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Diet formulated with black seed flour activates regulatory androgenic enzymes and neuroendocrine hormones in leachate-exposed reproductive damage of rat testes

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HIGHLIGHTS

- Mixed-metal intoxication in rat model inhibited steroidogenic proteins and neuroendocrine hormones.
- Dietary BSF at 10% and 20% increased steroidogenic enzymes neuroendocrine hormones in rats.
- BSF promoted spermatogenesis by potentiating seminiferous tubules and spermatocytes.

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ABSTRACT

Mixed environmental toxicants particularly mixed-metals had been implicated as testicular endocrine disruptors. Functional foods were applied in traditional medicine since decades for the treatment of several reproductive defects with limited scientific basis. The finding examines the steroidogenic proteins and neuroendocrine hormones linked to spermatogenesis and testicular wellness when exposed to toxicant-liquid from Elewi-odo vehicle-battery recycling site (EOMABRL) in rat, following treatment with black seed flour. Rats were distributed into six assemblages (n = 8): normal rats; normal rats administered with 10% and 20% black seed flour (BSF), respectively; testicular exposed rat; exposed rats treated with 10% and 20% supplementation of BSF, respectively; for 14 days. EOMABRL intoxication inhibited Δ^5 3β-HSD and Δ^5 17β-HSD activities and declined FSH, LH and testosterone levels. Diet supplemented with BSF at 10% and 20% significantly ($p < 0.05$) increased testicular steroidogenic enzymes (Δ^{5}-17β-HSD and Δ^{5}-17β-HSD) as well neuroendocrine hormones (LH, FSH) and testosterone levels in EOMABRL-intoxicated rats. Also, BSF prevented the alterations associated with spermatogenesis by potentiating seminiferous tubules (STs) and spermatocytes. We therefore concluded that diet formulated with BSF triggers the production of testicular endocrine hormones and hypothalamus pituitary–testicular axis via resuscitating germ cells in compromised rats.

1. Introduction

Chemicals from the environment are capable of altering biochemical pathways relating to male reproduction [1]. Reports from uncountable animal models have indicated that some types of environmental chemicals can interrupt the hypothalamic–pituitary–testicular axis as hormonal competitors or agonists and/or by interacting with the biological processes [2]. These environmental toxicants can, however, be broadly categorized into heavy metals: agricultural and industrial chemicals including leachate known as pro-reproductive abnormalities [3,4]. In addition, a number of hormone disruptors comprising 4-octylphenol (4-OPL) and quinones, could damage DNA single-strand, lipid peroxidation, and chromosomal aberrations as well as impair the degree of propagation of germ cells in gonads. Absolutely, due to industrialization and dietary malnutrition, studies have shown that 50% of fertility challenges are associated with male factors [9,10].

Nigella sativa (Black seed) is widely used in Asian traditional medicine and some part of Africa, as prevention against several diseases

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such as cough, inflammation, and diabetes [5,6]. The oil from black seed has been reported to exert hepatoprotective, nephroprotective, and cardioprotective functions [7,8] in mammals. Considering its multi-functioning ability, the present study hypothesized dietary inclusion with black seed flour may be effective against leachate (a mixture of metals) induced testicular dysfunctions and obliged as a pro-spermatogenic agent. Also, the effects of dietary supplement against mixed-environmental toxicants regarding the development of male reproductive systems are not well documented [11,12]. Interestingly, antioxidants, vitamins, minerals, flavonoids and phenolic compounds particularly gallic acid, quercitrin, quercetin, luteolin from plant origin were discovered to manage the myriads of reproductive dysfunctions [13,14].

Many unanswered questions in male reproductive dysfunctions by environmental toxicants advocated the development of better methods to manage reproductive upshots in mammals exposed to mixtures of chemicals [3]. The underlying mechanisms by which chemicals exposures elicit their toxic effects, particularly during spermatogenesis are still new. Also, metabolic food therapy against mixed metal poisoning is globally promoted. On the aforementioned bases, our study investigated the outcome of formulated diet from black seed flour against the steroid dehydrogenase activities and neuroendocrine hormonal levels in experimental animals exposed to liquid-toxicant from Elewi-Odo vehicle-battery recycling site (EOMABRS).

2. Materials and methods

2.1. Sampling site

Leachate: an environmental toxicant was isolated from a communal recycling site known as Elewi Odo vehicular battery recycling site. The site is positioned in the ancient city of Ibadan, Nigeria (latitude $7^0$25.08′ N and $7^0$25.11′ N and longitudes $3^0$56.45′ E and $3^0$56.42’ E). The recyclers were fond of dumping the unexploited motor-powered battery residues into the nearby flowing river of the area.

2.2. Sample collection and preparation of leachate

The sample was collected according to the technique of Siddique [15] and prepared following the standard procedure [16]. It was labelled as EOMABRL and kept at 4 °C for use.

2.3. Sample collection of black seed

The fresh samples of black seeds (*N. sativa*) were purchased from private agricultural seed shop, Ilorin, Kwara State, Nigeria. The seeds were certified at the Department of Biology, University of Ibadan, Nigeria. The check-receipt specimen was dropped at the University's Botany Herbarium.

2.4. Preparation of black seed flour

Approximate 500 g of the black seeds containing its polyphenols without corn oil was pulverized into powdered form. The powdered form of black seed was designated as black seed flour (BSF). The BSF was stored at room temperature before use.

2.5. Chemicals

The chemicals and reagents used were imported from Sigma (St Louis, MO, USA) and of analytical grade from the British Drug Houses (Poole, Dorset, UK). These include: 5, 5-Dithio-bis-2-nitrobenzoic acid (DTNB), Bovine Serum Albumin (BSA), Di-hydroxylepiandrosterone (DHEA), testosterone, nicotinamide adenosine dinucleotide (NAD+), GSH, H_2O_2, trichloroacetic acid (TCA) and thiobarbituric acid (TBA).

