In vitro anti-inflammatory effect of picrasmalignan A by the inhibition of iNOS and COX-2 expression in LPS-activated macrophage RAW 264.7 cells

FENG ZHAO[1], LEI CHEN[1], CHENCHEN BI[1], MENGLIN ZHANG[1], WEIHUA JIAO[2] and XINSHENG YAO[3]

[1]School of Pharmacy, Yantai University, Laishan, Yantai, Shandong 264005; [2]Department of Pharmacy, Changzheng Hospital, Second Military Medicinal University, Shanghai 200003; [3]Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, Guangdong 510632, P.R. China

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Abstract. *Picrasma quassioides* (*P. quassioides*) has been widely used as a traditional Chinese medicine for clearing fever and detoxification. Previous phytochemical studies have identified a novel dihydrobenzofuran-type neolignan, picrasmalignan A, isolated from the stems of *P. quassioides*. In the present study, the *in vitro* anti-inflammatory effects and molecular mechanisms of picrasmalignan A have been investigated in cultured RAW 264.7 cells, a mouse macrophage-like cell line. A Griess assay was used to demonstrate the inhibitory effect of picrasmalignan A on the overproduction of nitric oxide (NO). An enzyme-linked immunosorbent assay was used to determine the levels of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). The inhibitory effect on the enzymatic activity of cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) was observed by colorimetric and fluorimetric assays, respectively. Western blotting was conducted to detect the expression of iNOS and COX-2. Results showed that picrasmalignan A suppressed lipopolysaccharide-stimulated NO production and pro-inflammatory cytokine secretion, including TNF-α and IL-6, in a dose-dependent manner. It also significantly inhibited the expression and enzymatic activity of iNOS and COX-2. These results may demonstrate the anti-inflammatory mechanism of picrasmalignan A.

Introduction

Picrasma quassioides Benn. (of the Simaroubaceae family) is a traditional Chinese medicinal plant predominantly found in Southern China. The stems have been used for the treatment of inflammation, gastroenteritis, eczema and snakebites. However, the active constituents of this plant and their mechanisms of action in treating these diseases remain to be elucidated. Previous chemical investigation of this plant has led to the isolation of several alkaloids and quassinoids (aglycones), such as β-carbolines, canthin-6-ones and bis-β-carbolines (1-3). Studies aiming to elucidate the active constituents of *P. quassioides* have isolated and identified 48 compounds, including 25 β-carboline alkaloids, six canthinone alkaloids, 11 bis-β-carboline alkaloids, three lignins, two quassinoids and a flavonol (4-7). In the present study, the anti-inflammatory effects and molecular mechanisms of the neolignan, picrasmalignan A, were investigated.

Following inflammatory stimulation, macrophages produce nitric oxide (NO) and proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. These mediators are highly expressed in macrophages in numerous inflammatory diseases, including rheumatoid arthritis, atherosclerosis and hepatitis (8-10). NO, an important cellular second messenger, is produced via three types of nitric oxide synthase (NOS). Small quantities of NO produced by the constitutive NOS are essential for maintaining normal cellular function. Inducible NOS (iNOS) sustainably produces a high output of NO, which is one of the most important inflammatory reactions in activated macrophages (11).

In addition, two isoforms of cyclooxygenase, COX-1 and COX-2, which are encoded by separate genes, have been identified (12). The COX-1 isozyme is expressed at a constant level and does not fluctuate in response to various stimuli. By contrast, COX-2 is induced by lipopolysaccharide (LPS) and is the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs. Numerous studies have demonstrated that certain inducible enzymes (COX and iNOS), cytokines and their reaction products are involved in chronic inflammatory diseases (13-15).

Thus, the effect of picrasmalignan A on LPS-stimulated proinflammatory mediators (including NO, TNF-α and IL-6) in macrophages, and on the expression of iNOS and COX-2, was also investigated. In addition, the inhibitory effect of

Correspondence to: Professor Feng Zhao, School of Pharmacy, Yantai University, 30 Qingquan Road, Laishan, Yantai, Shandong 264005, P.R. China
E-mail: ytuzhaofeng@163.com

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picrasmalignan A on iNOS and COX-2 enzymatic activity in LPS-stimulated RAW 264.7 cells was demonstrated.

