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Bioassay-coupled LC-QTOF MS/MS to Characterize Constituents Inhibiting Nitric Oxide Production of *Thuja orientalis*

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Abstract – The ethyl acetate fractions prepared from the leaves of *Thuja orientalis* significantly inhibited nitric oxide (NO) production in lipopolysaccharide-stimulated BV2 microglial cells. According to bioassay-coupled LC-QTOF MS/MS, the components near 22 and 25 mins in the mass chromatogram highly inhibited NO production and were expected to be labdane diterpenes, and the active components were characterized via further isolation. The results of the NO production inhibitory assay of the isolated compounds correlated well with the results of bioassay-coupled LC-QTOF MS/MS. Among the identified constituents, NO production inhibitory activities of 16-hydroxy-labda-8(17),13-diene-15,19-dioic acid butenolide (**2**) and 15-hydroxypinusolidic acid (**3**) were newly reported. Taken together, these results demonstrated that LC-QTOF MS/MS coupled with NO production inhibition assay was a powerful tool for accurately predicting new anti-inflammatory constituents in the extracts from natural products. Moreover, it provided a potential basis for broadening the application of bioassay-coupled LC-QTOF MS/MS in natural product research.

Keywords – *Thuja orientalis*, bioassay-coupled LC-QTOF MS/MS, NO production inhibitory activity, labdane diterpene

Introduction

Thuja orientalis belongs to the Cupressaceae family and is native to Korea and China. It is used as a hemostatic agent and expectorant in Korea and as a treatment for cough and pneumonia in China.[1,2] *T. orientalis* contains labdane diterpenes such as pinusolide and isopimaric acid and flavonoids like quercetin and amentoflavone. The extracts and constituents possess anti-cancer, anti-inflammatory, antiviral, and antibacterial effects.[1,3,4] The methylene chloride fractions of the leaves of *T. orientalis* inhibited the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p38 mitogen-activated protein kinase (MAPK) signaling in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.[5] Moreover, the aqueous semen extracts of *T. orientalis* inhibited inducible nitric oxide synthase (iNOS), nitric oxide (NO), prostaglandin E2, interleukin-1β, and cyclooxygenase-2 (COX-2) by blocking the Jun N-terminal kinase/p38 MAPK and NF-κB pathways in BV2 cells.[6] Additionally, 15-methoxypinusolide and hinokiol isolated from the leaves of *T. orientalis* exhibited anti-inflammatory activity in BV2 microglial and RAW 264.7 cells.[7,8]

When LC-MS analysis is linked to a bioassay, the chemical information and the activity of the sample are simultaneously revealed. Therefore, analytical techniques such as HPLC and LC-MS are coupled with a bioassay using enzyme, cell membrane, or target components and widely employed for the rapid identification of active constituents from natural products.[9-12] In a previous study, LC-MS coupled with a cell-based assay method was established and verified.[13] Park et al. discovered constituents from the ethyl acetate (EtOAc) fractions of *Catalpa ovata* that inhibited NO production in the BV2 microglial cells when LC-MS was coupled with the NO production inhibition assay.[13] The BV2 cells are closely related to neurodegenerative diseases such as Alzheimer's and Parkinson's disease because of the overproduction of inflammatory mediators such as NO, prostaglandin E, and interleukin-6 (IL-6) during tissue damage or infection.[14-16] NO, an inflammatory mediator, plays a protective role in cells when present in a low concentration. However, when NO is overexpressed by iNOS, it produces various

Dedicated to Prof. Jinwoong Kim of the Seoul National University for his pioneering works on Pharmacognosy.

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inflammatory mediators and causes inflammation and damage to the cells.[15,17-19] Therefore, the characterization of the components that prevent the overproduction of NO is a good target for the treatment of inflammatory diseases.

In this study, previously reported anti-inflammatory constituents were verified, and new active constituents were identified from *T. orientalis,* using LC-QTOF MS/MS coupled with NO production inhibitory assay before isolation of compounds.

