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Research Paper

Analysis of cardiac stomach and medial portion of the posterior intestine of *Prochilodus lineatus* fed with extract of *Baccharis dracunculifolia* D.C. (Asteraceae)

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ABSTRACT

The leaves extract of *Baccharis dracunculifolia* is used in the treatment of diverse disorders, mainly digestive problems. This study aimed to use the histochemistry and morphometry of cardiac stomach and posterior intestine of *Prochilodus lineatus* to evaluate the effects of ingestion of the alcoholic extract of *B. dracunculifolia*. Sixty individuals were used, of which 32 were divided into two experimental groups in duplicate. These were randomly distributed in four 70-L aquariums (eight animals each). Both groups were fed for a maximum period of 21 days: control group (fed with regular commercial feed) and *B. dracunculifolia* group (fed with the feed enriched with extract of B. dracunculifolia). After treatment, 6 animals per aquarium were collected at 14 and 21 days and submitted to routine laboratory procedures. Histochemical and histomorphometric changes were evaluated with ImageJ® software. The results showed that the extract of *B. dracunculifolia*, added in the diet of curimbatás, can affect the morphometry of the villi of the middle portion of posterior gut and cardiac stomach, as well as increase the secretion by the caliciform cells of the studied regions.

Key words: Asteraceae; *Prochilodus lineatus*, cardiac stomach, posterior intestine, histochemistry; morphometric.

INTRODUCTION

Phytotherapy is a common form of treatment of a variety of health conditions across the globe (Lewith, 2000). Some of these compounds have potentially harmful side effects: recent examples include the use of ephedra linked to cardiovascular problems (Andraws et al., 2005).

Medicinal plant according to ANVISA is any plant or parts of it that contain substances or classes of substances responsible for therapeutic action (Resolução – RDC nº 10, de 09 de março, Brasília, 2018). According to WHO (World Health Organization), 65 to 80% of the world's population, especially in developing countries, rely on herbal products to treat their diseases (Rahman and Singhal, 2002). The use of these herbs is most often done by adults to supplement the treatment of a chronic disease (bvsms.saude.gov.br/bvs/publicacoes/ResumoExecutivoMedNatPratCompl1402052.pdf, 2018).

At present, we can find the commercialization of medicinal plants in free markets, popular markets, and other establishments in large and small Brazilian cities (Maciel and Constantin, 2002).

The uses of indiscriminate teas in children with hepatic, renal, or other diseases can pose serious consequences to their health without medical follow-up (Rang et al., 2007). The indiscriminate use of medicines and drug combinations increases the risk of mortality caused by the adverse effects and toxicity of these products (Wong, 2003).

The genus *Baccharis* includes more than 500 species, distributed from the United States to Argentina, with predominance in South America (Budel et al., 2005). *B. dracunculifolia* is traditionally used in the form of teas, to

combat gastric disorders and febrile conditions, besides being used in the treatment of wounds and inflammatory processes (Carvalho et al, 2011).

B. dracunculifolia is considered the main botanical source of green propolis, a product highly valued and commercialized in various pharmaceutical and cosmetic preparations, such as tablets, lozenges, dentifrices, lotions, facial creams, dyes, ointments, etc. (Bankova et al., 2000, Park et al., 2002).

Bacteriology, antimicrobial (Ferronatto et al., 2007), analgesic, antispasmodic, sedative, and cytostatic (Lorenzi and Matos, 2002) properties have been found in the essential oils of *B. dracunculifolia*. Moreover, according to Caetano (2012), the species *B. dracunculifolia* has anti-inflammatory, anti-protozoal, anthelmintic, antioxidant, anticancer, anticariogenic, cytotoxic, mutagenic (in high concentrations) and cicatrizing potentials.The intestine is an organ involved in important physiological functions, being the place of food digestion and absorption of nutrients (Caballero et al., 2003). The intestinal mucosa is fundamental in the digestive, absorptive and metabolic processes in teleostean fish and the increase in villus length implies an increase of the surface area for greater absorption of available nutrients (Caspary, 1992). This study aims to describe the effects of the extract of *B. dracunculifolia* on the cardiac stomach and medial portion of the posterior intestine of *Prochilodus lineatus* using histological approaches, as well as possible effects on fish in case of industrial dumps from *B. dracunculifolia*.

