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Research Paper

Subchronic toxicity of aqueous extract of *Alstonia boonei* de wild. (apocynaceae) stem bark in normal rats

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Abstract

Methodology: Wistar rats were randomly assigned into eight groups of five animals each: four male groups and four female groups. Each sex group had a control group receiving distilled water and three test groups receiving 200, 500 and 1000mg/kg respectively. Animal's body weights were recorded on the first day and once a week for the four experiment weeks. The hematological analysis included total WBC count, total RBC count, Hb, %HCT, MCV, MCH and MCHC. Biochemical/serum profile studies include TG, TC, ALT, AST, urea and TP. Tissue specimens of the liver, kidney and lung were subjected to histological examination using standard hematoxylin-eosin staining.
Results: In male rats, aqueous extract showed significant decreases in relative weight of liver with extreme significance P<0.001 at a dose of 200mg/kg (vs. control group), P<0.001 of lung at all the doses, P<0.05 (200 and 500mg/kg) and P<0.01 (1000mg/kg) in heart weight. In relative kidney weight, only the dose of 1000mg/kg showed a significant increase vs. normal control male rats. Unlike male rats, only relative kidney weight in female rats was significantly different from the control group in a dose-dependent manner. The aqueous extract treated male groups showed significant increases P<0.001 (1000mg/kg) of total WBC count and MCHC, significant decreases of %HTC (dose response manner), P<0.05 total RBC count (at doses of 500 and 1000mg/kg) and Hb P<0.01 (500mg/kg) vs. normal male rats. In female rats, the haematological study showed significant increase P<0.01 of total WBC count (at the doses of 500 and 1000mg/kg), significant decreases P<0.05 and P<0.01 of total RBC respectively at the doses of 200 and 1000mg/kg, significant decrease of Hb with extreme significance P<0.001 at the dose 1000mg/kg, %HTC also decrease dose response manner vs. control female rats. Biochemical study showed in male rats significant decreases in level of TG P<0.001 (at the doses of 200 and 500mg/kg) and urea, although it showed any dose-dependent effect vs. control male rats. AST also decreases (P<0.05) in male rats at the dose of 200mg/kg but significantly increase P<0.001 at the dose of 500mg/kg. In the female rats, biochemical study revealed significant increases in level of TG P<0.001 and urea P<0.01 at the dose of 200mg/kg and significant decreases in level of TG P<0.01, AST P<0.05 and urea P<0.05 at the dose of 500mg/kg (vs. control female rats). Microscopically, there were mild hepatic and renal tissue injuries supporting the hematological analysis.
Conclusion: The results indicated that aqueous extract of *Alstonia boonei* De Wild is toxic in high doses.

Keywords: *Alstonia boonei; Aqueous Extract; Rats; Subchronic Toxicity.*

1. Introduction

Alstonia boonei De Wild. belongs to the family of Apocynaceae, with about 50 species widely distributed in Africa, Asia and America's continents (Iwu 1993). *A. boonei* possess antihyperglycemic and antioxidant properties (Nkono *et al.* 2014), and it has been reported that the stem bark is used in traditional medicine to treat fever, painful micturition, insomnia, chronic diarrhea, rheumatic pains, as antivenom for snake bites and in the treatment of arrow poisoning (Olivier-Bever 1986, Asuzu & Anaga 1991).
In as much as many health problems have been solved using medicinal plants, a number of them presents toxic effects when not properly prepared or dispensed (Dalsiel 1997). As such a scientific approach needs to be applied towards the use of plant extracts in managing ailments, especially in the developing countries where the level of literacy is low and the status of health management is poor, and about 80% of the population patronized herbal drugs.
In our previous studies (Nkono *et al.* 2014), a single administration of *A. boonei* aqueous extract showed no toxicity, and the LD_{50} value was greater than 5000mg/kg. In their own studies with ethanolic stem bark extract of *A. boonei*, Oze *et al.* (Oze *et al.* 2007) reported moderate nephrotoxicity in guinea pigs. The kidney is the primary organ for clearance and excretion of xenobiotics, including drugs and drug products from the body. Kidney damage could arise due to the administration of plant extracts, but there is paucity of scientific information because the incidences of toxicity in local settings are hardly reported or documented.
The present work aims to evaluate the subchronic toxicity of *A. boonei* De Wild. stem bark aqueous extract in rats.

