Research Article

Antibacterial, Anti-Inflammatory, Antioxidant, and Antiproliferative Properties of Essential Oils from Hairy and Normal Roots of *Leonurus sibiricus* L. and Their Chemical Composition

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Essential oils obtained from the NR (normal roots) and HR (hairy roots) of the medicinal plant *Leonurus sibiricus* root were used in this study. The essential oil compositions were detected by GC-MS. Eighty-five components were identified in total. Seventy components were identified for NR essential oil. The major constituents in NR essential oil were β-selinene (9.9%), selina-4,7-diene (9.7%), *(E)*-β-caryophyllene (7.3%), myli-4(15)-ene (6.4%), and guaia-1(10),11-diene (5.9%). Sixty-seven components were identified in HR essential oil, the main constituents being *(E)*-β-caryophyllene (22.6%), and germacrene D (19.8%). The essential oils were tested for cytotoxic effect, antimicrobial, anti-inflammatory, and antioxidant activities. Both essential oils showed activity against grade IV glioma cell lines (IC_{50} = 400 μg/mL), antimicrobial (MIC and MFC values of 2500 to 125 μg/mL), and anti-inflammatory (decreased level of IL-1β, IL-6, TNF-α, and IFN-γ in LPS-stimulated cells). The essential oils exhibited moderate antioxidant activity in ABTS (EC_{50} = 98 and 88 μg/mL) assay. This is the first study to examine composition of the essential oils and their antimicrobial, antioxidant, antiproliferative, and anti-inflammatory activities. The results indicate that essential oils form *L. sibiricus* root may be used in future as an alternative to synthetic antimicrobial agents with potential application in the food and pharmaceutical industries.

1. Introduction

For millennia, plants have held great economic value, not only by playing a significant role in the food supply but also by acting as therapeutic agents [1]. Interest has recently grown in the application of plants as medicinal agents since several side effects have been found to be associated with synthetic drugs. Plants are known to have therapeutic ability

and contribute to reducing the risk of various inflammatory conditions and cancers and are the most important global source of drugs; about 50 percent of drugs produced in the world are of natural origin [2]. According to the World Health Organization, about 80% of people rely on traditional remedies such as herbal drugs, which are found in many modern medicinal formulations. Many naturally occurring agents in plant extracts or oils have shown antimicrobial, antioxidant, anticancer, and anti-inflammatory potential in several animal models and bioassay systems and have an effect on human disease [3–6].

One particularly interesting group of compounds are the essential oils obtained from various parts of plants. Although essential oils only represent a small fraction of plant's composition, they nevertheless confer the characteristics by which aromatic plants are used in the food, cosmetic, and pharmaceutical industries [7]. The antioxidant activity of essential oils is another biological property of great interest because they may preserve foods from the toxic effects of oxidants [8]. Moreover, their free radical scavenging ability may play an important role in some diseases' prevention such as brain dysfunction, cancer, heart disease, and immune system decline [9, 10].

One plant which shows a broad spectrum of biological activity is *Leonurus sibiricus* L. Modern pharmacological studies have shown that the active components in various parts of *Leonurus sibiricus* possess a wide range of pharmacological activities, with effects on the uterus, as well as bestowing more general cardioprotective, antioxidative, anticancer, analgesic, anti-inflammatory, neuroprotective, and antibacterial effects [11–14]. Earlier studies have demonstrated the anticancer and antioxidant activities of various parts of this plant [15]. The most interesting results for extract of *Leonurus sibiricus* were obtained for in vitro culture transformed and normal roots [16]. Literature data shows that transformation of the roots can encourage the production of valuable secondary metabolites [17]. Therefore, the present study was carried out to evaluate the antimicrobial, anti-inflammatory, antioxidant, and antiproliferative activities and identify the chemical profile of essential oils from *L. sibiricus* normal and hairy roots.

2. Materials and Methods

2.1. Plant Material of L. sibiricus Roots.
In this study, normal (NR) and hairy roots (HR) of *L. sibiricus* were tested. The hairy roots were obtained by infection of five-week-old in vitro shoots of *L. sibiricus* with *Agrobacterium rhizogenes* strain A4. Establishment of hairy root (HR) cultures has been described previously by Sitarek et al. [15].

2.2. Isolation and Analysis of Essential Oils.
The essential oils of NR and HR roots (about 50 g of each) were obtained by hydrodistillation, using a clevenger-type apparatus for 5 h. Chemical analysis of essential oils composition was performed by GC-MS method according to Makowczyńska et al. [18]. Apparatus details are as follows: Trace GC Ultra with FID and MS DSQ II detector and MS-FID splitter, with column Rtx-1 ms (Restek), 60 m × 0.25 mm i.d., and

film thickness 0.25 μm; temperature program, 50–310°C at 2°C/min; injector temp. 280°C; FID temp. 300°C; carrier gas helium and ionization voltage 70 eV; ion source temp. 200°C. The identification of the compounds was based on the comparison of their RIs and MS spectra with those stored in the computer libraries and literature data [19, 20]. The percentages were computed from FID response without the use of correction factors. The results are showed in Table 1.

