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Inhibitory effects of coumarin and acetylene constituents from the roots of *Angelica furcijuga* on D-galactosamine/lipopolysaccharide-induced liver injury in mice and on nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages

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Abstract—The methanolic extract (200 mg/kg, p.o. and i.p.), principal coumarin constituents (isoepoxypteryxin, anomalin, and praeroside IV), and a polyacetylene constituent (falcarindiol) (25 mg/kg, i.p.) from the roots of *Angelica furcijuga* protected the liver injury induced by D-galactosamine (D-GalN)/lipopolysaccharide (LPS) in mice. In in vitro experiments, coumarin constituents (hyuganins A–D, anomalin, pteryxin, isopteryxin, and suksdorfin) and polyacetylene constituents [(−)-falcarinol and falcarindiol] substantially inhibited LPS-induced NO and/or TNF-α production in mouse peritoneal macrophages, and isoepoxypteryxin inhibited D-GalN-induced cytotoxicity in primary cultured rat hepatocytes. Furthermore, hyuganin A, anomalin, and isopteryxin inhibited the decrease in cell viability by TNF-α in L929 cells.

1. Introduction

The roots of *Angelica furcijuga* KITAGAWA (Umbelliferae, 'Hyugatouki' in Japanese) have been used for hepatopathy, allergosis, inflammation, diabetes, and hypertension in Japanese folk medicine. In the course of characterization studies on this natural medicine, we previously reported 53 constituents including 11 new compounds isolated from the roots, flowers, and leaves of *Angelica furcijuga* collected in Miyazaki prefecture, Japan.[1–5] These constituents showed vasorelaxant effects,[3] protective effects on D-galactosamine (D-GalN)-induced cytotoxicity in primary cultured rat hepatocytes,[2] and inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages.[1,2] This paper deals with a full account of hepatoprotective and NO production inhibitory effects of the methanolic extract and principal constituents from the roots of *A. furcijuga*.

Keywords: *Angelica furcijuga*; Coumarins; Falcarindiol; Liver injury; Nitric oxide; Lipopolysaccharide; D-Galactosamine.
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2. Results and discussion

2.1. Protective effect of methanolic extract from the roots of *A. furcijuga* on liver injury induced by D-GalN/LPS in mice

The methanolic extract from the roots of *A. furcijuga* (collected in Miyazaki prefecture, Japan) showed an inhibitory effect on the increase in serum alanine aminotransaminase (sALT) and/or aspartate aminotransaminase (sAST), as a marker of liver injury, induced by D-GalN/LPS in mice at a dose of 200 mg/kg, p.o. and i.p., respectively (Table 1).

2.2. Effects of chemical constituents from the roots of *A. furcijuga* on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes and NO production in LPS-activated mouse peritoneal macrophages

D-GalN/LPS-induced liver injury is recognized to develop from immunological responses.[6] This type of liver injury occurs in two ways: first, depletion of uridine triphosphate and increased sensitivity of hepatocytes to TNF-α is induced by D-GalN; second, release of

Table 1. Protective effect of the MeOH extract from the roots of *Angelica furcijuga* on liver injury induced by D-GalN/LPS in mice

	Dose	*N*	sALT		sAST	
			Karmen unit	Inhibition (%)	Karmen unit	Inhibition (%)
	mg/kg, p.o.					
Normal	—	5	25 ± 2**	—	58 ± 6**	—
Control	—	11	7949 ± 1109	—	7568 ± 861	—
MeOH ext.	100	10	5831 ± 1462	26.7	5067 ± 1109	33.3
	200	9	4461 ± 768	44.0	4428 ± 637*	41.8
Hydrocortisone	20	10	223 ± 64**	97.5	348 ± 53**	96.1
	mg/kg, i.p.					
Normal	—	5	22 ± 3**	—	43 ± 5**	—
Control	—	12	6176 ± 637	—	6324 ± 649	—
MeOH ext.	100	9	5481 ± 889	11.3	5369 ± 900	15.2
	200	9	1131 ± 496**	82.0	1038 ± 332**	84.2
Hydrocortisone	20	10	105 ± 19**	98.7	133 ± 32**	98.6

Each value represents the mean ± SEM.
Significantly different from the control: *$p < 0.05$, **$p < 0.01$.

