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Anti-inflammatory Activity of New Compounds from *Andrographis paniculata* by NF-κB Transactivation Inhibition

WEN-WAN CHAO,[†] YUEH-HSIUNG KUO,*[,§,#] AND BI-FONG LIN*[,†]

[†]Department of Biochemical Science and Technology, and [§]Department of Chemistry, National Taiwan University, Taipei 10617, Taiwan, and [#]Tsuzuki Institute for Traditional Medicine, College of Pharmacy, China Medical University, Taichung 40227, Taiwan

Previous studies showed that the ethyl acetate (EtOAc) fraction of *Andrographis paniculata* (AP) possessed anti-inflammatory activity. This study further isolated these active compounds from bioactivity-guided chromatographic fractionation and identified eight pure compounds. Reporter gene assay indicated that 5-hydroxy-7,8-dimethoxyflavone (**1**), 5-hydroxy-7,8-dimethoxyflavanone (**2**), a mix of β-sitosterol (**3a**) and stigmasterol (**3b**), ergosterol peroxide (**4**), 14-deoxy-14,15-dehydroandrographolide (**5**), and a new compound, 19-*O*-acetyl-14-deoxy-11,12-didehydroandrographolide (**6a**), significantly inhibited the transcriptional activity of NF-κB in LPS/IFN-γ stimulated RAW 264.7 macrophages ($P < 0.05$). The two most abundant compounds, 14-deoxy-11,12-didehydroandrographolide (**7**) and andrographolide (**8**), had less inhibitory activity but exerted greater inhibitory activity by hydrogenation, oxidation, or acetylation to become four derived compounds, **9**, **10**, **11**, and **12**. All of the compounds significantly decreased TNF-α, IL-6, macrophage inflammatory protein-2 (MIP-2), and nitric oxide (NO) secretions from LPS/IFN-γ stimulated RAW 264.7 cells. Compounds **5**, **11**, and **12** exerted the strongest inhibitory effect on NF-κB-dependent transactivation in the RAW 264.7 cell, with IC_{50} values of 2, 2.2, and 2.4 μg/mL, respectively, providing encouraging results for bioactive compound development.

KEYWORDS: *Andrographis paniculata*; NF-κB; pro-inflammatory mediator; 19-*O*-acetyl-14-deoxy-11,12-didehydroandrographolide; macrophage inflammatory protein-2

INTRODUCTION

Andrographis paniculata (Burm. f.) Nees (Acanthaceae), known as Chuan-chin-lian in Chinese (AP), is widely used in Taiwan, China, India, and other Southeast Asian countries for treating infection, inflammation, common cold, fever, diarrhea, and liver disorders (*1*−*3*). Its major constituents, such as andrographolide, neoandrographolide, and 14-deoxy-11,12-didehydroandrographolide, have been studied for their anti-inflammatory and cardiovascular effects (*4*−*6*). Andrographolide, recognized as the most medicinally active phytochemical in AP, inhibits lipopolysaccharide (LPS)-induced NO production by suppressing inducible nitric oxide synthase (iNOS) (*4*) and nuclear factor kappa B (NF-κB) activation by blocking the binding of NF-κB oligonucleotide to nuclear proteins (*7*, *8*). A previous study has found that NF-κB-dependent luciferase reporter assay can be used as a prescreening tool to identify anti-inflammatory Chinese medicinal herbs (*9*). AP, one of the tested herbs screened by inhibition of NF-κB-dependent luciferase activity, also decreased ex-vivo NO and prostaglandin $(PGE)_2$ production by LPS plus interferon (IFN)-γ-stimulated peritoneal macrophages of mice (*10*).

Macrophages are one of the important immune cells involved in the innate immunity against microbial infection. When stimulated by pathogen- and host-derived molecules, such as LPS and IFN-γ, macrophages secrete inflammatory mediators and cytokines (*11*). One of the LPS-induced pathways involves NF-κB, which plays a role in the production of several constitutive cytokines such as TNF-α, IL-6, IL-12, MIP-2, and inflammation-related expression of the iNOS COX-2 genes. NF-κB signal transduction has become a major target in drug research (*12*). A previous study confirmed that the food components screened by in vitro cytokine secretions or NF-κB transactivation activity in murine macrophage cell line RAW 264.7 exert anti-inflammatory effect in an LPS-induced inflammatory murine model (*13*, *14*). The EtOAc extract of AP is also shown to inhibit LPS-induced acute inflammation in mice (*15*). The bioactive components are worthy of identification for future AP medicine study.

This study further isolated and identified the pure compounds from EtOAc fraction of AP and assayed their ability to inhibit NF-κB transactivation and pro-inflammatory mediators production in RAW 264.7 macrophages. The relationship between the structures of these compounds and the biological effect they elicit was also analyzed.

MATERIALS AND METHODS

General Experimental Procedures. NMR and mass spectrometry identified the chemical structures of the isolated active compounds. ^1H, ^{13}C, DEPT, and two-dimensional NMR spectra were acquired on a

*Authors to whom correspondence should be addressed [(B.-F.L.) telephone +886 2 3366 4451, fax +886 2 2362 1301, e-mail bifong@ntu.edu.tw; (Y.-H.K.) telephone +886 2 3366 1671, fax +886 2 2789 8654, e-mail yhkuo@ntu.edu.tw].

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Table 1. ^{13}C NMR and ^{1}H NMR Spectroscopic Data for Compound **6a** (100 MHz, 400 MHz in $CDCl_3$)

no.	δ_C	δ_H
1	38.2	1.12, 1.50 (m)[a]
2	27.6	1.63, 1.70 (m)
3	78.7	3.30 (dd, J = 4.4, 12.0 Hz)
4	42.2	
5	54.4	1.15 (m)[a]
6	23.2	1.80 (m)[a]
7	36.3	2.00, 2.40 (m)
8	147.4	
9	61.3	2.31 (d, J = 10.0 Hz)
10	38.4	
11	135.2	6.87 (dd, J = 10.0, 16.0 Hz)
12	120.7	6.10 (d, J = 16.0 Hz)
13	128.7	
14	142.6	7.15 (t, J = 2.1 Hz)
15	69.3	4.79 (d, J = 2.1 Hz)
16	171.7	
17	108.7	4.52 (s)
		4.77 (s)
18	22.2	1.14 (s)
19	64.7	4.13 (d, J = 11.6 Hz)
		4.33 (d, J = 11.6 Hz)
20	15.3	0.83 (s)
$CH_3\underline{C}OO-$	170.5	
$\underline{CH_3}COO-$	20.9	2.03 (s)

[a] Overlapped with each other.

