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SURVEY

Phytochemical study of the genus *Amphoricarpos*

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Abstract: Phytochemistry deals with the study of secondary metabolites produced by plants that synthesize these compounds for many reasons, including their own protection against attack of herbivores and plant diseases. Secondary metabolites are believed to represent plant adaptation to various environmental factors and that they enabled the survival of the species. Secondary metabolites of plants can have curative or toxic effects in humans and animals. Herbal medicine has a long tradition in folk medicine and until the early 20th century, when synthetic organic chemistry began to develop, plants were the main source of medicines. The two basic goals of our phytochemical research are: isolation and identification of new (biologically active) compounds – potential drugs, and chemotaxonomy (chemosystematics). In the following text through one selected example, the genus *Amphoricarpos* Vis., our phytochemical research is shown on both aspects.

Keywords: phytochemistry; amphoricarpolides; chemosystematics; metabolomics.

CONTENTS

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1. INTRODUCTION

Phytochemistry is the study of secondary metabolites produced by plants considering their structural compositions, the biosynthetic pathways, functions, mechanisms of actions in the living systems as well as their medicinal, industrial, and commercial applications. Phytochemistry is an important part of the range of disciplines, such as systematic botany, taxonomy, ethnobotany, conservation biology, plant genetic and metabolomics, evolutionary sciences, and plant pathology. The achievements in phytochemistry in the discovery of bioactive compounds is applied in pharmacy and pharmacognosy, complementary and alternative medicine, ethnomedicine, biochemistry, microbiology, bioinformatics, and computational chemistry. In the biotechnology and process engineering, nutrition and food sciences, as well as in organic chemistry, the expertise in phytochemistry is very important for improvement of processes yielding natural products. In the control of environmental pollution, for the application of bioremediation techniques, such as phytoremediation for the removal of harmful substances, the competence in phytochemistry is essential. Since phytochemistry is closely related to many biosciences, there are different opinions about the place of phytochemistry as a discipline. Some scientists consider it a subfield of botany and chemistry, while others believe that it should be a part of food and medical chemistry due to its wide application in drug discovery. In any case, it is difficult to single out phytochemistry as a discrete discipline.

To study chemical interactions in plants based on the chemical knowledge to successfully isolate components and determine molecular structure by studying their properties, it is necessary to know the basics of plant science, isolation, and identification of molecules from plants. Besides, knowledge and expertise on various analytical techniques for extraction, characterization and quality assessment are prerequisite. In addition, understanding natural products induction, metabolomics profiling (nuclear magnetic resonance (NMR), mass spectrometry (MS), micro-fractionation, natural products database, e-bioprospecting) is required. Expertise in the state-of-art techniques including the various extraction methods, for example, solvent extraction methods, supercritical fluid extraction, microwave-assisted extraction, chromatographic fingerprinting, and marker compound analysis is necessary. Advances in chromatographic techniques (liquid chromatography–MS; liquid chromatography–NMR), gas chromatography–MS, anti-microbial and antioxidant studies will help in a comprehensive analysis of natural product extracts. In the recent years, studies are being conducted in relation to the stress induction of natural products under metabolomics view (plant

metabolomics). The use of metabolomics in conjunction with direct NMR profiling approaches is important for the understanding metabolic reactions in plants.[1]

The aim of this paper is to present the basic goals of our phytochemical research on a single model example – gender *Amphoricarpos* Vis.:

1) isolation and identification of new (biologically active) compounds – potential drugs and

2) chemotaxonomy (chemosystematics).

The classification of the genus *Amphoricarpos*, an endemic species of the western part of the Balkan Peninsula, is somewhat vague. In the examination of European *Amphoricarpos* complex, Blečić and Mayer[2] reported two endemic species: *A. neumayeri* Vis. and *A. autariatus* Blečić *et* Mayer, the latter comprising two subspecies, ssp. *autariatus* and ssp. *bertisceus* Blečić *et* Mayer. The occurrence of *A. neumayeri* is limited to coastal Montenegro mountains over Boka Kotorska Bay, Orjen and Lovćen, whereas *A. autariatus* could be found throughout the wider area. The taxon growing on mountains of Bosnia, Herzegovina and northwest Montenegro was assigned as *A. autariatus* ssp. *autariatus* and the remaining one, mostly inhabiting mountain group Prokletije (situated between Montenegro, Kosovo, and Albania) and the mountains of north Greece was denoted as *A. autariatus* ssp. *bertisceus*. On the other hand, Webb[3] recognized only a single species, *A. neumayeri* Vis., divided in two subspecies, *i.e.*, ssp. *neumayeri* and ssp. *murbeckii* Bošnjak (syn. *Amphoricarpos autariatus* Blečić & E. Mayer).

Since the insight into secondary metabolites could provide additional information about the systematics of this taxon, a phytochemical study of plant species of the genus *Amphoricarpos* from different localities, collected over the years, was undertaken.

2. RESULTS AND DISCUSSION

2.1. Analysis of volatile components

Volatile components of plants of the genus *Amphoricarpos* from various localities[4] were obtained by steam distillation on a Likens–Nickerson continuous distillation–extraction apparatus. The volatiles were concentrated in dichloromethane and analyzed by GC-FID and GC–MS techniques. The components were identified based on the retention indices and comparison with reference spectra (Wiley and NIST databases). The relative percentage of the identified compounds was computed from GC-FID peak area, and the results are presented in the Supplementary material to this paper (Table S-I of the Supplementary material to this paper).