Materials and methods

Isolation and identification of picrasmalignan A. Picrasmalignan A was isolated and identified as previously demonstrated (4). Briefly, the stems of *P. quassioides* (100 kg) were extracted with 95% EtOH and the compounds were isolated from the ethanolic extract. The dried ethanolic extract (200 g) was suspended in water and partitioned with $CHCl_3$, AcOEt and BuOH to yield $CHCl_3$-soluble (128 g), AcOEt-soluble (8.12 g) and BuOH (20.8 g) fractions. The $CHCl_3$-soluble fraction was subjected to silica gel column chromatography eluted with cyclohexane, cyclohexane/AcOEt, AcOEt and MeOH to produce 10 fractions. Fraction 7 (19.2 g) was eluted with cyclohexane/AcOEt 5:5 and then purified by silica gel column chromatography ($CHCl_3$/MeOH gradient) to yield seven subfractions. The fourth subfraction was purified by silica gel column chromatography and high-performance liquid chromatography (HPLC) to yield picrasmalignan A (2.9 mg). Its chemical structure (Fig. 1) was identified by the chemical and physical properties, and spectra data.

Picrasmalignan A: white powder, [α] -13.2˚ (*c*0.5, MeOH); UV (MeOH) λmax (log ε) 208 (3.95), 283 (3.08), 337 (3.15) nm; IR (KBr) νmax 3404, 2942, 1661, 1597, 1497, 1482, 1383, 1330, 1278, 1213, 1138, 1031, 825 cm^{-1}; ^1H NMR (400 MHz, DMSO-*d*6) and ^{13}C NMR (100 MHz, DMSO-*d*6), listed in Table I; ESIMS (positive) *m/z* 557 $[M+Na]^+$, ESIMS (negative) *m/z* 569 $[M+Cl]^-$; HRESIMS *m/z* 535.1941 $[M+H]^+$ (calculated for $C_{30}H_{31}O_9$, 535.1963).

Reagents. RPMI-1640 medium and fetal bovine serum were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). LPS, dimethylsulfoxide (DMSO), MTT and DAF-FM DA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). The penicillin-streptomycin stock solution, mouse TNF-α ELISA, mouse IL-6 ELISA, Bradford protein assay and nitric oxide synthase assay kits were obtained from Yantai Science and Biotechnology Co., Ltd. (Yantai, China). The colorimetric COX (ovine) inhibitor screening assay kit, anti-murine iNOS polyclonal antibody and COX-2 (murine) polyclonal antibody were obtained from Cayman Chemical (Ann Arbor, MI, USA). β-actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Hydrocortisone (H4001; purity HPLC ≥98%; Sigma-Aldrich) was used as a positive control in all experiments.

Cell culture. Mouse monocyte-macrophage RAW 264.7 cells (ATCC TIB-71; American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS at 37˚C in a humidified incubator with 5% CO_2 and 95% air. The medium was replaced every 2 days. RAW 264.7 cells were passaged by trypsinization until 80% confluence was achieved.

Cell viability assay. RAW 264.7 cells were treated with picrasmalignan A at concentrations of 1-100 μM. The mitochondrial-dependent reduction of MTT to formazan was used to measure cell respiration as an indicator of cell viability. Briefly, following 24 h incubation, an MTT solution (final concentration, 200 μg/ml) was added and the cells were incubated for another 4 h at 37˚C. Following the removal of the supernatant, 100 μl DMSO was added to the cells to dissolve the formazan. The absorbance of each group was measured by a microplate reader at a wavelength of 570 nm (Biotek Synergy HT; BioTek Instruments, Inc., Winooski, VT, USA). The control group consisted of untreated cells, which were considered to be 100% viable. Results are expressed as the percentage of viable cells when compared with that of the control group.

NO analysis. NO levels were determined by measuring the quantity of nitrite in the cell culture supernatant using Griess reagent. RAW 264.7 cells were treated with LPS (1 μg/ml) with or without picrasmalignan A (10-100 μM) for 24 h. Cell culture supernatant (100 μl) was mixed with 100 μl Griess reagent and absorbance was measured at 540 nm. The nitrite concentrations were calculated using a standard calibration curve prepared from different concentrations of sodium nitrite.

