Evaluation of the toxicity of the aqueous stem bark extract of *Enantia chlorantha* on some reproductive and developmental parameters

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Abstract

*E. chlorantha* is widely used in African pharmacopeia and many patients, including pregnant women, use the bark aqueous extract to treat many diseases. The present work was carried out to investigate the possible reproductive and developmental toxicity that could result when the extract was given to pregnant rats during the period of organogenesis. Male rats were crossed overnight each with virgin females. Upon confirmation of mating (day 1 of pregnancy), *E. chlorantha* extract (0, 250, 500 and 1000 mg/kg BW) was administered once daily to four groups of 16 pregnant rats by gavage for 10 consecutive days (days 6–15 of gestation). On day 19 of gestation, 8 rats per group were sacrificed. The remaining females were allowed to deliver and pup development followed up to weaning. No dam deaths or abortions were observed.

No malformations were recorded at the extract doses of 500 and 1000 mg/kg BW, however, the subacute toxicity, observations showed that the extract had toxic effects on liver, kidney and lungs, notably, elevated serum AST and ALT levels, steatosis, intercellular hepatic edema, hepatic vein congestion and...
mild inflammatory leucocytic infiltration, hepatic and renal vein congestion, a collapse of the alveolar structure leading to the fusion of neighboring alveolar sacs at 1000 mg/kg dose. Given the high susceptibility of the developing fetus even to low doses of toxic substances, the above results prompted us to investigate the possible toxic effects of the widely-used extract of *E. chlorantha* when given during the period of organogenesis in female rats.

2. Material and Methods

2.1 Preparation of plant extract. The stem bark of *E. chlorantha* was harvested in Ambam, South region of Cameroon during the month of July. The plant was identified in comparison with the specimen n° 25918/SRFCAM, held at the Cameroon National Herbarium. The fresh stem-bark of *Enantia chlorantha* was cut up, dried and ground to a fine powder. A mixture of the powder and distilled water (10% w/v) was boiled for 20 minutes, and then cooled to room temperature. After filtration of the decoction obtained through Wattman filter paper number 3, the filtrate was evaporated at 40°C using a Raven convection air oven (Jencons PLS, UK). The yellowish dried solid obtained (4.53%) was stored at 4°C and used later for our pharmacological tests.

2.2 Phytochemical Tests. Phytochemical tests for major metabolites of the extract were performed. The aqueous extract of *Enantia chlorantha* was screened for the presence of biologically active compounds such as tannins, alkaloids, saponins, flavonoids, anthocyanins, phenols, quinones, coumarins, sterols, triterpenoids, glycosides, proteins. Based on the intensity of coloration, the lather or the precipitate formed during the reaction was noted. Secondary metabolites proportions were characterized as present (++), weakly present (+), and absent (−) when the test result was negative.

2.3 Mating procedure and treatment. The authorization for the use of laboratory animals in this study was obtained from the Cameroon National Ethics committee (Reg. No FWA-IRB00001954). Methods of [21]; [22], with a slight modification where used. Virgin females (140-190 grams) and experimented males were placed into a cage overnight (one male with 2 female per cage). The following morning, a pap smear was performed in other to confirmed mating. The microscopic observation of spermatozoan indicated that mating took place and that day were considered as day one of pregnancy. The pregnant rats were divided into four groups of 16 rats each. They respectively received by gavage, single doses (2.40 ± 0.03 and 2.30 ± 0.03) of the aqueous extract of *Enantia chlorantha* for 10 consecutive days, (days 6 to 15 of gestation period).

2.4 Evaluation of maternal behavioral aspect and physical changes: Daily recordings were done in all groups for the following: Mortality and morbidity, changes in fur, skin, eyes and mucous membrane, tremors, convulsions, salivation, diarrhea, lethargy, animal behavior, feeding pattern, changes in the level of motor activity, gait and posture, reactivity to handling or sensory stimuli, grip strength and bizarre behavior such as self-mutilation, walking backward.

