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PAPER

Chlorogenic acid abates oxido-inflammatory and apoptotic responses in the liver and kidney of Tamoxifen-treated rats

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Abstract

Plant-derived phenolics are utilized as chemopreventive agents to abate adverse toxic responses associated with drug-induced damages. Tamoxifen (TAM)—a chemotherapeutic agent—is used in managing all stages of hormone-dependent breast cancer. Notwithstanding TAM's clinical side effect—including hepatic toxicity—its use is commonplace. The present study investigates the effect of Chlorogenic acid (CGA: 25 and 50 mg kg^{-1}; *per os* (*p.o*)) reported to exhibit various beneficial properties, including antioxidative effect against TAM (50 mg/kg; *p.o.*)-induced hepatorenal toxicities in rats treated as follows: Control, CGA, or TAM alone, and rats co-treated with CGA and TAM for 2 weeks. Biomarkers of hepatorenal function, oxido-inflammatory stress, and hepatorenal histopathology were performed. We observed that TAM alone decreased relative organ weights (ROW), marginally impacted rat's survivability, and significantly ($P < 0.05$) increased hepatorenal toxicities and reactive oxygen and nitrogen species (RONS). TAM decreased ($P < 0.05$) antioxidant, anti-inflammatory cytokine (IL-10), besides increase in ($P < 0.05$) lipid peroxidation (LPO), pro-inflammatory cytokines (IL-1β, TNF-α), nitric oxide (NO), xanthine oxidase (XO), myeloperoxidase (MPO), and apoptotic caspases (Casp-3 and -9) levels. These biochemical alterations were accompanied by morphological lesions in experimental rats' liver and kidney. Conversely, that CGA dose-dependently relieved TAM-mediated toxic responses, restored antioxidants capacities, reduced oxidative stress, pro-inflammatory cytokines levels, and Casp-3 and -9 activities in experimental rats. Furthermore, CGA protected against lesions observed in the liver and kidney of rats treated with TAM alone. Overall, CGA blocked TAM-mediated hepatorenal injuries associated with pro-oxidative, inflammatory, and apoptotic mechanisms. CGA may serve as a chemoprotective agent boosting patients prognosis undergoing TAM chemotherapy.

Key words: Tamoxifen, chlorogenic acid, hepatorenal toxicity, oxido-inflammatory stress, antioxidant, apoptosis

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Introduction

Tamoxifen (Z-1-[4-(2-dimethylaminoethoxy)-phenyl]-1,2-diphenyl-1-butene; TAM) is used as a gold standard adjuvant treatment for both early and advanced breast cancers [1]. TAM is biotransformed by several drug-metabolizing enzymes including cytochrome P450 isoforms -MFO1, CYP3A, CYP2D6, CYP3A4; hydroxysteroid sulfotransferase subfamily enzymes in the liver into tamoxifen-*N*-oxide, *N*-desmethyl-tamoxifen, 4OH-tamoxifen, 4OH-N-desmethyl tamoxifen, α-hydroxylation products, and O-sulfonation [2]. TAM's primary mechanism of action is thought to be through the inhibition of estradiol binding at the ligand-binding domain of the estrogen receptor alpha (ERα) and inducing conformational changes that block the interaction of estrogen receptor with co-activator proteins [2, 3]. TAM also modulates signaling proteins including protein kinase C (PKC), mitogen-activated protein kinases (MAPK), proto-oncogene c-myc, c-jun N-terminal kinase (JNK), and induces the expression of pro-apoptotic proteins (Bax and Bak). Moreover, TAM increases mitochondrial permeability transition and the release of cytochrome c from the inner mitochondrial membrane, thereby resulting in apoptotic cell death [4–6]. With the widespread use of TAM to manage breast cancer, attention has been drawn to its deleterious effects on liver and kidney function. TAM-induced hepatic toxicities, including massive steatosis, multifocal fatty infiltration, fibrosis, cirrhosis, and necrosis [7–9], have been reported in human and rat models. In the kidney, TAM has been reported to cause grade II renal toxicity [10], and aberration in Wnt/β-catenin signaling [11] in mice model, thus leading to derangement of renal functions [12]. Hepatic and renal toxicities associated with TAM therapy is believed to be associated with the formation of reactive oxygen and nitrogen species (RONS) mediated oxidative stress. RONS, including superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), hypochlorous acid (HOCl), nitric oxide (NO), and peroxynitrite ($ONOO^-$) [3, 13], are capable of reacting with the cellular DNA, proteins, and lipids to form DNA-adducts, protein crosslink, and lipid peroxidation products [14, 15] in target organs. Consequently, these reactions create a permissive inflammatory environment, cell dysfunction/death, and impair the integrity and functionality of the liver and kidney cells.

Chlorogenic acid (5-O-caffeoylquinic acid, CGA) is a polyphenolic compound widely distributed in foods and herbs [16–18]. CGA's pharmacological potentials are widely documented in the pharmacopoeia, including antioxidant, anti-inflammatory, antilipidemic, anti-cancer, anti-diabetic, antihypertensive, and anti-neurodegenerative activities [19–25].

Our current interest is to minimize the side effects of TAM by supplementation with plant-derived phytochemicals in the management of breast cancer more effectively. Toward this end, the present study was carried out to investigate the effect of CGA against TAM-induced hepatorenal toxicities in albino Wistar rats. We evaluated the effects of TAM and co-administration of TAM and CGA on liver and kidney function, oxidative stress biomarkers, antioxidant enzyme activities, inflammatory and apoptotic mediator levels. We further explored histopathological alterations in experimental rats' liver and kidney to gain further mechanistic insight of the ameliorative effects of CGA on TAM-mediated hepatorenal toxicities. We observed that CGA impeded TAM-mediated liver and kidney injuries associated with pro-oxidative, inflammatory, and apoptotic mechanisms.

Materials and Methods

Chemicals

TAM, CGA, epinephrine, glutathione (GSH), thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), Griess reagent, 1-chloro-2, 4-dinitrobenzene (CDNB), xanthine, trichloroacetic acid, and bovine serum albumin (BSA) were purchased from Sigma (St Louis, Missouri, USA). Monosodium dihydrogen phosphate, disodium hydrogen phosphate, sodium carbonate, sodium hydroxide, copper sulfate, potassium iodide, sodium–potassium tartrate, and sodium chloride were obtained from BDH Ltd. (Poole, Dorset, UK) and William Hopkins Ltd. (Birmingham, UK). Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Lactate dehydrogenase (LDH), and gamma-glutamyl transferase (GGT) kits were obtained from Randox™ Laboratories Ltd. (Ardmore, Crumlin, Co., Antrim, UK). Enzyme-Linked Immunosorbent Assay (ELISA) kits for the assessment of Interleukin-1β (IL-1β), interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF-α), caspase-9 (Casp-9), and Caspase-3 (Casp-3) were obtained from Elabscience Biotechnology Company (Beijing, China). All other chemicals used for these experiments are of analytical grade.

Animal model

Healthy albino Wistar rats of 9 weeks old (sex: female; 204 ± 12 g, n = 50) were obtained from the Experimental Laboratory animal house, Faculty of Basic Medical Sciences, University of Ibadan. Experimental rats were housed in natural photoperiod (12/12 h light–dark) conditions in a well-ventilated rodent facility at the Department of Biochemistry. The rats were provided with rat pellets (Ladokun™ Feeds, Ibadan, Nigeria) and allowed free access to water. Rats were allowed to adapt (7 days) to their new environment preceding experimentation and adequately cared for as specified by 'Guide for the Care and Use of Laboratory Animals' published by the National Institute of Health. Also, experiments were performed following the University of Ibadan Ethical Use of Animal Committee's approval and following the United States National Academy of Sciences guidelines.

Experimental protocol

Experimental rats were randomly divided into five treatment cohorts (n = 10) after acclimatization, and were treated at random phases of the estrous cycle. The doses of TAM (50 mg/kg) and CGA (25 and 50 mg/kg) used in the current study were selected based on previously published data [26–28]. Different stock solutions of TAM (50 mg/mL) and CGA (50 mg/mL) were prepared freshly every other day and used in dosing the experimental rats by gavage or *per os* (*p.o.*). The gavage volumes for CGA (25 and 50 mg/kg) and TAM (25 or 50 mg/kg) were 100, 200, and 200 µl from the specific stock solution respectively. Cohorts of rats treated with TAM and CGA were treated separately using the appropriate volumes of TAM and CGA from the different stock solutions within 30 min of each other. The rats were dosed first with TAM before CGA in the specific treatment cohorts for 14 consecutive days as follows:

Group I: Control- administered corn oil-vehicle-(2 ml/kg; *p.o*) alone.

