Anti-inflammatory Activity of New Guaiane Type Sesquiterpene from *Wikstroemia indica*[1)]

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In our investigation of *in vitro* anti-allergic screening of medicinal herbal extracts, the ethyl acetate extract of the root of *Wikstroemia indica* was observed to inhibit nitric oxide (NO) production in a lipopolysaccharide (LPS) and recombinant mouse interferon-γ (IFN-γ) activated murine macrophage-like cell line, RAW 264.7. Fractionation of the active extract led to the isolation of one new guaiane type sesquiterpene, indicanone (1), and two known biflavonoids, sikokianin B (2) and sikokianin C (3). 1 inhibited NO production with IC_{50} values at 9.3 μM and also inhibit the inducible nitric oxide synhase (iNOS) gene expression. This is the first report of NO production inhibitory activity of *Wikstroemia indica* and supports the pharmacological use of it, which has been employed as an herbal medicine for the treatment of inflammation.

Key words *Wikstroemia indica*; Thymelaeaceae; guaiane; sesquiterpene; nitric oxide (NO); inducible nitric oxide synthase (iNOS)

Macrophages play major roles in host defense, immunity and inflammatory responses, once activated they result in pro-inflammatory cytokines, oxygen, and nitrogen species, which recruit activated immune and inflammatory cells to the site of lesion, thereby amplifying and perpetuating the inflammatory state. The inorganic free radical nitric oxide (NO) has been implicated in physiological and pathological processes such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation. NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). In the NOS family, inducible NOS (iNOS) in particular is involved in pathological overproduction of NO, and can be expressed in response to pro-inflammatory agents such as lipopolysaccharide (LPS) in various cell types including macrophages, endothelial cells, and smooth muscle cells. In inflammatory disease such as rheumatoid arthritis, excessive NO production by activated macrophages has been observed. Therefore, inhibition of iNOS activation and NO production may be of therapeutic benefit in various types of inflammation.[2,3)]

Wikstroemia indica (Thymelaeceae) is distributed in the southeast of China. It has long been used as a traditional crude drugs for the treatment of pneumonia, rheumatism, and bronchitis[4)] in China. Previous investigations of *E. lanulata* have yielded some flavonoids, lignans, and coumarins.[5—7)] However, no report on the isolation and characterization of anti-allergic constituents from this plant has been made.

The roots of *Wikstroemia indica* were extracted with 80% MeOH. The extract was then partitioned with hexane, ethyl acetate, *n*-butanol, and water, successively. The ethyl acetate fractions showed strong NO production inhibitory activity. Further bioassay-directed fractionation of this fraction led to the isolation of one new guaiane type sesquiterpene, Indicanone (**1**), and two known biflavonoids, sikokianin B (**2**), and sikokianin C (**3**). Here, we describe the isolation, structure elucidation and biological evaluation of these compounds.

1 was isolated as a colorless oil and assigned to possess a molecular formula of $C_{15}H_{20}O_2$ by HR-EI-MS ($[M]^+$, *m*/*z* 232.1464). Hydroxyl (3410 cm^{-1}) and unsaturated ketone (1665 cm^{-1}) absorptions were observed in the IR spectrum. The UV spectrum displayed an absorption maximum at 257 nm. The ^{13}C-NMR spectrum showed 15 carbon atoms which were classified as two methyls, six methylens, one methine, and six quaternary carbon atoms using distortionless enhancement by polarization transfer (DEPT) ^{13}C-NMR analysis. Furthermore, the ^{13}C-NMR chemical shifts suggested the presence of one carbonyl, one *exo*-methylene, two double bonds, one oxygen-bearing methylene. The detailed analysis of **1** using ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY) and heteronuclear multiplequantum coherence (HMQC) disclosed two partial structural units with correlated protons: CH_3–C=CH_2– (A) and –CH_2–CH–CH_2–CH_2– (B) (Table 1). In the heteronuclear multiplebond connectivity (HMBC) spectrum, the methyl proton signal at δ_H 1.79 (13-Me) was correlated with the carbon signals at δ_C 44.0 (C-7), 150.7 (C-11), and 109.8 (C-12) and the methine proton signal at δ_H 2.53 (7-H) with the carbon signals at δ_C 150.7 (C-11) (Table 1). These facts clearly indicated the combination of the partial structures A and B. Detailed analysis of **1** using HMBC spectrum showed two- and three-bond correlations between H-8 and C-10; between H-9 and C-1, 10, 14. In the NOESY spectrum, **1** showed significant NOE correlations between the oxygen-bearing methylene signal at δ_H 4.12 (H-14) and δ_H 2.92 (H-2). From these facts, the assignment at C-1, 2, 10, and 14 was confirmed. By the same way, HMBC correlations between H-6 and C-1, 4, and 5, between Me-15 and H-4 and H-5, and NOE correlations between Me-15 and

