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## Histopathological and biochemical changes associated with experimental cyanide-induced testicular toxicity in rats administered extracts of macrofungi

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### Abstract

This study examined how a methanolic extract of the macrofungal *Pleurotus ostreatus* protected rats' testicles from the toxicity caused by potassium cyanide (KCN). For 60 days, KCN was taken orally at a dose of 1.5 mg/kg body weight, either with or without *Pleurotus ostreatus* methanol extract (100, 200, and 400 mg/kg). Testicular histopathology, body and organ weight, organosomatic indices, serum testosterone, epididymal sperm count, testicular lipid peroxidation, and antioxidant enzyme activities (catalase and superoxide dismutase) were assessed. Lipid peroxidation was significantly increased by KCN intoxication, while antioxidant enzyme activities, serum testosterone levels, and the number of epididymal sperm were all significantly reduced. Additionally, testicular degenerations were noted. The co-administration of KCN and *Pleurotus ostreatus* extract resulted in a significant reduction in lipid peroxidation and an increase in antioxidant enzyme activities, serum testosterone concentration, epididymal sperm count, and testicular histology restoration. This finding implies that by strengthening the antioxidant defense system, *Pleurotus ostreatus* may be able to lessen the harmful effects of dietary cyanide toxicity on testicular function.

**Keywords:** Mushroom; Oxidative stress; *Pleurotus ostreatus*; Potassium cyanide; Testis.

## Introduction

Cyanide (CN), is a fast-acting metabolic poison whose pathogenesis involves the inhibition of the cytochrome c oxidase, the last oxidase in the mitochondrial electron transport chain to suppress aerobic ATP synthesis and cellular oxygen consumption, leading to death (Abraham & Kappas, 2008; Petrikovics et al., 2019; Lippner et al., 2022). It is a common environmental pollutant in industrialized countries where it is a waste product of different types of plastic or nitrile as well as domestic combustion fires (Hamel, 2011; Gensa, 2019; Lippner et al., 2022). Cyanide is also found naturally in many plant-based food such as bitter almonds and cassava (Jones, 1998; Rivadeneyra-Dominguez et al., 2013; Alitubeera et al., 2019; Arabizadeh et al., 2024). Incidentally, one of the most significant food crops in tropical nations is cassava where it forms important staple food and provides about 40% of all calories for over half a billion people, especially in Asia, Latin America, and Africa (Nweke et al., 2002; De Moura et al., 2015). Thus, human consumption of cassava or use as feed for livestock has been negatively impacted by cyanide toxicity, which is caused by the presence of harmful cyanogenic glycosides resulting in neuronal degeneration, kidney and liver damage, thyroid dysfunction, diabetes and testicular toxicity (Shivanoor & David, 2015; Oyewopo et al., 2021).

Chronic exposure to toxic pollutants is thought to be a major factor in the recent decline in fertility in both developed and developing nations, as evidenced by decreased sperm count and testicular function in men (Selvaraju et al., 2021). This is because humans and animals that live in industrial areas or eat food that contains some level of these chemicals are susceptible to exposure. Amongst the documented compounds that cause infertility is cyanide. Cyanide induced testicular toxicity is attributed to induction of oxidative stress and anaerobic cellular respiration (Hariharakrishnan et al., 2009; Baud et al., 2018; Haouzi et al., 2020). The major hindrance in the treatment of cyanide toxicity is the expensive nature of its antidote kits as well as side effects associated with the administration of known cyanide antidotes (Lippner et al., 2022). Due to these limitations, there is need for cheaper, safe and readily available products that could confer both protective as well as curative effects on cumulative cyanide toxicity.

Thus, in the management of infertility such as those associated with cyanide toxicity, various natural products and supplements have been employed. Studies have shown that a variety of mushroom extracts, which are extremely valuable dietary sources, possess antioxidant qualities (Bhambri et al., 2022; Rugolo et al., 2022; Łysakowska et al., 2023). Based on phytochemical analysis using HPLC, it has been reported that the mushroom *Pleurotus ostreatus* contains five phenolics and related compounds (gallic acid, chlorogenic acid, catechin, propyl gallate, and cinnamic acid) (Dkhil et al., 2020).