MATERIALS AND METHODS

Specimens

The *P. lineatus* juveniles used in this experiment (60.7 ± 1.3 g and 8.0 ± 1.5 cm) were purchased from Piscicultura Polettini, Mogi Mirim/SP, Brazil and transported to the Histology Laboratory of UNESP, Campus Rio Claro, Sao Paulo, Brazil. The animals were previously acclimatized in polyethylene boxes (500 liters) 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 from Rio Claro – SP, Brazil (22°22'30.0"S 47°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.

Ethanolic extract preparation

The leaf compound extraction followed the protocol established by ANVISA - (Brazilian Health Surveillance Agency) for the *Preparation mother tinctures from dry plants through maceration* (http://www.anvisa.gov.br/hotsite/farmacopeiabrasileira/arquivos/cp38_2010/x_metodos_preparacao_tintura.pdf/). For each treatment group, 1.5 mL (amount ingested in treatment in folk medicine) 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 aquarium (8 animals each) with air pumps, cooled with thermostat and covered with UV blocking material to reduce stress. Both groups were fed for a maximum of 21 days: Control group treated (with regular commercial food) and treated group (food added with *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 cardiac stomach and medial portion of the posterior intestine were fixed in bouin. Subsequently, the material was buffered in sodium phosphate solution pH = 7.4, dehydrated in increasing concentrations of alcohols, included in Leica historesin and sectioned in the Leica RM2245 microtome. The sections with 5 µm thickness, cross-section, were subjected to the specific reactions and consecutively form the assembly of the slides.

Histomorphometric analysis of intestinal villi and portions of the cardiac stomach

For histomorphometric analysis, the slides of the cardiac stomach and the medial portion of the posterior intestine

were stained with hematoxylin-eosin (HE) reaction. For the medial portion of the posterior intestine, 10 villi were measured per animal, totaling 120 villi per treatment, from which the height and total height of the villi were measured , corresponding to the distance from the apex of the villi to the beginning of the muscular layer and apex of the villi to the end of the serosa, respectively. The width of the villi and the thickness of their epithelium were also measured. For the cardiac stomach, there were also 10 regions in each analysis totaling 120 regions in each treatment, which included the total height of the apex of the mucosa up to the beginning of the muscular layer, respectively. The thickness of the muscular layer and mucosa were analyzed using the program ImageJ®.

Mucus quantification

For analysis of mucosal cells, eight sections from each individual were subjected to Alcian Blue pH 1.0 reaction for the identification of mucus sulfated acid substances; Alcian Blue pH 2.5 was used for the identification of sulfated and carboxylated acidic mucus and mucus neutral substances, evidenced by the periodic acid method Schiff (PAS) according to Paulete and Beçak (1976). From each cross section, 5 images (area corresponding to every fraction of the cut) and mucus present were isolated using ImageJ® software**.**

Quantification of total proteins and macrophages

For detection of total proteins, eight sections, in cross-section, of the cardiac stomach and medial portion of the posterior intestine of each animal were submitted to Xylidine Ponceau reaction according to Mello and Vidal (1980). For detection of macrophages, the same amount of cut was submitted to reaction (Gomori, 1949). For the analysis of total proteins of the medial portion of the posterior intestine, from each cross-section, 5 images were obtained (area corresponding to each fraction of the total cut) and the total proteins present were isolated using ImageJ ® software. The macrophages of the cardiac stomach and the medial portion of the posterior intestine were analyzed qualitatively and as the total proteins of the cardiac stomach.

Statistical analyzes

The data for analyzes were submitted to the Shapiro-Wilk test to verify the normality of the data and later to the ANOVA / Tukey test, while the parametric and non-parametric data to the Kruskal-Wallis / Dunn test, considering the significance of $p < 0, 05$. Statistical tests were performed with Biostat 5.0® software and GraphPad Prism 5.0® software.

