




Article

LC-ESI-MS/MS-MRM Profiling of Polyphenols and Antioxidant Activity Evaluation of Junipers of Different Origin

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Abstract: This study was aimed at identifying new efficient antioxidant juniper species and their metabolites, which are responsible for this activity. About 30 juniper representatives were assayed for antioxidant activity (DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical scavenging) and total polyphenol content (TPC). The most active species were identified, and their most abundant polyphenols were quantified by the LC-electrospray ionization (ESI)-MS/MS-multiple reaction monitoring (MRM) method. In the group of studied species, *J. ashei* (mountain cedar) leaf extract was outlined as the best antioxidant with the highest TPC. Catechin was revealed as the most abundant polyphenol in the *J. ashei* extract, contributing to its superior antioxidant properties. An in-depth analysis of antioxidant capacity was also performed. The higher metal-chelating activity was observed in the case of *J. sibirica* (0.83 mg DE/mL), whereas the lowest was observed for *J. communis* (3.2 mg dry extract (DE)/mL) extracts. All efficient antioxidant extracts were also able to inhibit lipoxygenase. EC₅₀ values ranged from 1.77 to 2.44 mg DE/mL. The most effective inhibitors were *J. ashei* and *J. formozana* extracts, which acted as uncompetitive lipoxygenase (LOX) inhibitors. The presented results have potential application in the pharmacy and cosmetics for the generation of antioxidant compositions based on naturally derived lead compounds for the prevention of oxidative-stress associated organ-degenerative diseases, cancer, or other free radical-induced disorders.

Keywords: antioxidant activity; flavonoids; *Juniperus* L.; *Juniperus ashei* J. Buchholz; LC-MS; polyphenolic acids

1. Introduction

The genus *Juniperus* L. (Cupressaceae) includes a great diversity of evergreen magnificent trees or small shrubs belonging to the Pinophyta division (Coniferophyta, Coniferae) of gymnosperm cone-bearing seed plants. This genus consists of about 50–67 species, depending on the taxonomic classification, and it includes over 220 cultivars. Junipers produce nearly 580 secondary metabolites, including anticancer lignans (podophyllotoxin and other derivatives), sesquiterpenes, diterpenes

(more than 220 structures), flavonoids, etc. Polyphenols are a large group of compounds, comprising flavonoids, stilbenes, polyphenolic acids, lignans, etc. These substances exhibit various biological activities, some of them with poorly understood mechanisms. The total polyphenol content of plant extracts correlates with their antioxidant activity. Antioxidants are important mediators of the cellular redox processes, whose balance is necessary for maintenance of the cellular homeostasis. Free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), appear in normal physiological processes or diseases, thus having a dual role in the cellular life and senescence. The beneficial effects of ROS/RNS are involved in the immune cell's defense response against infectious agents. In addition, mitochondrial electron transport chains lead to the production of adenosine triphosphate (ATP), which is the main source of energy for processes of the living cells, thus being essential for their life. On the other side, free radicals have oncogenic properties; however, they can act also as anticancer agents, inducing apoptosis of the cancer cells. Therefore, the existence of free radicals in living beings is tightly controlled by enzymes in a number of cellular signaling pathways in order to prevent cellular damage. Normally, there exists a balance between enzymatic and non-enzymatic antioxidants that maintains the cellular redox homeostasis. Flavonoids, carotenoids, vitamins (A, C, E), uric acid, glutathione, etc. are non-enzymatic antioxidants that prevent the cells from an excessive accumulation of deleterious free radicals in the living beings.

The literature survey revealed that *Juniperus* L. is an important medicinal plant due to the presence of many bioactive chemical constituents conditioning various medicinal and pharmacological properties, including an anti-inflammatory effect and its traditional uses to treat diseases [1,2]. The anti-inflammatory potential of aqueous, alcoholic, or hydro-alcoholic juniper extracts is usually documented in large survey studies, evaluating medicinal plants specific to each author's native region [3,4]. The evaluation of five Turkish *Juniperus taxa* aqueous and methanolic extracts for anti-inflammatory activity in hind paw oedema (carrageenin and prostaglandin-induced) showed a good anti-inflammatory activity [2], whereas the average anti-inflammatory potential of ethanolic extract of *J. communis* native to Russia was found [4]. The mechanism(s) for anti-inflammatory activity has not been fully explained, but it appears that nuclear factor- κ B (NF- κ B) inhibitory activity may determine such activities [5]. The results obtained by Fierascu et al. [6] indicates juniper microemulsion activity against pro-inflammatory cytokines (in the kaolin experimental model).

Some junipers representatives have been studied previously for their antioxidant activity and numerous secondary metabolites, contributing to this activity, have been identified [7–10]. In this regard, flavonoids apigenin, rutin, and luteolin were identified by LC-MS/MS in methanol extracts of cones and needles of *J. sibirica* Burgsdorf. from Serbia. The essential oil of Serbian *J. communis* L. var. *saxatilis* contained mainly the bicyclic monoterpene α -pinene, and the post-distillation waste contained rutin, contributing to its strong antioxidant activity. High content of the monoterpenes α -pinene and β -myrcene but less amounts of limonene and the bicyclic sesquiterpene δ -cadinene were found in the berry essential oil of *J. oxycedrus* ssp. *oxycedrus* L. from Lebanon. The berry essential oil of *J. communis* L. from Bulgaria was found to contain mainly monoterpenes α -pinene, β -pinene, myrcene, sabinene, and limonene. A study of the antioxidant activity of the galbuli essential oils of six Bulgarian juniper species determined the following order of their activity: *J. sibirica* > *J. excelsa* = *J. communis*. Our previous study of the antioxidant activity [7] of extracts (leaves and gabuli) of juniper species from Bulgaria (*J. communis* L., *J. deltoides* R. P. Adams, *J. excelsa* M. Bieb., *J. sabina* L., *J. pigmaea* K. Koch, *J. sibirica* Burgsd. and *J. virginiana* L.) revealed the best total polyphenol content and antioxidant activity for the leaf extracts of *J. sibirica* and *J. excelsa*. A great diversity of other junipers of various origin were also studied for their antioxidant activity and composition [11–17].

In accordance with the increasing interest in the identification of new powerful antioxidants for the pharmacy and cosmetics, the present research was aimed at the identification of plants with superior antioxidant properties and polyphenol content after screening about 30 *Juniperus* species of various origin around the world. The content of the antioxidant metabolites was determined by LC-ESI-MS/MS analysis of the studied extracts. The triple quadrupole MS detector was working in

the multiple reaction monitoring (MRM) scan mode, which ensures high sensitivity and selectivity of the analysis. Therefore, this method was employed as a very reliable and suitable tool for analyses of complex mixtures, e.g., plant extracts. Moreover, this technique enabled a fast distinguishing of compounds having the same parent ions but giving different fragment ions [18,19]. The present detailed study of the antioxidant properties of plenty of junipers around the world revealed the genus *Juniperus* as an invaluable natural source of efficient antioxidant agents. As a result, after the screening of a diversity of *Juniperus* species of various origin, extracts with excellent antioxidant activity and polyphenol content were distinguished and the metabolites responsible for these properties were identified. To our knowledge, *Juniperus ashei* J. Buchholz (mountain cedar) was determined for the first time as the representative with superior antioxidant activity and highest total polyphenol content in the group of assayed representatives of the genus *Juniperus*. The results of the present study have potential application in the generation of natural antioxidant compositions for the prevention of oxidative-stress associated organ-degenerative or other radical-induced diseases.