According to the GC-FID and GC–MS analyses, caryophyllene oxide is the main component in almost all samples. Based on the relative distribution of main components, three groups of samples are recognized.

In the sample OR04, *A. neumayeri* Vis. according to Blečić and Mayer[2], the main components are caryophyllene oxide (37.68 %), palmitic acid (9.93 %) and *β*-caryophyllene (6.80 %).

In the second group, caryophyllene oxide (28.53–36.68 %), decanal (12.83 to 13.58 %) and palmitic acid (7.50 to 8.18 %) are dominant. This group consists of samples that according to Blečić and Mayer[2] belong to *A. autariatus* ssp. *autariatus*.

The third group consists of samples belonging to *A. autariatus* ssp. *bertisceus*, in which results are inhomogeneous.

The results of analysis of volatile components are in support of the claims by Blečić and Mayer[2].

2.2. Analysis of nonvolatile components

Determination of sesquiterpene lactones content in plant species of the genus Amphoricarpos. Crude extracts of grounded aerial parts (BEM) of various *Amphoricarpus* species from different localities, collected over several years were analyzed by ^{1}H-NMR (Table I).[4] In all investigated samples the signals from the sesquiterpene lactones were present. Since the overall content of sesquiterpene lactones in the studied species was rather high (≥ 1–2 %, calculated per weight of the dried plant material), ^{1}H-NMR spectroscopy was applied to determine the total content of sesquiterpene lactones in the aerial parts of the examined plant species of the *Amphoricarpos* genus. The analysis was performed by comparison of the integral of the exomethylene H-13 proton of sesquiterpene lactones (δ ~6.2) with that of the two-proton singlet (δ ~7) of 2,6-*bis*(1,1-dimethylethyl)-4-methylphenol (BHT), used as internal standard.

The percentage of the lactones in the dry plant material was calculated as follows:

$$w_{\text{lakt}} = 100 \frac{m_{\text{BHT}} M_{\text{lakt}} 2 I_{\text{lakt}}}{M_{\text{BHT}} I_{\text{BHT}} m_{\text{sb}}} \quad (1)$$

w_{lakt} / %, content of lactone in dry plant; m_{BHT} / mg, the mass of BHT added to the plant material; M_{lakt}, average molar mass of lactone (270 g/mol); 2, the number of protons from which the BHT signal originates; I_{lakt}, the value of the integral of the exomethylene proton lactone, H-13; M_{BHT}, molar mass of BHT (220 g/mol); I_{BHT}, the value of the integral of the aromatic BHT protons, H-3 i H-5; m_{sb} / mg, mass of weighed dry plant material.

Quantitative ^{1}H-NMR analysis showed that plant species of the genus *Amphoricarpos* are characterized by a relatively high content of sesquiterpene lactones. The largest mass fraction of lactones is present in the samples of *A. neumayeri* Vis. – more than 1.50 %, calculated by weight of dry plant material. Samples of *A. autariatus* ssp. *autariatus* contain about 0.50 % of sesquiterpene lactones, and samples of *A. autariatus* ssp. *bertisceus* (according to Blečić and

Mayer) contain between 0.36 and 1.12 % of these compounds, depending on the location and time of collection of plant material.

TABLE I. Content of sesquiterpene lactones in crude extracts of aerial parts (BEM) of various species of the genus *Amphoricarpos*, based on quantitative ^{1}H-NMR analysis; dpm – dried plant material (aerial parts); BEM – petrol ether–diethylether–methanol (1:1:1 volume ratio)

Sample	m_{dpm} / mg	m_{BHT} / mg	I_{lact}	I_{BHT}	$w_{lact\ BEM}$ / %
OR04[a]	1061.4	7.264	51.19	48.81	1.76
OR06[a]	1020.0	4.728	58.98	41.02	1.60
OR07[a]	1030.0	8.274	53.39	46.61	2.26
KT04[b]	1029.4	2.238	49.35	50.65	0.52
KD05[b]	1041.4	2.179	50.05	49.95	0.52
KT05[b]	1013.0	2.179	52.01	47.99	0.57
PR01[c]	1041.8	2.775	48.27	51.73	0.61
ZEL02[c]	1002.7	2.903	51.35	48.65	0.75
VIS04[c]	1013.4	4.718	44.87	55.13	0.93
SINJ04[c]	1027.1	4.104	46.72	53.28	0.86
VIS05[c]	1055.1	7.264	38.18	61.82	1.04
VK05[c]	1002.5	4.100	45.22	54.78	0.83
PLK05[c]	1005.6	2.361	55.61	44.39	0.72
POP05[c]	1001.6	4.903	48.35	51.64	1.12
SINJ05[c]	1035.3	4.540	47.31	52.69	0.97
KOT06[c]	820.0	2.955	29.06	70.94	0.36
GR07[c]	530.0	2.955	54.46	45.54	0.52
VIS07[c]	1030.0	4.728	33.41	66.59	1.06

[a]*A neumayeri* Vis.; [b]*A. autariatus* ssp. *autariatus*; [c]*A. autariatus* ssp. *Bertisceus* (according to Blečić and Mayer[2])

Leaf-surface waxes of plant species of the genus Amphoricarpos. Intact air--dried leaves were sonicated with dichloromethane and extract filtered and evaporated. The solid residue was treated with *n*-hexane to separate non-polar from a more polar fraction.[4]

GC/FID and GC–MS analysis identification of the components of non-polar fraction was done based on the retention indices and comparison with reference spectra (Wiley and NIST databases). The relative percentage of the identified compounds was computed from GC/FID peak area. The results are given in Table II.

