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The Antitumor Properties of a Metal-free Ligand and Local Herbal Remedy against N-methyl-N-nitrosourea (MNU)-induced Carcinomas in Albino Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Author AA Osowole initiated the study and provided funds via the research grant. Author AA Oni managed the experimental, draft writing, and corrections of the manuscript. Author POP carried out the experimental work and prepared first draft. Author KCO carried out the histology, while author ATH conceptualized and supervised the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: We report a pilot study evaluating the antitumor properties of a Schiff base ligand, 3-[2,4-dihydro-1H-inden-4-yl imino)methyl] napthalen-2-ol] and aqueous herbal extract against N-methyl-N-Nitrosourea (MNU)-induced carcinomas in albino mice via hematology; and histology of the liver, thymus, spleen and small intestine.
Study Design: Twenty-three male and female mice respectively received a single intra-peritoneal dose of 60 mg/kg MNU. Seven males and seven females served as controls.

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Place and Duration of Study: Department of Zoology, University of Ibadan, Ibadan, Nigeria; January-July 2012.
Methodology: Five months post-induction, four induced males and four females of surviving mice were given a single intra-peritoneal dose (25 mg/kg) of ligand; three induced males and three females served as negative control. Three induced mice of both sexes respectively received daily doses of 136 mg/mL extract orally at an innoculation volume of 0.3 mL/mouse for 30 days (total dosage= 4.08 g/30 ml;) and monitored for two months. Depending on mortality, 1-3 mice/group were sacrificed at experiment termination and subsequently processed for hematology and histology.
Results: Tumor incidence was 13% (male) and 17% (female) respectively. There was no significant difference (*P*= .05) between hematology of control and statistically comparable experimental groups. Histology of control organs showed no abnormal features. Hepatocytes of ligand-treated mice showed cytoplasmic disintegration and presence of pleomorphic nuclei; while the extract-treated group showed progressive reversal towards normal with hepatocytic regeneration, fairly uniformly-shaped nuclei and bi-nucleate cells. Spleen of the ligand-treated group showed moderate lymphoid depletion, while the extract-treated group showed a regeneration of the central artery and red pulp with no visible lesion. The extract-treated thymus showed a gradual reversal of some of its Hassall's corpuscles. The small intestine of the ligand-treated mice showed gross erosion of the intestinal villi; while intestinal mucosa regeneration was observed in the extract-treated group.
Conclusion: The herbal extract may possess some antitumor properties, thus necessitating further investigations, while the ligand showed little or no activity *in vivo.*

Keywords: N-methyl-N-nitrosourea; albino mice; carcinomas; anti-tumor properties; ligand; herbal remedy.

1. INTRODUCTION

Cancer cells arise from the accumulation of many small alterations or mutations in the DNA of normal human and animal cells [1]. Chemotherapy is considered the most effective method of cancer treatment [2]. A number of natural and synthetic products have been experimented in scientific research in order to find potent anti-cancer agents. An ideal anticancer agent should incapacitate cancer cells without causing excessive damage to normal host cells. Unfortunately, currently available cancer chemotherapeutic agents subtly affect the host cells, especially the bone marrow, epithelial tissues and gonads [3].

Cis-platin, although considered effective in the treatment of a variety of cancers [4] has been reported to show a number of side effects, which include self-triggered drug / tumor resistance and the development of systemic toxicity. Furthermore, it is limited by its non- or poor oral bioavailability [5,6]. These drawbacks necessitated the need for an extensive search for other anti-tumor active inorganic complexes with improved pharmacological properties. Natural products have also been contemplated to be of exceptional value in the development of effective anticancer agents with minimum host cell toxicity [2]. Certain herbs used in folk and traditional medicine are considered as one of the main sources of cancer chemoprevention, drug discovery and development [7].

Results from earlier studies by our group on the *in vitro* antitumor activities of a Schiff base ligand (HL) and three metal complexes [(copper (II), zinc (II) and palladium (II) complexes], showed varying degrees of activity against colon carcinoma cells in comparison to the standard antitumor drug, cis-platin (CDDP) [8]. In order to designate a compound as an effective anti-cancer molecule, the *in vitro* and *in vivo* effects should be assessed and the results comparable [9]. Consequently, *in vivo* studies became necessary to evaluate the antitumor activity of the ligand and the metal complexes on carcinomas in albino mice.

This study therefore focused on the *in vivo* activity of the ligand (HL) 3-[2,4-dihydro-1H-inden-4-yl imino)methyl] napthalen-2-ol, on MNU-induced carcinomas. MNU is one of the carcinogens frequently used to induce cancers in experimental animals especially rats and mice, as it takes a relatively short time to induce carcinogenesis when compared to other carcinogens [10,11]. A single i.p dosage of 60 mg/kg MNU was used to induce cancers in the experimental mice. Previous work done by our group [12] had shown low tumor incidence

(2.5%) at the most commonly used dose of 50mg/kg MNU [13]. Hence, we chose a higher dosage of 60 mg/kg in this study. We also evaluated the efficacy of a locally used herbal remedy in cancer treatment (prepared from the powdered aqueous extract of four plant parts: *Anona senegalensis* leaves (sour sop), *Andrographic paniculata* leaves (king of bitters), *Curcuma sp.* (red ginger) and *Moringa oleifera* leaves on the treatment of MNU-induced carcinomas in albino mice. The hematological parameters and histology of the liver, thymus, spleen and small intestine were examined.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Sixty mice (thirty male and thirty female) of average weights of 25 and 21 g respectively, were obtained from the Department of Anatomy, University of Ibadan, Ibadan, Nigeria and used for the study. The mice were all six weeks old at the commencement of the study. Ten mice were placed into each of six iron cages underlain with wood shavings as beddings, and acclimatized for two weeks prior to the commencement of the experiment. They were maintained on 12 h light / 12 h dark cycle in a well ventilated room in the animal house of the Department of Zoology, University of Ibadan. They were fed with standard animal pellets and given drinking water *ad libitum.*

2.2 Chemical Carcinogen

The carcinogen N-methyl-N-nitrosourea (MNU) was purchased from Sigma Aldrich Chemicals Co. St Louise and used for tumour induction.

2.2.1 Preparation of carcinogen

A single intra-peritoneal (i.p) dosage of 60mg/kg of MNU was dissolved in 0.9% NaCl solution at a concentration of 10 mg MNU/ml of NaCl solution and given to the mice. Based on the average weights of 25.37 g and 21.23 g for male and female mice respectively, each male and female mouse thus received an i.p injection of 1.52 mg MNU in 0.152 saline solution, and 1.27 mg MNU in 0.127 ml saline solution respectively. These amounts were calculated based on the average weight of the mice as stated above and were equivalent to a dosage of 60 mg/kg MNU dissolved in NaCl solution at a concentration of 10mg MNU/ml of saline solution [13].

