Antioxidant and anti-inflammatory compounds isolated from Acer tegmentosum

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The chromatographic separation of methanol extract from Acer tegmentosum twigs led to the isolation of one phytosterol and five phenolic compounds. Using spectroscopic methods, the structures of these compounds were determined as β-sitosterol 3-O-β-D-glucopyranoside (1), ρ-hydroxyphenylethyl-O-β-D-glucopyranoside (Salidroside) (2), ρ-hydroxyphenylethyl alcohol (tyrosol) (3), 6'-O-galloylsalidroside (4), Quercetin 3-O-β-D-glucopyranoside (5), Quercetin (6). Compounds 1, 3, 4, 5 and 6 were isolated from the plant for the first time, but we also found that 2 and 4 were major compounds (high contents). We measured the antioxidant activity (radical/superoxide scavenging activity) and anti-inflammatory activity (nitric oxide (NO) scavenging activity in RAW 264.7, BV2 cell lines). The ethyl acetate fraction and compounds 4, 6 had both activities, but the hexane fraction and 2 had only anti-inflammatory activity. Together, all data indicate that A. tegmentosum extract is a source of functional food and treatments for neurodegenerative diseases.

Key words: Acer tegmentosum, antioxidant activity, anti-inflammatory activity, compound.

INTRODUCTION

Acer tegmentosum (Aceraceae) is a deciduous tree that grows in Korea, Russia and northern areas of China. In Korea, A. Tegmentosum has been used in traditional medicine for the treatment of hepatic disorders (Ahn, 1998). Its phytochemical constituents have been investigated in the past: Diaryheptanoids (Kubo et al., 1980), rhododendrol glycoside (Kubo et al., 1983) and tannins (Hatano et al., 1990) were isolated from the genus Acer. Some isoprenoids, flavonoids and other phenolic compounds were also reported from A. tegmentosum (Hur et al., 2006; Park et al., 2006; Hur et al., 2007). Among them, some were reported to have strong cytotoxic (ED50=1.32-3.85 µM) and anti-oxidative properties (Park et al., 2006; Hur et al., 2007). The methanolic extract of this plant suppresses the activity of 60kDa sphingomyelinase (Kim et al., 2005) and its 70% ethanol extract was reported to have anti-inflammatory activity (Yu et al., 2010). L-Arginine-derived NO is an intracellular mediator produced in mammalian cells by two types of nitric oxide synthase (NOS) (Forstermann et al., 1991). A constitutive NOS (cNOS) is Ca2+-dependent and releases small amounts of NO, which is required for physiological functions (Bredt and Snyder, 1990). The other form of inducible NOS (iNOS) is Ca2+-dependent and is induced by lipopolysaccharide (LPS) or some proinflammatory cytokines (TNF-α, etc) (Ryu et al., 2003). NO, produced in large amounts by iNOS, and its derivatives, such as peroxynitrite and nitrogen dioxide, play a role in inflammation and possibly also in the multistage process of carcinogenesis (Oshima et al., 1994). NO is also known to be responsible for the vasodilatation and hypotension observed in septic shock (Thiemermann and Vane, 1990). Therefore inhibitors of iNOS may be useful therapeutic agents in septic shock and inflammation. Pre-studies showed the isolated compounds, their antioxidant activities and the anti-inflammatory activities of the extract. Therefore, in this study, we isolated compounds from A. tegmentosum and confirmed the major compound. Also we checked their...
antioxidant activities and investigated the anti-inflammatory roles of the compounds in NO scavenging activity in two cell lines, RAW 264.7 and BV2 (microglial).

MATERIALS AND METHODS

Chemicals and apparatus

Ultraviolet (UV) spectra were recorded on an OPTIZEN 2120 spectrophotometer (Mecasy, Korea). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini 600 NMR spectrophotometer (Varian, USA) in methanol, dimethyl sulfoxide (DMSO), pyridine (Sigma, USA). Fast Atom Bombardment-Mass spectrometry (FAB-MS) data were obtained on a VG 70-VSEQ spectrophotometer (UK). Infrared (IR) spectra were recorded with the Jasco fourier transform infrared (FT/IR)-4100 (Japan). High-performance liquid chromatography (HPLC) used an Agilent 1100 (USA). Open column chromatography was carried out over silica gel by elution with chloroform to methanol to give compounds 1, 2, 3, 4, 5, and 6. Each fraction was further chromatographed on silica gel by elution with 20% methanol to give compounds 5 and sub-fractions EA_11_1~5. EA_11_4 was chromatographed on MCI gel by elution with 30% methanol to give compound 4.

