

Toxicological evaluation of *Lafoensia pacari* A. St.-Hil. (Lythraceae) stem bark extract: Acute and subchronic studies in mice

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This study evaluated the safety of *Lafoensia pacari* A. St.-Hil. (Lythraceae) stem bark hydroalcoholic extract (LPE) through acute and subchronic toxicological assessments in mice. In the acute toxicity evaluation, a single 2000 mg/kg oral dose of LPE was administered to mice and clinical observations were conducted for 14 days. For subchronic toxicity, LPE doses (6.25-1000 mg/kg) were administered orally for 28 days and biochemical, hematological, histopathological analyses and renal and liver expression of Ki-67 were carried out. The acute oral toxicity evaluation of LPE showed no toxicity in mice and it was classified as category 5 (LD₅₀>2000-5000 mg/kg). In a repeated dose 28-day toxicity study, LPE (100-1000 mg/kg) led to an increase in reticulocytes, which suggests a possible proliferative effect on blood cells. In addition, LPE (400-1000 mg/kg) of produced alterations in biochemical parameters, although no microscopic changes were found in the organs analyzed. A normal expression of the Ki-67 cell proliferation indicator was observed in the kidney and liver tissues, which suggests that LPE does not bring about changes in the proliferative activity of these organs. In conclusion, LPE should be used with caution, particularly in larger doses over longer periods and also in combination with other medication.

Keywords: *Mangava-brava*. *Lafoensia pacari*/evaluation. Medicinal plant. Natural product.

INTRODUCTION

Lafoensia pacari A. St.-Hil. (Lythraceae), popularly known as *mangava-brava* or *pacari*, is a medicinal plant found in the *Cerrado* biome, the Brazilian savanna. Its stem bark has been used to treat gastric ulcers, cancer, diarrhea, kidney diseases and inflammatory processes in Brazilian popular medicine (Proença, Oliveira, Silva, 2000; Solon *et al.*, 2000; Lima *et al.*, 2006a; Nascimento *et al.*, 2011).

Phytochemical analyses carried out on *Lafoensia pacari* stem bark hydroalcoholic extract (LPE) have demonstrated the presence of tannins, saponins, steroids, triterpenes and flavonoids (Galdino *et al.*, 2009; 2015).

In addition, several other phytochemicals have been identified in hydroalcoholic extract preparations, as well as their organic fractions, such as chalcones, aurones, free xanthenes, heterosides, leucoanthocyanidins, anthraquinones, 3-*O*- β -D-glucopyranoside, gallic acid and ellagic acid, which is the main active compound of this plant (Solon *et al.*, 2000; Lima *et al.*, 2006b; Rogerio *et al.*, 2006; Galdino *et al.*, 2009; 2015; Nascimento *et al.*, 2011; Sampaio *et al.*, 2011; Tamashiro Filho *et al.*, 2012).

Scientific studies have corroborated the popular use of *L. pacari* stem bark as an anti-inflammatory agent (Rogerio *et al.*, 2006; Matos *et al.*, 2008; Rogerio *et al.*, 2008a) through the inhibition of inflammatory parameters such as interleukin-5 and eosinophil migration (Rogerio *et al.*, 2003; 2008b; Rogerio, Sá-Nunes, Faccioli, 2010). *In vivo* studies have also reported antiulcer activity, associated, at least in part, with two different mechanisms: a dependent mechanism of the antioxidant and anti-

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secretory properties and inhibition of pro-inflammatory cytokines; and an independent mechanism of gastric/intestinal motility and mucus secretion (Tamashiro Filho *et al.*, 2012). Certain studies have also highlighted other significant activities, which include *in vitro* free radical scavenging (Solon *et al.*, 2000), anticancer (Marcondes *et al.*, 2014) and antimicrobial potential (Lima *et al.*, 2006a; 2006b; Silva Junior *et al.*, 2010; Pereira *et al.*, 2011; Queiroz e Silva *et al.*, 2012). In addition, analgesic and anti-oedematous effects (Rogerio *et al.*, 2006), antidepressant-like activity (Galdino *et al.*, 2009; 2015), sedating (Matos *et al.*, 2008), antinociceptive effects, independent of anti-inflammatory activity (Matos *et al.*, 2008; Nascimento *et al.*, 2011) and wound healing effect (Pereira *et al.*, 2018) have been shown.

