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Beta-caryophyllene ameliorates environmental toxicants-induced experimental nephrotoxicity through regulation of redox system and inflammation

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Kidneys play important roles in the detoxification and excretion of toxic metabolites making them vulnerable to xenobiotic toxicity. We investigated the possible nephrotoxicity induced in mice by environmental toxicants diethyl nitrosamine (DEN) and carbon tetrachloride (CCl_4) and their mitigation by supplementation of natural bicyclic sesquiterpene β-caryophyllene (BCP). DEN (1 mg/kg) and CCl_4 (0.2 mL/kg) were given to mice and then the treatment group received BCP (30 mg/kg). After 18 weeks, DEN/CCl_4-induced mice showed renal injury evidenced by significant increase in circulating kidney function markers as well as histopathological alterations. Concurrent supplementation of BCP significantly ameliorated kidney function markers and prevented renal tissue damage. BCP treatment suppressed lipid peroxidation and nitric oxide levels and enhanced the antioxidant defence mechanism in the kidneys. BCP successfully amended renal histopathological changes and improved kidney serum markers and electrolytes associated with kidney function in association with alleviation of the deteriorated renal oxidative state and antioxidant defence system. BCP modulated the expression of key inflammation markers (inducible nitric oxide synthase and cyclooxygenase-2) and apoptotic markers [caspase-3 and poly (ADP-ribose) polymerase] in the DEN/CCl_4-exposed kidneys. The study concludes that exposure to environmental toxicants DEN/CCl_4 caused extensive nephrotoxicity that was notably mitigated by BCP suggesting its effectiveness as an alternative therapy in renal dysfunctions.

Keywords: Apoptosis β-Caryophyllene, Carbon tetrachloride, Diethyl nitrosamine, Environmental toxicity, Inflammation, Nephrotoxicity, Oxidative stress

The kidney is the primary excretory organ for metabolic products and toxicants. Kidneys utilize many xenobiotic metabolizing enzymes that play important roles in the metabolization of medicines and foreign chemicals. Kidney illnesses are a global public health concern that can vary from minor infections to life-threatening renal failure. Kidneys maintain a marginal concentration of electrolytes in the body. Electrolytes are small inorganic ions prevalent in body fluid that are important in normal physiological functions, mainly sodium ion (Na^+), potassium ion (K^+), and calcium ion (Ca^+).Serum urea, creatinine, and uric acid are sensitive biomarkers for the assessment of renal function and for investigating drug-induced nephrotoxicity. The kidneys receive around 25% of the cardiac output, and the renal tubules have a high proclivity for drug uptake via transporter proteins or endocytosis. This can lead to elevated intracellular levels of numerous medications and chemicals, which are then metabolized, resulting in the creation of reactive oxygen species (ROS) and other hazardous metabolites[1]. Increased ROS generation and oxidative stress link to drug-induced kidney injury and tubular necrosis[2-5]. Diethyl nitrosamine (DEN) is categorized as an environmental carcinogen found in smoked pickled fish, tobacco, processed meat, dried milk, and alcoholic beverages[6]. DEN is a strong hepatocarcinogen that acts by transforming cytochrome P450-dependent monooxidase systems causing oxidative stress and cytotoxicity, mutagenicity, and carcinogenicity that are linked to the pathophysiology of drug-induced kidney injury and tubular necrosis[5,7]. Carbon tetrachloride (CCl_4) is another environmental toxicant thatdoes not occur naturally yet is released into the environment by human activities. CCl_4 is classified as a potential human carcinogen yet there are significant gaps in the available cancer studies in humans and animals. Thus, studies have explored their combined effects on the function and physiology of vital organs like liver and kidney.

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An increasing number of research show that antioxidant-rich foods and natural products have been beneficial against such injuries in the liver and kidney. Beta-caryophyllene (BCP) is a natural sesquiterpene found in the essential oils of many plants such as cloves, cinnamon, black pepper, and basil[8,9]. BCP has a variety of documented pharmacological effects, including anti-inflammatory, cytotoxic, antioxidant, anti-spasmodic, anti-cancer, anti-microbial, hypolipidemic, neuroprotective, and cytoprotective effects, making it a promising therapeutic target for a variety of diseases[10,11]. To the best of our knowledge, there haven't been many studies examining BCP's preventive benefits against DEN/CCl_4-induced nephrotoxicity. As a result, the goal of this work was to show that BCP can reduce oxidative stress, inflammation, and cell damage associated with DEN/CCl_4-induced nephrotoxicity in mice.

