Hepatoprotective Effect of Syringic Acid and Vanillic Acid on CCl_4-Induced Liver Injury

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The mycelia of the edible mushroom *Lentinula edodes* can be cultured in solid medium containing lignin, and the hot-water extracts (L.E.M.) is commercially available as a nutritional supplement. During the cultivation, phenolic compounds, such as syringic acid and vanillic acid, were produced by lignin-degrading peroxidase secreted from *L. edodes* mycelia. Since these compounds have radical scavenging activity, we examined their protective effect on oxidative stress in mice with CCl_4-induced liver injury. We examined the hepatoprotective effect of syringic acid and vanillic acid on CCl_4-induced chronic liver injury in mice. The injection of CCl_4 into the peritoneal cavity caused an increase in the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. The intravenous administration of syringic acid and vanillic acid significantly decreased the levels of the transaminases. Four weeks of CCl_4 treatment caused a sufficiently excessive deposition of collagen fibrils. An examination of Azan-stained liver sections revealed that syringic acid and vanillic acid obviously suppressed collagen accumulation and significantly decreased the hepatic hydroxyproline content, which is the quantitative marker of fibrosis. Both of these compounds inhibited the activation of cultured hepatic stellate cells, which play a central role in liver fibrogenesis, and maintained hepatocyte viability. These data suggest that the administration of syringic acid and vanillic acid could suppress hepatic fibrosis in chronic liver injury.

Key words hepatoprotection; *Lentinula edodes*; syringic acid; vanillic acid; polyphenol

The edible mushroom *Lentinula edodes* (shiitake) contains bioactive compounds that have immune-modulating, antitumor, antibacterial, antiviral, and antiparasitic effects.[1—4] The mycelia of *L. edodes* can be cultured in solid medium, and the hot-water extract (L.E.M.) is commercially available as a nutritional supplement. The main components of L.E.M. are sugars, proteins, and polyphenolic compounds. Polyphenols have protective effects against cancers, cardiovascular disease, and neurodegenerative disorders.[5—7] Among polyphenols, syringic acid and vanillic acid are enriched in the solid medium of cultured *L. edodes* mycelia.[8] *L. edodes* grown in lignocellulose secretes lignin-degrading peroxidase into the culture medium.[9] The mycelia-derived enzymes degrade the lignin to produce phenolic compounds, particularly syringic acid and vanillic acid. In our previous study, we demonstrated that these phenolic compounds had a hepatoprotective effect on concanavalin A (ConA)-induced liver injury in mice.[8] We intraperitoneally injected syringic acid or vanillic acid into mice shortly before a ConA injection into the tail vein, which greatly increased the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In addition, the inflammatory cytokines tumor necrosis factor (TNF)-α, interferon-γ (IFN-γ), and interleukin (IL)-6 in the serum increased rapidly, within 3 h of the ConA administration. The administration of syringic acid or vanillic acid significantly decreased the transaminase and inflammatory cytokine levels and suppressed the disorganization of the hepatic sinusoids. Since ConA-induced liver injury is a mouse model of immune-mediated liver injury that resembles viral and autoimmune hepatitis in humans, the phenolics appeared to have immunomodulating activity.

Polyphenols act as antioxidants by scavenging reactive oxygen species (ROS), which produce oxidative stress and can adversely affect many cellular processes. In the present study, we examined the possible hepatoprotective effects of two phenolic compounds, syringic acid and vanillic acid, on oxidative stress in chronic CCl_4-induced liver injury in mice. We found that both phenolic compounds could suppress oxidative damage, especially liver fibrosis caused by repeated administration of CCl_4.

MATERIALS AND METHODS

Reagents Syringic acid, vanillic acid, and CCl_4 were purchased from WAKO Pure Chemicals, Co., Ltd. (Osaka, Japan). The chemical structures of syringic acid and vanillic acid were shown in Fig. 1. L.E.M. was obtained from Kobayashi Pharmaceutical Co., Ltd. (Osaka, Japan). CCl_4 was dissolved in olive oil, and L.E.M., syringic acid, and vanillic acid were dissolved in phosphate buffered saline (PBS) for administration into mice. L.E.M., syringic acid, and vanillic acid were dissolved in culture medium for hepatocytes or hepatic stellate cells for *in vitro* experiments.

