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Positive Ki-67 Expression of Alkaloid Fractionate of *Cyperus esculentus* on Lead-induced Testicular Toxicity in Male Albino Rat

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ABSTRACT

The study investigated the positive Ki67 expression of alkaloid fractionates of *C.esculentus* on the testis of male albino rats. Forty adult male albino rats were grouped into four (4) (n=10). Group 1 was given saline only plus feed and water, and Group 2 was given 30 mg/kg body weight of lead acetate compound plus feed and water; Group 3 was given 50 mg/kg body weight of alkaloid fractionate of *C. esculentus* and 30 mg/kg body weight of lead plus feed and water and group 4 was given 100 mg/kg body weight of alkaloid fractionate of *C. esculentus* and 30 mg/kg body weight of lead acetate compound plus feed and water orally. This experiment was conducted for 4 weeks. The sacrifice of experimental animals was taken 24 hours after the experiment, and testes were harvested for histological and immunohistochemistry studies using the H&E and Ki67 immunostain, respectively. Histology and immunohistochemistry of the testes, sperm parameters, sex hormones, and levels of malondialdehyde (MDA), catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD) were the parameters used for the investigation.. According to the results, there was positive expression in Leydig cells and also in an increased series of spermatogenesis of the testes in the rats of groups administered with lead and alkaloid fractionate of *Cyperus esculentus* different doses 3 and 4 compared to lead acetate control group. A significant increase in the level of hormones suhc as total testosterone (TT), follicle-stimulating hormone (FSH), and luteinizing hormone (LH). levels in groups 3 and 4 ($p< 0.05$) compared to the lead acetate control group (2) was recorded. MDA levels significantly increased in the treated group(2). In groups treated with lead acetate and alkaloid fractionate of C. esculentus, positive expression was observed in the seminiferous epithelium and in sperm quality. In conclusion, This study demonstrated positive Ki-67 expression of alkaloid fractionate of *C. esculentus* on testicular lead-induced toxicity in albino rats.

INTRODUCTION

DLead is a known environmental toxin that negatively impacts the reproductive system [1]. It has an adverse effect on body system that may lead tohistological, biochemical and physiological abnormalities in our body system [1]. Exposure to lead can cause oxidative stress, apoptosis, reduced spermatogenesis. Chronic lead exposure is associated with decreased fertility and damage to testicular cells [1]. Toxicity of the lead in every society isvery common because of unavoidable exposure to environment and occupation [2]. The induction of oxidative stress is the mechanism that led tolead toxicity [3]. An imbalance in between removal and generation of ROS in cellular components of an organism causes to proteins, DNA, and membranes which result in oxidative stress [4]. Generation of ROS including superoxide radicals, hydrogen peroxide and hydroxyl radicals and lipid peroxides by lead result in oxidative stress [5].

Medicinal potentials of many plants have been screened by many researchers [6]. Plant parts and their products including fruits and vegetables serve as source of nutrients and balance diet [7]. The use of different parts and products of plants in disease treatment provides a safer therapy, affordable and very

commonly available [8,9]. *C. esculentus* is known for it diverse medicinal and nutritional benefits [1,9]. *C. esculentus* is known as 'Aki awusa' in Igbo language, 'Aya' in Hausa language and ofio or imumu in Yoruba language tribes of Nigeria. *C. esculentus* is consumed due believe of it aphrodisiac and fertility potentials [9]. *C. esculentus* was reported to have protective and amelioriativeeffect on testicular tissue [9, 10].

The qualitative phytochemical screening of *C. esculentus* tuber extracts revealed the presence of the following bioactive compounds: alkaloids, tannins, saponins, phenols, and steroids [9,11,12]. Alkaloids are natural organic nitrogen-containing bases. The diversity of alkaloids recorded has important physiological effects on our body system. Alkaloids are organic compounds found primarily in plants.

Alkaloids are known for their pharmacological properties, including antimicrobial and analgesic activities [13]. Alkaloids contribute to the plant's traditional medicinal uses and suggest potential for further exploration as a natural source of therapeutic agents [1]. Thus, this study was designed and carried out to investigate the Ki67 positive expression of alkaloid fractionate of *C. esculentus* on Lead-Induced testicular damage in male albino rats.

