Arch Biol Sci. 2018;70(4):613-620 https://doi.org/10.2298/ABS180309022G

Leaf micromorphology, antioxidative activity and a new record of 3-deoxyamphoricarpolide of relict and limestone endemic *Amphoricarpos elegans* Albov (Compositae) from Georgia

Milan Gavrilović[1,*], Vele Tešević[2], Iris Đorđević[3], Nemanja Rajčević[1], Arsena Bakhia[4], Núria Garcia Jacas[5], Alfonso Susanna[5], Petar D. Marin[1] and Peđa Janaćković[1]

[1] *University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac"*, Studentski trg 16, 11 000 Belgrade, Serbia

[2] *University of Belgrade, Faculty of Chemistry*, Studentski trg 12-16, 11000 Belgrade, Serbia

[3] *University of Belgrade, Faculty of Veterinary Medicine*, Bulevar oslobođenja 18, 11000 Belgrade, Serbia

[4] *Ilia State University, School of Natural Sciences and Engineering*, Cholokashvili Avenue 3/5, 0160 Tbilisi, Georgia

[5] *Botanic Institute of Barcelona (IBB,CSIC-ICUB)*, Pg. del Migdia s. n., 08038 Barcelona, Spain

***Corresponding author**: mgavrilovic@bio.bg.ac.rs

Received: March 9, 2018; **Revised**: May 5, 2018; **Accepted**: May 14, 2018; **Published online**: June 4, 2018

Abstract: We examined for the first time the leaf micromorphology, phytochemistry and biological activity of the rare and stenoendemic *Amphoricarpos elegans* Albov (Compositae) from Georgia. Scanning electron microscopy (SEM) revealed the presence of glandular trichomes on the leaves, which appeared as glandular dots that are considered the main sites of biosynthesis and accumulation of sesquiterpene lactones. Using high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy analyses, we identify and characterized 3-deoxyamphoricarpolide, a known sesquiterpene lactone for the genus *Amphoricarpos* Vis. Regarding chemotaxonomic significance, 3-deoxyamphoricarpolide represents a link between Balkan and Caucasian species of the genus. The antioxidative capacity of different leaf extracts, obtained using a Soxhlet extractor, was evaluated by two radical scavenging assays: DPPH (1,1-diphenyl-2-picrylhydrazyl) radical and the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), and ferric ion reducing antioxidant power (FRAP). The total phenolic and flavonoid contents were also determined. The highest antioxidative activity and the highest phenolic and flavonoid contents were detected in the methanol fraction, as a result of the contribution of not only phenols, but probably also lactones. The considerable antioxidative potential indicates possible applications in pharmacy and medicine.

Key words: *Amphoricarpos elegans*; glandular trichomes; 3-deoxyamphoricarpolide; antioxidative activity

INTRODUCTION

Compositae is one of the largest angiosperm families, containing more than 1600 genera and 23000 species with an almost global distribution [1]. In the Compositae, glandular trichomes of short-stalked capitate type, usually seen as glandular dots, are microcharacters that synthesize and accumulate sesquiterpene lactones [2]. Sesquiterpene lactones are secreted into the extracellular and subcuticular secretion storage space at the apical ends of multicellular trichomes and excreted to the plant surface. This type of gland is widely distributed in Compositae [2]. Compositae taxa produce a wide range of specialized metabolites with significant biological effects [3-4], and many are well-known medicinal plants showing antimicrobial and antioxidant activities due to the presence of phenolic compounds (e.g. flavonoids) and sesquiterpene lactones in different organs [5-12].

Sesquiterpene lactones are a large group of chemical compounds that have a broad range of functional groups. To date, the structures of more than 5000 natural sesquiterpene lactones have been reported [13]. Most of them are found in higher plants, mainly in Compositae (more than 90%), but they are also found in some fungi [14]. The most abundant sesquiterpene lactone types are germacranolides, guaianolides and

eudesmanolides, which are classified on the basis of their carbocyclic skeletons. A characteristic of these compounds is the γ-lactone function. Because of their biological activity and ecological functions, as well as their chemotaxonomic significance, sesquiterpene lactones are the subject of constant research worldwide [14-17].

The genus *Amphoricarpos* Vis. (Compositae-Cardueae-Carduinae) belongs to the *Xeranthemum* group [18], which includes annual plants, such as species of the genera *Xeranthemum* L., *Chardinia* Desf. and *Siebera* J. Gay, and perennial plants, including species of the genera *Amphoricarpos* Vis. and *Shangwua* Yu J. Wang, Raab-Straube, Susanna & J. Quan Liu [18]. The group is well characterized based on its molecular and morphological features [19]. Phylogenies based on plastid and nuclear analysis confirm that it is a natural group [20]. *Amphoricarpos* species are heterocarpic perennial chasmophytic plants, mountain endemics in the eastern Mediterranean (the Balkans, Anatolia and the Caucasus) [18].

