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Hepatoprotective Principles from the Rhizomes of *Picrorhiza kurroa*

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A methanol extract of rhizomes of *Picrorhiza kurroa* Royle ex Benth. (Plantaginaceae) showed hepatoprotective effects against D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced liver injury in mice. We had previously isolated 46 compounds, including several types of iridoid glycosides, phenylethanoid glycosides, and aromatics, *etc.*, from the extract. Among them, picroside II, androsin, and 4-hydroxy-3-methoxyacetophenone exhibited active hepatoprotective effects at doses of 50–100 mg/kg, *per os* (*p.o.*) To characterize the mechanisms of action of these isolates and to clarify the structural requirements of phenylethanoid glycosides for their hepatoprotective effects, their effects were assessed in *in vitro* studies on (i) D-GalN-induced cytotoxicity in mouse primary hepatocytes, (ii) LPS-induced nitric oxide (NO) production in mouse peritoneal macrophages, and (iii) tumor necrosis factor-*α* (TNF-*α*)-induced cytotoxicity in L929 cells. These isolates decreased the cytotoxicity caused by D-GalN without inhibiting LPS-induced macrophage activation and also reduced the sensitivity of hepatocytes to TNF-*α*. In addition, the structural requirements of phenylethanoids for the protective effects of D-GalN-induced cytotoxicity in mouse primary hepatocytes were evaluated.

Key words hepatoprotective effect, *Picrorhiza kurroa*, iridoid glycoside, picroside II, phenylethanoid glycoside, structural requirement

INTRODUCTION

According to the World Flora Online, *Picrorhiza kurroa* Royle ex Benth. is a small perennial herb belonging to the family Plantaginaceae,[1] and is mainly found in the northwest Himalayan region, from Kashmir to Kumaun, and Garhwal regions of India as well as in Nepal, growing at an elevation of 3000–5000 m a.s.l.[2,3] An extract of the rhizomes of *P. kurroa*, commonly known as "kutki," has been used for the treatment of liver and lung diseases, fever, skin lesions, worm infections, rheumatic diseases, urinary disorders, heart failure, and snake and scorpion bites in the Ayurvedic and Unani systems of medicine.[4–7] During characterization of the bioactive constituents of plants in Tibet and Xinjiang, both autonomous regions in China,[8–23] we had previously isolated 46 compounds including iridoid glycosides (**1**–**7**), phenylethanoid glycosides (**8**–**18**), aromatics (**19**–**29**), *etc.*, from the rhizomes of *P. kurroa* using normal phase silica gel column chromatography followed by HPLC; these isolates exhibited hyaluronidase and collagenase inhibitory activities.[8,9] Further studies on the bioactive properties of the plant material revealed that the principal constituents, picroside II (**2**), androsin (**20**), and 4-hydroxy-3-methoxyacetophenone (**24**) as well as their methanol extracts exhibited hepatoprotective activity on D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced liver injury in mice. This study reports the hepatoprotective effects and possible mechanisms of action of these isolates (**2**, **20**, and **24**). Furthermore, *in vitro* structural requirements of phenylethanoids related to the protective effects against D-GalN-induced cytotoxicity in mouse primary hepatocytes and tumor necrosis factor-*α* (TNF-*α*)-induced cytotoxicity in L929 cells were evaluated, and the mechanisms of action of hepatoprotective activity of these compounds were also assessed.

MATERIALS AND METHODS

Plant Material Rhizomes of *P. kurroa* cultivated in the Tibet Autonomous Region, China, were obtained in November 2006 from the China Meheco Corporation, Beijing, China. Identification of the plant material was confirmed by the Garden of Medicinal Plants, Kindai University, and by one of the authors (M.Y.). A voucher specimen (Lot. No. 1230HX) of this plant material is maintained in our laboratory.[8,9]

Chemical Constituents (1–30) Obtained from the Methanol Extract of *P. kurroa* Rhizome Procedures related to extraction and isolation of compounds (**1**–**30**) from the methanol extract of *P. kurroa* rhizomes have been reported previously.[8,9] In brief, a methanol extract (47.07% of dried rhizomes of *P. kurroa*) was partitioned using EtOAc–H_2O (1 : 1, v/v) to yield an EtOAc-soluble fraction (8.33%) and an aqueous phase. The latter was subjected to Diaion HP-20 column chromatography ($H_2O \rightarrow$MeOH) to yield H_2O- and MeOH-eluted fractions (21.25 and 17.49%, respectively). Further separation and purification procedures were carried out using normal-phase silica gel, reversed-phase octadecyl silica (ODS) column chromatography, and finally, HPLC to obtain 46 constituents from the EtOAc-soluble and the MeOH-eluted fractions, as described previously.[8,9] Among the isolates, pic-

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rosides I (**1**, 2.5703%), II (**2**, 5.1636%), III (**3**, 0.2416%), and IV (**4**, 0.1659%); 6-feruloylcatalpol (**5**, 1.4457%); minecoside (**6**, 0.3499%); picrorhizaoside A (**7**, 0.0196%); calceolarioside A (**8**, 0.0031%); plantamajoside (**9**, 0.5496%); isoplantamajoside (**10**, 0.0859%); scrosides A (**11**, 0.0436%), C (**12**, 0.0211%), D (**13**, 0.0054%), and E (**14**, 0.0223%); chionoside J (**15**, 0.0268%); kurroaosides A (**16**, 0.0926%), B (**17**, 0.0321%), and C (**18**, 0.0218%); picein (**19**, 0.1193%); androsin (**20**, 0.3340%); belalloside A (**21**, 0.0008%); scrophuloside A (**22**, 0.0034%); 4-hydroxyacetophenone (**23**, 0.0021%); 4-hydroxy-3-methoxyacetophenone (**24**, 0.0009%); vanillic acid (**25**, 0.0758%); *p*-coumaric acid (**26**, 0.0037%); caffeic acid (**27**, 0.0010%); ferulic acid (**28**, 0.0226%); 6-*O*-(*E*)-cinnamoyl *β*-D-glucopyranoside (**29**, 0.0263%); and 3,16-dihydroxy-4,4,9,14-tetramethyl-19-norpregn-5-en-20-one-2-*O*-*β*-D-glucopyranoside (**30**, 0.0329%), were examined in this study.

