

Chemical constituents from *Betula schmidtii* and their free radical scavenging, tyrosinase inhibitory, and neuroprotective activities

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Abstract

The study was performed to identify bioactive constituents from the inner bark of *Betula schmidtii*. Seven compounds were isolated through silica gel and Sephadex LH-20 column chromatography, and identified to be betulin (1), betulinic acid (2), β -sitosterol (3), lupeol (4), kojic acid (5), 1,7-bis-(*p*-hydroxyphenyl)-(3,4-dihydroxy)-hepan-5-one (6), and platyphyllenone (7) using EI-MS, 1D-, and 2D-NMR data. Compounds 5, 6, and 7 show antioxidant effect with IC₅₀ values of 18.23 ± 1.08, 29.37 ± 1.42, and 40.14 ± 1.75 μM in DPPH free radical scavenging activity. Compounds 4 and 5 show an antioxidant effect with IC₅₀ values of 28.21 ± 1.80 and 37.74 ± 1.02 μM in DCHF-DA intracellular ROS generations. Compounds 5, 6, and 7 also exhibited mushroom tyrosinase inhibitory activity with IC₅₀ values of 17.42 ± 1.08, 47.17 ± 1.92, and 26.64 ± 1.40 μM. Compound 1 showed cell viability of 24.50 ± 6.30 and 45.60 ± 3.10% at 10 and 25 μM, while compound 6 showed 11.20 ± 1.40% at 25 μM in glutamate-induced damaged mouse neuronal cells.

KEYWORDS

Betula schmidtii, free radical scavenging, neuroprotective, phytochemicals, ROS generations, tyrosinase inhibitory

INTRODUCTION

Biologically active compounds of natural products are known to contribute therapeutic agents against chronic diseases. The effect of active compounds can be strongly associated with molecular differences present in natural organisms. Many of the therapeutic potentials of natural compounds have not yet been identified, and further investigations into natural compounds and their efficacy may continue to result in new therapeutic drug candidates.¹ The genus *Betula* is known to be widely distributed over temperate and arctic regions including Korea, Japan, China, Nepal, Afghanistan, Mongolia, Russia, and Europe, and is also distributed in North and South America. Over the past few decades, many studies were conducted to investigate bioactive constituents for

therapeutic uses. In previous reports, alkaloids, arylbutanoids, diarylheptanoids, lignans, phenolic acids and glycosides, and terpenoids were isolated from the inner bark of *Betula* species.^{2–6} For a long time, *Betula* species have been recognized as having a wide range of medicinal properties, such as anti-arthritic, anti-cancer, anti-diabetic, anti-inflammatory, anti-microbial, anti-oxidant, anti-viral, and immunomodulatory activities.^{7–12} *Betula schmidtii* Regel. (Betulaceae) is commonly used in Korea, Japan, and Northern China as building material, furniture, dyes, and therapeutic resources.^{13–15} As part of our ongoing study for the identification of bioactive constituents, compounds 1–7 were isolated through extraction, solvent fractionation, and repeated silica gel and Sephadex LH-20 vacuum liquid column chromatography from the inner bark of *Betula schmidtii*.

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EXPERIMENTAL SECTION

General experimental procedure

Molecular fragmentation was performed by EI-MS spectrometry on a JMS-AX505WA HP5890 Series II, HP (Jeol, Tokyo, Japan). UV-visible spectra were analyzed by a JP/U3010 Spectrophotometer (Hitachi Ltd., Tokyo, Japan). NMR spectral analyses, such as ^1H - ^1H COSY, HMQC, and HMBC, were performed by a Varian UI500 FT-NMR Spectrometer (Varian Inc, Palo Alto, CA) at 500 MHz (^1H) and 125 MHz (^{13}C). Tetramethylsilane and CDCl_3 , CD_3OD - d_4 , and DMSO - d_6 were used as internal standard and NMR solvents. Silica gel 60 (70–230 mesh; Merck Millipore) and Sephadex LH-20 (25–100 mm; GE Healthcare Life Sciences, Uppsala, Sweden) were used for column chromatography (CC). Silica gel 60F₂₅₄ (0.25 mm layer thickness; Merck, Darmstadt, Germany) was used for thin layer chromatography (TLC). TLC plates were visualized by UV light and staining with FeCl_3 , 20% H_2SO_4 , and then heated at 70–80°C for 5 sec. All chemicals for NMR solvents, mushroom tyrosinase (EC 1.14.18.1), L-tyrosine, kojic acid, and ascorbic acid were purchased from Sigma-Aldrich Chemical Co (St. Louis MO, USA). Other reagents used were analytical grade.

Plant material

Air-dried inner bark of *Betula schmidtii* was presented by Life Trees Ltd. (Suwon, Kyunggi-Do, Republic of Korea) and taxonomically identified by Dr. Chun-Soo Na (Life Trees Ltd, Suwon).

