Morphometric Evaluation of the Seminiferous Tubules and the Antioxidant Protective Effects of Gallic Acid and Quercetin in the Testis and Liver of Butyl Phthalate Treated Rats

Sunny O. Abarikwu¹ · Godwin Simple¹ · Chimezie Samuel Onuoha¹

Received: 13 March 2018 / Accepted: 10 September 2018 / Published online: 15 September 2018
© Association of Clinical Biochemists of India 2018

Abstract The antioxidant protective effects of gallic acid (GAL) and quercetin (QUE) against oxidative stress induced by di-butyl phthalate (DnBP) in the liver and testis of rats were evaluated in this study. Adult albino Wistar rats (180–225 g) were treated with QUE or GAL (50 mg/kg) alone or in combination with DnBP (1 mL/kg) for 15 days. After treatment, tissue samples were taken for determination of glutathione and malondialdehyde levels, and superoxide dismutase and catalase activities. Serial sections of the testis and liver were stained with haematoxylin and eosin for microscopy and seminiferous tubular morphometry. As expected, DnBP induced oxidative stress was evident by increased malondialdehyde level in both organs. Co-treatment with GAL or QUE reversed the malondialdehyde by 45.42, 37.44 and 37.57%, 23.32% and catalase by 52.21, 70.15 and 85%, 38.14% in the testis and liver respectively whereas superoxide dismutase activity and glutathione level were differently modulated parallel to histopathological improvement in both tissues. The seminiferous tubular diameter, epithelial height, epithelial germ cell count and tubular length were significantly decreased by 11.09, 51.91, 40.65 and 11.10% respectively versus control values after DnBP treatments and were attenuated on co-treatment with GAL or QUE. Co-treatment with GAL afforded better protective effects in both tissues but QUE treatment alone appeared more effective than GAL on the investigated morphometric data. It seems likely that GAL or QUE prevented the tissue damage but the

antioxidant profiles of the liver and testis are different in response to the oxidative stress.

Keywords Morphometry · Quercetin · Gallic acid · Di-*n*-butyl phthalate · Oxidative stress · Antioxidant

Introduction

Oxidative stress plays an important role during tissue damage and is associated with several diseases such as carcinogenesis, inflammation, diabetes mellitus, and male factor infertility. Benzenedicarboxylic acid dibutyl ester, also known as butyl phthalate or di-*n*-butyl phthalate (DnBP) is widely used as a plasticizer in a variety of consumer products, such as plastic bags, food packaging, PVCs, cosmetics, industrial paints, solvent dye, and blood transfusion packs [1]. Because DnBP is bound non-covalently to their products, it can easily leach from these plastics and, consequently, be ingested. Therefore, the general population is exposed to phthalates through consumer products, diet and medical devices [2]. Oxidative stress is one of the mechanisms associated with phthalate esters-induced tissue atrophy in several tissues [2, 3]. These involve causing alterations in the antioxidant defense balance of tissues as well as inducing peroxidative damages [2].

Quercetin and gallic acid are naturally occurring flavonoids that are commonly found in fruits and vegetables. Quercetin is especially abundant in apples and onions where it is found as a glucoside [4] and gallic acid is found in gallnuts, grapes, tea leaves, oak bark, strawberries, pineapples, bananas, lemons, both in its free state and as part of the tannin molecule [5, 6]. Gallic acid has been demonstrated to have various biological activities such as

✉ Sunny O. Abarikwu
sunny.abarikwu@uniport.edu.ng; abarikwus@gmail.com

¹ Department of Biochemistry, University of Port Harcourt, Choba, Nigeria

antioxidant and anti-inflammatory effects [7] and may have beneficial effects to human health including chemoprevention of cancers and cardiovascular diseases [8]. Several studies with quercetin have also reported their interesting pharmacological properties in tissues including antioxidant and anti-inflammatory effects [9–12]. However, at high doses, both gallic acid and quercetin produces deleterious effects in tissues by acting as pro-oxidants [8, 13, 14].

The biological system is endowed with a panel of antioxidants and radical scavengers including glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) which protect tissues from oxidative damage and are associated with good health status [15]. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers [16]. Consequently there has been lots of interest in the use of phytochemicals and phenolics to optimise human health through exploiting of their antioxidative properties in several experimental models. For example, gallic acid extracted from rose flowers exhibits antioxidant effects in aged mice by restoring the activities of CAT and glutathione peroxidase [17]. Similarly onion extract rich in quercetin showed free radical scavenging properties and protected against low-density lipoprotein oxidation and lipid peroxidation in rats [18].

Thus, in the present study we wanted to compare the responses of the liver and testis to the antioxidant protective effects of gallic acid and quercetin against experimental oxidative stress induced by phthalate ester. For this purpose, malondialdehyde (MDA), an end product of lipid peroxidation; GSH, a key antioxidant, and CAT and SOD, primary defence enzymes in tissues were determined in the liver and testis of rats as well as the effect on testicular morphometry.

Materials and Methods

Test Chemicals

Quercetin, gallic acid, di-*n*-butyl phthalate were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All other reagents were commercially available and are of analytical grade.

