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Cytotoxic Constituents from *Notopterygium incisum*

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Abstract – The MeOH extract of *Notopterygium incisum* showed a strong cytotoxicity against B16 murine melanoma cell line. From this extract three furanocoumarins including bergamottin, isoimperatorin, notopterol and one polyacetylenic compound (falcarindiol) together with one phenylpropanoid (caffeic acid methyl ester) and one triterpenoid (pregnenolone) were isolated. The isolated compounds were evaluated for cytotoxic activity against four kinds of cancer cell lines, e.g. P388 (murine lymphocytic leukemia), B16 (murine melanoma), A549 (human lung carcinoma) and SK-OV-3 (human ovarian cancer). Among the isolates, falcarindiol and caffeic acid methyl ester expressed a significant antiproliferative activity against all tested cell lines.

Key words – *Notopterygium incisum*, Umbeliferae, cytotoxicity, furanocoumarin, falcarindiol, cinnamate.

Introduction

Notopterygium incisum is a perennial herb growing widely in Asian countries. Its underground parts have been used in the Orient for the treatment of headache, common cold, and rheumatism.[1] As regards the chemical constituents, sugars, amino acids, mono- and triterpenoids,[2] a polyacetylenic compound,[3] coumarins and phenolic compounds,[4] were reported to be present in the underground part of *N. incisum*. Biologically, *N. incisum* has been shown to have an analgesic activity[5] and inhibitory activity against 5-lipoxy- genase and cyclooxygenase.[6] However, there is no report on the cytotoxicity of *N. incisum*. As a part of our ongoing search for antitumor agents from medicinal plants, we found that the MeOH extract of the underground part of *N. incisum* showed a strong antiproliferative activity against B16 (murine melanoma) and P388 (murine lymphocytic leukemia) cell lines. We describe here the isolation, structure elucidation and biological evaluation of the constituents from the title plant.

Materials, Instruments and Methods

Reagents and Instruments – Unless otherwise stated, all materials, chemicals and solvents were of reagent grade and obtained from commercial sources; RPMI 1640 medium, Dulbeccos modified Eagles medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, DMSO, sulforhodamine B (SRB), PBS (phosphate buffer saline), trypsin-EDTA solution (100 mL), tris(hydroxymethyl)aminomethane (Tris-base) and other reagents used for cell culture and assay were purchased from GIBCO Co., Ltd. (Grand Island, NY). Melting points were determined on an Electrothermal melting point apparatus and are uncorrected. IR spectra were recorded on a Jasco Report-100 IR spectrometer. Proton NMR and carbon NMR spectra were recorded on a Varian-Gemini spectrometer at 300 MHz and 75 MHz, respectively, using tetramethylsilane as internal standard. Analytical thin layer chromatography was performed on a plastic sheet (0.2 mm) precoated with silica gel 60 F_{254} (E. Merck). Silica gel 60 (70-230 mesh, E. Merck) was used for column chromatography. Optical density was read using ELISA reader (Spectra Max 250, USA). An incubator purchased from Shellab Co. Ltd. (USA), was used for cell culture.

Plant materials – The plant material (rhizoma) was purchased from an oriental herbarium in Hanoi, Vietnam and identified by Professor Tran Cong Khanh, Department of Botany, Hanoi College of Pharmacy,

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Vietnam. Voucher specimens have been deposited in our laboratory at the College of Pharmacy, Chungnam National University, Taejon, Korea.

Cells and Cell Culture – P388 (murine lymphocytic leukemia), B16F10 (murine melanoma), A549 (human carcinoma) and SK-OV-3 (human ovarian carcinoma) cell lines were obtained from a Cancer Cell Bank at Korea Research Institute of Bioscience and Biotechnology (KRIBB) and cultured in DMEM supplemented with 10% FBS and L-glutamine (0.2 mM/mL).