Animals BALB/c mice and Sprague-Dawley rats were purchased from SLC (Shizuoka, Japan). The animals were housed in an air-conditioned room at 22 °C before the experiment. The animal experiments were conducted according to the ethical guidelines of Osaka University Graduate School

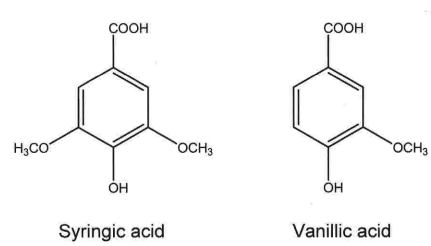

Fig. 1. Chemical Structures of Syringic Acid and Vanillic Acid

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of Pharmaceutical Sciences. The experimental protocol was submitted to the Committee on the Guidelines for Animal Experiments in Graduate School of Pharmaceutical Sciences, and the experiments were conducted after gaining the approval. Mice in the chronic liver injury model received intraperitoneal injections of CCl_4 (0.5 ml/kg body weight) and intravenously administered L.E.M., syringic acid, or vanillic acid (10 mg/kg body weight) twice a week for 4 weeks. Twenty-four hours after the L.E.M., syringic acid, or vanillic acid injection, the mice were anesthetized. Then, blood samples were collected to determine the transaminase activity, and the livers were excised for Azan staining and determination of hydroxyproline and malondialdehyde.

Assays Serum AST and ALT levels were measured by using an assay kit (Transaminase C-II, WAKO, Osaka, Japan).

Histological Analysis Liver specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut from the tissue blocks and mounted on slides. Azan staining was then performed to evaluate the extent of liver fibrosis.

Measurement of Hydroxyproline Content Hepatic hydroxyproline content was measured by using Kivirikko's method[10] with some modifications. Briefly, liver tissue (50 mg) was hydrolyzed with 6 mol/l HCl at 110 °C for 24 h in a glass test tube. After centrifugation at 3000 rpm for 10 min, 2 ml of the supernatant was neutralized with 8 N KOH. Two grams of KCl and 1 ml of 0.5 mol/l borate buffer were then added to the neutralized supernatant, followed by a 15-min incubation at room temperature and then a 15-min incubation at 0 °C. Freshly prepared chloramine-T solution was then added, and the sample was incubated at 0 °C for 1 h, followed by the addition of 2 ml of 3.6 mol/l sodium thiosulfate. The samples were incubated at 120 °C for 30 min. Then, 3 ml of toluene was added, and the samples were incubated for 20 min at room temperature. After centrifugation at 2000 rpm for 5 min, 2 ml of the supernatant was added to 0.8 ml buffer containing Ehrlich's reagent and incubated for 30 min at room temperature. The samples were then transferred to a plastic tube, and the absorbance was measured at 560 nm. The hydroxyproline content was expressed as micrograms of hydroxyproline per gram of liver.

Measurement of Malondialdehyde Lyophilized liver tissue (25 mg) was boiled for 30 min in a solution containing 250 ml of 1.15% KCl, 150 ml of 1% H_3PO_4, and 500 ml of 0.67% thiobarbituric acid. Two milliliters of *n*-butanol was added to the ice-chilled sample, and then the sample was stirred for 30 min. After centrifugation at $3000\times g$ for 10 min, the upper *n*-butanol phase was collected, and the amount of malondialdehyde was colorimetrically determined at 535 and 520 nm.

Isolation and Culture of Hepatic Stellate Cells Hepatic stellate cells (HSCs) were isolated from 10-week-old male Sprague-Dawley rats by digesting the liver with Pronase-E (Merck Darmstadt, Germany) and collagenase type I (WAKO Pure Chemicals Co., Osaka, Japan) as previously described.[11] Isolated HSCs were seeded at a density of 2×10^5 cells/cm^2 onto 24-well polystyrene culture plates (Asahi Techno Glass, Funabashi, Chiba, Japan) to observe the morphology and analyze fibrosis-related gene expression. Cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum.

