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HEPATOPROTECTIVE EFFECT OF TOFU PROCESSED FROM GERMINATED SOYBEAN ON CARBON TETRACHLORIDE INDUCED CHRONIC LIVER INJURY IN MICE

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Abstract:

The hepatoprotective activities of silk tofu made from germinated and non germinated soybeans at different doses of feeding against CCl_4 induced hepatic cell toxicity in mice was investigated in this study. The heptatoprotective activity was analyzed by assessing the ratio of liver weight to body weight (L/B), the levels of serum alanine aminotransferase (ALT), total cholesterols (TC), the hepatic malondehydyde (MDA), protein carbonyl (PC) and vitamin C levels as well as the histopathological analysis of liver tissue. All types of silk tofu significantly reduced the L/B value; ALT activity, total cholesterol, hepatic MDA and PC levels, beside, liver vitamin C content increased compared to CCl_4 intoxicated mice. Silk tofu made from germinated soybeans expressed higher hepatoprotective activity as compared to silk tofu made from non germinated soybeans. Mice fed with silk tofu made from germinated soybeans at the dose of 0.4 g/g body weight/day displayed all biochemical parameters as well as the liver tissue histopathological analysis that were similar to that of normal mice and silymarin treated mice. It was suggested that tofu specially made from germinated soybeans expressed great hepatoprotective effect.

Keywords: Liver injury, Carbon tetrachloride, Silk tofu, Germination, Antioxidants

Introduction

Chronic liver dysfunction or injury is one of the most serious health problems and be considered the major cause of human mortality in the world (Wood, 2010; Abdel-Wahhab *et al.*, 2011). Chronic liver diseases were described clinically through pathological processes of the liver, involving a process of progressive destruction and regeneration of the liver parenchyma. Finally, if left untreated, these processes will lead to cirrhosis and hepatocellular carcinoma (Hong *et al.*, 2015).

Generally, liver injury is considered a result of exposure to different environmental pollutants and xenobiotics e.g., thioacetamide, paracetamol, carbon tetrachloride, alcohol, etc. (Lazerow *et al.*, 2005; Ashraf *et al.*, 2012). These xenobiotic compounds mainly damage liver by producing the reactive oxygen species (ROS) that induce the toxicity by covalent binding and lipid peroxidation (Geesin *et al.*, 1990). Among these chemical hepatotoxins, CCl_4 had been frequently used to induces toxicity in rat liver which closely resembles human cirrhosis. It produces reactive free radicals trichloromethyl radical (CCl_3) and a proxy trichloromethyl radical (CCl_3O_2) when metabolized (Yang *et al.*, 2015). CCl_4 causes hepatocyte injury that is characterized by centrilobular necrosis that is followed by hepatic fibrosis (Yu *et al.*, 2002). Scavenging of free radicals by antioxidants could reduce the fibrosis process in the tissues (Thresiamma and Kuttan, 1996). Polyphenolic compounds from food materials are known to be excellent antioxidants in vitro because of the capacity to scavenge free radicals and protect antioxidant defense (Latha *et al.*, 1999). Beside, it is preferable due to lack of serious adverse effects.

Tofu is a phenolic rich soybean product accepted for consumption worldwide, mostly in Asian countries (Wu *et al.*, 2004). Tofu is rich in protein and a good source of vitamins, minerals, as well as antioxidants such as polyphenols, isoflavones, vitamins C and vitamin E (Poysa and Woodrow, 2002). It was also demonstrated to prevent acetaminophen-induced liver damage in rats (Yakubu *et al.*, 2013). A simple, efficiency and unexpensive process to enhance important antioxidants such as polyphenols, isoflavones, vitamin C and vitamin E in soybean is germination (Kaushik *et al.*, 2010; Paucar-Menacho *et al.*, 2010). Processing tofu from germinated soybean should be an effective mean to enhance the antioxidant compounds in the product that have a beneficiary effect to consumers. To demonstrate this, the protective effect of tofu produced from germinated soybean on the CCl_4 induced chronic liver damage in mice is investigated.

Materials and Methods

Germination of soybean seeds

Soybeans (*Glycine max* L., MTĐ 760 variety) were supplied from Department of Agricultural Genetic, College of Agricultural and Applied Biology, Cantho University.

