

Histopathological effects of Averno[®], an indigenous herbal formulation, on Cyclophosphamide - induced immunosuppressed male rats

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Abstract

Introduction: The study investigated the effect of an acclaimed immunoboosting herbal formulation, Averno[®], which contains *Aloe spp* on some basal physiological and pathophysiological profiles in immunomodulated male rats. **Materials and Methods:** Six groups of male rats were used for the study: control group received water and pelletized food *ad libitum*, Negative control Cyclophosphamide-treated group (30mg/kg i.p), two groups pre-treated with Cyclophosphamide (30mg/kg i.p) and followed by oral 200mg/kg and 400 mg/kg Averno[®] respectively, two groups orally pre-treated with 200mg/kg and 400 mg/kg Averno[®] respectively and followed by Cyclophosphamide (30mg/kg i.p) on the last three days. The experimental design was characterized by observations for behavioral changes in the rats, changes in body weight, food consumption, water intake and gross histopathological changes after sacrifice. **Results:** The results revealed the adverse effects in the cyclophosphamide pre-treated groups in the behavioral pattern, significant decrease in body weights at several *p-values*, significant decrease in food intake ($p < 0.05$), significant drop in water consumption and toxicological effects on the studied organs. The reverse was true for the Averno[®]-pretreated groups: significant increase in body weights, food intake and water consumption. The adverse effect of cyclophosphamide was most pronounced on the lungs with little or no amelioration of the adverse effects on the pulmonary milieu. However, there was remarkable recovery on the livers, kidneys and the hearts on treatment with Averno[®], particularly at the higher doses. **Conclusion:** Averno[®] showed tissue ameliorating potential except on the lungs, hence the need for therapeutic monitoring when administered to immunosuppressed subjects.

Keywords: Averno[®], Cyclophosphamide, body weight, food consumption, histological.

1 Introduction

The global demand for herbal medicinal products has significantly increased recently. The WHO fact sheet (WORLD..., 2012) reported an estimation that the world's population will exceed 7.5 billion in the next 10 to 15 years and the rise in population will occur mostly in the southern hemisphere, where approximately 80% of the population still relies on a traditional medicine based on herbal drugs for primary healthcare.

The immune system is one of the most complex biological systems in the body. The basic role of the immune system is to distinguish self from non-self (PATCHEN, D'ALESANDRO, BROOK et al., 1987). Immunomodulators, also known as immunoregulators, are categorized as immuno-suppressants and immunostimulants. Cyclophosphamide (CYP) is an oxazaphosphorine class of alkylating agent widely used in cancer chemotherapy (SCHMIDT and KOELBL, 2012). CYP is an anticancer and immunosuppressive agent with a very narrow therapeutic index, undergoing a complicated process of metabolic activation and inactivation (DE JONGE, HUITEMA, RODENHUIS et al., 2005; EKHART, GEBRETTENSAE,

ROSING et al., 2007). CYP is mainly used with other chemotherapeutic agents in the treatment of lymphomas, some of brain cancer, leukemia, and some solid tumors (YOUNG, WHISELL, NOBLE et al., 2006; SHANAFELT, LIN, GEYER et al., 2007). An earlier study has shown that biological actions of cyclophosphamide are dose-dependent (NICOLINI, MANCINI, FERRERI et al., 2004). CYP has been reported to show pancytopenia, hemorrhagic cystitis, graft versus-host disease syndrome, nausea and vomiting, cardiac toxicity and electrolyte disturbances as adverse effects (GOODMAN and GILMAN, 2008; MYTHILI and NAIR, 2004).

A survey by Bush, Rayburn, Holloway et al. (2007) reported that potential adverse drug-herb interactions were observed in 40 out of 100 patients receiving conventional therapy and taking an herbal product.

Averno[®], an indigenous herbal formulation indicated for immuno-suppression, contains Aloe vera and has enjoyed a great deal of use in Nigeria, West Africa. Many inhabitants co-administer this formulation with other drugs such as antibiotics, antimalarials and analgesics. Our earlier work (UKPO,

EHIANETA, ADEGOKE et al., 2013) on the formulation based on haematological and biochemical effects of Averon® on cyclophosphamide-induced immuno-suppression, showed the herbal formulation to be better suited for immunoprophylaxis than immunostimulatory. The indiscriminate co-administration of conventional drugs with herbals had informed this study in the light of specific endpoints of toxicity.

The data generated from this study will serve as a template for preclinical dossiers required by the National Agency for Food, Drug Administration and Control (NAFDAC) and World Health Organization (WHO) and in natural product development programmes prior to clinical trials in humans.

2 Materials and Methods

2.1 Experimental animals

Male Wistar rats weighing 125 ± 25 g were obtained from the Laboratory Animal Center of the College of Medicine, University of Lagos, Nigeria. The animals were kept in well-ventilated and hygienic compartments, maintained under standard environmental conditions and fed with standard rodent pellet (Livestock Feed PLC, Lagos, Nigeria) and water *ad libitum*. Animals were housed under standard conditions of ($25 \pm 5^\circ\text{C}$), 12:12hr light and dark cycle and humidity ($55 \pm 10\%$). The animals were cared for and used in accordance with the Institute of Laboratory Animal Research (ILAR) guidelines for care use of animals in experimental studies (NATIONAL..., 1996).

2.2 Test formulation and drugs

Averon® (Batch Number: AV002DG; Manufacture date: December 2011; Expiry date: December 2012; manufactured by the Pax Herbal Clinic and Research Laboratories Ltd. Benedictine Monastery, off Benin-Auchi Road, Ewu-Esan, Edo State, Nigeria) was the test product for the experiment. The formulation was labelled to contain Aloe species and acclaimed to be an immunobooster. Cyclophosphamide (Cycram®; Korea United Pharm. Inc. Chungnam, Korea) was used to induce immuno-suppression. Cycram® is labelled to contain 500 mg cyclophosphamide to be dissolved in 20 mL of water for injection according to the manufacturer's instructions.