Measurement of TNF-α and IL-6. RAW 264.7 cells were treated with LPS (1 μg/ml) with or without picrasmalignan A (10-100 μM) for 6 h. The culture supernatant (100 μl) was removed to determine the level of TNF-α and IL-6 using the respective mouse TNF-α and mouse IL-6 ELISA kits, according to the manufacturer's instructions.

Detection of iNOS, COX-2 and β-actin expression. RAW 264.7 cells were washed with cold phosphate-buffered saline (PBS) and lysed in western cell lysis buffer 24 h following treatment. Cell debris was removed by centrifugation. Following the determination of the protein concentration of each aliquot by a commercial Bradford protein assay kit, 30 μg total protein boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer was subjected to gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline with Tween 20 (TBST) at room temperature for 1 h. Following washing, the membranes were incubated in the respective primary antibody solution (anti-iNOS, anti-COX-2 and anti-β-actin antibodies) overnight at 4˚C. The membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody solution for 1 h at room temperature. The blots were washed again three times in TBST, detected using enhanced chemiluminescence (ECL) and exposed to photographic films. Images were collected and the bands corresponding to iNOS, COX-2 and β-actin were quantitated by densitometric analysis using DigDoc100 program (Genetic Technologies, Inc., Glencoe, MO, USA). Data regarding iNOS and COX-2 were normalized on the basis of the β-actin levels.

Assay of iNOS enzymatic activity. RAW 264.7 cells were treated with LPS (1 μg/ml) and picrasmalignan A (3-100 μM) for 2 h at 3˚C, the culture supernatant was removed and 100 μl NOS assay buffer were added to each well. Then 100 μl NOS assay reaction solution (50% NOS assay buffer, 39.8% MilliQ water, 5% L-Arginine solution, 5% 0.1 mM NADPH, 0.2%

Table I. NMR data of picrasmalignan A.

Position	δ_C	δ_H	^1H-^1H COSY	HMBC (H→C)
1	127.7, qC			
2	112.6, CH	7.30 (1H, s)		C-3, C-6, C-7, C-7'
3	144.1, qC			
4	150.6, qC			
5	130.1, qC			
6	118.5, CH	7.30 (1H, s)		C-2, C-4, C-7, C-8'
7	153.9, CH	7.64 (1H, d, *J* = 15.3 Hz)	H-8	C-2, C-6, C-9
8	126.1, CH	6.76 (1H, dd, *J* = 15.3, 7.8 Hz)	H-7, H-9	C-1, C-7, C-9
9	193.9, CH	9.59 (1H, d, *J* = 7.8 Hz)	H-8	C-8
1'	133.7, qC			
2'	110.8, CH	6.88 (1H, s)		C-1', C-4', C-6', C-7'
3'	143.5, qC			
4'	146.5, qC			
5'	129.7, qC			
6'	114.7, CH	6.92 (1H, s)		C-2', C-4', C-7'
7'	88.1, CH	5.60 (1H, d, *J* = 6.8 Hz)	H-8'	C-4, C-1', C-2', C-9'
8'	52.4, CH	3.55 (1H, m)	H-7', H-9'	C-4, C-1', C-5', C-7'
9'	62.6, CH_2	3.75 (1H, m)	H-8'	C-5, C-7'
1"	132.1, qC			
2"	118.9, CH	6.74 (1H, d, *J* = 1.0 Hz)		C-4", C-6", C-7"
3"	147.5, qC			
4"	147.5, qC			
5"	115.3, CH	6.75 (1H, d, *J* = 8.0 Hz)		C-1", C-3", C-6"
6"	110.4, CH	6.90 (1H, dd, *J* = 8.0, 1.0 Hz)		C-1", C-2", C-7"
7"	87.2, CH	5.44 (1H, d, *J* = 6.9 Hz)	H-8"	C-4', C-1", C-2"
8"	52.9, CH	3.46 (1H, d, *J* = 6.4 Hz)	H-7", H-9"	C-4', C-5', C-1"
9"	62.7, CH_2	3.64 (1H, t, *J* = 6.4 Hz)	H-8"	C-7", C-8"
3-OCH_3	55.9, CH_3	3.83 (3H, s)		C-3
3'-OCH_3	55.8, CH_3	3.76 (3H, s)		C-3'
3"-OCH_3	55.6, CH_3	3.73 (3H, s)		C-3"

Note: recorded at 400 MHz for ^1H, and at 100 MHz for ^{13}C, in DMSO-d_6, δ in ppm, *J* in Hz; C-atom numbering according to Fig. 1. COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; NMR, nuclear magnetic resonance; DMSO, dimethylsulfoxide.

