Antithrombotic Activities of Aqueous Extract from *Radix Ophiopogon japonicus* and Its Two Constituents

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To provide further pharmacological evidence for its clinical use in thrombotic diseases, the antithrombotic activities of the aqueous extract of *Radix Ophiopogon japonicus* (ROJ-ext) were studied in mouse and rat models. The results showed that ROJ-ext remarkably decreased length of tail thrombus in mice at 48 h and 72 h after carrageenan injection at doses of 12.5 and 25.0 mg/kg. Meanwhile, ROJ-ext markedly inhibited thrombosis induced by arterial-venous (AV) shunt (silk thread) in rats at doses of 6.25 and 12.5 mg/kg. Furthermore, ROJ-ext and one of its components, ruscogenin, significantly inhibited platelet aggregation induced by adenosine diphosphate (ADP) in rats by oral administration of 12.5 mg/kg or 0.7 mg/kg for three times, however, ophiopogonin D 1.4 mg/kg only showed slight inhibition. On the other hand, ophiopogonin D (0.5—2.0 mg/kg, *p.o.*) and ruscogenin (0.25—1.00 mg/kg, *p.o.*) produced dose-related inhibition of venous thrombosis induced by tight ligation of the inferior vena cava for 6 h in mice by once oral administration. The findings of this study indicate that an aqueous extract of *Radix Ophiopogon japonicus* (ROJ-ext) exerted significant antithrombotic activity and ruscogenin and ophiopogonin D are two of its active components, which supported its therapeutic use for thrombotic diseases.

Key words *Radix Ophiopogon japonicus*; antithrombotic activity; carrageenan; ophiopogonin D; ruscogenin

The plant *Ophiopogon japonicus* (Thunb.) Ker-Gawl. (Liliaceae), widely distributed in South-east Asia, especially in most areas of China,[2)] and first recorded in "shengnong Bengcaojing" (early in the third century B.C.), has been used in traditional Chinese medicine to treat inflammatory and cardiovascular diseases for thousands of years.[3)] Chemical studies have shown that this plant mainly includes saponins, polysaccharide and homoisoflavonoidal compounds.[4—10)] As the scientific evidence for its clinical efficacy, we formerly found that the aqueous extract of *Ophiopogon japonicus* (ROJ-ext) presented 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.[11)] Meanwhile, its cardiovascular activities, such as anti-ischaemia,[12)] anti-arrhythmic,[13)] inhibiting platelets aggregation,[14)] protecting endothelium from apoptosis,[15,16)] improving microcirculation, and so on have been confirmed in various assay.[17-21)] Furthermore, we also found that ROJ-ext significantly inhibited venous thrombosis, which is linked with its endothelial cell-protective and anti-adhesive activities.[22)] However, its antithrombotic activity has not been evaluated in other animal models and little is known about whether its two anti-inflammatory components showed antithrombotic effects.

Therefore, following our previous work,[11)] the present study was undertaken to investigate the antithrombotic effects of ROJ-ext and its two major components, ruscogenin and ophiopogonin D, by using several animal models so as to provide further evidence for its clinical use in the treatment of thrombotic diseases.

MATERIALS AND METHODS

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

Extraction and Isolation The aqueous extract of *Ophiopogon japonicus* (ROJ-ext) and its two components, ruscogenin and ophiopogonin D, were prepared according to the method described previously.[11)] In brief, 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, ethanol added to the final concentration of 75%, and the mixture left overnight. The supernatant was chromatographed on D101 resin column and eluted with water and 70% ethanol. The 70% ethanol elution was collected and concentrated in vacuum 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) produced ruscogenin (about 98 mg), ophiopogonin D (about 20 mg) (structures shown in Fig. 1), and 5 other compounds.

Drugs and Chemicals The drugs and reagents used in this study were as follows: aspirin (Jiangsu Hengsheng Pharmaceutical Factory, Nanjing, China); warfarin sodium (Shanghai Jiufu Chemical Co. Ltd., Shanghai, China); carrageenan (type I) and adenosine diphosphate (ADP) (Sigma Chemical Co., St. Louis, MO, U.S.A). Other reagents used were of analytical grade.

