JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Walnut Polyphenols Prevent Liver Damage Induced by Carbon Tetrachloride and D-Galactosamine: Hepatoprotective Hydrolyzable Tannins in the Kernel Pellicles of Walnut

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The polyphenol-rich fraction (WP, 45% polyphenol) prepared from the kernel pellicles of walnuts was assessed for its hepatoprotective effect in mice. A single oral administration of WP (200 mg/kg) significantly suppressed serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) elevation in liver injury induced by carbon tetrachloride (CCl_4), while it did not suppress D-galactosamine (GalN)-induced liver injury. In order to identify the active principles in WP, we examined individual constituents for the protective effect on cell damage induced by CCl_4 and D-GalN in primary cultured rat hepatocytes. WP was effective against both CCl_4- and D-GalN-induced hepatocyte damages. Among the constituents, only ellagitannins with a galloylated glucopyranose core, such as tellimagrandins I, II, and rugosin C, suppressed CCl_4-induced hepatocyte damage significantly. Most of the ellagitannins including tellimagrandin I and 2,3-*O*-hexahydroxydiphenoyl-glucose exhibited remarkable inhibitory effect against D-GalN-induced damage. Telliamgrandin I especially completely suppressed both CCl_4- and D-GalN-induced cell damage, and thus is likely the principal constituent for the hepatoprotective effect of WP.

KEYWORDS: Walnut; hydrolyzable tannin; polyphenol; carbon tetrachloride; D-galactosamine; liver injury; tellimagrandin I

INTRODUCTION

Walnuts, the seeds of *Juglans regia* L. (Juglandaceae), are rich in oil composed of unsaturated fatty acids (*1*), such as linoleic acid and oleic acid (*2*). Unlike unsaturated fatty acids in many other nuts, which are susceptible to oxidation leading to quality deterioration, walnut oils in the seed are stable against oxidation. Interestingly, the content of α-tocopherol, an antioxidant, is even lower in walnuts than in other nuts, such as almonds, hazelnuts, and peanuts (*3*). We then hypothesized that other antioxidants exist in walnuts that protect oils from oxidation. On subsequent investigation, we found indeed that the kernel pellicles of walnuts are rich in polyphenols, which are excellent antioxidants. In contrast, no polyphenol was detected in the seeds. Antioxidative activity-guided separation and purification led to the identification of 16 known hydrolyzable tannins (*4, 5*), three new ellagetannins (glansrins A, B, and C), two novel dicarboxylic acids (glansreginins A and B), and a new dimeric hydrolyzable tannin in the walnut kernel pellicles. These polyphenolic compounds are likely to protect the fatty acids from oxidation in walnuts. Besides, Colaric et al. determined poliphenolic compounds in the walnut kernel and thin skin separately and found that the pellicle is the major source of phenolics such as chlorogenic acid and ellagic acid (*6*). Recently, walnuts have attracted considerable attention for their pharmacological properties beneficial to lifestyle-related diseases such as arteriosclerosis (*7*), hypercholesterolemia (*3, 8, 9*), cardiovascular disease (*10, 11*), hypertriglyceridemia (*12, 13*), and diabetes mellitus (*14*). However, for walnut polyphenols of defined structures, only a few biological effects have been

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10.1021/jf8002174 CCC: $40.75
Published on Web 05/22/2008