Despite the above-mentioned scientific evidence, and the use of the *L. pacari* stem bark hydroalcoholic extract (LPE) in Brazilian popular medicine, the toxicity data on this plant are still scarce. With that in mind, this study evaluated the safety of LPE, through acute oral systemic toxicity and repeated dose 28-day toxicity studies in mice, since the most *L. pacari* preparations used by Brazilians are made using stem bark maceration in alcoholic preparations (Galdino *et al.*, 2015).

MATERIAL AND METHODS

Obtaining LPE

The stem bark was collected from *L. pacari* plants in Bela Vista, State of Goiás, Brazil (837 m, 16°58'54.2" S, 40°55'45.1" W). The botanical material was authenticated by Dr. José Realino de Paula, and a voucher specimen was deposited at the Federal University of Goiás Herbarium (UFG no. 27031). LPE was obtained as previously described by Nascimento *et al.* (2011). In brief, the LPE was obtained through drying of stem bark pieces at 30-40 °C for 48 h and powdered. After that, it was carried out maceration in a 70% hydroalcoholic solution for 72 h and subsequent filtration. The solvent was then evaporated until dry under reduced pressure, giving extract with a yield of 16.1% (w/w). In a previously published study, phytochemical analysis of LPE showed the presence of total phenols (18.9%), flavonoids (4%) and total tannins (16.7%), being ellagic acid one of the phytochemicals present in the extract (Nascimento *et al.*, 2011). The doses of LPE were immediately prepared by dilution in sunflower oil (vehicle) before administering to the animals, as recommended by OECD (2002).

Animals

The experimental protocols were approved by the Federal University of Goiás Research Ethics Committee for the Use of Animals (UFG N° 137/2009), in accordance with institutional procedures and guidelines of the Institutional Animal Care and Use Committee, which follows the recommendations of the Canadian Council on Animal Care (Olfert, Cross, McWilliam, 1993). Swiss male and female mice (body weight: 25-35 g; age: 7-10 weeks) were obtained from the Bioterium at the Federal University of Goiás. All efforts were realized to ensure the welfare of animals. They were kept in constant environmental conditions with light-dark cycles and controlled temperature, and water and food were provided *ad libitum*. There were daily checks of changes in activity and behavior as a clinical indication of animal suffering to determine when they should be humanely sacrificed (Hubrecht, Kirkwood, 2010). At the end of each experiment, the mice were anesthetized with intraperitoneally administered xylazine (10 mg/kg) and ketamine hydrochloride (100 mg/kg) and then euthanized by cervical dislocation (Hubrecht, Kirkwood, 2010).

Toxicity studies

Acute toxicity evaluation of LPE

The acute oral toxicity of LPE was investigated according to OECD Guideline 423 (2002). This guideline recommends an alternative acute toxic class method focused on reducing animals use without reducing the scientific integrity of data obtained. Male and female mice (n=3/group/sex) were randomly separated into control and treated groups. After three hours of food fasting, a single 2000 mg/kg dose of LPE (0.2 mL) was administered orally (gavage) to each animal. The control group only received LPE vehicle (sunflower oil) (0.2 mL). After exposure, clinical observations were conducted at 5, 15 and 30 min, and every hour up to the twelfth hour of the first day. Posteriorly, the mice were examined once a day for an additional 13 days. The safety of the 2000 mg/kg dose of LPE was subsequently confirmed in other male and female mice as recommended by OECD Guideline 423.

Subchronic toxicity evaluation of LPE

Female mice were used in the subchronic toxicity test based on OECD Guideline 407 – Repeated Dose 28-day Toxicity Study in Rodents (2008). The animals were randomly divided into five groups (n=5/group) and, after three hours of food fasting, they were treated daily with vehicle (sunflower oil) or doses of LPE (6.25, 25,

100, 400 or 1000 mg/kg). The extract and its vehicle were administered orally. During the test, the animals were observed daily and weighed weekly to adjust the dose when needed. After exposure, the mice were anesthetized and approximately 1 mL of blood was removed by cardiac puncture. The animals were immediately euthanized. Aliquots of blood samples were divided between a tube without anticoagulants and another containing EDTA for biochemical and hematological assays, respectively. In addition, organs were removed for macroscopic and histopathological analyses.

