Anthelmintic efficacy of dillapiole and the crude extract of Piper aduncum in the control of monogenoids in Colossoma macropomum

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Abstract

Piper aduncum, a medicinal plant, was evaluated for control of monogenoids and effects on the welfare of *Colossoma macropomum* using a crude ethanolic extract and its major constituent dillapiole. Treatments of 40 and 60 mg·L⁻¹ of the ethanolic extract were tested in in vitro and in vivo experiments with water and methanol controls. Dillapiole was evaluated at 28 and 33 mg·L⁻¹ with water and Tween-20 controls and a basal acclimated group. Parasitological, immunophysiological, biochemical and histological analyses were performed on 50 g fish. Both extract concentrations significantly reduced parasite load versus controls, yielding antiparasitic efficacies of 85.89% and 99.21% for 40 and 60 mg·L⁻¹, respectively. Control fish showed increases in hematocrit and hemoglobin, whereas fish treated with 60 mg·L⁻¹ extract exhibited hyperglycemia, elevated cortisol and reductions in total protein and Ca^{2+} and K^{+} ion levels, indicating physiological stress at higher extract concentration. In water controls, thrombocyte and leukocyte elevations correlated with heavy parasitism; these responses were attenuated in extract-treated groups. Dillapiole at 28 and 33 mg·L⁻¹ produced significant parasite reductions with efficacies of 52.44% and 80.24%, respectively. Dillapiole treatments induced increased thrombocytes, leukocytes and differential counts, suggesting stimulation of immune responses that favored recovery after therapeutic baths. Histological assessment showed milder tissue alterations in dillapiole-treated fish compared to controls. Results indicate that both *P. aduncum* extract and dillapiole effectively control monogenoid infestations in *C. macropomum*, combining antiparasitic action with moderate, concentration-dependent physiological impacts. These findings support further development of P. aduncum-based therapies for sustainable monogenoid management in tropical aquaculture globally.

1. INTRODUCTION

The intensification of aquaculture production has boosted the growth of the sector, but it has also predisposed fish to a greater susceptibility to pathogens, especially parasitosis. Furthermore, the supply of products and effective protocols remains insufficient to combat these diseases, which cause high rates of mortality and significant economic losses (Moraes et al. 2023; FAO 2024; Madsen & Stauffer 2024).

In this context, *Colossoma macropomum*, an endemic species of the Amazon and the main fish produced in the region, with increasing relevance in South America, Central America, the Caribbean and Asia (Valladão et al. 2018; Woynarovich & Van Anrooy 2019), depends on technological innovations and new therapeutic alternatives to sustain intensive cultivation systems. Several studies have indicated that the number of authorized drugs that can be used to control the main parasitoses in this species is very low, especially those caused by myxozoans (Vine et al. 2016), acanthocephalans (Andrade-Porto et al. 2021; Morais et al. 2023) and monogenoids (Affonso et al. 2023; Dias et al. 2015; Maciel & Affonso 2021; De Queiroz et al. 2022), which constitutses a major obstacle for increased production and productive expansion. In particular, monogenoids, ectoparasites of the skin and gills, are among the most common, and induce excessive mucus production, hyperplasia, lamella fusion and branchial necrosis, in addition to favoring secondary infections (Jerônimo et al. 2014; Noga 2010). Due to the negative impacts of these parasites on the productive sector, some chemotherapeutic products (hydrogen peroxide, formalin, mebendazole and praziquantel) have been evaluated for their therapeutic potential in *C. macropomum* (Andrade-Porto et al. 2017; Chagas et al. 2016; Hirazawa et al. 2016; Maciel & Affonso 2021). However, there is still a considerable range of variation in the results regarding their efficiency in reducing infestations; in addition, the prolonged use of some of these products can favor the development of resistant microorganisms, and serious risks of contamination of the host, the environment and consumers (FAO 2024; Ng et al. 2024; Huys et al. 2007).

To reduce the farmers dependence on conventional chemicals, the use of extracts, essential oils and substances isolated from plants has shown promise in the control of parasites in aquaculture (De Queiroz et al. 2022; Ng et al. 2024). For example, an extract from *Euphorbia fischeriana* controled *Dactylogyrus vastator* in *Carassius auratus* (Zhang et al. 2014); Acetone from *Bixa orellana* and oil from *Lipia alba* were effective against monogenoids in *C. macropomum* (Andrade et al. 2016; Soares et al. 2017); and oils from *Melaleuca alternifolia*, *Mentha piperita* and *Copaifera ducke* showed effectiveness against *Anacanthorus penilabiatus* and *Mymarothecium viatorum* in *Piaractus mesopotamicus* (Costa et al. 2017).

Piper aduncum, or monkey pepper, which is widely distributed in the tropics, is highlighted in studies due to its leaf extracts (yield > 17%, with ~ 41% dillapiole) and its essential oil (2.5–3.5%, up to 98% dillapiole), which are rich in prenylated compounds, flavonoids and sesquiterpenes (Corral et al. 2018; De Queiroz et al. 2022; Efdi et al. 2023). Its extracts have shown efficacy against monogenoids in *Arapaima gigas* and *C. macropomum* (Queiroz et al. 2022) and *Hysterothylacium* sp. in *A. gigas* (Corral et al. 2018),

while the dillapiole isolate exhibits parasiticidal, bactericidal, fungicidal and insecticidal actions (Flores et al. 2009; Almeida et al. 2009; Dal Picolo et al. 2014;), thus evidencing its potential for sustainable disease management.

Thus, with the aim of offering a sustainable phytopharmacological alternative, this study evaluated the anthelmintic efficacy of dillapiole and the ethanolic extract of *Piper aduncum* against monogenoids in juvenile *Colossoma macropomum*, in addition to investigating their effects on the welfare of the fish.

2. MATERIAL AND METHODS

2.1. Study site and Ethics Committee

The experiments were carried out at the Experimental Aquaculture Station of the Laboratory of Physiology Applied to Fish Farming (LAFAP), at the National Institute for Amazonian Research (INPA), Amazonas, Brazil. This work was carried out in accordance with the guidelines published by the National Council for Control of Animal Experimentation (CONCEA 2015) and was approved by the Ethics Committee on the Use of Animals (CEUA) of the National Institute for Amazonian Research (INPA) (Process No. 030/2016).

2.2 Acclimation of the fish and monitoring of water quality

Juvenile *C. macropomum* (10 ± 1.0 g) were obtained from a commercial fish farm, and then placed in six 500-L polyethylene tanks of (100 fish/tank), with a low water-renewal rate, constant aeration, temperature of 26.0 ± 0.5 ºC, dissolved oxygen of 6.5 ± 1.0 $mg{\cdot}L^{-1}$ and a pH of 6.5 ± 0.5. The fish were fed twice a day for 30 days with a 36% crude protein commercial feed until apparent satiety. At the end of this period, three fish from each tank were removed for parasitological screening before the execution of the experiments.

The following physical and chemical variables of the water were determined: dissolved oxygen (DO), temperature and electrical conductivity, with the aid of a digital oximeter (YSI, 85/10); pH, with a digital pH meter (YSI, 60/10); total ammonia concentration (NH_3 + NH_4^+), according to Verdouw et al. (1978); nitrite (NO_2^-) and carbon dioxide (CO_2), according to Boyd and Tucker (1992). The mean values (± SD) of the water quality variables were temperature 27.70 ± 0.07 ºC; pH 6.38 ± 0.04 (log H^+); dissolved oxygen 7.11 ± 0.20 $mg{\cdot}L^{-1}$; total ammonia 0.676 ± 0.066 $mg{\cdot}L^{-1}$; CO_2 17.92 ± 2.22 $mg{\cdot}L^{-1}$; nitrite 0.005 ± 0.001 $mg{\cdot}L^{-1}$; and electrical conductivity 51.60 ± 1.60 $\mu S{\cdot}cm^{-1}$. During the therapeutic baths, these variables did not present statistical differences between the treatments and were within the recommended limits for *C. macropomum* (Barroso et al. 2020).

