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Hepatoprotective effect of silymarin on chronic hepatotoxicity in mice induced by carbon tetrachloride

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Abstract
This study was designed to explore the ideal dose of CCl_4 for chronic hepatotoxicity in *Swiss albino* mice as well as to verify the effective dose of silymarin for protection. Five groups of mice were prepared, within them, group (I) was served as control. The animals of group (II), (III) and (IV) were treated with 10mL of CCl_4 solution (10, 20 and 30% CCl_4/olive oil, respectively)/kg b.w. for each three days, o.p. and during 6 weeks. Animals from group (V) were treated with silymarin by oral doses of 16mg/kg b.w. after one hour of every CCl_4 treatment as group (III). Serum alanine transaminase (ALT), total cholesterol (TC), liver weight/body weight ratio (L/B), liver tissue malondehydyde, protein carbonyls and histology properties were determined. The results showed that chronic hepatotoxicity induced by CCl_4 could be detected at CCl_4 concentration of 10mL CCl_4 solution (20% in olive oil)/Kg b.w. Beside, treatment with silymarin 16mg/Kg b.w. was verified as an effective dose for chronic hepatotoxicity protection.

Keywords: Hepatotoxicity, silymarin, alanine transaminase, malondehydyde, protein carbonyls

1. Introduction
Carbon tetrachloride (CCl_4) is one of the oldest hepatotoxic chemical agents and is most widely used for experimental induction of liver injury on animals [1]. CCl_4 is transformed to trichloromethyl free radicals that are capable to attack cellular macromolecules such as lipids, proteins, and DNA [2, 3]. These reactive free radicals initiate cell damage through two different mechanisms including of covalent binding to the membrane proteins and cause lipid peroxidation [4]. The results of intoxication with CCl_4 are cellular necrosis, oxidative stress and inflammation, which leads to hepatic damages, such as fibrosis, cirrhosis, and atrophy [5]. Antioxidants play a significant role in protecting liver from the toxic effect of various chemicals by preventing free radical formation [6].
Silymarin is the active constituent of *Silybum marianum* having antioxidant activity [7] and has been used for treatment in various toxic models of experimental liver diseases in laboratory animals, reduced liver toxicity caused by CCl_4 [8-10]. It inhibited free radical induced lipid peroxidation in microsomal and mitochondrial preparation of human red blood cells, thereby stabilizing the structure of the cell membrane [11]. According to Mourelle and Favari (1989) [12] Muriel and Mourelle (1990) [13], using silymarin with the dose of 15–800 mg/Kg body weight to dogs, mice and rats could prevent carbon tetrachloride induced liver damage.
The different concentrations of used CCl_4 could caused various degree of liver damage [14] and effective dose of silymarin is also required for treatment. This study intends to define concentration of CCl_4 which can cause chronic liver injury and to verify the effective dose of silymarin for treatment.

2. Materials and Methods
2.1 Animals
Male white mice (*Swiss albino* strain) were obtained from the Pasteur Institute, Ho Chi Minh city, Vietnam. They were 5 to 6 weeks old (25–30g) and allowed free access to pellet diet and water ad libitum to acclimatize for a week prior to experimentation. Mice were housed in plastic mesh cages in the laboratory of Department of Pharmacology, Cantho University of Medicine and Pharmacy, under ambient temperature and 12 h light and dark cycle.

2.2 Experimental protocol
Thirty mice were divided into five groups (each group consisted 6 mice). Group (I) as a normal control, animals were treated with olive oil (10mL/kg body weight per day, o.p. three days for once).

Group (II), (III) and (IV) animals were treated with 10mL of CCl_4 solution (10, 20 and 30% CCl_4/olive oil, respectively) per kg body weight (corresponding to 1, 2 and 3mL CCl_4/ kg body weight) for each three days, o.p. and during 6 weeks to induce chronic chemical liver injury [15]. Group (V) animals were treated firstly with 10mL of CCl_4 solution (20% CCl_4/olive oil) per kg body weight (corresponding to 2mL CCl_4/ kg body weight, o.p., similar to group III), one hour after CCl_4 induced hepatotoxicity they were treated simultaneously with silymarin (Sigma–Aldrich), by oral doses of 16mg/kg body weight [16], these treatment was carried out three days for once and for 6 weeks.

At the end of the experiments, blood and livers were collected immediately after the animals were sacrificed. Blood was determined the serum ALT and TC. The liver from each animal was weighed for determination of the L/B ratio and the biochemical and histology properties.

