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Research Article

Comparative study of the ethanol extracts of six *Acinos* Miller species: chemical composition, antimicrobial and antioxidative activities

Tatjana D. GOLUBOVIĆ[1]*, Gordana S. STOJANOVIĆ[2], Dušanka V. KITIĆ[3], Bojan K. ZLATKOVIĆ[4], Dragana R. PAVLOVIĆ[3], Snežana Č. JOVANOVIĆ[2], Jelena S. LAZAREVIĆ[3]

[1]*University of Niš, Faculty of Occupational Safety, Čarnojevića 10a, 18000 Niš, Serbia; acinos2002@gmail.com (*corresponding author)*
[2]*University of Niš, Faculty of Sciences and Mathematics, Department of Chemistry, Višegradska 33, 18000 Niš, Serbia; gocast@pmf.ni.ac.rs; coka.sslagalica@gmail.com*
[3]*University of Niš, Faculty of Medicine, Department of Pharmacy, Bul. Zorana Đinđića 81, 18000 Niš, Serbia; dkiticyu@yahoo.co.uk; anagard@medfak.ni.ac.rs; jelena217@yahoo.com*
[4]*University of Niš, Faculty of Sciences and Mathematics, Department of Biology and Ecology, Višegradska 33, 18000 Niš, Serbia; bojanzlat@yahoo.com*

Abstract

The ethanol extracts of selected *Acinos* Miller species were investigated in terms of chemical composition, antimicrobial and antioxidative activities. Qualitative and quantitative analyses of the extracts were performed using GC and GC-MS. Eighty-six constituents, accounting for 93.70-99.99% of the total composition of the extracts, were identified. The ethanol extracts of *A. majoranifolius, A. suaveolens* and *A. alpinus* were characterized by domination of monoterpenes, representing 85.03%, 57.39% and 28.02% of the total extracts, respectively. Fatty acids and their esters in the *A. arvensis* and *A. graveolens* extracts reached 28.97% and 30.75%. Also, *n*-alkanes were the major compounds found in *A. hungaricus* ethanol extract (30.98%). The extracts were characterized by determination of total polyphenols, flavonoids and tannins. Besides, the antioxidant activity of the investigated extracts was estimated by two assays: DPPH and FRAP test. The highest antioxidant activity was observed in the extract of *A. alpinus* which had high levels of all examined polyphenol classes. A disk diffusion method was employed for the determination of the antimicrobial activities of the ethanol extracts. Gram-positive: *Bacillus subtilis*, *Sarcina lutea*, *Staphylococcus aureus*, *Clostridium pyogenes*, *Enterococcus* sp., *Micrococcus flavus*; Gram-negative: *Klebsiella pneumoniae*, *Pseudomonas aerugionsa*, *Salmonella enteritidis*, *Proteus vulgaris*, *Escherichia coli* and fungal organisms: *Aspergillus niger*, *Candida albicans* and *Saccharomyces cerevisiae* were used as test microorganisms. The results of preliminary bioassay demonstrated that the *A. alpinus* ethanol extract could be a possible source of compounds with antioxidant and antimicrobial activity.

Keywords: antioxidant properties; chemical composition; extraction; solvent

Introduction

The *Acinos* Miller (Lamiaceae) is genus native to Europe, the Mediterranean, central Asia, North Africa, and North America. The genus *Acinos* is represented in Serbia and Montenegro by six species: *A. arvensis, A.*

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hungaricus, *A. alpinus, A. suaveolens, A. majoranifolius* and *A. graveolens* (Šilić, 1979; Zlatković and Ranđelović, 2004).