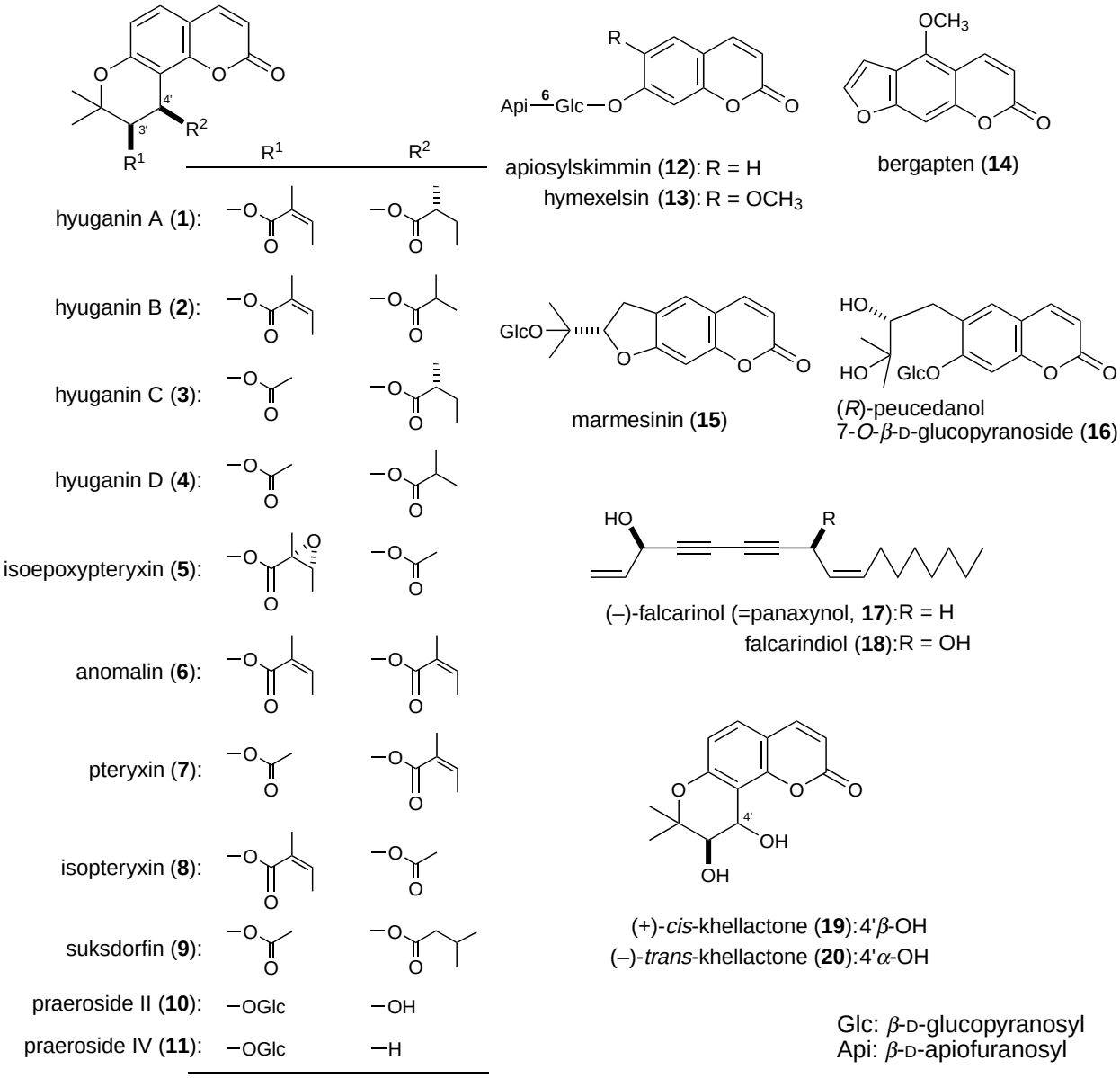

Figure 1. Chemical structures of compounds **1**–**20** from *Angelica furcijuga*.

proinflammatory mediators, such as TNF-α or peroxynitrate ('$ONOO^-$), from LPS-activated macrophages or Kupffer's cells. Apoptosis of hepatocytes induced by TNF-α is reported to be important in D-GalN/LPS-induced liver injury.[7]

In our previous study on hepatoprotective compounds from natural medicines, we reported saponin constituents (e.g., bupleurosides VI, IX, and XIII, and saikosaponin b_3) from *Bupleurum scorzonerifolium* and sesquiterpene constituents (e.g., curcumenolactones A and B, germacrone, and curdione) from *Curcuma zedoaria* with inhibitory effects on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes.[8,9] First, we examined the effects of the isolated constituents (**1–20**, Fig. 1) on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes. As shown in Table 2, only isoepoxypteryxin (**5**, IC_{50} = 29 μM) substantially inhibited D-GalN-induced cytotoxicity.

Next, effects of the isolated constituents on NO production, as a marker of activation of the macrophages, in LPS-activated mouse peritoneal macrophages were examined. As shown in Table 3, the 3′,4′-diacylated khellactone-type coumarins [hyuganins A (**1**, IC_{50} = 5.1 μM), B (**2**, 8.8 μM), C (**3**, 8.2 μM), and D (**4**, 4.2 μM), anomalin (**6**, 3.4 μM), isopteryxin (**8**, 8.8 μM), and suksdorfin (**9**, 11 μM)], except for isoepoxypteryxin (**5**), and two polyacetylenes [(−)-falcarinol (**17**, 4.8 μM) and falcarindiol (**18**, 4.4 μM)] showed potent inhibitory effects ($IC_{50} \leqq$ ca. 10 μM) without cytotoxic effects in the MTT assay. However, desacyl derivatives of **1–9**, (+)-*cis*-khellactone (**19**) and (−)-*trans*-khellactone (**20**), and the 3′-*O*-glucoside of **19** (**10**) showed weak activities. Furthermore, we previously reported that 3′-angeloyl-*cis*-khellactone (82 μM) with an acyl group at the 3′-position and laserpitin (>100 μM) with an acyl group at the 4′-position showed less activity than **6** (3.4 μM).[1] These findings suggested that both the 3′- and 4′-acyl groups were essential for the strong activity and the varieties of the 3′- and 4′-acyl groups were also important.

