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A SINGLE ORAL HIGH DOSE TOXICITY STUDY OF *KALANCHOE INTEGRA* VAR. *CRENATA* (ANDR.) CUF LEAF EXTRACT IN ICR MICE: HISTOPATHOLOGICAL AND BIOCHEMICAL CHANGES

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ABSTRACT

Background of Study: The *Kalanchoe* genus is a succulent perennial plant belonging to the class *Bryophyllum*. It thrives at temperate areas of the world. In Africa, over 200 species have been identified and many of these species have been used medicinally especially *Kalanchoe pinnata* and *Kalanchoe integra* in Ghana for the treatment of various ailments.

Aim/Objective: To evaluate the safety of the aqueous extract of the fresh leaves of *K. integra* during a single oral high dose of 5000 mg/kg b.wt in ICR mice, a study conducted by the fixed dose method.

Research methodology: Freshly prepared aqueous leaf extract were strained using muslin and freeze-dried, reconstituted and administered to ICR mice of either sex (21-25 g). The animals were kept under standard conditions and experimental procedure conducted in accordance with animal research guidelines. Blood samples for biochemical analysis were obtained by cardiac puncture. Gross and histopathological examinations were performed and statistical analysis was done using Graphpad Prism 5. **Results:** No deaths and slight weight increases ($p < 0.0001$) were recorded after the single dose administration of the extract after 14 days. Gross necropsy and histopathology did not reveal any abnormality. Urea levels reduced by 22.3% in males ($p < 0.0001$) but increased by 21% ($p < 0.0001$) in females compared to the control groups. Plasma creatinine level was decreased in both males 34.0% ($P = 0.08$) and females 34.3% ($p = 0.08$) while Alkaline Phosphatase (ALP) levels were significantly higher in the treated males by 19.5% ($P = 0.0001$) but reduced in females 17.9% ($P = 0.158$).

Conclusion: *Kalanchoe integra* appears to be non-toxic when administered to ICR mice during a single oral high dose of 5000 mg/kg⁻¹ BWT.

Key words: *Kalanchoe*, acute toxicity, histopathology

INTRODUCTION

Herbal medicine constitutes an important part of the human health care system in Ghana and most parts of the world since ancient civilization^{1,2,3}. There is an overwhelming belief worldwide that,

herbal medicines are safer and less toxic to the human body than the synthetic drugs based on the perception that natural substances are devoid of adverse effects (Briggs, 2007, Muzitano *et al*, 2009). However, there is little or no scientific

research to validate the safety and efficacy of most herbal products hence concerns have been raised regarding toxicity and adverse effects.^{1,3} Phytochemical screening of herbal medicines has shown presence of bioactive compounds with multiple pharmacological activities including hepatoprotective, antidiabetic, antihypertensive, hypolipidemic, anticancer, anti-inflammatory and immunomodulatory effects^{4,5,6,7,8,9}. *Kalanchoe sp* has been found to possess hypotensive and central nervous system depressant effect^{10,9}. The multiple pharmacological profiling has renewed interest in herbal medicine and therefore calls for a thorough scientific evaluation of medicinal plants⁴. The evaluation of the toxic effect of plant extract is necessary to ascertain treatment safety, define the intrinsic toxicity of the plant and the effects of acute overdose. Laboratory mice especially, females, are sensitive to toxic compounds in medicinal plants¹¹.

The *Kalanchoe* genus is a succulent perennial plant belonging to the class *Bryophyllum*. It thrives at temperate areas of the world. In Africa, over 200 species have been identified and many of these species have been used medicinally especially *Kalanchoe pinnata* and *Kalanchoe integra*¹³. The plant is used traditionally for the curative agent for many disease conditions such as peptic ulcer, upper respiratory tract infections, coughs and as anti-infective in Ghana^{14,15}. They grow widely along footpaths and forests in Ghana and is called by various local names (for example, the Ewes call it 'aflatoga', Fantes -'eporow', Twi and Ga - 'egoro' and 'tamiwu' respectively)¹⁴.

However, to the best of our knowledge, no report is available on the safety profile of *Kalanchoe integra*. This present study therefore evaluated the safety of the aqueous extract of the fresh leaves of *K. integra* with acute toxicity tests in ICR mice.

MATERIALS AND METHODS

Collection of plant material

Fresh leaves of *Kalanchoe integra* were collected from the Botanical Garden, University of Ghana, Legon in October 2011 and sent to the herbarium at the Botany Department, University of Ghana for identification and authentication.

