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Biochemical and Immunohistochemical Evaluation of *Aframomum alboviolaceum*'s Impact on Male Wistar Rat Testis

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Abstract

Background: Male reproductive health is a crucial aspect of overall well-being, with increasing concerns about fertility in recent years. Various factors, including diet, genetics, environmental toxins, and lifestyle changes, can impact male fertility. The search for natural compounds with potentially positive effects on male reproductive function has led to exploring traditional medicine and plant extracts. This research aimed to investigate the potential impact of *Aframomum alboviolaceum* on male fertility, focusing on its effects on testicular tissues.

Methodology: We employed a randomized controlled design involving 20 male Wistar rats divided into control, clomiphene, low-dose *A. alboviolaceum*, and high-dose *A. alboviolaceum* groups. *A. alboviolaceum* extract was prepared from freshly harvested leaves, and doses were administered based on body weight. The rats received treatments for 30 days, and assessments were conducted through immunohistochemical and biochemical analyses.

Results: Our findings indicate that *A. alboviolaceum* did not induce a significant reduction in Glutathione (GSH) levels, aligning with its potential positive impact on antioxidant enzymes, including Glutathione Peroxidase (GPX) and Catalase (CAT). Immunohistochemical evaluations revealed the presence of essential cell types in the testes, which are essential for continuous spermatozoa production. Observed immunoreactivity for Bcl-2 protein in the treated groups suggests a non-contributory role in cell death in the testes, as high-dose administration showed a significant presence of Leydig cells, Sertoli cells, spermatids, and spermatogonia. *A. alboviolaceum* demonstrates potential positive effects on male Wistar rat testicular activities, with protective impacts on GSH, GPX, and CAT levels.

Conclusion: These results show that *A. alboviolaceum* has the potential to influence fertility parameters as it does not negatively affect the testicular activities of male Wistar rats.

Keywords: Immunohistochemistry, Biochemical, Plant extract, Wistar rat

Introduction

Male reproductive health is a critical aspect of overall well-being, and concerns about fertility have grown in recent years (Agarwal et al., 2021). Various factors, including dietary, genetics, environmental toxins and lifestyle changes, can influence male fertility. The quest for natural compounds with potentially positive effects on male reproductive function has led to the exploration of traditional medicine and plant extracts. *Aframomum alboviolaceum* (*A. alboviolaceum*), a plant native to tropical African regions including Sierra Leone to Sudan, Zambia through Angola, Malawi, and Mozambique, Nigeria, has garnered attention for its potential therapeutic properties (Amadi et al., 2016; Inkoto et al., 2021).

It is a member of the *Zingiberaceae* family and the genus *Aframomum*; it is a perennial herbaceous plant with 8 mm diameter creeping rhizomes that are buried deep in the earth and

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up to 1.5 m tall, leafy shoots (Inkoto et al., 2021). Numerous substances, including flavonoids, phenolic acids, anthocyanins, coumarins, anthraquinones, tannins, alkaloids, iridoids, and triterpenes, have been found in *A. Alboviolaceum* extracts. The presence of these substances is an indication of a potential pro-fertility effect (Inkoto et al., 2021).

As stated in a recent report by the World Health Organization, infertility will affect a great deal of people at some point in their lives (Babakhanzadeh et al., 2020). About 17.5% of individuals worldwide, or one in six, are infertile, highlighting the urgent need to increase access to high-quality, cost-effective reproductive care for those who need it. One significant finding of the WHO report is that infertility affects everyone equally (Babakhanzadeh et al., 2020). These results suggest that the prevalence of infertility varies little by geography; however, males are found to be responsible for 20–30% of cases. The data indicates this is a major global health issue for high-, middle-, and low-income countries. The lifetime prevalence was 17.8% in high-income countries and 16.5% in low- and middle-income countries (Barratt et al., 2017). Moreover, due to the molecular makeup of its plasma membrane, the mammalian spermatozoon is highly susceptible to biochemical antioxidants. Mammalian sperm cells are particularly susceptible to oxidative stress (Geng et al., 2015; Shah & Khan, 2017).

Given this, our study aims to investigate the potential impact of *A. alboviolaceum* on male fertility, focusing on its effects on testicular tissues. The assessment will involve both immunohistochemical and biochemical analyses, with a specific emphasis on Glutathione (GSH), Glutathione Peroxidase (GPx), and Catalase (CAT) as markers of oxidative stress. Understanding the changes induced by *A. alboviolaceum* at the cellular and molecular levels will provide valuable insights into its potential therapeutic use in promoting male reproductive health.

