1.84 ± 0.06†	1.80 ± 0.04	1.38 ± 0.02	1.64 ± 0.03	1.36 ± 0.02	1.17 ± 0.01	2.21 ± 0.02†	0.32 ± 0.01	1.42 ± 0.54

*Means ± SD followed by different lowercases indicate significant difference among cultivars versus Mean.
†$p < 0.05$.

studies have demonstrated that myricetin exhibits favorable effects on bone health, either by decreasing bone resorption or by increasing osteoblastic activity and bone formation (57, 58). Rutin (quercetin-3-rutinoside), another flavonoid with high antioxidant activity that inhibits DNA oxidation (56), was also found in high amounts in walnut leaves, i.e., between 33.96 and 186.76 mg/100 g, while quercetin aglycone content was found in low concentrations, between 1.528 and 5.166 mg/100 g FW.

Conclusions

An RP-HPLC method with diode array detection was developed for determining 17 pharmacologically active phenolic compounds in walnut leaves. In previous studies, only a few individual phenolics had been determined in walnut leaves. The proposed HPLC assay showed good separation of the compounds and proved to be efficient, precise and accurate, therefore, it could be used for the simultaneous determination of phenolic acids, flavonoids and juglone present in walnut leaves.

The experimental results indicated that walnut leaves contain high concentrations of juglone, comparable to those obtained in previous studies.

Based on these results, ellagic acid was established as the dominating phenolic acid of walnut leaves. Although the other phenolic acids are present in much lower concentrations, they could contribute to a great deal to the antioxidant activity of walnut leaves.

Determinations of some flavonoids revealed high concentrations of myricetin, catechin hydrate and rutin, and low concentrations of quercetin and epicatechin aglycones.

The results show that composition is highly affected by differences in the plant genotypes.

References

1. Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L.; Polyphenols: Food sources and bioavailability; *The American Journal of Clinical Nutrition*, (2004); 79: 727–747.
2. Kvasnička, F., Čopíková, J., Ševčík, R., Krátká, J., Syntytsia, A., Voldřich, M.; Determination of phenolic acids by capillary zone electrophoresis and HPLC; *Central European Journal of Chemistry*, (2008); 6: 410–418.
3. Stalikas, C.D.; Extraction, separation, and detection methods for phenolic acids and flavonoids (review); *Journal of Separation Science*, (2007); 30: 3268–3295.
4. Scalbert, A., Williamson, G.; Dietary intake and bioavailability of polyphenols; *Journal of Nutrition*, (2000); 130: 2073S–2085S.
5. Tarnawski, M., Depta, K., Grejciun, D., Szelepin, B.; HPLC determination of phenolic acids and antioxidant activity in concentrated peat extract–a natural immunomodulator; *Journal of Pharmaceutical and Biomedical Analysis*, (2006); 41: 182–188.
6. Ziaková, A., Brandšteterová, E.; Validation of HPLC determination of phenolic acids present in some *Lamiaceae* family plants; *Journal of Liquid Chromatography & Related Technologies*, (2003); 26: 443–453.
7. Nikolova, M., Gevrenova, R.; Determination of phenolic acids in *Amaryllidaceae* species by high performance liquid chromatography; *Pharmaceutical Biology*, (2005); 43: 289–291.
8. Amaral, J.S., Valentão, P., Andrade, P.B., Martins, R.C., Seabra, R.M.; Do cultivar, geographical location and crop season influence phenolic profile of walnut leaves? *Molecules*, (2008); 13: 1321–1332.
9. Pereira, J.A., Oliveira, I., Sousa, A., Valentao, P., Andrade, P.B., Ferreira, I.C.F.R., *et al.*; Walnut (*Juglans regia* L.) leaves: Phenolic

