Chemical Composition, Enantiomeric Analysis, AEDA Sensorial Evaluation and Antifungal Activity of the Essential Oil from the Ecuadorian Plant Lepechinia mutica Benth (Lamiaceae)

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Authors

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Abstract

This study describes the GC-FID, GC/MS, GC-O, and enantioselective GC analysis of the essential oil hydrodistilled from leaves of *Lepechinica mutica* (*Lamiaceae*), collected in Ecuador. GC-FID and GC/MS analyses allowed the characterization and quantification of 79 components, representing 97.3% of the total sample. Sesquiterpene hydrocarbons (38.50%) and monoterpene hydrocarbons (30.59%) were found to be the most abundant volatiles, while oxygenated sesquiterpenes (16.20%) and oxygenated monoterpenes (2.10%) were the minor components. In order to better characterize the oil aroma, the most important odorants, from the sensorial point of view, were identified by Aroma Extract Dilution Analysis (AEDA) GC-O. They were α-Pinene, β-Phellandrene, and Dauca-5,8-diene, exhibiting the characteristic woody, herbaceus, and earthy odors, respectively. Enantioselective GC analysis of L. mutica essential oil revealed the presence of twelve couples and two enantiomerically pure chiral monoterpenoids. Their enantiomeric excesses were from a few percent units to 100%. Moreover, the essential oil exhibited moderate in vitro activity against five fungal strains, being especially effective against M. canis, which is a severe zoophilic dermatophyte causal agent of pet and human infections.

Introduction

The genus *Lepechinia* belongs to the family *Lamiaceae* and comprises about 43 species distributed from South-West USA to Chile. Sesquiterpenes, diterpenes, triterpenes, and flavonoids have been isolated from different species of this genus. Some species are used for their antitumor and insulin-mimetic properties, to treat uterine infections and stomach pains.[1] Regarding the essential oil components, 15 species of the genus Lepechinia have been studied so far, including *L. conferta*,[1] *L. floribunda*,[2-5] *L. graveolens*,[3] *L. caulescens*,[6] *L. paniculata*,[7] *L. betonicifolia*,[8] *L. meyeni*,[3][9] *L. salviaefolia*,[10] *L. bullata*,[11] *L. calycina*,[12] *L. schiedeana*,[13-15] *L. urbanii*,[16] *L. chamaedryoides*,[17] *L. radula*.[18] In Table 1, we have reported the major compounds from some *Lepechinia* species.

Table 1. Major components in the essential oils of genus *Lepechinia*

Major compound	*L. conferta*[1]	*L. floribunda*[2-5]	*L. graveolens*[3]	*L. caulescens*[6]	*L. paniculata*[7]	*L. betonicifolia*[8]	*L. meyenii*[3-9]	*L. salviaefolia*[10]	*L. bullata*[11]	*L. calycina*[12]	*L. schiedeana*[13-15]	*L. urbanii*[16]	*L. chamaedryoides*[17]	*L. radula*[18]
(−)-Palustrol								x						
(−)-Spirolepechinene									x					
1,8-Cineole		x								x				
Aromadendrene					x									
Borneol		x		x				x						
Bornyl acetate		x												
Camphene		x												
Camphor				x						x				
Ledol	x										x			
Ledyl acetate		x												
Limonene						x								
m-Cymene							x							
o-Cymene							x							
p-Cymene							x							
Viridiflorene					x									
α-Copaene												x		
α-Humulene			x											
α-Pinene						x	x							
β-Caryophyllene		x	x	x									x	x
β-Phellandrene	x							x					x	
β-Pinene							x							x
β-Selinene					x									
γ-Cadinene		x												
δ-Cadinene												x		
Δ^3-Carene	x						x			x	x	x	x	x

However, due to the heterogeneity of compounds identified in *Lepechinia* spp, it has not been possible to establish a typical metabolic pattern. The essential oils of seven different Ecuadorian plants of the family *Lamiaceae* have been studied so far, including two species belonging to the genus *Lepechinia*.[7][19] In 2002, Malagón et al. identified 54 compounds in the essential oil from L. mutica, collected at 'Cerro el Villonaco' (Loja, Ecuador), on the basis of their retention indices, referred to a series of homologous fatty acid methyl esters,[19] and the comparison of the mass spectra with an early collection of reference spectral data.[20]

