

# Protective Effects of Silymarin, Alone or in Combination with Chlorogenic Acid and/or Melatonin, Against Carbon Tetrachloride-induced Hepatotoxicity

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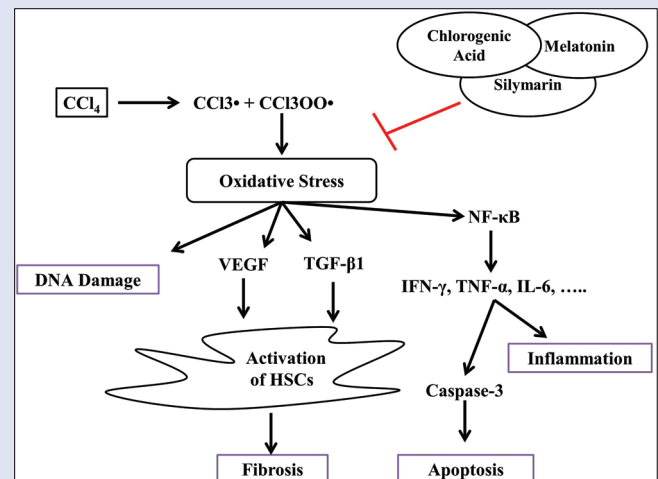
## ABSTRACT

**Objective:** The aim of this study was to evaluate the hepatoprotective effects of silymarin (SIL), alone and combined with chlorogenic acid (CA) and/or melatonin (ME), using a rat model of carbon tetrachloride (CCl<sub>4</sub>)-induced injury. **Materials and Methods:** Hepatotoxicity was induced by a single dose of CCl<sub>4</sub> (1 ml/kg, IP). One day after, rats were received SIL (200 mg/kg) alone or in combination with CA (60 mg/kg) and/or ME (20 mg/kg) for 21 days. **Results:** SIL significantly decreased serum alanine aminotransferase, inflammatory cytokines, and vascular endothelial growth factor levels. Histological alterations, fibrogenesis, oxidative DNA damage, inflammatory mediators, and caspase-3 activity were significantly attenuated in SIL treated CCl<sub>4</sub>-intoxicated rats. On the other hand, cytochrome P450 2E1 activity showed a significant decrease in the liver of CCl<sub>4</sub>-intoxicated rats, an effect that was reversed following treatment with SIL. All beneficial effects of SIL were markedly potentiated when combined with CA and/or ME. **Conclusions:** These data indicate that SIL, alone and combined with CA and/or ME, protected the liver against CCl<sub>4</sub>-induced hepatotoxicity via attenuating inflammation, oxidative DNA damage, apoptosis, and fibrotic changes. The significantly intensified hepatoprotective effects of SIL when combined with both CA and ME suggest a possible synergism. These synergistic effects need to be further confirmed using detailed studies.

**Key words:** Carbon tetrachloride, chlorogenic acid, inflammation, melatonin, silymarin

## SUMMARY

- Silymarin, chlorogenic acid and melatonin possess *in vivo* hepatoprotective activity
- Silymarin, chlorogenic acid and melatonin attenuate fibrogenesis, oxidative DNA damage, inflammation and apoptosis
- Chlorogenic acid and melatonin enhance the hepatoprotective effect of silymarin.



**Abbreviations used:** SIL: silymarin, CA: chlorogenic acid, ME: melatonin, CCl<sub>4</sub>: carbon tetrachloride, CYP2E1, cytochrome P450 2E1, ALT: alanine aminotransferase, IL-6: interleukin 6, IFN-γ: interferon gamma, VEGF: vascular endothelial growth factor, TNF-α: tumor necrosis factor alpha, CRP: C-reactive protein, 8-Oxo-dG: 8-Oxo-2'-deoxyguanosine, TGF-β1: transforming growth factor beta 1, HSCs: hepatic stellate cells.

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## INTRODUCTION

The liver plays a central role in many important body functions such as metabolism and bile secretion. Through detoxification and elimination, liver protects the body from exposure to toxic substances and drugs. Therefore, a healthy liver is essential to overall body health.<sup>[1]</sup> Excessive exposure of the liver to environmental toxins, drug overdose, alcohol, and chemotherapeutic agents can cause hepatotoxicity.<sup>[2]</sup> Hepatotoxicants are exogenous compounds that cause liver injury. Hepatotoxicants may include industrial chemicals, certain drugs, microcystins, dietary supplements, and herbal remedies.<sup>[3,4]</sup> Carbon tetrachloride (CCl<sub>4</sub>)-induced liver

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injury results from the toxic metabolites of  $\text{CCl}_4$  that cause centrilobular hepatic necrosis and steatosis and impair essential cellular processes.  $\text{CCl}_4$  is biotransformed by the cytochrome P450 system in liver microsomes, producing trichloromethyl free radical ( $\text{CCl}_3\bullet$ ) that can react with cellular molecules and impair crucial cellular processes.<sup>[5]</sup> In the presence of oxygen,  $\text{CCl}_3\bullet$  is converted to trichloromethylperoxy radical ( $\text{CCl}_3\text{OO}\bullet$ ) resulting in oxidative stress, lipid peroxidation, and loss of membrane integrity.<sup>[6]</sup> Therefore, antioxidant molecules might have the capacity to protect against  $\text{CCl}_4$ -induced hepatotoxicity.

Silymarin (SIL) is a flavonoid complex extracted from the seeds of *Silybum marianum* (milk thistle). It contains 4 the isomeric flavonoids silibinin, silydianin, isosilibinin, and silychristine.<sup>[7]</sup> SIL has been well demonstrated to exert multiple beneficial effects and thus used as a natural remedy for the treatment of hepatitis, jaundice, and cirrhosis. It protects against liver injury induced by radiation, alcohol abuse, ischemia, iron overload, environmental toxins, and  $\text{CCl}_4$ .<sup>[8-10]</sup> The antioxidant, anti-inflammatory, anti-apoptotic, and immunomodulating effects of SIL have also been reported.<sup>[11]</sup>

Chlorogenic acid (CA) (3-caffeoyl-D-quinic acid) is a polyphenolic compound found in coffee, beans, apples, potatoes, and other agricultural products. It is formed by esterification of quinic and caffeic acids.<sup>[12]</sup> Studies have demonstrated that CA exhibits multiple biological properties, including antioxidant, anti-inflammatory, anti-carcinogenic, anti-bacterial, and cholesterol lowering activities.<sup>[13-16]</sup> In addition, CA showed protective effects against lipopolysaccharide (LPS)<sup>[17]</sup> and acetaminophen<sup>[18]</sup> induced liver injury.