Isolation and Culture of Hepatocytes Hepatocytes were isolated from male BALB/c mice by perfusing the liver with collagenase, according to the method of Seglen.[12] Cells were seeded at a density of 1×10^5 cells/cm^2 into multi-well culture plates pre-coated with collagen type I (Asahi Techno Glass, Funabashi, Chiba, Japan). The basal medium consisted of 50 U/ml penicillin G, 50 μg/ml streptomycin (ICN Biochemicals, Inc., Costa Mesa, CA, U.S.A.), 1 μM insulin, 1 μM dexamethasone (WAKO Pure Chemicals Co., Osaka, Japan), and 10% fetal bovine serum in William's medium E (MP Biomedicals, Inc., Kayserberg, France). Six hours after the cells were seeded, the basal medium was replaced with medium containing L.E.M., syringic acid, or vanillic acid at a final concentration of 1.0 mg/ml without insulin and dexamethasone. Cells were then cultured for 24—48 h, and viable cells were counted after trypan blue staining.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) The HSCs were cultured for 7 d and the total RNA was extracted using High Pure RNA Isolation Kit (Roche, Mannheim, Germany). The gene expression of collagen 1 α(I) was analyzed using the following primers: forward 5′-TGCCGTGACCTCAAGATGTG-3′ and reverse 5′-CACAAGCGTGCTGTAGGTGA-3′. The gene expression of a smooth muscle actin (α-SMA) was analyzed using the following primers: forward 5′-CCGAGATCTCACCGACTACC-3′ and reverse 5′-TCCAGAGCGACATAGCACAG-3′. The gene expression of β-actin was analyzed using the following primers: forward 5′-CCCAGAGCAAGAGAGGCATC-3′ and reverse 5′-CTCAGGAGGAGCAATGATCT-3′.

The RT-PCR was examined using RNA PDR Kit (TaKaRa, Kyoto, Japan).

Statistical Analysis The data were analyzed for statistical significance by using Student's *t*-test and Dunnett's test.

RESULTS

Effect on CCl_4-Induced Chronic Liver Injury We examined the hepatoprotective effect of syringic acid and vanillic acid on CCl_4-induced chronic liver injury in mice. As shown in Fig. 2, after 4 weeks of CCl_4 treatment, the activities of blood AST and ALT increased 30-fold and 127-fold, respectively, compared with controls. The intravenous administration of syringic acid or vanillic acid significantly decreased the activities of AST and ALT. These results suggest that syringic acid and vanillic acid suppress the hepatic inflammation caused by repeated CCl_4 treatments. We also examined the effect of syringic acid and vanillic acid on liver fibrogenesis. Figure 3 shows typical Azan staining results, in which fibrous materials are stained blue. In the controls (Fig. 3A), hardly any blue staining was observed in the pericentral area. In contrast, the livers injured by chronic CCl_4 treatment displayed a considerable accumulation of fibrous materials (Fig. 3B). CCl_4 treatment for 4 weeks caused an excessive deposition of collagen fibrils that was sufficient for the evaluation of the antifibrogenic effect of syringic acid and vanillic acid. Based on the results of Azan staining, the syringic acid and vanillic acid treatments obviously suppressed collagen accumulation (Figs. 3D, E). To quantitatively evaluate the effect of syringic acid and vanillic acid on fibrogenesis, we

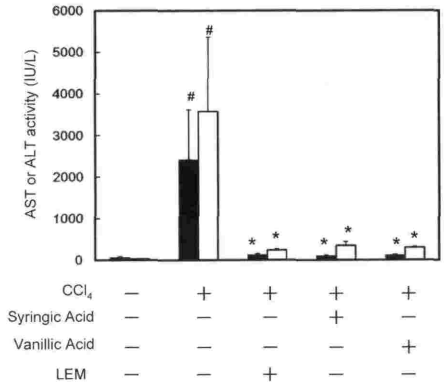

Fig. 2. Effect of Syringic Acid and Vanillic Acid on CCl_4-Induced Chronic Hepatic Injury

Mice received an intraperitoneal injection of CCl_4 and an intravenous injection of L.E.M., syringic acid, or vanillic acid twice a week for 4 weeks. The serum levels of AST (solid column) and ALT (open column) were determined. The values are mean±S.D (n=4). The data were analyzed by Student's t-test (# $p<0.05$, as compared to uninjured control mice) and Dunnett's method (* $p<0.05$, as compared to CCl_4-injured control mice).