compounds, antimicrobial activity and antioxidant potential of different cultivars; *Food and Chemical Toxicology*, 2007; 45: 2287–2295.
10. Asgary, S., Parkhideh, S., Solhpour, A., Madani, H., Mahzouni, P., Rahimi, P.; Effect of ethanolic extract of *Juglans regia* L. on blood sugar in diabetes-induced rats; *Journal of Medicinal Food*, (2008); 11: 533–538.
11. Carvalho, M., Ferreira, P.J., Mendes, V.S., Silva, R., Pereira, J.A., Jerónimo, C., *et al.*; Human cancer cell antiproliferative and antioxidant activities of *Juglans regia* L.; *Food and Chemical Toxicology*, (2010); 48: 441–447.
12. Amaral, J.S., Seabra, R.M., Andrade, P.B., Valentão, P., Martins, R.C., Pereira, J.A., *et al.*; Phenolic profile in the quality control of walnut (*Juglans regia* L.) leaves; *Food Chemistry*, (2004); 88: 373–379.
13. Cosmulescu, S., Trandafir, I.; Variation of phenols content in walnut (*Juglans regia* L.); *South Western Journal of Horticulture, Biology and Environment*, (2011); 2: 25–33.
14. Sharafati-Chaleshtori, R., Sharafati-Chaleshtori, F., Rafieian-Kopaei, M.; Biological characterization of Iranian walnut (*Juglans regia*) leaves; *Turkish Journal of Biology*, (2011); 35: 1–5.
15. Almeida, I.F., Fernandes, E., Lima, J.L.F.C., Costa, P.C., Bahia, M.F.; Walnut (*Juglans regia*) leaf extracts are strong scavengers of pro-oxidant reactive species; Food Chemistry, (2008); 106: 1014–1020.
16. Jakopic, J., Solar, A., Colaric, M., Hudina, M., Veberic, R., Stampar, F.; The influence of ethanol concentration on content of total and individual phenolics in walnut alcoholic drink; *Acta Alimentaria*, (2008); 37: 233–239.
17. Tűzen, M., Őzdemir, M.; Chromatographic determination of phenolic acids in the snowdrop by HPLC; *Turkish Journal of Chemistry*, (2003); 27: 49–54.
18. Soares, M.C., Ribeiro, E.T., Kuskoski, E.M., Gonzaga, L.V., Lima, A., Mancini Filho, J., *et al.*; Composition of phenolic acids content in apple (*Malus sp*) pomace; *Semina Ciencias Agrarias*, (2008); 29: 339–348.
19. Mattila, P., Kumpulainen, J.; Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection; *Journal of Agricultural and Food Chemistry*, (2002); 50: 3660–3667.
20. Wen, D., Li, C., Di, H., Liao, Y., Liu, H.; A universal HPLC method for the determination of phenolic acids in compound herbal medicines; *Journal of Agricultural and Food Chemistry*, (2005); 53: 6624–6629.
21. Krzek, J., Kaleta, J., Hibicka, U., Niedzwiedz, A.; Reversed-phase high-performance liquid chromatography determination of selected phenolic acids in propolis concentrates in terms of standardization for drug manufacturing purposes; *Journal of AOAC International*, (2006); 89: 352–258.
22. Kindt, M., Orsini, M.C., Costantini, B.; Improved high-performance liquid chromatography-diode array detection method for the determination of phenolic compounds in leaves and peels from different apple varieties; *Journal of Chromatographic Science*, (2007); 45: 507–514.
23. Ying, X., Wang, R., Xu, J., Zhang, W., Li, H., Zhang, C., *et al.*; HPLC determination of eight polyphenols in the leaves of *Crataegus pinnatifida* Bge. var. major; *Journal of Chromatographic Science*, (2009); 47: 201–205.
24. Mišan, A., Mimica-Dukić, N.M., Mandić, A.I., Sakač, M.B., Milovanović, I.L., Sedej, I.J.; Development of a rapid resolution HPLC method for the separation and determination of 17 phenolic compounds in crude plant extracts; *Central European Journal of Chemistry*, (2011); 9: 133–142.
25. Solar, A., Jakopic, J., Nour, V., Mikulic-Petkovsek, M., Veberic, R., Botu, M., *et al.*; Prohexadione-Ca induces reduction in bacterial blight severity and alteration in phenolic content in walnuts; *Journal of Plant Pathology*, (2012); 94: S1.47–S1.52.
26. Cao, Y.H., Wang, Y., Yuan, Q.; Analysis of flavonoids and phenolic acid in propolis by capillary electrophoresis; *Chromatographia*, (2004); 59: 135–140.
27. Kubán, P., Sterbová, D., Kubán, V.; Separation of phenolic acids by capillary electrophoresis with indirect contactless conductometric detection; *Electrophoresis*, (2006); 27: 1368–1375.
