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Comparative Analyses on Chemical Constituents and Biological Activities of *Laserpitium siler* L. from Serbia

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Abstract: The traditional European medicinal plant *Laserpitium siler* L. was studied for the first time for the chemical composition and *in vitro* biological potential of extracts from fruits and aerial parts. Eight extracts were subjected to spectrophotometric detection and LC-MS quantification of phenolics. The bioactivity assessment comprised antioxidant, antimicrobial, anti-denaturation, acetylcholinesterase, tyrosinase, α-amylase, and α-glucosidase inhibition assays. The spectrophotometric analyses showed that the alcoholic extracts contained the most phenolic acids and coumarins. Among the twenty quantified compounds in the methanolic extracts, chlorogenic acid (29.10 mg/kg in the fruits; 47.00 mg/kg in the aerial parts) and *p*-coumaric acid (7.50 mg/kg in the fruits; 9.50 mg/kg in the aerial parts) were the most abundant. The DPPH/ABTS/BBT tests showed the strongest antioxidant effect of the methanolic extract of the aerial parts. The results of the microdilution assay referred to stronger antibacterial than antifungal activity, with the essential oil being as effective as the antibiotics used. The extracts expressed promising activity against *P. aeruginosa* PAO1 biofilm formation and pyocyanin synthesis. The prevention of BSA denaturation by the ethanolic extracts was comparable to diclofenac (91.08%-fruits, 95.08%-aerial parts, and 95.60%-diclofenac). IC_{50} values showed greater enzyme inhibitory potential of aerial parts and the highest reduction of α-glucosidase activity.

Keywords: *Laserpitium siler*; phenolics; LC-MS; antioxidant; antimicrobial; *Pseudomonas aeruginosa*; BSA; enzyme inhibitory activity.

1. Introduction

The genus *Laserpitium* L. (Apiaceae) comprises about twenty perennial herbaceous and aromatic plant species. Although they occur in a wide area from the Canary Islands to Iran, they are mainly

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distributed in the Mediterranean region and southwest Asia [1]. These species are characterized by spindle-shaped roots that turn into a short, thickened rhizome in the upper part. They form small white or pink flowers and then schizocarpic fruits rich in essential oil [2, 3]. There are 14 *Laserpitium* species described in Flora Europaea, distributed in higher altitudes of southern and central Europe. The most common is *Laserpitium siler* L., or laserwort (syn: *Siler montanum* Crantz), which grows mainly on rocky meadows at about 1400 m altitude in submeridional-montane areas of central Europe [4]. It occurs in Serbia [3] and according to the Red List of the Flora of Serbia [5], it is considered to be at low risk. However, the root of this species, popularly known as "*raskovnik*", is intensively collected in eastern Serbia for its "miraculous powers" [6]. Numerous traditional uses of *Laserpitium* plants are known in Europe, especially in alpine and Russian medicine. The herb of *L. carduchorum* is used as a spice and for the treatment of urinary tract infections [7]. Gum resins are obtained from the roots of alpine *L. gallicum*, which are of medicinal importance in the treatment of tumors and warts [8]. The bitter herbal preparations from the roots and rhizomes of *L. latifolium* and *L. siler* are used in the treatment of gastrointestinal disorders to improve digestion. They are also applied in the treatment of heart and liver dysfunction, tuberculosis, rheumatism and as diuretics. Laserwort tonic is recommended for sleep disorders and severe depression [9-11]. Since ancient times, the fresh roots of *L. siler* have been used in the Balkans to cure cataracts and as an antidote for snakebites [12].

The wide application of *Laserpitium* species as herbal folk remedies led to studies of their chemical composition and examination of their bioactivity. Phenolic compounds and sesquiterpene lactones are the main active secondary metabolites responsible for the medicinal properties and traditional uses of these plants [7]. Numerous studies have shown that *L. siler* and other *Laserpitium* species are rich in sesquiterpene lactones of the eudesmanolides, germacranolides, and especially the guaianolides of the slovanolide type [8, 13-23, 24], which is of great chemotaxonomic importance. The sesquiterpene lactones are very complex and characteristic metabolites whose synthesis is very difficult, so they are often isolated from the plants. In this regard, Apiaceae species, including those of the genus *Laserpitium* [25], are one of their most important natural sources. The sesquiterpene lactones are derived from the sesquiterpene germacrenes A - D [26] and their lactone configuration (6b,7a/6b,7b) differs from that in the Asteraceae family (6a,7b) [27]. This structural difference serves as a phytochemical marker to distinguish the chemo-phylogenetic relationships in both families [25]. In addition, the tribe Laserpitieae could be chemosystematically identified by the presence of C-8 and C-10 oxidised guaianolides [16]. They are also typical of the genus *Laserpitium* and were identified as main constituents in the studied *Laserpitium* species from Serbia [13, 16]. As for chemotaxonomic guaianolides, therapeutic properties were demonstrated for α-methylene- and guaianolides with an α,β-unsaturated carbonyl residue. Their specific mechanism of action is related to these carbonyl groups through their interaction with thiols in peptides or proteins and enzymes [26, 27]. Their biological properties are primarily associated with anti-inflammatory and immunomodulatory [22, 25, 27], cytotoxic [21, 26] and antimicrobial activities [24, 27].

The presence of flavonoids, phenylpropanoids, oxygenated sesquiterpenoids, coumarins and daucans has also been determined in several species of this genus [14, 21, 23, 28-31]. These compounds are also known for many confirmed bioactivities such as hypotensive, antioxidant, anticoagulant, antimicrobial, antihyperglycaemic, anti-inflammatory, immunomodulatory, antiproliferative, antitumor, antidiabetic, cytotoxic and antiviral effects [22, 28, 29, 32-34]. Although phenolics are considered to be responsible for the bioactivity of Apiaceae species [35, 36, 37], this connection has so far only been published for *L. krapffi*. The authors related the content of phenolic acids and flavonoids to the antioxidant and cytotoxic properties of this species [9].

In addition, *L. siler* and other *Laserpitium* species contain considerable amounts of essential oils, the composition of which has been extensively analysed. According to many studies, the oils tested were mainly of the monoterpenoid chemotype, with a predominance of hydrocarbons [38-42]. The colorless or blue essential oils are often rich in α/β-pinene, sabinene, limonene, terpinen-4-ol, α-bisabolol, viridiflorol, and hamazulene [24, 38-40, 43-47]. *L. siler* is morphologically and chemically similar to *Laser trilobum* in terms of seed endosperm, fruit structure, and the presence of limonene and perillal in the essential oils of both species [48]. In fact, some of the analysed *L. siler* essential oils were of the limonene and perillaldehyde chemotype [43, 45], but the chemical profiles of this taxon differ

depending on the subspecies and origin. GC-MS and PCA analyses of the essential oils of *L. siler* subsp. *montanum* and subsp. *siculum* from five different geographical origins showed that the oils of subsp. *siculum* belong to one chemotype (perillaldehyde/chamazulene/sabinene), while subsp. *montanum* belongs to different chemotypes (*trans*-anethole, limonene, and sabinene) [47]. *L. siler* subsp. *garganicum* did not contain perillaldehyde or limonene, which could be considered as a chemotaxonomic feature according to the authors [39] and is a reason to consider this subspecies rather as a species of the genus *Laserpitium*. *L. siler* subsp. *siculum* possessed limonene along with β-phellandrene and β-terpinene as major oil constituents, but perillaldehyde was not reported in its composition [42]. In our earlier study on the oil composition of *L. siler*, limonene was identified in a significant percentage, but perillaldehyde was not detected. Besides α-pinene, the predominant compounds were *trans*-verbenol, *trans*-pinocarveol, and verbenone [49]. Therefore, this oil could be assigned to the pinene chemotype like some other *Laserpitium* oils [40-42, 49].

A wide spectrum of pharmacological effects of *Laserpitium* species includes immunostimulatory, anti-inflammatory, antitumor, antimicrobial, antioxidant, antimalarial, cytotoxic, neurotoxic, and many other activities [7, 9, 11, 21, 22, 39, 46, 50-52]. Although it is very common in traditional European medicine, there are insufficient data on the biological potential of this species to date. The biological effects of *L. siler* have been described in a few papers [11, 49, 52], but data on *L. siler* from Serbia are lacking and *L. siler* subsp. *siler* has not been investigated from the aspect of chemical composition and biological potential of the extracts. This prompted us to perform an *in vitro* screening of the activity of the laser herb to confirm its traditionally assumed curative properties. The present study was also inspired in part by our previous research demonstrating the significant antioxidant and antimicrobial potential of *L. ochridanum* [46].

The aim of this work was to determine the phenolic composition of extracts from the aerial parts and fruits of the plant. Quantification of phenolics in the methanolic extracts was performed by liquid chromatography/mass spectrometry. Further investigation included the determination of the *in vitro* bioactivity of the laserwort. The bioassays included (i) determination of antiradical activity, (ii) ability to reduce growth of pathogenic bacteria and micromycetes, (iii) inhibit biofilm and pyocyanin production in *P. aeruginosa*, (iv) prevent heat-induced BSA denaturation, and (v) examine inhibition of enzymes associated with diabetes and neurological disorders.

2. Materials and Methods

2.1. Plant Source

Plant material of *L. siler* subsp. *siler* was harvested in June 2018 in the fruiting stage near the town of Kosovska Mitrovica in Serbia (N 42º 59.26'; E 20º 49.42'; 489 m altitude). The species was collected and identified by Prof. Dr. Zoran Krivošej. The voucher of L. siler (BEOU 17869) is deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade-Faculty of Biology, Serbia. Separated and air-dried fruits (Fr) and aerial parts (AP-leaves and stems) were pulverised in a laboratory blender and extracted using the technique described later.