Experimental

General experimental procedures – NMR spectra were recorded on the Avance Dpx300 or Avance III HD 600 MHz spectrometer (Bruker Corporation, USA) using chloroform-*d*, methanol-d_4, or dimethyl sulfoxide-d_6 solvents, which were purchased from Cambridge Isotope Laboratories, Inc (USA). LC-QTOF MS/MS spectra were obtained on the Agilent 1260 Infinity series system and Agilent 6530 Accurate-Mass Q-TOF mass spectrometer (Agilent, USA). The optical density (OD) was measured on the SpectraMax 190 Microplate Reader (Molecular Devices, USA). HPLC-grade solvents (water and acetonitrile) were purchased from Thermo Fisher Scientific Korea, Ltd. (Korea). DMEM was purchased from HyClone Laboratories, Inc (USA). Penicillin-streptomycin, trypsin-EDTA, and FBS were purchased from GIBCO Inc. (USA). Dexamethasone, dimethyl sulfoxide, and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich Co. (USA). The Griess reagent was prepared using 1% sulfanilamide (Sigma Aldrich, USA), 0.1% naphthylethylenediamine dihydrochloride (Sigma Aldrich, USA), and 2% phosphoric acid (Wako Pure Chemical Industries Ltd., Japan). The WST-1 solution (EZ-cytox) was purchased from DAEIL LAB SERVICE Co. Ltd. (Korea).

Plant material – The leaves of *T. orientalis* were purchased from an oriental herbal market (Dongwoodang Pharmacy Co., Ltd., Yeongchen, Gyeongbuk, Republic of Korea). After identification by Dr. Ki Yong Lee, a professor at the College of Pharmacy, Korea University (Sejong, Republic of Korea), voucher specimens (KUP-HD025) were deposited at the Laboratory of Pharmacognosy, College of Pharmacy, Korea University.

Coupling of LC-QTOF MS/MS with NO production inhibition assay – LC-QTOF MS/MS was coupled with NO production inhibition assay and two runs were performed using a previously described method.[13] The chemical profile of the EtOAc fraction of *T. orientalis* was obtained by LC-QTOF MS/MS in the first run. In the second run, the bioassay was performed for the eluents collected at intervals of 30 s. LC-QTOF MS/MS analysis was performed on an Agilent 1260 series with an Agilent 6530 QTOF mass spectrometer. The LC system was equipped with a binary pump, an auto plate sampler, and a photodiode array detector. The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The mass spectrometer was equipped with the electrospray ionization (ESI) interface operating in the negative ion mode. Mass data were collected from m/z 50 to 1700, and MS/MS fragmentation was performed with collision energies of 10, 20, and 30 eV. The separation of the EtOAc fraction of *T. orientalis* was performed on a Kinetex C18 column (5 μm, 4.6 × 150 mm, Phenomenex) with binary gradient solvent conditions as follows: 5% B at 0-5 min and 5-95% B at 5-30 min. For identifying the activity using the bioassay, 20 μL of the sample (25 mg/mL) was injected into the LC-QTOF MS/MS, and 5 μL of the sample (1 mg/mL) was injected for chemical profiling. The effluent of the separation was manually collected in each well of a 96-well plate at intervals of 30 s each. Each well was then dried in a vacuum-dry oven (JEIO Tech, OV-12, Korea) at 40 ºC and dissolved in serum-free DMEM to evaluate the inhibition of NO production inhibition.

Extraction and isolation – The dried leaves of *T. orientalis* (3.1 kg) were extracted thrice with 80% MeOH for 60 min, yielding the total extract (327.3 g). The total extract was dissolved in water and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH. The EtOAc fraction (103.79 g) was subjected to silica gel column chromatography and eluted with *n*-hexane/EtOAc/MeOH (1:0:0 → 0:1:2) to yield 13 subfractions (E1–E13). E4 was subjected to silica gel column chromatography and eluted with *n*-hexane/EtOAc (1:0 → 0:1) to yield five subfractions (E4-1–E4-5). Among them, E4-4 was subjected to medium pressure liquid chromatography (MPLC) over silica gel using an Isolera One system (Biotage, USA) and eluted with CH_2Cl_2/MeOH (20:1 → 0:1) to yield six subfractions (E4-4-1–E4-4-6). Compound **2** (1.1 mg) was purified from E4-4-4 by RP C_{18} semi-preparative HPLC and eluted with 0.1% formic acid in water/0.1% formic acid in acetonitrile (ACN) (2.5:7.5 → 0.5:9.5, gradient, flow rate 1 mL/min). Based on the LC-QTOF MS/MS chromatograms of the EtOAc fraction, E9 was subjected to silica gel column chromatography and eluted with *n*-hexane/acetone (20:1 → 5:1) to yield 19 subfractions (E9-1–E9-19). E9-13 was subjected to MPLC over RP C_{18} silica gel and eluted with MeOH/water (1:16 → 1:0) to yield 18 subfractions (E9-13-1–E9-13-18). Compounds **1** (3.8 mg), **3** (8.1 mg), and **4** (10.9 mg) were purified from

