HETEROCYCLES, Vol. 75, No. 8, 2008, pp. 1983 - 1995. © The Japan Institute of Heterocyclic Chemistry
Received, 11th March, 2008, Accepted, 14th April, 2008, Published online, 17th April, 2008. COM-08-11385

BIOACTIVE CONSTITUENTS FROM CHINESE NATURAL MEDICINES. XXXI.[1] HEPATOPROTECTIVE PRINCIPLES FROM *SINOCRASSULA INDICA*: STRUCTURES OF SINOCRASSOSIDES A_8, A_9, A_{10}, A_{11}, AND A_{12}

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Abstract —The methanolic extract from the whole plant of *Sinocrassula indica* (Crassulaceae) was found to show hepatoprotective effect on D-galactosamine-induced cytotoxicity in primary cultured mouse hepatocytes. From the methanolic extract, five new acylated flavonol glycosides, sinocrassosides A_8 (**1**), A_9 (**2**), A_{10} (**3**), A_{11} (**4**), and A_{12} (**5**), were isolated together with 25 compounds. The structures of **1**—**5** were elucidated on the basis of chemical and physicochemical evidence. Among the isolated compounds, sinocrassosides A_1 (**6**), A_2 (**7**), and B_2 (**14**) were found to show potent hepatoprotective effect.

Sinocrassula indica (DECHE.) BERGER, a Crassulaceae biennial plant, is distributed on the mountain areas in China (*e.g.* Yunnan, Guangxi, Sichuan, Guizhou, and Hunan provinces of China). The whole plant of *S. indica* has been used for the treatment of hepatitis and otitis media in Chinese traditional medicine.[2] During the course of our studies on bioactive constituents from Chinese natural medicines,[1,3–25] we found that the methanolic extract of the whole plant of *S. indica* inhibited the increase in serum glucose levels in both sucrose- and glucose-loaded rats.[3] Furthermore, from the methanolic extract, 10 acylated flavonol glycosides, sinocrassosides A_1—A_7 and B_1—B_3 (**6**—**15**), were isolated together with 11 known flavonoids (**16**—**26**), two megastigmanes (**27**, **28**), L-phenylalanine, and guanosine.[3,4] As a continuing study on the constituents from *S. indica*, the methanolic extract of this herbal medicine was found to show hepatoprotective effect on D-galactosamine (D-GalN)-induced cytotoxicity in primary cultured mouse hepatocytes. From the methanolic extract, we additionally isolated five new acylated flavonol glycosides, sinocrassosides A_8 (**1**), A_9 (**2**), A_{10} (**3**), A_{11} (**4**), and A_{12} (**5**). This paper deals with the isolation and structure elucidation of **1**—**5** as well as hepatoprotective activities of the isolated compounds.

The methanolic extract from the dried whole plant of *S. indica* (7.7% from the dried plant) was partitioned into an EtOAc–H_2O (1:1, v/v) mixture to furnish an EtOAc-soluble fraction (2.5%) and an

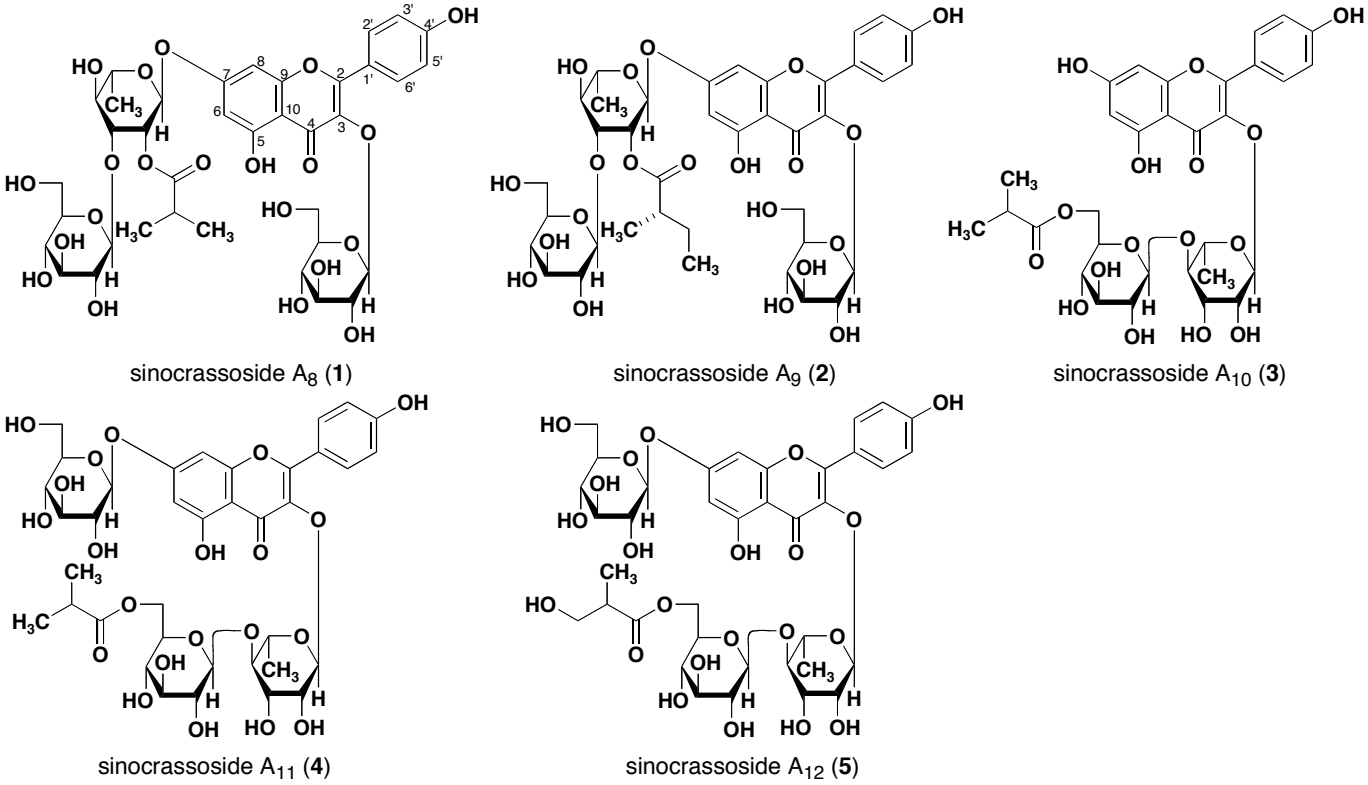


Chart 1

aqueous phase. The aqueous phase was further extracted with *n*-BuOH to give an *n*-BuOH-soluble fraction (1.7%) and a H_2O-soluble fraction (3.4%), which was described previously.[3] As shown in Table 1, the methanolic extract and EtOAc- and *n*-BuOH-soluble fractions were found to show hepatoprotective activities. From the EtOAc- and *n*-BuOH-soluble fractions, **1** (0.0014%), **2** (0.0035%), **3** (0.0048%), **4** (0.0013%), and **5** (0.0011%), were purified using normal- and reversed-phase silica gel chromatographies and finally HPLC.

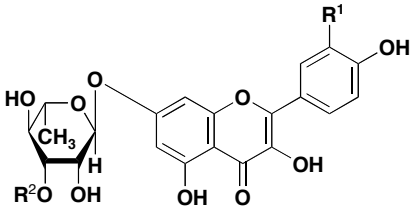
Table 1. Inhibitory Effects of the MeOH Extract from *S. indica* and Its Fractions on D-GalN-induced Cytotoxicity in Primary Cultured Mouse Hepatocytes

	Inhibition (%)				
	0 μg/ml	3 μg/ml	10 μg/ml	30 μg/ml	100 μg/ml
MeOH ext.	0.0 ± 3.4	3.6 ± 3.4	8.5 ± 3.0	16.9 ± 1.5**	37.2 ± 1.9**
EtOAc-soluble fraction	0.0 ± 1.5	9.0 ± 2.4	14.1 ± 3.2*	32.4 ± 1.7**	85.2 ± 5.1**
n-BuOH-soluble fraction	0.0 ± 1.0	2.7 ± 1.6	4.5 ± 2.6	16.0 ± 1.0**	31.6 ± 4.0**
H_2O-soluble fraction	0.0 ± 3.2	5.1 ± 3.4	5.1 ± 2.3	4.9 ± 2.5	11.3 ± 2.3

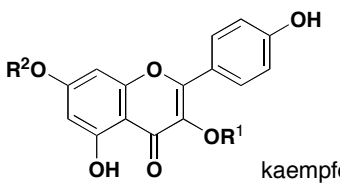
Each value represents the mean±S.E.M. (N=4). Significantly different from the control, *p<0.05, **p<0.01.