Recently, Wang et al., reported that polyacetylene in macrophage-like cell line RAW 2647 stimulated constituents (e.g., **17** and **18**) from *Saposhnikoikovia divaricata* and *Panax quinquefolium* were identified as active principles on the inhibition of NO production by inducible NO synthase in macrophage-like cell line RAW 264.7 stimulated by LPS and interferon-γ (IFN-γ).[10] In agreement with the report, **17** and **18** inhibited NO production in LPS-activated mouse peritoneal macrophages.

Previously, 1,1-dimethylallylcoumarins, scopoletin, and furanocoumarins were reported to inhibit NO production by inhibition of iNOS induction in RAW 264.7 cells stimulated by LPS and IFN-γ.[11–13] However, effects of khellactone-type coumarins on iNOS induction have not been reported so far. In the present study, the effects of two acylated khellactone-type coumarins (**1** and **6**), which showed strong inhibition against NO production, on iNOS induction were examined. iNOS protein was detected at 130 kDa after 12-h incubation with LPS by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)-Western blotting, and caffeic acid phenetyl ester (CAPE), an inhibitor of activation of NF-κB that leads to inhibition of iNOS expression, inhibited the iNOS induction. Both coumarins **1** and **6** suppressed the iNOS induction in a concentration-dependent manner similar to CAPE (Fig. 2).

Table 2. Inhibitory effect of the constituents from the roots of *Angelica furcijuga* on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes

	Inhibition (%)						IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	
Hyuganin A (**1**)	0.0 ± 0.6	0.6 ± 3.4	0.4 ± 2.3	0.3 ± 2.9	2.8 ± 2.7	−0.5 ± 2.1	
Hyuganin B (**2**)	0.0 ± 4.3	7.8 ± 7.1	3.7 ± 3.2	1.4 ± 3.1	−5.1 ± 2.6	9.8 ± 4.4	
Hyuganin C (**3**)	0.0 ± 0.5	−2.7 ± 0.7	−10.3 ± 0.6	−11.9 ± 0.3	−12.0 ± 0.5	−2.5 ± 0.3	
Hyuganin D (**4**)	0.0 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.2	−2.2 ± 0.2	
Isoepoxypteryxin (**5**)	0.0 ± 0.3	2.3 ± 0.4	6.1 ± 0.5	16.6 ± 0.4**	54.9 ± 0.2**	85.7 ± 1.0**	29
Anomalin (**6**)	0.0 ± 0.8	0.5 ± 2.8	0.2 ± 1.3	2.3 ± 1.7	3.1 ± 2.2	2.8 ± 1.9	
Pteryxin (**7**)	0.0 ± 4.3	−6.8 ± 1.6	−5.1 ± 1.9	8.5 ± 3.0	2.0 ± 0.4	4.4 ± 2.0	
Isopteryxin (**8**)	0.0 ± 11.0	8.3 ± 4.4	6.0 ± 8.9	8.5 ± 7.7	9.2 ± 9.2	13.2 ± 7.9	
Suksdorfin (**9**)	0.0 ± 7.8	−1.3 ± 5.4	9.2 ± 6.7	11.8 ± 4.2	14.5 ± 7.4	—	
Praeroside II (**10**)	0.0 ± 0.6	5.9 ± 0.9	2.0 ± 0.5	7.1 ± 0.7	−0.7 ± 0.7	8.6 ± 0.6	
Praeroside IV (**11**)	0.0 ± 0.8	−0.6 ± 0.7	−5.7 ± 0.9	−2.6 ± 0.7	0.0 ± 0.7	1.4 ± 0.5	
Apiosylskimmin (**12**)	0.0 ± 0.3	1.6 ± 0.2	−0.9 ± 0.3	−0.8 ± 0.6	−1.9 ± 0.3	−2.1 ± 0.1	
Bergapten (**14**)	0.0 ± 0.2	6.6 ± 0.4	11.2 ± 0.8	9.7 ± 0.4	17.5 ± 0.3**	22.5 ± 0.4**	
Marmesinin (**15**)	0.0 ± 11.0	0.0 ± 9.6	1.2 ± 4.9	2.3 ± 1.3	2.2 ± 2.2	16.3 ± 8.0	
16	0.0 ± 1.2	0.2 ± 1.2	0.7 ± 1.1	0.6 ± 0.8	1.2 ± 1.0	−5.8 ± 0.2	
(−)-Falcarinol (**17**)	0.0 ± 0.1	1.9 ± 0.5	1.7 ± 0.3	2.4 ± 0.2	0.6 ± 0.2	−0.6 ± 0.3	
Falcarindiol (**18**)	0.0 ± 4.8	−6.7 ± 2.7	−21.1 ± 1.6**	−23.6 ± 3.9**	−28.1 ± 3.4**	−32.9 ± 2.4**	
(+)-*cis*-Khellactone (**19**)	0.0 ± 0.1	−8.2 ± 0.4	1.3 ± 0.3	1.7 ± 0.3	5.3 ± 0.4	6.5 ± 0.5	
(−)-*trans*-Khellactone (**20**)	0.0 ± 0.5	−2.4 ± 0.4	−1.5 ± 0.5	2.4 ± 0.6	0.3 ± 0.5	1.5 ± 0.5	

Each value represents the mean ± SEM. (N = 4).
Significantly different from the control, $^{**}p < 0.01$.

