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Protective effects of *Citrullus lanatus* seed ethanol extract on aluminum chloride-induced testosterone, testicular and hematological changes in an experimental male rat model

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Abstract

The study was done to ascertain the protective potentials of ethanol seed extract of *Citrullus lanatus* on aluminum chloride-induced reproductive and hematological toxicities. Thirty mature male rats were used for the study. They were assigned into five groups (n = 6). Group 1 was treated daily with aluminum chloride (100 mg kg^{-1}) *per os* for 8 weeks. Group 2 was treated with aluminum chloride (100 mg kg^{-1}) and *C. lanatus* seed extract (CLSE) 200 mg kg^{-1} *per os* simultaneously for 8 weeks. Group 3 was served as a normal control and given distilled water as a placebo *per os* daily for 8 weeks. Group 4 was only treated with CLSE (200 mg kg^{-1}) for eight weeks. Group 5 was only treated with aluminum chloride (100 mg kg^{-1}) *per os* for 8 weeks and then treated with CLSE (200 mg kg^{-1}) *per os* for another 4 weeks. Testosterone level, testicular weight, sperm motility, gonadal sperm, and extragonadal sperm reserves showed significant increases in group 2 compared to groups 1 and 5. Optimum histoarchitectural protection of the seminiferous tubules was observed in group 2, which did not differ from normal ones. For the hematological parameters, optimum protection was also observed in group 2 compared to other groups. From the results, ethanol seed extract of *C. lanatus* demonstrated protective potentials against aluminum's harmful effects on the male reproductive system and hematology in an experimental male rat model.

Introduction

Reproductive toxicity in animals is a sub-clinical disease affecting production due to increasing industrialization, and other human activities. Infertility in herds due to aluminum toxicity is of substantial economic importance to farmers. This infertility of animals can be attributed to the increasing environmental accumulation of this heavy metal when animals contact it through field grazing.[1,2] Aluminum is the third most abundant element in the earth's crust, being highly abundant in the environment.[3] This increases the chances of exposure to man and animals.[4] This heavy metal accumulates and alters the integrity of cells altering their function.[5] Wide use of aluminum by industries to produce drugs, vaccines, cosmetics, food additives, preservatives, animal cages, and feeders also increase its chances of toxicity to animals.[6] Animals get exposed through inhalation and ingestion. Aluminum toxicity induces cognitive deficiency and dementia in the nervous system.[7] The reproductive toxicity of aluminum causes changes in cellular integrity of sperms through aluminum induced oxidative damage leading to sperm and seminal deficiencies.[8] Cannata has reported that aluminum is the leading causative agent of impaired reproductive function associated with testicular changes in male animals.[9] Decreased hemoglobin synthesis is an indicator of elevated systemic aluminum concentration leading to anemia.[10] Abou-Seif has concluded that elevated aluminum levels induce changes in the properties of red blood cell (RBC) membrane, causing peroxidation of membrane lipids and increased osmotic fragility.[10] It has been reported that an adequate intake of vitamin-rich foods helps minimize the oxidative stress of heavy metals.[11,12] *Citrullus lanatus* belongs to the *Cucurbitaceae* family, and its common name is watermelon. Its fruit is conventionally

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and readily consumed in our society and Nigeria at large. It is usually consumed fresh in slices, diced in mixed fruit salads or as juice, and can be blended with other fruit juices or made into wine. Some people eat the seeds alongside the fruit, while most persons spit it out. The *C. lanatus* seed, according to Deshmukh *et al*., has anti-inflammatory, antioxidant, anti-microbial, anti-ulcer, and laxative effects.[13] The *C. lanatus* seeds, according to a report by Khaki *et al*., can increase sperm motility, viability, and concentration in normal rats.[14] Olamide *et al*. have reported a significant decrease in the enlarged prostate in the study to know the effects of methanol seed extract of *C. lanatus* on experimentally induced benign prostate hyperplasia in adult male Wistar rats.[15] Hence, this study aimed to determine the protective effects of *C. lanatus* seed extract (CLSE)on the toxic effects of aluminum on the reproductive system and hematology of male rats.