2.3 Determination of ALT and total cholesterol of blood, biochemical and histology properties of liver

Blood and liver samples were sent to Cantho University Hospital for analysing of ALT and TC in serum and histology property in liver. The degree of fibrosis was evaluated in the liver tissue according to the Hepatitis Activity Index (HAI) [17] which scores of fibrosis were based on Knodell – Ishak scales from 0 to 22.

Biochemical properties of liver consist of protein carbonyls (PC) and lipid peroxidation product, measured as malondehydyde (MDA).

Protein carbonyls (PC) were measured by spectrophotometric method at the absorbance of 370 nm, using dinitro-phenylhydrazine (DNPH) reagent [18]. Results were calculated as nanomoles of carbonyl groups per milligram of protein (nmol/mg protein) using a molar extinction coefficient of 22,000 $M^{-1}cm^{-1}$. Total protein was determined by Bradford assay [19] that relies on the binding of the dye Coomassie Blue G250 to protein that has an absorbance maximum at 590 nm. The quantity of protein can be estimated by determining the amount of dye in the blue ionic form by measuring the absorbance of the solution at 595 nm.

Malondehydyde (MDA) of liver tissue was carried out using the modified method of Ohkawa *et al*. (1979) [20]. MDA is a product of lipid peroxidation that reacts with acid thiobarbituric (TBA) under acidic conditions forming a pink complex that absorbs at 532 nm. Malondialdehyde bis (Acros – Belgium) was used as the standard. The results are expressed as nmol/mg protein.

2.4 Statistical analysis

The data were submitted to analysis of variance (ANOVA) by Portable Statgraphics Centurion 15.2.11.0 and were expressed as mean values and standard deviation.

3. Results and discussion

The L/B ratio, ALT and TC of serum as well as MDA and PC of liver tissue from five groups of mice were presented in Table 1. Histological examination of liver tissues was displayed in Figure 1.

Table 1: Biochemical properties of liver blood and tissues of experiment mice groups

Groups	L/B (%)	ALT (U/L)	TC (mg/dL)	MDA (nmol/mg protein)	PC (nmol/mg protein)
I	3.43^{a}±0.14	46.67^{a}±2.66	131.88^{ab}±7.49	7.90^{a}±0.29	5.04^{a}±0.20
II	3.73^{a}±0.12	48.50^{a}±6.10	133.17^{b}±8.00	8.26^{a}±0.35	5.21^{a}±0.26
III	5.02^{c}±0.33	190.17^{b}±55.82	153.11^{c}±18.21	11.62^{b}±0.34	8.34^{b}±0.30
IV	5.80^{d}±0.43	407.50^{c}±109.37	156.33^{c}±12.63	14.45^{c}±0.25	11.20^{c} ± 0.78
V	4.15^{b}±0.30	61.33^{a}±18.61	117.69^{a}±13.99	8.22^{a}±0.32	5.44^{a}±0.44

(Means±SD, The values showing different superscripts within a row are significant different at p = 0.05)

All the L/B, ALT, TC, MDA and PC levels had the tendency to increase after carbon tetrachloride treatment as compared the normal group (oliu oil treatment). However, there was no significant different in biochemical properties between group II and group I (mice treated with 1mL CCl_4/Kg b.w.). CCl_4 concentration of 1mL/Kg b.w. seem to be not enough dose for experimental induction of chronic liver injury on *Swiss albino* mice. All these parameters of group III (mice treated with 2mL CCl_4/Kg b.w.) began to be significant different with control group. The higher concentration of CCl_4 treated was the more significant difference in these parameters. The treatment with silymarin after CCl_4 induced hepatotoxicity could help to remain all biochemical properties of mice (group V) closing to these values of control group (Table 1).

The ratio of liver weight to body weight (L/B) were higher in animals treated with 2 and 3mL CCl_4/Kg (group I.3 and I.4) than in control animals (group I.1). These increasing were 46.4 and 69.1%, respectively. Increasing in L/B in this study coincides with many previous results from studying of hepatotoxicity on mice by CCl_4 [21, 15, 22]. CCl_4 is a well-known hepatotoxic agent and the most remarkable pathological characteristics of CCl_4 induced hepatotoxicity are fatty liver, cirrhosis and necrosis [22]. It could result in an increasing of blood content, to the dilatation of central veins and sinusoids, swelling of hepatocytes resulted from the increase in water transport in cells and fatty liver or due to the increase in accumulation of fat in hepatocytes. All of these reasons could lead to increase in L/B of CCl_4 treated mice [23, 22]. Treatment with the silymarin (18mg/kg b.w.) reduced L/B to 82.7%, and formed the significant difference in L/B values of mice from group V that received from the combination of silymarin with CCl_4 (2mL/kg b.w.) and from the group treated with CCl_4 alone (group III). This result due to the protective effects of silymarin on CCl_4 caused damage.