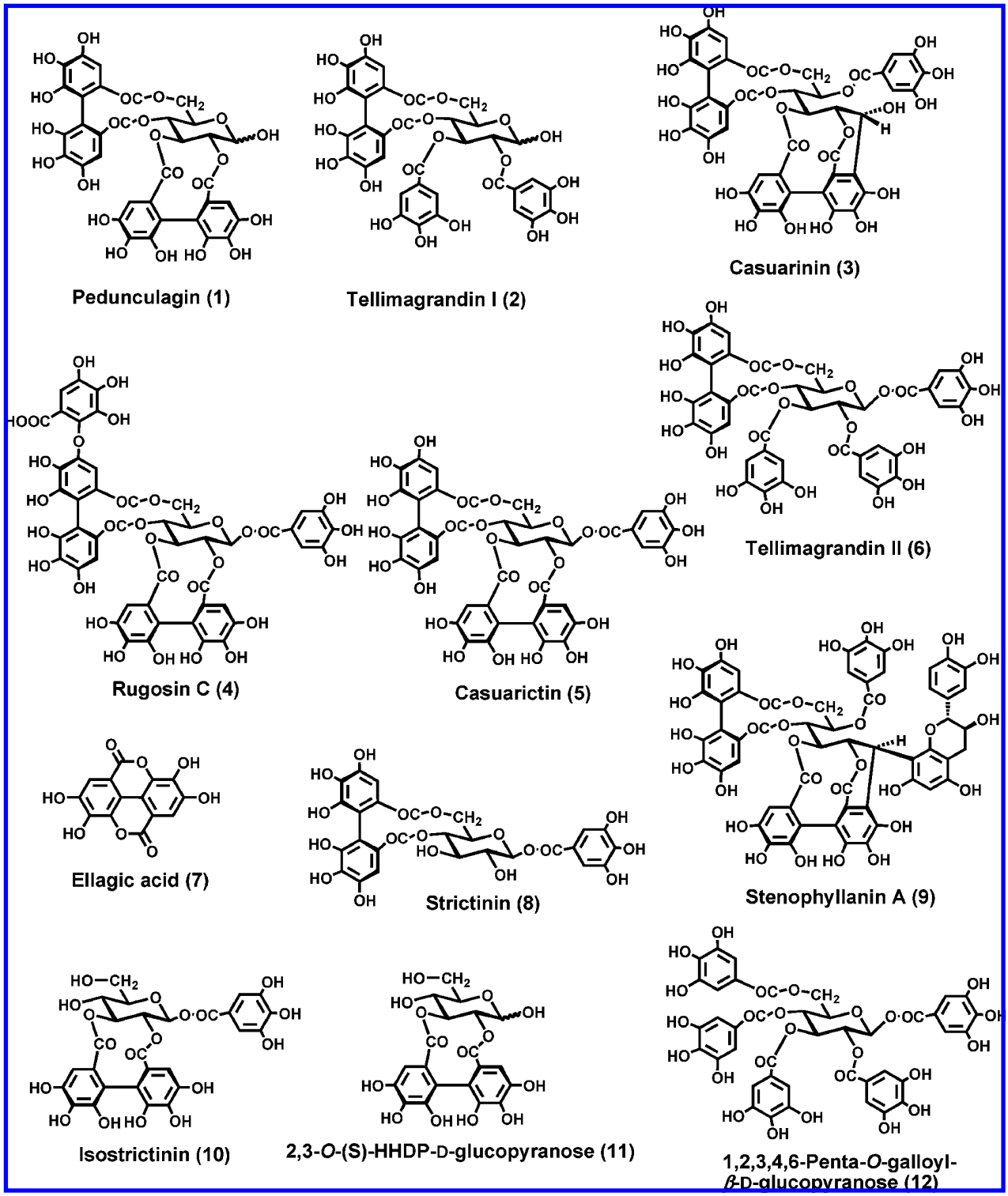

Figure 1. Polyphenolic compounds isolated from walnuts.

reported including antidiabetic (*15*), antioxidative (*16*), and low-density lipoprotein oxidation inhibitory activities (*17*). Hence in this study, we investigated the hepatoprotective effect of walnut polyphenols using an acute hepatitis model and identified the active principals using in vitro liver injury models.

MATERIALS AND METHODS

Reagents. Carbon tetrachloride (CCl_4), Transaminase CII Test Wako, D-galactosamine (GalN) hydrochloride, dimethylsulfoxide (DMSO), gallic acid, and curcumin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). William's E medium, fetal calf serum (FCS), penicillin and streptomycin mixture solution, and collagenase type I were obtained from Invitrogen Japan (Tokyo, Japan). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Dojindo Laboratories (Kumamoto, Japan).

Preparation of Walnut Polyphenol and Polyphenolic Constituents. Dried kernel pellicles (10 kg) of walnuts cultivated in China were powdered and extracted at 80 °C for 2 h with 50 L of 50% (v/v) ethanol. The solvent was subsequently evaporated. Yield of the spray-dried powder of walnut polyphenol extract (WP) was 10.5%, which contained 45% polyphenols as determined by the Folin-Ciocalteu method (ellagic acid equivalent). The contents of principal polyphenols were determined by HPLC equipped with the C30 column, 250 × 4.6 mm i.d. (Develosil

RPAQUEOUS, Nomura Chemical Co., Ltd., Aichi, Japan). The flow rate was fixed at 1 mL/min using the following eluents and linear gradient: solvent A (10 mM H_3PO_4:10 mM KH_2PO_4:CH_3CN = 45:45:10), solvent B (10 mM H_3PO_4:10 mM KH_2PO_4:CH_3CN = 30:30:40); 10 min at 100% A isocratic; from 0% B to 100% B in 30 min; 10 min at 100% B isocratic. The wavelength for UV detection was 280 nm, and each compound was identified by direct comparison with authentic samples. The contents of pedunculagin (**1**), tellimagrandin I (**2**), tellimagrandin II (**6**), and ellagic acid (**7**) in the WP extract were 5.8%, 2.8%, 1.4%, and 5.2%, respectively. Polyphenolic compounds (**Figure 1**) were isolated according to the previously reported method (*4*).

Animal Experiments. Male ddY mice (5 weeks old) and Wistar rats (8 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed in an air-conditioned room (23 ± 1 °C, 50 ± 10% R.H.) and fed a standard nonpurified diet (CE-2) (Clea Japan, Inc., Shizuoka, Japan) and tap water ad libitum. The experiments were performed in accordance with Japan Association for Laboratory Animal Science, 1987, Guidelines for Animal Experimentation.

