

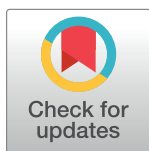
RESEARCH ARTICLE

Righting response and electrocardiographic evaluation of juvenile tambaqui (*Colossoma macropomum*) (Cuvier, 1818) exposed to immersion baths with different concentrations of bushy lippia essential oil (*Lippia alba*)

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Abstract

In recent years, the use of natural products as alternatives to synthetic anesthetic agents has gained notoriety in aquaculture. Among the essential oils studied, *Lippia alba* has attracted attention due to its diverse pharmacological properties, including sedative and anesthetic effects. This study aims to evaluate the anesthetic activity of *Lippia alba* essential oil (LAEO) to propose a therapeutic window for its use in tambaqui (*C. macropomum*). In this research, juvenile tambaqui ($10.9 \pm 5.9\text{g}$, $n = 90$) were submitted to anesthetic baths with LAEO at concentrations of $80\text{--}140\ \mu\text{L.L}^{-1}$ for behavioral assessment and electrophysiological recordings. The findings showed that all concentrations were effective in inducing the loss of posture reflex and its subsequent reversible return. However, electrocardiographic recordings at concentrations of 120 and $140\ \mu\text{L.L}^{-1}$ revealed alterations in the graph elements, indicating more intense bradycardia and atrioventricular block during anesthetic induction. Nevertheless, during the anesthetic recovery period, cardiac normality was restored at all tested concentrations, although more slowly at the highest concentration ($140\ \mu\text{L.L}^{-1}$). Thus, we conclude that this oil is safe for providing short-term anesthesia at concentrations between 80 and $100\ \mu\text{L.L}^{-1}$ in *C. macropomum* specimens.

Introduction

There is a growing number of studies investigating the application of natural products such as essential oils and their active substances as potential replacements for synthetic anesthetics in aquaculture [1,2]. *Lippia alba*, an aromatic plant from the Verbenaceae family widely cultivated in Brazil and commonly known as “lemon balm” has gained attention due to its diverse

pharmacological properties [3,4]. The anesthetic, antifungal, and antimicrobial activities of *L. alba* essential oil are well-documented by various authors [5–7]. The sedative and anesthetic actions of *L. alba* essential oil are directly related to the compounds citral (geranial and neral) and linalool, possibly through the modulation of the GABAergic system [8–10].

In light of this, the applicability of this essential oil as an anesthetic agent has been investigated in various fish species. A previous study reinforces the efficacy of this oil, noting that in addition to its anesthetic activity, it also reduces stress in tambacu (*Piaractus mesopotamicus* x *Colossoma macropomum*) during handling and transportation [11]. Furthermore, this essential oil has been associated with antioxidant effects that are crucial during periods of hyperoxia and hypoxia occurring during fish transport [12]. However, high doses of *L. alba* can lead to toxic effects, such as osmoregulatory changes and alterations in cortisol and glucose levels [13,14]. Therefore, it is evident that investigating different concentrations of this essential oil and its secondary effects on the animal is of significant importance.

There are numerous studies in the literature employing *L. alba* essential oil as an anesthetic agent in *C. macropomum*. In the study conducted by Souza et al. (2018) [15], the anesthetic efficacy of *L. alba* essential oil, chemotype linalool, was particularly evident, demonstrating effective sedation and anesthesia without compromising the recovery period. Additionally, a prior study establishes an anesthetic protocol for tambaqui using *L. alba* essential oil [5]. Although the anesthetic efficacy of *L. alba* is well-known, its systemic effects on *C. macropomum*, particularly its association with cardiac activity to delineate a safety margin for the use of this anesthetic, are not well understood.

Therefore, considering the potential properties of this essential oil as an anesthetic for fish and the lack of detailed information on its effects on the cardiac function of these animals, this study aims to provide an evaluation of the electrocardiogram of juvenile tambaqui subjected to anesthetic baths containing *L. alba* essential oil, in order to examine the risks and establish an appropriate therapeutic window for its use as an anesthetic.

Materials and methods

Experimental animals

The fish used for this research were 90 juveniles tambaquis, *Colossoma macropomum*, which were kept in aquariums (500 L) at the Experimental Animal Facility of the Laboratory of Pharmacology and Toxicology of Natural Products at the Federal University of Pará (LFTPN-IC-B-UFPA). The room had a controlled temperature (25 to 28°C) and a 12-hour light: 12-hour dark photoperiod, and the fish were fed commercial feed (32% protein) twice daily until satiation. Simultaneously, daily tank cleaning was performed through siphoning, and approximately 20% of the tank water was replaced with water from the same source. During the 15-day acclimation period, water quality variables such as temperature (°C), hydrogen potential (pH), dissolved oxygen (DO), ammonia (NH₃+), and total hardness were monitored and maintained within the following values: Temp 26.8°C; pH 7.5; DO > 5.0 mg.L⁻¹; NH₃+ 0.01 mg.L⁻¹; total hardness 70 NTU. This project was approved by the Ethics Committee on Animal Use of the Federal University of Pará (CEUA/UFPA) under protocol number 1024010224.

Acquisition, preparation, and preservation of the drug

Essential oil of *Lippia alba*, produced by the company Legeé óleos essenciais (Brasial-SP), with chromatographic data performed at the Laboratory of the University of Santa Cruz do Sul using a Gas Chromatograph with Mass Detector—Agilent Model MSD 5977B, showed the components described in Table 1. The essential oil of *Lippia alba* was diluted to 10% in 70% alcohol for use during the experiment.

Table 1. Chromatographic profile of *Lippia alba* essential oil, used in this study.

Identification	Relative area percentage (%)	Similarity (%)	Retention time (%)
Linalool	66,16	98,39	21,18
Eucalyptol	8,44	98,74	16,05
Caryophyllene	6,08	98,71	52,73
β -Copaene	4,14	98,85	56,06
β -Elemene	2,63	96,71	50,78
γ -Elemene	2,60	96,56	53,42
Sabinene (β -Thujene)	1,54	97,80	12,50
α -Pinene	1,38	98,44	10,67
Humuleno	1,01	97,16	54,78
Caryophyllene oxide	0,81	97,95	60,09
Germacrene B	0,62	97,60	59,24
Copaene	0,59	96,44	49,58
δ -Cadinene	0,52	95,46	57,70
Valence	0,36	93,09	55,00
β -Bourbonene	0,35	89,27	50,22
β -Myrcene	0,34	89,95	13,34
D-Limonene	0,29	91,50	15,84
(+)-epi-Bicyclosesquiphellandrene	0,27	88,34	50,64
Bicyclosesquiphellandrene	0,27	87,85	50,64
β -Copaen-4 α -ol	0,26	89,86	65,01
Linalool oxide	0,25	91,67	18,83
α -Murolene	0,24	95,81	56,89
γ -Cadinene	0,23	95,55	55,80
Ylangenal	0,22	93,04	62,60
Elemene isômero	0,21	93,64	56,72
trans-Linalool oxide (furanoid)	0,19	88,87	20,12

Indication of relative percentage (%), similarity (%), retention time (%), and registration in the Chemical Abstracts Service (CAS).