Melatonin (ME) or N-Acetyl-5-methoxytryptamine, a hormone produced by the pineal gland, is known to be involved in the modulation of circadian rhythms, seasonal reproduction, and immune function.<sup>[19,20]</sup> In addition, ME plays an important role in the improvement of sleeping disorders, migraine, and cardiovascular complications.<sup>[21]</sup> ME has also been reported to prevent hemorrhagic shock<sup>[22]</sup> and  $\text{CCl}_4$ -induced liver injury in rats.<sup>[20]</sup> *in vitro* and *in vivo* studies have suggested a protective effect of ME against oxidative stress by scavenging free radicals, stimulating the synthesis of endogenous antioxidants and up-regulating the expression of intracellular antioxidant enzymes.<sup>[23,24]</sup>

The individual protective effects of SIL, CA and ME against hepatotoxicants-induced liver injury have been investigated. However, studies demonstrating the hepatoprotective efficacy of these therapeutic agents in combination are scarce. This study tests the hypothesis that CA and/or ME may have a synergistic effect with SIL. Therefore, we used a rat model of  $\text{CCl}_4$ -induced hepatotoxicity and investigated several parameters following treatment with SIL alone and combined with CA and ME.

## MATERIALS AND METHODS

### Chemicals

SIL, CA, ME and  $\text{CCl}_4$  were purchased from Sigma Chemicals (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade and obtained from standard commercial supplies.

### Experimental animals

Male Wistar rats weighing between 160 and 180 g were obtained from the Experimental Animal Center, College of Pharmacy, King Saud University (Saudi Arabia). Animals were housed in special cages at controlled temperature of 20–22°C and humidity of 60% and fed a standard rat pellet chow with free access to tap water *ad libitum*. Rats were kept for 1 week before the experiment for acclimatization. All animal procedures were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the

Animal Care and Use Committee of the College of Pharmacy, King Saud University (Saudi Arabia).

### Experimental design

Forty-eight rats were randomly allocated into six equal groups, each consisting of 8 ( $n = 8$ ) animals as follows:

Group I (Control): Rats received a single intraperitoneal injection of corn oil and the vehicle gum acacia (2% w/v) by oral gavage for 21 successive days.

Group II ( $\text{CCl}_4$ ): Rats received a single intraperitoneal injection of 1 ml/kg body weight  $\text{CCl}_4$  in corn oil (1:1) and gum acacia by oral gavage for 21 successive days.

Group III ( $\text{CCl}_4$  + SIL):  $\text{CCl}_4$ -administered rats received SIL (200 mg/kg/day) dissolved in gum acacia by oral gavage for 21 successive days.

Group IV ( $\text{CCl}_4$  + SIL/CA):  $\text{CCl}_4$ -administered rats received SIL (200 mg/kg/day) and CA (60 mg/kg/day) dissolved in gum acacia by oral gavage for 21 successive days.

Group V ( $\text{CCl}_4$  + SIL/ME):  $\text{CCl}_4$ -administered rats received SIL (200 mg/kg/day) and ME (20 mg/kg/day) dissolved in gum acacia by oral gavage for 21 successive days.

Group VI ( $\text{CCl}_4$  + SIL/CA/ME):  $\text{CCl}_4$ -administered rats received SIL (200 mg/kg/day), CA (60 mg/kg/day) and ME (20 mg/kg/day) dissolved in gum acacia by oral gavage for 21 successive days.

SIL, CA, and ME were supplemented 24 h after  $\text{CCl}_4$  administration. The doses of  $\text{CCl}_4$ , SIL, CA and ME were selected according to the studies of Feng *et al.*,<sup>[25]</sup> Huang *et al.*,<sup>[26]</sup> Shi *et al.*,<sup>[27]</sup> and Abdel-Wahhab *et al.*,<sup>[28]</sup> respectively. The dosage was balanced weekly as indicated by any change in the body weight.

### Samples collection and preparation

At the end of the experiment, blood samples were collected, left to coagulate and centrifuged at  $1000 \times g$  for 10 min to separate serum. Sera were collected and stored at  $-20^\circ\text{C}$  until used. Rats were then sacrificed by decapitation and livers were quickly excised and washed thoroughly in ice-cold saline. Liver sample from each rat was homogenized (10% w/v) in cold phosphate buffered saline. The homogenates were centrifuged at  $4000 \times g$  for 15 min at  $4^\circ\text{C}$ , and the clear supernatants were collected and stored for analysis. Other liver samples were collected on 10% neutral buffered formalin for histopathological examination or kept at  $-80^\circ\text{C}$  for DNA isolation.

### Biochemical assays

#### Determination of alanine aminotransferase activity

Serum alanine aminotransferase (ALT) activity was determined by a coupled enzyme assay using reagent kit purchased from Sigma (USA) following the instructions provided.

#### Determination of inflammatory mediators and vascular endothelial growth factor levels

Serum interleukin 6 (IL-6), interferon gamma ( $\text{IFN-}\gamma$ ) and vascular endothelial growth factor (VEGF) and liver tumor necrosis factor alpha ( $\text{TNF-}\alpha$ ) levels were measured using specific enzyme-linked immunosorbent assay (ELISA) kits purchased from R and D systems (USA) following the manufacturer's instructions. Concentrations of the assayed proteins were determined spectrophotometrically at 450 nm using BioTek  $\mu$ Quant plate reader (BioTek Instruments, Winooski, VT, USA). Standard plots were constructed using standard corresponding proteins, and the concentrations for unknown samples were calculated from the standard plots.

Liver C-reactive protein (CRP) was measured using latex-enhanced immunonephelometry on a Behring BN II automated Nephelometer (Dade Behring), according to the manufacturer's instructions. In this assay, CRP present in the sample binds polystyrene beads coated with rat monoclonal antibodies and form aggregates which scatter light. The intensity of the scattered light reflects the concentration of CRP present in the sample.

#### Determination of cytochrome P450 2E1 activity

Microsomes fraction was prepared from homogenized liver samples following the method of Benson *et al.*<sup>[29]</sup> and used for cytochrome P450 2E1 (CYP2E1) determination using p-nitrophenol as a substrate.<sup>[30]</sup> CYP2E1 activity was normalized against protein content and presented as percentage of corresponding control. Protein content was determined using Bradford protein assay.<sup>[31]</sup>

#### Determination of caspase-3 activity

Caspase-3 activity was measured using the CasPACE assay system (Promega, Madison, WI, USA) according to the manufacturer instructions. The test is based on the ability of caspase-3 to release the yellow chromophore p-nitroaniline from the substrate Ac-DEVD-pNA. Caspase-3 activity was normalized against protein content and presented as a percentage of corresponding control.

