

## PRELIMINARY RESEARCH REGARDING *URTICA URENS* L. AND *URTICA DIOICA* L.

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### Abstract

The leaves of *Urtica urens* L. (dwarf nettle, *Uu*) and *Urtica dioica* L. (*Ud*) are included in the European Pharmacopoeia 7<sup>th</sup> edition, as a monograph called *Urticae folium* (collective drug), and both are traditionally used to treat diabetes mellitus. The insufficient data regarding the chemical composition of *Uu* leaves led us to approach their research, and that of *Ud* (harvested in the same period of growth), in order to select the highest quality raw material for obtaining pharmacologically active vegetal extracts. The phytochemical analysis consisted in: specific reactions, in order to identify the main active substances; spectrophotometric methods to quantify phenolcarboxylic acids, flavonoids, total phenolic compounds, tannins, carotenoids and sterols; chromatographic analysis HPLC/UV and HPLC/MS to identify and quantify phenolcarboxylic acids and flavonoids, respectively sterols. Sterols and phenolcarboxylic acids are the main active substances in both nettle species, but higher quantities are found in *Ud*. The carotenoids content is low. The chromatographic results indicated, for both species, the presence of caffeic acid, chlorogenic acid,  $\beta$ -sitosterol and stigmasterol. Rutin and ergosterol were present only in *Ud*, and campesterol only in *Uu*. Of these, sterols are known as peroxisome proliferator activated receptor gamma (PPAR- $\gamma$ ) (can lower blood glucose), and phenolcarboxylic acids as 3-hydroxy-3-methylglutaryl coenzyme-A (HMGCoA) inhibitors (may act as hypocholesterolaemic agents).

### Rezumat

Frunzele speciei *Urtica urens* L., urzica mică (*Uu*), alături de frunzele de *Urtica dioica* L., urzică (*Ud*) sunt admise de Farmacopeea Europeană 7.0, drept constituent al produsului *Urticae folium* (drog colectiv) și sunt utilizate tradițional în tratamentul diabetului zaharat. Datele, relativ puține, referitoare la compoziția sa chimică ne-au determinat să abordăm această tematică, comparativ cu frunzele de *Ud* (recoltate în aceeași perioadă), în vederea selectării materiei vegetale de cea mai bună calitate pentru obținerea unor extrakte standardizate, farmacologic active. S-au realizat: reacții chimice specifice pentru identificarea principalelor clase de principii active; determinarea spectrofotometrică a acizilor fenol-carboxilici, flavonelor, polifenolilor totali, taninului, carotenoidelor și sterolilor; analiza HPLC/UV a acizilor fenol-carboxilici, flavonelor și HPLC/MS a sterolilor. Acizii fenol-carboxilici și sterolii sunt clase majoritare în ambele specii, valorile înregistrate fiind mai mari pentru *Ud*. Carotenoidele sunt în cantități reduse. Cromatografie s-au identificat în ambele specii acizii cafeic și clorogenic,  $\beta$ -sitosterolul, stigmasterolul. Rutozida și ergosterolul se găsesc doar în *Ud*, iar campesterolul în *Uu*. Dintre aceștia, sterolii sunt citați în literatură ca agoniști ai receptorilor PPAR- $\gamma$  (peroxisome proliferator activated receptor gamma) (efect hipoglicemiant) și acizii fenol-carboxilici sunt inhibitori ai HMGCoA (3-hidroxi-3-metilglutaryl coenzima-A) - reductazei (efect hipocolesterolemiant).

**Keywords:** *Urtica* sp., polyphenols, sterols, HPLC/UV/MS

### Introduction

The leaves of *Urtica urens* L. (dwarf nettle, *Uu*) are traditionally used to treat diabetes mellitus. The European Pharmacopoeia 7<sup>th</sup> edition includes the leaves of *Uu* and *Urtica dioica* L. (*Ud*), in the monograph "*Urticae folium*" (collective drug) [32]. In folk medicine, *Uu* is used internally as a haematogenic remedy and diuretic. Traditionally, the herb is used for the treatment of arthritis,

rheumatism of the joints and muscles, and as a component of antidiabetic teas [1, 2].