2.2. Sample Preparation

Methanol (MeOH), ethanol (EtOH), methylene chloride (CH_2Cl_2), and ethyl acetate (EtOAc) crude extracts were obtained by ultrasound-assisted (US) extraction using 10 g of powdered plant material and 100 mL of the listed solvents. During the 24h extraction at room temperature, the extracts were subjected to US in the first and last hour of the procedure [46]. The characteristic blue essential oil was isolated using the Clevenger apparatus (European Pharmacopeia, 4th edition), in which 200 g of the mixed aerial parts and fruits were subjected to water distillation for 3 hours.

2.3. Solvents and Chemicals

The solvents and chemicals (analytical or HPLC grade) used in this work are listed in the Supporting information as S1.

2.4. Total Phenolic Contents

L. siler extracts (1 mg/mL) were subjected to spectrophotometric measurement of total phenolic (y=7.063x-0.015; r^2=0.991 for gallic acid (GA, mg)) [53], flavonoids (y=13x-0.019; r^2=0.995 for quercetin hydrate (QH, mg)) [54], flavonol (y=4.7488x + 0.0117; r^2=0.989 for QH, mg) [55, 56] and coumarin contents (y=0.2795x+0.1616; r^2=0.9941, for coumarin (C, mg)) [57, 58] using a Multiskan Sky Thermo Scientific plate reader. The amounts of target metabolites were quantified by equivalents of appropriate standards in each assay.

2.5. Liquid Chromatography-Mass Spectrometry Analysis

Stock solutions of crude MeOH extracts of AP and Fr were prepared in MeOH at C=20 mg/mL and subjected to phenolic chemical profiling. The details of the method, experimental conditions, and parameters of chromatographic separation and mass spectrometric quantification have been described previously [59, 60]. Quantification of the detected compounds (mg/kg dry weight of plant (DW)) was performed by comparison with MeOH solutions of available standards, whose stock solutions were prepared with mobile phase (acetonitrile and H_2O = 1:1) at a final Cs=0.025-1 g/L.

2.6. Antioxidant Activity

DPPH [61], ABTS [62] and BB [63] assays were performed to determine the antioxidant activity of the extracts of *L. siler* using Jenway 7315 UV/Visible spectrophotometer. The results were compared with the commercial standards BHA, BHT and ascorbic acid (Vit. C) and presented in tabular form.

2.7. Albumin Denaturation Test

The inhibitory effect of *L. siler* on the albumin denaturation process was examined on the reaction mixtures consisting of different combinations of solutions. The experimental samples of the extracts (C=0.1 mg/mL) were tested against diclofenac sodium solution (C=0.1 mg/mL) containing BSA solution. Absorbance measurements were performed at 255 nm using a Jenway 7315 UV/Visible spectrophotometer, and the results are expressed as the percentage of inhibition of BSA denaturation as described by Kar and Rahman [64, 65].

2.8. Treated Microbials

Antimicrobial activity was determined against pure control strains procured from the Mycological Laboratory, Institute of Biological Research "Siniša Stanković", Serbia. The bacterial strains used were as listed: *Bacillus cereus* (human isolate), *Enterococcus faecalis* (ATCC 19433), *Micrococcus flavus* (ATCC 10240), *Listeria monocytogenes* (NCTC 7973), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Proteus mirabilis* (ATCC 7002). The selected fungi for this experiment were: *Trichoderma viride* (IAM 5061), *Aspergilus ochraceus* (ATCC 12066), *Aspergilus versicolor* (ATCC 11730), *Aspergilus fumigatus* (human isolate), *Aspergilus niger* (ATCC 6275), *Penicillium verrucosum* var. *cyclopium* (Pvc-DS-11), *Penicillium ochrochloron* (ATCC 9112), *Penicillium funiculosum* (ATCC 10509).

2.9. Micro-well Dilution Test

A procedure of the microdilution technique [66-68] was performed using 5% DMSO extracts of *L. siler* (C=20 mg/mL) and pure essential oil (V=15-20 mL). Minimum inhibitory/bactericidal/fungicidal concentrations (MICs, MBCs, MFCs) were determined. A colorimetric viability assay was performed to determine the antibacterial results, while the growth of

micromycetes was observed with a binocular loupe. The results obtained were compared with the antibiotics streptomycin and ampicillin (C=1 mg/mL) in the antibacterial test, while fluconazole (C=1 mg/mL) served as the standard in the antifungal test.

2.10. Antibiofilm and Antipyocyanin Test

The anti- QS activity of *L. siler* was tested on *Pseudomonas aeruginosa* PAO1 grown in Luria-Bertani medium under the conditions described in our previous work [46]. The assay for determination of the inhibitory effect of the tested extracts on PAO1 biofilm formation was conducted using ½ of the MIC values previously determined in the microdilution method [69, 70] and the reading was taken at 625 nm (Sunrise™ - Tecan ELISA Reader). Pyocyanin production by *P. aeruginosa* was monitored in the presence of ½ of the MIC of the samples. Spectrophotometric measurement was performed at 520 nm (Shimadzu UV 1601, Kyoto, Japan), and results were expressed as percentages according to the following ratio: $(OD_{600} - OD_{520}/OD_{600}) \times 100$.

2.11. Enzyme Inhibitory Activity

The extracts of *L. siler* (C=0.25-5 mg/mL) were tested *in vitro* for their inhibitory effect on the selected enzymes. Inhibition of acetylcholinesterase (AchE, VI -S from *Electrophorus electricus*), was measured by the Ellman method, and galanthamine was used as a reference compound [71, 72]. Tyrosinase (Tyr, from fungi) was evaluated using L-3,4-dihydroxyphenylalanine as substrate and kojic acid as standard [72, 73]. The assay for inhibition of α-amylase (Amy, from porcine pancreas) was performed with iodine/potassium iodide [72, 74], whereas the assay for inhibition of α-glucosidase (Glu, type I from *Saccharomyces cerevisiae*, Sigma) was done by protocols of Zengin [72] and Hyun [75]. For both antihyperglycemic assays, acarbose served as a positive control. Absorbance values were recorded spectrophotometrically on a Multiskan Sky Thermo Scientific microplate reader. Results were calculated as inhibitory concentration IC_{50} (mg/mL) using the following equation: IC_{50} = (Abs (control-blank) - Abs (sample- blank))/Abs (control- blank) x 100.

2.12. Statistical Data

The obtained repeated measures of the tested extracts and analyses (both in triplicate) were expressed as their average values ± standard deviations (SD). One-way analysis of variance (ANOVA) and Tukey's HSD test with a = 0.05 were used (SPSS v. 18.0 programme).

3. Results and Discussion

3.1. Obtained Yields of the Samples

The obtained yields of the samples are presented in Table 1. It can be seen that the polar solvents extracted the aerial parts of the plant with greater intensity than fruits, while the use of methylene chloride and ethyl acetate resulted in greater amounts of fruit extracts. The obtained yield of essential oil from mixed aerial parts and fruits was 0.75 mL (0.375% v/v), while the oil density was ρ=0.9 g/mL. Like the oils of other *Laserpitium* representatives, the isolated essential oil of *L. siler* was blue in colour, which was due to the presence of chamazulene, a degradation product of guaianolides [7]. The dry extracts and essential oil were stored in amber vials and refrigerated until further analysis. In our previous study, using a double volume of solvents (200 mL) for the US extraction of the same amount of aerial parts of *L. ochridanum* (100 g), the obtained yields of the final dry extracts were lower than those obtained from *L. siler*. Also, the essential oil of *L. ochridanum* herbal parts, which was isolated by the same procedure, was obtained in a smaller amount than in the present study (0.11 % and 0.38 %, respectively) [46].

Table 1. Obtained yields of *L. siler* crude extracts and essential oil

***L. siler* Es[a] and EO[b]**			**Yields (g for Es) (mL for EO)**	**Yields (%) (w/w for Es) (v/v for EO)**
Es	Fr	MeOH	1.056[b]	9.72[b]
		EtOH	0.919[b]	9.31[b]
		CH_2Cl_2	0.459[a]	4.59[a]
		EtOAc	0.626[a]	6.23[a]
	AP	MeOH	2.374[c]	23.75[c]
		EtOH	2.180[c]	21.80[c]
		CH_2Cl_2	0.248[a]	2.48[a]
		EtOAc	0.233[a]	2.33[a]
EO		Fr and AP	0.75	0.375

Indicated letters mean significant difference in the same column ($p < 0.05$).
[a]10 g dry plant /100 mL solvent (ratio 1:10)
[b]200g dry plant/1000 mL distilled water

3.2. Secondary Metabolites Quantification

L. siler extracts were subjected to spectrophotometric quantification of various groups of secondary metabolites. In general, higher concentrations of secondary metabolites were measured in the aerial parts of the plant than in the fruits (Table 2). According to the results, the methanolic and ethanolic extracts of AP were the richest in total phenols, while the lowest concentration was recorded in the methylene chloride samples. Both alcoholic extracts of AP also had the highest concentration of coumarins, in contrast to Fr EtOAc extract. Furthermore, the MeOH and EtOH extracts of AP were richest in flavonoids, while the CH_2Cl_2 and EtOAc extracts of the fruits had the lowest contents. Less polar samples generally had lower amounts of flavonoids. The solvents used extracted higher amounts of flavonols from the fruits than from the aerial parts. Higher concentrations of flavonols (28.421-57.551 mg QHE/g DE) were measured in alcoholic extracts than in CH_2Cl_2 and EtOAc extracts (25.333-38.740 mg QHE/g DE). Compared with the previously determined quantities of phenolics from *L. ochridanum*, the concentration of phenolic compounds in the MeOH and EtOH samples of *L. siler* was slightly higher than in the *L. ochridanum* samples (171.39 and 169.69 mg GA /g DE; 141.30 and 111.28 mg GA /g DE, respectively).

