Free Radical Research, September 2006; 40(9): 986–992

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Antioxidant effects and hepatoprotective activity of 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *Morus bombycis* Koidzumi roots on CCl_4-induced liver damage

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Accepted by Professor J. Yodoi

(Received 21 February 2006; in revised form 28 April 2006)

Abstract

We investigated hepatoprotective activity and antioxidant effect of the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene that purified from *Morus bombycis* Koidzumi roots against CCl_4-induced liver damage in rats. The 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene displayed dose-dependent superoxide radical scavenging activity ($IC_{50} = 430.2$ μ*g/ml*), *as assayed by the electron spin resonance (ESR) spin-trapping technique. The increase in aspartate aminotransferase (AST) activities in serum associated with carbon tetrachloride (*CCl_4*)-induced liver injury was inhibited by 2,5-dihydroxy-4,3′-di(*β-D-*glucopyranosyloxy)-trans*-stilbene and at a dose of 400 ∼ 600 mg/kg samples had hepatoprotective activity comparable to the standard agent, silymarin. The biochemical assays were confirmed by histological observations showing that the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene decreased cell ballooning in response to CCl_4 treatment. These results demonstrate that the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene is a potent antioxidant with a liver protective action against CCl_4-induced hepatotoxicity.

Keywords: Morus bombycis *Koidzumi roots, 2,5-dihydroxy-4,3′-di(*β-*D-glucopyranosyloxy)*-trans-*stilbene, hepatoprotective, antioxidative,* CCl_4*, free radicals*

Abbreviations: *AST, aspartate aminotransferase;* CCl_4*, carbon tetrachloride; ROS, Reactive oxygen species;* CCl_3•*, trichloromethyl radical; ESR, electron spin resonance; RT, reverse transcription; DMPO, 5,5-dimethyl-1-pyrroline-oxide; TNF-α, tumor necrosis factor-α; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal*

Introduction

Reactive oxygen species (ROS) are contributed to the development of various diseases such as atherosclerosis, diabetes, and cancer [1,2]. Hence ROS scavengers reduced the incidence of free radical-mediated diseases. The use of antioxidants, both natural and synthetic, in the prevention and cure of various diseases is expanding, and there is considerable interest in the antioxidant activities of dietary antioxidants such as vitamins E and C, carotenoids, and plant polyphenolics. Furthermore, liver intoxication is increasing as a result of exposure to high levels of environmental toxins, as the liver has an important role in detoxification, and plants contain substances that can protect against hepatic injury.

Carbon tetrachloride (CCl_4) is metabolized to the trichloromethyl radical (CCl_3•) by the cytochrome P450

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Informa UK Ltd.
DOI: 10.1080/10715760600803823

system in liver microsomes, causing liver injury [3–5]. This reactive intermediate cause lipid peroxidation, and the breakdown of cellular membranes [6]. Antioxidants play an important role in protecting against CCl_4-induced liver injury [7]. Although several isoforms of cytochrome P450 can metabolize CCl_4, attention has focused largely on the cytochrome isoform, P450 2E1 (CYP2E1), which is ethanol-inducible [8]. Alterations in the activity of CYP2E1 affect susceptibility to hepatic injury from CCl_4 [9]. Therefore, carbon tetrachloride treatment has been used to evaluate the free radical scavenging action of antioxidants *in vivo*.

The mulberry, *Morus bombycis* Koidzumi are widely distributed in Asia and are used in traditional medicine on account of its apparent anti-inflammatory, antibiotic, and antioxidant effects, and lowering of blood hyperlipemia. Recently *M. bombycis* Koidzumi have been used for their anti-inflammatory, antioxidant [10], hepatoprotective effects [11]. However, the basis of the pharmacological effects of *M. bombycis* Koidzumi is obscure.

