HPLC-DAD-ESI/MS ANALYSIS OF ARNICA TM CONSTITUENTS

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Abstract: The herbal drugs obtained from *Arnica montana* flowers are known for their anti-inflammatory activity. However, there is no detailed data on chemical constituents of the whole *Arnica* plant. HPLC-DAD-ESI/MS analysis of the whole *Arnica montana* plant (*Arnica* TM), used in the preparation of allopathic and homeopathic medicinal products, has been conducted. The qualitative and quantitative analysis comprised phenolic acids, flavonoids and sesquiterpene lactones – helenalin and dihydrohelenalin esters identified earlier in *Arnica* flowers. The analyzed material showed to be a rich source of caffeoylquinic acid (CQA) derivatives, especially 3,5-di-O-caffeoylquinic acid. According to the literature data, the anti-inflammatory activity of extracts from the whole *Arnica montana* plant can be due to CQA presence as dominating compounds.

Keywords: polyphenols, *Arnica*, sesquiterpene lactones, simple phenols

Arnica flowers have been known for centuries as a herbal remedy, originally applied in phytotherapy internally (1, 2), and currently only externally (1-4). In many *in vitro*, *in vivo* and clinical studies, their anti-inflammatory activity has been confirmed, mainly due to the sesquiterpene lactone – helenalin, present in free form as well as ester derivatives (3, 5-15). The sesquiterpene lactones of *arnica* inhibit the synthesis of prostaglandins by inactivating the prostaglandin synthetase enzyme and cause a decrease in the biochemical parameters associated with inflammatory processes (3, 5-11). The mechanism of action at the molecular level of helenalin comprises the inhibition of activation of transcription factors such as NF- χ B, NF-AT stimulating the transcription of pro-inflammatory proteins, among others, cytokines IL-1, IL-8, TNF- α , acute phase proteins, COX, and iNO- synthetase (7, 9-11).

The chemical composition of *arnica* flowers is complex and includes, in addition to sesquiterpene lactones (12-15) also flavonoids (14-18) and phenolic acids (19-21), in particular derivatives of caffeoylquinic acids (CAQs) (20, 21). The role of the latter compounds in the biological activity of *arnica* flowers is not fully recognized. Some mono-CQA isomers have an affinity to protect against H₂O₂-induced apoptosis in PC12 cells by suppressing the mitochondrial membrane depolarization caused by

oxidative stress (22). Additionally, it was reported that 5-CQA and cynarin had a protective effect against t-BOOH-induced ROS generation in HepG2 cells (23). It was also stated, that 5-CQA prevents inflammatory responses in IL-1 α -stimulated human SW-1353 chondrocytes, a model for osteoarthritis (24, 25). *Arnica* flower flavonoids are involved in the anti-inflammatory action of this plant raw material, inhibiting the activity of pro-inflammatory mediators COX, 5-LOX, and hyaluronidase and elastase enzymes (8, 10).

Literature data on the chemical composition of the *Arnica montana* includes mainly information on the chemical constituents of dried (13-15, 18) or fresh flowers (12). In addition, the composition of sesquiterpene lactones in the leaves (26) was recognized, and phenolic acid complex and essential oil constituents were studied in the roots (27). There is no detailed data on the active compounds of the whole plant as raw plant material for the preparation of medicinal products – allopathic or homeopathic. In very large dilutions, tincture or liquid *arnica* extracts are used internally in homeopathy (3). Nowadays, the basic tools in the study of the composition of biologically active compounds in plants are hyphenated techniques, mainly high-performance liquid chromatography coupling with selective detectors DAD and MS (28).

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The aim of the study was to analyze by use of HPLC-DAD-ESI/MS elaborated methods the chemical composition of the extract from fresh whole *Arnica montana* L. plant prepared according to the homeopathic procedure (tincture mother TM).

MATERIALS AND METHODS

Chemicals and reagents

Standard compounds of apigenin, kaempferol, quercetin, isoquercetin, hyperoside, chlorogenic acid, caffeic acid, ferulic acid were obtained from Fluka (St. Gallen, Switzerland). Luteolin 7-*O*-glucoside, apigenin 7-*O*-glucoside, luteolin, astragalin, galangin, tectochrysin, genkwanin, isorhamnetin, quercetin 3-glucuronide, kaempferol 3-*O*-glucuronide from Extrasynthèse (Lyon, France). Hispidulin, pectolinarigenin were obtained from PhytoLab (Vestenbergsgreuth, Germany). Naringenin, isochlorogenic acid and p-coumaric acid were obtained from Koch-Light (Colnbrook, Great Britain). Chrysin and vanillic acid were obtained from Sigma-Aldrich (Saint Louis, USA). Helenalin was purchased from Abcam (Cambridge, Great Britain). Cynarin originated from the standard collection of the Department of Pharmacognosy of the Medical University of Gdańsk (Poland). Analytical-grade chloroform and methanol were obtained from POCH S.A. (Gliwice, Poland). Analytical-grade formic acid (89-91% purity) was purchased from Merck (Darmstadt, Germany). HPLC-grade acetonitrile (LC/MS) Lichrosolv was purchased from Sigma-Aldrich (Steinheim, Germany). Demineralized water was prepared by using Millipore Water Purification System (Molsheim, France).