The Caucasus is distinguished by different phytolandscapes and species genetic diversity due to its edaphic-climatic conditions, high hypsometric levels, well-expressed geographical isolation, etc. [21]. High endemism is characteristic of the Caucasus and it represents one of the world's biodiversity hotspots [22]. The flora of Georgia is represented by 4130 species of vascular plants, of which 4034 are angiosperms [23]; 1304 (32.3%) species are endemics for the Caucasus and 261 (6.6%) are endemics for Georgia [24]. In the Caucasus flora, there are 17 endemic genera, most of them represented by one species, *Amphoricarpos elegans* Albov [22]. Among the calciphytes, in the geographical province of Colchis, the most distinguished is *A. elegans* [21], which belongs to the group of "limestone endemics" of the western Caucasus Mountains [25].

The Balkan species of *Amphoricarpos* has been the subject of some phytochemical [26-28], biological activity [14,29,11], morphological [31] and taxonomic studies [32]. To the best of our knowledge, there is only one study describing the morphology and anatomy of *A. elegans* [31], but there are no data dealing with micromorphological, phytochemical and examinations of the biological activities of this species. Thus, *A. elegans* is almost unexplored. Therefore, the present study, for the first time, aimed to (i) examine leaf micromorphology, (ii) determine the total phenol and flavonoid contents and to evaluate the antioxidative potential of various leaf extracts, and (iii) to identify the most dominant sesquiterpene lactones of *A. elegans*.

MATERIALS AND METHODS

Plant material

The plant material (leaves) of *A. elegans* was collected in 2015 during the flowering period from plants growing in their natural habitat, Mt. Migaria, Samegrelo, Georgia (N 42.6479800; E 42.63992537). A voucher specimen was deposited in the Herbarium of the University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac" (accession number: BEOU 17420).

Micromorphological methods

Micromorphological analysis was carried out using scanning electron microscopy (SEM). Small parts of dry leaves were sputter-coated with gold for 180 s at 30 mA (BAL-TEC SCD 005) and observed using a JEOL JSM-6460LV electron microscope at an acceleration voltage of 20 kV.

Soxhlet extraction

Powdered dried leaves (1.00 g) were extracted in a Soxhlet extractor using pure *n*-hexane, ethyl acetate and methanol as solvents for 6 h. The solvent (3x120 mL) was successively changed after 2, 4 and 6 h. The extracts were evaporated to dryness.

Determination of total phenolics and flavonoids in the plant extracts

The phenolic contents of the methanol, ethyl acetate and *n*-hexane extracts were determined by UV spectroscopy [32]. The methanol solution of the extract (1 mg/mL) was used for the analysis. The reaction mixture was prepared by mixing 0.1 mL of extract solution with 0.5 mL of 10% Folin-Ciocalteu reagent and 0.4 mL of 7.5% $NaHCO_3$ and incubated for 120 min at room temperature. The absorbance was determined at λ_{max}=740 nm. Based on the measured absorbance,

the content of phenolics in the extracts was expressed in terms of the gallic acid (GA) equivalent (GAE) or mg of GA/g of extract. The flavonoid contents of the methanol, ethyl acetate and *n*-hexane extracts were determined by UV spectroscopy [33]. The sample contained 0.1 mL of the methanol solution of the extract at a concentration of 1 mg/mL, 0.41 mL 80% ethanol, and 0.01 mL and 0.1 mL of $Al(NO_3)_3x9H_2O$ and CH_3COOK each. The samples were incubated for 40 min at room temperature. Absorbance was determined at λ_{max}=415 nm. Based on the measured absorbance, the flavonoid contents of the extracts were expressed in terms of the quercetin (QU) equivalent (QUE), or mg of QU/g of extract.

Evaluation of antioxidant activity

DPPH assay

The ability of the plant extracts (dry methanol, ethyl acetate and *n*-hexane extracts) to scavenge the DPPH free radical was assessed by UV spectroscopy [34-35]. Dilutions of stock methanolic solution were made to obtain concentrations of 500, 350, 275, 250, 200, 100, 50 μg/mL DPPH. After 40 min in the dark at room temperature, the absorbance was measured at 517 nm. The control samples contained all the reagents except the extract. The percentage inhibition was calculated using the equation:

%inhibition=100x(A of control – A of the sample)/A of control).