Table 2. Total contents of phenolics (mean ± SD)

***L. siler* Es (C=1 mg/mL)**		**Phenolics mg GAE/g DE**	**Flavonoids mg QHE/g DE**	**Flavonols mg QHE/g DE**	**Coumarins mg CE/g DE**
Fr	MeOH	60.36 ± 1.00[b]	47.62 ± 0.11[b]	54.252 ± 1.197[c]	65.832 ± 2.530[c]
	EtOH	60.98 ± 1.20[b]	36.92 ± 0.33[b]	57.551 ± 1.053[c]	42.934 ± 2.066[b]
	CH_2Cl_2	42.40 ± 0.70[a]	23.96 ± 0.49[a]	38.740 ± 0.122[b]	52.236 ± 2.530[b]
	EtOAc	105.90 ± 1.20[c]	26.77 ± 0.11[a]	28.070 ± 0.729[a]	16.637 ± 4.131[a]
AP	MeOH	169.69 ± 0.50[d]	75.62 ± 0.11[c]	43.513 ± 0.973[b]	74.776 ± 4.131[c]
	EtOH	171.39 ± 1.10[d]	63.31 ± 0.44[c]	28.421 ± 0.677[a]	94.454 ± 8.764[d]
	CH_2Cl_2	23.64 ± 0.00[a]	35.54 ± 0.44[b]	25.333 ± 0.557[a]	30.769 ± 3.578[a]
	EtOAc	113.69 ± 0.90[c]	35.19 ± 0.38[b]	25.964 ± 0.172[a]	67.621 ± 3.578[c]

Indicated letters mean significant difference in the same column ($p < 0.05$).

The ethanolic sample of *L. ochridanum* [46] contained twice the amount of flavonoids than the ethanolic extract of *L. siler*. In another study on phenolic compounds in methanolic and etheric extracts of *L. krapffii* fruits [9], spectrophotometric measurements showed that the methanolic extract of the examined plant had significantly higher contents of phenols, flavonoids and phenolic acids than an etheric sample. Similarly, considering the polarity of the samples, the present results showed that MeOH

extracts of *L. siler* had higher contents of phenolics, flavonoids, flavonols, and coumarins than EtOAc and CH_2Cl_2 samples, except for the EtOAc extract of the fruits for phenols (Table 2). While the aerial parts of *L. siler* had high levels of phenols in our research, the flower extract of *L. zernyi* was found to have higher levels of phenols than the herbal extract, which additionally led to the better activity of this sample [76]. Coumarins are among the most potent metabolites of Apiaceae representatives [33] and their presence was confirmed spectrophotometrically in *L. siler*, especially in the alcoholic extracts of AP, where they were most abundant in the ethanolic sample (Table 2).

3.3. UHPLC-MS Quantification of Phenolics

Table 3 presents the results of UHPLC-MS for the total concentrations of phenolic compounds in the analysed extracts (mg/kg DW), obtained by comparison with reference compounds. A total of twenty phenolics were detected/quantified. The extracted-ion chromatograms (EICs) of all standard compounds used for quantification are shown at Supplemetary Figure 1, while Supplemetary Table 1 contains the retention times (min), molecular and fragment ions (*m*/*z*), linearity (Y=A+ BX), R^2, LOD (mg/L) and LOQ (mg/L) of the studied analytes. Nineteen metabolites were identified in the MeOH extract of Fr, while eighteen compounds were found in the MeOH extract of AP, including nine phenolic acids - hydroxybenzoic acid and hydroxycinnamic acid, and eleven flavonoids from the groups of flavonols, flavanones, and flavones. 5-O-Caffeoylquinic acid was present in the largest amount in both extracts. The content of this metabolite was 1.6 times higher in the aerial parts than in the fruits (47.00 and 29.10 mg/kg, respectively). In general, most of the compounds, fourteen out of twenty, were present in the aerial parts in higher concentrations than in the fruits. *p*-Coumaric acid was the second predominant metabolite, also present at higher levels in AP. The content of the flavonol glycoside rutin was more than thirty times higher in AP than in Fr, although it was present only in small amount in the plant (Table 3). In contrast, the content of the flavonol glycosides quercetin-3-O-glucoside and kaempferol-3-O-glucoside was ten times higher in the fruits. The flavone aglycones, luteolin and apigenin were also detected in higher concentrations in the fruit extract. Hydroxybenzoic acids, gallic acid and *p*-hydroxybenzoic acid, were detected in the aerial parts but not in the fruits, while the flavanone glycoside naringin was present only in the fruits. The UV chromatograms of summarized extracts, recorded at 254 nm, are shown in Supplementary Figure 2.

Various extracts of *Laserpitium* species are confirmed sources of different types of phenolic compounds. Thus, the presence of phenolic compounds in the 80% ethanolic extract of the leaves of *L. latifolium* was confirmed. It was found to contain various quercetin derivatives such as avicularin, isoquercitrin, quercitrin, rutin, as well as astragalin, and some other kaempferol glycosides. Phenylpropanoid compounds and their derivatives, such as latifolone, phenolcarboxylic acids, chlorogenic acid (5-O-caffeoylguinic) and neochlorogenic acid (3-O-caffeoylquinic acid) were also found in the leaves of *L. latifolium* [30]. The isoquercitrin, rutin, and astragalin were found in our samples, and a higher concentration of both was present in the aerial parts (4.60, 0.28, and 7.00 g/mL, respectively), while the phenylpropanoid latifolone was previously isolated by Stefanović [20] from *L. siler* collected in Serbia. Previously, the flavonoid artemetin was isolated from the chloroform extract of *L. siler* herb, which could not be detected in our analysis. In addition to apigenin and luteolin, chlorogenic acid was also previously detected in *L. zernyi* [76]. As mentioned earlier, HPLC analysis in our study revealed chlorogenic acid as the predominant phenolic compound in *L. siler*, while the content of apigenin and luteolin was higher in the fruits of the plant. Bogucka-Kocka [9] studied the methanolic and etheric extracts from the fruits of *L. krapffii* for the quantitative and qualitative content of phenolic acids and flavonoids, and protocatechuic acid and vanillic acid were the most abundant. Protocatechuic acid was also found in *L. siler* in our study (Table 3). The flavanone eriodictyol was detected in methanolic extracts of both *Laserpitium* species, in addition to *p*-coumaric and caffeic acids, while gallic acid was identified in both species, but it was present only in the aerial parts of *L. siler* (Table 3). The same authors concluded that astragalin, isoquercetin, nicotiflorin and 3-hydroxybenzoic acid were absent in the etheric extract, but still, these two extracts showed no significant difference in their composition with respect to phenolics [9].

Table 3. Quantitative data on individual phenolic compounds in laserwort samples

No.	Compounds		MeOH Es (mg/kg)	
			Fr	AP
1	Gallic acid	Hydroxybenzoic acids	< LOQ	1.18 ± 0.09
2	Protocatechuic acid		0.39 ± 0.01	4.20 ± 0.30
3	*p*-Hydroxybenzoic acid		< LOQ	1.89 ± 0.09
4	Gentisic acid		0.118 ± 0.003	0.91 ± 0.05
5	Caffeic acid	Hydroxycinnamic acids	0.57 ± 0.02	4.40 ± 0.30
6	*p*-Coumaric acid		7.50 ± 0.30	9.50 ± 0.60
7	Ferulic acid		0.63 ± 0.02	1.83 ± 0.09
8	Rosmarinic acid		0.030 ± 0.001	0.154 ± 0.009
9	5-*O*-Cafeoylquinic acid (chlorogenic acid)		29.10 ± 0.90	47.00 ± 4.00
10	Rutin (quercetin-3-*O*-rutinoside)	Flavonol glycoside	0.008 ± 0.000	0.28 ± 0.02
11	Kaempferol 3-*O*-glucoside (astragalin)		0.70 ± 0.02	7.00 ± 0.40
12	Quercetin 3-*O*-glucoside		2.82 ± 0.08	4.6 ± 0.3
13	Naringin	Flavanone glycoside	0.23 ± 0.01	< LOQ
14	Eriodictyol	Flavanone	0.046 ± 0.001	0.072 ± 0.003
15	Naringenin		0.061 ± 0.002	0.059 ± 0.004
16	Vitexin (apigenin-8-*C*-glucoside)	Flavone glycoside	0.264 ± 0.008	0.66 ± 0.03
17	Luteolin	Flavone	3.08 ± 0.09	2.02 ± 0.10
18	Apigenin		2.19 ± 0.06	1.13 ± 0.06
19	Hispidulin		0.91 ± 0.03	0.34 ± 0.02
20	Cirsimaritin		0.027 ± 0.001	0.024 ± 0.001

LOQ – limit of quantification.