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Experiment 1—Description of the behavioral experiment

For the behavioral evaluation, the animals (10.9 ± 5.9 g) were subjected to immersion baths with *Lippia alba* essential oil (LAEO). The groups were divided according to the following concentrations: a) $80 \mu\text{L.L}^{-1}$ group; b) $100 \mu\text{L.L}^{-1}$ group; c) $120 \mu\text{L.L}^{-1}$ group; d) $140 \mu\text{L.L}^{-1}$ group. Specific concentrations of this essential oil were tested by Silva et al. (2019). In contrast, the present study examines a broader range of concentrations to explore a more continuous gradient of effects.

For each treatment, $n = 9$ animals were used, totaling 36 animals. The behavioral assessment was conducted over 5 minutes for induction and recovery of anesthesia, evaluating the time to loss of posture reflex and recovery of posture reflex, respectively.

Experiment 2—Description of the electrophysiological experiment (electrocardiogram ECG)

The *C. macropomum* were randomly distributed into the following treatments: a) control group (baseline activity recording); b) vehicle group (only the vehicle was used to assess interference in the experiment at $1,400 \mu\text{L.L}^{-1}$) c) 80 LAEO group; d) $100 \mu\text{L.L}^{-1}$ LAEO group; e)

120 $\mu\text{L.L}^{-1}$ LAEO group; f) 140 $\mu\text{L.L}^{-1}$ LAEO group. All cardiac activity recordings lasted for 5 minutes. For each recording, $n = 9/\text{treatment}$ were used, totaling 54 animals. The analysis of electrocardiographic recordings was performed during induction and during anesthetic recovery.

Acquisition of the electrocardiogram (ECG)

For the analysis and monitoring of cardiac function, electrodes were made of 925 silver with a diameter and length of 0.3 mm and 10 mm, respectively, in a non-conjugated manner. The position used for fixing the reference electrode followed the indication of the cardiac vector and was fixed ventrally 2 mm before the end of the left opercular cavity (reference electrode). The recording electrode was inserted 2 mm before the end of the right opercular cavity (ventral). After the insertion of the electrodes, they were connected to a high-impedance amplifier (Grass Technologies, Model P511) for the acquisition of electrocardiographic records. With these, the following parameters were analyzed: heart rate (bpm), amplitude (mV), QRS complex duration (ms), and R-R (ms), P-Q (ms), and Q-T (ms) intervals.

Recording and analysis of records

The electrodes were connected to a digital data acquisition system through a high-impedance differential input amplifier (Grass Technologies, Model P511), configured for filtering from 0.3 to 300 Hz and amplification of 2000X, monitored by an oscilloscope (ProteK, Model 6510). The records were continuously recorded and digitized at a rate of 1 KHz on a computer with a data acquisition board (National Instruments, Austin, TX), and stored on a hard drive for further processing using specialized software (LabVIEW express). Record analysis was performed using Python version 2.7. For mathematical processing, the Numpy and Scipy libraries were used, and for graph creation, the Matplotlib library. The graphical interface was developed using the PyQt4 library (Souza-Monteiro et al., 2015). The amplitude graphs, in turn, highlighted the potential differences between the reference and recording electrodes. The signals from the records were obtained at 1000 samples per second.

Statistical analysis

After verifying the assumptions of normality and homogeneity of variances using the Kolmogorov-Smirnov and Levene tests, respectively, comparisons of mean power values were conducted using one-way ANOVA, followed by Tukey's test. GraphPad Prism[®] 8 software was used for the analyses, and a significance level of * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ was considered statistically significant in all cases.

Results

The behavioral analysis showed that LAEO caused a concentration-dependent loss of postural reflex. In this sense, the higher the dose, the shorter the latency for the onset of postural reflex loss, and a small increase in concentration resulted in a faster induction. However, animals subjected to treatments of 120 and 140 $\mu\text{L.L}^{-1}$ showed no difference in the time to induction ($p = 0.1178$). Fish exposed to 80 $\mu\text{L.L}^{-1}$ had a mean time to present postural loss of $247.1 \pm 12.35\text{s}$, which was higher than the other groups. The group treated with 100 $\mu\text{L.L}^{-1}$ ($182.9 \pm 32.72\text{s}$) had a longer induction time than the 140 $\mu\text{L.L}^{-1}$ group ($134.2 \pm 12.13\text{s}$). Fish treated with 100 $\mu\text{L.L}^{-1}$ were similar to the groups treated with 120 $\mu\text{L.L}^{-1}$ ($158.3 \pm 24.42\text{s}$) ($p = 0.10820$) (Fig 1A).

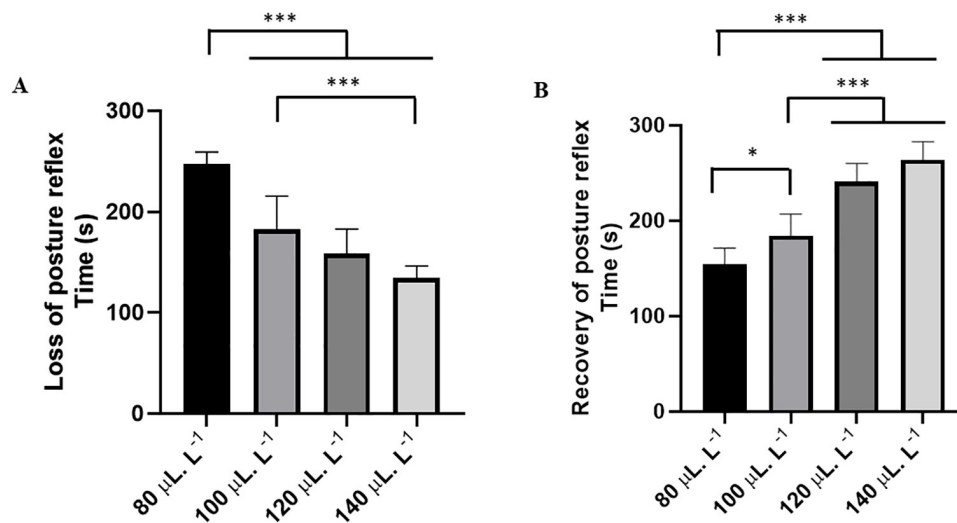


Fig 1. Mean latencies (s) for the loss of postural reflex in *Colossoma macropomum* during 5-minute immersion baths with different concentrations of *Lippia alba* essential oil (LAEO) (A). Recovery of postural reflex after exposure to different concentrations of LAEO (B). (ANOVA followed by Tukey's test; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

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The recovery of the postural reflex in the group exposed to $80 \mu\text{L.L}^{-1}$ occurred in 154.1 ± 17.34 s, which was shorter than the other groups: $100 \mu\text{L.L}^{-1}$ (184.4 ± 22.19 s), $120 \mu\text{L.L}^{-1}$ (240.8 ± 19.19 s), and $140 \mu\text{L.L}^{-1}$ (263.6 ± 19.38 s). The groups treated with 120 and $140 \mu\text{L.L}^{-1}$ were similar ($p = 0.0851$) (Fig 1B).

