

Sesquiterpene Lactones of *Amphoricarpos autariatus* ssp. *autariatus* from Montenegro - Antifungal Leaf - Surface Constituents

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Abstract: The composition of leaf cuticular neutral lipids of *Amphoricarpos autariatus* ssp. *autariatus* collected at canyon of river Tara (North Montenegro) was investigated by GC/MS (nonpolar fraction), LC-ESI TOF MS and ¹H NMR spectroscopy (more polar fraction). The nonpolar fraction (ca. 15% of the whole surface extract) contained C₂₇₋₃₃ *n*-alkanes, those with odd-number of carbons predominating. The LC-ESI MS and ¹H NMR of the more polar fraction revealed 13 sesquiterpene lactones, constituting ca. 97.5% of the lactone mixture, identified as the known guaianolides, so-called amphoricarpolides, found previously in the aerial parts of the genus. The lactone fraction exhibited considerable *in vitro* effect against eight fungi, *i.e.* *Aspergillus ochraceus*, *A. niger*, *A. versicolor*, *Penicillium funiculosum*, *P. ochrochloron*, *Trichoderma viride*, *Fusarium verticillioides* and *Fulvia fulvum*.

Keywords: *Amphoricarpos autariatus* ssp. *autariatus*; Asteraceae; Leaf-surface sesquiterpene lactones; Amphoricarpolides; Antifungal activity.

1. Plant Source

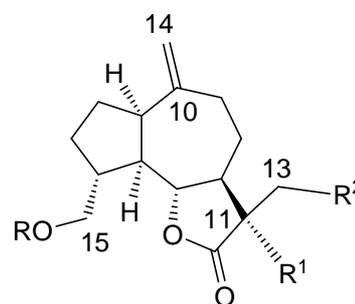
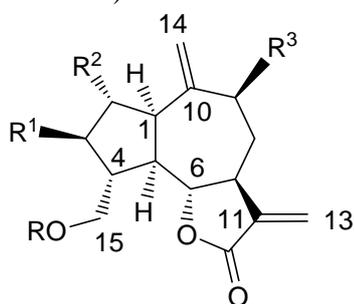
The genus *Amphoricarpos* Vis. (Asteraceae) is the high-land endemic of west part of Balkan peninsula, inhabiting cracks of carbonate rocks from central Bosnia to north-west Greece. The classification of the genus is somehow vague. Blečić and Mayer [1] reported two species: *A. neumayeri* Vis. and *A. autariatus* Blečić et. Mayer, the latter comprising two subspecies, ssp. *autariatus* and ssp. *bertisceus* Blečić et. Mayer. On the other hand, Webb [2] recognized only a single species, *A. neumayeri* Vis., divided in two subspecies, ssp. *neumayeri* and ssp. *murbeckii* Bošnjak.

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The aerial parts of *A. autariatus* ssp. *autariatus* [1] were collected at canyon of river Tara (north Montenegro) during the flowering (July) 2011. Voucher specimen (BEOU 16631) was deposited in the herbarium of the Botanical Garden “Jevremovac”, Faculty of Biology, University of Belgrade.

2. Previous Studies

Our previous phytochemical studies [3-4] of the aerial parts of *A. neumayeri* complex from Montenegro originating from the Orjen, Visitor and Karanfili mountains (the latter a part of mountain chains of Prokletije, North Albanian Alps) revealed twenty one sesquiterpene γ -lactones (**1** - **21**) with the same guaianolide skeleton (named amphoricarpolides). All of them exhibited α -oriented C(15)H₂OX group (X = H, acetyl or *i*-valeroyl). With exception of 11 α ,13-dihydroxy (**18** - **20**) and a 11 α -OH,13-chloro derivative **21** [5], all identified lactones contained 11(13)-double bond. In two of them (**17** and **18**) 10(14)-double bond was epoxidated. It also should be noted that the overall content of sesquiterpene lactones in the studied species was rather high (≥ 1 - 2%, calculated per weight of the dried plant material).



