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Aquaculture

Ventilatory frequency and anesthetic efficacy in silver catfish, *Rhamdia quelen*: a comparative approach between different essential oils

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ABSTRACT - This study investigated the efficacy of essential oils of *Lippia alba* (EOLA) citral chemotype and *Lippia origanoides* (EOLO) and their effects on ventilatory frequency (VF) of silver catfish, *Rhamdia quelen.* Fish were exposed to 50-300 μL L^{-1} of EOLA and 25-300 μL L^{-1} of EOLO to determine induction times to sedation, anesthesia, and recovery. Moreover, VF was determined in fish exposed to 5 or 10 μL L^{-1} of EOLA and of EOLO for 8 h. The increasing concentration of essential oils proportionally decreased sedation and anesthesia induction times. The highest EOLA concentration increased VF of fish from the control group at 1 h of exposure, but VF decreased at both EOLO concentrations after 2 h. The EOLA citral chemotype and EOLO were effective sedatives and anesthetics for silver catfish. However, EOLO was the most suitable sedative for additional studies regarding fish transport as it reduced VF and did not induce VF increase in silver catfish. The EOLA citral chemotype and EOLO are effective sedatives and anesthetics for silver catfish. Moreover, the EOLO is recommended for transport of silver catfish, because it maintains the ventilatory frequency constant, avoiding a possible metabolic stress.

Key Words: aquaculture, fish, physiology, *Rhamdia quelen*

Introduction

Fish maintained in culture systems and experimental laboratories are susceptible to stressful situations caused by capture, handling, or confinement, possibly causing behavioral, physiological, biochemical, and molecular changes, which can compromise production or experimentation (Barton and Iwama, 1991; Mommsen et al., 1999; Barton, 2002). In view of this, the use of anesthetics obtained from plants (extracts or essential oils) has been investigated, with the result that several of these substances are effective in reducing and/or minimizing stress responses (Cunha et al., 2010a; 2010b; 2011; Becker et al., 2012; 2013; Silva et al., 2012; 2013; Gressler et al., 2014; Parodi et al., 2014; Toni et al., 2014; 2015; Zeppenfeld et al., 2014; Salbego et al., 2014; 2015).

The genus *Lippia* (Verbenaceae) includes approximately 250 species of shrubs, small trees, and herbs and is widely distributed in southern and central American countries, Tropical Africa, the southern United States of America, India, and Australia (Bezerra et al., 1981; Terblanché and Kornelius, 1996; Singh et al., 2000; Day and McAndrew, 2003; Hennebelle et al., 2008). The essential oil (EO) of *Lippia alba* (Mill.) N.E. Brown linalool chemotype (EOLA) is a suitable anesthetic for several fish species (Cunha et al., 2010a, 2011; Toni et al., 2014, 2015; Hohlenwerger et al., 2016); the citral chemotype presented similar anesthetic effect for silver catfish, *Rhamdia quelen* (Quoy and Gaimard, 1824). The EO of *Aloysia tryphilla*, which contains citral as the main compound, also has anesthetic efficacy in silver catfish (Gressler et al., 2014; Parodi et al., 2014). However, no studies regarding fish anesthesia have been performed with *Lippia origanoides* Humboldt, Bonpland, and Kunth, popularly known in northern Brazil as “salva-de-marajó”, a shrub occurring in southern North America to northern South America (Stashenko et al., 2010). The EO of *Lippia sidoides*, a synonymy of *L. origanoides* (O’Leary et al., 2012), revealed anesthetic activity in silver catfish, but caused mucus loss and mortality (Silva et al., 2013).

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Therefore, the present study is the first to report the efficacy of EO of *L. origanoides* (EOLO) and EOLA as anesthetics in fish. Moreover, we analyzed ventilatory frequency to help understand the effects of these EO on fish behavior.

Material and Methods

Silver catfish (40.88±1.21 g, 17.02±0.16 cm) juveniles were obtained from a local fish culture and transferred to a laboratory. Fish were maintained in continuously aerated tanks (250 L) with controlled water parameters (mean±SEM): dissolved oxygen (6.73±0.07 mg L^{-1}), temperature (20.07±0.02 °C), pH (6.72±0.14), alkalinity (47.40±0.80 mg $CaCO_3$ L^{-1}), total ammonia nitrogen (0.6±0.02 mg N L^{-1}), and un-ionized ammonia (0.0042±0.0003 mg N L^{-1}). The photoperiod was 12 h light/12 h dark. We used a semi-static system and changed 50% of the water volume daily to remove uneaten food, residues, and feces. The juveniles were fed twice a day (5.0% biomass) with commercial feed (28% crude protein).

