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# Study on the effects of blueberry treatment on histone acetylation modification of $CCl_4$-induced liver disease in rats

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**ABSTRACT.** The objective of this study was to investigate the effects of blueberry treatment on histone acetylation modification of carbon tetrachloride ($CCl_4$)-induced liver disease in rats. Laboratory rats were randomly divided into control, hepatic fibrosis, blueberry treatment, blueberry intervention, and natural recovery groups. Rats in the model groups were treated with $CCl_4$ administered subcutaneously at 4- and 8-week intervals, and then executed. Both the 4- and 8-week treatment groups were treated with blueberry juice for 8 weeks, and then executed after 12 and 16 weeks, respectively. Following the experiment, four liver function and hepatic fibrosis indices were measured. Liver index

was calculated, hematoxylin-eosin staining was conducted, and H3K9, H3K14, and H3K18 expressions were evaluated among the nuclear proteins of the liver tissues. No differences in alanine transaminase were noted between the control and intervention groups, but significant differences were detected among the model, treatment, and natural recovery groups ($P < 0.01$). Significant differences were also observed in aspartate transaminase, hyaluronic acid, and collagen IV among the model, treatment, intervention, and natural recovery groups ($P < 0.01$, $P < 0.01$, $P < 0.01$). Liver index, and H3K9 and H3K14 expression were significantly different among the model groups ($P < 0.05$ and $P < 0.01$), whereas H3K18 expression was dramatically different among model, treatment, intervention, and natural recovery groups ($P < 0.01$). Following blueberry treatment, rat liver function and hepatic fibrosis improved, potentially indicating that blueberry components could regulate histone acetylation and improve liver pathologic changes in rats with $CCl_4$-induced disease.

**Key words:** SD rat; $CCl_4$; Liver damage; Hepatic fibrosis; Histone acetylation

## INTRODUCTION

The $CCl_4$-induced liver injury model has been widely used in the study of hepatic necrosis, hepatic fibrosis and cirrhosis etiology, histology research and liver-protecting drug development, and medicinal plant extract assessments (Lee et al., 2007; Ye et al., 2011). Continuous $CCl_4$ treatment increases liver cell damage, and results in pathological processes, such as lipid peroxidation, cytokine release, and calcium imbalance, in hepatic cells that had undergone apoptosis, regeneration, and gradually returned to normal. However, when necrotic liver cells exceed their regenerative capacity, it leads to liver cell damage, and even liver failure (Weber et al., 2003).

In previous studies, various extracellular matrix components were found to have increased in the liver while their degradation decreased, resulting in changes to liver tissue structure, liver fibrosis, and even cirrhosis (Sato et al., 2003; Tsukamoto, 2007; Brenner, 2009). The role of epigenetic regulation in liver injury involving liver fibrosis is still being explored. Thereby, in the present study, we evaluated the effects of blueberry compounds on protein acetylation of $CCl_4$-induced liver injury in rats; the findings would provide a reference for further studies.

## MATERIAL AND METHODS

### Experimental animals

One hundred male Sprague Dawley (SD) rats weighting approximately 160 ± 10 g were provided by the Guizhou Medical University Experimental Animal Center. Animal experiments strictly adhered to the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee.

## Reagents

Edible vegetable oil was purchased from a local market; blueberries were provided by the blueberry production center in Majiang County, Qiandongnan Prefecture, Guizhou Province. Histone acetylation antibodies were purchased from Abcam (Cambridge, MA, USA).

## Methods

### *Preparing rat models*

SD rats and food were provided by the Guizhou Medical University Experimental Animal Center. The rats were allowed to consume a free diet and were given sufficient feed and water. Following the experiments, blood tests were conducted to detect liver function, including four indicators of liver fibrosis, and HE staining was used to determine the successful preparation of the rat models. We used the protocols reported by Wang et al. (2015).