2.3 Screening and determination of in vitro concentrations

Initially, to determine the concentrations of crude ethanolic extract and dillapiole, a phytochemical (screening) experiment was performed. Collections, characterization, chemical analysis and toxicity tests of both compounds were performed as described by Queiroz et al. (2022). In the experiment, the anti-monogenoid activity of *P. aduncum* was evaluated in the juvenile *C. macropomum* after five-hour baths, in which four fish (45 ± 10 g) were euthanized by cerebral concussion (CONCEA 2018) and immediately immersed in two concentrations of crude ethanolic extract (20 and 200 $mg{\cdot}L^{-1}$, solubilized in methanol 0.1% v/v) and dillapiole (10 and 100 $mg{\cdot}L^{-1}$, solubilized in Tween-20 0.4% v/v). For each product, two control groups were paired: pure water and vehicle (methanol 0.1% or Tween-20 0.0004% v/v). Every 30 min, the gills were examined under a stereomicroscope (Zeiss, Stemi 2000-C) to record lethargy, detachment of the gills and parasite mortality (Fajer-Ávila et al. 2003), with the percentage of efficacy being calculated according to Martins et al. (2001) and Onaka et al. (2003).

With the results of this screening, four final concentrations for each product were selected for a quantitative *in vitro* assay: 40, 60, 80 and 100 $mg{\cdot}L^{-1}$ of the ethanolic extract (controls in water and methanol 0.1% v/v) and 15, 30, 60 and 90 $mg{\cdot}L^{-1}$ of dillapiole (controls in water and Tween-20 0.0004% v/v). For the assay, ten fish (45 ± 10 g) were euthanized (CONCEA 2018) and subjected to baths of the aforementioned concentrations, repeating the microscopic observation of lethargy, gill detachment and mortality at intervals of 30 min over five hours. The evaluation method remained identical, differing only in the amplitude of the doses tested in order to characterize the efficacy response curve of the ethanolic extract and the dillapiole.

2.4 In vivo therapeutic assays of the ethanolic extract and dillapiole in juvenile C. macropomum

2.4.1 Therapeutic baths

For the *in vivo* assay, we used the acute toxicity test (LC_{50-96h}) in non-parasitized *C. macropomum*, as described by Queiroz et al. (2022a), to ensure that the predicted doses would be sublethal). Based on the results obtained *in vitro*, 40 and 60 $mg{\cdot}L^{-1}$ for the crude ethanolic extract and 28 and 33 $mg{\cdot}L^{-1}$ for dillapiole were used in the *in vivo* therapeutic baths. For this, two tests were performed, each involving twelve 60-L aquariums (total capacity 80 L), containing ten juvenile *C. macropomum* (48.6 ± 5.0 g). The fish were submitted to two consecutive therapeutic baths of four hours, interspersed by three hours of recovery between baths, in 500-L tanks with continuous aeration and a photoperiod of 12 h/12 h, and a basal group (not parasitized and untreated) was used as a negative control. At the end of the second bath, five fish from each experimental unit were collected for parasitological analysis of the gills and five for histological examination (Marinho-Neto et al. 2019; Affonso et al. 2023). The remaining subjects remained in the 500-L fiber tanks for seven days, during which they were monitored daily for mortality, alterations in behavior and clinical signs of abnormal health.

2.4.2 Analysis of antiparasitic activity and identification of monogenoids

After the euthanasia of the fish, the gill arches were removed and individualized in Petri dishes for analysis under a stereomicroscope (Zeiss, Stemi 2000-C). After counting the parasites, the gills were fixed in 5% formalin (Eiras et al. 2010) for further counting and identification of the species of monogeneoids. The estimate of the percentage of efficacy of the treatments was calculated according to Martins et al. (2001) and Onaka et al. (2003). Parasitic indices followed the definitions of Bush et al. (1997).

The monogenoid species were identified according to De Kritsky et al. (1979), Thatcher and Krytsky (1983), Cohen and Kohn (2005), Belmont-Jégu et al. (2004) and Kritsky et al. (1996). For the study of the sclerotized structures (bars, hooks, anchors of the haptor, copulatory complex), the parasites were mounted on slides with a coverslip, using Grey & Wass for clarification (Thatcher 2006).

2.4.3 Clinical laboratory analysis

After the two therapeutic baths of 4 h each, blood samples were collected from nine fish per treatment via caudal vein puncture, using syringes with 10% EDTA, and then stored under refrigeration at 4°C. An aliquot of whole blood was used to determine hematocrit, hemoglobin concentration and hematimetric indices: mean corpuscular volume (MCV = Ht × 10/RBC); mean corpuscular hemoglobin (MCH = Hb × 10/RBC); total blood concentration (TBC); mean corpuscular hemoglobin concentration (MCHC = Hb × 100/Ht) and the erythrocyte count via the indirect method (Costa et al. 2021). Leukograms and thrombograms were obtained by blood extensions considering left shift when the proportion of band neutrophils exceeded 3% of the total leukocytes (Claudiano et al. 2019).

For the biochemical analyses, the determination of total proteins, albumin, magnesium and alanine aminotransferase (ALT) was performed in a semi-automatic analyzer, with the calculation of globulin and the albumin/globulin ratio (Claudiano et al. 2019), and glycemia according to Castro et al. (2014). For plasma ions (Ca^{2+}, Na^{+}, K^{+} and Mg^{2+}), the samples were diluted to 1:1000 (v/v) and quantified using flame spectrophotometry (Perkin-Elmer, AAS 1100B).

2.4.4 Histological analysis of the gills

After the baths with the crude extract or dillapiole, the gill structures of 36 fish from each experiment and nine from the basal sample were fixed in 5% buffered formalin for 24 h, thn transferred to 70% alcohol for 48 h. From each gill, the second gill arch was removed and embedded in paraffin (Sigma-Aldrich, Paraplast Plus) in the sagittal position, and serial cuts of 2–5 µm were obtained with the aid of a rotary microtome (Leica, RM 2245). To describe the histopathologies and obtain the histological alteration indices, three histochemical protocols were used: 1) eosin-hematoxylin 2) alcian blue and 3) Schiff's periodic acid. With the results, a descriptive and qualitative analysis was performed to classify the histopathological alterations in the gills of the animals: the mean alteration value (MAV), according to Schwaiger et al. (1997) and the histological alteration index (HAI), according to Poleksić and Mitrović-Tutundžić (1994).

2.5 Statistical analysis

The results of the parasitological, immunophysiological, histological and water quality analyses were submitted to statistical analysis. Results that presented a normal distribution (Shapiro-Wilk test) and homogeneity between deviations (Levene's test) were submitted to analysis of variance (ANOVA), followed by Tukey's test at 5% significance. Those that did not meet the parametric assumptions were submitted to the Kruskal-Wallis test, using the statistical program BioEstat 5.0.

3. RESULTS

3.1 In vitro anti-monogenoid activity of P. aduncum

The results of the phytochemical screening demonstrated that there was no mortality of monogenoids in the control groups (water and solvents) during the tests, nor any visible deterioration of the gill tissue of the fish exposed to the products.

In the experiment with the crude ethanolic extract of *P. aduncum*, there was a dose-and time-dependent effect on the antiparasitic efficacy. For the ethanolic extract, at the lowest concentration tested (40 $mg{\cdot}L^{-1}$), the efficacy gradually increased, reaching about 70% at the end of 5 h of exposure (Fig. 1a). At concentrations of 60 and 80 $mg{\cdot}L^{-1}$, efficacy was greater than 70% after 3 h, reaching values close to 100% with 5 h of exposure. The highest concentration evaluated (100 $mg{\cdot}L^{-1}$), with 4 h of exposure, showed an efficacy of 100%, evidencing faster action compared to the other doses (Fig. 1a). In the test with dillapiole alone, mortality was greater than 90% in the first 2 h at concentrations of 60 and 90 $mg{\cdot}L^{-1}$, reaching 100% after 3 h of exposure. At 30 $mg{\cdot}L^{-1}$, there was intermediate efficacy, with approximately 60% between 4 and 5 h of exposure (Fig. 1b). The lowest concentration (15 $mg{\cdot}L^{-1}$) showed low initial efficacy, with a progressive increase up to about 30% at the end of the experimental period (Fig. 1b).

The number of parasites adhered to the gill arches varied significantly between treatments ($p < 0.05$), with a significant reduction for the highest concentrations of both products (Fig. 1c and 1d). In the samples exposed to the crude ethanolic extract (Fig. 1c), the concentrations 40 and 60 $mg{\cdot}L^{-1}$ significantly reduced the mean monogenoid count when compared to the controls (water and methanol), with a decrease that was proportional to the increase in dose. For dillapiole, the concentrations 28 and 33 $mg{\cdot}L^{-1}$ also promoted a significant reduction in the number of parasites adhering to the gill arches, confirming the efficacy of the compound, even at intermediate doses (Fig. 1d).