The gas chromatography-olfactometry (GC-O) technique couples traditional gas chromatographic analysis with sensory detection. Thus GC-O may be considered a biological detection method for the characterization of the odor of compounds separated and quantified by GC chromatography.[21] Indeed, the correlation of the eluted peaks with specific odors affords to establish accurate retention indices or retention times for the odor active components, while peak areas in the GC-FID chromatogram are indicative of the relative abundance of the components. Moreover, GC-O in combination with mass spectrometry enables the identification of odor compounds with the aid of mass spectral information and leads to a partial correlation between the chemical nature of an odorant and its perceived smell.[21] To collect and process GC-O data and to estimate the relative odor potency of each aroma-active compound and thus its sensory contribution to the total odor of the oil, we used the dilution to threshold quantitative procedure known as the 'Aroma Extract Dilution Analysis' (AEDA).[22-24]

Another important point, related to the sensorial properties of the volatile fraction, is that many common constituents of essential oils are chiral and thus they may be present as one or both enantiomeric forms. Since the odor properties as well as the biological activity of the two stereoisomers may be greatly different, a complete characterization of odor-active components of an essential oil requires enantiomer recognition and enantiomeric excess (ee) and/or ratio (er) determination.[25][26] These separations are usually achieved by enantioselective gas chromatography, using a capillary column endowed with a chiral stationary phase, usually cyclodextrin derivatives, as chiral selectors.[25] Therefore, we decided to repeat the analysis of the oil by GC-FID and GC/MS techniques, and identified their components by comparison of their linear retention indices and mass spectra with an updated and more complete reference data collection.[27] Moreover, the essential oil was submitted, for the first time with this genus, to GC-O and enantioselective GC analyses to characterize better the pleasant minty smell of L. mutica essential oil and to identify odor active compounds.

Results and Discussion

GC/MS and GC-FID Analyses

The average and standard deviation of each oil component were calculated by six consecutive GC-FID analyses. The results of GC/MS and GC-FID analyses are reported in Table 2.

Table 2. Components of the essential oil from *L. mutica*

No.	Component[a]	t_R [b]	Calculated linear retention index[c]	Linear retention index from reference[27]	Δ*RI*	FID% area	σ
1	Tricyclene	4.07	921	926	5	Tr[d]	–
2	α-Thujene	4.17	924	928	4	0.09	0.06
3	α-Pinene	4.38	932	934	2	1.23	0.89
4	Camphene	4.88	946	949	3	0.75	0.80
5	Sabinene	5.65	969	971	2	0.24	0.15
6	β-Pinene	5.82	974	976	2	3.78	1.76
7	Oct-3-en-1-ol	6.08	974	983	9	0.07	0.07
8	Myrcene	6.27	988	989	1	0.52	0.28
9	*p*-Mentha-1(7),8-diene	6.73	1003	1002	−1	0.16	0.13
10	α-Phellandrene	6.82	1005	1003	−2	3.80	1.70
11	Δ^3-Carene	6.93	1008	1008	0	8.69	4.24
12	α-Terpinene	7.19	1014	1015	1	0.11	0.07
13	*p*-Cymene	7.31	1020	1019	−1	0.10	0.06
14	Sylvestrene	7.45	1025	1023	−2	0.29	0.18
15	o-Cymene	7.48	1022	1024	2		
16	Limonene	7.64	1024	1028	4	3.79	2.18
17	β-Phellandrene	7.69	1025	1030	5		
18	γ-Terpinene	8.64	1054	1057	3	0.23	0.12
19	*cis*-Sabinene hydrate	9.12	1065	1071	6	0.05	0.03
20	*p*-Mentha-2,4(8)-diene	9.43	1085	1080	−5	0.35	0.18
21	Terpinolene	9.56	1086	1083	−3	0.60	0.33
22	*trans*-Linalool oxide	9.67	1084	1086	2	Tr[d]	–
23	Linalool	10.20	1095	1102	7	0.20	0.09
24	Oct-1-en-3-yl acetate	10.44	1110	1109	−1	1.37	0.60
25	Camphor	11.58	1141	1145	4	Tr[d]	–
26	Borneol	12.46	1165	1172	7	0.25	0.05
27	4-Terpineol	12.71	1174	1180	6	0.14	0.02
28	α-Terpineol	13.23	1186	1196	10	0.11	0.02
29	Isobornyl acetate	15.81	1283	1281	−2	2.20	1.04
30	δ-Elemene	17.41	1335	1328	−7	Tr[d]	–
31	α-Cubebene	17.81	1348	1340	−8	0.57	0.08
32	α-Terpinyl acetate	17.89	1346	1342	−4		
33	α-Ylangene	18.57	1373	1361	−12	0.15	0.05
34	Isoledene	18.61	1374	1362	−12		
35	α-Copaene	18.81	1374	1367	−7	1.46	0.23
36	β-Bourbonene	19.08	1387	1375	−12	0.47	0.25
37	β-Cubebene	19.28	1387	1380	−7	0.15	0.04
38	α-Gurjunene	19.97	1409	1400	−9	1.94	0.37
39	α-Cedrene	20.26	1410	1407	−3	0.05	0.10
40	(*E*)-Caryophyllene	20.47	1417	1412	−5	4.55	2.16
41	Longifolene	20.74	1407	1418	11	0.15	0.07
42	β-Copaene	20.86	1430	1421	−9	0.50	0.08
43	β-Gurjunene	21.19	1431	1429	−2	1.47	0.78
44	*cis*-Muurola-3,5-diene	21.54	1448	1437	−11	0.45	0.36
45	α-Humulene	21.89	1452	1445	−7	1.20	0.47
46	Aromadendrene	22.05	1439	1449	10	0.56	0.10
47	*cis*-Cadina-1(6),4-diene	22.19	1461	1452	−9	0.99	1.36

