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Research Article

Combined Protective Effects of Quercetin, Rutin, and Gallic Acid against Cadmium-Induced Testicular Damages in Young-Adult Rats

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Cadmium (Cd) is a toxic metal that damages several tissues of animals and humans including the testis. The ameliorative effects of quercetin (QUE), rutin (RUT), and gallic acid (GAL) at 20 mg kg^{-1} body weight alone or in combination against testicular injury induced by Cd (24 mg kg^{-1} body weight) in male Wistar rats were evaluated in this study. Forty-two (42) rats were randomly grouped into six (6) groups: (1) vehicle control group, (2) Cd group, (3) RUT+Cd group, (4) GAL+Cd group, (5) QUE+Cd group, and (6) RUT+GAL+QUE+Cd group. At the end of the oral gavage of the tested chemicals, the rats were sacrificed, blood samples were collected, and testes were harvested and processed for biochemical assays. Cd exposure damaged the testis (smaller epithelium thickness and spermatogenesis index and sloughing of the epithelium); increased lipid peroxidation, glutathione *S*-transferase activity, and DNA fragmentation; and diminished glutathione reductase activity and serum testosterone level 40 days posttreatment. Treatment with the phenolics separately or in combination attenuated the effect of Cd on serum testosterone, glutathione reductase and glutathione *S*-transferase activities, lipid peroxidation, and percent fragmented DNA. The increased nitric oxide concentration in the QUE+Cd group was attenuated to control values in the combined (RUT+GAL+QUE+Cd) exposure group. Coadministration of the phenolics appears to have more substantial protective effects than their single effects against Cd-induced testicular DNA damage, glutathione *S*-transferase activity, and the recovery of testosterone levels and spermatogenesis index. Overall, the tested phenolics can reduce testicular damage more efficiently in their combined form than individual administration.

1. Introduction

Cadmium (Cd) is a widespread environmental pollutant that damages the gonads of animals. Humans are exposed to Cd through diet, water, and inhalation [1]. Occupationally, Cd exposure can arise from electroplating, plastic, and fertilizer manufacturing [2]. Cadmium exposure directly damages the testis of rats through inhibition of testosterone secretion and regulatory steroidogenic enzymes, disruption of the blood-testis barrier, induction of germ cell loss, testicular haemorrhage, and necrosis [3–5]. The high sensitivity of the testis to Cd toxicity is also affected by the expression of Cd transporters and metallothionein (MTs) and minerals—e.g., Zn^{2+} and Ca^{2+}-mediated cellular events [6]. At the molecular level, Cd complexes with MTs, but the binding does not reduce the toxic effects of Cd in the testis unlike in other tissues where MTs are induced under physiologic and pathological states to counteract Cd toxicity [7]. Additionally, Cd triggers an adaptive mechanism involving glutathione *S*-transferase activity in most Cd-vulnerable tissues that are necessary for removing Cd from the body [8, 9]. Although Cd is not a redox-active metal, it disrupts the homeostasis of redox-active minerals, e.g., Cu and Fe, and induces a higher level of hydroxyl radicals that initiate lipid peroxidation [5, 9–13].

Quercetin is the most universally consumed flavonoid present in appreciable amounts in many vegetables, fruits, and grains. Structurally, quercetin is a pentahydroxy flavone nucleus having hydroxyl groups attached at the 3-, 3′-, 4′-, 5-, and 7-positions that play significant roles in their antioxidant, antilipoperoxidative, and cytoprotective effects [14, 15]. Rutin (quercetin-3-O-rutinoside) is a glycoside consisting of the flavonoid quercetin and the sugar rutinose (α-L-rhamnopyranosyl-(1 ⟶ 6)-β-D-glucopyranose). It is present in a wide variety of citrus fruits. Rutin is reported to improve testosterone levels and suppress lipid peroxidative changes due to their antioxidant enhancing capacities in the testes in response to chemically induced oxidative stress [16–20]. Gallic acid is a monomeric phenolic compound that fits in the class of hydrolysable tannins. Structurally, gallic acid is a 3,4,5-trihydroxy benzoic acid. The polyphenolic constituent of gallic acid has been found in varieties of fruits such as grapes and blueberries. Gallic acid possesses antioxidant effects and has the capacity to scavenge reactive oxygen species [21–24].

We earlier established that both gallic acid and quercetin at comparable doses have antioxidant effects that could be influenced by experimental models (*in vivo* versus *in vitro)* [25]. Gallic acid has lower antioxidant potency than other flavonoids in the testicular system of a rat model [26], and the antioxidant capacity of quercetin could be improved when combined with other phenolic chemicals [27]. It is believed that some phenolics will have their antioxidant protective effects modified when administered concurrently with others, whereas some phenolic-based combinations are to be avoided [27–29]. In the present study, we explored the effects of gallic acid, rutin, and quercetin in order to understand their separate and combined antioxidant protective effects against Cd-induced oxidative injury in rats.

2. Materials and Methods

Quercetin, rutin, gallic acid, Cd, and all other chemicals were of analytical grade and were purchased from Sigma-Aldrich except otherwise stated.

2.1. Experimental Animals. Forty-two young adult male Wistar rats aged 8 weeks, weighing approximately 110 ± 5.9 g, were procured from the animal house of the Department of Biochemistry, University of Port Harcourt. The animals were allowed to acclimatise to their new surrounding 7 days prior to the start of the experiment with a schedule of 12 h light : 12 h dark cycle. They were housed in standard plastic cages with dry sawdust bedding, fed *ad libitum* with standard rat feed (Ladokun Feeds Ltd., Ibadan, Nigeria), and had access to clean drinking water throughout the duration of the experiment. The room where the animals were kept was well ventilated and maintained at a temperature of 23°C with 65% relative humidity. The study protocols and design including the methodology for the handling of the animals received the authorization of the Department of Biochemistry Ethical Committee for Research on Laboratory Animals (UPH/BCHREC2022/010) and were in accordance with the Standard Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication Number, 85-23).

2.2. Experimental Design. The experimental rats were randomly sorted into six (6) groups with seven (7) rats per group. The control group received not more than 0.2% of the vehicle, dimethyl sulfoxide; rats in the Cd group received a single dose of Cd (24 mg kg^{-1} body weight) by oral gavage during the 40-day duration of the study; rats in the RUT+Cd group received a single dose of Cd (24 mg kg^{-1} body weight) in the same manner as in the Cd group and were administered RUT (20 mg kg^{-1} body weight) twice a week by gavage for 40 days; rats in the GAL+Cd group received a single dose of Cd (24 mg kg^{-1} body weight) in the same manner as the Cd group and were administered GAL (20 mg kg^{-1} body weight) twice a week by gavage for 40 days; rats in the QUE+Cd group received a single dose of Cd (24 mg kg^{-1} body weight) in the same manner as the Cd group and were administered QUE (20 mg kg^{-1} body weight) twice a week by gavage for 40 days; and rats in the RUT+GAL+QUE+Cd group received a single dose of Cd (24 mg kg^{-1} body weight) in the same manner as the Cd group and were concurrently administered 20 mg kg^{-1} body weight of each of the three phenolics twice a week by gavage for 40 days. The animals in the individual and combined phenolics groups were pretreated with their respective phenolics for a week before coadministration with a single oral dose of Cd at 24 mg kg^{-1} body weight (b.w.).