The detailed count of the behavioral alterations caused by the crude ethanolic extract and dillapiole are represented in Tables 1 and 2, respectively. For the extract, a dose- and time-dependent effect is observed, with intense lethargy occurring in the first 30 min at concentrations of 80 and 100 $mg{\cdot}L^{-1}$, with mortality greater than 80% after 3 h and 100% at the end of 4 h of exposure. At 60 $mg{\cdot}L^{-1}$, some of the parasites remained alive until the third hour and, after 5 h, there was almost total death or detachment from the tissue. At 40 $mg{\cdot}L^{-1}$, the effect was more gradual, with a progressive increase in lethargy and partial mortality, with a significant number of parasites still alive at the end of the assay (Table 1).

Table 1

Number of gill monogenoids in *Colossoma macropomum* classified as alive, dead or exhibiting behavioral alterations during the *in vitro* test with the crude extract of *Piper aduncum* and different exposure periods.

Period	Crude extract	Alive	Behavioral alterations		Dead
	$mg.L^{-1}$		Lethargic	Detached	
0	0 (Water)	37	0	0	0
	0 (Methanol)	54	0	0	0
	40	70	0	0	0
	60	34	0	0	0
	80	37	0	0	0
	100	35	0	0	0
30 min	0 (Water)	37	0	0	0
	0 (Methanol)	54	0	0	0
	40	70	25	0	0
	60	34	10	0	0
	80	37	15	0	0
	100	33	20	0	2
1 h	0 (Water)	37	0	0	0
	0 (Methanol)	54	0	0	0
	40	70	25	0	0
	60	22	10	0	12
	80	25	15	11	12
	100	20	20	10	15
2 h	0 (Water)	37	0	0	0
	0 (Methanol)	54	0	0	0
	40	70	25	0	0
	60	22	13	9	12
	80	20	10	11	17
	100	20	20	10	15
3 h	0 (Water)	37	0	0	0
	0 (Methanol)	54	0	0	0
	40	60	40	10	10
	60	11	13	9	23
	80	8	3	20	29
	100	4	4	15	31
4 h	0 (Water)	37	0	0	0
	0 (Methanol)	54	0	0	0

Values are expressed as absolute counts per observation interval. Controls: water and Tween-20 (0.0004%).

	40	40	40	30	30
	60	11	0	20	23
	80	0	0	20	37
	100	0	0	0	35
5 h	0 (Water)	37	0	0	0
	0 (Methanol)	54	0	0	0
	40	40	20	50	30
	60	11	0	20	23
	80	0	0	20	37
	100	0	0	0	35

Values are expressed as absolute counts per observation interval. Controls: water and Tween-20 (0.0004%).

Table 2
Number of gill monogenoids in *Colossoma macropomum* classified as alive, dead or exhibiting behavioral alterations during the *in vitro* exposure to dillapiole isolated from *Piper aduncum* and different time intervals.

Period	Dillapiole (mg/L)	Alive	Behavioral alterations		Dead
			Lethargic	Detached	
0	0 (Water)	46	0	0	0
	0 (Water + Tween)	50	0	0	0
	15	65	0	0	0
	30	48	0	0	0
	60	40	0	0	0
	90	36	0	0	0
30 min	0 (Water)	46	0	0	0
	0 (Water + Tween)	50	0	0	0
	15	65	0	0	0
	30	48	0	0	0
	60	40	0	0	0
	90	4	4	0	32
1 h	0 (Water)	46	0	0	0
	0 (Water + Tween)	50	0	0	0
	15	65	0	0	0
	30	46	0	0	2
	60	35	20	5	5
	90	0	0	0	36
2 h	0 (Water)	46	0	0	0
	0 (Water + Tween)	50	0	0	0
	15	65	0	0	0
	30	46	20	5	2
	60	20	15	15	20
	90	0	0	0	36
3 h	0 (Water)	46	0	0	0
	0 (Water + Tween)	50	0	0	0
	15	65	0	0	0
	30	20	20	10	28
	60	0	0	0	40
	90	0	0	0	36
4 h	0 (Water)	46	0	0	0
	0 (Water + Tween)	50	0	0	0

Controls: water and water + Tween-20 (0.0004% v/v).

	15	65	0	0	0
	30	20	30	20	28
	60	0	0	0	40
	90	0	0	0	36
5 h	0 (Water)	46	0	0	0
	0 (Water + Tween)	50	0	0	0
	15	60	30	30	5
	30	20	20	20	28
	60	0	0	0	40
	90	0	0	0	36
Controls: water and water + Tween-20 (0.0004% v/v).					

In the test with dillapiole (Table 2), there was a faster and more intense action on the parasite when compared to the extract. At 90 $mg{\cdot}L^{-1}$, 89% of parasites were dead immediately after 30 min and 100% died at the end of 1 h of exposure. At 60 and 90 $mg{\cdot}L^{-1}$, severe lethargy was observed in the first hour and total mortality up to 3 h. At intermediate concentrations (15 and 30 $mg{\cdot}L^{-1}$), the effect was more limited and progressive, with part of the parasites becoming lethargic or detached and others still alive at the end of 5 h of exposure (Table 2). As shown in Tables 1 and 2, the remaining specimens showed reduced movement and lethargy from the first evaluation intervals.

3.2 *In vivo effect of dillapiole and the crude extract of P. aduncum in C. macropomum*

In the *in vivo* tests, three monogenoid species parasitizing *C. macropomum* were identified: *Anacanthorus spathulatus*, *Notozothecium janauachensi* and *Mymarothecium boegeri* (Table 3). In almost all the treatments with the crude ethanolic extract, *A. spathulatus* presented 100% prevalence, except in 60 $mg{\cdot}L^{-1}$ which showed a significant reduction in prevalence (66.6%) and lower mean abundance when compared to the controls ($p < 0.05$). The species *N. janauachensi* and *M. boegeri* occurred less frequently and did not present statistically significant differences in abundance between treatments ($p > 0.05$; Table 3).

Table 3. Prevalence and mean abundance of monogenean species (*Anacanthorus spathulatus*, *Notozothecium janauachensi* and *Mymarothecium boegeri*) recovered from the gill arches of *Colossoma macropomum* after *in vivo* therapeutic baths with the crude extract of and dillapiole from *Piper aduncum*.

Parasitic species	Crude extract								
	Control				Concentrations (mg.L^{-1})				"p"[1]
	Water		Methanol		40		60		
	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA	
A. spathulatus	100	36.55±7.5^{b}	100	34.11±7.5^{b}	100	22.22±6.4^{b}	66.6	3.55±2.49^{a}	*
M. boegeri	66.66	1.77±1.35	55.55	1.00±0.88	55.55	0.77±0.86	33.3	0.22±0.34	NS
N. janauachensis	77.00	1.22±0.74	66.66	1.00±0.66	55.55	0.55±0.61	33.3	0.33±0.44	NS
Parasitic species	Dillapiole								
	Control				Concentrations (mg.L^{-1})				"p"[1]
	Water		Tween-20		28		33		
	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA	
A. spathulatus	100	43.33±5.18^{c}	100	43.88±5.9^{bc}	100	22.22±3.58^{b}	100	13.88±4.32^{a}	***
M. boegeri	88.9	1.88±0.61^{b}	100	1.77±0.51^{b}	100	1.55±0.49^{ab}	55.6	0.44±0.49^{a}	*
N. janauachensis	77.8	1.44±1.037	100	1.77±0.69	100	1.22±0.30	77.8	0.88±0.39	NS

[1]Different letters on the same rows indicate statistically significant differences between treatments according to Tukey's test. * indicates $p < 0.05$; *** indicates $p < 0.01$; NS indicates no significant differences. P = prevalence; MA = mean abundance; N = 9 fish per treatment. Values are presented as mean ± standard deviation.

In the baths with dillapiole, the prevalence of *A. spathulatus* was 100% in all the treatments, while the mean abundance progressively decreased with increasing concentrations of the product (Table 3). A statistically significant difference ($p < 0.05$) between the controls and the 33 mg·L^{-1} treatment was also observed in this parasite species. For *M. boegeri*, prevalence and abundance were also reduced in the test with the highest concentration of dillapiole, presenting significant differences in relation to controls ($p < 0.05$. Table 3)).

The behavioral analysis indicated that the fish of the control treatments (water, methanol 0.1% and Tween-20 0.0004%) showed normal swimming and respiratory rate during the baths. In contrast, fish exposed to the highest concentration of the extract (60 mg·L^{-1}) and the highest concentration of dillapiole (33 mg·L^{-1}) showed behavioral alterations, including an increase in the frequency of opercular beats and a reduction in swimming activity. After the seven-day recovery period, no mortality was observed. The fish returned to normal feeding after the second day of recovery, presenting coloration and integrity of the fins similar to the basal group. However, individuals in the control groups exhibited clinical signs such as erosions on the fins and whitish spots.