Materials and Methods

Experimental design and animal model

The study utilized a randomized controlled design to investigate the impact of *A. alboviolaceum* on the testicular examination of male Wistar rats. The study was conducted on 20 rats (weighing 200g – 230g) over 30 days. The animals were kept in cages made of stainless-steel wire suspended from the ceiling and kept in a temperature-controlled environment (23–25°C) and humidity (40–60%). The animals were randomly divided into four groups of 5 rats, including the treatment groups receiving different doses of *A. alboviolaceum* extract and control groups receiving a vehicle solution. Each group of rats was allowed adequate access to water (ad libitum) and standard rodent chow (SRC) for four weeks with two weeks of acclimatization.

Research Design

S/N	GROUPS	DOSAGE	NO. OF ANIMALS
1	Control	Normal saline	5
2	Positive control	Clomiphene 50mg/kg	5
3	Low dose group	*A. alboviolaceum* 115mg/kg	5
4	High dose group	*A. alboviolaceum* 250mg/kg	5

Plant extract preparation

Freshly harvested leaves of *A. alboviolaceum* were collected and identified in the Botany Department, Faculty of Science, Ekiti State University. The fleshy leaves were picked, and the roots were removed. The freshly collected leaves of *A. alboviolaceum* were separated, chopped into small pieces, and air-dried at room temperature in a well-ventilated room for two weeks. The leaves were air-dried, and 100g of the dried leaves were obtained after blending. Fifty grams (50g) of the leaf powder was dispersed in 50 ml of distilled water in a glass bottle for 24 hours with intermittent vigorous shaking. The filtrate was separated from the shaft using Whatman's filter

paper. The filtrate was collected and stored in the refrigerator for subsequent use. The preliminary body weight of each rat was taken before the commencement of the experiment to determine the appropriate dose to be administered to each animal; the extract was then standardized to ensure consistency in dosage (Shirani et al., 2016). The dose was administered orally to each rat based on body weight for 30 days.

Sacrifice and Sample collection

Rats were weighed using a calibrated Leica model weighing scale after acclimatization (2 weeks), which was before the treatment period and then at intervals of 10 days post-treatment. At the end of the administration and feeding period, the animals were allowed to fast overnight, anaesthetized with ketamine (20mg/kg) and 5ml of whole blood sample was obtained through cardiac puncture into plain tubes (Eren et al., 2020). Dissection was immediately and carefully made through the anterior thoracic wall to sufficiently expose the heart from where a puncture was used to get blood samples. The blood sample was drawn and placed in tubes that contained Ethylene-diamine-tetra acetic acid (EDTA) and kept at a temperature of 4°C. Dissection was also made on the anterior inferior abdominal wall to harvest the testis, which is then fixed in a neutral buffered formalin for immunohistochemical examination (Angelopoulou et al., 2008).

Immunohistochemical Analysis

Testicular tissues were obtained post-euthanasia, embedded in paraffin wax, and fixed for 24 hours in 10% neutral buffered formalin. Glass slides were mounted with sections that were cut to a thickness of 4-5 µm. Heat-induced epitope retrieval (HIER) was used to retrieve the antigen using a citrate buffer (pH 6.0), and 3% hydrogen peroxide in methanol was used to quench the endogenous peroxidase activity. By blocking with 5% normal serum corresponding to the secondary antibody, non-specific binding was avoided (Alasmari et al., 2018; Angelopoulou et al., 2008).

Sections were then treated with certain primary antibodies against indicators associated with fertility, such as proliferating cell nuclear antigen (PCNA) and markers associated with oxidative stress. Following the manufacturer's instructions, antibodies were diluted and incubated at 4°C for a whole night. The sections were cleaned and then incubated for one hour at room temperature with the suitable secondary antibody coupled to horseradish peroxidase (HRP) (Alasmari et al., 2018).

Immunological reactivity was envisioned using 3,3'-diaminobenzidine (DAB) as a chromogen, and counterstaining was performed with hematoxylin. Images were captured by a light microscope with a digital camera, and image analysis software quantified immunostaining intensity, providing a semi-quantitative assessment of marker expression (Alasmari et al., 2018).

