Hydroethanolic extract from *Endopleura uchi* (Huber) Cuatrecasas and its marker bergenin: Toxicological and pharmacokinetic studies *in silico* and *in vivo* on zebrafish

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ARTICLE INFO

ABSTRACT

Endopleura uchi, is used for the treatment of inflammatory disease and related to the female reproductive tract. The aim of this study was to evaluate the acute toxicity of the *Endopleura uchi* stem bark hydroethanolic extract (EEu) in zebrafish, emphasizing the histopathological and biochemical parameters, as well as evaluating the *in silico* pharmacokinetic and toxicological parameters of the phytochemical/pharmacological marker, bergenin, as their metabolites. The animals were orally treated with EEu at a single dose of 75 mg/kg, 500 mg/kg, 1000 mg/kg and 3000 mg/kg. the oral LD_{50} of the EEu higher to the dose of 3000 mg/kg. Behavioral, biochemical and histopathological changes were dose dependent. *In silico* pharmacokinetic predictions for bergenin and its metabolites showed moderate absorption in high human intestinal absorption (HIA) and Caco-2 models, reduced plasma protein binding, by low brain tissue binding and no P-glycoprotein (P-Gp) inhibition. Their metabolism is defined by the CYP450 enzyme, in addition to bergenin inhibition of CYP2C9, CYP3A4 and CYP2C19. In the bergenin and its metabolites *in silico* toxicity test it have been shown to cause carcinogenicity and a greater involvement of the bergenin with the CYP enzymes in the I and II hepatic and renal metabolism's phases was observed. It is possible to suggest that the histopathological damages are involved with the interaction of this major compound and its metabolites at the level of the cellular-biochemical mechanisms which involve the absorption, metabolization and excretion of these possible prodrug and drug.

1. Introduction

Yellow uchi, scientifically known as *Endopleura uchi*, is a medicinal plant belonging to the family Humiriaceae. It is a species of the non-flooded forest, that presents high canopy and fruits very appreciated by the people, throughout the Brazilian Amazon [1]. The stem stumps are used in the form of tea, in traditional medicine for the treatment of arthritis, rheumatism, hypercholesterolemia and diabetes mellitus with

a large registry of traditional use in pathologies directed to the female reproductive tract, such as inflammation and uterine infections and as abortive [2]; Shanley & Gaia, 1998.

The medicinal plants are strategic sources with great importance for the discovery of new and effective agents with pharmacological effects [3]. However, popular and even traditional use is not enough to ethically validate natural products as effective and safe. In this sense, they are no different from another synthetic xenobiotic. Its recommendation or official authorization of its medicinal use must be based on experimental evidences, proving the safety of its use [4].

The bergenin is the major compound of the hydroethanolic extract of the stem barks of *Endopleura uchi*. Bergenin has been shown to have effects on osteoblastic cellular functions [5], as an immunomodulatory agent [6], effective in the treatment of acute lung injury [7], anxiolytic [8], antimalarial [9], antioxidante [10], anti-inflammatory agent [11,12] antinociceptive [13], antimicrobial [14] neuroprotective [15,16], anti-arthritic [17], hepatoprotective action [18] and anti-hepatotoxic [19]. It is also indicated as responsible, among the bioactive compounds of the extract, by the several biological activities reported. These results justify the growing interest in the study of this plant species.

Zebrafish is an effective model to test *in vivo* activity of many chemical compounds. When compared to rodents, the toxicology of zebrafish is much less expensive and can be carried out rapidly in large numbers of compounds. It can be inserted early in the pre-clinical development, which justifies the acceptance of studies with this animal species, by drug regulatory agencies [20].

According to Ducharme et al. [21], zebrafish can be used to accurately predict acute toxicity compared to inhalation in rats, rabbit skin pathways and oral exposure routes in rats. In addition, tissue changes in organs such as gills, kidneys, liver and intestines, caused by toxic effects of substances, can be evaluated using zebrafish [22]. Driessen et al. [23] compared zebrafish embryos to models traditionally used *in vivo* (rats and mice) and *in vitro* (primary human hepatocytes and rats and mice hepatocytes) in the liver toxicity tests.

The results showed that zebrafish is comparable to traditional models in identifying hepatotoxic activity. Similar results were observed for reproductive toxicity [21]. It is worth mentioning that the discovery of modern drugs is not only based on the pharmacological activities they can present, but it is also important to take into account the search for drugs with more favorable pharmacokinetic properties [24].

The evaluation of metabolic properties of a molecule can help to optimize the stability and consequently the *in vivo* half-life and the risk-benefit ratio of a drug. There is great interest in the development of computational tools to predict drug metabolism due to restrictions to study the human metabolism of xenobiotic compounds [25]. Zebrafish is also noted as a suitable model for the study of systems pharmacology, combining high yield potential with high genetic homology to superior vertebrates [26].

Elisavet et al. [27] studied cadmium exposure in zebrafish and the results were supported by histopathological studies that concluded that differentiated responses, could be linked to hormesis phenomena. In this study, histopathology was used to support the biochemical and compotarmental parameters observed in animals exposed to EEu.

It is important to note that this medicinal species is of great importance in traditional medicine, especially in the pan-Amazon region where it is widely used for the treatment of gynecological inflammatory processes. Therefore, despite the existence of phytochemical studies and some pharmacological studies, but it is of great relevance to carry out toxicology studies that can demonstrate the safety of the use of preparations based on this species, correlating with their main phytochemical and pharmacological markers.

Based on these descriptions and the potential of the traditional use of EEu, this study aimed to evaluate its acute toxicity in zebrafish emphasizing the histopathological and biochemical parameters, as well

as to evaluate the *in silico* pharmacokinetic and toxicological parameters of the phytochemical/pharmacological marker, bergenin, as well as its metabolites.

2. Material and methods

2.1. Collection and identification of botanical material

Endopleura uchi was collected in the Porto Grande City (N 0° 41′10.4″/ W 51° 29′39.8″, km 117, in the State of Amapá, Brazil. After identification of the botanical species, the samples were deposited in the Regional Herbarium of the Eastern Amazonian Embrapa IAN, under identification nº 196009.

2.2. Preparation of the extract

After harvesting the E. uchi stem bark, it was dissected in a circulating air heater at 40 °C, for 72 h and after, drying, crushed in a knife mill, thus obtaining the plant powder (1.632,87 g). Subsequently, this material was macerated in 70 % hydroethanolic solution, in a ratio of 1:5 at room temperature, for 10 days, under constant stirring. The macerate was then filtered and concentrated in a rotary evaporator at a temperature of 40 °C until complete evaporation of the solvent. The concentrated filtrate was subjected to lyophilization resulting in 2.65 % yield.

2.3. Analysis of EEu by HPLC-MS-MS

For EEu analysis was used an HPLC-MS system consisting of an Agilent 1100 Series HPLC and an Agilent 6410 Triple Quadrupole Mass Spectrometer. Ultra-High purity nitrogen was used as the drying gas. The sample was separated on a Zorbax SB-C18 column (5 µm, 4.5 X 150 mm; Agilent).

The separation was achieved using water (solvent A) and acetonitrile (solvent B) as the mobile phase. The gradient employed went from 90:10 (A:B) to 10:90 (A:B) in 10 min with a flow rate of 0.5 mL/min. Then, the gradient went from 10:90 (A:B) back to 90:10 (A:B) in 15.5 min using a flow rate of 1.5 mL/min. The system was operated at room temperature (20 °C). The detection was performed after electrospray ionization in negative ion mode. Dwell time was kept at 100 ms. The source temperature was kept at 650 °C, and the spray voltage was set at −4500 V. Ion source gas 1, ion source gas 2 and curtain gas were set at 45, 90 and 35, respectively. The MS-parameters were determined and optimized by post-column infusion of the compounds solution with a syringe pump. The ions were monitored for each molecule.

2.4. Experimental animals

Adult zebrafish (*Danio rerio*), a six-month-old AB wild-type of 3.7–4 cm long, weighing around 550 mg, were obtained from Acqua New Aquarium and Fish Ltda., located in Igarassu-PE, Brazil. After the arrival of the animals, they were kept for a period of quarantine and were maintained until the experiments were carried out on the Zebrafish Platform of the Laboratory of Research in Drugs, Biological Sciences and Health Department, Federal University of Amapá (UNIFAP), Brazil. The animals were kept in water under controlled conditions of temperature, feed and light/dark cycle, following the standards described by Souza et al. [28], Borges et al. [29]. This study was submitted and approved by the Committee on Ethics in Animal Use - CEAU of UNIFAP, receiving protocol number 003/2018.

2.5. Treatment groups and route of administration

After the acclimation period, the animals were randomly divided into a control group treated with saline solution and four groups treated at doses of 75 mg/kg, 500 mg/kg, 1000 mg/kg and 3000 mg/kg from

EEu. Each group was evaluated in three triplicates, each triplicate containing four animals, totalizing n = 12 animals/group. The animals were treated orally gavage as described by Borges et al. [29].

2.6. Determination of LD_{50}

For the determination of the lethal dose (LD_{50}), the animals were randomly collected and fasted for 24 h prior to the start of the experiments. Subsequently, they were administered orally at the doses of 75 mg/kg, 500 mg/kg, 1000 mg/kg and 3000 mg/kg of EEU. After the gavage procedure, they were distributed in aquariums containing water from the system.

2.7. Behavioral analysis and mortality

Once the administration of each dose occurred, the behavior and mortality of the animals corresponding to each group were recorded at 1 h, 3 h, 6 h, 24 h and 48 h after oral administration. The behavioral parameters observed were those described by Souza et al. [28]. The changes in zebrafish behavior were classified into three stages: I (higher swimming activity and presence of tail tremors), II (circular swimming and loss of balance), and III (loss of motility, rest at the bottom and death). Mortality was monitored continuously, and fish were considered dead when operculum movement and response to mechanical stimulation could no longer be detected.

2.8. Biochemical analysis

For biochemical analysis, blood samples from the various groups, including treated groups (doses of 75 mg/kg, 500 mg/kg, 1000 mg/kg and 3000 mg/kg) were collected after the animals were previously anesthetized. Then a diagonal incision was made with a steel blade between the caudal fin and the anal fin. Blood was collected with a 10 μl micropipette and stored in a eppendorf microtube.

Serum collection was performed shortly after the blood was centrifuged for ten minutes with a rotation of 2000 rpm in a Benfer Macro Centrifuge BF 10,887. The alanine aminotransferase ALT and aspartate aminotransferase AST parameters were determined on Bio-Plus equipment Bio-200 using the AST/GOT Liquiform and ALT/GPT Liquiform enzyme kits according to manufacturer Labtest instructions, respectively After the procedures described above, the animals were euthanized according to the guidelines for animal euthanasia AVMA, [30].

2.9. Histopathological analysis

2.9.1. Preparation of organs

For the preparation of organs (intestine, liver, kidney and gills), they were stored in identified cassettes and immediately fixed in bouin solution. Then, decalcification was carried out in 7 % EDTA solution for 24 h, dehydration in solutions with increasing concentrations of ethyl alcohol, diaphanization in xylol for clarification and inclusion with paraffin. The sections were obtained with a thickness of 5 μm in a microtome (Rotary Microtome Cut 6062, Slee Medical, Germany) and stained with Hematoxylin and Eosin following the technique described by Souza et al. [28]. The sections were analyzed under optical microscope (Olympus-micronal BX41) and photographed with digital camera ScopeImage 9.0 USB).