In Table 4, the hemato-biochemical results of the fish exposed to the crude extract at 60 mg·L^{-1} demonstrated hyperglycemia ($p < 0.05$) in relation to the other treatments and to the basal group, as well as significant decreases in total protein and plasma calcium levels at the highest concentrations ($p < 0.05$). Cortisol levels were significantly higher in fish treated with the extract, especially at a dose of 60 mg·L^{-1}, presenting statistical differences when compared to the controls and the basal group (Table 4). The mean values of thrombocytes and leukocytes were higher in the water control in comparison to the other treatments, except the basal one; there was eosinophilia in all the groups, which was not observed in the basal group, without differences for lymphocytes and monocytes ($p < 0.05$; Table 4).

Table 4
Blood parameters of *Colossoma macropomum* submitted to two four-hour baths with the crude extract of *Piper aduncum*.

Blood variables	Controls			Crude extract ($mg.L^{-1}$)		"p-value"[1]
	Basal	Water	Methanol	40	60	
Hematological parameters						
Hematocrit (%)	23.68 ± 1.85	24.5 ± 1.33	24.11 ± 1.01	22.72 ± 1.25	21.44 ± 0.5	NS
Hemoglobin (g/dL^{-1})	5.59 ± 0.60	6.72 ± 0.59	6.30 ± 0.53	6.13 ± 0.53	5.68 ± 0.29	NS
RBC ($x10^6.\mu L^{-1}$)	1.50 ± 0.22	1.47 ± 0.20	1.50 ± 0.28	1.52 ± 0.12	1.47 ± 0.20	NS
MCV(fL)	161.36 ± 19.1	169.69 ± 17.24	166.19 ± 25.93	150.11 ± 15.30	148.75 ± 20.17	NS
MCHC ($g.dL^{-1}$)	23.78 ± 2.63	27.62 ± 2.89	26.10 ± 1.60	27.01 ± 2.02	26.55 ± 1.54	NS
MCH (pg)	37.92 ± 4.79	46.62 ± 5.5	43.54 ± 7.67	40.84 ± 6.96	39.22 ± 4.50	NS
Biochemical parameters						
Glucose ($mg.dL^{-1}$)	89.44 ± 11.64^{b}	55.40 ± 5.66^{d}	64.25 ± 5.9^{cd}	71.75 ± 6.67^{bc}	121.69 ± 26.39^{a}	***
TP ($g.dL^{-1}$)	2.54 ± 0.44^{a}	2.23 ± 0.20^{ab}	2.32 ± 0.14^{a}	2.23 ± 0.11^{ab}	1.88 ± 0.15^{b}	*
Ca^{2+} (mg/L)	$1,215.55 \pm 21.72^{a}$	997.77 ± 124.69^{b}	862.22 ± 81.97^{b}	728.88 ± 72.34^{b}	550 ± 62.22^{c}	***
Mg^{2+} (mg/L)	86.66 ± 8.14	88.88 ± 10.37	84.44 ± 7.16	78.88 ± 5.92	80 ± 6.66	NS
Na^{+} (mg/L)	$4,012.47 \pm 408.13^{a}$	$3,618.915 \pm 204.06^{ab}$	$3,662.644 \pm 369.26^{ab}$	$3,400.27 \pm 310.96^{b}$	$3,986.84 \pm 430.92^{b}$	*
K^{+} (mg/L)	376.00 ± 102	423.33 ± 177.7	447.77 ± 118.51	232.22 ± 98.51	267.77 ± 131.35	NS
Cortisol (µdL)	67.53 ± 18.05^{ab}	47.63 ± 17.04^{ab}	42.93 ± 7.17^{b}	68.46 ± 6.99^{a}	69.24 ± 5.3^{a}	*
Innate immunity cell count						
Thrombocytes ($x10^3.\mu L^{-1}$)	93.00 ± 20.20^{ab}	155.66 ± 24.86^{a}	72.00 ± 11.6^{b}	66.55 ± 10.44^{b}	90.88 ± 30.06^{b}	*
Leukocytes ($x10^3.\mu L^{-1}$)	5.47 ± 2.73^{b}	10.59 ± 1.84^{a}	8.86 ± 1.62^{ab}	7.89 ± 1.84^{b}	8.52 ± 2.09^{ab}	*
Lymphocytes ($x10^3.\mu L^{-1}$)	1.76 ± 0.90^{ab}	2.79 ± 0.81^{a}	2.47 ± 0.76^{ab}	1.48 ± 0.56^{b}	1.82 ± 0.55^{ab}	*
Monocytes ($x10^3.\mu L^{-1}$)	0.97 ± 0.42^{ab}	1.30 ± 0.30^{ab}	1.52 ± 0.54^{a}	0.92 ± 0.22^{b}	1.23 ± 0.63^{ab}	*
Neutrophils ($x10^3.\mu L^{-1}$)	1.80 ± 0.96^{b}	3.43 ± 0.96^{a}	2.72 ± 0.74^{ab}	2.90 ± 1.02^{ab}	3.06 ± 1.29^{a}	*

[1]Different letters between the lines indicate statistical differences between treatments on Tukey's test (N = 9): * represents $p < 0.05$; *** represents $p < 0.01$; NS represents no statistical differences. Basal = acclimation. RBC = red blood cell count. MCV = mean corpuscular volume. MCHC = mean corpuscular hemoglobin concentration. MCH = mean corpuscular hemoglobin. TP = total proteins; Ca^{2} + = calcium, Mg^{2+}= magnesium, Na^{+}= sodium, K^{+}= potassium, SGC = special granulocytic cells. Means ± standard deviation.

Blood variables	Controls			Crude extract ($mg.L^{-1}$)		"p-value"[1]
	Basal	Water	Methanol	40	60	
Eosinophils ($x10^3.\mu L^{-1}$)	0.81 ± 0.59[b]	2.92 ± 0.70[a]	2.09 ± 0.59[a]	2.55 ± 0.57[a]	2.33 ± 1.64[a]	***
SGC ($x10^3.\mu L^{-1}$)	0.10 ± 0.08	0.08 ± 0.05	0.04 ± 0.03	0.03 ± 0.45	0.07 ± 0.05	NS

[1]Different letters between the lines indicate statistical differences between treatments on Tukey's test (N = 9): * represents $p < 0.05$; *** represents $p < 0.01$; NS represents no statistical differences. Basal = acclimation. RBC = red blood cell count. MCV = mean corpuscular volume. MCHC = mean corpuscular hemoglobin concentration. MCH = mean corpuscular hemoglobin. TP = total proteins; Ca^{2+} = calcium, Mg^{2+}= magnesium, Na^{+}= sodium, K^{+}= potassium, SGC = special granulocytic cells. Means ± standard deviation.

In Table 5, two 4-h baths with dillapiole (33 $mg \cdot L^{-1}$) reduced hematocrit, hemoglobin and MCH in comparison with the control ($p < 0.05$). There was also a reduction in glycemia, a decrease in calcium and potassium and an increase in sodium ($p < 0.05$), as well as lower plasma cortisol in the treated groups when compared to the controls ($p < 0.05$). In the leukogram, there was an increase in thrombocytes, total leukocytes and lymphocytes at the two highest concentrations, as well as neutrophilia and eosinophilia in the treated groups, which was not observed in the controls ($p < 0.05$; Table 5).

Table 5
Blood parameters of *Colossoma macropomum* submitted to two four-hour baths with dillapiole.