Dissolved oxygen and temperature were determined with a YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA); pH, with a DMPH-2 pH meter (Digimed, SP, Brazil); alkalinity, according to Boyd and Tucker (1992); total ammonia nitrogen levels, through the salicylate method (Verdouw et al., 1978); and un-ionized ammonia was obtained from a conversion table for fresh water.

The methodology of this experiment was approved by the local Ethical and Animal Welfare Committee (case no. 046/2010).

Leaves of *L. alba* were collected in Santarém (Pará, Brazil) in June 2012, and identification was performed by Dr. Fátima Salimena (voucher number CESJ 65276, Universidade Federal de Juiz de Fora, Minas Gerais, Brazil). Leaves of *L. origanoides* were collected in August 2008 in Alter do Chão (Santarém, Pará, Brazil), also identified by Dr. Salimena, and the voucher was deposited in Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) under the number IAN 184688.

Extraction of EO was performed by hydrodistillation for 3 or 6 h through a Clevenger-type apparatus (European Pharmacopoeia, 2007). About 100 g of fresh leaves were immersed in distilled water at a ratio of 1:10 (w/v), and the oil was separated from water after reaching room temperature. Average yield was 1.4% EOLA and 2.7% EOLO. Essential oils were transferred to glass flasks, filled to the top, and kept at a temperature of 10 ºC for further analysis.

Analysis of EOLA and EOLO by gas chromatography-mass spectrometry-thin ion chromatography was performed using an Agilent-6890 gas chromatograph coupled with an Agilent 5973 mass selective detector with a HP5-MS column (5% phenyl, 95% methylsiloxane, 30 m × 0.25 mm i.d. × 0.25 µm) as described by Silva et al. (2012). The chemical constituents of these EO were identified by comparison of the Kovats retention index and mass spectra with a mass spectral library and literature data (NIST/EPA/NIH, 2005; Adams, 2007; Mondello, 2011).

Fish were transferred to 1-L aquaria (n = 10 each concentration) and exposed to the following EO concentrations (in µL L^{-1}): 50, 100, 150, 200, and 300 of EOLA and 25, 50, 100, 200, and 300 of EOLO. All EO were previously diluted in ethanol (1:10). The concentrations tested were slightly different between the EO because their efficacy was variable (see results). Induction times of sedation and anesthesia as well as recovery were evaluated, and anesthesia stages were characterized as described by Small (2003), with a maximum observation time of 30 min. Silver catfish were placed in an aquarium with anesthetic-free water for recovery. We used a digital chronometer to record all times, expressed in seconds.

The second experiment determined ventilatory frequency (VF) using concentrations with potential application in transporting procedures. Ventilatory frequency was quantified at 0, 0.25, 1, 2, 4, and 8 h of exposure, as reported by Alvarenga and Volpato (1995): visual count of 20 successive buccal or opercular movements, recording the elapsed time with a digital chronometer. We used the following EO concentrations (previously diluted in ethanol and expressed in µL L^{-1}): 5 or 10 for EOLA and 5 or 10 for EOLO. Moreover, water (control) and ethanol groups were evaluated.

All results were expressed as mean±SEM. Evaluation of anesthetic activity was performed by regression analysis (concentration × time of anesthesia induction; concentration × time of recovery from anesthesia), using the SigmaPlot version 11.0 software. The homogeneity of variances of VF data was tested with Levene's test. These data did not show homoscedasticity and were subjected to Kruskal-Wallis ANOVA, followed by multiple comparisons of mean ranks for all groups. The software used was Statistica 7.0 (Stat Soft, Tulsa, OK), and the minimum significance level was set at $P<0.05$.

Results

The major components of EOLA were geranial (30.02%), neral (25.26%), and limonene (9.11%), while EOLO was mainly comprised of carvacrol (47.20%), thymol (12.80%), and p-cymene (9.70%) (Table 1).