Reagents LPS from *Salmonella enteritidis*, minimum essential medium (MEM), and William's E medium were purchased from Sigma-Aldrich Chemical (St. Louis, MO, U.S.A.), fetal bovine serum (FBS) was purchased from Life Technologies (Rockville, MD, U.S.A.), and other chemicals were procured from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Microplates (96-well) were purchased from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan).

Animals Male ddY mice (Kiwa Laboratory Animal Co., Ltd., Wakayama, Japan) were housed at a constant temperature of 23 ± 2 °C and were fed a standard laboratory chow (MF, Oriental Yeast Co., Ltd., Tokyo, Japan). The animals were fasted for 24 h prior to initiation of the experiment but were allowed free access to tap water. All experiments were performed using conscious mice unless mentioned otherwise. The experimental protocol was approved by the Experimental Animal Research Committee of the Kindai University (KAPR-2020-014).

Effects on D-GalN/LPS-Induced Liver Injuries in Mice The method described by Tiegs *et al.*[24] was modified and used for this study.[12,21,25–27] Briefly, male ddY mice weighing about 25–30 g were fasted for 20 h before initiating the experiment. D-GalN (350 mg/kg) and LPS (10 μg/kg) dissolved in saline were injected intraperitoneally to induce liver injury. Each test sample was administered orally 1 h before the D-GalN/LPS injection. Blood samples were collected from the infraorbital venous plexus 10 h after the D-GalN/LPS injection. Serum AST and ALT levels were determined using the Transaminase CII Test (FUJIFILM Wako). Test samples were suspended in 5% gum arabic solution, and the suspension was administered orally to the mice at 10 mL/kg in each experiment, while the vehicle was administered orally at 10 mL/kg to mice in the corresponding control group. Hydrocortisone was used as the reference compound.

Effects of Test Samples on Cytotoxicity Induced by D-GalN in Primary Cultured Mouse Hepatocytes Hepatoprotective effects of the test samples were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using primary cultured mouse hepatocytes.[12,13,21,25–27] Hepatocytes were isolated from male ddY mice (30–35 g) using a collagenase perfusion method. A suspension of 4×10^4 cells in 100 μL William's medium E containing FBS (10%), penicillin G (100 units/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well microplate and pre-incubated for 4 h at 37 °C under an atmosphere of 5% CO_2. After pre-incubation, the medium was replaced with 100 μL of fresh medium containing D-GalN (1 mM) and the test samples. After 44 h of incubation, 10 μL of MTT {5 mg/mL in phosphate buffered saline [PBS(–)]} was added to the medium. After 4 h of incubation, the medium was removed, and 80 μL of isopropanol containing 0.04 M HCl was added to dissolve the formazan produced in the cells. Optical density (O.D.) of the formazan solution was measured using a microplate reader at 570 nm (reference: 655 nm). Acteoside,[12,17] echinacoside,[12,17] isoacteoside,[12,17] and silybin,[17,26,27] were used as reference compounds. Inhibition of cytotoxicity (%) was calculated using the following formula.

$$\text{Inhibition (\%)} = \frac{\text{O.D.(sample)} - \text{O.D.(control)}}{\text{O.D.(normal)} - \text{O.D.(control)}} \times 100$$

Table 1. Inhibitory Effects of the Methanol Extract from the Rhizomes of *P. kurroa* and Its Fractions on D-GalN/LPS-Induced Liver Injuries in Mice

Treatment	Dose (mg/kg, *p.o.*)	*n*	sAST		sALT	
			(Karmen unit)	Inhibition (%)	(Karmen unit)	Inhibition (%)
Normal (vehicle)	—	7	100 ± 9[b]	—	25 ± 3[b]	—
Control (D-GalN/LPS)	—	10	6961 ± 1994	—	4960 ± 1313	—
MeOH extract	125	7	2709 ± 919	61.1	1961 ± 606	60.5
	250	7	1575 ± 532	77.4	1235 ± 425	75.1
	500	7	668 ± 173[a]	90.4	373 ± 83[a]	92.5
Control (D-GalN/LPS)	—	13	19080 ± 3230	—	11425 ± 1840	—
EtOAc-soluble fraction	125	7	6126 ± 1585[b]	67.9	3828 ± 976[b]	66.5
	250	7	2228 ± 588[b]	88.3	1632 ± 452[b]	85.7
MeOH-eluted fraction	125	7	12740 ± 2779	33.2	8730 ± 1789	23.6
	250	7	1826 ± 1009[b]	90.4	1529 ± 943[b]	86.6
H_2O-eluted fraction	250	7	11641 ± 4365	39.0	8225 ± 3015	28.0
Normal (vehicle)	—	5	95 ± 5[b]	—	19 ± 1[b]	—
Control (D-GalN/LPS)	—	8	9126 ± 1477	—	9830 ± 1650	—
Hydrocortisone[c]	10	7	627 ± 262[b]	94.2	247 ± 123[b]	97.7