No.	Component[a]	t_R [b]	Calculated linear retention index[c]	Linear retention index from reference[27]	Δ*RI*	FID% area	σ
48	Amorpha-4,11-diene	22.36	1449	1456	7	0.15	0.07
49	Dauca-5,8-diene	22.64	1471	1463	−8	0.38	0.09
50	*trans*-Cadina-1(6),4-diene	22.79	1475	1466	−9	0.99	0.12
51	*γ*-Muurolene	22.98	1478	1471	−7	0.92	0.23
52	*δ*-Selinene	23.29	1492	1478	−14	0.81	0.08
53	*cis*-*β*-Guaiene	23.42	1492	1481	−11	0.71	0.11
54	Bicyclogermacrene	23.62	1500	1486	−14	4.62	0.58
55	*epi*-Cubebol	23.74	1493	1489	−4		
56	*α*-Zingiberene	23.75	1493	1489	−4		
57	*α*-Muurolene	23.83	1500	1491	−9	0.91	0.17
58	(*E*,*E*)-*α*-Farnesene	24.34	1505	1503	−2	0.83	0.25
59	*γ*-Cadinene	24.44	1513	1505	−8	2.86	0.37
60	Cubebol	24.59	1514	1508	−6	0.36	0.21
61	*δ*-Cadinene	24.74	1522	1511	−11	6.96	0.99
62	*trans*-Calamenene	24.78	1521	1512	−9	0.15	0.04
63	*trans*-Cadina-1,4-diene	25.29	1533	1523	−10	0.37	0.10
64	*α*-Cadinene	25.48	1537	1527	−10	0.39	0.12
65	Selina-3,7(11)-diene	25.61	1545	1530	−15	0.14	0.04
66	Germacrene B	26.33	1559	1545	−14	0.18	0.06
67	Germacrene D-4-ol	27.31	1574	1567	−7	1.46	0.40
68	Caryophyllene oxide	27.42	1582	1569	−13	0.29	0.24
69	Globulol	28.11	1590	1584	−6	5.91	2.61
70	Viridiflorol	28.51	1592	1592	0	1.29	0.45
71	1,10-Di-*epi*-cubenol	29.71	1618	1617	−1	0.27	0.11
72	10-*epi*-*γ*-Eudesmol	29.96	1622	1622	0	0.54	0.15
73	Junenol	29.97	1618	1623	5	1.39	0.42
74	*α*-Acorenol	30.14	1632	1626	−6	0.09	0.15
75	*β*-Acorenol	30.78	1636	1639	3	0.47	0.81
76	*β*-Eudesmol	31.00	1649	1644	−5	4.47	1.93
77	*α*-Eudesmol	31.02	1652	1644	−8		
78	*α*-Cadinol	31.10	1652	1646	−6		
79	Shyobunol	32.83	1688	1681	−7	10.80	5.91
	Monoterpenes hydrocarbons					30.59	
	Oxygenated monoterpenes					2.07	
	Sesquiterpene hydrocarbons					38.54	
	Oxygenated sesquiterpenes					16.22	
	Others					9.88	
	Total identified					97.30	

[a]Compound identification methods: LRI and comparison of the mass spectrum with Adams. [b]t_R = Retention time (min). [c]Calculated linear retention index on a DB5-MS column. [d]Tr = trace (< 0.05%).

Table 2 reports the list of the identified components. Indeed, 78 compounds were identified, representing 95.60% the total oil sample. Sesquiterpene hydrocarbons (38.54%) and monoterpene hydrocarbons (28.89%) were the principal groups of compounds. Oxygenated sesquiterpenes (16.22%) and oxygenated monoterpenes (2.07%) were the minor groups. The most abundant components were shyobunol (10.80%), Δ3-carene (8.69%), δ-cadinene (6.96%), globulol (5.91%), (E)-caryophyllene (4.55%), β-pinene (3.78%), and γ-cadinene (2.86%). Δ3-Carene has also been found as one of the three main components in the oils *of L. conferta*,[1] *L. meyeni*,[3][9] *L. calycina*,[12] *L. schiedeana*,[13-15] *L. urbanii*,[16] *Sphacele chamaedryoides*, [17] *and L. radula*.[18]

