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ANTIMICROBIAL AND ANTIOXIDATIVE ACTIVITY OF VARIOUS LEAF EXTRACTS OF *AMPHORICARPOS* VIS. (ASTERACEAE) TAXA

Milan Gavrilović[1,*], Marina D. Soković[2], Milan Stanković[4], Petar D. Marin[1], Zora Dajić Stevanović[3] and Peđa Janaćković[1]

[1] *University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac"*, Studentski trg 16, 11000 Belgrade, Serbia

[2] *University of Belgrade, Institute for Biological Research "Siniša Stanković"*, Bulevar Despota Stefana 142, 11060 Belgrade, Serbia

[3] *University of Belgrade, Faculty of Agriculture*, Nemanjina 6, 11080 Belgrade, Serbia

[4] *University of Kragujevac, Faculty of Natural Sciences*, Radoja Domanovića 12, 34000 Kragujevac, Serbia

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

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Abstract: The antimicrobial and antioxidative activities of diethyl ether, 80% methanol and 50% acetone extracts of the leaves of three *Amphoricarpos* taxa: *A. neumayerianus, A. autariatus ssp. autariatus* and *A. autariatus ssp. bertisceus* (Asteraceae) from the Balkan Peninsula were investigated. The antimicrobial activity was determined by the broth microdilution assay against eight bacterial and eight fungal species. The *in vitro* antioxidative activity was assessed by the DPPH assay. The total phenolic and flavonoid contents were also determined. The most sensitive bacterial species were *Bacillus cereus* and *Staphylococcus aureus.* The best antibacterial potential was obtained for the methanol extract of *A. neumayerianus*, while the diethyl ether extract of this species showed the lowest effect. In general, the tested extracts showed higher activity than the commercial antibiotics streptomycin and ampicillin. Also, all micromycetes were sensitive to the tested extracts. The most sensitive was *Trichoderma viride*. The highest and lowest antifungal effect was determined in *A. a.* ssp. *autariatus* for the diethyl ether and acetone extracts, respectively. The highest total phenolic and flavonoid contents were determined in the methanol extract of *A. a. autariatus*. The best antioxidative activity was shown by the methanol extract of *A. a.* ssp. *autariatus* as comparing to matching extracts from the other two taxa.

Key words: *Amphoricarpos*; Asteraceae; leaf extracts; antimicrobial activity; antioxidative potential

INTRODUCTION

Asteraceae (Compositae) is a family of flowering plants whose species produce and accumulate a wide range of secondary metabolites with important biological activities [1,2]. Many Asteraceae species are known as medicinal plants showing antioxidant and antimicrobial activities due to the presence of phenolics (e.g. flavonoids) and other bioactive metabolites, such as sesquiterpene lactones [3-12]. The genus *Amphoricarpos* Vis. belongs to the family Asteraceae, tribe Cardueae, subtribe Carduinae, informal *Xeranthemum* group [13]. *Amphoricarpos* species are heterocarpic perennial chasmophytic plants, mountain endemics in the eastern Mediterranean (the Balkans, Anatolia and the Caucasus) [13]. There are three taxa distributed on the Balkan Peninsula: *A. neumayerianus* (Vis.) Greuter, *A. autariatus* ssp. *autariatus* Blečić & Mayer and *A. autariatus* ssp. *bertisceus* Blečić & Mayer [17,18]. Some authors have suggested that all Balkan populations should be treated as a single species – *A. neumayerianus* (Vis.) Greuter [14].

Phytochemical studies of the genus *Amphoricarpos* are scarce. To date, phytochemical investigation of this genus showed 13 new amphoricarpolides – guaianolides from *A. neumayeri* Vis. and nine new amphoricarpolides from two *Amphoricarpos* subspecies: *A. a.* ssp. *autariatus* and *A. a.* ssp. *bertisceus* [7,8,15]. This phytochemical data showed the

important differences between investigated taxa in composition and amounts of sesquiterpene lactones amphoricarpolides.

There are limited literature data dealing with the biological activity of *Amphoricarpos* species. Some previously published data describe the cytotoxic activity of *A. neumayerianus* [10]. There is one study dealing with the antifungal activity of leaf surface constituents of *A. a.* ssp. *autariatus* [12]. Also, a closely related species, *Xeranthemum annuum* L., is used in folk medicine [16].

