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NF-κB Activation by Compounds Found in *Platycodon grandiflorum* Extract

Hong, Sungwon[1], Yeonjoong Yong[1], Kyungrai Kang[1], Soon Young Shin[2], Young Han Lee[2], and Yoongho Lim[1]*

[1]*Division of Bioscience and Biotechnology, BMIC, RCD, Konkuk University, Seoul 143-701, Korea*
[2]*Institute of Biomedical Science and Technology, RCTC, Konkuk University, Seoul 143-701, Korea*

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Compounds extracted from *Platycodon grandiflorum* were evaluated for an activation effect on nuclear factor-kappa B (NF-κB). In its active state, NF-κB turns on the expression of genes related to cell proliferation or death. NF-κB activators promote growth of neuron cells and can be used to control neurodegenerative diseases. The biological activity of *P. grandiflorum* extracts toward NF-κB had not yet been studied. Although the biological activity of several compounds extracted from *P. grandiflorum* was evaluated, only three exhibited any significant activation effect on NF-κB.

Keywords: *Platycodon grandiflorum*, lobetyol, lobetyolin, NF-κB

Nuclear factor-kappa B (NF-κB), a transcription factor found in almost all animal cell types, plays an important role in regulating the immune response to infection and participates in the inflammatory response, cancer, autoimmune disease, and synaptic plasticity [8]. Mammalian NF-κB consists of five family members, NF-κB1, NF-κB2, Rel A, Rel B, and c-Rel, and all members have a Rel domain at the N-terminus [3]. In addition, NF-κB regulates genes that control proliferation and survival of cells [6]. In its active state, NF-κB turns on the expression of genes related to proliferation or death, and NF-κB activators are related to the growth of neuron cells and can be used to control neurodegenerative diseases [5].

Owing to consumer rejection of synthesized chemicals, natural products have become widely studied alternatives. In the current study, the extract of *Platycodon grandiflorum*, also known as *Doraji* in Korean or balloon flower in English, was evaluated to determine its activity on NF-κB. The roots of *P. grandiflorum* have been used as food and in traditional medicines as an expectorant and to treat hypertension and diabetes [9]. To the best of our knowledge, its biological activity toward NF-κB has not yet been determined.

The structures of the compounds isolated here were determined using nuclear magnetic resonance (NMR) spectroscopy and fast atom bombardment mass spectrometry (FAB-MS). ^{1}H NMR, ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT), correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bonded connectivity (HMBC) experiments were performed on a Bruker Avance 400 spectrometer system (Bruker, Karlsruhe, Germany) at 298 K. The ^{1}H experiments were performed with a 32 K time domain, and the ^{13}C NMR and DEPT experiments were performed with a 64 K time domain. All two-dimensional NMR experiments were collected with a 2,048×256 (t_2×t_1 time domain). The mixing time for TOCSY was 200 ms and the delay for the long-range coupling in HMBC was 62.5 ms. All NMR data were processed using XWINNMR (Bruker) [7].

The effects of compounds on NF-κB activity were analyzed by NF-κB-dependent reporter gene expression. HEK293 human embryonic kidney cells (1×10^5 cells/well) were plated on 12-well plates and transiently transfected with 50 ng of pNF-κB-Luc plasmid (Stratagene, Cedar Creek, TX, U.S.A.) containing five repeats of the NF-κB binding sites. Luciferase activity represented the transcriptional activation of NF-κB. To monitor the transfection efficiency, a pRL-null plasmid encoding *Renilla* luciferase was included in all transfections. At 24 h post-transfection, cells were treated with 20 μM of the B4-2-1, C5-4, or C5-5 fractions and further incubated for 8 h. The levels of firefly and *Renilla* luciferase activity were measured sequentially from a single sample using a Dual-Glo Luciferase Assay System (Promega Corp., Madison, WI, U.S.A.). Luminescence was measured with a Centro LB960 luminometer (Berthold Tech, Bad Wildbad, Germany). Firefly luciferase activity was normalized to the *Renilla* activity, and the luciferase activity in the untreated cells was considered as 1 [2].

