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HEPATOPROTECTIVE, SUPEROXIDE SCAVENGING, AND ANTIOXIDATIVE ACTIVITIES OF AROMATIC CONSTITUENTS FROM THE BARK OF *BETULA PLATYPHYLLA* VAR. *JAPONICA*

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Abstract : The 50% aqueous methanolic extract from the bark of *Betula platyphylla* SUKATCHEV var. *japonica* (MIQ.) HARA was found to show potent inhibitory activity on the liver-injury induced by CCl_4 or D-galactosamine (D-GalN)/lipopolysaccharide as well as O_2^- scavenging and antioxidative activities. From the 50% aqueous methanolic extract, two new diarylheptanoids named betulaplatosides Ia (**1**) and Ib (**2**) and a new arylbutanoid named betulaplatoside II (**3**) were isolated together with seventeen known aromatic constituents. **1**, **2**, and two known diarylheptanoids [(5*S*)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone 5-*O*-β-D-apiofurano-syl-(1→6)-β-D-glucopyranoside (**6**) and aceroside VIII (**7**)] showed protective activity against D-GalN-induced cytotoxicity in primary cultured rat hepatocytes. Furthermore, several aromatic constituents exhibited potent O_2^- scavenging and antioxidative activities.

Betula platyphylla SUKATCHEV var. *japonica* (MIQ.) HARA ("Shirakaba" in Japanese, Betulaceae) is widely distributed in Japan, China, Korea, Sakhalin, and Siberia. The bark of *B. platyphylla* has been used for pneumonia, choloplania, nephritis, and chronic bronchitis in Chinese traditional medicine. The chemical constituents of *Betula* species including *B. platyphylla* have already been identified as follows: betulin and several triterpenes from the outer bark, phenolic compounds such as diarylheptanoids and arylbutanoids from the inner bark, dammarane-type triterpenes from the leaves, dammarane-type triterpene caffeates and *p*-coumarates from the root bark.[1] However, the pharmacological property and bioactive constituents were left uncharacterized.

In the course of our studies on the hepatoprotective constituents of natural medicines,[2] the 50% aqueous methanolic extract from the bark of *B. platyphylla* var. *japonica* was found to show potent inhibitory activity on the liver injury induced by CCl_4 or D-galactosamine (D-GalN)/lipopolysaccharide (LPS) as well as O_2^- scavenging and antioxidative activities. From the 50% aqueous methanolic extract, new diarylheptanoid glucosides, betulaplatosides Ia (**1**) and Ib (**2**), and a new arylbutanoid glycoside, betulaplatoside II (**3**), were isolated together with seventeen known constituents. This paper deals with the isolation and characterization of inhibitors against D-GalN-induced hepatocytotoxicity, and O_2^- scavenging and antioxidative constituents from *B. platyphylla* var. *japonica*.

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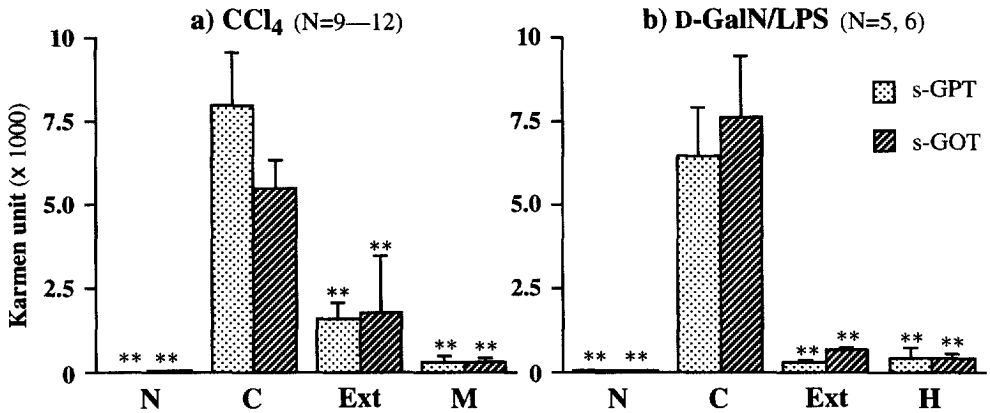


