



# Non-clinical toxicological evaluation of the ethanolic extract from *Zornia brasiliensis* Vogel (Fabaceae)

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## ABSTRACT

*Zornia brasiliensis* Vogel, Fabaceae, is used to treat venereal diseases and as diuretic in Brazilian northeast folk medicine. Herein, the toxicity profile of ethanolic extract from *Z. brasiliensis* aerial parts (ZBEE) was evaluated. Hemolysis assay was used to evaluate the *in vitro* toxicity, where as *in vivo* acute and repeated-dose (28 days) toxicity tests were performed on adult Swiss albino mice. Micronucleus test was used to evaluate genotoxicity. The concentration of ZBEE that produced 50% hemolysis (HC<sub>50</sub>) was 1,954 µg/ml, indicating low cytotoxicity. In acute toxicity assay, no deaths or behavioural changes in mice treated with 2,000 mg/kg ZBEE were recorded. Regarding the repeated-dose evaluation, after 28 days of treatment with ZBEE (250, 500, and 1,000 mg/kg, oral gavage), weak toxicity signs on biochemical, haematological, and neurological parameters were observed, especially for the highest dose tested. ZBEE (2,000 mg/kg) did not induce an increase in micronucleated erythrocytes number, suggesting no genotoxicity. In summary, ZBEE showed low nonclinical toxicity. These data support its safe use in the folk medicine and further studies to characterize the pharmacological activities of this medicinal plant.

## INTRODUCTION

Herbal medicine has been considered as an alternative form for treatment against several diseases. However, despite many people consider products obtained from nature safe, literature data have shown toxicological effects for medicinal plants. Then, toxicological studies should be done to ensure the safety of herbal drugs (Da Silva *et al.*, 2018; Kahaliw *et al.*, 2018).

*Zornia brasiliensis* Vogel (Fabaceae), popularly known as “urinária,” “urinana,” and “carrapicho,” is used in Brazilian northeast folk medicine to treat venereal diseases and as diuretic (Agra *et al.*, 2008). Literature data showed antioxidant and cytotoxic activities on *Artemia salina* larvae for the methanolic extract from *Z. brasiliensis* aerial parts (ZBEE) (David *et al.*, 2007). In addition, antinociceptive activity of 7-methoxyflavone, obtained

from *Z. brasiliensis* (Da Silva *et al.*, 2013), and antitumor effect for the leaf volatile oil were recorded (Costa *et al.*, 2015). Recently, the isolation of one previously undescribed C-glycosylated dihydrochalcone (zornioside), which was selectively cytotoxic for HL60 leukemia cells, and the presence of saponins in this species were reported (Nascimento *et al.*, 2018, 2019).

Nevertheless, despite the popular use for medicinal purposes, there are no toxicological reports in the literature for *Z. brasiliensis*. Herein, we evaluated the acute and repeated-dose toxicity of *Z. brasiliensis* in order to provide scientific data to support its safe use and further pharmacological tests.

## MATERIALS AND METHODS

### Vegetable sample

The aerial parts (composed of stem, leaves, and inflorescence) of *Z. brasiliensis* Vogel (Fabaceae) were collected in June (winter) 2016, in the city of Serra Branca (07°29'46" S and 36° 44'36" W, altitude: reaching 712 m elevation), located in the state of Paraíba, Brazil. An exsiccata is deposited in the Herbarium Arruda Câmara, in Campus I of the State University

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of Paraíba (UEPB), code 1,862. This study was registered in the National Management System of Genetic Patrimony and Associated Traditional Knowledge (SISGEN, Curitiba, Brazil) with registration number ADD107E. Ethanol extract from ZBEE was obtained from the plant material (5 kg) subjected to extraction with 95% ethanol by maceration [1:3, w (kg)/v (l)] at room temperature for 72 hours; this process was repeated four times. The final extraction yield was 11% (Nascimento *et al.*, 2018).

#### Quantification by high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD)

The substances 7-methoxyflavone and 5,7-dimethoxyflavone (Nascimento *et al.*, 2018) were quantified in the macerated crude ethanol extract, and they were considered as markers of the

extract. This quantification was performed by HPLC-DAD. For this, it was used as stationary phase a reverse-phase Kromasil® C4 column (250 mm × 4.6 mm ID, 3.5 μm filled with particles) with a Kromasil® C4 precolumn (4.6 mm ID × 3.0 mm, 3.5 μm filled with particles) and as mobile phase the solvents: water (0.1% formic acid) and acetonitrile (1:1, v/v) were used as the mobile phase in isocratic mode for 23 minutes, at a flow rate of 0.45 ml/minutes, the temperature of 40°C, and the detection was performed at 254 nm. The content of 7-methoxyflavone and 5,7-dimethoxyflavone in the *Z. brasiliensis* extract was 146.54 and 74.45 mg/g extract, respectively (Fig. 1).

