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Original Article

Acute toxicity of essential oils of *Aloysia triphylla* (L'Hér.) Britton, *Lippia gracilis* Schauer, and *Piper aduncum* L. in *Colossoma macropomum* (Cuvier, 1818)

Toxicidade aguda dos óleos essenciais de *Aloysia triphylla* (L'Hér.) Britton, *Lippia gracilis* Schauer e *Piper aduncum* L. em *Colossoma macropomum* (Cuvier, 1818)

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Abstract

The aim of this study was to determine the acute toxicity of the essential oils (EOs) of *Aloysia triphylla*, *Lippia gracilis* and *Piper aduncum* in juvenile tambaqui (*Colossoma macropomum*), and evaluate the possible histopathological alterations in their gills. For the acute toxicity tests, juvenile tambaqui (n=24/treatment) were distributed in six treatments with three replicates, which comprised the control and five EO concentrations of *A. triphylla* (60, 80, 100, 120 and 140 mg L^{-1}), *L. gracilis* (35, 40, 45, 50 and 55 mg L^{-1}) and *P. aduncum* (42.5, 45, 47.5, 50 and 52.5 mg L^{-1}), with an exposure period of 4 h. The mortality rate and severity of damage to the tambaqui gills were proportional to the increase in the concentration of the EO, with LC_{50}-4 h values estimated at 109.57 mg L^{-1} for *A. triphylla*, 41.63 mg L^{-1} for *L. gracilis* and 48.17 mg L^{-1} for *P. aduncum*. The main morphological damages observed in the gills of the tambaqui exposed to the three EOs, were Grade I: hypertrophy and hyperplasia of lamellar epithelial cells, lamellar fusion, epithelial detachment, capillary dilation and constriction, proliferation of chloride cells and mucosal cells and edema; in low frequency Grade II damage as epithelial rupture and lamellar aneurysm. Necrosis (Grade III damage) was observed only in gill lamellae exposed to *P. aduncum* EO (47.5, 50.0 and 52.5 mg L^{-1}). Concentrations of EOs below LC_{50}-4 h can be used sparingly, for short periods of exposure for the treatment of diseases in tambaqui breeding.

Keywords: lethal concentration, histology, natural products, tambaqui.

Resumo

O objetivo deste estudo foi determinar a toxicidade aguda dos óleos essenciais (OEs) de *Aloysia triphylla*, *Lippia gracilis* e *Piper aduncum* em juvenis de tambaqui (*Colossoma macropomum*), e avaliar as possíveis alterações histopatológicas em suas brânquias. Para os testes de toxicidade aguda, juvenis de tambaqui (n=24/tratamento) foram distribuídos em 6 tratamentos, com três repetições, sendo o controle e cinco concentração do OE de *A. triphylla* (60, 80, 100, 120 e 140 mg L^{-1}), *L. gracilis* (35, 40, 45, 50 e 55 mg L^{-1}) e *P. aduncum* (42,5, 45, 47,5, 50 e 52,5 mg L^{-1}), com exposição de 4 h. A taxa de mortalidade e a severidade dos danos nas brânquias de tambaqui foram proporcionais ao aumento da concentração do OE, com os valores de CL_{50}-4 h estimados em 109,57 mg L^{-1} para *A. triphylla*, em 41,63 mg L^{-1} para *L. gracilis* e em 48,17 mg L^{-1} para *P. aduncum*. Os principais danos morfológicos observados nas brânquias de tambaqui, expostos aos três OEs, foram os de grau I: hipertrofia e hiperplasia das células do epitélio lamelar, fusão lamelar, descolamento epitelial, dilatação e constrição capilar, proliferação de células de cloreto e de células mucosas e edema; em baixa frequência os de grau II como ruptura epitelial e aneurisma lamelar. Necrose (dano de grau III) foi observado somente nas lamelas branquiais expostas ao OE de *P. aduncum* (47,5, 50,0 e 52,5 mg L^{-1}). Concentrações do OEs abaixo dos valores de CL_{50}-4 h podem ser utilizados com parcimônia, em curtos períodos de exposição para o tratamento de doenças na criação de tambaqui.

Palavras-chave: concentração letal, histologia, produtos naturais, tambaqui.

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1. Introduction

Aquaculture stands out as the fastest growing animal production sector in the world, and is driven by population growth and the demand for healthy food (Valenti et al., 2021). In 2020, the total production of the sector was 87.5 million tons, with 54.4 million coming from inland waters and 33.1 million tons were farmed in marine environments (FAO, 2022). In Brazil, the production of fish was 860,355 tons in 2022, which is 2.3% higher than the previous year (Peixe BR, 2023). The production of native fish in 2022 contributed 31.04% to Brazilian production (267,060 tons), and most of this production (53.7%) came from the northern region and was primarily tambaqui (Peixe BR, 2023).

With the resumption of growth in native fish production and the intensification of production systems, it is important to monitor fish farms for the occurrence of parasitic diseases (Valenti et al., 2021). Fish losses can reach up to 20% in world production with estimated economic losses of about US$ 1.05 to US$ 9.58 billion/year (Shinn et al., 2015). In addition to the high mortality rates in the farming environment, there is also the impairment in reproductive performance and the negative impact on feed conversion, which affect the overall performance of fish (Tavares-Dias and Martins, 2017; Maciel et al., 2018; Pereira and Morey, 2018; Santos et al., 2018; Chagas et al., 2019).

