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Histopathological changes in female rabbits administered with aqueous extract of *Calotropis procera*

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Calotropis procera is an evergreen perennial shrub, which is found mainly in the arid regions and produces copious latex when cut. It has been reported to possess medicinal properties but equally pose deleterious effect in animals. In a bid to exploit its pharmacological properties, it was necessary to ascertain its level of safety. A toxicological evaluation of the aqueous extract of fresh leaves of the plant was therefore conducted in the more sensitive female rabbits of the same weight range. Low levels of phytochemicals (alkaloids, saponins, tannins, cardiac glycosides and flavonoids) were found, while elemental analyses showed traces of iron, lead, sodium, and potassium in concentrations of 0.23, 0.03, 0.82 and 9.5 mg/g, respectively. Acute toxicity study was conducted with oral administration of 200, 400, 800 and 1600 mg/kg of the extract once to groups I, II, III and IV, respectively with a 24 h observation period. Clinical signs such as mouth chewing, photophobia, bradycardia, coughing, vomiting and convulsion amongst others were noticed. Four rabbits died within 24 h and LD_{50} was estimated (940 mg/kg). 80, 40 and 20 mg/kg of the extract were administered daily to groups I, II, and III, respectively, during sub-acute toxicity study for 14 days. Grossly, catarrhal enteritis and mesenteric congestion of the small intestines, congestion of the lungs, hepatization and paleness of the liver, congestion and pallor of the kidney cortex, and congestion of the meninges were noticed. Histopathological examination of the tissues revealed mild pulmonary oedema and peribronchial lymphocytic infiltration of the lungs, hepatization of the liver, disruption of cardiac architecture, generalised cell necrosis and erosion of the villi of the small intestine. All the rabbits that survived gained weight, which is indicative of some nutrient value in the extract. It was concluded that the extract had dose-dependent deleterious effects on the tissues as higher dose groups were more affected. Hence, it is evident that sub-chronic toxicity studies would reveal greater lesions to better ascertain extent of damage.

Key words: *Calotropis procera*, phytochemical, histopathology, toxicity, lesions, tissues, organs.

INTRODUCTION

Calotropis procera is an evergreen shrub belonging to the category of spreading shrubs and to the class Angio-spermae. It grows mainly in the arid regions of Africa and the temperate and tropical regions of Asia. Its growth is favoured by open habitat with little competition (Adams, 1995). It releases a copious white sap (latex) when cut (Harkness and Wagner, 1989). It is a common plant in Nigeria but more abundant in the northern part of the country (Sofowora, 1984). It grows widely in the tropics, with disturbed vegetation and warm temperate regions (Sofowora, 1984).

Extracts, chopped leaves and latex have shown great promise as nematicides *in vitro and in vivo*, while poultices of the plant leaves heal rheumatism when applied to joints (Charu et al., 1997). Over 92 plants are used in ethno-veterinary practices, some of which have pesticidal and insect repellant activities (Aliu, 1996). *C. procera* has also been found to contain secondary metabolites which are of no apparent importance to the plant's own life but have prominent therapeutic as well as toxic effects on animal systems (Mgbojikwe, 2004). *C. procera* is also reported to have pharmacological properties including its traditional use in the treatment of leprosy, fever, diarrhoea, malaria and snake bites (Parrotta, 2001).

Many plants are however toxic, although their chemical and physiological characteristics are only understood empirically. Consumption of *C. procera* when fresh results in poisoning to livestock as well as humans (Lewis and Elvin-Lewis, 1977). Animals are exposed most especially during periods of starvation/drought or when livestock is moved from place to place in search of better pasture (Hall, 1977). *C. procera* contributes to a greater part of general malaise, dullness and inappetence in grazing animals and the cause which is seldom diagnosed is due to consumption of sub-clinical dose of the plant (Clarke and Clarke, 1977). *C procera* is described as an abortifacient and an anti-fertility agent (Malhi and Tridedi, 1972). Studies on the effects of the plant extract on the ultrastructure of the kidney as well as histology of the skin and reproductive organs of Wistar rats are reported (Al-Robai et al., 1993, Akinloye et al., 2001a, b).