In all analyzed samples the non-polar fraction contained *n*-alkanes with chain lengths ranging from 27 to 33 carbons, with over 90 % of odd-number of carbons.

There was no pattern in *n*-alkane distribution, so their analysis did not contribute to the classification of *Amphoricarpos* genus.

During the extraction of surface waxes with dichloromethane and re-extraction with *n*-hexane, it turned out that the fraction insoluble in hexane contained, to a large extent, sesquiterpene lactones.[4] They were separated from the other ingredients by methanol extraction (SL). By measuring the plant material and solid

extracts in each extraction phase, approximate mass fractions of lactone in whole dry leaves were obtained. The results are shown in Table III.

TABLE II. Results of GC/FID and GC/MS analyses of non-polar fraction of leaf-surface waxes

Sample	Content of *n*-alkanes, %							Total content of *n*-alkanes, %
	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	
OR04[a]	12.94	0.77	41.53	1.14	33.35	0.59	2.75	93.07
OR06[a]	1.43	2.77	17.45	1.77	44.18	1.46	26.36	95.42
OR07[a]	1.88	–	19.55	0.89	47.06	1.09	23.80	94.27
KT04[b]	10.54	0.74	35.81	1.22	39.11	0.50	3.31	91.23
KD05[b]	16.01	0.90	38.41	1.45	30.17	0.72	2.96	90.62
KT05[b]	19.63	1.32	42.03	1.76	29.47	–	2.46	96.67
PR01[c]	13.28	0.94	43.66	1.23	31.74	–	1.39	92.24
ZEL02[c]	13.35	0.64	42.73	1.02	33.88	0.59	2.54	94.75
VIS04[c]	15.50	0.90	43.40	1.09	30.96	–	2.07	93.92
SINJ04[c]	20.58	0.91	44.68	0.95	26.69	–	–	93.81
VIS05[c]	9.22	1.18	39.69	1.53	38.08	0.90	3.34	93.94
VK05[c]	12.52	1.02	42.91	1.53	35.81	0.44	3.08	97.31
PLK05[c]	15.82	0.77	46.97	1.17	28.20	–	2.45	95.38
POP05[c]	22.82	1.27	47.12	1.09	21.20	–	–	93.52
SINJ05[c]	12.67	0.99	42.13	1.86	31.69	1.10	2.65	94.10
KOT06[c]	1.95	1.71	17.42	1.76	44.86	1.50	26.31	95.51
GR07[c]	1.24	1.44	22.09	1.52	45.78	1.08	22.98	96.13
VIS07[c]	1.38	1.81	36.76	3.75	32.04	–	20.59	94.95

[a]*A neumayeri* Vis.; [b]*A. autariatus* ssp. *autariatus*; [c]*A. autariatus* ssp. *Bertisceus* (according to Blečić and Mayer[2])

TABLE III. Approximate percentage of sesquiterpene lactones on the leaf surface (LS) of plants of the genus *Amphoricarpos*

Sample	m_{leaves} / g	m_{lact} / mg	$w_{lact\ SL}$ / %
OR04[a]	1.04	18.3	1.76
OR06[a]	1.06	22.5	2.23
OR07[a]	1.01	33.9	3.36
KT04[b]	1.01	13.0	1.29
KD05[b]	1.26	16.2	1.28
KT05[b]	1.08	15.4	1.42
PR01[c]	0.98	10.2	1.04
ZEL02[c]	1.04	17.1	1.64
VIS04[c]	1.00	14.8	1.48
SINJ04[c]	1.05	16.8	1.60
VIS05[c]	1.04	16.7	1.60
VK05[c]	1.04	10.8	1.04
PLK05[c]	1.00	19.3	1.93
POP05[c]	1.02	16.1	1.58
SINJ05[c]	1.02	15.4	1.51
KOT06[c]	1.05	12.6	1.20
GR07[c]	0.52	5.3	1.02
VIS07[c]	1.06	17.6	1.66

[a]*A neumayeri* Vis.; [b]*A. autariatus* ssp. *autariatus*; [c]*A. autariatus* ssp. *Bertisceus* (according to Blečić and Mayer[2])

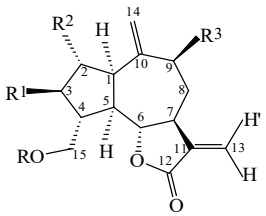
Considering these data and the previous results of ^{1}H-NMR quantifitation, it is shown that the largest mass fraction of lactones is present in the samples of *A. neumayeri* Vis., and the content of sesquiterpene lactones in *A. autariatus* ssp. *bertisceus* (according to Blečić and Mayer[2]) also varies depending on the locality and collection time of plants material.

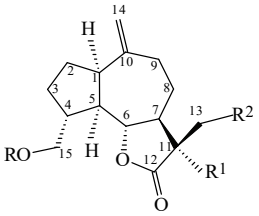
2.3. Isolation and characterization of secondary metabolites

Isolation of secondary metabolites begun with classic methods of extraction of aerial parts[5] followed by dry-flash, column and preparative thin-layer chromatography. In this manner, 23 new sesquiterpene lactones (SL, named amphoricarpolides, Table IV), all of them guaianolides, were isolated.[4,6,7] The structures of isolated SL were elucidated by detailed analysis of IR, NMR, and MS data.

