NIPES - Journal of Science and Technology Research
www.nipes.org

Aqueous Methanol Extract of *Gongronema latifolium* Leaf Protects Against Dibutyl Phthalate-Induced Alterations in Semen Parameters in Rat

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Article Info

Keywords: *Dibutylphthalate, Gongronema latifolium,Semen parameters,Antioxidants, Infertility*

Received 15 January 2025
Revised 16 February 2025
Accepted 17 February 2025
Available online 23 March 2025

https://doi.org/10.37933/nipes/7.1.2025.14

eISSN-2682-5821, pISSN-2734-2352

Abstract

Male infertility in humans and wildlife has been linked to environmental, lifestyle and dietary factors. Dibutyl phthalate (DBP), a plasticizer, is a ubiquitous environmental toxicant known to adversely affect male reproduction. This study investigated the mechanism of toxicity of DBP on the testes and evaluated the ameliorative potential of aqueous-methanol extract of Gongronema latifolium (GLE) leaf on DBP-induced testicular toxicity in male rats. Twenty-eight (28) male Wistar rats (180 ± 20 g) were randomly divided into four experimental groups of 7 rats each; Group 1 was the control, Group 2 was treated with DBP only (500 mg/kgbw), Groups 3and 4 received DBP (500 mg kg-1 body wt) and GLE (400 and 500 mg/kgbw. respectively). DBP and GLE were administered daily by gavage for 14 days. The results revealed that DBP significantly ($p < 0.05$) increased superoxide dismutase (SOD) with a concomitant increase in lipid peroxidation in the testes. It also significantly increased the number of deformed sperm cells, testicular cholesterol, plasma testosterone and T/LH ratio while, sperm count, sperm motility and plasma LH were significantly ($p < 0.05$) reduced relative to the control. Histopathological examination of the testes revealed DBP-induced distortion in testes architecture with marked degeneration of the seminiferous tubules and Leydig cells. Vascular damage, ulceration, and fibrosis of anterior pituitary-testicular artery and Leydig cell hyperplasia were also observed. Treatment with aqueous-methanol extract of Gongronema latifolium (GLE) leaf provided significant protection, with the 400 mg/kg body weight treatment being the most effective dose in this study.

1. Introduction

The incidence of male infertility is on the rise. Infertility affects 10–15% of couples and male factors account for nearly half of all infertility cases [1]. About 25% of male infertility cases are due to the decline in semen quality [2]. However, modern diagnostic methods continue to detect more organic causes of infertility. There are reports that environmental factors-induced oxidative stress may be an emerging factor in unexplained cause of male infertility [3], [4], [5]. Di-n-butyl phthalate (DBP) is a phthalic acid ester, commonly used in the industrial production of polyvinyl chloride (PVC) based products to make them softer and easy to mould into shape [2]. It is widely applied in making adhesives, personal care products and many other products including medical devices such as drip bags, some cosmetic formulations and children's toys [6]. Since DBP is non-covalently bound within PVC and its final product, they easily leach out into the environment, contaminating soil and water bodies, thereby posing a threat to human health [7], [8]. Human exposure occurs primarily through contaminated food and water, especially high-fat foods, which may be in contact with plastic, adhesives, or other packing materials that contain DBP [9]. The high annual production volume of DBP and its reported toxic effects on developmental and reproductive system has increased the public awareness and research into this plasticizer [10], [7]. Compared to adults, children are more vulnerable and sensitive to Phthalate exposure. Hence the strict restrictions by European Union, US, Australia, Canada, and China) on the use of phthalates as plasticizers in the

manufacture of toys and childcare articles, for children up to 36 months of age [11]. These group of children are at that stage in their development when they readily chew and /or suck these products. Reactive oxygen species (ROS) are normal consequences of biochemical reactions formed during the reduction of oxygen in cellular processes. Apart from the normal production of ROS, metabolism of environmental toxicants has also been shown to induce the generation of reactive oxygen species [12]. Although reactive oxygen species (ROS) are important for normal sperm function, their overproduction and/or impairment of the body's antioxidant defense system, may result in oxidative stress. Oxidative stress has been reported to be an important cause of sperm injury [13] and is suggested to be a new emerging factor in unexplained male infertility [3], [14]. Since spermatozoa are well endowed with membrane unsaturated fatty acids and contain very low levels of enzymatic antioxidant enzymes, they are highly susceptible to lipid peroxidation. It is therefore conceivable that intervention with antioxidants may help to reduce the toxic effect of ROS and consequently, improve male fertility.