2.3 Preparation of Test Compounds

The Schiff base ligand, 3-[2,4-dihydro-1H-inden-4-yl imino)methyl] napthalen-2-ol, (Fig. 1) was dissolved in 0.3ml Di-methyl sulphur oxide (DMSO) at a dosage of 25 mg/kg, and made up to 100 ml with saline water. The herbal extract was prepared as follows: 3.4 g powdered herbal extract formulation of the four plants was dissolved in 25 mL distilled water. This was equivalent to a concentration of 136 mg/ml of herbal extract. The inoculation volume was an average of 0.3 ml per mouse of this concentration.

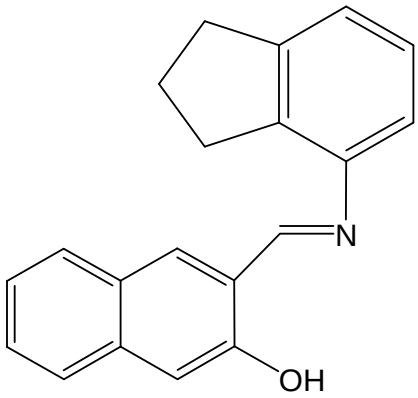

Fig. 1. Structure of the ligand, 3-[2,4-dihydro-1H-inden-4-yl imino)methyl]napthalen-2-ol

2.4 Experimental Design

2.4.1 Induction with the carcinogen

Twenty-three male and twenty-three female mice received a single dose of 60 mg/kg MNU via intra-peritoneal injection; while seven male and seven female mice served as controls. They were subsequently monitored for five months. During the last twelve weeks of the five month MNU induction period, just before the commencement of treatment, the mice were weighed weekly to note the changes in the weight of the induced male and female groups compared to their respective controls. The entire body of the mice was also palpated twice weekly over the entire five-month induction period to monitor tumour appearance. Tumor volumes were measured with slide callipers in accordance to the formula: $0.44 \times A \times B^2$, where A is the base diameter on the elongated side of the tumor and B is its perpendicular value [14].

2.4.2 Treatment with the ligand and herbal extract formulation

Five months after MNU induction, equal numbers of surviving male and female mice from MNU-induced groups were subdivided into three groups respectively (groups a-c below).

(a) Ligand treated animals comprising of four male and four female MNU induced mice respectively. Each mouse received a single dosage of 25 mg/kg metal free Schiff base ligand (HL) via intra-peritoneal injection at an inoculation volume of 0.3 ml/mouse. They were designated as ligand treated.

(b) Herbal extract treated animals comprising of three male and three female mice respectively. Each mouse was given 0.3ml of the herbal extract via oral gavage daily for a period of 30 days. They were designated as extract treated.

(c) Induced (no treatment or vehicle comprising three males and three females respectively).

The control group comprising of seven male and seven female mice was further subdivided into two (groups d-e below):

(d) One group of control were not induced with MNU nor given any placebo. This group was comprised of four males and four females respectively. They were simply designated as control.

(e) The last group comprising of three males and three females were administered 0.3 ml of vehicle only and designated as control vehicle.

Five weeks to the termination of the treatment period, male and female mice in the treatment groups (a-b) and controls (d-e) were monitored weekly to note the changes in their weight. At the end of the experimental period, male and female mice from each of the five groups (a-e) were sacrificed by cervical dislocation, dissected and processed for histological examination. Selected organs: liver, thymus, small intestine and spleen were removed and stored in 10% formal saline prior to histological examination. Blood samples were also taken from the retro-orbital sinus of the mice for haematological examination.

The number of mice sacrificed per group depended on the numbers of surviving male and female animals in each of the five groups at the end of the experimental period. Three male and three female mice were sacrificed from the [control male, control female (i.e. group d)], [induced male, induced female (i.e. group c)] and three males from the ligand-treated male group (i.e. group a).

Two mice were sacrificed from control (male with vehicle; i.e. group e). One male and one female mouse each was sacrificed from the extract-treated groups of both sexes (i.e. group b); and one mouse each from control (female with vehicle; i.e. group e) and ligand-treated female (i.e. group a).

2.5 Histo-pathological Analysis

The histo-pathological analysis was performed at the Department of Chemical Pathology, University of Ibadan using a Leica rotary microtome model 2125 RT, a Leica tissue embedding machine model 1160, a Raymond Lamb tissue floatation bath model E652, a hot plate model E18 and a Tissue-Tek II tissue processor model 4634. The prepared slides were viewed under an Olympus CX31 microscope attached to a HP pavilion computer monitor to provide identification record on the location and gross morphology of the lesions.

2.6 Haematological Parameter Analysis

Blood samples were taken from the retro-orbital sinus of the mice using heparinised 70 ml micro-hematocrit capillary tubes and stored in 0.5ml bottles containing ethylene diamine tetra acetic acid (EDTA). They were analysed for packed cell volume (PCV), red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb), platelet count, and lymphocyte; neutrophil, monocyte and eosinophil counts. WBC count was done by the method described by [15]; while RBC, PCV, and Hb counts were determined by the hemocytometer method [16]. These hematological parameters were analysed at the hematology section of the Department of Veterinary Medicine, University of Ibadan.

2.7 Statistical Analysis

Values of haematological parameters were expressed as Mean ± Standard Error of Mean (SEM). The data were analysed by one-way analysis of variance (ANOVA) using statistical package for social sciences (SPSS) and $P<0.05$ was considered significant. Mice with similar

numbers per group were subjected to statistical analysis (n=3). Exceptions were groups with only one surviving animal which could not be subjected to statistical analysis.

3. RESULTS

3.1 Mean Body Weight of the Various Experimental Groups of Albino Mice

The mean body weights of the control and induced groups of albino mice increased almost throughout the specified period of exposure, although the mean body weights (±SD) of the induced male (29.91±1.82 g) and induced female (24.19±0.43 g) dropped slightly at the eighth week (Fig. 2). However, in the subsequent period after treatment, there was no marked increase in the mean weights of the various experimental groups of albino mice (Figs. 3a-b). The mean weights of the ligand-treated male (34.28±0.53 g) was significantly higher ($p<0.05$) than the respective control male groups (30.48±0.77g); control (vehicle) (30.50±0.44 g) and higher but not significant at ($p<0.05$) than the induced male group (32.53±0.49 g). There was no significant difference ($p<0.05$) between the mean weight of the control female group (25.97±0.68 g) and that of the induced female group (23.75±0.63 g); but the mean weight of the ligand-treated group (28.41±0.55 g) was significantly higher ($p<0.05$) than the female control group only (25.97±0.68 g). Mortality occurred in extract treated animals immediately following oral administration of extract, hence only one animal was analysed for hematology. However, these results showed that the weight of the only surviving extract-treated male (36.10 g) was higher than the respective means of the control male (30.48±0.77 g) and induced male group (32.53±0.49 g); while the weight of the only surviving extract treated female (26.22 g) was also higher than that of the induced female group (23.75±0.63 g) and the female control groups (25.97±0.68 g). However, the mean weight of the female control (vehicle) group was highest at (27.30±0.53 g). Due to mortality in the extract treated group, values obtained were not amenable to statistical analysis.