Bruker DMX-400 spectrometer. ESI-MS and HREIMS were measured with JEOL Finnigan TSQ-46C and JEOL SX-102A mass spectrometers. Specific rotations were recorded on a JASCO DIP-1000 digital polarimeter, whereas IR spectra were recorded on a Perkin-Elmer 983 G spectrometer. The UV absorption was measured with a Hitachi S-3200 spectrometer. Extracts were initially fractionated on silica gel (Merck 70−230 mesh, 230−400 mesh, ASTM) and then purified with a semipreparative normal-phase HPLC column (250 × 10 mm Si, 5 μm, Purospher STAR) on an LDC Analytical-III system.

Plant Material. *A. paniculata* (Burm. f.) Nees (Acanthaceae) (AP) was purchased from a licensed Chinese herbal drug store in Taipei City and was authenticated (Sheng Chang Pharmaceutical, Co., Ltd., Taiwan) (*10*). The dried whole plant of AP (9 kg) was extracted with 95% ethanol (60 L) at room temperature for 2 weeks. After filtration, 95% ethanol was evaporated under vacuum to obtain a black syrup, which was suspended in water (1 L) and partitioned with EtOAc (1 L three times) to obtain EtOAc soluble layers.

Fractionation, Isolation, and Identification of Active Compounds from AP. The EtOAc-soluble fraction (316.9 g) was separated by silica gel column chromatography eluted by increasing the proportion of ethyl acetate (0−100% EtOAc) in *n*-hexane (Hex) and methanol in ethyl acetate (10−50% methanol) to give a total of 26 fractions. The 26 fractions were collected for bioassay-guided fractionation test by measuring their effect on NF-κB-dependent luciferase activity (*10*).

Compounds **1**, **2**, **3**, and **4** were isolated from active fractions (elution with 30−50% EtOAc/Hex). These were identified as 5-hydroxy-7,8-dimethoxyflavone (**1**) (0.1 g), 5-hydroxy-7,8-dimethoxyflavanone (**2**) (29 mg) (*16*), a mix (65.5 mg) of β-sitosterol (**3a**) and stigmasterol (**3b**) (*17*), and ergosterol peroxide (**4**) (10.2 g) (*18*) by comparison of their physical data with those reported in the literature. The following active fractions (elution with 50% EtOAc/Hex) afforded 14-deoxy-14,15-dehydroandrographolide (**5**) (1.18 g) (*19*) and a new compound, 19-*O*-acetyl-14-deoxy-11,12-didehydroandrographolide (**6a**) (89.4 mg). Two major components, 14-deoxy-11,12-didehydroandrographolide [eluted from 50% EtOAc/Hex) (**7**) (14.7 g) (*20*)] and andrographolide [eluted from 100% EtOAc) (**8**) (40.4 g) (*20*)], were purified by recrystallization.

New Compound Identification and Its Acetylation. The IR spectrum of compound **6a** also showed the presence of a hydroxyl group (3441 cm^{-1}) and an exocyclic double bond (891 cm^{-1}), as well as α,β-unsaturated γ-lactone and acetoxy (1736 cm^{-1}) groups. The ^{13}C NMR data (**Table 1**) of

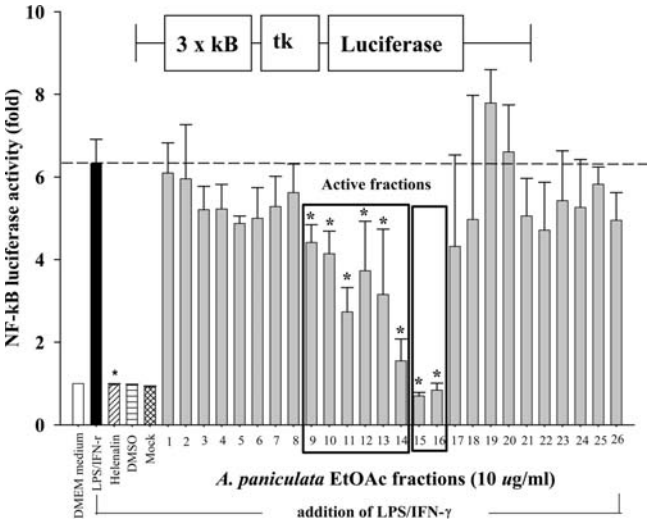

Figure 1. Bioactivity-guided chromatographic fractionation of EtOAc fractions of AP. RAW 264.7 macrophages transiently transfected with NF-κB reporter plasmids were pretreated with 26 fractions or helenalin (NF-κB inhibitor) for 1 h and then stimulated with LPS 100 ng/mL/IFN-γ 1000 units/mL for 8 h. NF-κB activity was estimated by the Dual-Glo Luciferase reporter assay. Data were the mean ± SD from three independent experiments. *, $P < 0.05$ versus LPS/IFN-γ treated group.

6a showed characteristic signals similar to those of **7**, including an exocyclic methylene at δ 147.4 and 108.7 (C-8 and C-17), a tertiary methyl at δ 22.2 (C-18), an acetoxymethylene at δ 64.7 (C-19), an angular methyl group at δ 20.9 (C-20), an α,β-unsaturated γ-lactone moiety at δ 128.7 (C-13), δ 142.6 (C-14), δ 69.3 (C-15), and δ 171.7 (C-16), and a pair of olefinic carbons at δ 135.2 (C-11) and δ 120.7 (C-12). The ^{1}H NMR signals (**Table 1**) [δ 2.03 (3H, s, CH_3COO-; 4.13, 4.33 (1H each, d, J = 11.6 Hz, H_2-19)] and ^{13}C NMR signals [δ 20.9 and 170.5 ($\underline{CH_3}\underline{C}OO-$)] revealed that the only difference between the two compounds was the presence of an additional acetyl group at C-19 in **6a**.

