Comprehensive combined chemical and pharmacognostic approach in the investigation of Montenegrin flora, with emphasis on endemic species: Past performance and future potential

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Herein, we have reviewed the comparative analysis relating pharmacognostic and chemical approaches in the investigation of the chosen plants from Montenegrin flora known for the constituents recognized as carriers of different biological activities. In addition, some of the mentioned extensively studied plants were with uncertain status in the systematics, as not being recognized as new species. The results of pharmacognostic studies with the thorough chemical analyses addressing the problem in positioning some of the investigated plants from *Swertia*, *Gentiana* and *Gentianella* genera belonging to family Gentianaceae in systematics, performed within the collaboration of two research groups from Institute for Medicinal Plants Research "Dr. Josif Pančić" and Faculty of Chemistry, the University of Belgrade under the leadership of Dr. Nebojša Menković and Prof. Dr. Slobodan Milosavljević were summarized. In addition, the complete chemical structure elucidation of sesquiterpene lactones present in *Anthemis* sp. as potential anti-inflammatory agents, applying the sophisticated 2D NMR techniques was reviewed. Further, the parthenolide content determination in *Tanacetum larvatum* revealed the possibility of the successful application of ^{1}H NMR techniques in quantification studies. The findings presented in the published literature stressed the importance of a combined chemical and pharmacognostic approach in the investigation of natural products originating from plants.

Key words: Combined pharmacognostic and chemical research; *Swertia*, *Gentiana*, *Gentianella*, *Anthemis* and *Tanacetum* species; oxygenated xanthones; sesquiterpene lactones, taxonomic markers; structure-activity investigation of secondary metabolites

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1. INTRODUCTION

Emerging trends in the current research and development strategies in pharmacy and medicines are prompting the interest in natural products as a source of chemical diversity for different types of medicines. Several different strategies based on chemical, pharmacognostic, and ethnopharmacological observations, resulting in consistent mapping for establishing the structure-activity relationship of drug-related chemicals, have been employed in exploring the biodiversity of the plants in the search for novel chemical structures and biological activities.

Given the numerous, naturally originated, chemically distinct molecules with significant and different biological functions, the intriguing question that might help in directing the investigation, is whether the naturally occurring, biologically active, and biosystematically important secondary metabolites exist in more than one particular species, recognized in traditional medicine. The second question is whether it would be of value to use unconventional and geographically limited plant sources that have never been systematically explored. In consent with such approaches, the rediscovery of known compounds recognized as carriers of the biological effects in traditionally used, well-characterized, and investigated medicinal plants, but in our investigations in endemic or in distantly related species, or characterization of the novel compounds found either in traditionally recognized sources or new ones, could represent the base for the novel systematic approach, comprising the study of the unique chemical fingerprints as the base for metabolomics investigations, as well. Further, in order to fully understand those problems associated with the complexity of correlation between the chemistry of the plants and their biological activities, one must possess the knowledge of the plants themselves, the chemical constituents in the plants, methods for their identification, aiming in pointing out the biochemistry pathways responsible for the certain profile of the secondary metabolites present in the species. Medicinal herbs have been remaining as the basis for the new drug discovery. According to World Health Organization, the demand for medicinal plants is US $14 billion per year and is likely to increase to more than US $5 trillion by 2050. However, the efficacy of many of them has not been established, lacking in the chemical, biochemical, physical, and biological determinants important for the precise definition of their place both in herbal medicine and in systematic.

The research on the Balkans' flora at the beginning of the 20th century (1927) revealed the presence of 6750 species, about 1750 being endemic. Afterward, a large number of new taxa have been described, and within the new species have been discovered, causing the increase of a total number of species identified, and thus endemics, as well (estimated at 7,500 species and close to 2,000 endemics). Interestingly, 3920 species are listed in the flora of Montenegro (taking into account the subspecies, the number amounts to 4140). According to the indicator of flora density (representing the number of species/area of the territory, which is 0.844), Montenegro takes second place in Europe (after Greece). The great complex of Montenegrin flora represents the challenge for both chemists and pharmacognostic investigators. Namely, out of 3600 species and subspecies detected on the territory of Montenegro, the total number of Balkan endemics taxa of the rank of species and subspecies is 372 (Vuksanović, 2016). The Durmitor massif has a total of about 1300 plant species, of which 122 are endemic. Prokletije has about 1400 plant species, 126 being endemic (Stevanović, 1999; Stevanović and Vasić, 1995). That was the reason that the joint research of different plant species, originated from Montenegro, had been undertaken by the Institute for Medicinal Plants Research and the research group of Professor Slobodan Milosavljevic from the Faculty of Chemistry, University of Belgrade. This review has the aim to give insight into the necessity of a combined approach to the investigation of plants, providing the detailed information on their chemistry that might be used in the proper determination of pharmacological properties, and their place in plant systematic, when it is impossible to distinguish two botanically similar species. Several secondary metabolites were chosen as the final targets for a comparative chemical survey – iridoids, xanthones, and sesquiterpene lactones being investigated most thoroughly within several plant genera – *Gentiana*, *Gentianella*, *Swertia*, *Anthemis*, and *Tanacetum*. Providing the chemical fingerprint of the investigated plants served as the base for not only prediction of their possible pharmacological properties, but as well might contribute to solving the dilemma existing in their chemotaxonomy. Taking into account that this review contemplated the investigations conducted in joint research of Chemical Faculty University of Belgrade and Institute for Medicinal Plants Research "Dr. Josif Pančić", the pharmacognostic and chemical investigation would point out the significance of their cooperation.

2. PHARMACOGNOSTIC STUDIES ON MONTENEGRIN FLORA

Professor of Natural Sciences Dr. Josif Pančić undertook the research of Montenegrin flora in the second half of XIX century, where his investigation addressed mainly its floristic elements (Pančić, 1874). Pančić reached Montenegro from Belgrade in July 1873 via Trieste and Kotor, Cetinje being the first stop. This detour was the consequence of the political and security issues in that period - namely, it was necessary to circumvent Sandžak, as part of the Ottoman Empire, located between Serbia and Montenegro. From Kotor Pančić, via Njeguš had gone to Cetinje from where he explored Lovćen (Jezerski vrh and Štirovnik). After that, he left for Durmitor, through Katunska nahija, Bjelopavlovići, Danilovgrad, and the Ostrog Monastery. Afterward, he explored the flora of Nikšić and Lukovo polje. Via Šavnik, he had arrived in Žabljak, from where he went to Durmitor, there visiting the sites on Crvena greda and the lake Crno jezero, as well as the peaks of Stouc and Sljeme. After he had visited the Morača Monastery via Tušina and Javorje, he stayed in Komovi for a short time, where he found rare and new species, of which the most significant were certainly the species: *Heliosperma macrantha* Pančić, *Valeriana bertiscea* Pančić, and *Hieracium naeglianum* Pančić, all having now the status in botany as "good species" (Pulević, 2006). From Komovi, he returned to Cetinje, via Morača, Zeta, and Skadasko Lake (Vir), afterward making the trip to Sutorman, and from Vir he arrived in Cetinje via the Crnojević River. From Cetinje, through Cuca and Grahovo polje, to Bijela gora, Vučji zub, and Orijen, then through Krivoši and Risan, he reached Kotor, closing the circle. Two years later, in 1875, in Belgrade, he published his famous work "Elenchus plantarum vascularum quas aestate a. 1873 in Montenegro" (Pančić, 1874), describing almost 1300 species, nine of them being new.

Having in a mind the directions of Dr. Josif Pančić investigation, the researchers from the Institute for Medicinal Plants Research "Dr. Josif Pančić" (further in the text "Institute"), followed his route, with the aim to explore the floral richness and diversity and to collect the plants (with specific attention to endemic species) (Figure 1). In order to enable the chemical characterization of the collected plant material, the tight cooperation with the research group of Prof. Slobodan Milosavljević from the Faculty of Chemistry, University of Belgrade, was established.

The investigation of Montenegrin flora by Institute's and prof. Milosavljević's research teams were undertaken on Orien, Bjelasica, Visitor, Hajla, Komovi, Prokletije, and Sinjajevina. Orjen was investigated from 2000 to 2007. The locations where the pharmacognostic research was conducted were: Kruševica, Vrbanj, Orjen sedlo, Orjen strane, Orjen vrh, Reovačka greda, Reovci, Zubački kabao, Jarčeva tvrđa, Ubli, Grab, Podstirovnik. The sub-Mediterranean and Mediterranean areas were explored, as well, from Herceg Novi to Kamen and Kruševica, then Luštica, and part from Risan to Crkvica. The mountain Bjelasica was explored in the period from 1998 to 2006, on localities: Svinjača, Mušovića rijeka, Jezerine, Klisura, katuni Vranjak, Potoci, Slađevac, Klisura, Melaja then Troglava, Ogorela glava and Zekova Glava, Pešića lake and wider surroundings, then Ocka, Šiško Lake, Kurikuće, Otaševa lica. In the period from 2001 to 2007, the following sites at Visitor were explored: Pepići, Pepićka reka, Zaparenik, Tomov Laz, Jagnjičar, Visitor Lake, Mramorje, Marašov katun, Kachuber, Valje, Velji vrh, Bandera vrh, Kupušnjak, as well as a number of unnamed localities especially on the slopes of the mountain. Hajla and Ahmica were investigated during the period of 1998 to 2007, especially Dramandol, then the area towards Ahmica from Ćafa Hajla, over Ujka's karst to the slopes and the top of Ahmica. The Čakor Mountain is located in the Northeast of Montenegro and geographically belongs to Prokletije. Representing the natural border between Adriatic and Black Sea basins, it is rich in flora. The investigation of this area was performed during 1998-2006 (Figure 2). The Čakor Mountain stretches north-south, while to the east it is open through the Rugova gorge towards Metohija, and to the west, towards the valley of the river Lim, it descends through the large amphitheater of the village Velika. In the south, it starts from the Dio saddle, then across the Čakor and Lijepi do passes spread to the north to Ćafa, the saddle between Mokra

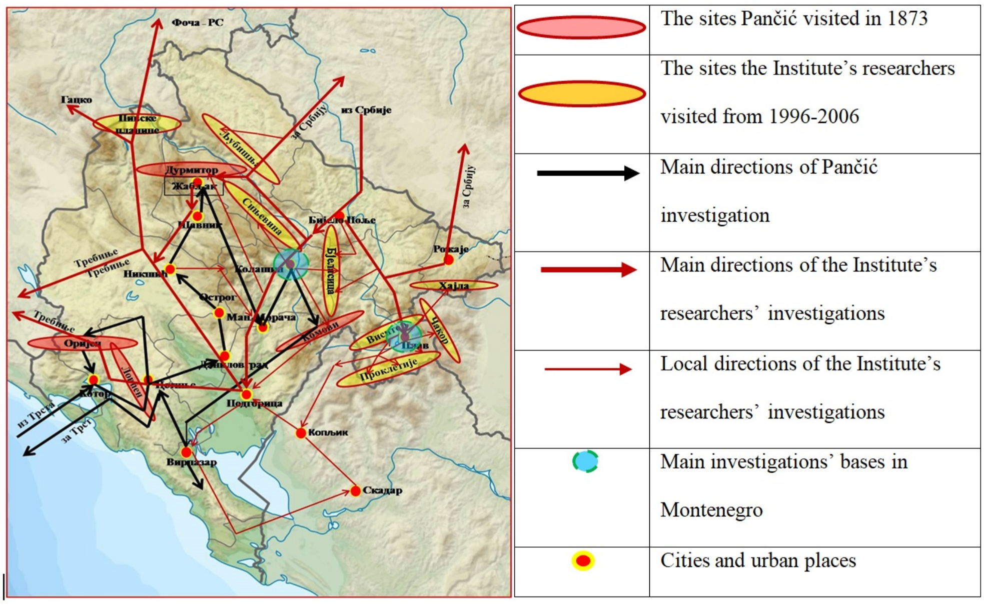


Fig. 1. A comparative presentation of the research routes of Dr. Josif Pančić undertaken in 1873 and research teams from Institute for Medicinal Plants Research "Dr. Josif Pančić" and Faculty of Chemistry, University of Belgrade during 1999-2007 in Montenegro

