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Anemonin, from *Clematis crassifolia*, potent and selective inducible nitric oxide synthase inhibitor

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Abstract

The aim of this study was to examine the anti-inflammatory effects of aerial part of *Clematis crassifolia* Benth. (Ranunculaceae) based on an iNOS inhibition in lipopolysaccharide (LPS) activated macrophages. Bioassay-guided fractionation and purification led to the isolation of ibotanolide B (**1**), calceolarioside B (**2**), *trans*-caffeic acid (**3**), anemonin (**4**) and 3′,4′,5,7-tetrahydroxy-6-C-glucopyranosylflavone (**5**). Their structures were elucidated on the basis of spectroscopic analysis. All these compounds inhibited NO production, detected as nitrite, in activated macrophages except **5**. Among them, anemonin (**4**) was the most potent. Analyses of reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting revealed that it decreased the expression of iNOS mRNA and protein in activated RAW 264.7 cells. In isolated rat thoracic aortic rings, anemonin prevented the vascular hyporeactivity to phenylephrine induced by LPS whereas it did not affect acetylcholine-induced endothelial NO-dependent relaxation, an index of endothelial NOS (eNOS) activity. These results indicated that the potential anti-inflammatory effect of anemonin, the naturally occurring selective iNOS inhibitor, may provide a rationale for the medical use of *Clematis crassifolia*.

Keywords: *Clematis crassifolia*; Ranunculaceae; Anemonin; Inducible nitric oxide synthase; Macrophage; Inflammation

1. Introduction

The reported biological activities of the genus *Clematis* (Ranunculaceae) include hepatic protective (Chiu et al., 1988) and hypotensive (Ho et al., 1989) effects, and anti-inflammatory (Yesilada et al., 1997; Li et al., 2003), anti-tumour (Qiu et al., 1999) activities. There are 21 species of *Clematis* found in Taiwan. *Clematis crassifolia* Benth., a scandent perennial woody vines with ternate compound leaves, is widely distributed in sunny places and forest margins at altitudes of 300–2300 m in north central part of this island (Huang, 1996). Its leaves and roots have been used in substitution for *Clematis chinensis* Osbeck. which has long been used in traditional Chinese medicine as analgesic, diuretic and anti-inflammatory agent. The phytochemical investigations on *Clematis chinensis* had revealed that its roots, a Chinese crude drug "Wei-Ling-Xian", are rich in triterpene saponins (Shao et al., 1995, 1996; Mimaki et al., 2004). However, the active ingredients and biological effects of *Clematis crassifolia* still remain unclear. Due to the positive anti-inflammatory activity it is regarded as the quality assurance of medicinal *Clematis chinensis* (Wei et al., 1991), and NO, produced in large quantity by activated macrophages via an iNOS, has been implicated in the pathogenesis of a variety of inflammatory mediated disorders (Kleinert et al., 2004). Thus, the present study was designed to find out the potential bioactive principles that were responsible for the anti-inflammatory effect of *Clematis crassifolia* leaves by inhibition the iNOS induction in LPS-activated murine macrophage RAW 264.7 cells. In association with bioassay-guided fractionation, a series

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of phytochemical examinations and structural elucidations was undertaken. In addition, we monitored the expression of iNOS mRNA and protein of active principle by RT-PCR and Western blot assay, respectively. The NO scavenging effect was measured in vitro using sodium nitroprusside. The vascular tension study was further performed in rat thoracic aortas to elucidate the selective inhibitory activity on iNOS.

2. Materials and methods

2.1. General experimental procedures

Optical Rotations were measured with JASCO P-1020 digital polarimeter at room temperature. IR spectra were recorded on a Thermo Mattson IR 300 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DMX-500 SB instrument using tetramethylsilane (TMS) as an internal standard. Chemical shifts are given in δ values (ppm) and coupling constants (J) are given in hertz (Hz). Fast atomic bombardment mass spectra (FAB-MS) obtained on Finnigan Thermo Quest MAT 95XL mass spectrometer. Extracts were chromatographed on silica gel (70–230 mesh, Merck), and further purified with a semi-preparative reversed phase HPLC column (250 mm × 10 mm, 5 μm, Thermo Hypersil BDS).

2.2. Plant material

The leaves of *Clematis crassifolia* Benth. were collected from the Tea Research and Extension Station, Nantou County, Taiwan in May 2004. A voucher specimen (No. 05192004) was identified by Dr. Tzong-Yuh Yang and deposited in the Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei, Taiwan.

2.3. Extraction and isolation

Fresh leaves (1.0 kg) of *Clematis crassifolia* were extracted three times with 5 l MeOH at room temperature for 2 weeks. The methanolic extract was concentrated in vacuum to give a black residue (85 g), which was re-dissolved in 85% aqueous methanol and then partitioned with *n*-hexane to generate two fractions: the aqueous methanol soluble fraction and *n*-hexane soluble fraction. Subsequently, the aqueous methanol soluble fraction was vacuum-evaporated to dryness (45 g) and further partitioned between EtOAc (500 ml × 2) and water (500 ml), and the remaining water solution was extracted three times with *n*-butanol (500 ml). Each primary fraction was then subjected to preliminary biological test as described in the following parts. The bioactive EtOAc fraction was evaporated to a black residue and redissolved in MeOH for chromatographic separation. The first separation step was carried out using gel permeation chromatography on a Sephadex LH-20 column (3 cm × 55 cm) and eluted by MeOH with a flow rate of 13 ml/min. Each fraction (15 ml) collected from the EtOAc fraction was checked for their compositions by TLC using EtOAc/HCO_2H/H_2O (85:10:15) for development. Fractions containing the same components were combined to give 11 secondary fractions, and each secondary fraction was checked its bioactivity again. Among the secondary fractions tested, secondary fractions VI–VIII suppressed NO production in LPS-activated RAW 264.7 cells. The secondary fraction VI (#fr.14–15) was further purified by repetitive HPLC on a reversed phase semi-preparative column with MeCN/H_2O (1:4) containing 0.1% trifluoroacetic acid (TFA) as eluent to obtain **5** (10.0 mg). The secondary fraction VII (#fr.16–18) was further purified by HPLC using the same column with MeCN/H_2O (15:85) containing 0.1% TFA as eluent to afford **4** (75.0 mg), and with MeCN/H_2O (1:4) containing 0.1% TFA as eluent to afford **1** (10.0 mg) and **2** (2.5 mg). The secondary fraction VIII (#fr.19–21) was further purified using the same chromatograph with MeCN/H_2O (15:85) containing 0.1% TFA as eluent to give **3** (5.0 mg) (Fig. 1).