Each value represents the mean ± S.E.M. Significantly different from the control, *a*) $p < 0.05$, *b*) $p < 0.01$. *c*) Commercial hydrocortisone was purchased from Sigma-Aldrich Chemical.[12,17,21,25–27]

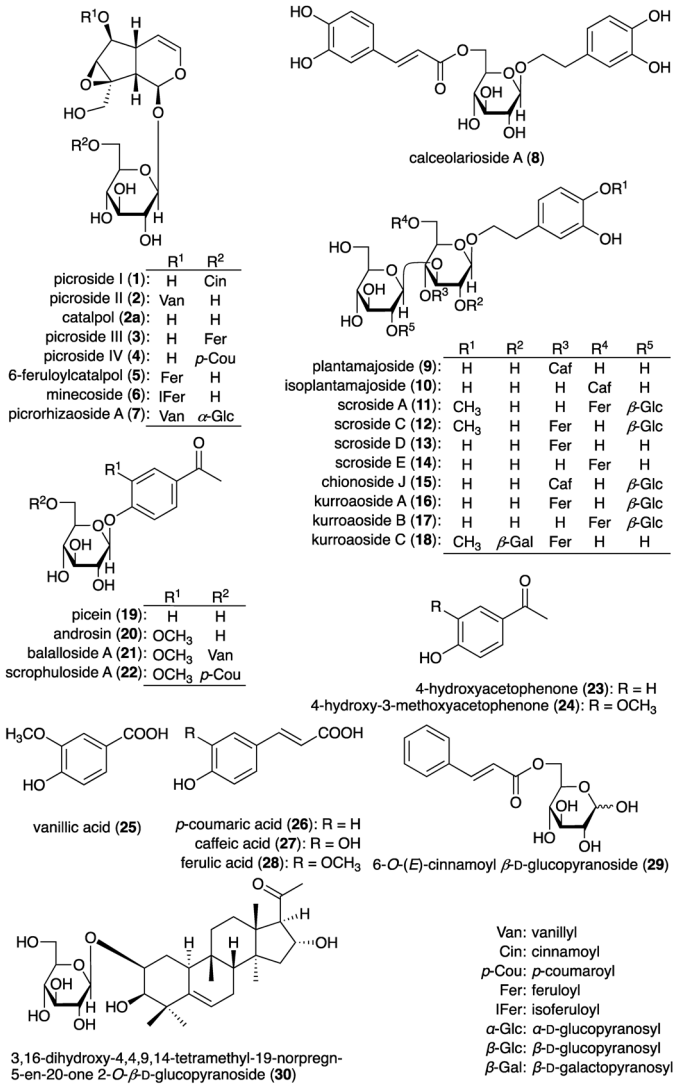

Fig. 1. Chemical Constituents (**1**–**30**) Obtained from Rhizomes of *P. kurroa* and a Related Compound Catalpol (**2a**)

Effects on Production of Nitric Oxide (NO) in LPS-Activated Mouse Peritoneal Macrophages Screening tests for NO production using TGC-induced mouse peritoneal macrophages were performed as described previously.[12,21,25–27] Briefly, peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice and suspended in 100 μL of RPMI 1640 supplemented with 5% FBS, penicillin G (100 units/mL), and streptomycin (100 μg/mL) and pre-cultured in 96-well microplates (5×10^5 cells/well) at 37 °C under 5% CO_2 for 1 h. After pre-incubation, the cells were cultured in 200 μL of medium containing 10 μg/mL LPS and various concentrations of test compounds for 20 h. After 20 h of incubation, NO production in each well was assessed by measuring the accumulation of nitrite (NO_2^-) in the culture medium using the Griess reagent. Cytotoxicity was determined using the MTT colorimetric assay after 20 h of incubation with the test compounds. Each test compound was dissolved in dimethyl sulfoxide (DMSO), and the solution was added to the medium (final DMSO concentration was 0.5%). N^G-Monomethyl-L-arginine (L-NMMA) and caffeic acid phenethyl ester (CAPE) were used as reference compounds.[21,25–27] O.D. was measured and inhibition of NO production (%) was calculated using the following formula; IC_{50} values were determined by plotting graphs ($N = 4$).

$$\text{Inhibition (\%)} = \frac{(A-B)}{(A-C)} \times 100$$

$A - C$: NO_2^- concentration (μg/mL or μM)

[A: LPS (+), sample (−); B: LPS (+), sample (+); C: LPS (−), sample (−)]

Inhibitory Effects against TNF-α-Induced Cell Death in L929 Cells L929 cells (RIKEN, Saitama, Japan) were maintained in Eagle's Minimum Essential Medium (E-MEM; FUJIFILM Wako) containing 10% FBS, 1% MEM Non-Essential Amino Acid Solution (FUJIFILM Wako), penicillin G (100 units/mL), and streptomycin (100 μg/mL) at 37 °C under a 5% CO_2 atmosphere. The cells were inoculated in a 96-well tissue culture plate (5×10^3 cells/well in 100 μL/well MEM). After 20 h of incubation, 100 μL of fresh medium containing 2 ng/mL TNF-α and each test sample were added to the medium (final concentration of TNF-α: 1 ng/mL). After 48 h of incubation, the viability of the cells was assessed by the MTT colorimetric assay as described above.[21,26,27] Each test compound was dissolved in DMSO, and the solution was

Table 2. Inhibitory Effects of Picrosides I (**1**) and II (**2**), Catalpol (**2a**), Androsin (**20**), and 4-Hydroxy-3-methoxyacetophenone (**24**) on D-GalN/LPS-Induced Liver Injuries in Mice

