Chapter 3

Histopathology in Zebrafish (*Danio rerio*) to Evaluate the Toxicity of Medicine: An Anti-Inflammatory Phytomedicine with Janaguba Milk (*Himatanthus drasticus* Plumel)

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Abstract

The zebrafish Danio rerio appears to be as an alternative experimental model mainly used on toxicological evaluations since the 1990s. In this chapter, we illustrate using a histopathological study the evaluation of a complex phytopreparation with janaguba milk (TPJM, used in popular medicine), which was administrated in zebrafish by immersion in water. We determined (1) lethal concentration 50 (LC_{50}) – 1188.54 μg/mL; (2) the behavioral changes; and (3) the acute administration of TPJM modifications (48 h) at concentrations 500, 750, 1000, and 1500 μg/mL, on the histopathological parameters of the gills, kidneys, and liver. Also the concentrations of 1000 and 1500 μg/mL caused significant damage to the gill tissue and produced a high rate of histological changes in the liver. The kidneys showed greater changes at concentrations of 750, 1000, and 1500 μg/mL. Based on the percentage of TPJM extracts that was only 1.85%, the LC_{50} was calculated as 475 mg/kg; according to traditional indication, only 6 tablespoons/day is consumed; and it is possible to infer that only 0.5 g of active ingredient is ingested by an adult user per day, corresponding to a dose of 7.14 mg/kg, which is far from the toxic effects, demonstrating low toxicity of TPJM.

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Keywords: *Danio rerio*, *Himatanthus drasticus*, toxicity, histopathology, traditional phytopreparation

1. Introduction

With the increasing pressure for animal welfare, many research groups have been increasingly restricting the use of mammals such as rodents and rabbits in the experimentations. Also, laws, discussions, projects, and committees aimed at reducing the number of animals used in toxicological experiments, forcing a reflection on the subject and the implementation of preliminary tests to draw conclusions that allow the reduction of animal use.

In this context, the zebrafish, *Danio rerio*, appears as an alternative experimental model. This specimen is now used as an experimental model in toxicology since the 1990s [1].

Some studies have demonstrated the importance of these animal species for the development of histopathological studies for the experimentation with new drugs. What has been observed are the relationships between the behavioral profiles within the tissue physiopathology in zebrafish, so it is possible to extrapolate the results to the human tissue changes.

Most of the natural products used in folk medicine do not have scientific backgrounds that prove and elucidate its action mechanism and ensure their use. The toxicological studies of TPJM have not been elucidated in order to measure its possible harm to human health.

Several species of the *Himatanthus* genus have been studied for their chemical composition. Therefore, various chemical classes and several substances of medicinal interest have been isolated and reported in the last years [2]. The metabolites isolated from *Himatanthus* and *Plumeria* species are mainly monoterpene, iridoid-type compounds with the structure of tetrahydrocyclo pentan-pyran [2].

The *H. drasticus* latex has popular knowledge and it is used against lung cancer and lymphatic, intestinal worms, fever, and gastric ulcers [3]. Studies developed by de Mesquita et al. [4] of the *H. drasticus* roots have indicated antiparasitic activity against *Leishmania donovani* promastigotes. Previous studies with the species *Himatanthus* demonstrated pharmacological activity against human epidermoid nasopharyngeal carcinoma [5].

Himatanthus drasticus, like other species of the genus *Himatanthus*, has rarely correlated their biological activity with the phytochemistry. Colares et al. [6] isolated and identified the triterpene lupeol cinnamate from the ethanolic bark extract, which is suggested that this metabolite presents an antitumor activity. Another research developed was the gastroprotective effect of the latex through gastric injury induced by ethanol and indomethacin [6].

Luz et al. [7] performed a phytochemical screening of the bark of *H. drasticus*, identifying alkaloids and tannins. It also presents coumarins with several biological activities, for example, antimicrobial, anti-inflammatory, antiviral, and antioxidant activity, besides the potential antifungal action and the hypocholesterolemic action of the saponins.

Other pharmacological studies conducted by de Sousa et al. [8] demonstrated the low toxicity of the crude methanol extract from the leaves of *H. drasticus* and the antitumor activity in experimental sarcoma 180. Mousinho et al. [9] investigated the antitumor effect of *H. drasticus* latex proteins, proving that it can be associated with the immunostimulatory properties. Lucetti et al. [10] studied the possible mechanism of the anti-inflammatory action of lupeol acetate, which probably involves the opioid system, and Colares et al. [6] described the antinociceptive activity evaluated in the writhing test induced by acetic acid.

The zebrafish has been useful for assessing the toxicity of extracts or products of these isolates [11]. In this chapter, we illustrate with a histopathological study the evaluation of a complex phytopreparation with janaguba milk (TPJM, or only janaguba milk, used in popular medicine), which was applied in zebrafish administrated by immersion in water.

2. Materials and methods

The initial project was submitted to the Ethics Committee on Animal Use of the Federal University of Amapá-CEUA-UNIFAP, Macapá, Amapá, Brazil, receiving approval and registration with the n°004/2015 protocol.

2.1. Obtention and yield of traditional phytopreparation with janaguba milk (Himatanthus drasticus): TPJM

The traditional phytopreparation with janaguba milk was purchased from a popular retailer located in the Crato-CE city, and according to the quality control report provided by the company (Number authenticity 0023-2014), the plant species used in the preparation is *Himatanthus drasticus* Plumel.

The yield of the extract of TPJM was estimated by lyophilization and expressed as a percentage. The lyophilized was analyzed by infrared spectrum and high-performance liquid chromatography (HPLC).

The Fourier-transformed infrared spectra (FT-IR) of the lyophilized TPJM were obtained on a Shimadzu spectrometer, FTIR-8400S model, operating in the Fourier transformer. The spectra were obtained in the region from 4000 to 400 cm^{-1} using KBr pellets (solid samples) with a 4.0 resolution and 64 scans.

For the HPLC chromatographic analysis, 3 mg of the lyophilized TPJM was added to 5 ml of hexane, the partition was performed with 5 ml of methanol, and the methanolic fraction was filtered through a membrane with a pore size of 0.45 μm (Millepore®) and analyzed on a HPLC (Shimadzu Corporation) equipped with auto-injector, diode array detector, and was scanned from 190 to 500 nm. Chromatographic conditions: chromatograms were obtained at 280 nm, the temperature was maintained at 30°C, a reverse phase column was used, Shimpack VP-ODS (150 × 4.6 mm; 5 μm), and the injection volume was 10 μL using a mixture of the phase A containing acidified water (24%) with acetic acid and methanol (70:30, v/v) and

phase B containing acetonitrile, in an isocratic system of proportions 70:30 (v/v) with a flow rate of 1 ml/min. The identification of the peak corresponding to gallic acid was performed in comparison with the standard substance.

2.2. Acute toxicity tests

2.2.1. Experimental animals

Authenticated *D. rerio* species were purchased from the company Acqua New Aquariums and Fish located in Itaguassu-PE, Brazil, under the authorization of the Protocol 526140011289802 (May 7, 2014), registered at IBAMA with No. 82957. The animals were kept in quarantine in a zebrafish platform on the Drug research laboratory (Laboratório de Pesquisa em Fármacos), UNIFAP, Brazil, until the experiments were made.

2.2.2. Assessment of the behavioral parameters of D. rerio under treatment with TPJM

For this study, a total of 90 animals were used, only adults who were 6–8 months were selected for the study. The animals were maintained at a temperature of 26 ± 2°C with a light:dark cycle of 10:14 h. Also we used standardized water for the maintenance of adult fish.

Adult fish were fed with commercial brine shrimp (*Artemia salina*). The animals were treated according to the guide for the care and use of experimental animals. The fish behavior was assessed by a human observer and filmed after 0, 3, 6, 12, 24, 27, 30, and 48 h.

