Anti-inflammatory Activities of Aqueous Extract from *Radix Ophiopogon japonicus* and Its Two Constituents

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To provide some pharmacological evidence for its clinical use in inflammatory diseases, anti-inflammatory effect of the aqueous extract from *Radix Ophiopogon japonicus* (ROJ-ext), a traditional Chinese herb, was examined in mouse and rat models. ROJ-ext significantly inhibited xylene-induced ear swelling and carrageenan-induced paw edema in mice when given orally at doses of 25 and 50 mg/kg. Moreover, ROJ-ext also remarkably suppressed carrageenan-induced pleural leukocyte migration in rats and zymosan A-evoked peritoneal total leukocyte and neutrophil migration in mice, while had no obvious effect on pleural prostaglandin E_2 level. Furthermore, two active compounds were isolated from ROJ-ext and identified as ruscogenin and ophiopogonin D. As the results, ROJ-ext, ruscogenin and ophiopogonin D dose-dependently reduced phorbol-12-myristate-13-acetate (PMA)-induced adhesion of HL-60 cells to ECV304 cells, with IC_{50} of 42.85 μg/ml, 7.76 nmol/l and 1.38 nmol/l, respectively. However, they showed no inhibitory effect on PMA-induced cyclooxygense-2 (COX-2) mRNA expression in ECV304 cells. Ruscogenin and ophiopogonin D also notably decreased zymosan A-induced peritoneal leukocyte migration, in comparison with ROJ-ext. These results demonstrate that ROJ-ext presents remarkable anti-inflammatory activity and ruscogenin and ophiopogonin D are two of its active components, which supported its traditional use in the treatment of various diseases associated with inflammation.

Key words *Ophiopogon japonicus*; ruscogenin; ophiopogonin D; anti-inflammatory activity; adhesion; cyclooxygenase-2

The plant *Ophiopogon japonicus* (THUNB.) (Liliaceae) widely distributed in South-east Asia, especially in most area of China,[1] has been widely used in traditional Chinese medicine to treat acute and chronic inflammatory diseases including pharyngitis, bronchitis, pneumonia and cough etc, as well as cardiovascular diseases such as arrhythmia, angina and thrombosis *etc*.[2] Chemical studies have shown that this plant includes saponins, polysaccharide and homoisoflavonoidal compounds.[3—11] As the scientific evidence for its clinical efficacy, its cardiovascular activities have been confirmed in various assays such as anti-ischaemia, anti-arrhythmic, inhibiting platelets aggregation, protecting endothelium from apoptosis, improving microcirculation, and so on.[12—24] However, there are few reports on its anti-inflammatory properties,[25—27] and its anti-inflammatory active components have not been adequately elucidated.

Therefore, in our present study, the anti-inflammatory effects of the aqueous extract from *Radix Ophiopogon japonicus* and its two components, ruscogenin and ophiopogonin D were investigated to provide some pharmacological evidence for its clinical use in inflammatory diseases.

MATERIALS AND METHODS

Plant Materials The dried tuber roots of *Ophiopogon japonicus* was purchased from Nanjing Medical Material Company (Nanjing, Jiangsu, China) and identified as *Ophiopogon japonicus* (THUNB.) by Professor Boyang Yu, one of the authors. The voucher specimen (BYY020516) was deposited at the Herbarium of China Pharmaceutical University.

Drugs and Chemicals The drugs and reagents used in this study were as follows: aspirin (Jiangsu Hengsheng Pharmaceutical Factory, Nanjing, China); dexamethasone (Dex, Nanjing 2nd Pharmaceutical Factory, Nangjing, China); Carrageenan (type I), zymosan A and phorbol-12-myristate 13-acetate (PMA, Sigma Chemical Co., St. Louis, MO, U.S.A.); RPMI 1640 medium (Gibco BRL, Life Technologies, Inc., New York, U.S.A.); newborn bovine serum (NBS, Hangzhou Sijiqing Biomaterial Co Ltd., Hangzhou, China) and 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, Ameresco, U.S.A.).