The chemical shifts and coupling patterns of five olefinic protons at δ 6.87 (1H, dd, J = 10.0, 16.0 Hz, H-11), 6.10 (1H, d, J = 16.0 Hz, H-12), 7.15 (1H, t, J = 2.1 Hz, H-14), and 4.52 and 4.77 (1H each, s, H-17) in **6a** were all similar to those of **7**. Thus, **6a** was identified as 19-*O*-acetyl-14-deoxy-11,12-didehydroandrographolide. It is colorless gum; $[\alpha]^{26}_D$ +5.2° (*c* 2.8, MeOH); UV (MeOH)λ_{max} nm (log ε) 249 (3.98); EIMS 70 eV m/z (relative intensity) 374 [M^+] (3), 314 (15), 296 (43), 121 (73), 91 (90), 133 (100); HREIMS m/z found 374.2104, calcd for $C_{22}H_{30}O_5$ 374.2088. The result was confirmed by treating **7** with Ac_2O in pyridine at room temperature for 1 h. After the usual workup, two components, **6a** (6.9 mg) and **6b** (72.6 mg), were isolated (*21*). Surprisingly, compound **6b** did not significantly inhibit NF-κB activity.

Synthesis of Analogues of 14-Deoxy-11,12-didehydroandrographolide (7) and Andrographolide (8). To study the structure−activity relationships (SAR), compounds **9**, **10**, **11**, and **12** were prepared from compounds **7** and **8**. The purification method and physical data of these derivatives are described below. Compound **7** (100 mg) dissolved in 30 mL of acetone with 10% Pd−C (15 mg) added was stirred under a hydrogen atmosphere for 1 h. After filtration and evaporation, the product was purified by HPLC with 50% EtOAc/Hex as the eluted solvent. The isolated products were a mix of hexahydro-14-dehydroxyandrographolide (**9**) (22.4 mg) and recovered **7** (70 mg). Via Jones oxidation in acetone, compound **7** yielded a new compound, 3,19-dioxolabda-8(17),11*E*,13-trien-16,15-olide (**10**).

Hexahydro-14-dehydroxyandrographolide (**9**; $C_{20}H_{34}O_4$). The signals from the major component were δ_H 0.75 and 1.18 (3H each, s, H_3-20, H_3-19), 0.82 (3H, d, J = 7.4 Hz, H_3-17), 2.40 (1H, m, H_3-13), 2.80 (2H, br s, −OH), 3.29, 4.14 (1H each, d, J = 11.1 Hz, H-19), 3.36 (1H, dd, J = 3.3, 10.9 Hz, H-3), 4.18 and 4.31 (1H each, m, H-15). The signals from the minor component were δ_H 0.68 and 1.16 (3H each, s, H-20, H-19), 0.83 (3H, d, J = 7.0 Hz, H-17), 2.40 (1H, m, H-13), 2.80 (2H, br s, −OH), 3.29, 4.14 (1H each, d, J = 11.1 Hz, H-19), 3.36 (1H, dd, J = 3.3, 10.9 Hz, H-3),

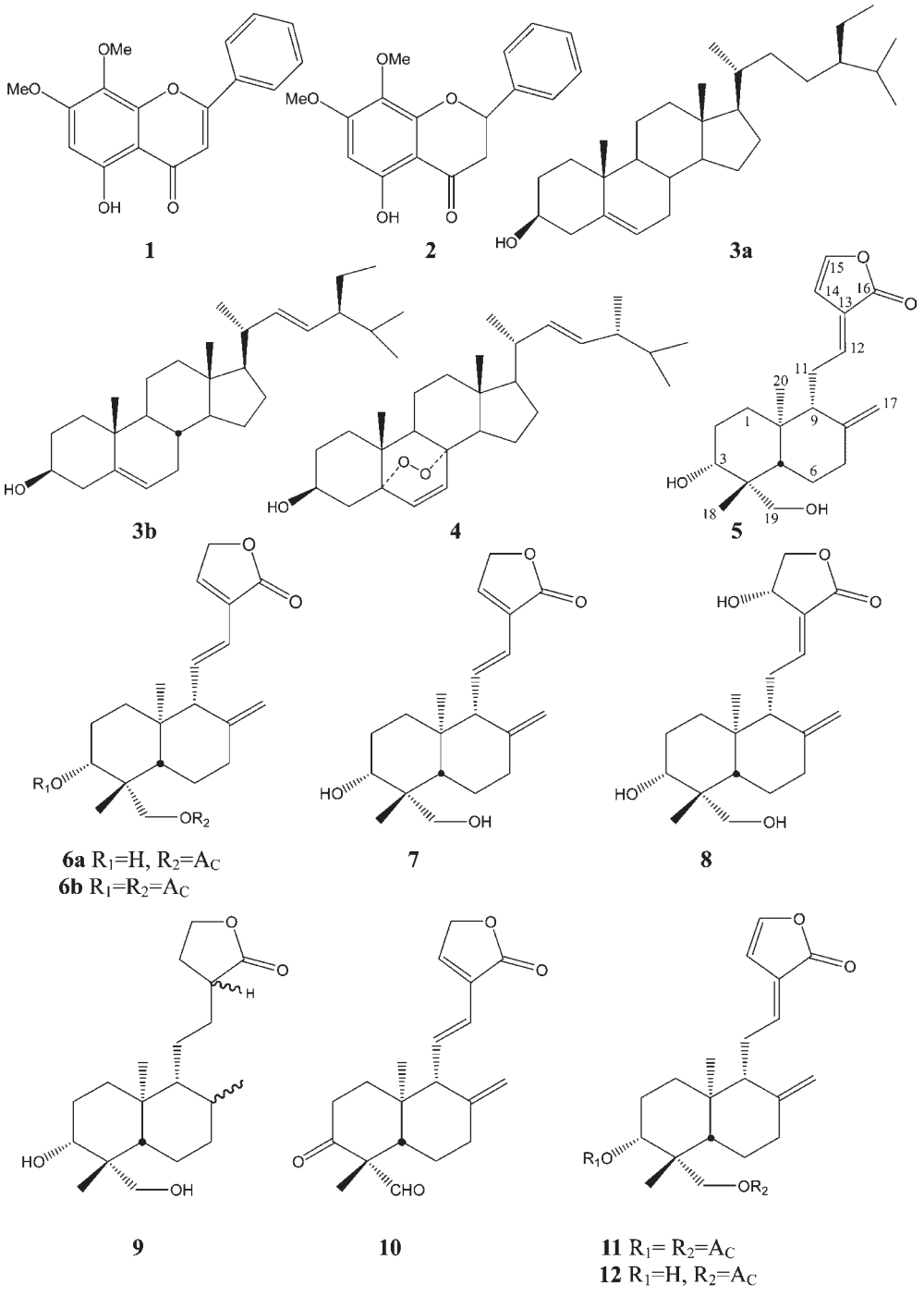

Figure 2. Chemical structures of compounds **1**−**12** isolated or synthesized from AP.