Table 3. Inhibitory effect of the constituents from the roots of *Angelica furcijuga* on NO production in LPS-activated mouse peritoneal macrophages

	Inhibition (%)						IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	
Hyuganin A (**1**)	0.0 ± 1.4	25.7 ± 3.1**	48.6 ± 2.8**	62.6 ± 3.5**	78.4 ± 0.3**	100.4 ± 0.4**,a	5.1
Hyuganin B (**2**)	0.0 ± 3.2	3.7 ± 3.9	17.8 ± 2.9**	47.4 ± 2.5**	85.2 ± 1.8**	101.9 ± 1.3**,a	8.8
Hyuganin C (**3**)	0.0 ± 2.5	32.4 ± 3.1**	39.5 ± 2.0**	43.3 ± 2.4**	74.4 ± 1.5**	93.8 ± 0.3**	8.2
Hyuganin D (**4**)	0.0 ± 1.6	35.2 ± 5.3**	46.2 ± 7.5**	54.1 ± 2.6**	68.9 ± 4.3**	86.2 ± 0.5**	4.2
Isoepoxypteryxin (**5**)	0.0 ± 2.7	14.2 ± 7.4	15.7 ± 5.2*	21.4 ± 6.2*	44.6 ± 2.1**	58.2 ± 2.5**	53
Anomalin (**6**)	0.0 ± 1.9	23.0 ± 5.2**	47.4 ± 5.1**	62.7 ± 3.4**	96.8 ± 1.6**	102.0 ± 0.6**,a	3.4
Pteryxin (**7**)	0.0 ± 3.3	14.0 ± 2.2	16.1 ± 5.4*	32.9 ± 6.4**	59.0 ± 1.2**	88.7 ± 0.7**	20
Isopteryxin (**8**)	0.0 ± 1.2	20.4 ± 2.1**	32.4 ± 3.0**	47.8 ± 3.4**	75.5 ± 2.2**	91.3 ± 1.0**	8.8
Suksdorfin (**9**)	0.0 ± 2.6	1.9 ± 6.5	13.9 ± 2.9	50.4 ± 3.3**	71.7 ± 3.5**	92.3 ± 2.0**	11
Praeroside II (**10**)	0.0 ± 4.8	8.5 ± 1.9	18.7 ± 0.7**	30.9 ± 5.3**	45.5 ± 3.5**	—	ca. 50
Praeroside IV (**11**)	0.0 ± 4.2	9.9 ± 2.1	12.6 ± 2.2*	12.4 ± 1.2*	16.2 ± 1.6**	20.6 ± 3.7**	
Apiosylskimmin (**12**)	0.0 ± 2.3	3.0 ± 10.2	7.2 ± 9.7	6.6 ± 5.0	20.3 ± 10.2	10.8 ± 3.6	
Bergapten (**14**)	0.0 ± 1.8	2.7 ± 7.6	7.0 ± 3.3	19.3 ± 6.5*	26.3 ± 2.1**	48.9 ± 2.4**	ca. 100
Marmesinin (**15**)	0.0 ± 4.9	16.4 ± 3.8	16.2 ± 3.3	18.2 ± 2.4	27.5 ± 7.2*	48.8 ± 1.9**	ca. 100
16	0.0 ± 4.9	10.1 ± 4.9	25.6 ± 5.9**	26.8 ± 1.4**	46.4 ± 2.8**	49.3 ± 2.2**	ca. 100
(−)-Falcarinol (**17**)	0.0 ± 3.5	29.4 ± 6.2**	37.8 ± 6.6**	70.0 ± 7.4**	96.1 ± 0.8**	—	4.8
Falcarindiol (**18**)	0.0 ± 2.4	24.4 ± 4.5**	42.3 ± 4.8**	65.4 ± 2.6**	102.9 ± 0.2**	107.3 ± **,a	4.4
(+)-*cis*-Khellactone (**19**)	0.0 ± 1.2	11.2 ± 1.8*	13.6 ± 1.8*	22.6 ± 2.9**	29.3 ± 2.9**	54.9 ± 2.1**	91
(−)-*trans*-Khellactone (**20**)	0.0 ± 1.9	7.1 ± 2.8	10.9 ± 2.9*	13.5 ± 3.4*	16.2 ± 1.4*	35.5 ± 2.7**	
CAPE	0.0 ± 4.0	−1.9 ± 3.3	25.5 ± 3.9**	97.5 ± 1.4**	104.1 ± 0.7**	—	ca. 4.0
L-NMMA	0.0 ± 1.1	4.4 ± 2.0	2.0 ± 1.6	17.7 ± 2.8**	52.3 ± 1.5**	79.2 ± 0.9**	28

Each value represents the mean ± SEM. ($N = 4$).
Significantly different from the control, $^{*}p < 0.05$, $^{**}p < 0.01$.
[a] Cytotoxic effect was observed.