Oral subacute evaluation of Cd toxicity in a previous study reported that a single oral dose of Cd (24 mg kg^{-1} b.w.) could induce oxidative stress and injury in the testicular tissues of rats much more than the intraperitoneal route [30]. Additionally, an oral dose of 24 mg Cd kg^{-1} b.w. which is approximately 1.2 mg kg^{-1} b.w. intraperitoneal dose is assumed to be a safe standard rate of absorption after Cd exposure [30, 31]. Our previous studies and those of others have established that quercetin, rutin, and gallic acid at low and comparable doses are capable of expressing antioxidant-inducing and protective effects separately in testicular systems of rats and other model systems [16, 25, 27, 32, 33]. For example, in the rat's testicular system, rutin expresses antioxidant-inducing potentials at lower doses (25 mg kg^{-1} b.w.) [16], and a low dose of gallic acid (20 mg kg^{-1} b.w.) expresses antioxidant protective function in the rat's cardiorenal systems [33]. Furthermore, in a pilot study, the oral dose (20 mg kg^{-1} b.w.) of the phytochemicals (gallic acid, quercetin, and rutin) is safe and expresses potent antioxidant-inducing properties in a normal rat model. No toxicity sign or change in behaviour was observed in the animals throughout the 40-day study and also in the pilot study during the dose selection of the phenolics. The 40-day period was chosen to cover the period for the transition of spermatogonia to spermatozoa in rats [31] and therefore allows for observable histological features of the rats' testis during the later stages of germ cell development [3, 16, 34, 35].

After 40 days of the administration, the animals were fasted during the night and sacrificed the next day under chloroform anaesthesia and decapitation. On decapitation,

the blood sample was collected into a plain specimen bottle until the animal stopped breathing and the blood stopped flowing. The serum sample was prepared after the collected blood samples have been spun at 4000 × g for 20 min at room temperature and thereafter used for evaluating testosterone concentration. The paired testes from each animal were put into labelled specimen bottles for histopathological analysis and preparation of the testicular homogenates that were used for biochemical assays. Testicular samples to be used for histology were quickly put in Bouin's solution for 8 h before they were dehydrated in ethanol (70%, 80%, 90%, 95%, and absolute alcohol) for 1 h. Dehydrated tissue samples were embedded in paraffin, cleared in xylene, sectioned at 5 μm, and stained with hematoxylin and eosin for observation under a light microscope.

2.3. Preparation of Tissue Homogenates and Postmitochondrial Supernatants. Seven testes from each group were sliced into smaller bits and homogenized in ice-cold sodium phosphate buffer (0.1 M, pH 7.4) using a mortar and pestle that were placed on a tray of ice to produce a 10% (*w*/*v*) homogenate. The samples were spun at 10,000 × g for 20 min, and the postmitochondrial supernatant was collected for examination of several biochemical parameters. Some portions of the supernatant samples were deproteinated with 20% trichloroacetic acid for reduced glutathione (GSH) and lipid peroxidation assays or 5% zinc sulfate for the Griess assay. The pellets were discarded after spinning at 5000 × g for 20 min at 4°C, and the supernatants were used for the biochemical assays.

2.4. Determination of GSH Redox System. The glutathione redox system consists of GSH and other antioxidants that use GSH to detoxify foreign chemicals and active oxygen metabolites including glutathione peroxidase (GSH-Px), glutathione reductase (GR), and glutathione *S*-transferase (GST). The glutathione peroxidase enzyme activity level was evaluated according to the method of Rotruck [36]. The absorbance of residual glutathione remaining after the glutathione peroxidase reaction was recorded in a spectrophotometer at 412 nm. The GSH-Px activity was extrapolated from a GSH standard curve (20-200 μg/ml) and expressed as μg GSH g tissue^{-1} ml^{-1}. The activity of glutathione reductase (GR) was measured as reported previously by Staal *et al.* [37]. The reaction was initiated by adding 1 mM NADPH to the reaction mixture containing 0.01 M oxidized GSH. The change in absorbance was read at 340 nm at 1 min intervals for 5 min against a substrate blank. Enzyme activity was computed from the molar extinction for NADPH (6.22×10^{-3} nmol ml^{-1}) and expressed as mU g tissue^{-1} ml^{-1}.

Reduced GSH concentration was monitored according to the method of Jollow *et al.* [38]. The reaction tube for the assay contains an aliquot of the sample, 0.2 M Na_2HPO_4, and 0.01 M Ellman's reagent. The absorbance of the yellow-coloured solution was recorded at 412 nm against a blank after 20 min. The GSH level was extrapolated from the calibration curve for GSH (20-200 μg/ml) and expressed as μg GSH g tissue^{-1} ml^{-1}.

The activity of GST was based on the protocol previously reported by Habig *et al.* [39]. The reaction cocktails were completed by adding 1-chloro-2,4-dinitrobenzene (0.02 M) to the assay tubes containing 100 mM GSH and potassium phosphate buffer (pH 6.5). The reaction was started by adding aliquots of the samples and was monitored at 340 nm wavelength. The absorbance was recorded at 1 min intervals for 3 min against a blank containing all reagents except the sample. The activity of the enzyme was computed from the molar absorbency index of 1-chloro-2,4-dinitrobenzene (9.6 mM^{-1} cm^{-1}) and expressed as U g tissue^{-1} ml^{-1}.

2.5. Determination of Lipid Peroxidation. The lipid peroxidation (LPO) level was quantified as reported previously by Varshney and Kale [40]. The assay tubes containing 150 mM Tris/KCl buffer (pH 7.4), thiobarbituric acid (0.75%), and an aliquot of deproteinated samples were put in a boiling water bath for the development of the pink-coloured chromophore in 45 min. The solution was allowed to cool before the absorbance was recorded versus blank at 532 nm.

2.6. Griess Assay for the Determination of Nitrite. In principle, when sulfanilamide is added to a nitrite-containing sample, nitrite ion in the reaction mixture reacts with it to form a diazonium salt, which combines with N-(1-naphthyl)ethylenediamine to form a pink-red azo dye [41]. To assay for nitrite concentration, an equal volume of protein-free supernatant samples and Griess reagent were combined together and kept in the dark for 20 min to allow a pink colour to develop. The absorbance of the solution was recorded at 540 nm, and the level of nitrite was computed from a nitrite calibration curve (0-500 μmol ml^{-1}) and expressed in μmol g tissue^{-1} ml^{-1}.

