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CHLOROFORM FRACTION OF *Ocimum gratissimum* (LINN) LEAF EXTRACT EXERTS CYTOPROTECTIVE AND MEMBRANE-STABILIZING POTENTIALS ON PLUMBAGIN-INDUCED INFERTILITY IN MALE WISTAR RATS

Salemcity A.J.[1]; Agboola O.O.[2]; Salemcity O.G.[3]; Effiong M.E.[4]; Ogungbemi O.J.[4]; Ikheloa O.[1]; Jacob P.I.[1]

[1]Department of Biochemistry, University of Medical Sciences, Ondo, Ondo State, Nigeria

[2]Department of Biochemistry, McPherson University

[3]Department of Chemistry, University of Medical Sciences, Ondo, Ondo State, Nigeria

[4]Department of Biochemistry, University of Ibadan, Ibadan, Oyo State, Nigeria

***Corresponding author:**

E-mail: asalemcity@unimed.edu.ng

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ABSTRACT

Background: The use of medicinal plants in combating infertility has been a traditional practice in enhancing male reproductive performance.
Objectives: This research was proposed to assess the impact of chloroform fraction of *Ocimum gratissimum* (CFOG) leaf extracts on sperm function, antioxidants status and histopathology in plumbagin-initiated male Wistar rats.
Methods: Twenty rats were categorized into four groups (n=5) as follows: normal control as well as sterile control were administered water (groups 1 and 2 respectively); plumbagin-induced sterile rats treated with 100mg/kg CFOG leaf extract and 400mg/kg CFOG (groups 3 and 4 respectively) through gavaging for fourteen days.
Results: 100 mg/kg and 400 mg/kg of CFOG notably increased the total antioxidant capacity, glutathione reduced levels alongside superoxide dismutase and catalase activities in a dose-reliant pattern but prevented plumbagin-mediated increase in lipid peroxidation. Furthermore, CFOG significantly enhanced the sperm parameters of the sterile rats and fostered cytoprotection as evident in the histology of the testes of the CFOG-administered rats. A significant rise was noticed in the estradiol and testosterone levels in CFOG–treated rats compared to the sterile control.
Conclusion: Therefore, it could be inferred that *Ocimum gratissimum* leaf possesses a capacity to mitigate plumbagin-induced male reproductive dysfunction owing to its ability to boost the antioxidant capacity of the cells and reverse spermatocellular damage.

Keywords: Plumbagin; Spermatocellular damage; Antioxidants; Infertility

1.0 Background

Sperm cells are highly susceptible to oxidative stress (Aitken, 2020). This is attributed to numerous sources of reactive oxygen species (ROS) generation in the sperm cells (Ahmadi *et al*., 2019). These sources include activities of L-amino oxidase, lipoxygenase, NADPH oxidase and the mitochondria, which are highly prone to leakage of electrons generated via the glycolytic pathway needed to meet the energy requirements of the cells amongst other endogenous and exogenous sources (Aitken, 2018). These free radicals at regulated levels play important physiological roles in the sperm cells, such as aiding enhanced sperm capacitation, acrosome reaction, and signalling processes to ensure fertilization and the stability of the mitochondrial capsule in the mid-piece (Calvert *et al.,* 2019). However, the reverse is mostly the case when the antioxidant capacity is overwhelmed. The imbalance in pro-oxidant -antioxidant status could lead to several pathological conditions, including diabetes mellitus and male infertility, among others (Ashibe *et al*., 2019).

Antioxidants play indispensable roles in maintaining male fertility; they help in scavenging the free radicals, thus maintaining a balance which keeps the male gametes free from damage (Kim *et al*., 2019). The endogenous sources of the antioxidant system in the sperms cells are mostly inadequate as a result of increased endogenous and exogenous sources of ROS (Alabi *et al*., 2020). This has led to an aggressive search for plants with high antioxidant potential to help in

augmenting the endogenous antioxidant level to combat the high incidence of male infertility.

Ocimum gratissimum is a well-known plant with inherent medicinal and nutritional properties. It is mostly consumed as a spice in the West African region (Sheneni *et al*., 2018). It is used in the treatment of various diseases such as respiratory tract infections, diarrhoea, headache, skin diseases, fever, and cough, amongst others (Imosemi, 2020). It is rich in various phytochemicals such as flavonoids, phenols, cardiac glycosides, tannins, saponins and its oil contains 1,8-cineol, eugenol, thymol, p-cimene which contribute to its high antioxidant capacity (Ojo *et al*., 2019).

Plumbagin, a crystalline bioactive compound identified as 2-methyl-5-hydroxy-1, 4-naphthoquinone, is a yellow pigment isolated from the roots of *Plumbago zeylanica*. It has been reported to possess numerous beneficial properties such as antimicrobial, anticarcinogenic, cardioprotective and antimalarial properties, etc (Shukla and Singh, 2015). However, it is known to cause excessive production of ROS such as H_2O_2 and O_2^- thereby causing depletion of intracellular glutathione levels, and mitochondrial proton leakage (Gani and Ganesan, 2013).