Group II: Treated with CGA dissolved in corn oil (50 mg/kg; *p.o*) alone.

Group III: Treated with TAM (50 mg/kg; *p.o.*)

Group IV: TAM + CGA1: Treated with (TAM: 50 mg/kg) and (CGA: 25 mg/kg; *p.o.*).

Group V: (TAM + CGA2): Treated with (TAM: 50 mg/kg) and (CGA: 50 mg/kg; *p.o.*).

Following the last treatment, on Day 15, the rats were weighed. Blood was collected from the retro-orbital venous plexus into plain pre-labeled sample bottles, before sacrificing by cervical dislocation. Subsequently, the clotted blood was centrifuged at 3000 g for 10 min at 4°C to obtain the serum. The liver and kidney were immediately excised, weighed, and processed for biochemical and histological analyses. The serum samples were stored (−20°C) until required for specific biochemical analysis.

Estimation of liver and kidney function indices

Analysis of serum activities of AST, ALT, ALP, GGT, and LDH and creatinine and urea levels in control, TAM-, and CGA-treated rats were performed using commercial kits from Randox™ Laboratories Limited (UK).

Assessment of oxidative stress indices

The liver and kidney samples from control, TAM-, and CGA-treated rats were homogenized in phosphate buffer (0.05 M, pH 7.4). The resultant tissue homogenates were then centrifuged at 12 000 g for 15 min at 4°C to obtain a clear supernatant, which was used to assess antioxidant, oxidative stress, inflammation, and apoptotic biomarkers. Hepatic and renal protein concentrations were evaluated according to the method of Lowry *et al.* [28]; total sulfhydryl group was assayed at 412 nm in line with the process of Ellman [29]; reduced GSH was determined at 412 nm according to the method described by Jollow *et al.* [30]; GST was assayed at 340 nm by the process of Habig *et al.* [31]; GPx activity was determined at 412 nm according to the method of Rotruck *et al.* [32]; SOD activity was determined at 480 nm by the method described by Misra and Fridovich [33]; CAT activity was determined at 240 nm using H_2O_2 as a substrate according to the method of Clairborne [34]; Xanthine oxidase was quantified at 290 nm by the method of Bergmeyer *et al.* [35]; and lipid peroxidation marker was quantified as malondialdehyde (MDA) at 532 nm according to the method described by Buege and Aust [36], and expressed as µmol MDA/mg protein.

Assessment of RONS

The levels of hepatic and renal RONS in control, TAM-, and CGA-treated rat samples were evaluated according to an established protocol, which is based on the RONS-dependent oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to dichlorofluorescein (DCF), according to Owumi and Dim [37]. Briefly, the reaction mixture (150 µl 0.1 M potassium phosphate buffer; 35 µl distilled water, 10 µl sample, and 5 µl freshly prepared DCFH-DA) was constituted with minimal exposure to air. Fluorescence emission of DCF produced from DCFH-DA oxidation was spectrophotometrically analyzed (wavelengths: 488 nm excitation; 525 nm emission) for 10 min at 30 s intervals using a Spectra Max 384 multimodal plate reader (Molecular Devices). DCF produced was expressed as a percentage over control.

Assessment of pro-inflammatory biomarkers and caspase-9 and caspase-3 activities

The level of hepatic and renal nitric oxide (NO) in control, TAM-, and CGA-treated rats was assessed using Griess reagent according to the established protocol of Green *et al.* [38]. Briefly, the reaction mixture consisting of an equal volume of sample and Griess reagent was incubated for 15 min before the absorbance was evaluated at 540 nm. The level of NO was extrapolated from the standard curve and then expressed as Units/mg protein. Moreover, myeloperoxidase (MPO) activity was evaluated according to the method described by Granell *et al.* [39]. Additionally, IL-1β, IL-10, TNF-α levels, as well as caspase-3 and caspase-9 activities were evaluated using commercially available ELISA Kits (Elabscience Biotechnology Company, Beijing, China) with the aid of a SpectraMax™ plate reader (Molecular Devices, CA, USA) as stated in the manufacturer's manual.

Histopathology

The liver and kidney samples were fixed using 10% phosphate-buffered formalin for 3 days and then embedded in paraffin after dehydration. Microtome cut tissue sections (4–5 µm) and fixed on charged microscopic glass slides were subsequently stained with hematoxylin and eosin (H & E) [40]. The tissue histology slides were blinded to a pathologist who examined them under a light microscope (Leica DM 500, Germany). The histopathological aberrations were scored semiquantitatively following established methods and as previously reported [41–44]: None (0); mild (1); mild–moderate (2); moderate (3); moderate to severe (4); and severe (5) [45]. Representative images were captured with a digital camera (Leica ICC50 E, Germany) attached to the microscope.

Statistical analysis

Data were analyzed by the one-way analysis of variance (ANOVA) and post hoc Tukey test (GraphPad Prism 5 Software, La Jolla, California, USA, www.graphpad.com) was used to ascertain significant differences in the treatment groups. *P* values < 0.05 were considered significant. The results were subsequently expressed as mean+/− standard deviation (SD).

Results

Effect of CGA on survivability and body and relative organ weight of TAM-treated rats

The survivability (Kaplan–Meyers) results, mean body weight, and relative liver and kidney weights are presented in Fig. 1 and Table 1. There is no statistical difference between initial and final body weight in control, CGA, TAM + CGA1, and TAM + CGA2 groups (Table 1). Compared to TAM alone, groups that received TAM + CGA (25 and 50 mg/kg) revealed slight alterations in final mean body weight. Cohorts of rat treated with TAM only, TAM + CGA1, and TAM + CGA2 exhibited weight losses of −11.39, −9.30, and − 4.30 g, respectively, indicating the protective role of CGA again TAM-induced alteration in body weight. There was no significant difference in the mean liver and kidney weight and the percentage relative liver and kidney weights.

Table 1: Effect of CGA on body weight change, relative liver and kidney weights of rats following treatment with TAM for 14 consecutive days

	Control	CGA	TAM	TAM + CGA1	TAM + CGA2
Initial body weight (IBW; g)	197.50 ± 18.45	203.90 ± 13.14	202.50 ± 18.45	201.30 ± 22.37	203.80 ± 8.50
Final body weight (FBW; g)	205.50 ± 20.61	210.50 ± 14.80	179.44 ± 19.91*	192.00 ± 31.34	199.50 ± 16.06
Liver weight (g)	5.67 ± 0.72	6.19 ± 0.81	5.71 ± 0.34	5.97 ± 1.12	5.45 ± 0.69
Kidney weight (g)	1.23 ± 0.17	1.21 ± 0.10	1.17 ± 0.15	1.19 ± 0.21	0.16 ± 0.07
Relative Liver weight (%)	2.75 ± 0.12	3.18 ± 0.25	3.20 ± 0.21	2.99 ± 0.15	2.89 ± 0.51
Relative Kidney weight (%)	0.60 ± 0.04	0.65 ± 0.04	0.65 ± 0.04	0.60 ± 0.03	0.61 ± 0.02

Values are expressed as mean ± SD of five rats per experimental group. *$P < 0.05$: IBW versus FBW. Chlorogenic acid (CGA); Tamoxifen (TAM).

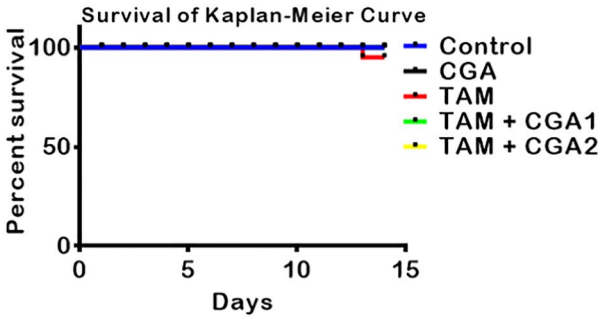

Figure 1: Experimental scheme and the effect of CGA on percentage rat's survival of rats treated with TAM for 2 weeks. Tamoxifen -TAM: 50 mg/kg; Chlorogenic acid -CGA: 25 and 50 mg/kg; TAM + CGA1, (50 + 25) mg/kg; TAM + CGA2, (50 + 50) mg/kg.