4.18 and 4.31 (1H each, m, H-15). Integration of the ^{1}H NMR signals revealed that the ratio of the two components was 3:1.

3,19-Dioxolabda-8(17),11E,13-trien-16,15-olide (***10***). **10** was obtained as an amorphous solid: $[\alpha]^{26}_D$ −0.28 (*c* 0.6, MeOH); UV (MeOH)λ_{max} nm (log ε) 250 (4.01); IR (KBr)ν_{max} 3050, 2729, 1735, 1725, 1719, 1650, 1204, 1049, 890 cm^{-1}; EIMS 70 eV *m*/*z* (relative intensity) 328 [M$^+$] (3), 296 (10), 109 (33), 73 (50), 59 (100); $C_{20}H_{26}O_4$; ^{1}H NMR spectrum ($CDCl_3$) δ_H 0.79 (3H, s, H_3-20), 1.27 (3H, s, H_3-18), 1.71 (1H, dd, J = 2.7, 12.9 Hz, H-5), 2.30 (1H, d, J = 11.0 Hz, H-9), 2.56 (1H, dt, J = 6.0, 15.7 Hz, H-2ax), 4.63, 4.87 (1H each, d, J = 1.2 Hz, H_2-17), 4.81 (2H, d, J = 1.6 Hz, H-15), 6.13 (1H, d, J = 16.1 Hz, H-12), 6.92 (1H, J = 16.1, 11.0 Hz, H-11), 7.17 (1H, t, J = 1.6 Hz, H-14), 9.69 (1H, s, H-19); ^{13}C NMR spectrum ($CDCl_3$) δ_C 38.9 (C-1), 35.9 (C-2), 208.9 (C-3), 56.5 (C-4), 56.7 (C-5), 23.9 (C-6), 36.9 (C-7), 146.5 (C-8), 60.4 (C-9), 39.2 (C-10), 134.8 (C-11), 121.9 (C-12), 128.9 (C-13), 143.2 (C-14), 69.6 (C-15), 172.2 (C-16), 110.6 (C-17), 17.3 (C-18), 203.1 (C-19), 13.9 (C-20).

Compound **8** (100 mg) was acetylated by the common method to yield 3,19-*O*-diacetylanhydroandrographolide (**11**) (57.4 mg) (*21*) and a new compound, 19-*O*-acetylanhydroandrographolide (**12**) (9 mg).

19-O-Acetylanhydroandrographolide (***12***). **12** was obtained as an amorphous solid: $[\alpha]^{26}_D$ −1.77 (*c* 1.18, MeOH); UV (MeOH)λ_{max} nm (log ε) 222 (3.98), 295 (3.90); IR (KBr)ν_{max} 3440, 3060, 1738, 1728, 1245, 1029, 890, 730, 600 cm^{-1}; EIMS 70 eV *m*/*z* (relative intensity) 374 [M$^+$] (2), 368 (32) 334 (100), 250(54); $C_{22}H_{30}O_5$; ^{1}H NMR spectrum ($CDCl_3$) δ 0.71 (3H, s, H_3-20), 1.13 (3H, s, H_3-18), 2.02 (3H, s, CH_3COO−), 3.31 (1H, dd, J = 4.0, 12.0 Hz, H-3), 4.09, 4.31 (1H each, d, J = 11.6 Hz, H_2-19), 4.40, 4.78 (1H each, s, H-17), 6.14, 6.97 (1H each, d, J = 3.5 Hz, H-14, H-15) and 6.66 (1H, t, J = 7.1 Hz, H-12); ^{13}C NMR spectrum ($CDCl_3$) δ 37.7 (C-1), 27.7 (C-2), 78.8 (C-3), 42.4 (C-4), 56.0 (C-5), 24.4 (C-6), 37.3 (C-7), 146.5 (C-8), 55.2 (C-9), 39.1 (C-10), 26.0 (C-11), 144.2 (C-12), 125.8 (C-13), 105 (C-14), 145.4 (C-15), 168 (C-16), 108.7 (C-17), 22.4 (C-18), 64.9 (C-19), 14.8 (C-20), 21.1 and 171.1 ($\underline{C}H_3\underline{C}OO$−).

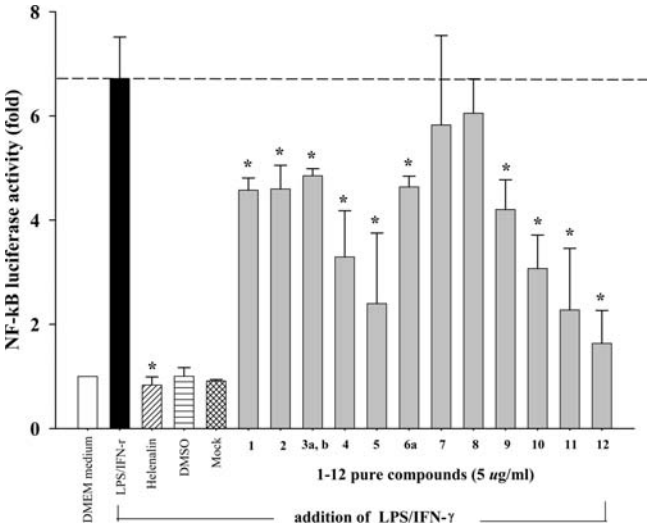

Figure 3. Inhibition of NF-κB-mediated transcription of the reporter gene by the eight compounds (**1**−**8**) isolated from *A. paniculata* and four products (**9**−**12**) synthesized from 14-deoxy-11,12-didehydroandrographolide (**7**) and andrographolide (**8**) in RAW 264.7 cells. RAW 264.7 cells transfected with NF-κB reporter plasmids were pretreated with 12 compounds or helenalin for 1 h and then stimulated with LPS/IFN-γ for 8 h. NF-κB activity was estimated by the Dual-Glo Luciferase reporter assay. Data were mean ± SD from three independent experiments. *, $P < 0.05$ versus LPS/IFN-γ-treated group.