Male infertility is usually treated with drugs such as clomiphene citrate, which triggers the pituitary gland to stimulate the synthesis of Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that invariably enhance sperm count, morphology and motility. However, there are adverse effects like blurred vision, acne, irritability and exacerbation of prostate cancer, which are associated with the use of this drug. Therefore, the search for medicinal plants is inevitable as an alternative or complementary to orthodox medicine. Hence, this research was meant to assess the attenuating effect of CFOG extract on plumbagin-induced male infertility in Wistar rats.

2.0 Materials and Methods

2.1 Materials

2.1.1 Harvesting of Plant Materials

Ocimum gratissimum leaves were purchased from Iya-Laje Market, Ondo, Ondo State, Nigeria. Authentication was carried out at University of Ibadan, Nigeria and given identification number, DPUI 1504.

2.1.2 Procedure to obtain the Extract

The leaves were cleaned, air-dried and minced using a blender. Subsequently, the powdered leaves obtained were immersed in absolute C_2H_5OH for three days. The decanted mixture was filtered through sieving cloth thrice to ensure thorough purification of filtrate which was then concentrated via rotatory evaporator (Salemcity *et al*., 2021). Lastly, the crude extract underwent successive fractionation with several solvents (n-C_6H_{14}, $CHCl_3$, $C_4H_8O_2$ and H_2O) in a partitioning funnel, thus producing different fractions.

2.2 Methods

2.2.1 Experimental Animals

The subjects used were male Wistar rats obtained from a Farm facility in Ogbomoso, Oyo State, Nigeria. These rats were sheltered at the animal facility of the University of Medical Sciences, Ondo State, Nigeria, where they passed through a two-week acclimatization period. Normal environmental situations were ensured, with limitless access to food and water during the study. After this, 8mg/kg of plumbagin was administered for two weeks before administration of the extract for another two weeks.

2.2.2 Experimental Design

In the experiment, a total of 20 rats with body weights ranging from 80 g to 100 g were used and divided into four groups as shown below:

Group 1 (Normal Control): Rats were administered water (vehicle).

Group 2 (8 mg/kg Plumbagin-Sterile control)

Group 3 (Plumbagin + $CFOG_{100mg/kg}$

Group 4: (Plumbagin + $CFOG_{400mg/kg}$)

2.2.3 Animal Euthanisia and Sample Collection

The rats were gently euthanized through cervical dislocation. Blood samples were obtained via cardiac puncture and centrifuged at 3,000 g for 300 seconds to separate the supernatant.

Open castration method was used and then the testicles were gently collected through the incision site. The testicles were then homogenized and centrifuged at 12,000 g. The left epididymis was collected for the sperm count.

2.2.4 Left testis antioxidant status

To obtain the post-mitochondrial fraction, which was used for the biochemical assays, the testes were homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 1.15 percent KCl. Subsequently, the ensuing homogenates were spun at 10,000 g for quarter-an hour at 4°C. Using H_2O_2 as a substrate, catalase (CAT) activity was measured at 240 nm via Claiborne's (1995) approach. The activity of superoxide

dismutase (SOD) was measured at 480 nm using the procedure outlined by Misra and Fridovich (1972). The concentration of reduced glutathione (GSH) was determined at 412 nm as outlined by Jollow et al., 1974). Chwatko (2013) procedure was used to determine the total antioxidant capacity (TAC) while lipid peroxidation was assessed as malondialdehyde (MDA) at 532 nm (Farombi et al., 2000).

2.2.5 Assessment of sperm increasing motility

Sperm cells were released onto a sterile, clean glass slide after the cauda epididymis was sliced using surgical blades. Following this, the sperm was thoroughly mixed and covered with a coverslip (24 x 24 mm) after being diluted with a 2.9% sodium citrate dihydrate solution that had been previously heated to 37 °C. By viewing at least ten microscopic fields under a phase contrast microscope at a magnification of X 200, the motility of sperm was assessed. Based on their motility, the sperm in the same field were sectioned into either progressive, non -progressive, or immotile. The information was presented as a proportion of the increasing motility of sperm (Zemjanis, 1970).

2.2.6 Evaluation of epididymal sperm count

This was calculated using the Wang (2003) approach. After being minced in nrmal saline, the epididymal sperm was filtered through nylon mesh. A 5µL sperm suspension aliquot was then combined with 95µL of diluents (0.25% trypan blue and 0.35% formalin with 5% NaHCO_3). The Neubauer chamber and a light microscope set at x100 were used to count the sperm cells in the epididymis after 10µL of the diluted sperm was placed on the hemocytometer and allowed to settle for five minutes in a humid chamber.

2.2.7 Assessment of sperm morphology and viability assay

The standard process of Gatimel et al. (2017) was followed in determining these parameters. A glass slide having some of the sperm suspension on it was covered with a cover slide. The smeared slides were stained with 1% eosin and 5% nigrosine in a 3% sodium citrate dehydrate solution to determine sperm motility, while a reagent comprising 0.1 g eosin and 0.3 g fast green dissolved in distil water and ethanol (2:1) was used to determine morphological defects. Each rat's 400 sperm cells were analyzed for morphological abnormalities.

2.2.8 Circulatory concentrations of pituitary and testicular hormonal assay

Using enzyme-linked immunosorbent assay kits specific for rats, testosterone and estradiol status were measured in accordance with the manufacturer's instructions.