Further 9 SL were isolated from extracts gained as a polar fraction (SL) during leaf-surface waxes investigation.[8,9] Given the great diversity of lactones isolated by classical separation methods, the study was continued by semi-preparative LC analysis. The SL isolated solely in this manner are presented in the Table IV.

TABLE IV. Sesquiterpene lactones (SL) isolated using classical extraction and separation methods, and semi-preparative LC





1–17, 23					18–22			
Lactone	R	R^1	R^2	R^3	Lactone	R	R^1	R^2
1	H	H	H	H	**18**[a]	H	OH	OH
2	H	OH	H	H	**19**	H	OH	OH
3	Ac	OH	H	H	**20**	Ac	OH	OH
4	Ac	OAc	H	H	**21**	Ac	OH	Cl
5[a]	Ac	OAc	H	H	**22**	H	OH	Cl
6	*i*-Val	OAc	OH	H				
7	Ac	OAc	OH	H				
8	Ac	OH	OH	H				
9	*i*-Val	OH	OH	H				
10	H	OAc	OH	H				
11	Ac	OAc	H	OH				
12	Ac	OAc	OAc	H				
13	*i*-Val	OAc	H	OH				
14	Ac	OH	H	OH				
15	*i*-Val	OH	H	OH				
16	Ac	H	H	H				
17[a]	H	H	H	H				
23[a]	Ac	OH	H	H				

TABLE IV. Continued

SL isoladed by semi-preparative LC								
24–31					**32**			
Lactone	R	R^1	R^2	R^3	Lactone	R	R^1	R^2
24[b]	H	H	H	H	**32**	Ac	H	OH
25[b]	Ac	H	H	H				
26[c]	Ac	H	H	H				
27	Ac	OAc	H	α-OH				
28	Sen	OAc	H	OH				
29	*i*-Val	OAc	H	H				
30	H	OH	H	OH				
31	H	OH	OH	H				

[a]10α(14)-Epoxy; [b]11(13)-epoxy; [c]10α(14)-epoxy

2.4. Statistical analysis of data obtained by phytochemical analyses of plant extracts of the genus Amphoricarpos

To gain insight into the possible pattern of synthesis of secondary metabolites in different species of plants of the genus *Amphoricarpos*, plant extracts were subjected to LC-DAD and LC-ESI–MS analyses.[4] The extracts were obtained by standard procedure[5] (BEM), and by dichloromethane extraction, after separation of non-polar fraction of surface waxes (SL). The results obtained by aforementioned analyses, as well as quantitative NMR and gravimetric analyses (Supplementary material, Table S-II), were subjected to statistical analysis.

Principal component analysis (PCA) was performed as *unsupervised* multivariate analysis for easier understanding of the structure of elements and characteristics in extensive tabular data. Discriminant analysis (DA) was employed to differentiate *a priori* defined groups and to assort the elements within the predefined groups. Cluster analysis (CA) was performed with the aim of identification of the relations among populations. Statistical analyses were all carried out with the software Statgraphics Plus (version 5.0; Statistical Graphics Corporation, USA) and Statistica (version 5, StatSoft. Inc., USA).

^{1}H-NMR, gravimetric and LC-DAD analyses, 18 populations. PCA explained 66.66 % of the total variance for the two main axes (axis 1, 40.10 %; axis 2, 26.56 %, Fig. 1).

The greatest influence on the formation of the first axis (axis 1) have the content of sesquiterpene lactones in BEM extracts of plants of the genus *Amphoricarpos* based on quantitative ^{1}H-NMR analysis ($w_{\text{lact BEM}}$), the content of sesquiterpene lactones in the whole dry leaves (SL extracts) based on gravimetric analysis ($w_{\text{lact SL}}$), and lactones **3**–**8** (in uniform proportion), and on the formation of the second axis (axis 2) lactones **1** and **19**. The plot of factor scores showed a tendency to form three groups. The first group consists of populations 1–3 characterized by higher content of lactones **3**, **4**, **7** and **8** (Table S-II, Supple-

mentary material). The second group consists of populations 4, 5 and 7 characterized by high content of lactones **9**, **11** and **13**. The third group is the least homogeneous and consists of other populations.

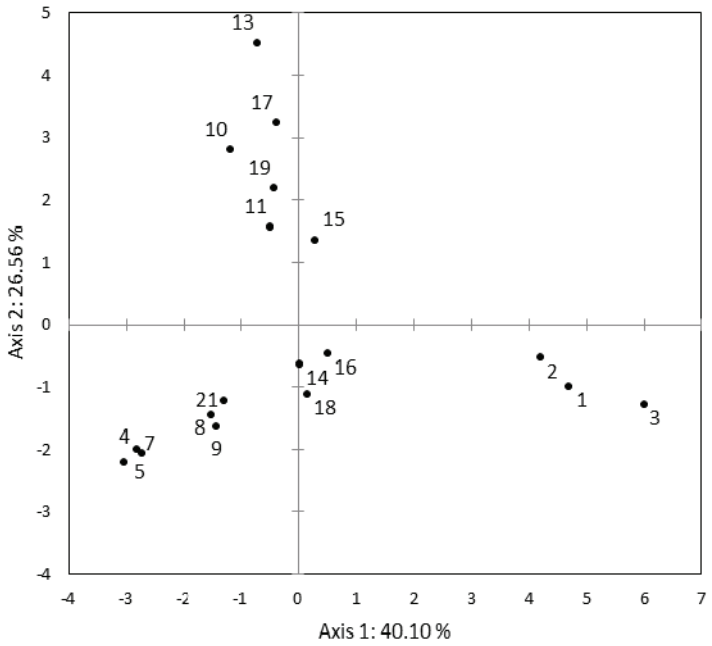


Fig. 1. Principle component analysis (PCA, Score plot) of 16 components (entries 1–16, Table S-II, Supplementary material) in 18 populations (1–5, 7–11, 13–19 and 21) of *Amphoricarpos* taxa.