Besides these compounds, the literature showed that many other constituents have been identified and isolated from *Laserpitium* representatives [15, 23, 24]. Milosavljević [13] published that many sesquiterpene lactones (guaianolide, laserpitin, acetylisomontanolide, silerolide, tarolide, etc.) were obtained from wild-growing *L. siler*, *L. marginatum*, *L. latifolium*, and *L. alpinum* from Serbia. Sesquiterpene lactones were also isolated from *L. siler* from Slovenia, in addition to some phytosterols and phenylpropane derivatives [14]. Most sesquiterpene lactones of *Laserpitium* genus belong to the guanolide type and are derivatives of slovanolide [19]. The endemic *L. zernyi* and *L. ochridanum* from the Balkan Peninsula possess the latifolone [77], which was previously identified in *L. siler* from Serbia [20]. In the study of Popović [21], the phenylpropanoids latifolon and laserin were the main compounds isolated from the chloroform extract of *L. latifolium*, while the chloroform extract of *L. garganicum* roots from Sardinia [23], served for isolation of the phenylpropane derivatives and sesquiterpene lactones. Furthermore, some coumarins [31] and aliphatic ketones were also obtained from *Laserpitium* species (*L. archangelica*, *L. siler*, *L. latifolium*) [18].

3.4. Antioxidant Capacity Measurements

The antiradical activity of *L. siler* was demonstrated by performing three antioxidant tests and the results were compared with corresponding standards (Table 4). Overall, the MeOH extract of AP can be considered the most potent antioxidant. In contrast, the methylene chloride samples showed no radical scavenging activity in the applied tests. Other investigated samples showed somewhat lower, moderate or weak activity compared to the commercial antioxidants used. In the DPPH assay, both MeOH extracts showed strong activity compared to the BHA standard. Ethyl acetate extracts had the lowest effect according to the IC_{50} values. Similar results were obtained in the ABTS test, where both the methanolic and ethanolic extracts of aerial parts were the most effective, while the Fr EtOAc extract

had the lowest measured activity. In the BBT assay, the methanolic extract of aerial parts and both ethanolic extracts best inhibited the formation of $COO^{\bullet}$ radicals and were most effective in preventing β-carotene bleaching. In combination with other antioxidant results, the high IC_{50} value obtained in this test for the ethyl acetate extract of the aerial parts indicates the lowest antiradical potential of this sample.

Table 4. Antioxidant results of *L. siler* extracts (mean ± SD)

***L. siler* Es/ Sds**		**DPPH (C=0.25-2 mg/mL) IC_{50} (mg/mL)**	**ABTS (C=1 mg/mL) mg vit.CE/g DE**	**BBT (C=1-12.5 mg/mL) IC_{50} (mg/mL)**
Fr	MeOH	0.713 ± 0.003^{a}	0.500 ± 0.023^{b}	10.702 ± 2.210^{c}
	EtOH	1.461 ± 0.018^{c}	0.233 ± 0.013^{a}	6.707 ± 0.014^{b}
	CH_2Cl_2	ND*	ND	ND
	EtOAc	9.638 ± 1.434^{d}	0.177 ± 0.020^{a}	8.165 ± 0.995^{b}
AP	MeOH	0.517 ± 0.001^{a}	0.609 ± 0.020^{b}	5.132 ± 0.033^{b}
	EtOH	1.17 ± 0.016^{c}	0.516 ± 0.039^{b}	6.779 ± 0.251^{b}
	CH_2Cl_2	ND	ND	ND
	EtOAc	8.195 ± 0.846^{d}	ND	14.045 ± 0.810^{c}
Sds	Vitamin C	0.031 ± 0.011^{a}		
	BHA	0.130 ± 0.012^{a}		0.017 ± 0.002^{a}
	BHT			0.019 ± 0.001^{a}
	Quercetin hydrate		2.752 ± 0.004^{c}	

Indicated letters mean significant difference in the same column ($p < 0.05$).
*The activity was not detected.

In our previous study [46], the EtOH extract of *L. ochridanum* AP was the richest in phenolics and flavonoids and exhibited the strongest activity in the DPPH test. In this research, the methanolic extract of the aerial parts displayed the same results for phenolic concentrations and DPPH values. Nevertheless, *L. ochridanum* can be considered as a more potent antioxidant, since all tested samples of this species had a stronger effect than the BHA standard. The most effective *L. siler* extract was four times weaker compared to this standard (Table 4). According to the IC_{50} values, the MeOH extract of *L. siler* AP was the strongest sample in the ABTS assay, while the aqueous extract of *L. ochridanum* was more effective than the methanolic sample in the ABTS assay [46]. Bogucka-Kocka [9] found that between the methanolic and etheric extracts of *L. krapffii* fruits, the former exhibited stronger antioxidant activity. However, the strength of the effect of the two extracts depended on the concentration, which is in agreement with our results. Moreover, the pooled FRAP, DPPH, and OH scavenging results for *L. zernyi* flower and herb methanolic extracts revealed that the flowers were stronger antioxidants [76]. Compared with the values of the methanolic extract of the aerial parts of *L. siler*, the extract of the flowers of *L. zernyi* was a slightly stronger anti-DPPH agent (IC_{50}=0.517 mg/mL and 0.390 mg/mL, respectively). Some constituents of *L. siler* found in our study previously have been shown to neutralize free radicals, such as luteolin 7-O-glucoside and apigenin, which show activity against DPPH radicals at very low concentrations [78, 79]. Chlorogenic acid, previously isolated from methanol extracts of *L. zernyi*, possessed concentration-dependent antioxidant activity [76].

Phytochemicals, especially naturally occurring phenolic compounds, have been shown to have health-promoting effects [80]. Since they show strong antioxidant by protecting the organism from the harmful effects of free radicals formed, they are considered as health protectors [81]. In many representatives of Apiaceae, it was found that the highest polyphenolic amounts dictate the highest antioxidant ability of plants [82, 83]. Thus, it has been shown that antioxidant activity and phenolic content are positively correlated in *Laserpitium* species [7, 9, 46, 76]. The antioxidant activity of *L. siler* found application in cosmetic preparations. The essential oil from roots, leaves, umbels, and seeds is used to maintain the quality of the skin by preventing chronological or photoaging caused by the formation of squalene peroxide free radicals by ultraviolet radiation from sunlight, which triggers lipid peroxidation of the skin. The cosmetic use of this species could be supported by the presence of

phenolics quercetin and rosmarinic acid, which play a protective role in preparations for damaged skin [54]. The heterosides rosmarinic acid and quercetin were found in the methanolic extract of *L. siler*, with higher amounts detected in the aerial parts (Table 2) which demonstrated higher antioxidant activity (Table 4).

3.5. Antimicrobial Activity

The ability of laserwort to control microbial contaminants and pathogens was defined through determined MIC, MBC, and MFC values, which are shown in Tables 5 and 6. The general observation is that the tested samples showed a more efficient impact on bacterial growth than on fungal growth. Furthermore, it was observed that the essential oil was more effective than the extracts, that the G+ strains were more sensitive than the G- strains, and that the aerial parts showed slightly stronger antimicrobial activity than the fruits. Nevertheless, all extracts were less effective than the standards. Only the essential oil showed strong antibacterial potential comparable to streptomycin and ampicillin. Considered individually, the methanolic and ethanolic samples of the fruits showed the strongest effects in the antibacterial test (Table 5). The essential oil was effective at low concentrations, while the highest amount of this sample was used against rod-shaped Gramme-negative *P. aeruginosa*, and this is the only bacterium against which the oil had lower activity than antibiotics. The lowest efficacy was detected for the methylene chloride extract of aerial parts, except for on *B. cereus* (MBC=1.88 mg/mL), while for other bacteria it was only effective at a maximum of 15 mg/mL tested. The MBCs showed that only EtOAc and both alcoholic extracts of aerial parts had similar values to the MBCs of the standards (≈ 3 mg/mL) against *B. cereus* and *S. aureus*. The least affected bacteria were *P. aeruginosa*, followed by *P. mirabilis* and *E. coli*. In contrast, the most affected were *B. cereus*, *M. flavus*, and *L. monocytogenes*.

The antifungal effect of the used extracts was lower than the antibacterial one, as the extracts studied showed lower inhibition of micromiceliar growth (Table 6). The fungicidal effect on the microfungi used was found at a ratio of MFCs=8.5-17 mg/mL. In general, the strongest effects in this assay were achieved at a minimum of 8.5 mg/mL, which was higher than the effective concentrations of the standard fluconazole. These effects were noted for the ethanolic extract of the fruits on the fungus *A. versicolor* and for methylene chloride of the aerial parts on *T. viride*. A methanolic extract of the aerial parts had the best inhibitory effect on fungi with a demonstrated ratio for MFCs of 10 to 17 mg/mL. The lowest fungistatic and fungicidal potential was shown by the ethyl acetate extract of aerial parts, which was active against all micromycetes at the highest concentration used. The most resistant fungus was *A. niger* (MFCs=17 mg/mL). *Aspergilus* species, *A. versicolor* and *A. fumigatus* proved to be slightly more sensitive fungal strains in this test. Nevertheless, *L. siler* was not comparable to mycotic fluconazole in the fungicidal treatment of eight selected micromycetes (Table 6).

