Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2016, 8(22):38-45
(http://www.derpharmachemica.com/archive.html)

Immunohistochemical Analysis of a Smooth Muscle Actin in Nephrotoxicity Induced Via CCl_4 in Rat Model: Role of Some Antioxidants

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ABSTRACT

NF-κB modulates coding genes for adaptive, innate immunity and inflammation. Some toxins activates α smooth muscle actin (α SMA), a marker of kidney myofibroblast, which is enhanced by Smads over expression. The current study estimated the efficacy of silymarine (SIL) in combination with dandelion (DA) and (-)-epigallocatechin-3-gallate (EGCG) against kidney damage induced via CCl4 in rats. This is demonstrated by the suppression of α-SMA, Smad-2 and NF-κB protein expressions in renal tissue. CCl4 was injected as a single intraperitoneal dose, followed by one month treatment with SIL, DA and EGCG. Serum urea and creatinine levels were significantly increased upon CCl4 intoxication whereas, uric acid level was slightly affected compared to the control values. Immunohistochemical analysis of kidney sections revealed that CCl4 induced an increase in immune response of NF-κB, α-SMA and Smad-2 protein expressions. While, treatment with the aforementioned natural products either alone or in combination showed mild immune reactivity in blood vessels and glomeruli with the combination regimen of the three antioxidants showing the most significant effect. This was also confirmed by histopathological examinations.

Keywords: (-)-Epigallocatechin-3-gallate, α-Smooth Muscle Actin, Smad-2, Dandelion, Immunohistochemistry

INTRODUCTION

Carbon tetrachloride (CCl_4) is a well-established hepatotoxic [1]. Additionally, it was demonstrated that liver is not the only target organ of CCl_4 and it causes free radical generation in other tissues like the kidney, lung and heart [2]. CCl_4 induces acute and chronic renal injuries. Silymarin was well known as a potent hepatoprotective agent but there are few studies concerning the protective effect of silymarin (SIL) on kidney functions [3] so the present study was designed to estimate the therapeutic role of silymarin, dandelion (DA) and EGCG either alone or in combination and their ability to elucidate novel mechanism as opposing apoptosis or attenuating the altered molecular signaling pathways such as NF-κB, α-SMA and Smad-2 which are targeted in the current study.

Nuclear factor-κB (NF-κB) is a protein complex profound in all animal cells. It controls DNA, cell survival and transcription of cytokines [4].

NF-κB including NF-κB1 p50/p105 and NF-κB2 p52/p100 are proteins functioning as dimeric transcription factors controlling the gene expression of innate and adaptive immunity, inflammation, B-cell development and stress responses. Disturbance in NF-κB was related to cancer, autoimmune and inflammatory diseases [5].

TGF-β, signaling via Smad-2, plays a crucial role in the morphological maturation of renal my fibroblasts [6]. Smad-2 over expression in addition to TGF-β induced more focal adhesions and elevated α-smooth muscle actin (α-SMA) organization in stressed fibers. A-SMA a marker of myo-fibroblast was over expressed in diabetic rats kidney [7].

High dose of CCl_4 could induce oxidative stress, inflammation and cellular necrosis leading to tissue injury and apoptosis. CCl_4 increased free radical production that may have a crucial role in tissue degeneration. CCl_4 elucidate its toxicity either via the production of ROS ($CCl_3OO\cdot$ & $CCl_3\cdot$) through NADPH–cytochrome P450 pathway leading to lipid peroxidation or via the activation of macrophages which produces profibrogenic and inflammatory markers [1]. Maintaining the balance between free

radicals and antioxidants is therefore important as well as inhibiting inflammatory mediators may serve as major mechanisms in preventing deleterious impact of CCl_4.

Silymarin has metabolic and cell-regulating effects, it regulates cell membrane permeability, inhibits 5-lipoxygenase pathway, scavengers ROS of the R-OH type and controls DNA-expression [8].

Dandelion (DA) (Taraxacum officinal) can treat cancers, hepatitis, and digestive diseases. DA extracts have antioxidant, anti-inflammatory and anti-apoptotic activity with no side effects, and is a potent antifibrogenic agents [9,10].

The protective effects of (-)-epigallocatechin-3-gallate (EGCG), a green tea polyphenol, against renal interstitial fibrosis in mice was previously observed. EGCG mitigated tubular, glomerular injury and alleviated renal fibrosis in mice. Furthermore, EGCG down regulated macrophage infiltration and inflammatory cytokine production. EGCG also caused an up-regulation in α-SMA expression correlated to TGF-β1 and Smad-2 phosphorylation [11].

