ORIGINAL ARTICLE

THE SELECTION OF TECHNOLOGICAL PARAMETERS IN ORDER TO OBTAIN AN EXTRACT WITH IMPORTANT ANTIOXIDANT ACTIVITY FROM STINGING NETTLE LEAVES

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Abstract

The aim of this study was to examine the effects of the plant stage development and the extraction parameters (solvent polarity and extraction procedure) on stinging nettle polyphenolic content and antioxidant activity, in order to select the optimal technological parameters for the obtaining of an extract with high antioxidant activity from stinging nettle leaves. The total polyphenolic (T.P.) content of the extracts was determined using Folin Ciocalteu's method. The antioxidant potential was assessed using the DPPH (1,1-diphenyl-2-picrylhydrazyl) and ABTS [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid] methods. The extract with the highest antioxidant effect was obtained by reflux extraction of leaves with 50% ethanol (water:ethanol = 50:50, v/v) (extract D 37.0 mg% tannic acid, IC500 for DPPH and ABTS are 50.39 µg/mL, respectively 91 µg/mL). The correlation factors of ABTS-T.P. and DPPH-T.P. were high for the immature leaves (0.94 and 0.96, respectively) and modest for mature ones (0.76 and 0.33). These results imply that T.P. are major contributors to the antioxidant activity of young nettle leaves. In conclusion, our research indicated that the optimal technological parameters for obtaining extracts with high antioxidant activity from stinging nettle leaves are: refluxing immature raw material using 50% ethanolic solution.

Rezumat

Scopul acestei cercetări a constat în stabilirea influenţei stadiului de dezvoltare al plantei precum şi a parametrilor de extracţie (polaritatea solventului, procedeul de extracţie) asupra conţinutului de polifenoli şi a acţiunii antioxidante a frunzelor de urzică, în scopul selectării parametrilor tehnologici optimi pentru obţinerea unui extract cu acţiune antioxidantă importantă din această materie primă. Conţinutul de polifenoli totali (P.T.) a fost determinat prin metoda Folin-Ciocâlteu, iar potenţialul antioxidant a fost determinat cu ajutorul metodelor DPPH (1,1-difenil-2-picrilhidrazil) şi ABTS (acid 2,2-azino-bis(3-etilbenzotiazolin)-6-sulfonic). Extractul cu cel mai intens efect antioxidant s-a obţinut prin refluxarea cu etanol 50% (apă:etanol = 50:50, v/v) a produsului vegetal imatur (extract D 37,0% acid tanic, IC50 pentru DPPH şi ABTS sunt 50,39 µg/mL, respectiv 91 µg/mL). Corelaţia între conţinutul P.T. şi neutralizarea radicalilor sintetici (DPPH şi ABTS) a fost foarte bună pentru extractele obţinute din produsul imatur (0,94 respectiv 0,96) şi modestă pentru produsul matur (0,33 şi 0,76). Rezultatele acestei corelaţii indică implicarea majoră a polifenolilor totali în imprimarea acţiunii antioxidante a produsului imatur. În concluzie, cercetarea noastră indică faptul că parametrii tehnologici optimi pentru obţinerea de extracte cu acţiune antioxidantă importantă din frunzele de urzică vie, sunt reprezentaţi de refluxarea produsului vegetal imatur cu etanol 50%.

Keywords: extraction parameters, solvent polarity, extraction procedure, antioxidant, phenolic acids, *Urtica dioica* L.

Introduction

Under stress, the human body produces more reactive oxygen species (ROS) (superoxide anion radicals, hydroxyl radicals and hydrogen peroxide), than enzymatic antioxidants (superoxide dismutase, glutation peroxidase and catalase). This excess of free radicals can lead to cellular damage and can be linked with cardiovascular diseases, cancer and degenerative diseases, such as Alzheimer's diseases [18]. A remedy for these problems can be the supplementation of human's diet with natural antioxidants [3, 29]. An example of antioxidant compounds are phenolic compounds, whose antioxidant effect was linked to health benefits [2, 29]. This "scavengers" perform as hydrogen donors or as metal chelators [18, 21]. The methods used to evaluate the antioxidant effect of vegetable extracts have different drawbacks, related to pH medium, solubility of the radicals or colour interference [27, 33]. Therefore, to avoid giving false results concerning antioxidant extract activity, it is recommended to use at least two antioxidant methods [24]. We chose the TEAC method, using the radicals ABTS and DPPH because both methods are simple and fast, and there is a good correlation between T.P. content (Folin-Ciocalteu method) and DPPH/ABTS results.

