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Hepatoprotective Principles from the Flowers of *Tilia argentea* (Linden): Structure Requirements of Tiliroside and Mechanisms of Action†

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Abstract—The methanolic extract from the flowers of *Tilia argentea* (linden) was found to show a hepatoprotective effect against D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced liver injury in mice. By bioassay-guided separation using in vitro D-GalN-induced damage to hepatocytes, five flavonol glycosides were isolated as the hepatoprotective constituents of the methanolic extract. Tiliroside, the principal flavonol glycoside, strongly inhibited serum GPT and GOT elevations at doses of 25–100 mg/kg (p.o.) in D-GalN/LPS-treated mice. By comparing the inhibitory effects of tiliroside with those of its components alone, the kaempferol 3-*O*-β-D-glucopyranoside moiety was found to be essential for the activity, and its effect was suggested to depend on the inhibition of tumor necrosis factor-α (TNF-α) production, decreased sensitivity of hepatocytes to TNF-α, and on the protection of hepatocytes against D-GalN.

The Tiliaceae plant *Tilia argentea* (synonym: *T. tomentosa*; common names: linden or lime), a well-known Occidental herb, is cultivated in Bulgaria and Albania. The dried flowers of this plant, which are commonly called 'silver linden flowers', have been widely used in herbal teas, and as a diuretic, stomachic, antineuralgic, and sedative in European countries.

In our continuing studies on hepatoprotective compounds from natural medicines,[2] we found that the methanolic extract from the flowers of *T. argentea* showed potent protective effects against D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced liver injury in mice and on D-GalN cytotoxicity in primary-cultured mouse hepatocytes. By bioassay-guided separation, six flavonol glycosides (**1**–**6**) were isolated from the methanolic extract. Among them, tiliroside (**1**), the principal flavonol glycoside, showed the most potent activity against D-GalN-induced cytotoxicity in hepatocytes. Oral administration of tiliroside (**1**) also exerted the protective effects on D-GalN/LPS-induced liver injury in mice. In this report, we describe the hepatoprotective effects of flavonol glycosides (**1**–**6**) from *T. argentea* in vitro and in vivo experiments. In addition, several structural requirements and mechanisms of action of tiliroside (**1**) for the hepatoprotective effects are discussed (Chart 1).

Results and Discussion

The dried flowers of *T. argentea* cultivated in Bulgaria were extracted with methanol under reflux. The methanolic extract (yield: 14.3% from the natural medicine) was partitioned into an ethyl acetate–water mixture to give an ethyl acetate-soluble fraction (3.6%) and water soluble fraction (10.7%). As shown in Table 1, the methanolic extract (250–1000 mg/kg, p.o.) was found to show inhibitory effects on the increase in serum GPT and GOT levels induced by D-GalN/LPS in mice. However, this extract did not inhibit liver injury induced by CCl_4 at high doses (500 and 1000 mg/kg, p.o.), which suggests that the methanolic extract did not prevent injury by $\cdot CCl_3$ and reactive oxidants that cause lipid peroxidation in cell membranes.

To clarify the active constituents in the methanolic extract, a bioassay-guided separation was performed using D-GalN cytotoxicity in primary-cultured mouse hepatocytes. The ethyl acetate-soluble fraction showed

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significant inhibition of D-GalN cytotoxicity in the hepatocytes, but the water-soluble fraction did not. Next, the ethyl acetate-soluble fraction was subjected to silica gel column chromatography to give 11 fractions (fr. 1–fr. 11). The active fractions 9 and 10, which showed strong inhibition against the D-GalN cytotoxicity at 100 μg/mL, were subjected to ODS column chromatography and finally HPLC to give six flavonol glycosides: Tiliroside (**1**,[3] yield 0.080%), kaempferol 3-*O*-β-D-glucopyranoside (**2**,[3] 0.0068%), quercetin 3-*O*-β-D-glucopyranoside (**3**,[4] 0.019%), kaempferol 3-*O*-α-L-rhamnopyranoside (**4**,[5] 0.013%), quercetin 3-*O*-α-L-rhamnopyranoside (**5**,[5] 0.0083%), and kaempferol 3,7-di-*O*-α-L-rhamnopyranoside (**6**,[6] 0.0068%). The effects of compounds **1**–**6** from *T. argentea* and related compounds [kaempferol (**7**), *p*-coumaric acid (**8**)] on D-GalN cytotoxicity were examined using primary-cultured mouse hepatocytes. Tiliroside (**1**), kaempferol 3-*O*-β-D-glucopyranoside (**2**), and quercetin 3-*O*-β-D-glucopyranoside (**3**) showed strong activity. However, glycosides (**4**, **5**, **6**) having an α-L-rhamnopyranosyl moiety at the 3-position and the components (**7**, **8**) of **1** showed weak activity (Table 2).

