Inhibitory Effects of Actinidiamide from Actinidia polygama on Allergy and Inflammation

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Actinidia polygama Max. was subjected to supercritical fluid extraction (SFE), and the resulting ethanol extract of marc (SFEM) was subjected to sequential fractionation with various solvents. Each extract and fraction was assayed for anti-inflammatory effect. The ethyl acetate fraction (EtOAc) contained the highest level (70.8% inhibition) of anti-inflammatory activity. In order to identify the active constituents, the EtOAc fraction was further fractionated by silica gel and ODS column chromatography. By activity-guided fractionation, an active ceramide was identified as the anti-inflammatory component, and its structure was determined by NMR and MS analysis. The novel ceramide was named actinidiamide, and was found significantly to inhibit nitric oxide (NO) production (30.6% inhibition at 1 μg/mL) in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and β-hexosaminidase release (91.8% inhibition at 1 μg/mL) in IgE-sensitized RBL-2H3 cells. Thus the presence of actinidiamide conveys allergy and inflammation treatment ability to A. polygama.

Key words: Actinidia polygama Max.; actinidiamide; anti-allergy; anti-inflammatory; nitric oxide (NO) production

Actinidia polygama Max. (silver vine) is used as a folk medicine in Korea for treating pain, gout, and inflammation. Only a few studies have provided detailed information on the medicinal effects of A. polygama fructus extracts. A water extract of A. polygama suppressed rat paw edema induced by carrageenan, inhibited the production of nitric oxide (NO) induced by lipopolysaccharide (LPS), and inhibited the expression of inducible NO synthase (iNOS) protein in RAW 264.7 macrophages. Moreover, it inhibited the vascular permeability induced by acetic acid and the edema induced by carrageenan in mouse and rat. It is not clear, however, whether α-linoleic acid is the main component responsible for reducing inflammation and allergic reactions.

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Materials and Methods

Plant sample. Dried fruit of A. polygama was purchased from a Chinese drug store in Yeongcheon City, Gyeongbuk, Korea. A voucher specimen (KHU-0090723) was deposited at the Laboratory of Natural Products Chemistry, Kyung Hee University, Yongin, Korea.

Instruments and reagents. Melting points were determined using a Fisher-John apparatus (Fisher Scientific, Pittsburgh, PA) and were not corrected. Optical rotation was measured on a Jasco P-1010 digital polarimeter (Jasco, Tokyo). The IR spectrum was collected on a Perkin-Elmer Spectrum One FT-IR spectrometer (Perkin-Elmer, Norwalk, CT). EIMS was recorded on a JEOI JMS 700 (JEOL, Tokyo). 1H NMR (400 MHz) and 13C NMR (100 MHz) spectra were assessed using a Varian Unity Inova AS 400 FT-NMR spectrometer (Palo Alto, CA). CHCl3-d1 with TMS as internal standard was purchased from Sigma (St. Louis, MO). Dinitrophenyl (DNP)-specific IgE, Earle’s basal salt solution (EBSS), trypsin solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and LPS were from Sigma (St. Louis, MO). Fetal bovine serum (FBS), minimal essential medium (MEM), and other cell culture reagents were from Gibco BRL (Grand Island, NY). DNP-bovine serum albumin was from Alpha Diagnostic International (San Antonio, TX). All other reagents were from Sigma Aldrich (St. Louis, MO).

Preparation of crude extract, marc of the supercritical fluid extract, and ethanol extract of it from A. polygama. One kg of dried A. polygama was ground and extracted 3 times with a 10-fold excess w/v of 70% ethanol at room temperature over 24 h. After filtering of the extract solutions, the combined filtrate was concentrated by rotary vacuum evaporator (Eyela Uni Trap UT-1000, Tokyo, Japan) at 55 °C. The ethanol extract was concentrated at room temperature over 24 h. The ethanol extract was concentrated to ensure that the decreased NO production was not due to

Determination of NO production. NO production was measured as reported by Matsuda et al.,24 with modifications. In brief, RAW 264.7 cells seeded in 24-well plates (2 × 10^5 cells/well) were sensitized with anti-DNP IgE (450 ng/mL) at 37 °C overnight. The cells were washed with Siraganian buffer and then incubated in 160 µL of the incubation buffer at 37 °C for 20 min. Then the cells were treated with 20 µL of extract for 10 min, followed by the addition of 20 µL of antigen (DNP-BSA, 10 µg/mL) at 37 °C for 10 min to stimulate the cells to granulate. The reaction was stopped by cooling in an ice bath for 10 min. Twenty-five µL of the supernatant was transferred into a 96-well plate and incubated with 25 µL of substrate (1 mM p-nitrophenyl-N-acetyl-d-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37 °C for 1 h. The reaction was stopped by adding 200 µL of stop solution (0.1 M Na2CO3/NaHCO3, pH 10.0), and the absorbance was measured at 405 nm using a microplate reader. The extract was dissolved in 0.1% DMSO and added to the incubation buffer.