#### Animals

Adult Swiss albino mice (*Mus musculus*), female and male (28–32 g), obtained from the Dr. Thomas George Bioterium

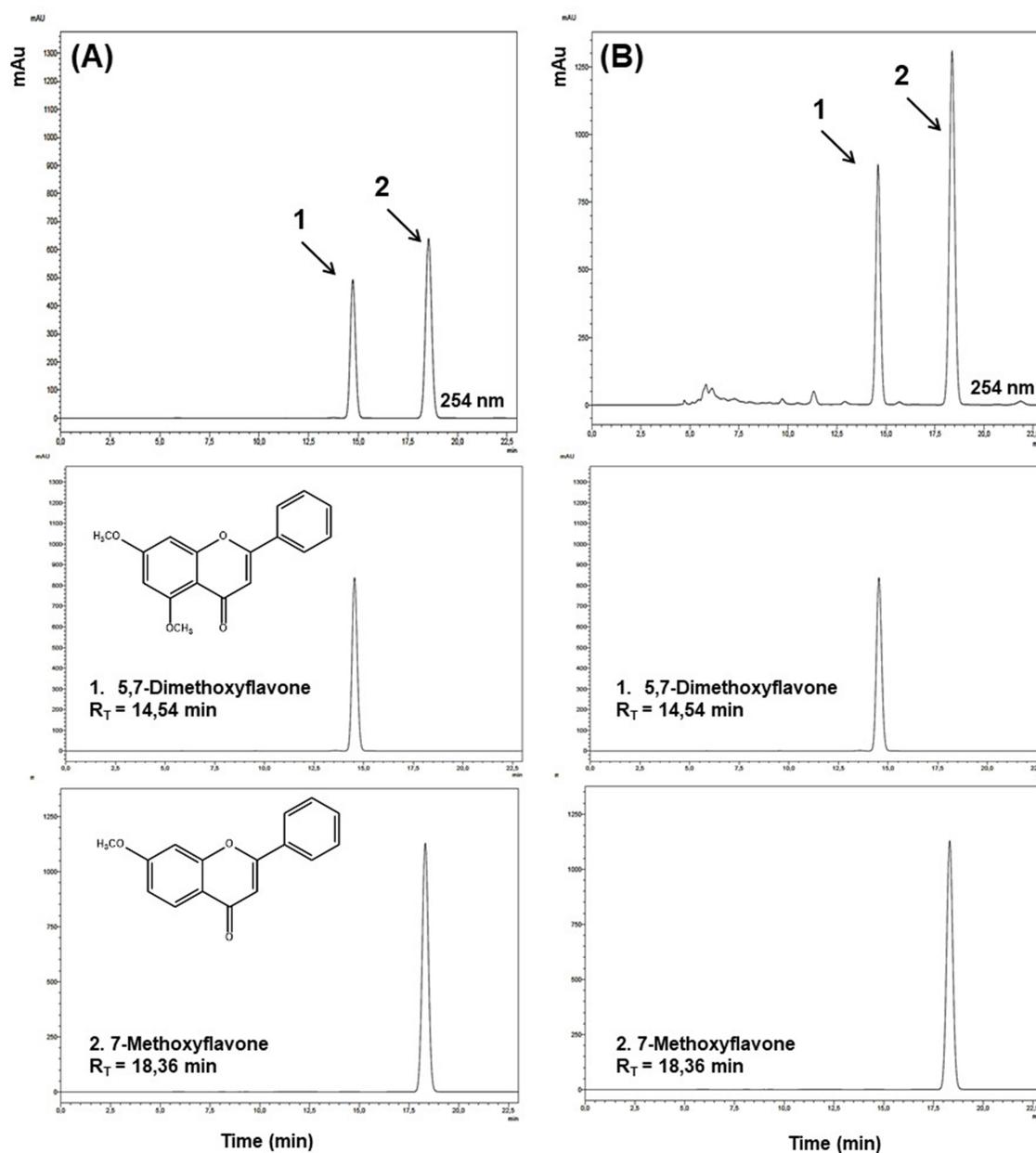


Figure 1. High-HPLC-DAD analysis of extract of *Zornia brasiliensis*. (A) Standard mixture (70 and 150 μg/ml of 5,7-dimethoxyflavone and 7-methoxyflavone, respectively). (B) Extract sample (2 mg/ml). RT = retention time.

Research Institute in Drugs and Medicines/Federal UEPB, Paraíba, Brazil, were used. Animals were randomly selected and separated by gender in rodent cages in a controlled environment (12/12 hours light–dark cycle, 24°C ± 1°C, 55% relative humidity) with food and water *ad libitum*. Animals were housed in groups of 6. The total number of animals/group changed according to the method ( $n = 6$ /gender/group for acute assay,  $n = 10$ /gender/group for repeated-dose assay, and  $n = 6$  males for micronucleus assay). All the study was approved by the Animal Ethics Committee of the Federal UEPB in December 2015 (CEUA/UFPB number 0701/14).

