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Antimicrobial activity of some essential oils against *Streptococcus agalactiae*, an important pathogen for fish farming in Brazil

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ABSTRACT

The antimicrobial activity of the essential oils from *Lippia alba, Lippia sidoides, Mentha piperita, Ocimum gratissimum* and *Zingiber officinale* was tested against *Streptococcus agalactiae*. The major compounds in the oils, identified by gas chromatography and mass spectrometry (GC/MS), were geranial (25.4%), neral (16.6%) and caryophyllene oxide (16.0%) in *L. alba*; thymol (76.6%), *p*-cymene (6.3%) and β-caryophyllene (5.0%) in *L. sidoides*; menthol (30.5%), menthyl acetate (14.6%), pulegone (14.2%) and menthone (12.9%) in *M. piperita*; eugenol (43.3%), 1,8-cineole (28.2%) and β-selinene (5.5%) in *O. gratissimum*; and geranial (23.2%), neral (16.7%) and 1,8-cineole (15.8%) in *Z. officinale*. All essential oils evaluated showed bactericidal action against *S. agalactiae* with minimum inhibitory concentration (MIC) ranging from 312.5–2,500 μg mL^{-1} and minimum bactericidal concentration (MBC) ranging from 416.7–2,500 μg mL^{-1}. In this study, *L. sidoides* essential oil showed the better results against *S. agalactiae*.

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KEYWORDS
Medicinal plants; essential oil composition; antimicrobial activity; group B *Streptococcus*

Introduction

Bacterioses are a limiting factor in fish farming, decreasing productivity, delaying fish growth and causing mortality in the world of aquaculture (1). The etiologic agents of disease in farmed fish in Brazil are *Aeromonas hydrophila, Flavobacterium columnare* and *Streptococcus* sp (2–5). The most commonly encountered *Streptococcus* species in fish is *Streptococcus agalactiae*, isolated from outbreaks in Nile Tilapia (*Oreochromis niloticus*) cultured in Brazil (2). This pathogen is frequently associated with meningoencephalitis and septicemia (6,7).

Antibiotics are commonly used for the treatment of bacterial diseases in aquaculture systems (1). However, the frequent use of antibiotics can lead to drug-resistant bacteria selection (8,9). Thus, essential oils and extracts from plants represent an alternative for the control of fish diseases (10,11). To evaluate this possibility, the essential oils of five different plants were tested. These species were selected since they are cultivated, easily obtained and used for food flavouring locally, and all have been reported as presenting antimicrobial properties.

The *Lippia* genus has about 200 species (12,13). *Lippia alba* ('erva-cidreira') is native to Brazil and the major compounds in its essential oil are citral, carvone and linalool (14,15). *Lippia sidoides* (pepper-rosmarin) exhibits in its essential oil up to 60% of thymol or mixtures of thymol and carvacrol (16,17). These *Lippia* species showed anaesthetic, antimicrobial and anti-parasitic activity (18–23).

Mentha piperita (peppermint) is an aromatic species from Europe and its essential oil contains menthol, menthofuran and pulegone as the main components. Antioxidant, immunostimulants, anti-parasitic and antimicrobial properties against Gram-positive and Gram-negative bacteria have been described (21,24–27).

Ocimum gratissimum (clove basil) is widely distributed in tropical regions and the major compound in the essential oil is eugenol. The oil was described as anesthetic, immunostimulant and anti-parasitic (28–30). The antimicrobial activity of *O. gratissimum* against *A. hydrophila* was registered by Sutili et al. (31).

Zingiber officinale (ginger) essential oil is rich in geranial, neral, cineole, borneol, zingiberene and bisabolene (32–34). Anti-inflammatory, antifungal, immunostimulants and antimicrobial activities were described for this oil (35–37). Ginger oil was shown to be effective against *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus* (38,39).

In this study, the chemical composition of the essential oils from *L. alba, L. sidoides, M. piperita, O.*

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gratissimum and *Z. officinale* was investigated, as well as their antimicrobial activity against *S. agalactiae*.

Materials and methods

Plant material

L. alba, L. sidoides, M. piperita, O. gratissimum and *Z. officinale* specimens were grown in the Medical Plant Collection of Embrapa Amazônia Ocidental in Manaus, Amazonas State, Brazil. The plants were identified and deposited in the IAN Herbarium of the Botany Laboratory of Embrapa Amazônia Oriental, Belém, in Pará State. After harvest in March 2016, the parts of the plants (leaves and inflorescences or rhizomes) were collected and dried at room temperature, in an open shed, in the shade and then stored for essential oil distillation.

