Acute and Subchronic Toxicity Studies of Roots Barks Extracts of *Calotropis procera* (Ait.) R. Br Used in the Treatment of Sickle Cell Disease in Burkina Faso

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Abstract: *Calotropis procera* Ait. (Family Asclepiadaceae) is a species widely used for the treatment of various diseases including sickle cell disease in Burkina Faso. It enter in the composition of FACA® , drug developed by Institute for Research in Health Sciences, Burkina Faso and used in the treatment of sickle cell disease. The objective of this study was to evaluate the toxic effects at short and long term of *Calotropis procera* root barks in some rodents. In the acute test, the limit test dose of 2000 mg/kg of aqueous and hydroalcoholic extracts were administered orally to NMRI mice and then observed individually 2 h post-dosing and at least once daily for 14 days. Sub-chronic toxicity was evaluated after a daily oral administration of 20 mg/kg body weight of aqueous extract for 3 and 6 weeks to Wistar rats. Biochemical and hematological assessments as well as body and relative organ weights of the rats were carried out. The limit dose of 2000 mg/kg did not cause any mortality or signs of acute toxicity in the mice tested during the observation period. In the sub-chronic tests, the results did not show any treatment–related abnormalities in terms of physiological, hematological parameters. However, on biochemical parameters, a slight but not significant (p˃0.05) elevation of ALT and AST were noticed in treated groups. Our results suggest that aqueous extract of *Calotropis procera* which contains many chemical compounds is relatively safe when administered orally and contribute to the safe use of this part of plant in pharmaceutical formulations.

Keywords: Biological parameters, *Calotropis procera* Ait., FACA®, mice, wistar rat

INTRODUCTION

Sickle cell anemia, a genetic disease that affects hemoglobin of red blood cells is a major public health problem in Burkina Faso and Africa, the prevalence rate reaching between 10 and 40% of the population in some areas (OMS, 2006).

However, there is currently no curative treatment against sickle cell disease (OMS, 2006).

Also, in Burkina Faso, as in many African countries, the available drugs are imported and are not accessible to the majority of the population due to the high cost (Guissou et al., 1995). Therefore; research and development of drugs against sickle cell disease based on traditional medicine; becoming a priority in Africa.

*Calotropis procera* (Ait.) R. Br. (Asclepiadaceae) commonly called “Pomme de Sodome” in french is a species widely used in traditional medicine for the treatment of various diseases including sickle cell disease, asthma, cancer (Nacoulma, 1996).

Scientific works carried out by “Institut de Recherche en Science de la Santé” (IRSS) in Burkina Faso have permit the development of a phytomedicinal drug, named FACA® and used for the treatment of sickle cell disease. FACA® is a mixture of roots barks powder of *Calotropis procera* (Ait.) R. Br (Asclepiadaceae) and *Zanthoxylum xanthoxyloides* Lam (Rutaceae). FACA® is revealed to have clinically an efficacy for the treatment and prevention of sickle cell crisis in children (Guissou et al., 1995; Nikiema et al., 2010).

*Calotropis procera* belonging to the family of Asclepiadaceae is a small tree, distributed in tropical and subtropical Africa and Asia (Millar and Morris, 1987).

Several authors reported that *Calotropis procera* have various pharmacological activities such as anti-inflammatory and analgesic activities (Basu and Chaudhuri, 1991), antibacterial activities (Mainasara et al., 2011), analgesic activities (Dewan et al., 2000) and antioxidant activities (Faruki et al., 2011).
However, toxic effects of latex and leaves of *Calotropis procera* have been reported. (Mahmoud *et al.*, 1979b; Pahwa and Chatterjee, 1988; Singhal and Kumar, 2009; Mahmoud *et al.*, 1979a).

Also *Calotropis procera* is well known to possess cardiac glycosides such as cardenolides which are cardiac poison (Van Quaquebeke *et al.*, 2005).

Considering the chronic treatment of sickle cell disease and the presence of cardenolides in the plant, it was necessary to evaluate the toxicity of these extracts.

The aims of this present work was therefore to evaluate the toxic effects at short and long term of *Calotropis procera* root barks to contribute to the safe use of this part of plant in pharmaceuticals formulations.

