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ESSENTIAL OILS AND EUGENOL AS ANESTHETICS FOR *Serrasalmus rhombeus**

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ABSTRACT

This study evaluated the periods of time of anesthetic induction and recovery of *Serrasalmus rhombeus* exposed to essential oils (EOs) of *Aloysia triphylla* and *Lippia alba* and eugenol, as well as if these anesthetics can be used for transport of this species through analysis of swimming behavior. Fish were placed in aquaria containing different concentrations of *A. triphylla* EO or *L. alba* EO or eugenol, posteriorly were transferred to aquaria containing only water to evaluate the recovery time. In the second experiment, behavior was analyzed during exposure to *A. triphylla* EO, *L. alba* EO or eugenol at 5 or 10 µL L^{-1}. The evaluations were carried out at 0, 1, 5, 10 and 15 min of exposure. Fish exposed to 150, 200 and 50 µL L^{-1} of *A. triphylla* EO, *L. alba* EO and eugenol, respectively, showed anesthetic induction time lower than 3 min and recovery time lower than 10 min. Concentrations of 50 µL L^{-1} of both EOs and 25 µL L^{-1} eugenol caused only sedation. Exposure to 5 and 10 µL L^{-1} EOs and eugenol decreased fish swimming time. Both EOs and eugenol were effective for anesthesia and can be used for transport of *S. rhombeus*.

Key words: anesthesia; behavior; black piranha; fish transport; Negro river.

ÓLEOS ESSENCIAIS E EUGENOL COMO ANESTÉSICO PARA *Serrasalmus rhombeus*

RESUMO

Esse estudo avaliou os períodos de tempo de indução e recuperação anestésica de *Serrasalmus rhombeus* expostos aos óleos essenciais (OEs) de *Aloysia triphylla* e *Lippia alba* e eugenol, bem como se esses anestésicos podem ser usados no transporte dessa espécie através da análise do comportamento natatório. Os peixes foram colocados em aquários contendo diferentes concentrações de OE de *A. triphylla* ou OE *L. alba* ou eugenol, posteriormente foram transferidos para aquários contendo somente água para avaliar o tempo de recuperação. No segundo experimento, comportamento foi analisado durante exposição aos OEs ou eugenol nas concentrações 5 ou 10 µL L^{-1}. As avaliações foram realizadas em 0, 1, 5, 10 e 15 min de exposição. Peixes expostos a 150, 200 e 50 µL L^{-1} de OE de *A. triphylla*, OE de *L. alba* e eugenol, respectivamente, apresentaram tempo de indução anestésica menor que 3 min e tempo de recuperação menor que 10 min. Concentrações de 50 µL L^{-1} de ambos OEs e 25 µL L^{-1} de eugenol causaram somente sedação. Exposição a 5 e 10 µL L^{-1} de OEs e eugenol diminuíram o tempo de natação dos peixes. Ambos os OEs e o eugenol foram efetivos para anestesia e podem ser utilizados para transporte de *S. rhombeus*.

Palavras-chave: anestesia; comportamento; piranha preta; transporte de peixes; rio Negro.

INTRODUCTION

There is a growing interest in the search for natural anesthetics, obtained from plants, which are economically viable and present low toxicity. The most widely used natural anesthetic is eugenol (4-allyl-2-methoxyphenol), the main compound from clove oil, which is obtained from plants of the genus *Eugenia* (ANDERSON *et al*., 1997). The anesthetic efficacy of eugenol was showed for several Neotropical species as *Piaractus mesopotamicus* (Holmberg, 1887) (GONÇALVES *et al*., 2008), *Astyanax bimaculatus* (Linnaeus, 1758) (SILVA *et al*., 2009), *Brycon amazonicus* (Spix & Agassiz, 1829) (VIDAL *et al*., 2007), *Rhamdia quelen* (Quoy & Gaimard, 1824) (CUNHA *et al*., 2010a), *Carassius auratus* (Linnaeus, 1758) (BITTENCOURT *et al*., 2012), *Centropomus parallelus* Poey, 1860 (SOUZA *et al*., 2012) and *Brycon hilarii* (Valenciennes, 1850)

(FABIANI *et al*., 2013). The efficacy of the essential oil (EO) of *Lippia alba* was also demonstrated for *Rhamdia quelen* (CUNHA *et al*., 2010b; HELDWEIN *et al*., 2012, 2014; TONI *et al*., 2014), *Hippocampus reidi* (Ginsburg, 1933) (CUNHA *et al*., 2011) and *Sparus aurata* (Linnaeus, 1758) (TONI *et al*., 2015), but the EO of *Aloysia triphylla* was studied only in *R. quelen* (PARODI *et al*., 2014) and *C. parallelus* (PARODI *et al*., 2016).