Materials and Methods

Animals and experimental protocols

The animal experimental procedures were approved by the institutional animal ethics committee. Male Balb/c mice, weighing about 10-15 g, were housed in cages at a temperature of 26-28 °C, relative humidity of 60% and 12 h light/dark cycle. The animals were fed with a standard food diet and had access to water *ad libitum*. For the study following groups were formed: Group I (Vehicle Control):animals were given intraperitoneal (i.p.) delivery of vehicle control [phosphate-buffered saline (PBS) and olive oil as vehicle control for DEN and CCl_4]; Group II (DEN+CCl_4): animals received a single dose of DEN [single dose 1 mg/kg body weight (b.w.), i.p.] and CCl_4 (0.2 ml/kg b.w., i.p., twice per week for 22 weeks); Group III (DEN+CCl_4+BCP): animals received DEN and CCl_4 as per Group II followed by BCP [30 mg/kg b.w., per oral (p.o), daily for 22 weeks]; and Group IV (BCP only): animals received only BCP (300 mg/kg b.w., p.o.). After 22 weeks of experimental duration, mice were sacrificed, and blood samples were collected. Kidneys were quickly removed and perfused with ice-cold saline. Kidneys' weights (g) were recorded and presented as weight change at the end of the experiment.

Biochemical assays for kidney function and oxidative stress

The assays of the kidney function markers such as sodium, potassium and chloride ions, creatinine, urea, and uric acid were performed from serum by using Erba Diagnostic kits (Mannheim, Germany) as per the manufacturer's instructions. The assay of oxidative stress associated biochemical parameters was performed from kidney tissues. Kidney samples were homogenized in cold PBS (10% w/v), centrifuged, and the clear homogenate was separated for assays. Estimation of the levels of malondialdehyde (MDA) levels as measures of lipid peroxidation, nitric oxide (NO), hydrogen peroxide (H_2O_2) and glutathione (GSH), and the activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione-S-transferase (GST) were performed as described in our previous studies[12-15].

Histopathological examinations

Kidneys' tissues were kept in 10% buffered formalin, embedded in paraffin wax, and cut into 5 µm thick slices from prepared blocks. For histological analysis, the paraffin embedded sections were de-paraffinized, washed, and stained with haematoxylin and eosin (H&E). The sections were then examined under a light microscope at 40x magnification.

Immunohistochemical evaluation

Kidney tissue sections were immune-stained with caspase-3, poly (ADP-ribose) polymerase (PARP), inducible nitric oxide synthase (iNOS;NOS-2) and cyclooxygenase-2 (COX-2) as described previously[13,14]. De-paraffinized sections were rehydrated and prepared for incubation with primary antibody followed by secondary antibody. The binding of antibody-antigen reaction was detected by using avidin-biotin complex (AB-kit, Santa Cruz Biotechnology, CA, USA). The immunostaining reaction was labelled with diaminobenzidine (DAB) as a chromogen and counterstained with Mayer's Haematoxylin. The immunostaining intensity was investigated by the EVOS-FL live cell microscopy system (Thermo Fisher, Inc., MA, USA).

Statistical analysis

All statistical data analyses were conducted by one-way ANOVA analysis of variance, and the results are presented as mean±SD (standard deviation) or SEM (standard error of the mean). Post hoc test was used for multiple comparisons between the treatment groups. *P*-P-values <0.05 were considered statistically significant. Graphical work and statistical calculations were done by GraphPad Prism 9.5.1.

Results

BCP improved kidneys physiology and function

Kidney being susceptible to toxicants showed an inflammatory enlargement in kidney weight (Fig. 1A). The absolute kidney weights were significantly increased in the DEN/CCl_4 group (0.4±0.02 g) as compared to the control, which indicated the chronic inflammatory renal pathophysiological state. DEN/CCl_4 causing an increase in the relative kidney weight may be attributed to their accumulation in the renal tubules, resulting in swelling of the kidneys and kidney damage. Kidneys are highly susceptible to damage by toxicants because of the high volume of blood flowing through it and the filtration of large amounts of toxins which can be concentrated in the kidney tubules[16]. Herein, we demonstrated the protective effect of the BCP against DEN/CCl_4-induced nephrotoxicity in mice.

In this research, upon induction of the mice with toxic environmental xenobiotics, DEN/CCl_4, the levels of various kidney function parameters, i.e., Na^+ (39.0±2.96), K^+ (7.0±0.59) and Ca^+ (8.5±0.21) were significantly ($P<$ 0.05) increased. After supplementation of BCP, the values of Na^+ (34.0±0.81), K^+ (5.0±1.00), and Ca^+ (6.8±0.15)