Gongronema latifolium (Benth), is a tropical rainforest plant found in countries such as Nigeria, Côte d'Ivoire, Sierra Leone, Ghana, Senegal, and some parts of Asia. It belongs to the family *Asclepiadaceae* and is primarily used for medicinal and nutritional purposes. *Gongronema latifolium* plant has been described as a reservoir of many natural antioxidants which possess free radical scavenging properties [16]. The leaf extract has been shown to normalize renal oxidative stress and lipid peroxidation in diabetic rats [16].

Although Dibutyl phthalate is reported to adversely affect male reproduction, leading to infertility, the mechanism by which this occurs is not clearly understood. Therefore, the current study explores the mechanism of toxicity of DBP on testicular function and attempts to find a natural/alternative remedy to the risk posed on fertility.

2.0. Materials and Method

2.1. Collection, Identification and Preparation of Plant Sample

Fresh leaves of *Gongronema latifolium,*bought from a local market in Benin City Nigeria, was used for this study. They were authenticated in the Department of Plant Biology and Biotechnology, Faculty of Life Sciences of the University of Benin, Benin-city by a Taxonomist. The leaves were identified based on their taxonomic characters and comparison with herbarium specimens. Fresh leaves of *G. latifolium* were sorted, rinsed with running water and air-dried at room temperature for three (3) weeks. The dried leaves were pulverized into fine powder with the use of a mechanical grinder. Thereafter, it was weighed and stored in an air tight, opaque container until use. The pulverized *G. latifolium* leaves (1000 g) were soaked in 6L of H2O: Methanol solution (20:80; v/v) in a glass jar and stirred intermittently for 72 hours. The resulting mixture was filtered and the filtrate was concentrated using a rotary evaporator. The crude extract was freeze-dried and stored in air-tight containers at 4°C until needed. The extraction gave a yield of 96.8 g of crude extract. Stock solution of 150 mg/ml was prepared subsequently by dissolving 3g of extract in 20 ml of 1% Tween 80. This was used for the treatment of animals as required.

2.2. Preparation of Stock Solution of Dibutyl Phthalate

Stock solution of 156.5 mg/ml was prepared by dissolving 7.5 ml of DBP in olive oil which served as the vehicle.

2.3. Experimental Animals

Twenty-eight male rats (Wistar strain), weighing 190.8 ± 18.0 g, were obtained from the Animal House of Anatomy Department, University of Benin. The animals were housed in well-ventilated cages with wire mesh floors to prevent coprophagia. Before the study commenced, animals were allowed an acclimatization period of one week and were allowed access to standard rat chow and water *ad libitum* throughout the duration of the study.

2.4. Experimental Design

The median lethal dose (oral) of Dibutyl Phthalate (DBP) was determined according to the method of Lorke, [17]. The animals were monitored for mortality and behavioral changes.

2.5. Chronic Toxicity Study

Experimental animals were divided randomly into 4 groups of 7 rats each. Group 1 served as control and received the vehicle (olive oil) 500 mL kg-1 body wt., Group 2 was treated with DBP (500 mgkg-1body wt.), while Group 3 and 4 received both DBP (500 mg kg-1 body wt) with aqueous- methanol extract of *G. latifolium* (400 mg Kg-1 and 500 mg

kg-1body wt. respectively). All procedures for animal handling and treatment were approved by the ethical committee of the Faculty of Life Sciences of the University of Benin, Benin City, Nigeria.

2.6. Animal Sacrifice

Twenty-four hours after the last treatment, the animals were sacrificed under chloroform anesthesia. The thoracic and abdominal cavities were opened, and blood was collected by cardiac puncture into heparinized tubes. The testes and vas deferens were excised, trimmed free of adhering fat and connective tissues, and weighed. One testis from each group was fixed in Bouin's fluid and used for histopathological study.

2.7. Preparation of Plasma Samples and Tissue Homogenate

Blood samples were collected in lithium heparin bottles and centrifuged at 1025 x g for 15min. The clear supernatant (plasma) was separated from the red blood cells and stored at -4 ^{o}C until needed for biochemical assays. The testes were homogenized in ice-cold normal saline solution using a ceramic crucible. The homogenate was centrifuged at 1000 x g for 10min, and the supernatant collected into plain bottles and stored at -4℃ until required for biochemical assays which was usually within 48 hours.