Some *Acinos* species have been used as spice and herbal teas as well as a cure for various disorders in traditional medicine (Carović-Stanko *et al*., 2016). Namely, some species of this genus are used in traditional medicine, especially in Mediterranean countries, as antispasmodics, antiseptics, tonics, diuretic, antipyretic and stimulants in the treatment of diarrhea, indigestion, obesity, respiratory diseases, coughs, melancholy, toothache, sciatica and neuralgia (Velasco-Negueruela *et al*., 1993; Kaya *et al*., 1999; Karousou *et al*., 2007). *Acinos* species had been investigated so far, considering that therapeutic application of medicinal plants is correlated with the presence of a wide range of secondary metabolites or phytochemicals with various pharmacological activities (Carović-Stanko *et al*., 2016). The chemical compositions of essential oils of *A. arvensis* (Souleles *et al*., 1988; Kaya *et al*., 1999; Jovanovic *et al*., 2005), *A. hungaricus* (Jovanovic *et al*., 2002; Chalchat *et al*., 2004), *A. alpinus* (Velasco-Negueruela *et al*., 1993; Kaya *et al*., 1999; Skaltsa *et al*., 1999), *A. rotundifolius* (Kaya *et al*., 1999), *A. graveolens* (Golubovic *et al*., 2010; Bidgoli *et al*., 2019), *A. majoranifolius* (Pavlovic, *et al*., 1983), *A. suaveolens* (Pavlovic *et al*., 1983; Kokkalou *et al*., 1988; Tumen *et al*., 1991; Kaya *et al*., 1999; Couladis *et al*., 2002) and *A. troodi* (Kaya *et al*., 1999) were investigated. The oil yield, the qualitative and quantitative composition of tannins and the qualitative analysis of the flavonoids have been reported in the literature (Stojanovic *et al*., 2009). Composition and content of fatty acids of *A. alpinus* and *A. hungaricus* had already been studied (Jovanovic *et al*., 2008). The antimicrobial activity of the *A. arvensis* essential oil was screened against *Escherichia coli*, *Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* All mentioned microorganisms, except for *Pseudomonas aeruginosa,* were susceptible to the oil (Jovanovic *et al*., 2005). It was found that *A. suaveolens* oil was active against *Escherichia coli*, *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hominis* and *Klebsiella pneumoniae* (Couladis *et al*., 2002). In addition, the antimicrobial activity of *A. graveolens* essential oil was investigated and it appeared that the oil was selective against Gram-negative and Gram-positive bacteria. The diluted oil sample inhibited the growth of Gram-negative bacteria, while the Gram-positive ones were highly unsusceptible. On the contrary, a significant reduction in *Candida albicans* growth was observed (Golubovic *et al*., 2010). The activity of the acetone extracts of *A. rotundifolius* root revealed promising results against mostly Gram-positive pathogens. The results indicated the presence of potent antimicrobial agents in the crude extract of this plant species. Also, authors pointed out that it was necessary to carry out a more profound investigation in order to determine the types of agents responsible for the antimicrobial effects in this medicinal plant (Ulukanli *et al*., 2005). As regards the antioxidant activity of *A. suaveolens,* the results indicated that the water extract of *A. suaveolens* had a protective effect against lipid oxidation (Triantaphyllou *et al*., 2001). Data about chemical composition, antimicrobial and antioxidant activities of *Acinos* extracts are very limited; therefore, after an extensive review of the available literature, the only relevant study of the methanol extracts was the study by Golubovic *et al*. (2014). Extraction yield and biological activity of the resulting extract are not only affected by the extraction technique but also by the extraction solvent (Hussain *et al*., 2011; Do *et al*., 2014; Truong *et al*., 2019). For this reason, the aim of the present study was to determine the chemical composition, as well as antimicrobial and antioxidative activities of *A. arvensis, A. hungaricus*, *A. alpinus, A. suaveolens, A. majoranifolius* and *A. graveolens* ethanol extracts in order to compare them with the methanol extracts and realize potentially most appropriate solvent for the extraction of active constituents responsible for antimicrobial and antioxidant efficiency of *Acinos* species.

Materials and Methods

Plant material

Aerial parts of *Acinos* species in flowering stage were collected in May (*A. graveolens*), June (*A. alpinus, A. majoranifolius* and *A. suaveolens*) and July (*A. hungaricus and A. arvensis*) at the following localities: Pčinja-Delinovica (*A. graveolens*), Šara, Gornje selo (*A. alpinus*), Pčinja, Trgovište (*A. suaveolens*), Orjen, Kotor-Njeguši (*A. majoranifolius*), Seličevica, Doljevac (*A. hungaricus*) and Rtanj, Zaječar (*A. arvensis*). The voucher specimens have been deposited at the Herbarium of the Department of Biology and Botanical Garden "Jevremovac", University of Belgrade (BEOU) and in Herbarium Moesicum Doljevac (HMD) under the accession numbers: *A. graveolens*-16140, *A. majoranifolius*-16059, *A. suaveolens*-16349 (BEOU), *A. hungaricus*-365, *A. alpinus*-366 i *A. arvensis*-367 (HMD).

Preparation of plant extracts

Aerial parts of investigated species were air-dried at room temperature, under shade, and then powdered to a fine grade by using a laboratory scale mill. Extracts were prepared by maceration with ethanol (reagent Ph. Eur) according to Ph. Eur. 6.0 (2008). Ethanol extracts of *A. hungaricus, A. alpinus, A. arvensis, A. suaveolens, A. graveolens* and *A. majoranifolius* were obtained after evaporation *in vacuo* at 40 °C and extracts yields were 12.52, 11.40, 11.28, 7.40, 8.80 and 11.20 (%, w/w), respectively.

GC and GC-MS analysis

Qualitative and quantitative analyses of the ethanol extracts were performed using GC and GC-MS. The GC analysis of the extracts was performed on a Hewlett-Packard HP-5890 Series II GC apparatus, equipped with split/splitless injector, and attached to an HP-5 column (25 m × 0.32 mm, 0.52 μm film thickness) and fitted to a flame ionization detector (FID). Carrier gas flow rate (H_2) was 1 ml/min, split ratio 1:30, injector temperature was 250 °C, detector temperature 300 °C, while column temperature was linearly programmed from 40 °C to 240 °C (at the rate of 4 °/min). The same analytical conditions as those mentioned for GC-FID were employed for GC-MS analysis, where Hewlett-Packard HP G 1800C Series II GCD system equipped with HP-5MS column (30 m x 0.25 mm, 0.25 μm film thickness) was used. Transfer line was heated to 260 °C. Mass spectra were acquired in EI mode (70 eV) within m/z range 40-400. The constituents were identified by comparing their mass spectra with those stored in MS libraries (Wiley 275, NIST05 and Adams2007), using different search engines (PBM, NIST 2.0), as well as calibrated AMDIS (ver. 2.64) for determination and comparison of retention indices. The quantitative data were collected electronically from the FID area percentage without using the correction factor (Adams, 2007).

