CHAPTER II

EXPERIMENTAL

2.1 Plant materials

Fruits of *H. perforata* were collected from Si Satchanalai, Sukhothai Province, Thailand in April 2010, while its roots were obtained from Takhli, Nakhonsawan Province, Thailand in August 2010 and March 2012, respectively. Plant materials were identified by Royal Forest Department, Bangkok, Thailand.

2.2 General experimental Procedures

2.2.1 Fourier transform infrared spectrophotometer (FT-IR)

The FT-IR spectra were measured with a Perkin-Elmer Model 1760X Fourier Transform Infrared Spectrophotometer. Solid samples were formally examined by incorporating the sample with potassium bromide (KBr) to form a pellet.

2.2.2 Mass spectrometer (MS)

HRESIMS spectra were obtained with a Bruker micrOTOF.

2.2.3 Melting point

Melting points were recorded on a Fisher-Johns melting point apparatus.

2.2.4 Nuclear magnetic resonance spectrometer (NMR)

The NMR spectra were recorded in chloroform-*d* ($CDCl_3$) and acetone-d_6 ($(CD_3)_2CO$) on a Bruker AV400 and Varain Mecury 400 plus spectrometer at 400 MHz for ^1H NMR and at 100 MHz for ^{13}C NMR using TMS (tetramethylsilane) as internal standard.

2.2.5 Optical rotation

Optical rotations were acquired on a Perkin-Elmer 341 polarimeter at 589 nm.

2.2.6 Ultraviolet-visible spectrophotometer (UV-vis)

UV data were recorded in MeOH on a CARY 50 Probe UV-visible spectrophotometer.

2.2.7 X-ray diffraction spectrometer

The crystal structure was solved by direct methods and using the SHELXS97 program. Crystallographic data, excluding structure factors, have been deposited at the Cambridge Crystallographic Data Centre.

2.2.8 Microplate spectrophotometer

The absorbance for biological assays was measured with a Biotek PowerWave XS2 microplate spectrophotometer.

2.2.9 CO_2 cell culture incubator

Cells using in present study were cultured in a Panasonic MCO-5AC CO_2 cell culture incubator.

2.2.10 Biosafety cabinet

All biological procedurs including cell passage, biological assays were worked in a biosafety cabinet BIOHAZARD Class II MICROTECH Model V6-T.

2.3 Chemicals

2.3.1 Solvents

All commercial grade solvents, used in this research such as hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), acetone and methanol (MeOH), were purified by distillation prior to use.

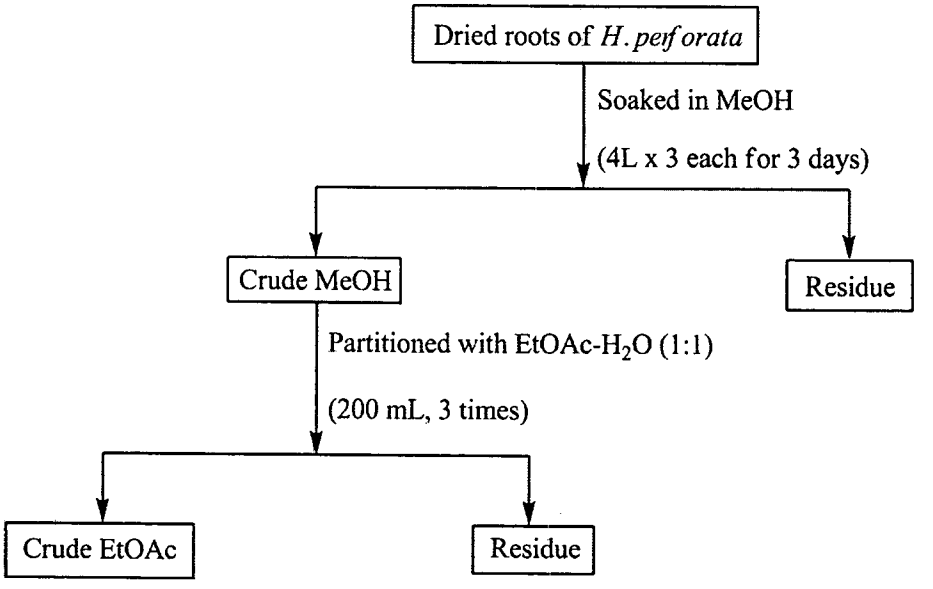
The deuterated solvents for NMR experiments are chloroform-*d* and acetone-d_6.