Statistical analysis. All experiments were performed 3–5 times. Data were expressed as mean ± standard error of the mean (SEM) or standard deviation (SD). A significant difference from the control for each experimental test condition was assessed by Student’s t-test for each paired experiment. A p-value < 0.05 was regarded as indicating statistical significance.

Results and Discussion

Inhibition of allergic inflammation by the EtOH extract and various SFEM fractions of A. polygama. To test the anti-inflammatory effects of the EtOH extract of A. polygama and various SFEM fractions, NO production and cytotoxicity were measured in unstimulated and LPS-stimulated RAW 264.7 cells. The cells were incubated with LPS (100 ng/mL) in the presence of various extracts for 24 h. As Table 1 shows, the EtOH and SFEM extracts decreased the NO concentration in the culture media to 81.0 ± 2.3% and 29.4 ± 1.5% LPS-stimulated cells at 100 µg/mL, respectively. Moreover, the EtOAc fraction from SFEM markedly suppressed NO production, to 29.2 ± 4.1% LPS-stimulated cells at 25 µg/mL. This fraction had a much stronger effect than the BuOH (83.2 ± 4.4%) and H2O (89.1 ± 7.0%) fractions. We tested cell viability by MTT assay to ensure that the decreased NO production was not due to
264.7 Cells

H3 Cells Induced by IgE with DNP-BSA

Fractions from

A. polygama

nulation, the release of

imposed.

significantly affected cell viability under the conditions

cell death. Neither the tested fractions nor 0.1% DMSO

Effects of the EtOH, SFEM, EtOAc, BuOH, and H2O

fractions against NO-production in LPS-stimulated

RBL-2H3 Cells Induced by IgE with DNP-BSA

Table 1. Effects of the EtOH, SFEM, EtOAc, BuOH, and H2O Fractions from A. polygama on LPS-Induced NO Production in RAW 264.7 Cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (ng/mL)</th>
<th>NO production (%) a</th>
<th>Cell survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>10</td>
<td>95.58 ± 0.91</td>
<td>95.32 ± 5.06</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>81.01 ± 2.23</td>
<td>102.73 ± 0.28</td>
</tr>
<tr>
<td>SFEM</td>
<td>10</td>
<td>63.38 ± 1.83</td>
<td>96.18 ± 4.23</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>29.42 ± 1.53</td>
<td>98.17 ± 1.71</td>
</tr>
<tr>
<td>EtOAc</td>
<td>25</td>
<td>29.16 ± 4.10</td>
<td>103.98 ± 4.58</td>
</tr>
<tr>
<td>BuOH</td>
<td>25</td>
<td>83.23 ± 4.71</td>
<td>95.32 ± 3.20</td>
</tr>
<tr>
<td>H2O</td>
<td>25</td>
<td>89.06 ± 7.04</td>
<td>88.88 ± 1.44</td>
</tr>
</tbody>
</table>

a Nitrite levels were measured in the supernatants. Data represent mean ± SEM of three independent experiments.

Table 2. Effects of the EtOH, SFEM, EtOAc, BuOH, and H2O Fractions from A. polygama on β-Hexosaminidase Release from RBL-2H3 Cells Induced by IgE with DNP-BSA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (μg/mL)</th>
<th>β-Hexosaminidase release (%) a</th>
<th>Cell survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>10</td>
<td>87.89 ± 1.18</td>
<td>98.20 ± 2.49</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>45.59 ± 7.77</td>
<td>108.27 ± 3.68</td>
</tr>
<tr>
<td>SFEM</td>
<td>10</td>
<td>76.24 ± 2.79</td>
<td>102.36 ± 1.09</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>50.27 ± 0.95</td>
<td>106.94 ± 2.14</td>
</tr>
<tr>
<td>EtOAc</td>
<td>100</td>
<td>43.06 ± 3.65</td>
<td>90.32 ± 11.50</td>
</tr>
<tr>
<td>BuOH</td>
<td>100</td>
<td>42.37 ± 0.87</td>
<td>114.04 ± 34.70</td>
</tr>
<tr>
<td>H2O</td>
<td>100</td>
<td>82.20 ± 5.56</td>
<td>123.42 ± 21.59</td>
</tr>
</tbody>
</table>

a The levels of β-hexosaminidase released were measured in the supernatants. Data represent mean ± SEM of three independent experiments.