E9-13-5 by RP C_{18} semi-preparative HPLC and eluted with 0.1% formic acid in water/0.1% formic acid in ACN (3:7 → 0.5:9.5, gradient, flow rate 1 mL/min). Compound **5** (6.0 mg) was purified from E9-13-6 by recrystallization. E12 was subjected to silica gel column chromatography and eluted with $CHCl_3$/MeOH/water (200:4:1 → 6:5:1) to yield 20 subfractions (E12-1–E12-20). Compounds **6** (106.4 mg) and **7** (8.5 mg) were obtained from single spots of E12-13 and E12-17 on the TLC plate respectively. E13 was subjected to silica gel column chromatography and eluted with $CHCl_3$/MeOH/water (100:4:1 → 6:5:1) to yield 20 subfractions (E13-1–E13-20). Compound **8** (44.0 mg) was purified from E13-17 using Sephadex LH-20 and eluted with MeOH. Compound **9** (2.7 mg) was obtained from E13-16 by recrystallization.

Cell culture – BV2 mouse microglial cells were obtained from the College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, Seoul, Korea. The cells were cultured in DMEM containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin and incubated at 37ºC under conditions of continuous 5% CO_2/95% air.

NO production inhibition activity – The NO production inhibition assay was conducted using a previously described method.[13] BV2 cells were seeded in 96-well plates (2×10^5 cells/mL) and incubated for 24 h. The medium of each well was replaced with serum-free DMEM, and samples were treated at different concentrations for 1 h. Cells were then treated with LPS (100 ng/mL) to induce NO production. After 24 h, the medium was collected, and the amount of NO was measured using the Griess reagent. The NO production inhibitory activity was expressed as (OD of LPS-treated cultures – OD of LPS-sample treated cultures)/(OD of LPS treated cultures – OD of control cultures) × 100. After evaluating the NO production inhibitory activity, cell viability was measured by the WST-1 assay and was expressed as (OD of LPS-sample treated cultures)/(OD of control cultures) × 100.

Results and Discussion

The NO production inhibition assay was conducted on the 80% MeOH extracts and each fraction of the leaves of *T. orientalis* using LPS-stimulated BV2 cells to select the sample for bioassay-coupled LC-QTOF MS/MS. The EtOAc fraction of *T. orientalis* leaves significantly attenuated NO production without affecting cell viability (Table 1).

To characterize the constituents inhibiting NO production from the EtOAc fraction of *T. orientalis*, LC-QTOF MS/MS was coupled with the NO production

Table 1. NO production inhibitory activity from extracts and fractions of *T. orientalis*

		NO inhibition (%)[c]	Viability (%)
Control		100.0 ± 0.2	100.0 ± 1.5
LPS (100 ng/mL)[a]		0.0 ± 4.4	91.8 ± 2.7
Dex (10 μg/mL)[b]		98.1 ± 0.7 ***	101.1 ± 3.7
80% MeOH extracts	1 μg/mL	2.6 ± 0.2	100.9 ± 2.4
	5 μg/mL	8.9 ± 5.0	104.8 ± 3.3
	10 μg/mL	12.0 ± 4.5	98.8 ± 1.3
n-hexane fraction	1 μg/mL	11.8 ± 1.4	105.5 ± 2.4
	5 μg/mL	76.0 ± 2.4 **	101.0 ± 2.5
	10 μg/mL	94.0 ± 3.6 ***	81.7 ± 6.4
EtOAc fraction	1 μg/mL	8.4 ± 2.0 *	96.3 ± 2.7
	5 μg/mL	35.5 ± 2.1 ***	102.2 ± 1.3
	10 μg/mL	87.3 ± 1.8 ***	105.6 ± 0.4
n-BuOH fraction	1 μg/mL	1.3 ± 0.9	97.5 ± 2.1
	5 μg/mL	0.3 ± 4.3	95.3 ± 4.2
	10 μg/mL	1.0 ± 0.6	99.1 ± 1.3
Aqueous fraction	1 μg/mL	2.8 ± 2.3	97.2 ± 3.0
	5 μg/mL	6.2 ± 2.8	101.5 ± 2.9
	10 μg/mL	17.1 ± 3.2 **	100.0 ± 0.7