2.2.3. Determination of LC_{50}

Concentrations of 500, 750, 1000, and 1500 μg/mL of the TPJM were tested in order to obtain the median lethal concentration (LC_{50}). Each concentration was performed by triplicate, using 15 animals per assay. The exposition time with the different solutions was 48 h.

2.2.4. Assessment of behavioral parameters

Animals were exposed to the concentrations of 500, 750, 1000, and 1500 μg/mL of the TPJM on a single tank for 48 h, all experiments were done by triplicate (n = 15), tank water was used as a control test. The behavior was assessed by a human observer and filmed after 0, 3, 6, 12, 24, 27, 30, and 48 h of exposure to TPJM. All the responses were characterized at three stages following the protocol previously described by Ribeiro [12].

2.2.5. Monitoring of mortality

Mortality was monitored continuously, and the fish were considered dead when the movement of the operculum and response to mechanical stimulation could no longer be detected. After the experiment, the remaining fish were euthanized by anesthesia by cooling, in agreement with the recommendation of the American Veterinary Medical Association Guidelines for the Euthanasia of Animals (2013 Edition Members).

2.2.6. Evaluation of the histopathological parameters

For the histopathology analysis, the organs (gills, liver, and kidney) were fixed in Bouin solution for 24 h according to the method described by Souza [13]. The analysis of slides was performed under an optical microscope Olympus BX41-Micronal and photographed with MDCE-5C USB 2.0 camera (digital) and by a scanning electron microscopy (microscope Hitachi TM3030PLUS).

2.2.6.1. Measurement of histopathological alterations

We considered some histopathological changes in the gills, liver, and kidneys. The damage was categorized using a semi quantitative analysis, and calculating the average medium assessment (VMA as previously described by Shwaiger [14]), calculated from semi quantitative analysis based on a scale of severity and occurrence of the lesions.

We also used the changes histological index (CHI) described by Poleksic and Mitrovic-Tutundzic [15]. This ratio was based on the branchial lesions, each type of injury being rated as the severity in stages I, II, and III.

Thus, the indices were calculated according to the following equation:

$$I = \frac{\sum_{i-1}^{na} ai + 10 \sum_{i-1}^{nb} bi + 102 \sum_{i-1}^{nc} ci}{N} \quad (1)$$

where: *a*: changes first stage; *b*: changes second stage; *c*: changes third stage; *na*: number of amendments considered to be the first stage; *nb*: number of modifications considered to be second-stage; *nc*: number of amendments considered to be the third stage; *N*: number of analyzed fish per treatment.

In this study, this equation was used to calculate the changing index not only in the gills but also liver and kidney [15]. In the present study, these relationships were extrapolated to the kidneys and the liver. The parameters considered in this chapter are established in **Table 1**.

2.3. Statistical analysis

The LC_{50} values over their 95% confidence intervals were calculated by probit analysis using GraphPad Prism Software Version 5.0. To analyze the histopathological findings a one-way

Values	Effects
0–10	Normal organ functionality
11–20	Organ with mild to moderate changes
21–50	Organ with moderate to severe changes
>100	Organ with irreversible damage

Table 1. Different stages in the histopathological changes seen in the zebrafish.

analysis of variance (ANOVA) followed by the Tukey-Kramer *post-hoc* test were conducted. Data were expressed as the mean ± SEM. Results with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ were considered statistically significant.

3. Results

The yield of the extracts of the TPJM obtained by lyophilization was 1.85%. The phytopreparation is composed of diluted exuding latex of janaguba (1:10). This concentration is similar as many of the products marketed commonly used on the folk medicine.

According to FT-IR analysis of the lyophilized TPJM, it is possible to observe an intense broadband of 3414.15 cm^{-1} suggesting the presence of fatty acids in the resin of *Himatanthus drasticus*. Note that the presence of aliphatic carbons in 2852.84 and 2924.21 cm^{-1} is characteristic of the carbon chains of terpenes (**Figure 1**). Lupeol triterpene class can be observed by characteristically the signals 1454.39 and 1377.23 cm^{-1} for the rings; the signal at 1695 cm^{-1} belongs of the stretching of carbon-carbon bond, suggesting the presence of compounds of the triterpene lupeol class [6].

The chromatogram obtained by HPLC of the methanolic fraction of the lyophilized TPJM contains several signals, the peak corresponding to gallic acid showed a retention time of 7.240 min (**Figures 2** and **3**), corroborating the study by Luz et al. [7] which identifies the presence of various classes of phenolic compounds.

The exposure by immersion of *D. rerio* to different concentrations of TPJM triggered significant behavioral changes in the fish only at higher concentrations (1000 and 1500 μg/mL). Such changes were classified into three stages: I, II, and III (**Table 2**).

The behavior of the fish was filmed for 2 min during the intervals of observation. The toxic action of TPJM depends on the concentration administered; at the two lower concentrations, the fish expressed only milder and less changes, whereas, at higher concentrations, severe behavioral changes were observed (**Table 2**).

Table 3 shows the percentage of dead animals by different concentrations of TPJM. These results demonstrate the susceptibility of *D. rerio* to the TPJM in preliminary testing concentrations, although between 20 and 100 μg/mL, no deaths were observed and neither at the concentrations of 500 and 750 μg/mL. The estimated LC_{50} value was calculated as 1188.54 μg/mL (**Figure 4**).

The changes in gill tissue of *D. rerio* by immersion exposure of TPJM are quantified in **Table 4**. Stage I of histopathological changes such as hypertrophy of the respiratory epithelium (composed of the lamellae) and hyperplasia of epithelial cells was present in all the concentrations (**Figure 5**).

The fusion of secondary lamellae was present in all tested concentrations and was more frequent the higher concentrations of 750, 1000, and 1500 μg/mL. Additionally, the complete

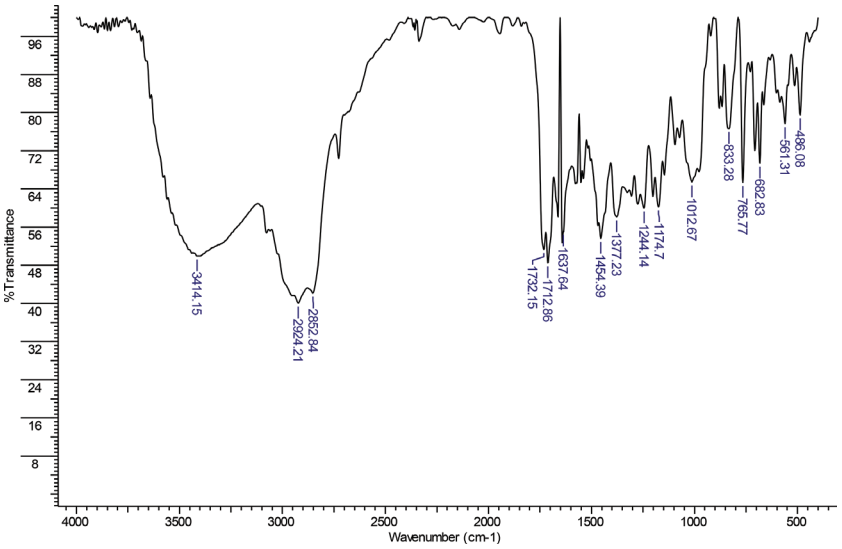


Figure 1. Infrared spectrum, corresponding to the lyophilized TPJM, an intense broadband 3414.15 cm^{-1} suggest the presence of fatty acids in the TPJM. Presence of aliphatic carbons in 2852.84 and 2924.21 cm^{-1} abundant in carbon chains of terpenes.

