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Original Research

The ameliorative role of silymarin on trichloroethylene-induced oxidative stress in rats

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Key Words
Liver; Rats; Silymarin; Trichloroethylene

Abstract
Silymarin, a chemical extracted from the herb milk thistle, is a widely known antioxidant, hepatoprotective and anti-carcinogenic. It suppresses the oxidative injury induced by trichloroethylene (TCE) which is considered as an environmental pollutant by increasing the endogenous antioxidant in animal tissues. Twenty four male albino rats were divided into *(1)* control, *(2)* silymarin-treated, *(3)* TCE-exposure, *(4)* and silymarin plus TCE combination groups. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGTP) and alkaline phosphatase (ALP) enzyme activities, and concentrations of total cholesterol (TC), triglycerides (TGs), low density lipoprotein cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c), urea and creatinine were analyzed. Additionally, lipid peroxidation (malondialdehyde, MDA), nitric oxide (NO), reduced glutathione (GSH) levels in liver as well as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities were measured. Liver Zn, Cu, Fe and Mn concentrations were also analyzed. TCE exposure increased serum ALT, AST, GGTP and ALP activities, TC, TGs, LDL-c, urea and creatinine concentrations, as well as MDA, NO, Zn, Cu, Fe and Mn concentration in liver, whereas it decreased SOD, GSH-Px activities, reduced GSH level in liver, and HDL-c in serum. Silymarin treatment significantly improved lipid peroxidation and oxidative injury induced by TCE. The study indicated that silymarin treatment ameliorate the harmful effect induced by TCE, taking in consideration the effect of silymarin as a free radical scavenger.

INTRODUCTION

Hepatitis viral infection, food additives, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, air and water pollutants are the major risk factors of liver toxicity [1]. Trichloroethylene (TCE) is an organic unsaturated solvent used in industrial field that induces toxicity in laboratory animals, and human hepatotoxicity, nephrotoxicity, cardiac and immune response disorder [2]. In TCE-related liver disease, free radicals play a part in the pathogenesis of liver damage. Acute and chronic TCE administration increases reactive oxygen species (ROS) production, lowers cellular antioxidant levels and enhances oxidative stress in many tissues, especially in the liver [2]. It undergoes metabolism by two major pathways, conjugation with glutathione (GSH) and cytochrome P450-dependent oxidation. The metabolites derived from P450 metabolism, including choral hydrate, trichloroacetate (TCA) and dichloroacetate (DCA), has been associated with pulmonary and hepatic toxicity as well as liver tumorigenesis [3]; also these metabolites are able to induce lipid peroxidation, oxidative DNA damage and oxidative stress, which implicate in their carcinogenesis [4].

Flavonoids belong to the family of benzo-gamma-pyrones including flavones, flavonones and isoflavonones. Most flavonoid functions in the human body are antioxidants. They help neutralize overly reactive oxygen molecules and prevent these reactive molecules damaging cell parts. Particularly in oriental medicine, flavonoids have been used in conjugation with their antioxidant, antitumor and protective properties [5].

Reports suggested that the number of hydroxyl (-OH) substitution is a critical factor in ROS scavenging activity of flavonoids; flavonoids with -OH groups exhibited more potent antioxidant activity [6]. The antioxidant nature of flavonoids is defined mainly by the presence of a B-ring catechol group (dihydroxy-lated B-ring) capable of readily donating hydrogen (electron to stabilize a radical species). The presence of 2,3 unsaturation in conjugation with 4-oxo- function in the C-ring and the presence of functional groups capable of binding transition metal ions such as iron and copper are also responsible for the antioxidant nature of the flavonoids [7].

Silymarin is a flavonoid extracted from the seed of *Silubum Marianum* L Gearth. The principle compo-nents are silybin (silibinin), silychristin, and silydinin [8]. It is commonly used as an herbal therapy; it has been effectively used for treating liver disease and acute liver injury partly due to its antioxidant activity [9]. Silymarin is an effective antioxidant, conserving GSH in liver cell while stabilizing the liver cell membranes against oxidative attack [10]; furthermore it reduces the inflammatory reaction, and inhibits the fibrogenesis in liver [11]. Based on the results of studies using methods of molecular biology, silymarin significantly reduces tumor cell proliferation, angioge-nesis as well as insulin resistance in male mice [12]. In addition, it exerts an anti-atherosclerotic effect and suppresses tumor necrosis factor induced protein production and mRNA expression due to adhesion molecule [10]. In a clinical trial silymarin lowered liver enzymes and improved antioxidant status, but did not consistently improve symptoms [13].