Preparation of crude extract

Fresh leaves of *K. integra* were washed thoroughly under tap water. Two hundred grams (200 g) of the leaves were macerated using Sanyo SM (G300) blender. The leaf extract (2 litres) was then strained using muslin cloth and freeze-dried (0.02mg/ml). The powdered samples were stored at 4°C and used within seven weeks after production.

Subjects and experimental design

Twenty ICR mice of either sex (21-25 g) from Noguchi Memorial Institute for Medical Research, Accra, Ghana were used for the study. The animals were kept on a 12-h light and 12-h dark schedule in a constant air-conditioned room at $25 \pm 1^{\circ}\text{C}$ with relative humidity $60 \pm 6\%$. During the acclimatization period, clinical observations of the animals were conducted as well as body weight measurement and the animals were found healthy. Females were nulliparous and non-pregnant. The animals used for the study were assigned into groups including a control group by the stratified random method according to their body weight³. They had free access to standard laboratory pellet diet (AIN-93G Formulation obtained from GAFCO – Ghana) and water. All experimental procedures were in accordance with animal research guidelines and approved by the protocol/ethical committee of the University of Ghana Medical School with protocol number MS-Et/M.4-P.4.2/2010-2011.

Prior to sacrifice, rats were euthanized by exsanguinations under ether anesthesia. Blood was sampled by cardiac puncture and all the

visible organs and tissues were macroscopically examined and harvested.

Route of administration

The administration route was oral (gavage) in accordance with the main route of intake of *K. integra* by humans for medicinal purposes.

Acute toxicity test

Five (5) mice constituted a group. Thus, two groups from both sexes consisting of control and a single oral high dose, 5000 mg/kg b.wt *K. integra* reconstituted as aqueous homogenous suspension were established. The high dose 5000 mg/kg b.wt was selected based on previous studies (unpublished data). In this study, no death resulted with the administration of three dose levels of the extract (1000 mg/kg, 2000 mg/kg and 4000 mg/kg).

Clinical observations

The observation period was 14 days post administration. Clinical signs of toxidromes (such as rising fur, sluggish movement, draping, tremors, excitability, miosis, mydriasis, twitching, salivation, food intake, morbidity, etc.) and mortality were observed after 0.5, 1, 3, and 6 h of administration. Thereafter, twice daily observations were made, up until the 14th day. Body weights were measured before dosing on the day of administration and every morning thereafter.

Laboratory examinations

Blood samples by cardiac puncture (15th day) were collected into plain tubes for biochemical examinations. Blood samples were centrifuged for 5min at 3000 rpm. The following biochemical assays were performed using SELECTRA JUNIOR VERSION 04 autoanalyzer for biochemical assays (VITAL SCIENTIFIC BV, NETHERLANDS). Creatinine, urea, aspartate amino transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP). Organs such as liver, kidney, lungs, spleen, testis, heart, brain and pancreas were examined macroscopically prior to harvesting. Harvested organs were

weighed and stored in formalin. Thus gross necropsy was performed but additionally, selected organs (liver, kidneys, and heart) were examined histologically.

Statistical analysis

The statistical analysis of the data was done using Graphpad Prism 5. Means $\pm$ SEM were determined for quantitative variables. Analysis of variance (ANOVA) was used to determine the existence of statistical significance between variables possibly with more than two outcomes. P values ≤ 0.05 were considered significant.

Thus all data are expressed as Means $\pm$ SEM. Two-way ANOVA was used and $p < 0.05$ were considered as significant, followed by Benferroni post hoc test.

Although the ANOVA was done for both sexes to check differences in various parameters based on sex, the Benferroni post hoc analysis was restricted to only control and treated group of same sex. This is based on literature that, females are more sensitive to xenobiotics and drugs than males due to hormonal imbalances¹¹. The test was just to confirm it. In the histopathological examinations, H & E (haematoxylin and eosin) stain were used with $\times 100$ magnifications.

RESULTS AND DISCUSSION

No deaths were recorded after oral administration of the extract. No physical signs of toxicity as evidenced by abnormal breathing, movement, etc. were observed. Observation of treated animals over the next 14 days showed no adverse effects of treatment. The oral LD₅₀ for the aqueous extract is thus greater than 5000 mg/kg³. In addition, gross necropsy finding did not show any adverse effect in both male and female mice organ treated with 5000 mg/kg b.wt single oral dose of *Kalanchoe integra* after day – 14 as compared to the controls. Also, both the treated males and females ICR mice gained a significant ($P < 0.0001$) weight over the course of this study compared to the controls (Fig 2).

