



Research Paper

Morphological changes in gills and gill rakers of *Prochilodus lineatus* treated with *Baccharis dracunculifolia* D.C. (Asteraceae)

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ABSTRACT

Jeffesson de Oliveira-Lima^{1*}, Bruno Fiorelini Pereira², João Rodolfo Tuckumantel Valim, Thiago Gazoni¹, Dimitrius Leonardo Pitol³ and Flavio Henrique Caetano¹

¹Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP Campus de Rio Claro, São Paulo, Brasil.

²Centro de Ciências Biológicas e da Saúde, Universidade Federal do Oeste da Bahia – UFOB Campus de Barreiras, Bahia, Brasil.

³Faculdade de Odontologia, Universidade de São Paulo – USP Campus de Ribeirão Preto, Ribeirão Preto, Brasil.

*Corresponding author. E-mail: jeffessonbiologia@mail.uft.edu.br. Tel: +55-193-526-4141.

The leaves of *Baccharis dracunculifolia* are used to treat gastric disorders and febrile conditions, besides their use in the treatment of wounds and inflammatory processes, among others. The objective of this study was to evaluate the toxic effects of the alcoholic extract of *B. dracunculifolia* by means of morphological analyzes of the gills and gill rakers of *Prochilodus lineatus* fish. Of the 60 subjects, 32 were used in two experimental groups in duplicates: Control group and treated group. After the treatment (21 days), 2 collections were done at 14 and 21 days. The histological and histochemical changes were evaluated by the Mean Value of Change (MVC), Histological Alteration Index (HAI) and ImageJ®. HAI and MVC indicated normal functioning, with changes localized in the organ. The results showed that the ingestion of the extract of *B. dracunculifolia* can affect the branchial epithelium and gill rakers, causing morphological alterations and an increase of mucous substances, as well as the number of lysosomes in the species studied.

Key words: Asteraceae; mucus; lysosomes; *P. lineatus*, gill.

INTRODUCTION

The Asteraceae family is the most numerous group within the Angiosperms, comprising about 1,100 genera and 25,000 species. About 98% of the genera are small plants and are found in several habitat types, but mainly in the tropical mountainous regions of South America (Joly, 1987).

Species of this genus are economically important to man as they help in the fight against erosion and can be used as ornamental plants. Although, they may also present as pests that are difficult to combat in pastures. However, they are majorly used in medicine, where several species are known (Simões-Pires et al., 2005).

B. dracunculifolia, native to Brazil, is considered an invasive pasture plant and has been eradicated from many regions. Its leaves have tectorial and glandular trichomes that, besides acting as a protective barrier to attack by predators, help in the interaction of this species with the bees that collect their resinous material (Sforcin et al., 2012). In addition, it has been shown to be the most

important botanical source for the production of green propolis (Park et al., 2002).

In medicine, the use of the branches and leaves of *B. dracunculifolia* to combat gastric disorders, febrile conditions and inflammatory processes has been reported (dos Santos et al., 2010). In its phytochemistry, there is an existence of flavonoid compounds, lipid diterpenes and clerodane (Verdi et al., 2005).

Recent studies have shown several biological activities, such as antiulcerogenic, immunomodulatory (Missima et al., 2007), cytotoxic, and anticariogenic effects (Leitão et al., 2004). Both green propolis and *B. dracunculifolia*, in the forms of ethanolic extract, were able to inhibit the oxidative metabolism of stimulated neutrophils, a concentration-dependent inhibition not related to toxic events (Simões-Ambrosio et al., 2010; Figueiredo-Rinhel et al., 2013).

However, it should be noted that species of the genus *Baccharis* have toxicity (Grance et al., 2008; Costa et al., 2008). Due to contact with water, it is possible that the

components of *B. dracunculifolia* present in the extract dissociate and affect the gills, since they have a large surface area and are in direct and permanent contact with agents diluted in water (Bernet et al., 1999). The aim of this study was to evaluate the toxic effects of the alcohol extract of *B. dracunculifolia*, by means of morphological analyzes of the gills and gill rakers of the fish (curimatá).

MATERIALS AND METHODS

Specimens

The *Prochilodus lineatus* juveniles used in this experiment (60.7 ± 1.3 g and 8.0 ± 1.5 cm) were purchased from Piscicultura Poletini, Mogi Mirim/SP, Brazil and transported to the Histology Laboratory of UNESP, Campus Rio Claro, Sao Paulo, Brazil. The animals were previously climatized in polyethylene boxes (500 L) with constant aeration and fed with appropriate commercial food (325 g of crude protein) once a day.