### Hemolysis test

Mouse erythrocytes were used to evaluate the hemolytic effect of ZBEE (Kang *et al.*, 2009). Swiss mice were anesthetized with sodium thiopental, 40 mg/kg, intraperitoneally (i.p.), and fresh blood was collected from the orbital plexus to obtain the erythrocytes. To avoid coagulation, the needle was heparinized (sodium heparin). Then, erythrocytes were suspended in phosphate-buffered saline (PBS) to make a 1% (v/v) solution, which was incubated with various concentrations of ZBEE (0–7,500 µg/ml) dissolved in cremophor (1.5%, v/v, in PBS). The plates were shaken for 60 minutes. After centrifugation and removal of the supernatant, Triton X-100 solution (0.1%) was added (200 µl/well) and the plate was shaken. The hemolysis induced by Triton X-100 (0.1%) was determined at 415 nm and served as the reverse test to set the HC<sub>50</sub> (concentration that produces 50% hemolysis) of ZBEE. The positive and negative controls were made (100% and 0% hemolysis, respectively) by incubating erythrocytes with 0.1% Triton X-100 in PBS and 1.5% of cremophor in PBS, respectively.

### Acute and repeated-dose toxicity assays

The study of acute and repeated-dose toxicity of ZBEE was carried out in accordance with the Brazilian Health Surveillance Agency (Anvisa) regulations (Brazil, Ministry of Health, 2013).

For acute toxicity assay, mice ( $n = 6$ /gender/group) received a single dose of 2,000 mg/kg ZBEE by oral gavage, and the control group was treated with the vehicle (Tween 80 at 12% (v/v) in saline). The activity on the Central Nervous System or Autonomic Nervous System was investigated at 0, 15, 30, and 60 minutes, 4 hours, and daily for 14 days. Food and water consumption was recorded daily, and the animal's weight was recorded at the beginning and at the end of treatment. The dose that killed 50% of animals (LD<sub>50</sub>) was estimated.

For repeated-dose toxicity test, Swiss mice ( $n = 10$ /gender/group), after 3 hours fasting, received ZBEE by oral gavage (250, 500, or 1,000 mg/kg), for 28 days, and the control group was treated with the vehicle [Tween 80 at 12% (v/v) in saline] under the same conditions. During the experiment, water and food intake and weight evolution were observed. On the day before the first administration (Basal) and the 14th and 28th days of administration, exploratory activity (open-field test) and motor activity (rotarod test) were evaluated.

One day after the last treatment, orbital plexus blood samples from all animals under anesthesia [sodium thiopental, 40 mg/kg, intraperitoneally (i.p.)] were obtained (Diehl *et al.*, 2001).

To avoid coagulation, the needle was heparinized (sodium heparin). Urea and creatinine levels and the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were quantified in serum samples using respective kits (Labtest®, Brazil) according to the manufacturer's instructions in Cobas Mira Plus®. The hematological analysis was performed using heparinized whole blood to determine the following parameters: hemoglobin (Hb) level, red blood cell (RBC) count, and hematocrit (Hct), and the red cell indices mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and total and differential leukocyte counts using a Hematoclin 2.8 Vet (Bioclin®). Then, after euthanasia by cervical dislocation, livers were removed and fixed in 10% (v/v) formaldehyde. Portions of the livers were cut into sections of 3 µm, stained with Masson's trichrome, and subjected to histopathological analysis.

### Micronucleus assay

For the micronucleus assay, mice ( $n = 6$  males/group) were subjected to a single oral gavage dose of 2,000 mg/kg ZBEE. Positive and negative control groups (cyclophosphamide, 50 mg/kg i.p. and Tween 80 at 12% (v/v) in saline, respectively) were added. After 48 hours treatment, blood extensions were made with peripheral blood samples collected from the tail (10 µl). After drying, the slides were stained with panoptic staining for further analysis under an optical microscope. Two thousand erythrocytes were counted in three blood extensions for each animal, and the number of micronucleated erythrocytes was recorded (OECD, 2016).

### Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) one-way followed by Tukey's test ( $p < 0.05$ ). For the comparison between the two groups, the Student *t*-test was used ( $p < 0.05$ ).

## RESULTS AND DISCUSSION

Toxicity assessment is an essential step in the development of new synthetic or natural medicines. Moreover, toxicity tests are necessary to ensure the safe use of medicinal plants in the folk medicine. Considering the popular use of *Z. brasiliensis* for medicinal purposes, in this study, we evaluated the acute and repeated-dose toxicity, besides the hemolytic and genotoxic effects.