Laboratory test

Hematological analysis

A hematological examination was undertaken using an ABX Micros 60 apparatus (Horiba, São Paulo, SP, Brazil). The parameters analyzed were red blood cells, hemoglobin, hematocrit, platelets and global leukocytes. Reticulocyte counting was also determined using brilliant cresyl blue dye (Nakage *et al.*, 2005). To prepare the slides, 50 μ L of the blood of each animal was incubated with the same amount of dye for 15 minutes at 37 °C. Then the number of reticulocytes per 1000 erythrocytes was determined microscopically (1000x magnification) in a blind test. A reticulocyte was identified as a red blood cell containing at least two granules of reticulum (Lamchiaghase, Pattanapanyasat, Muangsup, 2000).

Biochemical analysis

Blood samples without anticoagulants were centrifuged to obtain a serum pool, which was submitted to a biochemical examination. The following biochemical parameters were employed using LabmaxPlenno (Labtest, Belo Horizonte, MG, Brazil) equipment: alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine, albumin, total cholesterol and proteins, while the electrolytes (Na^+ and K^+) were evaluated using AVL 9180 Electrolyte Analyzer (Instrumed, Atlanta, GA, USA).

Necropsy and histopathology

A gross necropsy was conducted by visual inspection of all animals at the end of the treatment period. The following organs were collected, weighed and evaluated in terms of body weight percentage of each animal: brain, kidney, liver, heart, spleen and lung. These tissues were preserved in 10% buffered formalin to await the histopathological analysis.

For the histopathology evaluation, macroscopic cross sections of these organs were dehydrated in graded

ethanol (70-100%), cleared in xylene and then paraffin-embedded. Paraffin-embedded tissue sections of 5 μ m thickness were obtained using a microtome (Leica RM 2155, Heidelberg, BW, Germany). After mounting, the slides were dewaxed in xylene, hydrated using graded ethanol and stained with hematoxylin and eosin (H&E). The sections were observed with a light microscope (Axio Scope A1 Carl Zeiss, Jena, TH, Germany) using a 40x objective and photographed using an AxioCam MRc Carl Zeiss camera and AxioVs40 V 4.7.2.0 Carl Zeiss software.

Immunohistochemistry for detecting Ki-67

Paraffin-embedded liver and kidney sections of 3 μ m thickness were obtained and mounted on silane-coated microscope slides for immunohistochemistry to detect Ki-67 as described by Ávila *et al.* (2015).

Statistical analysis

The data were expressed as mean \pm standard deviation. Inter group variation was measured by the Analysis of Variance (ANOVA) followed by the Bonferroni test. Statistical significance was established as $p < 0.05$.

RESULTS

Acute oral toxicity evaluation of LPE

In the acute toxicity assay, the experimental groups of male and female mice were exposed to a single 2000 mg/kg dose of the LPE. During the 14 days after evaluation, only one female mouse appeared apathetic and died. The safety of this dose was subsequently defined through another experiment and no death or clinical signs of toxicity were observed in the mice. Thus, the DL_{50} of LPE was classified as category 5 ($\text{LD}_{50} > 2000 - 5000$ mg/kg) of the Globally Harmonized Classification System for Chemical Substances and Mixtures (GHS), in agreement with OECD Guideline 423 (2001). For the subsequent assay of subchronic toxicity assessment, females were used because of their possible susceptibility to LPE.

Subchronic toxicity evaluation of LPE

During the 28-day subchronic toxicity assay, neither death nor clinical signs of toxicity in the mice exposed to LPE (6.25, 25, 100, 400 or 1000 mg/kg) were observed. Moreover, no significant abnormality in feces, hair, body weight loss or behavior was identified in comparison with the control group.

In terms of the hematological analysis, the percentage of reticulocytes showed a significant increase for the higher doses of LPE, at 100, 400 and 1000 mg/kg ($p < 0.05$). The other parameters did not show any significant alterations for any of the extract doses (Table I).

The biochemical parameters of the pooled serum are summarized in Table II. In animals treated with the three lower doses of LPE (6.25, 25 and 100 mg/kg), the levels of total cholesterol, and hepatic and renal biomarkers were similar to those of the control values. On the other hand, the animals treated with 400 and 1000 mg/kg of LPE showed a tendency towards increase in levels of AST, ALT (especially at 1000 mg/kg), total proteins, albumin, urea and total cholesterol, while creatinine showed a tendency towards reduction when compared to those of the control values. In parallel, K^+ electrolyte levels were similar to those of the control group, whereas Na^+ levels were normal at lower doses. However, with doses of 400 and 1000 mg/kg, the levels of these electrolytes were reduced.