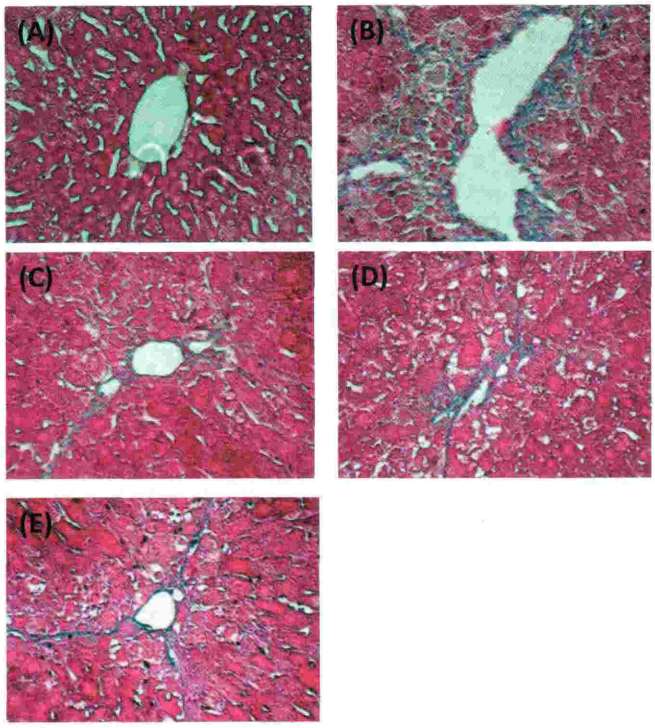

Fig. 3. Azan Staining of Liver Sections

Livers were excised from normal mice (A), CCl_4-injured control mice (B), L.E.M.-treated mice (C), syringic acid-treated mice (D), and vanillic acid-treated mice (E). Original magnification ×400.

measured the hepatic hydroxyproline content, which parallels the extent of fibrosis. After 4 weeks of CCl_4 treatment, the hepatic hydroxyproline content increased 4.6-fold as compared with the controls (Fig. 4). The intravenous administration of syringic acid or vanillic acid significantly decreased the hepatic hydroxyproline content. These data suggest that syringic acid and vanillic acid can suppress hepatic fibrosis in chronic liver injury. Next, we measured the amount of malondialdehyde in the liver samples as a marker of oxidative stress. The malondialdehyde content was drastically increased after 4 weeks of CCl_4 treatment, but the intravenous administration of syringic acid or vanillic acid significantly

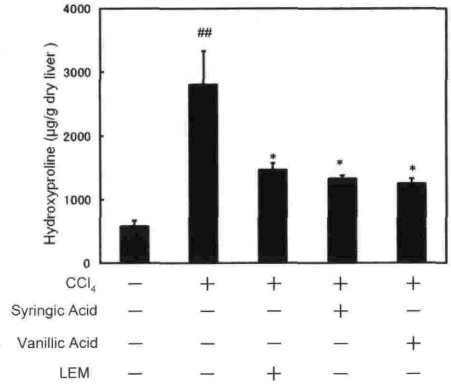

Fig. 4. Effect of Syringic Acid and Vanillic Acid on the Hydroxyproline Content of the Liver

The hydroxyproline content of the liver was measured after 4 weeks of treatments. The values are mean±S.D. (n=4). The data were analyzed by Student's t-test (## $p<0.01$, as compared to uninjured control mice) and Dunnett's method (* $p<0.05$, as compared to CCl_4-injured control mice).

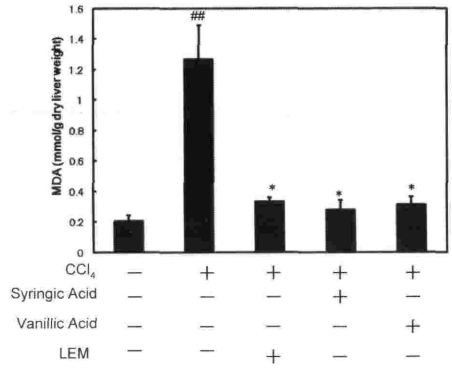

Fig. 5. Effect of Syringic Acid and Vanillic Acid on the Suppression of Oxidative Stress

The malondialdehyde content of the liver was determined after 4 weeks of experiments. The values are mean±S.D. (n=4). The data were analyzed by Student's t-test (## $p<0.01$, as compared to uninjured control mice) and Dunnett's method (* $p<0.05$, as compared to CCl_4-injured control mice).

decreased the malondialdehyde content to an almost normal level (Fig. 5). The protective effects of syringic acid and vanillic acid were almost comparable to that of L.E.M. (Figs. 2—5).

