

## Effect of Three Herbal Extracts on NO and PGE<sub>2</sub> Production by Activated Mouse Macrophage-like Cells

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**Abstract.** Three Chinese herbal extracts, *Drynaria baronii*, *Angelica sinensis* and *Cornus officinalis* Sieb. et Zucc (referred to as DB, AS, CO, respectively), were investigated for their possible anti-inflammatory activity. DB, AS and CO inhibited nitric oxide (NO) production by lipopolysaccharide (LPS)-activated mouse macrophage-like RAW264.7 cells. Western blot and RT-PCR analyses demonstrated that this was due to the inhibition of inducible NO synthase (iNOS) expression at both protein and mRNA levels. Electron-spin resonance spectroscopy showed that DB, AS and CO dose-dependently scavenged the NO radical produced by NOC-7 in the presence of carboxy-PTIO. In order to confirm the anti-inflammatory potency, effects on prostaglandin (PG) E<sub>2</sub> production and the expression of enzymes involved in the arachidonic acid pathway were next investigated. DB and CO effectively inhibited the PGE<sub>2</sub> production by LPS-stimulated RAW264.7 cells, although the extent of inhibition of PGE<sub>2</sub> production was slightly lower than that of NO production. AS only marginally inhibited the LPS-stimulated PGE<sub>2</sub> production. DB, AS and CO inhibited cyclooxygenase (COX)-2 expression at both protein and mRNA levels, but to much lesser extents as compared with that for iNOS expression. These data further substantiate the anti-inflammatory potency of DB, AS and CO.

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**Key Words:** Herbal extract, RAW264.7 cells, PGE<sub>2</sub>, COX-2, NO, iNOS, anti-inflammatory activity.

Chinese herbal extracts, such as *Drynaria baronii*, *Angelica sinensis* and *Cornus officinalis* Sieb. et Zucc (referred to as DB, AS, CO, respectively), have been reported to display diverse biological activities: the osteogenic (1) and bone resorption inhibitory (2) activities of DB; the radioprotective (3), antioxidative (4) and hematopoietic (3) activities of AS; and the antibacterial (5), anti-diabetic (6), antiarrhythmic (7) and antioxidant (8, 9) activities of CO. We have recently found that DB, AS and CO displayed potent radical scavenging activity against superoxide anion and hydroxyl radical (10). This suggests that these extracts might show anti-inflammatory activity. Previous studies of the anti-inflammatory activity of DB, AS and CO have been limited mostly to the inhibition of some inflammatory cytokines (11-13).

Therefore, we investigated here whether DB, AS and CO inhibit nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production by activated macrophages, and if so, whether they inhibit inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) expression that regulate the amount of NO and PGE<sub>2</sub>, respectively. For this purpose, mouse macrophage-like RAW264.7 cells, established from the peritoneal fluid of BALB/c mice (14) were used. Lipopolysaccharide (LPS)-activated RAW264.7 cells have provided a model system for the study of signal transduction during the macrophage activation (15, 16) and the search for substances that modify the macrophage function (17).

### Materials and Methods

**Materials.** The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM), phenol-red free DMEM (exogenously supplemented with glucose and glutamine so as to make the same concentration in DMEM) (GIBCO BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); LPS from *Escherichia coli* (Serotype 0111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chem Co., St. Louis, MO, USA); protease inhibitor cocktail (CALBIOCHEM, La

Jolla, CA, USA); 1-hydroxyl-2-oxo-3-*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) (Dojin, Kumamoto, Japan).

**Preparation of herbal extracts.** *D. baronii*, *A. sinensis* and *C. officinalis* Sieb. et Zucc were supplied by the Department of Pharmacology, School of Stomatology, The Fourth Military Medical University, Xi'an, China, and extracted for 2 hours twice with 12 volumes of water at 100°C. The supernatants, obtained after centrifugation at 3,000 rpm for 10 minutes, were lyophilized to a dried powder (referred to as DB, AS, CO, respectively). One g of dried powder was obtained from 3.3 g (DB), 2.2 g (AS) and 2.15 g (CO) solid raw material, respectively. Dried powder was dissolved in sterile distilled water at the concentration of 100 mg/ml, and stored at -30°C until use.

**Cell culture.** RAW264.7 cells (kindly supplied by Professor Ohmori, Meikai University) were cultured as an attached monolayer culture in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO<sub>2</sub> atmosphere (17, 18). When the cells were grown to confluency, they were detached by splashing the medium to them through the pipette, and seeded at a lower cell density. Cells were not collected by scraping them out from the plate with a rubber policeman, since the scraping procedure damaged the cells (unpublished data).