3.2 Hematological Examination

The hematological parameters in this study were not significantly different at p=.05, as observed in virtually all the experimental groups. The exceptions were in the extract treated male and female group, as well as in the female ligand treated group, which had only one surviving animal at the end of the experimental period (Table 1). The results showed that there was a reduction in the packed cell volume, hemoglobin, red blood cell count, white blood cell count, platelets and lymphocytes in the induced male group compared with those of the control male group, but these differences were not significant at p= .05. There was also a reduction in the levels of packed cell volume, hemoglobin, white blood cell count, platelets and lymphocytes; and increased levels in the red blood cell count of the ligand-treated male group when compared with the control male group. These differences were however not significant at p=.05. Values obtained for the only surviving extracted treated male showed a similar pattern as the ligand treated male group except for the packed cell volume.

Packed cell volume, hemoglobin, red blood cell count and lymphocytes showed reduced levels in the induced female group when compared to the control female group. These differences were however not significant at p=.05. On the other hand, the white blood cell count and platelets in the induced female group showed elevated levels when compared with the control female group. However, this was again not significant at p=.05. Packed cell volume, hemoglobin, red blood cell count, platelets and lymphocytes in the only surviving ligand treated female showed much lower levels when compared with these values in the control female group. In addition, the white blood cell count of the ligand-treated female showed increased levels, when compared with the control female group. The red blood cell count, white blood cell count, and lymphocytes in the only surviving extract treated female showed reduced levels, when compared to the levels of these parameters in the control female group. On the other hand, packed cell volume and hemoglobin in the extract treated female were elevated in comparison with the control female group (Table 1).

3.3 Histopathology

Histological sections of liver, spleen, thymus and small intestine derived from the various experimental groups of albino mice appeared normal in all control groups as expected, while certain abnormalities were observed in the induced male and female groups respectively. The ligand-treated groups generally did not show much reversal of these abnormalities as well as similar results in the extract-treated groups were seen.

3.3.1 Histopathological examination of liver

The histological section of the control group (Plate 1) showed distinct normal hepatocytes and binucleate cells, while the control (vehicle) dimethylsulphoxide (DMSO)-treated group of male mice shows phagocytic activity in response to the vehicle (DMSO), thus trying to mop it up (Plate 2). Abnormalities observed in the histological section of the induced male group (Plate 3) include pleomorphic or irregularly shaped nuclei and cytoplasmic degeneration. The histological section of the ligand-treated male group (Plate 4) shows pleomorphic nuclei and cytoplasmic disruption. However, the extract-treated male group (Plate 5) showed progressive reversals towards the normal (Plate 1) with hepatocytic regeneration and binucleate cells which are common in liver cells.

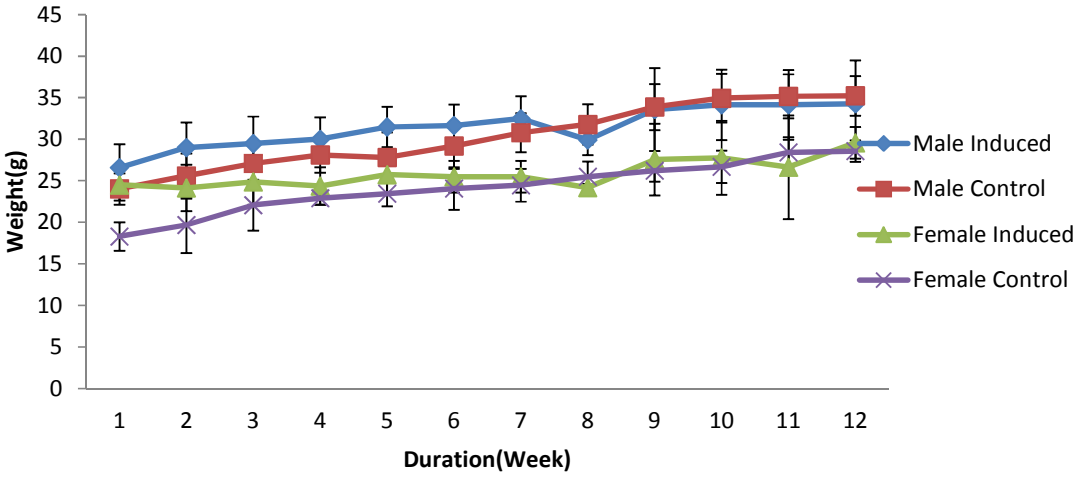

Fig. 2. The mean body weights of MNU-induced and control mice

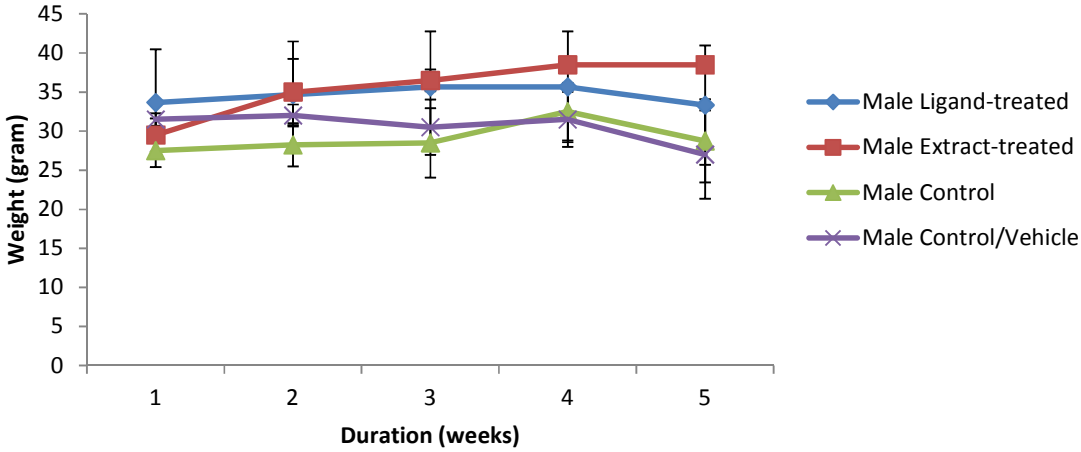

Fig. 3a. Mean body weights (Male) and standard deviation of various experimental groups of albino mice