Scientific literature reports the presence of flavonoids (patuletin, rutin and other heterosides of kaempferol, quercetin and isorhamnetin) [33], coumarins (scopoletin) [17], phenolcarboxylic acids, (0.5% caffeoylmalic acid in *Ud* and in small amounts or even absent in *Uu*; chlorogenic acid, caffeic acid, galic acid and ellagic acid) [16, 18, 33], sterols, ( $\beta$ -sitosterol) [6] and carotenoids, [5],

amino acids (glycine, alanine, leucine, serine, aspartic acid, glutamic acid), vitamins (vitamin K – 45 µg/m, folic acid) and minerals (calcium 12.262 mg%, copper 13 mg%, iron 839 ppm, magnesium 0.683 mg%, manganese 104 mg%, phosphorus 0.463 mg%, potassium 3.251 mg%, sodium 0.092 mg%) in both nettle leaves [5, 6, 19]. Hypoglycaemic and hypocholesterolaemic activities are cited for some of these compounds: sterols, phenolcarboxylic acids and flavonoids [9, 17, 26, 34, 35]. In the scientific literature they are cited as inhibitors of hepatic glucose production and stimulators of glucose transport (caffeic and chlorogenic acids) [15, 26], inhibitors of several enzymes like glucose-6-phosphatase, 3-hydroxy-3-methylglutaryl coenzyme-A (HMGCoA)-reductase (caffeic and chlorogenic acids) [15, 34], alpha-glucosidase (luteolin and luteolin-7-O-glucoside) [17] and aldose-reductase (patuletin, heterosides of kaempferol and quercetin) [13, 16, 19, 21, 29, 33, 34] or as PPAR-gamma agonists (β-sitosterol, phytol, α-carotene, lycopene) [9, 13]. Due to the fact that phytochemical data regarding nettle chemical composition are insufficient (*Uu*) or confusing (data referring mainly to the collective drug) [19], we consider that a research concerning the active substances, with hypoglycaemic and hypocholesterolaemic activities (total phenolic compounds, carotenoids, sterols), may explain the traditional uses of nettle as antidiabetic remedies.

## Materials and Methods

The aerial parts of the drugs were collected in May, 2009, from Hunedoara County, (Calan City for *Uu*) and from Dambovită County (Racari City for *Ud*) Romania. A voucher specimen of each species was deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, “Carol Davila” University of Medicine and Pharmacy, Bucharest. We used the leaves after stem removing.

### *Samples preparation*

#### *Phytochemical screening*

The raw materials were subjected to solvent extraction (diethyl-ether, methanol, water) using a solid-to-solvent ratio (1:10 – first extraction and 1:5 – second extraction) [7].

The obtained filtrates were codified for both nettle species based on the solvent used for extraction: SE (diethyl-ether), SM (methanol) and SA (aqueous).

#### *Spectrophotometric determination*

For polyphenols determination (phenolcarboxylic acids, flavonoids, total polyphenolic compounds, tannins), the raw materials were extracted with hydro-ethanolic mixtures (50% ethanol – phenolcarboxylic acids, total polyphenolic compounds, tannin, 50% methanol – flavonoids) for 30 minutes, using a reflux condenser. The solid-

to-solvent ratio used for the extraction was 1:200 – phenolcarboxylic acids, 1:333 – total polyphenolic compounds and tannins, 1:40 – flavonoids. For carotenoids extraction, the raw material were repeatedly extracted with diethyl-ether, for 30 minutes, using a solid-to-solvent ratio 1:10 (first extraction), 1:5 (second extraction and third extraction). The resulted filtrates were subdued to saponification with 10% potassium hydroxide solution in methanol (to release carotenoids from the ester combinations) [4]. For the extraction of free sterols, the raw materials were extracted with hexane (solid-to-solvent ratio = 1:200), for 2 hours using a reflux condenser. After filtering, the solutions were dry-evaporated and re-dissolved in ethanol (selective solvent) [22]. The esterified forms were obtained using the same technique (solid-to-solvent ratio = 1:200, heating at a reflux condenser for 2 hours) and a mixture of 20 mL ethanol and 5 mL of potassium hydroxide solution in methanol 500 g/L. After extraction, the filtrates were re-extracted three times with 15 mL hexane, dry-evaporated and re-dissolved in 20 mL absolute ethanol [20].

#### *HPLC determinations*

For the HPLC analysis of phenolcarboxylic acids, flavonoids, and sterols, ethanolic solutions were used. Also, the solutions were subjected to hydrolysis (phenolcarboxylic acids and flavonoids) or saponification (sterols) [11, 24].