Urtica dioica (Ud) is one of the most popular and cosmopolitan plants (present in Europe, Africa, Asia, America), whose dietary and therapeutic benefits have been known since ancient times [13]. In Ud's chemical composition, the polyphenolic compounds are prevailing: phenol-carboxylic acids [caffeoylmalic acid (1.6 g%), chlorogenic acid (0.5 g%), ferulic acid and neochlorogenic acid], flavonoids [rutin (0.3 - 0.8%), isoquercitrin (0.14 - 0.31 g%), astragalin, naringenin, isorhamnetin-3-O-glucoside, isorhamnetin-3,7-O-diglucoside, hyperoside], pro-anthocyanidins [pelargonidin, pelargonidin-3-O-xyloside and 3-O-dixyloside, peonidin-3-O-rutinoside and 3-O-(6-O-*p*-coumaroylglucoside), rosinidin-3-O-rutinoside], tannins [epicatechin (0.17 µg/g extract), epigalocatehin - gallate (0.82 - 1.38 µg/g extract)], coumarins [scopoletin (0.1 - 1 mg%)], sterols [β-sitosterol (290.03 mg%), stigmasterol (0.908 mg%)] and 0,01 % essential oil [hexahydrofarnesylacetone (31.20%), α-ionone (4.04%), β-ionone (11.86%) and farnesylacetone (1.26%)] [9, 12, 13, 16, 17, 22]. The antioxidant potential of Ud leaves has concerned many researchers [4, 5, 7, 10, 19], but the influence of plant stage development and extraction parameters (solvent polarity and extraction technique) on the antioxidant activity of the nettle extract hasn't yet been discussed. Therefore, the aim of this study was to examine the effects of plant stage development and extraction parameters (solvent polarity and extraction procedure) on stinging nettle polyphenolic content and antioxidant activity in order to select the optimal technological parameters for the obtaining of an extract with high antioxidant activity. Also, we evaluated the influence of total polyphenolic (T.P.) compounds on the antioxidant activity of extracts, using linear correlation between the antioxidant effect (determined by DPPH and ABTS assay) and the corresponding T.P. content.

Materials and Methods

The leaves of Ud were collected in two growth stages, immature (March) and mature (June) from Ghergani Village, Dâmbovița County, Romania, in 2009. The batches were codified immature (B_1) and mature (B_2). A voucher specimen of each batch was deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy, Bucharest. The solvents used were water, 50% ethanol (water:ethanol = 50:50, v/v) and absolute ethanol. The extraction techniques used were rotary shaking extraction (rotary shaker GFL 3005 at 300 rpm) and refluxation.

Preparation of extracts

200 mg (B_1)/600 mg (B_2) respectively, of raw material were brought into a flask with an acrylic stopper and treated with 20 mL/60 mL respectively, solvent (water, 50% ethanol); the flasks were tightly closed and shook using a magnetic stirrer, for 2 hours, at 300 rpm; after shaking, the flasks content was subjected to filtration. The filtrates were dried under vacuum.

200 mg of B_1 were shook with 20 mL absolute ethanol, using a magnetic stirrer, for 2 hours, at 300 rpm. The obtained solution was treated as previously described.

200 mg of B_1 were extracted for 2 hours with 20 mL of 50% etanol using a reflux condenser. After filtration, the solution was dried under vacuum.

Determination of total phenolic compounds. Determination of T.P. compounds and tannins, expressed as tannic acid equivalent, was performed according to Folin-Ciocalteu method [31] (modified by Humadi 2009) [11]. The results are expressed as tannic equivalents, using a standard curve, linear between 2.1 - 12.12 µg/mL. The standard curve equation is $y = 0.0605x + 0.0533$, $R^2 = 0.999$, where y = absorbance, and x = concentration.

Determination of antioxidant activity using the DPPH assay (1,1-diphenyl-2-picrylhydrazil) was employed according to Brand-Williams (1995) [1,8]. The reaction is based on the reduction of purple radical DPPH (1,1-diphenyl-2-picrylhydrazil) to 1,1-diphenyl-2-picrylhydrazine, coloured in yellow. The results were expressed as µg/mL extract that inhibits with 50% the DPPH absorbance (IC50).

Determination of antioxidant activity using ABTS assay [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] was performed according to Re (1999) [27]. Briefly, the pre-formed cation radical ABTS is reduced by donors of hydrogen, with the decolorizing of the solution [33]. The results were expressed as µg/mL of extract which inhibits with 50% the absorbance of ABTS (IC50).

All the determinations were made in duplicate and expressed as mean ± standard deviation (± SD). The software used for the correlation between T.P. values with DPPH/ABTS results is Origin 6.1.