2.3. Cell Culture.
Normal human astrocytes (NHA) were grown in AGM medium according to the manufacturer's protocol. The cells were seeded at a density of 4×10^5 cells per 25 cm^2 flask. Under these conditions, the cell cycle of this line was approximately 12 hours. Glioma cells in IV grade were derived from tumour patient. Condition and further procedures are described in a previous study by Sitarek et al. [15].

2.4. MTT Assay.
Cell viability was determined by MTT assay. Briefly, NHA (normal human astrocytes) and glioma cells in IV grade (4×10^5 cells) were cultured in four-well plates for 24 hours after treatment by each concentration (0–3200 μg/mL) of HR and NR essential oils of *L. sibiricus* root. Further procedures are described in a previous study by Sitarek et al. [15].

2.5. Antimicrobial Properties

2.5.1. Microbial Strains and Growth Conditions.
The bacterial strains *Enterococcus faecalis* (ATCC29212), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922 were incubated at 37°C in Mueller-Hinton medium (Biokar Diagnostics, Beauvais, France). The yeasts *Saccharomyces cerevisiae* ATCC 2601 and *Candida albicans* ATCC 10231 were grown in Sabouraud Dextrose Agar (Difco).

2.5.2. Microdilution Method.
The essential oil minimum inhibitory concentrations (MICs) were determined using the twofold serial broth microdilution assay [21]. For bacteria, the samples dissolved in DMSO were diluted in the range 500 to 0.48 μg/mL in Mueller-Hinton broth medium, while, for the yeasts, the samples were diluted in the range 5000 to 4.88 μg/mL in Sabouraud medium. The antimicrobial activity of DMSO was evaluated. Vancomycin, norfloxacin, and amphotericin B were used as controls. The lowest concentrations of the samples that inhibited the growth of the microorganisms after 24 hours of incubation at 37°C were adopted as the MIC values; these are presented in μg/mL. Microbial growth was measured at 620 nm using an Absorbance Microplate Reader (Thermo scientific Multiskan FC). Assays were carried out in triplicate for each tested microorganism.

2.5.3. Minimum Bactericidal Concentration (MBC) or Minimum Fungicidal Concentration (MFC).
Following the MIC determination, the MBC or MFC was found for each set

TABLE 1: Constituents of essential oils from NR and HR *Leonurus sibiricus* root.

Peak number	Constituent	RI_{exp}	RI_{lit}	NR	HR	Class of compounds
(1)	Hexanal	771	770	0.4	0.3	O
(2)	Hexanol	841	837	t	0.1	O
(3)	Heptanal	879	879	0.2	0.1	O
(4)	Benzaldehyde	936	941	t	t	O
(5)	α-Pinene	930	936	3.2	1.0	MH
(6)	Oct-1-en-3-one	952	956	0.3	0.3	O
(7)	Oct-1-en-3-ol	961	962	3.5	3.6	O
(8)	β-Pinene	972	978	0.5	0.1	MH
(9)	Pentylfuran	975	977	0.9	0.8	O
(10)	Octan-3-ol	983	981	t	0.2	O
(11)	Myrcene	982	987	0.4	0.1	MH
(12)	Phenylacetaldehyde	1009	1012	t	t	O
(13)	*p*-Cymene	1011	1015	0.1	t	MH
(14)	β-Phellandrene	1019	1023	0.1	t	MH
(15)	Limonene	1022	1025	0.9	0.3	MH
(16)	*(Z)*-β-Ocimene	1028	1029	0.6	t	MH
(17)	*(E)*-Oct-2-enal	1032	1034	—	0.1	O
(18)	*(E)*-β-Ocimene	1038	1141	0.2	0.1	O
(19)	*p*-Cymenene	1072	1075	0.1	t	MH
(20)	Terpinolene	1078	1082	0.1	0.0	MH
(21)	Nonanal	1072	1176	0.3	0.5	O
(22)	Linalool	1083	1186	0.5	0.4	MO
(23)	Menthone	1132	1136	0.1	0.1	MO
(24)	*(E)*-Non-2-enal	1135	1139	0.3	0.4	O
(25)	Nonanol	1147	1149	t	t	MO
(26)	Borneol	1148	1150	t	0.1	O
(27)	*p*-Cymen-9-ol	1156	1157	0.1	0.1	MO
(28)	Terpinen-4-ol	1157	1158	0.5	0.1	MO
(29)	Methyl salicylate	1168	1171	0.1	0.1	O
(30)	α-Terpineol	1171	1176	0.3	0.2	MO
(31)	Safranal	1176	1182	0.0	0.1	MO
(32)	Decanal	1181	1180	0.4	0.7	O
(33)	β-Cyclocitral	1192	1195	0.1	0.1	MO
(34)	*Trans*-chrysanthenyl acetate	1213	1217	0.1	0.1	MO
(35)	Thymol	1255	1267	0.3	0.6	MO
(36)	Decanol	1260	1264	—	0.1	O
(37)	Bornylacetate	1266	1270	0.1	0.1	MO
(38)	Undecanal	1285	1290	0.1	0.2	O
(39)	(2E,4E)-Deca-2,4-dienal	1286	1291	0.1	0.1	O
(40)	Bicycloelemene	1336	1338	1.7	3.0	SH
(41)	β-Damascenone	1361	1363	0.5	0.9	O
(42)	α-Ylangene	1373	1376	0.3	t	SH
(43)	**α-Copaene**	1376	1379	0.4	**5.0**	SH
(44)	β-Bourbonene	1383	1386	0.2	3.0	SH
(45)	β-Elemene	1389	1389	1.7	0.5	SH
(46)	**Myli-4(15)-ene**	1411	1418	**6.4**	0.5	SH
(47)	***(E)*-β-Caryophyllene**	1419	1421	**7.3**	**22.6**	SH

TABLE 1: Continued.

