Original Article

Elevated Levels of Gonadotrophic Hormones and Antioxidant Biomarker in Male Rats Following Administration of Hydromethanol Leaf Extract of *Justicia secunda* in Response to 2,4-Dinitrophenylhydrazine Induction

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ABSTRACT

Background: 2,4-Dinitrophenylhydrazine induces testicular toxicity and can result in reproductive dysfunction in male rats. **Aim:** This study investigated the effects of hydromethanolic leaf extract of *Justicia secunda* on phenylhydrazine (PHZ)-induced reproductive dysfunction in male Wistar rats. **Settings and Design:** Twenty rats (90–170 g) were grouped into five (A-E) (n = 4) with the approval of the research ethics committee. **Materials and Methods:** Group A (control) received 0.5 mL of normal saline, Groups B to E received PHZ, PHZ + Astymin (0.5 mL), PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg), respectively. All animals in Groups B to E received 2 mg/kg PHZ intraperitoneally for 2 days, and thereafter, administration of Astymin and *J. secunda* commenced in Groups C, D and E for 14 days using gavage. **Statistical Analysis Used:** The data were analysed using a one-way analysis of variance and the Bonferroni *post hoc* test. **Results:** Follicle-stimulating hormone (FSH) decreased significantly in PHZ, PHZ + Astymin and PHZ + *J. secunda* (0.2 mg/kg) and increased significantly in PHZ + *J. secunda* (0.5 mg/kg) than control. Luteinising hormone (LH) and testosterone significantly ($P < 0.001$) reduced in treated groups than control. Total cholesterol, triglyceride, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol and very-low-density lipoprotein-cholesterol were significantly reduced in the treated groups than the control. Tumour necrosis factor alpha (TNF-α) significantly ($P < 0.001$) increased in treated groups than in control. Testicular glutathione (GSH), glutathione peroxidase, catalase and malondialdehyde significantly increased in extract-treated groups compared to control. Superoxide dismutase significantly decreased in PHZ-treated group than in the control. **Conclusion:** PHZ administration caused testicular toxicity and altered biochemical markers, astymin treatment reduced male reproductive hormones, while *J. secunda* (0.5 mg/kg) increased FSH and LH, decreased TNFα levels and altered the concentration of testicular antioxidant markers. These alterations may be linked to the toxic effect of PHZ and could negatively affect spermatogenesis.

Keywords: *Astymin, Justicia secunda, phenylhydrazine, testosterone*

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Introduction

In recent years, the prevalence of some diseases, including anaemia and infertility has been on the increase worldwide; hence, need to find a global remedy.[1] In general, occupations involving the manufacture of some non-degradable chemicals, intensive exposure to radiation and use of toxic solvents are reported to be associated with reproductive dysfunction.[2] Alteration in the reproductive system may occur at the hypothalamic, anterior pituitary gland or gonads, and may likely lead to reproductive dysfunction.[3] Phenylhydrazine (PHZ), a strong oxidant agent used in industry, laboratory and therapeutic settings has been described to cause haemolytic anaemia, hypoxia, inflammation, alterations in the liver, kidney, central nervous system and autoimmune disturbances.[4] PHZ administration destroyed red blood cells by causing oxidative stress within erythrocytes and changes at the cellular level resulting in haemolytic anaemia.[5] *Justicia secunda* has been conventionally used as a blood tonic in Nigeria. Hence, this study investigates the reproductive impact of *J. secunda* in PHZ-induced toxicity in male Wistar rats.

Materials and Methods

Drugs and chemicals

Methanol, formalin and chloroform (Guangzhou JHD Chemical Reagent Co., Ltd. Shantou Guangdong, China), 2,4-dinitrophenyl-hydrazine (Sigma-Aldrich Limited Germany) and Astymin (Bayer Company, Inc., Leverkusen, Germany).

Hydro-methanolic preparation of *Justicia secunda*

Fresh leaves of *J. secunda* were obtained and sent to a Botanist in the Faculty of Pharmacy, Madonna University, for identification. It was dried at room temperature and ground to powder. Using an electronic weighing balance (Doran Scales, Inc., Batavia, Illinois, USA), 590 g of pulverised *J. secunda* was steeped in 1450 mL of methanol and 750 mL of water for 48 h. The obtained suspension was sieved and filtered using Whatman filter paper. The filtrate was then concentrated in a laboratory electronic incubator (Labotech International Co., Ltd., Tokyo, Japan) at a temperature of 60°C. The jelly-like residue was introduced into an air-tight container and stored in the refrigerator until used. The LD_{50} of *J. secunda* used for this study was 3800 mg/kg body weight, as reported by Onochie *et al*.[6] The choice of hydromethanolic extraction was to extract both hydrophilic and hydrophobic compounds of *J. secunda*.

Laboratory animals

Twenty male Wistar rats (90–170 g) were used for this study. The animals were kept in the animal house of the Department of Physiology, University of Calabar, Nigeria. The animals were kept in standard cages of 5 rats per cage. They were given *ad libitum* access to feed (AEC Agrosystem limited, Portharcourt, Rivers State, Nigeria) and fresh water and exposed to 12/12-h light/dark phase. They were acclimatised for 7 days and kept in line with laid down ethics for animal care approved by the National Committee for Research Ethics in Science and Technology (NENT), 2018. Before the study was initiated, ethical approval was obtained from the University of Calabar Animal Ethics Committee, ensuring alignment with the standard guidelines for the use of experimental animals. The study received the necessary approval, indicated by the ethical clearance registration reference number 040PHY3719. The research adhered strictly to the guidelines for use and care of laboratory animals, laid down by the research committee, which aligns with the standards outlined by the World Health Organisation (WHO).