Animal Model, Treatments and Collection of Samples

Adult male Wistar albino rats (*Rattus norvegicus*), weighing 180–225 g, were obtained from the animal house of the Department of Biochemistry, University of Port Harcourt, Nigeria. The animals were housed throughout the

experiment in plastic cages (each cage housing five animals) and allowed to acclimatize to laboratory environment for 1 week before the beginning of the experiment. The animals were maintained under 12-h light: 12-h dark cycles and were allowed free access to standard dry pellet diet and water *ad libitum*. The experiment was performed in accordance with the ethic regulations of the National Academy of Science on animal care and handling. The animals were divided into six groups, each containing five rats. The animals in group I was used as control group and received 2 mL/kg of corn oil by gavage. Animals in group II were administered di-*n*-butyl phthalate (DnBP) every other day for 15 days. The administered dose (1 mL/kg body weight) of DnBP and route of administration were based on previous published studies [2]. Animals in group III were first pre-treated with GAL (50 mg/kg body weight) [19] daily by gavage for 7 days and then for every other day for 15 days at the beginning of study. Animals in group IV were administered GAL (50 mg/kg body weight) followed by DnBP (1 mL/kg body weight) administration every other day for 15 days. Similarly, animals in group V were first pre-treated with QUE (50 mg/kg body weight) by daily oral gavage for 7 days, and then for every other day for 15 days at the beginning of study. From our preliminary studies, this dose is non-toxic for up to 30 days at 3 times per week by oral gavage, and has been reported previously to decrease oxidative stress in rats after 15 days [12]. Animals in group VI were administered QUE (50 mg/kg body weight) followed by DnBP (1 mL/kg body weight) administration three times a week for 15 days. The 50 mg/kg doses of both flavonoids were selected because in several *in vivo* studies were gallic acid and quercetin protected the antioxidant system of rat's tissues under several pathological conditions, the doses used are smaller than 100 mg/kg [3, 7, 19, 20]. Since, it was reported previously [3] that oral gavage of phthalate ester for 15 consecutive days increased MDA concentrations in the tissues, oxidative stress was induced in the present study with di-*n*-butyl phthalate. The animals were fasted overnight, after the last administration and killed by chloroform anaesthesia followed by cervical dislocation. Body weight was recorded prior to sacrifice. Blood samples were collected and the separated plasma was used for assay of biochemical markers of liver functions including, lactate dehydrogenase (LDH), alanine amino transaminase (ALT), and γ -glutamyl transferase (γ -GT). The paired testes and liver were excised from surrounding tissues, pat-dried between two sheets of filter paper and their wet weight was determined. The left testis and portion of each liver were processed for histology, whereas the right testis and the remaining portion of the liver were homogenized in ice-cold 0.1 M Tris-HCl buffer (pH 7.4) to produce 10% homogenate. The homogenate was centrifuged at 5000g, 4 °C for 15 min and the

supernatant collected was processed for determination of hepatic and testicular MDA, GSH, SOD, and CAT.

Determination of Hepatic and Testicular Malondialdehyde and Glutathione Levels

Lipid peroxidation level in the liver and testis homogenates were measured by the determination of MDA concentration as previously described [21]. The concentration of malondialdehyde was expressed as μmol of MDA per mL using 1, 1, 3, 3-tetramethoxypropane as standard. The absorbance was read at 532 nm. The concentration of GSH was determined in the liver and testis homogenates using Ellman's reagent 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) as a colouring reagent [22]. The absorbance was read at 412 nm by spectrophotometer. GSH concentration was calculated from a standard curve.

Determination of Hepatic and Testicular Superoxide Dismutase and Catalase Activities

Hepatic and testicular SOD was assayed in the liver and testis homogenates by the method of Misra and Fridovich [23], which involves the inhibition of epinephrine (0.01%) autoxidation at pH 10.2. One unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline to adrenochrome during 1 min. The absorbance was read at 480 nm against blank containing all the components except the enzyme source in a spectrophotometer. Catalase activity was estimated in the liver and testis, homogenates as previously described [24]. The specific activity of CAT was calculated using the molar extinction coefficient of H_2O_2 at 240 nm, 43.59 L/mol cm. One unit of catalase activity equals the amount of protein that converts 1 mmol H_2O_2 /min. Protein concentration of the tissue homogenate was determined by the Lowry method using bovine serum albumin as the standard [25].

Determination of Biochemical Markers of Hepatic Functions in the Plasma

The blood samples collected from the heart of the animals through cardiac puncture were centrifuged at 4000 rpm for 15 min. The separated plasma was used to evaluate the activities of alanine aminotransferase (ALT), γ -glutamyl-transferase (γ -GT), and lactate dehydrogenase (LDH) using Randox commercial kits (RANDOX Laboratories Ltd., Crumlin, United Kingdom) with an autoanalyzer (Lab-Tech, RE 1201007), and following the instructions provided by the manufacturers.

Histo-Pathological Examination

At necropsy, a portion of the liver of three rats per each experimental group was fixed with 10% buffered neutral formalin solution. The testis from three rats per experimental group were initially fixed with Bouin's solution for 8 h, and then transferred to 10% buffered formalin. After 48 h, formalin-fixed tissues were embedded in paraffin wax according to the routine procedure, and 5 μm -thick sections were cut with a rotary microtome. The sections were stained with haematoxylin and eosin for microscopy.

Testicular Morphometry

Morphometric analysis of the testis micrograph was performed using digital images of testis micrograph captured at different magnifications using a Labomed Lx 400 microscope (Labomed, USA) as previously described [26]. About 15 transverse sections of seminiferous tubules that were round or nearly round were chosen randomly and measured for each animal. The seminiferous tubular diameter and height of the seminiferous tubule epithelium were measured at $100\times$ magnification using the software image J version 1.50i (National Institute of Health, Bethesda, MA, USA). The seminiferous tubules length (TL, in meters) per testis and per gram of testis was estimated using the formula: $\text{TL} = \text{STV}/\text{R}^2$ (STV = seminiferous tubule volume; πR^2 = area of the seminiferous tubules cross section; R = tubular radius). The TL was divided by the testis weight in order to calculate the length of the seminiferous tubules per gram of testis. The gonadosomatic index (GSI) was calculated dividing the testis weight by the body weight, being the value multiplied by 100. The seminiferous epithelial germ cells count were obtained by counting 9633 points projected over 15 digital images (Labomed Lx 400, Auburn, USA) of the testis structure obtained from histological slides for each animal at $400\times$ magnification.

Statistical Analysis

Data are expressed as mean $\pm$ SD, and the statistical analysis was performed with the GraphPad Prism 6. All the statistical analyses were analyzed by ANOVA followed by Dunnett's test as a post hoc test. *p* values less than 0.05 were considered to indicate statistical significance.