Plant material and sample preparation

Arnica TM (series M6100663) was obtained from Boiron (France). *Arnica* TM tincture was pre-

pared from fresh *Arnica montana* L. plants (whole plants and parts of its roots) in proportion 1 : 10 (raw plant material : 96% ethanol). The water content in collected fresh plant material was 75.4%. *Arnica* plants were selected by qualified botanists and harvesters. Harvesting is regulated and confined to several mountainous regions in France (Vosges, Alps, Massif Central, Pyrenees, etc.) and Germany (Hesse, Bavaria). It takes place at the beginning of the flowering season in June or July depending on the altitude. The samples of *Arnica* TM were prepared by evaporating the solvent from a volume of 1 mL of the tincture and then dissolving the dry residue in 1 mL of methanol. The methanol solution was filtered through membrane filter prior to chromatographic analysis.

LC system

HPLC analysis was performed using an LC system by Shimadzu (Kyoto, Japan) consisting of two pumps LC-20AD, semi-micro mixer, CBM-20A system controller, CT0-20AC column thermostat, SIL 20AC_{XR} autosampler, UV-vis detector (Diode Array Detector) SPD-M20A, LCMS-2020 mass spectrometer with ESI ionization. Data were acquired and processed by LabSolution software.

HPLC-DAD analysis of simple phenols and polyphenols

Separation was carried out on a Kinetex C-PFP column (2.6 µm, 4.6 × 100 mm) (Phenomenex, USA), using gradient elution according to the program I (% B in A) : A – H₂O : HCOOH 100 : 0.1 (v/v), B – CAN : H₂O : HCOOH 50 : 50 : 0.1 (v/v/v) : 0 min – 12% B, 10 min – 20% B, 35 min – 43% B, 60 min – 100% B, 64 min – 100% B, 66 min – 12% B, 76 min – 12% B at flow rate of mobile phase 0.8 mL/min, column temperature – 20°C, injection volume: 1 µL (qualitative analysis), 6 µL and 1 µL of

Table 1. Validation parameters for HPLC determination of phenolic acids, flavonoids and sesquiterpene lactones in *Arnica* TM tincture.

Chemical compound	Range µg/mL	Calibration curve	R ²	LOD [µg/mL]	LOQ [µg/mL]
Chlorogenic acid	6.25 - 100 100 - 400 400 - 1000	y = 2043.5 x + 354.75 y = 1559.3 x + 50603 y = 2580.5 x - 306286	0.9993 0.9992 0.9907	0.702	2.340
Luteolin	3.125 - 100	y = 1525.3 x - 4222	0.9989	2.757	9.191
Quercetin	3.125 - 100	y = 5029.6 x - 6925.1	0.9997	0.372	1.240
Helenalin*	3.125 - 100	y = 3256.4 x + 12005	0.9982	0.638	2.126
Helenalin**	3.125 - 50	y = 2614.2 x + 341.29	0.9989	1.267	4.223

* HPLC determination of helenalin and dihydrohelenalin esters; ** HPLC determination of helenalin

Figure 1. HPLC separation of Arnica TM tincture: Kinetex C-18 and Kinetex PFP columns using gradient program I, UV λ -330 nm.

tincture diluted with methanol in proportion 1:4 (quantitative analysis). UV detection at λ -330 nm.

HPLC-DAD analysis of sesquiterpene lactones.

Helénalin

The analysis was carried out on a Kinetex C-18 column (2.6 μ m, 4.6 $\times$ 100 mm) (Phenomenex, USA) using a gradient elution according to the program II (% D in C): C – H₂O : MeOH 45 : 5 (v/v), D – MeOH: 0 min – 17% D, 20 min – 17% D, 25 min – 100% D, 35 min – 100% D, 40 min – 17% D, 55 min – 17% D at flow rate of mobile phase 0.8 mL/min, column temperature – 30°C, injection volume: 3 μ L, UV detection at λ -225 nm.

Helénalin and dihydrohelenalin esters

The analysis was carried out on two Kinetex C-18 columns (2.6 μ m, 4.6 $\times$ 100 mm) connected in series using a gradient elution according to the program III : C – H₂O : MeOH 50 : 50 (v/v), D – MeOH 0 min – 0% D, 51 min – 100% D, 70 min – 100% D, 71 min – 85% D at a mobile phase flow rate of 0.8 mL/min, column temperature 30°C and UV detection λ -225 nm. The injection volume was 6 μ L.

ESI-MS conditions

Mass spectra were acquired operating in positive (PI) ion modes. A full scan (range m/z 150–800 for simple phenols and polyphenols, m/z 250–400

for sesquiterpene lactones) and SIM (selected ion monitoring) techniques were used for monitoring specific signals. Operating parameters: interface voltage 5.0 kV, detector voltage 1.25 kV, heat block 200°C, DL temperature 250°C, nebulizing gas flow (N₂) 1.5 L/min, drying gas flow 15 L/min.