MATERIALS AND METHODS

Chemicals

All chemicals used were of high analytical grade, obtained from Sigma and Merck companies. SIL, DA and EGCG were obtained from Sigma Chemical Co. (Sigma, St. Louis, MO, USA).

Animals and treatments

Ninety male Wister albino rats, weighing 190-200 g, obtained from the animal house of National Research Center were used in this study. Animals were housed in cages kept at standardized conditions (22 ± 5°C, 55 ± 5% humidity, and 12 h light/dark cycle). They were allowed free access to water and pelleted standard chow diet.

All procedures relating to animal care and treatments strictly adhered to the ethical procedures and policies approved by Animal Care and Use Committee of National Research Center (12-038), and complied with the Guide for Care and Use of Laboratory published by the US National Institute of Health.

The rats were fasted overnight before treatment and were divided randomly into nine groups, of 10 rats each:

Group 1: Normal control rats.

Group 2: CCl_4-intoxicated rats.

Group 3: CCl_4-intoxicated rats treated with Silymarin alone.

Group 4: CCl_4 intoxicated rats treated with Dandelion.

Group 5: CCl_4 -intoxicated rats treated with EGCG.

Group 6: CCl_4 -intoxicated rats treated with Silymarin and Dandelion.

Group 7:CCl_4 -intoxicated rats treated with Silymarin and EGCG.

Group 8: CCl_4 -intoxicated rats treated with Dandelion and EGCG.

Group 9: CCl_4 -intoxicated rats treated with Silymarin, Dandelion and EGCG

A single dose of CCl_4 was injected intraperitoneally 1 ml/kg body in corn oil [11]. Silymarin (200 mg/Kg/day) [8], Dandelion (200 mg/Kg/day) [9] and EGCG (200 mg/Kg/day) [11,12] were orally given daily for 30 days, 24 h after CCl_4 administration [13]. After the end of the experimental period, blood samples were collected and kidney tissues were separated. Serum was separated by centrifugation at 3000 rpm (1006 g) for 10 min and used for biochemical serum analysis. A portion of kidney tissue was kept under 4% formalin for histopathological as well as Immunohistochemical analysis.

METHODS

Determination of serum urea, creatinine and uric acid

Serum urea, creatinine and uric acid were determined, following the instructions of the manufacturer. Diagnostic kits were purchased from Randox Company Chemical CO [12].

Histopathology study

Deparaffinized sections of 4 μm were stained with hematoxylin and eosin (H&E) and examined under light microscope [13].

Immunohistochemical analysis of α-SMA, Smad-2 and NF-ƙB.

Immunostaining of kidneys paraffin sections for detection of the abnormal immune reaction of different primary antibodies (α-SMA, Smad-2, and NF-κB) was performed using streptavidin-biotinylated horseradish peroxidase (S-ABC) method (Novalink Max Polymer detection system, Novocastra). The procedure involved endogenous peroxidase activity was inhibited by 3% H_2O_2

in distilled water for 5 min, and then the sections were washed in Tris-buffered saline (TBS) (Sigma, T 5030-100 TAB, PH 7.6) for 10 min. Non-specific binding of antibodies was blocked by incubation with protein block for 5 minutes (Novocastra). Sections were incubated with rabbit polyclonal or mouse anti-rat primary antibodies diluted 1:500 for 1 hour at room temperature. Sections were washed in Tris buffer for 3 times each for 3 minutes, incubated with biotinylatedantirabbit IgG (Novocastra) for 30 min. Then it was followed by washing in Tris buffer 3 times, each for 3 minutes, and then incubated with Novolink polymer (Novocastra) for 30 min. Then sections were washed in Tris buffer for 3 times, each for 3 minutes. Peroxidase was detected with the working solution of Diaminobenzidine (DAB) substrate (Novocastra) for 10 minutes. Finally, sections were washed in distilled water for 10 minutes, nuclei were stained with Mayer's hematoxylin and sections were mounted in DPX. For negative control sections, the same procedure was followed with the omission of incubation in primary antibodies [14-16].