2.3.2 Other chemicals

Silica gel 60 No. 7734 and No. 9385 (Merck), and Sephadex LH-20 (Pharmacia) were used for open column chromatography (CC). Silica gel 60 F_{254} (Merck) plates were used for thin layer chromatography (TLC). Spots were detected by ultraviolet light at wavelengths of 254 nm and dipped with $(NH_4)_6Mo_7O_{24}$ and 1% $CeSO_4$ solution in 10% aqueous H_2SO_4 following by heating.

2.4 Extraction and isolation

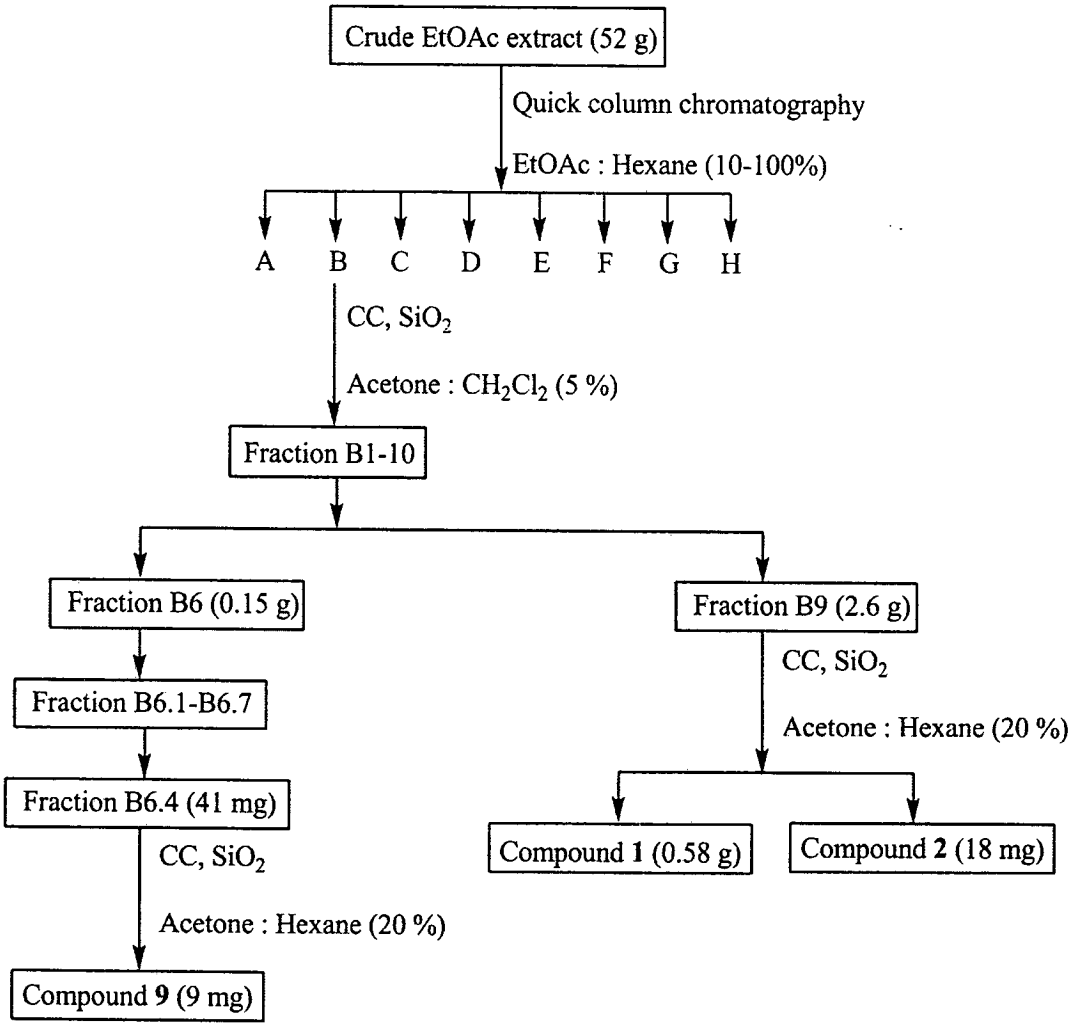
Dried roots of *H. perforata* were chopped into small pieces and soaked in MeOH for 3 days, three times (4L per each). After filtration, MeOH was evaporated and the residue was subsequently partitioned between H_2O and EtOAc (1:1). The organic layer was concentrated to dryness on a rotary evaporator under reduced pressure to yield EtOAc crude extract. The extraction procedure is shown in Scheme 2.1.