In the present study, we determined the antimicrobial activity of dry diethyl ether, 80% methanol and 50% acetone leaf extracts, and the total phenolic and flavonoid contents and antioxidative potential of dry methanol and liquid methanol and water leaf extracts from three *Amphoricarpos* taxa from the Balkan Peninsula. Our aim was to find a new potential source of biologically active compounds that could serve as a guide in future investigations for potential applications in pharmacy, medicine, agriculture and the food industry. This is the first report of antimicrobial and antioxidative activities of investigated *Amphoricarpos* taxa.

MATERIALS AND METHODS

Plant material

The leaves were collected during the flowering period from plants growing at the following localities: *A. neumayerianus* from Mt. Orjen, Montenegro (N 42°33'35,7"; E 18°33'05,1") in 2010, *A. a.* ssp. *autariatus* from Plužine, Montenegro (N 43°16'47,8"; E 18°50'53,7") in 2011 and *A. a.* ssp. *bertisceus* from Mt. Prokletije, Montenegro (N 42°30'27,7"; E 19°46'50,8") in 2011. In this study, we recognized three taxa of the *Amphoricarpos* genus [17,18]. Voucher specimens were deposited in the Herbarium of the University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac" (accession numbers: BEOU 16939, BEOU 16958 and BEOU 16926).

Preparation of extracts used for investigation of antimicrobial activity

All three samples (4 g of dry intact whole leaves) were extracted with diethyl ether for 24 h at room temperature. The extracts were filtered and the filtrates were evaporated until dry in a rotary evaporator (30.76 mg, 25.53 mg and 30.40 mg of crude dry extracts of *A. neumayerianus, A. a.* ssp. *autariatus* and *A. a.* ssp. *bertisceus* were obtained, respectively). The leaves used for extraction with diethyl ether (three samples) were powdered and each sample was extracted with 80% methanol for 24 h at room temperature. Each extract was filtered and the filtrates were evaporated until dry with a rotary evaporator (0.15 mg, 38.00 mg and 18.60 mg of crude dry extracts of *A. neumayerianus, A. a.* ssp. *autariatus* and *A. a.* ssp. *bertisceus* were obtained, respectively). Three new samples (4 g of dry leaves) were powdered and each was extracted with 50% acetone for 24 h at room temperature. Each extract was filtered and the filtrates were evaporated until dry with a rotary evaporator (15.60 mg, 12.13 mg and 11.64 mg of crude dry extracts of *A. neumayerianus, A. a.* ssp. *autariatus* and *A. a.* ssp. *bertisceus* were obtained, respectively).

Preparation of the extracts used for determination of total phenolic and flavonoid contents and antioxidative potential

One g of dried and powdered leaves of *A. neumayerianus*, *A. a.* ssp. *autariatus* and *A. a.* ssp. *bertisceus* was mixed with 20 mL of methanol and stored at room temperature. After 24 h, the liquid extracts were filtered with Whatman No. 1 filter paper, and the residue was re-extracted with an equal volume of solvent. After 48 h, the process was repeated. The combined liquid extracts were evaporated to dryness at 40°C using a vacuum evaporator. The obtained dry methanol extracts were kept in sterile sample tubes and stored at 4°C. Liquid methanol and water extracts were prepared by mixing 1 mg of dry powdered plant material and 1 mL of solvent. After 24 h, the obtained extracts were filtered using Whatman No. 1 filter paper and stored at 4°C.

Determination of antibacterial activity

Selected Gram (-) (*Enterobacter cloacae* human isolate, *Escherichia coli* ATCC 35210, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhimurium* ATCC 13311) and Gram (+) (*Bacillus cereus* clinical isolate, *Listeria monocytogenes* NCTC 7973, *Micrococcus flavus* ATCC 10240 and *Staphylococcus aureus* ATCC 6538) bacteria were used. The organisms were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia. The antibacterial assay was carried out by the microdilution method [19,20]. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0 x 10^5 CFU/mL. The inocula were prepared daily and stored at 4°C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. All experiments were performed in duplicate and repeated three times.

The minimum inhibitory and bactericidal concentrations (MICs and MBCs, respectively) were determined using 96-well microtiter plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0 x 10^5 CFU/mL. Each extract (diethyl ether, 80% methanol and 50% acetone) was dissolved in 5% DMSO to10 mg/mL in Tryptic Soy Broth (TSB) medium (100 µL) with a bacterial inoculum (1.0 x 10^4 CFU per well) to achieve the desired concentration. The microplates were incubated on a rotary shaker (160 rpm) for 24 h at 37°C. The lowest concentrations without visible growth (observed through a binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MIC). The MBC was determined by serial subcultivation of 2 µL into microtiter plates containing 100 µL of broth per well and further incubation for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating a 99.5% reduction in the viability of the initial bacterial inoculum. The optical density of each well was measured at 655 nm by a microplate manager 4.0 (Bio-Rad Laboratories) and compared with a blank and the positive control. The antibiotics streptomycin and ampicillin (1 mg/mL in sterile physiological saline) served as positive controls. All experiments were performed in duplicate and repeated three times.