2.8. Biochemical Analysis

Cholesterol level was determined according to the method of Artiss *et al* [18]. Testosterone and Luteinizing hormone were estimated by Enzyme-Linked Immunosorbent Assay (ELIZA) method, using rat specific commercially available test kits (product of Bioassay Technology Laboratory, Yangpu Dist. Shanghai, China and Calbiotech Inc. El Cajon, CA). The activity of superoxide dismutase (SOD) was estimated by the method of Marklund and Marklund [19], catalase activity was determined by the method of Cohen et al [20] while Malondialdehyde (MDA) was estimated by the method of Buege and Aust [21]. All reagents and chemicals used were of analytical grade.

2.9. Semen Analysis

2.9.1. Isolation of Sperm Cells

The Sperm cells were obtained from the vas deferens of the rats. A minimum of 36 mm length of the vas deferens at both extremities was ligated, cut, and placed in a sterile petri dish containing 6 µL of normal saline, already adjusted to 37+2°C. The vas deferens was teased to allow the sperm cells to flow out of it.

2.9.2. Morphology of Sperm Cells

A drop of the sperm suspension was placed on clean slides to make a smear. They were air dried and flooded with the Improved Eosin and Leishman stain for 15 mins. Excess stains on the slides were rinsed and the smears were air dried and analyzed under high power objective (Magnification x400). Two hundred sperm cells per animal were observed and classified as normal (long tail) and abnormal (short tail and hooked head) sperm cells. Total abnormalities were expressed in percentage incidence / rat [22]

2.9.3. Sperm Cell Motility and Count

A drop of the sperm suspension was taken from the petri dish and dispensed into a clean grease free slide and further covered with a transparent cover slip. The sperm cells were evaluated microscopically within 3-4 min of their isolation [22]. The motility was scored and expressed in percentage. A sperm cell is considered motile if it does not remain in the same location for 5-10 seconds. The spermatozoa were counted by haemocytometer using the improved Neubauer chamber [23].

2.10. Histopathology Study

The preparation of the histology sections of the testes and prostate was carried out using the hematoxylin and eosin staining method. The sections were observed under a light microscope according to the method of Bustos-Obregon and Gonzalez-Hormazabal [24].

2.11. Statistical Analysis

The data were analyzed by One-Way Analysis of Variance (ANOVA) using IBM-SPSS ver. 26. Duncan Post Hoc test was used to compare the differences between mean values of the groups. Values were considered significant at $P < 0.05$. The results were presented as mean ± standard deviation (SD).

3.0. Results

The effect of aqueous methanol extract of *G. latifolium* leaves (GLE) on Dibutyl phthalate (DBP)- induced alteration in male reproductive function was studied in rat. Acute toxicity study of Dibutyl phthalate using the Lorke method showed that the oral limit of 5000 $mgkg^{-1}$ body wt. did not cause death in rats within the 24 hours observation period (Table 1). However, at this dose, rats were inactive but recovered slowly.

Table 1: Determination of Median Lethal Dose (LD50) of Dibutyl Phthalate (DBP)

Phase	Doses (mg/kgbwt)	Number of behavioural rats/number animals	Observation
1	10	0/3	Normal
	100	0/3	Normal
	1000	0/3	Normal
2	1600	0/1	Normal
	2500	0/1	Normal
	2900	0/1	Normal
	5000	1/1	Inactive but alive

Results show a reduction in body weight of all the DBP-exposed rats relative to the control. Treatment with graded doses of GLE returned the body weight to levels that were not significantly ($P < 0.05$) different from the control. This pattern was also observed in the relative testes weights. However, GLE at the dose of 500 $mgkg^{-1}$ body wt. did not appear to have any beneficial effect on these gravimetric parameters in the testes (Table 2).

Table 2: The Effect of *Gongronema latifolium* leaf extract (GLE) on DBP-induced biochemical changes on gravimetric parameters.