2.7. Percent DNA Fragmentation Assay. DNA fragmentation was evaluated using diphenylamine as the substrate as earlier described by Wolozin *et al.* [42]. Tunica albuginea of the testes was removed, and the tubules were dispersed and homogenized in a cold DNA lysis buffer (Tris 10 mM, EDTA 1 mM, and 0.2% Triton X-100; pH 8.3). Testicular homogenates were put in a centrifuge tube and spun at 6000 g (4°C, 10 min) to obtain a nuclear pellet. The supernatants were eliminated, and the nuclear pellet was suspended in the lysis buffer and was then spun at 10,000 × g for 20 min at 4°C. The supernatant was collected, and the resulting pellets were resuspended in the lysis buffer. 25% trichloroacetic acid was put in the assay tubes whirled strongly and kept overnight at 4°C followed by centrifugation at 10,000 × g (4°C, 20 min). The supernatants and pellets were collected, and 5% trichloroacetic acid was added to the assay tubes followed by heating both supernatant and pellet samples at 90°C for 15 min. Diphenylamine (15 mg ml^{-1}) prepared in glacial acetic acid, containing 0.15 ml concentrated H_2SO_4 and 50 μl of acetaldehyde to make a 10 ml mixture, was put into the assay tube and kept overnight at room temperature. The absorbance of the blue-coloured solution that developed was read at 600 nm and expressed in percentages.

2.8. Determination of Testosterone Concentration. The level of free serum testosterone was measured by ELISA using the AccuBind™ testosterone kit (CAT NO. 3725-300) as per the instructions in the manufacturer's protocol (Monobind Inc., CA, USA).

2.9. Measurement of Cd and Fe Concentrations. Tissue slices were oven-dried at 60°C and weighed. The samples were digested in aqua regia (three parts of concentrated hydrochloric acid mixed with one part of concentrated nitric acid) at 120°C. When the tissues were dissolved in the acid mixtures, they were mixed with 10 ml of distilled water. The samples were then subjected to flame atomic absorption spectrometry for the measurement of Cd and Fe. Triplicate values were recorded for each analysed sample, and the variation in coefficient was usually less than 10%.

2.10. Spermatogenesis Score Index and Epithelium Thickness. Spermatogenesis in the seminiferous tubules was evaluated with "Johnsen's score." To achieve this, 50 circular or near-round seminiferous tubules were randomly selected from each section, and Johnson's score (scale of 1-10 based on the degree of spermatogenesis) was measured for each tubule [43]. Then, the mean of Johnson's score was calculated. The seminiferous epithelium thickness was measured from the tunica propria to the luminal border of each tubule at 100x magnification [31] using the straight-line tool of ImageJ software (National Institute of Health, Bethesda, MA, USA).

2.11. Statistical Analysis. All values in each of the experiments are represented as means ± standard deviation of the means. Comparisons between different means were done by analysis of variance and Tukey's post hoc test. Statistical difference was considered significant if $P < 0.05$. Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Quercetin, Rutin, and Gallic Acid Had No Effect on Biometric Variables in Cd-Exposed Rats. At the end of the study, the absolute and relative testis weight as well as the final body weight of the rats did not change substantially across all the groups (data not shown). The animals were also in a good state of general health as no symptom of morbidity or even death was observed throughout the study.

3.2. Quercetin, Rutin, and Gallic Acid Alleviated Cd-Induced Testicular DNA Fragmentation in Rats. The testes of Cd-intoxicated animals had higher percent fragmented DNA (212.49%) compared with the control group ($P < 0.0001$), suggesting that DNA damage was induced by Cd exposure. Importantly, the Cd-induced increase in testicular fragmented DNA was significantly decreased by 73.50% ($P < 0.0001$) in the RUT+GAL+QUE+Cd group. The RUT+Cd cotreatment has the least defensive effect against Cd-induced increase in percent fragmented DNA whereas the other cotreatment groups (GAL+Cd and QUE+Cd) have similar effects to the combined exposure group (RUT+GAL+QUE +Cd) (Figure 1).

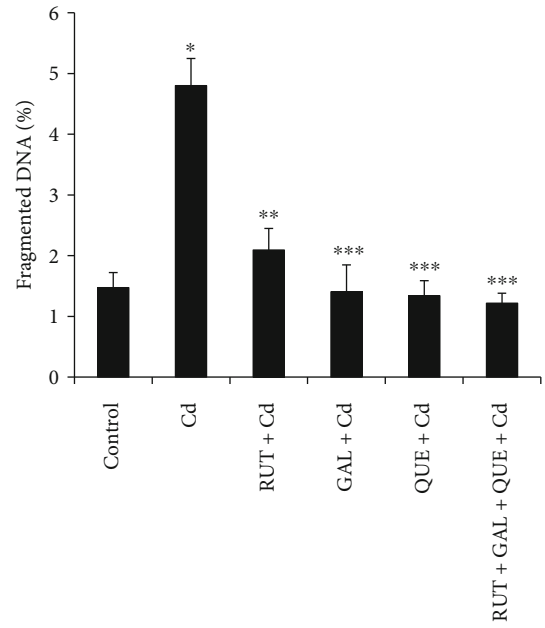

Figure 1: Protective effect of rutin (RUT), gallic acid (GAL), and quercetin (QUE) separately and in combination against Cd-induced DNA fragmentation in the rat's testis after exposure to a single dose of Cd for 40 days. *Significantly different from control ($P < 0.0001$), **significantly different from Cd groups ($P < 0.0001$), ***RUT+Cd vs. GAL+Cd ($P = 0.0356$), ***RUT+Cd vs. QUE +Cd ($P = 0.0179$), and ***RUT+Cd vs. RUT+GAL+QUE+Cd ($P = 0.0046$).