RESULTS

Intestinal histomorphometry

The water quality parameters (pH, ammonia, hardness, and temperature) were kept within acceptable levels by the species as described by Pereira et al. (2014). No behavioral change was observed between the control and treated groups.

The histomorphometric analysis of the cardiac stomach and medial portion of the posterior intestine demonstrated that at the 14 day, there was a significant increase in total villus height, villus height and villus width of the medial portion of the posterior intestine, and total height and thickness of the cardiac stomach muscle layer (Figure 1). After 21 days, a significant reduction in the height and width of the villi in the medial portion of the posterior intestine was observed (Table 1).

Mucus

It was observed a variation in the intensity secretion of mucus by caliciform cells (Figure 2). In the animals fed for 14 days, there was a significant increase of it in the medial portion of the posterior intestine (Figure 2a and b), whereas the cardiac stomach showed an increase, but not significant (Figure 3). At 21 day, there was a significant reduction in the medial portion of the posterior intestine (Figure 2c and d) and for the cardiac stomach, the increase was not significant with $p < 0.05$ for the ANOVA / Tukey test. No secretory labeling of acid mucus (AB pH 1.0; 2.5) was found in the cardiac stomach (Table 2).

Total proteins and macrophages

Total proteins in the medial portion of the posterior gut reduced ($p < 0.05$) after 21 days (ANOVA / Tukey test). In the cardiac stomach, there was an increase in both times. The results of the number of macrophages in the medial portion of the posterior intestine were not significant. In the cardiac stomach, there was an increase in the number of macrophages during 14 and 21 days of treatment (Figure 4). Lymphocytes were also observed in the villi of the mid portion of the posterior intestine**.**

DISCUSSION

Histomorphometric results was explained by Junqueira and Carneiro (2005), who postulate that the larger the intestinal villus size, the greater the nutrients absorption capacity. This confirms the results obtained the present study since the reduction of villi was observed during 21 days. This can be attributed to the decrease in the area of nutrient absorption, resulting from the addition of *B. dracunculifolia.*

