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Inducible Nitric Oxide Synthase Inhibitors of Chinese Herbs III. *Rheum palmatum*

Abstract

In this paper, the effects of bioactive compounds of *Rheum palmatum* L. on the inhibition of NO production from RAW 264.7 cells were explored. Seven main anthraquinone derivatives were isolated from the root of *R. palmatum,* and of these, emodin and rhein significantly inhibited nitrite production from lipopolysaccharide (LPS)-activated RAW 264.7 cells. The IC_{50} values for inhibition of nitrite production by emodin and rhein were 60.7 and 67.3 μM, respectively. After iNOS enzyme activity was stimulated by LPS for 12 h, treatment with emodin or rhein at 20 μg/ml for 18 h did not significantly inhibit NO production. The data show that the inhibitory activity of emodin and rhein is not due to direct inhibition of iNOS enzyme activity. However, expression of iNOS and the COX-2 protein was inhibited by emodin in LPS-activated RAW 264.7 cells, and PGE_2 production was reduced. Rhein also inhibited LPS-induced iNOS protein expression, but not COX-2 or PGE_2 production. On the other hand, inhibition effects on NO production from RAW 264.7 cells were enhanced and cytotoxic effects decreased by co-treatment with emodin and rhein. In conclusion, emodin and rhein are major iNOS inhibitors of *R. palmatum* and may possibly serve as bioactive substances for anti-inflammation effects.

Key words
Inducible nitric oxide synthase · *Rheum palmatum* · Polygonaceae emodin · Rhein · COX-2 · RAW 264.7 murine macrophages

Introduction

A number of traditional herb-derived medicines has been developed as anticancer drugs, free radical scavengers, etc. [1]. In this paper, we describe the bio-pharmacological compounds of the roots of *R. palmatum*, rhubarb (Polygonaceae). Rhubarb, an important traditional Chinese medicinal herb, has a strong antibacterial action and has been used for the treatment of bacterial dysentery [2]. Moreover, rhubarb also has been used as a crude drug and is employed as an antipyretic in the treatment of common fever by traditional doctors in Taiwan. The extract of rhubarb has been shown to exhibit anti-inflammatory activities [3]. The main constituents of rhubarb are anthraquinone derivatives including aloe-emodin, chrysophanol, emodin, physcion, rhein, and sennosides A and B (Fig. **1**). However, the exact mechanism of anti-inflammatory action of these compounds remains unclear. The possibility that these compounds exhibit their biological effects by blocking inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression, therefore, was examined in the present study.

Inducible NOS is an important pharmacological target in inflammation and mutagenesis research [4]. Therefore, inhibition of NO production by iNOS may have potential therapeutic value when related to inflammation and septic shock. NO is known to cause mutagenesis [5] and deamination of DNA bases [5] and to play an important role in the formation of carcinogenic *N*-nitroso compounds *in vivo* [6]. NO rapidly and spontaneously reacts with tri-

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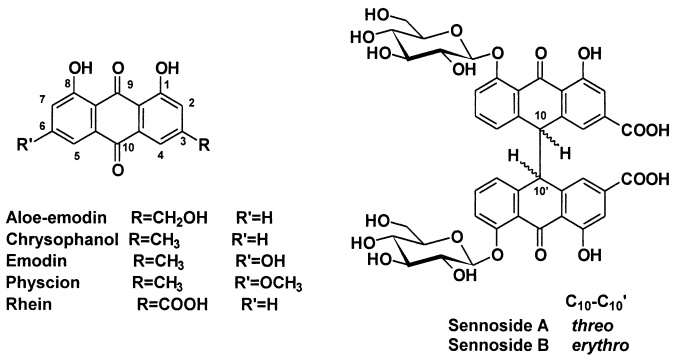

Fig. **1** Structures of anthraquinones of *Rheum palmatum* L.

plet oxygen ($^3O_2$) to form stable anions, nitrite, and nitrate [7]. These compounds non-enzymatically *N*-nitrosylate primary and secondary amines to produce carcinogenic nitrosamines [6]. Moreover, under inflammatory conditions, macrophages can greatly increase their production of both NO and the superoxide anion (O^{2-}) simultaneously, which rapidly react with each other to form the peroxynitrite anion ($ONOO^-$), and thus play a role in inflammation and also possibly in the multistage process of carcinogenesis [8]. The peroxynitrite anion activates the constitutive and inducible forms of cyclooxygenase (COX-1 and COX-2, respectively), which are rate-determining enzymes for prostaglandin biosynthesis during the inflammatory process [9]. Chronic inflammation of the colon increases the risk of colorectal cancer in rats [10]. On the basis of this evidence, iNOS inhibition has become a new approach for cancer chemoprevention.