2.5 Evaluation of maternal and fetal parameters. Gestating female were weighed daily and weight gains were noted. The presence of vaginal blood stains was check in order to detect any signs of abortion. On day 19 of the gestation, half of the females per group were sacrificed. Blood was collected in heparinized tubes for hematological parameters count. The fetuses were removed by opening the uterus and the following were noted: litter size, number of death fetuses, corpora lutea, implantations, fetal resorptions, uterine and placenta weight. Dam’s absolute and relative organs weights like lungs, liver, spleen, kidneys and heart were also measured. Live fetuses were examined for any external signs of deformities. Fetus weight, size and tail length were determined. Uterus and ovaries were kept into 10% formaldehyde solution for further histological studies.

2.6 Evaluation of neonatal developmental parameters. The remaining pregnant rats were allowed to give birth and, the following parameters were recorded: gestational period, litter size; sex ratio, delivery index (number of females delivering/number of pregnant females × 100), live-birth index (number of live offspring/number of offspring delivered × 100), viability index (number of live offspring at lactation day 4/number of live offspring delivered × 100) and weaning index (number of live offspring at day 21/number of live offspring born × 100) [22, 23]. Each young rat was check in order to detect any sign of abnormality: eyes opening (about 15 days postnatal), and ears (about 7 days postnatal), hair appearance, testis descent (about 3 weeks postnatal), vaginal opening (about 08 to 10 days postnatal).

2.7 Evaluation of the anthropometric parameters of the F1 offspring. Individual offspring were weighed on an electronic balance and measured (from crown to rump) with sliding feet. Tail was also measured. All these parameters were check on days 1; 5; 10; 15 and 20 postnatal.

2.8 Statistical analyses. One-way analysis of variance (ANOVA) was employed for statistical comparison. The difference between means was analyzed by Dunnet test. Values were represented as mean ± ESM. P < 0.05 was considered significant.

3. Results

3.1 Phytochemical Tests: Phytochemical tests of the aqueous extract of *E. chlorantha* revealed the presence of many phyto-constituents. Tannins, saponins, anthocyanins, acids, glycosides were present (++), alkaloids, ketones, flavonoids, sugars, coumarins, amino-acids and proteins were weakly presents (+), phenols, quinines, oils, sterols, triterpenoids and resins (-) were not detected.

3.2 Effect of *Enantia chlorantha* extract on behavioral aspect and physical changes on gestating females: During the experimental period, no female death was register. No abnormal morphological characteristics (eye, skin, fur etc.), neurological symptoms (convulsions trembling etc.), aggressiveness or abnormal behavior were observed.

3.3 Effect of *Enantia chlorantha* extract on maternal and fetal parameters: For the F1 offspring, no sign of external malformations was noticed.

Table 1 show that no abortion was recorded during the gestation period. No death fetuses were registered at parturition. Fetus sizes slightly increased in extract-treated groups (2.42 ± 0.02; 2.40 ± 0.03 and 2.30 ± 0.03, respectively.
for 250, 500 and 1000 mg/kg doses), compared with the control (2.06 ±0.22), but the differences were not statistically significant. In the extract-treated groups, there was no significant variation at the level of the number of corpora lutea, the number of implantations, litter size, placental weight, fetus weights and tail lengths compared with the control.

Table 1: Effects of *Enantia chlorantha* extract on fertility parameters and fetal anthropometric measures