3.3. Quercetin, Rutin, and Gallic Acid Alleviated Cd-Induced Deficits in the Testicular Glutathione Redox System in Rats. The enzymatic activity of GSH-Px in control and treated rats is presented in Figure 2(a). There was no significant statistical change in GSH-Px activity across the various groups at the termination of treatment of animals with the tested chemicals ($P > 0.05$). The enzyme activity of GR was diminished in rats treated with Cd relative to the control which had an activity of 73 mU/g tissue/ml ($P < 0.0001$). GR activity was increased to 56 mU/g tissue/ml in the RUT+Cd group relative to the Cd-treated animals (29 mU/g tissue/ml) ($P = 0.02$). There was a rise in GR activity in the GAL +RUT+QUE+Cd group (97.53 mU/g tissue/ml) by 237% when compared to the Cd group ($P < 0.0001$). GR activity was increased in these animals (GAL+RUT+QUE+Cd) by 75%, 36%, and 21% relative to the RUT+Cd, GAL+Cd, and QUE+Cd groups, respectively. The increase in GR activity in the QUE+Cd group was 180% higher than in the Cd group ($P < 0.0001$), 18% lower than in the GAL+RUT +QUE+Cd group ($P > 0.05$), and 45% higher than in the RUT+Cd group ($P = 0.032$). There was a maximum increase of 72 mU/g tissue/ml observed in the GAL+Cd relative to the Cd group ($P = 0.0001$), and the increase was 148% higher when compared with the Cd group (Figure 2(b)). There was also no significant statistical change in GSH concentration across the various treatment groups at the termination

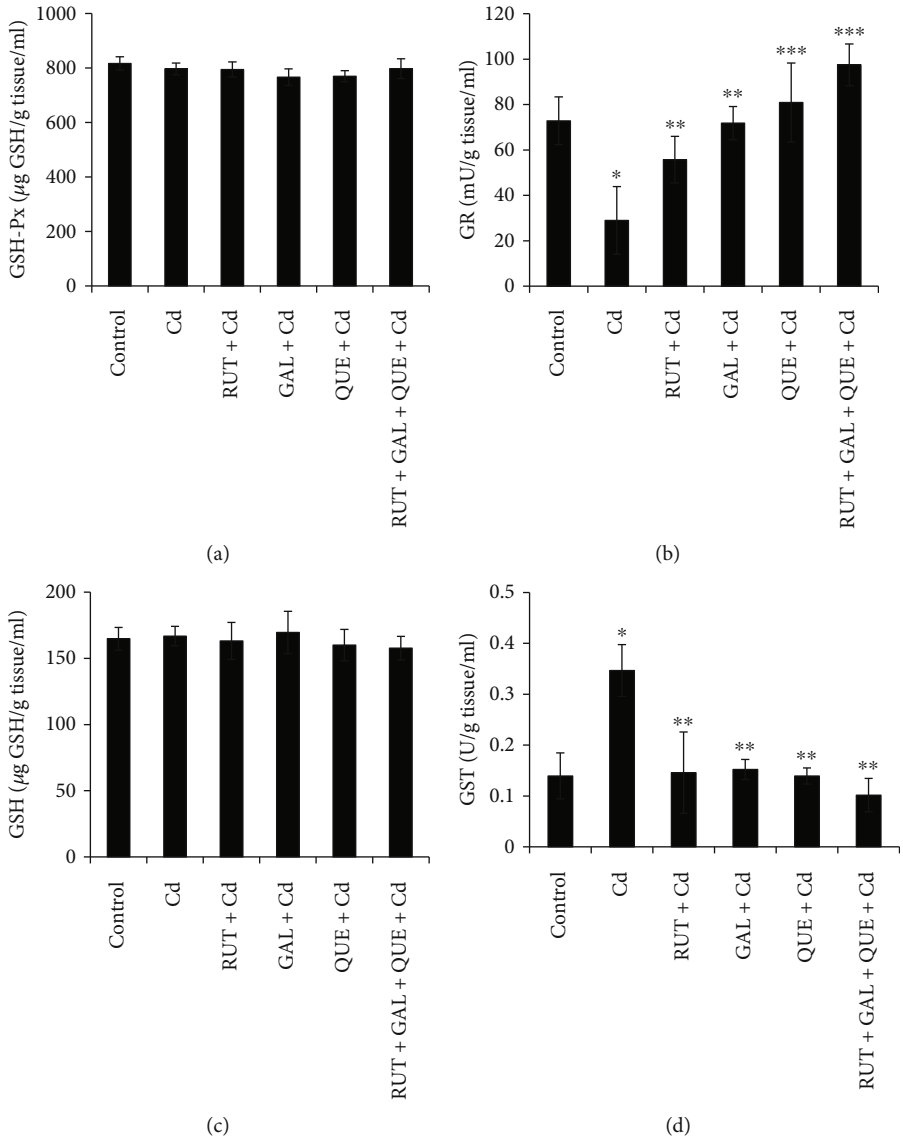

Figure 2: Protective effect of rutin (RUT), gallic acid (GAL), and quercetin (QUE) separately and in combination on the glutathione redox system of the rat's testis after exposure to a single dose of Cd for 40 days: GSH-Px enzyme activity (a), GR enzyme activity (b), GSH concentration (c), and GST enzyme activity (d) in the testes of rats. GR = *significantly different from control ($P < 0.0001$), ** significantly different from Cd ($P < 0.0001$), ***significantly different from RUT+Cd ($P = 0.032$), and ****significantly different from GAL+Cd ($P = 0.0275$) and RUT+Cd ($P = 0.0002$). GST = *control vs. Cd ($P < 0.0001$), **Cd vs. RUT+Cd ($P < 0.0001$), **Cd vs. GAL+Cd ($P < 0.0001$), **Cd vs. QUE+Cd ($P < 0.0001$), and **Cd vs. RUT+GAL+QUE+Cd ($P < 0.0001$). GSH-Px = glutathione peroxidase; GR = glutathione reductase; GSH = reduced glutathione; GST = glutathione *S*-transferase.

of the study ($P > 0.05$) (Figure 2(c)). The activity of GST was increased by 149% in the testis of Cd-exposed rats relative to the control group ($P < 0.0001$). There was a decrease in GST enzyme activity by 57%, 56%, and 60% in the RUT+Cd, GAL+Cd, and QUE+Cd groups, respectively, when compared with the Cd group ($P < 0.0001$). The animals administered RUT+GAL+QUE+Cd when compared to Cd alone could decrease GST activity by 71% ($P < 0.0001$) (Figure 2(d)).

3.4. Quercetin, Rutin, and Gallic Acid Suppressed Cd-Induced Testicular Lipid Peroxidation in Rats. A rise in the level of lipid peroxidation can reflect tissue damage and is considered an important parameter of oxidative damage. In the Cd-treated rats, the amount of testicular malondialdehyde (MDA) significantly increased by 37% as compared with normal controls ($P = 0.0164$), although the levels of testicular MDA were decreased by 51% in rats cotreated with RUT+Cd ($P < 0.0001$), by 36% in the QUE+Cd and GAL +Cd cotreated groups ($P = 0.001$), and by 32% after cotreatment with the combined phenolics compared to Cd ($P = 0.0034$) (Figure 3).

