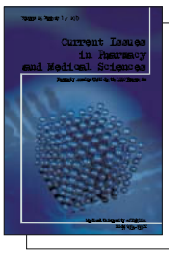

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Current Issues in Pharmacy and Medical Sciences

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The analysis of flavonoids in the flowering herbs of *Carduus acanthoides* L.

MAŁGORZATA KOZYRA*, KAZIMIERZ GŁOWNIAK, MAŁGORZATA BOGUSZEWSKA

Department of Pharmacognosy with Medicinal Plant Unit, Medical University of Lublin, Poland

ABSTRACT

The present paper discusses the analysis of flavonoids in the methanol extract from the flowering herbs of *Carduus acanthoides* (L.), where flavonois were indentified by TLC and SPE HPLC. The quantitative analysis was performed using spectrophotometric Christ-Müller's method. The samples containing flavonoids and those released after acid hydrolysis were investigated by 1D TLC on silica gel and polyamide. After purification by SPE, the samples were also analyzed by RP-HPLC. Apigenin-7-glucoside, luteolin-7-glucoside, kaempferol-3-rhamnoglucoside, kaempferol-3-glucoside, apigenin, luteolin and chlorogenic acid and p-coumaric acid were detected in the fractions of methanol extracts obtained from the plant. This method is very simple for the analysis and suitable for rapid screening of flavonoids in plants.

Keywords: *Carduus acanthoides* L., TLC, RP HPLC, flavonoids

INTRODUCTION

Carduus acanthoides L. belongs to the *Asteraceae* family. In Poland it usually occurs and grows in lowland and in lower mountain positions. The plant is famous for the occurrence of best-known group of secondary metabolites, i.e. flavonoids. The reported biological activity of flavonoids in the investigated plant displays the biological activity, such as larval growth inhibitor, antibacterial and insect attractant [10]. The extract of *Carduus crispus* L. is also present during blood glucose lowering and in analgesic composition [9].

Sterols, triterpenes (β-sitosterol, Δ-5-avenasterol, brassicasterol, campesterol, stigmasterol) and alkaloids (acanthoidyne, acanthoine, ruscopine) are other groups of secondary metabolites found in the *Carduus species*. Acanthoidine, for instance, indicates its hypotensive activity in dogs and human beings [1]. Besides the above mentioned metabolites, *Carduus acanthoides* L. also contains other flavonoids, such as luteolin, luteolin-7-O-galactoside, luteolin-7-O-digalactoside, luteolin-7-O-glucoside, as well as alkaloids, e.g. acanthoidine (I) and acanthoiddine (VI), both showing hypotensive activity in dogs [1,7].

Corresponding author
* Department of Pharmacognosy with Medicinal Plant Unit, Medical University of Lublin, 20-093 Lublin, ul. Chodźki 1, e-mail: mkozyra@pharmacognosy.org

DOI: 10.12923/j.2084-980X/26.1/ a.02

The aim of this paper was to investigate coumarin and flavonoid compounds that occur in *C. crispus* and *C. nutans*. In herbs of both species six coumarins (coumarin, umbelliferone, herniarin, esculetin, scopoletin, esculin) and four flavonoids (cinaroside, apigenine, luteolin, astragalin) were described [20]. Additionally, in *C. nutans* further flavonoids, i.e. kempferol-3-O-α-L-rhamnofuranoside and acacatin 7-β-D-glucopyranoside were identified by chromatography UV IR and NMR spectra. For the first time isorhamnetin, rutin, tilianin, apigenin and luteolin were isolated from dried leaf material with 80% MeOH for 24h and 50% MeOH for further 24h. The obtained fractions were investigated by TLC and separated on polyamide column. The compounds were identified by co chromatography [11,12,20] .

The flavonoids isolated from the methanolic extract of the aerial parts of *C. assoi* were identified by 13 CNMR as kaempferol, apigenin, tricin, luteolin, hispidulin 7-glucoside, luteolin 3'-rhamnoside, kaempferol-3-0-α-L- rhamnoside, kaempferol-3-0-β-D-glucoside [6]. Diosmetin and kaempferol 3-O-α-L-rhamnoside were isolated from the ether extract of the aerial parts of *C. pycnocephalus* and identified using different spectroscopic techniques. Additionally, glucoside, palustroide, kaempferol, apigenin, kaempferol 3,4'dimethyl ether 7-O-glucoside and apigenin-7-O-glucoside were identified [13,14].

