Chronic toxicity assessment of crude ethanolic extract of *Wissadula periplocifolia* (L.) C. Presl. leaves in albino Wistar rats

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**World Health Organization (WHO) has expressed a great interest in documenting the use of medicinal plant products from populations across the world (Macedo et al., 2007). Many developing countries have increased**

Wissadula periplocifolia (L.) C. Presl. is a medicinal plant widely distributed in USA, but it has not yet been investigated extensively. Some pharmacochemical studies were performed at the Federal University of Paraíba, and the components of the crude ethanolic extract were identified; one of them was a tiliroside, which has already been shown to have excellent anti-inflammatory activity that can inhibit the arachidonic acid cascade and can inhibit the release of prostaglandins, prostacyclins, and leukotrienes from leukocytes and neutrophils. This study aimed to verify the possible long-term toxicity of this plant. Chronic toxicity tests were performed, including all recommended parameters; Wistar rats of both sexes were treated with 3 doses of the ethanolic extract of *W. periplocifolia* (L.) C. Presl leaves, and 2 satellite groups and a control group were set. The platelet and white blood cell counts for all treated males were higher than those in the controls. The anatomopathological tests did not reveal any alterations which were consistent with those obtained from the biochemical tests.

**Key words:** Chronic toxicity test, medicinal plants, phytotherapeutic, *Wissadula periplocifolia* (L.) C. Presl.

**INTRODUCTION**

The World Health Organization (WHO) has expressed a great interest in documenting the use of medicinal plant products from populations across the world (Macedo et al., 2007). Many developing countries have increased
their documentation of ethno-medical data, and scientific research is being conducted using medicinal plants. However, the popularity and traditional use of medicinal plants are not sufficient to validate their effectiveness and safety. Therefore, evaluating the risks and benefits associated with their use is necessary by conducting toxicological studies (Lima et al., 2014).

Members of the family, Malvaceae are known to exert several pharmacological effects and are used mainly as anti-tumor agents and in the treatment of ulcers, wounds and, especially, inflammatory diseases (Chaves et al., 2013). Wissadula periploclifolia (L.) C. Presl. is a rarely studied species of the family Malvaceae. Previous studies have shown that this species has higher antioxidant potential than several plants from the same family (Oliveira et al., 2012); its extracts have been shown to exert antibacterial effects against Enterococcus faecalis (Teles et al., 2014).

The pharmacological potential of W. periploclifolia is related to its production of different secondary metabolites. Previous phytochemical studies on W. periploclifolia have suggested the presence of flavones, flavonoids, glycosylated phenolic acids, chlorophyll derivatives, steroids and triterpenes (Teles et al., 2014, 2015). However, many plant products and phytochemicals can produce toxic and adverse effects. Thus, investigating the potential toxicity of natural products used in popular medicines is necessary (Bussmann et al., 2011). Other studies indicated that the W. periploclifolia CEE showed no relevant toxicity in the pharmacological screening at a dose of 2000 mg/kg, and no deaths were observed during the acute study (Guedes et al., 2016). The results of other tests with W. periploclifolia demonstrated a significant anti-inflammatory activity, comparable to that of indomethacin which was used as standard. However, this effect was not CEE dose-dependent (Guedes et al., 2016).

The investigation of chronic toxicity with CEE of the leaves of W. periploclifolia (L.) C. Presl., by observing behavioral parameters, weight, temperature, hematological, biochemical, anatomy and pathological study is the objective of this assay.

MATERIALS AND METHODS

Plant materials

W. periploclifolia (L.) C. Presl plants were collected from Pedradu Boca (PB), municipality of Ararúna-PB, Brazil, in August, 2005. Botanical identification was performed by Prof. Dr. Maria de Fátima Agra of the Natural Products Research Center, UFPB. A specimen was archived in the herbarium of Prof. Lauro Pires Xavier at the Center of Exact and Natural Sciences, Federal University of Paraíba, under number 6498. The collection authorization number is SISBIO 46923-2.

