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Acylated phenylethanoid oligoglycosides with hepatoprotective activity from the desert plant *Cistanche tubulosa*[1]

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ABSTRACT

The methanolic extract from fresh stems of *Cistanche tubulosa* (Orobanchaceae) was found to show hepatoprotective effects against D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced liver injury in mice. From the extract, three new phenylethanoid oligoglycosides, kankanosides H_1 (**1**), H_2 (**2**), and I (**3**), were isolated together with 16 phenylethanoid glycosides (**4**–**19**) and two acylated oligosugars (**20**, **21**). The structures of **1**–**3** were determined on the basis of spectroscopic properties as well as of chemical evidence. Among the isolates, echinacoside (**4**, IC_{50} = 10.2 μM), acteoside (**5**, 4.6 μM), isoacteoside (**6**, 5.3 μM), 2′-acetylacteoside (**8**, 4.8 μM), and tubuloside A (**10**, 8.6 μM) inhibited D-GalN-induced death of hepatocytes. These five isolates, **4** (31.1 μM), **5** (17.8 μM), **6** (22.7 μM), **8** (25.7 μM), and **10** (23.2 μM), and cistantubuloside B_1 (**11**, 21.4 μM) also reduced TNF-α-induced cytotoxicity in L929 cells. Moreover, principal constituents (**4**–**6**) exhibited in vivo hepatoprotective effects at doses of 25–100 mg/kg, po.

1. Introduction

Cistanche tubulosa (SCHRENK) R. WIGHT (Orobanchaceae) is a perennial parasitic plant growing on roots of *Salvadora* or *Calotropis* species, and distributed in North Africa, Arabia, and Asian countries.[2] Stems of *C. tubulosa* as well as *Cistanche salsa* and *Cistanche deserticola* have traditionally been used for treatment of impotence, sterility, lumbago, and body weakness as well as a promoting agent of blood circulation.[2,3] Previously, several phenylethanoids, iridoids, monoterpenes, and lignans were isolated from Chinese and Pakistan *C. tubulosa*.[2,4–9] In the course of our characterization studies on bioactive constituents in this natural medicine, we previously reported that five iridoids, kankanosides A–D and kankanol, a monoterpene glycoside, kankanoside E, two phenylethanoid oligoglycosides, kankanosides F and G (**23**), and an acylated oligosugar, kankanose (**21**), were isolated from the methanolic extract of dried stems of *C. tubulosa*.[10,11] In addition, the methanolic extract and several isolates such as echinacoside (**4**), acteoside (**5**), cistanoside F (**20**), **21**, and kankanoside F were found to show vasorelaxant effects, that is, inhibitory effects against contractions induced by noradrenaline in isolated rat thoracic aorta.[11] In our continuing studies on constituents in the plant, a methanolic extract of fresh stems of *C. tubulosa* was found to show protective effect on liver injury induced by D-galactosamine (D-GalN)/lipopolysaccharide (LPS) in mice. From the methanolic extract, we have isolated three new phenylethanoid oligoglycosides named kankanosides H_1 (**1**), H_2 (**2**), and I (**3**) together with 16 phenylethanoid glycosides (**4**–**19**) and two acylated oligosugars (**20**, **21**). This paper deals with the isolation and structural elucidation of these new phenylethanoid oligoglycosides (**1**–**3**). Structural requirements of the phenylethanoid glycosides for the hepatoprotective activity are also discussed (Fig. 1).

2. Results and discussion

2.1. Protective effect of methanolic extract from stems of *C. tubulosa* on liver injury induced by D-GalN/LPS in mice

Fresh stems of *C. tubulosa* (cultivated in Urumuqi, Xinjiang Province, China) were extracted with methanol under reflux to yield a methanolic extract (8.36% from the fresh stems). As shown in Table 1, at doses of 250–500 mg/kg, po, the methanolic extract showed inhibitory effect on the increase in serum aspartate aminotransaminase (sAST) and alanine aminotransaminase (sALT), markers of liver injury, induced by D-GalN/LPS in mice.

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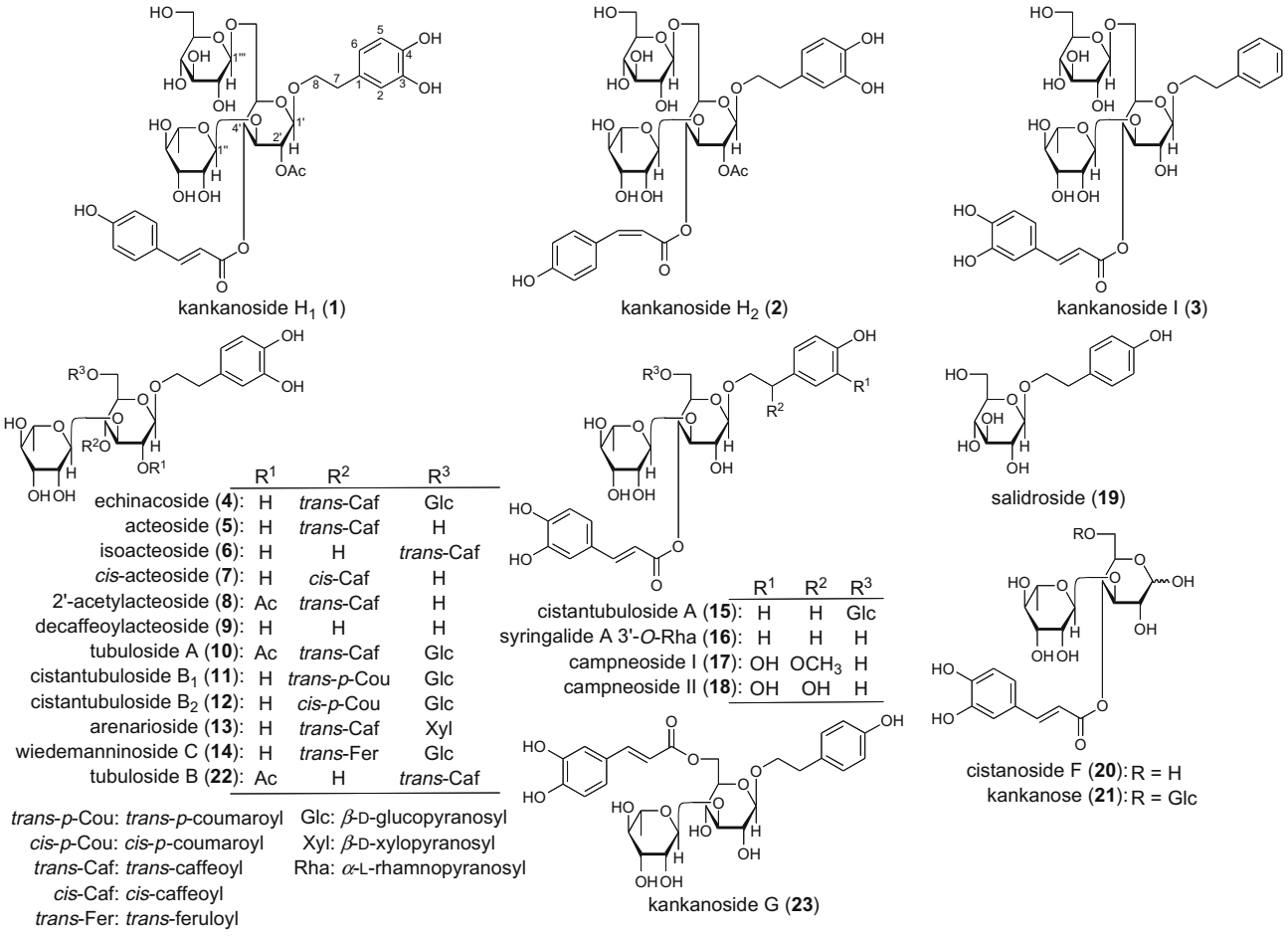

Figure 1. Chemical structures of compounds **1**–**23** from stems of *Cistanche tubulosa*.