CCl_4-induced liver injury was performed according to the method of Yoshikawa et al. (*18*). D-GalN-induced liver injury was performed according to a modified method of Matsuda et al. (*19*). WP suspended in water was given orally to 20-h fasted mice. One hour later, 5 mL/kg of 10% (v/v) CCl_4 diluted in olive oil or 350 mg/kg of D-GalN hydrochloride was subcutaneously injected into the mice. Blood samples were collected from the infraorbital venous plexus 20 h after CCl_4 and 22 h after D-GalN injection. Hepatic enzymes, glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in serum were determined by Transaminase CII Test Wako.

In Vitro Experiments. Hepatocytes were obtained from rats by a collagenase perfusion method. CCl_4- and D-GalN-induced cytotoxicity in primary cultured rat hepatocytes were performed according to the method of Yoshikawa et al. (*18*). One hundred microliters of hepatocyte suspension (4×10^4 cells) in William's E medium containing 10% FCS, penicillin (100 μg/mL), and streptomycin (100 μg/mL) was placed into each well of a 96-well culture plate and cultured for 4 h at 37 °C and 5% CO_2. The medium was replaced with 200 μL of a fresh one containing test reagents (dissolved in DMSO) and 5 mM CCl_4 or 10 mM D-GalN. The final DMSO concentration was adjusted to 1%. The cells were cultured for another 44 h. Ten microliters of MTT (5 mg/mL) solution in phosphate buffered saline was added, and the culture was continued for 4 h. The medium was removed, and 100 μL of isopropanol containing 0.04 M HCl was added. After dissolving the produced formazan, the absorbance at 570 nm was measured.

Statistics. The results are expressed as the means and SE. Significance of the differences was examined by one-way ANOVA followed by Dunnett's test. Differences with $p < 0.05$ were considered as significant.

RESULTS

Effect of WP on CCl_4- and D-GalN-Induced Liver Injury in Mice. In untreated mice, the hepatic enzymes, GOT and GPT in serum were 61 ± 6 and 19 ± 2 Karmen units, respectively (**Figure 2**). Intraperitoneal injection of CCl_4 induced intense serum GOT and GPT elevation (7391 ± 2340 and 4260 ± 1467 Karmen units, respectively) 20 h later. Oral treatment of WP (200 mg/kg) significantly ($p < 0.05$) suppressed serum GOT and GPT elevation by 63.7% and 62.1%, respectively. For comparison, curcumin (200 mg/kg) significantly ($p < 0.05$) suppressed GPT elevation by 45.5%. However, 22 h after the intraperitoneal injection of D-GalN, only slight serum GOT and GPT elevations (GOT: 114 ± 19, GPT: 38 ± 19 Karmen unit) were induced. In this model, neither WP (100 and 200 mg/kg) nor curcumin (200 mg/kg) suppressed the elevation of the two enzymes in serum.

Identification of Hepatoprotective Constituents in WP Using in Vitro Models. Incubation of rat hepatocytes with CCl_4 caused cell death after 44 h. In this model, WP (100 μg/mL) significantly ($p < 0.01$) suppressed cell damage by 42.1%

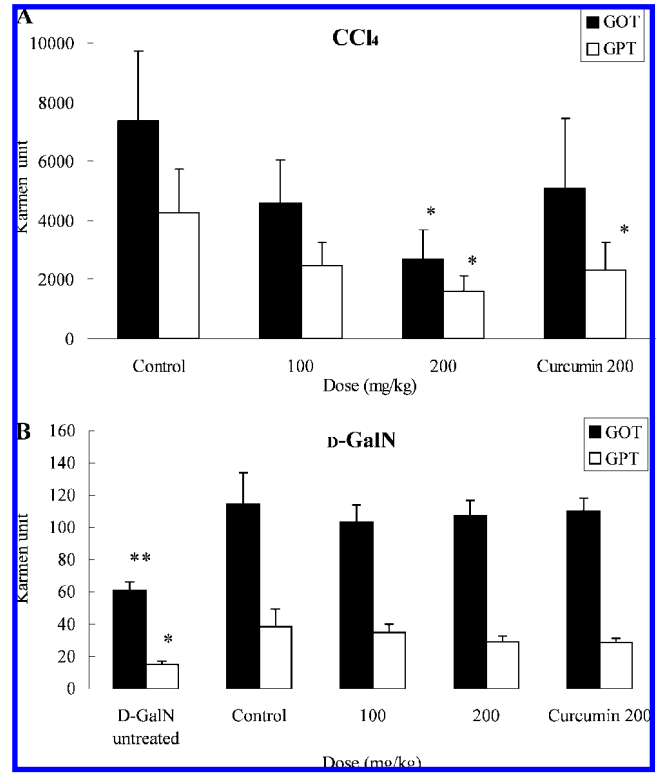

Figure 2. Effect of WP on (**A**) CCl_4- and (**B**) D-GalN-induced liver injury in mice. Each column represents the mean with the SE of 8 animals. An asterisk denotes significant difference from the control at *: $p < 0.05$. **: $p < 0.01$.