In this study, a 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene of *M. bombycis* Koidzumi roots evaluated the relationship between the pharmacological and antioxidant effects. The antioxidant effect of 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene was evaluated by electron spin resonance (ESR) spectrometry. CCl_4 hepatotoxicity in Wistar rats was used to study hepatoprotective effects by biochemical analyses and histopathological examination.

Materials and methods

Purification procedure of Morus bombycis *Koidzumi roots*

The *M. bombycis* Koidzumi roots were collected in the wild in the district of Korea. All samples were washed thoroughly in tap water, shade-dried, powdered (100 g) and extracted with 2 l of 80% methanol at room temperature for 1 h. The extract was filtered, dried by evaporation, dissolved in 500 ml distilled water and the dissolved material applied to an absorption column (MCI gel CHP20P: 2.5 × 40 cm). The fraction eluting with 20–30% methanol was dried by evaporation and dissolved in 10 ml of 70% methanol. It was then applied to a gel filtration column (Sephadex LH-20: 2.5 × 40 cm), and eluted with 70% methanol. Polyphenols were detected at wavelength 326 nm (UV–VIS and HPLC). The polyphenol fraction was collected, evaporated and identified the chemical structure using NMR.

Assay of superoxide anion scavenging activity

Using an ESR spectrometer, we analyzed the superoxide anion radical (O_2^-) formed from the spin adduct of O_2^- (DMPO–OOH) [12]. The superoxide radical O_2^- was generated in hypoxanthine HPX–xanthine oxidase reaction mixtures containing 180 μl of 0.1 M potassium dihydrogen phosphate (Kpi) buffer (pH 7.4), 4 μl of 50 mM hypoxanthine, 2 μl of 5 mM enetriaminepentaacetic acid (DETAPAC), 5 μl of various concentrations of purified compound, 2 μl of 100 μM catalase and 4 μl of 9 mM DMPO (5,5-dimethyl-1-pyrroline-oxide). The reactions were initiated by adding 3 μl of 25 units XOD to each sample. After 2 min, the spin adduct DMPO–OOH was measured with an ESR spectrometer (JEOL-JES-TM200, JEOL, Tokyo). ESR spectra were recorded at 37°C with the field set at 336.75 ± 5.0 mT for superoxide radicals, modulation frequency 100 kHz, modulation amplitude 0.79 × 0.1 mT, response time 0.1 s, sweep time 0.5 min, microwave power 2.0 mM (9.419 GHz), and receiver mode 1 st.

CCl_4-induced hepatotoxicity in rats

Male Wistar albino rats were purchased from the animal house of our institute and kept for 1 week on a commercial diet under environmentally controlled conditions (temperature 22 ± 3°C, relative humidity 55 ± 5%) with free access to water and food (Purina Rat Chow 5001, Ralston Purina, St Louis, MO, USA). A controlled 12 h light/dark cycle was maintained. Rats weighing 180–220 g were used for assessing CCl_4-induced hepatotoxicity. Liver damage was induced with a 1:1 (v/v) mixture of CCl_4 and olive oil, administered i.p. at a dose of 2 ml/kg body weight. The animals were divided into six groups of six rats each. Group 1 received normal saline (10 ml/kg, p.o.) as normal control. Group 2 was injected with CCl_4/olive oil alone (2 ml/kg). Groups 3–6 were administered of purified compounds from *M. bombycis* Koidzumi (100, 400, 600 mg/kg) and silymarin p.o., once, 30 min before i.p. injection of CCl_4/olive oil (2 ml/kg). Animals were sacrificed 24 h and 4 days after the administration of CCl_4.

Experimental data are expressed as means ± SE Duncan's multiple range test was applied for assessing the significance of differences between groups. $P < 0.05$ was regarded as significant.

Assay of serum AST activities

Twenty-four hours and 4 days after CCl_4 intoxication, animals were anaesthetized with ether, and blood was withdrawn from the carotid artery. The blood was centrifuged at 3000 rpm for 10 min at 4 to obtain serum aspartate aminotransferase (AST) activities measured with an auto-biochemistry-detector (EKTA Chem; DT60, DTSC module, DTE module, Johnson & Johnson Orthoclinical Diagnostics).

