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Original Article

Anesthesia or seizure-like behavior? The effects of two Amazonian plants, *Acmella oleracea* and *Piper alatabaccum* in zebrafish (*Danio rerio*)

Anestesia ou epilepsia? Os efeitos de duas plantas amazônicas, *Acmella oleracea* e *Piper alatabaccum* em peixe-zebra (*Danio rerio*)

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Abstract

Currently, available fish anesthetics can produce important side effects, including respiratory arrest and distress. Easy-to-implement alternatives with low toxicity are needed to ensure fish health as well as to help artisanal fisheries and fish sellers in handling and transporting fishes, and native plants seems to be the best alternative. We aimed to implement an anesthetic protocol using crude ethanolic extracts from flowers and leaves of two Amazonian plants, the *Acmella oleracea* and *Piper alatabaccum*. We first tested the extracts for anesthesia, using the zebrafish as model. Even though in some treatments the animals apparently entered deep anesthesia, many of them presented aberrant behaviors and even died. Thus, we performed new experiments testing the extracts effects on seizure-like behaviors of the fish. Only the leaf extract of *A. oleracea* has potential effects for fish anesthesia. Both the flower extract from this plant and the leaf extract from *P. alatabaccum* induced seizure-like behavior in the animals. In conclusion, besides bringing a possible new anesthetic protocol for fish, our work draws attention for the neurotoxic effects the anesthetic solutions may cause, since several studies defend other Piper species as anesthetic for fish and *A. oleracea* flowers' extract was already pointed as fish anesthetic.

Keywords: biodiversity, epilepsy, fish anesthesia, natural anesthetic.

Resumo

Atualmente, os anestésicos disponíveis para peixes podem produzir efeitos colaterais importantes, incluindo parada respiratória e sofrimento. Alternativas de fácil implementação e baixa toxicidade são necessárias para garantir a saúde dos peixes, bem como para auxiliar a pesca artesanal e os vendedores de pescado no manuseio e transporte do pescado, e as plantas nativas parecem ser a melhor alternativa. Nosso objetivo foi implementar um protocolo anestésico utilizando extratos etanólicos brutos de flores e folhas de duas plantas amazônicas, *Acmella oleracea* e *Piper alatabaccum*. Primeiro testamos os extratos para anestesia, usando o peixe-zebra como modelo. Embora em alguns tratamentos os animais aparentemente tenham entrado em anestesia profunda, muitos deles apresentaram comportamentos aberrantes e até morreram. Assim, realizamos novos experimentos testando os efeitos dos extratos em epilepsia dos peixes. Apenas o extrato de folhas de *A. oleracea* tem efeitos potenciais para anestesia de peixes. Tanto o extrato de flores desta planta quanto o extrato de folhas de *P. alatabaccum* induziram um comportamento semelhante a convulsões nos animais. Em conclusão, além de trazer um possível novo protocolo anestésico para peixes, nosso trabalho chama a atenção para os efeitos neurotóxicos que as soluções anestésicas podem causar, uma vez que vários estudos defendem outras espécies de Piper como anestésico para peixes e o extrato de flores de *A. oleracea* já foi apontado como anestésico para peixe.

Palavras-chave: biodiversidade, epilepsia, anestesia em peixe, anestésico natural.

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1. Introduction

Routine procedures in fish farming and in experiments using fish as model, such as biometry, transportation, blood sampling, can result on animal distress and pain, often causing death (Gimbo et al., 2008; Sneddon, 2012; Salbego et al., 2017). Thus, the use of anesthetics became a necessary strategy in order to guarantee the fish and human welfare, since the fish may jump or struggle hurting themselves and the handler during handling (Anschau et al., 2014; Posner et al., 2019).

Among the most commonly used anesthetics, tricaine methane sulfonate (MS-222) (Ross and Ross, 2008; Parker-Graham et al., 2020) quinaldine sulfate (Massee et al., 1995), and benzocaine (Ackerman et al., 2000; Uehara et al., 2019) are widely used in fish farms and research laboratories. However, those synthetic drugs can cause negative side effects in fish, including a loss of mucus, gill irritation, and corneal damage, as well as change in blood parameters, cortisol levels, and even mortality (Inoue et al., 2003; Gimbo et al., 2008; Babaiinezhad and Bahrekazemi, 2019; Basusta and Ozcan, 2019). Wong et al. (2014) showed that the zebrafish *Danio rerio* avoids the presence of MS-222 after an initial contact with the anesthetic, evidencing an aversive experience of the fish in contact to MS-222. Furthermore, synthetic anesthetics can cause harm to humans in case of misuse (Ackerman et al., 2000).