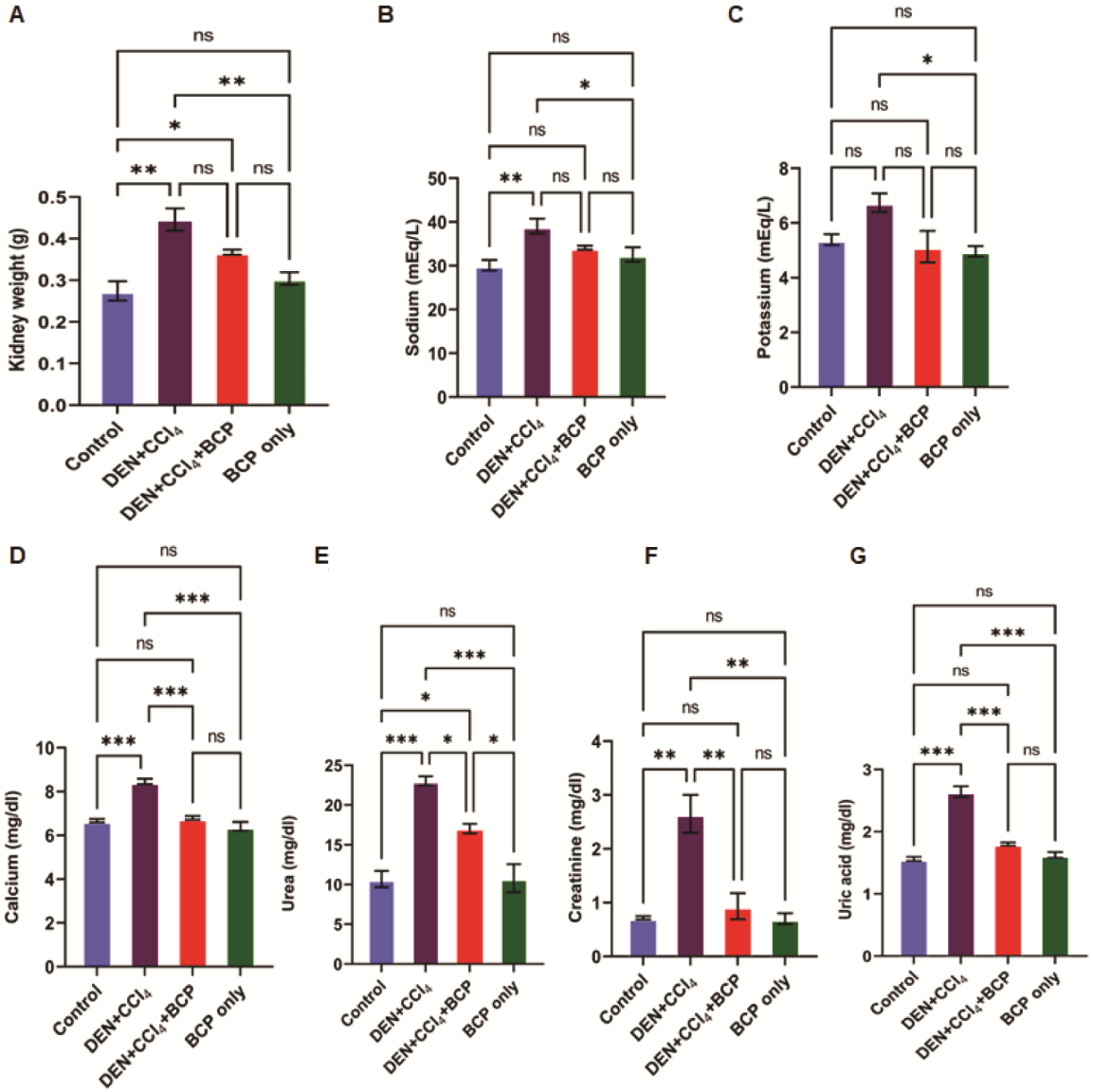

Fig. 1 — Effect of BCP on kidney physiology and function in DEN/CCl_4-exposed mice. (A) Kidney weight, (B) Sodium, (C) Potassium, (D) Calcium, (E) Urea, (F) Creatine, and (G) Uric acid. Values represented as mean±SEM. *$P<$ 0.05, **$P<$ 0.01 and ***$P<$ 0.001. *Abbreviations:* BCP, β-Caryophyllene; DEN, Diethyl nitrosamine; CCl_4, Carbontetrachloride;NS, non-significant

decreased in the DEN+CCl_4+BCP group of mice (Fig. 1 B-D). DEN/CCl_4-induced mice in the present study showed renal damage as evidenced by the significant increase in the circulating levels of creatinine, urea, and uric acid (Fig. 1 E-G). The DEN/CCl_4-induced mice showed renal damage as evidenced by the significant increase in the circulating levels of creatinine, urea, and uric acid. Our results showed that the levels of urea (23.0±0.57), creatinine (2.65±0.35), and uric acid (2.64±0.08) were significantly increased ($P< 0.01$) in the case of DEN/CCl_4 group. BCP supplementation was found to notably reduce these levels in a significant manner ($P< 0.01$) with the serum levels of urea (17.0±0.61), creatinine (0.93±0.24), and uric acid (1.80±0.03) as close to the control group. Our findings agree with the studies previously reporting that showed increased serum levels of creatinine, urea, and uric acid in DEN/PB-induced rats[17,18]. A relationship between nephrotoxicity and oxidative stress has been demonstrated in many experimental models[19] and the results in our study in relation to lipid peroxidation demonstrated a similar pattern as the organ function markers.