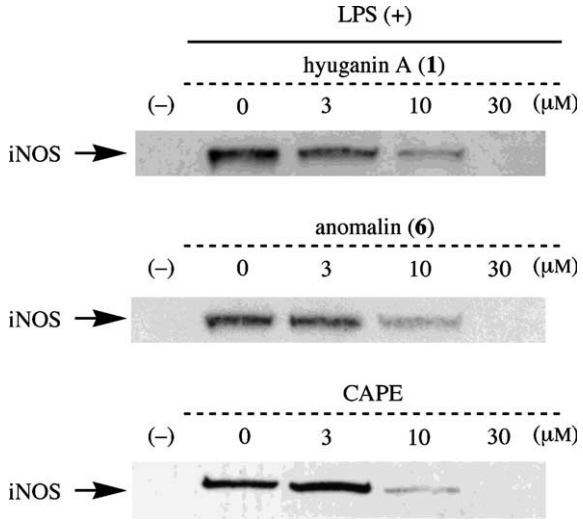

Figure 2. Effects of hyuganin A (**8**), anomalin (**12**), and CAPE on iNOS induction in LPS-activated mouse peritoneal macrophages.

2.3. Inhibitory effects of principal constituents (1, 5, 6, 8, 10, 11, and 18) from *A. furcijuga* on TNF-α production in LPS-activated macrophages and on TNF-α-induced cell death in L929

Next, effects of seven principal constituents (**1**, **5**, **6**, **8**, **10**, **11**, and **18**) on TNF-α production in LPS-activated macrophages were examined. As a result, compounds **1**, **5**, **6**, **8**, and **18** inhibited the release of TNF-α in the medium after 4-h incubation with LPS and test compounds. Especially, anomalin (**6**) and isopteryxin (**8**) showed stronger activity (Table 4). But, their inhibitory effects on production of TNF-α were weaker than those on production of NO. Praerosides II (**10**) and IV (**11**) having a glucopyranosyl moiety lacked the activity.

Furthermore, to clarify the effects of principal constituents on the sensitivity of hepatocytes to TNF-α, the effects of compounds **1**, **5**, **6**, **8**, **10**, **11**, and **18** on TNF-α-induced decrease in cell viability of L929 cells, a TNF-α-sensitive cell line,[14] were examined by the MTT assay. After incubation of L929 cells with 10 nM TNF-α for 20 h, cell viability of L929 without test sample reduced to ca. 60% compared to that of TNF-α-untreated cells. As shown in Table 5, hyuganin A (**1**), anomalin (**6**), and isopteryxin (**8**) inhibited the decrease in the cell viability, but other constituents (**5**, **10**, **11**, and **18**) lacked the activity. In the previous study, only acylated flavonol glycoside (e.g., tiliroside) from *Tilia argentea* with hepatoprotective activity has been reported to show such effect.[15]

2.4. Protective effects of principal constituents (1, 5, 6, 8, 10, 11, and 18) from *A. furcijuga* on liver injury induced by D-GalN/LPS in mice

Finally, we examined the effects of the principal constituents (**1**, **5**, **6**, **8**, **10**, **11**, and **18**) on the D-GalN/LPS-induced liver injury in mice. As shown in Table 6, isoepoxypteryxin (**5**), anomalin (**6**), and falcarindiol (**18**) significantly inhibited the increase in sALT and sAST at doses of 12.5 and/or 25 mg/kg, i.p., and isopteryxin (**8**) tended to inhibit the liver injury. However, hyuganin A (**1**), which showed inhibitory activity in vitro, did not show any effect in mice. Praeroside II (**10**), which showed only a weak activity for NO production in vitro, also did not inhibit the liver injury, but praeroside IV (**11**), which did not show any effect in vi-

Table 4. Inhibitory effects of the principal constituents (**1**, **5**, **6**, **8**, **10**, **11**, and **18**) from *Angelica furcijuga* on TNF-α production in LPS-activated mouse peritoneal macrophages

	Inhibition (%)					IC_{50} (μM)
	0 μM	3 μM	10 μM	30 μM	100 μM	
Hyuganin A (**1**)	0.0 ± 7.5	−4.5 ± 3.7	0.0 ± 8.0	37.2 ± 6.2**	87.9 ± 6.3**	57
Isoepoxypteryxin (**5**)	0.0 ± 6.9	−9.6 ± 5.0	3.0 ± 8.2	29.1 ± 3.7**	71.5 ± 4.8**	52
Anomalin (**6**)	0.0 ± 6.9	−6.2 ± 5.5	21.0 ± 6.1**	65.3 ± 6.4**	85.9 ± 6.2**	23
Isopteryxin (**8**)	0.0 ± 6.9	21.4 ± 4.4	34.3 ± 1.6**	70.7 ± 1.6**	94.4 ± 1.3**	20
Praeroside II (**10**)	0.0 ± 5.0	−11.8 ± 4.1	−15.9 ± 3.1	−11.9 ± 1.7	−11.3 ± 4.0	
Praeroside IV (**11**)	0.0 ± 5.0	−4.2 ± 4.0	1.6 ± 2.1	−4.2 ± 5.7	−7.8 ± 10.1	
Falcarindiol (**18**)	0.0 ± 9.3	—	—	32.2 ± 9.2**	102.1 ± 2.4**	ca. 48

Each value represents the mean ± SEM. ($N = 4$).
Significantly different from the control, $^{**}p < 0.01$.