Essential oil distillation

Essential oils from *L. alba, L. sidoides, M. piperita, O. gratissimum* and *Z. officinale* were obtained by hydrodistillation in a Clevenger-type apparatus for 2 h. In each distillation, 500 g of leaves and inflorescences or rhizomes were used. The essential oils collected were stored at 4°C until analysis. For use, the essential oils were dissolved in dimethylsulfoxide – DMSO (1:4) to prepare stock solutions, which were sterilized by filtration through a 0.22 μm membrane filter.

Essential oil composition

The chemical components of *L. alba, L. sidoides, M. piperita, O. gratissimum* and *Z. officinale* essential oils were analysed by gas chromatography (GC), and GC coupled to mass spectrometry (MS). Analyses were performed in an Agilent (Palo Alto, USA) 7890A gas chromatograph fitted with a 5% phenyl/95% methyl silicone (HP5, 30 m × 0.25 mm × 0.25 mm) fused silica capillary column. Oven temperature was programmed to increase from 60°C to 240°C at 3°C minutes^{-1}. Hydrogen was used as carrier gas (1.4 mL minutes^{-1}). The oils were diluted to 1% in dichloromethane and 1.0 mL of this solution was injected in split mode (1:100). The injector was kept at 250°C and the flame ionization detector (FID) at 280°C.

Mass spectra were obtained in an Agilent 5973N MSD system operating in electronic ionization mode (EI) at 70 eV, with scan mass range of 40–500 m z^{-1}. The sampling rate was 3.15 scans s^{-1}. The ion source was kept at 230°C, mass analyser at 150°C and transfer line at 260°C. All chromatographies were the same as described above. Helium was used as carrier gas (1.0 mL minutes^{-1}).

Linear retention indices were calculated by injecting a series of n-alkanes (C7–C26) into the same column, adopting the conditions used for GC analyses. Oil components were identified by a computer search using the 6th edition of the Wiley Registry of Mass Spectral Data, and comparison between calculated linear retention indices and data from the literature (40).

Antimicrobial activity

Microorganisms and culture conditions

The bacterium *S. agalactiae* (KJ561060) was isolated from a diseased tilapia (*Oreochromis niloticus*) at Aquaculture Center of the Paulista State University (CAUNESP), Jaboticabal, SP. The strain was stored in BHI (brain heart infusion, Himedia, Mumbai, India) supplemented with 30% sterile glycerol at −80°C in the Laboratory of Fish Culture at Embrapa Amazônia Ocidental. For reactivation, the strain was plated on TSA (trypticase soy agar, Himedia, Mumbai, India) supplemented with 5% sheep blood agar and incubated for 48 h at 35°C. Verification of strain purity was accomplished by Gram staining and visualization under a light microscope (1000 × magnification).

Antimicrobial assay

Antimicrobial activity was assayed by broth microdilution method according to procedures using 96-well plates, described by Clinical Laboratory Standard Institute (M7-A6) (41). Medium without inoculum or essential oils were used as negative controls, and medium with inoculum was used as positive control, under the same conditions. Cloramphenicol (Flucka BioChemika, St. Gallen, Switzerland) was used as positive control against bacteria (0.2 μg mL^{-1}). Microdilutions were performed for each oil type at final concentrations of 20,000, 10,000, 5,000, 2,500, 1,250, 625 and 312.5 μg mL^{-1}.

The results were based on visual growth of microorganism, which was confirmed by aseptical addition of the sterile aqueous solution of 0.5% triphenyl tetrazolium chloride (TTC) to the plate-wells and incubated at 35°C for 1 h. Results were obtained in triplicate and expressed as minimum inhibitory concentration (MIC), defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after 24 h of incubation. Bactericidal action of the essential oil were evaluated by adding 20 μL of the microbial culture, removed from wells with

concentrations equal to or higher than the MIC, inoculated on Mueller Hinton agar plates and incubated at 35°C for 24 h. After incubation, the plates were read and minimum bacterial concentration (MBC) was assigned to the lowest concentration without bacterial growth. Experiments were conducted in triplicate.

Statistical analysis

Data on MIC and MBC were evaluated by ANOVA and Tukey's test ($p < 0.05$) to compare the action of the essential oil plant species evaluated against *S. agalactiae*.

Results and discussion

Essential oil composition

The essential oil yields for *L. alba, L. sidoides, M. piperita, O. gratissimum* and *Z. officinale* were 0.30%, 4.36%, 1.10%, 2.36% and 0.68%, respectively. The chromatographic profiles of the essential oils are shown in Figures 1–5 and the chemical composition in Table 1.