There were variations in the plasma levels of urea, creatinine, ALP, ALT and AST (fig 3, 4, 5, 6, 7) in the treated groups compared to the control. For example, plasma urea level was significantly higher ($*P<0.05$) in control males compared to control females. Furthermore, administration of 5000 mg/kg b.wt *Kalanchoe integra* in both sexes did not show any significant difference in urea levels in both treated groups compared to the control groups. Additionally, plasma creatinine level was insignificantly lowered and increased ($P = 0.08$) in treated males and females respectively. However, there was a non-significant ($P = 0.158$) increase in ALP levels in treated females compared to other groups. The levels of ALP was significantly higher ($P = 0.0001$) in treated females whilst that of treated males were insignificant ($P>0.05$) compared to the control. This could be due to a hepato-biliary condition such as primary biliary cirrhosis. With the exception of growing animals or animals with bone diseases where an increase in ALP could be due to the osteoblasts' production, the elevated serum ALP is most likely to be due to hepatobiliary origin. Alanine transaminase (ALT) increased in both males 45.3% ($p>0.05$) and treated females 23% ($P=0.02$) compared to the control.

In female rats, 5000 mg/kg b.wt showed significant increase ($P= 0.02$) in levels of ALT compared to that of the controls. Finally, level of ALT in treated males was insignificant ($P> 0.05$) compared to the controls. Normally increases in ALT enzymes suggest a hepatic leakage or acute liver damage^{16,17}.

Though there is no evidence of any adverse effect in the use of *kalanchoe integra*, this is also not a guarantee of safety of the leaf extract since there is no monitoring of adverse reactions on herbal medications in general. The absence of scientific data to support its safety or otherwise and the popularity of the leaf extract in

Ghanaian traditional medicine prompted a safety evaluation which was clearly unavailable.

Histopathological studies did not reveal any abnormality as compared to the controls (fig 8). Haematoxylline and eosin (H&E) stained paraffin sections showed no significant morphological change in the kidneys, heart and liver of both the control and extract-treated mice evidenced by lack of fatty change or necrosis in the liver, and lack of swelling and proliferation of normally flat endothelial cells lining the glomerular capillaries. Torres confirms by observing that the serum levels of alanine-aminotransferase (ALT), aspartate-aminotransferase (AST), urea and alkaline phosphatase were unchanged in mice orally treated with supraoptimal *Kalanchoe pinnata* doses for 30 days, indicating the absence of chronic toxicity to the liver, heart or kidney.

The absence of these markers of injury suggests that the extract does not cause structural damage to the liver or the kidneys at the single high dose used in the present study¹⁸ thus contradicting previous studies conducted which claim the aqueous extract is the most toxic¹⁹. Thus more research into the location and composition of the plant need further clarification.

This could be due to a hepato-biliary condition such as primary biliary cirrhosis. With the exception of growing animals or animals with bone diseases where an increase in ALP could be due to the osteoblasts' production, the elevated serum ALP is most likely to be due to hepatobiliary origin. Alanine transaminase (ALT) increased in both males 45.3% ($p>0.05$) and treated females 23% ($P=0.02$) compared to the control.

CONCLUSION

The administration of an aqueous extract of *Kalanchoe integra* var. *crenata* (Andr.) Cuf leaves was accompanied by the absence of injury markers and did not cause any structural damage to the liver and kidneys. The LD₅₀ value

of the aqueous extract of *Kalanchoe integra* was beyond 5,000 mg/kg. Thus the extract appears to be non-toxic when administered orally to ICR mice.

CONFLICTING INTEREST

The authors hereby declare there is no any conflicting interest.