Searching for better alternatives for fish anesthesia, researches have performed experiments using secondary metabolites extracted from plants (Hoseini et al., 2019), such as menthol, which is extracted from essential oils of *Mentha arvensis* L. (Pádua et al., 2010; Sepulchro et al., 2016), and eugenol or clove oil, which is extracted from plant organs as stems, leaves and flowers of Indian clove *Syzygium aromaticum* L. (Roubach et al., 2005; Hamackova et al., 2006; Gonçalves et al., 2008; Fernandes et al., 2017). Both of those anesthetic solutions are nowadays the most used natural compounds for fish anesthesia (Roubach et al., 2005; Gonçalves et al., 2008; Mazandarani and Hoseini, 2017; Romaneli et al., 2018; Takatsuka et al., 2019; Ribeiro et al., 2019). However, in spite of their benefits, such as suppression of cortisol levels' in the Pallid sturgeon *Scaphirhynchus albus* (S. A. Forbes and R. E. Richardson, 1905) (Fenn et al., 2013), they can also implicate in negative side effects to the animals. Barbas et al. (2021), for example, showed that eugenol may cause seizurogenesis in *Colossoma macropomum* (Cuvier, 1818) juveniles, besides to being toxic to its brain. Moreover, those compounds require specific high-cost equipment and human skills for molecular isolation, making its production unfeasible for small and artisanal fish farmers; moreover, according to Summerfelt and Smith (1990), a good anesthetic must minimize the impacts on animal health and in human handlers, as well as be easily produced.

Aiming to make new alternatives of natural anesthetics and intending to minimize their impacts on the animals and human health during fish handling and to develop a new low-cost and effective protocol, easily accessible to small and artisanal producers, we evaluated the anesthetic effects of the crude extracts of two Amazonian plants, the "jambú" (*Acmella oleracea* (L.) R.K. Jansen) and the "joão brandinho" (*Piper alatabaccum* Trel. and Yunck, 1950).

Acmella oleracea is an herbaceous plant of the Asteraceae family, commonly used in the Northern region of Brazil as a culturally valuable alimentary item. It presents anti-inflammatory, analgesic, anesthetic, and antipyretic properties (Chakraborty et al., 2004, 2010). Studies have identified that spilanthol, the main component of its inflorescences (other bioactive constituents found in this plant include butylated hydroxytoluene, palmitic acid and Myristic acid (Leng et al., 2011)), can induce anesthesia in rats, mice (Chakraborty et al., 2004), frogs, and guinea pigs (Chakraborty et al., 2010), and can be used as a topical anesthetic in humans (Cerutti de Andrade et al., 2013), mice (in vivo) and pig (in vitro) (Freitas-Blanco et al., 2016). In fish, Barbas et al. (2016) suggest the waxy extract of flowers as anesthetic for *C. macropomum* juveniles' fish. However, *Acmella* extracts have been shown to produce toxic effects in fish as well. For example, an hydroethanolic extract of *A. oleracea* inflorescences induced behavioral abnormalities (spasms, tail tremors, loss of posture and motility, clonic-like seizures, bottom-dwelling, and death), associated with histopathological changes in the gills, liver, intestine, and kidney of zebrafish (Souza et al., 2019a). Likewise, the same extract produced reproductive toxicity, with changes in gonads and fertility as well as impaired embryonic development in animals from F1 generation (Souza et al., 2019a, b)

Piper alatabaccum is a Piperaceae family plant also found in the Northern region of Brazil, where its organs are used by riparian people and indigenous as local anesthetic, analgesic and anti-inflammatory for toothache, stomachache among others (personal communication). Although its constituents are already described (N-(3',4',5'-Trimethoxydihydrocinnamoyl-D3-pyridin-2-one, piplartine, piperovatine and 5,5',7-Trimethoxy-3',4'-methylenedioxyflavone (Azevedo et al., 2020)), available information about this species is rare on the literature. Most of the studies suggest it use as insecticide (Trindade et al., 2012; Santos et al., 2013).

2. Material and Methods

2.1. Vegetal species and preparation of the anesthetic crude solutions

The vegetable material was purchased on local market from Marabá - PA (*A. oleracea*) and in the countryside (Palmares II, 5.95 °S, 49.84 °W) of Parauapebas - PA, Brazil (*P. alatabaccum*). One specimen from each species was forwarded for specialist identification and are deposited in the herbarium Ezechias Paulo Heringer (Jardim Botânico de Brasília, DF) under the voucher numbers HEPH 37308 and HEPH 37309, respectively.

In the laboratory, the plants' leaves and flowers were washed, first in running and then distilled water, dried with paper towels, macerated in a pestle and crushed with knife crusher. The resulted material of each organ was weighed and extracted on 70% v/v ethanol in the ratios of 1:1 (1 g of the crushed vegetal material dissolved in 1 mL

of 70% ethanol) and 1:5 (1 g of the crushed vegetal material dissolved in 5 mL of 70% ethanol). Finally, the extracts were filtered and stored for 30 days in amber glasses and stored on average temperature of 4 °C. The methodology used for the extracts manufacturing was described as a protocol on Leite et al. (2019) protocol and is summarized on Figure 1.