It is known to have strong antioxidant properties and has been applied in the treatment of numerous illnesses. In Africa, cassava meals are prepared and eaten with soups usually prepared with mushroom sporophore as medicine and as a good substitute for meat protein (Ene-Obong & Camovale, 1992; Okhuoya et al., 2010; Ashaolu & Adeyeye, 2022). However, there is no preclinical scientific report on the effects of mushroom such as *Pleurotus ostreatus* on cyanide toxicity.

Therefore, the purpose of this study is to evaluate the potential protective effect of *Pleurotus ostreatus* extract against cyanide-induced testicular toxicity in rats.

## Materials and methods

### Materials

#### Chemicals and reagents

The source of the 95 percent pure Potassium cyanide (Sigma-Aldrich Chemical Co. Ltd, St. Louis, USA).

#### Mushroom

Fruiting bodies of the mushroom specie *Pleurotus ostreatus* was purchased from a commercial farm.

#### Preparation of hydro-methanol extracts of *Pleurotus ostreatus*

This was done using the procedure of (Elkhadragy & Abdel Moneim, 2017). Prior to being ground into a powder, the *Pleurotus ostreatus* fruiting bodies were allowed to dry at room temperature. After being manually shaken at 2-hour intervals for 72 hours, 500 grams (500 g) of the powdered material was soaked in 70% methanol before being filtered through Whatman paper (No. 1) and concentrated in an evaporator that rotates. At 4°C, the extract was kept.

#### Acute toxicity

In accordance with OECD guideline 423 for chemical testing, the acute toxicity of *Pleurotus ostreatus* was investigated (OECD, 2001). To conduct the study,  $150 \pm 10$  g rats were used. Five groups of five rats each received graded doses of the extract (625, 1250, 2500, and 5000 mg/kg bwt) intragastrically, while distilled water was given as a control. Rats were monitored for 14 days for indications of toxicity or death.

#### Experimental Animals

For the study, 35 male, healthy, 10- to 12-week-old Sprague Dawley outbred albino rats (*Rattus norvegicus*) weighing 180–200 g were employed. In the Experimental Laboratory Animal Unit, the rats were kept in standard 60 cm x 45 cm x 45 cm polypropylene cages with wood shavings as bedding. They were allowed to acclimate for two weeks at a temperature of  $25 \pm 4$  °C and a relative humidity of  $65 \pm 5$  percent, with a 12hour light

and dark cycle. They received an unlimited supply of water and a diet similar to that of commercial growers.

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## Experimental design

The Institutional Animal Ethical Committee approved the experiment, which was carried out in accordance with the Ethics and Regulations Guiding the Use of Research Animals. Following a two-week acclimatization period, the rats were randomly assigned using a completely randomized design into five groups of seven rats each. These groups were given normal saline as the control group (A), 1.5 mg/kg KCN only (B), 1.5 mg/kg KCN + 100 mg/kg PO (C), 1.5 mg/kg KCN + 200 mg/kg PO (D) and 1.5 mg/kg KCN + 400 mg/kg (E), every day for 60 days. Both the extract and KCN were administered orally at a volume of 5 mL/kg. The dose of potassium cyanide (KCN) used was 1/5<sup>th</sup> (1.5 mg/kg) of the known lethal dose (7.5 mg/kg) of potassium cyanide (Sabourin et al., 2016)

## Sample collection

Following an overnight fast at the conclusion of the experiment, two milliliters of blood were drawn from the rats' retro-orbital plexus in the morning and placed in anticoagulant-free tubes. The samples were centrifuged for 10 minutes at 1000 g in order to collect serum for the biochemical test. After the animals were dissected, samples of the right testis were cleaned of blood and immediately frozen in a freezer at -80°C for tissue malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD) assays. The left testis was then taken out and fixed in 10% buffered formalin after it had been weighed. In order to count the sperm, the epididymides were removed.