Experimental design and administration of drugs

The animals were allotted into five groups (n = 4). At the end of 7 days of acclimatisation, 2,4-dinitrophenyl-hydrazine, astymin and *J. secunda* extract administration began. The drugs were given orally using gavage [dose per rat outlined in Table 1], once, daily, to animals in treatment Groups (B to E), using the doses outlined in Table 1, whereas the control group was given feed and 0.5 mL normal saline as a vehicle. Astymin and *J. secunda* extract administration lasted for 14 days, whereas 2,4-dinitrophenyl-hydrazine was given intraperitoneally for 2 days; thereafter, the rats were anaesthetised with chloroform, and blood samples were collected from rats through the ocular puncture. The left testis of each rat was harvested for tissue antioxidant and pro-inflammatory cytokine

Table 1: Study design and drugs administration

Groups	Number of rats	Treatment
A (control)	4	Feed + 0.5 mL of normal saline as a vehicle throughout the experimental period
B	4	2 mg/kg bw of 2, 4-dinitro PHZ
C	4	2 mg/kg bw of 2, 4-dinitro phenyl-hydrazine + 0.5 mg/kg of astymin
D	4	2 mg/kg bw of 2, 4-dinitro PHZ +0.2 mg/kg of *J. secunda*
E	4	2 mg/kg bw of 2, 4-dinitro PHZ +0.5 mg/kg of *J. secunda*

PHZ=Phenylhydrazine, *J. secunda*=*Justicia secunda*

assessment. The samples were stored in an ice pack and immediately utilised for analysis of reproductive hormones, lipid profile parameters and testicular oxidative stress markers.

Measurement of serum reproductive hormones

Using chloroform anaesthesia, a 5 mL syringe and a 21G needle, blood was extracted through a heart puncture. The blood samples were added to plain-capped sample bottles, left for 2 h and then centrifuged using a bucket centrifuge for 5 min at 1000 rpm (B-Bran Scientific and Instrument Company, England). For this experiment, the settled serum was utilised. A rat-specific enzyme-linked immunosorbent assay (ELISA) kit technique was employed to detect the levels of serum testosterone, luteinising hormone (LH) and follicle-stimulating hormone (FSH), similar to what has been done by Mobisson *et al*., Khaki *et al*.[3,7] The rat-specific ELISA kits (reference numbers SL0668Ra for testosterone, SL0286Ra for FSH and SL1093Ra for LH) were acquired from Sunlong Biotech Limited, China.

Serum lipid profile assessment

The above-described process yielded the serum needed for this experiment. The Randox test was utilised to determine the serum levels of total cholesterol (TC) and different lipid fractions, as explained below.

Estimation of serum total cholesterol concentration

TC in plasma was measured using the method of Siedel *et al*.[8]

Evaluation of serum triglyceride concentration

The procedure described by Friedewald *et al*.[9] was used to calculate the serum triglyceride (TG) content of the samples. The reactions resulted in the production of a purple quinoneimine dye, which was measured colorimetrically at 540 nm.

Evaluation of high-density lipoprotein-cholesterol

High-density lipoprotein-cholesterol (HDL-C) was determined using the Friedewald *et al*.[9] method. Its principle is the same as previously described TC evaluation.

Evaluation of low-density lipoprotein-cholesterol level

Low-density lipoprotein-cholesterol (LDL-C) was measured using the difference between serum TC, the sum of HDL-C and TG as presented below:

LDL-c = TC-(VLDL-c + HDL-c)

Assessment of testicular oxidative stress markers

The Potter-Elvehjem homogeniser was used to homogenise each rat's left testis. Using a bucket centrifuge (B-Bran Scientific and Instrument Company, England), 20% (1/5 w/v) of the tissue homogenate was produced in 50 mm Tris-HCl buffer (pH 7.4) with 1.15% potassium chloride and centrifuged at 10,000 rpm at 4°C for 10 min. For the catalase (CAT) test, which used hydrogen peroxide as a substrate, the supernatant was collected. Reduced glutathione (GSH) was measured at 412 nm using the technique described by Luchese *et al*.[10] The activity of glutathione peroxidase (GPx) was examined using hydrogen peroxide as a substrate with regard to the Luchese *et al*.[10] method. Superoxide dismutase (SOD) was assayed using the method described by Misra and Fridovich.[11] The concentration of malondialdehyde (MDA) was determined in thiobarbituric acid reacting substances as described by Choudhary *et al*., Ohkawa *et al*.[12,13] After that, 0.2 mL of 8.1% sodium dodecyl sulphate was formed by the combined reaction, 1.5 mL of 20% acetic acid solution that was adjusted to pH 3.5 using sodium hydroxide and 1.5 mL of 0.8% thiobarbituric acid water solution were added to 0.2 mL of 10% (w/v) homogenate. The mixture was raised to 4.0 mL and heated for 60 min at 95°C using distilled water. After the tap water cooled, 1.0 mL of distilled water and 5.0 mL of the n-butanol and pyridine (15:1 v/v) mixture were added. The mixture was centrifuged at 4000 rpm. After removing the crude layer, the absorbance was measured up to 532 nm and compared to the MDA standards values. Normal absorption was used to measure value levels based on absorption values.