2. Materials and Methods

2.1. Chemicals and Reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl), ferrozine (3-(2-pyridyl)-5,6-bis-(4-phenyl-sulfonic acid)-1,2,4-triazine), lipoxygenase (LOX; from *Glycine max*, type 1B), ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), Folin–Ciocalteu's reagent (FC-reagent, 2N), gallic, caffeic, *o*-coumaric, *m*-coumaric, *p*-coumaric, ferulic, vanillic, protocatechuic, 3-hydroxybenzoic, salicylic, 4-hydroxybenzoic, syringic and formic acids, rutin, hyperoside, isorhamnetin-3-*O*-rutinoside, quercitrin, isoquercetin, kaempferol, luteolin, and taxifolin were purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA). Gentisic acid, myricetin, catechin, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, isorhamnetin-3-*O*-glucoside, naringenin-7-*O*-glucoside and eriodictyol were obtained from ChromaDex Co. (Irvine, CA, USA). Quercetin was provided by Fluka AG (Buchs, Switzerland). Astragalin, tiliroside, naringenin, apigenin, and kaempferol-3-rutinoside were from Roth AG (Karlsruhe, Germany). LC grade solvents were purchased from Fisher Scientific Inc. (USA) and Sigma-Aldrich Co. (Saint Louis, MO, USA). LC grade ultra-pure water was prepared by a Millipore Direct-Q3 water purification system (Bedford, MA, USA).

2.2. Plant Material

Evergreen junipers from the Arnold Arboretum, Harvard University, Boston, MA, USA, were collected in June 2017 (species No. 1–4, 7–10, 21–24, 26) or October 2018 (species No. 5, 6, 11–20) and their original sources accession summaries were reported (Table 1). *Juniperus sabina* var. *balkanensis* R. P. Adams and A. N. Tashev was collected in November 2017 from the eastern Rhodopes (peak Veikata), Bulgaria. The other junipers from the Balkan Peninsula region were collected as follows: *J. communis* L. was from the village Ognyanovo, Blagoevgrad Province, Rhodope Mountains (41°37'47.3" N; 23°47'14.5" E, 700 m a.s.l., April 2017); *J. excelsa* M. Bieb. was from the reserve Tisata, on the riverside of Struma (41°44'01.6" N; 23°09'22.5" E, 199 m a.s.l., April 2017). *J. sibirica* Burgsd. was from the Vitosha mountain on the outskirts of Sofia (42°34'59.6" N; 23°17'28.6" E, 1803 m a.s.l., April 2017); *J. pigmaea* C. Koch was from the Smolyan Province, Mursalitsa region of the Rhodope Mountains (41°38'40.8" N; 24°29'58.5" E, 1898 m a.s.l., May 2017); *J. deltoides* R. P. Adams was from the village Ognyanovo, Blagoevgrad Province, Rhodope Mountains (41°37'46.6" N; 23°47'15.4" E, 695 m a.s.l., April 2017); The voucher specimen from the Balkan Peninsula region were authenticated by A. N. Tashev (University of Forestry, Sofia, Bulgaria), according to R. P. Adams [20] and were deposited in the Herbarium (SOM) of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences.

Table 1. Antioxidant activity, evaluated as half-maximum 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging concentrations (DPPH-EC₅₀) [μ g dry extract (DE)/mL], and total polyphenols content (TPC) of the extracts [mg GAE/g DE].

No.	Specime No.	<i>Juniperus</i> L. Representatives	F/M	Organ Used	Original Sources	DPPH-EC ₅₀	TPC
1	AA 672-38*D	<i>J. x media</i> 'Pfitzeriana Aurea'	M	leaves	D. Hill Nursery, Dundee, Illinois, USA	340 \pm 6 ^b	94 \pm 8 ^h
2	AA 14834*B	<i>J. x media</i> 'Pfitzeriana'	M	leaves	Royal Botanic Gardens, Kew, UK	215 \pm 4 ^g	94 \pm 4 ^h
3	AA1675-66*A	<i>J. x media</i> 'Mint Julep'	F	leaves	Glover Nursery, Utah, USA	198 \pm 4 ^h	104 \pm 4 ^g
4	AA 284-63*A	<i>J. x media</i> 'Ozark Compact'	M	leaves	Phytotektor Nurs., USA, Tennessee	191 \pm 2 ^h	114 \pm 7 ^g
5	AA 74-42*C	<i>J. x media</i> 'Pfitz. Argentea'	M	leaves	Morris Arb., Pennsylvania, USA	148 \pm 2 ^j	134 \pm 1 ^e
6	AA 183-62*A	<i>J. x media</i> 'Old Gold'	M	leaves	Grootendorst Nurs., Holland	138 \pm 2 ^k	143 \pm 3 ^e
7	AA 280-98*A	<i>J. formosana</i> Hayata	F	leaves	wild, from Taiwan	86 \pm 1 ^o	189 \pm 4 ^c
8	AA 642-88*B	<i>J. pinchotii</i> Sudw.	M	leaves	wild, Kiowa, Oklahoma, USA	70 \pm 1 ^p	213 \pm 23 ^b
9	AA 276-86*A	<i>J. ashei</i> J. Buchholz	M	leaves	wild, Oklahoma, Murray, USA	49 \pm 1 ^r	263 \pm 25 ^a
10	AA 211-57*A	<i>J. scopulorum</i> 'Moon light'	M	leaves	D. Hill Nursery, Dundee, Illinois, USA	236 \pm 7 ^f	120 \pm 14 ^f
11	AA 981-87*A	<i>J. rigida</i> Siebold & Zucc.	M	leaves	wild, Japan	148 \pm 5 ^j	140 \pm 0.3 ^e
12	AA 20-89*A	<i>J. squamata</i> 'Meyeri'	F	leaves	Hicks Nurs., NY, Westbury, USA	150 \pm 5 ^j	136 \pm 5 ^e
13	AA 265-33*A	<i>J. chinensis</i> L.	M	leaves	California, USA	140 \pm 4 ^k	146 \pm 1 ^e
14	AA 14809*A	<i>J. chinensis</i> L.	F	leaves	Royal Botanic Gardens, Kew, UK	110 \pm 4 ^m	157 \pm 10 ^d
15	AA 14809*A	<i>J. chinensis</i> L.	F	galbuli	Royal Botanic Gardens, Kew, UK	186 \pm 5 ⁱ	94 \pm 0.3 ^h
16	AA 219-61*A	<i>J. chinensis</i> 'Plumosa Aurea'	M	leaves	Pennsylvania, USA	183 \pm 4 ⁱ	122 \pm 0.2 ^f
17	AA 1-51*A	<i>J. chinensis</i> 'Pfitz. Matthews Blue'	M	leaves	Interstate Nursery, Iowa, USA	182 \pm 3 ⁱ	113 \pm 6 ^g
18	AA 1746-81*A	<i>J. virginiana</i> L.	F	leaves	wild, Bald Head Cliff, Maine, USA, Ogunquit	135 \pm 2 ^l	133 \pm 5 ^e
19	AA 1746-81*A	<i>J. virginiana</i> L.	F	galbuli	wild, Bald Head Cliff, Maine, USA, Ogunquit	304 \pm 10 ^d	68 \pm 1 ^j
20	AA 14882*A	<i>J. virginiana</i> 'Glaucua'	F	galbuli	Rochester Park, New York, USA	325 \pm 11 ^c	70 \pm 1 ^j
21	AA 1136-61*A	<i>J. virginiana</i> 'Grey Owl'	F	leaves	Dominion Arb., Ottawa, Canada	251 \pm 2 ^e	97 \pm 0 ^h
22	AA 1136-61*A	<i>J. virginiana</i> 'Grey Owl'	F	galbuli	Dominion Arb., Ottawa, Canada	352 \pm 9 ^a	45 \pm 0.2 ^k
23	AA 4176-1*A	<i>J. communis</i> 'Oblonga Pendula'	M	leaves	Biltmore Estate, North Carolina, USA	258 \pm 7 ^e	91 \pm 3 ⁱ
24	AA 49-66*A	<i>J. communis</i> 'Laxa'	M	leaves	U. S. Natl. Arb., Washington	105 \pm 5 ⁿ	199 \pm 6 ^b
25	SOM 174400	<i>J. communis</i> L.	F	leaves	wild from Rhodopes, BG	154 \pm 4 ^j	132 \pm 4 ^e
26	AA 14868*E	<i>J. sabina</i> L.	F	leaves	wild, from Uzbekistan	217 \pm 4 ^g	101 \pm 2 ^g
27	SOM 177009	<i>J. sabina</i> var. <i>balkanensis</i>	F	leaves	wild, eastern Rhodopes, BG	130 \pm 2 ⁱ	161 \pm 3 ^d
28	SOM 174404	<i>J. excelsa</i> M. Bieb.	F	leaves	wild, Struma riverside, BG	103 \pm 2 ^m	169 \pm 7 ^d
29	SOM 174401	<i>J. sibirica</i> Burgsd.	F	leaves	wild from Vitoshka, BG	104 \pm 2 ^m	182 \pm 18 ^c
30	SOM 174402	<i>J. pigmaea</i> K. Koch	F	leaves	wild from Rhodopes, BG	140 \pm 3 ^k	138 \pm 4 ^e
31	SOM 174403	<i>J. deltoides</i> R. P. Adams	F	leaves	wild from Rhodopes, BG	154 \pm 5 ^j	135 \pm 7 ^e