Biochemical Analysis

After homogenising testicular tissues in ice-cold phosphate-buffered saline (PBS) to create a 10% (w/v) homogenate, the homogenate was then centrifuged at 2000g for 15 minutes, and the supernatant was obtained and kept at -20^{0}C until further examination. Assays were performed in duplicate, and mean values were utilized for analysis. Quality control samples with known activity were included to ensure assay reliability (Geng et al., 2015; Popoola et al., 2017).

Assay for Glutathione

Glutathione (GSH) levels were determined using a biochemical assay. The assay involved the reaction of GSH with a specific reagent to produce a coloured product, the intensity of which was measured using a spectrophotometer (Thermoscientific). GSH levels were quantified based on a standard curve (Shah & Khan, 2017).

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Assay for Glutathione peroxidase

Glutathione peroxidase (GPx) activity was assessed using a commercial assay kit based on the catalysis of cumene hydroperoxide reduction by GPx, coupled with the oxidation of GSH to oxidized GSH (GSSG). The rate of GSSG formation was measured using a spectrophotometer (Thermoscientific). A standard curve, generated with known GPx standards, was used to determine GPx activity in testicular homogenates by comparing absorbance values (Shah & Khan, 2017).

Assay for Catalase

Catalase activity was determined using a commercial assay kit (Calbiochem®) based on how catalase reacts with methanol when hydrogen peroxide is present. The formaldehyde produced was measured using a colourimeter at an appropriate wavelength, and the rate of formaldehyde production corresponded to catalase activity. A standard curve was produced using the kit's catalase standards at known concentrations. Catalase activity in testicular homogenates was quantified by comparing absorbance values to the standard curve.

Ethical Approval

Ethical approval was acquired for this experiment from the Ethics and Research Committee of the College of Medicine, Ekiti State University, Ado-Ekiti (ERCANA/2023/12/00l). The methods and procedures used in this experiment were entirely in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (National Research Council, 2011).

Data analysis

The data obtained were statistically evaluated using SPSS version 29. Values were recorded as mean ± standard deviation (SD).

Results

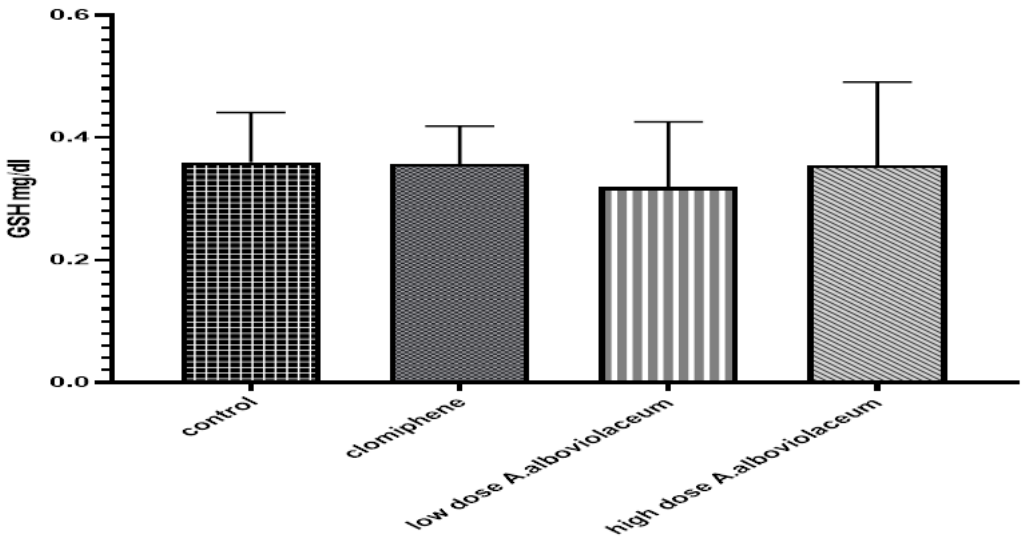

Figure 1: Mean value of GSH evaluation between experimental groups. Shows the comparison in the glutathione between control, clomiphene, low dose and high dose groups after administration. There is no significant decrease when the *A. alboviolaceum* administered group is compared with the control. P value set at 0.05