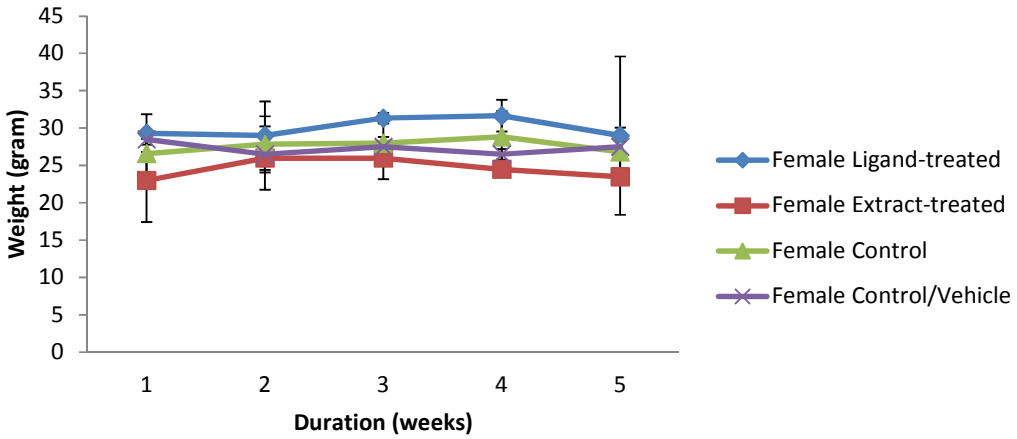

Fig. 3b. Mean body weights (Female) and standard deviation of various experimental groups of Albino Mice

3.3.2 Histopathological examination of thymus

The histological section of the control (plate 6) showed normal features of the thymus, particularly the hassall's corpuscles, while the DMSO-treated female control group (Plate 7) in addition to the hassall's corpuscles, also showed phagocytic activities as a result of the introduction of the vehicle (DMSO) which is seen as a foreign body. The histological section of the induced female group (Plate 8) shows a total distortion of the histology of the organ as the vital components such as the hassall's corpuscles and epithelial components are atrophied compared to the control (no vehicle) group (Plate 6). The extract-treated group (Plate 9) shows a gradual reversal towards normal, as traces of the hassall's corpuscles were observed along with some mild infiltration of phagocytic cells.

3.3.3 Histopathological examination of spleen

Plate 10 shows the normal histology of the control showing the red pulp. The histological section of the control/vehicle (DMSO-treated) female group (Plate 11) shows the red pulp and increased phagocytic activities due to the DMSO introduction. The histological section of the induced male group (Plate 12) shows a hyperplasia of the white pulp in the peri-arterial sheath, rupture of the central artery and a severe lymphoid depletion due to aggressive phagocytic activities. The section of the extract-treated male group shows a regeneration of the central artery and red pulp with no visible lesion, as well as phagocytic activities (Plate 13). The histological section of the ligand-treated male group shows a severe congestion at the red pulp and moderate lymphoid depletion with no distinct repair of the major parts of the spleen (Plate 14).

3.3.4 Histopathological examination of small intestine

Histological section of the control (Plate 15) showed moderate to severe erosion of the intestinal villi, while the induced female group (Plate 16) showed no visible lesion. The ligand-treated female (Plate 17) showed gross erosion of the intestinal villi. However, the small intestine of the extract-treated female had mildly stunted villi, mild secretion of mucinous glands, and its intestinal wall lining appears to be regenerating (Plate 18).

3.4 Tumor-bearing Mice

Tumors were observed in the induced male and female groups about the twentieth and twenty-fourth weeks after start of the study. It was estimated that 13% and 17.39% of the male and female induced groups had tumors respectively. Plate 19 shows the histological section of an induced female with a stomach tumor having hypochromatic and necrotic cells. There was also a tumor observed on the left hind limb of another induced male mouse which had a volume of 0.03 cm^3. An abdominal tumor of an induced female mouse had a volume of 0.27 cm^3, while another Induced female developed a tumor in the anal region. A tumor on the left thigh of an induced female mouse had a volume of 3.92 cm^3, while that observed on the right thigh of another ligand-treated female mouse was 4.65 cm^3.

Table 1. Hematological parameters of various experimental groups of albino mice

Exptal		PCV (%)	Hb (gm/dl)	RBC ($x10^6$/µl)	WBC ($x10^3$/µl)	Platelets ($x10^3$/µl)	Lympho. (%)	Neutrop. (%)	Monocyt. (%)	Eosinophils (%)
Male induced	Mean	32.33	11.40	7.20	14	110	42.66	48.33	0.33	0.33
n=3	SEM	1.20	0.31	0.36	2.72	10	2.18	2.91	0.33	0.33
	Minimum	30.00	10.80	6.48	8	100	40	43	0	0
	Maximum	34.00	11.80	7.64	18	130	47	53	1	1
Male control	Mean	39.00	12.93	9.55	14.8	113	53.00	47	0	0
n=3	SEM	6.65	2.25	2.58	2.16	17.63	9.07	9.07	0	0
	Minimum	27	8.90	5.28	12.60	80	35	36	0	0
	Maximum	50	16.70	14.22	19.20	140	64	65	0	0
Male induced/	Mean	35.33	11.66	10.24	9.66	100	47.33	51.66	0	1
treated	SEM	5.78	1.93	1.23	1.90	11.54	8.66	8.17	0	0.57
n=3	Minimum	25	8.20	7.82	6.40	80	30	43	0	0
	Maximum	45	14.90	11.88	13.00	120	56	68	0	2
*Male Induced/ extract n=1		39	12.9	10.64	10.60	100	40	60	0	0
Female induced	Mean	34.33	10.26	7.52	16.46	126.66	42.33	49	0.66	0.66
n=3	SEM	0.66	0.26	0.07	2.72	12.01	1.20	3.46	0.33	0.33
	Minimum	33	10	7.38	12.00	110	40	43	0	0
	Maximum	35	10.80	7.62	21.40	150	44	55	1	1
Female control	Mean	44.33	14.66	13.22	13.53	90	55	45	0	0
n=3	SEM	4.40	1.46	3.08	5.27	40.41	15.27	15.27	0	0
	Minimum	36	11.90	7.60	7.20	10	25	25	0	0
	Maximum	51	16.90	18.22	24.00	140	75	75	0	0
*Female induced/ treated n=1		15	4.0	4.86	16.40	50	29	70	0	0
*Female induced/ extract n=1		45	14.9	9.8	11.60	120	45	55	0	0

**Only one sample was analysed due to mortality*

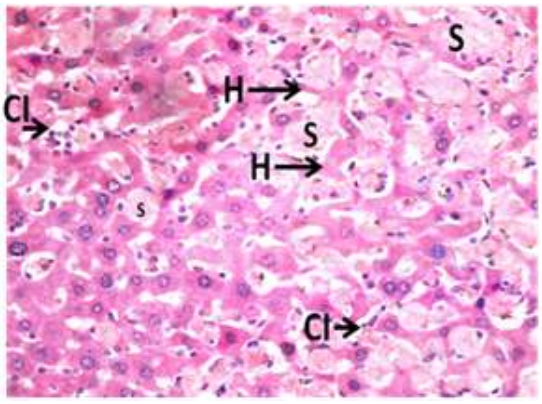

Plate 1. Control male (x400) with hepatocytes, H; the sinusoids S, are filled with pink staining material; mild cellular infiltration (Cl)