Table 1. Effects of WP and Constituents Isolated from Walnuts on Cell Damage in Rat Primary Cultured Hepatocytes Induced by CCl_4[a]

	inhibition (%)		
	10 (μg/mL)	30	100
WP	7.6 ± 0.2	13.2 ± 0.3	42.1 ± 1.8**
pedunculagin (**1**)	4.7 ± 0.2	3.4 ± 0.1	15.7 ± 0.7*
tellimagrandin I (**2**)	66.7 ± 9.4*	53.6 ± 2.8**	100.0 ± 6.7**
casuarinin (**3**)	3.1 ± 0.1	8.9 ± 0.2	12.0 ± 0.6
rugosin C (**4**)	15.8 ± 0.4	36.0 ± 1.4**	58.8 ± 0.7**
casuarictin (**5**)	12.6 ± 0.2	13.9 ± 0.8	48.3 ± 1.9**
tellimagrandin II (**6**)	35.5 ± 3.2	20.4 ± 1.2	54.8 ± 4.6**
ellagic acid (**7**)	−12.6 ± 0.5	−18.5 ± 1.3	–
strictinin (**8**)	7.3 ± 0.2	12.9 ± 0.9**	30.9 ± 0.6**
stenophyllanin A (**9**)	7.5 ± 0.2	27.2 ± 0.9**	37.8 ± 1.2**
isostrictinin (**10**)	5.1 ± 0.1	5.9 ± 0.3	24.0 ± 1.1**
2,3-HHDP-D-glucopyranose (**11**)	−10.6 ± 0.3	4.7 ± 0.3	4.1 ± 0.1
pentagalloyl glucose (**12**)	6.6 ± 0.4	22.8 ± 0.5**	27.1 ± 0.6**
gallic acid	19.5 ± 1.6	28.5 ± 1.5	21.5 ± 1.2
curcumin	8.5 ± 0.3	5.9 ± 0.1	–

[a] Each value represents the mean with the SE ($n = 6$). Asterisks denote significant differences from the control group at *: $p < 0.05$, **: $p < 0.01$. −, OD was not determined because of cell detachments due to cytotoxicity of the test sample.

(**Table 1**). We then fractionated and purified polyphenolic constituents and examined each of them for protective effect on CCl_4-induced hepatocyte damage. Among the compounds tested, tellimagranidin I (**2**) exhibited the most potent suppressing effect. Even at the lowest concentration tested (10 μg/mL), a significant suppression (66.7%) of cell damage was achieved, while 100 μg/mL tellimagranidin I (**2**) suppressed cell damage completely. Rugosin C (**4**), casuarictin (**5**), and tellimagrandin II (**6**) suppressed cell damage by approximately 50% at 100 μg/mL. Strictinin (**8**), stenophyllanin A (**9**), isostrictinin (**10**), and pentagalloylglucose (**12**) also showed weak suppressive

Table 2. Effects of WP and Constituents Isolated from WP on Cell Damage in Rat Primary Cultured Hepatocytes Induced by D-GalN[a]

	inhibition (%)		
	10 (μg/mL)	30	100
WP	34.0 ± 2.1*	92.5 ± 8.2**	104.0 ± 4.9**
pedunculagin (**1**)	19.0 ± 0.6	11.8 ± 0.8	6.5 ± 0.5
tellimagrandin I (**2**)	2.3 ± 0.1	42.5 ± 1.3*	100.0 ± 3.5**
casuarinin (**3**)	16.8 ± 0.5	9.3 ± 0.4	10.2 ± 0.7
rugosin C (**4**)	11.3 ± 0.5	2.8 ± 0.5	29.2 ± 2.8*
casuarictin (**5**)	0.0 ± 0.1	0.0 ± 0.1	86.7 ± 4.1**
tellimagrandin II (**6**)	4.4 ± 0.1	4.4 ± 0.3	80.2 ± 2.8**
ellagic acid (**7**)	14.3 ± 0.7	19.5 ± 0.5	16.5 ± 0.3
strictinin (**8**)	56.9 ± 5.7*	82.3 ± 5.9*	94.1 ± 2.8**
stenophyllanin A (**9**)	36.4 ± 2.4*	92.4 ± 4.9**	83.3 ± 3.8**
isostrictinin (**10**)	13.8 ± 1.0	42.5 ± 0.8*	80.0 ± 5.1**
2,3-HHDP-D-glucopyranose (**11**)	15.8 ± 0.8	92.0 ± 4.0**	121.0 ± 6.2**
pentagalloyl glucose (**12**)	−16.0 ± 1.9	37.0 ± 1.8*	80.2 ± 8.6**
gallic acid	60.5 ± 4.3**	63.6 ± 2.3**	58.2 ± 4.7**
curcumin	0.0 ± 0.1	33.3 ± 1.2*	47.7 ± 2.0*