Soybeans were cleaned and rinsed three times with cleaned water before being soaked for 12 hours at ambient temperature. The soaked beans were drained, rinsed and placed in a germination cabinet, which watered the seeds every 4 hours with cleaned water automatically, the time for watering was two minutes. The germination process was carried out at 25°C in dark condition for 42 hours.

Silk tofu preparation

Briefly, the germinated and non–germinated soybeans were rinsed and ground with hot water (water/dry weight of bean was 6/L, v/w) (Ndatsu and Olekan, 2012) by the crushing machine, the slurry was filtered through a three layers cheese cloth to obtain soy milk. Soy milk was boiled for 5 minutes and then cooled down 20ºC. GDL (Glucono-delta-lacton) 3g/L was added and mixed well. The soymilk was then filled to boxes, sealing them and they were immersed in water bath at 90ºC and 44 minutes for coagulation. The silk tofu products were stored at ≤ 5ºC for 1 day to analyse the total polyphenol content (TPC) antioxidant activities.

Determination of TPC and antioxidant activity of silk tofu

Tofu samples were freeze dried to fine powder before analysing. The extraction procedure for analysing was carried out by method of Duong *et al.* (2015).

Determination of the TPC

The TPC of tofus were estimated by Folin-Ciocalteu method (Jiang *et al.*, 2013). The total phenolic content of samples was expressed as milligrams garlic acid equivalents per gram of dry matter (mg GAE/g).

Determination of antioxidant activity

Antioxidant activity of silk tofu extracts were assessed by measuring their scavenging activity of stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. This procedure was described by Liu *et al.* (2011). Percentage of radical scavenging activity was plotted against the corresponding concentration of the extract (µg/mL) to obtain IC50 value in mg/mL. The results were showed in Table 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 were 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.

Experimental design

Forty-two mice were divided into seven groups (each group consisted 6 mice).

Group (1): Normal control group, animals were treated with olive oil (10mL/kg b.w., o.p. three days for once).

All other groups, mice were treated with 10mL (CCl_4 20% in oliu oil)/kg b.w., o.p. three days for once. In addition, they would be treated simultaneously in different ways, as followings:

Group (2): Control positive group (mice were treated with CCl_4 only).

Group (3): Control negative group, mice treated oral doses of 16mg silymarin/kg b.w. one hour after CCl_4 toxicititation.

Group (4) and (5): Mice were fed with silk tofu 0.2g/g b.w./day (ST low) and 0.4g/g b.w./day (ST high) respectively.

Group (6) and (7): Mice were fed with silk tofu made from germinated soybean 0.2g/g b.w./day (GST low) and 0.4g/g b.w./day (GST high) respectively.

The experiment was carried out during 6 weeks. At the end of the experiments, blood and livers were collected immediately after the animals were sacrificed. Blood was determined the ALT and TC in serum. The liver from each animal was determined the L/B, PC, MDA, vitamin C contents and histology properties.

Determination of serum ALT and TC, liver PC, MDA, vitamin C contents and histology properties

Determination of serum ALT, TC and liver histology property

Blood and liver samples were sent to Cantho University Hospital for analysing of serum ALT and TC by ARCHITECT–Ci4100 machine (Abbott Company, America) and hepatic histology property. The degree of fibrosis was evaluated in the liver tissue according to the Hepatitis Activity Index (HAI) (Ishak *et al.*, 1995) which scores of fibrosis were based on Knodell – Ishak scales from 0 to 22.

Determination of liver PC

The PC values were measured by spectrophotometric method at the absorbance of 370 nm, using dinitro-phenylhydrazine (DNPH) reagent (Levine RL, 1990). Results were calculated as nanomoles of carbonyl groups per milligram of protein (nmol/mg protein). Total protein was determined by Bradford assay (Bradford, 1976) 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.

Determination of liver MDA

The MDA levels of liver tissue were carried out using the modified method of Ohkawa *et al.* (1979). 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. Malonaldehyde bis (Acros–Belgium) was used as the standard. The results are expressed as nmol/mg protein.

Determination of the liver vitamin C content

Vitamin C contents in liver tissue were determined by the spectrophotometric method of George (2003) that is based on the reaction with 2,4-dinitrophenylhydrazine reagent. The optimum absorbance of reaction product color was 520 nm. A standard was prepared using of pure ascorbic acid. The results are expressed as µg/mg protein.

