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# *Lawsonia Inermis* Leaf Extract Mitigates Aluminium-Induced Testicular Toxicity in Wistar Rats: Immunohistochemical Study

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**Abstract**

**INTRODUCTION**

Apoptosis is a genetically controlled cell suicide pathway which plays an essential role in deleting excess, unwanted or damaged cells during development and tissue homeostasis. Phytochemical screening of the *Lawsonia inermis* leaf extracts had showed the presence of glycosides, phytosterol, steroids, saponins,

tannins and flavonoids.The aim of this study was to investigate the mitigating effect of *Lawsonia inermis* aqueous leaf-extract on aluminium-induced oxidative stress on the testes of adult Wistar rats' as determined by immunohistochemistry.

**MATERIALS AND METHODS**

Thirty five adult male Wistar rats, weighing between 100-196g. *Lawsonia inermis* extracts and aluminum chloride ($AlCl_3$) were dissolved in distilled water then administered for a period of three (3) weeks through the oral route with Five (5) rats per group. Group 1 (control): Given rat pellets and distilled water. Group 2: Given 60mg/kg/d extract of aqueous *Lawsonia inermis*. Group 3: Given 0.5mg/kg/d of $AlCl_3$. Group 4: Given 0.5mg/kg/d of $AlCl_3$ and 60mg/kg/d of aqueous *Lawsonia inermis* orally. Group 5: Given 0.5mg/kg/d of $AlCl_3$ and 75mg/kg/d of aqueous *Lawsonia inermis* orally. Group 6: Given 0.5mg/kg/d of $AlCl_3$ and 100mg/kg/d of aqueous *Lawsonia inermis* orally. Group 7: Given 0.5mg/kg/d of $AlCl_3$ and 5mg/Kg/d Ascorbic acid in distilled water orally. Twenty four hours after the last administration, the animals were weighed, anaesthesised with chloroform pituitary glands were located, removed and weighed using an electronic sensitive analytical balance.

**RESULTS**

The densities of Bax, ki 67 and Bcl2 were highest in the control rat followed by the rats given 60mg/kg extract of *Lawsonia inermis* while the rats given 0.5mg/kg/d of aluminum chloride were the lowest.

## CONCLUSION

The immunoreactivities of other rats increased according to the dosage. In conclusion, aluminum chloride decreased the immunoreactivities germ cells thus normal apoptosis was altered but *Lawsonia inermis* ameliorated/ mitigated this deleterious effect of aluminum chloride.

**Key Words: Apoptosis, Autophagy. Carcinogenesis, Entosis**

## INTRODUCTION

Multicellular organisms have several cells, and in other to maintain the homeostasis in the body of many of these organisms many of their cells die every day. We have different types of cells death: necrosis, apoptosis, autophagy and entosis. Necrosis is due to external factors such as toxins, infection, and/or trauma which elicits immune response while apoptosis is known as-programmed cell death which does not cause lysis of the cell, nor inflammatory response[1]. Autophagy is degradation of own cells by lysosomal reaction, while entosis involves cell destroying neighboring cell [2, 3]. Apoptosis is a physiological process which helps in maintaining homeostasis drilling the body by replacing old cells with new cells. It is said to be a genetically controlled cell suicide pathway.

Pathological dysfunction of the apoptotic pathway may result in carcinogenesis[4]. Some tumor cells interfere with the process of apoptosis leading to immortality. One of the genes that are implicated is: Bcl-2. Over expression of Bcl2 causes carcinogenesis[5, 6].

Carcinogenesis is induced when the programme cell death is impaired[7]. Bcl2 expression is high with tumors less than 550g and with favourable condition[8-10]. Bcl2 is an anti-apoptotic protein which inhibits apoptotic death while Bax protein is pro apoptosis which enhances the apoptotic death[11]. Ratio of Bcl2/Bax determines the induction as well as inhibition of apoptosis in human. When the ratio is greater than >1anti apoptotic signal is dominant and when the ratio is <1 apoptotic mechanism dominates. Apoptosis occurs in vertebrate body and there is high incidence in male organ (testis) about 75% of all the germ cells produce are renewed through apoptosis[12].