## Testicular organosomatic index

To weigh the testes, a sensitive Mettler weighing balance (made by Mettler Toledo, Switzerland) was employed. To calculate the testicular index, the testicular weights of the male rats were divided by their respective body weights (Afriyie, 2014).

## Sperm count

The conventional hemocytometric technique was used to evaluate the male rats' epididymal sperm reserves. In short, 90 mL of a solution containing 5 g NaHCO<sub>3</sub>, 1 mL of formalin (35 percent), and 25 mg of eosin per 100 mL of distilled water were used to dilute a sample of sperm suspension (0.5 mL). After that, each counting chamber received 10 µL of the diluted sperm suspension, which was left to stand for five minutes. Using the

formula Sperm count = Total number of sperm cells in 5 squares x 50 000 x 100 x 106/ mL, the concentration of epididymal sperm was calculated as millions of sperm cells per milliliter of suspension.

## Serum Testosterone Assay

According to the manufacturer's instructions, testosterone was quantitatively determined using enzyme-linked immunoabsorbent assay (ELISA) kits (Shanghai Yi Li Biological Technology, Shanghai, China).

## Oxidative Stress Markers

Testicular MDA levels were determined in accordance with Ohkawa et al. (1979). Superoxide dismutase (SOD) activity was measured using the previously described technique by Marklund and Marklund (1974), in nanomoles per milligram of protein, whereas catalase (CAT) activity was measured using the Aebi method (Aebi, 1984) in units per milligram of protein.

## Histopathology

The left testes fixed in 10% buffered formalin were prepared for histopathology. Using a microtome, sections of the samples were cut to a thickness of 5µ. Sections were then manually stained for light microscopy using hematoxylin and eosin (Survana et al., 2019). The Johnsen scoring system was used to assess the testicular lesions in each group (Johnsen, 1970). Briefly, testicular lesions were scored as: 1- No seminiferous epithelium; 2- No germinal cells, Sertoli cells only; 3- Spermatogonia only; 4- No spermatozoa or spermatids, few spermatocytes; 5- No spermatozoa or spermatids, many spermatocytes; 6- No spermatozoa, no late spermatids, few early spermatids; 7- No spermatozoa, no late spermatids, many early spermatids; 8- Less than five spermatozoa per tubule, few late spermatids; 9- Slightly impaired spermatogenesis with many late spermatids, disorganized epithelium; 10 Complete spermatogenesis with many spermatozoa present. Motic Images 2.0 digital camera was used to take photomicrographs (Motic China Group Ltd, China).

## Histomorphometry

Motic images plus 2.0 software (Motic China Group Ltd, China) was used to measure the germinal epithelial thickness (ET) and seminiferous tubule diameter (SD) of ten randomly chosen seminiferous tubules of hematoxylin and eosin (H and E) stained sections from each testis in micrometers.

## Statistical analysis

The SPSS statistics 23 program was used to perform the statistical analysis. For multiple comparisons, a one-way ANOVA and a post-hoc test ( $p < 0.05$ ) were used. Mean  $\pm$  Standard error of the mean (SEM) was used to express the values.

## Results

### Acute Toxicity

No animal deaths occurred at any of the extract dosages up to 5000 mg/kg. It was assumed that the extracts' oral LD50 was higher than 5000 mg/kg.

### Body weight, testicular weight and testicular index

The results in Table 1, displays the findings of the impact of *Pleurotus ostreatus* extract on the body weight, testicular weight, and testicular index of rats given potassium cyanide. Rats given cyanide alone (group B) had a lower body weight than the control group, but this difference was not statistically significant ( $p \geq 0.05$ ). The body weight of the rats intoxicated with potassium cyanide was not improved by treatment with the different doses of *Pleurotus ostreatus* extract, though, as the group given 400 mg/kg of *Pleurotus ostreatus* extract was significantly ( $p \leq 0.05$ ) lower than the control and untreated cyanide group B. Additionally, the testicular weight and testicular index of rats intoxicated with potassium cyanide were comparatively lower than the groups treated with the extract.

