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Anesthetic potential of essential oils from Brazilian native plants in *Rhamdia quelen* juveniles (silver catfish)

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The sedative and anesthetic actions of several essential oils (EO) on fish have been demonstrated, stimulating the search for new options for natural anesthetics. This work evaluated the safety and sedative and anesthetic efficacy of EOs from three native Brazilian plants, *Acmella oleracea* (jambu), *Aloysia hatschbachii* and *Cordia verbenacea* (whale herb) in juvenile *Rhamdia quelen* (silver catfish). Anesthetic induction and recovery protocols (20 to 400 mg L-1) and long exposure (48 h) from 10 to 100 mg L-1 were tested. The EOs performed sedative and/or anesthetic activities: AOOi at a concentration of 20 mg L-1, AOOl at 50 and 100 mg L-1, AHOl, and CVOL (only sedation) 50 mg L-1, as there were no important adverse effects and/or mortality. The results obtained indicate that *Cordia verbenacea* EO is the most promising as a sedative for juvenile silver catfish at a concentration of 50 mg L^{-1}.

Keywords: *Acmella oleracea*, *Aloysia hatschbachii*, Anesthesia, *Cordia verbenacea*, Sedation.

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As ações sedativas e anestésicas de diversos óleos essenciais (OE) em peixes têm sido demonstradas, estimulando a busca por novas opções de anestésicos naturais. Este trabalho avaliou a segurança e a eficácia sedativa e anestésica de OE de três plantas nativas brasileiras, *Acmella oleracea* (jambu), *Aloysia hatschbachii* e *Cordia verbenacea* (erva-baleeira) em juvenis de *Rhamdia quelen* (jundiá). Foram testados protocolos de indução e recuperação anestésica (20 a 400 mg L-1) e longa exposição (48 h) de 10 a 100 mg L-1. Os OEs realizavam atividades sedativas e/ou anestésicas: AOOi na concentração de 20 mg L-1, AOOl na concentração de 50 e 100 mg L-1, AHOl, e CVOL (somente sedação) 50 mg L-1 o AHOl (sedação e anestesia) e CVOL (sedação) na concentração de 50 mg L^{-1}, pois não houve efeitos adversos importantes e/ou mortalidade. Os resultados obtidos indicam que o OE de *Cordia verbenacea* é o mais promissor como sedativo para juvenis de jundiá na concentração de 50 mg L^{-1}.

Palavras-chave: *Acmella oleracea*, *Aloysia hatschbachii*, Anestesia, *Cordia verbenacea*, Sedação.

INTRODUCTION

Several basic procedures in fish research, such as biometrics and transport, can be stressful for fish when no sedative/anesthetic is used (Souza *et al*., 2019). Anesthetics of synthetic origin, such as tricaine methanesulfonate - MS-222, benzocaine and others, are expensive (Barbas *et al*., 2017) and can cause several adverse effects in fish, such as loss of mucus, tissue irritation, hypoxia, acidosis, and increased serum cortisol, among others (Zahl *et al*., 2011; Sneddon, 2012).

Thus, the anesthetics of natural origin stand out, more precisely essential oils (EO) and their isolated constituents (Souza *et al*., 2019), mainly because they are biodegradable and, as a rule, cause low rates of intoxication (Figueiredo *et al*., 2008). Furthermore, in most cases they are very close to what is expected from an ideal anesthetic for fish, that is, they have characteristics such as good availability, ease of use, and are safe for the environment, animal, and handler (Barbas *et al*., 2020). The increasing use of herbal as anesthetics in aquaculture is also due to their various health benefits to fish (Hoseini *et al*., 2019). Their low persistence in the environment reduces chemical contamination of surface waters, groundwater, and soils, as well as the organic matter available in them (Amani, James, 2007) while minimizing stress and fish mortality (Bhuvaneswari *et al*., 2015).

Fish anesthesia experiments consists in observing different stages. The first one is sedation, in which the fish present partial loss of reaction to external stimuli. However, increased concentrations of the anesthetic usually cause central nervous system (CNS) depression, resulting in loss of reflex activity and no reaction to external stimuli (Schoettger, Julin, 1967), not even if there is pressure in the caudal peduncle. Generally the lowest concentrations are only sedative and are recommended for transport. Anesthetic induction times close to 1 min can be used in low-stress procedures, such as blood collection (Hoseini *et al*., 2011; Hoseini, Ghelichpour, 2012; Hoseini, Nodeh, 2013). In fish surgeries, anesthetic concentrations with a long recovery time are the

most recommended (Roubach *et al*., 2005). For this study, the choice of the plant species to furnish the EOs was based on their botanical classification, as they belong to families that have representatives whose extractives showed promising activities for fish sedation, anesthesia, and/or analgesia, having been evaluated in other experimental models.

The genus *Acmella* (Asteraceae) is distributed in tropical and subtropical regions, consisting of more than 60 species (Sahu *et al*., 2011). The species *A. oleracea* stands out in Brazil, as it is cultivated throughout the year (Romão *et al*., 2015), generally in humid areas (Tiwari *et al*., 2011). Its flowers and leaves have a spicy flavor and when ingested they cause a sensation of numbness and tingling on the tongue (Wongsawatkul *et al*., 2008), being widely used in cooking in the northern region of Brazil. Its anesthetic activity was described in *Colossoma macropomum* (tambaqui) for the hexane flower extract (Barbas *et al*., 2017), however the EO was not evaluated to date. The EOs of some species of the genus *Aloysia* (Verbenaceae) showed sedative and anesthetic effects in fish: *A. tryphylla* (synonymy *A. citrodora*) (Gressler *et al*., 2012; Teixeira *et al*., 2016; Becker *et al*., 2017; Almeida *et al*., 2019; Parodi *et al*., 2013, 2016, 2020; Santos *et al*., 2022) and *A. gratissima* (Benovit *et al*., 2012, 2015). Besides anesthetic effects in *Epinephelus marginatus* (dusky grouper) (Fogliarini *et al*., 2017), the EO of *A. polystachya* also showed antidepressant and anxiolytic properties in *Danio rerio* (zebrafish) (Melo *et al*., 2019). The genus *Cordia* (Boraginaceae) is widely distributed in tropical and subtropical regions of the world and presents great variability, mainly in terms of floral, and fruit characteristics (Attar *et al*., 2018). In this genus, *C. verbenacea* stands out as a native aromatic shrub present throughout Brazil, with a greater abundance in the coastal region (Martim *et al*., 2021).