2.2.9 Light Microscopic Examination

Following a 24-hour fixation period with Bouin's solution (Avwioro, 2020), the testes and epididymis samples were prepared for histological analysis using Yoshida et al., (2009) procedure. The tissue processor (Leica TP 1020) was used to automatically dehydrate the fixed tissues at increasing alcohol concentrations. The tissues were then cleaned by xylene and embedded in paraffin wax. Subsequently, the tissues were sliced with a microtome into slices of 4-5 µm, placed on slides, and stained with hematoxylin and eosin. The slides were photographed with a Sony DSC-W 30 Cybershot and inspected with an Olympus CH light microscope (Olympus, Tokyo, Japan). The cytoplasm was dyed pink and the nuclei blue.

2.3 Statistical Analysis

One-way analysis of variance and Tukey post-hoc test were used to analyze the data by utilizing Graph Pad Prism 8 software, La Jolla, California, USA. Difference at $P< 0.05$ were considered significant.

3. Results

3.1 Chloroform fraction of *Ocimum gratissimum* leaf extract (CFOG) improved the sperm morphology in plumbagin-stimulated male rat sterility

Various sperm analysis parameters were examined in sterile rats treated with CFOG (Figure 1). A significant elevation was observed in sperm defects of the sterile group with no treatment in comparison to control. However, notable decrease was noticed in the sperm defects of the CFOG-treated groups in dose-dependent manner when compared to control.

3.2 Sperm capacitation was enhanced upon treatment with CFOG

Major increase in the percentage of sperm capacitation in both the acrosome-intact capacitated and uncapacitated sperm as well as a notable decline in percentage acrosome-reacted capacitated sperm in the untreated group relative to the control were observed. Intriguingly, acrosome-intact uncapacitated sperm cells decreased considerably following administration of CFOG doses (100 and 400mg/kg).

Conversely, significant rise was observed in acrosome-intact and acrosome-reacted capacitated sperm cells upon administration of CFOG to plumbagin pre-treated rats (Figure 2).

3.3 Stabilization of sperm membrane integrity, improvement of viability and motility by administration of CFOG to plumbagin-treated rats

In Figure 3, significant decreases were noticed in sperm integrity, viability and motility in PG group compared to the control. Meanwhile, intubation of CFOG considerably increases the parameters to near normal relative to control rats.

3.4 Serum Testosterone and Estradiol assays

There were significant decreases in testosterone and estradiol levels of PG group compared to the control. In contrast, it was observed that treatment with CFOG initiated significant dose-independent increases in testosterone while dose-dependent increase was noticed in estradiol to near normal relative to control rats (Figure 4).

3.5 Antioxidant assays

The TAC, GSH levels, SOD and CAT activities following administration of CFOG to PG-induced sterile male were investigated in Figure 5. There were significant decreases in all the parameters in PG rats compared to control. However, dose-dependent increases were observed in the antioxidant status of the CFOG-treated rats relative to PG-untreated group.

3.6 Inhibition of plumbagin-induced lipid peroxidation by CFOG

The results of malondialdehyde (MDA) status of PG rats treated with doses of CFOG were displayed in Figure 6. It could be noticed that notable elevation of MDA occurred in PG rats, while a significant dose-dependent decline was observed in CFOG-administered rats relative to normal control.

3.7 Effects of CFOG on the histological architecture of the testes of Plumbagin-induced sterility in male rats.

Plate 1 is a descriptive photomicrograph of the testes of rats. The control group shows normal architecture with no visible lesion while there is mild leydig cells hyperplasia with elevated maturation arrest inside seminiferous tubules in the PG-administered rats. Interestingly, administration of CFOG at both doses also resulted in normal architecture of the testes.

4.0 Discussion

The exogenous supply of antioxidants is highly sacrosanct in reducing the incidence of male infertility. Antioxidants aid in protecting the spermatozoa by reducing oxidative stress and improving its capacitation, functionality and architecture amongst others (Ojo *et al*., 2019). *Ocimum gratissimum* is an extensively munched plant for both medicinal and nutritional purposes. It is also found to be rich in bioactive phytoconstituents which possess numerous antioxidant potentials (Lawrence *et al*., 2019). The need to find safer alternatives in traditional medicine as opposed to the use of conventional drugs in combating the surge in male infertility has been on the increase.

The plasmalemma of sperm cells is full of polyunsaturated fatty acids (PUFA) for enhancing the flexibility and fluidity necessary for membrane fusion during fertilization (Griffin *et al*., *et al*., 2019). However, the PUFA contains double bonds which makes them highly susceptible to attack by free radicals thereby leading to the initiation of lipid peroxidative process and the production of MDA as an end-product of the cascade (Adedara *et al.,* 2017). As a result, MDA status of plumbagin-administered rat cells was evaluated. Our results showed that there was an elevated level of MDA in the untreated rats. This result corroborates the previous research conducted by Bello and co-workers (2021), which showed that plumbagin significantly increased the MDA level in rat liver mitochondria. However, a significant reduction of MDA level in animals treated with doses of CFOG probably stemmed from the presence of certain bioactive phytochemicals which could scavenge free radicals and prevent possible membrane damage that may result from ROS production in the spermatozoa. Therefore, this could be one of the modes of action of CFOG in ameliorating infertility in male animals.