In the previous study by Malagon of the oil from *L. mutica*, monoterpene hydrocarbons were found to be the main group of constituents (72%), among which β-phellandrene (30%), camphene (13%), limonene (8%), Δ3-carene (6%), and α-pinene (3%) were the most abundant ones.[19] The different chemical composition of the essential oil from L. mutica reported in the previous[19] and present works, may depend on many factors, such as the phenological status of the plant, different time and place of collection, distillation time, method of analysis. In fact, the plant material studied by Malagón et al. was collected in a different place and period (March 2000), giving a possible explication to the different composition.

AEDA GC-O

GC-O results were processed by means of AEDA technique, and are shown in Figures 1 and 2, and in Table 3. In Figure 1 each (red) signal in the aromagram represents the perception of the compound corresponding to the retention index reported in the underlying GC chromatogram; the intensity of each signal is proportional to the dilution corresponding to the FD factor; therefore, the greater is the signal intensity in the aromagram, the more important is the contribution of the corresponding compound to the olfactory profile of the essential oil. Figure 2 depicts the AEDA aromagram of the essential oil *from L. mutica* resulting from the FD factors of odor-active components from *L. mutica*.

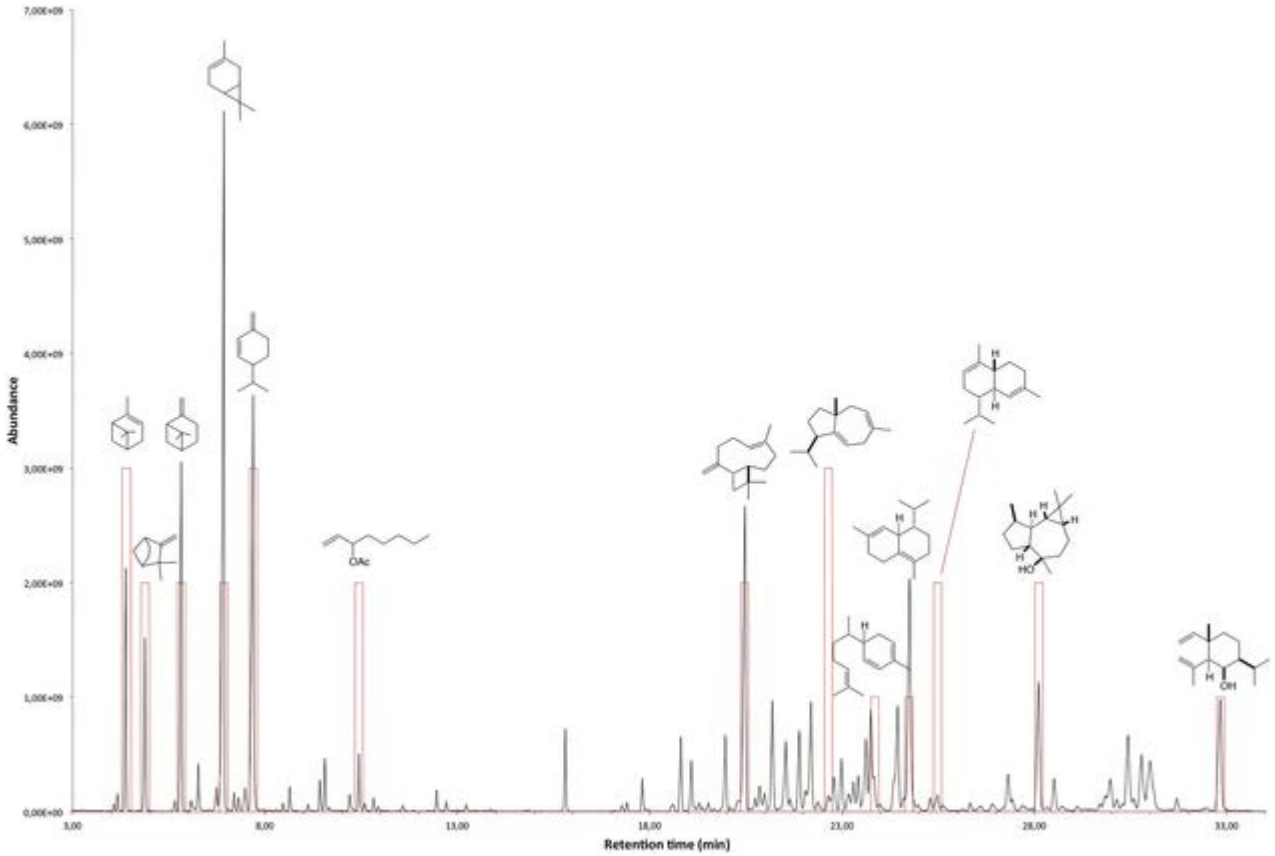

Figure 1. Superposition of the gas chromatogram and the aromagram of the essential oil.

