ORIGINAL ARTICLE

Chemometrics-based Approach in Analysis of *Arnicae* flos

Dimitrina Zh. Zheleva-Dimitrova, Vessela Balabanova, Reneta Gevrenova, Irini Doichinova[1], Antonina Vitkova[2]

Departments of Pharmacognosy and [1]Chemistry, Faculty of Pharmacy, Medical University, Sofia, 1000 Sofia, [2]Department of Plant and Fungal Diversity and Resources, Institute of Biodiversity and Ecosystem Research Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
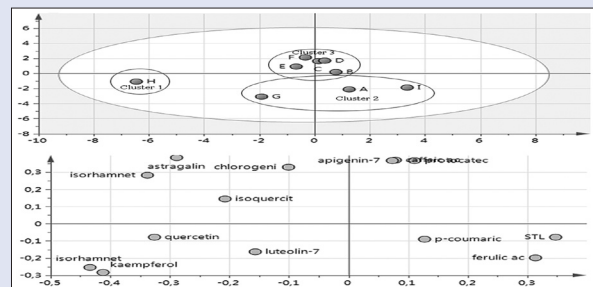
ABSTRACT

Introduction: *Arnica montana* flowers have a long history as herbal medicines for external use on injuries and rheumatic complaints. **Objective:** To investigate *Arnicae* flos of cultivated accessions from Bulgaria, Poland, Germany, Finland, and Pharmacy store for phenolic derivatives and sesquiterpene lactones (STLs). **Materials and Methods:** Samples of *Arnica* from nine origins were prepared by ultrasound-assisted extraction with 80% methanol for phenolic compounds analysis. Subsequent reverse-phase high-performance liquid chromatography (HPLC) separation of the analytes was performed using gradient elution and ultraviolet detection at 280 and 310 nm (phenolic acids), and 360 nm (flavonoids). Total STLs were determined in chloroform extracts by solid-phase extraction-HPLC at 225 nm. The HPLC generated chromatographic data were analyzed using principal component analysis (PCA) and hierarchical clustering (HC). **Results:** The highest total amount of phenolic acids was found in the sample from Botanical Garden at Joensuu University, Finland (2.36 mg/g dw). Astragalin, isoquercitrin, and isorhamnetin 3-glucoside were the main flavonol glycosides being present up to 3.37 mg/g (astragalin). Three well-defined clusters were distinguished by PCA and HC. Cluster C1 comprised of the German and Finnish accessions characterized by the highest content of flavonols. Cluster C2 included the Bulgarian and Polish samples presenting a low content of flavonoids. Cluster C3 consisted only of one sample from a pharmacy store. **Conclusion:** A validated HPLC method for simultaneous determination of phenolic acids, flavonoid glycosides, and aglycones in *A. montana* flowers was developed. The PCA loading plot showed that quercetin, kaempferol, and isorhamnetin can be used to distinguish different *Arnica* accessions.

Key words: *Arnica montana,* Asteraceae, chemometrics, flavonoids, high-performance liquid chromatography, phenolic acids

SUMMARY

• A principal component analysis (PCA) on 13 phenolic compounds and total amount of sesquiterpene lactones in Arnicae flos collection tended to cluster the studied 9 accessions into three main groups. The profiles obtained demonstrated that the samples from Germany and Finland are characterized by greater amounts of phenolic derivatives than the Bulgarian and Polish ones. The PCA loading plot showed that quercetin, kaempferol and isorhamnetin can be used to distinguish different arnica accessions.

Correspondence:

Dr. Reneta Gevrenova,
Department of Pharmacognosy,
Faculty of Pharmacy, Medical University-Sofia,
2 Dunav Str., 1000 Sofia, Bulgaria.
E-mail: rgevrenova@gmail.com
DOI : 10.4103/0973-1296.172958

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Arnica montana flowers have a long history as herbal medicines for external use on injuries and rheumatic muscle and joint complaints.[1] The plant is used in many cosmetic preparations due to its antiphlogistic and antiseptic properties.[2] The chemical constituents in *Arnica* comprise a complex of sesquiterpene lactones (STLs) (helenalin, dihydrohelenalin), flavonoids, and phenolic acids. [3-6] The main anti-inflammatory and analgesic activity of *Arnica* is based on STLs helenalin- and 11,13-dihydrohelenalinester.[7] However, flavonoids and phenolic acids are also crucial for several reasons. They show significant antioxidant and antibacterial effect[8,9] and are used to assure identity and purity of *A. montana* flowers according to the European Pharmacopoeia.[10]

Several high-performance liquid chromatography (HPLC) methods for the analysis of *A. montana* have been developed, determining phenolic acids, flavonoid glycosides, or flavonoid aglycones, using either ultraviolet (UV)[11,12] or mass spectrometry detection.[13,14] In addition, micellar electrokinetic capillary chromatography (MEKC) has been reported for the quantitative determination of phenolic derivatives in mountain *Arnica*.[15] Recently, nuclear magnetic resonance quantitative method for STLs has been developed.[16]

As a part of our ongoing study[17] on the quali-quantitative characteristics of *Arnicae* flos from different provenance, we investigated nine cultivated accessions from Bulgaria, Poland, Germany, Finland, and pharmacy store for their phytochemical content. Using a chemometric approach with principal component (PC) and hierarchical cluster (HC) analysis, it was possible to distinguish clearly each of the assayed accessions on the basis of their phenolic compounds and total content of STLs.

MATERIALS AND METHODS

Plant material

Arnicae flos from 9 different origins were selected for the assay. They included: 2 Bulgarian, 1 Polish cultivated collection, 2 cultivars, 3 botanical garden collections, and 1 purchased from a pharmacy store (identified as *Arnica chamissonis* by one of us, V. Balabanova) [Table 1]. The plant material was collected in the period 2002–2012.