Furthermore, the capacitation, functional and morphological parameters of the sperm were assessed in figures 1-3. The depletion in sperm viability, motility, count, percentage normal heads, capacitation and compromise in the membrane integrity in the Plumbagin-untreated animals, is in agreement with the finding that significant ROS was generated by Plumbagin (Bello *et al*., 2021). The ROS has been reported to cause membrane damage and eventual

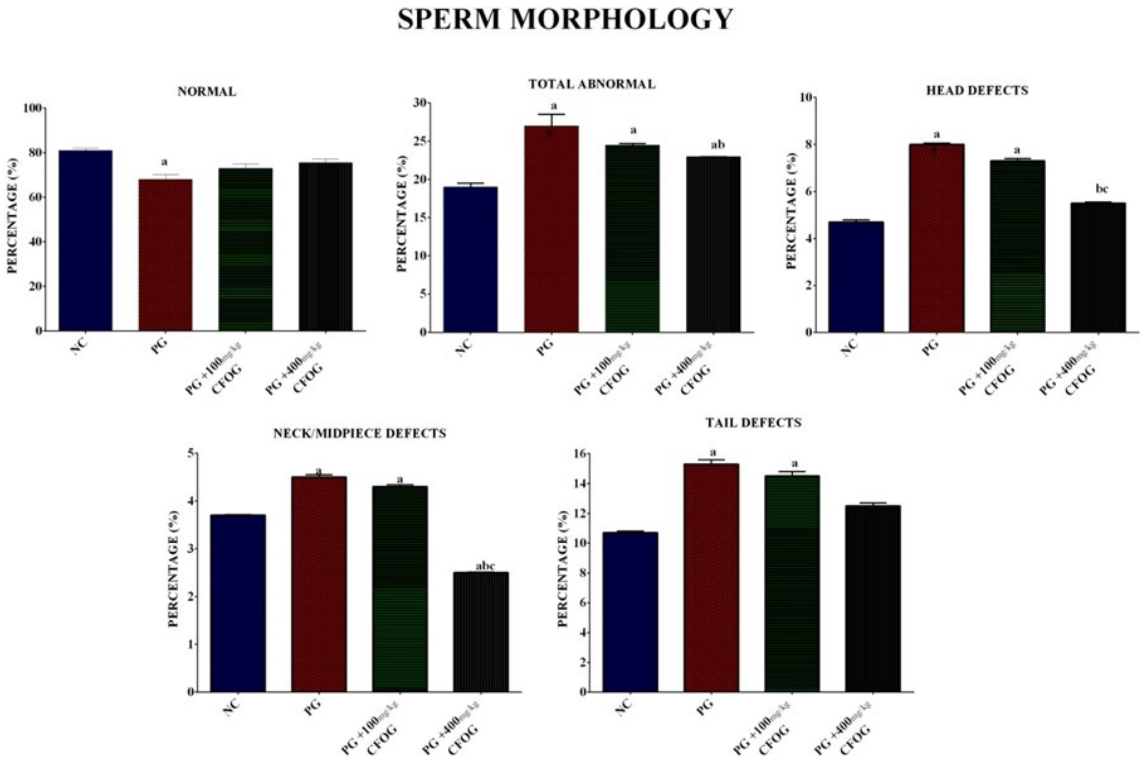

Figure 1: Effect of CFOG on sperm morphology in PG-induced infertility in Wistar rats

NC (Normal Control); PG (Plumbagin at 8 mg/kg); PG +100 mg/kg CFOG (Plumbagin + Chloroform fraction of leaf extract of *Ocimum gratissimum* (CFOG) at 100 mg/kg); PG + 400mg/kg CFOG (Plumbagin + Chloroform fraction leaf extract of *Ocimum gratissimum* (CFOG) at 400 mg/kg)

[a]Test groups vs normal control.

[b]Compared with PG group (P<0.05). ᶜVersus from PG +$CFOG_{100\ mg/kg}$ ($P < 0.05$).