Quantitative analysis and method validation

The HPLC methods developed for the purposes of quantitative analysis were validated by determining calibration curves, linear regression, limit of quantitation (LOQ), limit of detection (LOD), repeatability and recovery of the analyzed compounds, which were estimated according to Food and Drug Administration, Center for Drug Evaluation and Research Guidelines (29). Calibration curves were determined for the standard compounds. Linearity for the working concentrations of the standard compounds was evaluated by determining the correlation coefficient. Stock solutions of standard compounds were diluted in methanol (1 mg/mL) and the regression curves were determined basing on the analysis of plot of peak area for a concentration of compounds from 1 mg/mL to 3.125 µg/mL. LOQ and LOD were determined as the concentration of the standard compound equaling 10 × and 3 × of the signal-to-noise ratio, respectively. Calibration curves, correlation coefficients, LOQ and LOD values are presented in Table 1.

The intra- and inter-day repeatability of the methods developed were evaluated by analyzing continuous injections of the same sample six times

per day and once per day for six consecutive days respectively, and determined according to the relative standard deviation (RSD). The intra-day precision of simple phenols and polyphenols determination ranged from 0.28 to 5.05%, and the inter-day precision from 0.76 to 5.35%. The intra-day precision of helenalin determination was 2.68% and the inter-day precision was 6.11%. The intra-day precision of helenalin and dihydrohelenalin esters determination was 2.83% and the inter-day precision was 8.97%. Recovery was determined by adding known amount of standard compound corresponding to 25% and 50% (helenalin) and 75%, 125% (selected simple phenols and polyphenols standards) of its concentration in the sample, and calculating the percentage of recovery from the median sum of compound in the plant material as well as the added quantities of standard compound. The recovery for the helenalin was 90.2 to 96.2% and for simple phenols and polyphenols ranged from 92.3 to 112.2%.

RESULTS

In the preliminary chromatographic experiments, HPLC separation conditions were optimized dependently on a group of analyzed secondary metabolites by selection of two types of columns, namely Kinetex C-18 column (2.6 µm, 4.6 × 100 mm and 2.6 µm, 2.1 × 100 mm) and Kinetex PFP column (2.6 µm, 4.6 × 100 mm) (Fig. 1). A number of mobile phases containing a mixture of H₂O : CAN : HCOOH and H₂O : ACN or H₂O : MeOH

Figure 2. Chromatograms HPLC of Arnica TM tincture: Kinetex PFP column (2.6 µm, 4.6 × 100 mm), gradient program I, UV λ-330 nm.

Table 2. Polyphenols and simple phenols identified by HPLC-DAD-ESI/MS Arnica TM tincture (Boiron); Kinetex C-PFP column (2.6 μ m, 4.6 $\times$ 100 mm), gradient program I, UV λ -330 nm.

Peak number	Compound	t _R (min)	UV λ_{max} (nm)				ESI-MS (m/z) [M+H] ⁺ , [Ag+H] ⁺	Correlation with standard
1	Isomer of caffeoylquinic acid	4.6	234	243sh	296sh	326	355	
2	3,4-dihydrobenzoic acid (protocatechic acid)	6.0	259	292			155	0.8591
3	5- <i>O</i> -caffeoylquinic acid (chlorogenic acid)	10.5	234	243sh	296sh	324	355	0.943
4	Caffeic acid	12.2	232	241sh	294sh	322	181	0.9981
5	1,3-di- <i>O</i> -caffeoylquinic acid (cynarin)	16.6	230	243sh	299sh	325	517	0.9813
6	Patuletin 3- <i>O</i> -glucoside	24.5	256	269sh	348		495, 333	
7	Patuletin 3- <i>O</i> -glucuronide	24.7	256	267sh	347		509, 333	
8	Quercetin-3- <i>O</i> -glucoside (isoquercetin)	25.2	255	265sh	310sh	352	465	0.9813
9	Quercetin 3- <i>O</i> -glucuronide	25.5	255	264sh	310sh	353	479	
10	Eupafolin 7- <i>O</i> -glucoside	27.8	269	296sh	330		479, 317	
11	Isomer of dicaffeoylquinic acid	28.1	234	246sh	298sh	325	517	
12	Eupafolin 7- <i>O</i> -glucuronide	28.3	269	334			493, 317	
13	Kaempferol 3- <i>O</i> -glucoside	28.6	264	300sh	340		449	0.9953
14	Kaempferol 3- <i>O</i> -glucuronide	29.1	260	300sh	347		463	
15	3,5-di- <i>O</i> -caffeoylquinic acid	29.5	234	246sh	296sh	328	517	
16	1,5-di- <i>O</i> -caffeoylquinic acid	29.9	235	246sh	296sh	329	517	
17	4,5-di- <i>O</i> -caffeoylquinic acid	30.7	235	265sh	300sh	352	517	
18	1-methoxyoxaloyl-3,5-di- <i>O</i> -caffeoylquinic acid	31.6	235	241sh	298sh	329	603	
19	Isomer of di- <i>O</i> -caffeoylquinic acid	33.7	235	242sh	297sh	328	517	
20	Luteolin 3'- <i>O</i> -glucoside	34.0	267	301sh	331		449, 317	
21	Isomer of di- <i>O</i> -caffeoylquinic acid	34.4	230	240sh	297sh,	330		
22	Kaempferol 3- <i>O</i> -acetylglucoside	35.0	264	300sh	345		491	
23	Derivative of caffeic acid	35.5	232	245sh	300sh	330		
24	Tricaffeoylquinic acid	37.2	233	242sh	300sh	325	679	
25	Derivative of caffeic acid	42.1	232	243sh	300sh	329		
26	Derivative of caffeic acid	42.8	233	245sh	300sh	329		
27	6-Methoxyapigenin	44.6	272			333	317	
28	Apigenin	45.3	267	304sh	329		271	0.9231
29	Kaempferol	45.9	253sh	267sh	299sh	341	287	0.9231
30	Derivative of caffeic acid	47.0	235	241sh	307sh	330		