Serrasalmus rhombeus (Linnaeus, 1766), popularly known as black piranha or redeye piranha, is distributed across Amazon and Orinoco basins, Guiana Shield rivers and coastal rivers of northeastern Brazil (JÉGU and INGENITO, 2007). This species is an important fisheries resource (MPA, 2011) and has been exported as ornamental fish (ANJOS *et al*., 2009), but has powerful teeth that can cause serious injury in humans (MOL, 2006). Therefore, the use of anesthesia in the management of this fish is indicated to prevent injuries in the handlers and to reduce the effects of animal stress.

The aim of this study was to evaluate the anesthetic efficacy of EOs of *A. triphylla* and *L. alba* and eugenol in *S. rhombeus*. In addition, we also investigated the swimming behavior of *S. rhombeus* exposed to low concentrations of these anesthetics to indicate if they could be used in the water of transport of this species.

METHODS

Essential oils and eugenol

The plants *A. triphylla* and *L. alba* were cultivated at the campus of the Universidade Federal de Santa Maria in the city of Frederico Westphalen, southern Brazil. Voucher specimens (SMDB No. 11169 and 10050, respectively) were deposited in the herbarium of the Biology Department. Eugenol (99.9%, Maquira®) was purchased in a local drugstore.

Essential oil extraction and analysis

The oil extraction from the leaves of these plants was performed by hydro-distillation using Clevenger apparatus according to the European Pharmacopoeia (2007). Analysis was made by gas chromatography using an Agilent 7890A gas equipment coupled to an Agilent 5975C mass selective detector (GC-MS). The unit was equipped with a capillary column HP5-MS (Hewlett Packard, 5% fenilmetilsiloxane, 30 m x 0.25 mm, film thickness: 0.25 µm), and the ionization energy used was 70 eV. The parameters choose for the analysis were: He as gas carrier; split inlet 1:100; temperature program: 40°C for 4 minutes; 40 to 320°C at 4ºC min^{-1}; 1 mL min^{-1} of flow rate; and temperatures of injection and detection of 250°C. The chemical compounds identification was made by comparison of retention indexes, obtained by using a calibration curve of n-alkanes injected at the conditions mentioned for the samples, and the mass fragmentation patterns with NIST (2010) data.

Animals

Specimens of *S. rhombeus* (14.9 ± 0.51 cm, 110.9 ± 3.79 g, voucher number: INPA-ICT 53086) were collected during an expedition to Anavilhanas Islands of the Negro River, 110 km upstream from Manaus (2°23'41"S, 60°55'14"W). Fish were maintained in tanks with 50% water daily renewed, pumped directly from Rio Negro (29.8°C, pH 5.0), continuously aerated for a few hours before testing.

Experiment I: anesthesia induction and recovery in S. rhombeus exposed to A. triphylla and L. alba EOs and eugenol

The fish were transferred individually to aquaria containing 2 L (29.8 ± 0.46°C; pH 5.0 ± 0.1) of water with the *L. alba* (50, 100 and 200 µL L^{-1}) or *A. triphylla* EOs (50, 100 and 150 µL L^{-1}) or eugenol (25, 40 and 50 µL L^{-1}), first diluted in ethanol at a proportion of 1:10. These concentrations were chosen based in previous studies (CUNHA *et al*., 2010a, b; PARODI *et al*., 2014). A total of 14 compounds were identified in each essential oil. The main constituents of *A. triphylla* EO were limonene (21.69%) and geranial (24.32%) and of *L. alba* EO were linalool (66.35%) and eucalyptol (10.63%) (Table 1).

The time for anesthesia induction was evaluated according to SMALL (2003): stage I - corresponds to sedation, when the reactivity to external stimuli decreased; stage II – corresponds to partial loss of equilibrium and erratic swimming; and stage III – corresponds to total loss of equilibrium and cessation of locomotion. In recovery, the fish returns to regular swimming. Eight fish were used for each tested concentration and each fish was used only once. The maximum observation time was 15 min, since several studies indicated that sedation and anesthesia occur within this period (CÁRDENAS *et al*., 2016; HOHLENWERGER *et al*., 2016; PARODI *et al*., 2016; SENA *et al*., 2016; TEIXEIRA *et al*., 2017). Control experiment was performed using aquaria containing water and ethanol at a concentration equivalent to the highest dilution (1800 µL L^{-1}). After induction of anesthesia, fish were transferred to a tank containing only water to evaluate the recovery time. The animals were recovered when swimming regularly and reacting to external stimuli (the peduncle of the caudal fin was pressed with a glass rod).