MATERIALS AND METHODS

**Materials:**

- **Plant material:** Fresh roots of *C. procera* were collected in Roumtemga located at 25 km north-East of Ouagadougou, capital of Burkina Faso, savannah countries, in July 2010 (temperature about 30°C with high relative humidity).

  The plant sample was identified and authenticated at “Herbier National du Burkina (HNBU)” located at “Centre National de Recherche Scientifique et Technologique (CNRST)” where the voucher specimen has been deposited under number HB 8716.

  The barks were washed with tap water, dried under ventilation in the shade. The dried barks were pulverized using a mechanical grinder. The powder obtained was used for extracts preparation.

- **Animals care and treatment:** Male and female NMRI mice (average weight 27±4 g) and Wistar rats (either sex, average weight 160±42 g), procured from the Centre International de Recherche- Développement sur l’Élevage en zone Subhumide” (CIRDES), Burkina Faso were used for different experimental toxicology tests. They were housed in the animal cage with free access to water and standard laboratory pellet enriched with protein (29%).

  The animals were exposed under a controlled environment in animal house of “Unité de Formation et de Recherche en Science de la Santé” (University of Ouagadougou, Burkina Faso) at temperature of 23-25°C, 60% humidity and 12 h light-dark cycle two weeks before the use. The protocol of experimentation was approved by the local Ethical Committee for Animal Experimentation of University of Ouagadougou.

**Methods:**

- **Preparation of extracts:** A portion of *C. procera* root barks powder sample was weighed (250 g) and macerated in 2.5 L of solvent (distilled water and a mixture methanol/water (70/30 v/v) respectively for aqueous and hydroalcoholic extract) during 24 h at room temperature.

  The mixture was then filtered through cotton wool and the filtrate was centrifuged at 2000 rpm for five minutes. The collected supernatant of aqueous extract was then lyophilized, packaged in a bottle and stored in desiccator. For the hydroalcoholic extract, the collected supernatant was concentrated at 65°C under vacuum using a rotary evaporator before lyophilization and packaging in a bottle and stored in desiccator.

  **Acute toxicity test in mice:** Acute toxicity tests of aqueous and hydroalcoholic extracts of root barks of *Calotropis procera* were performed separately in male and female mice according to OECD guideline for chemicals tests (OECD, 2001). The limit test at dose level of 2000 mg/kg body weight was administered orally (gavage) to six fasted males and females mice per extract. The females were nulliparous and nonpregnant.

  The animals of different groups were individually observed for 120 min post-treatment and at least once daily for 14 days for mortality and signs of toxicity such as changes in skin and fur, eyes, mucus membranes, convulsion, salivation, diarrhea, lethargy, sleep and coma.

  **Subchronic toxicity test:**

  - **Experimental tests:** Sub-chronic toxicity study of aqueous extract of root barks of *Calotropis procera* were performed according to OECD guideline for chemicals tests with slight modification (OECD, 1998).

    Based on Lethal Dose 50 (LD<sub>50</sub>) values obtained from acute toxicity studies, the selection of dose for sub-chronic toxicity was carried out. The dose selected in this study is 20 mg/kg body weight. This dose corresponded at 1/100 of LD<sub>50</sub> obtained in the acute toxicity tests.

    A total number of 42 Wistar rats of both sex were randomly selected for the sub-chronic toxicity studies. The females were nulliparous and nonpregnant. The rats were divided into 3 groups and male and females were kept in separate polypropylene cages.

  - **Group I:** (5 males and 5 females) served as control and received a daily administration of vehicle (distilled water) for 6 weeks
  - **Group II:** (6 males and 6 females) received a daily administration of 20 mg/kg, body weight of aqueous extract for 3 weeks
  - **Group III:** (10 males and 10 females) received a daily administration of 20 mg/kg, body weight of aqueous extract for 6 weeks

  During the period of experimentation, all animals were observed twice a day for signs of toxicity and mortality. Individual body weights of animals were recorded daily (OECD, 1998).

  The animals were fasted over night prior to the terminal sacrifice, at which time the animals were anesthetized with ketamine and blood was collected via cardiac puncture into two vacutainers for each animals,
the first one containing Ethylene Diamine Tetraacetic Acid (EDTA) for hematology analysis and the second in dry vacutainer.