MATERIALS USED AND METHODOLOGY

Chemicals

Chemicals used for this study were purchased from Sigma-Aldrich (USA). The assay kits used for the analysis of Oxidative stress markers were purchased from registered and well-known distributors in Osogbo, Osun State.

Plant material collection and extraction of alkaloid from *Cyperus esculentus*

Cyperus esculentus tubers purchased at Ilesa Central Market, Ilesa, Osun State, Nigeria. The tuber identification and authentication were done to validate scientific merit at the Biological Science department, Faculty of Science, University of Ilesa, Ilesa, Osun State. *C. esculentus is* freshly procured and screened thoroughly to remove stones and debris. The tubers were dried at room temperature and weighed. Dried *C. esculentus* was ground to powder form using a mechanical grinder and stored in airtight containers at room temperature for subsequent analysis. Powdered *C. esculentus* was then defatted using petroleum ether in a Soxhlet extractor for about 2 hours to remove lipids and other nonpolar compounds. The defatted plant material was dried under reduced pressure at standard temperature for 2 hours and stored for further extraction [14].

The defatted powdered material was subjected to alkaloid extraction using an acid-base extraction method. The defatted powder was macerated in dilute sulphuric acid for 6 hours at room temperature, with intermittent shaking. The mixture was then filtered using Whatman filter paper No. 1, and the filtrate was collected. Basification followed acid extraction. The filtrate was basified by adding ammonia to liberate the free alkaloids; the mixture was stirred thoroughly to ensure complete basification. The organic layer collected was pooled and dried over anhydrous sodium sulphate. This was followed by concentration, in which organic solvent was removed at low pressure using a rotator evaporator at room temperature. Silica gel was used for the purification of the resulting alkaloid under column chromatography [14].

Alkaloid identification and Quantification was done to confirm the presence of alkaloids. Two drops of Mayer's reagent were added into the test tube containing 20g of the isolated extract. The presence of alkaloids was indicated by the observation of a white creamy precipitate [15].

Research design

Forty (40) albino male rats with an average weight of 150g were procured at the research animal house, Department of Anatomy, University of Ilesa, Ilesa, Osun, Nigeria. The rats were caged and fed and acclimatised for two weeks. The study was a randomized controlled trial involving four groups (n=10). Group 1 was given 1 mL of saline orally for four weeks. Group 2 was given 30 mg/kg of lead acetate orally for four weeks. Group 3 was given 50 mg/kg of alkaloid fractionate of *C. esculentus* followed by 30 mg/kg of lead acetate orally for four weeks. Group 4 was given 100 mg/kg of alkaloid fractionate of *C. esculentus* followed by 30 mg/kg of lead acetate orally for four weeks.

Collection of the samples

Following the administration of the last dose (day 28), 24 hours later, animals were anesthetized using thiopental administered intraperitoneally to ensure deep anesthesia. The absence of reflexes confirmed Anesthesia. After confirming anesthesia, animals were euthanized humanely. Blood samples were collected in EDTA tubes and stored at -80 degrees centigrade until analysis. The samples collected were centrifuged for 15 minutes at 4000 rpm to separate the plasma. The pelvis cavity of all the rats was opened up within the lower abdominal region to remove testes. Testes were removed, trimmed to remove fat, then weighed and fixed in Bouin's fluid for subsequent histological and immunohistochemical procedures [16].

Analysis of the specimen

Immediately following euthanasia, the scrotal region was surgically opened using sterile instruments to expose the reproductive organ. The epididymis was carefully isolated from the testis under sterile conditions. Semen was collected specifically from cauda epididymis which is the main site of sperm storage. The epididymis contents were then gently squeezed into a sterile Petri dish containing 2 mL pre-warmed physiological saline solution, phosphate-buffered saline (PBS) and then incubated at a temperature of 37 ºC [17].

A light microscope (manufactured by Leica DM750) was used to evaluate sperm motility as recommended by WHO [18]. The blood samples stored in the EDTA bottles were spanned at 2500 rpm for a period of 10 min using a bio-centrifuge (MSE, O-5122A product, manufactured in Germany). The level of hormones [total testosterone (TT), follicle-stimulating hormone (FSH), and luteinizing hormone (LH)] was measured using an ECOBAS-6000 hormone analyzer to provide quantitative results for hormone levels.