2.6. Total phenols and flavonoids examination

The total phenol of the black seed was determined according to the method of Singleton et al. [17]. Total flavonoid content was evaluated using a method reported by Meda et al. [18].

2.7. Decomposition of deoxyribose (Fenton's reaction)

The effect of the black seed extracts on Fenton's reaction was measured by the method of Halliwell et al. [19]. Briefly, the reaction mixture of 120 µl of 20 mM deoxyribose, 400 µl of 0.1 M phosphate buffer (pH 6.9) and 40 µl of 500 µm of FeSO4 was added to fresh methanol extracts ranging between 0 and 200 µl. The volume was made up to 800 µl with distilled water. It was incubated at 37 °C for 30 min and the reaction was discontinued by adding 0.5 ml of 2.8% TCA. Thereafter, 0.4 ml of 0.6% thiobarbituric acid (TBA) was added followed by incubation in boiling water for 1 h. The optical density was estimated at 532 nm in the spectrophotometer. The drop in absorbance of hydroxyl radical (OH•) on the addition of test sample in relation to the control was used to calculate the percentage OH• following the equation: % OH• = (A_{532} control – A_{532} sample)/A_{532} control x 100.

The calculations of IC_{50} were done from the % $OH^{\bullet}$ versus extract level using non-linear regression standard curve.

2.8. Estimation of DPPH free radical preventing power

The power of black seed extract to inhibit DPPH* was examined following the method of Cervato et al. [20]. Briefly, 1 mL of the extract was divided into several concentrations of 0.10, 0.20, 0.30, and 0.40 mg/mL. Each concentration of the extract was added with 60 μmol/L of DPPH*. The mixture was kept in the dark for 30 min, following the absorbance reading at 517 nm. Also, the reference DPPH* without the extract was measured and the % DPPH* preventing power of the extract was quantified as follow:

% scavenging power = [(A_{517} reference − A_{517} sample)/A_{517} reference] × 100; where A_{517} reference is the absorbance without the extract; and A_{517} sample is the absorbance with the extract.

2.9. Free radical-inhibiting activity on ABTS

The evaluation of antioxidant capacity of the black seed using the 2, 2′-azino-bis [3-ethylbenzthiazoline-6-sulphonic acid] (ABTS) as substrate was assessed by the method of Siddhuraju [21]. Briefly, 3 ml of 7 mM ABTS radical solution ($ABTS^{\bullet}$) was diluted with 1 ml of seed extract. The mixed-solution was incubated in the dark at 25 ^{O}C for 60 min. Thereafter, the optical density was read at 734 nm while its scavenging capacity was calculated using the equation: ((1 − Ab/A0) × 100%) (Ab and A0 are the absorbance of test and $ABTS^{\bullet}$ standard respectively.

2.10. Lipid peroxidation and thiobarbituric acid reactions (TBARS)

The assay of lipid peroxidation and thiobarbituric acid reactions (TBARS) was done following the modified method of Ohkawa et al. [22]. Concisely, the reaction mixture comprising 30 µl of 0.1 M pH 7.4 Tris-HCl buffer was mixed with100 µl low-speed supernatant (SI) fraction. More so, extract ranging between 0 and 100 µl and 30 µl of 70 µM freshly prepared sodium nitroprusside was added and made up with 300 µl with water. The mixture was incubated at 37 OC for 1 h. Thereafter, 300 µl of 8.1% SDS (sodium dodecyl sulfate) was added to the reaction mixture to develop a color reaction. Further addition of 600 µl of acetic acid/HCl (pH 3.4) mixture and 600 µl 0.8% TBA (thiobarbituric acid) was done. This mixture was incubated at 100 °C for 1 h to produce a pink color. The TBARS were quantified at 532 nm and juxtaposed with a standard curve of malondialdehyde (MDA). The decrease in absorbance of lipid peroxidation on the addition of test sample in relation to the control was used to calculate the % MDA

following the equation: % MDA = [(A_{532} control- A_{532} sample)/A_{532} control X100].

The calculations of IC_{50} were done from the % MDA versus extract level using non-linear regression standard curve.

2.11. Animal handling

Experimental male rats within 240 g were procured from general Animal Stock of the University of Ibadan, Nigeria. We kept the rats at a persistent temperature of 22 ± 2 °C on a 12 h light/dark cycle. All the rats had unrestricted access to food and water and were handled according to the institutional recommendations of Nigeria Academy.

2.12. Experimental protocol

The male experimental animals were familiarized for four weeks and unsystematically allocated into six sets of eight rats each (n = 8). Group 1 (CG): rat given distilled water; Group 2 (N_{10}TG): physiological rats fed with 10% supplementation of black seed flour (BSF); Group 3 (N_{20}TG): physiological rats fed with 20% supplementation of BSF; Group 4 (EOMABRL): testicular induced damage i.e. 1 ml of leachate was intraperitoneally (i.p) injected; Group 5 ($LITG_{10}$): induced rats treated with 10% supplementation of BSF; Group 6 ($LITG_{20}$): induced rats treated with 20% supplementation of BSF. The experiment lasted for 2 weeks (Table 1). Diet levels (10% and 20%) were selected because the crude protein content of black seed flour was taken into consideration. Also, previous study had made use of the same concentrations in diet formulation [23]. The feed intake was checked every day and body weight of the animals was calculated before and at the end of the experiment. Thereafter, the experimental rats were sacrificed 24 h after the last administration.