2. Materials and methods

2.1. Collection of plant material

Fresh stem bark of *A. boonei* was collected during November 2012 from Ombessa, Center Region of Cameroon. The plant was identified by Dr. B. Kengue and a voucher specimen (N°43368HNC) has been deposited at the National Herbarium of Cameroon (Nkono *et al.* 2014). After collection, fresh stem bark was washed thoroughly with water in order to remove any type of contamination, and then dried at room temperature. The dried stem bark was milled and preserved in airtight glass container at room temperature (25°C).

2.2. Extraction

A ratio of three hundred grams (300g) of *A. boonei* stem bark powder was weighed and extracted in 3000mL of distilled water by decoction for 30 min. The aqueous extract obtained by filtration (2500mL) was lyophilized and thereafter the paste (18.40g, yield: 9.46%, w/w) preserved at room temperature for further uses.

2.3. Experimental animals

Wistar rats weighted between 87-93g of either sex were used for subchronic oral toxicity study. Animals were maintained under standard husbandry conditions (temperature 25±2°C, 12h light: 12h dark cycle) and fed with standard pellet diet (Kamgang *et al.* 2005) and water ad-libitum. All animal experiments were handled according to the Cameroon National Ethics Committee (Ref. N° FWIRB 00001954) and all experiments have been examined and approved.

2.4. Subchronic oral toxicity

Toxicity studies were performed according to the OCDE guideline (OCDE 1995). Animals were divided into 8 groups of 5 animals each with 4 male groups (1, 3, 4, 5), and 4 female groups (2, 6, 7, 8). Vehicle and *A. boonei* were orally administered, daily for 4 weeks as shown below:
Groups 1 & 2: Control: received distilled water (10ml/kg b.w)
Groups 3 & 6: received *A. boonei* (200mg/kg b.w)
Groups 4 & 7: received *A. boonei* (500mg/kg b.w)
Groups 5 & 8: received *A. boonei* (1000mg/kg b.w)
The animals were sacrificed by cervical dislocation one week respectively after vehicle and aqueous extract administration, and arteriovenous blood was collected to the biochemical and haematological analyses, while liver, kidneys and lungs were collected to histopathological studies. The relative weight of internal organs was calculated as follow.

$$\text{Relative weight of organ} = \frac{\text{Organ weight}}{\text{Body weight}} \times 100 \quad (1)$$

2.5. Determination of biochemical and haematological parameters

Lipid profile (Total cholesterol and triglyceride), urea and total protein were estimated using diagnostic kits, Fortress-UK, transaminases activities (ALT and AST) were determined using the method of Reitman and Frankel (Reitman & Frankel 1957). The hematological studies (total WBC count, total RBC count, Hb, %HCT, MCV and MCHC) were conducted according to the method of Ghai (Ghai 2004).

2.6. Histopathological studies

Histopathological studies of the liver, kidneys and lungs were carried out on control and experimental groups. Biopsies of each organ were fixed in 10% formalin, embedded in paraffin, sliced 4 µm thick and stained with hematoxylin and eosin (H&E), and then assessed for pathological changes (Kasote *et al.* 2012).

2.7. Statistical analysis

The values were expressed as means ± standard errors (S.E.M). Statistical analysis were calculated using ‘‘Graph Pad, Prism’ software. One-way analysis of variance (ANOVA) followed by post-hoc test (Student-Newman-Keuls) were used to analyze the data. The criterion for statistical significance was $P<0.05$, and extreme significance was $P<0.001$.

3. Results

3.1. Variation of body weight

On male rats, after the first treatment week, *A. boonei* induced a significant ($P<0.001$) dose dependent decrease of the body weight gain (vs. control group) at all doses. From week2 to week4, *A. boonei* significantly $P<0.001$ and $P<0.01$ increased the body weight gain respectively at doses 200mg/kg (6.19%) and 500 mg/kg (5.87%) (Vs. control group). The dose of 1000mg/kg showed a significant ($P<0.05$) increase, only at week 3 (vs. control group) (Fig. 1A).
On female rats, *A. boonei*, at all doses, showed a significant ($P<0.001$) body weight gain decrease (vs. control group) during the 4 treatment weeks. The decreases were up to -9.47%, -10.52% and -14.73%, respectively for the doses 200, 500 and 1000mg/kg b.w. at the end of experimentation (Fig.1B).