Flavonoids are known for their vast occurrence in plants and they are reported to be an important group of substances. This group of compounds occurs in flowers as biological pigments providing colors from red to blue, both in flowers and leaves. They function as stress protectors in plant cells by scavenging reactive oxygen species produced by the photosynthetic electron transport system [16]. Because of their UV-absorbing properties, flavonoids also protects plant from the UV radiation of the sun [18]. Flavonoids reported in *Carduus spp*. display various biological activities: apigenin indicates its anti-inflammatory, antispasmodic, antibacterial and insect attractant UV – pigment activity; apigenin-7-O-neohesperidoside and diosmetin show an antimicrobial activity; hispidulin – cytotoxic activity; luteolin – enzyme inhibitor (α-glucosidase and á-amylase), larval growth inhibitor and antibacterial insect attractant as a UV – pigment activity; narnigenin – an antibacterial activity; quercetin – an antioxidant activity, and larval growth inhibitor [10]. All these compounds are of particular interest because of their various pharmacological activities (including antianginal, antihepatotoxic, antimicrobial, antiulcer, spasmolytic, antiallergic, antiinflammatory, antiviral, anticarcinogenic and antioxidant) [2,3,8,9,13].

Chromatographic methods are one of the most popular techniques applied in the analysis of natural mixtures. In this work, the separation of the components of the herbs of *Carduus acanthoides* L. in methanolic extract was performed using a variety of adsorbents, binary and ternary mobile phases TLC. The obtained extracts containing flavonoids were investigated by 1D TLC on silica gel and polyamide and SPE-HPLC. Quantitative analysis of flavonoids was carried out using a spectrophotometric method (i.e. Christ-Müller's method: Polish Pharmacopoeia VI, 2002) [5].

EXTRACTION AND CHROMATOGRAPHIC ANALYSIS

The investigation was performed on dried and powdered *Carduus acanthoides* L. (127g) collected in August 2010 near Lublin.

Plant material was dried at room temperature, powdered, macerated (24 h) and extracted with $CHCl_3$ in a Soxhlet apparatus. After chloroform extraction, the material was extracted exhaustively for 48 h with methanol (Polish Reagents, Gliwice, Poland) at 78°C. The obtained extract was concentrated under reduced pressure. The dry extract was dissolved in H_2O and heated for 24h. After the separation of ballast substances by filtration, a successive extraction was performed with Et_2O –fraction F_1, EtOAc-fraction F_2, n-BuOH– fraction F_3 and fraction after acid hydrolysis – F_4. After distilling off the solvents, the residues were diluted with MeOH. The Et_2O, EtOAc, n-BuOH extracts were investigated by TLC and SPE-HPLC. Several standards of flavonoids were used: apigenin-7-glucoside, kaempferol-3-rhamnoglucoside, kaempferol-3-glucoside, luteolin-7-glucoside, apigenin, luteolin. All flavonoids standards were purchased from Sigma – Aldrich (Steinheim, Germany).

One-dimensional TLC (1D-TLC) was performed on 200 x 100 x 0.1 mm silica gel plates, and 200 x 200 x 0.1 mm polyamide plates (E. Merck, Darmstadt, Germany). Each fraction and standard was spotted on 1D TLC plates and the plates were developed in horizontal DS chambers (CHROMDES, Lublin, Poland) using the following mobile phases: silica gel: ethyl acetate-formic acid-water (18:1:1, v/v/v), polyamide plates: ethyl acetate- formic acid- acetic acid - water (100:10:10:13, v/v/v/v). The obtained chromatograms were observed under UV light at λ= 366 nm and the light before and after derivatization after treatment with reagents: 1% methanolic solution of Naturtoffreagenz A; NP (Carl Roth Gmbh Karlsruhe) and 5% ethanolic solution Polietylenoglikol 400; PEG (Carl Roth Gmbh Karlsruhe), and monitored by video documentation camera Camag Reprostar 3, Switzerland (Table 1-2).