were evaluated. Kinetex PFP column and gradient program I comprising an increasing concentration of CAN : H₂O : HCOOH in H₂O : HCOOH from 12% to 100% within t_G 60 min was selected as providing the best separation of polyphenols and simple phenols present in *Arnica* TM tincture (Figs. 1 and 2).

For separation of triterpene lactones shown to be the most effective Kinetex C-18 column (2.6 μ m, 4.6 $\times$ 100 mm) and two elution programs characterizing by an increase of concentrations of MeOH in a mixture of H₂O : MeOH (50 : 50 (v/v) – program II or 45 : 5 (v/v)- program III) (Figs. 3 and 4).

The results of qualitative and quantitative analyzes obtained under the conditions of developed HPLC-DAD-ESI/MS methods are presented in Tables 2-5. Compounds were identified according to the obtained DAD and ESI spectral data, by comparison with authentic standards (t_R value) and literature data (14-19, 30).

Identification of simple phenols and polyphenols

In the examined plant material, based on the number of separated peaks, 30 compounds were identified as belonging to simple phenols and

polyphenols (Fig. 2 and Table 2). The analyses were carried out using 9 standard compounds from the group of phenolic acids: derivatives of benzoic acid: *p*-hydroxybenzoic, protocatechuic, gallic and vanillic; cinnamic acid derivatives: ferulic and caffeic, phenolic acids of an ester nature, namely: caffeic acid esters of quinic acid (*CQA*): chlorogenic acid (*5-O-CQA*), cynarin (*1,3-O-CQA*), isochlorogenic; and flavonoid compounds: from the group of flavones: luteolin, luteolin 7-*O*-glucoside, apigenin, apigenin 7-*O*-glucoside and from the group of flavonols: kaempferol, astragalin (kaempferol 3-*O*-

Figure 3. Chromatogram HPLC – TIC (+) and UV of sesquiterpene lactones (42-53) from Arnica TM tincture: Kinetex C-18 column (2.6 μ m, 4.6 $\times$ 100 mm), gradient program III, UV λ -225 nm.

Figure 4. Chromatograms HPLC of Arnica TM tincture (A) and helenalin standard (C) and UV spectra of helenalin in Arnica TM – 31 (B) and helenalin standard – S (D): Kinetex C-18 column (2.6 μ m, 4.6 $\times$ 100 mm), gradient program II, UV λ -225 nm.

glucoside), hyperoside (quercetin 3-*O*-galactoside), quercetin, isoquercetin (quercetin-3-*O*-glucoside) and isorhamnetin. Among the used standard compounds in Arnica TM tincture, the presence of cinnamic acid derivatives was confirmed: chlorogenic acid (compound **3**), caffeic acid (**4**), cynarin (**5**), benzoic acid derivatives: protocatechuic acid (**2**), from the flavone group: apigenin (**28**), flavonols: isoquercetin (**8**), kaempferol 3-*O*-glucoside (**13**), kaempferol (**29**) (Table 2). These compounds have been previously identified in *Arnica montana* and *Arnica chamissonis* flowers (14-19, 30). However, the presence of the following compounds from the group of phenolic acids and from the flavonol deriv-

atives, has not been confirmed in the analyzed material, namely: gallic acid, ferulic acid, p-hydroxybenzoic acid, p-coumaric acid, vanillic acid and apigenin 7-*O*-glucoside, quercetin, luteolin, luteolin 7-*O*-glucoside, isorhamnetin and hyperoside (15, 18).

