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An Anti-influenza Component of the Bark of *Alnus japonica*

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This study to identify anti-influenza components of the bark of *Alnus japonica* resulted in the isolation of four lupane-type triterpenes (**1**-**4**) and one steroid (**5**). Their structures were characterized on the basis of physicochemical properties, NMR evidence, and ESI-MS data compared with reported data in the literature. Betulinic aldehyde (**3**) exhibited a particularly strong anti-influenza effect against KBNP-0028 relative to a positive control.

Key words: *Alnus japonica*, Betulaceae, Lupane triterpene, Anti-influenza, KBNP-0028

INTRODUCTON

Alnus japonica Steudel (Betulaceae), an indigenous *Alnus* species in Korea, North China, and Japan, is a deciduous, ovate elliptic-leaved tree growing in wet lowlands and low mountainous areas. The bark of the *Alnus* species has been used in Korean folk medicine as a remedy for fever, hemorrhage, diarrhea, and alcoholism (Lee, 1966). Leaf and bark extracts of *A. japonica* have been used as a health food and to enhance immunity against influenza (Ra et al., 2009). Previous phytochemical investigations of *A. japonica* have led to the identification of numerous diarylheptanoids, along with several triterpenoids and flavonoids, some of which exhibited anti-inflammatory, anticancer, and antioxidative activities (Kim et al., 2005; Kuroyanagi et al., 2005; Lee et al., 1992; Wada et al., 1998). The current study reveals that the MeOH extract of *A. japonica* bark has a strong antiviral effect against an H9N2 subtype avian influenza virus, A/chicken/KBNP-0028/2000 (H9N2) (KBNP-0028) (Kwon et al., 2009). Subsequently, bio-guided chromatographic separation resulted in the isolation of four lupane-type triterpenes (**1**-**4**) and one steroid (**5**). Their antiviral activity was assessed based on *in vitro* egg-bit assays against KBNP-0028.

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MATERIALS AND METHODS

General procedures

Melting points were obtained with an Electrothermal 9100 melting point apparatus (Electrothermal Ltd.). Optical rotation was measured with a DIP-360 digital polarimeter (JASCO). NMR spectra were obtained on a DRX 500 (Bruker) and ECA 400 NMR (JEOL) spectrometers. ESI-MS data were obtained on an 1100 LC-MSD Trap spectrometer (Agilent).

Column chromatography (CC) was performed on silica gel (70-230 and 230-400 mesh; Merck) and YMC C-18 resins (30-50 μm; Fuji Silysia Chemical Ltd.). Thin layer chromatography analyses were run on Kieselgel 60 F_{254} (Merck 1.05715) and RP-18 F_{254s} (Merck) plates; spots were visualized by spraying with 10% aqueous H_2SO_4 followed by heating.

Plant material

The bark of *A. japonica* was collected in Yanzi Province, China, in September, 2006, and was taxonomically identified by Y. H. Kim (author). A voucher specimen (CNU 08102) has been deposited in the herbarium at the College of Pharmacy, Chungnam National University.

Extraction and isolation

Air-dried *A. japonica* bark (1.0 kg) was extracted with 3×3 L of 95% EtOH at room temperature, and the combined extracts were concentrated to dryness *in vacuo*. The EtOH residue (308 g) was suspended in 2.0 L of water and successively partitioned with CH_2Cl_2,

EtOAc, and *n*-BuOH (each 2.0 L×3) to obtain soluble fractions of CH_2Cl_2 (39 g), EtOAc (83 g), and *n*-BuOH (15 g). The CH_2Cl_2-soluble fraction, which was particularly active against KBNP-0028 (Table II), was fractionated over a silica gel column using a gradient of *n*-hexane-EtOAc (20:1-0:1, v/v) to yield seven fractions (1a-g). Fraction 1b was repeatedly separated on a silica gel column with *n*-hexane-EtOAc (6:1), followed by a YMC RP column with MeOH-acetone (6:1) to furnish compounds **1** (20 mg) and **3** (25 mg). Compound **5** (50 mg) was obtained from fraction 1b by further separation on a YMC RP column with MeOH-acetone (5:1). In a similar fashion, compound **4** (9 mg) was purified from fraction 1e by separation on a silica gel column with *n*-hexane-EtOAc (10:1). Finally, fraction 1f was separated on a silica gel column with $CHCl_3$-MeOH (20:1) to yield compound **2** (25 mg).

Lupeol (1)
White powder; m.p. 199-210°C; $[\alpha]_D^{20}$ +26° (*c* 0.80, $CHCl_3$); ESI-MS: *m/z* $[M+H]^+$ 427; ^1H NMR ($CDCl_3$, 400 MHz): δ 4.59 and 4.47 (each 1H, both s, H-29), 3.11 (1H, dd, *J* = 12.0, 5.2 Hz, H-3), 2.30 (1H, m, H-19), 0.67, 0.70, 0.74, 0.85, 0.88, 0.94, and 1.60 (each 3H, all s, H-24, 28, 25, 27, 23, 26, and 30); and ^{13}C NMR ($CDCl_3$, 100 MHz): see Table I.