One kg of dried *P. glandiflorum* root was purchased from Kyungdong Market in Seoul, Korea, and pulverized. The

*Corresponding author
Phone: +82-2-453-3760; Fax: +82-2-454-3760;
E-mail: yoongho@konkuk.ac.kr

powder was soaked three times in 4 l of methanol for 3 days. The methanol solution was filtered (No.2 filter paper; Whatman, Dawsonville, GA, U.S.A.) and dried with a rotary evaporator at reduced pressure and 40°C. The extract (118.66 g) was dissolved in 1 l of distilled water, and an equal amount of *n*-hexane was added. The organic component was removed with a separatory funnel, and the procedure was repeated with chloroform, ethylacetate, and *n*-butanol.

Each fraction was lyophilized (Operon, Kyunggi-do, Korea) and tested for activation of NF-κB. The chloroform (1.33 g) and *n*-butanol (31.97 g) fractions exhibited activation and were subjected to further separation. The chloroform fraction was refined *via* column chromatography on a silica gel 60 stationary phase (70-230 mesh; Merck, Whitehouse Station, NJ, U.S.A.) and eluted with a step-gradient of chloroform in methanol to obtain 14 fractions. The fifth fraction exhibited the highest activity and was separated further using preparative HPLC (Varian, Palo Alto, CA, U.S.A.) on a reversed-phase C18 column (Gemini, 5 μ, 10×250 mm; Phenomenax, St. Louis, MO, U.S.A.) with a UV/VIS detector (Varian, Palo Alto, CA, U.S.A.). The mobile phase consisted of 25% acetonitrile in water with a flow rate of 4.5 ml/min. Of the six fractions obtained, the fourth (C5-4) and the fifth (C5-5), at 30.7 min and 32.6 min, respectively, showed activity on NF-κB. These fractions were subjected to analytical HPLC (Agilent, Santa Clara, CA, U.S.A.) with a photodiode array detector and determined to be single compounds. Fraction C5-4 (4.5 mg) was dissolved in methanol-d4 and transferred to a 2.5-mm NMR tube. The ^{1}H NMR spectrum indicated two double bonds and two triple bonds. The ^{13}C NMR and DEPT spectra revealed the presence of one methyl group, three methylene groups, six methane groups, and four quaternary carbon atoms. One of the methylene groups and two methane groups were hydroxylated. Based on COSY, TOCSY, HMQC, and HMBC spectra, the molecular structure was identified as 4,12-tetradecadien-8,10-diyne-1,6,7-triol, shown in Fig. 1. The molecular mass, 234 g/mol, determined by FAB-MS analyses, agreed with the NMR data. This compound is also known as lobetyol, but its activity with regard to NF-κB has not previously been reported [4]. ^{13}C and ^{1}H NMR assignments are listed in Tables 1 and 2, respectively.

The same set of experiments was carried out on another single-component fraction, C5-5 (3.7 mg). The ^{1}H and ^{13}C NMR spectra were similar to those of C5-4. Based on the 1D and 2D NMR analyses, the molecular structure was identified as 4,8,12-triene-10-yne-1,6,7-triol (Fig. 2). The molecular mass, 236 g/mol, was determined by FAB-MS and matched the result obtained by NMR. Like C5-4, this compound was also known, but no activity with NF-κB has been reported [1]. ^{13}C and ^{1}H NMR assignments are listed in Tables 1 and 2, respectively.

Table 1. ^{13}C NMR assignments of three compounds isolated from *Platycodon grandiflorum*.

