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Original Article

Effects of Aqueous Leaf-extract of *Lawsonia Inermis* on Aluminium-Induced Oxidative Stress on the Histology and Histopathology of the Testes of Adult Wistar Rats

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Abstract

Lawsonia inermis is a medicinal plant which can be effective in treatment of many diseases.

It was reported that all isolated compounds of *Lawsonia inermis* exhibited antioxidant activity comparable to that of Ascorbic acid. The aim of this study was to investigate: 'The antioxidant effect of *Lawsonia inermis* aqueous leaf-extract on aluminium-induced Oxidative stress on the histology and histopathology of the Testes o adult Wistar rats' with the objectives on histology and histopathology of the Testes. Thirty five adult male Wistar rats, weighing between 100-196g and fifteen mice of the same weight range were used. *Lawsonia inermis* extracts and aluminium chloride ($AlCl_3$) were administered for a period of three (3) weeks 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 0.5 mg/kg/d of $AlCl_3$. Group 4: Given 0.5mg/kg/d of $AlCl_3$ and 60 mg/kg/d of aqueous *Lawsonia inermis* orally. Group 5: Given 0.5 mg/kg/d of $AlCl_3$ and 75 mg/kg/d of aqueous *Lawsonia inermis* orally. Group 6: Given 0.5 mg/kg/d of $AlCl_3$ and 100 mg/kg/d of aqueous *Lawsonia inermis* orally. Group 7: Given 0.5 mg/k/d of $AlCl_3$ and 5 mg/Kg/d Ascorbic acid in distilled water orally. Twenty four hours after the last administration, the animals were weighed, sedated with chloroform and Testes were located, removed and weighed using an electronic sensitive analytical balance. Aluminium toxicity caused Testicular histology to exhibit abnormalities, degenerative changes of seminiferous tubules. The intake of different doses of *Lawsonia inermis* aqueous leaf-extract resulted in testicular histology exhibiting classical histo-architectural appearance with intact seminiferous tubules; displaying normal seminiferous epithelia arrangements, and an enlarged portion of seminiferous epithelial diameter revealed germ cells at different spermatogenic phases. Bundles of spermatozoa were seen in the lumen of seminiferous tubules indicating spermiation. In conclusion, this study has revealed the anti-oxidant effect of aqueous leaf-extract of *lawsonia inermis* on aluminium-induced oxidative stress on testes of adult wistar rats.

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Introduction

Industrialization is one of major causes of proportional increase in environmental pollution. Some of these contaminants caused derailment in the biochemical and physiological parameters in man and Laboratory animals (1).

Aluminium is known to be the most abundant metal and the third most common element in the earth's crust (2, 3). It is found abundantly as trioxosilicate (IV) in the rocks and Clays. Chemically, it is often found in combination with silicon, fluorine, fluorine, oxygen, and other earth elements (4). It was reported that the oral bioavailability of aluminium can be as low as 0.1%; after absorption, it is distributed into the body of animal and man tissues, bones (5, 6). Aluminium ion is transported in the plasma by iron binding protein, transfering and it can enter the the brain, placental and fetus (2, 7).

It has been reported that aluminium has neurotoxicity effect on human body and it is implicated in Alzheimer's disease (8, 9, 10, and 11). Based on findings by Agency for Toxic Substances and Disease Registry 'ATSDR', exposure to high level of aluminium compounds may produce DNA damage (12).

Aluminium has direct effect on the male gonads, consequently, the Aluminium factory workers experience hypofertility (13, 14, 15). Toxic effects of aluminium poisoning can cause asthenospermia, hypospermia, teratospermia and reduction in sperm count (16).

Plant-base medicine has been wholly or partially a source of medical therapy for about 70% of the (17) with an estimated 80% of the world population currently seeks therapeutic solution from herbal medicine as primary health care and this has gained recognition in several nations of the world as well as the World Health Organisation (18, 19).

For over 9,000 years, Henna which has a botanical name *Lawsonia inermis* has been used as the substance for drawing tattoos on the body. Apart from using *Lawsonia inermis* cosmetically people use the plant as hair coloring agent in many parts of the world (20).

