

PRELIMINARY RESEARCH REGARDING THE THERAPEUTIC USES OF *URTICA DIOICA L.* NOTE I. THE POLYPHENOLS EVALUATION

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Abstract

The use of stinging nettle leaves for the treatment of diabetes mellitus without a scientific evaluation, persuaded us to study them, in order to obtain pharmacological active extracts. According to scientific data, polyphenols are known as HMG-CoA reductase inhibitors and have potent anti-glycation activity. These compounds are the main active principles in the *Urticae folium*.

The aim of this study was to evaluate the polyphenols' content (polyphenol-carboxylic acids = PPCA, and flavonoids) of stinging nettle leaves, harvested from culture and wild flora, originated from Racari, Dambovita district, Romania. Spectrophotometric and chromatographic methods were used. A new HPLC method for the identification and quantification of caffeic and chlorogenic acids from *Urticae folium* was also developed. The highest contents of polyphenolcarboxylic acids (expressed as % of chlorogenic acid) and flavonoids (expressed as % of rutin) were found in the leaves of wild nettles harvested in the month of March (4.2295% chlorogenic acid and 0.6237% rutin).

The polyphenols' content is slightly lower than the ones mentioned in the scientific literature. The content of PPCA decreases from March till September, both in wild and cultivated samples. The variation of the flavonoids' content differs with the origin of raw material (culture or spontaneous flora).

The new HPLC method for polyphenols' analysis is reproducible.

Rezumat

Utilizarea tradițională a produsului *Urticae folium* în diabet, fără o justificare științifică a acțiunii precum și menționarea flavonelor și acizilor fenolcarboxilici (compușii majoritari) ca inhibitori ai HMG-CoA reductazei și ai proceselor de glicozilare și lipoxidare avansată, ne-au determinat să abordăm cercetarea acestora, în vederea valorificării terapeutice sub formă de extracte selective. În acest scop am derulat o cercetare spectrofotometrică și cromatografică (HPLC) pentru a pune în evidență polifenolii prezenți în frunzele de urzică recoltate din flora spontană și de cultură, din localitatea Răcari, județul Dâmbovița. De asemenea, ne-am propus să separăm acizii cafeic și clorogenic și să-i determinăm cantitativ printr-o nouă metodă HPLC/UV.

Produsul vegetal cu cel mai mare conținut de acizi polifenolcarboxilici și flavone provine din locuri necultivate și este recoltat în luna martie (4,2295 g % acid clorogenic, 0,6237 g % rutozidă).

Comparativ cu literatura de specialitate consultată, produsul indigen analizat are un conținut mai mic de polifenoli. La începutul perioadei de vegetației predomină acizi fenolcarboxilici (AFC), aceștia scăzând spre maturitate, atât în plantele recoltate, cât și colectate. Conținutul de flavone diferă în funcție de proveniența produsului vegetal (floră spontană, cultură).

Metoda HPLC/UV de separare a acidului clorogenic de acid cafeic este reproductibilă.

Keywords: *Urtica dioica*, polyphenolcarboxylic acids, flavonoids, HPLC.

Introduction

In folk medicine, *Urtica dioica* is used internally as a hematogenic remedy, diuretic, in arthritis, in the rheumatism of the joints and muscles, and as a component of antidiabetic teas [1, 2]. The European Pharmacopoeia 6th edition includes the monograph „*Urticae folium*” [12]. The uses of stinging nettle leaves for the treatment of diabetes mellitus, without a scientific evaluation, persuaded us to study them, in order to obtain a pharmacological active vegetal extract.

According to scientific data, the following main active principles are reported for *Urticae folium*: 0.7 – 1.8 % flavonoids (isoquercitrin=kaempferol-3-O-rutinoside, rutin, astragalin), polyphenolcarboxylic acids (up to 0.5 % chlorogenic acid, up to 1.6 % caffeoylmalic acid, and others like caffeic acid and ferulic acid), sterols (β -sitosterol), coumarins (scopoletol), lectines (isoelectines, *Urtica dioica* Agglutinin), 0.3 % proanthocyanidins, 0.02 % carotenoids (beta-caroten, violaxanthin, zeaxanthin), 20 % minerals (0.6% potassium salts, 1.050 % calcium salts and 1 - 4 % silicic acid) [2 - 3, 5 – 6, 8, 10, 11]. Among these compounds, the hypoglycemic activity is cited for sterols, polyphenolcarboxylic acids and flavonoids [4].