Histamine is released from mast cells in response to an antigen or a degranulation inducer. β-Hexosaminidase is stored in secretory granules within mast cells and is released with histamine in response to an antigen. Hence β-hexosaminidase is a marker for histamine release from mast cells. There are several inhibitors of β-hexosaminidase release, including flavonoids,27 diarylheptanoids,24 sesquiterpenes,20 anthraquinones,29 and alkaloids.30

Purification and structural elucidation of an anti-inflammatory compound from the EtOAc fraction of A. polygama by activity-guided fractionation

The presence of a ceramide in the EtOAc layer of the 80% methanol extract of A. polygama was confirmed by silica gel TLC. As shown in Table 1, the EtOAc fraction exhibited the highest inhibitory activity among the fractions against NO-production in LPS-stimulated RAW 264.7 cells. Repeating the silica gel column chromatography of the ethyl acetate layer yielded purified compound 1.

Actinidiadime (1) is a white amorphous powder; m.p. 117–118 °C (CHCl3–MeOH); [α]D 25° = +30.8° (c 0.01, pyridine); IR (νmax, KBr, cm −1): 3,216, 3,011, 2,911, 1,643, 1,517, 1,471; HRFAB-MS (pos.) m/z 570.5097 [M + H]+; EI-MS m/z 569 [M]+, 316, 301, 277, 253, 197, 195, 181, 169, 155. For the 1H and 13C-NMR spectral data for the compound, see Table 3.

Compound 1 was isolated as a white amorphous powder from CHCl3-MeOH. The molecular formula of 1 was established as C34H42N2O3 (calculated 569.5019) based on its positive HRFAB-MS data for the [M + H]+ at m/z 570.5097. The IR absorption bands at 3,321 cm −1, 2,911 cm −1 and 1,643 cm −1 are characteristic of the hydroxyl, the amide carbonyl, and the olefinic group respectively.

The 1H NMR spectrum in pyridine-d5 of 1 (Table 3) displayed a low-field doublet at δH 8.55 (1H, J = 9.2 Hz, NH), two olefine methine, oxygenated, or other heteroatomized protons, and two methyl protons at δH
0.88 (6H, t, J = 6.4 Hz, H-18, 16') and a strong aliphatic methylene signal at δH 1.29–2.27, which confirms the presence of two long aliphatic chains. Signals of a trans-olefinic bond at δH 5.46 (1H, dt, J = 16.0, 6.8 Hz, H-5') and δH 5.55 (1H, dt, J = 16.0, 6.8 Hz, H-4'), and four characteristic signals of hydroxyl groups were observed at δH 4.59 (1H, m, H-2'), 4.73 (1H, dd, dd, J = 11.2, 4.8 Hz, H-1a), 4.39 (1H, dd, J = 11.2, 5.8 Hz, H-1b), 4.37 (1H, dd, J = 5.2, 3.7 Hz, H-3), and 4.26 (1H, m, H-4). Another low field signal was observed at δH 5.09 (1H, ddd, J = 5.2, 5.8, 9.2, H-2), indicating a methine proton vicinal coupling to the nitrogen atom of an amide linkage representing a sphingolipid skeleton.31) The 13C NMR (Table 3) and DEPT spectral data for 1 showed an amide carbonyl carbon at δC 175.20 (C-1'), two olefine methine carbons at δC 130.83 (C-5') and 130.69 (C-5), and five oxygenated and other heteroatomized carbons at δC 76.98 (C-3), 73.15 (C-4), 72.59 (C-2'), 62.20 (C-1), and 53.16 (C-2), which were assigned on the basis of HSQC data. In the aliphatic region, methylenes were observed between δC 29.76–30.21 and two terminal methyl groups at δC 14.43 (C-18 and C-16'). The low-field doublet at δH 8.55 (NH) had no correlation with any carbon in the HSQC spectrum of 1. The signal at δH 8.55 (NH) correlated with δC 5.09 (1H, m, H-2'), and that at δH 8.55 (NH) correlated with δC 62.20 (C-1), 53.16 (C-2), 76.98 (C-3), 175.20 (C-1'), and 72.59 (C-2') respectively in the 1H–13C COSY and HMBC spectra of 1.