***In Vitro* Cytotoxicity Assay** – Cytotoxicity assay was performed as follows: Cells at logarithmic phase were trypsinized, dispersed into single-cell suspension in DMEM supplemented with FBS in 5% and adjusted to a concentration of 5×10^4 cells/ mL of which, 180 mL was dispensed into each well of 96 well-plates. Cells were then incubated at 37°C under humidified atmosphere with 5% of CO_2 for 24 hr and test samples prepared in 20 mL of DMEM from stock solutions were added at various concentrations and further incubated for 72 hours. All samples were prepared such that the final concentration of dimethylsolfoxide was less than 0.2%. Cytotoxicity was measured by the SRB method[7] and the IC_{50} value was calculated using Probits method.[8] In brief, cells were fixed by gently layering 50 mL of cold trichloroacetic acid on the top of the growth medium in each well and incubated at 4°C for 1 hr and then washed five times with tap water. Plates were air-dried and stained with sulforhodamine B 0.4% (w/v) in acetic acid 1% for 15 ~30 minutes and rinsed four times with acetic acid 1% to remove unbound dye. Plates were air-dried and bound dye was solubilized with 100 mL of 10 mM unbuffered Tris-base (pH 10.5). Adsorbance was read with a microtiter plate reader (ELISA reader) at 540 nm. The IC_{50} value was the concentration of a sample that reduces adsorbance by 50% in comparison with a control (vehicle-treated well). The result was the average value of four independent measurements in which adsorbance readings were varied less than 5% and in this assay model, the IC_{50} values of the same sample from different experiments varied less than 30%.

Preliminary fractionation – The dried and powdered plant material (2 kg) was exhaustively extracted with hot MeOH (3 l x 3 times). The methanol extracts were combined and concentrated *in vacuo* to give a residue (125 gr) which was suspended in water and partitioned against ethyl acetate (EA). The EA extract was evaporated to give EA fraction (35 gr) which showed a strong antiproliferative activity against P388 leukemic cells with IC_{50} value of 7.85 μg/ml (MeOH extract displayed IC_{50} value of 11.89 μg/ml, the remain H_2O fraction showed IC_{50} value of > 30 μg/ml).

Isolation of compounds – The EA fraction (13.5 gr) was chromatographed on silica gel column using hexane-EA (9:1→5:5) as the eluting solvent and separated into 12 fractions [EA-1 (1.76 gr), EA-2 (0.20 gr), EA-3 (0.21 gr, EA-4 (0.10 gr), EA-5 (0.96 gr), EA-6 (0.25 gr), EA-7 (2.49 gr), EA-8 (1.77 gr), EA-9 (1.34 gr), EA-10 (0.35 gr), EA-11 (0.20 gr), EA-12 (3.9 gr)]. Among the EA-fractions, fractions EA-7, EA-8 showed strong growth inhibitory activity and fraction EA-3 expressed a moderate activity against P388 cells. Fraction EA-3 was rechromatographed on silica gel column eluting with hexane- CH_2Cl_2 (1:1→1:2) to give compound 1 (bergamottin, 55 mg). Compounds 2 (isoimperatorin, 310 mg) and 5 (notopterol, 523 mg) were crystallized out from fractions EA-5 and EA-9, respectively, using hexane-EA solvent system. Fraction EA-7 was further chromatographed on silica gel column eluting with hexane-CH_2Cl_2 (3:7→0:1) and CH_2Cl_2-EA (9:1→5:5) to give 7 fractions (EA-7-1~EA-7-7). The active fraction EA-7-5 eluted with CH_2Cl_2-EA (8:2) was purified on ODS column with H_2O-MeOH (1:2) to afford compound 3 (falcarindiol, 0.51 gr) and 4 (pregnenolone, 8 mg). Fraction EA-8 was rechromatographed on ODS column eluting with H_2O-MeOH (1:2→1:3) and separated into 6 fractions. The active fractions EA-8-3 and EA-8-4 displayed a similar spot on TLC were combined and further purified using Sephadex LH20 column eluting with MeOH-isopropanol (1:1) to give compound 6 (caffeic acid methyl ester, 26.8 mg). Description of the compounds 1-5 was omitted here since they were already isolated and elucidated from the same plant.[3-6,9,10]