Identification of flavonoids

Using DAD and MS detectors based on a comparison of literature data (16, 17, 30) and UV spectra obtained and *m/z* values of molecular $[M+H]^+$ and fragmentation $[Ag+H]^+$ ions in ESI spectra, in the tested plant material confirmed the presence of a number of flavones (Table 2), namely: eupafolin 7-*O*-glucoside (6-methoxyluteolin 7-*O*-glucoside)

(**10**) (16) and luteolin 3'-*O*-glucoside (**20**) (16). In the flavonols group based on UV spectra and *m/z* values of molecular ions, the following flavonols were identified: quercetin 3-*O*-glucuronide (**9**), kaempferol 3-*O*-glucuronide (**14**) (30) and kaempferol 3-*O*-acetylglucoside (**22**) (17). Compounds **6** and **7** are recognized as patuletin 3-*O*-glycosides (17, 30). The UV spectra of both compounds **6** and **7** were identical, which indicates the same glycosidation position in the flavonoid structure ($\lambda_{\max}$ 256, 269sh, 347 nm). The ESI-MS spectra of both compounds showed the presence of fragmentation ion $[\text{Ag}+\text{H}]^+$ at *m/z* 333 corresponding to the molecular weight of the patuletin (6-methoxyquercetin). In addition, the ESI-MS spectra of compounds **6** and **7** showed the presence of molecular ions $[\text{M}+\text{H}]^+$ at *m/z* 495 for **6** and *m/z* 509 for **7**, indicating that they are glycosidic forms of patuletin, respectively 3-*O*-glucoside patuletin and 3-*O*-glucuronide patuletin. Both compounds have previously been identified in the species *A. montana* and *A. chamissonis* by Merfort et al. (16, 30). The UV spectrum of compound **9** shows the absorption maxima characteristic of quercetin derivatives (31). The UV spectrum of **9** is identical to the spectra of **8** identified as isoquercetin (quercetin 3-*O*-glucoside). Based on the presence of the molecular ion at *m/z* 479, compound **9** was recognized as quercetin 3-*O*-glucuronide (30). Compound **10** was tentatively identified as eupafolin 7-*O*-glucoside (7-*O*-glucoside 6-methoxy-luteolin) taking into consideration the presence of two maxima of absorption in UV spectrum: at 330

nm (I maximum), 269 nm (II maximum) with an shoulder at 296 nm, as specific for flavone with an ortho-dihydroxy group in the side phenyl (31), and observing the presence at *m/z* 479 and *m/z* 317 molecular $[\text{M}+\text{H}]^+$ and fragmentation $[\text{Ag}+\text{H}]^+$ ions, respectively. Until now, the eupafolin 7-*O*-glucoside ester form, namely 6''-*O*-(2-methylbutyryl)-7-*O*-glucoside eupafolin has been only demonstrated in *A. chamissonis* flowers (17, 30).

Compound **22** exhibited UV spectra similar to the UV spectrum of kaempferol 3-*O*-glucoside (**13**). The presence of the molecular ion $[\text{M}+\text{H}]^+$ at *m/z* 491 indicated that in the structure of the compound is acetylglucose. Based on this data, **22** was recognized as kaempferol 3-acetylglucoside, which was previously isolated only from *Arnica chamissonis* flowers (16). In contrast, kaempferol 3-*O*-glucuronide (**14**) identified in *Arnica* TM, was earlier described both in flowers of *A. chamissonis* and *A. montana* (16,18). As with glucuronides and glucosides of patuletin and quercetin, also the retention time of kaempferol 3-*O*-glucuronide (t_R – 29.07 min) was higher compared to the kaempferol 3-*O*-glucoside (t_R – 28.56 min) (Table 2).

The UV spectrum of compound **20** indicates the absence of an ortho-dihydroxy group in the side phenyl, whereas the ESI-MS spectrum has a fragmentation ion $[\text{Ag}+\text{H}]^+$ at *m/z* 317 and molecular ion $[\text{M}+\text{H}]^+$ at *m/z* 449, what may indicate that this compound is a derivative of luteolin with attached sugar unit – glucose at C-3'-OH. Based on this, the compound was tentatively identified as luteolin 3'-*O*-

Table 3. Sesquiterpene lactones identified by HPLC-DAD-ESI/MS *Arnica* TM tincture (Boiron): Kinetex C-18 column (2.6 μm , 4.6 $\times$ 100 mm), gradient program II, UV λ -225 nm (compound 31), Kinetex C-18 column (2.6 μm , 4.6 $\times$ 100 mm), gradient program III, UV λ -225 nm (compounds 32-43).

Peak number	t_R (min)	UV $\lambda_{\max}$ (nm)	$[\text{M}+\text{H}]^+ / [\text{M}+\text{Na}]^+$	Compound
31	17.3	219	263	Helénalin*
32	18.8	210	351	Unknown lactone
33	19.6	212	371	11 α ,13-dihydro-2- <i>O</i> -tigloylflorilenalin
34	20.4	219	307/-	Acetyl-dihydrohelénalin
35	21.8	221	385/-	Unknown lactone
36	22.8	214	369/-	Unknown lactone
37	26.2	219	-/355	Helénalin isobutyrate
38	28.6	223	-/369	Dihydrohelénalin tyglinat
39	34.1	220	-/367+	Helénalin tyglinat
40	37.4	229	-/371	Dihydrohelénalin -2-methylbutyrate
41	40.1	227	-/371	Dihydrohelénalin isovalerat
42	43.5	219	-/369	Helénalin-2-methylbutyrate
43	46.4	220	-/369	Helénalin isovalerat

Table 4. Determined concentrations of identified compounds in *Arnica* TM tincture (Boiron) (mg/100g d.w. and mg/100 mL tincture).