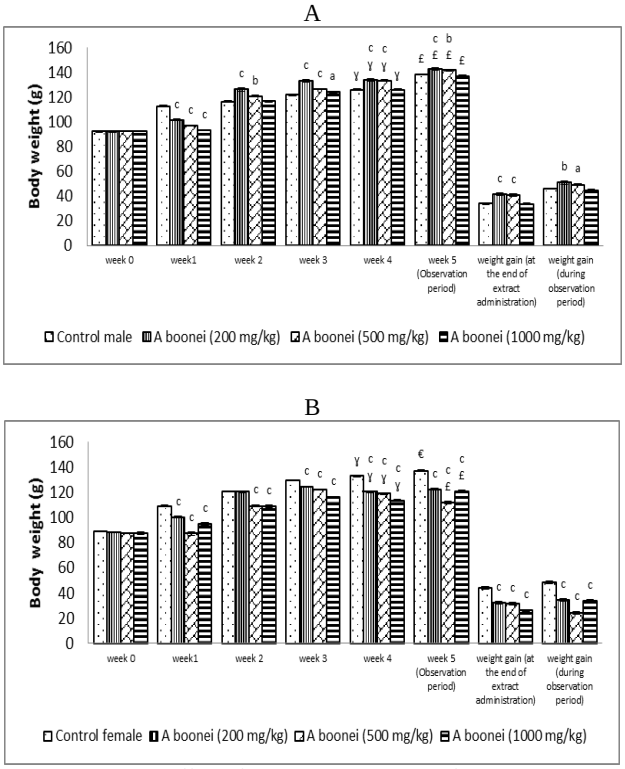

Fig. 1: Subchronic Effect of the Aqueous Extract of *A. boonei* on Body Weight in Male (A) and Female (B) Rats.
Test Drugs: Significant from Normal Control, [a]P<0.05; [b]P<0.01; [c]P<0.001. Significant From the Initial Value [€]P<0.01; [¥] P<0.001. Significant from the Week 4 Value [£] P<0.001. Mean ± S.E.M = Mean Values ± Standard Error of Means of Five Experiments.

3.2. Variation of internal organ weight

On male groups, while significant, *A. boonei* showed a weight gain decrease of all organs at all doses: of the liver, $P<0.001$ (200 and 500mg/kg b.w.) and $P<0.05$ (1000mg/kg b.w.), of the lungs

P<0.001 and the heart P<0.01 (200 and 500mg/kg b.w.) and P<0.05 (1000mg/kg b.w.) (Fig. 2A).

On female groups, *A. boonei* showed no significant (P>0.05) effect on the liver, lung and heart, while on the kidney, the extract, at all doses, showed significant increases (vs. control group), especially at the dose 1000mg/kg, which presented the highest increase value (0.88g/100g of kidney) (P<0.001) (Fig. 2B).