Chemicals such as potassium permanganate, praziquantel, albendazole, levamisole, sodium chloride, triclorfon, and copper sulfate, among others, are used in the treatment against different parasites that affect tambaqui (Campos et al., 2014; Farias et al., 2021; Tavares-Dias, 2021a; Tavares-Dias, 2021b; Tavares-Dias, 2021c; Sebastião et al., 2022b; Nunes et al., 2023), however their inappropriate use may present a risk to consumers, the fish and the environment, can cause parasitic or bacterial resistance, or may even have low applicability when used on a commercial scale (Kawsar et al., 2022; Sebastião et al., 2022a), which therefore reinforces the need to search for bioactive substances such as essential oils (EOs) (Soares et al., 2017a; Soares et al., 2017b; Ferreira et al., 2019; Dawood et al., 2022).

Essential oils (EOs) are used in human medicine due to their antioxidant, antibacterial, antifungal, and analgesic properties (Burt, 2004; Mucha and Witkowska, 2021), in addition to being used as flavorings in food, in perfume fragrances and in pharmaceutical products (Burt, 2004; Mucha and Witkowska, 2021). In aquaculture, the antibacterial and antiparasitic properties of EOs have been studied in several fish species, with proven efficacy against several pathogenic organisms (Sutili et al., 2015; Dawood et al., 2021), in addition to their use as an anesthetic (Brandão et al., 2021) or immunostimulant (Brum et al., 2017), among others. Furthermore, essential oils are more easily biodegradable, have a shorter residence time in water and are less likely to cause fish to become resistant to pathogens (Reverter et al., 2014; Tavares-Dias, 2018; Zhu, 2020; Dawood et al., 2021).

However, several steps are necessary in the choice of the most effective essential oil for pathological studies, and toxicity in particular is paramount since, just like any other drug, essential oils can have adverse effects if administered in high doses and can affect the development of fish and even cause their deaths (Tavares-Dias, 2018; Dawood et al., 2021). In this sense, acute toxicity tests are conducted to determine the average lethal concentration (LC_{50}), which is defined as the concentration that causes mortality of 50% of organisms during an exposure period of up to 96 h, and the side effects of exposure to the natural substance are also evaluated through physiological and histopathological analyses (Tavares-Dias, 2018; Miura et al., 2021). This information is essential in order to define safe concentrations for use in the efficacy tests of any drug or natural product.

As such, the aim of this study was to determine the acute toxicity of essential oils of *Aloysia triphylla* (L'Hér.) Britton, *Lippia gracilis* Schauer and *Piper aduncum* L. in juvenile *Colossoma macropomum* (Cuvier, 1818) and evaluate the possible histopathological alterations in their gills.

2. Materials and Methods

2.1. Extraction and chemical characterization of essential oils

Plant specimens of *A. triphylla*, *L. gracilis* and *P. aduncum* were obtained from the Medicinal Plants and Vegetables Division of Embrapa Amazônia Occidental, Manaus, Amazonas, Brazil. Leaves and inflorescences were collected and dried at room temperature and, after drying, the essential oils of the materials were extracted using the method of hydrodistillation in a Clevenger apparatus for about two hours. At the end of the extraction, samples of the essential oil were collected and analyzed for their chemical composition via gas chromatography and mass spectrometry, as described in Oliveira et al. (2021).

2.2. Fish acquisition and acclimation

Juvenile tambaqui (103.2 ± 36.9 g; 16.6 ± 2.8 cm) were acquired in commercial fish farms (Rio Preto da Eva, AM, Brazil), and were then transferred to Embrapa Amazônia Ocidental (Manaus, AM, Brazil) and acclimated for seven days in circular 1,000 L fiber tanks with a water circulation system, with constant aeration and with a heating central system that kept the water at 28 °C. During this period, the fish were fed with commercial extruded feed (Multifós®, Multi-Peixe 32% crude protein, 4-6 mm) until apparent satiety, twice a day. The composition of the feed was 100 g kg^{-1} moisture; 320 g kg^{-1} crude protein; 45 g kg^{-1} ethereal extract; 60g kg^{-1} crude fibre and 100 g kg^{-1} crude ash.

The physicochemical parameters of the water were monitored during the experiment using an oximeter (YSI Pro20, YSI Inc., USA) and pH meter (YSI F-1100, YSI Inc., USA). Alkalinity and hardness were determined via the titration method and total ammonia via the endophenol method (APHA, 1992). The mean values of the water quality parameters were as follows: oxygen 5.03 ± 0.4 mg L^{-1}; temperature 29.9 ±1.9 °C; pH 6.6 ± 0.3; alkalinity 95.16 ± 39.86 mg L^{-1}; hardness 10.8 ± 1.46 mg L^{-1} and ammonia 0.28 ± 0.016 mg L^{-1}.

This study was developed with the approval of the Ethics Commission for Animal Use (CEUA) of Embrapa Amazônia Occidental (Protocol No. 02/2019).