Forty-seven compounds were identified represented in the essential oil of *L. alba*, accounting for 89.5% of the oil composition, with geranial (25.4%), neral (16.6%) and caryophyllene oxide (16.0%) predominating. The monoterpenes geranial and neral are the main constituents of the citral chemotype (14,15). The main reason to variations on chemical constituents of *L. alba* essential oil is attributed to genetic factors (14,15).

In the essential oil of *L. sidoides*, twenty-one compounds were identified, accounting for 98.6% of its essential oil composition. The major compounds were thymol (76.6%), *p*-cymene (6.3%) and β-caryophyllene (5.0%). This profile was confirmed in others studies, with some variations in oil component proportions (42,43). The variations in content of *L. sidoides* essential oil are attributed to age and have low genetic control (43).

Nineteen compounds were identified in the essential oil of *M. piperita*, comprising 93.8% of the oil composition. Quantitatively, the most abundant were menthol (30.5%), menthyl acetate (14.6%), pulegone (14.2%) and menthone (12.9%). Similar results were observed for African peppermint essential oil, in which major compounds were menthol (39.3%) and menthone (25.2%), although the contents were higher than observed in our study (44).

In the essential oil of *O. gratissimum*, eighteen compounds were identified, accounting for 97.1% of the oil composition. The major compounds were eugenol (43.3%), 1,8-cineole (28.2%) and β-selinene (5.5%). Other reports have shown that chemical composition percentages of *O. gratissimum* were higher than ours with eugenol (68.8%) followed by methyl eugenol (13.2%) (45).

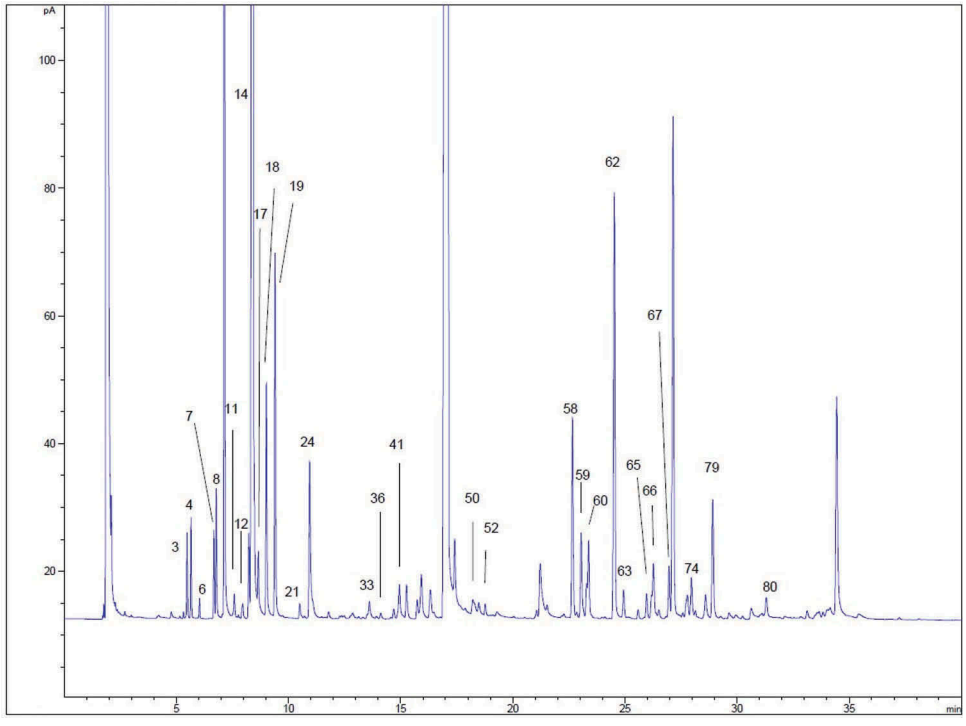

Figure 1. GC chromatogram of essential oil from *Lippia alba*.