Cardiac activity between the control and vehicle groups (not shown) was similar ($p = 0.9998$); therefore, we considered only the control group for comparisons between treatments. The control group exhibited a mean frequency of 91.78 ± 4.73 bpm, with sinus rhythm, and all cardiac elements were present in the electrocardiogram (Fig 2A). In a 30-second amplification, rhythmic cardiac activity is demonstrated, and the elements of the *Colossoma macropomum* normal activity graph can be observed, including the P wave, QRS complex, and T wave, allowing for the evaluation of intervals during immersion in LAEO and its recovery. Thus, we can identify atrial activity represented by P waves, ventricular activity by QRS complexes, and ventricular repolarization by T waves (Fig 2B and 2C). The graph elements that allowed for data evaluation are indicated in Fig 2C.

During treatment with LAEO at concentrations of $80 \mu\text{L.L}^{-1}$, $100 \mu\text{L.L}^{-1}$, $120 \mu\text{L.L}^{-1}$, and $140 \mu\text{L.L}^{-1}$, the ECG showed a concentration-dependent decrease in cardiac excitability (Fig 3A–3D). However, at higher treatment concentrations ($120 \mu\text{L.L}^{-1}$ and $140 \mu\text{L.L}^{-1}$), a pronounced decrease in cardiac activity was observed. Moreover, there was atrioventricular blockage at these treatment concentrations, as evidenced by repeated P waves without the presence of the QRS complex, indicating non-sinus rhythm (Fig 3C and 3D). Heart rate decreased by 32.44% in the group treated with $80 \mu\text{L.L}^{-1}$ and by 33.41% in fish exposed to $100 \mu\text{L.L}^{-1}$ compared to the control. Fish receiving $120 \mu\text{L.L}^{-1}$ and $140 \mu\text{L.L}^{-1}$ exhibited more intense bradycardia compared to the control, with a respective decrease in cardiac function of 61.25% and 62.71% (Fig 3).

Heart rate was significantly affected by increasing concentrations of LAEO. The control group had a mean of 91.78 ± 4.73 bpm, which was higher than the other groups. The group treated with $80 \mu\text{L.L}^{-1}$ had a mean of (62.00 ± 1.73 bpm) and was similar to the group treated with $100 \mu\text{L.L}^{-1}$ (61.11 ± 2.02 bpm) ($p = 0.9693$), while the group treated with $120 \mu\text{L.L}^{-1}$

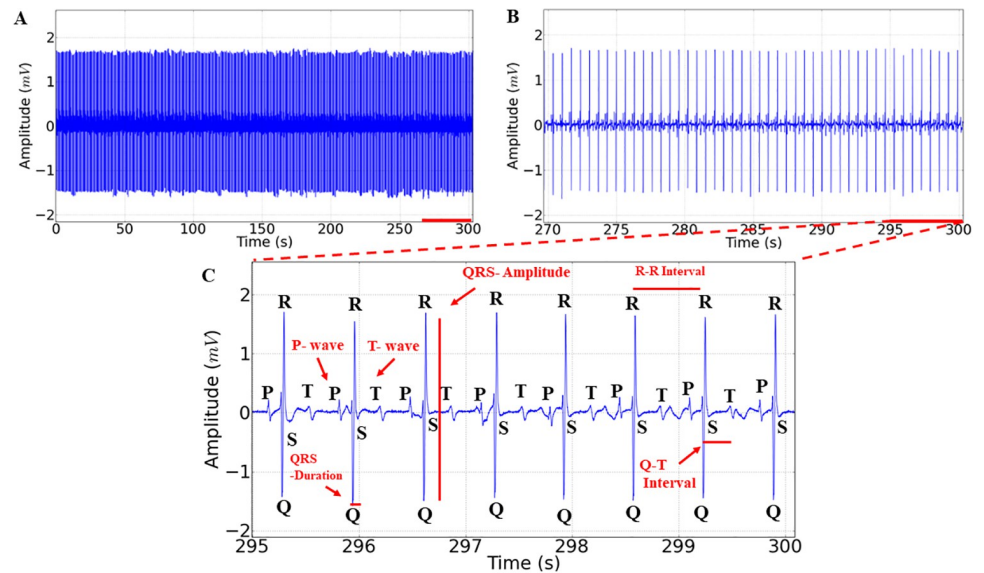


Fig 2. Electrocardiographic recording showing cardiac activity of the control group in juvenile *Colossoma macropomum* (A), amplification of the last 30 seconds of the 5-minute recording (270 to 300s) (B), the graph elements evaluated in the study demonstrating the P wave, QRS complex, and T wave, and variables such as heart rate (bpm), amplitude (mV), R-R, P-Q, and Q-T intervals (ms), and QRS complex duration (ms), are indicated in red (C).

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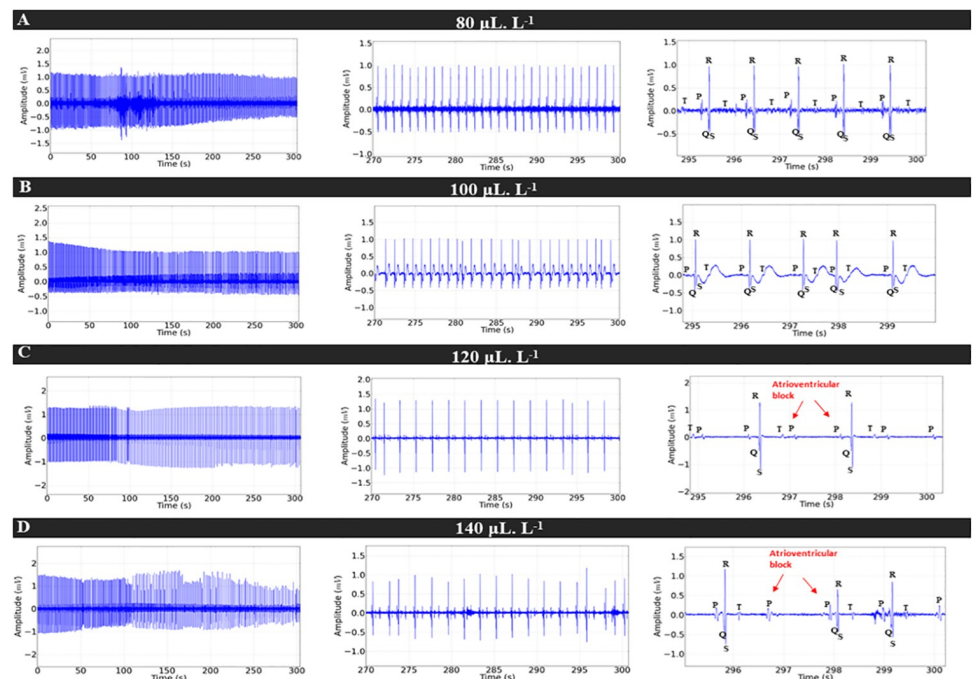


