Research Paper

Toxicological evaluation of hydroethanolic extract of *Helicteres sacarolha* A. St.- Hil. et al.

Sikiru Olaitan Balogun, Iberê Ferreira da Silva Jr., Edson Moleta Colodel, Ruberlei Godinho de Oliveira, Sérgio Donizeti Ascêncio, Domingos Tabajara de Oliveira Martins

Abstract

**Ethnopharmacological relevance:** *Helicteres sacarolha*, popularly known in Brazil as ‘rosquinha’, ‘sacarolhas’, ‘semente-de-macaco’, is widely distributed in different phytogeographic zones in Brazil. Preparations from its roots and leaves are employed in popular Brazilian medicine in the treatments of ailments such as peptic ulcer, hypertension among others. Cytotoxicity, acute oral and subchronic toxicity of the hydroethanolic extract of Helicteres sacarolha was investigated as well as the classes of phytochemical present in the extract.

**Materials and methods:** Hydroethanolic (70%) extract of *Helicteres sacarolha* (HEHs) was prepared by maceration. Potential cytotoxicity was evaluated in CHO-k1 cells. Acute administration of HEHs was done in mice as a single dose up to 5000 mg/kg and subchronic oral toxicity study for 30 days in Wistar rats at daily oral doses of 0, 250 and 750 mg/kg b.w. Clinical observations and toxicological related parameters were determined every 6 days. Blood was collected for biochemical and hematological analyses, while histopathological examinations were performed on selected organs. Selected secondary metabolites detected were quantified by UV-spectrophotometry and high performance liquid chromatography (HPLC).

**Results:** The extract was non-cytotoxic to CHO-k1 cells. In acute oral toxicity, there was no mortality or clinical alterations in the female mice, at all doses, except for the transient diarrhea observed at 5000 mg/kg acute. Doses up to 2000 mg/kg caused no mortality or treatment-related clinical manifestations in the male mice, but treatment-related alterations were however observed at 4000 mg/kg, with mortalities recorded at 5000 mg/kg. During the subchronic oral toxicity study, no mortality or treatment-related clinical signs were observed. Differences in relative organ weight, hematological parameters and histopathological observations between the treated and the control groups were considered not to be treatment-related. Spectrophotometric analysis revealed the presence of relatively high content of phenolics and flavonoids in HEHs. HPLC analysis confirmed the presence of the quantified compounds and demonstrated the presence of ellagic acid, morin and naringin.

**Conclusion:** Our results confirmed that HEHs have a broad safety margin for therapeutic use.

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1. Introduction

*Helicteres sacarolha* A. St.- Hil. et al., a Malvaceae, belongs to the pantropical genus *Helicteres* that is comprised of approximately 60 species, native to the tropics of both hemispheres (Goldberg, 2009). Members of the genus *Helicteres* are most abundant in Americas in which 38 species are distributed from Mexico, Central America, the Caribbean and South America through North Western Argentina and slightly South of the tropics line in Eastern Paraguay, Brazil. The genus members are also found in the old world, Southeast Asia and Australia (Goldberg, 2009). The members of the genus are shrubs or small trees of dry lowland areas. They are characterized by having distinctive fruits, which are spiral capsules, many seeded and with a long androgynophore in free or fused form. *Helicteres sacarolha* is widely distributed in Brazil and Bolivia (Cristóbal, 2001). It is not endemic, native plant present in the phytogeographic areas of the Amazon, Cerrado and Atlantic Forest. Its distribution spreads over the North, Northeast, Midwest and

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http://dx.doi.org/10.1016/j.jep.2014.09.013
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Southeast regions of Brazil (Cristóbal, 2001; Franceschini and Bawa, 2005). It is a shrub up to 1.5 m tall, branched from the base or erects fine branches. The fruits are small capsules (2–3 cm in length) with 10–30 seeds. The plant possesses xylopodes, which re-sprout in the beginning of the raining season (Franceschini and Bawa, 2005). It is popularly known in Brazil as ‘semente-de-macaco’, ‘saca-rolhas’, ‘rosquinha’ or ‘rosquinha-de-gato’ because of its fruits shape. Populations of Helicteres sacarolha are usually found growing in clumps.