Abbreviations: AA—specimen of the Arnold Arboretum; SOM—specimen of the IBER BAS Herbarium; F—female representative; M—male representative. Means ($n = 9$) followed by the different lowercase letters (a–r) in columns are significantly different at $p < 0.05$.

2.3. Extraction Procedure

The collected plant material was kept in vacuum bags in the freezer at $-20\text{ }^{\circ}\text{C}$ until extraction. Then, leaves or galbuli were separated, homogenized, weighed (5 g), and mixed with methanol (50 mL, 80% *v/v*) in an Erlenmeyer flask with a stopper. The suspension was stirred for 1.5 h in a shaker water bath at $20\text{ }^{\circ}\text{C}$. The mixture was filtered, and the extract was collected. The remaining solid material was subjected to a second extraction for 1.5 h with a new portion of 80% methanol (50 mL). After filtration, the solid mass was stirred again for 1.5 h in 80% methanol (25 mL). The extracts were combined and concentrated by a vacuum evaporator. The remaining residue was freeze-dried (24 h, at $-50\text{ }^{\circ}\text{C}$, 0.2 mbar) and kept in the freezer at $-20\text{ }^{\circ}\text{C}$ until analyses.

2.4. Total Polyphenol Content Determination

The total content of phenolic compounds (TPC) of the corresponding juniper extracts was determined according to the Folin–Ciocalteu method [21] with minor modifications. In brief, 20 μL of the extract [5 mg/mL in 80% (*v/v*) methanol] were mixed with distilled water (1.58 mL) and FC-reagent (100 μL) was added. The control sample contained the same reagents but without plant extract. After 3–5 min, 300 μL of sodium carbonate (20% *w/v*) were added, and the samples were kept at room temperature for 2 h. The absorbance at 765 nm was registered on a spectrophotometer.

The calibration curve was prepared using gallic acid standard. The TPC of the extracts was expressed in GAE (Gallic Acid Equivalents) according to the formula $C = c \cdot V/m$, where C is the concentration of phenolic compounds in mg GAE per gram dry extract (DE); c is the gallic acid concentration [mg/mL] from the calibration curve; m is the weight of plant extract [g]; V is the volume of plant extract [mL]. The TPC of each extract was determined by 2 independent analyses and was given as an average value $\pm\text{SD}$.

2.5. DPPH Radical Scavenging Method for Antioxidant Activity Determination

The antiradical activity was determined by the DPPH method [22]. One mL of the extract (at various concentrations) was mixed with 4 mL of DPPH solution (0.004% *w/v*) in a test tube. The control sample was prepared with the same reagents but without plant extract. The blank sample contained 80% (*v/v*) ethanol. The solutions were kept at room temperature for 1 h in the dark, and then, the absorption at 517 nm was measured. The percentage of the DPPH-scavenging was assessed according to the formula:

$$\% \text{ scavenging} = [(A_c - A_s) / A_c] \times 100 \quad (1)$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample. The half maximal inhibitory concentration of the extracts (DPPH- EC_{50}) was determined by interpolation of the dose–response curves. These EC_{50} values were calculated at fitted models as the concentration of the tested extract that gave 50% of the maximum inhibition of the initial DPPH concentration, based on a dose-dependent mode of action.

2.6. Ability to Quench ABTS Radicals

The ABTS free radical scavenging potential was determined according to Re et al. [23] with some modifications. The ability of samples to quench the ABTS free radical was assessed according to Formula (1).

The half maximal inhibitory concentration of the extracts (ABTS- EC_{50}) was determined by interpolation of the dose–response curves. These EC_{50} values were calculated at fitted models as the concentration of the tested extract that gave 50% of the maximum inhibition, based on a dose-dependent mode of action.

2.7. Metal Chelating Activity (CHP)

Metal chelating ability was determined according to Guo et al. [24]. The extract samples (100 µL) have been mixed with 20 µL of 2 mM FeCl₂ solution and 40 µL 5 mM ferrozine. The mixture was shaken vigorously and left standing at room temperature for 10 min. Then, absorbance of the solution was measured spectrophotometrically at 562 nm

The chelating power was assessed using the formula:

$$\% \text{ inhibition} = [1 - (A_s/A_c)] \times 100 \quad (2)$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample.

Antioxidant activity was determined as EC₅₀—the extract concentration providing 50% of activity was based on a dose-dependent mode of action.

2.8. Inhibition of Lipoyxygenase Activity (LOXI)

Lipoyxygenase activity was determined according to method described by Axelrod et al. [25] adapted for a microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek Instruments, Winooski, VA, USA) [26]. An increase in absorbance of 0.001 per minute at 234 nm was assumed as one unit of LOX activity. Antioxidant activity was expressed as EC₅₀—extract concentration provided 50% of activity was based on dose-dependent mode of action.