Statistical analysis

Data were expressed as means ± S.E.M. Statistical analysis was performed using Instat-3 computer program (Graph pad software Inc, San Diego, CA, USA). One way analysis of variance (ANOVA) by SPSS 12 program followed by Post HOC test was used to determine the differences between means of different groups. The level of significance was set at $p<0.05$ using Tukey's test.

RESULTS

Modulation of kidney function

The present study revealed that serum urea, uric acid and creatinine levels were significantly elevated in the CCl_4 intoxicated group ($p<0.001$), while the supplement of SIL either alone or in combination with DA and/or EGCG significantly downregulated the elevation of the kidney function biomarkers compared with CCl_4 intoxicated group (Table 1). Co-administration of the combination of the three antioxidants was the most effective one.

Table 1: Effect of Silymarin (SIL), Dandelion (DA) and Epigallocatechin gallate (EGCG) on serum urea, creatinine and uric acid levels in renal tissues of CCl_4 intoxicated rats

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)
Control	42.3 ± 3.7	0.32 ± 0.04	5.9 ± 0.18
CCl_4	100.3 ± 6.1***	1.44 ± 0.07***	8.7 ± 0.26***
SIL	45.5 ± 3.9#	0.46 ± 0.03###	7.8 ± 0.023
DA	44.6 ± 4.5#	0.44 ± 0.04###	6.6 ± 0.72
EGCG	45.9 ± 4.5#	0.56 ± 0.06###	7.3 ± 0.55
SIL+DA	46.8 ± 2.9#	0.54 ± 0.02###	7.3 ± 0.66
SIL+ EGCG	42.3 ± 3.7#	0.42 ± 0.01###	6.8 ± 0.72
EGCG+DA	44.5 ± 5.t#	0.45 ± 0.31###	6.7 ± 0.33
SIL+ EGCG+DA	43.3 ± 2.9#	0.43 ± 0.00###	7.4 ± 0.29

Values are expressed as mean ± S.E.M. (n=10); P-Value <0.05 is considered significant. Groups having *** are significantly different from control, while those having # are significantly different from CCl_4 intoxicated group

Modulation of histomorphological examination

Figures 1 and 2 shows H & E stained kidneys sections.

CCl_4 intoxication induced a marked renal fibrosis with a marked deposition of fibrous tissue and multiple foci of fibrosis. Kidney of rats exposed to CCl_4 and received Silymarin, DA and EGCG, showed a marked diminution of the abnormal of fibrous tissue. Kidney sections from rats exposed to CCl_4 and received the combination of either two or three of the tested antioxidants showed apparently normal kidney architecture.

Modulation of immunohistochemical analysis

Figures 3 and 4 shows anti-alpha smooth muscle immune-stained liver sections of rats' kidney tissue. Normal control rats showed weak immune reaction. While rats that received CCl_4 revealed renal fibrosis with strong intense immune positivity between renocytes. While kidneys exposed to CCl_4 and received SIL demonstrated a decrease of the abnormal immune reaction that becomes focally dispersed in few renocytes. Rat kidney exposed to CCl_4 and received DA showed marked a decrease in the immune reaction. While Rat kidney exposed to CCl_4 and received EGCG demonstrated a decrease of immune reaction but still there are many renocytes showed an intense immune reaction?

Rats exposed to CCl_4 and administered SIL and DA demonstrated mild renocytes immune reaction. While rats exposed to CCl_4 and received SIL and EGCG demonstrated moderate immune reaction in the renocytes. Rats exposed to CCl_4 and EGCG and Dan showed increased intensity of the normal distribution of the immune reaction. Whereas rats exposed to CCl_4 and received a mixture of SIL, DA and EGCG demonstrated a mild increase of the intensity of the normal vascular immune reaction.

Figures 5 and 6 shows anti Smad-2 immunostained in kidney sections of rats. Kidney sections from control rats showed a weak immune reaction. While rats received CCl_4 demonstrated renal degeneration with focal areas of strong intense immune positivity of the nuclei of renocytes. However, rat kidneys exposed to CCl_4 and received SIL, dandelion and EGCG each one alone showed a