Table 1: Investigated samples of *Arnicae flos*

Sample	Origin	Year
A	Cultivated, experimental field "Zlatni mostove", Vitosha Mt., Bulgaria	2012
B	Botanical Garden. Oulu University, Finland	2002
C	Cultivar "Marburg". Germany, grower	2002
D	Botanical Garden. Joensuu University, Finland	2002
E	Botanical Garden. Turku University, Finland	2002
F	Cultivar "ARBO". Germany, breeder	2002
G	Cultivated, experimental field, Poland	2011
H	Pharmacy store, origin Central America	2010
I	Cultivated, experimental field "Beglika", Rhodopi Mt., Bulgaria	2013

Sample preparation and determination of phenolic acids and flavonoids

Dried powdered flowers (0.50 g of each sample) were subjected to an ultrasound-assisted extraction with 80% methanol (v/v) (2 × 5 ml) for 30 min. The extracts were combined and made up to 10 ml in a volumetric flask. An aliquot (1 ml) of the extract was filtered through a 0.45 μm syringe filter disc (Polypure II, Alltech, Lokeren, Belgium) and 10 μL of the sample was subjected to HPLC analysis.

Isolation of sesquiterpene lactones by solid-phase extraction

The determination of STLs was performed according to the European Pharmacopoeia[10] with modification.[18] Plant material (0.50 g) was ultrasonicated (5 min) with CHCl$_3$ (10 ml) containing 1 mg/ml santonin as an internal standard. The sample was then filtered, rinsed (3 × 2 ml CHCl$_3$), evaporated to dryness (35°C), resuspended in MeOH (1 ml), and placed on a Bond Elut cartridge (500 mg, 3 ml) (Varian, MS, USA), previously conditioned with 3 ml methanol-water (3:2, v/v). The sample flask and cartridge were then rinsed with 1 ml methanol-water (3:2, v/v). The cartridge was allowed to drain dry, and the combined eluates were cooled at −20°C (30 min) filtered and were subjected to HPLC analysis.

Chemicals and reagents

The standards of protocatechuic (1), chlorogenic (2), caffeic (3), ferulic (4) and *p*-coumaric (5) acids, and luteolin 7-*O*-glucoside (6), isoquercitrin (7), apigenin 7-*O*-glucoside (8), astragalin (9), isorhamnetin 3-*O*-glucoside (10), quercetin (11), luteolin (12), and kaempferol (13) were purchased from Extrasynthese (Genay, France); santonin was supplied by Sigma-Aldrich (St. Louis, USA). HPLC-gradient grade solvents and analytical-grade chemicals were provided by Merck (Darmstadt, Germany). Water was double distilled. Solvents were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) and degassed in an ultrasonic bath before use. The stock standard solutions of analytes were prepared in methanol and stored at 4°C in the dark. The working standard solutions of appropriate concentration were prepared by diluting the stock standard solutions with methanol.

Chromatographic equipment and conditions

The chromatographic analyses were performed on a Varian (Walnut Creek, CA, USA) chromatographic system, which consisted of a tertiary pump model 9012, a Rheodyne injector with a 20 μL sample loop and a UV-VIS detector model 9050. The chromatograms of each sample were recorded at 280 and 310 nm to ensure reliable identification of the phenolic acids, and 360 nm for the flavonoids. A Varian Star Chromatography workstation running version 4.5 software (Varian, CA, USA) was used to control the HPLC system and to collect the data. The separation was carried out with a reverse-phase (RP) Hypersil ODS column (250 × 4.6 mm i.d.; 5 μm) (Shandon, Runcorn, UK) fitted with a precolumn (30 × 4.6 mm i.d.) dry packed with Perisorb RP-18 (30–40 μm) (Merck, Germany) and periodically changed.

The ternary solvent system consisted of solvent A (3% methanol in a 20 mM potassium dihydrogen phosphate buffer adjusted to a pH of 3.22 with *o*-phosphoric acid), solvent B (45% methanol in a 20 mM potassium dihydrogen phosphate buffer adjusted to a pH of 3.22 with *o*-phosphoric acid), and solvent C (methanol). Gradient program was performed as follows: 0 min – 90% A: 10% B, 20 min – 30% A: 70% B, 30 min – 25% A: 75% B, 45 min –15% A: 85% B, 55 min – 100% B, 65 min – 90% B: 10% C, 75 min – 80% B: 20% C, 85 min – 70% B: 30% C, 95 min – 70% B: 30% C and then return to the initial conditions in 5 min. The flow rate was 1.2 ml/min and the oven temperature was set at 35°C. The quantification of STLs was performed according to the method in European Pharmacopoeia 7[10] with slight modification[18] on a Luna C18 column (150 × 4.6 mm i.d., 5 μm (Phenomenex, USA).

Quantitative analysis and analytical performance

The analysis of phenolic compounds (1–13) was carried out using the external standard method. Because of the similar molecular structures, the responses of the flavonoid glycosides (5–9) and flavonoid aglycones (10–12) were related to isoquercitrin (6) and quercetin (10), respectively. External standard calibrations were established at five data points covering the concentration range of each analyte according to the level expected in the plant samples.

The concentrations of the analytes were as follows: 0.24; 0.062; 0.028; 0.012; 0.003 mg/ml for 1; 0.592; 0.213; 0.128; 0.071; 0.001 mg/ml for 2; 0.248; 0.149; 0.053568; 0.005357; 0.000536 mg/ml for 3; 0.226; 0.1356; 0.02712; 0.00488; 0.000488 mg/ml for 4; 0.23; 0.138; 0.0276; 0.004968; 0.000497 mg/ml for 5; 0.4; 0.24; 0.096; 0.0384; 0.00384 mg/ml за 7; 0.5; 0.3; 0.12; 0.048; 0.0048 mg/ml for 11.

Triplicate analyses were performed for each concentration and the peak area was detected at 280 and 310 nm (phenolic acids) and 360 nm (flavonoids). Calibration curves were constructed from peak areas versus analyte concentrations. Slope, intercept, and other statistics of calibration lines were calculated with a linear regression program using the Analytik-Software (Leer, Germany) STL statistics program. The regression equations were, respectively, for compounds 1–5, 7 and 11: y = 14179798x + 1353.9 (r^2 = 0.9986); y = 20841094.11x – 92597.94 (r^2 = 0.9966); y = 45393295x + 157746.4 (r^2 = 0.9953); y = 57405539x + 213692.3 (r^2 = 0.9954); y = 44439000x + 73828.51 (r^2 = 0.9956); y = 10824815x + 11363.42 (r^2 = 0.9994); y = 15692784x – 22958.3 (r^2 = 0.9994).