3.5. Quercetin, Rutin, and Gallic Acid Enhance Testicular Nitrite Concentration in Cd-Exposed Rats. Nitrite concentration was found to remain unchanged in the Cd group and

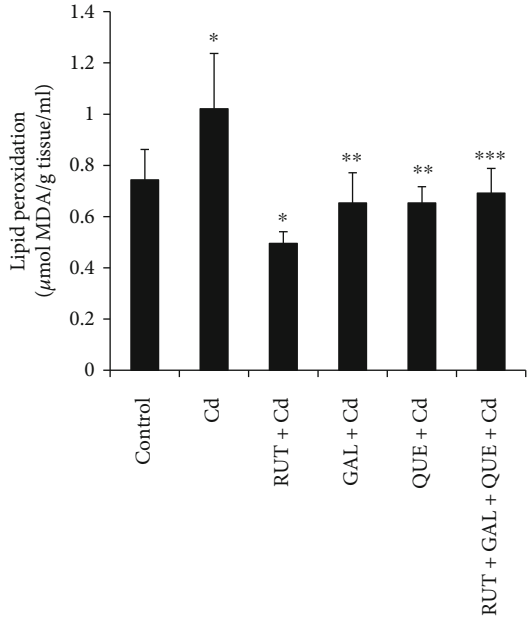

Figure 3: Protective effect of rutin (RUT), gallic acid (GAL), and quercetin (QUE) separately and in combination against Cd-induced level of lipid peroxidation (LPO) in the rat's testis after 40 days. *Control vs. Cd ($P = 0.0164$), *control vs. RUT+Cd ($P = 0.0392$), and **Cd vs. GAL+Cd ($P < 0.001$), Cd vs. QUE ($P = 0.001$), and RUT+Cd vs. RUT+GAL+QUE+Cd ($P = 0.0033$).

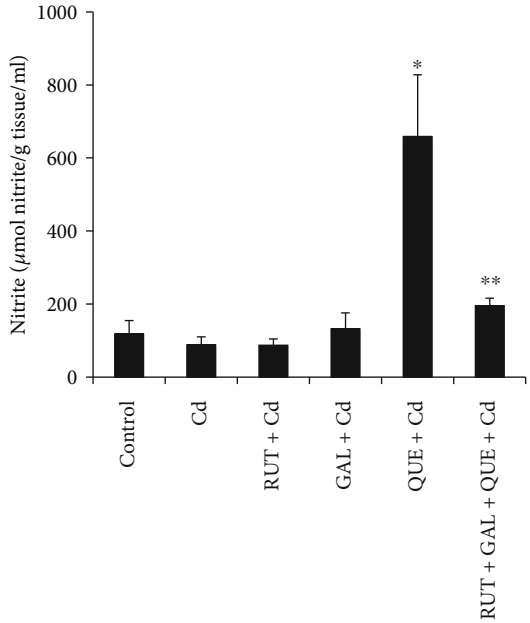

Figure 4: Protective effect of rutin (RUT), gallic acid (GAL), and quercetin (QUE) separately and in combination on nitrite concentration in the rat's testis after exposure to a single dose of Cd after 40 days. *Significantly different from control ($P < 0.0001$); **significantly different from Cd groups ($P < 0.0001$).

those of the RUT+Cd and GAL+Cd groups relative to control values ($P > 0.05$). There was an increase in nitrite concentration in the testes of animals treated with QUE+Cd relative to the control values ($P < 0.0001$) and other treatment groups ($P < 0.0001$). Nitrite concentration was found to increase by 455% in the QUE+Cd cotreatment group versus the control. The animals administered RUT+GAL+QUE +Cd when compared to QUE+Cd could decrease nitrite concentration by 72% ($P < 0.0001$) (Figure 4).

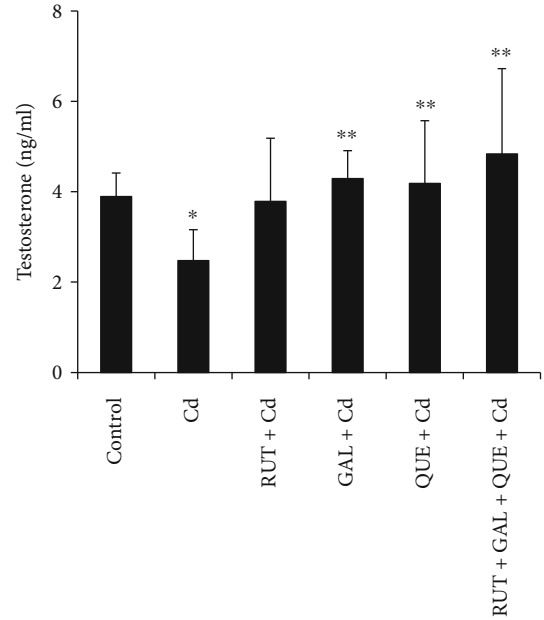

Figure 5: Protective effect of rutin (RUT), gallic acid (GAL), and quercetin (QUE) separately and in combination on serum testosterone level of rats after exposure to a single dose of Cd after 40 days. *Significantly different from control ($P = 0.006$), ** QUE+Cd vs. Cd ($P = 0.0381$), **GAL+Cd vs. Cd ($P = 0.0022$), and **RUT+GAL+QUE+Cd vs. Cd ($P = 0.00297$).

3.6. Quercetin, Rutin, and Gallic Acid Enhanced the Concentration of Serum Testosterone in Cd-Exposed Rats. The concentration of testosterone was significantly diminished by 57% in Cd-treated groups compared to the control values ($P = 0.006$). However, treatment with the phenolics was found to halt the Cd-induced decrease in the testosterone level. The testosterone level was found to be raised by 53% in the RUT+Cd group relative to the Cd group ($P = 0.095$). GAL+Cd and QUE+Cd administration was found to increase testosterone levels by 73% ($P = 0.0022$) and 69% ($P = 0.0381$), respectively, when analysed with Cd-treated rats. GAL+RUT+QUE+Cd cotreatment was found to increase testosterone concentration by 96% when compared to the Cd group ($P = 0.00297$). There were no statistically significant differences in the RUT+Cd, GAL+Cd, QUE+Cd, and RUT+GAL+QUE+Cd groups when they were compared with control values ($P > 0.05$) (Figure 5).

3.7. Quercetin, Rutin, and Gallic Acid Modulated Testicular Cd and Fe Concentrations in Cd-Exposed Rats. At the termination of the study, Cd was not detected in the testes of treated animals in all groups, but the concentration of Fe was noticed to slightly increase in the Cd-treated animals compared to the control values ($P = 0.0824$). The iron level was also found to be decreased in the RUT+Cd ($P = 0.0003$

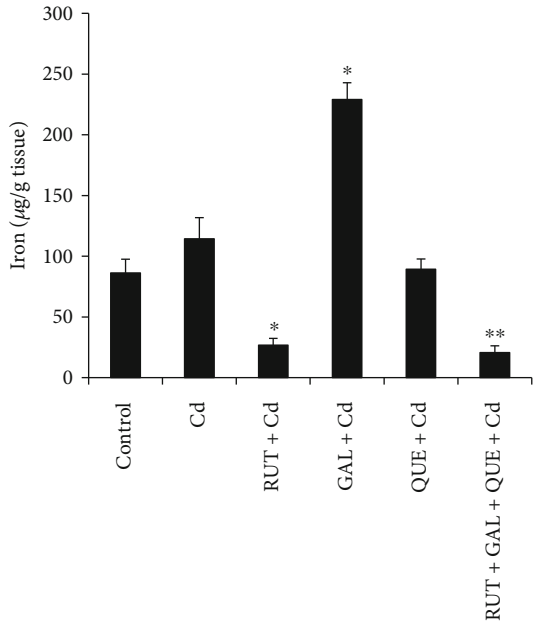

Figure 6: Protective effect of rutin (RUT), gallic acid (GAL), and quercetin (QUE) separately and in combination on iron (Fe) concentration in the rat's testis after exposure to a single dose of Cd after 40 days. *Cd vs. RUT+Cd ($P < 0.0001$), *Cd vs. GAL +Cd ($P < 0.0001$), and **Cd vs. RUT+GAL+QUE+Cd ($P < 0.0001$).