Estimation of tumour necrosis factor alpha level

The manufacturer's methodology was followed to estimate the amount of TNF-α in the testis supernatants. TNF-α was measured at a sensitivity limit of 4 pg/mL using a particular mouse TNF-α (BioLegend ELISA MAX™Deluxe kit, USA). The measurement was carried out using a microplate reader at a wavelength of 450 nm filter at room temperature following the BioLegend methodology. The testis's TNF-α level was determined using the TNF-α kits' standard curve. Next, the testis's TNF-α levels were expressed as pg/mg of protein. This method was employed by Wopara *et al*.[14]

Statistical analysis

Data were analysed using SPSS Version 17.0 by International Business Machines (IBM), New York, United States and an ExcelAnalyzer. One-way analysis of variance was utilised in comparing the differences within groups, followed by Bonferroni *post hoc* test. All results are presented as mean ± standard error of the mean, $n = 4$. The level of significance was set at $P < 0.05$.

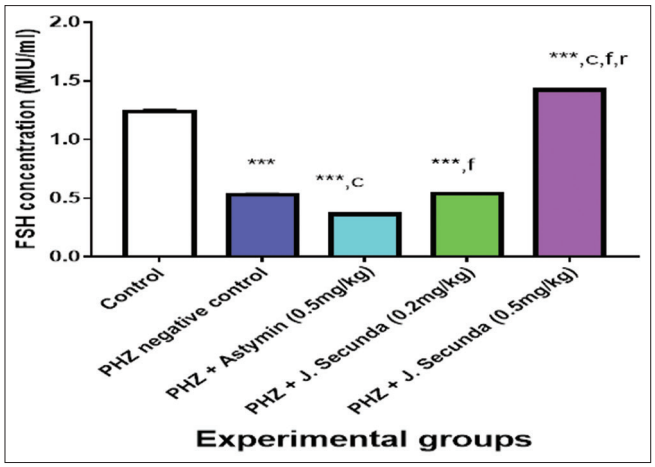

Figure 1: Comparison of follicle-stimulating hormone concentration of different experimental groups. Values are expressed as mean ± standard error of mean, $n = 4$. ***$P < 0.001$ versus control, $^{c}P < 0.001$ versus phenylhydrazine (PHZ) negative control, $^{f}P < 0.001$ versus PHZ + Astymin, $^{r}P < 0.001$ versus PHZ + *Justicia secunda* (0.2 mg/kg). FSH = Follicle-stimulating hormone, PHZ = Phenylhydrazine, *J. secunda* = *Justicia secunda*

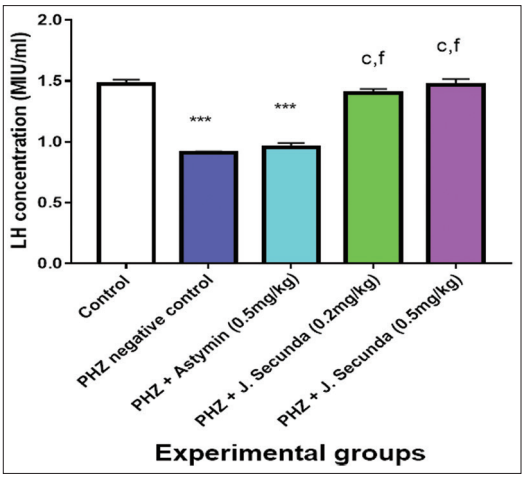

Figure 2: Comparison of luteinising hormone concentration of different experimental groups. Values are expressed as mean ± standard error of mean, $n = 4$. ***$P < 0.001$ versus control, $^{c}P < 0.001$ versus phenylhydrazine (PHZ) negative control. $^{f}P < 0.001$ versus PHZ + Astymin. PHZ = Phenylhydrazine, LH = Luteinising hormone, *J. secunda* = *Justicia secunda*

Results

Comparison of serum reproductive hormone concentrations in control and different experimental groups

In Figure 1, the serum concentration of FSH was significantly ($P < 0.001$) reduced in the group treated with PHZ, PHZ + Astymin and PHZ + *J. secunda* (0.2 mg/kg) compared to control. However, rats treated with PHZ + *J. secunda* (0.5 mg/kg) were significantly higher ($P < 0.001$) compared to control and other treated groups. In Figure 2, there was a significant ($P < 0.001$) reduction in serum concentration of LH in the group treated with PHZ and PHZ + Astymin compared to control. However, rats treated with PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) were significantly ($P < 0.001$) higher than other treated groups. In Figure 3, there was a significant ($P < 0.001$) reduction of testosterone in rats treated with PHZ, PHZ + Astymin, PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) compared to control. However, rats treated with PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) were significantly ($P < 0.05$) higher compared to the group treated with PHZ + Astymin.