The spermatogenic process occurs within the seminiferous tubule of the testis and it involves spermatogenesis maturation into spermatozoa. The cells production of spermatocytes within seminiferous tubules is dependent on the dynamic balance between programmed cell death and cell proliferation[13], as with other cells of the body. During embroyological development, excessive cells generated due to apoptosis[14], or over expression of Bax is often observed during the early stages of germ cell development of the testes[15].

Ki-67 expression is universally acceptable as proxy for cell proliferation activity and Ki-67 labelling index correlates with the growth of many human neoplasms[16]. Ki-67 is prominent during the cell cycle but not in GO phase. Ki-67 is located on chromosome 10. Both the sexual hormones and male germ cell apoptosis

regulate germ cell survival; however, excess or low hormones can induce cellular apoptosis in the testis[17].

Sertoli cells can serve as hormone regulators of spermatogenesis they have receptors for FSH and testosterone. Cessation of hormone secretion can lead to germ cell apoptosis[18]. Both FSH, testosterone and oestradiol assists during germ cell maturation. The homeostasis of germ cells is maintained by FSH and testosterone level[19, 20]. It has been reported that, excessive release of testosterone could result in death while testosterone deprivation elicits caspace activity which could induce DNA fragmentation in sertoli cells[21].

The potential of herbal medicines and medicinal plant in health care delivery, particularly in the third world is no longer in doubt having gained recognition in several nations of the world and by the World Health Organization (WHO) [22-24]. *Lawsonia inermis* commonly known as henna belongs to the family Lythraceae and genus Lythrum. Henna is a common name for a small shrub and for the dye that is obtained from its leaves. The shrub is also called alkanna mignonette tree, El-henna and Egyptian priest. Phytochemical screening of the henna leaf extracts has shown the presence of glycosides, phytosterol, steroids, saponins, tannins and flavonoids[24, 25]. According to Jain[26], the phytochemical investigation of henna leaf shows total ash (14.60%), acid insoluble ash (4.50%) and water soluble ash (3.0%). Alcoholic extract and aqueous extract carbohydrate, glycosides, tannins, phenolic compounds, gums and

mucilage were present in good quantity but saponins, alkaloids, phytosterols, fixed oils, fats, proteins, amino acids, volatile oils were absent. There is evidence of the plant having wound healing properties[27].

Aluminum is known to be the most abundant metal and the third most common element in the earth's crust2[8, 29]. It is found abundantly as trioxosilicate (IV) in rocks and Clays. Chemically, it is often found in combination with silicon, fluorine, oxygen, and other earth elements[30]. It was reported that the oral bioavailability of aluminum can be as low as 0.1%; after absorption, it is distributed into the body of animal and human tissues and bones[31-32]. Aluminum ion is transported in the plasma by the iron binding protein, transferrin and it can enter the the brain, placental and fetus[33, 34]. Aluminum has a longer half life in humans than in rodents.

Aluminium has been reported to have no biological function[35] but it has some important functions in most of the developing countries. Aluminum can be used in making utensils, cookware, cosmetics, cointainers, and aluminum foil; other primary sources of aluminum includesalt, yellow cheese, corn, herbs, teas and spices[36-39]. Medically, aluminum has been used in the production of antacids, vaccines and injectable allergens, as phosphate binders and in buffered aspirin[40-42]. It is also used as an additive in toothpaste and food[43]. Aluminum can also be found naturally or included in drinking water and food products[44]; In fact it is believed to not be toxic but it has adverse effects on human health[45].