The hemolysis test is an important assay used to assess the toxicity of new drugs. Considering that erythrocytes are cells without a nucleus and organelles, this assay aims to evaluate changes in red cell membranes. Then, erythrocytes of mammals are a useful model to study the cytotoxicity of new natural or synthetic drugs by cellular damage measure (Pagano and Faggio, 2015). Literature data have shown that CH50 values higher than 1,250 µg/ml suggest no hemolytic activity for plants (Dos Santos-Júnior *et al.*, 2010). Herein, ZBEE-induced hemolysis only on high concentrations, with HC50 value of 1954 (1,840–2,074) µg/ml, indicating no cytotoxicity to erythrocytes.

For ZBEE, on the acute study, no toxicity signs or deaths were observed after treatment (2,000 mg/kg). Then, LD<sub>50</sub> value is higher than 2,000 mg/kg. There was a decrease on food intake for males; however, this effect was not accompanied by changes in the

final weight of the ZBEE treated animals (Table 1). Then, these results together suggest weak acute toxicity for ZBEE.

Repeated-dose toxicity studies aim to characterize the toxicological profile of a new drug by repeated administration. Among the parameters evaluated on that assay are mortality, behavioral parameters, water and food intake, body weight, and clinical pathology (biochemical, hematological, and histopathological parameters) (ANVISA, 2013; OECD, 2008). For ZBEE, no death was recorded on 28-days assay. A decrease in food and water intake was observed. However, similar to acute assay, these effects did not induce any change in the final weight of the ZBEE treated animals (Table 2).

To evaluate the neurological effects of ZBEE, rotarod and open-field tests were used. In rodents, rotarod assay has been used to assess the motor deficits (Miyanishi *et al.*, 2019), and the open-field test is employed to evaluate anxiety, locomotion, and stereotypical behaviors, such as grooming and rearing (Prut and Belzung, 2003). During the repeated-dose assay, no effect on

rotarod assay was observed for ZBEE treated animals (Table 3), suggesting that ZBEE did not induce changes in psychomotor coordination. On open-field assay, no changes were observed in the number of rearing and grooming behaviors (Table 4). For female mice treated with 1,000 mg/kg ZBEE, there was an increase in ambulation ( $56.7 \pm 4.7$ ;  $p < 0.05$ ), only at the end of treatment (Table 4). Nevertheless, only this isolated data does not suggest a behavioral change. Instead, the neurological evaluation for ZBEE suggested no motor and behavioral changes.

Urea and creatinine serum levels are conventional circulating biomarkers for detecting nephrotoxicity. In addition, drug-induced liver injury, which is caused by drugs and herbal or dietary supplements, is evaluated using specific serum biomarkers, as ALT and AST activities (ANVISA, 2013; OECD, 2008). On biochemical evaluation, no change in serum urea was observed after ZBEE treatment (Tables 5 and 6). For creatinine levels, a decrease for all female mice groups was observed ( $p < 0.05$ ) (Table 6). However, despite these changes, the serum levels for

**Table 1.** Effects of ZBEE on feed and water consumption and weight evolution of mice subjected to a single dose (2000 mg/kg, v.o.).

Groups	Dose (mg/kg)	Sex	Feed intake (g)	Water intake (ml)	Starting weight (g)	Final weight (g)
Control	-	M	39.8 ± 1.0	50.9 ± 2.7	30.6 ± 0.5	35.5 ± 1.5
		F	37.8 ± 1.4	48.9 ± 1.9	28.3 ± 0.2	32.0 ± 0.7
ZBEE	2,000	M	24.7 ± 1.1 <sup>a</sup>	45.9 ± 2.8	30.2 ± 1.0	33.8 ± 2.4
		F	40.3 ± 1.3	55.4 ± 3.5	28.4 ± 0.4	32.3 ± 0.3

Data are expressed as the mean ± SEM from six animals/gender/group analyzed by Student *t*-test.

<sup>a</sup>Significantly different from the control group ( $p < 0.05$ ).

**Table 2.** Effects of ZBEE on feed and water consumption and weight evolution of mice subjected to repeated dose (28 days, v.o.).

Groups	Dose (mg/kg)	Sex	Feed intake(g)	Water intake (ml)	Startingweight (g)	Finalweight (g)
Control	-	M	40.6 ± 1.3	54.0 ± 2.1	29.6 ± 0.9	37.7 ± 1.2
		F	45.6 ± 1.7	43.8 ± 1.0	27.0 ± 0.6	33.2 ± 0.7
ZBEE	250	M	43.0 ± 1.5	55.1 ± 2.2	29.7 ± 0.6	38.4 ± 1.1
		F	27.1 ± 0.8 <sup>a</sup>	41.5 ± 1.5	25.4 ± 0.7	32.4 ± 1.3
ZBEE	500	M	32.4 ± 1.6 <sup>a</sup>	46.6 ± 1.4 <sup>a</sup>	26.9 ± 0.8	41.7 ± 1.3
		F	30.9 ± 0.9 <sup>a</sup>	42.9 ± 1.3	25.4 ± 0.5	36.2 ± 0.6
ZBEE	1,000	M	29.7 ± 1.0 <sup>a</sup>	45.2 ± 1.5 <sup>a</sup>	28.3 ± 0.7	39.2 ± 1.2
		F	39.4 ± 1.3 <sup>a</sup>	51.8 ± 1.8 <sup>a</sup>	25.6 ± 0.3	35.8 ± 0.8

Data are expressed as mean ± SEM from ten animals/gender/group analyzed by ANOVA followed by Tukey test.