Table 1. The plant species present at the research areas of Montenegro with the most prominent pharmacognosy, chemical, and economic significance

#	Specie	Family	Drug
1	*Abies alba* Mill.	Pinaceae	Raw fir leafs; cone; bark
2	*Achillea millefolium* L	Asteraceae	Herbaceous above-ground
3	*Agrimonia eupatoria* L.	Rosaceae	Herbaceous above-ground
4	*Alchemilla hybrida* Mill.	Rossaceae	Herbaceous above-ground
5	*Alchemilla vulgaris* L.	Rossaceae	Herbaceous above-ground
6	*Arctostaphyllos uva ursi* (L.) Spreng. (Figure 5)	Ericacae	Leaf
7	*Betula alba* L.	Betulaceae	Bark and leaf
8	*Epilobium angustifolium* L.	Oenotheraceae	Herbaceous above-ground part or only leaf
9	*Fragaria vesca* L.	Rosaceae	Leaf
10	*Gentiana asclepiadea* L.	Gentianaceae	Root
11	*Hypericum perforatum* L.	Hypericaceae	Herbaceous above-ground part
12	*Juniperus communis* L.	Cupressaceae	Fruit, wood and essential oils
13	*Orchis morio* L.	Orchidaceae	Tuber
14	*Origanum vulgare* L.	Lamiaceae	Tips of flowering branches and essential oils
15	*Pinus mhugo* Turra.	Pinaceae	Tips of branches and essential oils
16	*Polygonum bistorta* L.	Polygonaceae	Rhizome
17	*Primula elatior* L.	Primulaceae	Root and flower
18	*Rosa canina* L.	Rosaceae	Fruit
19	*Salix purpurea* L.	Salicaceae	Bark
20	*Taraxacum officinale* Weber	Asteraceae	Root, rarely entire plant with root
21	*Urtica dioica* L.	Urticaceae	Leaf and root and aboveground part
22	*Vaccinium myrtillus* L. (Figure 4)	Ericacae	Fruit, leaf and sprouts
23	*Veratrum album* L.	Liliaceae	Rhizome with roots
24	*Verbascum thapsus* L.	Liliaceae	Flower
25	*Viola tricoloris* L.	Violaceae	Above-ground part

Fig. 2. The main routes at the Čakor Mountain investigated by Institute's and Faculty of Chemistry research teams during 1998-2006

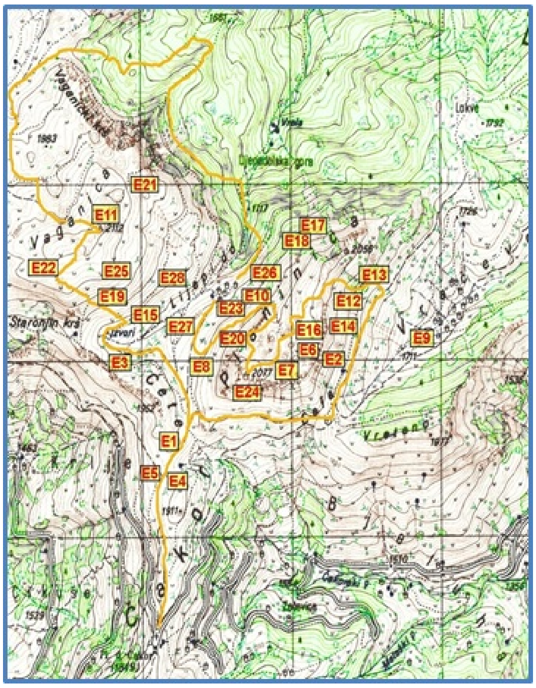


Fig. 3. The cartographic representation of the endemic species identified in investigated areas of the Mountain Čakor

and Vaganica. The mountain Čakor consists of the mountains Vaganica (the highest peak 2112 m), Planinica (2077 m and 2054 m), Vreteno (1961 m), and Đevojački Vrh (2057 m), interconnected bypasses and extending in a line in a north-south direction. Between Đevojački Krš, Planinica and Vreten, there is the Čakor pass (1849 m, being at the same time the lowest point of mountain). Lijepi do is located between Vaganica and Planinica, beginning at an altitude of 1915 m and gently descends towards Vaganička gora (1700 m) and Vraćevo. Lijepi do, with the northern slopes of Planinica and the eastern sides of Vaganica, as well as the tops of Vaganička gora represent the floristic center of the mountain region Čakor. Due to its geographical position, relief forms, rich and diverse flora, barrenness, and wilderness, the mountainous region of Čakor represent one of the most beautiful Montenegrin landscapes. In the foreground, west of Vaganica, the Visitor massif (2211 m) with Greben and Zeletina continues towards Jerinja glava and Bjelasica, in the background covered by gigantic Komovi. The northeastern part in the foreground consists of Cmiljevica and the mighty Hajla (2403 m), and at the end, the Golija and Kopaonik massifs (2017 m) can be seen in the distance.

During the research 1998-2006, the joint team of Institute and Prof. Milosavljević, evaluated the diversity of the Montenegrin flora, with the specific attention to those species characterized by prominent pharmacognosy and chemical significance (Menković et al., 2011; Tasić et al., 2009; Tešević et al., 2007; Trifunović et al., 2006; Zdunić, 2012; Zdunić et al., 2011). In addition, it was important to point out the species that could be exploited for economic purposes rationally and without endangering their survival due to their abundant presence in nature (Table 1). Within the plants detected at the investigated locations, the presence of the endemic species was confirmed and listed in Table 2 and Figure 3. The presented findings were in accordance with previously performed research and detected species (Braun-Blanquet, 1964; Josifović, 1975; Lakušić, 1968; 1971; 1974; 1990; Lakušić and Milojević, 1972; Lakušić et al., 1985; Lukić, 1985; Milojević et al., 1974; Pulević, 2005; Schilcher et al., 2007; Šilić, 1983; 1984; Tucakov et al., 1974).

Fig. 4. *Vaccinium myrtillus* L. Ericacae (Photo by Jeremić Miroslav)

Fig. 5. *Arctostaphyllos uva ursi* (L.) Spreng., Ericacae (Photo by Jeremić Miroslav, taken at the peak Planinice (2077 m), the Čakor Mountain)

Among numerous medicinal plants disclosed during the research conducted on mountains Prokletije and Komovi, it was revealed that *V. myrtillus* (Figure 4) was widespread throughout Montenegrin meadows and pastures, whose aerial parts, *Vaccinii herba* and *Vaccinii fructus* are official in European Pharmacopeia (Milojević et al., 1974). Apart from this plant, *A. uva ursi* (Figure 5) represent one of the most economically important species in Montenegrin flora, distributed in significant abundancy on Prokletije, Komovi, and Bjelasica (Gorunović and Lukić, 2001; Lakušić and Milojević, 1972).

The conducted studies were focused on the plant species, either those having the application in pharmaceutical, chemical,

Table 2. Identified endemic species at the investigated area of the mountains Čakor, Bjelasica, Prokletije and Durmitor

Code	Species	Family	Code	Species	Family
E1	*Acer heldreichii* Orph.	Aceraceae	E16	*Pancicia serbica* Vis.	Umbelliferae
E2	*Achillea abrotanoides* Vis.	Asteraceae	E17	*Pedicularis brachyodonta* Schloss. Et Vukot.	Scrophulariaceae
E3	*Achillea lingulata* W. et K.	Asteraceae	E18	*Pinus peuce* Grisebach	Pinaceae
E4	*Aconitum vulparia* Reich.†	Ranunculaceae	E19	*Plantago reniformis* G. Beck.	Plantaginaceae
E5	*Aconitum penteri* Hay.	Ranunculaceae	E20	*Potentilla montenegrina* Pant.	Rosaceae
E7	*Anthyllis jacquini* Kern	Fabaceae	E21	*Ranunculus scutatus* Waldst. & Kit	Ranunculaceae
E8	*Cerastium dinaricum* Beck et Szysz.	Caryophyllaceae	E22	*Sempervivum kosaninii* Praeger	Crassulaceae
E10	*Euphorbia myrsinites* L.	Euphorbiaceae	E23	*Tanacetum larvatum* (Pant.) Hayek	Asteraceae
E11	*Geum bulgaricum* Pančić	Rosaceae	E24	*Valeriana pancicii* Halacsy & Bald	Valerianaceae
E12	*Lilium albanicum* Gris	Liliaceae	E25	*Viola nicolai* Pant.	Violaceae
E13	*Onobrychis scardica* Griseb.	Fabaceae	E26	*Wulfenia blecicii* Lakušić	Scrophulariaceae
E14	*Oxytropis dinarica* Murb.	Fabaceae	E27	*Gentianella bulgarica* (Velen.) Holub	Gentianaceae
E15	*Oxytropis halleri* Koch	Fabaceae	E28	*Gentianella crispata* (Vis.) Holub	Gentianaceae

† *Aconitum vulparia* Reich. ssp. *pantoscekiaum* (Deg.& Bolu) Hay.

food, and cosmetic industries, at the same time known for their application in scientific and traditional medicine (official domestic and foreign pharmacopoeias) or being rare, endemic or specific for the investigated area (a potential source of new medicinal material, at the same time, providing the opportunity for comprehensive pharmacognostic and chemical research). Based on the above-mentioned requests, but being aware of the growing trend for research and exploitation of medicinal plants, this review was oriented mainly to the successfully applied pharmacognostic and chemistry approach in the research of several species, known as a rich source of either xanthones, or sesquiterpene lactones (Aljančić et al., 2008; Šavikin et al., 2015; Godjevac et al., 2004; Gođevac et al., 2006a;b; Janković et al., 2005; 2009; 2011; Krstić et al., 2004; Menković et al., 2002; 2014; Milosavljević et al., 1998; Šavikin-Fodulović et al., 2003; Šavikin et al., 2010; Tešević et al., 2007; Trifunović et al., 2006; Zdunić, 2012; Zdunić et al., 2011). Namely, the constant development of analytic methods, along with intensified pharmacological and clinical researches, has been opening the interesting field of quantitative structure-activity relationship (QSAR), stressing the importance of structure elucidation of isolated natural compounds from plants.

Xanthones, the secondary metabolites found in some higher plants, fungi, and lichens, due to their high taxonomic values aroused the noteworthy interest in order to research their pharmacological properties. In 1977, xanthone glycosides were described by Hostettmann and Wagner as an extensive group of natural xanthones. Based on the literature, about 650 xanthones are known from natural sources, the majority occurring in two plant families, Gentianaceae and Hypericaceae (Hostettmann and Wagner, 1977; Niaz and Khan, 2020). Iridoids represent one of the most important secondary metabolites in Gentianaceae, while Asteraceae is the family known for abundance in sesquiterpene lactones. With >1500 known members, the iridoids have been frequently investigated in plant chemosystematics studies, beginning with Dahlgren in the 1980s (Dahlgren, 1980). Apart from divergent findings in insects, the main distribution of iridoids is in the subclass Asteridae of the flowering plant family Gentianaceae, which is known for their presence in quite significant quantity. In addition, they are known to possess a variety of biological activities. The genus *Gentiana* comprises approximately 400 species. Many species have a wide range of pharmacological activities and have been used therapeutically for thousands of years. This review presents updated information concerning the recent progress on chemical and pharmacognostic analysis of some of the species belonging to Gentianaceae. Detailed and comprehensive data illustrate the numerous newly discovered monoterpene derivatives (xanthones and iridoids) and confirm the presence of the known ones responsible for therapeutic uses, stressing the existing structure-activity relationship.

