Researches Upon Indigenous Herbal Products for Therapeutic Valorification in Metabolic Diseases. Note I. Polyphenols' Analysis of *Rubi idaei folium*

Costea Teodora, Istudor Viorica, Nencu Ioana

Department of Pharmacognosy, Phytochemistry, Phytotherapy, Faculty of Pharmacy, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania

Introduction: Raspberry leaves (*Rubi idaei folium*) are a source of flavonoids, gallic tannins, phenolcarboxylic acids, sterols, vitamin C and oligoelements (selenium, vanadium). The leaves are not mentioned by the scientific literature for their possible use in metabolic diseases (diabetes, dyslipidaemia, hyperuricaemia), but among their compounds, polyphenols, sterols and vitamin C might be responsible for these properties. The aim of the study was to determine the optimum time for harvesting the leaves, in order to obtain pharmacological active extracts, with the highest content of flavonoids, phenolcarboxylic acids and tannins.

Material and methods: Qualitative (specific chemical reactions and thin layer chromatography, TLC) and quantitative (spectrophotometric methods and high liquid chromatography, HPLC) analyses upon leaves, harvested at different stages of development (before and after blossom, at fruits' ripening and at the end of vegetative state) were applied.

Results: Flavonoids, tannins and phenolcarboxylic acids were present in all vegetative states; rutin, hyperoside and chlorogenic acid were identified using TLC. The greatest amount of total polyphenols (16.0317 g% tannic acid), tannins (11.4376 g% tannic acid), flavonoids (2.6347 g% hyperoside) and phenolcarboxylic acids (2.4011 g% chlorogenic acid) were found in young leaves. Using HPLC, gallic, chlorogenic, caffeic, p-coumaric and ferulic acids, tannin, rutin, quercetin and catechin were identified in young leaves; rutin (0.0540 g%) and p-coumaric acid (0.03174 g%) were also quantified.

Conclusions: The highest content of flavonoids, phenolcarboxylic acids and tannins were found in leaves, harvested before blossom. The tannins content were higher, compared with scientific literature.

Keywords: *Rubi idaei folium*, quantitative analysis, ferulic acid, polyphenols

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Introduction

Raspberry leaves (*Rubi idaei folium*) are used as a folk medicine to treat wounds, diarrohea, colic pain and as an uterine relaxant, due to their antibacterial, astringent, antispasmodic and haemostatic properties [1–4]. Recent studies have shown their cytotoxic activity in human carcinomas [5]. According to the scientific literature, raspberry leaves have antioxidant properties [6,7], being a source of: 0.46–5% flavonoids (rutin = quercetin-3-O-rutinoside, hyperoside = quercetin-3-O-galactoside, tiliroside = kaempferol-3-O-β-D(6"E-p-coumaroyl) glucopyranoside and other heterosides of myricetin, kaempferol, quercetin, isorhamnetin); 2.06–6.89% gallic tannins as monomers and polymers (sanguiin H6, lambertianin C), phenolcarboxylic acids = AFC (gallic, chlorogenic, gentisic, ellagic, caffeic, ferulic, lithospermic, p-coumaric acids); sterols (β-sitosterol, stigmasterol); vitamin C and oligoelements (selenium = 19–381 μg/kg, vanadium = 138–1958 μg/kg) [6–13].

Raspberry leaves are not mentioned by the scientific literature for their potential use in metabolic diseases (diabetes, dyslipidaemia, alterations of protein metabolism – hyperuricaemia), but among their compounds, flavonoids, AFC, tannins and oligoelements have shown hypoglycemic and hypolipidaemic activities [14,15]. Myricetin acts as a potent inhibitor of xanthinoxidase's activity [16] and lithospermic acid raises glomerular filtration rate [1].

The aim of the present study was to determine the optimum time for harvesting the leaves, in order to obtain pharmacological active extracts (that might be used for metabolic diseases' treatment), with the highest content of AFC, flavonoids and tannins. Regarding this, qualitative (chemical reactions and thin layer chromatography, TLC) and quantitative analyses (spectrophotometric determinations and high liquid chromatography, HPLC) were applied.

Material and methods

The raw material consisted of raspberry leaves harvested from culture, in 2011, from Jilava, Ilfov county, Romania, in different stages of development: before blossom (end of April = group Z1), after blossom (June = group Z2), at fruits' ripening (July = group Z3), end of vegetative state (end of August = group Z4). The herbal products were dried in shadow and conserved in laboratory conditions.

For the qualitative analysis 2 g of herbal product from each group, was successively extracted with 30 mL of different solvents (ethyl ether, methanol and water). Specific chemical reactions were carried out, in order to identify active principles (flavonoids, AFC and tannins) [17].