2.13. Diet formulation

Experimental diets of two doses (10% and 20%) of BSF were formulated following the formulation procedure of Kumar et al. [24] (Table 1). Diet was prepared to contain 10% protein with casein, taking into consideration the crude protein content of the BSF. For instance, the quantity of casein required to obtain 10% protein in diet was derived from the following equation:

$$\frac{100 \text{ x } 10}{Y}$$

Table 1
Diet formulation for basal and supplemented diets for control and test groups.

Treatment	CG	N_{10}TG	N_{20}TG	EOMABRL	$LITG_{10}$	$LITG_{20}$
Casein	13.91	10.57	7.25	10.00	10.57	7.25
Mineral-Vitamin Premix	4.00	4.00	4.00	4.00	4.00	4.00
Corn oil	10.00	10.00	10.00	10.00	10.00	10.00
Corn starch	72.09	65.43	58.75	72.09	65.43	58.75
BSF	-	10.00	20.00	-	10.00	20.00
Total (g)	100.00	100.00	100.00	100.00	100.00	100.00

CG: Normal control group placed on a basal diet; N_{10}TG: normal control group placed on a diet supplemented with 10% of black seed flour; N_{20}TG: normal control group placed on a diet supplemented with 20% of black seed flour; EOMABRL: group induced with 100% of Elewi Odo municipal auto-battery recycling site leachate; $LITG_{10}$: group induced with 100% EOMABRSL plus diet supplemented with 10% of black seed flour; $LITG_{20}$: group induced with 100% EOMABRL plus diet supplemented with 20% of black seed flour.

Note: Casein = 71.88% protein; 1 g of the mineral-vitamin premix contains; 3200 i.u vitamin A, 600 i.u vitamin D_3, 2.8 mg vitamin E, 0.6 mg vitamin K_3, 0.8 mg vitamin B_1, 1 mg vitamin B_2, 6 mg niacin, 2.2 mg pantothenic acid, 0.8 mg vitamin B_6, 0.004 mg vitamin B_{12}, 0.2 mg folic acid, 0.1 mg biotin H_2, 70 mg choline chloride, 0.08 mg cobalt, 1.2 mg copper, 0.4 mg iodine, 8.4 mg iron, 16 mg manganese, 0.08 mg selenium, 12.4 mg zinc, 0.5 mg antioxidant.

Where *y* is the percentage crude protein in the casein.

2.14. Preparation of testicular post mitochondrion fraction

The isolated testes from rats were standardized by 4 vol in ice cold milieu, comprising of 1.15% KCl and 50 mM Tris-HCl buffer with a pH 7.4 using a motor driven Teflon-glass homogenizer. The supernatant was collected while the sediment was discarded. The sequestered supernatant was kept at 4 °C for several biochemistry analyses.

2.15. Determination of testicular Δ^5-3β-HSD activity

The reactive mixture of 250 µL of 100 µM sodium pyrophosphate buffer, pH 8.9, 10 µL ethanol with 30 µg of dehydroepiandrosterone and 240 µL of 25 mg % bovine serum albumin (BSA) were mixed with 250 µL of the testicular supernatant. The activity of Δ^5-3β-HSD was therefore determined subsequently the addition of 50 µL of 0.5 µM nicotinamide adenine dinucleotide (NAD^+) in a UV spectrophotometer at 340 nm against a blank (without NAD^+) [25]. One component of enzyme activity is corresponding to a variation in absorbance of 0.001/min at 340 nm.

2.16. Estimation of testicular $\Delta^5$17β-HSD activity

The reactive mixture of 250 µL of 440 µM sodium pyrophosphate buffer, pH 10.2,10 µL ethanol encompassing 0.3 µM testosterone (Sigma) and 240 µL of 25 mg% BSA (Bangalore Genei) were mixed with 250 µL of the testicular supernatant. The activity of Δ^5-17β-HSD was estimated following the addition of 50 µL of 0.5 µM NAD^+ in a UV spectrophotometer at 340 nm against a blank (without NAD) [25]. One component of enzyme activity is corresponding to a shift in absorbance of 0.001/min at 340 nm.

2.17. Measurement of neuroendocrine (LH and FSH) hormone

Serum LH and FSH levels were measured by the method of Watanabe et al. [26].

2.18. Serum testosterone level

Serum-free testosterone was estimated by the method of Biswas et al. [27].

2.19. Histological investigation

The testes were rapidly removed after sacrifice of rats and were immobilized with Bouin solution comprising 75% picric acid, 25% neutral buffered formalin and 5% acetic acid. Histologic segments were arranged, marked with hematoxylin and eosin and then examined under microscope.

2.20. Data analysis

Differences between assemblages were analyzed using one way analysis of variance (ANOVA) followed by Duncan multiple comparisons test by statistical software package for Social Science (SPSS) 17.0 The data are presented as treatment groups mean ± SD and the least significance difference (LSD) was accepted at $P < 0.05$.

3. Results

3.1. Polyphenols

Polyphenols and flavonoids including gallic acid, catechin, chlorogenic acid, caffeic acid, orientin, rutin, quercitrin, quercetin, luteolin and active ketones such as thymoquinone and dithymoquinone were

Table 2
Total phenolics and total flavonoids contents of free and bound phenolics extracts of *N. sativa* seeds.

Phenolic	Total phenolics (mg/g)	Total flavonoids (mg/g)
Free	46.18 ± 1.03[a]	4.53 ± 0.14[a]
Bound	31.72 ± 1.01[b]	2.96 ± 0.08[b]

Data represent the mean ± standard deviation of replicate readings. Values with the different lowercase superscript letter along the same column are significantly different ($P < 0.05$).

recently reported in black seeds using HPLC profile [28].