Fig 3. Cardiac activity in juvenile *Colossoma macropomum* during immersion bath in different concentrations of LAEO (left). Amplification of the recording in the last 30 seconds (270–300 s) (center), showing the amplification of the final 5 s of the recording (295–300 s) for identification of cardiac deflections (right), for treatment concentrations 80 $\mu\text{L.L}^{-1}$ (A), 100 $\mu\text{L.L}^{-1}$ (B), 120 $\mu\text{L.L}^{-1}$ (C), and 140 $\mu\text{L.L}^{-1}$ (D).

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(35.56 ± 2.40 bpm) was similar to the group treated with $140 \mu\text{L.L}^{-1}$ (34.22 ± 3.07 bpm) ($p = 0.8774$) (Fig 4A).

The mean QRS complex amplitude of the control group was 1.52 ± 0.21 mV, which was similar to the other groups. The group treated with $80 \mu\text{L.L}^{-1}$ (1.50 ± 0.31 mV), the group treated with $100 \mu\text{L.L}^{-1}$ (1.36 ± 0.24 mV), the group treated with $120 \mu\text{L.L}^{-1}$ (1.59 ± 0.19 mV), and the group treated with $140 \mu\text{L.L}^{-1}$ (1.49 ± 0.31 mV) were similar ($F(4,45) = 0.9945$; $p = 0.4204$) (Fig 4B).

The mean R-R interval of the control group was 656.6 ± 34.51 ms and was lower compared to the other groups. The group treated with $80 \mu\text{L.L}^{-1}$ had a mean R-R interval of 971 ± 29.28 ms, which was similar to the group treated with $100 \mu\text{L.L}^{-1}$ (974.4 ± 34.68 ms) ($p = 0.9999$). The groups treated with $120 \mu\text{L.L}^{-1}$ (1689 ± 113.0 ms) were similar to the group treated with $140 \mu\text{L.L}^{-1}$ (1762 ± 160.1 ms) ($p = 0.4503$) (Fig 4C).

The mean P-Q interval for the control group was 143.2 ± 1.92 ms and was similar to the groups treated with $80 \mu\text{L.L}^{-1}$ (175.2 ± 1.92 ms) and $100 \mu\text{L.L}^{-1}$ (167.3 ± 25.31 ms) ($p = 0.2868$). The groups treated with LAEO at concentrations of $120 \mu\text{L.L}^{-1}$ (255.7 ± 34.58 ms) and $140 \mu\text{L.L}^{-1}$ (253.6 ± 62.76 ms) were similar ($p = 0.9999$) (Fig 4D).

The mean duration of the QRS complex for the control group during induction was 38.32 ± 3.52 ms, which was similar to the groups treated with $80 \mu\text{L.L}^{-1}$ (37.22 ± 4.17 ms), $100 \mu\text{L.L}^{-1}$ (37.33 ± 3.31 ms), and $120 \mu\text{L.L}^{-1}$ (40.67 ± 2.83 ms) ($p = 0.5514$). The group treated with $140 \mu\text{L.L}^{-1}$ of LAEO (46.67 ± 2.95 ms) was higher than the other groups (Fig 4E).

For the control group, the mean Q-T interval during induction was 278.7 ± 4.79 ms, which was lower than the other groups. The groups treated with $80 \mu\text{L.L}^{-1}$ (352.1 ± 90.78 ms) and $100 \mu\text{L.L}^{-1}$ (362.4 ± 38.68 ms) were similar ($p = 0.9921$). The $120 \mu\text{L.L}^{-1}$ group (543.3 ± 36.82 ms) and $140 \mu\text{L.L}^{-1}$ group (569.2 ± 38.43 ms) were similar ($p = 0.8084$) (Fig 4F).

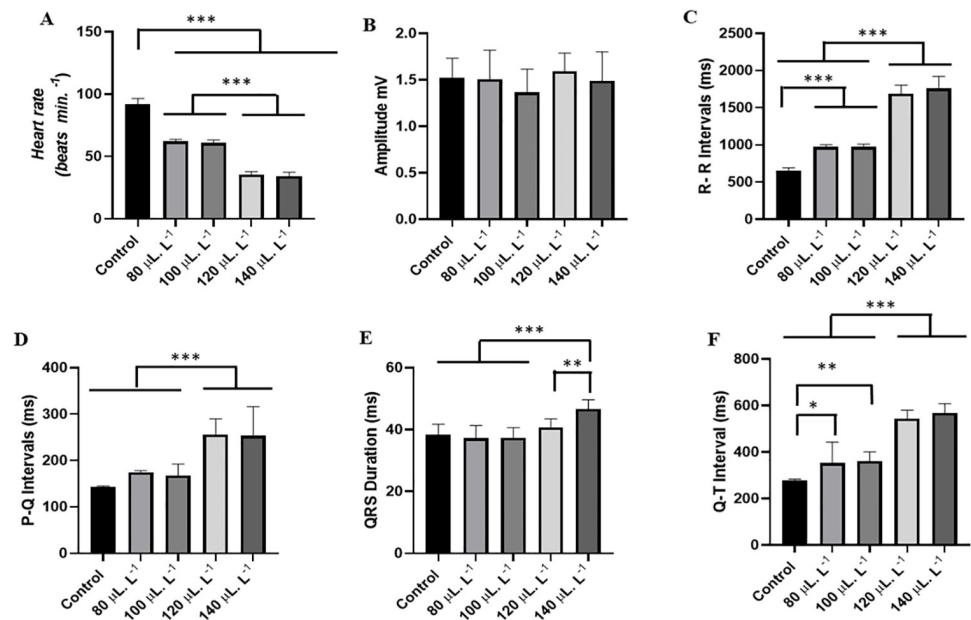


Fig 4. Mean values of heart rate in beats per minute (bpm) during immersion baths with LAEO treatments of $80 \mu\text{L.L}^{-1}$, $100 \mu\text{L.L}^{-1}$, $120 \mu\text{L.L}^{-1}$, and $140 \mu\text{L.L}^{-1}$ (A), mean values of QRS complex amplitude (mV) (B), mean values of R-R intervals (ms) (C), P-Q intervals (ms) (D), QRS complex duration (ms) (E), and Q-T intervals (ms) (F). (ANOVA followed by Tukey's test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; $n = 9$).