Endotoxins and a number of cytokines, including interferon and interleukins, bring about the expression of iNOS and COX-2 in macrophages [10]. Therefore, in the present investigation, NO released from lipopolysaccharide (LPS) stimulated murine macrophage RAW 264.7 cells was quantitatively analyzed using the Griess reaction. Moreover, effects on iNOS and COX-2 enzyme expression were detected by Western blot analysis, and the level of PGE_2 was measured with an ELISA method [11], [12]. The effects of the anthraquinone-derived activities of rhubarb were evaluated by examining NO production in LPS-activated RAW 264.7 macrophages. Structure-activity relationships on NO inhibitory potency of the anthraquinones are discussed.

Materials and Methods

Chemicals and cells

Dimethyl sulfoxide (DMSO), *N*-nitro-L-arginine-methyl ester (L-NAME), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide], trypan blue, LPS (*E. coli* serotype 0127 – 8B), and other chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics, L-glutamine, and trypsin-EDTA were purchased from GIBCO BRL (Grand Island, NY). The murine macrophage cell line, RAW 264.7, was obtained from American Type Cell Culture (ATCC; Rockville, MD, USA). Sennosides A and B were purchased from Extrasynthese (Genay Cedex, France). LiChrospher RP-18 gel (40 – 63 μm) was purchased from Merck (Darmstadt, Germany). MCI gel CHP-20P (75 – 150 μm) was purchased from Mitsubish (Japan).

Isolation of anthraquinones

Dried roots (90 g) of *R. palmatum* L. (Polygonaceae) were purchased from a traditional Chinese medicinal market in Taipei. A voucher of the plant material (RP-0001) is deposited in Graduate Institute of Pharmacognosy Science, Taipei Medical University. It was milled and refluxed with 50% aqueous ethanol (1 L) twice. The hot extract was filtered and concentrated with rotary evaporator to remove ethanol, which produced a dark-brown extract (21.1 g). An RP-18 gel column (2.5 × 40 cm) chromatography was eluted with aqueous MeOH stepwise ($H_2O \rightarrow$ 20% MeOH $\rightarrow$ 40% MeOH $\rightarrow$ 80% MeOH $\rightarrow$ MeOH). The 80% MeOH fraction was separated with RP-18 gel column (1.5 × 30 cm) eluted with MeOH/H_2O/H_3PO_4, 600 : 400 : 1 $\rightarrow$ 680 : 320 : 1. Fractionation of the eluate was detected by HPLC. Each fraction was then transferred to an MCI CHP-20P column (1 × 30 cm) eluted with aqueous MeOH to give aloe-emodin, rhein, emodin, chrysophanol, and physcion. The purity of all isolated compounds was shown to be more than 99% when compared with authentic samples by HPLC. The formula and molecular weights of aloe-emodin, rhein, emodin, chrysophanol, and physcion are $C_{15}H_{10}O_5$ (270.23), $C_{15}H_{18}O_6$ (284.21), $C_{15}H_{10}O_5$ (270.23), $C_{15}H_{10}O_4$ (254.23), $C_{15}H_{12}O_5$ (284.21), respectively [13].