Reagents: All chemical substances and reagents were of analytical grade, purchased from Sigma Aldrich (Selze, Germany) or Fluka (Buchs, Switzerland).

Results and Discussion

Influence of technological parameters upon nettle leaves T.P. content

Plant stage development. The influence of plant stage development upon T.P. content was studied extracting immature and mature nettle leaves with water (extracts A and E, respectively) and 50% ethanol (extracts B and F, respectively).

The extracts obtained from immature nettle had a higher content of T.P. than the ones obtained from mature nettle, regardless of the extraction solvent. Therefore in our next step we selected only the

immature nettle leaves. From another point of view, from Table I, we can state that maturation leads to a decrease of polyphenolic content. Also, we can speculate, based on the fact that the highest quantities of polyphenols from each batch are found in different solvents (50% ethanol for young nettle leaves and water for mature ones) (Table I), that the type of polyphenols changes with plant development.

Table I

The T.P. content (mg%) of the extracts

Extract	mg% tannic acid
A (immature leaves, water, magnetic stirring)	23.8 ± 0.72
B (immature leaves, 50% ethanol, magnetic stirring)	35.00 ± 0.57
C (immature leaves, absolute ethanol, magnetic stirring)	18.90 ± 1.00
D (immature leaves, 50% ethanol, reflux extraction)	37.0 ± 0.10
E (mature leaves, water, magnetic stirring)	17.50 ± 0.00
F (mature leaves, 50% ethanol, magnetic stirring)	10.50 ± 1.70

Solvent influence. Our obtained results indicated that hydroethanolic mixture has higher extraction activity of T.P. than water. Therefore, in our investigation regarding solvent influence upon T.P. content from immature nettle leaves, we used another solvent, absolute ethanol. For this purpose, the extract obtained from immature nettle leaves and absolute ethanol, was codified as C.

The results indicate that extract C has a lower T.P. content (18.90 mg% tannic acid) than extract B (35.00 mg% tannic acid). It can be concluded, that the hydroethanolic mixture has a higher extractive power for polyphenols from nettle leaves than does water or absolute ethanol. Thereby, in our next step we selected 50% ethanol and immature stinging nettle leves.

Extraction procedure. Regarding the extraction technique influence, we evaluated the effect of refluxation compared to magnetic stirring upon extract T.P. content. In this case, the extract obtained from immature nettle leaves refluxed with 50% ethanol was codified as D. We obtained 37 mg% tannic acid for D and 35 mg% tannic acid for B. This result indicates that, although shaking can increase the mass transfer between raw material and solvent, the influence of high temperature, is slightly more effective for obtaining extracts with high T.P. content. The high temperature may improve the solubility and diffusion coefficient in solution of phenol derivatives or may increase the mobilization of polyphenols from the cells into the solution, as a consequence of their release from their insoluble forms [34].

Influence of extraction technological parameters on nettle leaves antioxidant activity. The IC50 values are inversely correlated with the antioxidant activity of the extracts (Table II). The linearity of the free radical inhibition by the extracts is assessed by the correlation factors (R^2), given in Table II.

Table II

The antioxidant activity of the extracts expressed as IC50

Sample	IC50 (µg/mL)		R^2*	
	DPPH	ABTS	DPPH	ABTS
A	114.85 ± 11.80	122.00 ± 1.01	0.9967	0.9761
B	63.13 ± 2.60	103.00 ± 0.12	0.9904	0.99046
C	81.44 ± 11.22	173.00 ± 0.52	0.9776	0.9984
D	50.39 ± 3.266	91.00 ± 1.91	0.9700	0.9998
E	155.20 x10^3 ± 78.25	127.00 ± 0.21	0.9909	0.9904
F	731.2 ± 0.01	325.00 ± 0.95	0.9967	0.9925

*R^2 expressed the linearity of ABTS and DPPH inhibition by the extracts

Plant development stage. The plant development stage influences the antioxidant activity of the extracts. For this evaluation, we used the extracts codified as A, B (immature leaves) and E, F (mature leaves). The differences are more visible in DPPH assay than ABTS (DPPH: IC50 of B extract is 11 times lower than that of F extract, and of A is 1351 times lower than of E; ABTS: IC50 of A and E are very similar, and IC50 of B is three times smaller than that of F). An explanation of this difference may be due to the different reactivity of the two radicals in the medium. An example is DPPH lower reactivity compared to that of ABTS, towards hydrophilic antioxidants, like sugar derivatives, more soluble in water extracts than in ethanolic extracts [32]. Overall, the extracts obtained from immature nettle leaves, regardless of the extraction solvent, have higher antioxidant activity in both methods than the ones prepared from mature leaves.