**Determination of viable cell number.** RAW264.7 cells were inoculated at 1.5×10<sup>6</sup>/ml (100 µl) in a 96-microwell plate (Becton Dickinson) and incubated for 1-2 hours. Near-adherent cells were given with fresh culture medium and then treated for 24 hours with the indicated concentrations (0-50 mg/ml) of DB, AS or CO in phenol red-free DMEM supplemented with 10% FBS in the presence or absence of LPS (0.1 µg/ml). Viable cell number was then determined by MTT method. In brief, cells were incubated for 30 minutes with 0.2 mg/ml of MTT. After removal of the medium, the cells were lysed with DMSO, and the absorbance at 540 nm was recorded using a microplate reader. The 50% cytotoxic concentration (CC<sub>50</sub>) was determined from the dose-response curve (17, 18).

**Assay for NO production.** RAW264.7 cells were inoculated at 1.5×10<sup>6</sup>/ml (100 µl) in a 96-microwell plate and incubated for 1-2 hours. Near confluent cells were treated for 24 hours with the indicated concentrations (0-50 mg/ml) of DB, AS or CO in phenol red-free DMEM supplemented with 10% FBS in the presence or absence of LPS (0.1 µg/ml). The NO production by RAW264.7 cells was quantified by Greiss reagent, using the standard curve of NO<sub>2</sub><sup>-</sup>. To eliminate the interaction between sample and Greiss reagent, the NO concentration in the culture medium without the cells was also measured and subtracted from that obtained with the cells. The concentration that inhibited LPS-stimulated NO production by 50% (50% inhibitory concentration: IC<sub>50</sub>) was then determined from the dose-response curve (17). The efficacy of inhibition of NO production was evaluated by the selectivity index (SI), which was calculated using the following equation: SI=CC<sub>50</sub>/IC<sub>50</sub>.

**Assay for prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production.** RAW264.7 cells were inoculated at 1.5×10<sup>6</sup>/ml in a 96-microwell plate and incubated for 1-2 hours. Near-adherent cells were treated for 24 hours with different concentrations (0-5 mg/ml) of DB, AS or CO in the

presence of LPS (0.1 µg/ml). The culture supernatant was collected by centrifugation, and determined for PGE<sub>2</sub> concentration by EIA kit (Cayman Chemical Co, Ann Arbor, MI, USA) (19).

**Assay for iNOS and COX-2 protein expression.** RAW264.7 cells were inoculated at 1.5×10<sup>6</sup>/ml in 96-microwell plates (Becton Dickinson) and incubated for 1-2 hours. Near-adherent cells were treated for 24 hours with different concentrations of DB (0-3 mg/ml), AS (0-6 mg/ml) or CO (0-3 mg/ml) in the presence or absence of LPS (0.1 µg/ml). The cell pellets were lysed with 50µl of lysis buffer [10mM Tris-HCl (pH 7.6), 1% Triton® X-100, 150 mM NaCl, 5 mM EDTA-2Na, 2 mM phenylmethylsulfonyl fluoride and 1 × protease inhibitor cocktail] for 10 minutes on ice. The cell lysates were centrifuged at 16,000×g for 20 minutes at 4°C to remove the insoluble materials and the supernatant was collected. The protein concentrations of supernatant were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). An equal amount of the protein from cell lysates (10 µg) was mixed with 2 × sodium dodecyl sulfate (SDS)-sample buffer [0.1 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.01% bromophenol blue, 1.2% 2-mercaptoethanol], boiled for 10 minutes, and applied to SDS-8% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in phosphate-buffered saline [PBS (-)] plus 0.05% Tween 20 for 90 minutes and incubated for 90 minutes at room temperature with anti-COX-2 or anti-iNOS (dilution: 1:2,000 and 1:1,000, respectively; Santa Cruz Biotechnology, Delaware, CA, USA) or anti-actin antibody (1:6,000, Sigma), and then incubated with horseradish peroxidase-conjugated anti-goat, anti-rabbit or anti-mouse IgG for 60 minutes at room temperature. Immunoblots were developed with Western Lighting™ Chemiluminescence Reagent Plus (17).

**Assay for iNOS and COX-2 mRNA expression.** RAW264.7 cells were inoculated at 1.5×10<sup>6</sup>/ml in 24-well plates and incubated for 1-2 hours. Near-confluent cells were treated for 24 hours with different concentrations of DB (0-3 mg/ml), AS (0-6 mg/ml) or CO (0-3 mg/ml) in the presence or absence of LPS (0.1 µg/ml). The expression levels of iNOS and COX-2 mRNA were measured by RT-PCR. The primer sequence and size were: (i) iNOS: PCR product size 496 bp, sense primer 5'-CCCTTCCGAAGTTCTGGCAGCAGC-3', antisense primer 5'-GGCTGTCAGAGCCCTCGTGGCTTTGG-3'; (ii) COX-2: Product size 371 bp, sense primer 5'-TTTGTGTAGTCA TTCACC AGACAGAT-3', antisense primer 5'-CAGTATTGAGGAGAACAG ATGGGATT-3'; (iii) G3PDH: Product size 452 bp, sense primer 5'-ACCACAGTCCATGCCATCAC-3'; antisense primer 5'-TCCACCA CCCTGTTGCTGTGA-3' (17).