Group	Treatment	Mean-Body weight gain (g)	Testes weight(g)	Relative Testes weight (g)
I	Control	40.35 ± 2.74	2.71 ± 0.19	1.15 ± 0.16
II	DBP-only	29.77 ± 4.10[a]	1.99 ± 0.69[a]	1.00 ± 0.19
III	DBP + GLE400mg	30.63 ± 6.90	2.70 ± 0.25	1.20 ± 0.07
IV	DBP + GLE500mg	34.20 ± 8.74	1.82 ± 1.11[a]	0.53 ± 0.12[a*]

Values are mean ± SD (n = 6). Values with no superscript on the same column are statistically the same with control at ($P < 0.05$). Values with 'a' superscript on the same column differ significantly from control at ($P < 0.05$ Values with 'a*' superscripts on the same column differ significantly ($P < 0.05$) from both control and DBP-only treatment group.

The effect of GLE on DBP-induced changes in superoxide dismutase (SOD), catalase (CAT) and Malondialdehyde (MDA) is presented in Table 3. The DBP induced significant ($P < 0.05$) elevation in testicular SOD activity and MDA levels was restored to normal by GLE treatment.

DBP significantly ($P < 0.05$) increased total cholesterol in the testes but did not have any effect on the plasma cholesterol level when compared with the control (Table 4). GLE appears to potentiate this toxic effect of DBP on cholesterol levels in the testes. In general, there appears to be an inverse relationship between the effect of DBP on plasma testosterone and luteinizing hormone levels such that when the testosterone level is high, the luteinizing hormone level is low and *vice versa*. Treatment with GLE brought the testosterone and luteinizing hormone to levels that were not statistically different from the control. In the same pattern, DBP significantly ($P < 0.05$) elevated T/LH ratio.

The results further showed that DBP treatment caused significant ($P < 0.05$) oligozoospernia, in rats (Table 5), while treatment with GLE, especially at 400 $mgkg^{-1}$ body wt., increased sperm count to levels that were not statistically different from the control ($P > 0.05$). It is interesting to note that there was significant ($P < 0.05$) teratozoospermia in all the DBP-treated groups relative to the control. This condition was not reversed by treatment with the doses of GLE that were used in this study. Although DBP also reduced percentage progressive motility, this parameter in the GLE treated groups was adequately protected when compared with the control and DBP -only treatment group.

Table 3. The effect of *Gongronema latifolium* leaf extract (GLE) on DBP-induced biochemical changes in testicular Superoxide dismutase (SOD), Catalase(CAT) activities and Malondialdehyde (MDA) levels in rat

		Parameters		
Group	**Treatment**	**Superoxide Dismutase (U/g tissue)**	**Catalase (U/g tissue)**	**Malondialdehyde (mg/g tissue)**
I	Control	30.84 ± 5.67	1.39 ± 0.09	0.19 ± 0.01
II	DBP-only	62.50 ± 15.89[a]	1.97± 0.38	0.24 ± 0.02[a]
III	DBP+GLE $_{400\ mg}$	28.44 ± 6.57	1.69 ± 0.14	0.16 ± 0.01
IV	DBP+ GLE $_{500\ mg}$	21.71 ± 5.68	1.81 ± 0.44	0.20 ± 0.01

Values are mean ± SD (n=3)
Values with 'a' on the same column differ significantly from control ($P < 0.05$)
Values with no superscripts on the same column are statistically similar to the control

Table 4: The Effect of *Gongronema latifolium* leaf extract (GLE) on DBP-induced biochemical changes on the Levels of Cholesterol, Luteinizing hormone (LH), Testosterone (T), and its Ratio.

Group	**Treatment**	**Total Cholesterol (Testes) (mg tissue)**	**Total Cholesterol (Plasma) (mg/dL)**	**LH (Plasma) (mlU/ml)**	**Testosterone (Plasma) (ng/ml)**	**T/LH Ratio**
I	Control	0.60 ± 0.07	167.80 ± 1.11	1.40± 0.23	0.36 ± 0.11	0.26 ± 0.53
II	DBP-only	0.94 ± 0.10[a]	166.33 ± 3.46	0.73 ± 0.46[a]	1.75 ± 0.43[a]	2.42 ± 0.69[a]
III	DBP+GLE$_{400mg}$	1.24 ± 0.37[a*]	165. 08 ± 1.57	1.38 ± 0.19	0.74 ± 0.10	0.54 ± 0.08
IV	DBP+GLE$_{500mg}$	1.13 ± 0.51[a*]	163.72 ± 3.96	1.64 ± 0.53	0.99 ± 0.65[b]	0.78 ± 0.25

Values are mean ± SD (n = 5) Values with no superscript on the same column are statistically the same with control at ($P < 0.05$)
Values with 'a' superscript on the same column differ significantly from control at ($P < 0.05$).
Values with 'b' superscripts on the same column differ significantly ($P < 0.05$) from the DBP-only treatment group.
Values with 'a*' superscripts on the same column differ significantly ($P < 0.05$) from both control and DBP-only treatment group.