Antimicrobial activity

The *in vitro* antimicrobial activity of the ethanol extracts of *A. arvensis, A. hungaricus*, *A. alpinus, A. suaveolens, A. majoranifolius* and *A. graveolens* against a panel of laboratory control strains belonging to the American Type Culture Collections, Maryland, USA; Gram-positive: *Bacillus subtilis* (ATCC 6633), *Clostridium pyogenes* (ATCC 19404), *Enterococcus* sp. (ATCC 25212), *Micrococcus flavus* (ATCC 10240), *Sarcina lutea* (ATCC 9341), *Staphylococcus aureus* (ATCC 6538); Gram-negative: *Klebsiella pneumoniae* (ATCC 10031)*, Proteus vulgaris* (ATCC 8427), *Pseudomonas aeruginosa* (ATCC 27857), *Salmonella enteritidis* (ATCC 13076), *Escherichia coli* 95 (Institute of Immunology and Virology "Torlak", Serbia); fungal organisms *Aspergillus niger* (ATCC 16404), *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 9763) were determined using the disk diffusion assay recommended by NCCLS (1997). The following nutrient media were used: Antibiotic Medium 1 (Difco Laboratories, Detroit, MI, USA) for growing

the Gram-positive and Gram-negative bacteria and Tripton soy agar (TSA, Torlak, Belgrade, Serbia) for *A. niger* and *C. albicans*. The nutrient media were prepared following the manufacturer's instructions. All agar plates were prepared in 90 mm Petri dishes with 22 ml of agar, giving the final depth of 2 mm. On the solid media plates, a suspension of the tested microorganisms (0.1 ml, 10^8 cells per ml) was spread. Sterile filter paper discs ("Antibiotica Test Blättchen", Schleicher and Schuell, Dassel, Germany, 12.7 mm in diameter) were impregnated with 20 µl of the extracts; afterwards, they were placed on the inoculated plates. After standing at 4 °C for 2 h, these plates were incubated at 37 °C for 24 h for the bacteria, and at 30 °C for 48 h for the fungi. Standard disks of ampicillin, tetracycline, streptomycin+penicillin and nystatin (Institute of Immunology and Virology "Torlak", 30 µg of the active component) were used respectively as the positive controls. On the contary, disks impregnated with 50 µl of dimethylsulfoxide (DMSO) were used as the negative controls."Fisher-Lilly Antibiotic Zone Reader" (Fisher Scientific Co., USA) was used to measure the diameters of the inhibition zones in millimeters. Each test was performed in triplicate. In the end, the mean values have been presented.

Determination of total flavonoids

The content of total flavonoids was determined spectrophotometrically according to Crataegi folium et flores monograph in DAB 10 (1996). Total flavonoid aglycones, released after acid hydrolysis, were determined in $AlCl_3$-complex form, measuring absorbance at 425 nm. Results were expressed in % (w/w) of dry matter, as hyperoside content.

Determination of total phenolics

Folin-Ciocalteu colorimetric procedure was used to estimate the total phenolic content of extracts (Hagerman *et al*., 2000). Quantification was performed using a standard curve with (+)-catechin (concentration span of standard solutions was 0.1-1 mg ml^{-1}), whereas the total phenolics were determined as catechins equivalents (mg CE/g of extract). The results are presented in the form of a triple analysis.

Determination of total tannins

Total tannins content was determined using the same Folin-Ciocalteu procedure after removal of tannins by their absorption using insoluble binding agent (polyvinylpolypyrrolidone, PVPP). Assay was carried out with clear supernatant (Hagerman *et al*., 2000). The results were expressed as catechin equivalents (mg CE/g of extract), while the values are presented by means of triple analysis.

FRAP assay (in vitro ferric-reducing antioxidant power)

Ferric reducing antioxidant power (FRAP) assay was used to measure the total antioxidant activity. An intense blue colour appears when the ferric tripyridiltriazine (TPTZ-Fe^{3+}) complex is reduced to the ferrous tripyridiltriazine (TPTZ-Fe^{2+}) form in the presence of antioxidants at low pH (Pellegrini *et al*., 2003). Appropriately diluted extract or control solution (0.1 ml) was transferred into a test tube and 3.0 ml of freshly prepared FRAP reagent (25 ml acetate buffer: 300 mmol l^{-1}, pH 3.6, 2.5 ml 10 mmol l^{-1} TPTZ in 40 mmol l^{-1} HCl and 2.5 ml 20 mmol l^{-1} $FeCl_3 \bullet 6H_2O$) were added. Afterwards, the tubes were vigorously shaken and absorbance was recorded at 593 nm against a blank containing 0.1 ml of ethanol, after 5 min. Calibration curve of ferrous sulfate (100-1000 mmol l^{-1}) was used and the resulting FRAP values are expressed as mmol of ferric iron reduced per g of sample (mmol Fe^{2+}/g of extract).