28. Lu, Y., Breadmore, M.C.; Analysis of phenolic acids by non-aqueous capillary electrophoresis after electrokinetic supercharging; *Journal of Chromatography A*, (2010); 1217: 7282–7287.
29. Buiarelli, F., di Berardino, S., Coccioli, F., Jasionowska, R., Russo, M.V.; Determination of phenolic acids in olive oil by capillary electrophoresis; *Annali di Chimica*, (2004); 94: 699–706.
30. Rodríguez-Delgado, M.A., Pérez, M.L., Corbella, R., González, G., Garci'a Montelongo, F.J.; Optimization of the separation of phenolic compounds by micellar electrokinetic capillary chromatography; *Journal of Chromatography A*, (2000); 871: 427–438.
31. Verardo, V., Bendini, A., Cerretani, L., Malaguti, D., Cozzolino, E., Caboni, M.F.; Capillary gas chromatography analysis of lipid composition and evaluation of phenolic compounds by micellar electrokinetic chromatography in Italian walnut (*Juglans regia* L.): irrigation and fertilization influence; *Journal of Food Quality*, (2009); 32: 262–281.
32. Robbins, R.; Phenolic acids in foods: An overview of analytical methodology; *Journal of Agricultural and Food Chemistry*, (2003); 51: 2866–2887.
33. Naczk, M., Shahidi, F.; Extraction and analysis of phenolics in food; *Journal of Chromatography A*, (2004); 1054: 95–111.
34. Colaric, M., Veberic, R., Solar, A., Hudina, M., Stampar, F.; Phenolic acids, syringaldehyde, and juglone in fruits of different cultivars of *Juglans regia* L.; *Journal of Agricultural and Food Chemistry*, (2005); 53: 6390–6396.
35. Stampar, F., Solar, A., Hudina, M., Veberic, R., Colaric, M.; Traditional walnut liqueur—cocktail of phenolics; *Food Chemistry*, (2006); 95: 627–631.
36. Jakopic, J., Colaric, M., Veberic, R., Hudina, M., Solar, A., Stampar, F.; How much do cultivar and preparation time influence on phenolics content in walnut liqueur? *Food Chemistry*, (2007); 104: 100–105.
37. Anderson, K.J., Teuber, S.S., Gobeille, A., Cremin, P., Waterhouse, A.L., Steinberg, F.M.; Walnut polyphenolics inhibit in vitro human plasma and LDL oxidation; *Journal of Nutrition*, (2001); 131: 2837–2842.
38. Festa, F., Aglitti, T., Duranti, G., Ricordy, R., Perticone, P., Cozzi, R.; Strong antioxidant activity of ellagic acid in mammalian cells in vitro revealed by the comet assay; *Anticancer Research*, (2001); 21: 3903–3908.
39. Mertens-Talcott, S.U., Talcott, S.T., Percival, S.S.; Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells; *Journal of Nutrition*, (2003); 133: 2669–2674.
40. Papoutsi, Z., Kassi, E., Chinou, I., Halabalaki, M., Skaltsounis, L.A., Moutsatsou, P.; Walnut extract (*Juglans regia* L.) and its component ellagic acid exhibit anti-inflammatory activity in human aorta endothelial cells and osteoblastic activity in the cell line KS483; *British Journal of Nutrition*, (2008); 99: 715–722.
41. Mahmoodi, M., Eghbali, H., Hosseini Zijoud, S.M., Pourrashidi, A., Mohamadi, A., Borhani, M., *et al.*; Study of the effects of walnut leaf on some blood biochemical parameters in hypercholesterolemic rats; *Biochemistry and Analytical Biochemistry*, (2011); 1: 1–4.
42. Daniel, E.M., Krupnick, A.S., Heur, Y.H., Blinzler, J.A., Nims, R.W., Stoner, G.D.; Extraction, stability, and quantitation of ellagic acid in various fruits and nuts; *Journal of Food Composition and Analysis*, (1989); 2: 338–349.
43. Chrzanowski, G., Leszczyński, B., Czerniewicz, P., Sytykiewicz, H., Matok, H., Krzyzanowski, R.; Phenolic acids of walnut (*Juglans regia* L.); *Herba Polonica*, (2011); 57(2): 22–29.
44. Cosmulescu, S., Trandafir, I.; Seasonal variation of total phenols in leaves of walnut (*Juglans regia* L.); *Journal of Medicinal Plants Research*, (2011); 5(19): 4938–4942.
45. Chrzanowski, G., Leszczyński, B., Czerniewicz, P., Sytykiewicz, H., Matok, H., Krzyzanowski, R., *et al.*; Effect of phenolic acids from black currant sour cherry, walnut on grain aphid (*Sitobion avenae* F.) development; *Crop Protection*, (2012); 35: 71–77.