Materials and Methods

Aluminum chloride. The used aluminum chloride was purchased from Joe Chem Ventures Ltd., University of Nigeria, Nsukka, Nigeria.

Preparation of *C. lanatus* seed extract. Watermelon seeds were obtained from watermelon pods bought from Ikpa Industrial Market, Nsukka, Nigeria, and confirmed by a taxonomist of the Bioresources Development and Conservation Center, Nsukka, Nigeria. A voucher specimen was deposited in the center (No. 1783). The seeds were dried under a shade, finely ground using an electric blender, and extracted by cold maceration using 80.00% ethanol with intermittent shaking every 2 hr for 48 hr. It was filtered using No. 1 filter paper (Whatman, Maidstone, UK). The filtrate was dried in the oven at 37.00 ˚C and stored in a refrigerator throughout its use.

Experimental animals. Thirty male albino rats at 12 weeks of age weighing between 180 - 200 g were used for the study. These laboratory animals were procured from the Department of Veterinary Anatomy, University of Nigeria, Nsukka, Nigeria, and housed at room temperature (25.00 ± 2.00 ˚C) and 12 hr light/dark cycle in clean cages at the laboratory animal unit of the Department of Veterinary Physiology and Pharmacology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Nigeria. The animals were acclimatized for two weeks before the commencement of the study. The laboratory animals were well-fed *ad libitum* on commercial vital grower feed and clean water.

Ethical approval. Ethical guidelines for using experimental animals were observed and approved by the ethical committee of the University of Nigeria, Nsukka, Nigeria (approval Ref No. 2018/1815).

Experimental design. Group 1 was treated daily with aluminum chloride (100 mg kg^{-1}) *per os* for 8 weeks.[9] Group 2 was treated daily with aluminum chloride (100 mg kg^{-1}) and CLSE (200 mg kg^{-1}) simultaneously *per os* for 8 weeks.[14] Group 3 was served as a normal control and given distilled water as a placebo *per os* daily for 8 weeks. Group 4 was only treated with CLSE (200 mg kg^{-1}) orally for eight weeks. Group 5 was only treated with aluminum chloride (100 mg kg^{-1}) *per os* for 8 weeks and then treated with CLSE (200 mg kg^{-1}) *per os* for another 4 weeks.

Determination of testosterone level. At the end of the study period, serum samples from the rats were subjected to testosterone assay according to the method of Guo *et al.* using enzyme linked immunosorbent assay test kit (Cayman Chemical, Ann Arbor, USA).[16]

Determination of testicular weight. At the end of the study period, rats from each group were euthanized using pentobarbital sodium (Euthathal®; 180 mg kg^{-1}; Merial Animal Health Ltd., Dublin, Ireland). The testis from each rat was carefully dissected out, trimmed off of extraneous tissues, and weighed with a weighing balance (Nikon, Tokyo, Japan).

Determination of sperm motility and count. A drop of sperm sample from the caudal epididymis was placed on a pre-warmed slide, covered with a coverslip, mounted, and viewed under 40× microscopic magnification. Sperm cells moving in a unidirectional movement were counted, and those showing jerky movements, circular motion, or backward movements were disregarded.[17] The gonadal and extra-gonadal sperm counts were determined using the method described by Pant and Srivastava.[18] The testis and epididymis were crushed for gonadal and extra-gonadal sperm reserves, respectively, with ceramic mortar and pestle. Each crushed sample was diluted with 10.00 mL of normal saline, filtered with a nylon sieve, and 0.10 mL of the sperm solution was made up to 1.00 mL with white blood cell diluting fluid. This was then used to charge the Neubauer chamber and viewed under a microscope at 10×. The sperm cells were counted in 4 corner squares, estimated in 169 squares, and multiplied by 10.