Histopathological observation

Sections were taken from each lobe of the liver immediately after the blood was collected. The tissues

were fixed in 10% neutral formalin for 24 h, dehydrated in graded (50–100%) alcohols, embedded in paraffin, cut into 4–5 μm sections and stained with haematoxylin–eosin for microscopic assessment.

$FeCl_2$–ascorbic acid-stimulated lipid peroxidation in rat liver homogenates

Young male Wistar albino rats weighing 180 g were used to prepare liver homogenates [13]. The rats were killed by decapitation and their liver tissues quickly removed. About 2 g portions of liver tissue were sliced and homogenized in ice cold 10 ml of 150 mM KCl–Tris–HCl buffer (pH 7.2). Reaction mixtures were composed of 0.35 ml of liver homogenate, 0.1 ml of Tris–HCl buffer (pH 7.2), 0.05 ml of 4 mM $FeCl_2$ 0.05 ml of 0.1 mM AA and 0.05 ml of different concentration of purified samples. The mixtures were incubated at 37°C for 1 h in capped tubes; then 0.5 ml of HCl (0.1 N), 0.2 ml of SDS (9.8%), 0.7 ml of distilled water and 2 ml of TBA (0.6%) were added with vigorous shaking. The tubes were placed in a boiling water bath (100°C) for 30 min. After cooling, the flocculent precipitate was removed by adding 5 ml of *n*-BuOH and centrifuging at 3000 rpm for 15 min. Thereafter, the absorbance of the supernatant was measured at 532 nm. The lipid peroxidation products were measured by the formation of thiobarbituric acid-reactive material, malonidialdehyde (MDA) [14]. 1,1,3,3-tetraethoxypropan was used as a standard for the calibration of MDA. Protein content was determined by the Lowry method [15].

RT-PCR analysis of CYP2E1 and TNF-α

Total RNA was isolated from rat liver homogenates with a Trizol RNA isolation kit (Invitrogen). cDNA was synthesized from the total RNA with an AMV RNA PCR kit (Takara, Japan). Reverse transcription (RT) reactions were carried out in 2.5 mM $MgCl_2$, 10 × PCR buffer, RNase free dH_2O, 10 mM dNTP, 0.25 unit AMV reverse transcriptase, 2.5 pmol/μl oligo dT in a final volume of 20 μl. RT conditions were 10 min at 30°C, 30 min at 50°C for annealing, 2 min at 95°C, 5 min at 5°C in a final volume of 50 μl containing 2 μl of RT reaction mixture, 2.5 mM $MgCl_2$, 10 × PCR buffer, 2.5 mM dNTP, 0.05 μM each of the oligonucleotide primers and 2.5 unit *Taq* DNA polymerase (Takara, Japan). The primers and the sized of expected PCR products were summarized in Table I. PCR conditions were denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min before a final extension at 72°C for 7 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel, followed by staining with ethidium bromide.

Results

Superoxide radical scavenging activity

The 50% scavenging concentration (IC_{50}) of 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *M. bombycis* Koidzumi roots was 450.2 μg/ml and measurement of ESR signals reduced the DMPO–OOH signal in a dose-dependent manner (Figure 1). Comparison of the ESR signals generated by different concentrations of 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *M. bombycis* Koidzumi roots with the manganese oxide (Mn^{2+}) signal as internal standard was strongly inhibitory at 500 μg/ml. The superoxide radical scavenging activities of different constituents are shown in Figure 2.