## Groups

The SD rats were randomly divided into 10 groups, with each group comprising 10 rats. The liver fibrosis model group was injected with a 40% $CCl_4$ solution combined with vegetable oil at a concentration of 0.3 ml/100 g once every 3 days to establish the liver fibrosis model; rats in this group were executed after 8 weeks (N = 10). The normal control group was subcutaneously administered the same amounts of vegetable oil during the fourth week; all individuals in this group were killed after the eighth week (N = 10). The 4-week blueberry treatment group was injected during the fourth week, and then administered a 5-ml blueberry solution twice after the eighth week and over another 4 weeks, for a total duration of 12 weeks (N = 10). The 8-week blueberry treatment group was injected during the fourth week, and then administered a 5-ml blueberry solution twice after the eighth week and over another 8 weeks (N = 10). The 4-week blueberry intervention group was orally administered 5 ml blueberry juice twice a day for 4 weeks before model preparation; they were then treated with $CCl_4$ and blueberry juice simultaneously for another 4 weeks (N = 9). The 8-week blueberry intervention group was orally administered 5 ml blueberry juice twice a day for 4 weeks before model preparation; they then were treated with $CCl_4$ and blueberry juice simultaneously for another 8 weeks (N = 9). The 4-week spontaneous recovery group was given a normal diet for 8 weeks following the 4-week model creation period (N = 10). The 8-week spontaneous recovery group was also given a normal diet for 8 weeks following the 4-week model creation period (N = 9).

### *Specimen collection*

Rat specimens were weighed after chlorine aldehyde hydration, and then fixed on anatomical plates treated with a conventional disinfectant. Left iliac blood vessels were excised from the left iliac veins along the left inguinal region, and the liver was separated and excised; the liver was then weighed to calculate liver index.

### *Pathological analysis*

The unity of the left hepatic lobe was fixed in 4% formalin and analyzed via HE

staining in the Department of Pathology of the Affiliated Hospital of Guizhou Medical University. Liver function and liver fiber were investigated in the Biochemistry Laboratory of the aforementioned institution, and any liver disease present was also noted. Residual liver specimens were stored at -80°C.

### *Plasma, nuclear, and cytoplasmic protein collection*

A Bioworld nuclear and cytoplasmic protein extraction kit was used to extract nuclear proteins from 60-mg samples of rat liver tissue, which were then measured using a bicinchoninic acid (BCA) protein assay kit.

### *Western blot*

SDS-PAGE was used to analyze nuclear and plasma proteins (40 μg). Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane following electrophoresis, and then incubated with the primary antibody, H3K9 (ab8898, Abcam), and secondary antibodies H3K14 (ab52946, Abcam), H3K18 (ab1191, Abcam), H3 (ab1791, Abcam). They were detected using an enhanced chemiluminescence (ECL) reagent. A Bio-Rad gel imager was used to observe and analyze the images. Each experiment was repeated three times to detect related protein expression.

### *Statistical analysis*

Data were analyzed using SPSS 20 statistical software. All data are reported as means ± standard deviation. Single factor analysis of variance (ANOVA) was used to compare differences among groups; the least significant difference (LSD) method was used to compare differences between two groups. Differences were considered statistically significant when $P < 0.05$.

## RESULTS

### Animal status

No deaths occurred in the 4-week groups during the model creation process; however; among the 8-week groups, one rat died in the intervention group and one died in the natural recovery group.

### Liver indices at different times throughout the modeling process

The rats from two groups were weighed before execution. After execution, the liver was isolated and weighed, and then, the liver index was calculated. After 4 weeks, separate liver indices of rats were 0.03 ± 0.02, 0.04 ± 0.00, 0.03 ± 0.00, 0.03 ± 0.00, and 0.03 ± 0.00 in the control, model, blueberry treatment, blueberry intervention and natural recovery groups, respectively; liver indices were 0.03 ± 0.00, 0.03 ± 0.00, 0.04 ± 0.00, 0.03 ± 0.01, and 0.03 ± 0.00 after 8 weeks, respectively. Differences ($P < 0.05$) were observed between the 4- and 8-week model groups, whereas no differences ($P > 0.05$) were observed in the 4- and 8-week

normal, treatment, intervention, and natural recovery groups. In the normal control group, the liver was rosy in color and had a smooth surface, uniform texture, and soft and good elasticity. In the 4-week model group, the liver was brownish yellow with a larger volume, likely due to swelling. In the 8-week model group, the liver was reddish-brown with granular nodules on the surface and had a hard texture.