**NO radical-scavenging activity.** The radical intensity of DB, AS and CO was determined at 25°C, using electron-spin resonance (ESR) spectroscopy (JEOL JES REIX Tokyo, Japan; X-band, 100 kHz modulation frequency, microwave power 5 mW, gain 400) (17). The radical intensity of NO, produced from the reaction mixture of 20 µM carboxy-PTIO and 60 µM NOC-7, was determined in 0.1 M phosphate buffer, pH 7.4 in the presence of 30% DMSO. When NOC-7 and carboxy-PTIO were mixed, NO was oxidized to NO<sub>2</sub> and carboxy-PTIO was reduced to carboxy-PTI, which produces seven-line signals. Samples were added 3 minutes after mixing. The NO radical intensity was defined as the ratio of the signal intensity of the first peak of carboxy-PTI to that of MnO, and expressed as the ratio

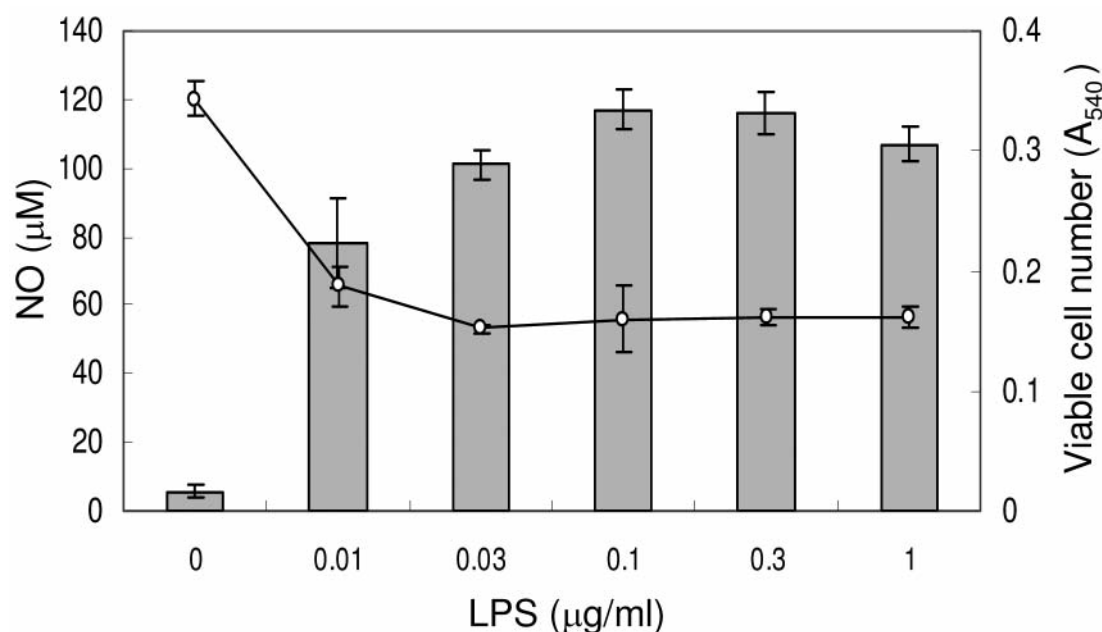


Figure 1. Optimal concentration of LPS for the induction of NO production by RAW264.7 cells. RAW264.7 cells were incubated for 24 hours with the indicated concentrations of LPS, and the viable cell number and extracellular concentration of NO were then measured. Each value represents the mean±SD of triplicate assays.

to the height of MnO an external marker. The concentration that reduced the NO radical intensity by 50% (IC<sub>50</sub>) was determined from the dose-response curve (17).

## Results

**Optimal concentration of LPS.** We previously reported that LPS (0.1 µg/ml) enhanced the NO (17) and PGE<sub>2</sub> (19) production by RAW264.7 cells. We first confirmed that 0.1 µg/ml of LPS was the optimum concentration for the induction of NO production by RAW264.7 cells and also for growth inhibition (Figure 1). This concentration of LPS was used in the subsequent experiments.