Extraction and Isolation The dried tuber roots of *Ophiopogon japonicus* (10 kg) were extracted with boiling distilled water (2×100 l). The extract was combined and concentrated to dryness in vaccum. The residue was dissolved with water, and then added with ethanol at the final concentration of 75% overnight. The supernatant was chromatographed on D101 resin column and eluted with water and 70% ethanol. The 70% ethanol elution was collected, concentrated in vaccum to give 45 g of the extract (ROJ-ext). The dosage of this extract was indicated as the powder.

ROJ-ext (20 g) was then chromatographed on a silica gel column with chloroform–methanol (5 : 1) as an eluent and gave a dried fraction. Repeated chromatography over a silica gel and Sephadex LH-20 eluting with chloroform–methanol–water (65 : 20 : 5) to obtain ruscogenin (about 98 mg) and ophiopogonin D (about 20 mg) (structures shown in Fig. 1), and other five compounds. The tested drugs were dissolved in distilled water for *in-vivo* assay and in 0.1% DMSO of RPMI-1640 for *in-vitro* assay.

Animals Male Sprague–Dawley rats weighing 250—280 g and male ICR mice weighing 25—30 g were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). Animals were free to standard pellet food and water with a 12 h light/dark cycle. This

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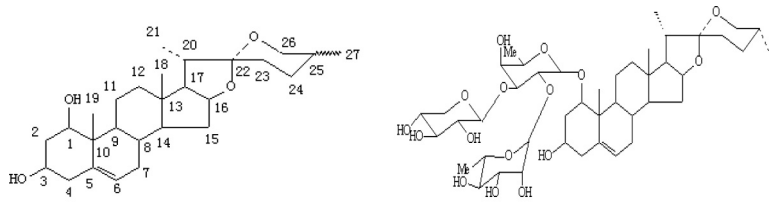

25(R, S)-ruscogenin Ophiopogonin D

Fig. 1. Structures of Two Components from *Radix Ophiopogon japonicus*

study complied with current ethical regulations on animal research (National Research Council of U.S.A., 1996) and all animals used in the experiment received humane care.

Xylene-Induced Ear Swelling in Mice The tested samples including aspirin as a positive-control were given orally to the mice fasted for 10 h. One hour later, each animal received 30 μl of xylene on the anterior and posterior surfaces of the right ear lobe. The left ear was considered as control. Two hours later, the animals were sacrificed by cervical dislocation and both ears were removed. Circular sections were taken, using a cork borer with a diameter of 7 mm, and weighed. The degree of ear swelling was calculated based on the weight of left ear without xylene.[28)]

Carrageenan-Induced Paw Edema in Mice Mice were given a subcutaneous injection of carrageenan in sterile saline (400 μg/paw) into the right paw. The contra lateral paw received the same volume of sterile normal saline and served as a control. ROJ-ext (25, 50 mg/kg) and aspirin (50 mg/kg) were administered orally 1 h before carrageenan injection. The thickness of the paw was measured with a screw micrometer at different time-points (0, 1, 3, 5, 24 h) after carrageenan injection.[29)]

Carrageenan-Induced Pleurisy in Rats Rats were lightly anaesthetized with ether, and 0.5 ml of 1% carrageenan dissolved in sterile normal saline or normal saline alone was injected into the right pleural space through the chest skin. ROJ-ext (25 and 50 mg/kg) and dexmethasone (5 mg/kg) were administered orally 1 h before carrageenan injection. Seven hours later, animals were killed by cervical dislocation, the chest was carefully opened and the pleural exudates were collected. Any exudates contaminated with blood were discarded. The leukocytes in the exudates were suspended in phosphate buffer saline (PBS) and counted with an optical microscope. 0.3 milliliter of the supernatant was added with 2.0 ml of 0.5 mol/l potassium hydroxide–methanol solution and reacted under 50 °C for 20 min, and then diluted with 1.0 ml of methanol and its absorbance was determined at 278 nm to indicate the content of PGE_2 as described.[30)]