Next, we examined the effects of the principal constituent, tiliroside (**1**), on D-GalN/LPS-induced liver injury in mice. Compound **1** significantly suppressed serum GPT and GOT elevations at doses of 25–100 mg/kg as shown

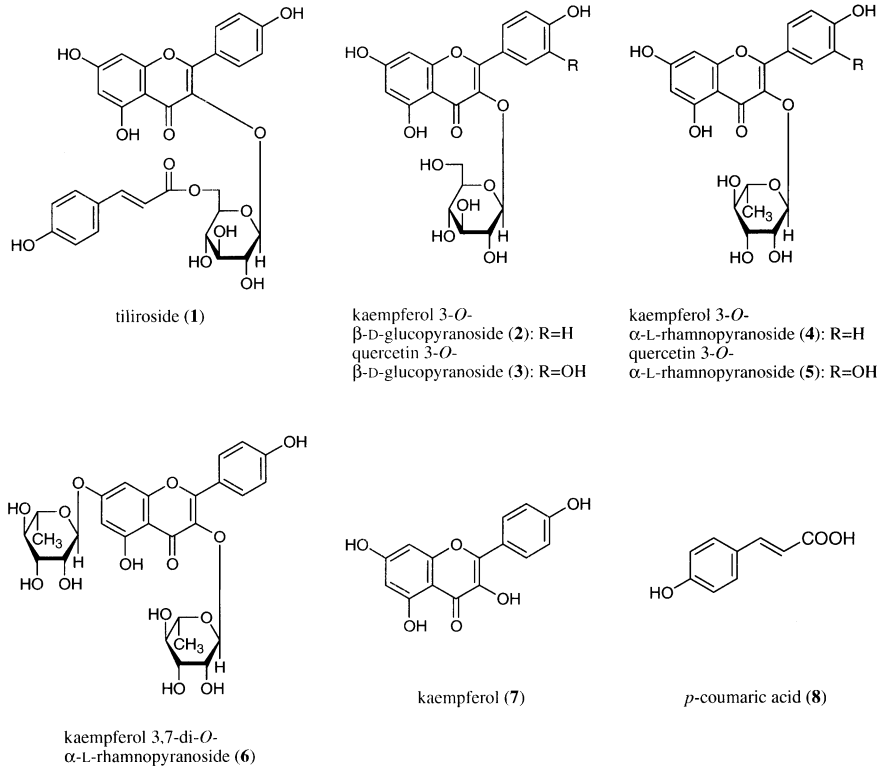

Chart 1.

Table 1. Inhibitory effects of the methanolic extract (MeOH ext.) from *T. argentea* on liver injury induced by D-GalN/LPS or CCl_4

	Dose (mg/kg, p.o.)	*n*	Serum GPT (Karmen unit)	Serum GOT (Karmen unit)	Mortality
(D-GalN/LPS-induced liver injury)					
Untreated control	—	5	20±1**	87±3**	0/5
Control	—	8	10,019±2221	8005±1635	4/12
MeOH ext.	250	7	6633±2219	4148±1022	1/8
	500	8	2893±725	2961±655	0/8
	1000	8	2498±836*	1412±830**	0/8
Hydrocortisone	20	8	311±14**	486±21**	0/8
(CCl_4-induced liver injury)					
Untreated control	—	5	19±1**	81±4**	0/5
Control	—	10	9825±1299	9571±1122	0/10
MeOH ext.	500	10	11,445±1021	10,994±805	0/10
	1000	10	10,479±916	9676±525	0/10
Malotilate	200	10	2652±922**	2159±358**	0/10

Male ddY mice were fasted for 20 h, and samples suspended in 5% acacia were given orally. One hour later, a mixture of D-GalN (350 mg/kg) and LPS (10 μg/kg) or 10% (v/v) CCl_4 in olive oil (5 mL/kg) was injected into mice (D-GalN/LPS: i.p., CCl_4: s.c.). Blood samples were collected 10 h (D-GalN/LPS) or 20 h (CCl_4) after injection.