	R	R ¹	R ²	R ³
1	H	H	H	H
2	H	OH	H	H
3	Ac	OH	H	H
4	Ac	OAc	H	H
5^a	Ac	OAc	H	H
6	<i>i</i> -Val	OAc	OH	H
7	Ac	OAc	OH	H
8	Ac	OH	OH	H
9	<i>i</i> -Val	OH	OH	H
10	H	OAc	OH	H
11	Ac	OAc	H	OH
12	Ac	OAc	OAc	H
13	<i>i</i> -Val	OAc	H	OH
14	Ac	OH	H	OH
15	<i>i</i> -Val	OH	H	OH
16	Ac	H	H	H
17^a	H	H	H	H

^a10 α (14)-Epoxy

	R	R ¹	R ²
18^a	H	OH	OH
19	H	OH	OH
20	Ac	OH	OH
21	Ac	OH	Cl

^a10 α (14)-Epoxy

3. Present Study

Our previous examinations were mostly concentrated on identification of secondary metabolites of the genus, but their possible ecological role was not discussed. It is very well known that plants produce a diverse array of secondary metabolites, many of which have antifungal activity, some of these existing in healthy plants in their biologically active forms. Asteraceae is among the principal families whose species accumulate secondary metabolites, including sesquiterpene lactones, with a vast array of important biological activities, deposited in glandular trichomes on the leaf

surface, playing a defensive role against predators (herbivores and microbial pathogens) [5-8]. This prompted us to undertake a study of the secondary metabolites from the leaf surface of a member of the *Amphoricarpos* complex, not studied before, *i.e.* *A. autariatus* ssp. *autariatus* (originating from the canyon of river Tara, north Montenegro), involving identification of their constituents and antifungal assays of the extracts against eight fungi. Owing to the continuing development of microbial resistance in medicine and agriculture, a discovery of new antimicrobial substances is also an important objective [9].

Intact air-dried leaves (300 g) were sonicated with CH₂Cl₂ (2 L) at room temperature for 5 min. After filtration and evaporation of the solvent, the residue (4.41 g, 1.47%) was treated with *n*-hexane (3 x 100 mL) to separate nonpolar alkanes from a more polar fraction. The yields of nonpolar fraction (containing almost exclusively *n*-alkanes) was 0.66 g (0.22 %). The more polar residue (3.6 g, 1.2%) contained sesquiterpene lactones as the major constituents (Supporting information and Table 2). A combination of dry-column flash chromatography and column chromatography on silica gel, described previously [3-4] yielded amphoricarpolides **2-8**, **11-15** and **20**, identified by comparison of their spectra with those published.

GC-FID and GC-MS analysis of the n-alkane fractions: The experimental details regarding the analysis of the *n*-alkane are presented in the supporting information. Identification of the components was done on the basis of the retention index and comparison with reference spectra (Wiley and NIST databases). The percentage (relative) of the identified compounds was computed from GC peak area. According GC-MS and GC FID, the *n*-alkane fraction contained chain lengths ranging from 27 to 33 carbons. Over 90% of them had odd-numbers of carbons (Table 1).

Table 1. The content (%)^a of *n*-alkanes in nonpolar leaf-surface extract of *A. autariatus* ssp. *autariatus*

C ₂₇	C ₂₈	C ₂₉	C ₃₀	C ₃₁	C ₃₂	C ₃₃	Total %
19.63	1.32	42.03	1.76	29.74	-	2.46	96.94

^aBased on relative area in GC FID.

The occurrence of *n*-alkanes is not surprising, since it is well known that such hydrocarbons are present in the waxes of the majority of higher plants.

LC-DAD-ESI HR MS TOF and ¹H NMR analysis of the lactone fraction: The major (insoluble in *n*-hexane) part of the surface extract, *i.e.* 1.2%, calculated per weight of dried leaves, analysed by means LC-DAD-ESI TOF MS and ¹H NMR spectroscopy (Supporting information), contained almost exclusively amphoricarpolides, typical for the genus. The identification of the majority of the constituents (**2 - 8**, **11 - 15** and **19**), constituting *ca.* 97.5% of the lactone fraction (according to the peak areas calculated from total ion current LC-ESI MS TOF chromatograms, Table 2), was based on identity of the spectra of the isolated lactones with those of the amphoricarpolides isolated previously from the samples collected at other localities [3-6]. Among them, amphoricarpolides **11** (36.74 %) and **13** (48.73 %) hydroxylated at C-9, were the major constituents. It should also be noted that these lactones have shown considerable cytotoxic activities against human cervical cancer (HeLa) and murine melanoma (B16) cell lines [10].