Determination of antifungal activity

The fungi *Aspergillus fumigatus* (ATCC 1022), *A. versicolor* (ATCC 11730), *A. ochraceus* (ATCC 12066), *A. niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *P. ochrochloron* (ATCC 9112) and *Candida albicans* (ATCC 10231) used in this study were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia. The micromycetes were maintained on malt agar and the cultures were stored at 4°C and subcultured once a month [21]. The antifungal assay was performed using a modified microdilution technique [19,20]. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of about 1.0 x 10^5 in a final volume of 100 µL per well. The inocula were stored at 4°C until use. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contamination and to check the validity of the inoculum. Determinations of MICs were performed using a serial dilution technique in 96-well microtiter plates. The examined extracts (diethyl ether, 80% methanol and 50% acetone) were added in a concentration of 10 mg/mL in malt agar (MA) medium with the inoculum. The microplates were incubated on a rotary shaker (160 rpm) for 72 h at 28°C. The lowest concentrations without visible growth (examined with a binocular microscope) were defined as MICs. The minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 µL of the tested extracts dissolved in medium for 72 h in microtiter plates containing 100 µL of broth per well at 28°C; this was followed by further incubation for 72 h. The lowest concentration with no visible growth was defined as the MFC, indicating 99.5% reduction in the viability of the initial inoculum. The fungicides bifonazole and ketoconazole served as positive controls (1-3500 µg/mL). All experiments were performed in duplicate and repeated three times.

Determination of total phenolics

The phenolic contents of the dry methanol and liquid methanol and water extracts were determined by

spectrophotometry [22]. The methanol solution of the extract (1 mg/mL) was used for the analysis. The reaction mixture was prepared by mixing 0.5 mL of extract solution with 2.5 mL of Folin-Ciocalteu reagent and 2 mL of 7.5% $NaHCO_3$ and incubated for 15 min at 45°C. The absorbance was determined at λ_{max}=765 nm. Based on the measured absorbance, the content of phenolics in the extracts was expressed in terms of the gallic acid (GA) equivalent (GAE) or mg of GA/g of extract.

Determination of flavonoids

The flavonoid contents of the dry methanol and liquid methanol and water extracts were determined by spectrophotometry [23]. The sample contained 1 mL of the methanol solution of the extract at a concentration of 1 mg/mL and 1 mL of 2% $AlCl_3$. The samples were incubated for 1 h at room temperature. The absorbance was determined at λmax=415 nm. Based on the measured absorbance, the flavonoid contents of the extracts were expressed in terms of the rutin (RU) equivalent (RUE), or mg of RU/g of extract.

Evaluation of antioxidant activity

The ability of the plant extracts (dry methanol and liquid methanol and water extracts) to scavenge the 1,1-dyphenyl-2-picrylhydrazyl (DPPH) free radical was assessed by spectrophotometry [24,25]. Dilutions of stock methanolic solution were made to obtain concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99, 0.97 μg/mL DPPH. After 30 min in the dark at room temperature, the absorbance was measured at 517 nm. The control samples contained all the reagents except the extract. The percentage inhibition was calculated using the equation: % inhibition=100x(A of control – A of sample)/A of control). IC50 values were estimated from the % inhibition *vs.* the concentration sigmoidal curve, using nonlinear regression analysis. The antioxidant efficiency of the extract increased with the decrease of IC50 values.

Data analysis

All data are presented as the means±standard deviations where appropriate. All statistical analyses were performed using Microsoft Excel software.