Each value represents the mean±SEM. Asterisks denote significant differences from the control at $*p<0.05$, $**p<0.01$.

in Table 3. Since compound **1** is composed of kaempferol (**7**), *p*-coumaric acid (**8**), and D-glucose, we compared it with the effects of kaempferol 3-*O*-β-D-glucopyranoside (**2**), **7**, and **8** to clarify the essential moiety for the activity of **1**. Thus, kaempferol 3-*O*-β-D-glucopyranoside (**2**) significantly suppressed serum GPT and GOT elevations at doses of 50 and 100 mg/kg, but its effect was almost half that of **1** (ED_{50} for serum GPT, **1**: 24 mg/kg, **2**: 43 mg/kg). On the other hand, kaempferol (**7**) showed weak inhibition, and *p*-coumaric acid (**8**) lacked any significant effect on liver injury at a dose of 100 mg/kg. These in vivo findings were similar to those of the in vitro experiments described above. On the basis of this evidence, the kaempferol 3-*O*-β-D-glucopyranoside moiety in **1** was found to be essential for the activity, and the *p*-coumaric acid moiety was required to enhance the activity of **2**.

D-GalN/LPS-induced liver injury is recognized to develop from immunological responses.[7] This type of liver injury occurs in two ways: First, depletion of uridine triphosphate and increased sensitivity of hepatocytes to TNF-α induced by D-GalN; Second, release of pro-inflammatory mediators, such as TNF-α, from LPS-activated macrophages (Kupffer's cells). Apoptosis of hepatocytes induced by TNF-α is reported to be important in D-GalN/LPS-induced liver injury.[8]

To clarify the effects of tiliroside (**1**) and its components (**2**, **7**, **8**) on the sensitivity of hepatocytes to TNF-α, the effects of **1**, **2**, **7**, and **8** on the TNF-α-induced decrease in cell viability of L929 cells, a TNF-α-sensitive cell line,[9] were examined by MTT assay. As shown in Table 4, tiliroside (**1**) at a high concentration (100 μM) strongly inhibited the decrease in viability induced by TNF-α,

Table 2. Inhibitory effects of MeOH extract, AcOEt and H_2O fractions, and flavonol glycosides (**1**–**6**) from *T. argentea* on D-GalN-induced cytotoxicity in primary-cultured mouse hepatocytes

	Inhibition (%)					
	0	3	10	30	100 (μg/mL)	
MeOH extract	0.0±0.5	3.1±0.4	6.3±0.5**	10.6±0.4**	30.1±1.8**	
H_2O fraction	0.0±0.8	1.1±0.6	1.3±0.4	2.8±0.8	5.5±0.9	
AcOEt fraction	0.0±1.0	3.0±0.5	6.5±0.6**	16.3±0.9**	35.0±0.6**	
Fr. 9 from AcOEt fraction	0.0±0.7	8.9±0.8*	20.9±3.1**	54.8±3.1**	101.8±1.8**	
Fr. 10 from AcOEt fraction	0.0±0.9	7.3±1.2	16.1±1.9**	32.4±1.8**	93.4±3.8**	
	0	3	10	30	100	IC_{50} (μM)
Tiliroside (**1**)	0.0±2.5	13.5±3.3**	33.0±1.2**	67.4±3.5**	106.0±2.2**	14
Kaempferol 3-*O*-β-D-glucopyranoside (**2**)	0.0±1.1	4.0±0.8	24.6±2.0**	72.4±3.5**	98.7±2.7**	17
Quercetin 3-*O*-β-D-glucopyranoside (**3**)	0.0±1.1	8.1±1.6*	29.7±1.8**	80.5±3.7**	97.6±1.6**	15
Kaempferol 3-*O*-α-L-rhamnopyranoside (**4**)	0.0±0.7	1.8±1.2	5.0±1.7	16.8±1.4**	68.9±3.4**	63
Quercetin 3-*O*-α-L-rhamnopyranoside (**5**)	0.0±1.6	7.6±1.8	14.9±1.3**	32.1±1.1**	62.7±0.8	66
Kaempferol 3, 7-di-*O*-α-L-rhamnopyranoside (**6**)	0.0±1.9	4.0±1.9	2.1±2.2	8.2±1.3*	38.1±2.4**	>100
Kaempferol (**7**)	0.0±1.0	0.1±1.3	6.8±1.6*	10.3±2.1**	15.2±1.2**	>100
p-Coumaric acid (**8**)	0.0±0.6	1.6±0.6	1.2±0.7	2.2±0.3	4.3±0.4**	>100

Mouse hepatocytes (4×10^4 cells/well) in William's E medium supplemented with 10% FCS were seeded in a 96-well microplate and cultured for 4 h. Medium was replaced with the fresh medium containing 1 mM D-GalN and a test sample, and the hepatocytes were cultured for 44 h. Viability was assessed by MTT assay. Each value represents the mean±SEM ($n=4$). Asterisks denote significant differences from the control at $^*p<0.05$, $^{**}p<0.01$.