IC_{50} values were estimated from the % inhibition vs. the concentration sigmoidal curve, using nonlinear regression analysis. The antioxidant effects of the extract increased with the decrease in IC_{50} values.

ABTS assay

The ABTS radical scavenging method [36] with some modifications was used. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate and its reduction in the presence of hydrogen donating antioxidants is measured by UV spectroscopy at 734 nm. The reaction mixture of 5 mg of ABTS was dissolved in potassium persulfate before the experiment. This solution was dissolved in distilled water in order to calibrate working solution absorbance to 0.700 at 734 nm. The sample concentration was 1 mg/mL. The reaction mixture was prepared by mixing 15 μL of a test sample and 4-1.5 mL ABTS. After a 4-min incubation at room temperature, the absorbance of the mixture was measured at 734 nm. The radical scavenging activity for each extract was determined based on the linear calibration curve of ascorbic acid and was expressed as mg ascorbic acid/g of dry extract (mg Vitamin C/g d.e.).

FRAP assay

The reducing power of methanol, ethyl acetate and *n*-hexane extracts was determined using the FRAP assay [37]. This assay is based on the reducing power of a compound (antioxidant). The potential antioxidant is expected to reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}); the latter forms a blue complex (Fe^{2+}/2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), which increases the absorption at 593 nm. Briefly, the FRAP reagent was prepared by mixing acetate buffer (200 μL, pH 3.6), a solution of 20 μL TPTZ and 20 μL $FeCl_3$. The sample solution (30 μL) and the reagent (0.9 mL FRAP) were mixed thoroughly and incubated at 37°C. Absorbance was taken at 593 nm after 5 min. A standard calibration curve was prepared using different concentrations of $FeSO_4\times7H_2O$. All solutions were freshly prepared. The results were expressed in mmol Fe/mg dried extract (mmol Fe/mg d.e.).

All the assays described above were performed using a Perkin Elmer Lambda Bio UV-Vis spectrometer.

Extraction and Isolation for HPLC and NMR analysis

Intact air-dried leaves (1.00 g) were sonicated in CH_2Cl_2 at room temperature for 10 min. After filtration and evaporation of the solvent, the residue was treated with methanol (MeOH)/hexane 1:1. The lower MeOH layer was separated in separation funnel, and the hexane layer was extracted with MeOH. The combined MeOH extracts were evaporated and the dry residue was used for HPLC and NMR analyses. NMR spectra were recorded in a low-dielectric solvent, $CDCl_3$, on a Bruker AVANCE III (500 MHz). Chromatographic analysis of the combined MeOH extracts was performed on a 1100 series liquid chromatograph (Agilent Technologies)

equipped with a diode array detector (DAD; λ=210 nm) and autosampler. The separation column was a Zorbax Eclipse XDB C18 (250×9.4 mm; 5 μm). The column heater was set at 20°C and the mobile phase flow rate was maintained at 0.5 mL min-1. The gradient (solvent A water, solvent B, 100% acetonitrile) was 0-5 min, 10% B; 5-20 min, 10-35% B; 20-30 min, 35% B, 30-40 min, 35-50% B; 40-60 min, 50% B; 60-61 min, 50-10% B; 61-66 min, 10% B. The injection volume was 1 μL.

Statistical analysis

All the experiments were carried out in triplicate. The results are expressed as mean values and standard errors of the mean. The existence of significant differences among the results for total phenol and flavonoid contents and the antioxidant properties of the extracts were analyzed by analysis of variance (ANOVA) [38]. The IC50 values obtained in the antioxidant assays established the regression equation between the sample concentrations and the scavenging effect.

RESULTS

Leaf micromorphology

Scanning electron micrographs of the adaxial and abaxial leaf blade of *A. elegans* are presented in Fig. 1. Epidermal cells show sinuous anticlinal walls (Fig. 1B). Densely distributed, uniseriate, nonglandular curly trichomes were noticed on both sides on the leaf blade, but there were much more on the abaxial side (Fig. 1C and D); thus, the leaves are velvety underneath. Rare glandular trichomes were observed on the adaxial side (Fig. 1A and B). The glandular trichomes are of the capitate type, seen as glandular dots (Fig. 1A and B). On the abaxial side, glandular trichomes were not recorded as the abaxial side was densely covered with nonglandular curly trichomes.

