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SHORT COMMUNICATION

QUANTITATIVE ANALYSIS OF FLAVONOIDS AND PHENOLIC ACIDS FROM INFLORESCENCES AND AERIAL PARTS OF SELECTED *CIRSIUM* SPECIES USING ASE METHOD

MAŁGORZATA KOZYRA* and KRYSTYNA SKALICKA-WOŹNIAK

Department of Pharmacognosy with Medicinal Plant Laboratory, Medical University, 1 Chodźki St., 20-093 Lublin, Poland

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According to the literature, plants from the *Cirsium* genus are rich in phenolic compounds (1–6). Phenolic acids are known to have various biological activities, especially fungistatic, bacteriostatic, antioxidant, anticancer, choleretic, potential sedative – hypnotic, antianxiety and anticonvulsant activity (1, 8, 9). Flavonoids display vasoprotective, hepatoprotective, anti-inflammatory, anticarcinogenic, and free radical-scavenging properties. Recent studies have shown that apigenin exhibits anti-proliferation effects on several forms of cancer cells such as prostate cancer cells, breast cancer cells, leukemia cells, colon cancer cells. Compared with other flavonoid substances, apigenin is characterized by low toxicity and non-mutagenesis (9).

So far, flavonoids and phenolic acids have not been studied in such plants as *Cirsium decussatum* Janka, *Cirsium eriophorum* (L.) Scop., *Cirsium ligulare* Boiss. Therefore, it is the first time when qualitative and quantitative analysis of flavonoids and phenolic acids in the flower and herb of *Cirsium* spp. has been carried out. The aim of this study was to carry out the separation of the active components of inflorescences and leaves of *Cirsium decussatum* Janka, *Cirsium eriophorum* (L.) Scop., *Cirsium ligulare* Boiss. using accelerated solvent extraction method. The obtained extracts were combined and purified by SPE. The SPE-eluates were analyzed by RP-HPLC. Quantitative analyses of flavonoids and phenolic acids were also carried out. Standard deviation was calculated for all the results, which allows to claim that the results are statistically significant. The isolation and separation of natural compounds (including phenolic acids) from plants is a very important problem in phytochemical analysis (10–14). Flavonoids were quantitatively analyzed for the first time. The obtained methanolic extracts proved to be very rich in flavonoids, some of which have not been identified.

EXPERIMENTAL

Plant material

Inflorescences and leaves were collected in 2009 in the Medicinal Plant Garden, Department of Pharmacognosy, Lublin, Poland. They were dried in air at room temperature and immediately powdered according to accepted normal procedures.

Plant material (1 g) was placed in the stainless-steel cell of Dionex (Sunnyvale, CA, USA) ASE 100 accelerated solvent extractor using methanol as solvent.

The extraction conditions were optimalized, giving the best parameters of extraction for: methanol concentration 70%, temperature: 85°C, number of cycles: 3. Extraction was performed at 100 bar.

Purification procedures

All the methanolic extracts were concentrated under reduced pressure, dissolved in small portion of methanol, and transferred to a 10 mL graduated flask. Solid phase extraction (SPE) is a popular procedure used for the isolation, purification and pre-

* Corresponding author: e-mail: mkozyra@pharmacognosy.org

concentration of organic compounds present in biological material. It is often considered an alternative to other methods. In this study, SPE was used for the isolation of flavonoids and phenolic acids from the *Cirsium* genus**.** Samples containing phenolic acids were purified from fatty components and chlorophylls with SPE. Samples were evaporated to dryness, dissolved in 30% aqueous methanol and applied to octadecyl BakerBond SPE microcolums (500 mg, 3 mL, J.T. Baker Phillipsburg, NJ, USA) previously activated with 10 mL of methanol and then 10 mL of water. The isolated compounds were obtained by the elution of columns with 7 mL methanol : water, 80 : 20 (v/v), under reduced pressure (SPE-12G chamber, J.T. Baker USA, Groß-Gerau, Germany). The eluates obtained were free from ballast compounds and contained aglycones and phenolic acids (15).