Table 3. Inhibitory effects of tiliroside (**1**) and related compounds (**2**, **7**, **8**) on liver injury induced by D-GalN/LPS

	Dose (mg/kg, p.o.)	*n*	s-GPT (Karmen unit)	s-GOT (Karmen unit)	Mortality
Control	—	15	9657±1728	7633±1366	5/20
Tiliroside (**1**)	12.5	9	6761±2041	5031±1388	0/9
	25	9	4242±1412*	3442±825*	0/9
	50	9	3196±1047*	2709±823*	0/9
	100	9	568±115**	637±80**	0/9
Kaempferol 3-*O*-β-D-glucopyranoside (**2**)	12.5	6	7490±2175	6148±1781	3/9
	25	9	7636±2134	6020±1679	0/9
	50	9	3886±1241*	3332±685*	0/9
	100	9	1629±589**	1103±298**	0/9
Control	—	10	10128±939	7474±682	3/13
Kaempferol (**7**)	50	9	7501±1494	5867±690	0/9
	100	7	5810±939*	4660±569*	2/9
p-Coumaric acid (**8**)	50	8	6934±774	6328±670	1/9
	100	8	6570±918	6175±726	1/9

Each value represents the mean±SEM. Asterisks denote significant differences from the control at $^*p<0.05$, $^{**}p<0.01$.

Table 4. Inhibitory effects of tiliroside (**1**) and related compounds (**2**, **7**, **8**) on the decrease in viability of L929 cells induced by TNF-α

	Inhibition (%)				
	0	3	10	30	100 (μM)
Tiliroside (**1**)	0±1	11±4	12±4	18±3**	44±2**
Kaempferol 3-*O*-β-D-glucopyranoside (**2**)	0±4	10±2	10±3	11±4	19±3**
Kaempferol (**7**)	0±2	10±2*	13±2*	17±3**	11±2*
p-Coumaric acid (**8**)	0±3	8±3	18±5**	18±4**	8±2

L929 cells (10^4 cells/well/100 μL) were cultured in medium with 10 ng/mL TNF-α. After incubation for 20 h, viability of the cells was assessed by MTT assay. Each value represents the mean±SEM ($n=4$). Asterisks denote significant differences from the control at $*p<0.05$, $**p<0.01$.

but **2**, **7**, and **8** showed weak activity. This finding suggests that the reduction in TNF-α sensitivity is partly involved in the protective effect of **1**.

Finally, the effects of **1** and **2** on increases in serum TNF-α levels induced by D-GalN/LPS in mice were examined. As shown in Table 5, **1** and **2** (12.5 to 100 mg/kg, p.o.) significantly suppressed increase in serum TNF-α in mice. In addition, in an in vitro experiment, mouse peritoneal macrophages were cultured with 10 μg/mL LPS for 4 h, and the TNF-α concentration in the medium was determined. TNF-α concentration in the medium without test sample (control group) was 308±8.4 pg/mL, and that without LPS (unstimulated group) was 0.5±0.8 pg/mL ($n=8$). Compounds **1** and **2** thus strongly inhibited TNF-α levels in the medium (Table 6). These findings indicate that the inhibition of TNF-α production is important in the protective effects of **1** and **2**.

Table 5. Inhibitory effects of tiliroside (**1**) and kaempferol 3-*O*-β-D-glucopyranoside (**2**) on serum TNF-α elevations induced by D-GalN/LPS in mice

	Dose (mg/kg, p.o.)	*n*	Serum TNF-α (pg/mL)
Normal (untreated control)	—	4	9±4**
Control (D-GalN/LPS)	—	5	210±65
Tiliroside (**1**)	12.5	4	84±24*
	25	4	66±22*
	50	4	52±7**
	100	4	37±6**
Kaempferol 3-*O*-β-D-glucopyranoside (**2**)	25	4	56±14**
	50	4	40±5**
	100	4	45±4**

Blood samples were collected 1.5 h after D-GalN/LPS injection. Amount of TNF-α was assessed by ELISA. Each value represents the mean±SEM. Asterisks denote significant differences from the control at $*p<0.05$, $**p<0.01$.