[a]Negative control: LPS (100 ng/mL)
[b]Positive control: dexamethasone (10 μg/mL)
[c]NO production inhibition was expressed as a percentage compared to the LPS group (mean ± SD, n=6).
*p<0.05, **p<0.01, ***p<0.001

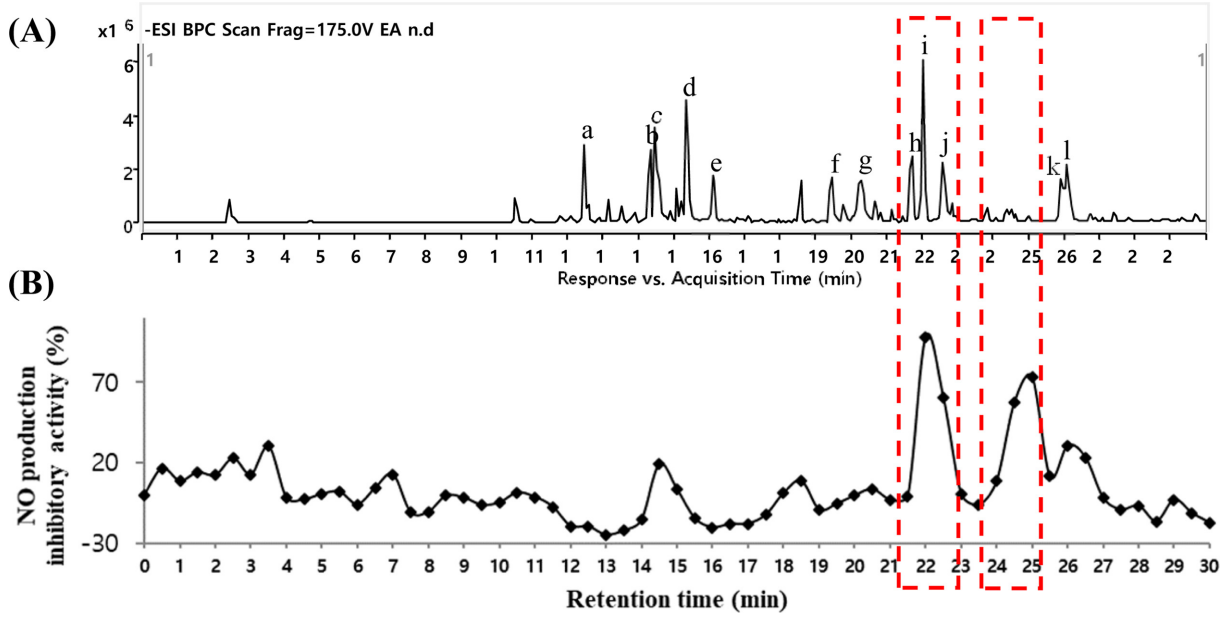


Fig 1. Coupling LC-QTOF MS/MS with NO production inhibition assay of the EtOAc fraction of *T. orientalis*. [A] Mass chromatogram in the negative mode. [B] NO production inhibitory activity of eluents collected at 30 s intervals.