TLC were performed for flavonoids and AFC identification, using ready-made silicagel GF_{254}, 10×20 cm plates (kept at 105°C for 1 h before use) and ethyl acetate – for-

Correspondence to: Teodora Costea
E-mail: teodoracostea85@yahoo.com

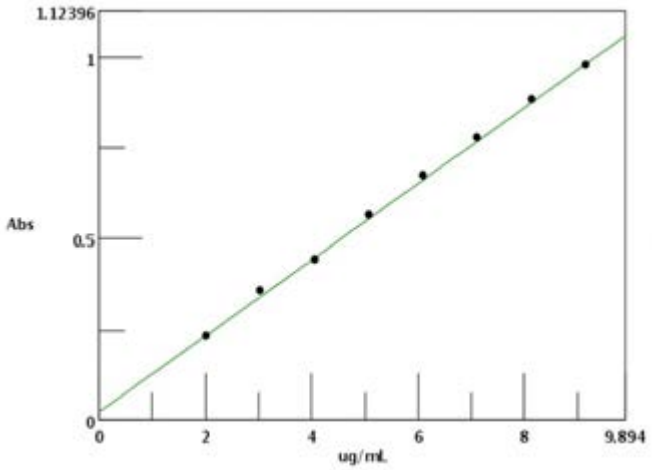

Fig. 1. Standard calibration curve for tannic acid. Absorbance = A + B x conc, where A = 0.0257, B = 0.1044, R^2 = 0.999494

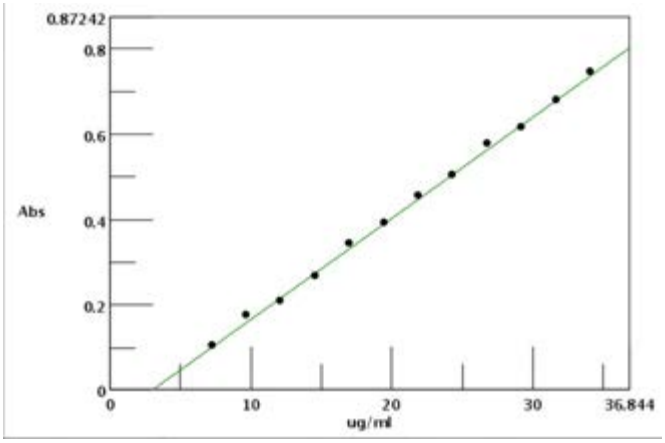

Fig. 2. Standard calibration curve for hyperoside. Absorbance = A + B x conc, where A = -0,0703 B = 0,0237, R^2 = 0,999187

mic acid – glacial acetic acid – water (100:11:11:26) as the mobile phase.

Samples (SMZ1, SMZ2, SMZ3, SMZ4) were prepared from 1 g of herbal product from all groups, treated with 10 mL methanol on a boiling water bath, with ascending condenser, for 10 min. Standard references consisted of methanolic solutions of 2.5 mg rutin and hyperoside (SR, SH), 1 mg of chlorogenic and cafeic acids (SC, SCC) dissolved in 10 mL methanol and quercitrin 1mg/mL (SQ). All standards were purchased from Roth. 10 µL of samples and 5 µL of standard solutions were applied as bands. The migration distance was 16 cm and NEU/PEG reagent (10 g/L methanolic solution of diphenylboriloxiethylenamine and 50 g/L methanolic solution of polyethylenglycol 400) was used for identification (Fluka) [18]. The chromatogram was observed in UV light (366 nm), using a Camag Reprostar Lamp with Epson Photo PC 850.

For all spectrophotometric determinations, 0.5 g of powdered leaves (Z1, Z2, Z3, Z4 groups) were twice extracted with 50 mL ethanol 50% (v/v) on a reflux condenser for 30 min. After cooling, the liquid was filtered through a plug of absorbent cotton in a volumetric flask and made up to 100 mL by rising of the flask and filter. The assay for total polyphenols and tannins (λ = 763 nm) was performed according to Makkar method with Folin Ciocalteu reagent; the content being expressed as g% tannic acid [19]. In order to evaluate the flavonoid content, a spectrophotometric method was used, based on the chelating reaction with aluminium chloride, according to the Romanian Pharmacopoeia 10th edition, (the monograph *Cynarae folium*) [20]. The flavonoid content was expressed as g% hyperoside. The assay for AFC (λ = 525 nm) was performed using the method from European Pharmacopoeia 7th edition, based on the formation of oxymes with Arnow reagent (the monograph *Fraxini folium*); results were expressed in g% chlorogenic acid [21]. For all determinations, UV-VIS JascoV-530 spectrophotometer was used. The content of total polyphenols, tannins, flavonoids and AFC were determined using calibration standard curves (Figures 1–3) of: tannic acid (2.04–9.18 µg/mL, R^2 = 0.9994, n = 8), hyperoside (7.32–34.16 µg/mL, R^2 = 0.9918, n = 12) and chlorogenic acid (0.0113–0.0527 mg/mL, R^2 = 0.9998, n = 6). Standard deviation function (STDEVA) in Microsoft Excel 2003 were used in statistically results calculation.

