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p-Coumaric acid ameliorates ethanol-induced kidney injury by inhibiting inflammatory cytokine production and NF-κB signaling in rats

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ABSTRACT

Objective: To examine the effects of *p*-coumaric acid on ethanol-induced kidney injury in Swiss Wistar rats.

Methods: Ethanol (25% v/v) was used to induce nephrotoxicity in rats. *p*-Coumaric acid was orally administered at 50, 100, or 200 mg/kg body weight. The levels of oxidative parameters were determined; pro-inflammatory biomarkers were analyzed by Western blotting and apoptotic protein was analyzed by immunohistochemistry.

Results: Ethanol treated rats showed decreased levels of antioxidants and aberrant production of pro-inflammatory cytokines (IL-6, IL1β, TNF-α), NF-κB activation and imbalance of pro- and anti-apoptotic proteins (Bcl-2, Bax, caspase 3). Meanwhile, *p*-coumaric acid restored antioxidant levels and decreased the levels of inflammatory cytokines, NF-κB, and pro-apoptotic proteins and increased Bcl-2 expression.

Conclusions: *p*-Coumaric acid ameliorates ethanol-induced kidney injury by restoring antioxidant production and suppressing cellular apoptosis and inhibiting NF-κB expression. *p*-Coumaric acid should be further investigated as a promising candidate for ethanol-induced kidney toxicity.

1. Introduction

The kidney is a very important organ of the human system. It is involved in eliminating harmful toxic wastes and maintaining water, intracellular fluids, minerals and electrolytes in our system. Malfunction of the kidney results in severe illness or even death. Kidney has been affected by various factors such as drugs, virus, and heavy alcohol consumption[1]. Alcohol is one of the causative agents for several illnesses which include liver-toxicity, cardiovascular disease, and nephrotoxicity in the modern world. World Health Organization has estimated that about 2 billion peoples have alcohol consuming habits worldwide. Moreover, 76 million people are presently addicted to consumption of alcohol and they are affected by alcohol-mediated kidney damage[2]. Centers for Disease Control and Prevention have reported that excessive intake of alcohol is considered as the third most cause of noncurable death in the developed countries[3]. Alcohol can cause overproduction of harmful reactive oxygen species in organs and leads to tissue damage or oxidative stress[4]. Alcohol-mediated overproduction of free radicals incites lipid peroxidation, and stimulates cell damage leading to acute and chronic kidney nephrotoxicity[5].

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The plenty of experimental evidence has proved that alcohol-mediated hepatotoxicity and nephrotoxicity were associated with overproduction of inflammatory cytokines and oxidative stress[6,7]. The production of tumor necrosis factor (TNF-α) and inflammatory interleukins are considered as the earliest inflammatory events in alcohol-mediated tissue damage. This triggers other cytokines productions that need inflammatory cells to kill parietal cells, podocytes, cuboidal epithelial cells, mesangial cells, and induce healing responses[8]. Nuclear factor-κB (NF-κB) is one of the major transcription factors that play a vital role in various pathological processes including inflammation and apoptosis. NF-κB is activated by a variety of inflammatory stimuli such as interleukine, tumor necrosis factor, and other biological factors[9]. Many dietary phenolic derivatives strongly inhibit cytokine production and NF-κB signaling activation in experimental animals[10].

An elimination and reversible agent might be excellent strategies for medical intervention of kidney damaged patients. Therefore, pharmacological strategies are required to prevent or reverse kidney damage[11]. Many researchers have focused on multiple therapeutic interventions to overcome this disease, by developing transcription factor inhibitors, therapies for cell-based therapies and antiviral, synthesis of nanoparticles and plant-based natural phytonutrients[12].

Kidney transplantation is one of the emergent surgical options, regrettably, the lack of sufficient donors and organ rejection restrict this medical surgical procedure. The modern investigation focused on renal protection properties of plant-based nutraceutical products because of their molecular structural aspect, a lot of pharmacological properties as well as minimal toxicity[13]. A recent study documented that phytochemicals have drawn attention towards health management as superior sources for chemotherapeutic drug development in treating a wide variety of diseases[14,15].