Extraction, fractionation, and isolation

Air-dried inner bark of *Betula schmidtii* (5.97 kg) was extracted with 80% ethyl alcohol (EtOH) in hot water. The extract was filtered, and the filtrate was evaporated to give EtOH extracts (652.82 g). The EtOH extract was suspended in water (1 L), and partitioned with *n*-hexane, chloroform (CHCl_3), ethyl acetate (EtOAc), and *n*-butyl alcohol (*n*-BuOH), yielding 1.84, 18.26, 42.16, and 122.78 g, respectively. Silica gel column chromatography (CC) (2 × 30 cm) of *n*-hexane-soluble fraction (1.0 g) was performed using *n*-hexane and a mixture of *n*-hexane-EtOAc (100:0 → 1:3) in a gradient system yielded thirty-five subfractions (H1 ~ H35). Fraction H13 (0.24 g) was subjected to silica gel CC (1 × 25 cm) using *n*-hexane-EtOAc (100:0 → 1:2) to give ten subfractions (H13-1 ~ H13-10). Fraction H13-3 ~ 4 (0.14 g) was subjected to Sephadex LH-20 vacuum liquid column chromatography (VLC) (2 × 12 cm) with MeOH as eluent, yielded Compound **1** (51.0 mg). Fraction H29 (0.18 g) was subjected to silica gel CC (1 × 30 cm) using *n*-hexane and a mixture of *n*-hexane-EtOAc (100:0 → 1:3) to give twenty subfractions (H29-1 ~ H29-20). Fraction H29-12 (0.07 g)

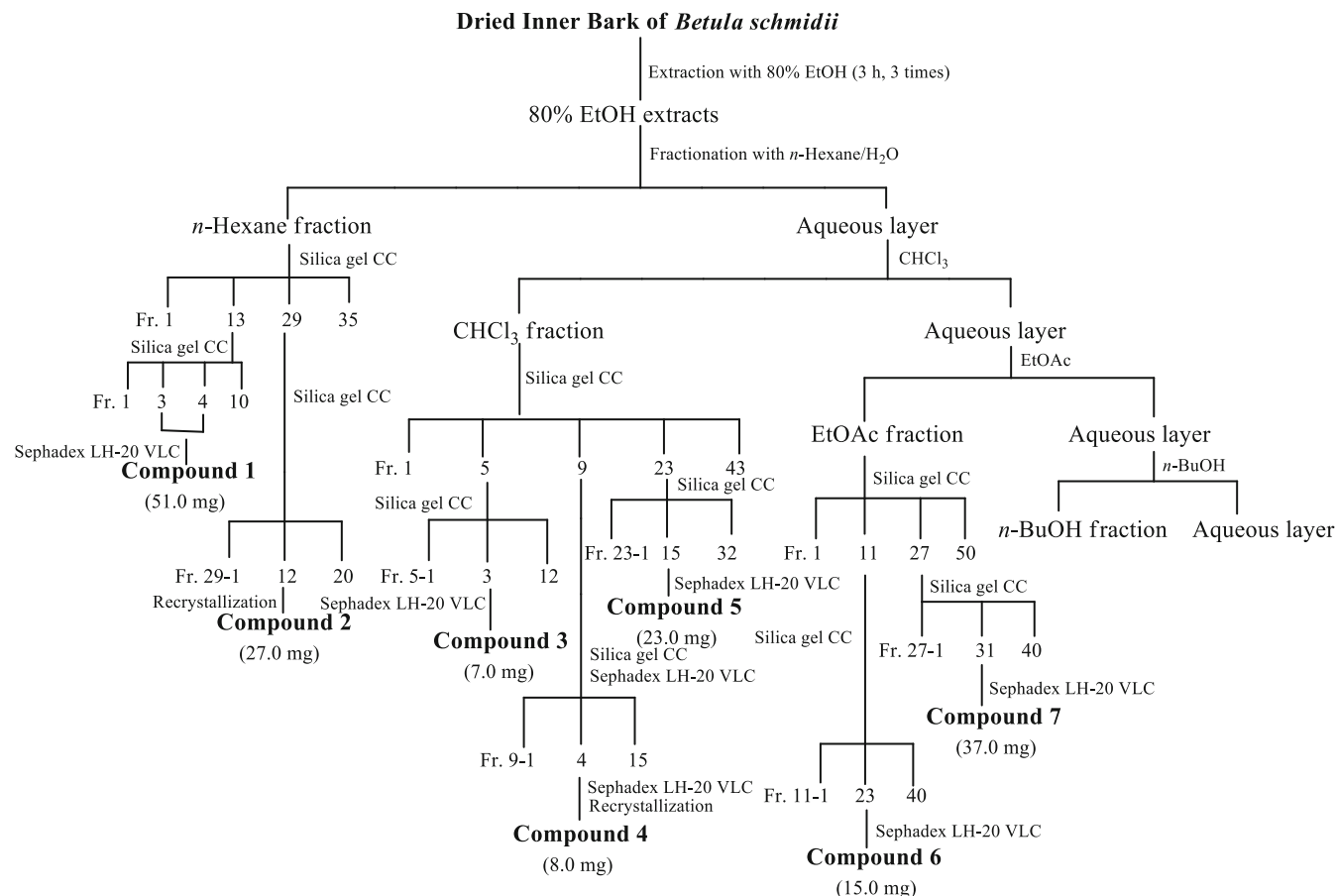
was recrystallized using MeOH yielded compound **2** (27.0 mg). Silica gel CC (5 × 30 cm) of CHCl_3 -soluble fraction (20 g) was carried out using CHCl_3 and a mixture of CHCl_3 -MeOH (100:0 → 10:1) to yield forty-three subfractions (C1 ~ C43). Fraction C5 (0.8 g) was subjected to silica gel CC using *n*-hexane and a mixture of *n*-hexane-EtOAc (100:0 → 5:1) to give twelve subfractions (C5-1 ~ C5-12). Fraction C5-3 was subjected to Sephadex LH-20 VLC (2 × 10 cm) eluted with MeOH yielded compound **3** (7.0 mg). Fraction C9 (1.0 g) was subjected to silica gel CC (1.5 × 25 cm) using CHCl_3 and a mixture of CHCl_3 -MeOH (100:0 → 9:1) to give fifteen subfractions (C9-1 ~ C9-15). Fraction C9-4 (0.17 g) was subjected to Sephadex LH-20 VLC (1.2 × 8 cm) and recrystallized using MeOH yielded compound **4** (8.0 mg). Fraction C23 (1.8 g) was subjected to silica gel CC (1 × 15 cm) using CHCl_3 and a mixture of CHCl_3 -MeOH (100:0 → 9:1) to give thirty-two subfractions (C23-1 ~ C23-32). Fraction C23-15 (0.14 g) was subjected to Sephadex LH-20 VLC (0.8 × 8 cm) eluent with MeOH yielded compound **5** (23.0 mg). Silica gel CC of EtOAc-soluble fraction (10 g) was carried out using CHCl_3 and a mixture of CHCl_3 -MeOH (100:0 → 3:1) yielded fifty subfractions (E1 ~ E50). Fraction E11 (2.2 g) was subjected to silica gel CC (3 × 30 cm) using a mixture of *n*-hexane-EtOAc (20:1 → 1:2) to give forty subfractions (E11-1 ~ E11-40). Fraction E11-23 (0.12 g) was subjected to Sephadex LH-20 VLC (1.2 × 12 cm) eluted with MeOH yielded compound **6** (15.0 mg). Fraction E27 (1.4 g) was subjected to silica gel CC (2 × 25 cm) using a mixture of *n*-hexane-EtOAc (10:1 → 1:4) to give forty subfractions (E27-1 ~ E27-40). Fraction E27-31 was subjected to Sephadex LH-20 VLC (1.2 × 10 cm) eluted with MeOH yielded compound **7** (37.0 mg) (Scheme 1).