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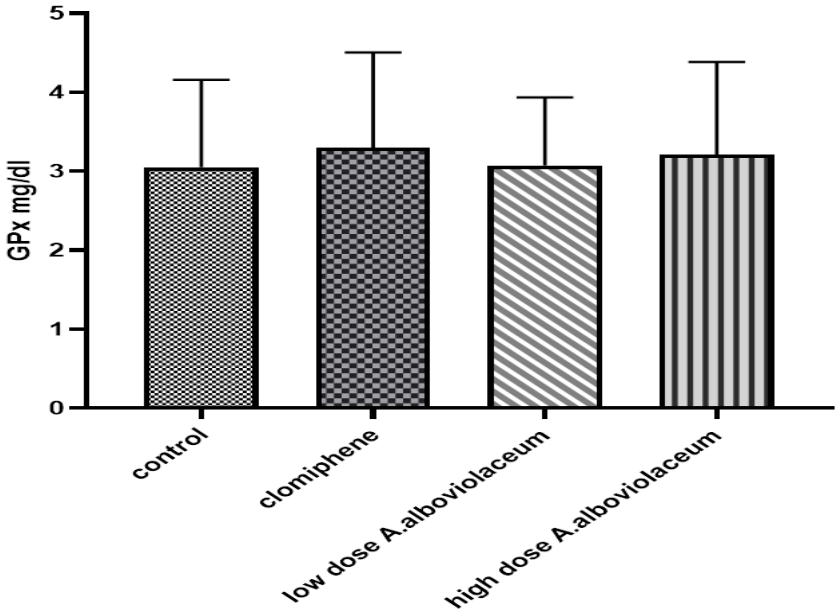

Figure 2: Mean value of Glutathione peroxidase activity between groups. Shows the comparison in the glutathione peroxidase between control, clomiphene, low dose and high dose groups after administration. There is no significant decrease when the *A. alboviolaceum* administered group is compared with the control. P value set at 0.05

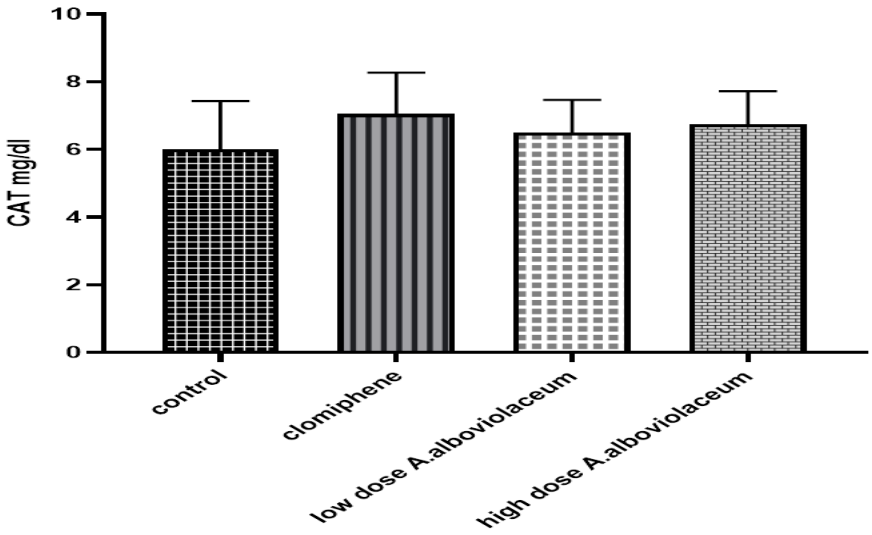

Figure 3: Mean value of Catalase activity between groups. Shows the comparison in the catalase activity between control, clomiphene, low dose and high dose groups after administration. There is no significant decrease when the *A. alboviolaceum* administered group is compared with the control. P value set at 0.05