## Comparison of liver function in each group

There was no difference ($P = 0.065$) in alanine transaminase (ALT) in comparisons between normal and intervention groups, whereas the remaining groups showed significant differences ($P < 0.05$). There were also significant differences among the groups ($P < 0.01$) when compared with the model group; the intervention group was significantly different from the treatment group ($P < 0.01$), but showed no differences with the natural recovery group ($P = 0.149$), whereas the intervention group showed dramatic differences with the natural recovery group ($P < 0.05$). The model group demonstrated significant differences in aspartate transaminase (AST) with the normal group ($P < 0.01$). Results are shown in Figure 1.

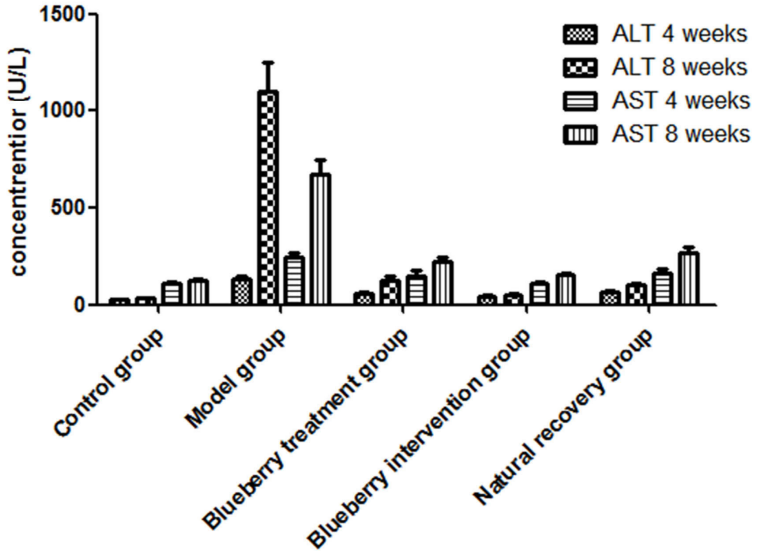


**Figure 1.** Comparison of plasma ALT and AST levels among different groups of rats. ALT: 4- and 8-week model, treatment and natural recovery groups were different ($P < 0.01$); AST: 4- and 8-week model, treatment, intervention and natural recovery groups ($P < 0.01$).

## Comparison of liver fibrosis in each group

Compared with the normal group, there were notable differences in hyaluronic acid (HA) levels among the remaining groups ($P < 0.05$). Significant differences in collagen IV (Col IV) between the normal group and the remaining groups ($P < 0.01$) were also observed, whereas there were no significant changes or statistical differences in laminin (LN) or N-terminal collagen III propeptide (PC III NP) levels among the groups ($P > 0.05$). Results are shown in Figure 2.