Plant collection

The twig of A. Tegmentosum was purchased from Kyoung-dong market, Korea, in December 2009 and was identified by professor Whang Wan Kyunn. A voucher specimen of the plants was deposited at the Pharmaceutical Botany Laboratory in the College of Pharmacy, Chung-ang University.

Extraction and isolation

Dried twig (2.5 kg) of A. teggentosum (Acerceae) was extracted with methanol under sonication. After filtration, the methanol solution was evaporated under vacuum to yield a methanol extract (393 g). The extract was partitioned between hexane and water to give a hexane-soluble fraction (17.9 g). The water-soluble fraction was partitioned consecutively with ethyl acetate and butanol to give 66.0 and 30.0 g, respectively. Based on activity-guided fractionation, the ethyl acetate fraction was chromatographed on silica gel by elution with chloroform to methanol to give compounds 1 (220 mg), 3 (8 mg) and sub-fractions EA_1~11. EA_11 was chromatographed on MCI gel by elution with 30% methanol to give compound 4 (300 mg) and sub-fractions EA_11_1~5. EA_11_4 was chromatographed on MCI gel by elution with 20% methanol to give compounds 5 (11 mg) and 6 (7 mg). The butanol fraction was chromatographed on silica gel by elution with chloroform to methanol to give 2 (1000 mg). As a result, six compounds were isolated from A. teggentosum methanol extract (Figure 1).

β-Sitosterol 3-O-β-D-glucopyranoside (1)

Colorless amorphous powder; IR (KBr) cm⁻¹: 3426 (OH), 1464(CH2), 1374 (CH2), 1074 (C-O); FAB-MS (pos.) m/z: 577[M+H]+; "H-NMR and "C-NMR data were consistent with literature values (Chang et al., 1981).

P-Hydroxyphenylethyl-O-β-D-glucopyranoside (salidroside) (2)

Colorless needle crystal; IR (KBr) cm⁻¹: 3310 (OH), 1517 (C=C), 1442 (CH2), 1072 (C-O); FAB-MS (pos.) m/z: 301[M+H]+; "H-NMR and "C-NMR data were consistent with literature values (Jorn et al., 2002).

ρ-Hydroxyphenylethyl alcohol (tyrosol) (3)

Yellow needle crystal; IR (KBr) cm⁻¹: 3392 (OH), 151 (C=O), 1450 (CH2), 1051 (C-O); FAB-MS (pos.) m/z: 199[M+H]+; "H-NMR and "C-NMR data were consistent with literature values (Sarker et al., 2000).

6'-O-Galloylsalidroside (4)

Colorless amorphous powder; IR (KBr) cm⁻¹: 3402 (OH), 1701 (C=O), 1619 (C=C), 1449 (CH2), 1078 (C-O); FAB-MS (pos.) m/z: 453[M+H]+; "H-NMR and "C-NMR data were consistent with literature values (Nonaka et al., 1982).

Quercetin 3-O-β-D-glucopyranoside (5)

Yellow amorphous powder; FAB-MS (pos.) m/z: 453[M+H]+; "H-NMR and "C-NMR data were consistent with literature values (Harborne and Mabry, 1982).

Quercetin (6)

Yellow amorphous powder; FAB-MS (pos.) m/z: 303[M+H]+; "H-NMR and "C-NMR data were consistent with literature values (Harborne and Mabry, 1982).

Cell culture

RAW 264.7 (macrophage) and BV2 (microglia) cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco) in a 5% CO2 atmosphere. The cells were used to measure viability and NO production.