Table 2 shows a significant ($P < 0.001$) reduction of TC in rats treated with PHZ, PHZ + Astymin, PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda.* However, the group treated with PHZ + *J. secunda* (0.5 mg/kg) was significantly ($P < 0.001$) lowered compared to PHZ + *J. secunda* (0.2 mg/kg) treated group. Furthermore, the group treated with PHZ + *J. secunda* (0.2 mg/kg) was significantly ($P < 0.001$) higher than other treated groups. TG concentration was significantly ($P < 0.001$) reduced in groups treated with PHZ, PHZ + Astymin, PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) compared to control. The group treated with PHZ + *J. secunda* (0.5 mg/kg) was significantly ($P < 0.001$) lower compared to PHZ + Astymin and PHZ + *J. secunda* (0.2 mg/kg) treated groups. However, the rat group treated with PHZ + *J. secunda* (0.2 mg/kg) was significantly ($P < 0.001$) higher than other treated groups. High-density lipoprotein significantly ($P < 0.05$) reduced in the group treated with PHZ compared to the control. However, groups treated with PHZ + Astymin, PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) were significantly ($P < 0.001$) increased compared to control. Furthermore, the group treated with PHZ + *J. secunda* (0.5 mg/kg) was significantly ($P < 0.01$) lower compared to groups treated with PHZ + Astymin, PHZ + *J. secunda* (0.2 mg/kg). Low-density lipoprotein significantly ($P < 0.001$) reduced in groups treated with PHZ, PHZ + Astymin, PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) compared to control. However, rats treated with PHZ + *J. secunda* (0.5 mg/kg) were significantly ($P < 0.001$) lower compared to other treated groups. However, rats treated with PHZ + *J. secunda* (0.2 mg/kg) were significantly ($P < 0.001$) higher compared to other treated groups. Very low-density lipoprotein significantly ($P < 0.001$) reduced in groups treated with PHZ, PHZ + Astymin, PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) compared to control.

However, the group treated with PHZ + Astymin was significantly ($P < 0.001$) lower compared to PHZ-treated group. However, the group treated with PHZ + *J. secunda* (0.2 mg/kg) was significantly ($P < 0.001$) higher compared to PHZ and PHZ + Astymin treated groups.

Comparison of testicular tumour nephrotic factor alpha concentration in control and different experimental groups

In Figure 4, TNFα significantly ($P < 0.001$) increased in groups treated with PHZ, PHZ + Astymin and PHZ + *J. secunda (*0.2 mg/kg) compared to control. However, the group treated with PHZ + *J. secunda* (0.5 mg/kg) significantly decreased compared to PHZ, PHZ + Astymin and PHZ + *J. secunda* (0.2 mg/kg) treated groups.

In Figure 5, testicular reduced GSH significantly ($P < 0.001$) decreased in PHZ negative control compared to control. Groups treated with PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) were significantly ($P < 0.001$) increased compared to control. Furthermore, the group treated with PHZ + astymin, PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) were significantly ($P < 0.001$) higher compared to PHZ negative control. Groups treated with PHZ + *J. secunda (*0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) were significantly ($P < 0.001$) higher compared to PHZ + astymin group. Rats treated with PHZ + *J. secunda (*0.5 mg/kg) were significantly ($P < 0.001$) increased compared to PHZ + *J. secunda* (0.2 mg/kg). In Figure 6, testicular GPx was significantly ($P < 0.05$) increased in groups treated with PHZ + *J. secunda* (0.5 mg/kg) compared to control. Furthermore, the group treated with PHZ + *J. secunda (*0.5 mg/kg) was significantly higher compared to other treated groups. In Figure 7, testicular CAT was significantly ($P < 0.01$) decreased in groups treated with PHZ negative control and PHZ + Astymin compared to control. Rats treated with PHZ + *J. secunda* (0.5 mg/kg)

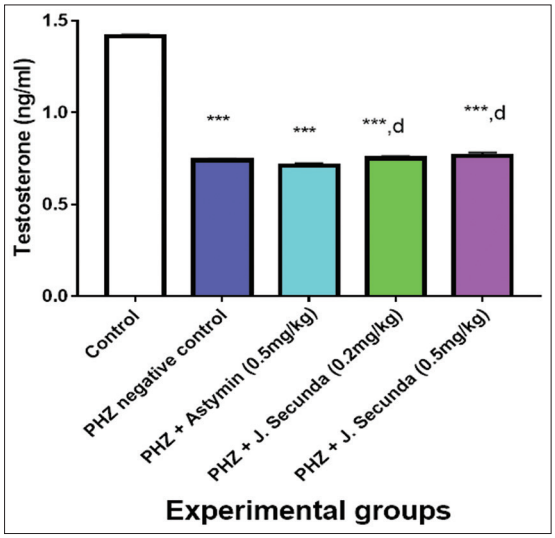

Figure 3: Comparison of serum lipid profile concentrations in control and different experimental groups. Values are expressed as mean ± standard error of mean, $n = 4$. ***$P < 0.001$ versus control, $^{d}P < 0.05$ versus phenylhydrazine + Astymin *Justicia secunda* (0.5 mg/kg) compared to control. PHZ = Phenylhydrazine, *J. secunda* = *Justicia secunda*