Table 1
Inhibitory effects of the methanolic extract from stems of *C. tubulosa* on D-GalN/LPS-induced liver injury in mice

Treatment	Dose (mg/kg, po)	*n*	sAST		sALT	
			(Karmen unit)	Inhibition (%)	(Karmen unit)	Inhibition (%)
Normal (vehicle)	—	7	86 ± 5[b]	—	28 ± 6[b]	—
Control (D-GalN/LPS)	—	11	10,714 ± 1520	—	6823 ± 1011	—
MeOH extract	250	8	4653 ± 1698[b]	56.6	3632 ± 1527	46.8
	500	8	2049 ± 556[b]	80.9	1318 ± 397[b]	80.7
	1000	8	904 ± 272[b]	91.6	701 ± 226[b]	89.7

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

2.2. Chemical constituents from stems of *C. tubulosa*

A methanolic extract from the fresh stems of *C. tubulosa* was subjected to Diaion HP-20 column chromatography (H_2O→MeOH) to give H_2O- and MeOH-eluted fractions (5.63% and 2.73%, respectively). The MeOH-eluted fraction was subjected to SiO_2 and ODS column chromatographies and finally HPLC to furnish three new phenylethanoid oligoglycosides, kankanosides H_1 (**1**, 0.0008%), H_2 (**2**, 0.0001%), and I (**3**, 0.0007%) together with 16 phenylethanoid glycosides, echinacoside[2,11] (**4**, 0.45%), acteoside[2,11] (**5**, 0.28%), isoacteoside[2,11] (**6**, 0.041%), *cis*-acteoside[12,13] (**7**, 0.0007%), 2′-acetylacteoside[2,11] (**8**, 0.0038%), decaffeoylacteoside[14] (**9**, 0.0002%), tubuloside A[2,11] (**10**, 0.013%), cistantubulosides B_1[9] (**11**, 0.0020%), B_2[9] (**12**, 0.0003%), arenarioside[15] (**13**, 0.0006%), wiedemanninoside C[16] (**14**, 0.0008%), cistantubuloside A[9] (**15**, 0.0009%), syringalide A 3′-*O*-α-L-rhamnopyranoside[4] (**16**, 0.0017%), campneosides I[17,18] (**17**, 0.015%) and II[17,18] (**18**, 0.0005%), and salidroside[11] (**19**, 0.0027%), and two acylated oligosugars, cistanoside F[11] (**20**, 0.0004%) and kankanose[11] (**21**, 0.0004%).

2.3. Structures of kankanosides H_1 (1), H_2 (2), and I (3)

Kankanoside H_1 (**1**) was obtained as a white powder with negative optical rotation ($[\alpha]_D^{24}$ −45.3 in MeOH). Its IR spectrum showed absorption bands at 3416, 1736, 1638, 1605, 1518, 1070, and 1040 cm^{-1} ascribable to hydroxyls, ester carbonyls, ether functions, and aromatic rings. The positive- and negative-ion FABMS spectra of **1** showed quasimolecular ion peaks at m/z 835 $(M+Na)^+$ and m/z 811 $(M-H)^-$, respectively, and the molecular formula was determined as $C_{37}H_{48}O_{20}$ by high-resolution positive-ion

FABMS measurement. The ^{1}H and ^{13}C NMR spectra of **1** (CD_3OD, Tables 2 and 3), which were assigned by various NMR experiments,[19] showed signals assignable to two methylenes [δ 2.70 (2H, m, H_2-7), 3.66, 4.07 (1H each, both m, H_2-8)], *ortho*- and *meta*-coupled ABC-type aromatic protons [δ 6.52 (1H, dd, J = 1.8, 7.8 Hz, H-6), 6.65 (1H, d, J = 1.8 Hz, H-2), 6.67 (1H, d, J = 7.8 Hz, H-5)], two β-D-glucopyranosyl moieties [δ 4.29 (1H, d, J = 7.8 Hz, *terminal*-Glc-H-1), 4.54 (1H, d, J = 8.2 Hz, *inner*-Glc-H-1)], and a α-L-rhamnopyranosyl moiety [δ 1.06 (3H, d, J = 6.0 Hz, Rha-H_3-6), 4.80 (1H, br s, Rha-H-1)] together with an acetyl group [δ 1.98 (3H, s)] and a *trans*-*p*-coumaroyl group {an *trans*-olefin [δ 6.34, 7.67 (1H each, both d, J = 16.0 Hz, H-8 and 7)] and *ortho*-coupled A_2B_2-type aromatic protons [δ 6.80, 7.46 (2H each, both d, J = 8.7 Hz, H-3,5 and 2,6)]}. Connectivities between the oligoglycoside and acyl moieties in **1** were characterized by a HMBC experiment, which showed long-range correlations between the following proton and carbon pairs: *inner*-Glc-H-1 and C-8 (δ_C 71.9); *inner*-Glc-H-2 [δ 4.88 (1H, dd-like)] and the acetyl carbonyl carbon (δ_C 171.4); *inner*-Glc-H-4 [δ 5.08 (1H, dd, J = 9.6, 9.6 Hz)] and the *p*-coumaroyl carbonyl carbon (δ_C 168.2); Rha-H-1 and *inner*-Glc-C-3 (δ_C 80.5); and *terminal*-Glc-H-1 and *inner*-Glc-C-6 (δ_C 69.3) (Fig. 2). Finally, alkaline hydrolysis of **1** with 5% potassium hydroxide (KOH) liberated *trans*-*p*-coumaric acid, which was identified by HPLC analysis, together with a deacylated product. The deacylated product was successively treated with 1.0 M hydrochloric acid (HCl) to liberate L-rhamnose and D-glucose, which were identified by HPLC analysis using an optical rotation detector.[10,11] Thus, the structure of kankanoside H_1 was elucidated to be 2-(3,4-dihydroxyphenyl)ethyl *O*-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-2-*O*-acetyl-4-*O*-*trans*-*p*-coumaroyl-β-D-glucopyranoside (**1**).