Lawsonia inermis is a medicinal plant which can be effective in treating gonorrhea, herpes infection, rheumatic, wound, neuralgic agent and can also be used as antidiabetic drug (21, 22).

The main compound is Lawsone (2-hydroxy-1, 4 napthoquinone; $C_{10}H_6O_3$, m.p.190º decomp). It is the principal colouring compound and varies from 1% to 20% (23). It was reported that all isolated compounds exhibited antioxidant activity comparable to that of ascorbic acid (2.5 mM) (24, 25). According to Nasir (23) the dyeing property of henna depends mainly on its principal colouring matter known as lawsone, 2- hydroxy-1:4 napthaquinone (C10H6O3, m.p.190º decomp), the yield of which varies from 1% to 20%. The active ingredients of *Lawsonia inermis* are: napthoquinone derivatives, phenolic compounds, terpenoids, sterols, aliphatic derivatives, xanthones, coumarin, fatty acids, amino acids and other constituents.

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 ethical 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 50ºc in an electric oven (GallenKamp). After drying it was stored in the refrigerator at 4^0c until needed for use.

Drugs and Chemicals

Aluminium Chloride and Ascorbic acid were bought in Mich-Deson Hospital Equipment store, Upper Taiwo, Ilorin. The histological staining was done in

Anatomical-pathology Department, University Teaching Hospital Ilorin.

Acute Toxicity Test (LD_{50}) and Dosage of the Extract Administer

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 1000 mg/kg body weight of the extract after oral administration in mice (three animals per group) (OECD-OCDE 425 Guide). Using 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 0.75 g while the safe dose is 0.1 g/kg b.w.

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

Breeding of the Animals

Thirty five adult male Wistar rats were used, with an average weight of 100-196 g. 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.

Experimental design

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 60 mg/kg/d extract of *Lawsonia inermis* and pellets.

Group 3: (n = 5): Given 0.5 mg/kg/d of aluminium chloride in distilled water and pellets.

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

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

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

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

Collection and processing of the samples

Twenty four hours after the last administration, the animal were weighed and thereafter sacrificed by the use of chloroform as a sedative. Abdominal cavity was opened by a midline abdominal incision and the reproductive organs (Testes) were removed.

Histological and Histochemical Procedures

Tissue specimens were taken from the Testes of each of the 7 groups of rats and were immersion-fixed in 4% buffered paraformaldehyde for 24 hours, after which each was cut transversely into small (3-5mm thick) slabs and further fixed in a change of the same fixative for another 15 hours. Testes for

Periodic Acid-Schiff Reaction with Haematoxylin (PAS-H) were fixed in Bouin's fluid. Trimming was done on the fixed tissue specimens and washed in tap water for 12 hours. Serial alcohols (methyl, ethyl and absolute) were used for dehydration of the tissue samples. Tissue specimens were cleared in xylene and embedded in paraffin. The paraffin blocks were sectioned at 5 micron thickness by rotary microtome. The obtained tissue sections were collected on glass slides and stained using Haematoxylin and Eosin stains, and Periodic Acid-Schiff Reaction with Haematoxylin.

Results

A. Histological Observation:

Effect of the *Lawsonia inermis* leaves extract and aluminium chloride on the Histology of the Testis (H&E) for each groups are shown plates 1 & 2 below.

Plates:

Group 1: As shown in plates 1 & 2, all features of normal testicular histology are observable in control.

Group 2: As shown in plates 1 & 2, all features of normal testicular histology are observable in rats given 60 mg/kg extract of *Lawsonia inermis*.

Group 3: As shown in plate 1 & 2, testicular histology exhibits abnormalities, the seminiferous tubules were degenerative (A); widened interstitial spaces and oedematous changes (B) separation of basement membrane from underlying layer with infiltrations by mononuclear cells (C).