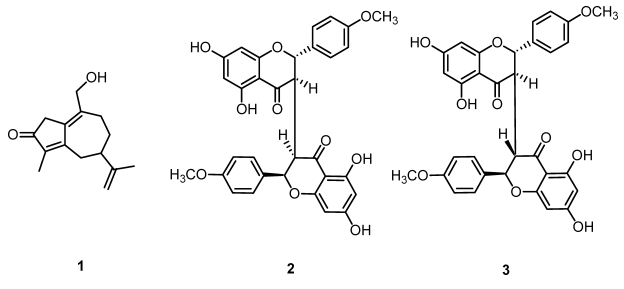

Chart 1. Structures of Compounds from *W. indica*

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Table 1. ^{1}H- and ^{13}C-NMR Data for Compound **1** (500 MHz, 125 MHz in $CDCl_3$)[a]

	δ_H	δ_C	^{1}H–^{1}H COSY	HMBC
1		133.5		
2	2.92 br s	39.3		C-1, 3, 4, 5, 10
3		203.6		
4		139.9		
5		167.2		
6	2.80 dd (15.4, 8.9)	34.0	H-7	C-1, 4, 5, 11
	2.86 dd (15.4, 4.5)			
7	2.53 m[b]	44.0	H-6, 8	C-5, 6, 9, 11
8	1.79 m	33.3	H-7, 9	G-6, 10, 11
	1.98 m			
9	2.50 ddd (17.1, 8.6, 3.1)	29.0	H-8	C-1, 7, 10, 14
	2.66 m[b]			
10		139.6		
11		150.7		
12	4.71 br s	109.8	H-13	C-7, 11, 13
	4.63 br s			
13	1.79 s	20.6	H-12	C-7, 11, 12
14	4.12 br s	65.2		C-1, 9, 10
15	1.71 s	8.4		C-3, 4, 5

a) Assignments confirmed by decoupling, ^{1}H–^{1}H COSY, HMQC, HMBC, and NOESY spectra. *b*) Overlapped signal.

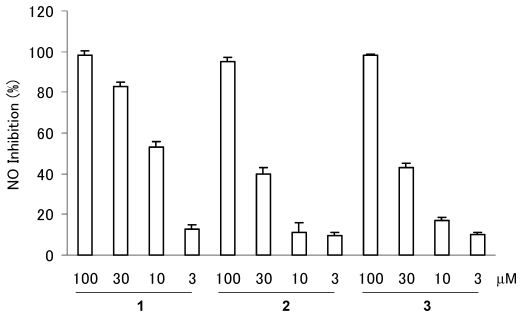

Fig. 1. Inhibitory Effect of Compounds **1**, **2**, and **3** on NO Production Stimulated by LPS and IFN-γ

RAW 264.7 cells were treated with LPS/IFN-γ alone or together with each compounds at concentrations indicated. After 16 h incubation, the supernatants were tested by Griess assay and the inhibitory rates were calculated. The experiment was performed four times and the data are expressed as mean±S.D. values.

H-6 firmly established the linkage of C-15, 4, 5, 6, and 1. The signal of C-2 appear at δ_C 39.3, due to the presence of a ketone group at C-3. Analysis of the HMBC spectrum (between Me-15 and C-3; between H-2 and C-3) established the connectivity of C-3 to C-2 and C-4. Thus, the structure of Indicanone was formulated as **1**. It is a new guaiane type sesquiterpene. Other proton correlations in the HMBC spectrum also supported this elucidation (Table 1).

2 and **3** were known compounds, whose structure were elucidated by comparisons with the literature.[8]

1 is thought to be an effective prevention agent on NO production for **1** showed stronger inhibition than quercetin on NO production (IC_{50}=9.3 μM). Quercetin exhibited a similar effect (IC_{50}=24.8 μM). Quercetin is reported to have an inhibitory effect on the production of NO by LPS stimulated macrophage cell RAW 264.7.[9,10] The cytotoxic effects of these compounds were measured with MTT assay. **1** (3—30 μM) didn't show any cytotoxic effect, but **2** and **3** (100 μM) demonstrated significant cytotoxicity with LPS/IFN-γ treatment for 24 h. When the concentrations of **2** and **3** were diluted to 30 μM, the cytotoxic effects were weakened, but the

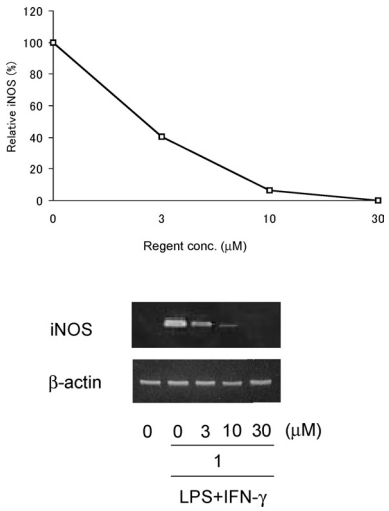

Fig. 2. Inhibitory Effect of Compound **1** on iNOS Expression Stimulated by LPS and IFN-γ

RAW 264.7 cells were stimulated with LPS/IFN-γ alone or together with compound **1** at concentrations indicated. The untreated and treated cells were collected and examined by RT-PCR. The experiment was repeated twice and a representative result is shown.