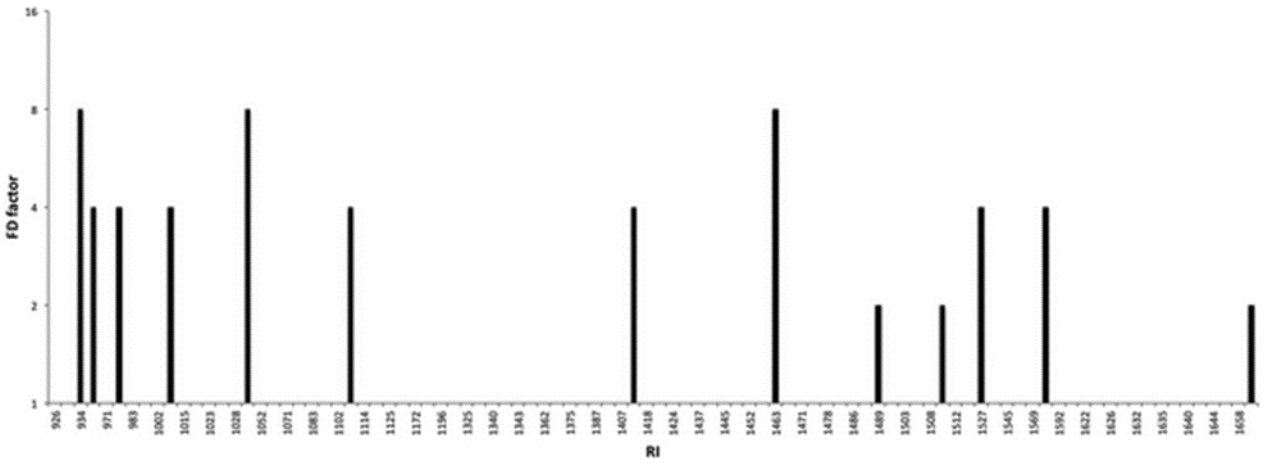

Figure 2. AEDA Aromagram of the essential oil from *L. mutica*.

Table 3. Components of the olfactory profile of *L. mutica* essential oil

Odour	AEDA (FD)	Compound	Calculated *LRI*
Woody	8	*α*-Pinene	934
Vanilla	4	Camphene	949
Woody	4	*β*-Pinene	976
Lemon	4	Δ^3-Carene	1008
Herbaceous	8	*β*-Phellandrene	1030
Lavender	4	Oct-1-en-3-yl acetate	1109
Woody	4	(*E*)-Caryophyllene	1412
Earthy	8	Dauca-5,8-diene	1463
Woody	2	*α*-Zingiberene	1489
Woody	2	*δ*-Cadinene	1511
Woody	4	*α*-Cadinene	1527
Herbaceous	4	Globulol	1584
Woody	2	Shyobunol	1681

The most important compounds from the olfactory point of view are reported in Table 3. Thus, the main odor contributors, according to AEDA analysis, were α-pinene, having a strong woody odor, β-phellandrene, endowed with a characteristic herbaceus tonality, and dauca-5,8-diene possessing a typical earthy odor.

According to the AEDA analysis, the importance of odorous compounds does not correspond to the component percentage in the oil. For example, shyobunol and Δ3-carene, which are among the most abundant compounds, are not among the most powerful odorants; instead, the opposite is true, for example, for β-phellandrene and dauca-5,8-diene.

Enantioselective GC/MS Analysis

Enantiomer components and enantiomeric excesses (ee) of *L. mutica* essential oil were determined as the mean value of two enantioselective GC/MS analyses,[26][28] performed on samples obtained from two different distillation processes. Since enantiomers have the same chemical properties, the enantiomeric distribution (unlike the chemical composition) should not be affected by the distillation process. For this reason, the enantioselective analysis can be performed as the mean value of two replicates. Twelve couples and two enantiomerically pure chiral monoterpenoids were detected (Table 4) and baseline separated. (+)-Camphor and (−)-borneol were detected as enantiomerically pure compounds, while (−)-camphene, (−)-α-pinene, (−)-β-pinene were present in mixture with their enantiomers but with a very high ee value. In contrast, the enantiomeric excesses of (−)-sabinene, (+)-β-phellandrene, (−)-limonene, (+)-linalool, and (−)-α-terpineol were only moderate, while terpinen-4-ol and trans-linalool oxide were almost racemic. These results further confirm that secondary metabolites can be present in plants as enantiomeric mixtures.