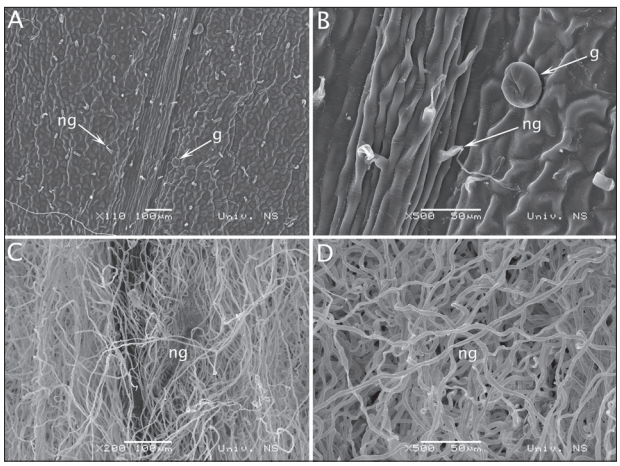

Fig. 1. Scanning electron micrographs of the adaxial and abaxial leaf blade of *Amphoricarpos elegans*. **A, B** – rare uniseriate, curly nonglandular and glandular trichomes on the adaxial leaf blade surface; **C, D** – densely distributed, uniseriate, curly nonglandular trichomes on the abaxial leaf blade surface.

Total phenol and flavonoid content

The total phenol contents varied significantly between the extracts, with the methanol extract containing the largest amount of phenolics (Table 1). No phenols or flavonoids were detected in the *n*-hexane fraction.

Antioxidative activity

Since there were no flavonoids or phenols present in the *n*-hexane fraction (Table 1), ethyl acetate and methanol fractions were used in the antioxidative assays. In all three assays, the methanol extract showed the highest antioxidative potential. As there is little to no difference between the total flavonoid contents in both extracts, most of the antioxidative potential can be attributed to phenols. However, the antioxidative potential of the methanol fraction, as determined by the DPPH assay, was more than ten times higher than that of the ethyl acetate fraction.

Identification and characterization of 3-deoxyamphoricarpolide

In the HPLC chromatogram of the *A. elegans* extract, one peak is dominant (Fig. 2). After comparison with chromatograms and retention characteristics of previously isolated and characterized amphoricarpolides from Balkan species of *Amphoricarpos*, it was concluded that this dominant peak is 3-deoxyamphoricarpolide [26].

The ^{1}H NMR spectrum of the *A. elegans* extract (Fig. 3) confirmed the results of HPLC; namely, the signals from H-14, H-14', H-13, H-13', H-6, H-1 and H-9β are in agreement with the previously isolated and characterized compound from Balkan *Amphoricarpos* taxa [26].

Table 1. Total phenolic and flavonoid contents and antioxidant activity of *n*-hexane, ethyl acetate and methanol leaf extracts of *Amphoricarpos elegans*.

Extracts	Yield	Total phenols	Total flavonoids	DPPH	ABTS	FRAP
	[%]	GAE µg/mg	QUE µg/mg	IC50 mg/mL	AAEC mg/mL	Fe^{2+} mmol/mg
n-hexane	6.65	-	-	-	-	-
Ethyl acetate*	2.32	19.9^{a}±12.8	86.6^{a}±8.9	3.50	0.03^{a}±0.01	0.13^{a}±0.01
Methanol*	9.67	96.3^{b}±5.5	96.7^{a}±7.7	0.24	0.07^{b}±0.01	0.32^{b}±0.01

*The samples were analyzed in triplicate (n=3) and expressed as the mean±standard deviation, except for the DPPH assay (IC50). Means followed by different superscripts (a-b) within the same column indicate statistically significant differences based on ANOVA ($p<0.05$)

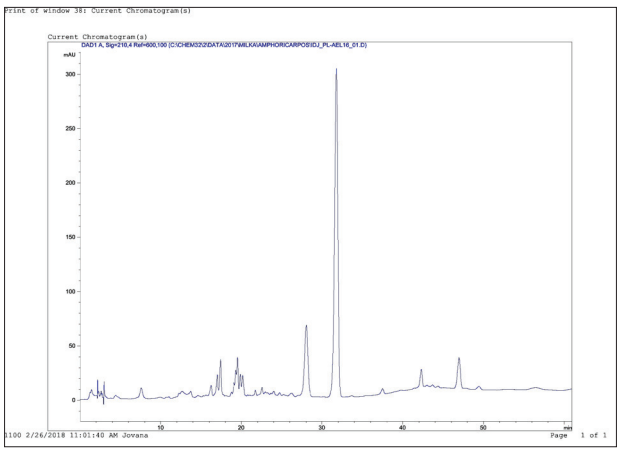

Fig. 2. HPLC chromatogram of the methanol extract of *Amphoricarpos elegans*.