HPLC analysis

The HPLC system consisted of a Shimadzu Model LC-10AT system equipped with a Shimadzu Model SIL-9A auto injector and a Shimadzu Model SPD-10A detector. Peak areas were calculated with a Shimadzu Model C-R8A recorder. The mobile phase was composed of MeOH/H_2O/H_3PO_4 with 720 : 280 : 1. A LiChrospher 100 RP-18e reversed-phase column (125 × 4 mm I.D., Merck, Darmstadt, Germany) and a LiChrospher 100 RP-18e guard column (4 × 4 mm I.D., Merck) were used. The flow-rate was 1.0 ml/min with UV absorbance detection 254 nm. The analysis involved 10 μl of sample solution. The operating temperature was maintained at 40 °C with a SHIMADZU Model CTO-10A oven.

Sample preparation

Test solutions of anthraquinones (4 mg/ml) were prepared by dissolving each compound in DMSO, and then they were stored at 4 °C until use. Serial dilutions of the tested solutions with culture medium were prepared before *in vitro* assays.

NO production by RAW 264.7 cells

The murine macrophage cell line, RAW 264.7, was cultivated in DMEM medium supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. The cells (0.2 ml, 3×10^5 cells/ml) were placed in 96-well plates and treated with LPS (100 ng/ml) and tested compounds. After 18 h, the level of nitrite was measured as described below. Tested compounds dissolved in DMSO were diluted with culture medium to appropriate concentrations. The final concentration of DMSO was adjusted to 0.5% (v/v).

iNOS activity assay

The enzyme preparation was obtained from RAW 264.7 cells cultured in a 100-mm plate after activation with LPS (1 μg/ml) for

12 h. Cells were collected and washed twice with PBS to remove LPS. RAW 264.7 cell suspensions (0.2 ml, 3×10^5 cells/ml) were placed in 96-well plates, and indicated compounds were added. L-NAME as a specific inhibitor of NO synthase enzyme activity was used as a positive control, while 0.5% DMSO was used as a solvent control [11]. After 12 h, the amount of nitrite was measured by the Griess reaction as described below.

Cell viability

Mitochondrial respiration, an indicator of cell viability, was assayed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. Cells in 96-well plates were incubated with MTT (0.25 mg/ml) for 4 h. The cells were solubilized in 0.04 N HCl in isopropanol. The extent of the reduction was measured by absorbance at 600 nm [11].

Measurement of nitrite formation

Nitrite, as an indicator of NO synthesis, was determined in cell culture supernatants by the Griess reaction [11]. After incubation of cells for 18 h, the supernatants (0.1 ml) were added to a solution of 0.1 ml Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diaminedihydrochloride in 5% H_3PO_4) to form a purple azodye. Using $NaNO_2$ to generate a standard curve, nitrite production was measured by an absorption reading at 530 nm.

Western blot analysis

RAW 264.7 cells (2 ml, 3×10^5 cells/ml), grown in 6-well plates to confluence, were incubated with or without LPS in the absence or presence of tested samples for 18 h, respectively. Cells were washed with ice-cold phosphate-buffered saline and stored at –70 °C until further analysis. Protein samples were prepared and resolved by denaturing SDS-PAGE using standard methods [11]. The proteins were transferred to a nitrocellulose membrane, and Western blotting was performed using polyclonal rabbit IgG antibody against inducible NO synthase (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-651), polyclonal goat IgG antibody against COX-1 (sc-1752), COX-2 (sc-1745), and monoclonal mouse IgM antibody against α-tubulin (sc-8035). Goat anti-rabbit, goat anti-mouse, or donkey anti-goat antibodies conjugated to alkaline phosphatase (sc-2007, sc-2022, and sc-2008) and BCIP/NBT (BCIP/NBT, Gibco) were used to visualize protein bands.

Measurement of PGE_2 production

RAW 264.7 cells were culture with tested compounds and 100 ng/ml LPS for 18 h. One hundred microliters of supernatant of culture medium was collected for the determination of PGE_2 concentrations with an ELISA kit (Amersham Pharmacia Biotech, UK) [11].

Statistical analysis

Each experiment was performed at least in triplicate. Results are expressed as the mean value ± standard deviation (SD). Statistical analysis was performed using unpaired Student's *t*-test. *p* values < 0.05 were considered significant.