Ibotanolide B (**1**). Amorphous white powder; $[\alpha]_D^{25} = -52.8^\circ$ (c 0.65, MeOH); UV (MeOH): λ_{max} (log ε) = 220 (4.2), 245 (3.9, sh), 287 (4.0, sh), 302 (4.0, sh), 329 (4.1); IR (KBr): ν_{max} = 3382, 1680, 1599, 1512, 1278 1200, 1072 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz): δ_H 2.62 (t, J = 7.0 Hz), 3.40 (t, J = 8.6 Hz), 3.47, 3.50, 3.63 (t, J = 7.0 Hz), 3.67 (m), 4.35 (dd, J = 7.3, 11.9 Hz), 4.55 (dd, J = 1.7, 11.9 Hz), 4.69 (d, J = 7.3 Hz), 6.29 (d, J = 16.0 Hz), 6.51 (dd, J = 1.8, 8.2 Hz), 6.70 (d, J = 1.8 Hz), 6.80 (d, J = 8.2 Hz), 6.95 (dd, J = 1.7, 8.2 Hz), 7.03 (d, J = 8.2 Hz), 7.06 (d, J = 1.7 Hz), 7.58 (d, J = 16.0 Hz); ^{13}C NMR (CD_3OD, 125 MHz): δ_C 39.6 (t), 64.2 (t), 64.6 (t), 71.9 (d), 74.9 (d), 75.8 (d), 77.5 (d), 104.5 (d), 115.0 (d), 115.2 (d), 116.6 (d), 117.7 (d), 119.0 (d), 121.4 (d), 123.0 (d), 127.7 (s), 136.2 (s), 145.1 (s), 146.9 (s), 147.2 (d), 148.2 (d), 149.7 (s) and 168.9 (s); FABMS (NBA): m/z = 501 $[M + Na]^+$.

Calceolarioside B (**2**). Amorphous white powder; ^{1}H NMR (CD_3OD, 500 MHz): δ_H 2.80 (m), 3.22 (t, J = 8.1 Hz), 3.35, 3.38, 3.52, 3.70 (m), 3.95 (m), 4.31 (d, J = 8.1 Hz), 4.35, 4.49 (d, J = 11.7 Hz), 6.28 (d, J = 15.9 Hz), 6.64 (d, J = 8.2 Hz), 6.76 (d, J = 8.1 Hz), 6.88 (d, J = 8.1 Hz), 7.05 (d, J = 8.2 Hz), 7.06 (s), 7.55 (d, J = 15.9 Hz); ^{13}C NMR (CD_3OD, 125 MHz): δ_C 36.5 (t), 64.7 (t), 71.8 (d), 72.4 (t), 75.1 (d), 75.4 (d), 78.0 (d), 104.6 (d), 114.9 (d), 115.1 (d), 116.2 (d), 116.5 (d), 123.1 (d), 127.7 (s), 130.6 (s), 130.9 (d), 146.8 (s), 147.2 (d), 149.6 (s), 156.8 (s) and 169.1 (s).

Trans-caffeic acid (**3**). Amorphous white powder; ^{1}H NMR (CD_3OD, 500 MHz): δ_H 6.21 (d, J = 15.9 Hz), 6.76 (d, J = 8.1 Hz), 6.92 (dd, J = 1.8, 8.1 Hz), 7.02 (d, J = 1.8 Hz), 7.52 (d, J = 15.9 Hz); ^{13}C NMR (CD_3OD, 125 MHz): δ_C 114.8 (d), 115.1 (d), 116.5 (d), 122.9 (d), 127.7 (s), 146.8 (s), 146.9 (d), 149.6 (s) and 169.8 (s).

Anemonin (**4**). Amorphous white powder; ^{1}H NMR (CD_3OD, 500 MHz): δ_H 2.35 (m), 2.61 (m), 6.18 (d, J = 5.3 Hz), 8.08 (d, J = 5.3 Hz); ^{13}C NMR (CD_3OD, 125 MHz): δ_C 24.3 (t), 91.9 (s), 121.1 (d), 156.4 (d) and 173.3 (s).

3′,4′,5,7-Tetrahydroxy-6-C-glucopyranosylflavone (**5**). Amorphous yellow powder; $[\alpha]_D^{25} = -25.0^\circ$ (c 0.65, MeOH); UV (MeOH): λ_{max} (log ε) = 269 (4.2), 348 (4.1); IR (KBr): ν_{max} = 3317, 1650, 1614, 1489, 1353, 1265 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz): δ_H 3.41 (ddd, J = 1.6, 5.5, 8.5 Hz), 3.44 (dd, J = 8.0, 10.5 Hz), 3.46 (dd, J = 8.5, 10.5 Hz), 3.73 (dd, J = 5.5, 12.1 Hz), 3.87 (dd, J = 1.6, 12.1 Hz), 4.16 (dd, J = 8.0, 8.0 Hz), 4.90 (d, J = 8.0Hz), 6.46 (s), 6.52 (s), 6.88 (d,

J = 8.5 Hz), 7.35 (d, J = 2.0 Hz), 7.35 (dd, J = 2.0, 8.5 Hz); ^{13}C NMR (CD_3OD, 125 MHz): δ_C 62.9 (t), 71.8 (d), 72.6 (d), 75.3 (d), 80.1 (d), 82.6 (d), 95.2 (d), 103.9 (d), 105.1 (s), 109.2 (s), 114.1 (d), 116.8 (d), 120.3 (d), 123.5 (s), 147.1 (s), 151.2 (s), 158.7 (s), 162.1 (s), 165.1 (s), 166.3 (s) and 184.0 (s); FABMS (NBA): m/z = 449 $[M+H]^+$.

2.4. Cell culture

RAW 264.7 cells (a transformed murine macrophage cell line) obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) were maintained by once-weekly passage in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and penicillin–streptomycin.