In order to exploit its pharmacological properties, toxicity investigation was carried out to ensure safety. Based on these reports, and the lack of statistical significance on haematological and biochemical parameters for acute and sub-acute toxicity studies (Jato et al., 2009), this study was carried out to investigate the effects of the extract at tissue levels in rabbits. As objectives, we sought to answer the questions: At what dose and to what extent is the plant toxic, what are the target organs, and at what dose do we have no observable effect?

MATERIALS AND METHODS

Animals

Twenty seven (27) female New Zealand rabbits of the same weight range and 8 to 10 week old, obtained from the Small Unit of the Diagnostic Department of the National Veterinary Research Institute (NVRI) were used in this study. Female rabbits are more sensitive and responded quickly to foreign agents, while 8 to 10 week old are more resistant, and so would form a good basis for toxicity evaluation. Fifteen (15) of the rabbits were divided into 5 groups of 3 animals each for acute toxicity study, with Group V serving as the control. Twelve (12) rabbits were also divided equally into Groups I to IV for sub-acute toxicity study, with Group IV serving as the control. They were fed daily on pelleted feeds obtained from the 'Dagwom' Farm of NVRI, with water given *ad libitum*. Faecal samples were examined for two weeks to ensure the absence of infectious agents prior to commencement of the experiment. Temperature was also taken daily during each observation to ensure that the animals did not record abnormal fluctuations.

Weight analysis

The animals were weighed (in kg) each morning and evening using a scale balance before feeding. Weight changes were determined by difference with previous weights and the average for all groups gave the overall change.

Plant collection

Fresh leaves of *C. procera* were obtained from Fadan Karshe in Kaduna and identified at the Federal College of Forestry in Jos, Nigeria.

Preparation of the aqueous extract

3.0 kg of fresh leaves were then blended into a pulp using a blender (ATO MSE Mix – Guangzhou Sunmile Industries Co. Ltd). It was then filtered and the filtrate was dried at 50°C using an oven (Gallenkamp 300 plus – Gallenkamp) to obtain a dry powder. 1 g of the powder was then dissolved in 100 ml of distilled water to obtain a stock solution for daily administration. Fresh leaves were used because the dry form is reported to be harmless when consumed, while aqueous extraction was carried out due to the high solubility of the extract in water (Mgbojikwe, 2004).

Elemental analysis

At the Nigerian Mining Corporation (NMC), Jos, 0.2 g of the powdered extract was weighed and put into a clean Kjerdhal flask. 5 ml of concentrated nitric acid (HNO_3), 1 ml of concentrated sulphuric acid (H_2SO_4) and 1 ml concentrated perchloric acid were added. The mixture was heated for digestion until it turned colourless. After digestion, the clear extract was filtered and the filtrate was made up to 100 ml in a volumetric flask with distilled water. It was then analysed for iron, lead, copper, chromium, cobalt, sodium,

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potassium and arsenic using the Atomic Absorption Spectro-photometer, (AAS, Unicam Solar 969 - Unicam), (Haswell, 1991).

Phytochemical screening

Phytochemicals like alkaloids, saponins, tannins, cardiac glycosides and flavonoids were tested for the crude extract using standard procedures.

Test for alkaloids

0.5 g of the plant extract was stirred with 3 ml of 1% aqueous hydrochloric acid (HCl) in a steam bath. This was filtered and 1 ml of the filtrate was treated with a few drops of picric acid solution. The reaction was observed for formation of precipitate which indicates presence of alkaloids (Trease and Evans, 1989).

Test for saponins

0.5 g of the plant extract was shaken with distilled water in a test-tube. Frothing appearance or foaming which persists on warming was taken as preliminary evidence for the presence of saponin (Trease and Evans, 1989).

Test for tannins

0.5 g of the plant extract was stirred with 1 ml of distilled water. This was filtered and ferric chloride reagent was added to the filtrate. The reaction was observed for a green, blue-black or blue-green precipitate, which indicates the presence of tannin (Trease and Evans, 1989).

Keller Killiani test for cardiac glycosides

100 g of the plant extract was dissolved in 1 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under-layed with 1 ml of concentrated sulphuric acid. A brown ring obtained at the interphase indicates the presence of a deoxysugar which is characteristic of cardenolides or cardiac glycosides, (Trease and Evans, 1989).