Sesquiterpene lactones (SLs) represent one of the biggest groups of secondary metabolites, one of the largest biogenetically homogenous groups of natural products known. Currently, the Dictionary of Natural Products holds over 11000 entries on sesquiterpene lactones. They represent the class of terpenoids with more than 30 skeleton subtypes and several substitutional features, known not only as carriers of different biological activities (antitumor, allergenic, phytotoxic, antimicrobial, insecticidal, etc.) but as well as chemotaxonomic markers. Namely, in the early 1960s, major emphasis was on the use of SLs as taxonomic markers in systematic biochemical studies (Djordjević et al., 2004; Milosavljević et al., 1999a; 1998; Tadić et al., 2009). Although the species-rich family of Asteraceae is known for significant SLs content, this group of natural compounds might be found in some other plant families, like Lauraceae, Apiaceae, Burseraceae, and Magnoliaceae, as well as in some fungi. Here, *Anthemis* sp. and *Tanacetum* sp. will be presented as the representatives, taking into consideration that those genera were thoroughly investigated regarding their chemical composition, with the connection to taxonomic significance (Aljančić et al., 2010; 2001; Bulatović et al., 1997a; 2006; 1997b; Tadić et al., 2010; Vajs et al., 1999; 2000).

3. THE COMPARATIVE STUDIES

3.1. The comparative study on *Swertia perennis* and *S. punctata*

In the flora of Europe, there is only one species of the genus *Swertia*, *S. perennis*, which belongs to the Pentamera section, the Alteae subsection, and the Perennis series. *S. perennis* is an arcto-tertiary relict of mountain flora. The Perennis series includes two more vicarious species: *S. iberica* (Caucasian floral element) and *S. obtusa*, which is a widespread Asian species. Although only *S. perennis* is officially recognized in European Flora. Tan and Vladimirov (2001) claimed recently that *S. punctata* growing on the moistly terrains of the Western Stara Planina mountain, situated between Bulgaria and Serbia, represented the well and precisely defined plant species differing from *S. perennis* occurring in Rila, Vitosha and Pirin mountains (Bulgaria), but missing in Serbia. *S. punctata* is also described by Jovanović-Dunjić (1973) as the only species of the genus occurring in Serbia. According to Tutin et al. (1972), the existence of the species *S. punctata*, *S. alpestris* and *S. obtusa* outside the species *S. perennis* has been denied, with the explanation that the described differences in morphological characteristics (leaf arrangement, cup shape, flower color, hair length on nectary, etc.) are not sufficient for differentiation at the subspecies level, given that the species appear sporadically throughout Europe. They also gave the data that the populations of *S. perennis* and *S. punctata* in the Balkans differentiated. In their work, the authors presented the diagnostic characteristics of each species individually.

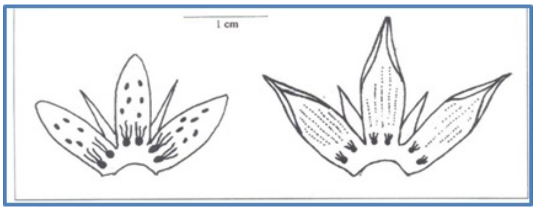


Fig. 6. Dissected corollas of *S. punctata* (left) and *S. perennis* (right)

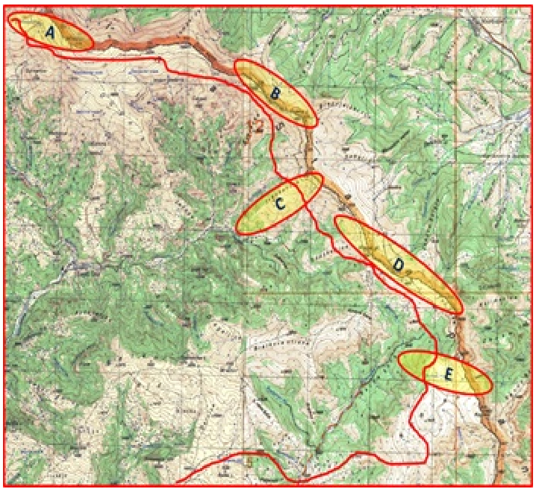


Fig. 7. Distribution of *S. punctata* on Stara Planina: Tri kladenca (Midžor, A.), Golema Čuka (B), Ivankovica (C), Vražja Čuka (D), Tri Kladenca - Kopren (E)

To confirm this finding, a thorough investigation of the chemical properties of both species was carried out, giving a new perspective in order to separate these two species within *Swertia* genera (Stevanović, 1999). In the review, the comparison of the botanical (Figure 6, Table 3), but as well as the chemical characteristics of the two species, *S. punctata* and *S. perennis*, performed by the prof. Milosavljevic and Menkovic's group of researchers will be presented (Menković et al., 2002).
Figures 7 and 8 presents the distribution of the species *S. punctata* L., Gentiananceae with common name "picobojka on Stara Planina", confirmed by pharmagnostic investigations undertaken within several projects financed by Ministry of Education, Technology and Technological Development, Serbia. The red line (Figure 7) represented the research route of Dr. Josip Pančić from 1880, performed on Stara Planina, in the direction of Rosomač-Ržana-Vrtop-Kopren-Tri čuke-Vražja Glava, Ivankovica-Krvave Bare and Toplo Do, when the species *S. punctata* was first described. The results were published as addition to the Flora of the Principality of Serbia (1973) (Pančić, 1874).
After thorough extraction of the plant material collected (*G. punctata* and *G. perennis* at Stara planina- Kopren and Žugića bare – Durmitor, respectively), the isolated and purified compounds (Figure 9) were subjected to chemical analysis, and

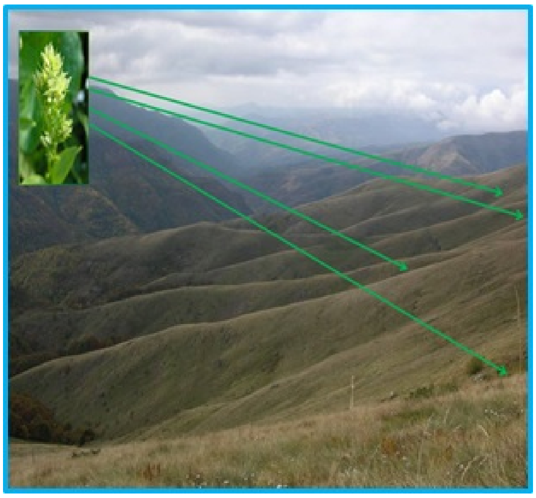


Fig. 8. View from Ivankovica (1780 m) towards Topli do (on the left side of the massif of Bratkova strana, 1980 m), where the most abundant areal of *S. punctata* was localized

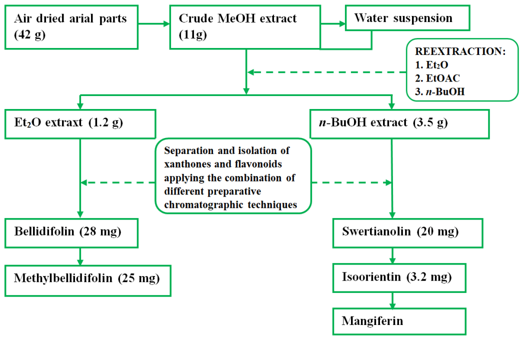


Fig. 9. Scheme of extraction procedure of the aerial part of *S. punctata*

their structure were elucidated, applying ^{1}H and ^{13}C NMR techniques, as well as LC-MS methodology, revealing, besides the already known (Figure 10), the presence of two new components, until now unknown in the plant kingdom (Figure 11) (Menković et al., 2002). The comparative review of pharmacognostic and chemical characteristics of *S. punctata* and *S. perenis* (Tables 4 and 5) revealed the differences that might be considered as the starting point in the distinct separation of these two species within *Swertia* genera.
The complete chemical composition of methanol extract of *S. punctatae folium* was achieved by applying the LC-MS analysis (Figure 12). Sample preparation comprised the dissolution of extract in methanol (c=10 mg/mL) prior HPLC analysis, all the samples being filtered through a Teflon Millipore filter type HV 0.45 μm. In all analyzed samples the quantification of secoiridoid, flavonoid, and xanthone complex, present in Gentianaceae, was carried out. The analysis was conducted on Agilent MSD TOF coupled with Agilent 1200 series RR liquid chromatograph, equipped with column LiChrospher 100 RP 18e, 150 $\times$ 4.0 mm i.d., (5.0 μm). Injection volume was always 5 μl of prepared extracts, and the flow rate was 0.995 mL/min. Mobile phases were: A – water (with 0.2 % HCOOH), B – MeCN, and the acceptable separations of the compounds were achieved using gradient elution (98–90 % A (0–5 min); isocratic 90 % A (13 min); 90–85 % A (18-20 min); isocratic 85 % A (for 5 min), 85-70 % A (25-30), 70-30 % A (30-40 min), isocratic 30 % A for 10 min, 30-98 % A (50-51 min), isocratic 98 % A for 4 min. MSD conditions were as follows: drying gas (N_2) flow 12 L/min; nebulizer pressure 45 psig; drying gas temperature 350 °C; capillary voltage, 4000 V; fragmentor voltage, 140 V;

Table 3. Main differences between *S. punctata* and *S. perennis*

Characters	*Swetria punctata*	*Swetria perennis*
Stem height	20-65 cm	15-45 cm
Stem diameter at base	(2.5-) 3-7 mm	1.0-2.5 (-3.5) mm
Stem colour	green	greenish-purple, rarely green
Number of flowers/inflorescences	(15-) 30-65 (-101)	5-15 (-24)
Corolla colour	greenish-yellow with blackish-violet dots	bluish- violet
Petal position at anthesis	erect-patent to almost erect	patent
Petal shape	oblong-lanceolate to linear, obtuse at apex; margin not enrolled	lanceolate to linear
Sepal/petal ratio	2/3 or more than 2/3 of petals	1/2 to 2/3
Length of fimbnae	3-5 times diam. of nectary	1-3 times diam. of nectary
Balkan distribution	Carpathians, Western Stara Planina Mt, Kosovo	Carpathians, Rila, Pirin and Vitosha Mts

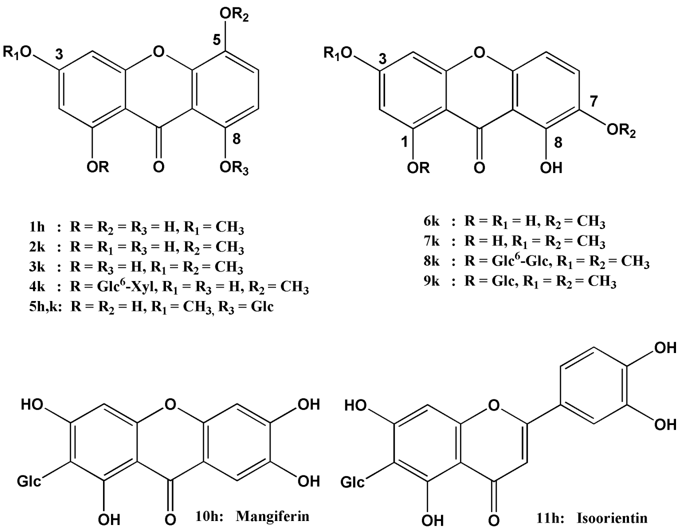
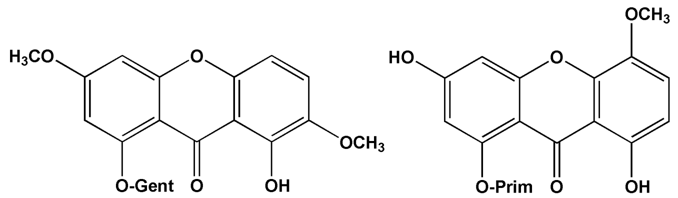