2.3 Preparation of test formulation and drug

Averon®, a liquid formulation, was filtered with white cloth and the filtrate oven-dried at controlled temperatures of 40°C , until completely dried and the residue re-constituted for experimental use with distilled water. The immunosuppressant, Cyclophosphamide, was reconstituted with water for injection to obtain a working concentration of 25 mg/mL.

2.4 Acute toxicity studies

The acute toxicity study was conducted in line with the OECD guidelines. Five male Swiss albino rats were fasted overnight, followed by administration of a single bolus dose (2000 mg/kg) per oral of Averon® and then observed over 14 days for mortality and physical/behavioural changes. The animals did not show any mortality at the dose of 2000 mg/kg and hence its 1/10th dose, that is, 200 mg/kg and 1/5th dose, that is, 400 mg/kg were used as the therapeutic doses of the Averon® formulation to represent low and high doses respectively.

2.5 Experimental design

All animals were acclimatized for 10 days and were divided into six groups, each consisting of eight animals. Group I served as the negative control while Group II (positive control group) was treated with cyclophosphamide, 30 mg/kg intraperitoneally on days 19-21. Groups III and IV were treated with cyclophosphamide, 30 mg/kg i.p. on days 1-3, followed by oral administration of Averon® 200 mg/kg and 400 mg/kg respectively on days 4-21. Groups V and VI were administered Averon® 200 mg/kg and 400 mg/kg orally respectively for days 1-21, followed by cyclophosphamide (30 mg/kg i.p) on days 19-21, one hour after the administration of the respective oral treatment.

Throughout the study period, each group was provided with 120 g of pelletized animal feeds and 300 mL of distilled water daily. The weight change per week, food intake and water consumed per day were noted appropriately.

2.6 Immunosuppressant dose determination

A preliminary study was carried out to determine the dose of Cyclophosphamide that can exert immunosuppression without causing mortality in the rats. Three groups of 5 animals each were selected. Group 1 was administered 50 mg/kg intraperitoneally (i.p) while Group 2 received 30 mg/kg (i.p) and Group 3 was administered 10 mg/kg (i.p). The animals were observed for a period of 7 days and haematological parameters were checked to choose the appropriate dose for the study.

2.7 Gross necropsy and histopathology

At end of the experimental period, the animals were fasted overnight (water allowed), anaesthetized, weighed and sacrificed. Four organs were collected from each representative animal and preserved in 10% buffered neutral formalin for further processing for histopathology. Histopathological evaluation of lungs, liver, kidneys and hearts were performed. This process involved preparing tissues for microtomy; they were further treated with several reagents to process the tissues for routine paraffin wax embedding. Sections of the embedded tissues were made at $5\mu\text{m}$ and stained with Haematoxylin and Eosin (H and E) (MBAKA and OWOLABI, 2011). The stained tissues were examined under light microscope at high power magnification for changes in organ architecture and photomicrographs were taken.

2.8 Statistical analysis

Data were analysed with a statistical software (Graph pad Prism 5) and values were expressed as Mean \pm SEM and differences between the groups were statistically determined by analysis of variance (ANOVA) followed by Dunnett's test. Statistically significant levels were considered at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

3 Results

Table 1 showed the body weight (bwt) progression pattern throughout the experimental period. There was a 22.8% increase in body weight in Group I which was steady over the experimental period, consequent to food and water intake only. Group II showed a 22.0% increase in body weight that decreased by the third week. The highest appreciation in weight was noticed in groups VI (58.9%) and V (53.6%) which demonstrated the ability of Averon® to increase body

weight even after the administration of cyclophosphamide. Statistically, the weight increase was significant ($p<0.001$) in group VI while decreasing markedly ($p<0.001$) in groups III and IV. This decrease is possibly due to the effect of cyclophosphamide as is evidenced by the weight-increasing effect of Averon® observed in Groups III (2.3%) and IV (4.8%), although seemingly minimal following prior administration of cyclophosphamide. In all, there were significant changes in weight in Groups III and IV throughout the experimental period. Our study therefore showed the weight-increasing effect of Averon® to be dose-dependent.

The effect of Averon® on the food consumption pattern on the cyclophosphamide-treated animals is shown in Table 2. Within the first 2 weeks, Groups I, II, V and VI which were not treated with cyclophosphamide showed a 100% consumption of the animals feed. However, on administration of cyclophosphamide, the food consumption decreased variably by 38.1% (II), 22.8% (III), 10.9% (IV), and 5.9% (V) and 10.8% (VI) as against 0% in the negative control group. The food consumption pattern and appetite stimulation on administration of Averon® and cyclophosphamide in this study can be trended as: $I > V > VI \geq IV > III > II$. This also indicates no statistical significant difference between groups V and VI, which is in effect due to the prophylactic administration of Averon® (Table 2).

Figure 1 showed the effect of Averon® on water consumption pattern. In similar manner like the food consumption, cyclophosphamide was observed to have affected the rate of water consumption by decreasing the water intake of the affected animals. The animals in Groups II and III had the lowest water intake over the 3 weeks study period, averaging 130.72 ± 10.16 mL and 118.57 ± 7.94 mL respectively. The decrease was most likely as a result of the cyclophosphamide effect. Animals in Groups V and VI recorded the highest volume of water consumption with the quantity consumed comparable between the two groups. Of the 300 mL of water provided daily, the average daily water intake was 268.9 ± 11.11 mL and 267.87 ± 8.47 mL for Groups V and VI respectively.