NF-κB-Dependent Luciferase Activity Assay. The NF-κB-promoted luciferase reporter was used to assay the activity of NF-κB transactivation. A reporter plasmid, 3x-κB-tk-luc, had three copies of an NF-κB binding site in the upstream thymidine kinase promoter and a luciferase reporter gene in the pGL_2 vector (Mock) (Promega Corp., Madison, WI) (*22*). The RAW 264.7 cells were seeded on 24-well plates (Nunc, Roskilde, Denmark) at 5×10^4 cells/well in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (GIBCO, Grand Island, NY). The cells were cotransfected with 0.3 μg of 3x-κB-tk-luc reporter gene plasmid and 0.1 μg of Renilla luciferase reporter plasmid pRL-tk (Promega) for 48 h using the ExGen 500 in vitro transfection reagent (Fermentas, Hanover, MD).

After transfection, the cells were preincubated with various fractions or compounds from the AP EtOAc extract, the NF-κB-DNA binding inhibitor helenalin (10 μM, Calbiochem-Novabiochem Corp., San Diego, CA), or its solvent dimethyl sulfoxide (DMSO) for 1 h and then stimulated with LPS (100 ng/mL, Sigma, St. Louis, MO) plus IFN-γ (1000 units/mL, Sigma) for 8 h. The supernatant was collected for cytokine and NO assays. Luciferase level was analyzed using the Dual-Glo Luciferase reporter assay system (Promega) (*9*). There was no cytotoxicity of those fractions or compounds at the concentrations used in this study as confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide, (MTT, Sigma) assay for cell viability (*10*).

TNF-α, IL-6, and MIP-2 Measurements. The collected cell supernatants were assayed for TNF-α, IL-6, and MIP-2 productions using commercial ELISA kits (PharMingen, San Diego, CA; and R&D Systems, Inc., Minneapolis, MN). Cytokine concentration was measured according to the manufacturer's protocol.

NO Determination by Griess Assay. Griess reagent was freshly prepared from reagents A (1% sulfanilamide in 2.5% phosphoric acid) and B (0.1% *N*-1-naphthylethylenediamide dihydrochloride in 2.5% phosphoric acid) at a ratio of 1:1. Each liquor of 50 μL of cell supernatant was incubated with 50 μL of Griess reagent in a 96-well plate for 10 min, and the plate was read on an ELISA reader at 540 nm. The NO concentrations were determined using a standard curve (*23*).

Statistical Analysis. The data were expressed as mean ± SD. The significant difference compared to the control group was obtained by Student's *t* test using the SAS statistical software (SAS/STATA version 8.2; SAS Institute, Cary, NC). The correlation was analyzed by simple correlation of the SAS program. Differences were considered to be significant if P was < 0.05.

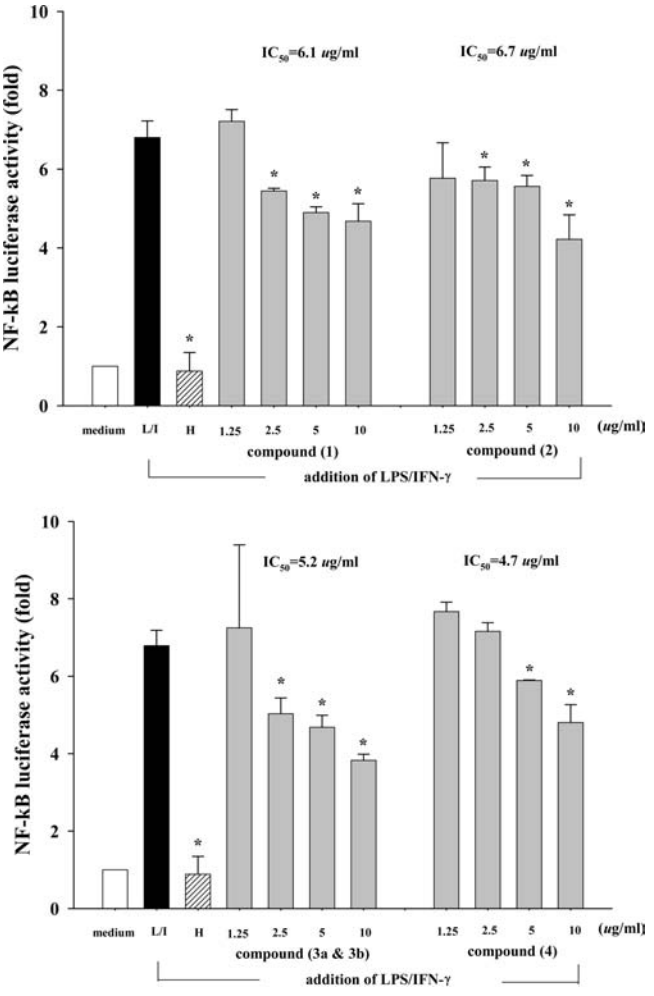

Figure 4. Effect of 5-hydroxy-7,8-dimethoxyflavone (**1**), 5-hydroxy-7,8-dimethoxyflavanone (**2**), β-sitosterol and stigmasterol (**3a** and **3b**, respectively), and ergosterol peroxide (**4**) on NF-κB-dependent luciferase reporter activity in RAW 264.7 cells activated by LPS/IFN-γ (L/I). RAW 264.7 cells transfected with NF-κB reporter plasmids were pretreated with **1**, **2**, **3**, and **4** or helenalin (H) for 1 h and then stimulated with L/I. NF-κB activity was estimated by the Dual-Glo Luciferase reporter assay. Data were mean ± SD from three independent experiments. *, $P < 0.05$ versus L/I-treated group.

RESULTS

Identification of Active Fractions from AP EtOAc Extract Using LPS/IFN-γ-Induced NF-κB Transcriptional Activation. The AP EtOAc extract subjected to silica column chromatography was eluted into 26 fractions according to polarity. There was no cytotoxicity of AP EtOAc fractions up to 10 μg/mL by cell viability (data not shown). Activation of RAW 264.7 cells with LPS/IFN-γ increased NF-κB transactivated luciferase activity (**Figure 1**). Helenalin, a NF-κB binding inhibitor, thoroughly suppressed reporter activity. No activation by DMSO or Mock indicated that these activations were not resulted from the sample's solvent and basic vector. Pretreatment of AP EtOAc fractions 9−16 significantly decreased LPS/IFN-γ-activated NF-κB-dependent luciferase activity ($P < 0.05$). Thus, fractions 9−14 were combined, as well as 15 and 16, for further fractionation and isolation of active pure compounds.