Kankanoside H_2 (**2**) was isolated as a white powder with negative optical rotation ($[\alpha]_D^{25}$ −52.4 in MeOH). Its molecular formula $C_{37}H_{48}O_{20}$ was found to be the same as that of **1** by high-resolution positive-ion FABMS measurement. The spectroscopic properties of **2** were very similar to those of **1**, except for signals due to the *cis*-*p*-coumaroyl group [δ 5.79, 6.95 (1H each, both d, J = 12.8 Hz, H-8 and 7), 6.76, 7.73 (2H each, both d, J = 8.7 Hz, H-3,5 and 2,6)]. Furthermore, HMBC experiment on **2** also revealed similar modes of long-range correlations as those detected for **1** between the following proton and carbon pairs: *inner*-Glc-H-1 [δ 4.51 (1H, d, J = 7.8 Hz)] and C-8 (δ_C 72.0); *inner*-Glc-H-2 [δ 4.88 (1H, m)] and the acetyl carbonyl carbon (δ_C 171.4); *inner*-Glc-H-4 [δ 4.98 (1H, dd, J = 9.6, 9.7 Hz)] and the *p*-coumaroyl carbonyl carbon (δ_C 166.7); Rha-H-1 [δ 4.77 (1H, d, J = 1.4 Hz, Rha-H-1)] and *inner*-Glc-C-3 (δ_C 80.7); and *terminal*-Glc-H-1 [δ 4.28 (1H, d, J = 7.8 Hz)] and *inner*-Glc-C-6 (δ_C 69.4) (Fig. 2). By the degradation study, **2** gave *cis*-*p*-coumaric acid and two same sugars as those obtained in the case of **1**. Consequently, the structure of kankanoside H_2 was elucidated to be 2-(3,4-dihydroxyphenyl)ethyl *O*-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-2-*O*-acetyl-4-*O*-*cis*-*p*-coumaroyl-β-D-glucopyranoside (**2**).

Kankanoside I (**3**) was also isolated as a white powder with negative optical rotation ($[\alpha]_D^{25}$ −62.2 in MeOH). Its molecular formula, $C_{35}H_{46}O_{18}$, was determined by positive- and negative-FABMS and HRFABMS measurements. The spectroscopic properties of **3** (CD_3OD, Tables 2 and 3) were superimposable on those of echinacoside (**4**), except for signals due to the aglycone part {{two methylenes {δ 2.95 (2H, dd, J = 7.3, 7.8 Hz, H_2-7), [3.79 (1H, m), 4.10 (1H, dt, J = 16.9, 7.3 Hz), H_2-8]}, monosubstituted aromatic protons [δ 7.18 (1H, m, H-4), 7.26–7.28 (4H, m, H-2,6, 3,5)]}}. Alkaline hydrolysis of **3** with 5% KOH liberated *trans*-caffeic acid, and the deacylated product was successively treated with 1.0 M HCl to liberate L-rhamnose and D-glucose. Finally, connectivities of the acyl group and the sugar moieties in **3** were clarified by the HMBC experiment, as shown in Figure 2. On the basis of above-mentioned evidence, the structure of kankanoside I was elucidated to be 2-phenylethyl *O*-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-4-*O*-*trans*-caffeoyl-β-D-glucopyranoside (**3**).

Table 2
^{1}H NMR (600 MHz, CD_3OD) data for kankanosides H_1 (**1**), H_2 (**2**), and I (**3**)

Position	1	2	3
	δ_H (J Hz)	δ_H (J Hz)	δ_H (J Hz)
2	6.65 (d, 1.8)	6.63 (d, 1.8)	7.26–7.28 (m)
3			7.26–7.28 (m)
4			7.18 (m)
5	6.67 (d, 7.8)	6.65 (d, 8.2)	7.26–7.28 (m)
6	6.52 (dd, 1.8, 7.8)	6.52 (dd, 1.8, 8.2)	7.26–7.28 (m)
7	2.70 (2H, m)	2.69 (2H, m)	2.95 (2H, dd, 7.3, 7.8)
8	3.66 (m)	3.62 (m)	3.79 (m)
	4.07 (m)	4.04 (m)	4.10 (dt, 16.9, 7.3)
8-*O*-Glc			
1′	4.54 (d, 8.2)	4.51 (d, 7.8)	4.40 (d, 8.2)
2′	4.88 (dd-like)	4.88 (m)	3.99 (dd, 8.2, 9.2)
3′	4.01 (m)	3.94 (dd, 9.2, 9.6)	3.81 (m)
4′	5.08 (dd, 9.6, 9.6)	4.98 (dd, 9.6, 9.7)	5.01 (dd, 9.6, 10.1)
5′	3.84 (m)	3.75 (ddd, 2.3, 5.9, 9.7)	3.76 (m)
6′	3.66 (dd, 6.0, 11.9)	3.62 (m)	3.56 (m)
	3.95 (dd, 1.8, 11.9)	3.91 (dd, 2.3, 12.0)	3.94 (dd, 1.8, 11.8)
3′-*O*-Rha			
1″	4.80 (br s)	4.77 (d, 1.4)	5.18 (d, 1.8)
2″	3.50 (m)	3.46 (m)	3.53 (m)
3″	3.62 (m)	3.62 (m)	3.92 (m)
4″	3.25 (m)	3.24 (m)	3.26 (m)
5″	3.50 (m)	3.53 (m)	3.50 (m)
6″	1.06 (3H, d, 6.0)	1.11 (3H, d, 6.0)	1.08 (3H, d, 6.0)
6′-*O*-Glc			
1‴	4.29 (d, 7.8)	4.28 (d, 7.8)	4.28 (d, 7.4)
2‴	3.24 (m)	3.18 (m)	3.20 (m)
3‴	3.34 (m)	3.34 (m)	3.31 (m)
4‴	3.24 (m)	3.20 (m)	3.24 (m)
5‴	3.22 (m)	3.18 (m)	3.24 (m)
6‴	3.64 (dd, 5.1, 12.0)	3.62 (m)	3.60 (dd, 5.0, 11.9)
	3.83 (br d, ca. 12)	3.80 (dd, 2.3, 12.3)	3.80 (m)
2′-*O*-Ac			
	1.98 (3H, s)	1.97 (3H, s)	
4′-*O*-Acyl			
2	7.46 (d, 8.7)	7.73 (d, 8.7)	7.05 (d, 2.3)
3	6.80 (d, 8.7)	6.76 (d, 8.7)	
5	6.80 (d, 8.7)	6.76 (d, 8.7)	6.77 (d, 8.2)
6	7.46 (d, 8.7)	7.73 (d, 8.7)	6.95 (dd, 2.3, 8.2)
7	7.67 (d, 16.0)	6.95 (d, 12.8)	7.59 (d, 16.0)
8	6.34 (d, 16.0)	5.79 (d, 12.8)	6.27 (d, 16.0)

2.4. Effects of chemical constituents from the stems of *C. tubulosa* on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes

D-GalN/LPS-induced liver injury is recognized to develop via immunological responses.[19] This type of liver injury is reported to occur in two forms. First, sensitivity to TNF-α by depletion of uridine triphosphate in hepatocytes is increased by D-GalN. Second, pro-inflammatory mediators, such as NO and TNF-α, are released from LPS-activated macrophages (Kupffer's cells). Apoptosis of hepatocytes by TNF-α is reported to have an important role in D-GalN/LPS-induced liver injury.[20]

In our previous studies on hepatoprotective compounds from natural medicines, we reported that several constituents from *Hovenia dulcis*,[21] *Bupleurum scorzonerifolium*,[22,23] *Curcuma zedoaria*,[24–26] *Angelica furcijuga*,[27,28] *Betula platyphylla* var. *japonica*,[29] *Pisum*

Table 3
^{13}C NMR (150 MHz, CD_3OD) data for kankanosides H_1 (**1**), H_2 (**2**), and I (**3**)