## MATERIALS AND METHODS

**Collection and Preparation of Extracts**

The plant was obtained from Isanlu-Isin in Kwara State, Nigeria and identified professionally with the Herbarium number **UPH/P/114** by the Taxonomist in the Department of Plant Science and Biotechnology, University of Port-Harcourt, Rivers State, Nigeria. The Research ethics Committee of the same institution approved this work on 25th February, 2016 with reference number **UPH/CEREMAD/REC/04.** The plant leaves were washed with water, cut into pieces, dried in a cool environment. The dried plant leaves were pulverized into coarse powder in a grinding machine. The filtrate was concentrated using Rotary evaporator (Buchi) and further concentrated to dryness at 50°c in an electric oven (GallenKamp).After drying it was stored in the refrigerator at $4^0$c until needed for use.

**Drugs and Chemicals**

Aluminium Chloride and Ascorbic acid were bought from Mich-Deson Hospital Equipment store, Upper Taiwo, Ilorin. The histological staining was done in the Anatomical-pathology Department of the University Teaching Hospital, Ilorin and immunohistochemical staining was done in University College Hospital, Ibadan.

**Acute Toxicity Test ($LD_{50}$):** Fifteen mice were used to conduct the above test to determine the safe dosages and lethal dosage.

They were grouped into five (5), with three (3) mice per group. The acute toxicity of the Aqueous Extract of *Lawsonia inermis* extract was assessed by $LD_{50}$ calculation, using a limit dose test at a limit dose of 1000mg/kg body weight of the extract after oral administration in mice (three animals per group) (OECD-OCDE 425 Guide).

**Determination of the Dosage of the Extract to Administer:** The choice of dosage based on the acute toxicity test ($LD_{50}$) above, the safe dose of *Lawsonia inermis*is 0.1g/Kg or 100mg/Kg body weight. The highest dose is 100mg/Kg, medium dose is 75mg/Kg and the lowest dose is 60mg/Kg.

**Breeding of the Animals:** Thirty five adult male Wistar rats and fifteen mice were used, with an average weight of 100-196g. The rats, after procurement, were housed in cages (made from wood, wire gauze and net) under natural light and dark cycles at room temperature in the animal house of the Faculty of Basic Medical Sciences, University of Ilorin. The floor of the cages were made with wood to make it comfortable for the rats and it was covered with sawdust to provide a soft floor for the rats and to make cleaning of the cage convenient when littered. They were fed with rat pellets purchased from approved stores by University of Ilorin, and water was given *ad libitum.* They were grouped and left to acclimatize for 2 weeks before the study commences.

**Grouping**: The total numbers of animals were thirty five. They were grouped into one (1) control and six (6) experimental groups with

consideration towards size variation. Using a feeding tube (size-6), distilled water, and *Lawsonia inermis* extracts were administered to the control and treated animals respectively for a period of three (3) weeks.

Group 1 (control): (n = 5): Given rat pellets and distilled water.

Group 2: (n = 5): Given 60mg/kg/d extract of *Lawsonia inermis* and pellets.

Group 3: (n = 5): Given0.5mg/kg/d of aluminum chloride in distilled water and pellets.

Group 4: (n = 5): Given 0.5mg/kg/d of aluminum chloride and low dose 60mg/kg/d of *Lawsonia inermis* in distilled water orally.

Group 5: (n = 5): Given 0.5mg/kg/d of aluminum chloride and medium dose 75mg/kg/d of *Lawsonia inermis* orally.

Group 6: (n = 5): Given 0.5mg/kg/d of aluminum chloride and high dose 100mg/kg/d of *Lawsonia inermis* in distilled water orally.

Group 7: (n=5): Given 0.5mg/k/d of aluminum chloride and 5mg/Kg/d Ascorbic acid in distilled water orally.

**Animal Sacrifice and Sample collection:** Twenty four hours after the last administration, the animal were weighed and thereafter sacrificed by the use of chloroform as an anesthetic. Abdominal cavity was opened by a midline abdominal incision and the reproductive organs (Testes) were removed.

**Immunohistochemical Staining Method**

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The two testes were embedded in paraffin blocks.These tissues were sectioned serially, at least two non-serial sections were stained with hematoxylin and eosin (H&E). The Leydig cells were identified by staining for 3b-HSD. The 5mm sections were mounted, then dewaxed and rehydrated.