Structures of Sinocrassosides A_8 (1), A_9 (2), A_{10} (3), A_{11} (4), and A_{12} (5)

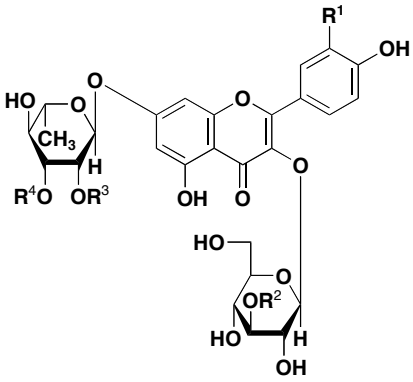
Sinocrassoside A_8 (**1**) was obtained as a yellow powder and exhibited a negative optical rotation ($[\alpha]_D^{23}$ – 44.5° in MeOH). In the UV spectrum of **1**, absorption maxima were observed at 266 (log ε 4.33) and 350 (4.26) nm. The IR spectrum of **1** showed absorption bands at 1721, 1655, and 1599 cm^{-1} assignable to ester carbonyl and γ-pyrone functions and aromatic ring in addition to strong absorption bands at 3432 and 1074 cm^{-1} suggestive of glycoside moieties. In the positive- and negative-ion FAB-MS of **1**, quasimolecular ion peaks were observed at *m/z* 849 $(M+Na)^+$, and 825 $(M–H)^–$, and high-resolution



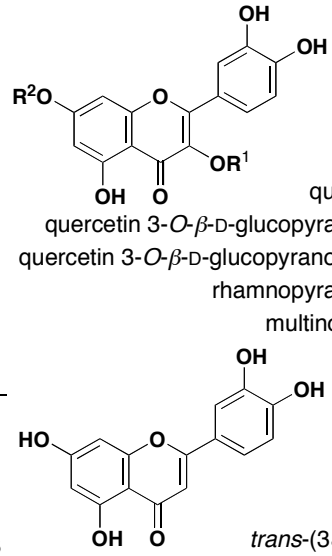
	R^1	R^2
sinocrassoside A_1 (**6**):	H	IB
sinocrassoside A_2 (**7**):	H	(*S*)-2MB
sinocrassoside B_1 (**13**):	OH	IB
sinocrassoside B_2 (**14**):	OH	(*S*)-2MB



	R^1	R^2
kaempferol (**16**):	H	H
kaempferol 3-*O*-β-D-glucopyranoside (**17**):	Glc	H
kaempferol 7-*O*-α-L-rhamnopyranoside (**18**):	H	Rha
kaempferol 3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**19**):	Glc	Rha
multiflorin B (**20**):	Rha(4→1)Glc	H
21:	Rha(4→1)Glc	Rha
1a:	Glc	Rha(3→1)Glc
4a:	Rha(4→1)Glc	Glc

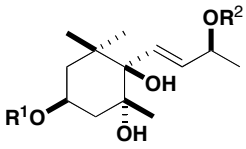


	R^1	R^2	R^3	R^4
sinocrassoside A_3 (**8**):	H	Ac	H	H
sinocrassoside A_4 (**9**):	H	H	H	IB
sinocrassoside A_5 (**10**):	H	Ac	IB	H
sinocrassoside A_6 (**11**):	H	Ac	(*S*)-2MB	H
sinocrassoside A_7 (**12**):	H	Ac	H	(*S*)-2MB
sinocrassoside B_3 (**15**):	OH	H	H	(*S*)-2MB



	R^1	R^2
quercetin (**22**):	H	H
quercetin 3-*O*-β-D-glucopyranoside (**23**):	Glc	H
quercetin 3-*O*-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside (**24**):	Glc	Rha
multinoside A (**25**):	Rha(4→1)Glc	H

luteolin (**26**)



trans-(3*S*,5*R*,6*R*,9*S*)-megastigman-7-ene-3,5,6,9-tetraol 3-*O*-β-D-glucopyranoside (**27**): R^1 = Glc, R^2 = H

trans-(3*S*,5*R*,6*R*,9*S*)-megastigman-7-ene-3,5,6,9-tetraol 9-*O*-β-D-glucopyranoside (**28**): R^1 = H, R^2 = Glc

Glc: β-D-glucopyranosyl
Rha: α-L-rhamnopyranosyl

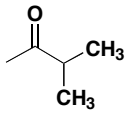
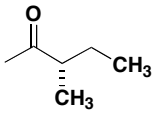
Ac =  IB =  (*S*)-2MB = 

Chart 2

FAB-MS analysis revealed the molecular formula of **1** to be $C_{38}H_{46}O_{21}$. Alkaline hydrolysis of **1** with 10% aqueous potassium hydroxide (KOH)–50% aqueous 1,4-dioxane (1:1, v/v) furnished the desacyl-derivative (**1a**), together with an organic acid, isobutyric acid (IB), which was identified by HPLC analysis of its *p*-nitrobenzyl derivative.[3,4,13,15,16,21] Acid hydrolysis of **1a** with 1.0 M hydrochloric acid (HCl) liberated kaempferol (**16**), together with L-rhamnose and D-glucose, which were identified by HPLC analysis using an optical rotation detector.[1,3–8,11–16,18–22,24,25] The ^{1}H- (DMSO-d_6, Table 2) and ^{13}C-NMR (Table 3) spectra of **1a**, which were assigned by various NMR experiments,[26] showed signals assignable to *meta*-coupled and A_2B_2 type aromatic protons [δ 6.48, 6.86 (1H each, both d, J = 2.2 Hz, 6, 8-H), 6.89, 8.08 (2H each, both d, J = 8.9 Hz, 3',5', 2',6'-H)] together with two β-D-glucopyranosyl and a α-L-rhamnopyranosyl moieties [δ 1.14 (3H, d, J = 5.8 Hz, Rha-6-H_3), 4.48 (1H, d, J = 7.6 Hz, 7-*O*-*terminal*-Glc-1-H), 5.48 (1H, d, J = 7.3 Hz, 3-*O*-Glc-1-H), 5.59 (1H, br s, Rha-1-H)]. The connectivities of glycopyranosyl parts were determined by a heteronuclear multiple-bond correlations (HMBC) experiment on **1a**. Namely, long-range correlations were observed between the following protons and carbons [3-*O*-Glc-1-H and 3-C (δ_C 133.4), Rha-1-H and 7-C (δ_C 161.2), and 7-*O*-*terminal*-Glc-1-H and