Results

DnBP, GAL, and QUE Do Not Affect Body and Organ Weights

Animals were observed each day during treatment to ensure good health. All rats in the treated group appeared to be in good health. To further determine if there was general toxicity associated with the various treatments, body weight, liver and testis weights of the rats were measured. There were no significant differences in body and testis weights between the treated and control groups at 15 days exposure period (Table 1). Additionally, no statistically significant differences ($p > 0.05$) in the mean organ weights of the liver were found for animals after 15 days exposure period (data not shown).

GAL and Not QUE Induce GSH in the Liver and Testis and Protect Against DnBP-Induced Increase in GSH Level in the Testis but Not in Liver

After 15 days of treatments with the tested chemicals, no significant differences were found on GSH level in the liver of the DnBP, QUE, or DnBP + QUE groups. The GSH level in the liver was significantly higher in the GAL and GAL + DnBP groups than in the control group (Fig. 1). In the testis, GSH level was significantly higher in the GAL, DnBP, QUE, and QUE + DnBP groups than in control group, whereas there was significant decrease in GSH level between the DnBP group and the GAL + DnBP group (Fig. 1).

GAL and QUE Co-treatment Protect Against DnBP-Induced Increase in MDA Level in the Liver and Testis

MDA level was significantly higher in the liver and testis of the DnBP-treated animals than in the control animals. There was no significant difference in MDA level due to GAL treatment between the GAL group and control group in the liver and testis as well as between the QUE group and control group in the liver and testis. Tissue MDA levels were significantly lower in the GAL + DnBP group than in the DnBP group in the liver and testis as well as in the QUE + DnBP group compared to the DnBP group in the liver and testis (Fig. 2).

GAL and Not QUE Co-treatment Protect Against DnBP-Induced Increase in SOD Activity in the Testis but Not the Liver

The activity of SOD was significantly lower in the liver and was significantly higher in the testes of rats treated with DnBP than in the control group. No significant differences due to GAL or QUE treatment were found on SOD activities in the liver and testis. SOD activities were significantly higher in the GAL + DnBP group in the liver and significantly lower in the testis than in the DnBP group. In the QUE + DnBP group, SOD activity was significantly higher in the liver and no significant difference in SOD activity was observed in the testis between the QUE + DnBP group and the DnBP group (Fig. 3).

Table 1 Morphometry of the testis of control and experimental adult rats at the end of study

Parameters	Control	DnBP	GAL	GAL + DnBP	QUE	QUE + DnBP
Tubular diameter (μm)	221.57 $\pm$ 30.13	196.98 $\pm$ 42.84 ^a	227.01 $\pm$ 50.13	235.38 $\pm$ 56.37 ^b	251.88 $\pm$ 33.63	241.74 $\pm$ 20.81 ^b
Epithelial height (μm)	80.49 $\pm$ 12.80	38.71 $\pm$ 7.86 ^a	77.54 $\pm$ 10.67	76.47 $\pm$ 18.05 ^b	85.78 $\pm$ 13.22	73.56 $\pm$ 12.91 ^b
Germinal epithelial cell number	461.67 $\pm$ 87.56	274 $\pm$ 46.03 ^a	407.33 $\pm$ 93.61	376.67 $\pm$ 83.51 ^b	467.67 $\pm$ 55.16	406.67 $\pm$ 36.02 ^b
Testis weight (g)	1.65 $\pm$ 0.41	1.78 $\pm$ 0.55	2.1 $\pm$ 0.83	1.79 $\pm$ 0.74	1.87 $\pm$ 0.62	1.82 $\pm$ 0.58
Body weight (g)	214 $\pm$ 12.94	215 $\pm$ 13.69	220 $\pm$ 11.18	223 $\pm$ 4.47	215 $\pm$ 13.69	214 $\pm$ 12.94
Gonado-somatic index (%)	0.796 $\pm$ 0.17	0.847 $\pm$ 0.14	0.974 $\pm$ 0.18	0.803 $\pm$ 0.23	0.873 $\pm$ 0.21	0.875 $\pm$ 0.29
Seminiferous tubular length (m)	47.01 $\pm$ 2.13	41.79 $\pm$ 4.23 ^a	48.17 $\pm$ 3.41	49.94 $\pm$ 5.12 ^b	53.44 $\pm$ 6.17	51.29 $\pm$ 7.91 ^b
Seminiferous tubular length/gm testis	28.67 $\pm$ 4.12	23.48 $\pm$ 3.14 ^a	29.37 $\pm$ 2.83	27.91 $\pm$ 2.67 ^b	28.58 $\pm$ 4.57	28.18 $\pm$ 4.93 ^b

Data are presented as the mean $\pm$ SD ($n = 15$) except for testis weight, gonado-somatic index and body weight ($n = 5$)

QUE, quercetin; GAL, gallic acid; DnBP, di-*n*-butyl phthalate

^aVersus control animals

^bVersus DnBP treated animals ($p < 0.05$)

Fig. 1 Effects of quercetin (QUE) and gallic acid (GAL) on the level of glutathione (GSH) in the liver (a) and testis (b) of rats after 15-day of oral treatment with di-*n*-butyl phthalate (DnBP). Values are mean $\pm$ SD; $n = 5$, * $p < 0.05$ versus control; ** $p < 0.05$ versus DnBP

Fig. 2 Effects of quercetin (QUE) and gallic acid (GAL) on the level of malondialdehyde (MDA) in the liver (a) and testis (b) of rats after 15-day of oral treatment with di-*n*-butyl phthalate (DnBP). Values are mean $\pm$ SD; $n = 5$, * $p < 0.05$ versus control; ** $p < 0.05$ versus DnBP

GAL was More Effective than QUE in Lowering DnBP-Induced Increase in CAT Activities in the Live and Testis

The activity of CAT was significantly higher in the testis and liver of rats treated with DnBP than in the control group. GAL or QUE treated animals also showed higher activity of CAT in the liver but not testis than the animals in the control group. In the combine exposure groups, CAT activities in the testis and liver were significantly lower in the GAL + DnBP group than in the DnBP group. The activity of CAT in both tissues of the QUE + DnBP treated animals was significantly lower than the DnBP group (Fig. 4).