The chemical shifts of the allylic methylene carbons in 1 were located at δC 35.85 (C-3') and 33.42 (C-6') based on the correlation between HMBC signals from the olefinic signals at δH 5.46 (H-5') and δH 5.55 (H-4') to these two carbon signals (Fig. 1) respectively. The C=C bond configuration was determined to be trans based on the vicinal coupling constants (J4,5 = 16.0 Hz). These trans-geometries were also deduced from the chemical shifts of allylic carbons C-3' and C-6' (δC 33.85, 33.42 respectively).32)

Further investigation of the EIMS showed important fragment ions at m/z 181 and 155, suggesting a double bond at C-4'. The lengths of the amide chain and the fatty alcohol were determined by El-MS (Fig. 2). The El-MS of 1 showed a molecular ion at m/z 569 [M]+. Significant fragment ions at m/z 253 [M]+, 312 [M]+, 197 [M]+, and 316 [M–amide chain]+ in the El-MS indicated an amino alcohol chain of 16 carbon atoms. The relative stereochemistry of 1 at C-2, C-3, C-4, and C-2' was proposed to be 2S, 3S, and 4R, since the proton and carbon signals were in good agreement with natural and synthetic ceramides reported in the literature.33) Also, in that because the optical rotation ([α]D25 = +30.8°) compared with reported sphingolipids,34) compound 1 had a sphingosine moiety with a (2S,3S,4R,4E) geometry. Thus the structure was identified as (2S,3S,4R,4E)-2N-{[(2'R)-2'-hydroxy-hexadec-4'-enyl]octadecane-1,3,4-triol, and the chemical was named actinidiamide (Fig. 1).

Inhibitory effects of actinidiamide from A. polygama on allergy and inflammation

Next we evaluated the effects of the novel ceramide actinidiamide on NO production in LPS-stimulated RAW 264.7 cells and on β-hexosaminidase release in RBL-2H3 cells. Ceramides are a family of sphingolipids that have antiproliferative and proapoptotic activities,35–37) but, their role in anti-inflammatory processes is poorly understood.

As shown in Fig. 3, NO production was inhibited by actinidiamide (in 0.08% pyridine) in a dose-dependent manner. At a concentration of 2 μg/mL, NO production decreased to 21.7%, and actinidiamide was not cytotoxic. Pyrimidine (0.08%), used as a reference, did not affect cell viability or NO production. The inhibitory effect of actinidiamide on β-hexosaminidase release is shown in Fig. 4. Actinidiamide inhibited β-hexosaminidase release in a dose-dependent manner and had a significant effect at 0.5 (31.5 ± 10.3%) and 1 μg/mL (8.2 ± 3.1%) without cytotoxicity. These results suggest that actinidiamide isolated from the EtOAc fraction of SFEM from A. polygama potently regulates allergic inflammation.

In summary, we found that the EtOAc extract of SFEM from A. polygama markedly inhibited NO pro-
Inhibitory Effects of Actinidiamide from *Actinidia polygama*

Fig. 4. Cytotoxicity and Inhibitory Effects of Actinidiamide from *A. polygama* on β-Hexosaminidase Release from RBL-2H3 Cells Induced by IgE with DNP-BSA.

RBL-2H3 cells (2 × 10⁵ cells) were sensitized with 450 ng/mL of DNP-specific IgE overnight, and were pretreated with varying doses of actinidiamide over 10 min. Cells were stimulated with 10 μg/mL of DNP-BSA for 10 min. β-Hexosaminidase release was assayed in the culture media of the cells. Values represent mean ± SD of three independent experiments. **p < 0.01 vs. IgE alone.

production without cytotoxicity in LPS-stimulated RAW 264.7 cells. The active component of the EtOAc fraction was isolated by activity-guided fractionation steps, and was identified as the novel ceramide, actinidiamide. Moreover, actinidiamide inhibited NO production in RAW 264.7 cells (0.1–2 g/mL) and degranulation in RBL-2H3 cells induced by DNP-BSA and IgE (0.1–1 g/mL) in a dose-dependent manner. At a concentration of 1 μg/mL, actinidiamide inhibited NO production and β-hexosaminidase by 31 and 92% respectively. The strong inhibitory activities of actinidiamide might explain the anti-inflammatory effects of the *A. polygama* extract.

Acknowledgments

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References