3.2. Free and bound phenolic acids and flavonoids

The key active compounds responsible for testicular wellness of black seed were quantified. The Table 2 showed the results of phenolic acids and flavonoids. As observed, the total soluble-free phenolic (46.18 mg/g) considerably ($p < 0.05$) abundant than total bound polyphenolic fraction (31.72 mg/g). Similarly, total soluble-free flavonoids (4.53 mg/g) noticeably ($p < 0.05$) higher than total bound flavonoids (2.96 mg/g) (Table 2).

3.3. Effect of soluble-free and bound phenolics from black seeds on DPPH, Fe^{2+} chelation, hydroxyl (OH•) radical levels and lipid peroxidation-in vitro

The result of soluble-free and bound phenolics on DPPH, chelation of Fe2+, OH• production, and Fenton reaction - in vitro is shown in Table 3. Soluble free phenolics from black seed remarkably scavenged stable DPPH• (IC50 = 113.86 µg/ml) better than bound phenolics (IC50 = 129.62 µg/ml) (Table 3). Also, the soluble free (IC50 = 18.36 µg/ml) phenolics from black seed reduced Fe3+ to Fe2+ than bound (IC50 = 22.17 µg/ml) phenolics. Similarly, the treatment with phenolics (IC50 = 30.43 µg/ml) profoundly repressed the OH• level than bound phenolics (IC50 = 38.25); and the pattern of its inhibition was dose-dependent. Lastly, soluble free phenolics treated with rat testes significantly ($P < 0.05$) decreased lipid peroxidation (IC50 = 49.12 µg/ml) than the bound phenolics (IC50 = 57.46 µg/ml). Generally, the inhibition potential of soluble-free phenolics from black seed is more effective to scavenge free radicals than the bound phenolics in the order of Fe2+ chelation > OH• production > Fe2+ induced lipid peroxidation > DPPH.

3.4. Effect of soluble-free and bound phenolics from black seeds on ABTS• radical and reducing power

Table 4 showed the effect of soluble and bound polyphenols from black seed on ABTS• scavenging radical and reducing power. The soluble free phenolics (0.69 mM TEAC/g) remarkably ($p < 0.05$) scavenged ABTS free-radical than the bound polyphenolics (0.59 mM TEAC/g). Also, soluble free phenolics (28.43 mg GAE/g)) from black seed had demonstrated positive reducing prowess than bound phenolics (20.06 mg GAE/g)). Commonly, soluble-free phenolics from black seed are more effectual to scavenge ABTS stable radicals as well as having higher reducing power than bound phenolics.

Table 4
$ABTS^{*+}$ scavenging ability and reducing power of free and bound phenolics oil of *N. sativa* seeds.

Phenolics	$ABTS^{*+}$ scavenging ability (mM TEAC/g)	Reducing power (mg GAE/g)
Free	0.64 ± 0.03[a]	28.43 ± 0.93[a]
Bound	0.59 ± 0.02[b]	20.06 ± 0.88[b]

Data represent the mean ± standard deviation of replicate readings. Values with the different lowercase superscript letter along the same column are significantly different ($P < 0.05$).

Table 5
Characterization of heavy metals in EOMABRL in comparison with WHO limit [10].

Metal	Level	WHO Limit	% increase
Cu	0.341	2.00	-
Zn	0.010	3.00	-
Cd	0.006	0.003	100
Mn	7.842	0.40	1860
Co	0.049	0.05	-
Cr	0.068	0.05	36
Fe	2.667	0.30	789
Ni	0.051	0.02	150
Pb	0.015	0.01	50

All values are in milligrams per liter. Least observable effective concentration (LOEC) set by the World Health Organization [57]; **EOMABRSL**: Elewi Odo municipal battery recycling site leachate; **% increase**: Percent increase compared with the WHO permissible limits in drinking water.

3.5. Composition of inorganic substances in EOMABRL

The composition of the leachate from our laboratory previously characterized by Atomic Absorption Spectrophotometer (AAS) revealed several inorganic metals including Cu, Zn, Pb, Cd, Mn, Co, Cr, Fe, and Ni (Table 5).

3.6. Effect of dietary supplementation of black seed flour on testicular Δ^5 3β-HSD and Δ^5 17β-HSD activities in EOMABRL-induced testicular damage

Figs. 1 and 2 showed the effect of BSF on steroidogenic enzymes (Δ5 3β and Δ5 17β hydroxysteroid dehydrogenase activities) in testicular tissues, respectively of the exposed animals. It was shown that intraperitoneal intubation with EOMABRL caused the activity of Δ5 3β hydroxysteroid dehydrogenase to be reduced by 32%, 35%, and 42%, respectively in relation to the physiological control group (CG), N10TG and N20TG (Fig. 1). However, co-management with BSF (LITG10 and LITG20) prevented this alteration by increasing the activity of Δ5 3β HSD enzymes, compared with the EOMABRL-treated rats (Fig. 1). In addition, our results also demonstrated that the Δ5 17β hydroxysteroid dehydrogenase activity was considerably reduced by 29%, 37%, and 39%, respectively in relation to CG, N10TG, and N20TG groups following EOMABRL intoxication in male rats (Fig. 3). Co-treatments with black seed flour of LITG10 and LITG20 resulted in an increase of Δ5 17β hydroxyl steroid dehydrogenase activity by 29% and 38%, respectively in relation to EOMABRL-induced rats.

Table 3
IC_{50} values (µg/mL) of free and bound phenolics oil of *N. sativa* seeds for DPPH*, Fe^{2+} chelation, OH* production and Fe^{2+}-induced lipid peroxidation.

Extract	DPPH*	Fe^{2+} chelation	OH* production	Fe^{2+}-induced lipid peroxidation
Free	113.86 ± 2.04[b]	18.36 ± 0.74[b]	30.43 ± 1.28[b]	49.12 ± 1.83[b]
Bound	129.62 ± 2.16[a]	22.17 ± 0.85[a]	38.25 ± 1.66[a]	57.46 ± 1.97[a]

Results are expressed as mean ± standard deviations (SD) of triplicate determinations. Values with the different lowercase superscript letter along the same column are significantly different ($P < 0.05$).