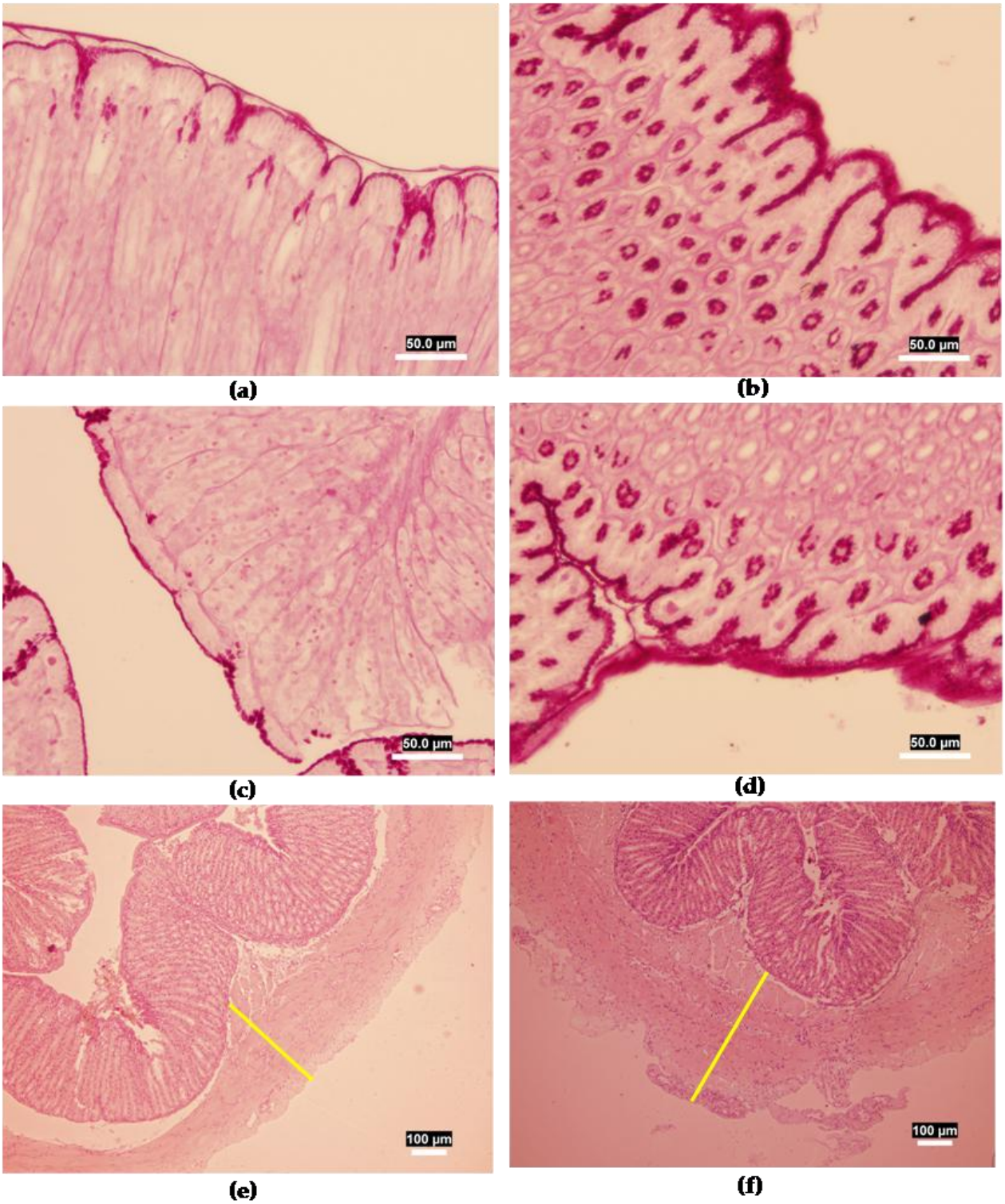

Figure 1: Changes in the levels of neutral mucus is morphometry of the cardiac stomach of *P. lineatus*. (A) Control 14 days. (B) Treated 14 days. (C) Control 21 days. (D) Treated 21 days. PAS technique. (E) Control 14 days. (F) Treated 14 days. Note the increase in mucus during 14 and 21 days of treatment and increase of the muscular layer in 14 days of treatment. HE technique.

Caliciform cells proliferate to increase mucus production when aggression due to pathogens occurs (Schwarz et al, 2011). This study demonstrated the increase in the area occupied by the mucus after 14 days of feeding. This

Table 1: Means in μm of the morphometry of the medial portion of the posterior intestine and cardiac stomach. Averages, standard deviation (S ±) and significance (*).

Histomorphometric	Control 14 days	Treated 14 days	Control 21 days	Treated 21 days
Posterior intestine				
Total villus height	601.4± 24.80	731.4 ± 21.77*	720.9± 27.14	635.8± 17.79
Height of villi	500.7± 20.94	611.2± 20.85*	615.0± 26.61	512.6± 15.59*
Width of villi	132.0± 6.509	157.0± 6.360*	158.4± 7.843	120.9± 4.019*
Thickness of villus epithelium	65.24± 3.999	69.37± 2.687	67.06± 3.619	56.71± 1.874
Cardiac stomach				
Thickness of the mucosa	333.8±39.56	329.9± 36.90	270.3± 26.07	313.7± 20.18
Thickness of muscle layer	348.6± 16.92	592.2± 47.11*	360.9± 11.97	441.3± 42.93
Total height	687.5± 37.00	888.9± 38.20*	659.5± 32.83	768.0± 48.86

Table 2: Mean in μm of the area occupied by glycoproteins and total proteins of the medial portion of the posterior intestine and cardiac stomach of *P. lineatus*. Averages, standard deviation (±) and significance (*).