### Sperm count

In comparison to the control and *Pleurotus ostreatus* extract treated groups, the untreated potassium cyanide-intoxicated rats' mean epididymal sperm count was significantly ( $p < 0.05$ ) lower. Nonetheless, compared to the untreated potassium cyanide-intoxicated group, the mean epididymal sperm count of the groups treated with *Pleurotus ostreatus* extract was comparatively higher (Table 2).

### Serum testosterone

While the mean serum testosterone concentration of the groups treated with *Pleurotus ostreatus* extract was comparatively higher than the mean serum testosterone concentration of rats intoxicated with potassium cyanide, the mean serum testosterone concentration of the untreated potassium cyanide intoxicated group was significantly ( $p < 0.05$ ) lower than that of the control and *Pleurotus ostreatus* extract treated groups (Table 2).

### Oxidative stress markers

In comparison to the control group, the MDA level was significantly ( $p < 0.05$ ) higher in the untreated potassium cyanide intoxicated group B, whereas the MDA level was comparatively lower in the groups treated with *Pleurotus ostreatus* extract. Additionally, compared to the control group, the untreated potassium cyanide intoxicated group had lower CAT and SOD activity, whereas the group treated with *Pleurotus ostreatus* extract had non-relatively higher CAT and SOD activity (Table 3)

### Histomorphometry

The untreated potassium cyanide intoxicated group showed a lower ( $p > 0.005$ ) mean epithelial thickness and tabular diameter compared to the control. However, the *Pleurotus ostreatus* extract treated groups showed a relatively higher mean epithelial thickness and tubular diameter compared to the untreated potassium cyanide intoxicated group as presented in Table 2.

**Table 1:** Rats with Experimental Cyanide Intoxication and the Effects of *Pleurotus Ostreatus* Extract on Body Weight, Testicular Weight, and Testicular Index.

Experimental groups	Initial weight (g)	Final weight (g)	Weight gain (g)	Testis weight (g)	Testicular index (%)
A (Control)	126.83 ± 1.19	190.67 ± 3.18	63.83 ± 2.99 <sup>a</sup>	2.70 ± 0.04 <sup>a</sup>	1.50 ± 0.07 <sup>a</sup>
B (1.5 mg/kg KCN only)	127.10 ± 0.51	190.00 ± 3.51	62.90 ± 3.36 <sup>a</sup>	2.55 ± 0.06 <sup>b</sup>	1.34 ± 0.01 <sup>b</sup>
C (1.5 mg/kg KCN+100 mg/kg PO)	127.33 ± 0.64	186.00 ± 5.03	58.67 ± 4.58 <sup>ab</sup>	2.64 ± 0.03 <sup>ab</sup>	1.42 ± 0.03 <sup>ab</sup>
D (1.5 mg/kg KCN+200 mg/kg PO)	127.87 ± 1.00	185.67 ± 2.85	57.80 ± 1.90 <sup>ab</sup>	2.59 ± 0.03 <sup>ab</sup>	1.40 ± 0.02 <sup>ab</sup>
E (1.5 mg/kg KCN+400 mg/kg PO)	127.30 ± 0.53	181.33 ± 0.67	54.03 ± 1.12 <sup>b</sup>	2.65 ± 0.02 <sup>ab</sup>	1.46 ± 0.01 <sup>a</sup>

Significance was assessed using a one-way ANOVA and the Post-hoc Test. Mean ± SEM (n = 7) values with distinct superscripts a, b in the same column is significantly ( $p < 05$ ) different. PO stands for *Pleurotus ostreatus*; g for gram; KCN for potassium cyanide; mg for milligrams; and kg for kilogram

**Table 2:** Impact of *Pleurotus ostreatus* extract on Testicular Histo-morphometric Alterations, Serum Testosterone levels, and Sperm Count in Rats Experimentally Intoxicated with Cyanide.