2.3. Assessment of acute toxicity of essential oils

The acute toxicity tests were performed in circular tanks with a capacity of 70 liters that had a static system with constant aeration of the water and maintenance of the temperature at 29.9 ± 1.9 °C, using a thermostat positioned on the side of each tank. The fish were acclimated in these experimental units (n=8/tank) for seven days. The concentrations of EOs were determined based on data already published in the literature (Oliveira et al., 2018; Brandão et al., 2021; Queiroz et al., 2022), and followed by preliminary tests in which the lowest concentration capable of promoting 100% mortality and the highest concentration that does not cause fish mortality were established. The definitive tests were conducted in a completely randomized design, with six treatments and three replicates per plant; the six being made up of the control (only tank water) and concentrations of *Aloysia triphylla* EOs (60, 80, 100, 120 and 140 mg L^{-1}), *Lippia gracilis* (35, 40, 45, 50 and 55 mg L^{-1}) and *Piper aduncum* (42.5, 45, 47.5, 50 and 52.5 mg L^{-1}). The essential oil was diluted in ethyl alcohol, in a ratio of 1:10 (mass/volume). The period of exposure to essential oils was set at four hours, as recommended by Malheiros et al. (2016), since it offers a good margin of safety for short-term therapeutic baths.

In the definitive tests, after four hours of exposure to the different concentrations of the essential oils, the mortality rate was calculated and the behavior of the fish was recorded in relation to the following parameters: loss of balance, changes in opercular movement and irregular swimming. The fish were considered dead when the opercular beats and the caudal fin had stopped and when they no longer responded to mechanical stimuli from the external environment, as per Ferreira et al. (2019). With the mortality data, the mean lethal concentration values (LC_{50}-4 h) for each essential oil were calculated using the statistical method of Trimmed Spearman Karber (Hamilton et al., 1978) and, from this information, the EOs were classified as to their toxicity to tambaqui, based on the criteria of Zucker (1985), which establishes that compounds with LC_{50} values <0.1 mg L^{-1} are considered very highly toxic, between 0.1 and 1.0 mg L^{-1} are highly toxic, in the range <1.0 and ≤10 mg L^{-1} are moderately toxic, between >10 and ≤100 mg L^{-1} are slightly toxic and >100 mg L^{-1} are practically non-toxic.

2.4. Histopathological analysis

After the toxicity test and after euthanasia, the gills of six fish from each treatment with the *A. triphylla*, *L. gracilis* and *P. aduncum* EOs, were collected from the 2 gill arches. The collected samples (12 per treatment) were fixed in 5% buffered formalin for 24 h, subsequently dehydrated in an ascending series of ethanol (70, 80, 90 and 100%), diaphanized in xylol, impregnated and embedded in liquid paraffin at 60 °C. Slices of a thickness of 5 µm (two from each sample) were performed with the aid of semi-automatic microtome (Leica RM2245) and stained with hematoxylin and eosin. The secondary lamellae were examined under an optical microscope (Leica, DM 500) coupled to an image capture system. The semiquantitative analysis of gill damage was performed according to the method established by Poleksic and Mitrovic-Tutundzic (1994) using the histological alteration index (HAI) in the formula: $HAI = 10^{0}\, \Sigma I + 10^{1}\, \Sigma II + 10^{2}\, \Sigma III$, where: stage I damage – mild, does not compromise the functioning of the organ; Stage II damage – moderate, compromises the normal function of the organ and Stage III damage – severe, is irreversible.

2.5. Statistical analysis

The logistic regression model was used to determine the LC_{50}-4 h of essential oils, as well as for the equation and the mortality curve. The values of the histological alteration index did not meet the assumptions of normality of Shapiro Wilk and homoscedasticity of Levene, so Kruskal-Wallis nonparametric statistics were applied, followed by Dunn's test to compare the indices of histopathological alterations between treatments. The significance level used in all tests was 95% (p-value ≥ 0.05), and the software R, version 4.0.2 was used.

3. Results

The main compounds found in the EOs of *A. triphylla*, *L. gracilis* and *P. aduncum* are shown in Figure 1. These EOs have as majority compounds β-pinene (22.1%), *trans*-pinocamphone (13.1%) and *trans*-pinocarveol acetate (7.6%) for *A. triphylla*, carvacrol (42.2%), *p*-cymene (11.2%) and γ-terpinene (10.7%) for *L. gracilis*, dillapiole (80.7%), *trans*-caryophyllene (4.6%) and myristicin (2.9%) for *P. aduncum*.

In the toxicity tests, there were no records of fish mortalities in the control group during the period of the experiment. For *A. triphylla*, mortalities increased starting from the concentration of 80 mg L^{-1}, after 200 minutes of exposure, which represents a 12.5% mortality rate, and 100% mortality rate was found at the highest concentration (140 mg L^{-1}), with onset of mortalities after 25 minutes of application of the EO (Figure 2A). For *L. gracilis*, mortalities occurred at the lowest concentration (35 mg L^{-1}), from 120 minutes of exposure to the EO, representing a mortality rate of 29.17% of the total number of individuals. At the highest concentration of *L. gracilis* (55 mg L^{-1}), the fish began to die after 30 minutes of exposure, reaching a rate of 100% at 240 minutes, this being the maximum time established (Figure 2B). Similarly, for *P. aduncum*, fish mortalities started at the lowest concentration (42.5 mg L^{-1}) with 40 minutes of exposure. In the highest concentration of *P. aduncum* (52.5 mg L^{-1}), the first mortality record occurred after 25 minutes of exposure, reaching the rate of 100% before the maximum exposure time (240 minutes) (Figure 2C).