The aim of this study was to determine the optimal time for harvesting the leaves (cultivated and wild nettles), in order to obtain extracts with the highest content in polyphenolcarboxylic acids and flavonoids. Considering this, spectrofotometric methods were applied.

Another purpose of the current study was to identify the polyphenolic compounds by using two different HPLC methods.

Materials and Methods

The raw material (nettles leaves) originated from Romania, Racari Village, Dambovita District. The samples were harvested in March, June and

September 2009, from wild flora (noted Am, Ai, As), and from culture (noted Bm, Bi, Bs). The herbal products were naturally dried in the shade and stored in controlled laboratory conditions.

In order to evaluate the flavonoids' content it was used a spectrophotometric method, based on the chelating reaction with aluminium chloride, according to the Romanian Pharmacopoeia 10th edition, the monograph *Cynarae folium* [13]. The polyphenolcarboxylic acids' content was evaluated using a spectrophotometric method based on the formation of oxymes in the presence of sodium nitrite and sodium hydroxide, according to the European Pharmacopoeia 6th edition, the monograph *Fraxini folium* [12]. A spectrophotometer Jasco V-530 was used.

The first HPLC experiment for flavonoids and polyphenolcarboxylic acids were carried out using an Agilent HPLC Series system (Agilent U.S.A) equipped with degasser, binary gradient pump, column thermostat, autosampler and UV detector. The HPLC system was integrated with Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). For the separation, a reverse-phase analytical column was used (Zorbax SB-C18 100 x 3.0 mm i.d, 3.5 μ m particle); the working temperature was 48°C. The mobile phase was a binary gradient prepared from methanol and a solution of acetic acid 0.1% (v/v). The elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35 minutes; isocratic elution followed for the next 3 minutes with 42 %. The flow rate was 1 mL min⁻¹ and the injection volume was 5 μ L [7, 9]. The detection of the compounds was performed on both UV and MS mode. The UV detector was set at 330 nm for the first 17.5 min., then at 370 nm. The MS system operated using an electrospray ion source in negative mode. The chromatographic data were processed using ChemStation and DataAnalysis software from Agilent, USA.

The following standards were used: caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, *p*-cumarinic acid, ferulic acid, sinapic acid, hyperoside, isoquercitrin, rutin, miricetin, fisetin, quercitrin, quercetin, patuletine, luteolin, kaempferol and apigenin (fig. 1). Calibration curves in the 0.5-50 μ g mL⁻¹ range had a good linearity ($R^2 = 0.999$, $n = 5$).

In order to quantify chlorogenic and caffeic acids a second HPLC method was carried out, using a Jasco HPLC MD-2015 equipped with degasser, binary gradient pump, column thermostat, and UV detector. For the separation, a reverse-phase analytical column was employed (Nucleosil - C18 25 x 0.4 mm i.d, 5 μ m particle). The mobile phase was a binary gradient prepared from acetonitrile and a solution of phosphoric acid 0.1% (v/v). The elution followed a linear gradient, beginning with 10% acetonitrile

and ending at 35% acetonitrile, for 13 minutes [14]. The flow rate was 1.5 mL min⁻¹ and the injection volume was 20 µL. The UV detector was set at 310 nm. The chromatographic data were processed using Chrompass from Jasco, Japan. The following standards were used: caffeic acid, chlorogenic acid (fig. 2). Calibration curves in the 8 – 170 µg mL⁻¹ range had good linearity ($R^2 = 0.998$, $n = 5$) [14].

Sample preparation (for both HPLC analysis). For extraction of polyphenolic compounds, 2.5 g of powdered leaves (batch Am) with ethanol 50 % were heated on a reflux condenser for 30 minutes. After cooling and filtration the extract was completed up to 25 mL in a volumetric flask (SE₁).

For the extraction of free aglycons, a volume of 1 mL HCl 2N was added to 1 mL extract and heated on a reflux condenser at 80°C, for 40 minutes (SE₂).

Results and Discussion

The spectrophotometric results (table I) showed the highest content of polyphenolcarboxylic acids (PPCA) in leaves of wild nettle harvested in the month of March.