Histopathological examination of the testes of the DBP-only treatment group revealed severe testicular damage, vascular ulceration and fibrosis of the anterior pituitary-testicular artery, Leydig hyperplasia and focal tubular degeneration (Figure1). Treatment with GLE at 400 $mgkg^{-1}$ body wt., effectively protected the testicular architecture but at GLE 500 $mgkg^{-1}$ body. wt. treatment, the testicular structure showed focal tubular degeneration, Leydig cell hyperplasia and a few normal seminiferous tubules.

Table 5: The effect of *Gongronema latifolium* leaf extract (GLE) on DBP-induced biochemical changes in rat semen

Group	Treatment	Sperm Count (x10^6 cells/mm^3)	Progressive Motility (%)	Immotile Sperm (%)	Abnormal Morphology (%)
I	Control	433.33 ± 32.15	65.00 ± 5.77	20.00 ± 0.00	10.00 ± 0.00
II	DBP-only	206.67 ± 70.24[a]	53.33 ± 5.77[a]	46.67 ± 11.55[a]	30.00 ± 0.00[a]
III	DBP+GLE400mg	303.33 ± 37.86	60.00 ± 0.00	30.00 ± 0.00	31.67 ± 2.89[a]
IV	DBP+GLE500mg	243.33 ± 110.15[b]	75.00 ± 5.00[a*]	85.00 ± 15.00[a*]	26.67 ± 5.77[a]

Values are mean ± SD (n = 3)
Values with no superscript on the same column are statistically the same with control at ($P < 0.05$).
Values with 'a' superscript on the same column differ significantly from control at ($P < 0.05$).
Values with 'a*' superscripts on the same column differ significantly ($P < 0.05$) from both control and DBP-only treatment group.

3.1. Discussion

This study showed that the oral lethal dose (LD_{50}) of DBP for rat is greater than 5000 $mgkg^{-1}$bwt. However, there was an observable color change (dark brown) in the urine of treated rats. Fennell, *et al.*[25], reported that DBP is rapidly metabolized and excreted in the urine. Hence, the obvious color change in urine could signify rapid metabolism and excretion of the compound. The oral lethal dose, (LD_{50}) of DBP in rats has been reported to be greater than 8000 $mgkg^{-1}$bwt [26]. Thus, suggesting that DBP may not be acutely toxic.

Evaluation of organ weight is a recognized indicator of chemically induced change or impairment in the normal function of organs Sellers *et al*, [27]. The weight of reproductive organs in sexually mature animals is therefore an important tool in evaluating reproductive toxicity. The data reported in the current study showed DBP-induced reduction in relative body and testes weights respectively, when compared with the control. It is possible that the reduction in testes weight observed in this study is a consequence of the reduction in the mass of differentiated spermatogenic cells as suggested by Ihsan *et al* [28]. This hypothesis is supported by the result of the histopathological study of the testes (Fig.1: i & iv) which indicated severe testicular damage and focal tubular degeneration on exposure to DBP. It is interesting to note that the DBP+GLE400 treatment group (Fig 1: iii), which had a relative testes weight that was not significantly ($p < 0.05$) different from the control, also showed normal testes structure and tubules on histological examination.