2.2. Ethics statement, animal species and experimental design

The zebrafish (*Danio rerio*, Hamilton 1822) species was used in the experiments. This species was selected because of its consolidation as biological model for laboratory tests (Wyatt et al., 2015; Harper and Lawrence, 2016), including those involving anesthetic solutions (Grush et al., 2004; Matthews and Varga, 2012; Collymore et al., 2014; Wong et al., 2014; Collymore, 2020), in addition to its easy reproduction in laboratory (Zon and Peterson, 2005; Harper and Lawrence, 2016) and increasing use in all scientific research fields.

In total 175 sexually mature zebrafish specimens were used in this study, 55 on Experiment 1 and 120 on Experiments 2 and 3. Animals weighted 320.85 ± 39.14 g and measured 3.40 ± 0.17 cm total length on average. All experimental procedures imposed on animals were approved by the Animal Use Ethics Committee of Federal University of South and Southeast of Pará, (CEUA-UNIFESSPA, project number 23479.010692/2021-16), and all procedures used were consistent with the established guidelines of the National Council for the Control of Animal Experimentation (CONCEA), and the study was divided in three experiments: First the anesthetic potential of the vegetal extracts was tested (Experiment 1), following tests for seizure-like behavior (Experiment 2) and measurements of nitrite levels in telencephalic and head kidney tissue (Experiment 3).

2.2.1. Experiment 1 – Anesthetic potential of the vegetal crude extracts

This experiment was divided in 11 treatments with five animals each (Table 1). Summarizing, the anesthetic solution of eugenol (biodinâmica) was used as the positive control treatment (Treatment 1, T1). Its solution was prepared according to the manufacturer instructions (20 mL of Eugenol diluted in 100 mL 70% ethanol).

The 1:1 ratio *A. oleracea* flowers' extract was divided in Treatment 2 (T2), in which 1 mL of the flowers' extract was used and; Treatment 3 (T3), in which 2 mL of flowers' extract were used. For the 1:1 ratio *A. oleracea* leaves' extract, three treatments were performed: Treatment 4 (T4), using 1 mL of the leaves' extract; Treatment 5 (T5), in which 2 mL of the leaves' extract were used and; Treatment 6 (T6), in which 3 mL of the leaves' extract were tested.

As the 1:1 ratio *A. oleracea* flowers' extracts caused animal death after anesthesia (Table 1), we also tried 1:5 *A. oleracea* flowers' extracts in two different treatments: Treatment 7 (T7), using 1 mL of the flowers' extract and; Treatment 8 (T8), in which 2 mL of flowers' extract were used.

Treatment 9 (T9) was performed using 2.5 mL of the 1:1 ratio *P. alatabaccum* leaves' extract. In the Treatment 10 (T10), 2.5 mL of the 1:5 ratio leaves' extract were used. Since for this species the flowers are rarely found, only the leaves were used in the study.

The negative control treatment was performed (Treatment 11, T11) using 1 mL of 70% ethanol, because ethanol was used as vehicle to prepare the crude extracts. Details of the treatments are organized on Table 1.

Each animal was used only once (five per treatment) and was considered a replicate. Initially, the crude extract solutions were diluted in five-liters-aquarium. Following, the physical and chemical parameters of water (temperature (using a digital thermometer), pH and hardness (using the TDS&EC meter (hold)) and dissolved ammonia, oxygen and nitrite using the Labcon-Kit test) were taken aiming to prevent any bias. The animals were starved for 24 hours before the experiment to avoid any behavioral change (Dametto et al., 2018) and were exposed to anesthetic bath for the maximum time of 5 minutes, where they were observed regarding the anesthesia stages according to Ross and Ross (2008) (Table 2). Fish behavior during anesthesia were recorded using a video camera (Sonyr DCR-DVD610), which was positioned in the front of the aquarium, therefore allowing observation. After the anesthesia, the animals passed through a usual procedure

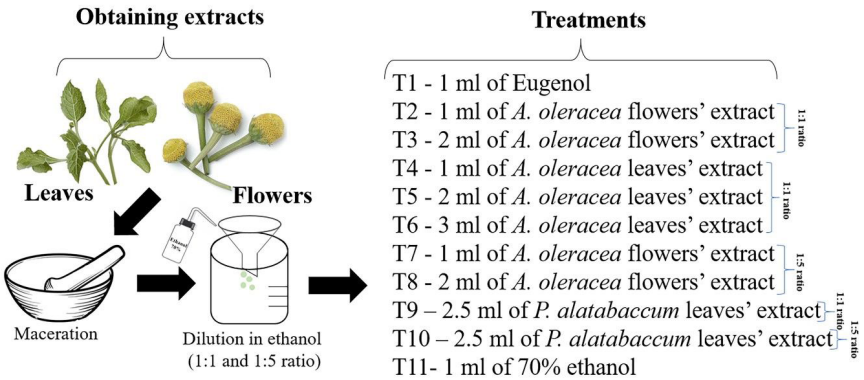

Figure 1. Diagram illustrating the procedure for extraction and production of the vegetal crude extracts.

Table 1. Data of anesthesia experiment using the *Acmella oleracea* and *Piper alatabaccum* crude extracts in the zebrafish.