The experimental model chosen for this study was the silver catfish, a species of fish native to South America, more specifically living in the rivers and equatorial rivers of Brazil (Koerber, Reis, 2020) and one of the main experimental models for studying anesthetics obtained from natural sources (Souza *et al*., 2019). This work aimed to evaluate the sedative and/or anesthetic potential of EOs from three promising native Brazilian plants in terms of yield, chemical content, and/or activities, which have not been tested on fish regarding their sedative and anesthetic properties to date. In this way, their safety and efficacy profiles were established and concentration-response curves were provided.

MATERIAL AND METHODS

Collection of plant material and essential oil extraction. Two of the plants used to extract the EOs were cultivated in cities from Rio Grande do Sul State, and the third EO was purchased commercially (Tab. 1). These EOs were obtained by hydrodistillation for 3 h using a modified Clevenger apparatus (European Pharmacopeia, 2010) and then transferred to amber glass bottles, sealed, and stored at -4°C.

Obtaining essential oils and analyzing their chemical compositions. The qualitative analysis of the composition and percentage of the EOs components was

TABLE 1 | Native plant species used to obtain essential oils. *Cities located in Rio Grande do Sul State, southern Brazil. [1]Geographic locations of harvest; [2]Supplier company.

Species (common name)	Family	Plant organ used	Tested sample abbreviation	Locations for obtaining plants
Acmella oleracea (jambu)	Asteraceae	Leaves Inflorescences	AOOl AOOi	Cultivated (*ex situ*) São João do Polêsine* 29º40'53"S 53º31'32"W[1]
Aloysia hatschbachii (unknown)	Verbenaceae	Leaves	AHOl	Cultivated (*ex situ*) Frederico Westphalen* 27º23'26"S 53º25'43"W[1]
Cordia verbenacea (erva-baleeira)	Boraginaceae	Leaves	CVOl	Laszlo Aromatologia Eireli (Brazil)[2]

carried out by gas chromatography in an Agilent 7890A hyphenated system, equipped with a 5975C series mass selective detector. The analysis parameters were as follows: split injection mode 1:50; carrier gas: He, with a flow of 1 mL min^{-1}; DB5-MS fused silica capillary column (5% phenylmethylsiloxane, 30 m x 0.25 mm, film thickness: 0.25 µm); oven heating program: 40°C, (Ti) for 4 min, 40-320°C at 4°C/min; injector, detector and interface temperature: 250 °C. The components of the EOs were identified by comparing their mass spectra fragmentation patterns and Kovats retention indices (KI) with literature data and the equipment library (Nist, 2008; Adams, 2011; Silva, 2015; Garlet *et al*., 2019a). Kovats indices were determined through a calibration curve of a homologous series of n-alkanes (C8-C40), injected under the same conditions as the samples. Quantification of compounds was performed by gas chromatography with flame ionization detection on an Agilent 7890A chromatograph. The analysis parameters were the same as mentioned above, with the exception of splitless injection, as well as the detector temperature (300 °C).

Fish maintenance. Silver catfish, *Rhamdia quelen* (Quoy & Gaimard, 1824), juveniles (voucher number of Universidade Federal do Rio Grande do Sul, UFRGS 29744) (4.52 ± 1.66 g and 7.49 ± 1.22 cm) were purchased from a fish farm in Santa Maria, RS, and transported to the Laboratório de Fisiologia de Peixes. Fish were acclimated for two weeks in 250 L tanks with constant aeration, protected from light, at 22 °C, fed with commercial feed (Supra juvenil, 32% CP, Alisul Alimentos S.A., São Leopoldo, RS, Brazil), supplied until satiety three times a day (8, 13, and 18 h). Daily, 10% of the water in the tanks was replaced 30 min after feeding, to remove feces and food remains. Dissolved oxygen levels (6.67 ± 0.20 mg L^{-1}) and temperature (22.1 ± 0.85 °C) were measured daily with an YSI55 oximeter and pH with a pH meter (7.52 ± 0.24 units, DMPH-2, Digimed, Brazil). Before the experiments, the fish were fasted for 12 h and the EOs were previously diluted in 95% ethanol (1:10) and added directly to the aquarium water. Fish were exposed individually to the EOs in aquariums (11.5 cm high x 12.5 cm wide x 17.5 cm long) containing 1 L of aerated water. Through the experiments, clinical and behavioral signs compatible with central depression were evaluated. The adverse effects (clinical and behavioral signs) observed were recorded and evaluated by a Veterinary professional, and these were recorded based on the apparent individualized

visualization of the experimental models. At the end of the protocols, euthanasia was performed by immersion in eugenol (100 mg L^{-1}), followed by spinal cord transection just behind the opercula (Balko *et al*., 2018).

Sedative and/or anesthetic induction and recovery. The EOs were tested at the following concentrations (n = 8 each EO and concentration tested): EO of *A. oleracea* inflorescences (AOOi) - 20, 80, and 100 mg L^{-1}; EO of leaves of *A. oleraceae* (AOOl) - 50, 100, 200, and 300 mg L^{-1}; EO of *A. hatschbachii* leaves (AHOl) - 50, 100, and 300 mg L^{-1}, and EO of *C. verbenacea* leaves (CVOl) - 50, 80, 100, 200, 300, and 400 mg L^{-1}. The EOs were initially evaluated in pilot tests, at a concentration of 100 mg L^{-1}. If 100 mg L^{-1} induced the S4 stage, lower concentrations were tested. If S4 was not reached, the concentrations to be tested were increased. Eugenol (50 mg L^{-1}) (Cunha *et al*., 2010) was used as a positive control. Sedative and/ or anesthetic induction and recovery were evaluated using the steps described by Gomes *et al*. (2011): S2 - deep sedation (loss of reaction to external stimuli); S3a - partial loss of balance (animals swim sideways); S3b - total loss of balance (loss of the ability to swim, but the fish respond to pressure on the caudal peduncle, descending to the bottom of the aquarium); S4 - anesthesia (loss of reflexes; fish do not respond to pressure stimuli on the caudal peduncle) and S5 - bulbar collapse (cessation/death of respiratory movements).

When the animals reached the S4 stage, or within a maximum time of 30 min, they were transferred to recovery in 1 L aerated aquariums. To determine recovery times, the time elapsed until the fish returned to normal swimming behavior was observed. Each animal was used only once, and sedation and anesthesia induction and recovery times were measured with a digital stopwatch.