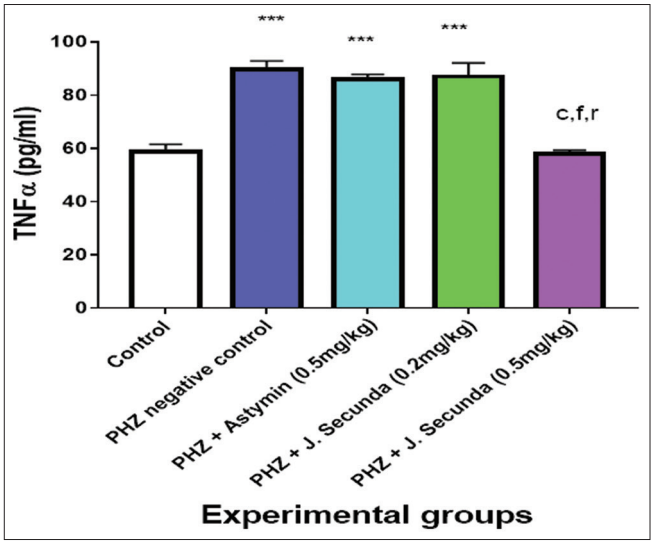

Figure 4: Comparison of testicular tumour necrotic factor-alpha in control and different experimental groups. Values are expressed as mean ± standard error of mean, $n = 4$. ***$P < 0.001$ versus control, $^{d}P < 0.05$ versus phenylhydrazine + Astymin. PHZ = Phenylhydrazine, *J. secunda* = *Justicia secunda*, TNFα = Tumour necrosis factor-alpha

Table 2: Comparison of serum lipid profile concentrations in control and different experimental groups

Parameters (mmol/L)	Group A control	Group B PHZ negative control	Group C PHZ + astymin (0.5 mg/kg)	Group D PHZ + *J. secunda* (0.2 mg/kg)	Group E PHZ + *J. secunda* (0.5 mg/kg)
TC	4.70±0.07	2.92±0.08***	2.95±0.13***	3.52±0.06***,^{c,f}	2.12±0.06***,^{c,f,r}
TG	1.77±0.02	1.46±0.01***	1.13±0.02***,^{c}	1.60±0.02***,^{c,f}	1.05±0.01***,^{c,e,r}
HDL	1.71±0.01	1.51±0.01*	1.38±0.06***	1.40±0.03***	1.15±0.07***,^{e,q}
LDL	3.91±0.06	2.08±0.06***	2.09±0.06***	2.81±0.10***,^{c,f}	1.42±0.01***,^{c,f,r}
VLDL	0.80±0.01	0.65±0.01***	0.51±0.01***,^{c}	0.71±0.01***,^{b,f}	0.49±0.00***,^{c,r}

*$P<0.05$ versus control, ***$P<0.001$ versus control, $^{b}P<0.01$ versus PHZ negative control, $^{c}P<0.001$ versus PHZ negative control, $^{e}P<0.01$ versus PHZ + astymin, $^{f}P<0.001$ versus PHZ + astymin, $^{r}P<0.001$ versus PHZ + *J. Secunda* (0.2 mg/kg), $^{q}P<0.01$ versus PHZ + *J. Secunda* (0.2 mg/kg). Values are expressed as mean±SEM, n=4. TC=Total cholesterol, TG=Triglyceride, HDL=High-density lipoprotein, LDL=Low-density lipoprotein, VLDL=Very LDL, PHZ=Phenylhydrazine, SEM=Standard error of mean, *J. secunda*=*Justicia secunda*

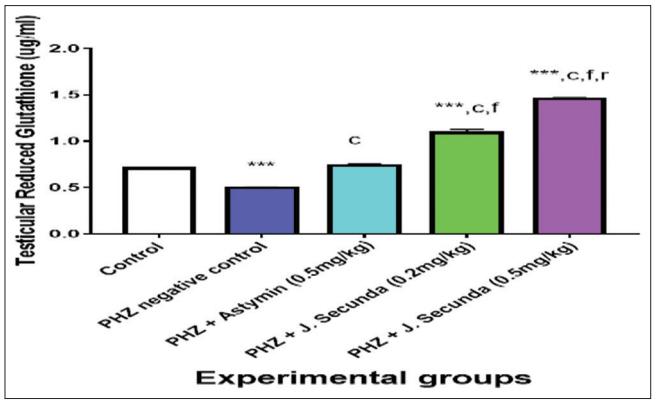

Figure 5: Comparison of testicular reduced glutathione of different experimental groups. Values are expressed as mean ± standard error of mean, n = 4. ***P < 0.001 versus control, ^{c}P < 0.001 versus phenylhydrazine (PHZ) negative control, ^{f}P < 0.001 versus PHZ + Astymin, ^{r}P < 0.001 versus PHZ + *Justicia secunda* (0.2 mg/kg). PHZ = Phenylhydrazine, *J. secunda* = *Justicia secunda*