Histopathological evaluation. The rats' testes specimen were taken from all groups, fixed by immersion into Bouin's fluid, dehydrated in graded ethanol concentration, cleared in xylene, and embedded in paraffin wax. Then, 5.00 µm sections were cut, picked up with slides, and stained with Hematoxylin and Eosin for light microscopy.[19] Photomicrographs were captured using Motic Images Plus software (version 2.0; Novex, Groningen, The Netherlands).

Packed cell volume (PCV) evaluation. The hematocrit technique, according to Schalm *et al*., was used.[20] Blood samples from the rats were collected into EDTA-contained sample bottles. Capillary tubes were filled with the blood, spun at 10,000 rpm for 5 min with the microhematocrit centrifuge (Leja, Amsterdam, The Netherlands), and read with a reader. Values were expressed in percentage.

Evaluation of RBC count. A Hemocytometer technique, according to Dacie and Lewis, was used.[21] Blood samples from the rats were collected into EDTA-contained sample bottles. The blood was drawn to the 0.50 mark on the stem of the red cell pipette, and red blood diluting fluid was sucked up to the 101 marks immediately above the bulb rotating the pipette in the process, while the outside of the pipette was wiped out with a piece of clean gauze. The tip of the pipette was closed with a thumb and mixed well by shaking. The sample was used to charge the Neubauer chamber and viewed under a microscope at 40×, and RBCs were counted in five tertiary squares.

Evaluation of total leukocyte count. A Hemocytometer technique, according to Dacie and Lewis, was used.[21] The sample was prepared as in RBC count, used to charge the Neubauer chamber, and viewed under a microscope at 10×, and the white blood cells (WBCs) were counted in four corner squares.

Determination of hemoglobin concentration. Hemoglobin concentration was evaluated according to the technique of Schalm et al.[20] Drapkin solution (5.00 mL) was added to a clear test tube. Then, 0.02 mL of the blood sample was added to the solution and mixed thoroughly. The mixture was allowed to react for 20 min, and the absorbance was read at 540 nm against a reagent blank on a digital colorimeter (Shimadzu, Tokyo, Japan). The blood sample's hemoglobin concentration was obtained by multiplying the sample's absorbance with the calibration factor derived from the absorbance and the standard's concentration. Values were recorded in g dL^{-1}.

Hematological indices. Mean corpuscular volume (MCV) was determined by finding the ratio of the hematocrit to the RBC count, mean corpuscular hemoglobin concentration (MCHC) was determined by finding the average concentration of hemoglobin in an average of RBC, and mean corpuscular hemoglobin (MCH) was calculated by dividing the total mass of hemoglobin by the number of RBC in a volume of blood.

Statistical analysis. The software, SPSS (version 22.0, SPSS Inc., Chicago, USA), was used for analysis. Data from the study were analyzed using One Way Analysis of Variance. The variant means were separated using Duncan multiple range test. The level of significance was accepted at $p < 0.05$.

Results

Reproductive parameters. The testosterone level of group 2 increased significantly ($p < 0.05$) compared to groups 1 and 5 but did not differ significantly ($p > 0.05$) compared to groups 3 and 4. The testosterone level of group 4 increased slightly above that of group 3; although, in a non-significant manner (Fig. 1). Animals in group 1 showed a significant decrease ($p < 0.05$) in testicular weight compared to groups 2, 3, and 4 but showed no significant difference ($p > 0.05$) compared to group 5. The testicular weight of group 2 increased significantly ($p < 0.05$) compared to groups 1 and 5. The testicular weight of group 4 significantly increased ($p < 0.05$) compared to group 3 (Table 1). Groups 2, 3, and 4 showed significant increases ($p < 0.05$) in sperm motility compared to groups 1 and 5. There was no significant difference ($p > 0.05$) in sperm motility of group 1 compared to group 5, and there was no significant difference ($p > 0.05$) in sperm motility among groups 2, 3, and 4 (Table 1). The gonadal sperm reserves of group 2 were slightly higher than those of groups 1 and 5; although, not significantly ($p>0.05$). The gonadal sperm reserve of group 4 significantly increased ($p < 0.05$) compared to group 3 (Table 1). The extra-gonadal sperm reserves of group 2 increased significantly ($p < 0.05$) compared to groups 1 and 5 and did not differ significantly ($p > 0.05$) from those of normal animals (Table 1).