Position	**1**	**2**	**3**
	δ_C	δ_C	δ_C
1	131.7	131.8	140.0
2	117.2	117.2	130.0
3	146.0	146.0	129.4
4	144.6	144.6	127.2
5	116.3	116.3	129.4
6	121.3	121.3	130.0
7	36.3	36.3	37.2
8	71.9	72.0	72.1
8-*O*-Glc			
1′	101.7	101.7	104.2
2′	75.1	75.1	76.1
3′	80.5	80.7	81.6
4′	70.7	70.8	70.4
5′	74.8	74.9	74.7
6′	69.3	69.4	69.4
3′-*O*-Rha			
1″	103.3	103.5	103.1
2″	71.8	71.9	72.1
3″	72.6	72.6	72.3
4″	73.6	73.7	73.8
5″	70.8	70.8	70.6
6″	18.4	18.2	18.5
6′-*O*-Glc			
1‴	104.7	104.8	104.7
2‴	74.9	74.9	75.1
3‴	77.8	77.9	77.8
4‴	71.5	71.5	71.5
5‴	77.9	78.0	77.9
6‴	62.6	62.6	62.6
2′-*O*-Ac			
1	20.9	20.9	
2	171.4	171.4	
4′-*O*-Acyl			
1	127.0	127.5	127.6
2	131.4	134.4	115.3
3	116.9	115.9	146.8
4	161.5	160.5	149.8
5	116.9	115.9	116.5
6	131.4	134.4	123.3
7	148.0	147.6	148.2
8	114.7	115.7	114.7
9	168.2	166.7	168.5

sativum,[30] *Salacia reticulata*,[31] *Tilia argentea*,[32] *Anastatica hierochuntica*,[33] *Panax notoginseng*,[34] *Cyperus longus*,[35] *Erycibe expansa*,[36] *Camellia sinensis*,[37] *Sedum sarmentosum*,[38,39] *Sinocrassula indica*,[40] *Hedychium coronarium*,[41] and *Piper chaba*[42,43] showed hepatoprotective effects on the liver injury induced by D-GalN/LPS in mice and/or inhibitory effect on D-GalN-induced cytotoxicity in primary cultured hepatocytes. Since the methanolic extract from the stems of *C. tubulosa* showed hepatoprotective effects on D-GalN/LPS-induced liver injury in mice (vide ante), inhibitory effect of the constituents on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Table 4, echinacoside (**4**, IC_{50} = 10.2 μM), acteoside (**5**, 4.6 μM), isoacteoside (**6**, 5.3 μM), 2′-acetylacteoside (**8**, 4.8 μM), tubulosides A (**10**, 8.6 μM) and B[11] (**22**, 14.6 μM), and kankanoside G[11] (**23**, 14.8 μM) showed strong activity. Their activities were greater than that of commercial silybin (38.8 μM),[33–43] as a positive control.[44,45] The structural requirements of the phenylethanoid glycosides for the activity were as followings; (1) the aglycone part was essential for the activity [**4** >> kankanose (**21**, >100 μM), **5** >> cistanoside F (**20**, >100 μM)]; (2) the aglycone having the 3,4-dihydroxy group showed stronger activity than that having the 4-hydroxy group (**6** > **23**); (3) the 6′-*O*-β-D-glucopyranosyl moiety reduced the activity (**4** < **5**, **10** < **8**); (4) the 8-*O*-β-D-glucopyranosyl part having the 4′-*O*-caffeoyl group showed stronger activity than that having the 6′-*O*-caffeoyl group (**5** ≧ **6**, **8** > **22**); and (5) introduction of the 2′-*O*-acetyl moiety reduced the activity (**8** ≦ **5**, **22** < **6**).

Next, effects of the principal constituents (**4**–**6**) on NO and TNF-α productions, as markers of macrophage activation in LPS-activated mouse peritoneal macrophages were examined.[43,46,47] As the result, these constituents (**4**–**6**) showed neither NO nor TNF-α production inhibitory activities (IC_{50} >100 μM, data not shown). Thus, these compounds were found not to affect the overproductions of NO and TNF-α from LPS-activated macrophages.

2.5. Effects of the chemical constituents on TNF-α-induced cytotoxicity in L929 cells

In order to clarify the effects of the constituents on the sensitivity of hepatocytes to TNF-α, TNF-α-induced decrease in viability of L929 cells, a TNF-α-sensitive cell line,[48] was examined using the MTT assay. Without test samples, the cell viability was reduced to ca. 60% after incubation of the cells with 20 ng/mL TNF-α for 44 h compared to the case without TNF-α. As shown in Table 5, echinacoside (**4**, IC_{50} = 31.1 μM), acteoside (**5**, 17.8 μM), isoacteoside (**6**, 22.7 μM), 2′-acetylacteoside (**8**, 25.7 μM), tubuloside A (**10**, 23.2 μM), and cistantubuloside B_1 (**11**, 21.4 μM) inhibited the decrease of the cell viability, and their activities were found greater than those of piperine[42,43] and silybin. The structural requirements of the phenylethanoid glycosides for the activity were as followings; (1) the aglycone part was essential for the activity [**4** >> kankanose (**21**, >100 μM)]; (2) the aglycone having the 3,4-dihydroxy group showed stronger activity than that having the 4-hydroxy

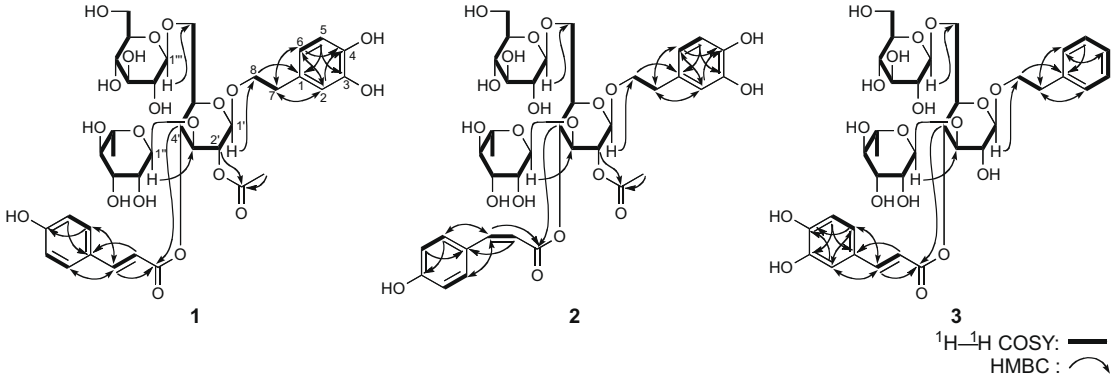

Figure 2. ^{1}H–^{1}H COSY and HMBC correlations for **1**–**3**.

Table 4
Inhibitory effects of the methanolic extract and its constituents from stems of *C. tubulosa* on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes

	Inhibition (%)					
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	IC_{50} (μg/mL)
MeOH extract	0.0 ± 1.8	9.1 ± 2.9[a]	17.3 ± 1.9[b]	29.2 ± 1.4[b]	53.0 ± 2.4[b]	97.3
MeOH-eluted fraction	0.0 ± 1.5	13.6 ± 2.5[a]	26.6 ± 4.1[b]	40.2 ± 3.6[b]	73.5 ± 0.9[b]	34.9
H_2O-eluted fraction	0.0 ± 1.5	−1.8 ± 2.9	0.0 ± 2.8	−2.1 ± 2.1	−8.7 ± 1.5	
	0 μM	3 μM	10 μM	30 μM	100 μM	IC_{50} (μM)
Kankanoside H_1 (**1**)	0.0 ± 1.8	8.7 ± 3.2	16.4 ± 4.2[a]	20.4 ± 2.2[b]	34.0 ± 2.4[b]	
Kankanoside H_2 (**2**)	0.0 ± 0.6	4.4 ± 1.1	11.6 ± 1.3[b]	18.2 ± 1.9[b]	26.3 ± 0.9[b]	
Kankanoside I (**3**)	0.0 ± 0.6	3.9 ± 0.6	13.6 ± 0.3[b]	25.9 ± 1.7[b]	27.7 ± 2.5[b]	
Echinacoside (**4**)	0.0 ± 2.1	32.8 ± 1.4[b]	46.7 ± 4.3[b]	67.7 ± 1.7[b]		10.2
Acteoside (**5**)	0.0 ± 2.4	40.9 ± 1.3[b]	71.8 ± 2.3[b]	119.2 ± 5.4[b]		4.6
Isoacteoside (**6**)	0.0 ± 4.4	43.7 ± 2.1[b]	57.3 ± 2.2[b]	101.2 ± 5.9[b]		5.3
2′-Acetylacteoside (**8**)	0.0 ± 1.9	41.9 ± 3.2[b]	58.4 ± 5.3[b]	95.2 ± 3.2[b]		4.8
Tubuloside A (**10**)	0.0 ± 3.7	31.1 ± 1.6[b]	50.2 ± 4.6[b]	74.6 ± 0.9[b]		8.6
Cistantubuloside B_1 (**11**)	0.0 ± 1.0	3.1 ± 1.2	10.3 ± 1.7[b]	18.5 ± 1.6[b]	31.2 ± 2.7[b]	
Wiedemanninoside C (**14**)	0.0 ± 0.5	4.5 ± 1.7	11.5 ± 0.9[b]	20.6 ± 2.6[b]	39.4 ± 2.8[b]	
Cistantubuloside A (**15**)	0.0 ± 1.9	3.0 ± 1.5	8.2 ± 3.4	17.0 ± 4.1[b]	15.3 ± 3.4[b]	
Syringalide A 3′-*O*-Rha (**16**)	0.0 ± 1.3	9.7 ± 0.7	21.4 ± 1.5[b]	35.7 ± 4.0[b]	55.7 ± 6.1[b]	71.2
Salidroside (**19**)	0.0 ± 1.8	0.9 ± 0.6	1.4 ± 1.4	−0.7 ± 1.8	0.2 ± 1.3	
Cistanoside F (**20**)	0.0 ± 1.5	2.0 ± 0.7	4.0 ± 2.6	7.7 ± 3.9	21.2 ± 0.8[a]	
Kankanose (**21**)	0.0 ± 2.8	−4.9 ± 1.3	−1.3 ± 2.9	−7.9 ± 2.1	−2.8 ± 2.8	
Tubuloside B (**22**)	0.0 ± 4.4	8.6 ± 2.3	33.6 ± 4.5[b]	75.4 ± 2.8[b]		14.6
Kankanoside G (**23**)	0.0 ± 3.0	12.6 ± 3.6[a]	33.3 ± 3.3[b]	72.7 ± 4.1[b]		14.8
Silybin[c]	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] Commercial silybin was purchased from Funakoshi Co., Ltd (Tokyo, Japan).

group [**4** > cistantubuloside A (**15**, >100 μM), **5** > syringalide A 3′-*O*-α-L-rhamnopyranoside (**16**, >100 μM), **6** > kankanoside G (**23**, >100 μM)]; (3) the 6′-*O*-β-D-glucopyranosyl moiety reduced the activity (**4** < **5**); (4) the 8-*O*-β-D-glucopyranosyl part having the 4′-*O*-caffeoyl group showed stronger activity than that having the 6′-*O*-caffeoyl group [**5** > **6**, **8** > tubuloside B (**22**, >100 μM)]; and (5) introduction of the 2′-*O*-acetyl moiety reduced the activity (**8** < **5**, **22** < **6**). These requirements were found similar to those observed in the previous chapter concerning the inhibitory effects on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes.

These in vitro findings suggest the following possible mechanisms of action for the hepatoprotective effect of the constituents of stems of *C. tubulosa*: (i) decreasing of D-GalN-induced cytotoxicity (**4**–**6**, **8**, **10**, **22**, and **23**), and (ii) decreasing of TNF-α-induced cytotoxicity (**4**–**6**, **8**, **10**, and **11**).

2.6. Protective effects of echinacoside (4), acteoside (5), and isoacteoside (6) against liver injury induced by D-GalN/LPS in mice and their mode of action

Finally, we examined the effect of principal phenylethanoid glycosides, echinacoside (**4**), acteoside (**5**), and isoacteoside (**6**) on the D-GalN/LPS-induced liver injury in mice. As shown in Table 6 and **4**–**6** significantly inhibited the increase of sAST and sALT induced by D-GalN/LPS in mice at doses of 25–100 mg/kg, po. On the other hand, **4**–**6** did not affect the overproduction of TNF-α from LPS-activated macrophages. Furthermore, **4**–**6** significantly inhibited the death of L929 cells caused by TNF-α, indicating a decrease in the sensitivity of L929 cells to TNF-α (vide ante). These findings suggest that **4**–**6** reduce the sensitivity of these cells to the TNF-α-induced cytotoxicity. Many compounds that inhibit cell death induced by D-GalN and production of TNF-α have been reported,[24,26,32] but there reported few compounds that selectively reduce the sensitivity of hepatocytes to TNF-α.[42,43,49,50]

In conclusion, from fresh stems of *C. tubulosa*, three new phenylethanoid glycosides, kankanosides H_1 (**1**), H_2 (**2**), and I (**3**) together with 16 known phenylethanoid glycosides (**4**–**19**) and two acylated oligosugars (**20**, **21**) were isolated. Structural requirements for their protective effects on hepatocyte injury induced by D-GalN, and cytoprotective effects on L929 cells caused by TNF-α were as followings; (1) the aglycone part was essential for the activity; (2) the aglycone having the 3,4-dihydroxy group showed stronger activity than that having the 4-hydroxy group; (3) the 6′-*O*-β-D-glucopyranosyl moiety reduced the activity; (4) the 8-*O*-β-D-glucopyranosyl part having the 4′-*O*-caffeoyl group showed stronger activity than that having the 6′-*O*-caffeoyl group; and (5) introduction of the 2′-*O*-acetyl moiety reduced the activity. Furthermore, principal phenylethanoid glycosides, echinacoside (**4**), acteoside (**5**), and isoacteoside (**6**), inhibited the increase in sAST and sALT at doses of 25–100 mg/kg po in D-GalN/LPS-treated mice, and those inhibitory effects were suggested to be dependent on the reduced sensitivity of hepatocytes to TNF-α. The detail mechanisms of action of the phenylethanoid glycosides should be studied further.

3. Experimental

3.1. General

The following instruments were used to obtain spectral and physical data: specific rotations, Horiba SEPA-300 digital polarimeter ($l = 5$ cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; ^1H and ^{13}C NMR spectra, JEOL JNM-ECA600 (600 and 150 MHz) and JEOL JNM-ECS400 (400 and 100 HMz) spectrometers with tetramethylsilane as an internal standard; FABMS and high-resolution FABMS, JEOL JMS-SX 102A mass spectrometer; HPLC detector, Shimadzu RID-10A refractive index, Shimadzu SPD-10A UV–vis, and Shodex OR-2 optical rotation detectors. HPLC column, Cosmosil $5C_{18}$-MS-II and πNAP (Nacalai Tesque Inc., 250 × 4.6 mm id) and (250 × 20 mm id) columns were used for analytical and preparative purposes, respectively.