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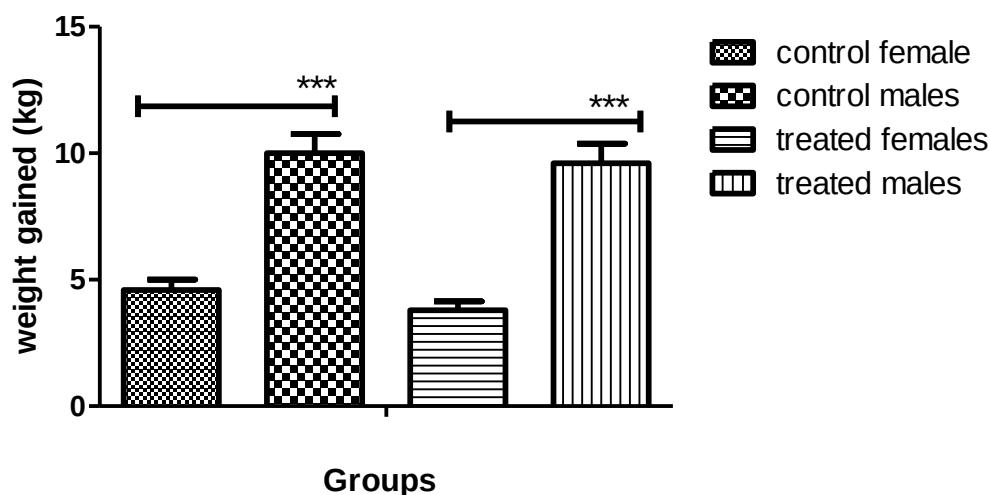
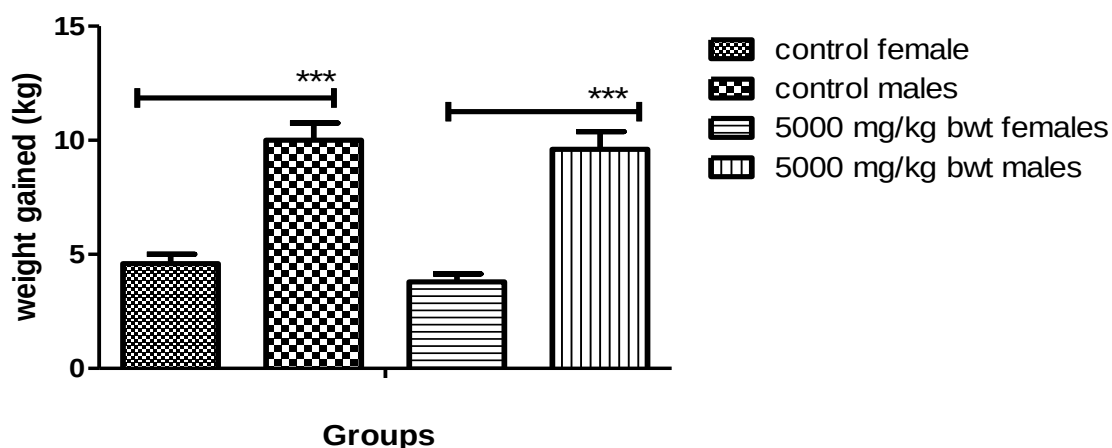
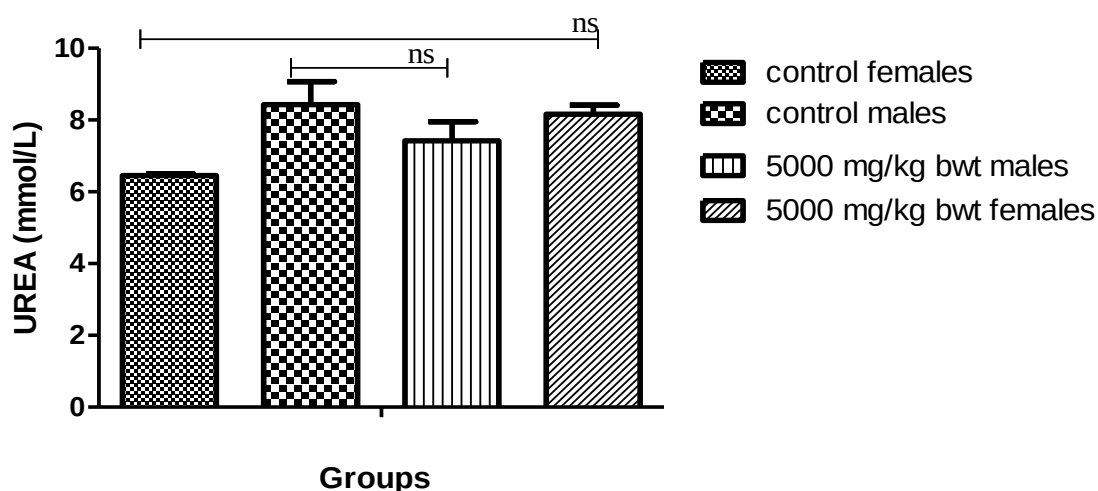