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.

Results and Discussion

The L/B, serum ALT and TC values from seven experimental mice groups were presented in Table 2. The MDA, PC and vitamin C contents in mice liver tissues from these groups were showed in Table 3. Histological examination of mice liver tissues was displayed in Figure 1.

The L/B ratio were increased 60% in mice treated with CCl_4 (Control positive group) as compared to that of control mice. Feeding mice with silk tofu (ST low, ST high, GST low and GST high) reduced the L/B values to 8.3; 10.0; 13.6 and 23.7% respectively. In which, the L/B values of mice from ST high, GST low and GST high groups similar to L/B value of mice treated with silymarin (Control negative group), whose L/B value was remained closing to L/B value of normal group (Table 2).

Serum ALT increased 344% in mice treated with CCl_4 comparing to ALT of control mice. ALT value of mice treated with silk tofu (ST low, ST high, GST low and GST high) restricted the increase in serum ALT (the decreasing of 46.7, 61.7, 61.0 and 70.9% respectively) as compared to that of mice treated with CCl_4. Within them, tofu made from germinated soybeans (GST low and GST high) showed the higher effective in the ALT restoration. Specially, the ALT value in mice fed with high dose (0.4g/g b.w./day) of silk tofu made from germinated soybeans was similar to that of normal control group and control negative group (Table 2).

A significant increase in serum TC levels (43.9%) were observed in CCl_4 treated mice, compared to the control group. Four groups of mice fed with silk tofu attenuated the increased levels of serum TC that resulted from the treatment previously with CCl_4. The TC value from mice group fed with high dose of silk tofu made from germinated soybeans was not significant different with TC values from normal control group and control negative group (Table 2).

In this study, CCl_4 treatment markedly increased (50.2%) the hepatic MDA level as compared with the normal control group. Treatment with silk tofus significantly reversed this change. MDA levels in mice from ST low, ST high, GST low and GST high groups reduced 9.6, 16.0, 15.9 and 23.5% respectively as compared to hepatic MDA level of control positive group. The MDA value from mice group fed with high dose of silk tofu made from germinated soybeans was not significant different with MDA value from control negative group (Table 2).

The present study detected a significant increasing (64.9%) in liver PC content of the CCl_4 treated mice as compared to control mice. The PC levels in four mice groups fed with tofu decreased significantly when compared with that of control positive group. Tofu made from germinated soybeans also displayed as the more effective agents in the reversion of the change in PC content caused by CCl_4 toxication.

The level of vitamin C in liver of CCl_4 control group significantly decreased in comparison with the normal control group (54.6%). After application of silymarin as well as silk tofu as ST low, ST high, GST low and GST high groups the increase the levels of hepatic vitamin C by 101.0, 49.5, 74.9, 73.8 and 95.9% respectively, as compared to that of CCl_4 treatment group.

The results of liver histopathology from Figure 1 of seven *Swiss albino* mice groups were described more detailed in Table 4.

Table 1. The TPC and IC50 values of silk tofus made from germinated and non–germinated soybeans

	Silk tofu (Germinated soybeans)	Silk tofu (Non–germinated soybeans)
TPC (mg GAE/g d.w.)	3.39^{b}±0.03	2.45^{a}±0.09
IC50 (mg d.w./mL)	14.09^{a}±0.12	15.37^{b}±0.14

(Means ±SD, the values showing different superscripts within a row are significant different at $P<0.05$)

Table 2. The L/B, serum ALT and TC values of experimental mice groups

Groups	L/B (%)	ALT (U/L)	TC (mg/dL)
1. Normal control group	3.30^{a}±0.20	48.83^{a}±0.02	105.51^{a}±12.61
2. Control positive group	5.28^{e}±0.15	216.83^{d}±37.94	151.83^{d}±32.63
3. Control negative group	4.25^{bc}±0.47	58.83^{a}±8.16	106.79^{ab}±7.97
4. ST low group	4.84^{de}±0.49	115.67^{c}±18.89	130.60^{c}±8.94
5. ST high group	4.75^{cd}±0.44	83.00^{b}±4.00	120.95^{bc}±3.15
6. GST low group	4.56^{cd}±0.71	84.50^{b}±3.89	120.95^{bc}±3.15
7. GST high group	4.03^{b}±0.35	63.17^{a}±7.41	104.22^{a}±4.23