Group 4: As shown in plates 1 & 2, all features of testicular histology of wistar rats given 0.5 mg of aluminium chloride and 60 mg/kg extract of *Lawsonia inermis*; showing few area of degeneration in the seminiferous tubules (A); Widening interstitial space (B) and Testicular necrosis (E).

Group 5: As shown in plates 1 & 2, all features of testicular histology of wistar rats given 0.5 mg of aluminium chloride and medium dose 75 mg/kg of *Lawsonia inermis.* An enlarged portion of seminiferous epithelial diameter reveals germ cells at different spermatogenic phases; Area of Vacoulation (V) and Testicular necrosis (E).

Group 6: As shown in plates 1 & 2, the portion of seminiferous epithelial diameter reveals Clusters of germ cells at different spermatogenic phases. Also visible were few areas of Vacuolation (V) and Testicular necrosis (E).

Group 7: As shown in plates 1 & 2, all features of testicular histology of wistar rats given 0.5 mg of aluminium chloride and 5 mg/kg Ascorbic acid 'Group 7' showing structure of normal histo-architectural appearance with seminiferous epithelial diameter reveals clusters of germ cells at different spermatogenic phases. Also visible were area of testicular necrosis (E) and vacuolation (V).

B. Histopathological Observation:

Modified Johnsen's score for Testicular Histopathology

Modified Johnsen's Scores as described by Holstein (26); The scores in percentage for Testicular Histopathology for each groups are shown in Table I and plate 3 below.

10 represents intact spermatogenesis with many spermatids matured and spermiation zones.

9 describes modestly reduced spermatogenesis: few mature spermatids, few spermiation zones.

8 implies a distinct reduction in spermatogenesis; that is decreased mature spermatids, no spermiation zones.

7 is awarded when there is considerably reduced spermatogenesis: immature spermatids only.