Test for flavonoids

2 g of the plant extract was completely detanned with acetone (Segelman and Sofia, 1971). The acetone was evaporated in a water bath and the residue was then extracted in warm water. The mixture was then filtered while still hot and the filtrate allowed to cool. Lead acetate solution was added to 5 ml of the detanned water extracted. Appearance of a yellow coloured precipitate indicates presence of flavonoids.

Dosing of the animals and determination of LD_{50}

200, 400, 800 and 1600 mg/kg of the extract were given by oral gavage to Groups I, II, III and IV, respectively, within 24 h in acute toxicity study. Group V was given only water *ad libitum*. LD_{50} was then estimated graphically from a plot of percentage response against dose. For sub-acute toxicity study, 20, 40 and 80 mg/kg were given daily to Groups I, II and III, respectively, for 14 days period. Group IV, the control was given only water for the same study period.

Necropsy and histopathology

All the rabbits that died immediately on administration of the extract and those that were humanely air-embolized were physically observed for clinical signs and then dissected for postmortem examination. They were examined grossly for lesions and tissues and organs were cut for histopathology. Histopathology was carried out through fixation, embedding, sectioning, staining and micro-scopic examination.

Fixation

The tissues were fixed in a fixative, a process that stabilizes the tissues to prevent decay. The fixative used was neutral buffered formalin (10% formaldehyde in phosphate buffered saline (PBS).

Embedding

Wax embedding was done in which the samples were immersed in multiple baths of progressively more concentrated ethanol to dehy-drate the tissue using an autotechnicon. This was followed by clearing with xylene and finally impregnation in hot molten paraffin wax. During this process, paraffin wax replaces the water; and soft, moist tissues were turned into a hard paraffin block, which was then placed in a mould containing more molten wax and allowed to cool and harden.

Sectioning

The tissues were then trimmed to appropriate sizes by removal of excess molten wax and then attached to labeled wooden blocks. The ice on the wooden blocks was then ice cold so that the tissues could give straight ribbons during sectioning. Tissues were sectioned into very thin (2 to 5µm) sizes using a microtome (RM2235 – Leica Microsystems). The slices appeared as slender ribbons and were put on the surface in a water bath with temperature below that of the paraffin wax to relax the ribbons. These slices which were usually thinner than an average cell, were then placed on a glass slide for staining. Egg albumin was used to attach tissues to slide.

Staining

For clear vision under a light microscope, tissue sections were stained with hematoxylin and eosin (H&E). Hematoxylin colours nuclei blue, while eosin colours the cytoplasm pink. This was done to give contrast to the tissue being examined, as without staining, it is very difficult to see differences in cell morphology.

Microscopic examination of tissues

After preparation, the slides were stained with eosin and hema-toxylin and examined microscopically using the X10, X40 and X100 magnifications for any pathological changes that might have occurred as a result of the extract.

RESULTS

Weight analysis

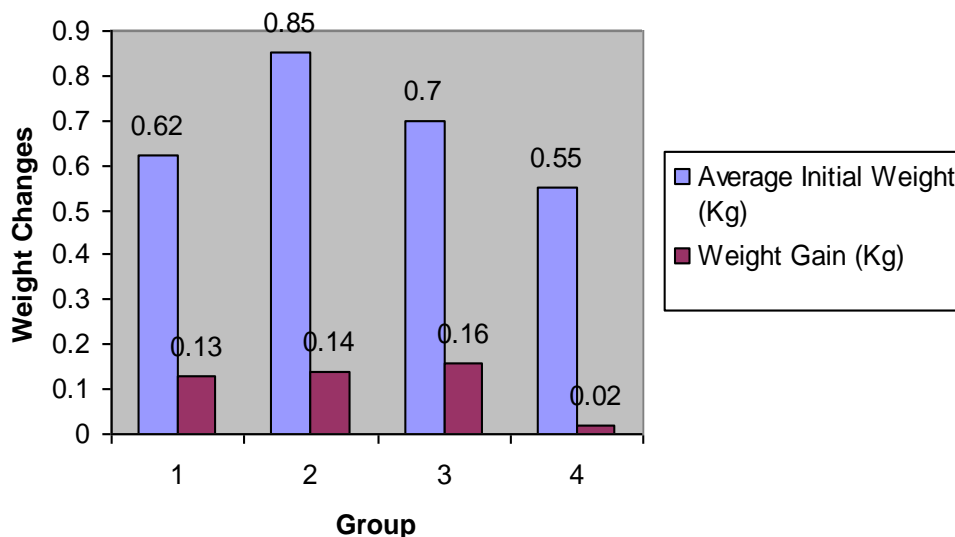The rabbits recorded average weight gain of about 0.01 kg (16%), as they got bigger by the day (Figure 1).