Fig. 10. The distribution of identified compounds in aerial (h) and underground part (k) in *S. perennis* extracts (**1**, bellidifolin; **2**, isobellidifolin; **3**, methylbellidifolin (swerchirin); **4**, isobellidifolin-1-*O*-primveroside (new compound); **5**, bellidifolin-8-*O*-glucoside (swertianolin); **6**, isoswertianin; **7**, methylswertianin; **8**, methylswertianin-1-*O*-gentiobioside (new compound); **9**, norswertianin-1-*O*-glucoside; **10**, mangiferin; **11**, isoorientin)



1-*O*-gentiobiosyl methylisogentiakochainin **1-*O*-primverosyl isobellidifolin**

Fig. 11. New compounds isolated from *S. punctata* (not present in *S. perennis*)

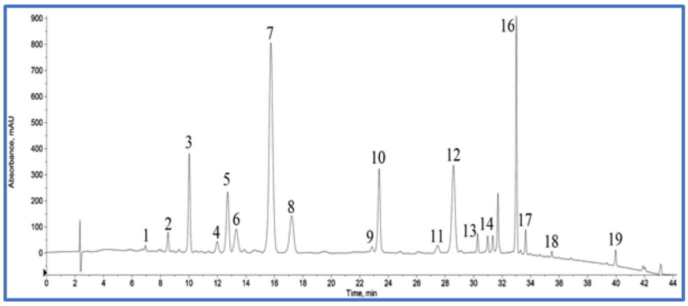


Fig. 12. Fingerprint of *S. punctata* folium MeOH extract analysed by LC-MS; **1**, eustomorussid; **2**, secologanosid; **3**, loganic acid; **4**, septemfidosid; **5**, swertiamarin; **6**, homomangiferin; **7**, gentiopicrin; **8**, swerosid; **9**, isosapoarin; **10**, mangiferin; **11**, isoorientin-2''-*O*-glucoside; **12**, isoorientin; **13**, swertisin; **14**, isovitexin; **15**, swertianolin; **16**, swerchirin (methylbellidifolin); **17**, not identified; **18**, demethylbellidifolin; **19**, bellidifolin

skimmer, 60 V; Oct RF voltage 250 V; positive mode, mass range *m/z* 100–2500; 10,000 transients/scan.

As reviewed by Pant et al. (2000) xanthones bellidifolin, metilbelidifolin, methylswertianin, and mangiferin exhibited various biological activities such as: hypoglycemic, hepatoprotective, and anti-inflammatory, whereas methylswertianin exhibited antituberculous and antioxidant activities, as well. This makes *S. punctata* attractive as a source of medicinal raw material, but since its population is scarce and endangered, as reported recently in the Red Book of Serbian Flora (Stevanović, 1999) our efforts are now concentrated on finding out an alternative way for biomass production.

Based on the previously published work of Tan and Vladimirov (2001), it is suggested that *S. punctata* was well and precisely-defined plant species differing from *S. perennis*. In our work, the thorough chemical analysis of *S. punctata* (Table 6) revealed the presence of two new xanthone derivatives, namely 1-*O*-genciobioside of methyisogentiakochianin (rare 1,3,7,8-substitution, 1-*O*-gentiobiosyl-3,7-dimethoxy-8-hydroxyxanthone) and 1-*O*-primverosidyl isobelidifoline (1,3,5,8-substitution, 1-*O*-primeverosyl-3,8-dihydroxy-5-methoxyxanthone), isolated from the root of this plant for the first time (Menković et al., 2002). The findings that *G. punctata* represents species different from *S. perennis* (Table 7) is of importance for systematic genera *Gentiana*. In addition, as the only areal of its distribution in the Balkans, further speculation is that it likely represents the stenoendemic.

3.2. The comparative study on *Gentiana kochiana* and *G. dinarica*

G. kochiana and *G. dinarica* were collected on Čakor and Prokletije, respectively. The chemical profiling regarding these two species of *Gentiana* genera published by Balijagić et al. (2011), revealed the significant differences between them, which had been reviewed herein (Tables 8-12; Figures 13-17). Interestingly, the aerial part of *G. dinarica* contained secoiridoids and flavonoid C-glycosides (Tables 11,12; Figure 14), while xanthone ingredients were absent, while *G. kochiana* was rich in iridoids and xanthones (Table 10; Figure 13). Nevertheless, the root of *G. dinarica* contained xanthones and flavonoids, isoorientin, and its derivatives, which were present in the aerial parts, as well (Table 12). In the root, a significant amount of amarogentin was found. Worth noting is that xanthone constituents were not present in the form of aglycones.

Table 4. *Swertia punctata* botanical data

Swertia punctata Baumg.- common name: pikobojka
General distribution
Central Balkans
Floral element
Central Balkan mountain endemic
Habitat
Distributed in the range from 1500 to 2000 m above sea level in the zone of subalpine peat pastures and meadows and mountain ores, mainly on silicate and serpentine.
Distribution in the Central Balkans
Serbia - Stara Planina
Macedonia - Korab (Kula e Ziberit 1700 to 2400 m)
Bulgaria - Balkans (parts towards Serbia), Vitosha
Taxonomic status
Section: Pentamere
Swertia punctata Baumg.

Table 5. *Swertia perennis* botanical data

Swertia perennis L. - common name: plava pikobojka
General distribution
Alps, Northern Europe, North America, Balkan, Apennine and Iberian Peninsula, Carpathians, Asia Minor
Floral element
Boreal-Subatlantic (European)
Glacial relic
Habitat
Distribution in the range from 1000 to 2000 m above sea level in the zone of mountain subalpine peat bog, wet raisins, but also on brown rocky and wet runkers.
Distribution in the Central Balkans
Serbia – not found
Macedonia – not found
Bulgaria - Vitosha, Rila, Pirin
Montenegro - Žugića Bare (Žabljak)
Taxonomic status
Section: Pentamere
Swertia perennis L.

From the root were isolated norswertianin-1-*O*-primveroside, norswertianin-8-*O*-primveroside, gentioside and amarogentin, as pharmacologically active constituents (Table 11; Figures 14 and 15) (Krstić-Milošević, 2008).

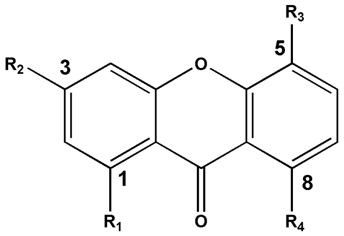
The bitter principles (secoiridoids) (Figure 15), the usual constituents of the genus, stimulate the secretion of gastric juices and bile, thus improving the appetite and digestion. In addition, they also exhibit other biological activities. Namely, sweroside is antihepatitic drug, and swertiamarin and sweroside inhibit the growth of *Bacillus cereus*, *B. subtilis*, *Citrobacter freundii* and *Escherichia coli*. While swertiamarin was also active against *Proteus mirabilis* and *Serratia marcescens*, sweroside inhibited the growth of *Staphylococcus epidermidis*. The recent investigation revealed that secoiridoids sweroside and swertiamarine possess significant general toxicity in the brine shrimp lethality test (Krstić-Milošević, 2008). Gentiopricrin has shown spasmolytic activity, in a concentration-dependent manner, the spontaneous contractions of isolated guinea-pig ileum. Contractions induced by histamine, acetylcholine, $BaCl_2$, and KCl on the ileum were also significantly blocked by this monoterpene glycoside, which suggests that interference with calcium influx into the smooth muscle cells might occur (Tovilović-Kovačevicć et al., 2020). Recent studies have confirmed the radioprotective effect of infusions of the root of the *G. dinarica*, as well as individual xanthone components, such as norswertianin-1-*O*-primveroside and norswertianin-8-*O*-primveroside, a new compound isolated from the root of *G. dinarica*. In addition, the significant antioxidant activity of isolated compounds was established, with the most prominent being norswertianin and norswertianin-1-*O*-primveroside.

The chemical analysis of *G. kochiana* aerial parts extracts revealed a significant amount of xanthone aglycones gentiakochianin, gentiacaulein and decussatin, while in root extract gentiakochianin and decussatin were not detected (Table 10; Figure 13). Xanthone *O*-glycosides, gentiacaulein-1-*O*-primveroside, gentiacaulein-1-*O*-glucoside, decussatin-1-*O*-primveroside and gentiakochianin-7-*O*-primvroside were present both in the aerial parts and roots. The most dominant xanthone *O*-heteroside both in the aerial parts and roots is gentiacaulein-1-*O*-primveroside. From aerial parts isolated gentiakochianin, gentiacaulein and their glycosides 1-*O*-primveroside and 1-*O*-glucoside, respectively, were pharmacologically active.

In traditional medicine, *G. kochiana* has been used as antipyretic, spasmolytic, immunostimulant, and gastrostimulant (*amara pura*). The neuropharmacological effects of diethylether extract of *G. kochiana* and the isolated xanthones gentiacaulein and gentiakochianin on CNS activity in rodents (Tomić et al., 2005) comprised the inhibition of monoaminoxidase (MAO) A and MAO B, thus indicating some antidepressant therapeutic potential of the tested substances. In a pharmacological study involving antiglioma action of xanthones from *G. kochiana*, gentiacaulein and gentiakochianin were shown to be active

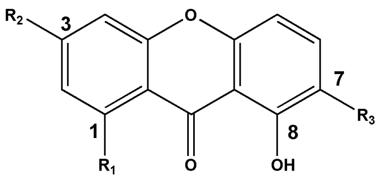
Table 6. Chemical profile of the species *S. punctata*

1,3,5,8 – substitution



#	Compounds Name	Plant part
1	Bellidifolin (R_1=R_3=R_4=OH; R_2=OCH_3)	Roots/Aerial
2	Isobellidifolin (R_1=R_2=R_4=OH; R_3=OCH_3)	Roots
3	Methylbellidifolin (Swerchirin) (R_1=R_4=OH; R_2= R_3=OCH_3)	Roots/Aerial
4	Isobellidifolin-1-*O*-primveroside (R_1=*O*-primverosyl; R_4=OH; R_2= R_3=OCH_3)	Roots
5	Bellidifolin- 8-*O*-glucoside (Swertianolin) (R_1= R_3=OH; R_4=*O*-glucosyl; R_2 =OCH_3)	Roots/Aerial

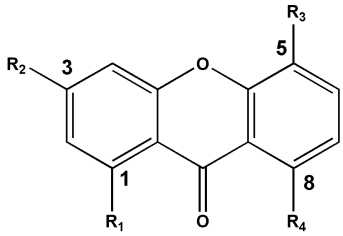
1,3,7,8 – substitution



#	Compounds Name	Plant part
6	Isoswertianin (R_1= R_2=OH; R_3=OCH_3)	Roots
7	Methylswertianin (R_1= OH; R_2=R_3=OCH_3)	Roots
8	Methylswertianin-1-*O*-gentiobioside (R_1= *O*-gentiobiosyl; R_2=R_3=OCH_3)	Roots
9	Norswertianin- 1-*O*-glucoside (R_1=*O*-glucosyl; R_2=R_3=OH)	Roots

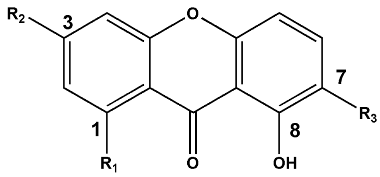
Table 7. Chemical profile of the species *S. perennis*

1,3,5,8 – substitution



#	Compounds Name	Plant part
1	Bellidifolin (R_1=R_3=R_4=OH; R_2=OCH_3)	Roots/Aerial
2	Bellidifolin-8-*O*-glucoside (Swertianolin) (R_1= R_3=OH; R_4=*O*-glucosyl; R_2 =OCH_3)	Roots/Aerial

1,3,7,8 – substitution



#	Compounds Name	Plant part
3	Norswertianin (R_1=R_2=R_3=OH)	Roots/Aerial
4	Norswertianin- 1,3-diglucoside (R_2= OH; R_1=R_3=*O*-diglucosyl)	Aerial
5	Decussatin (R_1=R_2=R_3=OCH_3)	Aerial

principles responsible for the *in vitro* antiglioma action of ether and methanolic extracts of the plant (Isaković et al., 2008). The assessment of structure-activity relationship in a series of structurally related xanthones from *G. kochiana* and *Gentianella austriaca* revealed dihydroxylation at positions 7, 8 of the xanthone nucleus as the key structural feature responsible for the ability to induce microtubule-associated G2/Mcell block and apoptotic cell death in glioma cells (Isaković et al., 2008).