The photomicrograph of normal renal tissue (Figure 2) showed the cortical area in which were the renal corpuscles formed by a dense rounded mass of glomeruli separated from surrounding structures by Bowman's spaces. The convoluted tubules were also well defined separated by normal interstitium. The photomicrograph of negative control (Figure 3) treated with only cyclophosphamide, showed infiltration and distension of the portal tract with mixed inflammatory cells while the convoluted tubules showed normal appearance. In Figure 4, diffuse interstitial inflammation was observed after treatment with Averon® at 200mg/kg bwt. In Figures 5, 6 and 7, pathological changes were not observed both at the renal corpuscles and at the interstitium,

The normal lung tissue (Figure 8) showed the alveolar ducts and sacs surrounded by the interstitium with ill-defined interstitial capillaries. In Figure 9, the animals fed with cyclophosphamide showed severe acute pulmonary congestion with the alveolar air sac filled with red blood cells and edema fluid while those treated with Averon® at 200 mg/kg bwt after the administration of cyclophosphamide (Figure 10) showed severe cellular inflammation and pulmonary congestion. In Figure 11, after treatment with Averon® at 400 mg/kg bwt, pulmonary necrosis/pneumonia was observed. Similarly in Figure 12 there was also pulmonary congestion with alveolar air sac filled with red blood cells. In Figure 13 the lung tissue was devoid of pathological changes.

The photomicrograph of normal hepatic tissue (Figure 14) showed the portal tracts with normal cords of hepatic cells demarcated by unremarkable hepatic sinusoids. The treatment with cyclophosphamide (Figure 15) resulted in marked sinusoidal congestion and oedema. Also there was focal accumulation of neutrophils, plasma cells and lymphocytes within the hepatic parenchyma. In Figure 16, there was marked hydropic or vacuolar degeneration and sinusoidal congestion. In Figures 17, 18 and 19, no pathological changes occurred, also indicating that the herbal formulation had histo-protective

Table 1. Effect of Averon® on body weight progression of rats in the experimental groups.

Animal group (n=6)	Pre-dose weight	Week 1 (g)	Week 2 (g)	Week 3 (g)
I	142.5 ± 4.79	152.5 ± 4.79	172.5 ± 4.79	175.0 ± 6.46
II	147.5 ± 2.50	160.0 ± 0.00	180.0 ± 4.08	155.0 ± 6.46
III	107.5 ± 2.50***	102.5 ± 6.29***	102.5 ± 11.09***	110.0 ± 0.00***
IV	105.0 ± 1.89***	100.0 ± 0.00***	110.0 ± 7.07***	110.0 ± 0.00***
V	105.0 ± 1.89***	118.8 ± 1.25***	137.5 ± 1.64**	161.3 ± 2.27
VI	131.3 ± 3.98	157.5 ± 3.66	184.3 ± 3.69	208.6 ± 2.61***

Values are expressed as Mean ± SEM, One Way ANOVA followed by Dunnet's Test. **= $p<0.01$; ***= $p<0.001$. Treated groups were compared with negative control group.

Table 2. Effect of Averon® on average food consumption pattern in the experimental groups.

Animal group (n=6)	Week 1 (g)	Week 2 (g)	Week 3 (g)
I	120.0 ± 0.00	120.0 ± 0.00	120.0 ± 0.00
II	120.0 ± 0.00	120.0 ± 0.00	74.29 ± 21.59**
III	72.14 ± 17.45**	61.43 ± 8.85***	55.71 ± 3.69***
IV	65.71 ± 13.43***	48.57 ± 4.59***	58.57 ± 3.40***
V	120.0 ± 0.00	120.0 ± 0.00	112.9 ± 4.74
VI	120.0 ± 0.00	120.0 ± 0.00	107.0 ± 8.37

Values are expressed as Mean ± SEM, One Way ANOVA followed by Dunnet's Test. **= $p<0.01$; ***= $p<0.001$. Treated groups were compared with negative control group.

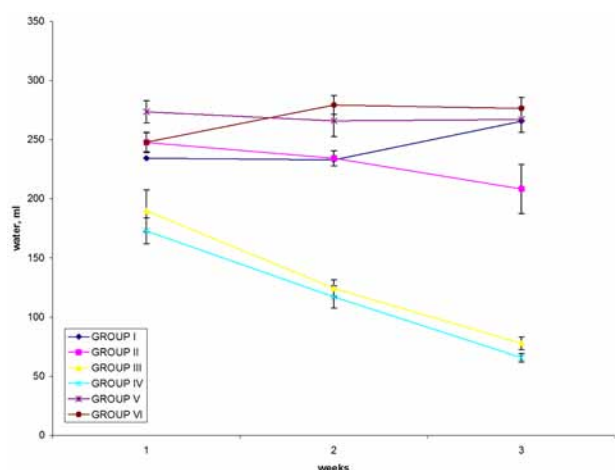


Figure 1. A plot of water consumption pattern of the experimental groups.