DPPH assay (radical scavenging capacity)

The free radical scavenging activities were tested on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical according to the method described by Cuendet *et al*. (1997). Various concentrations of samples (4 ml) were

mixed with 1ml of an ethanol solution of $DPPH^{\cdot}$ (0.05 mM). The mixture was shaken vigorously and left in the dark at room temperature for 30 min. Assay is based on measurement of the loss of DPPH deep violet colour at 517 nm after reaction with test sample. Inhibition of DPPH free radical (I) was calculated, in percentage, according to:

% I = (Ac – As/Ac) x 100

where Ac is the absorbance of the control reaction (containing ethanol instead of test solution), and As is the absorbance of the sample. BHT and BHA were used as reference compounds. IC_{50} value, which denotes the concentration of sample that is required to scavenge 50% of DPPH free radicals was calculated. Concentrations are expressed in $\mu g\ ml^{-1}$. All reagents and solvents used in this investigation were of analytical grade. Spectrophotometric measurements were performed using the apparatus Specol 11 UV–Vis spectrophotometer (Carl Zeiss, Jena, Germany).

Statistical analysis

The results are presented as mean ± standard deviation.

Results and Discussion

Chemical composition of the ethanol extracts

The chemical composition of examined ethanol extracts of *A. arvensis*, *A. hungaricus*, A. *alpinus*, *A. suaveolens*, *A. majoranifolius* and *A. graveolens* is presented in Table 1. Overall, eighty-six constituents found in six extracts were identified: 22 in *A. majoranifolius* extract (97.64% of the total extract), 25 in *A. suaveolens* extract (97.64% of the total extract), 28 in *A. arvensis* extract (96.50% of the total extract), 29 in *A. alpinus* extract (99.99% of the total extract), 31 in *A. graveolens* extract (95.68% of the total extract) and 18 compounds in *A. hungaricus* extract (93.70% of the total extract).

GC and GC-MS analysis of *A. majoranifolius* ethanol extract showed that the main compounds of the ethanolic extract were pulegone (45.50%), isomenthone (24.29%) and piperitenone (7.80%). The most abundant group of compounds were monoterpenes (85.03%). The class of oxygenated monoterpenes had the highest representation in the ethanol extract (84.10%). The content of sesquiterpenes and diterpenes were 7.11% and 1.68%, respectively. The chemical composition of *A. majoranifolius* ethanol extract is similar to *A. majoranifolius* methanol extract in terms of their quantity and quality. Golubovic *et al.* (2014) discovered that the main group of compounds of *A. majoranifolius* methanolic extract was monoterpenes (81.15%). According to them, the most abundant compounds were pulegone (45.69%), isomenthone (20.90%) and piperitenone (5.13%).

Pulegone (22.49%) and isomenthone (11.51%) were dominant compounds in the *A. suaveolens* ethanol extract. The ratio of monoterpenes (57.39%) and sesquiterpenes (17.61%) was similar to the methanol extract (Golubovic *et al*., 2014). Monoterpene, as the dominant group of compounds, contain 56.02% of oxygen derivatives, while hydrocarbons comprise only 1.37%. The difference between the composition of the ethanol and methanol extracts is the fact that ethanol extract contains hexadecanoic acid (10.35%), caryophyllene oxide (9.11%) and myrthenol (6.49%). The main compounds of the *A. suaveolens* methanol extract were pulegone (40.29%), isomenthone (12.78%) and piperitenone (10.71%). The main group of compounds was monoterpenes (76.13%) and all of them belong to oxygenated fraction. Sesquiterpene hydrocarbons (10.31%) were the main group of sesquiterpenes (Golubovic *et al*., 2014).

The main compounds of the *A. alpinus* ethanol extract were thymol (11.01%), *n*-docosane (10.08%) and hexadecanoic acid (9.15%). The presence of germacrene D (8.66%), menthyl-acetate (7.73%) and pulegone (7.59%) was also significant. The ratio of monoterpenes and sesquiterpenes were 28.02% and 23.77%,

respectively. Both groups are dominated by oxygen derivatives. In the study of Golubovic *et al*. (2014), the main compounds of the *A. alpinus* methanol extract were thymol (31.50%), borneol (14.59%) and spathulenole (14.59%). Also, there was a significant content of germacrene D (9.61%), caryophyllene oxide (9.13%) and hexadecanoic acid (8.39%). The main group of compounds was monoterpenes (49.31%), among which the oxygenated monoterpenes were present in largest percentages (48.31%). Besides, the significant part of sesquiterpenes (37.41%) belongs to oxygenated fraction (25.76%) (Golubovic *et al*., 2014).