#### *Spectrophotometric determinations*

The total phenolcarboxylic acids content was measured using a spectrophotometric method with Arnov's reagent. The results were expressed as acid chlorogenic equivalents, using the equation of the calibration curve of chlorogenic acid ( $y = 0.0123 + 18.1890x$ ,  $y$  = Absorbance and  $x$  = Concentration mg/mL, corresponding to the determined absorbance with  $R^2 = 0.9998$ ; the linearity of the calibration curve was 11.3-52.8 mg/mL) [32]. The flavonoid content was determined using the spectrophotometric aluminium chloride method, and the results were expressed as rutin equivalents using an equation that was obtained from the calibration curve of rutin ( $y = 0.0002 + 0.3150x$ ,  $R^2 = 0.9997$ , 5-35 µg/mL) [31]. The total polyphenolic and tannin content (indirect method, based on the tannin precipitation with the hide powder) was determined using Folin-Ciocalteu assay [28]. Tannic acid was used to obtain the calibration curve and the equation was used ( $y = 0.0533 + 0.0605x$ ,  $R^2 = 0.99908$ , 1.21-9.68 µg/mL) for the quantification of the total polyphenolic and tannin content [10, 23]. The carotenoids were determined using a spectrophotometric method based on the carotenoid absorbance at  $\lambda = 460$  nm [4]. The results were expressed as β-carotene equivalents using the equation of the calibration curve of β-carotene ( $y = 0.0355 + 0.224x$ ,  $R^2 = 0.999$ ,

6-30 µg/mL). Finally, the sterols determination was based on the formation of dehydration products with multiple conjugated double bonds in the presence of concentrated sulphuric acid and ferric chloride (catalyst) [8, 25]. The results expressed as stigmasterol equivalents were quantified using the equation of a stigmasterol standard curve ( $y = 0.0186 + 0.0013x$ ,  $R^2 = 0.9992$ , 100-700 mg/mL).

The results of the spectrophotometric determination are expressed as Mean  $\pm$  standard deviation upon two independent replicates. A spectrophotometer Jasco V-530, 2005 was used.

#### HPLC determination

Apparatus: Jasco HPLC MD-2015 equipped with degasser, binary gradient pump, column thermostat, and UV detector; Agilent HPLC Series system (Agilent U.S.A) equipped with degasser, binary gradient pump, column thermostat, autosampler, UV detector and integrated with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). The chromatographic conditions were previously described by Ibrahim K. et al. (2011), Nencu et al. (2012) [11, 24]. The standards used as polyphenols and sterols were chlorogenic acid, caffeic acid, rutin, respectively ergosterol, stigmasterol,  $\beta$ -sitosterol, campesterol and stigmasterol. The standard calibration curves (5 calibration points for polyphenols and 7 for sterols) had a good linearity ( $R^2 > 0.999$ ). The ranges were 37-375 µg/mL for polyphenols and 0.08-8 µg/mL for sterols [3, 12].

## Results and Discussion

The results of the phytochemical screening indicate the same active substances in both nettles leaves: sterols and carotenoids (in SE solutions), coumarins, tannins, flavonoids, phenolcarboxylic acids, total polyphenolic compounds, (in SM solutions), water-soluble polysaccharides (mucilages) and sugars (in SA solutions). For *Uu* the spectrophotometric quantitative determination indicated total polyphenolic compounds content is very similar to that found in scientific literature (1.44 g% acid tannic) [13], the phenolcarboxylic acids compounds are prevailing; the flavonoids content is low, only slightly higher than that found by Jimoh et al. (2010) (0.46 mg quercetol/g dry vegetal product, equivalent with 0.09 g% rutin) [14]; the carotenoids have the smallest content compared to all other active substances (Table I); the sterols (free and esterified) are, along with total polyphenolic compounds, the main active substances of the *Uu* leaves.

For *Ud*, the results of the spectrophotometric quantitative determination indicated mainly the same pattern: sterols are found in higher amounts than total polyphenolic compounds (phenolcarboxylic

acids are prevailing); carotenoids also have lower concentrations, but higher than in *Uu*.

Both species show the same trend of accumulation of active compounds, but their content is higher for *Ud* than *Uu*. The difference between the content of active substances from leaves of *Uu* and *Ud* is high for carotenoids (10 times more in *Ud* than *Uu*), medium for total polyphenolic compounds (approx. 2 times more total polyphenolic compounds for *Ud*) or low for phenolcarboxylic acids and flavonoids (no more than 0.02% between the two nettles).