The male reproductive system contains an array of antioxidant enzymes which continuously inactivate reactive oxygen species (ROS) [29]. Maintenance of a fine balance between the pro- oxidant and antioxidant system is paramount for normal testicular function [30]. Oxidative stress occurs in situations where there is an increase in ROS, reduction in the availability of antioxidant or both. It is well documented that the major routes of phthalate-induced testicular toxicity involves decreased steroidogenesis via the alteration of the HPT axis, induction of oxidative stress and apoptosis (31, 32). Phthalate exposure increases the production of oxidants (O^2, OH and H_2O_2) and downregulates antioxidant enzymes and Nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway. Nrf2 is considered, in recent years, to be a master regulator of the cellular antioxidant defense system responsible for the neutralization of ROS and consequent restoration of redox homeostasis. It is interesting to note that dysregulation of Nrf2 signaling is associated with infertility in mammals [33]. Upregulation of Nrf2 is therefore a strategy for reducing oxidative stress due to phthalate exposure. In the testes of mammals, superoxide dismutase (SOD) plays a key role in both male germ cell protection and differentiation [2]. Although in our study, DBP did not affect CAT, it significantly increased SOD activity in the testes relative to the control. Mohammad *et al.* [34] and

Zhou *et al.* [2] have reported that reduced SOD and increased CAT activities normally occur with Phthalate toxicity, particularly at high doses of the toxicant. However, some studies [9], [35] reported an increase in SOD activity on exposure to phthalate. In the current study, it is possible that the DBP-induced generation of ROS in the testes was so high that it could not readily be cleared by the SOD secreted in response to the ROS. Hence the high MDA level observed in the DBP- only treatment group. Phthalate induced disruption of spermatogenesis is known to be influenced mainly by oxidative stress and mitochondrial dysfunction