B. dracunculifolia leaves

The *B. dracunculifolia* leaves used in this experiment were collected in Rio Claro – SP, Brazil ($22^{\circ}22'30.0''S$ $47^{\circ}28'31.5''W$) and identified by Ms. Daniela de Oliveira, technician of “Herbário Rioclarense”. Exsiccates of the vegetal material was deposited and registered in the herbarium under number 58140. The nomenclature used herein is according to Joly (1987) and Dr. Marco Antonio Assis of the Botany Department of UNESP, campus Rio Claro.

Ethanol extract preparation

The leaf compound extraction followed the protocol established by ANVISA - (Brazilian Health Surveillance Agency) for the preparation of mother tinctures from dry plants through maceration (http://www.anvisa.gov.br/hotsite/farmacopeiabrasileira/arquivos/cp38_2010/x_metodos_preparacao_tintura.pdf). The leaves were macerated with grain alcohol, 30 and 70%, for nine days. The product of the 30% maceration was mixed to the 70% and vice-versa. After nine days, all the leaf compounds were obtained, those soluble in alcohol of 70 and 30%. For each treatment group, 1.5 mL of the extract was added to 1.2 g of commercial food (http://www.poytara.com.br/tropicais_diaadia.html). The material was kept in microbiological incubator at 37°C for alcohol evaporation and stored in amber jars.

Control and treatment groups

Sixty individuals were used, of which 32 were divided into two experimental groups in duplicate: Control group and

B. dracunculifolia treated group. The animals were randomly distributed into four 70-L tanks (8 animals each) with air pumps, cooling, thermostat (to maintain a constant temperature) and covered with UV blocking material to reduce stress. Both groups were fed for a maximum of 21 days: Control group with regular commercial food and *B. dracunculifolia* treated group with the food comprising *B. dracunculifolia* extract. The animals were collected 14 and 21 days after the experiment (21-day feeding period), 6 individuals per treatment were collected and anesthetized with benzocaine solution (0.1 g of benzocaine in 1 mL of ethanol for each 100 mL of deionized water) and euthanized according to the procedures established by the Ethics Committee on Animal Use (CEUA) – UNESP, Rio Claro, process 10/2017. The fish were kept in the semi-static system and the water physical and chemical parameters (ph, ammonia, hardness and temperature) were measured at each collection.

Histological processing

After treatment, fragments of the gills and gill rakers were fixed in 10% formalin. Subsequently, the material was buffered in sodium phosphate solution with pH = 7.4, dehydrated in increasing concentrations of alcohols, included in Leica historesin and sectioned in the Leica RM2245 microtome. The sections with 6 µm thickness were subjected to the specific reactions and consecutively produce the assembly of the slides.

Gill morphology analysis

Morphological changes were evaluated semi-quantitatively by the Calculation of the Mean Value of Change (VMC), based on the incidence of lesions, according to Schwaiger et al. (1997), in which a numerical value for each animal is assigned according to the scale: grade 1 (absence of histopathological alteration), grade 2 (occurrence of localized lesions) and grade 3 (lesions widely distributed by the organ) and Histological Alteration Index (HAI), based on the severity of each lesion. Stage I alterations (do not compromise the functioning of the organ), stage II (more severe and impair the normal functioning of the organ), and stage III, (very severe and irreversible) (Poleksic and Mitrovic-Tutundzic, 1994). For each animal, the AHI was calculated using the formula: $IAH = (1 \times SI) + (10 \times SII) + (100 \times SIII)$, where I, II and III equal the number of stages I, II and III changes, respectively.

The mean AHI was divided into 5 categories according to Poleksic and Mitrovic-Tutundzic (1994): 0-10 = normal tissue functioning; 11-20 = slight to moderate tissue damage; 21-50 = moderate to severe modification of tissue; 51-100 = severe tissue modification; greater than 100 irreparable tissue damages.

Table 1: Frequency of histological changes found in the gills of *P. lineatus*.

Gill	Stage	Control 14 days	Control 21 days	Treated 14 days	Treated 21 days
Lamellar hyperplasia	I	+	+	+	++
Epithelial detachment	I	0+	0+	+	+
Vascular congestion	I	0+	0+	+	+
Secondary lamellae fusion	I	0+	0+	+	+
Lamellae aneurysm	II	0+	0	0+	0+

0 = no change 0+ = rarely frequent + = frequent ++ = very frequent +++ = extremely frequent.