p-Coumaric acid (PCA) is a hydroxycinnamic acid in phenolic nature and is derived from a fraction of plants materials[16]. The main sources for phenolic compounds are widely distributed in plant-derived products such as apples, pears, potatoes, beans and beverages like phenolic-rich tea, beer and chocolate[17]. Many researchers have evidenced that the phenolic compound strongly suppressed chronic inflammation and cell proliferation by enhancing immune and antioxidant levels in many experimental models[18,19]. Notably, PCA has various pharmacological properties such as strong anti-oxidant, antitumor, anti-inflammatory, anti-angiogenic, anti-diabetic, anti-microbial and immunomodulatory activities[20-23]. Recently, we have reported that PCA inhibited apoptosis, protected testicular dysfunction and improved sperm fertility in the ethanol-induced experimental model[24]. However, effect of PCA on alcohol-mediated nephrotoxicity and its molecular mechanism have still not yet been studied. Therefore, for the first attempt, we investigated that role of PCA in EtOH-induced inflammation and cell death by suppressing pro-inflammatory cytokines and NF-κB in experimental rats.

2. Materials and methods

2.1. Chemicals and antibodies

PCA, and monoclonal primary antibodies such as TNF-α and NF-κB were obtained from Sigma-Aldrich. The molecular grade ethanol (>99% purity) grade was purchased from Merck Chemicals, Bangalore. The primary anti-monoclonal antibodies such as IL-6, IL-8, IL1β, IκB, β-actin and secondary antibodies such as goat anti-rabbit and goat antirat were obtained from Santa Cruz, CA, USA. All other solvents and chemicals for biochemicals and histological experiments were used in the molecular grade in this work.

2.2. Animals

Male Wistar Albino rats (120-150 g) were obtained from the National Institute of Nutrition, Hyderabad, India. Experimental animals were carefully maintained under sterilized conditions at (23 ± 2) ℃, (40 ± 5)% relative humidity and alternating 12-h light/dark rotation at the Animal Home, Bharathidasan University, Tiruchirappalli. Sterile water and standard pellet were provided for rat diet (Mysore Snack Feed Ltd, Mysore, India). The Institutional Animal Ethics Committee (IAEC) has approved (No: BDU/ IAEC/ 2017/ NE/ 03/ Dt. 21. 03. 2017) this experimental protocol.

2.3. Experimental design

A total of 30 Wistar Albino rats were used for this experiment and were randomly divided into five experimental groups, with 6 rats in each group. The group Ⅰ served as normal control and received distilled water. Group Ⅱ, Ⅲ, Ⅳ& Ⅴ were treated orally with sequentially (per week) increased dose of EtOH 25% v/v (5, 8, 10, 12 g/kg b.wt/week in each group) for 28 d[25]. On the third and fourth week, the group Ⅲ, Ⅳ and Ⅴ were administered orally with PCA at three different concentrations (50, 100 and 200 mg/kg b.wt) based on acute oral toxicity study respectively, 1 h prior to EtOH treatment. At the end of the experimental period (28 d), animals were sacrificed after overnight fasting and the kidney samples were collected for various biochemical, histological and immunohistochemical analysis.

2.4. Biochemical determinations

Thiobarbituric acid reactive substance (TBARS), lipid hydroperoxides (LOOH) and conjugated dienes (CD) are considered as products of lipid peroxidation and were measured by previous method[26-28]. Non-enzymatic antioxidants like reduced glutathione (GSH), vitamin C and vitamin E were measured by standard biochemical estimation previously described[29-31]. Superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione-s-transferase (GST) and glutathione peroxidase (GPx)

as enzymatic antioxidants were measured by the standard method as previously described[32–36].

2.5. Immunoblot analysis

The protein expression of inflammatory cytokines, NF-κB transcription factors and inhibitory kappa B proteins (IκB)-α were measured by Western blotting analysis. The obtained results were normalized to housekeeping protein marker β-actin expression. Following the protein estimation, the treated test samples were separated using 10% SDS-PAGE gel electrophoresis and the proteins from the gel were transferred to PVDF membrane and treated with appropriate primary and secondary antibodies to detect the protein markers as per the method described previously[37]. The band intensities were quantified by scanning densitometry using Image-studio software (LI COR, USA), and normalized to respective β-actin loading control.

2.6. Immunohistochemical assay

The protein expressions of apoptotic markers including Bcl-2, Bax and caspase 3 were analyzed by immunohistochemical assay. Appropriate antigens present in the kidney tissue sections were observed with specific type of primary antibodies. Immunohistochemical images of Bcl-2, Bax and caspase 3 protein expressions were studied as per the protocol described previously[24].