Table 5. Inhibitory effects of the principal constituents (**1**, **5**, **6**, **8**, **10**, **11**, and **18**) from *Angelica furcijuga* on decrease in viability of L929 cells by TNF-α

	Inhibition (%)					IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	
Hyuganin A (**1**)	0.0 ± 3.3	−1.3 ± 4.1	8.6 ± 1.7	50.3 ± 2.3**	138.9 ± 6.5**	ca. 10
Isoepoxypteryxin (**5**)	0.0 ± 3.4	2.6 ± 7.6	2.8 ± 9.5	3.3 ± 7.8	16.1 ± 4.6	
Anomalin (**6**)	0.0 ± 3.4	1.2 ± 2.3	9.4 ± 2.3	50.1 ± 0.9**	127.0 ± 7.7**	ca. 10
Isopteryxin (**8**)	0.0 ± 5.2	−6.0 ± 7.1	−1.5 ± 5.7	18.9 ± 6.8	69.3 ± 6.3**	ca. 20
Praeroside II (**10**)	0.0 ± 2.4	−1.6 ± 5.8	7.1 ± 7.8	2.1 ± 4.2	−7.5 ± 1.9	
Praeroside IV (**11**)	0.0 ± 5.7	−7.5 ± 4.9	0.2 ± 3.4	2.1 ± 4.2	−7.5 ± 1.9	
Falcarindiol (**18**)	0.0 ± 1.0	−8.4 ± 2.2	0.1 ± 3.0	4.0 ± 1.6	19.1 ± 1.3**	>30

Each value represents the mean ± SEM. ($N = 4$).
Significantly different from the control, $^{**}p < 0.01$.

Table 6. Protective effects of the constituents from the roots of *Angelica furcijuga* on liver injury induced by D-GalN/LPS in mice

	Dose	N	sALT		sAST	
	(mg/kg, i.p.)		Karmen unit	Inhibition (%)	Karmen unit	Inhibition (%)
Normal	—	5	22 ± 4		43 ± 5	
Control	—		4951 ± 769	—	5055 ± 641	—
Hyuganin A (**1**)	12.5	8	4827 ± 1361	2.5	5157 ± 1295	−2.0
	25	9	4697 ± 907	5.1	4955 ± 885	2.0
Isoepoxypteryxin (**5**)	12.5	9	1380 ± 530**	74.5	1453 ± 366**	74.6
	25	9	1126 ± 384**	79.2	1173 ± 297**	79.5
Anomalin (**6**)	12.5	8	2336 ± 749	52.8	2685 ± 585	46.9
	25	9	1195 ± 168**	75.9	1761 ± 182**	65.2
Isopteryxin (**8**)	12.5	9	2935 ± 978	40.7	2869 ± 667	43.2
	25	9	2325 ± 459	53.0	2648 ± 431	47.6
Falcarindiol (**18**)	12.5	8	2001 ± 685*	64.3	2164 ± 880	63.8
	25	6	1862 ± 929*	66.8	1436 ± 697**	76.3
Control	—		4951 ± 769	—	5055 ± 641	—
Praeroside II (**10**)	12.5	8	4317 ± 854	12.8	4865 ± 834	3.8
	25	9	4206 ± 770	15.1	4171 ± 634	17.5
Praeroside IV (**11**)	12.5	10	2699 ± 589	45.5	3098 ± 572	38.7
	25	9	2260 ± 477*	54.4	2356 ± 399*	53.4
Hydrocortisone	20	10	160 ± 45**	96.8	229 ± 34**	95.5

Each value represents the means ± SEM.
Significantly different from the control: $^{*}p < 0.05$, $^{**}p < 0.01$.

tro, significantly inhibited the liver injury at a dose of 25 mg/kg, i.p.

The results from the in vitro experiments suggested that the inhibition of production of TNF-α and NO from LPS-activated macrophages [anomalin (**6**), isopteryxin (**8**), and falcarindiol (**18**)], the protective effects against D-GalN-induced cell damage [isoepoxypteryxin (**5**)], and both the inhibition of production of TNF-α and NO from LPS-activated macrophages and reduction in TNF-α sensitivity of heparocytes [anomalin (**6**) and isopteryxin (**8**)] were involved in the in vivo protective effects of **5**, **6**, **8**, and **18**.