In other to perform, antigen retrieval was performed by pressure cooking slides in 0.01M citrate buffer (pH 6.0) about 5minutes and then incubated in 3% (vol/vol) of $H_2O_2$ in methanol to block endogenous peroxidase activities for 30 minutes, then slides were washed in Tris-buffered saline [TBS; 0.05M Tris, 0.85% (wt/vol) NaCl (pH 7.4)]. No specific binding sites were blocked with an appropriate normal serum diluted at 1:5 in TBS containing 5% (wt/vol) BSA.

For proper incubation of tissue overnight, addition of goat polyclonal antibody against 3b-HSD incubation at 4 _C and 3b-HSD antibody was recommended for detection of 3b-HSD1 or 3b-HSD2 in mouse tissues. It was then wash in Tris-buffered saline (TBS).

Moreso, the incubation of slide was for 30 minutes;using the suitable secondary antibody conjugated to biotin rabbit anti-goat (Santa Cruz Biotechnologies). Dilution into 1:500 in the blocking mixture. Incubated with horseradish peroxidase-labeled avidin-biotin complex for 30minutes, followed by application of diaminobenzidine for immunostain develop.

Lastly, counterstained with hematoxylin, dehydrated then mounted, using Pertex mounting medium. The number of 3b-HSD-positive cells was counted in 12 randomly

selected fields from each slide at a magnification of x400.

**Immunohistochemical detection of BCL-2**

Immunohistochemical detection of BCL-2 was done using avidin-biotin-peroxidase complex with monoclonal antibodies against BCL-2. The section was cooled at room temperature for 20 minutes, followed by retrieval and then incubated for 30 minutes with 10% normal horse serum.

Excess serum were decanted away, the sections were then incubated with primary antibody for 20 hours at room temperarure; After that, DO7 monoclonal antibody was used at a 1:100 dilution. The sections were subsequently incubated with pre-diluted biotinylated anti-mouse immunoglobulin for 30 minutes at 37℃.

The sections were washed with PBS, and allowed to react with peroxidise-conjugated at dilution of 1:500 for 30 minutes at 37℃. Lastly, PBS, peroxidise activity was evaluated with 3,3'-diaminobenzidine tetrahydrochloride (DAB), and the sections were counterstained with Harris hematoxylin.

**Immunohistochemical detection of BAX**

The avidin-biotin-peroxidase complex was employed to achieve immunohistochemical procedure. Following antigen retrieval and cooling for 20minutes, incubation of section was done with 10% normal horse serum (Vectastain Elite kit) for 30minutes. After decanting away the excess serum, sections were incubated with primary antibody for 2hours at 37℃. The monoclonal antibody BAX (policlonal, Dako®, 1:50), was used at 1:500 dilution (Pharmingen, USA). The

sections were subsequently incubated with pre-diluted biotinylated anti-mouse immunoglobulin (Vectastain Elite kit) for 30 minutes at 37℃. After washing with PBS, the sections were reacted with peroxidase-conjugated streptoavidin (Dako, Denmak) diluted at 1:500 for 30 minutes at 37℃. After washing with PBS, peroxidase activity was evaluated with 3, 3'-diaminobenzidine tetrahydrochloride (DAB), and the sections were counterstained with Harris hematoxylin.

**RESULTS**

**Plate A**: Bax, Immunoreactivities micrograph for different groups. The arrows show some cells in the seminiferous tubules which are immunopositive, most are elongated spermatids, spermatozoa and few spermatocytes, spermatogonia. Magnification x400.

**Plate B**: BCL-2 Immunoreactivities micrograph for different groups. The arrows show some cells in the seminiferous tubules which are immunopositive, most are elongated spermatids, spermatozoa and few spermatocytes, spermatogonia. Magnification x400.