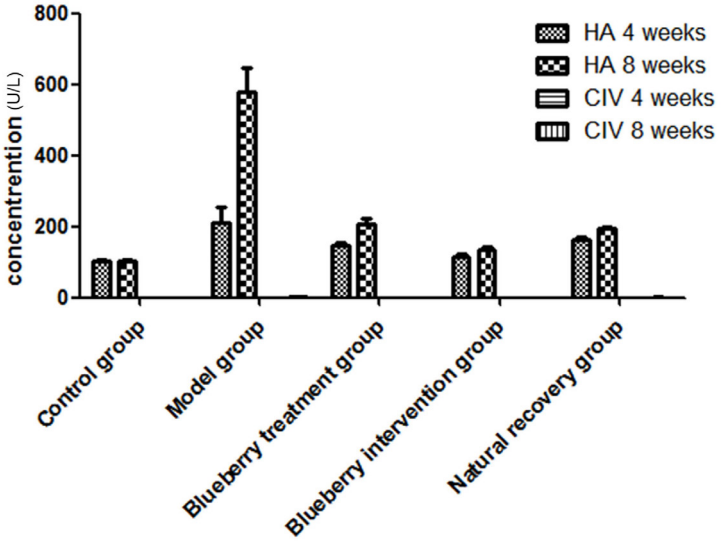


**Figure 2.** Comparison of plasma hepatic fibrosis levels among different groups of rats. HA and CIV: 4- and 8-week model, treatment, intervention, and natural recovery groups were different ($P < 0.01$).

## HE staining of liver samples in each group

According to liver pathological sectioning results, normal cells showed a smooth surface and uniform size, and had no fat vacuoles. In the 4-week model group, the hepatic lobule was normal, but indicators of moderately severe edema and fatty degeneration in some liver cells, visible focal necrosis, and periportal infiltration of a small number of chronic inflammatory cells were also present. In contrast, the 8-week model group showed normal liver damage. The treatment group showed that the collagen fibers were extended and connected to each other. In the 4-week treatment group, degeneration was found to be reduced in the liver cells, although a small amount of focal necrosis was present. The 8-week treatment group showed that the collagen fibers were obviously extended, but not throughout the entire liver. In the 4-week intervention group, hepatic lobules were normal, liver cells showed mild edema, and there were only a few areas of focal necrosis. The 8-week intervention group revealed collagen fibers extending outward from the portal area and visible fatty degeneration. The 4-week natural recovery group included remnant hepatic lobules and liver cells indicating moderate edema and fatty degeneration. There was little periportal infiltration of inflammatory cells in this group. In the 8-week natural recovery group, the collagen fibers were stretched and connected (Figure 3).

## H3K9, H3K14, and H3K18 expression

There were significant differences in H3K9 and H3K14 protein expression between the 4- and 8-week model groups ($P < 0.01$). Additionally, there were significant differences in H3K18 protein expression among the model, treatment, and intervention groups ($P < 0.01$). Expression levels of the three proteins in the normal group were lower, higher, and higher than that in the model, treatment, and intervention groups, respectively (Figures 4 and 5).

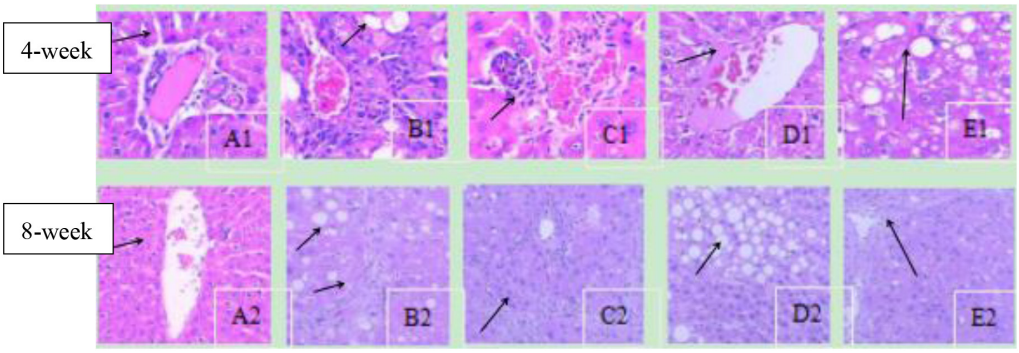


**Figure 3.** H&E staining of liver sections from rats in each group. A1, normal group: H&E staining, 100X, normal hepatic cells. B1, model group: H&E staining, 100X, severe edema of liver cells. C1, treatment group: H&E staining, 100X, mild fatty degeneration of liver cells. D1, intervention group: H&E staining, 100X, mild edema of liver cells. E1, natural recovery group: H&E staining, 100X, moderate edema of liver cells. A2, normal group: H&E staining 100X, normal hepatic cells. B2, model group: H&E staining, 100X, liver leaflet structure destruction. C2, treatment group: H&E staining, X 100, liver structure completely destroyed. D2, intervention group: H&E staining, 100X, collagen fibers to mild portal area l extension. E2, natural recovery group: H&E staining, 100X, collagen fibers obviously extended.