Table 1. Chemical composition (%) of ethanol extracts of *Acinos* species

			Percentage (%)					
Compound	AI	RI	*A. majoranifolius*	*A. suaveolens*	*A. alpinus*	*A. arvensis*	*A. graveolens*	*A. hungaricus*
α-Thujene	924	923.2	0.93	1.37	-	-	-	-
α-Pinene	932	931.4	tr.	tr.	tr.	tr.	tr.	tr.
Sabinene	969	969.6	tr.	tr.	-	-	-	tr.
β-Pinene	974	972.2	tr.	tr.	tr.	1.08	tr.	-
Myrcene	988	988.2	-	tr.	-	-	-	-
δ-3-Carene	1008	1006.4	-	-	-	-	1.98	-
α-Terpinene	1014	1013.2			1.30			-
Limonene	1024	1022.4	tr.	-	-	-	-	-
β -Phellandrene	1025	1026.1	-	-	tr.	1.02	-	-
1,8-Cineole	1026	1031.2	-	-	5.52	4.25	3.95	5.56
Linalool	1095	1095.0	-	-	tr.	-	-	-
Menthone	1148	1146.8	0.81	tr.	-	-	-	-
Isomenthone	1158	1156.6	24.29	11.51	tr.	-	-	-
Borneol	1165	1164.8	-	-	1.20	tr.	-	-
Menthol	1167	1167.0	1.07	-	-	-	-	-
Terpinen-4-ol	1174	1173.8	2.81		-			-
α-Terpineol	1186	1185.8	-	2.55	-	-	-	-
Myrtenol	1194	1192.4	-	6.49	-	-	-	-
Pulegone	1233	1231.2	45.50	22.49	7.59	tr.	1.97	8.01
Piperitone	1249	1249.2	0.90	1.48	-	-	-	-
Thymol	1289	1290.0	-	-	11.01	1.00	1.20	-
1-Tridecene	1290	1292.2	-	-	-	-	-	-
Menthyl acetate	1294	1294.1	-	-	7.73	-	-	-
Carvacrol	1298	1296.9	-	-	1.40	-	-	-
δ-Elemene	1335	1334.2	-	-	-	tr.	1.01	-
Piperitenone	1340	1340.4	7.80	10.08	-	-	-	-
α-Cubebene	1345	1344.8	-	-	-	-	-	-
α-Terpinyl acetate	1346	1346.2	-	-	tr.	-	-	-
Thymol acetate	1349	1348.3	-	-	-	-	-	-
Piperitenone oxide	1366	1368.0	0.92	1.42	-	-	-	-
β -Bourbonene	1387	1386.4	-	-	tr.	-	tr.	3.50
β -Elemene	1398	1397.1	-	-	-	tr.	1.10	-
α-Gurjunene	1409	1402.1	-	-	-	1.01	1.01	-
α-*cis*-Bergamotene	1411	1410.1	-	-	-	-	1.00	-
β -Cariophyllene	1417	1417.0	4.00	3.60	-	-	tr.	-
β -Ylangene	1419	1418.2	-	-	-	-	-	-
β-Copaene	1430	1428.4	-	-	-	-	-	-
Mint furanon 1	-	1435.0	tr.	tr.	-	-	-	-
Mint furanon 2	-	1440.0	1.74	2.73	-	-	-	-
cis -β-Farnesene	1440	1441.0	-	-	-	.	tr.	-
α-Humulene	1452	1452.4	1.34	2.40	tr.	-	-	-
(E)- β- Farnesene	1454	1455.0	-	-	-	-	-	-
Alloaromadendrene	1458	1458.6	-	-	1.10	-	-	-
γ-Murolene	1478	1477.8	-	-	-	-	-	-
Germacrene D	1484	1486.3	-	1.49	8.66	3.30	5.00	-
(E) -β-Ionon	1487	1486.8.4	-			2.95		-
Valencene	1496	1495.2	-			2.04		-
Bicyclogermacrene	1500	1500.4	-	-	-	3.35	4.98	-
β-Bisabolene	1505	1505.2	-			3.28		-
Germacrene A	1509	1508.8	-			4.30		-
γ-Cadinene	1513	1511.1	-			2.10		-
Spathulenol	1577	1576.3	-	-	tr.	-	-	3.53
Caryophyllene oxide	1582	1576.9	1.77	9.11	6.41	-	4.91	4.38
1-Hexadecene	1588	1588.6	-	-	-	-	-	-
Globulol	1590	1590.2	-	-	4.40	-	-	4.48

Salvial-4(14)-en-1-one	1594	1594.0	-	-	3.20	-	-	-
Humulene epoxide II	1608	1606.4	-	1.01	tr.	-	2.50	-
1,10-di-epi- Cubenol	1620	1618.2	-	-	-	-	1.80	-
τ-Muurolol	1644	1642.1	-	-	-	-	1.03	-
δ-Cadinol	1652	1650.9	-	-	-	-	-	-
(E)-Bisabolol-11-ol	1667	1667.2	-	-	-	-	-	-
8-Cedren-13-ol	1688	1687.6	-	-	-	-	-	-
(2Z.6E)-Farnezol	1722	1724.0	-	-	-	-	-	-
n-Hexadecanol	1874	1874.2	-	-	-	-	-	-
Phytol	1942	1940.2	0.23	0.51	-	7.65	7.93	7.69
Isophytol	1946	1946.6	-	-	-	-	-	4.28
Hexadecanoic acid	1959	1960.0	0.94	10.35	9.15	7.40	8.23	6.22
(Z)-9,17-Octadecadienal	-	1988.0	-	-	-	-	-	7.60
1-Eikosene	1987	1988.2	-	-	5.50	2.39	-	4.27
Ethyl hexadecanoate	1992	1994.2	-	-	-	10.75	8.40	3.20
(Z.E)-Geranyl linalool	1998	1998.2	-	-	6.50	5.28	-	-
n-Eicosane	2000	2001.1	-	-	1.62	5.30	4.58	-
(E.E)- Geranyl linalool	2026	2025.5	-	-	-	5.10	-	-
n-Octadecanol	2077	2075.0	-	-	-	-	-	-
Methyl linoleate	2095	2093.2	-	-	-	5.20	4.30	-
n-Heneicosane	2100	2101.2	-	-	1.64	-	8.70	11.03
Linoleic acid	2132	2131.0	-	-	6.08	5.62	8.82	-
Oleic acid	2141	2140.4	1.14	1.45	-	-	1.00	-
1-Docosene	2189	2188.2	-	-	-	-	1.03	-
n-Docosane	2200	2200.3		-	10.08	5.63	1.50	5.11
Sclareol	2222	2220.0	1.45	-	-	-	-	-
n-Tricosane	2300	2300.6	-	-	-	-	5.20	5.74
n-Pentacosane	2500	2501.0	-	-	-	-	2.55	9.10
Octacosane	2800	2800.0				6.50		-
Triacontane	3300	3301.2		4.01				-
Tetratriacontane	3400	3400.6		3.59				-
Terpenoids			92.37	75.51	58.29	47.71	41.37	41.43
Monoterpenes			85.03	57.39	28.02	10.30	9.10	13.57
Sesquiterpenes			7.11	17.61	23.77	19.38	24.34	15.89
Monoterpene hydrocarbons			0.93	1.37	1.03	2.10	1.98	-
Oxygenated monoterpenes			84.10	56.02	26.72	8.20	7.12	13.57
Sesquiterpene hydrocarbons			5.34	7.49	9.76	19.38	14.10	3.50
Oxygenated sesquiterpenes			1.77	10.12	14.01	-	10.24	12.39
Diterpenes			1.68	0.51	6.50	18.03	7.93	11.97
Others			3.82	22.13	41.70	48.79	54.31	52.27
Total			97.64	97.64	99.99	96.50	95.68	93.70