CGA abates hepatic and renal toxicities in TAM-treated rats

The effect of CGA on biomarkers of liver and kidney toxicities in the serum of rat cohorts treated with TAM for 14 consecutive days is presented in Fig. 2. Relative to the control, TAM-treated rats exhibited significant ($P < 0.05$) increase in the activities of ALT, ALP, LDH, GGT, and AST-biomarkers of liver function, as well as creatinine and urea-kidney function biomarkers levels. In contrast, rat cohorts co-treated with TAM + CGA at 25 and 50 mg/kg exhibited decreases ($P < 0.05$) in the serum of biomarkers of hepatorenal dysfunction compared to the rats treated with TAM alone. The protective effect of CGA is dose dependent— CGA at 50 mg/kg protected more than CGA at 25 mg/kg (CGA2 > CGA1) on hepatorenal toxicity rats caused by TAM only treatment.

CGA abates TAM-induced hepatic and renal oxidative damage in rats

The influence of CGA on the antioxidant status, oxidative and nitrosative stress by TAM-induced toxicities in rats' liver and kidney is presented in Figs 3–5. TAM only markedly decreased ($P < 0.05$) liver and kidney activities of GPx, GST, and GSH (Fig. 3). CGA—at 25 and 50 mg/kg; co-treatment with TAM markedly reverse ($P < 0.05$) the observed changes by increasing the quantities of GPx, GST, and GSH dose-dependently (CGA2 > CGA1). TAM only caused a similar decrease ($P < 0.05$) in liver and kidney activities of CAT and SOD as well as the TSH levels (Fig. 4) relative to control. SOD and CAT activities and TSH level increased ($P < 0.05$) in rat cohorts co-treated with CGA + TAM compared to TAM alone in a dose-dependent manner, except in the kidney where SOD activities and TSH level were not significantly affected at CGA1. Additionally, TAM treatment alone increased ($P < 0.05$) the levels of LPO and RONS, and XO activity in the liver and kidney of rats compared to the control (Fig. 5). The observed increases of prooxidant elements of LPO, RONS, and XO in the liver and kidney of rat cohorts treated with TAM alone were reduced ($P < 0.05$) in rat following CGA + TAM co-treatment, except in the liver where XO activities were not significantly affected at CGA1.

CGA suppresses TAM-mediated increase in biomarkers of inflammation and apoptosis in the liver and kidney tissues of rats

The impact of CGA on pro-inflammatory, anti-inflammatory, and apoptotic biomarkers in the liver and kidney of rats treated with TAM is presented in Figs 6–8. Treatment with TAM alone caused increase ($P < 0.05$) in the kidney and liver activity of MPO and NO level compared to the control rats (Fig. 6). Co-treatment with CGA + TAM reversed the MPO and NO status of rats' liver and kidney relative to TAM only treated rat cohorts. IL-β and TNF-α level in rats' liver and kidney treated with TAM only also increased ($P < 0.05$) with concurrent lessening of IL-10 level compared to the control (Fig. 7). Conversely, rat cohorts co-treated with TAM + CGA (at 25 and 50 mg/kg) exhibit reduced ($P < 0.05$) pro-inflammatory—IL-β and TNF-α—biomarker levels with a simultaneous increase in IL-10 level compared to TAM only treated rats. Additionally, TAM only treated rats showed increases ($P < 0.05$) in liver and kidney activities of Casp-9 and Casp-3 (Fig. 8) compared to the control cohort. The observed changes in initiator and executioner caspases' activities were reduced ($P < 0.05$) following CGA + TAM co-treatment relative to rat cohorts treated with TAM alone.

CGA abates hepatorenal lesions in rats treated with TAM

The representative photomicrographs showing the effect of CGA on TAM-induced histological damages in the liver and kidney of experimental rats are presented in Figs 9 and 10; at ×400 (top row) and ×100 (bottom row) magnification. The liver and kidney of control and rats treated with CGA alone appeared normal with well-preserved tissue morphological architecture. The liver of rats treated with TAM alone showed severe disseminated sinusoidal congestion (red arrow) and infiltration of inflammatory mononuclear cells (yellow notched arrow), couples with fibroplasia of the periportal region (blue arrow). The kidney of rats treated with TAM alone showed multiple area of glomerulonecrosis and tubular desquamation (green arrow) and the presence of inflammatory cells (black notched arrow). However, the liver and kidney of rats co-treated with CGA and TAM dose-dependently reduced TAM-mediated alterations in the liver and kidney with visible improvement in the liver and kidney cyto-architecture at CGA1, and histological features were somewhat similar to controls at

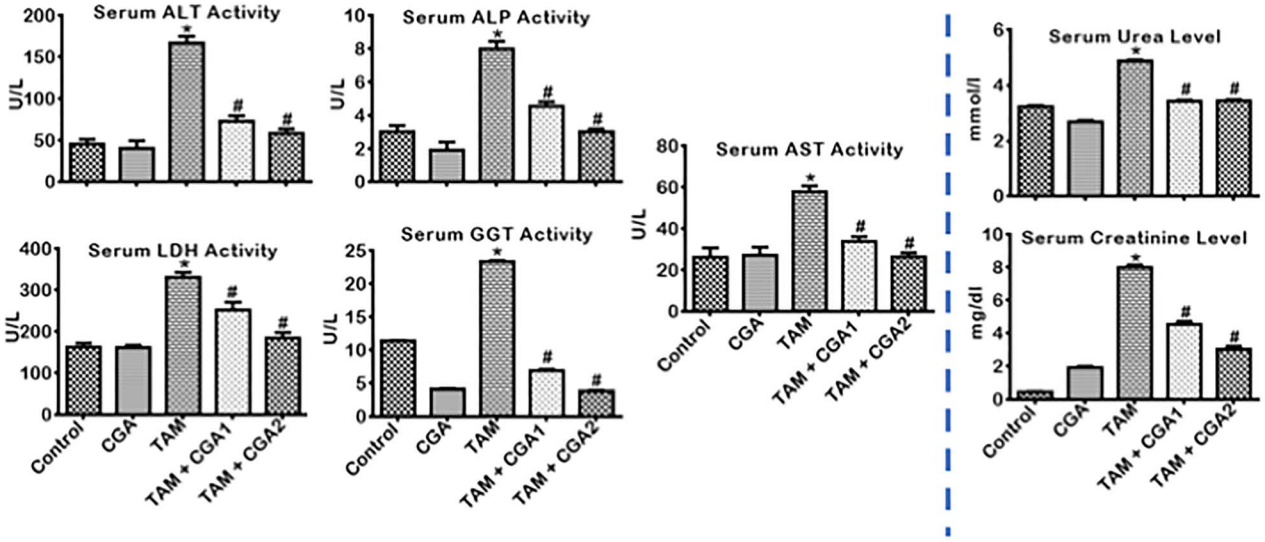

Figure 2: Effect of CGA on biomarkers of hepatic and renal function in rats treated with TAM for 2 weeks. Tamoxifen -TAM: 50 mg/kg; Chlorogenic acid -CGA: 25 and 50 mg/kg; TAM + CGA1, (50 + 25) mg/kg; TAM + CGA2, (50 + 50) mg/kg. Each bar represents mean ± SD of 10 rats. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus TAM only. SD: standard deviation; ALP: alkaline phosphatase; AST: aspartate amino transferase; ALT: alanine amino transferase; GGT: gamma glutamyl transferase; LDH: lactate dehydrogenase.