Quantification of mucous substances

For the analysis of the mucus substances, five fields of six cuts in each animal were analyzed. In each field were counted the markings of mucus substances between regions of 10 secondary lamellae. The cuts were submitted to Alcian Blue pH 1.0 + PAS reaction for the identification of mucus sulfated substances, hexoses, and sialic acids. Mucus neutral substances were evidenced by the periodic acid method Schiff (PAS) (Gustavo et al., 2014). The mucus substances in the gill rakers were quantified with the aid of ImageJ® software, with the plugins -Threshold Color.

Quantification of lysosomes

For this analysis, 6 cuts of 6 µm were obtained from each individual and subjected to the technique adapted from Gomori (1950). Of these six cuts, five fields were photographed and the lysosome markings present were counted with the aid of ImageJ®.

Statistical analyzes

The data obtained in the analyzes were subjected to Shapiro-Wilk test to verify the normality of the groups and later to the ANOVA / Tukey test, for parametric data and those that did not present Kruskal-Wallis / Dunn normality, considering the significance of $p < 0.05$. Statistical tests were performed with Bioestat 5.0® software and GraphPad Prism 5.0® software.

RESULTS

Gill morphology

The gills of the specimens presented the following changes: lamellar hyperplasia, epithelial detachment, vascular congestion, lamellar aneurysm and partial fusion of secondary lamellae. The frequency of histological changes in the gills is shown in Table 1.

The VMC and AHI obtained in the gill analyzed were not significantly higher than the control in the ANOVA / Tukey test (Figure 1).

Mucus

In the gills, the mucus sulfated substances, hexoses, and sialic acids did not present significant differences during treatment and a reduction was observed during 21 days of treatment. However, the mucus sulfate, hexoses, sialic and neutral acids presented significant elevation with $p < 0.05$ for the Kruskal-Wallis / Dunn test (Figures 2 and 3, and Table 2).

Lysosomes

The animals treated for 21 days showed a significant increase in the number of lysosomes as compared with the control group with $p < 0.05$ for the ANOVA / Tukey test (Table 2).

Morphology of gill rakers and mitochondrial rich cells

The gill rakers of *P. lineatus* fed with ration enriched with *B. dracunculifolia* extract showed blood cell infiltration (congestion) and epithelial decharacterization (Figure 4-B and C). Cells rich in mitochondria were not observed in using Von Kossa's technique, however, the presence of calcium in the light brown mucous cells along the epithelium was observed (Figure 4-E and F).

In the gill rakes, the mucus sulfate substances, hexoses and sialic acids presented significant differences with only 14 days of treatment. The mucus sulfated acidic substances, hexoses, sialic acids and neutrally presented significant differences for the ANOVA / Tukey test, during 14 and 21 days of treatment (Table 3) (Figure 5).

DISCUSSION

Histopathological analyzes are important for assessing organ sensitivity to xenobiotics. The degree of the lesions is directly related to their pathological potential, thus, the way the lesion affects the function of the organ and the survival capacity of the animal is taken into account when it comes to the importance of the lesions (Bernet et al., 1999). In this study, the occurrence of localized lesions such as lamellar hyperplasia, epithelial detachment, vascular congestion,

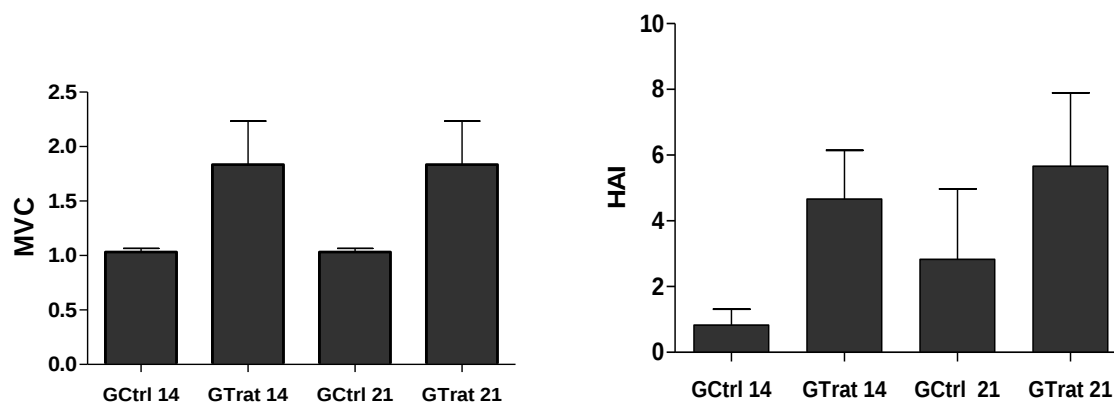


Figure 1: Mean values of VMC and AHI in the gills of *P. lineatus*. A significant difference was considered at $p < 0.05$. (*) significant difference in relation to the control group.