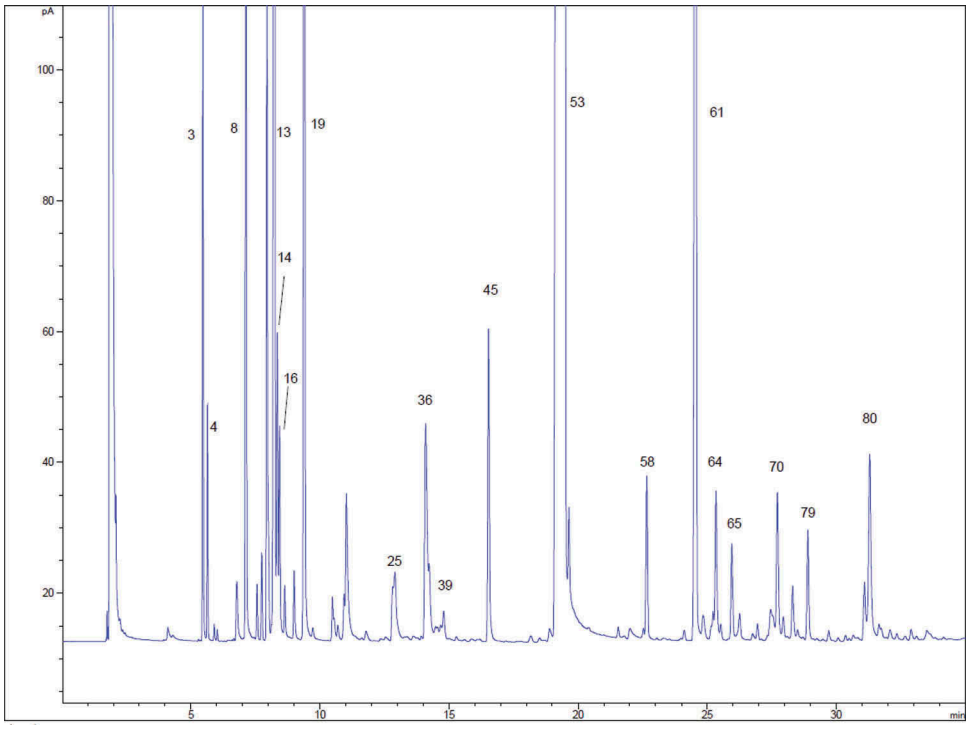

Figure 2. GC chromatogram of essential oil from *Lippia sidoides*.

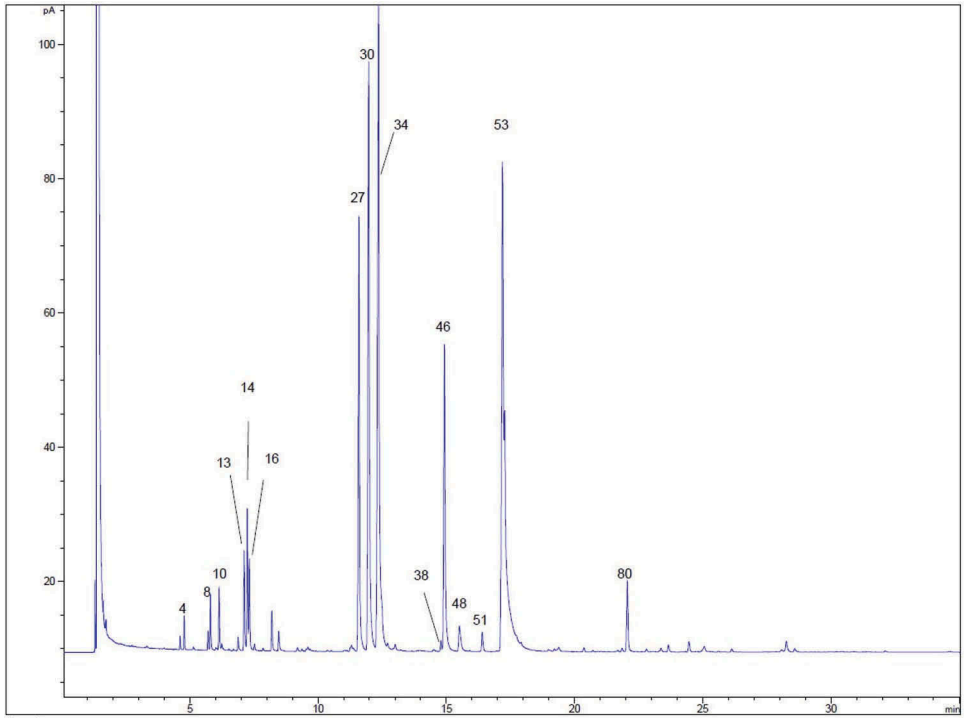

Figure 3. GC chromatogram of essential oil from *Mentha piperita*.