[a] Each value represents the mean with the SE ($n = 6$). Asterisks denote significant differences from the control group at *: $p < 0.05$, **: $p < 0.01$.

activity at 100 μg/mL. However, pedunculagin (**1**), casuarinin (**3**), ellagic acid (**7**), 2,3-hexahydroxydiphenoyl (HHDP)-D-glucopyranose (**11**), and gallic acid did not show suppressive activity.

In the in vitro D-GalN model, WP significantly suppressed cell damage at 10 to 100 μg/mL in a concentration-dependent manner (**Table 2**). Tellimagrandin I (**2**), **5**, **6**, **8**, **9**, **10**, **11**, and **12** significantly suppressed cell damage at 100 μg/mL by more than 80%. Rugosin C (**4**) at 100 μg/mL suppressed cell damage slightly. Pedunculagin (**1**), **3**, and **7** did not suppress cell damage in this model.

Only ellagic acid was cytotoxic for the rat hepatocyte primary culture at the highest concentration tested (100 μg/mL). None of the other polyphenolic compounds of WP exhibited cytotoxicity at the highest concentration tested (100 μg/mL).

DISCUSSION

In this study, WP was found to suppress CCl_4-induced liver injury in mice when orally administrated. The suppressive effect of WP at 200 mg/kg was nearly comparable to that of curcumin, which has been previously reported to be effective in this model (*20, 21*). Several ellagitannins of pomegranate such as methyl (*S*)-flavogallonate (*22*), punicalagin (*22, 23*), and punicalin (*23*) have been reported to be hepatoprotective in a CCl_4-induced rat liver injury model. WP contains ellagitannins (**Figure 1**), and thus, the hepatoprotective activity in the same model found in this study is in concordance with the previous findings. Peroxidation of hepatic lipids, production of proinflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6], and activation of nuclear factor (NF)-κB are involved in this induction of liver injury (*20*). WP may protect hepatocytes by inhibiting these injury-induction-related events.

However, WP did not suppress liver injury induced by D-GalN in vivo. Usually, D-GalN is injected intraperitoneally in combination with lipopolysaccharide to induce liver injury in mice and rats (*19, 24, 25*), where D-GalN is thought to enhance the sensitivity of the cells to LPS. In our initial experiments, a combination of D-GalN and LPS was used to induce liver injury in mice, which led to serum GOT and GPT elevation to 942 and 847 Kalmen units, respectively. However, several mice died due to endotoxin shock. WP (100−500 mg/kg) did not suppress this liver injury. Hence, to exclude the interference of exogenous endotoxin, we refrained from using LPS in the mouse model. Consequently, the injection of D-GalN alone led to only a slight increase in serum GOT and GPT. In this model, D-GalN reacts with uridine 5′-diphosphate to form uridine 5′-diphosphate galactosamine, which reduces intracellular uridine 5′-triphosphate in liver (*26*). Accordingly, the synthesis of RNA and protein is blocked, and a disorder of sugar metabolism occurs, which finally leads to hepatocyte membrane damage. Also the activation of Kupffer cells by endotoxin derived from the gut is thought to induce liver parenchymal damage. Oral administration of WP (200 mg/kg) did not suppress liver injury in this model, implying that WP did not affect the action of D-GalN leading to liver failure and sensitization of Kupffer cells to endotoxin. Similarly, curcumin did not suppress liver injury induced by D-GalN in this study. However, Morikawa et al. (*27*) reported that curcumin significantly suppressed serum GOT and GPT elevation induced by D-GalN and LPS. The different effect of curcumin in D-GalN and D-GalN/LPS models may be explained by this compound suppressing only the action of LPS. Chen and Zheng (*28*) found that curcumin suppressed gene expression of connective tissue growth factor in hepatic stellate cells, leading to hepatic fibrosis. The suppression was reversed by LPS, an activator of NF-κB. Thus, curcumin likely suppresses LPS-induced NF-κB activation, leading to hepatocyte damage. Moreover, curcumin was reported to suppress LPS-induced elevation of several hepatic enzymes including GOT and GPT in serum (*29*). These findings suggest that curcumin suppresses only the action of LPS but not that of D-GalN.