Preparation of the extract

The leaves (3 kg) were dried in an oven at 40°C for 96 h and then ground to powder in a mechanical mill; subsequently, the powder was macerated with ethanol (EtOH) for 3 days. This procedure was repeated to maximize extraction. The ethanolic extract was concentrated using a rotary evaporator at 50°C, and 705 g of crude EtOH extract (CEE) was obtained. The CEE (200 g) was solubilized in EtOH: water (9:1) solution.

Animals

Adult albino Wistar rats (Rattusnor vegicus), males and nulliparous non-pregnant females, weighing between 200 and 300 g, were supplied by the vivarium, courtesy Prof. Thomas George, UFPB.

The animals were grouped in polyethylene cages, maintained under controlled temperature at 27±2°C, without the use of any medication, and provided food (Purina® ration pellets) and drinking water ad libitum; water was provided in graduated polyethylene bottles placed in metal grids in the upper part of the cages. The animals were maintained under a 12 h light-dark cycle. Before the start of the experiment, the animals were placed in the working environment for at least 30 min. The experimental protocol was approved by the Ethics Committee for Animal Experimentation (CEUA) of the UFPB (Process no., 169/2015).

Toxicological assay

The animals were divided into 6 groups; one control and 5 test groups, containing 20 animals each (10 males and 10 females) and treated orally (gavage) daily in the morning for 90 days. The control group was administrated water, which was the vehicle used for the preparation of W. periploclifolia CEE. The 3 treatment groups received the following doses of CEE: the lowest dose of 10 mg/kg, an intermediate dose (3×) of 30 mg/kg, and a higher dose (9×) of 90 mg/kg. The minimum dose was calculated from a previous study of carrageenan paw edema induced by W. periploclifolia CEE (Guedes et al., 2016). The fifth and sixth groups (satellite groups) received doses of 30 and 90 mg/kg, respectively. All animals were killed 30 days after the end of the experiment to assess the reversal of possible toxicities condition.

The effects of prolonged administration of the W. periploclifolia CEE were evaluated using the following parameters: temperature, water and food consumption, weight evolution, exploratory activity (open-field test), and motor activity of the animals (Rota-rod test). Hematological and biochemical assays and anatomy pathological examinations were performed using rat organs. The results were compared with those obtained from the control group, according to previous studies (Castello et al., 2011).

Temperature

The body temperature was measured using a digital thermometer (modelMC-3BC®, OMRON, China). Temperature was measured by lubricating the thermometer’s thermosens or with petroleum jelly and introducing it up to 5 cm in the rectum, reaching the colon. This parameter was measured weekly in all animals.

Consumption of water and food and weighted assessment

The consumption of water and food pellets was evaluated in all the animals. Every day, 200 g of food and graduated bottles containing 250 mL of water were placed in the cages. On the following day, in
the morning, the water volume ingested by the animals was measured and assessed cumulatively. The consumed food was evaluated in a similar manner. The animals were weighed daily to calculate the administered dose.

Behavioral evaluation

Open-field test

The open-field apparatus (Insight, Brazil) was used for behavioral evaluation following the protocol described by Carlini et al. (1986) to analyze the exploratory activity of animals by spontaneous movement (locomotion). The test registered the number of crossings with 4 paws between the grid and across the square crossings of the field and quantified self-cleaning, rinsing, and defecation (number of fecal cakes) behaviors.

The parameters were used as the index to determine the influence of CEE on the emotional behavior of the animals; it indicates the changes in the central nervous system. Every fortnight, one hour after CEE administration, the parameters described above were assessed in the treated and control animals (3 min for each animal) (Mansur et al., 1971).

Rota-rod test

Further, every fort night, one hour after CEE administration, the treated and control animals were placed on a rotating rod of the Rota-rod apparatus (Acceler Rota-Rod (Jones & Roberts) for rats; 7750: Ugo Basile, Italy), which was rotated at a constant speed of 9 rpm, and the riding time on the device was recorded with 3 repetitions.