**Plate C:** Ki-67 Immunoreactivities micrograph for different groups.. The red arrows show some cells in the seminiferous tubules which are immunopositive, most are elongated spermatids, spermatozoa and spermatogonia. Magnification x400.

**Figure 1.0:** Densities of Bax, Bcl-2 and Ki-67 Immunopsitive cells per tubules

**Table 1.0:** Densities of Bax, Bcl-2 and Ki-67 Immunopositive cells per tubules for the wistar rats

Immunoreactivities of cells were demonstrated in the table and figure 1.0 above, the densities of Bax, ki 67 and Bcl2 were highest in the control rat followed by the rats given 60mg/kg extract of *Lawsonia inermis* while the rats given 0.5mg of aluminum chloride per kg of body weight were the lowest. The immunoreactivities of other rats increased according to the dosage.

## DISCUSSION

In this current study by the oral route, the animals showed dose-dependent signs of toxicity, ranging from lack of appetite, depression, immobility and respiratory distress to death. $LD_{50}$ for *Lawsonia inermis* extract is 0.75g while the safe dose is 0.1g/Kg b.w.

The immunoreactivities of the cells were demonstrated in the table and figure 1.0 above the densities of both Bax, ki 67 and Bcl2 were highest in the control rat followed by the rats given 60mg/kg extract of *Lawsonia inermis* while the rats given 0.5mg of aluminum chloride per kg of body weight were the lowest. The immunoreactivities of other rats increased according to the dosage, that is: immunoreactivities of the rats that received low dose of *Lawsonia inermis* aqueous leaf-extract and aluminum chloride were lower compared to the highest dose with aluminum chloride .This implies, *Lawsonia inermis* aqueous leaf-extract ameliorates/ mitigate the

oxidative stress induced by aluminum chloride toxicities.

The immunoreactivities of the cells demonstrated in the table and figure 1.0 above the densities of Bax, ki 67 and Bcl2, shows that the rats given 60mg/kg extract of *Lawsonia inermis* were equally having high density. According to Inder [46], *Lawsonia inermis* contains many dietary elements including both micro and micronutrients (carbohydrate, glycosides, tannins, phenolic compounds, gums, mucilage, saponins, alkaloids, phytosterols, fixed oils, fats, proteins, amino acids, D-Glucopyranoside, methyl (51.73%) and 1, 4-Naphthalenedione, 2-hydroxy- 'Synonyms: Henna glycosides', phytosterol, steroids, saponins, tannins and flavonoids). This suggests its ability to influence many physiological processes. Many of the elements found in *Lawsonia inermis* have been demonstrated to have positive roles in spermatogenesis and testicular steroidogenesis.

The immunoreactivities of the rats increased according to the dosage, it was obvious in our study that the rats given low dose of *Lawsonia inermis* aqueous extract and aluminum chloride were lower compared to the rats given medium, highest dose of aluminum chloride. According to Shaha[17] reported that sexual hormones and programme cell death regulate germ cells. Shortage and over-production of hormones can cause cellular programme cell death in the testis. Alteration of hormone can lead to apoptosis[18]. During seminiferous tubule maturation, testosterone alongside the synergistic action of FSH and

oestradiol assist in ensuring germ cell survival while oestradiol on its own acts as an inhibitor, with a pro-apoptotic effect. Ruwanpura reported sudden alteration of FSH could lead to spermatogonia[47].

Excess testosterone can cause as reported by Zhou[20]. In contrast, withdrawal of testotesrone stimulates the activity caspase which cause fragmentation of DNA in Sertoli cells and weak effect in germ cells [21]. Ki-67 expression is widely accepted as a measure of cell proliferative activity and the Ki-67 labeling index correlates with the growth of many human neoplasms[16]. Ki-67 is expressed during the G1, S, G2 and M phase of continuously cycling cells, but is absent in G0 cells. Ki-67 expression is more prominent during cell cycle of sperm cells. Rodrigue reported that toxicant influences survival rate of the germ cells and these induce programme cell death[48]. These could be as a result of toxicant or oxidative stress induced by aluminum chloride but table 1; plates A, B and C, show that addition of different doses of *Lawsonia inermis* ameliorated deleterious effect of aluminum chloride.