Experimental groups	Sperm count (10 <sup>6</sup> cells/ml)	Testosterone (ng/ml)	SD (µm)	ET (µm)
A (Control)	291.03 ± 3.38 <sup>a</sup>	3.34 ± 0.16 <sup>a</sup>	174.11 ± 9.03	86.77 ± 4.43
B (1.5 mg/kg KCN only)	271.87 ± 0.75 <sup>b</sup>	1.74 ± 0.06 <sup>b</sup>	148.22 ± 14.66	75.51 ± 6.14
C (1.5 mg/kg KCN + 100 mg/kg PO)	286.47 ± 4.81 <sup>a</sup>	1.93 ± 0.03 <sup>b</sup>	169.69 ± 8.87	82.84 ± 5.51
D (1.5 mg/kg KCN + 200 mg/kg PO)	288.00 ± 0.68 <sup>a</sup>	2.02 ± 0.32 <sup>b</sup>	171.57 ± 8.76	85.63 ± 4.24
E (1.5 mg/kg KCN + 400 mg/kg PO)	286.93 ± 6.10 <sup>a</sup>	1.98 ± 0.34 <sup>b</sup>	170.08 ± 8.94	85.04 ± 4.12

One-way ANOVA and the Post-hoc Test were used to determine significance. Mean ± SEM (n = 7) values with different superscripts a, b in the same column is significantly (p < 05) different. SD; seminiferous tubule diameter, ET; germinal epithelial thickness, g for gram, KCN for potassium cyanide, PO for *Pleurotus ostreatus*, mg for milligrams, and kg for kilograms.

**Table 3:** Impact Of *Pleurotus Ostreatus* Extract on Antioxidant Enzyme Activity and Lipid Peroxidation in the Testis of Rats Experimentally Intoxicated with Cyanide.

Experimental groups	MDA (nmol/L)	CAT (U/mg protein)	SOD (U/mg protein)
A (Control)	22.74 ± 2.23 <sup>a</sup>	41.19 ± 3.97 <sup>a</sup>	19.45 ± 0.33 <sup>a</sup>
B (1.5 mg/kg KCN only)	34.79 ± 2.94 <sup>b</sup>	22.86 ± 0.81 <sup>b</sup>	15.55 ± 0.63 <sup>b</sup>
C (1.5 mg/kg KCN + 100 mg/kg PO)	29.10 ± 3.63 <sup>ab</sup>	27.21 ± 0.59 <sup>b</sup>	15.82 ± 0.70 <sup>bc</sup>
D (1.5 mg/kg KCN + 200 mg/kg PO)	29.15 ± 2.66 <sup>ab</sup>	27.52 ± 4.81 <sup>b</sup>	15.83 ± 0.80 <sup>bc</sup>
E (1.5 mg/kg KCN + 400 mg/kg PO)	27.79 ± 3.02 <sup>ab</sup>	34.20 ± 0.29 <sup>b</sup>	16.11 ± 0.81 <sup>ac</sup>

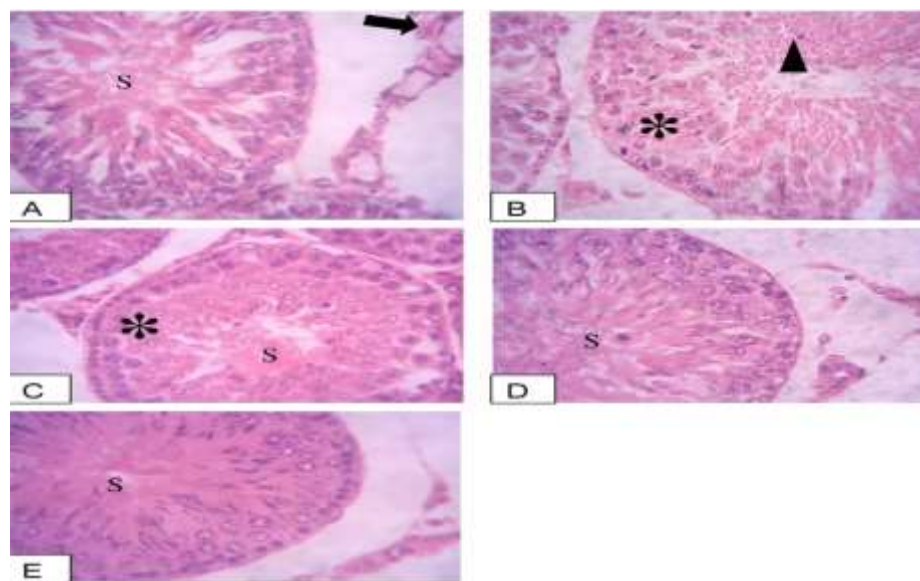
Significant differences (p≤ 0.05) exist between mean values with different superscripts in the same column. PO stands for *Pleurotus ostreatus*; g for gram; KCN for potassium cyanide; mg for milligrams; and kg for kilograms