BCP restored histopathological alterations in kidneys

Histopathological examination of the kidney sections revealed normal histological structure of the renal tissues in both control and BCP-only supplemented mice groups (Fig. 2). The histological abnormalities caused by DEN/CCl_4 treatment included adenoma, dysplastic renal tubules with karyomegalic nuclei, glomerular tuft atrophy, inflammatory cell infiltration, protein cast in the lumen of renal tubules, and renal tubule vacuolation. Concomitant treatment of the DEN/CCl_4-induced mice with BCP showed marked amelioration of histopathological alterations in kidney tissues. Histopathological examination of the renal tissues of the DEN/CCl_4 treated mice demonstrated dilated blood vessels with intense haemorrhaging and some hyaline lumens. Additionally, renal sections revealed confined urinary gaps that might be caused by oedema. However, certain glomeruli still seemed smaller and there was still some observable haemorrhage after BCP treatment, even though there was a general improvement in the renal architecture and the recovery of several tissue damage markers. Similar observations were reported with the use of natural supplementation against CCl_4-induced nephrotoxicity in mice[20]. Our findings were supported by observations with hesperidin preventing DEN/phenobarbital (PB)-induced nephrotoxicity in rats[18] and cisplatin and trichloroethylene-induced nephrotoxicity in rats[21-23].

BCP suppresses apoptotic and inflammatory changes in kidneys

Immunohistochemical staining of kidneys was performed with apoptotic markers (*viz*., caspase-3 and PARP) and inflammation markers (viz., iNOS and COX2). The tissue damages were correlated with analyzing the expression of caspase-3, which is an executioner caspase that initiates apoptosis in the

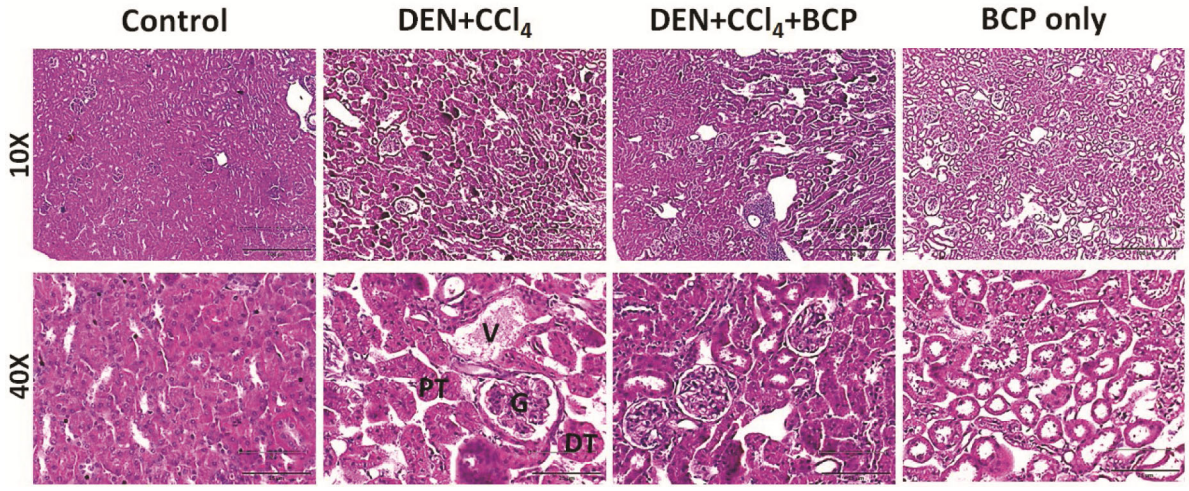

Fig. 2 — Effect of BCP on nephro-histopathological alteration induced by DEN/CCl_4. H&E staining and histological analysis (10x and 40x) ofkidney sections showing histological structures of renal parenchyma, glomerulus (G), proximal tubules (PT) and distal tubules (DT). Magnification 10X and 40X. *Abbreviations:* BCP, β-Caryophyllene; DEN, Diethyl nitrosamine; CCl_4, Carbontetrachloride; H&E,Haematoxylin eosin