<sup>a</sup>Significantly different from control group ( $p < 0.05$ ).

**Table 3.** Effects of ZBEE on psychomotor coordination by rotarod treadmill apparatus of mice subjected to repeated dose (28 days, v.o.).

Groups	Dose (mg/kg)	Sex	Time spent on rotarod apparatus (s)		
			Basal	14 days	28 days
Control	-	M	155.6 ± 15.6	171.6 ± 6.2	161.5 ± 12.4
		F	168.9 ± 4.5	168.4 ± 5.6	178.6 ± 1.4
ZBEE	250	M	166.0 ± 9.1	167.4 ± 10.2	176.8 ± 2.2
		F	165.8 ± 4.5	173.6 ± 2.6	172.4 ± 4.1
ZBEE	500	M	164.6 ± 9.6	153.4 ± 6.0	174.8 ± 5.1
		F	160.6 ± 9.6	161.1 ± 7.4	173.0 ± 3.4
ZBEE	1,000	M	166.6 ± 6.3	164.5 ± 12.3	170.9 ± 5.1
		F	151.8 ± 9.5	161.1 ± 8.2	165.2 ± 10.6

Data are expressed as mean ± SEM from ten animals/gender/group analyzed by ANOVA followed by Tukey test.

**Table 4.** Effects of ZBEE on open-field behavior test on mice subjected to repeated dose (28 days, v.o.).

Groups	Dose (mg/kg)	Sex	Number of rearing behaviors (n°)		
			Basal	14 days	28 days
Control	-	M	4.5 ± 1.2	4.0 ± 2.0	7.3 ± 1.6
		F	4.6 ± 1.2	6.8 ± 1.7	7.0 ± 1.3
ZBEE	250	M	2.3 ± 0.8	8.3 ± 2.9	3.2 ± 1.2
		F	2.5 ± 1.0	8.3 ± 4.1	4.1 ± 1.4
ZBEE	500	M	5.5 ± 1.5	7.2 ± 2.5	3.2 ± 1.0
		F	3.2 ± 1.3	5.6 ± 2.7	3.5 ± 1.5
ZBEE	1,000	M	5.1 ± 1.9	2.3 ± 1.1	3.9 ± 1.4
		F	2.0 ± 1.8	5.0 ± 1.6	6.2 ± 2.0
Grooming (s)					
			Basal	14 days	28 days
Control	-	M	16.7 ± 6.6	11.6 ± 2.9	7.6 ± 2.1
		F	6.9 ± 2.7	14.3 ± 2.9	16.0 ± 5.5
ZBEE	250	M	18.2 ± 3.7	16.8 ± 5.0	7.8 ± 2.0
		F	11.0 ± 2.3	9.1 ± 2.3	8.6 ± 2.9
ZBEE	500	M	16.3 ± 3.9	10.2 ± 5.6	9.6 ± 2.9
		F	17.3 ± 5.9	11.5 ± 2.6	5.3 ± 1.6
ZBEE	1,000	M	25.3 ± 8.8	14.9 ± 2.9	13.1 ± 2.4
		F	11.3 ± 3.3	16.7 ± 2.9	11.5 ± 2.3
Ambulance (n° of quadrants)					
			Basal	14 days	28 days
Control	-	M	61.4 ± 4.2	35.4 ± 4.2	38.0 ± 5.1
		F	60.7 ± 5.6	50.2 ± 5.3	36.6 ± 3.4
ZBEE	250	M	58.0 ± 7.9	43.8 ± 3.9	37.0 ± 1.4
		F	40.9 ± 9.5	39.3 ± 4.9	42.6 ± 3.8
ZBEE	500	M	46.7 ± 3.6	29.2 ± 5.3	36.3 ± 2.4
		F	44.0 ± 5.6	34.1 ± 4.5	36.4 ± 2.9
ZBEE	1,000	M	42.2 ± 3.1	35.6 ± 5.0	40.3 ± 3.1
		F	45.4 ± 7.8	43.9 ± 4.3	56.7 ± 4.7*

Data are expressed as mean ± SEM from ten animals/gender/group analyzed by ANOVA followed by Tukey test.