The following experimental conditions were used for chromatography: normal-phase silica gel column chromatography (CC), silica gel 60 N (Kanto Chemical Co., Ltd, 63–210 mesh, spherical, neutral); reversed-phase silica gel CC, Diaion HP-20 (Nippon Rensui) and Chromatorex ODS DM1020T (Fuji Silysia Chemical,

Table 5
Inhibitory effects of the methanolic extract and its constituents from stems of *C. tubulosa* on TNF-α-induced cytotoxicity in L929 cells

	Inhibition (%)					
	0 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	IC_{50} (μg/mL)
MeOH extract	0.0 ± 1.4	17.6 ± 8.1	40.5 ± 5.3[b]	58.3 ± 4.6[b]	47.9 ± 4.4[b]	18.4
MeOH-eluted fraction	0.0 ± 7.5	21.5 ± 8.3	58.4 ± 4.9[b]	76.0 ± 2.3[b]	77.4 ± 15.1[b]	9.0
H_2O-eluted fraction	0.0 ± 0.7	9.1 ± 2.0	18.6 ± 6.2	11.7 ± 3.7	−31.1 ± 4.5	
	0 μM	3 μM	10 μM	30 μM	100 μM	IC_{50} (μM)
Echinacoside (**4**)	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 (**5**)	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 (**6**)	0.0 ± 1.2	−4.6 ± 3.5	19.0 ± 2.6	61.9 ± 5.9[b]	102.4 ± 8.7[b]	22.7
2′-Acetylacteoside (**8**)	0.0 ± 3.1	2.3 ± 5.0	8.9 ± 6.6	64.1 ± 4.9[b]	107.3 ± 10.4[b]	25.7
Tubuloside A (**10**)	0.0 ± 2.4	14.7 ± 4.6[a]	36.2 ± 4.8[b]	55.2 ± 2.8[b]	101.9 ± 2.2[b]	23.2
Cistantubuloside B_1 (**11**)	0.0 ± 3.9	−14.7 ± 17.2	31.0 ± 4.4[b]	32.8 ± 10.8[b]	122.7 ± 13.7[b]	21.4
Cistantubuloside A (**15**)	0.0 ± 2.3	2.8 ± 1.2	3.6 ± 0.5	4.6 ± 1.6	11.2 ± 1.1[a]	
Syringalide A 3′-*O*-Rha (**16**)	0.0 ± 2.9	4.5 ± 1.0	4.6 ± 1.4	13.3 ± 3.3	22.2 ± 6.4[b]	
Campneoside I (**17**)	0.0 ± 2.0	7.7 ± 2.9	−8.8 ± 8.5	1.9 ± 5.8	7.5 ± 3.1	
Salidroside (**19**)	0.0 ± 6.1	−1.2 ± 7.9	−8.3 ± 10.5	−5.4 ± 5.1	−1.0 ± 4.8	
Kankanose (**21**)	0.0 ± 1.9	−1.1 ± 1.2	2.2 ± 1.8	1.3 ± 1.8	0.8 ± 0.1	
Tubuloside B (**22**)	0.0 ± 4.9	10.7 ± 4.7	13.4 ± 4.7	36.4 ± 13.3[a]	39.2 ± 6.3[b]	
Kankanoside G (**23**)	0.0 ± 2.8	1.3 ± 0.9	4.7 ± 0.5	3.1 ± 2.6	2.9 ± 1.4	
Piperine[42,43]	0.0 ± 1.3	5.5 ± 1.6[a]	5.3 ± 1.4[a]	10.6 ± 0.9[b]	41.8 ± 1.4[b]	
Silybin[c]	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] Commercial silybin was purchased from Funakoshi Co., Ltd (Tokyo, Japan).

Table 6
Inhibitory effects of principal constituents (**4**–**6**) from stems of *C. tubulosa* on D-GalN/LPS-induced liver injury in mice

Treatment	Dose (mg/kg, po)	*n*	sAST		sALT	
			(Karmen unit)	Inhibition (%)	(Karmen unit)	Inhibition (%)
Normal (vehicle)	—	5	58 ± 6[b]	—	25 ± 2[b]	—
Control (D-GalN/LPS)	—	12	11,768 ± 1621	—	5484 ± 666	—
Echinacoside (**4**)	25	8	4562 ± 1413[a]	61.2	3084 ± 1117	43.8
	100	8	3914 ± 1181[b]	66.7	2634 ± 920	52.0
Acteoside (**5**)	25	8	5736 ± 3048[a]	51.3	3047 ± 1462	44.4
	100	8	3703 ± 1594[b]	68.5	2220 ± 1045[a]	59.5
Isoacteoside (**6**)	25	8	6339 ± 1950	46.1	3278 ± 1021	40.2
	100	8	3425 ± 848[b]	70.9	2265 ± 567[a]	58.7
Normal (vehicle)	—	5	95 ± 5[b]	—	19 ± 1[b]	—
Control (D-GalN/LPS)	—	8	9126 ± 1477	—	9830 ± 1605	—
Hydrocortisone	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]$p<0.01$.

Ltd, 100–200 mesh); normal-phase TLC, pre-coated TLC plates with silica gel $60F_{254}$ (Merck, 0.25 mm); reversed-phase TLC, pre-coated TLC plates with silica gel RP-18 F_{254S} (Merck, 0.25 mm); reversed-phase HPTLC, pre-coated TLC plates with silica gel RP-18 WF_{254S} (Merck, 0.25 mm), detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4, followed by heating.

3.2. Plant material

Fresh stems of *C. tubulosa* cultivated at Urumuqi, Xinjiang Province, China were collected in September 2007. The plant material was identified by one of the authors (M.Y.). A voucher of the plant material is on file in our laboratory.

3.3. Extraction and isolation

The fresh stems of *C. tubulosa* (2.98 kg) were finely cut and extracted three times with methanol under reflux for 3 h. Evaporation of the solvent under reduced pressure provided a methanolic extract (249.1 g, 8.36%). The methanolic extract was subjected to Diaion HP-20 CC (5.0 kg, $H_2O \rightarrow$MeOH) to give H_2O- and MeOH-eluted fractions (167.84 g, 5.63% and 81.21 g, 2.73%, respectively). The MeOH-eluted fraction (61.00 g) was subjected to normal-phase silica gel CC [1.8 kg, $CHCl_3$–MeOH–H_2O (15:3:0.4→10:3:0.5→6:4:1, v/v/v)→MeOH] to give seven fractions [Fr. 1 (1.12 g), 2 (9.56 g), 3 (0.89 g), 4 (10.69 g), 5 (8.84 g), 6 (12.52 g), and 7 (4.60 g)]. The fraction 2 (9.56 g) was separated by reversed-phase silica gel CC [400 g, MeOH–H_2O (10:90, v/v)→MeOH→acetone] to give five fractions [Fr. 2–1 (55.9 mg), 2–2 (4.48 g), 2–3 (3.42 g), 2–4 (1.16 g), and 2–5 (31.9 mg)]. The fraction 2–3 (1.00 g) was subjected to HPLC [Cosmosil $5C_{18}$-MS-II, CH_3CN–1% aqueous AcOH (7:93, v/v)] to give seven fractions [Fr. 2–3–1 (66.5 mg), 2–3–2 (20.4 mg), 2–3–3 (26.0 mg), 2–3–4 (136.6 mg), 2–3–5 (380.6 mg), 2–3–7 (84.9 mg)]. The fraction 2–3–4 (106.6 mg) was further purified by HPLC [Cosmosil πNAP, CH_3CN–1% aqueous AcOH (5:95, v/v)] to give salidroside (**19**, 13.7 mg, 0.0027%). The fraction 4 (10.69 g) was separated by reversed-phase silica gel CC [500 g, MeOH–H_2O (30:70, v/v)→MeOH→acetone] to give four fractions [Fr. 4–1 (878.2 mg), 4–2 (7.06 g), 4–3 (1.57 g), and 4–4 (792.8 mg)]. The fraction 4–2 (1.50 g) was subjected to HPLC [Cosmosil $5C_{18}$-MS-II, CH_3CN–1% aqueous AcOH (20:80, v/v)] to give five fractions {Fr. 4–2–1 (42.9 mg), 4–2–2 [= campneoside I (**17**, 67.0 mg, 0.014%)], 4–2–3