3.3. The comparative study on *Gentianella albanica*, *G. crispata* and *G. austriaca*

Methanol extracts of the aerial parts of three *Gentianella* species, *G. albanica*, *G. crispata*, and *G. austriaca*, were analyzed by reversed-phase HPLC with photodiode array detection (HPLC/DAD). The chromatograms indicated considerable similarity between the investigated species, revealing almost the same constituents (Figure 16). All species were characterized by the presence of three classes of compounds typical for Gentianaceae, such as secoiridoids, C-glucoflavones and xanthones (Janković, 2005). The main isolated and identified compounds were: demethylbellidofolin, bellidifolin, corymbiferin, demethylbellifolin-8-*O*-glucoside, bellidifolin-8-*O*-glucoside, corymbifertin-1-*O*-glucoside, veratriloside, lanceoside, swertisin, campestroside, mangiferin and isoorientin. The chromatogram of *G. austriaca* was presented as a representative example for thorough chemical analyses (Figure 17, Table 13). The main comparative characteristics for *G. crispata* and Balkan endemic species *G. albanica* are given in Tables 14 and 15.

The *G. austriaca* aerial part has the same profile as *G. albanica*, having one more compound, campestroside (Figure 17). The roots of these three investigated *Gentianella* species contained the same xanthone profile, except again for campestroside, and flavonoids, but the content of secoiridoids was detected to be higher in *G. austriaca*. The presence of veratriloside and lanceoside were confirmed for the genus *Gentianella* for the first time (Table 13). Demethylbellidifolin, bellidifolin as well as their 8-*O*-glycosides were recognized as the carrier of pharmacological effects. Demethylbellidifolin, demethylbellidifolin-8-*O*-glucoside, bellidifolin-8-*O*-glucoside, and swertisin exhibited radioprotective effect; bellidifolin and demethylbellidifolin possess cardioprotective properties, while bellidifolin, bellidifolin-8-*O*-glucoside, swertizin, and isoorientin, besides antioxidant properties showed significant hypoglycemic activity. Overall, xanthones are recognized as hepatoprotective, hypolipidemic, anticholinesterase, and cerebral vasodilator agents (Jiang et al., 2021; Tchamo Diderot et al., 2006).

Recently, a pharmacological study (Janković et al., 2008) involving radioprotective effects of *G. austriaca* ether and methanolic fractions and polyphenolic constituents (demethylbellidifolin and its 8-*O*-glucoside, as well as bellidifolin-8-*O*-glucoside and swertisin) in human lymphocytes was under-

Table 8. *Gentiana kochiana* botanical data

Gentiana acaulis L., common name: velemun
syn. *G. kochiana* E.P.Perrier & Songeon
General distribution
Iberian and Balkan Peninsula, Alps and Carpathians, Central European Mountains
Floral element
Central and southern Europe
Habitat
Mountain ores with acid silicate substrate, mostly southern exposures
Distribution in the Central Balkans
Serbia: Kopaonik, Golija, Stara planina
Macedonia: Pelister, Karadzic
Montenegro: Bjelasica, Visitor, Chakor, Hajla
Taxonomic status
Section: Megalanthe
Aggregate: *G. acaulis* L.

Table 9. *Gentiana dinarica* botanical data

Gentiana dinarica Beck., common name: dinarian velemun
General distribution
Apennine and Balkan Peninsula, Balkan-Dinaric Mountain floral element (endemic)
Floral element
Western Balkan Peninsula
Habitat
Occurs exclusively on carbonate substrates, from 800 to 2300 m
Distribution in the Central Balkans
Serbia: Mount Tara, Koritnik
Croatia: Velebit
Montenegro: Greben, Maja Rusolija, Mokra Gora
B& H: Trebevic, Vran-Blidinje, Bjelašnica
Taxonomic status
Section Megalanthe
Aggregate: *G. dinarica* Beck.

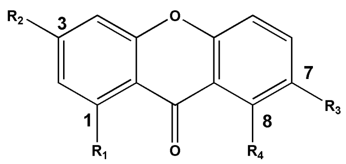
taken. Both extracts showed better protection in the treatment of human lymphocytes after γ -irradiation than isolated compounds. These results suggested that the antioxidative properties of polyphenols tested might be responsible for contributing to the radioprotective effects of *G. austriaca*.
Interestingly, in the Chinese province of Mongolia, the herb "guixincao", *G. acuta* (Michaux.) Hulten, Gentianaceae (Figure 18) has been used as a medicinal tea, for colds, to cleanse the blood of harmful substances and pathogens. Besides, it has been known for its diuretic effect. This species has a very similar xanthone profile as our investigated *G. austriaca*, with demethylbellidifoline and bellidifoline and their corresponding heterosides as dominant constituents. This plant exhibits various biological activities such as: antioxidant, anti-inflammatory, antibacterial, hypoglycemic, antitumor, cardioprotective (Ren et al., 2019). Contrary, in European traditional medicine there is no data that *G. austriaca* was used.

3.4. The comparative study on *Anthemis carpatica* and *A. montana*

Sesquiterpene lactones (SLs), one of the most prevalent groups of the secondary metabolites in the Asteraceae family, are known for their antimicrobial, antitumoral, and anti-inflammatory activities, effects on the central nervous and cardiovascular systems as well as allergenic potency. One of the most attractive properties that SLs possess, is their anti-inflammatory potential. Inflammation represents a patho-

Table 10. Chemical profile of the species *G. kochiana*

1,3,7,8 – substitution



1	Gentiakochianin (R_1=R_3=R_4=OH; R_2=OCH_3)	Aerial
2	Gentiokochianin-7-primveroside (R_1=R_4=OH; R_3=O-primverosyl; R_2=OCH_3)	Roots/Aerial
3	Gentiacaulein (R_1=R_3=OH; R_2=R_4=OCH_3)	Roots/Aerial
4	Gentiacaulein-1-primveroside (R_1=*O*-primvesrosyl; R_2=R_4=OCH_3; R_3=OH)	Roots/Aerial
5	Gentiacaulein-1-glycoside (R_1=*O*-glucosyl; R_2=R_4=OCH_3; R_3=OH)	Roots/Aerial
6	Decussatin (R_1=OH; R_2=R_3=R_4=OCH_3)	Aerial
7	Decussatin-1-primveroside (R_1=*O*-primverosyl; R_2=R_3=R_4=OCH_3)	Roots/Aerial

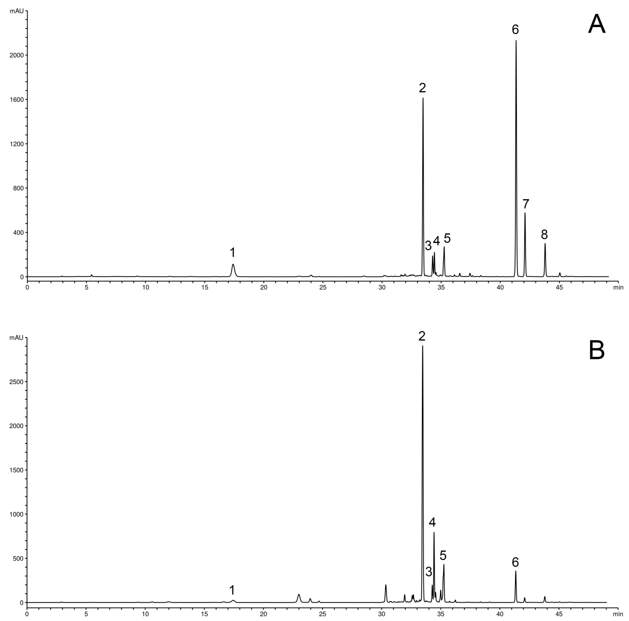


Fig. 13. HPLC chromatogram of *Gentiana kochiana* extracts; A - aerial parts; B - roots; **1**, secoiridoid derivative; **2**, gentiacaulein-1-*O*-primveroside; **3**, gentiacaulein-1-*O*-glucosyl, **4**, decussatin-1-*O*-primveroside; **5**, 1,8-dihydroxy-3-metoxy-7-*O*-primveroside; - **6**, gentiacaulein; **7**, gentiakochianin; **8**, decussatin

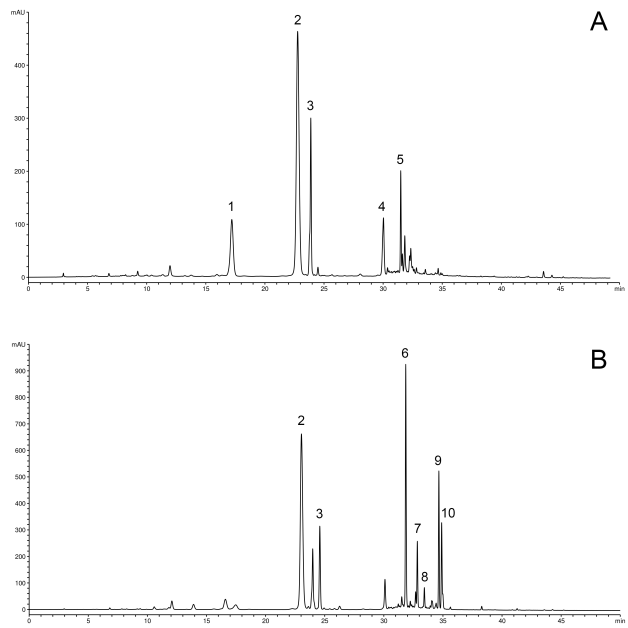
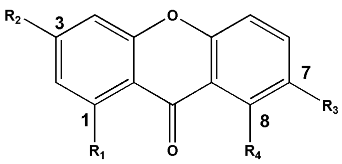


Fig. 14. HPLC chromatogram of *Gentiana dinarica* extracts; A - aerial parts; B - roots; **1**, swertiamarin; **2**, gentiopicrin; **3**, isoorientin-4′-*O*-glycoside; **4**, isoorientin; **5**, isovitexin; **6**, norswertianin-1-*O*-primveroside; **7**, norswertianin-8-*O*-primveroside; **8**, norswertianin-1-*O*-glucoside; **9**, gentioside; **10**, amarogentin (secoiridoid)