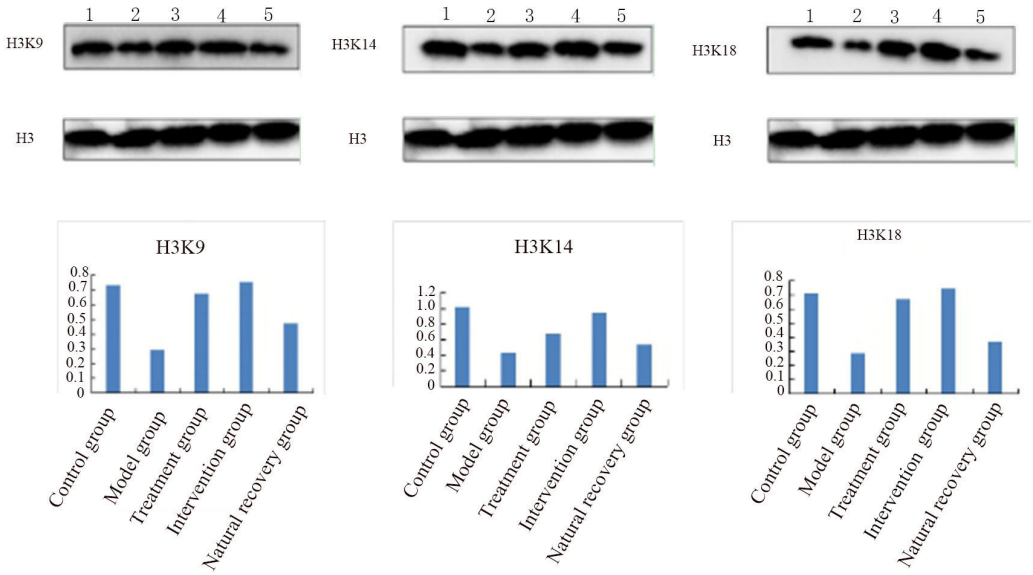


**Figure 4.** Expression of H3K9, H3K14, and H3K18 proteins in liver cells following 4-week separation of liver cytoplasm. 1. Normal group. 2. Model group. 3. Blueberry treatment group. 4. Blueberry intervent group. 5. Natural treatment group.

