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ORIGINAL ARTICLE

Nuclear Factor Kappa B Activation and Peroxisome Proliferator-activated Receptor Transactivational Effects of Chemical Components of the Roots of *Polygonum multiflorum*

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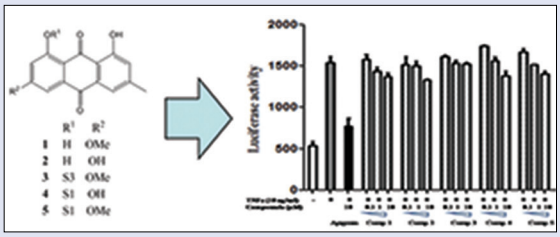
ABSTRACT

Background: *Polygonum multiflorum* is well-known as "Heshouwu" in traditional Chinese herbal medicine. In Northeast Asia, it is often used as a tonic to prevent premature aging of the kidney and liver, tendons, and bones and strengthening of the lower back and knees. **Objective:** To research the anti-inflammatory activities of components from *P. multiflorum*. **Materials and Methods:** The compounds were isolated by a combination of silica gel and YMC R-18 column chromatography, and their structures were identified by analysis of spectroscopic data (1D, 2D-nuclear magnetic resonance, and mass spectrometry). The anti-inflammatory activities of the isolated compounds 1–15 were evaluated by luciferase reporter gene assays. **Results:** Fifteen compounds (1–15) were isolated from the roots of *P. multiflorum*. Compounds 1–5 and 14–15 significantly inhibited tumor necrosis factor-α-induced nuclear factor kappa B-luciferase activity, with IC_{50} values of 24.16–37.56 μM. Compounds 1–5 also greatly enhanced peroxisome proliferator-activated receptors transcriptional activity with EC_{50} values of 18.26–31.45 μM. **Conclusion:** The anthraquinone derivatives were the active components from the roots of *P. multiflorum* as an inhibitor on inflammation-related factors in human hepatoma cells. Therefore, we suggest that the roots of *P. multiflorum* can be used to treat natural inflammatory diseases.

Key words: Human hepatoma cells, nuclear factor kappa B, peroxisome proliferator-activated receptors, *Polygonaceae*, *Polygonum multiflorum*

SUMMARY

- This study presented that fifteen compounds (1–15) isolated from the roots of Polygonum multiflrum exert signifiant anti inflmmatory effects by inhibiting TNF α induced NF κB activation and PPARs transcription.



Abbreviation used: NF κB: Nuclear factor kappa B, PPARs: Peroxisome proliferator activated receptors, PPREs: Peroxisome proliferator response elements, TNF α: Tumor necrosis factor α, ESI-MS: Electrospray ionization mass spectrometry, HepG2: Human hepatoma cells.

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INTRODUCTION

Nuclear factor kappa B (NF-κB) is a protein complex that controls DNA transcription. This complex comprises a family of structurally related eukaryotic transcription factors that promote the expression of over 150 genes involved in a variety of cellular processes.[1,2] It is found in nearly all animal cell types and is involved in several cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens.[3-6] Five members of the NF-κB family (p50, p52, p65/Rel A, c-Rel, and Rel B) form 15 transcription factors through homo- and hetero-dimerization.[7,8] In the previous study, NF-κB was determined to play an important role in the transcriptional regulation of numerous cytokines and adhesion molecules. It is the most extensively studied transcription factor in the immune system.[9] Furthermore, in most cell types, inactive NF-κB complexes are sequestered in the cytoplasm via noncovalent interactions with inhibitory proteins known as inhibitor kappa B. Therefore, activation of NF-κB causes transcription at the κB site, which is involved in several diseases including inflammatory disorders and cancer. Hence, inhibition of NF-κB signaling is an important therapeutic target for the treatment of such diseases.[10]