***In Vitro* Effect on HSC Activation and Hepatocyte Viability** We examined the direct effect of syringic acid and vanillic acid on the activation of HSCs, which play a central role in liver fibrogenesis, using the monolayer culture. HSCs are activated during the monolayer culture to transform into proliferating myofibroblast-like cells. As shown in Fig. 6A, HSCs were activated after 7 d of culture to be fibroblastic cell-type. The addition of syringic acid or vanillic acid dose-dependently suppressed the activation (Fig. 6B). HSCs maintained their quiescent state by the addition of more than 0.5 mg/ml of the respective compound. Next, the effect of syringic acid and vanillic acid on gene expression of Type I collagen and α-SMA, which are markers of activated HSCs, was examined. HSCs were cultured for 7 d in the presence or absence of syringic acid or vanillic acid, and the gene expression was analysed by RT-PCR. As shown in Fig. 7, syringic acid and vanillic acid remarkably suppressed the expression of collagen and α-SMA genes, indicating that the phenolic compounds directly act on HSCs and suppress the activation

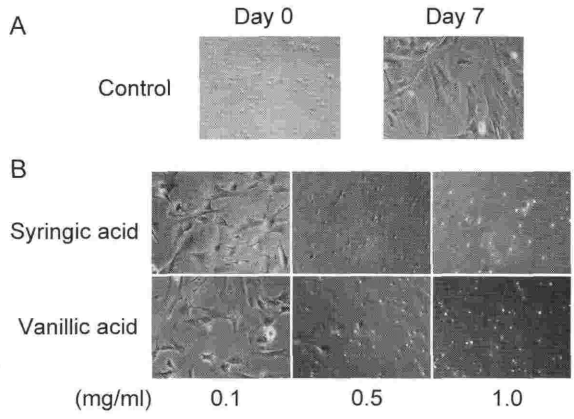

Fig. 6. Phase-Contrast Micrographs of Cultured HSCs

Freshly isolated HSCs were cultured for 7 d in the absence (A) and presence (B) of syringic acid or vanillic acid at the indicated concentration. Original magnification ×200.

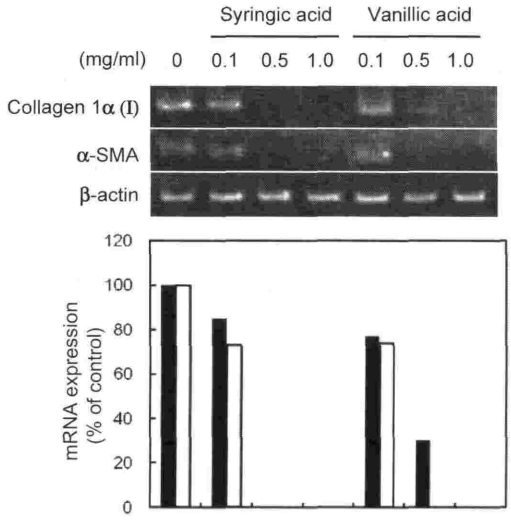

Fig. 7. RT-PCR Analysis of Gene Expression Relating to HSC Activation

Bottom figure shows the relative expression of collagen 1 α(I) (closed bar) and α-SMA (open bar) compared with the non-addition control.

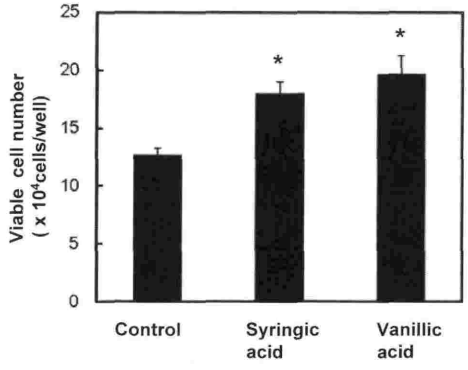

Fig. 8. Effect of Syringic Acid and Vanillic Acid on Viability of Cultured Hepatocytes

Freshly isolated hepatocytes were cultured for 24 h in the absence or presence of 1.0 mg/ml syringic acid or vanillic acid. Viability was measured by trypan blue exclusion test. The data were analyzed by Dunnett's method ($*p<0.05$, as compared to control).

to maintain the quiescent state. We then examined the effect of the compounds on liver parenchymal hepatocytes using the primary culture (Fig. 8). Hepatocytes were isolated and cultured in the presence or absence of syringic acid or vanillic acid. After 24 h of culture, viable cells were counted using trypan blue exclusion test. The addition of syringic acid or vanillic acid significantly maintained viability of cultured hepatocytes. These results suggested that syringic acid or vanillic acid might suppress liver fibrogenesis and inflammation by inhibiting HSC activation and protecting hepatocytes, respectively in chronically liver injured mice.