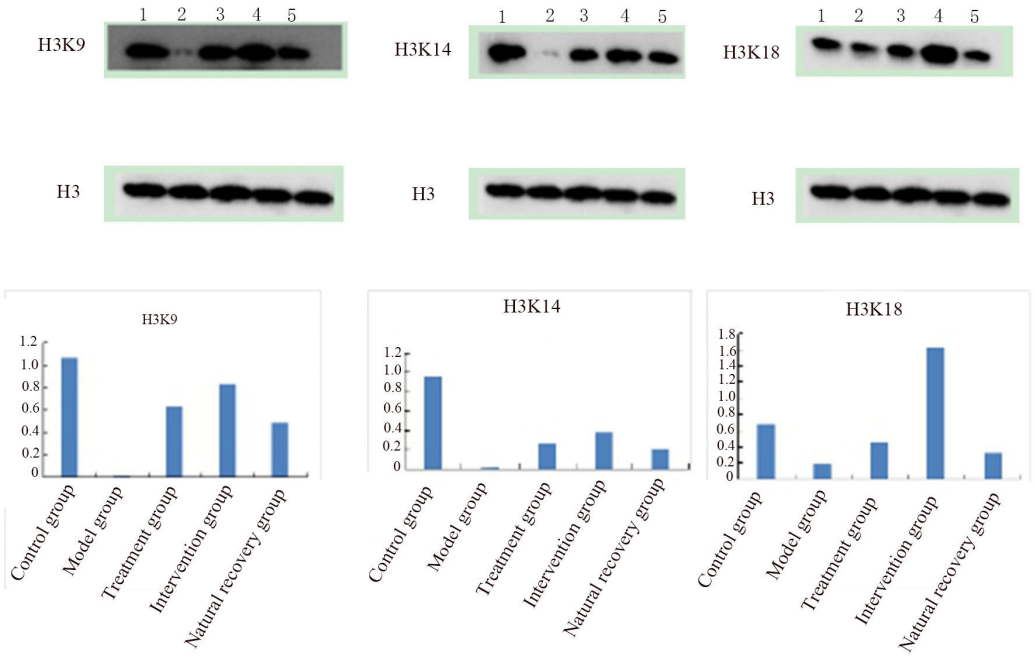


**Figure 5.** Expression of H3K9, H3K14, and H3K18 proteins in liver cells following 8-week separation of liver cytoplasm. 1. Normal group. 2. Model group. 3. Blueberry treatment group. 4. Blueberry intervention group. 5. Natural treatment group.

## DISCUSSION

The $CCl_4$-induced liver injury model serves as a classic liver disease model for the evaluation of liver fibrosis in rats. Hepatocyte injury and necrosis are used to induce the aggregation, activation and release of inflammatory cells, which in turn causes large amounts of extracellular matrix (ECM) to be present in the liver, leading to the development of hepatic fibrosis (Sato et al., 2003; Carloni et al., 2014). This also corresponds to intermediate links in the development of the "chronic liver disease-liver fibrosis-cirrhosis" model (Das and Vasudevan, 2008).

Histone acetylation occurs when an acetyl group is transferred to lysine histone H3 or to a histone H4 that is otherwise rich in lysine; the positive charge, which is conducive to the binding of transcription factors and DNA, promotes transcription with the help of histone acetyltransferase (HAT; Santos-Rosa and Caldas, 2005; Binda, 2013). Previous studies have reported (Tiwari et al., 2009) that alcohol may inhibit histone deacetylase (HDAC) activity while selectively enabling expression of acetylase H3K9 through increases in HAT liver cell activity.

Not only are blueberries rich in anthocyanins, flavonoids and other biologically active substances, but their nutritional components also contain antioxidants, combinations of which contribute to an inherent resistance to a variety of diseases (Kong et al., 2003). In fact, Barros et al. (2006) found that blueberries were not only rich in anthocyanins, but also contained substances that functioned as both antioxidants and gene-protectors, thereby preventing DNA

damage. Thus, the use of blueberries and blueberry-derived compounds could improve the functioning of multiple organs, and could provide many related beneficial effects, including antioxidant responses in scavenging oxygen-free radicals and disease reduction (Rimando et al., 2004).

In this study, we found that the blueberry intervention group showed more obvious improvements than the other groups did. Liver function significantly improved, as evaluated via liver HE staining, and measuring ALT, AST, and two important indicators of liver fiber. The expression of H3K9, H3K14, and H3K18 protein also improved. Compared with the model group, the expression of H3K9, H3K14, and H3K18 increased significantly in the intervention group. These effects may be caused by certain anti-inflammatory components in blueberries that improved the liver injury and hepatic fibrosis induced by CCl4. Additionally, long-term administration of blueberry compounds may increase the body's antioxidant production capability and improve histone acetylation, suggesting that the primary effective components in blueberries may function as deacetylase inhibitors. Further research is needed to study these mechanisms.