Abbreviations: "tr." - traces (< 0.1%); "-" - not detected; "AI" - Retention index (according to Adams 2007); "RI" - Retention index (experimentally determined by calibrated AMDIS)

Ethyl-hexadecanoate (10.75%), phytol (7.65%) and isophytol (7.40%) are the main constituents of the *A. arvensis* ethanol extract. The characteristic of the ethanol extract was high percentage of fatty acids and esters (28.97%). In addition, the presence of alkanes was significant (17.43%). The content of sesquiterpenes, diterpenes and monoterpenes were 19.38%, 18.03% and 10.30%, respectively. The main compounds of the *A. arvensis* methanol extract were germacrene D (8.97%), hexadecanoic acid (8.73%), β-bourbonene (6.14%) and *(E,E)*-geranyl-linalool (5.89%). The main group of compounds was sesquiterpenes (36.81%), whereas the sesquiterpene hydrocarbons (28.45%) were prevailing. On the contrary, the monoterpenes were present in traces. However, the amount of diterpenes (14.41%) was significant (Golubovic *et al*., 2014).

The main compounds of the *A. hungaricus* ethanol extract were alkanes, *n*-heneicosane (11.03%) and *n*-pentacosane (9.10%). The main group of compounds was alkanes (30.98%). The content of terpenes was as follows: sesquiterpenes (15.89%), monoterpenes (13.57%) and diterpenes (11.97%). An earlier study reported *n*-docosane (11.05%) as the main compound of investigated methanol extract (Golubovic *et al*., 2014). Globulol (8.29%), phytol (7.83%), caryophyllene oxide (7.51%), isophytol (6.53%) and pulegone (5.93%) were also present in high percent. The main group of compounds were sesquiterpenes (40.22%), particularly

oxygenated fraction (33.70%). The ratio of diterpenes and monoterpenes was 14.36% and 16.32%, respectively. Furthermore, the number of alkanes (17.14%) was significant (Golubovic *et al*., 2014).

One of the main features of the *A. graveolens* ethanol extract was high percentage of esters and fatty acids (30.75%). The main compounds were linoleic acid (8.82%), hexadecanoic acid (8.23%), ethyl hexadecanoate (8.40%), as well as methyl linoleate (4.30%). The related literature provided the descriptions of the *A. graveolens* methanol extract chemical composition. Moreover, the composition and abudances of major components in present study is correlated with the previous study (Golubovic *et al*., 2014). Namely, the main constituent of *A. graveolens* methanol extract was hexadecanoic acid (14.94%). The class of compounds with highest abundance was sesquiterpenes (38.96%), whereas monoterpenes were present in low content (13.24%). Moreover, the presence of alkanes (11.84%) should be emphasized (Golubovic *et al*., 2014).

Phenolic content of ethanol extracts

The quantity of phenolic compounds in ethanol extracts of investigated *Acinos* species is presented in Table 2.

Table 2. Total content of polyphenols, tannins, flavonoids and *in vitro* assays of antioxidant capacity of *Acinos* species ethanol extracts

Ethanol extract	Total flavonoids (%)	Total polyphenols (mg CE/g)	Total tannins (mg CE/g)	FRAP value (mmol Fe^{2+}/g)	IC_{50} (μg ml^{-1})
A. alpinus	0.80 ± 0.01	140.12 ± 6.17	105.89 ± 4.32	7.40 ± 0.21	37.51 ± 0.30
A. arvensis	0.60 ± 0.01	62.74 ± 1.71	37.50 ± 0.37	5.70 ± 0.14	85.84 ± 1.32
A. hungaricus	0.80 ± 0.01	131.76 ± 9.15	91.43 ± 7.60	6.97 ± 0.32	49.48 ± 0.53
A. graveolens	0.70 ± 0.03	82.72 ± 2.67	54.73 ± 1.2	6.22 ± 0.14	64.45 ± 0.39
A. suaveolens	0.50 ± 0.01	32.75 ± 2.10	20.77 ± 1.14	3.68 ± 0.03	49.39 ± 0.58
A. majoranifolius	0.60 ± 0.01	60.98 ± 2.18	37.42 ± 0.32	3.55 ± 0.01	78.46 ± 0.47