Long-term exposure protocol. In this experiment, fish (n = 8 each EO and concentration tested) were exposed individually and at the same time to each EO for up to 48 h, and were observed for 5 min at times 0, 10, 20 and 30 min, 1, 2, 3, 6, 12, 24 and 48 h, to check possible adverse effects and mortality. The concentrations (Tab. 2) were chosen according to the adverse effects presented by some of the evaluated EOs in sedative and/or anesthetic induction and recovery experiments and/or because only sedative concentrations were detected, aiming to evaluate possible bulbar collapse or intensification of adverse effects. Furthermore, stimulation was applied to the caudal peduncle with a glass rod, in specimens that appeared to be at the S4 stage. The control used in this protocol was ethanol, which had no effect in silver catfish (Heldwein *et al*., 2012). Ethanol was used to evaluate whether it really did not cause adverse effects and/ or mortality in fish. Eugenol was not used in this protocol, as it was not necessary to compare adverse effects and/or mortality.

TABLE 2 | Concentrations used in long-term exposure protocols. AOOi (*Acmella oleracea* inflorescences EO), AOOl (*A. oleracea* leaves EO), AHOl (*Aloysia hastschbachii* leaves EO), and CVOl (*Cordia verbenacea* leaves EO), (n = 8).

OEs - Sample abbreviations	Concentrations (mg L^{-1})
AOOi	10, 25 and 30
AOOl	10, 25 and 70
AHOl	20, 50 and 100
CVOl	50, 80, 90 and 100

Statistical analysis. Comparisons between the different concentrations of each EO were performed using the Kruskal-Wallis test for non-parametric data followed by the Dunn test, using the Prism version 9.0 software. The significance level considered was 95% ($p < 0.05$). To construct the concentration-response curves, the parameters "log (agonist) *vs*. answer – Find E Canything" available in the software were applied. The indicated parameter was EC50, therefore, the concentration of the agonist (X) that offers an average response between minimum and maximum was considered. In this way, the data were obtained according to the following equation:

$$Y = Minimum + (Maximum-Minimum) / (1 + 10^{(LogEC50-X)}).$$

RESULTS

Chemical composition of essential oils. The major compounds of each EO were β-ocymene (for AOOi), β-caryophyllene (for AOOl), eucalyptol (for AHOl), and α-pinene (for CVOl) (Tab. 3).

TABLE 3 | Chemical composition of the essential oils of *Acmella oleracea* (AOOi - inflorescences, AOOl - leaves), *Aloysia hastschbachii* (AHOl - leaves), and *Cordia verbenacea* (CVOl - leaves). Subtitle: [a]RI = Retention index; [b]Experimental; [c]Literature Adams *et al.* (2011) and NIST (2023).

RI[a] E[b]	RI[a] L[c]	Compound	Composition (%)			
			AOOi	AOOl	AHOl	CVOl
929	939	α-Pinene	–	–	1.3	34.8
970	969	Sabinene, (*Z*)-	0.7	–	1.4	–
974	975	β-Pinene	1.2	–	–	–
989	988	β-Myrcene	3.1	–	–	–
1027	1028	Limonene	–	–	1.3	1.3
1028	1026	β-Phellandrene	11.2	–	-	–
1029	1031	Eucalyptol	–	–	42.7	–
1036	1037	β-Ocimene	40.1	0.5	-	–
1098	1098	Sabinene hydrate	–	–	0.5	–
1193	1190	α-Terpineol	–	–	1.7	–
1388	1392	Elemene	–	–	6.9	2.7
1417	1417	β-Caryophyllene	36.5	69.0	4.1	–
1453	1452	a-Humulene	0.8	1.7	–	3.8
1461	1471	Dehydro-sesquicineole	–	–	0.7	–
1479	1480	Germacrene D	3.5	25.8	0.6	–
1492	1491	α-Farnesene	–	2.2	–	–
1493	1491	β-Guaiene	–	–	8.7	–
1504	1505	α-Bisabolene, (*Z*)-	–	–	2.0	–
1561	1560	Eremophila ketone	–	–	4.7	–
1568	1575	Cedrene epoxide	–	–	6.2	–
1574	1571	Spathulenol	–	–	2.6	2.8
1592	1590	Isoaromadendrene epoxide	–	–	1.6	–
1640	1641	Cedrenal	–	–	1.1	–
1643	1649	Methyl jasmonate	–	–	0.9	–
1653	1644	Selin-3,11-dien-6-a-ol	–	–	1.6	–
1655	1654	Cadinol	–	–	0.6	–
1665	1670	a-Caryophylene	–	–	0.7	–
1678	1677	Nerolidy acetate	–	–	0.7	–
1704	1703	Tridecenol acetate	–	–	1.1	–
1721	1718	Farnesol	–	–	5.1	–
1886	1844	Spilanthol	2.56	–	–	–
1957	1949	Cembrene A	–	0.5	–	–
2087	2082	Kaur-16-ene	–	–	–	–
Identified components			99.6	99.7	98.8	45.4
Unidentified components			0.4	0.3	1.2	54.6

Anesthetic induction and recovery protocol. Sedation (S2) with eugenol 50 mg L^{-1} was achieved in 23.5 ± 6.6 s and anesthesia (S4) in 205.8 ± 32.3 s, with a recovery time of 533.5 ± 117.1 s.

Essential oils from inflorescences (AOOi) and leaves (AOOl) of *Acmella oleracea*. Silver catfish exposed to 20 mg L^{-1} of AOOi took longer to reach stages S2, S3a and S3b than those subjected to 80 and 100 mg L^{-1}. Furthermore, 80 mg L^{-1} took less time to reach S4 than those subjected to 20 and 100 mg L^{-1}. Only fish anesthetized with 20 and 80 mg L^{-1} recovered within the 30 min evaluation time (Tab. 4). Considering the AOOl concentrations evaluated, the time to reach the S2 stage was inversely proportional to the increase in concentration. The concentration of 100 mg L^{-1} took longer to reach the anesthetic stage (S4) than 50, 200 and 300 mg L^{-1}. However, the concentration of 100 mg L^{-1} was the one that recovered in the shortest time compared to the concentrations of 200 and 300 mg L^{-1}. However, it did not differ from 50 mg L^{-1} in terms of anesthetic recovery time (Tab. 4).

***Aloysia hatschbachii* leaves essential oil (AHOl).** The 100 mg L^{-1} concentration took longer to reach S2 than the 300 mg L^{-1} concentration, but did not differ from 50 mg L^{-1}. To reach stages S3a and S3b, the concentration of 300 mg L^{-1} took the least time. However, to achieve deep anesthesia the concentration that took the longest was 100 mg L^{-1}, but this did not differ from 50 mg L^{-1}. The concentrations of 50 and 100 mg L^{-1} were those that achieved anesthetic recovery the fastest (Tab. 4).