Hematological parameters. Group 2 showed a significant increase ($p < 0.05$) in PCV compared to animals in groups 1 and 5 (Table 2). The RBC of animals in group 2 showed a significant increase ($p < 0.05$) compared to group 1 but showed no significant difference ($p > 0.05$) compared to group 5. The RBC of group 2 showed no significant difference ($p > 0.05$) than groups 3 and 5. Group 4 showed a slight increase in PCV and RBC compared to group 3; although, not significantly (Table 2). The hemoglobin concentration of animals in group 2 increased significantly ($p < 0.05$) compared to that of group 1 but did not differ significantly ($p > 0.05$) from that of group 5. There was no significant difference ($p > 0.05$) in the hemoglobin concentration of groups 3 and 4. The WBCs of group 2 increased significantly compared to groups 1 and 5, but did not differ significantly from those of normal animals (Table 2). The MCV, MCH, and MCHC of group 2 increased significantly compared to group 1, but did not differ significantly compared to group 5 (Table 2).

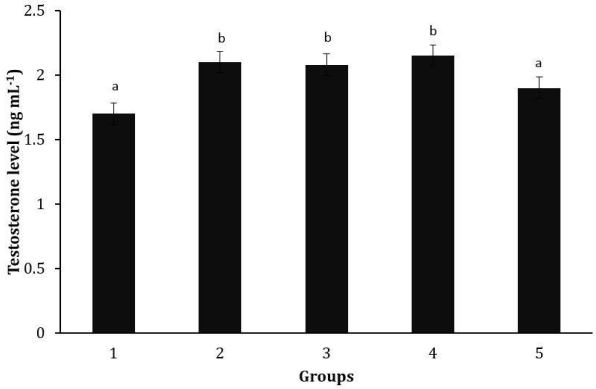

Fig. 1. Effects of *Citrullus lanatus* seed extract (CLSE) on testosterone levels of the male rats. 1: Aluminum chloride alone, 2: Aluminum chloride and CLSE simultaneously, 3: Distilled water, 4: CLSE alone, 5: Aluminum chloride alone for 8 weeks, then CLSE for another 4 weeks. [ab] Different letters indicate significant differences in each row ($p < 0.05$).

Table 1. Effects of *Citrullus lanatus* seed extract on testicular weight, sperm motility, and gonadal and extra-gonadal sperm reserves.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5
Testicular weight (g)	2.30 ± 0.00^{a}	2.60 ± 0.00^{b}	2.80 ± 0.00^{c}	2.97 ± 0.03^{d}	2.27 ± 0.07^{a}
Sperm motility (%)	20.00 ± 5.77^{a}	65.00 ± 2.89^{b}	70.00 ± 5.77^{b}	76.67 ± 3.33^{b}	30.00 ± 0.00^{a}
Gonadal sperm reserve ($\times 10^{6}$)	0.32 ± 0.61^{a}	0.35 ± 0.70^{a}	1.06 ± 0.12^{b}	8.98 ± 0.18^{c}	0.32 ± 0.06^{a}
Extra-gonadal sperm reserve ($\times 10^{6}$)	5.07 ± 0.42^{a}	20.35 ± 1.78^{b}	23.17 ± 2.99^{b}	19.22 ± 0.24^{bc}	13.52 ± 3.17^{c}

^{abcd} Different superscripts indicate significant differences in each row ($p < 0.05$).