DnBP Treatment Results to Changes in the Morphology of the Testis and Liver which were Blocked by GAL and QUE

The morphologic structure of the testes in the control and all treated groups are shown in Fig. 5. The germinal epithelium of tubules in the control group appears normal with no visible lesion and showed orderly arrangement of the seminiferous tubular cells (Fig. 5a). The seminiferous tubules of GAL, QUE, DnBP + GAL-or DnBP + QUE-treated animals were similar to control and had most of the germ cells well represented while there was reduction in germ cells in the testes of DnBP-treated animals because of loss of tubular germ cells and thin epithelial height

Fig. 3 Effects of quercetin (QUE) and gallic acid (GAL) on the activity of superoxide dismutase (SOD) in the liver (a) and testis (b) of rats after 15-day of oral treatment with di-*n*-butyl phthalate (DnBP). Values are mean $\pm$ SD; $n = 5$, * $p < 0.05$ versus control; ** $p < 0.05$ versus DnBP

Fig. 4 Effects of quercetin (QUE) and gallic acid (GAL) on the activity of catalase (CAT) in the liver (a) and testis (b) of rats after 15-day of oral treatment with di-*n*-butyl phthalate (DnBP). Values are mean $\pm$ SD; $n = 5$, * $p < 0.05$ versus control; ** $p < 0.05$ versus DnBP

(Fig. 5b–f). Also most of the cells present in the tubules of DnBP treated animals are round spermatids and spermatocytes, and the tubular lumen seems expanded. The seminiferous epithelium also seems degenerated and with no spermatozoa in the lumen of some tubules. Furthermore, empty vacuolar spaces were observed between Sertoli cells in places of tubular germ cells (spermatogonia, spermatids and spermatocytes). For the liver morphology, as expected, there were no visible lesions seen in the liver sections of the control, GAL-treated, and QUE-treated animals. The liver of DnBP-treated animals showed severe periportal cellular infiltration by mononuclear cells and degeneration of hepatocytes. The infiltration by mononuclear cells was reduced in the liver sections of DnBP + GAL or

DnBP + QUE treated animals suggesting protective effects of both flavonoids on liver pathology (Fig. 6).

GAL and QUE Co-treatment Protect Against DnBP-Induced Changes in Testicular Morphometry

In morphometric measurements, the tubular diameter and germinal epithelium height decreased significantly ($p < 0.05$) in DnBP-treated animals. The tubular diameter and germinal epithelium height in the other experimental groups were similar to control values (Table 1). There was no significant variation in the gonado-somatic index between control and treated groups at the end of study (Table 1). In addition, a reduction in the seminiferous

Fig. 5 Cross sections of the testes of treated and control animals at the end of study. Control (a) Testis of control animals showed no visible lesions; Testis of Di-*n*-butyl phthalate treated animals (b) showed depleted epithelial height (arrow head) and azoospermia in the tubular lumen (long arrow), cytoplasmic vacuoles (short arrow). Also the lumen appears wider (L). Testis of gallic acid treated animals (c) appears similar to control and showed normal seminiferous tubules

(arrow head); Testis of gallic acid + di-*n*-butyl phthalate treated animals (d) showed normal seminiferous tubules (arrow head); seminiferous tubules of quercetin treated animals (e) are normal with no visible lesion seen (arrow head); Testis of quercetin + treated animals (c) appears similar to control with no visible lesion and showed normal seminiferous tubules with most of the germ cells well represented (arrow head). Hematoxylin and eosin, mag $\times 400$

tubular length per testis and length of the seminiferous tubules per gram of testis was significant in the DnBP experimental group ($p < 0.05$). These morphometric indexes were close to control values in the GAL, QUE, DnBP + GAL-or DnBP + QUE-treated animals.

GAL and QUE Protect Against DnBP-Induced Increase in Plasma LDH and γ -GT Activities but Not Against Increase in ALT Activity

The activities of LDH, γ -GT and ALT which are indices of hepatic function were significantly higher in the DnBP-treated rats than in the control group. No significant difference in the levels of these hepatic markers was found in the plasma between GAL-treated or QUE-treated rats and the control group. The activities of LDH and γ -GT were

significantly lower in the GAL + DnBP group and the QUE + DnBP group than in the DnBP group, but there was no significant difference found in ALT activity between the GAL + DnBP group and the QUE + DnBP group versus the control group (Fig. 7).

Discussion

Phytochemicals are widely known to have beneficial effects against chemically-induced tissue pathologies. In the present study, we compare the effects of same dose of QUE and GAL (50 mg/kg body weight) against DnBP-induced oxidative damage in the liver, and testis of rats. We used this dose with the purpose of achieving protective effects of QUE and GAL in multiple tissues under the same

Fig. 6 Cross sections of the liver of animals at the end of study. Control (a) no visible lesion seen; di-*n*-butyl phthalate treated animals (b) severe periportal cellular infiltration by mononuclear cells (long arrow); degenerated hepatocytes (starred); Gallic acid treated rats (c): no visible lesion seen; Gallic acid + di-*n*-butyl phthalate treated

animals (d): mild and diffuse cellular infiltration by mononuclear cells (long arrow); Quercetin treated rats (e): no visible lesions seen; Quercetin + di-*n*-butyl phthalate treated rats (f): mild cellular infiltration by mononuclear cells (long arrow). Hematoxylin and eosin, mag $\times 400$