) and RUT+GAL+QUE+Cd ($P = 0.0001$) cotreated groups and was raised in the GAL+Cd group ($P < 0.0001$) compared to the control (Figure 6).

3.8. Quercetin, Rutin, and Gallic Acid Attenuated Cd-Induced Histopathological Injuries in the Testes of Rats. To confirm the protective effects of the phenolics on Cd-induced lesions in the testes, we examined the histological changes in the rat's testes as previously described [3, 44]. As presented in Figure 7, rats in the Cd-intoxicated group have some small-sized tubules, decreased germ cell layers, no defined tubular lumina, spermatocytic arrest in some tubules, sloughing and disorganization of the epithelium, and spermatogenic hypoplasia whereas only mild disarrangement of the seminiferous epithelium was found in the phenolics-cotreated rats after both separate and combined administration, suggesting a recovery of the tubular epithelial disruption, although few empty vacuolar spaces were still found in the QUE+Cd cotreated animals which were significantly alleviated by the coadministration of gallic acid, rutin, and quercetin to Cd-intoxicated rats. As expected, histopathological analysis of the seminiferous tubules showed that the microanatomy of the testes of rats in the control groups was normal. Similarly, germ cells at all stages of maturation were seen in the germinal epithelium and the well-defined tubular lumina filled with sperms. In the interstitial spaces between neighbouring tubules, blood vessels, Leydig cells, and other interstitial cells of different shapes and sizes were present and bound by seminiferous tubules.

3.9. Quercetin, Rutin, and Gallic Acid Enhanced Spermatogenesis Score Index and Seminiferous Tubule Epithelium Thickness of Cd of Treated Rats. The Johnsen score was found to significantly decrease in the Cd group compared to the control values ($P < 0.0001$). The spermatogenesis score index was higher in the testes after separate administration of the phenolics compared to the Cd values but was lower than the control value (Figure 8(a)). The Johnsen score was raised by 88% in the RUT+Cd, GAL +Cd, and QUE+Cd groups relative to the Cd values ($P < 0.0001$). When Johnsen scores were compared to control values, they were found to be lower in the RUT+Cd ($P = 0.0025$), GAL+Cd ($P = 0.0298$), and QUE+Cd ($P = 0.0298$) groups. Combined administration of the phenolics (RUT+GAL+QUE+Cd) was found to increase the Johnsen score by 125% compared to the Cd values ($P < 0.0001$) and by 20% compared to the RUT+Cd values ($P = 0.0186$).

Seminiferous tubule epithelium density was significantly decreased in the Cd group compared to the control ($P < 0.0001$) and could be increased in the RUT+Cd ($P = 0.0026$), GAL+Cd ($P = 0.0017$), QUE+Cd ($P = 0.0001$), and RUT+GAL+QUE+Cd ($P < 0.0001$) groups relative to the Cd group (Figure 8(b)). Furthermore, epithelium thickness was significantly increased in the RUT+GAL+QUE +Cd group compared to the RUT+Cd group by 23% ($P = 0.0451$). There were no statistically significant changes when the epithelium thickness of RUT+GAL+QUE+Cd rats was compared with GAL+Cd values ($P = 0.0654$) and QUE +Cd ($P = 0.4156$). Epithelium thickness was also found to be significantly decreased in the RUT+Cd ($P = 0.0039$) and GAL+Cd ($P = 0.006$) groups compared to the control values but did not reach a statistical threshold in the QUE+Cd ($P = 0.0654$) and RUT+GAL+QUE+Cd ($P = 0.8972$) groups.

4. Discussion

Intake of flavonoids has proven to be potent in the protection of tissues against oxidative damage induced by noxious environmental chemicals and metals. In the current study, Cd administration was noted to induce oxidative toxicity in the testes of rats by elevating the enzymatic action of GST, LPO level, and percent fragmented DNA and also by suppressing the enzymatic activity of GR and the concentration of nitrite and testosterone level. This was also supported by the histological changes observed in the testes of the animals.

The increase in DNA fragmentation induced by Cd supports the fact that Cd could cause damage to the DNA of the rat's testis as described earlier by Emad *et al. [46]*. Cd could induce oxidative stress in susceptible tissues such as the testis and exert toxic effects on the DNA validating the fact that impaired antioxidant systems in the gonads make the DNA vulnerable to the damaging effects of Cd [1]. Coadministration of the phenolics attenuated the DNA damage caused by Cd, and the three phenolics (RUT+GAL+QUE) had better potency than individual treatments against the Cd-induced increase in DNA damage. This observation agrees with the reports of Gelen *et al. [47]* which suggested that phenolic

