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ORIGINAL ARTICLE

Potential impact of silymarin in combination with chlorogenic acid and/or melatonin in combating cardiomyopathy induced by carbon tetrachloride

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Abstract The aim of this study was to investigate the effective role of silymarin either alone or in combination with chlorogenic acid and/or melatonin against the toxic impact of carbon tetrachloride (CCl4) induced cardiac infarction. CCl4 (1.2 ml/kg body weight) was administered as a single dose intraperitoneally. The results revealed that the administration of silymarin alone or in combination with chlorogenic acid (CGA) and/or melatonin for 21 consecutive days, 24 h after CCl4 injection to rats, markedly ameliorated the increases in serum markers of cardiac infarction, including troponin T and creatine kinase-MB (CK-MB), as well as increases in the pro-inflammatory biomarkers, including interleukin-6 (IL-6), interferon-γ (IFN-γ) in serum and tumor necrosis factor-α (TNF-α) and C-reactive protein in cardiac tissue compared to CCl4 intoxicated rats. The used agents also successfully modulated the alteration in vascular endothelial growth factor (VEGF) in serum and the oxidative DNA damage and the increase in the apoptosis marker caspase 3 in cardiac tissue in response to CCl4 toxicity. The present biochemical results are supported by histo-pathological examination. The current results proved that treatment with silymarin in combination with CGA and melatonin was the most effective one in ameliorating the toxicity of CCl4 induced cardiac damage and this may support the use of this combination as an effective drug to treat cardiac damage induced by toxic agents.
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1. Introduction

Carbon tetrachloride (CCl4) is a xenobiotic hazardous hepatotoxin (Xiao et al., 2012). But in addition to hepatic damage, it also causes disorders in tissues other than liver including heart by generating free radicals (Ozturk et al., 2003; Jayakumara

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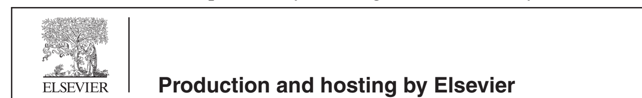
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et al., 2008; Botsoglou et al., 2009). CCl4 at a high dose often rapidly causes cellular necrosis, oxidative stress and inflammation which leads to acute tissue injury and apoptotic organ failure (Karakus et al., 2011; Shi et al., 2012). The toxicity of CCl4 resulting from increased free radical production may play an important role in degenerative processes in the tissues. The toxicity of CCl4 includes two steps. The first one is the production of free radicals (CCl3* and CCl3OO*) 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 (Shi et al., 2012).

Maintaining the balance between free radicals and antioxidants is therefore important as well as inhibiting inflammatory mediators may serve as major mechanisms in preventing damage impact induced by toxic agents. The implication of oxidative stress and inflammation in the etiology and progression of several acute and chronic clinical disorders has led to the suggestion that agents with antioxidant and anti-inflammatory properties may have health benefits. Several antioxidant agents, including silymarin, antioxidant vitamins (C and E), and melatonin have been reported to reduce CCl4-induced toxicity (Donder et al., 1999; Turkdogan et al., 2001; Shaker et al., 2011).

Silymarin is an active extract isolated from the milk thistle plant *Silybum marianum*. Silymarin consists of a mixture of three bioflavonoids found in the fruit, seeds and leaves of the plant: silybin, silydianin and silychristin (Pepping, 1999). Silybin is the main component (60–70%) and is believed to have the most biological activity. Silymarin has been shown to have potential impact in many liver disorders, including oxidative stress, injury and fibrosis induced by CCl4 (Shaker et al., 2011). It also has protective effect against reperfusion-induced myocardial infarction in rats (Rao and Viswanath, 2007). Silymarin can prevent lipid peroxidation, inhibit low-density lipoprotein oxidation and scavenge reactive oxygen species (Post-White et al., 2007). Silymarin also has anti-inflammatory effects which may relate its ability to inhibit the transcription factor nuclear factor-kB (NF-kB), which contributes to the production of proinflammatory mediators such as interleukin (IL)-1 and IL -6, TNF-α, lymphotoxin, granulocyte macrophage, colony-stimulating factor (GM-CSF) and interferon (IFN)-γ (Deep and Agarwal, 2007).

Chlorogenic acid (CGA) is a type of polyphenol and is found in many foods, including coffee, berries, potatoes, carrots, wine, apples, and various herbs (Stoclet et al., 2004). It has many pharmacological activities as anti-inflammatory, antidiabetic, antitumor and antiulcerogenic actions. (Feng et al., 2005; Kim et al. 2011; Shimoyama et al., 2012; Yang et al., 2012). A recent study showed that CGA can protect mice from lipopolysaccharides (LPS) -induced hepatotoxicity or lung injury (Xu et al., 2012; Zhang et al., 2010). Another study showed that CGA can inhibit LPS-induced inflammatory cytokines release in RAW264.7 cells (Shan et al., 2009). CGA can also efficiently inhibit CCl4 induced liver fibrosis in rats (Shi et al., 2012). It has the ability to regulate cytokine and chemokine release, suppress cellular apoptosis and has protective beneficial effect against the injury of different organs including heart in murine sepsis (Lee et al., 2012).