Table 1 - Chemical composition of the essential oils of *Lippia alba* (EOLA) and *Lippia origanoides* (EOLO)

Compound	Relative percentage (%)		RI exp	RI lit
	EOLA	EOLO		
Hexenal<2E->	0.06	-	846	846
Thujene<alpha->	0.10	-	926	924
Benzaldehyde	0.04	-	958	952
Sabinene	0.35	-	973	969
Dimethyl-4-heptanone<3.5->	0.04	-	975	973
Hepten-2-one<6-methyl-5->	1.40	-	984	981
Myrcene	0.31	1.10	990	988
Phellandrene<alpha->	0.10	-	1005	1002
Isoamyl isobutyrate	0.03	-	1014	1007
Terpinene<alpha->	0.18	-	1016	1014
Cymene<para->	1.35	-	1024	1020
Limonene	9.11*	0.20	1029 (EOLA) 1026 (EOLO)	1024
Cineole<1.8->	0.04	-	1031	1026
Ocimene<(E)-beta->	0.45	-	1046	1044
Bergamal	3.13	-	1058	1051
Sabinene hydrate<cis->(IPP vs OH)	0.08	-	1066	1065
Terpinolene	0.04	-	1088	1086
NI	0.08	-	1097	-
Linalool	0.95	2.90	1099 (EOLA) 1098 (EOLO)	1095
Pinene oxide<alpha->	0.12	-	1104	1099
NI	0.15	-	1138	-
Geijerene	0.12	-	1142	1138
Isocitral<exo->	0.26	-	1143	1140
Necrodol<trans-alpha->	0.25	-	1149	1144
Citronellal	0.34	-	1152	1148
Borneol	0.10	-	1165	1165
Borneol	0.06	-	1174	1165
Terpinen-4-ol	0.27	1.50	1177 (EOLA) 1176 (EOLO)	1174
9 Piperitol<cis->	0.05	-	1200	1195
Carveol<trans->	0.05	-	1218	1215
Citronellol	1.42	-	1229	1223
Mentha-1(7),8-dien-2-ol<cis-p->	0.14	-	1234	1227
Neral	25.26*	-	1244	1235
Carvone	0.07	-	1245	1239
Geraniol	0.17	-	1254	1249
Geranial	30.02*	-	1274	1264
Citronellyl acetate	0.04	-	1353	1350
Eugenol	0.13	-	1357	1356
Neryl acetate	0.11	-	1364	1359
Copaene<alpha->	0.13	-	1377	1374
Geranyl acetate	0.35	0.60	1383 (EOLA) 1382 (EOLO)	1379
Bourbonene<beta->	0.11	-	1386	1382
Cubebene<beta->	0.31	-	1391	1387
Elemene<beta->	0.30	-	1393	1389
Sesquithujene	0.16	-	1406	1405
Cedrene<alpha->	0.17	-	1413	1410
Funebrene<beta->	0.06	-	1415	1413
Caryophyllene(E-)	0.43	-	1420	1417
Copaene<beta->	0.14	-	1430	1430
Humulene<alpha->	0.10	-	1454	1452
Farnesene<E-beta->	0.15	-	1457	1454
Aromadendrene<allo->	0.20	-	1462	1458
Muurolene<gamma->	4.33	-	1482	1478
Amorphene<gamma->	1.14	-	1496	1495

Continues...

Table 1 (Continued)