#### Determination of 8-Oxo-2'-deoxyguanosine levels

DNA was extracted from frozen liver samples using DNA purification kit (Promega, USA). Eight-Oxo-2'-deoxyguanosine (8-OxodG) levels were determined using OxiSelect™ Oxidative DNA Damage ELISA Kit where the 8-OxodG content in unknown samples was determined by comparison with predetermined 8-OxodG standard curve.

#### Comet assay

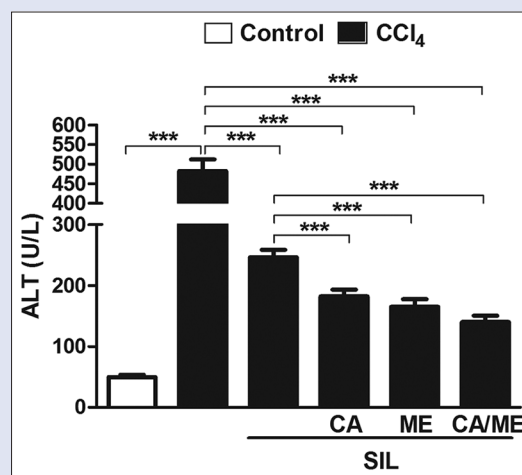
The alkaline comet assay (single gel electrophoresis) was performed according to the method of Singh *et al.*<sup>[32]</sup> Briefly, a small piece of liver was minced in cold Hank's balanced salt solution, digested with collagenase, mixed with low melting point agarose at ratio of 1:7 and spread onto microscope slides precoated with normal melting point agarose. The slides were covered with a third layer of low melting point agarose, and a cover slip was applied to spread the sample. The slides were placed in lysis buffer for 20 min and subsequently electrophoresed at 4°C in alkaline electrophoretic solution (pH >13) for 30 min at 30 v. The slides were then normalized, stained with ethidium bromide and examined using fluorescence microscope. For each sample, 100 comets were randomly selected and measured for tail length and %DNA in tail.

#### Histopathological study

Small pieces of liver were fixed by 10% neutral buffered formalin and then embedded into paraffin, sectioned (5–6 µm), and mounted on glass microscopic slides using the standard histopathological technique. The sections were stained with hematoxylin and eosin (H and E) and Masson's Trichrome stains, and then examined by light microscopy.

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA), and all statistical comparisons were made using the one-way analysis of variance test followed by Tukey's test *post hoc* analysis. Results were expressed as mean ± standard error of the mean and the value of  $P < 0.05$  was considered statistically significant.



**Figure 1:** Effects of silymarin alone or in combination with chlorogenic acid and/or melatonin on serum alanine aminotransferase activity in carbon tetrachloride-intoxicated rats. Data are mean ± standard error of the mean ( $n = 8$ ). \*\*\* $P < 0.001$ . SIL: Silymarin; CA: Chlorogenic acid; ME: Melatonin; ALT: Alanine aminotransferase; SEM: Standard error of mean

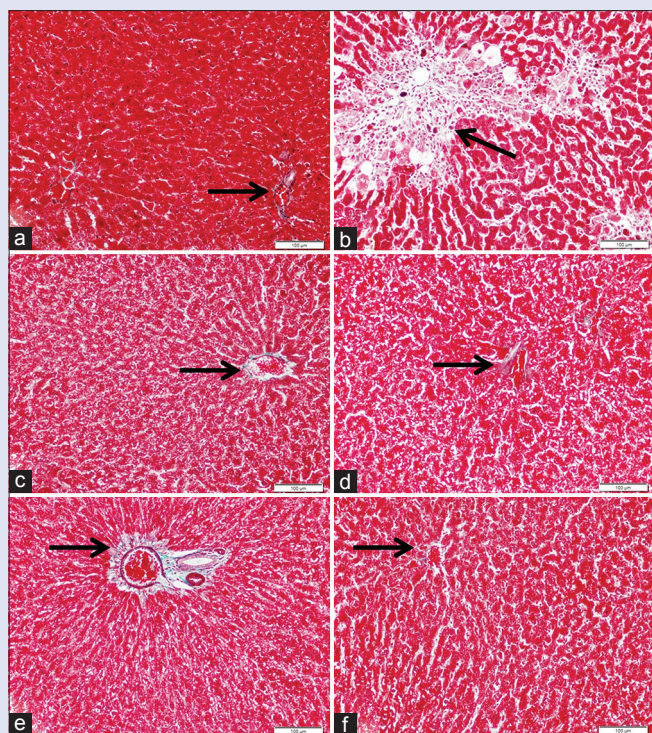
## RESULTS

### Silymarin alone or in combination with chlorogenic acid and/or melatonin prevent carbon tetrachloride-induced liver injury and fibrogenesis

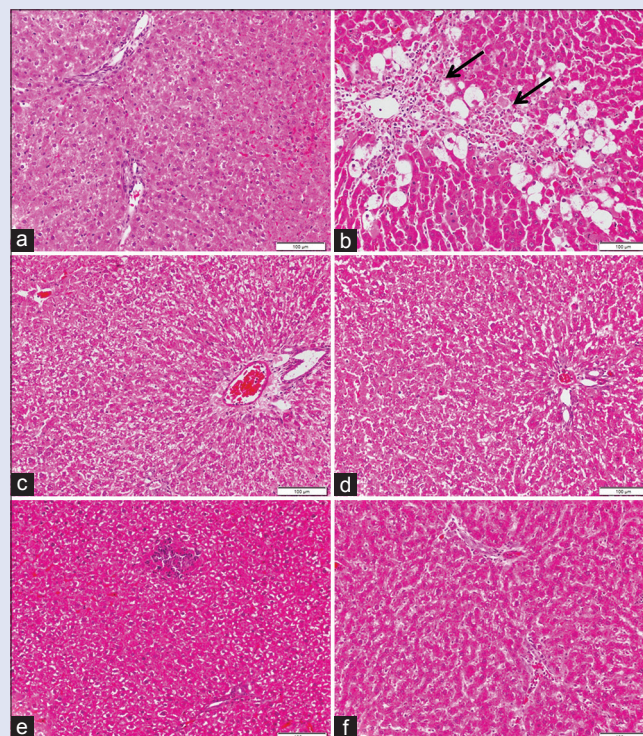
Data represented in Figure 1 show the effects of SIL alone and in combination with CA and/or ME on serum ALT activity, the main liver activity biomarker, in CCl<sub>4</sub>-intoxicated rats. Administration of CCl<sub>4</sub> produced a significant ( $P < 0.001$ ) increase in serum ALT activity when compared with the control rats. Concurrent administration of SIL significantly ( $P < 0.001$ ) decreased serum ALT activity in CCl<sub>4</sub>-intoxicated rats. Treatment of CCl<sub>4</sub>-intoxicated rats with SIL combined with CA, ME or both significantly ( $P < 0.001$ ) ameliorated serum ALT activity when compared with either CCl<sub>4</sub> or SIL treated rats.