Table 1. Flavonoids in the extracts of *Carduus acanthoides* L. 1D TLC

Standarts			*Carduus acanthoides* L.			
flavonoids	R_f	Colour UV 254	F_1	F_2	F_3	F_4
apigenin-7-glucoside	0.68	yellow /green	++	+	+	-
kaempferol-3-rhamnoglucoside	0.66	yellow/ orange	++	++	-	-
kaempferol -3- glucoside	0.64	yellow	++	++	-	-
luteolin -7-glucoside	0.54	orange	++	++	+	-
apigenin	0.29	Yellow/ green	++	-	-	+/-
luteolin	0.26	orange	++	-	-	+/-

Average R_F values of identified flavonoids separated by 1D TLC with mobile phase ethyl acetate-formic acid-acetic acid-water (100:10:10:13, v/v/v/v).
Stationary phase: Polyamide
Derivatization: NP/PEG.
F_1 – diethyl ether extract; F_2 – ethyl acetate extract; F_3 – n-butanol extract; F_4 – extract after hydrolysis with 2M HCL.

Table 2. Flavonoids in the extracts of *Carduus acanthoides* L. 1D TLC.

Standarts			*Carduus acanthoides* L.			
flavonoids	R_f	Colour UV 254	F_1	F_2	F_3	F_4
apigenin-7-glucoside	0.38	yellow /green	+	+	+	-
kaempferol-3- rhamnoglucoside	0.18	yellow/ orange	+	+	+/-	-
kaempferol-3-glucoside	0.35	yellow	+/-	+/-	-	-
luteolin-7-glucoside	0.31	orange	++	+	+	-
apigenin	0.94	yellow/ green	+	-	-	+/-
luteolin	0.88	orange	+	-	-	+/-

Average R_F values of identified flavonoids separated by 1D TLC with mobile phase ethyl acetate-formic acid-water (18:1:1, v/v/v/v).
Stationary phase: Silica gel
Derivatization: NP/PEG.
F_1 – diethyl ether extract; F_2 – ethyl acetate extract; F_3 – n-butanol extract; F_4 – extract after hydrolysis with 2M HCL