Treatment (Ethanol ratio)	Concentration (mL/L)	Plant organ	Anesthesia (min.)	Recovery (min.)	Mortality (n)
Eugenol	1	-	0.44	5	0
A. oleracea (1:1)	1	Flower	4.2	1.24	2
A. oleracea (1:1)	2		4.33	4.33	5
A. oleracea (1:1)	1	Leaves	*	*	0
A. oleracea (1:1)	2		*	*	1
A. oleracea (1:1)	3		4.25	0.18	1
A. oleracea (1:5)	1	Flower	*	*	0
A. oleracea (1:5)	2		5.02	1.49	1
P. alatabaccum (1:1)	2.5	Leaves	2.08	0	0
P. alatabaccum (1:5)	2.5		3.53	6.43	5
70% Ethanol	1	-	*	*	*

*No anesthesia.

Table 2. Stages of anesthesia and recovery.

	Stages of Anesthesia	Description	Recovery
1	Light sedation	Response to stimulus, with swimming and reduced breathing.	Immobilized body, but opercular movements just beginning
2	Light anesthesia	Reduced stimulus response.	Regular opercular movements and beginning of partial body movement
3	Deep anesthesia	Partial loss of balance - Analgesia.	Total recovery of body equilibrium
4	Surgical anesthesia I	Total balance loss and breath almost absent.	
5	Surgical anesthesia II	Total loss of reaction to stimulus.	

of Biometry (fish were weighed (g) and measured (total and standard length, cm), and then they were placed in a recovery tank (5 liters). The time elapsed from the moment of deep anesthesia and complete recovery of swimming activity was also recorded (Table 1). In order to confirm the anesthetic solutions safety, fish survival was observed for 48 hours after the experimentation.

2.2.2. Experiment 2 – Epileptic seizure potential of the vegetal crude extracts

For this experiment, a 5 liters water aquarium was used and 8 treatments, using 15 animals each, were performed: T1, negative Control (water); T2, commercial anesthetic (Eugenol, 1 mL per liter of water); T3 (2 mL of the 1:1 ratio *A. oleracea* flowers' extracts per liter of water); T4 (2.5 mL of the 1:1 ratio *P. alatabaccum* leaves' extract per liter of water); T5 (2.5 mL of 70% Ethanol per liter of water); T6 (2 mL of 70% Ethanol per liter of water); T7, positive control (5 µl of Cortland's saline solution, i. p.) and; T8 (400 mg/kg pilocarpine) (Table 3).

The treatments 1 to 6 were accomplished as the treatments from the Experiment 1. Thus, the animals were exposed to the anesthetic bath. However, here they were observed and recorded using the video camera for the maximum time of 15 minutes. The behavior scored regarded the epileptic seizure scores according to Mussulini et al. (2013) (Table 4). The camera was positioned in the front of the aquarium, therefore allowing observation and tracking. Following the treatments, each animal was euthanized on ice followed by decapitation. Their encephalon and head kidney were then collected and fixed in PBS for the oxidative stress experiment (Experiment 3).

The treatments 7 and 8 were performed by intraperitoneal (i.p.) injection using a Hamilton microsyringe (Sigma Aldrich). To assess the profile of epileptic seizures, an injection of pilocarpine (400 mg / kg) was used as positive control in treatment 8-T8. This dose is enough to induces the clonic and tonic-clonic convulsions in normal animals (Pinto, 2015). For this, the animals were anesthetized in cold water (12 °C). After the injection, they were isolated in aquarium for 1 minute. Then, they were transferred for the experimental aquarium and the procedure was the same as that described for the other treatments. As pilocarpine was diluted in Cortland's saline solution (40 mg / 1 mL), another control treatment (T7), using only this vehicle (5 µl, i.p.) was also performed

2.2.3. Experiment 3 – Effects of the vegetal crude extracts on nitrite levels in head kidney and brains

The encephalon and cephalic kidney collections were performed by the following protocol.

Table 3. Data of epilepsy experiment using the *A. oleracea* and *P. alatabaccum* crude extracts in the zebrafish. The data show the average time, in minutes, the animals entered each stage.

Epilepsy Stages	Water (5L)	Eugenol 1 mL/L	Ethanol (2.5 mL/L)	Ethanol (1 mL/L)	*A. oleracea* (1 mL/L)	*P. alatabaccum* (2.5 mL/L)	Pilocarpine (0.005 µl)	Cortland´s (0.005 µl)
0	-	-	0.60a	0.39a	0.11b	0.10b	0.78b	2.89a
1	-	-	1.44b	1.81b	0.24c	0.39bc	3.04a	5.74a
2	-	-	4.18b	3.79b	0.45c	0.61c	4.78b	7.37a
3	-	-	6.35a	6.83a	0.72b	1.28b	6.48a	6.25a
4	-	-	9.90a	8.36a	1.03b	2.55b	9.02a	-
5	-	-	-	-	2.57a	2.15a	12.25b	-
6	-	-	-	-	-	-	-	-

Different letters in the same row indicate differences among the treatments.