Table 2. Effects of *Citrullus lanatus* seed extract on hematological parameters.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5
Packed cell volume (%)	19.67 ± 1.20^{a}	33.67 ± 0.67^{b}	36.33 ± 0.33^{c}	37.33 ± 0.33^{c}	28.33 ± 0.88^{d}
Red blood cell ($\times 10^{6}$)	5.38 ± 0.66^{a}	6.96 ± 0.10^{b}	7.68 ± 0.29^{abc}	8.52 ± 0.23^{c}	6.43 ± 0.46^{ab}
Hemoglobin (g dL^{-1})	8.22 ± 1.00^{a}	12.25 ± 0.29^{b}	15.28 ± 1.04^{c}	16.87 ± 0.27^{c}	11.70 ± 1.02^{b}
White blood cell ($\times 10^{3}$)	7.83 ± 0.69^{a}	17.27 ± 1.08^{b}	14.50 ± 0.14^{bc}	14.75 ± 0.84^{bc}	13.68 ± 1.60^{c}
Mean corpuscular volume	28.04 ± 2.77^{a}	39.92 ± 1.70^{bd}	47.54 ± 2.63^{cd}	50.29 ± 2.95^{c}	38.55 ± 3.42^{b}
Mean corpuscular hemoglobin	13.39 ± 0.96^{a}	16.89 ± 0.20^{b}	21.70 ± 1.46^{c}	24.14 ± 0.59^{c}	16.55 ± 0.53^{b}
Mean corpuscular hemoglobin concentration	36.16 ± 0.39^{a}	44.32 ± 0.58^{b}	45.61 ± 1.18^{b}	51.35 ± 3.65^{c}	41.98 ± 0.02^{b}

^{abcd} Different superscripts indicate significant differences in each row ($p < 0.05$)

Histopathological findings. In group 1, there was desquamation of the seminiferous epithelium showing thinness of the basal laminae. There was widened inter-tubular connective tissue devoid of Leydig cells, and the lumen of the seminiferous tubules was wide with few germinal cells of the spermatogenic cycle (Fig. 2A). In group 2, signs of protection are evident as well-defined tubular lining, intertubular connective tissue, and cell content. Spermatozoa and elongated spermatids are discernible in the tubular lumen (Fig. 2B). Tubular epithelial appears thicker than those in Figure 2A, with a wider diameter. Figures 2C and 2D, representing groups 3 and 4, are comparable with the well-defined histo-architecture of the seminiferous tubule and its content. In group 5, evidence of recovery was obvious with re-population of tubular germ cells, even though the tubular lumen is still wide and devoid of mature sperm cells and spermatids in some seminiferous tubules (Fig. 2E).

Discussion

The significant increase in the testosterone levels of group 2 compared to groups 1 and 5, which did not differ significantly from those of the normal animals, may be responsible for the significant increases in testicular weight and extragonadal sperm reserve of group 2 compared to groups 1 and 5. Testosterone is very vital in male reproduction due to its role in the maintenance of normal sperm development.[22]

The significant decreases in the testicular weight of groups 1 and 5 compared to group 2 may be due to atrophy of the testis caused by aluminum toxicity. This result is in tandem with El-Ashmawy *et al.* and Bataineh *et al.* suggesting that aluminum causes a decrease in testis weight.[23,24] The CLSE protected the testicles from the aluminum toxicity; hence, a significant increase in group 2 was seen. It was also observed that there was a significant