Preparations from its root and leaves are used in Brazilian popular medicine in the lowering of hypertension, alleviation of gastric ulcer symptoms, syphilis, tooth eruption, liver problems, inflammation of the ovaries, amenorrhea and in blood deparation (Truitt et al., 2005; Borba and Macedo, 2006; Silva et al., 2010; Bieski et al., 2012).

In Brazilian popular medicine, about 10 g of the dry leaves is soaked in 11 of water to make an infusion. In the treatment of gastric ulcer, a cup (150 mL) of the resulting solution is taken twice a day for 20–30 days. There is no report of toxicity by the local population that uses the plant as medicine (Bieski et al., 2012).

Despite the use of this plant in folk medicine by several traditional communities, there have been no reports on the toxicological or phytochemical investigation of this species until now to the best of our knowledge. The present work is aimed at evaluating the potential toxicity of Helicteres sacarolha using in vitro and in vivo models of acute and sub-chronic toxicities, in addition to its phytochemical analysis.

2. Materials and methods

2.1. Experimental animals and housing conditions

Albino mice Mus musculus, Swiss-Webster strain (25–30 g) and albino rats Rattus norvegicus, Wistar strain (180–200 g) were used in the in vivo studies. Animals were provided by the Central Animal House of the Federal University Mato Grosso (UFMT). The animals were kept in a room where the temperature was maintained at 22 ± 3 °C under a 12 h light–dark cycle. They were provided with standard laboratory chow (Purina, Labina®; Goiás, Brazil) and water ad libitum. The protocol for this study was according to the ethical principles of animal experimentation and was approved by the UFMT Ethics Committee for Animal Use under no. 23108.060195/13-5.

2.2. Plant material

2.2.1. Plant collection

The leaves of Helicteres sacarolha were collected from the Bahia do Campo Community Poconé municipal, located in Mato Grosso State, Brazil in the month of March 2013. Three flowered voucher specimens were herborized and were identified at Herbarium of the Federal University of Mato Grosso (UFMT), Brazil. The same was deposited in the collection of the Herbarium with register no. 40.722. The plant name was checked with www.theplantlist.org, on the 14th of August 2014. The plant collection was done based on an earlier ethnopharmacological study (Bieski et al., 2012). In order to access the traditional knowledge associated with genetic resources for research purposes from the traditional communities, an ethical clearance (approval no. 247) from the Brazilian Ministry of Environment, under the auspices of the Council for Genetic Heritage Management (CGEN/MMA), was obtained.

2.2.2. Plant extract preparation

Ten kilograms (10 kg) of the fresh leaves of Helicteres sacarolha were cleared and dried in the oven (model TE-394/4 Tecnal, Brazil) at 40 ± 1 °C, for a period of 72 h, milled using an electric miller (model TE-625 Tecnal, Brazil) and thereafter sieved (mesh size No. 40). The powdered plant material was extracted by maceration in 70% hydroethanolic solution (1:10 w/v) for 7 days in order to obtain the aqueous ethanolic extract. After extraction, the solvent was partially evaporated under reduced pressure (600 mmHg) at 40 °C in a rotary evaporator (model 801, Fisatom, Brazil), and residual solvent was eliminated in a hot air circulating oven (model TE-394/4 Tecnal, Brazil) at 40 ± 1 °C. The extract was then lyophilized (Lyophilizer model LL 1500, Heto, Italy) to obtain HEHs, which was bottled and kept in a fridge (model 350 L, Brastemp, Brazil) at 4 ± 1 °C. The extract yield per gram of dried powdered leaves was 10%. HEHs was dissolved in distilled water at the time of use.

2.3. Quantitative analysis of selected phytochemical constituents

2.3.1. Quantification of total phenolics content

Quantification of the total phenols was performed by the Folin–Ciocalteu method as described by Amorim et al. (2008) using tannic acid as a standard. Total phenols were determined by interpolation of the absorbance of the samples against a calibration curve constructed with different concentrations of tannic acid standard and expressed as mg tannic acid equivalents (TAE) per gram of lyophilized extract (mg TAE/g). All experiments were performed in triplicate.