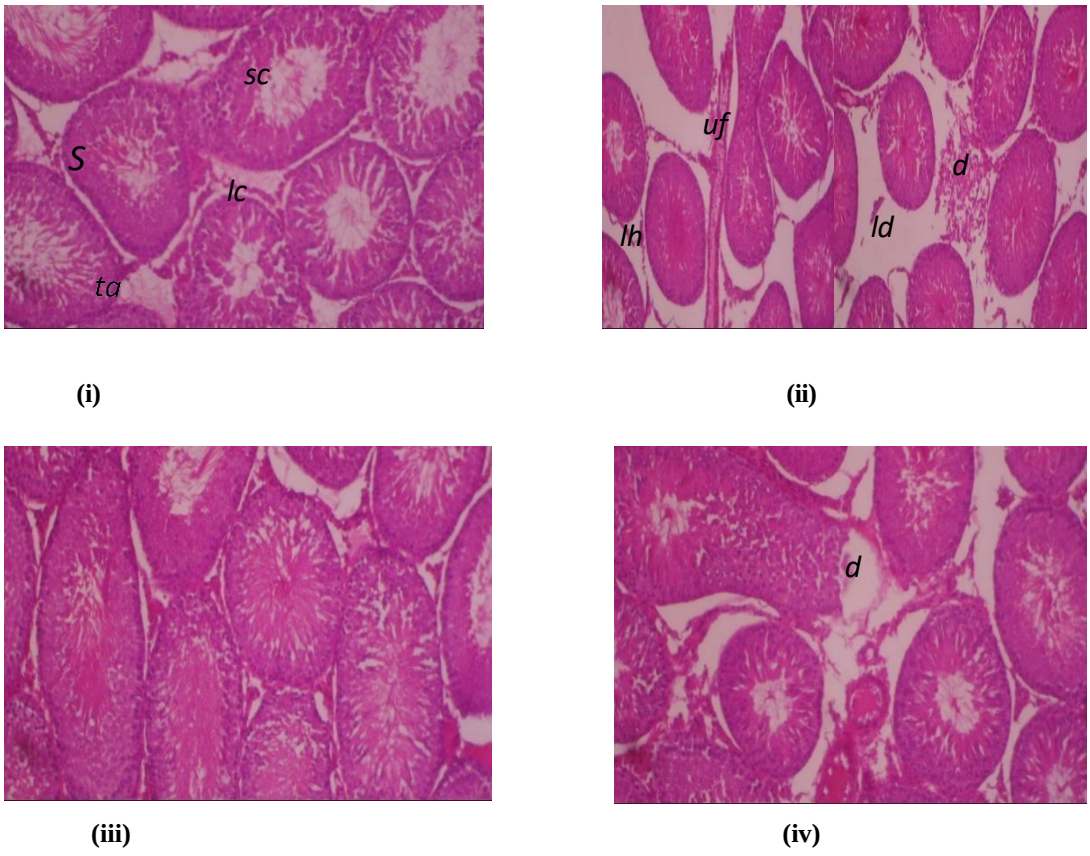

Figure 1. Histology section through the testes of rats treated with DBP and GLE (**HE x100) (i);** Control group showing normal testes structure composed of seminiferous tubules (s) lined by developing spermatocytes in normal sequential maturation, anterior pituitary testicular artery (ta), interstitial cells of Leydig (Ic) and Sertoli cells (sc). **(ii)** group given DBP-only showing severe testicular damage; vascular ulceration and fibrosis (uf) of the anterior pituitary-testicular artery, Leydig hyperplasia (lh), Focal tubular degeneration (d) and Leydig cell degeneration (ld). **(iii);** group given DBP +GLE400mg showing normal testes structure and normal tubules. **(iv);** group given DBP+GLE500mg showing testes structure of; Focal tubular degeneration (d), Leydig cell hyperplasia (lh) and a few normal tubule

Malondialdehyde (MDA) is a biomarker of lipid peroxidation and is formed as a result of degradation of peroxides of unsaturated fatty acids [36]. MDA causes asymmetric distribution of lipid membrane components by penetrating cell membrane structure and increasing oxidative stress. Consequently, lipid peroxidation damages cell membrane structure, and disrupts cell membrane fluidity and permeability [36]. This may have led to the Leydig cell and focal tubular degeneration observed in Fig.1 (ii) and (iv). Several reports have shown an increasing number of natural products such as phytochemicals that are able to enhance the activation of Nrf2 and consequently upregulate antioxidant enzymes and proteins [32], [37], [38]. Analysis of *G. latifolium* leaf extract has revealed high levels of flavonoids and phenols [39]. Among other phytochemicals, flavonoids and polyphenols have been shown to activate the Nrf2 signaling pathway [40], [41]. In our current study, it appears that GLE treatment at 400 $mgkg^{-1}$bwt was able to protect testicular lipids from peroxidation. It is therefore conceivable that these phytochemicals in *G. latifolium* leaf extract could have acted as agonists of Nrf2 and consequently brought the activity of the antioxidant enzymes in our study as well as the

malondialdehyde levels to normal. The antioxidant property of *G. latifolium* has earlier been reported [42], [43].

The activity of sperm cells reflects their quality. Sperm quality assessment is crucial for the evaluation of male reproductive health. In this study, DBP significantly reduced sperm count, sperm motility and increased sperm deformity, resulting in Azoospermia, oligozoospermia, asthenozoospermia and teratozoospermia respectively. This result agrees with previous reports [2], [9] that DBP caused significant changes in sperm quality. It is known that testes, spermatozoa are extremely sensitive to ROS-induced damage [44] because of their high content of polyunsaturated fatty acids which makes them highly sensitive to lipid peroxidation and oxidative stress [45]. In the current study, the alterations observed in the antioxidant system of the DBP-treated rat testes, corresponded with the observed negative changes in sperm quality. Asadi *et al* [36] explained that oxidative stress contributes to the elevation of abnormal sperm quality and consequently, infertility by causing sperm DNA fragmentation. Seminiferous tubules are the site for spermatogenesis and damage or degeneration of these tubules, will lead to reduced sperm production [46]. It is therefore possible that the decrease in sperm count observed in the DBP-only treatment group, might be due to the degeneration of the seminiferous tubules and consequently decreased spermatogenesis. This is moreso as the histopathology results also showed vascular ulceration of the anterior pituitary testicular artery (Fig 1ii). These changes are indicative of rupture of the blood vessels which may have inhibited the flow of nutrients and caused death of the sperm cells. Nutrients which are required for the optimum maturation of sperm cells are carried and distributed in the blood. It is therefore conceivable that increased levels of ROS and lipid peroxidation (LPO) with consequent degeneration of the blood vessels and seminiferous tubules, may be some of the mechanisms by which DBP causes toxic effect on semen quality and consequently, male fertility. Though the level of toxicant (DBP) administered to the test groups in this study was constant (500 $mgkg^{-1}$bwt), increasing the extract (GLE) from 400 to 500 $mgkg^{-1}$bwt, as seen in the group treated with GLE500mg, did not protect semen quality (Table 4). This inconsistent protective effect of the high dose of GLE might not be surprising since some dietary phytochemicals have been reported to exhibit both antioxidant and prooxidant activities depending on their concentration and cellular microenvironment [47].