This situation is clearly illustrated by cluster analysis (Fig. 2). At the higher distance of the cluster (upper gray line), two groups of taxa stand out corresponding to *A. neumayeri* (populations 1–3) and *A. autariatus* (the other 18 populations to the right). At the lower distance (bottom gray line) three groups of taxa stand out corresponding to *A. neumayeri* (populations 1–3), *A. autariatus* ssp. *autariatus* (populations 4, 5 and 7) and *A. autariatus* ssp. *bertisceus* (the other 12 populations).

The groundedness for the formation of the three groups previously suggested by PCA was verified by DA analysis (Fig. 3). The clear formation of the three groups obtained by DA analysis correspond to the defined taxa: *A. neumayeri* (group 1), *A. autariatus* ssp. *autariatus* (group 2) and *A. autariatus* ssp. *bertisceus* (group 3). Over 98 % of discrimination is explained by the first function (dis1), and lactones **4** and **7**, and the contents of sesquiterpene lactones in BEM ($w_{\text{lact BEM}}$) and SL ($w_{\text{lact SL}}$) plant extracts have the greatest influence on this group separation (based on standardized coefficients for canonical variables).

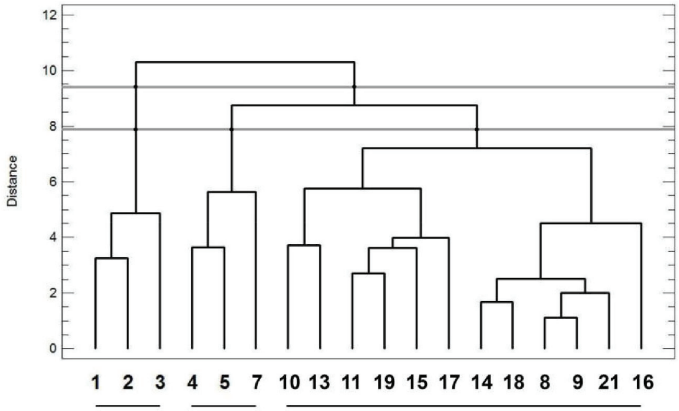


Fig. 2. Cluster analysis (CA) based on a 'furthest-neighbor method' (Euclidean distance) of the 18 studied populations (1–5, 7–11, 13–19 and 21). The numbers on the vertical axis refer to distance level, calculate based on differences between population contents of lactones. The numbers on the horizontal axis refer to the 18 studied populations.

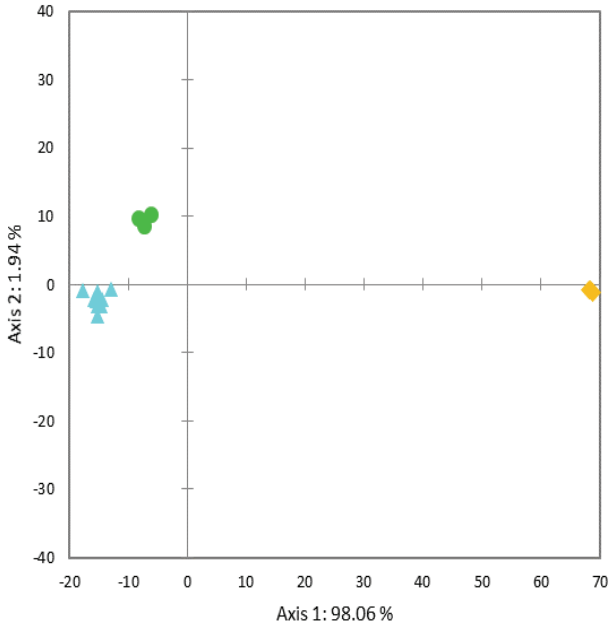


Fig. 3. Discriminant analysis (DA) of 16 components (entries 1–16, Table S-II, Supplementary material) of 18 populations (1–5, 7–11, 13–19 and 21) of *Amphoricarpos* taxa. Populations 1–3 (squares); populations 4, 5 and 7 (circles); populations 8–11, 13–19 and 21 (triangles).

LC–MS analysis, 20 populations. PCA explained 59.84 % of the total variance for the two main axes (axis 1, 40.39 %; axis 2, 19.45 %, Fig. 4).