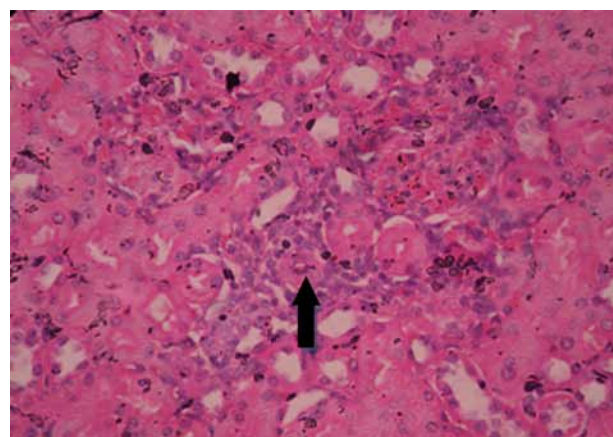


Figure 4. Photomicrograph of a cross section of cortical region of the renal tissue administered with cyclophosphamide & Averaon® (200mg/kg) indicated diffused (black arrow) interstitial inflammation. (H&E stain) Mag. X400.

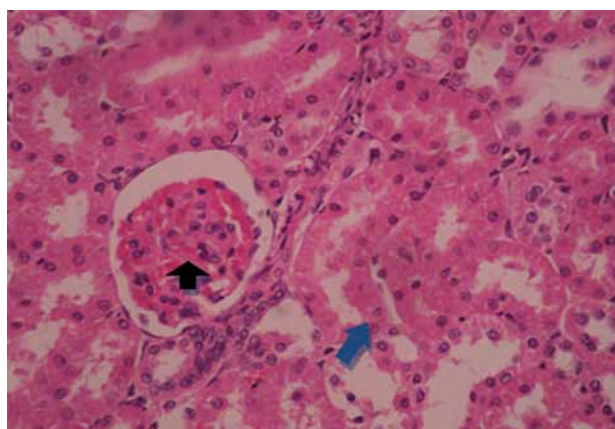


Figure 2. Photomicrograph of a cross section of cortical region of the renal tissue of the control indicating renal corpuscles (black) and convoluted tubules (blue). (H&E stain) Mag. X400.

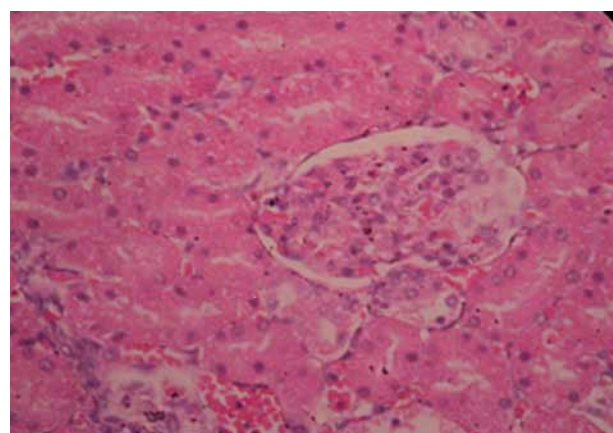


Figure 5. Photomicrograph of a cross section of cortical region of the renal tissue administered with cyclophosphamide & Averaon® (400mg/kg) indicated normal appearance. (H&E stain) Mag. X400.

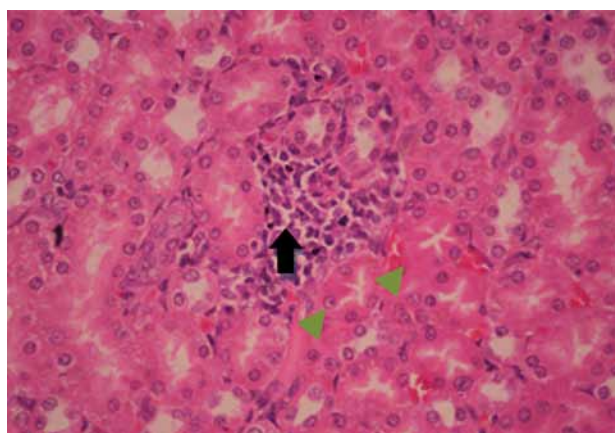


Figure 3. Histological section of the kidney from a rat fed with cyclophosphamide showed a portal tract (thick arrow) that was infiltrated and distended by mixed inflammatory cells. The tubules (arrowhead) are unremarkable. (H&E stain) Mag. X400.

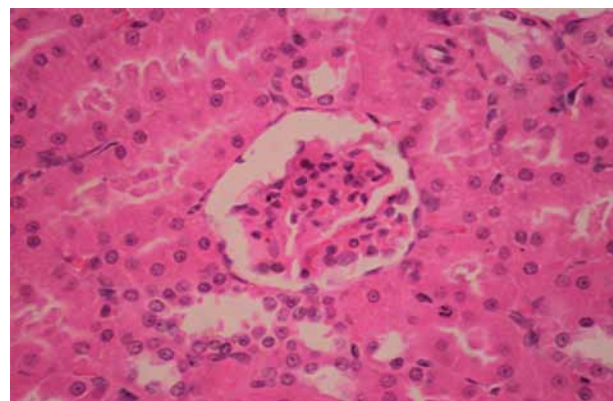


Figure 6. Photomicrograph of a cross section of cortical region of the renal tissue administered with Averaon® (200mg/kg) followed with cyclophosphamide. No lesion observed. (H&E stain) Mag. X400.

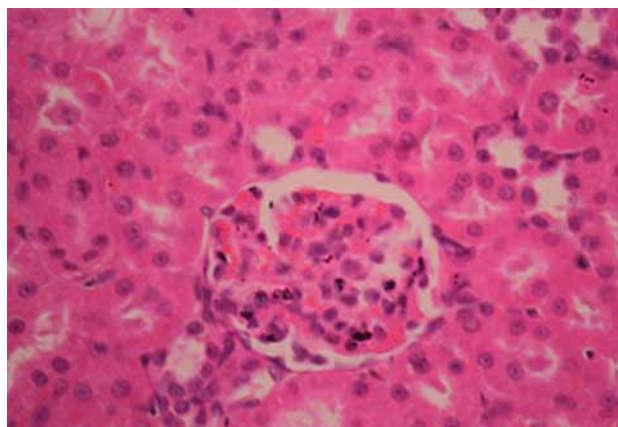


Figure 7. Photomicrograph of a cross section of cortical region of the renal tissue administered with Averon® (400mg/kg) followed with cyclophosphamide. No pathological changes occurred. (H&E stain) Mag. X400.