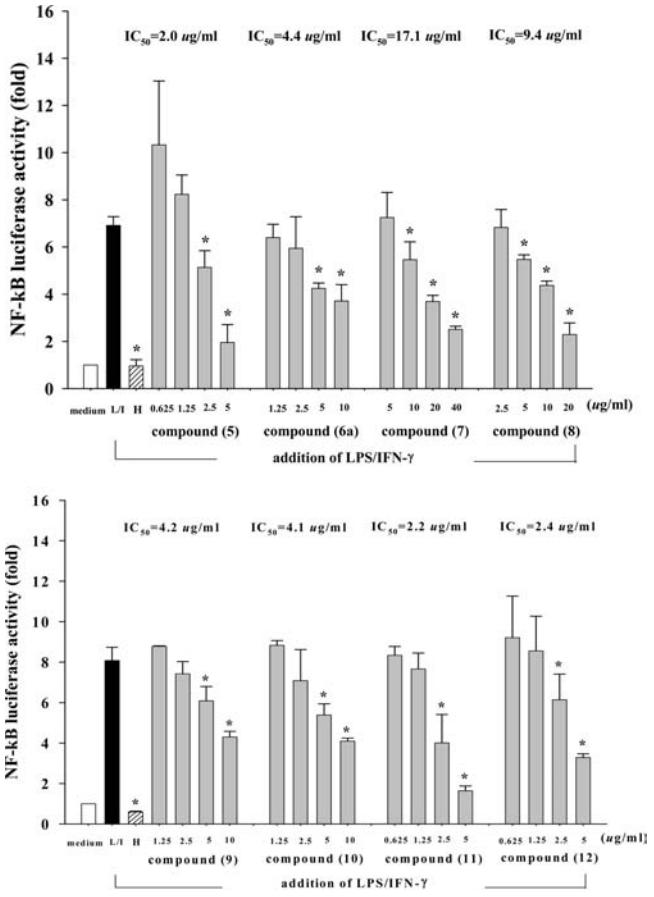

Figure 5. Effect of 14-deoxy-14,15-dehydroandrographolide (**5**), 19-*O*-acetyl-14-deoxy-11,12-didehydroandrographolide (**6a**), 14-deoxy-11,12-didehydroandrographolide (**7**), andrographolide (**8**), hexahydro-14-dehydroandrographolide (**9**), 3,19-dioxolabda-8(17),11*E*,13-trien-16,15-olide (**10**) 3,19-*O*-diacetylanhydroandrographolide (**11**), and 19-*O*-acetylanhydroandrographolide (**12**) on NF-κB dependent luciferase reporter activity in RAW 264.7 cells activated by LPS/IFN-γ. NF-κB activity was estimated by the Dual-Glo Luciferase reporter assay. Data were the mean $\pm$ SD from three independent experiments. $*$, $P < 0.05$ versus LPS/IFN-γ-treated group.

Identification of Pure Compounds 1−12 from AP EtOAc Extract and Their Effects on LPS/IFN-γ-Induced NF-κB Transcriptional Activation. The 12 pure compounds and their inhibitory effects are shown in **Figures 2** and **3**, respectively. The compounds 5-hydroxy-7,8-dimethoxyflavone (**1**), 5-hydroxy-7,8-dimethoxyflavanone (**2**), a mix composed of β-sitosterol (**3a**) and stigmasterol (**3b**), ergosterol peroxide (**4**), 14-deoxy-14,15-dehydroandrographolide (**5**), and a new compound, 19-*O*-acetyl-14-deoxy-11,12-didehydroandrographolide (**6a**), significantly inhibited NF-κB transcriptional activity ($P < 0.05$).

In contrast, 14-deoxy-11,12-didehydroandrographolide (**7**) and andrographolide (**8**), two major AP compounds, did not show significant inhibition. Further attempts to modify compounds **7** and **8** by hydrogenation, oxidation, or acetylation derived four compounds: hexahydro-14-dehydroxyandrographolide (**9**), 3,19-dioxolabda-8(17),11*E*,13-trien-16,15-olide (**10**), 3,19-*O*-diacetylanhydroandrographolide (**11**), and 19-*O*-acetylanhydroandrographolide (**12**) were also reported (**Figure 2**). Their inhibitory effects on NF-κB-dependent luciferase activity were greater than those of original compounds **7** and **8** (**Figure 3**).

To evaluate the anti-inflammatory activity, the pure compounds **1**−**12** were further tested at various concentrations. Compounds **1**, **2**, a mix of **3a** and **3b**, and **4** significantly inhibit NF-κB transcriptional activity (**Figure 4**). Compounds **1** and **2** with similar chemical structures also had similar IC_{50} values, 6.1 and 6.7 μg/mL, respectively. A mix of **3a**/**3b** and compound **4** with similar structure also showed similar IC_{50} values of 5.2 and 4.7 μg/mL, respectively.

14-Deoxy-14,15-dehydroandrographolide (**5**) and a new 19-*O*-acetyl-14-deoxy-11,12-didehydroandrographolide (**6a**) showed significant and greater inhibitory activities, with IC_{50} values of 2.0 and 4.4 μg/mL, respectively (**Figure 5**). The known major constituents of AP, compounds **7** and **8**, showed significant inhibitory effects at higher concentrations and, thus, higher IC_{50} values of 17.1 and 9.4 μg/mL, respectively. The synthesized compounds derived from **7**, compounds **9** and **10**, had IC_{50} values of 4.2 and 4.1 μg/mL, respectively. Other synthesized compounds derived from **8**, compounds **11** and **12**, showed greater inhibitory potential with lower IC_{50} values of 2.2 and 2.4 μg/mL, respectively.