Table 11. Chemical profile of the species *G. dinarica*

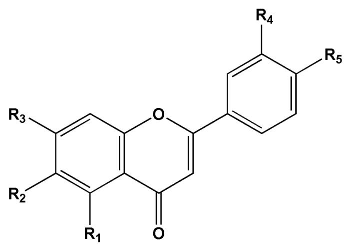
1,3,7,8 – substitution



1	Norswertianin-1-*O*-primveroside (R_1=*O*-primverosyl; R_2=R_3=R_4=OCH_3)	Roots
2	Norswertianin-8-*O*-primveroside (R_1=OH; R_2=R_3=OCH_3; R_4=*O*-primverosyl)	Roots
3	Norswertianin-1-*O*-glucoside (R_1=*O*-glucosyl; R_2=R_3=R_4=OH)	Roots
4	Gentioside (R_1=OH; R_2=*O*-primverosyl; R_3=OCH_3; R_4=H)	Roots

Table 12. C-flavonoids detected in aerial part of *G. dinarica*

1,3,7,8 – substitution



1	Isoorientin-4′-*O*-glucoside (R_1=R_3=R_4=OH; R_2=glucosyl; R_5=*O*-glucosyl)	Aerial
2	Isoorientine (R_1=R_3=R_4=R_5=OH; R_2=glucosyl)	Aerial
3	Isovitexine (R_1=R_3=R_5=OH; R_2=glucosyl; R_8=H)	Aerial

physiological process, comprising the integrated response of many defense systems of the body to the invasion of a foreign body of any kind. The great interest in inflammation is due to the involvement of its components in many serious diseases, including cancer, Alzheimer's disease, and acquired immunodeficiency syndrome (AIDS). The anti-inflammatory mechanisms include inhibition of the production of cytokines, lipid mediators, and other related molecules, modulation of pro- and antioxidant contents, and regulation of intracellular signaling pathways. These mechanisms are involved in several inflammatory diseases; thus, determining the chemical structure of the SLs that could reduce and/or help to control inflammatory diseases and their symptoms might properly direct the investigation for novel compounds with the desired pharmacological properties. In addition, the chemotaxonomic importance of SLs, together with various mentioned biological activities, is the major reason for the continuing interest in these compounds.

In order to perform the necessary chemotaxonomic and pharmacological inquires, the structure elucidation of pure and isolated SLs should be carried out. Figure 19 represents the schematic display of simple methodologies used for isolation and purification of SLs present in the investigated aerial part of *Anthemis carpatica* Willd. and *A. montana* L., Asteraceae. The investigation had been carried out at the Faculty of Chemistry, University of Belgrade, under Prof. Slobodan Milosavljevic supervision (Bulatović et al., 1998). The applied extraction and purification techniques yielded thirty-one SLs of the same guaiadienolide type, all of them exhibiting an exomethylene 11(13) double bond, and one of germacranolide type (Figures 20,21). According to proven chemotaxonomic importance and various biological activities, structure elucidation plays an important role in all of the studies concerning this class of

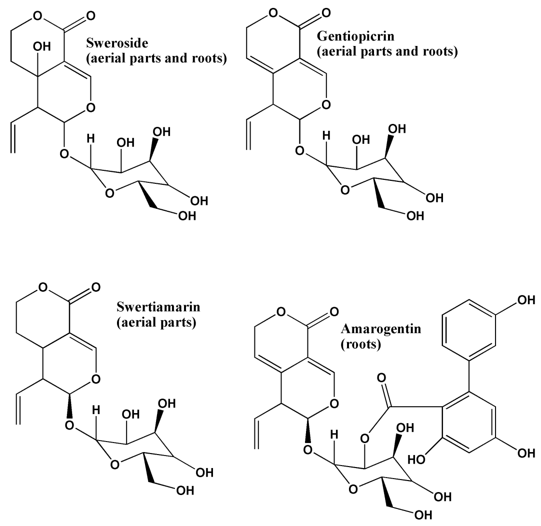
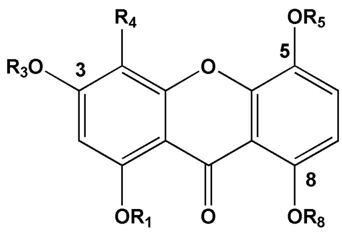


Fig. 15. Secoiridoids detected in aerial part of *G. dinarica*

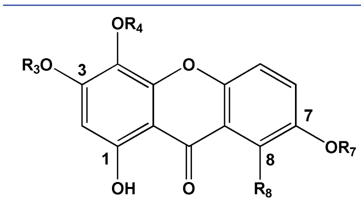
Table 13. The structures of the main identified γ-pyrone compounds in aerial parts of *G. austriaca*

3,5,8 – substitution



#	Compounds Name	Plant part
1	Demethylbellidifolin (R_1=R_3=R_4=R_5=R_8=H)	Aerial
2	Corymbiferin (R_1=R_3=R_8=H; R_4=OCH_3; R_5=CH_3)	Aerial
3	Demethylbellidifolin-8-*O*-glucoside (R_1=R_3=R_4=R_5=H; R_8=glucosyl)	Aerial
4	Bellidifolin (R_1=R_4=R_5=R_8=H; R_3=CH_3)	Aerial
5	Bellidifolin-8-*O*-glucoside (R_1=R_4=R_5=R_8=H; R_3=CH_3; R_8=glucosyl)	Aerial
6	Corymbiferin-1-*O*-glucoside (R_1=R_8=glucosyl; R_3=CH_3; R_4=OCH_3; R_5=CH_3)	Aerial

1,3,7,8 – substitution



#	Compounds Name	Plant part
7	Veratriloside (R_3= R_4=CH_3; R_7=glucosyl; R_8=H)	Aerial
8	Lanceoside (R_3=R_7=CH_3; R_4=glucosyl; R_8=OH)	Aerial

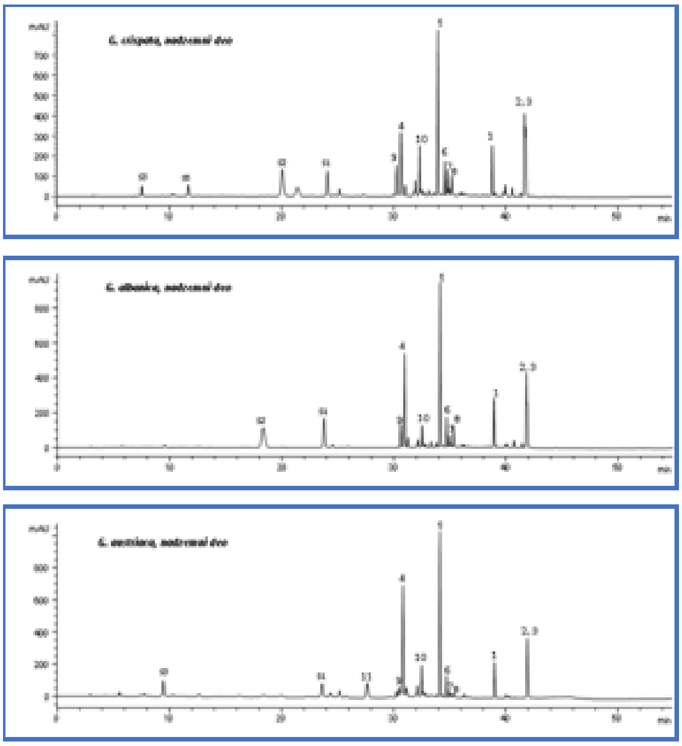


Fig. 16. Comparative fingerprint chromatograms of aerial parts of *G. crispata*, *G. albanica* and *G. austriaca*

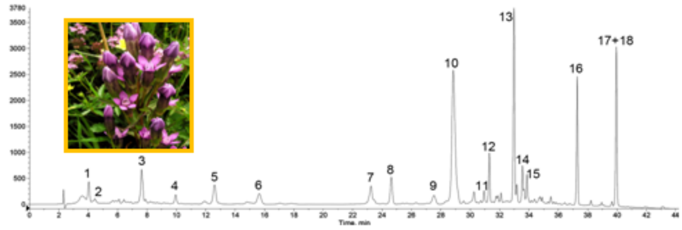


Fig. 17. "Fingerprint" chromatogram of *G. austriaca* (A. & J. Kern.) Holub, Gentianaceae; **1**, eustomorussid; **2**, eustomosid; **3**, campestroside isomer; **4**, loganic acid; **5**, swertiamarin; **6**, gentiopicrin; **7**, mangiferin; **8**, campestroside; **9**, lanicerin; **10**, demethylbellidifolin-8-*O*-glucoside; **11**, isovitexin; **12**, swertisin; **13**, bellidifolin-8-*O*-glucoside; **14**, corymbiferin-1-*O*-glucoside; **15**, veratriloside; **16**, demethylbellidifolin; **17**, bellidifolin; **18**, corymbiferin

Fig. 18. *Gentianella acuta* (Michaux.) Hulten Gentianaceae

compounds. In the characterization of the chemical structures of the isolated natural compounds, Dixon (2005) proposed that several phases may be delineated, such as a) determination of the enzymatic steps leading to the common precursors of the metabolic pathway; b) the use of molecular biology to probe the changes in gene expression associated with the

Table 14. *Gentianella albanica* botanical data

Gentianella albanica (Jáv.) Holub

General distribution

Central Balkans (Albania, Montenegro, Serbia)

Geological background

Carbonate

Habitat

Elevation: 2300 - 2530 m above sea level

Alpine carbonate ores Oxytpodion dinaricae (*Elyno-Edraianthetum alpini*, *Elyno-Leontopodietum* Rudski)

Floral element

Central Balkan mountain endemic

Distribution in the Central Balkans

Montenegro - Komovi, Prokletije (Maja Karanfili, Hajla, Žljeb).

Taxonomic status

Section: Gentanella

Gentianella albanica (Jáv.) Holub

Table 15. *Gentianella crispata* botanical data

Gentianella crispata (Vis.) Holub

General distribution

Balkan Peninsula - Bulgaria, Albania, Montenegro, Dalmatia, Bosnia and Herzegovina.

Habitat

Dry and poor habitats (pastures and meadows) in the Alpine region

Floral element

It belongs to the Pontic-Balkan floral element

Distribution in the Central Balkans

Serbia: Kosovo and Metohija

Taxonomic status

Section: Gentanella

Gentianella crispata (Vis.) Holub

plasticity of natural products metabolism; c) the emergence of functional genomics, providing a more accurate picture of the diversity of the genes/enzymes involved in the metabolism of secondary metabolites; d) the exploitation of genetic engineering for optimizing the secondary metabolites profiles in plants; e) the employment of pharmacological and clinical studies supporting the traditional application of plants.

Increased interest in the study of natural products as potential drugs and rapidly changing research strategies have been potentiating the role of pharmacognosy in the wider context of pharmaceutical research. To present the usefulness of combined pharmacognostic and chemistry approaches to medicinal plant investigations, here, we will present the research performed on two *Anthemis* species, similar in their habitat but different in chemical components belonging to SLs. Applying different sophisticated techniques for the identification of the unknown isolated compounds from plant material, interesting results had been obtained. Generally, the most widely used method is NMR spectroscopy. ^{1}H NMR spectral data analysis is usually starting point since it is one of the most informative techniques for this purpose. The ^{1}H NMR analyses of purified SLs from *A. carpatica* and *A. montana* extract revealed the existence of three types of guaianolides, marked as Δ^2, Δ^3, and Δ^4-group, depending on the position of a double bond in a five-membered, non-lactone ring (Figure 23).