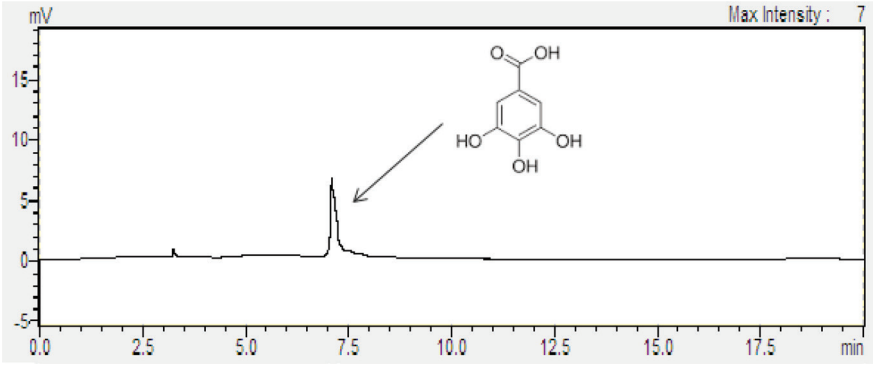


Figure 2. Chromatographic obtained of the gallic acid standard reference with retention peak in 7.240 min.

fusion of all the secondary lamellae is visible only in the higher concentrations of TPJM (**Figure 5**).

The changes in blood vessel lamellar, stage I, were common at all concentrations (**Table 4**). The rupture of blood vessels and subsequent hemorrhage were observed in every treatment. The presence of a lamellar aneurysm in only one concentration was observed (1000 μg/mL of TPJM) (**Figure 5**). These changes are rarely observed in normal conditions. Although the infiltration of

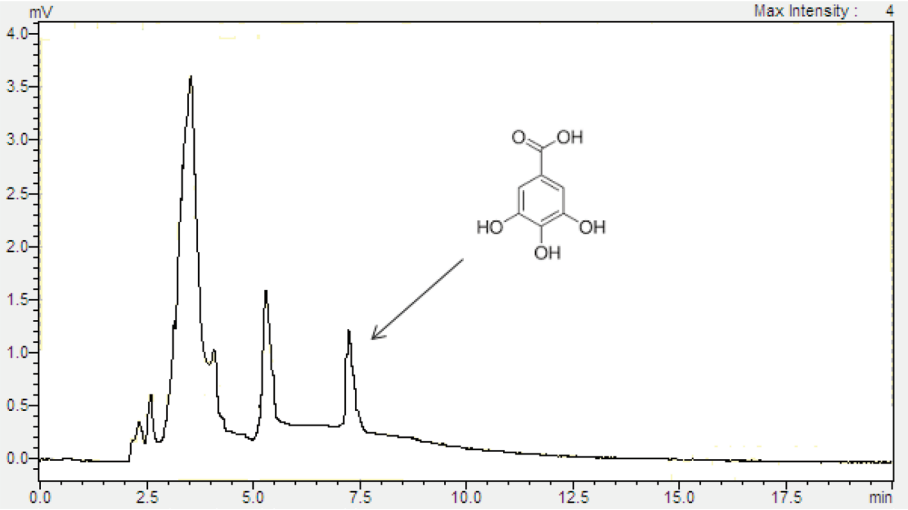


Figure 3. Chromatographic profile of the methanolic fraction from the lyophilized TPJM, with retention peak in 7.240 min equivalent to gallic acid.

		500 μg/mL of TPJM	750 μg/mL of TPJM	1000 μg/mL of TPJM	1500 μg/mL of TPJM	Control
Stage I	0′	1	1	1	1 and 3	
Stage II	0′		1	1 and 2	1 and 2	
Stage III	0′				2 and 3	
Stage I	3 h			1	2	
Stage II	3 h			1	1 and 2	
Stage	3 h			2 and 3	2 and 3	
Stage I	9 h			1		
Stage II	9 h					
Stage III	9 h		2		2	
Stage I	24 h			1		
Stage II	24 h					
Stage III	24 h			2 and 3	2 and 3	
Stage I	27 h			1		
Stage II	27 h					
Stage III	27 h				2	
Stage I	33 h			1		
Stage II	33 h					
Stage III	33 h				2	
Estágio I	48 h			1		
Estágio II	48 h					
Estágio III	48 h				2	

Stage I: (1) increase swimming activity, (2) tail tremors; stage II: (1) circular swimming movement, (2) loss of posture; stage III: (1) loss of motility; (2) animal deposition in the base of the beaker, (3) death.

Table 2. Effects of the concentrations of TPJM on ***D. rerio*** behaviors in different hours of observations.

Concentration of TPJM	500 μg/mL	750 μg/mL	1000 μg/mL	1500 μg/mL	Control
Number of animals death	0	0	4	13	0
Percentagen (%)	0	0	26.7	86.6	0

Table 3. Percentage of animal death with the concentrations of TPJM on ***D. rerio***.

leukocytes in the lamellar epithelium was also not diagnosed, it may lead to aneurysm or even hemorrhage epithelial disruption [16].

In stage III, the necrotic alteration on the *D. rerio* specimens was manifested in the groups treated with 1000 and 1500 μg/mL causing the death of the animals. Cellular degeneration was presented only at the concentrations higher than 750 μg/mL (**Figure 5**).

The quantitative results (**Table 4**) and qualitative results (**Figure 5**) indicate that the exposition caused changes on the gills of *D. rerio*, compared to the control. Furthermore, it is possible to note that concentrations of 1000 and 1500 μg/mL were responsible for the higher IAH (**Figure 6** and **Table 5**).

According to the gills, IAH even with stage III changes such as necrosis can also be classified as functionally organs. **Table 5** shows that the gills are functionally normal in all treatments with TPJM.

The *D. rerio* livers showed histopathological changes in hepatocytes, blood vessels, and bile canaliculi. The *D. rerio* hepatocytes have typical aspects described as in most vertebrates. In **Table 6**, we quantified the occurrence of liver abnormalities in the fish exposed to different concentrations of TPJM. Also, qualitative data show changes in **Figures 7** and **8**. All of the stage I alterations were present in the various treatment concentrations. It may be noted in

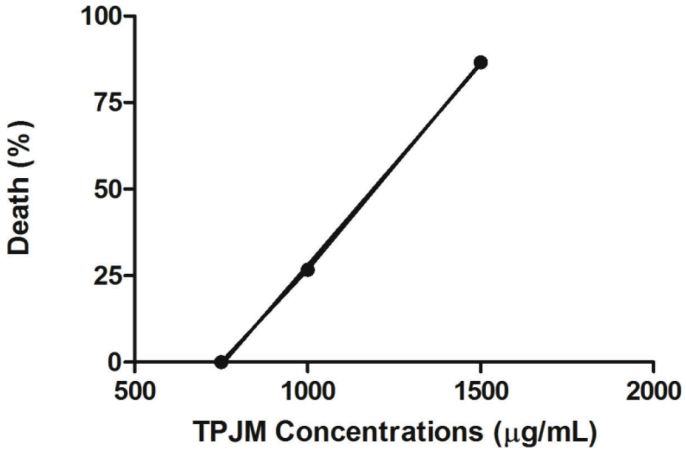


Figure 4. Effects of administration of different TPJM concentrations (500, 750, 1000, 1500 μg/mL) on D. rerio n = 15/group, LC50 = 1188.54 μg/mL.