Histopathological examination of the H and E-stained liver sections of control rats showed the normal histological structure of the hepatocytes [Figure 2a]. On the other hand, sections in the liver of rats treated with CCl<sub>4</sub> showed focal areas with massive degeneration, necrosis, and inflammatory cellular infiltration [Figure 2b]. Liver of CCl<sub>4</sub>-intoxicated rats treated with SIL revealed moderate improvement of hepatic cellular degeneration [Figure 2c]. CCl<sub>4</sub>-induced rats treated with SIL combined with CA [Figure 2d] or ME [Figure 2e] showed marked improvement of hepatocellular degeneration, but still there are scattered areas of degeneration and focal areas of cellular infiltration. Rats received CCl<sub>4</sub> and treated with SIL, CA, and ME showed apparently normal liver tissue [Figure 2f].

To demonstrate the effects of CCl<sub>4</sub> and treatment with SIL alone or combined with CA and/or ME on collagen deposition, liver sections were stained with Masson's trichrome. Control rats showed little collagen fibers, especially in the portal area [Figure 3a] while CCl<sub>4</sub>-intoxicated rats showed large patches of fibrous tissue [Figure 3b]. Treatment of the CCl<sub>4</sub>-intoxicated rats with SIL [Figure 3c] and its combination with either CA [Figure 3d] or ME [Figure 3e] decreased fibrous tissue deposition but still some scattered areas of collagen deposition in the portal area. The combined triple treatment (SIL, CA and ME) significantly reduced collagen deposition and showed apparently normal collagen distribution within the liver tissue of CCl<sub>4</sub>-intoxicated rats [Figure 3f].



**Figure 2:** Photomicrographs of H and E-stained liver sections of (a) control rats, (b) carbon tetrachloride-intoxicated rats showing focal areas with massive degeneration, necrosis and inflammatory cellular infiltration, (c) carbon tetrachloride-intoxicated rats treated with silymarin alone revealing moderate improvement of hepatic cellular degeneration, (d) carbon tetrachloride-intoxicated rats treated with silymarin and chlorogenic acid showing marked improvement of hepatocellular degeneration but still there are scattered areas of degeneration, (e) carbon tetrachloride-intoxicated rats treated with silymarin and melatonin with few areas of little hepatic cells degeneration and focal areas of cellular infiltration and (f) carbon tetrachloride-intoxicated rats treated with silymarin, chlorogenic acid, and melatonin showing apparently normal liver tissue



**Figure 3:** Photomicrographs of Masson's trichrome-stained liver sections of (a) control rats showing normal little collagen fibers specially in the portal area, (b) carbon tetrachloride-intoxicated rats showing large patches of fibrous tissue, (c) carbon tetrachloride-intoxicated rats treated with silymarin alone, (d) silymarin and chlorogenic acid, (e) silymarin and melatonin showing small patches of collagen deposition and (f) carbon tetrachloride-intoxicated rats treated with silymarin, chlorogenic acid, and melatonin showing apparently normal collagen distribution within liver tissue

### Silymarin alone or in combination with chlorogenic acid and/or melatonin reduce serum vascular endothelial growth factor levels in carbon tetrachloride-intoxicated rats

$\text{CCl}_4$ -administered rats exhibited a significant ( $P < 0.001$ ) increase in serum levels of VEGF as represented in Figure 4. Treatment with SIL or its combination either with CA or with ME significantly ( $P < 0.05$ ) decreased serum VEGF levels when compared with the  $\text{CCl}_4$ -intoxicated rats. Treatment of the  $\text{CCl}_4$ -intoxicated rats with SIL, CA and ME potentially ( $P < 0.001$ ) decreased serum VEGF. Compared with SIL alone, no significant differences on serum VEGF levels were recorded when combined with CA and/or ME.

### Silymarin alone or in combination with chlorogenic acid and/or melatonin attenuate carbon tetrachloride-induced inflammation

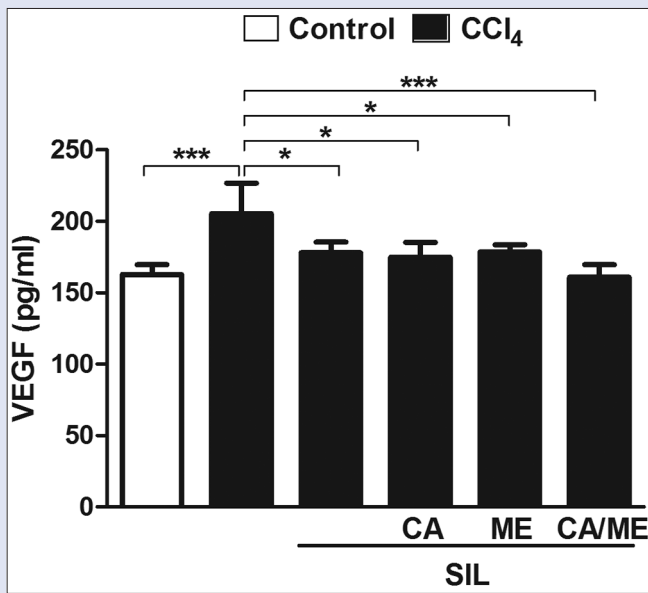
$\text{CCl}_4$ -intoxicated rats showed a significant ( $P < 0.001$ ) increase in liver levels of the pro-inflammatory cytokine  $\text{TNF-}\alpha$ , as represented in Figure 5a. Oral administration of SIL alone significantly ( $P < 0.01$ ) decreased levels of  $\text{TNF-}\alpha$  in the liver of  $\text{CCl}_4$ -induced rats. Treatment

of the  $\text{CCl}_4$ -intoxicated rats with SIL combined with CA and/or ME produced a marked decrease ( $P < 0.001$ ) in  $\text{TNF-}\alpha$  when compared with the  $\text{CCl}_4$  control rats. The combination of SIL and CA significantly ( $P < 0.05$ ) alleviated serum  $\text{TNF-}\alpha$  levels when compared with SIL alone. Treatment of the  $\text{CCl}_4$ -intoxicated rats with SIL combined with either ME or ME and CA produced a more potent effect ( $P < 0.001$ ) on liver  $\text{TNF-}\alpha$  levels when compared with SIL alone.