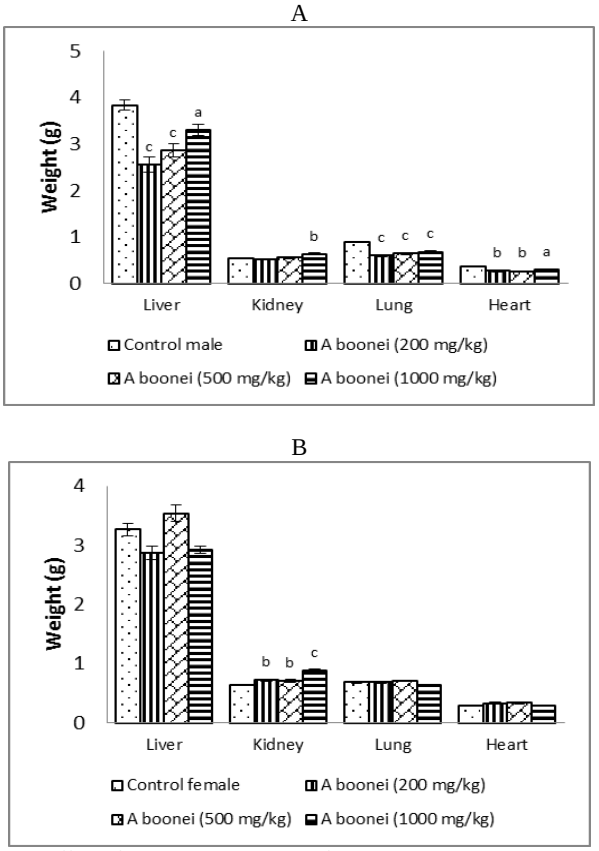

Fig. 2: Effect of the Aqueous Extract of *A. boonei* on Relative Weight of the Internal Organ in Male (A) and Female (B) Rats.
Test Drugs: Significant from Normal Control, [a]P<0.05; [b]P<0.01; [c]P<0.001 Mean ± S.E.M = Mean Values ± Standard Error of Means of Five Experiments.

3.3. Serum biochemical parameters

The results of the effect of graded doses of aqueous extract of *A. boonei* stem bark on serum biochemical parameters of male and female rats in oral route are shown in Figure 3.

The *A. boonei* stem bark presented various changes on biochemical parameters according to sex group. On both males and females, the extract failed to induced variations on TC (mg/dL) and ALT (U/L) (P>0.05) (Fig.3 A&B).

For other parameters, on male groups, *A. boonei* induced a significant (P<0.001) decrease of TG at doses 200mg/kg (134.6mg/dL) and 500mg/kg b.w. (132.8mg/dL), a significant (P<0.001) increase of AST at the dose 500mg/kg b.w., a significant decrease of urea at all doses (no dose-dependent effect), especially at the dose 500mg/kg b.w. (P<0.01) (49.15mg/dL), a significant decrease (P<0.01) of TP only at the dose of 1000mg/kg b.w.(vs. control group) (Fig.3 A).

On female groups, *A. boonei* induced dual effects on TG and urea according to doses: at the dose 200mg/kg (b.w.), the extract increased both parameters values 145.1mg/dL and 61.3mg/dL 3mg/dL respectively to TG (P<0.001) and urea (P<0.01) and decreased them at dose 500mg/kg b.w. 120.1mg/dL for TG (P<0.01) and 49.01mg/dL for urea (P<0.05). The extract also decreased AST significantly (P<0.05) only at the dose 200mg/kg b.w. (Fig. 3B).