Table 2. ^{1}H-NMR (500 MHz) Data of **1**—**3** and **1a**

	1	**1a**	**2**	**3**
position	***δ*H *(J Hz)***	***δ*H *(J Hz)***	***δ*H *(J Hz)***	***δ*H *(J Hz)***
6	6.53 (d, 2.1)	6.48 (d, 2.2)	6.54 (d, 2.1)	6.23 (d, 2.1)
8	6.92 (d, 2.1)	6.86 (d, 2.2)	6.92 (d, 2.1)	6.43 (d, 2.1)
2',6'	8.09 (d, 8.9)	8.08 (d, 8.9)	8.09 (d, 8.9)	7.76 (d, 8.9)
3',5'	6.90 (d, 8.9)	6.89 (d, 8.9)	6.90 (d, 8.9)	6.94 (d, 8.9)
5-OH	12.62 (br s)	12.62 (br s)	12.61 (br s)	12.59 (br s)
	(3-*O*-*Glc*)	(3-*O*-*Glc*)	(3-*O*-*Glc*)	(3-*O*-*Rha*)
1	5.49 (d, 7.3)	5.48 (d, 7.3)	5.49 (d, 7.0)	5.17 (br s)
2	3.21 (dd, 7.3, 8.9)	3.21 (dd, 7.3, 8.9)	3.20 (dd, 7.0, 8.3)	4.06 (br s)
3	3.19 (dd, 8.6, 8.9)	3.19 (dd, 8.9, 8.9)	3.18 (dd, 8.3, 8.9)	3.74 (dd, 3.4, 9.5)
4	3.10 (m)	3.10 (dd, 8.9, 9.2)	3.10 (m)	3.37 (dd, 9.2, 9.5)
5	3.10 (m)	3.09 (m)	3.10 (m)	3.31 (dq, 9.2, 6.1)
6	3.34 (dd, 6.0, 12.5)	3.34 (dd, 6.0, 11.6)	3.35 (dd, 6.5, 11.2)	0.86 (d, 6.1)
	3.58 (dd, 2.0, 12.5)	3.57 (dd, 2.0, 11.6)	3.58 (dd, 2.1, 11.2)	
				(3-*O*-Rha^4—^{1}*Glc*)
1				4.36 (d, 7.9)
2				3.02 (dd, 7.9, 8.5)
3				3.20 (dd, 8.5, 9.5)
4				3.07 (dd, 9.2, 9.5)
5				3.35 (m)
6				4.02 (dd, 7.3, 11.6)
				4.30 (dd, 1.8, 11.6)
	(7-*O*-*Rha*)	(7-*O*-*Rha*)	(7-*O*-*Rha*)	
1	5.70 (br s)	5.59 (br s)	5.70 (br s)	
2	5.29 (dd, 1.9, 3.7)	4.11 (br s)	5.29 (dd, 1.9, 3.7)	
3	4.00 (dd, 3.7, 9.5)	3.77 (dd, 3.1, 8.9)	4.00 (dd, 3.7, 9.5)	
4	3.50 (dd, 9.2, 9.5)	3.50 (dd, 8.9, 9.2)	3.50 (dd, 9.5, 9.5)	
5	3.61 (dq, 9.2, 6.1)	3.52 (dq, 8.9, 5.8)	3.61 (dq, 9.5, 6.1)	
6	1.18 (d, 6.1)	1.14 (d, 5.8)	1.18 (d, 6.1)	
	(7-*O*-Rha^3—^{1}*Glc*)	(7-*O*-Rha^3—^{1}*Glc*)	(7-*O*-Rha^3—^{1}*Glc*)	
1	4.46 (d, 7.6)	4.48 (d, 7.6)	4.46 (d, 7.6)	
2	3.00 (dd, 7.6, 8.9)	3.08 (dd, 7.6, 8.9)	3.00 (dd, 7.6, 8.9)	
3	3.19 (dd, 8.6, 8.9)	3.20 (dd, 8.9, 8.9)	3.19 (dd, 8.9, 9.6)	
4	3.03 (dd, 9.5, 9.6)	3.10 (dd, 8.9, 9.2)	3.01 (dd, 8.6, 8.9)	
5	3.17 (m)	3.17 (m)	3.17 (m)	
6	3.42 (dd, 6.0, 11.5)	3.47 (dd, 6.0, 12.2)	3.40 (dd, 6.5, 11.4)	
	3.66 (dd, 2.0, 11.5)	3.68 (dd, 2.0, 12.2)	3.68 (dd, 2.4, 11.6)	
acyl				
2	2.62 (qq, 6.7, 6.7)		2.45 (ddq, 7.0, 7.0, 7.0)	2.47 (qq, 7.0, 7.0)
3	1.14 (d, 6.7)		1.50 (ddq, 7.0, 14.0, 7.4)	1.05 (d, 7.0)
			1.64 (ddq, 7.0, 14.0, 7.4)	
4	1.15 (d, 6.7)		0.93 (dd, 7.4, 7.4)	1.06 (d, 7.0)
5			1.12 (d, 7.0)	

Measured in DMSO-d_6

Rha-3-C (δ_C 80.6)]. Consequently, the structure of **1a** was determined to be kaempferol 3-*O*-β-D-glucopyranosyl-7-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranoside. The proton and carbon signals in the ^{1}H- (DMSO-d_6, Table 2) and ^{13}C-NMR (Table 3) spectra[26] of **1** were found to be similar to those of **1a**, except for the signals due to an isobutyryl group [δ 1.14, 1.15 (3H each, both d, J = 6.7 Hz, IB-3, 4-H_3), 2.62 (1H, qq, J = 6.7, 6.7 Hz, IB-2-H)]. Comparison of the ^{13}C-NMR data for **1** with those for **1a** indicated an acylation shift at the Rha-2-position of **1** [**1**: δ_C 94.7 (Rha-1-C), 70.5 (Rha-2-C), 77.7 (Rha-3-C); **1a**: δ_C 98.1 (Rha-1-C), 69.5 (Rha-2-C), 80.6 (Rha-3-C)]. In the HMBC experiment of **1**, a long-range correlation was observed between the Rha-2-proton [δ 5.29 (1H, dd, J = 1.9, 3.7 Hz)] and the isobutyryl ester carbonyl carbon (δ_C 175.8). Thus the position of the isobutyryl group in **1** was clarified. On the basis of those findings, the structure of sinocrassoside A_8 was determined as kaempferol 3-*O*-β-D-glucopyranosyl-7-*O*-β-D-glucopyranosyl-(1→3)-α-L-(2-*O*-isobutyryl)-rhamnopyranoside (**1**).

Sinocrassoside A_9 (**2**) was also isolated as a yellow powder with negative optical rotation ($[\alpha]_D^{25}$ –52.6° in MeOH). The UV spectrum of **2** showed absorption maxima at 266 (log ε 4.31) and 350 (4.24) nm.

Table 3. ^{13}C-NMR (125 MHz) Data of **1**—**5** and Related Compounds (**1a** and **4a**)

	1	**1a**	**2**	**3**	**4**	**4a**	**5**
Position	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C	δ_C
2	156.7	156.7	156.7	157.2	157.8	157.8	157.8
3	133.4	133.4	133.5	134.3	134.6	134.5	134.6
4	177.6	177.5	177.6	177.6	177.8	177.8	177.8
5	160.8	160.8	160.8	161.1	160.8	160.8	160.8
6	99.4	99.4	99.5	98.7	99.3	99.3	99.4
7	160.6	161.2	160.6	164.3	162.9	162.9	162.7
8	94.6	94.6	94.7	93.7	94.6	94.6	94.3
9	155.8	155.8	155.8	156.5	156.0	156.0	156.1
10	105.9	105.6	105.9	104.0	105.7	105.7	105.8
1'	120.6	120.5	120.7	120.4	120.2	120.2	120.2
2',6'	130.9	130.9	130.9	130.5	130.6	130.6	130.5
3',5'	115.1	115.1	115.1	115.4	115.4	115.5	115.3
4'	160.1	160.2	160.1	159.9	160.1	160.1	160.1
	(3-*O*-*Glc*)	(3-*O*-*Glc*)	(3-*O*-*Glc*)	(3-*O*-*Rha*)	(3-*O*-*Rha*)	(3-*O*-*Rha*)	(3-*O*-*Rha*)
1	100.7	100.6	100.7	101.8	101.8	101.8	101.8
2	74.1	74.1	74.1	69.7	69.6	69.6	69.6
3	76.3	76.3	76.3	70.2	70.2	70.2	70.2
4	69.8	69.8	69.8	81.7	81.8	81.8	81.8
5	77.5	77.5	77.5	68.7	68.8	68.6	68.9
6	60.8	60.7	60.8	17.1	17.1	17.2	17.2
				(3-*O*-Rha^4-^{1}*Glc*)	(3-*O*-Rha^4-^{1}*Glc*)	(3-*O*-Rha^4-^{1}*Glc*)	(3-*O*-Rha^4-^{1}*Glc*)
1				104.3	104.3	104.6	104.6
2				74.2	74.2	74.3	74.4
3				76.2	76.2	76.5	76.5
4				70.0	70.0	69.7	69.8
5				73.7	73.7	76.9	73.9
6				63.4	63.5	60.7	63.4
	(7-*O*-*Rha*)	(7-*O*-*Rha*)	(7-*O*-*Rha*)		(7-*O*-*Glc*)	(7-*O*-*Glc*)	(7-*O*-*Glc*)
1	94.7	98.1	94.7		99.8	99.6	99.5
2	70.5	69.5	70.5		73.0	72.8	72.8
3	77.7	80.6	77.8		76.3	76.3	76.0
4	70.7	70.4	70.6		69.5	69.4	69.8
5	69.3	68.9	69.3		77.1	77.1	76.9
6	17.8	17.8	17.8		60.5	60.5	60.9
	(7-*O*-Rha^3-^{1}*Glc*)	(7-*O*-Rha^3-^{1}*Glc*)	(7-*O*-Rha^3-^{1}*Glc*)				
1	104.2	104.5	104.4				
2	73.9	73.9	73.9				
3	76.0	76.1	76.0				
4	70.0	69.8	70.1				
5	76.6	76.7	76.5				
6	61.2	60.9	61.4				
acyl							
1	175.8		175.4	176.0	175.8		174.1
2	33.3		40.1	33.1	33.1		42.0
3	18.5		26.2	18.6	18.6		63.1
4	18.8		11.1	18.6	18.6		13.3
5			16.1				