Peak number	Constituent	RI$_{exp}$	RI$_{lit}$	NR	HR	Class of compounds
(48)	β-Duprezianene	1427	1423	0.7	1.1	SH
(49)	α-Guaiene	1443	1440	1.6	—	SH
(50)	Isogermacrene D	1447	1445	—	0.2	SH
(51)	(E)-β-Farnesene	1447	1448	—	0.1	SH
(52)	α-Humulene	1452	1455	2.2	3.3	SH
(53)	β-Ionone	1465	1468	0.2	0.5	O
(54)	γ-Gurjunene	1469	1472	0.5	—	SH
(55)	**Selina-4,7-diene**	1469	1470	**9.7**	—	SH
(56)	**Selina-4,11-diene**	1475	1475	**4.4**	—	SH
(57)	**Germacrene D**	1477	1479	**1.2**	19.8	SH
(58)	cis-β-Guaiene	1482	1488	1.3	—	SH
(59)	**β-Selinene**	1484	1486	**9.9**	0.9	SH
(60)	Pentadec-1-ene	1488	1486	—	0.7	O
(61)	Bicyclogermacrene	1492	1494	—	2.1	SH
(62)	α-Muurolene	1492	1496	2.1	—	SH
(63)	(E,E)-α-Farnesene	1494	1498	—	0.6	SH
(64)	γ-Cadinene	1506	1507	0.2	0.2	SH
(65)	**Guaia-1(10),11-diene**	1515	1517	**5.9**	—	SH
(66)	δ-Cadinene	1519	1520	—	1.1	SH
(67)	trans-Calamenene	1517	1517	0.3	0.1	SH
(68)	α-Calacorene	1527	1528	0.3	0.1	SH
(69)	(E)-α-Bisabolene	1531	1530	1.2	1.1	SH
(70)	Nerolidol	1546		0.3	0.5	SO
(71)	Guaia-6,9-dien-4-β-ol	1564	1465	0.5	—	SO
(72)	Spathulenol	1567	1572	—	1.6	SO
(73)	**Caryophyllene epoxide**	1570	1578	**4.5**	**8.0**	SO
(74)	Humulene epoxide II	1598	1602	0.6	1.2	SO
(75)	α-Corocalene	1600		1.2	—	SH
(76)	Caryophylla-3(15),7(14)-dien-6-ol	1620	1636	0.4	0.4	SO
(77)	T-Muurolol	1625	1633	0.2	0.3	SO
(78)	β-Eudesmol	1635	1641	0.9	0.9	SO
(79)	α-Cadinol	1637	1643	0.4	0.5	SO
(80)	Cadalene	1646	1659	t	—	SH
(81)	Myristic acid	1742	1748	0.3	0.2	O
(82)	Farnesylacetone	1888	1890	0.1	0.2	O
(83)	Methyl palmitate	1902	1901	0.1	0.2	O
(84)	Palmitic acid	1945	1942	2.4	2.2	O
(85)	Phytol	2095	2114	0.4	1.3	O
	Total identified			86.5	96.1	
	Monoterpene hydrocarbons MH			6.0	1.5	
	Oxygenated monoterpenes MO			2.1	1.9	
	Sesquiterpene hydrocarbons SH			60.2	64.8	
	Oxygenated sesquiterpenes SO			6.8	13.4	
	Other O			10.8	14.0	

The concentrations of the main compounds were signified in bold.
RI: relative retention index on Rtx-1 ms column, RI$_{lit}$: relative retention index of literature, NR: essential oil of normal roots of *L. sibiricus*, and HR: essential oils of hairy roots of *L. sibiricus*.

of wells. A loopful of broth was collected from the wells which did not show any growth, and this was inoculated on sterile Mueller-Hilton medium broth for bacteria and Sabouraud medium for yeasts by streaking; these media were then incubated at 37°C for 24 hours. After incubation, the lowest concentration at which no visible growth was observed was recorded as MBC (for bacteria) or MFC (for yeasts).

2.6. Anti-Inflammatory Activity. Levels of IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α in supernatant were measured by ELISA. The astrocytes cells (NHA) were subcultured in 6-well plates (5×10^5 cells/mL) and incubated with NR and HR essential oil (15, 30, and 60 μg/mL) or DMSO (vehicle) for 24 hours and incubated with LPS (1 μg/mL) for 24 h. After treatments, cell-free supernatant was collected, and IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α were measured using ELISA kits, in accordance with the manufacturer's instructions (Qiagen, USA). The absorbance was determined using a microplate reader. Results represent the mean ± SD of three independent experiments.