**Inhibition of NO production.** All three herb extracts failed to stimulate RAW264.7 cells to produce detectable amounts of NO, but did inhibit the LPS-stimulated NO production dose-dependently (Figure 2). The cytotoxicity of DB was the highest (CC<sub>50</sub>=11.5 and >50 mg/ml in the absence or presence of LPS, respectively), followed by CO (19.5 and 24.2 mg/ml) and then AS (20.6 and 32.2 mg/ml). DB most efficiently inhibited the LPS-stimulated NO production with the highest selectivity SI (IC<sub>50</sub>=0.93 mg/ml; SI>53.8), followed by CO (IC<sub>50</sub>=2.1 mg/ml, SI=11.7) and AS (IC<sub>50</sub>=12.6 mg/ml, SI=2.6) (Table I).

It should be noted that DB at the highest concentration (50 mg/ml) induced NO production even in the absence of LPS. CO showed similar NO-inducing activity, but to a much lesser extent. This was not due to the interference of

Table I. Inhibition by DB, AS, CO of LPS-induced NO production.

	CC <sub>50</sub> (mg/ml)		IC <sub>50</sub> (mg/ml)	SI=CC <sub>50</sub> /IC <sub>50</sub>
	LPS (-)	LPS (+)	LPS (+)	
DB	11.5	>50	0.93	>53.8
AS	20.6	32.2	12.6	2.6
CO	19.5	24.2	2.1	11.7

Each value represents the mean of three independent experiments.

Table II. Inhibition by DB, AS, CO of LPS-induced stimulated PGE<sub>2</sub> production

	CC <sub>50</sub> (mg/ml)	IC <sub>50</sub> (mg/ml)	SI=CC <sub>50</sub> /IC <sub>50</sub>
	LPS (+)	LPS (+)	
DB	>50	0.72	>69.5
AS	32.2	>10	<3.2
CO	24.16	0.58	41.9

Each value represents the mean of triplicate assays.

NO determination by the brownish color they have, since we have subtracted the value of absorbance without cells from the value with the cells to reduce the contribution of the color.