Experiment II: fish behavior through exposure to low concentrations of A. triphylla and L. alba EOs and eugenol

The fish were placed into tanks containing 20 L of water and *A. triphylla* or *L. alba* EOs or eugenol at 5 and 10 µL L^{-1}. These concentrations were 2.5 and 5-fold lower than the lowest eugenol concentration tested, which induced stage II (partial loss of equilibrium) (see results) and also based on studies carried out by BECKER *et al*. (2012, 2013) and PARODI *et al*. (2014). Four fish per aquaria were used for each concentration (in triplicate). The fish were filmed for 20 s for analysis of the total swimming time and equilibrium (partial or total loss of equilibrium or normal) at 0, 1, 5, 10 and 15 min of exposure. Control experiments were performed using aquaria containing only water and aquaria containing ethanol at a concentration equivalent to that used in the highest dilution of the EOs (90 µL L^{-1}).

Table 1. Chemical composition of essential oils.

Essential Oil	RI* Experimental	RI Literature[a]	Chemical Compound	Percent Composition
A. tryphilla	989	986	5-Hepten-2-one, 6-methyl	2.085
	991	986	β-Pinene	0.499
	1026	1026	Limonene	21.694
	1049	1048	β -cis-Ocimene	0.717
	1229	1228	cis-Geraniol	2.218
	1241	1247	cis-Carveol	18.533
	1256	1259	Linlyl Acetate	2.703
	1271	1269	Geranial	24.317
	1417	1415	β-Caryophyllene	5.323
	1483	1483	α-Curcumene	3.251
	1495	1487	(-)-Alloaromadendrene	1.136
	1577	1578	Spathulenol	2.617
	1582	1583	Caryophyllene Oxide	6.793
	1640	1639	T-Cadinol	1.411
			Identified compounds	**93.297**
L. alba	971	969	Sabinene	0.817
	992	996	β-Pinene	0.972
	1027	1026	Limonene	1.992
	1028	1030	Eucalyptol	10.633
	1100	1101	Linalool	66.347
	1143	1146	Camphor	0.516
	1204	1205	Trans-Dihydrocarvone	1.183
	1242	1252	Carveol	1.135
	1272	1270	Geranial	0.764
	1273	1270	Neral	0.361
	1418	1419	Aromadendrene	3.480
	1480	1482	Germacrene D	2.784
	1556	1558	Germacrene B	2.219
	1582	1582	Spathulenol	1.340
			Identified Compounds	**94.543**

*RI = Retention Index. [a]NIST, 2010.

Statistical analyses

All data are expressed as mean ± SEM. Homogeneity of variances among treatments was tested by Levene's test. The data from time to induction of anesthetic stages presented homogeneous variances and comparisons between the different concentrations were assessed using one-way ANOVA and Tukey's test. The data from swimming time did not exhibit homogeneous variances; therefore, comparisons between the different treatments and times were assessed using the non-parametric Scheirer-Ray-Hare extension of the Kruskal-Wallis test followed by the post-hoc Nemenyi test. The analysis was performed using the Statistica 7.0 software (Stat Soft. Inc.) and the minimum significance level was set at $P<0.05$.

RESULTS

The concentrations of 50 μL L^{-1} *A. triphylla* EO, 50 μL L^{-1} *L. alba* EO and 25 μL L^{-1} eugenol only induced sedative effect within 15 min, but all other assay concentrations induced all stages of anesthesia. Fish exposed to 150, 200 and 50 μL L^{-1} of *A. triphylla* and *L. alba* EO and eugenol, respectively, reached deep anesthesia (stage 3) significantly faster than the lower concentrations (Figure 1). The increasing concentration of *L. alba* EO increased proportionally recovery time (Figure 1B). The *A. triphylla* EO increased recovery time up to 100 μL L^{-1} and eugenol only at the highest concentration (50 μL L^{-1}) (Figures 1A, C). The fish showed at the first contact with the anesthetics in both tests small jumps and swimming bursts in the first experiment. The application of 1800 μL L^{-1} ethanol alone did not produce any anesthetic effect.