Animals Male Sprague–Dawley rats weighing 280—340 g and male ICR mice weighing 25—30 g were obtained from the Experimental Animal Center of China Pharmaceutical University (Nanjing, China). They were kept in plastic

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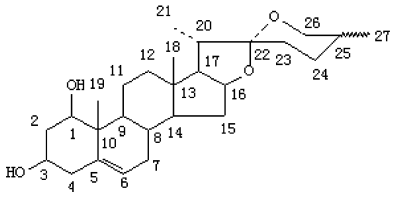
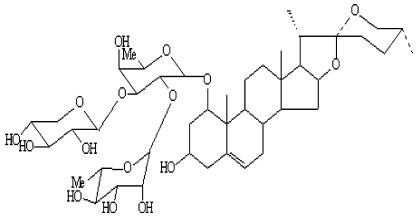


25(R, S)-ruscogenin



Ophiopogonin D

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

cages at 22±2 °C with free access to pellet food and water and on a 12 h light/dark cycle. This study complied with current ethical regulations on animal research (National Research Council of USA, 1996) and all animals used in the experiment received humane care.

Carrageenan-Induced Tail Thrombosis in Mice Tail thrombosis in mice was induced by carrageenan according to the reported method.[23] Ten microliters of 4% carrageenan in sterile saline solution was injected into the subplantar region of the right hind paw of the mice. ROJ-ext of 12.5 and 25.0 mg/kg, aspirin 50 mg/kg, or vehicle was administered orally 1 h before carrageenan injection and 23 h, 47 h and 71 h after injection, respectively. Measurement of the length of black tail (thrombosis) was carried out at 24 h, 48 h and 72 h after injection.

Arterial-Venous Shunt (Silk Thread Model) in Rats Rat arterial-venous shunt (silk thread model) was prepared according to a previous technique, with slight modification.[24] In brief, fasted male rats were anesthetized with chloral hydrate (350 mg/kg, i.p.). Two 10 cm-long polyethylene tubes (1 mm i.d.), linked by a central part (8 cm-long; 2 mm i.d.) containing a 5-cm silk thread and filled with saline solution containing heparin 0.5 mg/kg, were placed between the right carotid artery and the left jugular vein. This central part of the shunt was removed after 15 min of blood circulation and the silk thread supporting the thrombus was extracted. The wet weight of the thrombus was determined. ROJ-ext of 6.25 and 12.5 mg/kg, aspirin 1.0 mg/kg, or vehicle was administered as single oral doses 1 h before thrombosis induction.

Assay of *ex Vivo* Platelet Aggregation in Rats[25] Six male rats in each group were used after overnight fasting. Rats were orally administered ROJ-ext, ophiopogonin D, or ruscogenin at doses of 12.5 mg/kg, 1.4 mg/kg and 0.7 mg/kg or vehicle for 3 d. Blood was collected by cardiac puncture into a plastic flask containing 3.28% sodium citrate (1 : 9 v/v) 1 h after final sample treatment. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 1000 rpm for 5 min and further centrifuged at 3000 rpm for 10 min to prepare platelet poor plasma (PPP). Platelet aggregation was measured by turbimetry using a TXN-98 Platelet Aggregometer (Shanghai Tongyong Ltd., Shanghai, China) according to the method of Born and Cross.[26] Briefly, rat PRP (200 μl) was incubated at 37 °C for 3 min in the aggregometer with stirring at 1200 rpm and then stimulated with 10 μM of ADP in phosphate buffer solution. Changes in light transmission were recorded for 5 min. The maximal aggregation rate was recorded.

Table 1. Effect of ROJ-ext on Tail Thrombosis Induced by Carrageenan in Mice

Group	Dose (mg/kg)	Thrombus length (mm)		
		24 h	48 h	72 h
Control		42.6±7.1	52.0±7.1	49.5±7.6
ROJ-ext	12.5	26.7±4.0 (37.3)	24.8±3.4 (52.3)**	23.5±3.8 (52.5)*
ROJ-ext	25.0	24.6±3.0 (42.3)	19.8±3.1 (57.7)**	22.0±3.5 (60.0)**
Aspirin	50.0	24.3±4.8 (42.9)	23.3±5.1 (55.1)**	22.4±4.8 (54.7)*

ROJ-ext at doses of 12.5 and 25.0 mg/kg and aspirin 50.0 mg/kg were administered orally respectively 1 h before carrageenan injection and then given for another three days. Tail thrombus length was measured 24 h, 48 h and 72 h after injection in mice. Data are expressed as the mean±S.E.M. (n=8—10); numbers in parentheses indicate percentage inhibition by the drugs. *p<0.05, **p<0.01, compared with control.