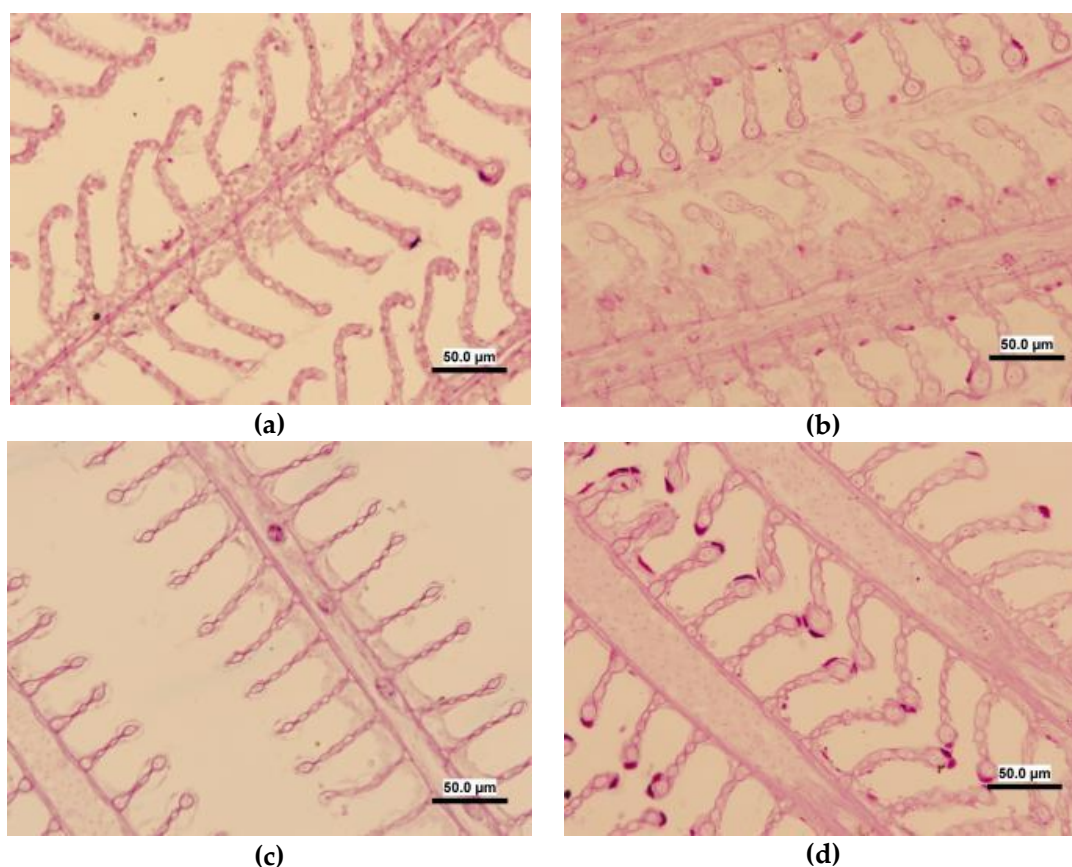


Figure 2: Changes in the gills of *P. lineatus*. (A) Control group 14 days. (B) Group treated 14 days. (C) Control group 21 days. (D) Group treated 21 days. PAS technique. Note the higher amounts of neutral polysaccharides in the group treated with *B. dracunculifolia*.

lamellar aneurysm and partial fusion of secondary lamellae (AHI) showed the normal functioning of the organ. With localized lesions, all of these changes are reversible characteristics.