Treatment	Dose (mg/kg, *p.o.*)	*n*	sAST		sALT	
			(Karmen unit)	Inhibition (%)	(Karmen unit)	Inhibition (%)
Normal (vehicle)	—	6	162 ± 36[b)]	—	127 ± 50[b)]	—
Control (D-GalN/LPS)	—	12	5867 ± 2038	—	3066 ± 1049	—
Picroside I (**1**)	50	7	4856 ± 1771	17.7	2506 ± 855	19.0
	100	7	4366 ± 1599	26.3	2547 ± 848	17.6
Normal (vehicle)	—	9	81 ± 9[b)]	—	44 ± 14[b)]	—
Control (D-GalN/LPS)	—	10	7415 ± 1394	—	4318 ± 873	—
Picroside II (**2**)	50	10	1944 ± 819[a)]	74.7	1166 ± 472[a)]	74.0
	100	10	1169 ± 430[a)]	85.9	737 ± 314[a)]	84.5
Normal (vehicle)	—	6	69 ± 8[b)]	—	31 ± 7[b)]	—
Control (D-GalN/LPS)	—	6	4607 ± 1392	—	2594 ± 922	—
Catalpol (**2a**)	100	8	3757 ± 1407	18.7	2166 ± 858	16.7
Normal (vehicle)	—	7	108 ± 24[b)]	—	31 ± 7[b)]	—
Control (D-GalN/LPS)	—	7	7926 ± 1863	—	9281 ± 2703	—
Androsin (**20**)	50	6	3190 ± 1309[a)]	60.6	3702 ± 1725[a)]	60.3
	100	6	1910 ± 615[a)]	77.0	2056 ± 640[a)]	78.1
Normal (vehicle)	—	7	63 ± 4[b)]	—	23 ± 6[b)]	—
Control (D-GalN/LPS)	—	7	4999 ± 513	—	3907 ± 522	—
4-Hydroxy-3-methoxyacetophenone (**24**)	50	7	3519 ± 721	30.0	2630 ± 517	32.9
	100	7	2498 ± 843[a)]	50.7	1752 ± 562[a)]	55.5

Each value represents the mean ± S.E.M. Significantly different from the control, *a*) $p < 0.05$, *b*) $p < 0.01$.

added to the medium (final concentration in DMSO 0.5%). Acteoside,[12,17)] echinacoside,[12,17)] isoacteoside,[12,17)] and silybin,[17,26,27)] were used as reference compounds.

Statistical Analysis Values are expressed as mean ± standard error of the mean (S.E.M.). One-way ANOVA followed by Dunnett's test was used for statistical analysis. Probability (p) values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Protective Effects of the Methanol Extract Prepared from Rhizomes of *P. kurroa* and Its Fractions on Liver Injury Induced by D-GalN/LPS in Mice Infection with hepatitis C virus and chronic consumption of alcohol are the major causes of liver injury, cirrhosis, and hepatocellular carcinoma worldwide. TNF-α mediates organ injury through the induction of cellular inflammatory responses. In the liver, the biological effects of TNF-α have been implicated in hepatic injuries associated with hepatic toxins, ischemia/reperfusion, viral hepatitis, alcoholic liver disease, and alcohol-related disorders.[28–30)] Therefore, TNF-α is considered an important target for the discovery of anti-inflammatory and hepatoprotective agents. Liver injury in the D-GalN/LPS-induced liver injury model is recognized to occur *via* the effects of immunological responses.[31)] This model causes liver injury in two steps. First, the expression of inhibitors of apoptosis proteins (IAPs) is inhibited by administration of D-GalN through depletion of uridine triphosphate in hepatocytes. Second, proinflammatory mediators such as NO, reactive oxygen species (ROS), and TNF-α are released from LPS-activated macrophages (Kupffer cells). Apoptosis of hepatocytes induced by TNF-α plays an important role in D-GalN/LPS-induced liver injury.[32)] In our previous study related to compounds obtained from natural medicines possessing hepatoprotective activity, we reported that several naturally occurring compounds such as sesquiterpenes,[33–36)] triterpenes,[21,37)] limonoids,[26)] coumarins,[38)] phenylethanoids,[12,17)] stilbenoids,[27)] and acid amides,[25,39,40)] exhibited significant protective effects against liver injuries induced by D-GalN/LPS in mice. Continuing the search for hepatoprotective principles from natural medicines, the effects of the methanol extract of rhizomes of *P. kurroa* in this model were examined. As shown in Table 1, the methanol extract of *P. kurroa* rhizome was found to significantly inhibit the increase in serum levels of aspartate transaminase (sAST) and alanine transaminase (sALT), which serve as markers of acute liver injury,[41–43)] at a dose of 500 mg/kg *per os* (*p.o.*) Through bioassay-guided separation, the EtOAc-soluble and methanol-eluted fractions were found to be the active fractions, which significantly inhibited sAST and sALT levels at doses of 125 and 250 mg/kg, *p.o.*, respectively, whereas the H_2O-eluted fraction did not exhibit significant activity.