Application of the sophisticated two-dimensional nuclear magnetic resonance (2D NMR) methods, including some new variations of the known techniques (e.g., ^{13}C NMR spectra editing using Heteronuclear Single Quantum Correlation) in combination with other spectrometric methods (e.g. chemical ionization mass spectrometry), demonstrated the power of structure-guided screening as a complementary method to assay-guided screening, enabling complete structural assignment and determination of relative stereochemistry of natural products. Such an approach enables the possibility to determine the novel rather than known plant constituents. ^{1}H and ^{13}C NMR assignment and structure determination of both major and minor components of isolated SLs were based on the characteristic chemical shifts and couplings obtained by the first-order analysis combined with 2D NMR measurements, such as Double Quantum Filtered Correlated Spectroscopy (DQF COSY), Total Correlated Spectroscopy (TOCSY), Nuclear Overhauser Effect Spectroscopy (NOESY), HSQC (Heteronuclear Single Quantum Correlation) and HMBC (Heteronuclear Multiple Band Correlation) performed on isolated compounds. Thus, this combination of high-performance separations techniques with structurally informative spectroscopic methods (MS and NMR) could allow extracts to be screened not just for biologically active compounds, but at the same time for chemically significant structural classes (Bulatović et al., 1998; Da Costa et al., 2004; Djordjević et al., 2004; Juranić et al., 1998; Milosavljević et al., 1999b; 2004; 1998; Schmidt, 1996; Yoshioka, 1974).

To demonstrate the importance of employing the above-mentioned methods in complete structure elucidation of the isolate SLs, special attention was paid to lactone belonging to Δ^2-group of isolated lactones, lactone 5 (8-*O*-isobutyryl-

9-*O*-acetylanthemolide B). It was studied utilizing 2D NMR methods (DQF COSY, TOCSY, NOESY, HSQC, and HMBC). Although the application of COSY method enabled the identification of 8α,9α-diacyloxyguaia-2,11(13)-dienolide gross structure (Figure 24), and the presence of tertiary OH and OOH groups, the TOCSY, NOESY, and HMBC spectra were necessary to determine the acylation pattern, as well to establish unambiguously the position of tertiary C-4 and C-10 oxygen functions. In the case of lactone 18 (Figure 25), the application of the mentioned 2D NMR methods revealed the existence of two conformers. Namely, in addition to employing the first-rate NMR analyses, the DQF COSY (Figure 25A), as well as ^{13}C data measured in HSQC (Figure 25B) enabled the identification of compound 18 as 9α-acetoxycumambrin A. As the previously used scalar H,H-coupling networks measurements revealed the interesting conformational exchange present in 18, the HSQC and COSY analyses performed at the low-temperature regime, gave the evidence of the existence of two conformers - the distorted chair conformation of 7-membered ring in lactones 18A and 18B, with (pseudo)axial and (pseudo)equatorial 10β-methyl, respectively.

4. STUDIES ON MONTENEGRIN ENDEMIC *TANACETUM LARVATUM* USING ^{1}H NMR FOR PARTHENOLIDE QUANTIFICATION

Plants from the genus *Tanacetum* L. (Asteraceae) have been used in traditional medicine from ancient times, of which *Tanacetum parthenium* (L.) Shultz-Bip. (feverfew) has been known as a remedy for the treatment of various diseases, including migraine, arthritis, fever, vertigo, menstrual disorders, stomachache, and psoriasis (Ernst and Pittler, 2000; Wider et al., 2015). Additionally, *T. vulgare* L. (common tansy) and *T. microphyllum* DC. are useful in the treatment of various inflammatory disorders (Silván et al., 1998; Zhang et al., 2005). A recent investigation of *T. larvatum* extract suggested the application of this species as an alternative or supplementary herbal remedy for the treatment of inflammatory diseases due to its anti-inflammatory and anti-ulcer activities (Petrović et al., 2003). The secondary metabolites that mediate these pharmacological effects are mainly biologically active sesquiterpene lactones, such as parthenolide and hydroxyachillin. Parthenolide, found in significant amounts in feverfew, has been indirectly linked to the anti-migraine action of feverfew preparations, as well as to anti-tumor and anti-inflammatory properties (Wen et al., 2002). The mode of parthenolide activity comprises the inhibition of prostaglandin production and 5-hydroxytryptamine secretion as mediators of inflammation (Kang et al., 2001; Mittra et al., 2000). A recent investigation confirmed its gastric anti-ulcer properties due to its ability to restore the reduction of sulfhydryl groups within the gastric mucosa and to increase mucosal PGE2 level (Ernst and Pittler, 2000; Wider et al., 2015).

Because of the assumed importance of parthenolide content for pharmacological activities, supported by the studies already performed, hence, we investigated the parthenolide content in *T. larvatum* (Gris.) Kanitz., Asteraceae, endemic species originated from Sinjajevina, Montenegro (Josifović, 1975; Pantocsek, 1873). The collected material was subjected to quantitative ^{1}H NMR and HPLC analysis (Figure 26). Bearing in mind that *T. parthenium* native to Serbia does not contain parthenolide, of chemotaxonomic importance are the intriguing results concerning the parthenolide content and antioxidant activity of *T. larvatum*, which were comparable, even higher, than those of *T. parthenium*. The present data support further investigation of this species as a new commercial source of parthenolide or potential herbal remedy with antimigraine, anti-inflammatory, and gastroprotective effects (Aljančić et al., 2010; 2001; Bulatović et al., 2006; Tadić et al., 2010).

CONCLUSIONS

By modernizing and renewing the proven science, pharmacognosy represents the strategic connection between biology and chemistry. This multidisciplinary approach in the discovery of novel and unique molecules with the biological potential to target specific impairments in human organisms is necessary. On the other hand, the chemical composition of plants represents the specific characteristics that might be used for the investigated plant to ascertain the specific place in plants systematics. The classification of plants according to their chemical constitution is defined as chemotaxonomy. Namely, various dilemmas regarding the correct systematic classification of plants have been solved by performing the chemotaxonomic analysis. A wide variety of studies include the chemotaxonomic classification of secondary metabolites; among the most investigated compounds being xanthones and sesquiterpene lactones. In this review, the importance of employing the combined chemical and pharmacognostic approach to the investigation of Montenegrin flora, with emphasis on endemic species was stressed. The chemotaxonomic dilemma regarding the systematic classification of *Swertia perennis*, *Gentiana kochiana* and *G. dinarica*, as well as *Gentainella albanica* and *G. austriaca*, might be solved by applying the chemical approach. In addition, the structure-activity investigation enables the recognition of the pattern that might be successfully used to classify a set of str-

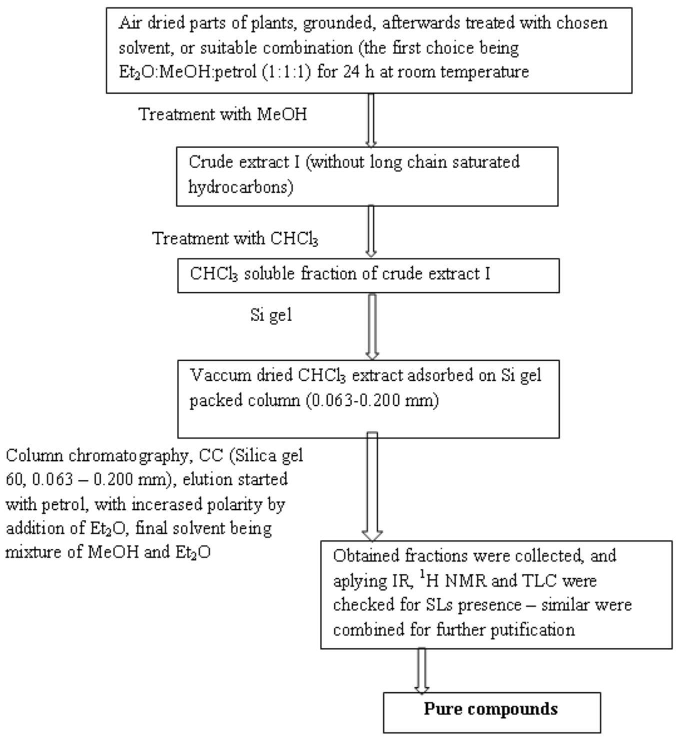
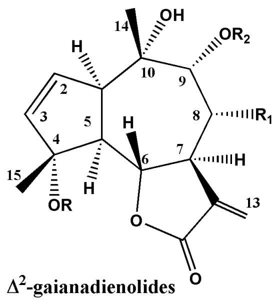
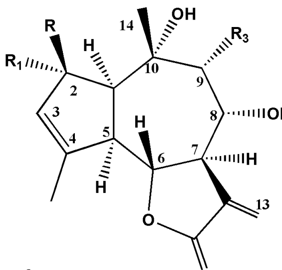


Fig. 19. The schematic presentation of the isolation and purification of identified sesquiterpene lactones from *A. carpatica* and *A. montana*



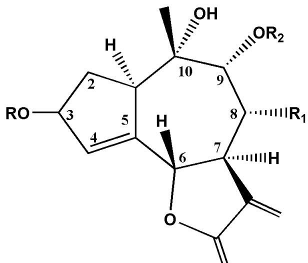
Δ^2-gaianadienolides

	R	R_1	R_2
1^{c}	H	H	Ac
$2^{c,m}$	OH	OAc	H
3^{c}	OH	OH	Ac
$4^{c,m}$	OH	OAc	Ac
$5^{c,m}$	OH	O-*i*-But	Ac
6^{c}	H	O-*i*-But	Ac
7^{c}	OH	H	Ac
$8^{c,m}$	OH	OH	H
9^{c}	OH	OTig	Ac
10^{m}	OH	OAng	Ac



Δ^3-gaianadienolides

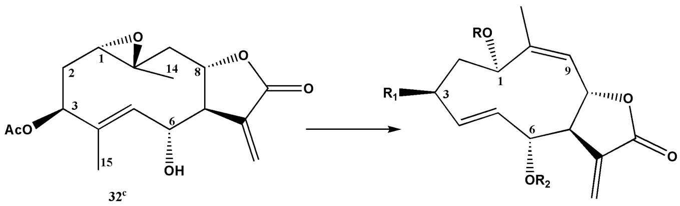
	R	R_1	R_2	R_3
$11^{c,m}$	H	H	H	OAc
$12^{c,m}$	H	H	Ac	OH
13^{c}	H	H	Ac	OAc
14^{c}	H	OOH	*i*-But	OAc
15^{c}	H	H	H	H
16^{c}	OH	H	H	H
17^{c}	H	H	*i*-But	OAc
18^{m}	OAc	H	*i*-But	OAc
19^{m}	OAc	H	H	OAc
20^{m}	OAc	H	Ac	OAc
21^{m}	OAc	H	H	O-*i*-But
22^{m}	OAc	H	H	O-*i*-Val
23^{m}	OAc	H	H	O-2-MeBut



Δ^4-gaianadienolides

	R	R_1	R_2
24^{c}	H	H	Ac
$25^{c,m}$	H	OAc	Ac
26^{c}	OH	OAc	Ac
27^{c}	H	OAc	H
28^{c}	H	OProp	Ac
29^{c}	H	O-*i*-But	Ac
30^{c}	H	OTig	Ac
31^{c}	OH	O-*i*-But	Ac

Fig. 20. Sesquiterpenoids isolated from two *Anthemis* sp.; c - sesquiterpenes isolated from *A. carpatica*, m - sesquiterpene isolated *A. cretica* subsp. *cretica* c,m - sesquiterpene isolated from both investigated species



32^{c}

	R	R_1	R_2
33^{c}	H	OAc	H
34^{c}	Ac	OAc	Ac
35^{c}	H	H	Ac
36^{c}	H	H	Ac

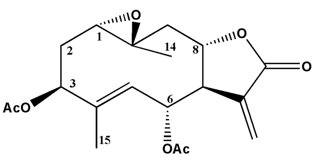


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Fig. 21. New germacranolides isolated from *A. carpatica*