2.3.2. Quantification of total flavonoid content

The quantification of total flavonoids was performed accordingly as previously described (Peixoto-Sobrinho et al., 2008) with slight modifications and using rutin as standard. The total flavonoid contents were determined using a calibration curve constructed with different concentrations of rutin standard and expressed as milligrams of rutin equivalents (RE) per gram of lyophilized extract (mg RE/g).

2.4. HPLC analysis

The HPLC system (Shimadzu, Tokyo, Japan) consisted of a chromatograph (LC-10 Awp series) equipped with a pump (LC-10 AD), a degasser (DGU-14A) to pump the mobile phase, rhodemyne manual injector (20 μL loop) and class integrator (LC-10A), a UV–vis (SPD-10A) detector and a column oven (CTO 10A). The extract solutions and standards were prepared with methanol and filtered through a Millipore® (0.45 mM pore size) membrane. The separation was carried out by a gradient system, using a reverse-phase Phenomenex Luna 5 mm C18 (2) (250 × 4.6 mm²) column with direct-connect C18 Phenomenex Security Guard Cartridges (4 × 3.0 mm²) filled with similar material as the main column. Mobile phase A was 0.2% formic acid in Milli-Q water and mobile phase B was 0.2% formic acid in methanol. Program gradient: 0–0.01 min, 5% B; 0.01–5 min, 25% B, 5–10 min, 45% B; 10–16 min 45% B, 16–20 min, 80% B, 20–25 min 80% B, 25–30 min 80% B, 30–35 min, 100% B; 35–35.1 min, 100% B. Flow rate: 1 mL/min, temperature: 35 °C. UV detection was done at 280 nm and 340 nm.

The compounds were identified by comparing the retention times of samples with the authentic standards, such as naringin, rutin, ellagic acid, myricetin, morin, quercetin. (±)-naringenin and luteolin (Sigma®). Concentrations of the compounds were expressed in micrograms per milligram of extract (μg/mg) by correlating the area of the analyte with the calibration curve of standards built in concentrations of 125–1000 μg/mL.
2.5. Toxicological evaluation

2.5.1. Cytotoxicity evaluation

Potential cytotoxicity of HEHs was evaluated following a described method (Nakayama et al., 1997). Briefly, CHO-k1 cells were exposed to different concentrations (3.12–200 μg/ml) of HEHs for up to 72 h. Thereafter, the cells were washed and the cell viability was assessed by using Alamar Blue. Aliquots of 200 μL of stock Alamar Blue solution were added to each well (containing 200 μL of medium [10% final solution]) and incubated for 5 h. The absorbance at 540 nm and 620 nm was read on a plate reader. Doxorubicin (Sigma) was used as a positive control.

2.5.2. Acute toxicity screening test

The effect of HEHs on the general behavior of conscious animals was evaluated in mice, as previously described (Malone and Robichaud, 1962) with slight modification. Briefly, male and female mice (n=6/group) kept in metabolic cages (Ugo Basile, model 41800, Italy), were treated daily by oral gavage with the vehicle (distilled water, 0.01 mL/g) and HEHs (250 and 750 mg/kg b.w.) for 30 days. In order to keep constant the doses administered, adjustments in dosage were made based on an animal’s body weight gain every 3 days. Body weight and intake of food and water were determined every 3 days and grouped every 6 days (D1, D2, D3, D4 and D5) over the 30-day period. Signs and symptoms of behavioral alterations were recorded, including skin, eyes, gastrointestinal, respiratory, central nervous system and peripheral alterations, including any other general changes. At the end of the treatments, animals were anesthetized with intraperitoneal injection of pentobarbital sodium at a dose of 50 mg/kg on the 30th day after a 12 h overnight fasting. Blood was collected via vena cava for biochemical and hematological analyses. Animals were then sacrificed by cervical dislocation and the organs (liver, heart, lung, kidneys, stomach and spleen) were removed. The relative weights were calculated (weight of the organ/body weight x 100) and subjected to a complete necropsy, including external body macroscopic examinations. The organs were fixed in 10% formalin solution for histopathological analysis.