Melatonin (N-acetyl-5-methoxytryptamine) is formed mainly in the pineal gland of most mammals including man. This hormone has important functions. It is a powerful anti-inflammatory, anti-apoptosis, free radical scavenger and broad-spectrum antioxidant which has been tested in previous experimental studies (Zhaoa et al., 2008; Cuesta et al., 2011). Melatonin also was reported to have protective impact on myocardial infarction (Chen et al., 2003). The anti-inflammatory activity of melatonin was reported through the inhibition of NF-κB (Alonso et al., 2006) which is known to be one of the crucial transcription factors required for maximal transcription of a wide array of pro-inflammatory molecules, including TNF-α, IL-1β, IL-6 and other mediators (Ali and Mann, 2004). It also has a protective action against oxidative DNA damage (Zhaoa et al., 2008).

This study was designed to investigate the potential impact of silymarin either alone or in combination with CGA and/or melatonin on cardiac damage induced by inflammation, oxidative DNA damage and apoptosis in response to CCl4 toxicity in rats.

2. Materials and methods

2.1. Chemicals

All chemicals used were of high analytical grade, product of Sigma and Merck companies. Silymarin, chlorogenic acid and melatonin were purchased from Sigma Chemical Co. (Sigma, St. Louis, MO, USA).

2.2. Animals and treatments

Animal experiments were performed with approval from the local ethics committee. Sixty healthy male albino rats (120–150 g) were supplied by the Experimental Animal Center, College of Pharmacy, King Saud University. Animals were kept in special cages and maintained on a constant 12-h light/12-h dark cycle with air conditioning and a controlled temperature of 20–22 °C and humidity of 60%. Rats were fed a standard rat pellet chow with free access to tap water *ad libitum* for 1 week before the experiment for acclimatization. Animal utilization protocols 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. After 1 week of acclimation, the rats were fasted overnight before treatment and randomly divided into six groups, each of ten rats as follows.

- Group 1: Normal healthy animals (not receiving any medication).
- Group2: CCl4-intoxicated animals.
- Group3: CCl4 intoxicated animals and treated with silymarin.
- Group 4: CCl4 intoxicated animals and treated with silymarin and CGA.
- Group 5: CCl4 intoxicated animals and treated with silymarin and melatonin.
- Group 6: CCl4 intoxicated animals and treated with a combination of silymarin, CGA and melatonin.

CCl4 was administered to the rats intraperitoneally as a mixture of CCl4 and corn oil (1:1, 1 ml/kg body weight; 0.5 ml/kg body weight as CCl4, Yachi et al., 2010). Silymarin

(200 mg/kg/day, Li et al., 2012a), CGA (60 mg/kg/day, Shi et al., 2009) and melatonin (20 mg/kg/day, Laliena et al., 2012) were dissolved in gum acacia (2% w/v) and given orally once daily for 21 successive days, 24 h post CCl4 injection. After the experimental period, blood samples were collected from each animal in all groups into sterilized tubes for serum separation. Serum was separated by centrifugation at 3000 rpm for 10 min and used for biochemical serum analysis. After blood collection, the rats of each group were sacrificed under ether anesthesia, and their hearts were collected, weighed and washed using chilled saline solution. The hearts were minced and homogenized in ice-cold bi-distilled water to yield 10% homogenates. The homogenates were centrifuged for 15 min at 4000 rpm at 4 °C, and the supernatants were used for biochemical tissue analysis. Four hearts from each group were kept in 10% formalin for histo-pathological examination.

2.3. Biochemical serum analysis

Troponin T concentration was determined using a Siemens Dimension Xpand Plus instrument (IL, USA). Creatine kinase-MB (CK-MB) was estimated spectrophotometrically using a standard enzyme kit supplied by Spinreact, S.A.-Spain (Cat.No.1001055). The concentration of InF-γ and IL-6 was determined by enzyme-linked immunosorbent assay (ELISA) kits (R&D ELISA Kit, USA) following the manufacturer's instructions. The level of vascular endothelial growth factor (VEGF) was determined at 492 nm by quantitative colorimetric sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems, UK) in accordance with the manufacturer's instructions. Concentrations were calculated using a standard curve generated with specific standards provided by the manufacturer.

2.4. Biochemical assay of heart tissue

The concentration of TNF-α was determined using commercially available ELISA assays following the instructions supplied by the manufacturer (DuoSet kits; R&D Systems, Minneapolis, MN, USA). CRP was measured with latex-enhanced immunonephelometry on a Behring BN II Nephelometer (Dade Behring). In this assay, polystyrene beads coated with rat monoclonal antibodies bind CRP present in the sample and form aggregates. The intensity of the scattered light is proportional to the size of the aggregates and thus reflects the concentration of CRP present in the sample. The intra-assay and inter-assay coefficients of variation for CRP were 3.3% and 3.2%, respectively. The lower detection limit of the assay was 0.15 mg/L.