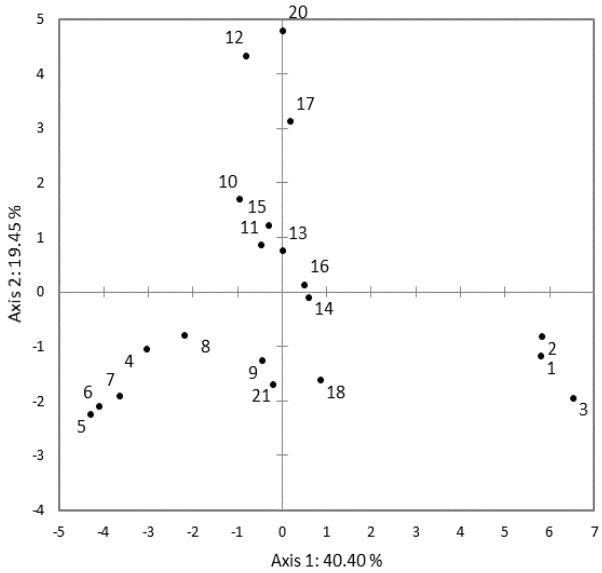


Fig. 4. Principle component analysis (PCA, Score plot) of 23 components (entries 17–39, Table S-II, Supplementary material) in 20 populations (1–18, 20–21) of *Amphoricarpos* taxa.

Lactones **3**, **4** and **8** in both, BEM and SL extracts (in uniform proportions) have the greatest influence on the formation of the first axis (axis 1), and lactone **17** in BEM extracts on the formation of the second axis (axis 2). The plot of factor scores showed a tendency to form three groups. The first group consists of populations 1, 2 and 3 characterized by a higher content of lactones **3**, **4**, **7** and **8** in both, BEM and SL extracts (Table S-II). The second group consists of populations 4–7 characterized by a higher content of lactones **11**, **13** and **14** in BEM, and lactone **13** in SL extracts. The third group is heterogeneous and consists of other populations. This situation is clearly illustrated by cluster analysis (Fig. 5).

Identical to the previous cluster analysis, three groups stand out. The basis of the formation of the three groups previously suggested by PCA was verified by DA analysis (Fig. 6).

The clear formation of the three groups obtained by DA analysis correspond to the defined taxa: *A. neumayeri* (group 1), *A. autariatus* ssp. *autariatus* (group 2) and *A. autariatus* ssp. *bertisceus* (group 3). Over 90 % of discrimination is explained by the first function (dis1) and the greatest influence on this group separation has lactone **11** in BEM extracts (and with this component highly correlated properties **14** in BEM, and **11** and **13** in SL extracts; $r > 0.7$, $P < 0.001$) and **3** in BEM extracts (and with this component highly correlated properties **8** in BEM, and **3** and **8** in SL extracts; $r > 0.7$, $P < 0.001$; based on correlation properties and standardized coefficients for canonical variables, not shown, r = correlation coefficient, P = probability).

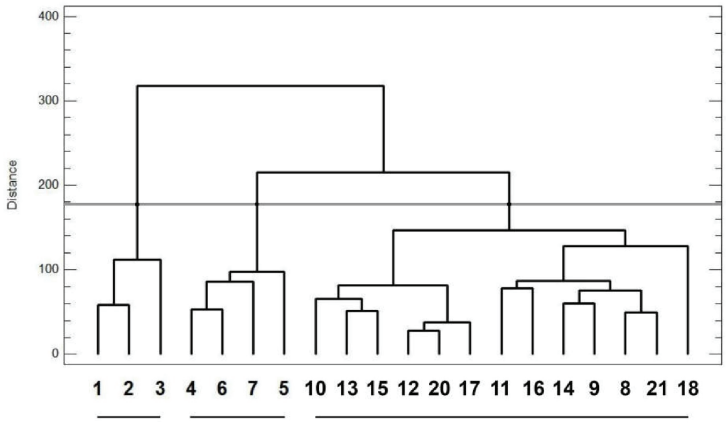


Fig. 5. Cluster analysis (CA) based on a 'furthest-neighbor method' (Euclidean distance) of the 20 studied populations (1–18, 20–21). The numbers on the vertical axis refer to distance level, calculate based on differences between population contents of lactones. The numbers on the horizontal axis refer to the 20 studied populations.

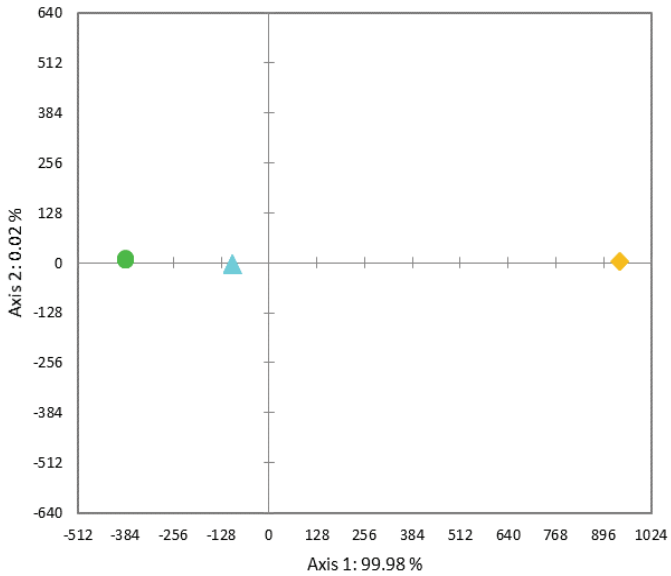


Fig. 6. Discriminant analysis (DA) of 23 components (entries 17–39, see Table S-II, Supplementary material) in 20 populations (1–18, 20 and 21) of *Amphoricarpos* taxa. Populations 1–3 (square); populations 4–7 (circle); populations 8–18, 20 and 21 (triangle).