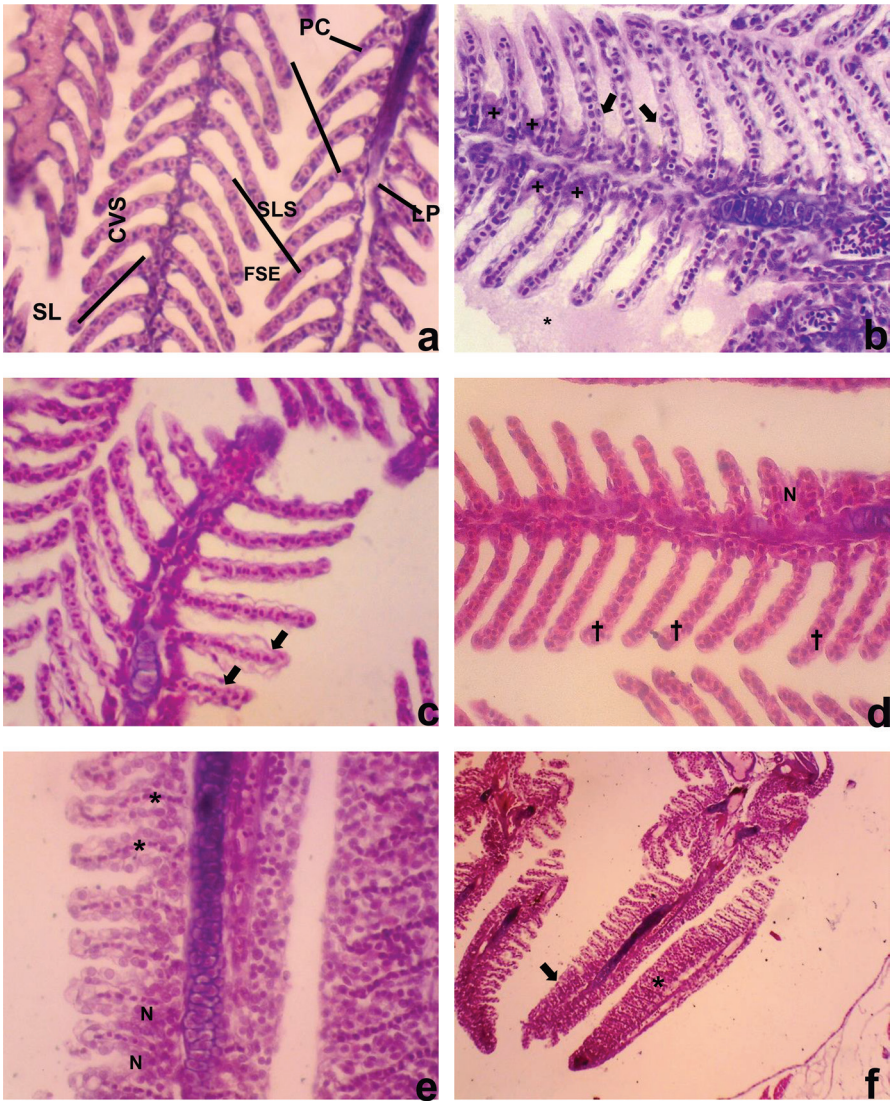


Figure 5. Histopathological changes in the gills of *D. rerio* exposed to different concentrations of TPJM. (a) Normal gill filament on longitudinal histological section. PC—pillar cells; FSE—filament stratified epithelium; SLS—secondary lamellar squamous epithelium; SL—secondary lamellae; CVS—central venous sinus (400×); (b) gill filament on longitudinal histological section exposed to 500 μg/mL of TPJM. Hyperplasia of epithelial cells in the secondary lamellae (+), presence of chloride (black arrow) and mucus secretion (*) (400×). (c) Gill filament on longitudinal histological sections exposed to 750 μg/mL. Epithelial Detachment (black arrow) (400×). (d) Gill filament on longitudinal histological section exposed to concentration of 1000 μg/mL. Vascular congestion, dilation of capillaries (†) and necrosis of respiratory epithelial cells (N). (e) Gill filament on longitudinal histological section exposed to 1500 μg/mL of TPJM. Necrosis of respiratory epithelial cells (N) and capillary derangements (*) (400×). (f) Gill filament on longitudinal histological section exposed to 1500 μg/mL. Fusion of some secondary lamellae (black arrow) and fusion of all secondary lamellae (*) (100×).

Alterations	Stage	Control	500 μg/mL of TPJM	750 μg/mL of TPJM	1000 μg/mL of TPJM	1500 μg/mL of TPJM
HTEC	I	0	86.6	100	100	100
TEpi	I	0	0	0	0	0
DEC(LS)	I	0	86.6	100	100	100
HECBSL	I	0	100	100	100	100
HECSL	I	0	93.3	100	100	100
FPLS	I	0	40	66.6	66.6	100
LeuELS	I	0	0	0	0	0
HP/HTCM	I	0	60	66.6	60	93.3
HP/HTCC	I	0	13.3	93.3	60	100
CCSL	I	0	86.6	93.3	86.6	93.3
MuLS	I	0	26.6	26.6	20	93.3
DiC	I	0	100	100	93.3	100
CDe	I	0	66.6	100	93.3	100
VC	I	0	100	100	93.3	100
Par	I	0	0	0	0	0
CFSSL	I	0	26.6	60.3	60.3	100
CFALSL	II	0	0	0	0	53.3
CD	II	0	0	66.6	73.3	100
ER	II	0	0	0	66.6	100
Hem	II	0	0	0	0	0
An	II	0	0	0	20	0
Fib	III	0	0	0	0	0
Nec	III	0	0	0	66.6	86.7

Each value represents, in percentage, number of damage fishes in relation to total fishes (N = 15) for each concentration. HTEC = hypertrophy of epithelial cells; TEpi = thinning of epithelium; DELC(LS) = displacement or lifting up of epithelial cells (LS); HECBSL: hyperplasia of epithelial cells at the basis of secondary lamellae; HECSL = hyperplasia of epithelial cells on secondary lamellae; LeuELS = presence of leukocytes; HP/HTCM = hyperplasia/hypertrophy of mucous cells; HP/HTCC = hyperplasia/hypertrophy of chloride cells; CCSL = presence of chloride cells on secondary lamellae; MuLS = presence of mucous cells on secondary lamellae; DiC = dilatation of capillaries; CDe = capillary disarrangement; VC = vascular congestion; Par = presence of parasites; CFSSL = complete fusion of some secondary lamellar; CFASL = complete fusion of all secondary lamellae; CD = cellular degeneration: ER = epithelial rupture; Hem = hemorrhage; An = aneurism; Fib = fibrosis; Nec = necrosis.

Table 4. Occurrence of alterations in percentage on gills of treated groups exposed to different concentrations of TPJM.

particular the disruption of hepatic cords, loss or atypical contour of hepatocytes, intense vacuolation, and decreased glycogen amount.

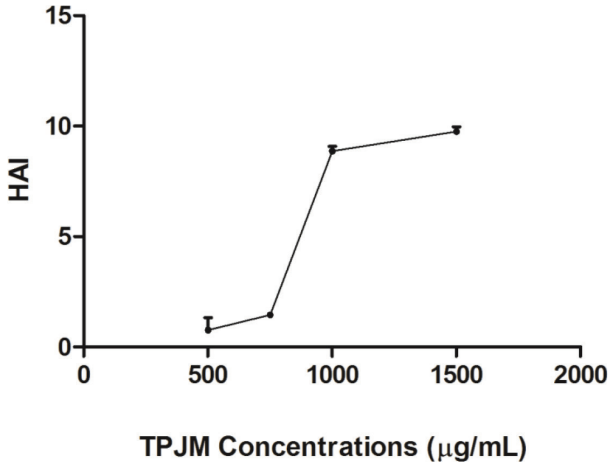


Figure 6. .Mean HAI obtained from histopathological alterations observed on *D. rerio* gills exposed to TPJM (500, 750, 100 and 1500 μg/mL). Each point represents mean ± SEM (N = 15/group). ANOVA followed by Tukey-Kramer test: 500 μg vs. control $p < 0.05$; 750 μg/mL vs. control $p < 0.001$; 1000 vs. control $p < 0.001$; 1500 vs. control $p < 0.001$.