2.9.2. Assessment of histopathological changes

The analysis of the tissue alterations of the intestine, liver, kidney and gills were performed according to Souza et al. [28], Carvalho et al. [22] and Borges et al. [29]. The Index of Histopathological Changes (HAI) was calculated from the levels of tissue changes observed in gills, liver, kidney and intestine. Alterations were classified as levels I, II and III, and the HAI value indicated whether the organ was normal (0–10),

moderately altered (11–20), moderate to severe (21–50) or containing severe irreversible changes (> 100) [31], Ringolin-Sá, 1999, [32]).

Data on the total histological changes of the gills, kidneys, intestine and liver were calculated in relation to the total HAI values corresponding to each organ and expressed as a percentage.

2.10. Statistical analysis

The results obtained in the various analyzes were expressed as average ± average standard error (average ± ASE) of each experimental group. For the statistical analysis of the results of the experimental parameters of the histopathological study, one-way ANOVA test (Kruskal-Wallis) was used followed by Dunnett's test. For the analysis of the biochemical parameters, one-way ANOVA test followed by Tukey post-hoc. Statistical analyzes were performed using the GraphPad Prism program (version 5.03). Results with $p < 0.05$ were considered statistically significant.

2.11. In silicostudy

2.11.1. Prediction of bergenin metabolism using Metatox and SMARTCyp software

The Metatox virtual platform, which uses algorithms to predict the possible sites of metabolism of a molecule, from phase 1 reactions (oxidation, reduction and hydroxylation) and phase 2 (conjugation) [33,34]. In the first step of the generation of metabolites, MetaTox calculated the estimation of the types of reactions that occurred with a substrate in its entirety. The values obtained for the metabolite probability of formation (PC) were estimated from the PR (probability "for the reaction to be real") - estimated the chance that this predicted class of reaction occurred in the experiment, PI (probability "for the reaction to be impossible") - estimated the chance that this predicted class of reaction did not occur in the experiment, T-estimated the chance that the considered atom was modified during the biotransformation reaction and, PF - estimated the chance that the atom considered was not modified during the considered biotransformation reaction [33,34]. The probability of the parent compound is equal to one, P0 = 1.

The calculation of the probability of formation of the metabolite was performed by the following equation:

$$PC = \frac{P_R P_T}{P_R\, P_T + P_I P_F}$$

Bergenin was submitted to an online server, the MetaTox (http://www.way2drug.com/mg/), through a two-dimensional drawing in a database of several thousand xenobiotic biotransformations observed in humans and experiments with cells and tissues. It included information on 15 classes of phase I and phase II reactions of xenobiotic metabolism. These reactions were used to identify the patterns of different transformations of the fragments database. In addition, the estimate of organ specific carcinogenicity and adverse effects for metabolites generated and parent compounds in MetaTox was added.

2.11.2. In silicoevaluation of the pharmacokinetic and toxicological parameters of bergenin and its metabolites

The online Protox server (http://tox.charite.de/protox_II/index.php site = compound_search_similarity) was used to obtain values for LD_{50}, risk class, molecular mass (MM), hydrogen bonding acceptors (HBA), hydrogen bonding donors (HBD) and number of rotational bonds pertaining to bergenin and its metabolites. Another online server was also used, the PreADMET (https://preadmet.bmdrc.kr/), which predicted pharmacokinetic properties value: human intestinal absorption (HIA), cell penetrability *in vitro*using the Caco-2 cell model, plasma protein binding (PPB (%), brain-blood partition coefficient BBB $_{(C.brain/C.blood)}$, interaction with P-glycoprotein (P-gp), CYP450, Madin-Darby Canine Kidney Cells $P_{MDCK(nm/sec)}$ and LogP, besides the predictions of toxicological properties as mutagenicity and carcinogenicity in rats and
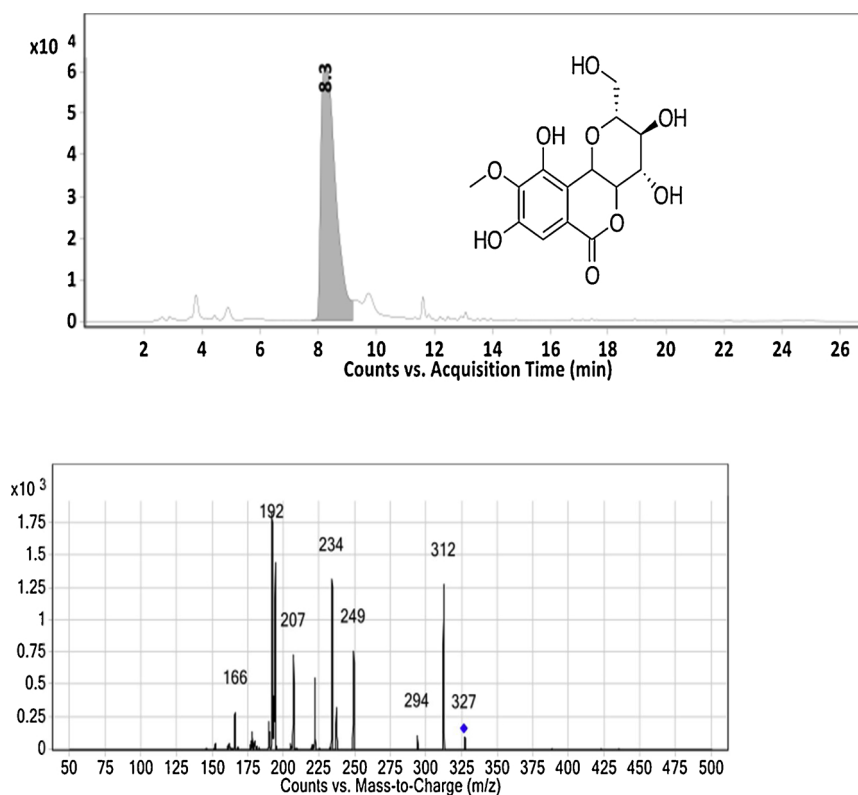
Fig. 1. TIC chromatogram of *Endopleura uchi* bark extract (EEu), HPLC/MS analysis and fragmentation pattern. Bergenin is the major compound of the extract.

mice, development (using Medaka fish as a parameter) [35] and the risk of cardiac toxicity due to inhibition of the ether-a-go-related (hERG) human gene.

Subsequently, the compounds were also evaluated using Derek software [36] to obtain additional data related to toxicity.

3. Results

3.1. EEu HPLC-MS-MS analysis

The major compound identified by HPLC-MS-MS in EEu, was the Bergenin (Fig. 1). This compound exposed the presence of a peak with retention time of 8.3 min and ion peak at *m/z* 328.27, which was consistent with the molecular formula $C_{14}H_{16}O_9$. – which was identified as bergenin.

3.2. Behavioral analysis and mortality

Oral treatment of the zebrafish groups with EEu at doses of 75 mg/kg, 500 mg/kg and 1000 mg/kg was not able to cause deaths of the animals. The dose that received 3000 mg/kg (higher dose) induced only 8.3 % death in relation to the total number of animals in the group (Table 1). There were no deaths in the control group treated with saline

Table 1
Quantity and percentage of animals killed after treatment with EEu orally at doses of 75 mg/kg, 500 mg/kg, 1.000 mg/kg and 3.000 mg/kg in Zebrafish.

Dose mg/kg	Number of dead animals	Percentage
Control Group	0/12	0 %
75 mg/kg	0/12	0 %
500 mg/kg	0/12	0 %
1.000 mg/kg	0/12	0 %
3.000 mg/kg	1/12	8.3 %

N = 12 animals/group.

solution. Therefore, the oral LD_{50} of the EEu higher to the dose of 3000 mg/kg.

The animals' behavior after exposure to EEu was classified into three stages (stage I, stage II and stage II). All groups were evaluated up to 48 h after exposure. Among the animals that received doses of 75 mg/kg and 500 mg/kg the presence of increased swimming activity (stage I) was observed only during the first hour after treatment. These data corroborate the behavior of the saline treated animals, regarding the control group. It is noteworthy that in the other hours these groups did not present behavioral changes (Table 2).

In relation to the animals treated with the dose of 1000 mg/kg, during the first hour after exposure to EEu, changes were observed such as increased swimming activity, tail tremors (stage I) and circular swimming movement characteristic of stage II behavioral change. Stage I changes were also noted in the third hour of analysis. After this period, the animals maintained their behavior unchanged until the end of the observation period (48 h) (Table 2).

During the first and third hour after treatment with EEu at a dose of 3000 mg/kg, the animals presented behavioral changes of stage I (increased swimming activity and tail tremors) and stage II (circular swimming movement, loss of posture). In the sixth hour after treatment, there was persistence of the behavioral alterations of stage II and the appearance of alterations of stage III (Table 2), causing the death of 1 animal of the group (8.3 %) (Table 1). After this period, the animals recovered and remained stable during the 24 h and 48 h analyzes.

3.3. Biochemical analysis

It was observed the elevation of the enzymatic activities of both ALT and AST that occurred in a directly proportional doses. As for ALT, there was a significant increase in the groups treated with the highest doses (1000 mg/kg and 3000 mg/kg) in relation to the control group ($p < 0.05$, one-way ANOVA followed by Tukey post-hoc). There was also a significant increase in the group treated with the highest dose (3000 mg / kg) in the treated groups at doses of 75 mg/kg, 500 mg/kg

Table 2
Behavioral changes in different fields of observation after oral treatment with EEu at doses of 75 mg/kg, 500 mg/kg, 1.000 mg/kg and 3.000 mg/kg in Zebrafish.

Behavioral Parameters	*D. rerio* with behavioral parameter change per group				
	Control Group	EEu 75 mg/kg	EEu 500 mg/kg	EEu 1.000 mg/kg	EEu 3.000 mg/kg
1 h					
Stage I	X	X	X	X	X
Stage II	–	–	–	X	X
Stage III	–	–	–	–	–
3h					
Stage I	–	–	–	X	X
Stage II	–	–	–	–	X
Stage III	–	–	–	–	–
6h					
Stage I	–	–	–	–	–
Stage II	–	–	–	–	X
Stage III	–	–	–	–	X
24 h					
Estage I	–	–	–	–	–
Estage II	–	–	–	–	–
Estage III	–	–	–	–	–
48 h					
Estage I	–	–	–	–	–
Estage II	–	–	–	–	–
Estage III	–	–	–	–	–

Stage I: increased swim activity, tail tremors; Stage II: Circular swimming movement, loss of posture; Stage III: death. (n = 12 animals/group).

and 1000 mg/kg (Table 3) (p < 0.05, ANOVA one-way followed by post-hoc Tukey).

In terms of AST activity, there was a significant increase in the groups treated with the doses of 75 mg/kg, 500 mg/kg, 1.000 mg/kg and 3000 mg/kg in relation to the control group. The groups treated at doses of 500 mg/kg, 1000 mg/kg and 3000 mg/kg differed significantly from the group treated with the lowest dose (75 mg/kg). The groups treated with the 1000 mg/kg and 3000 mg/kg doses differed significantly from the 500 mg/kg dose group (Table 3) (p < 0.05, one-way ANOVA followed by Tukey post-hoc).