2.5. NO measurement

Cell aliquots (5×10^5 cells/ml) were grown to confluence on 24-well plates for 24 h. Then the medium was changed to serum-free media for another 4 h to render the attached cells quiescence. To assess the effects on LPS-induced NO production, EtOAc fraction of extract (0.4–5.0 μg/ml), compounds **1**–**5** (100 μM), vehicle, positive controls N^{ω}-nitro-L-arginine (L-NNA), a non-selective NOS inhibitor, or aminoguanidine, a selective iNOS inhibitor, at 100 μM, was added in the presence of LPS (200 ng/ml) to the cells for further 24 h. The nitrite concentration in the culture medium was determined spectrophotometrically as an index of NO production (Green et al., 1982). Results are expressed as percentage of inhibition calculated versus vehicle plus LPS-treated cells.

2.6. Cell viability assay

A redox indicator, alamarBlue, used to measure the cytotoxicity was reported (Kwack and Lynch, 2000). After culture supernatant removed for NO measurement described as above, and then a solution of 10% alamarBlue in DMEM added to each well containing RAW 264.7 cells. The plates were incubated at 37 °C in a humidified 5% CO_2 for 3 h. Following incubation, the absorbance of the alamarBlue was read spectrophotometrically at dual wavelengths of 570 and 600 nm against the blank prepared from cell-free wells. The absorbance in cultures treated with LPS plus vehicle was regarded as 100% cell viability.

2.7. Western blot assay

RAW 264.7 cells were prepared as previously described (Wang et al., 2007). After quiescence, the cells challenged by LPS (200 ng/ml) for 24 h in the presence of EtOAc fraction (0.4–5.0 μg/ml), anemonin (2.5–30 μM) or vehicle were analyzed by Western blotting for iNOS protein expression. The cell lysates were sonicated and centrifuged at 3000 g for 20 min at 4 °C. The cytoplasmic protein concentration in the supernatants was determined by the protein-dye method of Bradford (1976). Western blot analyses were performed according to standard procedures as described previously (Wang et al., 2007). Immunoreactive bands were visualized with a chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ). The protein expression was quantified by scanning densitometry (Camera Image Systems, LAS-3000, Fujifilm, Tokyo, Japan).

2.8. RNA extraction and RT-PCR assay for iNOS and GAPDH mRNA

Cells were seeded at 5×10^6 cells per 6-cm culture dishes. After quiescence, the cells were exposed for 6 h to anemonin (2.5–30 μM) or vehicle in the presence of LPS (200 ng/ml). The total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA concentration and purity were assessed by optical density ratios at 260/280 nm. Total RNA was diluted in RNase-free water and stored at −80 °C. The PCR primers for rat iNOS were 5′-TGTCTCTGGGTCCTCTGGTCAAA and 5′-TGGCTTGCCCTTGGAAGTTTCTC, generating a 384-bp product. The primers for the rat GAPDH (housekeeping gene) were 5′-TCCACCACCCTGTTGCTGTA and 5′-ACCACAGTCCATGCCATCAC, generating a 452-bp product as a control for PCR amplification. The reverse transcription (RT) was performed using Advantage™ RT-for-PCR kit (Clontech, Heidelberg, Germany) to synthesize first-strand cDNA from 5 μg of total RNA at 42 °C for 60 min then 94 °C for 5 min. The cDNA was diluted to final volume of 100 μl by adding DEPC-H_2O. Each PCR amplification reaction mixture (50 μl) contained 2.5 μl of cDNA, 10 mM dNTPs, 0.5 μM of each primer, 5 μl of 10× PCR buffer, 1.5 mM $MgCl_2$ and 2.5 units of Bio Taq DNA polymerase (Bioman Scientific Co. LTD, Taipei, Taiwan). The PCR amplifications were performed in the PCR Robocycler (Gradient 96, Stratagene, La Jolla, CA). PCR parameters: preheat at 94 °C for 5 min followed by 30 cycles for iNOS and 25 cycles for GAPDH (94 °C for 45 s, annealing at 56 °C for 45 s and elongation at 72 °C for 2 min); the reaction ended with 7 min incubation at 72 °C and chilled to 4 °C. PCR products were analyzed on 2% agarose gel and visualized by UV transillumination. The band intensities were quantified by densitometer (ImageMaster VDS, Pharmacia Biotech, San Francisco, CA).

2.9. Animals

Male Sprague–Dawley rats weighing 280–350 g were obtained from the Laboratory Animal Center, National Yang-Ming University, Taipei, Taiwan, and maintained in individual ventilated cages with a constant temperature of 20–22 °C, relative humidity 55% and light with 12:12 h light-dark cycles. Standard laboratory fodder (Purina Mills, Richmond, IN) and drinking water were provided ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committees of National Research Institute of Chinese Medicine and were conducted in accordance with the National Institutes of Health Animal Care standards (NIH publication #85-23, revised in 1985).

2.10. Vascular tension experiment

The tension measurement was essentially in line with our previously published method (Wang et al., 1996; Ko et al., 2006). In brief, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and killed by exsanguination. The chest of each rat was opened, and the central portion of the thoracic aorta was isolated, cleaned of surrounding tissues, and divided into four (3-mm-long) rings. Rings taken from the same rat were used for different experiments or for control, respectively. Each value of number in the results and figure legends represents the number of rings obtained from the different rats. To assess the effects on iNOS activity, aortic rings without endothelium were random placed in 24-well plate with 1 ml MEM containing LPS (300 ng/ml) plus anemonin (5–30 μM) or vehicle, and incubated at 37 °C for 6 h before tension study. The endothelium-denuded aortic rings were then fixed isometrically in organ chamber containing an oxygenated Krebs' solution under passive tension of 1.8 g. Vascular tension was recorded via a force displacement transducer (Grass FT03, Quincy, MA) connected to a Gould 3400S polygraph and displayed with a data acquisition system (PowerLab, ADInstruments Pty Ltd.). The concentration–response curves generated by phenylephrine (1.0 nM to 100 μM) were constructed and compared. To investigate the effect of anemonin on eNOS function, the functional integrity of endothelium was confirmed by an observation of more than 98% relaxation in response to acetylcholine (1 μM) in tissues pre-contracted with phenylephrine (0.3 μM). Cumulative concentrations of acetylcholine (10 nM–10 μM) were applied during the sustained phase (considered as 100%) of phenylephrine (0.3 μM)-induced contraction in endothelium-intact aortic rings. Following washing and recovery for 30 min, similar experiments were carried out in the presence of anemonin (10, 100 μM), L-NNA (100 M), aminoguanidine (100 μM) or vehicle for 20 min. The construction of concentration–response curves for acetylcholine was based on the percentage of relaxation of the agonist-induced contraction. A complete relaxation was considered attained when the pre-contracted rings returned to the base line position.