The blood samples contained in dry vacutainers were centrifuged at 3000 rpm for 10 min using a table centrifuge and the sera obtained were kept in sterile tubes and stored at -4°C for later biochemical assays.

After the blood collection, internal organs including liver, heart, kidneys, lungs, stomach, testicles and ovaries were collected, weighed to determine relative organs weights and examined for gross pathology.

Blood analysis: Hematological parameters were performed using the blood samples contained in EDTA tube.

Red Blood Cell count (RBC), White Blood Cell count (WBC), platelet count (PLT), hemoglobin (HGB), hematocrit (HCT), plateletcrit (PTC), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Mean Platelet Volume (MPV), Red cell Distribution Width (RDW) and Platelet Distribution Width (PDW) were determined on a semiautomatic cell counter (Hospitex Diagnostic, model: Hema screen 13, Italy).

Blood chemistry tests were performed on a semiautomatic biochemistry analyzer (Hospitex diagnostic, screen master LIHD113, Italy). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREAT) and total protein were determined.

Histopathological evaluation: Tissue samples liver, kidneys, lungs and stomach were fixed in 10% buffered formalin solution. After routine processing, the paraffin sections of each tissue were cut at 5 μm thickness and stained with haematoxylin and eosin for histopathological examination. Microscopic slides were analyzed qualitatively under light microscope.

Statistical analysis: Results were expressed as Means±Standard deviations (SD). Means and standard deviations were calculated separately for males and females. The statistical data were processed with Graph Pad Prism. All groups were compared by using one-way analysis of variance (ANOVA), followed by comparison of the treated groups to control by Dunnett’s multiple comparison tests. Differences were considered to be statically significant at p<0.05.

RESULTS

Acute toxicity study of the plant extracts: In acute toxicity study carried out in mice, the limit test at dose level of 2000 mg/kg body weight in single oral administration of aqueous and hydroalcoholic extract did not cause any death after 72 h post-treatment in males and females mice. Also any behavioral changes including changes in skin and fur, eyes, mucus

<table>
<thead>
<tr>
<th>Day</th>
<th>Sex</th>
<th>Control</th>
<th>3 Weeks</th>
<th>6 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>00-07</td>
<td>M</td>
<td>5.8±1.30</td>
<td>3.5±0.84</td>
<td>10.1±3.87</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6.0±2.35</td>
<td>8.17±1.72</td>
<td>6.5±2.76</td>
</tr>
<tr>
<td>07-14</td>
<td>M</td>
<td>5.2±2.17</td>
<td>4.33±1.97</td>
<td>8.6±3.98</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5.4±2.61</td>
<td>5.67±2.25</td>
<td>7.4±2.99</td>
</tr>
<tr>
<td>14-21</td>
<td>M</td>
<td>4.6±2.88</td>
<td>3.83±1.17</td>
<td>7.6±3.89</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5.8±2.39</td>
<td>5.83±1.07</td>
<td>5.6±2.91</td>
</tr>
<tr>
<td>21-28</td>
<td>M</td>
<td>4.2±2.95</td>
<td>5.4±3.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>3.8±1.92</td>
<td>4.4±1.35</td>
<td></td>
</tr>
<tr>
<td>28-35</td>
<td>M</td>
<td>5.0±2.88</td>
<td>5.0±2.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>3.4±1.34</td>
<td>3.6±1.96</td>
<td></td>
</tr>
<tr>
<td>35-42</td>
<td>M</td>
<td>4.8±2.39</td>
<td>3.8±2.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>3.6±1.52</td>
<td>3.8±1.32</td>
<td></td>
</tr>
</tbody>
</table>

Mean and standard deviation are presented (a: n = 5; b: n = 6; c: n = 10); No statistical difference between the control and treated groups; (p>0.05) One-way ANOVA followed by Dunnett’s multiple comparison tests; M = Male; F = Female

Table 2 presents the mean relative organ weights of control and daily treated rats with aqueous extract of plant in sub-chronic oral toxicity test