2.7. Histological observation

After the treatment with PCA and /or ethanol, rat kidney was collected and it was fixed with 10% formaldehyde to embedded in tissue paraffin. The processed kidney tissue sections were stained with standard haematoxylin and eosin (H&E). The H & E stained slides sections were analyzed under a bright field microscope (Olympus CH20i, Japan). The brief methodologies of histopathology were performed as previously described[24].

2.8. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical variances were assessed using by One way ANOVA followed by Duncan's Multiple Range Test with SPSS 11.0 version software package. The value $P<0.05$ was considered as statistical significance. All experiments were performed by three independent investigations.

3. Results

3.1. Effect of PCA on LPO and antioxidants in rat kidney

Table 1 illustrates the lipid peroxidative markers, enzymatic, and non-enzymatic antioxidants levels in kidney tissues of control and experimental animals. The lipid markers, TBARS, LOOH, and CD were significantly increased in EtOH exposed rats. Moreover, SOD was significantly increased, while CAT, GR, GPx, GSH, GST, vitamin-E and vitamin-C levels were significantly ($P<0.05$) depleted in EtOH-induced rat kidney as compared to untreated normal rats. PCA (50, 100, 200 mg/kg b.wt) significantly decreased TBARS, LOOH, CD, SOD levels, and restored the antioxidants levels as near to control rats. Additionally, the effect was more significant in higher dose PCA (200 mg/kg b.wt) as compare to other treatment doses.

3.2. Effect of PCA on histopathological changes in kidney tissues of EtOH–induced rats

Figure 1 shows the effect of PCA on histopathological changes in kidney tissues. The sections of untreated control rat kidney showed normal renal glomerular, and tubular structure (Figure 1a); while EtOH treated rats displayed stern tubular epithelial cell disintegration (Figure 1b). PCA (50, 100, 200 mg/kg b.wt) treatment groups showed well reverted histopathological changes remarkably in kidney tissue section (Figure 1c, d, e).

Table 1. Levels of TBARS, LOOH, CD and antioxidants in kidney tissues.

Treatment	Control	Alcohol	Alcohol + PCA (mg/kg b.wt)		
			50	100	200
TBARS (mmol/100g of tissue)	3.57 ± 0.11^{a}	6.43 ± 0.22^{d}	5.45 ± 0.16^{c}	4.22 ± 0.14^{b}	3.56 ± 0.11^{a}
LOOH (mmol/100g of tissue)	74.40 ± 5.16^{a}	127.76 ± 10.12^{d}	102.75 ± 8.30^{c}	85.14 ± 6.13^{b}	75.03 ± 5.16^{a}
CD (mmol/100g of tissue)	27.65 ± 1.54^{a}	42.10 ± 2.21^{d}	35.24 ± 1.94^{c}	30.24 ± 1.66^{b}	27.52 ± 1.54^{a}
SOD (U^{a}/mg protein)	3.75 ± 0.13^{a}	6.87 ± 0.26^{d}	5.68 ± 0.20^{c}	4.67 ± 0.18^{b}	3.67 ± 0.13^{a}
CAT (U^{b}/mg protein)	49.17 ± 6.17^{d}	31.67 ± 4.14^{a}	40.56 ± 5.24^{c}	33.16 ± 4.64^{b}	48.78 ± 6.17^{d}
GPx (U^{c}/mg protein)	23.36 ± 1.11^{d}	10.26 ± 0.58^{a}	15.65 ± 0.75^{c}	12.77 ± 0.63^{b}	22.88 ± 1.11^{d}
GR (mg/g tissue)	27.23 ± 1.67^{d}	13.67 ± 0.84^{a}	20.78 ± 1.12^{c}	15.44 ± 0.92^{b}	27.34 ± 1.67^{d}
GST (U^{d}/mg protein)	7.86 ± 0.67^{d}	2.34 ± 0.29^{a}	4.99 ± 0.43^{c}	3.49 ± 0.34^{b}	7.74 ± 0.67^{d}
GSH (μg/mg tissue)	23.49 ± 0.98^{d}	12.92 ± 0.41^{a}	17.87 ± 0.62^{c}	14.87 ± 0.58^{b}	23.46 ± 0.98^{d}

U^{a} - Enzyme concentration required for 50% inhibition of NBT reduction/min; U^{b}- μmole of hydrogen peroxide consumed/min; U^{c}- μmol of reduced glutathione consumed/min; U^{d}- μmoles of GSH-cDNB conjugate formed/minute. Values do not share common superscript letter (a, b, c, d) between groups differ significantly at $P < 0.05$. TBARS: thiobarbituric acid reactive substance. LOOH: lipid hydroperoxides. CD: conjugated dienes. SOD: superoxide dismutase. CAT: catalase. GPx: glutathione peroxidase. GR: glutathione reductase. GST: glutathione-s-transferase. GSH: glutathione.