In all concentrations in which mortalities occurred, the fish showed behavioral changes such as erratic swimming, lethargy, agglomeration on the surface of the tanks, (where there is more aeration), loss of hydrodynamic balance, and muscle spasms. As for clinical signs, these were observed only in the highest concentrations, and included excessive production of mucus in the skin and gills, opaque gills and corneal opacity. On the other hand, in the concentrations of EOs in which the lowest mortality rates were recorded, a sedative effect of these EOs was observed. The fish showed partial loss of balance, without reacting to external stimuli, but recovered at the end of the exposure period when they returned to normal rearing conditions.

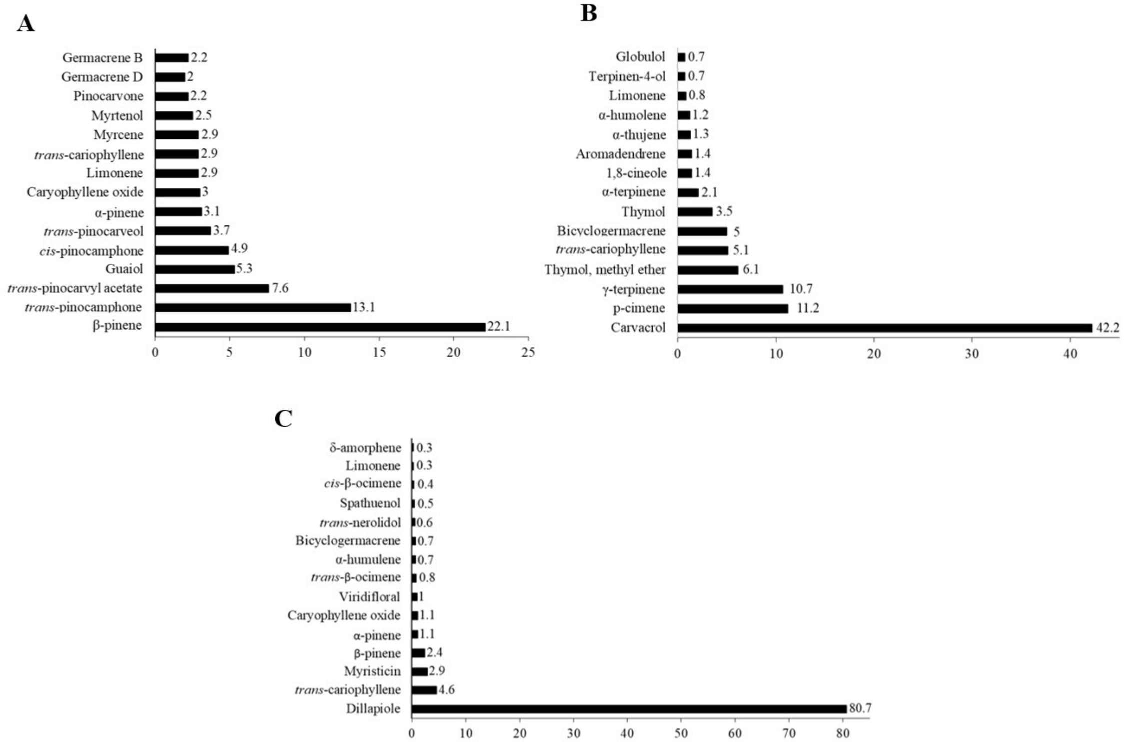

Figure 1. Graphic representation of the major compounds of the essential oils of *Aloysia triphylla* (A), *Lippia gracilis* (B) and *Piper aduncum* (C).