Lp.	Compound	<i>Arnica</i> TM Boiron	
		Amount [mg/100 g d.w.]	Amount [mg/100 mL tincture]
1	Isomer of caffeoylquinic acid	41.5 ± 1.8	4.61 ± 0.20
2	3,4-dihydrobenzoic acid (protocatechic acid)	6.04 ± 0.15	0.672 ± 0.017
3	5-O-caffeoylquinic acid (chlorogenic acid)	41.2 ± 1.1	4.58 ± 0.12
4	Caffeic acid	16.68 ± 0.57	1.857 ± 0.064
5	1,3-di-O-caffeoylquinic acid (cynarin)	12.82 ± 0.39	1.427 ± 0.043
6	Patuletin 3-O-glucoside	7.33 ± 0.14	0.815 ± 0.015
7	Patuletin 3-O-glucuronide	4.41 ± 0.25	0.491 ± 0.028
8	Quercetin 3-O-glucoside (isoquercetin)	5.19 ± 0.11	0.577 ± 0.012
9	Quercetin 3-O-glucuronide	6.91 ± 0.19	0.769 ± 0.021
10	Eupafolin 7-O-glucoside	14.7 ± 1.7	1.64 ± 0.18
11	Isomer of dicafeoylquinic acid	55.0 ± 2.7	6.12 ± 0.30
12	Eupafolin 7-O-glucuronide	7.76 ± 0.41	0.864 ± 0.046
13	Kaempferol 3-O-glucoside	6.56 ± 0.29	0.730 ± 0.032
14	Kaempferol 3-O-glucuronide	7.55 ± 0.13	0.840 ± 0.014
15	3,5-di-O-caffeoylquinic acid	450 ± 13	50.1 ± 1.4
16	1,5-di-O-caffeoylquinic acid	31.5 ± 1.1	3.51 ± 0.12
17	4,5-di-O-caffeoylquinic acid	6.80 ± 0.32	0.757 ± 0.036
18	1-methoxyoxaloyl-3,5-di-O-caffeoylquinic acid	33.7 ± 1.1	3.75 ± 0.13
19	Isomer of di-O-caffeoylquinic acid	37.1 ± 1.3	4.13 ± 0.15
20	Luteolin 3'-O-glucoside	8.69 ± 0.23	0.968 ± 0.026
21	Isomer of di-O-caffeoylquinic acid	5.09 ± 0.09	0.567 ± 0.010
22	Kaempferol 3-O-acetylglucoside	12.00 ± 0.35	1.335 ± 0.039
23	Derivative of caffeic acid	5.35 ± 0.23	0.596 ± 0.026
24	Tricafeoylquinic acid	8.65 ± 0.28	0.962 ± 0.031
25	Derivative of caffeic acid	36.1 ± 1.7	4.017 ± 0.193
26	Derivative of caffeic acid	7.84 ± 0.39	0.872 ± 0.044
27	6-Methoxyapigenin	19.6 ± 1.6	2.178 ± 0.176
28	Apigenin	2.30 ± 0.15	0.256 ± 0.016
29	Kaempferol	0.55 ± 0.01	0.061 ± 0.001
30	Derivative of caffeic acid	*	*
Total flavones		53.0 ± 6.7	5.91 ± 0.44
Total flavonols		42.9 ± 3.5	5.62 ± 0.16
Total phenolic acids		803 ± 110	88.527 ± 2.88

*not quantitatively determined

glucoside. The above-mentioned compounds with the exception of eupafolin 7-*O*-glucoside have been previously identified in arnica flowers [16,17,31]. Among the free aglycones in the *Arnica* TM tincture, only 6-methoxyapigenin (compound **27**) was recognized (Fig. 2).

For the first time, the presence of eupafolin 7-*O*-glucoside (**12**) in *Arnica montana*, as well as

kaempferol 3-acetylglucoside (**28**) and luteolin 3'-*O*-glucoside (**25**) was indicated (Fig. 2). The last two compounds have been detected so far only in *Arnica chamissonis* flowers (16).