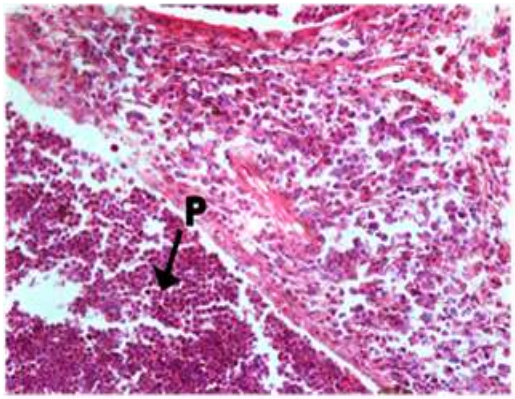

Plate 2. Control/vehicle male mouse liver: showing phagocytic activities P (x400)

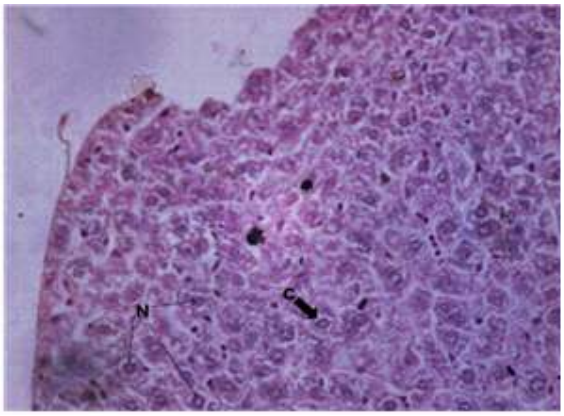

Plate 3. Induced male liver showing pleomorphic nuclei N and cytoplasmic degeneration C (x400)

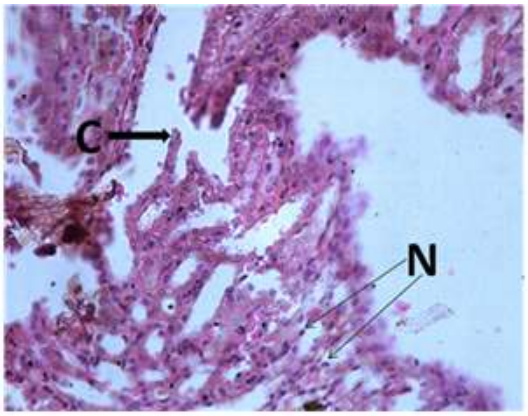

Plate 4. Ligand-treated male liver showing cytoplasmic disintegration C and irregularly shaped nuclei N (x400)

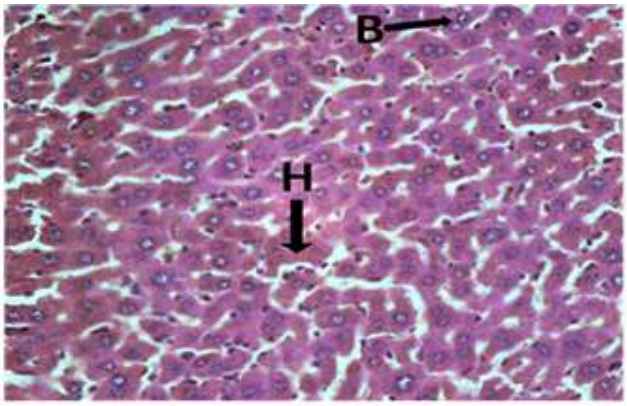

Plate 5. Extract-treated male liver showing binucleate cell B and hepatocytic regeneration H (x400)

4. DISCUSSION

The present study is aimed at determining the antitumor properties of a metal-free ligand and an herbal extract on N-methyl-N-nitrosourea induced carcinomas in albino mice. The study showed that both ligand-treated and extract-treated male and female groups were higher in

mean body weight than the corresponding induced and control groups. This is in contrast with anticancer studies which showed that novel Co(II), Ni(II), Cu(II) ligands and cisplatin reduced the body weight gain of tumor-bearing mice [17,18]. It should be noted that only 13% and 17% of all the MNU-induced male and female mice in this study had tumors respectively and thus, these proportions might not be high enough to significantly influence the body weights of the whole induced male and female groups. The low survival rate in the extract-treated male and female groups might probably be due to lack of expertise during the (route of administration) (oral) of the herbal extract as oral administration of mice requires expertise [19]. They died immediately following the daily oral administration on the fourth day of treatment. However, the others appeared healthy and survived to the end of the study and these were the ones used for hematological and histopathological analysis.

4.1 Hematological Studies of Mice

The PCV, hemoglobin and WBC of the surviving extract-treated male and female groups were within the range of the corresponding standard normal values. The extract-treated male and female animals showed a rise in packed cell volume compared to the induced groups, which brought their values close to the packed cell volume of the male and female control groups (Table 1). There was also a rise in hemoglobin values in extract-treated male and female animals compared to the induced group and these values differed only marginally from their respective controls (Table 1). Studies by Perumal et al. [20] showed a significant increase in hemoglobin content in the group treated with 400 mg/kg ethanolic extract of *Artemisia nilagirica* compared with carcinoma bearing mice. Carcinoma-bearing mice had reduced hemoglobin levels when compared to the normal control group. A similar pattern was replicated in

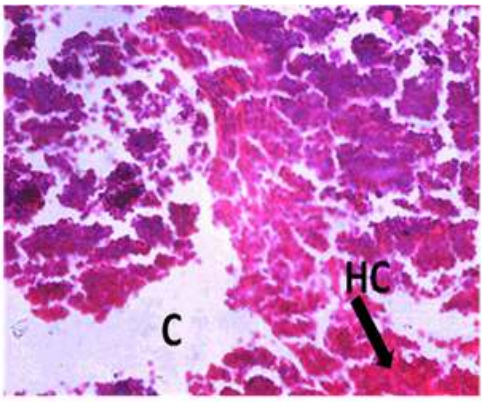

Plate 6. Control female thymus (x400)

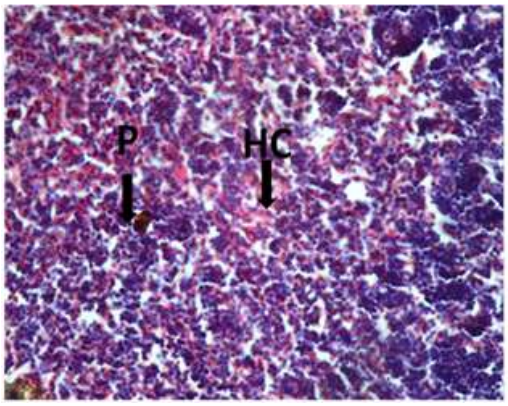

Plate 7. Control/vehicle female thymus showing phagocytic activities P and Hassall's corpuscles HC (x400)

