RESEARCHES REGARDING OBTAINING SELECTIVE EXTRACTS WITH HYPOGLYCEMIANT PROPERTIES FROM VEGETAL INDIGENOUS PRODUCTS (*CICHORII HERBA* AND *FRAXINI FOLIUM*) NOTE III. PHENOLIC COMPOUNDS ANALYSIS FROM *FRAXINI FOLIUM*

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Abstract

The aim of this study was to establish the polyphenolic derivatives content (phenolcarboxylic acids, flavones and tannin) from *Fraxini folium* (Ash leaves) harvested at different stages of development compared to those from S.C. Phytotherapy Bucharest S.A, Romania, and to verify their presence by high performance liquid chromatography coupled with mass spectrometry and UV (HPLC/MS/UV). Leaves harvested in June had the highest content in polyphenolic derivatives. By HPLC/MS and HPLC/UV were identified luteolin, caftaric and gentisic acids not mentioned in the consulted literature.

Rezumat

Obiectivul cercetării a constat în determinarea dinamicii de acumulare a derivaţilor polifenolici (acizi fenolcarboxilici, flavone şi taninuri) din *Fraxini folium* (frunze de frasin) recoltat în diferite stadii de dezvoltare, comparativ cu produsul provenit de la S.C. Fitoterapia S.A. România, precum şi în verificarea prezenţei acestora prin cromatografie de lichide de înaltă performanţă cuplată cu spectrometrie de masă şi UV (HPLC/MS/UV). Frunzele recoltate în iunie au arătat cel mai mare conţinut în derivaţi fenolici. Prin HPLC/MS/UV s-au identificat luteolina şi acizii caftaric şi gentizic, nemenţionaţi în literatura de specialitate consultată.

Keywords: *Fraxini folium*, phenolic compounds, HPLC/MS/UV.

Introduction

Ash leaves (*Fraxini folium*) are containing 0.1 to 0.9% flavones (including rutin, hyperoside (quercetin 3-O-galactoside), quercetin, isoquercitrine (quercetin 3-O-glucoside) and astragalin (keampferol-3-O-

glucoside) , tannin, 16 to 28% mannitol, 10 to 20% mucilages, coumarin derivatives (coumarin, fraxin, esculetin, fraxetin, isoesculetin), iridoids (syringoxide, deoxy-syringoxidin), 2.5 to 3.2% phenolic acids (caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid, protocatehic acid, syringic acid, and vanillic acid) and are used in traditional medicine as a diuretic [1-2, 10, 12-13, 16]. Some of these constituents are mentioned in the literature for their inhibitory effect on the aldose-reductase (ARI), on 11-β hydroxysteroid dehydrogenase type 1 (11-βHSD_1) and advanced glycation end products (AGE), which are involved in the pathogenesis of diabetes and aging processes [3]. Considering that the presence of phenolic derivatives could be involved in regulating glucose metabolism and vascular disorders induced by this offset, we aimed at achieving selective extracts, standardized flavones, tannin and phenolic acids. *Fraxini folium* is not mentioned in the literature for hypoglycemiant properties.

Materials and Methods

The study material was supplied by S. C. Phytotherapy Bucharest, Romania (batch FF) and was spontaneously harvested from Buftea (Ilfov county) in 2011, in different periods of vegetation (June – batch F1, July – batch F2, and August – batch F3). 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*. The polyphenolcarboxilic 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* [4-5, 7, 14-15]. The standard calibration curves were obtained using rutin and respectively caffeic acid. For the spectrophotometric determinations there were used UV-VIS Cecil Series 2000 and Jasco-V 530 spectrophotometers [4-5, 7, 14]. The total phenolic content was assessed according to the method reported by Singleton *et al* (1999) by using Folin-Ciocâlteu reagent with some modifications and gallic acid as reference substance [8-9]. A spectrophotometer Jasco-V 530 was used. All determinations were performed in triplicate. Results are expressed as a mean ± standard deviation.

Establishing standard curve of gallic acid

In a series of 10 mL volumetric flasks were introduced volumes of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 mL 0.0108% gallic acid solution. To each flask it was added distilled water up to 1 mL, 1 mL Folin Ciocâlteu reagent diluted 1 : 1 with water and filled to mark with 10% sodium carbonate

solution. After standing for 40 minutes absorbances were mesured at λ =765 nm using a spectrophotometer Jasco-V 530. The data obtained were used for the calibration curve which was drawn according to the formula: **Y= A + B** * **Conc** , A = -0.0094, B = 0.1382 and which is linear in the concentration range (1.08 to 7.56 µg/mL) and r^2 = 0.999370 (Figure 1).