The chemical analyses of sesquiterpene lactones (^{1}H-NMR, gravimetric, LC/DAD and LC–MS) combined with multivariate data analysis (PCA, DA and CA) discriminated three taxa of *Amphoricarpos* populations: 1) *A. neumayeri* – taxon characterized by higher content of lactones **3**, **4**, **7** and **8**, 2) *A. autariatus* ssp.

autariatus – taxon characterized by higher content of lactones **9**, **11** and **13** and 3) *A. autariatus* ssp. *bertisceus* – the least homogeneous taxon. Lactones **3**, **4**, **7** and **11**, as well as the contents of sesquiterpene lactones in BEM ($w_{\text{lact BEM}}$) and SL ($w_{\text{lact SL}}$) plant extracts have the greatest influence on taxon separation. Based on this, it can be concluded that the chemotaxonomic status of *Amphoricarpos* in Montenegro coincides with the taxonomic status previously defined by Blečić and Mayer[2].

2.5. Metabolomic study of the genus Amphoricarpos

In addition to the above (phytochemical) research, *Amphoricarpos* taxa was the subject of metabolomic analysis. Metabolomics is a new scientific multidisciplinary area that encompasses various aspects of biology, chemistry and mathematics. It uses modern spectroscopic and chromatographic techniques (NMR, IR, MS, GC, LC) and statistical (multivariate) data analysis (PCA, PLS, OPLS-DA, etc.) to measure quantitatively and qualitatively as many metabolites, in the studied organism, as possible and thus obtain a clear metabolic picture under the given conditions. Our metabolic study of the genus *Amphoricarpos* had two objectives: 1) new knowledge about the taxonomic status of the genus and 2) the development of a simple method for identification biologically active compounds in the plant extract.

2.6. Chemotaxonomic study of the genus Amphoricarpos *using metabolomics*

Experimental procedure of this metabolomic study included the following phases: 1) sample collection, 2) rapid sample drying using anhydrous silica gel, 3) preparing the extracts for NMR analysis, 4) recording ^{1}H-NMR spectra and 5) statistical processing of recorded spectra.[10]

1) The leaves specimens (58 in total) of all three taxa (according to Blečić and Mayer[2]) were collected in July 2014 during the flowering period in Montenegro, at seven locations (Sinjajevina, Lovćen, Tara Canyon, Planinica, Visitor, Vratlo, Orjen).

2) Immediately after collection, the samples were hermetically sealed in plastic bags together with anhydrous silica gel, with the aim of removing water as quickly as possible and thus prevent enzymes from chemically altering the sample.

3) Dried leaves were chopped in the laboratory mill under liquid nitrogen and then extracted with a mixture of deuterated methanol and D_2O (1:1) with the addition of buffer and internal NMR standard – TSP-d_4.

4) All ^{1}H-NMR spectra were recorded at 500 MHz under the same conditions. Typical sample spectra of all three examined taxa are shown in Fig. S-1 of the Supplementary material.

Identification of extract components in ^{1}H-NMR spectra was based on comparison with the spectra of reference compounds, previously isolated in our laboratories, and with the spectra of known compounds from the spectrum library.

Overlapping NMR signals were separated using two-dimensional (2D) NMR methods (COSY, TOCSY, ROESY, HSQC and HMBC).

As major chemotaxonomic markers amphoricarpolides hydroxylated in 2*α*-(**8**) and 9*β*-positions were detected (lactones **11** and **13**, Table II).

5) Statistical processing of multivariate NMR spectra analysis, using PCA methods and OPLS/DA, was performed using SIMCA software (version 14, Umetrics, Umeå, Sweden).

The application of these methods made clear differentiation of three supposed taxa – two species of which one is divided into two subspecies, as can be seen from the graph in Fig. 7.