The serum ALT increased 307.5 and 773.2% respectively in mice treated with 2 and 3mL CCl_4/Kg comparing to ALT of control mice (Table 1). The serum activity of ALT is used as an indication of the extent of liver damage due to the release of large quantities of it into the bloodstream [24]. The ALT is found outside of the mitochondria of the liver. CCl_4 induces the peroxidation of lipids that damage the membranes of liver cells and organelles and results in the release of ALT into the circulating blood [25]. Administration of CCL_4 significantly increased the serum levels of liver enzymes (ALT), which are indices of liver cell damage and leakage of enzyme from cells [26]. The rising in ALT activity is almost always due to hepatocellular damage [27]. Essawy *et al*. (2012) reported that serum ALT for *Swiss albino* mice treated with CCl_4 at a dose level 1.9 ml/kg b.w increased 328.8% comparing to ALT value of control mice [28]. ALT value of mice treated with

silymarin was similar to ALT value of mice from control group (Table 1). The restoration towards normal level of serum ALT indicated that silymarin acted against the damaging effects of free radicals produced by CCl_4. Silymarin preserved the structural integrity of hepatocellular membrane and protected the liver from the harmful effects of this hepatotoxin. The tendency of this result was also found from earlier studies [29, 30].

The liver is the major site for the synthesis and metabolism of cholesterol [31]. Distinct alterations in lipid metabolism have been reported in CCl_4 induced hepatotoxicity in rats [32]. The present results expressed the significant increasing in levels of blood total cholesterol in CCl_4 inducted mice. The TC value increased 16.1 and 18.3% respectively in mice treated with 2 and 3mL CCl_4/Kg comparing to TC value of control mice (Table 1). CCl_4 increases the transport of acetate into the liver cell, resulting in increased acetate availability, for this reason, the cholesterol synthesis from acetate was also increased[33]. Silymarin also attenuated the increased levels of blood total cholesterol of mice treated previously with CCl_4. In this study, blood total cholesterol of mice in group V (silymarin treatment) was similar to that of control group. This rule was consistent with the results from other researchers [34-36].

The malondialdehyde (MDA) level in liver tissue was assessed as an indicator of lipid peroxidation in oxidative liver damage. MDA is one of lipid peroxidative product and for several decades it has been used as a biomarker of lipid peroxidation [37]. In addition, the increase of MDA has been considered a key feature in liver injury [38]. In this study, CCl_4 treatment markedly increased the hepatic MDA level compared with the normal group. These increasing were 47.1 and 82.9% respectively in mice treated with 2 and 3mL CCl_4/Kg comparing to MDA level of control mice (Table 1). Saad (2013) [39] found that hepatic MDA level of *Swiss albino* mice with CCl_4 induced hepatic injury was 6.42 ± 0.52 (µM/mg protein) while the liver MDA level of control mice was 4.96 ± 0.28 (µM/mg protein). Treatment with silymarin significantly reversed these changes. In this study, MDA level in mice from group V (silymarin treatment) was maintained similar to hepatic MDA level of control group (Table 1). It was due to its antioxidant activity owing to the presence of silymarin. It was also proven that the decreased MDA level in mice tissue due to silymarin treatment by many authors [40].

Another aspect as regards to oxidation of proteins, protein oxidation may play a role in the pathogenesis of CCl_4 induced liver injury [41]. Free radical-mediated oxidation of proteins results in the formation of carbonyl groups [42]. For this reason, protein carbonyl (PC) content is widely used as a indicator for measuring of oxidative damage [43]. The present study was able to detect a significant increasing in the liver carbonyl protein contents in the CCl_4 treated mice comparing to control mice. These increasing were 65.5 and 122.2% in mice treated with 2 and 3mL CCl_4/Kg respectively comparing to PC of control mice (Table 1). In the model of chronic liver injury from the study of Sundari *et al*. (1997) [41], a great increase in liver PC was observed approximately 3 fold compared to control rat liver and the author suggested that the accumulation of oxidised proteins in the liver may be an early indication of CCl_4 liver injury. In our study, silymarin significantly reversed the elevation of liver protein carbonyls level that was similar to liver PC level of control group. The reason of situation was due to the antioxidant activity of silymarin against lipid peroxidation in mice liver. Silymarin can contribute to the antioxidant defenses in different ways. Firstly, by direct free radical scavenging. Secondly, by preventing free radical formation by inhibiting specific enzymes responsible for free radical production, and thirdly, by participating in the maintenance of optimal redox status of the cell by activating antioxidant enzymes [44]. The present results in liver PC changes of silymarin treated mice are consistent with previous reports [45, 46].