2.11. NO-scavenging activity of anemonin in sodium nitroprusside solution

The method was essentially the same as reported previously (Mirkov et al., 2004). To estimate possible NO-scavenging activity of anemonin, sodium nitroprusside solution (5 mM)

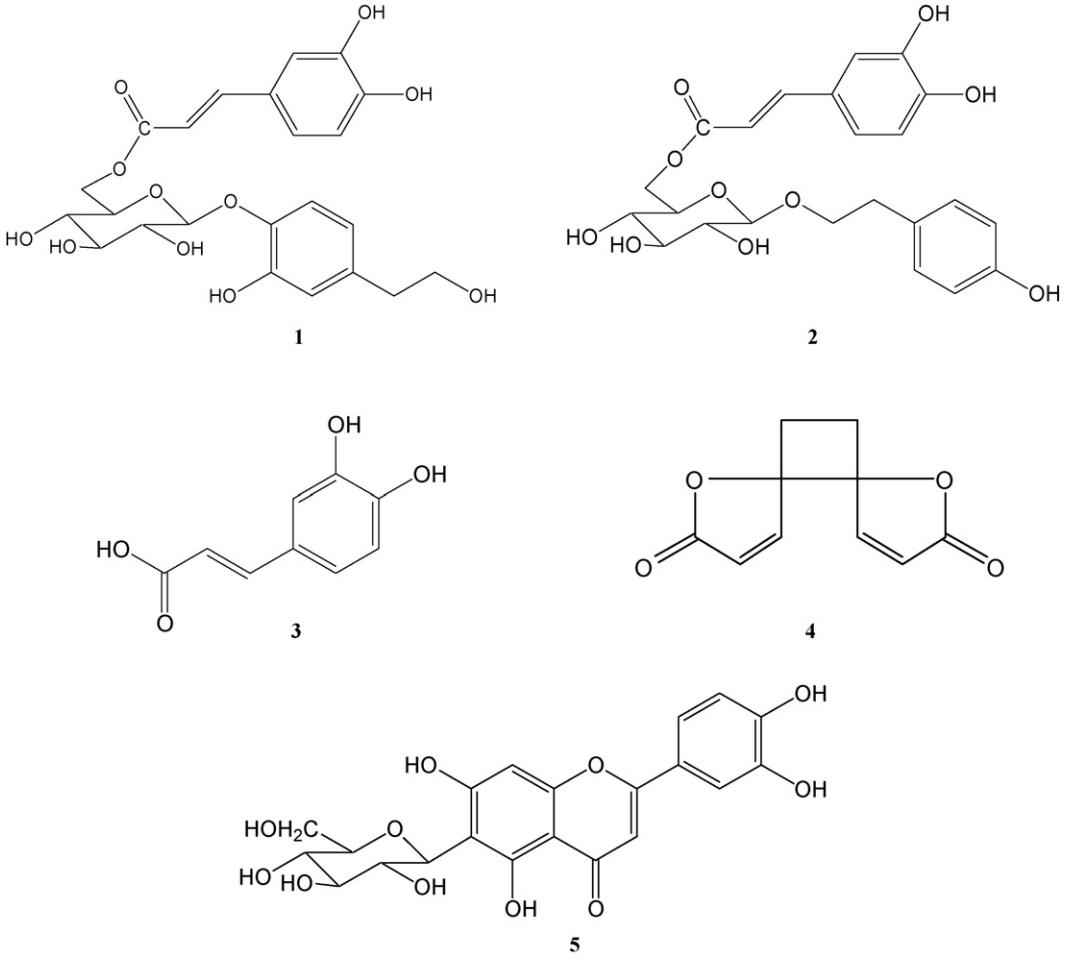

Fig. 1. Structures of ibotanolide B (**1**), calceolarioside B (**2**), *trans*-caffeic acid (**3**), anemonin (**4**) and 3′,4′,5,7-tetrahydroxy-6-C-glucopyranosylflavone (**5**) from *Clematis crassifolia*.

were incubated in combination with different concentrations of anemonin (2.5–30 μM) or vehicle in microcentrifuge tubes. Sodium nitroprusside is an inorganic complex where NO is found as NO^+ and light irradiation is necessary for release of NO (Feelisch and Stamler, 1996). Therefore, incubation mixtures were incubated on light, at room temperature, and nitrite levels were determined at 0, 30, 60, 90, 120 and 150 min using Griess reagent as described above.

2.12. Reagents

The following drugs were used: acetylcholine, aminoguanidine, dimethyl sulfoxide, L-NNA, LPS (*Escherichia coli* Serotype 055:B5) and phenylephrine, from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA); alamarBlue, from Biosource International Co. (Camarillo, CA); mouse monoclonal antibody against mouse iNOS from Transduction Laboratories (San Diego, CA). EtOAc fraction of *Clematis crassifolia* and compounds **1**–**5** were dissolved in dimethyl sulfoxide to make stock solutions, and kept at −30 °C. The final concentration of the vehicle in the solution never exceeded 0.1% and had no effects on NO production, iNOS mRNA and protein expressions, cell viability and vascular tension assay.

2.13. Statistic analysis

For each experimental series, data are given as mean ± S.E. and *n* represents the number of independently performed experiments. All data were analyzed by an IBM-compatible statistical software package (SPSS for Windows, Ver. 10.0). The significance of the concentrations and sample treatments was determined by two-way analysis of variance (ANOVA) with repeated measures. If there were significant interactions, the simple main effect of each factor would be assessed using Kruskal–Wallis nonparametric ANOVA. Post hoc comparisons were carried out between means, according to the suitability. *P* value less than 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Effects of EtOAc fraction of Clematis crassifolia and its derived compounds on cell viability

The alamarBlue assay was used to examine whether the amount of the test specimens used in this study caused cell damage. Our results indicated that either EtOAc fraction of *Clematis crassifolia* (0.4–5.0 μg/ml) or vehicle had no adverse effects on the growth of RAW 264.7 macrophages in the presence of LPS (200 ng/ml) (data not shown). However, a 5.86 ± 1.92% reduction in mitochondrial reductase activity was noted with compound **1** (100 μM) when compared with the vehicle-treated group (Fig. 3A). Both **2** and **5** in the presence of LPS showed a significant cytotoxicity at the concentration used (100 μM). The cell viability were reduced by 22.02 ± 4.31% and 11.73 ± 3.34%, respectively. In contrast, compounds **3** and **4**, at the concentration of 100 μM, did not interfere with the survival percentage of macrophages in the presence of LPS.