The increase in cell volume of hepatocytes is observed with treatment with TPJM and reflects the hyperfunctional condition of the liver (**Figures 7** and **8**). The increase in hepatic metabolism may also lead to necrosis whereas the core becomes hypertrophic or even degenerates (histopathologic change stage III); both changes are found in the three higher concentrations.

The biliary stagnation was another stage of manifestation I (**Table 6**) observed in treatments with TPJM at all concentrations. However, cholestasis was seen on the 750, 1000, and 1500 μg/mL treatments; it is characterized by a manifestation of a pathophysiological condition (**Figures 7** and **8**) assigned to metabolic failure or the excretion of bile pigments, because the metabolites excreted as bilirubin need to be solubilized in water, a process which occurs only through conjugation with glucuronic acid.

Triplicate	Control	500 μg/mL of TPJM	750 μg/mL of TPJM	1000 μg/mL of TPJM	1500 μg/mL of TPJM
01	0.0	0.80	1.46	9.46	9.53
02	0.0	0.86	1.46	8.8	10.2
03	0.0	0.67	1.46	8.87	9.53
Mean	0.0	0.77	1.46	9.04	9.75
Average standard error	±0.0	±0.56	±0.0	±0.21	±0.22

Table 5. Mean of histological alteration index (HAI) of ***D. rerio*** gills after exposure do different concentrations of TPJM in triplicate (n = 15 animals/group).

Alterations	Stage	Control	500 μg/mL of TPJM	750 μg/mL of TPJM	1000 μg/mL of TPJM	1500 μg/mL of TPJM
HCD	I	0	93.3	100	100	100
OCLA	I	0	100	100	100	100
ONLA	I	0	100	80	100	100
ICV	I	0	100	100	93.3	100
INV	I	0	60	73.4	80	100
CV	I	0	66.7	60	80	100
DRFN	I	0	93.3	100	80	100
IRFBV	I	0	53.4	80	80	100
IRVBV	I	0	20	20	46.7	100
GR	I	0	80	53.4	80	100
BS	II	0	46.7	86.7	93.3	100
Hyp	II	0	6.7	66.7	100	100
DBV	II	0	60	86.7	93.3	100
DBC	II	0	26.7	73.4	93.3	100
NV	II	0	0	66.7	60	100
CD	II	0	0	6.7	20	40
ND	II	0	0	0	26.7	66.7
NA	III	0	0	6.7	0	20
CDis	III	0	0	33.5	80	80

Each value represents, in percentage, number of damage fishes in relation to total fishes (N = 15) for each concentration. HCD = hepatic cordon disarrangement; OCLA = outline cell loss or atypia; ONLA = outline nuclear loss or atypia; ICV = increase in cell volume; INV = increase in nuclear volume; CV = cytoplasmic vacuolation; DRFN = decrease in relative frequency of nuclei; IRFBV = increase in the relative frequency of blood vessels; IRVBV = increase on relative volume of blood vessels; GR = glycogen reduction; BS = biliary stagnation; Hyp = hyperemia; DBV = disruption of blood vessels; DBC = degeneration bile canaliculi; NV = nuclear vacuolation; CD = cytoplasmic degeneration; ND = nuclear degeneration; NA = nuclear atrophy; CDis = cellular disruption.

Table 6. Occurrence of alterations in percentage on liver of treated groups exposed to different concentrations of TPMJ.

The most frequent stage II changes (**Table 6**) were hyperemia, degeneration, and vacuolization (nuclear/cytoplasmic). The redness may indicate an adaptation process which leads to an increased blood flow to the liver tissue, facilitating the transport of macrophages to the damaged tissue and also the growth of the oxygenation of these areas or may indicate a secondary mechanism of detoxification. Changes such as nuclear and cell degeneration and cell strain may indicate contour dysfunctions induced by a toxic agent, since metabolically active areas of the liver are reduced, leading to a possible reduction in the overall functions performed by this organ.

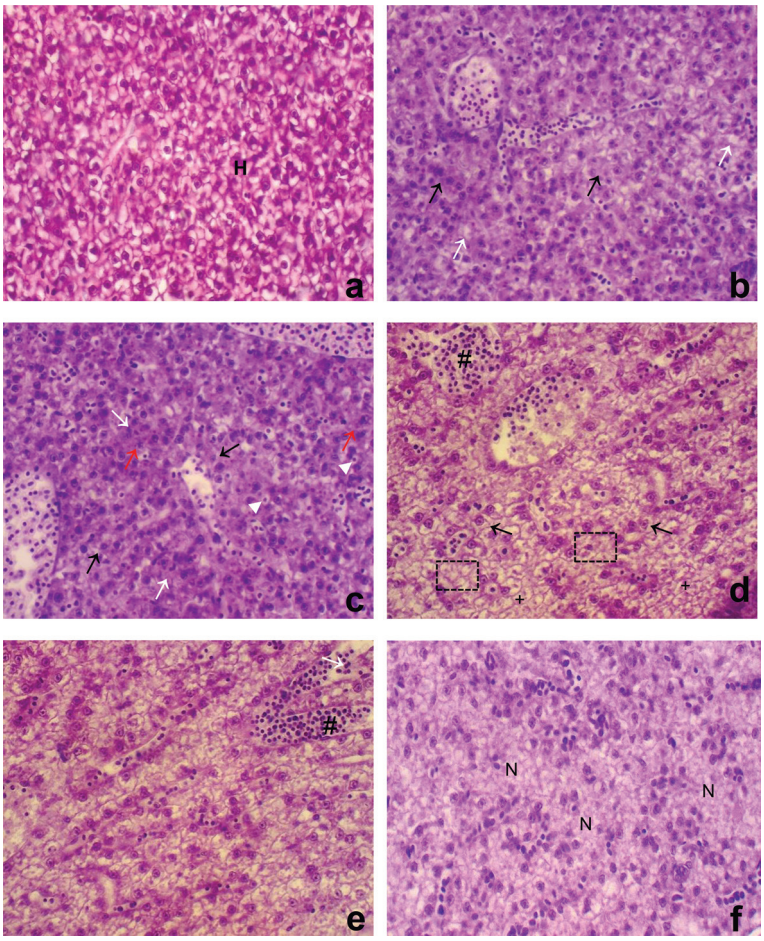


Figure 7. Histopathological changes in the livers of *D. rerio* exposed to different concentrations of TPJM. (a) Normal liver on longitudinal histological section. Hepatocytes (H) (400×). (b) Liver on longitudinal histological section exposed to 500 μg/mL of TPJM. Outline cell atypia (black arrows) and derangement of the hepatic cords (white arrows) (400×). (c) Liver on longitudinal histological section exposed to 750 μg/mL of TPJM. Nuclear atrophy (black arrows), cytoplasmic degeneration (white arrows), biliary stagnation (white arrow heads) and nuclear degeneration (red arrows) (400×). (d) Liver on longitudinal histological section exposed to 1000 μg/mL of TJPM. Glycogen reduction (+), decrease in the relative frequency of nuclei (dotted area), nuclear vacuolation (black arrows) and hyperemia (#). (e) Liver on longitudinal histological section exposed to 1500 μg/mL of TPJM. Hyperemia (#) and vessel rupture (white arrow) (400×). (f) Liver on longitudinal histological section exposed to 1500 μg/mL of TJPM. Focal necrosis (N) (400×).

As for stage III changes (**Table 6**), these were noted in the three major concentrations and are represented by the rupture vessels and overall or focal necrosis.