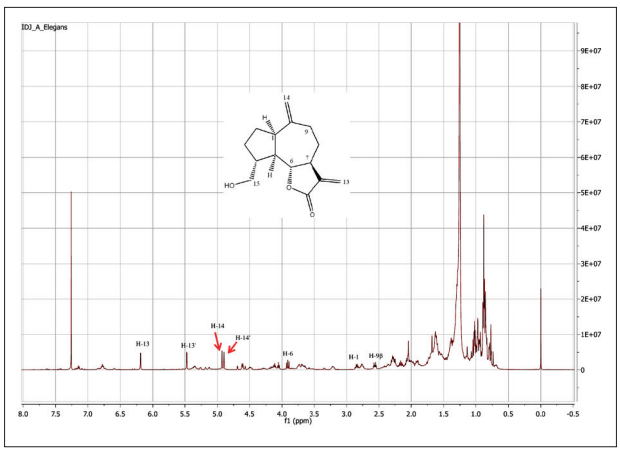

Fig. 3. ^{1}H NMR spectrum of the methanol extract of *Amphoricarpos elegans*.

DISCUSSION

Cavities, ducts, idioblasts and glandular trichomes are found inside and on the leaves of many Compositae members [39-41]. In the whole Compositae family, essential oils, resins, alkaloids, lipids, tannins, pectin-like substances, flavonoids and sesquiterpene lactones are the main products located mostly in ducts and glands [42]. Based on the extraction of the main classes of metabolites of Compositae, glandular trichomes are considered the main sites of biosynthesis and accumulation of sesquiterpene lactones, assuming important roles in the biological activity, ecology and chemotaxonomy of the family [12,16,43-48].

In Compositae, especially in the tribe Cardueae, specialized metabolites predominantly include lipophilic constituents (particularly sesquiterpene lactones), while hydrophilic compounds are scarcely represented [18]. Guaianolide-type sesquiterpene lactones representing one of the largest groups among the sesquiterpene lactones from Compositae, generally exhibit a low complexity [26]. The common feature of all guaianolides isolated from *Amphoricarpos*, and named amphoricarpolides, is that they have a unique oxygenation pattern that is not found in any Cardueae nor in other tribes of Compositae. All previous phytochemical studies [26-28] of *Amphoricarpos* taxa from the western Balkans revealed thirty amphoricarpolides in total (with the same guaianolide skeleton). Our investigation of the rare and stenoendemic species *A. elegans* from western Caucasus shows that 3-deoxyamphoricarpolide is dominant. This finding, as regards the chemotaxonomic significance of amphoricarpolides, represents a link between Balkan and Caucasian species of the same genus. Since *A. elegans* is a stenoendemic species, we used a very small amount of plant material (only 1 g) for lactone isolation, in comparison to previous investigations, where much more of the plant material was used [26,27]. This obstacle resulted in the identification and characterization of only the dominant sesquiterpene lactone with a guaianolide skeleton – 3-deoxyamphoricarpolide.

Literature on the biological activities of Balkan *Amphoricarpos* species is scarce; the cytotoxic activity of *A. neumayerianus* [14] and the antifungal activity of the leaf-surface constituents of *A. autariatus* ssp. *autariatus* [29] have been described. Also, our previous research on the antimicrobial and antioxidative activities of various leaf extracts of three Balkan *Amphoricarpos* taxa showed a very high antimicrobial and a considerable antioxidative potential [11]. The highest total flavonoid and phenolic contents, as well as the best antioxidative activity (DPPH assay) were observed in the methanol extract of *A. autariatus* ssp. *autariatus*. This finding is in accordance with current results, as we also show that the methanol extract of *A. elegans* possessed the highest total flavonoid and phenolic contents and exhibited the best antioxidative activity. The antioxidative potential of the methanol fraction, as observed in the DPPH test, was over ten times higher than of the ethyl acetate fraction. This cannot be contributed only to phenols since the amount of total phenolic compounds was only four times higher in the methanol fraction, and this could be linked to the proven presence of lactones.

Guaianolide-type sesquiterpene lactones produce effects related to the prevention and regulation of oxidative cellular damage and inflammation [16]. While the molecular mechanism of the antioxidative protection of sesquiterpene lactones is unknown, their antioxidant effects appear to be associated with the biosynthesis of endogenous antioxidants (e.g. prostaglandins) [17]. Previous studies have shown that the cyclic esters of hydroxycarboxylic acids containing the sesquiterpene lactones are responsible for their biological activities [49]. It has also been shown that the biological activity displayed by the majority of sesquiterpene lactones is due to the presence of α-methylene-γ-lactones and an α,β-unsaturated cyclopentenone ring [50]. For these reasons, our results regarding the antioxidative potential of *A. elegans* extracts and the finding of 3-deoxyamphoricarpolide are of importance for future research into biological activity, therapeutic action and new plant sources of medicinal substances.