2.4.1. Assay of caspase 3 activity

Caspase-3-like protease was assayed according to the method described by Vaculova and Zhivotovsky (2008).

2.4.2. Comet assay

The comet assay, or single cell gel electrophoresis, is a widely used technique for measuring and analyzing DNA breakage in individual cells. The method of Singh et al. (1988), which involves the unwinding of DNA under alkaline conditions, was used in this study. At least fifty cells per sample were submitted to analysis. The parameters measured to analyze the electrophoretic patterns were the tail length as measured from the middle of the head to the end of the tail, tail moment and the relative DNA content in the tail. The tail moment was defined by the percentage of DNA in the tail multiplied by the length between the center of the head and tail which was defined by Olive et al. (1990).

2.5. Histopathological examination

Small pieces of heart were fixed by 4% formalin and then embedded into paraffin, sectioned for 5–6-µm thickness, and mounted on glass microscopic slides using standard histopathological technique. The sections were stained with hematoxylin-eosin and examined by light microscopy.

2.6. Statistical analysis

Data were analyzed by comparing values for different treatment groups with the values for individual controls. Results are expressed as mean ± SE. Significant differences among values were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test.

3. Results

Serum cardiac biomarkers, namely troponin T and CK-MB, in the normal and different experimental groups intoxicated with CCl4 are shown in Table 1. The toxicity of CCl4 induced pronounced increases in these biomarkers compared with normal animals ($P \leqslant 0.001$), and the intake of either silymarin alone or in combination with CGA and/or melatonin significantly down-modulated the deviation in these markers versus CCl4 intoxicated group.

The levels of some immunological pro-inflammatory biomarkers, including InF-γ and IL-6 in sera and TNF-α and CRP in cardiac tissue in the normal and different experimental groups intoxicated with CCl4 are illustrated in Tables 2 and 3, respectively. These biomarkers were dramatically elevated in rats intoxicated with CCl4 compared with the normal ones ($P \leqslant 0.001$). The intake of the studied agents 24 h after CCl4 injection for 21 consecutive days, markedly inhibited the induced inflammatory mediators compared with intoxicated animals.

The level of VEGF (angiogenic factor) significantly increased in the sera of rats intoxicated with CCl4 compared with normal animals (Table 2). Administration of silymarin alone or in combination with CGA and/or melatonin, markedly reduced the dramatic increase in this biomarker in the sera of CCl4-intoxicated rats compared with intoxicated untreated animals ($P \leqslant 0.001$).

The effect of the administration of CCl4 on cardiac DNA of rats is shown in Table 4 and Fig. 1. A significant increase in the tail length, DNA% (tail DNA content) and tail moment was shown in the cardiac tissue of rats intoxicated with CCl4. Administration of the current agents in CCl4-intoxicated rats significantly mitigated their cardiac tissue from DNA damage as indicated by a decrease in the above markers of DNA damage compared with intoxicated untreated rats ($P \leqslant 0.001$). Table 4 also shows that the cardiac apoptosis biomarker caspase 3 was significantly up-regulated in rats administered

Table 1 Effect of silymarin alone or in combination with CGA and/or melatonin treatment on serum markers of cardiac infarction in rats intoxicated with CCl4.

Parameter	CK-MB (U/L)	Trponine T (Pg/ml)
Control	34.84 ± 1.19	21.66 ± 1.14
CCL_4	100.7 ± 2.38[a]	41.22 ± 1.17[a]
CCl4 + Silymarin	59.70 ± .43[b*]	37.93 ± 0.67[a**]
CCl4 + Silymarin + CGA	53.74 ± 2.17[b*$$]	30.59 ± 0.44[b*$$]
CCl4 + Silymarin + Melatonin	54.65 ± 1.73[b*$$]	31.03 ± 0.45[b*$$]
CCl4 + Silymarin + CGA + Melatonin	40.53 ± .87[b*$]	25.65 ± 1.60[b*$]

Data are presented as mean ± SD of 6 rats.
[a] $P \leqslant 0.001$ compared with the normal group.
[b] $P \leqslant 0.01$ compared with the normal group.[*] $P \leqslant 0.001$ compared with the CCl4-intoxicated group.
[**] $P \leqslant 0.05$ compared with the CCl4-intoxicated group.
[$] $P \leqslant 0.01$ compared with the silymarin group, using ANOVA followed by the Bonferroni's test.
[$$] $P \leqslant 0.05$ compared with the silymarin group, using ANOVA followed by the Bonferroni's test.

Table 2 Effect of silymarin either alone or in combination with CGA and/or melatonin on the levels of serum inflammatory and angiogenic markers in CCl4 intoxicated rats.