Blood variables	Basal	Control		Dillapiole ($mg.L^{-1}$)		p-value[1]
		Water	Tween 20	28	33	
Hematological parameters						
Hematocrit (%)	23.68 ± 1.85^{b}	25.55 ± 2.38^{a}	23.94 ± 1.53^{ab}	21.72 ± 1.08^{bc}	20.22 ± 1.62^{c}	***
Hemoglobin (g/dL^{-1})	5.59 ± 0.60^{ab}	6.85 ± 0.97^{a}	6.17 ± 0.87^{ab}	5.59 ± 0.85^{ab}	4.95 ± 0.54^{b}	*
RBC ($x10^{6}.\mu L^{-1}$)	1.50 ± 0.22	1.51 ± 0.14	1.71 ± 0.21	1.60 ± 0.17	1.58 ± 0.14	NS
MCV(fL)	161.36 ± 19.11	170.71 ± 16.83	142.70 ± 15.98	137.87 ± 13.99	129.19 ± 15.70	NS
MCHC ($g.dL^{-1}$)	23.78 ± 2.63	24.83 ± 2.99	25.11 ± 4.28	25.82 ± 4.36	24.54 ± 2.37	NS
MCH (pg)	37.92 ± 4.79^{ab}	42.31 ± 6.62^{a}	38.36 ± 6.84^{ab}	35.53 ± 6.39^{ab}	31.76 ± 5.12^{b}	*
Biochemical parameters						
Glucose ($mg.dL^{-1}$)	89.44 ± 11.64^{a}	49.58 ± 8.57^{c}	50.23 ± 10.87^{c}	66.36 ± 13.20^{bc}	81.70 ± 14.04^{bc}	*
TP ($g.dL^{-1}$)	2.54 ± 0.44	2.35 ± 0.16	2.36 ± 0.14	2.50 ± 0.19	2.51 ± 0.16	NS
Ca^{2+} (mg/L)	$1,215.55 \pm 21.72^{a}$	$1,215.55 \pm 69.38^{a}$	$1,166.66 \pm 97.77^{ab}$	$1,013.33 \pm 53.33^{b}$	1045.55 ± 151.85^{b}	*
Mg^{2+} (mg/L)	86.66 ± 8.14	91.11 ± 8.41	91.11 ± 15.06	92.22 ± 10.86	83.33 ± 17.77	NS
Na^{+} (mg/L)	$4,012.47 \pm 408.13^{ab}$	$3,658.177 \pm 263.20^{b}$	$3,561.662 \pm 353.20^{b}$	$3,984.90 \pm 330.8^{ab}$	$4,198.169 \pm 108.15^{a}$	*
K^{+} (mg/L)	376.66 ± 102.22^{ab}	554.44 ± 128.39^{a}	487.77 ± 183.70^{a}	236.66 ± 77.77^{b}	186.66 ± 106.66^{b}	*
Cortisol (µdL)	67.53 ± 18.05^{a}	59.54 ± 4.26^{b}	55.93 ± 10.30^{b}	67.22 ± 3.73^{a}	46.14 ± 14.47^{b}	*
Innate immunity cell count						
Thrombocytes ($x10^{3}.\mu L^{-1}$)	89.66 ± 25.40^{c}	195.00 ± 47.33^{ab}	161.55 ± 25.62^{bc}	178.66 ± 59.40^{ab}	259 ± 58.22^{a}	*
Leukocytes ($x10^{3}.\mu L^{-1}$)	5.47 ± 2.73^{c}	13.68 ± 2.48^{a}	$11.55 \pm 1.1.70^{bc}$	15.81 ± 2.28^{a}	14.361 ± 2.044^{ab}	***
Lymphocytes ($x10^{3}.\mu L^{-1}$)	1.76 ± 0.70^{b}	3.00 ± 0.79^{ab}	2.33 ± 0.60^{b}	3.57 ± 0.96^{a}	3.17 ± 0.91^{a}	*
Monocytes ($x10^{3}.\mu L^{-1}$)	0.97 ± 0.42^{b}	1.39 ± 0.28^{ab}	2.07 ± 0.54^{a}	2.72 ± 0.71^{a}	1.49 ± 0.43^{ab}	*
Neutrophils ($x10^{3}.\mu L^{-1}$)	1.80 ± 0.96^{b}	5.28 ± 1.27^{a}	3.56 ± 1.01^{ab}	5.76 ± 0.76^{a}	7.09 ± 1.70^{a}	*

[1]Different letters between the lines indicate statistical differences between treatments on Tukey's test (N = 9): * represents $p < 0.05$; *** represents $p < 0.01$; NS represents no statistical differences. Basal = acclimation. RBC = red blood cell count. MCV = mean corpuscular volume. MCHC = mean corpuscular hemoglobin concentration. MCH = mean corpuscular hemoglobin. TP = total proteins; Ca^{2} + = calcium, Mg^{2+}= magnesium, Na^{+}= sodium, K^{+}= potassium, SGC = special granulocytic cells. Means ± standard deviation.

Blood variables	Basal	Control		Dillapiole (mg.L^{-1})		p-value[1]
		Water	Tween 20	28	33	
Eosinophils (x10³.µL^{-1})	0.81 ± 0.29^{c}	3.90 ± 0.99^{a}	3.26 ± 1.08^{ab}	3.68 ± 0.94^{a}	2.57 ± 0.77^{b}	***
SGC (x10³.µL^{-1})	0.005 ± 0.006	0.006 ± 0.005	0.013 ± 0.009	0.006 ± 0.007	0.002 ± 0.004	NS

[1]Different letters between the lines indicate statistical differences between treatments on Tukey's test (N = 9): * represents $p < 0.05$; *** represents $p < 0.01$; NS represents no statistical differences. Basal = acclimation. RBC = red blood cell count. MCV = mean corpuscular volume. MCHC = mean corpuscular hemoglobin concentration. MCH = mean corpuscular hemoglobin. TP = total proteins; Ca^{2} + = calcium, Mg^{2+}= magnesium, Na^{+}= sodium, K^{+}= potassium, SGC = special granulocytic cells. Means ± standard deviation.

The histopathological alterations index (HAI) showed significant differences ($p < 0.05$) between the treatments with 40 and 60 mg·L^{-1} of the extract and the control with methanol (Fig. 2a). In the dillapiole experiments, the HAI was higher in the control groups, but did not differ statistically in relation to the treatments and the basal group (Fig. 2b).

Quantitative evaluation of histological parameters indicated variations in the index/mean of the lesions between the crude extract and dillapiole treatments (Fig. 2c–d). In the crude extract, the mean values ranged from 1.33 to 1.96, with lower means in the treated groups (40–60 mg·L^{-1}) compared to the controls with water/methanol; there was no dose-dependent effect nor statistically significant differences with the controls ($p > 0.05$; Fig. 2c). Similarly, the mean values of the histological alterations in the gills of fish treated with dillapiole remained similar between the concentrations tested and did not show significant differences when compared to basal group (Fig. 2d).

Histopathological evaluation of *C. macropomum* gills exposed to the ethanolic extract revealed the classification of lesions with different frequency patterns between the treatments (Fig. 3). In the basal group, it was possible to observe branchial lamellae with preserved morphology, without evident alterations (Fig. 3a), or presenting mild and discrete alterations, such as small areas of hypertrophy and focal hyperplasia (Fig. 3b). In the controls with water and methanol, the frequency and intensity of lesions increased, with characteristic alterations, including hyperplasia of the lamellar epithelium with partial fusion of secondary lamellae (Fig. 3c and 3d), abundant presence of monogenoids adhered to the branchial surface (Fig. 3c–d), dilatation of the marginal canal (Fig. 3e) and a marked proliferation of mucosal cells, evidencing the impact of the parasitic infestation. At concentrations of 40 and 60 mg·L^{-1} of crude extract, there was a reduction in the frequency and severity of these alterations, with decreased hyperplasia and better lamellar organization compared to the controls (Fig. 3f–h). Some samples showed areas with reduced epithelial thickness and a slight presence of aneurysmal lamellae (Fig. 3h) and a smaller number of mucous cells and few residual parasites adhered to the arches (Fig. 3i). In general, the presence of parasites was high in the controls, while in the treatments with 40 and 60 mg·L^{-1} of the extract and in the basal group the occurrence was low or sporadic (Fig. 3a, 3b, 3g–i).

In the histopathological evaluation of the fish of the dillapiole assay, the fish of the control groups (water and Tween-20) showed a higher frequency of epithelial hyperplasia and hypertrophy, associated with partial fusion of the secondary lamellae and an abundant presence of parasites adhered to the gill filaments (Fig. 4c–d). These alterations also included dilatation of the marginal canal and intense proliferation of mucous cells in the controls. The therapeutic baths reduced the frequency and intensity of these lesions, especially in the bath with 33 mg·L^{-1}, which showed more preserved lamellar architecture and fewer mucocytes (Fig. 4f). At the concentration of 28 mg·L^{-1}, the pattern was intermediate, there were still areas of hyperplasia, but these were less extensive than in the controls (Fig. 4e). The presence of parasites was high in the control groups and practically absent in the fish treated with the highest concentration of dillapiole. In the basal group, gills with preserved morphology or mild and focal alterations were observed (Fig. 4a-b).

4. DISCUSSION

The results of the present study unequivocally demonstrate that the crude ethanolic extract of *P. aduncum* and its major compound, dillapiole, have a highly effective anti-monogenoid activity, evidenced by the dose- and time-dependent action in the *in vitro* assays and by the substantial reduction of the parasitic load in the *in vivo* assays. These findings, gathered in a pioneering way, provide consistent data on the potential of these substances as herbal alternatives in the control of branchial parasites in *C. macropomum*.