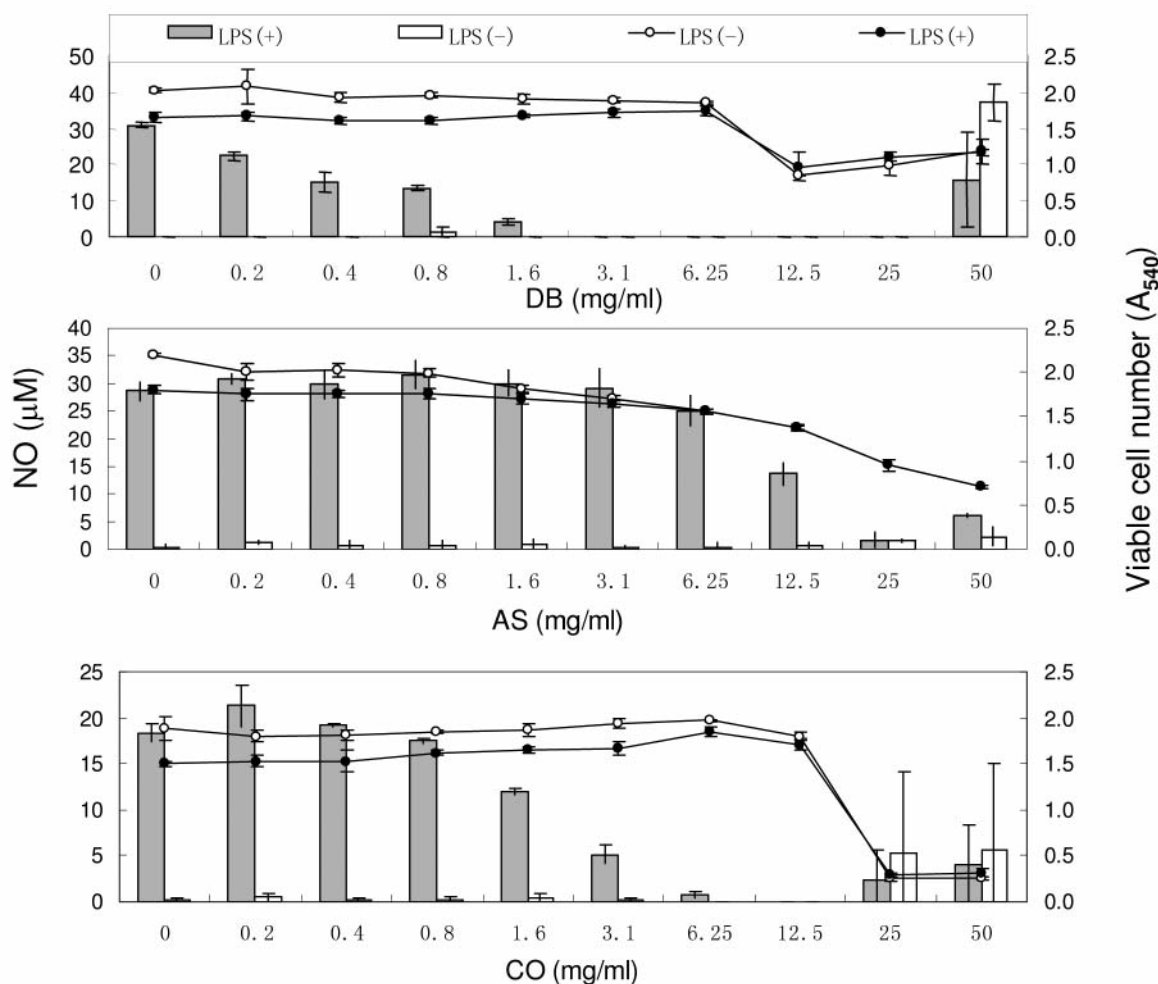


Figure 2. Inhibition by herbal extracts of LPS-stimulated NO production. RAW264.7 cells were incubated for 24 hours with the indicated concentrations of DB, AS or CO in the presence or absence of  $0.1 \mu\text{g/ml}$  LPS and the viable cell number (absorbance at 540 nm determined by MTT method) (circles) and extracellular concentration of NO (determined by Griess Method) (bars) were then measured. Each value represents the mean  $\pm$  SD of three independent experiments.

**Effects on  $\text{PGE}_2$  production.** All three herb extracts dose-dependently inhibited the  $\text{PGE}_2$  production by LPS-activated RAW264.7 cells (Figure 3). DB most efficiently inhibited the LPS-stimulated  $\text{PGE}_2$  production with the highest SI value ( $\text{IC}_{50}=0.72 \text{ mg/ml}$ ;  $\text{SI}=69.5$ ), followed by CO ( $\text{IC}_{50}=0.58 \text{ mg/ml}$ ,  $\text{SI}=41.9$ ) and AS ( $\text{IC}_{50}>10.0 \text{ mg/ml}$ ,  $\text{SI}<3.2$ ) (Table II).

**Effects on iNOS and COX-2 protein expression.** We next investigated whether the inhibition of NO or  $\text{PGE}_2$  production by herbal extracts was due to the reduced expression of iNOS or COX-2 protein expression. Western blot analysis showed that this was indeed the case. It is apparent that DB (1 mg/ml), CO (1 mg/ml) and AS (2 mg/ml) almost completely inhibited LPS-stimulated iNOS protein expression (Figure 4). DB tended to inhibit LPS-stimulated COX-2 protein expression,

but to much lesser extent than that observed for iNOS protein expression (Figure 4). However, AS and CO did not apparently affect the COX-2 protein expression.

**Effect on iNOS and COX2 mRNA expression.** RT-PCR experiments demonstrated that the reduced expression of iNOS and COX-2 proteins was due to the inhibition of mRNA expression (Figure 5). DB (0.1 mg/ml), CO (0.3 mg/ml) and AS (2 mg/ml) almost completely inhibited iNOS mRNA expression. DB (0.1 mg/ml) and CO (1 mg/ml) almost completely inhibited the COX-2 mRNA expression, whereas AS was much less active.