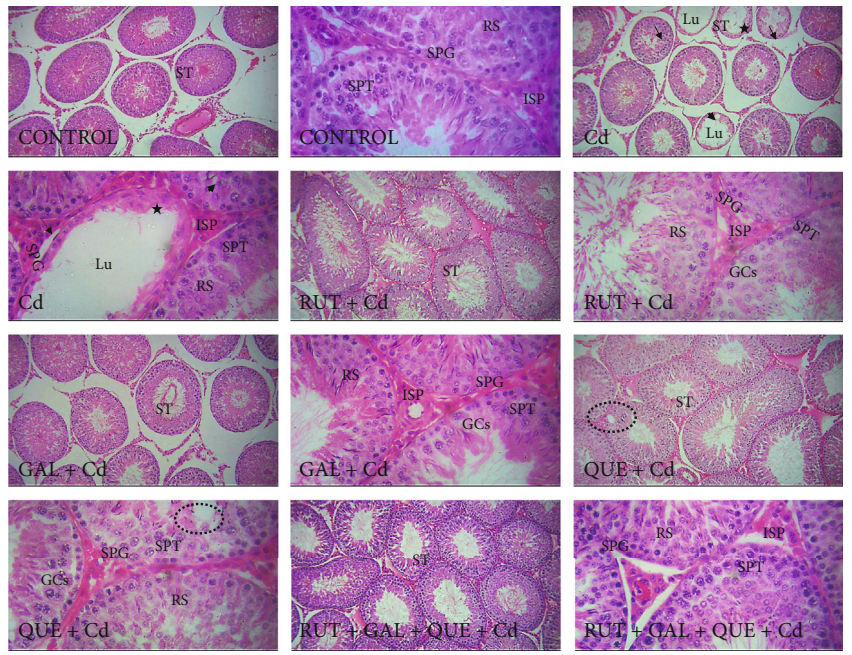

Figure 7: The histopathological features in Cd-intoxicated testes included reduction of germ cell layers (starred), germ cell aplasia, thin basement membrane (arrow with dotted lines), undefined lumen (Lu), atrophied tubular epithelium (arrow), and vacuolated epithelium (arrowhead), whereas the testis damages were significantly alleviated by the coadministration of quercetin, gallic acid, and rutin either in combination or separately to Cd-intoxicated rats. Some histologic features in these groups included few vacuolated epithelia (dotted circles) and few germ cell layers (GCs). As expected, most of the seminiferous tubules in the control animals are without damage and showed normal spermatogenic activity. Some Leydig cells were presented in the interstitial spaces between neighbouring tubules of the testes. ST = seminiferous tubules; ISP = intertubular spaces; SPG = spermatogonia; SPT = spermatocytes; RS = round spermatids; hematoxylin and eosin; smaller magnification = 100x Mag., larger magnification = 400x Mag.

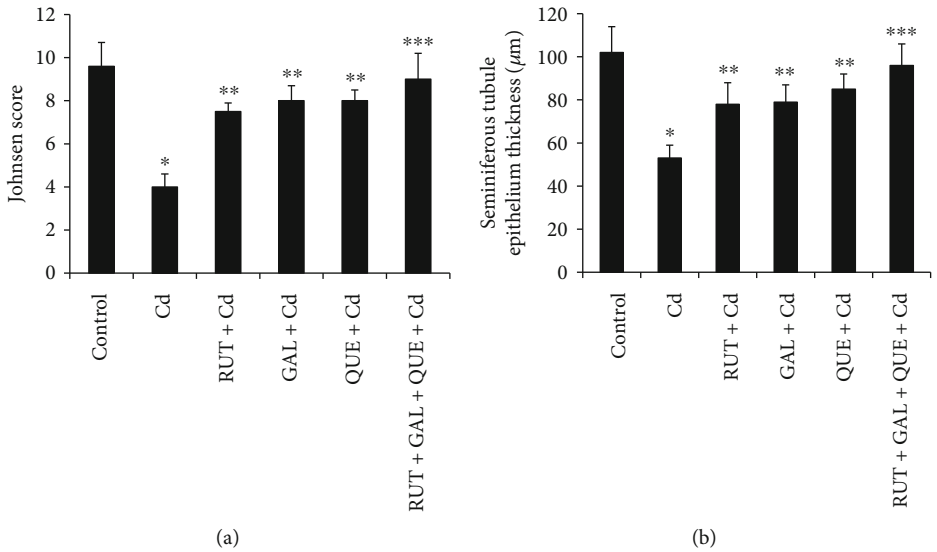

Figure 8: Protective effect of rutin (RUT), gallic acid (GAL), and quercetin (QUE) separately and in combination on the (a) spermatogenesis score index and (b) seminiferous tubule epithelium height (SEH) of the rat's testis after exposure to a single dose of Cd for 40 days. Johnsen score = *control vs. Cd ($P < 0.0001$), **significantly different from Cd group ($P < 0.0001$), ***RUT+Cd vs. RUT+GAL+QUE+Cd ($P = 0.0186$). SEH = *control vs. Cd ($P < 0.0001$), **Cd vs. RUT+Cd ($P = 0.0026$), **Cd vs. GAL+Cd ($P = 0.0017$), **Cd vs. QUE+Cd ($P = 0.0001$), and ***RUT+Cd vs. RUT+GAL+QUE+Cd ($P = 0.0451$).

combination was a better treatment strategy than a single treatment regimen for lowering DNA injury due to oxidative stress. It is assumed that phenolics through their catechol and hydroxyl groups could bind to metals to inactivate them in order to achieve this synergistic effect on the recovery of DNA integrity [47, 48].

Cd exposure was also found to increase the activity of GST in conformity with the report of Mouro *et al.* [49] confirming the participation of GST in the inactivation of Cd [50]. From this present study, the separate treatment with the phenolics recovered GST activity, but the combination of the phenolics (RUT+GAL+QUE) showed a greater potency. Glutathione reductase is a component of the GSH redox system which serves to maintain GSH testicular concentration in the reduced form [51]. In the present study, Cd exposure was found to decrease GR activity in corroboration with the reports of Shukla *et al. [53]*. It appears that the decline of GR activity was compensated by the elevated GST activity in order to maintain the tissue GSH level in the experimental model system despite the increase in lipid peroxidation [32, 53]. This sort of event happens when GSH concentrations were kept at stability between synthesis and exhaustion [51] and/or when exposure happens with a low dose of the toxicant [51]. To support this assumption, we did not detect the testicular level of Cd after 40 days of a single oral Cd dose (24 mg/kg b.w). In studies where these changes happen (e.g., changes in tissue GSH), the animals were exposed to Cd at a daily dose that was smaller than was used in the current study, and the duration of exposure was for a longer time [4–6, 51, 54]. In the present study, Cd was administered as a single oral dose and the posttreatment effect was evaluated after 40 days during which Cd was undetected in the testes of the rats. This could have been a contributory factor for the unchanged GSH testicular concentration. It is therefore rational to assume that the unchanged GSH concentration was responsible for the unaltered GSH-Px activity [51, 52]. It must also be stated that discrepancies in Cd toxicity studies on antioxidant systems are dependent on multiple factors including experimental design and/or condition of the study such as Cd dose used, Cd concentration detected, and when tissues were harvested, e.g., posttreatment [6, 16]. The GSH system constitutes a surveillance system in response to stimuli that induce oxidative stress in many mammalian model systems and is therefore a reliable marker for studying Cd-induced oxidative injury [16, 51]. Single and concurrent administration of the phenolics increased GR and decreased GST activities toward the control values suggesting their antioxidant protective effects on the GSH redox system.