Twenty-seven compounds were identified in the essential oil of *Z. officinale*, comprising 98.9% of the oil composition. The main compounds were geranial (23.2%), neral (16.7%) and 1,8-cineole (15.8%) and in minor levels, α-zingiberene (2.1%), β-sesquiphellandrene (1.1%) and *ar*-curcumene (1.0%). However, in the commercial ginger essential oil, the compounds geranial, neral, α-curcumene and 1,8-cineol were not found, but they were present in this study. The dominant components of this commercial

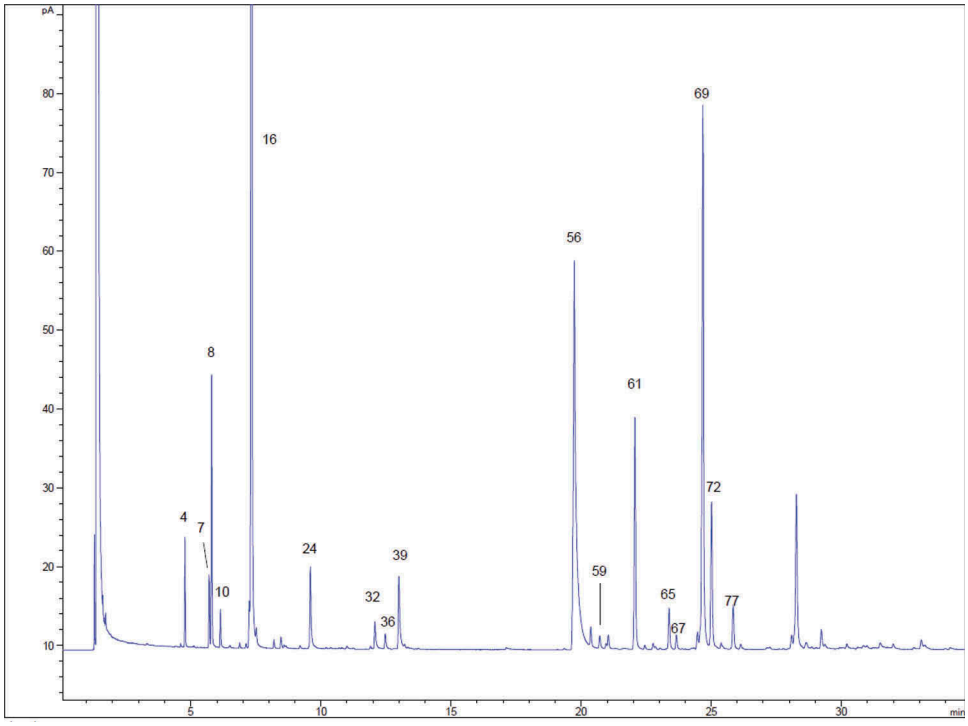

Figure 4. GC chromatogram of essential oil from *Ocimum gratissimum*.

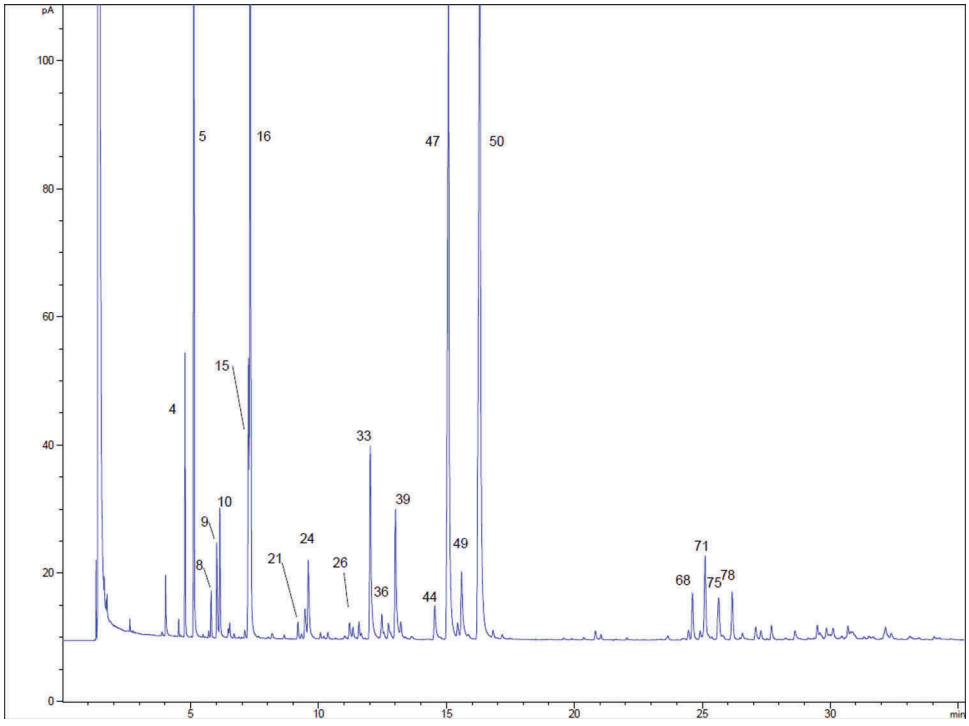

Figure 5. GC chromatogram of essential oil from *Zingiber officinale*.