For each sample, the complete assay procedure was carried out in triplicate, and the standard deviation was calculated. The examined compounds were assigned in the HPLC chromatograms by comparing individual peak retention times with these of authentic references standards, as well as by spiking techniques. The repeatability was established by injecting the standard solution (0.003 mg/ml[1]; 0.001 mg/ml[2]; 0.000536 mg/ml[3]; 0.000488 mg/ml[4]; 0.000497 mg/ml [5]; 0.00384 mg/ml [7]; 0.0048 mg/ml [11]) 6 times. The reproducibility was determined over 10 days by three injections per day of the same solution.

The limits of detection (LODs) were calculated according to the expression 3.3 σ/S, where σ was the standard deviation of the response and S the slope of the calibration curve.[19] Limits of quantification (LOQs) were established from the expression 10 σ/S. In order to check the percentage recovery, known quantities of standards of assayed phenolic

acids and flavonoids were added to known amounts of *Arnicae* flos (*A. montana*-Vitosha). Recoveries of the analytes were examined at levels between 0.001 mg/ml [5] and 1.092 mg/ml [1]. The fortified samples were then extracted and analyzed with the proposed sample preparation procedure and HPLC method. The percentage recovery was determined by subtracting the values obtained for the control matrix preparation from those samples that had been prepared with added standards, dividing by the amount added and multiplying by 100.

Statistical analysis

Data were expressed as mean ± standard deviation (SD).

Hierarchical clustering

Clustering is a process of dividing set of entities into subsets in which the members of each subset are similar to each other but different from members of other subsets.[20] In the present study, a hierarchical clustering (HC) using the agglomerative algorithm[20] was applied. According to this algorithm, the clusters are built from the bottom-up, first by merging individual items into clusters, and then by merging clusters into superclusters, until the final merge brings all items into a single cluster.

The HC was applied as implemented in SIMCA-P 13.0 (2012). The distance between clusters was calculated by Ward method,[21] that is, at each step, the pair of clusters with minimum between-cluster distance is merged.

Principal component analysis

Principal component analysis (PCA) is a multivariate data analysis designed to represent large, multidimensional data sets in a limited, but visually interpretable, number of dimensions, usually two to five, referred to as principal components (PC), such that an overview of the data is obtained. This overview may reveal groups of observations, trends, and outliers. It also uncovers the relationships between observations and variables and between the variables themselves.[22] The results from PCA can be visualized on different plots: Scores and loadings. Score plots (observation projects on PC) visualize groupings of the accessions based on their compositions. Accessions with the similar composition are clustered together. Loading plots (variable projections on PC) visualize the contributions of the phenolic compounds in the clustering of accessions. PCA was used as implemented in SIMCA-P 13.0 (2012).

RESULTS

High-performance liquid chromatography analysis and analytical performance

The efficiency of the extraction procedure for phenolic compounds (1–13) was tested using different techniques and by sequentially varying the composition of the solvents (80% aqueous methanol; 60% aqueous methanol, isopropanol). The results shown in Figure 1 demonstrate that the highest yield of the studied phenolic acids and flavonoids was achieved by ultrasound assisted extraction with 80% methanol. Hence, the plant materials were exhaustively extracted twice with 5 ml solvent for 30 min each time. Preliminary HPLC experiments were performed on a reversed-phase column C18 with solvent systems consisting of an organic phase (either acetonitrile or methanol) and 20 mM phosphate buffer (adjusted to a pH of 3.22 with *o*-phosphoric acid). Various proportions of methanol or acetonitrile, ranging from 45% to 85% (v/v) were tested for separating the analytes. A fairly good separation was obtained after adjustment of the gradient program using ternary solvent system consisting of 3% methanol in a 20 mM phosphate buffer at pH 3.22 (solvent A), 45% methanol in a 20 mM phosphate buffer at pH of 3.22 (solvent B) and methanol (solvent C).

With the initial mobile phase concentration set at 90% A: 10% B followed by a several steps to 100% B over 55 min and a gradient program to 30% B: 70% C for 20 min, separation of the phenolic acids (1–5) and flavonoids (6–13) was achieved with a good resolution. Typical HPLC chromatograms of an *Arnica* sample and a standard mixture are presented in Figure 2a and b, respectively. Chromatograms were recorded at 280 and 310 nm (1–5), and 360 (6–13) according to the UV absorption maxima of the phenolic acids and flavonoids, respectively. In respect to the analytical performance, for triplicate analysis for both standards and plant sample, RSDs of the retention times were ≤2.16 (for 6). The instrument precision was composed of repeatability and reproducibility studies of the assayed compounds [Table 2]. The relative SD (RSDs) of the repeatability and the reproducibility were ≤3.54% (for 3) and ≤4.86% (for 7), respectively. In the calibration experiments, all compounds showed acceptable linearity with correlation coefficients (r^2) higher than 0.99 within the range of concentrations investigated. The recovery of the phenolic acids (1–5) and flavonoids (6) was checked by an addition of a standard solution mixture (from 0.0001 mg/ml for 4–1.092 mg/ml for 1) at a concentration close to that expected in the real plant samples. The
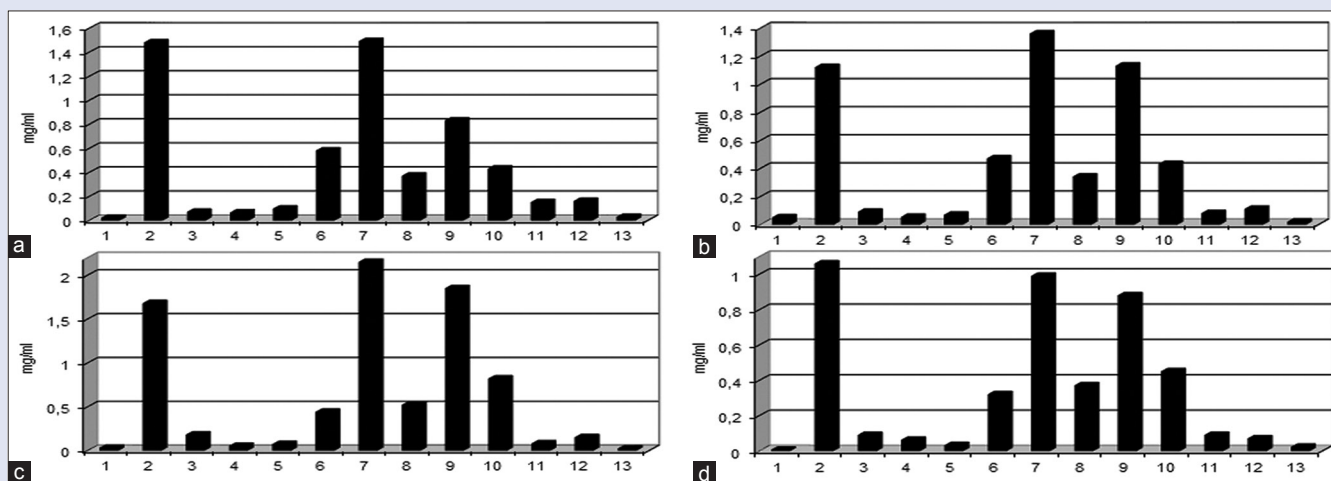