HPLC were also performed for group Z1, using a Dionex apparatus with pump P580, column RP8 lichrosorb 4.6×200 mm, 10 µm particle, using as detector Diode areas UV-VIS 3404. The mobile phase consisted of methanol and phosphoric acid 0.01 M (gradient), the flow rate was 1 mL/min, the injection volume 20 µL, the temperature 20–25°C and the time for analysis 70 min. The UV detector was set at 280 nm. The sample (SEZ1) was prepared from 5 g powdered herbal product (batch Z1) extracted with ethanol 50% (v/v) on a reflux condenser for 30 min. After cooling, the liquid was completed to 100 mL in a volumetric flask. Gallic, chlorogenic, caffeic, p-coumaric, ferulic acids, rutin, quercetin, catechin and tannin (Sigma-Aldrich) were used as standards. The chromatographic data were processed using Chromeleon (c) Dionex version 6.40 SP1, build 711 [22].

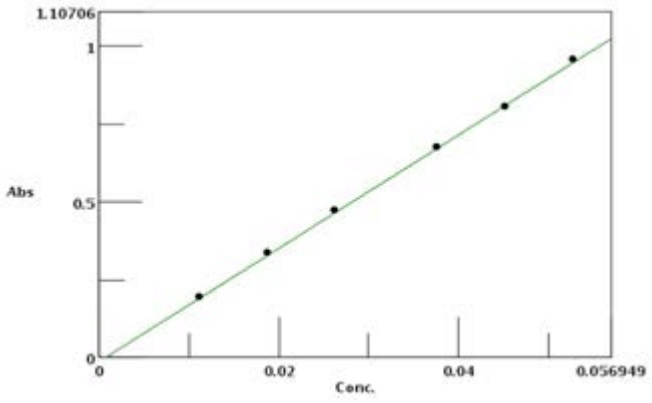

Fig. 3. Standard calibration curve for chlorogenic acid. Absorbance = A + B x conc, where A = -0,012, B = 18.1890, R^2 = 0.999858

Table I. Results of of polyphenols' spectrophotometric determinations

Group of herbal products	Total polyphenols (g% tannic acid)	Tannins (g% tannic acid)	Phenolcarboxylic acids (g% chlorogenic acid)	Flavonoids (g% hyperoside)
Z1	16.0317 ± 0.1729	11.4376 ± 0.3169	2.4011± 0.11653	2.6347 ± 0.2406
Z2	11.3827 ± 0.2935	8.1924 ± 0.3012	1.7428 ± 0.1443	1.8991 ± 0.2110
Z3	11.1100 ± 0.1866	7.8051 ± 0.2911	2.1820 ± 0.1018	2.2985 ± 0.1856
Z4	9.6172 ± 0.2046	6.2200 ± 0.1593	2.2081 ± 0.1510	2.2148 ± 0.1515

Results were expressed as average ± standard deviation

Results

Flavonoid aglycones were identified in etheric solutions, flavonoid heterosides, AFC and tannins were identified in methanolic and aqueous solutions. Analysing the TLC chromatogram (Figure 4) one can note the presence of several spots corresponding to compounds with flavonoidic behaviour (yellow fluorescence) or to AFC (blue fluorescence). Among these compounds, rutin (R_f = 0.48), hyperoside (R_f = 0.73) and chlorogenic acid (R_f = 0.67) were identified in all samples; caffeic acid (R_f = 0.93) only in SMZ1 sample and quercitrin was not identified.

The results of the spectrophotometric determinations are shown in Table I.

The HPLC analysis of SEZ1 solution (Figure 5, Table II) showed the presence of gallic, chlorogenic, caffeic, ferulic, p-coumaric acids, rutin, catechin, tannin and quercetin. Among these compounds, rutin (0.0540g%) and p-coumaric acid (0.03174 g%) were quantitatively determined.