Results

Effects of anthraquinones on NO produced by RAW 264.7 cells

We screened 50% EtOH extracts of several Chinese herbs including rhubarb for iNOS inhibition on RAW 264.7 cells. The extract of rhubarb at 300 μg/ml could inhibit 67.8% of NO production from LPS-stimulated RAW 264.7 cells. Five major components, aloe-emodin, rhein, emodin, chrysophanol, and physcion, were isolated from rhubarb and the inhibition effects on NO production were observed. The inhibition effects of these compounds on the generation of NO were examined in lipopolysaccharide (LPS, 100 ng/ml)-stimulated RAW 264.7 macrophages. Nitrite production was measured by the Griess method. Primary screening tests were done at a sample concentration of 20 μg/ml, and cytotoxicity was detected at this concentration by the MTT assay. As shown in Table **1**, emodin and rhein resulted in more than 50% inhibition of NO production at 20 μg/ml and low cytotoxic effects at the same concentration. However, aloe-emodin showed the strongest cytotoxic effect compared to the other compounds and the inhibition percentage of cell viability (CI%) was 66.9%. The amount of NO production at 5–40 μg/ml was continuously measured, and the IC_{50} values for the above compounds were determined. Emodin and rhein dose-dependently reduced the induction of NO products as shown in Figs. **2A** and **3A**, and the IC_{50} values were 60.7 and 67.3 μM, respectively, as described in Table **1**.

Effects of emodin or rhein on iNOS enzyme activity

It is unknown whether the reduction in nitrite accumulation by emodin or rhein is a result of inhibition of iNOS expression or inhibition of its enzymatic activity. The effects of emodin or rhein were compared with that of L-NAME, a specific inhibitor of NO synthase enzyme activity. RAW 264.7 cells were activated by LPS (1 μg/ml) for 12 h, after which the medium was replaced with

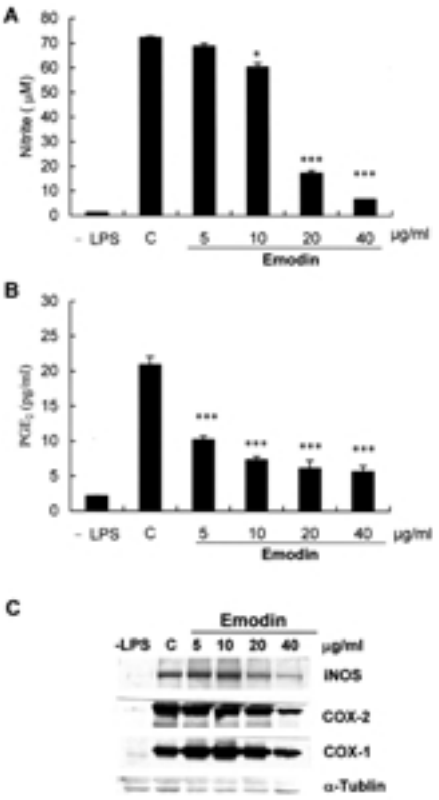

Fig. **2** Dose-dependent inhibition by emodin of nitrite and PGE_2 production and iNOS and COX expression by LPS-activated RAW 264.7 cells. Inhibition of nitrite production was measured by the Griess reaction (**A**), PGE_2 production by an ELISA kit (**B**), and iNOS and COX expressions by Western blot analysis (**C**). Statistical analysis was done by Student's *t*-test. ** $p < 0.01$; *** $p < 0.001$, significantly different from the DMSO-treated group. –LPS indicated without treatment LPS, C indicated DMSO-treated group in the presence of LPS.