Concerning the data on testicular MDA concentration, rutin cotreatment (RUT+Cd) had a better outcome than the other treatment regimens and was found to even decrease testicular MDA concentrations below the basal level. This confirms the antilipoperoxidative effect of RUT as previously described by Russo *et al.* [55]. We must point out that, though rutin and quercetin belong to the same flavonoid class, they may not always exert similar biological effects in normal and diseased states. For instance, there are studies where rutin exerted anti-inflammatory activity whereas quercetin was either not effective against or worsened the inflammatory response [56] and where rutin exerted stronger protection against nitrosative stress but weaker antioxidant and anti-inflammatory activities than quercetin [57]. In fact, the pharmacological effect of rutin under pathological states may differ from those of quercetin and gallic acid as a result of the rutinoside moiety which is crucial for some of its pharmacological effects and might influence its antioxidant effect [57]. However, other possibilities could also exist where phenolics could antagonize their anti-lipoperoxidative effects during combined administration [58]. To support this speculation, nitrite concentration that was elevated in the quercetin cotreated group (QUE +Cd) was found to drop to the basal level during concurrent administration of the phenolics (QUE+RUT+GAL+Cd). This further supports the hypothesis that phenolics administered concurrently could antagonize their anti-lipoperoxidative effects *in vivo* [59]. Additionally, the hydroxyl groups in multiple locations in the phenolics can interact with themselves or with other compounds and exhibit many effects including antagonistic, additive, or synergistic effects [58]. We recently observed antagonistic effects when rutin was coadministered with kolaviron, a potent biflavonoid with a rich antioxidant effect [28]. These antagonistic interactions were observed to lead to changes in their antioxidant capacities [58, 60, 61]. Additionally, phenolic acid mixtures containing gallic acid and other antioxidants exhibit differences in their reaction kinetics and could be another factor for the antioxidant antagonism observed in the present study when the phenolics were administered concurrently [62]. Therefore, understanding how phenolics with antioxidant effects work together *in vivo* will improve the understanding of their application in the development of antioxidant cocktail regimens for the treatment of biological model systems of testicular injury (preclinical and clinical) under pathological states that are associated with oxidative injury.

Histological changes and androgenic effects of Cd are established as early indicators of the immediate effect of Cd on the male gonads of rats [3]. Low testosterone concentration observed after Cd exposure in the present study corroborated the findings of previous studies and was explained by the fact that Cd suppresses the capacity of Leydig cells to secrete testosterone [63, 64]. The histological features that were observed in the testes of these rats included small-size tubules, decreased epithelium density, germ cell hypoplasia, and sloughing of the epithelium. Many other studies have also confirmed these abnormal features in rats [3, 10, 30, 32, 65] and reflect the fact that in our experimental model, Cd indeed caused testicular injury.

It is believed that increased MDA concentrations are associated with testosterone deficiency, DNA damage, and elevated reactive oxygen species formation in gonadal tissues that are under various pathological states and could be responsible for the impaired sperm quality associated with Cd effects on the testes and spermatogenesis [31, 66–70]. Interestingly, cotreatment with the phenolics both singly or concurrently could reverse Cd-induced decline in testosterone levels and improve the morphology of the testis. Although concurrent administration of the phenolics (RUT +GAL+QUE) was more potent than their separate effects on rescuing testosterone level, the morphology of the testis of the QUE+Cd-treated animals that showed few vacuolar spaces were better after concurrent administration of the phenolics, confirming that quercetin administration after

Cd exposure has a weaker protective effect on the histology of the rats' testes [27] that was improved after cotreatment with the phenolics. Because some phenolic chemicals exhibit androgenic effects, their intake could improve reproductive hormones in both preclinical and clinical experimental model systems [71]. This has been found to be true in several model systems because phenolics with chemical structures that resemble cholesterol and other steroids could alter the synthesis and secretion of androgens by Leydig cells [71]. For instance, naturally occurring flavonoids, e.g., quercetin and rutin, that have a 5,7-dihydroxychromen-4-one skeletal backbone appear to raise the entry of substrate-producing steroids into the mitochondria, contributing to elevated androgen production [71]. Furthermore, several studies including those established in our laboratories have also found that intake of quercetin, rutin, and gallic acid could also increase testosterone levels in rats under various diseased states [16, 18, 54, 72, 73] thereby supporting our present findings. It is therefore a plausible assumption that where the intake of individual phenolic chemicals could partially reverse a chemically induced decline in testosterone levels, a multimodal treatment regimen approach could offer better stimulatory effects on the testosterone level and recovery of the histology of the testes in both animals and humans [16, 27, 71].

The inverse relationship between endogenous Fe and exogenous Cd administration has been established in most studies involving animals and humans [3, 74, 75]. It is believed that high Cd accumulation in tissues tends to increase the elimination of Fe and vice versa [44, 74]. However, the manner via which Fe-dependent metabolism regulates Cd tissue accumulation and toxicity still remains unclear [3, 76]. Because a single oral Cd dose of $24\,mg\,kg^{-1}$ b.w. during a 40-day period was insufficient to be accumulated in the testis [5, 76], it allowed us to assume that the effect of Cd seen in the present study is not due to a direct effect on the testis but indirectly through altering the GSH redox system and testosterone concentration [3]. As a result, the raised tissue level of Fe in the Cd-treated animals was in natural response of the animals to the harmful effect of Cd [74, 75]. Similarly, the low level of Fe that was observed on treatment with the regimen containing rutin either as single or concurrent administration or the high level of Fe on treatment with the regimen containing GAL as single administration confirms the diverse modulatory effects of the phenolics on Fe metabolism which could be responsible for their antioxidant and antilipoperoxidative effects [32]. This appears plausible because gallic acid intake interferes with Fe absorption thereby modulating its availability for oxidative processes [77]. Furthermore, rutin possesses better Fe chelating properties than quercetin [78–80]. Incidentally, rutin cotreatment demonstrated a lower lipoperoxidative effect than the other phenolic groups, suggesting that the low endogenous Fe was the reason for the low lipid peroxidation status in the testes of the animals [80–82].

In conclusion, a multimodal treatment regimen of the tested phenolics is a better treatment strategy than a single treatment approach in a Cd model of testicular injury associated with oxidative damage. Combined administration of the antioxidative phytochemicals protected against Cd-induced testicular oxidative damage by reducing toxic effects on the DNA, raising testosterone concentration and Johnsen scores and recovery of the GSH redox system, and preventing testicular injuries more efficiently than the single treatment regimen of the phenolic chemicals. Although the separate application of the phenolics in the testicular system of a rat model has diverse effects, there also exist the possibilities that their antilipoperoxidative effects *in vivo* could be reduced during their concurrent administration due partly to diminished endogenous Fe concentrations. Therefore, the clinical applicability of these findings in humans will need to be tested in future research.

Data Availability

Data is available on request.

Disclosure

This study has been done as part of the MSc dissertations of Nebeolisa E. Chisom and Chijioke B. Chukwuebuka in the Biochemistry Department, University of Port Harcourt, Choba, Nigeria.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

Investigation, statistical analysis of data, and draft manuscript were worked on by CNE and CCB; investigation, conceptualization, review of the draft manuscript, and supervision and data curation were worked on by SOA; supervision and investigation were worked on by EEO; and supervision, investigation, and editing of the draft manuscript were worked on by SCO.

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