Measured in DMSO-d_6

The IR spectrum of **2** showed absorption bands at 3432, 1728, 1655, 1599, and 1079 cm^{-1} ascribable to hydroxyl, ester carbonyl, γ-pyrone, and ether functions and aromatic ring. The molecular formula $C_{38}H_{48}O_{21}$ of **2**, was determined by the quasimolecular ion peaks in positive- and negative-ion FAB-MS [*m*/*z* 863 $(M+Na)^+$ and *m*/*z* 839 $(M-H)^-$] and by high-resolution FAB-MS. Alkaline hydrolysis of **2** with 10% KOH–50% aqueous 1,4-dioxane (1:1, v/v) furnished **1a** together with *S*-(+)-2-methylbutyric acid [(*S*)-2MB], which was identified by HPLC analysis using an optical rotation detector.[3,4] The proton and carbon signals in the ^{1}H- (DMSO-d_6, Table 2) and ^{13}C-NMR (Table 3) spectra[26] of **2** were very similar to those of **1** {an aglycone part [δ 6.54, 6.92 (1H each, both d, J = 2.1 Hz, 6, 8-H), 6.90, 8.09 (2H each, both d, J = 8.9 Hz, 3',5', 2',6'-H)], two β-D-glucopyranosyl and a α-L-rhamnopyranosyl moieties [δ 1.18 (3H, d, J = 6.1 Hz, Rha-6-H$_3$), 4.46 (1H, d, J = 7.6 Hz, 7-*O*-*terminal*-Glc-1-H), 5.49 (1H, d, J = 7.0 Hz, 3-*O*-Glc-

1-H), 5.70 (1H, br s, Rha-1-H)]} except for the signals due to an acyl moiety [δ 0.93 (3H, dd, J = 7.4, 7.4 Hz, (*S*)-2MB-4-H_3), 1.12 (3H, d, J = 7.0 Hz, (*S*)-2MB-5-H_3), 1.50, 1.64 (1H each, both ddq, J = 7.0, 14.0, 7.4 Hz, (*S*)-2MB-3-H_2), 2.45 (1H, ddq, J = 7.0, 7.0, 7.0 Hz, (*S*)-2MB-2-H)]. The position of (*S*)-2-methylbutyryl part in **2** was confirmed by the HMBC experiment, which showed a long-range correlation between the Rha-2-proton[δ 5.29 (1H, dd, J = 1.9, 3.7 Hz)] and the (2*S*)-2-methylbutyryl ester carbonyl carbon (δ_C 175.4). Consequently, the structure of sinocrassoside A_9 was determined as kaempferol 3-*O*-β-D-glucopyranosyl-7-*O*-β-D-glucopyranosyl-(1→3)-α-L-[2-*O*-(2*S*)-2-methylbutyryl]rhamnopyranoside (**2**).

Sinocrassoside A_{10} (**3**) was obtained as a yellow powder with negative optical rotation ($[\alpha]_D^{26}$ –88.9° in MeOH). The IR spectrum of **3** showed absorption bands at 1736, 1655, 1610 cm^{-1} ascribable to ester carbonyl and γ-pyrone functions and broad bands at 3432 and 1068 cm^{-1}, suggestive of glycoside moiety. In the positive- and negative-ion FAB-MS of **3**, quasimolecular ion peaks were observed at *m/z* 687 $(M+Na)^+$ and *m/z* 663 $(M–H)^–$, and high-resolution FAB-MS analysis revealed the molecular formula of **3** to be $C_{31}H_{36}O_{16}$. On alkaline hydrolysis of **3** with 10% KOH–50% aqueous 1,4-dioxane (1:1, v/v), multiflorin B (**20**)[27] was obtained together with isobutyric acid, which was identified by HPLC analysis of its *p*-nitrobenzyl derivative. The ^{1}H- (DMSO-d_6, Table 2) and ^{13}C-NMR (Table 3) spectra[26] of **3** showed signals assignable to two methyls [δ 1.05, 1.06 (3H each, both d, J = 7.0 Hz, IB-3, IB-4-H_3)], a methane [δ 2.47 (1H, qq, J = 7.0 Hz, IB-2-H)], *meta*-coupled and A_2B_2-type aromatic protons [δ 6.23, 6.43 (1H each, both d, J = 2.1 Hz, 6, 8-H), 6.94, 7.76 (2H each, both d, J = 8.9 Hz, 3',5', 2',6'-H)], together with a rhamnopyranosyl moiety [δ 0.86 (3H, d, J = 6.1 Hz, Rha-6-H_3), 5.17 (1H, br s, Rha-1-H)], and a glucopyranosyl part [δ 4.36 (1H, d, J = 7.9 Hz, Glc-1-H)]. Comparison of the ^{13}C-NMR data for **3** with those for **20**[27] revealed an acylation shift around the Glc-6-position of **3** [**3**: δ_C 73.7 (Glc-5-C), 63.4 (Glc-6-C); **20**: δ_C 76.9 (Glc-5-C), 61.1 (Glc-6-C)]. Furthermore, in the HMBC experiment of **3**, long-range correlation was observed between the Glc-6-protons [δ 4.02 (1H, dd, J = 7.3, 11.6 Hz), 4.30 (1H, dd, J = 1.8, 11.6 Hz)] and the isobutyryl ester carbonyl carbon (δ_C 176.0). Consequently, the position of the isobutyryl ester moiety in **3** was determined and the structure of sinocrassoside A_{10} was elucidated as kaempferol 3-*O*-β-D-(6-*O*-isobutyryl)glucopyranosyl-(1→4)-α-L-rhamnopyranoside (**3**).