DISCUSSION

The physiological functions of plant-derived phenolic compounds have been extensively reported.[7,13,14] Syringic acid and vanillic acid possess antimicrobial, anti-cancer, and anti-DNA oxidation properties.[15—17] We recently found that syringic acid and vanillic acid could act as immunomodulators in mice with ConA-induced liver injury.[8] In the present study, we show that syringic acid and vanillic acid have protective effects in mice with CCl_4-induced liver injury. Both phenolic compounds dramatically suppressed liver fibrogenesis in the chronic CCl_4-treatment model. When these phenolics are orally administered to hamsters, they are adsorbed and appear in the blood within 40 min.[18] Although these compounds are intravenously administered in the present study, oral administration could also elicit the hepatoprotective effect. The syringic acid and vanillic acid contents in L.E.M. are 450 and 378 μg/g, respectively. Thus, the contents are relatively small, but these compounds are commercially available at low prices. Therefore, syringic acid and vanillic acid might be promising oral agents for the prevention of liver disease.

We evaluated the hepatoprotective effect of phenolic compounds in mice with CCl_4-induced liver injury. After intravenous administration, CCl_4 is introduced into the liver, where it is toxic to hepatocytes. Cytochrome P-450 in the endoplasmic reticulum of hepatocytes catalyzes the dehalogenation to produce an unstable complex trichloromethyl radical,[19] resulting in the extensive necrosis of hepatocytes that leads to liver inflammation. In the present study, the transaminase level in the serum was drastically increased by CCl_4 treatment. Generation of ROS degrade polyunsaturated lipids to form malondialdehyde, which is a marker of oxidative stress. The chronic CCl_4 treatment significantly increased the malondialdehyde content of the liver. Syringic acid and vanillic acid clearly suppressed the transaminase and malondialdehyde levels in CCl_4-treated mice. Since both of these compounds have 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity,[8] the suppression of ROS generation appears to be responsible for the hepatoprotective effect. Moreover, the CCl_4-induced liver fibrogenesis was suppressed by the administration of syringic acid and vanillic acid. The activation of HSCs is responsible for the development of liver fibrosis.[20,21] During liver injury with persistent inflammation, HSCs are activated to differentiate into proliferating myofibroblast-like cells and overproduce extracellular matrix, leading to fibrogenesis. Since HSCs are activated spontaneously during cell culture,[22] we examined the effect of syringic acid and vanillic acid on the activation of primary cell cultures of rat HSCs. Both of these compounds clearly inhibited the change from spherical to spindle shape and the expression of α-smooth muscle actin and collagen Type Iα genes, which are the markers of HSC activation. We also

examined the effect of syringic acid and vanillic acid on the maintenance of hepatocyte viability *in vitro*. Both of these compounds significantly maintained the viability of primary cell cultures of hepatocytes. Thus, syringic acid and vanillic acid could directly exert a physiological effect on hepatocytes and HSCs. Both phenolic compounds might affect CCl_4 metabolism to inhibit the generation of cytotoxic trichloromethyl radical in the liver. However, the direct effects of syringic acid and vanillic acid on HSCs and hepatocytes were shown in this study, and the protective effect was also shown in ConA-induced liver injured mice in our previous study.[8] Moreover, these phenolic compounds have strong radical scavenging activity. These results suggest that during the repeated treatment of CCl_4, these compounds could protect hepatocytes and HSCs from CCl_4-induced oxidative stress to suppress liver inflammation and fibrogenesis.

The hot-water extracts from cultured mycelia of *L. edodes* have versatile physiological effects and might contain promising seed compounds for pharmaceutical development. We have shown that syringic acid and vanillic acid have anti-oxidative and immunomodulating activities. In addition to these phenolics, L.E.M. could contain novel compounds with pharmaceutical potential. We are currently trying to isolate bioactive components from L.E.M.

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