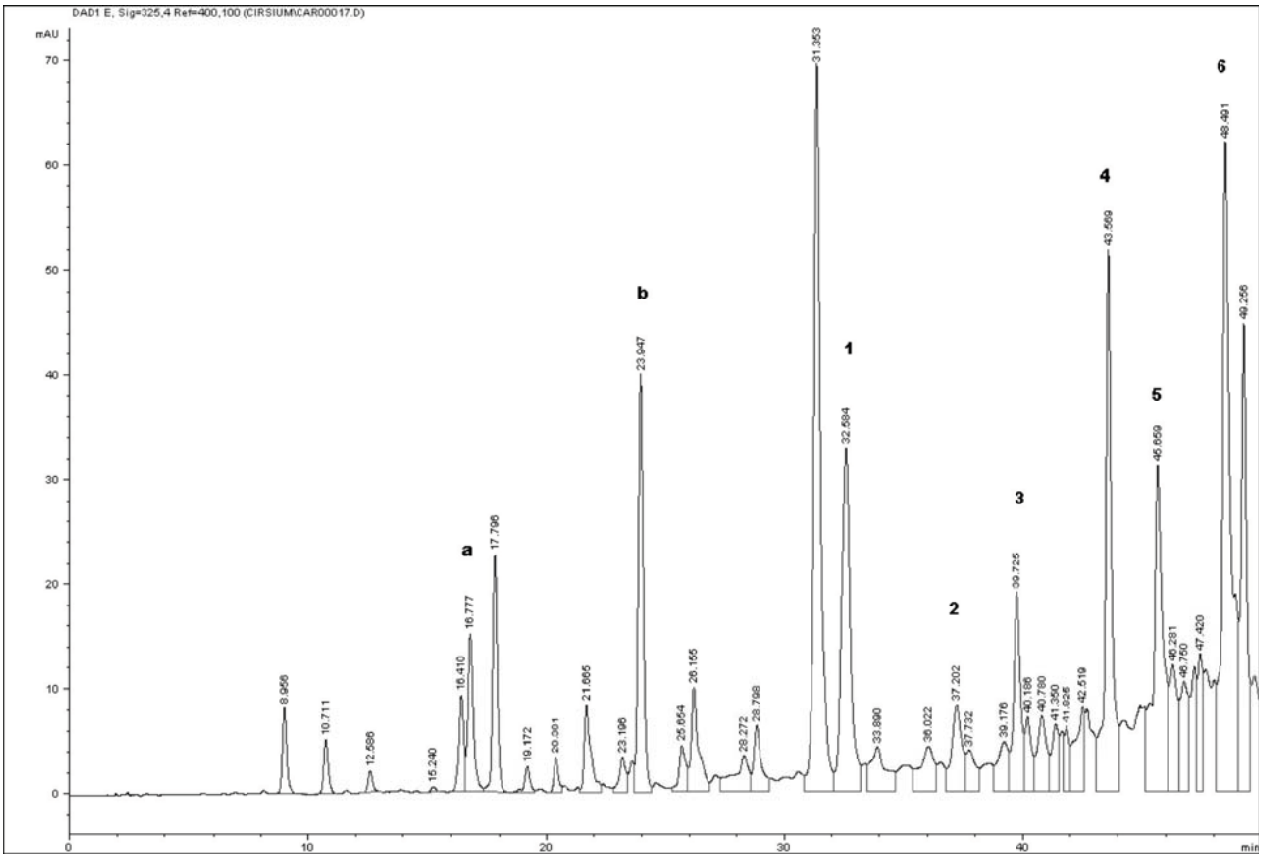

Fig. 1. LC chromatogram of extract F1- diethyl ether extract of the flowering herb of *C. acanthoides*.
Peaks: 1. luteolin-7-glucoside, 2. apigenin -7-glucoside, 3. kaempferol 3-glucoside, 4. kaempferol -3- rhamnoglucoside, 5. luteolin 6. apigenin, a-chlorogenic acid, b- p-coumaric acid