This study has been designed to evaluate the antioxidant activity of silymarin against TCE-induced oxidative damage in male albino rats.

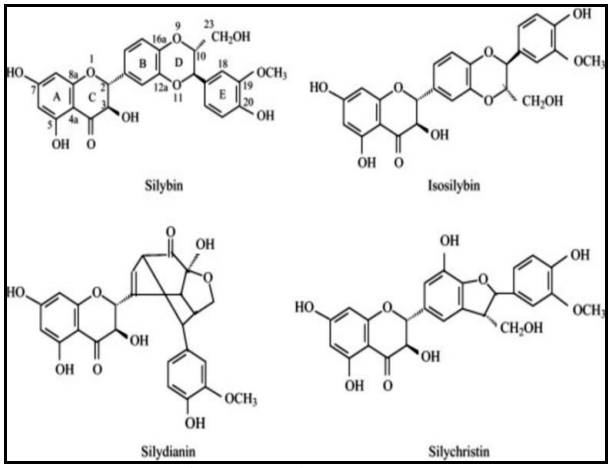

Figure 1. Structure of 4 isomers of silymarin

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma Chemical (St. Louis, MO, USA). Kits used in this experiments were purchased from Bio-Diagnostics (Worcestershire, UK).

Animals and treatment

Male Wistar rats, weighing 150-180 g, were purchased from the animal breeding unit of the National Research Center, Giza, Egypt. Rats were housed under appropriate conditions of controlled humidity, temperature and light. The animals were allowed free access to water and fed a standard pellet rat diet. The rats were acclimatized in the animal facility of the National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt, for at least one week before subjecting them to experimentation. The study was conducted in accordance with the guidelines set by the European Economic Community (EEC) regulations (Revised Directive 86/609/EEC) and approved by the Ethical Committee at NCRRT. Rats were segregated into groups of six animals each. Each group was subjected to one of the following treatments:

-*Control animals:* The animals supplied with 0.1 ml/kg maze oil as a vehicle.

-*Silymarin treated group:* silymarin was supplied to a certain group of animals as a single oral (p.o.) dose (70 mg/kg b.wt) [14].

-*Trichloroethylene administered group:* animals in TCE group were administered intraperitoneally (i.p.) with a single dose of TCE (500 mg/kg b.wt diluted with 0.1 ml/kg maze oil) [15].

-*Curative group*: animals were administered single dose of TCE (500 mg/kg b.wt diluted with 0.1 ml/kg maze oil, i.p.); thirty minutes later, these animals were treated with a single dose of silymarin (70 mg/kg b.wt, p.o.). Animal groups were sacrificed one and three days post TCE exposure and/or silymarin treatment, blood and liver samples were collected for biochemical analysis.

Biochemical analysis

The blood samples were collected directly from the animals by heart puncturing. The blood samples were centrifuged using Hettich Universal 16 centrifuge (Andreas Hettich GmbH, Tuttlingen, Germany) at 3000 rpm for 15 min. and the clear serum was collected and stored in a refrigerator. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGTP) and alkaline phosphatase (ALP) as well as concentrations of total cholesterol (TC), triglycerides (TGs), low density lipoprotein cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c), urea and creatinine in serum were analyzed.

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Liver was excised from the rats and the homogenization was carried out using a homogenizer (Universal Laboratory Aid Type MPW-309; Mechanika Precyzyjna, Warsaw, Poland). One portion is used to analyze malondialdehyde (MDA), nitric oxide (NO), reduced glutathione (GSH) levels as well as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities.

ALP activity was assayed using the method of Belfield and Goldberg [16]. The quantitative determination of GGTP activity was done using a commercial kit according to the method of Szasz [17]. ALT and AST activities were determined by kits according to the method of Reitman and Frankel [18]. LDL-c concentration was determined according to the method of Castelli [19], while HDL-c concentration was estimated according to Lopes-Virella *et al* [20]. TC concentration was determined according to the method of Meiattini and coworkers [21]. TGs concentration was determined according to the method of Bucolo and David [22]. Urea content present in the serum sample was determined according to the method of Patton and Crouch [23]. Creatinine content present in the sample was determined according to the method of Folin [24]. MDA was assayed with the method of Yoshioka and colleagues [25]. NO concentration was measured by using the method of Moshage *et al* [26]. GSH content was determined using the method of Ellman [27]. Superoxide dismutase activity was estimated with the method of Marklund and Marklund [28]. The activity of GSH- Px was measured according to the method of Gross *et al* [29]. All biochemical assays were performed with a Hελios UV-visible spectrophotometer (Thermo Spectronic, Cambridge, UK).