3.4. Evaluation of histopathological parameters

3.4.1. Intestine

Following histopathological analysis of the intestinal tissue, it was possible to observe several changes of levels I and II and absence of level III changes (necrosis) in the groups treated with the doses of 75 mg/kg and 500 mg/kg of EEu (Fig. 2B and C, respectively). Level I alterations were: dilation of the vessels present in the villi, detachment of the epithelial lining of the intestinal villi, villous atrophy, epithelial cell hypertrophy, goblet cell hyperplasia, stromal lymphocytic infiltration, reduction in lamina propria, vacuolization of enterocytes, leukocyte infiltration, shortening of lamina propria, cell degeneration and distancing of the lamina propria. The level II alterations were: displacement of the lamina propria, desquamation of the intestinal mucosa, partial fusion of villi and hemorrhage in the lamina propria. The dose of 500 mg/kg also showed degeneration in the villi as a level II

alteration (Fig. 2C). Fig. 6 and Table 4 show that the HAI scores were 13.2 and 15.7 and the percentage of total alterations were 32.1 % and 38 %, respectively, showing that the organs remained moderately altered. The results did not differ significantly in relation to the control group.

In the intestines of the animals belonging to the 1.000 mg/kg and 3.000 mg/kg groups, in addition to the level I and II alterations, tissue necrosis (level III) was also identified (Fig. 2D and E). The HAI values were 30.5 and 40 (Fig. 6), respectively, showing that the alterations found influenced the normal functioning of these organs, since the organs were moderately to severely altered. Both groups showed a significant percentage of alterations in the intestinal tissue with 73.9 % and 96.9 %, respectively (Table 4). However, it should be noted that only the group treated with the highest dose (3000 mg/kg) differed significantly from the control group (p < 0.05, one-way ANOVA (Kruskal-Wallis) followed by the Dunnett post hoc test). In the control group, no tissue changes were found, thus, the organ was characterized as normal (Fig. 2A).

3.4.2. Liver

The control group did not present alterations in the hepatic tissue, remaining normal (Fig. 3A). In relation to the groups treated with doses of 75 mg/kg and 500 mg/kg, the most frequent alterations were the loss and atypia of the cellular contour, loss or atypia of the nuclear contour and intense cytoplasmic vacuolization (level I changes). Regarding to the level II changes, they presented hyperemia and nuclear degeneration as common alterations. However, the group treated with the 500 mg/kg dose also presented nuclear atrophy, cytoplasmic degeneration and cellular disruption (Fig. 3B and C, respectively). The HAI values were 5.5 and 10.1 (Fig. 6), respectively, showing that the alterations found did not influence the normal functioning of these organs. The percentages of total alterations were 11.6 % and 21.3 %, respectively, of total changes (Table 4).

The most frequent tissue changes in the 1.000 mg/kg dose group were loss of cell atypia, nuclear contour loss or atypia, intense cytoplasmic vacuolization, and nuclear vacuolation (level I changes). Level II changes were nuclear atrophy, cytoplasmic degeneration, nuclear degeneration, and hyperemia. It also presented necrosis as a level III alteration (Fig. 3D). The HAI of this group was 34 (Fig. 6), indicating that the organ had moderate to severe changes, with a percentage of 71.4 % of total alterations (Table 4). In contrast, the group treated with the 3.000 mg/kg dose also presented changes at levels I, II and III (Fig. 3E). However, the HAI (44) was higher, differing significantly from the control group (p < 0.05, one-way ANOVA (Kruskal-Wallis) followed by Dunnett's post hoc test) (Fig. 6). The percentage of total alterations of 92.4 % was also high, with a value of almost 100 % in relation to the total liver changes (Table 4).

3.4.3. Kidney

In the histopathological evaluation of the kidney, the control group remained as a normal organ (Fig. 4A). The groups treated with the doses of 75 mg/kg and 500 mg/kg showed changes of level I (tubular cell hypertrophy, tubular disorganization, increase of the Bowman's capsule and decrease in the relative frequency of glomeruli), and level II (tubular degeneration, cytoplasmic degeneration of tubular cells, nuclear degeneration of tubular cells, hyperemia and rupture of blood

Table 3
Changes in AST and ALT enzymatic activity in zebrafish treated orally, with the control group and doses of 75 mg/kg, 500 mg/kg, 1.000 mg/kg and 3.000 mg/kg.

Organs	Control	75 mg/kg	500 mg/kg	1.000 mg/kg	3.000 mg/kg
ALT	39.0 ± 6.00	75.5 ± 8.50	80.0 ± 2.00	96.0 ± 9.16[a]	898.5 ± 7.50[a,b,c,d]
AST	55.5 ± 7.50	725 ± 613.0[a]	824.0 ± 8.50[a,b]	1231.0 ± 7.50[a,b,c]	1268 ± 11.0[a,b,c]

Values represent the average ± E.P.M. The ANOVA test was applied, followed by the Tukey test. [a]p < 0.05 compared to the control group. [b]p < 0.05 compared to the 75 mg/kg dose. [c]p < 0.05 compared to a dose of 500 mg/kg. [d]p < 0.05 compared to a dose of 1.000 mg/kg.
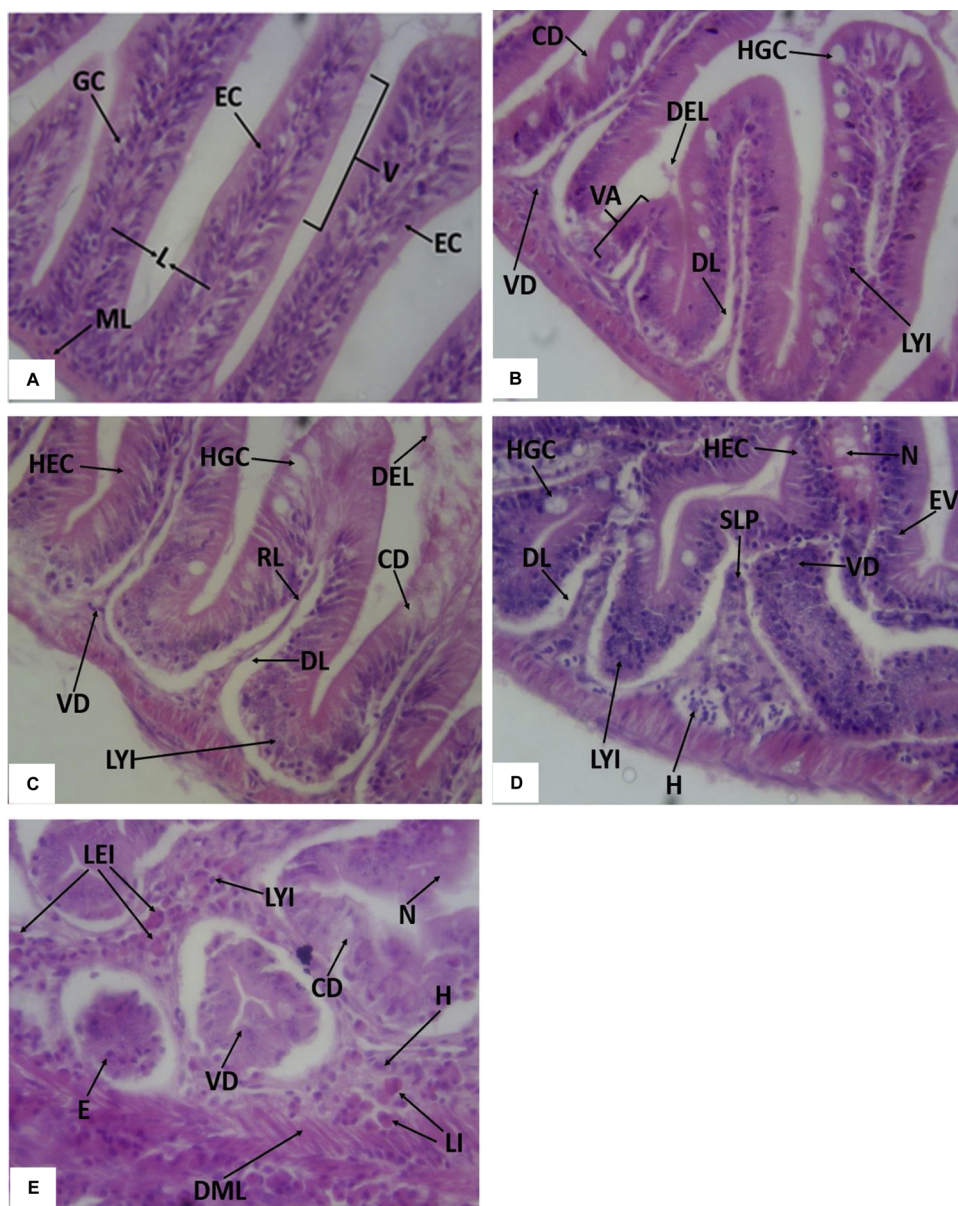
Fig. 2. Longitudinal cross section of zebrafish intestine treated with different doses of EEu. **A:** Control showing GC goblet cells, EC enterocytes, V villi, L lamina propria, ML muscle layer; **B:** EEu 75 mg / kg showing HGC goblet cell hypertrophy, DEL epithelial lining detachment, VD vessel dilatation, LYI lymphocyte infiltration, DL displacement of lamina propria, CD cell degeneration, VA atrophy of villi; **C:** EEu 500 mg/kg showing HGC goblet cell hypertrophy, DEL epithelial lining detachment, VD vessel dilatation, LYI lymphocyte infiltration, DL displacement of lamina propria, CD cell degeneration, HEC hypertrophy of epithelial cell, RL reduction of the lamina propria; **D:** EEu 1000 mg/kg showing HGC goblet cell hypertrophy, DEL epithelial lining detachment, LYI lymphocyte infiltration, DL displacement lamina propria, CD cell degeneration, HEC epithelial cell hypertrophy, RL reduction of lamina propria, SLP shortening of the lamina propria, N necrosis, RV villi degeneration, EV enterocytes vacuolization; **E:** EEu 3.000 mg/kg showing LYI lymphocyte infiltration, CD cell degeneration, LI leukocyte infiltration, DML degeneration of muscle layer, RV villi degeneration, E edema, N necrosis, H hyperemia. Flushed with HE, magnification of 400 × .

Table 4
Percentage of total histopathological changes presented in gills, liver, kidney and intestine of D. rerio after treatment with EEE at doses 75 mg/kg, 500 mg/kg, 1.000 mg/kg and 3.000 mg/kg.

Organs	Control	75 mg/kg	500 mg/kg	1.000 mg/kg	3.000 mg/kg
Intestine	0 %	32.1 %	38 %	73.9 %	96.9 %
Liver	0 %	11.6 %	21.3 %	71.4 %	92.4 %
Kidney	0 %	19.6 %	24.7 %	65.8 %	99.1 %
Gill	0 %	10.3 %	10.9 %	53.1 %	59.2 %

The percentage was determined on the number of animal per group (n = 12). According to Poleksic and MitrovicTutundzic (1994), Rigolin-Sá [84] and Takashima & Hibiya [85].

vessels) (Fig. 4B and C, respectively). Fig. 6 shows that the HAI scores were 9.9 and 12.5 demonstrating that the organ remained normal and moderately altered, respectively. The percentage of total changes was 19.6 % and 24.7 %, respectively, of total changes (Table 4). The group treated with the dose of 1.000 mg/kg presented the organ with moderate to severe changes (Fig. 4D), since the HAI was 33.2 (Fig. 6) and a percentage of 65.8 % (Table 4). Differently from the doses of 75 mg/kg

and 500 mg/kg in this group, the presence of necrosis in the organ, a level III change, was observed. Level I changes were mild tubular hyaline degeneration, tubular disorganization, glomerular disorganization, increased Bowman's capsule space, and dilation of the glomerular capillaries. Level II changes were: tubular degeneration, glomerular degeneration, cytoplasmic degeneration of tubular cells, nuclear degeneration of tubular cells, hyperemia and rupture of blood vessels.