Histopathology

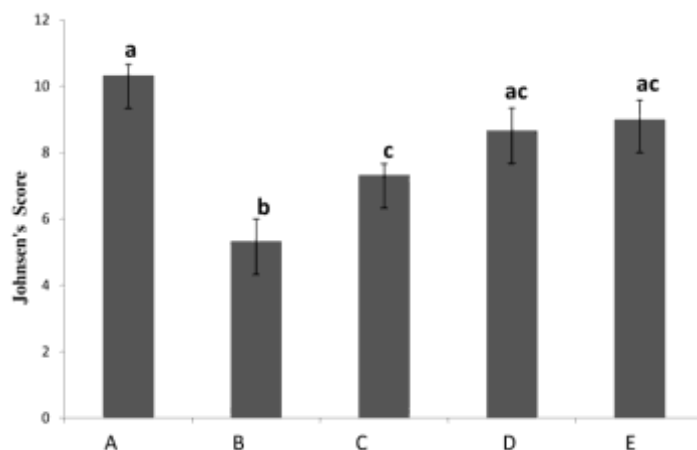
Sections of control rats' testes examined under a microscope revealed a thick layer of spermatogenic cells and normal germinal epithelial cells. Rats treated with *Pleurotus ostreatus* extract showed similar histological characteristics in their testes. However, the testes of the group that was intoxicated with potassium cyanide without treatment displayed degeneration of the germinal epithelial cells, with few layers and a low number of germinal epithelial cells (Figure 1). In contrast to the group that

was intoxicated with potassium cyanide without treatment, the *Pleurotus ostreatus* extract treatment improved spermatogenesis and preserved testicular histoarchitecture. In comparison to the other groups, the untreated potassium cyanide intoxicated group's mean Johnsen's scores were significantly (p < 0.05) lower. However, when compared to the rats in the untreated potassium cyanide intoxication group, the groups treated with *Pleurotus ostreatus* extract had significantly (p < 0.05) higher Johnsen's scores (Figure 2).





**Figure 1:** Photomicrograph of the testis from the experimental groups A (control) showing normal typical seminiferous tubules (S) and interstitium (arrow); B (1.5 mg/kg KCN only) moderately low population of cells in the germinal epithelium (asterisk) and residual bodies (arrowhead); C (1.5 mg/kg KCN + 100 mg/kg PO) shows mild reduced population of cells in the germinal epithelium (asteriks) while D (1.5 mg/kg KCN + 200 mg/kg PO) and E (1.5 mg/kg KCN + 400 mg/kg PO) shows normal seminiferous tubules (S), H and E stain  $\times 400$ .



**Figure 2:** Johnsen's score of testicular histopathology of experimental groups A (control); B (1.5 mg/kg KCN only); C (1.5 mg/kg KCN + 100 mg/kg PO); ssD (1.5 mg/kg KCN + 200 mg/kg PO) and E (1.5 mg/kg KCN + 400 mg/kg PO). Data are means  $\pm$  SE. <sup>a</sup> Significant difference vs. untreated potassium cyanide intoxicated groups B and C (3.5 mg/kg KCN + 100 mg/kg PO); <sup>b</sup> significant difference vs. all groups, <sup>c</sup> significant difference vs. control and untreated potassium cyanide intoxicated group B. KCN=Potassium cyanide; PO=Pleurotus ostreatus; mg=milligrams; kg=kilograms.