RESULTS AND DISCUSSION

Antimicrobial activity

The results of *in vitro* antibacterial activity of dry diethyl ether, 80% methanol and 50% acetone leaf extracts of *Amphoricarpos* taxa against eight bacterial species are presented in Table 1. Inhibitory activity was achieved at concentrations ranging from 0.007-0.20 mg/mL; a bactericidal effect was obtained at 0.015-0.25 mg/mL. The most sensitive bacterial strains were *Bacillus cereus* and *Staphylococcus aureus,* while *Listeria monocytogenes* and *Pseudomonas aeruginosa* were the most resistant to the tested extracts. Both commercial antibiotics exhibited lower activity than the tested extracts. The MIC and MBC susceptibilities of streptomycin for the examined microorganisms ranged from 0.04-0.26 mg/mL and 0.09-0.52 mg/mL, respectively, and for ampicillin 0.25-0.74 mg/mL and MBCs of 0.37-1.24 mg/mL, respectively. Among the tested taxa, the antibacterial activities were ranked as follows: of the methanol extracts, the strongest activity was exhibited by the extract of *A. neumayerianus* leaves; of the diethyl ether extracts the strongest activity was displayed by *A. a.* ssp. *bertisceus*, and of the 50% acetone extracts the strongest activity was exhibited by *A. a.* ssp. *autariatus*. The antibacterial potential of the tested extracts was as follows (the numbers refer to different extracts, as stated in Table 1): 1>3>5>6>8>7>4>9>2. The highest and lowest antibacterial potential was demonstrated by the *A. neumayerianus* methanol and diethyl ether extracts, respectively.

The results of *in vitro* antifungal activity of diethyl ether, 80% methanol and 50% acetone extracts of *Amphoricarpos* taxa against eight fungal species are presented in Table 2. All microfungi were sensitive to the tested extracts. The extracts inhibited all micromycetes at 0.001-0.4 mg/mL (MIC) and completely arrested growth (MFC) at 0.02-0.8 mg/mL. The most sensitive species was *Trichoderma viride*, while *Candida albicans* was the most resistant to the extracts. Commercial antimycotics, bifonazole (MIC 0.10-0.20 mg/mL; MFC 0.20-0.25 mg/mL) and ketoconazole (MIC 0.15-2.50 mg/mL; MFC 0.20-3.50 mg/mL) were in general less active than extracts 1, 3, 5 and 6. Extracts 2, 4, 7, 8 and 9 showed lower antifungal

Table 1. Minimum inhibitory (MIC) and bactericidal concentrations (MBC) of the different leaf extracts of *Amphoricarpos* Vis. taxa (mg/mL).