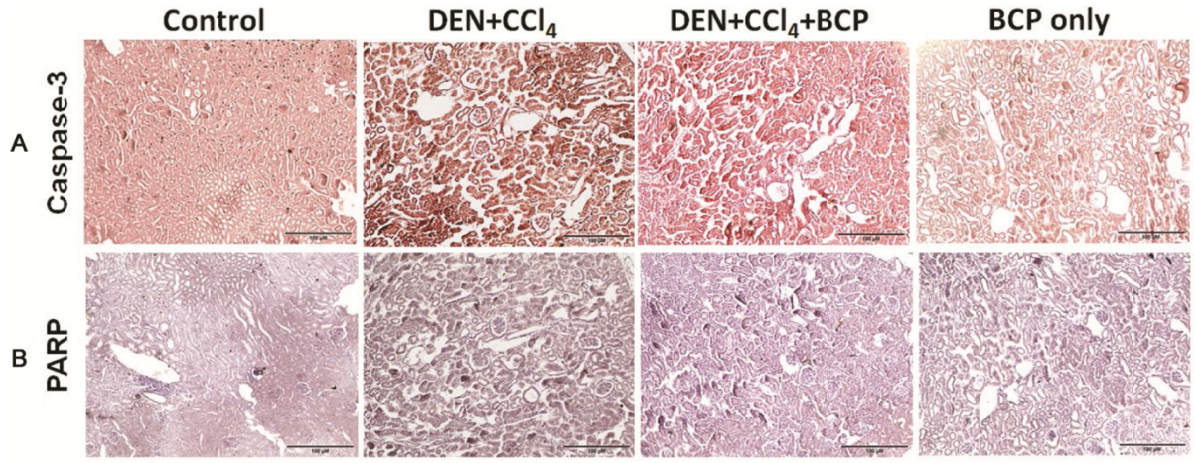

Fig. 3 — Effect of BCP on expression of apoptotic markers in DEN/CCl_4-treated mice. IHC staining of renal tissues for (A) Caspase-3 and (B) PARP. *Abbreviations:* BCP, β-Caryophyllene; DEN, Diethyl nitrosamine; CCl_4, Carbontetrachloride; IHC, immunohistochemistry; PARP, Poly (ADP-ribose) polymerase

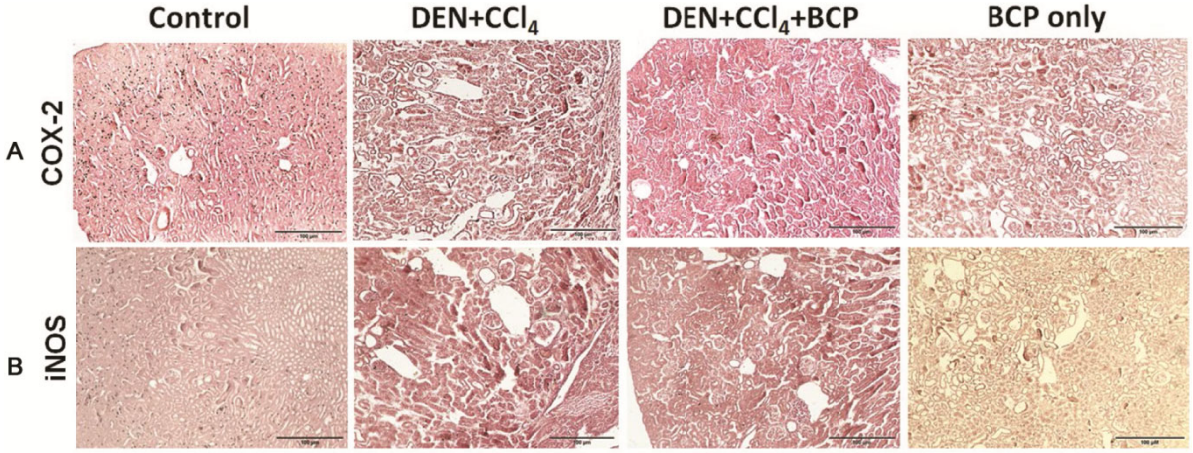

Fig. 4 — Effect of BCP on expression of inflammation markers in DEN/CCl_4-treated mice. IHC staining of renal tissues for (A)iNOS and (B) COX-2. *Abbreviations:* BCP, β-Caryophyllene; DEN, Diethyl nitrosamine; CCl_4, Carbontetrachloride; IHC, immunohistochemistry; iNOS, Inducible nitric oxide synthase; COX-2, Cyclooxygenase-2

kidney by catalyzing the cleavage of its nuclear substrate PARP. The effect of DEN/CCl_4 exposure in mice kidneys was assessed for apoptotic changes by immunohistochemistry (IHC) of caspase-3 and PARP (Fig. 3A & B). The control group showed normalized expression of caspase-3 and PARP, which were elevated multi-fold in the DEN/CCl_4-treated group. The BCP treatment to DEN/CCl_4-exposed mice showed drastic reduction in the expression of caspase-3 and PARP. Caspases are the type of cysteine protease that regulates programmed cell death through the mitochondrial pathway of apoptosis by activating DNA damage, whereas the extrinsic pathway is activated in response to ligands binding to receptors present on the cell surface[24]. Caspase-3 is an effector caspase that participates in both the extrinsic and intrinsic pathways of cell apoptosis. It initiates the death cascade and is the convergence point of different signaling pathways[25]. Our results of IHC showed that BCP alleviated the effect of DEN/CCl_4-induced nephrotoxicity. Expression of caspase-3 was found to be elevated by DEN/CCl_4-exposed mice which signifies its apoptotic inducing, whereas BCP treatment notably downregulated the expression of caspase-3 indicating the apoptosis regulation a mechanism of preventing nephrotoxicity.