Table 6 and **Figures 7** and **8** show that the concentrations of TPJM (750, 1000, and 1500 μg/mL) caused significant changes when compared to the control; also no alterations were observed

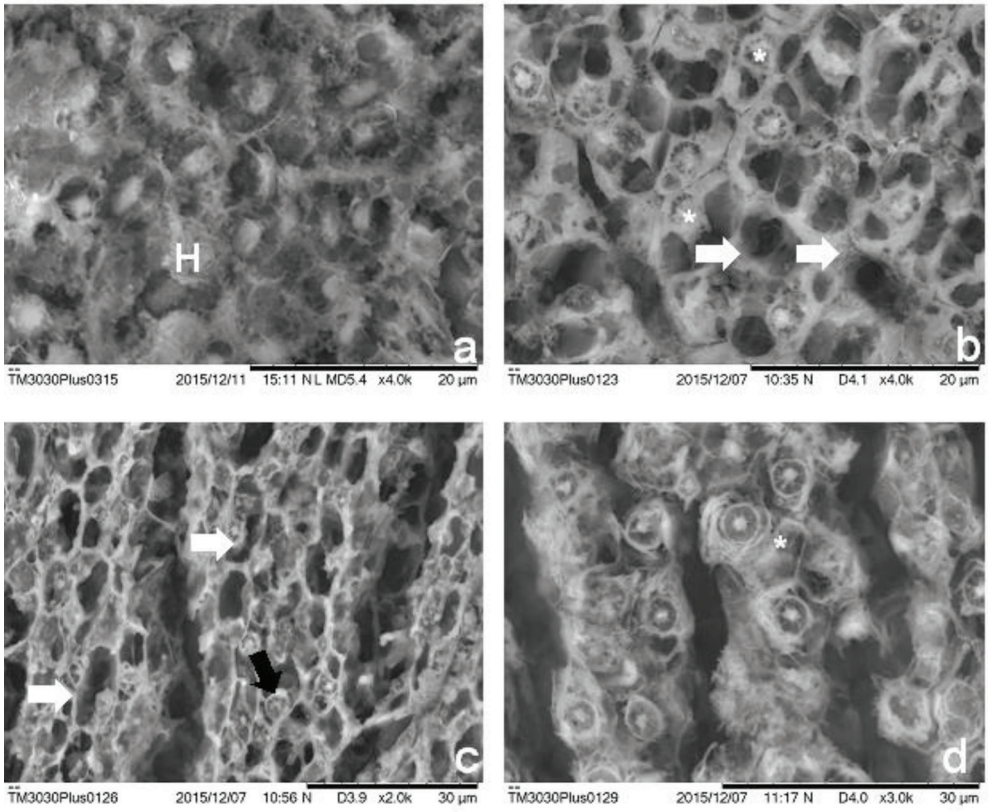


Figure 8. Histopathological analysis by scanning electron microscopy of the livers of *D. rerio* exposed to different concentrations of TPJM. (a) Normal liver on longitudinal histological section. Hepatocytes (H) (3000×). (b) Liver on longitudinal histological section exposed to 750 µg/mL of TPJM. Outline cell atypia (*), nuclear atypia (white arrow) (4000×). (c) Liver on longitudinal histological section exposed to 1000 µg/mL of TJPM. Outline cell atypia (white arrow), nuclear vacuolation (black arrow) (2000×). (d) Liver on longitudinal histological section exposed to 1500 µg/mL of TPJM. Nuclear degeneration (*) (3000×).

at 500 µg/mL. Interestingly, when comparing the IAH liver with the ones exposed with TPJM, it is also possible to observe that only the comparison between the concentrations of 750 versus 1000 µg/mL was not significant (**Table 7**).

The pathological changes in the kidneys of *Danio rerio* were evaluated in the lymphoid tissue, glomeruli, and renal tubules and blood vessels. **Table 8** shows the quantitation of kidney histopathological changes and **Figures 7** and **8** show changes in the qualitative data. Thus, virtually all stage I changes were observed in the various treatment concentrations.

Necrosis was ranked in this study as change stage III, being observed at the three highest exposure concentrations of TPJM (**Table 8**).

Triplicate	Control	500 μg/mL of TPJM	750 μg/mL of TPJM	1000 μg/mL of TPJM	1500 μg/mL of TPJM
01	0.0	1.2	10.7	11.3	18.7
02	0.0	2.7	10.7	12	12
03	0.0	3.9	11.3	10.7	18.7
Mean	0.0	2.6	10.9	11.3	16.5
Average standard error	±0.0	±0.78	±0.2	±0.38	±2.23

Table 7. Mean of histological alteration index (HAI) of ***D. rerio*** liver after exposure do different concentrations of TPJM in triplicate (n = 15 animals/group).

Alterations	Stage	Control	500 μg/mL of TPJM	750 μg/mL of TPJM	1000 μg/mL of TPJM	1500 μg/mL of TPJM
LCO	I	0	60	80	100	100
LDH	I	0	93.3	100	100	100
HTC	I	0	93.3	93.3	100	100
TDis	I	0	93.3	100	100	100
GDis	I	0	93.3	86.7	93.3	100
IBCS	I	0	80	46.7	33.3	20
DBCS	I	0	26.6	73.3	60	93.3
DGC	I	0	20	53.3	26.6	33.3
PRT	I	0	20	13.3	0	40
DRFG	I	0	46.7	60	80	86.7
DilBV	I	0	60	93.3	73.3	100
ITL	I	0	60	46.7	46.7	66.7
TO	I	0	100	73.3	93.3	100
SDTH	II	0	13.3	73.3	80	100
TD	II	0	86.7	93.3	100	100
GD	II	0	33.3	20	93.3	100
CDTC	II	0	86.7	73.3	100	100
NDTC	II	0	33.3	60	100	100
PLTBC	II	0	53.3	60	13.3	100
Hyp	II	0	6.7	73.3	93.3	100
RBV	III	0	0	0	0	40
Nec	III	0	0	53.3	66.7	100

Each value represents, in percentage, number of damage fishes in relation to total fishes (N = 15) for each concentration. LCO = loss of cellular outline or atypical cellular outline on lymphoid tissue; LDH = low degeneration of hyaline; HTC = hypertrophy of tubular cells; TDis = tubular disorganization; GDis = glomerular disorganization; IBCS = increase on Bowman´s capsule space; DBCS = decrease on Bowman´s capsule space; DGC = dilatation of glomerular capillaries; PRT = presence of regenerated tubules or "new" nephrons; PSG = presence of several granules PAS positive on tubular cells; DRFG = decrease in the relative frequency of glomerulus; DilBV = dilatation of blood vessels; ITL = increase in tubular lumen; TO = tubular obstruction; SDTH = severe degeneration on tubular hyaline; TD = tubular degeneration; GD = glomerular degeneration; CDTC = cytoplasmic degeneration of tubular cells; NDTC = nuclear degeneration of tubular cells; PLTBC = presence of lymphoid tissue on Bowman´s capsule; Hyp = hyperemia; RBV = rupture of blood vessels; Nec = necrosis.

Table 8. Occurrence of alterations in percentage on kidney of treated groups exposed to different concentrations of TPMJ.

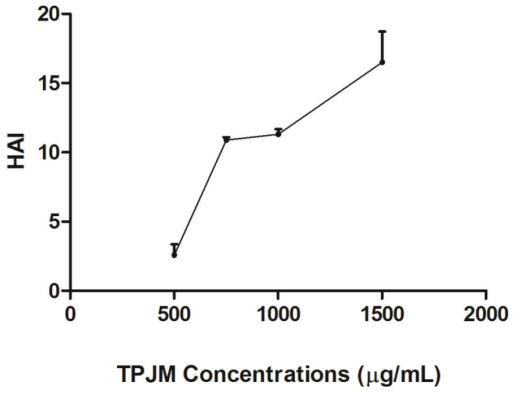


Figure 9. Mean HAI obtained from histopathological alterations observed in liver *D. rerio* exposed to TPJM concentrations (500, 750, 1000 and 1500 μg/mL). Each point represents mean ± SEM (N = 15/group). ANOVA followed by Tukey-Kramer: 500 μg vs. control no significative; 750 μg/mL vs. control $p < 0.001$; 1000 vs. control $p < 0.001$; 1500 vs. control $p < 0.001$.