Figure 1: Yields (mg/g) of compounds 1–13 obtained with different extraction methods from flowers of *Arnica montana*: a: ultrasound extraction with 80% (v/v) aqueous methanol; b: ultrasound extraction with 60% (v/v) aqueous methanol; c: extraction with 80% (v/v) aqueous methanol and stirring; d: ultrasound extraction with isopropyl alcohol
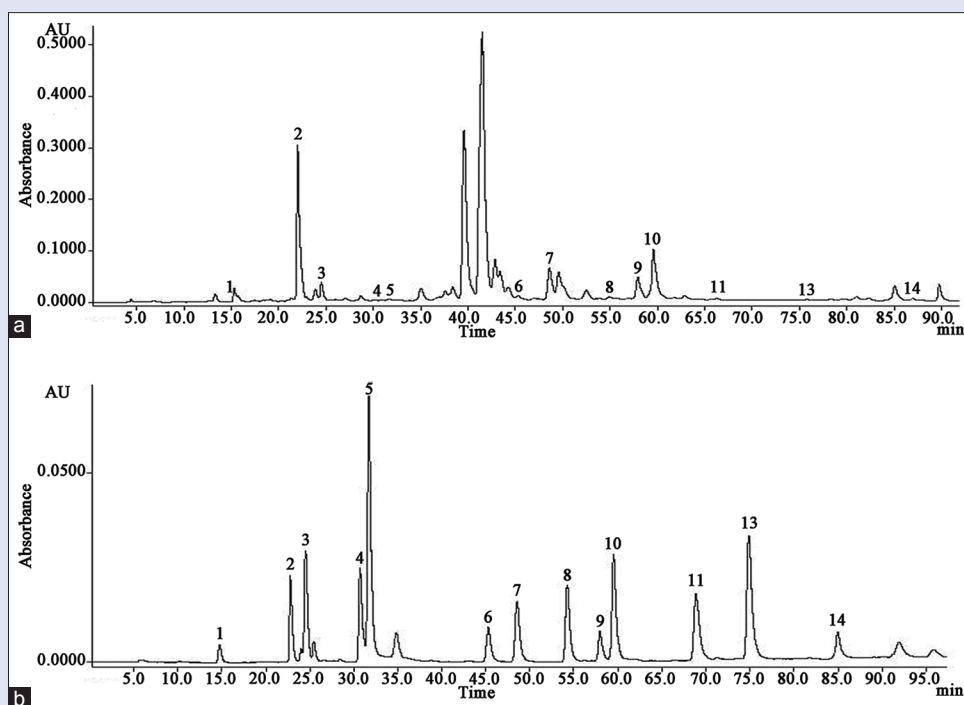
Figure 2: High-performance liquid chromatography chromatograms of: (a) Sample from Vitosha mountain, (b) standard mixture of phenolic compounds at 280 nm. Key to peaks identified: protocatechic (1), chlorogenic (2), caffeic (3), ferulic (4) and p-coumaric (5) acids, and luteolin 7-O-glucoside (6), isoquercitrin (7), apigenin 7-O-glucoside (8), astragalin (9), isorhamnetin 3-O-glucoside (10), quercetin (11), luteolin (12) and kaempferol (13)

Table 2: Repeatability, reproducibility, limits of detection, and quantification obtained for the compounds assayed

Analyte	RSDa	RSDb	LOD (µg/ml)	LOQ (µg/ml)
Protocatechic acid (1)	2.09	4.67	0.19	0.57
Chlorogenic acid (2)	1.25	3.70	0.51	1.53
Caffeic acid (3)	3.54	4.36	0.33	0.99
p-coumaric acid (4)	2.20	2.60	0.83	2.51
Ferulic acid (5)	1.16	2.30	0.20	0.61
Isoqueritrin (7)	1.91	4.86	1.22	3.70
Quercetin (11)	1.54	3.13	0.51	1.54

RSD: Relative standard deviation; LOD: Limits of detection; LOQ: Limits of quantification

mean recovery was ≥86.29% ±0.16% (for 3). The LODs and LOQs values were <1.22 and 3.70 mg/ml for isoquercitrin (7) [Table 2].

The content of phenolic acids and flavonoids in the assayed samples is shown in Table 3.

The highest total content of the assayed phenolic acids was found in the accession originating from Botanical Garden Joensuu (D) (2.36 mg/g dw), while Polish *Arnica* (G) demonstrated the lowest quantity (0.82 mg/g dw) [Table 3]. Chlorogenic acid was the dominant phenolic acid in the studied samples being present in amounts between 0.68 ± 0.04 (G) and 2.06 ± 0.17 mg/g dw (D). The highest level of caffeic acid (2) was found in cultivar ARBO (0.162 ± 0.076 mg/g dw), whereas in the Polish *Arnica* (G), the content was under the limit of quantification. *p*-coumaric acid (4) and ferulic acids (5) were present in the highest amounts in Bulgarian accession (A) [Table 3]. However, they occurred in relatively lower concentration in the specimen from a pharmacy store.