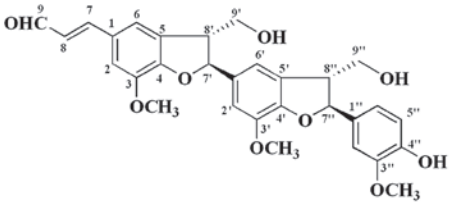

Figure 1. Chemical structure of picrasmalignan A, demonstrating C-atom numbering.

DAF-FM DA) was added to each well and incubated for 2 h at 37˚C. Fluorescence was measured with a fluorescence plate reader (Biotek Synergy HT; BioTek Instruments, Inc.) at excitation 485 nm and emission 528 nm.

Assay of COX-2 enzymatic activity. COX-2 activity was determined by a colorimetric COX inhibitor screening assay

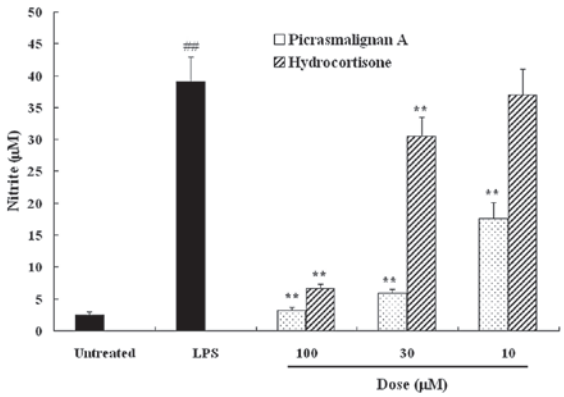

Figure 2. Inhibitory effect of picrasmalignan A on the NO production induced by LPS. RAW 264.7 cells were treated with 1 μg/ml LPS with or without picrasmalignan A (100, 30 and 10 μM) for 24 h. Nitrite concentrations were measured in triplicate. Data are presented as the mean ± SD from three separate experiments. **P<0.01 vs. the LPS treatment group; $^{\#\#}$P<0.01 vs. the untreated group. NO, nitric oxide; LPS, lipopolysaccharide.

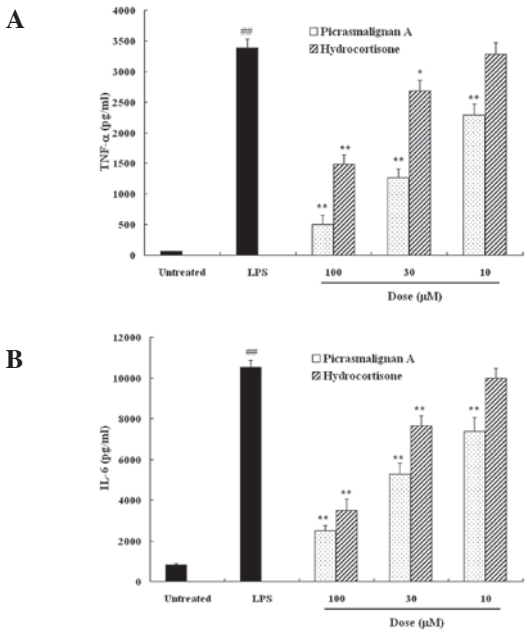

Figure 3. Inhibitory effect of picrasmalignan A on the (A) TNF-α and (B) IL-6 release in RAW 264.7 cells. RAW 264.7 cells were treated with 1 μg/ml LPS with or without picrasmalignan A (100, 30 and 10 μM) for 6 h. TNF-α and IL-6 levels were measured in triplicate. Data are presented as the mean ± SD. *P<0.05 and **P<0.01 vs. the LPS treatment group; $^{\#\#}$P<0.01 vs. the untreated group. TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; LPS; lipopolysaccharide.