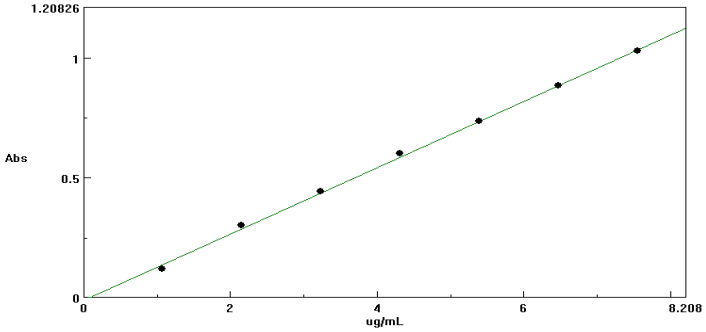

Figure 1
Standard curve of gallic acid solution 0.0108%, λ=765 nm

Preparation of test solution: 1g of powdered herbal material from each batch was refluxed with 50 mL of 50% methanol for 2 times successively for 30 minutes; the extractive solution was filtered into a 100 mL volumetric flask and was brought to the mark with 50% methanol. Samples of 0.2mL-0.8mL test solution were used during the experiment. Absorbances were mesured at λ =765 nm using a spectrophotometer Jasco-V 530.

HPLC/MS

The HPLC determinations of flavonoids and phenolcarboxilic acids were carried out using an Agilent HPLC Series system (Agilent U.S.A.) equipped with degasser, binary pump, column thermostat, autosempler 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 µm 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 5µL. 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*-cumaric acid, ferulic acid, sinapic acid, hyperoside, isoquercitrin, rutin, miricetin, fisetin, quercitrin, quercetin, patuletine, luteolin, kaempferol and apigenin (Figure 2). Calibration curves in the 0.5-50 μg mL^{-1} range had a good linearity (r^2= 0.999, n = 5) [6, 11].

Sample preparation. For extraction of polyphenolic compounds, 1g of powedered leaves (batch F1) was refluxed with 100mL of 50% methanol for 30 minutes. Extraction solution was filtered into a 100mL volumetric flask and filled to the mark (SE-1). For extraction of free aglycons, 50mL of SE-1 was hydrolysed with 50mL of 2 N HCl through a water bath maintained at 80°C for 60 minutes (SE-2).

Results and Discussion

The spectrophotometric results (Table I) showed the highest content of active principles in the leaves of bach F1 (harvested in June). So, the sample F1 was further selected for HPLC analysis.

Table I

Results of spectrophotometric determination

Batch	flavones (g% rutin) ± STD	phenolcarboxilic acids (g% caffeic acid)± STD	phenolcarboxilic acids (g% chlorogenic acid) ± STD	polyphenols (g% gallic acid) ± STD	tannin (g% gallic acid) ± STD
F1	0.2596±0.019	3.2815±0.231	2.5518±0.122	2.5056±0,149	0.4383±0.014
F2	0.1388±0.017	2.7632±0.064	2.1666±0.047	1.7821±0,182	0.3909±0.209
F3	0.1352±0.020	2.7015±0.198	2.4000±0.071	2.4765±0,390	0.4175±0.193
FF	0.2462±0.042	3.1981±0.058	2.4818±0.077	2.4438±0,199	0.4018±0.039

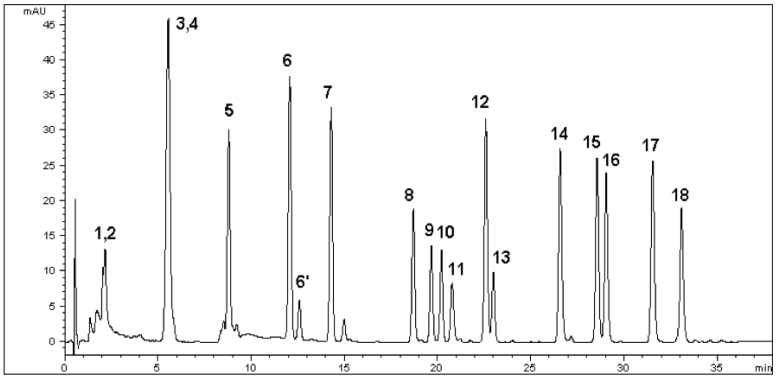

Figure 2

The standards cromatogram, UV detection at λ=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).