CRP showed a significant ( $P < 0.001$ ) increase in the liver of  $\text{CCl}_4$ -intoxicated rats when compared with the control group [Figure 5b]. Oral administration of SIL and its combination with CA and/or ME produced a significant ( $P < 0.001$ ) decrease in liver CRP levels. While the combination of SIL with CA or ME produced no significant change in liver CRP levels compared with SIL-treated group, its combination with both CA and ME significantly ( $P < 0.05$ ) reduced CRP levels.

IL-6 exhibited a significant ( $P < 0.001$ ) elevation in the serum of  $\text{CCl}_4$ -intoxicated rats when compared with the corresponding control rats [Figure 5c]. Treatment of the  $\text{CCl}_4$ -intoxicated rats with SIL significantly ( $P < 0.01$ ) decreased serum IL-6. In addition, SIL in combination with CA and/or ME produced a marked ( $P < 0.001$ ) decrease in serum levels of IL-6 in  $\text{CCl}_4$ -intoxicated rats. While its combination with either CA or ME showed a nonsignificant ( $P > 0.05$ ) effect, SIL combined with both agents significantly ( $P < 0.05$ ) decreased IL-6 levels as compared with SIL alone.

Serum  $\text{IFN-}\gamma$  exhibited nearly the same behavioral pattern.  $\text{CCl}_4$  administration produced a significant ( $P < 0.001$ ) increase in serum



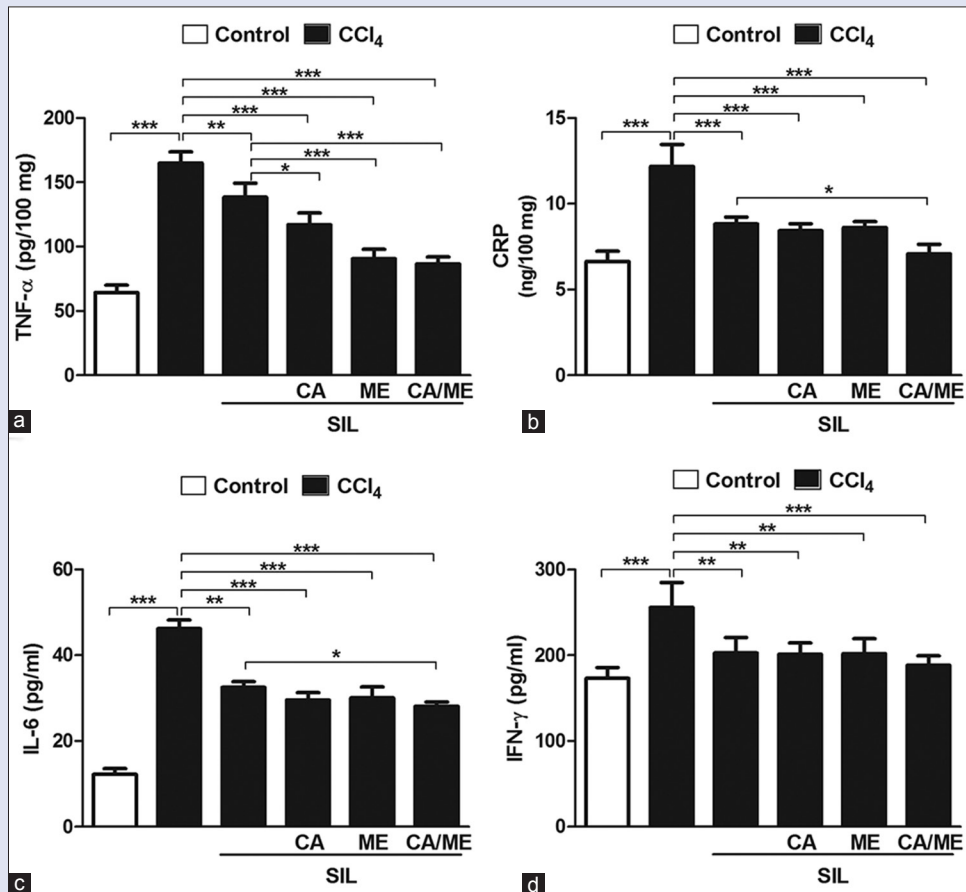
**Figure 4:** Effects of silymarin alone or in combination with chlorogenic acid and/or melatonin on serum vascular endothelial growth factor levels in carbon tetrachloride-intoxicated rats. Data are mean  $\pm$  standard error of the mean ( $n = 8$ ). \* $P < 0.05$  and \*\*\* $P < 0.001$ . SIL: Silymarin; CA: Chlorogenic acid; ME: Melatonin; VEGF: Vascular endothelial growth factor; SEM: Standard error of mean

levels of IFN- $\gamma$  when compared with the control rats as depicted in Figure 5d. Concurrent treatment of the CCl<sub>4</sub>-intoxicated rats with SIL or SIL combined with either CA or ME significantly ( $P < 0.01$ ) ameliorated serum IFN- $\gamma$ . When combined with both CA and ME, SIL produced a significant ( $P < 0.001$ ) amelioration of serum IFN- $\gamma$  in CCl<sub>4</sub>-intoxicated rats.