<table>
<thead>
<tr>
<th></th>
<th>Negative control 0 mg/kg</th>
<th>Extract 250 mg/kg</th>
<th>Extract 500 mg/kg</th>
<th>Extract 1000 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant dam</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Abortion</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Implantations</td>
<td>10.75 ± 1.26</td>
<td>9.63 ± 0.50</td>
<td>9.00 ± 0.32</td>
<td>9.63 ± 0.75</td>
</tr>
<tr>
<td>Resorptions a(b)</td>
<td>3 (13)</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Number of fetus/dam</td>
<td>9.13 ± 0.64</td>
<td>9.38 ± 0.38</td>
<td>8.75 ± 0.4</td>
<td>9.25 ± 0.73</td>
</tr>
<tr>
<td>Fetus death/dam</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Placental absolute weight (g)</td>
<td>0.39 ± 0.03</td>
<td>0.37 ± 0.02</td>
<td>0.36 ± 0.01</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>Fetus weight (g)</td>
<td>1.75 ± 0.16</td>
<td>1.58 ± 0.07</td>
<td>1.51 ± 0.05</td>
<td>1.50 ± 0.05</td>
</tr>
<tr>
<td>Fetus size (cm)</td>
<td>2.06 ± 0.22</td>
<td>2.42 ± 0.02</td>
<td>2.40 ± 0.03</td>
<td>2.30 ± 0.03</td>
</tr>
<tr>
<td>Tail length (cm)</td>
<td>0.74 ± 0.07</td>
<td>0.66 ± 0.03</td>
<td>0.63 ± 0.02</td>
<td>0.60 ± 0.03</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. a = number of dam with resorptions; (b) = total number of resorptions/group

On day 19 of the gestation, no significant variation in pregnant female weight where noticed in the different groups. Figure 1 shows a non-significant increase in body weights at the 1000 mg/kg dose of extract (14.34 ± 1.61 g) in comparison with the control (10.36 ± 1.16 g), on day 1-5 interval, and a slight decrease of the body weight at the same extract dose (19.26 ± 1.93 g), in comparison with the control (23.14 ± 2.15 g), on day 6-15 interval. The body weight gains significantly increased (p<0.01) with 250 mg/kg extract dose at 16-19 interval (20.41 ± 1.21), in comparison with the negative control (15.31 ± 0.55). In spite of the observations above, from day 1 to day 19, there was no significant weight variation in extract treated groups, in comparison with the control group. Figure 2 shows that there is a significant decreased in heart and spleen weight at 250 mg/kg extract dose compared with the negative control, and a significant decreased (p< 0.05) in liver weight at the 1000 mg/kg compared with the negative control. No significant variation were noted in the uterus and the ovary weight.

Fig 1: Effects of *Enantia chlorantha* extract on maternal weight gain (g). 1a) Maternal weight (g); 1b) maternal weight gain (g). (Values are expressed as mean ± SEM.

Fig 2: Effect of *Enantia chlorantha* extract on dam’s relative organs weight values are expressed as mean ± SEM. *p < 0.05; **p < 0.01 are considered significant.
Table 2 shows that there was no significant variation of hematological parameters in the extract treated groups in comparison with the control.

Table 2: Effects of *Enantia chlorantha* extract on maternal hematological parameters