Position (C no.)	δ of ^{13}C		
	C5-4	B2-4-1	C5-5
1	60.1	62.3	62.5
2	32.1	33.0	33.2
3	28.3	29.9	29.9
4	132.1	139.0	134.6
5	128.9	126.6	130.7
6	74.1	81.9	76.9
7	66.1	66.7	76.4
8	83.3	81.2	142.8
9	68.9	71.3	112.5
10	76.8	78.2	87.0
11	71.9	72.6	89.9
12	109.2	110.6	112.2
13	144.8	145.4	140.4
14	18.6	18.9	18.8
6-O-Glc-1'		100.8	
2'		74.9	
3'		78.1	
4'		71.7	
5'		78.2	
6'		62.9	

As discussed above, the *n*-butanol layer exhibited an activation effect on NF-κB. To isolate the responsible compound, the fraction was further separated by column chromatography with Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) with a step-gradient elution consisting of chloroform and methanol. When elution conditions were 65:35 chloroform:methanol, seven fractions were collected. The fourth fraction was separated on a preparatory HPLC column (C18 reversed-phase Phenomenex) with a mobile phase of 60% methanol in water at a flow rate of 4.5 ml/min. The peak at 21.6 min was separated again using the same column with a mobile phase of 40% methanol at

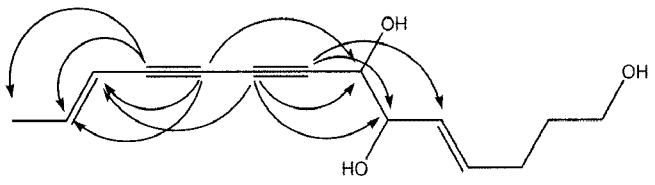

Fig. 1. The molecular structure of fraction C5-4 and the long-range correlations obtained from the HMBC data.

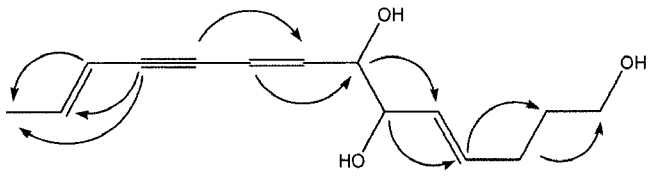

Fig. 2. The molecular structure of fraction C5-5 and the long-range correlations obtained from HMBC data.

Table 2. ^{1}H NMR assignments of three compounds isolated from *Platycodon grandiflorum*.

Position	δ of ^{1}H (mult, *J*, Hz)		
	C5-4	B2-4-1	C5-5
1	3.58 (dd, 6.8, 6.8)	3.65 (dd, 6.5, 6.5)	3.65 (dd, 6.5, 6.5)
2	1.64 (m)	1.65 (m)	1.78 (dd, 1.7, 6.5)
3	2.15 (dd, 6.8, 7.2)	2.19 (dd, 6.5, 15.4)	2.13 (ddd, 6.5, 13.5, 13.5)
4	5.67 (m)	5.91 (m)	5.72 (ddd, 6.5, 6.5, 15.2)
5	5.46 (m)	5.46 (dd, 8.2, 15.4)	5.84 (ddd, 1.4, 6.5, 15.2)
6	3.98 (dd, 6.8, 6.8)	4.16 (dd, 7.5, 8.2)	3.89 (dd, 6.5, 6.5)
7	4.21 (d, 6.8)	4.42 (d, 7.5)	3.98 (ddd, 1.4, 6.0, 6.5)
8			6.04 (dd, 6.0, 15.8)
9			6.09 (m)
12	5.71 (m)	5.57 (dd, 1.7, 15.8)	5.61 (dd, 1.7, 13.9)
13	6.37 (dq, 6,8, 15.3)	6.33 (ddd, 6.8, 6.8, 15.8)	6.09 (ddd, 6.8, 6.8, 13.9)
14	1.81 (dd, 1.8, 6.8)	1.80 (dd, 1.7, 6.8)	1.78 (dd, 1.7, 6.8)
6-O-Glc-1'		4.31 (d, 7.4)	
2'		3.25 (dd, 9.6, 9.6)	
3'		3.18 (dd, 9.6, 8.8)	
4'		3.29 (d, 11.9))	
5'		3.31 (m)	
6'		3.58 (dd, 6.5, 6.5) 3.85 (dd, 2.1, 11.9)	

