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HEPATOPROTECTIVE AND NITRIC OXIDE PRODUCTION INHIBITORY ACTIVITIES OF COUMARIN AND POLYACETYLENE CONSTITUENTS FROM THE ROOTS OF *ANGELICA FURCIJUGA*

Hisashi Matsuda, Toshiyuki Murakami, Tadashi Kageura, Kiyofumi Ninomiya, Iwao Toguchida, Norihisa Nishida, and Masayuki Yoshikawa*

Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan

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Abstract : The methanolic extract from the roots of *Angelica furcijuga* KITAGAWA was found to exhibit protective effects on liver injury induced by D-galactosamine (D-GalN) and lipopolysaccharide (LPS). From the methanolic extract, seventeen coumarins, two phenylpropanoids, and two polyacetylenes were isolated and examined their *in vitro* and *in vivo* hepatoprotective effects and inhibitory activity of NO production in macrophages. A acylated khellactone, isoepoxypteryxin, showed protective activity against D-GalN-induced cytotoxicity in primary cultured rat hepatocytes. On the other hand, six acylated khellactones (hyuganins A, B, C, and D, anomalin, isopteryxin) and two polyacetylenes [(-)-falcarinol and falcarindiol] strongly inhibited NO production induced by LPS in cultured mouse peritoneal macrophages, and also other acylated khellactones (isoepoxypteryxin, pteryxin, and suksdorfin) and a coumarin glycosides (praeroside II) were found to show the activity. By comparison of the inhibitory activities for acylated khellactones with those for other coumarins, acyl groups were found to be essential to exerting potent activity.

The roots of *Angelica furcijuga* KITAGAWA (Umbelliferae, Japanese name "Hyugatouki") has been used as remedies for hepatopathy and allergosis in Japanese folk medicine, but its pharmacological property and bioactive constituents were left uncharacterized. In the course of our studies on the hepatoprotective constituents of natural medicines,[1] the methanolic extract from the fresh roots of *A. furcijuga* was found to show potent inhibitory activity on the liver injury induced by D-galactosamine (D-GalN) and lipopolysaccharide (LPS), which was known to be immunologically induced liver injury. Furthermore it has been shown that both hepatocytotoxicity of D-GalN and activation of macrophages stimulated by LPS are important to exhibit this liver injury.[2]

We isolated seventeen coumarins including four new acylated (+)-*cis*-khellactones [hyuganins A (**2**), B (**3**), C (**4**), and D (**5**)], two new phenylpropanoids, and two polyacetylenes from the methanolic extract of *A. furcijuga* with the hepatoprotective activity. This communication deals with the isolation and characterization of inhibitors from *A. furcijuga* against D-GalN/LPS-induced liver injury (*in vivo*), D-GalN-induced hepatocytotoxicity, and NO production as an index of activation of macrophages stimulated by LPS (*in vitro*). In addition, we describe the structure-requirement of khellactone-type coumarin for the activities.

Chemicals

Isolation of Chemical Constituents from the Roots of A. furcijuga The fresh roots of *A. furcijuga* (4.0 kg,

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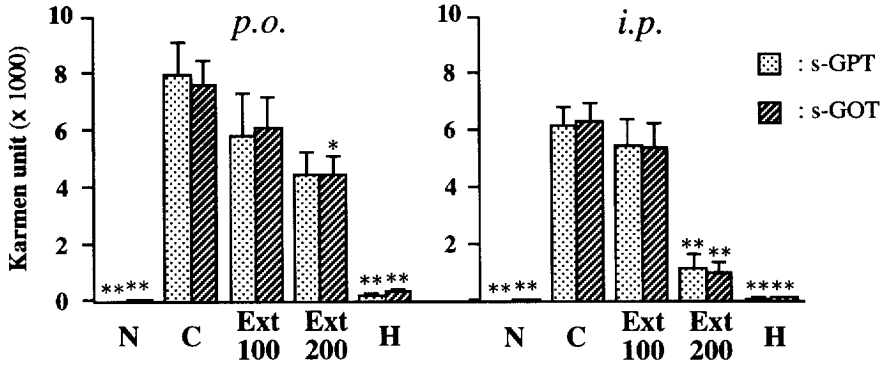


Fig. 1. Inhibitory Effects of Methanolic Extract from the Roots of *Angelica furcijuga* on D-GalN/LPS-Induced Liver Injury in Mice

N : normal group, **C** : control group, **Ext 100** : MeOH ext. 100 mg/kg, **Ext 200** : MeOH ext. 200 mg/kg, **H** : hydrocortisone 20 mg/kg.