ginger are zingiberene, β-bisabolene, β-sesquiphellandrene and < ar> curcumene, and these compounds are identified in this study at minor proportions (33).

In general, several factors affect both the yield and composition of essential oils, including geographical origin, climate conditions, seasonality, stage of plant

Table 1. Chemical composition (%) of *Lippia alba, Lippia sidoides, Mentha piperita, Ocimum gratissimum* and *Zingiber officinale* essential oils.

	Components	*L. alba*	*L. sidoides*	*M. piperita*	*O. gratissimum*	*Z. officinale*	AI	RI*
1	2-heptanone	-	-	-	-	1.0	889	890
2	tricyclene	-	-	-	-	0.3	921	922
3	α-thujene	0.2	0.3	-	-	-	924	929
4	α-pinene	0.3	0.1	0.3	-	2.9	932	932
5	camphene	-	-	-	-	11.3	946	947
6	benzaldehyde	0.2	-	-	-	-	952	965
7	sabinene	0.1	-	-	0.7	-	969	975
8	β-pinene	0.4	-	0.6	2.8	0.6	974	976
9	6-methyl-5-hepten-2-one	-	-	-	-	1.2	981	985
10	myrcene	2.0	1.1	0.4	0.7	1.8	988	988
11	α-phellandrene	0.5	-	-	-	0.3	1002	1005
12	α-terpinene	0.3	0.7	-	-	-	1014	1019
13	*p*-cymene	0.7	6.3	0.3	-	-	1020	1022
14	limonene	-	0.4	3.2	-	-	1024	1025
15	β-felandrene	-	-	-	-	4.0	1025	1028
16	1,8-cineole	-	0.7	1.6	28.2	15.8	1026	1030
17	*cis*-ocimene	0.6	-	-	3.7	-	1032	1038
18	*trans*-ocimene	0.3	-	-	-	-	1044	1052
19	γ-terpinene	0.4	2.0	-	-	-	1054	1061
20	*cis*-sabinene hydrate	0.2	-	-	-	-	1065	1071
21	terpinolene	-	-	-	-	0.3	1086	1088
22	*p*-cymenene	0.7	-	-	-	-	1089	1091
23	epoxy myrcene	0.5	-	-	-	-	1090	1095
24	linalool	1.5	-	-	1.3	1.8	1095	1100
25	ipsdienol	-	0.6	-	-	-	1140	1148
26	camphor	0.3	-	-	-	0.4	1141	1142
27	menthone	-	-	12.9	-	-	1148	1151
28	citronellal	-	-	-	-	0.3	1148	1152
29	methyl campheniol	0.2	-		-	-		1154
30	menthofurane	-	-	1.5	-	-	1159	1160
31	*neo*-menthol	-	-	2.4	-	-	1161	1163
32	δ-terpineol	-	-	-	0.4	-	1162	1166
33	borneol	0.3	-	-	-	4.3	1165	1164
34	menthol	-	-	30.5	-	-	1167	1175
35	umbelulone	-	0.2	-	-	-	1167	1176
36	terpinen-4-ol	1.2	1.0	-	0.4	0.6	1174	1178
37	(*E*)-isocitral	-	-	-	-	0.5	1177	1182
38	*neo*-isomenthol	-	-	1.0	-	-	1184	1190
39	α-terpineol	-	0.2	-	1.1	2.8	1186	1190
40	myrtenal	-	-	-	-	0.5	1195	1190
41	*trans*-dihydrocarvone	0.5	-	-	-	-	1200	1197
42	β-fenchyl alcohol	0.2	-	-	-	-		1208
43	citronellol	-	-	-	-	1.0	1223	1228
44	nerol	0.2	-	-	-	-	1227	1231
45	thymol methyl ether	0.6	1.0	-	-	-	1232	1236
46	pulegone	-	-	14.2	-	-	1233	1237
47	neral	16.6	-	-	-	16.7	1235	1242
48	piperitone	-	-	1.5	-	-	1249	1250
49	geraniol	0.2	-	-	-	1.9	1249	1254
50	geranial	25.4	-	-	-	23.2	1264	1272
51	*neo*-menthyl acetate	-	-	0.8	-	-	1271	1271
52	bornyl acetate	0.3	-	-	-	-	1287	1289
53	thymol	-	76.6	-	-	-	1289	1296
54	menthyl acetate	-	-	14.6	-	-	1294	1292
55	β-cubebene	0.2	-	-	-	-	1387	1289
56	eugenol	0.1	-	-	43.3	-	1357	1357
57	cyclosativene	0.2	-	-	-	-	1369	1370
58	α-copaene	1.0	0.4	-	-	-	1374	1375
59	β-bourbonene	0.2	-	-	0.9	-	1387	1381
60	β-elemene	2.0	-	-	0.8	-	1389	1389
61	(*E*)-caryophyllene	-	-	0.3	3.7	-	1417	1415
62	β-caryophyllene	6.6	5.0	-	-	-		1417
63	β-gurjunene	0.4	-	-	-	-	1431	1431
64	aromadendrene	0.5	0.4	-	-	-	1439	1436
65	α-humulene	0.6	0.3	-	0.6	-	1452	1450
66	*allo*-aromadendrene	0.7	-	-		-	1458	1460
67	γ-muurolene	0.2	-	-	0.9	-	1478	1477
68	*ar*-curcumene	-	-	-	-	1.0	1479	1481
69	β-selinene	1.7	-	-	5.5	-	1489	1482
70	ledene	-	0.3	-	-	-		1492