BACTERIA	1 ANMeOH MIC MBC	2 AAAMeOH MIC MBC	3 AABMeOH MIC MBC	4 ANEt$_2$O MIC MBC	5 AAAEt$_2$O MIC MBC	6 AABEt$_2$O MIC MBC	7 ANAcet MIC MBC	8 AAAAcet MIC MBC	9 AABAcet MIC MBC	Streptomycin MIC MBC	Ampicillin MIC MBC
Staphylococcus aureus	0.007±0.0001^{a} 0.060±0.002^{b}	0.125±0.060^{f} 0.250±0.030^{e}	0.045±0.000^{cd} 0.060±0.003^{b}	0.060±0.003^{d} 0.125±0.060^{d}	0.070±0.003^{e} 0.150±0.000^{d}	0.015±0.002^{b} 0.030±0.001^{a}	0.0625±0.003^{de} 0.125±0.030^{d}	0.0625±0.000^{de} 0.125±0.030^{d}	0.125±0.030^{f} 0.250±0.030^{e}	0.040±0.003^{c} 0.090±0.0001^{c}	0.250±0.010^{g} 0.370±0.030^{f}
Bacillus cereus	0.007±0.000^{a} 0.015±0.002^{a}	0.125±0.010^{f} 0.250±0.020^{f}	0.015±0.002^{b} 0.030±0.006^{b}	0.060±0.003^{d} 0.125±0.030^{d}	0.070±0.003^{de} 0.150±0.030^{de}	0.015±0.003^{b} 0.030±0.006^{b}	0.0625±0.0060^{d} 0.125±0.030^{d}	0.030±0.006^{c} 0.0625±0.003^{c}	0.062±0.000^{d} 0.125±0.030^{d}	0.090±0.003^{e} 0.170±0.000^{e}	0.250±0.060^{g} 0.370±0.030^{g}
Micrococcus flavus	0.015±0.002^{a} 0.060±0.003^{a}	0.125±0.030^{f} 0.250±0.060^{c}	0.060±0.000^{d} 0.125±0.010^{b}	0.200±0.010^{h} 0.250±0.030^{c}	0.030±0.006^{b} 0.060±0.003^{a}	0.045±0.006^{c} 0.060±0.003^{a}	0.0625±0.003^{d} 0.125±0.060^{b}	0.080±0.003^{e} 0.125±0.060^{b}	0.125±0.060^{f} 0.250±0.030^{c}	0.170±0.030^{g} 0.340±0.060d	0.250±0.020^{i} 0.370±0.000^{e}
Listeria monocytogenes	0.060±0.006^{c} 0.125±0.010^{b}	0.125±0.060^{d} 0.250±0.000^{c}	0.060±0.006^{c} 0.125±0.000^{b}	0.030±0.000^{a} 0.060±0.003^{a}	0.060±0.002^{c} 0.125±0.020^{b}	0.045±0.000^{b} 0.060±0.006^{a}	0.0625±0.002^{c} 0.125±0.060^{b}	0.030±0.003^{a} 0.125±0.030^{b}	0.030±0.003^{a} 0.125±0.060^{b}	0.170±0.020^{e} 0.340±0.020^{d}	0.370±0.060^{f} 0.490±0.060^{e}
Pseudomonas aeruginosa	0.007±0.0006^{a} 0.030±0.003^{a}	0.125±0.060^{f} 0.250±0.060^{e}	0.015±0.003^{b} 0.030±0.006^{a}	0.060±0.006^{e} 0.125±0.010^{d}	0.035±0.003^{c} 0.070±0.003^{c}	0.045±0.003^{d} 0.060±0.000^{b}	0.0625±0.002^{e} 0.125±0.000^{d}	0.0625±0.003^{e} 0.125±0.030^{d}	0.125±0.000 0.250±0.060^{e}	0.170±0.010^{g} 0.340±0.060^{f}	0.740±0.010^{h} 1.240±0.300^{g}
Salmonella typhimurium	0.070±0.000^{d} 0.080±0.003^{d}	0.125±0.030^{e} 0.250±0.010^{f}	0.015±0.003^{a} 0.030±0.003^{a}	0.030±0.003^{b} 0.060±0.002^{b}	0.035±0.000^{b} 0.070±0.003^{c}	0.015±0.003^{a} 0.030±0.006^{a}	0.0625±0.0030^{c} 0.125±0.060^{e}	0.0625±0.003^{c} 0.125±0.060^{e}	0.125±0.010^{e} 0.250±0.000^{f}	0.170±0.030^{f} 0.340±0.000^{g}	0.370±0.040^{g} 0.490±0.060^{h}
Escherichia coli	0.030±0.006^{a} 0.125±0.030^{b}	0.125±0.020^{e} 0.250±0.030^{c}	0.045±0.006^{b} 0.060±0.003^{a}	0.125±0.010^{e} 0.250±0.020^{c}	0.045±0.006^{b} 0.060±0.006^{a}	0.045±0.003^{b} 0.060±0.003^{a}	0.0625±0.003^{c} 0.125±0.000^{b}	0.080±0.009^{d} 0.125±0.030^{b}	0.125±0.060^{e} 0.250±0.010^{c}	0.170±0.010^{f} 0.340±0.060^{d}	0.250±0.060^{g} 0.490±0.000^{e}
Enterobacter cloacae	0.030±0.000^{a} 0.0625±0.006^{a}	0.125±0.060^{e} 0.250±0.060^{c}	0.030±0.006^{a} 0.060±0.009^{a}	0.125±0.030^{e} 0.250±0.060^{c}	0.030±0.006^{a} 0.060±0.000^{a}	0.045±0.002^{b} 0.060±0.000^{a}	0.0625±0.003^{c} 0.125±0.030^{b}	0.080±0.006^{d} 0.125±0.030^{b}	0.125±0.060^{e} 0.250±0.060^{c}	0.260±0.060^{f} 0.520±0.010^{d}	0.370±0.000^{g} 0.740±0.090^{e}

Different letters in each line indicate significant differences (p<0.05). ANMeOH – dry 80% methanol extract *A. neumayerianus*; AAAMeOH – dry 80% methanol extract *A. a. ssp. autariatus*; AABMeOH – dry 80% methanol extract *A. a. ssp. bertisceus*; ANEt$_2$O – dry diethyl ether extract *A. neumayerianus*; AAAEt$_2$O – dry diethyl ether extract *A. a. ssp. autariatus*; AABEt$_2$O – dry diethyl ether extract *A. a. ssp. bertisceus*; ANAcet – dry 50% acetone extract *A. neumayerianus*; AAAAcet – dry 50% acetone extract *A. a. ssp. autariatus*; AABAcet - dry 50% acetone extract *A. a. ssp. bertisceus.* MICs and MFCs (mg/mL), mean value of two measurements. Bifonazole was used as a stock solution 1 mg mL^{-1}. Ketoconazole was used as a stock solution 1 mg mL^{-1}.