Table 2. Chemical profile of the EtOAc fraction of *T. orientalis*

Peak No	Expected compounds	t_R (min)	Observed *m/z*	Calculated *m/z*	Molecular formula $[M-H]^-$	MS/MS fragments (*m/z*)	UV (λ_{max}, nm)	Isolated compound No
a	Unidentified	12.414	289.0715	289.0738	$C_{15}H_{13}O_6$	245 $[M-C_2H_4O-H]^-$		
b	Unidentified	14.289	509.2027	509.2028	$C_{25}H_{33}O_{11}$	473 $[M-36-H]^-$		
c	Myricitrin	14.414	463.0878	463.0882	$C_{21}H_{19}O_{12}$	316 $[M-C_6H_{11}O_5-H]^-$	260, 352	**8**
	5,7,8,3',4'-Pentahydroxyflavone 7-*O*-β-D-xylopyranoside	15.093	433.0826	433.0776	$C_{20}H_{18}O_{11}$	301 $[M-C_5H_8O_4-H]^-$		**9**
d	Quercetin	15.289	447.0930	447.0933	$C_{21}H_{19}O_{11}$	301 $[M-C_6H_{10}O_4-H]^-$	258, 349	**7**
e	Afzelin	16.038	431.0980	431.0984	$C_{21}H_{19}O_{10}$	285 $[M-C_6H_{10}O_4-H]^-$	265, 347	**6**
f	Biflavone	19.475	537.0823	537.0827	$C_{30}H_{17}O_{10}$	375 $[M-C_6H_{10}O-H]^-$	269, 333	
g	Biflavone	20.225	537.0823	537.0827	$C_{30}H_{17}O_{10}$	375 $[M-C_6H_{10}O-H]^-$	268, 330	
h	16-Hydroxy-8(17),13-labdadien-15,16-olid-19-oic acid	21.662	347.2747	347.1864	$C_{20}H_{27}O_5$	303$[M-CO_2-H]^-$	220	**2**
i	15-Hydroxypinusolidic acid	22.166	347.2749	347.1864	$C_{20}H_{27}O_5$	303$[M-CO_2-H]^-$	220	**3**
	Acacetin	22.654	283.0632	283.1612	$C_{16}H_{12}O_5$	268 $[M-15-H]^-$	219, 338	**5**
j	Biflavone	22.791	551.2108	347.1864	$C_{20}H_{27}O_5$		267, 332	
	Pinusolidic acid	23.841	331.1863	331.1915	$C_{20}H_{27}O_4$	294 $[M-37-H]^-$		**1**
	15-Methoxypinusolidic acid	25.029	361.1972	361.2020	$C_{21}H_{29}O_5$	283 $[M-78-H]^-$		**4**
k	Labdane diterpene	25.849	317.2118	317.2122	$C_{20}H_{29}O_3$			
l	Labdane diterpene	26.037	317.2118	317.2122	$C_{20}H_{29}O_3$	299$[M-H_2O-H]^-$		

inhibition assay (Fig. 1). The mass chromatogram of the EtOAc fraction was obtained in the negative ionization mode, and the structure of each peak was inspected by MS, UV, and MS/MS spectra (Fig. 1A and Table 2). Fig. 1B shows the inhibitory effects of the separated eluent on NO production. The components near 22 and 25 min on the mass chromatogram highly inhibited NO production. They were assumed to be labdane diterpenes (Table 2).

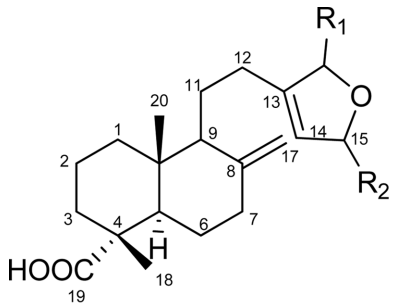
Four labdane diterpenes including compounds (**1-4**) and five flavonoids were isolated from the EtOAc fraction

Table 3. NO production inhibitory activity of isolated compounds **1-9** from *T. orientalis extracts*

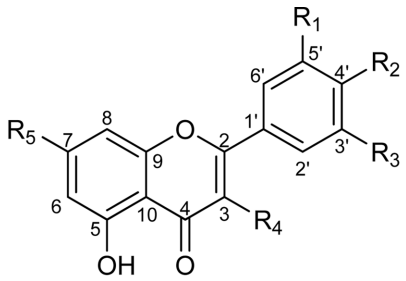
Compound		NO inhibition (%)[c]	Viability (%)
Control		100.0 ± 0.4	100.0 ± 2.0
LPS 100 ng/ml[a]		0.0 ± 2.3	104.0 ± 3.3
Dex 10 μM[b]		67.0 ± 2.2 ***	106.9 ± 2.8
1	1 μM	-23.5 ± 3.5	99.8 ± 3.2
	3 μM	-17.5 ± 9.2 **	99.5 ± 2.2
	5 μM	-12.4 ± 0.3 *	102.2 ± 1.9
	15 μM	3.3 ± 0.1	104.0 ± 2.5
2	1 μM	66.0 ± 0.7 ***	98.3 ± 1.9
	3 μM	86.8 ± 1.5 ***	99.3 ± 4.1
	5 μM	96.5 ± 0.6 ***	99.5 ± 3.0
	15 μM	102.5 ± 0.2 ***	106.7 ± 0.7
3	1 μM	-1.0 ± 2.6	101.8 ± 0.8
	3 μM	22.6 ± 2.1 **	97.1 ± 2.2
	5 μM	67.8 ± 4.0 **	97.1 ± 1.3
	15 μM	100.3 ± 1.0 ***	93.9±1.6
4	1 μM	-20.4 ± 0.4**	99.7 ± 2.7
	3 μM	24.5 ± 4.2*	103.2 ± 3.3
	5 μM	78.9 ± 3.1***	97.0 ± 2.4
	15 μM	99.3 ± 1.4***	95.3 ± 2.5
5	1 μM	-11.0 ± 0.8 **	101.0 ± 1.8
	3 μM	-14.1 ± 3.7	100.2 ± 1.9
	5 μM	-7.1 ± 1.7 ***	98.8 ± 4.8
	15 μM	25.8 ± 3.5 ***	94.2 ± 4.9
6	1 μM	-21.4 ± 0.6 *	92.0 ± 2.0
	3 μM	-25.8 ± 0.7	96.7 ± 2.5
	5 μM	-22.9 ± 8.9	96.6 ± 2.5
	15 μM	-7.7 ± 0.5 **	93.1 ± 2.1
7	1 μM	-30.1 ± 3.1 **	93.6 ± 4.3
	3 μM	-13.5 ± 7.7	100.6 ± 3.3
	5 μM	-1.3 ± 5.4	93.1 ± 3.8
	15 μM	4.2 ± 5.1	95.9 ± 0.1
8	1 μM	0.3 ± 3.8*	100.5 ± 2.4
	3 μM	0.7 ± 1.9	101.8 ± 2.8
	5 μM	2.3 ± 2.9	102.8 ± 4.2
	15 μM	23.3 ± 3.9 ***	101.5 ± 1.4
9	1 μM	-0.9 ± 3.7	102.2 ± 3.0
	3 μM	-0.8 ± 5.4	105.4 ± 5.1
	5 μM	3.7 ± 2.7	102.3 ± 4.9
	15 μM	8.2 ± 1.9	105.4 ± 5.3