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During the recovery from LAEO at $80 \mu\text{L.L}^{-1}$, $100 \mu\text{L.L}^{-1}$, $120 \mu\text{L.L}^{-1}$, and $140 \mu\text{L.L}^{-1}$, a slow reversibility was observed, with sinus bradycardia being more pronounced in the group treated with the highest concentration, indicating that recovery was slow and concentration-dependent (Fig 5). The increase in heart rate gradually approached that of the control group. Accordingly, for the group treated with $80 \mu\text{L.L}^{-1}$, the heart rate value relative to the control group was 90.79% (Fig 5A). For the other groups, the values were 92.49% for $100 \mu\text{L.L}^{-1}$ (Fig 5B), 91.04% for $120 \mu\text{L.L}^{-1}$ (Fig 5C), and 70.46% for $140 \mu\text{L.L}^{-1}$ (Fig 5D). No arrhythmias were observed during the recovery period, and the reversal of LAEO effects on the heart was gradual and dependent on the concentration used (Fig 5).

During the recovery period, the control group exhibited an average heart rate of 91.78 ± 4.73 bpm, which was higher than the other groups: the $80 \mu\text{L.L}^{-1}$ group with an average of 83.33 ± 2.23 bpm, the $100 \mu\text{L.L}^{-1}$ group with 84.89 ± 3.01 bpm, the $120 \mu\text{L.L}^{-1}$ group with 83.56 ± 2.78 bpm, and the $140 \mu\text{L.L}^{-1}$ group with 64.67 ± 2.82 bpm. The groups treated with $80 \mu\text{L.L}^{-1}$, $100 \mu\text{L.L}^{-1}$, and $120 \mu\text{L.L}^{-1}$ were similar ($p = 0.8447$) (Fig 6A).

The amplitude of the QRS complex during recovery for the control group was 1.52 ± 0.21 mV, which was similar to the other groups: $80 \mu\text{L.L}^{-1}$ (1.59 ± 0.22 mV), $100 \mu\text{L.L}^{-1}$ (1.47 ± 0.23 mV), $120 \mu\text{L.L}^{-1}$ (1.43 ± 0.23 mV), and $140 \mu\text{L.L}^{-1}$ (1.57 ± 0.29 mV) ($F(4, 45) = 0.7171$; $p = 0.584$) (Fig 6B).

The mean R-R interval during recovery for the control group was 656.6 ± 34.51 ms, which was shorter than that of the other groups. The $80 \mu\text{L.L}^{-1}$ group with 720.0 ± 19.69 ms, the $100 \mu\text{L.L}^{-1}$ group with 702.0 ± 24.22 ms, and the $120 \mu\text{L.L}^{-1}$ group with 718.4 ± 24.66 ms were similar ($p = 0.7043$). The group treated with $140 \mu\text{L.L}^{-1}$ had a mean R-R interval of 929.0 ± 40.88 ms, which was longer than that of the other groups (Fig 6C).

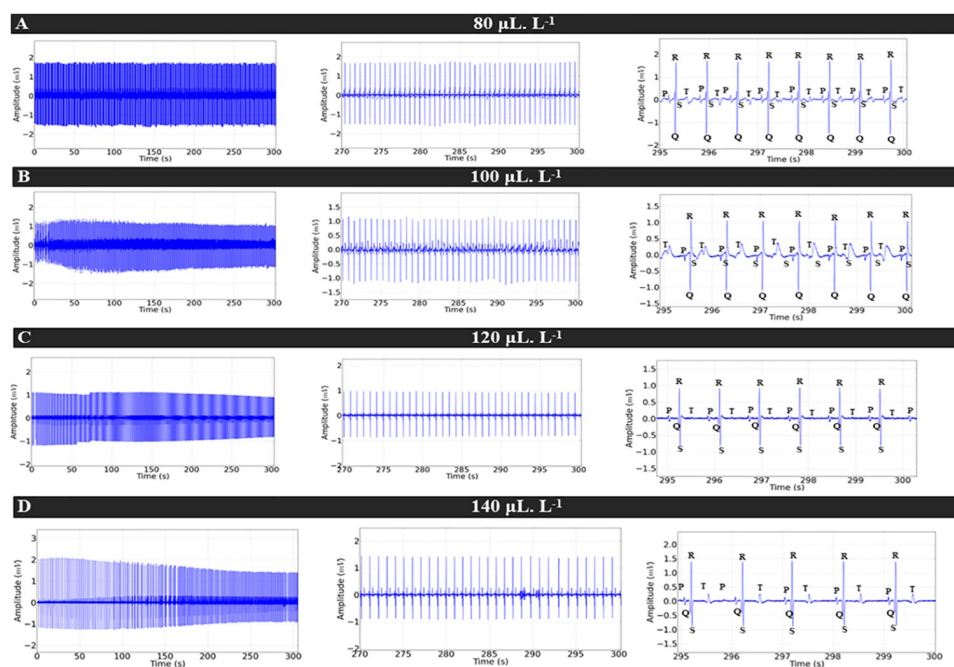


Fig 5. Cardiac activity in juvenile *Colossoma macropomum* during recovery after immersion baths with different concentrations of LAEO (left). Amplification of the recording in the last 30 seconds (270–300 s) (center), showing the magnification of the last 5 seconds of the recording (295–300 s) for identification of cardiac events (right), during the recovery period after immersion baths with the following concentrations of LAEO: $80 \mu\text{L.L}^{-1}$ (A), $100 \mu\text{L.L}^{-1}$ (B), $120 \mu\text{L.L}^{-1}$ (C), and $140 \mu\text{L.L}^{-1}$ (D).

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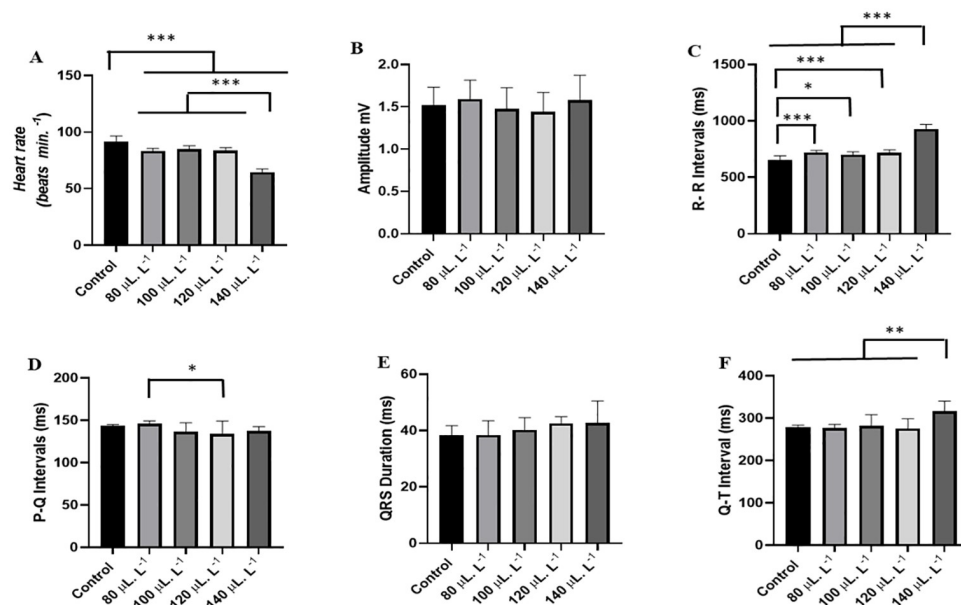