The renal tissue of the group treated with the highest dose 3000 mg / kg had moderate to severe changes Fig. 4E), differing significantly from the control group ($p < 0.05$, one-way ANOVA (Kruskal-Wallis) followed by Dunnett's post hoc test.). The HAI was 50.0 and the percentage of changes in the renal tissue was expressive, with a value of 99.1 %, showing that this organ was already quite compromised (Fig. 6 and Table 4, respectively). This group, besides presenting all changes of level I, II and III in relation to the 1000 mg/kg group, also presented other changes of level I (atypical cellular contour, tubular cell hypertrophy, tubular obstruction, tubular lumen enlargement and decrease in the relative frequency of glomeruli) and II (severe tubular hyaline degeneration, presence of lymphoid tissue in Bowman's capsule and blood vessels' dilation).
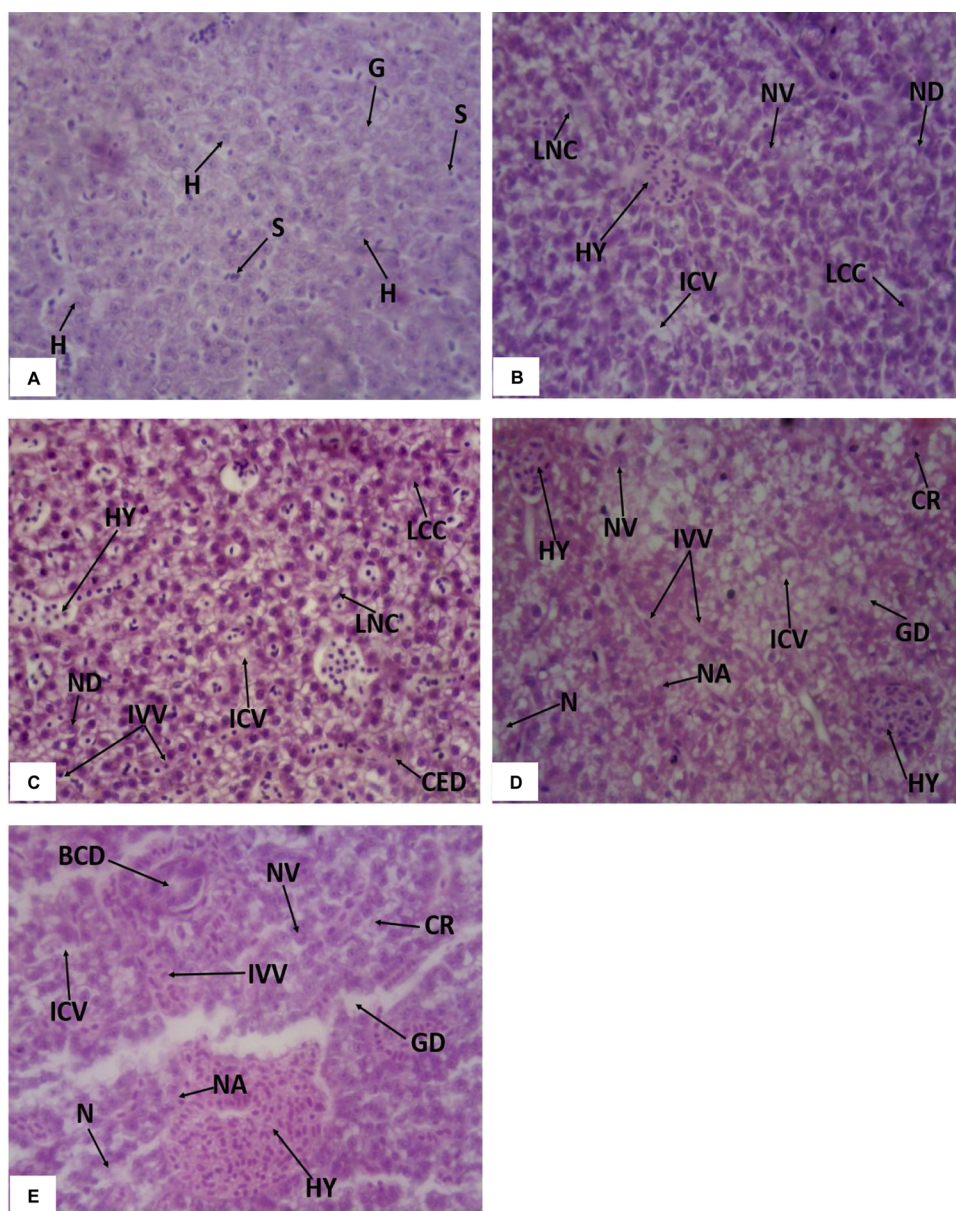
Fig. 3. Longitudinal cross section of zebrafish liver treated with different doses of EEu. **A:** Control showing H Hepatocyte, S Sinusoid, G glycogen; **B:** EEu 75 mg / kg showing ICV intense cytoplasmic vacuolization, LCC loss of cell contour, LNC loss of nuclear contour, ND nuclear degeneration, HY hyperemia, NV nuclear vacuolization; **C:** EEu 500 mg/kg showing ICV intense cytoplasmic vacuolization, LCC loss of cell contour, LNC loss of nuclear contour, ND nuclear degeneration, HY hyperemia, CED cytoplasmic degeneration, IVV increase in vessel volume; **D:** EEu 1000 mg / kg showing HY hyperemia, NV nuclear vacuolization, IVV increased vessel volume, N necrosis, NA nuclear atrophy, ICV intense cytoplasmic vacuolization, CR cell rupture, GD glycogen decrease. **E:** EEu 3000 mg/kg showing BCD bile canaliculi degeneration, ICV intense cytoplasmic vacuolization, NV nuclear vacuolization IVV increased vessel volume HY hyperemia, NA: nuclear atrophy, CR cell rupture, GD glycogen decrease, N necrosis. Flushed with HE, magnification of 400 × .

3.4.4. Gills

In the histopathological study of the gills, the control group treated with saline did not present histopathological alterations (Fig. 5A). The HAI value was zero and the percentage of tissue changes was also zero (Fig. 6 and Table 4, respectively). The group treated with the dose of 75 mg/kg of EEu presented level I changes (epithelial cell dislocation, partial fusion of the secondary lamellae, complete fusion of some secondary lamellae, dilation of the capillaries, derangement of the capillaries and vascular congestion) and only a level II change, in this case, epithelial rupture (Fig. 5B). The HAI value was 6.6 (Fig. 6), indicating that the organ remained normal, with a percentage of 10.3 % of the total alterations (Table 4).

In the group treated with the 500 mg/kg dose, epithelial cell displacement, epithelial cell hyperplasia at the base of the secondary lamellae, partial melting of the secondary lamellae, presence of chloride cells in the secondary lamellae, dilation of the capillaries, disarrangement of the capillaries and vascular congestion, such changes correspond to changes in level I. However, it presented two level II alterations, such as epithelial rupture and complete fusion of all secondary lamellae (Fig. 5C). The HAI value was 7.0 (Fig. 6), showing that in this group this organ also remained normal, with a value of 10.9 % of the total alterations (Table 4).

Treatment at doses of 1.000 mg/kg and 3.000 mg/kg were able to cause changes in gill tissue at levels I, II and III (necrosis) (Fig. 5D and E), different from the doses of 75 mg/kg and 500 mg/kg which did not show level III changes. The HAI of these groups were 34 and 37.9 (Fig. 6), indicating that these organs had moderate to severe changes, corresponding to a percentage of 53.1 % and 59.2 %, respectively, of the total alterations (Table 4). However, only the highest dose (3000 mg/kg) differed significantly from the control group (p < 0.05, one-way ANOVA (Kruskal-Wallis) followed by Dunnett's post hoc test).

3.5. Prediction of bergenin metabolism using Metatox and SMARTCyp software

Fig. 7 shows the bergenin metabolites predicted by the MetaTox software and the reactions to form them.

The metabolic chemical reactions predicted by the CYP450 enzyme (Fig. 7) included phase I reactions such as hydrogenation and dehydrogenation and phase II reactions such as O-glucuronidation, O-dealkylation, methylation and O-sulfation.

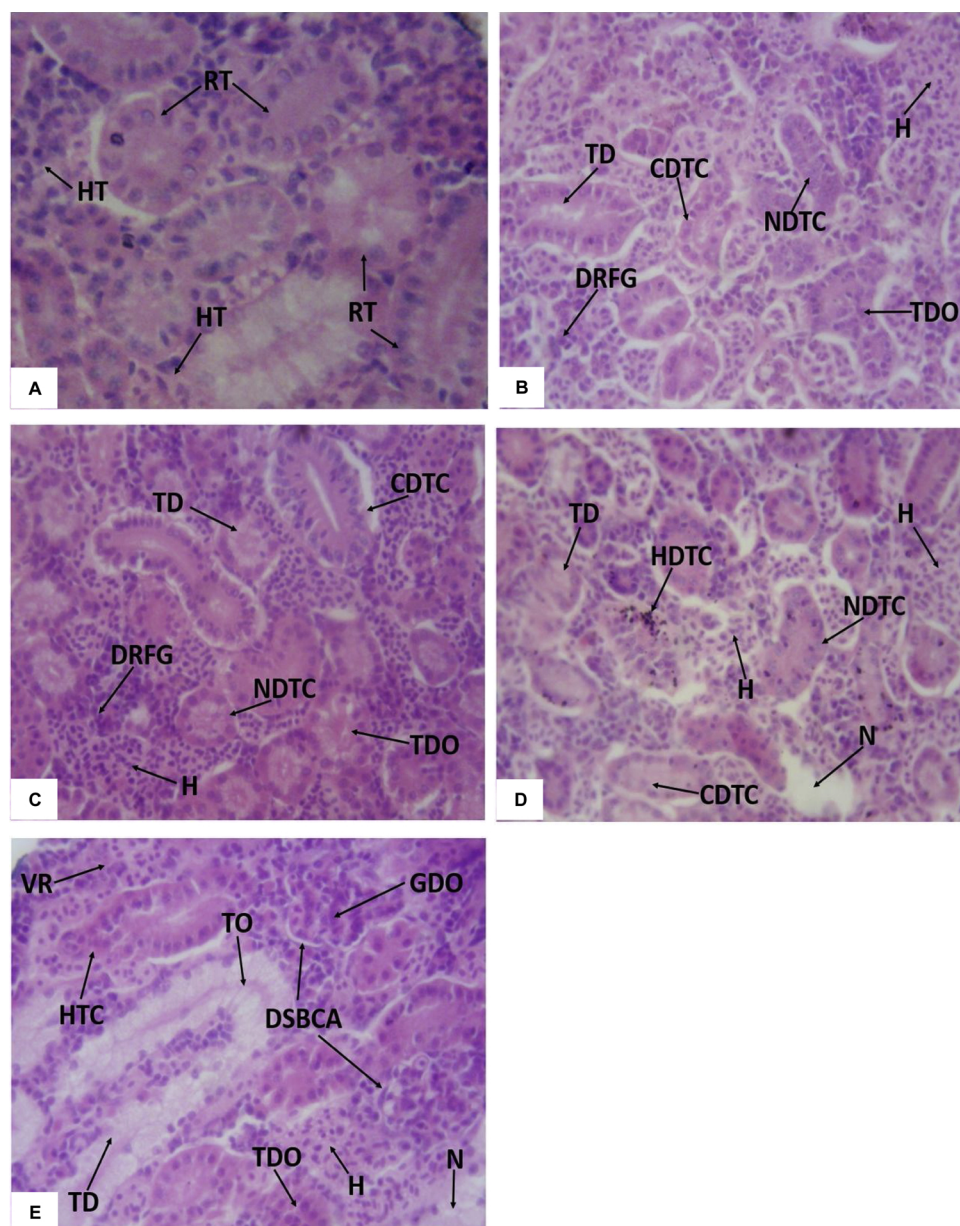According to Table 5, the metabolite M1 showed O-glucuronidation