Effects of Pure Compounds 1−12 on TNF-α, IL-6, MIP-2, and NO Secretions. To investigate the effects of pure compounds on pro-inflammatory mediators, the levels of TNF-α, IL-6, MIP-2, and NO secreted by LPS/IFN-γ-stimulated RAW 264.7 cells pretreated with compounds **1**−**12** were determined. The results showed that these pure compounds significantly decreased TNF-α, IL-6, MIP-2, and NO production in a dose-dependent manner (**Tables 2** and **3**), except for compounds **1** and **7** on IL-6. There were significant correlations between NF-κB-dependent luciferase activity and TNF-α ($r = 0.56$, $P < 0.05$), MIP-2 ($r = 0.75$, $P < 0.01$), and NO ($r = 0.69$, $P < 0.01$) productions. The IC_{50} values of each compound for inhibition of the pro-inflammatory cytokines were similar to those for NF-κB transcriptional activation (data not shown).

DISCUSSION

To investigate the potential of bioactive chemicals contained in natural health products as effective drug therapies, methods involving gene expression analysis, cell membrane chromatography, and trans-activated reporter gene assay have been developed and proposed as rapid throughput screening systems (*10*, *24*, *25*). NF-κB plays a pivotal role in the pathogenesis of inflammation, such that a variety of drugs designed to treat human inflammatory disease focus on inhibiting NF-κB activation (*12*). Many natural compounds or herbal extracts reportedly exhibit anti-inflammatory activities that generally involve NF-κB activation (*14*, *26*). Phytochemicals, especially flavonoids, are currently of interest because of their important biological and pharmacological properties, including inhibition of NF-κB activation (*27*, *28*).

TNF-α is the most important cytokine mediator linked to the pathogenesis of septic shock (*29*). TNF-α is a known potent cytokine for inducing MIP-2 (*30*), which is a heparin-binding chemokine also called CXCL2 that has binding sites for NF-κB within the regulatory region of gene (*31*). The correlation is the greatest in NF-κB transactivation and MIP-2 secretions among pro-inflammatory mediator test in this study. IL-6 is also an inflammatory cytokine mainly synthesized by macrophages that plays a role in the acute phase response (*32*), whereas NO is an important mediator of acute and chronic inflammation. Bacterial LPS acts on macrophages to release TNF-α, and the secreted TNF-α or LPS then stimulates cells to produce IL-6. The induction of these cytokines is also dependent on NF-κB activation. Overproduction of TNF-α and NO results in excessive inflammatory reactions deleterious to the human body (*33*). Therefore, inhibition of NO production is an important therapeutic target in the development of anti-inflammatory agents.

Table 2. Effects of Pure Compounds **1**−**6** Isolated from AP EtOAc Fraction on Pro-inflammatory Mediator Production from LPS/IFN-γ-Stimulated RAW 264.7 Macrophage Cells*

		pro-inflammatory mediators			
treatment		TNF-α (pg/mL)	IL-6 (ng/mL)	MIP-2 (ng/mL)	NO (μM)
DMEM medium only		nd[b]	0.24 ± 0.01	nd	2.32 ± 0.45
LPS/IFN-γ		968 ± 0	0.78 ± 0.04	1.15 ± 0.14	10.4 ± 0.88
helenalin (10 μM)		nd	0.29 ± 0.02*	nd	2.76 ± 0.81*
compound **1** (μg/mL)	1.25	1000 ± 13	0.85 ± 0.01	1.20 ± 0.25	9.05 ± 1.35
	2.5	800 ± 14	0.72 ± 0.09	0.82 ± 0.10	8.60 ± 1.48
	5	455 ± 50*	0.68 ± 0.04	0.43 ± 0.05*	6.95 ± 1.98
	10	300 ± 1*	0.52 ± 0.19	0.34 ± 0.02*	3.25 ± 0.35*
compound **2** (μg/mL)	1.25	994 ± 8	0.79 ± 0.13	1.02 ± 0.01	10.2 ± 1.20
	2.5	815 ± 21	0.70 ± 0.10	0.90 ± 0.11	8.00 ± 1.41
	5	510 ± 14*	0.65 ± 0.06	0.68 ± 0.10*	6.15 ± 2.94
	10	358 ± 60*	0.48 ± 0.25	0.53 ± 0.05*	4.00 ± 1.41*
compound **3a, b** (μg/mL)	1.25	1003 ± 4	0.90 ± 0.03	1.06 ± 0.09	10.5 ± 0.71
	2.5	910 ± 13	0.71 ± 0.07	0.91 ± 0.04	8.25 ± 1.27
	5	633 ± 47*	0.68 ± 0.06	0.69 ± 0.04*	6.00 ± 0.94*
	10	306 ± 8*	0.31 ± 0.04*	0.30 ± 0.00*	3.36 ± 0.59*
compound **4** (μg/mL)	1.25	1115 ± 6	0.79 ± 0.06	1.02 ± 0.04	10.5 ± 2.12
	2.5	808 ± 11	0.68 ± 0.02	0.90 ± 0.02	9.15 ± 0.71
	5	613 ± 3*	0.60 ± 0.11	0.61 ± 0.08*	5.82 ± 1.08*
	10	373 ± 36*	0.33 ± 0.03*	0.31 ± 0.01*	3.74 ± 0.37*
compound **5** (μg/mL)	0.625	983 ± 18	0.98 ± 0.05	1.25 ± 0.08	11.5 ± 0.08
	1.25	845 ± 53	0.69 ± 0.08	0.98 ± 0.16	8.90 ± 0.19
	2.5	410 ± 14*	0.35 ± 0.03*	0.35 ± 0.07*	3.81 ± 0.26*
	5	290 ± 57*	0.28 ± 0.01*	0.27 ± 0.04*	3.16 ± 0.23*
compound **6a** (μg/mL)	1.25	1005 ± 3	0.81 ± 0.01	1.08 ± 0.04	9.90 ± 0.14
	2.5	905 ± 21	0.62 ± 0.10	0.89 ± 0.06	8.05 ± 1.10
	5	585 ± 7*	0.40 ± 0.06*	0.49 ± 0.04*	4.09 ± 1.29*
	10	334 ± 26*	0.32 ± 0.10*	0.29 ± 0.04*	3.37 ± 0.90*

* Values are expressed as mean ± SD of three independent experiments performed in triplicates. *, $P < 0.05$ versus LPS/IFN-γ-treated group. [b] Not detectable.