Laboratory assessment of hematological parameters

After the chronic toxicity experiment, blood samples were collected from the brachial plexus of the animals treated with CEE (10, 30 and 90 mg/kg/day) and the control group. After a12 h fasting period, blood was collected in tubes containing EDTA for the assessment of hematological parameters (hemogram and platelet count) and in tubes containing separator gel; they were centrifuged for 10 min at 3,500 rpm to obtain serum, which was subjected to biochemical analysis for glucose, urea, creatinine, transaminases, aspartate amino transferase (AST), alanine amino transferase (ALT), cholesterol, triglycerides, uric acid, alkaline phosphatase, total proteins and fractions, calcium, magnesium, sodium and potassium levels.

Anatomy-pathological examination

The test animals were killed by administering high levels of the anesthetic (xylazine and ketamine at 30 and 255 mg/kg, respectively) followed by cervical action as recommended by the scientific community, and the viscera were collected and examined macroscopically, with resection and consecutive weighing. The heart, liver and kidney were sectioned by sagittal incision, and the lungs were subjected to perfusion through the trachea by using 10% formaldehyde.

The organs of the animals treated with the highest dose (90 mg/kg) were sectioned and immersed in a fixing solution. After 12 h of fixation, the samples for histopathological processing were obtained by paraffin embedding and stained using hematoxylin and eosin and subjected to Masson trichrome.

Satellite groups

Thirty percent of the male and female animals treated with 30 and 90 mg/kg CEE were kept alive for 30 days after the remaining were killed, to assess the reversibility of the possible toxic effects caused by the administration of CEE.

RESULTS

Administration of CEE at the stipulated doses did not cause mortality in any of the animals, but bone marrow signs of toxicity were observed.

Temperature

Small changes in body temperature were observed in both sexes. As compared to that in the control group, the temperature increased during the first week after treatment with10 mg/kg CEE in males, whereas it increased during the third week at doses of 30 and 90 mg/kg in females.

Consumption of water and food

Water consumption significantly increased in the fourth week in males treated with 90 mg/kg CEE, whereas it increased in the tenth week in females after treatment with 30 mg/kg CEE and in the tenth, eleventh and twelfth weeks in females treated with 90 mg/kg CEE. The cumulative water and food consumption in males and females is shown in Figures 1, 2 and 3.

Weight evolution

No change was observed in the weight of control rats or those treated with the CEE.

Behavioral (open-field test) and motor (Rota-rod test) assessment

In the open-field test, changes were observed only in 2 instances in males treated with 90 mg/kg on the first day, in which an increase in behavioral activity was noted. However, in the Rota-rod test, the riding time on the rotating rod decreased only in one of the males treated with 90 mg/kg on the fourth day. All other results were normal in all the groups.

Clinical blood assessment

At 90 days in the treated groups and 120 days in the 2 satellite groups, whole blood was collected for biochemical measurements and hematological assessment.
**Figure 1.** Water consumption by male Wistar rats treated with *W. periplocitolia* CEE during the chronic toxicity test. The values are expressed as mean ± standard error of the mean (n = 10) One-way analysis of variance (ANOVA)/Tukey test (*P*<0.05).

**Figure 2.** Water consumption by female Wistar rats treated with *W. periplocitolia* CEE during the chronic toxicity test. The values are expressed as mean ± standard error of the mean (n=10) One-way analysis of variance (ANOVA)/Tukey test (*P*<0.05).

**Hematological parameters**
Statistically significant changes were observed in the hematological parameters. In both sexes, the hematocrit value of the satellite groups treated with 30 and 90 mg/kg reduced. However, the hematocrit value was slightly higher in males than in females treated with 30 and 90 mg/kg CEE; this increased the mean corpuscular hemoglobin concentration (MCHC) in the 90 mg/kg satellite group as compared to that of the 90 mg/kg female treatment group. Significant differences in leucopenia mainly at the expense of neutropenia were noted in the male treated groups and the satellite groups, but no tin females as we can see in the Table 1.

**Biochemical parameters**
Some parameters (total protein, albumin, calcium and uric acid) were significantly different in the treated groups than in the control group as we can see in Table 2.