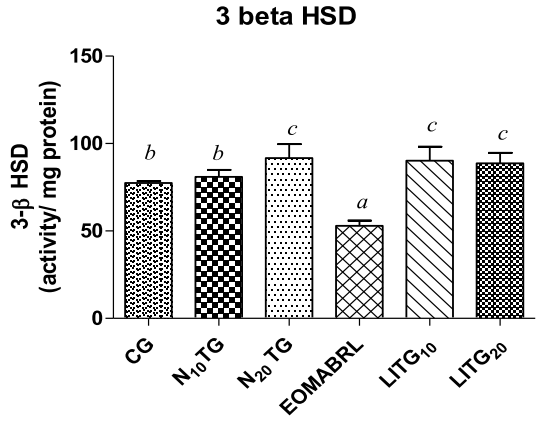


Fig. 1. Effect of dietary supplementation of black seed flour on 3-β HSD activity in EOMABRL induced testicular damaged rat. Values represent mean ± SEM, (n = 8); Values with different superscript are significantly (P < 0.05) different. CG: Normal control group placed on a basal diet; N_{10}TG: normal control group placed on a diet supplemented with 10% of black seed flour; N_{20}TG: normal control group placed on a diet supplemented with 20% of black seed flour; EOMABRL: group induced with 100% of Elewi Odo municipal auto-battery recycling site leachate; $LITG_{10}$: group induced with 100% EOMABRL plus diet supplemented with 10% of black seed flour; $LITG_{20}$: group induced with 100% EOMABRL plus diet supplemented with 20% of black seed flour.

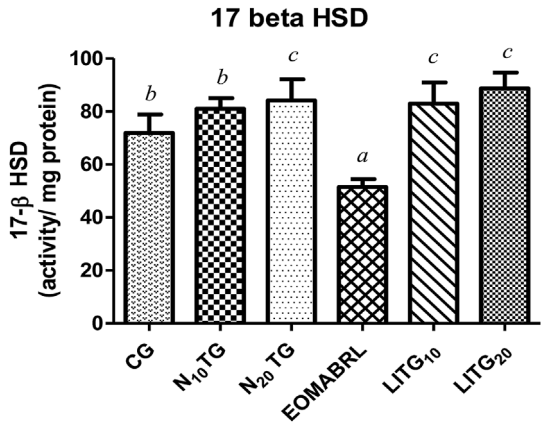


Fig. 2. Effect of dietary supplementation of black seed flour on 17-β HSD activity in EOMABRL induced testicular damaged rat Values represent mean ± SEM, (n = 8); Values with different superscript are significantly (P < 0.05) different. CG: Normal control group placed on a basal diet; N_{10}TG: normal control group placed on a diet supplemented with 10% of black seed flour; N_{20}TG: normal control group placed on a diet supplemented with 20% of black seed flour; EOMABRL: group induced with 100% of Elewi Odo municipal auto-battery recycling site leachate; $LITG_{10}$: group induced with 100% EOMABRL plus diet supplemented with 10% of black seed flour; $LITG_{20}$: group induced with 100% EOMABRL plus diet supplemented with 20% of black seed flour.

3.7. Effect of dietary supplementation of black seed flour on neuroendocrine hormone levels in EOMABRL-induced testicular damage

As observed in Fig. 3, EOMABRL intoxication in male rats lowered the serum luteinizing hormone (LH) levels by 24% and 34%, respectively in comparison with the CG and control- N_{10}TG; while no significant increase (10%) was observed between the control-treated group (N_{20}TG) and EOMABRL induced group (Fig. 3). However, co-treatments ($LITG_{10}$ and $LITG_{20}$) with dietary supplementation of black seed flour remarkably elevated serum LH level by 42% and 52%, correspondingly (Fig. 3). Similarly, as observed in Fig. 4, the concentration of serum follicle stimulating hormones (FSH) was depleted in EOMABRL induced testicular damage by 26% and 27%, respectively; while

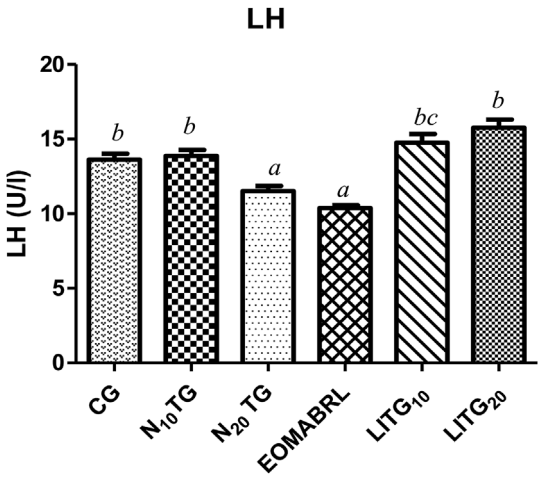


Fig. 3. Effect of dietary supplementation of black seed flour on luteinizing hormone (LH) level in EOMABRL induced testicular damaged rat. Values represent mean ± SEM, (n = 8); Values with different superscript are significantly (P < 0.05) different. CG: Normal control group placed on a basal diet; N_{10}TG: normal control group placed on a diet supplemented with 10% of black seed flour; N_{20}TG: normal control group placed on a diet supplemented with 20% of black seed flour; EOMABRL: group induced with 100% of Elewi Odo municipal auto-battery recycling site leachate; $LITG_{10}$: group induced with 100% EOMABRSL plus diet supplemented with 10% of black seed flour; $LITG_{20}$: group induced with 100% EOMABRL plus diet supplemented with 20% of black seed flour.