**NO-scavenging activity.** The amount of NO is regulated by both synthesis and degradation. We next investigated the

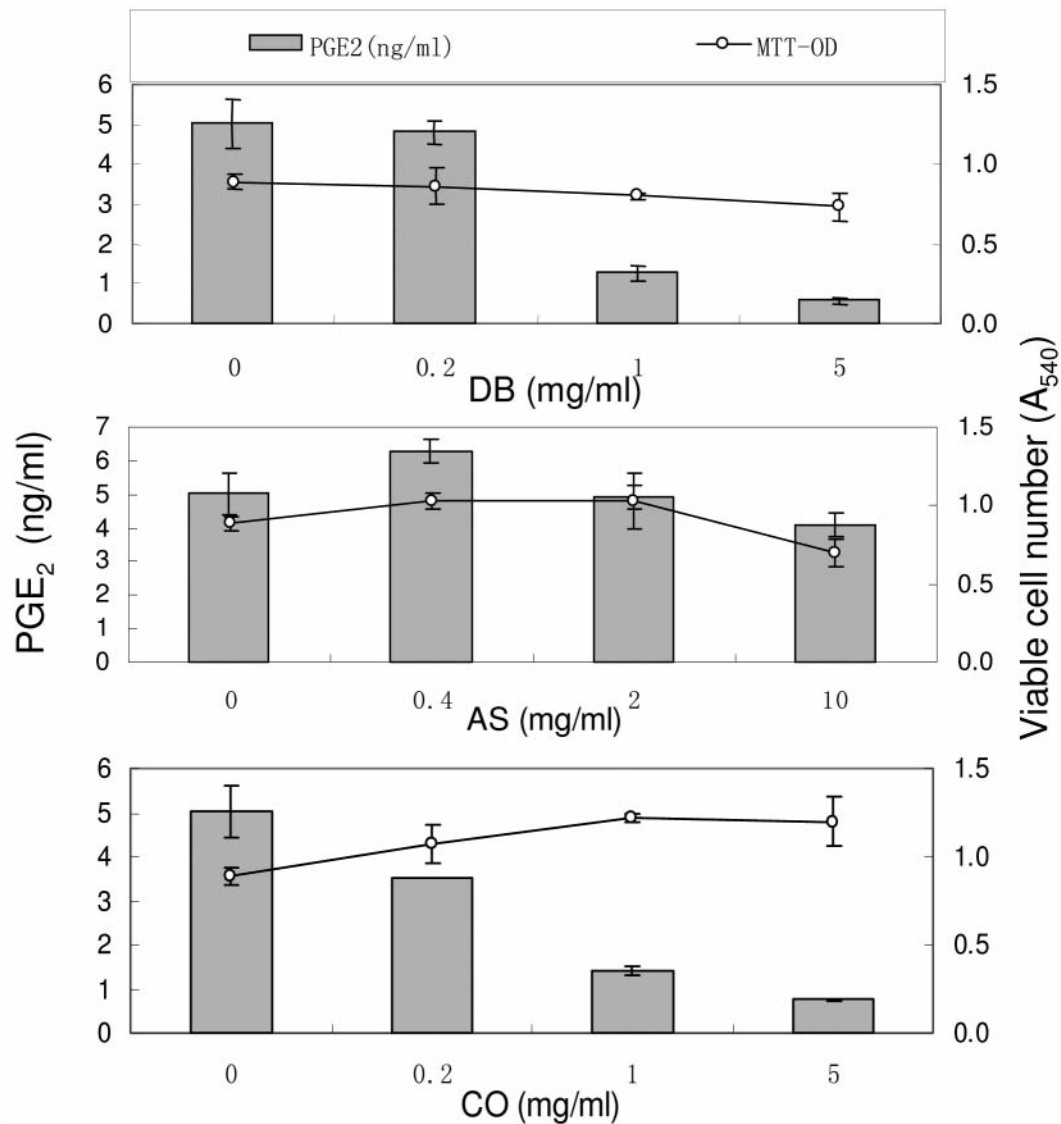


Figure 3. Inhibition by herbal extracts of LPS-stimulated PGE<sub>2</sub> production. RAW264.7 cells were incubated for 24 hours with different concentrations of herbal extracts in the presence of 0.1  $\mu$ g/ml LPS, and then the viable cell number (absorbance at 540 nm determined by MTT method) (circles) and extracellular PGE<sub>2</sub> concentration (determined by EIA) (bars) were determined. Each value represents the mean  $\pm$  SD of three independent experiments.

possibility that the reduced amount of NO recovered from the LPS-stimulated RAW264.7 cells in the presence of herbal extracts might be due to their direct scavenging activity against NO. The *in vitro* experiment with ESR spectroscopy shows that this was the case: DB, AS and CO dose-dependently reduced the radical intensity of NO, generated from NOC in the presence of carboxy-PTIO, with extrapolated IC<sub>50</sub> values of >0.30, >3.0 and 0.29 mg/ml, respectively, lower than their corresponding IC<sub>50</sub> values for inhibition of NO production (0.93, 12.6 and 2.1 mg/ml, respectively) (Figure 6).

## Discussion

NO plays a crucial role in the vascular, neural, endocrine and immune systems, however, excessive NO can injure tissue (20). Nitric oxide synthase (NOS) catalyzes the production of NO from L-arginine. There are three isoforms of NOS: the neural form (nNOS), the endothelial form (eNOS) and the inducible form (iNOS) (21). Whilst nNOS and eNOS activities are constitutive and calcium dependent, iNOS activity is calcium independent and induced by pro-inflammatory stimuli (21, 22). The present