**Anatomy pathological study**
Macroscopically, the organs showed no significant anatomical changes, with minimal variations in weight. None of the animal organs showed histological peculiarities, except the lung [Figure 4A, B, C, D] that showed minor changes such as discreet pulmonary infiltrates, which was related to the oral administration by gavage.

**DISCUSSION**
Toxicological studies involving evaluations of efficacy and
Figure 3. Food consumption by male Wistar rats treated with *W. periploclia* CEE during the chronic toxicity test. The values are expressed as mean ± standard error of the mean (n=10). One-way analysis of variance (ANOVA)/Tukey test (*P<0.05).

Table 1. Hematological parameters of Wistar rats treated with *W. periploclia* CEE during the chronic toxicity test.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10 mg/kg</th>
<th>30 mg/kg</th>
<th>90 mg/kg</th>
<th>30 mg/kg satellite</th>
<th>90 mg/kg satellite</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red blood cells (10^6)/µL</td>
<td>8.43±0.19</td>
<td>8.35±0.17</td>
<td>8.25±0.22</td>
<td>8.51±0.14</td>
<td>7.87±0.11</td>
<td>6.61±0.83</td>
</tr>
<tr>
<td>Hemoglobin(g/dL)</td>
<td>16.5±0.5</td>
<td>1.9±0.3</td>
<td>15.6±0.4</td>
<td>16.3±0.2</td>
<td>15.9±0.4</td>
<td>14.0±1.8</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>45.8±1.3</td>
<td>45.2±0.9</td>
<td>44.5±0.7</td>
<td>44.4±0.7</td>
<td>41.5±0.6</td>
<td>34.3±4.5*</td>
</tr>
<tr>
<td>MCV (µg)</td>
<td>53.1±0.6</td>
<td>53.2±0.3</td>
<td>53.6±0.6</td>
<td>53.4±0.5</td>
<td>52.7±0.5</td>
<td>51.9±0.9</td>
</tr>
<tr>
<td>MHC (µg)</td>
<td>19.2±0.3</td>
<td>18.7±0.2</td>
<td>19.2±0.3</td>
<td>19.1±0.2</td>
<td>2.2±0.5</td>
<td>19.6±0.3</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>36.1±0.3</td>
<td>35.1±0.3</td>
<td>35.9±0.3</td>
<td>35.8±0.2</td>
<td>38.4±0.5*</td>
<td>37.8±0.3*</td>
</tr>
<tr>
<td>Leukocytes (mm^3)</td>
<td>6573±360.1</td>
<td>3789±281.1*</td>
<td>3087±471.7*</td>
<td>3054±31.2*</td>
<td>4550±1245</td>
<td>2920±209.3*</td>
</tr>
<tr>
<td>Neutrophils (mm^3)</td>
<td>1948±183.3</td>
<td>1155±111.7*</td>
<td>927.0±191.2*</td>
<td>800.1±132.8*</td>
<td>1228±399.2</td>
<td>898.7±118.9*</td>
</tr>
<tr>
<td>Eosinophils (mm^3)</td>
<td>8.94±8.94</td>
<td>11.55±8.893</td>
<td>9.62±9.62</td>
<td>13.28±5.802</td>
<td>16.70±16.70</td>
<td>6.00±6.00</td>
</tr>
<tr>
<td>Lymphocytes (mm^3)</td>