Peroxisome proliferator-activated receptors (PPARs) is a member of the nuclear receptor superfamily as transcription factors regulating the expression of genes.[11] It plays essential roles in the regulation of cellular metabolism, inflammatory, and immune responses. There are three isoforms: PPARα, PPARβ, and PPARγ have been identified. PPARs regulate the expression of genes involved in the regulation of glucose, lipid, and cholesterol metabolism by binding to specific peroxisome proliferator response elements (PPREs) in the enhancer sites of regulated genes.[12-14] Accordingly, modulate the function of PPARs are attractive for the treatment of tissues with high catabolic rates for fatty acids and peroxisome metabolism, and it has become a target for the prevention and treatment of obesity, insulin resistance, metabolic syndromes, inflammation, and cardiovascular disease.[15] In the present study, the effects of compounds 1–15 from *Polygonum*

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multiflorum on tumor necrosis factor-α (TNF-α) induced NF-κB transcriptional activity and PPARs transcriptional activity were evaluated in human hepatocarcinoma human hepatoma cells (HepG2) cells.

P. multiflorum belongs to the *Polygonaceae* family, is one of the most important traditional Chinese herbs, and listed in the official Chinese Pharmacopeia. It has long been used in the preparation of herbal medicines in many oriental countries such as China, Japan, and Korea.[16] This herb exerts many significant effects, such as antioxidant, and antitumor properties, improves cardiovascular symptoms, enhances immune function, reduces cholesterol, and inhibits atherosclerosis.[17,18] *P. multiflorum* root extracts and some monomeric compounds isolated from *P. multiflorum* roots were reported to exert anti-inflammatory,[19] antioxidant,[20] anti-HIV,[21] and liver protective effects.[22] However, the effects of chemical components from *P. multiflorum* on NF-κB and PPARs transcriptional inhibitory activity have not yet been reported. In the present study, fifteen compounds were isolated from the roots of *P. multiflorum* and their anti-inflammatory activities were evaluated to determine their therapeutic potential.

MATERIALS AND METHODS

General experimental procedures

Optical rotations were determined using a Jasco DIP–370 automatic polarimeter. The Fourier Transform Infrared spectra were measured using a Jasco Report–100 infrared spectrometer. The nuclear magnetic resonance spectra were recorded using a JEOL ECA 600 spectrometer (^{1}H, 600 MHz; ^{13}C, 150 MHz). Electrospray ionization mass spectrometry was recorded using an Agilent 1200 LC MSD trap spectrometer. Column chromatography was performed using a silica gel (Kieselgel 60, 70–230, and 230–400 mesh, Merck, Darmstadt, Germany), YMC RP-18 resins, and thin layer chromatography was performed using precoated silica-gel 60 F_{254} and RP-18 F_{254S} plates (both 0.25 mm, Merck, Darmstadt, Germany); the spots were detected under ultraviolet light and using 10% H_2SO_4.

Plant material

Dried roots of *P. multiflorum* were purchased from the herbal company, Naemome Dah, Ulsan, Korea, in November 2011, and identified by Prof. Young Ho Kim, College of Pharmacy, Chungnan National University. A voucher specimen (CNU11103) was deposited at the herbarium of the College of Pharmacy, Chungnam National University in Korea.