Groups	InF-γ (pg/ml)	IL-6 (pg/ml)	VEGF (Pg/ml)
Control	1.79 ± .081	12.92 ± 0.83	1.66 ± .053
CCL_4	3.57 ± .07[a]	42.31 ± 2.10[a]	2.9 ± .088[a]
CCl4 + Silymarin	2.3 ± .06[b*]	31.92 ± 0.52[a*]	1.89 ± .016[b*]
CCl4 + Silymarin + CGA	2.02 ± .04[c*$]	21.98 ± 0.52[b*$]	1.82 ± .024[b*$$]
CCl4 + Silymarin + Melatonin	2.01 ± .02[c*$]	20.53 ± 1.1[b*$]	1.80 ± .02[b*$$]
CCl4 + Silymarin + CGA + Melatonin	1.81 ± .05[*$]	13.28 ± 0.7[*$]	1.69 ± .028[*$]

Data are presented as mean ± SD of 6 rats.
[a] $P \leqslant 0.001$ compared with the normal group.
[b] $P \leqslant 0.01$ compared with the normal group.
[c] $P \leqslant 0.05$ compared with the normal group.
[*] $P \leqslant 0.001$ compared with the CCl4-intoxicated group.
[$] $P \leqslant 0.01$ compared with silymarin group, using ANOVA followed by the Bonferroni's test.
[$$] $P \leqslant 0.05$ compared with silymarin group, using ANOVA followed by the Bonferroni's test.

Table 3 Effect of silymarin either alone or in combination with CGA and/or melatonin on levels of inflammatory markers in cardiac of CCl4 intoxicated rats.

Groups	TNFα (pg/100 mg)	CRP (ng/100 mg)
Control	50.34 ± 0.98	6.97 ± 0.13
CCL_4	93.84 ± 1.47[a]	12.67 ± 0.58
CCl4 + Silymarin	65.82 ± 0.87[c*]	9.80 ± 0.15[b*]
CCl4 + Silymarin + CGA.	58.32 ± 1.15[c*$$]	8.72 ± 0.2[c*$]
CCl4 + Silymarin + Melatonin	56.93 ± 0.49[c*$$]	8.60 ± 0.6[c*$]
CCl4 + Silymarin + CGA + Melatonin	50.8 ± .20[*$]	7.2 ± 0.3[*$$]

Data are presented as mean ± SD of 6 rats.
[a] $P \leqslant 0.001$ compared with the normal group.
[b] $P \leqslant 0.01$ compared with the normal group.
[c] $P \leqslant 0.05$ compared with the normal group.
[*] $P \leqslant 0.001$ compared with the CCl4-intoxicated group.
[$] $P \leqslant 0.01$ compared with sil group, using ANOVA followed by the Bonferroni's test post-ANOVA.
[$$] $P \leqslant 0.05$ compared with sil group, using ANOVA followed by the Bonferroni's test post-ANOVA.

CCl4. Administration of the studied agents to CCl4-intoxicated rats beneficially down-modulated the increase in cardiac caspase 3 in relation to intoxicated rats ($P \leqslant 0.001$).

The myocardial structure of normal and different groups of CCl4 intoxicated rats was examined by H&E staining (Fig. 2A–F) and masson's trichrome (Fig. 3A–F) staining respectively. Normal untreated rats showed normal cardiac fibers (Fig. 2A) and normal collagen fibers (Fig. 3A). Myocardial damage induced by CCl4 is observed by focal areas with massive degeneration (Fig. 2B) and scattered areas of fibrosis stained in blue (Fig. 3B). Administration of silymarin 24 h post CCl4 injection showed mild improvement in the cardiac histological picture (Fig. 2C) and few areas of fibrosis (Fig. 3C). Administration of silymarin either with CGA (Fig. 2D and Fig. 3D) or melatonin (Fig. 2E and Fig. 3E) showing few scattered areas of little degeneration and collagen

Table 4 Effect of silymarin alone or in combination with CGA and/or melatonin on markers of DNA damage using the comet assay and the apoptosis marker, caspase-3 in the cardiac tissues of CCl4 intoxicated rats.

Groups	Tail length (μm)	DNA% in tail	Unit tail moment	Caspas-3
Control	1.41 ± 0.039	1.88 ± 0.094	1.41 ± 0.19	86.32 ± 1.91
CCL_4	4.42 ± 0.15[a]	3.82 ± 0.08[a]	4.8 ± .15[a]	182 ± 3.12[a]
CCl4 + Silymarin	2.92 ± 0.06[a*]	2.93 ± 0.06[b*]	2.72 ± 0.06[b*]	94.41 ± .98[b*]
CCl4 + Silymarin + CGA.	2.5 ± 0.03[a*$]	2.44 ± 0.05[b*$]	2.18 ± 0.024[b*$]	90.79 ± 1.23[*]
CCl4 + Silymarin + Melatonin	2.4 ± 0.04[a*$]	2.29 ± 0.15[b*$]	2.05 ± 0.03[b*$]	89.99 ± .92[*$]
CCl4 + Silymarin + CGA + Melatonin	1.48 ± 0.05[*$$]	1.92 ± 0.02[*$$]	1.54 ± .05[*$$]	86.49 ± 1.01[b*$]

Data are presented as mean ± SD of 6 rats.
[a] $P \leqslant 0.001$ compared with the normal group.
[b] $P \leqslant 0.01$ compared with the normal group.
[*] $P \leqslant 0.001$ compared with the CCl4-intoxicated group.
[$] $P \leqslant 0.05$ compared with the silymarin group, using ANOVA followed by the Bonferroni's test.
[$$] $P \leqslant 0.01$ compared with the silymarin group, using ANOVA followed by the Bonferroni's test.

deposition respectively. Administration of a combination of the three agents showing more or less normal cardiac tissue (Fig. 2F) and normal collagen distribution within cardiac tissue (Fig. 3F).