Figure 1. Weight changes in sub-chronic toxicity studies.

Preparation of the aqueous extract

0.086 kg of dry powdered extract was obtained from the 3.0 kg fresh leaves, giving a percentage yield of 2.5%.

Elemental analysis

Elemental analysis indicated trace amounts of iron, lead, sodium and potassium at concentrations of 0.21 mg/g, 0.03 mg/g, 0.80 mg/g and 9.3 mg/g, respectively. Chromium, molybdenum, cobalt and arsenic were not detected.

Phytochemical screening

Saponins, tannins, alkaloids, cardiac glycosides and flavonoids were detected, with cardiac glycosides being in higher concentration. Anthraquinone was not detected.

Dosing of animals and determination of LD_{50}

Besides deaths recorded within 24 h, survivors exhibited some clinical signs (Table 1). From the deaths recorded, LD_{50} was determined to be 940 mg/kg. Percent mortality showed that cumulative percentage mortality increased with increase in dose (Table 2).

Necropsy and histopathology

Grossly, lesions were observed in the lungs, small intestine, kidney, heart, liver and the brain in both acute and sub-acute toxicity studies with greater lesions in sub-acute. Unlike appearing well fleshed as at the start of the study, catarrhal enteritis, mesenteric congestion of the intestines, congestion of the lungs with hepatization, pale and shrunken liver, congestion and pallor of the kidney cortex, and congestion of the meningis were observed. Histopathology revealed cellular lymphocytes infiltration and oedema of the renal tubules of medulla (Slide a) and engorgement and dilation of hepatic sinusoid with red blood cells, cellular lymphocytic infiltration of the liver (Slide b). Also, there was mild pulmonary oedema (congestion) and peribronchial lymphocytic infiltration of the lungs (Slide c) and erosion of the villi of the small intestine (Slide d). The cardiac architecture of the heart was also slightly disrupted, with generalized necrosis and cellular infiltration (lymphocyte and neutrophils) (Figure 2).

DISCUSSION

The rabbits experienced dose-dependent increase in weight. Only 0.086 kg of the fresh leaves gave the dry powdered extract. Trace amounts of iron, potassium, sodium and lead were detected, while phytochemicals such as saponins, flavonoids, tannins, alkaloids and cardiac glycosides were found. Upon administration of the aqueous extract, the rabbits exhibited clinical signs while others died instantly. Gross and histopathological examinations revealed lesions. Much is reported about the pharmacological and poisonous properties of *C. procera* (Nsekuye, 1994; Basu et al., 1997), but there is not much literature on possible toxicity of the aqueous extract on organs and systems of an animal.

The chewing movement of the mouth observed is also

Table 1. Clinical signs observed on administration of the aqueous extract of *C. procera* (acute toxicity studies).

Group/ dose (mg/kg)	Average fasting weight (kg)	Average Volume administered (m/s)	Onset of toxicity (mins) after	Mortality $^x/_y$ (mins)	Observation
I (200)	1.1	2.1	5.7	$^0/_3$ (-)	Chewing movement of the mouth, fast and abnormal heartbeat.
II (400)	1.2	4.7	2.0	$^0/_3$ (-)	Chewing movements of the mouth, engorged blood vessels of the ears, depression, photophobia, epistotonus and rapid abnormal breathing.
III (800)	1.3	10.4	3.7	$^1/_3$ (1277)	Chewing movements of the mouth, engorged blood vessels of the ears, bradycardia, prostration, depression, serous discharge from the anus after four hours, pawing, sneezing, coughing, difficult abdominal breathing.
IV (1600)	1.7	17.5	2.3	$^3/_3$ (5.7)	Gasping, difficult abdominal breathing, dog sitting, epistotonus, excitation, prostration, frothy vomiting, staggering, gasping, convulsion and circling movements
V (Water)	0.9	14.4	-	-	No observable changes

Mortality $^x/_y$: x = Death in the group and y = number of animals per group.