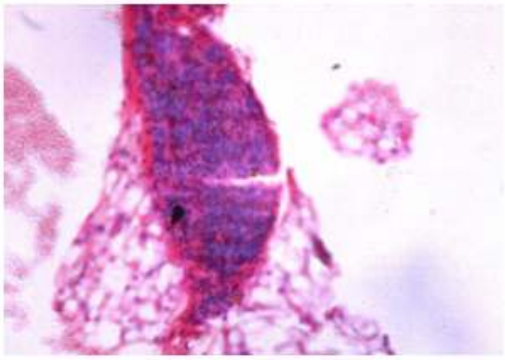

Plate 8. Induced female thymus (x400)

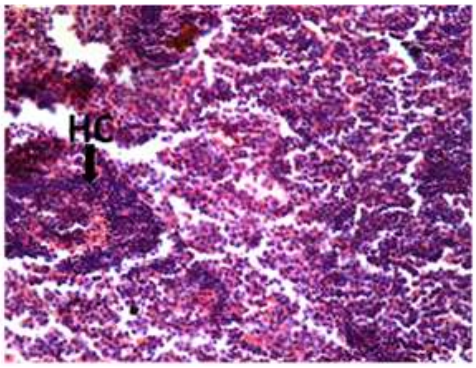

Plate 9. Extract-treated female thymus showing Hassall's corpuscles (x400)

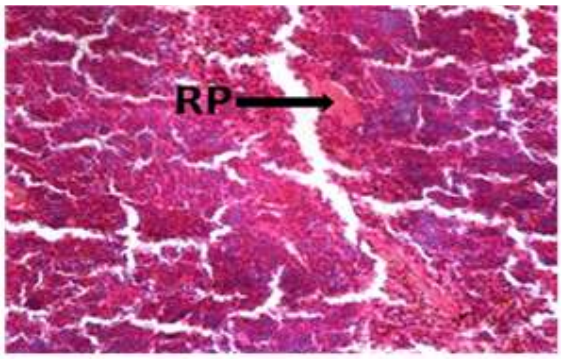

Plate 10. Histological section of control male showing Red Pulp (x400)

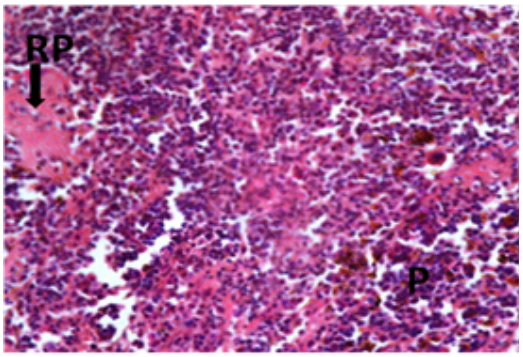

Plate 11. Histological section of control/vehicle female spleen showing red pulp (RP) and phagocytic activities P (x400)

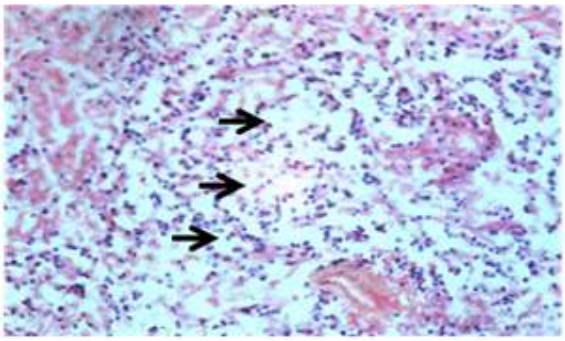

Plate 12. Histological section of induced male spleen showing lymphoid depletion (arrows) (x400)

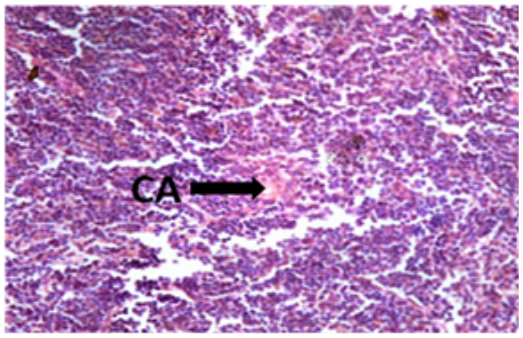

Plate 13. Histological section of extract-treated male spleen showing central artery CA (x400)

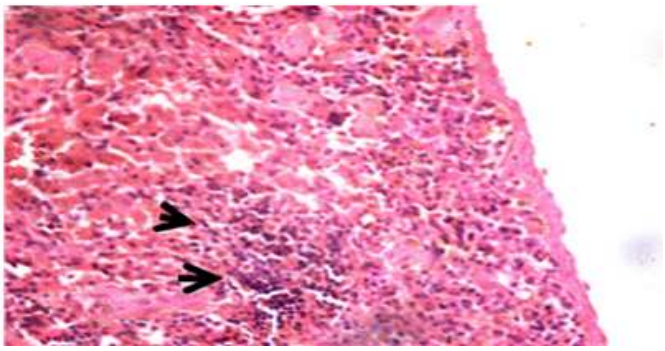

Fig. 14. Histological section of ligand-treated male spleen showing severe congestion at the red pulp and moderate lymphoid depletion (arrows) (x400)

the red blood cell counts of surviving extract-treated male and female animals as RBC count was increased compared to the induced groups and values obtained were within close range of the control group. The white blood cell counts in the extract-treated male and female animals were also lower compared to the induced and control groups (Table 1). The insignificant decrease in the WBC count of extract-treated animals shows that the herbal extract is not likely to compromise the principal function of white blood cells, which is to defend the body against infectious diseases or foreign materials. Perumal et al. [20] equally observed that Ehrlich's Ascites

Carcinoma (EAC)-bearing mice had significantly higher total white blood cell counts compared with the non tumor-bearing control mice while EAC-induced mice treated with *Artemisia nilagirica* had lower WBC counts compared to the control (non-tumor-bearing) mice.