***Cordia verbenacea* leaves essential oil (CVOl).** An inversely proportional relationship was observed between CVOl concentration and induction time to reach S2, which was achieved for all concentrations studied. Stages S3a and S3b were not reached within 30 min in fish exposed to 50 and 80 mg L^{-1}, and at higher concentrations there was no difference between them. The S4 stage was induced between 200 to 400 mg L^{-1}, also without differences between concentrations. The recovery times between the two lowest concentrations evaluated did not differ from each other and were below 20 min, while the fish subjected to concentrations of 100 to 400 mg L^{-1} did not recover within the maximum observation period (Tab. 4).

Long exposure

Essential oil from inflorescences (AOOi) and leaves (AOOl) of *Acmella oleracea*. For AOOi, the concentration of 10 mg L^{-1} induced the S4 stage in fish from 30 min to 2 h; subsequently, silver catfish reached the S5 stage, with total mortality. At 25 mg L^{-1}, the fish reached the S4 stage in 20 min, but within 30 min some individuals were in the S5 stage and at 1 h, 87.5% of the animals were dead. After 3 h, all fish reached the S5 stage. At 30 mg L^{-1}, the fish reached S4 stage from 20 min to 2 h, and at 3 h, all were in the S5 stage (Fig. 1A).

The concentration of 10 and 25 mg L^{-1} of AOOl sedated part of the animals at 10 min and at 20 and 30 min all the fish were in the S2 stage. When exposed to 10 mg L^{-1} from 1h onwards, all animals showed normal behavior. After 10 min of exposure to 70 mg L^{-1}, the animals were sedated (S2), while at 20 min 50% of the fish were still in the S2 stage, 37.5% reached the S3a stage, and 12.5% showed normal behavior. After 2 h,

TABLE 4 | Anesthetic induction and recovery times (s) in *Rhamdia quelen* juveniles exposed to essential oils of *Acmella oleracea* inflorescences (AOOi) and leaves (AOOl), *Aloysia hatschbachii* leaves (AHOl), and *Cordia verbenacea* leaves (CVOl). Mean ± standard deviation of the mean. Different letters in the same row indicate a significant difference between concentrations (n = 8); (-) indicates stage not reached; (-*): indicates no recovery in the maximum observation time (30 min).

Concentrations (mg L^{-1})						
	AOOi					
Stages	**20**		**80**		**100**	
S2	54.9 ± 20.7 [a]		16.1 ± 3.6 [b]		18.7 ± 6.7 [b]	
S3a	123.8 ± 41.7 [a]		42.2 ± 13.5 [b]		45.7± 12.4 [b]	
S3b	146.8 ± 45.2 [a]		81.4 ± 20.5 [b]		58.7 ± 10.4 [b]	
S4	153.4 ± 48.2 [a]		97 ± 24.6 [b]		159.8 ± 56.4 [a]	
Recovery	954.6 ± 483.7 [a]		1293 ± 376 [a]		-*	
	AOOl					
Stages	**50**	**100**	**200**		**300**	
S2	224 ± 95.4 [a]	73.7 ± 49 [a,b]	37.4 ± 19.5 [b,c]		14 ± 7,6 [c]	
S3a	320 ± 218.6 [a,b]	394 ± 142.1 [a]	134.6 ± 40.1 [b,c]		62.4 ± 19.7 [c]	
S3b	263.9 ± 234.7 [a,b]	401.1 ± 141.4 [a]	188.8 ± 55.5 [b,c]		86.6 ± 26.6 [c]	
S4	211 ± 240.8 [b]	414 ± 135.5 [a]	196,7 ± 57.2 [b]		102.9 ± 46.5 [b]	
Recovery	1172 ± 394.1 [bc]	1147 ± 402.5 [c]	1525 ± 233.2 [ab]		1609 ± 208.5 [a]	
	AHOl					
Stages	**50**		**100**		**300**	
S2	91.7± 72.4 [ab]		167.7 ± 120 [a]		64.5 ± 15 [b]	
S3a	314.2 ± 140.1 [a]		450 ± 160.5 [a]		113.8 ± 32.2 [b]	
S3b	530.7 ± 116.8 [a]		679.1 ± 174 [a]		235.9 ± 39.2 [b]	
S4	596.9 ± 232.4 [ab]		795.1 ± 181.5 [a]		449.9 ± 157.7 [b]	
Recovery	813.1 ± 169.5 [b]		933.4 ± 527.9 [b]		1599 ± 292 [a]	
	CVOl					
Stages	**50**	**80**	**100**	**200**	**300**	**400**
S2 S3a	746 ± 25.9 [a]	711± 77.2 [a]	78.5 ± 7.7 [a,b]	23.2 ± 5.3 [b,c]	21.3 ± 2.9 [b,c]	7.7 ± 1.4 [c]
	-	-	436 ± 88.5 [a]	347± 44.8 [a]	310 ± 32.4 [a]	122 ± 25.6 [a]
S3b	-	-	935 ± 608.1 [a]	627 ± 51.3 [a]	695 ± 78.6 [a]	474 ± 101 [a]
S4	-	-	-	1340 ± 118 [a]	1287 ± 36 [a]	711 ± 134 [a]
Recovery	968.9 ± 19 [a]	1169 ± 130 [a]	-*	-*	-*	-*

the S2 stage was visualized in 62.5% of the fish, and the S3b and S4 stages were detected in the remaining fish. From this moment on, the central depression decreased and 12 h after the start of the experiment, all fish showed normal behavior (Fig. 1B).

Essential oil from leaves of *Aloysia hatschbachii* (AHOl). At 20 mg L^{-1}, fish were in the S2 stage from 10 min to 3 h after the start of the experiment. From 3 to 6 h, 87.5% of the fish exposed to this concentration remained sedated (S2), and 12 h after the start of the experiment, they showed normal behavior. After 10 min at 50 mg L^{-1}, 75% of the fish were in the S4 stage and the remaining fish in the S3b stage. After 20 min, 87.5% of the fish were in the S4 stage and the remaining ones in the stage S3b. All fish were in the S4 stage after 30 min and from this time onwards, the central depressant effect gradually regressed and after 12 h, most fish showed normal behavior. The concentration of 100 mg L^{-1} induced the S4 stage in all fish from 10 min to 2 h, and a 3 h, all fish were at S5 (Fig. 1C).