Further results revealed an intense immuneexpression of iNOS and COX-2 in the glomerular podocytes, tubular epithelium, and mononuclear cells in the sections treated with DEN/CCl_4 than the control (Fig. 4A & B). The BCP treatment to DEN/CCl_4-exposed mice showed a

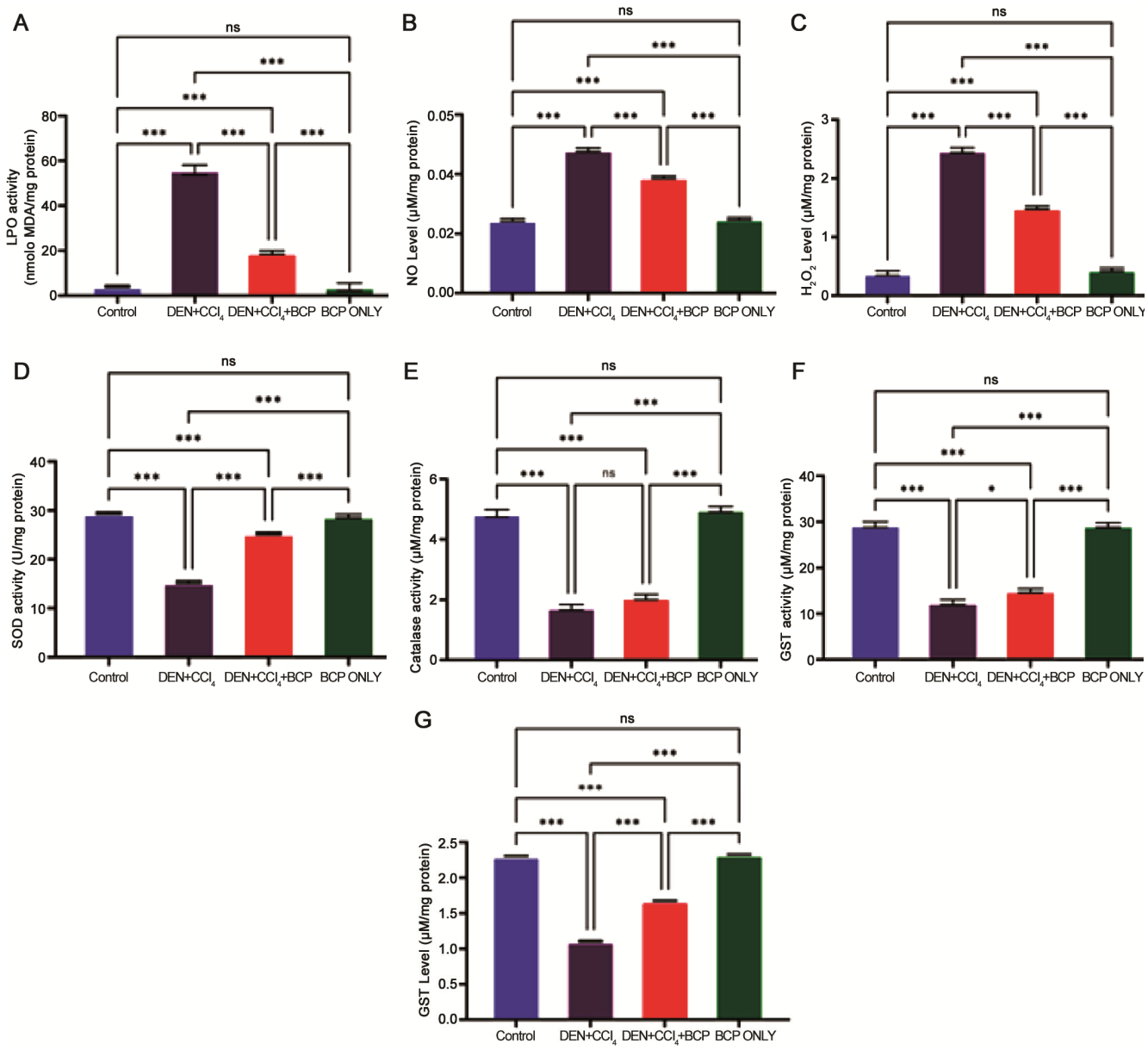

Fig. 5 — Effect of BCP on oxidative stress and antioxidant defences markers in kidney of DEN/CCl_4-induced mice.(A) LPO, (B) NO, (C) H_2O_2, (D) SOD, (E) Catalase, (F) GST, (G) GSH. Values represented as mean±SEM. *P< 0.05, **P< 0.01 and ***P< 0.001. *Abbreviations:* BCP, β-Caryophyllene; DEN, Diethyl nitrosamine; CCl_4, Carbontetrachloride; LPO, Lipid peroxidation; NO, Nitric oxide;H_2O_2, Hydrogen peroxide; SOD, Superoxide dismutase; GST, Glutathione-S-transferase; GSH, Glutathione;SEM, Standard error of the mean; NS, non-significant

remarkably lowered expression of iNOS and COX-2 suggesting a potent anti-inflammatory response of BCP in inflamed kidneys due to DEN/CCl_4 exposure. These results suggest that DEN/CCl_4 caused excessive apoptosis which led to histological degenerations and the same was mitigated by BCP supplementation.