Table 2. Percentage mortalities on administration of the aqueous extract of *C. procera* (acute toxicity studies).

Group	Dose Regimen (mg/kg)	Survival ($^x/_y$)	Mortality ($^x/_y$)	Mortality (%)	Cumulative mortality (%)
I	200	$^3/_3$	$^0/_3$	0.0	0.0
II	400	$^3/_3$	$^0/_3$	0.0	0.0
III	800	$^2/_3$	$^1/_3$	33.3	33.3
IV	1600	$^0/_3$	$^3/_3$	100.0	133.3

($^x/_y$): x, animals that survived and y, number of animals per group.

reported by Dada et al. (2002) and considered to be a taste of palatability. The fast and abnormal heartbeat, bradycardia and gasping for breathe are indicative of some damage inflicted on the heart and this can be attributed to cardiac glycosides. Engorgement of blood vessels of the ears shows that there was circulatory disturbance. The lesions observed in the lungs, liver, brain and kidney grossly confirmed the clinical signs observed. Catarrhal enteritis reported by Mahmoud et al. (1979) was also observed but diarrhoea reported by Dada et al. (2002) in Wistar rats was not noticed.

Respiratory difficulties are indicative of lymphocytic cellular infiltration of the lungs due to presence of blood cells and fluid in air sacs. Disruption of cardiac architecture, with generalized necrosis of the myocardium could be attributed to cardiac glycosides which have the ability to inhibit the membrane potential through obstructtion of the Na^+/K^+ ATPase pump (Al-Robai et al., 1993). Hepatic oedema with slight necrosis of portar triad and hyperplasia of the bile duct was found to run across the groups, indicating that the liver was damaged but not markedly. Previous study by Kaneko and Cornelius,

(1980) indicates that alanine amino tran-sferase is found in high concentrations in hepatic tissues of dogs, cats and primates and elevation of its activity in plasma indicates hepatocellular damage. Another study reports that increase in plasma enzyme activities often seen following liver damage does not indicate an increase in liver ability to synthesize that enzyme but rather a loss of material from damaged hepatocytes. For there to be any increased level of serum enzymes in blood, the liver must be greatly damaged (Jato et al., 2009). This dispels toxicity due to high levels of biochemical and haematological parameters.