Sinocrassosides A_{11} (**4**) and A_{12} (**5**) were obtained as a yellow powder with negative optical rotations (**4**: $[\alpha]_D^{26}$ –10.5°; **5**: $[\alpha]_D^{26}$ –11.3°, both in MeOH). The molecular formulas of **4** ($C_{37}H_{46}O_{21}$) and **5** ($C_{37}H_{46}O_{22}$) were determined from the positive- and negative-ion FAB-MS and by high-resolution FAB-MS. Alkaline hydrolysis of **4** and **5** with 10% KOH–50% aqueous 1,4-dioxane (1:1, v/v) gave the common desacyl-derivative (**4a**), together with isobutyric acid from **4** and 3-hydroxyisobutyric acid (3HIB) from **5**, which was identified by HPLC analysis, respectively. Acid hydrolysis of **4a** with 1.0 M HCl liberated **16**, L-rhamnose, and D-glucose, which were identified by HPLC analysis. The ^{1}H- (DMSO-d_6, Table 4) and ^{13}C-NMR (Table 3) spectra[26] of **4a** indicated the presence of the following functions: a kaempferol part [δ 6.46, 6.76 (1H each, both d, J = 1.8 Hz, 6, 8-H), 6.92, 7.78 (2H, d, J = 8.9 Hz, 3',5', 2',6'-H)], an α-L-rhamnopyranosyl moiety [δ 0.92 (3H, d, J = 6.1 Hz, Rha-6-H_3), 5.27 (1H, br s, Rha-1-H)], and two glucopyranosyl groups [δ 4.30 (1H, d, J = 7.9 Hz, 3-*O-terminal*-Glc-1-H), 5.07 (1H, d, J = 7.1 Hz, 7-*O*-Glc-1-H)]. In the HMBC experiment of **4a**, long-range correlations were observed

between the following proton and carbon pairs [Rha-1-H and 3-C (δ_C 134.5), 3-*O-terminal*-Glc-1-H and Rha-4-C (δ_C 81.8), and 7-*O*-Glc-1-H and 7-C (δ_C 162.9)]. Thus, the structure of **4a** was clarified as kaempferol 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-*O*-β-D-glucopyranoside.

Table 4. ^{1}H-NMR (500 MHz) Data of **4**, **5** and **4a**

	4	**4a**	**5**
Position	***δ*H *(J Hz)***	***δ*H *(J Hz)***	***δ*H *(J Hz)***
6	6.46 (d, 1.8)	6.46 (d, 1.8)	6.45 (d, 2.1)
8	6.77 (d, 1.8)	6.76 (d, 1.8)	6.76 (d, 2.1)
2',6'	7.78 (d, 8.6)	7.78 (d, 8.9)	7.77 (d, 8.5)
3',5'	6.94 (d, 8.6)	6.92 (d, 8.9)	6.94 (d, 8.5)
5-OH	12.59 (br s)	12.58 (br s)	12.60 (br s)
	(3-*O-Rha*)	(3-*O-Rha*)	(3-*O-Rha*)
1	5.19 (br s)	5.27 (br s)	5.21 (br s)
2	4.06 (br s)	4.06 (br s)	4.06 (br s)
3	3.74 (dd, 3.4, 9.5)	3.69 (dd, 3.4, 9.5)	3.76 (dd, 3.4, 9.5)
4	3.38 (dd, 9.2, 9.5)	3.41 (dd, 9.2, 9.5)	3.38 (dd, 9.2, 9.5)
5	3.31 (dq, 9.2, 6.1)	3.32 (dq, 9.2, 6.1)	3.31 (dq, 9.2, 6.1)
6	0.87 (d, 6.1)	0.92 (d, 6.1)	0.93 (d, 6.1)
	(3-*O*-Rha^4—^{1}*Glc*)	(3-*O*-Rha^4—^{1}*Glc*)	(3-*O*-Rha^4—^{1}*Glc*)
1	4.35 (d, 7.9)	4.30 (d, 7.9)	4.31 (d, 7.9)
2	3.02 (dd, 7.9, 8.6)	2.98 (dd, 7.9, 8.6)	3.00 (dd, 7.9, 8.6)
3	3.19 (dd, 8.6, 9.2)	3.17 (dd, 8.6, 9.2)	3.15 (dd, 8.6, 9.2)
4	3.07 (dd, 9.2, 9.5)	3.06 (dd, 9.2, 9.5)	3.06 (dd, 9.2, 9.5)
5	3.35 (m)	3.05 (m)	3.75 (m)
6	4.02 (dd, 7.4, 11.6)	3.69 (dd, 5.5, 11.2)	3.95 (dd, 6.0, 11.6)
	4.29 (dd, 1.8, 11.6)	3.76 (dd, 1.8, 11.2)	4.43 (dd, 1.8, 11.6)
	(7-*O-Glc*)	(7-*O-Glc*)	(7-*O-Glc*)
1	5.07 (d, 7.4)	5.07 (d, 7.1)	5.07 (d, 7.4)
2	3.30 (dd, 7.4, 9.5)	3.29 (dd, 7.1, 9.5)	3.30 (dd, 7.4, 9.5)
3	3.32 (dd, 9.2, 9.5)	3.31 (dd, 9.2, 9.5)	3.32 (dd, 9.2, 9.5)
4	3.17 (dd, 9.2, 9.3)	3.17 (dd, 8.3, 9.2)	3.17 (dd, 8.3, 9.2)
5	3.47 (m)	3.47 (m)	3.47 (m)
6	3.45 (dd, 5.5, 11.2)	3.44 (dd, 5.5, 11.2)	3.45 (dd, 5.5, 11.2)
	3.71 (dd, 1.8, 11.2)	3.62 (dd, 1.8, 11.2)	3.71 (dd, 1.8, 11.2)
acyl			
2	2.48 (qq, 7.0, 7.0)		2.51 (ddq, 7.0, 7.0, 7.0)
3	1.05 (d, 7.0)		3.36 (dd, 7.0, 12.8)
			3.50 (dd, 7.0, 12.8)
4	1.05 (d, 7.0)		0.98 (d, 7.0)
5			

Measured in DMSO-d_6

The proton and carbon signals in the ^{1}H-(DMSO-d_6, Table 4) and ^{13}C-NMR (Table 3) spectra[26] of **4** and **5** were found to be similar to those of **4a**, except for the signals due to an acyl group: [**4**: δ 1.05, 1.05 (3H each, both d, J = 7.0 Hz, IB-3, IB-4-H$_3$), 2.48 (1H, qq, J = 7.0, 7.0 Hz, IB-2-H); **5**: δ 0.98 (3H, d, J = 7.0 Hz, 3HIB-4-H$_3$), 2.51 (1H, ddq, J = 7.0, 7.0, 7.0 Hz, 3HIB-2-H), 3.36, 3.50 (1H each, both dd, J = 7.0, 12.8 Hz, 3HIB-3-H$_2$)]. In the HMBC experiment of **4** and **5**, long-range correlation was observed between the 6-protons of the 7-*O-terminal*-glucopyranosyl part [**4**: δ 3.45 (1H, dd, J = 5.5, 11.2 Hz), 3.71 (1H, dd, J = 1.8, 11.2 Hz); **5**: δ 3.45 (1H, dd, J = 5.5, 11.2 Hz), 3.71 (1H, dd, J = 1.8, 11.2 Hz)] and the acyl ester carbonyl carbon (**4**: δ_C 175.8; **5**: δ_C 174.1). Consequently, the structures of sinocrassosides A$_{11}$ and A$_{12}$ were elucidated as kaempferol 3-*O*-β-D-(6-*O*-isobutyryl)glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-*O*-β-D-glucopyranoside and kaempferol 3-*O*-β-D-(6-*O*-3-hydroxyisobutyryl)glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-*O*-β-D-glucopyranoside, respectively.

Protective Effects on D-GalN-induced Cytotoxicity in Primary Cultured Mouse Hepatocytes

Since the methanolic extract of *S. indica* was found to show protective effect on D-GalN-induced cytotoxicity in primary cultured mouse hepatocytes (Table 1), the inhibitory effects of the constituents from this herbal medicine were also examined. As shown in Table 5, sinocrassosides A$_1$[3] (**6**, IC$_{50}$ = 19.2 μM), A$_2$[3] (**7**, 11.6 μM), and B$_2$[3] (**14**, 23.2 μM), kaempferol 3-*O*-β-D-glucopyranoside[3] (**17**, 16.8 μM),

kaempferol 7-*O*-α-L-rhamnopyranoside[3] (**18**, 12.5 μM), and kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-7-*O*-α-L-rhamnopyranoside[3] (**21**, 21.2 μM), were found to show strong inhibitory activity. The activities of 3- or 7-*O*-mono-glycosides (**6**, **7**, **14**, **17**, **18**, **23**) were stronger than those of corresponding aglycones, kaempferol (**16**, 51.8 μM) and quercetin (**22**, 48.0 μM).