CCl_4-induced hepatotoxicity

The protective properties of 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *M. bombycis* Koidzumi roots were examined in rats treated with CCl_4. In these studies, one dose of 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *M. bombycis* Koidzumi roots was administered 30 min before the CCl_4. We then examined serum AST activities, and histological changes of the liver 24 h and 4 days after the i.p. injection of CCl_4. Serum AST activities increased markedly in the CCl_4-treated rats (Figure 2) and treatment with 100, 400, and 600 mg/kg 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene or 50 mg/kg silymarin protected against this hepatotoxicity; the levels of ALT and AST returned almost to baseline by 4 days. Histological examination of livers in the CCl_4-treated rats revealed massive and severe hepatocyte necrosis in the centrilobular zone with influx of inflammatory cells. Injection of CCl_4: olive oil (1:1) induced ballooning degeneration,

Table I. Oligonucleotide primers and sizes of expected PCR products.

cDNA amplified	Primers sequence	Amplicon (bp)
CYP2E1	Sense: 5′-ACCACCAGCACAACTCTGAGATATGG-3′ Antisense: 5′-CAATTCCATGCGGGCCAGGCCTTCTCC-3′	436
TNF-α	Sense: 5′-CGAGTGACAAGCCCGTAGCC-3′ Antisense: 5′-GGATGAACACGCCAGTCGCC-3′	735
β-action	Sense: 5′-CATCCCCCAAAGTTCTAC-3′ Antisense: 5′-CCAAAGCCTTCATACATC-3′	347

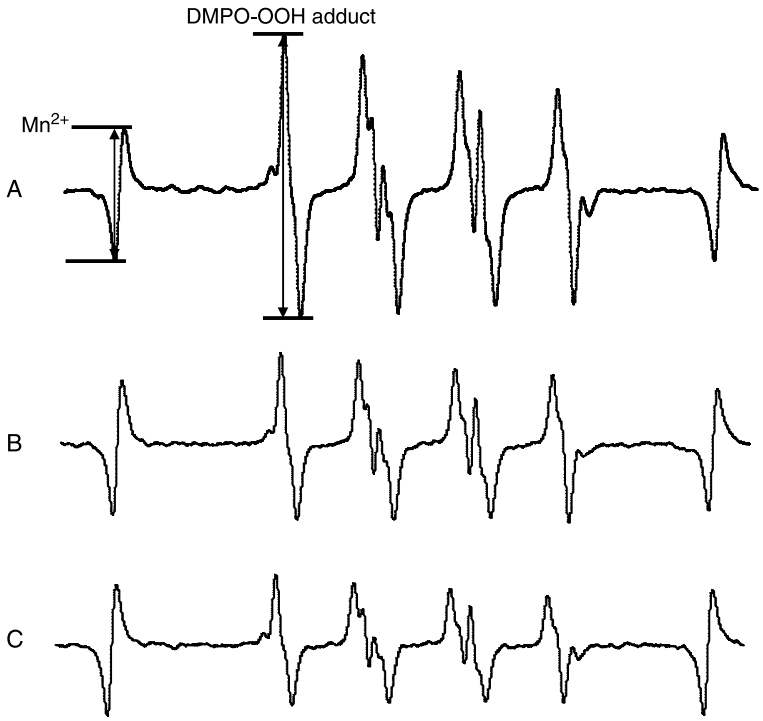

Figure 1. ESR signal of the standard compound, manganese oxide (Mn^{2+}), and the superoxide radical (DMPO–OOH) peak without SOD, and inhibitory effect of different concentration on the ESR signal of the superoxide radical. (A) Control (B) 300 μg/ml (C) 500 μg/ml.

centrilobular necrosis, bridging necrosis, and injury to the hepatocytes, and the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene had a significant liver protective effect against this hepatotoxicity. Ballooned hepatocytes were of variable size but much larger than normal hepatocytes, and they occasionally appeared as confluent areas (Figure 3).