As seen in Table 2, the highest content of total polyphenolic constituents (140.12 ± 6.17 and 131.76 ± 9.15 mg CE/g of extract) was determined in *A. alpinus* and *A. hungaricus* extracts, respectively. The lowest amount (32.75 ± 2.10 mg CE/g of extract) was detected in *A. suaveolens* extract. The ethanol extracts of all examined species had almost identical flavonoids content. Flavonoids are represented as percentage range from 0.50 ± 0.01% (*A. suaveolens*) to 0.80 ± 0.01% (*A. alpinus* and *A. hungaricus*). The tannin fraction represents more than a half of the total polyphenolic compounds (from 20.77 ± 1.14 in *A. suaveolens* to 105.89 ± 4.32 mg CE/g of extract in *A. alpinus* extract). These results coincide with the previous studies on the content of phenolic compounds in methanol extracts of *Acinos* species (Golubovic *et al*., 2014). Namely, the highest level of total polyphenolic constituents was found to be 121.96 ± 3.03 mg CE/g (*A. alpinus*) and 121.29 ± 2.46 mg CE/g (*A. hungaricus*) compared to the lowest content 73.64 ± 3.80 mg CE/g (*A. majoranifolius*) and 76.62 ± 1.02 mg CE/g (*A. suaveolens*). Also, tannins were dominant polyphenolic fraction in methanol extracts. Due to the variety of bioactive compounds in plant species and their differing solubility, the optimal solvent for extraction mostly depends on the specific plant materials and the compounds which should be isolated. For this reason, it is rather challenging to recommend a proper solvent for individual plant materials. Methanol, ethanol, as well as their mixtures with water are reported for the ability to extract bioactive compounds, especially the phenolic ones (Sultana *et al*., 2009; Do *et al*., 2014). The amounts of total phenolic compounds were higher in *A. alpinus* (140.12 ± 6.17 mg CE/g) and *A. hungaricus* (131.76 ± 9.15 mg CE/g) ethanol extracts in comparison with methanol extracts of this species (121.96 ± 3.03 mg CE/g and 121.29 ± 2.46 mg CE/g, respectively) (Golubovic *et al*., 2014). This is in accordance with previous research on total phenolic content

in the *P. rhoeas* L. extracts, where Kostic *et al*. (2010) concluded that *P. rhoeas* ethanol extract had higher phenolic content than methanol extracts. Do *et al.* (2014) stated that higher total phenolic content was obtained in the ethanol, rather than in the methanol extract of *Limnophila aromatica.* On the other hand, *A. graveolens, A. arvensis, A. majoranifolius* and *A. suaveolens* ethanol extracts had lower total phenolic content (82.72 ± 2.57, 62.74 ± 1.71, 60.98 ± 2.18, 32.75 ± 2.10 mg CE/g, respectively) compared to their methanol counterparts (Golubovic *et al*., 2014). When it comes to extraction, methanol was identified as more effective solvent than ethanol, resulting in the higher extraction yield as well as the higher content of phenolic and flavonoid compounds (Amensour *et al*., 2009; Sultana *et al*., 2009; Nsor-Atindana *et al*., 2012; Stojković *et al*., 2014; Kamkar *et al*., 2014; Aksay, 2016; Truong *et al*., 2019).

The significant differences concerning total phenolic content may be related to different polarity of the used solvents which have extracted entirely selective compounds.

Antioxidant capacity of ethanol extracts

The results of antioxidant activities of analysed extracts are presented in Table 2. All extracts demonstrated a considerable radical scavenging activity and ferric ion reduction ability (in DPPH and FRAP assay, respectively). The IC_{50} value is one of the important indicators in determination of antioxidant capacity. The lowest IC_{50} pointed the strongest capacity of the extracts to act as DPPH radicals scavengers. Radical scavenging activity of tested extracts is lower but comparable with IC_{50} value of BHT (14.00 ± 0.51). The results obtained in this study show that DPPH scavenging activity decrease as follow: *A. alpinus* > *A. suaveolens* > *A. hungaricus* > *A. graveolens* > *A. majoranifolius* > *A. arvensis.* The strongest antioxidant activity has been recorded by *A. alpinus* extract (7.40 mmol Fe^{2+}/g in FRAP test and IC_{50} value of 37.51 μg ml^{-1} in DPPH test), which contained the highest amount of total polyphenons and tannins (Table 2). Beside the *A. hungaricus* extract (0.80%), *A. alpinus* extract has the highest content of flavonoids (0.80%). For this reason, the combined activities of various compounds may have influence on the antioxidant properties of this extract. Since thymol is recognized for its antioxidant activity, it could contribute to the noticeable antioxidant activity of *A. alpinus* extract (Kulisic *et al*., 2004; Shan *et al*., 2005; Wojdylo, 2007; Alia *et al*., 2012; Alizadeh, 2013; Agili; 2014). These results coincide with the previous studies on the antioxidant activities of methanol extracts of *Acinos* species (Golubovic *et al*., 2014). When it comes to methanol extracts, the strongest antioxidant activity has been recorded by *A. alpinus* extract (6.98 mmol Fe^{2+}/g in FRAP test and IC_{50} value of 24.10 μg ml^{-1} in DPPH test). Radical scavenging activity of *A. alpinus* ethanol extract is slightly lower than the methanol one (IC_{50} values are 37.51 μg ml^{-1} and 24.10 μg ml^{-1}, respectively). The same ratio, according IC_{50} values, was detected in the following extracts: *A. suaveolens, A. hungaricus, A. graveolens* and *A. arvensis.* It was observed that only in *A. majoranifolius,* IC_{50} value of ethanolic extract was lower than in methanol extract.