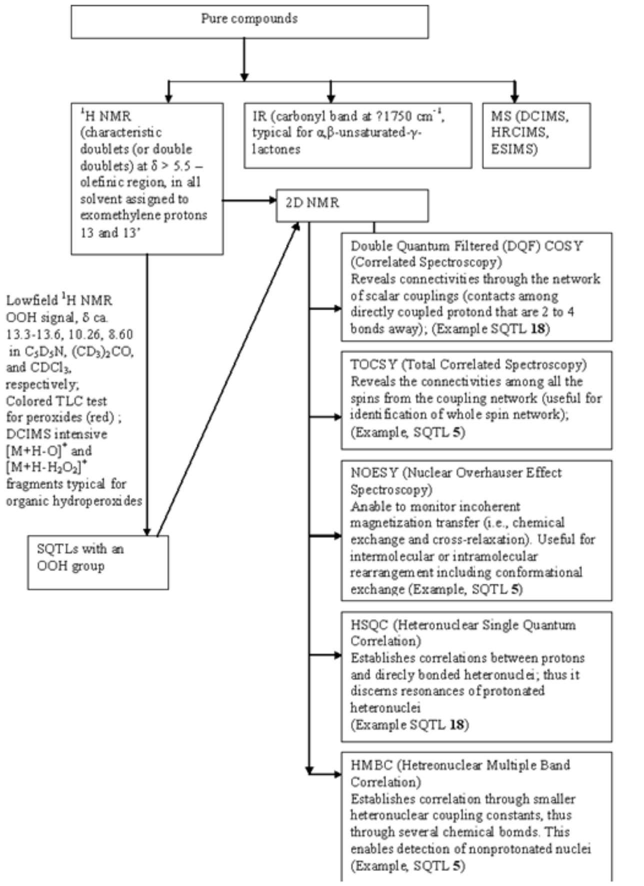

Fig. 22. Methods used to elucidate the structure of SLs isolated from *A. carpatica* and *A. montana* (Tadić et al., 2009)

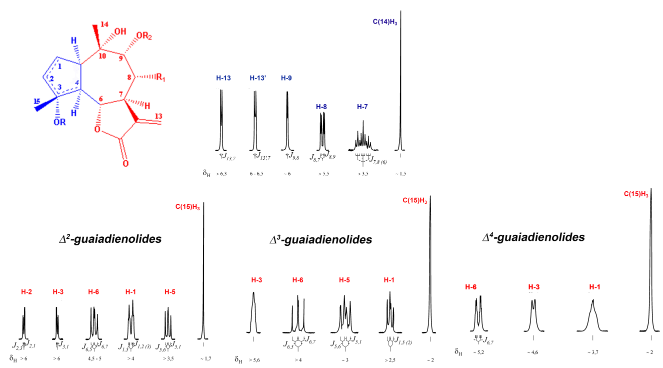

Fig. 23. Characteristic chemical shifts in ^1H (C_5D_5N) NMR spectra and couplings assigned to SLs isolated from *A. carpatica* and *A. montana*; red highlight in the guaiadienolides structure common for isolated SLs; blue common for Δ^2-lactones, Δ^3-lactones; and Δ^4-lactones

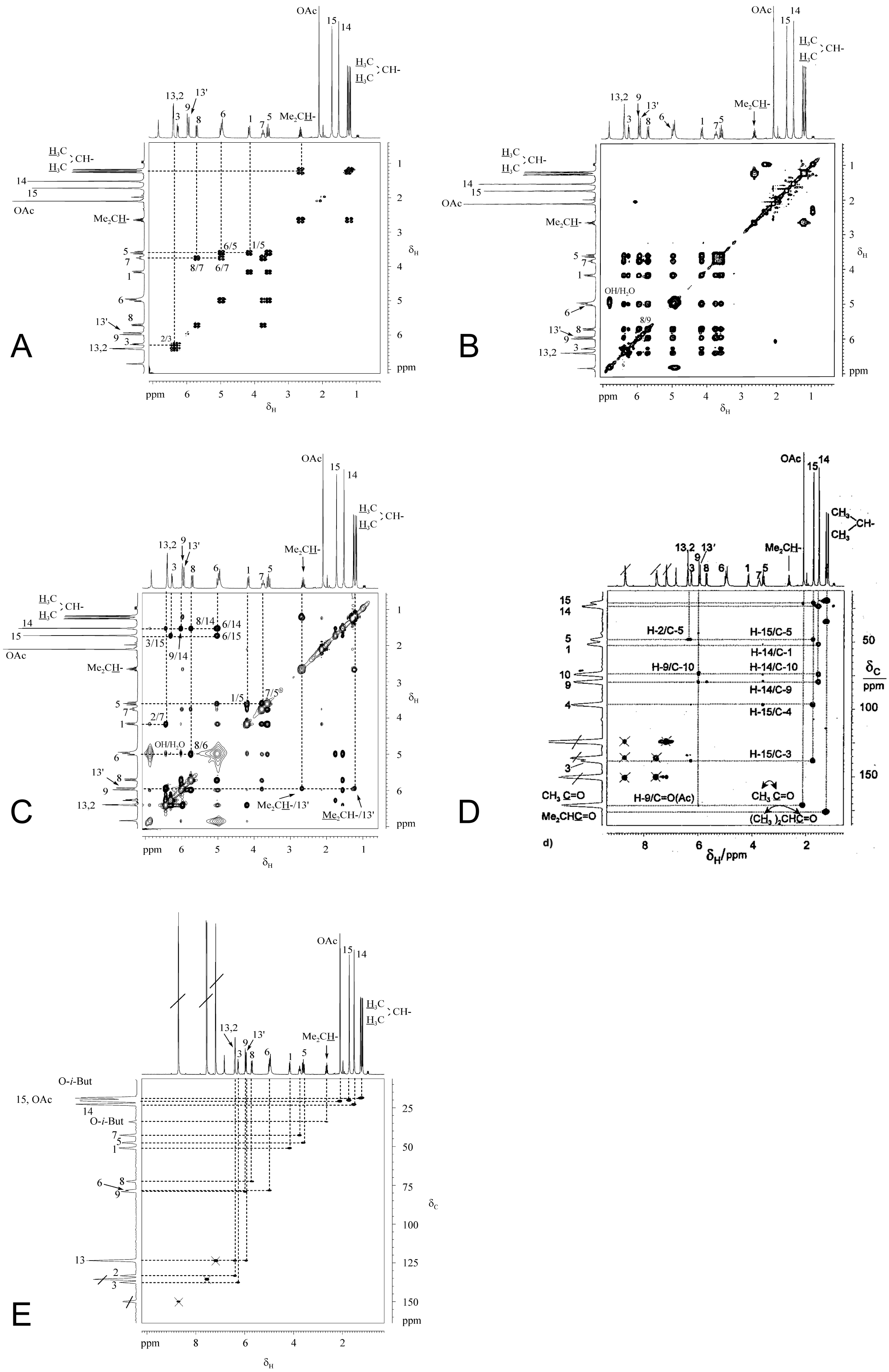

Fig. 24. Structure elucidation of lactone 5: A, DQF COSY (in C_5D_5N); B, TOCSY spectrum in C_5D_5N; correlation due to a chemical exchange OOH/OH not shown; C, NOESY spectrum in C_5D_5N; D, Long-range heteronuclear C,H-correlation (HMBC) spectrum in C_5D_5N; E, One bond heteronuclear C,H-correlation (HSQC) spectrum in C_5D_5N

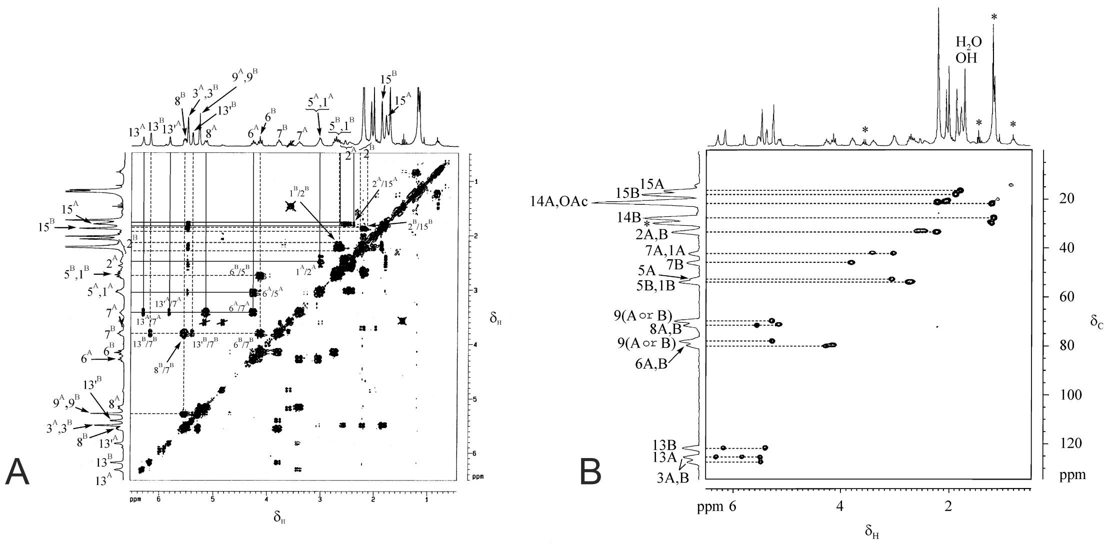

Fig. 25. DQF COSY (A) and HSQC (B) of lactone 18 in $CDCl_3$ at -30 °C

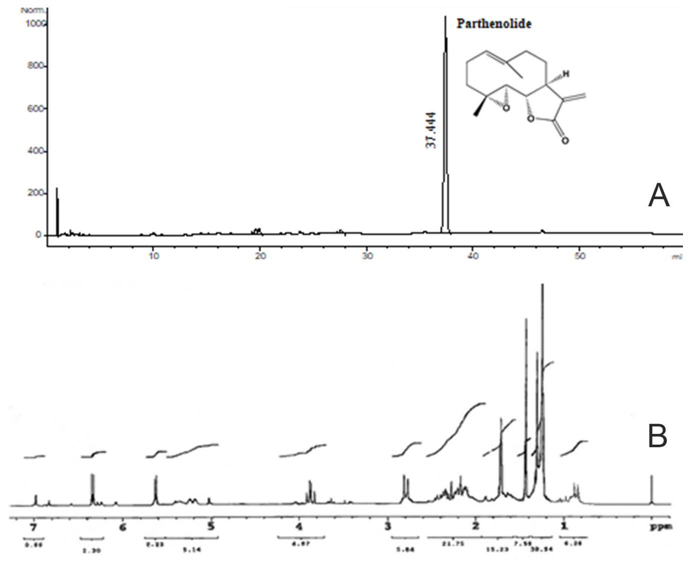

Fig. 26. The HPLC (A) and ^{1}H-NMR (B) fingerprint profile of *T. larvatum* extract from Mountain Sinjajevina, Montenegro

ucturally diverse compounds as therapeutic agents. Taking into account the numerous activities that sesquiterpenes possess, thorough determination of their presence in some *Anthemis* species plants was reviewed, as well. The investigation of the Montenegrin endemic species, *T. larvatum* revealed that this species is rich in parthenolide, a secondary metabolite known for its antimigraine activity. The application of simple ^{1}H NMR techniques in quantification studies of parthenolide content was reviewed. The presented investigation gave an insight into the usefulness of these integrated pharmacognostic and chemical approaches in the investigation of natural products that originated from plants.

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