Measurement of cell viability

Cellular viability was measured by using a MTT based colorimetric assay (Woerdenbag et al., 1994). Cells in 48 well plates (1.5x10⁶ cell/ml) were exposed to fractions and compounds at 37°C for 24 h. MTT solution (5 mg/ml in 10% FBS DMEM) was added (concentration: 0.5 mg/ml) and further incubated for 4 h. After aspirating the supernatant from the wells, 100 µL of DMSO were added for dissolution of formazan crystals. The absorbance of each well was then read at 570 nm on an ELISA reader.

DPPH scavenging activity

The DPPH radical scavenging activity was determined by slightly modified method (Cos et al., 2002). Briefly, solutions of each sample at varying concentrations were added to a solution of 0.1 mM DPPH (dissolved in anhydrous ethanol) and the reaction mixture (total volume: 200 µL) was shaken vigorously. After incubating at 37°C for 20 min, the absorbance of the test mixtures was measured.
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Figure 1. Chemical structures of compounds 2 and 4 isolated from AT methanol extract.

Nitrobluetetrazolium (NBT)/xanthine oxidase superoxide radical scavenging assay

The scavenging potential for superoxide radicals was determined via a hypoxanthine/xanthine oxidase system coupled with NBT reduction (Choi et al., 2008). The reaction mixture (400 µL) contained 50 mM potassium phosphate buffer (pH 7.4), 0.6 mM hypoxanthine, 1mM ethylene diamine tetraacetic acid (EDTA), 0.2 mM NBT and test compound was incubated by adding 100 µL of xanthine oxidase (100 µU/ml) for 20 min at 37°C. Allopurinol was used as a xanthine oxidase inhibitor and ascorbic acid and pyrogallol (natural phenolic compounds) were positive controls. Absorbance was measured at 540 nm using ELISA reader. The radical scavenging activity of each sample was expressed as the IC\(_{50}\) (50% Inhibition Concentration) value.

Measurement of inhibitory effect on NO production

NO released from macrophages was assessed by the determination of NO concentration in culture supernatant. Samples (100 µL) of culture media were incubated with 150 µL of griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine in 2.5% phosphoric acid solution) at room temperature for 10 min in 96 well microplates (Green et al., 1982). Absorbance at 540 nm was read using an ELISA reader. Standard calibration curves were prepared using sodium nitrite. L-NMMA (L-Monomethylarginine), an inhibitor of NOS, was used as a positive control.

RESULTS AND DISCUSSION

Antioxidative activity of extracts and compounds

Antioxidative activity was determined by using the DPPH radical scavenging assay and the NBT/xanthine oxidase superoxide radical scavenging assay. The ethyl acetate, butanol and water fractions dose-dependently removed radicals (Table 1). In the DPPH radical scavenging assay, the IC\(_{50}\) values of the ethyl acetate, butanol and water fractions were 34.8, 56.3 and 48.9 µg/ml, respectively, while those of the positive controls, ascorbic acid and pyrogallol, were 26.8 and 5.37 µg/ml, respectively. In the NBT/xanthine oxidase superoxide radical scavenging assay, the IC\(_{50}\) values of the ethyl acetate, butanol and water fractions were 18.4, 35.3 and 73.1 µg/ml, respectively, and those of the positive controls, ascorbic acid and pyrogallol were 76.7 and 5.3 µg/ml. We confirmed that the ethyl acetate fraction had strong antioxidant activity and the butanol and water fractions had mild antioxidant activities. Using activity-guided fractionation, we isolated six compounds from ethyl acetate and butanol fractions. Compounds 4, 5 and 6 dose-dependently removed radicals (Table 2). In the DPPH radical scavenging assay, the IC\(_{50}\) values of 4, 5 and 6 were 24.7, 49.3 and 36.5 µM, respectively, while those of the positive controls, ascorbic acid and pyrogallol, were 76.7 and 24.5 µM. In NBT/xanthine oxidase superoxide radical scavenging assay, the IC\(_{50}\) values of 4, 5 and 6 were 68.4, 59.5 and 57.6 µM, respectively, while those of the positive controls, ascorbic
acid and pyrogallol, were 436.2 and 41.9 µM. The antioxidant activities of 4, 5 and 6 were greater than that of ascorbic acid and similar to that of pyrogallol.