(Mean s±SD, the values showing different superscripts within a column are significant different at P<0.05)

Table 3. The liver tissue MDA, PC and vitamin C values of experimental mice groups

Groups	MDA (nmol/mg protein)	PC (nmol/mg protein)	Vitamin C (µg/mg protein)
1. Normal control group	7.77^{a}±0.65	5.04^{a}±0.18	10.69^{e}±0.81
2. Control positive group	11.67^{e}±0.30	8.31^{e}±0.30	4.85^{a}±0.47
3. Control negative group	8.58^{b}±0.42	5.44^{b}±0.44	9.75^{d}±0.83
4. ST low group	10.55^{d}±0.52	7.38^{d}±0.39	7.25^{b}±0.18
5. ST high group	9.80^{c}±0.17	6.75^{c}±0.25	8.48^{c}±0.41
6. GST low group	9.81^{c}±0.38	6.50^{c}±0.22	8.43^{c}±0.33
7. GST high group	8.93^{b}±0.38	5.22^{b}±0.24	9.50^{b}±0.47

(Means ±SD, the values showing different superscripts within a column are significant different at P<0.05)

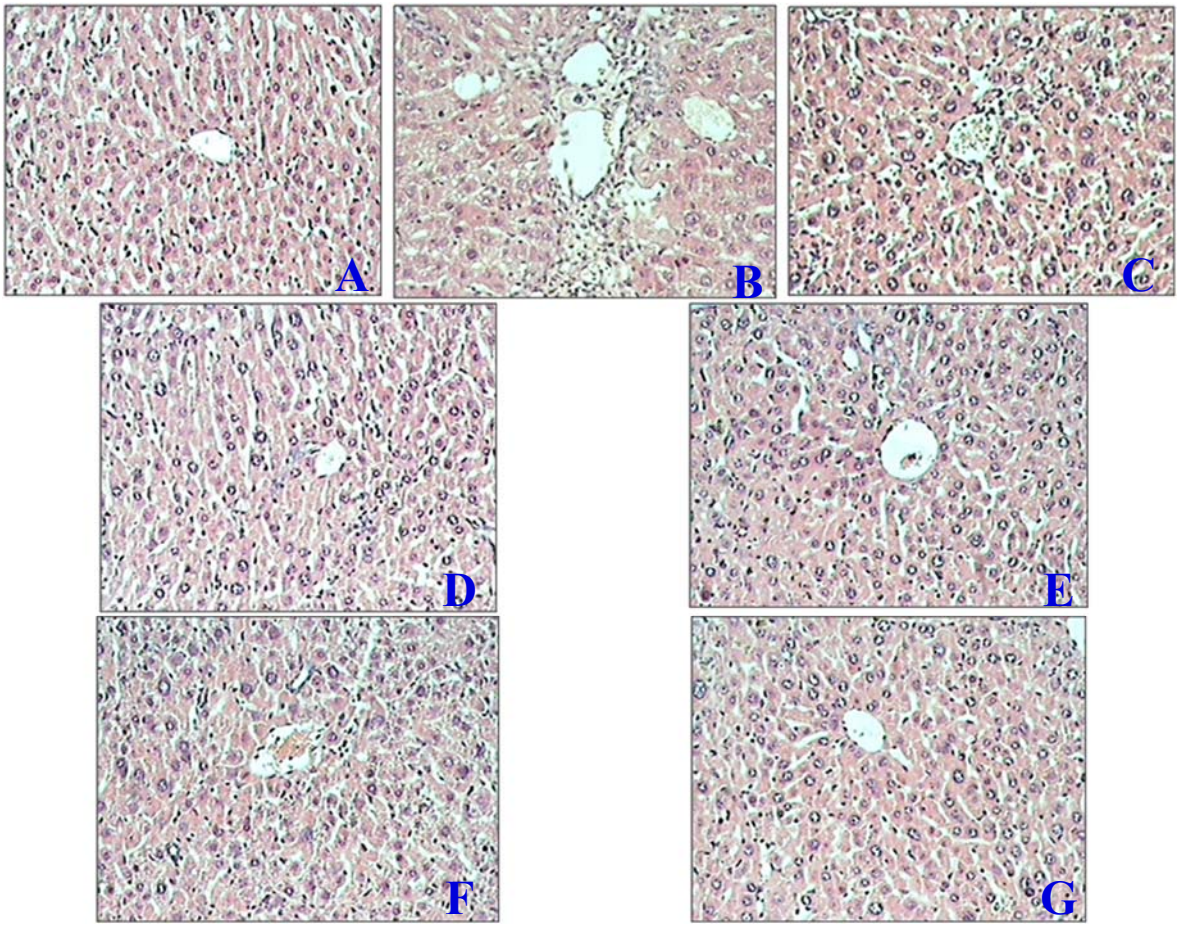
Table 4. Liver histopathology description and chronic hepatitis degrees of *Swiss albino* mice from seven experimental groups