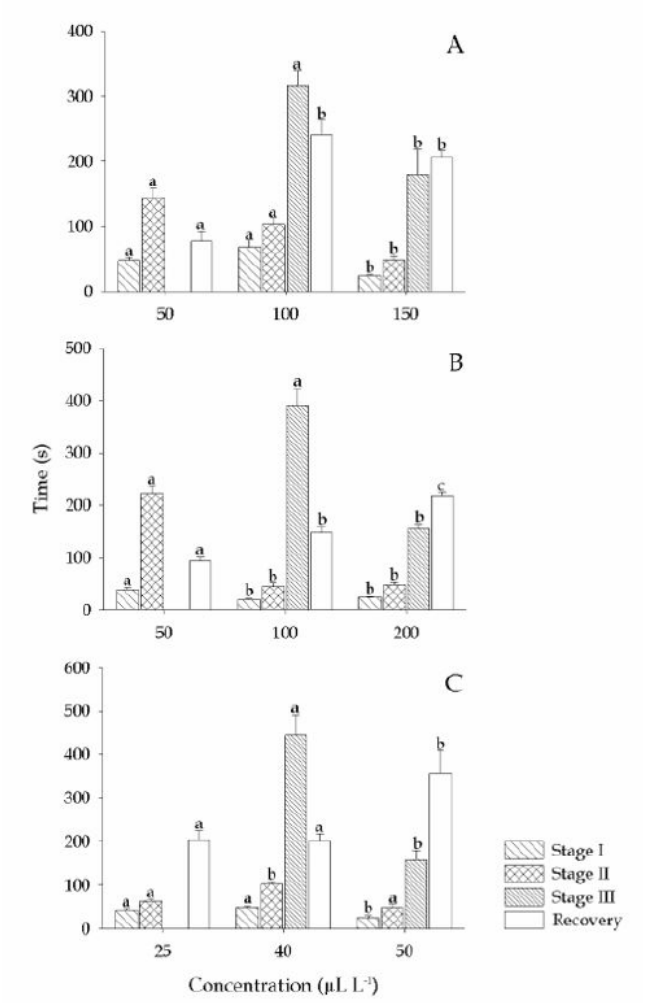

Figure 1. Time required for induction and recovery of anesthesia in *Serrasalmus rhombeus* using *Aloysia triphylla* (A) and *Lippia alba* (B) essential oils and eugenol (C). Different letters indicate a significant difference in the same stage based on one-way ANOVA and Tukey's test ($P < 0.05$).

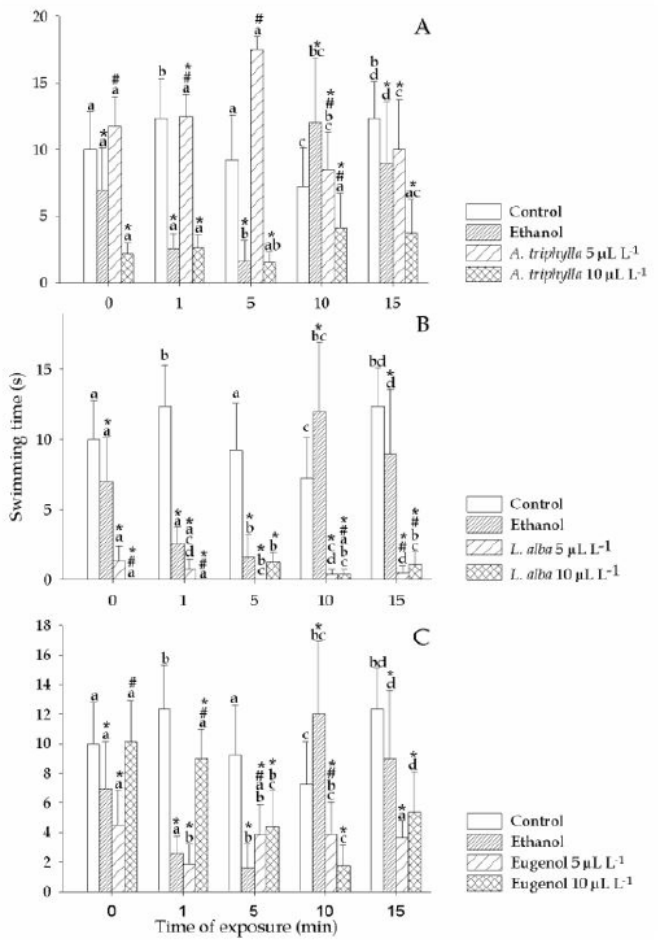

Figure 2. Swimming time of *Serrasalmus rhombeus* exposed to low concentrations of *Aloysia triphylla* (A) and *Lippia alba* (B) essential oils and eugenol (C). Different letters indicate significant difference between times of exposure. *indicates significant difference from control fish. # indicates significant difference from ethanol-exposed fish ($P < 0.05$).