Zymosan A-Induced Peritonitis in Mice The peritonitis was induced by injection of zymosan A in 0.5 ml PBS (0.1 M, pH 7.4). The tested drugs were administered orally 1 h before zymosan A injection. Four hours later, animals were killed by CO_2 exposure and peritoneal cavities were washed with 3 ml of PBS containing 3 mM ethylenediaminetetracetic acid (EDTA) and 25 U ml^{-1} heparin. Aliquots of the lavage fluids were stained with Turk's solution (0.01% crystal violet in 3% acetic acid) and differential cell counts performed with a light microscope by Wright's–Giemsa's staining according to the reported method.[31)]

Cells and Cell Culture Human umbilical vein endothelial cell line (ECV304) and human pro-myelocytic leukemia cell strain (HL-60) were purchased from Shanghai Cell Collection of China Academy of Science. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated newborn bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

MTT Assay for Cell Viability Assay For detection of cytotoxicity, the MTT test was used.[32)] This colorimetric assay is based on the conversion of the tetrazolium salt MTT to formazan crystals by intact mitochondria. The measured absorbance at 570 nm is therefore directly proportional to the number of vital cells. MTT was dissolved in PBS to final concentration of 5 mg/ml. ECV304 cells were incubated with tested drugs for 3 h, and washed twice. Then MTT was added for further 3 h at 37 °C before formazan crystals were dissolved with DMSO. The plate was agitated for 10 min on plate shaker and absorbance was measured with an ELISA reader at 570 nm.

Adhesion Assay for HL-60 to ECV304 Cells Adhesion assay was performed according to the report with some modifications.[33)] Briefly, ECV304 cells were pretreated with drugs or medium for 1 h, and then activated with PMA 10 ng/ml for 2 h. At the end of culture, the supernatant was aspirated, and 100 μl RPMI 1640 medium (as blank) or HL-60 cells (1×10^5) were added. The cells were co-incubated at 37 °C for 30 min and non-adherent cells were removed by washing three times with RPMI 1640 medium. Then cell viability was determined by MTT assay. The wells that were added with MTT without previous washing were regarded as the absorbance of total cells. The results were expressed as the mean adhesion percentage (% adhesion) determined as (absorbance of tested wells-absorbance of blank)/(absorbance of total wells-absorbance of blank)$\times$100 from triplicate wells and the experiments were repeated three times.

RNA Preparation and Polymerase Chain Reaction[34)] Total RNA was extracted from PMA-activated or naive ECV304 cells using Tripure reagent (Roche) as described by the manufacturer. Single-stranded cDNA was synthesized from 2 μg of total RNA by reverse transcription using 0.5 μg primer of oligo$(dT)_{18}$. Following cDNA synthesis, amplification was performed using the following primers (Genebase, Shanghai, China): GAPDH, 5′-ACATCTGCTGGAAGGTGGAC and 3′-GGTACCACCATGTACCCAGG, COX-2, 5′-CTTACAATGCTGACTATGGCTAC and 3′-GTCGTGAAGTGCGTAGTCA. PCR cycle conditions were: 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min for 30 cycles. After

amplification, PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide dying.

Statistical Analysis Results were expressed as mean ±S.E.M. Data were analyzed by a one-way ANOVA, followed by Student's two tailed-*t*-test for comparison between two groups, and Dunnett's test when the data involved three or more groups. $p<0.05$ was considered to be significant.

RESULTS

Effect of ROJ-ext on Xylene-Induced Ear Swelling in Mice ROJ-ext remarkably inhibited xylene-induced mice ear swelling in a dose-dependent manner (Fig. 2). The inhibitory effect of ROJ-ext at 50 mg/kg was similar to that of the non-steroidal anti-inflammatory drug aspirin at 50 mg/kg.