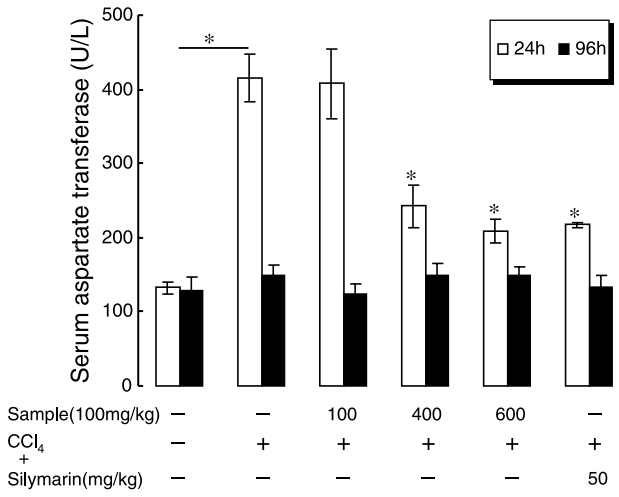

Figure 2. Serum aspartate transaminase levels in various groups of rats treated with 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *Morus bombycis* Koidzumi followed by induction of necrosis by carbon tetrachloride. Rats were sacrificed 24 h and 4 days after treatment. Data are expressed as means ± SE, that are significantly differenced from the control rats at $P < 0.05$ ($n = 6$).

$FeCl_2$–ascorbic acid induced lipid peroxidation in rat liver homogenates

Rat liver homogenates were treated with Fe^{2+}/ascorbic acid ($FeCl_2$–AA) to cause non-enzymatic lipid peroxidation and the action of the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene was determined. On incubation for 1 h at 37, the presence of $FeCl_2$-AA caused an increase in the level of MDA. At 300 μg/ml, purified compound from *M. bombycis* Koidzumi inhibited its formation by 63.4% which was almost the same as the inhibition achieved with 100 μg/ml vitamin E (Table II).

*Effect of 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from* Morus bombycis *Koidzumi roots on CYP2E1 and TNF-α expression*

CCl_4 is metabolized to the trichloromethyl free radical (CCl_3·) by CYP2E1. To measure expression of CYP2E1 mRNA, we performed RT-PCR using total RNA isolated from liver homogenates. This showed that the level of CYP2E1 mRNA was increased in liver homogenates of each of the CCl_4-treated groups (Figure 4) and this effect was reduced by pretreatment with the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *M. bombycis* Koidzumi roots or silymarin (Figure 4, lane 3–6).

Tumor necrosis factor-α (TNF-α) have been shown as important cytokines in liver regeneration induced in partially hepatectomized rats [16]. The level of TNF-α