2.7. Reverse Transcriptase-PCR Analysis. Total RNA was isolated from astrocyte cells according to the manufacturer's instructions using a Total RNA Isolation System (Blirt, Poland). cDNA was generated by Reverse Transcription System (A&A Biotechnology, Poland), and 10 μL of the cDNA product was used as a template in the PCR reaction. Briefly, the PCR amplification consisted of 35 cycles (94°C, 1 min; 55–65°C, 1 min; 72°C, 2 min) with the commercial oligonucleotide primer sets for human IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α. These primer sets yield PCR products of 93, 262, 74, 296, 75, and 406 bp for IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α, respectively. The final PCR products were electrophoresed on 1.5% agarose gels. Further procedures are described in a previous study by Sitarek et al. [15].

2.8. Antioxidant Activity

2.8.1. Evaluation of DPPH Radical Scavenging Assay. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was assessed according to Blois [22] with some modifications. Briefly, a series of dilutions of NR and HR essential oils were made. Following this, 1.5 mL of each concentration was mixed with 1.5 mL of a 0.2 mmol/L methanolic DPPH solution. After a 30-minute incubation period at 25 C, the absorbance at 520 nm (maximum absorbance of DPPH) was recorded spectrophotometrically as $A_{\text{(sample)}}$. A blank reading was taken using a cuvette containing solution without the test material, and the absorbance was measured as $A_{\text{(blank)}}$. The free radical scavenging activity of each solution was then calculated as percentage inhibition according to the following equation:

$$\% \text{ inhibition} = 100(A_{\text{(blank)}} - A_{\text{(sample)}})/A_{\text{(blank)}}$$

The antioxidant activity of the essential oil was expressed as EC$_{50}$, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH concentration. BHA was used as a standard. All measurements were performed in triplicate.

2.8.2. ABTS Radical Cation Scavenging Assay. The capacity of the samples to scavenge the ABTS (2,20-azinobis-3-ethylbenzothiazoline-6-sulphonate) radical cation was determined according to Re et al. [23] with some modifications. The ABTS radical cations were generated by mixing a 7 mmol/L of ABTS at pH 7.4 (5 mmol/L NaH$_2$PO$_4$, 5 mmol/L Na$_2$HPO$_4$, and 154 mmol/L NaCl) with 2.5 mmol/L potassium persulfate (final concentration). The mixtures were then stored in the dark at room temperature for 16 hours. Following this, the mixture was diluted with water to give A_{734} value of 0.69 ± 0.03 units using a spectrophotometer. For the sample, solutions of the essential oil in methanol were allowed to react with fresh ABTS solution; the absorbance was then measured six minutes after the initial mixing. BHA was used as a standard. The free radical scavenging capacity was expressed as EC$_{50}$ (μg/mL), which was determined using the same equation used for the DPPH method. All measurements were performed in triplicate.

2.9. Statistical Analysis. The results are presented as the mean ± standard deviation of at least three separate experiments. Statistical differences were determined by one-way ANOVA. Results with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Chemical Composition of NR and HR of Essential Oils from L. sibiricus Roots. In this study we demonstrated qualitative and quantitative essential oil compositions from NR and HR of *L. sibiricus*. Essential oils from NR and HR were obtained with yields of 0.06% and 0.04%, respectively. Eighty-five components of NR and HR root essential oils were identified (Table 1). NR and HR essential oils contained a complex mixture consisting mainly of sesquiterpene hydrocarbons (60.2% and 64.8%, resp.), oxygenated sesquiterpenes (6.8% and 13.4%, resp.), and monoterpene hydrocarbons (6.0% and 1.5%, resp.). We had a little portion of oxygenated monoterpenes (2.1% and 1.9%) (Table 1). For NR essential oil, seventy components were identified. The major constituents in NR essential oil were β-selinene (9.9%), selina-4,7-diene (9.7%), *(E)-β*-caryophyllene (7.3%), myli-4(15)-ene (6.4%), and guaia-1(10),11-diene (5.9%). In HR essential root oil, sixty-seven components were identified. The main constituents of HR essential oil were *(E)-β*-caryophyllene (22.6%), germacrene D (19.8%), and α-copaene (5%).