Effect of ROJ-ext on Carageenan-Induced Paw Edema in Mice As shown in Fig. 3, ROJ-ext significantly inhibited paw edema in mice in a dose-dependent manner when given orally at 1, 3 and 5 h after carrageenan injection at doses of 25 and 50 mg/kg and the inhibition rate of the latter was comparable to that of aspirin 50 mg/kg.

Effect of ROJ-ext on Carageenan-Induced Pleurisy in Rats In rat carrageenan-induced pleurisy model, ROJ-ext exerted a dose-dependent inhibition on leukocyte migration to the thoracic cavity, which the inhibitory rate is 40% and 68% at the doses of 25 and 50 mg/kg, respectively. However, ROJ-ext at both doses didn't show remarkable effect on PGE_2 content in pleural exudates, in contrast to a significant efficacy posed by dexamethasone 5 mg/kg, which served as a control drug (Figs. 4A, B).

Effect of ROJ-ext on Zymosan A-Induced Leukocyte Migration in Mice Intraperitoneal injection of zymosan A produced significant leukocytes, mainly neutrophil accumulation into mouse peritoneal cavities. And similarly, ROJ-ext at doses of 25 and 50 mg/kg and dexamethasone 5 mg/kg showed remarkable inhibitory effects on leukocytes, mainly neutrophil migration induced by zymosan-A in mice (Fig. 5).

Effects of ROJ-ext and Its Two Components on Normal

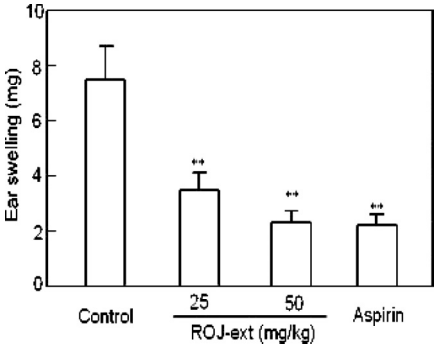

Fig. 2. Effect of the Aqueous Extract from *Radix Ophiopogon japonicus* (ROJ-ext) on Xylene-Induced Ear Swelling in Mice

ROJ-ext at doses of 25 and 50 mg/kg and aspirin 50 mg/kg were administered orally respectively 1 h before the xylene attack. Each value represents the mean±S.E.M. of 10 mice. ** $p<0.01$ significantly different from the control group.

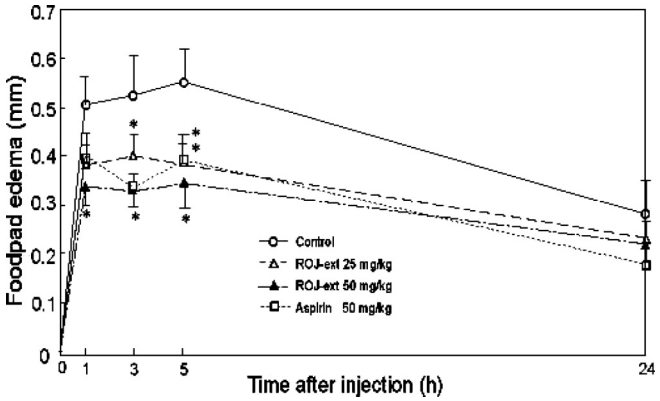

Fig. 3. Effect of the Aqueous Extract from *Radix Ophiopogon japonicus* (ROJ-ext) on Carrageenan-Induced Paw Edema in Mice

ROJ-ext at doses of 25 and 50 mg/kg and aspirin 50 mg/kg were administered orally respectively 1 h before injection of carrageenan. Each value represents the mean±S.E.M. of 10 mice. * $p<0.05$ significantly different from the control group.