In general, Apiaceae species most often exhibit moderate antimicrobial properties [83]. The microdilution method used in the present work to determine the antimicrobial potential of *L. siler* showed that this species has lower activity against the microbial strains used compared to previous results for Macedonian *L. ochridanum* [46]. In both studies, we indicated stronger antibacterial than antifungal potential. Also, the essential oils were the strongest antimicrobial agents, showing efficacy at the level of the standards used. *L. siler* oil reduced the growth of the bacteria used the most, while *L. ochridanum* oil was the most successful in inhibiting micelle growth. MeOH and EtOH extracts were the most prominent compared to antibiotics, just as in this study (Table 5). However, *L. ochridanum* extracts showed slightly better antifungal effect with lover MFC values. Another similarity is that *B. cereus* and *A. versicolor* were the most susceptible strains [46]. Lencher [11] reported that the hexane and dichloromethane extracts of *L. siler* roots expressed activity against fast growing mycobacteria at a concentration of MIC=64 mg/mL. Compared to the aerial parts and fruits presented here, the roots of this species have better antimicrobial activity. Dastan [7] tested different Es and EOs of *L. carduchorum* for their antibacterial potential and the tested oils showed modest to strong inhibitory activity on the bacteria used, except for *P. aeruginosa*, which was the most resistant bacterium, similar to this study. In their work, *B. cereus* was also the most sensitive microorganism (MIC=7.5 mg/mL). Compared to our results, *L. siler* had higher activity on this microorganism (MBC=0.47-3.75 mg/mL for Es and MBC=0.0063 mg/mL for EO). The essential oil of *L. carduchorum* in ripening phase reduced the

growth of *S. aureus* and *E. coli* slightly weaker than ampicillin, as well as that of *L. siler* EO (Table 5). The strong effect of the oil was associated with α-pinene and limonene as major constituents of the oil [7]. The general conclusion is that the significant antimicrobial potential of *Laserpitium* oils is due to the synergy of the main terpenoids of the oils [39]. It has been published that oils from leaves and flowers of *L. zernyi* exhibit strong inhibition of some microorganisms [41]. The antifungal activity of *L. garganicum* EO was observed on *T. viride*, *Penicillium pinophilum, P. chrisogenum*, and *A. niger* [39]. *A. niger* was stopped by the oil at MIC of 0.125 L/mL and above, with the same level sensitivity to all applied concentrations. Our results indicated that the fungus *A. niger* was inhibited by all extracts only at the maximum concentration of 17 mg/mL, so the oil of *L. garganicum* significantly stronger reduced *A. niger* growth. In this test, the aerial part had a slightly greater antimicrobial potential than the fruits. A similar conclusion was published by Popović [76], who observed that the extract of the aerial parts inhibited the growth of bacteria more effectively than the flower extract. The authors attributed the bactericidal and fungicidal effects of the methanolic extracts of AP to the detected chlorogenic acid and flavones. However, it was assumed that polyphenols were not the only contributors to the potential of this species [76]. As the main compounds of *L. siler*, sesquiterpene lactone showed promising inhibitory activity on some mycobacteria. It was concluded that in addition to sesquiterpene lactones, falcarinol and other polyacetylenes probably contribute to the antimicrobial activity [11].

Regarding the essential oils of *Laserpitium* species, they were found to have strong antifungal effects mainly due to the high content of oxygenated components [39], while the monoterpenes showed different antimicrobial effects [41]. The oils of the pinene chemotype of *L. latifolium* and *L. ochridanum* showed comparable antibacterial activity to the positive control thymol, which was also shown for the oil of *L. siler* in our previous [49] and in the present study (Table 5). Indeed, all tested oils demonstrated *in vitro* antimicrobial activity against a wide range of bacterial and fungal strains, which can be attributed to a high content of α-pinene and/or β-pinene [39, 40, 49, 84]. Limonene, which was present at over 4% in our oil sample [49], is also considered to be a strong antifungal principle [40]. Another compound, terpinen-4-ol, present at more than 2% in the oil of *L. siler*, is also a very potent antimicrobial agent [41]. The main terpenoids of our previously analysed *L. siler* are also the originators of many other preventive/therapeutic properties. For example, α- and β-pinene have anticoagulant, antitumor, antimalarial, antioxidant, and anti-inflammatory effects [84], while limonene possesses antioxidant, anticancer, toxic, and antiparasitic properties [85]. Limonene or phellandrene has been reported to possess insecticidal activity and to be toxic to stored product species [42]. Verbenone is an oxygenated monoterpene with allelochemical activity in flowering plants [86]. This ketone has promising antidiabetic potential as it inhibits α-amylase and α-glucosidases *in vitro* and improves glucose uptake and inhibits haemoglobin glycation *in vivo* [87]. As mentioned, *L. siler* essential oil has been recommended for use in cosmetic preparations. The administration and cosmetic use of *L. siler* oil has been patented due to its strong antioxidant role in preventing skin ageing or the development of skin/scalp disorders caused by oxidative stress [88]. It is also proposed as a novel agent for the prevention and/or treatment of skin diseases associated with constriction of the cutaneous capillary circulation due to its abundant and effective compounds such as perillaldehyde, α-pinene, chamazulene, etc. [89]. However, a study on essential oils and decoctions of eight aromatic plants, including Apiaceae species, suggests that the compounds present in the decoctions (high phenolic content) are more active than the essential oils (mainly monoterpenoids) in terms of free radical scavenging, anti-inflammatory (5-lipoxygenase), and anti-acetylcholinesterase activities [90]. Since our essential oil exhibited weak antiradical activity, further tests in this study were performed only with extracts of the plant.

Table 5. Antibacterial activity of *L. siler* (mean ± SD)

Bacteria/ *L. siler* samples, Sds			***B. cereus***	***M. flavus***	***L. monocytogenes***	***S. aureus***	***E. faecalis***	***E. coli***	***P. aeruginosa***	***P. mirabilis***
Fr	MeOH	MIC	0.47 ± 0.02^{a}	1.25 ± 0.00^{a}	0.47 ± 0.01^{a}	0.94 ± 0.12^{a}	3.75 ± 0.21^{a}	10.00 ± 1.21^{b}	15.00 ± 1.23^{c}	10.00 ± 0.03^{b}
		MBC	0.63 ± 0.03^{a}	3.75 ± 0.11^{a}	1.88 ± 0.07^{a}	2.50 ± 0.03^{a}	7.50 ± 0.32^{b}	15.00 ± 0.33^{c}	16.00 ± 0.88^{c}	15.00 ± 0.11^{c}
	EtOH	MIC	0.94 ± 0.00^{a}	1.25 ± 0.08^{a}	1.25 ± 0.02^{a}	1.88 ± 0.20^{a}	3.75 ± 0.28^{b}	10.00 ± 1.22^{c}	10.00 ± 0.49^{c}	10.00 ± 0.63^{c}
		MBC	1.25 ± 0.11^{a}	2.50 ± 0.12^{a}	1.88 ± 0.24^{a}	3.75 ± 0.30^{a}	7.50 ± 0.99^{b}	15.00 ± 0.14^{c}	15.00 ± 0.05^{c}	15.00 ± 0.96^{c}
	CH_2Cl_2	MIC	1.88 ± 0.24^{a}	7.50 ± 0.84^{c}	5.00 ± 0.00^{b}	7.50 ± 0.11^{c}	5.00 ± 0.37^{b}	3.75 ± 0.03^{b}	7.50 ± 1.03^{c}	10.00 ± 0.12^{d}
		MBC	3.75 ± 0.32^{a}	10.00 ± 0.32^{b}	7.50 ± 0.01^{b}	10.00 ± 0.02^{b}	10.00 ± 0.01^{b}	5.00 ± 0.07^{a}	10.00 ± 0.98^{b}	15.00 ± 0.14^{c}
	EtOAc	MIC	0.94 ± 0.58^{a}	5.00 ± 0.02^{b}	2.50 ± 0.36^{a}	5.00 ± 0.00^{b}	7.50 ± 0.00^{b}	7.50 ± 1.14^{b}	10.00 ± 0.09^{c}	10.00 ± 0.74^{c}
		MBC	1.88 ± 0.06^{a}	7.50 ± 0.25^{b}	5.00 ± 0.36^{a}	10.00 ± 0.78^{b}	15.00 ± 0.03^{c}	15.00 ± 0.77^{c}	15.00 ± 1.11^{c}	15.00 ± 0.36^{c}
AP	MeOH	MIC	0.47 ± 0.08^{a}	1.88 ± 0.05^{a}	0.94 ± 0.47^{a}	1.88 ± 0.20^{a}	5.00 ± 0.16^{b}	10.00 ± 0.68^{c}	10.00 ± 1.11^{c}	10.00 ± 2.01^{c}
		MBC	0.94 ± 0.03^{a}	3.75 ± 0.24^{a}	1.88 ± 0.22^{a}	2.50 ± 0.07^{a}	7.50 ± 0.46^{b}	15.00 ± 0.55^{c}	15.00 ± 0.98^{c}	15.00 ± 1.39^{c}
	EtOH	MIC	0.63 ± 0.11^{a}	3.75 ± 0.09^{a}	1.88 ± 0.08^{a}	1.88 ± 0.00^{a}	3.75 ± 0.33^{a}	10.00 ± 0.14^{b}	10.00 ± 0.17^{b}	10.00 ± 0.16^{b}
		MBC	1.25 ± 0.18^{a}	5.00 ± 0.21^{b}	3.75 ± 0.74^{a}	3.75 ± 0.00^{a}	7.50 ± 0.13^{b}	15.00 ± 0.27^{c}	15.00 ± 0.58^{c}	15.00 ± 0.49^{c}
	CH_2Cl_2	MIC	0.94 ± 0.07^{a}	10.00 ± 0.32^{c}	7.50 ± 0.55^{b}	7.50 ± 0.47^{b}	10.00 ± 0.55^{c}	10.00 ± 0.25^{c}	10.00 ± 1.25^{c}	10.00 ± 0.78^{c}
		MBC	1.88 ± 0.00^{a}	15.00 ± 1.02^{b}	15.00 ± 0.42^{b}	15.00 ± 0.52^{b}	15.00 ± 0.68^{b}	15.00 ± 2.02^{b}	15.00 ± 0.77^{b}	15.00 ± 1.47^{b}
	EtOAc	MIC	0.31 ± 0.13^{a}	7.50 ± 1.14^{b}	5.00 ± 0.10^{b}	7.50 ± 0.30^{b}	7.50 ± 0.54^{b}	3.75 ± 0.31^{a}	0.94 ± 0.09^{a}	10.00 ± 0.23^{c}
		MBC	0.47 ± 0.01^{a}	10.00 ± 2.01^{bc}	7.50 ± 0.01^{b}	10.00 ± 0.01^{c}	10.00 ± 0.36^{c}	5.00 ± 0.74^{a}	1.25 ± 0.20^{a}	15.00 ± 0.14^{d}
	EO	MIC	0.0032 ± 0.00^{a}	0.006 ± 0.00^{a}	0.21 ± 0.03^{a}	0.85 ± 0.01^{a}	0.42 ± 0.01^{a}	0.21 ± 0.02^{a}	6.75 ± 0.01^{b}	0.85 ± 0.47^{a}
		MBC	0.0063 ± 0.00^{a}	0.0135 ± 0.02^{a}	1.35 ± 0.11^{a}	6.75 ± 0.09^{b}	0.85 ± 0.01^{a}	0.42 ± 0.01^{a}	27.00 ± 1.00^{c}	1.69 ± 0.02^{a}
Streptomycin		MIC	0.24 ± 0.04^{a}	0.12 ± 0.02^{a}	0.47 ± 0.08^{a}	1.50 ± 0.33^{b}	0.50 ± 0.16^{b}	0.12 ± 0.00^{a}	0.06 ± 0.00^{a}	0.24 ± 0.06^{a}
		MBC	0.47 ± 0.09^{a}	0.24 ± 0.05^{a}	0.94 ± 0.10^{a}	3.00 ± 1.05^{b}	1.50 ± 1.22^{b}	0.24 ± 0.01^{a}	0.24 ± 0.02^{a}	0.47 ± 0.10^{a}
Ampicillin		MIC	0.24 ± 0.11^{a}	0.47 ± 0.14^{a}	0.31 ± 1.12^{c}	2.00 ± 0.17^{b}	0.12 ± 0.03^{a}	0.06 ± 0.01^{a}	0.03 ± 0.00^{a}	0.06 ± 0.02^{a}
		MBC	0.47 ± 0.13^{a}	0.94 ± 0.00^{a}	0.60 ± 0.78^{c}	3.00 ± 0.36^{b}	0.24 ± 0.07^{a}	0.12 ± 0.00^{a}	0.06 ± 0.01^{a}	0.12 ± 0.04^{a}