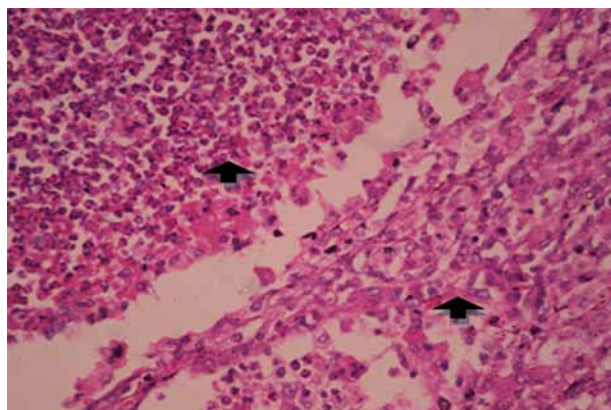


Figure 10. Photomicrograph of a cross section of lung tissue of a rat administered with cyclophosphamide & Averon® (200mg/kg) which showed severe cellular inflammation and pulmonary congestion. Mag. X400.

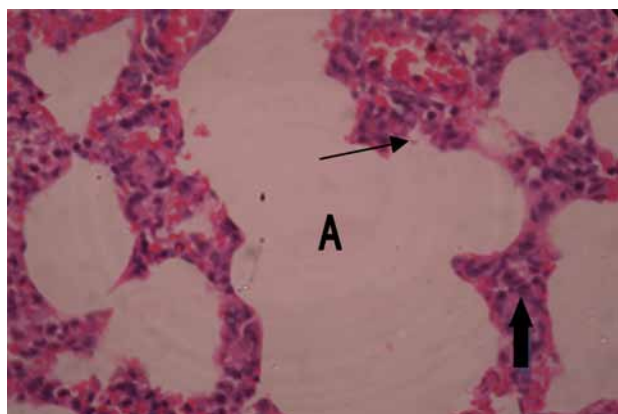


Figure 8. Photomicrograph of a cross section of lung tissue showed alveolar air spaces A, surrounded by normal interstitium (thick arrow). Few interstitial capillaries (thin arrow) are seen. Mag. X400.

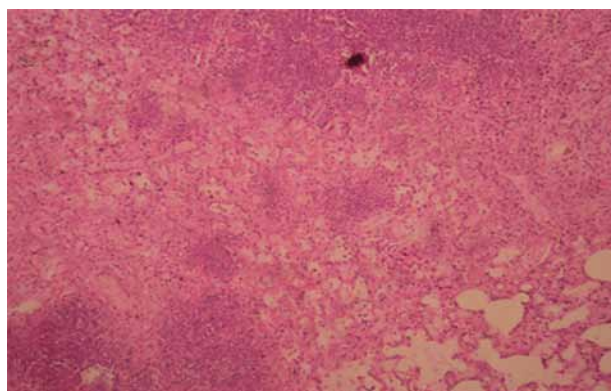


Figure 11. Photomicrograph of a cross section of lung tissue of a rat administered with cyclophosphamide & Averon® (400mg/kg) which indicated pulmonary necrosis/pneumonia. Mag. X400.

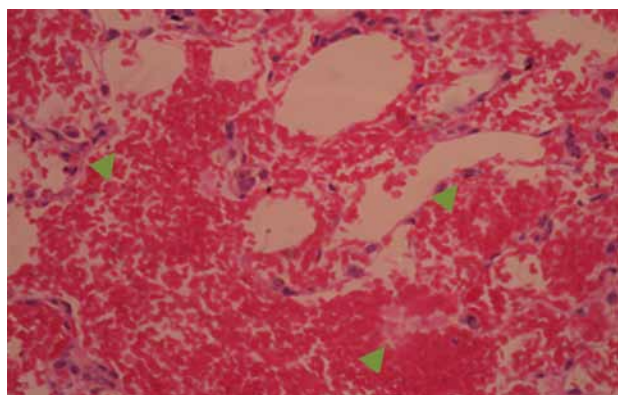


Figure 9. Photomicrograph of a cross section of lung tissue of a rat fed with cyclophosphamide showed alveolar air spaces (green arrow head) filled with red blood cells and edema fluid. Mag. X400.

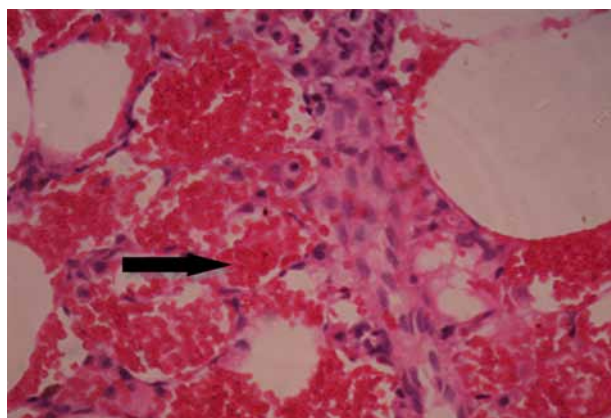


Figure 12. Photomicrograph of a cross section of lung tissue administered with Averon® (200mg/kg) followed with cyclophosphamide showed infiltration of red blood cells (arrowed) into the alveolar air sac. Mag. X400.