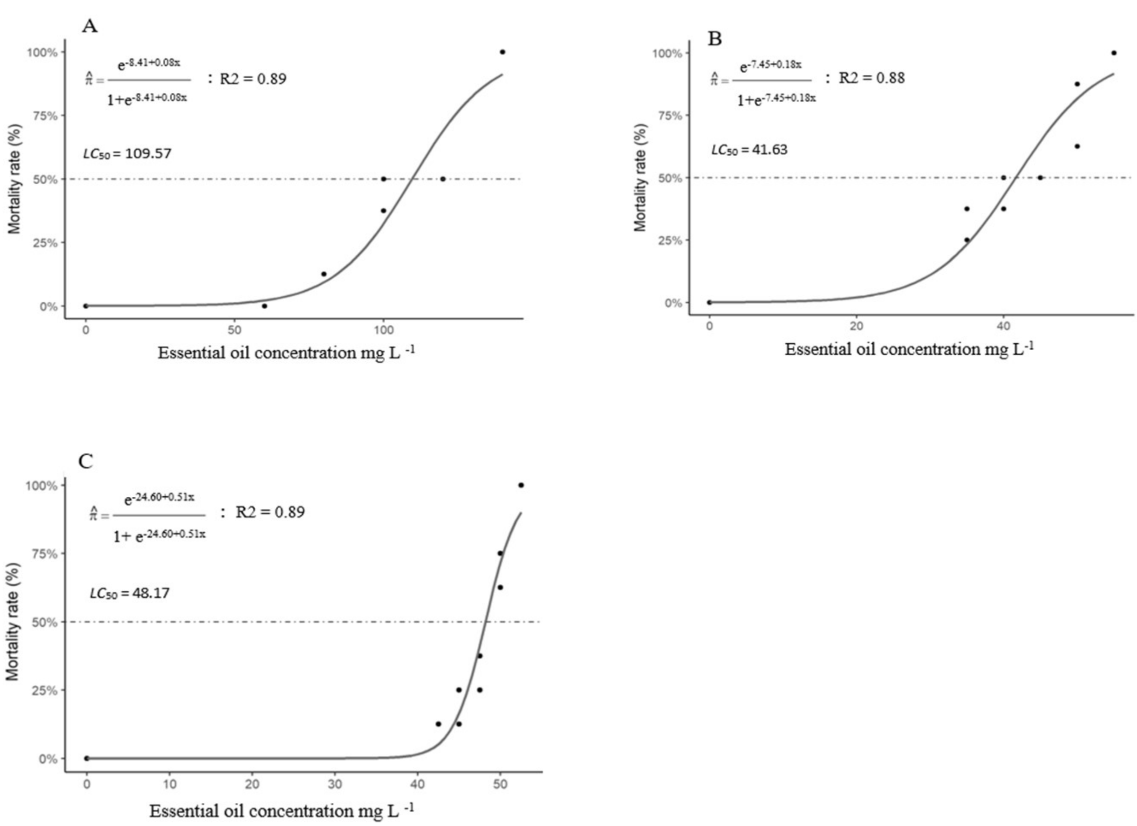

Figure 2. Mortality rate (%) of juvenile tambaqui, *Colossoma macropomum*, subjected to different concentrations of essential oils of *Aloysia triphylla* (A), *Lippia gracilis* (B) and *Piper aduncum* (C) for 4 h, and concentration estimates lethal mean (LC_{50}).

The LC_{50}-4 h of *A. triphylla* EO for tambaqui was 109.57 mg L^{-1}, with a lower limit of 99.54 mg L^{-1} and an upper limit of 113.02 mg L^{-1}, considering a range of 95%. For *L. gracilis*, the LC_{50}-4 h was 41.63 mg L^{-1}, with lower and upper limits of 38.82 and 46.18 mg L^{-1}, respectively. Regarding the EO of *P. aduncum*, the LC_{50}-4 h was 48.17 mg L^{-1}, with a lower limit of 47.53 mg L^{-1} and an upper limit of 49.63 mg L^{-1}. The equations of the dose-response relationship of the three EOs are presented in Figure 2.

By applying the criterion of Zucker (1985), the estimated LC_{50}-4 h values for the essential oils of *P. aduncum* and *L. gracilis* were classified as slightly toxic and *A. triphylla* as practically non-toxic. Histopathological changes in the gills such as hypertrophy and hyperplasia of lamellar epithelial cells, lamellar fusion, epithelial detachment, capillary dilation and constriction, proliferation of chloride cells and mucosal cells, edema, epithelial rupture, lamellar aneurysm and necrosis were observed in fish exposed to different concentrations of the essential oils evaluated (Figure 3 and Table 1). In the treatments with the EOs of the three plants, the most frequent damage to the gill lamellae was classified as Grade I damage, such as hyperplasia of the lamellar epithelium and lamellar fusion, and with low frequency, Grade II damage, such as rupture of the lamellar epithelium (Table 1), and Grade III damage, necrosis, was observed in the gill lamellae of fish exposed to *P. aduncum* EO at the highest concentrations specifically where it shows a higher rate of histopathological changes (Figure 4), but despite this occurrence, the frequency was low in relation to the others (Table 1).