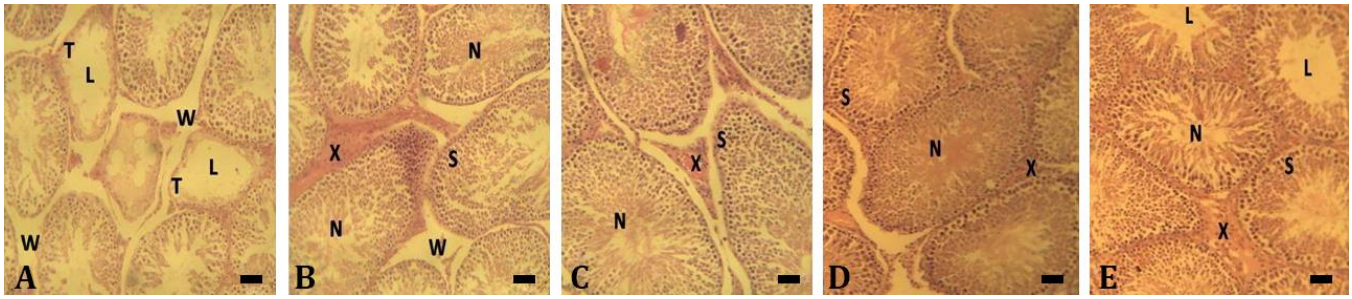

Fig. 2. A) Microscopic cross-section of the testes from group 1 (aluminum only) showing loss of germ cells (L), the thinness of the basal laminae (T), and widened inter-tubular connective tissue, devoid of Leydig cells (W), **B)** Testicular tissue of group 2 (aluminum and *Citrullus lanatus* seed extract [CLSE]) with evidence of protection showing intact tubular lining (S), intact inter-tubular connective tissue (X) and numerous germ cells (N); although there is still little trace of widened inter-tubular connective tissue (W), **C)** Microscopic cross-section of the testes from group 3 (normal healthy rats given distilled water), **D)** Testicular tissue of group 4 (normal healthy rats given CLSE alone), **E)** Microscopic cross-section of the testis of group 5 (aluminum and later CLSE). Testicular tissues of figures C and D are comparable showing seminiferous tubules with numerous germ cells (N), intact tubular lining (S) and intact inter-tubular connective tissue (X). Testicular tissue of Figure E shows evidence of healing and repopulation of germ cells in the seminiferous tubules (N), intact tubular lining (S) and intact inter-tubular connective tissue (X); even though the tubular lumen is still wide and devoid of structurally mature sperm cells and spermatids in some seminiferous tubules (L), (H&E, Scale bars = 100 µm).

increase in testicular weight of group 2 compared to group 5, showing that treatment with CLSE from the first day of aluminum toxicity induction produces a better amelioration than allowing the toxicity to occur before commencement of treatment with CLSE. The significant increase in testicular weight of group 4 compared to group 3 shows that CLSE improves testicular weight in normal animals.

The sperm motility of animals in groups 2 and 4 did not differ significantly from that of normal animals but significantly increased compared to groups 1and 5. This can be attributed to the antioxidant property of the CLSE, which may have inhibited reactive oxygen attack and lipid peroxidative stress of aluminum in group 2. Oxidative stress and lipid peroxidation lead to changes in spermatozoa membranes leading to decreases in sperm motility, concentration and viability.[25,26] Aluminum decreases sperms viability by affecting the sperm cells DNA, damaging acrosomes and rendering them non-viable.[27,28]