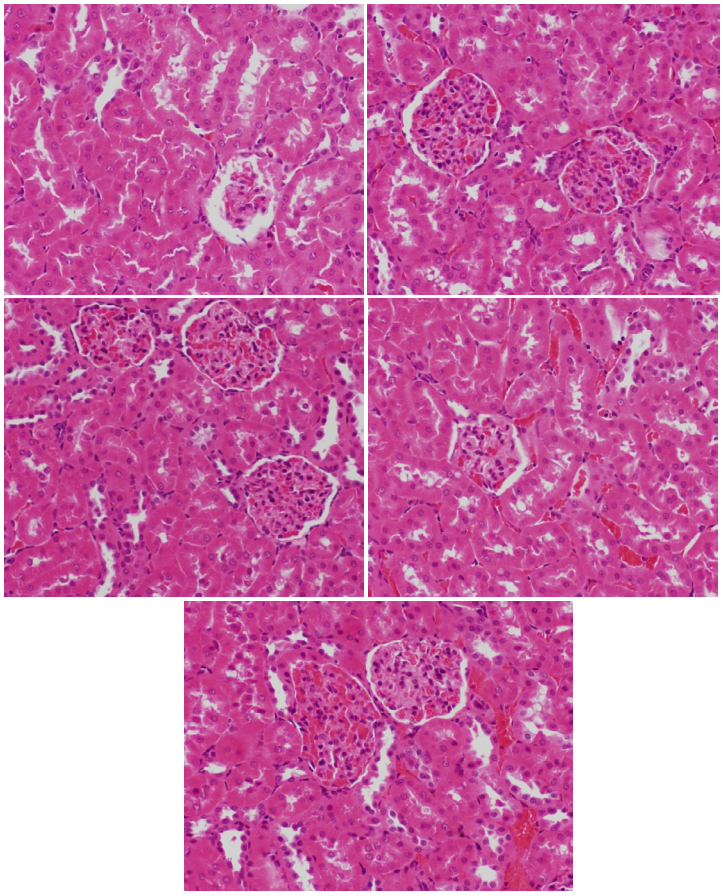

Figure 1: H&E stained kidney sections of rats, (A) received CCl_4 showing scattered patches of tubular epithelial degeneration (arrows) and glomerular sclerosis (star) (B) kidney tissue from normal control rat with normal tubules (arrow) and normal renal corpuscle (star) (C) kidney from rat exposed to CCl_4 and received SIL showing improvement of tubular degeneration (arrow) and glomerular sclerosis (star). (D) kidney section from rat exposed to CCl4 and received DA showed healing of tubular degeneration except of few cells with pyknotic nuclei (arrow) and mild improvement of the glomerular sclerosis (star). (E) Kidney section from rat exposed to CCl_4 and received EGCG showing marked improvement of tubular degeneration (arrow), and healing of the sclerosis (star) (X400)

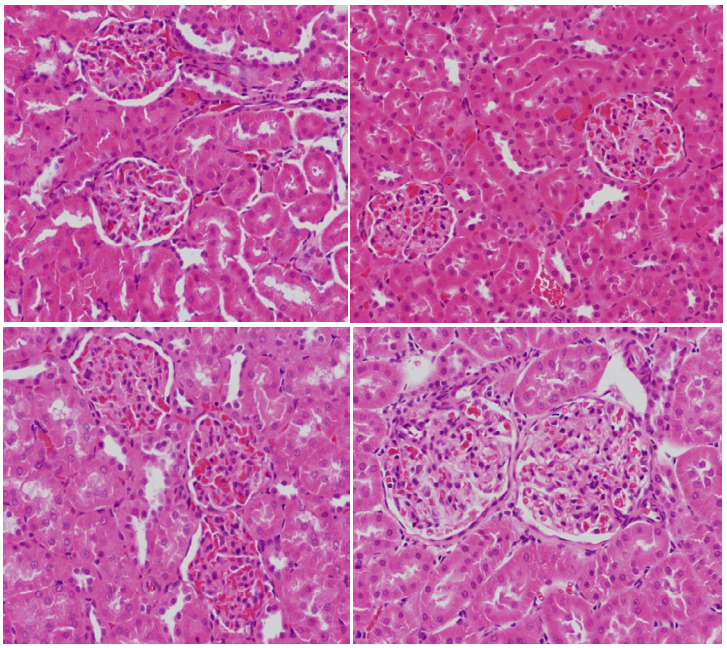

Figure 2: Showing H&E stained sections of kidney from rat exposed to CCl_4 and administered SIL and DA (F), (G) rat exposed to CCl_4 and received SIL and EGCG, (H) rat exposed to CCl_4 and EGCG and DA showing disappearance of most of tubular degenerations (arrows), and glomerular sclerosis while (I) represent liver section of rat exposed to CCl_4 and received SIL, EGCG and DA showing disappearance of tubular degeneration (arrow) and renal corpuscle (X400)