In our previous studies, each active fraction (the EtOAc-soluble and methanol-eluted fractions) was subjected to normal-phase silica gel and reversed-phase ODS column chromatography, and finally to HPLC to obtain a total of 46 compounds including iridoid glycosides (**1**–**7**), phenylethanoid glycosides (**8**–**18**), and aromatics (**19**–**29**), *etc*., as shown in Fig. 1.[8,9)]

Among the isolates, the principal acylated iridoid glycosides, such as picrosides I (**1**) and II (**2**), and acetophenone derivatives, androsin (**20**) and 4-hydroxy-3-methoxyacetophenone (**24**) were examined for *in vivo* hepatoprotective activity as described above. Among them, picroside II (**2**) was found to show hepatoprotective activity at a dose of 50 mg/kg, *p.o.*, as shown in Table 2. Although it has been previously reported that intraperitoneal administration of picroside II (**2**) ameliorates the adverse effects in the same acute liver injury

Table 3. Inhibitory Effects of the Methanol Extract, Its Fractions, and Constituents on D-GalN-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes

	Inhibition (%)					IC_{50} (μg/mL)
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	
MeOH extract	0.0 ± 0.3	1.8 ± 0.5[a)]	4.4 ± 0.2[b)]	8.2 ± 0.7[b)]	9.5 ± 0.3[b)]	
EtOAc-soluble fraction	0.0 ± 0.4	0.7 ± 0.2	2.9 ± 0.2[b)]	6.2 ± 0.4[b)]	6.9 ± 0.6[b)]	
MeOH-eluted fraction	0.0 ± 0.1	3.5 ± 0.3[b)]	7.1 ± 0.4[b)]	10.5 ± 0.8[b)]	16.7 ± 0.3[b)]	
H_2O-eluted fraction	0.0 ± 0.1	2.4 ± 0.2[b)]	5.0 ± 0.4[b)]	9.4 ± 0.4[b)]	13.1 ± 0.6[b)]	
	Inhibition (%)					IC_{50} (μM)
	0 μM	10 μM	30 μM	100 μM	300 μM	
Picroside I (**1**)	0.0 ± 2.4	−11.9 ± 1.4	−9.5 ± 4.4	−15.9 ± 3.4	−14.1 ± 3.3	
Picroside II (**2**)	0.0 ± 10.0	7.8 ± 8.7	11.9 ± 2.9	41.0 ± 7.1[b)]	82.7 ± 9.4[b)]	167.3
	Inhibition (%)					IC_{50} (μM)
	0 μM	3 μM	10 μM	30 μM	100 μM	
Catalpol (**2a**)	0.0 ± 1.2	−1.0 ± 1.2	−0.3 ± 0.3	0.0 ± 0.4	3.2 ± 1.2	
Picroside III (**3**)	0.0 ± 2.5	0.2 ± 2.2	6.2 ± 4.5	17.4 ± 2.1[a)]	31.8 ± 5.2[b)]	
Picroside IV (**4**)	0.0 ± 3.0	−8.0 ± 3.2	−3.2 ± 4.4	8.1 ± 5.6	33.1 ± 8.1[b)]	
6-Feruloylcatalpol (**5**)	0.0 ± 3.2	7.5 ± 4.6	13.7 ± 6.1	21.7 ± 7.6	38.6 ± 5.8[b)]	
Minecoside (**6**)	0.0 ± 2.2	6.8 ± 3.0	16.7 ± 11.6	13.5 ± 3.9	28.6 ± 2.6[a)]	
Picrorhizaoside A (**7**)	0.0 ± 1.6	2.5 ± 5.3	5.4 ± 3.9	3.1 ± 2.3	−1.9 ± 1.3	
Calceolarioside A (**8**)	0.0 ± 1.9	3.2 ± 1.8	8.8 ± 2.5	36.6 ± 2.6[b)]	96.6 ± 4.4[b)]	50.2
Plantamajoside (**9**)	0.0 ± 2.4	28.3 ± 5.8[b)]	44.5 ± 1.8[b)]	59.2 ± 6.7[b)]	83.8 ± 4.9[b)]	21.2
Isoplantamajoside (**10**)	0.0 ± 3.2	18.3 ± 4.2	37.9 ± 6.4[b)]	61.7 ± 6.0[b)]	102.1 ± 6.2[b)]	21.8
Scroside A (**11**)	0.0 ± 2.0	4.4 ± 2.6	5.3 ± 1.9	8.8 ± 0.9	22.9 ± 4.8[b)]	
Scroside C (**12**)	0.0 ± 0.8	2.4 ± 1.0	2.6 ± 2.2	3.3 ± 1.2	25.3 ± 4.5[b)]	
Scroside D (**13**)	0.0 ± 2.9	3.2 ± 2.8	6.7 ± 1.6	22.2 ± 6.0[b)]	90.4 ± 6.0[b)]	56.7
Scroside E (**14**)	0.0 ± 1.0	8.4 ± 1.5	10.7 ± 2.5	17.7 ± 4.7[b)]	65.1 ± 5.5[b)]	76.7
Chionoside J (**15**)	0.0 ± 0.8	0.1 ± 1.5	8.8 ± 1.4	13.5 ± 1.2	23.9 ± 1.9[b)]	
Kurroaoside A (**16**)	0.0 ± 1.8	29.0 ± 7.2[b)]	35.4 ± 5.1[b)]	42.9 ± 1.5[b)]	48.9 ± 2.9[b)]	
Kurroaoside B (**17**)	0.0 ± 5.1	19.3 ± 4.4[a)]	19.6 ± 1.0[a)]	39.5 ± 5.7[b)]	38.3 ± 3.4[b)]	
Kurroaoside C (**18**)	0.0 ± 2.1	10.0 ± 4.0	16.5 ± 5.7	42.8 ± 6.5[b)]	61.8 ± 2.7[b)]	70.7
Picein (**19**)	0.0 ± 5.2	0.1 ± 2.4	−0.9 ± 3.1	5.2 ± 2.5	12.2 ± 6.5	
Androsin (**20**)	0.0 ± 4.8	2.8 ± 2.9	5.8 ± 2.9	14.1 ± 1.4[a)]	18.6 ± 3.4[b)]	
Belalloside A (**21**)	0.0 ± 2.3	3.1 ± 1.9	1.8 ± 2.2	3.6 ± 1.3	24.2 ± 5.1[b)]	
Scrophuloside A (**22**)	0.0 ± 1.7	1.4 ± 2.3	−2.4 ± 0.5	−2.2 ± 0.6	7.7 ± 2.3[a)]	
4-Hydroxyacetophenone (**23**)	0.0 ± 1.8	0.9 ± 1.6	7.4 ± 1.1[a)]	24.9 ± 1.0[b)]	34.5 ± 2.9[b)]	
4-Hydroxy-3-methoxyacetophenone (**24**)	0.0 ± 1.8	5.6 ± 2.2	9.8 ± 5.9	56.3 ± 9.5[b)]	108.6 ± 7.2[b)]	41.4
Vanillic acid (**25**)	0.0 ± 0.9	−1.2 ± 1.0	−0.7 ± 0.7	2.4 ± 0.3	2.4 ± 0.5	
p-Coumaric acid (**26**)	0.0 ± 0.5	−0.7 ± 0.4	0.0 ± 0.3	1.3 ± 0.5	2.9 ± 0.2[b)]	
Caffeic acid (**27**)	0.0 ± 5.9	10.6 ± 4.0	18.7 ± 2.4[a)]	42.0 ± 2.3[b)]	63.4 ± 5.1[b)]	68.8
Ferulic acid (**28**)	0.0 ± 1.7	6.0 ± 1.4	5.6 ± 2.8	14.8 ± 2.1[a)]	19.6 ± 6.7[b)]	
6-*O*-(*E*)-Cinnamoyl β-D-glucopyranoside (**29**)	0.0 ± 1.6	6.1 ± 2.0	2.5 ± 1.5	3.7 ± 0.5	6.9 ± 1.8[a)]	
3,16-Dihydroxy-4,4,9,14-tetramethyl-19-norpregn-5-en-20-one-2-*O*-β-D-glucopyranoside (**30**)	0.0 ± 2.2	13.9 ± 3.9	24.1 ± 5.8[a)]	28.2 ± 2.8[b)]	26.6 ± 8.0[b)]	
Echinacoside[c)]	0.0 ± 2.1	32.8 ± 1.4[b)]	46.7 ± 4.3[b)]	67.7 ± 1.7[b)]		10.2
Acteoside[c)]	0.0 ± 2.4	40.9 ± 1.3[b)]	71.8 ± 2.3[b)]	119.2 ± 5.4[b)]		4.6
Isoacteoside[c)]	0.0 ± 4.4	43.7 ± 2.1[b)]	57.3 ± 2.2[b)]	101.2 ± 5.9[b)]		5.3
Silybin[d)]	0.0 ± 0.3	4.8 ± 1.1	7.7 ± 0.7	45.2 ± 8.8[b)]	77.0 ± 5.5[b)]	38.8