Products of lipid peroxidation were quantified using measuring TBARS as described by Niehaus [19]. The non-enzymatic antioxidants GSH and CAT were estimated according to the Ellman method [20], which involved monitoring the decomposition of hydrogen peroxide. SOD activity in the testis was also evaluated using the procedure described by Marklund [21]. The testicular tissues removed were fixed in Bouin's solution 4 hours before the histological technique using H&E stain and Ki67 stain using a light microscope.

Ki67 as a marker of proliferation

Ki67 is a cellular marker used to measure cell proliferation. It is expressed during active cell cycle phases but absent in resting cells. Monitoring Ki67 expression helps assess the regenerative capacity of testicular tissue in the face of toxic insult. Immunohistochemistry for Ki67 was performed on testicular tissue sections to evaluate cell proliferation levels. Additionally, histological analysis of the testis was conducted to assess overall tissue architecture and damage.

Ethical approval from the ethical committee

Clearance and approval from the ethical committee for this research were obtained from the University of Ilesa, Ilesa, Osun State.

Statistical analysis

Descriptive statistical analysis was conducted using SPSS version 28 (IBM Corp, Armonk, NY). Data were expressed as mean ± standard deviation. A significant difference was taken with a p-value of <0.05with one-way ANOVA.

RESULTS

Sperm analysis

According to **Table 1**, sperm parameters and motility significantly increased in the groups treated with alkaloid fractionate of *C. esculentus* compared with lead control.

Table 1. Sperm parameters, effect of alkaloid fractionate of *Cyperus esculentus* on sperm parameters.

Group	Sperm count (x10^6)	Non-Motile sperm (%)	Active sperm (%)
1	678.33±0.57	15.00±0.00	65.23±0.58
2	470.33±0.57*	100.00±0.00*	1.03±0.05*
3	40.33±0.57*#	75.10±0.17*#	10.03±0.05*#
4	44.33±0.57*#	75.00±0.00*#	11.03±0.06*#

Values are expressed as mean ± standard deviation. *p< 0.05 and control, # p<0.05 significant difference from Lead.

Sexual hormones on the effect of alkaloid fractionate of *Cyperus esculentus*

According to **Table 2**, in Groups 3 and 4 that received alkaloid fractionate of *C. esculentus* at 50 mg/kg and 100 mg/kg, respectively, a significant increase in TT, FSH, and LH levels with $p < 0.05$ compared with lead control was observed.

Table 2. Sexual hormones on the effect of alkaloid fractionate of *Cyperus esculentus.*

Group	TT (ng/ml)	FSH(ng/ml)	LH(ng/ml)
1	5.05±0.03	0.81±0.01	8.20±0.02
2	2.04±0.02*	0.30±0.02*	7.70±0.00*
3	3.02±0.01*#	0.20±0.01*	7.71±0.00*
4	3.10±0.00*#	0.60±0.01*#	6.30±0.00*#

Values are expressed as mean ± standard deviation.*p< 0.05 and control, # p<0.05 significant difference from lead, number of animals in a group= 10. TT: Testosterone, FSH: Follicle Stimulating Hormone, LH: Luteinizing Hormone.
Key:TT-testosterone
LH-luteinizing hormone
FSH-follicle stimulating hormone

Oxidative markers, the effect of alkaloid fractionate of *C. esculentus* on oxidative markers.

According to **Table 3**. alkaloid fractionate of *Cyperus esculentus* elevated the levels of SOD, CAT, and GSH significantly (p<0.05) in groups 3 and 4 when compared to the lead control while MDA level was significantly decreased (p<0.05) in groups 3 and 4 compared to lead control.

Table 3. Oxidative markers, the effect of alkaloid fractionate of *C. esculentus* on oxidative markers.

Group	Superoxide Dismutase (U/mg)	Catalase (U/mg)	Malondialdehyde (nmol/mg)	Glutathione (nmol/mg)
1	26.10±0.00	13.30±0.01	0.52±0.03	2.30±0.03
2	12.60±0.00*	8.30±0.00*	3.40±0.01*	1.10±0.02*
3	21.30±0.00*#	16.00±0.00*#	1.10±0.02*#	1.40±0.01*
4	28.50±0.00*#	12.30±0.00*#	1.20±0.00*#	1.90±0.02*#

Values are expressed as mean ± standard deviation.*p< 0.05 and control, # p<0.05 significant difference from lead, number of animals in a group= 10.