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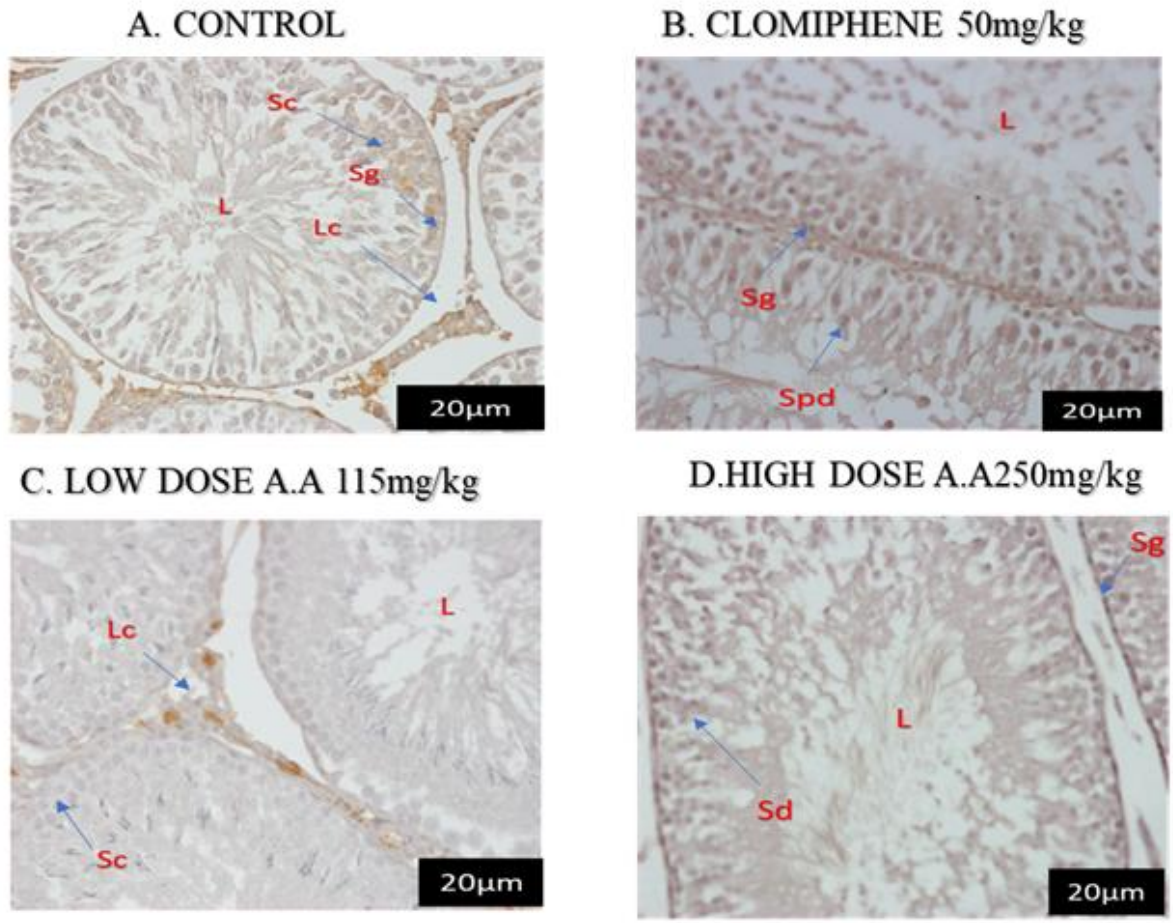

Figure 4: Image A-D show the photomicrograph of the immunoreactivity of Bcl-2 in the Wistar rat testes in each group respectively (x400 Mag). Cells observed in the seminiferous tubules are Spermatogonia (Sg), spermatids (Sd), Sertoli (Sc), Leydig (Lc) and Lumen (L).

Discussion

Glutathione (GSH), glutathione peroxidase (GPX), and catalase (CAT) are closely linked to fertility and sperm health due to their roles in oxidative stress regulation and protection of reproductive tissues (Hussein et al., 2020). An increase in antioxidants including GSH, GPX, and CAT is generally beneficial for fertility, as it helps protect sperm from oxidative damage and supports optimal reproductive function. Conversely, a decrease in antioxidants may contribute to oxidative stress, impairing sperm quality and increasing the risk of infertility (Corrêa et al., 2019; Mohamed & Arafa, 2013).

The GSH mean values in the testis of the control, clomiphene, low-dose, and high-dose groups are presented in Figure 1. There were no notable variations seen between the high and low-dose groups when compared with the clomiphene and control groups. While this suggests that *A. alboviolaceum* did not induce a significant reduction in GSH levels, it also means it did not have any detrimental effect on the testicular cell and could influence fertility. This is because the levels of the antioxidant enzymes in the high-dose group match up to the level seen in clomiphene

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which is a known substance to improve fertility (Scovell & Khera, 2018). This aligns with the work of Ramasamy et al. (2014).