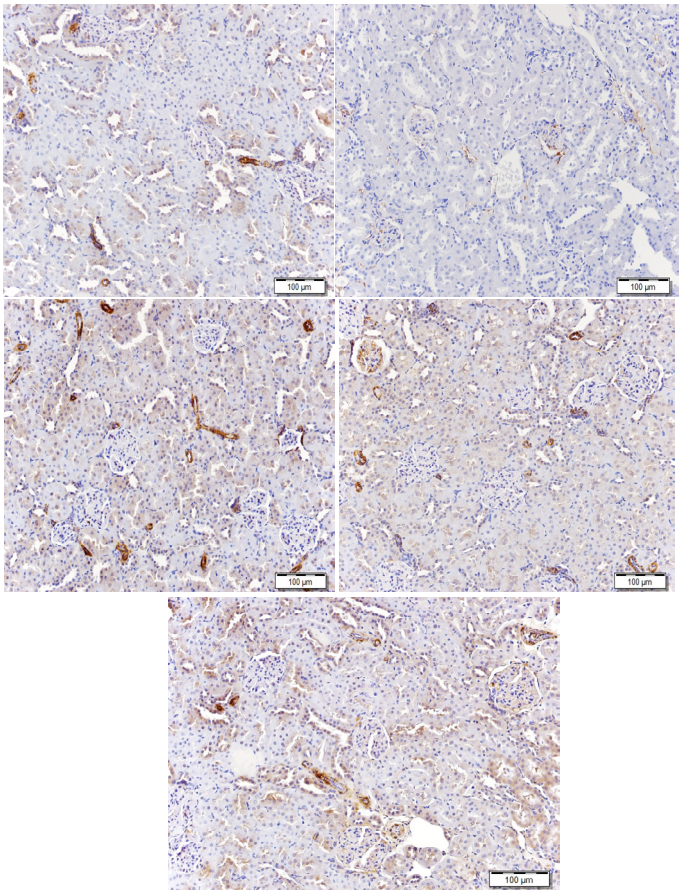

Figure 3: Showing anti *α*- SMA immunostained kidney sections of rats (A) received CCl_4 showing strong intense immune reaction of the wall of many blood vessels (arrows), and cells of some glomeruli (arrow). (B) Kidney tissue from normal control rat showing normal weak immune reaction of few blood vessels (arrow) and no reaction of glomerular cells (star). (C) Kidney from rat exposed to CCl_4 and received SIL, (D) kidney section from rat exposed to CCl_4 and received DA, and (E) kidney section from rat exposed to CCl_4 and received EGCG showing decrease of the abnormal immune reaction of the glomeruli (stars) while the wall of the interstitial blood vessels show strong immune reaction (arrows). Scale bar=100 µm

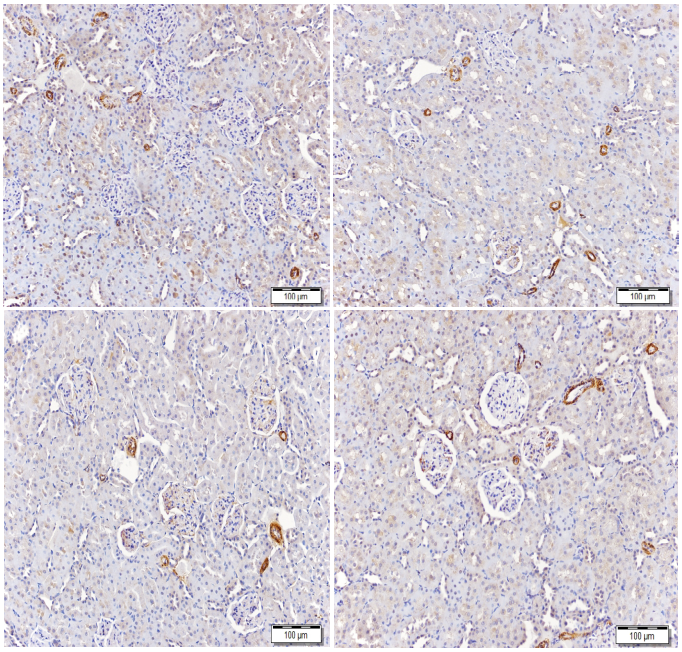

Figure 4: Showing anti α-smooth muscle (α-SMA) immune stained sections of kidney from rat (F) exposed to CCl_4 and administered SIL and DA, (G) rat exposed to CCl_4 and received Silymarin and EGCG (H) rat exposed to CCl_4 and EGCG and DA, (I) rat exposed to CCl_4 and received mixture of SIL, DA and EGCG demonstrating mild decrease of immune reaction of smooth muscles of blood vessels (arrows) glomeruli (stars). Scale bare=100 µm.