Male ddY mice weighing 27—29 g were used. After 20 h of fasting, D-GalN (350 mg/kg) and LPS (10 μg/kg) was injected *i.p.* to produce liver injury. Each test sample was administered orally (*p.o.*) or intraperitoneally (*i.p.*) 1 h before D-GalN/LPS injection. Blood samples were collected 10 h after D-GalN/LPS injection. Each column represents the mean with S.E. (N=9—12, $*p<0.05$, $**p<0.01$).

cultivated in Miyazaki prefecture, Japan) was extracted with methanol under reflux. The methanolic extract was subjected to HP-20 (H_2O→MeOH→acetone), silica gel (hexane-AcOEt→$CHCl_3$-MeOH-H_2O) and ODS (MeOH) column chromatography and finally HPLC (YMC-pack R&D-ODS-5-A, MeOH-H_2O, CH_3CN-H_2O) to give hyuganins A (**2**, 0.011% from the fresh roots),[3] B (**3**, 0.002%),[3] C (**4**, 0.014%),[3] and D (**5**, 0.002%),[3] isoepoxypteryxin (isoepoxybuterixin, **6**, 0.040%),[4] pteryxin (**7**, 0.017%),[4] suksdorfin (**8**, 0.014%),[4] and anomalin (**9**, 0.013%),[4] isopteryxin (**10**, 0.040%),[4] praerosides II (**11**, 0.012%)[4] and IV (**13**, 0.019%),[4] marmesinin (**14**, 0.001%),[4] bergapten (**15**, 0.001%),[4] apiosylskimmin (**16**, 0.030%),[4] (*R*)-peucedanol 7-*O*-β-D-glucopyranoside (**17**, 0.004%),[4] hyuganosides II (**18**, 0.003%)[3] and III (**19**, 0.001%),[3] (-)-falcarinol (panaxynol, **20**, 0.002%),[4] and falcarindiol (**21**, 0.005%)[4] together with hyuganoside I (0.001%),[3] hymexelsin (0.001%),[4] β-sitosterol (0.032%), and (+)-pinoresinol *O*-β-D-glucopyranoside (0.0001%).[4]

Alkaline hydrolysis of hyuganins A—D (**2**—**5**) and isoepoxypteryxin (**6**) with 5% aq. KOH in dioxane furnished (+)-*cis*- and (-)-*trans*-khellactones (**1**, **12**)[4] together with the corresponding organic acids. The organic acids were derived to the *p*-nitrobenzyl esters, which were identified by HPLC analysis.

Bioassay Methods

In Vivo Experiment :

The D-GalN/LPS-induced liver damage was performed according to our previous report.[1e] Each test compound was suspended in 0.5% CMC-Na, and given orally (*p.o.*) or intraperitoneally (*i.p.*) before 1 h of D-GalN/LPS treatment.

In Vitro Experiment : [1e]

Protective Effect on D-GalN-Induced Cytotoxicity : The hepatoprotective effects of these constituents were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using

primary cultured rat hepatocytes after incubation of D-GalN (1 mM) and a test compound for 44 h. Each test compound was dissolved in DMSO and was added to the medium at the concentration of 1—100 μM (N=4).

NO Production from Macrophages Stimulated by LPS and Cytotoxicity : Mouse peritoneal macrophages were used. Cells were incubated with RPMI 1640 medium containing 10% fetal calf serum, LPS (10 μg/ml) and a test compound for 20 h and nitrite which accumulated in the culture medium was measured spectrophotometrically using Griess reagent as nitric oxide. Each test compound was dissolved in DMSO and was added to the medium at the concentration of 0.3—100 μM (N=4).

Statistical Analysis :

Each value was expressed as the mean±S.E. *in vivo* experiments, and the statistical significance was assessed by one-way analysis of variance following Dunnett's test, and IC_{50} *in vitro* experiments was determined graphically.

Results and Discussion

The hepatoprotective effects of the methanolic extract from *A. furcijuga* were examined by monitoring the inhibitory activity on the increase of serum GPT and GOT induced by D-GalN/LPS in mice (Fig. 1). The methanolic extract (200 mg/kg, *p.o.* and *i.p.*) inhibited the increase of serum GPT and GOT. Next, the chemical constituents of the methanolic extract [four new acylated khellactones (**2**—**5**), five known acylated khellactones (**6**—**10**), (+)-*cis*- and (-)-*trans*-khellactones (**1**, **12**), other known coumarins (**11**, **13**—**17**), two new phenylpropanoids (**18**, **19**), and polyacetylenes (**20**, **21**) were evaluated against D-GalN-induced hepatocytotoxicity and LPS-induced NO production in macrophages.