The content of PPCA decreased from March till September, both in wild and cultivated samples. On the other hand, the variation of flavonoids content (table I) didn't show the same pattern. For wild nettles, the flavonoid's content decreased with the maturization, but for the cultivated ones the flavonoid's content was slightly modified from March till September. The content of polyphenols (polyphenolcarboxylic acids and flavones) was lower than that cited in the scientific literature [2, 11]. All these spectrophotometric results indicated that young wild nettle leaves have the highest content of polyphenols. So, the sample **Am** was further selected for HPLC analysis.

Table I
Results of spectrophotometric determination

Nr. crt.	Batch	Flavonoids (% , rutin)	Polyphenolcarboxylic acids (% , chlorogenic acid)	Reference (FE 6)
1	Am	0.6237 ± 0.0241	4.2295 ± 0.0170	0.3 % acid chlorogenic acid (sum of acid chlorogenic and caffeoylmalic acid)
2	Ai	0.12162 ± 0.0090	1.07222 ± 0.041677	
3	As	0.0399 ± 0.0040	0.6626 ± 0.0029	
4	Bm	0.10354 ± 0.00784	2.9447 ± 0.0754	
5	Bi	0.10352 ± 0.001517	1.05682 ± 0.021381	
6	Bs	0.08324 ± 0.004142	0.7206 ± 0.02585	

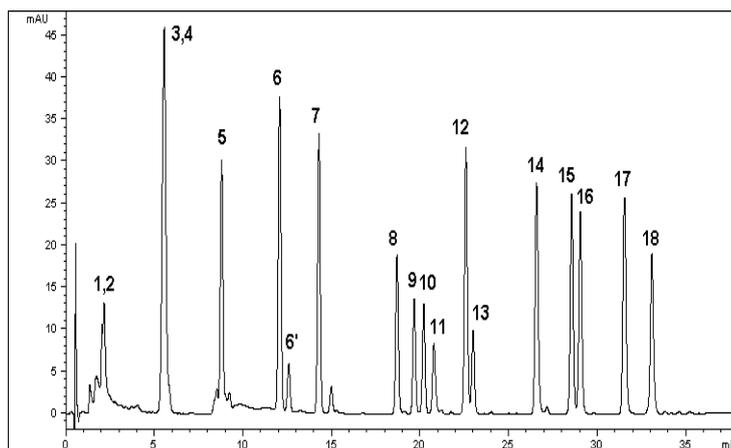


Figure 1

The standards chromatogram, UV detection at 330 and 370 nm: caftaric acid (1), gentisic acid (2), caffeic acid (3), chlorogenic acid (4), *p*-cumaric acid (5), ferulic acid (6), sinapic acid (7), hyperoside (8), isoquercitrin (9), rutin (10), miricetin (11), fisetin (12), quercitrin (13), quercetin (14), patuletine (15), luteolin (16), kaempferol (17) and apigenin (18).

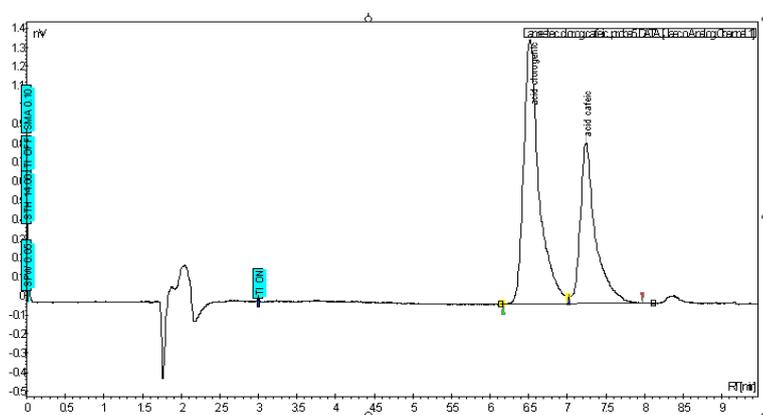


Figure 2

The standard chromatogram for chlorogenic and caffeic acids separation; UV detection at 310 nm.

The results of HPLC/MS showed the presence of caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, ferulic acid, rutin and isoquercitrin in the non-hydrolyzed solution SE₁, luteolin, quercetol and kaempferol in the hydrolyzed solution SE₂ respectively. Because the content of ferulic acid in the hydrolyzed solution was higher than that in non-hydrolyzed solution, we concluded that this compound exists both in the free form and in the glycosidic and esterified forms (e.g. chinic-, tartaric- or malic-esters) (Table II).