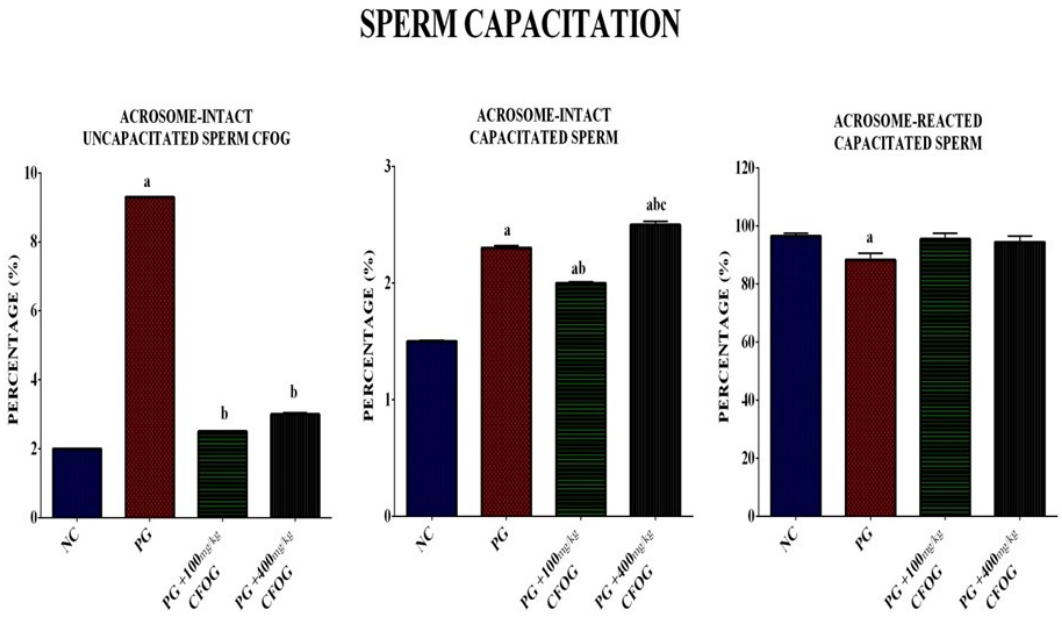

Figure 2: Effect of CFOG on sperm capacitation in plumbagin-triggered infertility in Wistar rats

NC (Normal Control); PG (Plumbagin at 8 mg/kg); PG +100 mg/kg CFOG (Plumbagin + Chloroform fraction leaf extract of *Ocimum gratissimum* (CFOG) at 100 mg/kg); PG + 400mg/kg CFOG (Plumbagin + Chloroform fraction leaf extract of *Ocimum gratissimum* (CFOG) at 400 mg/kg)

[a]Test groups vs normal control.

[b]Compared with PG group (P<0.05). ᶜVersus from PG +$CFOG_{100\ mg/kg}$ ($P < 0.05$).

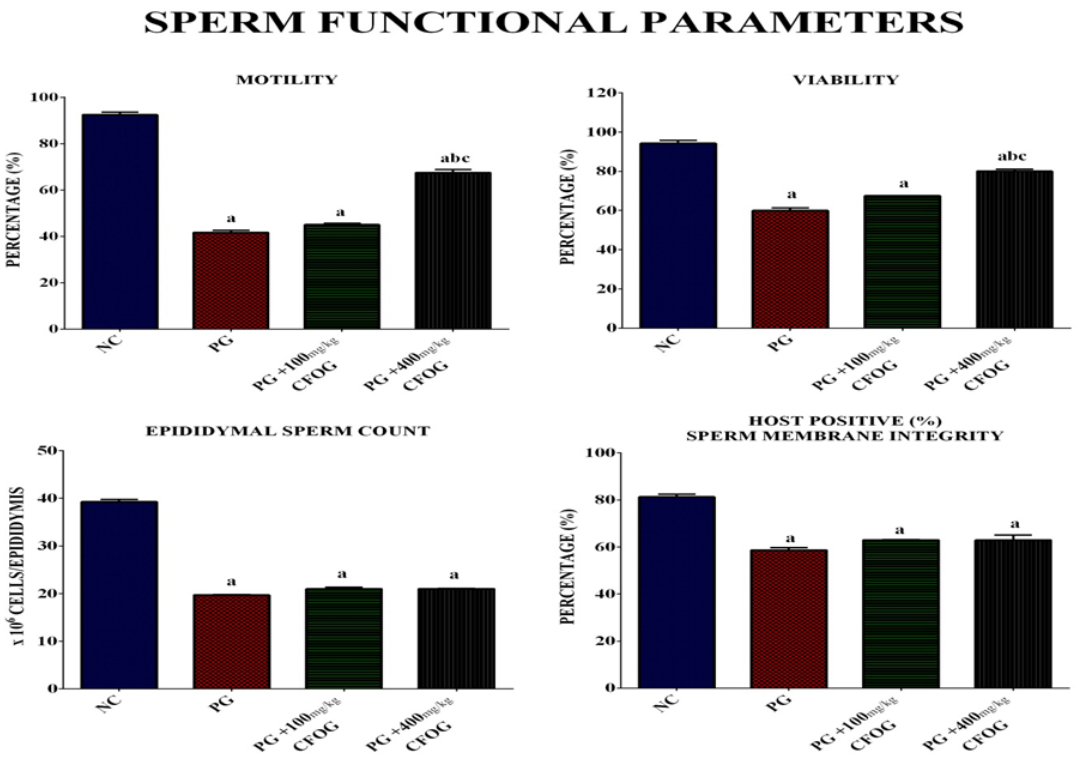

Figure 3: Effect of CFOG on sperm parameters in plumbagin-induced infertility in Wistar rats

NC (Normal Control); PG (Plumbagin at 8 mg/kg); PG +100 mg/kg CFOG (Plumbagin + Chloroform fraction leaf extract of *Ocimum gratissimum* (CFOG) at 100 mg/kg); PG + 400mg/kg CFOG (Plumbagin + Chloroform fraction leaf extract of *Ocimum gratissimum* (CFOG) at 400 mg/kg)
[a]Test groups vs normal control.
[b]Compared with PG group (P<0.05). [c]Versus from PG + $CFOG_{100\ mg/kg}$ ($P < 0.05$).