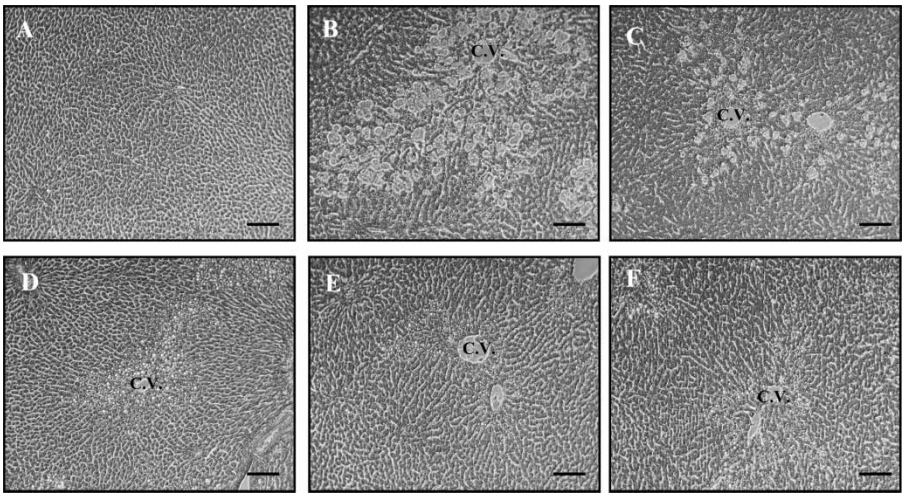

Figure 3. Section of liver of a CCl_4-treated rat showing ventral vein (C.V.) and hepatic cells (hematoxylin–eosin stain, original magnification, 100x). Rats were sacrificed 24 h after the administration of CCl_4. A, control group; B, CCl_4/olive oil (1:1, 2 ml/kg); C, the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *Morus bombycis* Koidzumi (100 mg/kg) + CCl_4; D, (400 mg/kg) + CCl_4; E, (600 mg/kg) + CCl_4; F, Silymarin (50 mg/kg) + CCl_4.

gene expression increased in liver homogenates of each of the CCl_4-treated groups and this effect was reduced by pretreatment with the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *M. bombycis* Koidzumi or silymarin (Figure 4, lane 3–6).

Discussion

Free radicals have been implicated in the pathogenesis of alcohol-induced liver injury in humans and CCl_4-induced liver injury in rats. The most extensively studied aspect of free radical-induced liver injury is lipid peroxidation. It is known that hepatic cytochrome P450 undergoes significant destruction during metabolism of CCl_4 [17,18]. In CCl_4-induced hepatic injury, CCl_4 is reduced by cytochrome P450 to a trichloromethyl free radical (CCl_3·) intermediate, which catalyzes the lipid peroxidation [19]. Our results demonstrate that, following an initial rapid rise at 24 h, serum ALT activity returned to the control level by 4 day after treatment (Figure 2), which coincided with the repair of liver damage, as judged by examination of histological changes (Figure 3).

The metabolism of CCl_4 initiates the peroxidation of polyunsaturated fatty acids producing α,β-unsaturated aldehydes, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). Liver injury was progressive to 24 h as lipid peroxidation and hepatocellular necrosis increased [20].

In vitro lipid peroxidation in liver homogenates occurs by enzymatic and non-enzymatic processes. The former process is NADPH-dependent, while the latter is induced by ascorbate in the presence of Fe^{2+}/Fe^{3+}, and even occurs in boiled liver homogenates. We showed that 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *M. bombycis* Koidzumi roots inhibit $FeCl_2$–AA-stimulated lipid peroxidation (MDA) in rat liver homogenates (Table II).

ROS and free radicals play an important role in the etiology and pathogenesis of a variety of diseases such as hypertension and cardiovascular disease [21–23]. Any compound, natural or synthetic, with antioxidant properties that might contribute to the partial or total alleviation of this damage, could have a significant role in maintaining health when continuously taken as a component of the diet or as a drug. Removing the

Table II. The inhibitory effects of 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene of *Morus bombycis* Koidzumi roots on $FeCl_2$–ascorbic acid induced lipid peroxidation in a rat liver homogenate *in vitro*⋆.

Groups	Concentration (μg/ml)	MDA (nmol/mg protein)	Inhibition rate (%)
Normal	–	0.325 ± 0.112	–
$FeCl_2$–AA	–	1.001 ± 0.184	–
$FeCl_2$–AA + sample	50	0.634 ± 0.080	20.7
$FeCl_2$–AA + sample	100	0.644 ± 0.064	27.7
$FeCl_2$–AA + sample	300	0.246 ± 0.104⋆	63.4
$FeCl_2$–AA + V_E	100	0.112 ± 0.077⋆	67.9

⋆ Values are expressed as means ± SD ($n = 5$). Significance $P < 0.05$, compared to $FeCl_2$–AA.