In conclusion, the methanolic extract from the flowers of *T. argentea* was found to show a hepatoprotective effect on D-GalN/LPS-induced liver injury in mice. By bioassay-guided separation, five flavonol glycosides were isolated as the active components. In particular, the principal flavonol glycoside tiliroside (**1**) strongly inhibited serum GPT and GOT elevations in D-GalN/LPS-treated mice. By comparing the inhibitory activity of **1** with that of its components alone, the kaempferol 3-*O*-β-D-glucopyranoside moiety was found to be essential for the activity. Its effect was suggested to depend on the inhibition of TNF-α production, decreased sensitivity to TNF-α, and on the protection of hepatocytes against D-GalN.

Materials and Methods

Extraction and isolation

The dried flowers of *T. argentea* (4.0 kg, cultivated in Bulgaria and purchased from Tochimoto Tenkaido Co. Ltd., Osaka, Japan) were extracted three times with methanol under reflux for 3 h. Evaporation of the solvent under reduced pressure gave the methanolic extract (570 g, 14.3% from the natural medicine). The extract (500 g) was partitioned into an ethyl acetate (AcOEt)–water (H_2O) mixture to give an AcOEt soluble fraction (144 g, 4.1%) and water soluble fraction (356 g, 10.2%). The AcOEt soluble fraction (80.6 g) was subjected to silica gel column chromatography [SiO_2 2.5 kg, *n*-hexane–MeOH (10:1→ 1:1, v/v)→$CHCl_3$–MeOH (5:1→1:1, v/v)→MeOH] to give eleven fractions (fr. 1–fr. 11). The two active fractions 9 (14.1 g, 0.72%) and 10 (7.8 g, 0.40%) were subjected to ODS column chromatography (MeOH–H_2O) and finally HPLC [YMC-pack R&D ODS-5-A, 250×20 mm i.d., MeOH–H_2O (45% MeOH)] to give tiliroside (**1**, 1367 mg, 0.080%), kaempferol 3-*O*-β-D-glucopyranoside (**2**, 116 mg, 0.0068%), quercetin 3-*O*-β-D-glucopyranoside (**3**, 371 mg, 0.019%), kaempferol

Table 6. Inhibitory effects of tiliroside (**1**) and kaempferol 3-*O*-β-D-glucopyranoside (**2**) on TNF-α production from LPS-activated macrophages

	Inhibition (%)						
	0	0.1	0.3	1	3	10	IC_{50} (μM)
Tiliroside (**1**)	0±4	37±1**	48±3**	51±2**	63±2**	71±1**	0.64
Kaempferol 3-*O*-β-D-glucopyranoside (**2**)	0±4	11±2	39±2**	55±2**	61±2**	64±3**	1.1

Mouse peritoneal macrophages (5×10^5 cells/well) in RPMI 1640 medium containing 10 μg/mL LPS and test sample, and the cells were cultured for 4 h, and TNF-α levels in the medium were determined by ELISA. Each value represents the mean±SEM ($n=4$). Asterisks denote significant differences from the control at $**p<0.01$.

3-*O*-α-L-rhamnopyranoside (**4**, 222 mg, 0.013%), quercetin 3-*O*-α-L-rhamnopyranoside (**5**, 162 mg, 0.0083%), and kaempferol 3,7-di-*O*-α-L-rhamnopyranoside (**6**, 133 mg, 0.0068%).

Reagents

Lipopolysaccharide (LPS, from *Salmonella enteritidis*), RPMI 1640, minimum essential medium (MEM), and William's E medium were purchased from Sigma Chemical (St. Louis, MO, USA); 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl tetrazolium bromide (MTT) was from Dojin (Kumamoto, Japan); fetal calf serum (FCS) was from Life Technologies (Rockville, MD, USA); and other chemicals were from Wako Pure Chemical Industries (Osaka, Japan). The 96-well microplate was purchased from Nalge Nunc International (Naperville, IL, USA).

Animals. Male ddY mice were purchased from Kiwa Laboratory Animal Co., Ltd., Wakayama, Japan. The animals were maintained at a constant temperature of $23\pm2\,^{\circ}C$ and were fed a standard laboratory chow (MF, Oriental Yeast Co., Ltd., Japan) for a week. Test samples were suspended in 5% acacia solution, and the solution was administered orally at 10 mL/kg in each experiment, while the vehicle was given orally at 10 mL/kg in the corresponding control group.