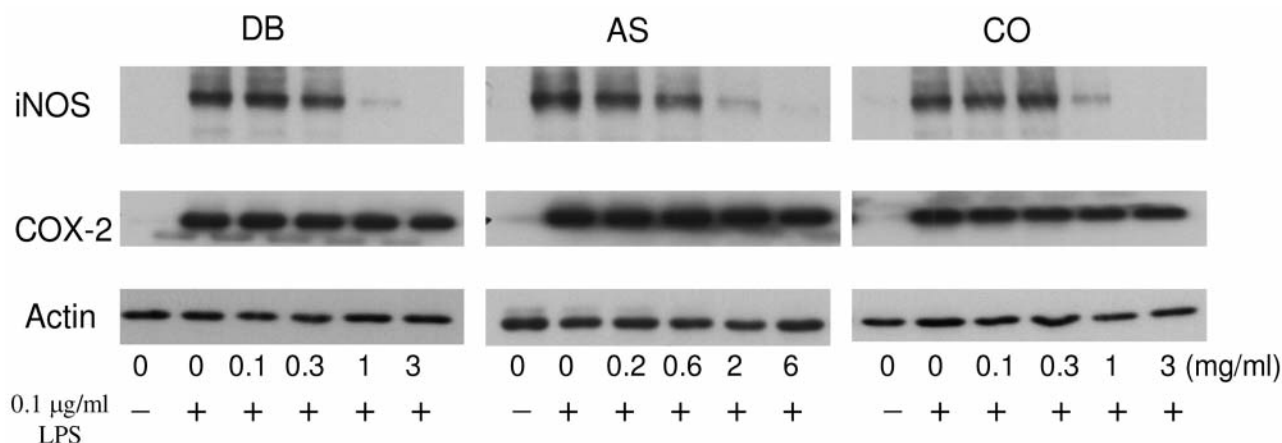


Figure 4. Inhibition of iNOS and COX-2 protein expression by herbal extracts in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated for 24 hours with the indicated concentrations of DB (0-3 mg/ml), AS (0-6 mg/ml) or CO (0-3 mg/ml) in the presence or absence of LPS (0.1 µg/ml) and processed for Western blot analysis for iNOS, COX-2 and actin (internal marker) protein expression. Representative patterns of Western blot analysis of three independent experiments are shown.

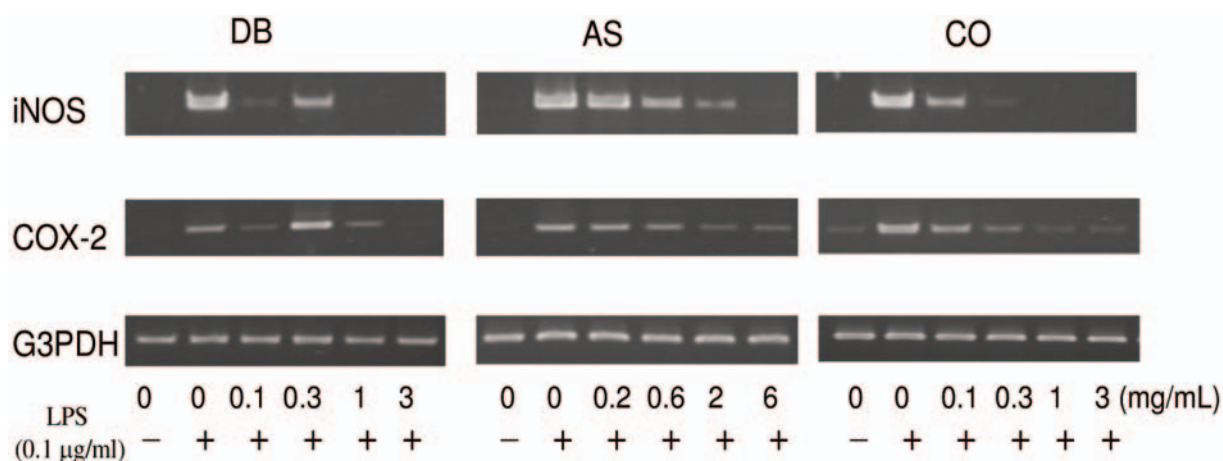


Figure 5. Inhibition of iNOS and COX-2 mRNA expression by herbal extracts in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated for 24 hours with the indicated concentrations of DB, AS or CO in the presence or absence of LPS (0.1 µg/ml) and processed for RT-PCR analysis for iNOS, COX-2 and G3PDH (internal marker) mRNA expression. Similar data were obtained in another two experiments.

study demonstrates that non-cytotoxic concentrations of DB, AS and CO inhibited the NO production by LPS activated macrophage-like RAW264.7 cells. This was due to the inhibition of iNOS protein and mRNA expression, since the same concentrations of herbal extracts inhibited both NO production and iNOS expression (at protein and mRNA levels) to comparable extents (Figure 5). The NO scavenging activities of DB, AS and CO may further reduce the active concentration of NO in the culture medium. These extracts may thus be expected to alleviate NO-mediated diseases and exert an anti-inflammatory effect.