Evaluating the toxic effects of drugs on animals, as well as testing their effect directly on parasites, *in vitro*, are fundamental steps that allow us to determine the therapeutic concentrations to be used, as well as enable us to understand their mechanism of action on parasites when applied *in vivo* (Affonso et al. 2022; de Queiroz et al. 2022). The *in vitro* tests showed that both the crude extract and dillapiole showed a progressive lethality that was proportional to the increase in concentration and exposure time. Specifically, the extract demonstrated 100% efficacy at a concentration of 100 $mg \cdot L^{-1}$ after 4 h, and dillapiole, in turn, reached the same level of efficacy in the first hour at a concentration of 90 $mg \cdot L^{-1}$, confirming the faster and more intense action of the isolated compound. These results corroborate previous observations by de Queiroz et al. (2022), who also identified the dose-dependent action of *P. aduncum* extract against monogenoids in *A. gigas* and *C. macropomum*, although previous studies have not directly compared the extract to the dillapiole isolate under controlled laboratory conditions.

The comparison between the two products suggests that dillapiole plays a central role in anthelmintic action, a hypothesis supported by the phytochemical analyses that indicate its predominance (up to 98%) in the essential oil (Almeida et al. 2009; de Queiroz et al. 2022). However, it cannot be ruled out that other secondary metabolites present in the crude extract, including chromenes, flavonoids, alkaloids and monoterpenes (Efdi et al. 2023), exert synergistic or potentiating effects on the parasites, increasing the biological activity of the product, as already proposed by Corral et al. (2018) and Andrade et al. (2016).

The mechanism of action of dillapiole on monogenoids remains unclear. However, studies into phenylpropanoids demonstrate that these molecules have the ability to interact with the lipids of cell and mitochondrial membranes, compromising the integrity of the lipid bilayer, increasing ion permeability and leading to cell death (Carson et al. 2002). In addition, dillapiole is recognized as a potent inhibitor of cytochrome P450 complex enzymes (Omura and Sato, 1964; Mukerjee et al. 1979), and interferes with oxidative and biotransformative processes of the organisms. This metabolic inhibition may have contributed to the lethal efficacy on the parasites, which was observed even at low concentrations and during reduced exposure times, a desirable characteristic for the rapid control of infestations in an intensive farming environment.

The relevance of these findings becomes even more evident when one considers that alternatives for monogenoid control have limitations that are associated with environmental toxicity, residue formation and potential induction of resistance (Guardone et al. 2022). In Brazilian aquaculture, chemotherapeutic treatments, such as formalin and albendazole, have legal restrictions and prolonged withdrawal periods (Cordeiro et al. 2021). In this context, plant products, such as dillapiole and the crude extract of *P. aduncum*, represent a strategic alternative for safer and more effective disease management and are aligned with the principles of sustainability.

In the *in vivo* test, the applications of therapeutic baths in successive doses resulted in a significant reduction in the prevalence and mean abundance of *A. spathulatus*, the main parasite species identified in the fish studied, in addition to lower occurrences of *N. janauachensi* and *M. boegeri*. This pattern confirms that chronic infestation in *C. macropomum* can be efficiently controlled with 4-h bathing protocols at moderate concentrations, a strategy already proposed by Andrade et al. (2016) for plant extracts. The positive effects on the parasitic load were accompanied by the recovery of feeding behavior and integrity of the fins, especially noticeable in the groups treated with dillapiole at 33 $mg \cdot L^{-1}$ and with the crude extract at 60 $mg \cdot L^{-1}$, demonstrating a positive effect on animal welfare.

The hematological and biochemical analyses provided important information about the tolerance of fish to the treatments. While in the crude extract hyperglycemia and increased cortisol levels occurred at higher concentrations, dillapiole showed lower glycemic elevation and a more controlled response to stress (Affonso et al. 2009). This finding may be associated with the anesthetic effect described for concentrated dillapiole, which reduces oxygen consumption, basal metabolism and, consequently, the release of catecholamines in the face of stressful stimuli (Lemos et al. 2023). Furthermore, treatment with 33 $mg \cdot L^{-1}$ of dillapiole resulted in decreased erythrocyte parameters, including a drop in mean corpuscular hemoglobin and hematocrit, suggesting a picture of iron deficiency anemia, potentially resulting from interference in the absorption of iron and essential ions (Marshall 2002; Evans et al. 2005). The concomitant drop in calcium and potassium levels reinforces this hypothesis, since the active transport of these ions by the mitochondrial cells of the gills is fundamental for ionic balance and the physiological integrity of the fish (Perry et al. 2003; Baldisserotto 2025).

The leukocyte parameters revealed distinct modulations: in the fish exposed to baths with the crude extract. There was an improvement in response and, in both treatments (*P. aduncum* extract and dillapiole), there was an inflammatory response that is

characteristic of parasitosis, with the elevation of neutrophils and eosinophils – cells associated with defense against parasites and inflammatory processes (Claudiano et al. 2019). These findings suggest that both phytochemicals can exert immunomodulatory action, thus expanding their applicability as prophylactic agents (Mansoori et al. 2024).

The histological findings corroborate the effectiveness of the treatments. In the controls, the presence of hypertrophy, hyperplasia, lamellar fusion and necrosis, and the abundance of parasites are alterations that are compatible with the response to intense infestations (Vanhove et al. 2024). In fish treated with the crude extract and dillapiole, especially in the highest concentrations, the frequency and severity of the lesions were reduced, as well as the histopathological alterations index (HAI) when compared to the controls. This confirms that the reduction in the parasitic load is associated with tissue preservation and lower respiratory impairment, unlike what was observed in treatments with synthetic agents such as glyphosate, which, although effective in eliminating parasites, caused extensive lesions in the gills.

As a result, this work advances previous studies by demonstrating that the use of dillapiole and the crude extract of *P. aduncum* not only promotes the effective removal of monogenoids, but also favors homeostasis and animal welfare. This is evidenced by: (i) an organized inflammatory response that is typical of parasitoses (neutrophilia/eosinophilia), (ii) a serum biochemical profile maintained within physiological ranges, (iii) a hormonal modulation (cortisol) that is compatible with the maintenance of homeostasis, (iv) branchial histopathological findings with reduced scores/no relevant differences when compared to the controls and (v) adequate behavior during and after baths, even after successive therapeutic cycles. Together, this confirms that the extract and dillapiole are viable and promising herbal alternatives, able to integrate biosecurity and disease control programs for *C. macropomum* in intensive systems, while also being aligned with the demand for sustainable aquaculture and the reduction in the use of conventional chemotherapeutics (Ali et al. 2021; Norbury et al. 2022; Freitas et al. 2023).

5. CONCLUSION

The results suggest that both products, dillapiole and the crude ethanolic extract of *P. aduncum*, have an effective and safe anthelmintic effect in the control of the monogenoids *A. spathulatus*, *N. janauachensis* and *M. boegeri* in juvenile *C. macropomum*, with up to 99% efficacy when using 60 $mg{\cdot}L^{-1}$ of the extract and 85% when using 33 $mg{\cdot}L^{-1}$ of dillapiole. In addition to the significant reduction of the parasitic load, the treatments preserved the physiological homeostasis of the fish, which showed behavioral recovery and integrity of the gill tissues after recovery. Taken together, the findings support the practical applicability of these herbal medicines as biosecurity components in intensive farming systems, with the potential to reduce the use of conventional chemotherapies.

Declarations

Data availability statement: No data are available for this study.