From the current results, it can observed that ingestion of the combination of silymarin, CGA and melatonin to CCl4 intoxicated rats was the most effective one in modulating the deviation in studied biochemical biomarkers and histomorphological pictures of cardiomyopathy compared with silymarin alone or in combination with either CGA or melatonin .

4. Discussion

The current study revealed that CCl4 injection to rats induced cardiotoxicity as indicated by elevations in serum cardiac damage biomarkers, namely troponin T and CPK-MB. Troponins regulate the calcium-mediated interaction between actin and myosin (Adamcova et al., 1997). Most intracellular troponin T is bound to the myobrils in the cardiac myocyte; however, a small percentage exists in a cytosolic pool (Kemp et al., 2004). The importance of this pool is as the source of cytosolic troponins released 4–6 h after myocardial injury. Continuing breakdown of the myobrils in damaged myocytes results in the prolonged elevation of the concentration of troponin T in blood (Maynard et al., 2000). Troponin T and CK-MB are released by damaged heart tissues and are frequently used as diagnostic markers for predicting adverse cardiovascular events, such as death or myocardial infarction (Kemp et al., 2004; Santos et al., 2011).

Administration of silymarin only or in combination with CGA and/or melatonin to CCl4 intoxicated rats markedly reduced the serum biomarkers of myocardial infarction compared with intoxicated rats. The combination of the three agents was the most effective one. This result may predict the beneficial role of these agents in protection against myocardial injury induced by CCl4 toxicity. This good impact of the studied agents against tissue damage induced by toxic agents was previously documented in experimental animal models (Chlopcíková et al., 2004; Rao and Viswanath, 2007; Dominguez-Rodriguez et al., 2012).

The present study also showed marked increases in immunological pro-inflammatory biomarkers, including InF-γ and IL-6, in rat sera intoxicated with CCl4 accompanied with increases in TNF-α and CRP in their cardiac tissue compared with the normal group. The increase in the inflammatory cytokines by CCl4 may be related to the activation of nuclear factor-kB which contributes to the production of inflammatory cytokines (Li et al., 2012a). The induction of such biomarkers may play a principle role in cardiac infarction and dysfunction induced by CCl4 toxicity. This is supported by previous study which reported that TNF-α is implicated in the pathophysiology of myocardial failure (Irwin et al., 1999). The up-regulation of this cytokine promotes the production of other cytokines and IL-6, the chief stimulator of the production of CRP, inducing inflammatory heart disease (De Ferranti and Rifai, 2007). IL-6 triggers the activation of transcription factors that bind to DNA elements and stimulate increased transcription of CRP, resulting in a rise in its level (Patel et al., 2007).

Thus, a protective strategy that attenuates the production of inflammatory mediators could predispose to remote organ dysfunction.

Intake of silymarin alone or in combination with CGA and/or melatonin to CCl4 intoxicated rats, effectively ameliorated the increases in the inflammatory immunological biomarkers, InF-γ and IL-6 in sera, as well as TNF-α and CRP in cardiac tissue of CCl4 intoxicated rats, suggesting that their cardioprotective effect against myocardial infarction may be related to their anti-inflammatory and immunomodulatory beneficial actions. Treatment with the silymarin in combination with CGA and melatonin was the most influential one in reducing the levels of these markers .The immunomodulatory and the anti-inflammatory actions of silymarin, CGA or melatonin were previously proven through inhibiting nuclear factor-kappa B (NF-kB) action which is known to be one of the critical transcription factors required for maximal transcription of a wide array of pro-inflammatory molecules, including TNF-α, IL-1β, IL-6 and other mediators (Ali and Mann, 2004; Feng et al., 2005; Polyak et al., 2007). Also, CGA was found to have the ability to attenuate the increases in these inflammatory cytokines in sera of CCl4- intoxicated rats (Shi et al., 2012).