Table 4. Enantiomeric analysis of the components of *L. mutica* essential oil

Enantiomer	t_R	Enantiomeric distribution [%]	*ee* [%]
(−)-(1*S*,4*R*)-Camphene	9.57	98.48	96.96
(+)-(1*R*,4*S*)-Camphene	10.24	1.52	
(+)-(1*R*)-*α*-Pinene	9.76	5.70	88.61
(−)-(1*S*)-*α*-Pinene	9.82	94.31	
(+)-(1*R*)-*β*-Pinene	10.88	1.88	96.25
(−)-(1*S*)-*β*-Pinene	11.29	98.13	
(+)-(1*R*,5*R*)-Sabinene	12.19	32.97	34.06
(−)-(1*S*,5*S*)-Sabinene	12.93	67.03	
(−)-(*R*)-*α*-Phellandrene	14.38	6.85	86.31
(+)-(*S*)-*α*-Phellandrene	14.54	93.16	
(−)-(*R*)-*β*-Phellandrene	15.90	9.12	81.77
(+)-(*S*)-*β*-Phellandrene	16.60	90.89	
(−)-(*S*)-Limonene	16.44	70.94	41.88
(+)-(*R*)-Limonene	17.39	29.06	
(+)-(1*R*)-Camphor	20.94	> 99%	100
(+)-*trans*-Linalool oxide (furanoid)	18.56	52.89	5.77
(−)-*trans*-Linalool oxide (furanoid)	18.91	47.12	
(−)-(*R*)-Linalool	23.13	27.83	44.35
(+)-(*S*)-Linalool	23.97	72.18	
(−)-(2*R*)-Borneol	24.25	> 99%	100
(+)-(*S*)-Terpinen-4-ol	27.21	44.72	10.57
(−)-(*R*)-4-Terpinen-4-ol	27.47	55.29	
(−)-(*S*)-*α*-Terpineol	29.83	73.64	47.28
(+)-(*R*)-*α*-Terpineol	30.52	26.36	

Antifungal Activity

Different biological properties have been attributed to the essential oils isolated from a few Lepechinia species. They include the antimicrobial activity against *Paenibacillus* larvae for the oil from *L. floribunda*,[2][4][5] in vitro anti-*Vibro cholera* activity for the oil from *L. caul*escens,[6] repellent activity against *Tribolium castaneum*[8] and high total antioxidant activity (TAA) in the DPPH assay for the oil from *L. schiedeana*,[13-15] and insecticidal activity against *Drosophila melanogaster* for the oil from *L. chamaedryoides*.[29] To the best of our knowledge, the biological activity of L. mutica essential oil (Table 5) has not yet been investigated. We tested the antifungal activity of the oil against three severe human fungal pathogens*, Candida albicans, Trichophytum rubrum* and *Microsporum canis*, and two potent plant pathogens, *Pyricularia oryzae* and *Fusarium graminearum*. Compared to the positive controls (amphotericin B and voriconazole), the essential oil exhibited moderate activity against *M. canis* and *T. rubrum*, having MIC values ranging between 2.2 and 4.5 mg/ml.

Table 5. MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration) values (mg/ml) of the essential oil hydrodistilled from *L. mutica*

Sample	*Candida albicans* (human pathogen)	*Trichophyton rubrum* (human pathogen)	*Microsporum canis* (human pathogen)	*Pyricularia oryzae* (plant pathogen)	*Fusarium graminearum* (plant pathogen)
Oil	*MIC* > 9 mg/ml	2.2 < *MIC* ≤ 4.5 mg/ml; *MFC* > 9 mg/ml	2.2 < *MIC* ≤ 4.5 mg/ml; 4.5 < *MFC* ≤ 9 mg/ml	*MIC* > 9 mg/ml	*MIC* > 9 mg/ml
Amphotericin B [a]	*MIC* = 0.001 mg/ml	*MIC* = 0.5 mg/ml	*MIC* = 0.0005 mg/ml	n.d.	n.d.
Voriconazole [a]	*MIC* = 0.00006 mg/ml	*MIC* = 0.5 mg/ml	*MIC* = 0.00025 mg/ml	n.d.	n.d.
Flutriafol PESTANAL ® [a]	n.d.	n.d.	n.d.	*MIC* = 0.04 mg/ml	n.d.