NO production inhibitory activities were also significantly weakened [inhibition (%) <50]. Thus **2** and **3** are not thought to be effective prevention agents on NO production. Furthermore, The RT-PCR analysis in the present study indicated that LPS/IFN-γ treatment increased the level of iNOS mRNA expression, and that guaiane type sesquiterpenes **1** inhibited this increase in a concentration-dependent manner (Fig. 2). On the basis of above-mentioned evidence, the active guaiane type sesquiterpene, **1** may be useful for the treatment of various inflammatory diseases.

Recently, Jin and coworkers have reported that some guaiane type sesquiterpenes significantly inhibited LPS-induced NF-κB activation.[11] NF-κB is an important transcription factor involved in the regulation of the expression of inflammatory NF-κB target genes such as TNF-α, iNOS, and COX-2,[12,13] This report is well in agreement with our findings suggesting **1** maybe inhibited iNOS gene expression through a NF-κB pathway.

Experimental

General Procedures The UV spectrum was obtained in MeOH on a Shimadzu UV-160 spectrophotometer, and the IR spectrum was recorded on a JASCO IR A-2 spectrophotometer. Optical rotations were measured in MeOH on a JASCO DIP-360 polarimeter. The NMR spectra were recorded on a JEOL GL-500 spectrometer, with TMS as an internal standard. The mass spectra (MS) were obtained on a JEOL GCmate spectrometer. Column chromatography was carried out with silica gel (Wako gel C-300, Wako Pure Chemical Industry Ltd.). Thin-layer chromatography (TLC) was performed on Merck TLC plates (0.25 mm thickness), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in ethanol solution and then heating on a hot plate. HPLC was performed on a JASCO PU-2089 apparatus equipped with a JASCO UV-2075. A Senshu Pak PEGASIL Silica 60-5 (10×250 mm i.d.) column and a Senshu Pak PEGASIL ODS 2 were used for preparative purposes.

Plant Materials The dried root of *W. indica* was collected in Guangdong Province, People's Republic of China, in October 1998 and was identified by Professor Weichun Wu (Department of Medical Plants, Shenyang Pharmaceutical University, People's Republic of China). Voucher specimens have been deposited at the Department of pharmacognosy, College of Pharmacy, Nihon University.

Extraction and Isolation The dried roots of *W. indica* (2.3 kg) were extracted twice with 80% methanol. Evaporation of the solvent under reduced

pressure from the combined extract gave the 80% MtOH extract 286 g (NO inhibitory effect 100 μg/ml, 38.2%). The extract was dissolved and suspended in water (2 l) and partitioned with hexane (3×2 l), ethyl acetate (3×2 l), and *n*-butanol (3×2 l). The amounts extracted were 6.5 g (76.1%), 62.0 g (86.9%), and 25.0 g (−6.4%), respectively, and the residual aqueous extract yielded 88.3 g (3.8%).

The ethyl acetate fraction was subjected to sephadex LH-20 column chromatography (13×20 cm, eluted with MeOH : H_2O 30 : 70—90 : 10). The column chromatographic fractions (200 ml each) were combined according to TLC monitoring into four portions. Fraction 2, was subjected to silica gel column chromatography (3×21 cm, eluted with chloroform and methanol in increasing polarity). The column chromatographic fractions (100 ml each) were combined according to TLC monitoring into eleven portions. Portion three was isolated and further purified by HPLC (Senshu pak PEGASIL ODS, 10×250 mm, MeOH : H_2O, 7 : 3) to give **1** (5.6 mg). Fraction 4, was subjected to silica gel column chromatography (3×21 cm, eluted with $CHCl_3$: MeOH 90 : 10—70 : 30). The column chromatographic fractions (100 ml each) were combined according to TLC monitoring into seven portions. Portion four was isolated and further purified by HPLC (Senshu pak PEGASIL ODS Al, 10×250 mm, MeOH : H_2O, 7 : 3) to give **2** (8.9 mg) and **3** (13.4 mg).