Extraction and isolation

Dried roots of *P. multiflorum* (3.0 kg) were extracted with 70% EtOH 3 times under refluxing. The 70% EtOH extract (500.0 g) was suspended in H_2O (2.8 L) and partitioned with CH_2Cl_2 and EtOAc to yield CH_2Cl_2 fraction (a), EtOAc fraction (b), aqueous fraction (c), respectively. The CH_2Cl_2 extract (14.0 g) was subjected to silica gel column chromatography with a gradient of *n*-hexane-EtOAc (25:1–0:1) to give five fractions (A1–A5). Fraction A3 was further chromatographed on a silica gel column using a gradient of *n*-hexane-EtOAc (6:1–0:1) to give three subfractions (A3.1–A3.3), then subfraction A3.3 was chromatographed on a silica gel column with *n*-hexane-EtOAc (3:1–1:5) to obtain four subfractions (A3.3.1–A3.3.4), further purification of the subfraction A3.3.2 and A3.3.4 led to compounds 1 (20.0 mg) and 2 (917.3 mg). Fraction A5 was column chromatographed over silica gel, eluting with CH_2Cl_2-MeOH (10:1–1:1) to provide six subfractions (A5.1–A5.6), then subfraction A5.4 was further chromatographed on a reverse–phase (RP) chromatography column with MeOH–H_2O (1:1–2.5:1) to give compounds 3 (5.0 mg), 14 (39.0 mg), and 15 (53.0 mg). The EtOAc extract (103.0 g) was chromatographed over silica gel column with a gradient of CH_2Cl_2–MeOH (20:1–1:1) to yield 4 fractions (B1–B4). Fraction B1 was chromatographed on a silica gel chromatography column with CH_2Cl_2–MeOH (50:1–5:1) to yield 6 subfractions (B1.1–B1.6); subfraction B1.2 was further chromatographed on RP chromatography column with acetone–MeOH–H_2O (0.2:0.5:1) to yield compounds 12 (210.0 mg) and 13 (40.0 mg). Subfraction B1.4 was separated by a RP chromatography column using acetone–MeOH–H_2O (0.2:0.5:1–1:1:1) as eluents, further purified by chromatography column over silica gel, to obtain compounds 4 (75.0 mg), 6 (17.0 mg), 7 (180.0 mg), 10 (35.0 mg), and 11 (52.0 mg). Compounds 5 (13.0 mg), 8 (290.0 mg), and 9 (28.0 mg) were isolated from fraction B1.5 using a RP chromatography column with MeOH–H_2O (1:5–2:1).

Cell culture and reagents

HepG2 cells were maintained in Dulbecco's modified Eagles' medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 10 μg/mL streptomycin at 37°C and 5% CO_2. Human TNF-α was purchased from ATgen (Seoul, Korea).

Cytotoxicity assay

A cell-counting kit (CCK)-8 (Dojindo, Kumamoto, Japan) was used to analyze the effect of compounds on cell toxicity according to the manufacturer's instructions. HepG2 cells were cultured overnight in a 96-well plate (~1×10^4 cells/well). Cell toxicity was assessed after the addition of compounds in a dose-dependent manner. After 24 h of the treatment, 10 μL of the CCK-8 solution was added to triplicate wells and incubated for 1 h. The absorbance at 450 nm was measured to determine the viable cell numbers.

Nuclear factor kappa B-luciferase assay

The luciferase vector was first transfected into human hepatocarcinoma HepG2 cells. After a limited amount of time, the cells were lysed, and luciferin, the substrate of luciferase, was introduced into the cellular extract along with Mg^{2+} and an excess of ATP. Under these conditions, luciferase enzymes expressed by the reporter vector could catalyze the oxidative carboxylation of luciferin. Cells were seeded at 2×10^5 cells per well in 12-well plates and grown. After 24 h, cells were transfected with inducible NF-κB luciferase reporter and constitutively expressing Renilla reporter. After 24 h of transfection, medium was changed to assay medium (Opti-MEM + 0.5% FBS + 0.1 mM NEAA + 1 mM sodium pyruvate + 100 units/mL penicillin + 10 μg/mL streptomycin), and cells were pretreated for 1 h with either vehicle (dimethyl sulfoxide [DMSO]) and compounds, followed by 1 h of treatment with 10 ng/mL TNF-α for 20 h. Unstimulated cells were used as a negative control (−); apigenin was used as a positive control. Dual luciferase assay was performed 48 h after transfection, and promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization.

Peroxisome proliferator response elements-luciferase assay

HepG2 were seeded at 1.5×10^5 cells per well in 12-well plates and grown for 24 h before transfection. An optimized amount of DNA plasmid (0.5 μg of PPRE-Luc and 0.2 μg of PPAR-inpCMV) was diluted in 100 μL of DMEM. All cells were transfected with the plasmid mixture using WelFect M Gold (WelGENE Inc.) as described by the manufacturer. After 30 min of incubation at room temperature, the DNA plasmid

solution (100 μL) was introduced and mixed gently with cells. After 24 h of transfection, the medium was changed to (transfection optimized medium, Invitrogen) containing 0.1 mM NEAA, 0.5% charcoal-stripped FBS, and the individual compounds (test group), DMSO (vehicle group), or rosiglitazone (positive control group). The cells were then cultured for 20 h. Next, the cells were washed with PBS and harvested with 1 × passive lysis buffer (200 μL). The intensity of emitted luminescence was determined using an LB 953 Autolumat (EG and G Berthold, Bad Wildbad, Germany).