Stasis-Induced Venous Thrombosis in Mice Thrombus formation was induced according to a previous report with some modifications.[27] Briefly, mice were anesthetized by intraperitoneal injection of chloral hydrate at a dose of 350 mg/kg. The abdomen of each animal was opened surgically, and after careful dissection, the inferior vena cava was exposed and dissected free from the surrounding tissue. Venous thrombosis was induced by tight ligation of the inferior vena cava just below the left renal venous branch using a cotton thread. The abdominal cavity was closed provisionally and the stasis was maintained for 6 h. The cavity was then reopened, the ligated segment was opened longitudinally and the clot was harvested. To quantify the weight of the formed thrombus, the clot was dried at 60 °C for 24 h and then weighed. Ophiopogonin D (0.5—2.0 mg/kg), ruscogenin (0.25—1.00 mg/kg) and warfarin 2.0 mg/kg were administered orally respectively 1 h or 18 h before the ligation.

Statistical Analysis Results are expressed as the mean ±S.E.M. Data were analyzed by 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 Tail Thrombosis Induced by Carrageenan in Mice The results in Table 1 showed marked tail thrombus at 24 h, became the longest at 48 h, and then tended to decrease at 72 h after carrageenan injection. A part of the tail became necrotic and fell off. In contrast to this, ROJ-ext at doses of 12.5 and 25.0 mg/kg and aspirin 50.0 mg/kg markedly inhibited thrombosis at 48 h and 72 h

Table 2. Effect of ROJ-ext on Thrombus Formation Induced by Arterio-Venous Shunt in Rats

Group	Dose (mg/kg)	Thrombus weight (mg)	Inhibition (%)
Control		32.3±2.8	
ROJ-ext	6.25	18.7±2.4**	42.1
ROJ-ext	12.5	12.7±1.7**	60.7
Aspirin	1.0	10.6±1.6**	67.2

ROJ-ext at doses of 6.25 and 12.5 mg/kg and aspirin 1.0 mg/kg were administered orally 1 h before the shunt. Thrombus was obtained 15 min after the shunt in rats. Data are expressed as the mean±S.E.M. ($n=8$). **$p<0.01$, compared with control.

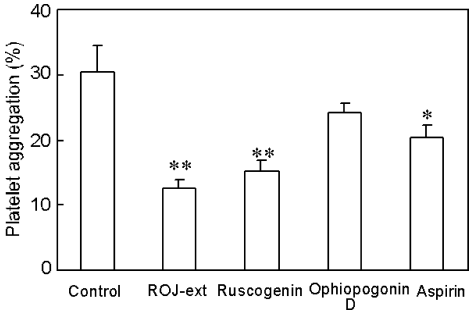


Fig. 2. Effects of the Aqueous Extract from *Radix Ophiopogon japonicus* (ROJ-ext) and Its Two Components on Platelet Aggregation Induced by ADP in Rats *in Vivo*

ROJ-ext 12.5 mg/kg, ruscogenin 0.7 mg/kg, ophiopogonin D 1.4 mg/kg, and aspirin 1 mg/kg were administered orally for 3 d. Platelet aggregation was induced by 10 μM of ADP and measured by turbidimetry. Each value represents the mean±S.E.M. of 6 rats. *$p<0.05$, **$p<0.01$, compared with control.

and slightly inhibited thrombosis at 24 h after carrageenan injection.

Effect of ROJ-ext on Arterial-Venous Shunt (Silk Thread) Thrombosis in Rats ROJ-ext at single oral doses of 6.25 and 12.5 mg/kg significantly inhibited thrombus formation induced by arterio-venous shunt (silk thread) in rats, and the inhibition percentage of the latter was similar to that of aspirin 1.0 mg/kg (as shown in Table 2).

Effect of ROJ-ext and Its Two Components on Platelet Aggregation in Rats As shown in Fig. 2, ROJ-ext and ruscogenin significantly inhibited platelet aggregation induced by ADP in rats by oral administration 3 times at doses of 12.5 and 0.7 mg/kg, respectively, while ophiopogonin D at the dose of 1.4 mg/kg showed slight inhibition. Meanwhile, aspirin also markedly inhibited platelet aggregation.