Free radical scavenging activity

DPPH assay was performed to determine the free radical-scavenging activity of each sample. The radical-scavenging activity was evaluated as a percentage of DPPH discoloration compared to control. Relative radical scavenging activity (%) was calculated by the equation, $[1 - \text{absorbance of solution with sample} / \text{absorbance of solution with control}] \times 100$. Ascorbic acid was tested as the positive control. The test result was expressed as the scavenged 50% of the free radicals from the reaction mixture.¹⁶

Intracellular ROS generation

The intracellular ROS was determined using the ROS-sensitive fluorescence indicator 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich; St. Louis, MO, USA) as the ROS-sensitive fluorescence indicator.¹⁷ Samples at final concentration of 0.5 mg/mL were added to kidney post-mitochondrial fraction in 50 mM potassium phosphate buffer, and incubated at 37°C for 5 min. The solution was



SCHEME 1 Extraction, fractionation, and isolation of **1–7**.

mixed with DCFH-DA (5 µg/mL) in phosphate buffer. When the nonfluorescent DCFH-DA diffuses into cells, mitochondrial esterase hydrolyzes DCFH-DA to DCFH. DCFH is oxidized to fluorescent, 2',7'-dichlorofluorescein (DCF) by ROS generated. The fluorescence of DCF was measured at 485 nm (an excitation wavelength) and 530 nm (an emission wavelength) for 30 min on a microplate fluorescence spectrophotometer FL 500 (Bio-Tek Instruments, USA). Trolox was used as the positive control. ROS generated was measured by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA).

Mushroom tyrosinase inhibition

Mushroom tyrosinase inhibition activity of samples was determined using L-tyrosine as substrate according to the methods described with slight modifications.^{18,19} DMSO buffer and kojic acid solution were used as the blank and the positive control, respectively. The activity was measured using the ELISA reader at 490 nm, and the inhibition rate of tyrosinase activity was calculated as the following equation:

$$\% \text{ inhibition} = (A_2 - A_1) - (B_2 - B_1) / (A_2 - A_1) \times 100.$$

A1, absorbance at 490 nm of blank reference at 0 min; A2, absorbance at 490 nm of blank at 10 min. B1, absorbance at 490 nm of test sample at 0 min; B2, absorbance at 490 nm of test sample at 10 min. Results were expressed as IC₅₀.

Neuroprotective activity

HT-22 mouse neuronal cell line was used for evaluation of glutamate-induced toxicity by MTT assay.^{20,21} Cells were cultured in DMEM supplemented with 10% FBS, 1% streptomycin/penicillin at 37 °C in 5% CO₂ Incubator. After culturing for 14 days, cells were treated with 100 µM glutamate for 24 h to induce neurotoxicity. The cells were treated with each test sample, dissolved in DMSO at the final concentration of 0.1%, and then incubated for 1 h at 37 °C with 5% CO₂. After incubation, MTT (5 mg/mL) was added to 10% of the culture medium, and after 3 h of incubation at 37 °C, DMSO was added to dissolve the formazan formed, then the absorbance was measured at 540 nm. The cell viability was expressed as percentage of absorbance difference between test samples and control.

TABLE 1 ^{13}C NMR (125 MHz, CDCl_3) spectral data of **1–4**.

Carbon No.	1	2	3	4
1	37.58	37.94	36.71	38.29
2	26.94	26.02	29.90	28.21
3	77.82	77.81	72.03	79.23
4	38.01	37.97	42.54	38.95
5	54.72	54.59	141.0	59.31
6	17.50	17.40	121.93	18.55
7	33.62	33.41	32.14	34.52
8	39.91	39.82	31.96	42.72
9	49.85	49.76	50.42	55.54
10	38.01	36.28	36.37	37.23
11	20.12	20.0	21.32	25.38
12	24.84	24.71	40.01	27.65
13	36.32	37.47	42.54	37.32
14	41.64	41.61	57.01	48.21
15	26.06	31.12	24.53	27.68
16	28.88	31.42	28.51	34.21
17	46.91	56.61	56.30	48.54
18	48.50	48.38	12.08	54.32
19	49.72	46.17	19.27	50.68
20	149.90	149.86	131.12	151.18
21	29.82	28.80	19.01	29.92
22	33.51	36.28	34.19	40.23
23	28.42	26.92	26.34	29.24
24	14.43	13.69	46.08	15.59
25	15.14	15.12	29.40	16.34
26	14.97	14.94	20.04	16.10
27	13.70	13.01	19.62	14.82
28	58.74	178.24	23.31	18.20
29	18.20	18.27	12.21	21.16
30	108.59	108.50		109.54

Statistical analysis

All measurements were performed in triplicate, and values were expressed as the mean $\pm$ the standard deviation. Results were subjected to analysis of variance followed by Tukey's test to analyze differences between conditions. A p -value of <0.05 was determined to be statistically significant.