Lamellar epithelial detachment, hyperplasia and partial or total fusion of the secondary lamellae, observed in the gills of *P. lineatus*, are alterations that decrease the surface area of the gills and increase the diffusion barrier to the

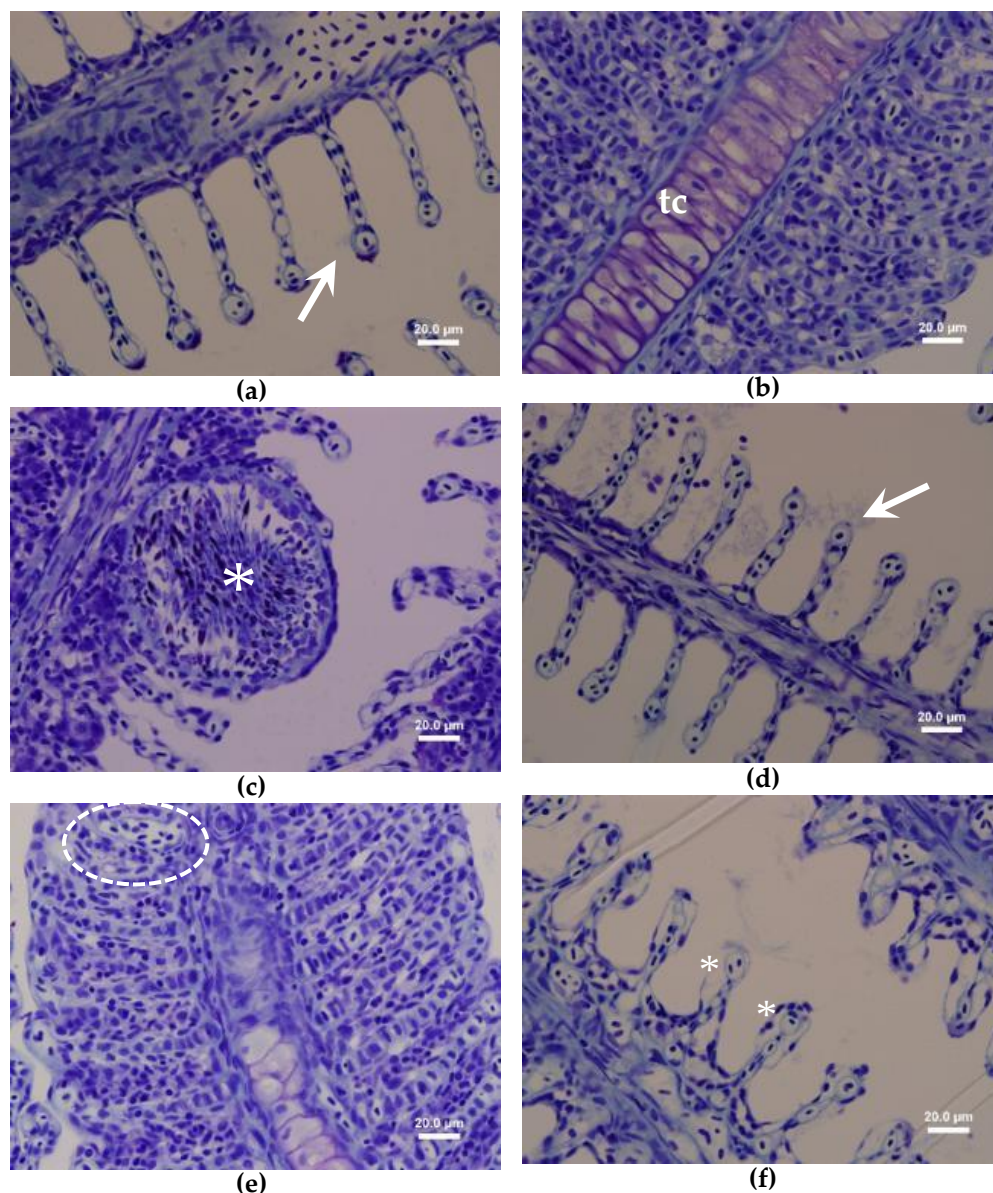


Figure 3: Most frequent histological changes in the gills of *P. lineatus*. (A) control 14 days: gill filament without significant changes - secondary lamella (arrow). (B) *B. dracunculifolia* 14 days: lamellar hyperplasia and fusion of secondary lamellae, (tc) cartilaginous tissue. (C) *B. dracunculifolia* 14 days: aneurysm (asterisk). (D) control 21 days: gill filament without significant changes - secondary lamella (arrow). (E) *B. dracunculifolia* 21 days: congestion (circle), lamellar hyperplasia and fusion of secondary lamellae. (F) *B. dracunculifolia* 21 days: epithelial detachment (asterisk). Blue toluidine technique.