3.2. Effects of EtOAc fraction of Clematis crassifolia and its derived compounds on LPS-induced NO production

To investigate the anti-inflammatory effects of *Clematis crassifolia*, EtOAc fraction of extract and five derived compounds were tested with regard to their effect on NO production in LPS-activated macrophages. Without LPS, RAW 264.7 cells released

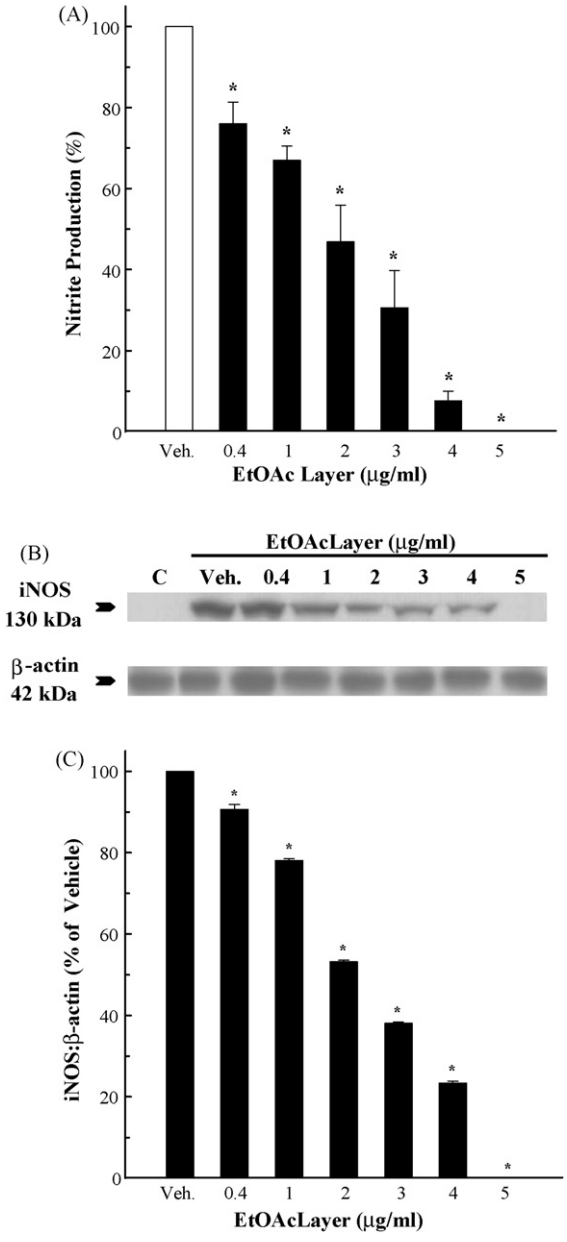

Fig. 2. The effects of the EtOAc fraction of *Clematis crassifolia* on NO production and iNOS protein expression in LPS-activated RAW 264.7 cells. Vehicle representing as 100% is equal to 45.55 ± 0.52 μM of NO produced in the medium per well of cells. $n = 6$ in each group (A). One of three representative Western blotting experiments is shown (B). Results were generated as integrated intensity units by densitometry and expressed as percentage of vehicle treatment (C). $^*P < 0.05$ when compared with vehicle-treated cells.

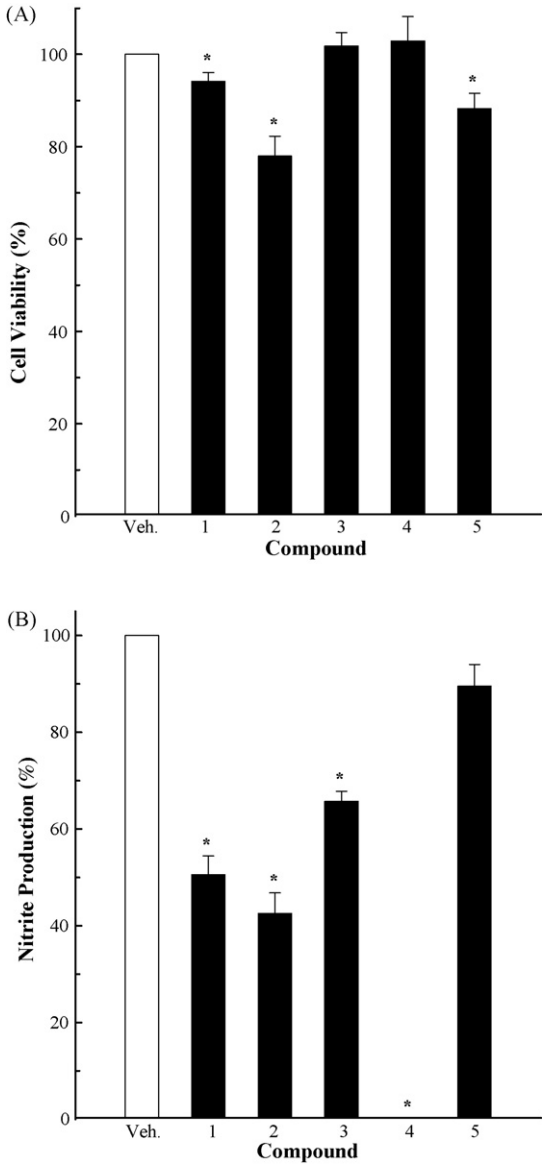

Fig. 3. The effects of compounds **1–5** on cell viability (A) and NO production (B) in LPS-activated RAW 264.7 cells. Vehicle representing as 100% is equal to 43.35 ± 1.67 μM of NO produced in the medium per well of cells. $n = 5–6$ in each group. $^*P < 0.05$ when compared with vehicle-treated cells.

undetectable levels of NO, measured as nitirite concentration, after 24 h incubation (data not shown). When LPS (200 ng/ml) was added to RAW 264.7 cells, NO production was dramatically increased to 41–46 μM for the 24 h incubation period. Vehicle did not affect the NO production induced by LPS. As shown in Fig. 2A, when EtOAc fraction of *Clematis crassifolia* (0.4–5.0 μg/ml) together with LPS for 24 h, this extract significantly attenuated NO production in a concentration-related manner, with an IC_{50} value of 1.60 ± 0.28 μg/ml. The inhibitory results have also been obtained for compounds **1–4** at a concentration of 100 μM while **5** did not show any significant effect on NO production (Fig. 3B). The data indicated that **4** was the markedly potent which completely reversed NO production induced by LPS. Such an inhibitory effect was