2.5.3. Subchronic toxicity studies

Subchronic toxicity was evaluated as previously proposed (Chan et al., 1982). Animals (n=6/group), kept in metabolic cages (Ugo Basile, model 41800, Italy), were treated daily by oral gavage with stock Alamar Blue solution were added to each well (containing 200 μL of medium [10% final solution]) and incubated for 5 h. The absorbance at 540 nm and 620 nm was read on a plate reader. Doxorubicin (Sigma) was used as a positive control.

2.5.4. Hematological analysis

Blood samples were collected in Vacutainer® tubes containing EDTA and hematological tests, which included hematocrit (Ht), hemoglobin (Hb), total platelets, erythrocytes, total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, and basophils were performed with an automatic cell counter (Cell Dyn 3700, Abbott Laboratories, Minnesota, USA).

2.5.5. Biochemical analysis

For biochemical analysis, blood, without additive, was centrifuged at 3000 x g for 10 min at 4 °C. Serum was separated and stored at −20 °C until analysis. Biochemical parameters, which are glucose, creatinine, urea, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total cholesterol, triglycerides, uric acid, albumin, and total proteins were determined by colorimetric assays with Labtest® kits (Minas Gerais, Brazil).

2.5.6. Histopathological analysis

Organ samples from each animal were further processed for histopathology. Samples were routinely processed and sectioned at 5 μm, stained by hematoxylin and eosin techniques and analyzed under a light optical microscope. Vital organs such as liver, kidneys and lungs were preserved for histopathological analysis. The organs were fixed in 10% formalin, dehydrated in ethanol and clarified in xylene. After processing, the tissues were embedded in paraffin and then sectioned to a thickness of 5 μm using a Hyrax M60 (Carl Zeiss Microlmaging GmbH, Germany).

The sections were stained with hematoxylin and eosin. The tissues were examined under a microscope in a random order and blind to the original animal or group. The renal injury was based on degeneration of Bowman space and glomeruli, degeneration of proximal and distal tubules, vascular congestion and interstitial edema. The criteria for liver injury were vacuolization of hepatocytes and pyknotic hepatocyte nuclei, number of Kupffer cells and enlargement of sinusoids. Moreover, the histopathological change of lungs was based on congestion, edema, inflammation and hemorrhage.

2.6. Data analysis

The data for relative organ weights, hematology and serum biochemistry were analyzed statistically. Bartlett’s test was used to test for homogeneity of variance between groups. When no significant heterogeneity was detected, one-way analysis of variance (ANOVA) was applied. If parameters were found to demonstrate significant differences between groups, it was followed by Student–Newman–Keuls multiple comparison. The p < 0.05 level was considered as significant. GraphPad Prism® version 5.01 for Windows (GraphPad Software, USA) was used for statistical analysis. The IC50 was determined from a linear regression curve relating the percentage of inhibition vs the logarithm of the tested concentrations and assuming a confidence level of 99% (p < 0.01) for the curve obtained. Approximate LD50 was calculated with geometric means of transition doses (OECD, 2008). For in vitro assays that do not involve statistical analysis, we used the mean ± SEM of three independent experiments performed in duplicate.

3. Results

3.1. Quantification of total phenolics content

The equation and correlation coefficient of the linearity obtained for the calibration curve used to quantify total phenols were y=0.0689x+0.0143 and R²=0.9732, respectively. The mean absorbance values revealed a significant concentration of phenolic compounds 199.0±3.84 mg TAE/g of HEHs.

3.2. Quantification of total flavonoid content

The equation and correlation coefficient of the linearity obtained for the calibration curve used to quantify total flavones were y=0.0217x−0.0067 and R²=0.9928, respectively. The mean absorbance values revealed a significant concentration of flavonoids 144.01±0.94 mg RE/g of HEHs.

3.3. HPLC fingerprinting

Analysis by HPLC confirmed the presence of phenolic compounds detected in the preliminary phytochemical analysis and quantified spectrophotometrically in HEHs. The chromatographic profile (fingerprint) obtained by HPLC of HEHs is shown in Fig. 1.
Naringin (time 24.5 min) was detected in quantities above the detection of the calibration curve, ellagic acid (time 26.4 min) at a concentration of 60.802 mg/g totaling 6% of the dry extract and morin (time 26.7 min) at a concentration of 3.663 mg/g totaling 0.3% of the dry extract.