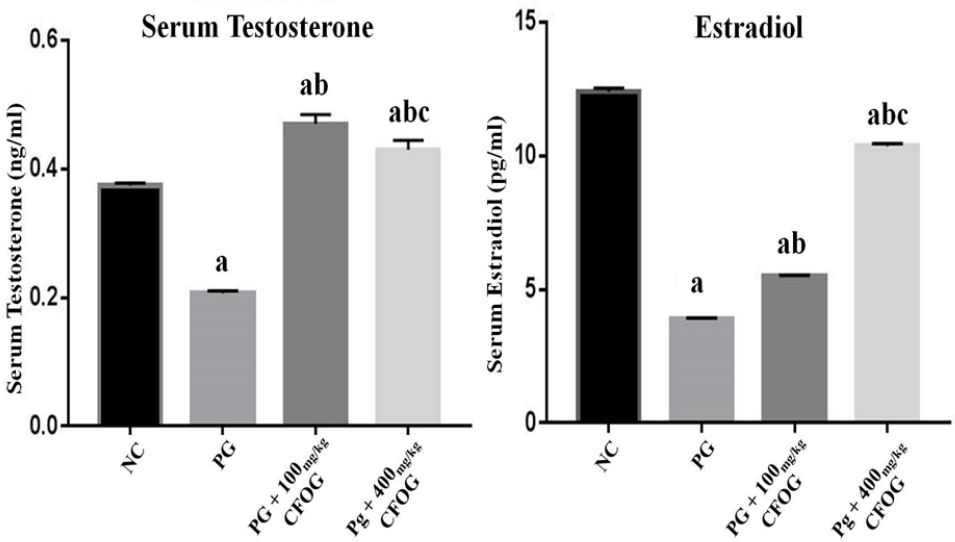

Figure 4: Effect of CFOG on serum testosterone and estradiol in plumbagin-initiated infertility in male Wistar rats

NC (Normal Control); PG (Plumbagin at 8 mg/kg); PG +100 mg/kg CFOG (Plumbagin + Chloroform fraction of leaf extract of *Ocimum gratissimum* (CFOG) at 100 mg/kg); PG + 400mg/kg CFOG (Plumbagin + Chloroform fraction leaf extract of *Ocimum gratissimum* (CFOG) at 400 mg/kg)
[a]Test groups vs normal control.
[b]Compared with PG group (P<0.05). [c]Versus from PG + $CFOG_{100\ mg/kg}$ ($P < 0.05$).

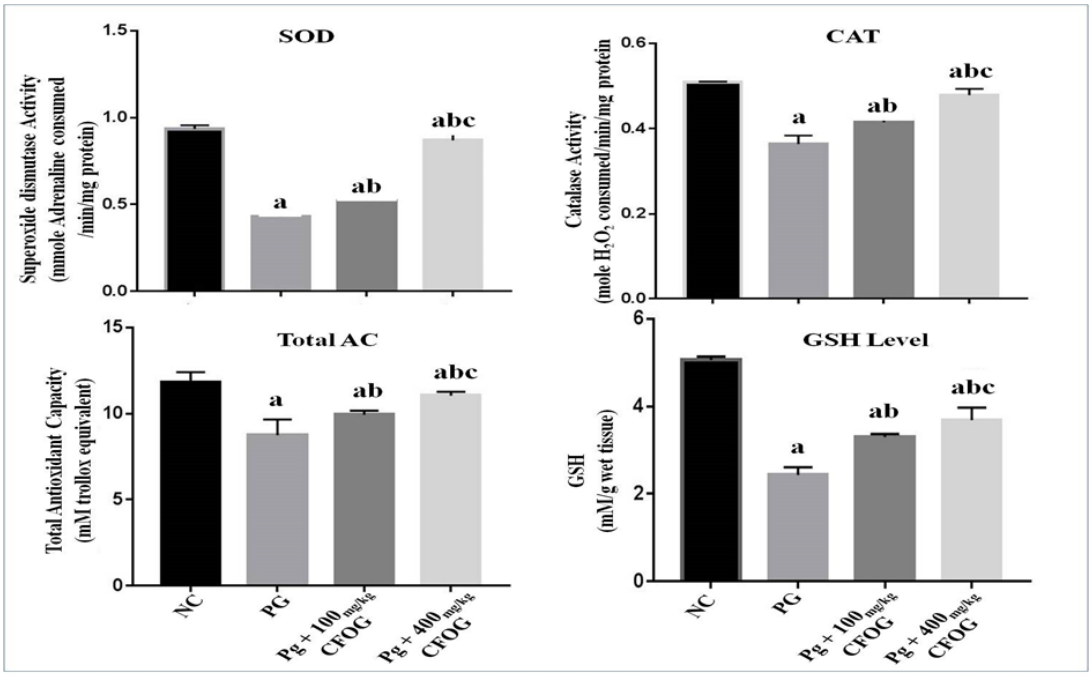

Figure 5: Effect of CFOG on superoxide dismutase, catalase activities, total antioxidant capacity and GSH status in plumbagin-initiated infertility in Wistar rats

NC (Normal Control); PG (Plumbagin at 8 mg/kg); PG +100 mg/kg CFOG (Plumbagin + Chloroform fraction leaf extract of *Ocimum gratissimum* (CFOG) at 100 mg/kg); PG + 400mg/kg CFOG (Plumbagin + Chloroform fraction leaf extract of *Ocimum gratissimum* (CFOG) at 400 mg/kg)
[a]Test groups vs normal control. [b]Compared with PG group (P<0.05). [c]Versus from PG + $CFOG_{100\ mg/kg}$ (P < 0.05).