Table 2. Minimum inhibitory (MIC) and fungicidal (MFC) concentrations of various leaf extracts of Amphoricarpos Vis. taxa (mg/mL).

FUNGI	1 ANMeOH MIC MFC	2 AAAMeOH MIC MFC	3 AABMeOH MIC MFC	4 ANEt$_2$O MIC MFC	5 AAAEt$_2$O MIC MFC	6 AABEt$_2$O MIC MFC	7 ANAcet MIC MFC	8 AAAAcet MIC MFC	9 AABAcet MIC MFC	Bifonazole MIC MFC	Ketoconazole MIC MFC
Aspergillus fumigatus	0.025±0.003^{a} 0.050±0.006^{a}	0.200±0.030^{d} 0.400±0.060^{d}	0.10±0.003^{c} 0.20±0.003^{c}	0.200±0.000^{d} 0.400±0.000^{d}	0.050±0.006^{b} 0.100±0.030^{b}	0.025±0.0030^{a} 0.050±0.0060^{a}	0.200±0.030^{d} 0.400±0.060^{d}	0.400±0.000^{f} 0.800±0.060^{f}	0.300±0.030^{e} 0.400±0.060^{d}	0.150±0.030^{cd} 0.200±0.060^{c}	0.200±0.000^{d} 0.500±0.000^{e}
Aspergillus versicolor	0.006±0.0002^{a} 0.025±0.000^{a}	0.100±0.006^{e} 0.200±0.000^{d}	0.05±0.006^{d} 0.10±0.003^{c}	0.400±0.030^{h} 0.800±0.060^{g}	0.0125±0.002^{b} 0.025±0.003^{a}	0.025±0.0020^{c} 0.050±0.0000^{b}	0.200±0.000^{f} 0.400±0.020^{e}	0.100±0.030^{e} 0.400±0.020^{e}	0.300±0.020^{g} 0.400±0.010^{e}	0.100±0.010^{e} 0.200±0.000^{d}	0.200±0.006^{f} 0.500±0.030^{f}
Aspergillus ochraceus	0.030±0.0003^{c} 0.060±0.0003^{c}	0.200±0.003^{e} 0.400±0.060^{e}	0.025±0.000^{b} 0.05±0.003^{b}	0.400±0.060^{f} 0.800±0.030^{f}	0.030±0.006^{c} 0.060±0.003^{c}	0.0125±0.003^{a} 0.025±0.006^{a}	0.400±0.030^{f} 0.800±0.060^{f}	0.400±0.060^{f} 0.800±0.030^{f}	0.400±0.000^{f} 0.800±0.060^{f}	0.150±0.000^{d} 0.200±0.000^{d}	0.150±0.060^{d} 0.200±0.030^{d}
Aspergillus niger	0.025±0.006^{b} 0.050±0.002^{a}	0.200±0.030^{e} 0.400±0.010^{d}	0.05±0.002^{c} 0.10±0.090^{b}	0.400±0.010^{g} 0.800±0.000^{f}	0.006±0.0001^{a} 0.050±0.003^{a}	0.050±0.003^{c} 0.100±0.060^{b}	0.300±0.060^{f} 0.400±0.030^{d}	0.400±0.000^{g} 0.800±0.010^{f}	0.400±0.060^{g} 0.800±0.060^{f}	0.150±0.060^{d} 0.200±0.060^{c}	0.200±0.060^{e} 0.500±0.003^{e}
Trichoderma viride	0.020±0.003^{b} 0.030±0.002^{b}	0.050±0.000^{c} 0.200±0.003^{d}	0.025±0.003^{bc} 0.05±0.001^{c}	0.100±0.030^{d} 0.200±0.030^{d}	0.001±0.000^{a} 0.020±0.000^{a}	0.025±0.003^{bc} 0.050±0.000^{c}	0.050±0.000^{c} 0.200±0.000^{d}	0.100±0.010^{d} 0.400±0.000^{e}	0.050±0.003^{c} 0.800±0.030^{f}	0.150±0.030^{e} 0.200±0.000^{d}	1.000±0.090^{f} 1.000±0.060^{g}
Penicillium funiculosum	0.020±0.003^{a} 0.030±0.000^{a}	0.100±0.001^{c} 0.200±0.006^{d}	0.05±0.000^{b} 0.10±0.010^{c}	0.100±0.010^{c} 0.200±0.000^{d}	0.025±0.006^{a} 0.050±0.003^{b}	0.025±0.003^{a} 0.050±0.003^{b}	0.200±0.010^{d} 0.400±0.020^{e}	0.200±0.030^{d} 0.400±0.000^{e}	0.100±0.000^{c} 0.200±0.030^{d}	0.200±0.010^{d} 0.250±0.006^{de}	0.200±0.030^{d} 0.500±0.000^{f}
Penicillium ochrochloron	0.006±0.0006^{a} 0.050±0.003	0.100±0.000^{d} 0.200±0.030	0.05±0.003^{c} 0.10±0.030	0.200±0.060^{e} 0.400±0.060	0.006±0.0003^{a} 0.050±0.006	0.025±0.000^{b} 0.050±0.003	0.400±0.060^{f} 0.800±0.030	0.200±0.060^{e} 0.400±0.030	0.200±0.030^{e} 0.400±0.060	0.200±0.030^{e} 0.250±0.001	2.500±0.100^{g} 3.500±0.300
Candida albicans	0.025±0.000^{a} 0.050±0.003^{a}	0.400±0.060^{f} 0.800±0.060^{f}	0.10±0.010^{d} 0.20±0.000^{c}	0.400±0.000^{f} 0.800±0.000^{f}	0.050±0.003^{c} 0.100±0.010^{b}	0.030±0.001^{b} 0.050±0.006^{a}	0.200±0.000^{e} 0.400±0.000^{e}	0.200±0.030^{e} 0.400±0.010^{e}	0.200±0.000^{e} 0.400±0.000^{e}	0.100±0.000^{d} 0.200±0.000^{c}	0.200±0.003^{e} 0.300±0.006^{d}

