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Acute and sub-acute toxicity of *Terminalia fagifolia* Mart. & Zucc. (Combretaceae) in rodents

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*Terminalia fagifolia* Mart & Zucc is a typical plant from Brazilian savanna, whose stem bark is used in popular medicine for relief of gastrointestinal discomfort. A phytochemical study showed the presence of large amounts of antioxidants (flavonoids and triterpenes). In acute toxicity tests, five groups of female mice were orally treated with stem bark extract from *T. fagifolia* or fractions by gavage at doses 2 g/kg/day (n = 5) and general behavior, adverse effects and mortality were recorded for up to 14 days. In sub-acute toxicity assays, animals received *T. fagifolia* ethanol extract or fractions at doses of 80, 240 or 720 mg/kg/day (n = 6) for 30 days and biochemical and morphological parameters were determined. The acute treatment in mice produced 40% of death. The estimate for the median lethal oral dose was 2 g/kg (LD₅₀ < 5 g/kg). Sub-acute treatment with *T. fagifolia* failed to change body weight gain, food and water consumption and biochemical profiles or reproductive parameters in female Wistar rats. In addition, no changes in macroscopic and microscopical aspect of organs were observed. Our results showed that acute or sub-acute administration of *T. fagifolia* has no significant toxicity, suggesting that it is safe for use as herbal medicine.

Key words: Toxicity, herbal medicine, reproductive toxicity, rodents.

INTRODUCTION

One of the great legacies of miscegenation of the Brazilian population is knowledge about the use of plants to treat illnesses. The Brazilian savanna has 5% of the flora and fauna of the world. Their medicinal potential is known historically by indigenous peoples, Portuguese colonists, African slaves and the hinterland communities, which have passed their knowledge from generation to generation. This conjuncture of knowledge and biodiversity, provides good scenario for the development of research aimed at discovering new drugs from native species (Ferreira et al., 2011). Vochysiaceae, Fabaceae, and Combretaceae families are among the 59 already identified in the savannah area. The family Combretaceae includes 18 genera, *Combretum* and *Terminalia* being the most abundant, with about 370 species and 200, respectively (Almeida et al., 1998).

*Terminalia fagifolia* Mart. & Zucc. known as chapadeiro, cachaporra, cachaporra-do gentio and pau de bicho is a Brazilian savannah tree, its stem bark is widely used by local people for relief of gastric discomfort, treatment of tumors and ulcers (Almeida et al., 1998). Fitoquimos and biological studies with this species showed that the
ethanolic extract of the bark has antioxidant and cytotoxic activity, as well as the occurrence of flavonoids (flavanones, flavan and chalcones), 1,3-diarilpropanos, pentacyclic triterpenes glycosylated and non-glycosylated and the steroid sitosterol (Garcez, 2006). The ethanol extract of leaves showed antioxidant potential in the DPPH assay, this activity was higher in hydroalcoholic fraction, as evidenced by the values of effective concentration able to reduce the DPPH by 50% (EC₅₀) (Ayres, 2009).

This characterization of phytochemical and antioxidant performance of *T. fagifolia* signal their full therapeutic potential. However, it is possible that antioxidants protect a given system and another made vulnerable, or even induce lesions in other systems, thus allied with the study on the medication, it is essential to study toxicological effects according to recommendations of the OECD.

Several species of *Terminalia* and their isolated compounds have been targeted for research on pharmacological activities. Studies by Manna et al. (2006) demonstrated that the aqueous extract of *Terminalia arjuna* prevented hepatotoxic and nephrotoxic effects induced by carbon tetrachloride (CCl₄). The methanol extract of *T. arjuna* showed gastroprotective activity in trials of gastric ulcer induced by diclofenac sodium in rats (Devi et al., 2007).