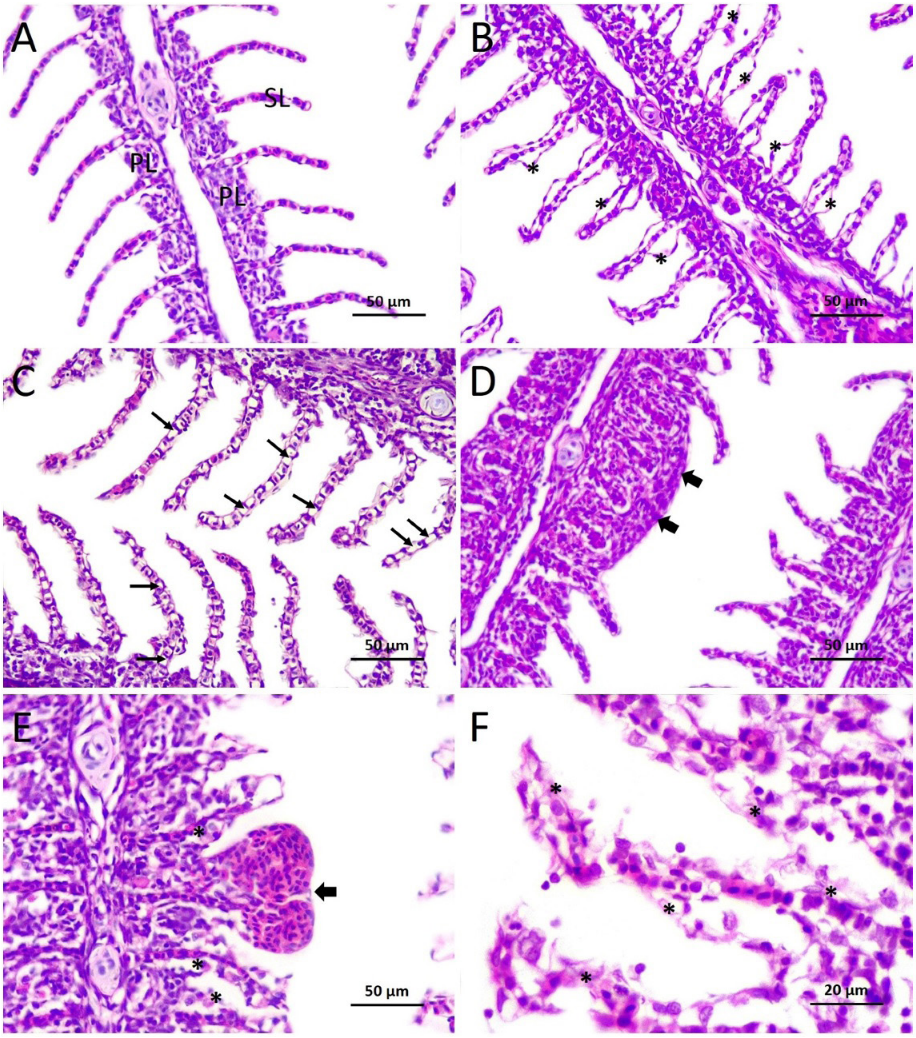

Figure 3. Major tissue damage observed in the gills of *Colossoma macropomum*, after 4 h of exposure to essential oils of *Aloysia triphylla*, *Lippia gracilis* and *Piper aduncum*. A: control treatment, with details of primary lamellae (PL) and secondary lamellae (SL). B: epithelial detachment (asterisks) at 40 mg L^{-1} concentration of *L. gracilis* essential oil. C: capillary dilation in the gills of fish exposed to 50 mg L^{-1} of *P. aduncum* essential oil (arrow). D: lamellar fusion observed at the concentration of 35 mg L^{-1} of the essential oil of *L. gracilis* (thick arrow). E: lamellar aneurism (arrow) and proliferation of mucous cells (asterisks) in the gills of fish exposed to 60 mg L^{-1} of *A. triphylla*. F: necrosis observed in the gills of fish exposed to 50 mg L^{-1} of the essential oil of *P. aduncum*, with areas of cytoplasmic degeneration (asterisks).

There was no difference between the mean values of the histopathological alteration index (HAI) recorded for the treatments with *A. triphylla*, *P. aduncum* and *L. gracilis* EOs (Figure 4). Significantly higher mean HAI values were observed in the treatment with 50 mg L^{-1} compared to the other treatments (Figure 4C and Table 1).

Considering the scale of tissue damage, the most frequent values of the histopathological alteration index (HAI) are between 11 and 20, and correspond to mild to moderate organ damage (75% for *A. triphylla*, 27.7% for *L. gracilis* and 60% for *P. aduncum*) (Table 2). HAI values between 21 and 50, which are indicative of moderate to severe alterations, were observed with a low percentage for the three EOs (Table 2). Severe and irreparable alterations, indicated by HAI values between 50 and 100 and greater than 100, were observed in the gills of the tambaqui with the use of the EO of *P. aduncum* (3.33% and 18.33%, respectively) (Table 2).

Table 1. Relative frequency (%) of histological damage to gills of *Colossoma macropomum* after exposure to essential oils of *Aloysia triphylla*, *Lippia gracilis* and *Piper aduncum*.

Histopathological changes	Degree	Essential oils		
		Aloysia triphylla	***Lippia gracilis***	***Piper aduncum***
Hypertrophy of lamellar epithelium	Grade I	12.28	13.72	12.13
Hyperplasia of lamellar epithelium	Grade I	12.85	13.90	12.58
Lamellar fusion	Grade I	10.20	11.27	8.60
Detachment of the epithelium	Grade I	12.85	13.90	11.96
Capillary dilation	Grade I	9.64	12.59	9.92
Capillary constriction	Grade I	3.96	8.08	5.50
Chloride cell proliferation	Grade I	12.85	13.72	12.58
Proliferation of mucous cells	Grade I	8.50	4.13	7.79
Edema	Grade I	6.23	2.44	5.56
Epithelial rupture (hemorrhage)	Grade II	2.64	3.94	1.37
Lamellar aneurysm	Grade II	7.93	2.25	8.21
Necrosis	Grade III	0	0	3.77
Total (%)		100	100	100

Table 2. Relative frequency (%) by the histopathological alteration index (HAI) scale observed in *Colossoma macropomum* gills after exposure to essential oils of *Aloysia triphylla*, *Lippia gracilis* and *Piper aduncum*.