Table 2: Mean in μm^2 of the area occupied by the mucous substances and lysosomes of the gills of *P. lineatus*.

Gill	Control 14 days	Treated 14 days	Control 21 days	Treated 21 days
AB pH 1.0 + PAS	4.684 $\pm$ 0.6355	7.474 $\pm$ 1.678	7.000 $\pm$ 1.591	5.316 $\pm$ 1.087
AB pH 2.5 + PAS	8.000 $\pm$ 1.051	17.26 $\pm$ 2.733*	5.632 $\pm$ 1.269	20.37 $\pm$ 3.112*
PAS	8.300 $\pm$ 4.772	12.83 $\pm$ 5.879*	5.867 $\pm$ 4.718	12.27 $\pm$ 3.591*
Lysosomes	184.5 $\pm$ 13.49	216.5 $\pm$ 15.44	144.6 $\pm$ 12.44	201.0 $\pm$ 18.30*

Averages, standard deviation ($\pm$) and significance (*). Note the higher presence of mucous substances, lysosomes in the group treated with *B. dracunculifolia*.

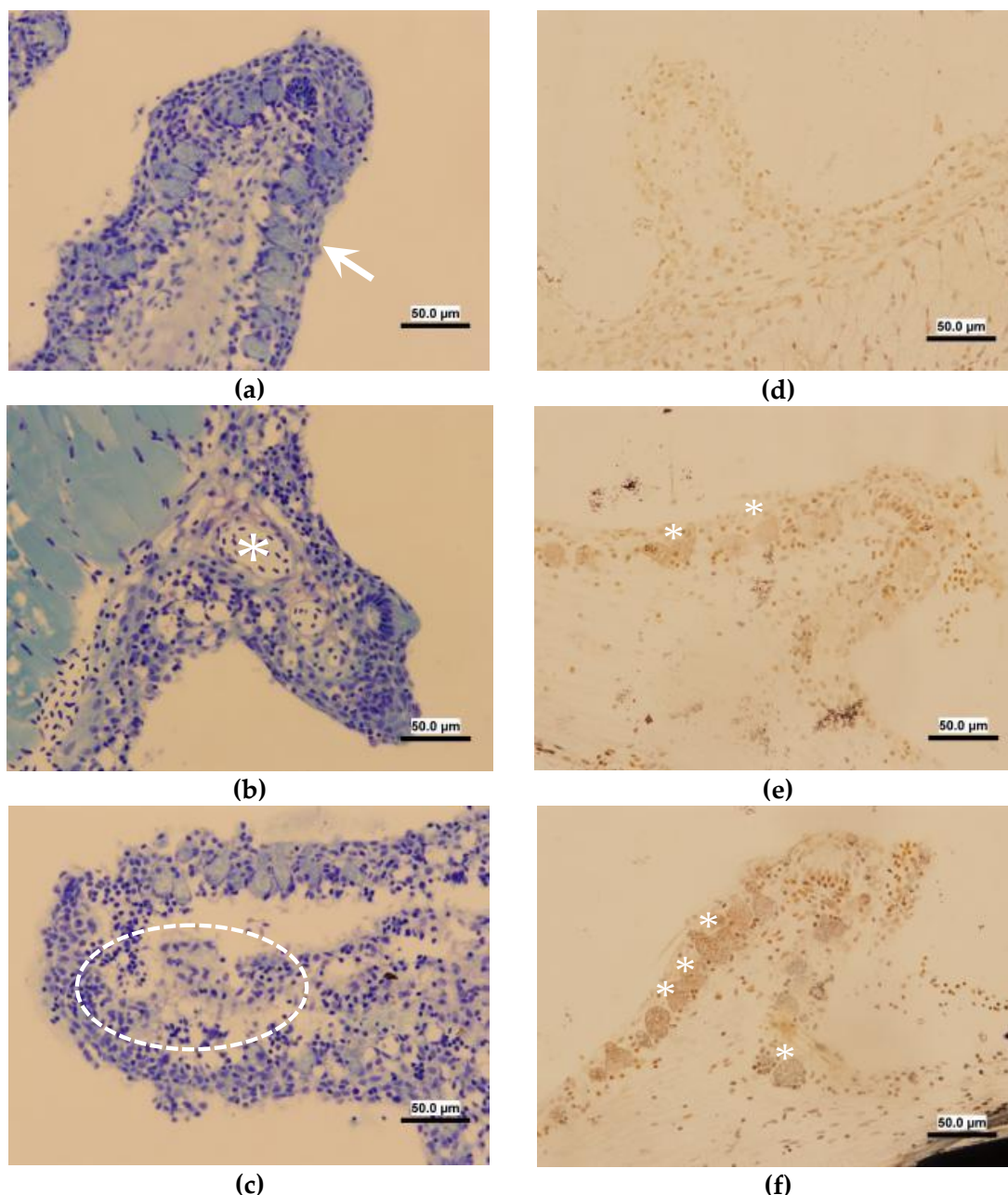


Figure 4: Changes in gill rakers of *P. lineatus*. (A) Control group 14 days without changes: mucosal cell (arrow). (B) Treated group 14 days: Blood cell infiltrates or congestion (asterisk). (C) Decomposition of the gill raker structure (circle): Treated group 21 days. Toluidine blue technique. (D) Control group 21 days: no change. (E) Treated group 14 days: mucus cells with the positive reaction to calcium (asterisk). (F) Treated group 21 days: mucus cells with the positive reaction to calcium (asterisk). Von Kossa Technique.

Table 3: Means in μm^2 of the area occupied by mucous.

Gill rakers	Control 14 days	Treated 14 days	Control 21 days	Treated 21 days
Alcian Blue pH 1.0 + PAS	206.8 $\pm$ 25.27	485.3 $\pm$ 128.7*	262.0 $\pm$ 26.48	368.3 $\pm$ 54.39
Alcian Blue pH 2.5 + PAS	188.2 $\pm$ 31.82	374.2 $\pm$ 45.08*	262.4 $\pm$ 36.16	435.1 $\pm$ 55.60*
PAS	754500 $\pm$ 76370	1431000 $\pm$ 17840*	617100 $\pm$ 73420	1427000 $\pm$ 192900*

Means, standard deviation ($\pm$) and significance (*). Note the higher presence of mucus in the treated group *B. dracunculifolia*.