2.9. LC-ESI-MS/MS-MRM Analyses

The identification and quantification of phenolic acids, flavonoid aglycones, and flavonoid glycosides was carried out using liquid chromatography coupled to electrospray ionization triple quadrupole mass spectrometry. The method for simultaneous analysis of all mentioned polyphenol groups was partly founded on the basis on previous experiments [19,27]. The LC-MS system consisted of a 1200 Series LC system (Agilent Technologies, Santa Clara, CA, USA), which was coupled with a 3200 QTRAP mass spectrometer with Turbo V™ source and an electrospray ionization (ESI) probe (AB Sciex, Redwood City, CA, USA). The sample or standards' solutions at a volume of 3 µL were injected on an Eclipse XDB-C18 column (4.6 mm × 150 mm; 5 µm; Agilent Technologies, Santa Clara, CA, USA), whose temperature was maintained at 25 °C. The mobile phases were 0.1% HCOOH in milli-Q water (solution A) and 0.1% HCOOH in acetonitrile (solution B). The gradient elution (flow rate 400 µL min⁻¹) was used: 0–1.5 min 13% B; 1.5–2 min 13–20% B; 2–4.5 min 20% B; 4.5–5 min 20–25% B; 5–8 min 25% B; 8–9 min 25–33% B; 9–11 min 33% B; 11–13 min 33–60% B; 13–16 min 60% B; 16–18 min 60–80% B; 18–21 min 80% B; 23–28 min conditioning with 13% B. QTRAP worked in multiple reaction monitoring (MRM) mode. Optimal mass analyzer conditions and the selection of product ions were determined experimentally (results are given in Table S1). MS parameters were set as a capillary temperature of 500 °C, negative ionization mode source voltage −4500 V, curtain gas at 30 psi, nebulizer gas at 55 psi. MS data acquisition and processing was performed using Analyst 1.5 software (AB Sciex, Redwood City, CA, USA) analytes. The calibration curves were generated using peak areas of the most intense MRM transitions. The linearity ranges, LOD (limit of detection; at a signal-to-noise ratio of 5:1) and LOQ (limit of quantification; at a signal-to-noise ratio of 10:1) values for all analytes were established (Table S2). LC-MS assay was performed in triplicate for each standard solution and sample.

2.10. Statistical Analysis

Obtained data were presented as means ± standard deviations. Moreover, one-way ANOVA and Tukey's test were performed ($\alpha = 0.05$) for the data obtained from 3 independent extracts in 3 parallel experiments ($n = 3$). Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA) was used for the analysis.

3. Results

The present study was designed to analyze the total polyphenol content and antioxidant activity of juniper extracts in order to reveal species of different origin around the world, exhibiting the highest activity, and to identify their secondary metabolites responsible for these properties. The first step of the study aimed at identifying juniper extracts with the highest total polyphenol content, which was correlated with their half-maximum DPPH-radical scavenging concentrations (DPPH-EC₅₀). The comparison of these experimental variables revealed that the leaf extracts demonstrated higher antioxidant properties than the corresponding galbuli extracts, as it was shown by analysis of the leaf and galbuli extracts of *J. chinensis* L., *J. virginiana* L., as well as cultivars *J. virginiana* ‘Glauc’ and *J. virginiana* ‘Grey Owl’. Using the Folin–Ciocalteu method for quantitative polyphenol determination, several juniper leaf extracts were found to exhibit highest TPC values: *J. ashei* J. Buchholz > *J. pinchotii* Sudw. ≈ *J. communis* Laxa ≥ *J. formosana* Hayata. The original sources summaries of the 31 studied juniper samples are shown in Table 1.

The DPPH-EC₅₀ values of all studied extracts were correlated with their TPC values, deriving a polynomial function, where greater TPC and lower DPPH-EC₅₀ denote higher antioxidant activities (Figure 1).

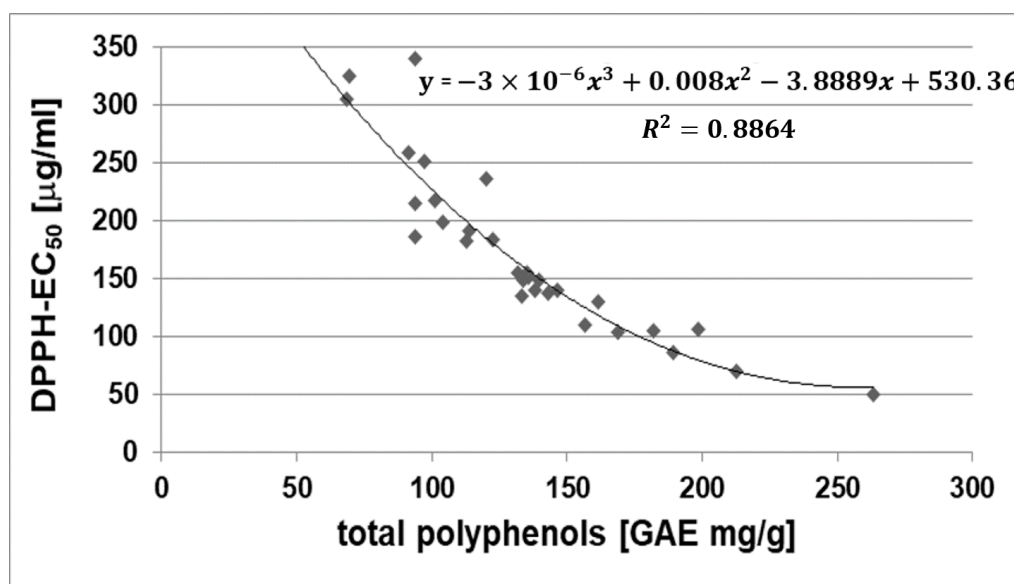


Figure 1. Correlation of the total polyphenol content with the DPPH-radical scavenging half-maximum concentrations (DPPH-EC₅₀) of the studied juniper extracts. Lower EC₅₀ values denote higher activity.

The juniper species from which the leaf extracts were characterized as the best sources of antioxidants and a rich polyphenol source (based on their TPC and DPPH-EC₅₀ values) were subjected to LC-ESI-MS/MS (liquid chromatography/electrospray ionization mass spectrometry) metabolite analysis. The method designed for the simultaneous analysis of flavonoid aglycones, flavonoid glycosides, and phenolic acids was partly founded on the basis of the previous experiments [10,18]. The LC-MS conditions are presented in Tables S1 and S2 (in the Supplementary Material). The optimization of the experimental method allowed the separation of 23 different compounds belonging to several polyphenol structural subclasses (hydroxybenzoic and hydroxycinnamic acids, dihydroflavonols, flavanones, flavan-3-ols, flavonols, flavones) (Table 2). Polyphenolic profiles of the studied species were qualitatively and quantitatively differentiated. It was observed that phenolic acids constitute a relatively small part of all polyphenols, with protocatechuic acid as a dominant representative (up to 43.3 ng/mg DE). It can be seen that the majority of them belong to hydroxybenzoic acid derivatives (salicylic, gallic, protocatechuic, 4-OH-benzoic). Only one hydroxycinnamic acid derivative was found, i.e., *p*-coumaric acid. The highest amount of phenolic acids was found in *J. sibirica* extract.