Histochemistry	Control 14 days	Treated 14 days	Control 21 days	Treated 21 days
Posterior intestine				
Acid	9.120 ± 0.9153	18.48 ± 2.458*	13.28 ± 1,452	7.640 ± 1.923
Sulphated acid	8.320 ± 1.092	23.16 ± 3.065*	14.20 ± 1,998	8.320 ± 1.876
Neutral	798000± 86810	1257000± 157900*	1021000± 105200	583600± 59330*
Total Proteins	2576000± 256900	2910000± 346300	3019000± 370200	1816000± 309200*
Cardiac stomach				
Neutral	4714000± 564300	5225000± 802700	4298000±796400	4817000± 688500
Acid	-	-	-	-
Sulphated acid	-	-	-	-

suggests that the increasing mucus secretion avoid the absorption of toxic substances present in *B. dracunculifolia* extract. The glycoproteins secreted by caliciform cells have important functions in protection against infections, because it prevents the contact of microorganisms with epithelial cells and has a bactericidal effect due to the presence of lysozyme and low molecular weight fatty acids (Noga, 1996).

However, when fed for 21 days, a significant secretion reduction was observed, suggesting that *B. dracunculifolia* may compromise the intestinal epithelium, since the release of these polysaccharides protects fish health, by acting on the immune system and preventing colonization of pathogenic bacteria in the gastrointestinal tract (Spring, 2001; Refstie et al., 2010).

The Xylidine Ponceau technique demonstrated the reduction in total protein levels. This reduction must have occurred due to the action of *B. dracunculifolia* on glycoproteins in the gut, confirming its toxicity. According to Kopoor et al. (2005), intestinal digestion depends on the secretion of glycoprotein substances from goblet cells, the proteolytic action of pancreatic secretion and also on intracellular digestion which is associated with the presence of gastric glands.

Other researchers also reported intestinal damage in fish fed with ingredients of plant origin, in rainbow trout, as soy consumption caused changes in the posterior intestine, with a decrease in height of the simple fold (Sørensen et al., 2011). Bureau et al. (1998) reported irregularities in the form of enterocytes, the presence of large vacuoles in the apical cytoplasm of enterocytes, among other changes in the intestinal epithelium of rainbow trout (*Oncorhynchus mykiss* and *Oncorhynchus tshawytscha*), after the consumption of diets containing soybean meal and quinoa saponin.

When *Corydoras paleatus* was exposed to organophosphorus, the occurrence of alterations in the intestinal mucosa with disorganized villi, an inflammatory process and lipid vacuolization of enterocytes, were observed. In addition, epithelial cell necrosis, desquamation, lymphocyte infiltration and enterocyte hyperplasia were also observed. Such modifications were only described