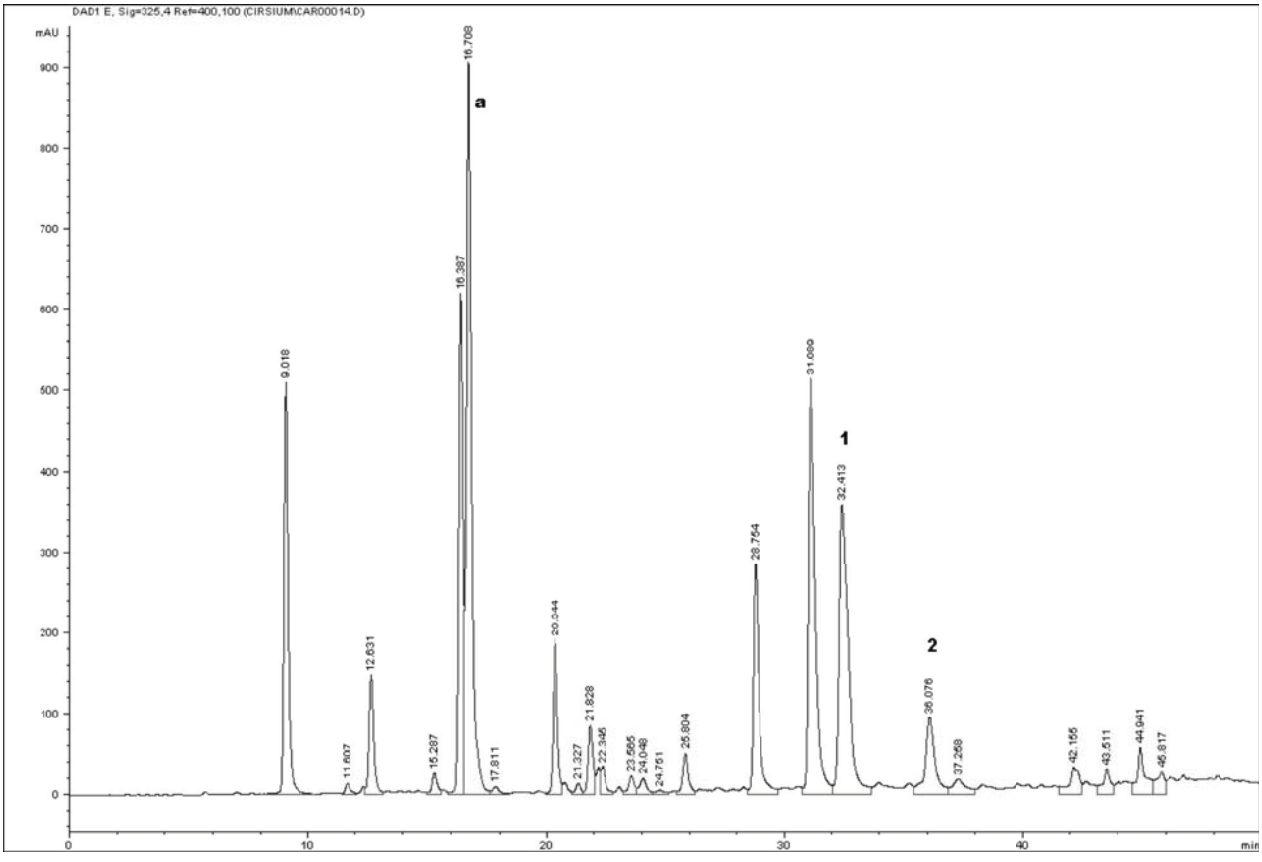

Fig. 2. LC chromatogram of extract F2- ethyl acetate extract of the flowering herb of *C. acanthoides.*
Peaks: 1. luteolin-7-glucoside, 2. apigenin -7-glucoside, a-chlorogenic acid