RP-LC analysis

LC was performed with an Agilent 1100 system coupled with an autosampler, a column thermostat; and diode array detector (DAD). Compounds were separated on 250 × 4.6 mm stainless-steel column packed with 5 μm Hypersil XDB- C18 (ZORBAX Eclipse), using a stepwise mobile phase gradient prepared from 1% aqueous acetic acid (component A) and methanol (component B) (v/v). The gradient was: 0 min, 2% B in A; 8 min 5% B in A; 12 min 10% B in A; 20 min 25% B in A; 35 min 45% B in A; 40 min 60% B in A, 45 min 75% B in A. The mobile phase flow rate was 1 mL/min, the sample injection volume was 10 μL, and elution was performed at 25°C. The LC pumps, autosampler, column oven, and DAD were monitored and controlled by use of HP Chem. Station rev.10.0 software (Agilent).

The identity of compounds examined was performed by the comparison of retention times (t_R) and UV spectra with standard substances analyzed under the same conditions. The qualitative and quantitative analysis was performed. Retention times were compared with those of standards, using UV spectra (λ = 254, 280 and 320 nm) as a comparative parameter. Quantitative determination was performed at the wavelength of maximum absorption of flavonoids and chlorogenic acid – 320 nm.

Recovery, repeatability and precision

Each extract was injected in triplicate on the same day. The RSD (relative standard deviation, %) of retention times and peak arrays were used as the measure of precision. The method of precision was evaluated by use of intra-day and inter-day tests. Intra-day experiments were performed by replicate analysis of three aliquots of the same sample on the same day, inter-day tests were performed on three consecutive working days in the same way as intraassay experiments (16). Peak area of each of the extract components was measured three times.

Calibration procedure

Each calibration plot was prepared three times after chromatography of five different concentrations (1, 0.75, 0.5, 0.25, 0.1 mg per 10 mL for all the flavonoids and chlorogenic acids except kaempferol-3-rhamnoglucoside, for which the additional concentration of 0.05 mg per 10 mL. was also

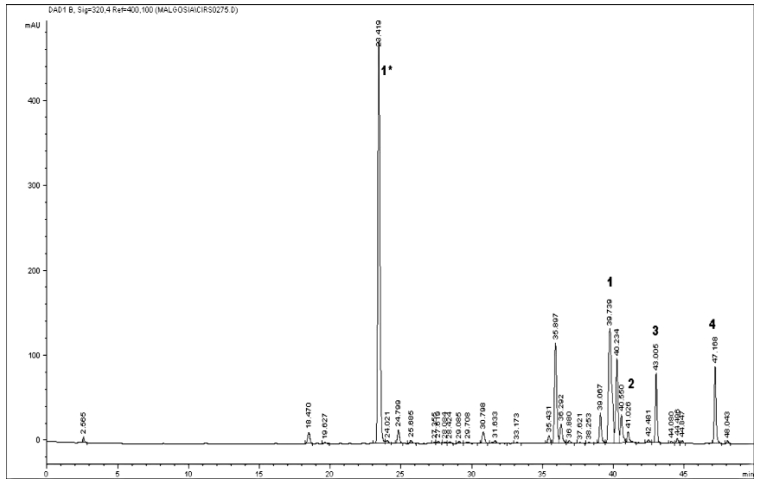

Fig. 1. HPLC chromatogram obtained from analysis of the flower of *C. ligulare* Boiss.
peaks: 1*. chlorogenic acid, 1. apigenin 7-glucoside, 2. kaempferol 3-rhamnoglucoside, 3. kaempferol 3-glucoside, 4. apigenin

used). Quantification was performed by comparing the chromatographic peak areas for the external standard. The calibration plots were characterized by their regression coefficients, the slopes of plots *(b)* and the intercepts of the plots with the *y*-axis *(a)*. Calibration equations for flavonoids were: apigenin 7-glucoside y = 487.26x – 355.12 R^2 = 0.992; apigenin y = 966.45x – 739.91 R^2 = 0.9923; kaempferol 3-rhamnoglucoside y = 157.51x – 144.64 R^2 = 0.9917; and for phenolic acid: chlorogenic acid y = 646.75x – 629.77 R^2 = 0.9919.

RESULTS AND DISCUSSION

Calibration plots for the phenolic acids were highly linear ($R^2 > 0.991$) in the concentration range 0.05–1.00 mg per 10 mL (n = 3). The obtained methanol extracts proved to be very rich in flavonoids, some of which have not been identified. Therefore, more research is required. In the investigated inflorescences and aerial parts of leaves of *Cirsium decussatum* Janka, *Cirsium eriophorum* (L.) Scop., *Cirsium ligulare* Boiss. qualitative HPLC