The immunoreactivities of the cells demonstrated in the table and figure 1; also plates A, B & C, above shows that the densities of Bax, ki 67 and Bcl2 were very low in the rats given 0.5mg of aluminum chloride per kg of body weight. Oxidative stress induced by aluminum chloride may disrupt molecular machinery that control germ cell cycle especially at the *stage* of morphological differentiation producing failure of mature spermatids to disengage from their relation

with Sertoli cells. It is not entirely clear if the mammalian testis is equipped with the ability to mount an active fight back to ongoing testicular insult. Endocrine disruptors affect hormones regulation and alters normal apoptosis according to McGlyn[49]. Usually, when there is hormonal disturbance, sperm production goes into recession and future recovery is dependent on the nature, the degree, and the target of the insult[50, 51].

## CONCLUSION

The effect of *Lawsonia inermis* aqueous leaf-extract on spermatogenesis is registered molecularly and this was indicated by increase in BCL2, Bax and Ki-67 densities while the negative effects of aluminum toxicity activate BCL2, Bax and Ki-67 -dependent pathway(s) which often result in apoptosis of cells from lethally damaged cells. Aluminum chloride decreased the immunoreactivities germ cells thus normal apoptosis was altered but *Lawsonia inermis* ameliorated/ mitigated this deleterious effect of aluminum chloride.

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days suppressive model on chloroquine sensitive *Plasmodium berghei* (ANKA strain) infected mice with chloroquine as standard control drug. Phytochemical analysis was by using standard methods. Student's t-test of significance ($p < 0.05$) was used for data analysis**.**

**Results:** The LF (yield 0.46 % w/w ; $LD_{50}$ > 5000 mg/kg bw; supressive activity (10 and 1000 mg/kg bw): 71.69 % and 70.91 %) and HF (yield 0.41 % w/w; $LD_{50}$ > 5000 mg/kg bw; supressive activity (10 and 1000 mg/kg bw): 69.3 % and 70.4 % ) were both non-toxic and significantly ($p < 0.5$) suppresive *in vivo* compared to the untreated group though not as active when compared to chloroquine (100 % suppresion). Mice treated with the higher dose of the HF, showed lesser weight reduction and higher survival rate compared to the lower dose. Phytochemicals: (LF:Fixed oils and triterpenoids and HF :amino acids, and carbohydrates) were present with saponins, phenolics compounds, cyanogenic glycosides, anthraquinones and alkaloids absent in both fractions.

**Conclusion:** This observed *in vivo* antiplasmodial activity corroborated the earlier reported *in vitro* pLDH activity of this edible mushroom and is a further evidence of its nutraceutical potential in the management of malaria.

**Keywords:** *Pleurotus ostreatus*, nutraceuticals, malaria, triterpenoids, amino acids,

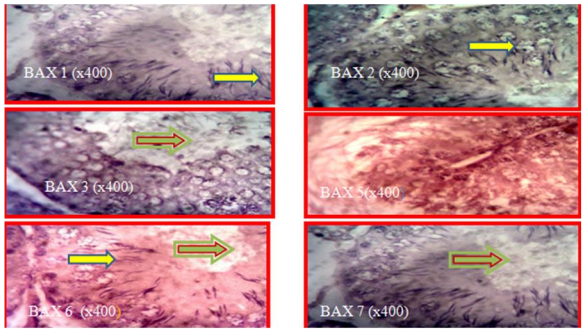


Plate A: Bax, Immunoreactivities micrograph for different groups. The arrows show some cells in the seminiferous tubules which are immunopositive, most are elongated spermatids, spermatozoa and few spermatocytes, spermatogonia. Magnification x400.