Compound	Relative percentage (%)		RI exp	RI lit
	EOLA	EOLO		
Muurolene<alpha->	0.19	-	1501	1500
Bisabolene<(Z)-alpha->	0.13	-	1509	1506
Cadinene<delta->	0.45	-	1524	1522
Calamenene<cis->	0.09	-	1536	1528
Copaen-11-ol<alpha->	0.06	-	1541	1539
Elemol	5.24	-	1551	1548
Nerolidol<E->	0.71	-	1564	1561
Globulol	0.66	-	1598	1590
Cubenol<1,10-di-epi->	0.05	-	1621	1618
Eremoligenol	0.11	-	1630	1629
Eudesmol<gamma->	0.39	-	1632	1632
Hinesol	0.05	-	1647	1640
Eudesmol<beta->	0.54	-	1651	1649
Eudesmol<alpha->	0.52	-	1654	1652
Bulnesol	0.05	-	1675	1670
Germacra-4(15),5,10(14)-trien-1-alpha-ol	0.13	-	1686	1685
Bergamotol<(Z)-alpha-trans->	0.26	-	1697	1690
Curcumenol	0.14	-	1738	1733
Geranyl isobutanoate	0.19	-	1514	1514
NI	0.28	-	1516	-
Selinene<7-epi-alpha->	0.11	-	1521	1520
Isocitral<Z->	1.10	-	1163	1160
Isocitral<E->	1.51	-	1182	1177
NI	0.23	-	1194	-
(Z)-Hexen-3-ol	-	0.20	854	859
α-Thujene	-	0.60	926	924
α-Pinene	-	0.40	934	932
1-Octen-3-ol	-	0.10	976	974
α-Terpinene	-	0.50	1016	1014
p-Cymene	-	9.70*	1025	1020
1,8-Cineole	-	1.30	1032	1026
γ-Terpinene	-	0.20	1056	1054
Umbellulone	-	0.30	1169	1167
Thymol methyl ether	-	1.30	1234	1232
Thymol/carvacrol isomer (MW=150)	-	0.40	1282	-
Thymol	-	12.80*	1292	1289
Carvacrol	-	47.20*	1299	1298
Thymol acetate	-	0.40	1351	1349
Carvacrol acetate	-	0.60	1372	1370
(E)-Caryophyllene	-	2.30	1418	1416
trans-α-Bergamotene	-	0.20	1434	1432
α-Humulene	-	0.30	1455	1452
p-Methoxythymol	-	7.40	1487	1484
β-Bisabolene	-	0.30	1506	1505
(Z)-α-Bisabolene	-	0.20	1508	1506
p-Methoxycarvacrol (tent.)	-	1.30	1555	-
Caryophyllene oxide	-	1.60	1584	1582
2-phenylethyl tyglate	-	0.20	1587	1584
Humulene epoxide II	-	0.20	1611	1608
α-Eudesmol	-	0.10	1656	1653
α-Bisabolol	-	0.20	1686	1685
Unidentified sesquiterpenes	-	1.20	-	-
Identified compounds	97.50	97.80		
Unidentified compounds	0.74	-		

NI - not identified component; RI experimental - calculated Kovats retention index; RI literature - reference Kovats retention index (Adams (31), NIST (32), Mondello (33)).
* Relative percentage of main compounds.

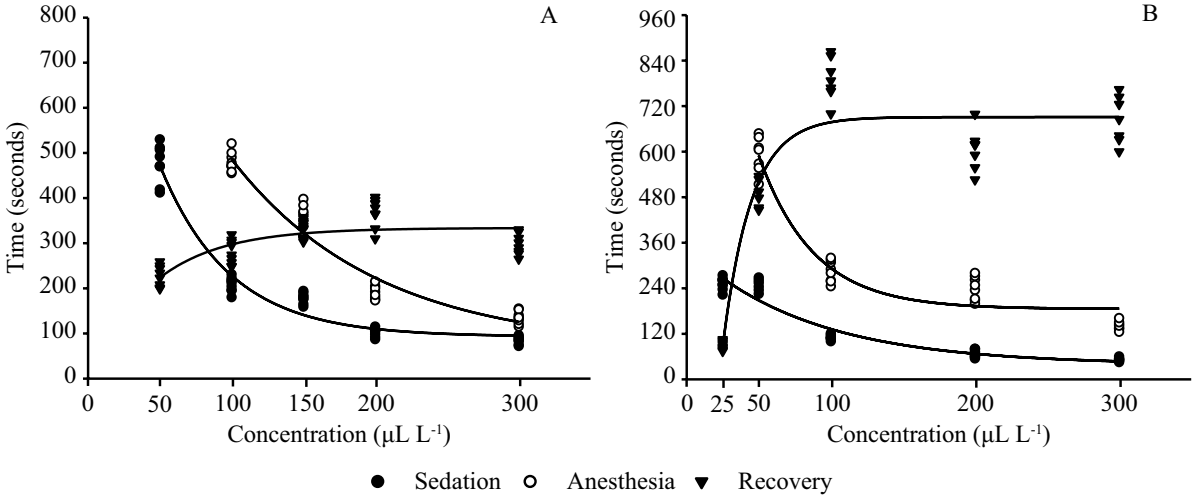
Time to induce sedation with EOLA decreased to around 150 μL L^{-1}, i.e., higher concentrations did not reduce time to induce sedation, and increasing concentration of EOLA proportionally decreased anesthesia induction time (Figure 1A). The lowest concentration of EOLO (25 μL L^{-1}) was only sedative to silver catfish, and equation revealed that concentrations higher than 300 μL L^{-1} do not decrease time to induce sedation with EOLO. Increasing concentrations of EOLO proportionally decreased anesthesia induction times, but concentrations higher than 150 μL L^{-1} did not decrease time to induce anesthesia with EOLO (Figure 1B). Recovery time increased proportionally up to around 150 and 100 μL L^{-1} EOLA and EOLO, respectively (Figure 1).