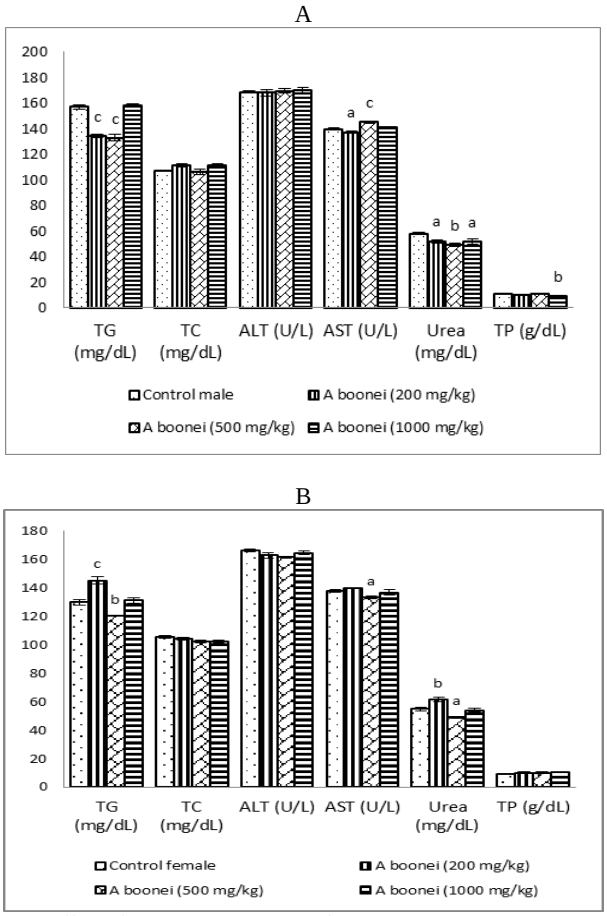

Fig. 3: Effect of the Aqueous Extract of *A. boonei* on Serum Biochemical Parameters in Male (A) and Female (B) Rats.
Test Drugs: Significant from Normal Control, [a]P<0.05; [b]P<0.01; [c]P<0.001. Mean ± S.E.M = Mean Values ± Standard Error of Means of Five Experiments.

3.4. Haematological parameters

The results of the haematological study in male rats (Fig. 4A) indicated a significant decreases (P<0.05) in the total RBC count at the doses of 500 and 1000mg/kg b.w, a significant decrease (P<0.01) in Hb at the dose of 500mg/kg, although it showed any dose-dependent effect. In the same order, the percentage of HTC also decreased significantly but unlike the total RBC count and the Hb, it was dose-dependent. The extract also caused a significant increase in the total WBC count P<0.001 and MCHC P<0.05 only at the dose of 1000mg/kg b.w of the aqueous extract and significant decreased P<0.01 in the total WBC count only at the dose of 200mg/kg b.w (vs. control group).

The results of the haematological study of the female rats as showed in Figure 4B indicate a significant increases P<0.01 in the total WBC count at doses of 500 and 1000mg/kg b.w. (vs. control group), significant decreases the total RBC count P<0.05 and P<0.01 respectively at the doses of 200 and 1000mg/kg b.w. (vs. control group), Hb also decrease significantly but non-dose dependent unlike the HTC percentage which decreased significantly dose-dependent manner (vs. control group).

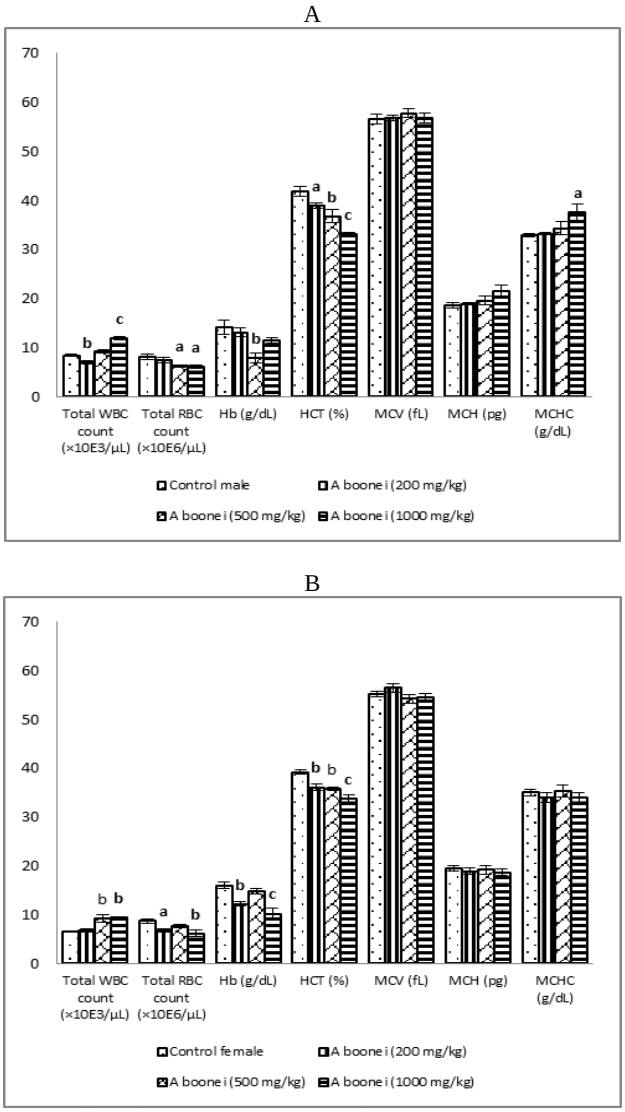

Fig. 4: Effect of the Aqueous Extract of *A. boonei* on Haematological Parameters in Male (A) and Female (B) Rats.
Test Drugs: Significant from Normal Control, [a]P<0.05; [b]P<0.01; [c]P<0.001. Mean ± S.E.M = Mean Values ± Standard Error of Means of Five Experiments.