<table>
<thead>
<tr>
<th></th>
<th>WBC (10³/uL)</th>
<th>RBC (10⁶/uL)</th>
<th>Plt (10³/uL)</th>
<th>% Lymphocytes</th>
<th>MCHC (g/dl)</th>
<th>MCH (pg)</th>
<th>% HCT</th>
<th>HGB (g/dl)</th>
<th>MCV (fl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>11.46 ± 0.28</td>
<td>5.21 ± 0.16</td>
<td>329.13 ± 23.7</td>
<td>73.79 ± 3.39</td>
<td>35.06 ± 1.25</td>
<td>20.8 ± 1.02</td>
<td>29.89 ± 0.82</td>
<td>10.03 ± 0.29</td>
<td>56.8 ± 0.74</td>
</tr>
<tr>
<td>Extract 250 mg/kg</td>
<td>12.30 ± 0.26</td>
<td>5.07 ± 1.20</td>
<td>398 ± 48.97</td>
<td>81.81 ± 2.66</td>
<td>29.30 ± 1.22</td>
<td>20.1 ± 1.98</td>
<td>30.49 ± 0.89</td>
<td>8.3 ± 0.55</td>
<td>56.7 ± 0.70</td>
</tr>
<tr>
<td>Extract 500 mg/kg</td>
<td>11.74 ± 0.25</td>
<td>4.58 ± 0.14</td>
<td>441.00 ± 45.6</td>
<td>79.06 ± 3.24</td>
<td>30.08 ± 1.31</td>
<td>21.46 ± 0.75</td>
<td>30 ± 0.91</td>
<td>9.38 ± 0.42</td>
<td>67.31 ± 2.38</td>
</tr>
<tr>
<td>Extract 1000 mg/kg</td>
<td>11.16 ± 0.65</td>
<td>5.23 ± 0.26</td>
<td>343 ± 34.8</td>
<td>76.9 ± 3.48</td>
<td>31.12 ± 0.98</td>
<td>17.43 ± 0.53</td>
<td>29.18 ± 1.23</td>
<td>9.6 ± 0.36</td>
<td>56.38 ± 1.02</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. Red blood cell count (RBC), White blood cell (WBC), Lymphocyte percentage (Lymp %), Platelet count (Plt), Hemoglobin level (Hb), Hematocrit level (HCT), Mean Cell Volume (MCV), Mean Cell Haemoglobin level (MCH) and Mean Cell Haemoglobin Concentration (MCHC).

3.4. Effect of *Enantia chlorantha* extract on neonatal developmental parameters.

There was no significant variation in the duration of gestation. The number of pups and the number of male at birth were high at 1000 mg/kg extract dose compared with all the other groups. The viability and lactation indices at the extract dose of 1000 mg/kg slightly decreased compared with the negative control (Table 3).

Table 3: Effect of *Enantia chlorantha* extract on neonatal developmental parameters in rats

<table>
<thead>
<tr>
<th></th>
<th>Negative control 0 mg/kg</th>
<th>Extract 250 mg/kg</th>
<th>Extract 500 mg/kg</th>
<th>Extract 1000 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of gestation (days)</td>
<td>22.75 ± 0.16</td>
<td>22.50 ± 0.27</td>
<td>22.25 ± 0.25</td>
<td>22.25 ± 0.25</td>
</tr>
<tr>
<td>Number of pups/dam</td>
<td>8.88 ± 0.35</td>
<td>8.25 ± 0.16</td>
<td>8.50 ± 0.73</td>
<td>9.25 ± 0.37</td>
</tr>
<tr>
<td>Sex ratio</td>
<td>0.47 ± 0.05</td>
<td>0.66 ± 0.04</td>
<td>0.67 ± 0.05</td>
<td>0.85 ± 0.12</td>
</tr>
<tr>
<td>Delivery index</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Birth live index</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Viability 4 index</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>94.05</td>
</tr>
<tr>
<td>lactation 21 index</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>94.05</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM for duration of gestation, number of pups and the sex ratio. 

**Delivery index:** (Number of females delivering/number of pregnant female) × 100. 

**Birth-Live index:** (Number of females delivered/number of pregnant animal) × 100. 

**Viability index:** (Number of living offspring at lactation day 4/number of living offspring delivered) × 100. 

**Lactation index:** (Number of living offspring at day 21/number of living offspring born) × 100.

3.5. Effects of *Enantia chlorantha* extract on anthropometrics parameters of F1 generation.

On day 20, a non-significant decreased of pups weight and a significant tiny tail (p<0.01) was observed at 1000 mg/kg dose in comparison with the negative control (Figure 3).
Fig 3: Effects of Enantia chlorantha extract on anthropometrics parameters of F1 generation. Values are expressed as mean ± SEM. (3a): weight; (3b): crown-rump length (cm); (3c): tail length (cm).