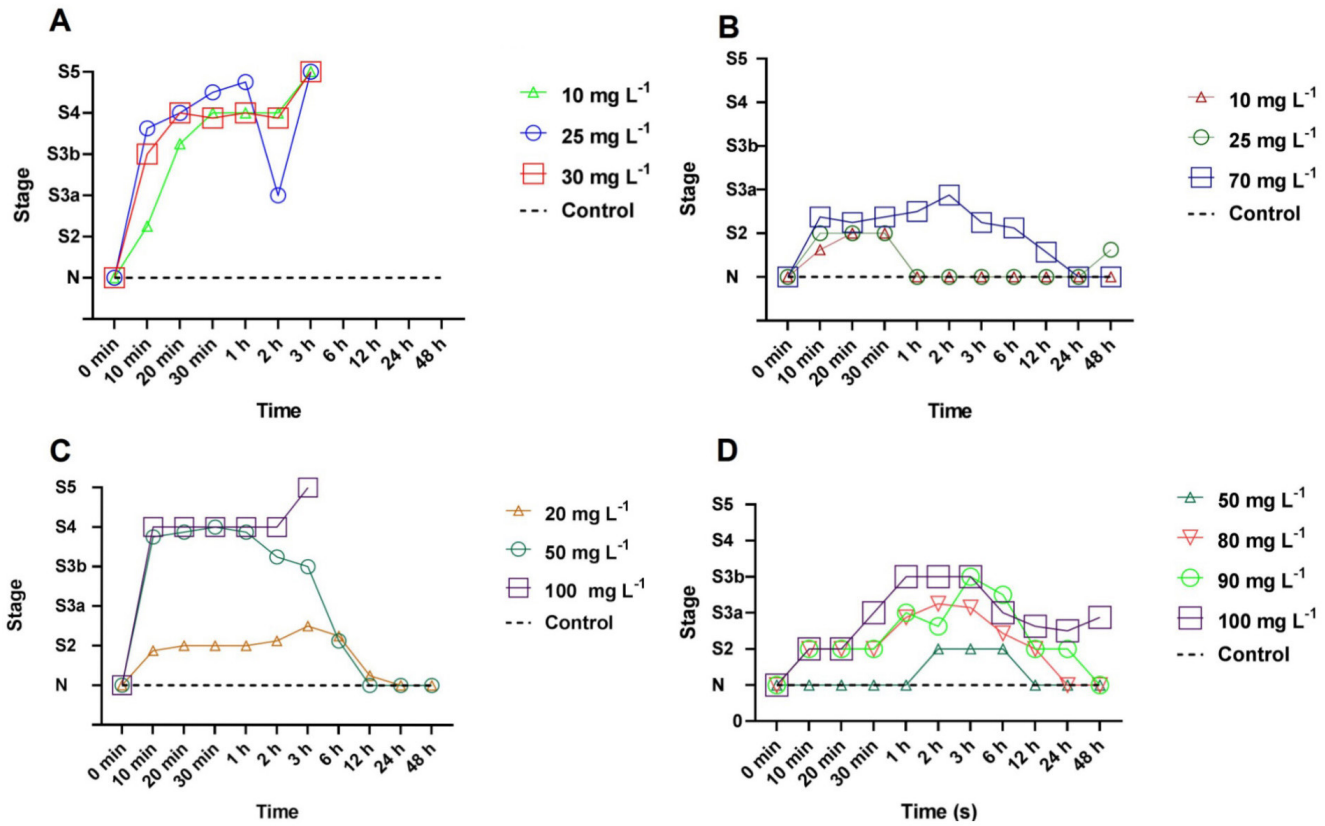

FIGURE 1 | Stages of anesthesia observed over time in *Rhamdia quelen* (silver catfish) exposed to essential oil of inflorescences (**A**) and leaves (**B**) of *Acmella oleracea*, leaves (**C**) of *Aloysia hatscbachii* and leaves (**D**) of *Cordia verbenacea*. N - Normal behavior, S2 - sedation, S3a - partial loss of balance, S3b - total loss of balance, S4 - anesthesia, and S5 - bulbar collapse (n = 8).

Essential oil from leaves of *Cordia verbenacea* (CVOl). The fish subjected to 50 mg L^{-1} of CVOl did not show behavioral changes up to 1 h after the start of the experiment (Fig. 1D). In the evaluation at 2, 3 and 6 h, 100% of the fish were in S2 stage. However, in the evaluation after 12 h until the last evaluation (48 h), 100% of the animals showed normal behavior. At a concentration of 80 mg L^{-1}, sedation (S2) was induced in 10 min. and lasted until 30 min. After 1 h from the beginning of the experiment, 62.5% of the fish were in the S3a stage and, after 2 h, the percentage of fish in this stage rose to 75%. In the 3-h assessment, 50% of the fish were in stage S3b, and the remaining ones were distributed between stages S3a and S2. After 6 h, the central depressant effect decreased, with 75% of animals in S2. In the evaluation 12 h after the start of the experiment, 100% of the fish were in S2 stage, and in the evaluations after 24 and 48 h, all returned to normal behavior. The 90 mg L^{-1} concentration followed the same pattern as 80 mg L^{-1} until 30 min., with all fish in S2. In the evaluation after 1 h, 100% of the animals were in S3a, and after 2 h, 62.5% of the animals continued in this stage and the remaining animals were in S2. After 3 h, 100% of the fish were in S3b, and in the next evaluation, 75% remained in S3b, with the other fish in S2. From this time on, the central depressant signs began to decrease and, at the last evaluation, all fish had returned to normal behavior. At 100 mg L^{-1}, after 10 and 20 min. 100% of the fish were

in stage S2, in 30 min. 100% of the fish were in S3a and in the following evaluation, 100% were in S3b, remaining in this stage until 2 and 3 h after the beginning of the experiment. However, in the evaluation at 6 h, all animals regressed to stage S3a. From this time onwards, signs of central depression decreased until the 24-h assessment. However, at the end of the experiment (48 h), 12.5% of the animals were in S3a, 25% in S4 and the remaining fish were in S2.

Concentration-effect curves obtained for the essential oils tested

Essential oils from inflorescences (AOOi) and leaves (AOOl) of *Acmella oleracea*. The time for the induction of stages S2, S3a decreased as the AOOi concentration increased. The opposite was observed for the recovery time, which increased as the applied concentration increased. Considering the results presented above, this study suggests a concentration of 20 mg L^{-1}, represented in the graph by log = 1.3, as the most recommended. At this concentration, stage S4 was reached in an average time of 153 s, with the recovery time being the shortest detected for this oil at the concentrations evaluated (Fig. 2A). Another relevant aspect, which reinforces the concentration of 20 mg L^{-1} as good for anesthetizing silver catfish, is the fact that it is the only one that did not cause adverse effects. Higher concentrations, such as 80 and 100 mg L^{-1}, caused undesirable effects on fish.