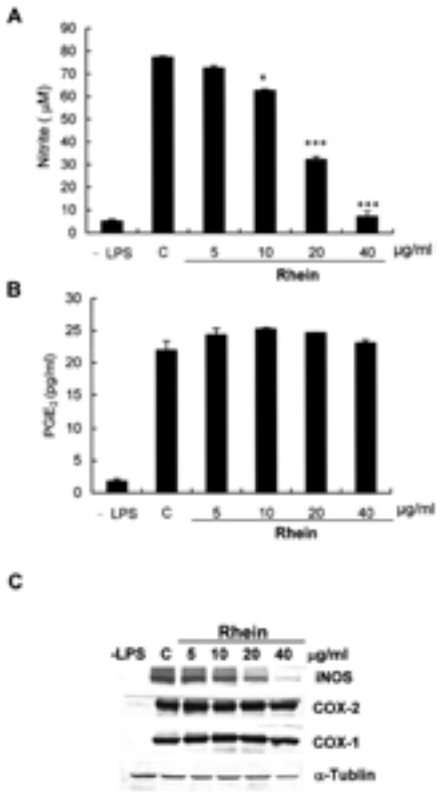

Fig. **3** Dose-dependent inhibition by rhein of nitrite, PGE_2 production and iNOS and COX expression by LPS-activated RAW 264.7 cells. Inhibition of nitrite production was measured by the Griess reaction (**A**), PGE_2 production by an ELISA kit (**B**), and iNOS and COX expressions by Western blot analysis (**C**). Statistical analysis was done by Student's *t*-test. ** $p < 0.01$; *** $p < 0.001$, significantly different from the DMSO-treated group. –LPS indicated without treatment LPS, C indicated DMSO-treated group in the presence of LPS.

Table **1** Inhibition effects of anthraquinone derivatives on NO production by RAW 264.7 cells

Compound	*NO production Inhibition (%)*	*IC_{50} (μM)*	*CI (%)*
Aloe-emodin	45.5 ± 4.2	–	66.9 ± 1.1
Chrysophanol	9.6 ± 6.8	–	30.5 ± 1.9
Emodin	62.5 ± 7.3	60.7 ± 8.5	13.9 ± 1.5
Physcion	25.0 ± 0.9	–	35.6 ± 3.2
Rhein	60.3 ± 1.2	67.3 ± 5.3	15.1 ± 1.4
Sennoside A	12.1 ± 3.7	–	18.8 ± 2.8
Sennoside B	17.8 ± 5.8	–	7.1 ± 10.6
L-NAME	53.5 ± 0.8	450 ± 9.5	18.7 ± 3.2

RAW 264.7 cells were treated with tested compounds at 20 μg/ml, and results are expressed as the mean ± S.D. of three experiments.
L-NAME (500 μM), an iNOS activity inhibitor, was used as a positive control.
CI%: inhibition of cell viability (%).
–: $IC_{50} > 100$ μM.

fresh medium containing test samples. Emodin, rhein (both at 20 μg/ml), or the control solvent (0.5% DMSO) weakly inhibited iNOS activity in activated RAW 264.7 macrophages. In contrast, L-NAME significantly inhibited nitrite accumulation by more than 50% at 200 μM (Table **2**). According to the above results, we suggest that emodin or rhein do not exhibit a direct inhibitory effect on the enzymatic activity of inducible NO synthase.

Effects of emodin or rhein on iNOS and COX enzyme expressions

The effects of tested compounds on the induction of iNOS and COX enzyme expressions were checked using a Western blotting technique. As shown in Fig. **2C**, emodin concentration-dependently reduced the induction of iNOS and COX-2 enzyme expression at 5~40 μg/ml. However, rhein significantly inhibited the expression of iNOS but not COX-2, as shown in Fig. **3C**. Neither emodin nor rhein influenced the expression of COX-1. In addition, PGE_2 production of LPS-activated RAW 264.7 cells was measured in the presence of emodin or rhein. In Figs. **2B** and **3B**, the data show that emodin, but not rhein, significantly reduced PGE_2 production.

Table **2** Effects of indicated compounds after LPS induction of iNOS in RAW 264.7 cells

LPS pretreatment of cells	*Addition to LPS-treated RAW 264.7 cells*	*NO production inhibition (%)*	*CI%*
LPS (1 μg/ml), 12 h	DMSO, 0.5%	10.1 ± 5.2	6.1 ± 4.2
	Emodin, 20.0 μg/ml	9.4 ± 4.0	17.5 ± 2.0
	Rhein, 20.0 μg/ml	0 ± 3.5	15.7 ± 3.5
	L-NAME, 200.0 μM	77.5 ± 3.8	14.4 ± 10.4

Results are expressed as the mean ± S.D. of three experiments.
DMSO (0.5%) was used as a solvent in this experiment.
CI%: inhibition of cell viability (%).
L-NAME (200.0 μM), an iNOS activity inhibitor, was used as a positive control.