Indicated letters mean significant difference in the same row ($p < 0.05$).

Table 6. Antifungal activity of *L. siler* (mean ± SD)

Fungi/ *L. siler* samples, Sd			***T. viride***	***P. funiculosum***	***P. ochrochloron***	***P. verrucosum var. cyclopium***	***A. ochraceus***	***A. versicolor***	***A. fumigatus***	***A. niger***
Fr	MeOH	MIC	12.00 ± 0.32^{c}	12.00 ± 0.41^{c}	12.00 ± 0.21^{c}	4.25 ± 0.11^{a}	12.00 ± 0.10^{c}	8.50 ± 0.94^{b}	8.50 ± 0.07^{b}	8.50 ± 0.77^{b}
		MFC	17.00 ± 0.02^{b}	17.00 ± 0.96^{b}	17.00 ± 0.20^{b}	12.00 ± 0.32^{a}	17.00 ± 1.12^{b}	12.00 ± 0.00^{a}	17.00 ± 1.32^{b}	17.00 ± 0.96^{b}
	EtOH	MIC	12.00 ± 1.06^{c}	12.00 ± 0.55^{c}	12.00 ± 0.00^{c}	12.00 ± 0.00^{c}	12.00 ± 0.86^{c}	6.00 ± 0.07^{a}	8.50 ± 0.23^{b}	12.00 ± 0.22^{c}
		MFC	17.00 ± 0.11^{c}	17.00 ± 0.64^{c}	17.00 ± 1.25^{c}	17.00 ± 0.96^{c}	17.00 ± 0.77^{c}	8.50 ±0.78^{a}	12.00 ± 0.69^{b}	17.00 ± 0.87^{c}
	CH_2Cl_2	MIC	8.50 ± 0.78^{a}	12.00 ± 0.75^{b}	12.00 ± 1.32^{b}	12.00 ± 1.14^{b}	12.00 ± 0.87^{b}	12.00 ± 0.03^{b}	8.50 ± 0.04^{a}	12.00 ± 0.10^{b}
		MFC	12.00 ± 0.03^{a}	17.00 ± 0.85^{b}	17.00 ± 1.11^{b}	17.00 ± 0.78^{b}	17.00 ± 0.00^{b}	17.00 ± 0.50^{b}	12.00 ± 1.14^{a}	17.00 ± 1.01^{b}
	EtOAc	MIC	12.00 ± 0.99^{b}	12.00 ± 0.00^{b}	12.00 ± 1.62^{b}	12.00 ± 0.69^{b}	12.00 ± 0.68^{b}	12.00 ± 0.50^{b}	8.50 ± 0.50^{a}	12.00 ± 0.08^{b}
		MFC	17.00 ± 0.16^{b}	17.00 ± 0.48^{b}	17.00 ± 2.21^{b}	17.00 ± 0.08^{b}	17.00 ± 0.22^{b}	17.00 ± 0.09^{b}	12.00 ± 0.03^{a}	17.00 ± 0.33^{b}
AP	MeOH	MIC	8.50 ± 0.22a	8.50 ± 0.36a	10.00 ± 0.18b	12.00 ± 0.74c	8.50 ± 0.36a	10.00 ± 0.87b	10.00 ± 0.98b	12.00 ± 2.41c
		MFC	15.00 ± 1.25b	10.00 ± 0.47a	15.00 ± 1.00b	15.00 ± 0.65b	12.00 ± 0.66a	15.00 ± 1.00b	17.00 ± 0.93c	17.00 ± 0.22c
	EtOH	MIC	10.00 ± 1.80a	8.50 ± 0.78a	12.00 ± 0.35b	12.00 ± 0.44b	12.00 ± 0.02b	12.00 ± 0.67b	10.00 ± 0.87a	15.00 ± 0.17c
		MFC	15.00 ± 0.54b	10.00 ± 0.23a	15.00 ± 2.03b	17.00 ± 1.23b	17.00 ± 2.01b	17.00 ± 0.66b	12.00 ± 0.15a	17.00 ± 0.11b
	CH_2Cl_2	MIC	6.00 ± 0.50^{a}	6.00 ±0.30^{a}	12.00 ± 0.06^{c}	6.00 ± 0.78^{a}	12.00 ± 0.88^{c}	8.50 ± 1.00^{b}	12.00 ± 0.41^{c}	15.00 ± 0.01^{d}
		MFC	8.50 ± 0.10^{a}	12.00 ± 0.77^{b}	17.00 ± 1.52^{c}	12.00 ± 0.02^{b}	17.00 ± 0.04^{c}	12.00 ± 0.02^{b}	17.00 ± 1.23^{c}	17.00 ± 0.98^{c}
	EtOAc	MIC	12.00 ± 0.78^{b}	12.00 ± 0.56^{b}	12.00 ± 0.03^{b}	12.00 ± 0.08^{b}	12.00 ± 0.54^{b}	8.50 ± 0.63^{a}	12.00 ± 0.76^{b}	15.00 ± 1.52^{c}
		MFC	17.00 ± 0.55^{b}	17.00 ± 0.69^{b}	17.00 ± 0.74^{b}	17.00 ± 0.28^{b}	17.00 ± 0.05^{b}	12.00 ± 0.01^{a}	17.00 ± 0.96^{b}	17.00 ± 0.57^{b}
Fluconazole		MIC	1.00 ± 0.12^{d}	0.25 ± 0.00^{b}	1.00 ± 0.07^{d}	0.20 ± 0.36^{a}	0.50 ± 0.04^{c}	0.13 ± 0.34^{a}	0.50 ± 0.52^{c}	0.25 ± 0.01^{b}
		MFC	1.50 ± 0.13^{c}	0.50 ± 0.09^{a}	1.50 ± 0.10^{c}	0.30 ± 0.63^{a}	1.00 ± 0.00^{b}	0.50 ± 0.11^{a}	1.00 ± 0.09^{b}	1.00 ± 0.04^{b}

Indicated letters mean significant difference in the same row ($p < 0.05$).