Fig. 1. Inhibitory Effects of 50% Aq. Methanolic Extract from the Bark of *B. platyphylla* var. *japonica* on CCl_4- or D-GalN/LPS- Induced Liver Injury in Mice

N : normal group, **C** : control group, **Ext** : 50% aq. MeOH ext. 200 mg/kg, *i.p.*, **M** : malotilate 100 mg/kg, *i.p.*, **H** : hydrocortisone 20 mg/kg, *i.p.*

Male ddY mice weighing 27—29 g were used. After 20 h of fasting, 10% (v/v) CCl_4/olive oil was injected subcutaneously at 5 mL/kg or D-GalN (350 mg/kg) and LPS (10 μg/kg) were injected intraperitoneally (*i.p.*) to produce liver injury. Each test sample was administered *i.p.* 1 h before D-GalN/LPS or CCl_4 injection. Blood samples were collected 10 h after D-GalN/LPS injection or 20 h after CCl_4 injection. Each column represents the mean with S.E. (**$p<0.01$).

Isolation and Structure Elucidation

Isolation of Chemical Constituents from the Bark of B. platyphylla var. japonica : The dried bark of *B. platyphylla* var. *japonica* collected at Nagano prefecture in Japan was extracted with 50% aqueous methanol under reflux. The 50% aqueous methanolic extract was purified by a combination of ordinary- and reversed-phase silica gel column chromatography, and finally HPLC (YMC-pack R&D-ODS-5-A, MeOH-H_2O, *i*-PrOH:H_2O) to give two new diarylheptanoid glucosides, betulaplatoside Ia (**1**, 0.0009%) and Ib (**2**, 0.0002%), and a new arylbutanoid glycoside, betulaplatoside II (**3**, 0.0009%), together with seventeen known constituents such as a diarylheptanoid [1,7-bis(4-hydroxyphenyl)-3-hepten-5-one (**4**, 0.0008%)],[1i] three diarylheptanoid glycosides [platyphylloside (**5**, 0.089),[1b] (5*S*)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone 5-*O*-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (**6**, 0.0077%),[1e] aceroside VIII (**7**, 0.042%)[1i]], five arylbutanoid glycosides [rhododendrin (**8**, 0.0058%),[1f] epirhododendrin (**9**, 0.0058%),[1f] apiosylrhododendrin (**10**, 0.0065%),[1e] apiosylepirhododendrin (**11**, 0.029%),[1f] (2*R*)-4-(4-hydroxyphenyl)-2-butanol 2-*O*-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside (**12**, 0.0002%)[1e]], four lignan glycosides [(+)-lyoniresinol 3α-*O*-β-D-glucopyranoside (**13**, 0.0024%),[1g] (-)-lyoniresinol 3α-*O*-β-D-glucopyranoside (**14**, 0.0001%),[1d] nudiposide [= (-)-lyoniresinol 3α-*O*-β-D-xylopyranoside] (**15**, 0.0007%),[1h] (+)-5'-methoxyisolariciresinol 3α-*O*-β-D-glucopyranoside (**16**, 0.00003%)[1d]], (+)-catechin (**17**, 0.0007%),[1i] (+)-catechin 7-*O*-β-D-xylopyranoside (**18**, 0.0013%),[1i] 3,4,5-trimethoxyphenyl β-D-glucopyranoside (0.0034%),[1c] and 3,4,5-trimethoxyphenol β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside (0.0007%).[1h]