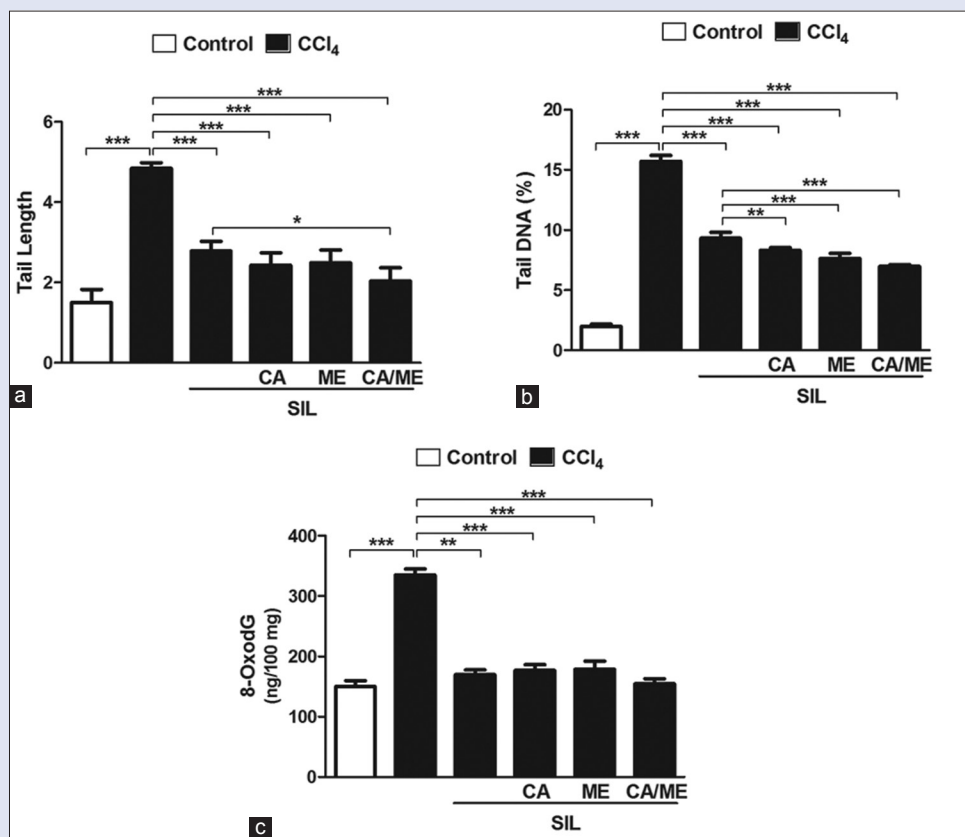
### Silymarin alone or in combination with chlorogenic acid and/or melatonin prevent carbon tetrachloride-induced oxidative DNA damage

Comet assay data represented in Figure 6a and b show the effects of SIL alone and in combination with CA and/or ME on DNA fragmentation in the liver of CCl<sub>4</sub>-intoxicated rats. Rats received CCl<sub>4</sub> showed a significant ( $P < 0.001$ ) DNA damage as indicated by the significant increase in comet tail length and DNA% when compared with the control group. SIL, alone or combined with CA and/or ME, significantly ( $P < 0.001$ ) attenuated DNA damage in CCl<sub>4</sub>-intoxicated rats. While the combination of SIL with either CA or ME produced no effect on comet tail length, both combinations significantly decreased tail DNA% when compared with SIL alone. On the other hand, the triple combination significantly decreased both comet tail length ( $P < 0.05$ ) and DNA% ( $P < 0.001$ ) when compared with SIL monotherapy.

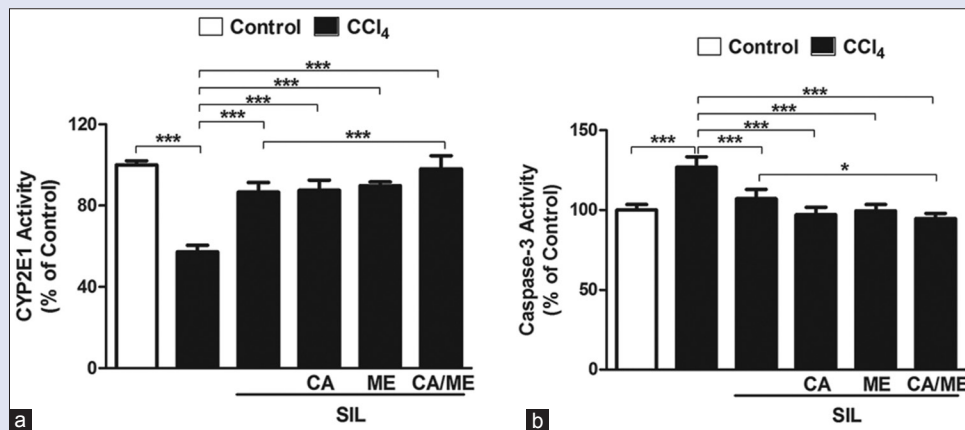
To further investigate the protective effects of SIL, CA and ME on CCl<sub>4</sub>-induced oxidative DNA damage, liver 8-OxodG was assayed.



**Figure 5:** Effects of silymarin alone or in combination with chlorogenic acid and/or melatonin on liver tumor necrosis factor alpha (a) and C-reactive protein (b), and serum interleukin-6 (c) and interferon gamma (d) levels in carbon tetrachloride-intoxicated rats. Data are mean  $\pm$  standard error of the mean ( $n = 8$ ). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . SIL: Silymarin; CA: Chlorogenic acid; ME: Melatonin; TNF- $\alpha$ : Tumor necrosis factor alpha; CRP: C-reactive protein; IL-6: Interleukin 6; IFN- $\gamma$ : Interferon gamma; SEM: Standard error of mean



**Figure 6:** Effects of silymarin alone or in combination with chlorogenic acid and/or melatonin on Comet tail length (a) and tail DNA% (b), and 8-Oxo-2'-deoxyguanosine levels (c) in liver of carbon tetrachloride-intoxicated rats. Data are mean  $\pm$  standard error of the mean ( $n = 8$ ). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . SIL: Silymarin; CA: Chlorogenic acid; ME: Melatonin; 8-Oxo-dG: 8-Oxo-2'-deoxyguanosine; SEM: Standard error of mean



**Figure 7:** Effects of silymarin alone or in combination with chlorogenic acid and/or melatonin on CYP2E1 (a) and caspase-3 (b) in liver of carbon tetrachloride-intoxicated rats. Data are mean  $\pm$  standard error of the mean ( $n = 8$ ). \* $P < 0.05$  and \*\*\* $P < 0.001$ . SIL: Silymarin; CA: Chlorogenic acid; ME Melatonin; CYP2E1: Cytochrome P450 2E1; SEM: Standard error of mean

8-Oxo-dG showed a significant ( $P < 0.001$ ) increase in the liver of CCl<sub>4</sub>-intoxicated rats when compared with the corresponding control group [Figure 6c]. Oral treatment of CCl<sub>4</sub>-intoxicated rats with SIL significantly ( $P < 0.01$ ) decreased 8-Oxo-dG levels in the liver. When combined with CA and/or ME, SIL produced a significant ( $P < 0.001$ ) decrease in liver 8-Oxo-dG levels.

### Silymarin alone or in combination with chlorogenic acid and/or melatonin ameliorate activity of cytochrome P450 2E1 and caspase-3 in liver of carbon tetrachloride-intoxicated rats

CCl<sub>4</sub>-administered rats showed a significant ( $P < 0.001$ ) decrease in hepatic CYP2E1 activity, as represented in Figure 7a. Treatment with

SIL alone significantly ( $P < 0.001$ ) restored hepatic CYP2E1 activity in  $\text{CCl}_4$ -intoxicated rats. Oral administration of SIL combined with CA and/or ME produced marked improvement ( $P < 0.001$ ) of CYP2E1 activity in liver of  $\text{CCl}_4$ -intoxicated rats. The combination of SIL, CA and ME significantly ( $P < 0.001$ ) alleviated hepatic CYP2E1 activity when compared with SIL alone.