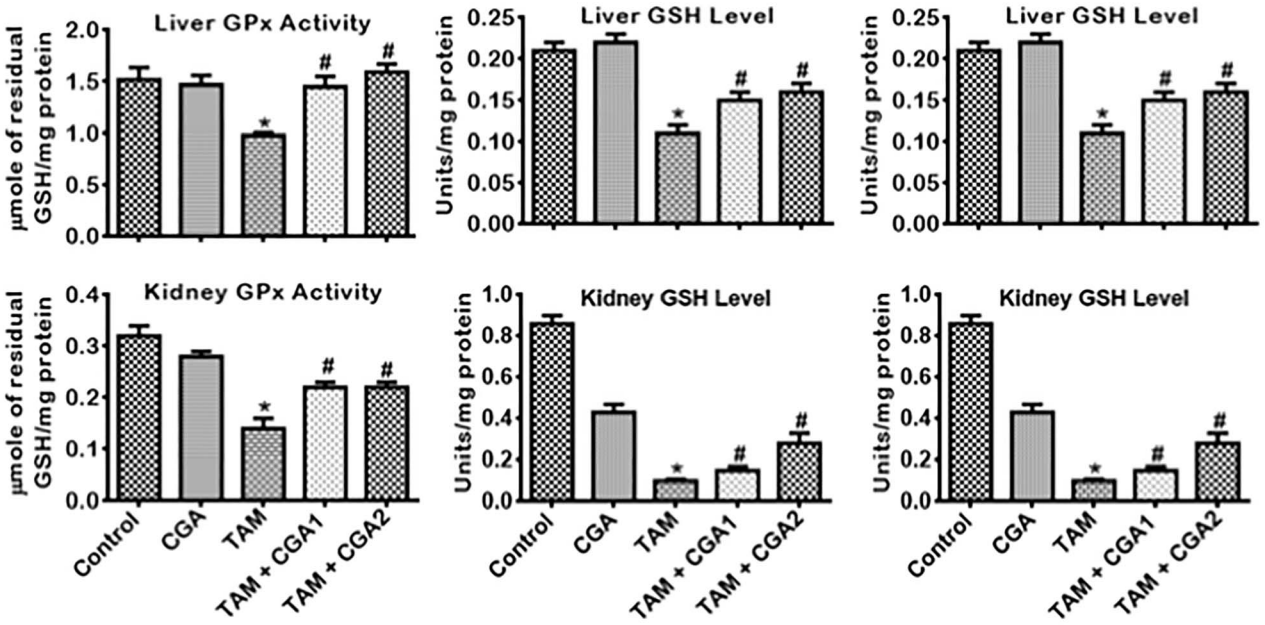

Figure 3: Effect of CGA on GSH-dependent enzyme activities and GSH level in the liver and kidney of rats treated with TAM for 2 weeks. Tamoxifen -TAM: 50 mg/kg; Chlorogenic acid -CGA: 25 and 50 mg/kg; TAM + CGA1, (50 + 25) mg/kg; TAM + CGA2, (50 + 50) mg/kg. Each bar represents mean ± SD of 10 rats. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus TAM only. SD: standard deviation; GPx: glutathione peroxidase; GST: glutathione S-transferase; GSH: glutathione.

higher CGA dose (CGA2), but with mild disseminated congestion in the kidney. Table 2 depicts semiquantitative values of hepatic and renal lesions frequencies identified in experimental rats.

Discussion

TAM is one of the most commonly prescribed chemotherapeutic agents for all stages of hormone-dependent breast malignancy [6]. Despite its several side effects, especially toxicity to the liver [8, 9] and kidney [10, 12] demonstrated in human and rat models, the usage of TAM in breast cancer therapeutic is a common practice in the clinical setting. Recent experimental studies revealed that plant-derived phytochemicals mediate antioxidant, anti-inflammatory, anti-cancer, antiapoptotic effects [19, 20, 22]. Thus, such plant-derived compounds are good natural source of chemopreventive agents against toxic responses from many antineoplastic agents. CGA is a phenolic compound present in several plant species, reported to protect against a wide range of xenobiotic-mediated toxicities, mechanistically by reducing ROS generation, and inflammatory responses. The present study

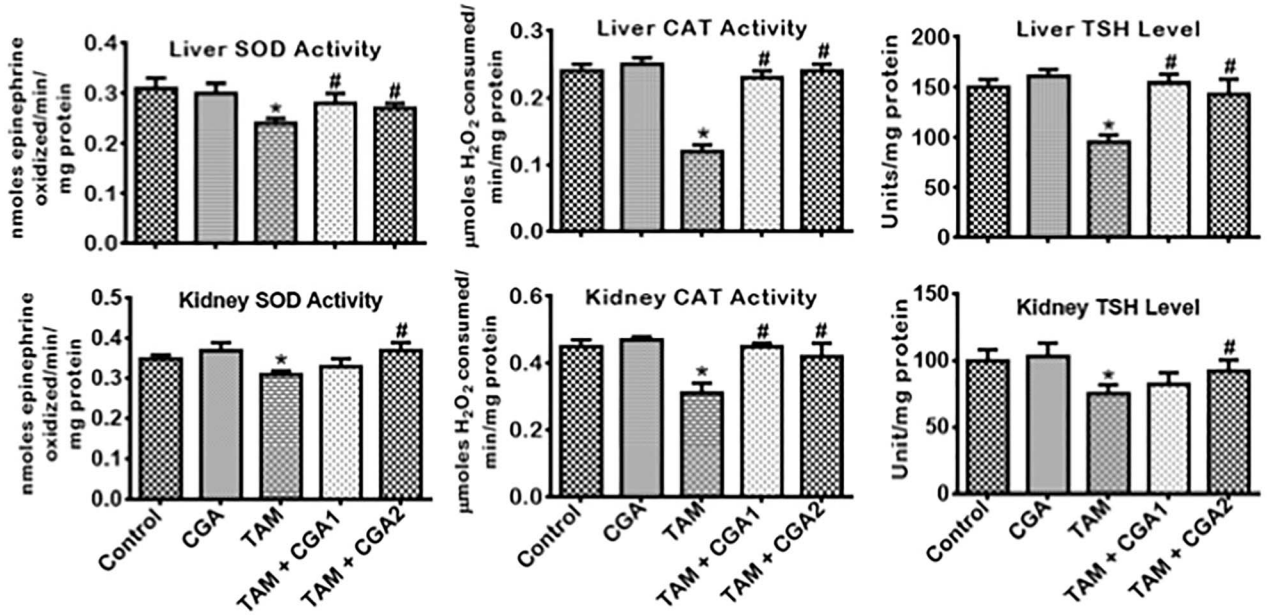

Figure 4: Effect of CGA on SOD and CAT activities and TSH level in the liver and kidney of rats treated with TAM for 2 weeks. Tamoxifen -TAM: 50 mg/kg; Chlorogenic acid -CGA: 25 and 50 mg/kg; TAM + CGA1, (50 + 25) mg/kg; TAM + CGA2, (50 + 50) mg/kg. Each bar represents mean ± SD of 10 rats. * $P < 0.05$ versus control; # $P < 0.05$ versus TAM only. SD: standard deviation; SOD: superoxide dismutase; CAT: catalase; TSH: total sulfhydryl group.

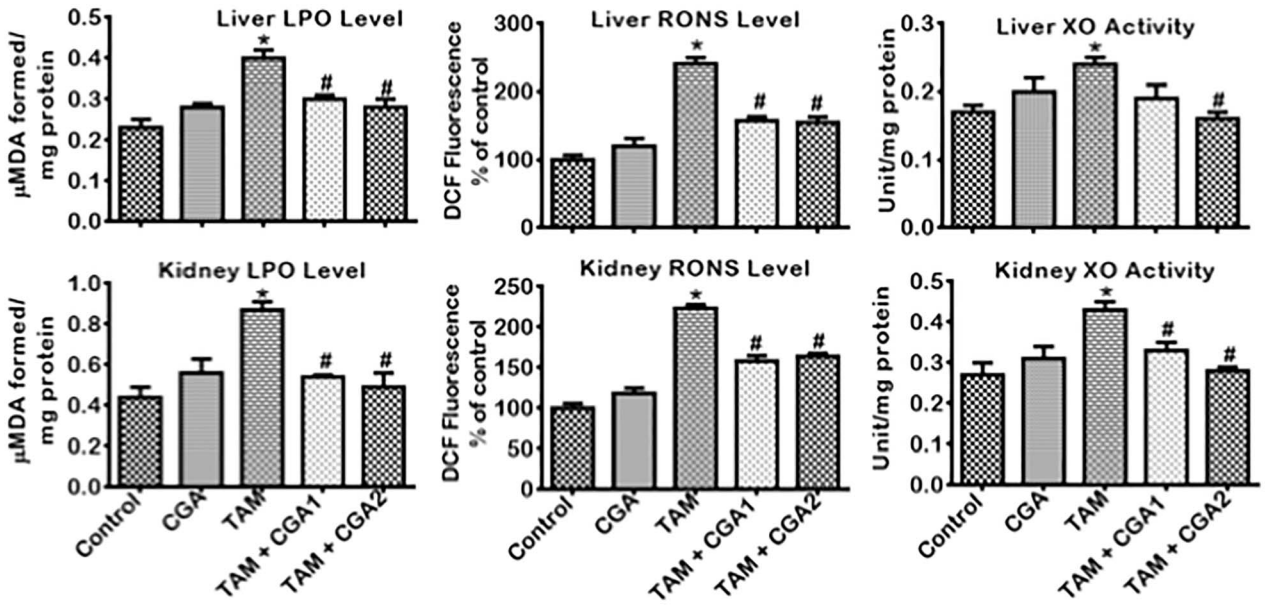

Figure 5: Effect of CGA on the levels of RONS and LPO and the activity of XO in the liver and kidney of rats treated with TAM for 2 weeks. Tamoxifen -TAM: 50 mg/kg; Chlorogenic acid -CGA: 25 and 50 mg/kg; TAM + CGA1, (50 + 25) mg/kg; TAM + CGA2, (50 + 50) mg/kg. Each bar represents mean ± SD of 10 rats. * $P < 0.05$ versus control; # $P < 0.05$ versus TAM only. SD: standard deviation; RONS: reactive oxygen/nitrogen species; LPO: lipid peroxidation; XO: xanthine oxidase.