Betulin (1)-White powder; EI-MS (positive ion mode): m/z 443.13 $[\text{M} + \text{H}]^+$; ^1H NMR (CDCl_3 , 500 MHz): δ 0.70 (3H, s, H-26), 0.78 (3H, s, H-25), 0.90 (3H, s, H-24), 0.99 (3H, s, H-27), 1.22 (3H, s, H-22), 1.63 (3H, s, H-29), 2.20 (1H, dt, $J = 11.5$, 6.3 Hz, H-19), 3.10 (1H, dd, $J = 11.1$, 5.0 Hz, H-3), 3.23 (1H, d, $J = 11.1$ Hz, H-28), 4.62 (1H, s, H-30a), 4.66 (1H, s, H-30b); ^{13}C NMR (CDCl_3 , 125 MHz): Table 1.

Betulinic acid (2)-White powder; EI-MS (positive ion mode): m/z 457.16 $[\text{M} + \text{H}]^+$; ^1H NMR (CDCl_3 , 500 MHz): δ 0.69 (3H, s, H-24), 0.85 (1H, t, H-28), 0.85 (3H, s, H-23), 0.89

(3H, s, H-25), 0.90 (3H, s, H-26), 1.00 (3H, s, H-27), 1.63 (3H, s, H-29), 2.21 (1H, dt, $J = 11.0$, 6.3 Hz, H-19), 3.10 (1H, t, $J = 7.95$ Hz, H-21), 4.52 (1H, br s, H-30b), 4.67 (1H, br s, H-30a); ^{13}C NMR (CDCl_3 , 125 MHz): Table 1.

β -Sitosterol (3)-White powder; EI-MS (positive ion mode): m/z 415.17 $[\text{M} + \text{H}]^+$; ^1H NMR (CDCl_3 , 500 MHz): δ 0.61 (3H, s, H-18), 0.74 (3H, d, $J = 6.8$ Hz, H-27), 0.77 (3H, d, $J = 6.8$ Hz, H-26), 0.78 (3H, t, $J = 7.5$ Hz, H-29), 0.85 (3H, d, $J = 6.5$ Hz, H-21), 0.94 (3H, s, H-19), 3.45 (1H, m, H-3), 5.28 (1H, br d, $J = 5.1$ Hz, H-6); ^{13}C NMR (CDCl_3 , 125 MHz): Table 1.

Lupeol (4)-White powder; EI-MS (positive ion mode): m/z 427.20 $[\text{M} + \text{H}]^+$; ^1H NMR (CDCl_3 , 500 MHz): δ 0.69 (3H, s, H-18), 0.72 (3H, s, H-26), 0.76 (3H, s, H-25), 0.87 (3H, s, H-27), 0.90 (3H, s, H-24), 2.30 (1H, s, H-19), 1.55 (2H, s, H-28), 0.96 (3H, s, H-29), 1.61 (3H, m, H-23), 3.19 (1H, dd, $J = 11.3$, 5.0 Hz, H-3), 4.58 (1H, br s, H-30b), 4.69 (1H, br s, H-30a); ^{13}C NMR (CDCl_3 , 125 MHz): Table 1.

Kojic acid (5)-White powder; EI-MS (positive ion mode): m/z 143.10 $[\text{M} + \text{H}]^+$; ^1H NMR ($\text{CD}_3\text{OD}-d_4$, 500 MHz): δ 4.41 (2H, s, H-1), 4.85 (1H, s, CH_2OH), 6.49 (2H, s, H-6), 7.95 (1H, s, H-3); ^{13}C NMR ($\text{CD}_3\text{OD}-d_4$, 125 MHz): δ 59.79 (C-1), 109.35 (C-6), 139.60 (C-3), 146.0 (C-5), 169.04 (C-2), 175.47 (C-4).

1,7-bis-(p -Hydroxyphenyl)-(3,4-dihydroxy)-hepan-5-one (6)-Yellowish powder; EI-MS (positive ion mode): m/z 331.19 $[\text{M} + \text{H}]^+$; ^1H NMR ($\text{DMSO}-d_6$, 500 MHz): δ 2.46 (2H, t, $J = 6.4$ Hz, H-6), 2.66 (2H, t, $J = 6.4$ Hz, H-7), 2.75 (2H, t, $J = 6.4$ Hz, H-1), 2.79 (2H, t, $J = 7.8$, 16.2 Hz, H-2), 6.04 (2H, d, $J = 16.2$ Hz, H-2), 6.69 (4H, dt, $J = 7.8$, 16.5 Hz, H-2',6',2'',6''), 6.97 (4H, dd, $J = 7.8$, 16.5 Hz, H-3',5',3'',5''); ^{13}C NMR ($\text{DMSO}-d_6$, 125 MHz): δ 29.29 (C-1), 33.14 (C-7), 34.28 (C-6), 41.31 (C-2), 114.78 (C-3'), 114.79 (C-3'), 128.91 (C-2''), 128.98 (C-2'), 130.24 (C-5), 131.07 (C-4), 131.64 (C-1'), 131.81 (C-1''), 147.90 (C-3), 155.19 (C-4''), 155.23 (C-4'), 201.52 (C-5).