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sex</th>
<th>Control</th>
<th>3 Weeks</th>
<th>6 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>M</td>
<td>2.9±0.44</td>
<td>2.91±0.20</td>
<td>2.88±0.34</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>2.85±0.22</td>
<td>3.22±0.15</td>
<td>3.19±0.26</td>
</tr>
<tr>
<td>Heart</td>
<td>M</td>
<td>0.36±0.05</td>
<td>0.33±0.03</td>
<td>0.38±0.05</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.34±0.05</td>
<td>0.34±0.03</td>
<td>0.37±0.03</td>
</tr>
<tr>
<td>Kidneys</td>
<td>M</td>
<td>0.62±0.01</td>
<td>0.53±0.02</td>
<td>0.57±0.09</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.56±0.06</td>
<td>0.53±0.03</td>
<td>0.57±0.05</td>
</tr>
<tr>
<td>Lung</td>
<td>M</td>
<td>0.52±0.12</td>
<td>0.49±0.06</td>
<td>0.54±0.07</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.48±0.07</td>
<td>0.52±0.03</td>
<td>0.57±0.05</td>
</tr>
<tr>
<td>Stomach</td>
<td>M</td>
<td>2.81±0.94</td>
<td>2.38±0.54</td>
<td>2.37±0.64</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>2.78±1.14</td>
<td>2.58±0.52</td>
<td>2.45±0.42</td>
</tr>
<tr>
<td>Testicles</td>
<td>M</td>
<td>1.73±0.24</td>
<td>1.58±0.19</td>
<td>1.95±0.43</td>
</tr>
<tr>
<td>Ovaries</td>
<td>F</td>
<td>0.09±0.03</td>
<td>0.10±0.02</td>
<td>0.09±0.02</td>
</tr>
</tbody>
</table>

Mean and standard deviation are presented (a: n = 5; b: n = 6; c: n = 10); No statistical difference between the control and treated groups; (p>0.05) One-way ANOVA followed by Dunnett’s multiple comparison tests; M = Male; F = Female

Subchronic toxicity study of aqueous extract in rats: Daily administration of aqueous extract to males and females Wistar rats during 3 and 6 weeks at the dose of 20 mg/kg/day induced no mortality in either sex. No evidence of treatment-related gross toxicity was identified during clinical observation of rats exposed to the extract.

Effect of extract on physical parameters: The mean weekly body weight gain of control and daily treated rats with aqueous extract of root barks of the plant during 3 and 6 weeks is presented in the Table 1. Statistical analysis revealed that there were no significant differences in body weight between control and treatment groups (p>0.05).
Histopathological examination: Histological examination of liver, kidney, stomach and lungs on groups (p>0.05). No significant change between treated and control groups (p>0.05).

Results show that there were no statistically significant differences between different values of treatment and rats. However there were no statistically significant elevation of alanine aminotransferase (ALT) after 3 and 6 weeks of treatment in the treated weeks of treatment and aspartate aminotransferase (AST) after 3 and 6 weeks of treatment in the treated groups (p>0.05). This result indicates a slight optical microscope showed that there were not most histological changes in treated rats compared to controls.

However, slight congestions of stomach, lungs and liver, a dilatation of central veins of liver and lungs alveoli were observed in some rats after 3 and 6 weeks of treatment (Fig. 1).

### DISCUSSION

Many investigations on C. procera have reported that the plant has numerous pharmacological properties. The toxicity of different parts including the aerial parts and the latex of the plant has already been evaluated. However, there is little information about the toxicity of root bark which was the subject of our study.

In this present study of acute toxicity in NMRI mice, the limit test at dose level of 2000 mg/kg body weight in single oral administration of aqueous and alcoholic extract did not cause any mortality and signs of toxicity during the period of observation in both sex. These results indicate that both extracts of the plants have a LD_{50} higher than 2000 mg/kg and suggests that the plant has numerous pharmacological properties.
these extracts are products which have low oral toxicity according to classification of Hodge and Sterner (1943) and United Nation (2011).

Compared to digoxin which is a reference cardenolide, the LD$_{50}$ of the aqueous extract of the root bark of the plant is 250 times greater than digoxin that is 7.8 mg/kg by oral route on mice.