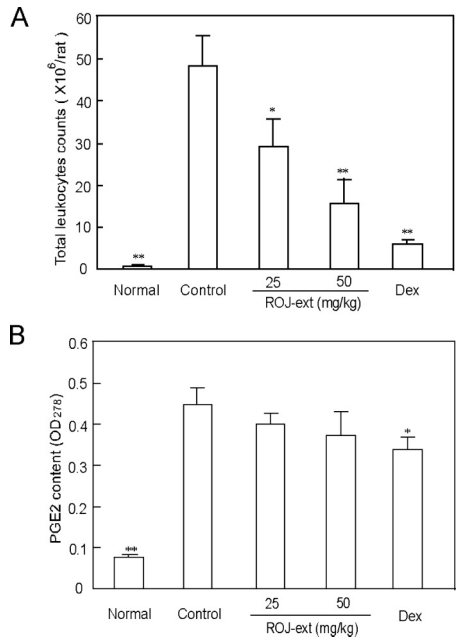

Fig. 4. Effect of the Aqueous Extract from *Radix Ophiopogon japonicus* (ROJ-ext) on Carrageenan-Induced Pleural Leukocytes Migration (A) and PGE_2 Content (B) in the Pleural Cavity and in Rats

ROJ-ext at doses of 25 and 50 mg/kg and dexmethasone 5 mg/kg were administered orally respectively 1 h before injection of carrageenan. PGE_2 content were expressed as the absorbance at 278 nm. Each value represents the mean±S.E.M. of 8 rats. * $p<0.05$, ** $p<0.01$ significantly different from the control group.

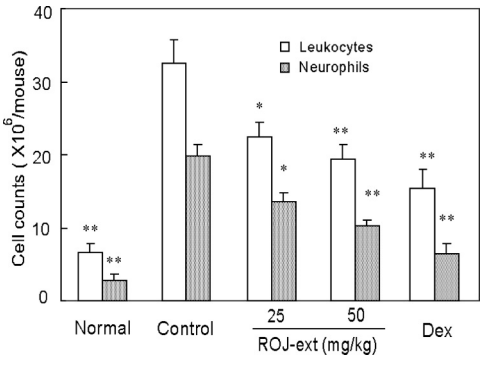

Fig. 5. Effect of the Aqueous Extract from *Radix Ophiopogon japonicus* (ROJ-ext) on Zymosan A-Induced Peritoneal Leukocytes Migration in Mice

ROJ-ext at doses of 25 and 50 mg/kg and dexmethasone 5 mg/kg were administered orally respectively 1 h before injection of zymosan A. Each value represents the mean±S.E.M. of 8 mice. * $p<0.05$, ** $p<0.01$ significantly different from the control group.

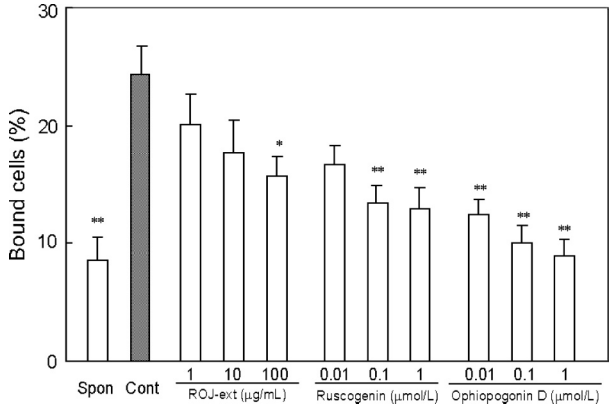

Fig. 6. Effect of the Aqueous Extract from *Radix Ophiopogon japonicus* (ROJ-ext) and Its Two Components on PMA-Induced Adhesion of HL-60 to ECV304 Cells

ECV304 cells were pretreated with ROJ-ext at concentrations of 1, 10 and 100 μg/ml, ruscogenin or ophiopogonin D at concentrations of 0.01, 0.1 and 1 μmol/l for 1 h, and then activated by PMA (10 ng/ml) for 2 h of co-incubation. Then the cells were washed three times with RPMI 1640 medium and incubated with HL-60 cells (1×10^5/well) for 30 min. Data were expressed as the mean±S.E.M. of three independent experiments and each experiment included triplicate sets. Spon: spontaneous adhesion without PMA stimulation. $*p<0.05$, $**p<0.01$ significantly different from the control group.