In order to identify the active principal constituents, we examined the activity of WP and constituents in suppressing hepatocyte damage induced by CCl_4 in vitro. We aimed to find candidate compounds that can be further studied for their hepatoprotective activity in vivo. After confirming the hepatoprotective activity of WP in vitro, each of the constituent polyphenols was then evaluated. Pedunculagin (**1**) and ellagic acid (**7**), the major constituents of WP, were nearly inactive. Tellimagrandin I (**2**), the third major constituent of WP, was most potent in suppressing cell damage and thus is the principal compound for the hepatoprotective activity of WP in the in vitro CCl_4 model. Previously, Hikino et al. (*30*) examined the antiheptatotoxic activity of a number of tannins including **1**−**7**, **11**, **12**, and gallic acid on rat hepatocyte damage induced by CCl_4. The inhibitory activity of **1**, **4**, **6**, and **7** on CCl_4-induced hepatocyte damage was similar to that in our study. However, **2** suppressed only 19% of cell damage in the study of Hikino et al., while a 100% suppression was found in our study. In addition, activities of **3**, **5**, **12**, and gallic acid reported in the two studies differ by 20 to 35%. Furthermore, Hikino et al. reported that the antihepatotoxic activity of hydrolyzable tannins tended to depend on the number of galloyl groups. However, comparing the inhibitory potential and the number of galloyl groups of **2**, **5**, **6**, and **12** (**Table 1**) in our study did not provide supporting evidence for this hypothesis. The differences in results of the two studies may be in part explained by different experimental conditions. In the study of Hikino et al., the cells were treated with CCl_4 and each test sample for only 90 min, and cytotoxicity was evaluated by deviated GPT. In contrast, we treated the hepatocytes with CCl_4 and each test sample for 44 h and used the MTT assay for evaluation of cytotoxicity. Hikino et al. further excluded a clear structure−activity correlation among hydrolyzable tannins. However, we found evidence suggesting that in some compounds, the hepatoprotective activity is related to the structure. For example, **1** and **2**

have very similar structure with the only difference that **1** has two HHDP groups, while in **2**, one HHDP group was replaced by two galloyl groups. Interestingly, while **1** was inactive, **2** was the most potent constituent in protecting hepatocytes from damage, indicating that a galloyl group is essential for hepatoprotective activity. Tellimagrandin II (**6**) with an additional galloyl group at the anomeric center of **2** retained activity. Casuarinin (**3**) with a ring-opened glucose and gallic acid itself did not have the activity. However, **9** with the same structure except for a catechin bond to the position **1** of the ring-opened glucose had the activity. These results suggest that the galloylated glucopyranose core is essential for the expression of hepatoprotective activity. In fact, ellagitannins **4**, **5**, **8**, **10**, and **12**, which are congeners of the galloylatyed glucopyranose, all exhibited hepatoprotective activity. In contrast, 2,3-HHDP-D-glucopyranose (**11**) lacking a galloyl group did not show this activity.

We examined possible correlations between the hepatoprotective activity and antioxidative activity of WP constituents. Fukuda et al. (*4*) reported antioxidative activity in tellimagrandin I (**2**), **3**−**6**, and **9** with IC50 values of less than 1 μg/mL. However, while hepatoprotective activities were found in **2**, **4**−**6**, and **9**, casuarinin (**3**) was inactive even at 100 μg/mL. In addition, **7** and **11** were antioxidative with IC50 values of 1.2 and 2.1 μg/mL, respectively, while being ineffective against hepatocyte damage at 100 μg/mL. Hence, the hepatoprotective activity of hydrolyzable tannins in WP is not necessarily correlated with their antioxidative activity. The action mechanism of some of these compounds in CCl_4-induced hepatocyte damage may be different from that of scavenging free radical stress. In conclusion, tellimagrandin I (**2**) was the most potent hepatoprotective constituents among hydrolyzable tannins in WP and should be further investigated in vivo.

WP did not suppress D-GalN-induced liver injury in vivo. However, WP significantly suppressed D-GalN-induced hepatocyte damage in vitro. This discrepancy may be due to different effect of original polyphenols in vitro and that of their metabolites in vivo. Further studies revealing a more detailed mechanism of metabolism of polyphenols may help to clarify the difference between these in vitro and in vivo observations. In addition, different parameters were measured for the evaluation of hepatocyte damage in vivo and in vitro. Elevation of serum GOT and GPT in vivo and cytotoxicity in vitro may reflect different aspects of hepatocyte damage. To identify hepatoprotective constituents in WP, we evaluated the effect of each of the isolated polyphenols on D-GalN induced hepatocyte damage. At 100 μg/mL, most of the ellagitannins and gallic acid except **1**, **3**, **4**, and **7** exhibited even more potent hepatoprotective effect than the positive control curcumin. Unlike for CCl_4-induced cell damage, 2,3-HHDP-D-glucopyranose (**11**), with a relatively simple structure, showed the strongest suppressive activity at 100 μg/mL. Tellimagrandin I (**2**) also significantly suppressed cell damage at 30 and 100 μg/mL. Although a definite structure-activity correlation could not yet be concluded, tannins with a 2,3-HHDP-glucose moiety likely have significant protective effect against D-GalN-induced hepatocyte damage. In contrast, the addition of an HHDP or equivalent (valoneoyl) group at *O*-4/*O*-6 of the glucose residue seems to diminish the hepatoprotectivity of the compounds in this *in vitro* model.