Table 2. Qualitative and quantitative LC-ESI-MS/MS analysis of polyphenols contained in extracts from leaves of selected *Juniperus* representatives. ESI: electrospray ionization.

Compound	<i>J. ashei</i>	<i>J. pinchotii</i>	<i>J. communis</i> 'Laxa'	<i>J. formozana</i>	<i>J. sibirica</i>	<i>J. excelsa</i>	<i>J. sabina</i> var. <i>balkanensis</i>
Phenolic acids [ng/mg DE]							
Gallic	BQL	BQL	BQL	BQL	BQL	BQL	BQL
Protocatechuic	26.78 ± 0.11 ^b	43.30 ± 0.21 ^a	42.65 ± 0.78 ^a	27.35 ± 0.28 ^b	44.03 ± 0.18 ^a	BQL	26.85 ± 0.00 ^b
4-Hydroxybenzoic	0	0	BQL	BQL	BQL	0	0
<i>p</i> -coumaric	BQL	BQL	5.95 ± 0.07 ^d	BQL	11.63 ± 0.04 ^c	0	BQL
Salicylic	0	BQL	BQL	0	BQL	BQL	0
Flavonoid aglycones [ng/mg DE]							
Catechin	2227.50 ± 3.54 ^a	1013.00 ± 2.83 ^f	1727.50 ± 3.54 ^b	1530 ± 7.07 ^d	1822.50 ± 10.61 ^b	1295.25 ± 6.72 ^e	1592.00 ± 11.31 ^c
Taxifolin	BQL	5.63 ± 0.18 ⁿ	6.25 ± 0.21 ⁿ	0	11.03 ± 0.32 ⁿ	0	0
Myricetin	BQL	BQL	0	0	BQL	BQL	0
Luteolin	BQL	BQL	1.78 ± 0.01 ^p	BQL	3.82 ± 0.13 ^o	1.41 ± 0.04 ^p	BQL
Eriodictyol	0	0	0	BQL	BQL	0	0
Quercetin	BQL	4.68 ± 0.03 ^o	114.50 ± 0.71 ^g	5.69 ± 0.02 ⁿ	29.05 ± 0.21 ⁱ	BQL	BQL
Apigenin	8.15 ± 0.28 ^m	18.35 ± 0.35 ^k	29.88 ± 0.39 ⁱ	23.33 ± 0.39 ^j	58.88 ± 1.59 ^h	12.63 ± 0.04 ^l	29.83 ± 0.39 ⁱ
Flavonoid glycosides [ng/mg DE]							
Quercetin-3- <i>O</i> -rutinoside (Rutin)	BQL	0	2555.00 ± 21.21 ^a	105.50 ± 1.41 ⁿ	2375.00 ± 7.07 ^b	95.75 ± 1.77 ^w	882.50 ± 10.61 ^c
Hyperoside	0	33.3 ± 0.14 ^o	0	BQL	0	0	0
Luteolin-7- <i>O</i> -glucoside	28.50 ± 0.07 ^p	5.83 ± 0.18 ^u	10.93 ± 0.18 ^s	19.85 ± 0.21 ^r	12.88 ± 0.39 ^s	14.40 ± 0.21 ^s	25.80 ± 0.21 ^p
Isoquercetin	10.65 ± 0.14 ^s	57.75 ± 1.77 ^m	115.00 ± 0.71 ^j	24.23 ± 3.92 ^p	199.75 ± 1.06 ^f	35.90 ± 0.57 ^o	847.50 ± 10.61 ^c
Kaempferol-3- <i>O</i> -rutinoside	0	0	219.00 ± 1.41 ^f	9.95 ± 0.42 ^s	310.25 ± 1.06 ^e	7.35 ± 0.21 ^t	136.50 ± 2.83 ^h
Isorhamnetin-3- <i>O</i> -rutinoside (Narcissoside)	0	0	78.50 ± 0.71 ^l	33.55 ± 0.42 ^o	122.25 ± 0.35 ⁱ	0	19.70 ± 0.42 ^r
Astragalin	BQL	BQL	9.73 ± 0.11 ^s	BQL	1.59 ± 0.03 ^y	BQL	38.48 ± 0.25 ^o
Isorhamnetin-3- <i>O</i> -glucoside	0	0	3.73 ± 0.04 ^u	BQL	BQL	BQL	BQL
Quercitrin	372.25 ± 0.35 ^d	152.75 ± 3.89 ^g	49.83 ± 0.25 ^m	2660 ± 21.21 ^a	122.00 ± 1.41 ⁱ	2515.00 ± 14.14 ^a	2385.00 ± 7.07 ^b
Apigenin-7- <i>O</i> -glucoside	47.75 ± 0.42 ^m	13.70 ± 0.35 ^s	95.00 ± 2.83 ^k	36.30 ± 0.14 ^o	74.75 ± 0.35 ^l	25.20 ± 0.14 ^p	76.50 ± 1.41 ^l
Naringenin-7- <i>O</i> -glucoside	46.30 ± 0.49 ^m	17.10 ± 0.21 ^r	24.50 ± 0.28 ^p	41.38 ± 0.25 ⁿ	14.98 ± 0.25 ^s	6.18 ± 0.11 ^t	31.43 ± 0.39 ^o

BQL—below the quantification limit (peak detected, concentration > limit of detection, but < limit of quantification). Means ($n = 9$) followed by the different lowercase letters (a–y) in each group of compounds are significantly different at $p < 0.05$.

As a result of the comparative LC-ESI-MS/MS analysis of the best antioxidant extracts, catechin was revealed as the abundant flavonoid aglycone (1013.00–2227.50 ng/mg DE), with the highest amount found in the *J. ashei* leaves extract. The contents of other individual aglycones (apigenin, quercetin, luteolin, taxifolin, myricetin and eriodictyol) were much lower (to 114.50 ng/mg). It can be noticed that in the case of the studied juniper samples, the total aglycone contents and profiles are species-specific. The greatest differentiation was observed in phenolic glycosides. The highest amount of these metabolites was determined in the leaves of *J. communis*, *J. sibirica*, *J. formozana*, and *J. sabina*. In the case of *J. ashei* and *J. pinchotii*, glycosides represented a relatively small portion of all polyphenols. Quercetin derivatives (e.g., rutin, isoquercetin, quercitrin) were found to be predominant reaching concentrations ≥ 2375.00 ng/mg. Rutin was the major flavonoid glycoside in *J. communis* 'Laxa' and *J. sibirica*, while quercitrin was dominant in *J. formozana*, *J. excelsa*, and *J. sabina* var. *balkanensis*. Several other glycosides including luteolin, kaempferol, isorhamnetin, apigenin, and naringenin derivatives were detected in the samples. However, *J. ashei* and *J. pinchotii* extracts did not have isorhamnetin glycosides and there was only a trace amount of one kaempferol glucoside (astragalin). In addition to the above-mentioned and confirmed analytes, some isobars of naringenin 7-glucoside, apigenin-7-glucoside, isorhamnetin-3-glucoside, astragalin, luteolin-7-glucoside, kaempferol-3-rutinoside, kaempferol, quercetin, naringenin, myricetin, catechin, and isorhamnetin were detected during LC-MS analyses. However, their reliable identification could not be performed.