3.4. Cytotoxicity evaluation

Treatment of CHO-k1 cells with increasing concentrations of HEHs had no effect on the cell survival (IC50 4.200 μg/mL) up to 72 h. By contrasts, doxorubicin used as a standard was highly cytotoxic (IC50 1.770.06 mg/ml) to CHO-k1 cells.

3.5. Acute toxicity

In the present study, the male mice proved to be more sensitive to the toxicological effect of HEHs than the female mice. The male mice tolerated the dose up to 2000 mg/kg. Doubling the dose produced clinical signs and at a further increase of 25% in the dose, death occurred in 80% of the male mice (Table 1). A disparate picture was obtained for the females. The female mice tolerated the dose up to 4000 mg/kg and only presented transient diarrhea at 5000 mg/kg. Therefore, whereas no mortality was observed in the female mice at 5000 mg/kg, therefore the approximate LD50 for the male was 4472 mg/kg, indicating about 3 fold sensitivity difference between the genders in this mouse strain, with females being the more resistant sex in this study.

3.6. Subchronic toxicity

3.6.1. General conditions and behavior

There were no HEHs treatment-related mortalities recorded in rats after 30 days of oral dosing. None of the animals showed any obvious morbidity or clinical symptoms of toxicity such as changes in the skin and fur, eyes, respiratory rate, autonomic (salivation, perspiration, and piloerection), and central nervous system (ptosis, drowsiness, abnormal gait, tremor and convulsion) effects throughout the experimental period.

3.6.2. Body weight, weight gain, food and water consumption, feces and urine excretion

In this study, all rats in both the control and treated groups attained significant weight gain (46–53%, p < 0.001) after 30 days of the experiment compared to their baseline weight. However, there was no significant difference in both the body weight and accumulated body weight gain in each week throughout the study period when other treatment groups were compared to the control. Likewise, there were no significant difference in the water consumption, food and water consumption, and feces and urine excretion.

Table 1

<table>
<thead>
<tr>
<th>Male group</th>
<th>Dose (mg/kg, p.o.)</th>
<th>Behavioral alterations/toxicity signs</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>No alteration</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>HEHs</td>
<td>500</td>
<td>No alteration</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>No alteration</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>No alteration</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td>Moderate reduction in motility, diarrhea</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>Reduced motility, passivity, diarrhea, analgesia</td>
<td>4/5</td>
</tr>
</tbody>
</table>

Table 2

Effect of subchronic oral administration of the hydroethanolic extract of *Helicteres sacarolha* (HEHs) on relative weight of the internal organs of rats after 30 days of treatment.

<table>
<thead>
<tr>
<th>Organsa</th>
<th>Vehicle</th>
<th>HEHs (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>250</td>
</tr>
<tr>
<td>Brain</td>
<td>0.61 ± 0.01</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>Liver</td>
<td>3.92 ± 0.10</td>
<td>4.15 ± 0.22</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.28 ± 0.10</td>
<td>0.49 ± 0.19</td>
</tr>
<tr>
<td>Right kidney</td>
<td>0.46 ± 0.01</td>
<td>0.47 ± 0.00</td>
</tr>
<tr>
<td>Left kidney</td>
<td>0.47 ± 0.01</td>
<td>0.47 ± 0.00</td>
</tr>
<tr>
<td>Lung</td>
<td>0.62 ± 0.03</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>Heart</td>
<td>0.42 ± 0.01</td>
<td>0.40 ± 0.01</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.57 ± 0.02</td>
<td>0.63 ± 0.03</td>
</tr>
</tbody>
</table>

The values represent mean ± S.E.M. for 6 animals/group. One way ANOVA followed by Student–Newman–Keuls test.

a Relative organ weights in %.

* p < 0.05 vs vehicle.

** p < 0.01 vs vehicle.
intake and feed consumption, fecal and urine excretions between the treated groups and the control (data not shown).