<td>3759±371.5</td>
<td>2380±147.9*</td>
<td>2017±283.2*</td>
<td>2038±250.3*</td>
<td>2989±818.0</td>
<td>1854±150.3*</td>
</tr>
<tr>
<td>Monocytes (mm^3)</td>
<td>644.8±71.1</td>
<td>242.0±39.9</td>
<td>133.2±41.5*</td>
<td>111.0±12.1*</td>
<td>316.1±143.0</td>
<td>161.8±24.0</td>
</tr>
<tr>
<td>Platelets(10^9/mm^3)</td>
<td>647.6±0.31</td>
<td>609.6±0.24</td>
<td>497.4±0.30*</td>
<td>456.4±0.32*</td>
<td>622±0.47</td>
<td>585.2±0.73</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Red blood cells (10^6)/µL</td>
<td>7.75±0.12</td>
<td>7.46±0.15</td>
<td>7.25±0.60</td>
<td>7.52±0.12</td>
<td>6.38±0.63</td>
<td>6.14±0.73</td>
</tr>
<tr>
<td>Hemoglobin(g/dL)</td>
<td>15.4±0.2</td>
<td>14.4±0.3</td>
<td>15.7±0.7</td>
<td>14.8±0.3</td>
<td>13.0±1.2</td>
<td>12.8±1.4</td>
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<tr>
<td>Hematocrit (%)</td>
<td>40.9±0.7</td>
<td>39.3±0.7</td>
<td>40.6±0.5</td>
<td>39.8±0.6</td>
<td>33.9±3.5*</td>
<td>32.6±3.5*</td>
</tr>
<tr>
<td>MCV (µg)</td>
<td>52.8±0.2</td>
<td>52.7±0.4</td>
<td>52.0±0.4</td>
<td>53.0±0.4</td>
<td>53.0±0.6</td>
<td>53.4±1.3</td>
</tr>
<tr>
<td>MHC (µg)</td>
<td>19.6±0.1</td>
<td>19.4±0.2</td>
<td>19.2±0.3</td>
<td>19.6±0.3</td>
<td>20.4±0.3</td>
<td>20.9±0.4</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>37.0±0.2</td>
<td>36.8±0.5</td>
<td>36.9±0.3</td>
<td>37.0±0.4</td>
<td>38.5±0.5</td>
<td>39.1±0.6*</td>
</tr>
<tr>
<td>Leukocytes (mm^3)</td>
<td>3784±399.7</td>
<td>4017±461.5</td>
<td>3206±573.5</td>
<td>4322±973.6</td>
<td>3908±916.6</td>
<td>2600±668.4</td>
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<tr>
<td>Neutrophils (mm^3)</td>
<td>1125±113.9</td>
<td>1447±168.5</td>
<td>850.0±209.4</td>
<td>1893±888.3</td>
<td>1219±248.4</td>
<td>739.5±246.5</td>
</tr>
<tr>
<td>Eosinophils (mm^3)</td>
<td>10.9±7.1</td>
<td>6.1±4.4</td>
<td>2.5±2.5</td>
<td>2.7±2.7</td>
<td>14.1±14.1</td>
<td>11.1±11.1</td>
</tr>
<tr>
<td>Lymphocytes (mm^3)</td>
<td>2436±280.3</td>
<td>2336±266.8</td>
<td>2178±511.5</td>
<td>2299±262.1</td>
<td>2419±585.6</td>
<td>1654±343.6</td>
</tr>
<tr>
<td>Monocytes (mm^3)</td>
<td>213.0±32.8</td>
<td>232.6±61.6</td>
<td>118.3±29.3</td>
<td>127.4±31.4</td>
<td>255.8±74.3</td>
<td>195.3±88.0</td>
</tr>
<tr>
<td>Platelets(10^9/mm^3)</td>
<td>480.6±63.6</td>
<td>603.1±18.2</td>
<td>530.2±24.2</td>
<td>517.6±28.5</td>
<td>46.7±50.4</td>
<td>490.0±80.5</td>
</tr>
</tbody>
</table>