Fig. 4. Longitudinal cross section of zebrafish kidney treated with different doses of EEu. **A:** Control showing RT renal tubules, HT hematopoietic tissue; **B:** EEu 75 mg/kg showing TD tubular degeneration, DRFG decreased relative frequency of glomeruli, CDTC cytoplasmic degeneration of tubular cells, NDTC nuclear degeneration of tubular cells, H hyperemia, TDO tubal disorganization; **C:** EEu 500 mg/kg showing TD tubular degeneration, DRFG decreased relative frequency of glomeruli, CDTC cytoplasmic degeneration of tubular cells, NDTC nuclear degeneration of tubular cells, H hyperemia, TDO tubal disorganization; **D:** EE 1000 mg/kg showing TD tubular degeneration, CDTC cytoplasmic degeneration of tubular cells, NDTC nuclear degeneration of tubular cells, H hyperemia, N necrosis, HDTC hyaline degeneration of tubular cells; **E:** EEu 3000 mg/kg showing TD tubular degeneration, H: hyperemia, N necrosis, HTC tubular cell hypertrophy, GDO glomerular disorganization, TO tubular obstruction, VR vessel rupture, TDO tubular disorganization, DSBCA decrease space in Bowman's capsule. Flushed with HE, magnification of 400 × .

reaction with the probability of occurrence of 0.9885. The M3 metabolite presented the *O*-dealkylation reaction, with a reaction probability of 0.9878. The metabolite M6, presented *O*-sulfation reaction, with probability of 0.9719. Methylation, hydrogenation and dehydrogenation were predicted to occur in metabolites M8, M12 and M15, respectively, with the probability of 0.9594, 0.9402 and 0.8670 to occurr.

3.6. In silico evaluation of pharmacokinetic and toxicological parameters of bergenin and its metabolites

The results showed that bergenin follows the Lipinski rule or "rule of five," since logP, molecular mass (MM), hydrogen bonding acceptors (HBA), hydrogen bonding donors (HBD) were : -0.7805 (logP ≤ 5), 326.3 (MM ≤ 500), 8 (HBA ≤ 10) and 0 (HBD ≤ 5), respectively (Table 6). Metabolites M3, M5, M8, M12, M15 and M16 also conformed to this rule (Table 6).

Human intestinal absorption (HIA) and cell penetrability *in vitro* using the Caco-2 cell model were used to describe intestinal absorption, in addition to bergenin, the five M3, M5, M8, M15 and M16 metabolites were moderately absorbed (30 % < HIA < 70 %), nine

metabolites (M1, M2, M4, M6, M7, M9-M14 and M17) as being poorly absorbed (HIA ≤ 30 %) and no metabolites were classified as being well absorbed (HIA ≥ 70 %), see Table 7. Regarding to the cell penetrability in Caco-2, bergenin and 14 of its metabolites (M1-M5, M7-M9, M11-M13 and M15-M17) presented values corresponding to average permeability (4–70 nm/s) and three metabolites (M6, M10 and M14) showed low permeability for Caco-2 (< 4 nm/s) (Table 7).

In relation to the distribution properties, the following parameters were used: plasma protein binding (PPB (%)), brain/blood partition coefficient BBB (C.brain/C.blood) and interaction with P-glycoprotein (P-gp). In this study, the calculated PPB values ranged from 27.70 % to 46.26 %. Thus, bergenin and all its metabolites (M1-M17) were classified as weakly bound to plasma proteins (PPB < 90 %) (Table 7). Based on the C_{brain}/C_{blood} ratio, in general, all chemicals fall into three categories: high absorption to the central nervous system (CNS) (C_{brain}/C_{blood} value greater than 2.0), medium absorption to CNS (C_{brain}/C_{blood} between 2.0 and 0.1) and low absorption at the CNS (C_{brain}/C_{blood} value less than 0.1) [37]. Thus, the ratio between Cbrain / Cblood suggests low uptake to CNS for bergenin and all of its metabolites (M1-M17) (Table 7). The *in silico* study revealed that bergenin and its metabolites
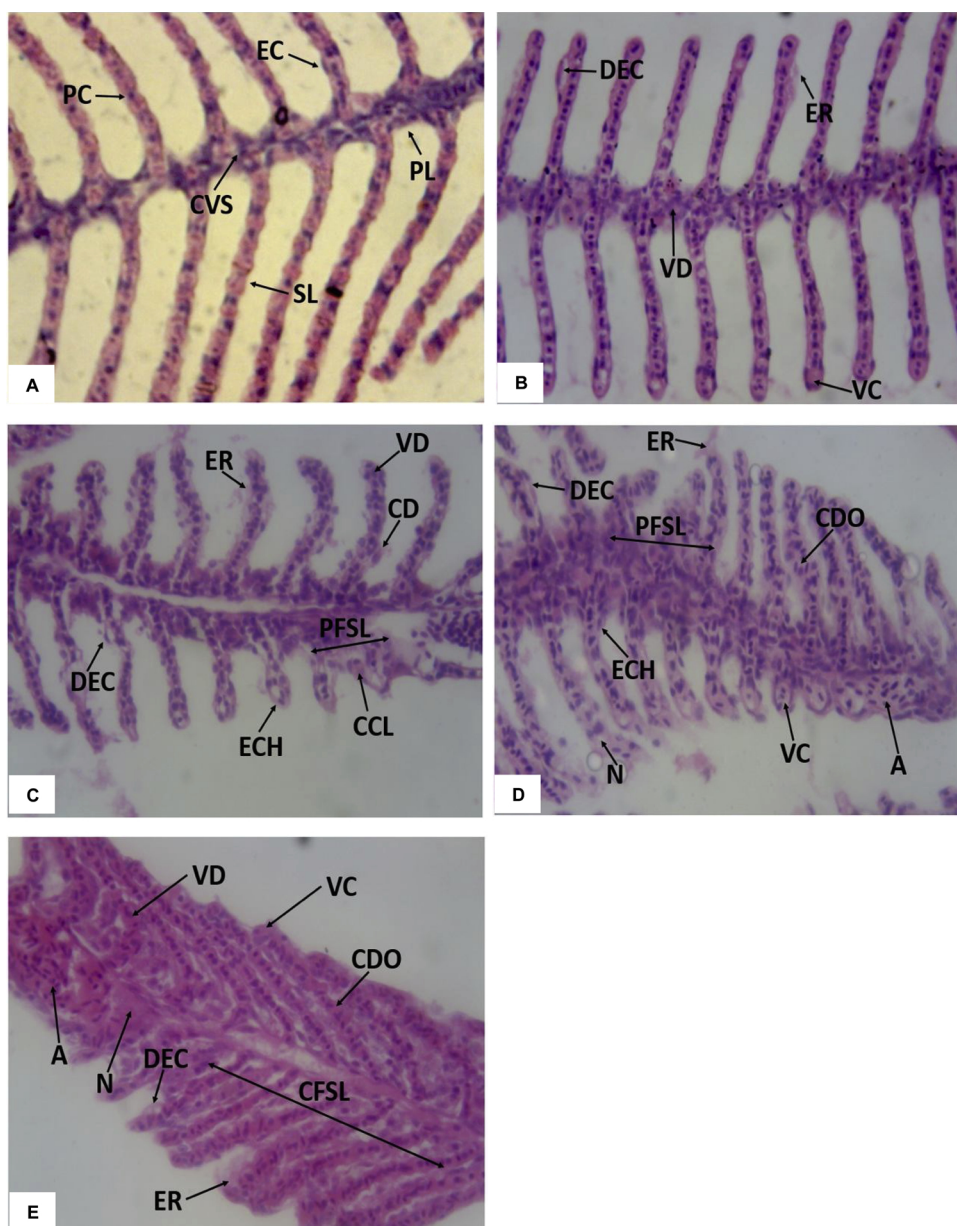
Fig. 5. Longitudinal cross-section of zebrafish gills treated with different doses of EEu. **A**: Control showing EC epithelial cell, PC cell pillar, CVS: central venous sinus, PL primary lamella, SL secondary lamella; **B**: EEu 75 mg/kg showing DEC epithelial tissue displacement, ER epithelial rupture, VC vascular congestion, VD vascular dilatation; **C**: EEu 500 mg/kg showing DEC displacement of epithelial cells, CCL presence of chloride cells on secondary lamellae, ER epithelial rupture, ECH epithelial cell hyperplasia, PFSL partial fusion of secondary lamellae, CD cell degeneration, VD vascular dilatation; **D**: EEu 1000 mg/kg showing DEC displacement of epithelial tissue, ER epithelial rupture, ECH epithelial cell hyperplasia, PFSL partial fusion of secondary lamellae, CDO capillaries disorganization, A aneurysm, VC vascular congestion, N necrosis; **E**: EEu 3000 mg/kg showing DEC displacement of epithelial tissue, ER: epithelial rupture, CFSL complete fusion of secondary lamellae, CDO capillaries disorganization, A aneurysm, VC vascular congestion, VD vascular dilatation, N necrosis. Flushed with HE, magnification of 400 × .
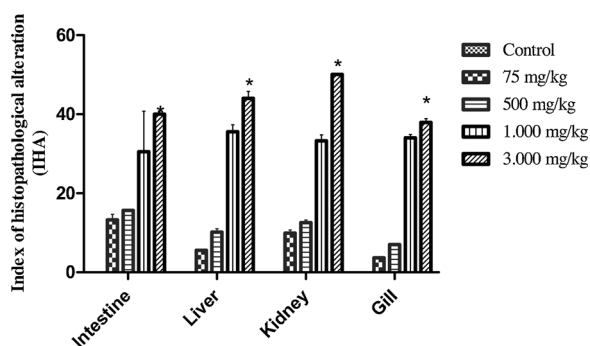

Fig. 6. Average of the histopathological changes index HAI of the intestine, liver, kidney and gills of zebrafish after treatment with the EEu oral route, in triplicate at doses of 75 mg / kg, 500 mg / kg, 1000 mg / kg and 3000 mg / kg and the control group.

are not inhibitors of P-gp.