Atomic absorption analysis

Liver tissues were digested with a mixture of HNO_3 and H_2O_2 (5:1). Digestion was done by using a microwave oven model MLS-1200 MEGA Milestone (Bergamo, Italy). Then digested samples were diluted with deionized water to a fixed volume [30]. Selected elements (Fe, Cu, Zn and Mn) were estimated quantitatively by an atomic absorption spectrophotometer (Unicam 939*)* by using hollow cathode lamps in air/acetyelene flame [31]. Concentration of elements in tissues was calculated by using calibration curve prepared from their stock solution (1 mg). The concentration of elements per grams of wet tissues [32] could be determined by the following equation:

$$\mathbf{C1 = C2 / W \times D}$$

C1; concentration of the element per gram wet tissue (µg/g)

C2; concentration of the sample solution (µg/ml)

W; sample weight

D; dilution factor of the sample

Statistical analysis

To assess the significant level of influence caused by silymarin treatment in TCE administered rats, one way analysis of variances (ANOVA) followed by Tukey's multiple comparison test was used. Data represents means values and standard deviation of at least three independent experiments. Statistical analysis was performed by using Graph-Pad software, San Diago, CA, USA) Differences were considered statistically significant when the P value was less than 0.05.

RESULTS

Trace element homeostasis

Concerning the effects on trace element homeostasis, exposure to TCE produced an elevation in liver Zn, Cu and Fe that became significantly on the 3rd post exposure day recorded as 29%, 30% and 45%, respectively, while Mn showed initial significant decline on the 1st post TCE exposure day that elevated and became significantly increased on the 3rd post exposure day (-28% and 22%, respectively). Administration of silymarin after TCE provided significant protection to these altered liver trace elements (Table 1).

Table 1. Effect of silymarin treatment on liver Zn (µg/g tissue), Cu (µg/g tissue), Fe (µg/g tissue) and Mn (µg/g tissue) concentrations of rats received TCE

Treatment	Control group		TCE group		Sily group		TCE & Sily group	
	1 day	3 days	1 day	3 days	1 day	3 days	1 day	3 days
Zn (µg/g tissue)	35.38 ± 3.82	39.06 ± 2.35	29.32 ± 3.01	50.57 ± 10.21[a]	35.38 ± 4.7	38.39 ± 3.26	30.47 ± 1.93	42.21 ± 3.39
Cu (µg/g tissue)	3.19 ± 0.29	3.26 ± 0.29	3.74 ± 0.54	4.25 ± 1.03[a]	2.71 ± 1.15	3.25 ± 1.13	3.11 ± 0.49[b]	3.67 ± 0.81
Fe (µg/g tissue)	238.2 ± 0.59	233.9 ± 20.87	286.27 ± 57.95	339 ± 39.07[a]	224 ± 4.79	244 ± 6.62	234.16 ± 3.9	291.8 ± 8.52[a]
Mn (µg/g tissue)	1.88 ± 0.049	1.68 ± 0.15	1.34 ± 1.47[a]	2.06 ± 0.29[a]	1.51 ± 0.69	1.42 ± 0.55	1.56 ± 1.01	1.5 ± 0.424[b]

Data are represented as mean ± SD from at least three independent experiments; [a]significantly different from the control group ($P < 0.05$), [b]significantly different from the TCE group ($P < 0.05$); Sily, silymarin.

Liver function enzymes

As presented in Fig.2, the activity of liver enzymes were significantly increased in the serum of TCE administered rats; compared to controls, ALT, AST, GGTP and ALP activities increased by 45%, 103%, 41% and 66%, respectively, on the 3rd post TCE exposure day. Administration of silymarin after TCE exposure was beneficial in recovering the liver enzymes activity.

Lipid profiles

The levels of serum TC, TGs, LDL-c were increased by TCE exposure through the experimentation period to 48%, 61% and 151% of control levels, respectively, after 3 days of TCE exposure. Serum HDL-c significantly decreased with a maximum level of 58% compared to control group on the 1st day of TCE exposure (Fig.3). Silymarin treatment significantly ameliorated these changes and the lipid profile levels became more or less similar to controls.