3.5. Histopathology of liver, kidney and lung

The results of microscopic examination of liver sections of animals from control group showed normal liver architecture (Fig. 5A). On the liver sections of male rats, only *A. boonei* at the dose of 1000mg/kg b.w. (Fig. 5D) exhibited dilatation of capillary caused by infiltration of macrophages.
On female rats, the dose of 500mg/kg b.w. showed necrosis, congestion and dilatation, lymphocytic and granulocytic infiltration of portal area (Fig. 5F) while, the dose of 1000mg/kg b.w. showed pyknotic and nuclear degeneration characterized by lymphocytic and granulocytic infiltrations (Fig. 5G).

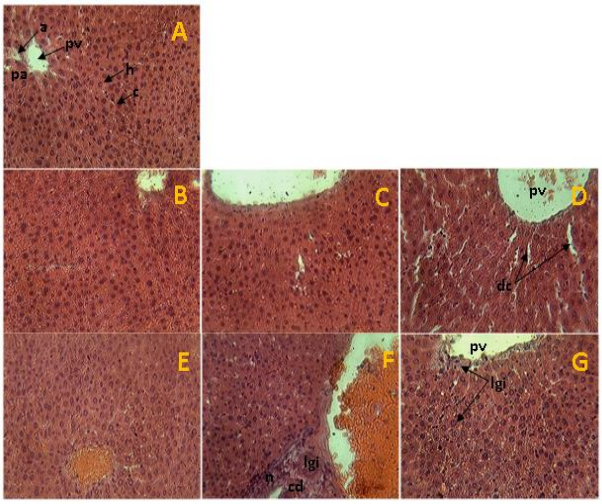

Fig. 5: Histological Appearance (H&E× 400) of the Liver of rat, one Week after the Treatment end for: Control (A), Male 200 (B), 500 (C) and 1000 (D) mg/kg b.w. of *A. boonei* and Female 200 (E), 500 (F) and 1000 (G) mg/kg b.w. of *A. boonei*. A: Shows the Normal Appearance of Artery (a), Portal Vein (pv), Portal Area (pa), Hepatocyte (h) and Capillary. B, C and E: Show no Morphological Difference When Compared With the Control. D: Shows Dilatation of Capillaries (dc). F: Showing's Necrosis (n), Congestion and Dilatation (cd), Lymphocytic and Granulocytic Infiltrations (lgi) of Portal Area. G: Showing's Lymphocytic and Granulocytic Infiltrations in the Central Portions of the Hepatic Lobules and in the Portal Area.

On the kidneys, sections treated with *A. boonei* 1000mg/kg b.w. induced damage only in female rats, characterized constriction of the glomerulus and increase of the Bowman space. Moreover, hyperplasia of cells around tubules appeared with hemorrhage and lymphocytic infiltration, as well as necrosis of the cells (Fig. 5G),