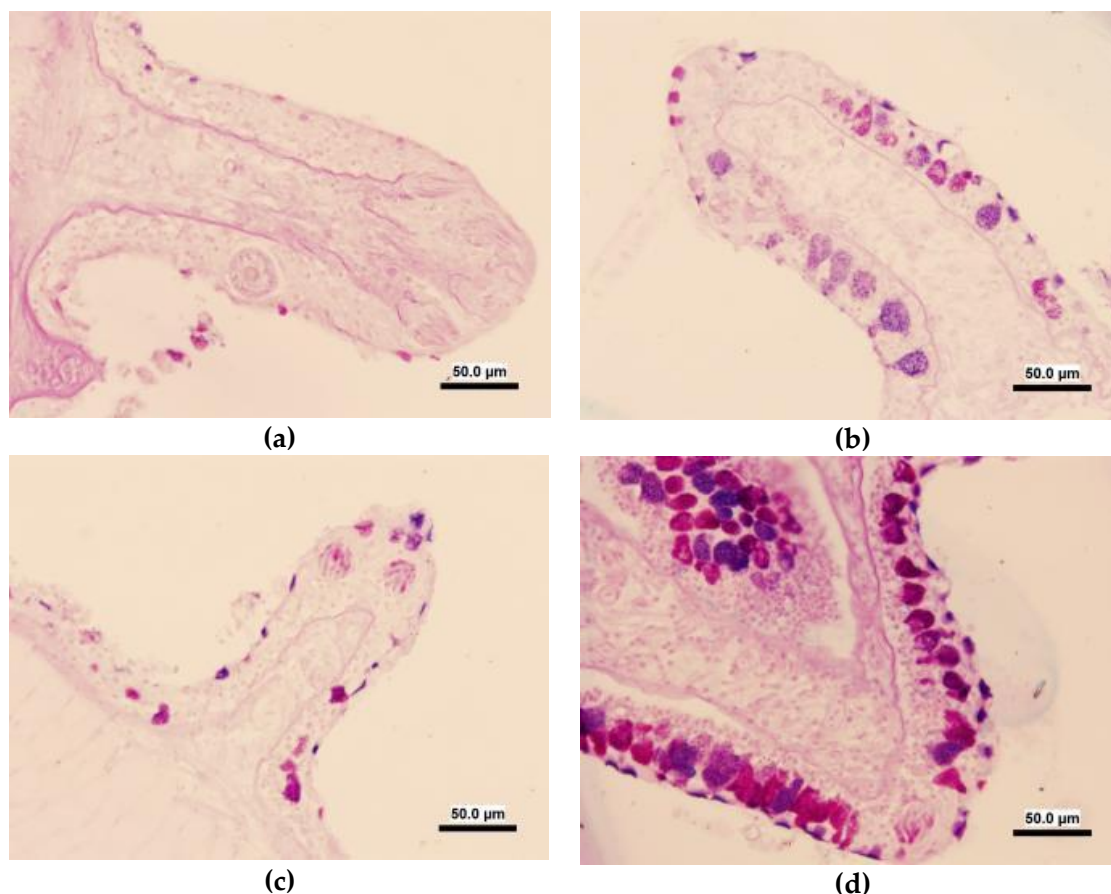


Figure 5: Changes in gill ratios of *P. lineatus*. (A) Control group 14 days. (B) Treated group 14 days. (C) Control group 21 days. (D) Treated group 21 days. Alcian Blue pH 2.5 + PAS technique. Note the highest amounts of sulfated acidic mucus in the group treated with *B. dracunculifolia*.

pollutant; however, it hinders the process of exchange of gases, showing that animals are exposed to substances that compromise their health (Fernandes and Mazon, 2003).

An aneurysm on the gill lamellae are lesions resulting from ruptures of the pillar cells caused by contaminants (Winkaler et al., 2007). This can cause the interruption/reduction of the gas exchange capacity and consequently the weakening of the fish.

Studies on the effects of plants on fish have been carried out by several researchers. Taiwo et al. (2008) tested the effect of the aqueous extract of Aloe vera leaves on various organs of the fish, including the gills, and concluded that this plant contains substances toxic to these animals. In gills of Nilotic tilapia exposed to the aqueous extract of *Carica papaya* filament and lamella degeneration, vacuolization of the gill arches and total removal of the lamellae were observed (Ayotunde and Ofem, 2008).

In rats, the toxicity of *B. dracunculifolia* has been confirmed through behavioral alterations and a decrease in polychromatic and monochromatic erythrocytes (Rodrigues et al., 2009).

Our study also confirms the toxicity of *B. dracunculifolia*, with a significant increase in the number of mucus cells and lysosomes. Exposure to pollutants can influence the proliferation of mucus cells with different types of mucus substances that proliferated after fish exposure to stressors (Alberto et al., 2005). Studies in Germany' rivers have found a fusion of secondary lamellae, the proliferation of mucus cells and mitochondria-rich cells and lysosomal proliferation in gills (Gernhöfere et al., 2001). According to Bernet et al. (1999), the neutral polysaccharides play important roles, acting as a barrier in the protection against particles, pathogens, and compounds toxic to fish.