Table 2. The content (%)^a of amphoricarpolides in the examined leaf-surface extracts of *A. autariatus* ssp. *autariatus* according to LC-ESI TOF MS

2	3	4	6	7	8	11	12	13	14	15	19	Total
0.29	0.15	0.43	<0.05	0.11	0.37	36.74	5.29	48.73	4.83	0.63	<0.05	97.6

^aRelative areas calculated from total ion current chromatograms

Antifungal-Activity Assay: The results of antifungal activity of the surface lactone extract against eight fungi tested by microdilution method (supporting information) are presented in Table 3. It can be seen that the spectrum of the activities is very wide, depending on fungal species, with MIC in the range of 0.01 - 0.25 mg/mL, and MFC ranging from 0.01 to 0.30 mg/mL. The commercial antifungal agent, bifonazole, showed MIC at 0.10 - 0.20 mg/mL and MFC at 0.20 - 0.25 mg/mL. Ketoconazole showed fungistatic activity at 0.20 - 2.50 mg/mL and fungicidal effect at 0.30 - 3.50

mg/mL. The studied sample showed higher activity than bifonazole against *A. versicolor*, *F. verticillioides* and *F. fulvum*, as well as against *Penicillium* species. In the case of other fungi the samples showed slightly lower or similar activity as bifonazole.

Table 3. Minimum inhibitory (MIC) and fungicidal concentrations (MFC), mg/mL, of the leaf-surface extracts of *A. autariatus* ssp. *autariatus*

Fungus	Extract	Bif ^a	Ketoc ^a
	MIC (MFC)	MIC (MFC)	MIC (MFC)
<i>Aspergillus niger</i>	0.20 (0.25)	0.15 (0.20)	0.20 (0.50)
<i>A. ochraceus</i>	0.25(0.30)	0.15 (0.20)	0.20 (0.50)
<i>A. versicolor</i>	0.05 (0.10)	0.10 (0.20)	0.20 (0.50)
<i>Penicillium funiculosum</i>	0.01 (0.15)	0.20 (0.25)	0.20 (0.50)
<i>P. ochrocloron</i>	0.01 (0.15)	0.15 (0.20)	1.0 (1.0)
<i>Trichoderma viride</i>	0.20 (0.25)	0.20 (0.25)	2.5 (3.5)
<i>Fusarium verticillioides</i>	0.01 (0.01)	0.10 (0.20)	0.20 (0.30)
<i>Fulvia fulvum</i>	0.05 (0.05)	0.10 (0.20)	0.20 (0.50)

^aBif and Ketoc: positive controls, bifunazole and ketoconazole, respectively

Our previous investigation of sesquiterpene lactones showed that some guaianolides possessed very high antifungal activity [11]. The biological activity of sesquiterpene lactones is generally attributed to the alkylating property of the α -methylene- γ -lactone moiety, and the presence of other alkylating sites (epoxides and conjugated carbonyl groups) may enhance their biological activities. Moreover, their lipophilicity seems to play an important role in antifungal activity. Since the chemical composition of the fungal cells walls is highly lipophilic, they generally represent strong barriers for the penetration of hydrophilic compounds, and the transport of polar compounds through the outer lipid layer of fungi is retarded [12].

The fact that these results were obtained in assays of the members of four families of fungi suggests that *Amphoricarpos* secondary metabolites, namely amphoricarpolides, may function as a defense against a variety of fungi. In the complex series of defensive barriers set by plants against the fungi, the studied sesquiterpene lactones seems to play an important role. Accumulated in trichomes they may enhance the resistance to microbial pathogens and at the same time with co-occurring *n*-alkanes increase hydrophobic quality of the plant surface.

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Supporting Information

Supporting Information accompanies this paper on <http://www.acgpubs.org/RNP>

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