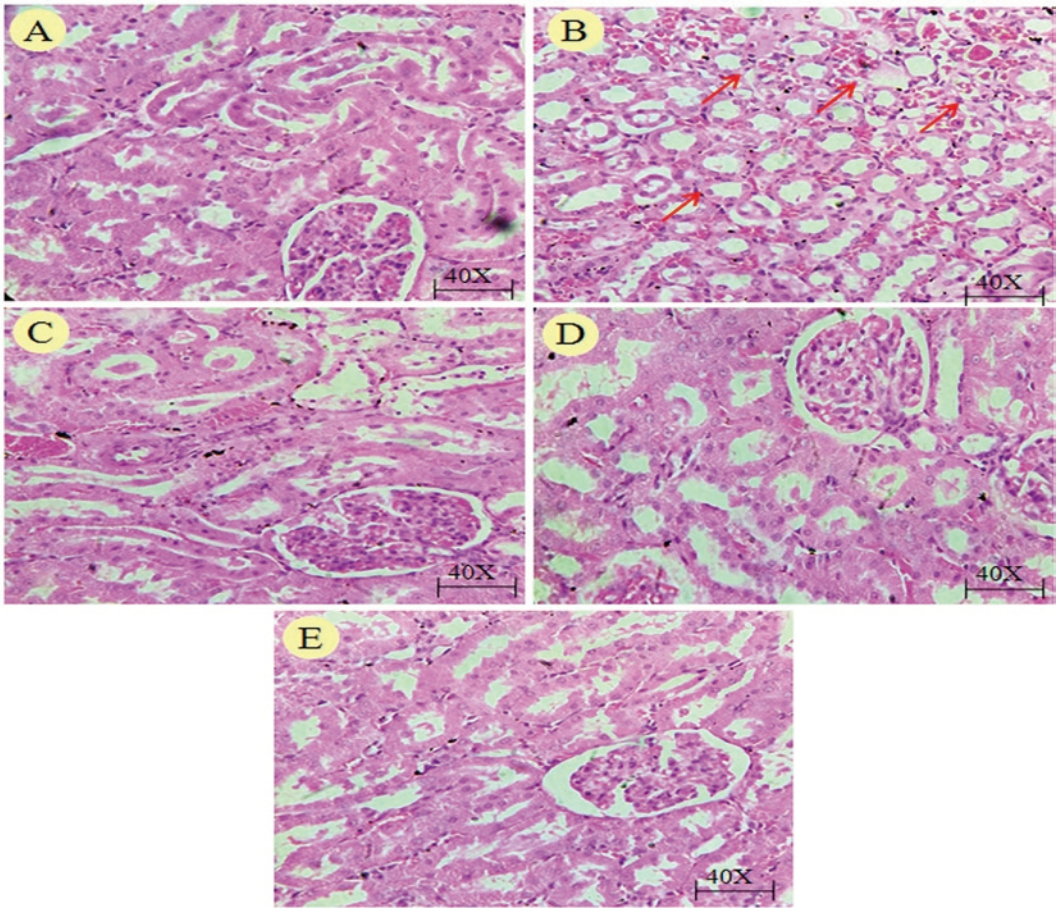


Figure 1. Histological alteration of control and experimental animals assessed by haematoxylin and eosin staining (H&E). (A) Normal kidney histology. (B) In rats with ethanol (EtOH) induced kidney injury, there are severe degenerative alterations in the tubules, congestion of blood vessels and diffused inflammatory cell infiltration. (C) EtOH + *p*-coumaric acid (PCA) (50 mg/kg b.wt) treated rat kidney shows few tubules containing fat vacuoles. (D) EtOH + PCA (100 mg/kg b.wt) treated rat kidney shows slight congestion of peritubular capillaries and normal glomeruli. (E) EtOH + PCA (200 mg/kg b.wt) treated rat kidney shows normal histology.