3.2. MTT Viability on Normal Human Astrocytes and Glioma Cells after Treatment with Essential Oils of L. sibiricus NR and HR. First, to test the viability of cells after treatment with LPS, NR, and HR essential oils of *L. sibiricus* roots, MTT assay was performed in NHA and glioma in IV grade cells (Figures 1(a) and 1(b)). LPS did not affect cell viability in
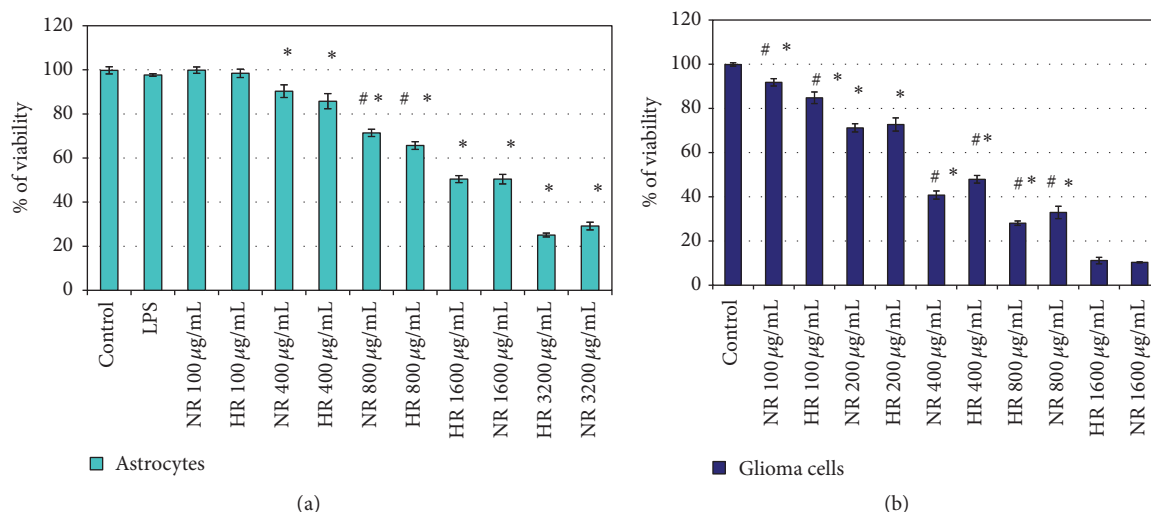
(a)

(b)

Figure 1: Viability of NHA cells (a) and IV grade glioma cells (b) treated with NR or HR essential oil from *L. sibiricus* root for 24 h. The values are the mean ± SD for triplicate. $^{*}p < 0.05$ versus control. $^{\#}p < 0.05$ NR versus HR.

tested concentration (1 μg/mL) after 24 h (data not shown). Our results showed that viability was similar for both essential oils. The IC_{50} value was considered as the concentration of 1600 μg/mL for NR and HR essential oils which caused a 50% decrease in cell viability relative to the negative control, comprising cell culture and DMSO without either of the essential oils (Figure 1(a)). Additionally, the NR and TR oils were found to have a cytotoxic effect on IV grade glioma cells at the IC_{50} concentration of 400 μg/mL. The tested NR and HR root essential oils of *L. sibiricus* did not affect viability of the NHA cells in IC_{50} concentration for IV grade of glioma cells (Figure 1(b)).

3.3. Antimicrobial Activity.

The antimicrobial activities of the essential oils from NR and HR of *L. sibiricus* root were quantitatively assessed by determination of the MIC and MBC/MFC, as given in Table 2. MIC and MBC/MFC values of oils from NR and HR of *L. sibiricus* roots were in the range of 125 to 2500 μg/mL for MICs and 500 to 5000 μg/mL for MBC/MFCs for all tested strains. Antibacterial activity was shown by NR and HR oils of *L. sibiricus* roots against all bacterial strains with MICs values in the range of 125–250 μg/mL, but the highest value was observed against *P. aeruginosa* (125 μg/mL). *L. sibiricus* oils were found to have substantial antifungal activity against *S. cerevisiae* and *C. albicans* with MIC values of 1250 and 2500 μg/mL, according to microdilution assay, and for MFC values of 2500 and 5000 μg/mL (Table 2). No significant differences were observed between the NR and HR essential oils of *L. sibiricus* roots.

3.4. Antioxidant Activity.

The antioxidant activities of NR and HR essential oils of *L. sibiricus* root were assessed by free radicals (DPPH and ABTS) scavenging assays. No significant differences were observed between NR and HR essential oils.

In the ABTS assay, EC_{50} values were determined to be 92.40 ± 1.99 μg/mL for NR oil and 88.22 ± 1.35 μg/mL for HR oil, respectively. In turn, EC_{50} values were 500.50 ± 1.37 μg/mL for NR oil and 489.20 ± 1.45 μg/mL for HR oil in DPPH assay. Both tested oils showed similar antioxidant effectiveness but better activity was observed for the ABTS method. The results were presented in Table 3.

3.5. Effects of HR and NR Essential Oils from L. sibiricus Roots on IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α Level.

The study examined the influence of NR and HR essential oils of *L. sibiricus* roots on the LPS-stimulated production of IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α in NHA cells. Our results showed that NR and HR root oils decreased level of LPS-stimulated IL-1β, IL-6, TNF-α, and IFN-γ compared to controls (Figure 2). This effect was observed at various concentrations (15, 30, and 60 μg/mL) of NR and HR essential oils and was dependent on the dose. No differences in IL-2 and IL-4 level were observed with regard to controls for NR or HR essential oils, and no significant differences were observed between the two essential oils.

3.6. Effects of NR and HR Root Oils of L. sibiricus IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α on mRNA Expression.

Based on the previous results, the optimal concentrations for both essential oils were selected for RT-PCR analysis to determine whether NR and HR oils of *L. sibiricus* root can regulate IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α mRNA expression. NHA cells were incubated with LPS and then in the presence of 60 μg/mL NR and HR root oils. As shown in Figure 3, the expression of IL-1β, IL-6, IFN-γ, and TNF-α genes was found to be lower than in LPS-stimulated cells for both essential oils. No changes in expression were found for IL-2 or IL-4.