Different letters in each line indicate significant differences (p<0.05). ANMeOH – dry 80% methanol extract *A. neumayerianus*; AAAMeOH – dry 80% methanol extract *A. a. ssp. autariatus*; AABMeOH – dry 80% methanol extract *A. a. ssp. bertisceus*; ANEt$_2$O – dry diethyl ether extract *A. neumayerianus*; AAAEt$_2$O – dry diethyl ether extract *A. a. ssp. autariatus*; AABEt$_2$O – dry diethyl ether extract *A. a. ssp. bertisceus*; ANAcet – dry 50% acetone extract *A. neumayerianus*; AAAAcet – dry 50% acetone extract *A. a. ssp. autariatus*; AABAcet – dry 50% acetone extract *A. a. ssp. bertisceus*. MICs and MBCs (mg/mL), mean values of two measurements.
Streptomycin was used as a stock solution 0.1 mg mL^{-1}. Ampicillin was used as stock solution 0.1 mg mL^{-1}.

activity than the examined commercial antimycotics, with the exception of *T. viride* and *Penicillium* species, where extracts 2, 4, 7, 8 and 9 showed higher inhibitory activity. Extract 2 also displayed a stronger effect than ketoconazole against all fungi, except *C. albicans* and *Aspergillus ochraceus*. The antifungal activities can be presented as follows: for the methanol extract, the extract from *A. neumayerianus* exhibited the strongest activity, for the diethyl ether extract, the strongest activity was displayed by the *A. a.* ssp. *autariatus* extract, while for the acetone extract the strong activities were provided by extracts from the subspecies *A. neumayerianus* and *A. a.* ssp. *bertisceus*. The antifungal potential can be presented as follows: 5>1>6>3>2>7>9>4>8. The diethyl ether and acetone extracts of *A. a.* ssp. *autariatus* had the highest and lowest antifungal effects, respectively.

Growth of the tested microorganisms responded differently to different extracts, indicating that either the various components of the extracts have different modes of action or that the metabolism of some microorganisms was capable of more effectively overcoming the effect of the tested compounds, or to adapt to it. This could explain the lower antifungal than antibacterial activity of extracts 2, 4, 7 and 9. Previous studies have described different compounds (sesquiterpene lactones) from different *Amphoricarpos* taxa [7,8,15]. Together with our results, this suggests that different antimicrobial activities among investigated *Amphoricarpos* taxa are the result of the different composition of secondary compounds that have an important role in biological activity.

Total phenolic and flavonoid contents

The results of total phenolic content determination of the tested plant extracts are provided in Table 3. The total phenolic content was expressed as gallic acid equivalents (GAE), which ranged from 11.90±0.19 to 67.23±0.70 mg/g. Higher concentrations of phenolic compounds were determined in the methanol extracts of all tested taxa than in methanol and water infusions. The highest total phenolic content was measured in the methanol extract of *A. a.* ssp. *autariatus* (67.23±0.70 mg GA/g). The flavonoid content in the methanol and water extracts was expressed in terms of rutin equivalents (Table 4). The flavonoid concentrations in the methanol extracts and methanol liquid and water liquid extracts ranged from 9.79±0.43 to 88.69±1.33 mg RU/g. High concentrations of flavonoids were determined in the methanol extracts of all tested taxa. The highest concentrations of flavonoids were found in the methanol extract of *A. a.* ssp. *autariatus* (88.69±1.33 mg RU/g).