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Figures

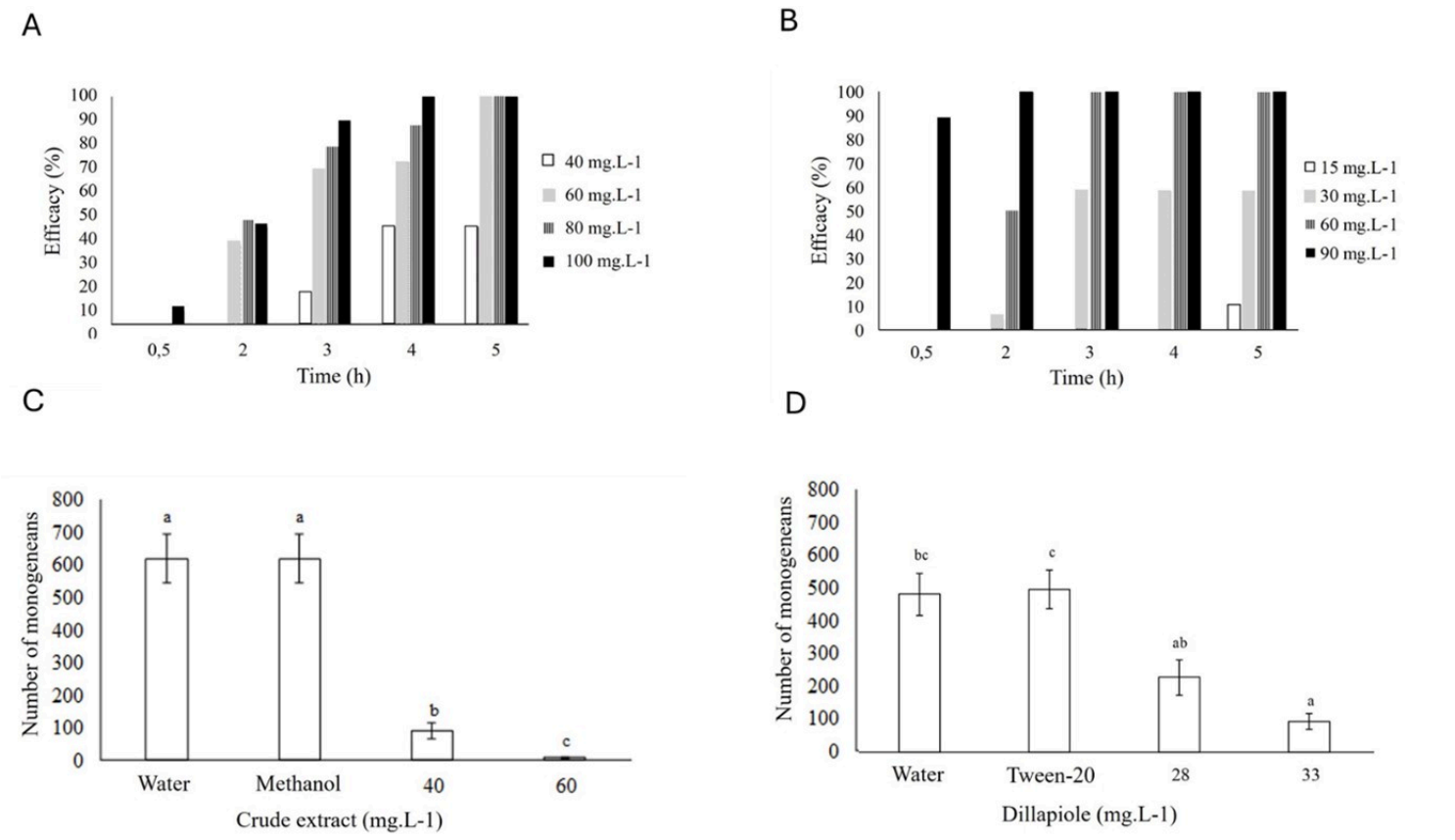

Figure 1

In vitro efficacy of *Piper aduncum*-based products against gill monogenoids of *Colossoma macropomum*. (A) Percentage of parasite mortality over time after exposure to the crude ethanolic extract at concentrations of 40, 60, 80, and 100 mg·L⁻¹ compared to controls (water and methanol 0.1%). (B) Percentage of parasite mortality over time after exposure to dillapiole at concentrations of 15, 30, 60, and 90 mg·L⁻¹ compared to controls (water and Tween-20 0.0004%). (C) Mean number of monogenoids adhered to gill arches after exposure to crude extract. (D) Mean number of monogenoids adhered to gill arches after exposure to dillapiole. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$). Values represent means ± standard error.

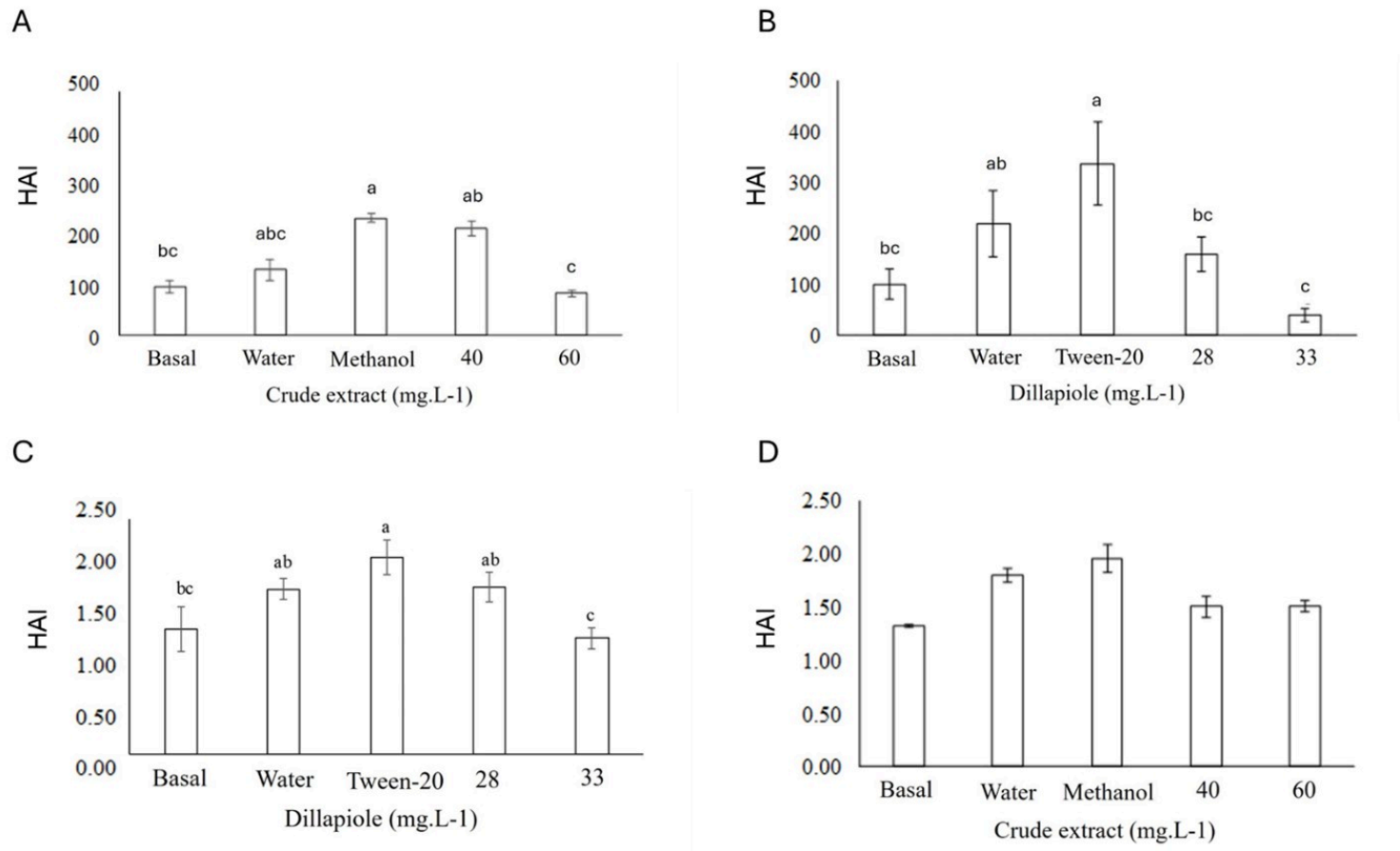

Figure 2

Mean histological alterations and histopathological alteration index (HAI) in the gills of *Colossoma macropomum* after *in vivo* baths with *Piper aduncum*-based treatments. (A) Histopathological alteration index in treatments with crude extract. (B) Histopathological alteration index in treatments with dillapiole. (C) Mean values of histological alterations in treatments with crude extract. (D) Mean values of histological alterations in treatments with dillapiole. Bars represent mean ± standard deviation. Different letters above the columns indicate statistically significant differences between treatments, according to ANOVA, followed by Tukey's test ($p < 0.05$). HAI — Histopathological alteration index.