Unlike ferulic acid, *p*-coumaric acid was identified only in the hydrolyzed solution, so it may be present as an acyl-derivative (Fig.3).

Due to the lack of standards, we could not establish if kaempferol comes from kaempferol-3-O-rutinoside or from kaempferol-3-O-galactoside. Scientific literature doesn't mention the presence of luteolin (5,7,3',4' - tetrahydroxyflavone), gentisic and caftaric acid in the leaves of *Urtica dioica*, therefore those compounds are mentioned here for the first time. Four polyphenols (chlorogenic acid, caffeic acid, gentisic acid and caftaric acid) cannot be quantified in the current chromatographic conditions due to overlapping.

Table II
Quantification of polyphenols by HPLC/MS and HPLC/UV

Compound	HPLC / MS (method I)		HPLC / UV (method II)	
	SE ₁	SE ₂	SE ₁	SE ₂
caftaric acid	+	-	NA	NA
gentisic acid	+	-	NA	NA
caffeic acid	+	-	7.0040	13.61
chlorogenic acid	+	-	60.079	21.59
<i>p</i> -coumaric acid	-	2.8724	NA	NA
ferulic acid	1.0004	4.399	NA	NA
sinapic acid	-	-	NA	NA
hyperoside	-	-	NA	NA
isoquercitrin	1.2745	-	NA	NA
rutin	4.649	-	NA	NA
miricetin	-	-	NA	NA
fisetin	-	-	NA	NA
quercitrin	-	-	NA	NA
quercetin	-	1.6031	NA	NA
patuletine	-	-	NA	NA
luteolin	-	0.1303	NA	NA
kaempferol	-	0.2247	NA	NA
apigenin	-	-	NA	NA

„+” identified by MS analysis, „-” unidentified by MS analysis; „NA” non-applicable

Chlorogenic acid and caffeic acid are important polyphenolcarboxylic acids from nettle leaves, therefore we developed a second HPLC method in order to quantify them. Unfortunately, the pair gentisic acid / caftaric acid remained undetermined. The results obtained by this second HPLC method (table II) indicated that the content of chlorogenic acid was higher than the content of caffeic acid in the non-hydrolyzed solution, but smaller in the hydrolyzed one. This fact is due to the liberation of caffeic acid from the chlorogenic structure during hydrolysis. The increase of caffeic acid content

is not proportional with the decrease of chlorogenic acid content (molecular weight of acid caffeic / molecular weight of chlorogenic acid = 1/2). This fact indicated that during hydrolysis chlorogenic acid undergoes chemical degradation, other than the cleavage of the esteric bound. The scientific data confirm this hypothesis [1].

Another source of caffeic acid can be caffeoylmalic acid, the main polyphenol cited for nettle's leaves. This acid may be responsible for the main peak in the sample's chromatogram (1.0578 % - calculated by procentual area using chlorogenic calibration curve) and may explain the high content of polyphenolcarboxylic acids found by the spectrophotometric method. Due to the lack of standard this compound remained unidentified (Fig. 4).