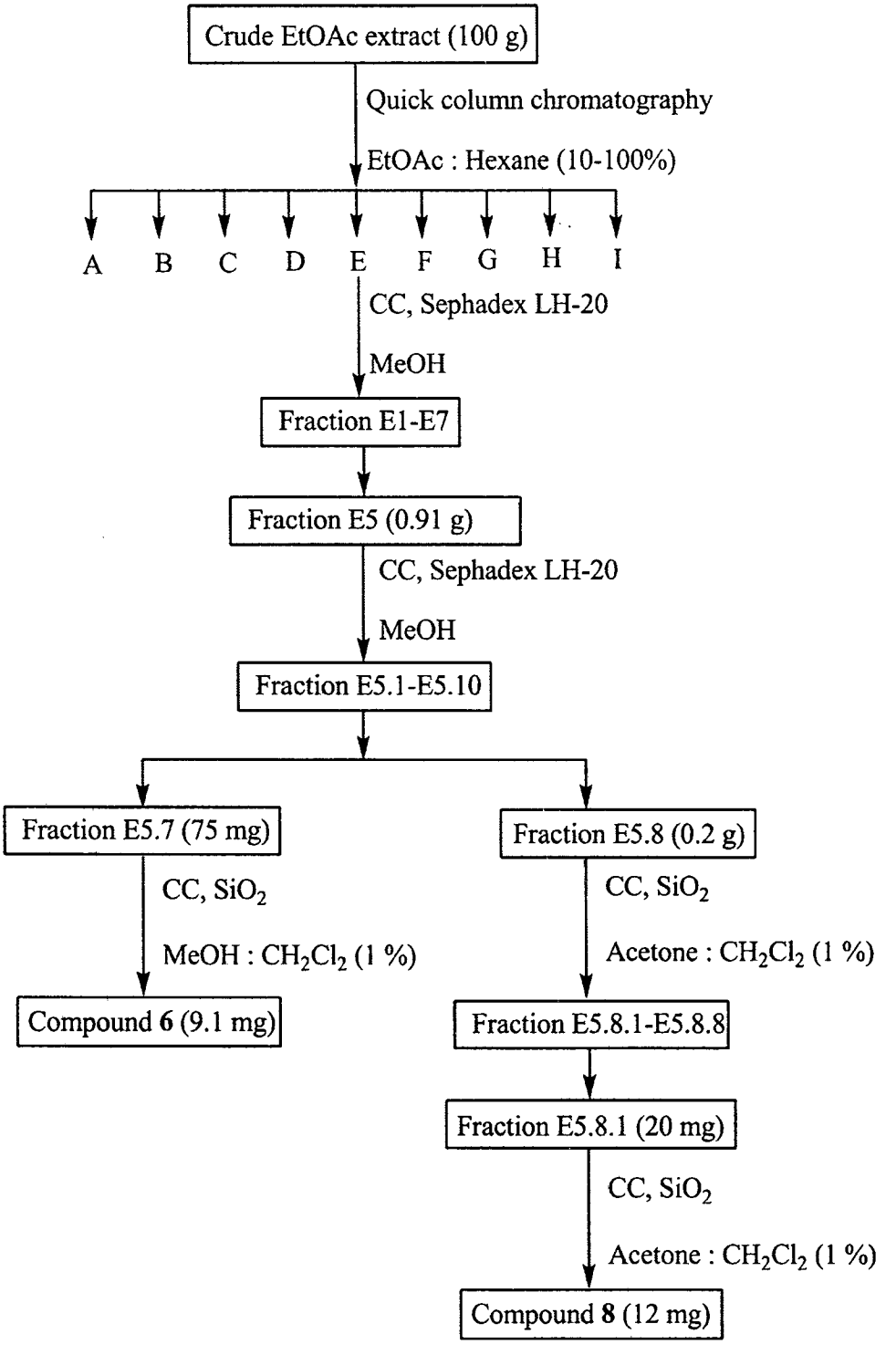
Scheme 2.1 The extraction procedure of *H. perforata* roots. (April 2010)