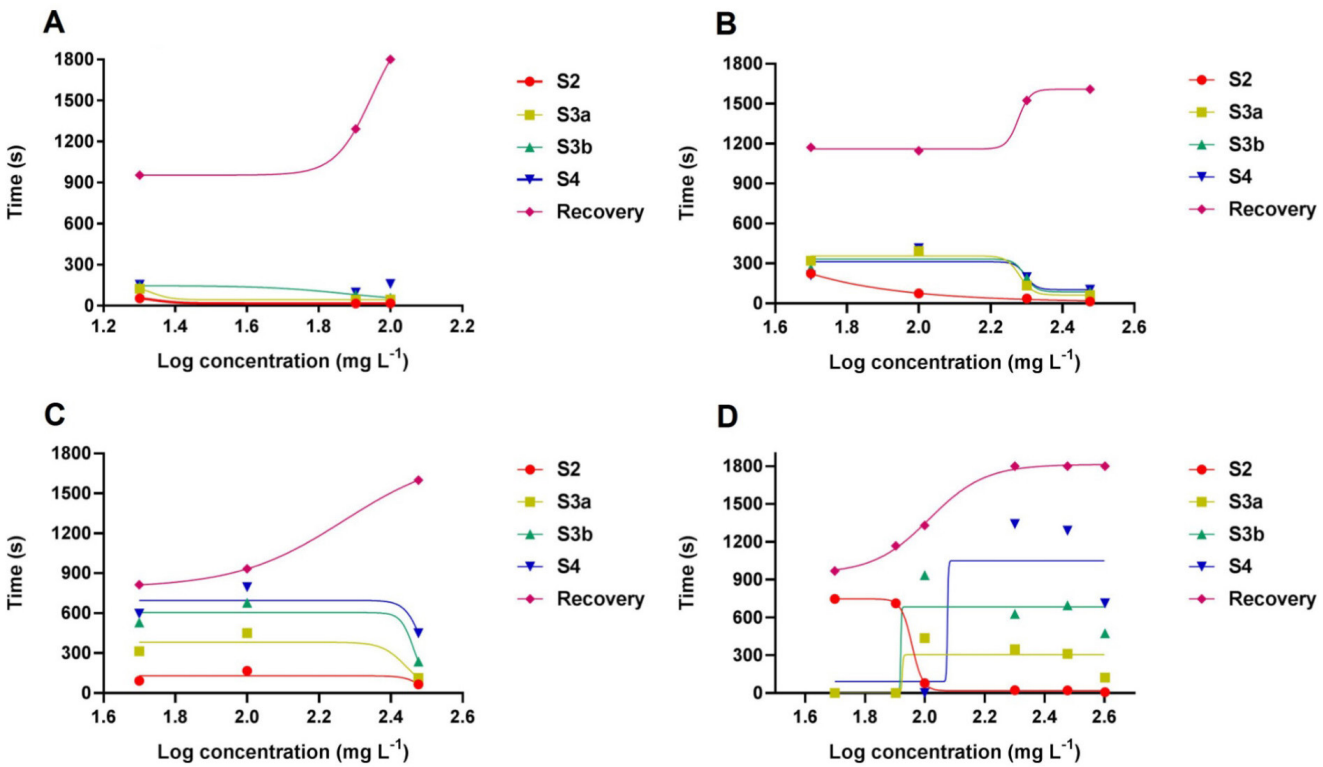

FIGURE 2 | Graphic representation for the studied concentrations of the essential oils of *Acmella oleracea* inflorescences (**A**) and leaves (**B**), *Aloysia hatschbachii* leaves (**C**), and *Cordia verbenacea* leaves (**D**). The graphs were constructed from the equation described in item 2.7. The concentrations are represented in log form, being 20 mg L^{-1} (log = 1.3); 30 mg L^{-1} (log = 1.47); 50 mg L^{-1} (log = 1.69); 80 mg L^{-1} (log = 1.9); 100 mg L^{-1} (log = 2.00); 200 mg L^{-1} (log = 2.3); 300 mg L^{-1} (log = 2.47), and 400 mg L^{-1} (log = 2.6).

All AOOl concentrations evaluated showed a sedative effect and the shortest average time to sedation was detected at 300 mg L^{-1} (log = 2.47) (Fig. 2B). Furthermore, according to the generated curve, the higher the concentration, the shorter the response time. For stages S3a, S3b and S4, curves with similar patterns were obtained. However, at concentrations of 50 mg L^{-1} (log = 1.69) and 100 mg L^{-1} (log = 2.00) the curves are constant, showing a decrease in response time in the case of higher concentrations. Although apparently the concentration of 300 mg L^{-1} (log = 2.47) is the best in terms of response time, there is also an increase in recovery time with increasing concentration. Therefore, the most appropriate AOOl concentrations for use in silver catfish are 50 mg L^{-1} (log = 1.69) or 100 mg L^{-1} (log = 2.00) and only for sedation.

Essential oil from leaves of *Aloysia hastschbachii* (AHOl). Regarding the signs of anesthesia induction/ CNS depression, the concentration-response curves for AHOl show a constant pattern (Fig. 2C). Furthermore, the lowest concentrations showed a similar pattern between them, such as concentrations of 50 mg L^{-1} (log = 1.69) and 100 mg L^{-1} (log = 2.00), with a decrease in induction time for the highest concentration (300 mg L^{-1}; log = 2.47). However, the recovery time at this concentration increased and, in addition, the animals presented adverse effects. Thus, among the concentrations applied, the lowest may be indicated for silver catfish juvenile, as they have shorter recovery time and times to reach anesthetic induction stages similar to 100 mg L^{-1}.

Essential oil from leaves of *Cordia verbenacea* (CVOl). At higher concentrations, CVOl showed a pattern of decreasing induction times for S2 stage, as the concentration increased. Through the curve (Fig. 2D) it is possible to infer that the concentration of 400 mg L^{-1} (log = 2.6) induces this stage with an average time of 7.72 s. Stage S3a was not reached at concentrations of 50 mg L^{-1} (log = 1.69) and 80 mg L^{-1} (log = 1.9). However, the estimated curve for this stage generated a constant line, from the concentration of 100 mg L^{-1} (log = 2.0) to 400 mg L^{-1} (log = 2.6). Stage S3b was very similar to the previous one, also not being reached at concentrations of 50 mg L^{-1} (log = 1.69) and 80 mg L^{-1} (log = 1.9). At this stage, a constant pattern was also maintained, observing a smooth drop in time due to the increase in concentration. On the other hand, stage S4 was only reached from a concentration of 100 mg L^{-1} (log = 2.0), with a reduction in time also being observed in this case because of the increase in concentrations. This pattern was not strong enough to change the pattern of the generated concentration-response curve. Thus, considering the induction of anesthesia stages, the curve indicates that the higher the concentration applied, the better and faster the response. In the case of the concentration-recovery response curve, it is clear that the higher the concentration applied, the longer it will take the fish to recover. In this way, at a concentration of 200 mg L^{-1} (log = 2.3) the maximum acceptable time for stage S4 is reached.