Scale of HAI	Essential oil			Interpretation
	Aloysia triphylla	***Lippia gracilis***	***Piper aduncum***	
0-10	20.00	63.88	13.33	Normal functioning of the organ
11-20	75.00	27.77	60.00	Mild to moderate organ damage
21-50	4.16	8.33	5.00	Moderate to severe alterations in the organ
50-100	0.00	0.00	3.33	Severe alterations in the organ
> 100	0.00	0.00	18.33	Irreparable damage to the organ

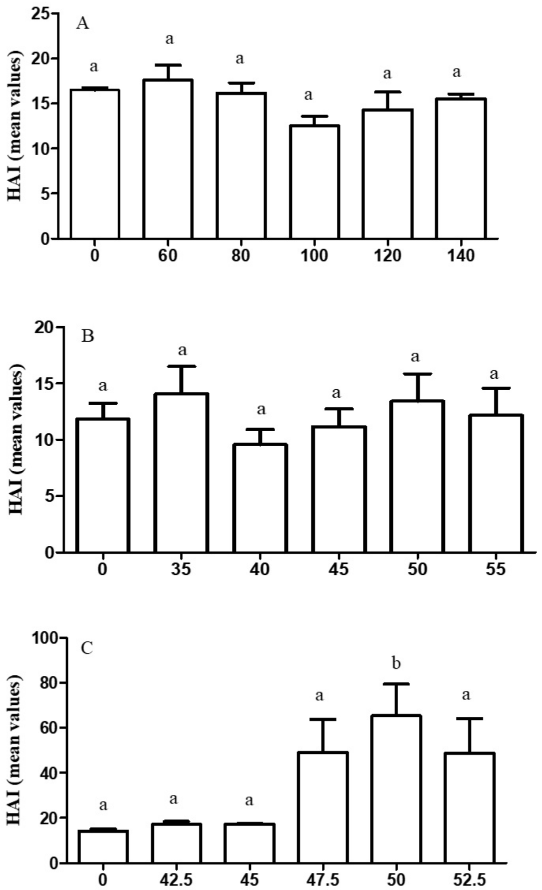

Figure 4. Mean values of the histopathological alteration index (HAI) in the gills of *Colossoma macropomum* exposed to essential oils of *Aloysia triphylla* (A), *Lippia gracilis* (B) and *Piper aduncum* (C). Different letters on the bar indicate difference between treatments according to Dunn's test (p<0.05).

4. Discussion

In addition to possessing information about their chemical composition, it is also necessary to know the toxic potential *A. triphylla*, *L. gracilis* and *P. aduncum* EOs have for fish, especially for native species, for which information is scarce (Malheiros et al., 2016; Oliveira et al., 2018; Ferreira et al., 2019; Barriga et al., 2020).

Toxicity data for the use of *P. aduncum* essential oil in fish are scarce. The available information is on the use of the ethanol extract of *P. aduncum* for tambaqui in an exposure period of 12 h, with an LC_{50}-12 h of 79 mg L^{-1} and a confidence interval of 66 to 92 mg L^{-1} with low toxicity (Queiroz et al., 2022). In this study, an LC_{50}-4 h with the use of *P. aduncum* EO was established at 48.17 mg L^{-1} for tambaqui, showing a difference in fish tolerance, due to differences in protocols and products obtained (ethanol extract and essential oil) of *P. aduncum*. In addition, dillapiole, identified as the main chemical component, was present in 80.7% of the EO of *P. aduncum* used in this study, while for the ethanolic extract it represented 13.46% (Queiroz et al., 2022). On the other hand, for organisms of different trophic levels (plants, algae and nematodes), Miura et al. (2021) reported that the EO of *P. aduncum* (dillapiole, corresponding to 75.5% of the EO) affects the growth and mortality of these organisms, with greater sensitivity presented by microcrustaceans. This set of information underscores the importance of ecotoxicological assessments in establishing safe concentrations for target and non-target organisms.

Plants of the genus *Lippia* have been used for anesthetic induction in fish and in the treatment of diseases (Barriga et al., 2020; Brandão et al., 2021; Monteiro et al., 2021). Regarding the EO of *L. origanoides*, the mean lethal concentration at 96 h for tambaqui was established at 15.2 mg L^{-1} (Oliveira et al., 2018). This value is lower than that observed in this study for the EO of *L. gracilis*, which also has carvacrol (49.7%) in its composition and has values close to those of the composition of the EO used in this study (42.2%). Regarding LC_{50}, the difference in the results may be related to the length of exposure, which in this study was 4 h, thus allowing tambaqui to tolerate a higher concentration LC_{50}-4 h of 41.63 mg L^{-1}, as well as the combined effect of the other constituents of the EO. In another toxicity study using the EO of *L. grata*, heterotypic of *L. gracilis*, tambaqui tolerated 700 mg L^{-1} for 30 minutes, and an anesthetic effect of this OE was observed after five minutes of exposure, but without mortality of the fish. Therefore, this concentration is recommended for use in therapeutic baths (Barriga et al., 2020). Differences in the evaluation protocols, in the size of the fish, which for *L. grata* was 30 g, are reasons for the difference found in the tolerance of tambaqui to the EO, since the percentage of the main chemical constituents of EO of *L. grata*, carvacrol (48.12%) and *p*-cymene (24.39%), were close to those found in the present study for *L. gracilis* (42.2% and 11.2%, respectively).