Table 3. Effects of Pure Compounds **7**−**12** Isolated or Synthesized from AP EtOAc Fraction on Pro-inflammatory Mediators Produced by LPS/IFN-γ-Stimulated RAW 264.7 Macrophage Cells

		pro-inflammatory mediators			
treatment		TNF α (pg/mL)	IL-6 (ng/mL)	MIP-2 (ng/mL)	NO (μM)
DMEM medium only		nd[b]	0.24 ± 0.01	nd	2.32 ± 0.45
LPS/IFN-γ		968 ± 0	0.78 ± 0.04	1.15 ± 0.14	10.4 ± 0.88
helenalin (10 μM)		nd	0.29 ± 0.02*	nd	2.76 ± 0.81*
compound **7** (μg/mL)	5	995 ± 7	0.98 ± 0.01	1.30 ± 0.28	9.50 ± 0.71
	10	865 ± 35	0.80 ± 0.06	0.91 ± 0.01	8.52 ± 1.59
	20	702 ± 4*	0.72 ± 0.10	0.85 ± 0.04*	4.74 ± 1.78*
	40	425 ± 35*	0.65 ± 0.10	0.71 ± 0.01*	3.29 ± 1.00*
compound **8** (μg/mL)	2.5	990 ± 14	0.89 ± 0.05	1.05 ± 0.07	9.10 ± 0.14
	5	890 ± 14	0.68 ± 0.08	1.03 ± 0.04	8.90 ± 0.35
	10	734 ± 48*	0.60 ± 0.10	0.91 ± 0.02*	4.10 ± 0.69*
	20	329 ± 44*	0.49 ± 0.01*	0.45 ± 0.07*	3.06 ± 0.04*
compound **9** (μg/mL)	1.25	1004 ± 4	1.05 ± 0.06	1.15 ± 0.07	10.0 ± 0.04
	2.5	925 ± 64	0.80 ± 0.11	0.91 ± 0.01	8.90 ± 1.77
	5	595 ± 7*	0.68 ± 0.04	0.60 ± 0.01*	4.24 ± 1.08*
	10	325 ± 35*	0.38 ± 0.00*	0.31 ± 0.01*	3.84 ± 0.93*
compound **10** (μg/mL)	1.25	1130 ± 28	0.72 ± 0.02	1.01 ± 0.01	10.7 ± 1.14
	2.5	928 ± 39	0.59 ± 0.10	0.88 ± 0.02	9.25 ± 1.20
	5	775 ± 7*	0.42 ± 0.03*	0.67 ± 0.01*	5.21 ± 1.71*
	10	406 ± 8*	0.34 ± 0.01*	0.30 ± 0.01*	3.65 ± 0.92*
compound **11** (μg/mL)	0.625	978 ± 39	0.73 ± 0.10	0.90 ± 0.08	10.5 ± 0.66
	1.25	525 ± 36	0.61 ± 0.06	0.50 ± 0.02	8.95 ± 1.05*
	2.5	363 ± 18*	0.36 ± 0.06*	0.34 ± 0.06*	4.41 ± 1.54*
	5	287 ± 40*	0.28 ± 0.03*	0.31 ± 0.03*	3.89 ± 0.16*
compound **12** (μg/mL)	0.625	999 ± 2	0.92 ± 0.07	1.14 ± 0.17	9.27 ± 1.03
	1.25	690 ± 14	0.80 ± 0.09	0.96 ± 0.09	9.05 ± 1.68
	2.5	443 ± 60*	0.62 ± 0.12*	0.50 ± 0.06*	4.35 ± 1.44*
	5	302 ± 16*	0.32 ± 0.02*	0.33 ± 0.02*	3.75 ± 0.31*

* Values were expressed as mean ± SD of three independent experiments performed in triplicates. $P < 0.05$ versus LPS/IFN-γ-treated group. [b] Not detectable.

Because inflammatory responses are mainly controlled through regulation of NF-κB (*34*), this study shows that compounds **1**−**12** inhibit NF-κB-dependent transcription and also inhibit TNF-α, IL-6, MIP-2, and NO productions, suggesting potential in vivo anti-inflammatory effects (*14*, *15*). The aerial parts of plant are used to extract active phytochemicals. Previous studies on the chemical composition of AP showed a rich source of diterpenoids and flavonoids, including andrographolide, 14-deoxy-11,12-didehydroandrographolide (*20*), and 5-hydroxy-7, 8-dimethoxyflavone (*16*). Over the past 30 years, the active constituent of this herb, andrographolide, a diterpenoid lactone, has been used clinically in China. This drug is effective in relieving symptoms of fever and pain due to bacterial and viral infections and inflammation (*35*). Aside from NF-κB pathways used in this study, other possible mechanisms of the anti-inflammatory effects of andrographolide have been proposed to be activated protein kinase, or p38 MAPKs pathways (*36*, *37*).

This study identified eight components isolated from AP, which can be divided into three categories according to their chemical structures. Compounds **1** and **2** are flavonoids, **3a**, **3b**, and **4** are steroids, and **5**, **6a**, **7**, and **8** are diterpenoids (labdane-type) as are synthesized products **9**, **10**, **11**, and **12** derived from **7** and **8**. The IC_{50} values of the flavonoids are about 6.4 μg/mL, indicating that they are more effective inhibitors than **7** and **8**. However, the steroids exhibit the greatest inhibitory activity (IC_{50} values of approximately 5.0 μg/mL). The activity of compound **6a**, different from **7** with an additional acetyl group at C-19, is 4-fold greater than that of compound **7**.

Analysis of the results from the in vitro study indicates that compound **5** is the most efficacious inhibitor of NF-κB activity (IC_{50} = 2.0 μg/mL). In its structure, the only difference is the side chain with an α-alkylidene-β,γ-unsaturated-γ-lactone. Therefore, this side chain may play an essential functionality in inhibiting NF-κB luciferase activity. This finding is confirmed on the basis of the IC_{50} values of synthesized analogues of compounds **11** and **12**, two compounds that have activities similar to that of compound **5** and which contain the same α-alkylidene-β, γ-unsaturated-γ-lactone.

In conclusion, we have identified pure compounds of AP that exert anti-inflammatory effects not only on NF-κB-dependent luciferase activity but also on its downstream inflammatory mediators, including TNF-α, IL-6, MIP-2, and NO. Among these, 14-deoxy-14,15-dehydroandrographolide (**5**), 3,19-*O*-diacetylanhydroandrographolide (**11**), and 19-*O*-acetylanhydroandrographolide (**12**) possess the most potent inhibitory activity on NF-κB transactivation and inflammatory mediators. This study provides evidence that these compounds might be potential anti-inflammatory drugs that warrant further research and development.

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