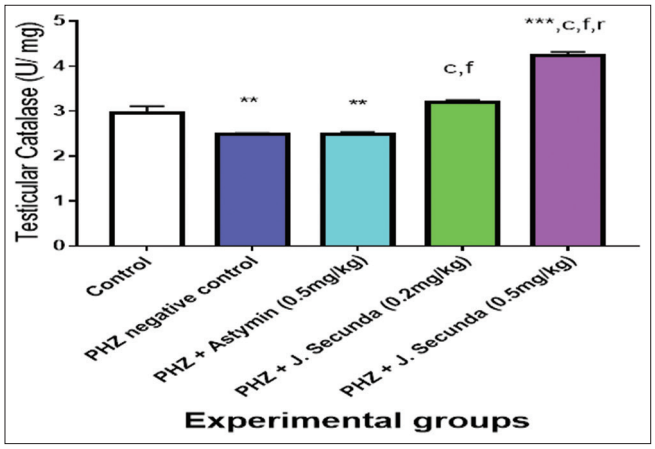

Figure 7: Comparison of testicular catalase of different experimental groups. Values are expressed as mean ± standard error of mean, n = 4. **P < 0.01, ***P < 0.001 versus control, ^{c}P < 0.001 versus phenylhydrazine (PHZ) negative control, ^{f}P < 0.001 versus PHZ + Astymin, ^{r}P < 0.001 versus PHZ + *Justicia secunda* (0.2 mg/kg). PHZ = Phenylhydrazine. *J. secunda* = *Justicia secunda*

significantly (P < 0.001) increased compared to control. Groups treated with PHZ + *J. secunda (*0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) significantly increased compared to PHZ negative control. Furthermore, the group treated with PHZ + *J. secunda* (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) were significantly higher compared to PHZ + Astymin treated group. The group treated with PHZ + *J. secunda* (0.5 mg/kg) was significantly higher (P < 0.001) compared to PHZ + *J. secunda* (0.2 mg/kg) treated group. In Figure 8, testicular SOD was significantly (P < 0.05) decreased in groups treated with PHZ compared to control. However, groups treated with PHZ + Astymin significantly (P < 0.01) increased compared to PHZ negative control. PHZ + *J. secunda* (0.2 mg/kg) and PHZ + J. *secunda* (0.5 mg/kg) treated groups were significantly decreased compared

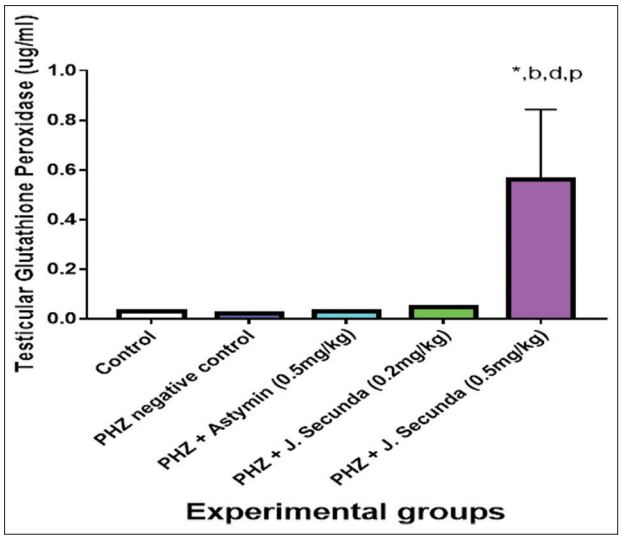

Figure 6: Comparison of testicular glutathione peroxidase of different experimental groups. Values are expressed as mean ± standard error of mean, n = 4. *P < 0.05 versus control, ^{b}P < 0.01 versus phenylhydrazine (PHZ) negative control, ^{d}P < 0.05 versus PHZ + Astymin, ^{p}P < 0.05 versus PHZ + *Justicia secunda* (0.2 mg/kg). PHZ = Phenylhydrazine, *J. secunda* = *Justicia secunda*

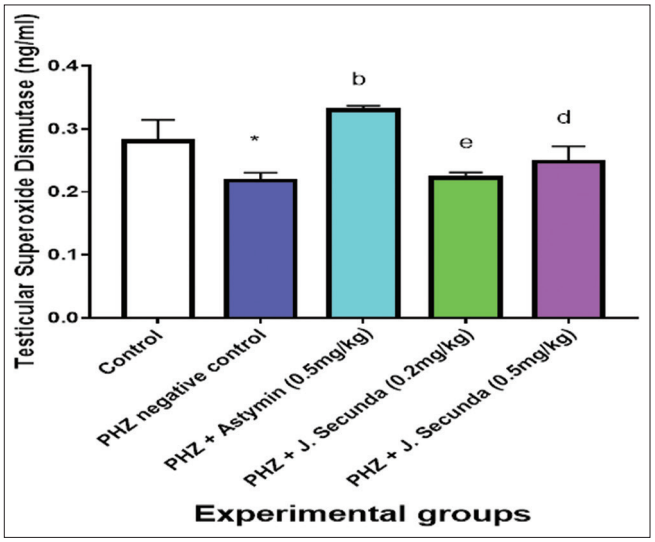

Figure 8: Comparison of testicular superoxide dismutase of different experimental groups. Values are expressed as mean ± standard error of mean, n = 4. *P < 0.05 versus control, ^{b}P < 0.01 versus phenylhydrazine (PHZ) negative control, ^{d}P < 0.05 versus PHZ + Astymin. PHZ = Phenylhydrazine, *J. secunda* = *Justicia secunda*

to groups treated with PHZ + Astymin. In Figure 9, testicular MDA was significantly (P < 0.001) increased in groups treated with PHZ negative control, PHZ + J. secunda (0.2 mg/kg) and PHZ + J. secunda (0.5 mg/kg) compared to control. Furthermore, the group treated with PHZ + Astymin was significantly (P < 0.001) decreased compared to the PHZ negative control. However, the group treated with PHZ + *J.* secunda (0.2 mg/kg) and PHZ + *J. secunda* (0.5 mg/kg) was significantly (P < 0.01) increased compared to PHZ + astymin.