Fig 6. Mean values of cardiac parameters during recovery from exposure to different concentrations of LAEO: 80 $\mu\text{L.L}^{-1}$, 100 $\mu\text{L.L}^{-1}$, 120 $\mu\text{L.L}^{-1}$, and 140 $\mu\text{L.L}^{-1}$. Mean values of heart rate (bpm) (A); mean values of QRS complex amplitude (mV) (B); mean values of R-R interval (ms) (C); P-Q intervals (ms) (D); QRS complex duration (ms) (E); and Q-T interval (ms) (F). (ANOVA followed by Tukey's test; * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$; $n = 9$).

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The P-Q interval during recovery for the control group was 143.2 ± 1.92 ms, which was similar to the 80 $\mu\text{L.L}^{-1}$ group (146.1 ± 3.10 ms), the 100 $\mu\text{L.L}^{-1}$ group (138.6 ± 15.39 ms), the 120 $\mu\text{L.L}^{-1}$ group (136.6 ± 10.53 ms), and the 140 $\mu\text{L.L}^{-1}$ group (137.2 ± 5.31 ms) ($F(4, 40) = 3.044$; $p = 0.0279$) (Fig 6D).

The duration of the QRS complex during recovery was similar across all groups: the control group (38.22 ± 3.52 ms), the group treated with 80 $\mu\text{L.L}^{-1}$ (38.44 ± 5.05 ms), 100 $\mu\text{L.L}^{-1}$ (40.22 ± 4.41 ms), 120 $\mu\text{L.L}^{-1}$ (42.56 ± 2.35 ms), and 140 $\mu\text{L.L}^{-1}$ (42.67 ± 7.79 ms) ($F(4, 40) = 1.67$; $p = 0.175$) (Fig 6E).

During the recovery period, the Q-T interval for the control group was 278.7 ± 4.79 ms, similar to that of fish exposed to 80 $\mu\text{L.L}^{-1}$ (276.4 ± 8.77 ms), 100 $\mu\text{L.L}^{-1}$ (281.9 ± 26.42 ms), and 120 $\mu\text{L.L}^{-1}$ (275.0 ± 23.64 ms) ($p = 0.9448$). The group treated with 140 $\mu\text{L.L}^{-1}$ (316.2 ± 23.96 ms) had a longer Q-T interval compared to the other groups (Fig 6F).

Discussion

In recent years, the use of essential oils as an alternative to synthetic anesthetic agents has been gaining ground in fish farming. Anesthetic agents such as Aqui-S, which contains isoeugenol as its active ingredient, have already been approved for aquaculture application in some countries [16,17]. However, there is a noticeable search for other anesthetic agents within the scientific community. The essential oil of *L. alba* is recognized for its anesthetic capacity through the modulation of the GABAergic system, as previously suggested [8]. This essential oil exhibits great variability in its constituents, which is not only related to genetic factors but also to biotic and abiotic factors, typically classified by its major compound, with the most common being citral, linalool, and carvone [18,19]. The study performed by Souza et al. (2018) [15] investigated the application of two chemotypes of *L. alba* essential oil (citral and linalool) as an

anesthetic agent in *Rhamdia quelen*. Their results showed that the citral chemotype was stressful for this species, suggesting the use of the linalool chemotype, as it did not cause major alterations in the hypothalamus-pituitary-interrenal (HPI) axis when administered. Therefore, for the purpose of investigating safety and animal welfare, as well as possible side effects, our study was conducted with the linalool chemotype.

Our study confirmed anesthetic induction using juveniles tambaqui with *L. alba* within 4 minutes at the lowest concentration ($80 \mu\text{L.L}^{-1}$) and under 3 minutes at higher concentrations, the recovery period remained below 5 minutes for all concentrations tested. These induction and recovery periods are recommended in the literature for short-term anesthesia, which reinforces the application of this essential [20,21]. This time interval should also be considered due to the absorption and clearance rates in fish. Over a prolonged period, the absorption rate may equal the clearance rate, resulting in a steady state that does not produce the expected anesthetic effect, which could compromise the proper induction of anesthesia [20]. Our result differed from the study conducted by Silva et al. (2019), at a concentration of $100 \mu\text{L.L}^{-1}$ of *L. alba* essential oil, the induction time reached 8 minutes, which may be explained by the difference in the weight and developmental stage of the fish. Thus, anesthetic susceptibility to a specific dosage, even among fish of the same species, depends on biological factors such as age, body weight, sexual maturity, and other physiological factors [22,23]. In agreement with this, Ribeiro et al. (2015) [24] investigated the anesthetic efficacy of eugenol in *Oreochromis niloticus* during early life stages and found that group IV (2.62 g) had longer induction times at concentrations of $50\text{--}125 \text{ mg.L}^{-1}$ compared to group V (11.64 g). In this context, the study by Park et al. (2018) [25] investigated the anesthetic efficacy of eugenol in juvenile and adult *Epiplatys akarra*, finding that the ratio of recovery to induction time was higher in juveniles compared to adults. Therefore, the life stage and weight of the animal undergoing anesthesia must be considered when determining the appropriate concentration of the anesthetic agent.

Furthermore, electrophysiological evaluations have been emphasized in anesthetic monitoring by several authors [26–28]. This is the first work to report the electrocardiographic evaluation of juveniles tambaqui subjected to *L. alba* essential oil. Our results confirm a reduction in cardiac activity of 32.44% to 62.71% during the anesthetic induction process in this species. On the other hand, a previous study that used menthol as an anesthetic agent in tambaqui recorded a significant decrease in heart rate even at lower concentrations than those tested in this work [26]. Additionally, the study carried out by Araújo et al. (2023) [29], which subjected tambaquis to anesthetic baths with geraniol and citronellol, recorded a significant reduction in QRS complex amplitude during electrocardiography, especially when using citronellol, which was not observed when using *L. alba* essential oil regardless of the concentration tested. This decrease in amplitude recorded in the electrocardiogram is related to the loss of cardiac muscle contractility. Thus, the loss of excitability tissue resulting from sodium channel blockade can result not only in conduction deceleration but also in excitability, reflecting the reduction in amplitude and prolongation of QRS [30]. Our work did not register this decrease in QRS complex amplitude, the animals subjected to the anesthetic presented amplitudes similar to the control.