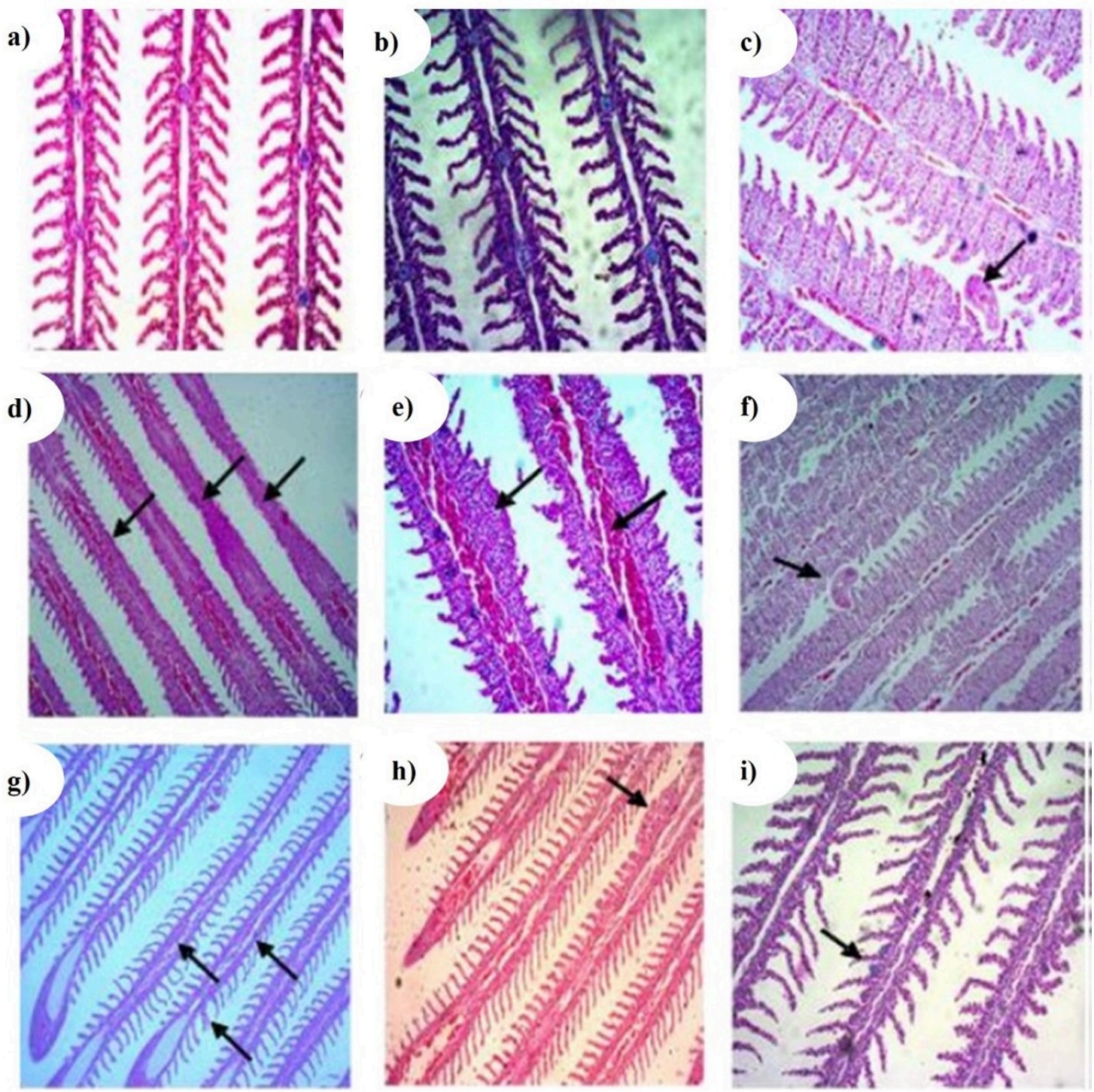

Figure 3

Histopathological alterations in the gills of *Colossoma macropomum* exposed to the ethanolic extract of *Piper aduncum*(paraffin sections, 20 µm thick, objective lens ×40).

a) Basal group showing preserved gill architecture without lesions.

b) Basal group with mild focal epithelial alterations.

c) Control (water): hyperplasia with partial fusion of secondary lamellae and presence of parasites.

d) Control (water): hyperplasia with lamellar fusion and abundant gill parasites.

e) Control (water): hyperplasia with lamellar fusion and dilation of the marginal channel.

f) Control (methanol): hyperplasia with lamellar fusion and parasite adhesion.

g) 40 mg·L⁻¹: reduction of hyperplasia.

h) 40 mg·L⁻¹: reduction of hyperplasia and presence of lamellar aneurysm.

i) 60 mg·L⁻¹: reduction of hyperplasia and increased mucous cell proliferation.

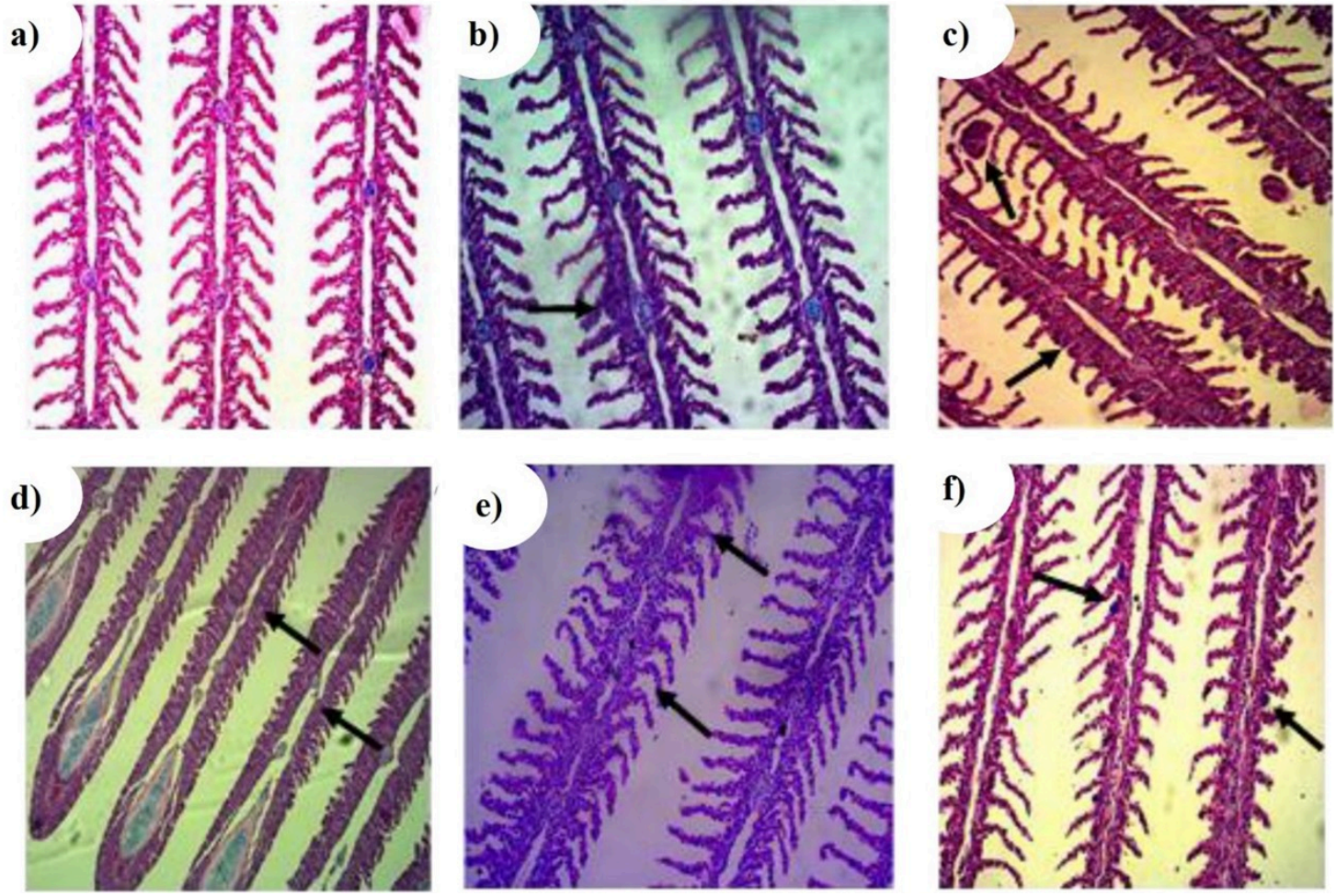

Figure 4

Histopathological alterations in the gills of *Colossoma macropomum* exposed to dillapiole (paraffin sections, 20 µm thick).

a) Basal group showing preserved gill architecture without lesions.

b) Basal group with mild epithelial alterations.

c) Control (water): hyperplasia with partial fusion of secondary lamellae and presence of parasites.

d) Control (Tween-20): hyperplasia with partial lamellar fusion and widening of the marginal channel.

e) 28 mg·L⁻¹: reduction of lamellar hyperplasia compared to controls.

f) 33 mg·L⁻¹: reduction of hyperplasia and increased mucous cell proliferation.