3.6. Antibiofilm and Antipyocyanin Activity

Antibiofilm and antipyocyanin activities were determined using *P. aeruginosa* PAO1 as the biomonitoring organism and half of the previously determined MICs for extracts in the antibacterial assay. In both tests, the effects obtained were compared with the activities of ampicillin and streptomycin. The results shown in Table 7 are expressed as percentages. The strongest inhibition of biofilm synthesis was observed after addition of subMIC of the methanolic extract of AP. Its activity was twice as strong as the activity of the antibiotics used. However, the best reduction in biofilm formation was achieved by streptomycin. Similar results were registered for methanolic and ethanolic fruit extracts. Ethyl acetate extracts showed activity in the range of ampicillin. The ability of the extracts to reduce or stop the production of the toxic pyocyanin varied among the samples. The synthesis of this toxin was most effectively suppressed by MeOH extracts of Fr (19.36%) and AP (36.45%). In contrast, the least inhibition was measured in the presence of methylene chloride from fruits, but still more effective than the two standards used (Table 7). Compared to the effects of the ethanolic extract of *L. ochridanum*, which reduced the biofilm formation of *P. aeruginosa* in a range from 8.63% to 63.88%, the ethanolic extract of *L. siler* was less effective, reducing the biofilm formation by the fruits and the aerial parts by 30.36% and 41.01%, respectively. Previously, the aqueous extract of *L. ochridanum* had shown no effect on biofilm formation, but all *L. siler* samples had the activity on this virulence factor. It was concluded that fewer polar metabolites were active in reducing pyocyanin synthesis [46]. The inhibitory ability of *L. latifolium* extracts on biofilm formation of clinical isolates of *Candida albicans* and *Candida krusei* was clear. All the isolated compounds of these extracts had higher potential in preventing biofilm synthesis of the candidas used, but isomontanolide was the most active, followed by laserpitin and montanolide, and all compounds showed more pronounced potential in inhibiting biofilm than fluconazole [24]. The production of pyocyanin was lower in the presence of ethanolic (23.46%) and aqueous (18.07%) extracts of *L. ochridanum*, and both extracts prevented pyocyanin production more successfully than antibiotics [46]. Their activity was similar to that of *L. siler* MeOH extract, but other samples of *L. siler* had less effect on pyocyanin than *L. ochridanum* (36.45%-89.19%).

Table 7. Biofilm and pyocyanin production of PAO1 in addition of *L. siler*

***L. siler* Es/Sds**		**Biofilm formation (%) 0.5 MIC**	**Pyocyanin production (%) 0.5 MIC**
Fr	MeOH	41.26 ± 0.07^{b}	19.36 ± 0.12^{a}
	EtOH	41.01 ± 0.44^{b}	52.54 ± 0.72^{c}
	CH_2Cl_2	34.54 ± 0.16^{c}	89.19 ± 0.47^{d}
	EtOAc	29.53 ± 0.42^{b}	45.09 ± 7.19^{c}
AP	MeOH	48.71 ± 0.52^{c}	36.45 ± 0.59^{b}
	EtOH	30.36 ± 0.07^{bc}	39.24 ± 4.82^{b}
	CH_2Cl_2	33.22 ± 1.83^{c}	62.26 ± 7.50^{d}
	EtOAc	23.5 ± 0.76^{b}	72.08 ± 0.89^{d}
Sds	Ampicillin	84.81 ± 2.62^{c}	137.00 ± 5.90^{e}
	Streptomycin	21.56 ± 1.23^{b}	132.48 ± 3.40^{e}

Indicated letters mean significant difference in the same column ($p < 0.05$).

3.7. Anti- Anti-inflammatory Activity

The initial screening of anti-inflammatory bioactivity was performed by determining the prevention of denaturation of BSA. The calculated results in percent are listed in Table 8. All samples were tested at C=0.1 mg/mL. The range of albumin denaturation protection was from 75.86% for the ethyl acetate extract of the fruits to 95.08% for the ethanolic extract of aerial parts. The most effective agents against denaturation were ethanolic extracts active at the level of the anti-inflammatory agent diclofenac. The evaluation of anti-inflammatory plants includes the BSA denaturation assay as one of the first proposed *in vitro* screenings, designed to measure the effect on heat destabilised albumin [22,

32]. Heating of albumin can lead to the disintegration of protein structure by breaking the bonds between molecules. It has been shown that denaturation of BSA leads to the formation of protein aggregates different from those of the parent albumin. These aggregates also occur spontaneously at low concentrations in untreated albumin and have been shown to act as antigens in the development of immunoinflammatory diseases [9]. It has been demonstrated that some plants can slow down the thermal degradation of BSA. Since laserwort species are used in traditional medicine to cure inflammation and infection, it is believed that the main metabolites are responsible for their healing properties [22, 32]. In a recent study [82], a strong positive correlation was found between the total flavonoid content and the rate of inhibition of protein denaturation, and the authors hypothesized that flavonoids are the key anti-inflammatory metabolites. In addition, the total phenolic and flavonoid contents in some other Apiaceae species and their anti-inflammatory potential were strongly positively correlated. In particular, the authors pointed out the strong inhibition by parsley extracts, which were as effective as diclofenac sodium. Moreover, parsley most markedly prevented hypotension-induced lysis and stabilized the membrane of erythrocytes, which was one of the tests used to determine anti-inflammatory potential [82]. This statement can be supported by the results of Popović [76], who demonstrated that flavonoids inhibit the enzymes phospholipase A2 and cyclooxygenase and thus the synthesis of inflammatory mediators, which contribute to the inhibition of inflammation, allergy or fever. According to the results of Ranjbar [91], celery root extracts possessed potent anti-inflammatory and antinociceptive effects after intraperitoneal injection in mice and had the highest flavonoid content (0.0625 mg QHE/ g DE). Compared with the flavonoid content of the most potent anti-inflammatory laserworth extract (35.19 mg QHE/ g DE), the flavonoid content of celery was significantly lower. In our study, the ethanolic extract of the aerial parts has a very high potential comparable to that of diclofenac, and it had high concentrations of phenols and coumarins (Tables 2 and 8). Many polyphenols isolated from laserwort also showed anti-edematous activity [92].

Table 8. Anti-inflammatory activity of *L. siler* on BSA denaturation.

***L. siler* Es (C=0.1 mg/mL)**		**Anti-inflammatory activity (%)**
Fr	MeOH	81.45 ± 1.40^{b}
	EtOH	91.06 ± 0.00^{d}
	CH_2Cl_2	86.05 ± 0.00^{c}
	EtOAc	75.86 ± 1.40^{a}
AP	MeOH	85.61 ± 0.00^{c}
	EtOH	95.08 ± 6.98^{d}
	CH_2Cl_2	84.62 ± 1.40^{b}
	EtOAc	84.11 ± 1.40^{b}
Sd	Diclofenac	95.60 ± 0.00^{d}

Indicated letters mean significant difference in the same column ($p < 0.05$).

In the carrageenan-induced inflammation model, *L. zernyi* MeOH extracts possessed the ability to reduce this inflammatory symptom [76]. The authors concluded that luteolin 7-O-glucoside, apigenin 7-O-glucoside, chlorogenic acid, and polyphenols are generally carriers of high antioxidant or anti-edematous activity. According to Table 3, chlorogenic acid is the most important compound in our methanolic samples, especially in the aerial parts, and the phenolics and flavonoids are the highest in methanolic and ethanolic extracts (Table 2), which support the results obtained previously [76]. Indeed, chlorogenic acid has been shown to inhibit inflammatory mediators and manifest antioxidant and anticarcinogenic effects [81]. Trilobolide and archangelolide present in *Laserpitium* species have confirmed remarkable immunobiological potential [22], and sesquiterpene lactone trilobolide and daucan ester have been obtained from *L. siler* and *L. archangelica* as potent activators of cytokine secretion [9]. Bioactive coumarins inhibit cyclooxygenases at comparable potency to ibuprofen, aspirin, and naproxen [34]. The coumarin content was highest in the EtOH extract of *L. siler* AP and probably also contributed to the overall potential of this extract in preventing albumin desintegration. The mechanism of action of anti-inflammatory drugs has not been clearly defined [9], but it has been

suggested that most of the anti-inflammatory activity is associated with the polar compounds. These results, as well as other previous and recent publications, reinforce the folk use of Apiaceae species against inflammatory ailments.

3.8. Enzyme Inhibitory Activity

Inhibition of targeted disease-related enzymes by plants as a more economical and effective alternative is considered as one of the main therapeutic strategies for the treatment of banded health complications and disorders [93]. This study was carried out to test the efficacy of *L. siler* in regulating the activity of enzymes that are important in therapeutic practice. The summarized results of these tests are presented in Table 9. According to the available data, the inhibition of selected enzymes is dependent on concentration. The tested plant possessed the highest activity on α-glucosidase, while the lowest effect was observed on tyrosinase activity. The results of acetylcholinesterase inhibition assay presented separately, showed that the aerial parts had higher activity than the fruits. The best result was obtained for methanolic and ethyl acetate extracts of AP, which caused suppression of the enzyme with IC_{50} values of 1.76 and 1.84 mg/mL, respectively. Still, both samples were less active than standard galantamine (Table 9). As for the reduction of tyrosinase activity, AP showed a slightly stronger effect on this enzyme, with the ethanolic extract appearing to be the most effective. The most effective extract against α-amylase activity proved to be ethyl acetate extract of aerial parts which showed 3.5 times less activity than the blood sugar-lowering agent acarbose. Again, the samples obtained from the aerial parts were more effective. As mentioned above, the highest potential of the tested extracts was found against α-glucosidase activity. As in the previous test, the ethyl acetate extract of aerial parts expressed a significant reduction of this enzyme. This was achieved with a concentration twice that of the effective concentration of acarbose. The fruit and aerial parts samples expressed similar activity in this assay.