CONCLUSION

This study represents a contribution to the leaf micromorphology, antioxidative activity and phytochemistry of an unexplored relict and limestone endemic species, *Amphoricarpos elegans* (Compositae) from Georgia. We showed the presence of glandular trichomes, the main sites of biosynthesis and accumulation of sesquiterpene lactones. Also, our phytochemical investigation provides a new record of 3-deoxyamphoricarpolide, which is a component of the related *Amphoricarpos* taxa from the Balkan Peninsula. The considerable antioxidative potential of this species indicates the need to continue the study of other species of the genus *Amphoricarpos* and also other species from related Cardueae genera (Compositae) and their potential application in pharmacy and medicine.

Funding: Financial support was provided by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project Nos. 173029 and 172053).

Author contributions: MG, NR, PJ drafted the manuscript; AB collected the plant material and drafted the manuscript; VT, IĐ, NGJ, AS, PM and PJ supervised the research and critically reviewed the manuscript. All authors read and approved the final manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest.

REFERENCES

1. Anderberg AA, Baldwin BG, Bayer RG, Breitwieser J, Jeffrey C, Dillon MO, Eldenäs P, Funk V, Garcia-Jacas N, Hind DJN, Karis PO, Lack HW, Nesom G, Nordenstam B, Oberprieler Ch, Panero JL, Puttock C, Robinson H, Stuessy TF, Susanna A, Urtubey E, Vogt R, Ward J., Watson LE. Compositae. In: Kubitzki K, editor. The families and genera of vascular plants. Vol. 8. Springer-Verlag Berlin Heidelberg; 2007. p. 61-588.
2. Robinson H. An introduction to micro-characters of Compositae. In: Funk VA, Susanna A, Stuessy TF, Bayer RJ, editors. Systematics, Evolution and Biogeography of Compositae. Vienna (Austria): IAPT; 2009. p. 89-100.
3. Taleb-Contini HS, Schorr K, Da Costa BF, Camilo D, de Oliveira R. Detection of flavonoids in glandular trichomes of *Chromolaena* species (Eupatorieae, Asteraceae) by reversed-phase high-performance liquid chromatography. Braz J Pharm Sci. 2007;43:2.
4. Fritz E. and Saukel J. Anatomy of subterranean organs of medicinally used Cardueae and related species and its value for discrimination. Sci Pharm. 2011;79:157-74.
5. Farrag NM, Abd El Aziz EM, El-Domiaty MM, El Shafea AM. Phytochemical investigation of *Centaurea araneosa* growing in Egypt. J Pharm Sci. 1993;2:29-45.