3.6.3. Relative organ weights

Administration of HEHs for 30 days did not produce any abnormal change in the relative body weights, with the exception of the spleen and the lungs of rats in the 750 mg/kg group, which differed significantly, increasing by about four folds and 33% respectively when compared with the control group as shown in Table 2.

3.6.4. Biochemical and hematological data

In the biochemical parameters evaluated, all parameters remained unchanged as non-significant variations were observed. As shown in Table 3, subchronic oral administration with HEHs induced significant increases in the leukocyte and basophil counts at 750 mg/kg when compared to the control. Significant decreases were also noticed in the segmented neutrophil, monocyte and hematocrit counts in the 250 and 750 mg/kg groups respectively.

3.6.5. Histopathological examination

Histopathological analysis of the kidney, liver, heart, stomach, basophil and the brain did not reveal any difference among groups. In one animal of the 250 mg/kg treated group, mild lung inflammation with perivascular lymphocyte infiltration was observed, while 50% of the vehicle control group presented lung hyperplasia (BALT), a light perivascular infiltrate of mononuclear cells. In addition, bronchiolitis was also observed in the lungs of the vehicle treated group.

4. Discussion

The search for, and the assessment of in vitro and in vivo models that are suitable to predict adverse effects in humans exposed to chemicals represent an important issue in toxicological pharmacology (Olson et al., 2000). This is quite true in the case of preclinical toxicity testing in terms of safety concerns as well as economic gains in the development of medicines destined for human use (Jordan et al., 2010). The present study evaluated the potential toxicity of Helicteres sacarollia, a medicinal plant commonly used in the treatment of common ailments, particularly in gastric disturbances (Bieski et al., 2012).

According to the United States National Cancer Institute plant screening program, a plant extract is generally considered to have an active cytotoxicity effect if the IC50 value following incubation between 48 and 72 h is < 30 μg/ml (Suffness and Pezzuto, 1990). Thus, HEHs may be considered non-cytotoxic. We thus proceeded to examine the potential toxicity of the extract using in vivo animal models.

Acute toxicity is usually an initial study performed: to serve as the basis for classification and labeling, to provide initial information on the mode of toxic action of a substance, to help arrive at a dose of a new compound and to help in dose determination in animal studies (Ukwuani et al., 2012).

Gender discrimination was observed in the acute toxicity test at higher doses of HEHs (Table 1). There is ample evidence in scientific literature that shows gender discrimination with regard to responses to groups of drugs (Czerniak, 2001; Sin et al., 2007; Kadokar et al., 2012). The gender discrimination observed in the mice may be due to one or more of the several factors already described. These factors may include gender-based differences in the hepatic metabolism (which may be due to differences in expression of sex-specific cytochrome P450s), pharmacokinetics parameters (plasma clearance and volume) and differences in both phase I and phase II metabolism (Czerniak, 2001).

In order to explain the significant difference among groups, limitrophic values obtained from analysis of control group (higher and lower values) were employed. In the case where normal range of control group did not explain the significant difference, reference values published in the literature were utilized (Smith et al., 2002; Palmeiro et al., 2003).

In subchronic toxicity study, no treatment-related clinical symptoms or deaths were recorded during the course of the experiment. Comparison of organ weights between treated and untreated groups of animals has conventionally been used to evaluate the toxic effect of the test substance in toxicological experiments (Michael et al., 2007).

Significant differences in the relative organ weights of the spleen and the lungs were observed in the 750 mg/kg treated group with respect to the control. Rat spleen serves as the major site for the destruction of red blood cells (Bivin and Crawford, 1979). Thus, the higher relative weight in the spleen of this experimental group may be due to increased destruction of the RBCs. However, there was no corresponding decrease in the RBCs. Thus these were judged to be of little toxicological significance, since no remarkable treatment related histological alterations in these two organs were observed (Tasaki et al., 2008; Mu et al., 2011).