Effects of Ruscogenin and Ophiopogonin D on Venous Thrombosis in Mice The inferior vena cava ligation procedure led to dramatic thrombus formation. After 6 h of stasis, a red thrombus was present below the ligature in almost all mice administered vehicle. As shown in Table 3, both ophiopogonin D (0.5—2.0 mg/kg, *p.o.*) and ruscogenin (0.25—1.00 mg/kg, *p.o.*) produced dose-related inhibition of venous thrombosis in mice. Their ED_{50} values were calculated to be 1.39 and 1.05 mg/kg, respectively.

Table 3. Effects of Ophiopogonin D and Ruscogenin on Thrombus Formation Induced by Ligation of Inferior Vena Cava in Mice

Group	Dose (mg/kg)	Thrombus weight (mg)	Inhibition (%)
Control		1.11±0.12	
Ophiopogonin D	0.50	0.82±0.12	26.1
	1.00	0.55±0.07**	50.4
	2.00	0.51±0.04**	54.0
Ruscogenin	0.25	1.06±0.06	4.5
	0.50	0.86±0.04	22.5
	1.00	0.62±0.09**	44.1
Warfarin	2.00	0.50±0.05**	54.9

Ophiopogonin D at doses of 0.50, 1.00, and 2.00 mg/kg, ruscogenin at doses of 0.25, 0.50, and 1.00 mg/kg and warfarin at 2.00 mg/kg were administered orally 1 h or 18 h before ligation. Thrombus was obtained 6 h after ligation in mice. Data are expressed as the mean±S.E.M. of data from 18 mice. **$p<0.01$, compared with control.

DISCUSSION AND CONCLUSION

In this paper, we continued to observe the antithrombotic properties of the aqueous extract from *Radix Ophiopogon japonicus* in several animal thrombosis models. As an experimental model of peripheral obstructive disease, carrageenan-induced thrombosis in mice was used on the basis of its advantages of simple induction in small laboratory animals and easy observation and quantification without killing the animals.[27] The results showed that ROJ-ext significantly inhibited tail thrombus formation at 48 h and 72 h after carrageenan injection, an efficacy that was similar to that of aspirin (Table 1). Such data provided some evidence for its use in peripheral obstructive disease. On the other hand, it has been reported that the aggregation of platelets and the aggregation of red blood cells (RBC) are often increased in a carrageenin-induced thrombosis model,[29] which suggests that the antithrombotic activity of ROJ-ext might be related to the inhibition of aggregation of platelets and RBC.

Next, we investigated the antithrombotic activity of ROJ-ext in an AV shunt model in rats, in which, in addition to fibrin and erythrocytes, platelets make a significant contribution.[30] ROJ-ext also exerted a remarkable inhibitory effect (Table 2), which further confirmed its antithrombotic activity related to inhibition of platelet function. Next, we examined the *in vivo* effect of ROJ-ext and its two major components on platelet aggregation induced by ADP, and found that ROJ-ext and ruscogenin significantly inhibited platelet aggregation induced by ADP in rats by oral administration 3 times of 12.5 mg/kg or 0.7 mg/kg (Fig. 2), which proved that ROJ-ext inhibited arterial thrombosis and ruscogenin was one of its active components, and meanwhile suggested there were other components in ROJ-ext with inhibitory effects on platelet aggregation.

On the other hand, as mentioned in the introduction, we previously found that ROJ-ext significantly inhibited stasis-dependent venous thrombosis by tight ligation of the inferior vena cava in mice, and appeared to be slightly more potent than in the carrageenan model (71.6% *vs.* 60.0%). Therefore, we investigated the activities of two components (ruscogenin and ophiopogonin D) from ROJ-ext in the venous thrombosis model in mice. Considering the molecular mass of ruscogenin was almost half that of ophiopogonin D, we observed the effects of ruscogenin at doses of 0.25—1.00 mg/kg, and ophiogonin D at doses of 0.50—2.00 mg/kg. The results showed that they all produced dose-dependent inhibition of venous thrombus formation in mice, which suggests these two anti-inflammatory components from ROJ-ext were also its two antithrombotic constituents.

Overall, ROJ-ext ameliorates experimental thrombosis in

several animal models, which supported its therapeutic applications for thromboembolic diseases, and its activity can be partly ascribed to ophiopogonin D and ruscogenin. Further studies on its other active components and molecular mechanisms are in process.

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