(*Continued*)

Table 1. (Continued).

	Components	*L. alba*	*L. sidoides*	*M. piperita*	*O. gratissimum*	*Z. officinale*	AI	RI*
71	α-zingiberene	-	-	-	-	2.1	1493	1494
72	α-selinene	1.3	-	-	1.7	-	1498	1496
73	menthalactone	-	-	5.3	-	-		1495
74	α-muurolene	0.4	-	-	-	-	1500	1501
75	(*E,E*)-α-farnesene	-	-	-	-	1.2	1505	1508
76	γ-cadinene	0.2	-	-	-	-	1513	1505
77	7-*epi*-α-selinene	-	-	-	0.4	-	1520	1513
78	β-sesquiphellandrene	-	-	-	-	1.1	1521	1522
79	δ-cadinene	1.2	0.3	-	-	-	1522	1521
80	caryophyllene oxide	16.0	0.7	0.4	-	-	1582	1572
81	viridiflorol	-	-	2.0	-	-	1592	1584
82	humulene epoxide II	1.1	-		-	-	1608	1612
	Total identified	89.5	98.6	93.8	97.1	98.9		

Note: RI = Retention Index in HP-5, AI = Arithmetic Index.

development, procedures adopted for plant processing and oil extraction, and plant chemotype (46).

Antimicrobial activity

Essential oils of *L. alba, L. sidoides, M. piperita, O. gratissimum* and *Z. officinale* showed antimicrobial activity against *S. agalactiae*, with MIC ranging from 312.5–2,500 μg mL^{-1} and MBC from 416.7–2,500 μg mL^{-1} (Table 2). It was demonstrated that all essential oils evaluated showed bactericidal activity against *S. agalactiae*.

The highest antimicrobial activity was exhibited by *L. sidoides* essential oil (MIC = 312.5 μg mL^{-1} and MBC = 416.7 μg mL^{-1}), and the lowest activity by *O. gratissimum* essential oil (MIC = 2,500 μg mL^{-1} and MBC = 2,500 μg mL^{-1}). Therefore, the most effective oil against *S. agalactiae* was *L. sidoides*, followed by *Z. officinale, M. piperita, L. alba* and *O. gratissimum* (Table 2).

The essential oil of *L. alba* at a concentration of 1,666.7 μg mL^{-1} inhibited *S. agalactiae* growth. Sutili et al. (47) and Majolo et al. (22) described the bacteriostatic and bactericidal activity of *L. alba* against Gram-negative bacteria such as *A. hydrophila*. In the present study, this oil was effective against *S. agalactiae*, which is Gram-positive. The essential oil of another *Lippia* species, *L. sidoides*, showed the highest activity against *S. agalactiae*. Similar results were observed by Majolo et al. (22) in tests against *A. hydrophila*, where they attributed the effect to the thymol contained in the oil. A stronger antimicrobial action can be obtained using isolated oil compounds, as shown by Heo et al. (48), who obtained MIC from 10–80 μg mL^{-1} using thymol against *A. salmonicida*. Earlier investigations corroborate the antimicrobial activity of essential oil of *Lippia* species (*L. origanoides, L.* aff. *gracillis* and *L. grandis*) against Gram-negative and Gram-positive bacteria, but these reports indicating that Gram-positive bacteria are more sensitive to the essential oils compared to Gram-negative bacteria (49–51).