[a]Positive reference antifungal compound. n.d. = not determined

Conclusions

The chemical composition of the essential oil hydrodistilled from the leaves of L. mutica determined in this work resulted to be quite different from that previously found by Malagón et al.[19] In this investigation we identified 79 components of the essential oil, which represented 97.30% the total sample. Sesquiterpene hydrocarbons (38.54%) and monoterpenes hydrocarbons (30.59%) were the prevalent groups of compounds, while oxygenated sesquiterpenoids (16.22%) and oxygenated monoterpenoids (2.07%) were present in minor amounts. The most abundant components were shyobunol (10.80%), Δ3-carene (8.69%), δ-cadinene (6.96%), globulol (5.91%), and (E)-caryophyllene (4.55%), while the most sensorially important components, determined by AEDA GC-O, were α-pinene, β-phellandrene, and dauca-5,8-diene, possessing typical woody, herbaceus, and earthy odors, respectively. Enantioselective GC/MS analysis of L. mutica essential oil revealed the presence of twelve enantiomeric couples and two enantiomerically pure monoterpenes. Their enantiomeric excesses varied from a few percent units to virtually 100%. Moreover, the essential oil exhibited moderate in vitro activity against five fungal strains, being especially effective against M. canis, which is a severe zoophilic dermatophyte causal agent of pet and human skin infections (tinea). This oil might be used for an alternative antifungal treatment of infected men and animals, as well as for the remediation of indoor environments contaminated by infected hair shedding, which is a potential source of new infections.

Experimental Section

Plant Material and Preparation of the Essential Oil

The collection of *L. mutica* leaves, authorized by the Ministry of Environment of Ecuador (MAE) (authorization No. 001-IC-FLO-DBAP-VS-DRLZCH-MA), was performed in the Quilanga region of the Loja Province, Ecuador, in November and December 2009. The plant was identified by Bolivar Merino, 'Herbarium of the Universidad Nacional Loja'. A voucher specimen, with the No. PPN-la-005, has been deposited with the Herbarium of the 'Universidad Técnica Particular de Loja'. The essential oil, d20 = 0.916 ± 0.026, n20 = 1.4867 ± 0.0009, [α]D20 = −5.8 (neat), was obtained in 0.40 ± 0.12% yield (w/w) by steam distillation of fresh leaves (approximately 10.36 kg) in a Clevenger-type apparatus for four hours. Subsequently, the essential oil was tagged and stored in a brown vial at 4 °C until analysis.

GC/MS Analysis

Qualitative analysis of the essential oil (six replicates) was performed by GC/MS using an Agilent Chromatograph (Model 6890N series), coupled to a mass spectrometer detector (model Agilent series 5973 inert). The spectrometer, controlled by the data system MSD-Chemstation D.01.00 SP1, operated at 70 eV; electron multiplier 1600 V; scan rate: 2 scan/s; mass range: 40 – 350 m/z. A non-polar capillary column, DB-5MS 5%-phenyl-methylpolysiloxane, 30 m × 0.25 mm, thickness 0.25 µm film, was used. Samples were dissolved in dichloromethane. An autosampler (series 7673) was used. Helium was the carrier gas at a flow rate of 1.0 ml/min in constant flow mode; the detector and injector temperatures were set at 250 °C. The injector operated in split mode (split ratio 20:1). The oven temperature was set at 60 °C for 5 min, then increased to 110 °C, with a gradient rate of 5 °C/min, followed by an increase to 148 °C with a gradient of 2 °C/min. A third gradient rate of 20 °C/min increased the temperature to 250 °C, which was hold for 2.4 min. The ion source temperature was 250 °C. Chemical components of L. mutica essential oil were identified by comparing their EI-MS spectra with the spectra of compounds having close retention indices (RI) reported in the Adam's comprehensive work.[27] Retention indices were determined, according to Van Den Dool and Kratz,[30] on the basis of the retention times of a homologous series of hydrocarbons C10 – C25 (TPH-6RPM of CHEM SERVICE), which were analyzed by GC under the same conditions.

GC-FID Analysis

The analysis of the essential oil (six replicates) was carried out on an Agilent Technologies chromatograph (model 6890N series), using a flame ionization detector (FID). The percentage content of each oil component was computed from the corresponding GC-FID peak area without applying any correction factor. The analytical parameters were the same as the GC/MS analysis.

GC-O Analysis by Incremental Dilution Technique (AEDA)

GC-O-MS analysis was performed using an Agilent Technologies chromatograph (model 6890N series), and a Gerstel Olfactory Detection Port ODP 3. The same analytical parameters as those used in the GC/MS analysis were applied. GC-O analysis was performed by injecting, at incremental dilutions (1, 2, 4, 8 times with respect to the original solution of 200 µl/ml corresponding to FD = 1), the essential oil dissolved in CH_2Cl_2, while two trained panelists signaled the perceived odors by pushing a button, without seeing the chromatogram in progress.[25] Furthermore, the panelists were asked to describe the odor perceived at the sniffing port in the two analyses of the oil. During AEDA, stepwise dilutions of the original oil were performed and the diluted samples were then evaluated by GC-O to provide flavour dilution (FD) factors, i.e., the highest oil dilution at which the odor of analyzed compound could distinctively be detected. The overall results obtained with AEDA have been reported in an aromagram (Figures 1 and 2), showing the exponential (2x) FD values against the retention indices (RI).