[a]Negative control: LPS (100 ng/mL)
[b]Positive control: dexamethasone (10 μM)
[c]NO production inhibition was expressed as a percentage compared to the LPS group (mean ± SD, n=6).
*p<0.05, **p<0.01, ***p<0.001

of *T. orientalis* (Fig. 2) to confirm their biological activity. After comparing the spectroscopic data of the isolated compounds with the reference spectra, they were identified as pinusolidic acid (**1**),[20,21] 16-hydroxy-labda-8(17),13-diene-15,19-dioic acid butenolide (**2**),[3,20] 15-hydroxypinusolidic acid (**3**),[3,22] 15-methoxypinusolidic



1 R_1 = O R_2 = H
2 R_1 = OH R_2 = O
3 R_1 = O R_2 = OH
4 R_1 = O R_2 = OCH_3



5 R_1 = H R_2 = OCH_3 R_3 = H R_4 = H R_5 = OH
6 R_1 = H R_2 = OH R_3 = H R_4 = O-rha R_5 = OH
7 R_1 = H R_2 = OH R_3 = OH R_4 = O-rha R_5 = OH
8 R_1 = OH R_2 = OH R_3 = OH R_4 = O-rha R_5 = OH
9 R_1 = H R_2 = OH R_3 = OH R_4 = H R_5 = O-xyl

Fig 2. Chemical structures of compounds **1-9**.

acid (**4**),[2] acacetin (**5**),[23] afzelin (**6**),[24] quercitrin (**7**),[25] myricitrin (**8**),[26] and 5,7,8,3',4'-pentahydroxyflavone-7-O-β-D-xylopyranoside (**9**).[27] The NO production inhibitory activity of the isolated compounds was evaluated by the Griess assay at concentrations of 1, 3, 5, and 15 μM (Table 3). Compounds **2–4** exhibited significant inhibition of NO production in a concentration-dependent manner without causing cytotoxicity. This bioassay result correlated well with the data predicted from the bioassay-coupled LC-QTOF MS/MS. Compound **4** has been reported to inhibit NO production by reducing the concentrations of tumor necrosis factor-α (TNF-α) and iNOS in the BV2 cells and decreasing inflammatory cytokines such as TNF-α, IL-6, and COX-2.[7] The NO production inhibitory activities of compounds **2** (peak h) and **3** (peak i) were found for the first time in this study.

The EtOAc fraction of *T. orientalis* leaves inhibited NO production in LPS-stimulated BV2 cells. The results of the newly identified NO production inhibitory constituents (compounds **2** and **3**) from the bioassay-coupled LC-QTOF MS/MS matched well with the experimental results. Taken together, our findings indicated that coupling the LC-QTOF MS/MS with a bioassay was a powerful tool for the rapid characterization of new active compounds in extracts before the isolation of natural products. Furthermore, this study provided a potential basis for expanding the application of LC-QTOF MS/MS in natural product research.

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