In-Depth Analysis of Antioxidant Activity

All tested samples showed strong antiradical activity. Taking into account the ability to scavenge ABTS free radicals, samples can be ordered as follows: *J. ashei* > *J. sibirica* > *J. excelsa* > *J. formozana* \approx *J. pinchotii* > *J. communis* \approx *J. sabina*. The activity (expressed as EC₅₀) ranged from 331.64 ± 5 μ g DE/mL (*J. ashei*) to 798.71 ± 10 μ g DE/mL (*J. sabina*) (Figure 2A). All tested extracts were also able to chelate metal ions. The higher activity was observed in the case of *J. sibirica* (0.83 mg DE/mL) whereas the lowest was observed for *J. communis* (3.2 mg DE/mL). In terms of this activity, samples can be ordered as follows: *J. sibirica* > *J. formozana* > *J. sabina* > *J. pinchotii* > *J. excelsa* > *J. ashei* > *J. communis* (Figure 2B). All extracts were also able to inhibit LOX. In the case of this activity, the smallest differences between extracts were observed. EC₅₀ values ranged from 1.77 mg DE/mL to 2.44 mg DE/mL. The most effective inhibitors were extracts from *J. ashei* and *J. formozana*, whereas the lowest activity was observed in the case of *J. communis* extract (Figure 2C).

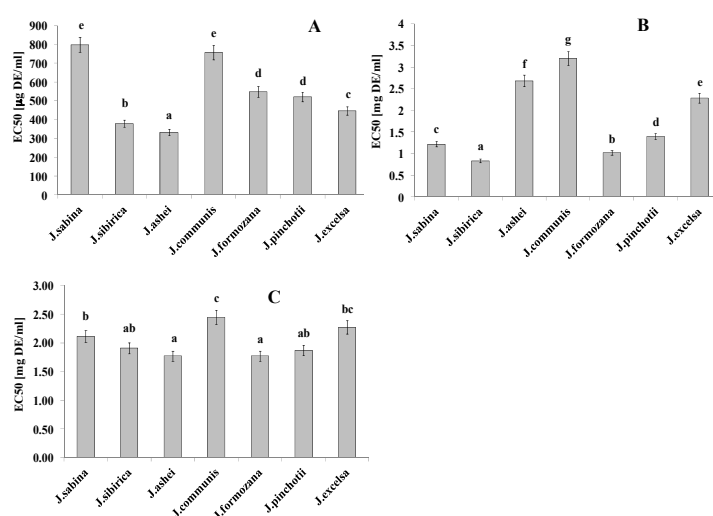


Figure 2. Antioxidant activity of extracts from leaves of previously selected juniper representatives. (A)—antiradical activity against 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS), (B)—chelating power, (C)—ability to lipooxygenase inhibition. Standard deviation (SD) is shown as error bars. Values (\pm SD) with different letters (a–g) are significantly different at $p < 0.05$.

Interestingly, despite the similar inhibitory activity, the extracts have different modes of action. The most effective samples acted as uncompetitive LOX inhibitors (Figure 3). Extracts from *J. pinchotii* and *J. excelsa* demonstrated a non-competitive mode of action, whereas the weakest inhibitor—extract from *J. communis*—acted as a competitive LOX inhibitor (Figure 3D,F).

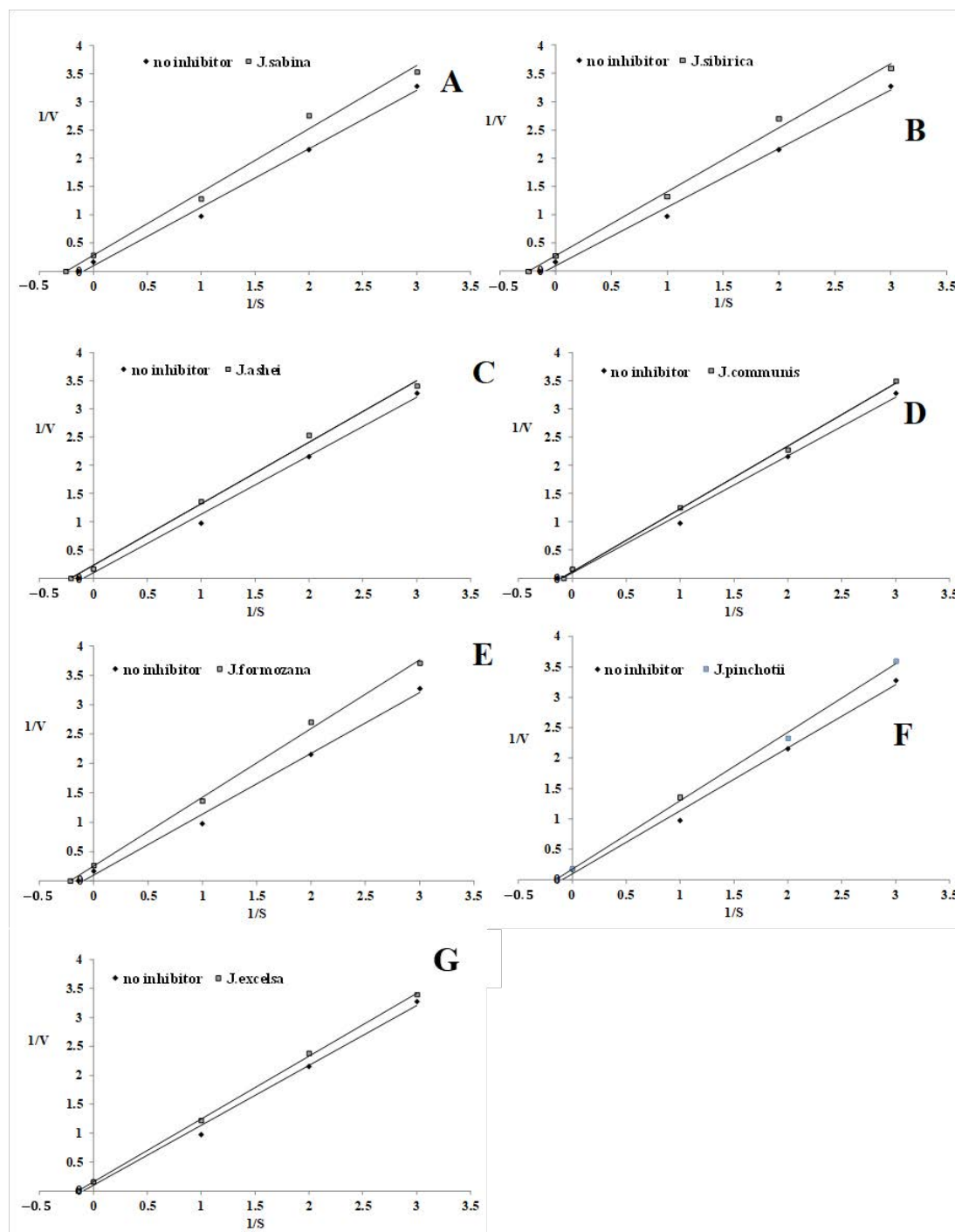


Figure 3. Mode of lipoxygenase (LOX) inhibition by extracts from leaves of previously selected juniper representatives. (A) *J. sabina*, (B) *J. sibirica*, (C) *J. ashei*, (D) *J. communis*, (E) *J. formozana*, (F) *J. pinchotii*, (G) *J. excelsa*. V—rate of enzymatic reaction expressed as the absorbance unit (see methodology), S—the substrate concentration (see Methodology).