Table 3. The antimicrobial activity[a] of ethanol extracts of *Acinos* species

Microorganism	*E. coli*		*S. enteritidis*		*P. aeruginosa*		*K. pneumoniae*		*S. aureus*		*S. lutea*		*B. subtilis*		*A. niger*		*S. cerevisiae*		*C. albicans*		*C. pyogeny*		*Entero-coccus sp.*		*M. flavus*		*P. vulgaris*	
	C[b]	S[c]	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S
A. majoranifolius	19	22	18	22	18	21	-	20	21	24	17	22	21	24	-	20	26	-	-	19	19	21	18	22	19	25	18	23
A. suaveolens	19	22	19	22	17	21	-	20	19	22	21	23	20	23	19	22	31	-	-	20	18	22	18	22	18	23	20	24
A. graveolens	19	21	19	23	17	21	20	22	21	24	18	21	18	21	-	20	30	-	19	22	21	24	20	23	19	24	19	23
A. alpinus	20	23	14	17	20	23	21	23	22	26	19	23	21	24	19	22	19	-	16	19	21	25	20	24	21	26	21	24
A. arvensis	-	17	21	24	-	17	-	19	17	21	17	20	19	22	-	19	-	17	-	17	19	24	18	21	-	22	17	22
A. hungaricus	19	22	19	22	17	20	19	21	19	22	18	22	20	23	-	19	34	-	-	20	20	24	19	23	19	21	20	23
Ampicillin	-	19	-	18	-	17	-	-	-	19	-	20	-	20	-	-	-	-	-	-	-	17	-	18	-	18	-	19
Tetracycline	30	-	28	-	27	-	27	-	29	-	31	-	30	-	23	-	-	-	24	-	27	-	28	-	31	-	26	-
Streptomycin+ Penicillin	23	-	19	-	21	-	20	-	20	-	22	-	23	-	20	32	-	-	18	32	18	-	20	-	19	-	18	
Nystatin	nt		nt		nt		nt		nt		nt		nt		17	-	17	-	18	-	nt		nt		nt		nt	
DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] The antimicrobial activity (diameters of growth inhibition zone measured in mm), including disc diameter, 12.7 mm; [b] bacteri-and fungicidal zones; [c] bacteri- and fungistatic zones. Values for static zones represent the extra millimeters around the cidal zone (or the sole disk if no cidal activity) in which the growth of microorganisms was inhibited but in which the microorganisms were not killed; nt-not tested; - - the zone of inhibition was not observed

Antimicrobial activity of the ethanol extracts

Moderate antimicrobial activity is exhibited by almost all of the ethanol extracts (Table 3). In comparison to others, *A. alpinus* extract showed stronger antimicrobial activity which can be correlated to its main component - thymol (Kordali *et al.*, 2008; Mathela *et al.*, 2010; Mihajlov-Krstev *et al*., 2011; Alia *et al*., 2012; Ho *et al*., 2012; Alizadeh, 2013). The study by Thompson *et al*. (2003) proved the considerable antimicrobial activity of phenolic derivatives (such as thymol and carvacrol), as well as their biosynthetic precursors (γ-terpinene and *p*-cymene). In all examined plant species, ethanol extracts are considered unselective (approximately equally active against Gram-negative and Gram-positive bacteria). Ethanol extracts of *A. majoranifolius*, *A. suaveolens, A. hungaricus* and *A. graveolens* species exert fungicidal activity against *S. cerevisiae*. The fungi *A. niger* and *C. albicans* are resistant towards ethanol extracts of the majority of analyzed plant species, whereas *K. pneumoniae* is resistant to three analyzed ethanol extracts. Also, weaker antimicrobial activity is recognized in ethanol extracts rather than in essential oils (dilution 1:10); however, ethanol extracts seem to be more active than the essential oils (dilutions 1:20 and 1:30) (Jovanovic, 2002; Jovanovic *et al*., 2005; Stojanovic *et al*., 2009; Golubovic, 2010).

The research results referring to ethanol extracts mainly coincide with the methanol ones for the selected plant species (Golubovic *et al*., 2014).

Conclusions

The highest phenolic and flavonoid content, as well as antioxidative activity, according to all assays used, was found in *A. alpinus* ethanol extract. The combined activities of various compounds may have influence on the antioxidant properties of this extract. Moderate antimicrobial activity is exhibited by almost all of the ethanol extracts of *Acinos* species. In comparison to others, *A. alpinus* extract showed stronger antimicrobial activity which can be correlated to its main component-thymol. The preliminary bioassay results indicated that

the ethanol extract of *A. alpinus* could be a possible source of antioxidant and antimicrobial compounds. The research results referring to ethanol extracts mainly coincide with the previously studied methanol ones for the examinated plant species.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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