Fig. 7 Effects of quercetin (QUE) and gallic acid (GAL) on the plasma indices of liver functions of rats after 14-day of oral treatment with di-*n*-butyl phthalate (DnBP). Values are mean $\pm$ SD; $n = 5$, * $p < 0.05$ versus control; ** $p < 0.05$ versus DnBP

experimental conditions. In the present study, experimental rats treated with DnBP for 15 days, showed marked decrease in the activity of SOD and increase in CAT

activity and MDA level in the liver but there were no effects on hepatic GSH level. In the testis, DnBP treatment increased GSH and MDA levels, as well as SOD and CAT

activities. There are previous studies that have also reported that DnBP-induces oxidative damage in several tissues [27–30], and support our present findings. The histopathological damage in the liver of the DnBP-treated animals was further confirmed by the rise in plasma levels of ALT, LDH and γ -GT. These marker enzymes are sensitive indicators of hepatocyte injury that are usually released into the blood stream after liver injury [19]. The increased activities of these enzymes in the plasma are indicative of cellular leakage and loss of the functional integrity of hepatic tissues [31]. This result is consistent with earlier reports [32–34]. The recovery to the control level in the plasma activities for LDH and γ -GT in the QUE + DnBP-treated rats further supports the protective effects of QUE and GAL against oxidative damage in the liver [35, 36] and may account for the hepatoprotective effect of GAL and QUE.

The diameter of the seminiferous tubules was 221.57 μ m, epithelium height was 80.49 μ m, and the seminiferous tubular length and its value per gram weight of testis are 47.01 m and 28.67 m/g for the control animals, and are similar to values previously published for rats and mice [37, 38]. Furthermore, the altered testicular morphometric indexes and histological changes in the testis caused by DnBP treatment and the protective effects of GAL and QUE justify the beneficial effects of both phytochemicals on testicular function. The histo-morphometric data showed that both GAL and QUE blunted the effects of DnBP on spermatogenesis and morphometry of the testis. The tubules of the DnBP treated animals had no spermatozoa [2], and most of the tubular germ cells especially elongated spermatids that were lost were recovered in the testes of GAL + DnBP or QUE + DnBP treated animals. Treatment with DnBP also reduced the tubular diameter, seminiferous epithelial height, tubular length and tubular length per gram testis. The seminiferous tubular epithelium and its cytoarchitectural components are important for efficient spermatogenesis [39]. The decreased tubular diameter and epithelial height by DnBP suggested that the loss of cells from the seminiferous epithelium disturbed cellular associations in the germinal epithelium. The altered antioxidant enzyme profiles leading to oxidative stress in DnBP treated animals could also affect normal germ cell association [2, 30, 40, 41]. Therefore, it seems that DnBP inhibits spermatogenesis and induces injury to the testis by oxidative damage. Another reason for the decreased germ cell numbers could be lack of the lack of testosterone to support germ cell development or the result of functional damage to the basal membrane [2, 30]. This has been reported for many chemicals that impairs testicular function [16, 37, 38]. It was also observed in the present study that QUE and GAL can modulate the effects of DnBP on spermatogenesis and testicular morphometric

parameters. Consequently, the germinal epithelium height and seminiferous tubule diameter, tubular length and tubular length per gram testis significantly increased in the QUE + DnBP and GAL + DnBP treated animals in comparison with the DnBP-treated group. Although the protection afforded by both phenolics were similar in reducing lipid peroxidation, QUE alone was more effective, though not statistically significant than GAL on most of the investigated morphometric indexes, suggesting that the effects of phytochemicals under pathological situations in vivo are different from their effects under normal condition within the biological system. For instance, in the present study, QUE increased tubular diameter (251.88 ± 33.63 vs. 227.01 ± 50.13), epithelial height (85.78 ± 13.22 vs. 77.54 ± 10.67), epithelial germ cell count (467.67 ± 55.16 vs. 407.33 ± 93.61) and seminiferous tubular length (53.44 ± 6.17 vs. 48.17 ± 3.41) in comparison with the GAL treated group. These results corroborate previous findings of the positive effects of QUE [42] and GAL [43] on the seminiferous tubules of rats as well as on spermatogenesis. Similarly, Taepongsorot et al. [44] reported that tubular diameter was increased by QUE treatment in a dose dependent manner, further supporting our present data. It is reported that QUE acting through estrogenic receptors induces the proliferation of cells that might be responsible for the increase in tubular diameter, epithelial height and germ cell numbers [45, 46]. Similarly, the affinity of GAL for estrogen receptors [47] and role of estrogen metabolism related enzymes in the mechanism of the protective effects of GAL against endocrine disruptors have been reported [48].

In the present study, it was also observed that co-treatment of DnBP with GAL increased GSH level and SOD activity and decreased CAT activity and MDA level in the liver whereas the testis showed significant increase in GSH activity, decrease in SOD and CAT activities and MDA level and consequently protected the histo-pathological changes in both the testis and liver of treated animals. In contrast, DnBP-treated rats that were treated with QUE showed no effect on GSH, increased SOD and decreased MDA level and CAT activity in the liver whereas in the testis, the GSH level and SOD activity remained high while CAT activity and MDA were decreased compared to DnBP treated animals. Despite the differences in the antioxidant profiles in both tissues of GAL + DnBP and QUE + DnBP treated animals, peroxidative damages evidenced by high MDA levels induced by DnBP were blocked by either GAL or QUE. Therefore the antioxidant property of GAL and QUE could be beneficial in preventing oxidative damage due to exogenous sources in multiple tissues. MDA is the end biomarker for lipid peroxidation and their levels in tissues reflect the extent of oxidative damage [49]. Several findings have also demonstrated that GAL and