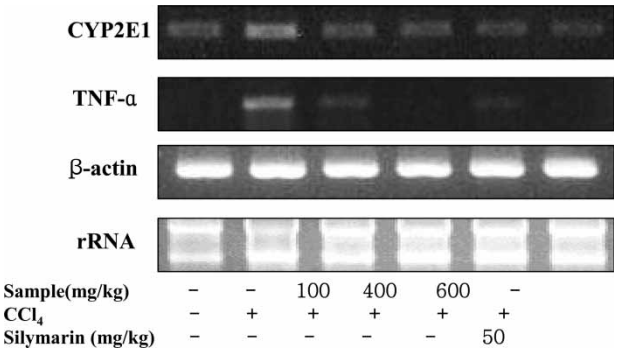

Figure 4. Inhibitory effect of the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *Morus bombycis* Koidzumi on the level of cytochrome P450 2E1 mRNA and TNF-α mRNA. CYP2E1 and TNF-α mRNA was detected by RT-PCR using total RNA from the livers of rats that had been exposed to (1) control, (2) CCl_4 /olive oil (1:1, 2 ml/kg), and (3)–(5) the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene (100, 400, 600 mg/kg) after administration of CCl_4 and (6) silymarin (50 mg/kg) after administration of CCl_4. All data were obtained from at least 3 rats.

superoxide radical is probably one of the most effective defenses against disease.

Studies on hepatoprotective models show that CCl_4 is first metabolized to the highly reactive free radical CCl_3· by cytochrome P450 in the endoplasmic reticulum of the liver [24]. In the presence of oxygen, this free radical promotes auto-oxidation of the fatty acids present in the phospholipids of the cytoplasmic membrane [25] leading to functional and morphological changes in the cell membrane. It is thought that the metabolic activation of CCl_4 to its toxic metabolites is mainly mediated by CYP2E1 [26], and pretreatment of rats with CYP2E1 inhibitors can protect against CCl_4-induced hepatotoxicity [27]. In the present study, we found that hepatic P450 2E1 transcripts increased in rats treated with CCl_4 alone; the addition of *M. bombycis* Koidzumi roots reduced this decline in hepatic P450 2E1 by 24 h after CCl_4 treatment and almost restored the normal level of P450 2E1 mRNA by 4 days after CCl_4 exposure.

The increase of TNF-α mRNA occurring very early during the regenerative response, has clearly implicated TNF-α as a major effector of signal pathways of liver regeneration [28]. Mitochondrial derived ROS generation has been contributed to tumor necrosis factor-a (TNF-α)-induced cytotoxicity [29,30]. Figure 4 showed that the level of TNF-α gene expression increased in liver homogenates of each of the CCl_4-treated groups and this effect was reduced by pretreatment with the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *M. bombycis* Koidzumi or silymarin (Figure 4, lane 3–6). Therefore, it is possible that *M. bombycis* Koidzumi roots restores hepatic P450 2E1 function and these effects were associated with hepatocyte protection against oxidative injury, since this cytokine is considered to activate death signals in hepatocytes in the setting of oxidative stress. The metabolic activation of CCl_4 is mediated through CYP2E1 [8,21,22] and CYP2E1 gene knockout mice were resistant to CCl_4-induced liver damage [23]. There is shown a good relationship between CYP2E1 and the level of protection against CCl_4-induced hepatotoxicity in the liver homogenates of *Lycium chinense* Miller fruit-pretreated group [31].

Silymarin, used here as a positive control, is a flavonoid extracted from the milk thistle *Silybum marianum* that has demonstrated protective effects against oxidative peroxidation in several experimental models and in human hepatic damage [32,33]. It also protects against hepatotoxicity produced by CCl_4 [34,35]. The results of the present study show that the 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *M. bombycis* Koidzumi roots has a preventive action against CCl_4-induced hepatotoxicity similar to silymarin.

In conclusion, we have identified a 2,5-dihydroxy-4,3′-di(β-D-glucopyranosyloxy)-*trans*-stilbene from *M. bombycis* Koidzumi roots, that has free radical scavenging activities, a liver protective effect against CCl_4-induced hepatotoxicity and expressional regulation of CYP2E1 and TNF-α.

Acknowledgements

This study was supported by High-Technology Development Project (0903013-1-3), Ministry of Agriculture and Forestry, Republic of Korea.

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