Clinical chemistry tests are used in toxicology studies to generate data that are crucial for evaluating altered organ function or damage in experimental animals (Smith et al., 2002; Boehm et al., 2007). In the present subchronic toxicity study of HEHs with

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**Table 3**

Effect of subchronic oral administration of hydroethanolic extract of Helicteres sacarollia (HEHs) on biochemical and hematological parameters in rats after 30 days of treatment.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle (mg/kg p.o.)</th>
<th>HEHs (mg/kg p.o.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>250</td>
</tr>
<tr>
<td></td>
<td></td>
<td>750</td>
</tr>
<tr>
<td><strong>Biochemical parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>209.00 ± 21.74</td>
<td>238.67 ± 22.45</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>31.33 ± 2.17</td>
<td>35.83 ± 1.14</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.30 ± 0.01</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>80.33 ± 4.25</td>
<td>87.50 ± 17.67</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>56.00 ± 3.90</td>
<td>53.33 ± 18.55</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>439.17 ± 18.04</td>
<td>412.50 ± 37.83</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>81.83 ± 5.72</td>
<td>80.17 ± 5.97</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>80.33 ± 18.04</td>
<td>61.50 ± 4.01</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>2.28 ± 0.47</td>
<td>3.25 ± 0.82</td>
</tr>
<tr>
<td>Total proteins (g/dL)</td>
<td>5.53 ± 0.15</td>
<td>5.58 ± 12.12</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.13 ± 0.09</td>
<td>4.18 ± 0.10</td>
</tr>
</tbody>
</table>

| **Hematological parameters**        |                      |                   |
| Leukocytes (x 10³/mm³)              | 7.08 ± 0.28          | 6.72 ± 0.49       |
| Segmented neutrophil (%)            | 22.06 ± 0.91         | 18.10 ± 0.64      |
| Lymphocytes (%)                     | 74.52 ± 0.91         | 77.55 ± 0.61      |
| Monocytes (%)                       | 0.49 ± 0.2           | 0.55 ± 0.03       |
| Eosinophils (%)                     | 2.64 ± 0.39          | 3.13 ± 0.31       |
| Basophils (%)                       | 0.29 ± 0.12          | 0.49 ± 0.20       |
| Erythrocytes (x 10³/mm³)            | 5.70 ± 0.18          | 5.82 ± 0.05       |
| Hemoglobin (g/dL)                   | 17.32 ± 0.51         | 17.40 ± 0.16      |
| Hematocrit (%)                      | 51.94 ± 1.53         | 52.20 ± 0.47      |
| MCV (FL)                            | 56.46 ± 0.41         | 56.70 ± 0.27      |
| MCH (pg)                            | 30.39 ± 0.26         | 29.91 ± 0.95      |
| MCHC (g/dL)                         | 33.82 ± 1.38         | 33.39 ± 0.16      |
| Platelets (x 10³/mm³)               | 919.20 ± 27.93       | 930.74 ± 24.77    |

Values represent mean ± SEM (n = 6/group). One-way ANOVA followed by Student–Newman–Keuls test.

* Mean corpuscular volume.
* Mean corpuscular hemoglobin.
* Mean corpuscular hemoglobin concentration.
* p < 0.05 vs vehicle.
** p < 0.01 vs vehicle.
*** p < 0.001 vs vehicle.
daily gavage at 250 and 750 mg/kg/day for 30 days, there were no alterations in any of the clinical chemistry parameters, suggesting that there were no alteration or damage of organ function.

Evaluation of hematological parameters represents an important and relevant risk evaluation as the changes in the hematological system have a higher predictive value for human toxicity, when the data are translated from animal studies (Olson et al., 2000). In addition, hematological analyses provide information about the hematopoietic system and immunological responses (Igwebuike and Obidike, 2007).

The hematological profile of treated rats showed no significant difference with control group, except in few cases that occurred in a sporadic manner. The leukocytes count was significantly elevated in the higher dose group while the neutrophil level was lesser in the lower dose group. However, these values were considered normal since they are within the reference values (Melo et al., 2013). The differences found in the hemoglobin and hematocrit values are within the normal range relative to the limitrophic values of the control group (14.9–18.5 g/dl and 44.70–55.40%, respectively).

Leukocyte differential count showed significant difference in the percentage of monocytes and the basophils. These changes were only observed in the higher dose, but were not accompanied by any clinical or histopathological changes. It is therefore unlikely to be of toxicological relevance since clinical conditions (monocytosis and basophilia) were not manifested in any of the rats. Additionally, the levels of eosinophils were maintained in the physiological range, indicating the absence of allergic process to HEHs.