After decapitation, the skin and cranial bones were removed, exposing the brain. To avoid damaging the olfactory bulbs and telencephali, the dissection started at the level of the junction between the spinal cord and the brainstem, which was gently lifted with an insulin needle (1.60 mm x 50 mm), and the ventral roots of the cranial nerves were sectioned using microdissection forceps. The brain was then sectioned at the height of the habenula, separating the telencephalon and the olfactory bulb from the rest of the tissues. To extract the head kidney, which contains interrenal glands, the animals' body was placed on an immobilization bed in dorsal decubitus. The abdominal cavity was opened using a scalpel and the other organs were removed to facilitate the access to the organ of interest. The gland was sectioned at its cephalic portion and appropriately stored in 500 µl of PBS.

The tissue levels of nitrite and/or nitrate (NOx-) were determined by Griess reaction (Griess, 1865). It is based on a two steps diazotization reaction, in which the acidified nitrite produces a nitrosating agent that reacts with sulphanilic acid to produces the diazonium ion. This ion is then coupled to N-(1-Naphythyl)-ethylenediamine, forming an azo-derived cromophoric that absorbs light around 540 nm (Griess, 1865; Hetrick and Schoenfisch, 2009). Because the Griess test shows relatively low sensibility to NOx- (Hetrick and Schoenfisch, 2009), a pool of the tissues (five telencephalons and five cephalic kidneys) was used. After dissection five samples of each tissue were mechanically homogenized in PBS (NaCl 0.8%, KCl 0.02%, K2HPO4 0.02 M, pH 7.3). For the essay performance, equal volumes of the sample and Griess (phosphoric acid 5%, N-(1-Naphythyl)-ethylenediamine 0.1% and sulfanilamide 1%) were mixed and incubated in the dark for 10 minutes in room temperature. The product absorbance was gauged in 543 nm spectrophotometer and the nitrite concentration in each sample was determined by interpolation in sodium nitrite standard curve. The essays were performed in triplicate.

2.3. Statistics

Differences in latencies to reach deep anesthesia or total recovery were assessed by log-rank tests of Kaplan-Maier estimates of time to event. Since treatment with ethanol (vehicle), *A. oleracea* leaves at the lower concentrations (1 and 2 mL/L), and *A. oleracea* flowers (1:5) at both concentrations did not induce anesthesia, the data was removed from the statistical analysis, but are reported in the Results section. When p-values were < 0.05, tests were followed by pairwise comparisons with the log-rank test, with p-values Bonferroni-corrected for multiple comparisons.

Table 4. Epileptic seizure scores based on Mussulini et al. (2013).

Escores	Behavior
0	Short swim
1	Increased swimming activity and high frequency of opercular movement
2	Burst swim and erratic movements
3	Circular movement
4	Clonic seizure-like behavior
5	Tonic seizure-like behavior
6	Death

Differences in latencies to reach each seizure scores were assessed by log-rank tests of Kaplan-Maier estimates of time to event. When p-values were < 0.05, tests were followed by pairwise comparisons with the log-rank test, with p-values Bonferroni-corrected for multiple comparisons. Pairwise comparisons were planned, with each treatment being compared to its specific vehicle, pilocarpine, and the other extract. Since we were interested mainly in latencies to reach scores 4 (clonic seizures) and 5 (tonic-clonic seizures), only these data are shown in figures.

3. Results

3.1. Experiment 1

The physical and chemical parameters of treatments' water, took just before the begging of the experiments, were in accordance with the ideal recommendation for fish survival as can be seen on Table 5, and then, did not interfere in the anesthesia results.

Table 5. Physical chemical parameters of the treatments.

	Analyzed parameters					
Treatments	**pH**	**Ammonia (ppm)**	**Oxygen (ppm)**	**Nitrite (ppm)**	**Hardness (ppm)**	**Temperature (°C)**
Flower (*A. oleracea*)	7.2	0.25	8.3	0	4	24.2
Leaves (*A. oleracea*)	7.2	0.27	7.9	0	4	24.8
Leaves (*P. alatabaccum*)	7.1	0.21	9	0	4	25
Eugenol	7.3	0.13	6	0	4	27
70% Ethanol	7.1	0.63	7	0	6	26

The results of the experiment 1 are organized on Table 1. Summarizing, treatment with ethanol (vehicle), *A. oleracea* leaves at the lower concentrations (1 and 2 mL/L), and *A. oleracea* flowers (1:5) at both concentrations did not induce anesthesia in less than 5 min. A significant effect of treatment on anesthesia latencies was observed (Figure 2a; $\chi^2 = 137.53$, df = 10, $p < 0.001$). Due to right censoring of all data in groups in which anesthesia was not induced in less than 5 min., these were removed from pairwise comparisons. Eugenol induced anesthesia quickly, with a median latency of 0.44 min. *Acmella oleracea* flowers (1:1) showed significantly higher latencies to reach deep anesthesia than eugenol at both concentrations ($p = 0.002$ for both), with median latencies of 4.2 min (1 mL/L) and 3.25 min (2 mL/L). *Acmella oleracea* leaves (1:1) at the highest concentration (3 mL/L) showed significantly higher latencies to reach deep anesthesia than eugenol ($p = 0.002$), with a median latency of 4.25 min; as observed above, lower concentrations did not induce anesthesia in less than 5 min. *Piper alatabaccum* (2.5 mL/L) also showed significantly higher latencies than eugenol at both concentrations ($p = 0.002$ for 1:1, $p < 0.001$ for 1:5), with median latencies of 3.53 min (1:1 ratio) and 2.12 min (1:5 ratio).