With respect to the flavonoids, astragalin (9) was present in the highest amounts followed by isoquercitrin (6) and isorhamnetin 3-glucoside (10), while content of the flavonoid aglycons 11, 13, and 14 was considerably

lower for all samples [Table 4]. *Arnica* from Turku University, Finland (E) demonstrated the highest total amount of studied flavonoids (7.695 mg/g dw), whereas the lowest content was established in Bulgarian accession I (1.543 mg/g dw).

Here, we noticed that *Arnicae* flos from a pharmacy store contained a high level of quercetin [Table 4]. However, kaempferol and isorhamnetin were not evidenced; luteolin was present only in this sample.

The levels of STLs were calculated as dihydrohelenalin tiglate equivalents and achieved up to 1.73% ± 0.21% in Bulgarian accession I [Table 3]. Plants cultivated in Bulgaria were the richest samples in STLs, while they demonstrated the lowest levels of total flavonoids and significantly low content of phenolic acids. In contrast, commercial product (H) was characterized by high content of phenolic acids (1.81 mg/g) and significantly low amounts of STLs (0.25% ± 0.06%) and flavonoids (3.06 mg/g). Furthermore, only H does not satisfy the requirements of pharmacopoeia with regard to the content amounts of STLs.

Hierarchical clustering on phenolic compounds and sesquiterpene lactones

The HC of 9 *Arnica* accessions on the basis of their content of phenolic compounds and STLs is given in Figure 3. Three well-defined clusters are distinguished which suggests three patterns of chemical composition. The first cluster C1, placed on the left side of the dendrogram consisted of only one sample – this from the pharmacy store (H). The sample was the poorest in the content of caffeic and ferulic acids, luteolin 7-glucoside, apigenin 7-glucoside, isorhamnetin, kaempferol, and STLs [Tables 3 and 4]. The second cluster C2, placed in the middle of the dendrogram included the Bulgarian and Polish samples (A, G, I). The accessions were grouped by their moderate concentration of astragalin ranged from 0.44 ± 0.03 mg/ml (I) to 1.58 ± 0.02 (G). The third cluster C3, placed on the right side of the dendrogram, consisted of the following accessions cultivated in Germany and Finland: B, C, D, E, F. The compound most associated with this cluster was astragalin being present from 2.34 ± 0.51 mg/ml (E) to 3.37 ± 0.29 mg/ml (D).

Table 3: Content (mg/g) of phenolic acids and STLs (%) in different *Arnica* samples (*n*=3)

Sample	Protocatechuic acid (1)	Chlorogenic acid (2)	Caffeic acid (3)	*p*-coumaric acid (4)	Ferulic acid (5)	Total (1-5)	STL percentage
A	0.020±0.006	1.48±0.10	0.073±0.012	0.064±0.005	0.096±0.013	1.73	1.44±0.30
B	0.099±0.001	1.03±0.004	0.066±0.001	0.048±0.006	0.096±0.049	1.34	1.27±0.03
C	0.11±0.002	1.50	0.061	0.058	0.062	1.79	0.77±0.18
D	0.098±0.003	2.06±0.17	0.079±0.040	0.050±0.005	0.075±0.011	2.36	1.15±0.15
E	0.08±0.007	1.51±0.36	0.062±0.042	0.058±0.003	0.062±0.013	1.77	1.18±0.08
F	0.14±0.011	1.39±0.07	0.162±0.076	0.052±0.002	0.046±0.009	1.79	1.17±0.14
G	0.013±0.001	0.68±0.04	-	0.056±0.003	0.071±0.007	0.82	0.86±0.16
H	0.069±0.013	1.66±0.87	0.017±0.002	0.045±0.001	0.015	1.81	0.25±0.06
I	0.12±0.01	0.79±0.12	0.03±0.02	0.05±0.002	-	0.99	1.73±0.21

STLs: Sesquiterpene lactones

Table 4: Content (mg/g) of flavonoids in different *Arnica* samples (*n*=3)

Sample	Luteolin-7-glucoside (6)	Isoquercitrin (7)	Apigenin-7-glucoside (8)	Astragalin (9)	Isorhamnetin-3-glucoside (10)	Quercetin (11)	Luteolin (12)	Kaempferol (13)	Isorhamnetin (14)	Total (6-14)
A	0.58±0.15	1.49±0.23	0.37±0.03	0.83±0.07	0.43±0.08	0.15±0.030	-	0.16±0.02	0.028±0.016	4.038
B	0.45±0.03	1.49±0.09	0.71±0.005	2.52±0.29	1.03±0.008	0.19±0.122	-	0.15±0.02	0.022±0.004	6.562
C	0.41±0.01	1.92±0.03	1.21±0.08	2.50±0.001	1.22±0.068	0.11±0.021	-	0.09±0.01	0.025±0.01	7.485
D	0.38±0.02	0.93±0.04	1.11±0.03	3.37±0.29	0.93±0.052	0.07±0.008	-	0.18±0.02	0.026±0.011	6.996
E	0.46±0.09	1.82±0.45	1.00±0.22	2.34±0.51	1.833±0.376	0.06±0.0001	-	0.15±0.04	0.035±0.018	7.695
F	0.42±0.09	2.12±0.12	0.80±0.095	2.86±0.35	1.08±0.128	0.10±0.061	-	0.17±0.007	0.033±0.002	7.583
G	0.45±0.04	1.93±0.02	0.66±0.01	1.58±0.02	0.75±0.009	0.18±0.006	-	0.48	0.053±0.004	6.083
H	-	1.74±0.19	0.10±0.02	-	-	1.15±0.198	0.07±0.001	-	-	3.06
I	0.33±0.004	0.27±0.03	0.17±0.0001	0.44±0.03	0.22±0.01	0.03±0.01	-	0.06±0.004	0.023±0.008	1.543
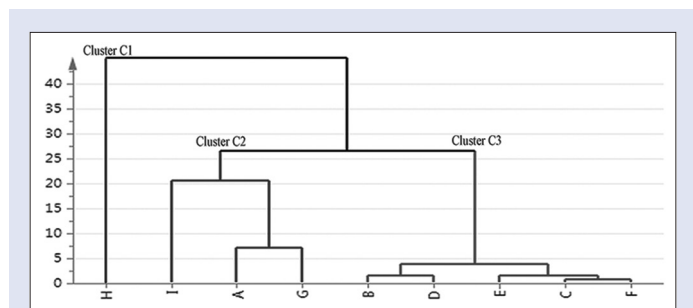
Figure 3: Hierarchical clustering of 9 *Arnica* accessions on the basis of the phenolic compounds assayed and total STLs. The content of phenolic compounds is reported in Table 1. Key to accessions identities: A-Cultivated, experimental field "Zlatni mostove", Vitosha Mt., Bulgaria; B - Botanical Garden, Oulu University, Finland; C - Cultivar "Marburg", Germany, grower; D - Botanical Garden, Joensuu University, Finland; E - Botanical Garden, Turku University, Finland; F - Cultivar "ARBO", Germany, breeder; G - Cultivated, experimental field, Poland; H - Pharmacy store, origin Central America; I - Cultivated, experimental field "Beglika", Rhodopi Mt., Bulgaria