The *Terminalia chebula* is used in Ayurvedic medicine for its astringent, anthelmintic, expectorant, laxative effects, among others (Pfundstein et al., 2010). Pretreatment of animals with *T. chebula* Gertner reduced the production of ferric nitrotriacetic acid, inducer of oxidative stress and renal tumorigenesis (Prasad et al., 2007) and showed hepatoprotective activity in the model of toxicity induced by rifampicin, pyrazinamide and isoniazid in rodents (Tasduq et al., 2006). *In vitro* studies using different concentrations of extracts and fractions of this species showed a reduction in the concentration of cytochrome P450 in rat liver, revealing anti-apoptotic activity (Ponnusankar et al., 2011) and also promoted the proliferation and activation of matrix metalloproteinase-2 (MMP-2) in fibroblasts, justified by the antioxidant activity of the extract (Manosroi et al., 2010). In studies by Srivastav et al. (2010) for evaluation of reproductive toxicity in males of another plant of the same gender, were orally administered 100 mg/kg of extract of *T. chebula* for 60 days and observed a potent antispermatogenic effect and a sharp reduction of fertility in rats undergoing treatment.

Therefore, *T. fagifolia* Mart. & Zucc provide important pharmacological effects with great therapeutic potential, nevertheless no reports were found in the literature reports of toxicological studies, so this work is a pioneering study that aims to evaluate the acute and sub-acute toxicity of the extract and fractions of stem bark from *T. fagifolia*, including aspects of reproduction, thus contributing to a safer use of the plant by the population and the development of herbal medicines derived from this species.

### MATERIALS AND METHODS

#### Plant

Samples of stem bark of *T. fagifolia* Mart. & Zucc. were collected in the savanna biome of the region between Northeastern and Northern Brazil in the municipality of Timon, Maranhão, Brazil, in November 2006. The voucher specimen was identified by Garden Maria de Souza and deposited in Graziella Barroso Herbarium of Federal University of Piauí, Brazil, with a record 21,691-TEPB.

#### Extraction

The stem bark of *T. fagifolia* (1950 g) were air-dried, crushed (knife mill Marconi, São Paulo, Brazil) and submitted to maceration process six times with ethanol PA (Vetec, Rio de Janeiro, Brazil) at room temperature for 72 h. After removal of the solvent on the rotary evaporator (Quimis P4482, São Paulo, Brazil) at 55°C under reduced pressure and lyophilization, was obtained 416.7 g of the ethanolic extract of *T. fagifolia* (EETF). A portion of the extract (256.3 g) was taken for toxicological activity tests and remaining material (160 g) was suspended in MeOH/H₂O (2:1) and submitted to partition with ethyl acetate PA. The organic phase was concentrated (99.86 g), suspended in MeOH/H₂O (9:1) and then was extracted with hexane providing hydroalcoholic (FHA-EETF, 91.96 g) and hexane (FHEX-EETF, 4.19 g) phases. The EETF and fractions from the partition were lyophilized. The lyophilized extract was kept at room temperature until use as a suspension in distilled water.

#### Animals

Adult non-pregnant female Wistar rats (*Rattus norvegicus* var. *albinus*) weighing 180 to 220 g and female mice (*Mus musculus* var. Swiss) created and maintained in the Animal Facility for Experimentation of the Department of Veterinary Morphology, Center for Agricultural Sciences, Federal University of Piauí, Brazil. The animals were kept under 12-h light and 12 h of darkness, in individual cages in a room with air conditioning and free access to water and food (FRI-LAB Rats - Fri-Ribe).

The procedures that required euthanasia of the animals were performed according to CFMV Resolution Number 714 of 20 June 2002. The experimental procedures were approved by the Ethics Committee on Animal Experiments of UFPI, protocol number 04/2011.

The experimental protocols were elaborated and developed based on the principle of the three R's (Refine, Reduce and Redesign). That is, the lowest number of animals possible was used in order to determine statistical differences; the protocols developed do not overlap regarding the objective of the study. Moreover, the animals were handled by trained researchers in our laboratory only when necessary. They were not exposed to any kind of pain or stress caused by noise, lack of food, water, or variation in temperature.