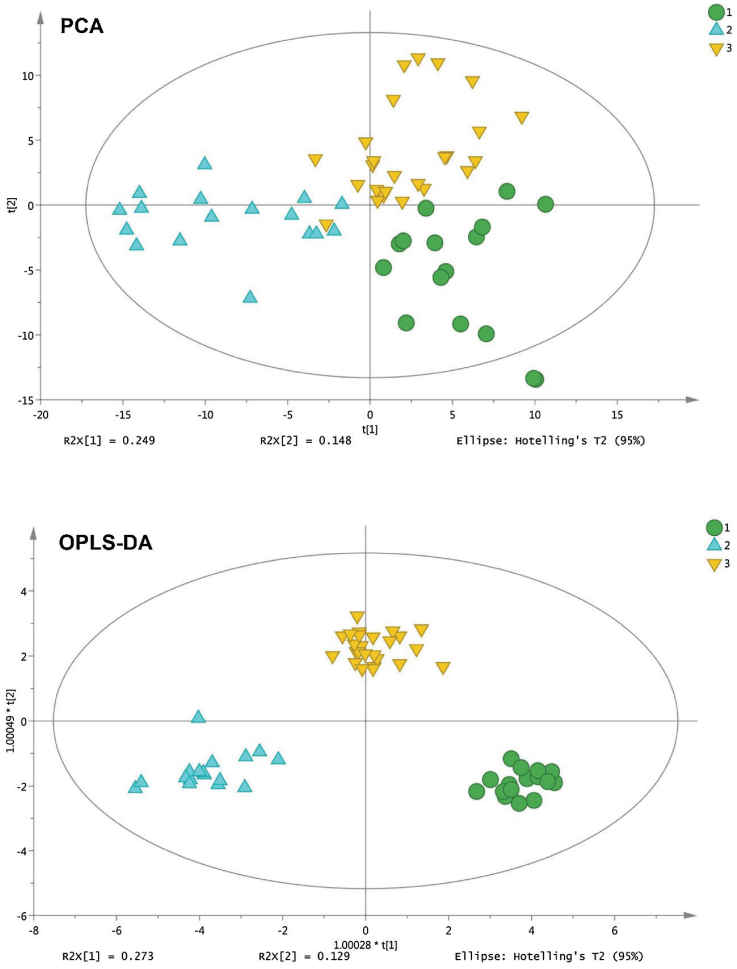


Fig. 7. Results of statistical PCA and OPLS/DA analysis of NMR spectra of extracts of the genus *Amphoricarpos*: 1 – *A. autariatus* ssp. *autariatus*, 2 – *A.autariatus* ssp. *bertisceus*, 3 – *A. neumayeri*.

The score t1 (first component) explains the largest variation of the *X* space, followed by t2 (second component); T2 – significance level of Hotelling's ellipse; R2X[1] – fraction of *X* variation modeled in the component; R2X[2] – cumulative R2X up to the specified component; 1.00028 t[1] – score of the first prediction component scaled proportional to R2X; 1.00049 t[2] – score of the second prediction component scaled proportional to R2X.

Based on the content of major chemotaxonomic markers, that is 2*α*-OH (**8**), 9*β*-OH (**11** and **13**) amphoricarpolides, the differentiation between the two types is demonstrated. Lactone **13** predominates in *A. neumayeri*, while **11** and **8** are characteristic of *A. autariatus.* At the same time different ratio of chlorogenic and malic acid content enabled the separation of two *A. autariatus* subspecies. In the subtype *bertisceus* chlorogenic acid is in excess, while in the subspecies *autariatus* malic acid is dominant. These results are in agreement with the conclusions which were previously given by Blečić and Mayer,[2] on the basis of morphological characteristics of the genus.

2.7. Metabolomic identification of cytotoxic metabolites from A. autariatus *ssp.* autariatus *by application of chromatography/spectroscopy/*in vitro *tests combinations*

A combination of dry-column flash chromatography (DCFC), two spectroscopic methods (NMR and FTIR) and cytotoxicity tests (on HeLa and A549 cervical cancer cells and lung cancer cells) was applied.[11]

Powdered dried leaves of *A. autariatus* ssp. *autariatus* were extracted at room temperature with a mixture of CH_2Cl_2-MeOH (1:1). The extract was separated into 13 fractions by DCFC (SiO_2, eluent CH_2Cl_2/MeOH, gradient 100/0 → 80/20). This chromatographic separation was performed in triplicate. All separated fractions were analyzed using two spectroscopic methods, ^{1}H-NMR and FTIR. At the same time, the *in vitro* cytotoxic effect of each fraction on HeLa and A549 cells was investigated. To correlate chemical composition of fractions, with the results of cytotoxic activity testing, an OPLS analysis was applied. According to the NMR analysis, the highest contribution to the cytotoxic activity was recorded for the fractions containing signals of sesquiterpene *γ*-lactones with characteristic guaianolide skeleton (lactones **11** and **13**, Table IV) which is in line with our previous cytotoxicity study of some amphoricarpolides isolated from the genus *Amphoricarpos*.[12] The results obtained by correlating FTIR data with cytotoxic activity confirmed the results obtained by NMR measurements. These results showed that the sesquiterpene *γ*-lactones may play a major role in cytotoxic activity of the studied extract. This is not unexpected since it is known that the conjugated *α*-methylene-*γ*-lactone group could be responsible for the cytotoxic functions.[13] To finally prove the result obtained from the OPLS models, two identified constituents of the active fractions (lactones **11** and **13**) were tested for cytotoxic activity on HeLa and A549 cell lines. Both reference compounds

exhibited considerable cytotoxic activity, corresponding to the activity obtained from the most effective fractions.

Coupling DCFC chromatography technique to NMR an FTIR based metabolomics and multivariate data analysis revealed the possibility for of the identification of biological active compounds, without prior isolation. This procedure provides a fast method for identification of biologically active compounds combining chromatography and NMR or FTIR spectroscopy techniques with bioassays and multivariate data analysis.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/11002, or from the corresponding author on request.

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ИЗВОД

ФИТОХЕМИЈСКО ИСТРАЖИВАЊЕ БИЉНОГ РОДА *Amphoricarpos*

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Фитохемија се бави изучавањем секундарних метаболита које биљке синтетишу из много разлога, укључујући сопствену заштиту од напада биљоједа и од биљних болести. Сматра се да секундарни метаболити представљају адаптацију биљака на различите факторе околине и да су управо они омогућили опстанак врста. Секундарни метаболити биљака могу испољити лековито или токсично дејство на људе и животиње. Лечење биљем има дугу традицију у народној медицини и све до почетка 20. века, када је почела да се развија синтетичка органска хемија, биљке су биле главни извор лекова. Основни циљеви наших фитохемијских истраживања обухватају: изоловање и идентификацију нових (биолошки активних) једињења – потенцијалних лекова, и хемотаксономију (хемосистематика). У тексту су кроз један одабран пример – род *Amphoricarpos* Vis. – приказана оба наведена аспекта наших фитохемијских истраживања.

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