Principal component analysis on phenolic compounds and sesquiterpene lactones

The first three PCs explain 32%, 26%, and 16% of the variance, respectively. The PCA score plot for the first two PCs is given in Figure 4. The clusters correspond to those generated by the HC. The loading plot for the first two PCs [Figure 4] points quercetin, kaempferol, and isorhamnetin as the compounds with the highest contribution in PC1. Apigenin 7-glucoside, isorhamnetin 3-glucoside, and astragalin have the highest contribution in PC2, while luteolin 7-glucoside and protocatechuic acid – in PC3 (data not shown).

DISCUSSION

In the present study, five phenolic acids, four flavonol glycosides, and four flavonol aglycones were analyzed in 9 collections of *Arnicae* flos from different origins. In addition, the total content of STLs was determined. Based on the content of phenolic derivatives and STLs, the studied accessions were clustered, and the dominant compound responsible for the clustering was identified. HPLC analyses showed that Finish D and E, and Bulgarian I accessions displayed the highest total content of the studied phenolic acids, flavonoids, and STLs, respectively. The HC distinguished three well-defined clusters: Cluster C1 contains the sample from a pharmacy store, cluster C2 – accessions from Bulgaria and Poland, and cluster C3 – accessions from Finland and Germany.

The obtained results revealed that the dominant phenolic derivatives are chlorogenic acid, isoquercitrin, and astragalin, which was in agreement with the literature.[23] The highest total level of studied flavonoids recorded in the sample from the Botanical Garden, Turku University (E) was in the same order of magnitude as that given by Bomme, 1999 (7.00 mg/g),[24] Ganzera *et al.*, 2008 (0.6–1.7%),[15] Dall'Acqua *et al.*, 2011 (0.70–1.42%).[25] On the other hand, other authors reported significantly higher values (13.18–23.45 mg/g;[11] 10.4–24.4 mg/g;[26] 9.6–24.4 mg/g;[27] 13.18–23.45 mg/g[28]).

Luteolin was found only in the sample H acquired by the pharmacy store, and its content (0.07 mg/g) agreed with that reported by Craciunescu *et al.*, 2012 (0.077 mg/g).[29] In addition, the authors provided evidence for a higher quercetin content (1.881 mg/g) as compared to our results. The amount of isoquercitrin determined in this study was lower than the levels found by Albert *et al.*, 2009 (9.7–11.7 mg/g)[12] and Pljevljakušić, 2012 (7.8–13.9 mg/g),[23] while the amount of astragalin was within the range of reported values (2.2–3.7 mg/g),[12] except for the Bulgarian accessions A and I.
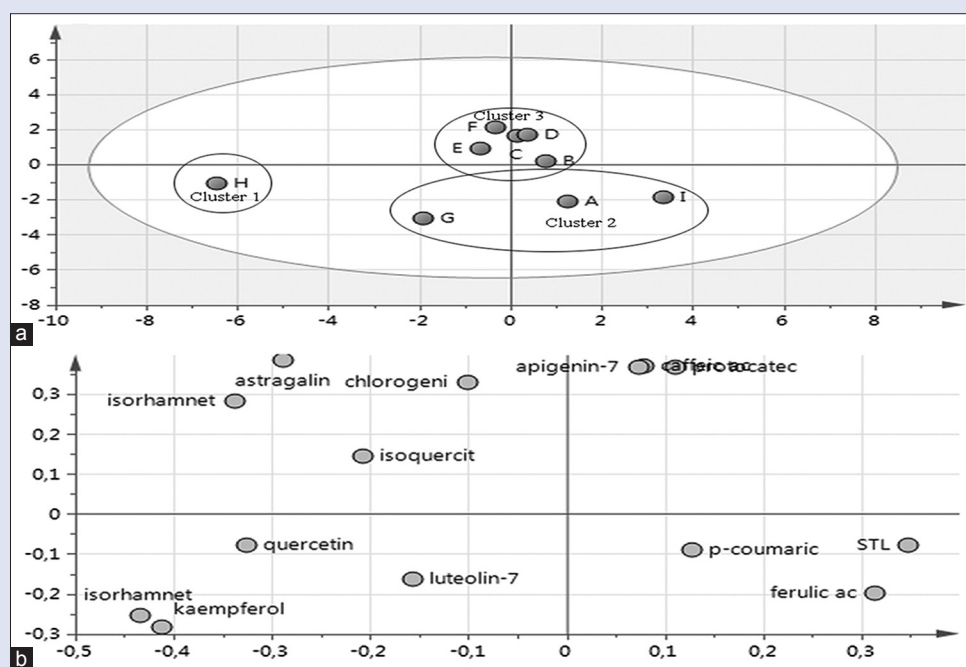
Figure 4: A principal component analysis on the phenolic compounds studied and total sesquiterpene lactones in *Arnica* collection: (a) Principal component analysis score plot; (b) Principal component analysis loading plot

Several differences were observed in the quantitative pattern of phenolic acids as compared with already published data.[15,25-27] In our study, the amounts of chlorogenic and *p*-coumaric acids were generally higher than those reported by Craciunescu *et al.*, 2012 (0.329 mg/g chlorogenic acid; 0.038 mg/g *p*-coumaric acid).[29] On the other hand, chlorogenic acid was up to 3 orders of magnitude lower than that established by Albert *et al.*, 2009 (4.4–6.7 mg/g)[12] and Pljevljakušić, 2012 (1.9–6.57 mg/g).[23] The content of caffeic acid was in agreement with the literature.[15,29] The difference could be due to the methods of sample preparation and quantification used as well as the number and variety of phenolic acids included in the amounts stated.