BCP improved antioxidant defence system in kidneys

Oxidative stress is described as an imbalance in the aggregation and generation of free radicals, as demonstrated by changes in biomarkers such as lipid peroxidation (LPO) and nitric oxide (NO) which were measured in the current study (Fig. 5). Data evaluated by one-way ANOVA analysis showed a significant effect on oxidative stress parameters (LPO and NO). The post hoc multiple comparison test showed that the levels of LPO and NO were significantly increased (P< 0.05) in the DEN/CCl_4-exposed group as compared to the control. The BCP treatment to the DEN/CCl_4-exposed group significantly decreased

($P< 0.05$) the levels of oxidative stress markers in the kidney. Notably, BCP causing decreases in the level of oxidative stress markers may be due to its free radical scavenging and redox potential properties. Furthermore, antioxidant enzymes (SOD, CAT, and GST) and non-enzymatic moieties (GSH) were assayed (Fig. 4C-F) which are capable of stabilizing or catalyzing the transformation of free radicals into stable molecules before their impacts on cellular organelles. GSH content in the kidney homogenate of DEN/CCl_4-induced mice showed a significant ($P< 0.001$) decrease when compared with the control group. On the other hand, BCP (30 mg/kg) markedly ameliorated GSH content in the kidney of DEN/CCl_4-induced mice. The activity of SOD, catalase, and GST was significantly ($P< 0.001$) declined in the kidney of DEN/CCl_4-induced mice when compared with the corresponding control mice. Concurrent treatment of the DEN/CCl_4-induced mice with BCP significantly ($P< 0.001$) alleviated the activity of SOD and GST. Mice receiving only BCP showed non-significant ($P> 0.05$) changes in kidney GSH, SOD, and GST when compared with the control mice. All these factors deformed the cell membrane leading to tissue injury[26] as observed with the histopathological changes in kidneys induced by DEN/CCl_4.

Antioxidants either sequester the redox-active metals and prevent the formation of free radicals or they may scavenge the ROS directly to ameliorate oxidative stress[27]. Levels of various enzymatic and non-enzymatic antioxidants were assessed in DEN/CCl_4-exposed mice treated with BCP. The striking decrease in antioxidants (SOD, CAT, GSH, and GST) in DEN/CCl_4-exposed mice, in the present investigation, elicits strong evidence for the involvement of oxidative damage as a mechanism of DEN/CCl_4-induced nephrotoxicity. This may be due to ROS produced from the metabolism of DEN that caused decreases in the activities of renal antioxidant enzymes. Our results agree with other reports showing DEN decreased GSH content and reduced activities of SOD, CAT, and GST[3,28,29]. DEN/CCl_4 indicated amarked reduction in GSH content due to the impairment of H_2O_2 clearance and promotion of hydroxyl radical ($^{\bullet}OH$) formation which leads to oxidative stress. The effective restoration of LPO and enhancement of GSH content were observed after the treatment with BCP. SOD is a metalloenzyme that catalyzes the dismutation of superoxide radicals and converts it into H_2O_2. SOD activity was significantly decreased after the treatment with DEN/CCl_4 whereas administration of BCP restored the decreased activity of SOD. Catalase is another antioxidant enzyme thatis responsible for the reduction of H_2O_2 to water and oxygen thus preventing the damage of cells from oxidative stress. DEN/CCl_4 decreased the activity of CAT which was protected significantly with BCP treatment.

Discussion

In recent years, with the increase in industrialization and modernization, the level of toxic chemicals present in the environment has increased to a greater extent. Hence, the search for compounds that prevent their toxic effect is very important. In this regard, the use of complementary and alternative medicine has shown a promising approach that has been explored in this study. DEN/CCl_4 administration induced an increase in the relative kidney weight which can be attributed to the accumulation of DEN/CCl_4 in the renal tubules, resulting in swelling of the kidneys and kidney damage. Accumulation of DEN/CCl_4 in kidney tissue causes damage totubular cells resulting in dehydration and thus a decrease in the body weight gain. Serum urea, creatinine, and uric acid are sensitive biomarkers for the assessment of renal function and for investigating drug-induced nephrotoxicity.DEN/CCl_4-induced mice showed renal damage as evidenced by the significant increase in the circulating levels of creatinine, urea, and uric acid. Serum creatinine level has been reported to reveal glomerular function and its increase is an indicator of renal failure[30]. Urea is a byproduct of protein metabolism and is used as a marker of kidney injury, and uric acid has been proposed as a potential risk factor for new-onset kidney diseases[17]. These findings are in agreement with the previous study reporting increased serum levels of creatinine, urea, and uric acid in DEN/PB-induced rats[18].