Enantioselective GC Analysis

Enantioselective GC/MS analysis was performed (two replicates) using a Shimadzu QP2010 GC/MS system. The mass spectrometer operated in electron impact ionization mode at 70 eV, with a mass range of m/z 35 – 350 full scan mode. The ion source temperature was set at 200 °C. Helium was the carrier gas at a flow rate of 1.0 ml/min. The injector operated in split mode (split ratio 20:1) at 200 °C, with a transfer line at 230 °C. The oven temperature was set at 50 °C for 2 min, and then increased to 220 °C, with a gradient rate of 2 °C/min, which was hold for 2.0 min. A chiral capillary column, 30% 2,3-diethyl-6-tert-butyldimethylsilyl-β-CDX dissolved in Silicon PS 086 (25 m × 0.25 mm × 0.25 µm) from Mega (Legnano, MI, Italy), was used. Oil samples were dissolved in cyclohexane. The enantiomer order in the enantioselective GC/MS analysis was obtained by separated injections of enantiomerically pure standards.

Physical Properties of the Essential Oil

The relative density, refractive index, and optical activity of the essential oil were determined as the means of three different experiments done at 20 °C by using a pycnometer (5 ml) and an analytical balance (METTLER AC100), a refractometer (model ABBE), and a Perkin–Elmer 241 polarimeter, respectively.

In Vitro Evaluation of Antifungal Activity

The in vitro antifungal activity of the oil was tested against different strains belonging to the fungal collection deposited at the *Laboratory of Mycology, University of Pavia, Candida albicans (C.P. Robin) Berkhout, Microsporum* canis E. Bodin ex Guég., *Trichophyton rubrum* (Castell.) Sabour., *Fusarium graminearum* Schwabe, and *Pyricularia oryzae* Cavara. The first three fungi were isolated from human patients suffering of cutaneous mycoses, while the last two fungi were isolated, respectively, from plants of rice (*Oryza sativa* L.) with blast disease and plants of barley (*Hordeum vulgare* L.) infected by FHB (*Fusarium* Head Blight). All fungi were cultured and maintained on Sabouraud agar (Oxoid, Basingstoke, UK) before performing the antifungal tests.

The Minimum Inhibitory Concentration (MIC) of the essential oil was determined by broth-microdilution method using 96 well flat-shaped microtitre plates (Sigma–Aldrich), according to Gadd[31] and the Clinical and Laboratory Standards Institute[32][33] procedures, with minor modifications. The MIC is defined as the lowest drug concentration completely inhibiting observable fungal growth compared to the control. The essential oil was added to the liquid culture medium RPMI 1640 in micro-wells at a final concentration from 10 to 1 µl/ml. 0.002% Tween 80 (v/v) was included to enhance oil solubility. Inoculum suspensions were prepared by transferring fungi in 2 ml of sterile water with 0.85% NaCl (API BioMerieux) adjusted to 0.5 McFarland by nephelometric measurement. At first, the filamentous fungi were homogenously disrupted by vortex in glass tubes containing sterile water and sterile broken cover glasses. Total volume in each micro-well was 100 µl. Incubating temperature was 25 °C, except for C*. albicans* (cultured at 37 °C). Test plates were examined after 24 and 48 h for *C. albicans*, and after 5 days for *T. rubrum, M. canis, P. oryzae*, and *F. graminearum*. Amphotericin B, voriconazole (ATB Fungus 3, BioMerieux), and flutriafol PESTANAL®, containing the antifungal compound (R,S)-2,4-difluoro-α-(1H-1,2,4-triazol-1-ylmethyl)benzhydryl (Sigma–Aldrich), were used as positive controls. To determine the Minimum Fungicidal Concentration (MFC) by broth-microdilution method, the initial inoculum was sub-cultured from microwell plates containing the extract where no fungal growth was observed (100% inhibition) in fresh culture medium, free of the essential oil, and Petri plates were examined for 10 days at 24 h interval.[31-33] The MFC is defined as the sample lowest concentration causing total reduction of the initial inoculum on culture medium. All bioassays were performed in triplicate.

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Author Contribution Statement

J. R., G. G., and J. M. collected the plant material. M. J. and J. M. performed the olfactometric analysis. J. R., C. C., and G. G. performed the chemical and enantiomeric analyses. J. R., G. V., and C. B. wrote and revised the article. J. R. and G. G. elaborated the analytical data. M. R. and M. L. G. performed the biological activity test.

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