Conversely, the apoptosis marker caspase-3 showed a significant ( $P < 0.001$ ) increase in liver of  $\text{CCl}_4$ -intoxicated rats, as represented in Figure 7b. Oral treatment with SIL either alone or combined with CA and/or ME significantly ( $P < 0.001$ ) reduced hepatic caspase-3 activity. The combination of SIL with CA or ME did not affect liver caspase-3 when compared with SIL alone. On the other hand, the combination of SIL, CA and ME significantly ( $P < 0.05$ ) decreased liver caspase-3 activity when compared with SIL monotherapy.

## DISCUSSION

In this investigation, we evaluated the protective effects of SIL alone and combined with CA and/or ME against liver injury induced by  $\text{CCl}_4$ , a frequently used model mimicking the liver damage caused by hepatotoxins in human. Our results showed that a single intraperitoneal dose of  $\text{CCl}_4$  produced liver damage in rats, demonstrated by a significant elevation in serum ALT activity as well as the histopathological alterations. Elevated level of serum ALT is indicative of cellular leakage and loss of functional integrity of cellular membrane in liver. The elevated serum ALT activity is consistent with the findings of several previous studies.<sup>[10,33,34]</sup> A number of histopathological alterations including necrosis, degenerated hepatocytes, inflammatory cells infiltration, dilated hepatic sinusoids, steatosis, and large patches of fibrous tissue are evident in  $\text{CCl}_4$ -intoxicated rats. The present findings demonstrate that treatment of the intoxicated rats with SIL, alone and combined with CA and/or ME, potentially attenuated  $\text{CCl}_4$ -induced hepatocellular injury. Several studies have clearly reported that SIL,<sup>[10,34]</sup> CA<sup>[27]</sup> and ME<sup>[35]</sup> alleviated serum transaminases and histopathological alterations in rats. Interestingly, combination of SIL with CA or ME significantly ameliorated serum ALT and liver histological findings when compared with SIL alone. When combined with both CA and ME, SIL produced more potent amelioration, suggesting a possible synergy.

On liver injury, the damaged cells release a variety of cytokines, which then trigger the release of more inflammatory mediators from Kupffer cells.<sup>[36]</sup> Kupffer cells and activated hepatic stellate cells (HSCs) have been reported to secrete chemokines, pro-inflammatory cytokines and adhesion factors which play central roles in inflammation and liver fibrosis.<sup>[37]</sup> In accordance,  $\text{CCl}_4$ -intoxicated rats exhibited marked increase in liver TNF- $\alpha$  and CRP, and circulating IL-6 and IFN- $\gamma$ , demonstrating inflammation. Further,  $\text{CCl}_4$  administration induced the formation of patches of fibrous tissue in the liver of the rats. The increased inflammatory mediators could be explained by  $\text{CCl}_4$ -induced activation of nuclear factor-kappa B (NF- $\kappa$ B) leading to the induction of pro-inflammatory cytokines<sup>[38]</sup> such as TNF- $\alpha$  and IL-6. These inflammatory responses are usually accompanied by biliary obstruction<sup>[39]</sup> and are implicated in increased markers of end-organ injury and death. TNF- $\alpha$  is a pro-inflammatory cytokine contributes to the pathological complications observed in several diseases<sup>[14]</sup> and activates the pro-apoptotic caspase cascade.<sup>[40]</sup> Therefore, inhibition of TNF- $\alpha$  synthesis or activity could attenuate liver injury induced by various toxicants. A recent study conducted by AlSaid *et al.*<sup>[41]</sup> reported significant up-regulation of TNF- $\alpha$  and IL-6 mRNA expression that was observed in the  $\text{CCl}_4$ -administered rats. In the same context, Ebaid *et al.*<sup>[20]</sup> demonstrated increased TNF- $\alpha$  mRNA expression in the liver tissue of a rat model of  $\text{CCl}_4$ -induced hepatotoxicity.

Oral treatment of the  $\text{CCl}_4$ -intoxicated rats with SIL, alone or combined with CA and/or ME, markedly ameliorated liver TNF- $\alpha$  as well as serum

IL-6 levels. These findings could be attributed to the anti-inflammatory and immunomodulatory effects of SIL, CA and ME. The anti-inflammatory efficacy of SIL in  $\text{CCl}_4$ -induced rats has been reported in a number of studies.<sup>[10]</sup> Previous work conducted by Shalan *et al.*,<sup>[42]</sup> Shaker *et al.*<sup>[43]</sup> and Rasool *et al.*<sup>[34]</sup> clearly demonstrated that SIL possesses anti-inflammatory potential and can attenuate  $\text{CCl}_4$ -induced histopathological changes, such as necrosis, ballooning, and inflammatory infiltration of lymphocytes. Since  $\text{CCl}_4$ -induced liver injury is thought to be mediated partly through the action of inflammatory cytokines on hepatocytes, the anti-inflammatory function of CA may contribute to its hepatoprotective effects. It has been shown that CA inhibits staphylococcal exotoxin-induced production of inflammatory cytokines in human peripheral blood mononuclear cells.<sup>[44]</sup> Furthermore, CA presented anti-inflammatory activity in an animal model of carrageenan-induced inflammation.<sup>[45]</sup> Furthermore, CA has been reported to regulate the expression of inflammatory cytokines in LPS-activated macrophages<sup>[15]</sup> and mice.<sup>[17]</sup> The anti-inflammatory effect of CA has been recently shown to be mediated through inhibition of toll-like receptor 4 signaling pathway in  $\text{CCl}_4$ -treated rats.<sup>[27]</sup> ME has been demonstrated to decrease the mRNA expression of TNF- $\alpha$ , IL-1  $\beta$ , and NF- $\kappa$ B in aged mice.<sup>[46]</sup> ME inhibited the expression of NF- $\kappa$ B and decreased production of pro-inflammatory cytokines from Kupffer cells in liver tissue of fibrotic rats.<sup>[47]</sup> ME also reduced cytokine levels in surgical neonates<sup>[48]</sup> and in  $\text{CCl}_4$ -induced rats.<sup>[20]</sup> The study conducted by Ebaid *et al.*<sup>[20]</sup> reported that ME treatment produced significant down-regulation of TNF- $\alpha$  and IFN- $\gamma$  mRNA expression in the liver of  $\text{CCl}_4$ -intoxicated rats. The anti-inflammatory effect of the treatment agents was further confirmed by the decreased serum IFN- $\gamma$  and liver CRP levels in  $\text{CCl}_4$ -induced rats. An interesting finding in the present investigation is the potentiated anti-inflammatory effect of SIL when combined with CA and ME.