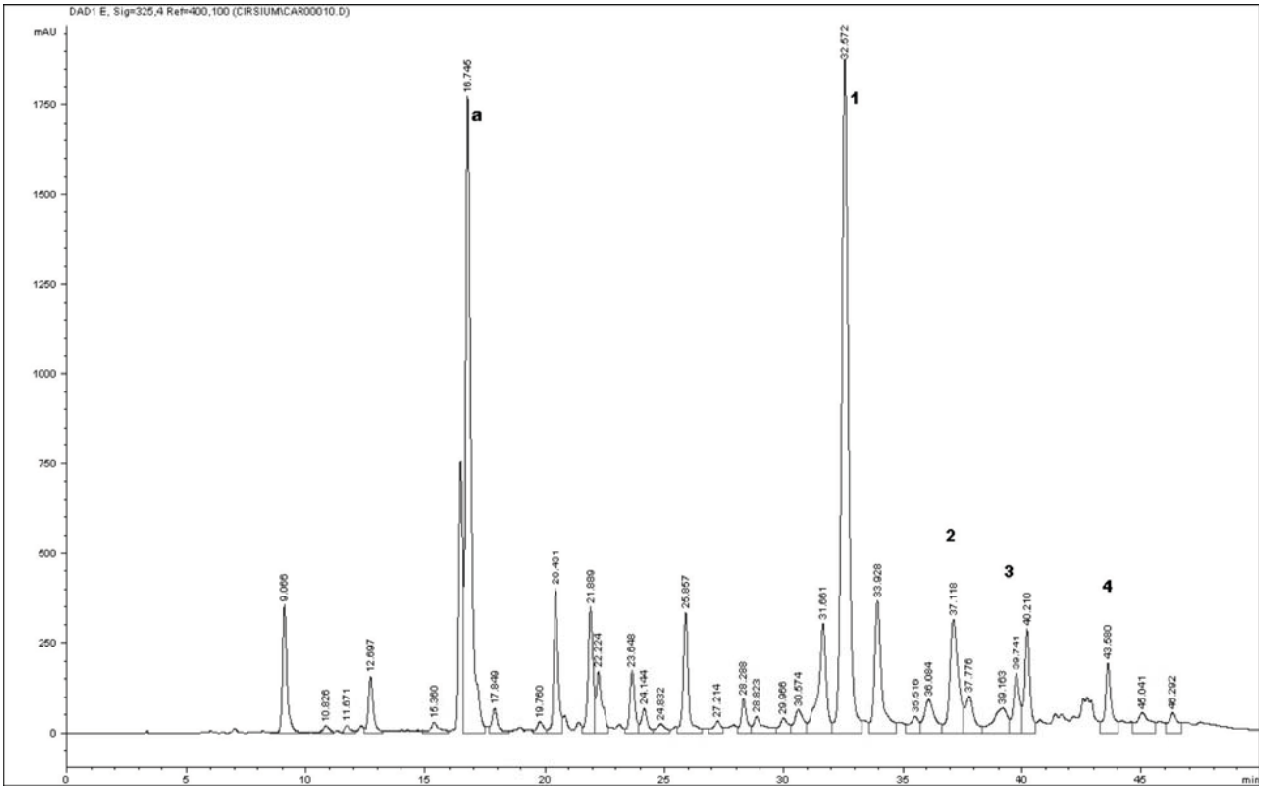

Fig. 3. LC chromatogram of extract F3 – n-butanol extract of the flowering herb of *C. acanthoides*.
Peaks: 1. luteolin-7-glucoside, 2. apigenin -7-glucoside, 3. kaempferol 3-glucoside, 4. kaempferol -3- rhamnoglucoside, a-chlorogenic acid

Samples containing flavonoids were purified from ballasts and chlorophylls by SPE before HPLC analysis. The samples were evaporated to dryness, dissolved in 30% aqueous methanol and applied to octadecyl BakerBond SPE microcolums (500 mg, 3 mL, J.T. Baker), previously activated with 10 mL methanol and then 10 mL water. Flavonoids were obtained by the elution of the columns with 10 mL water-methanol, 30:70 under reduced pressure (SPE-12G chamber, Baker USA). After the purification by SPE the samples were analyzed by RP-HPLC on a 250 x 4.6 mm i.d.; d_p= 5 μm Hypersil ODS column, eluted with gradient mobile phase prepared from 1% aqueous acetic acid (component A) and methanol (component B) (v/v). The gradient was as follows: 0 min 10% B in A; 2 min 10% B in A; 8 min 15% B in A; 25 min 35% B in A; 35 min 40% B in A; 45 min 60% B in A, 47 min 60% B in A. A Hewlett-Packard model 1100 Liquid Chromatograph equipped with a 20 μL sample injector (Rheodyne) and a variable wavelength DAD detector were used. Chromatography was performed at 25°C and the flow rate was 1 mL/min. The identity of compounds examined was performed by the comparison of retention times (t_R) and UV spectra with standard substances purchased from Sigma and analyzed under the same conditions. The qualitative analysis was performed. The retention times were compared with those of standards, using UV spectra as a comparative parameter (Fig. 4). The qualitative determination was performed at the wavelength of maximum absorption of flavonoids. The quantitative determination was performed at the wavelength of maximum absorption of flavonoids and chlorogenic acid – 320 nm.

Quantitative analysis of flavonoids was made by Christ-Muller's method. The absorbance measurement was carried out using UV-VIS spectrophotometer (Helios beta, Warsaw, Poland), λ= 425 nm. All solvents for analytical grade were purchased from POCH (Gliwice, Poland). The content of the flavonoids was determined for quercetin.

RESULTS AND DISCUSSION

As shown in Table 1-2, the use of 1D TLC in the analysis of fractions F_1, F_2, F_3 and F_4, the following flavonoids were identified: apigenin-7-glucoside, luteolin-7-glucoside, kaempferol-3-rhamnosideglucoside, kaempferol-3-glucoside, apigenin, luteolin in diethyl ether extracts F_1, apigenin-7-glucoside, luteolin-7-glucoside, kaempferol-3-rhamnosideglucoside, kaempferol-3-glucoside in ethyl acetate extract F_2, apigenin-7-glucoside, luteolin-7-glucoside in n-butanol extract F_3 and apigenin, luteolin in extracts after acid hydrolysis F_4.