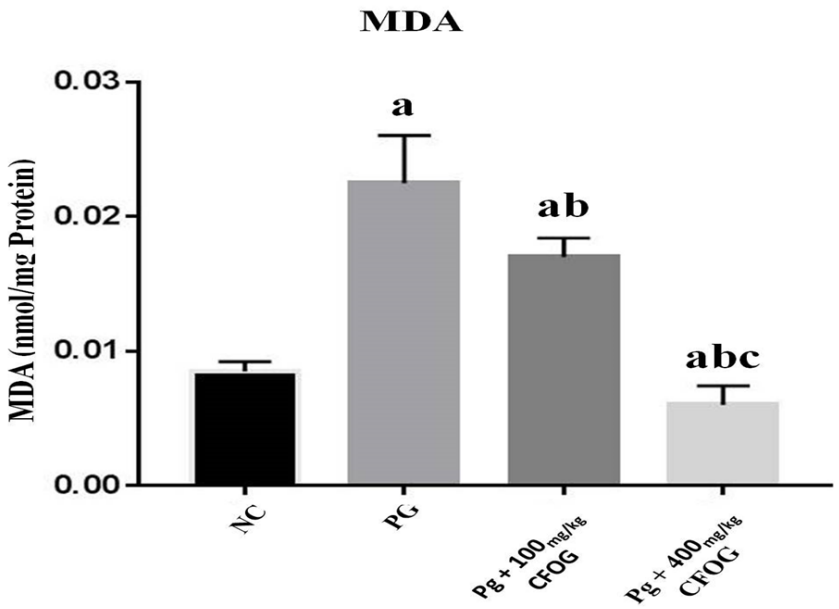

Figure 6: Effect of CFOG on MDA level in plumbagin-stimulated infertility in male Wistar rats

NC (Normal Control); PG (Plumbagin at 8 mg/kg); PG +100 mg/kg CFOG (Plumbagin + Chloroform fraction leaf extract of *Ocimum gratissimum* (CFOG) at 100 mg/kg); PG + 400mg/kg CFOG (Plumbagin + Chloroform fraction leaf extract of *Ocimum gratissimum* (CFOG) at 400 mg/kg)
[a]Test groups vs normal control. [b]Compared with PG group (P<0.05). [c]Versus from PG + $CFOG_{100\ mg/kg}$ (P < 0.05).

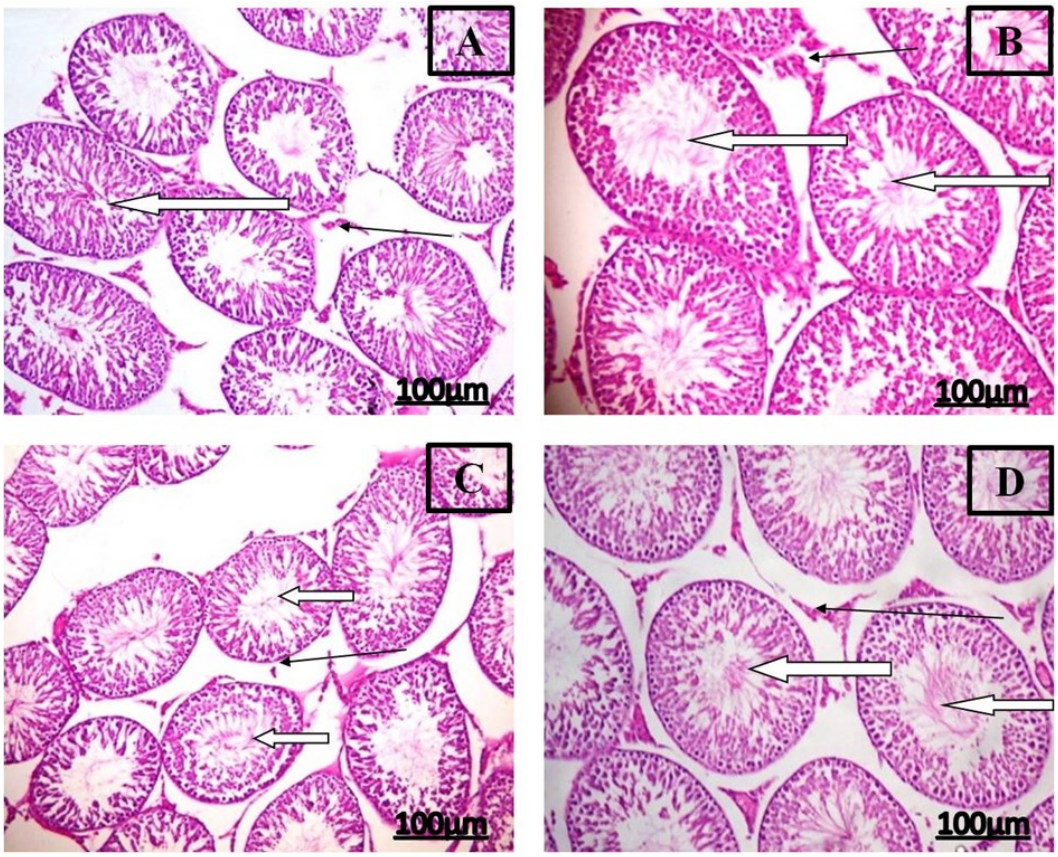

Plate 1: Representative histopathological sections of the testes (X 100).
Plate **A:** Photomicrograph of testes of normal control. Plate **B:** Photomicrograph of testes of PG treated rats. Plate **C:** Photomicrograph of testes of PG rats administered with 100 mg/kg CFOG. Plate **D:** Photomicrograph of testes of PG rats treated with 400 mg/kg CFOG.

cell death. In contrast, as the ROS was mopped up by administration of CFOG to the rats, the enervating consequences of free radical production such as elevated morphological defects and membrane destruction, were subsequently prevented. Thus, it shows that CFOG contains efficacious antioxidant capability that could impede such debilitating occurrences (Agbodjento *et al*., 2019).