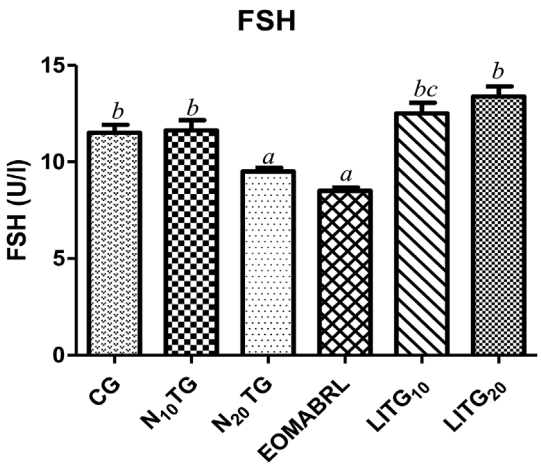


Fig. 4. Effect of dietary supplementation of black seed flour on follicle stimulating hormone (FSH) level in EOMABRL induced testicular damaged rat. Values represent mean ± SEM, (n = 8); Values with different superscript are significantly (P < 0.05) different. CG: Normal control group placed on a basal diet; N_{10}TG: normal control group placed on a diet supplemented with 10% of black seed flour; N_{20}TG: normal control group placed on a diet supplemented with 20% of black seed flour; EOMABRL: group induced with 100% of Elewi Odo municipal auto-battery recycling site leachate; $LITG_{10}$: group induced with 100% EOMABRL plus diet supplemented with 10% of black seed flour; $LITG_{20}$: group induced with 100% EOMABRL plus diet supplemented with 20% of black seed flour.

no significant increase (11%) between N_{20}TG and EOMABRL induced group (Fig. 4). Hence, co-managements ($LITG_{10}$ and $LITG_{20}$) with dietary supplementation of black seed flour remarkably elevated serum FSH levels by 45% and 59%, correspondingly (Fig. 4).

As shown in Fig. 5, the testosterone level in EOMABRL-exposed testicular rats was remarkably depleted in comparison with CG and treated (N_{10}TG and N_{20}TG) groups by 34% 38% and 42%, respectively; indicating that the exposure of EOMABRL caused observable endocrine

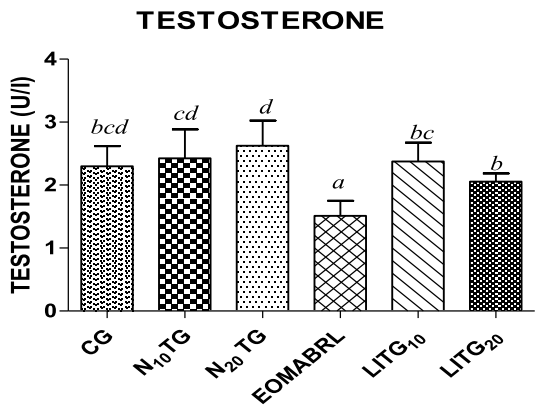


Fig. 5. Effect of dietary supplementation of black seed flour on testosterone level in EOMABRL induced testicular damaged rat. Values represent mean ± SEM, (n = 8); Values with different letters are significantly ($P < 0.05$) different. CG: Normal control group placed on a basal diet; N_{10}TG: normal control group placed on a diet supplemented with 10% of black seed flour; N_{20}TG: normal control group placed on a diet supplemented with 20% of black seed flour; EOMABRSL: group induced with 100% of Elewi Odo municipal auto-battery recycling site leachate; $LITG_{10}$: group induced with 100% EOMABRL plus diet supplemented with 10% of black seed flour; $LITG_{20}$: group induced with 100% EOMABRL plus diet supplemented with 20% of black seed flour.

disruption. However, it was found that the decrease was increased by co-treatment ($LITG_{10}$ and $LITG_{20}$) with black seed flour by 57% and 36%, respectively.

3.8. Effect of dietary supplementation of black seed flour on seminiferous tubules (STs) leydig cells (LDCs) and spermatocytes in EOMABRL-induced testicular damage

Although, the group of animal intraperitoneally intoxicated with EOMABRL showed numerous, closely-packed, uniformly-sized seminiferous tubules (STs) but depleted in spermatogenic cells (DASCs) including Leydig/Sertoli cells (as discovered in Fig. 6). This indicated necrosis of the resultant spermatocytes (NSCs). Also, it was discovered that the germinal epithelial cells were reduced with widened lumens. Interestingly, experimental animals that were control-treated and induced co-treated with dietary supplementation of flour showed spermatocytes. Observation also depicted numerous, closely-packed seminiferous tubules (STs) that have the regular outline and contain numerous amounts of spermatogenic cells (SCs) as well as having slightly reduced amounts of spermatogenic cells and germinal epithelium with increased lumens.

4. Discussion

Currently, prevention of sub-acute testicular damage interrelated with mixed-metal poisoning coupled with organic pollutants is particularly scanty. However, the quest for unique food therapies is modern. Recent studies have implicated the polyphenols as one of the paramount curative agents for several pathological disorders [29,30]. Biologically, they act as principal mediators for the survival of human wellness and promoters of male reproductive functionality [31]. The significant upsurge and decrease of both free and bound phenolics, respectively (Table 2) are the indication of strong glycosidic bonds of bound phenolics [32,33]. Also, the content of polyphenols and flavonoids were higher in free phenolic fractions than the bound fraction (Table 2). This suggests that both free-soluble polyphenols and flavonoids of black seeds were significant contributors to the release of neuroendocrine hormones. This observation aligned with the report of Barb et al. [34] which discovered that flavonoids were strong activators of the hypothalamic-pituitary-gonadal neuron to release the gonadotropin-releasing hormone (GnRH) in pigs.