46. Solar, A., Colaric, M., Usenik, V., Stampar, F.; Seasonal variation of selected flavonoids, phenolic acids and quinones in annual shoots of common walnut (*Juglans regia* L.); *Plant Science*, (2006); 170: 461–543.
47. Cosmulescu, S., Trandafir, I., Achim, G., Baciu, A.; Juglone content in leaf and green husk of five walnut (*Juglans regia* L.) cultivars; *Notulae Botanicae Horti Agrobotanici*, (2011); 39: 237–240.
48. Aithal, B.K., Kumar, M.R., Rao, B.N., Udupa, N., Rao, B.S.; Juglone, a naphthoquinone from walnut, exerts cytotoxic and genotoxic effects against cultured melanoma tumor cells; *Cell Biology International*, (2009); 33: 1039–1049.
49. Zhang, C.J., Zhang, Z.H., Xu, B.L., Wang, Y.L.; Recent advances in the study of pin1 and its inhibitors; *Acta Pharmacologica Sinica*, (2008); 43: 9–17.
50. Gîrzu, M., Fraisse, D., Carnat, A.P., Carnat, A., Lamaison, J.L.; High performance liquid chromatographic method for the determination of juglone in fresh walnut leaves; *Journal of Chromatography A*, (1998); 805: 315–318.
51. Thakur, A., Cahalan, C.; Geographical variation of *Juglans regia* L. in juglone content: Rapid analysis using micro plate reader; *Current Science*, (2011); 100: 1483–1485.
52. Babula, P., Huska, D., Hanustiak, P., Baloun, J., Krizkova, S., Adam, V., *et al.*; Flow injection analysis coupled with carbon electrodes as the tool for analysis of naphthoquinones with respect to their content, functions in biological samples; *Sensors*, (2006); 6: 1466–1482.
53. Cheniany, M., Ebrahimzadeh, H., Vahdati, K, Preece, J.E., Masoudinejad, A., Mirmasoumi, M.; Content of different groups of phenolic compounds in microshoots of *Juglans regia* cultivars and studies on antioxidant activity; *Acta Physiologiae Plantarum*, September 11, 2012: 10.1007/s11738-012-1087-7.
54. Ross, J.A., Kasum, C.M.; Dietary flavonoids: Bioavailability, metabolic effects, and safety; *Annual Review of Nutrition*, (2002); 22: 19–34.
55. Lugasi, A., Hóvári, J., Sági, K.V., Bíró, L.; The role of antioxidant phytonutrients in the prevention of diseases; *Acta Biologica Szegediensis*, (2003); 47: 119–125.
56. Ferrari, C.K.B.; Functional foods, herbs and nutraceuticals: Towards biochemical mechanisms of healthy aging; *Biogerontology*, (2004); 5: 275–289.
57. Kuo, P.L.; Myricetin inhibits the induction of anti-Fas IgM-, tumor necrosis factor-alpha- and interleukin-1beta-mediated apoptosis by Fas pathway inhibition in human osteoblastic cell line MG-63; *Life Sciences*, (2005); 77: 2964–2976.
58. Hsu, Y.L., Chang, J.K., Tsai, C.H., Chien, T.T., Kuo, P.L.; Myricetin induces human osteoblast differentiation through bone morphogenetic protein-2/p38 mitogen-activated protein kinase pathway; *Biochemical Pharmacology*, (2007); 73: 504–514.