Moreover, the present study observed the onset of atrioventricular block at higher concentrations of *L. alba* essential oil. This event has been reported in other studies with tambaquis using both synthetic anesthetics and plant-derived compounds. Atrioventricular block is characterized by irregular conduction of the action potential between the atrium and ventricle, resulting in dyssynchrony between these chambers [31]. A previous study demonstrated the occurrence of advanced cardiac blocks was preceded by an increase in the P-R interval and the presence of a non-conducted P wave occurring during or before the T wave of the preceding QRS complex [32]. Thus, these findings suggest that the ventricle had not yet completed

repolarization when the atrium contracted again, it is proposed that the 2:1 atrioventricular block results from a prolonged ventricular refractory period that exceeds the sinus rate. In the present study, the onset of second-degree atrioventricular block was observed, along with prolongation of the P-Q, QRS, and Q-T intervals at concentrations of 120 and 140 $\mu\text{L.L}^{-1}$, leading to potential dyssynchrony in conduction between the atrium and ventricle. Additionally, Araújo et al. (2023) indicated a potential atrioventricular block in their experiment due to the prolongation of the QRS complex, RR, and QT intervals. Souza et al. (2024) [33] reported atrioventricular block in juveniles of tambaqui subjected to immersion baths with tricaine at higher concentrations (200 and 250 mg.L^{-1}). In contrast, some studies with tambaqui using lidocaine hydrochloride and menthol as anesthetic agents did not record occurrences of atrioventricular block [26,34]. Therefore, it is evident that this species is highly susceptible to cardiac alterations, and exploring various anesthetic agents and their concentrations is necessary to ensure the safety and well-being of the animal during the anesthetic process.

Despite the cardiac events during anesthetic induction, the recovery process showed reversibility of these parameters to normal levels. It was noticeable that at the highest concentration of *L. alba* essential oil, some parameters remained slightly below those of the control group, such as heart rate and consequently the R-R interval. Furthermore, the Q-T interval, which relates to ventricular activity, remained altered at the concentration of 140 $\mu\text{L.L}^{-1}$. However, the animals had a smooth recovery without alterations in the electrocardiogram trace. In contrast, a prior study identified P wave apiculation during anesthetic recovery using *Nepeta cat-aria* essential oil at a concentration of 200 $\mu\text{L.L}^{-1}$, this alteration indicates atrial overload in blood pumping to the ventricle [35]. Thus, the essential oil tested in this study allows for the reversibility of cardiac parameters to normal levels during the anesthetic recovery period.

In conclusion, *Lippia alba* essential oil proved to be an efficient and reversible anesthetic during the conducted analyses. However, the evaluation of cardiac function showed that with the deepening of the anesthetic plan, there were alterations in the morphographic elements, indicating changes in ventricular function, with the presence of atrioventricular block during treatment with higher concentrations of LEOA. In this sense, *Lippia alba* essential oil proves to be safe at doses between 80 and 100 $\mu\text{L.L}^{-1}$ to provide short-term anesthesia without major risks to hemodynamics in juvenile tambaquis.

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References

1. Martins T, Valentim A, Pereira N, Antunes LM. Anaesthetics and analgesics used in adult fish for research: A review. *Lab Anim*. 2018 Dec 4; 53(4):325–41. <https://doi.org/10.1177/0023677218815199> PMID: 30514148
2. Aydin B, Barbas LAL. Sedative and anesthetic properties of essential oils and their active compounds in fish: A review. *Aquaculture*. 2020 Apr; 520:734999.
3. Malik S, Odeyemi SW, Pereira G, Mamede L, Hamid HA, Atabaki N, et al. New insights into the biotechnology and therapeutic potential of *Lippia alba* (Mill.) N.E.Br. ex P. Wilson. *Journal of Essential Oil Research*. 2021 Jun 17; 33(6):523–35.
4. Kampke EH, Eduarda M, Marques FM, Fronza M, Scherer R, Lemos MF, et al. Genotoxic effect of *Lippia alba* (Mill.) N. E. Brown essential oil on fish (*Oreochromis niloticus*) and mammal (*Mus musculus*). *Environ Toxicol Pharmacol*. 2018 Apr 1; 59:163–71.
5. Batista ES, Brandão FR, Majolo C, Inoue LAKA, Maciel PO, Oliveira MR, et al. *Lippia alba* essential oil as anesthetic for tambaqui. *Aquaculture*. 2018 Oct; 495:545–9.
6. Sales G, Medeiros S, Soares I, Sampaio T, Bandeira M, Nogueira N, et al. Antifungal and Modulatory Activity of Lemon Balm (*Lippia alba* (MILL.) N. E. BROWN) Essential Oil. *Sci Pharm*. 2022 May 11; 90(2):31.
7. Majolo C, Pilarski F, Chaves FCM, Bizzo HR, Chagas EC. Antimicrobial activity of some essential oils against *Streptococcus agalactiae*, an important pathogen for fish farming in Brazil. *Journal of Essential Oil Research*. 2018 Jul 30; 30(5):388–97.
8. Heldwein CG, Silva LL, Reckziegel P, Barros FMC, Bürger ME, Baldissierotto B, et al. Participation of the GABAergic system in the anesthetic effect of *Lippia alba* (Mill.) NE Brown essential oil. *Braz J Med Biol Res*. 2012 May 7; 45(5):376–472.
9. Siqueira-Lima PS, Passos FRS, Lucchese AM, Menezes IRA, Coutinho HDM, Lima AAN, et al. Central nervous system and analgesic profiles of *Lippia* genus. *Rev Bras Farmacogn*. 2019 Jan; 29(1):125–35.
10. Silva HNP, Carvalho BCF, Maia JLS, Becker AG, Baldissierotto B, Heinzmann BM, et al. Anesthetic potential of the essential oils of *Lippia alba* and *Lippia organoides* in Tambaqui juveniles. *Cienc Rural*. 2019 May 14; 49(6).
11. Sena AC, Teixeira RR, Ferreira EL, Heinzmann BM, Baldissierotto B, Caron BO, et al. Essential oil from *Lippia alba* has anaesthetic activity and is effective in reducing handling and transport stress in tambacu (*Piaractus mesopotamicus* × *Colossoma macropomum*). *Aquaculture*. 2016 Dec 1; 465:374–9.
12. Azambuja CR, Mattiazzi J, Riffel APK, Finamor IA, Garcia LO, Heldwein CG, et al. Effect of the essential oil of *Lippia alba* on oxidative stress parameters in silver catfish (*Rhamdia quelen*) subjected to transport. *Aquaculture*. 2011 Sep 1; 319(1–2):156–61.
13. Maia JLS, Sousa EMO, Silva HNP, Pinheiro MTL, Mourão RHV, Maia JGS, et al. Hydrolate toxicity of *Lippia alba* (Mill.) N. E. Brown (Verbenaceae) in juvenile tambaqui (*Colossoma macropomum*) and its potential anaesthetic properties. *Aquaculture*. 2019 Mar 30; 503:367–72.
14. Simões-Bueno LN, Copatti CE, Gomes LC, Val AL, Amanajás RD, Caron BO, et al. Linalool chemotype essential oil from *Lippia alba* in the anesthesia of fat snook (*Centropomus parallelus*): ventilatory rate, biochemical, antioxidant, and oxidative status parameters. *Neotrop Ichthyol*. 2024 Apr 19; 22(1):e230114.
15. Souza CF, Descovi S, Baldissiera MD, Bertolin K, Bianchini AE, Mourão RHV, et al. Involvement of HPI-axis in anesthesia with *Lippia alba* essential oil citral and linalool chemotypes: gene expression in the secondary responses in silver catfish. *Fish Physiol Biochem*. 2018 Aug 17; 45(1):155–66.
16. Meinertz JR, Greseth SL, Schreier TM, Bernardy JA, Gingerich WH. Isoeugenol concentrations in rainbow trout (*Oncorhynchus mykiss*) skin-on fillet tissue after exposure to AQUI-S™ at different temperatures, durations, and concentrations. *Aquaculture*. 2006 Apr 1; 254(1–4):347–54.
17. Li Y, She Q, Han Z, Sun N, Liu X, Li X. Anaesthetic Effects of Eugenol on Grass Shrimp (*Palaemonetes sinensis*) of Different Sizes at Different Concentrations and Temperatures. *Sci Rep*. 2018 Jul 20; 8(1).