Each value represents the mean ± S.E.M. ($N = 4$). Significantly different from the control, *a*) $p < 0.05$, *b*) $p < 0.01$. *c*) Echinacoside, acteoside, and isoacteoside were isolated from the stems of the desert plant, *Cistanche tubulosa*.[12,17] *d*) Commercial silybin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan).[17,26,27]

model,[44] to the best of our knowledge, the present study is the first to report its hepatoprotective effect *via* the oral route. However, its desacyl derivative, catalpol (**2a**), and a common iridoid glycoside having an acyl group at a different position, picroside I (**1**), did not display hepatoprotective effects at the same dose. These results suggest that the presence and position of the acyl group of the iridoid glycoside is essential for its activity. As for the acetophenone derivatives, androsin (**20**) and 4-hydroxy-3-methoxyacetophenone (**24**) were also found to show hepatoprotective activities at doses of 50 and 100 mg/kg, *p.o.*, respectively. Previously, a similar hepatoprotective activity of 4-hydroxy-3-methoxyacetophenone (**24**) has been reported.[45] As a new finding in this study, androsin (**20**), a 4-*O*-β-D-glucopyranosyl derivative of 4-hydroxy-3-methoxyacetophenone (**24**), showed stronger hepatoprotective activity than that of the aglycone (**24**). Notably, the activity of the iso-

Table 4. Inhibitory Effects of the Methanol Extract, Its Fractions, and Constituents on TNF-α-Induced Cytotoxicity in L929 Cells