The present study also demonstrated a significant increase in the angiogenic factor VEGF in the sera of rats intoxicated with CCl4. The increase in this angiogenic factor was documented in response to CCl4 toxicity by some authors (Shi et al., 2009). Previous data stated that the expression of various

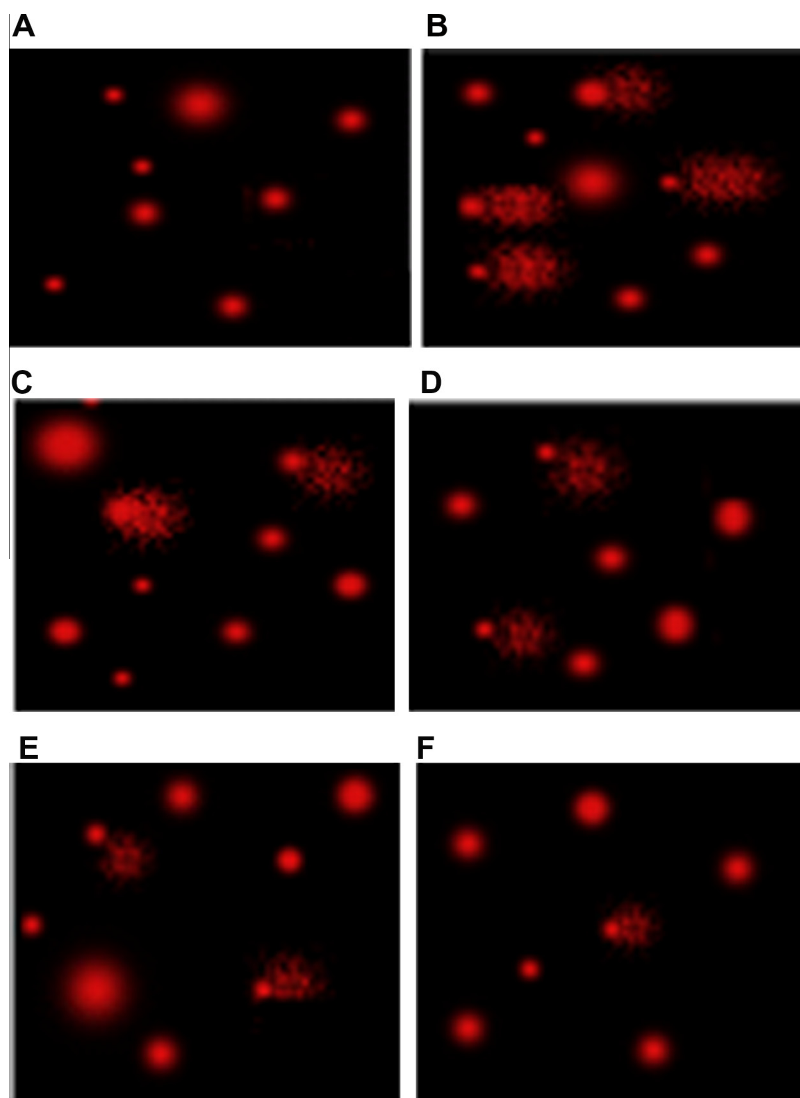

Figure 1 DNA damage in the cardiac tissues of CCl4 intoxicated rats and the effect of silymarin alone or in combination with CGA and/or melatonin treatment on the level of DNA damage. Comet assay showing the degree of DNA damage in the cardiac tissues of (A) normal control group, (B) group intoxicated with the CCl4, (C) intoxicated group treated with silymarin, (D) intoxicated group treated with silymarin and CGA (E) intoxicated group treated with silymarin and melatonin, (F) intoxicated group treated with a combination of silymarin, CGA and melatonin.

inflammatory tissue factors, cytokines, and chemokines stimulate VEGF synthesizing cells such as platelets, immune cells, and inflammatory cells (Verheul et al., 2000; Lingen, 2001). It is a part of the system that restores the oxygen supply to tissues when blood circulation is inadequate (Prior et al., 2004). However, other studies stated that TNF-α and VEGF expressions were significantly linked. Both TNF-α and VEGF may promote a procoagulant state by increasing the expression of tissue factors on endothelial cells and/or monocytes (Clauss et al., 1996; Mechtcheriakova et al., 2001). Increased tissue factor expression is thought to play a significant role in the development of multi-organ system failure in acute injury (Mechtcheriakova et al., 2001). The current result may suggest the possibility of TNF-α and VEGF synergistically to potentiate cardiac infarction and/or systemic organ dysfunction. Ingestion of silymarin alone or in combination with CGA and/or melatonin markedly reduced the dramatic increase in this angiogenic biomarker in the sera of CCl4-intoxicated rats. The three agents were synergistically beneficial in attenuating this marker, suggesting their anti-angiogenic potential action. The angio-preventive beneficial effect of these agents was also proved by some studies (Shi et al., 2009; Park et al., 2010; Deep et al., 2012).

The damaging effect of CCl4 on DNA has been shown in previous studies (Iwai et al., 2002; Makni et al., 2012). The comet assay is a sensitive and a simple assay for detecting DNA damage at the level of individual cells (Singh et al., 1988).