Structures of Betulaplatosides Ia (1), Ib (2), and II (3) : The UV and IR spectra of betulaplatosides Ia [**1**, a white powder, $[\alpha]_D^{25}$ -11.5° (MeOH)] and Ib [**2**, a white powder, $[\alpha]_D^{24}$+4.4° (MeOH)] were similar to one another and showed the presence of aromatic ring and hydroxyl functions. The positive-ion and negative-ion FAB-MS of **1** and **2** showed the same quasimolecular-ion peaks at *m/z* 479 $(M+H)^+$, *m/z* 501 $(M+Na)^+$, and

m/z 477 $(M-H)^-$ and the molecular formula $C_{25}H_{34}O_9$ was determined by high-resolution MS measurement. The ^1H- and ^{13}C-NMR (CD_3OD) (Table 1) spectra of **1** and **2** showed signals due to two disubstituted benzene rings, two benzyl methylenes, three methylenes, and two oxygenated methines together with a β-D-glucopyranoside moiety. Comparison of the physical data for **1** and **2** with those for platyphylloside (**5**)[1b] led us to confirm the 3-dihydro-structures of **5** for **1** and **2**. Acid hydrolysis of **1** and **2** with 5% aq. H_2SO_4-dioxane liberated D-glucose, which was identified by GLC analysis of the TMS thiazolidine derivative.[3] Finally, reduction of **5** with $NaBH_4$ in methanol gave **1** and **2** in a 2:1 ratio, which furnishd optical active **1a** and meso-type **2a** by enzymatic hydrolysis with β-glucosidase. On the basis of this evidence, the absolute stereostructures of betulaplatosides Ia (**1**) and Ib (**2**) were expressed to be as (3*S*,5*S*)-1,7-bis(4-hydroxyphenyl)-3,5-dihydroxyheptane-5-*O*-β-D-glucopyranoside (**1**) and its 3*R*-epimer (**2**).

Acid hydrolysis of betulaplatoside II [**3**, a white powder, $[\alpha]_D^{26}$ -48.5° (EtOH), $C_{21}H_{32}O_{11}$, UV λ_{max} MeOH nm (log ε): 280 (3.5), 224 (4.1), IR (KBr) cm^{-1}: 3414, 2926, 1618, 1516, 1074, 833] liberated D-glucose and L-arabinose, while enzymatic hydrolysis of **3** provided (+)-rhododendrol (**9a**).[1f] The ^1H- and ^{13}C-NMR (CD_3OD) (Table 1) spectra of **3** showed signals assignable to a (+)-rhododendrol moiety [δ 3.76 (m, 2-H)] together with a β-D-glucopyranosyl [δ 4.31 (d, *J*=7.9 Hz, 1''-H)] and α-L-arabinofuranosyl [δ 4.94 (br s, 1'''-H)]. The oligosaccharide structure of **3** was characterized by HMBC experiment, which showed long-range correlations between the 1'''-proton and the 6''-carbon and between the 1''-proton and the 2-carbon. Consequently, the absolute stereostructure of betulaplatoside II (**3**) was determined as shown.

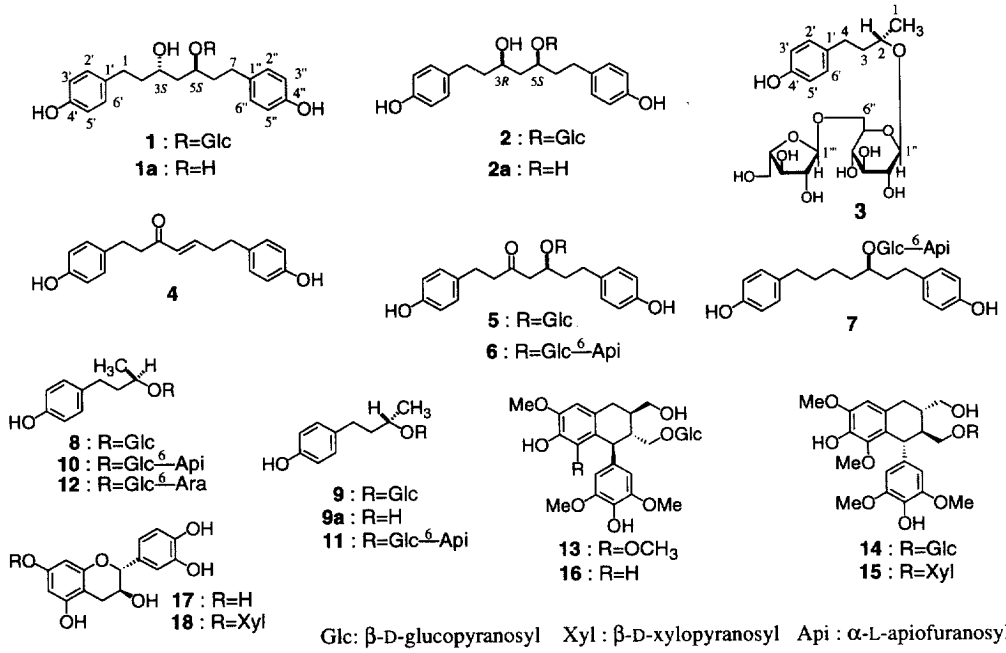
Table 1. ^{13}C-NMR of Betulaplatosides Ia (1), Ib (2), and II (3)