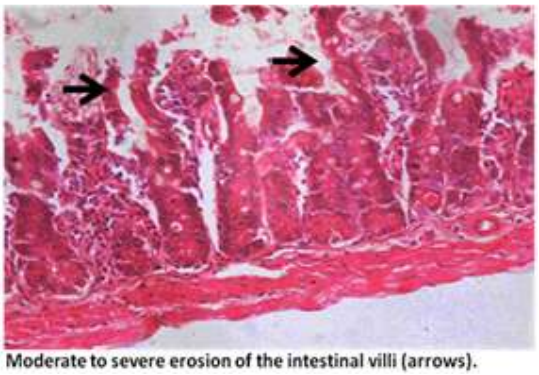

Plate 15. Histological section of control small intestine (x400)

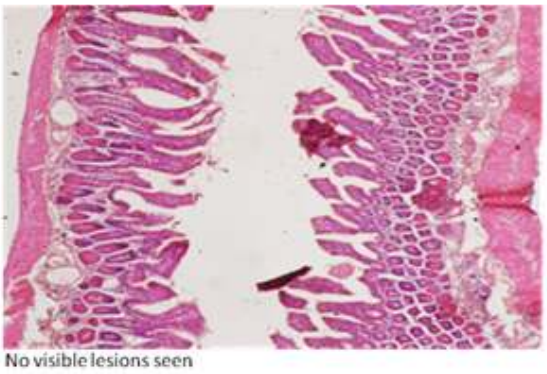

Plate 16. Histological section of induced female small intestine (x400)

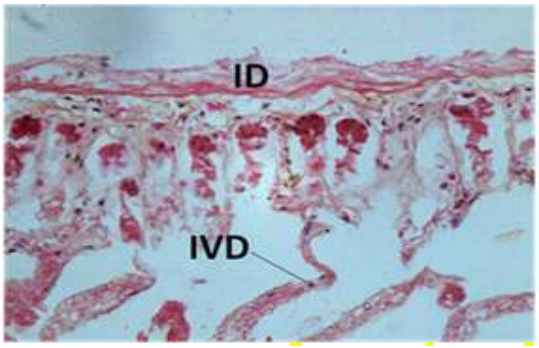

Plate 17. Histological section of ligand-treated female small intestine showing intestinal wall degeneration ID and Intestinal villi disruption IVD (x400)

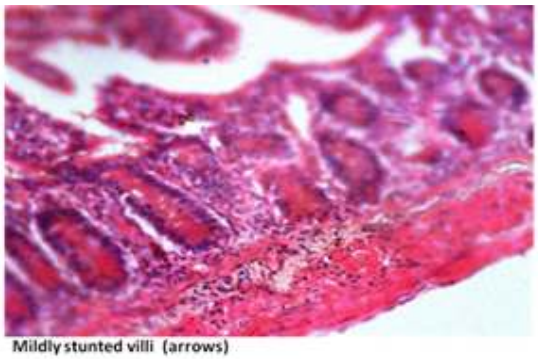

Plate 18. Histological section of extract- treated female small intestine (x400).

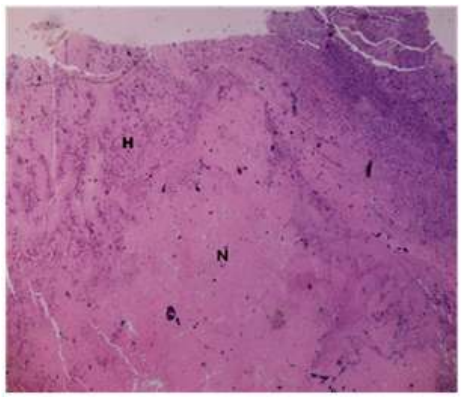

Plate 19. Female induced with stomach tumor showing hypochromatic cells H and Necrotic cells N

The lymphocyte percentage of the extract-treated female animal was only higher than that of the induced group, while the extract-treated male had a lower lymphocyte percentage than both induced and control groups (Table 1). Increased lymphocyte percentage may suggest an improvement from an immunosuppressive effect. Perumal et al. [20] also observed an increase in percentage lymphocyte when the ethanolic extract of *Artemisia nilagirica* was used to treat EAC bearing mice. Both extract-treated male and female animals had higher percentages of neutrophil when compared with the induced and control groups.

Restoration of hematological parameters (particularly packed cell volume, hemoglobin concentration, red blood cell count and white blood cell count) in this study by the herbal extract may indicate its ability to counter the effect of MNU-induced toxicity in mice [21]. A similar observation by Gowry and Vadivel [22] suggests that the herbal extract may have a protective effect on the hematopoietic system. The reduction in RBCs or hemoglobin percentage in tumor-bearing mice may cause anemia, which may occur due to iron deficiency, hemolytic or myelopathic condition [23]; Adhvaryu et al. [24] also observed that these hematological parameters were restored to near normal levels by the Ayurvedic herbs (used in traditional Hindu system of healing) – *Curcuma longa* L., *Tinospora cordifolia* (wild) and *Zizyphus mauritiana*.

The findings from this study demonstrate the indirect inhibitory effect of the herbal extract, which is probably mediated by the enhancement and deactivation of either macrophages or cytokine production [20]. The ability of herbal extracts to reduce white blood cell counts of tumor-induced mice has also been observed by other workers [25,20]. This suggests that the herbal extract might contain flavonoids which have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis [26,27]; they also possess antimutagenic and antimalignant effects [28]. Other compounds which possess potent antitumor properties are some protein and polysaccharide fractions in *Cucurbita maxima* fruits and seeds [29]. Further studies using larger sample sizes of extract-treated mice are however necessary to confirm these findings, considering the technical expertise required for oral route of drug administration.

The packed cell volume, hemoglobin and red blood cell counts of the ligand-treated male group were increased when compared to the induced group and tended towards that of the control group; while those of the ligand-treated female group were very low compared to the control group. This report is in line with that of Sunil et al. [18] who observed that hemoglobin levels and red blood cell counts were improved in Schiff base (100 mg/kg body weight of mice) treated groups. Sathisha et al. [17] also observed a similar trend of decreased red blood cell count and hemoglobin in tumor-induced mice, which increased significantly ($p<0.05$) after treatment with cisplatin and some metal complexes of thiocarbohydrazone ligands. Furthermore, the general hematological parameters of tumor bearing rats after treatment with a Schiff base copper complex showed statistically significant increase in the red blood cells and hemoglobin as compared to tumor bearing controls [9].

The white blood cell counts of the ligand-treated male and female groups were reduced compared to the induced groups. Sunil et al. [18] also observed that an optimal dose of 100mg/kg body weight of schiff bases was able to reduce white blood cell count in the Schiff base-treated groups of tumor-induced mice. Sathisha et al. [17] reported that all the metal complexes of thiocarbohydrazone ligands were able to reverse the tumor-induced rise in total counts of white blood cells. Furthermore, Chakraborty et al. [9] observed that total white blood cell counts which increased significantly in a tumor model, were found to be close to normalcy after treatment with a Schiff base copper complex $[Cu(Pyimpy)Cl_2]$. Although the platelet counts of the induced male group was lower than the control groups, the platelet counts of the ligand-treated male and female groups were still lower than the induced and control groups, a condition known as thrombocytopenia. The lymphocyte percentage of the ligand-treated male group was higher compared to the induced group while that of the ligand-treated female group was lower. Neutrophil percentages of the ligand-treated male and female groups were higher compared to their respective induced groups. This rise in lymphocyte percentage of the ligand-treated male group and neutrophil percentage in both ligand-treated male and female groups is in line with the report of Sathisha et al. [17]. Minor variations in the overall hematological values might be due to differences in the strains of mice, sampling, methods of analysis and nutrition [30]. Age might also be responsible for variations in

the hematological values of mice. Magnani et al. [31] reported that older mice have a reduced red cell count than the young ones but have the same hematocrit compared with the younger ones.