[= acteoside (**5**, 1.21 g, 0.26%)], 4–2–4 (41.6 mg), and 4–2–5 (181.3 mg)}. The fraction 4–2–4 (41.6 mg) was further purified by HPLC [Cosmosil πNAP, CH_3CN–1% aqueous AcOH (18:82, v/v)] to give isoacteoside (**6**, 19.1 mg, 0.0040%) and *cis*-acteoside (**7**, 3.4 mg, 0.0007%). The fraction 4–3 (1.57 g) was purified by HPLC [Cosmosil 5C_{18}-MS-II, CH_3CN–1% aqueous AcOH (20:80, v/v)] to give 11 fractions [Fr. 4–3–1 (30.4 mg), 4–3–2 (55.2 mg), 4–3–3 (= **17**, 22.1 mg, 0.0010%), 4–3–4 (= **5**, 224.6 mg, 0.010%), 4–3–5 (27.4 mg), 4–3–6 (43.6 mg), 4–3–7 (= **6**, 825.0 mg, 0.037%), 4–3–8 [= syringalide A 3′-*O*-α-L-rhamnopyranoside (**16**, 37.6 mg, 0.0017%)], 4–3–9 (39.8 mg), 4–3–10 [= 2′-acetylacteoside (**8**, 85.4 mg, 0.0038%)], and 4–3–11 (64.6 mg)]. The fraction 4–3–6 (43.6 mg) was further purified by HPLC [Cosmosil πNAP, CH_3CN–1% aqueous AcOH (18:82, v/v)] to give kankanosides H_1 (**1**, 17.0 mg, 0.0008%) and H_2 (**2**, 3.3 mg, 0.0001%). The fraction 4–3–9 (39.8 mg) was further purified by HPLC [Cosmosil πNAP, CH_3CN–1% aqueous AcOH (18:82, v/v)] to give kankanoside I (**3**, 15.4 mg, 0.0007%) and **6** (3.1 mg, 0.0001%). The fraction 5 (8.84 g) was separated by reversed-phase silica gel CC [400 g, MeOH–H_2O (20:80→30:70, v/v)→MeOH→acetone] to give seven fractions [Fr. 5–1 (870.2 mg), 5–2 (478.9 mg), 5–3 (3.72 g), 5–4 (979.9 mg), 5–5 (1.19 g), 5–6 (1.27 g), and 5–7 (130.1 mg)]. The fraction 5–1 (870.2 mg) was purified by HPLC [Cosmosil πNAP, CH_3CN–1% aqueous AcOH (5:95, v/v)] to give decaffeoylacteoside (**9**, 5.1 mg, 0.0002%) and cistanoside F (**20**, 8.1 mg, 0.0004%). The fraction 5–3 (3.72 g) was purified by HPLC [Cosmosil 5C_{18}-MS-II, CH_3CN–1% aqueous AcOH (15:85, v/v)] to give six fractions [Fr. 5–3–1 (128.6 mg), 5–3–2 [= kankanose (**21**, 9.4 mg, 0.0004%)], 5–3–3 (64.6 mg), 5–3–4 (72.3 mg), 5–3–5 [= echinacoside (**4**, 2.54 g, 0.11%), and 5–3–6 (318.5 mg)]. The fraction 5–3–4 (72.3 mg) was further purified by HPLC [Cosmosil πNAP, CH_3CN–1% aqueous AcOH (10:90, v/v)] to give campneoside II (**18**, 10.6 mg, 0.0005%). The fraction 5–4 (979.9 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, CH_3CN–1% aqueous AcOH (15:85, v/v)] to give nine fractions [Fr. 5–4–1 (28.6 mg), 5–4–2 (90.5 mg), 5–4–3 (99.7 mg), 5–4–4 (= **4**, 25.7 mg, 0.0012%), 5–4–5 (16.4 mg), 5–4–6 (318.5 mg), 5–4–7 (21.6 mg), 5–4–8 (= **5**, 204.4 mg, 0.0091%), and 5–4–9 (134.7 mg)]. The fraction 5–4–6 (318.5 mg) was purified by HPLC [Cosmosil πNAP, CH_3CN–1% aqueous AcOH (15:85, v/v)] to give cistantubulosides B_1 (**11**, 44.9 mg, 0.0020%), B_2 (**12**, 7.6 mg, 0.0003%), and A (**15**, 21.1 mg, 0.0009%) and wiedemanninoside C (**14**, 17.2 mg, 0.0008%). The fraction 5–5 (1.19 g) was purified by HPLC [Cosmosil 5C_{18}-MS-II, CH_3CN–1% aqueous AcOH (18:82, v/v)] to give tubuloside A (**10**, 300.3 mg, 0.013%) and arenarioside (**13**, 5.8 mg, 0.0006%). The fraction 6 (12.52 g) was separated by reversed-phase silica gel CC [600 g, MeOH–H_2O (20:80→30:70, v/v)→MeOH→acetone] to give four fractions [Fr. 6–1 (553.8 mg), 6–2 (10.69 g), 6–3 (1.40 g), and 6–4 (17.8 mg)]. The fraction 6–2 (500.0 mg) was purified by HPLC [Cosmosil 5C_{18}-MS-II, CH_3CN–1% aqueous AcOH (25:75, v/v)] to give **4** (353.1 mg, 0.34%).

3.3.1. Kankanoside H_1 (1)

A white powder, $[\alpha]_D^{24}$ −45.3 (*c* 1.06, MeOH). High-resolution positive-ion FABMS: Calcd for $C_{37}H_{48}O_{20}Na$ $(M+Na)^+$: 835.2637. Found: 835.2633. UV [MeOH, nm (log ε)]: 226 (4.33), 293 (sh, 4.36), 317 (4.52). IR (KBr): 3416, 1736, 1638, 1605, 1518, 1070, 1044 cm^{-1}. ^1H NMR (600 MHz, CD_3OD) *δ*: given in Table 2. ^{13}C NMR (150 MHz, CD_3OD) δ_C: given in Table 3. Positive-ion FABMS: *m*/*z* 835 $(M+Na)^+$. Negative-ion FABMS: *m*/*z* 811 $(M-H)^-$.

3.3.2. Kankanoside H_2 (2)

A white powder, $[\alpha]_D^{25}$ −52.4 (*c* 0.27, MeOH). High-resolution positive-ion FABMS: Calcd for $C_{37}H_{48}O_{20}Na$ $(M+Na)^+$: 835.2637. Found: 835.2644. UV [MeOH, nm (log ε)]: 228 (4.26), 290 (sh, 4.22), 316 (4.37). IR (KBr): 3420, 1717, 1638, 1605, 1508, 1159, 1067 cm^{-1}. ^1H NMR (600 MHz, CD_3OD) *δ*: given in Table 2. ^{13}C NMR (150 MHz, CD_3OD) δ_C: given in Table 3. Positive-ion FABMS: *m*/*z* 835 $(M+Na)^+$. Negative-ion FABMS: *m*/*z* 811 $(M-H)^-$.