4. Discussion

Polyphenols contribute to a wide range of biological properties of herbal samples. They can be major active ingredients or a valuable addition, demonstrating a synergistic effect with other natural molecules or synthetic drugs [28]. Therefore, sources of bioactive polyphenols are still searched and evaluated. Moreover, the content and profile of polyphenols is an important phytochemical and chemotaxonomic information as well. The present study was designed to analyze the total polyphenol content and antioxidant activity of juniper extracts in order to reveal species of different origin, exhibiting highest activity, and to identify the phytochemicals (secondary metabolites) responsible for these properties. Antioxidant activity, particularly antiradical activity, is very important due to the deleterious role of free radicals in biological systems and food. Plants rich in secondary metabolites, including phenolic compounds, have antioxidant activity related to their redox properties. The DPPH screening is one of the most widely used tests for the evaluation of the antioxidant activity of the extracts [29]. Thus, the first step of the study was aimed at identifying juniper extracts with highest TPC content correlated with high antiradical potential measured as DPPH-radical scavenging ability.

This screening enabled the designation of the most promising juniper extracts for further detailed studies of polyphenolic profile and biological assays. It was found that all samples contain large amount of polyphenols (45–263 mg GAE/g of dry extract (DE)). For several juniper leaf extracts, TPC values were found to be outstanding (>180 mg/g DE): *J. ashei* J. Buchholz $>$ *J. pinchotii* Sudw. $>$ *J. communis* Laxa \geq *J. formosana* Hayata. $>$ *J. sibirica* Burgsd. It could be noticed that the leaf extracts demonstrated higher antioxidant properties than the corresponding galbuli extracts, as it was shown by analysis of the leaves and galbuli extracts of *J. chinensis* L., *J. virginiana* L., as well as cultivars *J. virginiana* ‘Glaucua’ and *J. virginiana* ‘Grey Owl’. TPC values were significantly higher than those reported for *Juniperus phoenicea* L. leaves (6.3 and 9.6 mg/g) by Ghouti et al. [17]. On the other hand, the TPC values found for galbuli (“berries”) were lower than those reported for *J. communis* and *J. oxycedrus* [29]. It can be noticed that the TPC values of juniper samples are strictly species and organ-dependent. Moreover, differences in phenolic content may be related to the different growing or/and climate conditions. All samples had radical scavenging action in the DPPH radical scavenging assay test (Table 1). Determined EC₅₀ values ranged from 49 to 352 μ g DE/mL. The lowest values (indicating the highest activity) were found for *J. ashei*, *J. pinchotii*, and *J. formosana* (49–86 μ g DE/mL). *Juniperus* samples (e.g., *J. communis*, *J. formosana*, and *J. sibirica* ethanolic extracts) were previously reported to be effective radical scavengers with EC₅₀ 21.39–28.55 μ g/mL [29,30]. Interestingly, it was observed that juniper ethanol extracts are more effective than essential oils obtained from the corresponding plant materials [30].

After identification of the best antioxidant plants in the group of all assayed junipers, the LC-ESI-MS/MS-MRM technique was employed for the qualitative and quantitative determination of the secondary metabolites (phenolic acids, flavonoid aglycones, flavonoid glycosides), which is responsible for the highest activity of best antioxidant extracts. It was observed that phenolic acids constitute a relatively small part of all polyphenols, with protocatechuic acid as a dominant representative (up to 43.3 ng/mg DE). The majority of them belong to hydroxybenzoic acid derivatives (4-OH-benzoic, salicylic, protocatechuic, gallic). Only one hydroxycinnamic acid derivative was found, i.e., *p*-coumaric acid. The highest amount of phenolic acids was found in *J. sibirica* extract, whereas *J. excelsa* contained only trace amount of these compounds. Mrid et al. [15] have also observed that hydroxybenzoic derivatives constitute the biggest part of *Juniperus oxycedrus* leaf phenolic acids. However, they found that salicylic and 4-OH benzoic acid were the most abundant ones.

The highest concentration of phenolic glycosides was determined in the leaves of *J. communis*, *J. sibirica*, *J. formosana*, and *J. Sabina*, whereas in the case of *J. ashei* and *J. pinchotii*, glycosides represented a relatively small portion of all polyphenols. Quercetin derivatives (e.g., rutin, isoquercetin, quercitrin) were found to be predominant, reaching concentrations ≥ 2375.00 ng/mg. Quercetin, apigenin, luteolin and their glucosides, isorhamnetin glucosides, and catechin were previously reported in juniper samples [6,15,17,30,31]. In the study of Sahin Yaglioglu and Eser [32], catechin was found (206.654 μ g/g extract), while rutin was found (110.203 μ g/g extract) for *Juniperus oxycedrus* L. subsp. *oxycedrus* needles.

We did not find free kaempferol, naringenin, or myricetin glucosides previously identified in some species [11,13,31]. However, we have detected free myricetin. Moreover, kaempferol 3-O-rutinoside was found in *J. communis* 'Laxa', *J. formozana*, *J. sibirica*, *J. excelsa*, and *J. sabina* var. *balkanensis*, and kaempferol 3-glucoside (astragalin) with naringenin glucoside was found in all the studied species. All the studied juniperus species are very diverse in terms of polyphenolic profile. Moreover, the polyphenolic profile established for *J. communis* 'Laxa' differed greatly from the one recently reported by Dziedzinski et al. [31] for *J. communis* shoot collected in Poland. Similarly, polyphenolic profiles of other studied juniper extracts are very diverse in terms of composition [6,11,17]. Therefore, it can be assumed that the species, variety, and origin strongly influence the phytochemical composition of juniper plant material. The major chemical components in the *J. excelsa* leaves were monoterpene hydrocarbons, sesquiterpene hydrocarbons, and oxygen-containing sesquiterpenes [32].

Other major phenolic constituents found in extracts of *J. excelsa* species are coumarins, lignans, sesquiterpenes, abietane, labdane and pimarane diterpenes, flavonoids, flavone glycosides, and tannins [33]. In leaves and seed cones extracts of *J. excelsa*, catechin, quercitrin, epicatechin, rutin, apigenin, and amentoflavone were present in substantial amounts. Phenolic acids were found in small amounts (0.26% and 0.48% of dry weight (DW) of leaves and seed cones, respectively); the dominant compounds were 2,5-dihydroxybenzoic and *p*-hydroxybenzoic acid. Furthermore, lignans, mainly matairesinol, were present in moderate amounts, primarily in seedcones, while coumarins were found in small amounts in both *J. excelsa* extracts [34].