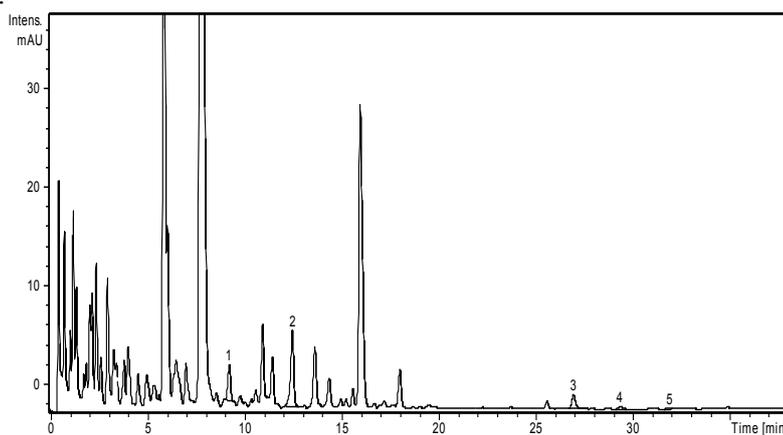


Figure 3
The chromatogram of
the hydrolyzed solution (SE₂) method 1

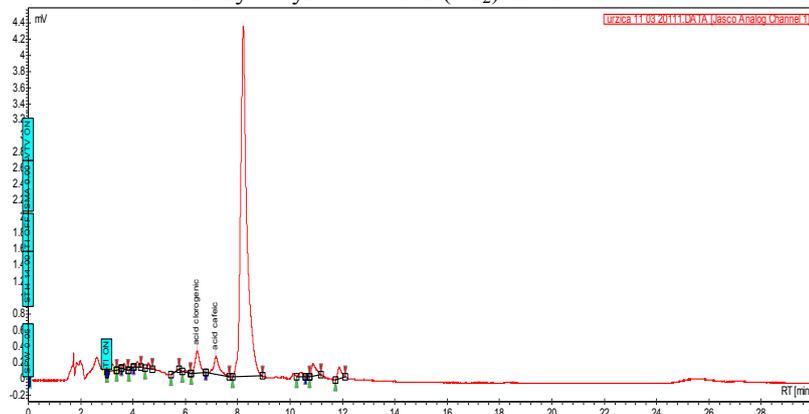


Figure 4
The chromatogram of
the unhydrolyzed solution (SE₁), method 2

Conclusions

The polyphenols' content in *Urticae folium* vegetal product is lower than the one mentioned in the scientific literature, but more diverse, depending on the the month of harvesting. The highest content of polyphenols was found in the leaves of wild nettles, harvested in March. The two HPLC methods made possible to identify and quatify phenolcarboxilic acids and flavonoids. Gentisic acid, caftaric acid and luteolin are new compounds reported in the nettle leaves, unmetioned by the scientific literature.

References

1. Bruneton J., Pharmacognosie, Phytochemie, Plantes medicinales, second edition, Ed Tec&Doc- Paris, 1999, 213, 229, 230.
2. Fleming Th., PDR for Herbal Medecines, Ed. Thompson, 2004, 792-795.
3. Harborne B.I, Baxter H., Moss P.G., Phytochemical Dictionary, second edition, Ed. Taylor – Francis, 1999, 4421, 658, 1664, 1962, 1964, 1960.
4. Istudor V. – Fitoterapia bolilor metabolice – Ed. Tehnoplast Company 2008, București, 43.
5. Ji T.F., Liu C.H., Wang A.G., Zang J.B., Su Y.L., Yuan L., Feng X.Z. - Studies on the chemical constituents of *Urtica dioica* L. grown in Tibet Autonomous Region, *Journal of Chinese medicinal materials*, 2007, 30(6), 662-664.
6. Kavtaradze N. Sh., Alaniya M D - Anthocyan Glycosides from *Urtica dioica* - Chemistry of Natural Compounds, 2003, 39 (3), 315.
7. Khalaf. I, Vlase L., Lazăr D., Corcioavă A., HPLC-UV-MS Study of Polyphenols from *Glycyrrhiza Glabra* – *Farmacia*, 2010, 58 (4), 416 – 420.
8. Kudritsata S. E., Filman G. M., Zagorodskaya L. M., Chikovanii D. M. - Carotenoids of *Urtica dioica* - *Chemistry of Natural Compounds*, 1986, 22 (5), 604-605.
9. Hanganu D, Vlase L, Olah N., LC/MS analysis of isoflavones from *Fabaceae* species extracts, *Farmacia*, 2010, 58(2), 177-183.
10. Wichtl M, Anton R., Plantes therapeutiques, Ed. TEC&DOC, Paris, 2003, 618.
11. XXX - European Scientific Cooperative of Phytotherapie, Second Edition Completely revised and expanded- Ed. ESCOP Exter & GTV Stuttgart & TNY New York, 2003, 521 – 527.
12. XXX - European Pharmacopoeia, 6 th edition, Council of Europe, Strassbourg, 2006, 4928,4929.
13. XXX - Farmacopeea Română, Ed. a X-a Ed. Medicală, București, 1993, 334-335, 1016, 1057-1058, 1063.
14. XXX. - United States Pharmacopeia and National Formulary (USP 30-NF 25), Rockville, 28 (4), United States Pharmacopeia Convention; 2007, 914.

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