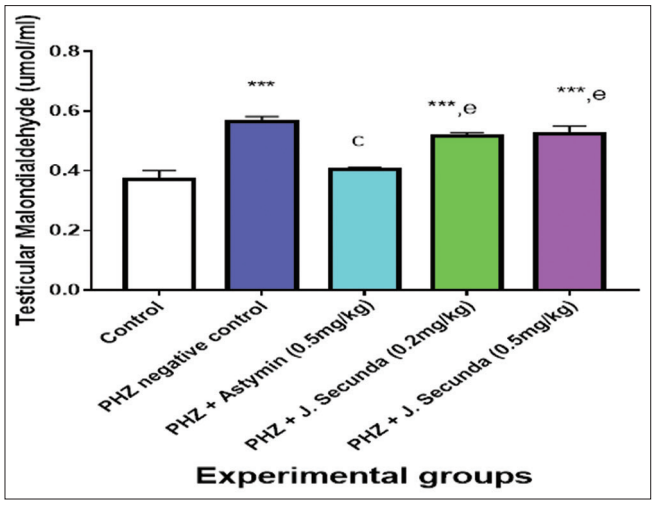

Figure 9: Comparison of testicular malondialdehyde of different experimental groups. Values are expressed as mean ± standard error of mean, $n = 4$. ***$P < 0.001$ versus control, $^{c}P < 0.001$ versus PHZ negative control, $^{e}P < 0.01$ versus PHZ + Astymin. PHZ = Phenylhydrazine, *J. secunda* = *Justicia secunda*

Discussion

The incidence of some diseases, including cancer, diabetes or cardiovascular problems as well as infertility, has reached a perilous point universally, hence, need to find a global remedy.[1] Synthetic drugs topped the list, but their long-term use raises serious concerns. Hence, the importance of finding efficient drugs points out the opportunity to study natural products, especially un-investigated plants.[1] Therefore, this study investigated the reproductive impact of *J. secunda* on PHZ-induced toxicity in male Wistar rats. The parameters assessed in this study include; serum FSH, LH, testosterone, TC, TG, HDL-C, LDL-C, very-low-density lipoprotein-cholesterol (VLDL-C), TNF-α and testicular oxidative stress markers (GSH, GPx, CAT, SOD and MDA).

The significant increase in FSH in the group treated with PHZ + *J. secunda* (0.5 mg/kg) could be linked to decreased LH and testosterone in this group, which may have sent a feedback effect to the anterior pituitary gland to increase FSH to signal spermatogenesis. It may also be linked to the FSH-enhancing property of *J. secunda*. The significant increase in FSH in *J. secunda*-treated rats is in agreement with the report by Okpara *et al*.,[15] who also reported a significant increase in FSH in *J. secunda*-treated female rats. Furthermore, the decreased FSH in PHZ control, PHZ + Astymin and PHZ + *J. secunda* (0.2 mg/kg) may be linked to the toxic effect of PHZ in these groups as well as a decreased concentration of LH and testosterone in these groups reported possible adverse effects of *J. secunda* in reproduction. Furthermore,[16,17] reported a possible reproductive defect in PHZ-treated mice. The significant decrease in the serum concentrations of LH and testosterone in this study could be linked to a possible adverse effect of the drugs administered which may have triggered testicular toxicity in the male rats. The decline in the testosterone levels may be a possible indication of reduced spermatogenesis. This result is in contrast with the report by Okpara *et al*.[15] that reported increased LH and Estrogen in *J. secunda*-treated female rats.

The significant decrease in the serum concentration of lipid parameters in this study could be linked to decreased concentration of testosterone in this study. Cholesterol serves as a substrate for the synthesis of steroid hormones.[3] This significant decrease in the serum concentration of lipid parameters corresponds with the report by Karimipour *et al*.,[17] who reported that PHZ elevated reactive oxygen species (ROS), caused lipid peroxidation and alleviated GSH. These were suggested to be the effects of the antioxidant administered (Vitamin C). Furthermore Adegoke *et al*.,[18] reported a significant decrease in the TGs, cholesterol, HDL-C and LDL-C caused by *Trypanosoma brucei* infection which induces oxidative stress. *J. secunda* also caused a significant increase in the levels of TC, TG, LDL-C and VLDL-C, while causing a significant decrease in HDL-C. which corresponds with a report by Onochie *et al*.[6] This increase in lipid parameters could be linked to the anti-inflammatory and antioxidant properties of *J. secunda,* as reported by Onoja *et al*.[19] Therefore, based on these results, it could be suggested that *J. secunda* possesses negative effects on the serum lipid profile of male Wistar rats, which is in correlation with a study by Onochie *et al*.,[6] that reported that the ethanol leaf extract of *J. secunda* causes elevated levels of blood urea, creatinine and lipid profile, as well as detrimental effects on the heart and kidneys on Wistar rats. The study provides a scientific justification for the advice that plant extracts should not be used excessively or for an extended length of time to treat any kind of illness.