In terms of metabolism, bergenin demonstrated a scenario of inhibition for CYP2C9, CYP3A4 and CYP2C19 (Table 8). All metabolites (M1-M17) were able to inhibit CYP2C9. Metabolites M1-M5, M7, M9-M12 and M14-M17 were shown to be inhibitors of CYP3A4. However, only the metabolites M6, M10, M13 and M14 proved to be substrates for CYP3A4. Metabolites M2-M5, M8, M10-M12 and M14-M16 have been shown to be candidates for CYP2C19 inhibitors. Bergenin and its metabolites were not able to inhibit CYP2D6 (inhibition and substrate) (Table 8).

Concerning to renal clearance, bergenin and all metabolites had low permeability in the PMDCK system (nm/sec) (< 4nn/sec). No compound showed high or medium permeability (> 70nn/sec and 4-70nn/sec, respectively) (Table 7). The results concerning the predictions of the toxicological properties (mutagenicity and carcinogenicity) of bergenin and its metabolites, are described in Table 9. The mutagenicity predictions were evaluated using the Ames test, while in the carcinogenicity rats and mice were used as animal model. Only the metabolites M6, M10, M13, M14, M16 and M17 showed mutagenic predictions,
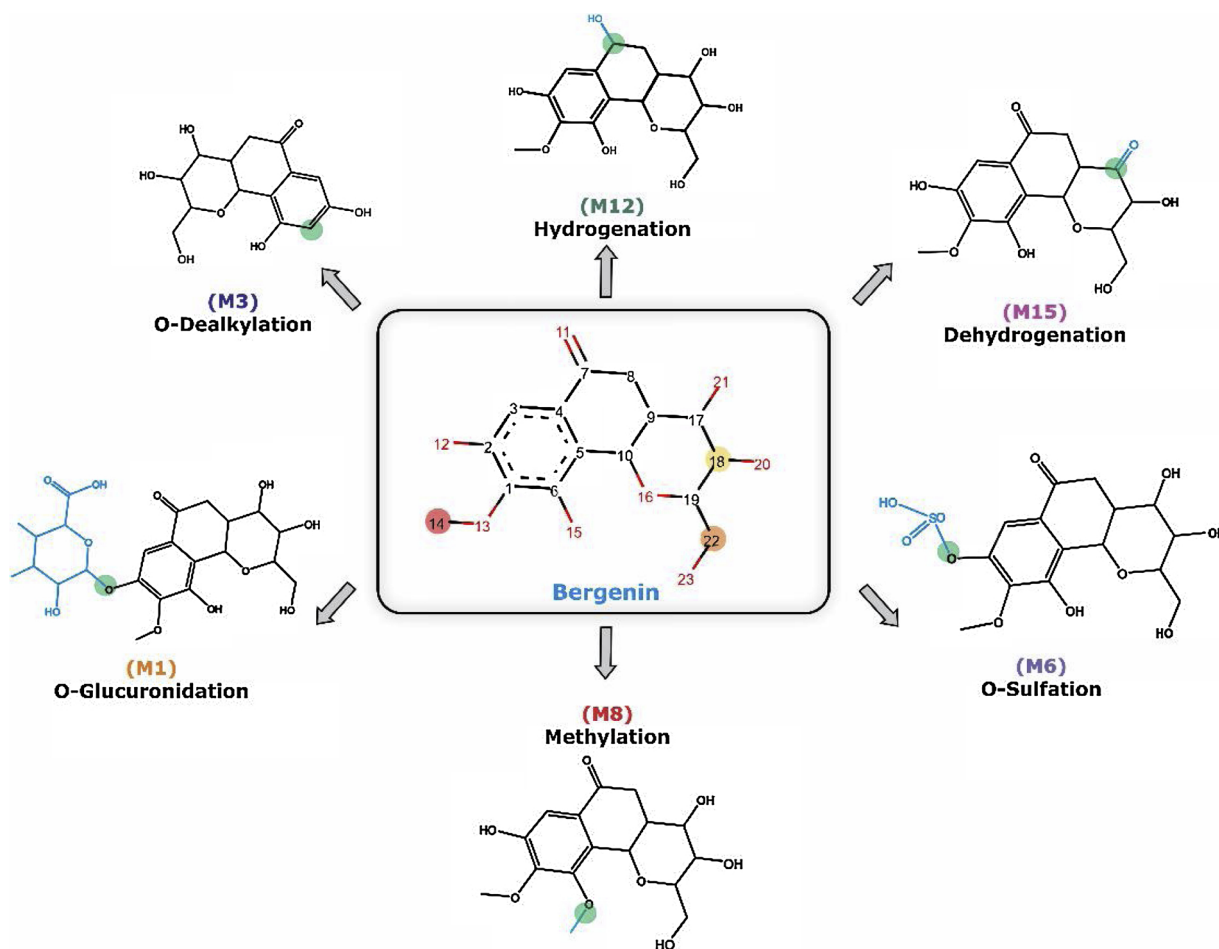
Fig. 7. Prediction in the Metatox of Bergenin and its metabolites and their respective chemical reactions.

Table 5
Probable metabolites of Bergenin, chemical reactions and probable value of the reaction occur.

	Phase Type	Chemical reaction	Probability (PR)
M1	Phase II Biotransformation	O-Glucuronidation*	0,9885
M3	Reaction	O-Dealkylation	0,9878
M6		O-Sulfation*	0,9719
M8		Methylation*	0,9594
M12	Phase I Biotransformation Reaction (Reduction)	Hydrogenation	0,9402
M15	Phase II Biotransformation Reaction I (Oxidation)	Dehydrogenation	0,8670

 * Glucuronidation, sulfation, and methylation reactions involve activated or "high energy" cofactors, as reported by [83].

whereas the compound bergenin, and the metabolites M1-M5, M7-M9, M11, M12 and M15 showed non-mutagenic predictions. With respect to the carcinogenicity test in mice and rats, bergenin and all metabolites (M1-M17) were predicted to be negative, showing that there is evidence of carcinogenic activity (Table 9).

As shown in Table 9, bergenin and 13 metabolites (M2, M3, M5, M6, M8, M10 and M12-M17) showed a low probability of being hERG type K$^+$ channel blockers. No metabolite had a high and medium probability of causing this effect. Five metabolites (M1, M4, M7, M9 and M11) were classified as ambiguous. In addition, in the evaluation of toxicity in marine organisms, no compounds were found to be very toxic (< 1 mg/L), 9 metabolites (M5, M6, M8, M10 and M13-M17) were toxic ($1-10$ mg/L), while bergenin and four metabolites (M1-M3 and M12) were found to be harmful (between $10-100$ mg/L), the metabolites

Table 6
Application of the Lipinski rule for bergenin and its metabolites.

Compound Name	LogP	MM	HBA	HBD	Nº of rotable bonds
Bergenin	− 0.7805	326.3	8	0	2
M1	− 2.5495	502.42	14	0	5
M2	− 0.7251	312.27	0	14	1
M3	− 1.0473	296.27	7	0	1
M4	− 2.2030	502.42	14	0	5
M5	− 0.6418	340.33	8	0	3
M6	− 0.8324	406.36	11	0	4
M7	− 2.7436	502.42	14	0	5
M8	− 0.8358	340.33	8	0	3
M9	− 2.2517	502.42	14	0	5
M10	− 0.3421	406.36	11	0	4
M11	− 2.2517	502.42	14	0	5
M12	− 0.7722	328.31	8	0	2
M13	− 1.0264	406.36	11	0	4
M14	− 0.3908	406.36	11	0	4
M15	− 0.6088	324.28	8	0	2
M16	− 0.5413	324.28	8	8	2
M17	− 0.3908	406.36	11	0	4

M4, M7, M9 and M11 were classified as non-toxic (> 100 mg/L) in development (Medaka) (Table 9).

Table 10 shows that bergenin had an LD$_{50}$ of 290 mg/kg and it was in level III of toxicity class. The metabolites presented LD$_{50}$ ranging from 100 mg/kg to 1500 mg/kg and most were classified as level III risk, except for metabolites M8 and M15, which were classified as level IV.

Additional toxicological data for bergenin and its 17 metabolites

Table 7
Predictions of pharmacokinetics parameters from the bergenin and its metabolites.

Pharmacokinetics Parameters					
Compound Name	Absorption		Distribution			Excretion
	IHA (%)	Caco-2	PPB (%)	BBB (C.brain/C.blood)	P-gp Inhibition	$P_{MDCK(nm/sec)}$
Bergenin	39.1307	20.2132	37.1203	0.0945	Non	0.8770
M1	3.0572	15.0635	27.4118	0.0290	Non	0.4115
M2	23.4932	19.9929	40.2462	0.0537	Non	0.7157
M3	44.4436	16.6918	41.3494	0.0807	Non	0.6497
M4	3.0490	15.8324	30.0301	0.0586	Non	1.2137
M5	57.8432	20.5098	38.1198	0.0361	Non	1.1729
M6	18.9740	0.8803	40.6750	0.0093	Non	0.4233
M7	3.0573	10.8887	27.7034	0.0272	Non	0.3955
M8	57.8448	18.9566	38.1624	0.3599	Non	1.1729
M9	3.0491	11.3573	30.3502	0.0683	Non	0.4787
M10	18.9232	0.8523	40.9797	0.0098	Non	0.4084
M11	3.0491	16.8085	30.2472	0.0430	Non	0.7886
M12	29.3358	20.1940	35.5294	0.0576	Non	0.8318
M13	18.9744	10.3192	42.6803	0.0609	Non	0.6379
M14	18.9237	2.1127	46.2691	0.0099	Non	0.4002
M15	47.8975	20.2287	39.1300	0.4987	Non	0.9387
M16	47.8976	20.2287	37.8860	0.5137	Non	0.8922
M17	18.9237	4.6238	39.2378	0.0571	Non	0.6897

tested using the Derek software are shown in Table 11. Bergenin did not present any toxicological warnings to humans, rats and mice. The M3 metabolite showed a probability of causing damage to human chromosomes (*in vitro)* and rodents (mice and rats) inducing molecular structural alterations due to the presence of the catechol pharmacophore group. The M4 metabolite showed toxicity to the skin sensitivity in rodents (rats and mice), possibly due to the presence of the Resorcinol group or precursor. The metabolites M7 and M14 also presented skin sensibility in rodents, however, due to the presence of Catechol or precursor.

4. Discussion

The phytochemical analysis indicates bergenin as the major compound (Fig. 1), which is in accordance with other studies [11,38] reporting bergenin as the major compound of this plant.

Among the detrimental side effects caused by medicinal plants are hepatotoxicity, nephrotoxicity, genotoxicity, carcinogenic and abortive effects [39]. For instance, some plant species reported to have a nephrotoxic or hepatoxic effect are *Piper methysticum* [40,41], *Cayaponia tayuya [*42], and *Symphytum officinale* L. [39].