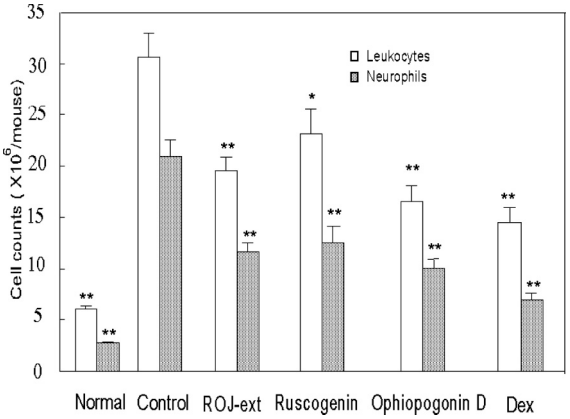

Fig. 7. Effect of the Aqueous Extract from *Radix Ophiopogon japonicus* (ROJ-ext) and Its Two Components on Zymosan A-Induced Peritoneal Leukocytes Migration in Mice

ROJ-ext 50 mg/kg, ruscogenin 5 mg/kg, ophiopogonin D 1 mg/kg and dexmethasone 5 mg/kg were administered orally respectively 1 h before injection of zymosan A. Each value represents the mean±S.E.M. of 8 mice. $*p<0.05$, $**p<0.01$ significantly different from the control group.

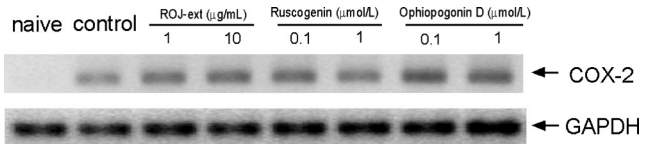

Fig. 8. Effect of the Aqueous Extract from *Radix Ophiopogon japonicus* (ROJ-ext) and Its Two Related Components on PMA-Induced Cox-2 mRNA Expression of ECV304 Cells

Cells were lysed and total RNA was prepared for RT-PCR analysis of gene expression. cDNA of ECV304 cells either treated, stimulated with PMA (10 ng/ml) for 9 h, or pretreated for 1 h and cotreated with PMA with ROJ (1.0, 10 μg/ml), ruscogenin or ophiopogonin D (0.1, 1 μmol/l) for 9 h were subjected to PCR. COX-2 sequences (240 bp) were detected by agrose gel electrophoresis. PCR of GAPDH was performed to correct for uneven loading of cDNA.

and PMA-Induced Adhesion of HL-60 Cells to ECV304 Cells PMA treatment remarkably increased the adhesion of HL-60 cells to ECV304 cells. Against this, ROJ-ext produced a concentration-dependent decrease at concentrations of 1—100 μg/ml with IC_{50} of 42.85 μg/ml. Ruscogenin and ophiopogonin D also inhibited such adhesion at concentrations of 0.01—1 μmol/ml, and their IC_{50} is 7.76 nmol/l and 1.38 nmol/l, respectively. (Fig. 6). Meanwhile, the tested drugs had no obvious effect on HL-60 adherence to resting ECV304 cells and had no cytotoxicity on ECV304 cells when pretreated for 3 h (data not shown).

Effects of ROJ-ext and Its Two Components on Zymosan A-Induced Leukocyte Migration in Mice *In vivo* anti-inflammatory activities of the two compounds were observed in zymosan-A induced peritonitis. The results showed that ruscogenin at 5 mg/kg and ophiopogonin D at 1 mg/kg significantly decreased peritoneal leukocytes and neutrophil count, with the similar potency to that of the aqueous extract at 50 mg/kg (Fig. 7).

Effects of ROJ-ext and Its Two Components on PMA-Induced COX-2 mRNA Expression PMA remarkably enhanced COX-2 mRNA expression in ECV304 cells. However, the extract and its two components had no remarkable effects on PMA-induced COX-2 mRNA expression at the tested concentration when given 1 h before stimulation (Fig. 8).