Platyphyllenone (7)-Brownish powder; EI-MS (positive ion mode): m/z 297.18 $[\text{M} + \text{H}]^+$; ^1H NMR ($\text{DMSO}-d_6$, 500 MHz): δ 2.42 (2H, t, $J = 6.4$ Hz, H-1), 2.62 (2H, t, $J = 6.4$ Hz, H-2), 2.67 (2H, t, $J = 6.4$ Hz, H-7), 2.78 (2H, t, $J = 6.4$ Hz, H-6), 6.09 (1H, d, $J = 16.4$ Hz, H-4), 6.65 (4H, dt, $J = 7.8$, 16.4 Hz, H-3',5',3'',5''), 6.83 (1H, d, $J = 16.4$ Hz, H-3), 6.89 (4H, dt, $J = 7.8$, 16.4 Hz, H-2',6',2'',6''); ^{13}C NMR ($\text{DMSO}-d_6$, 125 MHz): δ 29.44 (C-7), 33.47 (C-1'), 34.55 (C-2), 34.60 (C-1), 41.84 (C-6), 115.73 (C-3',5',3'',5''), 129.81 (C-2',6',2'',6''), 131.0 (C-4), 131.6 (C-1'), 131.9 (C-1''), 147.47 (C-3), 156.12 (C-4',4''), 199.86 (C-5).

RESULTS AND DISCUSSION

Dried inner bark of *Betula schmidtii* was extracted with 80% EtOH to give dark yellow extracts, and then fractionated with *n*-hexane, CHCl_3 , EtOAc, and *n*-BuOH, respectively, to yield solvent fractions. Compounds **1**, **2**, and **3–5**, and **6**, **7** were isolated from *n*-hexane, CHCl_3 , and EtOAc-soluble fractions, respectively, using repeated silica

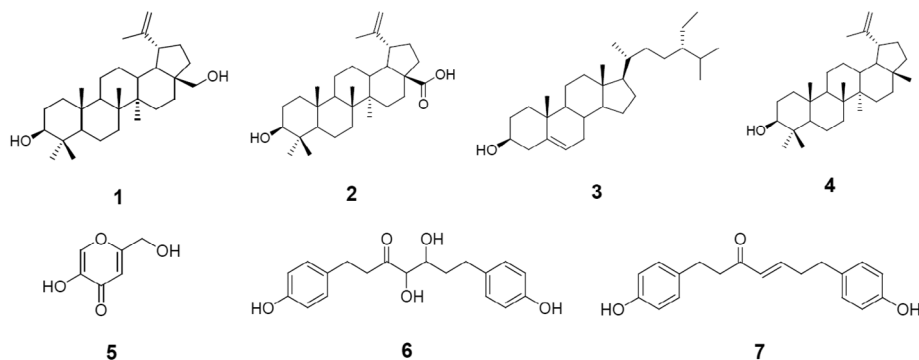


FIGURE 1 Chemical structure of 1–7.

gel CC and Sephadex LH-20 VLC (Scheme 1). The structure of compounds 1–7 were determined by comparing ^1H NMR, ^{13}C NMR, ^1H - ^1H COSY, HMQC, HMBC, and EI-MS spectral data with those in the literatures to be betulin (1), betulinic acid (2), β -sitosterol (3), lupeol (4), kojic acid (5), 1,7-bis-(*p*-hydroxyphenyl)-(3,4-dihydroxy)-hepan-5-one (6), and platyphyllenone (7) (Figure 1). Compound 1 was obtained in a white powder. The molecular ion peak $[\text{M} + \text{H}]^+$ was at m/z 443.13 in the positive-ion EI-MS spectrum, and as result of ^1H NMR and ^{13}C NMR spectrum, it was determined that molecular formula to be $\text{C}_{30}\text{H}_{50}\text{O}_2$. The ^1H NMR structure represented signals for six methyl groups at δ_{H} 0.70, 0.78, 0.90, 0.99, 1.22, and 1.63, two exomethylene protons at δ_{H} 4.62 and 4.66, one oxygenated proton at δ_{H} 3.10, oxygenated methylene proton at δ_{H} 3.23. In the ^{13}C -NMR, thirty carbon signals appeared, with two olefinic carbons at δ_{C} 108.59 and 149.90, one oxygenated methylene carbon at δ_{C} 58.74, six methy carbons at δ_{C} 13.70, 14.43, 14.97, 15.14, 17.50, and 26.94. These spectral data and published paper²² implied that 1 was to be a betulin (lup-20(29)-en-3 β ,28-diol). Betulin, also called betulinol, betulol, betulinic alcohol, birch camphor or lupendiol, is a pentacyclic triterpenoid belonging to the lupan group. Many pharmacological properties of betulin have been reported for anti-bacterial, anti-tumor, anti-viral, hypolipidemic activity. in *Betula* species. The wide use of betulin in medicine or industry requires the development of methods to effectively isolate betulin from *Betula* species.²³ In our experiments, 5.1% of betulin was isolated as pure compound from dried inner bark of *Betula schmidtii*. Compound 2 was purified as a white powder form, with molecular ion peak $[\text{M} + \text{H}]^+$ at m/z 457.16 in the positive-ion EI-MS spectrum, and as a result of ^1H NMR and ^{13}C NMR spectrum, it was determined that molecular formula to be $\text{C}_{30}\text{H}_{48}\text{O}_3$. The ^1H NMR structure represented signals for six methyl groups at δ_{H} 0.69, 0.85, 0.89, 0.90, 1.0, and 1.63, two exomethylene protons at δ_{H} 4.52 and 4.67, one methylene proton at δ_{H} 3.10. In the ^{13}C NMR, thirty carbon signals appeared, including one carbonyl carbon at δ_{C} 178.24, one oxygenated carbon at δ_{C} 77.81, six methy carbons at δ_{C} 13.01, 13.69, 14.94, 15.12, 18.27, and 26.92. These