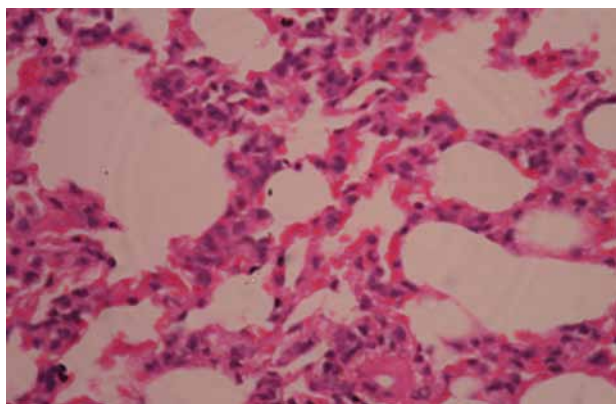


Figure 13. Photomicrograph of a cross section of lung tissue administered with Avera® (400mg/kg) followed with cyclophosphamide showed no lesion. Mag. X400.

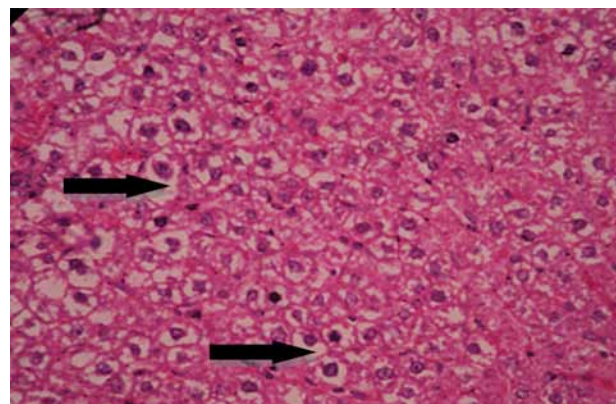


Figure 16. Photomicrograph of a cross section of hepatic tissue administered with cyclophosphamide & Avera® (200mg/kg) indicated severe hepatocytes vacuolization (black arrowed). (H&E stain) Mag. X400.

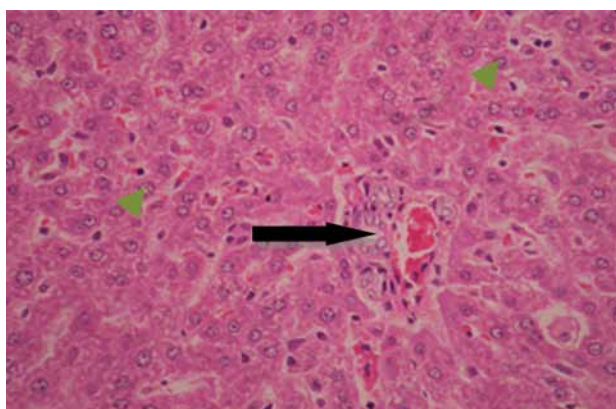


Figure 14. Photomicrograph of a cross section of hepatic tissue of the control showing portal tract (thick arrowed) and normal hepatocytes (arrow head) separated by ill-defined sinusoids (thin arrow). (H&E stain) Mag. X400.

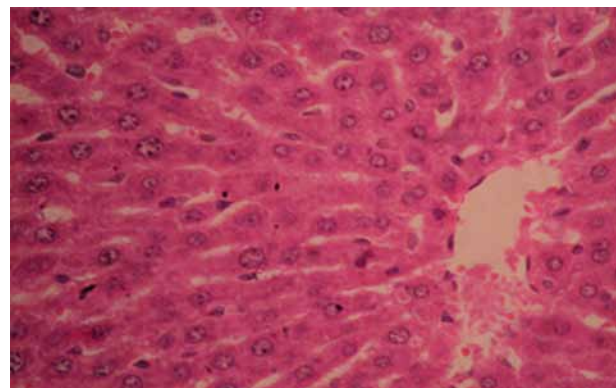


Figure 17. Photomicrograph of a cross section of hepatic tissue administered with cyclophosphamide & Avera® (400mg/kg) indicated no lesion. (H&E stain) Mag. X400.

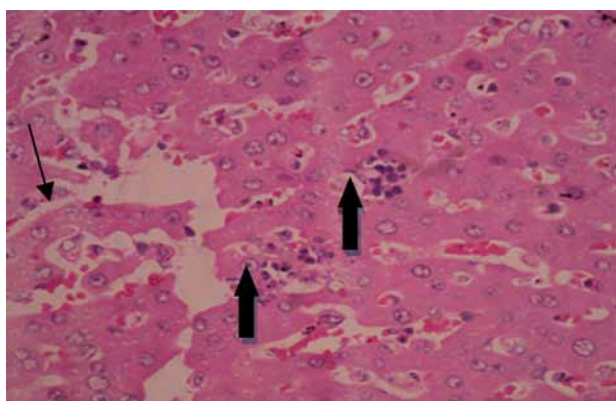


Figure 15. Photomicrograph of a cross section of hepatic tissue fed with cyclophosphamide showed marked sinusoidal (thin arrowed) congestion and oedema, and focal accumulation of neutrophils, plasma cells and lymphocytes (thick arrowed). (H&E stain) Mag. X400.