Kidney function

As demonstrated in Fig.4, toxic effects on kidney function upon TCE exposure and subsequent treatment. TCE intoxication imposes deleterious effects on kidney function evidenced by significant increase in serum urea and creatinine levels; this increase was recorded as 60% and 44%, respectively, compared with the controls. Treatment with silymarin provided significant protection to these altered serum kidney function profile variables.

Liver oxidative stress status

Fig.5 shows results of TCE exposure effects on some hepatic oxidative stress variables and subsequent treatment. Liver MDA and NO levels increased significantly, recorded 24% on the 3rd day and 52% on the 1st day post TCE exposure. A reverse trend was observed as GSH level showed a decrease on TCE exposure group. Interestingly, treatment with silymarin alone recorded a significant increase in GSH content on the 1st day of silymarin treatment (57%) as compared to control level. However, silymarin was beneficial in improving liver reduced GSH, while MDA and NO levels were still significantly higher as compared to control groups.

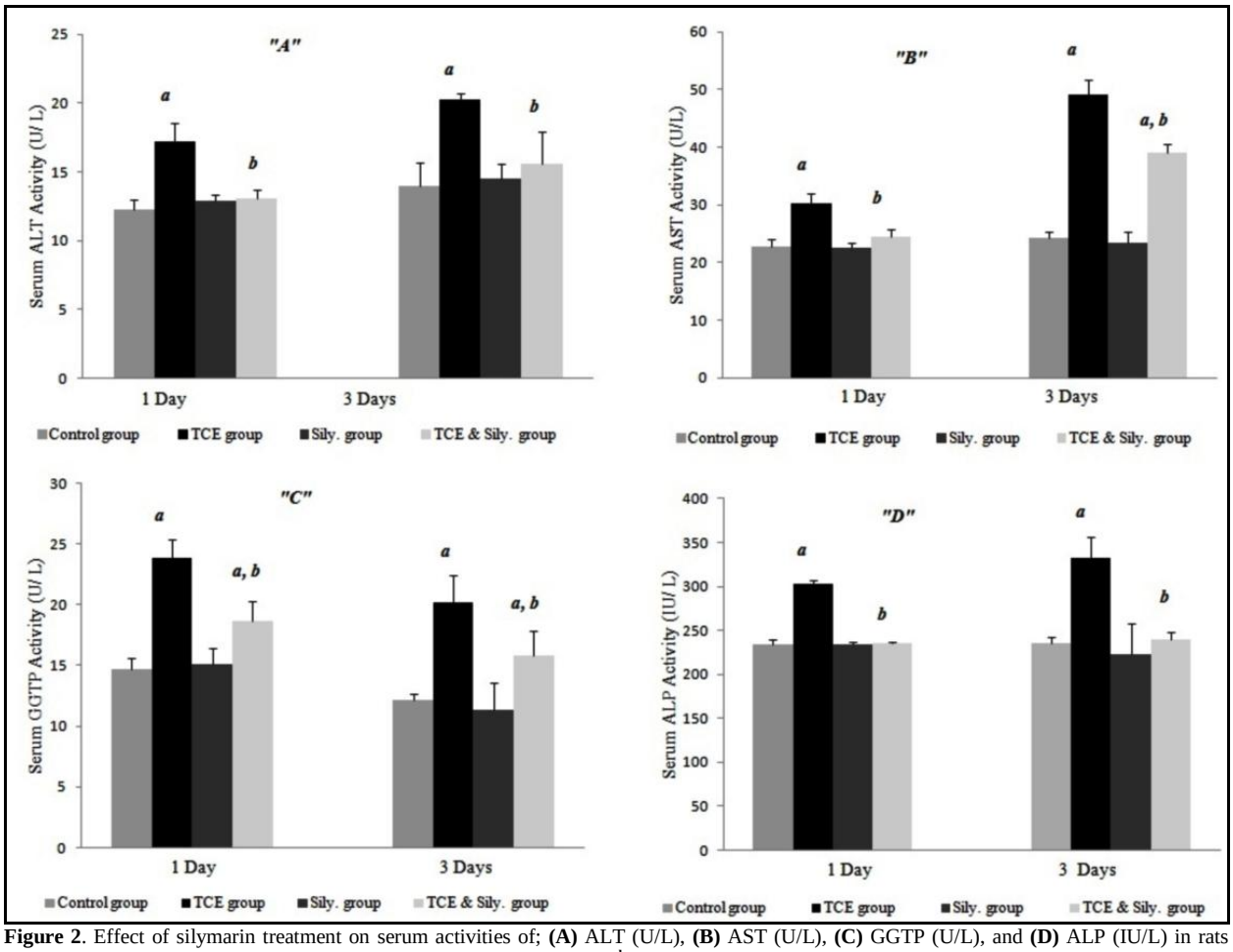

Figure 2. Effect of silymarin treatment on serum activities of; **(A)** ALT (U/L), **(B)** AST (U/L), **(C)** GGTP (U/L), and **(D)** ALP (IU/L) in rats received TCE; [a]significantly different from the control group ($P < 0.05$), [b]significantly different from the TCE group ($P < 0.05$); Sily, silymarin.

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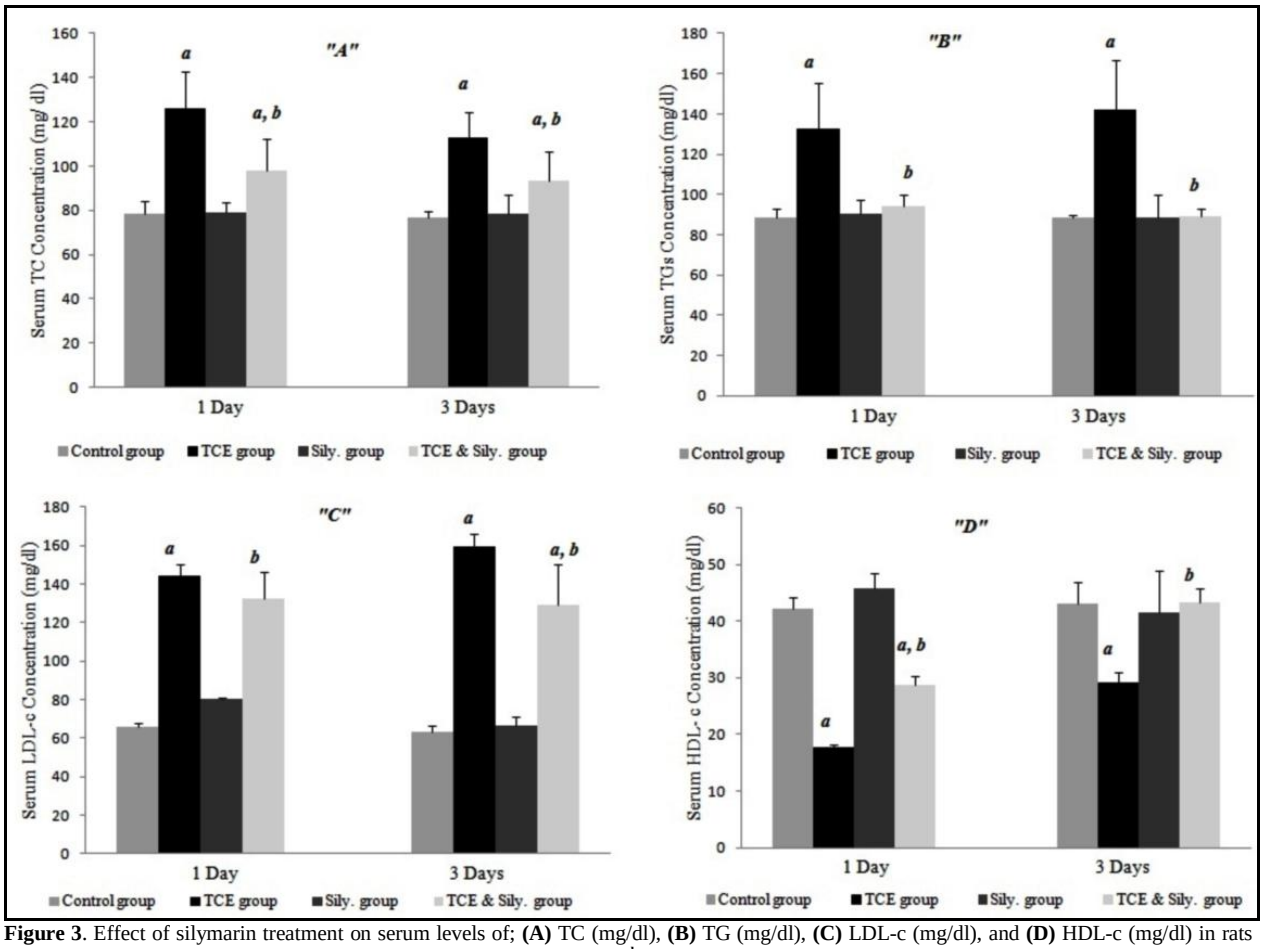

Figure 3. Effect of silymarin treatment on serum levels of; **(A)** TC (mg/dl), **(B)** TG (mg/dl), **(C)** LDL-c (mg/dl), and **(D)** HDL-c (mg/dl) in rats received TCE; [a]significantly different from the control group ($P < 0.05$), [b]significantly different from the TCE group ($P < 0.05$); Sily, silymarin.