Our results are similar to Mossa et al. (1991) which showed that ethanol extract of the aerial parts of C. procera does not cause oral toxicity in mice at doses up to 3000 mg/kg.

Other authors have found that the aqueous decoction and ethanolic extract of the roots of C. procera of Mali at doses of 1500 and 2000 mg/kg caused a mortality of 20-40% of Wistar rats (Circosta et al., 2001). The difference with our results may be explained by the extraction procedure, the type of animal used, but also the soil factors that can influence the chemical composition of extracts.

The subchronic toxicity study showed that the daily oral administration of aqueous extract at 20 mg/kg/day body weight during 3 and 6 weeks did not cause any death and clinical signs of toxicity. The mean weekly body weight gain and relative organ weights of treated groups were similar to control group. Body weight is known to be one of the most sensitive indicators of adverse effects. In this study all animals’ body weight were increased during the administration period suggesting that aqueous extract did not influence the animal’s weight gain. These results go in agreement with the results of other researchers who also observed this weight gain with the aqueous extract of the leaves of C. procera on rabbits (Pouokam et al., 2011; Shahat and Shihata, 2012).

Hematological profile is important to know in the treatment of sickle cell disease.

In this present subchronic toxicity study we found that there were no significant changes in the hematological parameters between the control and the experimental groups. According to some authors, there is a correlation of toxicity in hematological, gastrointestinal and cardiovascular adverse effects between animals and humans (Olson et al., 2000).

The hematopoietic system is one of the most sensitive targets for toxic chemicals and an important index of physiological and pathological status in human and animal (Li et al., 2010).

Hematological indices in animals are important to determine the toxicity risk since the changes in the blood system have a higher predictive value for human toxicity. The hematological indices obtained in this study suggest that the aqueous extract of plant is not
toxic on hematological parameters as they do not affect the circulating blood cells or their production. Thus, the ingestion of aqueous extract shall not have adverse effects on cellular components of blood in sickle cell patients.

Our results were similar to Dada et al. (2002) who have found that the daily oral administration of latex of *C. procera* on rat during 7 and 14 days has no significant effects on blood parameters.

For the biochemical parameters, a slight elevation of transaminases ALT and AST were noticed but there were not statistically significant differences. Our result is in the line of data from other authors who reported the slight elevation of serum enzymes in rat treated with latex of *C. procera* (Dada et al., 2002).

The results of analytes of the renal function (Whitby et al., 1997) confirmed these results. The normal values of creatinine obtained suggest that kidney were not greatly damaged and liver were not greatly damaged respectively.


Our result indicate that the liver was not greatly damaged to release significant quantities of the enzyme into the blood due to the quantity of extract administered (Odutola, 2000). The amount of enzyme released into blood is directly proportional to the number of damaged cells and the interval of time between injury and the test (Adedeji et al., 2002).

The slight congestion and dilatation of central veins of liver seen in histological examination could explained the slight elevation of transaminases ALT and AST observed in this study.

Concerning the values of creatinine and total protein, there were no significant change between treated and control groups meaning that kidney and liver were not greatly damaged respectively.

Creatinine is a serum metabolite that is indicative of the renal function (Whitby et al., 1987). The normal values of creatinine obtained suggest that kidney were not damaged. The normal cytoarchitecture of kidney found in histological examination confirmed these results.

The dose used in this subchronic toxicity study is at least 10 times higher than the dose used in the FACA®. The overall results obtained in subchronic toxicity study indicates that the aqueous extract of root bark is tolerated in oral repeat administration and there would be less risk of toxicity in sickle cell patients under treatment FACA®.

**CONCLUSION**

Our results have suggested that aqueous and hydroalcohol extracts of *C. procera* root bark are relatively safe when administered orally and could justify the use of this part of the plant in the treatment of various diseases including sickle cell disease.

These results contribute to reassure people in the safe use of FACA® in the treatment of sickle cell disease. But it is necessary to complete the toxicological evaluation of products derived from this plant including FACA® by pharmacovigilance monitoring of patients under treatment.

**ACKNOWLEDGMENT**

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**REFERENCES**


**Endnote:**

1: Center specializing in livestock breeding of laboratory animals for research.