\*Significantly different from control group ( $p < 0.05$ ).

creatinine were within normal parameters for the species (Gad, 2007). There was an increase in AST activity for male mice at 1,000 mg/kg ZBEE ( $243.5 \pm 15.9$  U/l;  $p < 0.05$ ) (Table 5). For ALT activity, there was an increase for all female mice groups (Table 6) and for 1,000 mg/kg ZBEE male mice group ( $p < 0.05$ ) (Table 5), suggesting the occurrence of hepatic toxicity. Then, the liver histological examination was performed to investigate this effect. The liver histological analyses of the group subjected to ZBEE (1,000 mg/kg) were within normal histological limits, as shown in zone 3 (Z3) of the hepatocyte acinus of the liver which presents terminal hepatic venule surrounded by converging hepatocyte plates and sinusoids and in turn presents normal-sized hepatocytes without nuclear changes in addition to normalities without connective tissue (Fig. 2). These findings suggest that the biochemical changes are minor and do not alter the tissue structure.

Hematological parameters analysis is often employed to determine the safety profile, including plant extracts, on the blood constituents. The parameters investigated after ZBEE treatment are important tools in ascertaining the toxicological profile of

new drugs (ANVISA, 2013; OECD, 2008). On hematological evaluation, for male mice, an increase in the number of erythrocytes was observed ( $10.3 \pm 0.3$   $10^6/\text{mm}^3$  and  $11.1 \pm 0.4$   $10^6/\text{mm}^3$ ;  $p < 0.05$ ), in hemoglobin concentration ( $17.5 \pm 0.5$  g/dl and  $18.7 \pm 0.5$  g/dl;  $p < 0.05$ ), and in hematocrit ( $60.2\% \pm 1.5\%$  and  $60.3\% \pm 2.6\%$ ;  $p < 0.05$ ), for 250 and 500 mg/kg groups, respectively (Table 5), indicating the increase of RBCs. For female mice groups, a decrease in eosinophils count was observed ( $0.4 \pm 0.2$  and  $0.0 \pm 0.0$ , at 500 and 1,000 mg/kg ZBEE, respectively;  $p < 0.05$ ), in addition to a decrease in monocytes ( $1.3 \pm 0.4$ ;  $p < 0.05$ ) at 1,000 mg/kg ZBEE (Table 6). Literature reports have shown that eosinophils, basophils, and monocyte counts are usually very low and variable. Thus, it is improbable to detect toxicological effects on these cell types. Therefore, these isolated changes have no clinical significance.

During the new drugs safety assessment, the genotoxicity is also commonly evaluated. The micronucleus assay has been added in many genotoxic test guidelines of synthetic and natural new drugs (Araldi *et al.*, 2015). Although the trend of the safety

**Table 5.** Effects of ZBEE on biochemical and hematological parameters of peripheral blood of male mice subjected to repeated dose (28 days. v.o.).

Parameters	Control	ZBEE (mg/kg)		
		250	500	1,000
<i>MALE</i>				
RBCs (10 <sup>6</sup> /mm <sup>3</sup> )	9.0 ± 0.4	10.3 ± 0.3 <sup>a</sup>	11.1 ± 0.4 <sup>a</sup>	8.3 ± 0.5
Hemoglobin (g/dl)	13.5 ± 1.1	17.5 ± 0.5 <sup>a</sup>	18.7 ± 0.5 <sup>a</sup>	14.3 ± 0.9
Hematocrit (%)	45.3 ± 3.5	60.2 ± 1.5 <sup>a</sup>	60.3 ± 2.6 <sup>a</sup>	54.3 ± 2.2
VCM (fm <sup>3</sup> )	56.4 ± 1.4	58.0 ± 1.1	56.2 ± 2.0	68.3 ± 6.7
HCM (pg)	16.6 ± 0.5	17.0 ± 0.3	16.9 ± 0.5	16.5 ± 0.2
CHCM (g/dl)	29.5 ± 0.2	29.4 ± 0.4	30.1 ± 0.7	23.9 ± 2.2 <sup>a</sup>
Total leukocytes (10 <sup>3</sup> /mm <sup>3</sup> )	5.1 ± 0.3	6.5 ± 0.8	6.0 ± 0.6	6.8 ± 0.7
Lymphocytes	53.2 ± 5.2	63.4 ± 3.4	63.3 ± 4.6	66.4 ± 3.9
Neutrophils	42.5 ± 4.9	30.0 ± 3.1	30.8 ± 4.2	31.4 ± 5.0
Monocytes	4.3 ± 0.7	5.7 ± 0.7	5.7 ± 0.7	3.57 ± 0.9
Eosinophils	0.0 ± 0.0	0.4 ± 0.3	0.5 ± 0.3	0.0 ± 0.0
AST (U/L)	155.7 ± 3.0	191.3 ± 13.7	207.5 ± 3.5	243.5 ± 15.9 <sup>a</sup>
ALT (U/L)	36.50 ± 6.1	56.6 ± 9.1	61.3 ± 7.1	89.5 ± 17.1 <sup>a</sup>
Urea (mg/dl)	43.0 ± 1.3	57.3 ± 2.4	53.3 ± 6.5	50.6 ± 3.6
Creatinine (mg/dl)	0.4 ± 0.08	0.6 ± 0.08	0.6 ± 0.03	0.4 ± 0.09

Data are expressed as mean ± SEM from ten animals/group analyzed by ANOVA followed by Tukey test.