Statistical analysis

All measurements were performed independently at least triplicate. Data were expressed as the mean ± standard deviation. Statistical significance is determined by one-way analysis of variance followed by Dunnett's multiple comparison test $P < 0.05$.

RESULTS AND DISCUSSION

In the current study, five anthraquinones (1–5), two torachrysones (6 and 7), four stilbene glycosides (8–11), two flavanols (12 and 13), and two sterols (14 and 15) were isolated from methanol extracts of *P. multiflorum* roots [Figure 1]. Their structures were elucidated by comparing spectroscopic data to published data. The compounds were identified as follows: Physcion (1),[23] emodin (2),[24] physcion-8-*O*-*β*-D-(6'-*O*-acetyl)-glucoside (3),[25] emodin- 8-*O*-*β*-D-glucoside (4),[26] physcion-8-*O*-*β*-D-glucoside (5),[25] torachrysone-8-*O*-*β*-D-glucoside (6),[27] torachrysone-8-*O*-*β*-D-glucoside-6'-*O*-gallate (7),[28] (*Z*)-2,3,5,4'-tetrahydroxystilbene-2-*O*-*β*-D-glucoside (8),[29] (*E*)-2,3,5,4'-tetrahydroxystilbene-2-*O*-*β*-D-glucoside (9),[29] (*E*)-2,3,5,4'-tetrahydroxystilbene-2-*O*-*β*-D-xyloside (10),[28] (*E*)-2,3,5,4'-tetrahydroxystilbene-2-*O*-*β*-D-(6'-*O*-acetyl)-glucoside (11),[28] (+)- catechin (12),[30] (+)-catechin-3-*O*-gallate (13),[30] *β*-sitosterol (14),[31] and *β*-sitosterol-3-*O*-*β*-D-glucoside (15).[31]

Compounds 1–15 were evaluated for cytotoxicity according to the manufacturer's instructions using a CCK-8 (Dojindo, Kumamoto, Japan) assay, as described in "Materials and Methods." Results indicated that compounds 1–15 caused no significant cytotoxicity in HepG2 cells at the tested concentrations [Figure 2].

HepG2 cells were treated with 10 ng/mL TNF-α, which resulted in increased transcriptional activity relative to untreated cells. Transfected HepG2 cells were pretreated with 0.1, 1, and 10 μM of each compound, followed by stimulation with TNF-α [Figure 3]. Apigenin was used as a positive control (IC_{50}: 1.64 ± 0.19). The anti-inflammatory activities of compounds 1–15 were evaluated by the inhibition of a TNF-α-induced NF-κB luciferase reporter in HepG2 cells. Compounds 1–5 and 14–15 significantly inhibited TNF-α-induced NF-κB transcriptional activity, with IC_{50} values of 30.25, 25.63, 37.56, 24.16, 25.71, 28.78, and 31.56 μM, respectively. In contrast, compound 8 exhibited weak inhibitory activity, with an IC_{50} value of 50.20 μM. We continuously investigated the effects of compounds 1–15 on PPAR activity using a nuclear transcription PPRE cell-reporter system. HepG2 cells were treatment with 0.1, 1,