The values are expressed as mean±standard error of the mean (n=10). One-way analysis of variance/ Tukey test *P<0.05. MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV mean corpuscular volume
safety are fundamental to the development of medicinal plant products. In a previous study, the changes in the organs and body weights of rats were considered clear indications of damage caused by the test substance (Berenguer-Rivas et al., 2013). Treatment with *W. periploclolia* CEE reduced the consumption of water and food in females and males at some doses as compared to that in the control group. However, the weights of the animals did not vary significantly between the control and treated groups; thus, CEE was thought to have low toxicity after chronic exposure (Berenguer-Rivas et al., 2013). With regard to motor and behavioral tests, the animals were active and responsive to stimuli, without any clinical signs that could be associated with local or systemic toxic effects. Normality was noted, and the behavior of the animals remained normal (Castello Branco et al., 2011).

The hematopoietic system is one of the more sensitive
targets for toxic compounds and is an important index to measure the physiological and pathological states of treated animals. Changes in these parameters in animal studies have a greater predictive value for human toxicity to a substance (Olson et al., 2000). For the assessed hematological parameters, statistically significant alterations were observed as compared to published data (Castello Branco et al., 2011).

However, in the red series of both sexes, the hematocrit value decreased in the 30 and 90 mg/kg satellite groups, which was slightly more pronounced in males than in the satellite groups. These alterations increased the MCHC in the 90 mg/kg satellite group as compared to that of the 90 mg/kg group of females, which was as expected, since erythrocytes are known to remain viable in the peripheral blood for 90 to 120 days (Castello Branco et al., 2011). Unlike that in previous studies (Castello et al., 2011; Giknis and Clifford, 2006), significant difference was noted among males of the 10, 30, and 90 mg/kg groups and those of the 30 and 90 mg/kg satellite groups, which

Figure 4. A and B: Photomicrographs of bronchial parenchyma of normal female and male controls, showing no significant change. C and D: Photomicrographs of the bronchial parenchyma of a male and a female from the treated groups showing mucous infiltrates in the membranes.
showed leukopenia mainly at the expense of neutrophilia; this was not observed in females. Regarding platelet count, no statistically significant changes were noted among females whereas, in males, a significant reduction was observed in the groups treated with 30 and 90 mg/kg CEE, although this count was within the normal limits for the rats studied (Castello Branco et al., 2011; Giknis and Clifford, 2006).

The CEE had been shown to have a potential-inflammatory effect (Telles et al., 2015) and can inhibit the arachidonic acid cascade, thereby inhibiting the release of prostaglandins, prostacyclins, and leukotrienes from leukocytes and neutrophils (Guedes et al., 2016). The CEE of W. periplocifolia also included a potential-inflammatory substance, tiliroside, which according to previous studies, selectively inhibits the spinal release of platelets and granulocytes in male rats after prolonged use (Telles et al., 2015).

Some enzymes and proteins (ALT, AST, gamma-glutamyl transferase and bilirubin; OCDE2008b) (Brandt et al., 2009) and biomarkers of renal function, such as blood creatinine, urea and nitrogen, can be considered indicators of hepato cellular alterations (Lameire et al., 2005). Of the 12 biochemical indicators of transaminases, AST and ALT are the 2 enzymes that are associated with hepato cellular damage and are thus used as biomarkers to predict possible toxicity (Huang et al., 2006; Ojiako and Nwanyo, 2006). Although, both are common hepatic enzymes, and their concentrations in hepatocytes is high, only ALT is noticeably specific to liver function, since AST is mainly present in the myocardium, skeletal muscle, brain and kidneys (Sacher and McPherson, 1991; Withawaskul et al., 2003). No statistically significant difference was noted in the hepatic or renal parameters between the treated and control groups. Some parameters (total protein, albumin, calcium and uric acid) were statistically significant from those in the control group. Initially, hypoalbuminemia was noted in males treated with 90 mg/kg CEE and in females treated with 30 mg/kg CEE as compared to that in rats of the 90 and 30 mg/kg satellite groups. This might have been caused by the reduction in food intake. The reduction in albumin also justifies the reduction in calcium, since 50% of calcium is bound to albumin in the peripheral blood, and its levels are measured and interpreted by comparing the levels of free calcium and those bound to albumin. However, according to Giknis and Clifford (2006), the reduction noted in this study was not clinically significant and could be considered as a physiological variation in both males and females (Castello Branco et al., 2011).

Qualitative macroscopic analyses revealed that none of the tested doses produced changes in the vital and reproductive organs of the treated animals. Similarly, in the histopathological analysis, no changes suggesting toxic effects were noted. These results were in accordance with the obtained data from biochemical analyses. The chronic toxicity study showed the occurrence of major and significant changes primarily at the level of blood parameters and warrants additional studies for confirming these results in the long term.

Conflict of Interests

The authors have not declared any conflict of interests.

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