**Table I**

The results of the spectrophotometric determinations

| Active substance        | <i>Urtica urens</i> L. | <i>Urtica dioica</i> L. |
|-------------------------|------------------------|-------------------------|
| PCA (g% chlorogenic)    | 1.2706 $\pm$ 0.0854    | 1.2686 $\pm$ 0.0586     |
| F (g %rutin)            | 0.1930 $\pm$ 0.1001    | 0.2130 $\pm$ 0.0070     |
| TPC (g% tannic acid)    | 1.6855 $\pm$ 0.0760    | 3.5760 $\pm$ 0.3436     |
| T (g% tannic acid)      | 0.100 $\pm$ 0.0010     | 0.2470 $\pm$ 0.0234     |
| C (g% $\beta$ -caroten) | 0.0372 $\pm$ 0.009     | 0.2757 $\pm$ 0.0117     |
| FS (g% stigmasterol)    | 0.9583 $\pm$ 0.0708    | 1.1896 $\pm$ 0.1814     |
| ES (g% stigmasterol)    | 0.5669 $\pm$ 0.043     | 3.3593 $\pm$ 0.2953     |

Legend: phenolcarboxylic acids - PCA, flavonoids - F, total phenolic compounds - TPC, tannins - T, carotenoids - C, free sterols - FS, esterified sterols - ES

The HPLC/UV analysis indicated the presence of chlorogenic and caffeic acids in both nettles leaves, but rutin was only presented in *Ud* leaves. The quantification of chlorogenic and caffeic acids was possible only for *Ud* leaves. The results showed that chlorogenic acid is the main compound, followed by caffeic acid and rutin. Due to the lower quantities of chlorogenic and caffeic acids (found below the method's detection limit) from *Uu* leaves, their HPLC quantification was not possible. These results confirm data from European Scientific Cooperative on Phytotherapy (ESCOP) (Figure 1, Figure 2, and Table II) [33].

**Table II**

The results of the HPLC/UV determinations

| Compound         | <i>Urtica urens</i> L. |    | <i>Urtica dioica</i> L. |       |
|------------------|------------------------|----|-------------------------|-------|
|                  | mg%                    |    |                         |       |
|                  | FP                     | PG | FP                      | PG    |
| Caffeic acid     | +                      | -  | 5.650                   | 10.87 |
| Chlorogenic acid | +                      | -  | 17.90                   | -     |
| Rutin            | -                      | -  | 2.500                   | -     |

Legend: FP - free polyphenols, PG - polyphenolic glycosides, + UV identified, - UV unidentified

The presence of other phenolcarboxylic acids like gallic acid or ellagic acid [19] in the chemical composition of *Uu* (but remained unidentified due to the lack of standards), may explain similar results found in spectrophotometric determinations of phenolcarboxylic acids for both nettle species (Table III). The low amount of chlorogenic and caffeic acids in *Uu* and *Ud* may be explained by the moment of harvesting. There is no data regarding

*Uu*, but for *Ud* a sudden drop of phenolcarboxylic acids content in the moment of blooming is specified [27, 30]. The HPLC/MS analysis showed the presence of stigmasterol,  $\beta$ -sitosterol and campesterol, as free forms and only  $\beta$ -sitosterol as esterified forms in *Uu*. For *Ud*, the sterols found in the free forms are ergosterol and  $\beta$ -sitosterol. Only  $\beta$ -sitosterol is found as ester. In both nettle species, the free sterols are prevailing and  $\beta$ -sitosterol is the main sterol. Stigmasterol is found in higher quantities in *Uu* than *Ud*. Although the sterolic content (the sum of sterols quantified by HPLC) was greater in *Ud* leaves, there is a small difference