In the second experiment, fish exposed to 10 µL L^{-1} EO of *A. triphylla*, 5 and 10 µL L^{-1} EO of *L. alba* and 5 µL L^{-1} of eugenol presented lower swimming time than control fish in all evaluated times. The fish did not show loss of equilibrium at both concentrations of EOs and eugenol tested. Ethanol-exposed fish initially decreased swimming time, but returned to the control level after 10 min (Figure 2).

DISCUSSION

Aloysia triphylla EO used in this study presented in its chemical composition limonene and geranial as main constituents. Unlike the present study, other studies detected citral, which is composed of geranial and neral, as main constituents of this EO (FIGUEIREDO *et al*., 2004; SARTORATTO *et al*., 2004; PAULUS *et al*., 2013). *Lippia alba* EO presented as the main constituent linalool, as observed in previous studies (HELDWEIN *et al*., 2012; TONI *et al*., 2015).

The anesthetic and sedative effects of eugenol are well established in rodents and fish (CUNHA *et al*., 2010a; FREIRE *et al*., 2006). On the other hand, the central depressor effects detected for the EOs of *A. triphylla* and *L. alba* are due to the association of different components, and resulted from additive and/or synergistic activities. For some of their constituents, as linalool and spathulenol, anesthetic and sedative effects were already described in *Rhamdia quelen* (BENOVIT *et al*., 2015; HELDWEIN *et al*., 2014). Additionally, some components detected in the EOs showed sedative and/ or anxiolytic like properties in mice, as limonene, geranial, 1,8-cineole and ß-pinene

(GOMES *et al*., 2010; GUZMÁN-GUTIÉRREZ *et al*., 2012; VALE *et al*., 2002).

The present study demonstrates that eugenol and the EOs of *A. triphylla* and *L. alba* presented anesthetic effect in *S. rhombeus*. According to PARK *et al*. (2009), the suitable anesthetic induction time is about 3 min and at most 10 minutes for recovery. Following this premise, the best concentrations of the EOs of *A. triphylla* and *L. alba* and eugenol to anesthetize *S. rhombeus* were 150, 200 and 50 µL L^{-1}, respectively. The time required to induce deep anesthesia at these concentrations was approximately 164 s. In relation to the recovery time, all concentrations of EOs and eugenol remained in the suggested range by PARK *et al*. (2009), the largest time being 355 s. The concentration of the EO of *A. triphylla* to anesthetize both albino and gray strains of *R. quelen* at 24°C and pH 7.0 within 3 min was between 400-800 µL L^{-1} (PARODI *et al*., 2014). The lowest concentration of the EO of *L. alba* that induced anesthesia in *R. quelen* within 3 min at 21°C and pH 7.0 was 400 mg L^{1} (around 500 µL L^{-1} because the density of this EO is of approximately 0.8) (CUNHA *et al*., 2010a), and in *H. reidi* was 450 µL L^{-1} (CUNHA *et al*., 2011). Therefore, for both EOs deep anesthesia can be obtained with lower concentration in *S. rhombeus* than in these other species studied, probably due to the higher temperature used in the present study as found by GOMES *et al*. (2011). In contrast, *S. rhombeus* exposed to 50 µL L^{-1} did not reach anesthesia stage, but *H. reidi* did (CUNHA *et al*., 2011). TONI *et al*. (2015) observed that the lowest concentration required to induce deep anesthesia in *Sparus aurata* at 38 ppt salinity, 18°C, within 3 min was 200 µL L^{-1} EO of *L. alba*, the same concentration found to *S. rhombeus*. As for *S. rhombeus*, all concentrations tested in *S. aurata* showed recovery time lower than 10 min (TONI *et al*., 2015).