18. Gomes AF, Almeida M, Leite MF, Schwaiger S, Stuppner H, Halabalaki M, et al. Seasonal variation in the chemical composition of two chemotypes of *Lippia alba*. *Food Chem.* 2019 Feb 1; 273:186–93.
19. Filho JCFS, Nizio DAC, Oliveira AMS, Alves MF, Oliveira RC, Luz JMQ, et al. Geographic location and seasonality affect the chemical composition of essential oils of *Lippia alba* accessions. *Ind Crops Prod.* 2022 Nov 1; 188:115602–2.
20. Ross LG, Ross B. *Anaesthetic and Sedative Techniques for Aquatic Animals*. 3rd ed. Oxford: Blackwell Publishing Ltd; 2008.
21. Park MO, Hur WJ, Im SY, Seol DW, Lee J, Park IS. Anaesthetic efficacy and physiological responses to clove oil-anaesthetized kelp grouper *Epinephelus bruneus*. *Aquac Res.* 2008 Jun; 39(8):877–84.
22. Zahl IH, Samuelsen O, Kiessling A. Anaesthesia of farmed fish: implications for welfare. *Fish Physiol Biochem.* 2011 Dec 9; 38(1):201–18. <https://doi.org/10.1007/s10695-011-9565-1> PMID: 22160749
23. Vergneau-Grosset C, Benedetti ICC. Fish Sedation and Anesthesia. *Vet Clin North Am Exot Anim Pract.* 2022 Jan 1; 25(1):13–29. <https://doi.org/10.1016/j.cvex.2021.08.001> PMID: 34823688
24. Ribeiro PAP, Miranda-Filho KC, Melo DC, Luz RK. Efficiency of eugenol as anesthetic for the early life stages of Nile tilapia (*Oreochromis niloticus*). *An Acad Bras Cienc.* 2015 Mar 1; 87(1):529–35.
25. Park IS, Lee TH, Lim SG. Anesthetic efficacy and physiological responses of clove oil on juvenile and adult red spotted grouper, *Epinephelus akarra*. *Fish Aquatic Sci.* 2018 Jul 22; 21(1).
26. Cantanhêde SM, Amado LL, Alho da Costa BMP, Barbas LAL, Torres MF, Hamoy AO, et al. Menthol exposure induces reversible cardiac depression and reduces lipid peroxidation in the heart tissue of tambaqui *Colossoma macropomum*. *Aquaculture.* 2021 Aug 30; 541:736847.
27. Costa BMPA, Torres MF, da Silva RA, Aydın B, Amado LL, Hamoy M, et al. Integrated behavioural, neurological, muscular and cardiorespiratory response in tambaqui, *Colossoma macropomum* anaesthetized with menthol. *Aquaculture.* 2022 Nov 15; 560:738553.
28. Vilhena CS, Silva RA, Costa BMPA, Torres MF, Mello VJ, Noronha RCR, et al. Cardiac response in tambaqui *Colossoma macropomum* anaesthetised with *Piper divaricatum* essential oil. *Fish Physiol Biochem.* 2022 Oct 1; 48(5):1413–25.
29. Araújo ERL, Torres MF, Hamoy M, Barbas LAL, Sampaio LA. Cardiac response of tambaqui *Colossoma macropomum* anaesthetised with geraniol and citronellol. *Aquaculture.* 2023 Feb 25; 565:739101.
30. Berger V, Gabriel L, Lilliu E, Hackl B, Marksteiner J, Hilber K, et al. Modulation of cardiac ventricular conduction: Impact on QRS duration, amplitude and dispersion. *Eur J Pharmacol.* 2023 Feb 15; 941:175495. <https://doi.org/10.1016/j.ejphar.2023.175495> PMID: 36621601
31. Vornanen M, Badr A, Haverinen J. Cardiac arrhythmias in fish induced by natural and anthropogenic changes in environmental conditions. *J Exp Biol.* 2024 Aug 9; 227(20). <https://doi.org/10.1242/jeb.247446> PMID: 39119881
32. Zena LA, Ekström A, Morgenroth D, McArley T, Gräns A, Axelsson M, et al. Ischemia-induced alterations in the electrocardiogram of salmonid fish. *Aquaculture.* 2024 Feb 25; 581:740482.
33. Souza ASL, Paz CA, Barbosa GB, Santos MF, Conceição RM, Paz APS, et al. Behavioral and electrocardiographic evaluations in tambaqui (*Colossoma macropomum*) submitted to immersion bath in different concentrations of Tricaine methanesulfonate (MS-222). *Aquaculture.* 2024 Mar 1; 583:740575–5.
34. Vieira LR, Pereira YLG, Diniz LA, Nascimento CP, Silva ALM, Azevedo JEC, et al. Graded concentrations of lidocaine hydrochloride in the modulation of behavioral, cardiac, and muscular responses of the Amazon freshwater fish tambaqui (*Colossoma macropomum*). *Aquaculture.* 2023 Jan 1; 563:738985–5.
35. Santos MF, Nascimento LM, Paz CA, Câmara TM, Motomya YKM, Ferreira RC, et al. Behavioral and electrophysiological study in *Colossoma macropomum* treated with different concentrations of Nepeta cataria oil in an immersion bath revealed a therapeutic window for anesthesia. *Fish Physiol Biochem.* 2024 May 27:1–15.