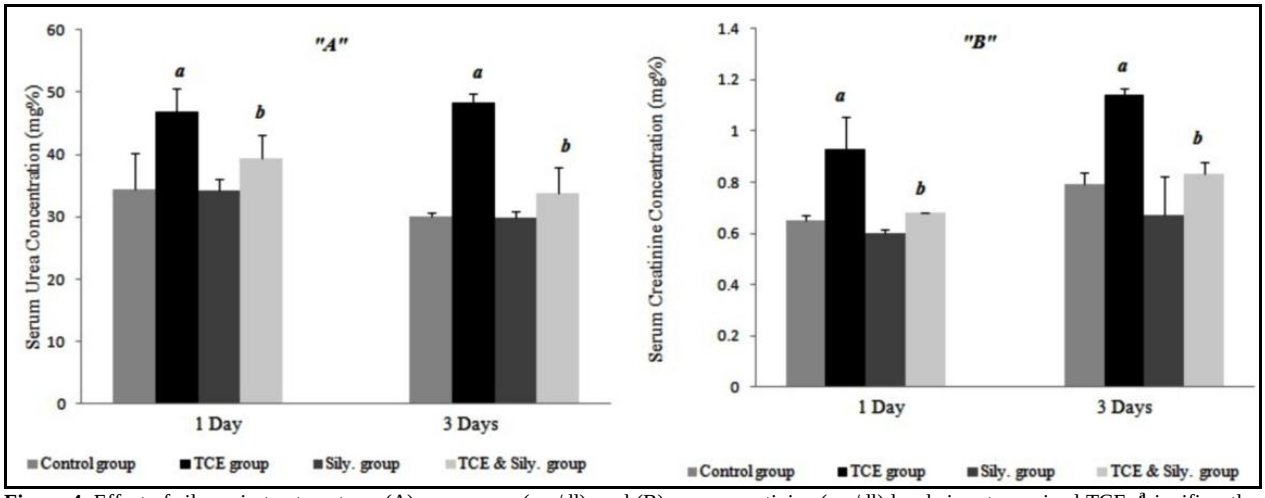

Figure 4. Effect of silymarin treatment on; (A) serum urea (mg/dl), and (B) serum creatinine (mg/dl) levels in rats received TCE; [a]significantly different from the control group ($P < 0.05$), [b]significantly different from the TCE group ($P < 0.05$); Sily, silymarin.

Antioxidant enzymes

Fig.6 illustrates the toxic effects on some hepatic antioxidant enzymes upon TCE administration and subsequent treatment. TCE exposure imposes deleterious effects on SOD and GSH-Px enzymes in liver as evidenced by a significant depletion of GSH-Px and SOD activities; the inhibited SOD and GSH-Px activities showed significant protection following treatment with silymarin suggesting improved antioxidant defense system.