Groups	Descriptions	Scores (HAI)	Degrees of chronic hepatitis
(1)	Liver tissues presented with normal histological structure, hepatocytes and venous sinusoids are arranged as interconnected plates (Figure 1.A)	0	No inflammation
(2)	Appearing many 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 at serious level (Figure 1.B).	10	Moderate chronic hepatitis
(3)	Necrotic cells could not be found in lobules, but there was very little inflammatory and necrotic cells at portal area (Figure 1.C).	3	Very mild chronic hepatitis
(4)	There was little inflammatory and necrotic cells in lobules and portal area (Figure 1.D).	4	Mild chronic hepatitis
(5)	Moderate appearance of necrosis in lobules and portal area, there was little inflammatory cells at portal area (Figure 1.E).	8	Mild chronic hepatitis
(6)	Necrotic cells could not be found in lobules, but there was very little inflammatory and necrotic cells at portal area (Figure 1.F).	3	Very mild chronic hepatitis
(7)	There was little inflammatory and necrotic cells in lobules and portal area (Figure 1.G).	4	Mild chronic hepatitis

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 (Huo *et al.*, 2011). 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 (Robins *et al.*, 1979; Huo *et al.*, 2011). Increasing in L/B coincides with many previous results from studying of hepatotoxicity on mice by CCl_4 (Domitrović *et al.*, 2009; Huo *et al.*, 2011).

It is well documented that CCl_4 enhanced lipid peroxidation (Abdel-Wahhab *et al.*, 2006; El Denshary *et al.*, 2012). The CCl_4 induces the peroxidation of lipids that damage the membranes of liver cells and organelles. This results in the release of ALT that is found outside of the mitochondria of the liver into the circulating blood (Shankar *et al.*, 2008) leading to increasing the levels of liver enzymes (ALT). The rising in ALT activity is almost always due to hepatocellular damage (Ravikumar *et al.*, 2005). Essawy *et al.* (2012) reported that serum ALT of *Swiss albino* mice treated with CCl_4 at a dose level 1.9 mL/kg b.w increased 328.8% when compared with ALT value of control mice.

Distinct alterations in lipid metabolism have been reported in CCl_4 induced hepatotoxicity in rats (Singhal and Gupta, 2012). The liver is the major site for the synthesis and metabolism of cholesterol (Yang *et al.*, 2011). 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 (Boll *et al.*, 2001). Sarhan *et al.* (2012) reported that TC levels in *Sprague Dawley* male rats much higher after the treatment with CCl_4 for 8 weeks.



(H and E staining, magnification x 100)

Figure 1. Micrographs from representative liver tissues collected from mice from group (1) (Figure 1.A); group (2) (Figure 1.B), group (3) (Figure 1.C), group (4) (Figure 1.D), group (5) (Figure 1.E), group (6) (Figure 1.F) and group (7) (Figure 1.G).

The result of the peroxidation of lipids induced by CCl_4 is the formation of MDA and its level in liver tissue was assessed as an indicator of lipid peroxidation in oxidative liver damage (Nielsen *et al.*, 1997). The present results in liver MDA increasing of CCl_4 treated *Swiss albino* mice are consistent with previous study (Saad, 2013). Another aspect as regards to oxidation of proteins. Protein oxidation may play a role in the pathogenesis of CCl_4 induced liver injury (Sundari *et al.*, 1997) and the accumulation of oxidised proteins in the liver may be an early indication of CCl_4 liver injury. The PC that is product from the free radical-mediated oxidation of proteins (Robinson *et al.*, 1999), is widely used as a indicator for measuring of oxidative damage (Luo and Wehr, 2009). The advantage of using protein carbonyl as a marker may be due to the relatively early formation and stability of oxidized proteins (Dalle-Donne *et al.*, 2003). The result in the increase of hepatic PC due to CCl_4 treatment from this study coincided with the results of Sundari *et al.* (1997) in the model of chronic rat liver injury.