As regards organ weight analysis, only animals treated with 1000 mg/kg of LPE showed a significant reduction in liver weight ($p < 0.001$), while for the other organs analyzed (brain, kidney, heart, spleen and lung) no changes were observed in the groups treated when compared to the control (Table III). In addition, macroscopy and histopathology evaluation presented no abnormality in organs of the groups treated. These included the kidney and liver, the two major organs of metabolism and excretion of xenobiotics. In these, no changes were observed in the Ki-67 expression, which shows that the LPE probably did not alter renal or hepatic proliferative activity (Figure 1).

DISCUSSION

Several studies have highlighted the medicinal properties of *L. pacari*, corroborating its use in popular medicine. Phenolic compounds (e.g. flavonoids and tannins, and in particular the main active compound of LPE,

TABLE I - Effects of daily oral treatment of female mice with LPE (6.25, 25, 100, 400 or 1000 mg/kg/day) during 28 days in the hematological parameters

Groups	Red blood cell ($10^6/mm^3$)	Reticulocyte (%)	Hemoglobin (g/dL)	Hematocrit (%)	Platelets ($10^3/mm^3$)	Global leukocytes ($10^3/mm^3$)
Control	4.54 ± 0.64	1.66 ± 0.39	6.31 ± 0.46	21.94 ± 2.86	404.44 ± 41.73	4.54 ± 0.64
6.25 mg/kg	4.68 ± 0.19	2.0 ± 0.20	6.35 ± 0.37	22.98 ± 1.21	456.0 ± 42.0	4.68 ± 0.19
25 mg/kg	5.19 ± 0.23	2.10 ± 0.20	6.76 ± 0.47	24.70 ± 1.52	396.20 ± 70.96	5.19 ± 0.23
100 mg/kg	4.54 ± 0.25	2.75 ± 0.07 ^a	6.15 ± 0.43	21.30 ± 1.15	430.25 ± 66.75	4.54 ± 0.25
400 mg/kg	4.10 ± 0.31	2.60 ± 0.53 ^a	6.14 ± 0.39	19.66 ± 1.23	339.0 ± 64.0	4.10 ± 0.31
1000 mg/kg	4.66 ± 0.29	2.85 ± 0.30 ^a	5.85 ± 0.28	22.63 ± 1.08	368.33 ± 117.88	4.66 ± 0.29

The data are expressed as the mean ± standard deviation of five animals. ^a $p < 0.05$ vs. control. One-way ANOVA and Bonferroni's test.

TABLE II - Evaluation of serum pool biochemical parameters after daily oral treatment of female mice with LPE (6.25, 25, 100, 400 or 1000 mg/kg/day) during 28 days

Groups	ALT (U/L)	AST (U/L)	Albumin (g/dL)	Total Proteins (g/dL)	Urea (mg/dL)	Creatinine (mg/dL)	Electrolytes (mEq/L)		Total Cholesterol (mg/dL)
							K^+	Na^+	
Control	34	201.5	2.3	5.8	50.5	0.33	5.0	161	90
6.25 mg/kg	34	218	2.1	5.6	51	0.35	*	*	98
25 mg/kg	32	183	1.9	5.1	45	0.33	4.8	168	74
100 mg/kg	41	184	2.2	5.8	56	0.36	5.5	172	94
400 mg/kg	51	210	2.9	7.3	57	0.31	5.6	152	125
1000 mg/kg	43	309	2.6	6.7	64	0.21	5.1	152	122

Data represent the analysis of a sample pool, since the reduced amount of serum obtained. The asterisk (*) represents electrolyte levels not detected in the sample

TABLE III - Relative organ weights of female mice after daily oral treatment with LPE (6.25, 25, 100, 400 or 1000 mg/kg/day) during 28 days

Groups	Brain (%)	Kidney (%)	Liver (%)	Heart (%)	Spleen (%)	Lung (%)
Control	1.31 ± 0.12	1.10 ± 0.04	4.68 ± 0.26	0.61 ± 0.19	0.33 ± 0.04	0.84 ± 0.16
6.25 mg/kg	1.43 ± 0.05	1.06 ± 0.06	4.80 ± 0.19	0.42 ± 0.02	0.38 ± 0.09	0.95 ± 0.31
25 mg/kg	1.30 ± 0.10	1.12 ± 0.06	4.56 ± 0.24	0.44 ± 0.03	0.37 ± 0.05	0.92 ± 0.15
100 mg/kg	1.25 ± 0.15	1.03 ± 0.07	4.71 ± 0.14	0.55 ± 0.11	0.36 ± 0.05	0.82 ± 0.06
400 mg/kg	1.21 ± 0.16	1.0 ± 0.07	4.39 ± 0.32	0.49 ± 0.04	0.29 ± 0.03	0.97 ± 0.16
1000 mg/kg	1.50 ± 0.10	1.01 ± 0.06	4.15 ± 0.17 ^a	0.59 ± 0.16	0.25 ± 0.04	0.86 ± 0.10

Data are expressed as the mean ± standard deviation of five organs animals. ^ap<0.001 vs. control. Two-way ANOVA and Bonferroni test.