3.6. Histological results
Figure 4 and Figure 5 present respectively ovarian and uteri tissue of female rats sacrificed on day 19 of gestation. With higher extract doses congestions and edema are observed. The extract dose of 500 mg/kg resulted in cystic glandular hyperplasia on uteri, represented by an abundance of dilated glands (G) with large lumens. At dose 1000 mg/kg, there is persistent cystic glandular hyperplasia as seen at dose 500 mg/kg, but in addition there is an increase in glandular epithelial cell proliferation in multiple layers and some of the cells are shed into the glandular lumen (epitheliosis) (Ep). The stroma is also more congested (C) and oedematous (O)

Fig 4: Ovarian tissue of female rats sacrificed on day 19 of gestation. 4a) Extract (1000 mg/kg) (H&E x 100); Extract (1000 mg/kg) (H&E x 200). At the extract dose of 1000 mg/kg, congestions (C) are observed.
Fig 5: Effects of the aqueous stem bark extract of *E. chlorantha* on uterine tissue of female rats sacrificed on day 19 of gestation. 5a) Extract (250 mg/kg) (H&E x 200); 5b) Extract (500 mg/kg) (H&E x 200); 5c) Extract (500 mg/kg) (H&E x 200); 5d) Extract (1000 mg/kg) (H&E x 100); 5e) Extract (1000 mg/kg) (H&E x 100); 5f) Extract (1000 mg/kg) (H&E x 200). Dilated glands (G). Glandular epithelial cell proliferation into the glandular lumen (epitheliosis) (Ep). Congestion (C) oedema (O).

4. Discussion
In the present study, we evaluated the toxic effects of the stem bark aqueous extract of *Enantia chlorantha* on some reproductive and developmental parameters, specifically after the extract was given during the organogenesis phase of gestation. No visible toxicological signs or deaths were observed in the dams. At the level of skin, eyes and fur, no abnormal morphological characteristics were noted, suggesting that tegumentary and ocular systems were not attained during this period. Aggressiveness, abnormal behavior, convulsions, salivation and trembling were not observed. Suggesting that nervous system was not attained.

The administration of extract during the period of organogenesis (days 6-15) did not present any symptoms of maternal toxicity manifested by changes in body weight, relative organs weight, body weight gain, hematological parameters. These findings suggested that any fetal effects observed would have resulted from the toxicity of the extract and not from its maternal intolerance since there are correlations between maternal and developmental toxicity [24]. Fetal malformations are defined as those structural anomalies that alter general body conformity, interfere or disrupt with body function, or are generally thought to be incompatible with life [25]. At the level of fetuses of the females that were sacrificed on day 19 of gestation, no external malformations or deaths were observed in extract-treated and control groups, meaning that the extract did not have teratogenic effects at that level. Slight but not statistically significant differences were observed in placenta weight, fetus body weight and size in treated groups compared with the control. In the first generation (F1) offspring of the females admitted to give birth, from the birth to the day 21 of growth, no observations of toxicity were recorded. No significant difference was observed in number of corpora lutea, the number of implantation, litter size, placental weight, in the extract group, compared with the control, suggesting that the extract did not affect fertility parameters.