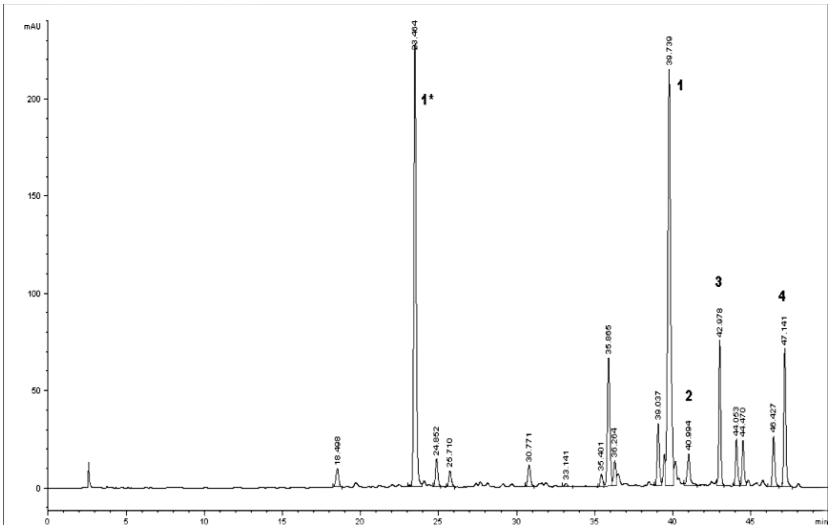

Fig 2. HPLC chromatogram obtained from analysis of the flower of *C. eriophorum* (L.) Scop.
peaks: 1*. chlorogenic acid, 1. apigenin 7-glucoside, 2. kaempferol 3-rhamnoglucoside, 3. kaempferol 3-glucoside, 4. apigenin

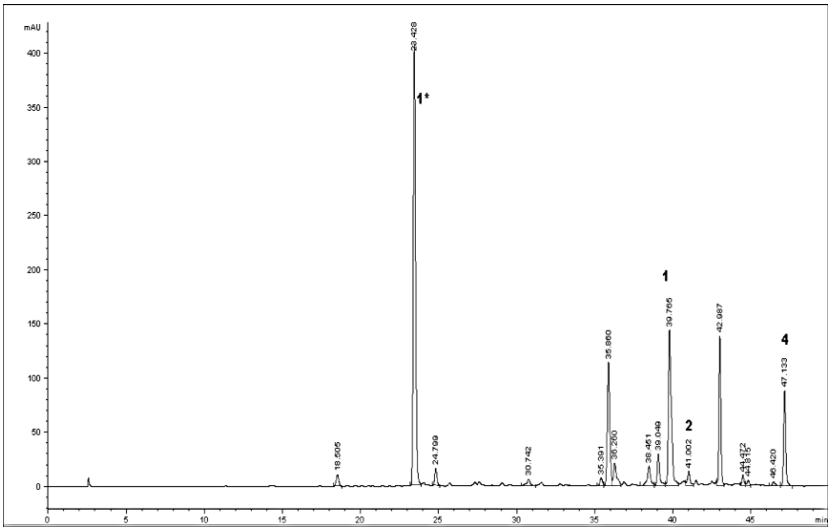

Fig 3. HPLC chromatogram obtained from analysis of the flower of *C. decussatum* Janka.
peaks: 1*. chlorogenic acid, 1. apigenin 7-glucoside, 2. kaempferol 3-rhamnoglucoside, 4. apigenin

analysis was performed for selected identified compounds. Flavonoids, such as apigenin 7-glucoside, kaempferol 3-glucoside, kaempferol 3-rhamnoglucoside, apigenin, and chlorogenic acid were identified. Because we have not standard of kaempferol 3-glucoside we do not determinate this compound quantitatively.

Standard deviation was calculated for all the results, which allows to claim that the results are statistically significant. HPLC analysis of extracts enabled the identification of four flavonoids and one phenolic acid. Typical chromatograms obtained from the extracts from inflorescences are shown in Figures 1–3. The analysis of methanol extracts of three inflorescences and leaves from the *Cirsium* genus, as well as their quantitative analysis were carried out for the first time. The concentration of flavonoids was varied in selected investigated inflorescences and leaves. The amounts of all flavonoids and phenolic acid were estimated by HPLC. Chlorogenic acid was found in every fraction studied in different proportions, depending on species and organ. The acid was predominant in the inflorescences, mainly in the flowers of *C. ligulare* where amount was twice higher than in inflorescences of *C. eriophorum* (50.28 [mg/g] and 24.92 [mg/g], respectively). The leaves of *C. decussatum* were richer source of this acid (47.01 mg/g comparing to *C. ligulare* 26.34 mg/g and *C. eriophorum* – only 9.73 mg/g) (Table 1.) The results of the study suggest that chlorogenic acid may by responsible for the activity and applications of plants of the *Cirsium* genus. This is important because this acid is known for its antioxidant activity (16–18). Nazaruk et al. (19) performed studies concerning simultaneous identification of eight phenolic acids and three flavonods aglycones in Et_2O-fractions of inflorescences and leaves of five species of *Cirsium* genus – *C. arvense, C. oleraceum, C. palustre, C. rivulare and C. vulgare*. The contents of phenolic acids, expressed as caffeic acid, determined by the spectroscopic method with Arnov's reagent, were higher in leaves than in flowers heads.