a flow rate of 4.0 ml/min. The peak at 28.2 min was due to a single compound, B4-2-1 (21.7 mg), and exhibited activity toward NF-κB. ^{1}H and ^{13}C NMR, DEPT, COSY, TOCSY, HMQC, and HMBC experiments revealed similar spectra to those obtained for C5-4, with the exception of six carbon peaks corresponding to glucose between 60 and 100 ppm. HMBC spectra indicated a long-range connectivity between H-1 of glucose and C-6 of lobetyol. The molecular structure was therefore determined as 6-*O*-β-D-glucopyranosyl-tetradeca-4,12-dien-8,10-diyne-1,6,7-triol, or lobetyolin, with a molecular mass of 396 g/mol (Fig. 3). Like the other NF-κB active compounds, lobetyolin is a known compound, but an activation effect with NF-κB has not been reported [4]. ^{13}C and ^{1}H NMR assignments are listed in Tables 1 and 2, respectively.

The effects of these compounds on NF-κB activation were evaluated by transiently transfecting HEK293 cells with a pNF-κB-Luc reporter construct and then treating the cells with the C5-4, C5-5, or B4-2-1 fractions. B4-2-1 and C5-5 produced a 2.5- and 2-fold increase, respectively, in NF-κB-dependent reporter gene expression (Fig. 4). In contrast, treatment with C5-4 had little effect on NF-κB activity. These results demonstrated that both B4-2-1 and C5-5 trigger the transcriptional activity of NF-κB.

The extract from *P. grandiflorum* was evaluated for activation effects on NF-κB. Three of the isolated compounds exhibited the desired activity. Although these three compounds were not novel, their activation effects on NF-κB had not been reported. Compounds C5-4 and C5-5 differed by only one bond. Conjugation of the two triple bonds was not conducive to NF-κB activation, whereas glycosylation increased activation efficiency. The relationship between the position of glycosylation and the activation of NF-κB will be the subject of future work.

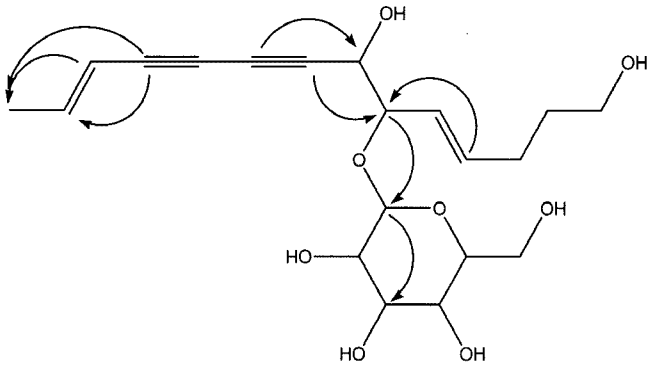

Fig. 3. The structure of fraction B4-2-1 and the long-range correlations obtained from the HMBC data.

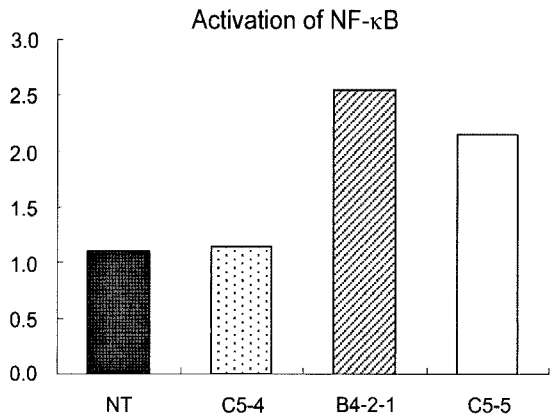

Fig. 4. Activation effects of the three isolated compounds on NF-κB.

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