Cholesterol is secreted into the seminal plasma by the prostate. Male reproductive function has been shown to be dependent on efficient cholesterol homeostasis [48]. Cholesterol serves as a precursor for testosterone in the testicular Leydig cell under the influence of pituitary gonadotropin LH. The mechanism by which DBP stimulates increase in testicular cholesterol level is not known. However, DBP causes oxidative stress in the testes. It is therefore possible that the secretion and build-up of cholesterol in the testes is a biological event, intended to protect sperm cells from oxidative stress and damage. Adaikpoh and Obi [49] had earlier suggested this explanation in relation to the elevated cholesterol in the testes of cadmium exposed rats.

The male reproductive function is mainly controlled by the hypothalamic-pituitary-testicular axis (HPT-axis) which spans through the testes. The gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus which acts on the pituitary gland to secrete luteinizing hormone. LH acts on the testicular Leydig cells to stimulate the secretion of testosterone (from cholesterol) which initiates and regulates spermatogenesis [50]. Thus, the alterations in these plasma reproductive hormone levels can be used as an important indication of the disruption of the HPT- axis and consequently testicular function. The current study showed a significant decrease in LH level in the DBP-only treatment group. This effect is probably due to the failure of the pituitary gland to secrete LH. This result correlates with that obtained from the histopathological study Fig.1(ii) which indicated severe testicular damage due to ulceration and fibrosis of the anterior pituitary-testicular artery. Boa *et al*.[51] also reported decreased serum level of LH from altered pituitary function, following exposure to DBP. This collaborates with other reports [52], [53] that DBP affects pituitary hormone-producing cells at both prepubertal and adult stages in males. One would have expected Testosterone level to be decreased following the reduction in LH levels but in our study, DBP significantly increased the level of plasma testosterone. Since the histopathology result in our study, Fig 1(ii) revealed Leydig cell hyperplasia (increase in the number and size of Leydig cell), it is conceivable that this may have led to the increased secretion of testosterone observed. Akingbemi *et al* [54] earlier reported elevated serum testosterone levels and hyperplasia in Di-ethylhexyl phthalate - treated (DEHP) rats. They opined that those chemicals that stimulate or inhibit endogenous hormone production for prolonged periods can induce tumor in the relevant large organs. Hormonal imbalance can alter or impair spermatogenesis and result in abnormal sperm qualities including sperm concentration, motility, and morphology [55]. Our study showed that at 400 mgkg-1 body weight, GLE effectively restored LH and testosterone to levels that were

not significantly different from the control. The present study also identified the association of testicular function with the ratios of the androgenic hormone (testosterone) and the gonadotropic hormone (LH) to see the flipped condition in various groups relative to control. DBP significantly altered the Testosterone/LH ratio which may have contributed to the damages seen in this study. Shoaib *et al*.[56], reported that disturbance in the LH and Testosterone ratios cause infertility since these hormones function in synergy to maintain a feedback control system.

4.0. Conclusion

This study has demonstrated that DBP caused testicular toxicity in rats by increasing ROS, lipid peroxidation, degeneration of the blood vessels and seminiferous tubules, alterations in the gonadotropic (LH) and androgenic (testosterone) hormones and consequently, distortions of the pathways responsible for sperm production. Treatment with GLE at a dose of 400 $mgkg^{-1}$ body wt. provided significant protection from DBP-induced testicular toxicity in rat, possibly by activating the Nrf2 signaling pathway.

4.1. Limitations and Clinical Implications

G. latifolium, which is commonly found in tropical Africa, is useful for its medicinal and nutritive properties. It has been studied extensively for its anti-diabetic properties. However, little is known about its effect on male infertility. Further investigation of the relative contributions of GLE in the management of male infertility will therefore be a major focus for future research in this important but neglected area. One of the limitations of the current study is the short duration of treatment with GLE. Future research is needed to determine the long-term effect of GLE treatment and elucidate clearly, the molecular mechanism of its action against Phthalate – induced alterations in semen parameters and consequently, male infertility. It might also be necessary to determine the effect of GLE doses lower than 400 mg /kg bwt., as this will help to confirm the therapeutic potential of lower doses of the herbal extract. The results obtained will establish the therapeutic and cost-effective use of GLE in the management of male infertility and hopefully, bring succor to couples that are going through male factor- related infertility.

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