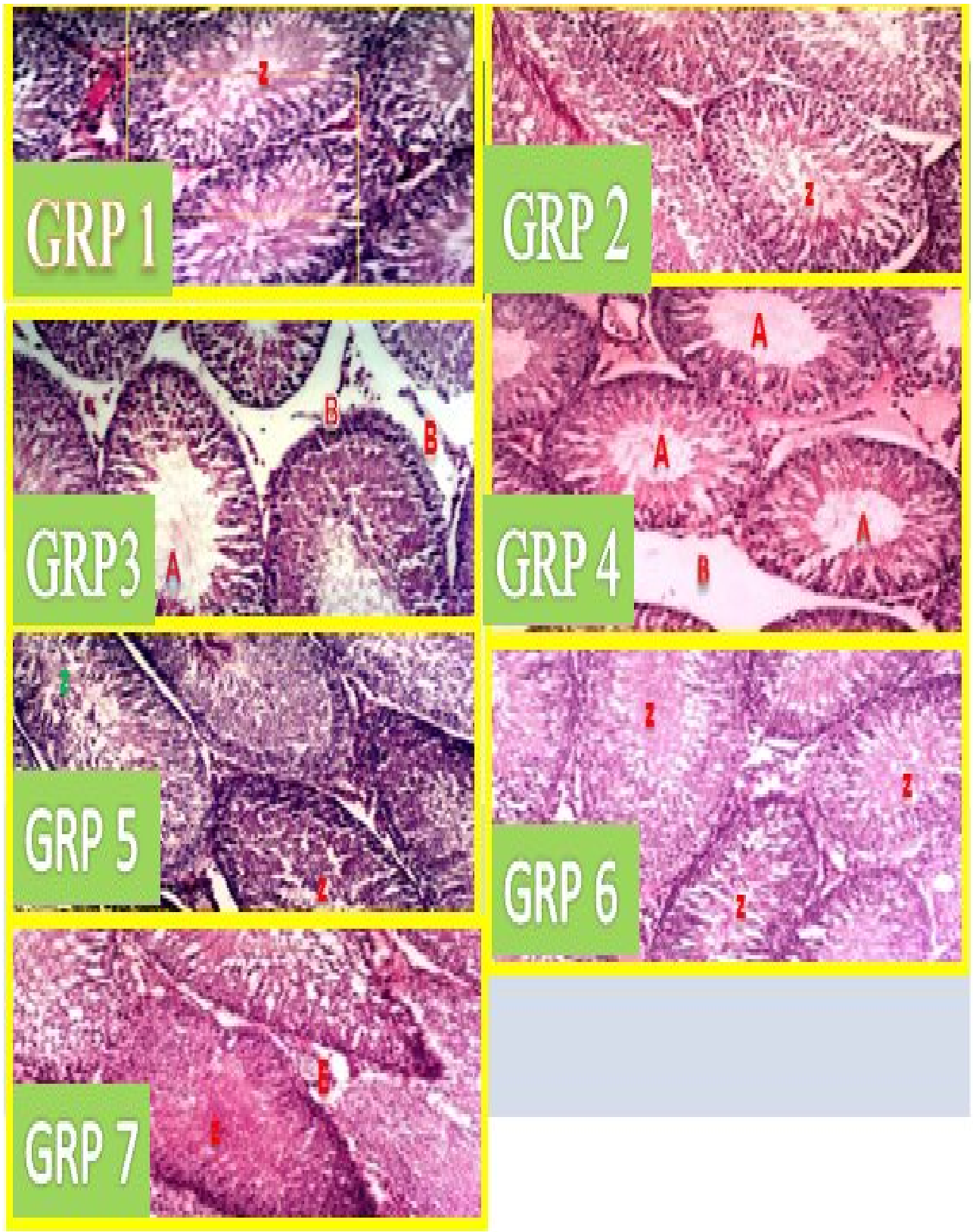

Plate 1 : Representative testicular micrograph of wistar rat showing structure of seminiferous tubules (Group 1-7): degenerative changes of seminiferous tubules (A); Widening interstitial space and diffuse edematous changes(B); Spermatozoa (Z), Stain is H&E and Magnification x100.