In the acute oral toxicity test in zebrafish, no death was observed until 48 h of analysis after treatment with doses of 75 mg/kg, 500 mg/kg, and 1000 mg/kg, and even with doses up to 3000 mg/kg was determine the oral LD_{50} of the EEu higher to the dose of 3000 mg/kg. Hence, the extract of *Endopleura uchi* (per oral) can be considered nontoxic up this concentration [43]. These results are in accordance with Politi et al. [44], who evaluated the toxicity of *Endopleura uchi* powdered barks extract in mice and reported that up to 2000 mg/kg the extract caused no signs of toxicity or death (Table 1). However, in this mentioned study was not evaluated injury at the histopathological or biochemical levels, like in the present study.

The analysis of behavioral parameters is essential to understand the physiological aspects of the animal subjected to the toxicity test [45]. According to Carvalho et al. [22], the organs of zebrafish have a different sensibility to toxic agents, which together with its homology toward humans, is considered an ideal model to perform toxicity tests.

The observation of behavioral changes may indicate that the animal is in contact with a foreign substance [22]. The behaviors are classified in three stages (I, II, and II). The excitability is one of the first changes observed when the animal is exposed to a xenobiotic compound, which can culminate in loss of posture, fall to the bottom of the tank, and eventually, death. In this study was observed during the first hour in animals treated with doses of 75 mg/kg and 500 mg/kg, an increase of swimming activity, a stage II behavioral alteration (Table 2). It is important to observe, however, that this behavioral alteration was also observed in the control group.

As stated by Borges et al. [29], this may be due to the stress of the animal caused by the oral treatment since in the next hours no other behavioral alterations were observed. Animals treated with doses of 1000 mg/kg and 3000 had stage II behavioral alterations for a more extended period compared to the previous doses (Table 2). Also, the group treated with the highest dose (3000 mg/kg) had stage III alterations, evidencing that the higher doses caused a higher imbalance in the animals' homeostasis. Other studies report behavioral changes after exposure to a range of compounds [22,28,29,46].

In this study, it should be considered that a drug to be clinically useful, must have a dosage schedule that usually involves the administration of several doses in the acute, subchronic, or chronic treatment phase. Therefore, the exploitation of single-dose toxicity cannot override effects that can arise with the administration of multiple doses over

Table 8
Metabolic (Phase I) characteristics from the bergenin and its metabolites.

Metabolism						
Compound Name	Phase I – Cytochrome P450 (CYP450)					
	2C19 (Inhibition)	2C9 (Inhibition)	2D6 (Inhibition)	2D6 (substrate)	3A4 (Inhibition)	3A4 (substrate)
Bergenin	Inhibitor	Inhibitor	Non	Non	Inhibitor	Weakly
M1	Non	Inhibitor	Non	Non	Inhibitor	Weakly
M2	Inhibitor	Inhibitor	Non	Non	Inhibitor	Inhibitor
M3	Inhibitor	Inhibitor	Non	Non	Inhibitor	Weakly
M4	Inhibitor	Inhibitor	Non	Non	Inhibitor	Weakly
M5	Inhibitor	Inhibitor	Non	Non	Non	Weakly
M6	Non	Inhibitor	Non	Non	Non	Substrate
M7	Non	Inhibitor	Non	Non	Inhibitor	Weakly
M8	Inhibitor	Inhibitor	Non	Non	Non	Weakly
M9	Non	Inhibitor	Non	Non	Inhibitor	Weakly
M10	Inhibitor	Inhibitor	Non	Non	Inhibitor	Substrate
M11	Inhibitor	Inhibitor	Non	Non	Inhibitor	Weakly
M12	Inhibitor	Inhibitor	Non	Non	Inhibitor	Weakly
M13	Non	Inhibitor	Non	Non	Non	Substrate
M14	Inhibitor	Inhibitor	Non	Non	Inhibitor	Substrate
M15	Inhibitor	Inhibitor	Non	Non	Inhibitor	Weakly
M16	Inhibitor	Inhibitor	Non	Non	Inhibitor	Weakly
M17	Non	Inhibitor	Non	Non	Inhibitor	Weakly

Table 9
Predictions of toxicity parameters from the bergenin and its metabolites.

Compound Name	Mutagenicity (Ames test)	Carcinogenicity		hERG (inhibition)	Medaka
		Carcino_Mouse	Carcino_Rat		
Bergenin	non-mutagen	negative	negative	low_risk	10.3546
M1	non-mutagen	negative	negative	ambiguous	95.0245
M2	non-mutagen	negative	negative	low_risk	13.5837
M3	non-mutagen	negative	negative	low_risk	10.1477
M4	non-mutagen	negative	negative	ambiguous	144.5310
M5	non-mutagen	negative	negative	low_risk	7.5648
M6	mutagen	negative	negative	low_risk	8.4673
M7	non-mutagen	negative	negative	ambiguous	103.5850
M8	non-mutagen	negative	negative	low_risk	7.3608
M9	non-mutagen	negative	negative	ambiguous	176.5060
M10	mutagen	negative	negative	low_risk	6.1800
M11	non-mutagen	negative	negative	ambiguous	201.8730
M12	non-mutagen	negative	negative	low_risk	15.2790
M13	mutagen	negative	negative	low_risk	8.5193
M14	mutagen	negative	negative	low_risk	5.2069
M15	non-mutagen	negative	negative	low_risk	7.6870
M16	mutagen	negative	negative	low_risk	8.7887
M17	mutagen	negative	negative	low_risk	5.4330

Table 10
Toxicity prediction from the bergenin and its metabolites.

Compound Name	LD$_{50}$	Toxicity Class
Bergenin	290 mg/kg	III
M1	100 mg/kg	III
M2	290 mg/kg	III
M3	290 mg/kg	III
M4	215 mg/kg	III
M5	290 mg/kg	III
M6	290 mg/kg	III
M7	100 mg/kg	III
M8	1500 mg/kg	IV
M9	215 mg/kg	III
M10	290 mg/kg	III
M11	215 mg/kg	III
M12	215 mg/kg	III
M13	215 mg/kg	III
M14	290 mg/kg	III
M15	1.500 mg/kg	IV
M16	290 mg/kg	III
M17	290 mg/kg	III

some time. However, one must evaluate the responses found, mainly those of histopathological level, with a single dose in the acute treatment phase.

The gastrointestinal absorption is a significant aspect influencing the bioavailability of orally consumed drugs. Considering that the movement of a molecule through the body is affected by its diffusion, several molecular properties can influence the passive absorption of a drug [47]. Hence, a molecule should be in accordance with the Lipinski Rules to be a good drug candidate – that is, log P not higher than 5,

molecular mass lower than 500, number of hydrogen bond acceptors lower or equal to 10, and number of hydrogen bond donors lower or equal to 5. Molecules that do not follow more than one of these rules may probably have poor bioavailability. In this study, bergenin and the metabolites M3, M5, M8, M12, M15, and M16 (Table 6) are in accordance with the rules, suggesting that these compounds are probably the most suitable as potential drugs.

Through *in silico* analysis, the pharmacokinetic profile of bergenin and its compounds were evaluated. Bergenin and the metabolites M3, M5, M8, M15, and M16 had the best values of intestinal absorption (HIA), meaning that they are moderately absorbed (Table 7). Caco-2 are cells derived from the human carcinoma and are often used to test drugs intestinal absorption of orally consumed drugs [48,49]. In this study, the *in silico* prediction of bergenin and its metabolites are of medium permeability, as shown in Table 7. This is in accordance with Qin et al. [50], who reported that the oral absorption of bergenin is limited *in vitro* (Caco-2 cells) and *in vivo* (rats), and the main route of absorption is passive diffusion.

The intestinal toxicity is essential since the gastrointestinal mucosa is the first barrier in which an orally consumed xenobiotic compound pass through [51]. The exposure of the gastrointestinal tract to toxic xenobiotic compounds can damage its mucosa and impair the cellular development, and hence, its physiology and function [22]. According to Ryu et al. [51], the zebrafish is a promising animal to evaluate the intestinal toxicity and can even replace the rat model or the *in vitro* Caco-2 cells.

Wang et al. [52] highlight the main similarities between the zebrafish intestine with the human's, among them are the genes responsible for the metabolism of carbohydrates, lipids transport, and

Table 11
Toxicity results given by the software Derek.

Compound Name	Toxicity Prediction Alert (Lhasa prediction)	Toxicophoric Group	Toxicity Alert
Bergenin	–	–	–
M3	Chromosome damage *in vitro* in human Chromosome damage *in vitro* in mouse Chromosome damage *in vitro* in rat	Catechol	Plausible
M4	Skin sensitisation in mouse Skin sensitisation in rat	Resorcinol or precursor	Plausible
M7	Skin sensitisation in mouse Skin sensitisation in rat	Catechol or precursor	Plausible
M14	Skin sensitisation in mouse Skin sensitisation in rat	Catechol or precursor	Plausible

energy generation. Also, the absorption is performed by column-shaped enterocytes, which are the most abundant cells in the intestinal epithelium. There is also the occurrence of goblet cells, the second most frequent type in this epithelium (Menke et al., 2011).

As previously mentioned, bergenin and its metabolites are moderately absorbed, increasing the period of contact between this compound with the intestine. This can explain the occurrence of enterocytes vacuolization – very frequent in animals treated with doses of 1000 and 3000 mg/kg – since the intestine is one of the organs accountable for biotransformation, and the local toxicity of the enterocytes can damage its structure [53].

The occurrence of cellular degeneration and necrosis in the highest doses (1000 and 3000 mg/kg) can explain the increased leukocytes infiltration in the mucosal layer since those alterations manage to attract inflammatory cells to trigger the inflammatory process (Jungueira & Carneiro, 2013).

In all groups treated with EEu was observed alterations in the lamina propria; however, the alterations were higher as the doses increased. Overall, animals treated with doses of 75 and 500 mg/kg had fewer histological changes while animals treated with doses of 1000 and 3000 mg/kg had moderate to severe histological changes (73.9 % and 96.9 % of tissue alteration, respectively; Table 4).

The liver of zebrafish has several cell types parallel with those from mammals; this organ is accountable of bile production, neutralization and elimination of toxic compounds [46,54]. In zebrafish, the parenchyma is homogenous, and the hepatocytes are arranged circularly around a network of sinusoids leading to the central vessel [55]. These hepatocytes are the most abundant cells, and like in superior vertebrates, they store lipids, glycogen, and iron [46,54].

The groups treated with EEu at 75 mg/kg and 500 mg/kg had few hepatocytes changes, hyperemia, and vessel changes, the doses of 500 mg/kg also had increased vessels relative volume. Hyperemia is a mechanism to increase the blood flow to the liver, increase the release of nutrients and oxygen to a stressed area, preventing hypoxia [22,29].

The doses of 75, 500, 1000, and 3000 mg/kg resulted in intense cytoplasmic vacuolization. This was also reported by Simonato et al. [56] in fishes exposed to pollutants such as petroleum. In the doses of 1000 and 3000 mg/kg had increased frequency of level II tissue changes and occurrence of level III tissue changes, evidencing the vulnerability of hepatic cells as the treatment dose increase. The group treated with EEu at 1000 mg/kg had more severe tissue changes in the hepatocytes compared to the groups treated with the doses 75 mg/kg and 500 mg/kg. Moreover, the group treated with 3000 mg/kg had moderate to severe tissue changes, and the organ was highly injured, and could negatively impact the animals' homeostasis compared to the control group.

Due to the results from the liver histopathology, we further assessed the enzymes ALT and AST, indicators of liver damage. As shown in Table 3, a significant increase in these enzymes activity was detected caused by the treatment with EEu. Overall, the results of histopathology and enzymatic activity show that the treatment could damage the liver proportionately to the dose, especially for 1000 and 3000 mg/kg.