The solvent influence was evaluated using the same extracts as in T.P. evaluation (A, B, C). The analysis indicated that B had a higher antioxidant activity than A and C; in both antioxidant assays (IC50 for B was 63.13 µg/mL in DPPH method and 103 µg/mL in ABTS method). This result indicated that 50% ethanol is a better solvent for the

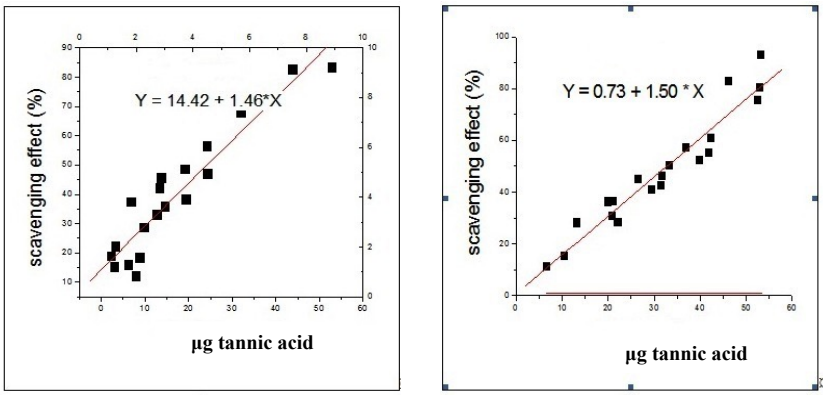
antioxidant compounds than water or absolute ethanol. This is probably due to the preferential solubilisation of phenolcarboxylic acids (mainly chlorogenic acid) and heterosidic flavonoids in the 50 % ethanolic solution, compared to water and absolute ethanol [30, 35]. For aqueous and absolute ethanol extracts, the antioxidant activity was different depending on the radical used. Considering the DPPH method, the absolute ethanolic extract (C) had a higher antioxidant activity than the aqueous extract (A). In the case of ABTS method, the results state the opposite. This behaviour could be explained by the fact that DPPH reacts better in absolute ethanolic medium with lipophilic antioxidants than in aqueous medium. Also, in the case of ABTS, the radical is not affected by the medium polarity [26].

Extraction method. From Table II, it can be seen that the extract obtained by refluxation (D) had a higher antioxidant activity than the one prepared by shaking (B). The same findings were obtained for T.P. determination, but here the differences between the results are more evident. Therefore, one can state that extraction at high temperature has a positive influence on the antioxidant activity of the extracts, probably due to the increase of their T.P. content [23, 28].

In the scientific literature the antioxidant activity reports of nettle extracts vary widely. For the DPPH determination, the IC50 varies between 11.7 µg/mL and 335 mg/mL or, in the case of Pieroni *et al.*, 2.5 mg/mL didn't show any scavenging activity [10, 15, 20, 21, 25]. Compared to these results, the antioxidant activity of our nettle extracts, with well-defined polyphenolic content (from both batches) fall within these limits. The results obtained for the ABTS method are lower to those reported in the scientific literature [5, 14]. We suppose that this behaviour is due to using different quality of the raw material, extraction techniques or different drug-to solvent ratio.

Correlation between T.P. and percentage of inhibition by ABTS/DPPH assay

The correlation between the T.P. content of extracts and the inhibition percentage of free radical (DPPH and ABTS) were performed individually for each type of raw material. The correlation factors between T.P.-DPPH and T.P.-ABTS, for the immature nettle leaves are 0.94 (Figure 1A) and 0.96 (Figure 1B). For the mature leaves, the correlation factors indicate lower values, 0.33 for T.P.-DPPH and 0.76 for T.P.-ABTS.



A B

Figure 1.
The correlation between T.P. content of extracts and the inhibition percent, for immature nettle leaves, obtained by: A) DPPH method; B) ABTS method

The good correlations found for immature nettle leaves extracts indicate that T.P. have a major contribution to their scavenging effect. In mature nettle leaves, the antioxidant effect, can be partially explained by the T.P. content (there is a significant correlation T.P.-ABTS), but other compounds (α-tocoferol, or fatty acids that are found in mature leaves in higher quantities than in young nettle leaves) [6, 7] may act as antioxidants.

Conclusions

In conclusion, our research indicated that the optimal technological parameters for obtaining extracts with high antioxidant activity from stinging nettle leaves are: refluxing immature raw material using 50% ethanolic solution.

The T.P. have a major contribution to the antioxidant effect of immature leaves and modest for the mature ones, other compounds being involved in the antioxidant activity of mature nettle leaves.

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