The EtOAc crude extract (52 g) of roots (2 kg, collected in April 2010) was fractionated by quick column chromatography on a siliga gel column eluted with EtOAc-hexane gradient (10-100%) to yield eight fractions (A-H). Fraction B (4.5 g) was subjected to siliga gel column using acetone-CH_2Cl_2 (5-10%) as eluent to give 10 fractions (B1-10). The fraction B6 (0.15 g) was separated on a siliga gel column eluted with acetone-CH_2Cl_2 (5-10%) to afford seven fractions (B6.1-B6.7), then faction B6.4 (41 mg) was repeatedly chromatographed on a siliga gel column (acetone-hexane, 20%) to give compound **9** (9 mg). The fraction B9 was purified by a column of siliga gel using acetone-hexane (20%) to obtain two limonoids, which were eluted in the order compounds **1** (0.58 g) and **2** (18 mg). The isolation of fraction B is described in Scheme 2.2.

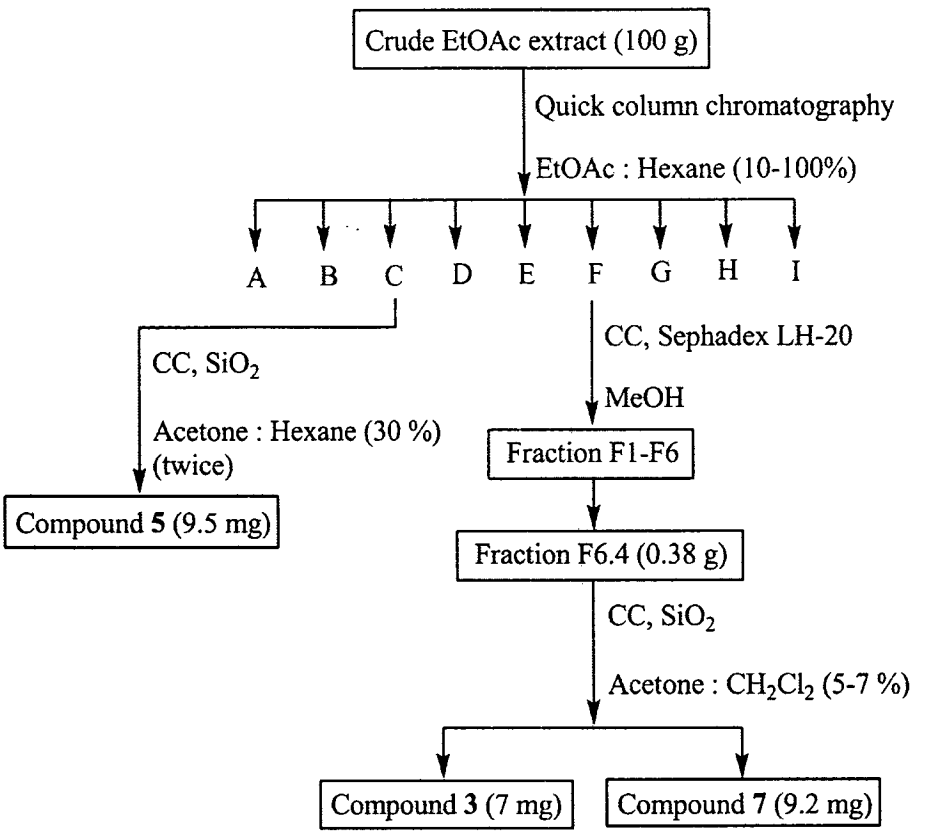
Isolation of the extract of *H. perforata* roots (2 kg) collected in March 2012 is shown in Schemes 2.3 and 2.4. The EtOAc crude extract (100 g) was quickly chromatographed on a column of siliga gel eluted with EtOAc-hexane gradient (10-100%) to yield eight fractions (A-I). Fraction C (1 g) was chromatographed on a siliga gel column eluted with acetone-hexane (30%) and repeatedly purified by the same condition to obtain compound **5** (9.5 mg). Fraction E (3.5 g) was subjected to Sephadex LH-20 column chromatography using MeOH as eluent to give seven fractions (E1-E7). The fraction E5 (0.91 g) was rechromatographed on a Sephadex LH-20 column to afford 10 fractions (E5.1-E5.10). Compound **6** (9.1 mg) was purified by a siliga gel column (MeOH-CH_2Cl_2, 1%) from fraction E5.7 (75 mg). The fraction E5.8 (0.2 g) was subjected to a siliga gel column eluted with acetone-CH_2Cl_2 (5%) to give eight fractions (E5.8.1-E5.8.8). Fraction E5.8.1 (20 mg) provided compound **8** (12 mg) by separating on a siliga gel column using acetone-CH_2Cl_2 (1%). Fraction F was rechromatographed on a Sephadex LH-20 column to give six fractions (F1-F6). Fraction F6.4 (0.38 g) was further subjected to column chromatography on siliga gel (5-7% acetone-CH_2Cl_2) to afford compounds **7** (7.1 mg) and **3** (7 mg) which were recrystallized by MeOH.