Clinical and/or behavioral signs observed. Adverse effects recorded with AOOi were high excitability, spasms, and convulsions. Furthermore, silver catfish juveniles showed accelerated mouth movements, indicating respiratory distress. In the case of higher concentrations, congested gills were also observed for AOOi (100 mg L^{-1}) and AOOl (300 mg L^{-1}). When the fish have reached stage S4 during anesthetic induction, they were removed from the water for biometry. Then they showed intense agitation,

but apparently returned to stage S4 as soon as they were transferred to the anesthetic recovery aquarium, without reacting to stimuli in caudal peduncle. It is noteworthy that in the anesthetic induction and recovery protocol, no deaths were noted. Mortality was detected only in the long exposure protocol.

At the highest concentration of AHOl evaluated (300 mg L^{-1}), 37.5% of the fish showed regurgitation and marked loss of mucus. Furthermore, after the anesthetic induction and recovery protocol, the animals were observed for another 48 h in aquariums containing only water and oxygenation, with one death being observed in the animals subjected to a concentration of 300 mg L^{-1}. For none of the CVOL concentrations evaluated, adverse or behavioral effects were observed.

DISCUSSION

Long exposure tests with AOOi, at concentrations of 10, 25, and 30 mg L^{-1}, took all tested animals to the S5 stage. Thus, although the results regarding the induction time for stages S2 and S4 are very satisfactory, the concentrations tested showed that they are not suitable for procedures involving long exposure times, as they caused fish death. Therefore, the use of AOOi for transport at these concentrations must be discarded and lower concentrations should be tested. In this case, concentrations of around 2 mg L^{-1} could have been studied, as at a concentration of 20 mg L^{-1} they achieved deep anesthesia in around more than 2 min. These additional tests were not performed due to the impossibility of obtaining additional amounts of AOOi at this moment. The low yield of AOOi, added to the adverse effects and mortality of all fish in long exposure experiments indicated that this EO is not promising and could suggest its exclusion from future studies.

However, the observation of adverse effects in long-term exposure experiments alone does not justify excluding an essential oil/extract from investigation. To evaluate this issue, we must consider that synthetic drugs, such as MS-222, have also shown negative physiological effects on silver catfish (Gressler *et al*., 2012) and yet it is considered a reference anesthetic for aquatic organisms (Williams *et al*., 2009). Furthermore, benzocaine, when tested as an anesthetic in tambaquis, caused agitation in these fish (Gomes *et al*., 2001). Likewise, Barbas *et al*. (2016) described the occurrence of agitation in tambaquis after using the waxy extract of *A. oleracea* inflorescences by immersion. This work is the first to establish sedative and anesthetic activity for the EO of *A. oleracea* inflorescences in experiments with fish, especially silver catfish.

The presence of N-alkylamides such as spilanthol in this plant implies good results to obtain anesthesia. However, it must be remembered that several factors are linked to good results in anesthetic induction, such as the presence of constituents with anesthetic and analgesic potential in the collected plant, the species and size of the fish under study, the concentration used and also water quality parameters (Gomes *et al*., 2011; Bowker *et al*., 2015). The quality parameters of the water used can directly influence the time needed for the fish to reach each stage (Gimbo *et al*., 2008), and it can be one of several factors which influences the anesthetic effectiveness (Olsen *et al*., 1995; Stehly, Gingerich, 1999). This is because the recovery of fish exposed to anesthesia is faster at higher temperatures, which are also associated to higher metabolic rates. On the other

hand, at lower temperatures, anesthetic induction time may be longer (Hikasa *et al*., 1986; Hoskonen, Pirhonen, 2004). Furthermore, factors such as the part of the plant used to extract the active constituents, the composition of the extract/OE, the method of obtaining it and even the time needed to carry out the extraction can influence the levels of efficacy and safety of the essential oil (Lee *et al*., 2001). In this context, the standard pharmacopeial method for the extraction of essential oils was used.

Spilanthol (N-Isobutyl-2*E*, 6*Z*, 8*E*-decatrienamide) was detected in AOOi in proportion of 2.57%. According to Dias *et al*. (2012), this compound is found mainly in inflorescences, which is in agreement with the results of this work, as in AOOl this compound was not detected. Spilanthol has several proven beneficial activities, such as analgesic, anti-inflammatory and did not show significant cytotoxic activities (Rios *et al*., 2007; Wu *et al*., 2008) when isolated from *A. oleracea* extract and tested in mice. Spilantol is considered to have high anesthetic and analgesic potential (Nomura *et al*., 2013). Although spilanthol is one of the minor AOOi components, according to a review by Spinozzi *et al*. (2022) its anesthetic activity is well established and is the result of increased GABA release, activation of the GABAergic, serotonergic and opioid systems. The interaction with the vanilloid receptors TRPV1 and TRPA1 and the blockade of voltage-gated Na^+ channels also contribute to this action. In this context, the time taken to induce anesthesia in silver catfish was very encouraging, although this compound was in low concentration in AOOi. However, the effects observed for an EO often result from the collaborative action of several components. The major compounds found in this EO were β-ocimene (40.12%), β-caryophyllene (36.52%) and β-phellandrene (11.25%). No information was found in the literature about a possible CNS depressant action of β-ocimene. However, anti-inflammatory, analgesic and anxiolytic activities have been described for β-caryophyllene (Galdino *et al*., 2012). On the other hand, β-phellandrene showed genotoxicity in *in vitro* and *in vivo* tests female SPF ICR mice, however at much higher concentrations than those used in this study (Cheng *et al*., 2017).

Essential oil from *Acmella oleracea* leaves (AOOl), at a concentration of 300 mg L^{-1}, caused adverse effects on fish, but much weaker than the effects detected for AOOi. At this concentration, AOOl only caused fish excitability. However, of the concentrations used in the long exposure protocols (10, 25 and 70 mg L^{-1}), the first only lead fish to sedation and did not cause any visible adverse effect, which, when subjected to 10 mg L^{-1}, presented recovered at the end of the protocol. However, at concentrations of 25 and 70 mg L^{-1}, 12.5% of the animals reached the S5 stage. Therefore, we believe that the absence of notable adverse effects, such as those observed in AOOi, may be due to the absence of spilanthol in the composition of AOOl. Spilanthol is also recognized as having insecticidal properties (Pandey *et al*., 2011; Barbosa *et al*., 2016). Therefore, the toxic effects observed could be linked to this compound.