<sup>a</sup>Significantly different from control group ( $p < 0.05$ ).

**Table 6.** Effects of ZBEE on biochemical and hematological parameters of peripheral blood of female mice subjected to repeated dose (28 days. v.o.).

Parameters	Control	ZBEE (mg/kg)		
		250	500	1000
Female				
RBCs (10 <sup>6</sup> /mm <sup>3</sup> )	9.0 ± 0.2	8.6 ± 0.4	8.6 ± 0.3	9.1 ± 0.1
Hemoglobin (g/dl)	14.4 ± 0.4	13.7 ± 0.4	13.5 ± 0.4	14.3 ± 0.3
Hematocrit (%)	51.1 ± 1.4	48.6 ± 1.4	47.0 ± 1.5 <sup>a</sup>	51.1 ± 1.5
VCM (fm <sup>3</sup> )	56.7 ± 0.9	56.8 ± 1.6	54.6 ± 1.3	56.3 ± 1.2
HCM (pg)	16.0 ± 0.3	16.0 ± 0.3	15.7 ± 0.2	16.5 ± 0.2
CHCM (g/dl)	28.3 ± 0.5	28.3 ± 0.4	28.8 ± 0.7	28.0 ± 0.4
Total leukocytes (10 <sup>3</sup> /mm <sup>3</sup> )	3.6 ± 0.2	6.5 ± 0.8	6.0 ± 0.6	6.8 ± 0.7
Lymphocytes	76.0 ± 2.2	78.4 ± 4.9	74.8 ± 2.6	79.6 ± 1.4
Neutrophils	18.0 ± 2.1	16.9 ± 4.7	20.0 ± 2.5	18.4 ± 1.1
Monocytes	4.1 ± 0.4	4.4 ± 0.9	3.4 ± 0.4	1.3 ± 0.4 <sup>a</sup>
Eosinophils	1.4 ± 0.3	0.8 ± 0.3	0.4 ± 0.2 <sup>a</sup>	0.0 ± 0.0 <sup>a</sup>
AST (U/l)	164.7 ± 10.6	182.7 ± 13.4	180.1 ± 16.7	151.1 ± 11.5
ALT (U/l)	24.2 ± 2.4	56.8 ± 9.0 <sup>a</sup>	63.7 ± 6.8 <sup>a</sup>	41.1 ± 1.3 <sup>a</sup>
Urea (mg/dl)	40.3 ± 1.3	46.5 ± 6.7	50.9 ± 7.1	43.3 ± 3.3
Creatinine (mg/dl)	0.6 ± 0.05	0.2 ± 0.05 <sup>a</sup>	0.2 ± 0.02 <sup>a</sup>	0.2 ± 0.03 <sup>a</sup>

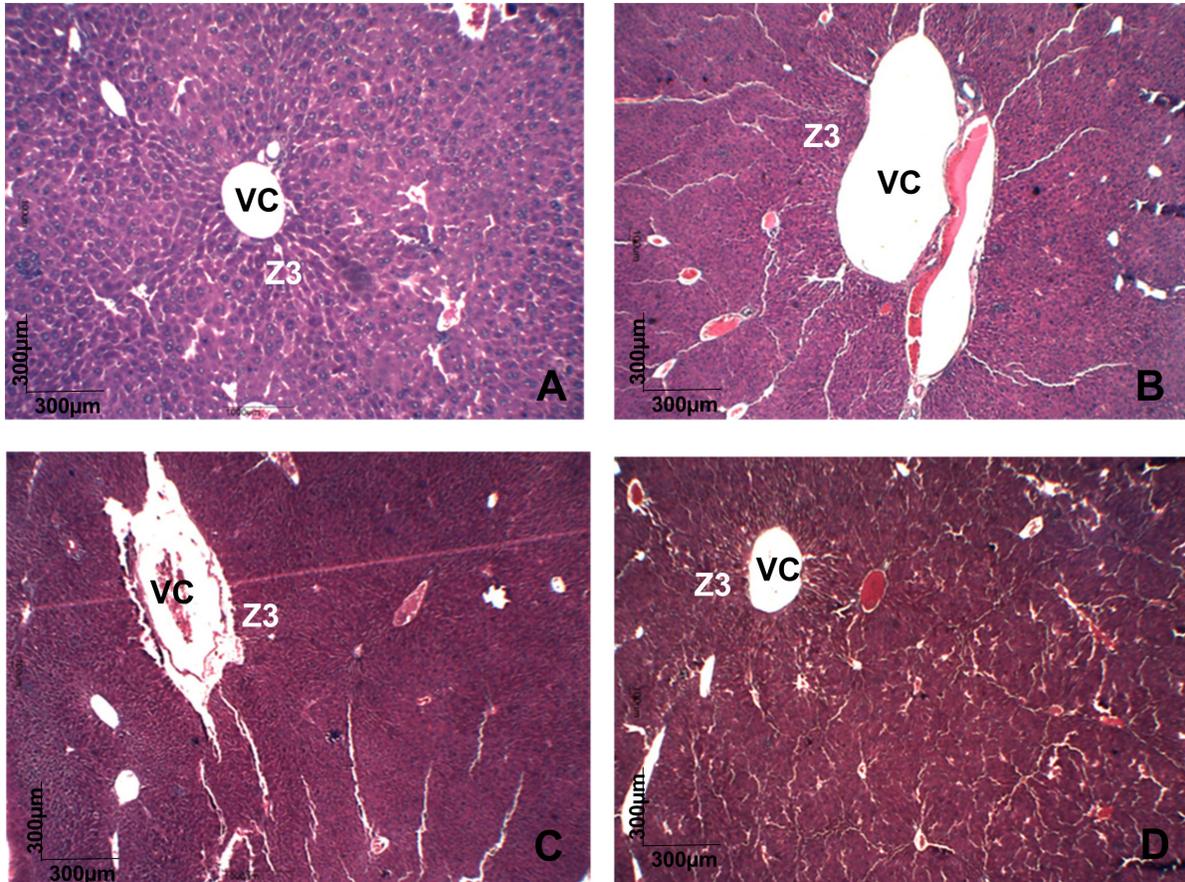
Data are expressed as mean ± SEM from ten animals/group analyzed by ANOVA followed by Tukey test.