Increased DNA migration accompanies the DNA fragmentation associated with the cell death arising from a non- DNA-mediated process or apoptosis (Tice and Strauss, 1995). With an increasing number of breaks, DNA pieces migrate freely into the tail of the comet, and in extreme cases (the apoptotic cell), the head and the tail are well separated. Tail length, percentage of total DNA in the tail and tail -DNA moment,

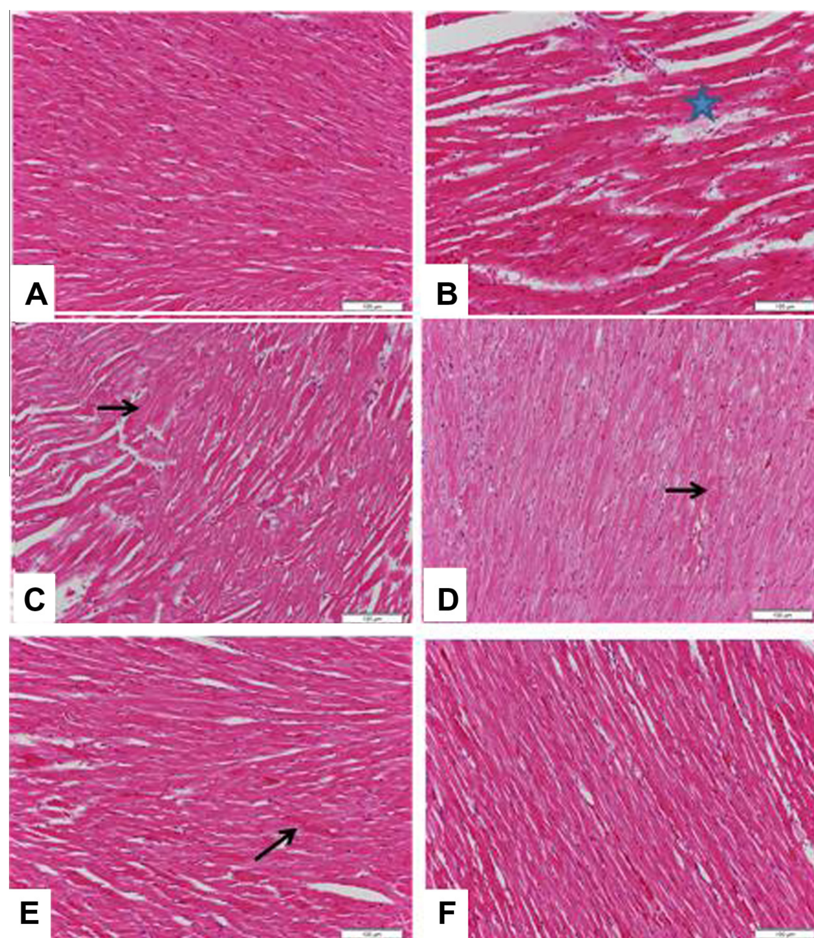

Figure 2 Light photomicrograph of rat cardiac stained with H&E; scale bar = 100 μm in which (A) is normal myocardium, (B) CCl4 intoxicated rats, showing focal areas with massive degeneration (star), (C) CCl4 intoxicated rats and received silymarin showing marked improvement of myocardial degeneration (arrow), (D) CCl4 intoxicated rats that received both silymarin and CGA, showing few areas of little degeneration (arrow), (E) CCl4 intoxicated rats that received silymarin and melatonin showing few areas of little degeneration (arrow),while (F) CCl4 intoxicated rats that received a combination of silymarin, CGA and melatonin showing more or less normal cardiac tissue.

reflect DNA damage though the percentage of tail DNA generally seems to be the most useful, as it is directly related to the frequency of breaks over a wide range of damage (ColLins et al., 1996).

As documented by some studies, the current study revealed that CCl4 induced cardiac DNA damage as evidenced by a significant increase in the tail length, DNA% in the tail and tail - DNA moment in cardiac of rats versus normal ones (Iwai et al., 2002; Makni et al., 2012). Some studies demonstrated that the over production of nitric oxide (NO) induced by inflammatory cytokines in response to tissue injury has the major role in DNA damage (Liu et al., 1996; Filep et al., 1997). Also, it was found that, the increase of TNF-α level stimulated the production of reactive oxygen species (ROS), which mediate DNA damage, in cultured cardiac myocytes (Suematsu et al., 2003).

Adminsteration of silymarin alone or in combination with CGA and/or melatonin to CCl4 -intoxicated rats, effectively attenuated the deviation in the above markers of DNA damage in their cardiac tissue. The combination of the three agents was the potential one that ameliorated these markers to near their normal levels. Recent study documented that silymarin possesses substantial protective effect against DNA damage and apoptosis induced by the environmental pollutant benzo(a)pyrene (Perumal Vijayaraman et al., 2012). Also, it was proved that CGA and melatonin have DNA damage protective effects (Fischer et al., 2012; Xu et al., 2012).

Apoptosis represents a key event after oxidative DNA damage (Sharma et al., 2009). In line with some investigators, the result in the present study revealed that CCl4 induced apoptosis in cardiac muscles as shown by markedly increased activity of the apoptosis biomarker caspase 3 in cardiac tissue of rats intoxicated with CCl4, suggesting that apoptosis might contribute to this toxin induced DNA damage (Karakus et al., 2011).