In addition to treatments that did not induce anesthesia, treatment with the lowest ratio of *P. alatabaccum* (1:5) also led to death of all animals during the recovery time. Therefore, these data were removed from pairwise comparisons. Significant differences between treatments were found in recovery time (Figure 2b; $\chi^2 = 34.78$, df = 9, $p < 0.001$). Median latency to fully recover from anesthesia in the eugenol group was 4.56 min. No significant differences in latency to fully recover were found between any treatments and eugenol (*A. oleracea* flowers, 1:1 ratio, 1 mL/L: median latency 1.25 min, $p = 0.492$; *A oleracea* flowers, 1:1 ratio, 2 mL/L, 4.33 min, $p = 0.214$; *A. oleracea* flowers, 1:5 ratio, 2 mL/L: median latency 1.49 min, $p = 0.492$; *A. oleracea* leaves, 1:1 ratio, 3 mL/L: median latency 1.05 min, $p = 0.712$; *P. atabalaccum*, 1:5 ratio, 2.5 mL/L: median latency 6.43 min, $p = 0.902$).

3.2. Experiment 2

Since many fish died during experiment 1 and other presented awkward behaviors (tremors, burst swim, erratic and circular movements, Supplementary material 1), different from that fish submitted to the commercial anesthetic (eugenol, Supplementary material 2), we decided to test the epileptic seizure potential of the vegetal extracts (Table 3).

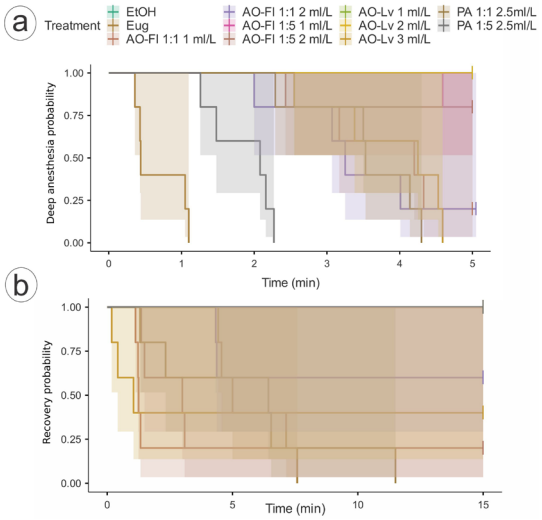

Figure 2. Graphical representation for both Deep anesthesia (a) and Recovery (b) probability in minutes assessed by log-rank tests of Kaplan-Maier estimates. Abbreviations: AO-Fl = *Acmella oleracea* flowers; AO-Lv = *Acmella oleracea* leaves; EtOH = Ethanol; Eug = Eugenol; PA = *Piper alatabaccum*.

Latencies to reach all seizure stages above 0 were significantly different between groups (Table 3). Pilocarpine significantly reduced latencies to reach score 1 in relation to Cortland's salt solution ($p = 0.036$). *Acmella oleracea* ($p = 0.005$) significantly reduced latencies to reach stage 1 in relation to its vehicle ($p = 0.04$) and water ($p < 0.001$). *Piper alatabaccum* ($p = 0.015$) did not produce significant reductions in score 1 latencies in relation to its vehicle ($p = 0.079$), but an effect was observed in relation to water ($p < 0.001$). No differences were found in score 1 latencies between plants ($p = 0.915$).

Acmella oleracea ($p < 0.001$) and *Piper alatabaccum* ($p = 0.001$) significantly reduced latencies to reach score 2 in relation to Pilocarpine and Cortland's salt solution ($p = 0.004$). *Acmella oleracea* significantly reduced latencies to reach stage 2 in relation to its vehicle and water (both $p < 0.001$). *Piper alatabaccum* significantly reduced score

2 latencies in relation to its vehicle ($p = 0.004$) and water ($p < 0.001$). No differences were found in score 2 latencies between plants ($p = 0.799$)

Acmella oleracea and *P. alatabaccum* (both $p < 0.001$) significantly reduced latencies to reach score 3 in relation to Pilocarpine and Cortland's salt solution ($p = 0.001$). *Acmella oleracea* significantly reduced latencies to reach stage 3 in relation to its vehicle and water (both $p < 0.001$). *Piper alatabaccum* significantly reduced score 3 latencies in relation to its vehicle and water (both $p < 0.001$). No differences were found in score 3 latencies between plants ($p = 0.549$).