#### Acute toxicity

Healthy female mice fasted overnight, but with access to water *ad libitum*, were randomly divided into five groups (n=5). The first group (control group) received distilled water orally. Groups 2 to 5 were orally treated with EETF, aqueous fraction (FA-EETF), hydroalcoholic fraction (FHA-EETF) and hexane fraction (FHEX-EETF) with up to dose 2 g/kg of body weight. Animals were observed for general behavioral and body weight changes.
hazardous symptoms and mortality for a period of 14 days after treatment. The acute toxicity was estimated according to the method described by the Organisation for Economic Co-operation and Development (OECD) Test Guidelines (OECD, 2001).

### Sub-acute toxicity

The method was performed according to the OECD Test Guidelines with no modification (OECD, 1995). Healthy female Wistar rats were randomly divided into six groups by treatment (n=6). Animals received water-vehicle orally (control group) or EETF with doses of 80, 240 or 720 mg/kg/day, FAQ-EETF and FHA-EETF with dose 240 mg/kg/day for 30 consecutive days. Food consumption and water intake were monitored daily. The body weight was recorded weekly and the percentage of weight gain of each animal was calculated by the difference of the final weight and the initial weight (g) divided by the initial weight x 100. To evaluate the toxicity of EETF and fractions during the estrous cycle all the rats were examined daily between 8:00 and 9:00 h, initially for seven days to verify whether they were cycling normally and only those with regular cycles were included in the experiment. Then the rats were submitted to the pharmacological experimental design. The stage of the estrous cycle of each rat was recorded daily during the 30 days of treatment. The interval between estrus of each rat was calculated from the number of days between estrus divided by the total days of treatment. At the end of the treatment, animals were fasted overnight, but allowed access to water ad libitum. They were then anesthetized with a combination of ketamine (50 mg/kg) and xylazine (11.5 mg/kg), blood collection was performed by puncturing heart, in bottles without anticoagulant (BD-Vacuette) for evaluation of biochemical parameters.

### Biochemical analysis

For biochemical analysis, blood was centrifuged at 20,000 g for 5 min to obtain serum; which was stored at -20°C until following parameters were determined: aspartate aminotransferase (AST); alanine aminotransferase (ALT); and creatinine and urea. Dosages were made using semi-automated biochemical analyzer (RA-50, Bayer Germany), using kits Lab test (Labtest Diagnostica, Lagoa Santa, MG, Brazil).

### Morphological study

The animals were euthanized with excess of the same anesthetic mixture previously described and necropsied. The organs: heart, liver, kidneys, spleen, ovaries and uterus were then removed, weighed and evaluated macroscopically as the morphology and color. The absolute masses were then converted to relative masses (mg/100 g body weight).

### Statistical analysis

The results are expressed as mean ± standard error of mean (SEM). Variance in data for body weights, food and water consumption, hematology, serum biochemistry and relative organ weights was checked for homogeneity by analysis of variance (one-way ANOVA) followed by Dunnet post hoc test for comparison between the control and treatment groups. A probability level of less than 5% (p < 0.05) was considered significant. Statistical analyses were performed with the aid of the software GraphPad Prism® 5.0.

### RESULTS

#### Acute toxicity

In the experimental model adopted for acute toxicity with EETF, FHA-EETF, EETF FAQ-or-FHXHR EETF at doses of 1 and 2 g/kg, mortality was not observed. The acute treatment has not altered other evaluated parameters such as weight gain, water consumption and feed intake. The estimate for the median lethal oral dose was LD<sub>50</sub> > 2 g/kg.

#### Sub-acute toxicity

No toxicity signs (such as piloerception, alteration in the locomotor activity or diarrhea) or deaths were recorded during the 30 consecutive days of treatment via oral route with EETF with doses of 80, 240 or 720 mg/kg/day, FAQ-EETF and FHA-EETF with dose 240 mg/kg/day for 30 consecutive days. Food consumption and water intake were monitored daily. The body weight was recorded weekly and the percentage of weight gain of each animal was calculated by the difference of the final weight and the initial weight (g) divided by the initial weight x 100. To evaluate the toxicity of EETF and fractions during the estrous cycle all the rats were examined daily between 8:00 and 9:00 h, initially for seven days to verify whether they were cycling normally and only those with regular cycles were included in the experiment. Then the rats were submitted to the pharmacological experimental design. The stage of the estrous cycle of each rat was recorded daily during the 30 days of treatment. The interval between estrus of each rat was calculated from the number of days between estrus divided by the total days of treatment. At the end of the treatment, animals were fasted overnight, but allowed access to water ad libitum. They were then anesthetized with a combination of ketamine (50 mg/kg) and xylazine (11.5 mg/kg), blood collection was performed by puncturing heart, in bottles without anticoagulant (BD-Vacuette) for evaluation of biochemical parameters.