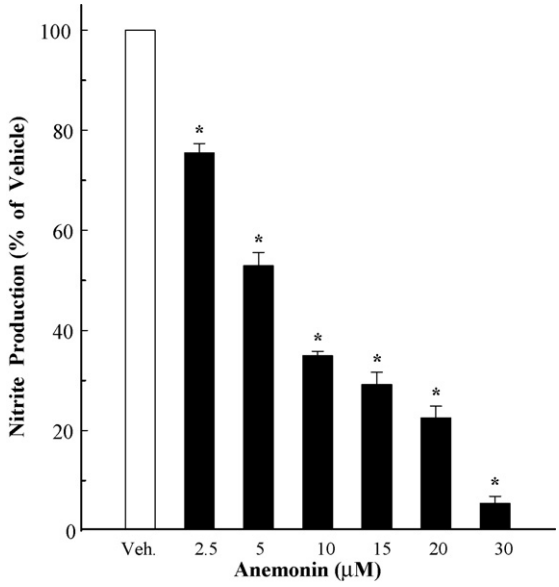

Fig. 4. The effects of anemonin on NO production in LPS-activated RAW 264.7 cells. Vehicle representing as 100% is equal to 43.35 ± 1.67 μM of NO produced in the medium per well of cells. $n = 7–8$ in each group. $^*P < 0.05$ when compared with vehicle-treated cells.

in a concentration-dependent manner with an IC_{50} value of 5.37 ± 0.39 μM (Fig. 4).

3.3. Effects of EtOAc fraction of Clematis crassifolia and anemonin on LPS-induced iNOS protein expression

To evaluate if the inhibitory effect of EtOAc fraction of *Clematis crassifolia* on NO production was related to a modulation of iNOS induction, we evaluated iNOS protein expression by Western blot analysis. In un-stimulated RAW 264.7 cells, 130 kDa of iNOS protein expression was undetectable (Fig. 2B). LPS treatment significantly elevated the level of iNOS protein in macrophages while EtOAc fraction of extracts (0.4–5.0 μg/ml) was able to reverse such increase. A representative blot and statistical results from these experiments are shown in Fig. 2B and C. Western blot analysis of cell lysates incubated with anemonin (2.5–30 μM) showed a significant and concentration-related suppression of iNOS protein level as measured by densitometer scans (Fig. 5A and B).

3.4. Effects of anemonin on LPS-induced iNOS mRNA expression

The levels of iNOS and GAPDH mRNA expression were measured by semi-quantitative RT-PCR. The mRNA expression of the housekeeping gene, GAPDH, was not influenced by LPS or test specimens treatments. As shown in Fig. 6A, in the resting RAW 264.7 macrophages, the expression of iNOS mRNA was hardly detectable while it was dramatically induced in cultures treated with LPS at 6 h of incubation period. The LPS-induced expression of iNOS mRNA was gradually decreased with increasing concentrations of anemonin (2.5–30 μM) (Fig. 6B).

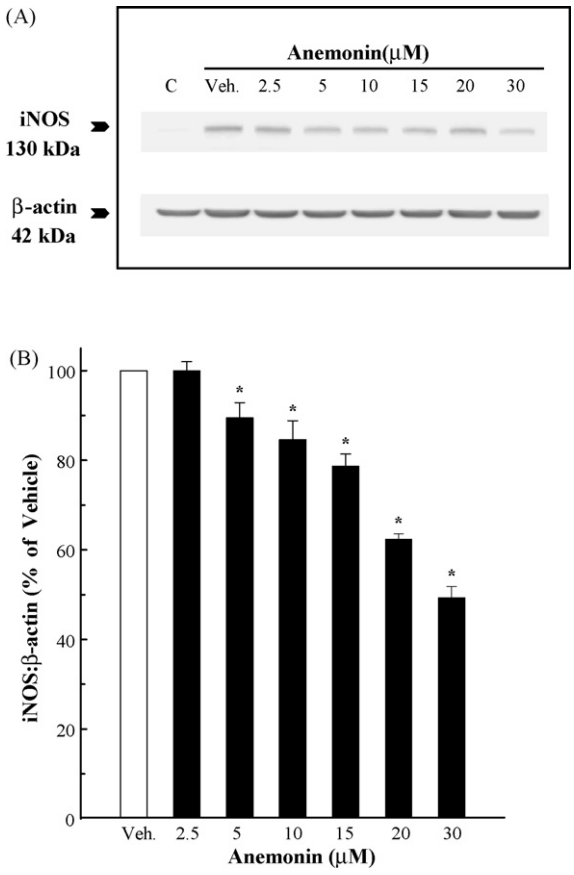

Fig. 5. Effect of anemonin on LPS-induced iNOS protein expression. One of three representative Western blotting experiments is shown (A). Results were generated as integrated intensity units by densitometry and expressed as percentage of vehicle treatment (B). *$P < 0.05$ when compared with vehicle-treated cells.

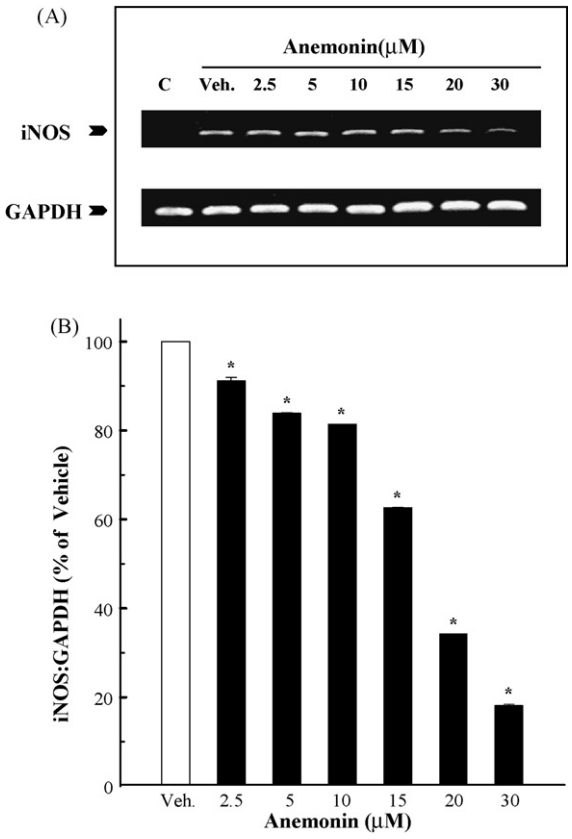

Fig. 6. Effect of anemonin on LPS-induced iNOS mRNA expression. One of three representative RT-PCR experiments is shown (A). Results were generated as integrated intensity units by densitometry and expressed as percentage of vehicle treatment (B). When no S.E. is shown, it was smaller than the symbol for the mean. *$P < 0.05$ when compared with vehicle-treated cells.