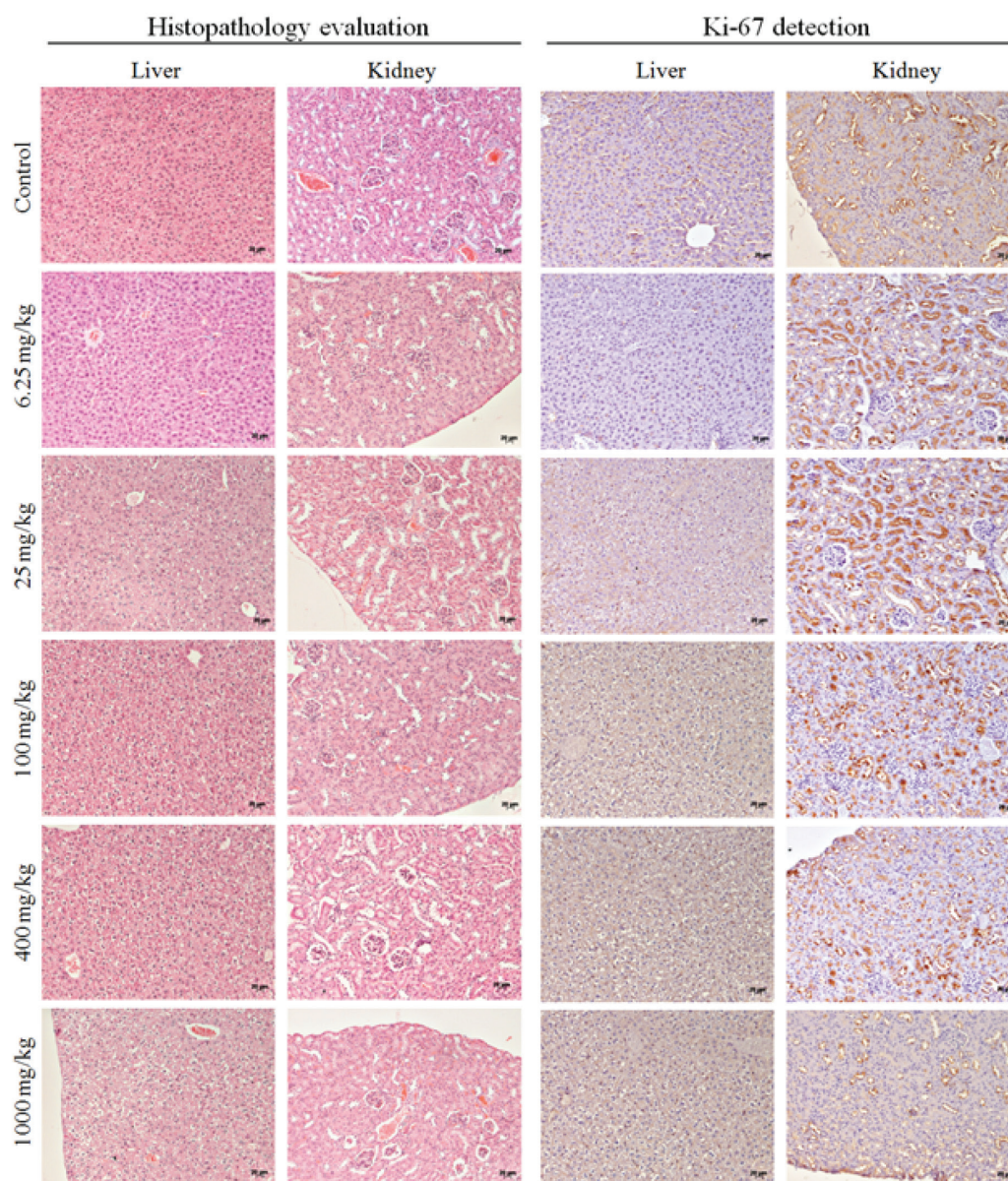


FIGURE 1 - Histopathology and Ki-67 detection representative photomicrographs of kidney and liver sections of mice treated orally with LPE (6.25, 25, 100, 400 or 1000 mg/kg/day) for 28 days.

ellagic acid) have been shown to be fundamental for the biological activities found in *L. pacari* (Solon *et al.*, 2000, Sampaio *et al.*, 2011). However, no toxicity studies had been well conducted to demonstrate the safety of this plant.