Flavonoids are also predominant compounds in inflorescences. The main compound is apigenin 7-glucoside present in *C. ligulare* (27.75 mg/g and 23.55 mg/g), respectively, in *C. decussatum* and in very small amount in *C. eriophorum* (3.06 mg/g).

Table 1. Flavonoids' and phenolic acids content (*C*) of extracts obtained from the investigated plants.

		Apigenin	Apigenin 7-glucoside	Kaempferol 3- rhamnoglucoside	Chlorogenic acid
C. ligulare Boiss. inflorescences	*C*	5.29	27.75	3.50	50.28
	± SD	6.96	18.24	3.71	11.70
	RSD	0.13	0.065	0.10	0.02
C. ligulare Boiss. leaves	*C*	0.39	1.91	ND	26.34
	± SD	1.72	6.23		20.41
	RSD	0.43	0.33		0.08
C. decussatum Janka inflorescences	*C*	5.24	23.55	3.63	44.87
	± SD	2.53	3.26	25.32	11.14
	RSD	0.05	0.01	0.70	0.02
C. decussatum Janka leaves	*C*	0.82	3.97	ND	47.01
	± SD	1.26	25.12		4.43
	RSD	0.15	0.63		0.01
C. eriophorum (L.) Scop. inflorescences	*C*	4.24	3.06	4.73	24.92
	± SD	3.72	8.62	5.55	29.59
	RSD	0.09	0.28	0.12	0.12
C. eriophorum (L.) Scop. leaves	*C*	0.34	1.23	ND	9.73
	± SD	5.99	8.12		27.38
	RSD	1.78	0.66		0.28

Each value is the mean (mg per 1 g dry sample) from three replicate analyses. SD = standard deviation, RSD = relative standard deviation, ND = not determined.

The amounts of kaempherol 3-rhamnoglucoside were similar in the investigated inflorescences but was not identified in leaves. Flowers of *C. ligulare* and *C. decussatum* contain the similar amounts of apigenin. In leaves of *C. decussatum* concentration of this flavonoids was higher than in another investigated species. (0.82 mg/g) (Table1). These identified compounds: apigenin-7-glucoside, kaempferol 3-glucoside, kaempferol 3-rhamnoglucoside, apigenin, may by responsible for the activity and applications of plants of the *Cirsium* genus. Flavonoids are of particular interest because of their various pharmacological activities (including antianginal, antihepatotoxic, antimicrobial, antiulcer, spasmolytic, antiallergic, antiinflammatory, antiviral, anticarcinogenic and antioxidant) (1, 8, 15–17).

This is the first report of simultaneous quantification of three flavonoids and one phenolic acid in inflorescences and aerial parts (leaves) in different species of *Cirsium*. In the investigated aerial parts of flowering leaves of *Cirsium decussatum* Janka, *Cirsium eriophorum* (L.) Scop., *Cirsium ligulare* Boiss. have been identified: kaempferol 3-rhamnoglucoside, kaempferol 3-glucoside, apigenin, apigenin 7-glucoside and chlorogenic acid. (Figs. 1–3) The methanol extract is very rich in flavonoids but some of the flavonoids were not identified. Therefore, more research is required. The analysis of methanol extracts as well as their quantitative analysis have been carried out for the first time.

Calibration plots for the phenolic acids were highly linear ($R^2 > 0.991$) in the concentration range 0.05–1.00 mg per 10 mL (n = 3).

Standard deviation was calculated for all of the results leading to a conclusion that the results are statistically significant. The result of our investigation enabled us to establish a simple RP-HPLC method. ASE and SPE proved to be an inexpensive but very efficient methods for rapid isolation, separation and identification of the flavonoids and phenolic acids present in the extracts examined.

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