The propensity of both free and bound polyphenolic compounds of BSF to inhibit DPPH (1, 1-diphenyl-2-picrylhydrazyl) steady radical are very potent (Table 3). The free-soluble polyphenols were efficacious as the prophylactic agent of free radicals (DPPH•) than the bound phenols. This suggests that several functional groups (OH and COOH) are located at the glycoside moiety of the free polyphenols when related to the bound polyphenol. The previous investigation supported the finding that soluble–free phenolics have higher DPPH radical scavenging ability [29]. Also, Fe2+ chelating ability of soluble–free phenolic fraction showed better efficacy than bound phenolics (Table 3). Empirically, the fractions of black seeds act as chelating agents against free radical assaults. The high Fe2+ chelating ability of soluble–free phenolic fraction suggests black seeds as a source of dietary antioxidants for male reproductive health. Additionally, the prophylactic capacity of black seed on hydroxyl radical (OH•) production was considerably higher in free-soluble polyphenols than bound. Similarly, free soluble phenols of this finding showed better inhibitory outcome on MDA testes than bound phenols. The better inhibition exhibited by the free phenols was associated to its greater antioxidant action as demonstrated by its greater Fe2+ chelating ability, DPPH free-radical defensive knack, highly hydroxyl radicals mopping and inhibition of testicular MDA contents (Table 3). Increased MDA levels in testicular tissue is an indication of over-accumulation of lipid peroxides, initiating abnormal penile erection and infertility among patients with defective cell membrane [31,35,36].

As shown in this study, the free-soluble phenolic fraction was efficiently better to reduce Fe3+ to Fe2+ than bound phenolics (Table 4). The high reducing power of free-soluble phenolic fraction suggests black seeds as a source of dietary antioxidants for male reproductive health. Correspondingly, the free-soluble phenolic fraction from black seed inhibited ABTS stable radical than bound polyphenols (Table 4). The recent study demonstrated that antioxidants from plant foods offer resistance by scavenging ABTS radicals via several mechanisms to prevent male reproductive syndromes [37]. However, synergistic or additive interaction of polyphenols from black seed flour is able to inhibit the various generations of stable radicals, through the prevention of testicular oxidation of Fe2+ in Fenton's reaction [38]. Thus, promoting the normal operation of the testes. Studies have shown that the accumulation of free radicals in the testicular tissue initiates erectile dysfunction [36,39].

The control of steroidogenesis is primarily at the point of gene transcription of P450 side chains [40,41] and committed step [42,43]. However, regarding testicular steroidogenesis, Δ5-3β-HSD and Δ5-17β-HSD are essential rates limiting production of spermatogenesis and fertility in males [44]. As observed, the depleted activities of Δ5-3β-HSD and Δ5-17β-HSD (Figs. 1 and 2) demonstrated the subacute adverse effect of the mixture of metals on male gonadal steroidogenic activity [44]. This elucidates that expression of the gene in respect of Cyt p450c 17, steroidogenic acute regulatory protein as well as the influx of cholesterol into mitochondria was altered [43]. However, 1 ml of mixed-metal is potent to induce Leydig cell PGF2α production, shifting testicular steroidogenic acute regulatory (StAR) protein [44], inhibiting LH from binding to its receptor and lowering the level of testosterone (Fig. 5). The up-regulation of Δ5-3β-HSD and Δ5-17β-HSD activities by BSF inclusion provides possible steroidogenic-mediated spermatogenesis occasioned by the conjugated structures of the active compounds [45]. The previous finding had reported flavonoids as the analog precursor of cholesterol [45] while, luteolin, rutin, and quercetin are bi-flavonoids, that react directly with Δ5-3β-HSD and Δ5-17β-HSD as co-factors to up-regulate spermatogenesis [46].

The dire regulation of GnRH hormonal levels plays a significant role in attenuating testicular toxicity during the incidence of mixed-metal intoxication [47,48]. The depleted neuroendocrine hormones (LH and FSH) on exposure to mixed metal (EOMABRL) reveals that the mixture