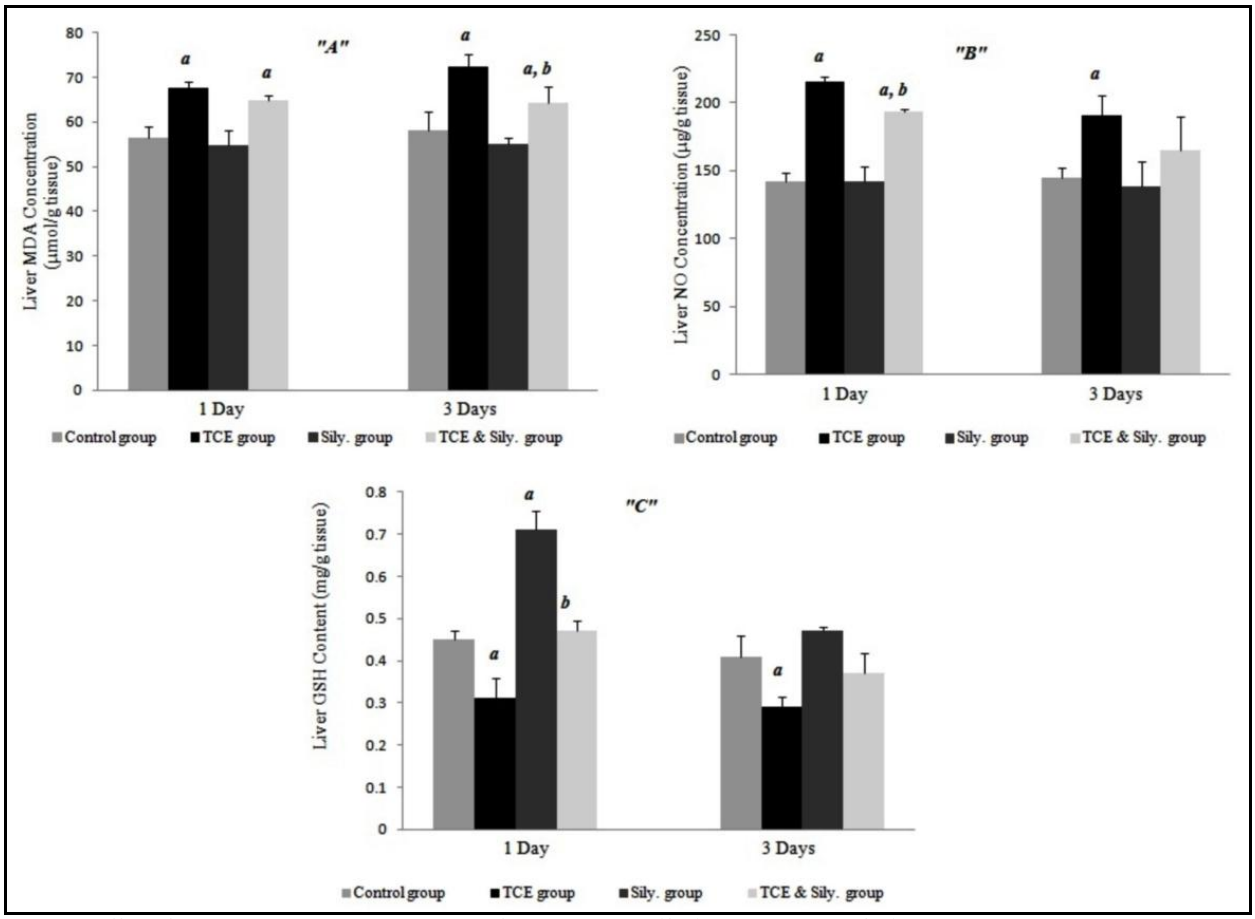

Figure 5. Effect of silymarin treatment on liver levels of; (A) MDA (µmol/g tissue), (B) NO (µg/g tissue), and (C) GSH (mg/g tissue) in rats received TCE; [a]significantly different from the control group ($P < 0.05$), [b]significantly different from the TCE group ($P < 0.05$); Sily, silymarin.