In the first observation of this study, the acute oral systemic toxicity evaluation of a single 2000 mg/kg dose of LPE showed no toxicity in either the male or female mice studied. Thus, the LD_{50} of LPE was classified as GHS category 5 ($LD_{50} > 2000 - 5000$ mg/kg), as proposed by the OECD evaluation criteria (2002). Although products classified in this category are commonly considered as having low acute toxicity, continued use can produce different biological effects and therefore should be investigated. In this regard, a repeated dose 28-day toxicity study of LPE (6.25, 25, 100, 400 or 1000 mg/kg) was evaluated through clinical, biochemical, histopathological/immunocytochemical and hematological analyses to examine possible toxic effects in tissues, specifically hepatic and renal effects (OECD, 2008).

The results indicated that a daily oral administration of 6.25, 25 or 100 mg/kg of LPE for 28 days did not alter the biochemistry, hematology or organs of the animals exposed. In addition, the data suggested that 400 and 1000 mg/kg of LPE induced alterations in the biochemical parameters of the animals, especially in transaminase levels, hematological parameters and in liver weight. However, these two higher doses do not represent the amount commonly used in folk medicine and, in this study, they only illustrate an overdose condition, as recommended by OECD (2008). In a study of the therapeutic assessment of LPE in the eradication of *Helicobacter pylori*, capsules with 500 mg powder of 20% methanolic extract of *L. pacari* were orally administered twice a day for 14 days to patients with dyspeptic complaints and positive for *H. pylori*. This treatment did not eradicate *H. pylori*; however, it was well-tolerated and individuals reported relief of symptoms (Menezes *et al.*, 2006).

Macroscopic evaluations of the organs of animals treated with LPE (6.25-1000 mg/kg) did not show any alteration. Furthermore, no change was observed in the expression of the Ki-67 cell proliferation indicator in the kidney or liver tissues, which suggests that LPE did not promote changes in the proliferative activity of these organs. Several studies have reported the protective effects of ellagic acid, the main active compound responsible for the biological effects of LPE, and of other *Lythraceae* family species on liver (Baravalia, Chanda, 2011; García-Niño, Zazueta, 2015; Kang *et al.*, 2016; Salem *et al.*, 2016) and kidney (Priya, Sabu, Jolly, 2007; Basha, Saumya, 2013; El-Garhy *et al.*, 2014; Ayhanci *et al.*, 2015) injuries triggered by xenobiotic-induced oxidative stress.

In terms of the hematological analysis, animals treated with 100, 400 and 1000 mg/kg of LPE showed a significant increase in reticulocytes, which demonstrates a possible proliferative effect on blood cells. In another study, Lima *et al.* (2013) presented the antigenotoxic and anticytotoxic activities of LPE on reticulocytes obtained from mouse bone marrow through the micronucleus assay. Corroborating these findings, Grossi *et al.* (2014) also showed that ellagic acid did not reveal any negative effect on the bone marrow hematopoietic cells. Moreover, a 90-day subchronic toxicity study showed no mortality, clinical signs, biochemical or histopathological changes in F344 male or female rats treated with different doses of ellagic acid (Tasaki *et al.*, 2008).

In conclusion, the acute oral toxicity evaluation of the LPE showed no toxicity in either female or male mice, showing $LD_{50} (> 2000 - 5000$ mg/kg) classified as GHS category 5. In addition, the results indicate that a daily oral administration of two higher doses of LPE (400 and 1000 mg/kg) for 28 days did not produce hematotoxicity, but biochemical alterations in the liver and renal function, without any macroscopic/microscopic or proliferative changes, were found. In view of the fact that there is no scientific knowledge about the toxicological profile of the *L. pacari* plant, it should be used with caution, particularly in larger doses over longer periods and also in combination with other medication.

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DECLARATION OF INTEREST

The authors declare no conflict of interest.

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