### Morphological analysis

The relative organ weights were not altered by extract and fractions of <i>T. fagifolia</i> treatment (Table 3). The macroscopic analysis of the target organs of the treated animals did not show significant change in color and texture when compared with the control group.

### DISCUSSION

As shown in the results of acute toxicity test, the stem...
bark extract and fractions of *T. fagiflora* Mart. & Zucc were practically non-toxic, because of the result of the dose of 2 g/kg and the document of OECD does not recommend the test for acute toxicity using a dose of 5000 mg/kg, as a way to protect the animal's life (OECD, 2001).

However, the acute toxicity experiment guided the choice of doses to be used in the sub-acute toxicity protocols. We used three doses (80, 240 and 720 mg/kg) in a geometric progression of ratio, in order to establish the highest dose that does not induce toxic effect level (NOAEL) and lowest dose that induces a toxic effect without leading to death (LOAEL), and obeying 1000 mg/kg the limit for toxicological testing in accordance with

Table 2. Effects of *Terminalia fagifolia* ethanolic extract (EETF), hexane fraction (FHA-EETF) and aqueous fraction (FAQ-EETF) by oral route on Biochemical parameters in female Wistar rats treated for 30 consecutive days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>EETF 80 mg/kg</th>
<th>EETF 240 mg/kg</th>
<th>EETF 720 mg/kg</th>
<th>FHA-EETF 240 mg/kg</th>
<th>FAQ-EETF 240 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.288±0.0441</td>
<td>0.312±0.0227</td>
<td>0.288±0.0295</td>
<td>0.233±0.0422</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>350.6±94.4</td>
<td>180.7±13.1</td>
<td>238.7±42.1</td>
<td>167.6±18.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>57.9±6.10</td>
<td>54.3±2.95</td>
<td>45.1±7.84</td>
<td>38.4±5.84</td>
<td>52.4±3.75</td>
<td>52.5±4.40</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>96.4±27.2</td>
<td>194.7±76.9</td>
<td>165.8±27.7</td>
<td>132.6±64.9</td>
<td>85.6±24.18</td>
<td>77.37±30.8</td>
</tr>
<tr>
<td>Urea</td>
<td>105.6±7.0</td>
<td>90.3±6.54</td>
<td>88.4±10.30</td>
<td>102.2±12.29</td>
<td>40.43±2.51</td>
<td>41.0±2.68</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM (n=6/group). AST, aspartate aminotransferase; ALT, alanine aminotransferase. Have no significant difference (p<0.05).