Betulin (2)
White powder; m.p. 250-252°C; $[\alpha]_D^{20}$ +22° (*c* 0.60, $CHCl_3$); ESI-MS: *m/z* $[M+H]^+$ 443; ^1H NMR ($CDCl_3$, 500 MHz): δ 4.68 and 4.56 (each 1H, both s, H-29), 3.80 and 3.34 (each 1H, both d, *J* = 11.0 Hz, H-28), 3.20 (1H, dd, *J* = 11.5, 4.5 Hz, H-3), 2.40 (1H, m, H-19), 0.77, 0.83, 0.98, 0.99, 1.03, and 1.69 (each 3H, all s, H-24, 25, 23, 27, 26, and 30); and ^{13}C NMR ($CDCl_3$, 125 MHz): see Table I.

Betulinic aldehyde (3)
White powder; m.p. 192-193°C; $[\alpha]_D^{20}$ +19° (*c* 0.50, $CHCl_3$); ESI-MS: *m/z* $[M+H]^+$ 441; ^1H NMR ($CDCl_3$, 400 MHz): δ 9.60 (1H, s, H-28), 4.68 and 4.56 (each 1H, both s, H-29), 3.12 (1H, dd, *J* = 11.2, 4.8 Hz, H-3), 2.80 (1H, m, H-19), 0.68, 0.75, 0.84, 0.89, 0.90, and 1.63 (each 3H, all s, H-24, 25, 23, 27, 26, and 30); and ^{13}C NMR ($CDCl_3$, 100 MHz): see Table I.

3-Acetoxybetulinic aldehyde (4)
White powder; m.p. 190-191°C; $[\alpha]_D^{20}$ +14° (*c* 0.50, $CHCl_3$); ESI-MS: *m/z* $[M+H]^+$ 483; ^1H NMR ($CDCl_3$, 400 MHz): δ 9.60 (1H, s, H-28), 4.68 and 4.56 (each 1H, both s, H-29), 4.42 (1H, dd, *J* = 11.2, 4.8 Hz, H-3), 2.80 (1H, m, H-19), 2.10 (3H, s, $COC\underline{H}_3$), 0.71, 0.78, 0.86, 0.89, 0.90, and 1.63 (each 3H, all s, H-24, 25, 23, 27, 26, and 30); and ^{13}C NMR ($CDCl_3$, 100 MHz): see Table I.

β-Sitoserol (5)
White needles; m.p. 139-140°C; $[\alpha]_D^{20}$ -37° (*c* 0.10, $CHCl_3$); ESI-MS: *m/z* $[M+H]^+$ 415; ^1H NMR ($CDCl_3$, 400 MHz): δ 5.32 (1H, br t, *J* = 5.2 Hz, H-6), 3.50 (1H, dd, *J* = 11.2, 4.8 Hz, H-3), 0.98 (3H, s, H-19), 0.90 (3H, d, *J* = 6.4 Hz, H-21), 0.84 (3H, t, *J* = 7.2 Hz, H-29), 0.80 (3H, d, *J* = 6.8 Hz, H-27), 0.79 (3H, d, *J* = 6.8 Hz, H-26), 0.65 (3H, s, H-18); and ^{13}C NMR ($CDCl_3$, 100 MHz): see Table I.

Table I. ^{13}C NMR Data of **1**-**5**

Position	1	2	3	4	5
1	38.7	38.9	38.7	39.2	33.1
2	27.4	27.6	27.3	27.0	33.5
3	79.0	79.2	78.9	80.9	71.4
4	38.8	39.0	38.8	38.8	39.4
5	52.3	55.5	52.2	52.3	140.3
6	18.3	18.5	18.2	18.2	121.3
7	34.3	34.4	34.3	34.3	31.5
8	40.8	41.1	40.8	40.8	31.5
9	50.4	50.6	50.4	50.3	49.7
10	37.2	37.5	37.1	37.1	36.1
11	20.9	21.0	20.7	20.7	20.7
12	25.1	25.4	25.5	25.5	41.9
13	38.0	37.4	38.6	38.6	41.9
14	42.8	42.9	42.5	42.5	56.4
15	28.0	27.3	29.2	29.2	23.9
16	35.6	29.4	28.8	28.7	27.9
17	43.0	48.0	59.3	59.3	55.6
18	48.3	49.0	48.0	47.9	11.6
19	48.0	48.0	47.5	47.5	19.4
20	151.0	150.7	149.7	150.4	35.7
21	30.0	29.9	29.8	29.8	18.6
22	40.0	34.2	33.2	33.2	39.4
23	27.4	28.2	27.3	27.3	25.6
24	15.4	15.6	15.32	15.3	45.4
25	15.9	16.2	15.8	15.9	29.0
26	16.1	16.3	16.1	16.1	19.0
27	14.5	15.0	14.2	14.2	18.4
28	18.0	60.8	206.7	206.7	22.9
29	109.3	109.9	110.1	110.6	11.5
30	19.3	19.3	19.0	19.0	
$\underline{C}OCH_3$				171.2	
$CO\underline{C}H_3$				21.7	