QUE inhibits lipid peroxidation and tissue injuries by blocking formation of free radicals and oxidative stress [3, 7, 19, 20]. It was also found in the present study that treatment with both GAL and QUE alone increased GSH level in both tissues, and treatment with GAL alone increased CAT activity in the liver but not in the testis. This effect was not associated with increased MDA formation, and might be related to the antioxidant effects of the flavonoids in both tissues [50]. Additionally, tissues are generally affected by exogenous toxic agents including phthalate esters that induce reactive oxygen species formation and oxidative stress [2, 41]. The oxidative stress affects testicular lipid peroxidation which in turn leads to tissue damage [2, 16]. It seems that QUE and GAL or their metabolites have the capacity to reach the rat testis and block DnBP-induced oxidative stress [12, 43]. QUE and GAL exerts its antioxidant effects by various mechanisms like scavenging free radicals, chelating metal ions and blocking of lipid peroxidation [16, 43]. Our results allowed us to conclude that both phytochemicals had protective effects on the investigated tissues but that the tissues respond differently to the antioxidant effects of GAL and QUE. One possible reason for this is the different pharmacology of these chemicals at their target sites [51, 52]. Some tissues might require lower concentration of phytochemicals than others to exhibit antioxidant effects. For instance, doses of QUE lower than was used in the present study decreased GSH in the liver but not in the kidney under pathological conditions even though MDA level was recovered to the control level [52]. Furthermore, the distribution of phytochemicals to tissues as a function of their intestinal absorption is an important factor that modulates the pharmacological activities of phytochemicals in experimental models [51]. It is reported that phytochemicals which decreased oxidative stress act primarily by direct induction of antioxidant enzymes including CAT whereas as those that increased oxidative stress act primarily by increasing production of H_2O_2 and therefore any increased activity of CAT or GSH level is presumed to be secondary, and reflect an attempt to compensate partially for the increased oxidative stress [53]. This strategy of inducing antioxidant enzymes in response to oxidative insult is a way by which the biological systems develop tolerance to a subsequent larger insult [50, 54]. Because we observe increased GSH levels only in the testis of QUE-treated animals and increased GSH levels in both tissues as well as increased CAT activity in the liver of GAL-treated animals indicated that QUE has better antioxidant efficiency in the testis than in the liver and that GAL has better antioxidant protective effects than QUE in vivo in multiple tissues under the same experimental conditions. The increased GSH level and CAT activity can convert H_2O_2 to O_2 and H_2O , preventing the accumulation of free radicals

Fig. 8 Molecular structures of tested phytochemicals **a** Gallic acid: (3,4,5-trihydroxybenzoic acid), **b** Quercetin: 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one

[10]. Consequently phytochemicals such as GAL and QUE could protect oxidative tissue damage by increasing the levels of antioxidant system e.g. GSH and CAT in the target tissue. The antioxidant protective effects of phytochemicals are reported to be related to their structure amongst other factors including concentration at target tissues [8]. For instance, the several OH-groups and double bonds in QUE and the OH group at para-position to the carboxylic group in GAL (Fig. 8) are essential for blocking the chain reaction of lipid peroxidation and defence of tissues against oxidative stress through a resonance effect that maintain their antioxidant efficiency [55].

In conclusion, the present study demonstrates that DnBP-induced toxicity involves oxidative damage in the testis and liver, and that co-treatment with GAL or QUE can be effective in inhibiting the oxidative stress and histological changes, by enhancing the antioxidant defence system of the tissues and blocking of oxidative stress. Although the antioxidant profiles of the tissues are different under co-treatment with the phytochemicals, lipid peroxidation levels were inhibited, suggesting that QUE and GAL, in the doses used, may afford the same degree of protection against DnBP toxicity. However, QUE alone, at the same dose with GAL, could potentially be better antioxidant in the testis than in the liver, and better than GAL in improving the indexes of testicular morphometry. Further studies are required to elucidate the mechanism of QUE and GAL as therapeutic agents against toxic effects of phthalate esters on testicular morphometry and spermatogenesis.

Acknowledgements We thank Mr Christian Kingsley Nwaughu for the collection of blood samples from the animals used in this study, as well as Messrs Tochukwu Anthony Nnadiukwu and Ohanador Olisa Robinson for their technical assistance in the handling of the equipment used in this study.

Authors' Contribution SOA designed the study, supervised experiments, interpreted data and wrote the manuscript; GS performed experiments and participated in the design; CSO supervised experiments and participated in the design.

Funding This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Compliance with Ethical Standards

Conflict of interest All authors of this manuscript are aware of this submission and declare no conflict of interest.

Ethical Approval All applicable institutional and international guidelines for the care, handling and use of animals were followed.