6. Font Quer P. Plantas Medicinales: El Dioscórides Renovado. 15th ed. Madrid: Labor; 1995. 1033 p.
7. Barrero AF, Herrador MM, Arteaga P, Cabrera E, Rodríguez-García I, García-Moreno M, Grávalos DG. Cytotoxic activity of flavonoids from *Carthamus arborescens*, *Ononis natrix* ssp. *ramosissima* and *Centaurea malacitana*. Fitoterapia. 1997;68:281-3.
8. Baba Aissa F. Flore d'Algérie et du Maghreb. Substances végétales d'Afrique, d'orient et d'occident. Encyclopédie des plantes utiles. Rouiba (Algeria): EDAS, Librairie moderne; 1999.
9. Vallejo JR, Pardo de Santayana M, Peral D, Carrasco MC, López D. Uso medicinal de *Atractylis gummifera* L. en Guadiana del Caudillo (Badajoz, Espana). Toxicidad y especies afines. Rev Fitoterapia. 2008;8:161-9.
10. Vallejo JR, Peral D, Gemio P, Carrasco MC, Heinrich M, Pardo de Santayana M. *Atractylis gummifera* and *Centaurea ornata* in the Province of Badajoz (Extremadura, Spain). Ethnopharmacological importance and toxicological risk. J Ethnopharmacol. 2009;126:366-70.
11. Gavrilović M, Soković MD, Stanković M, Marin PD, Stevanović ZD, Janaćković P. Antimicrobial and antioxidative activity of various leaf extracts of *Amphoricarpos* Vis. (Asteraceae) taxa. Arch Biol Sci. 2016;68:803-10.
12. Sokovic M, Ciric A, Glamoclija J, Skaltsa H. Biological activities of sesquiterpene lactones isolated from the genus *Centaurea* L. (Asteraceae). Curr Pharm Design. 2017;23:2767-86.
13. Milosavljević S, Bulatović V, Stefanović M. Sesquiterpene lactones from the Yugoslavian wild growing plant families Asteraceae and Apiaceae. J Serb Chem Soc. 1999;64:397-442.
14. Atrrog BAA, Natic M, Tosti T, Opsenica Milojković D, Djordjević I, Tešević V, Jadranin M, Milosavljević S, Lazić M, Radulović S, Tešić Z. Lipophilicity of some guaianolides isolated from two endemic subspecies of *Amphoricarpos neumayeri* (Asteraceae) from Montenegro. Biomed Chromatogr. 2008;23:250-6.
15. Repetto MG, Llesuy S. Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. Braz J Med Biol Res. 2002;35:523-34.
16. Da Costa F, Terfloth L, Gasteiger J. Sesquiterpene lactone-based classification of three Asteraceae tribes: a study based on self-organizing neural networks applied to chemosystematics. Phytochemistry. 2005;66:345-53.
17. Repetto MG, Boveris A. Bioactivity of sesquiterpenes: compounds that protect from alcohol-induced gastric mucosal lesions and oxidative damage. Mini Rev Med Chem. 2010;10:615-23.
18. Susanna A, Garcia-Jacas N. Cardueae (Carduoideae). In: Funk VA, Susanna A, Stuessy TF, Bayer RJ, editors. Systematics, Evolution and Biogeography of Compositae. Vienna (Austria): IAPT; 2009. p. 293-313.
19. Susanna A, Garcia-Jacas N. Tribe Cardueae Cass. (1819). In: Kubitzki K, editor. The Families and Genera of Vascular Plants. VIII Flowering Plants-Eudicots. Springer-Verlag Berlin Heidelberg; 2007. p. 123-46.
20. Barres L, Sanmartín I, Anderson CL, Susanna A, Buerki S, Galbany-Casals M, Vilatersana R. Reconstructing the evolution and biogeographic history of tribe Cardueae (Compositae). Am J Bot. 2013;100:867-82.
21. Shetekauri S, Kutateladze L. Diversity, ecotopology and hypsometric distribution of the endemic flora in high-mountain phytolandscapes of the Caucasus. Earth. 2017;6:38-48.
22. Akhalkatsi M, Ekhvaia J, Asanidze Z. Diversity and genetic erosion of ancient crops and wild relatives of agricultural cultivars for food: implications for nature conservation in Georgia (Caucasus). In: Tiefenbacher J, editor. Perspectives on Nature Conservation-Patterns, Pressures and Prospects. Rijeka, Croatia: InTech; 2012. p. 51-92.
23. Nakhutsrishvili G. The vegetation of Georgia (Caucasus). Camerino: Dipartimento di Botanica ed Ecologia dell' Univ; 1999. p. 74. (Braun-Blanquetia; Vol. 15).
24. Schatz G, Shulkina T, Nakhutsrishvili G, Batsatsashvili K, Tamanyan K, Ali-zade V, Kikodze D, Geltman D, Ekim T. Development of Plant Red List Assessments for the Caucasus Biodiversity Hotspot. In: Zazanashvili N, Mallon D, editors. Status and Protection of Globally Threatened Species in the Caucasus. Tbilisi: CEPF, WWF. Contour Ltd; 2009. p. 188-92.
25. Linczevsky IA. Compositae Tribes Echinopsideae and Cynareae. In: Schischkin BK, Bobrov FG, editors. Flora of the USSR. Vol. 27. Moscow, Leningrad: Akademiya Nauk SSSR; 1962. p. 74-9.
26. Djordjević I, Vajs V, Bulatović V, Menković N, Tešević V, Macura S, Janaćković P, Milosavljević S. Guaianolides from two subspecies of *Amphoricarpos neumayeri* from Montenegro. Phytochemistry. 2004;65:2337-45.
27. Djordjević I, Jadranin M, Vajs V, Menković N, Tešević V, Macura S, Milosavljević S. Further Guaianolides from *Amphoricarpos neumayeris* ssp. *murbeckii* from Montenegro. Z Naturforsch. 2006;61b:1437-42.
28. Cvetković M, Đorđević I, Jadranin M, Vajs V, Vučković I, Menković N, Milosavljevic S, Tešević V. Further Amphoricarpolides from the surface extracts of *Amphoricarpos* Complex from Montenegro. Chem Biodivers. 2014;11:1428-37.
29. Jadranin M, Djordjević I, Tešević V, Vajs V, Menković N, Soković M, Glamočlija J, Milosavljević S. Sesquiterpene Lactones of *Amphoricarpos autariatus* ssp. *autariatus* from Montenegro - Antifungal Leaf - Surface Constituents. Rec Nat Prod. 2013;7:234-8.
30. Petit DP. Generic interrelationships of the Cardueae (Compositae): A cladistic analysis of morphological data. Plant Syst Evol. 1997;207:173-203.
31. Caković D, Stešević D, Schönswetter P, Frajman B. How many taxa? Spatiotemporal evolution and taxonomy of *Amphoricarpos* (Asteraceae, Carduoideae) on the Balkan Peninsula. Org Divers Evol. 2015;15:429-45.
32. Singleton VL, Orthofer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent oxidants and antioxidants. Method Enzymol. 1999;299:152-78.
33. Matejić J, Džamić A, Mihajilov-Krstev T, Ranđelović V, Krivošej Z, Marin P. Total phenolic content, flavonoid concentration, antioxidant and antimicrobial activity of methanol extracts from three *Seseli* L. taxa. Cent Eur J Biol. 2012;7:1116-22.