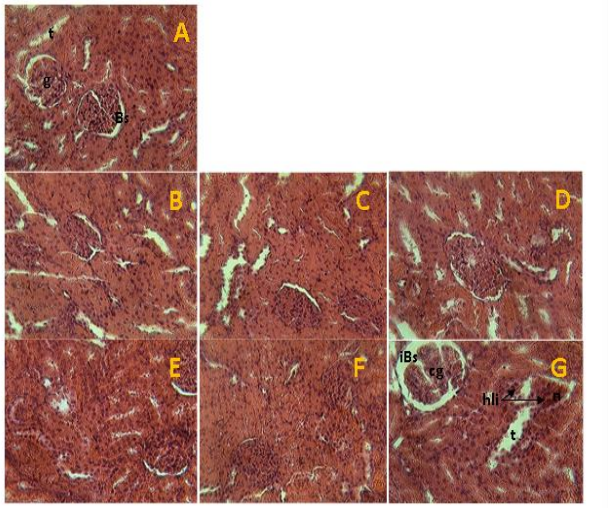

Fig. 6: Histological Appearance (H&E× 400) of the Kidney of Rat, One Week after the Treatment End for: Control (A), Male 200 (B), 500 (C) and 1000 (D) mg/kg b.w. of *A. boonei*) and Female 200 (E), 500 (F) and 1000 (G) mg/kg b.w. of *A. boonei*).B, C, D, E and F Showing No Difference in Morphology Glomeruli (g), Tubules (t) and Bowman Space (Bs) vs. Control (A). G Showing Constriction of the Glomerulus (cg) and Increase of the Bowman Space (iBs), Enlargement of the Cells Around The Tubules With Hemorrhage and Lymphocytic Infiltration (hli), and Necrosis (n).

Histopathological results of the effects of *A. boonei* on male (Fig. 7B, C&D) and female groups (Fig. 7E, F&G) showed no difference in the morphology of alveoli, bronchi and bronchial wall at all doses vs. control (Fig. 7A).

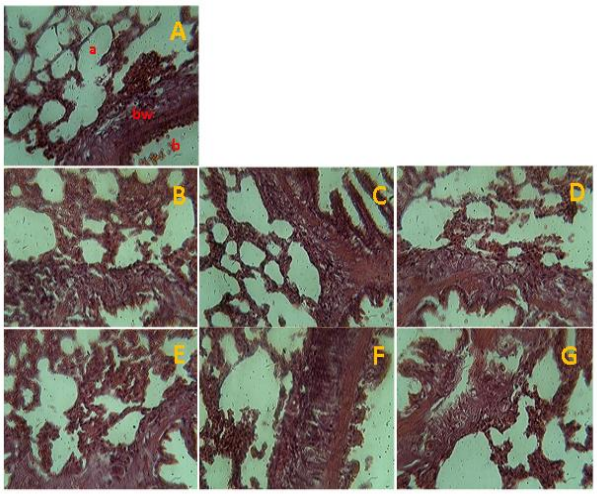

Fig. 7: Histological Appearance (H&E× 400) of the Lungs of rat, one Week After the Treatment end for: Control (A), Male 200 (B), 500 (C) and 1000 (D) mg/kg b.w. of *A. boonei)* and Female 200 (E), 500 (F) and 1000 (G) mg/kg b.w. of *A. boonei*). B, C, D, E, F and G Showing no Difference in Morphology of Alveoli (a), Bronchi (b) and Bronchial Wall (bw) vs. Control (A)

4. Discussion

The present study was carried out to evaluate the subchronic toxicity of stem barks of *A. boonei* aqueous extract on male and female rats. The stem barks extract of *A. boonei* was reported to have a therapeutic importance in folk medicine. Apart from the lack of information about the adverse or toxicity effects of this plant extract, and despite its wide-spread use in folk medicinal or traditional practice, there is lack of information underlying biochemical mechanism responsible for some of the observable and reported properties of this plant.

The subchronic oral toxicity of aqueous extracts of *A. boonei* stem bark did not cause mortality in male and female rats. Nkono *et al.* (2014) reported that the acute oral toxicity of *A. boonei* aqueous extract at the doses of 2000mg/kg b.w., and 5000mg/kg b.w showed no toxicity signs. All animals included in the test were healthy after the 14-day observation period. The LD_{50} value of *A. boonei* was considered greater than 5000mg/kg b.w. The highest dose of the extract used for this study (1000mg/kg b.w.) was not greater than 1/5 of the highest dose (5000mg/kg b.w.) used in the acute oral toxicity study of Nkono *et al.* (2014).

In a dose-dependent manner, the aqueous extract of *A. boonei* stem bark significantly increased the percentage of the body weight gain in male rats vs. control. However, significantly reduced dose dependent the percentage of the body weight gain of female rats. This result suggested that, the decrease in the body weight of female rats treated for 4 weeks with the aqueous extract could be due to the activities of the echitamine and echitamidine, potent constituents of *A. boonei*, which have earlier been reported to have diuretic and hypotensive properties (Kucera *et al.* 1972, Maurica 1993). The diuretic activities of these constituents could have resulted in loss of water and electrolytes and consequently, loss in body weight in the female rats (Raji *et al.*, 2005).