Table 3. Effects of *Terminalia fagifolia* ethanolic extract (EETF), hexane fraction (FHA-EETF) and aqueous fraction (FAQ-EETF) on relative organ weight (mg/100g) in female Wistar rats treated by oral route for 30 consecutive days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Heart</th>
<th>Uterus</th>
<th>Ovary</th>
<th>Stomach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3254±99.44</td>
<td>677.2±15.46</td>
<td>341.2±18.81</td>
<td>343.4±6.40</td>
<td>242.1±27.18</td>
<td>51.61±3.322</td>
<td>640.9±27.16</td>
</tr>
<tr>
<td>EETF 80 mg/kg</td>
<td>3246±121.7</td>
<td>675.4±25.76</td>
<td>385.6±23.34</td>
<td>329.4±10.61</td>
<td>279.1±34.05</td>
<td>55.62±3.833</td>
<td>686.5±31.10</td>
</tr>
<tr>
<td>EETF 240 mg/kg</td>
<td>3579±164.6</td>
<td>664.8±25.73</td>
<td>374.2±23.03</td>
<td>324.5±12.94</td>
<td>228.7±30.26</td>
<td>48.44±2.878</td>
<td>677.4±22.88</td>
</tr>
<tr>
<td>EETF 720 mg/kg</td>
<td>3525±131.3</td>
<td>708.4±19.39</td>
<td>358.5±10.41</td>
<td>330.3±8.244</td>
<td>206.8±15.80</td>
<td>44.17±3.851</td>
<td>742.3±29.74</td>
</tr>
<tr>
<td>FHA-EETF 240 mg/kg</td>
<td>3384±141.8</td>
<td>735.1±53.18</td>
<td>416.0±28.18</td>
<td>328.8±12.34</td>
<td>261.8±38.79</td>
<td>54.25±3.714</td>
<td>592.0±28.06</td>
</tr>
<tr>
<td>FAQ-EETF 240 mg/kg</td>
<td>3226±43.65</td>
<td>657.2±24.52</td>
<td>344.1±24.33</td>
<td>300.2±9.607</td>
<td>211.8±6.336</td>
<td>43.05±4.65</td>
<td>661.8±6.60</td>
</tr>
</tbody>
</table>

Values represent the mean ± SEM (n=6/group). Have no significant difference (p<0.05).
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ALP,
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ABREVIATIONS
AST, Aspartate transaminase; ALT, alanin transaminase;
ALP, alkaline phosphatase; EETF, ethanolic extract of
Terminalia tagifolia; FAq-EETF, aqueous fraction of
ethanolic extract of Terminalia tagifolia; FHA-EETF,
hydroalcoholic fraction of ethanolic extract of Terminalia
tagifolia; NOAEL, no observed adverse effect level;
LOAEL, lowest observed adverse effect level; OECD,
organization for economic cooperation and development;
LD50, median lethal dose.

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Terminalia chebula (fruit) prevents liver toxicity caused by sub-

OECD 407 (OECD, 1995).
In nulliparous rats, treatment with EETF, FHA-EETF and
and PAq-EETF for 30 days did not cause changes in
length, sequence of phases of the estrous cycle and
repeatability of the following cycles, compared to control
group. These data indicate that the treatment did not
cause disturbances in the hypothalamic-pituitary-ovarian
preserving reproductive function (Andrews et al., 2002).
Signs of systemic toxicity were evaluated from the
reduction of body weight of experimental animals, reduced water consumption and food intake, behavioral
changes, apathy and other changes that may signal, for
example, liver injury. Only EETF females treated with
doses of 80 and 720 mg/kg showed a reduction in weight
gain body mass compared to vehicle group, no change in
the consumption of food and water could be seen. It was
also observed that the intermediate dose (240 mg/kg)
cause no reduction in mass gain, showing that the effect
was not dose dependent. Considering that in the
experiment with males (data not shown) there was no
reduction of the gain in the same doses of the experiment
with females and not observed any signs of significant
systemic toxicity within 30 days of treatment with EETF
and fractions, it can be considered that the doses studied
did not induce significant toxic effects.
Toxic effects on biological systems can be observed by
the standard serum urea, creatinine (mg/dl) whose
changes provide evidence of renal overload, acute renal
failure, or even increase in protein catabolism
(Vijayalakshmi et al., 2000; Adebayo et al., 2003).
Changes in aminotransferases enzymes (AST and ALT
U/L) and alkaline phosphatase are important indicators of
injury in liver cells. A drug does not cause liver damage
without interfering with normal activity of their enzymes
(Alía et al., 2003). The biochemical profile of animals
showed statistical similarity between groups and that all
parameters evaluated were within the reference values
(Rodrigues et al., 2006), which does not indicate
significant levels of hepatic or renal toxicity in sub-acute
treatment.
The ethanol extract of stem bark of T. tagifolia showed
low systemic toxicity and no effect was observed on
estrus cycle within the limits of doses used in this
experiment, which are also of toxicological limits.