3.3. Effect of PCA on pro-inflammatory cytokines and transcription factor

The pro-inflammatory cytokines and transcription factor NF-κB play a major role in inflammatory progression. Figure 2 shows a significant elevation in the expression of IL-1β, IL-6, TNF-α, NF-κB-p65 (P<0.05) and a significant decrease of IκB-α in EtOH-induced kidney injury rats as compared to untreated normal rats. Consequently, administration of PCA (50, 100, 200 mg/kg b.wt) significantly decreased IL1β, IL-6, TNF-α, NF-κB p65 and inhibited phosphorylation of IκB-α expression level in EtOH- treated rats.

3.4. Effect of PCA on apoptotic marker expression

The expressions of Bcl-2, Bax and caspase-3 were showed in Figure 3. EtOH-induced rats showed overexpression of Bax, caspase-3, and reduced Bcl-2 expression levels as compared to untreated control. PCA (50, 100, 200 mg/ kg b.wt) significantly increased Bcl-2 expression and decreased Bax, caspase-3 expression levels as compared to EtOH-induced rats.

4. Discussion

Chronic kidney disease (CKD) is a foremost health issue in developing countries and this complication is associated with oxidative stress[38]. Chronic alcohol intake has been reported to induce oxidative stress *via* active multiple mechanisms. The pathological changes of alcohol-induced kidney disease are categorized by morphological changes with minimal injury to more detrimental kidney damage[39]. Hence, CKD must be treated by medical surgery. Although a lot of advanced chemotherapies can exclusively inhibit CKD progression, but they frequently cause harsh side effects. Sun *et al*[40] suggested that medicinal plant-derived phytochemicals may offer superior preventive effects against CKD with minimal toxicity to existing medicinal therapy.

Highly generative free radicals are typically short-lived molecules which is the by-products of alcohol metabolism. They are known to cause cellular oxidative damage unless the body can clear them *via* antioxidants. Oxidative stress occurs when homeostatic biological processes fail and free radical generation is much beyond the ability of the body's defenses, thus leading to cellular injury and organelle tissue damage[41]. Ethanol metabolism generates ROS by inducing