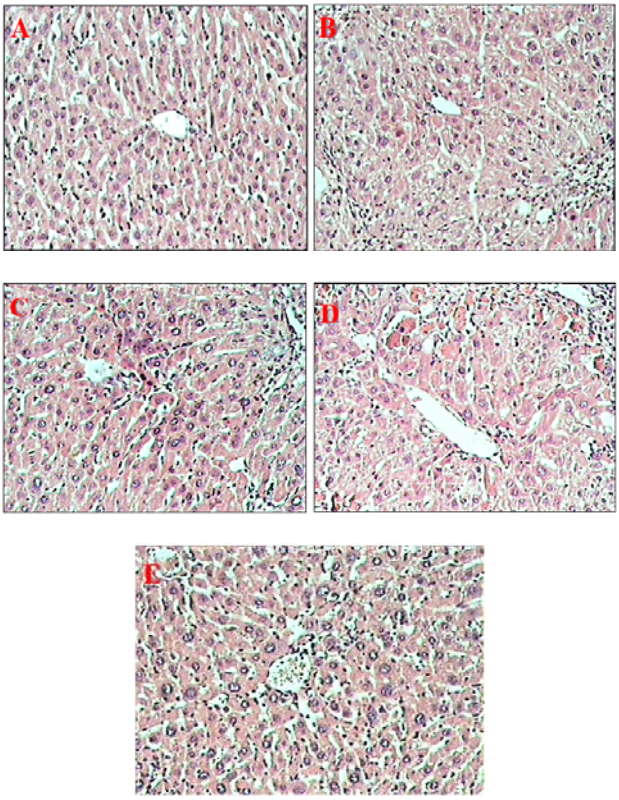

Fig 1: Micrographs from representative liver tissues collected from mice treated with oliu oil (control, A); 1mL CCl_4/Kg (B), 2mL CCl_4/Kg (C), 3mL CCl_4/Kg (D) and 2mL CCl_4/Kg + Silymarin 18mg/Kg (E). (H and E staining, magnification x 100)

The Effects of CCl_4 concentrations and silymarin treatment on the liver histopathology of *Swiss albino* mice were described in Table 2.

Table 2: Liver histopathology description and chronic hepatitis degrees of *Swiss albino* mice treated with CCl_4 and silymarin

Groups	Descriptions	Scores (HAI)	Degrees of chronic hepatitis
I	Liver tissues presented with normal histological structure, hepatocytes and venous sinusoids are arranged as interconnected plates (Figure 1.A)	0	No inflammation
II	Appearing very little inflammatory cells as well as necrotic cells in the lobules, widening of portal area, the disarrangement of hepatocytes and venous sinusoids around the central lobules (Figure 1.B).	4	Mild chronic hepatitis
III	The expression was more serious level comparing to that of group II (Figure 1.C).	10	Moderate chronic hepatitis
IV	Liver tissue expressed serious damage, the structure of the liver is degenerated, the necrotic alterations were observed in the hepatocytes surrounding the central veins. Many inflammatory as well as fibrosis cells infiltration was detected around portal area (Figure 1.D).	18	Serious chronic hepatitis
V	Liver tissues displayed nearly normal histological structure, hepatocytes and venous sinusoids are arranged as interconnected plates. Necrotic cells could not be found in the lobules, but there was very little inflammatory cells (Figure 1.E).	3	Very mild chronic hepatitis

4. Conclusion

Liver is the most important primary target organ for CCl_4 induced toxicity in many species. CCl_4 when metabolized in the body is changed into a very reactive free radicals that then induce hepatic damage. The peroxidation and damage occurred to biomolecules (lipid and protein) that were proven through the results of this study. In addition, the treatment with silymarin could protect the hepatocellular damage induced by CCl_4 due to its antioxidant activity. To develop the chronic heptato toxicity induced by CCl_4 in *Swiss albino* mice, CCl_4 concentration of 2mL/Kg b.w. was ideal dose for injury detecting, and silymarin 16mg/Kg b.w. was considered effective dose for injury treatment.

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