	1	2	3
C-1	32.0	32.3	22.2
C-2	41.2	41.3	77.4
C-3	69.9	68.4	40.1
C-4	42.9	43.0	31.7
C-5	79.7	78.4	
C-6	38.6	39.2	
C-7	31.4	31.6	
C-1'	134.4	134.6	134.6
C-2', 6'	130.3[a)]	130.3	130.4
C-3', 5'	116.0[b)]	116.1	116.1
C-4'	156.2[c)]	156.3	156.3
C-1''	134.7	134.7	
C-2'', 6''	130.4[a)]	130.5	
C-3'', 5''	116.1[b)]	116.1	
C-4''	156.3[c)]	156.3	
Glc-1	103.7	104.3	104.3
-2	75.2	75.5	75.4
-3	78.2	78.3	78.1
-4	71.6	71.7	72.1
-5	77.8	77.9	76.5
-6	62.8	62.8	68.2
Ara-1			110.0
-2			83.2
-3			79.0
-4			86.0
-5			63.1

a), b), c) : Assignments may be interchangeable.

Bioassay Methods

Hepatoprotective Activity : CCl_4- or D-GalN/LPS-induced liver damage in mice was performed according to our previous report.[2] Each test compound was suspended in 0.5% CMC-Na, and the solution was given intraperitoneally (*i.p.*) before 1 h of CCl_4 or D-GalN/LPS treatment. The hepatocytoprotective 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 with D-GalN (1 mM) and a test compound for 44 h.[2] Each DMSO solution of test compound was added to the medium at the concentration of 3—100 μM (N=4).



Glc: β-D-glucopyranosyl Xyl : β-D-xylopyranosyl Api : α-L-apiofuranosyl

Table 2. Effects of 50% Aq. Methanolic Extract and Constituents from *B. platyphylla* var. *japonica* on D-GalN-Induced Cytotoxicity in Primary Cultured Rat Hepatocytes

sample	inhibition (%)			
	12.5 μg/ml	25 μg/ml	50 μg/ml	100 μg/ml
50% aq. MeOH ext.	16.0±1.9**	18.0±2.1**	37.5±3.9**	53.4±4.6**

sample	inhibition (%)			
	3 μM	10 μM	30 μM	100 μM
diarylheptanoids				
betulaplatoside Ia (**1**)	9.9±3.2	7.4±3.2	21.7±5.8**	37.4±2.2**
betulaplatoside Ib (**2**)	16.2±5.0	14.1±2.0	27.3±4.1**	74.7±7.6**
6	24.9±2.9**	49.4±4.5**	71.4±2.2**	112.9±3.1**
aceroside VIII (**7**)	28.3±3.3**	38.4±5.5**	43.9±5.8**	55.3±2.6**
arylbutanoids				
betulaplatoside II (**3**)	10.1±2.7	3.6±1.9	9.6±2.7	5.4±2.0
rhododendrin (**8**)	4.7±1.1	10.1±5.2	4.9±2.4	-0.8±0.1
apiosylrhododendrin (**10**)	1.4±2.9	-1.6±0.6	2.1±1.4	-2.3±1.0
apiosylepirhododendrin (**11**)	3.6±3.0	1.7±2.0	0.7±1.8	-4.5±1.1
12	3.4±1.8	6.2±1.8	7.5±2.5	6.8±0.2
others				
13	22.3±6.4	24.8±8.8*	24.1±3.8*	26.9±7.1*
nudiposide (**15**)	8.3±1.2	7.6±2.9	10.5±1.8**	28.9±1.6**
18	4.8±2.2	8.8±2.8	8.5±1.4	9.4±3.0