References

- Muczynski V, Lecureuil C, Messiaen S, Guerquin MJ, N'tumba-Byn T, Moison D, et al. Cellular and molecular effect of MEHP involving LXR α in human fetal testis and ovary. *PLoS ONE*. 2012;7:e48266.
- Farombi EO, Abarikwu SO, Adedara IA, Oyeyemi MO. Kolaviron and curcumin ameliorate di-*n*-butylphthalate-induced testicular toxicity in rats. *Basic Clin Pharmacol Toxicol*. 2007;100:43–8.
- Abd-Allah MF, Aly HAA, Mokhlis HAM, Abdel-Aziz AH. Quercetin attenuates di-(2 ethylhexyl) phthalate-induced testicular toxicity in adult rats. *Hum Exp Toxicol*. 2016;35:232–43.
- Abarikwu SO, Pant AB, Farombi EO. Quercetin decreases steroidogenic enzyme activity, NF- κ B expression and oxidative stress in cultured Leydig cells exposed to atrazine. *Mol Cell Biochem*. 2013;373:19–28.
- Kuppan G, Balasubramanyam J, Monickaraj F, Srinivasan G, Mohan V, Balasubramanyam M. Transcriptional regulation of cytokines and oxidative stress by gallic acid in human THP-1 monocytes. *Cytokine*. 2010;49:229–34.
- Li D, Liu Z, Zhao W, Xi Y, Niu F. A straightforward method to determine the cytotoxic and cytopathic effects of the functional groups of gallic acid. *Process Biochem*. 2011;46:2210–4.
- Prasad L, Khan TH, Jahangir T, Sultana S. Effect of gallic acid on renal bio-chemical alterations in male Wistar rats induced by ferric nitriloacetic acid. *Hum Exp Toxicol*. 2006;25:523–9.
- Choi EJ, Lee BH, Lee K, Chee KM. Long-term combined administration of quercetin and daidzein inhibits quercetin-induced suppression of glutathione antioxidant defences. *Food Chem Toxicol*. 2005;43:793–8.
- Kaindl U, Eyberg I, Rohr-Udilova N, Heinze C, Marian B. The dietary antioxidants resveratrol and quercetin protect cells from exogenous pro-oxidative damage. *Food Chem Toxicol*. 2008;46:1320–6.
- Ansari MA, Abdul HM, Joshi G, Opii WO, Butterfield DA. Protective effect of quercetin in primary neurons against Abeta (1–42): relevance to Alzheimer's disease. *J Nutr Biochem*. 2009;20:269–75.
- Abarikwu SO, Farombi EO. Quercetin ameliorates atrazine-induced changes in the testicular function of rats. *Toxicol Ind Health*. 2016;32:1278–85.
- Baltaci BB, Uygur R, Caglar V, Aktas C, Aydin M, Ozen OA. Protective effects of quercetin against arsenic-induced testicular damage in rats. *Andrologia*. 2016;48:1202–13.
- Farombi EO, Abarikwu SO, Adesiyun AC, Oyejola TO. Quercetin exacerbates the effects of sub-acute treatment of atrazine on reproductive tissue antioxidant defence system, lipid peroxidation and sperm quality in rats. *Andrologia*. 2013;45:256–65.
- Abarikwu SO, Durojaiye M, Alabi A, Asonye B, Akiri O. Curcumin protects against gallic acid-induced oxidative stress, suppression of glutathione antioxidant defences, and hepatic and renal damage in rats. *Ren Fail*. 2015;38:321–9.
- Abarikwu SO, Durojaiye MA, Alabi AF, Akiri OF. Combined administration of curcumin and gallic acid inhibits gallic acid-induced suppression of steroidogenesis, sperm output, antioxidant defences and inflammatory responsive genes. *J Steroid Biochem Mol Biol*. 2014;143:49–60.
- Jahan S, Ain QU, Ullah H. Therapeutic effects of quercetin against bisphenol A induced testicular damage in male Sprague Dawley rats. *Syst Biol Reprod Med*. 2016;62:114–24.
- Li L, Ng TB, Gao W, Li W, Fu M, Niu SM. Antioxidant activity of gallic acid from rose flowers in senescence accelerated mice. *Life Sci*. 2005;77:230–40.
- Jaiswal N, Rizvi SI. Onion extract (*Allium cepa* L.) quercetin and catechin up-regulate paroxonase 1 activity with concomitant protection against low density lipoprotein oxidation in male Wistar rats subjected to oxidative stress. *J Sci Food Agric*. 2014;94:2752–7.
- Vijaya Padma P, Sowmya T, Arun Felix R, Baskaran P, Poornima P. Protective effect of gallic acid against lindane induced toxicity in experimental rats. *Food Chem Toxicol*. 2011;49:991–8.
- Olayinka ET, Ore A, Ola OS, Adeyemo OA. Protective effect of quercetin on melphalan-induced oxidative stress and impaired renal and hepatic functions in rat. *Chemother Res Pract*. 2014;2014:936526.
- Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*. 1979;95:351–8.
- Sedlak J, Lindsay RH. Estimation of total, protein bound, and non-protein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem*. 1968;25:192–205.
- Misra HP, Fridovich I. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem*. 1972;247:3170–5.
- Clairborne A. Catalase activity. In: Greewald AR, editor. *Handbook of methods for oxygen radical research*. Boca Raton: CRC Press Ltd; 1995, pp. 237–42.
- Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. *J Biol Chem*. 1951;193:265–75.
- Morais DB, Barros MS, Paula TAR, Freitas MBD, Gomes MLM, Matta SLP. Evaluation of the cell population of the seminiferous epithelium and spermatogenic indexes of the bat *Sturmira lilium* (Chiroptera: Phyllostomidae). *PLoS ONE*. 2014;9:e101759.
- Ryu JY, Lee E, Kim HJ, Park H, Im JY, Kim J, et al. Alterations of di(*n*-butyl) phthalate-induced oxidative stress in the testis of hypothyroid rats. *Toxicol Environ Chem*. 2008;90:113–26.
- Zhou D, Wang H, Zhang J. Di-*n*-butylphthalate (DBP) exposure induces oxidative stress in epididymis of adult rats. *Toxicol Ind Health*. 2011;27:65–71.
- Zuo HX, Li JQ, Han B, Ke CJ, Liu XD, Zhang YC, et al. Di-(*n*-butyl)-phthalate-induced oxidative stress and depression-like behaviour in mice with or without ovalbumin immunization. *Biomed Environ Sci*. 2014;27:268–80.
- Aly HA, Hassan MH, Beshbishy HA, Alahdal AM, Osman AM. Dibutyl phthalate induces oxidative stress and impairs spermatogenesis in adult rats. *Toxicol Ind Health*. 2016;32:1467–77.
- Abarikwu SO, Benjamin S, Ebah SG, Obilor G, Agbam G. Protective effect of *Moringa oleifera* oil against HgCl₂-induced hepato- and nephro-toxicity in rats. *J Basic Clin Pharmacol Physiol*. 2017;28:337–45.
- Kwack SJ, Han EY, Park JS, Bae JY, Ahn IY, Lim SK, et al. Comparison of the short term toxicity of phthalate diesters and monoesters in sprague-dawley male rats. *Toxicol Res*. 2010;26:75–82.
- Ferguson KK, Loch-Carusio R, Meeker JD. Exploration of oxidative stress and inflammatory markers in relation to urinary