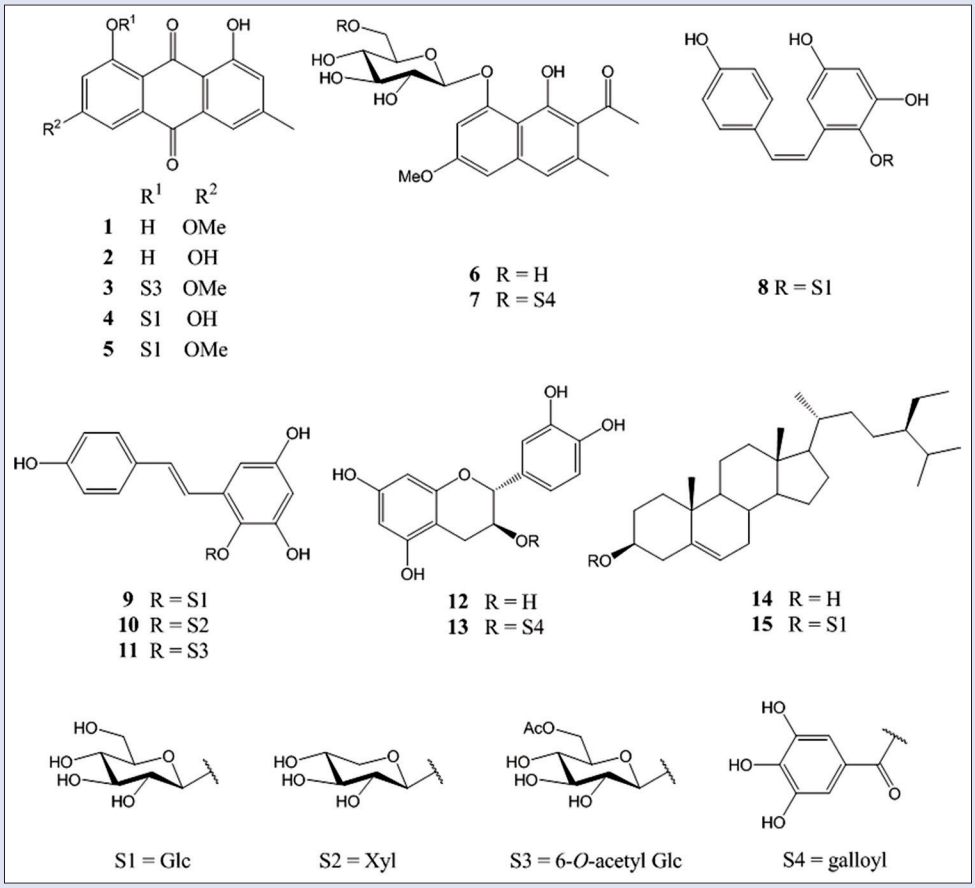


Figure 1: Structures of compounds 1–15 from the roots of *Polygonum multiflorum*

and 10 μM of compounds, The results showed that compounds 1–5 greatly enhanced PPARs transcriptional activity with EC_{50} values of 18.26–31.45 μM [Table 1], whereas compounds 6–15 were inactive (EC_{50} >50 μM).

Consistent with the structure–activity relationship of the isolated compounds (1–15), the anthraquinone derivatives (1–5) exerted significant effect inhibitory activities on NF-κB transcription, with IC_{50} values of 24.16–37.56 μM. In addition, the sterol derivatives (14 and 15) also displayed potent inhibitory activities, with IC_{50} values of 28.78 and 31.56 μM. These results indicate that the anthraquinone and sterol derivative components from *P. multiflorum* may play an important role in TNF-α induced NF-κB transcriptional activity. Compared with compounds 9–11, compound 8 is a cis-form stilbene glycoside that displays obvious NF-κB inhibitory activity at the same concentration, suggesting that all cis-form stilbene glycosides exhibit increased NF-κB transcriptional inhibitory activity. Moreover, the anthraquinone derivatives (1–5) exerted obvious activated PPAR transcriptional activity in a dose-dependent manner. Other compounds (6–15) did not exhibit significant activity. It suggests that anthraquinone derivatives from *P. multiflorum* are the active ingredient for activation of PPAR transcription. These results may be useful for determining the structure – function relationship of the useful components of *P. multiflorum.*

Table 1: PPARs transactivational activities of compounds 1-15

Compounds	EC_{50} [a] (μM)[b]
1	31.45±2.12
2	25.32±2.64
3	23.68±3.01
4	18.26±4.32
5	28.56±2.06
Rosiglitazone[c]	1.60±0.17