Further evaluation of the GPX and CAT enzymes in the testes of the Wistar rats as presented in Figure 2 reveals the mean values for GPX and CAT in the testes of Wistar rats across the control, clomiphene, low-dose, and high-dose groups. There was an increase in the level of these enzymes observed in the high-dose group compared to the normal control and also near levels when compared with the clomiphene groups. These findings suggest that *A. alboviolaceum* could positively impact the activities of antioxidant enzymes GPX and CAT in the testes. This also agrees with the work of Shah and Khan (2017).

It is also important to consider the multifaceted nature of fertility. Numerous factors contribute to fertility, including hormonal regulation, sperm quality, and overall reproductive health. Existing literature supports the idea that plant compounds, including those found in *Aframomum* species, can influence fertility parameters (Roozbeh et al., 2021). *A. alboviolaceum* may contribute to improved sperm quality and motility, as reported in studies on other plant extracts (Roozbeh et al., 2021). A decrease in antioxidants may lead to elevated oxidative stress, which can result in damage to sperm cells, including DNA fragmentation, additionally, Lower levels of antioxidants may compromise sperm motility, viability, and overall function, potentially reducing fertility (Eren et al., 2020).

In this study, upon further immunohistochemical evaluations, cells observed in the seminiferous tubules are Spermatogonia (Sg), Spermatids (Sd), Sertoli (Sc), Leydig (Lc) and Lumen (L). The coordinated activity of these cell types is essential for the continuous production of spermatozoa, which is critical for male fertility (Khalaf et al., 2019; Yahyazadeh et al., 2020). Proper hormonal regulation, including testosterone from Leydig cells, supports the progression of spermatogenesis. The structural and supportive roles of Sertoli cells contribute to the nurturing environment necessary for germ cell development (Yahyazadeh et al., 2020). Disturbances or dysfunction in any of these cell types can impact spermatogenesis, leading to fertility issues (Yahyazadeh et al., 2020).

Visualization of testicular tissue from the control and clomiphene group shows moderate immunoreactivity for Bcl-2 protein which plays a crucial role in preventing cells from experiencing programmed cell death (apoptosis) resulting in the presence of spermatogonia and Leydig cells in the basement membrane, Sertoli cells close to the basement membrane (Figure 4). While present in all varieties of tubular cells, Bcl-2 protein exhibited a highly comparable and selective expression in cells in proximity to the tubular lumen, indicating the potential involvement of these proteins in the interaction among the luminal milieu, spermatogonia, and Leydig cells near the basement membrane. In the human testis, these are the cells that exhibit the majority of spontaneous apoptotic events (Ahmed et al., 2017; Ozen et al., 2008).

The groups administered *A. Aframomum*, showed a reaction linked to the expression of Bcl-2 protein indicating that the plant does not contribute to cell death (apoptosis) in the testis. The expression of the sperm cells can be said to be dose-dependent as the presence of Leydig cells, Sertoli cells, spermatids, and spermatogonia were significantly observed in the high-dose testicular tissue. (Figure 4). This agrees with previous studies (Xu et al., 2005; Zhao et al., 2017).

Conclusion

A. alboviolaceum may hold the potential to influence fertility parameters as it does not have a negative effect on the testicular activities of male Wistar rats. The testicular cells were protected from death (apoptosis) by the expression of the Bcl-2 protein. Additionally, it appears to have a protective and beneficial effect on GSH, GPX, and CAT levels. A combination of *A. alboviolaceum* with other fertility-boosting agents could also be considered. Further research could be carried out to assess the phytochemicals supporting the pro-fertility effect in *A. alboviolaceum* extract

with a focus on the expression of genes in the testicular tissue as well as the ultra-structural effect.

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Declarations of interest
None declared.

Authors' contributions
AMA, FOB and OHA conceived and designed the study. Each author participated in the execution of the studies and the gathering of the data. COF and AAF conducted data analysis and wrote the initial draft of the manuscript. Each author reviewed and edited the final draft.

Data Availability
Data generated during the study, including raw data and analysis results, will be made available upon request to ensure transparency and facilitate further scientific inquiry. This comprehensive approach in the materials and methods section ensures the transparency and reproducibility of the study, allowing other researchers to understand and replicate the experimental procedures.

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