**Cell viability**

The cytotoxicity of extracts and compounds was evaluated based on their effects on cell growth (MTT test). Results showed that, at all concentrations ranging from 10 to 40 (µg/ml: extracts, µM: compounds), extracts and compounds did not affect cell growth (data not shown), nor did they exhibit any toxicity in this concentration range in the two cell lines (RAW 264.7, BV2 microglia).

**Inhibitory effect on NO production**

All fractions and compounds were evaluated for inhibitory activity against NO production in order to determine their anti-inflammatory activity. Among the fractions, the NO scavenging activities of the ethyl acetate and hexane fractions were greater than those of the other fractions (Table 3). In RAW 264.7 cells, the IC50 value of each fraction was 21.4 (hexane), 10.5 (ethyl acetate), 85.9 (butanol) and 128.8 (water) µg/ml, respectively. In BV2 cells, the IC50 value of each fraction was 20.6 (hexane), 22.0 (ethyl acetate), 32.4 (butanol) and 31.4 (water) µg/ml, respectively. Among the compounds, in RAW 264.7 cells, only compound 6 only had NO scavenging activity; the IC50 value for 6 was 12.3 µM, while the others were more than 100 µM. In BV2 cells, 6 also had the best activity, but the other compounds, especially 1, 2, 4 and 5 had activities (Table 4). The IC50 values of 1, 2, 4 and 5 were in 30 to 35 µM range; thus, 40 µM of these compounds showed about 60% NO scavenging activity. As a result, we confirmed that the ethyl acetate and hexane fractions and compound 6 had NO scavenging activity in two cell lines. We also found that all fractions and compounds showed activities in BV2 cells.

**Conclusion**

Compounds in the dried twigs of *A. tegmentosum* were extracted with methanol and partitioned with hexane, ethyl acetate and butanol. Subsequently, we isolated compounds from the ethyl acetate and butanol fractions and confirmed the presence of six compounds...
especially the ethyl acetate fraction) could be a source of

stress scavenging agents in the brain, so these findings

increase the possibility that A. tegmentosum extract

(especially the ethyl acetate fraction) could be a source of

functional food or treatments for neurodegenerative
diseases, such as the Alzheimer’s disease.

ACKNOWLEDGEMENTS

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Table 3. IC50 values for NO production inhibition by fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>IC50 value 1(µg/ml)</th>
<th>IC50 value 2(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>21.4±1.01</td>
<td>20.6±1.95</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>10.5±0.95</td>
<td>22.0±3.84</td>
</tr>
<tr>
<td>Butanol</td>
<td>85.9±2.61</td>
<td>32.4±5.65</td>
</tr>
<tr>
<td>Water</td>
<td>128.8±1.32</td>
<td>31.4±12.50</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>3.2±0.57</td>
<td>1.52±0.72</td>
</tr>
</tbody>
</table>

1The NO scavenging activities in RAW 264.7 cell line; 2The NO scavenging activities in BV2 cell line. Values represent mean±S.D. of six determinations. Values bearing different superscripts in same column are significantly different (p<0.05).

Table 4. IC50 values for NO production inhibition by compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 value 1(µM)</th>
<th>IC50 value 2(µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(β-sitosterol 3-O-β-D-glucopyranoside)</td>
<td>141.7±0.96</td>
<td>32.9±9.09</td>
</tr>
<tr>
<td>2(Salidroside)</td>
<td>562.7±0.86</td>
<td>32.0±3.12</td>
</tr>
<tr>
<td>3(tyrosol)</td>
<td>156.7±0.26</td>
<td>41.8±1.64</td>
</tr>
<tr>
<td>4(6'-O-galloylsalidroside)</td>
<td>127.5±1.59</td>
<td>34.4±2.17</td>
</tr>
<tr>
<td>5(Quercetin 3-β-D-glucopyranoside)</td>
<td>145.7±2.10</td>
<td>34.2±2.04</td>
</tr>
<tr>
<td>6(Quercetin)</td>
<td>12.3±0.21</td>
<td>20.9±6.93</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>7.21±0.74</td>
<td>6.25±0.78</td>
</tr>
</tbody>
</table>

1The NO scavenging activities in RAW 264.7 cell line; 2The NO scavenging activities in BV2 cell line. Values represent mean±S.D. of six determinations. Values bearing different superscripts in same column are significantly different (p<0.05).