The use of different mobile phases in TLC method allowed for separation of flavonoids in fractions F_1-F_4 obtained from the flowering herbs of *Carduus acanthoides*. The obtained flavonoids differed from one another.

HPLC confirmed the presence of the compounds in the extracts obtained from *Carduus acanthoides* L. (Fig. 1-3).

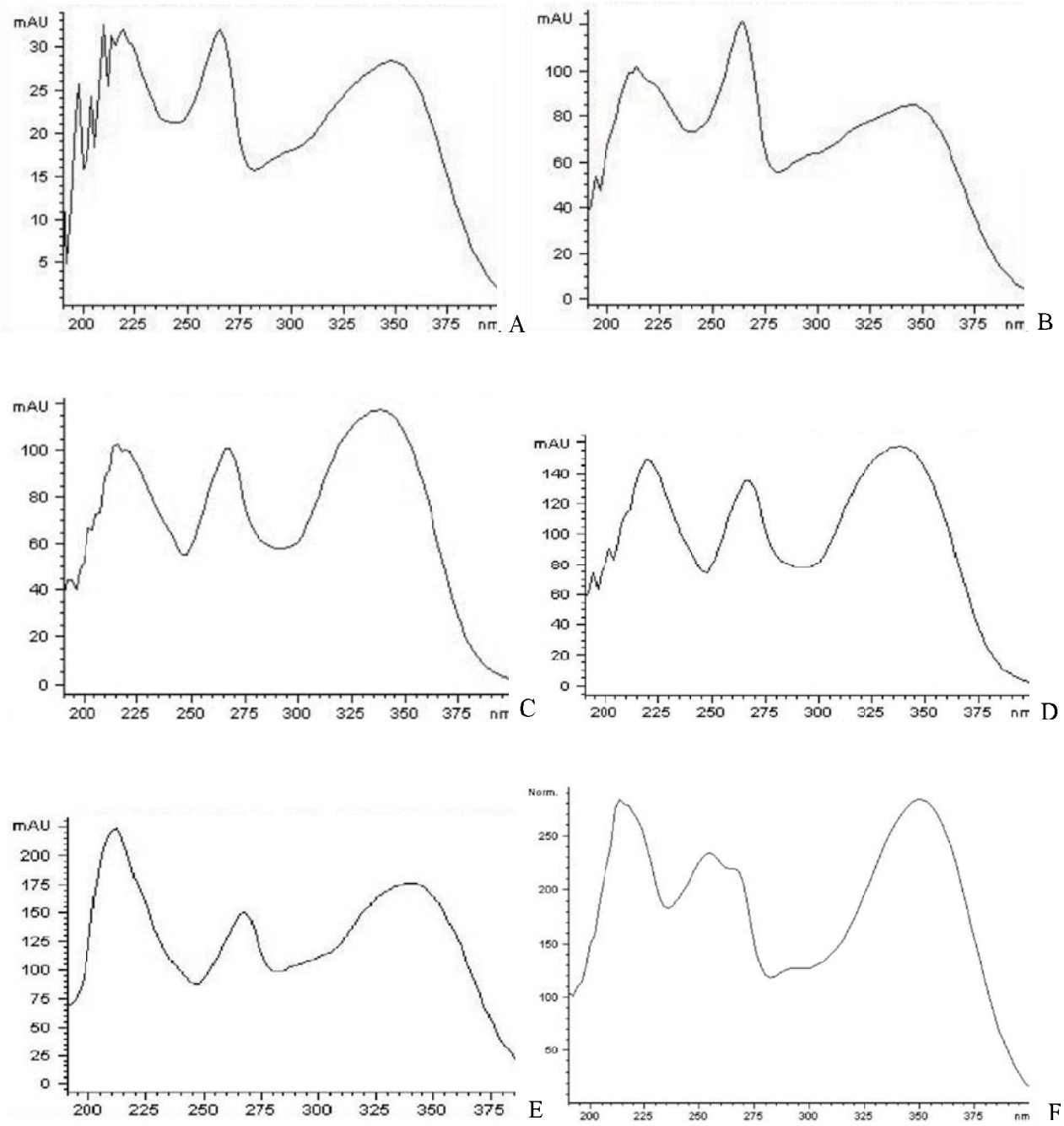

Fig. 4. UV spectra (λ=200–400 nm) of the compound and standards, present in the methanolic extract from flowering herbs scanned with DAD.
A – kaemferol-3-rhamnoglucoside, B – kaemferol 3-glucoside, C – luteolin-7-glucoside, D – apigenin-7-glucoside, E – apigenin, F – luteolin

Slight differences in the profiles of flavonoids were observed using two chromatographic methods.

Silica gel and polyamide are the best sorbents for the separation of flavonoids. Three- step development gives better separation on polyamide. In the flowering herbs the following flavonoids were detected: apigenin-7-glucoside, luteolin-7-glucoside, kaempferol-3-rhamnoglucoside, kaempferol-3-glucoside, apigenin, luteolin. The isolation and separation of natural compounds from plants (including phenolic compounds) is an important analytical problem in phytochemistry. Standard procedures based on TLC still play an important role in the isolation, purification and simple identification such as the fingerprint of phenolic compounds [2,4,15,17,19,21]. The extraction of flavonoids from plant material and their further purification for SPE-HPLC analysis is usually a complex procedure because of the presence of various nonpolar ballast compounds in biological extracts (e.g. chlorophylls, oils, sterols etc.), which can cause damage of analytical columns and interfere with the process of chromatographic determination. Therefore, Solid Phase Extraction, a popular procedure used for isolation, purifi-

cation and preconcentration of organic compounds present in biological material had been used before HPLC was applied [4].

In the investigated flowering herbs of *Carduus acanthoides* L. qualitative HPLC analysis was performed for selected identified compounds. Flavonoids, such as apigenin-7-glucoside, luteolin-7-glucoside, kaempferol-3-rhamnoglucoside, kaempferol-3-glucoside, apigenin, luteolin and chlorogenic acid and p-coumaric acid were identified.