Table 2. Antimicrobial activity of essential oils of bioactive plants.

Species	MIC (μg.mL^{-1})	MBC (μg.mL^{-1})
Lippia alba	1,666.7 ± 721.7ab	1,666.7 ± 721.7ab
Lippia sidoides	312.5 ± 0.0b	416.7 ± 180.4b
Mentha piperita	1,250 ± 0.0ab	1,250 ± 0.0ab
Ocimum gratissimum	2,500 ± 0.0a	2,500 ± 0.0a
Zingiber officinalis	625 ± 0.0ab	833.3 ± 360.8ab

Note: Values are given as mean± SD. Means followed by different letter in a column differs statistically by Tukey test ($p < 0.05$). MIC = minimum inhibitory concentration, MBC = minimum bactericidal concentration

The MIC obtained with *M. piperita* essential oil was 1,250 μg ml^{-1}. Mahboubi & Kazempour (26) found high activity of *M. piperita* essential oil against Gram-positive and Gram-negative bacteria, and the oil they tested had similar composition to that identified in the present study, with menthol (36.9%), menthone (28.8%) and methyl acetate (4.5%) as the main components. Menthol isolated from *M. piperita* was found to have high antimicrobial activity against seven microorganisms, the highest inhibitory effect being observed against *Streptococcus mutans* (52). In an investigation of the antimicrobial potential of other *Mentha* species using the disc diffusion method, Stanisavljević et al. (53) found a significant inhibitory effect of *M. longifolia* essential oil against the Gram-positive bacteria *Bacillus subtilis, Micrococcus luteus* and *Enterococcus faecalis*.

The MIC of *O. gratissimum* essential oil against *S. agalactiae* was 3,200 μg ml^{-1}. Other studies showed higher antimicrobial activity of *O. gratissimum* essential oil against both Gram-positive (*S. aureus, Bacillus* spp.) and Gram-negative bacteria (*E. coli, Pseudomonas aeruginosae, S. typhi, Klebisiella pneumoniae, Proteus mirabilis*) (45). Sutili et al. (31) found *O. gratissimum* essential oil MIC from 200–1,600 μg ml^{-1} against the Gram-negative bacteria *A. hydrophila*. For commercial

isolated eugenol, MIC ranged from 1,600–3,200 μg ml^{-1} (54), which is similar to the value obtained for *S. agalactiae* treated with *O. gratissimum* essential oil containing eugenol as the major compound (43.3%). The mechanism action of eugenol involves the rapid inhibition of the energy metabolism of Gram-positive bacteria (55).

Essential oil of *Z. officinale* also showed antimicrobial action against *S. agalactiae*, with MIC of 625 μg ml^{-1} and MCB of 833.3 μg ml^{-1}. Moderate activity of essential oil of *Z. officinale* var. *rubrum* Theilade was observed against the Gram-positive bacteria *Bacillus licheniformis, Bacillus spizizenii* and *S. aureus* and the Gram-negative bacteria *E. coli, K. pneumoniae* and *Pseudomonas stutzeri*. The major components of essential oil extracted from *Z. officinale* rhizomes are camphene (14.5%), geranial (14.3%) and geranyl acetate (13.7%) (56). The antimicrobial activity of *Z. officinale* is attributed to components such as zingiberene and geranial (57). In the present study, the major compounds of *Z. officinale* essential oil were geranial (23.2%), neral (16.7%) and 1,8-cineol (15.8%), as well as α-zingiberene (2.1%) at minor levels, and they likely participated in antimicrobial activity by synergetic association with other active compounds. And this fact is important to be highlighted for all essential oils evaluated in this study.

Conclusion

The antimicrobial activity of essential oils of *L. alba, L. sidoides, O. gratissimum, M. piperita* and *Z. officinale* against *S. agalactiae* was confirmed in this study and the greatest inhibitory effect was obtained with *L. sidoides* essential oil. The results suggest that essential oils could be applied to treat bacterial fish diseases. However, further investigations are required to evaluate other strains of *S. agalactiae* and isolate the bioactive components of these oils and assess their individual and combined effects, as well as their toxicity and effective dose.

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