Identification of caffeic acid esters

In the studied plant material, a number of compounds with a similar UV spectrum to caffeic acid

have been demonstrated (232, 241sh, 294sh, 322) (Table 2 and Fig. 2) (20, 21). These were compounds: **1**, **15**, **16**, **17**, **18**, **19**, **21**, **23**, **24**, **25**, **26** and **30**. Based on the value of molecular ion at m/z 603 $[M+H]^+$ in the ESI-MS spectrum, **18** was identified as 1-methoxyoxaloyl-di-*O*-caffeoylquinic acid (20,21). On the basis of the presence of molecular ion $[M+H]^+$ at m/z 517 in the ESI-MS spectra and the elution sequence (32) the structures of the following compounds were initially characterized as di-*O*-caffeoylquinic acid isomers – 3,5-di-*O*-CQA (**15**), 1,5-di-*O*-CQA (**16**), 4,5-di-*O*-CQA (**17**). In contrast, compound **24** based on the m/z 679 molecular ion $[M+H]^+$ value may be a tricaffeoylquinic acid. In addition to the chlorogenic acid, the presence of the mono-caffeoylquinic isomer – compound **1** [e.g. cryptochlorogenic (4-*O*-caffeoyl) acid or neochlorogenic (3-*O*-caffeoyl) acid] was shown, whose molecular ion $[M+H]^+$ at m/z 355, corresponds to the molecular weight of chlorogenic acid which is 5-*O*-caffeoylquinic acid.

HPLC analysis using the MS and DAD detectors did not reveal the presence of some of the phenolic acids identified earlier in *A. montana* flower, e.g. tricaffeoyl-oxaloyl-quinic acid, di-*O*-caffeoyl-dimethoxyoxaloyl-quinic acid, acetyl-feruloyl-methoxyoxaloyl-quinic acid, di-*O*-caffeoyl-fumaroyl-quinic acid (20, 21).

The obtained separations of the majority of extract components from *Arnica* TM, confirm the better separation efficiency of simple phenols and polyphenols on the PFP type column, compared to C-18 (Fig. 1). The separation process on Kinetex PFP, in comparison to Kinetex C-18, includes 5 different mechanisms of interactions for polar and non-polar compounds, including hydrophobic interactions, δ - δ (aromatic rings), electrostatic, steric and associated with the formation of hydrogen bonds (33).

Identification of triterpene lactones

The analyses were carried out on two Kinetex C-18 columns (2.6 μ m, 4.60 $\times$ 100 mm) connected in series using gradient elution according to the program III. Bergonzi et al. (34) analyzing the chemical

composition of *Arnica montana* tincture noted the limitations of the European Pharmacopoeial method of sesquiterpene lactones determination. The method requires to carry out the complex purification step of the extract (35) prior to separation on a short HPLC column (150 mm). In our study, the connection in series of two columns (2 $\times$ 100 mm) enabled effective separation of sesquiterpene lactones in raw arnica extract (Figure 3) in the similar time as in the analysis of the arnica flower extract preceded by its purification on a column (13, 36). Moreover, compared to the method used by Schmidt et al. (26) to determine sesquiterpene lactones in arnica leaves, the extended analysis time in our method enabled the separation of helenalin isobutyrate and helenalin tyglinat. As a result, 12 compounds in the *Arnica* TM tincture were identified based on their UV and mass spectra (**32-43**) as belonging to the group of sesquiterpene lactones, including esters of helenalin (compounds **37**, **39**, **42**, **43**), esters of 11,13 α -dihydrohelenalin (compounds **38**, **40**, **41**) and acetyl-dihydrohelenalin (compound **34**) (13-15, 18, 28). In addition, based on the molecular ion at m/z 371 $[M+Na]^+$ in obtained ESI mass spectrum, compound **33** was found to be 11 α ,13-dihydro-2-*O*-tigoylflorilenalin, previously identified in fresh flowers of *A. montana* (12). This compound, in contrary to helenalin and dihydrohelenalin classified as 10 α -methylpseudoguaianolides, belongs to another group of sesquiterpene lactones, namely 1,5-*trans*-guaianolides (12). Three other compounds **32**, **35**, **36** had UV spectra with maximum absorption in the characteristic range of α , β -unsaturated sesquiterpene lactones (205-225 nm) (36), however their molecular weights calculated on the basis of m/z molecular ion values, not corresponded to the molecular masses of other sesquiterpenes identified so far in the arnica flowers (12). Given the low t_R value of helenalin (6.7 min) and the presence of a number of interfering compounds from the matrix, it was not possible to determine this compound in the *Arnica* TM tincture under described above HPLC conditions (Fig. 3). For these purposes gradient elution program has been changed in terms of its profile and decrease of elution power of used solvent

Table 5. Determined concentrations of sesquiterpene lactones, helenalin and dihydrohelenalin esters and free helenalin in *Arnica* TM (Boiron).

Content of sesquiterpene lactones in <i>Arnica</i> TM tincture [mg/100 g d.w.] $\pm$ SD		
Total	Helenalin and dihydrohelenalin esters	Helenalin
99.4 $\pm$ 3.4	88.2 $\pm$ 6.4	0.591 $\pm$ 0.015

mixture – changes in concentration of MeOH in a mixture of MeOH : H₂O from proportion 50 : 50 v/v to 45 : 5 v/v (program II). Helenalin (**31**) was effectively separated on Kinetex C-18 columns (2.6 µm 4.60 × 100 mm) with *t_R* 17.3 min (Fig. 4). The identity of the analyzed compound was confirmed by the comparison with the UV and ESI-MS spectra of the helenalin standard.