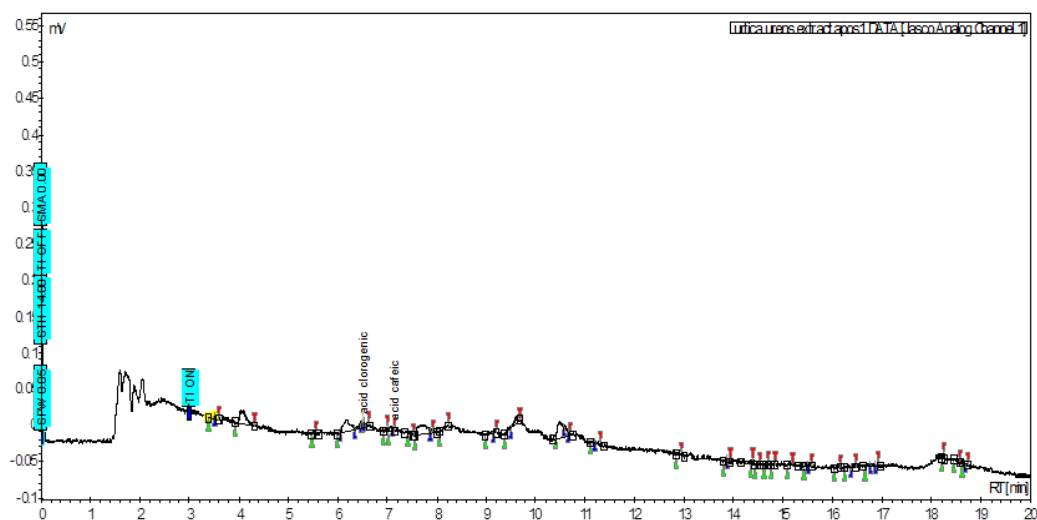
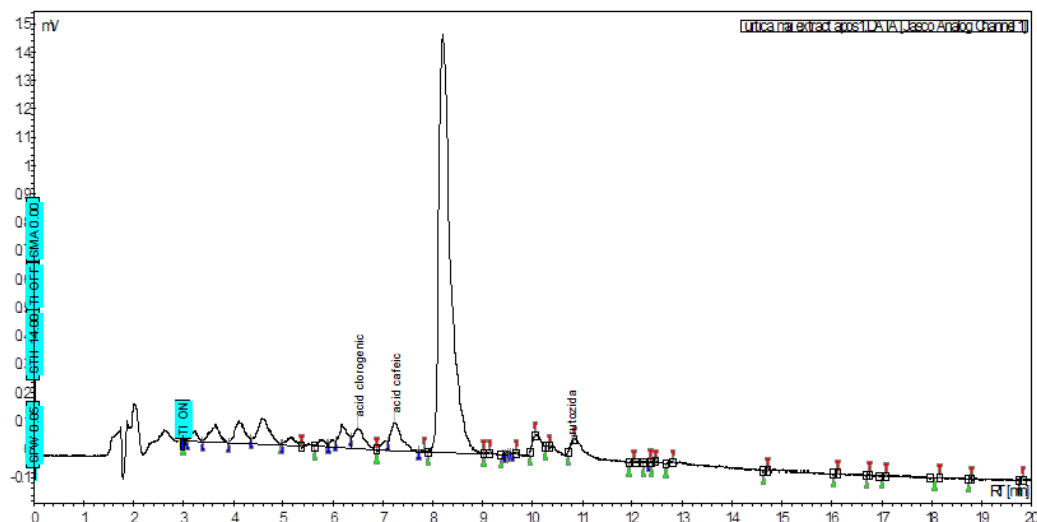
between the sterol content of *Uu* and *Ud*. Of course, further research is needed regarding the dynamics of accumulation of these active substances.

**Table III**

The results of the HPLC/MS determinations

| Compound            | <i>Urtica urens</i> L. |         | <i>Urtica dioica</i> L. |       |
|---------------------|------------------------|---------|-------------------------|-------|
|                     | mg%                    |         |                         |       |
|                     | FS                     | ES      | FS                      | ES    |
| Stigmasterol        | 7.5026                 | -       | 0.9080                  | -     |
| $\beta$ -sitosterol | 178.27                 | 28.2138 | 290.03                  | 35.22 |
| Campesterol         | 7.2078                 | -       | -                       | -     |
| Ergosterol          | -                      | -       | 0.2474                  | -     |

Legend: FS - free sterols, ES - esterified sterols

**Figure 1.**HPLC chromatogram of *Urtica urens* L. alcoholic solution**Figure 2.**HPLC chromatogram of *Urtica dioica* L. alcoholic solution

## Conclusions

The leaves resulted from flowering specimens of *U. urens* L. are a source of sterols (mainly  $\beta$ -sitosterol). The leaves of *U. dioica* are richer in phenolic compounds, sterols and carotenoids than

that of *U. urens*. The sterols can reduce the pathological elevated blood glucose found in diabetes mellitus through the activation of PPAR- $\gamma$  receptors. The main phenolic compounds, phenolcarboxylic acids can give a hypocholesterolaemic effect acting

as HMGCoA inhibitors. The other derivatives (flavonoids, tannins) and carotenoids can also contribute as antioxidants. The presence of sterols is mentioned only in the collective drug; therefore the present data provides a modest contribution to sterols study from *U. urens* leaves. Also, our research establishes the identity and quantity of sterols from *U. dioica* leaves. The results lead us to continue our research in order to obtain pharmacologically active standardized extracts.

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