- phthalate metabolites: NHANES 1999–2006. *Environ Sci Technol.* 2012;46:477–85.
34. Majeed KA, Ur Rehman H, Yousaf MS, Zaneb H, Rabbani I, Tahir SK, et al. Sub chronic exposure to low concentration of dibutyl phthalate affects anthropometric parameters and markers of obesity in rats. *Environ Sci.* 2017;24:25462–7.
 35. Abo-Salem OM, Abd-Ellah MF, Ghonaim MM. Hepatoprotective activity of quercetin against acrylonitrile-induced hepatotoxicity in rats. *J Biochem Mol Toxicol.* 2011;25:386–92.
 36. Nabavi SF, Nabavi SM, Habtemariam S, Moghaddam AH, Sur-eda A, Jafari M, et al. Hepatoprotective effect of gallic acid isolated from *Peltiphyllum peltatum* against sodium fluoride-induced oxidative stress. *Ind Crops Prod.* 2013;44:50–5.
 37. Dehghani F, Hassanpour A, Poost-Pasand A, Noorafshan A, Karbalay-Doust S. Protective effects of L-carnitine and homogenized testis tissue on the testis and sperm parameters of busulfan-induced infertile male rats. *Iran J Reprod Med.* 2013;11:693–704.
 38. Nna VU, Ujah GA, Mohamed M, Etim KB, Igba BO, Augustine ER, et al. Cadmium chloride-induced testicular toxicity in male Wistar rats; prophylactic effect of quercetin, and assessment of testicular recovery following cadmium chloride withdrawal. *Biomed Pharmacother.* 2017;94:109–23.
 39. Azu OO, Naidu EC, Naidu JS, Masia T, Nzemende NF, Chuturgoon A, et al. Testicular histomorphologic and stereological alterations following short-term treatment with highly active antiretroviral drugs (HAART) in an experimental animal model. *Andrology.* 2014;2:772–9.
 40. Zhou D, Wang H, Zhang J, Gao X, Zhao W, Zheng Y. Di-*n*-butyl phthalate (DBP) exposure induces oxidative damage in testes of adult rats. *Syst Biol Reprod Med.* 2010;56:413–9.
 41. Zhang L-D, Li C-H, Chong T, Gao M, Yin J, Fu D-L, et al. Pre-pubertal exposure to genistein alleviates di-(2-ethylhexyl) phthalate induced testicular oxidative stress in adult rats. *Biomed Int.* 2014;2014:598630.
 42. Ciftci O, Aydin M, Ozdemir I, Vardi N. Quercetin prevents 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin-induced testicular damage in rats. *Andrologia.* 2012;44:164–73.
 43. Oyagbemi AA, Omobowale TO, Saba AB, Adedara IA, Olowu ER, Akinrinde AS, et al. Gallic acid protects against cyclophosphamide-induced toxicity and epididymis of rats. *Andrologia.* 2016;48:393–401.
 44. Taepongsorat L, Tangpraputgul P, Kitana N, Malaivijitnond S. Stimulating effects of quercetin on sperm quality and reproductive organs in adult male rats. *Asian J Androl.* 2008;10:249–58.
 45. Vander Woude H, Tar Veld MG, Jacob N, Vaan Der Saag PT, Murk AJ, Riet Jen IM. The stimulation of cell proliferation by quercetin is mediated by estrogen receptor. *Mol Nutr Food Res.* 2005;49:763–71.
 46. Jahan S, Iftikhar N, Ullah H, Rukh G, Hussain I. Alleviative effect of quercetin on rat testis against arsenic: a histological and biochemical study. *Syst Biol Reprod Med.* 2015;61:89–95.
 47. Hidalgo M, Martin-Santamaria S, Recio I, Sanchez-Moreno C, de Pascual-Teresa B, Rimbach G, et al. Potential anti-inflammatory, anti-adhesive, anti/estrogenic, and angiotensin-converting enzyme inhibitory activities of anthocyanins and their gut metabolites. *Genes Nutr.* 2012;7:295–306.
 48. Dogrul NB, Kiliccalan I, Samet AE. The protective effect of gallic acid against endocrine disrupting damages of bisphenol A and possible mechanism. *J Contemp Med Educ.* 2016;4:39.
 49. Abarikwu SO, Njoku RC, Lawrence CJ, Charles IA, Ikewuchi JC. Rutin ameliorates oxidative stress and preserves hepatic and renal functions following exposure to cadmium and ethanol. *Pharm Biol.* 2017;55:2161–9.
 50. Nelson SK, Bose SK, Grunwald GK, Myhill P, McCord JM. The induction of human superoxide dismutase and catalase in vivo: a fundamentally new approach to antioxidant therapy. *Free Radic Biol Med.* 2006;40:341–7.
 51. Bouayed J, Bohn T. Exogenous antioxidants-Double-edged swords in cellular redox state: health beneficial effects at physiologic doses versus deleterious effects at high doses. *Oxid Med Cell Longev.* 2010;3:228–37.
 52. Abarikwu SO. Protective effect of quercetin on atrazine induced oxidative stress in the liver, kidney, brain and heart of adult Wistar rats. *Toxicol Int.* 2014;21:148–55.
 53. Abarikwu SO, Pant AB, Farombi EO. Dietary antioxidant, quercetin, protect Sertoli-germ cell co-culture from atrazine-induced oxidative damage. *J Biochem Mol Toxicol.* 2012;26:477–85.
 54. Abarikwu SO, Akiri OF, Durojaiye MA, Alabi AF. Combined effects of repeated administration of Bretmont Wipeout (Glyphosate) and Ultrazin (Atrazine) on testosterone, oxidative stress and sperm quality of Wistar rats. *Toxicol Mech Methods.* 2015;25:70–80.
 55. Lu Z, Nie G, Belton PS, Tang H, Zhao B. Structure-activity relationship analysis of antioxidant ability and neuroprotective effect of gallic acid derivatives. *Neurochem Int.* 2006;48:263–74.