Table II. Antiviral activity of the extracts and compounds **1**-**5** against the influenza virus, KBNP-0028, in the egg-bit assay

Sample	EC_{50} (μg/mL)[a]	CC_{50}(μg/mL)[b]	SI[c]
MeOH ext.	62.5	ND[d]	ND
CH_2Cl_2 ext.	31.3	ND	ND
EtOAc ext.	93.4	ND	ND
BuOH ext.	>100	ND	ND
1	>100	>250	ND
2	>100	>250	ND
3	12.5	23.4	1.9
4	>100	>250	ND
5	>100	>250	ND
Oseltamivir[e]	0.063	ND	ND

[a]EC_{50}: 50% effective concentration; [b]CC_{50}: 50% cytotoxicity concentration; [c]SI: selection index; [d]ND: not determined; [e]Positive control.

Antiviral assay

Virus and virus propagation

KBNP-0028 was propagated in the allantoic cavity of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE; Hy-Vac) (Kwon et al., 2009).

Antiviral testing

To test the anti-influenza activity of the isolated compounds, an egg-bit assay was used as reported previously (Fulton and Armitage, 1951) with slight modification. Briefly, egg-bits were prepared from 10- to 11-day-old SPF ECE. Each egg-bit was placed into a well of a 24-well culture plate. The allantois was infected with 100 μL of KBNP-0028 solution corresponding to 100 times the 50% egg-bit infection dose ($EBID_{50}$) and allowed to incubate for 30 min. One milliliter of 199+F10 (1:1; Gibco-BRL) medium containing 0.075% sodium bicarbonate and gentamicin (100 μg/mL) was added to each well. *A. japonica* isolates were evaluated for antiviral activity at 125, 100, 50, 25, 12.5, 6.3, and 3.1 μg/mL in duplicate, with incubation for 5 days. Plate hemagglutination tests were performed by mixing 25 μL of the culture fluid with the same volume of washed chicken red blood cells (0.1%). The isolate concentration required to reduce the degree of hemagglutination of KBNP-0028 by 50% relative to control wells without isolate (EC_{50}) was calculated by plotting the percent hemagglutination inhibition versus isolate concentration.

Cytotoxic testing

To test cytotoxicity, chicken embryo fibroblast (CEF) cells were treated with isolate concentrations of 250, 125, 62.5, 31.3, and 15.6 μg/mL and cell viability was measured with a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Mosmann, 1983).