The intake of silymarin alone or in combination with CGA and/or melatonin to CCl4 -intoxicated rats pronouncedly down-modulated the increase in heart caspase 3. This result may suggest that the benefits of using agents in protecting cardiomyopathy induced by CCl4 might be associated with their strong anti-apoptotic impact. The mechanism of their antiapoptotic effect may be related to their ability to prohibit DNA damage induced by CCl4. This result in accordance with Patel et al. (2010) who documented that silymarin reduced apoptotic cell death associated with hepatotoxicity. CGA can also suppress cellular apoptosis (Cho et al., 2009). Besides,

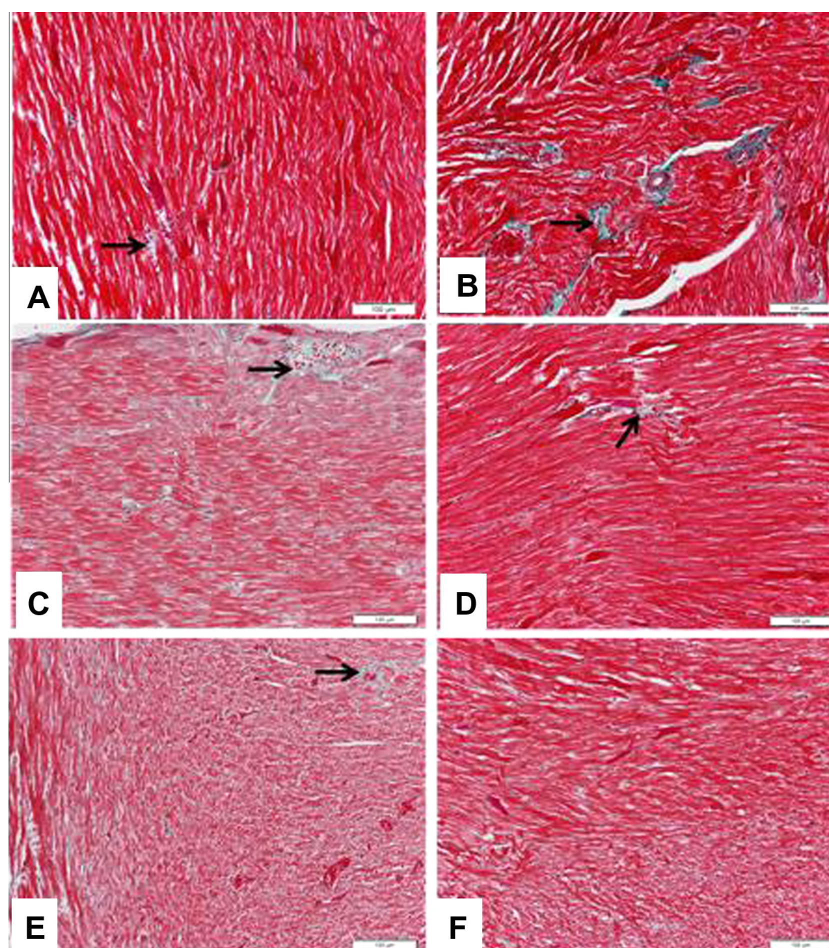

Figure 3 Light photomicrograph of rat cardiac stained with Masson's trichrome; scale bar = 100 μm in which (A) showing normal perivascular collagen fibers (arrow), (B) cardiac of CCl4 intoxicated rats showing scattered areas of fibrosis stained in blue (arrow), (C) cardiac of CCl4 intoxicated rats that received silymarin modulated cardiac fibrosis as shown by few areas of collagen deposition (arrow), (D and E) CCl4 intoxicated rats that received both silymarin with either CGA or melatonin respectively, showing a marked decrease in collagen deposition (arrow), (F) CCl4 intoxicated rats that received a combination of silymarin, CGA and melatonin showing apparently normal collagen distribution within the cardiac tissue.

melatonin can diminish apoptosis of the liver after ischemia/reperfusion (Kireev et al., 2012).

The biochemical results of the current study were supported by histopathological observations. The damaging effect of CCl4 on cardiac tissue was observed in histomorphologic pictures by focal areas with great degeneration and excess collagen accumulation. Similar observation was supported by Li et al. (2012b). Administration of silymarin alone showed mild improvement which was more evident on the administration of silymarin with either CGA or melatonin. However ingestion of the combination of the three agents was the most effective one in modulating the histomorphologic picture of cardiac to more or less normal. The modulating effect of the used agents on collagen deposition may indicate the anti- fibrotic benefit of the used agents which was documented by previous studies (Kim et al., 2012; Li et al., 2012b; Shi et al., 2012).

In conclusion, the present findings showed that although silymarin alone or in combination with either CGA or melatonin was beneficial in ameliorating the inflammatory response-induced cardiac infarction as well as oxidative DNA damage and apoptosis caused by the toxic effects of CCl4, the combination of the three agents was the most effective one and this may support the use of this combination as an effective drug to treat cardiomyopathy.

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