	Inhibition (%)					IC_{50} (μg/mL)
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	
MeOH extract	0.0 ± 1.0	−0.4 ± 1.5	2.4 ± 4.1	1.0 ± 3.9	2.1 ± 2.2	
EtOAc-soluble fraction	0.0 ± 1.0	3.1 ± 1.2	7.1 ± 1.5	12.6 ± 3.3[b]	21.6 ± 1.9[b]	
MeOH-eluted fraction	0.0 ± 4.6	−1.3 ± 1.2	1.7 ± 0.5	−0.6 ± 2.1	2.6 ± 1.2	
H_2O-eluted fraction	0.0 ± 1.6	10.7 ± 2.6[b]	16.4 ± 1.8[b]	10.6 ± 2.3[b]	3.2 ± 1.8	
	Inhibition (%)					IC_{50} (μM)
	0 μM	3 μM	10 μM	30 μM	100 μM	
Picroside I (**1**)	0.0 ± 1.8	2.7 ± 0.6	1.5 ± 2.1	−0.4 ± 0.8	−4.8 ± 1.1	
Picroside II (**2**)	0.0 ± 2.1	−2.5 ± 0.7	−3.5 ± 1.8	−2.8 ± 0.9	−3.6 ± 0.6	
Picroside III (**3**)	0.0 ± 1.3	−1.6 ± 0.7	−1.1 ± 0.5	−4.4 ± 1.4	−7.5 ± 1.2	
Picroside IV (**4**)	0.0 ± 0.3	−3.9 ± 1.5	−1.2 ± 1.1	−4.2 ± 1.4	−3.7 ± 1.7	
6-Feruloylcatalpol (**5**)	0.0 ± 6.7	2.0 ± 13.2	2.6 ± 12.8	19.2 ± 9.2[b]	30.1 ± 8.0[b]	
Minecoside (**6**)	0.0 ± 1.4	2.6 ± 1.6	0.7 ± 0.9	−1.4 ± 1.2	−3.7 ± 0.4	
Picrorhizaoside A (**7**)	0.0 ± 1.9	−1.9 ± 0.6	−4.0 ± 3.2	−5.0 ± 1.2	−10.9 ± 2.2[b]	
Calceolarioside A (**8**)	0.0 ± 0.8	4.4 ± 1.5	12.1 ± 2.4[b]	12.6 ± 2.1[b]	21.6 ± 1.3[b]	
Plantamajoside (**9**)	0.0 ± 11.7	1.7 ± 6.7	13.1 ± 8.1	10.3 ± 6.9	18.8 ± 8.5	
Isoplantamajoside (**10**)	0.0 ± 8.8	6.7 ± 5.0	12.9 ± 4.7	30.7 ± 5.1	79.1 ± 20.4[b]	60.2
Scroside A (**11**)	0.0 ± 1.8	0.1 ± 1.5	−2.1 ± 2.4	−1.0 ± 2.0	−5.9 ± 1.2	
Scroside C (**12**)	0.0 ± 0.9	−0.1 ± 1.1	−1.8 ± 0.8	0.9 ± 1.7	−6.4 ± 1.4[b]	
Scroside D (**13**)	0.0 ± 0.8	−2.5 ± 1.0	−0.3 ± 1.3	4.6 ± 3.1	4.9 ± 1.1	
Scroside E (**14**)	0.0 ± 2.8	1.0 ± 1.3	−3.0 ± 0.9	4.4 ± 3.2	16.2 ± 2.4[b]	
Chionoside J (**15**)	0.0 ± 4.2	−0.9 ± 1.2	−3.1 ± 2.4	−2.5 ± 4.7	4.5 ± 2.3	
Kurroaoside A (**16**)	0.0 ± 1.9	−1.4 ± 0.5	−0.2 ± 1.6	12.0 ± 10.3	12.8 ± 3.2[a]	
Kurroaoside B (**17**)	0.0 ± 3.1	17.2 ± 12.9	27.7 ± 12.2	43.3 ± 9.3[a]	58.3 ± 8.9[b]	72.4
Kurroaoside C (**18**)	0.0 ± 0.7	0.6 ± 1.3	−2.9 ± 1.1	−2.5 ± 1.2	−5.5 ± 0.8	
Picein (**19**)	0.0 ± 6.8	3.8 ± 7.1	−2.6 ± 8.2	−2.1 ± 8.8	11.8 ± 9.4	
Androsin (**20**)	0.0 ± 1.8	−0.1 ± 2.3	−0.6 ± 1.9	−2.6 ± 2.4	−8.5 ± 0.9	
Belalloside A (**21**)	0.0 ± 4.4	0.6 ± 0.6	−1.0 ± 3.1	−0.3 ± 3.0	−5.7 ± 1.5	
Scrophuloside A (**22**)	0.0 ± 1.9	−0.1 ± 1.2	−4.9 ± 1.9	−4.4 ± 3.0	−18.6 ± 1.8[b]	
4-Hydroxyacetophenone (**23**)	0.0 ± 0.9	−2.8 ± 1.0	−0.7 ± 1.1	1.6 ± 0.6	1.9 ± 1.8	
4-Hydroxy-3-methoxyacetophenone (**24**)	0.0 ± 1.1	−4.9 ± 0.5	−5.4 ± 0.8	−1.5 ± 1.3	−3.2 ± 2.2	
Vanillic acid (**25**)	0.0 ± 1.2	−4.3 ± 1.1	−4.9 ± 1.2	−4.2 ± 2.2	−4.4 ± 1.0	
p-Coumaric acid (**26**)	0.0 ± 2.4	1.2 ± 1.3	−2.1 ± 1.4	2.1 ± 1.6	−2.2 ± 2.3	
Caffeic acid (**27**)	0.0 ± 0.9	0.1 ± 0.9	−0.4 ± 1.9	1.9 ± 1.4	18.4 ± 2.4[b]	
Ferulic acid (**28**)	0.0 ± 1.9	−4.6 ± 1.3	−2.6 ± 0.7	0.6 ± 1.5	−4.1 ± 1.9	
6-*O*-(*E*)-Cinnamoyl β-D-glucopyranoside (**29**)	0.0 ± 1.9	−1.4 ± 2.1	−6.0 ± 2.5	−8.1 ± 3.4	−17.7 ± 1.7[b]	
3,16-Dihydroxy-4,4,9,14-tetramethyl-19-norpregn-5-en-20-one-2-*O*-β-D-glucopyranoside (**30**)	0.0 ± 0.6	−2.7 ± 3.1	3.1 ± 1.2	1.9 ± 1.3	−0.6 ± 1.1	
Echinacoside[c]	0.0 ± 4.8	5.2 ± 3.5	22.5 ± 1.6[b]	45.7 ± 6.0[b]	80.4 ± 4.5[b]	31.1
Acteoside[c]	0.0 ± 1.1	16.4 ± 1.3[a]	24.1 ± 4.6[b]	58.4 ± 2.5[b]	91.9 ± 5.3[b]	17.8
Isoacteoside[c]	0.0 ± 1.2	−4.6 ± 3.5	19.0 ± 2.6	61.9 ± 5.9[b]	102.4 ± 8.7[b]	22.7
Silybin[d]	0.0 ± 2.6	5.3 ± 2.8	22.0 ± 3.8[b]	48.0 ± 4.1[b]	50.8 ± 3.9[b]	60.4

Each value represents the mean ± S.E.M. ($N = 4$). Significantly different from the control, *a*) $p < 0.05$, *b*) $p < 0.01$. *c*) Echinacoside, acteoside, and isoacteoside were isolated from the stems of the desert plant, *Cistanche tubulosa*.[12,17] *d*) Commercial silybin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan).[17,26,27]

lates (**2** and **20**) was equivalent to that of curcumin[17,21,26,27,33–36] and more potent than that of silybin,[17,26,27] which are well-known naturally occurring hepatoprotective products.