Figure 3a presents Kaplan-Meier estimates for latencies for score 4. Pilocarpine significantly reduced latencies to reach score 4 in relation to Cortland's salt solution, but latencies were lower in *A. oleracea* and *P. alatabaccum* in relation to pilocarpine (all $p < 0.001$, S3). *Acmella oleracea* significantly reduced latencies to reach stage 4 in relation to its vehicle and water (both $p < 0.001$, S3). *Piper alatabaccum* significantly reduced score 4 latencies in relation to its vehicle and water (both $p < 0.001$). No differences were found in score 4 latencies between plants ($p = 0.329$).

Figure 3b presents Kaplan-Meier estimates for latencies for score 5. Pilocarpine significantly reduced latencies to reach score 5 in relation to Cortland's salt solution, but latencies were lower in *A. oleracea* and *P. alatabaccum* in relation to pilocarpine (all $p < 0.001$). *Acmella oleracea* significantly reduced latencies to reach stage 5 in relation its vehicle and water (both $p < 0.001$, S3). *Piper alatabaccum* significantly reduced score 5 latencies in relation to its vehicle and water (both $p < 0.001$, S3). No differences were found in score 5 latencies between plants ($p = 0.176$).

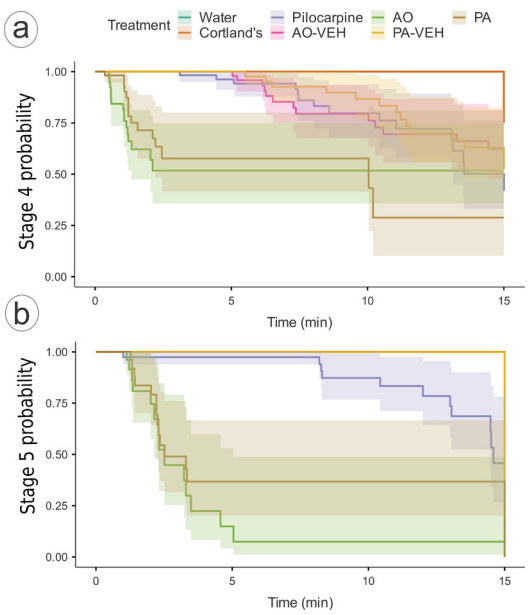

Figure 3. Kaplan-Meier estimates for the epileptic seizure latencies for score 4 (a) and 5 (b) (Mussulini et al., 2013) of the vegetal extracts. Abbreviations: AO = *Acmella oleracea*; AO-VEH = *Acmella oleracea* vehicle; PA = *Piper alatabaccum*; PA-VEH = *Piper alatabaccum* vehicle.

3.3. Experiment 3

The results obtained through the spectrophotometry of the NOx- analyte revealed no significantly increase of nitrite or nitrate (NOx-;) levels in both, the encephalon and cephalic kidney tissues among the treatments (Figure 4).

4. Discussion

The effectiveness of anesthesia in fish using metabolites derived from plants has already been shown and has been used very frequently (Zon and Peterson, 2005; Roohi and Imanpoor, 2015; Barbas et al., 2016; Baldisserotto et al., 2018; Romaneli et al., 2018; Tomi et al., 2018; Can et al., 2019; Aydin and Barbas, 2020; Nozu and Nakamura, 2019), since they are supposed to reduce or eliminate the negative side effects caused by the synthetic anesthetics (Fenn et al., 2013). In common, however, both the natural and synthetic known anesthetics demand laboratorial or industry production to be manufactured and only then became available for the researchers and fish farmers, often at high values, which can derail the production cost or even became inaccessible for small fish farmers. Thus, in this study we tried to make available a new anesthetic protocol based on crude extracts of Amazonian native plants that was effective, low-cost, safe and easily produced. In spite of the good results for the *A. oleracea* leaves' extract, both *A. oleracea* flowers and *P. alatabaccum* extracts, which in the first moment, apparently, present anesthetic potential, in fact, induced seizure-like behavior in the fish.

The anesthetic effect of *A. oleracea* = *S. acmella* has already been shown by Barbas et al. (2016) in tambaqui (*C. macropomum*) using the essential oil extracted from flowers. Posteriorly, his group also showed (Barbas et al., 2020) the same extract failed to attenuate the stress caused to juveniles of the same species during transportation. In this study, we showed the *A. oleracea* flowers' extract

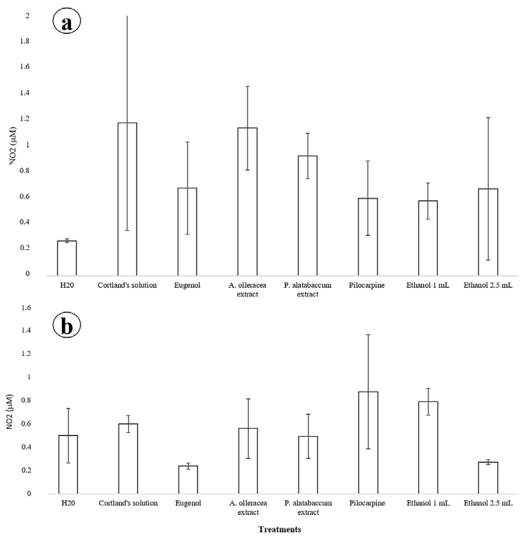

Figure 4. Spectrophotometry analysis of the NOx- analyte for the cephalic kidney (a) and encephalon (b) in zebrafish after the treatments.