TABLE 2: Antimicrobial activity of the essential oils obtained from NR and HR roots of *L. sibiricus*.

Plant material	Staphylococcus aureus		Pseudomonas aeruginosa		Escherichia coli		Enterococcus faecalis		Saccharomyces cerevisiae		Candida albicans	
	MIC (μg/mL)	MBC	MIC (μg/mL)	MBC	MIC (μg/mL)	MBC	MIC (μg/mL)	MBC	MIC (μg/mL)	MBC/MFC	MIC (μg/mL)	MBC/MFC
HR essential oil	250	>500	125	>500	250	>500	250	>500	1250	2500	1250	>5000
NR essential oil	250	>500	125	>500	250	>500	250	>500	2500	>5000	1250	1250
Positive control	7.82 VAN	>500	<0.48 NOR	>500	0.98 NOR	>500	1.95 VAN	>500	<0.48 ANF	>5000	<0.48 ANF	>5000

VAN: Vancomycin, NOR: Norfloxacin, ANF: Amphotericin B. Data represent the median values in triplicate.

Table 3: Antioxidant activity of the essential oils obtained from NR and HR roots of *L. sibiricus*.

Oil plant material	Antioxidant activity	
	ABTS	DPPH
	(EC$_{50}$, μg/mL)	(EC$_{50}$, μg/mL)
HR essential oil	88.22 ± 1.35	489.20 ± 1.45
NR essential oil	92.40 ± 1.99	500.50 ± 1.37
Positive control/BHA	6.25 ± 0.09	4.25 ± 0.10

BHA: butylated hydroxyl anisole, DPPH (2,2-diphenyl-1-picrylhydrazyl), and ABTS (2,20-azinobis-3-ethylbenzothiazoline-6-sulphonate). Data represent the mean values ± SD in triplicate.
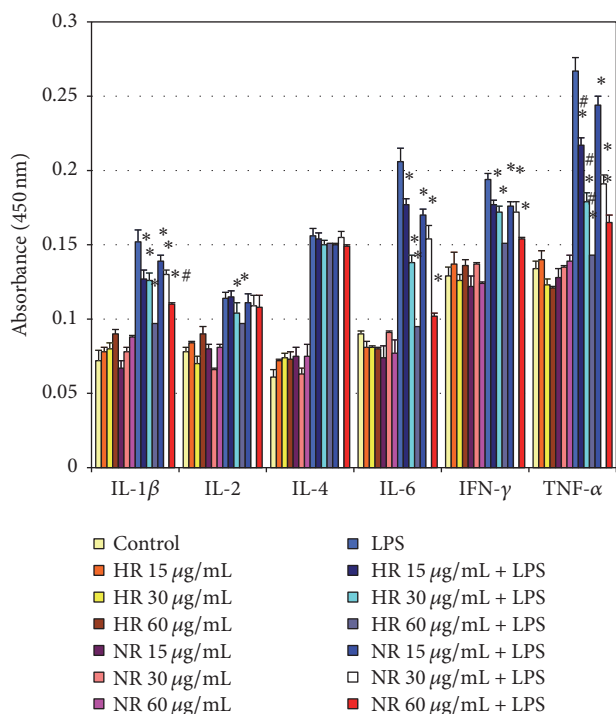

Figure 2: Effect of NR and HR essential oil of *L. sibiricus* root on the production of cytokines IL-6, IL-1β, TNF-α, IL-2, IL-4, and IFN-γ by LPS induced in NHA cells in various concentrations (15–60 μg/mL). The supernatants were collected and analysed by ELISA kits. The data are presented as mean ± SD of three independent experiments. *$p < 0.05$: treated group significantly different from LPS-treated group. #$p < 0.05$ NR versus HR.

4. Discussion

Natural plant products have been used since ancient times, and their use is now increasing. Some essential oils are known to have various health benefit properties and have been used as alternative remedies for the prevention and treatment of many infectious diseases: their antibacterial, antifungal, anti-inflammatory, anticancer, and antioxidative activities make them particularly suitable for this role. It is known that the efficacy of the essential oil depends on many environmental and genetic factors. It has been proven that the main factors responsible for the diverse chemical

composition of essential oils include climatic conditions, geographic origin, time of collection, distillation conditions, correct farming practices, and the part of the plant from which oil is extracted [24, 25]. An important characteristic of essential oils and their components is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable, resulting in the leakage of ions and other cell contents [26]. The antimicrobial, anticancer, anti-inflammatory, and antioxidative properties of essential oils have been evaluated by many other researchers [27, 28] and their results support the findings of the present investigation. The present study investigated the chemical profile of essential oils derived from the normal and hairy roots of *Leonurus sibiricus* and evaluated their antimicrobial, anti-inflammatory, antioxidant, and antiproliferative activities.

The essential oils were isolated from the *L. sibiricus* herb or its leaves according to a previous study [29]. This study is the first to identify the essential oil compositions of *L. sibiricus* normal and hairy roots. Dambolena et al. [29] report that β-caryophyllene and α-humulene were two of three main constituents, amounting in total to 75–80% in both oils (leaves and herb), with β-cubebene as the third. In turn, Almeida et al. found germacrene D to be the most dominant compound in leaf oil. In other species of the genus *Leonurus*, *L. japonicus* sesquiterpenes (45.4%) with β-caryophyllene and its oxide are the main constituents of essential oil isolated from the herb [30].