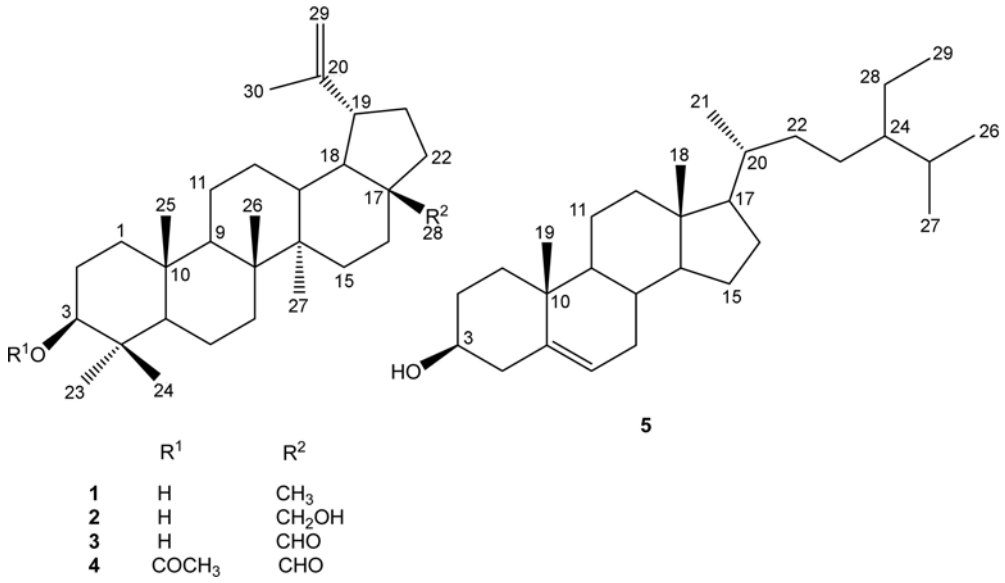
RESULTS AND DISCUSSION

The chromatography described above resulted in the isolation of four lupane-type triterpenes (**1**-**4**) and one steroid (**5**) from the CH_2Cl_2 fraction of the *A. japonica* bark extract (Fig. 1). Compound **1** was obtained as a white powder. Its ^1H NMR spectrum showed one oxymethine proton at δ_H 3.11 (1H, dd, J = 12.0, 5.2 Hz, H-3), two geminal olefinic protons at δ_H 4.47 and 4.59 (each 1H, both s, H-29), together with seven methyl signals at δ_H 0.67, 0.70, 0.74, 0.85, 0.88, 0.94, and 1.60 (each 3H, all s, H-24, 28, 25, 27, 23, 26, and 30). The ^{13}C NMR spectrum of **1** (Table I) contained 30 carbon signals, including one oxygenated carbon atom at δ_C 79.0 ppm (C-3) and two olefinic carbon atoms corresponding to a >C=CH_2 group at δ_C 151.0 (C-20) and 109.3 (C-29), typical of a lupane triterpene. In addition, a quasimolecular peak at m/z $[M+H]^+$ 427 was observed in the ESI-MS spectrum of **1**. Based on these results and previously published spectra (Sholichin et al., 1980), compound **1** was identified as lupeol.

The ESI-MS spectrum of **2** exhibited a molecular peak at m/z $[M+H]^+$ 443. The ^1H- and ^{13}C-NMR spectra of **2** (Table I) resembled those of **1** except for the presence of a hydroxyl methylene group [δ_H 3.34 and 3.80 (each 1H, both d, J = 11.0 Hz, H-28); δ_C 60.8 (C-28)] in place of the methyl group in **1**. Thus, compound **2** was identified as betulin (Sholichin et al., 1980).

Compound **3** was obtained as a white powder. Its ESI-MS spectrum exhibited a molecular peak at m/z $[M+H]^+$ 441. The ^1H and ^{13}C NMR spectra (Table I) of **3** were identical to **1** except for the presence of a formyl group [δ_H 9.60 (1H, s, H-28); δ_C 206.7 (C-28)] in place of the methyl group at C-28. A detailed comparison of these NMR data with those reported by Sholichin et al. (1980) led to the conclusion that compound **3** was betulinic aldehyde.

The NMR spectra of compound **4**, also a white powder, was almost identical to those of **3** except for the presence of one acetyl group [δ_C 171.2 ($\underline{C}OCH_3$) and 21.7 ($CO\underline{C}H_3$); δ_H 2.10 (3H, s, $COC\underline{H}_3$)]. This finding was further confirmed by a molecular peak at m/z $[M+H]^+$ 483, 42 amu more than that of **3**. The structure could be further refined as 3-OAc by the downfield shift of H-3 to δ_H 4.42 (1H, dd, J = 11.2, 4.8 Hz) relative to that of **3**. Compound **4** was therefore identified as 3-acetoxybetulinic aldehyde (Hiroya et al., 2002). To the best of our knowledge, the current study is the first report on the isolation of compounds



	R^1	R^2
1	H	CH_3
2	H	CH_2OH
3	H	CHO
4	$COCH_3$	CHO

Fig. 1. Structures of **1**-**5**

1-4 from *A. japonica*.

Compound **5** was identified as β-sitosterol based on its physicochemical properties, NMR spectra (^{1}H and ^{13}C NMR), ESI-MS spectra, and by comparison with published spectra (Chang et al., 1981).

Compounds **1**-**5** were screened for *in vitro* antiviral activity against KBNP-0028 using the egg-bit assay. Oseltamivir (Roche), an approved antiviral drug, was used as the positive control with an EC_{50} of 0.063 μg/mL. Betulinic aldehyde (**3**) showed the highest antiviral activity, with an EC_{50} of 12.5 μg/mL. The other compounds were considerably less active, with percentages of hemagglutination less than 50% at concentrations up to 100 μg/mL (Table II). Structurally, the presence of 3β-OH and 28-CHO groups in the lupane-type triterpenes might be necessary for anti-KBNP-0028 activity. The cytotoxic concentration that inhibited the viability of chicken embryo fibroblast (CEF) cells by 50% (CC_{50}) was calculated from the results of a MTT assay. Compound **3** was relatively toxic to CEF cells, with a CC_{50} of 23.4 μg/mL (SI value of 1.9) (Table II), which limits its therapeutic potential considerably.

In conclusion, the present study confirms the anti-influenza properties of *A. japonica* and helps explain, in part, the use of *A. japonica* extracts in folk remedies for influenza.

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