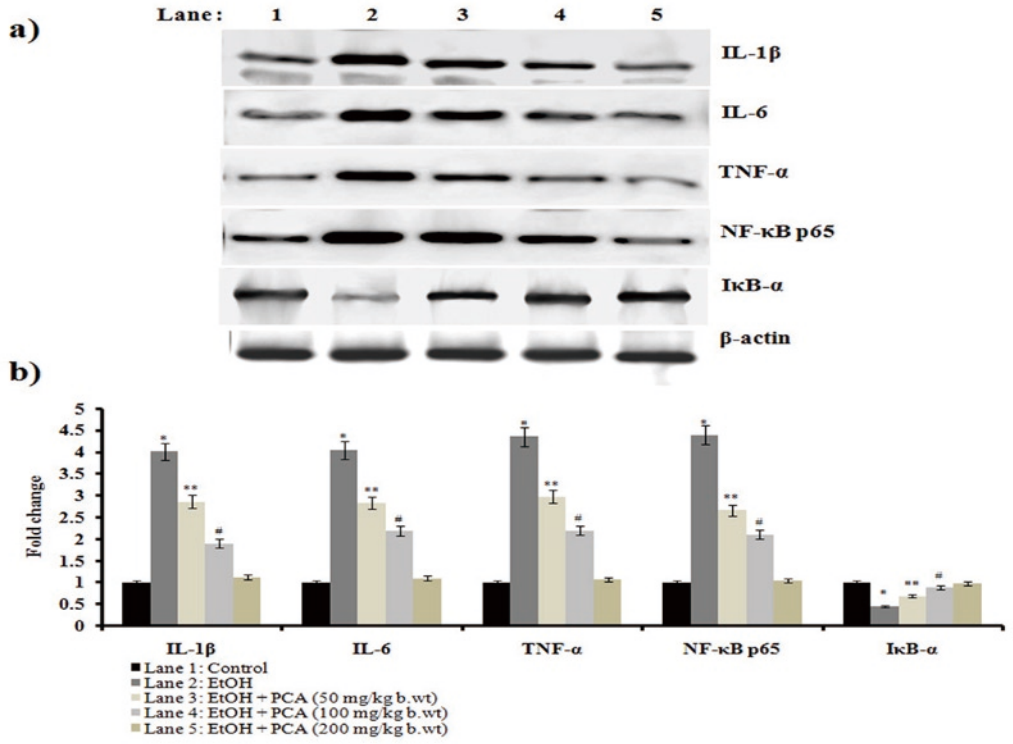


Figure 2. Effect of PCA on inflammatory cytokines and NF-κB expression. Expression of IL-1β, IL-6, TNF-α, NF-κB, IκB-α were analysed by Western blot. β-actin serves as control loading and it confirms equal loading of protein samples. Densitometry data represented as mean ± SD from three independent experiments. Values do not share common superscript letter (*, **, #) between groups differ significantly at $P < 0.05$ (DMRT).

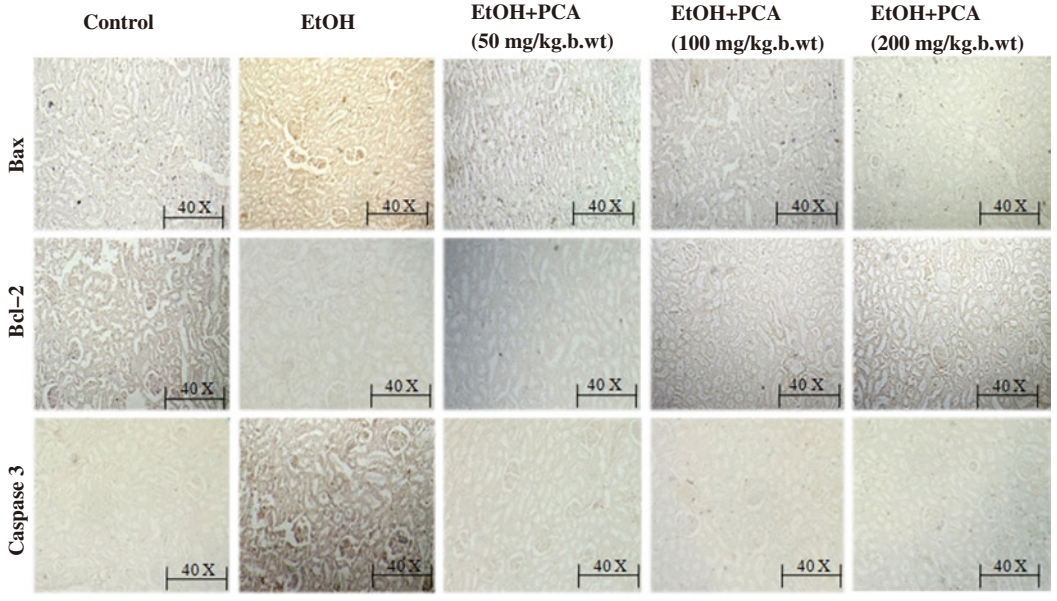


Figure 3. Effect of PCA on apoptosis in kidney tissue. Bax and caspase-3 are overexpressed while Bcl-2 is downregulated in the kidney tissues of rats with ethanol-induced kidney injury. EtOH + PCA (50,100 and 200 mg/kg b.wt) treated rats showed decreased Bax and caspase-3 expression and increased Bcl-2 levels as compared to EtOH-induced rats (Positive staining indicated as brown spot).

lipid peroxidation and decreases cellular antioxidant levels[42]. In our report, increased levels of TBARS, LOOH, CD, SOD and decreased antioxidants levels such as GPx, CAT, GR, GST, GSH, vitamin-C and vitamin-E were observed in alcohol-administered rat kidney tissue. EtOH exposure resulted in high overproduction of free radicals such as superoxide radicals, hydroxyethyl radical, peroxyl radical ($ROO^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2)[43]. These EtOH mediated radicals are possible to react quickly with lipid molecules which in turns enhance lipid peroxidation byproduct TBARS[44]. Many pieces of research evidenced that elevated LPO has considered as a toxic sign of EtOH intake. In addition, wide damage to tissue by the production of free radicals associated LPO can result in the loss of membrane organisation and tissue damage[45,46]. Similarly, after EtOH administration, structural integrity of kidney tissue in rarts was observed in histopathological studies. Herein, administration of PCA reverts pathological alteration in EtOH-induced rat as compared to normal. In the previous study, Navaneethan and Rasool[46] documented that PCA significantly protected the biochemical changes like decreased LPO levels and enhanced antioxidants levels as well as recovered histopathology alterations in cadmium chloride-induced nephrotoxicity in rats. Another study documented that natural phytochemical ingredients of silymarin strongly enhanced antioxidants and decreased lipid peroxidation levels in EtOH-induced live injury rats[22]. As above mentioned, PCA effectively scavenged free radicals mediated LPO, reverted tissue alteration and boosted antioxidant status.