Kidneys maintain the concentration of electrolytes levels in the body, mainly Na^+, Cl^-, K^-, HCO_3, and H^+, amongst which sodium content is the major osmotic solute in the extracellular fluid (ECF). Renal regulation of these ions is controlled by renal sympathetic, atrial natriuretic peptide, and aldosterone actions which may result in reabsorption or excretion of these ions at the distal tubule of the nephrons. However, nephrological defects caused by xenobiotics such as CCl_4 and DEN may truncate these functions and result in irregular distribution of these

ions in the ECF[31]. Histopathological changes provide signatory mechanistic impacts of DEN on renal dysfunctions as shown by renal injury induced by DEN/CCl_4. Histological alterations induced by DEN/CCl_4 included congestion and atrophy of glomerular tuft, oedema, cloudy swelling in convoluted tubules, thickening of the blood vessels, pyknosis, fibrosis, and inflammatory cells infiltration, which were notably restored by BCP treatment. Our findings are supported with the observations by hesperidin preventing DEN/PB-induced nephrotoxicity in rats[18] and cisplatin and trichloroethylene-induced nephrotoxicity in rats[21-23].

Oxidative stress is the result of an increase in free radicals and ROS along with LPO as one of the important markers of oxidative stress[32]. The level of LPO was found to be drastically increased in kidney tissues after the administration of DEN/CCl_4 while BCP treatment showed a marked diminution in LPO levels. This may be due to the antioxidant properties of BCP which scavenged free radicals thereby inhibitingLPO. The elevated level of LPO is linked with the loss of membrane fluidity, an increase in the permeability of cell membrane to ions, and the failure of antioxidant defence systems for scavenging free radicals. All these factors deformed the cell membrane leading to tissue injury [26] as observed with the histopathological changes in kidneys induced by DEN/CCl_4. Antioxidants either sequester the redox-active metals and prevent the formation of free radicals or they may scavenge the ROS directly to ameliorate oxidative stress[27]. Levels of various enzymatic and non-enzymatic antioxidants were assessed in DEN/CCl_4-exposed mice treated with BCP. The striking decrease in antioxidants (SOD, CAT, GSH, and GST) in DEN/CCl_4-exposed mice, in the present investigation, elicits strong evidence for the involvement of oxidative damage as a mechanism of DEN/CCl_4-induced nephrotoxicity. This may be due to ROS produced from the metabolism of DEN that causes decreases in the activities of renal antioxidant enzymes. GSH is an important antioxidant which plays an important role in stopping the injury to cellular components caused by free radicals and peroxides. DEN/CCl_4 indicated amarked reduction in GSH content due to the impairment of H_2O_2 clearance and promotion of $^{\bullet}OH$ formation which leads to oxidative stress. BCP significantly attenuated the LPO and NO production in the renal tissue probably because of its antioxidant capacity to scavenge oxygen free radicals in the kidney tissue cells of mice.

The mitochondrial pathway of apoptosis is activated due to DNA damage, while the extrinsic pathway is activated in response to ligands binding to receptors present on the cell surface[25]. Our results of immune histochemistry evaluations showed that BCP alleviated the effect of DEN/CCl_4-induced nephrotoxicity. Expression of caspase-3 was found to be elevated by DEN/CCl_4-exposed mice which signifies its apoptotic inducing, whereas BCP treatment notably downregulated the expression of caspase-3 indicating the apoptosis regulation, a mechanism of preventing nephrotoxicity.

Conclusion

Kidneys are vital organs in maintaining physiological homeostasis and by detoxification and excretion of toxic metabolites. Kidneys are highly likely to be targeted by various drugs and xenobiotics that are metabolized in the liver and are exposed through the kidneys making them vulnerable to nephrotoxicity. This study investigated the possible nephrotoxicity induced by environmental toxicants DEN and CCl_4 and their mitigation by supplementation of natural bicyclic sesquiterpene BCP in mice. DEN/CCl_4werefound to be potent nephrotoxic substances that led to oxidative stress by depleting the activities of antioxidant enzymes, kidney function markers production, and stimulated apoptosis. The treatment with BCP significantly attenuated the DEN/CCl_4-induced renal toxicity and histopathological alterations. Thus, BCP is identified and advocated for its uses as an effective therapeutic agent for potential treatment of drug-induced nephrotoxicity.

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Conflict of interest

The author declares no conflicts of interest.

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