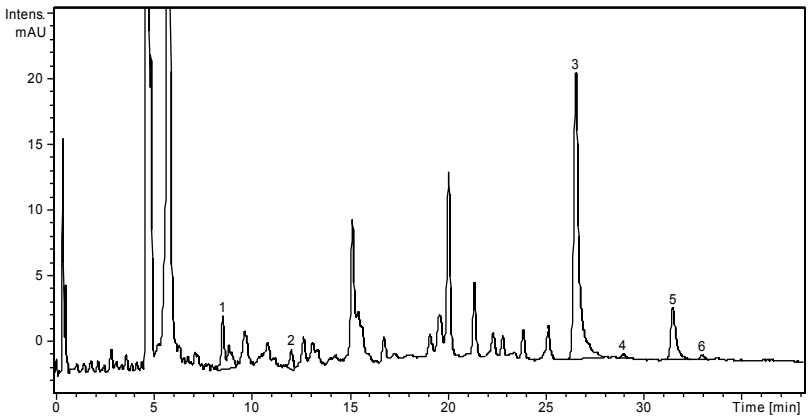

Figure 3
The chromatogram of the unhydrolyzed solution SE-1

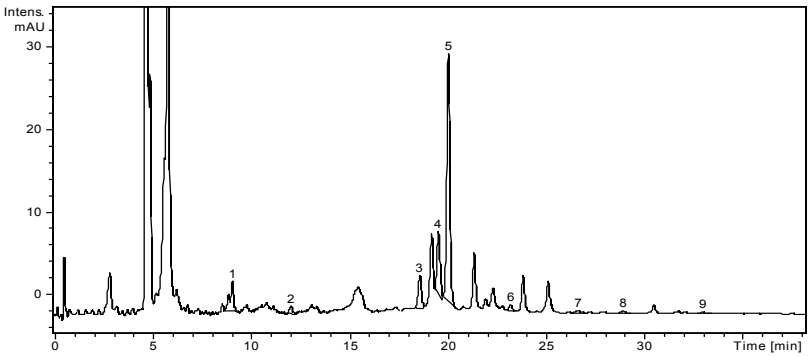

Figure 4
The chromatogram of the hydrolyzed solution SE-2

The results of HPLC/MS analysis showed the presence of caftaric acid, gentisic acid, chlorogenic acid, *p*-cumaric acid, ferulic acid, rutin, hyperoside, isoquercitrin, quercitrin, apigenin, and small amounts of luteolin and quercetin in the non-hidrolyzed solution SE-1 (Figure 3); caffeic acid, ferulic acid, *p*-cumaric acid, luteolin, kaempferol and large amounts of quercetol and in the hydrolyzed solution SE-2 (Figure 4). Four polyphenols (caffeic acid, caftaric acid, gentisic acid and chlorogenic acid) could not be quantified in the current chromatographic conditions due to overlapping (Table II). Ferulic acid and *p*-cumaric acid were found after hydrolisys, but in smaller quantities than before hydrolysis. This behavior shows a degradation either by oxidation, or by demetilation of caffeic acid (detected only after hydrolysis by HPLC/MS) which in turn oxidizes. Caffeic acid could derive from hydrolysis of chlorogenic acid. The increased amount of

quercetin in SE-2 was due to the release of rutin, isoquercitrin or hyperozide. Due to the lack of standards, we could not establish if kaempferol comes from keampferol-3-O-glucoside (astragalin mentioned in composition) or from another compounds (kaempferol-3-O-galactoside). Scientific literature doesn't mention the presence of luteolin (5,7,3',4'-tetrahidroxyflavone), gentisic and caftaric acid in the leaves of *Fraxini folium*, therefore those compounds are mentioned here for the first time.

Table II

Quantification of polyphenols by HPLC/UV/MS (mg/100g vegetal product, raw)

Compound	HPLC/MS		HPLC/UV	
	SE-1	SE-2	SE-1	SE-2
Caftaric acid	+	-	-	-
Gentisic acid	+	+	-	-
Chlorogenic acid	+	+	-	-
p-cumaric acid	5.6852	4.2913	+	+
Ferulic acid	1.0118	0.9098	+	+
Caffeic acid	-	+	-	-
Hyperozide	9.9286	-	+	-
Isoquercitrin	25.3544	-	+	-
Rutin	98.1922	-	+	-
Quercitrin	2.9735	-	+	-
Quercetin	0.6779	21.9729	+	+
Luteolin	0.5199	0.3299	+	+
Apigenin	0.7658	0.5799	+	+
Kaempferol	-	4.9913	-	+

where: + positive, -negative

Conclusions

Identification by a HPLC method of luteolin, caftaric and gentisic acid in *Fraxini folium* is a personal contribution to the knowledge of chemical composition of this vegetal product (these compounds are not quoted in literature in the chemical composition). *Fraxini folium* (batch F.1 harvested in June) had the highest content in polyphenolic derivatives. Considering that the presence of phenolic compounds could be involved in regulating glucose metabolism and vascular disorders induced by diabetes mellitus, we obtained and characterised selective extracts, standardized flavones, tannin and phenolic acids.

Acknowledgements

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