By means of SPE – HPLC**,** one-dimensional TLC and quantitative analysis in the flowering herbs of *Carduus acanthoides* L were determined for the first time. The total content (mg $100g^{-1}$of dry material of flavonoids in flowering herbs) amounted to 14 mg.

CONCLUSION

The present paper discusses the analysis of flavonoids obtained from the flowering herbs of *Carduus acanthoides* L. using 1D TLC and RP HPLC methods. For the separation, difference mobile phases and stationary phases were used. All obtained results were satisfactory. The results were confirmed by RP HPLC analysis. It should be stressed that 1D TLC is not only inexpensive but also a suitable method and is therefore often used for rapid separation and identification of flavonoids present in the *Carduus* species extracts.

Literature [1] describes the presence of such flavonoids as luteolin, luteolin-7-O-galactoside, luteolin-7-O-digalactoside, luteolin- 7-O-glucoside in the investigated plant. In our work luteolin, luteolin- 7-O-glucoside and additionaly apigenin-7-glucoside, kaempferol-3-rhamnoglucoside, kaempferol-3-glucoside, apigenin and chlorogenic acid and p-coumaric acid were detected. In addition, the quantitative analysis in the flowering herbs of *Carduus acanthoides* L was determined for the first time. The total content (mg $100g^{-1}$of dry material of flavonoids in flowering herbs) amounted to 14 mg.

The identified compounds indicate their therapeutically activities. Luteolin and apigenin, among others, were found to be strong inhibitors of IL-4 and IL13 production basophiles. Due to this activity, the flavonoids mentioned may ameliorate allergic symptoms or prevent the onset of allergic diseases. These compounds display a central nervous system activity with anxiolytic – like effects [3,8].

Moreover, literature [10] discusses such flavonoids as luteolin, quercetin, galactoside and glucoside luteolin that occur in *Carduus acanthoides* L. They are are known as enzyme inhibitor (α- glucosidase and α-amylase), larval growth inhibitor, antibacterial, insect attractant as UV-pigment.

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