examined the protective effect of CGA against TAM-induced hepatorenal toxicities in cohorts of rats treated for 2 weeks. Treatment with TAM alone altered mean body weight, and relative liver and kidney weights. Arnold *et al.* [46] and Elsea *et al.* [47] reported that reduction in weight may be brought about by cytokine (IL-6) stimulation, and growth hormones downregulation, and correlated to anorexia nervosa, lethargy, breakdown of body cell mass, and depletion of nitrogen. On the contrary, the co-administration of CGA at different doses abated TAM-induced body weight loss in the experimental rats. This finding may be attributed to CGA-mediated inhibition of the activation and propagation of IL-6 [48, 49].

Liver and kidney function biomarkers are relied upon in the proper diagnosis of hepatotoxicity and nephrotoxicity, and any significant increase in the levels of biomarkers of dysfunction of the liver and kidney correlates to injury to these organs. In this study, we observed that TAM markedly increased the activities of ALT, AST, ALP, LDH, and GGT in the liver of rats and the levels of

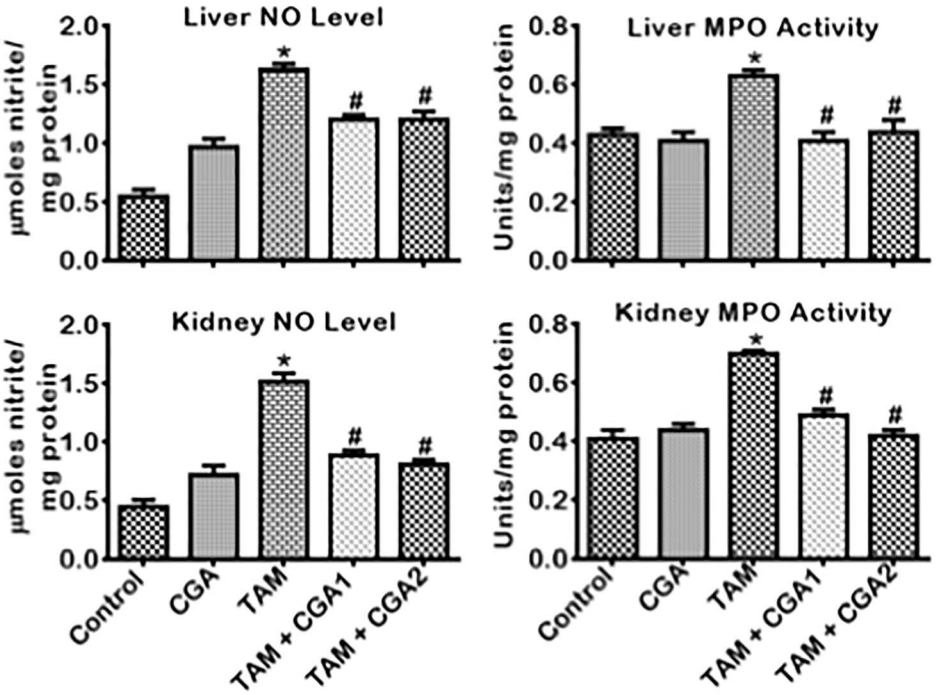

Figure 6: Effect of CGA on NO level and MPO activity in the liver and kidney of rats treated with TAM for 2 weeks. Tamoxifen -TAM: 50 mg/kg; Chlorogenic acid -CGA: 25 and 50 mg/kg; TAM + CGA1, (50 + 25) mg/kg; TAM + CGA2, (50 + 50) mg/kg. Each bar represents mean ± SD of 10 rats. * $P < 0.05$ versus control; $^{\#}P < 0.05$ versus TAM only. SD: standard deviation; NO: nitric oxide; MPO: myeloperoxidase.

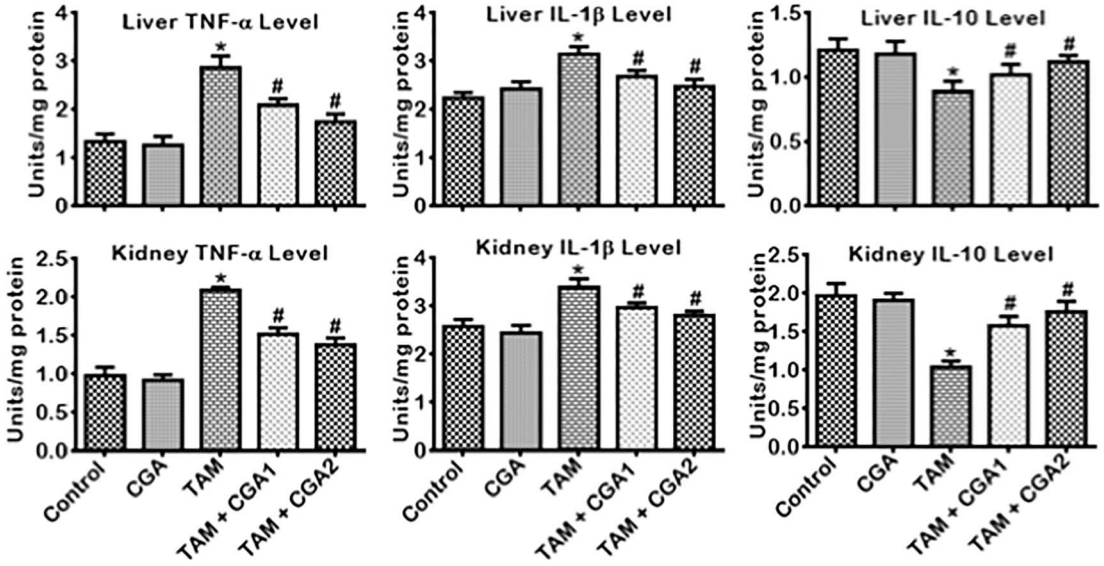

Figure 7: Effect of CGA on TNF-α, IL-1β and IL-10 levels in the liver and kidney of rats treated with TAM for 2 weeks. Tamoxifen -TAM: 50 mg/kg; Chlorogenic acid -CGA: 25 and 50 mg/kg; TAM + CGA1, (50 + 25) mg/kg; TAM + CGA2, (50 + 50) mg/kg. Each bar represents mean ± SD of 10 rats. * $P < 0.05$ versus control; $^{\#}P < 0.05$ versus TAM only. SD: standard deviation; TNF-α: tumor necrosis factor-alpha; IL1β: interleukin-1Beta; IL-10: interleukin-10.

creatinine and urea in the kidneys compared to the control and CGA only. These observations agree with the observation by Gao *et al.* [50], who demonstrated TAM's hepatotoxic potential in mice. ALT is a gold standard for the detection of liver injury. It is located primarily in the liver and, together with AST which is localized in the liver and other organs such as the brain, heart, and skeletal muscles, plays an essential role in the metabolism of amino acid and gluconeogenesis. ALT and AST catalyze the transfer of amino group from alanine and aspartic acid to α-ketoglutarate to yield glutamic acid and pyruvic acid or oxaloacetic acid [51].