spectral data and published paper^{24,25} implied that 2 was to be a betulinic acid (lup-20(29)-en-28-oic acid). Betulinic acid is generally derived from plant sources, such as birch, eucalyptus, and plane trees. Betulinic acid has gained considerable interest owing to a variety of biological and pharmacological activities, anti-inflammatory, antibacterial, antiviral, antidiabetic, antimalarial, anti-HIV, and antitumor effects.^{26–29} Betulinic acid is widely distributed in various plant sources, such as eucalyptus and birch trees. Betulinic acid has been studied extensively due to its various biological and pharmacological activities such as anti-inflammatory, antibacterial, antiviral, antidiabetic, antimalarial, anti-HIV, and antitumor effects.^{26–29} However, betulinic acid present in low concentrations in plants has limitations in industrial application. In order to solve these problems, research on new chemical synthesis and bio-transformation is being actively conducted in recent years. Due to its high clinical application potential, insufficient supply of betulinic acid from plant sources is a major challenge for large markets.³⁰ Compound 3 was purified as a white powder with molecular ion peak $[\text{M} + \text{H}]^+$ at m/z 415.17 in the positive-ion EI-MS spectrum, and as result of ^1H NMR and ^{13}C NMR spectrum, it was determined that molecular formula to be $\text{C}_{29}\text{H}_{50}\text{O}$. The ^1H NMR structure represented signals for six methyl groups at δ_{H} 0.61, 0.74, 0.77, 0.78, 0.85, and 0.94, one olefinic and one oxygenated methine signals at δ_{H} 5.28 and 3.45, respectively. In the ^{13}C NMR, twenty-nine carbon signals appeared, including one olefinic methine carbon at δ_{C} 121.93, one oxygenated carbon at δ_{C} 72.03, six methyl carbons at δ_{C} 12.08, 12.21, 19.01, 19.27, 19.62, and 20.04. These spectral data and published paper³¹ implied that 3 was to be a β -sitosterol (17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol). β -Sitosterol, is the major plant sterols similar to chemical structure of animal cholesterol. It is found large amounts in lipid rich plants, such as nuts, vegetable oil, avocados, and seeds.³² It has been reported that β -sitosterol has antinociceptive,³³ anxiolytic and sedative,³⁴ analgesic,³⁵ immunomodulatory,³⁶ antimicrobial,³⁷ anticancer,³⁸ anti-inflammatory,³⁹ protective effect against NAFLD,⁴⁰ lipid

lowering,⁴¹ hepatoprotective,⁴² protective effect on respiratory diseases,⁴³ wound healing,⁴⁴ antioxidant,⁴⁵ and anti-diabetic⁴⁶ activities. Compound **4** was purified as a white powder. The molecular ion peak $[M + H]^+$ at m/z 427.20 in the positive-ion EI-MS spectrum, and as result of ^1H NMR, and ^{13}C NMR spectrum, it was determined that molecular formula to be $\text{C}_{30}\text{H}_{50}\text{O}$. The ^1H NMR structure represented signals for seven methyl groups at δ_{H} 0.69, 0.72, 0.76, 0.87, 0.90, 0.96, and 1.61, two exomethylene protons at δ_{H} 4.50 and 4.62. In the ^{13}C NMR, thirty carbon signals appeared, including two olefinic carbons at δ_{C} 109.54 and 151.18, one oxygenated carbon at δ_{C} 79.23. These spectral data and published paper^{47,48} implied that **4** was to be a lupeol (lup-20(29)-en-3 β -ol). Lupeol (3- β -lup-20(29)-3 n-3-ol) is a pentacyclic triterpene widely distributed in the diverse plant kingdom. It has been reported that lupeol has a great area of pharmacological activities, antioxidant, anti-inflammatory, anticancer, anti-microbial, and antidiabetic. In addition, lupeol shows potential to decrease neuroinflammation and neurodegeneration, and to protect various tissues and organs.^{49–55} It is also reported to attenuate cataract in rats.⁵⁶