Scheme 2.2 The isolation from fraction B of EtOAc extract of *H. perforata* roots. (collected in April 2010)

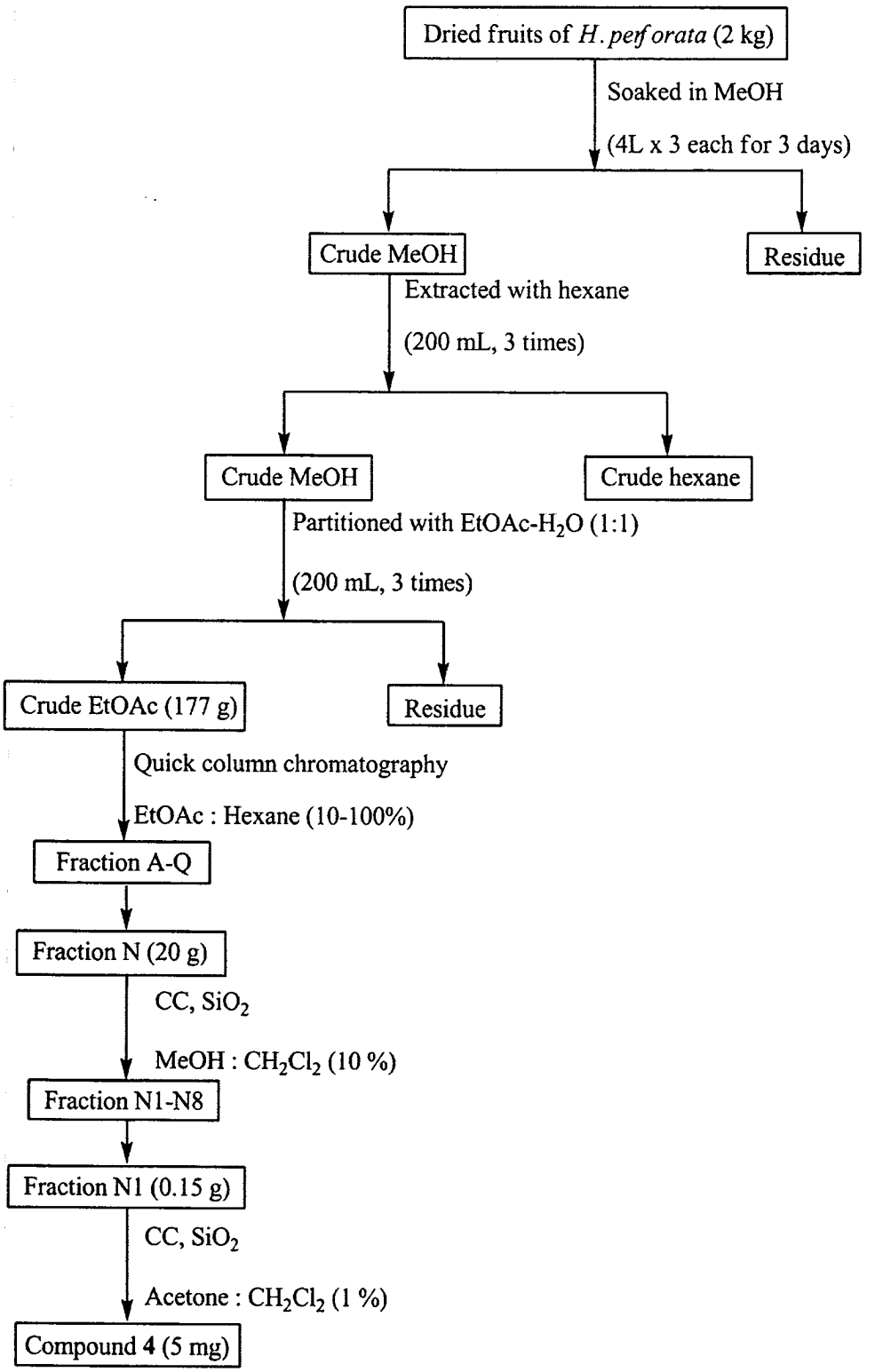


Scheme 2.3 The isolation of EtOAc extract of *H. perforata* roots from fraction E (collected in March 2012).



Scheme 2.4 The isolation of EtOAc extract of *H. perforata* roots from fractions C and F (collected in March 2012).

The MeOH residue of dried fruits (2 kg) of the same plant was deffated with hexane before partitioned in H_2O-EtOAc as shown in Scheme 2.5. EtOAc crude extract (177 g) was done with quick chromatography on a siliga gel column eluting with EtOAc-hexane gradient (10-100%) to afford 17 fractions (A-Q). The fraction N (20 g) was separated on a column of siliga gel (10% MeOH-CH_2Cl_2) to give eight fractions (N1-N8), then fraction N1 (0.15 g) was rechromatographed by elution with acetone-CH_2Cl_2 (5%) on siliga gel column and further recrystallized from MeOH to obtain compound **4** (5 mg). The extraction and isolation procedure of *H. perforata* fruits is presented in Scheme 2.5.



Scheme 2.5 The extraction and isolation procedure of *H. perforata* fruits.

2.5 Biological activity

2.5.1 Nitric oxide inhibitory assay

Murine macrophage J774.A1 cell lines were purchased from Cell Line Service (CLS) and were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin G (100 units/mL) and streptomycin (1 μg/mL). The cell lines were seeded in 24-well plates with 1×10^5 cells/well and allow to adhere for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. The cells were pretreated with various concentrations of test compounds or vehicle (DMSO) for 2 h, and then activated with 1 μg/mL of lipopolysaccharide (LPS) from *Escherichia coli* for 20 h. The culture supernatant (50 μL) of each well was collected and the concentration of nitric oxide (NO) was further measured by using Griess reagent. Those collected supernatants were added 50 μL of 1% sulfanilamide, per well, incubated under the dark condition at