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

Duong Thi Phuong Lien¹, Cao Thi Kim Hoang², Nguyen Thi Hanh², Duong Xuan Chu², Phan Thi Bich Tram¹ and Ha Thanh Toan³

¹Cantho University, College of Agriculture and Applied Biology, Vietnam
²Cantho University of Medicine and Pharmacy, Pharmacy Faculty, Department of Pharmacology, Vietnam
³Cantho University, Biotechnology Research and Development Institute, Vietnam

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Abstract:
The hepatoprotective activities of silk tofu made from germinated and non germinated soybeans at different doses of feeding against CCl₄ induced hepatic cell toxicity in mice was investigated in this study. The hepatoprotective activity was analyzed by assessing the ratio of liver weight to body weight (L/B), the levels of serum alanine aminotransferase (ALT), total cholesterol (TC), the hepatic malondehyde (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 CCl₄ 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, CCl₄ had been frequently used to induces toxicity in rat liver which closely resembles human cirrhosis. It produces reactive free radicals trichloromethyl radical (CCl₃) and a proxy trichloromethyl radical (CCl₃O₂) when metabolized (Yang et al., 2015). CCl₄ 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 (Poya and Woodrow, 2002). It was also demonstrated to prevent acetyaminophen-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 CCl₄ induced chronic liver damage in mice is investigated.

Materials and Methods

Germination of soybean seeds

Soybeans (Glycine max L., MTD 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 20°C. 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 90°C and 44 minutes for coagulation. The silk tofu products were stored at ≤5°C 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 (CCl4 20% in olive 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 CCl4 only).

Group (3): Control negative group, mice treated oral doses of 16mg silymarin/kg b.w. one hour after CCl4 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

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 CCl₄ (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 CCl₄ comparing to ALT of control mice. ALT value of mice treated with 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 CCl₄. 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 CCl₄ 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 CCl₄. 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, CCl₄ treatment markedly increased (50.2%) the hepatic MDA level as compared with the normal control group. Treatment with silk tofu 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 CCl₄ 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 CCl₄ intoxication.

The level of vitamin C in liver of CCl₄ 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 CCl₄ 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 |
|-----------------|-----------------|-----------------|
| TPC (mg GAE/g d.w.) | 3.39±0.03 | 2.45±0.09 |
| IC50 (mg d.w./mL) | 14.09±0.12 | 15.37±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

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

(Mean ±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

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

(Mean ±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

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

CCl₄ is a well known hepatotoxic agent and the most remarkable pathological characteristics of CCl₄ 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 CCl₄ 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 CCl₄(Domitrović et al., 2009; Huo et al., 2011).

It is well documented that CCl₄ enhanced lipid peroxidation (Abdel-Wahhab et al., 2006; El Denshary et al., 2012). The CCl₄ 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 CCl₄ 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 CCl₄ 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). CCl₄ 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 CCl₄ for 8 weeks.
The result of the peroxidation of lipids induced by CCl₄ 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 CCl₄ 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 CCl₄ induced liver injury (Sundari et al., 1997) and the accumulation of oxidised proteins in the liver may be an early indication of CCl₄ 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 CCl₄ 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 CCl₄ indicated was detected. CCl₄ 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 CCl₄ 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 CCl₄. 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 CCl₄ intoxication.

Feeding mice with various forms of silk tofu (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 CCl₄ 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 CCl₄ (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 CCl₄. Beside, the authors stated that soy extracts 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 tofu exhibited hepatoprotective activity against CCl₄ 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 hepatoprotective 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.

References


tetrachloride-induced hepatocyte injury bioactivation of CCl₄ by cytochrome P450 and effects on lipid homeostasis. *Zeitschrift für Naturforschung*, 56(1-2), 111-121.