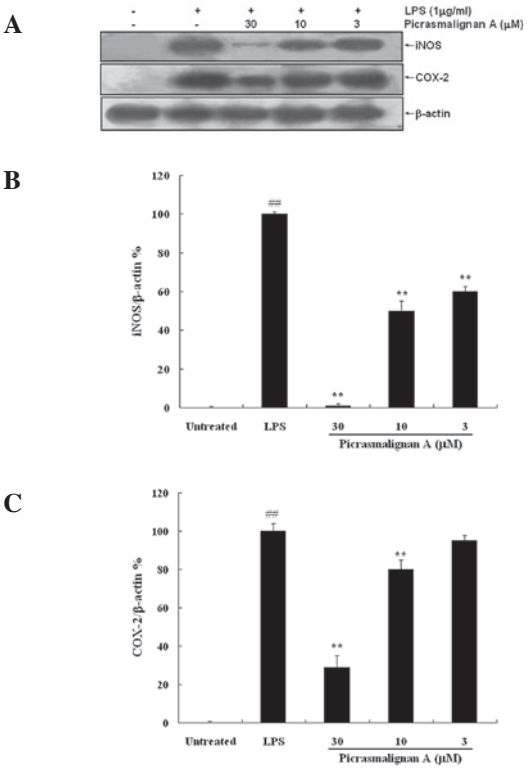

Figure 4. RAW 264.7 cells were treated with LPS 1 μg/ml with or without picrasmalignan A (30, 10 and 3 μM) for 24 h and the expression of iNOS protein and COX-2 was assessed by western blot analysis. (A) Detection of β-actin was carried out to confirm the equal loading of proteins. Densitometric analysis of (B) iNOS protein and (C) COX-2 protein expression represent the mean of three separate experiments. Data were normalized on the basis of β-actin levels. **P<0.01 vs. the LPS group; $^{\#\#}$P<0.01 vs. the untreated group. LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2.

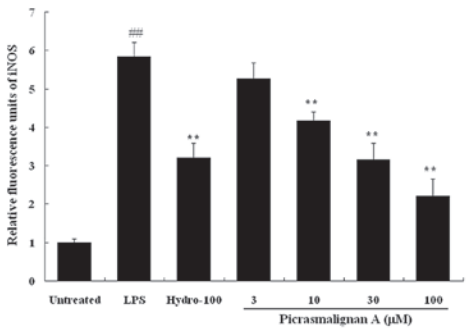

Figure 5. Effect of picrasmalignan A and hydrocortisone on the iNOS enzymatic activity, tested by a fluorimetric assay. RAW 264.7 cells were treated with LPS (1 μg/ml) with or without picrasmalignan A (3, 10, 30 and 100 μM) or 100 μM hydrocortisone for 2 h. The fluorescence was measured at an excitation of 485 nm and emission of 528 nm. The levels of iNOS enzymatic activity were plotted as relative fluorescence units. The experiment was repeated twice and the same result was obtained. **P<0.01 vs. the LPS group; $^{\#\#}$P<0.01 vs. the untreated group. LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase.

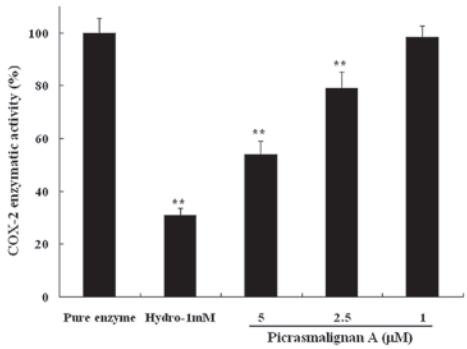

Figure 6. Effect of picrasmalignan A and hydrocortisone on the COX-2 enzymatic activity, tested by a colorimetric assay. The levels of COX-2 enzymatic activity were plotted as relative units compared with that of the pure enzyme. The experiment was repeated twice and the same result was obtained. **P<0.01 vs. the pure enzyme group. COX-2, cyclooxygensase 2.

kit according to the manufacturer's instructions. Briefly, 160 μl assay buffer and 10 μl heme were added to the background wells, while 150 μl assay buffer, 10 μl heme and 10 μl COX-2 enzyme were added to the 100% initial activity wells. Picrasmalignan A (10 ml; final concentrations, 1, 2.5 and 5 mM) was added to the sample wells and 10 μl DMSO was added to the background wells. The plate was carefully shaken for a few seconds and incubated for five min at 25˚C. The colorimetric substrate solution (20 μl) followed by arachidonic acid (20 μl) were added to each well. The plate was again shaken carefully for a few seconds and incubated for 5 min at 25˚C. The absorbance at 590 nm was read by a microplate reader and the inhibition ratio of COX-2 enzymatic activity was calculated according to the manufacturer's instructions.