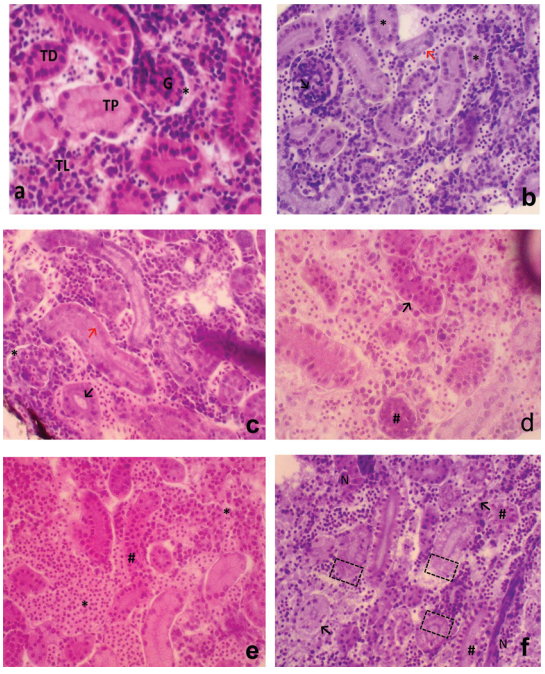


Figure 10. Histopathological changes in the kidney of *D. rerio* exposed to different concentrations of TPJM. (a) Normal kidney on longitudinal histological section. Glomerulus (G). Intercapsular space (*). Lymphoid tissue (TL), distal tubule (TD), proximal tubule (TP). (b) Kidney on longitudinal section exposed to 500 μg/mL of TPJM. Dilated glomerular capillaries (black arrow), tubular obstruction (*) and tubular disorganization (red arrow) (400×). (c) Kidney on longitudinal section exposed to 750 μg/mL of TPJM (400×). Decrease on Bowman´s capsule space (*), increase in tubular lumen (black arrow), cytoplasmic degeneration (red arrow). (d) Kidney on longitudinal section exposed to 1000 μg/mL of TPJM (400×). Glomerular degeneration (#) and degeneration of tubular hyaline (black arrow). (e) Kidney on longitudinal section exposed to 1000 μg/mL of TPJM (400×). Tubular degeneration (#) and hyperemia (*). (f) Kidney on longitudinal section exposed to 1500 μg/mL of TPJM. Nuclear degeneration (dotted area), tubular degeneration (#), necrosis (N) and degeneration of tubular hyaline (black arrow).

These histopathological changes were noted at all concentrations of TPJM treatment in an increasing manner about the percentage of occurrence, while the other histopathological changes related to Bowman's capsule (AECB) were not manifested (**Figures 10** and **11**).

The appearance of blood cells, blood cell aggregates, or foreign matter in Bowman's space can also occur [17, 18]. Sometimes the excess of red blood cells in the capillary can lead to the rupture of these vessels and, in this case, it is common to find cells in Bowman's space. Rupture of the capillaries due to the excess of erythrocytes has been observed at the concentration of 1500 μg/mL and it is classified as a stage III change (**Table 9**).

All of these observations can be explained by the phytochemical composition of *H. drasticus*, which has phytochemical markers such as plumeride and isoplumeride, which are typically lipophilic compounds.

The concentrations that caused mortality showed kidneys with mild-to-moderate changes. The concentration of 750 μg/mL has caused changes in this level, demonstrating that there is already kidney damage at this concentration (**Figure 12**).

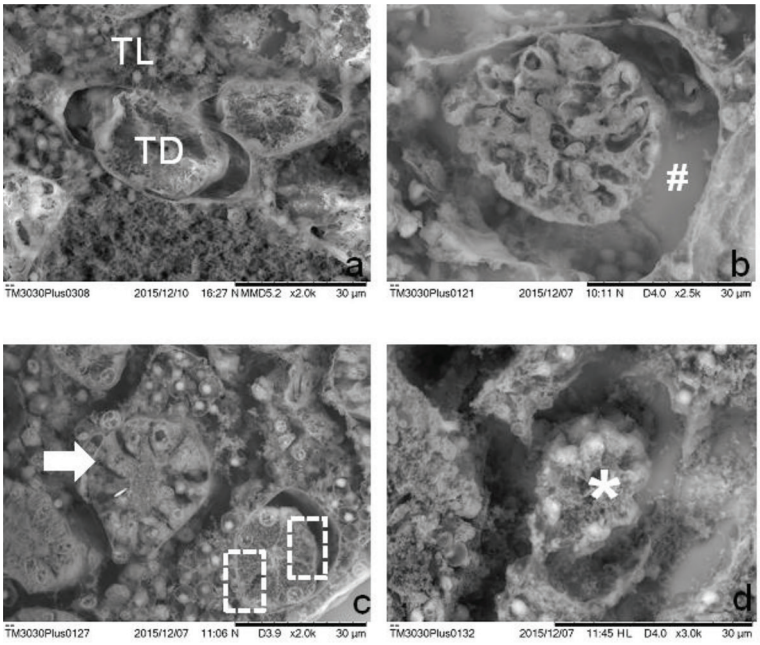


Figure 11. Histopathological analysis by scanning electron microscopy of the kidney of *D. rerio* exposed to different concentrations of TPJM. (a) Normal kidney on longitudinal histological section. Lymphoid tissue (TL) and distal tubule (TD) (2000×). (b) Kidney on longitudinal section exposed to 750 μg/mL of TPJM. Increase on Bowman's capsule space (#) (2500×). (c) Kidney on longitudinal section exposed to 1000 μg/mL of TPJM. Degeneration of tubular hyaline (arrow), nuclear degeneration of tubular cells (dotted area) (2000×). (d) Kidney on longitudinal section exposed to 1500 μg/mL of TPJM. Glomerular degeneration (*) (3000×).

Triplicate	Control	500 μg/mL of TPJM	750 μg/mL of TPJM	1000 μg/mL of TPJM	1500 μg/mL of TPJM
01	0.0	2.73	11.53	12.2	18.67
02	0.0	3.4	12.06	11.33	18.86
03	0.0	4.2	11.53	11.53	18.67
Mean	0.0	3.44	11.70	11.68	18.73
Average standard error	±0.0	±0.42	±0.17	±0.26	±0.06

Table 9. Mean of histological alteration index (HAI) of ***D. rerio*** kidney after exposure do different concentrations of TPJM in triplicate (n = 15 animals/group).

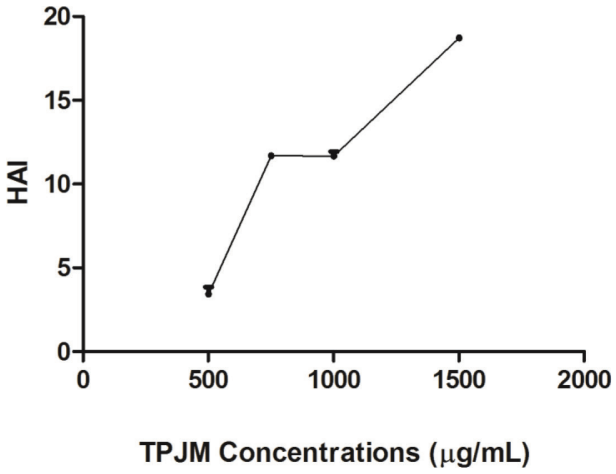


Figure 12. Mean HAI obtained from histopathological alterations observed on *D. rerio* kidneys exposed to TPJM (500, 750, 1000 and 1500 μg/mL). Each point represents the mean ± SEM (N = 15/group). ANOVA followed by Tukey-Kramer test: 500 μg/mL vs. control $p < 0.001$; 750 μg/mL $p < 0.001$; 1000 μg/mL vs. control $p < 0.001$; 1500 μg/mL vs. control $p < 0.001$.