Compound **5** was purified as a white powder with molecular ion peak $[M + H]^+$ at m/z 143.10 in the positive-ion EI-MS spectrum, and as result of ^1H NMR and ^{13}C NMR spectrum, it was determined that molecular formula to be $\text{C}_6\text{H}_6\text{O}_4$. The ^1H NMR structure represented signals one methylene signals at δ_{H} 4.41, and two methine groups at δ_{H} 6.49 and 7.95. In the ^{13}C NMR, six carbon signals appeared, including one carbonyl and methylene carbon at δ_{C} 175.47 and 59.79, respectively. These spectral data and published paper⁵⁷ implied that **5** was to be a kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone). Compound **6** was purified as a yellowish powder. The molecular ion peak $[M + H]^+$ at m/z 331.19 in the positive-ion EI-MS spectrum, and as result of ^1H NMR and ^{13}C NMR spectrum, it was determined that molecular formula to be $\text{C}_{19}\text{H}_{22}\text{O}_5$. The ^1H NMR spectrum represented signals for four methylene groups at δ_{H} 2.46, 2.66, 2.75, and 2.79, two oxygenated proton signals at δ_{H} 1.16 and 1.18, two oxygenated protons at δ_{H} 6.04, eight aromatic protons at δ_{H} 6.69 and 6.97. In the ^{13}C NMR, nineteen carbon signals appeared, including four methylene carbons in heptanoid carbon at δ_{C} 29.29, 33.14, 34.28, and 41.31, one carbonyl carbon at δ_{C} 201.52, two oxygenated aromatic carbons at δ_{C} 155.19 and 155.23, including eight aromatic carbons at δ_{C} 114.78, 114.79, 128.91, 128.98, 130.24, 131.64, 131.81, and 147.90, two oxygenated carbons in heptanoid carbon at δ_{C} 131.07 and 147.90. These spectral data and published paper⁵⁸ for partial structure implied that **6** was to be a 1,7-bis-(*p*-hydroxyphenyl)-1-(3,4-dihydroxy)-hepan-5-one. To the best of our knowledge, compound **6** was isolated and identified for the first time in *Betula* species. Compound **7** was purified as a brownish powder. The molecular ion peak $[M + H]^+$ at m/z 297.18 in the positive-ion EI-MS spectrum, and as result of ^1H NMR and ^{13}C NMR spectrum, it was

determined that molecular formula to be $\text{C}_{19}\text{H}_{20}\text{O}_3$. The ^1H NMR spectrum represented signals for four methylene groups at δ_{H} 2.42, 2.62, 2.67, and 2.78, two methine groups at δ_{H} 6.09 and 6.83. In the ^{13}C NMR, nineteen carbon signals appeared, including four methylene carbons at δ_{C} 29.44, 34.55, 34.60, and 41.84, two methine carbons at δ_{C} 131.0 and 147.47, two oxygenated aromatic carbons at δ_{C} 156.12 and one carbonyl carbon at δ_{C} 199.86, respectively. These spectral data and published paper^{10,25,58} implied that **7** was to be a platyphyllenone (1,7-bis-(4-hydroxyphenyl)-4-hepten-3-one). Compound **7** is reported to present anti-metastatic and apoptotic in oral and lung cancers.⁵⁹

Compounds **1–7** were investigated antioxidant, whitening activity, and neuroprotective activities using DPPH free radical scavenging, DCHF-DA ROS generations, mushroom melanogenesis inhibitory, and glutamate-induced toxicity in HT-22 mouse neuronal cells. As shown in Table 2, antioxidant activities were shown in compounds **5**, **6**, and **7**, with IC_{50} values of 18.23 ± 1.08 , 29.37 ± 1.42 , and 40.14 ± 1.75 μM , respectively, in DPPH free radical scavenging activity. It is reported with one more phenol moiety of compounds **5**, **6**, and **7** increases the resonance stability, this stability may have influenced the inhibitory effect.^{60,61} It has been studied since 1954, that the oxidative stress may be highly harmful to cells, tissues, and organs.⁶² Under normal physiological conditions, cells are in a state of redox homeostasis that maintains a balance between cellular oxidants and antioxidants. Excess free radicals can oxidize DNA and proteins, resulting in changes in their structure and function. Gene mutations may occur due to cross-linking or mismatches of base pairs in the DNA chain.⁶³ ROS, such as hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot\text{OH}$), superoxide (O_2^-), and singlet oxygen ($^1\text{O}_2$), may affect the function of antioxidant enzymes and lead to cell destruction.⁶⁴ Our study indicates that compound **4** exerted as total ROS generators rather than scavengers. Free radicals and ROS have been studied to be associated with several degenerative diseases, including inflammation and some liver diseases.⁶⁵ Numerous signs of liver damage have been demonstrated to be associated with oxidative stress.⁶⁶ The generation of ROS was assessed using the ROS-sensitive fluorescence indicator DCFH-DA. When the nonfluorescent DCFH-DA diffuses into cells, mitochondrial esterase hydrolyzes DCFH-DA to DCFH. DCFH is oxidized to DCF by ROS generated. In this study, compounds **4** and **5** show the antioxidant effect with IC_{50} values of 28.21 ± 1.80 and 37.74 ± 1.02 in DCHF-DA intracellular ROS generations, respectively.

Some studies have suggested antioxidant properties that act as moderate radical scavengers but as membrane stabilizers.^{67–69} It has been studied that β -sitosterol reduced the levels of free radicals and hydrogen peroxide in RAW 264.7 cells and stimulated the enzymatic antioxidant. It was also studied that β -sitosterol could revert the impairment in glutathione/oxidized glutathione ratio by

TABLE 2 DPPH free radical scavenging, ROS generations, and tyrosinase inhibitory activities of **1–7**.^a

Compounds	DPPH scavenging activity	Intracellular ROS generations	Tyrosinase inhibitory activity
1	– ^c	– ^c	– ^c
2	– ^c	– ^c	– ^c
3	– ^c	– ^c	– ^c
4	– ^c	28.21 ± 1.80*	– ^c
5	18.23 ± 1.08*	37.74 ± 1.02*	17.42 ± 1.08*
6	29.37 ± 1.42*	– ^c	47.17 ± 1.92*
7	40.14 ± 1.75	– ^c	26.64 ± 1.40*
Vitamin C ^b	4.70 ± 1.51*		
Trolox ^b		10.30 ± 0.61*	
Kojic acid ^b			15.23 ± 1.04*

^aResults are the IC₅₀ ± SD (μM). Values are presented as means of three independent experiments.^bPositive control.^cCompounds did not show DPPH, ROS radical scavenging, and tyrosinase inhibition at the same condition (IC₅₀ value >200 μM).