The anti-inflammatory effects of SIL, CA and ME could be explained regarding their antioxidant and radical scavenging activities. During its biotransformation by CYP2E1,  $\text{CCl}_4$  produces highly reactive  $\text{CCl}_3\text{OO}\cdot$  and superoxide anion free radicals. NF- $\kappa$ B has been well-documented to be up-regulated by free radicals, leading to production of pro-inflammatory cytokines.<sup>[49]</sup> These cytokines, particularly TNF- $\alpha$ , activate the pro-apoptotic caspase cascade.<sup>[40]</sup> In the present investigation,  $\text{CCl}_4$  induced a marked increase in hepatic caspase-3. As the central place of detoxification, liver is constantly exposed to cell stress which disrupts the balance of inflammatory cytokines that promote or prevent injury.<sup>[50]</sup> Under stress, hepatocytes become more susceptible to the lethal effects of Fas ligand, IFN $\gamma$  and TNF- $\alpha$ . Subsequently, TNF and Fas receptor-associated death domain proteins activate caspase-8 which can start apoptosis through a direct activation of caspases-3, 6, and 7.<sup>[51]</sup> Treatment of the  $\text{CCl}_4$ -intoxicated rats with SIL alone or in combination with CA and/or ME significantly decreased hepatic caspase-3 activity. These results support the beneficial effect of the three treatment agents in counteracting hepatotoxicity induced by  $\text{CCl}_4$ . In consistent with our findings, Patel *et al.*<sup>[52]</sup> and Al-Rasheed *et al.*<sup>[10]</sup> reported elevated caspase-3 activity in doxorubicin and  $\text{CCl}_4$ -induced rats, respectively, and the ameliorative effect of SIL. Similarly, acetaminophen-induced liver injury in rats has been associated with up-regulated caspase-3, an effect that was reversed following treatment with CA.<sup>[53]</sup> In addition, studies showed that ME attenuated apoptosis in  $\text{CCl}_4$ <sup>[54]</sup> and acetaminophen-treated rats.<sup>[55]</sup> Further, Ebaid *et al.*<sup>[20]</sup> reported that ME treatment reduced FAS expression in the liver of  $\text{CCl}_4$ -intoxicated rats.

$\text{CCl}_4$ -induced oxidative stress acts as a stimulus for the process of fibrogenesis in experimental animals and humans.<sup>[56]</sup> Apoptosis has also been considered as one of the events involved in the fibrosis of the liver.<sup>[57]</sup> In this study, increased level of collagen deposition in the liver of  $\text{CCl}_4$ -intoxicated rats is evident from the Masson's trichrome special staining. This was concomitant with the increased inflammatory cytokines which are known to activate HSCs, major collagen-producing cells in injured liver.<sup>[58]</sup> These

findings were further confirmed by the increased serum levels of VEGF. Increased expression of VEGF and its receptors has been reported in experimentally-induced cirrhosis<sup>[59]</sup> and CCl<sub>4</sub>-induced fibrosis.<sup>[60]</sup> Moreover, VEGF has been suggested to contribute to the development of liver fibrosis through inducing the proliferation of sinusoidal endothelial cells and HSCs.<sup>[61]</sup> In this study, we found that serum levels of VEGF were decreased in CCl<sub>4</sub>-induced rats treated with SIL alone or combined with CA and/or ME. Therefore, it seems that attenuated production of VEGF by SIL and CA and/or ME plays a central role in decreasing collagen production in HSCs.

Free radicals produced during the biotransformation of CCl<sub>4</sub> can covalently bind to macromolecules such lipids and induce an increase in free peroxide and lipoperoxide radicals. These free radicals can cause oxidative DNA damage including genetic mutations, strand breakage, formation of DNA adducts, and chromosomal alterations.<sup>[53]</sup> In addition, the free radicals can increase 8-OxodG concentration in tissues of experimental animals.<sup>[62]</sup> Data of this study indicated CCl<sub>4</sub>-induced DNA oxidative damage and strand breakage as showed from the results of 8-OxodG and comet assays. 8-OxodG is a predominant form of free radical-induced nuclear and mitochondrial DNA oxidative damage and has thus been used as a biomarker for oxidative stress.<sup>[63]</sup> Comet assay is a sensitive tool used for the detection of DNA damage.<sup>[64]</sup> Administration of SIL, alone or combined with CA and/or ME, effectively decreased 8-OxodG and DNA strand breaks in the liver of CCl<sub>4</sub>-intoxicated rats, which could be associated with their antioxidant effects.

The protective effects of SIL, CA and ME on CCl<sub>4</sub>-induced hepatotoxicity could be also connected to their ability to increase the activity of CYP2E1. Multiple studies have reported that in both human and rodents the biotransformation of CCl<sub>4</sub> is principally mediated by CYP2E1.<sup>[5]</sup> In this context, the study of Wong *et al.*<sup>[65]</sup> demonstrated the resistance to CCl<sub>4</sub> hepatotoxicity in CYP2E1 knockout mice. Here, we found that CCl<sub>4</sub> administration induced a considerable decrease in hepatic CYP2E1 activity, an effect that was reversed following treatment with SIL either alone or combined with CA and/or ME. In consistent with our findings, the expression<sup>[65,66]</sup> and activity<sup>[10]</sup> of hepatic CYP2E1 showed significant decrease in CCl<sub>4</sub>-treated rodents. Recently, SIL has been reported to ameliorate hepatic CYP2E1 activity in CCl<sub>4</sub>-intoxicated rats.<sup>[10,66]</sup> Interestingly, the ameliorative effect of SIL on CYP2E1 activity was significantly increased when combined with both CA and ME.

## CONCLUSION

This study demonstrates that SIL alone and combined with CA and/or ME protected the liver against CCl<sub>4</sub>-induced injury via attenuating inflammation, oxidative DNA damage, apoptosis, and fibrotic changes. The effects of SIL were significantly intensified when combined with both CA and ME. These findings suggest a synergistic effect between SIL, CA, and ME. This synergism plays an active role in their protective mechanism against CCl<sub>4</sub>-induced liver injury. However, the hypothesized synergistic effects need to be further confirmed using detailed pharmacological, toxicological, and clinical studies.

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## Conflicts of interest

There are no conflicts of interest.

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