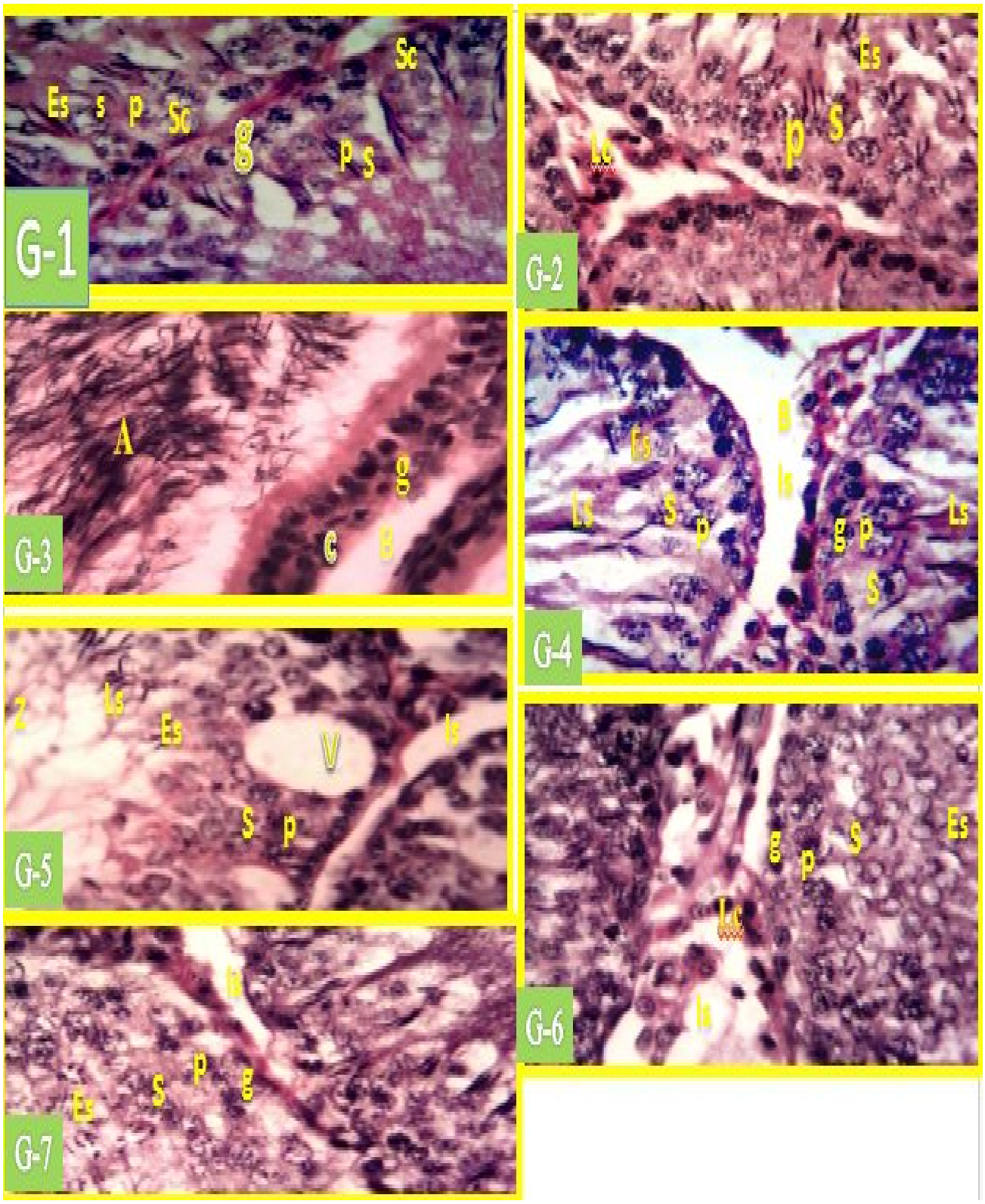

Plate 2 : Representative testicular micrographs of wistar rat; Groups 1-7. {Grp 3-showing degenerative changes of seminiferous tubules (A); Widening interstitial space and diffuse edematous changes (B) with mononuclear cells infiltrations besides basement membranes separating from the underlying layers(C)}. *Sc* = Sertoli cells, *Lc* = Leydig cell, *g* = spermatogonia, S = Secondary spermatocytes, *P* = Primary spermatocytes, Es = Early spermatid, Ls = Late spermatids, *z* = spermatozoa; Stain is H&E and Magnification x400.