As a result of the indispensable roles of antioxidants in combating ROS generation, the cell antioxidant levels were evaluated via investigation of activities of SOD and CAT, TAC and the GSH levels. The SOD and CAT are two important endogenous enzymes which scavenges O_2^- and H_2O_2 respectively (Lawrence *et al*., 2019). Glutathione is a non-enzymatic antioxidant that can react with ROS directly. It is also a co-factor of glutathione peroxidase, an enzyme which triggers decrease of H_2O_2 and other hydroperoxides and in turn protects the sperm cells from oxidative damage (Ebong *et al*., 2014).

Interestingly, the results from our study revealed that the enzymes activities like SOD and CAT were considerably low in the animals administered only Plumbagin. This is very consistent with previous results that plumbagin stimulates excessive free radical production. This overwhelming ROS generation leads to the evident SOD and CAT activities reduction noticed in PG rats. Oxidative stress ensued as the aftermath, and consequently, membrane damage occurs. A report revealed that one of the mechanisms through which plumbagin induces toxicity is via the production of hydrogen peroxides and superoxide anions (Gani and Ganesan, 2013). However, our study reveals that the activities of these enzymes were significantly enhanced by co-treatment with doses of CFOG. This shows that the plant extract could exhibit cytoprotective capability through its ability to foster the antioxidant defence system. Improved GSH and TAC levels observed were suggestive of free radicals scavenging capacity of the plant extract. Previous studies have reported that excessive ROS production are considered as one of the triggers of male sterility (Alahmar, 2019). That is why the physicians usually

advise the infertile men to desist from wearing very tight clothing to package the scrotum, because ROS can be produced in the process to destroy the sperm cells, reduce viability and count.

Aside from oxidative stress, hormonal imbalance has been seen as a non-oxidative mechanism by which male infertility is propagated (Darbandi *et al*., 2018). Spermatogenesis and testosterone production are key determinants of fertility and are regulated by the FSH and the LH produced by the stimulation of the anterior pituitary by the hypothalamus gonadotropin-releasing hormone (GnRH) (Adedara *et al*., 2017). Estradiol also plays a key role in managing male infertility (Darbandi *et al*., 2018). In this study, our findings show that there was a marked decline in the circulatory levels of testosterone and estradiol in the plumbagin-administered rats. This further suggests an interference in the cellular processes involved in spermatogenesis alongside the pituitary-testicular axis regulation of reproductive function. However, a remarkable restoration in the testosterone and estradiol levels comparable to the control in a dose-dependent manner by CFOG suggests its ability to protect sperm cells via pituitary-testicular axis, thus regulating processes involved in spermatogenesis as well as the preservation of the Leydig cells from injury.

We further elucidated the protective mechanism of CFOG in the histopathological examination of the testes. The administered plumbagin resulted in damage to the Leydig cells alongside elevated maturation arrest in the seminiferous tubules. This was also responsible for the reduction in the circulating hormonal levels. Notably, CFOG's capacity to reverse spermatocellular damage (including Leydig cell impairment) and impede elevated maturation arrest indicates it contains bioactive compounds that facilitate these protective effects. A similar result obtained in testosterone levels well corroborates the efficacy of this plant extract in ameliorating male infertility.

Conclusion

Ocimum gratissimum leaf confers protection on sperm cells against infertility via oxidative and non-oxidative mechanisms. The oxidative mechanism includes maintenance of physiological levels of ROS via reducing its generation and boosting the antioxidant status of the cells, which in turn helps to prevent lipid peroxidation and maintain the morphology, capacitation and functionality of the sperm cells. The non-oxidative mechanisms include its influence on hormonal balance by acting on the pituitary-testicular axis and protecting the Leydig and Sertoli cells from damage.

Thus, *Ocimum gratissimum* can serve as a potent agent in combating the upsurge of male infertility due to its intrinsic antioxidant, anti-inflammatory and cytoprotective potentials.

Ethics approval and consent to participate

The approval for this research was obtained from the Animal Care and Use Research Ethical Committee at the University of Medical Sciences, Ondo, Ondo State, Nigeria.

Consent for publication

All authors gave their consent for the publication of this manuscript.

Availability of data and materials

The data generated for this study are available.

Competing interests

There was no potential conflict of interest reported by the author(s).

Funding

There was no funding for the study. The financial contribution was borne by the authors.

Authors' contribution

AJ designed the study and monitored the process of the experiments. OO proof-read and reviewed the manuscript. ME wrote the manuscript. OG and OJ did the statistical analysis. OI and PI were involved in the feeding of the animals and running of different assays.

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