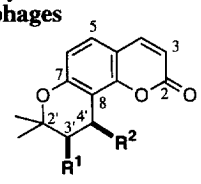
A principal acylated khellactone [isoepoxypteryxin (**6**)] only inhibited the D-GalN-induced cytotoxicity in cultured rat hepatocytes. On the other hand, four new acylated khellactones [hyuganins A (**2**), B (**3**), C (**4**), and D (**5**)], two known acylated khellactones [anomalin (**9**), isopteryxin (**10**)], and two known polyacetylenes [(-)-falcarinol (**20**), falcarindiol (**21**)] strongly inhibited the LPS-induced NO production in macrophages, and also other acylated khellactones [isoepoxypteryxin (**6**), pteryxin (**7**), and suksdorfin (**8**)] and a coumarin glycosides [praeroside II (**11**)] were found to show the activity. But, (+)-*cis*-khellactone (**1**) and other known coumarins (**12**—**17**) and a new phenylpropanoid (**18**) showed a weak activity. These constituents showed no cytotoxicity except for **21** at 100 μM against the hepatocytes and the macrophages by using MTT assay.

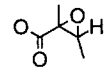
Above results indicated the following structural requirements of khellactone-type coumarins for the activities: 1) the acyl groups at the 3' and 4' position of (+)-*cis*-khellactone were essential to inhibit the LPS-induced NO production in macrophages, and 2) the chemical structure of acyl groups were important not only to exhibit the protective activity against hepatocytotoxicity of D-GalN, but also to inhibit the NO production.

Next, inhibitory effects of principal constituents isoepoxypteryxin (**6**), anomalin (**9**), isopteryxin (**10**), and (-)-falcarindiol (**21**), which exhibited the hepatocytoprotection or inhibition of NO production *in vitro*, on D-GalN/LPS-induced liver injury were examined. These constituents (**6**, **9**, **10**, and **21**) significantly inhibited the increase of serum GOT and GPT at the dose of 25 mg/kg (Fig. 2), and they were also found to reduce the remarkable necrosis induced by D-GalN/LPS in histological examination (data were not shown).

This study seems to be a first report for hepatoprotective and NO production inhibitory activities of coumarin and polyacetylene constituents. It is well known that acylated coumarins and polyacetylenes are also contained

Table 1. Inhibitory Effects of Khellactone-Type Coumarins (1—11) from *Angelica furcijuga* on D-GalN-Induced Cytotoxicity in Primary Cultured Rat Hepatocytes and on LPS-Induced NO Production in Mouse Peritoneal Macrophages

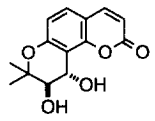


	R^1	R^2	D-GalN-Induced Hepatocytotoxicity IC_{50} (μM)	LPS-Induced NO Production IC_{50} (μM)
(+)-*cis*-khellactone (**1**)	OH	OH	>100 (6.5)	91
hyuganin A (**2**)			>100 (-0.5)	5.1
hyuganin B (**3**)			>100 (10)	8.8
hyuganin C (**4**)			>100 (-2.5)	8.2
hyuganin D (**5**)			>100 (-2.2)	4.2
isoepoxypteryxin (**6**)			29	53
pteryxin (**7**)			>100 (4.4)	20
suksdorfin (**8**)			>100 (-3.4)	11
anomalin (**9**)			>100 (2.8)	3.4
isopteryxin (**10**)			>100 (13)	8.8
praeroside II (**11**)	O-Glc	OH	>100 (8.6)	51

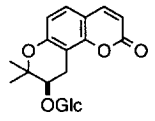
Glc : β-D-glucopyranosyl

( ) : Values in parenthesises represent the inhibition (%) at 100 μM.

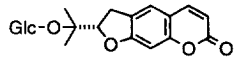
in various important Chinese natural medicines such as Angelicae Radix (*Angelica acutiloba*, Umbelliferae), Bupleuri Radix (*Bupleurum falcatum*, Umbelliferae), Aurantii Fructus Immaturus (*Citrus auranticum*, Rutaceae), and Ginseng Radix (*Panax ginseng*, Araliaceae), which are frequently prescribed for hepatoprotective and antiinflammarory purposes in Chinese traditional preparations. Anomalin (**9**) and falcarindiol (**21**) having hepatoprotective and NO production inhibitory activities may be responsible constituents of Angelicae Radix, Bupleuri Radix, Aurantii Fructus Immaturus, and Ginseng Radix. Since