Compound 6 – Colorless prisms. EI-MS *m/z*: 194.0515 (M^+) (Cal. for $C_{10}H_{10}O_4$, 194.0579). IR (KBr, ν_{max}

cm^{-1}): 3350, 3300, 3020, 2950, 1680, 1620, 1580, 1460, 1250, 1080, 980. ^{1}H-NMR ($CDCl_3$) δ : 3.75 (3H, s, -OCH_3), 6.25 (1H, d, J=16.0 Hz, H-7), 6.78 (1H, d, J=8.0 Hz, H-5), 6.94 (1H, dd, J=2.0, 8.0 Hz, H-6), 7.04 (1H, d, J=2.0 Hz, H-2), 7.54 (1H, d, J=16.0 Hz, H-8). ^{13}C-NMR (DMSO) δ : 52.0 (-OCH_3), 114.9 (C-7), 115.2 (C-2), 116.5 (C-5), 122.9 (C-6), 127.4 (C-1), 146.8 (C-3), 146.9 (C-80), 149.5 (C-4), 169.8 (C=O).

Methylation of 6 – To a 0°C solution of **6** (10 mg) in dry MeOH was added dropwise diazomethane which was generated in situ from Diazald®. After a reaction completed, the mixture was concentrated in vacuo and purified on silica gel to give 8.1 mg of 3, 4-dimethoxycinnamic acid methyl ester (6a). Colorless needles. EI-MS *m/z*: 222 (M^+). IR (KBr, ν_{max} cm^{-1}): 3015, 2930, 16750, 1610, 1590, 1460, 1250, 1080, 980. ^{1}H-NMR ($CDCl_3$) δ : 3.75 (3H, s, -$COOCH_3$), 3.99 (3H, s, 3-OCH_3), 4.01 (3H, s, 4-OCH_3), 6.23 (1H, d, J=15.8 Hz, H-7), 6.98 (1H, d, J=7.2 Hz, H-5), 7.04 (1H, dd, J=1.8, 7.8 Hz, H-6), 7.12 (1H, d, J=1.8 Hz, H-2), 7.61 (1H, d, J=15.8 Hz, H-8).

Results and Discussion

The dried and powdered underground part of *N. incisum* was exhaustively extracted with hot MeOH. The MeOH extract, after concentration in vacuo to a dry mass and resuspension in water, was partitioned against EA. Subsequent bioassay followed revealed that the activity of the MeOH extract was almost located in EA fraction. Repeated chromatography on silica gel, ODS and Sephadex LH-20 led to the isolation of six compounds. The structures of compounds 1 (bergamottin), 2 (isoimperatorin), 3 (falcarindiol), 4 (pregnenolone) and 5 (notopterol), which had been reported from *N. incisum* previously, were elucidated directly on the basis of physical and spectral data comparison with literature values.[3-6,9,10]

Compound 6 was shown to have a molecular weight of 194 (EI-MS). IR spectrum revealed strong absorptions at 3350 and 3300 cm^{-1} and 1680 cm^{-1}, suggesting the presence of hydroxyl and carbonyl groups, respectively. The peaks at 1600 and 1580 cm^{-1} were evidence for presence of aromatic ring in the molecule. ^{1}H-NMR displayed two doublet peaks at 6.25 and 7.54 ppm with coupling constant of 16 Hz, attributed for a *trans* α,β-unsaturated carbonyl function. Thus, six double bond equivalents shown by the molecular formula were fully accounted for by the aromatic ring and the α,β-unsaturated carbonyl function, building a cinnamoyl moiety of the molecule. The peaks appeared at 52.0 ppm in ^{13}C- NMR and 3.75 ppm as a singlet in ^{1}H-NMR certified the methyl ester (OCH_3) group. With these data, we could construct a molecular formula of $C_{10}H_8O_2$ (MW, 160). The rest of the molecular weight of 194 was 34 corresponding to two hydroxyl groups. The two doublets at 6.78 (1H, d, J=8.0 Hz), 7.04 (1H, d, J=2.0 Hz) and a doublet of doublets at 6.94 (1H, dd, J=2.0, 8.0 Hz) showed that the aromatic ring is 1,3,4-trisubstituted. The chemical shift and coupling constant of the peak at 7.04 (1H, d, J=2.0 Hz) exhibited that it is flanked by a hydroxyl group and an α,β-unsaturated carbonyl; thus it should be H-2. The doublet of doublets peak at 6.94 ppm (1H, dd, J=2.0, 8.0 Hz) should be H-6 and the peak at 6.78 ppm (1H, d, J=8.0 Hz) should be H-5. Thus, two hydroxyl groups must assume 3,4-positions of the cinnamoyl moiety. To further confirm the presence of two hydroxyl groups and certify the methoxy group functionality, compound 6 was methylated. The methylated compound showed molecular weight of 222, indicating the introduction of two methyl groups in the structure, which appeared at 4.01 and 3.99 ppm. These chemical shifts appearing at lower fields compared with an ester methoxy group (3.75 ppm in compound 6), should correspond to methoxy groups on the aromatic ring. Based on all of these evidences, 6 was determined to be caffeic acid methyl ester which has been reported from *Angelica japonica*.[10] However, this is the first report on the presence of **6** from *N. incisum*.