In conclusion, the methanolic extract, and principal coumarin and polyacetylene constituents (**5**, **6**, **8**, **11**, and **18**) from the roots of *A. furcijuga* protected the D-GalN/LPS-induced liver injury in mice. In addition,

the possible mechanisms of action including the inhibitory effect on activation of macrophages, the protection of hepatocytes, and reduction in TNF-α sensitivity of heparocytes were deduced. It is well known that acylated coumarins and polyacetylenes are also contained in various important Chinese natural medicines such as Angelicae Radix (*Angelica acutiloba*, Umbelliferae), Bupleuri Radix (*Bupleurum falcatum*, Umbelliferae), Aurantii Fructus Immaturus (*Citrus auranticum*, Rutaceae), and Ginseng Radix (*Panax ginseng*, Araliaceae), which are frequently prescribed for hepatoprotective and antiinflammatory purposes in Chinese traditional preparations. Since overproduction of NO is known to be a cause of inflammation and endotoxin-induced shocks and TNF-α also plays a critical role in the pathogenic mechanisms of a number of chronic inflammatory diseases, including rheumatoid arthritis, Crohn's disease, and psoriasis,[16,17] these coumarins (**6**, **8**) and a polyacetylene (**18**) may be effective in the treatment of inflammation and endotoxic shock. On the other hand, since compound **11** lacked the activity in vitro, the metabolites of **11** might be effective in the treatment of D-GalN/LPS-induced liver injury, or other mechanisms exist. The detailed mechanisms of action of the active constituents should be studied further.

3. Experimental

3.1. Extraction and isolation

The extraction and isolation of **1**–**18** and from the fresh roots of *A. furcijuga* KITAGAWA (cultivated in Miyazaki prefecture, Japan) were described in our previous paper[2,3,5] [hyuganins A (**1**, 0.011% from the fresh roots), B (**2**, 0.002%), C (**3**, 0.014%), and D (**4**, 0.002%), isoepoxypteryxin (**5**, 0.040%), anomalin (**6**, 0.013%), pteryxin (**7**, 0.017%), isopteryxin (**8**, 0.040%), suksdorfin (**9**, 0.003%), and praerosides II (**10**, 0.012%) and IV (**11**, 0.019%), apiosylskimmin (**12**, 0.030%), hymexelsin (**13**, 0.001%), bergapten (**14**, 0.001%), marmesinin (**15**, 0.001%), (*R*)-peucedanol 7-*O*-β-D-glucopyranoside (**16**, 0.004%), (−)-falcarinol (**17**, 0.002%), and falcarindiol (**18**, 0.045%)]. (+)-*cis*-Khellactone (**19**) and (−)-*trans*-khellactone (**20**) were derived by alkaline hydrolysis of hyuganins A–D (**1**–**4**) with 5% aqueous potassium hydroxide (KOH) as reported previously.[3]

3.2. Bioassay

3.2.1. Reagents. Lipopolysaccharide (LPS, from *Salmonella enteritidis*), N^G-monomethyl-L-arginine (L-NMMA), minimum essential medium (MEM), and William's E medium were purchased from Sigma; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was from Dojin; RPMI 1640 and fetal calf serum (FCS) were from Life Technologies (Rockville, MD, USA); protease inhibitor cocktail (Complete Mini) was from Boehringer–Mannheim; anti-mouse iNOS antibody (monoclonal) was from BD Transduction Laboratories; anti-mouse IgG antibody conjugated to horseradish peroxidase and the enhanced chemiluminescense (ECL) kit, TNF-α ELISA kit (Tumor necrosis factor alpha mouse, ELISA system) were from Amersham Biosciences; thioglycolate (TGC) medium was from Nissui Seiyaku; Nitrocellulose membranes (0.25 μm) were purchased from Bio-Rad; and other chemicals were from Wako Pure Chemical Industries. Ninety six-well microplate was purchased from Nalge Nunc International.

3.2.2. Animals. Male ddY mice and Wistar rats were purchased from Kiwa Laboratory Animal (Wakayama, Japan). The animals were maintained at a constant temperature of 23 ± 2 °C and were fed a standard laboratory chow (MF, Oriental Yeast, Japan) for 1 week. Test samples were suspended with 5% acacia solution or 1% sodium carboxymethylcelullose, and the solution was administered at 10 mL/kg in each experiment, while vehicle was administered at 10 mL/kg in the corresponding control group.

3.2.3. Protective effect on D-GalN/LPS-induced liver injury in mice. The method described by Tiegs et al.[18] was modified as described previously.[8,9,15] Briefly, male ddY mice weighing about 25–27 g (5–6 weeks old) were fasted for 20 h before the experiment. D-GalN (350 mg/kg) and LPS (10 μg/kg) dissolved in saline were injected intraperitoneally to produce liver injury. The test sample was given orally or intraperitoneally 1 h before D-GalN/LPS injection. Blood samples were collected from the infraorbital venous plexus 10 h after D-GalN/LPS injection. sALT and sAST levels were determined by the Reitman–Frankel method (commercial kit, S.TA-Test Wako, Wako Pure Chemical Industries Co. Ltd.). Hydrocortisone was used as a reference compound.