Histopathological analysis revealed the presence of mild peri-vascular mononuclear cell infiltrates and bronchioliitis in the lungs of only one animal in the lower dose group, suggesting a chronic inflammation process. These alterations are not treatment related, since similar observations were noted in the control group. This type of alterations are not uncommon in rats subjected to this kind of assay (Tasaki et al., 2008; Beserra et al., 2010). Although no histopathological changes and clinical constituents were observed in the present subchronic toxicity study, caution should be taken in using the extract at high doses.

UV-Spectrophotometric quantification of HEHs demonstrated significant amount of the flavonoids and the phenolics, while HPLC confirmed the presence of and quantified selected phenolics, precisely, ellagic acid, morin and naringin. Ellagic acid is known for its wide array of biological and pharmacological properties. It has antioxidant and free radical scavenging effects, among others (Beserra et al., 2011; Rosillo et al., 2012). It is an approved food additive in Japan, due to its antioxidant property (Tasaki et al., 2008). A number of studies have addressed the potential toxicity of ellagic acid, and has been shown to be non-toxic even at high doses (Tasaki et al., 2008; Beserra et al., 2010). This may have contributed substantially to the non-toxic effect of HEHs observed in the present study, as ellagic acid is 6% of the dry extract. A study by Li et al. (2013) demonstrated that naringin at a single dose of 16 g/kg and 13 weeks oral treatment at up to 1250 mg/kg had no adverse effects on Sprague-Dawley rats. In addition, naringin is known to protect against oxidative damage, cancer, inflammation, gastric ulcer and neurotoxicity, among others, in both in vitro and in vivo assays (Borrelli and Izzo, 2000; Li et al., 2013). Morin, also a phenolic compound detected in HEHs has received attention recently due to its desirable pharmacological properties (Kim et al., 2004; Bellik et al., 2012; Costa et al., 2012). A 13-week toxicological study on morin (Cho et al., 2006) demonstrated significant alterations in some biochemical parameters at different doses in male and female rats, including increases in the relative liver and kidney weights. In the same study, no observed adverse effect level (NOAEL) in Fl44 rats fed morin supplemented feed was 299 and 356 mg/kg b.w./day for males and females, respectively. In the present study, the equivalent doses of morin were 0.75 and 2.25 mg/kg at the doses of HEHs of 250 and 750 mg/kg, respectively. Therefore, it is unlikely that morin will cause any toxic effect in the present study.

The NOAEL of HEHs is therefore calculated to be above 750 mg/kg b.w. The human equivalent dose (HED) of 750 mg/kg in the rats using body surface area (Reagan-Shaw et al., 2007) is 121.62 mg/kg. In popular medicine, an adult takes 2 cups (300 ml/day) of the leaves infusion of Helicteres sacarolha for 20–30 days. This is equivalent to 360 mg of the extract, translating to an intake of 5.14 mg/kg per day for an adult of 70 kg weight, considering that an infusion of 10 g in 1 L of water rendered 1.2 g (preliminary data). Thus, the HED is 23 times more than the approximate dose used by the population. The maximum dose tested in the acute toxicity study that caused no behavioral alteration in mice of both sexes was 2000 mg/kg, while the maximum dose in the subchronic study realized in rats was 750 mg/kg. The HED in both cases was, respectively, about 32 times and 23 times more than the dose ingested by humans, thus indicating high safety margin of HEHs in the form being utilized.

5. Conclusion

In summary, based on the toxicological parameters evaluated in this current study, we conclude that HEHs have a broad safety margin for therapeutic use. Thus, the present study indicates that HEHs is safe in rats at a NOAEL of up to an oral dose of 750 mg/kg b.w./day for 30 days. The results presented here will be of use in future development of Helicteres sacarolha as an alternative in the treatment of common ailments, especially those indicated for Helicteres sacarolha by the Community.

Acknowledgments

We wish to express our gratitude to CNPq, CAPES and INAU (704792/2009) for their financial assistance. We are also grateful to Dr. Germano Guarin Neto of UFMT Herbarium for technical assistance with plant identification.

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