Additionally, despite all the scientific evidence on the effects of *A. oleracea*, its sedative and anesthetic activity is still controversial, since, despite the good results for the *A. oleracea* flower extract described by Leite *et al*. (2022), the authors argue that the extract induced seizure-like behavior in the fish. It cannot be ruled out the possibility that other compounds are causing the adverse effects. Studies with the EOs must be further developed, because if the results are promising for other aquatic species and even for silver catfish, the oils from this species may have potential for the development of an anesthetic for aquatic animals.

However, the limiting factor in this case is the very low EOs yield of this species, especially from inflorescences. To overcome this bottleneck, one of the alternatives would be to invest in conventional breeding processes or those involving genetic engineering, aiming to increase the production of essential oil and/or the concentration of potentially active substances (Cappellari *et al*., 2019; Silva-Santos *et al*., 2023).

Although AHOl caused marked loss of mucus in induction and long-term exposure protocols at higher concentrations, at concentrations of 20 and 50 mg L^{-1}, no adverse effects or mortality were observed. Therefore, the use of concentrations above 50 mg L^{-1} are not recommended for juvenile silver catfish, since mucus is one of the most important protective substances associated with fish skin (Seriani *et al*., 2015; Adorian *et al*., 2020). The EO of this plant, described as a recent occurrence in the State of Rio Grande do Sul (Araujo *et al*., 2020), led all animals exposed to the immersion bath at a concentration of 100 mg L^{-1} to the S5 stage in the long exposure protocol.

The genus *Aloysia* has species of high importance for aquaculture, such as *Aloysia triphylla*, whose EO has anesthetic and growth-stimulating activity when added to the diet (Daniel *et al*., 2014; Zeppenfeld *et al*., 2014, 2016, 2017), in addition to antibacterial and antispasmodic activities (Merétika *et al*., 2010). Another important fact is the chemical composition of AHOl, since one of the major compounds is eucalyptol /1,8-cineole (42.78%), which is present in oils from other species with consolidated importance for aquaculture, such as *Lippia alba*, which has an anesthetic effect in several aquatic species (Cunha *et al*., 2010; Becker *et al*., 2012). Other components were also detected in percentages above 5%, such as β-guaiene (8.71%) and elemene (6.94%). Thus, this study demonstrated that low concentrations may be promising for use as a sedative and anesthetic in animal production.

The anesthetic activity of eucalyptol had previously been reported for *Cyprinus carpio* (Mazandarani *et al*., 2017), *Oncorhynchus mykiss* (Mirgahed *et al*., 2018) and *Salmo caspius* (Mirgahed *et al*., 2022). For some of the secondary constituents of AHOl, central depressant effects have also been reported in the literature. For farnesol, which occurs in AHOl at a rate of 5.1%, Jeevan *et al*. (2023) described the modulation of $GABA_A$ receptors, which is the site of action of several substances of natural origin with an anesthetic effect in fish (Helwein *et al*., 2012, Garlet *et al*., 2019a,b). Another minor component whose anaesthetic activity in silver catfish was previously proven by our research group is spathulenol, present in this oil in a proportion of 2.6% (Benovit *et al*., 2015).

Cordia verbenacea is well known and used in folk medicine, mainly due to the properties of its leaves. In this sense, its anti-inflammatory, anti-ulcer and anti-rheumatic actions are already known (Sertié *et al*., 1988; Roldão *et al*., 2008). Furthermore, in Brazil there is already an herbal medicine for topical use registered in ANVISA as an anti-inflammatory, produced from the EO of this plant (Nizio *et al*., 2015). Another important factor is that no toxic activities have been described to date due to the use of extracts or substances isolated from this plant, when applied orally or topically (Basile *et al*., 1989; Oliveira *et al*., 1998; Bayeux *et al*., 2002; Carvalho *et al*., 2004; Sertié *et al*., 2005; Passos *et al*., 2007; Roldão *et al*., 2008). In this study, no adverse effects were observed for CVOl, both in induction and in long exposure experiments in juvenile silver catfish. Regarding chemical constituents, the EO under study presented α-pinene (34.8%) as its main constituent. For a-pinene, the major component of CVOl, it was proven to bind to the benzodiazepine site of the GABAa receptor, thus increasing the affinity of GABA

to its binding site (Yang *et al*., 2016) and reinforcing its inhibitory action. Another component detected in low proportions in this oil, and which had its anesthetic and sedative effect described in silver catfish is spathulenol, whose effectiveness was similar to eugenol (Benovit *et al*., 2015). As CVOL showed sedative effects and no toxicity at 50 mg L^{-1}, this recommended concentration for transport could also have an additional anti-inflammatory effect, due to the presence of a-humulene (Fernandes *et al*., 2007).

It is worth highlighting that when exploring experimental concentrations for anesthesia in fish, some factors can influence the anesthetic action (Sneddon, 2012), and this influence can be seen in some induction times. This is because as concentration increased, the time to reach the stage also increased. However unusual, a similar pattern was observed with silver catfish sedated with the methanolic extract of *Condalia buxifolia* (Becker *et al*., 2013). Apparent incoherent results were observed in previous studies with complex mixtures of plant extracts and can be explained by the interaction between the components of these mixtures (Efferth, Koch, 2011). These interactions can result in potentiation, additive effect, synergism or antagonism. Antagonistic substances may not reach the effective concentration when the essential oil is used at low concentrations, but their effect is detected in higher concentrations by increasing the induction time. In addition to the pharmacodynamic interactions explained above, pharmacokinetic interactions may also occur between the different active components.

Considering the efficacy and safety data obtained in this work, all essential oils tested showed some compatible level of CNS depression in silver catfish juveniles. Some samples caused adverse effects and/or mortality. Additional evaluations are necessary, considering other concentrations and the implementation of protocols to determine cortisol and/or additional secondary markers of stress response, among other evaluations, such as how much EOs can affect the cardiovascular system and long-term development of juveniles, for example. For silver catfish juveniles, CVOl at a concentration of 50 mg L^{-1} was sedative and showed no adverse effects. AOOi can be used at a concentration of 20 mg L^{-1}, without adverse effects and AOOl can be used at concentrations of 50 and 100 mg L^{-1} for sedation and/or anesthesia. Finally, AHOl can be used at a concentration of 50 mg L^{-1} for sedation and/or anesthesia, allways considering the same fish species, development stage and water quality parameters. The CNS depressant effects observed for the evaluated EOs are due to the association of different components.

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ETHICAL STATEMENT

The present study is registered in Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SISGEN) under number A6FA8B7 and was approved by the UFSM Ethics Committee, under number 6037240221.

COMPETING INTERESTS

The author declares no competing interests.

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