room temperature for 10 min. After that 50 μL of 0.1% *N*-1-napthylethylenediamine dihydrochloride (NED) were added incubated under the dark condition for further 10 min. The absorbance was measured at 540 nm with a microplate reader. Nitrite level in the samples was calculated from the standard curve created from known concentrations of sodium nitrite [12].

2.5.2 Cytotoxicity assay

To determine the cell viability of the active compounds, the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) colorimetric method was performed [12]. The cells were seeded in 96-well plate with 1×10^4 cells/well and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. After treatment with samples or vehicle (DMSO) for 24 h, MTT solution (10 μL, 5 mg/mL in phosphate buffer saline (PBS)) was then added to each well and incubated further for 4 h. The medium was removed and DMSO (100 μL/well) was added to dissolve the produced formazan crystals and the absorbance was measured at 540 nm using a microplate reader. Cells treated with only DMSO were used as a positive control.

2.5.3 Western Blot Analysis

After treatment of the cells with the indicated concentrations of harperfolide (**2**) and stimulation with LPS (1 μg/mL) in the same manner as 2.5.1, cells were washed with cold PBS and lysed with M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, USA). Lysates were centrifuged at 5000 rpm for 5 min, and supernatants were collected. Protein concentration was determined using the microBCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as standard. Thirty micrograms (μg) of protein were separated on sodiumd sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidenefluoride (PVDF) membrane. After blocking with 3% skim milk in PBS containing 0.05% Tween 20, the membrane was successively probed with primary antibody (Cell Signaling Technology), with iNOS at 4 ^{o}C overnight and with β-actin for 1 h at room temperature. The signals were detected using the chemiluminescent method [13].