4.2 Histopathology

4.2.1 Histological study of liver

The liver is an important organ in toxicological studies and serves as a major site of detoxification in the body for all drugs and toxins [32]. It remains a major target organ for carcinogens [33]. The histological section of the control (with vehicle) of male mice shows the activity of phagocytes in response to the vehicle (DMSO), thus trying to mop it up (Plate 2) compared to that of the male control group (Plate 1) where there were distinct normal hepatocytes, binucleate cells and portal tracts. Phagocytes are cells that occur throughout the body generally for the purpose of defending the organism as a whole from potentially harmful invaders. The histological section of the ligand-treated male group (Plate 4) shows uniformly-sized nuclei with cytoplasmic disruption; there were also pleomorphic nuclei (irregularly-shaped nuclei). This is in contrast with the report of Sunil et al. [18] who observed that treatment of ascetic carcinoma bearing mice with a schiff base (100 mg/kg) showed only mild central vein dilation suggesting reduced hepatotoxicity compared to the standard (cisplatin) treated group. These results suggest that the histology of the liver did not improve upon treatment with the metal-free ligand, perhaps because the dosage (25 mg/kg body weight of mouse) was too low for any visible improvement. It might also be due to the absence of an active metal base. El-Refaiy and Eissa [34] reported that zinc (a metal) had a protective effect against cadmium-induced liver damage, as it has been demonstrated that zinc has a protective effect on histological damage by maintaining membrane integrity due to its direct action on free radicals [35]. The ligand-treated female group also showed phagocytic activities and mild infiltration with plasma cells and lymphocytes. However, the extract-treated male group (Plate 5) showed progressive reversals towards normal (Plate 1). This suggests that at a dosage of 136 mg/ml aqueous extract of this herbal product, hepatotoxicity was reduced and tended towards normal compared to the induced groups as the extract-treated male and female groups show hepatocytic regeneration, mild phagocytic infiltration, fairly uniformly-shaped nuclei, binucleate cells which are common in liver cells.

4.2.2 Histological section of thymus

The histological section of the thymus of the control (with vehicle) female group (Plate 7) shows phagocytic activities in response to the vehicle which is seen as a foreign body. This naturally occurs because the thymus gland plays an important role in the development of immune responses especially in early life and is a site of T-lymphocyte formation and antibody production [36]. The histological section of the induced female group (Plate 8) shows a reduction or shrinkage in the size of the thymus – known as atrophy; a total distortion of the histology of the organ as the vital components such as hassall's corpuscles and epithelial components are atrophied compared to the control group (Plate 6). This might be as a result of prolonged protein malnutrition and immunosuppression by drugs and chronic viral infection [37]. The extract-treated group (Plate 9) showed a gradual reversal of some of the histological components showing traces of the hassall's corpuscles; it also shows mild infiltration with plasma cells and lymphocytes which are phagocytic cells.

4.2.3 Histological section of spleen

The histological section of the spleen of the control/vehicle (DMSO-treated) group (plate 11) shows increased phagocytic activities with plasma cells and lymphocytes indicative of a response to the foreign material (DMSO). The histological section of the induced male group (Plate 12) shows a hyperplasia of white pulp in the periarterial sheath, rupture of the central artery and a severe lymphoid depletion due to aggressive phagocytic activities. Similar spleen changes were induced by Balani et al. [38], and all these changes may be attributed to a loss of infiltration efficiency. The splenic white pulp is an important site of lymphocyte traffic where the formation of plasma cells occurs [39]. Tumor cells and their secretions may result in the depletion of the white pulp of the spleen [40]. The section of extract-treated male group shows a regeneration of the central artery and the red pulp with no visible lesion (Plate 13); however, there is also presence of phagocytic activities. Khalaf [41] reported that such regeneration may result in the disappearance of transplanted tumor. The red pulp is a blood filter that removes foreign materials and damaged and weak erythrocytes; it is a storage site for iron,

erythrocytes and platelets. It is a site of hematopoiesis in fetal and neonatal animals [42]. The histological section of the ligand-treated group shows a severe congestion at the red pulp and moderate lymphoid depletion with no distinct repair of the major parts of the spleen as seen in plate 14. This might be as a result of the immune response of the lymphoid tissue (mainly spleen) against tumor cell proliferation [43]. This shows that the herbal extract was more effective in reversing the tumorigenic or toxic effects of MNU in the induced mice than the metal-free ligand.

4.2.4 Histological section of small intestine

Histological section of the induced male group (plate 16) shows that the intestinal wall lining (longitudinal wall of muscularis propria) appears normal with no visible lesion. There is increased mucin secretion probably in response to the presence of a carcinogen to cover up the eroding portion – this might be a symptom of an inflammation [44]. The ligand-treated female (Plate 17) showed mild presence of plasma cells and lymphocytes and gross erosion of intestinal villi. Gross erosion or reduction of the intestinal villi in the ligand-treated female group might be a symptom of apoptosis. Tamaki et al. [45] reported that mice treated repeatedly with 5-fluorouracil (an anticancer drug) at a dosage of 50mg/kg body weight showed a rapid increase in diarrhoea symptoms and a steady decrease in the height of villi. However, the small intestine of the extract-treated female showed mildly stunted villi and mild secretion of the mucinous glands, while the lining of the intestinal wall appeared to be regenerating (Plate 18).

5. CONCLUSION

This is a pilot study that has revealed that MNU (at a dosage of 60 mg/kg body weight) caused varying degrees of abnormalities and tumors in swiss albino mice. There were no significant differences (p=.05) in comparable hematological parameters as a result of the MNU induction. Treatment with herbal extract showed improvement in the histology of the liver (hepatocytic regeneration), spleen, thymus (hassall's corpuscles) and small intestine (regeneration of intestinal wall lining) while treatment of induced groups with ligand did not show much improvement compared with the induced. This might be due to the low dosage (25 mg/kg body weight) and the absence of active metals in its components. There is a need for further *in vivo* investigations on the antitumor properties of the ligand, 3-[2,4-dihydro-1H-inden-4-yl imino)methyl] napthalen-2-ol, using higher doses, in order to establish its efficacy or otherwise. There is also a need for further studies on varying dosages of this herbal extract using larger sample sizes of carcinogen-induced mice.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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