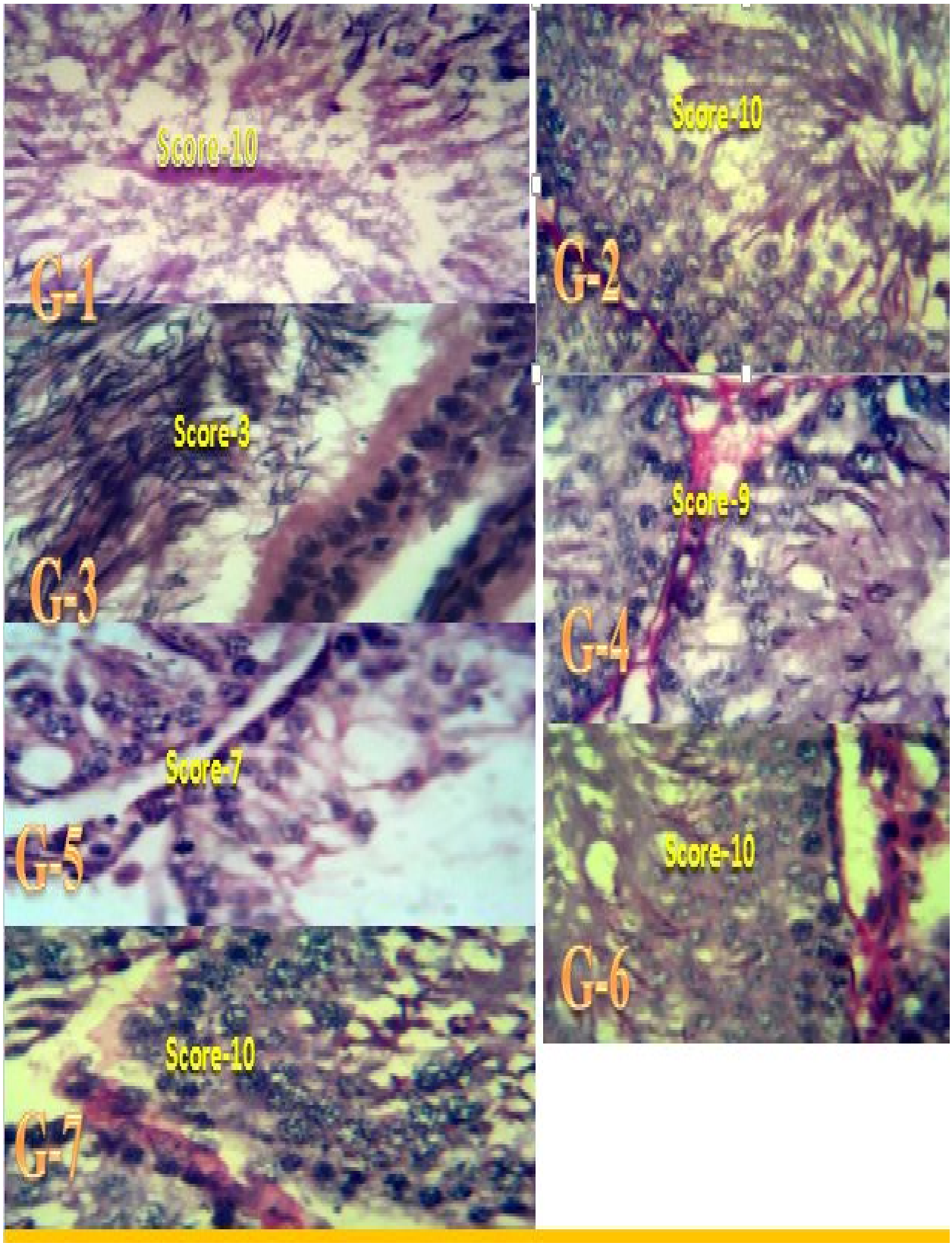

Plate 3 : Modified Johnsen's score 10, 10, 3, 9, 7, 10 and 10 for Testicular Histopathology photomicrographs; Group 1, 2, 3, 4, 5, 6 and 7. Staining is PAS-H and Magnification x400.

TABLE I: Modified Johnsen's score in percentage for Testicular Histopathology for each groups.

Group/Score	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Group 1						5.0	16.0	79.0
Group 2							10.0	90.0
Group 3	47.5	7.5			2.5	17.5	10.0	15.0
Group 4					1.5	8.5	10.0	80.0
Group 5			6.7	1.7	1.7	3.5	3.5	81.5
Group 6								100.0
Group 7								100.0

6 means severely reduced spermatogenesis: immature spermatids are few, seminiferous epithelial height low.

5 indicates that spermatogenesis at the stage of primary spermatocytes has been arrested: spermatocytes on the border of the luminal region.

4 implies arrest of spermatogenesis at the stage of primary spermatocytes: primary spermatocytes present are few.

3 describes that spermatogonia stage was arrested: Presence of A type spermatogonia do not develop into next germ cell.

2 means only sertoli cells are present.

1 awarded when neither germ cells nor Sertoli cells are found.

Discussion

The testicular histology of rats in the control group exhibited classical histo-architectural appearance with intact seminiferous tubules displaying normal seminiferous epithelia arrangements, also all the germ cells, intact basement membrane of the seminiferous tubules were all represented. The inter-tubular arrangement of the Leydig cell and other non-Leydig extratubular components are of the classical type described for normal testicular histology. Sertoli cells are observable, including spermatogonia at the basement membrane. Also visible are Secondary spermatocyte migrating to adluminal compartment. Primary spermatocytes with enlarged nuclei are observable too, with early spermatids and Late spermatids. Bundles of spermatozoa are seen in the lumen of seminiferous tubule. Cluster of Leydig cells are seen in the interstitial space. This pattern of normal testicular micro-architecture was also observed in animals that received 60 mg/kg extract of *Lawsonia inermis*. There seem to be some positive implications for the observation of normal histological appearance. Bundles of spermatozoa are seen in the lumen of seminiferous tubule indicating spermiation. It shows normal testicular micro-anatomy with full presence of phase of spermatogenesis, well arranged basement membranes, well distributed Leydig cells, and seminiferous tubules filled with sperm cells.