All isolates were evaluated for cytotoxic activity against a small panel of cancer cell lines including B16 (murine melanoma), P388 (murine lymphocytic leukemia), A549 (human lung carcinoma), and SK-OV-3 (human ovarian cancer). The IC_{50} values are shown in Table I. All furanocoumarins and pregnenolone were found inactive. Falcarindiol, which had been found to

Table I. IC_{50} values of the isolated compounds against tumor cell lines

Compounds	Cytotoxicity (IC_{50} values,[1] μg/ml) against cancer cell lines[2]			
	B16	P388	A549	SK-OV-3
Bergamottin	>30	>30	>30	>30
Isoimperatorin	>30	>30	>30	>30
Falcarindiol	1.29	0.83	2.18	1.95
Pregnenolone	>30	27.15	>30	>30
Notopterol	>30	>30	>30	>30
Caffeic acid methyl ester	2.89	1.20	1.75	1.85
5-FU	3.98	0.20	5.47	4.67
Etoposide	0.86	2.12	1.17	1.21
Adriamycin	0.10	0.06	0.09	0.15

[1] The concentration that causes a 50% reduction in cell growth percentage. [2] B16, murine melanoma; P388, murine lymphocytic leukemia; A549, human lung carcinoma, and SK-OV-3, human ovarian cancer.

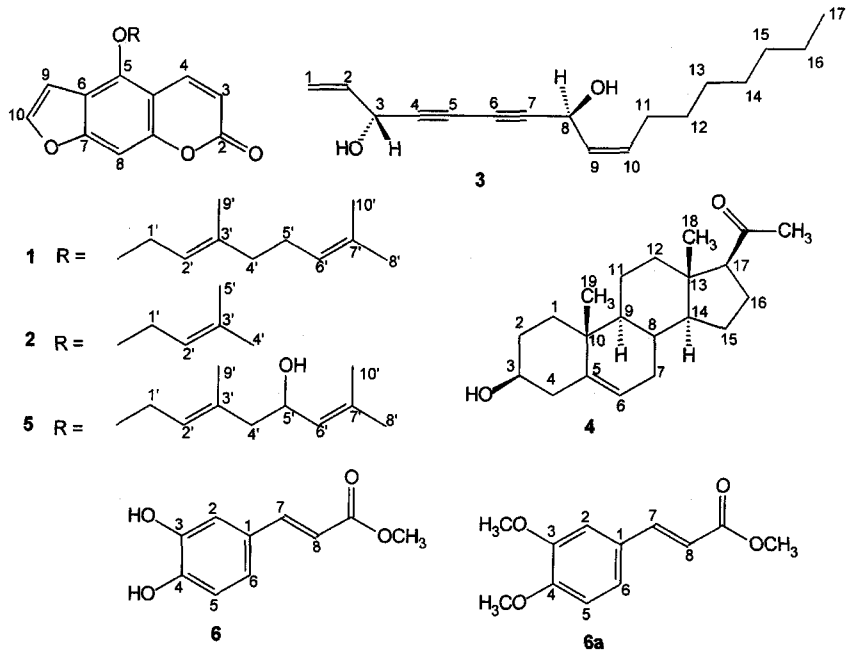

Fig. 1. Structures of the isolated compounds

be cytotoxic,[10] showed a significant cytotoxic activity in all tested cell lines. It is noteworthy that the compound 6 exhibited a higher cytotoxicity than 5-FU. The cytotoxicity of **6** was also comparable to that of etoposide but lower than that of adriamycin.

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