Histological findings

Fig. 1. shows hematoxylin and eosin (H&E) stained sections of testicular tissue across experimental groups. Panel 1A (control group) displays normal histoarchitecture of the seminiferous tubules with intact germinal epithelium, organized spermatogenic series, and clearly visible Leydig cells in the interstitial space. In contrast, panel 1B (lead-exposed group) reveals severe structural distortion, including disrupted tubular morphology, disorganization of the germinal epithelium, and reduced germ cell density. Panels 1C and 1D (treatment groups) exhibit progressive restoration of seminiferous tubule structure, with improved organization of spermatogenic cells and reduced pathological changes compared to the lead-exposed group.

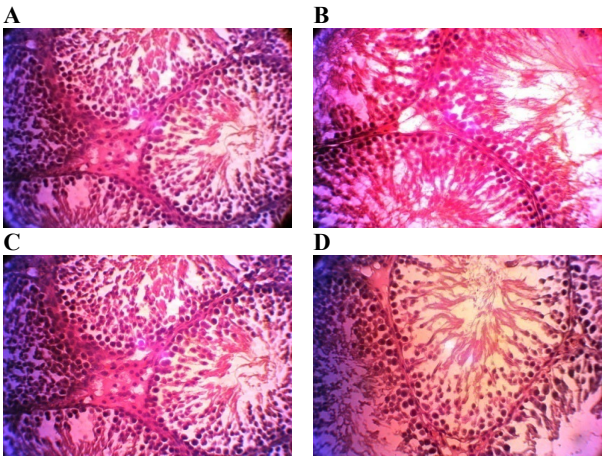

Fig. 1. Histological sections of testicular tissue stained with hematoxylin and eosin (H&E), viewed at 200× magnification. (A) Normal control group showing intact seminiferous tubule architecture with a well-organized spermatogenic series, prominent Sertoli cells, and clearly identifiable Leydig cells in the interstitial space. (B) Lead-exposed group displaying severe distortion of the seminiferous tubules, disorganized germinal epithelium, and noticeable depletion of germ cells, indicating testicular damage. (C) Treatment group 1 showing partial restoration of tubule structure, with moderate reorganization of the spermatogenic layers and improved cellularity. (D) Treatment group 2 exhibiting near-normal seminiferous tubule morphology, improved germ cell arrangement, and reduced histopathological damage compared to the lead-exposed group.

Immunohistological findings

Fig. 2 shows the immunohistochemical staining of testicular tissue sections using the Ki-67 antibody. Positive Ki-67 expression, indicative of active cell proliferation, is observed in groups 3 (Panel C) and 4 (Panel D), as evidenced by the presence of brown-stained nuclei within the seminiferous tubules.

In contrast, group 2 (Panel B), representing the lead-exposed control group, shows markedly reduced or absent Ki-67 expression, indicating impaired proliferative activity. Panel A represents the normal control with clear structural integrity and baseline Ki-67 expression.

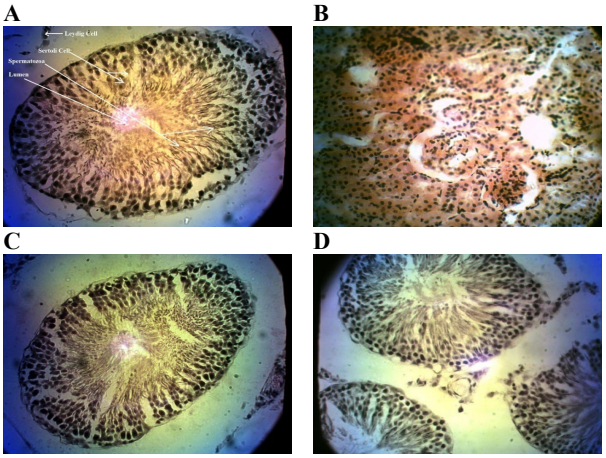

Fig. 2. Immunohistochemical staining of testicular tissue using Ki-67 antibody. (A) Control group showing normal seminiferous tubule architecture with active Ki-67 expression in spermatogonia. (B) Group 2B showing disrupted tubule structure and absence of Ki-67 expression, indicating reduced proliferative activity. (C) Group 2C showing restoration of tubular structure with moderate Ki-67 expression. (D) Group 2D showing improved seminiferous tubule organization with strong Ki-67 positivity, indicating enhanced cell proliferation. Ki-67 positive nuclei are primarily localized in basal germ cells. Images captured under light microscopy.