The acidic mucus presents a higher viscosity and plays the role of fixing the organic and inorganic material of small size, besides defending the organ, against lesions and proliferation of pathogens. The increase of the acid mucus in the gill and branchial rakers indicates the possible defense response against toxic compounds present in *B. dracunculifolia*, as well as the number of lysosomes, possibly due to the need to combat the toxic agents present the extract. Pereira et al. (2017) observed a reduction in

the number of lysosomes, which it was associated with severe metabolic dysfunction because it prevents organelles and defective structures from being recycled.

In addition to changes in the number of mucous substances in the mucosal cells, there were also changes in the morphology of the gill rakers. This is in agreement with the finding of Valim (2017), who observed the loss of the integrity of the gill rakers, indicating that it is characterized by the detachment of superficial cells.

It should be noted that flavonoids found in plants demonstrated genotoxic effect at high concentrations (Ferguson, 2001; Pereira et al., 2006). The caffeic acid found in extracts of *B. dracunculifolia* (Resende et al., 2007; Munari et al., 2008) induced DNA damage in rats (Pereira et al., 2006). In this regard, we believe that the parameters affected in curimatás may have occurred by components that are the same or similar to those previously mentioned.

CONCLUSIONS

During feeding with the extract of *B. dracunculifolia*, ration compounds dissociated and after reaching the gills and the gill rakers, alters their morphology, causing mucus and morphological changes. Therefore, the gill and gill rakers of *P. lineatus* are sensitive indicator in determining the toxic effects of *B. dracunculifolia* extract.

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