Watermelon is rich in phytonutrients (phenolics, carotenoid compounds, and some amino acids), having antioxidant functions.[11] The significant increases in testicular weight and gonadal sperm reserve of group 4 compared to group 3 showed that CLSE might have boosted spermatogenesis in normal animals. This agrees with the report of Khaki *et al*. showing that CLSE enhances reproductive parameters in normal animals.[29] The significant increase in the extra-gonadal sperm reserve of group 2 compared to groups 1 and 5 showed that CLSE protected the sperm cells in group 2, and treatment with CLSE from the first day of aluminum toxicity induction produced a better amelioration than allowing the toxicity to occur before commencement of treatment with CLSE. However, the testis' histopathological findings elucidated the results of the testicular weights, sperm motility, and sperm reserves. Photomicrograph of group 1 testicular section showed seminiferous tubules with a widened tubular lumen and thinner tubular epithelium due to depletion of germ cells; this might have led to the meager testicular weight, sperm motility, and gonadal and extra-gonadal sperm reserve values observed in this group. This agrees with studies done by Buraimoh *et al.* and Thirunavukkarasu *et al*. reporting that aluminum treatment in rats causes changes in seminiferous tubules and spermatogenic and Sertoli cells degeneration.[30,31] There was also an erosion of intertubular connective tissue in group 1; thus, Leydig cells' loss was noticeable. This may have led to the decrease in testosterone level observed in this group. Leydig cells secret testosterone when stimulated by luteinizing hormone.[32] Testosterone then stimulates genes in Sertoli cells inducing spermatogonia differentiation and type a spermatogonia formation. The conversion of primary spermatocytes into secondary spermatocytes is dependent on testosterone.[31] The testicular section of group 2 showed seminiferous tubules with thick epithelial lining and normal lumen, evident of the healthy testis. Co-administration of CLSE with aluminum in group 2 may have protected the deleterious effect of aluminum on the testis; thus, no observable change in testicular morphology was recorded.

Further, the recorded significant increases in sperm motility, testicular weight, and extra-gonadal sperm reserve compared to groups 1 and 5 were observed. This may be attributed to the antioxidant activity of *C. lanatus*.[33] The testis section in group 5 showed evidence of recovery with repopulation of tubular germ cells, even though the tubular lumen is still wide and devoid of structurally mature sperm cells and spermatids in some seminiferous tubules. This suggests healing of the damaged seminiferous tubules as they are repopulated with germ cells with sound epithelial linings. The CLSE treatment given to this group may have possibly tried to re-instate spermatogenesis as a significant increase was recorded in the extra-gonadal sperm reserve of group 5 compared to that of group 1.

For hematology, groups 3 and 4 showed no significant difference in PCV but showed a significant increase compared to other groups. The significant decreases in PCV of groups 1 and 5 compared to group 2 may be due to changes in the RBC membranes caused by aluminum. Aluminum toxicity has been reported to cause anemia.[34,35] It was also reported that chronic treatment with high aluminum levels interferes with iron metabolism.[36-38] Morphological changes in RBC membrane have been reported in rats treated with aluminum-citrate.[2,39] Watermelon may have limited the lipid peroxidative effect in the RBC membranes of aluminum treated animals; thus, the PCV value of group 2 was significantly higher compared to groups 1 and 5. This showed that watermelon protected the RBCs against oxidative stress of aluminum toxicity. The result also suggests that it prevented toxicity from occurring when given from induction day than allowing the toxicity to occur before treatment. The significant increases in PCV and RBC of group 2 compared to group 1 and increases in PCV and RBC of group 4 compared to group 3 may be due to boosted erythropoiesis through an elevated level of testosterone in groups 2 and 4. Testosterone has been reported to boost erythropoiesis through increased synthesis of erythropoietin in the kidney.[40] The significant decrease in hemoglobin concentration of group 1 compared to groups 2 and 5 may be due to aluminum interference with iron metabolism, and such interference may have been protected by CLSE in groups 2 and 5.

The significant increase in the WBC of group 2 compared to groups 1 and 5 may be due to immune stimulation produced by the adjuvant action of aluminum and CLSE. The significant decreases in MCV and MCHC of group 1 compared to group 2 may be due to aluminum induced anemia in group 1.

In conclusion, the results showed that CLSE protected the animals from aluminum-induced reproductive dysfunction and hematological changes. The results also showed that CLSE improved reproductive parameters and hematology in normal male rats. Finally, the results showed that CLSE prevented aluminum toxicity from occurring when given from the first day of induction than allowing the toxicity to occur before treatment. This suggests that *C. lanatus* supplementation in livestock feed can protect the animals from harmful effects of aluminum on reproductive organs and hematology; hence, it can lead to sound reproductive performance and good health.

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Conflict of interest

The authors declare that no conflict of interest was encountered with the study.

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