Bulgarian *Arnica* accessions (cluster C2) shared high levels of STLs, while their total amounts of flavonoids and phenolic acids were substantially lower as compared with cluster C3. In contrast to these results, the commercial sample H (cluster C1) was characterized by a high content of phenolic acids and a considerably small amount of STLs and flavonoids [Tables 3 and 4]. Furthermore, only sample H, claimed to be mountain *Arnica*, had no satisfactory results according to the requirements of the European Pharmacopoeia (content of lactones over 0.4%).[10] Nevertheless, our phytochemical investigation and morphological identification proved it as *Arnica chamissonis*. This species has similar flavonoid pattern and medicinal characteristic as mountain *Arnica*, and it is considered to be equivalent of *A. montana*.[4,30] Our data were lower in comparison to those from the *A. chamissonis* flower heads (0.62%) reported by Todorova *et al.*, 2008.[31]

In this study, the content of STLs in accessions with Bulgarian provenance agreed with that reported for *Arnica* by Pljevljakušić, 2014 (from 4.6 mg/g to 13.9 mg/g),[23] and was intermediate between the values reported by Heldmaier, 2007 (0.64%)[32] and Clauser *et al.* 2012 (up to 2.31%).[27] As regards previously studied samples with Bulgarian origin, our results showed predominantly larger quantities of STLs (9.5 mg/g lacton mixture helenalin and dihydrohelenalin esters).[16] With respect to the examined cultivars C and F (cluster C3), they gave values within the range of literature (0.8–1.3%).[11]

In this study, plant material was with different provenance and collected in different years. In general, altitudinal variation and temperature have been proved to be key factors affecting secondary metabolite profile of *Arnica* flowers.[11,12,28] These principal parameters vary considerably by region and year, giving notions of chemical composition. At this respect, previous studies on mountain *Arnica* have associated a higher content in flavonoids with a high altitude. Consisting with the findings of Spitaler *et al.*, 2008,[28] a high mountain altitude was chosen for cultivation of Bulgarian accessions A and I (1 400 m a.s.l. and 1 500 m a.s.l., respectively, and latitude 42.62° and 41.85°, respectively). In contrast, Finish samples were at low altitudes (59–117 m a.s.l.), as well as German and Polish ones (170 m a.s.l.), but on different Northern latitude (51–64°). It should be concluded that although Finland and Germany are geographically closer and samples set showed similar distribution in the cluster C2, a certain relationship between the distribution of the accessions into the clusters and geographical origin could not defined. Therefore, it is more appropriate to consider the proposed method as a good technique for standardization of *Arnica* flowers and the clustering as a possibility of monitoring of *Arnica* plant material.

CONCLUSION

HC and PCA clustering on phenolic compounds 1–13 and the total amount of STLs in collections of *Arnicae* flos from different origins tended to cluster the studied 9 accessions into three main groups. The profiles obtained demonstrated that the samples from Germany and Finland are characterized by greater amounts of phenolic derivatives than the Bulgarian and Polish ones. Bulgarian accessions showed the tendency of a higher content of STLs. The pharmacy store sample was characterized by the lowest contents of the majority of the studied compounds (caffeic and ferulic acids, luteolin 7-glucoside, apigenin 7-glucoside, isorhamnetin, kaempferol and STLs), and a higher amount of individual chlorogenic acid and total phenolic acids assayed. Regardless of the modest sample set used in this work, the chemometric approach has been proved absolutely valid to distinguish *A. chamissonis* flowers. In future works, studies of larger accessions number should be done for the complete chemometric method allowing initial selection of *Arnica* plant material.

Acknowledgments

Financial support and sponsorship

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Willuhn G. Arnica flowers: Pharmacology, toxicology and analysis of the sesquiterpene lactones-their main substances. In: Lawson LD, Bauer R, editors. Phytomedicines of Europe, ACS Symp. Ser. 691. Amer Chem Soc; 1998. p. 118-132.

2. Wagner S, Merfort I. Skin penetration behaviour of sesquiterpene lactones from different *Arnica* preparations using a validated GC-MSD method. J Pharm Biomed Anal 2007;43:32-8.

3. Kos O, Lindenmeyer MT, Tubaro A, Sosa S, Merfort I. New sesquiterpene lactones from *Arnica* tincture prepared from fresh flower heads of *Arnica montana*. Planta Med 2005;71:1044-52.

4. Merfort I, Wendisch D. Flavonoidglycoside aus *Arnica montana* und *Arnica chamissonis*. Planta Med 1987;53:434-7.

5. Merfort I. Caffeoylquinic acids from *Arnica montana* and *Arnica foliosa*. Phytochemistry 1992;31:2111-3.

6. Ebert M, Merfort I, Willuhn G. Flavonoid distribution in *Arnica* subgenera Montana and austromontana. Phytochemistry 1988;27:3849-51.

7. Klaas CA, Wagner G, Laufer S, Sosa S, Della Loggia R, Bomme U, *et al.* Studies on the anti-inflammatory activity of phytopharmaceuticals prepared from *Arnica* flowers. Planta Med 2002;68:385-91.

8. dos Santos MD, Almeida MC, Lopes NP, de Souza GE. Evaluation of the anti-inflammatory, analgesic and antipyretic activities of the natural polyphenol chlorogenic acid. Biol Pharm Bull 2006;29:2236-40.