## Discussion

This investigation assessed how well *Pleurotus ostreatus* extract protected against testicular pathology in potassium cyanide intoxicated group of rats. The cyanide induced low body and testicular weights as well as testicular index observed in this study are consistent with previous reports in experimental

animals (Shivanoor & David, 2015; Nourani et al., 2021; Oyewopo et al., 2021). This may be attributed to the suppression of food intake due to metabolic acidosis. Cyanide inhibition of oxidative phosphorylation with cellular hypoxia and the depletion of ATP lead to metabolic acidosis which is known to cause loss of muscle mass (Carnauba et al., 2017). The low testicular weight was also reflected in the low epididymal sperm

count observed in the untreated potassium cyanide intoxicated group of rats compared to the control.

*Pleurotus ostreatus* extract did not show a protective effect on body and testicular weights. The lower body weights observed in all the rats that were given the *Pleurotus ostreatus* extract compared to the control, which could be because of bioactive components in the extract. Previous research on animals in the literature demonstrated that eating mushrooms decreased body weight Neyrinck, et al. (2009) and Ganesan & Xu (2018), due to its inhibition of adipogenesis through the action of  $\beta$ -glucan (Handayani et al., 2011; Sheng et al., 2019).

The testicular tissue of potassium cyanide intoxicated rats treated with *Pleurotus ostreatus* extract had lower MDA level and higher activities of SOD and CAT in comparison to those that are not treated. Well-known free radical scavengers, flavonoids, alkaloids, and phenolic acids (gallic acid, chlorogenic acid, catechin, propyl gallate, and cinnamic acid) are all present in *Pleurotus ostreatus* extract (Dkhil et al., 2020; Effiong et al., 2024). Consequently, the balance between oxidants and antioxidants is improved in potassium cyanide intoxicated rats that received *Pleurotus ostreatus* extract compared to the untreated potassium cyanide intoxicated group. The pituitary's hormonal secretion, which controls the different functional units in the testis, facilitates normal spermatogenesis. Therefore, the pituitary-secreted gonadotropin hormones regulate testosterone, which continues to be the most significant hormone involved in male reproduction or spermatogenesis. LH from the pituitary gland stimulates the Leydig cells to secrete more testosterone (Dirican and Kalender, 2012). In the current investigation, serum concentrations of testosterone were significantly lower after cyanide intoxication. This is consistent with previous report in rats administered high dose of cyanide in drinking water (Shivanoor and David, 2015). This could be explained by cyanide's toxicity to Leydig cells, which results from tissue hypoxia caused by cyanide and oxidative stress-induced damage to plasma membranes (Shivanoor & David, 2015; Nourani et al., 2021). Rats intoxicated with cyanide had a significantly higher level of serum testosterone after receiving *Pleurotus ostreatus* extract than rats intoxicated with potassium cyanide, but this was insufficient to raise the testosterone concentrations to those in the control group.

Rats intoxicated with potassium cyanide showed decreased seminiferous tubular diameter and epithelial thickness when compared to the control and *Pleurotus ostreatus* extract-treated groups. *Pleurotus ostreatus* extract was used in this study to improve the histological structure of rats intoxicated with potassium cyanide; this may be because the extract has antioxidant properties (Stastny et al., 2022).

## Conclusion

The results of the biochemical and histopathologic examinations showed that *Pleurotus ostreatus* had protective effect against cyanide- induced testicular toxicity. This suggests that dietary inclusion of *Pleurotus ostreatus* in cyanogenic food like cassava diets could protect against possible deleterious effects of cyanide intoxication in man and animals.

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## Conflict of Interest

The authors declare that there are no competing interests.

## Ethical approval

This study was approved by the Faculty of Veterinary Medicine Institutional Animal Care and Use Committee (Approval Reference Number: IACUC-2023-05103).

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