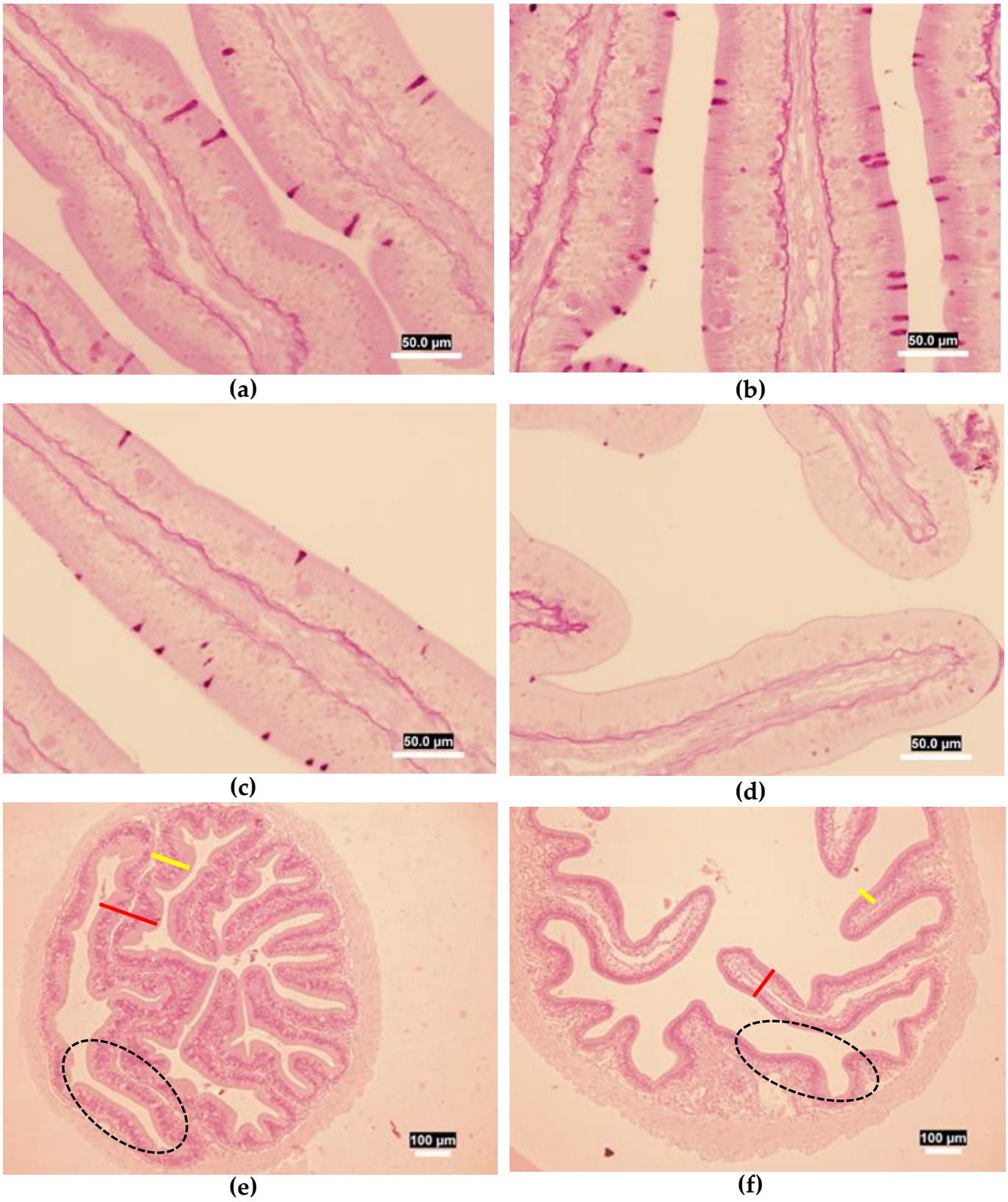

Figure 2: Changes in the neutral polysaccharide levels of the villi of the medial portion of the posterior gut of *P. lineatus*. (A) Control 14 days. (B) Treated 14 days. (C) Control 21 days. (D) Treated 21 days. Note the increase in treated animals for 14 days and a drastic reduction after 21 days of treatment. PAS technique. (E) Control 21 days. (F) Treated 21 days. Note the reduction in epithelial thickness (yellow dash) and villus width (red dash) and a decrease of caliciform cells (circle). HE technique.