Synergistic effects of emodin with rhein on inhibition of NO production in RAW 264.7 cells

Inhibition effects of NO production in RAW 264.7 cells were enhanced by co-treatment with emodin and rhein. Moreover, the effects of emodin were enhanced by rhein, which decreased the cytotoxic effects. In Table **3**, the combined NO inhibition effect of 4 μg/ml emodin and 16 μg/ml rhein was significantly stronger than that of 20 μg/ml emodin or rhein individually. In contrast, when the dosage of emodin was larger than rhein, synergic effects of emodin with rhein did not occur. On the other hand, the

Table **3** Cytotoxic effects and NO production inhibition of co-treatment of RAW 264.7 cells with emodin and rhein

Concentration (μg/ml)			
Emodin	*Rhein*	*NO production inhibition (%)*	*CI%*
5	0	27.1 ± 13.3	1.1 ± 0.9
10	0	36.7 ± 15.0	5.1 ± 6.8
20	0	62.5 ± 7.3	13.9 ± 1.5
0	5	0.0 ± 6.9	3.7 ± 4.6
0	10	31.3 ± 5.9	6.9 ± 0.4
0	20	60.3 ± 1.2	15.1 ± 1.4
5	5	41.2 ± 3.5	0.2 ± 3.2
4	16	75.2 ± 2.1	2.3 ± 1.6
5	15	71.3 ± 7.4	6.8 ± 2.1
10	10	70.3 ± 4.1	14.8 ± 4.6
15	5	49.9 ± 2.9	6.0 ± 5.0
16	4	42.1 ± 5.6	5.6 ± 2.2

RAW 264.7 cells were co-treated with emodin and rhein, and results are expressed as the mean ± S.D. of three experiments.
CI%: inhibition of cell viability (%).

cytotoxic effects of RAW 264.7 cells also decreased by co-treatment with 4 μg/ml emodin and 16 μg/ml rhein (Table **3**). When RAW 264.7 cells were treated with emodin or rhein at 10 μg/ml, the NO production inhibition was 36.7 ± 15% and 31.3 ± 5.9%, respectively. Summation effects of NO production inhibition in RAW 264.7 cells (Table **3**) were enhanced by co-treatment with emodin 10 μg/ml and rhein10 μg/ml (70.3 ± 4.1%).

Discussion

Rhubarb, *R. palmatum*, is a Chinese herb and a well-known purgative agent. Traditional doctors clinically use it to promote digestion, relieve dyspepsia, and purge heat in Taiwan. Moreover, anthraquinone derivatives are well known to be contained in the root of *R. palmatum*. As the present results show, NO production inhibition effects of emodin and rhein were stronger than those of the other anthraquinones, i. e., aloe-emodin, chrysophanol, physcion, and sennosides A and B, at 20 μg/ml (Table **1**). Aloe-emodin exhibited moderate NO production inhibition, but its cytotoxic effect was strongest among those anthraquinones. Thus, NO production inhibition by aloe-emodin can be attributed to a decrease in the viability of RAW264.7 cells. Anthraquinones, based on the position of the hydroxy group substitution, are divided into the emodin-type (1,8-dihydroxy-anthraquinone) and alizarin-type (1,2-dihydroxy-anthraquinone). As shown in Fig. **1**, aloe-emodin, chrysophanol, emodin, physcion, and rhein belong to emodin-type anthraquinones. It is interesting to note how the characteristics of the substitution of functional groups affects NO production inhibition. According to Table **1**, some structure-activity relationship can be deduced.