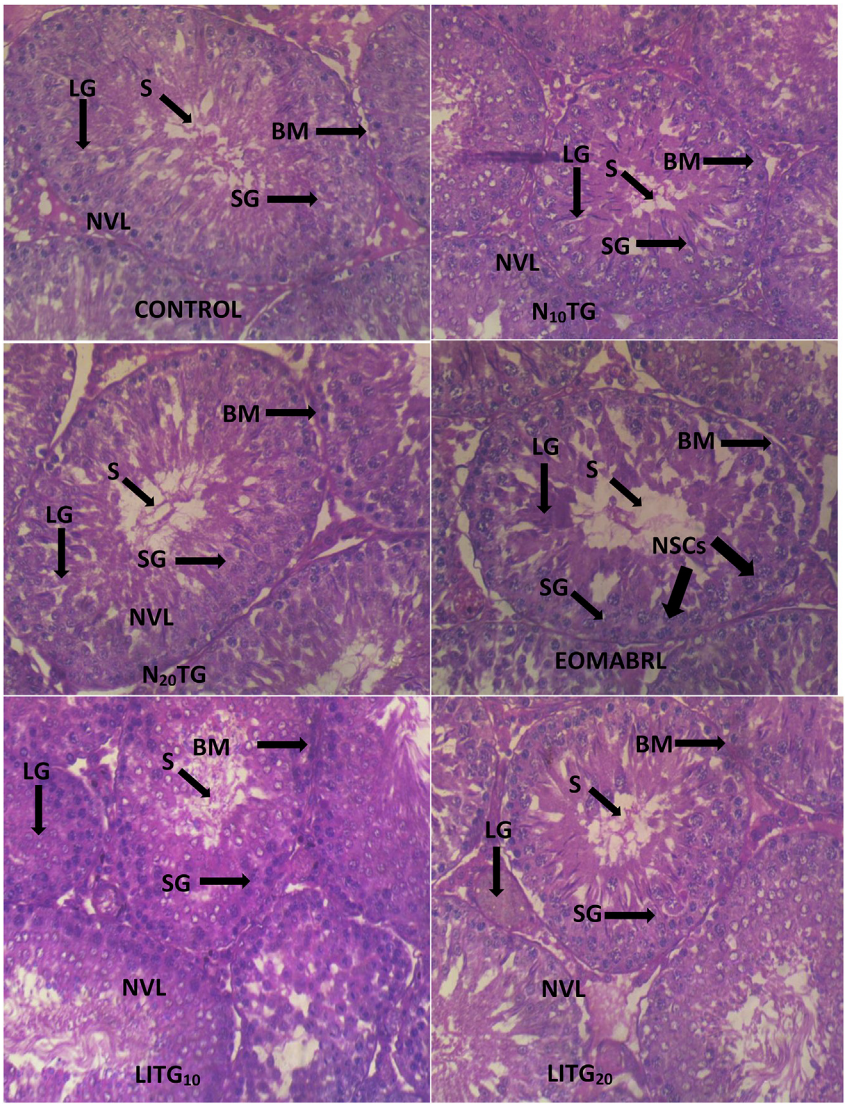


Fig. 6. Testicular histopathology changes on rats administered with dietary supplementation of black seed flour in EOMABRL-induced damage. (Original magnification X 400); **CG:** showed numerous, large, closely-packed seminiferous tubules (STs) that have regular outline and contain numerous amounts of spermatogenic cells (SCs) i.e. no visible lesion (NVL) to spermatocytes **N_{10}TG:** depicted numerous, large, closely-packed seminiferous tubules (STs) that have regular outlines and contain numerous amounts of spermatogenic cells (SCs) i.e. no visible lesion (NVL) to spermatocytes **N_{20}TG:** portrayed numerous, uniformly-sized, seminiferous tubules (STs) that have regular outlines and contain numerous amounts of spermatogenic cells (SCs) i.e. no visible lesion (NVL) to spermatocytes **EOMABRL:** There are numerous, closely-packed, uniformly-sized seminiferous tubules (STs) that have regular outlines with depleted amounts of spermatogenic cells (DASCs) i. e necrosis of the spermatocytes (NSCs). Also, the germinal epithelial cells were reduced and the lumen widened. **$LITG_{10}$**: This group showed numerous, closely-packed seminiferous tubules (STs) that have regular outline and contain numerous amounts of spermatogenic cells (SCs) i.e. no visible lesion (NVL) to spermatocytes **$LITG_{20}$**: There are numerous, uniformly-sized, closely-packed seminiferous tubules (STs) that have regular outline with slightly reduced amounts of spermatogenic cells and germinal epithelium but lumens were increased i.e. no visible lesions (NVL) to spermatocytes (S = sperm, LG = Ley dig cells, SG = Spermatogonia, BM = Basement membrane).

of toxicants was able to abrogate the expression of the GnRH gene in vivo. Consistent with our study, polychlorobiphenyls (PCBs) mixtures and DDT down-regulated the expression of GnRH gene in the testicular animal model [49,50] and low stimulation of neuroendocrine hormones [51,52]. Conversely, up-regulation of LH and FSH levels by dietary BSF on 1 ml EOMABRL intoxicated rats suggests that flavonoids essentially thymoquinone versus dithymoquinone from BSF has a protective effect against testicular poisoning [46,53,54]. In addition, some investigations have associated flavonoids such as rutin and luteolin as regulatory agents in reproductive wellness [46,54]. Furthermore, elevation of testosterone flow caused by dietary BSF against EOMABRL poisoning mediates spermatogenesis and prevention of testosterone level linked to the pathogenesis of erectile dysfunction [55]. Other study depicted that a depleted level of testosterone could trigger the dysfunction of the pituitary gland, cholesterol content and lipid levels, resulting in prostate cancer and chronic kidney failure [12].

Sub-acute exposure (14 days) of rats to EOMABRL depleted the amounts of spermatogenic cells including impairment of Leydig cells with necrosis of the spermatocytes. Also, the remarkable reduction of germinal epithelial cells that are instrumental to the production of spermatozoa confirmed the testicular damage in rat initiated by additive, synergistic, competitive interaction of mixed metals [3]. Pharmacologically, the stoppage of necrotic spermatocytes and restoration of more spermatogenic cells particularly Leydig cells by 10 and 20% BSF treatment in EOMABRL intoxicated rats was attributed to the active components in BSF [54]. Rutin and thymoquinone are powerful flavonoids, known as the chief mediators in the homeostasis of testicular functions, including penile erection, hyperactive sexual desire, physiological ejaculation and stimulation of the spermatogenic processes [54,56].

5. Conclusion

EOMABRL intoxication depleted Δ5-17β-HSD and Δ5-17β-HSD activities with a decrease in the levels of neuroendocrine hormones (LH, FSH) and testosterone levels. Both bound and free-soluble polyphenols from black seed inhibit DPPH, ABTS, OH radicals and testicular lipid peroxidation with strong ability to chelate and reduce Fe3+ to Fe2+. However, a diet supplemented with BSF at 10% and 20% increased the serum hormones with respect to spermatogenesis. The study hence underlines BSF as functional food against male reproductive dysfunction in EOMABRL poisoning and suggests some possible mechanisms by which its polyphenols prevent mixed metal-induced testicular toxicity in rats.

Conflicts of interest

The authors declared no conflict of interest.

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