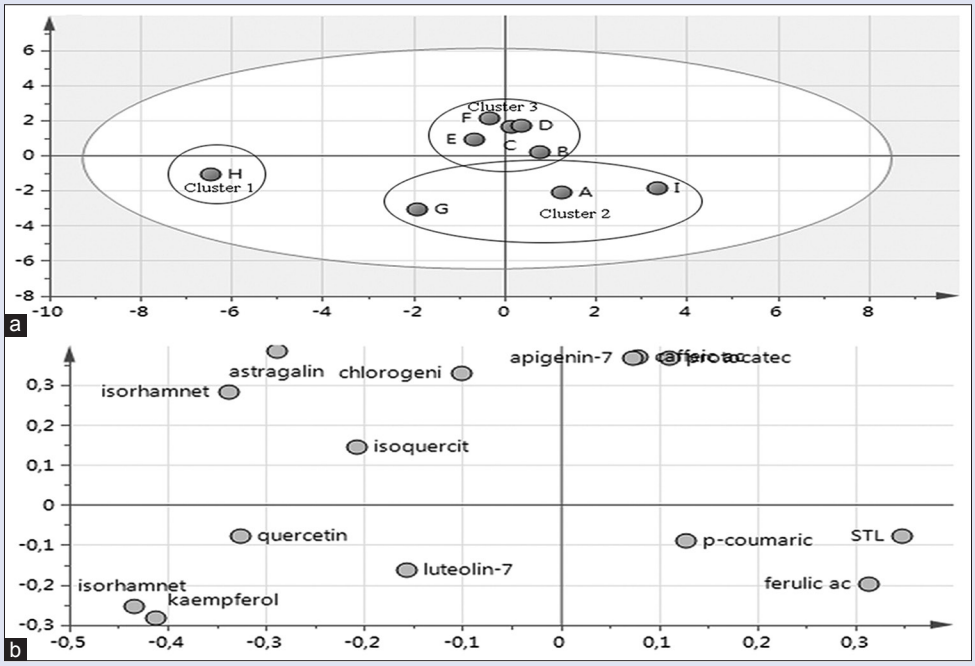

Figure 4: A principal component analysis on the phenolic compounds studied and total sesquiterpene lactones in *Arnica* collection: (a) Principal component analysis score plot; (b) Principal component analysis loading plot

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.

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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 properties 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.

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