In the present study, β-caryophyllene (22.6% of total) and germacrene D (19.8%) were found to be the main constituents in HR essential oil, being present in higher concentrations in HR than in NR (7.3%, 1.2%, resp.) essential oil. Additionally, the analysis of NR essential oil revealed other compounds including selina-4,7-diene, selina-4,11-diene, and guaia-1(10),11-diene which were not detected in HR essential oil. The other main components in NR essential oil were β-selinene and myli-4(15)-ene. In addition, β-selinene was present in ninefold higher concentrations, and myli-4(15)-ene sixfold higher, in NR essential oil than in HR oil. Despite the different contents, no significant differences were found regarding biological activities.

Although many workers have examined the antioxidant activity of essential oils by DPPH and ABTS assay on a range of species [31, 32], none have discussed the antioxidant activity of essential oil from NR and HR of *L. sibiricus* root. Our studies showed both oils to have the low reactivity to DPPH assay (EC$_{50}$ = 500 μg/mL and EC$_{50}$ = 489 μg/mL) and moderate reactivity to ABTS assay (EC$_{50}$ = 92 μg/mL and EC$_{50}$ = 88 μg/mL), which may be associated with the high concentrations of sesquiterpene hydrocarbons in NR and TR essential oils (60.2% and 64.8%, resp.). Our findings are consistent with those of Andrade et al., which indicate that the low antioxidant activity of essential oils of *Siparuna guianensis* results from the high content (59.4%) of sesquiterpene and monoterpene hydrocarbons [33]. The DPPH test is based on the use of a very crowded radical; however, the use of a low crowded radical such as ABTS can be more suitable [32]: the ABTS assay gives better results for expressing antioxidant
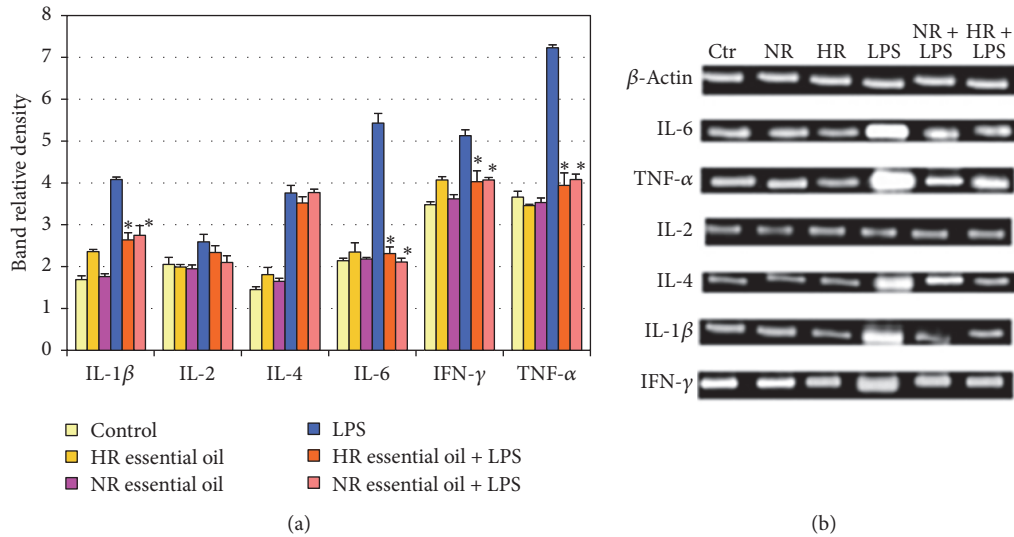
(a)

(b)

Figure 3: Impact of NR and HR essential oil of *L. sibiricus* root on gene expression. NHA cells were treated with LPS and next NR and HR essential oil for 24 hours. (a) The bar graph gives the relative density. (b) Expression of mRNA levels of IL-1β, IL-2, IL-4, IL6, IFN-γ, and TNF-α genes. mRNA values are mean ± SD, $^*p < 0.05$ compared to LPS group. Data were obtained as the mean of three replications.

property because it is sensitive, requires a short reaction time, and can be used in both organic and aqueous solvent systems [32]. The difference between the results of the DPPH and ABTS tests may be attributed to the mechanism of the involved reaction: the reactions of the ABTS radical involve electron transfer and take place at a much faster rate compared to DPPH radicals, whose amount of discoloration is attributed to the hydrogen donating ability of the test compounds [31, 32].