Ventilatory frequency of the control group decreased significantly 15 min after fish were placed in the aquaria and kept unchanged for 8 h. Silver catfish exposed to ethanol also progressively reduced VF up to 4 h, with significantly higher values than those for control fish after exposure of 15 min to 1 h. Fish exposed to EOLA presented lower VF than control and/or ethanol groups in some measurements, but the highest EOLA concentration also increased VF at 1 h. Ventilatory frequency values of fish exposed to both concentrations of EOLO from 15 min to 8 h were significantly lower than those in control and ethanol groups in all measurements, with the exception of 1 h for the highest concentration of EOLO, in which the values were similar to those in the control (Table 2).

Discussion

Essential oil of *Lippia alba* citral chemotype used in the present study had citral (55.28% geranial + neral) as major constituent, therefore belonging to the citral chemotype (Hennebelle et al., 2008), which was previously observed in another city of Minas Gerais, albeit with a lower citral content (Oliveira et al., 2006). The lowest EOLA concentration used in the present experiment (50 μL L^{-1}) sedated silver catfish within 8 min, and a concentration of 200 μL L^{-1} was needed to anesthetize this species within 3 min, in agreement with the results observed by Souza et al. (2017) in the same species. *A. triphylla* EO, which has citral as its main compound (72% E-citral + Z-citral) (Parodi et al., 2012) also sedated silver catfish within 8 min at 50 μL L^{-1} (Parodi et al. 2014), but a concentration of 200 μL L^{-1} induced anesthesia after 6 to 10 min, depending on fish size (Gressler et al., 2014, Parodi et al., 2014). Even considering fish size, EOLA citral chemotype was more efficient in anesthetizing silver catfish than *A. triphylla* EO, which can probably be explained by the presence of limonene (around 9%) in EOLA. Limonene is the main compound (97.66%) of the *Citrus sinensis* EO, which has an anxiolytic effect in Wistar rats (Faturi et al., 2010).

The main compound of EOLO was carvacrol (47.20%), which corresponds to the carvacrol chemotype of *L. origanoides* (Stashenko et al., 2010). The anesthetic effect of EOLO in silver catfish was expected because,



The following equations were fitted to the data: *L. alba* (sedation: $y = 92.938 + 1066.002e^{(-0.021x)}$, $r^2 = 0.959$; anesthesia: $y = 67.446 + 1127.087e^{(-0.010x)}$, $r^2 = 0.952$; recovery: $y = 1.727 + 331.991*(1-e^{(-0.022x)})$, $r^2 = 0.577$), and *L. origanoides* (sedation: $y = 38.636 + 306.390e^{(-0.012x)}$, $r^2 = 0.932$; anesthesia: $y = 185.427 + 1512.793e^{(-0.026x)}$, $r^2 = 0.929$; recovery: $y = -1457.050 + 2148.272*(1-e^{(-0.051x)})$, $r^2 = 0.906$).

Figure 1 - Times required for induction and recovery from anesthesia in silver catfish, *Rhamdia quelen*, exposed to the essential oils of *Lippia alba* (A) and *Lippia origanoides* (B).