[a]EC_{50}: The concentration of a tested compound that gave 50% of the maximal reporter activity; [b]The values are mean±SD (n=3). Compounds 6-13 were inactive (EC_{50}>50 μM) at tested concentrations; [c]Rosiglitazone, positive control (10 μM). SD: Standard deviation; PPARs: Peroxisome proliferator-activated receptors

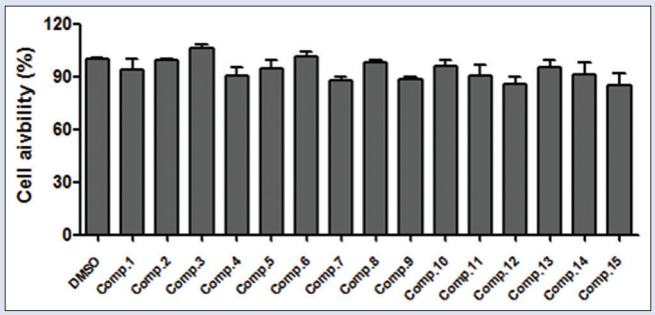


Figure 2: Cytotoxic effects of compounds 1–15 on human hepatoma cells all values are means ± standard deviation (n = 3,/P < 0.05). Dimethyl sulfoxide as control group. human hepatoma cells 2 were cultured overnight in 96-well plates and treated with 10 μM for 24 h. Cell viability was assessed using MTS assays. The results are expressed in terms of percentage relative cell viability

CONCLUSION

These results led us to conclude that fifteen compounds (1–15) isolated from the roots of *P. multiflorum* exert significant anti-inflammatory effects by inhibiting TNF-α induced NF-κB activation and PPARs transcription. Interestingly, the results of this study indicate that anthraquinone and sterol derivatives of *P. multiflorum* exhibit strong anti-inflammatory activities by inhibiting TNF-α induced NF-κB activation; anthraquinone derivatives from *P. multiflorum* are activated PPAR transcription. Therefore, we suggest that the roots of *P. multiflorum* can be used to treat natural inflammatory diseases. However, further studies on potential anti-inflammatory effects and benefits of anthraquinone, sterol, and stilbene derivative components from *P. multiflorum* are warranted.

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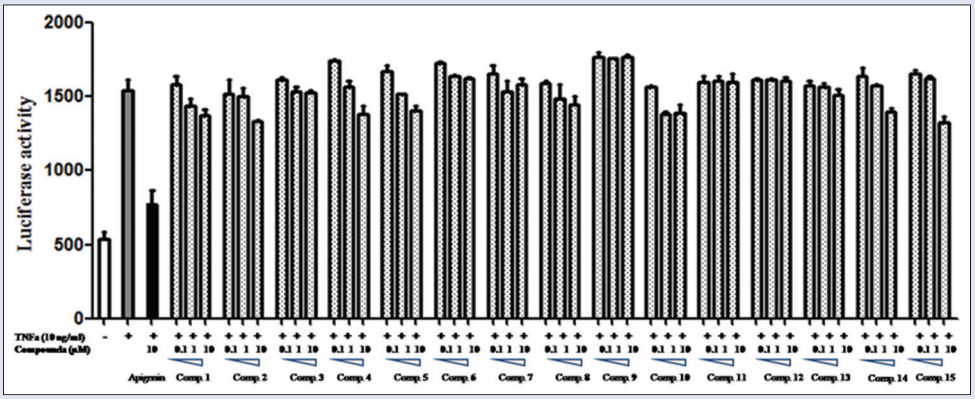


Figure 3: Effects of compounds 1 – 15 on the tumor necrosis factor-α induced nuclear factor kappa B-luciferase reporter activity in human hepatoma cells. The values are means ± standard deviations (n = 3). Apigenin, positive control (10 μM). Statistical significance is determined by one-way analysis of variance followed by Dunnett's multiple comparison test, P < 0.05 versus control

Conflicts of interest

There are no conflicts of interest.

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