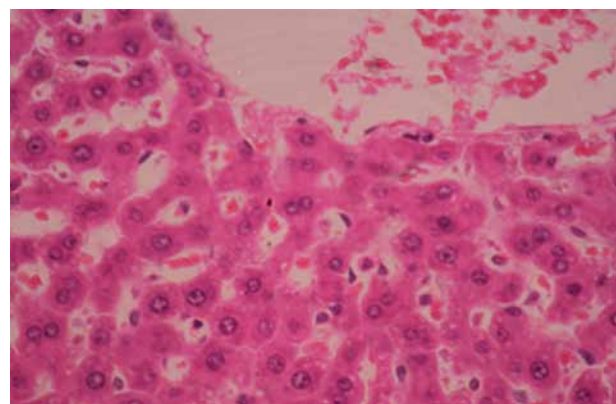


Figure 18. Photomicrograph of a cross section of hepatic tissue administered with Avera® (200mg/kg) followed with cyclophosphamide showed no lesion. Mag. X400.

effect on hepatocytes which was strongly indicative of the histo-protective effect of Averon® on the hepatocytes.

The photomicrograph of normal cardiac tissue (Figure 20) indicated a cross section of myocardium. The nuclei (myocytes) appeared deeply stained in the interstitium while the intercalated disc was unmarked. In Figure 21 there was focal accumulation of lymphocyte and eosinophils within the interstitium. In Figures 22 and 23 there were mild interstitial congestion. In Figures 24 and 25, no abnormal changes were observed. This again showed the histo - protective potential of Averon® on the cardiac tissues.

4 Discussion

The result of the pilot study on the dose of cyclophosphamide appropriate for the study is at variance with the 50 mg/Kg cyclophosphamide used in the study by Ahmad, Rashid, Bhatia et al. (2012). All animals survived the experimental period. However, cyclophosphamide-treated animals showed general signs of deterioration such as writhing reflex, hunched posture, pilo-erection, hair loss, lethargy, shivers and reduced activity. The decrease observed in body weight can be attributed to the administered cyclophosphamide following the comparable

weight increase in both Groups I and II. This was indicative of the weight-depreciating effect of cyclophosphamide in the absence of a chemoprotective agent. The increase in volume of water intake by Groups V and VI infer the thirst stimulating effect of Averon®

The clinical signs as typified by reduced activity are reflected in the significant decrease in the average food consumption pattern in Groups III and IV. Studies have shown that appetite regulation is an immensely complex process involving the gastrointestinal tract, hormones, the central and autonomic nervous systems (WYNNE, STANLEY, MCGOWAN et al., 2005). The appetite-stimulating effect of Averon® might not be conclusively dose-dependent as it did not indicate any significant statistical difference in the prophylactic administration (V and VI) between 200 and 400 mg/kg of the herbal formulation.

Cyclophosphamide, an immunosuppressant agent (SCHMIDT and KOELBL, 2012) exerted varied pathological effects on the vital organs of the experimental animals which corroborated with some of its toxic effect earlier reported (GOODMAN and GILMAN, 2008; MYTHILI and NAIR, 2004).

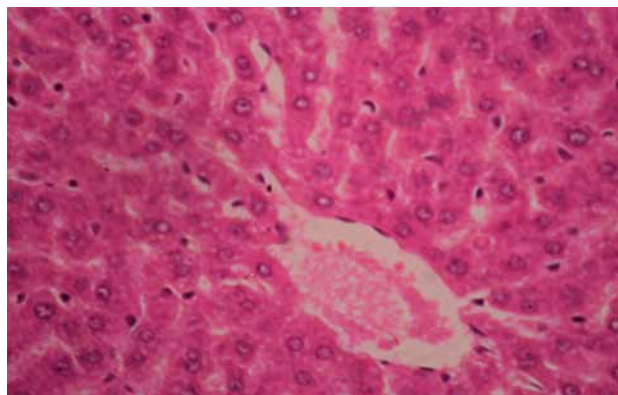


Figure 19. Photomicrograph of a cross section of hepatic tissue administered with Averon® (400mg/kg) followed with cyclophosphamide showed no pathological changes .Mag. X400.

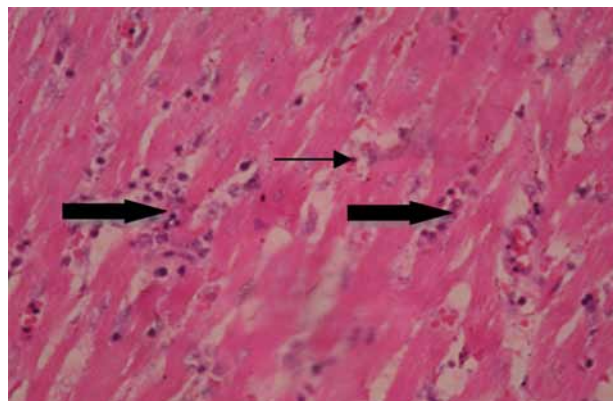


Figure 21. Photomicrograph of a cross section of heart showing focal accumulation of lymphocytes (thick arrowed) and eosinophils (thin arrowed) within the interstitium. (H&E stain) Mag. X400.

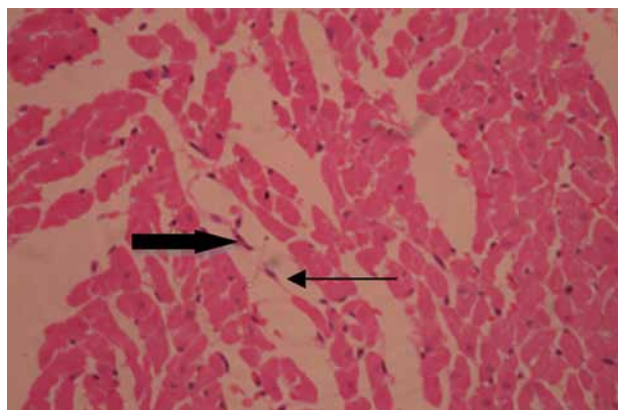


Figure 20. Photomicrograph of a cross section of myocardium (heart) showing myocytes (thick arrowed) separated by an unremarkable interstitium (thin arrow). (H&E stain) Mag. X400.