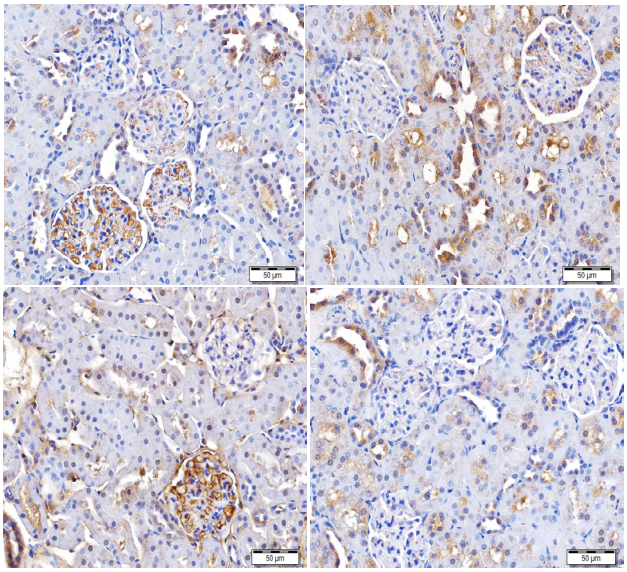

Figure 5: Showing anti NF-κB immune stained sections of kidney from rat (F) exposed to CCl_4 and administered SIL and DA demonstrating few tubules with positive immune stained cells (arrow) while there are many glomeruli with positive immune reaction (stars) in addition to few glomeruli with negative reaction (red triangle). (G) Rat exposed to CCl_4 and received SIL and EGCG also shows many tubules with positive immune reaction (arrows), glomeruli without positive reaction (red triangle) and some immunopositive glomeruli (star). (H) Rat exposed to CCl_4, EGCG and DA showed some glomeruli with positive immune reaction (star), some glomeruli with intense immunopositivity (red triangle), while few tubules show immune reactivity (arrow). (I) Rat exposed to CCl_4 and received mixture of SIL, DA and EGCG showing few tubules with weak immune reaction (arrow) while glomeruli have no positivity (red triangles). Scale bare=50 µm