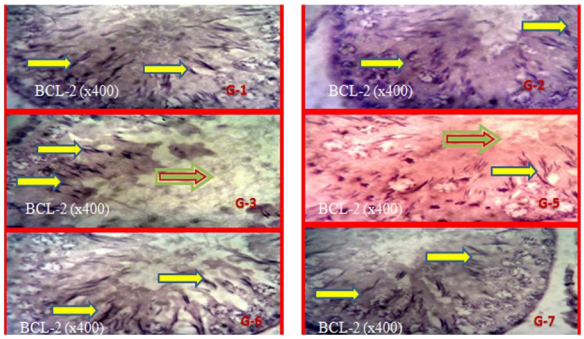


Plate B: BCL-2 Immunoreactivities micrograph for different groups. The arrows show some cells in the seminiferous tubules which are immunopositive, most are elongated spermatids, spermatozoa and few spermatocytes, spermatogonia. Magnification x400.

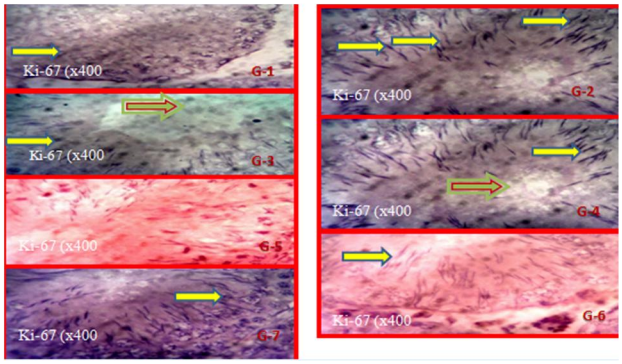


Plate C: Ki-67 Immunoreactivities micrograph for different groups.. The red

arrows show some cells in the seminiferous tubules which are immunopositive, most are elongated spermatids, spermatozoa and spermatogonia. Magnification x400.

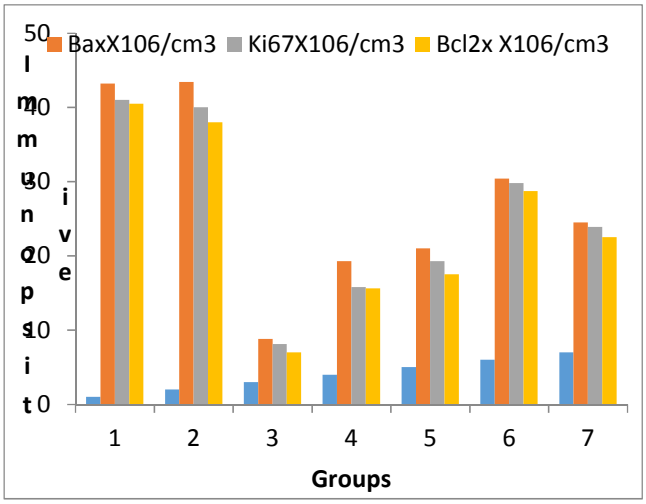


**Figure 1.0: Densities of Bax, Bcl-2 and Ki-67 Immunopsitive cells per tubules**

**Table 1.0:** Densities of Bax, Bcl-2 and Ki-67 Immunopositive cells per tubules for the wistar rats

| Group | BaxX106/cm3 | Ki67X106/cm3 | Bcl2x X106/cm3 |
|---|---|---|---|
| GRP 1 | 43.2± 2.2 | 41± 2.1 | 40.5± 1.9 |
| GRP 2 | 43.4± 2.3 | 40± 1.4 | 38± 1.1 |
| GRP 3 | 8.8± 2.1 | 8.1± 0.6 | 7± 0.4 |
| GRP 4 | 19.3±1.1 | 15.8±0.9 | 15.6±0.9 |
| GRP 5 | 21±1.3 | 19.3± 1.3 | 17.5±1.0 |
| GRP 6 | 30.4±2,0 | 29.8±1.9 | 28.7±1.8 |
| GRP 7 | 24.5±1.4 | 23.9±1.4 | 22.5±1.1 |

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