Table 5. Inhibitory Effects of Constituents from *S. indica* on D-GalN-induced Cytotoxicity in Primary Cultured Mous Hepatocytes

	Inhibition (%)					IC_{50}
	0 μM	3 μM	10 μM	30 μM	100 μM	(μM)
sinocrassoside A_9 (**2**)	0.0 ± 3.1	1.7 ± 1.4	8.3 ± 3.8	14.8 ± 3.1*	18.9 ± 3.7**	
sinocrassoside A_{10} (**3**)	0.0 ± 2.6	-6.3 ± 1.0	-5.7 ± 3.9	12.9 ± 2.2	74.5 ± 1.7**	59.4
sinocrassoside A_1 (**6**)	0.0 ± 2.9	5.1 ± 3.3	13.0 ± 3.5*	85.9 ± 5.7**	-7.0 ± 6.6[#]	19.2
sinocrassoside A_2 (**7**)	0.0 ± 0.8	5.1 ± 1.2	22.4 ± 3.4*	99.8 ± 9.3**	13.1 ± 9.3	11.6
sinocrassoside A_4 (**9**)	0.0 ± 2.3	6.5 ± 2.4	18.9 ± 2.7**	46.8 ± 1.9**	126.2 ± 6.3**	42.9
sinocrassoside A_5 (**10**)	0.0 ± 2.4	-5.8 ± 2.4	-0.5 ± 2.4	29.5 ± 4.4**	94.2 ± 2.4**	38.6
sinocrassoside A_7 (**12**)	0.0 ± 2.2	3.5 ± 1.8	14.8 ± 1.9*	39.2 ± 3.9**	78.7 ± 1.1**	38.2
sinocrassoside B_1 (**13**)	0.0 ± 1.3	2.9 ± 1.0	7.3 ± 1.6*	32.9 ± 1.1**	51.4 ± 0.9**	86.1
sinocrassoside B_2 (**14**)	0.0 ± 1.0	7.4 ± 4.3	16.2 ± 2.1*	63.3 ± 6.4**	-13.1 ± 2.8[#]	23.2
sinocrassoside B_3 (**15**)	0.0 ± 3.3	4.1 ± 4.3	14.8 ± 3.8	53.3 ± 8.4**	108.5 ± 10.0**	32.9
kaempferol (**16**)	0.0 ± 2.2	3.0 ± 2.0	13.7 ± 4.5	39.6 ± 2.1**	64.5 ± 6.6**	51.8
kaempferol 3-*O*-Glc (**17**)	0.0 ± 1.1	4.0 ± 0.8	24.6 ± 2.0**	72.4 ± 3.5**	102.5 ± 2.7**	16.8
kaempferol 7-*O*-Rha (**18**)	0.0 ± 4.1	10.4 ± 1.8	28.5 ± 4.9*	80.8 ± 11.2**	124.8 ± 7.5**	12.5
19	0.0 ± 1.1	3.8 ± 2.1	8.1 ± 0.6*	24.6 ± 1.5**	59.7 ± 3.1**	72.9
multiflorin B (**20**)	0.0 ± 2.3	-1.4 ± 2.1	-0.3 ± 2.4	3.2 ± 2.4	14.6 ± 4.1*	
21	0.0 ± 2.3	15.0 ± 3.4*	26.3 ± 2.8**	55.0 ± 0.7**	86.9 ± 4.0**	21.2
quercetin (**22**)	0.0 ± 1.1	1.3 ± 1.7	7.4 ± 3.0	21.5 ± 3.0**	79.7 ± 2.7**	48.0
quercetin 3-*O*-Glc (**23**)	0.0 ± 1.4	3.1 ± 1.6	12.1 ± 0.9*	30.1 ± 3.6**	104.1 ± 7.3**	32.0
24	0.0 ± 3.2	2.3 ± 2.9	9.0 ± 3.7	11.6 ± 0.9*	16.7 ± 1.7**	
multinoside A (**25**)	0.0 ± 0.8	5.9 ± 1.5	8.1 ± 2.3*	22.7 ± 1.2**	47.3 ± 1.6**	
luteolin (**26**)	0.0 ± 0.4	3.0 ± 2.5	6.9 ± 2.7	38.8 ± 1.3**	-42.7 ± 0.4[#]	
27	0.0 ± 1.9	2.5 ± 2.7	0.8 ± 1.8	11.2 ± 3.3*	8.2 ± 3.2	
sinocrassoside B_4[28]	0.0 ± 2.6	10.4 ± 5.4	8.9 ± 3.3	28.8 ± 2.4**	38.3 ± 2.7**	
sinocrassoside B_5[28]	0.0 ± 0.7	7.7 ± 0.9	16.3 ± 0.6**	28.6 ± 3.1**	66.9 ± 1.6**	55.2
sinocrassoside C_1[28]	0.0 ± 1.8	0.9 ± 1.4	8.2 ± 0.2	26.5 ± 3.6**	63.5 ± 6.2**	63.6
sinocrassoside D_1[28]	0.0 ± 5.2	-1.1 ± 2.0	3.5 ± 3.0	15.9 ± 1.4**	51.5 ± 0.9**	95.0
sinocrassoside D_3[28]	0.0 ± 2.1	6.8 ± 1.8	11.1 ± 1.0*	20.4 ± 1.3**	30.6 ± 2.0**	
silybin[a,29]	0.0 ± 0.3	4.8 ± 1.1	7.7 ± 0.7	45.2 ± 8.8**	77.0 ± 5.5**	38.8

Each value represents the mean±S.E.M. (N=4). Significantly different from the control, *p<0.05, **p<0.01.
[a]Commercial silybin was purchased from Funakoshi Co., Ltd. (Tokyo, Japan).
[#]Cytotoxicity

EXPERIMENTAL

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l = 5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrophotometer; EI-MS and high-resolution MS, JEOL JMS-GCMATE mass spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JEOL EX-270 (270 MHz) and JNM-LA500 (500 MHz) spectrometers; ^{13}C-NMR spectra, JEOL EX-270 (68 MHz) and JNM-LA500 (125 MHz) spectrometers with tetramethylsilane as an internal standard; HPLC detector, Shimadzu RID-6A refractive index and SPD-10A*vp* UV-VIS detectors; and HPLC column, YMC-Pack

ODS-A (YMC Co., Ltd. 250 × 4.6 mm i.d. and 250 × 20 mm i.d.) columns were used for analytical and preparative purposes, respectively.

The following experimental conditions were used for chromatography: normal-phase column chromatography; Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150–350 mesh), reversed-phase column chromatography; Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100–200 mesh); TLC, pre-coated TLC plates with Silica gel 60F_{254} (Merck, 0.25 mm) (normal-phase) and 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) (reversed-phase) and detection was achieved by spraying with 1% $Ce(SO_4)_2$-10% aqueous H_2SO_4, followed by heating.