Indicanone (**1**): Colorless oil; $[\alpha]_D^{23}$: +14.3° (c=0.11, MeOH); UV (MeOH) λ_{max} (log ε) 257 (3.71), 205 (3.40); IR (KBr) ν_{max} 3410, 1665, 1407, 1370, 1038 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; HR-EI-MS *m/z*: 232.1464 (Calcd for $C_{15}H_{20}O_2$, 232.1463).

Inhibitory Activity on NO Production from Activated Macrophages-Like Cell Line, RAW 264.7 The cells were seeded at 1.2×10^6 cells/ml onto 96-well flat bottom plate (Sumitomo Bakelite, #8096R, Tokyo) and then incubated at 37 °C for 2 h. Next, the test extract was added to the culture simultaneously with both *Escherichia coil* LPS. (100 ng/ml) and recombinant mouse IFN-γ (0.33 ng/ml). Then cells were incubated at 37 °C for approximately 16 h and subsequently chilled on ice. One hundred microliters of the culture supernatant was placed in duplicate in the wells of 96-well flat-bottomed plates. A standard solution of $NaNO_2$ was placed in alternate wells on the same plate. To quantify nitrite, 50 μl of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-1-naphthyletylenediamide dihydrochloride) was added to each well. After 10 min the reaction products were colorimetrically quantified at 550 nm using a Model 3550 Microplate Reader (BIO-RAD) and the background absorbance (630 nm) was subtracted. Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method.

Reverse Transcriptase-Polymerase Chain Reaction Analysis of iNOS mRNA The cells were seeded at 1.2×10^6 cells/ml onto 96-well falt bottom plate and then incubated at 37 °C for 2 h. Then the test compound was added to the culture simultaneously with both LPS (100 ng/ml) and recombinant mouse IFN-γ (0.33 ng/ml). Then cells were incubated at 37 °C for approximately 8 h. Total RNA was isolated from the cell pellet using a RNA isolation kit (QIAGEN, Hilden, Germany). Total RNA (250 ng) was reverse-transcribed into cDNA by oligo $(dT)_{12-18}$ primer. The PCR samples contained 30 μl of the reaction mixture, comprised of 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM dNTPs, 0.6 units of Ampli Taq GOLD (Applied Biosystems, CA, U.S.A.), and 0.4 μM of sense and antisense primers. The sense primer for iNOS was 5′-ACCTACTTCCTGGACATTACGACCC-3′ and the antisense primer was 5′-AAGGGAGCAATGCCCGTACCAGGCC-3. The sense primer for β-actin was 5′-TGGATCCTGTGGCATCCATGAAAC-3′ and the antisense primer was 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′. The PCR reaction was performed under the following conditions: 25 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1.5 min, using a thermal cycler (GeneAmp PCR Systems 9700; PE Applied Biosystems, U.S.A.). The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. The bands in the gel were then photographed.

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References and Notes

1) Part 10 in the series Anti-allergic Agents from Natural Sources. Part 9: Daikonya A., Katuski S., Kitanaka S., *Chem. Pharm. Bull.*, **52**, 1326—1329 (2004).
2) Titheradge M. A., *Biochimica et Biophysica Acta*, **1411**, 437—455 (1999).
3) Nussler A. K., Billiar T. R., *J. Leukoc. Biol.*, **54**, 171—175 (1993).
4) "Zhong Hua Ben Cao," Vol. 5, Shanghai Xin Hua Press, Shanghai, 1999, pp. 425—426.
5) Lee K. H., Tagahara K., Suzuki H., Wu R. Y., Haruna M., Hall I. H., Huang H. C., Ito K., Iida T., Lai J. S., *J. Nat. Prod.*, **44**, 530—535 (1981).
6) Suzuki H., Lee K. H., Haruna M., Iida T., Ito K., Huang H. C., *Phytochemistry*, **21**, 1824—1825 (1982).
7) Hu K., Kobayashi H., Dong A. J., Iwasaki S., Yao X. S., *Planta Med.*, **66**, 564—567 (2000).
8) Nunome S., Ishiyama A., Kobayashi M., Otoguro K., Kiyohara H., Yamada H., Omura S., *Planta Med.*, **70**, 76—78 (2004).
9) Rao M. K., Ghosh B., *Int. J. Immunopharmacol.*, **21**, 435—443 (1999).
10) Kim H. K., Cheon B. S., Kim Y. H., Kim S. Y., Kim H. P., *Biochem. Pharmacol.*, **58**, 759—765 (1999).
11) Jin H. Z., Lee J. H., Lee D., Hong Y. S., Kim Y. H., Lee J. J., *Phytochemistry*, **65**, 2247—2253 (2004).
12) Wong H. R., Menendez I. Y., *Biochem. Biophys. Res. Commun.*, **262**, 375—380 (1999).
13) Pahl H. L., *Oncogene*, **18**, 6853—6866 (1999).