ROS scavenging.⁷⁰ Baskar et al.⁷¹ reported that treatment of β -sitosterol enhanced the enzymatic and non-enzymatic antioxidant in colon carcinogenic rat, which showed the potential of β -sitosterol as a chemopreventive drug for colon cancer. Manisha et al.⁷² reported the antioxidant potential of β -sitosterol in vitro through DPPH free radical scavenging activity. Unlike these, β -sitosterol did not show DPPH and ROS radical scavenging in our experiments (IC₅₀ value >200 μM). Melanogenesis is involved in the first two steps of melanin biosynthesis, which consists of the hydroxylation of L-tyrosine and the oxidation of the product of this reaction, L-DOPA, to the corresponding o-quinone.⁷³ In many cases, melanin synthesis inhibitors act by inhibiting tyrosinase activity.^{74,75} Tyrosinase (EC 1.14.18.1) is a copper-containing metalloenzyme mediating the oxidation of tyrosine to L-DOPA or o-diphenol (3,4-dihydroxyphenylalanine) and subsequently oxidizing L-DOPA to dopaquinone.⁷⁶ The resulting dopaquinone induces melanin formation which is responsible, in some cases, for skin disorders like hyperpigmentation. In addition to the production of melanin, it has been reported that the highly reactive DOPA-quinones are also related to damage neurons and cause cell death.^{77–79} Plants are rich sources of bioactive chemicals that are mostly free from harmful side effects. Therefore, there is an increasing interest in finding tyrosinase inhibitors from plants.⁸⁰ *Betula* leaf extracts have been used in some skincare products. The potential of ethanolic extract of *Betula pendula* leaves was evaluated on mushroom tyrosinase activity for development of skin whitening agents.⁸¹ Compounds **5**, **6**, and **7** exhibited inhibitory activities on mushroom tyrosinase with IC₅₀ values of 17.42 ± 1.08, 47.17 ± 1.92, and 26.64 ± 1.40 μM, respectively. On the other hand, compounds **1–4** did not show mushroom tyrosinase inhibitory effect (IC₅₀ value >200 μM). In our experiments, kojic acid exhibited tyrosinase inhibitory activity with the IC₅₀ from 15.23 ± 1.04 to 17.42 ± 1.08 μM, since it differs purity of compounds. It has long been confronted to isolate

TABLE 3 Neuroprotective activity of **1–7**.^a

Compounds	10 μM	25 μM
1	24.5 ± 6.3*	45.6 ± 3.1*
2	– ^c	– ^c
3	– ^c	– ^c
4	– ^c	– ^c
5	– ^c	– ^c
6	– ^c	11.2 ± 1.4*
7	– ^c	– ^c
Glutamate-treated ^b	0	0

^aResults are the cell viability (%). Values are presented as means of three independent experiments.^bPositive control.^cCompounds did not show neuroprotective activity at the same condition.

compounds with high purity from natural resources for pharmaceutical industry.²³ The highest analytical grade (≥99.0%) (HPLC) was used as the positive control. Results showed that compounds **5–7** were capable of inhibiting L-DOPA oxidation. It has been suggested that the inhibitory activity of phenolic compounds can be associated with the structural similarity to chalcones.⁸² It is demonstrated that the inhibitory activities of phenolic compounds might be due to the structural similarity to chalcones.⁸² Kojic acid is popular for its applications in pharmaceuticals, cosmetics, agriculture, food, and chemical industry. Kojic acid may act as a brightening ingredient in whitening skincare or bodycare products, and also in dental care products. With increasing demands and industrial supply, more studies are required for designing and developing products based on kojic acid.⁵⁷

Many studies have been conducted on natural neuroprotective products and antidepressants using nerve cells including HT-22 cells.^{83,84} Recently, it has been reported betulin and betulinic acid have protective effects on neurons.^{85–87} We expected that the fraction or compounds **4**, and **5** with higher antioxidant activity could be effective in neuroprotection because reducing oxidative stress is a

main protective mechanisms. As results, compound **1** showed cell viability of $24.5 \pm 6.3\%$ at $10 \mu\text{M}$, and $45.6 \pm 3.1\%$ at $25 \mu\text{M}$, while compound **6** showed $11.2 \pm 1.4\%$ at $25 \mu\text{M}$ in glutamate-induced damaged mouse neuron HT-22 cells (Table 3). Further study is needed to continue in animal model with ameliorated memory deficits induced by scopolamine to identify therapeutic and cognitive enhancer. It has been expected that the inner bark of *B. schmidtii* could have beneficial effects, as other compounds identified are also known or expected for various efficacy.

CONCLUSIONS

Natural products have long been used as traditional medicine in many parts due to high pharmaceutical effects in treating diseases. The ethnopharmacological studies of *Betula* species have not been sufficiently established in spite of its long history as traditional medicine. Therefore, extensive research is required to increase its therapeutic potentials. In this study on the phytochemical constituents from *Betula schmidtii*, seven compounds were isolated. The chemical structure of the isolated four triterpenoids, one phenolic acid, and two diarylheptanoids were determined using the positive-ion EI-MS, 1D-, and 2D NMR (^1H - ^1H COSY, HMQC, HMBC) spectral analysis. Among these isolates, compounds **5**, **6**, and **7** might be useful in antioxidant and anti-tyrosinase activities in vitro. Compound **1** shows mild cognitive enhancing activity in glutamate-induced damaged mouse neuron HT-22 cells. These results indicate *Betula schmidtii* could be candidate for bioactive natural product resources. Further studies are needed to evaluate *in vivo* efficacies of compounds **1**, **5**, **6**, and **7**.

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