ALP, LDH, and GGT are nonspecific biomarkers for the diagnosis of hepatocellular damage. However, elevated levels of these biomarkers in the serum of experimental animals treated with a potent hepatotoxin could serve as a secondary diagnostic marker for liver disease. While ALP is part of a family zinc-metalloenzymes that are highly localized in the

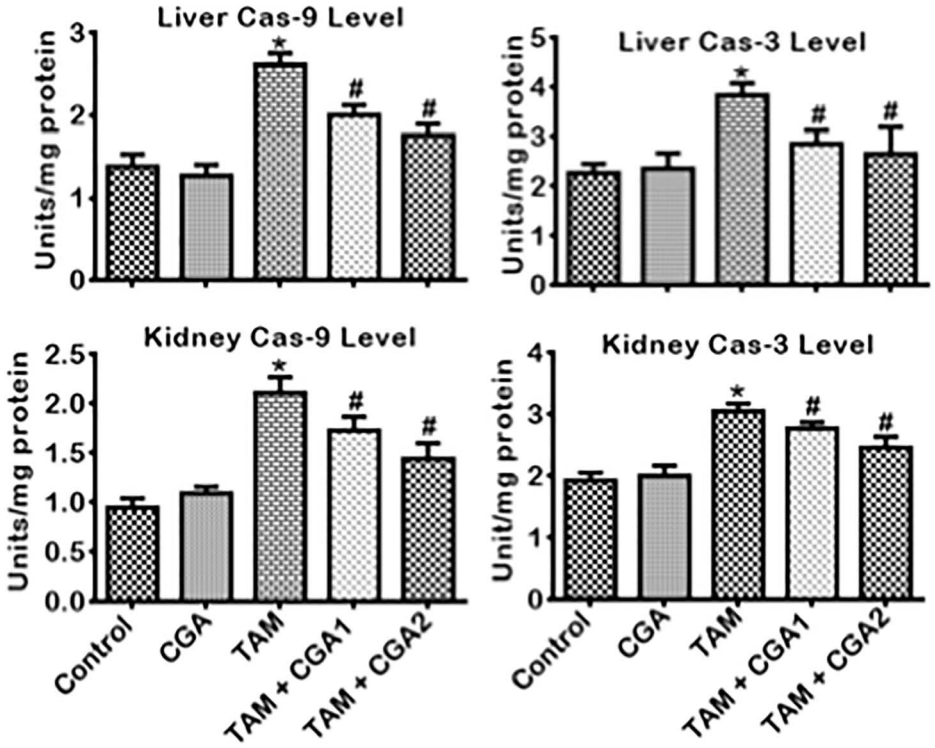

Figure 8: Effect of CGA on Caspase 3 and Caspase 9 activities in the liver and kidney of rats treated with TAM for 2 weeks. Tamoxifen -TAM: 50 mg/kg; Chlorogenic acid -CGA: 25 and 50 mg/kg; TAM + CGA1, (50 + 25) mg/kg; TAM + CGA2, (50 + 50) mg/kg. Each bar represents mean ± SD of 10 rats. * $P < 0.05$ versus control; # $P < 0.05$ versus TAM only. SD: standard deviation.

microvilli of the bile canaliculus as well as several other tissues including bone, intestines, and placenta; GGT is a glycoprotein that catalyzes the transfer of a gamma-glutamyl group from peptides to other amino acids, and is localized on membranes of cells with high secretory or absorptive activities like liver, kidney, pancreas, intestine, and prostate, testicles, spleen, heart, and brain. Significant elevation in these biomarkers is indicative of the cholestatic pattern of hepatic injury [52, 53]. LDH is an intracellular enzyme that catalyzes pyruvate's reversible transformation to lactate with a concurrent generation of NAD^+ to drive the glycolytic pathway and NADH for energy generation anaerobes. It is localized in tissues that utilize glucose energy, and therefore not organ-specific. An elevated level of LDH is an indication of cell necrosis, megaloblastic anemia, shock, and liver diseases [54]. Also, creatinine and urea are byproducts of the cellular breakdown of creatine phosphate in the myocytes, and protein metabolism is used to predict the same integrity and functionality of the kidney of experimental rats. The elevated level of creatinine and urea is indicative of the onset of nephrotoxicity. The diagnostic precision of the two biomarkers differs. Creatinine is a better predictor of renal damage than urea [55] as it provides an accurate measure of glomerular filtration rate [56]. Contrary to the preceding, CGA protected against hepatic and renal injury exemplified by decrease serum levels of hepatic transaminases and urea and creatinine, respectively, since. CGA protective effect averted injury-mediated leakage of transaminases into circulation and the excessive breakdown of creatine phosphate and amino acids in the body dose-dependently. CGA at both doses (25 and 50 mg/kg) tested brought about the reduction in serum urea level, but this reduction in urea level in the presence of TAM was not affected by the doses of CGA administered. The implication of this is that in some instances, the low doses of CGA (25 mg/kg) were not able to avert the effects of TAM, but confers partial protection.

The generation of ROS in the liver and kidney of experimental animals is necessary for normal physiologic and biochemical processes. However, increased ROS production beyond the scavenging potential of endogenous antioxidants has been shown to initiate oxidative stress leading to lipid peroxidation, the formation of DNA adducts, protein crosslinks as well as suppression of endogenous antioxidant levels in the liver and kidney [12, 13]. Compared to the control and CGA alone, TAM caused a significant increase in the levels of LPO and RONS activity with concurrent decrease in CAT, SOD, GPx, and GST activities as well as GSH and TSH levels. Increase in activities and levels of prooxidant elements following TAM administration and bioactivation to 4-hydroxytamoxifen (4-OHTAM) [54–58] results from concurrent production of RONS in the absence of RONS scavengers. Prooxidants also interact with membrane lipids, forming lipid peroxidation products, including malondialdehyde (MDA) and 4-Hydroxy-2-Nonenal (4-HNE) [59, 60]. Moreover, 4-OHTAM may be further biotransformed via P450-mediated oxidation into para-quinone methide, forming a stable adduct with nitrogenous bases of DNA [57]. The formation of DNA adducts by paraquinone methide may result in transition and transversion mutations in the liver and kidney cells. The elevated level of RONS in our study further revealed the oxidative and electrophilic tendency TAM. Owumi and Dim [37] opined that increased levels of RONS in the liver and kidney tissues of rats exposed to foreign compounds are an indication of increased generation of free radical species and suppression of the antioxidant defense system.

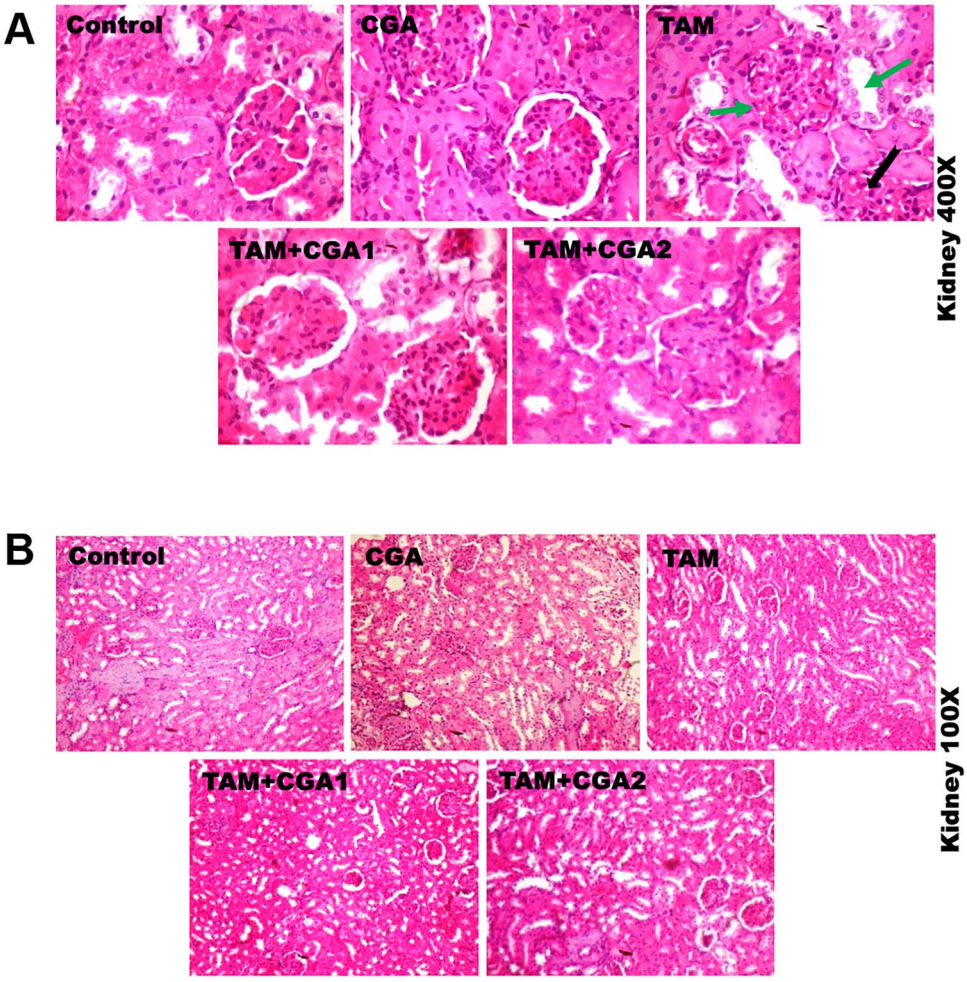

Figure 9: Representative photomicrographs of experimental rat liver. Control rats showed normal liver morphology. Liver of rats treated with TAM alone presenting severe disseminated congestion (red arrow), and infiltration of inflammatory cells (yellow notched arrow), couples with fibroplasia of the periportal region (blue arrow). However, the liver of rats co-treated with CGA + TAM dose-dependently reduced TAM-mediated alterations in the liver to a degree somewhat similar to control rat cohort with visible improvement in the live cyto-architecture at CGA1, and histological features are similar to controls at higher CGA dose (CGA2). H and E stained. Magnification: top row ×400; bottom row ×100.