Figure 1. Average weight changes in ICR mice treated with 5000mg/kg *Kalanchoe integra*

Table 1 Changes in organ weights during *Kalanchoe integra* administration

ORGAN/AVERAGE WEIGHT (GM)	Control female	Control male	Treated female	Treated males	P values
Lungs	1.03 ± 0.07	0.61 ± 0.02***	0.76 ± 0.02	0.74 ± 0.08	0.0006
Liver	1.50 ± 0.07	1.96 ± 0.10**	1.32 ± 0.07*	1.90 ± 0.09***	0.0001
Spleen	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	
Heart	0.10 ± 0.00	0.16 ± 0.02	0.10 ± 0.00	0.14 ± 0.02	0.06
Kidney	0.46 ± 0.02	0.36 ± 0.02	0.30 ± 0.03**	0.40 ± 0.02	0.002
Brain	0.42 ± 0.02	0.36 ± 0.02	0.34 ± 0.02	0.32 ± 0.02*	0.033

**Figure 2.** Body weight gained after day - 14. Single oral dose administration of *Kalanchoe integra* showed significant increase ($P < 0.0001$) in body weight gained after day – 14 compared to that of controls**Figure 3.** Plasma urea levels in treated mice vs control. Plasma urea level was significantly higher ($*P < 0.05$) in control males compared to control females. Administration of 5000 mg/kg b.wt *Kalanchoe integra* in both sexes did not show any significant difference in urea levels in both treated and the control groups after 14 days.

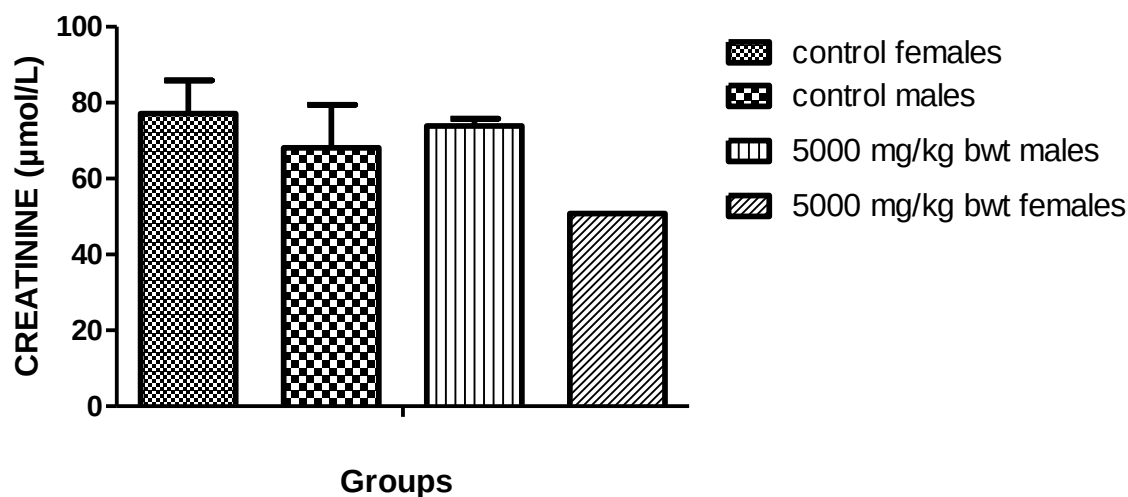


Figure 4. Plasma creatinine levels in treated mice vs control. Plasma creatinine level was insignificantly lowered and increased ($P = 0.08$) in treated males and females respectively after day -14.