A slight but not significant decreased were observed in fetal body weight of extract-treated groups compared with the control. Gestational weight gain increased is associated with higher rates of complications of pregnancy and delivery [26]. In this study, from day 1 to day 19 of gestation, maternal weight gain remained slightly high in all the extract treated groups, but the difference was not significant compared with the negative control. This result suggests that the extract did not negatively impact the maternal weight gains and thus, did not lead to pregnancy complications. Generally, simple and sensitive indices of toxicity after exposure to toxic substances are the reductions in body weight gain and internal organ weights [27]. Significant decreased (*p*<0.05) in heart and spleen weight at 250 mg/kg extract dose compared with the negative control, and significant decreased (*p*<0.05) in liver...
weight at the 1000 mg/kg compared with the negative control were noticed. No significant variation was noticed at the level of the other organs notably the uterus and the ovaries. Normal levels of blood parameters can be modified by any toxic substance, even from plants [28, 29]. Hematological studies can easily reveal anomalies in body metabolic processes, and the blood profile usually furnished vital information on the response of the body to injury, deprivation and/or stress [30]. In this study, no significant difference in maternal hematological parameters was observed between the extract-treated groups and the control meaning that the extract did not altered those parameters and did not caused anemia. In developmental toxicity studies, the major effects of prenatal exposure of a chemical compound are manifested before birth through abortions, or at the time of birth through, malformations, embryo lethality and growth retardation [25, 31]. None of these signs were noted in this study, suggesting that the extract did not affect pup’s development. Histological sections show dose-dependent estrogenic manifestations, with a very marked trophic effect at the dose of 1000 mg / kg. Histological sections of the uterus, presented no abnormality with the extract dose of 250 mg/kg group compared with the control. In contrast, for the 500 and 1000 mg/kg doses, cystic glandular hyperplasia was observed and was amplified at 1000 mg/kg, accompanied by an invasion of the glandular lumen by epithelial cells, congestions and edema in the stroma. These observations at doses of 500 and 1000 mg/kg are manifestations of estrogenic hyper stimulation due to hypersecretion of estrogen. These observations can be explained either as normal physiological changing into a gestational uterus at the moment of parturition or as signs of excessive stimulation of estrogen synthesis and/or action by the aqueous extract of E. chlorantha. The endometrium plays a key role in the acceptance of the embryo and is of paramount importance for female fertility. Normal functioning of the epithelial cells near the lumen is critical for attachment and implantation of the embryo [33]. Secretions of the uterine epithelium play an essential role during pregnancy [33, 34], and the endometrial glands provide an important source of nutrients and growth factors for the establishment of the uterine epithelium. Estrogenic activity usually results in morphological, histological, and biochemical changes in the uterus [35]. Estrogens have long been known to be involved in mammals in many fundamental biological processes such as sex hormone regulation, progesterone receptor biosynthesis, cell development and differentiation [36]. In the uterus, the proliferation of epithelial cells changes during the estrus cycle and pregnancy [37, 38]. As a prerequisite for embryo implantation, luminal epithelial cells stop proliferating during early pregnancy [39]. Abnormal proliferation of epithelial cells affects the functioning of the uterus and pregnancy, leading to implantation failure or pathological conditions such as endometrial hyperplasia, which in the long term may develop into cancer [39, 40]. Excessive and prolonged use of estrogen can cause cancers of many organs in both humans and animals. Usually the consequence of hyper-estrogenism are Hyperplasia of the endometrium [41]. In this study, at the dose of 500 mg/kg, estrogenic hyperstimulation was characterized by a mild, non-atypical, single glandular hyperplasia. At a dose of 1000mg/kg, glandular hyperplasia was more pronounced, but still without atypia. All these observations suggest that the aqueous extract of E. chlorantha could have estrogenic properties. In fact, phytochemical screening of the aqueous extract of Enantia chlorantha revealed the presence of compounds and the oestrogenic activity of some of these compound like flavonoids had been demonstrated [42]. The most potent phytoestrogens are members of the flavonoid family. Phytoestrogens are chemicals that derived from plants and have estrogen-like activities [43]. Previous studies show that high doses of flavonoids cause a reduction in the estrogenic effect through interaction with cytochrome P450 or by blocking CYP19, a very important enzyme in estrogen biosynthesis. When administered in high doses, phytoestrogens can cause hormonal imbalance that may even compete with estrogen and behave as antiestrogens [44, 45]. The observations above suggest that at high doses of Enantia chlorantha extract, the hyper-stimulatory effects and resulting glandular hyperplasia can in the long run generate more negative effects like an endometrial cancer. More investigations are needed to confirm this assertion.

5. Conclusion
The aqueous extract of E. chlorantha at low doses do not show macroscopic and histological toxic effects on gestating dams, fertility parameters, and on the birth parameters and development of F1 offspring up to weaning. Higher doses can be estrogenic and have toxic effects especially in the uterus of the dams.

6. Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

7. References
44. P. Hodek, P.Trefil, M. Stibozova, “Flavonoids-potent