The results from this study suggest that *A. boonei* could cause impairment of the kidney at high doses in the male than in female rats, with more advanced sensitivity in female rats. The kidney is the primary organ for clearance and excretion of xenobiotics including drugs and drugs' products from the body. The study of the effect of the plant extract on the kidney is essential because of the cardinal role that the organ plays in plasma clearance, some detoxification process, homeostasis and excretion of xenobiotics (Awodele *et al.* 2010). *A. boonei* stem bark (200, 500 and 1000mg/kg b.w) significantly reduced the relative weight of heart, liver and lungs of male rats vs. control rats. This result is similar to studies of Ojo *et al.* (2014) who reported the hepatoprotective effect of the ethanolic extract of *A. boonei* stem bark. Although in female rats there was no significant difference in the same doses.

Serum biochemical markers are generally employed to asses liver function. Estimation of serum enzymes AST and ALT is the quantitative marker for the determination of type of liver and body diseases. It is established that ASAT can be found in the liver, cardiac muscle, skeletal muscle, and so forth, whereas ALAT is predominantly present in the liver (Rej 1997, Tsague *et al.* 2015). In the present study, the increased level of serum AST in *A. boonei* at 500mg/kg b.w. ($P<0.001$) male group indicate an increased permeability of hepatocytes. The decreased level of AST $P<0.05$ both in male and female rats respectively at doses of 200 and 500mg/kg b.w. of aqueous extract and non-significant difference of ALT ($P>0.05$) vs. control groups at all doses (male and female) suggest that *A. boonei* aqueous extract supports the beneficial effects of this plant on liver function (Ojo *et al.* 2014).

The increase of total WBC count may be a consequence of inflammatory reactions produced in damaged tissues of extracts treated animals and the anemia (reduction of red blood cells and %HCT) may be due to bone marrow suppression (Mohajeri et al 2007). In our study, *A. boonei* aqueous extract at 1000mg/kg b.w. increased the level of total WBC count $P<0.001$ and decreased %HCT $P<0.001$ in male rats. A similar result was observed in female rats at the dose of 1000mg/kg b.w., increased level of total WBC count $P<0.01$ and decreased %HCT $P<0.001$. This result infers that, a subchronic administration of aqueous extract of *A. boonei* at a high dose could produce an inflammatory reactions and anemia.

The histopathological observations of liver and kidney samples provided the supportive evidence for the haematological analysis. The aqueous extract at the dose of 1000mg/kg b.w showed the dilatation of capillaries of the liver of the male rats while, the aqueous extract at the doses of 500 and 1000mg/kg b.w causes necrosis, lymphocytic and granulocytic infiltrations of the portal area in the liver of female rats. It has also been observed in the kidneys of the female rats the enlargement of the cells around the tubules with hemorrhage and the lymphocytic infiltration. Centrolobular degeneration and necrosis of hepatocyte is particularly common, as this portion of the lobule receives the least oxygenized blood and is therefore, susceptible to hypoxia. Periportal degeneration and necrosis may be occurred following exposure to toxins (Cullen 2007). Thus, we can suggest that the histopathological findings of the liver in our study reflect the direct and typical toxic action of the plant extract as well as anemia in extract treated rats (Mohajeri *et al* 2007). There was no change in histopathological observations of lungs in all animals groups treated with aqueous extract of *A. boonei* stem bark.

5. Conclusion

The present study concludes that the aqueous extract of *A. boonei* De Wild stem bark is toxic in high doses. Thus, reduction in the given doses could prevent the toxic effects produced. Therefore, aqueous extract of *A. boonei* stem bark appears to have no order of toxicity when ingested in therapeutic amounts. Finally, it's suggested that the toxic chemical constituents of the aqueous extract of *A. boonei* stem bark has to be identified in order to study how to neutralize them.

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Competing interests

Authors have declared that no competing interests exist.

Ethical approval

All authors hereby declare that ‘‘Authorization for the use of laboratory animal’’ was obtain from the Cameroon National Ethics Committee (Ref. N° FWIRB 00001954), all experiments have been examined and approved.

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