Plant Material

This item was described in a previous report.[3]

Extraction and Isolation

Fractions 6-1 (9980 mg) and 7-2 (340 mg) obtained from the EtOAc-soluble fraction and fractions 4-8 (970 mg) and 5-8 (1310 mg) from the *n*-BuOH-soluble fraction of the methanolic extract from the whole plant of *S. indica* (9.9 kg, cultivated in Guangxi province, China) as reported previously.[3]

Fraction 6-1 (1180 mg) from the EtOAc-soluble fraction was purified by HPLC [MeOH-H_2O (52:48, v/v)] to give sinocrassoside A_{10} (**3**, 14.7 mg, 0.0048%) together with sinocrassosides A_3 (**8**, 4.6mg, 0.0015%), A_4 (**9**, 10.0 mg, 0.0032%), and B_3 (**15**, 11.3 mg, 0.0037%), kaempferol 3-*O*-*β*-D-glucopyranoside (**17**, 14.0 mg, 0.0046%), and kaempferol 3-*O*-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranoside (**19**, 32.9 mg, 0.0070%). Fraction 7-2 (340 mg) from the EtOAc-soluble fraction was subjected to HPLC [MeOH-H_2O (60:40, v/v)] to give sinocrassoside A_9 (**2**, 22.3 mg, 0.0006%). Fraction 4-8 (970 mg) from the *n*-BuOH-soluble fraction was further purified by HPLC [MeOH-H_2O (55:45, v/v)] to give sinocrassosides A_8 (**1**, 72.0 mg, 0.0014%) and A_9 (**2**, 150.4 mg, 0.0029%). Fraction 5-8 (300 mg) from the *n*-BuOH-soluble fraction was further purified by HPLC [MeOH-H_2O (40:60, v/v)] to give sinocrassosides A_{11} (**4**, 15.8 mg, 0.0013%) and A_{12} (**5**, 13.1 mg, 0.0011%) together with kaempferol 3-*O*-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranoside (**19**, 17.9 mg, 0.0015%), kaempferol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-7-*O*-*α*-L-rhamnopyranoside (**21**, 9.6 mg, 0.0008%), quercetin 3-*O*-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranoside (**24**, 35.3 mg, 0.0030%), and multinoside A (**25**, 27.6 mg, 0.0023%).

Sinocrassoside A_8 (**1**): a yellow powder, $[\alpha]_D^{23}$ –44.5° (*c* 2.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{38}H_{46}O_{21}Na$ $(M+Na)^+$: 849.2429. Found: 849.2438. UV [MeOH, nm (log *ε*)]: 266 (4.33), 350 (4.26). IR (KBr): 3432, 1721, 1655, 1599, 1074 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6) *δ*: given in Table 2. ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C: given in Table 3. Positive-ion FAB-MS *m/z*: 849 $(M+Na)^+$. Negative-ion FAB-MS *m/z*: 825 $(M–H)^–$.

Sinocrassoside A_9 (**2**): a yellow powder, $[\alpha]_D^{25}$ –52.6° (*c* 1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{38}H_{48}O_{21}Na$ $(M+Na)^+$ 863.2586. Found: 863.2581. UV [MeOH, nm (log *ε*)]: 266

(4.31), 350 (4.24). IR (KBr): 3432, 1728, 1655, 1599, 1079 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: given in Table 2. ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C: given in Table 3. Positive-ion FAB-MS *m/z*: 863 $(M+Na)^+$. Negative-ion FAB-MS *m/z*: 839 $(M-H)^-$.

Sinocrassoside A_{10} (**3**): a yellow powder, $[\alpha]_D^{26}$ –88.9° (*c* 1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{31}H_{36}O_{16}Na$ $(M+Na)^+$ 687.1904. Found: 687.1901. UV [MeOH, nm (log ε)]: 265 (4.31), 347 (4.16). IR (KBr): 3432, 1736, 1655, 1610, 1068 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: given in Table 2. ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C: given in Table 3. Positive-ion FAB-MS *m/z*: 687 $(M+Na)^+$. Negative-ion FAB-MS *m/z*: 663 $(M-H)^-$.

Sinocrassoside A_{11} (**4**): a yellow powder, $[\alpha]_D^{26}$ –10.5° (*c* 0.70, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{37}H_{46}O_{21}Na$ $(M+Na)^+$ 849.2429. Found: 849.2436. UV [MeOH, nm (log ε)]: 266 (4.40), 343 (4.23). IR (KBr): 3432, 1719, 1655, 1603, 1074 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: given in Table 4. ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C: given in Table 3. Positive-ion FAB-MS *m/z*: 849 $(M+Na)^+$. Negative-ion FAB-MS *m/z*: 825 $(M-H)^-$.

Sinocrassoside A_{12} (**5**): a yellow powder, $[\alpha]_D^{26}$ –11.3° (*c* 1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{37}H_{46}O_{22}Na$ $(M+Na)^+$ 865.2378. Found: 865.2381. UV [MeOH, nm (log ε)]: 265 (4.31), 344 (4.13). IR (KBr): 3432, 1719, 1655, 1605, 1075 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: given in Table 4. ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C: given in Table 3. Positive-ion FAB-MS *m/z*: 865 $(M+Na)^+$. Negative-ion FAB-MS *m/z*: 841 $(M-H)^-$.

Alkaline Hydrolysis of 1—5

A solution of **1**—**5** (each 5.0 mg) in 10% aqueous potassium hydroxide (KOH)—50% aqueous 1,4-dioxane (1:1, v/v, 1.0 mL) was stirred at 37 °C for 1 h. Removal of the solvent under reduced pressure gave a reaction mixture. A part of the reaction mixture was dissolved in $(CH_2)_2Cl_2$ (2.0 mL) and the solution was treated with *p*-nitrobenzyl-*N*-*N*'-diisopropylisourea (10 mg), then the whole was stirred at 80 °C for 1 h. The reaction solution was subjected to HPLC analysis [column: YMC-Pack ODS-A, 250 × 4.6 mm i.d.; mobile phase: MeOH–H_2O (70:30, v/v); detection: UV (254 nm); flow rate: 0.9 mL/min] to identify the *p*-nitrobenzyl esters of 3-hydroxyisobutyric acid (**a**, t_R 10.5 min) from **5**, isobutyric acid (**b**, t_R 11.5 min) from **1**, **3**, and **4**, and 2-methylbutyric acid (**c**, t_R 16.9 min) from **2**, which were carried out by comparison of their retention time with those of commercially obtained standard samples. The rest of the reaction mixture was neutralized with Dowex HCR W2 (H^+ form) and the resin was removed by filtration. Evaporation of the solvent from the filtrate under reduced pressure yielded a product. A part of the product was subjected to HPLC analysis [column: YMC-Pack ODS-AQ, 250 × 4.6 mm i.d.; mobile phase: CH_3CN–0.1% aqueous H_3PO_4 (20:80, v/v); detection: optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan)]; flow rate: 1.0 mL/min] to identify *S*-(+)-2-methylbutyric acid [**c'**, t_R 15.4 min (positive)] from **2**, which was carried out by comparison of its retention time and optical rotation with that of commercially obtained standard sample. The rest of the product was subjected to HPLC [MeOH–H_2O (50:50 v/v)] to give kaempferol 3-*O*-β-D-glucopyranosyl-7-*O*-β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranoside (**1a**, 2.0 mg from **1**, 2.0 mg from **2**), multiflorin B[27] (**20**, 1.5 mg form **3**), kaempferol 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-*O*-β-D-glucopyranoside (**4a**, 1.2 mg from **4**, 1.4

mg from **5**).

Kaempferol 3-*O*-*β*-D-glucopyranosyl-7-*O*-*β*-D-glucopyranosyl-(1→3)-*α*-L-rhamnopyranoside (**1a**): a yellow powder, $[\alpha]_D^{26}$ –52.5° (*c* 1.00, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{39}H_{48}O_{21}Na$ $(M+Na)^+$ 863.2586. Found: 863.2581. UV [MeOH, nm (log *ε*)]: 266 (4.31), 350 (4.24). IR (KBr): 3432, 1655, 1599, 1075 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6) *δ*: given in Table 2. ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C: given in Table 3. Positive-ion FAB-MS *m*/*z*: 863 $(M+Na)^+$. Negative-ion FAB-MS *m*/*z*: 839 $(M-H)^-$.

Kaempferol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-7-*O*-*β*-D-glucopyranoside (**4a**): a yellow powder, $[\alpha]_D^{21}$ –98.1° (*c* 0.20, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{33}H_{40}O_{20}Na$ $(M+Na)^+$ 779.2011. Found: 779.2007. UV [MeOH, nm (log *ε*)]: 265 (4.17), 343 (4.02). IR (KBr): 3432, 1655, 1603, 1074 cm^{-1}. ^{1}H-NMR (500 MHz, DMSO-d_6) *δ*: given in Table 4. ^{13}C-NMR (125 MHz, DMSO-d_6) δ_C: given in Table 3. Positive-ion FAB-MS *m*/*z*: 779 $(M+Na)^+$. Negative-ion FAB-MS *m*/*z*: 755 $(M-H)^-$.