Another determinant of oxidative and electrophilic stress is XO. XO, together with xanthine dehydrogenase (XDH), forms the structural motif of xanthine oxidoreductase complex (XOR). XO catalyzes the oxidation of hypoxanthine to xanthine and then uric acid in the metabolism of purines with concomitant release of $O_2^{\cdot -}$ and H_2O_2 [61–63] in the liver and kidney cells. To maintain redox balance and tissues homeostasis, SOD, CAT, GPx, GST, GSH, and TSH protect against ROS and RNS. SOD mediates the dismutation of $O_2^{\cdot -}$ into H_2O_2, while CAT and GPx converts H_2O_2 into water and alcohols [64]; GST removes reactive intermediates capable of generating deleterious ROS [65], while GSH and TSH detoxify peroxides with their thiol residues [66, 67]. Treatment with TAM increases XO activity in the liver and kidney of the rats; XO is known to tilt redox balance in favor of prooxidants. The alteration in redox state of the experimental rats was abated by CGA augmentation of rat's antioxidant defenses. CGA mediated reversals in the decreases of antioxidant enzyme, GSH and total thiol did not completely eliminate TAM toxicity nor restore these biomarkers of oxidative health to levels comparable to baseline -control. However, the observed improvement in anti-oxidative stress biomarkers implies that CGA within the limits of this study can mitigate the toxic effect of TAM with promising strategies to further explore to completely eliminate TAM toxicity. Our findings corroborate recent reports demonstrating the protective effect of CGA against lipopolysaccharide-induced hepatic toxicities in rats [68, 68], and sodium arsenite mediated kidney damages in mice [26].

Pro-inflammatory markers and ROS play essential roles in liver and kidney injuries in experimental animals [6, 26]. Typically, a balance between the pro-inflammatory and anti-inflammatory biomarkers is required to maintain organ and also systemic homeostasis in the liver and kidney of healthy individuals [69–71]. An imbalance in pro- and anti-inflammatory biomarkers can trigger toxic responses in the liver and kidney [72, 73] from an increase in pro-inflammation and decrease in anti-inflammatory mediators. TAM mediates a negative shift in the balance between the pro-inflammatory and the anti-inflammatory biomarkers. Pro-inflammatory biomarkers—NO, MPO, IL-1β, and TNF-α—were significantly increased by TAM, with a concomitant reduction in the level of IL-10 compared to the control and CGA alone treated groups. This imbalance is attributable to TAM-mediated toxicity. It can trigger the expression of the Toll-like receptors (TLRs) among other patterns of recognition receptors in the cells of the liver and kidney [74, 75],

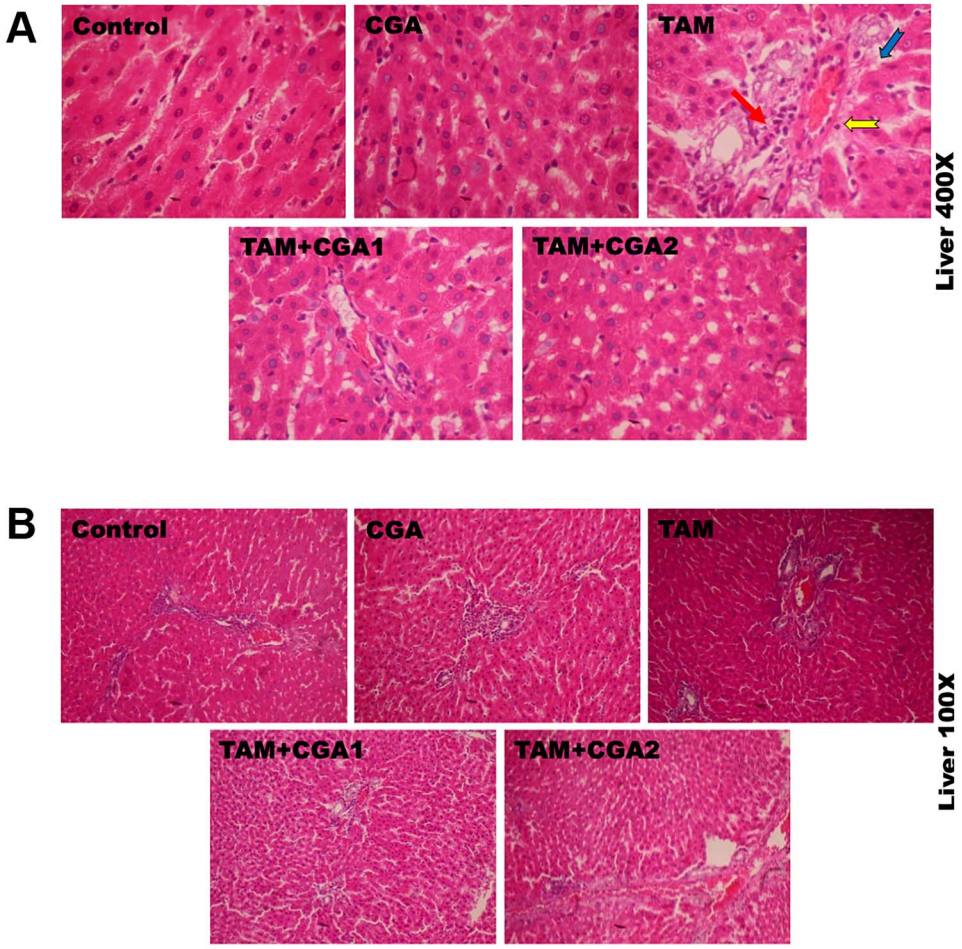

Figure 10: Representative photomicrographs of experimental rat kidney. Control rats showed normal kidney morphology. Kidney of rats treated with TAM alone showing glomerulonecrosis and tubular desquamation (green arrow), and presence of inflammatory cells (black notched arrow). The kidney of rats co-treated with CGA + TAM dose-dependently abated TAM-mediated injuries in the kidney with visible improvement in the kidney cyto-architecture at CGA1, and histological features were somewhat similar to controls at higher CGA dose (CGA2), except with mild disseminated congestion in the kidney. H and E stained, magnification: top row ×400; bottom row ×100.

Table 2: Frequency of histological alterations identified in the liver and kidney of rats treated with CGA and TAM for 14 consecutive days

Parameters	Control	TAM alone	CGA alone	TAM + CGA1	TAM + CGA2
Liver					
Congestion of vessels	0	3	0	2	1
Focal area of inflammation	0	4	0	2	1
Fatty infiltration	0	4	0	1	1
Kidney					
Large vacuoles	0	3	0	2	1
Focal area of necrosis	0	4	0	1	1
Inflammatory cells	0	4	0	1	1
Hepatic degeneration	0	4	0	2	1

Chlorogenic acid -CGA: 25 and 50 mg/kg; Tamoxifen -TAM: 50 mg/kg n = 10. Values are indicative of the severity of histological changes observed in their tissues. Scores: 0—within normal limit; 1—change barely exceed that within normal limit; 2—easily identified lesion with limited severity; 3—prominent lesions with potential for severity; 4—complete severe changes as possible in organ.

consequently activating specific transcription factors including mitogen-activating protein (MAP) kinases, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and further production of pro-inflammatory cytokines such as IL-1β and TNF-α capable of mediating acute and chronic liver disorder [76, 77].