34. Tekao T, Watanabe N, Yagi I, Sakata K. A simple screening method for antioxidant and isolation of several antioxidants produced by marine Bacteria from fish and shellfish. Biosci Biotechnol Biochem. 1994;58:1780-3.
35. Kumarasamy Y, Byres M, Cox PJ, Jasapars M, Nahar L, Sarker SD. Screening seeds of some scottish plants for free radical scavenging activity. Phytother Res. 2007;21:615-21.
36. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Bio Med. 1999;26:1231-7.
37. Li HB, Wong CC, Cheng KW, Chen F. Antioxidant properties in vitro and total phenolic contents in methanol extracts from medicinal plants. Lebensm-Wiss Technol. 2008;41:385-90.
38. Hammer Ø, Harper DAT, Ryan PD. PAST: paleontological statistics software package for education and data analysis. Palaeontol Electronica. 2001;4:1-9.
39. Milan P, Hayashi AH, Appezzato-da-Glória B. Comparative leaf morphology and anatomy of three Asteraceae species. Braz Arch Biol Techn. 2006;49:135-44.
40. Duarte MR, Budel JM, Matzenbacher NI. Menarim DO. Microscopic diagnosis of the leaf and stem of *Lucilia nitens* Less., Asteraceae. Lat Am J Pharm. 2011;30:2070-5.
41. Camilotti JG, Biu CC, Farago PV, Santos VLPD, Franco CRC, Budel JM. Anatomical characters of leave and stem of *Calea serrata* Less., Asteraceae. Braz Arch Biol Techn. 2014;57:867-73.
42. Bartoli A, Galati BG, Tortosa RD. Anatomical studies of the secretory structures: glandular trichomes and ducts, in *Grindelia pulchella* Dunal (Astereae, Asteraceae). Flora. 2011;206:1063-8.
43. Spring O, Benz T, Ilg M. Sesquiterpene lactones of the capitate glandular trichomes of *Helianthus annuus*. Phytochemistry. 1989;28:745-9.
44. Spring O. Trichome microsampling of sesquiterpene lactones for the use of systematic studies. Recent Adv Phytochem. 1991;25:319-45.
45. Spring O, Zipper R, Reeb S, Vogler B, Da Costa FB. Sesquiterpene lactones and a myoinositol from glandular trichomes of *Viguiera quinqueremis* (Heliantheae; Asteraceae). Phytochemistry. 2001;57:267-72.
46. Sakamoto HT, Gobbo-Neto L, Cavalheiro AJ, Lopes NP, Lopes JLC. Quantitative HPLC analysis of sesquiterpene lactones and determination of chemotypes in *Eremanthus seidelii* MacLeish & Schumacher (Asteraceae). J Brazil Chem Soc. 2005;16:1396-401
47. Appezzato-da-Glória B, Da Costa FB, Silva VC, Gobbo-Neto L, Rehder VLG, Hayashi AH. Glandular trichomes on aerial and underground organs in *Chrysolaena* species (Vernonieae - Asteraceae): structure, ultrastructure and chemical composition. Flora. 2012;207:878-87
48. Lusa MG, Da Costa FB, Appezzato-da-Glória B. Histolocalization of chemotaxonomic markers in Brazilian Vernonieae (Asteraceae). Bot J Linn Soc. 2016;182:581-93.
49. Raola VK and Chakraborty K. Biogenic guaianolide-type sesquiterpene lactones with antioxidative and anti-inflammatory properties from natural mangrove hybrid *Rhizophora annamalayana*. Nat Prod Res. 2017;31:2719-29.
50. Chaturvedi, D. Sesquiterpene lactones: structural diversity and their biological activities. In: Tiwari VK, Mishra BB, editors. Opportunity, Challanges and Scope of Natural Products in Medicinal Chemistry. Trivandrum: Research Signpost; 2011. p. 313-34.