Acid Hydrolysis of 1a and 4a

A solution of **1a**, and **4a** (1.0 mg both) in 1 M HCl (2.0 mL) was heated under reflux for 3 h. After cooling, the reaction mixture was extracted with EtOAc. The EtOAc-soluble fraction was subjected to HPLC analysis under the following conditions, respectively: HPLC column, YMC-Pack ODS-A, 4.6 mm i.d. × 250 mm (YMC Co., Ltd., Ktoyo, Japan); detection, UV (254 nm); mobile phase, MeOH-1% aqueous AcOH (60:40, v/v); flow rate 1.0 mL/min]. Identification of kaempferol (**22**, t_R 15.1 min) present in the EtOAc-soluble fraction was carried out by comparison of their retention time with that of authentic sample, respectively. On the other hand, the aqueous layer was subjected to HPLC analysis under the following conditions, respectively: HPLC column, Kaseisorb LC NH_2-60-5, 4.6 mm i.d. × 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**) present in the aqueous layer was carried out by comparison of their retention time and optical rotation with those of an authentic samples. t_R: (**i**) 7.8 min (negative optical rotation) and (**ii**) 13.9 min (positive optical rotation), respectively.

Bioassay Method

Protective Effect on Cytotoxicity Induced by D-GalN in Primary Cultured Mouse Hepatocytes

The hepatoprotective effects 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.[44] Hepatocytes were isolated from male ddY mice (30—35 g) by collagenase perfusion method. The cell suspension at 4×10^4 cells in 100 *μ*L William's E medium containing fetal calf serum (10%), penicillin G (100 units/mL), and streptomycin (100 *μ*g/mL) was inoculated in a 96-well microplate, and precultured for 4 h at 37 ˚C under a 5% CO_2 atmosphere. The fresh medium (100 *μ*L) containing D-GalN (2 mM) and a test sample were added and the hepatocytes were cultured for 44 h. The medium was exchanged with 100 *μ*L of the fresh medium, and 10 *μ*L of MTT (5 mg/mL in phosphate buffered saline) solution was

added to the medium. After 4 h culture, the medium was removed, 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.

Inhibition (%) = [(O.D.(sample)—O.D.(control))/(O.D.(normal)—O.D.(control))] × 100

Statistics

Values were expressed as means±S.E.M. For statistical analysis, one-way analysis of variance followed by Dunnett's test was used. Probability (P) values less than 0.05 were considered significant. ID_{50} values were estimated based on linear regressions of probit-transformed values of inhibition (%).

ACKNOWLEDGMENTS

M. Yoshikawa and H. Matsuda were supported by the 21st COE Program, Academic Frontier Project, and a Grant-in Aid for Scientific Research from MEXT (the Ministry of Education, Culture, Sports, Science and Technology of Japan). T. Morikawa and N. Ninomiya were supported by High-tech Research Center Project (2007-2011) and a Grant-in Aid for Scientific Research from MEXT.

REFERENCES AND NOTES

1. Part XXX: M. Yoshikawa, S. Nakamura, X. Li, and H. Matsuda, *Chem. Pharm. Bull.*, in press.
2. Shanghai Scientific and Technologic Press, 'Dictionary of Chinese Traditional Medicines', Shogakkan, Tokyo, 1985, pp. 364.
3. M. Yoshikawa, T. Wang, T. Morikawa, H. Xie, and H. Matsuda, *Chem. Pharm. Bull.*, 2007, **55**, 1308.
4. T. Morikawa, H. Xie, T. Wang, H. Matsuda, and M. Yoshikawa, *Chem. Biodiversity*, submitted.
5. H. Matsuda, T. Morikawa, J. Tao, K. Ueda, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2002, **50**, 208.
6. T. Morikawa, H. Matsuda, I. Toguchida, K. Ueda, and M. Yoshikawa, *J. Nat. Prod.*, 2002, **65**, 1468.
7. J. Tao, T. Morikawa, I. Toguchida, S. Ando, H. Matsuda, and M . Yoshiakwa, *Bioorg. Med. Chem.*, 2002, **10**, 4005.
8. T. Morikawa, J. Tao, H. Matsuda, M. Yoshikawa, *J. Nat. Prod.*, 2003, **66**, 638.
9. J. Tao, T. Morikawa, S. Ando, H. Matsuda, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2003, **51**, 654.
10. H. Matsuda, T. Morikawa, H. Xie, and M. Yoshikawa, *Planta Med.*, 2004, **70**, 847.
11. B. Sun, T. Morikawa, H. Matsuda, S. Tewtrakul, L. J. Wu, S. Harima, and M. Yoshikawa, *J. Nat. Prod.*, 2004, **67**, 1464.
12. T. Morikawa, B. Sun, H. Matsuda, L. J. Wu, S. Harima, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2004, **52**, 1194.
13. H. Xie, T. Wang, H. Matsuda, T. Morikawa, M. Yoshikawa, and T. Tani, *Chem. Pharm. Bull.*, 2005, **53**, 1416.
14. T. Morikawa, H. Xie, H. Matsuda, and M. Yoshikawa, *J. Nat. Prod.*, 2006, **69**, 881.

15. T. Morikawa, H. Xie, H. Matsuda, T. Wang, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2006, **54**, 506.
16. H. Xie, T. Morikawa, H. Matsuda, S. Nakamura, O. Muraoka, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2006, **54**, 669.
17. M. Yoshikawa, H. Matsuda, T. Morikawa, H. Xie, S. Nakamura, and O. Muraoka, *Bioorg. Med. Chem.*, 2006, **14**, 7468.
18. H. Matsuda, S. Sugimoto, T. Morikawa, K. Matsuhira, E. Mizoguchi, S. Nakamura, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2007, **55**, 106.
19. M. Yoshikawa, T. Morikawa, Y. Zhang, S. Nakamura, O. Muraoka, and H. Matsuda, *J. Nat. Prod.*, 2007, **70**, 575.
20. T. Morikawa, Y. Zhang, S. Nakamura, H. Matsuda, O. Muraoka, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2007, **55**, 435.
21. K. Ninomiya, T. Morikawa, Y. Zhang, S. Nakamura, H. Matsuda, O. Muraoka, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2007, **55**, 1185.
22. Y. Zhang, T. Morikawa, S. Nakamura, K. Ninomiya, H. Matsuda, O. Muraoka, and M. Yoshikawa, *Heterocycles*, 2007, **71**, 1565.
23. S. Nakamura, X. Li, H. Matsuda, K. Ninomiya, T. Morikawa, K. Yamaguti, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2007, **55**, 1505.
24. S. Nakamura, X. Li, H. Matsuda, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2008, **56**, 536.
25. X. Li, S. Nakamura, H. Matsuda, and M. Yoshikawa, *Chem. Pharm. Bull.*, 2008, **56**, 612.
26. The ^{1}H- and ^{13}C-NMR spectra of **1**—**5**, **1a**, and **4a** were assigned with the aid of distortionless enhancement by polarization transfer (DEPT), homocorrelation spectroscopy (^{1}H-^{1}H COSY), heteronuclear multiple quantum coherence (HMQC), and HMBC experiments.
27. K. Yamasaki, R. Kasai, Y. Masaki, M. Okihara, and O. Tanaka, *Tetrahedron Lett.*, 1977, **14**, 1231.
28. These new compounds were also isolated from the methanolic extract of *S. indica*. The detail of isolation and structure elucidation of these compounds were described in ref. 30.
29. M. Yoshikawa, F. Xu, T. Morikawa, K. Ninomiya, and H. Matsuda, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 1045.
30. T. Morikawa, H. Xie, T. Wang, H. Matsuda, and M. Yoshikawa, *Chem. Pharm. Bull.*, submitted.