The essential oils showed a pronounced effect against grade IV glioma cell line, with IC$_{50}$ values of 400 μg/mL. A novel aspect of our findings is that they demonstrate that NR and HR essential oils from *L. sibiricus* roots have cytotoxic effects. Legault and Pichette showed that β-caryophyllene has an anticancer activity against the cell line MCF-7 [34]. This compound was identified in the HR and NR essential oils. In turn, de Oliveira et al. found the oils to have anticancer effects in various cancer cell lines, with germacrene D as one of the main compounds in the essential oils [35]. However, previous studies indicate that the anticancer activity of the essential oil might be due to the synergic effects of all the terpenes in the oil; alternatively there may be some other active compounds responsible for the anticancer activity of the essential oil, which deserves attention in future study. The MTT assay found that the NR and HR essential oils from *L. sibiricus* root used in the present study did not affect the viability of NHA cells when applied in the concentration range 0–800 μg/mL.

The essential oils of many plants are known to have antimicrobial activity [4, 36, 37], which could act as a chemical defence against various pathogens. The present study examines the antimicrobial activities against all tested strains, which may be attributed to the presence of sesquiterpenes such as (E)-β-caryophyllene or germacrene D in the tested

oils. It is known that β-caryophyllene and germacrene D possess strong antimicrobial activity [36]. Dahham et al. showed that β-caryophyllene has selective antibacterial activity and more pronounced antifungal activity [38]. Xiong et al. note that the oil of *Leonurus japonicus possesses* antimicrobial activity against Gram-positive bacteria but not against Gram-negative bacteria [36]. In contrast, our results showed antimicrobial activity against Gram-positive and Gram-negative bacteria. If the MBC of a pure compound is no more than four times the MIC value, it can be regarded as bactericidal; hence these essential oils appear to offer potential as a bactericide [39]. However, it is difficult to compare our data with other studies because the variables that influence the results are the essential oil composition and antimicrobial test method used. Moreover, the standard criteria for the evaluation of the activity of the plant essential oils are missing and therefore the results obtained by different authors vary widely. Due to the high antimicrobial activity of essential oils, they may have valuable use as food preservatives. To our knowledge, this is the first report to confirm antimicrobial properties of essential oils derived from *L. sibiricus* root.

The next step in our study was to evaluate the anti-inflammatory activities of essential oils from *L. sibiricus* root on NHA cells. This effect has been proven by ELISA examination of LPS-induced production of pro- and anti-inflammatory cytokines such as TNF-α, IL-6, IL-2, IL-4, IL-1β, and INF-γ and treatment with essential oils. Additionally, the study examined the expression of genes such as TNF-α, IL-6, IL-2, IL-4, IL-1β, and INF-γ on mRNA level. As inflammation is an important aspect of many human diseases, its reduction could be of therapeutic interest. Glial cells such as astrocytes and microglia are important immune cells in the brain [40]. The activation of microglia as

resident microphages initiates the release of several potentially cytotoxic substances including reactive oxygen intermediates, nitric oxide, and proteases [41]. These components are believed to contribute to the progressive damage observed in neurodegenerative diseases; hence, reducing their levels in activated microglia can ameliorate neurodegenerative disease symptoms [42, 43]. Many cytokines and inflammatory factors, neurotransmitters, and neuropeptides are known to affect IL-6 regulation in brain cells. In addition, IL-1β and TNF-α have been observed to induce IL-6 in cultured cortical neurons and astrocytes, the latter involving the NFκB and the PKC pathway [44]. LPS has commonly been used as an inflammogen for activating cells in several in vivo and in vitro cell models [45]. While LPS normally induces IL-6 in both astrocytes and microglia, TNF-α only induces IL-6 in astrocytes; it does not do so in microglia [46]. Species-specific effects may exist, since, in human cells in vitro, LPS affects microglia to a greater degree than astrocytes with regard to the production of TNF-α, IL-1β, and IL-6; however, IL-1β is a potent stimulator of IL-6 production in astrocytes [47]. Therefore, the inhibition of normal human astrocyte activation and reduction of the release of pro- and anti-inflammatory mediators represent a promising strategy for the prevention of neurodegenerative diseases. Our findings are the first to report that NR and HR essential oils from *L. sibiricus* root suppressed TNF-α, IL-6, IL-2, IL-4, and IL-1β production induced by LPS stimulation in a dose-dependent manner. Furthermore, essential oil was found to suppress the expression of TNF-α, IL-6, IFN-γ, and IL-1β genes in LPS-activated NHA cells. We suspect that this is due to the major compounds identified in the essential oils, that is, sesquiterpenes such as *(E)-β*-caryophyllene and germacrene D; however, the literature indicates that this may be due to the synergistic effect of the constituents in the oil. Similarly, Baylac and Racine note that sesquiterpenes such as β-caryophyllene exhibited activity in the in vitro 5-lipoxygenase assay in the inflammation process [48].

5. Conclusion

Our findings detail the constituents of the essential oil of normal and hairy roots (NR and HR) of *L. sibiricus* and indicate their promising use as pharmaceuticals, with antimicrobial, antioxidant, anti-inflammatory, and antiproliferative activities. Our results clearly show that these essential oils are active against grade IV glioma cells, inhibit the growth of food pathogens, have moderate antioxidant activity in ABTS test, and suppress LPS-stimulated NHA activation by inhibiting pro- and anti-inflammatory mediators. The results presented here can be considered as the first information on biological properties of NR and HR essential oils of *L. sibiricus* roots. Therefore, this plant may be used to safely extend the shelf life of food and may be an interesting candidate in phytotherapy.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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