may, in fact, cause seizure-like behavior. Souza et al. (2019a) also showed that hydroethanolic extracts of *A. oleracea* flowers induced clonus in zebrafish. In spite the different methods used, since Barbas' group used the essential oil extracted from flowers and we used their crude extract, we believe to be the spilanthol the responsible for that effect, since it appears in higher concentrations in the flowers (Ramsewak et al., 1999) and it is the main compound associated with the possible anesthesia.

Studies of reproductive toxicity in zebrafish using hydroethanolic extract of *A. oleracea* flowers as well as spilanthol, showed a spawning interruption and reduction of mature cells in male and female gonads, which resulted in fertility changes and lethality of the embryos treated with spilanthol (Souza et al., 2020). In addition to the neurotoxic, *A. oleracea* flowers' extract at the higher concentration (2 mL / L^{-1}) caused 100% mortality in T3 in the first 12 hours after the anesthetic bath. Although, the first experiment we performed, suggest anesthesia induction was achieved in some of the animals immersed in the low flowers' extract concentration (T2 and T8), the high mortality (8/20) caused and the seizure-like behavior presented by the animals in the experiment 2, suggest further studies must be accomplished before the use of *A. oleracea* flowers' extract for anesthesia in fish.

On the other hand, the leaves' crude extract caused lower mortality (3/15), and in the concentration of 3 mg / L^{-1} it induced the anesthesia of 100% of the animals in approximately 4 minutes, which is considered an ideal time for anesthesia in fish; recovery was also into the time required to not cause any problems to the animal (Ross and Ross, 2008). Moreover, the behavior of fish submitted to the leaves' extract was similar to that treated with the Eugenol oil (Supplementary material 2), which may signalize the anesthesia was really achieved.

Piper alatabaccum leaves' extract, initially, also seemed to anesthetize zebrafish animals. However, all the fish from T10 died after the recovery time and, in the experiment 2, all the fish showed the seizure-like behaviors, similar to those provoked by pilocarpine administration. This effect is possibly associated with one of its compounds called piperovatine, which is found in several species of Piper (Gottlieb et al., 1981; Facundo et al., 2005; Souza and Lorenzi, 2008; Andrade et al., 2009; Tan and Nishida, 2012; Santos et al., 2013) and is derivative from Eugenol (Tan and Nishida, 2012).

The anesthetic effectiveness of other Piper plants have been demonstrated in some fishes, as the oil of *Alpinia galanga* L. (Willd) for *Cyprinus carpio* (Linnaeus, 1758) (Khumpirapang et al., 2018) and the essential oil of *P. divaricatum* (Mey) in *C. macropomum* juveniles (Vilhena et al., 2019), but they were mainly showed for mammals as rats (Sell and Carlini, 1976), rabbits (Barbosa, 1988; Carlini et al., 1981) and mice (Yano et al., 2006). Methyleugenol a substance that is present in the essential oils of different Piper plants is also an agonist of $GABA_A$ receptors (Ding et al., 2014). Nevertheless, none of those studies possibly looked at the possibility the behavior identified as anesthesia could be, in fact, seizure-like behavior. Indeed, criteria for seizure-like behavior and anesthesia effects are phenomenologically similar (see Tables 2 and 4).

In conclusion, we suggest further studies using the crude extract of *A. oleracea* leaves and its potential use as anesthetic for fish, since to the best of our knowledge, this is the first work to show the effectiveness of crude extracts of plant as a fish anesthetic. Moreover, a better look about at the use of the flowers' extract of *A. oleracea*, as well as those using plants containing piperovatine as putative anesthetics for fish must be performed. Even Eugenol already established as a commercial fish anesthetic, when used in dosages above 10^{-3} mol/L in vivo and in vitro, which is considered safe and low toxic (Taylor et al., 1964; Almeida, 2004), provokes different types of toxicity in body, such as dermatitis, allergic reactions, hepatic dysfunction, intravascular coagulation widespread and severe hypoglycemia in rabbits (Hume, 1983). Since the fish physiological responses to any compound, including anesthetics (Readman et al., 2017), is species-specific, and the anesthetic concentration used to induce the depth anesthesia may also vary according to individual's weight and length, appropriate concentration must be assessed before its use for anesthetize other species.

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Supplementary Material

Supplementary material accompanies this paper.

Supplementary material 1. Recording of seizure-like behaviors of Danio rerio submitted to Piper alatabaccum extract.

Supplementary material 2. Recording of anesthesia behaviors of Danio rerio submitted to Acmella oleracea extract.
This material is available as part of the online article from 10.1590/1519-6984.266010.