Statistical analysis. All results are presented as the mean ± standard deviation. Statistical comparison was conducted using Student's t-test following analysis of variance (ANOVA). P<0.05 was considered to indicate a statistically significant difference.

Results

RAW 264.7 cells were treated with various concentrations of picrasmalignan A for 24 h and the cell viability was tested by an MTT assay as described in Materials and methods. Picrasmalignan A did not demonstrate cytotoxicity in the range of 1-100 μM (data not shown). RAW 264.7 cells were then treated with 1 μg/ml LPS with or without the indicated concentrations of picrasmalignan A or hydrocortisone. After 24 h, nitrite concentrations were determined as an indicator of the NO production. As shown in Fig. 2, picrasmalignan A significantly inhibited the LPS-induced production of NO to an even greater extent than the positive control hydrocortisone, a commonly used anti-inflammatory drug. RAW 264.7 cells were treated with 1 μg/ml LPS with or without the indicated concentrations of picrasmalignan A or hydrocortisone for 6 h, and the resulting pro-inflammatory cytokine levels of TNF-α and IL-6 in the supernatant were determined by an ELISA assay. As shown in Fig. 3, LPS-induced TNF-α and IL-6 release were significantly suppressed by picrasmalignan A in a dose-dependent manner. Furthermore, the inhibitive action of picrasmalignan A was more potent than that of the positive control, hydrocortisone. These results demonstrated that picrasmalignan A significantly inhibited the expression of LPS-induced proinflammatory mediators, such as NO, TNF-α and IL-6, in macrophages, which may be responsible for its anti-inflammatory effect.

As the excess production of NO is correlated with the upregulation of inducible nitric oxide synthase (iNOS) expression, the expression of iNOS and COX-2 was investigated by western blot analysis. As shown in Fig. 4A, treatment with 30 μM picrasmalignan A almost completely inhibited the expression of iNOS and significantly inhibited the LPS-induced overexpression of COX-2. The density of bands corresponding to the iNOS and COX-2 proteins were normalized on the basis of β-actin and are shown in Fig. 4B and 4C, respectively.

The inhibitory effects of picrasmalignan A on the activity of the iNOS and COX-2 enzymes were determined. As shown in Fig. 5, treatment with 1 μg/ml LPS led to an ~6-fold increase of iNOS enzymatic activity within 2 h. The indicated concentrations of picrasmalignan A (3, 10, 30 and 100 μM) markedly inhibited the iNOS enzymatic activation in RAW 264.7 cells and demonstrated dose-dependency. Picrasmalignan A (2.5 and 5 mM) also significantly inhibited the activity of the COX-2 enzyme, similarly to that of the positive control, hydrocortisone (Fig. 6).

Discussion

P. quassioides is a commonly used traditional Chinese medicine predominantly used to treat fever and inflammation.. It has previously been demonstrated that β-carboline alkaloids (the predominant active constituents of this medicinal plant) exert anti-inflammatory effects through the inhibition of the iNOS pathway but independent of the COX-2 pathway (16). In the present study, another type of natural compound neolignan, picrasmalignan A, was further examined for its possible anti-inflammatory molecular mechanism. The results demonstrated that picrasmalignan A blocked various LPS-induced macrophage responses including the increased production of inflammatory mediators (NO, TNF-α, IL-6), the overexpression of iNOS and COX-2, and the activity of iNOS and COX-2 enzymes. To the best of our knowledge, this is the first study to demonstrate the anti-inflammatory effect of neolignan agents. These results also suggested that neolignan and β-carboline alkaloids coexist in *P. quassioides* and may exert the anti-inflammatory effect through different pathways.

Acknowledgements

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