As shown in plates 1 and 2 (Group 3), testicular histology exhibits abnormalities, slight degenerative changes of seminiferous tubules; these changes include: widening of interstitial spaces with infiltration of oesinophilic cells, edematous vacuolated fluids, necrosis and diffuse edematous, aside the displacement membrane there were mononuclear cells infiltrations. Severe hypospermatogenesis or absolute arrest of spermatogenesis as bundle of spermatozoa seen is distorted compare to those of baseline.

In these current findings, the degenerative changes were reversed by *Lawsonia inermis* aqueous leaf-extract. Low dose of *Lawsonia inermis* aqueous leaf-extract with aluminium chloride showed mild recovery from severe histological changes.

As shown in plates 1 and 2 (Group 5), all features of testicular histology of wistar rat given 0.5 mg of aluminium chloride and medium dose 75 mg/kg of *Lawsonia inermis* aqueous leaf-extract. An enlarged portion of seminiferous epithelial diameter reveals germ cells at different spermatogenic phases. Sertoli cells are observable, including spermatogonia at the basement membrane. Also visible are Secondary spermatocyte migrating to adluminal compartment. Primary spermatocytes with enlarged nuclei are observable too, with early spermatids and late spermatids. Bundles of spermatozoa are seen in the lumen of seminiferous tubule indicating spermiation. Cluster of Leydig cells are seen in the interstitial space.

Specifically, and as shown Testicular histology of wistar rat given 0.5 mg of aluminium chloride and high dose 100 mg/kg of *Lawsonia inermis* aqueous leaf-extract. Plates 1 and 2 (Group 6), shows all features of normal testicular micro-anatomy with full presence of phase of spermatogenesis, well arranged basement membranes, Cluster of Leydig cells are well distributed in the interstitial space and seminiferous tubules filled with sperm cells. Sertoli cells are observable, including spermatogonia at the basement membrane. Also visible are Secondary spermatocyte migrating to adluminal compartment. Primary spermatocytes with enlarged nuclei are observable too, with early spermatids and late spermatids. Bundles of spermatozoa are seen in the lumen of seminiferous tubule indicating spermiation. This pattern of normal testicular micro-architecture was also observed in animals that received 0.5 mg of aluminium chloride and 5 mg/kg Ascorbic acid, as shown in plate 1 and 2 (Group 7).

A number of studies have evaluated the roles of *Lawsonia inermis* but not all of these studies have suggested a beneficial role for antioxidants therapy in male infertility. These findings proved that *Lawsonia inermis* aqueous leaf-extract could also perform the role of antioxidant relatively close to ascorbic acid.

Modified Johnsen's Scores as described by Holstein (26) was used to describe or analyse testicular histology qualitatively and quantitatively. Histological sections stained with Periodic acid-Schiff then counterstained with Hematoxylin (PAS-H); Modified Johnsen score was employed in histopathological evaluations of testicular tissue in other to determine effect of *lawsonia inermis* leave extract in aluminium-induced oxidative stress on the Histology of the testis. The Johnsen score grades seminiferous tubules on a scale of 1-10 so that the best histological appearance with evidence of full spermatogenesis is rated 10 while the worst histological appearance with absent seminiferous tubules is given a score of 1.

The current study summarized the score allocated to each seminiferous tubule in each of the groups (Table I). This score provides a means to lessen the often subjective qualitative description of testicular histology. While the use of qualitative description persists, Johnsen score provides objective basis for comparison between treated groups with control**.** Quantitative analysis of seminiferous tubules based on Johnsen's score was, therefore, applied to grade the effect of *lawsonia inermis* leaf-extract in aluminium- induced oxidative stress on the Spermatogenesis.