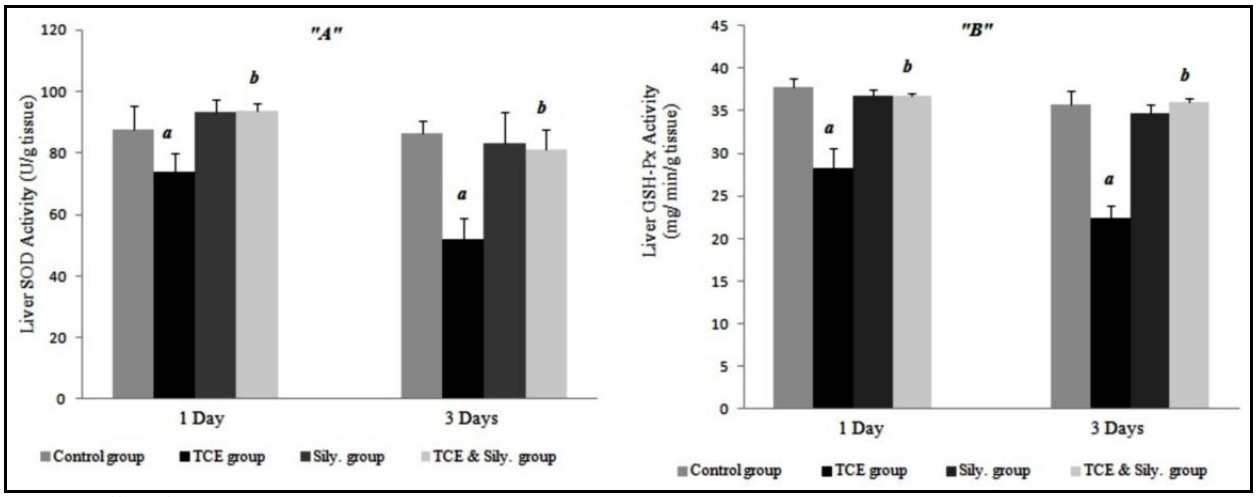

Figure 6. Effect of silymarin treatment on; (A) liver SOD (U/g tissue), and (B) liver GSH-Px (mg/min/g tissue) activities in rats received TCE; [a]significantly different from the control group ($P < 0.05$), [b]significantly different from the TCE group ($P < 0.05$); Sily, silymarin.

DISCUSSION

Previous studies and present results demonstrated the oxidative stress damage induced by TCE exposure [33, 34]. In the present study TCE intoxication imposes deleterious effects on liver and kidney functions as well as lipid profile and some trace elements homeostasis. The hepatotoxicity induced by TCE is dependent on its metabolites derived from P450 including choral hydrate, TCA and DCA [35]. The elevation of both ALP and GGTP activities observed in the present result reflects a liver damage [36].

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According to Brown *et al* [37] the increase in serum TC and LDL-c concentrations, and liver dysfunction after TCE exposure may be due to the effect of LDL receptors or oxidized cholesterol toxicity caused by free radicals, beside the major metabolic consequence of oxidative stress, that associated with the deposition of triglycerides in liver [38] The present study confirmed the recent findings of Gharib [34] and Khan *et al* [39] that TCE significantly increased urea and creatinine in blood. Moreover, oxidative markers measured as lipid peroxidation and NO concentration in liver tissue increased markedly followed by a decrease in liver reduced GSH level, SOD and GSH-Px activities. The depletion of GSH enhances utilization of protein thereby increasing the urea level that is accompanied by an increased creatinine level suggested previously by Gharib [34]. Furthermore, Chen *et al* [40] stated that the decrease of GSH-Px and SOD may be due to the decrease in antioxidant defense system and elevation of free radicals. The increase in liver NO concentration caused by TCE exposure attributed to the activation of nitric oxide synthase and expression of NO mRNA [41].

On the other hand, TCE exposure caused an increase in the liver levels of Zn, Cu and Fe, while Mn showed a highly significant decrease on the first day accompanied by an increase on the 3rd day post TCE treatment. According to Dashti *et al* [42] the increase in Zn and Cu content could be due to the severity of cellular damage observed microscopically in the liver. Moreover, Keen et al [43] stated that Mn is eliminated from the body mainly in the bile. Thus, the impaired liver function may lead to decrease Mn secretion.

Silymarin belongs to flavonoids which are naturally occurring substances that exhibit various pharmacological and therapeutic applications [44]. Due to their phenolic structures they have antioxidant effects and inhibit free radical mediated processes [45]. Hepatoprotective effects are achieved via several mechanisms including inhibition of lipid peroxidation [46] stimulation of RNA polymerase and subsequent protein synthesis leading to enhanced hepatocyte regeneration [47] and enhanced liver detoxification via inhibition of phase I detoxification [48], enhanced glucuronidation and protection from GSH depletion [49].

Results from the present study showed that silymarin treatment ameliorated TCE induced oxidative damage. According to Mansour *et al* [50] the antioxidant effect of silymarin attributed to its structure; silymarin possess a hydroxyl group at C_5 in addition to a carbonyl group in C_4, which may form a chelate with ferrous ions. This chelation can raise the activity level of most active scavengers. The free hydroxyl group at C_5 and C_7 on silymarin structure may also favour the inhibition of lipid peroxidation by reacting with peroxy radicals (see Fig.1). Furthermore, the presence of functional groups are capable of binding to iron and copper [45]; this may be an explanation why silymarin could ameliorate the increase of Fe and Cu concentration induced by TCE exposure observed in the present study. This ability of silymarin leads to a significant increase in the cellular antioxidant defence machinery by ameliorating the deleterious effects of free radical reaction and by increase in GSH content, which is important in maintaining the ferrous state [50, 51].

In conclusion, silymarin significantly ameliorated the oxidative damage induced by exposure to TCE due to the increased antioxidant capacity.

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