Quantification of active biologically compounds from *Arnica* species

The contents of the compounds are given in mg of the compound per 100 g of analyzed dried plant material, constituting the whole plant used to obtain the *Arnica* TM tincture calculated from the data provided by the manufacturer – Boiron (France) (Table 4 and 5). Moreover, in Table 4, the content of individual compounds in 100 mL of Boiron's tincture was showed. The content of individual flavonols was calculated on quercetin, flavones expressed as luteolin and caffeoylquinic acids/phenolic acids calculated as chlorogenic acid (Table 4). The content of free helenalin and other sesquiterpene lactones, including esters of helenalin and dihydrohelenalin was calculated on helenalin standard (Table 5).

Flavonoids

In terms of the dried herbal material constituting the whole *A. montana* plant, the estimated flavonols content was 42.9 ± 3.5 mg/100 g d.w. and flavones 53.0 ± 6.7 mg/100 g d.w. (Table 4). The dominant flavonoids were determined as 6-methoxyapigenin (**27**) (19.6 ± 1.6 mg/100 g d.w.), eupafolin-7-*O*-glucoside (**10**) (14.7 ± 1.7 mg/100 g d.w.) and kaempferol-3-*O*-acetylglucoside (**22**) (12.0 ± 0.35 mg/100 g d.w.) (Table 4).

Sesquiterpene lactones

A. montana TM Boiron proved to be rich in sesquiterpene lactones – 99.4 ± 3.4 mg/100 g d.w (40) (Table 5). The content of helenalin and dihydrohelenalin esters was determined as 88.2 ± 6.4 mg/100 g d.w. and free helenalin as 0.591 ± 0.015 mg/100 g d.w. (Table 5). The determined content of sesquiterpene lactones by us in *Arnica* TM tincture and whole *Arnica* plant may be higher because dihydrohelenalins possess weaker absorbances at the 225 nm wavelength. According to Perry et al. (13), this problem also applies to the European Pharmacopoeia method, and its use means that sesquiterpene lactones content in arnica samples rich in helenalin esters can be almost twice as high as their real content. To solve this problem, the authors (13) pro-

posed taking into account the differences between the values of response factors (based on relative absorbance in relation to helenalin methacrylate) experimentally established for individual sesquiterpene lactones – helenalin and dihydrohelenalin esters. Because the *Arnica* TM contains several sesquiterpene lactones with the unknown structure the use of the method developed by Perry et al. (13) was impossible.

Phenolic acids

Among the identified compounds, the *A. montana* TM tincture chemical composition is significantly dominated by 3,5-di-*O*-caffeoylquinic acid (50.1 ± 1.4 mg/100 mL, which is equal to 450 ± 13 mg/100 g d.w.). Another dominating compound is chlorogenic acid (4.58 ± 0.12 mg/100 mL). In addition, at a concentration of about 4 mg/100 mL each, numerous caffeic acid derivatives are also present (Table 4). The content of compounds belonging to phenolics in the starting plant material for the production of *Arnica* TM was characterized as 803 ± 110 mg / 100 g d.w. (Table 4).

CONCLUSIONS

The analysis of the chemical composition of the tincture from *Arnica montana* whole plant was conducted for the first time, showing mainly quantitative differences between the whole *Arnica* plant and *Arnica* flowers (13, 38-39).

The tincture of *Arnica* TM (Boiron) is a rich source of caffeic acid derivatives (Table 4), especially CQAs – mono- and di-*O*-caffeoylquinic acids. Among them 3,5-di-*O*-caffeoylquinic acid (compound **15**) is a dominating compound and presenting in higher concentration than it was shown in *Arnica* flowers (38-39).

On the other hand, the whole *A. montana* plant contains a smaller amount of sesquiterpene lactones, estimated at about 0.1% (99.4 ± 3.4 mg / 100 g of dry matter), compared to at least 0.4% in the flowers of *Arnica* required by the European Pharmacopoeia (35). However, the determined content is significantly higher comparing to the content of sesquiterpene lactones, described in the monograph of *Arnica montana* whole plant in French Pharmacopoeia (at least 0.01% calculated on dihydrohelenalin tiglate) (40). Compared to the chemical composition of arnica flowers, the whole plant also contains a smaller amount of flavonoids (about 0.1%) compared to the Polish Pharmacopoeia V norm (at least 0.4% of flavonoids expressed as quercetin) (41).

The high content of caffeic acid derivatives, which have hepatoprotective (5-*CQA*, cynarin), anti-inflammatory and antioxidant activity confirms the use of *Arnica* TM as an anti-inflammatory drug. Although more research is needed to determine the relationship between activity and the chemical structure of various *CQAs* derivatives.

Conflict of interest

The authors declare no conflicts of interest.

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