Hepatoprotective activity of several hydrolyzable tannins in D-GalN-induced damage of rat primary cultured cells was also reported by Hikino et al. (*30*). However, their results were profoundly different from ours. In their study, **2**, **5**, **6**, **12**, and gallic acid suppressed less than 30% of hepatocyte damage at 100 μg/mL, whereas ellagic acid (*7*) suppressed 56% of the damage at the same concentration. Hikino et al. used enzyme inhibition of GPT, while we used suppression of cytotoxicity as the measure for the hepatoprotective activity of the compounds, which may account for the difference in the results.

In conclusion, we found the hepatoprotective activity of WP against CCl_4-induced liver injury in mice. Further in vitro studies revealed that polyphenolic constituents having both HHDP and galloyl groups on the glucopyranose core were effective in protecting hepatocytes from CCl_4-induced damage. In addition, 2,3-HHDP glucose (**11**) and tellimagrandin I (**2**) suppressed hepatocyte damage induced by D-GalN in vitro. These compounds are promising candidates as potent hepatoprotective reagents for further in vivo studies.

LITERATURE CITED

(1) Mukuddem-Petersen, J.; Oosthuizen, W.; Jerling, J. C. A systematic review of the effects of nuts on blood lipid profiles in humans. *J. Nutr.* **2005**, *135*, 2082–2089.

(2) Maguire, L. S.; O'Sullivan, S. M.; Galvin, K.; O'Connor, T. P.; O'Brien, N. M. Fatty acid profile, tocopherol, squalene and phytosterol content of walnuts, almonds, peanuts, hazelnuts and the macadamia nut. *Int. J. Food Sci. Nutr.* **2004**, *55*, 171–178.

(3) Amaral, J. S.; Alves, M. R.; Seabra, R. M.; Oliveira, B. P. Vitamin E composition of walnuts (*Juglans regia* L.): a 3-year comparative study of different cultivars. *J. Agric. Food Chem.* **2005**, *53*, 5467–5472.

(4) Fukuda, T.; Ito, H.; Yoshida, T. Antioxidative polyphenols from walnuts (*Juglans regia* L.). *Phytochemistry* **2003**, *63*, 795–801.

(5) Ito, H.; Okuda, T.; Fukuda, T.; Hatano, T.; Yoshida, T. Two novel dicarboxylic acid derivatives and a new dimeric hydrolyzable tannin from walnuts. *J. Agric. Food Chem.* **2007**, *55*, 672–679.

(6) Colaric, M.; Veberic, R.; Solar, A.; Hudina, M.; Stampar, F. Phenolic acids, syringaldehyde, and juglone in fruits of different cultivars of *Juglans regia* L. *J. Agric. Food Chem.* **2005**, *53*, 6390–6396.

(7) Ros, E.; Núñez, I.; Pérez-Heras, A.; Serra, M.; Gilabert, R.; Casals, E.; Deulofeu, R. A walnut diet improves endothelial function in hypercholesterolemic subjects. *Circulation* **2004**, *109*, 1609–1614.

(8) Tapsell, L. C.; Owen, A.; Gillen, L. J.; Baré, M.; Pach, C. S.; Kennedy, M.; Batterham, M. Including walnuts in a low-fat/modified-fat diet improves HDL cholesterol-to-total cholesterol ratios in patients with type 2 diabetes. *Diabetes Care* **2004**, *27*, 2777–2783.

(9) Iwamoto, M.; Sato, M.; Kono, M.; Hirooka, Y.; Sakai, K.; Takeshita, A.; Imaizumi, K. Walnuts lower serum cholesterol in Japanese men and women. *J. Nutr.* **2000**, *130*, 171–176.

(10) Feldman, E. B. The scientific evidence for a beneficial health relationship between walnuts and coronary heart disease. *J. Nutr.* **2002**, *132*, 1062S–1101S..

(11) Morgan, J. M.; Horton, K.; Reese, D.; Carey, C.; Walker, K.; Capuzzi, D. M. Effects of walnut consumption as part of a low-fat, low-cholesterol diet on serum cardiovascular risk factors. *Int. J. Vitam. Nutr. Res.* **2002**, *72*, 341–347.

(12) Zibaeenezhad, M. J.; Rezaiezadeh, M.; Mowla, A.; Ayatollahi, S. M. T.; Panjehshahin, M. R. Antihypertriglyceridemic effect of walnut oil. *Angiology* **2003**, *54*, 411–414.

(13) Zibaeenezhad, M. J.; Shamsnia, S. J.; Khorasani, M. Walnut consumption in hyperlipidemic patients. *Angiology* **2005**, *56*, 581–583.