D-GalN-induced cytotoxicy. Hepatocytes were isolated from male ddY mice (35–38 g) by the collagenase perfusion method. A cell suspension of 4×10^4 cells in 100 μL William's E medium containing fetal calf serum (FCS, 10%), penicillin (100 units/mL), and streptomycin (100 μg/mL) was inoculated on a 96-well microplate, and pre-cultured for 4 h at 37 °C under a 5% CO_2 atmosphere. The medium was replaced with fresh medium containing D-galactosamine hydrochloride (1 mM) and a test sample, and the hepatocytes were cultured for 44 h. The medium was replaced with 100 μL of fresh medium, and 10 μL of MTT [5 mg/mL in phosphate-buffered saline (PBS)] solution was added. After a 4 h culture, the medium was removed, and 100 μL of isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced by the cells. The optical density (OD) of the formazan solution was measured by a microplate reader at 570 nm. Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration was 0.5%).

TNF-α production by LPS-activated macrophages. Peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice (about 30 g) by washing with 6–7 mL of PBS, and cells (5×10^5 cells/200 μL/well) were suspended in 200 μL of RPMI 1640 supplemented with FCS (10%), penicillin (100 units/mL) and streptomycin (100 μg/mL), and pre-cultured in 96-well microplates at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing, and the adherent cells were cultured in fresh medium containing 10 μg/mL LPS and various concentrations of test sample. Four hours later, TNF-α levels in the media were determined by ELISA (commercial kit: Tumor necrosis factor alpha mouse, ELISA system, Amersham Pharmacia Biotech, UK). Cytotoxicity was determined by MTT colorimetric assay as described above.

Sensitivity of L929 cells to TNF-α. L929 cells, a TNF-α-sensitive cell line, was obtained from Dainippon Pharmaceutical (Osaka, Japan). After a 20 h incubation in MEM with 10 ng/mL TNF-α, viability of the cells was assessed by MTT colorimetric assay. Briefly, a suspension of 10^4 cells in 100 μL MEM containing FCS (10%), penicillin (100 units/mL), and streptomycin (100 μg/mL) was inoculated on a 96-well microplate. After a 20 h culture at 37 °C under a 5% CO_2 atmosphere, the medium was replaced with fresh medium containing TNF-α (10 ng/mL) and a test sample, and the cells were cultured for 20 h. Cell viability was determined by MTT colorimetric assay as described above.

D-GalN/LPS-induced liver injury in mice. The method described by Tiegs et al.[10] was modified and used for this experiment. Male ddY mice weighing 25–30 g were used. After 20 h of fasting, a mixture of D-galactosamine hydrochloride and LPS dissolved in saline was injected intraperitoneally at a dose of 350 mg/kg and 10 μg/kg, respectively, to induce liver injury. Each test sample was given orally 1 h before D-GalN/LPS injection. Blood samples were collected from the infraorbital plexus 10 h after administration of D-GalN/LPS, and serum GPT and GOT levels were determined by the Reitman-Frankel method (S.TA-test Wako, Wako Pure Chemical Industries). Hydrocortisone was used as a reference compound.

Determination of serum TNF-α levels. In a pre-examination, blood samples were collected several hours after D-GalN/LPS injection, and serum TNF-α levels were determined using the commercial kit (Amersham Pharmacia Biotech). Serum TNF-α levels peaked at 90 min after D-GalN/LPS injection, as reported previously.[11] Therefore, serum TNF-α levels at 90 min after the injection were compared.

CCl_4-induced liver injury. Male ddY mice weighing 20–25 g were used. After 20 h of fasting, a mixture of 10% (v/v) CCl_4 in olive oil was injected subcutaneously (s.c.) at a dose of 5 mL/kg to induce liver injury. Each test sample was given orally 1 h before CCl_4 injection. Blood samples were collected from the infraorbital plexus 20 h after injection, and serum GPT and GOT were determined. Malotilate (Daiichi Pharmaceutical Industry, Tokyo, Japan) was used as a reference compound.

Statistical analysis. Values are expressed as means ±SEM. One-way analysis of variance following Dunnett's test for multiple comparison analysis were used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

References and Notes

1. This paper is 'Medicinal Flowers V' in our serial studies.
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