In the present study, the decrease in the liver vitamin C level induced by CCl_4 indicated was detected. CCl_4 generated ROS causing the feed-back inhibition or oxidative inactivation of enzyme protein leading the decrease antioxidants (such as GSH) in plasma and tissue (Pigeolet *et al.*, 1990). This resulted subsequently in reduction of other antioxidants such as ascorbic acid and aggravate the cells to further damage (Al-Assaf, 2014).

The above changes related to CCl_4 induced liver injury expressed an indication of structural and functional defects in liver cells that was proved in histopathological examination (Figure 1.B and Table 4). It was clearly established that necrosis and inflammatory cells were observed in the liver sections of animals treated with CCl_4. These damages observed on the liver architecture were

expression of moderate chronic hepatitis and they might be associated with the production of oxidative stress caused by CCl_4 intoxication.

Feeding mice with various forms of silk tofus (ST low, ST high, GST low and GST high) had tendency to reduce L/B, serum ALT and TC as well as the hepatic MDA and PC values. However, the vitamin C content increased and finally, the liver injury was improved through the histopathological examination (Figure 1.A, C, D, E, F and G). This histopathological observation could be attributed to the potent antioxidant activities of tofu polyphenol compounds that are potent free radical scavengers in the body system. Tofu made from germinated soybeans showed greater effective than that of tofu made from ungerminated soybeans in protection against CCl_4 induced hepatic toxicity. Especially, feeding mice with tofu made from germinated soybeans at the dose of 0.4g/g b.w./day (GST high) remained the biochemical properties of mice liver as closing to that of mice from normal control group and mice treated with silymarin. Interestingly, intact hepatic cell architectures were observed in mice from this group and this normal histological structure was similar to liver cell sections of the normal control and control negative group (Figure 1.A, C and F).

Phenolic compounds in soybeans and soy products were natural antioxidants which functions as a potent neutralizer of free radical species in the body and they acted against the liver damaging effects of free radicals produced by CCl_4 (Yakubu *et al.*, 2013; Yakubu and Mohammed, 2016). Tejasari *et al.* (2014) proved through both the histopathologic observations and statistical analyses that the administration of soy extract can provide protection against mouse liver tissue damage where injury is induced by CCl_4. Beside, the authors stated that soys inhibit the initiation of both the extrinsic and intrinsic apoptotic processes in pathways in hepatocytes is what ultimately could play a role in improving survival in conditions in a state of liver injury (Tejasari *et al.*, 2014).

Germination involves physiological changes, synthesis and breakdown of macromolecules, improving the digestibility and nutritive value of soybeans (Fernandez-Orozco *et al.*, 2008). This process enhances levels of important antioxidants such as polyphenols, isoflavones, vitamin C and vitamin E as compared to ungerminated soybeans (Paucar-Menacho *et al.*, 2010). So, the potential of free radical scavenging of germinated soybeans as well as products from them were increased. In this study, TPC content of silk tofu made from germinated soybeans was 1.38 folds (Table 1) higher than that of silk tofu made from non–germinated soybeans. So, IC50 value of silk tofu made from germinated soybeans was lower than IC50 value of silk tofu made from non–germinated soybeans (by 91.7%, Table 1). For this reason, silk tofu from germinated soybean showed the greater hepatoprotective effects as compared to that of silk tofu from non–germinated soybeans.

Conclusion

The present study demonstrated that all silk tofus exhibited hepatoprotective activity against CCl_4 intoxication in mice. The liver protection ability of silk tofu may be associated with their free radical scavenging and antioxidant capacities. Specially, silk tofu made from germinated soybeans may be more efficacious hepatopreventive agent. Therefore, supplementation of tofu as well as food products made from germinated soybeans in our diets can be highly recommended as it can be used as a functional food to prevent liver injury.

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