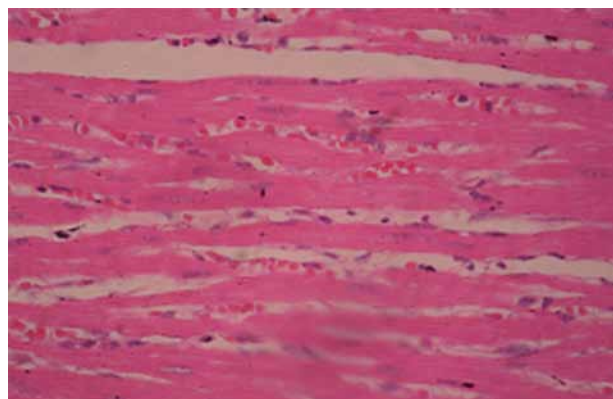


Figure 22. Photomicrograph of a cross section of heart showing mild congestion within the interstitium. (H&E stain) Mag X400.

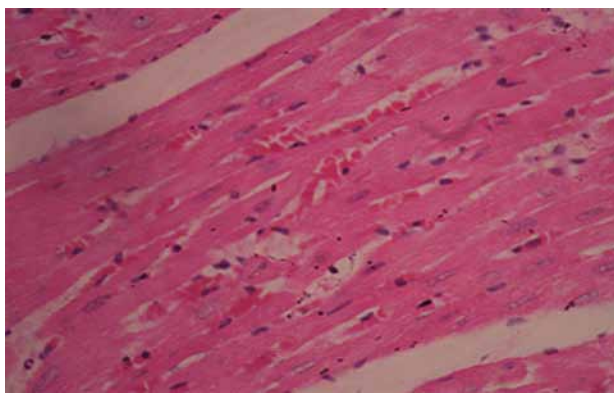


Figure 23. Photomicrograph of a cross section of heart showing moderate congestion in the interstitium. (H&E stain) Mag. X400.

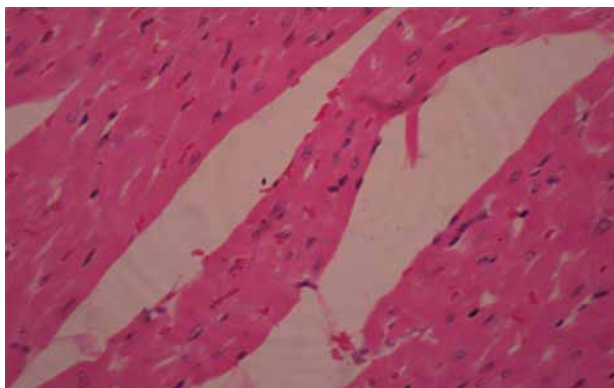


Figure 24. Photomicrograph of a cross section of myocardium showing no pathological changes. (H&E stain) Mag. X400.

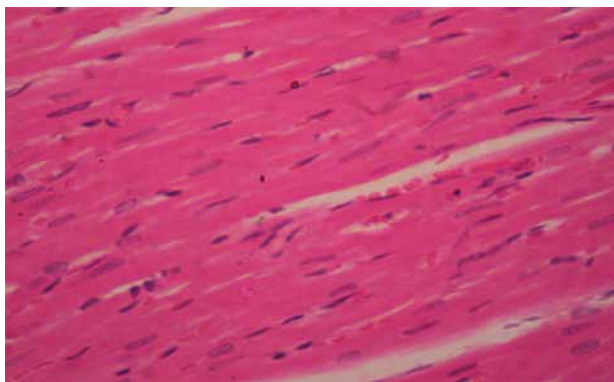


Figure 25. Photomicrograph of a cross section of myocardium showing no lesion. (H&E stain) Mag. X400.

In Figure 2, it was obvious that the prophylactic treatment (Groups V & VI) exhibited histoprotective effect on the renal tissues when compared with the CYP treatment alone (Group II). Also, the highest dose of the herbal formulation administered after pre-treatment with CYP significantly attenuated cyto-toxic effect of the immunosuppressive agent on the kidney. Pulmonary congestion resulting from the cyclophosphamide was countered by the prophylactic administration of Averon® at 400 mg/kg. This was indicative of the histo-protective potential of the higher dose of Averon® on the pulmonary

tissues. The photomicrographs in Figures 8-13, showed the erythrocyte infiltration into the lungs as well as pulmonary necrosis and pneumonia.

The effect of the chemical compound, cyclophosphamide was most profound in the lungs such that Averon®, an herbal formulation indicated for immunosuppression could not significantly ameliorate the adverse effects on the pulmonary milieu. However, there was remarkable recovery in the liver, kidney and the heart after treatment with the Averon®, particularly at the higher doses. From the tissue histology, Averon® showed to be better suited for histo-prophylaxis since it effectively protected the organs against the harmful effect of the cyclophosphamide except in the lungs. Our results again agree with those of an earlier work by Ukpo, Ehianeta, Adegoke et al. (2013), supporting Averon® to be better suited as prophylactic herbal formulation.

5 Conclusions

The herbal formulation improved the feeding habit as well as water consumption of the animals which consequently led to weight gain. Although Averon® ameliorated the toxic effect of cyclophosphamide, it was however better suited for histo-prophylaxis than healing activity. There was remarkable recovery of the liver, kidney and heart tissues after treatment with the herbal formulation, particularly at the higher doses, however, the effect of cyclophosphamide was most pronounced on the lungs such that Averon® could not significantly ameliorate the adverse effects on the pulmonary milieu.

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