Also, IRF5, MAPK, and NF-κB can trigger the inducible nitric oxide synthase (iNOS) activation to produce NO in the liver [15, 78] and kidney [79, 80]. Uncontrolled generation of NO at high levels will lead to the production of numerous RNS, which can mediate a wide range of physiological and pathological effects in the liver and kidney [81]. Additionally, high levels of NO can

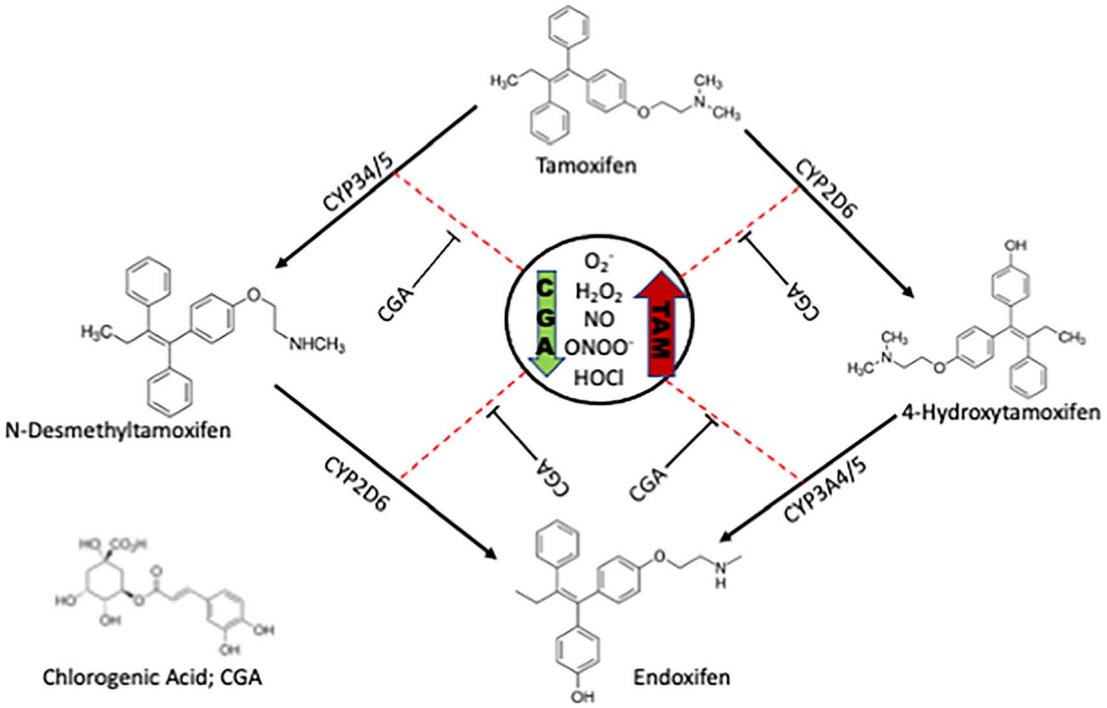

Figure 11: Plausible mechanisms of CGA-mediated amelioration of TAM-induced injury in rats' hepatorenal system. Note that Cytochrome P450's (CYP's), known to biotransform TAM to various isoforms, here was used as a model to illustrate the consequence of induction of Phase II drug metabolizing enzymes is abrogated by CGA to produce less prooxidant radicals. The red arrow indicates upregulation and the green arrow indicates downregulation.

readily react with $O_2^{\cdot-}$ forming highly reactive $ONOO^-$ which could induce lipid peroxidation or indirectly react with reduced GSH as well as nitrosative and oxidative modifications to proteins, nucleic acids, and lipids with simultaneous evolution of oxidative products which accumulate in the cells of the liver and kidney and cause their damage [82, 83]. MPO is a heme-containing enzyme found in mammalian neutrophils. It is known to catalyze the hydrogen peroxide mediated peroxidation of halide ions and the pseudohalide thiocyanate with concomitant release of HOCl. HOCl can react with several biomolecules, especially those containing thiols, nitrogen compounds, or unsaturated double carbon bonds, and trigger the dysfunction of the liver's cells and the kidney [84, 85].

The induction of inflammation was further manifested in our studies following the significant suppression of IL-10 in TAM's liver and kidney tissues. IL-10 is an anti-inflammatory cytokine that controls inflammation and inhibits dendritic cells' activation and differentiation and macrophages [86, 87]. On the other hand, rats treated with CGA (25 and 50 mg/kg) exhibited reduced pro-inflammatory markers and cytokines (NO, MPO, IL-1β, and TNF-α) with concurrent increase in the level of IL-10. The anti-inflammatory property of CGA has recently been reported [48], and CGA may have abated inflammation by inhibiting some inducers—NF-κB and iNOS—of inflammatory and oxidative responses in experimental rats.

Uncontrolled oxidative and nitrosative stress and inflammation in the liver and kidney cells, following exposure to foreign substances, can alter their architectural buildup, thereby making them susceptible to self-destruction. In our study, TAM significantly increased activities of Casp-9 and Casp-3 in the liver and kidney tissues. This signifies that TAM may have induced apoptosis via either the extrinsic or intrinsic pathways of apoptosis. Accordingly, sustained oxidative and inflammatory responses will mobilize Calpain to activate p38 resulting in the phosphorylation of p53, which then upregulates the expression of pro-apoptotic genes, including Bax and Bak. These proteins are translocated into the liver and kidney cells' mitochondria, thereby inducing mitochondrial permeability transition with a simultaneous release of cytochrome C (Cyt. C). The assemblage of Cyt C with apoptotic protease activating factor 1 (APaf-1) and procaspase-9 will lead to the formation of apoptosome and active Casp-9 (initiator caspase), which then activates procaspase-3 into active Casp-3 (the executioner caspase). Active Caps-3 eventually commits the liver and kidney cells to apoptosis by cleaving the substrate and recognizing the DxxD motif [3, 5, 8]. However, rats treated with CGA (25 and 50 mg/kg) exhibited a significant decrease in apoptotic markers of Casp-9 and Caps-3 in the liver and kidney tissues. Earlier studies have also demonstrated the antiapoptotic effect of CGA [88–90].

Assessment of the liver and kidney tissues' biochemical changes following TAM-induced hepatorenal injury without evaluating the histopathological changes will be clinically incorrect. To this end, we investigated the effect of TAM on the morphology of the liver and kidney tissues. The outcomes revealed severe disseminated congestion and infiltration of inflammatory cells in the liver and showed a focal area of necrosis and inflammatory cells in the kidney. This may be due to the increased generations of oxygen radicals such as O^{2-}, OH, and H_2O_2, capable of damaging these organs' cells via lipid peroxidation, the formation of protein crosslinks, and DNA double-strand breaks [6, 91]. CGA dose-dependently reduced TAM-mediated alterations in the liver and kidney with visible improvement in the live and kidney cyto-architecture at CGA1, and histological features were somewhat similar to controls

at higher CGA dose (CGA2). However, there were still mild disseminated congestion in the kidney and the presence of inflammatory cells at TAM + CGA1.

The outcomes of the histopathology of the liver and kidney were similar to the biochemical findings on the protective effect of CGA against TAM-induced hepatorenal toxicities.

In conclusion, the present study reveals that the administration of TAM significantly alters the integrity and functionality of the cells of the liver and kidney by elevating the biomarkers of the dysfunction of the liver and kidney in the serum, as well as oxidative, inflammatory, and apoptotic markers with a concomitant decrease in mean body weight, anti-inflammatory markers, and antioxidant defense system. However, CGA demonstrated a protective activity by increasing the antioxidant defense system, mean body weight, with a concurrent decrease in serum liver biomarkers, as well as markers of oxidation, inflammation, and apoptosis. Based on these findings, we propose that the protective effect of CGA against TAM-induced hepatorenal toxicities could be a combination of the anti-oxidative, anti-inflammatory, and antiapoptotic effects of CGA as depicted in our proposed mechanism of action presented in Fig. 11. Therefore, CGA supplementation in the diet of patients undergoing TAM chemotherapy may be necessary to circumvent possible clinical sequelae. However, additional study is necessary to elucidate the molecular mechanisms of the anti-oxidative, anti-inflammatory, antiapoptotic, anti-hepatotoxic, and anti-nephrotoxic effects of CGA.

Supplementary Data

Supplementary data is available at TOXRES Journal online.

CRediT roles

S.E.O., A.K.O., U.O.A., and J.K.O. conceptualized the experiments, carried out the research and preliminary data analysis. S.E.O. and U.O.A. supervised the investigation. S.E.O. checked the generated data for error. The manuscript was written and revised by S.E.O., A.K.O., and U.O.A.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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