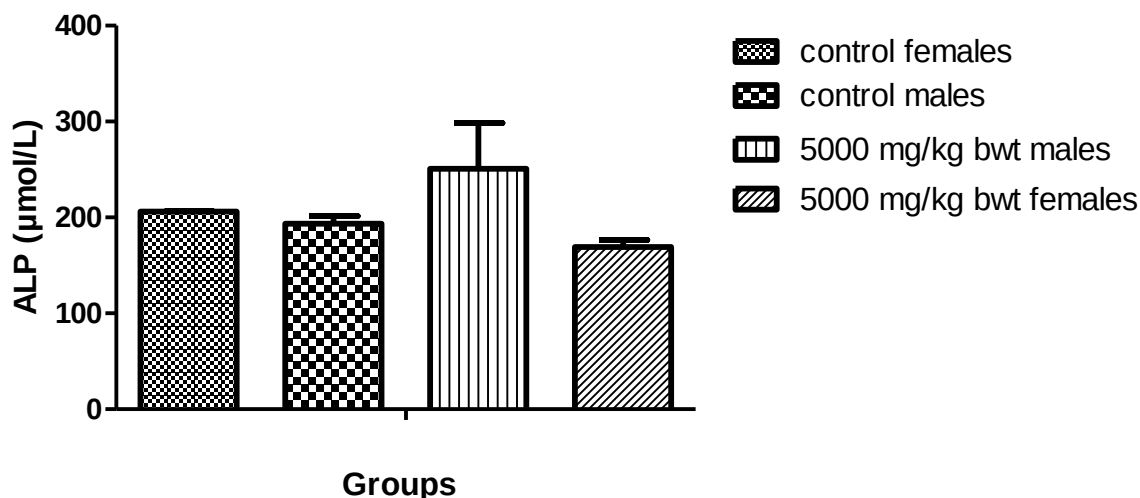


Figure 5. Plasma alkaline phosphatase (ALP) levels in treated mice vs control. There was a non-significant ($P = 0.158$) increase in ALP levels in treated females compared to other groups after day - 14.

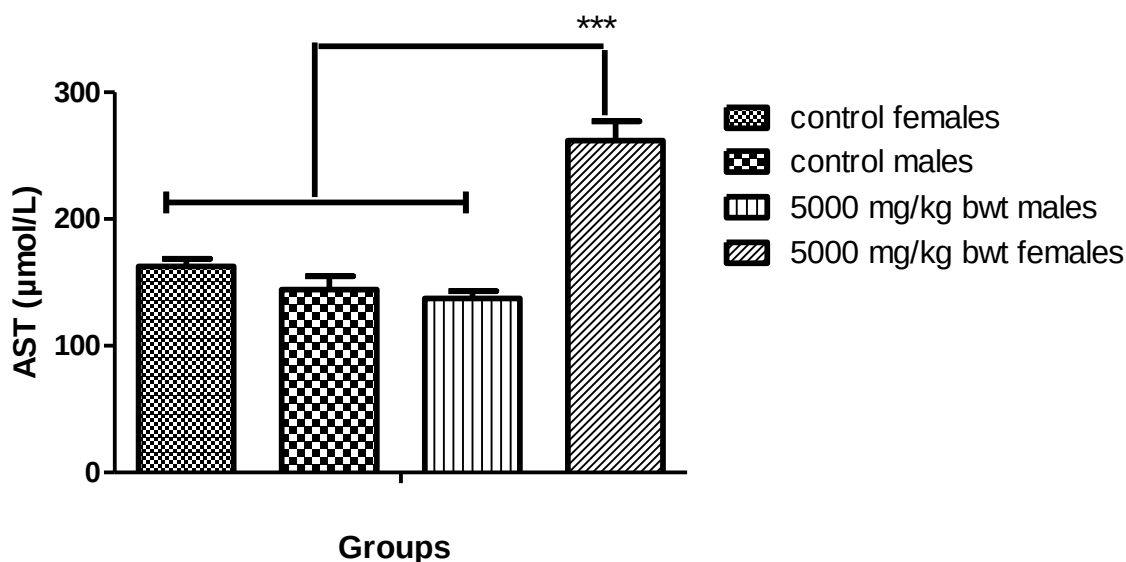


Figure 6. Plasma aspartate aminotransferase (AST) levels in treated mice vs control. The levels of ALP was significantly higher ($P = 0.0001$) in treated females whilst that of treated males were insignificant ($P > 0.05$) compared to the control after day - 14.