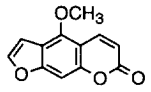
(-)-*trans*-khellactone (**12**)



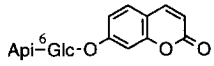
praeroside IV (**13**)



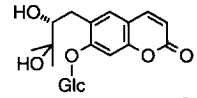
marmesinin (**14**)



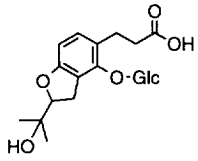
bergapten (**15**)



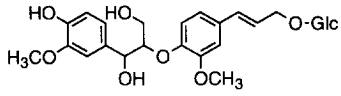
apiosylskimmin (**16**)



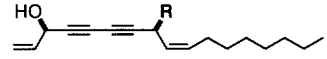
(*R*)-peucedanol 7-*O*-β-D-glucopyranoside (**17**)



hyuganoside II (**18**)



hyuganoside III (**19**)



(-)-falcarinol (panaxynol, **20**) : R=H
falcarindiol (**21**) : R=OH

Glc : β-D-glucopyranosyl Api : β-D-apiofuranosyl

Table 2. Inhibitory Effects of Six Coumarins (12—17), Two phenyl Propanoids (18, 19), and Two Polyacetylenes (20, 21) from *Angelica furcijuga* on D-GalN-Induced Cytotoxicity in Primary Cultured Rat Hepatocytes and on LPS-Induced NO Production in Mouse Peritoneal Macrophages

Compounds	D-GalN-Induced Hepatocytotoxicity IC_{50} (μM)	LPS-Induced NO Production IC_{50} (μM)
(-)-*trans*-khellactone (**12**)	>100 (1.5)	>100 (36)
praeroside IV (**13**)	>100 (1.4)	>100 (21)
marmesinin (**14**)	>100 (16)	>100 (49)
bergapten (**15**)	>100 (23)	>100 (49)
apiosylskimmin (**16**)	>100 (-2.1)	>100 (36)
17	>100 (5.8)	>100 (49)
hyuganoside II (**18**)	>100 (0.6)	>100 (32)
hyuganoside III (**19**)	>100 (-0.2)	>100 (-26)
(-)-falcarinol (**20**)	>100 (-0.6)	4.8
falcarindiol (**21**)	>100 (-33)	4.4

( ) : inhibition (%) at 100 μM

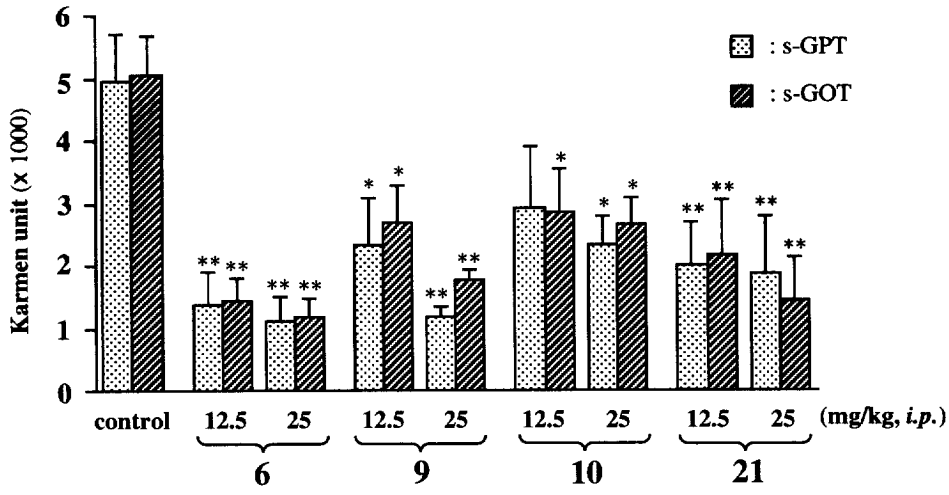


Fig. 2. Inhibitory Effects of Acylated Khellactones (6, 9, 10) and Falcarindiol (21) from the Roots of *Angelica furcijuga* on D-GalN/LPS-Induced Liver Injury in Mice

Each column represents the mean with S.E. (N=8—10, $*p<0.05$, $**p<0.01$).

overproduction of NO is well known to be a cause of inflammation, immunological responses and endotoxin-induced shocks,[5] these coumarins and polyacetylenes (**2**—**5**, **9**, **10**, **20**, and **21**) may be effective for inflammation and endotoxic shocks.

References and Notes

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