(*p<0.05, **p<0.01)

Table 3. O_2^- Scavenging and Antioxidative Activities of Constituents from the Bark of *B. platyphylla* var. *japonica*

sample	O_2^- scavenging activity		antioxidative activity	
	conc. (μg/ml)	inhibition (%)	conc. (μg/ml)	inhibition (%)
50% aq. MeOH ext.	20	87.3	200	90.6
diarylheptanoids				
betulaplatoside Ia (**1**)	20	1.8	200	61.8
betulaplatoside Ib (**2**)	20	1.0	200	56.3
4	20	58.6	200	90.9
platyphylloside (**5**)	20	13.6	200	82.5
6	20	11.7	200	33.6
arylbutanoids				
betulaplatoside II (**3**)	20	4.0	-	-
apiosylrhododendrin (**10**)	20	1.8	200	93.5
apiosylepirhododendrin (**11**)	20	10.8	200	21.8
12	20	5.1	200	98.2
others				
13	20	67.3	200	96.8
nudiposide (**15**)	20	58.8	200	87.2
(+)-catechin (**17**)	20	93.8	200	37.0
18	20	92.9	200	50.1
α-tocopherol	-	-	200	56.7
BHA (*tert*-buthyl-4-hydroxyanisol)	-	-	200	88.0
BHT (*tert*-buthyl-4-hydroxytoluene)	-	-	200	97.0

O_2^- Scavenging Activity : O_2^- scavenging activity was measured using nitroblue tetrazolium (NBT) methods.[4] Briefly, the reaction mixture containing 100 μM xanthine, 100 μM EDTA, 25 μM NBT, 0.005% BSA, and xanthine oxidase in 40 mM carbonate buffer (pH 10.2) was incubated with or without each test compound for 20 min at 25°C. After incubation, formazan formation was monitored at 560 nm.

Antioxidative Activity : Antioxidative activity was measured using TBA method.[5] Briefly, the mixture containing 0.5% linoleic acid in 20 mM phosphate buffer (pH 7.0) was incubated with or without the extract or each test compound at 40°C. Aliquots of reaction mixture were incubated with thiobarbituric acid, and malondialdehyde formation was monitored at 532 nm.

Results and Discussion

The hepatoprotective effects of the 50% aqueous methanolic extract were examined by monitoring the inhibitory activity on the increase of serum GPT and GOT by either CCl_4 or D-GalN/LPS. The extract (200 mg/kg, *i.p.*) inhibited the increase of serum GPT and GOT in both experimental models (Fig. 1). Furthermore, we examined the inhibitory effect of its constituents on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes. As shown in Table 2, diarylheptanoids **1**, **2**, **6**, and **7** showed the inhibitory activity in a concentration-dependent manner, while other phenolic compounds exhibited little effect.

Results of O_2^- scavenging and antioxidative activities of the 50% aqueous methanolic extract and each compound are summarized in Table 3. The 50% aqueous methanolic extract showed strong O_2^- scavenging activity, and its constituents, **4**, **13**, **15** **17**, and **18**, showed marked activity. Diarylheptanoids (**1**, **2**, **4**—**6**), arylbutanoids (**10**, **12**), and lignan glucosides (**13**, **15**) were found to show marked inhibitory activity on lipid peroxidation as well as α-tocopherol, BHA and BHT. These O_2^- scavenging and antioxidative activities seemed to be active constituents against CCl_4- and D-GalN/LPS-induced liver injury, because O_2^- radical and lipid peroxidation are appeared to be important in these liver injury processes.[6,7] Diarylheptanoids **1**, **2**, and **6** with hepatoprotective activity against D-GalN-intoxication had no O_2^- scavenging activity but showed antioxidative activity.

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