Table 9. Inhibitory activity of *L. siler* on selected enzymes (IC_{50} mg/mL)

***L. siler* Es (C=0.25-5 mg/mL) Sds (C= 0.00625-1 mg/mL)**		**AChE**	**Tyr**	**Amy**	**Glu**
Fr	MeOH	5.954 ± 0.024^{c}	5.522 ± 0.516^{b}	6.252 ± 0.050^{b}	4.250 ± 0.181^{d}
	EtOH	3.671 ± 0.014^{b}	7.154 ± 0.365^{c}	9.239 ± 0.285^{c}	1.145 ± 0.091^{b}
	CH_2Cl_2	2.605 ± 0.108^{b}	7.168 ± 0.145^{c}	4.956 ± 0.080^{b}	1.491 ± 0.069^{b}
	EtOAc	2.510 ± 0.002^{b}	6.424 ± 0.036^{b}	9.174 ± 0.168^{c}	2.737 ± 0.000^{c}
AP	MeOH	1.760 ± 0.344^{a}	4.973 ± 0.100^{b}	4.189 ± 0.011^{b}	3.454 ± 0.063^{c}
	EtOH	2.223 ± 0.036^{b}	4.891 ± 0.089^{b}	5.831 ± 0.076^{b}	1.985 ± 0.154^{b}
	CH_2Cl_2	4.939 ± 0.001^{c}	9.909 ± 0.201^{c}	4.514 ± 0.045^{b}	5.655 ± 0.159^{d}
	EtOAc	1.837 ± 0.038^{a}	5.690 ± 0.076^{b}	2.919 ± 0.006^{a}	0.479 ± 0.016^{a}
Sds	Galantamine	0.130 ± 0.005^{a}			
	Kojic acid		0.180 ± 0.001^{a}		
	Acarbose			0.832 ±0.057^{a}	0.296 ± 0.013^{a}

Indicated letters mean significant difference in the same column ($p < 0.05$).

Inhibition of acethyl- and butyrylcholinesterase, tyrosinase, α-amylase, and α-glucosidase is one of the most important approaches for the treatment of Alzheimer's (AD) and Parkinson's diseases, as well as for the regulation and control of type 2 diabetes mellitus. Since the aforementioned enzymes are linked with the development and progression of these diseases, and commercial drugs sometimes have severe side effects, various plant extracts and metabolites with inhibitory activity are being investigated as potential new therapeutics of natural origin [94]. AChE has been associated with the development of adverse neurological conditions and the pathogenesis of neurodegenerative illnesses such as AD [83]. The multiple biological activities of polyphenols and their ability to prevent degenerative conditions are well documented and strongly supported by some studies [92]. Therefore,

many researchers have also studied the representatives of Apiaceae in order to find a new source of active compounds against the mentioned health problems. Zengin [83] published that the most potent agent against neurodegenerative enzymes was *Chaerophyllum macrospermum*, which strongly inhibited AChE (4.53 mg GE /g DE) and BChE (3.22 mg GE /g DE). The present findings revealed that the strongest *L. siler* extracts were twice as potent as the anti-Alzheimer agents in this test (Table 9). In the work of Bahadori [95], the dichloromethane extract of *Prangos gaubae* from the parsley family possessed the highest neuroprotective effects against these two enzymes. The dichloromethane extract from the aerial parts of *L. siler* was also twice as potent as the methanol extract, but the extracts obtained from the fruits showed opposite results, probably due to the different chemical composition of the active polyphenolics. n-Hexane extracts from *P. ferulacea* inhibited AChE activity most effectively, and the furanocoumarin heraclenin isolated from this fraction showed similarly high activity [34]. Some Umbelliferae species such as *Ferula* plants are traditionally used as nerve remedies and are very rich in coumarins and terpenoids. Terpenoids present in Apiaceae species are effective against AChE activity. The monoterpenes carvacrol, α-pinene, and β-pinene isolated from essential oils of Apiaceae species inhibited AChE. However, the strongest carvacrol had about 130 times less AChE inhibition than carbaryl [96]. The monoterpenoid tschimgine was active against AChE and the sequiterpene acorenone B, previously found in *Chaerophyllum hirsutum*, inhibited both AChE and BChE at low concentrations [83]. The neuroprotective effect of our samples was evident at much higher concentrations compared to these compounds.

Among numerous enzymes, tyrosinase is the most important enzyme associated with melanogenesis and skin pigmentation in humans. This physiological process is important for protection against the harmful and carcinogenic effects of sun exposure. When melanin is overproduced, the resulting accumulation of this pigment leads to various physiological skin conditions that can eventually lead to skin diseases. The activity of this enzyme has been linked to the development of Parkinson's disease. It is also crucial for the browning of vegetables, fruits and mushrooms, which is the second undesirable phenomenon caused by this enzyme, along with hyperpigmentation of human skin. Therefore, many plants and their metabolites are being studied as depigmenting, skin lightening and anti-tanning agents in the pharmaceutical-cosmetic, medical or food industries. Some flavonoids and their derivatives have been shown to be very potent inhibitors of this enzyme, and a strong correlation between its inhibition and the decrease in melanin production in melanocytes has been established. So far, many natural compounds did not show strong effects on tyrosinase. The methanol extract of *Prangos ferulacea* was assessed with six other Apiaceae species against this enzyme and showed the strongest potential against tyrosinase with an IC_{50} value of 131.97 mg KAE /g DE [83]. Table 9 shows that *L. siler* can be considered as a weak anti-tyrosinase agent compared to kojic acid. The same is true for *P. ferulacea* and other extracts tested, as the ratio of their activity was significantly lower than the activity of the standard.

The common disease diabetes mellitus is fought in patients with antidiabetic drugs that delay the degradation, digestion and absorption of starch and reduce the level of sugar in the blood. The results of their effectiveness are the control of postprandial glucose levels and the prevention of hyperglycemia [83, 94]. This is achieved by inhibiting the activities of certain digestive enzymes to an optimal extent [93]. Among all enzymes, pancreatic α-amylase and digestive α-glucosidase are the most important [94], and common methods for determining *in vitro* antidiabetic activity include inhibition of these two enzymes [80]. *Laserpitium* species have not been tested for the activity of these enzymes, but some Apiaceae species showed different potentials when it comes to diabetes control. In the study of Deveci [97], the hexane extract of *Ferula elaeochytris* (Apiaceae) showed the best *in vitro* activity on the enzyme α-glucosidase among seven medicinal plants tested from different families. In addition, the authors concluded that hexane extracts of these plants exhibited more notable α-amylase inhibitory activity than methanolic extracts and that they responded to a greater extent to α-Amy than to α-Glu. Laserwort samples expressed better activity on the enzyme α-glucosidase than on α-amylase, but similar to *Ferula elaeochytris*, the highest inhibition of these digestive enzymes was registered for the least polar ethyl acetate extract of the fruits. The dichloromethane extract from the roots of *Ferulago bracteata* had comparable activity on α-glucosidase as acarbose (IC_{50}=0.95 mg/mL) [33]. Compared to our results, dichloromethane extract

from the aerial parts of *L. siler* had slightly lower inhibitory activity than *F. bracteata* (IC_{50}=1.49 mg/mL), while the fruits were the least active and thus not comparable to acarbose activity (Table 9). Phenolic compounds have been shown to be effective antidiabetic agents, and intake of phenolics via food/beverage/food supplements has been recommended as preventive against the development of type 2 diabetes [80]. Various phenolic compounds such as hydroxycinnamic acids, flavan-3-ols, flavanols, or anthocyanins inhibit these enzymes [93]. The coumarins of Apiaceae plants have also been suggested as potential antidiabetic agents, the uptake of which leads to better absorption of glucose from the blood. Karakaya [33] published that the coumarins osthole, imperatorin, prantschimgin, peucedanol-20 benzoate, bergapten, xanthotoxin, umbelliferone, and grandivitinol showed moderate to higher activity compared to acarbose, but none of them showed significant activity against α-amylase. In this study, the ethyl acetate extract of the aerial parts with significant amounts of phenols and coumarins had the strongest hypoglycemic effect. The amounts of these metabolites were higher than in most samples studied, except for the methanolic and ethanolic extracts AP. The effect on α-amylase was also less pronounced (Table 2).

4. Conclusion

Considering the results obtained, *L. siler* is rich in phenolic compounds, especially alcoholic extracts of the aerial parts. Among the twenty compounds detected, the phenylpropanoid 5-O-cafeoylquinic acid was the most important dominant compound, followed by the phenolic *p*-coumaric acid. The methanolic extract of aerial parts stood out as the most potent antioxidant in all the testes used, while the essential oil proved to be effective against the growth of several bacterial species, as potent as the antibiotics used. Among the extracts, the alcoholic samples of the fruits were the most potent antibacterial agents, which was further enhanced by the strong suppression of bacterial biofilm formation by these samples. Production of the toxin pyocyanin by *P. aeruginosa* was reduced the most in percentage terms by the methylene chloride extract of the fruits. In contrast, *L. siler* showed no significant inhibition of the micromycetes used. According to the BSA test, both ethanolic extracts can be considered suitable for further anti-inflammatory studies since the effects of these samples were as strong as those of diclofenac. Finally, laserwort expressed anti-diabetic action. It can be considered a natural antidiabetic and anti-inflammatory agent, especially in alcoholic infusions. Although many valuable results have been obtained, they are still preliminary studies and *in vitro* methods. Comprehensive *in vivo* and clinical methods are essential to confirm the potential of *L. siler* or its compounds biologically and pharmacologically.

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Supporting Information

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