The lowest eugenol concentration necessary to anesthetize *S. rhombeus* within 3 min was 50 µL L^{-1}. This is the same lowest eugenol concentration to anesthetize *R. quelen* (at 21°C and pH 7) (CUNHA *et al*., 2010b), *B. amazonicus* (*B. cephalus*) (VIDAL *et al*., 2007) and *P. mesopotamicus* (at 25 °C) (GONÇALVES *et al*., 2008). *Centropomus parallelus* needed a similar eugenol concentration range to anesthetize and recover within the proposed periods: 25 – 62.5 mg L^{-1} (23.6 – 59 µL L^{-1}, because the density of eugenol is approximately 1.06) at 21°C (SOUZA *et al*., 2012). *Brycon hilarii* needed higher concentrations than *S. rhombeus*, in the range of 100 – 300 mg L^{-1} (94.3 – 283 µL L^{-1}), for induction and recovery at the suitable time at 25°C (FABIANI *et al*., 2013). In contrast, BITTENCOURT *et al*. (2012) verified that 75 mg L^{-1} (70.7 µL L^{-1}) eugenol required more than 3 min to anesthetize *C. auratus* (higher concentrations were not tested). The 5.0-7.0 pH range at 23°C did not change time of induction to eugenol in *R. quelen* exposed to 40 mg L^{-1} (37.7 µL L^{-1}), but at pH 7.0 and 30°C eugenol anesthetized this species within 225-275 s (depending on size of the fish) (GOMES *et al*., 2011). *S. rhombeus* needs a higher time (443 s) to anesthetize at this eugenol concentration.

In the second experiment, the exposure of the fish to low concentrations of the anesthetics was performed to verify the possibility of using these products in the transport of *S. rhombeus*. The sedation stage, with lower responsiveness to external stimuli and metabolic rate, but without losing equilibrium, is recommended for transporting fish (SUMMERFELT and SMITH, 1990; PIRHONEN and SCHRECK, 2003).

Ethanol initially reduced swimming activity, but it returned to control level after 10 min. Ethanol enhances the action of several GABA receptors subtypes (WALLNER *et al*., 2003), which may be related to the decreased swimming activity in *S. rhombeus*. This effect may have been fast due to ethanol evaporation. Both concentrations of EO of *A. triphylla* can be tested for transport of *S. rhombeus*, and the best concentration apparently is 10 µL L^{-1}, which reduced swimming activity through the 15 min observation. The addition of 30 to 50 µL L^{-1} of EO of *A. triphylla* in transport water (lower concentrations were not tested) reduced ions loss, plasma cortisol levels and ammonia excretion in *R. quelen*, suggesting lower physiological damage resulting from transport (PARODI *et al*., 2014; ZEPPENFELD *et al*., 2014). Both EO of *L. alba* concentrations can be tested for the transport of *S. rhombeus*. Similar concentrations (10 and 20 µL L^{-1}) of *L. alba* EO are recommended for the transport of *R. quelen* because they improved blood and ionoregulatory parameters (BECKER *et al*., 2012). Both concentrations of eugenol can be tested for the transport of *S. rhombeus*, and the best concentration seems to be 5 µL L^{-1}, which reduced swimming activity at all observation times. The addition of 1 – 3 µL L^{-1} of eugenol is recommended for the transport of *R. quelen* because they decreased non-ionized ammonia levels, ion loss and mortality (BECKER *et al*., 2012, 2013). The determination of the swimming activity allowed a more precise analysis of the sedative effects of the anesthetics tested, because the identification of the decreased reactivity to external stimuli proposed by SMALL (2003) for stage I is rather subjective. However, since this study analyzed swimming activity for only 15 min, it would be interesting that further studies investigate if the effects on swimming activity and equilibrium caused by the essential oils and eugenol can last for several hours, as well as if they can improve water, blood and ionoregulatory parameters as observed in the transport of silver catfish as observed by BECKER *et al*. (2012, 2013), PARODI *et al*. (2014) and ZEPPENFELD *et al*. (2014).

CONCLUSION

In conclusion, both *A. triphylla* and *L. alba* EOs and eugenol are effective for anesthetic induction within 3 min at the concentrations 150, 200 e 50 µL L^{-1}, respectively. For fast sedation, the recommended concentration is 50 µL L^{-1} for both EOs and 25 µL L^{-1} for eugenol. The concentrations of 5 and 10 µL L^{-1} of both EOs and eugenol reduced fish swimming activity and are indicated for studies of transportation of *S. rhombeus* but for eugenol and the EO of *A. triphylla* the best concentrations are 5 and 10 µL L^{-1}, respectively.

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