<sup>a</sup>Significantly different from control group ( $p < 0.05$ ).

evaluation of chemicals faces to animal welfare and *in vitro* systems are more welcome than the *in vivo* systems, the *in vivo* test systems are paid more attention in the field of genotoxicity because of its weight of evidence (Hayashi, 2016). Herein, ZBEE (2,000 mg/kg) did not alter the number of micronucleated erythrocytes in peripheral blood ( $7.83 \pm 0.75$ ), comparing to the control group ( $8.33 \pm 0.99$ ), suggesting no genotoxicity

(Table 7). In contrast, literature data have shown genotoxic effects for other *Zornia* species, for example, *Zornia diphylla* (Belcavello *et al.*, 2012).

There are few literature reports about toxicological and pharmacological properties of *Zornia* species (Arun Kumar *et al.*, 2012a, 2012b, 2014; Laxane *et al.*, 2008, 2011; Rojas *et al.*, 1999; Sawant *et al.*, 2014; Talluri *et al.*, 2018). In addition,



**Figure 2.** Histopathology of liver of different groups stained with Masson's trichrome. All groups showing a normal histological picture of terminal hepatic venule (VC) surrounded in zone 3 (Z3) of the hepatocyte acinus showed no changes in cell size or connective tissue. (A) cControl male mice. (B) cControl female mice. (C) ZBEE 1,000 mg/kg male mice. (D) ZBEE 1,000 mg/kg female mice. VC =: hepatic venule; Z3 =: zone 3.

**Table 7.** Effect of ZBEE on micronucleated erythrocytes number in peripheral blood of mice subjected to a single-dose ZBEE or cyclophosphamide.

Groups	Dose (mg/kg)	Number of micronucleated erythrocytes
Control	–	8.33 ± 0.99
Cyclophosphamide	50	17.67 ± 1.39*
ZBEE	2,000	7.83 ± 0.75

Data are expressed as mean ± SEM from six animals/gender/group analyzed by ANOVA followed by Tukey test.

\*Significantly different from control group ( $p < 0.05$ ).

these species are widely used in the folk medicine (Arunkumar *et al.*, 2014; Laxane *et al.*, 2011; Sawant *et al.*, 2014). Despite this, only one toxicological study for *Zornia* species has been described (Arunkumar *et al.*, 2012b). Therefore, data presented in the present study for *Z. brasiliensis* contribute to the knowledge of the *Zornia* genus toxicological profile, as well as to the safe use in the folk medicine.

## CONCLUSION

*Zornia brasiliensis* has weak toxicity after treatment of single or repeated doses, providing data for its safe use in the folk medicine and encouraging further pharmacological studies with this medicinal plant.

## AUTHORS' CONTRIBUTIONS

Tatianne Batista, Renata Abrantes, Aline Xavier, João Carlos Pita, Monalisa Brito, Adriano Alves, Anne Dayse Silva, Yuri Nascimento, Sócrates Santos, Josean Tavares, Marcelo Silva, and Marianna Sobral contributed to the conception or design of the work or the acquisition, analysis, or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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## CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## ETHICAL APPROVALS

All the study was approved by the Animal Ethics Committee of the Federal UEPB in December 2015 (CEUA/UFPB number 0701/14).

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