3.3.3. Kankanoside I (3)

A white powder, $[\alpha]_D^{25}$ −62.2 (*c* 1.00, MeOH). High-resolution positive-ion FABMS: Calcd for $C_{35}H_{46}O_{18}Na$ $(M+Na)^+$: 777.2581. Found: 777.2585. UV [MeOH, nm (log ε)]: 246 (3.97), 295 (sh, 4.04), 334 (4.23). IR (KBr): 3415, 1717, 1647, 1603, 1508, 1070, 1046 cm^{-1}. ^1H NMR (600 MHz, CD_3OD) *δ*: given in Table 2. ^{13}C NMR (150 MHz, CD_3OD) δ_C: given in Table 3. Positive-ion FABMS: *m*/*z* 777 $(M+Na)^+$. Negative-ion FABMS: *m*/*z* 753 $(M-H)^-$.

3.4. Alkaline and acid hydrolysis of 1–3

Solutions of **1**, **2**, and **3** (each 1.5 mg) in 5% aqueous potassium hydroxide (KOH, 0.5 mL) were stirred at 40 °C for 1 h. Each solution was neutralized with Dowex HCR W2 (H^+ form), and the resins were removed by filtration. Evaporation of the solvent under reduced pressure yielded the corresponding deacylated products, which were subjected to HPLC analysis [column: Cosmosil πNAP, 250 × 4.6 mm id; mobile phase: CH_3CN–1% aqueous AcOH (15:85, v/v); detection: UV (254 nm); flow rate: 1.0 mL/min] to be identified as *trans*-caffeic acid (t_R 9.9 min from **3**), *trans*-*p*-coumaric acid (t_R 17.1 min from **1**), and *cis*-*p*-coumaric acid (t_R 17.8 min from **2**), respectively. Then each was dissolved in 1.0 M HCl (1.0 mL) and heated at 80 °C for 3 h. After being cooled, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form), and the resins were removed by filtration. After removal of the solvent under reduced pressure, the residue was separated by Sep-Pak C18 cartridge column (H_2O→MeOH). The H_2O-eluted fraction was subjected to HPLC analysis under the following conditions: HPLC column, Kaseisorb LC NH_2-60-5, 4.6 mm id × 250 mm (Tokyo Kasei Co., Ltd, Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd, Tokyo, Japan); mobile phase, CH_3CN–H_2O (85:15, v/v); flow rate 0.8 mL/min]. Identification of L-rhamnose (i) and D-glucose (ii) liberated from **1**, **2**, or **3** in the H_2O-eluted fraction were carried out by comparison of their retention times and optical rotation with those of authentic samples [i, t_R 9.9 min (negative)] and [ii, t_R 17.9 min (positive)].

3.5. Bioassay

3.5.1. Reagents

LPS (from *Salmonella enteritidis*), minimum essential medium (MEM), and William's E medium were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA); fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA, USA); and other chemicals were from Wako Pure Chemical Industries, Co., Ltd (Osaka, Japan). 96-Well microplates were purchased from Sumitomo Bakelite Co., Ltd (Tokyo, Japan).

3.5.2. Animals

Male ddY mice were purchased from Kiwa Laboratory Animal Co., Ltd, (Wakayama, Japan). The animals were housed at a constant temperature of 23 ± 2 °C and were fed a standard laboratory chow (MF, Oriental Yeast Co., Ltd, Tokyo, Japan). All of the experiments were performed with conscious mice unless otherwise noted. The experimental protocol was approved by the Experimental Animal Research Committee of Kinki University.

3.5.3. Protective effects on D-GalN/LPS-induced liver injury in mice

The method described by Tiegs et al.[51] was modified and used for this experiment. Briefly, male ddY mice weighing about 25–30 g were fasted for 20 h before the experiment. D-GalN (350 mg/kg) and LPS (10 μg/kg) dissolved in saline were injected intraperitone-

ally to produce liver injury. Each test sample was given orally 1 h before the D-GalN/LPS injection. Blood samples were collected from the infraorbital venous plexus 10 h after D-GalN/LPS injection. sAST and sALT levels were determined using the Reitman–Frankel method (commercial kit, Transaminase CII-Test Wako, Wako Pure Chemical Industries, Co., Ltd). Hydrocortisone was used as a reference compound. Test samples were suspended with 5% Arabic gum 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.

3.5.4. Protective effects on cytotoxicity induced by D-GalN in primary cultured mouse hepatocytes

The hepatoprotective effect of the constituents were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using primary cultured mouse hepatocytes.[33–43] Hepatocytes were isolated from male ddY mice (30–35 g) by collagenase perfusion method. A cell suspension at 4×10^4 cells in 100 μL William's E medium containing FBS (10%), penicillin G (100 units/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well microplate and pre-cultured for 4 h at 37 °C under a 5% CO_2 atmosphere. The medium was added with 100 μL of the fresh medium containing D-GalN (2 mM) with or without the test sample and the hepatocytes were cultured for 44 h. The medium was exchanged with 100 μL of the fresh medium, and 10 μL of MTT [5 mg/mL in phosphate buffered saline (PBS)] solution was added to the medium. After 4 h of incubation, the medium was removed, and 100 μL of isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density (O.D.) of the formazan solution was measured by microplate reader at 570 nm (reference: 655 nm). Inhibition (%) was obtained by following formula.

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

3.5.5. Effects on production of NO in LPS-stimulated mouse peritoneal macrophages

Screening test for NO production using TGC-induced mouse peritoneal macrophages was performed as described previously.[43,46,47]

3.5.6. Effects on production of TNF-α in LPS-stimulated mouse peritoneal macrophages

TNF-α released in the medium was determined as described previously[28] with a slight modification. Briefly, TGC-induced mouse peritoneal macrophages (5×10^5 cells/well) were collected from the peritoneal cavities of male ddY mice, suspended in 100 μL of RPMI 1640 supplemented with 5% FBS, 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 with PBS, and 100 μL of fresh medium containing various concentrations of test compound was added. After 10 min, 100 μL of the medium containing 10 μg/mL LPS was added and incubated for 4 h. TNF-α production in each well was determined using an ELISA kit (Mouse TNF-α ELISA kit, Invitrogen). Each test compound was dissolved in DMSO, and the solution was added to the medium (final DMSO concentration 0.5%).

3.5.7. Inhibitory effects on TNF-α-induced cell death in L929 cells

L929 cells (Dainippon Pharmaceuticals, Osaka, Japan) were maintained in Minimum Essential Medium Eagle (MEM, Sigma–Aldrich) containing 10% FBS, 1% MEM Non-Essential Amino acids (Invitrogen), penicillin G (100 units/mL), and streptomycin (100 μg/mL) at 37 °C under a 5% CO_2 atmosphere. The cells were inoculated in 96-well tissue culture plate [3×10^4 cells/well in 100 μL/well in MEM]. After 44 h of incubation in the medium TNF-α (20 ng/mL) with or without the test sample, the viability of the cells was assessed by the MTT colorimetric assay (vide ante).[49,50] Each test compound was dissolved in DMSO, and the solution was added to the medium (final concentration in DMSO 0.5%).

3.6. Statistics

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

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