The significant increase in the concentration of testicular tumour necrosis factor-alpha (TNF-α) in this study could be the possible inflammatory effect of PHZ, which is a potential inflammatory agent that can affect various cells and tissues in the body. It causes inflammation by inducing oxidative stress, which is an imbalance between reactive nitrogen species and ROS generation and elimination. Oxidative stress, in turn, triggers inflammatory responses by activating signalling pathways, such as nuclear factor-kappa B and inducing the expression of pro-inflammatory cytokines, including interleukin-6 (IL-6), IL-1β and TNF-α.[20] As such, TNF-α is a promoter of lipid peroxidation and

is known to be elevated in inflammatory diseases, such as rheumatoid arthritis, Crohn's disease and sepsis.[21] Haemolytic anaemia can cause inflammation by releasing haemoglobin and other substances into the bloodstream, which can activate the complement system, a part of the immune system that enhances inflammation and phagocytosis. Haemolytic anaemia can also cause hypoxia or low oxygen levels in tissues, which can induce inflammation by stimulating the production of hypoxia-inducible factor 1 alpha, a transcription factor that regulates genes involved in inflammation and angiogenesis.[22] The significant increase in the concentration of TNF-α is in correlation with the report by El-Gayar *et al*.[23] which reported that PHZ injection increased the serum levels of TNF-α in rats after 24 h, suggesting that TNF-α was involved in the inflammatory response to haemolysis. They concluded that PHZ-induced haemolytic anaemia stimulated the production of pro-inflammatory cytokines, such as TNF-α, IL-2 and interferon-γ, which might play a role in the immune response to haemolysis. Furthermore, in this study, the hydromethanol extract of *J. secunda* caused a significant decrease in TNF-α, which is in correlation with a report by Kings-Ogbonna and Anyasor,[24] which reported there was a significant ($P < 0.05$) decrease in serum levels of TNF-α, IL-6 and COX-2 in *J. secunda* leaf aqueous fraction treated rats compared with untreated control animals. This decrease could be linked to its anti-inflammatory properties, as reported by Onoja *et al*.[19] suggesting that *J. secunda* possesses anti-inflammatory, antinociceptive and antioxidant activities. Although,[25] revealed that in both *in vitro* and *in vivo* settings, methanol extract of *J. secunda* leaves showed significant anti-inflammatory effects.

Sperm analysis was deliberately not considered in the study, as the study focused on alternative endpoints that are more directly related to the research question or the mechanism of action of the herbal extract-hormonal concentrations and antioxidant biomarkers were deemed more relevant to assessing the efficacy of the extract in restoring reproductive functionality in male rats with dysfunctional reproductive systems induced by 2,4-Dinitrophenylhydrazine. Furthermore, sperm analysis has commonly been seen to yield variable results due to factors such as sample handling, staining techniques and observer subjectivity. It was, therefore, important to focus more on reliable or consistent variables. The assay of sperm analysis on animals involves invasive procedures and may cause discomfort or distress to the animals. The potential benefits of including sperm analysis did not justify the additional stress or risk to the animals; it was opted of the study design.

Testicular MDA concentration was significantly increased in PHZ control compared to normal control. This is an indication of lipid peroxidation, which is evident with a significant decrease in the activities of SOD, CAT and GPx and reduced GSH which are antioxidant enzymes. This could be a possible sign of oxidative damage. This is in correlation with a report by Anbara *et al*.[26] which suggest that PHZ-induced haemolytic anaemia and hepatic damage in Wistar rats, reduced their testicular steroidogenesis and increased semen oxidative stress, decreased levels of SOD, CAT, GPx and GR, GSH and testosterone in the testes and increased the levels of MDA, ROS and sperm DNA fragmentation. It was found that in *J. secunda* treated groups, there was a significant increase in the levels of GSH, GPx and CAT and a significant decrease in the level of MDA with no significant difference in the level of SOD. This could be linked to the suggested antioxidant properties of the *J. secunda* extract. This is in correlation with a report by Karimipour *et al*.,[17] that while sperm characteristics and DNA integrity are negatively impacted by PHZ, Vitamin C, a strong antioxidant, can mitigate these effects and increase the rate of *in vitro* fertilisation. However, Vitamin C alone cannot fully restore *in vitro* embryonic development and fertility potential.

As such, *J. secunda*, which may also have antioxidant properties as reported by Ejovi and Hamilton-Amachree,[27] suggests that the leaves of *J. secunda* may serve as a substantial source of natural antioxidant supply that could aid in halting the development of several oxidative stresses.

Conclusion

The administration of PHZ triggered testicular toxicity, causing notable shifts in biochemical markers. These observed changes, stemming from the toxic effects of PHZ, possess the capacity to negatively affect spermatogenesis. In contrast, treatment with Astymin and the extracts of *J. secunda* leaves resulted in heightened levels of reproductive hormones and increased concentrations of testicular antioxidant markers. However, the potential reversibility of these toxic effects by PHZ through the administration of this plant suggests promising prospects for mitigating the impact on reproductive function.

Author contributions

S.K. M designed the study and wrote the study protocol. S. K. M, J.B. M, F.U. I, U.L. I and B.E. E performed laboratory experiments and literature searches. S.K. M drafted the manuscript and worked on data analysis. All authors read and approved the final manuscript.

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Conflicts of interest

There are no conflicts of interest.

Data availability statement

All data generated or analysed during this study are included in this article.

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