NF-κB, a principal transcription factor, regulates an array of pro-inflammatory mediators and cytokines which participates in pathogenesis of aggressive inflammatory disorders[47,48]. Moreover, TNF-α and interleukins (IL) are well-known cytokines that play a key role in the inflammatory process. NF-κB stimulation or phosphorylation is crucial for pro-inflammatory cytokine production[49]. Thus, NF-κB is regulated by IκB that sequesters NF-κB in the cytoplasm. Signaled phosphorylation, ubiquitination, and proteolysis degradation of IκB lead to activation of NF-κB[50]. Those free liberated NF-κB relocate into the nucleus, where it binds to the specific promoter region and in turn promotes the expression of cytokines and inflammatory progress. The release of pro-inflammatory mediators and cytokines (IL-1β, IL-6, TNF-α) are associated with the elevated risk of numerous pathological kidney nephrotoxicity[51,52]. Increased expression of NF-κB, IL-1β, IL-6 and TNF-α were observed in kidney tissue of EtOH induced rats. Dietary nutritional agents have well anti-inflammatory properties. The previous study evidenced that phenolic agent of curcumin and [6]-shogaol inhibits oxidative stress mediated NF-κB activation and induction of pro-inflammatory cytokines[37]. Our study shows that PCA significantly decreased the production of IL-1β, IL-6, TNF- α protein expression in EtOH-induced rat. Previous study reported that PCA from *Oldenlandia diffusa* inhibits inflammatory cell infiltration by suppressing of TNF-α and IL-6 in rats of arthritis model[53]. Moreover, PCA could possibly inhibit lipo polysaccharides mediated several transduction factors such as MAPKs, NF-κB and cytokine productions in experimental animals[54]. Thus, decreased levels of these cytokines would diminish further activation of the NF-κB translocation and thus alleviate inflammatory response.

Thus chronic inflammation could be associated with apoptosis, which is important for understanding the development of therapeutic drugs for specific organ damage[55]. Chronic oxidative stress induces imbalance in Bax/Bcl-2 ratio and dysfunction of the apoptotic mechanism leading to cell death[56]. Liu *et al*[6] documented that EtOH could cause oxidative stress-mediated apoptotic cell death by elevated pro-apoptotic (Bax, caspase-3) and decreased anti-apoptotic (Bcl-2) protein expression. Another study evidenced that chronic EtOH exposure induced overproduction of free radical, and caused DNA strand breaks in the experimental model[57]. Increased Bax, caspase-3 and decreased Bcl-2 expression in EtOH-induced experimental rats were observed in our study. In the other hand, supplementation of PCA significantly decreased Bax, caspase 3 and increased anti-apoptotic molecule Bcl-2 in EtOH-induced rats. A previous study documented that, plant-derived phenolic compound like fisetin inhibits alcohol-induced oxidative stress, lipid peroxidation and hepatic apoptotic cell death[58]. Another research documented that polyphenolic agents effectively inhibit oxidative stress and cytokine-mediated apoptosis in the different types of experimental model[59,60].

5. Conclusion

Herein, we conclude that the PCA can ameliorate EtOH mediated nephrotoxicity. Chronic EtOH-induction increases oxidative stress by elevating lipid peroxidation, and decreasing antioxidants in rats. Furtherly, this oxidative stress causes kidney tissue damage response to the production of pro-inflammatory cytokines and NF-κB signaling activation, subsequently induces inflammation and apoptosis in EtOH-induced rats. Our data evidenced that PCA effectively inhibits oxidative stress cytokine production and NF-κB transcriptional activation, thereby inhibits inflammation and apoptosis in EtOH-induced rats. Based on this evidence, PCA has strong anti-inflammatory and anti-apoptotic potential and it may be used to treat alcohol-related disease.

Conflict of interest statement

Authors declare that there are no competing interests.

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