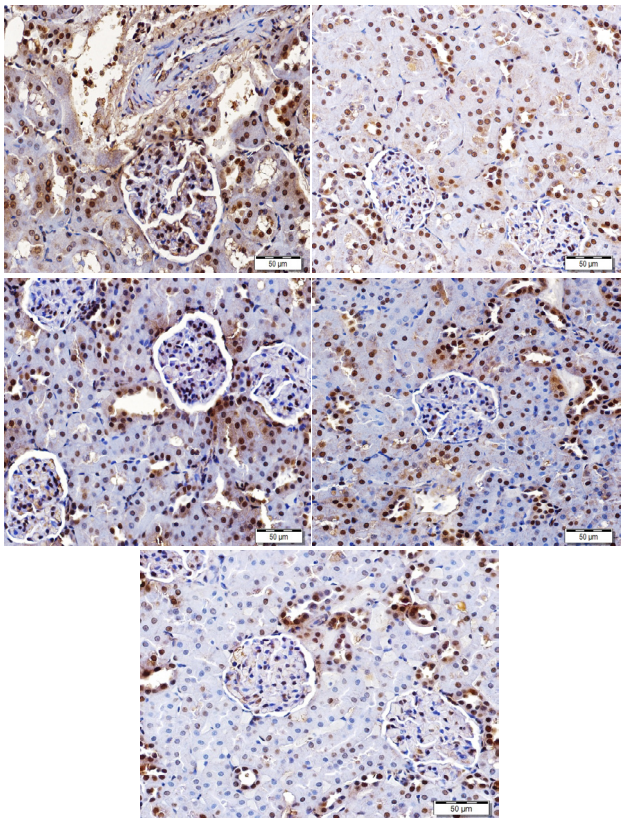

Figure 6: Showing anti Smad2 immune stained sections of kidney from rat (A) exposed to CCl_4 demonstrating strong positive immune reaction of the nuclei and cytoplasm of tubular epithelial cells (arrows). Many cells of the glomeruli show positive reaction of both nuclei and cytoplasm (star). (B) Kidney from normal control rat shows moderate immune reaction of nuclei of some tubular epithelial cells (arrow), while the glomerular cells have not immune reactivity (red triangle). (C) Kidney section from rat exposed to CCl_4 and received SIL shows decrease of immune reaction of tubular epithelial cells (arrow) and appearance of many glomeruli without immune reaction (red triangle), but still there are few glomeruli show immune reaction (star). (D) Kidney from rat exposed to CCl_4 and received Dand showing absence of immune reactivity of the glomeruli (red triangle) and decrease of tubular epithelial cells positivity (arrow). (E) kidney from rat exposed to CCl_4 and received EGCG showing marked decrease of tubular epithelial cells immune positivity (arrow) while the glomeruli have no immune reaction (red triangle). Scale bare=50 µm.

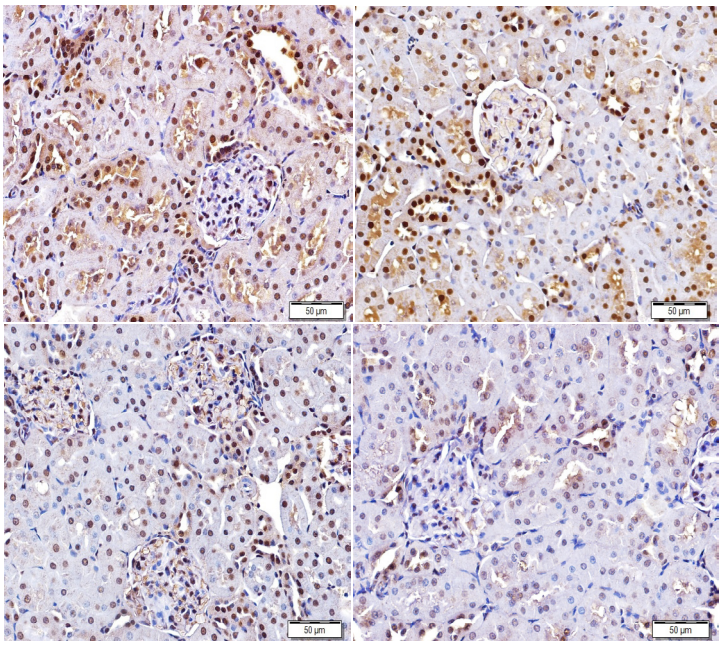

Figure 7: Showing anti Smad2 immune stained sections of kidney from rat (F) exposed to CCl_4 and administered SIL and DA demonstrating many tubules with positive epithelial cells nuclei and cytoplasmic immune reactivity (arrow) while there is decrease of glomerular immune reaction (star). (G) Rat exposed to CCl_4 and received Silm and EGCG showed moderate decrease of tubular epithelial cells immune reaction (arrow), also the glomeruli show few nuclei with positive immune positivity (star). (H) Rat exposed to CCl_4 and EGCG and DA show many tubules without immune reaction (arrow) and glomeruli with weak immunopositivity (star). (I) kidney from rat exposed to CCl_4 and received mixture of SIL, DA and EGCG showing very few tubules with positive immune reaction of the nuclei (arrow, while the glomeruli show negative immune reaction (red triangle). Scale bare=50 µm.

decrease of the abnormal immune reaction that become focally dispersed in few renocytes nuclei. Moreover, rats exposed to CCl_4 and administered the combination of two antioxidants demonstrated moderate immune positivity of renocytes nuclei and cytoplasm while rats exposed to CCl_4 and received the combination of three antioxidants SIL, DA and EGCG showed few renocytes with a positive immune reaction of the nuclei while their cytoplasm's have no reaction.