Chrysophanol (9.6%), emodin (62.5%), and physcion (25.0%), with a methyl group at the C-6 position, have different activities. No substitution (chrysophanol) or having a methoxy group (physcion) at the C-3 position enhanced the cytotoxicity but did not significantly affect NO production inhibition. However, emodin, with a hydroxy group at C-3, showed high activity. Structural modification in chrysophanol (9.6%), aloe-emodin (45.5%), and rhein (60.3%), with a different oxidation state of the methyl group and lacking a substitution at C-3, resulted in dramatic effects. Chrysophanol and aloe-emodin have moderate and the strongest cytotoxicity, respectively, but rhein has little. Among them, only rhein possesses strong NO production inhibition activity. Anthraquinones have a flat-type structure, thus the C-3 and C-6 positions can be convertible in the emodin-type. Comparing the structural characteristics of emodin and rhein, the two anthraquinones with the strongest NO production inhibition, acidic substitution such as with a phenolic or carboxylic group at C-3 or C-6 may contribute to the potential activity. Sennosides A and B, dianthrone glycosides, with two carboxylic groups at C-3 and C-3′, showed no significant effect which may be due to the stereo-shading effect of the bulky structure and glycoside substitution at C-8 and C-8′. Antioxidant, metal-chelating, and hydroxyl radical scavenging of anthraquinones may contribute to their bioactivities. However, it is not easy to relate the present data to antioxidant activity because, in addition to chrysophanol, almost all anthraquinones show inhibition of lipid peroxidation [14].

Previous studies showed that emodin only inhibited iNOS expression, not COX and PGE_2 production [12]. Because, the level of PGE_2 production induced from RAW 264.7 cells was too high (control group is 3 ng/ml), the cell culture condition was modified and induced a low PGE_2 amount (control group is 30 pg/ml) from RAW 264.7 cells in this study. The inhibiting effects of emodin on PGE_2 production and COX expression could be more evidently observed. However, emodin can inhibit TNF-induced NF-κB activity in human vascular endothelial cells [15]. Unlike previous studies, we suggest that emodin inhibitions iNOS and COX expression on RAW 264.7 cells.

Furthermore, the effect of emodin on inhibition of NO production by RAW 264.7 cells was synergistically enhanced by rhein, which decreased the cytotoxic effect (Table **3**). Emodin also exhibited an anti-inflammatory effect on carrageenan-induced edema in rats [16]. Therefore, emodin and rhein may be the anti-inflammation substances in *R. palmatum*.

Chinese herbs are composed of active components that exhibit the ability to regulate cell activities. Previous treatments of disease with herbs were empirical more than theoretical. Therefore, clarification of the mechanisms of action of components in herbs is important in developing their applications. Emodin and rhein are active constituents of the anti-inflammation effects of *R. palmatum*. Pharmacological studies have demonstrated that emodin possesses anticancer, antibacterial, diuretic, anti-inflammation, and vasorelaxant effects [17], [18]. Rhein could induce necrosis in tumor cells or inhibit IL-1β-induced NO production by human osteoarthritic chondrocytes [19]. According to the present results, emodin can inhibit iNOS and COX protein expression and NO and PGE_2 production by LPS-activated RAW 264.7 cells (Fig. **2**). However, rhein only can inhibit iNOS protein expression and NO production (Fig. **3**). Therefore, the inhibition effects on NO production by emodin were enhanced by inhibition of iNOS protein expression by rhein. The synergistic effect of emodin combined with rhein on inhibition of NO production constitutes the anti-inflammatory mechanism of extracts of rhubarb.

Macrophages play a major role in host defenses against infection and tumor development, and this activity is regulated through the production of several mediators [20]. In particular, the production of NO by macrophages mediates killing or growth inhibition of tumor cells, bacteria, fungi, and parasites [20]. However, over-expression of iNOS by various stimuli, resulting in the over-production of NO, contributes to the pathogenesis of septic shock and some inflammatory and auto-immune diseases [20]. It has been suggested that the interaction between NO and superoxide ($O_2^{\cdot-}$) which yields peroxynitrite ($ONOO^-$) and its conjugated acid, peroxynitrous acid (ONOOH), dramatically enhances the toxicity of either NO or $O_2^{\cdot-}$ alone. Therefore, it would be valuable to develop potent and selective inhibitors of iNOS for potential therapeutic use. Thus emodin and rhein, which inhibit the expression of iNOS and are easily synthesized might be useful for the prevention of various diseases. The findings presented here might therefore provide a scientific basis for the use of plant extracts containing emodin and rhein and related compounds against inflammatory diseases in phytotherapy.

Acknowledgements

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