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Study on the distribution and metabolism in Majiang blueberry anthocyanins *in vivo* distribution, Southeastern Branch of J word (2015) No. 062; experimental study on the effects of blueberry extract on liver cancer cell protein acetylation, Guizhou Branch of LH word {2015} No. 7433; blueberry research on liver cancer cell proliferation, apoptosis and histone acetylation affected, #gzwjkj2015-1-037; experimental study on the effect of blueberry extract on the acetylation of liver fibrosis, Southeastern Branch of J word (2016) No. 081; experimental study on the effect of blueberry extract on the acetylation of liver fibrosis, Guiyang science and technology contract (2016) No.1001.

## REFERENCES

Barros D, Amaral OB, Izquierdo I, Geracitano L, et al. (2006). Behavioral and genoprotective effects of Vaccinium berries intake in mice. *Pharmacol. Biochem. Behav.* 84: 229-234. http://dx.doi.org/10.1016/j.pbb.2006.05.001

Binda O (2013). On your histone mark, SET, methylate! *Epigenetics* 8: 457-463. http://dx.doi.org/10.4161/epi.24451

Brenner DA (2009). Molecular pathogenesis of liver fibrosis. *Trans. Am. Clin. Climatol. Assoc.* 120: 361-368.

Carloni V, Luong TV and Rombouts K (2014). Hepatic stellate cells and extracellular matrix in hepatocellular carcinoma: more complicated than ever. *Liver Int.* 34: 834-843. http://dx.doi.org/10.1111/liv.12465

Das SK and Vasudevan DM (2008). Genesis of hepatic fibrosis and its biochemical markers. *Scand. J. Clin. Lab. Invest.* 68: 260-269. http://dx.doi.org/10.1080/00365510701668516

Kong JM, Chia LS, Goh NK, Chia TF, et al. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry* 64: 923-933. http://dx.doi.org/10.1016/S0031-9422(03)00438-2

Lee CH, Park SW, Kim YS, Kang SS, et al. (2007). Protective mechanism of glycyrrhizin on acute liver injury induced by carbon tetrachloride in mice. *Biol. Pharm. Bull.* 30: 1898-1904. http://dx.doi.org/10.1248/bpb.30.1898

Rimando AM, Kalt W, Magee JB, Dewey J, et al. (2004). Resveratrol, pterostilbene, and piceatannol in vaccinium berries. *J. Agric. Food Chem.* 52: 4713-4719. http://dx.doi.org/10.1021/jf040095e

Santos-Rosa H and Caldas C (2005). Chromatin modifier enzymes, the histone code and cancer. *Eur. J. Cancer* 41: 2381-2402. http://dx.doi.org/10.1016/j.ejca.2005.08.010

Sato M, Suzuki S and Senoo H (2003). Hepatic stellate cells: unique characteristics in cell biology and phenotype. *Cell Struct. Funct.* 28: 105-112. http://dx.doi.org/10.1247/csf.28.105

Tiwari BK, O'Donnell CP, Patras A and Cullen PJ (2009). Stability of anthocyanins and ascorbic acid in sonicated strawberry juice during storage. *Eur. Food Res. Technol.* 228: 717-724. http://dx.doi.org/10.1007/s00217-008-0982-z

Tsukamoto H (2007). Epigenetic mechanism of stellate cell trans-differentiation. *J. Hepatol.* 46: 352-353. http://dx.doi.org/10.1016/j.jhep.2006.11.002

Wang YL, Lv HY and Zhang Q (2015). Effect of flavonoid compounds extracted from Iris species in prevention of carbon tetrachloride-induced liver fibrosis in rats. *Genet. Mol. Res.* 14: 10973-10979. http://dx.doi.org/10.4238/2015.September.21.9

Weber LW, Boll M and Stampfl A (2003). Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit. Rev. Toxicol.* 33: 105-136. http://dx.doi.org/10.1080/713611034

Ye JF, Zhu H, Zhou ZF, Xiong RB, et al. (2011). Protective mechanism of andrographolide against carbon tetrachloride-induced acute liver injury in mice. *Biol. Pharm. Bull.* 34: 1666-1670. http://dx.doi.org/10.1248/bpb.34.1666