The toxicity from EEu could be, in part, due to the products of the compounds metabolism at these concentrations. The major compound, bergenin, can be transformed by the cytochrome P450 into metabolites with toxic activity in the hepatocytes, accountable for the tissue injury at these concentrations.

The *in silico* assays shows the metabolite M1 is formed in a phase II biotransformation reaction, glucuronidation (Fig. 7), which is one of the major routes of xenobiotics transformation in mammals [57]. M3, in turn, is formed by O-dealkylation [33,34]; both M1 and M3 have hepatotoxicity as an adverse effect, which is in accordance with the ALT and AST results (Table 3).

It is worth noticing that bergenin was predicted to inhibit two of the major CYPs involved in the drug's metabolism from the human liver: CYP2C9 and CYP3A4. All the metabolites were CYP2C9 inhibitors, and

the majority were CYP3A4 inhibitors (Table 8). These results are in accordance with Dong et al. [58], who evaluated the effects of bergenin in the eight principal isoforms of CYP450 in human hepatic microsomes and reported that bergenin managed to inhibit the activities of CYP3A4, 2E1, and 2C9. Hence, we suggest that the histopathological changes (also AST and ALT activity, evidenced by changes of these hepatic biomarkers [59]) observed in this study are due to the CYP-inhibitor activity of bergenin. In fact, drug-induced liver injury (DILI) can be caused not only by the drug itself but also by its metabolites [60,61]. Moreover, these results together suggest that bergenin, the main phytochemical compound of EEu, should not be consumed concomitantly with drugs dependent on the mentioned CYPs due to its inhibition.

The main function of the kidney in freshwater teleosts is to eliminate the vast quantities of water that enter through their gills. It is also the primary organ accountable to eliminate drugs and their metabolites [46,62].

In this study, few tubule alterations were found in animals treated with doses of 75 and 500 mg/kg; however, in animals treated with 1000 and 3000 mg/kg, the alterations were higher. Just like in the gills, liver, and intestine, the tissue changes found in the kidney were dependent on the dose of EEu. According to Meletti [63], the majority of kidney tubules changes are found in a specific group of cells, the epithelial cells, and could eventually lead to necrosis, as observed in the groups treated at 1000 and 3000 mg/kg.

The treatment with EEu at 3000 mg/kg produced all vessel alterations in this organ. Hyperemia was also observed in animals treated with the lowest doses, however, in the kidney of animals treated at 3000 mg/kg this alteration was more severe, which could negatively impact the normal function of the organ. Other tissue changes were observed in all the groups treated with EEu, such as increased space around the Bowman capsule, decreased Bowman capsule, and decreased frequency of glomeruli. According to Meletti [63], these alterations are associated with others such as glomerular degeneration, dilated vessels, and hyperemia; all of them observed in this study.

The metabolism and depuration of drugs rely mostly – if not all – on the liver, with a lesser contribution of the kidney (Kathleen et al., 2013). It is possible, however, that the histopathological changes found in the kidneys are also due to the metabolites predicted *in silico*. For instance, M15 is formed through a phase I reaction (dehydrogenation, Fig. 8) by the cytochrome P450 [64–66], in this reaction, a single bond is transformed into a double bond where a hydroxyl group is turned into a carbonyl [57] (Fig. 8), and the products of this reaction may have nephrotoxicity as a side effect. Moreover, the products of O-dealkylation (a phase II reaction, Fig. 8), such as the metabolite M3 also can be nephrotoxic [33,34].

The *in silico* results indicates that bergenin and all of its metabolites have a low permeability in Madin-Darby Canine Kidney cells (P_{MDCK}) (Table 7), then, it is reasonable to assume that these compounds may affect the glomerular filtration and renal clearance. Considering that these cells have morphological and physiological aspects similar to the cells from the mammals' collector and distal tubules (Collares-Buzato et al., 2002), it is possible that the tissue changes found in the kidneys from animals treated with the highest doses (1000 and 3000 mg/kg) are a result of this low permeability of bergenin and its metabolites.

The gills of zebrafish are formed by four bilateral gill arches. One of the first tissue alterations observed in this tissue when exposed to toxic compounds is the displacement of epithelial cells. In this study, all the doses (75, 500, 1000, and 3000 mg/kg) could induce this tissue alteration related to the animal physiological adaptation. However, along with the fusion of lamellae (found in all the groups as well), these tissue changes are reversible.

In the gills of animals treated with EEu at 500, 1000 and 3000 mg/kg was observed hyperplasia of the epithelial cells; this tissue alteration is a mechanism of defense but can eventually hamper the respiration [28,67]. Lamellae blood vessels alteration is a level I tissue alteration and was observed in all groups, however, only in the groups treated at
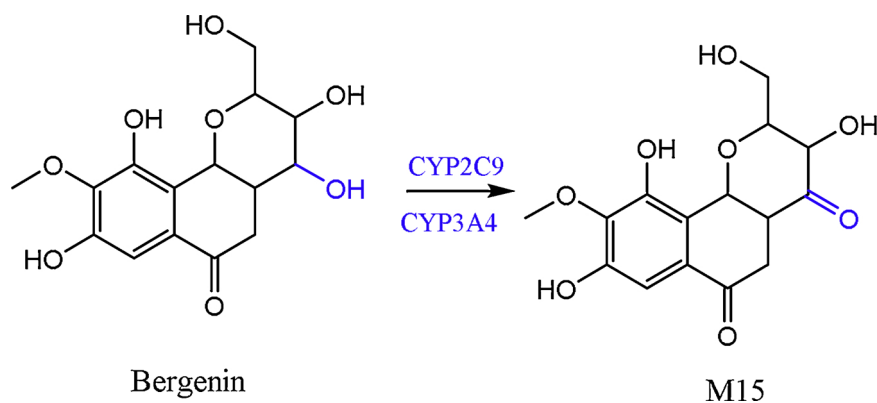
Fig. 8. Example of dehydrogenation reaction catalyzed by the cytochrome P450.

1000 and 3000 mg/kg was observed lamellae aneurysm (a level II tissue change), which is indicative of pillar cells death. The pillar cells, found in the secondary filaments, have an essential role in the maintenance of blood flow in this organ [46].

The highest doses (1000 and 3000 mg/kg) could also induce cell degeneration and necrosis (level II and III alterations, respectively), which are related to loss of function in the gill tissue [29]. Necrosis is an unplanned death of cells (unlike apoptosis) that can be induced by chemical compounds; these necrotic cells swell, their membrane dilates and finally, the cells break open, letting its content in the extracellular space [54].

In general, the doses of 1000 and 3000 mg/kg did not induce death (except for one fish treated at 3000 mg/kg) up to 48 h of observation. However, for a more extended observation period, it is possible that all of these fish would eventually die due to necrosis of the gills. It is worth noticing that the drug was orally administered, and hence, it reached the gills from the circulation, not from the exterior of the fish.

The Plasma Protein Binding (PPB), is a necessary parameter to predict the efficacy and toxicity of a drug [68]. The *in silico* prediction of bergenin and its metabolites were classified as weakly connected to plasma proteins (Table 7). Drugs with such low affinity to bind to albumin are limited to circulate through the tissues and reach the site of action. However, this property does not prevent the drug from performing its activity because free molecules are needed to exert their pharmacological activity [69]. On the other hand, a study performed by Zhang et al. [70] about bergenin and the human serum albumin (HAS) reported that bergenin could connect to this molecule through hydrophobic interaction in microemulsions.

According to Schinkel et al. [71], the passage of drugs into the brain can lead to adverse side effects in the CNS. The *in silico* results indicates that bergenin and all of its metabolites have low absorption in the blood-brain barrier, and are consequently inactive in the CNS (Table 7). Hence, the behavioral alterations observed are probably not due to the treatment effects in the CNS.

P-glycoproteins (P-GP) are membrane proteins accountable to remove potential cytotoxic compounds from the cells and can ultimately, make the cells resistant to certain drugs [71]. However, the *in silico* results indicates that bergenin and its metabolites do not interact with P-GPs (Table 7).

In the *in silico* toxicological analysis, bergenin and its metabolites were assessed for potential mutagenicity and carcinogenicity. Assess mutagenic activity is essential to screen safer drugs for treatment [72]; in this study, bergenin and most of its metabolites (M1-M5, M7-M9, M11, M12, and M5) were not predicted to be mutagenic (Table 9). As for the carcinogenic activity, bergenin and all of its metabolites were predicted to be carcinogenic in mice and rats (Table 9). Assess the mechanism in which these molecules could induce cancer is essential considering that bergenin is indicated for several therapeutic uses. The metabolism prediction of bergenin had phase I and II biotransformation

reactions, for instance, in the metabolites M3, M6, M8, M12, and M15. The occurrence of these reactions forming these molecules could be carcinogenic (Fig. 7), corroborating the previous result. This carcinogenic activity would be induced over the hematopoietic system and pituitary gland of female mice; vascular system, stomach and kidney of male rats; and kidney of male mice [33,34].

Bergenin and the metabolites M1-M3 and M12 were predicted to be harmful in the development of medaka fish (Table 9). As for the interaction with hERG-type K$^+$ channels (Table 9), neither bergenin or its metabolites were predicted to act as blockers. These channels are essential during the repolarization of the cardiac action potential [73].

The *in silico* results suggest an LD$_{50}$ of 290 mg/kg (Table 10), however, this is not in accordance with the *in vivo* results reported by Nazir et al. [17], where bergenin had LD$_{50}$ superior to 2000 mg/kg in mice, and also in this study, where the LD$_{50}$ could not be determined in the tested doses in zebrafish. Also, in the Derek server, bergenin was not predicted to have toxicological risks, but the presence of some toxic chemical groups should be considered such as catechol and resorcinol in the metabolites M3, M4, M7, and M14 (Table 11); these groups can induce chromosome damages in human and rodents, and also affect skin sensibility [74–82].

This was the first study assessing *in vivo* the acute toxicity of EEu, with an emphasis in biochemical parameters and histopathology of the intestine, gills, liver, and kidney. Further analysis *in silico* was performed on bergenin, the major compound, and its metabolites. Despite the relevant potential of bergenin for therapeutic uses, more toxicological studies are needed for this molecule.

5. Conclusion

The effects of orally administered EEu was evaluated in zebrafish using different doses, and overall, the extract had minimal toxicity in low doses, in accordance with other reports, while in higher doses only tissue alterations were observed, mainly in the liver and intestine. In *in silico* analysis, bergenin was predicted to interact with CYP enzymes of phase I and II biotransformation reactions, and the histological changes found in these tissues, corroborated by biochemical markers, could be due to the action bergenin metabolites at these concentrations.

Based on this study, it is concluded that potential drugs candidates using active principles of this plant should consider the doses administered since, in higher doses, tissue and biochemical changes were observed that could impair the organ physiology.

Authorship contributions

Participated in research design: BMSH, BLSO, CBRS, AN and JCTC.
Conducted experiments: BMSH, KRTP, LBS, RSB, RCA, HOC and GMGA.
Performed data analysis: ACMP and BMSH.

Wrote or contributed to the writing of the manuscript: BMSH, AN and JCTC.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2020.01.011.

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