3.2.4. Protective effect on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes. Effects of test compounds on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes were examined as described previously.[8,9,15] Hepatocytes were isolated from male Wistar rats (130–160 g) by the collagenase perfusion method.[19] The cell suspension at 4×10^4 cells in 100 μL William's E medium containing calf serum (10%), penicillin (100 U/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well microplate and precultured for 4 h at 37 °C under a 5% CO_2 atmosphere. The medium was exchanged with a fresh medium containing D-GalN (1 mM) and a 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 culture, 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 (OD) of the formazan solution was measured by a microplate reader at 570 nm (reference: 655 nm). Inhibition (%) was calculated by the following formula and the IC_{50} was determined graphically ($N = 4$):

$$\begin{aligned}\text{Inhibition}(\%) = [&(\text{OD}(\text{sample}) \\ &- \text{OD}(\text{control}))/(\text{OD}(\text{normal}) \\ &- \text{OD}(\text{control}))] \times 100\end{aligned}$$

3.2.5. NO production from macrophages stimulated by LPS. NO production inhibitory activity was performed as described previously.[1,20,21] Peritoneal exudate cells were collected from the peritoneal cavities of male ddY mice by washing with 6–7 mL ice-cold PBS, and cells (5×10^5 cells/well) were suspended in 200 μL RPMI 1640 supplemented with 5% fetal calf serum, penicillin (100 U/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 the cells with PBS, and the adherent cells were cultured in fresh medium containing 10 μg/mL LPS and test compound (1–100 μM) for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using the Griess reagent. Cytotoxicity was determined by the MTT colorimetric assay. Briefly, after 20-h incubation with test compounds, MTT (10 μL, 5 mg/mL in PBS) solution was added to the wells. After 4-h culture, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured with a microplate reader at 570 nm (reference, 655 nm). CAPE and L-NMMA were used as reference compounds. Each test compound was dissolved in dimethylsulfoxide (DMSO), and the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated by the following formula and the IC_{50} was determined graphically ($N = 4$):

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

$A - C$: NO_2^- conc.(μM)[A : LPS(+), sample(−);

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

3.2.6. Detection of iNOS. Detection of iNOS was performed as described previously.[20] TGC-Induced peritoneal exudate cells (7.5×10^6 cells/3 mL/dish) were pre-cultured in culture dishes (6 cm i.d.) for 1 h. After washing, the medium was exchanged with fresh medium containing 5% FCS, 20 μg/mL LPS, and test compound. After 12-h incubation, cells were collected in lysis buffer [150 mM NaCl, 10 mM Tris, protease inhibitor cocktail (1 tab/50 mL), 0.1% Triton X-100, and 2 mM EGTA] and sonicated. After determination of protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension was boiled in Laemmli buffer. For SDS–PAGE, aliquots of 40 μg of protein from each sample were subjected to electrophoresis in 7.5% polyacrylamide gels. Following electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose membrane. The membrane were incubated with 5% nonfat dried milk in Tris-buffered saline (T-TBS, 100 mM NaCl, 10 mM Tris, and 0.1% Tween 20, pH 7.4) and probed with mouse monoclonal IgG (dilution of 1:1000) against iNOS. The blots were washed in T-TBS and probed with secondary antibody, anti-mouse IgG antibody conjugated to horseradish peroxidase (dilution of 1:5000). Detection was performed using an ECL™ and X-ray film (Hyperfilm-ECL™, Amersham Biosciences). CAPE was used as a reference compound.

3.2.7. TNF-α production from LPS-activated macrophages. TNF-α released in the medium was determined as described previously[15] with a slight modification. TGC-Induced peritoneal exudate cells (5×10^5 cells/well) were suspended in 200 μL RPMI 1640 supplemented with FCS (5%), penicillin (100 U/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 the cells, and the adherent cells were cultured in fresh medium containing 10 μg/mL LPS and various concentrations of the test sample. Four hours later, TNF-α levels in the medium were determined by the ELISA kit. Inhibition (%) was calculated by the following formula and the IC_{50} was determined graphically ($N = 4$):

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

$A - C$: TNF-α conc.(pg/mL)

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

C : LPS(−), sample(−)].

3.2.8. Sensitivity of L929 cells for TNF-α. Sensitivity of L929 cells against TNF-α was performed by the method given previously.[15] L929 cells, a TNF-α-sensitive cell line, were obtained from Dainippon Pharmaceutical (Osaka, Japan). After 20-h incubation in minimum essential medium (MEM) with 10 ng/mL TNF-α, viability of the cells was assessed by the MTT colorimetric assay. Briefly, the cell suspension of 10^4 cells in 100 μL MEM containing FCS (10%), penicillin (100 U/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well microplate. After culturing for 20 h at 37 °C under a 5% CO_2 atmosphere, the medium was exchanged with a fresh medium containing TNF-α (10 ng/mL) and a test sample, and the cells were cultured for 20 h. Cell viability was determined by the MTT colorimetric assay as described above. Inhibition (%) was calculated by the following formula and the IC_{50} was determined graphically ($N = 4$):

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

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

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