The anti-inflammatory potency of DB, AS and CO was further substantiated by our finding that these extracts inhibited the production of PGE<sub>2</sub>, an inflammatory mediator (23-25), by LPS-stimulated RAW264.7 cells. PG synthesis begins with the liberation of arachidonic acid (AA), the prime precursor, from membrane phospholipids by phospholipase A<sub>2</sub> (PLA<sub>2</sub>). Subsequently, cyclooxygenase (COX) catalyzes the rate-limiting reactions for PG synthesis comprising the *bis*-cyclooxygenation of AA to form PGG<sub>2</sub> and the peroxidative reduction of the intermediate to PGH<sub>2</sub> (26). The cPLA<sub>2α</sub> is activated by pro-inflammatory cytokines or growth factors and catalyzes the release of AA

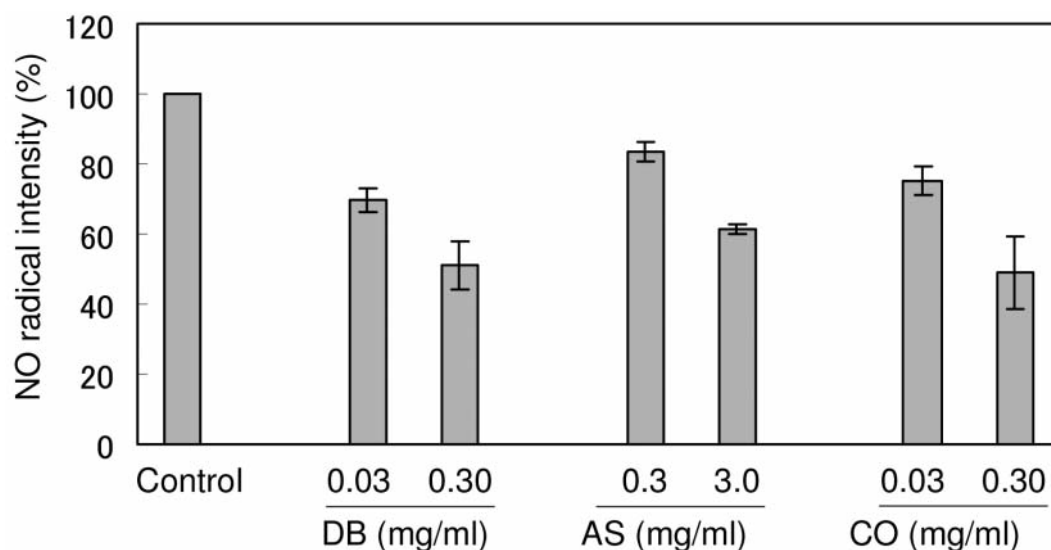


Figure 6. NO radical-scavenging activity of herbal extracts. Each value represents the mean  $\pm$  S.D. of three independent experiments. IC<sub>50</sub> values were determined by the extrapolation of the data. The reducing activity of DB, AS and CO against C-PTI was corrected.

from the cell surface membrane (27, 28). It is known that COX has two isoforms, COX-1 (constitutive isoform) and COX-2 (inducible isoform) (29). It was unexpected that DB, AS and CO inhibited COX-2 expression to much lower extent, as compared with that of iNOS expression. It remains to be investigated whether these extracts inhibit the expression of PGE<sub>2</sub> synthase (30-32), or the activation of COX-2 by S-nitrosylation (33).

The present study demonstrated that among three herbal extracts, DB most effectively inhibited NO and PGE<sub>2</sub> production, and iNOS and COX-2 expression at both the protein and mRNA level. DB produced NO at the highest concentration (Figure 2). Furthermore, DB produced a broad ESR signal and had synergistic action with vitamin C (10), properties similar to those observed for lignin carbohydrate complex that stimulates the NO production (34). It remains to be investigated whether DB contains both inhibitors and stimulators for iNOS and COX-2 expression.

We observed that all herbal extracts (DB, AS, CO) had little or no hormetic effect (growth stimulatory effect observed at lower concentrations) (35, 36) against RAW264.7 cells, further confirming our recent finding that these extracts enhanced the growth of human oral normal and tumor cells at most 23% at their lower concentrations (10).

In conclusion, the present study demonstrated that three herbal extracts (DB, AS and CO) not only reduced LPS-stimulated NO and PGE<sub>2</sub> production, but also down-regulated the mRNA and protein expression of COX-2 and iNOS, further substantiating the previous reports of anti-inflammatory effects of DB, AS and CO (11-13).

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Received December 23, 2008

Revised March 9, 2009

Accepted April 2, 2009