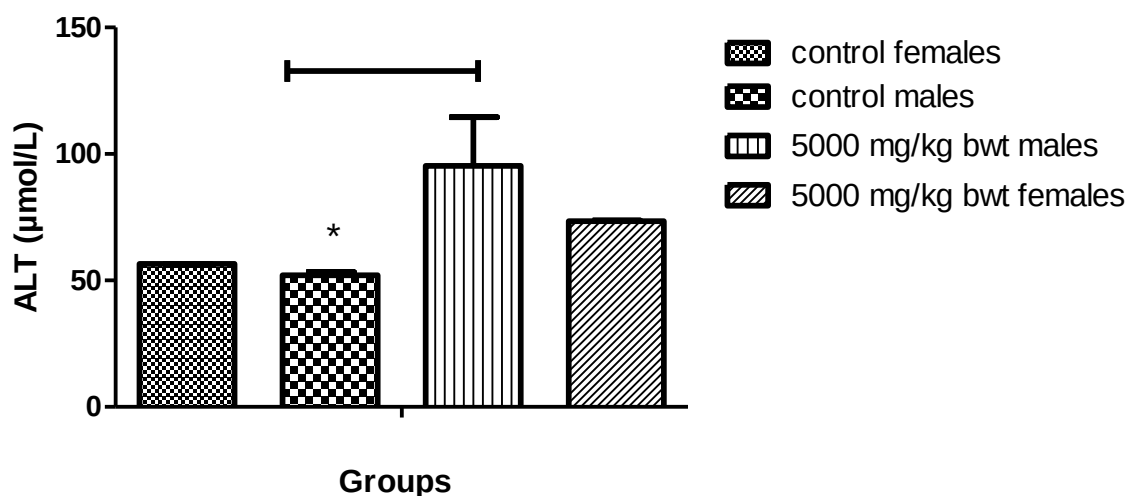
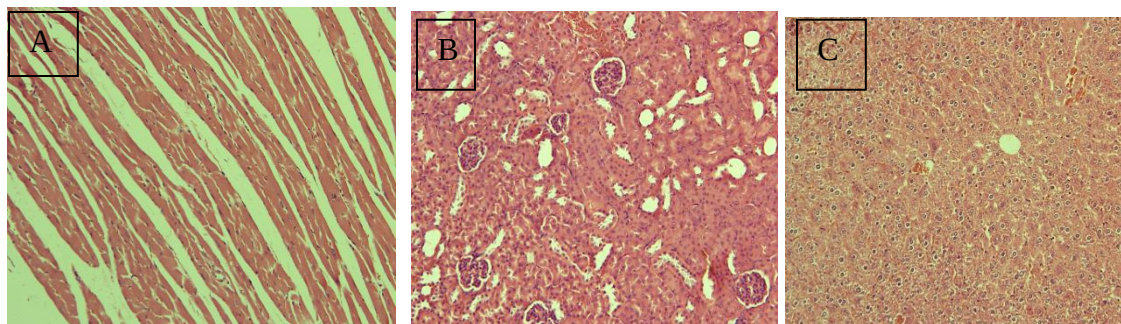


Figure 7. Plasma alanine aminotransferase (ALT) levels in treated mice vs control. 5000 mg/kg b.wt females showed significant increase ($P = 0.02$) in levels of ALT compared to that of the controls. Level of ALT in treated males was insignificant ($P > 0.05$) after day - 14.

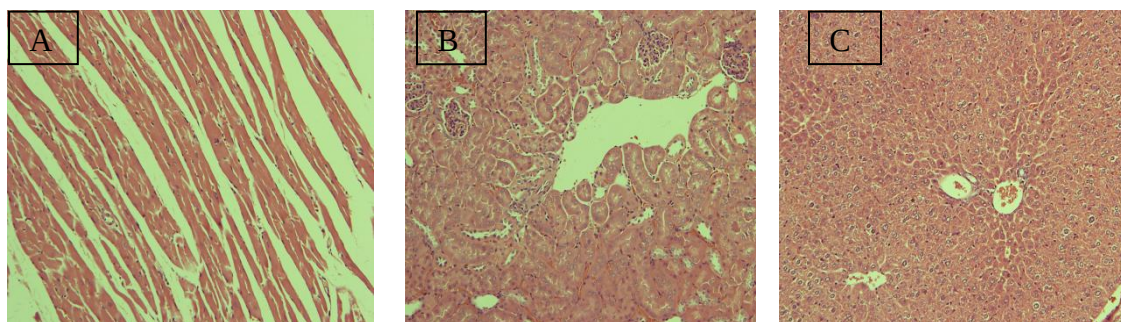


a) Controls

Keys: A (heart),

B (Kidney)

C (Liver).



b) 5000 mg/kg High Dose

Figure 8. Histopathological examination during *Kalanchoe integra* administration (5000 mg/kg).

Comment: No lesions in all micrographs.