The antioxidant activity of a given plant extract depends on the plant's phytochemical profile. Some of the phenolics (anthocyanidin, catechins, flavonoids), tannins (ellagic and gallic acids), phenyl isopropanoids (caffeic, coumaric, ferulic acids), lignans, catechol, and many others act as antioxidants [35]. There are many reports in the available literature on the antioxidant activity of juniper fruit [5]. The aqueous and ethanolic extracts from *J. communis* L. fruits demonstrated a strong total antioxidant capacity at the concentrations of 20, 40, and 60 µg/mL. At these concentrations, both extracts of juniper fruit demonstrated effective reducing power, metal-chelating activity, as well as antiradical potential in various scavenging assays. These fruit extracts effectively inhibited the peroxidation of the linoleic acid [36]. However, most studies on the antioxidant potential of juniper concern essential oils [37,38]. The in vitro antioxidant capacity of juniper oil has been observed using various free radical scavenging tests. It is mainly attributed to electron transport, which makes juniper essential oil a powerful antioxidant. Due to the increased activity of various antioxidant enzymes, the possibility of blocking the oxidation process was also confirmed in living models [39].

Little is known about the bioactivity of juniper leaf extracts. During investigations performed by Manel et al. [4], solvents of increasing polarity such as methanol, petroleum ether, chloroform, ethyl acetate, and water (MeE, PEE, ChlE, EtAE, and AqE) were used for preparation extracts from the leaves of *J. thurifera* L. The antiradical activity of MeE, ChlE, EtAE, and AqE extracts was assessed by the DPPH assay. The three extracts of EtAE, AqE, and MeE showed high scavenging potential ($IC_{50} = 29.348$ µg/mL, 37.538 µg/mL, and 52.573 µg/mL, respectively), while the lowest antiradical activity was demonstrated by the ChlE ($IC_{50} = 70.096$ µg/mL) [13]. The EtOH extract from *J. communis* ssp. *nana* leaves showed the best antiradical activity against DPPH ($91.40 \pm 0.54\%$), which was followed by the leaf EtOH extract of *J. foetidissima* ($91.21 \pm 0.45\%$). All of the extracts demonstrated ferric ion-chelating effect below 50% [1]. In our study, the activity antiradical activity (against ABTS free radicals; expressed as EC_{50}) ranged from 331.64 ± 5 (*J. ahei*) to 798.71 ± 10 µg DE/mL (*J. sabina*) (Figure 2A). The higher chelating power was observed in the case of *J. sibirica* (0.83 mg DE/mL) whereas the lowest was observed for *J. communis* (3.2 mg DE/mL) (Figure 2B).

The mechanisms of inflammation mediated by metabolites of lipoxygenase and nitric oxide play key roles in physiological immune response [40].

Lipoxygenases (LOXs) are enzymes involved in the production of inflammatory mediators such leukotrienes and prostaglandins [41]. Human LOX isoenzymes (5-, 12-, and 15-lipoxygenases) are extremely unstable at physiological temperature in the cell-free lipoxygenase assay; therefore, a good

solution is to use commercially available and the physiological temperature-stable LOX derived from *Glycine max* L. for the LOX inhibitory assay [42].

LOX inhibition is a prospective method for the treatment of inflammation [43]. Furthermore, the additional advantage of this type of experiment is avoidance of the undesirable in vivo tests on experimental animals (carrageenan-induced paw edema in rats) [44]. The LOX-inhibitory potential of pure chemicals and plant extracts is well known and extensively studied [45,46]. Samples tested in our study are able to inhibit LOX. EC₅₀ values ranged from 1.77 to 2.44 mg DE/mL. The most effective inhibitors were extracts from *J. ashei* and *J. formozana*, whereas the lowest activity was observed in the case of *J. communis* extract (Figure 2C). Extracts from *J. pinchotii* and *J. excelsa* demonstrated a non-competitive mode of action, whereas the weakest inhibitor—extract from *J. communis*—acted as a competitive LOX inhibitor (Figure 3). The extracts from *J. sibirica* Burgsdorf. extracts demonstrated 12-LOX inhibitory activity (IC₅₀ = 4.85 mg/mL for needles and 1.34 mg/mL for cones). The cones extract showed a better anti-inflammatory activity compared to the needles [44]. Our results indicate that all tested species exhibit a strong antioxidant activity and sufficient ability to inhibit the LOX enzyme included in inflammation processes. No correlation was found between the total phenolics content and the anti-inflammatory capacity, which suggests a synergistic effect of compounds contained in the extracts. The literature data also suggest a synergistic action of various compounds and do not attribute the biological effects (antioxidant, antifungal, or anti-inflammatory) to a single compound [6].

In the group of all assayed juniper species, the leaf extract of *Juniperus ashei* J. Buchholz (mountain cedar) was distinguished as the representative with highest total polyphenol content and best antioxidant properties. To our knowledge, this is the first determination of *J. ashei* as a juniper representative with superior antioxidant activity in the genus *Juniperus*. The mountain cedar is a dioecious plant that is found naturally in north-eastern Mexico and south-central USA, including Missouri and central Texas. The original source of *J. ashei* in this study is collected from a wild representative in the Murray County, Oklahoma, United States (Table 1). Using LC-ESI-MS/MS-MRM analysis, catechin was revealed as its most abundant antioxidant metabolite than in the other studied juniper extracts, and therefore, it was considered as the metabolite contributing to the superior antioxidant properties of the *J. ashei* J. Buchholz leaves extract.

5. Conclusions

To our knowledge, this is the first detailed antioxidant activity evaluation of *Juniperus* L. representatives of various origins around the world, which was accomplished with a quantitative and qualitative analysis of the polyphenol compounds of selected best antioxidant samples. As a result of the study, the most promising species for potential application were identified. For the first time, *J. ashei* J. Buchholz was distinguished as the species with superior antioxidant activity in the group of studied junipers. The identified juniper extracts and polyphenols with superior antioxidant activity have potential application in the pharmacy and cosmetics as sources of lead compounds for the generation of antioxidant compositions for the prevention of oxidative-stress-associated organ-degenerative diseases. We hope our findings will accelerate interest from the wider research community in the involvement of the *Juniperus* organs in different plant-related products. It should be mentioned that the observed differences in the biological activity and polyphenol content may be partially related to the collection site, climate, altitude, sampling season, soil parameters, etc. Therefore, once the most promising species have been identified, the impact of pedoclimatic conditions and sampling season on their chemical constituents and bioactivity should be investigated. Moreover, the safety of the application of juniper-based natural products should be established.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/10/24/8921/s1>, Table S1: Optimized parameters for the LC-ESI-MS/MS qualitative analysis of phenolic acids and flavonoids. Table S2: Analytical parameters applied for LC-MS/MS quantitative method.

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D.I., S.B. and G.A.; investigation, M.O., D.I., U.G.-D., S.B. and G.K.; resources, A.T., R.N., U.G.-D.; data curation, M.O., D.I., U.G.-D. and G.A.; writing—original draft preparation, M.O., R.N., D.I., U.G.-D.; writing—review and editing, M.O., R.N., D.I., U.G.-D., G.A. and A.T.; visualization, M.O., D.I. and U.G.-D.; supervision, R.N., U.G.-D., G.A. and A.T.; project administration, M.O., R.N., D.I., U.G.-D., G.A., A.T.; funding acquisition, R.N., A.T., G.A. and U.G.-D. All authors have read and agreed to the published version of the manuscript.

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