Figure 7 show anti Smad-3 immunostained liver sections. Kidney sections from control rats showed a weak immune reaction. While rats received CCl_4 demonstrated renal degeneration with focal areas of strong intense immune positivity of the nuclei of renocytes. However, rat kidneys exposed to CCl_4 and received SIL, dandelion and EGCG each one alone showed a decrease of the abnormal immune reaction that become focally dispersed in few renocytes nuclei. Moreover, rats exposed to CCl_4 and administered the combination of two antioxidants demonstrated moderate immune positivity of renocytes nuclei and cytoplasm while rats exposed to CCl_4 and received the combination of three antioxidants SIL, Dan and EGCG showed few renocytes with a positive immune reaction of the nuclei while their cytoplasm's have no reaction.

DISCUSSION

The production of inflammatory mediators and irreversibly binding to reactive aldehydes, nucleic acid hypomethylation, and loss of calcium are possible mechanisms of CCl_4 toxicity [4].

The present study revealed that CCl_4 significantly elevated kidney diagnostic markers including urea, creatnine and to a lesser extent uric acid. However, treatment with EGCG, dandlion and Silymarin significantly reduced their levels with the combination regimen showing the most significant effect.

In harmony, CCl_4-induced nephrotoxicity is a dose-dependent manner, indicated by both diagnostic indicators of kidney damage and histopathological analysis and induced a profound elevation in ROS and oxidative stress, as evidenced by the increase of lipid peroxidation level and the depletion of the total antioxidant capacity (TAC) level in the kidney. Furthermore, protein expression by Western blot analysis showed that CCl_4 significantly elevated the production of renal interleukin-6 and tumor necrosis factor-α [2].

Previous study demonstrated that SIL has metabolic and cell-regulating effects, namely regulation of cell membrane permeability, inhibition of the 5-lipoxygenase pathway, scavenging ROS, via suppression of nuclear factor (NFκB). The protective effect of silymarin on kidney cells against oxidative damage was previously demonstrated. That protection occurs via elevating the thiol status (GSH) in the kidney [3].

DA is a member of the Asteraceae family; treat liver, gallbladder, kidney, and joint problems. DA is traditionally considered an alternative medicine and is used as diuretic in the treatment of eczema and cancer.

Dan extracts have been used to treat cancers, hepatitis, and digestive diseases. Dan is shown to have anti-inflammatory, antioxidant, and anticarcinogenic activities [17].

There is good evidenced from previous studies that EGCG have a role in protection against degenerative diseases. It was reported that EGCG has protective effects of against biomarkers for cancer, cardiovascular disease, and other degenerative diseases. EGCG could act as antitumorigenic agents and as immune modulators in immune dysfunction caused by transplanted tumors or by carcinogen treatment. EGCG relieves hepatoma- and prevents hepatoxicity. It modulates lipid and glucose metabolism in type 2 diabetes, and could alleviate kidney diseases [11].

Here in, it was observed that treatment with EGCG, Dandelion and Silymarin either alone or in combination significantly down regulated renal immunohistochemical analysis of NFKB, Smad-2 and α-SMA as compared to CCl_4 intoxicated rats.

It was previously reported that CCl_4 at a high dose often causes cellular necrosis, oxidative stress and inflammation which leads to acute tissue injury and apoptotic organ failure [4]. The toxicity of CCl_4 resulting from increased free radical production may play an important role in degenerative processes in the tissues. The toxicity of CCl_4 includes two steps. The first one is the production of free radicals (CCl_3· and CCl_3OO·) through the metabolism of NADPH – cytochrome P450 system, which induces lipid peroxidation. The second phase involves the activation of tissue macrophages which is accompanied by the production of inflammatory and profibrogenic mediators [1].

TGF-β, signaling via Smad-3, plays an important role in the morphological and functional maturation of renal my fibroblasts. Smad-2 and Smad-3 signal via independent pathways. Smad3-overexpressing cells as well as TGF-β produce more focal adhesions and increased (α-SMA) organization in stress fibers, and Smad-3 also regulates cytoskeletal organization in HSC [6].

Previous studies revealed that the appearance of α-smooth-muscle-actin (α-SMA)-positive cells during renal fibrosis induced by CCl_4 injection [7].

Conclusion: that co-administration of DA along with SIL and EGCG demonstrates a mild immune reactivity of the kidney against the overexpression α Smooth Muscle actin and Smad-2 which may lead to cell death. So this combination is a potential candidate for the suppression of the fibrotic process and hence cell death so it can be applied as a potent anti-fibrotic agent.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ACKNOWLEDGMENT

Is directed to Dr. Nermin Ali, professor of pathology, pathology department faculty of dentist, Cairo university for histopathological aid.

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