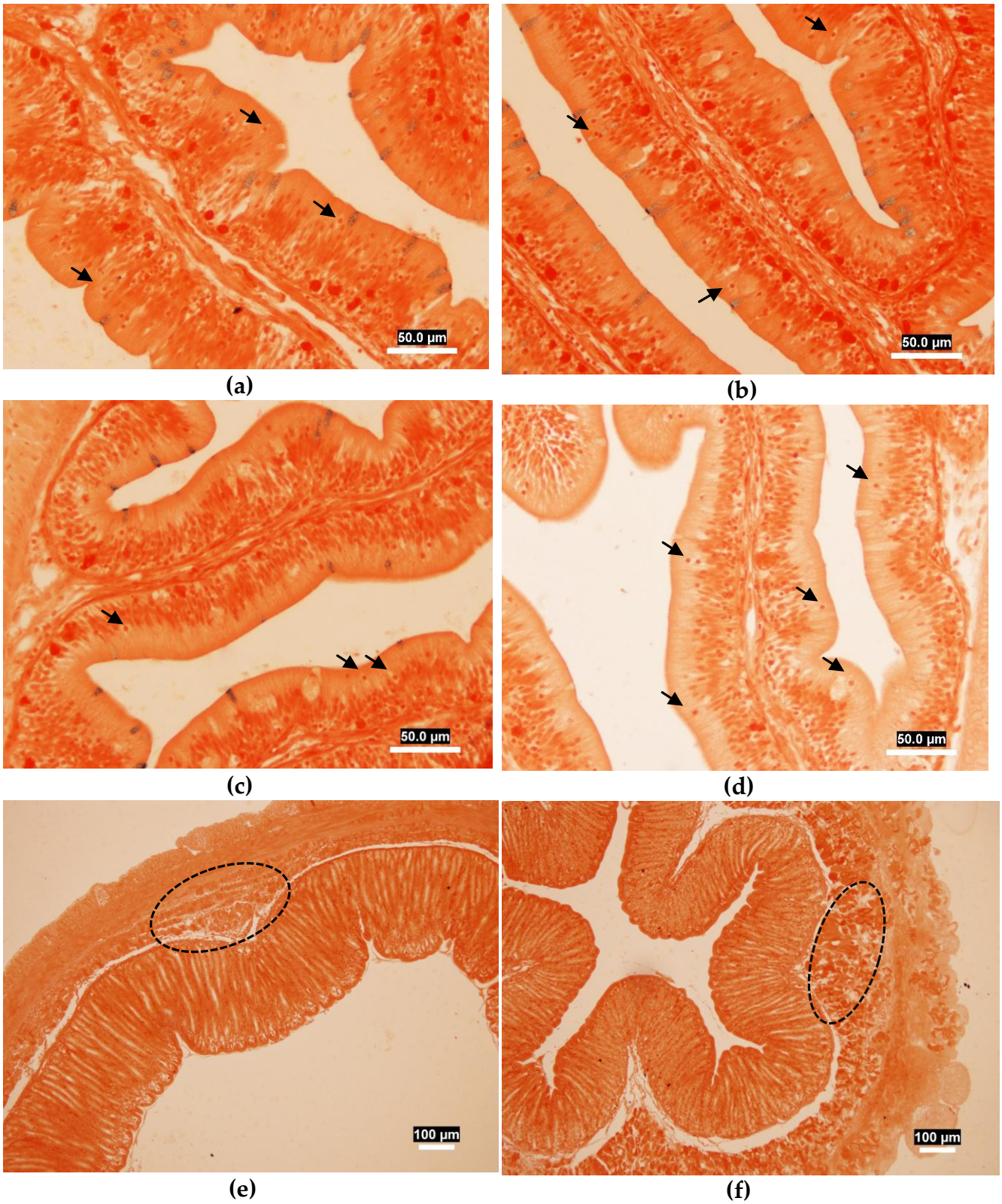

Figure 3: Changes in total protein levels of villi of the medial portion of the posterior gut of *P. lineatus*. (A) Control for 14 days. (B) Treated 14 days. (C) Control 21 days. (D) Treated 21 days. (E) Control 14 days: Portion of the cardiac stomach-areas with protein concentration (circle). (F) Traded 14 days: Portion of the cardiac stomach-areas with protein concentration (circle). Note the increase of protein concentration in treated animals in 14 days and a reduction after 21 days of treatment. Note the presence of lymphocytes (arrows). Xylidine Ponceau Technique.