3.5. Phenylephrine-induced vasocontraction responses

Anemonin given individually did not alter the baseline tension of the aortic rings (data not shown). Phenylephrine (1 nM to 100 μM) produced a concentration-dependent contraction in endothelium-denuded aortic rings, with a maximal contraction of 3.39 ± 0.26 g (Fig. 7A). Contractile response to phenylephrine was significantly shifted to the right and the maximal contraction was significantly decreased to 1.60 ± 0.23 g in aortic rings incubated with LPS (300 ng/ml) for 6 h. Vehicle co-treatment did not affect the responses caused by LPS. Co-incubation with anemonin (5–30 μM) significantly reversed the hyporeactivity to phenylephrine induced by LPS in concentration dependent manner, with a maximal contraction of 3.09 ± 0.14 g.

3.6. Acetylcholine-induced vasorelaxing responses

Acetylcholine (10 nM–10 μM) produced a concentration-dependent relaxation in the precontracted aortic rings with intact endothelium (Fig. 7B). When the concentration of acetylcholine reached to 10 μM, a marked relaxation occurred ($99.19 \pm 0.81\%$). The vasorelaxing effect was not affected in the presence of vehicle. L-NNA (100 μM) completely abolished acetylcholine-evoked vasorelaxation whereas aminoguanidine (100 μM) did not influence the effect evoked by acetylcholine. This vasorelaxing effect also has no change in the presence of anemonin (10, 100 μM) for 20 min. The maximal relaxation was 100%, which was similar to aminoguanidine yielding maximal relaxation of $98.08 \pm 1.92\%$.

3.7. NO-scavenging activity of anemonin in sodium nitroprusside solution

The test specimens were evaluated for their scavenging effects on NO derived from sodium nitroprusside. When solutions of sodium nitroprusside in the presence of vehicle were incubated at room temperature of up to 150 min, they generated a time dependent nitrite production of about 20.61 ± 0.78 μM/h. Co-incubation of sodium nitroprusside with anemonin (30 μM) for 150 min did not affect the level of nitrite, in comparison to the nitrite levels obtained when sodium nitroprusside plus vehicle. The accumulation of nitrite upon decomposition of sodium nitroprusside after 150 min incubation also showed no change in the presence of anemonin (2.5–30 μM) (data not shown).

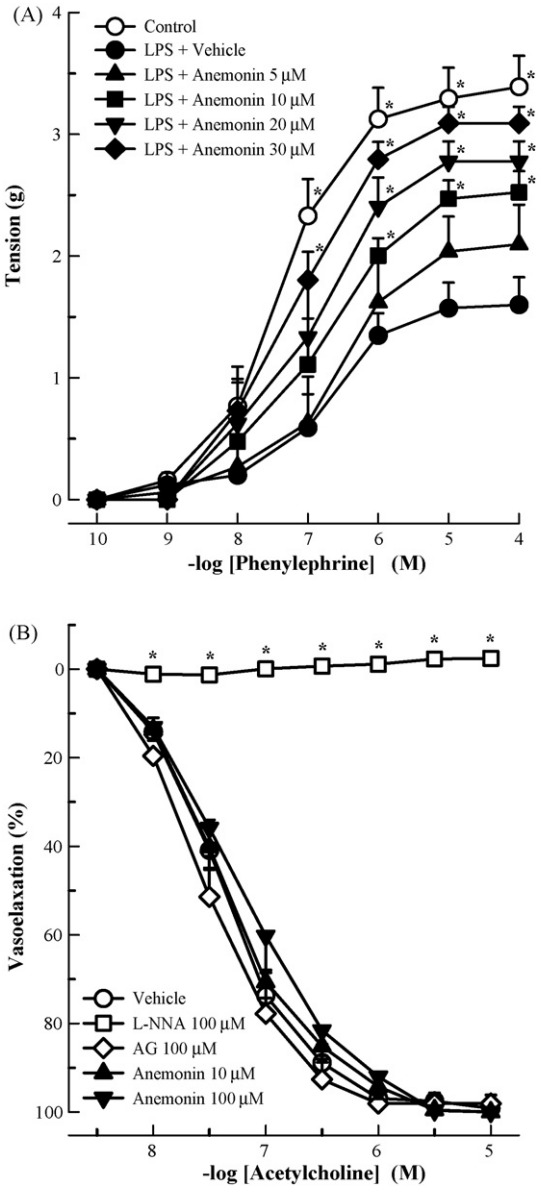

Fig. 7. (A) Effects of anemonin on phenylephrine (1 nM to 100 μM)-induced concentration dependent contraction in endothelium-denuded aortic rings. $n = 6–7$ in each group. $^*P < 0.05$ when compared with LPS plus vehicle group. (B) The vasorelaxing effects of acetylcholine (10 nM to 10 μM) in the presence of anemonin (10, 100 μM), vehicle, L-NNA (100 μM) or aminoguanidine (AG, 100 μM) for 20 min in rat aortic rings with intact endothelium precontracted with phenylephrine (0.3 μM). When no S.E. is shown, it was smaller than the symbol for the mean. $n = 4–5$ in each group. $^*P < 0.05$ when compared with vehicle-treated cells.

4. Discussion

The present study has clearly demonstrated that the EtOAc fraction of *Clematis crassifolia* and its derived active anemonin were able to inhibit iNOS-mediated NO signaling pathway upon LPS stimulation. Such an effect could be contributed to protect against endotoxin-induced response in activated macrophages by selectively modulating the expression and activity of iNOS instead to a direct effect on NO scavenging activity. The findings seem to provide a rationale for the medicinal use of *Clematis crassifolia* and are presumed to be responsible for replacement of *Clematis chinensis* for anti-inflammatory effects.