Table 3. Total phenolic content in extracts of *Amphoricarpos* taxa, expressed as gallic acid (GA) equivalents (GAEs; mg of GA/g of extract)

Taxon	Dry methanol extract	Liquid methanol extract	Liquid water extract
A. neumayerianus	41.60±1.04	14.96±0.48	11.90±0.19
A. a. ssp. *autariatus*	67.23±0.70	25.10±0.50	19.71±0.46
A. a. ssp. *bertisceus*	50.74±0.93	20.15±1.10	16.68±0.21

Each value is the average of three analyses ± standard deviation.

Table 4. Flavonoid content in extracts of *Amphoricarpos* taxa expressed as rutin (RU) equivalent (RuE; mg of Ru/g of extract).

Taxon	Dry methanol extract	Liquid methanol extract	Liquid water extract
A. neumayerianus	48.22±0.41	15.97±0.31	9.79±0.43
A. a. ssp. *autariatus*	88.69±1.33	21.14±0.22	13.14±0.12
A. a. ssp. *bertisceus*	54.61±0.45	17.50±0.14	10.16±0.28

Each value is the average of three analyses ± standard deviation.

Table 5. Antioxidant activity of extracts of *Amphoricarpos* taxa expressed in terms of IC_{50} values (μg/mL).

Taxon	Dry methanol extract	Liquid methanol extract	Liquid.water extract
A. neumayerianus	365.52±1.12	748.35±2.05	1041.62±2.35
A. a. ssp. autariatus	170.01±1.58	565.02±1.45	676.18±1.84
A. a. ssp. bertisceus	226.42±1.79	570.26±1.99	684.98±1.60

Each value is the average of three analyses ± standard deviation.

Antioxidative activity

The antioxidant potential of dry methanol and liquid methanol and water extracts from *Amphoricarpos* taxa is expressed in terms of IC_{50} (μg/mL) values. The IC_{50} values for the antioxidant potential of the extract

and liquid extracts are provided in Table 5. The antioxidant activity values ranged from 170.01±1.58 to 1041.62±2.35 μg/mL. The largest capacity to neutralize DPPH radicals was measured in the dry methanol extract from *A. a.* ssp. *autariatus* (170.01±1.58 μg/mL). It correlated with the high total phenolic content and flavonoid concentrations in the dry methanol extract of *A. a.* ssp. *autariatus*. The antioxidant activities of plant extracts have been examined in a number of papers that underscore a linear correlation between antioxidant activity and the total phenolic content [26]. The antioxidative potential observed in *A. a.* ssp. *autariatus* was more pronounced than in the other two taxa. This finding suggests that this subspecies either contains different phenolic and flavonoid compounds or that it possesses the same ingredients that are involved in antioxidative activity, but at higher concentrations.

Using different extracts with different compositions of potentially active compounds with different biological activities could provide a guideline for the selection of the most active extracts and compounds in future investigations. Our research showed that extracts from three closely related taxa have different biological activities due to the presence of different secondary compounds (i.e., sesquiterpene lactones and phenolics). Therefore, further phytochemical and taxonomic investigations of *Amphoricarpos* species are needed. Additional research may also resolve some uncertainties regarding the phylogenetic relationships in this very interesting and complex genus.

Since the results of our research revealed a very high antimicrobial potential of *Amphoricarpos* taxa, this species could serve as an excellent source of potential antimicrobial substances.

The marked antioxidative potential and strong antimicrobial effects observed in extracts of *Amphoricarpos* species render them a promising natural source of biologically active substances, and point to future research of other species of the genus *Amphoricarpos* and related genera of the tribe Cardueae (Asteraceae) and their potential application in pharmacy, medicine, agriculture and the food industry.

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Authors' contributions: MG is the main author who contributed the original data and the designed all the studies conceived in the project, in cooperation with PJ and MS, who carried out the field research, organized and analyzed the data and wrote the manuscript. MDS, MS and PM contributed in designing the research and analyzed the data. ZDS supervised the research project and reviewed several drafts of the manuscript. PJ, as the main supervisor of the research project, carried out the field research and reviewed several drafts of the manuscript. All authors read and approved the final manuscript.

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