4. Discussion

Previously reported by Vale [19] indicates that he obtained the yield of the ethanol extract of the bark *Himatanthus articulatus* equivalent to 16.82% by lyophilization, in comparison with other studies [20], and obtained a yield of 3.64% from the *H. articulatus* extract [21] and 20.12% of the methanol extract of *H. drasticus* leaves by maceration.

The stress signal manifestation and "*leakage*" (increased swimming activity) were present in all concentrations even at the beginning of the experiment. Within the behavioral changes observed were increased swimming activity, tremors on the axis of the tail, loss of mobility,

loss of posture, animal deposition tank bottom, and ultimately death. In addition to these manifestations, fish shallow breathing was also observed; this indicates a malfunction on the condition of the animal [22]. Other authors also report it as a defense mechanism against stressful conditions or elements present on their environment [23], which is an attempt to decrease the likelihood of death, or else there may be a metabolic cost-saving behavior to maintain the overall physiological homeostasis.

The behavioral changes described by Ribeiro [12], at the concentration of 170 μg/mL ethanolic extract of Jambu, are similar with the two highest concentrations of TPJM (1000 and 1500 μg/mL).

According to Barreto [24], the gills are especially sensitive to toxic substances dissolved in the aqueous environment because of their direct contact with the water in the gas exchange. Since Hoffman et al. [25] report that the gills are sites of potential absorption for toxic agents present in the water, and they are considered as the main site for the absorption of toxic agents, due to their characteristics such as large surface contact, the small diffusion distance, and the large countercurrent flow between water and blood.

According to Rigolim-Sá [26], the proliferation of the respiratory epithelium-like massive hyperplasia and lamellar fusion, among other changes, is the first gills' defense mechanism that promotes increased water-blood barrier enhancing the detoxification process. Epithelial proliferation is an initial response of the respiratory system. These defense mechanisms are typical because they reduce or even entirely prevent the passage of water between the secondary lamellae. This loss of respiratory surface can cause death by anoxia [27–29].

Meletti and Rocha [16] reported that the mucous cells and chloride cells might become hyperplastic and/or hypertrophic as a result of toxic agents present in the water. The presence of chloride cells and mucous cells in the secondary lamellae, as well the hypertrophy and hyperplasia, was observed at all concentrations, which demonstrate the potential change of the standard metabolism of these cell types when TPJM was administered due to proliferation expression proteins or the enhancement on their transport activity [1].

Takashima and Hibya [17] reported that many pathogenic agents might lead to epithelial swelling, vacuolization, and necrosis of secondary lamellae as observed in this study. However, this amendment undermines the primary function of the gills, whose function is to carry out the gas exchange, endangering the homeostasis of the body [16].

The hepatic metabolism of the *D. rerio* is similar to mammals and rodents. This fish metabolizes drugs using similar pathways used by humans. They have a wide range of cytochrome P-450 enzymes, allowing metabolic reactions, including hydroxylation, conjugation, oxidation, demethylation, and deethylation. Also, the liver synthesizes the bile salts, stores the glycogen, and produces vitellogenin, a protein present in the skin that surrounds the egg [30, 31].

Meletti and Rocha [16] points out that the vacuolation in hepatocytes can be an indirect measure but the not very precise amount of glycogen or lipids contained in this cell type.

Silva [32] reports that a reduction of glycogen in hepatocytes may represent the beginning of a nonspecific response to stress in fish induced by different chemical compounds. All stage I changes were present in various concentrations of treatment. It may be noted in particular the disruption of hepatic cords, loss or atypia contour of hepatocytes, intense vacuolation, and decreased glycogen like the observations shown (**Figures 7–9**).

However, the reduction of bilirubin binding capacity of this acid may explain the liver dysfunction [17, 33].

Robins and Contran [34] state that necrosis in liver tissue is related to intoxication processes, indicating that the severity of the injury is proportional to the type of, duration of the inflammation, the severity of aggression, and physiological state. Poleksic and Karan [35] observed necrosis in carp hepatocytes and exposed the importance of histopathology to analyze the damage similar to the observations with the treatment with the NSAID drug paracetamol [36].

The kidneys are the main route of excretion for metabolites of various xenobiotics on the zebrafish. It represents one of the common routes of elimination due to the urine formation and the excretion on an aqueous environment, some by glomerular filtration, others by resorption, or by secretion of processes tubular [32].

The kidneys receive large blood flow; the presence of chemicals in the blood can lead to some pathological changes in the Bowman's capsule, such as the abnormal proliferation of epithelial cells and thickening of the basal membrane, resulting in the reduction of Bowman's space. This change can impair the filtration process of the whole blood and renal function [17, 18].

The tubular obstruction is a histopathologic change of stage I. According to Meletti and Rocha [16], this change is classified as a type and "cloudy swelling," which is characterized by the presence of tubular epithelial cells or swollen with hypertrophied fine granules of eosinophils in the cytoplasm.

The hyaline degeneration was also observed at the concentrations tested. This modification is characterized by the presence of massive eosinophil granules, which according to Takashima and Hibyia [17] and Hilton and Lauren [18] can be produced inside the cell or formed by reabsorption of excess protein substances possibly formed by the glomerulus. Meletti and Rocha [16] affirms that many of the tubular changes observed in the kidneys of *D. rerio* are caused by metabolic disorders by toxic agents. Also, according to Takashima and Hibyia [17], most tubular degenerative changes are often culminating in necrosis.

The dilation of the glomerular capillaries and the glomerular degeneration were frequent changes in all groups exposed to TPJM. According to Takashima and Hibyia [17], the occurrences of these changes are manifested on pathological conditions due to changes of the basal

lamina and are typically accompanied by changes in podocyte and endothelial cells, such as hyperplasia.

The occurrence of new nephrons or tubules regeneration was unusual in groups treated with TPJM (**Figure 10**). Preexisting renal tubules can often be regenerated after being damaged by some diseases or even toxic agents [17]. According to Hinton and Laurén [18], tubular regeneration in fishes can be a good indication of adaptation and recovery. Also, after kidney damage induced by toxic agents, there may be entirely new nephron production [17, 37]. However, one can find new nephrons in unpolluted water fish, without the presence of toxic or xenobiotic agents (**Figures 11** and **12**).

All this evidence provides for the first time some information of the histopathological changes on the main detoxification organs of *D. rerio* by the exposition of TPJM, although further analysis is needed to be done in order to correlate the phytochemical composition with the toxic effects or the therapeutical ones.

5. Conclusions

The exposure of *Danio rerio* in concentrations of 500, 750, 1000, and 1500 μg/mL of TPJM for 48 h concludes that the TPJM can be considered low toxicity when compared to products already tested in the same model, because their mortality and histopathological changes were observed at high concentrations, and based on the percentage of extractives TPJM which was 1.85%, LC_{50} equal to dose 475 mg/kg, and according with the traditional statement, which is 6 tablespoons/day, it can be inferred that only 0.5 g of active ingredients are ingested by an adult user per day, corresponding to a dose of 7.14 mg/kg, which is a far lethal dose, and the doses producing histopathologic lesions, demonstrating the low toxicity of TPJM. However, discretion is advised for the use of this phytopreparation due to the poisoning risks existing in high doses, especially face of health problems that are indicated (lung cancer, lymphatic cancer, intestinal worms, fever, and gastric ulcers).

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Conflict of interest

The authors declare that there are no conflicts of interest.

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