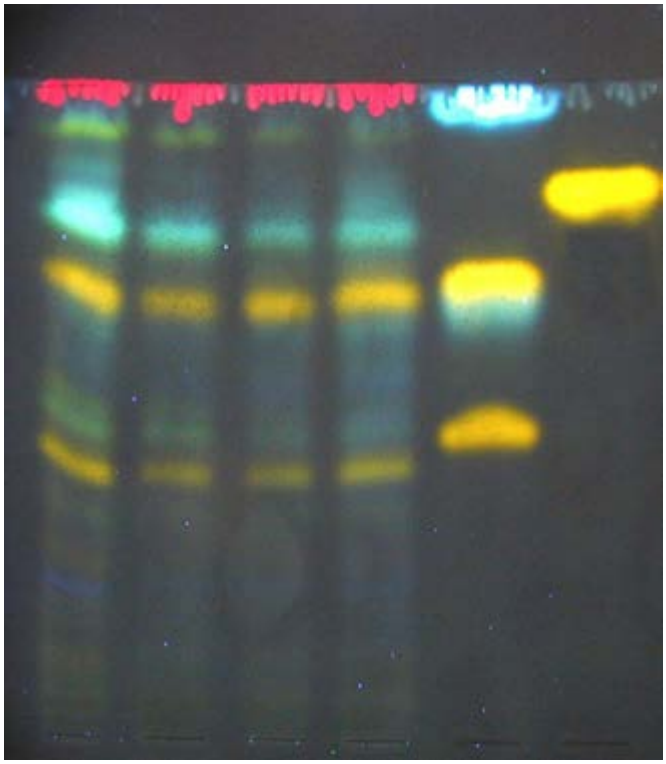

Fig. 4. TLC chromatogram for flavonoids and polyphenolcarboxylic acids (UV 366 nm after spraying the plate with NEU/PEG reagent). 1 = SMZ1, 2 = SMZ2, 3 = SMZ3, 4 = SMZ4, 5 = SCC + SH + SC + SR (from the top downwards), 6 = SQ

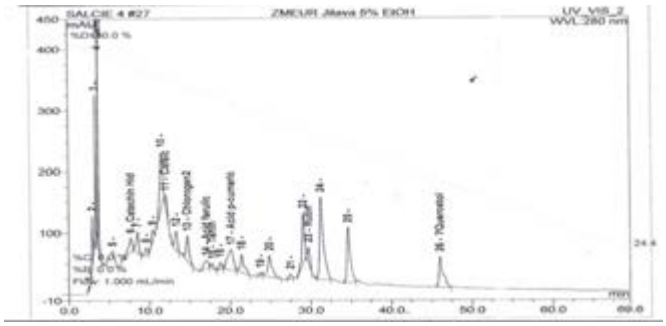

Fig. 5. HPLC chromatogram for SEZ1 solution

Discussion

Flavonoids, tannins and AFC were present in all vegetative states. Hyperoside, rutin and chlorogenic acid were identified in all samples by TLC. The greatest amount of active compounds was observed in young leaves (group Z1). Similar quantities are reported in scientific literature, except

Table II. Compounds identified at HPLC analysis of SEZ1 solution

No.	Retention time (minutes)	Relative peak area (%)	Compound
1.	2.55	0.22	NI
2.	2.95	2.72	NI
3.	3.36	3.04	NI
4.	3.68	10.71	gallic acid
5.	5.43	4.41	NI
6.	7.71	2.63	NI
7.	8.54	2.67	NI
8.	9.60	0.84	NI
9.	10.43	1.44	NI
10.	11.52	8.64	NI
11.	12.04	0.85	caffeic acid
12.	13.32	2.28	NI
13.	14.70	3.48	chlorogenic acid
14.	17.03	1.84	ferulic acid
15.	17.77	0.51	tannin
16.	18.80	0.86	NI
17.	20.11	5.73	p-coumaric acid
18.	21.42	3.21	NI
19.	23.76	0.37	NI
20.	24.85	4.35	NI
21.	27.47	0.58	NI
22.	29.07	6.74	NI
23.	29.72	1.53	rutin
24.	31.25	15.42	NI
25.	34.61	8.99	NI
26.	46.02	5.92	quercetin

NI = not identified

tannins content, which were higher [3,4,9]. Tannins and total polyphenols quantities slowly decreased until the end of vegetative state. At fruits' ripening, flavonoids and AFC content raised slowly, probably counterbalancing the decreased in tannins, which accumulated in fruits. These data have not been mentioned before, by scientific literature. Using HPLC, we have identified a series of compounds in young leaves, also mentioned by scientific literature [6–8,10]. The HPLC chromatogram revealed the presence of other peaks, unfortunately, due to lack of standards we weren't able to identify them; future research is needed.

Conclusions

Raspberry leaves are a considerable source of flavonoids, tannins and AFC; the highest content of these compounds was found in young leaves (harvested before blossom) and these will be further used for obtaining pharmacological active extracts. Using HPLC analysis we have identified a series of polyphenols, also cited by the scientific literature.

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