Congestion of the kidneys suggests the failure of the kidney to excrete potassium, leading to increased levels of potassium in the extracellular fluid (Morag, 1989) and blood urea. Catarrhal enteritis seen grossly is confirmed histopathologically by erosion of the villi of the small intestine with lymphocytic infiltration. Meningitis indicates that there is obstruction of communication with the brain, hence lack of coordination, circling movement, staggering and convulsion. The continuous increase in weight of both control and test groups observed in rabbits has also
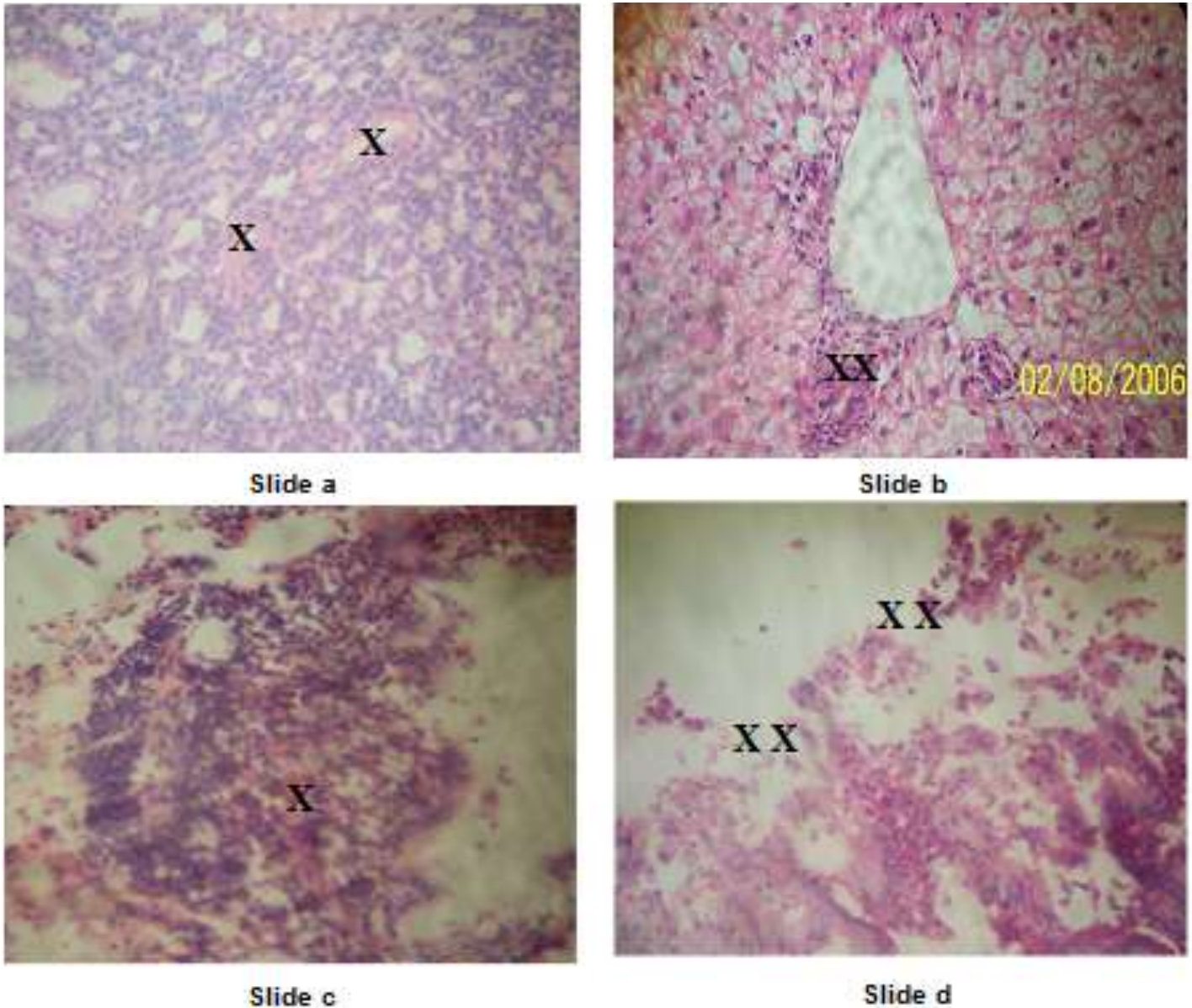
Figure 2. Histopathological studies. Slide a) Kidney: Cellular infiltration and oedema of the renal tubules of the medulla, with congestion, marked engorgement of renal blood vessels with red blood cells. *(Hematoxylin-Eosin, x40)* b) Liver: Generalized necrosis, perivascular cellular infiltration and hepatic oedema with fluid in the sinusoid. *(Hematoxylin-Eosin, x40)* c) Lung: Peribronchial lymphocytic infiltration of blood into the alveoli of the lungs. (*Hematoxylin-Eosin, x40*) d) Small Intestine: Erosion of the Villi with lymphocytic infiltration. *(Hematoxylin-Eosin, x40).* X - Lesion to tissues. X - Mild Lesion and XX – Severe Lesions.

been reported in Wistar rats by Ajagbonna et al. (1999) and could rather be attributed to the presence of some nutrients, making the extract palatable. This however opposes the weight loss reported in Wistar rats by Dada et al. (2002).

It was therefore concluded that the crude extract of *C. procera* affects organs when administered to rabbits and depends on the duration for which it is consumed. The results of sub-chronic toxicity studies could improve, since this might allow enough time for significant damage on the liver and other organs, thereby ascertaining the extent of damage and paving the way for possible conclusions of the pharmaceutical prospects.

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