DISCUSSION AND CONCLUSION

In this paper, we first examined the anti-inflammatory effect of the aqueous extract from *Radix Ophiopogon japonicus* (ROJ-ext) in mouse and rat inflammatory models. Xylene-induced neurogenous edema partially associated with substance P was selected because it is a common inflammatory model for evaluating vascular permeability.[35] The results showed that ROJ-ext remarkably inhibited xylene-induced ear swelling in a dose-dependent manner (Fig. 2), which suggested it might reduce the release of substance P or antagonize its action. Meanwhile, the carrageenan-induced paw inflammation has been accepted as a useful phlogistic tool for evaluating systemic anti-inflammatory agent, which has been demonstrated that bradykinin, substance P and prostaglandin are implicated and a diffuse cellular infiltrate with predominance of neutrophils are revealed in carrageenan mouse paw edema.[36,37] ROJ-ext showed a dose-dependent inhibitory activity on carrageenan-induced paw inflammation over a period of 5 h, which indicated its action against neutrophils migration and release of histamine, serotonin and kinins in early phase, and prostaglandin in later phase (Fig. 3).

Considering the crucial role of the migration of leukocytes to inflammatory area in the progress of inflammation,[38] we next observed the activity of ROJ-ext on carrageenan and zymosan A-induced leukocyte migration. The migration of leukocytes to the thoracic cavity induced by carageenan in rats and to the celiac cavity induced by zymosan A in mice were significantly enhanced when the stimulators were used. Against the increase, the treatment with ROJ-ext showed a remarkable inhibition (Figs. 4A, 5). However, it had no marked effect on PGE_2 content in pleural exudates (Fig. 4B), which indicated that ROJ-ext mainly acted on the pathway of leukocytes migration. These findings suggest that ROJ-ext exerts significant anti-inflammatory activity *via* inhibiting

vascular permeability and leukocyte transmigration, which might be connected with reduction of release of inflammatory mediator or inhibition of adhesion molecular expression *etc*.

To explore its possible active components, we then observed the activities of two compounds, ruscogenin and ophiopogonin D isolated from the aqueous extract. It has been widely accepted that vascular endothelial cells play an important and indispensable role in the inflammatory reaction, which provided traffic signals for leukocytes adhesion and transmigration.[39] In an *in vitro* model testing the leukocyte adherence to endothelial cells, the two components significantly inhibited the adhesion of HL-60 cells to ECV304 cells activated by PMA, in comparison with the aqueous extract (Fig. 6), while they had no remarkable effect on HL-60 adherence to resting ECV304 cells. Moreover, the possibility that the drugs caused cell attachment inhibition directly *via* its cytotoxicity can also be ruled out by an unaffected viability of ECV304 cells in the presence of these agents (data not shown). To further confirm the inhibitory effect of these two compounds on leukocyte adhesion, we next examined their efficacy *in vivo* and found that they also markedly inhibited leukocyte migration induced by zymosan A in mice (Fig. 7). A similar inhibition was also observed with the total extract. These results revealed that the anti-inflammatory activity of ROJ-ext was at least partly due to these two components. In view of the participation of COX-2 activity in zymosan A or carrageenan-induced models,[40,41] we further observed the effects of the aqueous extract and two components on PMA-induced COX-2 mRNA expression in ECV304 cells (Fig. 8). However, they had no obvious inhibitory effects on COX-2 mRNA expression at tested concentrations, supporting the observation of unchanged PGE_2 content by the extract. Further molecular mechanisms of anti-inflammatory activity of these agents remain to be elucidated in the further studies.

In conclusion, the extract from *Radix Ophiopogon japonicus* was confirmed to exert potent anti-inflammatory actions, which provided experimental evidence for its therapeutic efficacy in the treatment of various inflammatory disorders. Meanwhile, its anti-inflammatory activity might be partly ascribable to its two components, ruscogenin and ophiopogonin D. Other active constituents remained to be studied in the future.

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