A. triphylla is one of the EOs that has been widely studied in aquaculture with respect to its anesthetic activity (Santos et al., 2017; Almeida et al., 2019). For tambaqui, with the use of 150 mg L^{-1} of *A. triphylla* EO, for 4.7 minutes, the fish reached anesthetic induction, without fish mortality (Brandão et al., 2021). In this toxicity study, there was no record of mortality of tambaquis within the first minutes or hours of exposure to *A. triphylla* EO, as reported by Brandão et al. (2021); however, after 3 h of exposure to the highest concentrations (120 and 140 mg L^{-1}) and 4 h at the other concentrations (80 and 100 mg L^{-1}), fish mortalities were recorded, with determination of LC_{50}-4h for tambaqui at 109.57 mg L^{-1}. Compared to the other EOs (*L. gracilis* and *P. aduncum*), tambaqui showed greater tolerance to the EO of *A. triphylla*, in whose composition the compounds β-pinene (22.1%), *trans*-pinocamphone (13.1%) and *trans*-pinocarvil acetate (7.6%) are present. According to the criterion of Zucker (1985), the EO of *A. triphylla* is classified as practically non-toxic, while the EOs of *L. gracilis* and *P. aduncum* were classified as slightly toxic, and attention should be paid to its use in disease treatment protocols for tambaqui.

Different types of morphological damage were observed in this study in the gills of fish exposed to the EOs of *A. triphylla*, *L. gracilis* and *P. aduncum*, with more frequent Grade I damage such as hypertrophy and hyperplasia of the lamellar epithelium, lamellar fusion, epithelial detachment and proliferation of chloride cells.

These same damages were observed by Brandão et al. (2021) in tambaqui exposed to the EOs of *A. triphylla*, *Lippia sidoides* and *M. piperita,* and by Soares et al. (2017b) in tambaquis exposed to the EO of *L. sidoides*. Alterations such as hypertrophy and lamellar epithelial hyperplasia are considered a common and nonspecific response to subacute to chronic gill damage (Strzyzewska et al., 2016) and, together with the other alterations, may be indicative of a stress condition, as they evidence the functional activation of the osmoregulation organ (Spanghero et al., 2019; Vieira et al., 2019). In this study, Grade II damage, such as lamellar epithelial rupture and lamellar aneurysm, occurred at a low frequency and were recorded after exposure to the three EOs evaluated. These damages are more severe and can compromise the functioning of the gills, which is the organ responsible for gas and ion exchange, but they are repairable if adverse conditions are controlled (Bernet et al., 1999).

The semi-quantitative analysis of gill damage, evidenced by the mean HAI values, showed that the fish exposed to the EO of *A. triphylla* presented mild to moderate damage (75%), a pattern also observed by Brandão et al. (2021) and one which showed normal functioning (75%) and mild to moderate damage to the tambaqui gills (16.7%) with the use of the same EO, during anesthetic induction, at a concentration of 150 mg L^{-1}. For the EO of *L. gracilis*, the HAI ranged from 9.6 to 14.1, with normal functioning (63.88%) and mild to moderate damage to the gills of the fish (27.77%). These results are similar to those observed with the EO of *L. origanoides*, which has carvacrol as the majority compound of this EO (49.7%) and in a percentage close to that of the EO of *L. gracilis*, with the description of moderate to severe damage, with the use of 40 mg L^{-1} for 30 minutes. However, after 24 h of recovery, these were classified as mild to moderate damage (Soares et al., 2017b). In the highest concentrations of *P. aduncum* (47.5, 50 and 52.5 mg L^{-1}), the gills were affected to a greater extent, with HAI values ranging from 48.83 to 65.33. At the concentration of 50 mg L^{-1} severe and irreversible alterations in the gills of fish were observed, such as necrosis (Grade III damage). Severe and irreparable damage was observed in the gills of tambaqui exposed to the EOs of two species of *Lippia*, with the use of EO of *L. alba* for 30 minutes at concentrations of 100 and 150 mg L^{-1}; the HAI ranged between 66 and 121 and, with the EO of *L. sidoides* (20 mg L^{-1} for 15 minutes), whose mean value of HAI was 119.5 and, in both cases, there was an occurrence of lamellar necrosis (Soares et al., 2016; Soares et al., 2017b).

It is important to highlight that the occurrence of aneurysm is due to the collapse of the pillar cell system and that it can culminate in the rupture of the lamellar epithelium (Grade II damage). These changes are reversible if the predisposing condition is adjusted, but if the exposure to the aggressor agent is prolonged the damage can progress to Grade III, (Poleksic and Mitrovic-Tutundzic, 1994; Winkaler et al., 2007). These alterations were observed in this study in the highest concentrations of *P. aduncum*, *L. gracilis* and *A. triphylla* EOs, and are responsible for the highest degree of gill impairment and consequent reduction in respiratory capacity of the fish, which can lead to their mortality. Therefore, the use of histological information in conjunction with the LC_{50}-4 h values of the EOs evaluated in this study is of great importance for the establishment of safe concentrations to be used in protocols for the treatment of tambaqui diseases.

The essential oils of *L. gracilis* and *P. aduncum* were classified as mildly toxic and *A. triphylla* as practically non-toxic to tambaqui. According to the histological alteration index, most of the damage to the gills of fish with the use of essential oils were categorized as mild to moderate, but with a low frequency of severe damage such as aneurysms, in addition to irreversible damage such as necrosis in the highest concentrations of *P. aduncum* essential oil. These essential oils can be used sparingly, in low concentrations and short exposure periods.

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