DISCUSSION

Over the years, different parts of medicinal plants have been used in the curative and prevention of many diseases [22,23]. Herbal remedy is well known by ancient people in treatment of ailments such as infertility and increase sexual performance [24]. Antioxidant potential of alkaloid has been reported [25]. The results indicate that alkaloid fractionate of *C. esculentus* mitigate the detrimental effects of lead-induced testicular toxicity. The increased Ki67 expression after alkaloid treatment suggests that this compound may promote cell proliferation, potentially aiding in the recovery of spermatogenesis and restoration of testicular function.

The protective effect could be attributed to the antioxidant properties of alkaloids, which likely reduce oxidative stress caused by lead acetate, allowing for better cell survival and regeneration. Lead affects sperm count and motility through several key mechanism, which can severely impact male fertility [26]. Lead exposure increases production of reactive oxygen species (ROS) in the testis, causing oxidative damage to sperm cells. Oxidative stress harms cellular structures like lipids, proteins, and DNA, which are critical for sperm function [27]. There is a correlation between Increase formation of ROS and decrease sperm motility.

TT is crucial in the development and growth of the male reproductive system and essential for several biological processes, both during fetal development and throughout life and responsible for secondary sexual characteristics [17]. TT stimulates the production of sperm (spermatogenesis) in the testes. Without adequate TT, sperm production would be impaired, leading to infertility [17]. In this study, increased serum TT and LH levels in the groups treated withalkaloid fraction of *C.esculentus* was observed when compared with lead control group. This significant incrementin the serum levels of TT and LH observed was dose dependent. Rapidly dividing interstitial cells leads to increase in serum levels of TT and LH [9]. FSH induction ofsertoli cells to secrete androgen-binding proteins is the responsibility of FSH [18].

According to the results of this study showed, lead acetate reduced oxidative stress markers such as GSH, CAT, and SOD. However, the increase in MDA, lipid peroxidation by-product in this study is as a result of increase in the generation of free radicals, OH and H_2O_2 in the testis of the lead-exposed rat [28, 29]. Alkaloidfractionate of *C. esculentus*increased testicular antioxidant enzymes and decreased MDA levels in this study.

The result of this study showed negative expression of Ki-67 on histological of testicular tissue of rats in lead treated group. It led to shortening of the seminiferous epithelium, with significant lower of spermatogenic cells. Lead is known as toxic agent that can cause structural damage to the seminiferous tubules which are responsible for sperm production. This includes degeneration of germ cells, atrophy of seminiferous tubules, and disruption of the blood-testis barrier.The sertoli and Leydig cells, crucial for supporting spermatogenesis and producing TT, respectively, may also suffer from lead-damage [30,31]. Reduction in cellularity of the interstitium in the testes of the rats treated with lead alone resulted in low level of serum TT and ultimately led to poor spermatogenesis observed in this study.

Alkaloid fraction of *Cyperus esculentus*resulted in positive expression of Ki-67 inhistoarchitecture of the testis, increased the proliferative activity of spermatogonia, and maintained cells of the spermatogenic series. Ki-67 reflect the proliferative activity of a cell population positively or negatively.Positive reaction of Ki67 expression observed in groups 3 and 4 when compared with lead control. The novel of aspect of this study lies in demonstrating the protective role of alkaloid fractionate of *C. esculentus* in enhancing cell proliferation, as indicated by Ki67, which hasn't been extensively explored in this context before.

CONCLUSSION

In conclusion, our study demonstrated a distinct pattern of Ki67 expression in the testis. These findings enhance our understanding of testicular function and pathology. Our results suggest that alkaloid fractionate of *C.esculentus* has positive expression on Ki67 on testis which could serve as a valuable marker for diagnosis and therapeutic targeting in testicular condition. The study demonstrates that alkaloid fractionate of *C. esculentus* can restore ki67 expression and promote cell proliferation in the testis after lead-induced toxicity. This suggest a potential role of alkaloid in counteracting environmental reproductive toxicants.

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