(14) Gillen, L. J.; Tapsell, L. C.; Pach, C. S.; Owen, A.; Batterham, M. Structured dietary advice incorporating walnuts achieves optimal fat and energy balance in patients with type 2 diabetes mellitus. *J. Am. Diet. Assoc.* **2005**, *105*, 1087–1096.

(15) Fukuda, T.; Ito, H.; Yoshida, T. Effect of the walnut polyphenol fraction on oxidative stress in type 2 diabetes mice. *BioFactors* **2004**, *21*, 251–253.

(16) Li, L.; Tsao, R.; Yang, R.; Liu, C.; Zhu, H.; Young, J. C. Polyphenolic profiles and antioxidant activities of heartnut (*Juglans ailanthifolia* var. *cordiformis*) and Persian walnut (*Juglans regia* L.). *J. Agric. Food Chem.* **2006**, *54*, 8033–8040.
(17) Anderson, K. J.; Teuber, S. S.; Gobeille, A.; Cremin, P.; Waterhouse, A. L.; Steinberg, F. M. Walnut polyphenolics inhibit *in vitro* human plasma and LDL oxidation. *J. Nutr.* **2001**, *131*, 2837–2842.
(18) Yohikawa, M.; Ninomiya, K.; Shimoda, H.; Nishida, N.; Matsuda, H. Hepatoprotective and antioxidative properties of *Salacia reticulata*: preventive effects of phenolic constituent on CCl_4-induced liver injury in mice. *Biol. Pharm. Bull.* **2002**, *25*, 72–76.
(19) Matsuda, H.; Ninomiya, K.; Shimoda, H.; Yoshikawa, M. Hepatoprotective principles from the flowers of *Tilia argentea* (Linden): structure requirements of tiliroside and mechanisms of action. *Bioorg. Med. Chem.* **2002**, *10*, 707–712.
(20) Reyes-Gordillo, K.; Segovia, J.; Shibayama, M.; Vergara, P.; Moreno, M. G.; Muriel, P. Curcumin protects against acute liver damage in the rat by inhibiting NF-κB, proinflammatory cytokines production and oxidative stress. *Biochim. Biophys. Acta* **2007**, *1770*, 989–996.
(21) Liu, Y. G.; Chen, H. C.; Jiang, Y. P. Protective effect of curcumin on experimental liver injury in mice. *Zhongguo Zhong Yao Za Zhi* **2003**, *28*, 756–758.
(22) Marzouk, M. S.; El-Toumy, S. A.; Moharram, F. A.; Shalaby, N. M.; Ahmed, A. A. Pharmacologically active ellagitannins from *Terminalia myriocarpa*. *Planta Med.* **2002**, *68*, 523–527.
(23) Lin, C. C.; Hsu, Y. F.; Lin, T. C.; Hsu, F. L.; Hsu, H. Y. Antioxidant and hepatoprotective activity of punicalagin and punicalin on carbon tetrachloride-induced liver damage in rats. *J. Pharm. Pharmacol.* **1998**, *50*, 789–794.
(24) Lehmann, V.; Freudenberg, M. A.; Galanos, C. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. *J. Exp. Med.* **1987**, *165*, 657–663.
(25) Freudenberg, M. A.; Keppler, D.; Galanos, C. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. *Infect. Immun.* **1986**, *51*, 891–895.
(26) Nakagiri, R.; Oda, H.; Kamiya, T. Small scale rat hepatocyte primary culture with applications for screening hepatoprotective substances. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 1629–1635.
(27) Morikawa, T.; Matsuda, H.; Ninomiya, K.; Yoshikawa, M. Medicinal food stuffs. XXIX. Potent protective effects of sesquiterpenes and curcumin from Zedoariae Rhizoma on liver injury induced by D-galactosamine/lipopolysaccharide or tumor necrosis factor-α. *Biol. Pharm. Bull.* **2002**, *25*, 627–631.
(28) Chen, A.; Zheng, S. Curcumin inhibits connective tissue growth factor gene expression in activated hepatic stellate cells *in vitro* by blocking NF-κB and ERK signaling. *Br. J. Pharmacol.* **2008**, *153*, 557–567.
(29) Kaur, G.; Tirkey, N.; Bharrhan, S.; Chanana, V.; Rishi, P.; Chopra, K. Inhibition of oxidative stress and cytokine activity by curcumin in amelioration of endotoxin-induced experimental hepatotoxicity in rodents. *Clin. Exp. Immunol.* **2006**, *145*, 313–321.
(30) Hikino, H.; Kiso, Y.; Hatano, T.; Yoshida, T.; Okuda, T. Antihepatotoxic actions of tannins. *J. Ethnopharmacol.* **1985**, *14*, 19–29.

Received for review January 22, 2008. Revised manuscript received April 4, 2008. Accepted April 4, 2008.

JF8002174