Effects on D-GalN-Induced Cytotoxicity in Primary Cultured Mouse Hepatocytes The methanol extract of the rhizomes of *P. kurroa* and several major constituents (**2**, **20**, and **24**) showed hepatoprotective effects against D-GalN/LPS-induced liver injury in mice as described above. Inhibitory effect of the isolates (**1**–**30**) on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes was examined using the MTT assay. As shown in Table 3, several acylated phenylethanoid glycosides, such as calceolarioside A (**8**, $IC_{50} = 50.2\,\mu$M); plantamajoside (**9**, 21.2 μM); isoplantamajoside (**10**, 21.8 μM); scrosides D (**13**, 56.7 μM) and E (**14**, 76.7 μM); and kurroaoside C (**18**, 70.7 μM) reduced D-GalN-induced cytotoxicity in primary mouse hepatocytes. Structural requirements of the phenylethanoid glycosides for their activity are as follows: (1) compounds with a caffeoyl group showed stronger activity than those with a feruloyl group (**9** > **13**, **10** > **14**); (2) the glycosyl moiety with a disaccharide showed stronger activity than that with monosaccharide (**10** > **8**) or trisaccharide [**9** > chionoside J (**15**, > 100 μM)]. These findings reinforced those observed in our previous reports regarding structural requirements of the phenylethanoid glycosides obtained from *Cistanche tubulosa* [*e.g.*, echinacoside ($IC_{50} = 10.2\,\mu$M), acteoside (4.6 μM), and isoacteoside (5.3 μM), *etc.*] for hepato-

protective activity.[12,17] However, the iridoid glycoside, acetophenone derivative, and phenylpropanoid constituents did not show any notable activity, except for picroside II (**2**, 167 μM), 4-hydroxy-3-methoxyacetophenone (**24**, 41.4 μM), and phenylpropanoid caffeic acid (**27**, 68.8 μM).

Effects on LPS-Induced NO Production in Mouse Peritoneal Macrophages The effects of the isolates (**1**–**30**) from the rhizomes of *P. kurroa* on NO production were examined to estimate the macrophage activation levels in LPS-treated mouse peritoneal macrophages. These isolates (**1**–**30**) did not affect the overproduction of NO in LPS-induced macrophages ($IC_{50} > 100\,\mu$M) (Supplementary Table S1).

Effects on TNF-α-Induced Cytotoxicity in L929 Cells To clarify the effects of the isolates (**1**–**30**) on the sensitivity of hepatocytes to TNF-α, a decrease in cell viability of a TNF-α-sensitive cell line, L929 cells,[46] induced by TNF-α was assessed. Therefore, it is possible to estimate the concentration of TNF-α in the medium from the intensity of cytotoxic activity of L929 by TNF-α. In the absence of a test sample, cells incubated with 1 ng/mL TNF-α for 48 h were compared with those not incubated with TNF-α. Among them, acylated phenylethanoid glycosides isoplantamajoside C (**10**, 60.2 μM) and kurroaoside B (**17**, $IC_{50} = 72.4\,\mu$M) were found to inhibit the decrease in cell viability; this activity was equivalent to that of silybin ($IC_{50} = 60.4\,\mu$M)[21,26,27] as shown in Table 4. However, the activities of these phenylethanoid glycosides (**10** and **17**) were weaker than those observed in our previous findings observed in *Cistanche tubulosa* [*e.g.*, echinacoside ($IC_{50} = 31.1\,\mu$M), acteoside (17.8 μM), and isoacteoside (22.7 μM), *etc.*].[12,17]

CONCLUSION

In conclusion, a methanol extract from the rhizomes of *P. kurroa* was shown to have hepatoprotective effects against D-GalN/LPS-induced liver injury in mice. From the extract, acylated iridoid glycoside picroside II (**2**) and its acetophenone derivatives, androsin (**20**), and 4-hydroxy-3-methoxyacetophenone (**24**), exhibited significant hepatoprotective effects at doses of 50–100 mg/kg, *p.o.* We characterized the mechanisms of action of these isolates (**2**, **20**, and **24**) and found that they were likely to decrease the cytotoxicity caused by D-GalN without inhibiting LPS-induced macrophage activation and also reduced the sensitivity of hepatocytes to TNF-α. In addition, structural requirements of phenylethanoids for the protective effects of D-GalN-induced cytotoxicity in mouse primary hepatocytes are as follows: (1) compounds with a caffeoyl group showed stronger activity than those with a feruloyl group; (2) the glycosyl moiety with a disaccharide showed stronger activity than that with monosaccharide or trisaccharide. These findings reinforce the findings observed in our previous reports regarding the structural requirements of phenylethanoid glycosides obtained from *Cistanche tubulosa* for hepatoprotective activity.[12,17] Nevertheless, detailed mechanisms of action as well as structural requirements of phenylethanoid glycosides should be studied further.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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