During infection and inflammation, overexpression of iNOS may be intimately involved in the pathogenesis of many diseases and play a key role in the regulation of immune responses. At the site of an acute inflammatory reaction, all the conditions were met for the generation of NO and for a role of this compound as an inflammatory mediator. To verify whether *Clematis crassifolia* possessed an anti-inflammatory activity justifying the traditional medicinal use of the plant, an in vitro system was used to evaluate its effects on NO production and iNOS protein expression in LPS-induced RAW 264.7 cells, a murine macrophage-like cell line. The observed effect does not seem to be related to the cytotoxicity, since the EtOAc fraction of *Clematis crassifolia* showed no impairment of cell viability. Subsequently, this fraction was further separated and purified using Sephadex-20 column chromatography and reversed phase HPLC by the guidance of NO production assay to yield five compounds including ibotanolide B (**1**) (Masao et al., 1989), calceolarioside B (**2**) (Iossifova et al., 1999), *trans*-caffeic acid (**3**) (Todd et al., 1993), anemonin (**4**) (Mahran et al., 1968), and 3′,4′,5,7-tetrahydroxy-6-C-glucopyranosylflavone (**5**) (Santos et al., 2001) which were identified by their spectroscopic evidences.

In an attempt to evaluate the potential of these compounds, the isolated pure compounds were also tested for their activities on iNOS and cell viability in LPS-stimulated RAW 264.7 cells. Results reveal that both ibotanolide B (**1**) and calceolarioside B (**2**) are able to suppress NO synthesis up to 50–60% but showed somewhat impairment of cell viability. Although *trans*-caffeic acid (**3**) did not exhibit an appreciable cytotoxicity, however, the inhibitory activity on NO production was moderate. In contrast to the other compounds, the effect of compound **5** doesn't seem to be linked to NO production, although it weakly reduced cell viability in the presence of LPS. Of all the compounds tested, anemonin (**4**) has to be considered the active compound present in the EtOAc fraction of *Clematis crassifolia*; in fact it has been able to completely inhibit NO production but without cell toxicity, events currently believed responsible for the anti-inflammatory response. Similar results were found in rat intestinal microvascular endothelial cells as previous report (Duan et al., 2006). Under the same conditions, the positive inhibitors aminoguanidine, a selective iNOS inhibitor, and L-NNA, a non-selective NOS inhibitor, exhibited an inhibition of 85.21 ± 0.87 % and 46.16 ± 3.07 %, respectively, similar to our previous report (Wang et al., 2007).

In order to determine if the observed inhibitory effect of anemonin on the inflammatory mediators was directly related to the modulation of iNOS induction, we examined its protein expression levels by Western blot analysis in activated RAW 264.7 cells. The inhibitory effect of anemonin on iNOS protein expression could account, at least in part, for the suppression of NO production. The expression of iNOS is mainly regulated at the transcriptional level. To investigate this suppression mechanism of anemonin, the mRNA expression of iNOS was further

analyzed. According to our preliminary RT-PCR experiments, the most increases of iNOS mRNA expression were obtained after LPS stimulation for 6 h. After 6 h treatment, anemonin markedly suppressed the LPS-induced iNOS mRNA levels in a concentration-dependent manner without affecting mRNA expression for GAPDH, a house-keeping gene product. In general, the results are consistent with the profile of the inhibitory effect of anemonin on NO release.

Various studies have provided evidence that LPS-elicited induction of arterial iNOS results in subsequent overproduction of NO, which causes vascular hyporesponsiveness to vasoconstrictors, vascular damage, and disseminated intravascular coagulation, ultimately leads to multiple organ dysfunction and death (Rees et al., 1998; Karima et al., 1999). Evidence in the literature suggests that non-selective NOS inhibitors may increase organ ischemia and mortality in experimental models of endotoxic shock (Harbrecht et al., 1992). The results might be attributable to the inhibition of eNOS, which is constitutively present in the endothelial cells and produces physiological levels of NO, leading to the maintenance of vascular tone via its antioxidant and vasorelaxant effects (Moncada et al., 1991). It was therefore postulated that pharmacological inhibition of selective iNOS might be of therapeutic value to improve the vascular hyporeactivity in the LPS-induced inflammation. Anemonin may be described as such a potential candidate based on experiments performed in isolated aortic rings. Our data confirms that aortic rings treated with LPS indeed showed a reduced contractile response to the α_1-adrenergic vasoconstrictor, phenylephrine. Anemonin significantly prevented the vascular hyporeactivity to phenylephrine induced by LPS and thus functional improvement of vascular tone. In addition, it is well established that NO is the major endogenous endothelium-derived vasodilator in large-conduit vessels (Chataigneau et al., 1999). Studies examining the effect on modification of eNOS function can be performed by the acetylcholine-evoked relaxation in isolated endothelium intact aortic rings. In accordance with previous other studies, L-NNA completely inhibited acetylcholine-evoked vasorelaxation whereas aminoguanidine did not change the effect induced by acetylcholine as seen in our results. In the presence of anemonin, acetylcholine-induced endothelial NO-dependent vasorelaxation, an index of eNOS activity, was also not affected. These findings suggest that anemonin, unlike L-NNA, did not modify the activity of eNOS. This compound exhibits significant and comparable selectivity for the inhibition of iNOS and may be developed as a potential therapeutic strategy for anti-inflammation, without changes in vascular tension and systemic blood pressure. In parallel with iNOS assay, we investigated scavenging capacity of anemonin toward NO radicals. According to previous reports (Kagota et al., 2004; Mirkov et al., 2004), we used sodium nitroprusside as donor of NO, which generated by photochemical decomposition and eventually led to stable nitrite ions. Obtained results showed that anemonin did not affect the accumulation of nitrite formed during the reaction of NO with oxygen even at the maximum concentration tested for 150 min; thus, its mode of action presumably does not involve NO scavenging modulation.

5. Conclusion

The anti-inflammatory effect of the EtOAc fraction of *Clematis crassifolia* is related to modulation of iNOS expression which could be linked to the presence of anemonin. Anemonin is a potent selective iNOS inhibitor and has potential in the prevention and treatment of disorders caused by an increased expression of iNOS, although further study is still warranted.

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