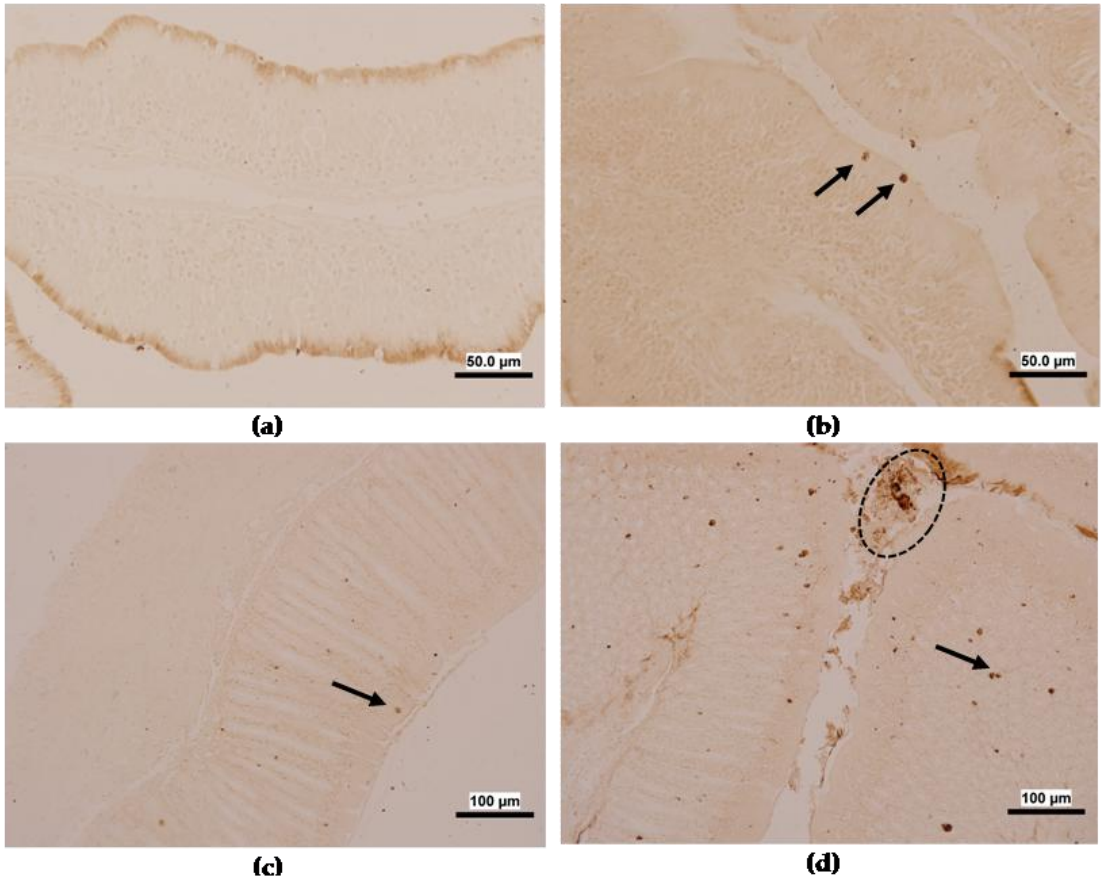

Figure 4: Changes in the number of macrophages of the cardiac stomach and medial portion of the posterior intestine of *P. lineatus*. (A) Control for 14 days. (B) Treated 14 days -Maghage (arrow). (C) Control 21 days. (D) Treated 21 days - macrophage (arrow) and mucus (circle). Note the increase in macrophages in the cardiac stomach after 21 days of treatment. Gomori technique.

after administration of the insecticide through contaminated food.

In this study, we observed an increase in the number of macrophages that is possibly due to the need to combat the toxic agents present in *B. dracunculifolia*. For *Platichthys flesus*, collected from disturbed environments, Köhler et al. (2002) observed that the liver had a decrease in glycogen, accumulation of cytoplasmic lipids, elevation in the number of apoptotic cells and large aggregates of macrophages. For lysosomes, it was found that both number increase and decrease due to lysosomal fusion. Pereira et al. (2014) evidenced that groups exposed to contaminants, presented mobilization of macrophages, however, the number of lysosomes decreased.

In addition, among the enterocytes of *P. lineatus*, the presence of lymphocytes was observed in this study, indicating a possible immunological defense cell barrier. Intraepithelial lymphocytes (cytotoxic T cells) may be involved in oral immunological tolerance and also in the immune response of the intestinal mucosa in fish (Eto et al., 2013). B lymphocytes appear late in the intestinal mucosa, whereas the early onset of T lymphocytes in this organ suggests that it may be a site of extra-thymic differentiation of these leukocytes (Cain et al., 2000; Rombout et al., 2010).

Conclusions

The extract of *B. dracunculifolia* added in the diet of *P. lineatus* can affect the histomorphometry of the villi of the medial portion of posterior gut and cardiac stomach, as well as increase the secretion by the caliciform cells of the studied regions. In the case of contamination of the aquatic environment by industrial dumps linked to by-products produced by *B. dracunculifolia*, it will possibly result in toxicity to the species.

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