According to Table I above, the control rats, 79% have intact spermatogenesis with many mature spermatids and zones of spermiation, they were graded 10. 16% of the rats were scored 9 that is: they were described as having modestly reduced spermatogenesis:- reduced number of mature spermatids, a few zones of spermiation; While 5% were scored 8 which implies a distinct reduction in spermatogenesis: few mature spermatids, no spermiation. While the rats given 60 mg/kg extract of *Lawsonia inermis* were scored 9 for 10% and 10 for 90%. By considering the two groups, there were matured spermatids and clusters of spermatozoa in rats given 60 mg/kg extract of *Lawsonia inermis* than the control rat. This implies consumption of *Lawsonia inermis* enhanced Follicular stimulating hormone, Luteinizing hormone, Testosterone level of the rats. These hormones are extremely critical to spermatogenesis. The most common androgen in males is testosterone, with a primary function of sperm production and development of masculine characteristics. Hence, zone of spermiation and mature spermatids were more in rats with *Lawsonia inermis* aqueous leaf-extract than the control group.

The current study summarizes the score allocated to seminiferous tubule of the rats given 0.5 mg of aluminum chloride per kg of body weight were scored 3, 4, 7, 8, 9 and 10 which are: 47.5%, 7.5%, 2.5%, 17.5%, 10% and 15%, table 1. While score 3 were the highest 47.7%, which means 47.5% depicts arrest at the stage of spermatogonia: A type spermatogonia multiply but do not develop to maturing cells of spermatogenesis.

The rats given 0.5 mg of aluminium chloride per kg of body weight affected the spermatogenesis. If this effect could be seen in sertoli, cells it may not be

reversible. Concomitant feeding of rats with 0.5 mg of aluminum chloride per kg of body weight and low dose 60 mg/kg of *Lawsoni ainermis* provided ameliorating effect or detoxification effect against heavy metal poisoning. This was greatly seen when comparing the results of aluminum poisoned group with this recent group with addition of *Lawsonia inermis*. The scores are: 7, 8, 9, 10 which are: 1.5%, 8.5%, 10% and 80%.

According to Nasir (23) *Lawsonia inermis* contain flavonoids which are a good antioxidant. The broad therapeutic effects of flavonoids can be largely attributed to their antioxidant properties. In line with the current finding *Lawsonia inermis* ameloriate/ detoxify the effect of aluminium poison and according to Table I, score 10 was the highest having 80%, which means majority of the seminiferous tubules have intact spermatogenesis with many mature spermatids and zones of spermiation.

The rats given 0.5 mg of aluminium chloride per kg of body weight and medium dose 75 mg/kg of *Lawsonia inermis* were scored: 5, 6, 7, 8, 9 and 10; According to Table I, score 10 was the highest having 81.5%, which means majority of the seminiferous tubules have intact spermatogenesis with many mature spermatids and zones of spermiation.

Lastly, the rats given 0.5 mg of aluminium chloride per kg of body weight with highest dose 100 mg/kg of *Lawsonia inermis* and also the rats given 0.5 mg of aluminium chloride per kg of body weight with 5 mg/kg Ascorbic acid were having the same score 10, according to table 1, 100%; which means all the seminiferous tubules have intact spermatogenesis with many mature spermatids and zones of spermiation. However, in order to protect the cells from oxidative radical elements, a complex and sophisticated antioxidant protection system evolved. Meanwhile, it is necessary to point out that Johnsen score was found to be of limited application as far as the whole histological appearance of the testis was concerned. For instance Johnsen score could not grade the presences of cellular debris, apoptosis, autophagic and residual bodies.

Conclusion

In this current study full knowledge of negative effect of aluminium has been demonstrated as it affects the testicular architecture where stress impacts were most felt. The therapeutic effect of *Lawsonia inermis* aqueous leaf-extract in amelioration was well elucidated. Indeed, *Lawsonia inermis* aqueous leaf-extract has proved to be a potent antioxidant.

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