Table 2 - Effects of the essential oils of *Lippia alba* (EOLA) and *Lippia origanoides* (EOLO) on ventilatory frequency (opercular or buccal movements min^{-1}) of silver catfish (*Rhamdia quelen*)

Time of exposure (h)	Group					
	Control		EOLA		EOLO	
	Water	Ethanol	5 μL L^{-1}	10 μL L^{-1}	5 μL L^{-1}	10 μL L^{-1}
0	72.30±2.43ABa	79.21±2.56Aa	78.60±1.39Aa	69.60±2.08Bb	75.16±1.98ABa	43.70±2.03Cab
0.25	48.24±1.27Bb	70.86±2.29Ab	42.34±2.20BCb	38.99±1.90CDc	29.45±1.08Ebc	32.24±1.45DEbc
1	48.57±1.92Cb	62.44±1.98Bc	46.35±1.93Cb	85.91±1.93Aa	36.31±1.71Db	52.04±2.28Ba
2	55.32±1.96ABb	51.76±1.67ABd	44.14±2.27BCb	66.50±2.21Ab	30.84±0.92Cb	32.08±1.89Cbc
4	49.52±1.32Ab	42.09±2.02Ae	45.69±2.26Ab	43.45±2.32Ac	30.17±1.44Bb	25.41±1.09Bc
8	48.83±2.21Ab	44.79±1.33Ae	39.64±2.30Ab	40.04±1.63Ac	21.45±1.22Bc	27.91±1.18Bc

SEM - standard error of the mean.
Values are means ± SEM.
Different uppercase letters in the rows indicate significant differences between groups in the same time ($P<0.05$).
Different lowercase letters in the columns indicate significant differences between times in the same group ($P<0.05$).

in a study by Silva et al. (2013), *L. sidoides* EO (67.89% carvacrol) induced anesthesia in this species. Time to induce sedation with the lowest EOLO concentration in this experiment was similar, but time to induce anesthesia (around 1 min with 200-300 μL L^{-1}) was much shorter than in the study of Silva et al. (2013) (around 20 min with 150-300 μL L^{-1}). In addition, the results of our study differed in several other aspects from those of Silva et al. (2013): we observed no involuntary contractions and jumping behavior toward the surface, and all fish recovered within 10-11 min, even at the highest concentration tested. As carvacrol inhibits acetylcholinesterase activity *in vitro* (Jukic et al., 2007), Silva et al. (2013) hypothesized that this compound could be mainly responsible for involuntary contractions and jumping behavior of silver catfish exposed to *L. sidoides* EO. However, carvacrol had an anxiolytic-like effect in one study (Melo et al., 2010) and increased the latency for the development of convulsions in mice (Quintans-Júnior et al., 2010). It also blocks neuronal excitability by a direct inhibition of the voltage-gated sodium current (Joca et al., 2012), which is similar to the mechanism of action of eugenol (Cho et al., 2008), an effective anesthetic in silver catfish (Cunha et al., 2010b).

Ventilatory frequency of control fish decreased a few minutes after they were placed in aquaria. This was expected, because the presence of the anesthetic in the water may provoke a transitory stress which increases VF levels, as observed previously in this species (Becker et al., 2012). The same was observed in silver catfish exposed to 10-20 μL L^{-1} EOLA chemotype linalool (Becker et al., 2012). The use of anesthetic concentrations (150-450 μL L^{-1}) of EOLA chemotype linalool also induced the same VF response (Toni et al., 2014). The EOLA chemotype citral, used in the present experiment, reduced VF in some measurements. In addition, anesthesia with this EO also prevented the increase of plasma cortisol in silver catfish caused by handling (Souza et al., 2017; 2018). However, anesthesia with EOLA citral chemotype increased protein carbonylation levels in the kidney and liver of silver catfish, indicating that this EO may provoke renal and hepatic damage (Souza et al., 2018). There are no studies regarding VF of fish exposed to this EO or to *A. triphylla* EO. It is therefore not clear if *A. triphylla* EO reduced metabolism, because, at the end of 5 h of transport of silver catfish, CO_2 levels of the water were lower, but O_2 levels remained unchanged (Parodi et al., 2014). Essentia oil of *L. origanoides* was the most effective EO to reduce VF and apparently did not induce any initial stress response.

Conclusions

The essential oils of *Lippia alba* citral chemotype and *Lippia origanoides* are effective sedatives and anesthetics for silver catfish. Moreover, the essential oils of *Lippia origanoides* are recommended for transport of silver catfish, because they maintain the ventilatory frequency constant, reducing a possible metabolic stress.

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