9. Gawlik-Dziki U, Swieca M, Sugier D, Cichocka J. Comparison of antioxidant properties of infusions from different parts of *Arnica montana* and *Arnica chamissonis* var. *Foliosa* Less. Herb Pol 2009;55:60-71.

10. Council of Europe, European Directorate for the Quality of Medicines. European Pharmacopoeia. 7th ed. Strasburg: Council of Europe (COE)-European Directorate for the Quality of Medicines (EDQM); 2011. p. 1053-6.

11. Spitaler R, Schlorhaufer PD, Ellmerer EP, Merfort I, Bortenschlager S, Stuppner H, *et al.* Altitudinal variation of secondary metabolite profiles in flowering heads of *Arnica montana* cv. ARBO. Phytochemistry 2006;67:409-17.

12. Albert A, Sareedenchai V, Heller W, Seidlitz HK, Zidorn C. Temperature is the key to altitudinal variation of phenolics in *Arnica montana* L. cv. ARBO. Oecologia 2009;160:1-8.

13. Schmidt T, Merfort I, Matthiesen U. Resolution of complex mixtures of flavonoid aglycones by gas chromatography-mass spectrometry data. J Chromatogr 1993;634:350-5.

14. Pietta PG, Mauri PL, Bruno A, Merfort I. MEKC as an improved method to detect falsifications in the flowers of *Arnica montana* and *A. chamissonis*. Planta Med 1994;60:369-72.

15. Ganzera M, Egger C, Zidorn C, Stuppner H. Quantitative analysis of flavonoids and phenolic acids in *Arnica montana* L. by micellar electrokinetic capillary chromatography. Anal Chim Acta 2008;614:196-200.

16. Staneva J, Denkova P, Todorova M, Evstatieva L. Quantitative analysis of sesquiterpene lactones in extract of *Arnica montana* L. by 1H NMR spectroscopy. J Pharm Biomed Anal 2011;54:94-9.

17. Balabanova V, Gevrenova R, Zheleva-Dimitrova D. HPLC determination of phenolic acids in *Arnicae* flos. C R Acad Bulg Sci 2013;66:1547-52.

18. Douglas JA, Smallfield BM, Burgess EJ, Perry NB, Anderson RE, Douglas MH, *et al*. Sesquiterpene lactones in *Arnica montana*: A rapid analytical method and the effects of flower maturity and simulated mechanical harvesting on quality and yield. Planta Med 2004;70:166-70.

19. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. Validation of analytical procedures: Text and methodology Q2 (R1); 1995.

20. Barnard JM, Downs GM. Clustering of chemical structures on the basis of two-dimensional similarity measures. J Chem Inf Comput Sci 1992;32:644-9.

21. Ward JH Jr. Hierarchical grouping to optimize an objective function. J Am Stat Assoc 1963;58:236-44.

22. Eriksson L, Johansson E, Kettaneh-Wold N, Wold S. Multi- and Megavariate Data Analysis. Umea: Umetrics Academy; 2001. p. 43.

23. Pljevljakušić D. Influence of growing conditions on morphological and chemical proparties and biological effects of extracts of *Arnica montana* L. Doctoral dissertation. Belgrade: University of Belgrade, Faculty of Agriculture; 2012.

24. Bomme U. Anbau und Zuchtung von *Arnica montana* L. Z Arznei Gewurzpflanzen 1999;4:202-3.

25. Dall'Acqua S, Inniniocenti G,. Ferretti V, Aiello N, Scartezzini F, Vender C. Quali-quanitative analysisi of *Arnica Montana* wild accessions compared in field-results of the second year. International Symposium on Medicinal, Aromatic and Nutracentical Plants from Mountains Areas (MAP-Mountain 2011). Acta Hortic. 2011; 955: 325-7.

26. Aiello N, Bontempo R, Vender C, Ferretti V, Innocenti G, Dall'Aqua S. Morpho-quantitative and qualitative traits of *Arnica montana* L. wild accessions of Trento, Italy. Ind Crop Prod 2012;40:199-203.

27. Clauser M, Aiello N, Scartezzini F, Innocenti G, Dall'Acqua S. Differences in the chemical composition of *Arnica montana* flowers from wild populations of north Italy. Nat Prod Commun 2014;9:3-6.

28. Spitaler R, Winkler A, Lins I, Yanar S, Stuppner H, Zidorn C. Altitudinal variation of phenolic contents in flowering heads of *Arnica montana* cv. ARBO: A 3-year comparison. J Chem Ecol 2008;34:369-75.

29. Craciunescu O, Constantin D, Gaspar A, Toma L, Utoiu E, Moldovan L. Evaluation of antioxidant and cytoprotective activities of *Arnica montana* L. and *Artemisia absinthium* L. ethanolic extracts. Chem Cent J 2012;6:97.

30. Cassels AC, Walsh C, Belin M, Cambornac M, Robin JR, Lubrano C. Establishment of a plantation from micropropagated *Arnica chamissonis*, a pharmaceutical substitute for the endangered *A. montana*. Plant Cell Tissue Organ Cult 1999;56:139-44.

31. Todorova M, Staneva J, Evstatieva L. Phytochemical study of *Arnica chamissonis* subsp. *foliosa* (Nutt.) Maguire. C Rend Acad Bulg Sci 2008;61:451-4.

32. Heldmaier M. Phytochemische Charakterisierung Öliger Extrakte aus Pflanzlichen Drogen. Dissertation, Hamburg; 2007.
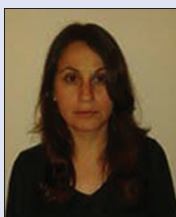
ABOUT AUTHORS

Dimitrina Zh. Zheleva-Dimitrova and Vessela Balabanova, Assistant professor, Department of Pharmacognosy, Faculty of Pharmacy, Medical University-Sofia, 2 Dunav Str., 1000 Sofia, Bulgaria

Vessela Balabanova, PhD, Assist. Prof. Department of Pharmacognosy, Faculty of Pharmacy, Medical University-Sofia, 2 Dunav Str., 1000 Sofia, Bulgaria Images are provided in additional files.

Dimitrina Zh. Zheleva-Dimitrova

Vessela Balabanova