Natural Product Sciences
10(3) : 109-113 (2004)

Antithrombosis of Chungpesagantang and Its Ingredients

Eun-Kyung Park[1], Yeo-Ock Han[2], Myung Joo Han[2], and Dong-Hyun Kim[1]*

[1]College of Pharmacy, Kyung Hee University, and [2]Department of Food and Nutrition, Kyung Hee University, 1, Hoegi, Dongdaemun-ku, Seoul 130-701, Korea

Abstract – The possibility of Chungpesagantang, which has been recommended for stroke patients in Korean Sasang Constitutional Clinics, and its ingredients as a novel antithrombotic agent was evaluated. Chungpesagantang potently inhibited ADP and collagen-induced rat platelet aggregation *in vitro* as well as *ex vivo* in a dose-dependent manner. Puerariae Radix and Rhei Rhizoma potently inhibited ADP-induced rat platelet aggregation *ex vivo*. However, Puerariae Radix did not inhibit both *in vitro* ADP and collagen-induced rat platelet aggregations. Chungpesagnatang and its ingredients except Rhei Rhizoma did not affect certain plasma clotting times, such as APTT, PT, and TT. Chungpesagantang and its ingredients Raphani semen and Scutellariae Radix showed significant protection against death due to pulmonary thrombosis in mice.

Keywords – Chungpesagantang, antithrombosis, Puerariae Radix, Rhei Rhizoma

Introduction

Platelets play an important role in the pathogenesis of thrombosis. The interactions between the platelets and blood vessel walls are important in the development of thrombosis and cardiovascular diseases, such as myocardial infraction stroke, and arteriosclerosis (Mustard, 1975; Mustard and Packham, 1990; Dinerman and Mehta, 1990). Once the blood vessels are damaged, platelet aggregation occurs rapidly to form hemostatic plugs or arterial thrombi at the sites of vessel injury or in regions where the blood flow is disturbed. These thrombi are the source of thromboembolic complications of arteriosclerosis, heart attacks, stroke, and peripheral vascular disease (Packham, 1994; Stein and Fuster, 1989; MacMahon and Fuster, 1989). Therefore, the inhibition of platelet function represents a promising approach for the prevention of thrombosis. A number of antiplatelet herbal medicines and their polyprescriptions have been evaluated for their effects in preventing the development of thrombosis or its recurrence.

As part of our continuing search for biological active anti-stroke agents from medicinal resources, we investigated Chungpesagantang, Sunghyangjunggisan and Yankyuksan-whatang because it has been used frequently for patients who suffer from stroke in Korea (Lee, 1996; Bae *et al.*, 1987; Park *et al.*, 2002). Kang *et al.* (2001) and Park *et al.* (2002) reported that Chungpesagantang exhibited antithrombotic activity the most potently. However, studies on antithrombotic activities of its ingredients have not been studied. In the present study, we evaluated the antithromotic activity of ingredients of Chungpesagantang and examined the possibility of ingredients of Chungpesagantang as a novel antithrombotic agent by determining its inhibitory effect on platelets induced by various aggregating agents *in vitro* and *ex vivo*, and its anti-thrombotic effect *in vivo*.

*Author for correspondence
Fax: +82-2-957-5030; E-mail dhkim@khu.ac.kr

Materials and Methods

Materials – Adenosine 5'-diphosphate (ADP), epinephrine, collagen, bovine serum albumin, prothrombin, thromboplastin, thrombin, dimethyl sulfoxide (DMSO), heparin, acetyl salicylic acid, and aPPT, PT and TT assay kits were purchased from Sigma Chemical Co. (USA). Human plasma was donated by Dr. N.-J. Kim (East-West Medical Research Institute, Kyung Hee University). The other chemicals were of analytical reagent grade.

Plant materials and extraction – The rhizome of *Scutellaria baicalensis*, the rhizome of *Angelica tenussima*, the rhizome of *Cimicifuga heracleifolia*, and the rhizome of *Angelcia dahurica* were produced in China and the rhizomes of *Pueraria thunbergiana*, the rhizome of *Planticodon gradiflorum* and the semen of *Raphanus satius* were in Korea. They were purchased from Kyungdong Crude Drug Market, Soul, Korea, 1999. They were identified by N.-J. Kim (East-West Medical Research Institute, Kyung Hee University) and

Table 1. Composition of the ingredients of Chungpesagantang and extracted yields

Herbal medicine	Composition (g)	Extract Yield (%)	Voucher Specimen
Puerariae Radix	16	22.7	KHP990702-1
Scutellariae Radix	8	35.4	KHP990702-2
Angelicae Tenuissimae Radix	8	19.2	KHP990702-3
Raphani Semen	4	10.7	KHP990702-4
Platycodi Radix	4	35.9	KHP990702-5
Cimicifugae Rhizoma	4	7.2	KHP990702-6
Angelicae Dahuricae Radix	4	25.1	KHP990702-7
Rhei Rhizoma	4	11.3	KHP990702-8
Chungpesagantang	52	19.4	

voucher specimens (990702-1 990702-8) were kept at College of Pharmacy, Kyung Hee University. One hundred grams of herbal medicines (100 g) or Chungpesagantang (Table 1) were extracted twice with 500 ml of boiling water. After evaporation, each extract were used.

Animals – Male Sprague-Dawley rats (male, 180-220 g) and ICR mice (male, 20-24 g) were purchased from Sam Yook Animal Co. (Korea) and acclimatized for one week at a temperature of 22±1°C and a humidity of 55±5% with free access to a commercial pellet diet obtained from Samyang Co. (Korea) and drinking water before the experiments. The animal experiments were carried out in accordance with international guidelines.

Preparation of platelets – Blood was collected from the rats by cardiac puncture into a flastic flask containing 2.2% sodium citrate (1:9 v/v). The platelet-rich plasma (PRP) was prepared by centrifuging the blood at 120×g for 15 min, and then again at 850×g for 10 min to prepare the platelet poor plasma (PPP) (Teng and Ko, 1988). The supernatants were pooled and centrifuged at 600×g for 15 min at room temperature. Then, platelet pellets were gently resuspended in Tyrode-HEPES buffer.

***In vitro* antiplatelet aggregation** – The platelet aggregation was measured by turbimetry using a dual channel Whole Lumini-Ionized Calcium Aggregometer (490-X, Chrono-Log Co., Ltd, Havertown, PA, USA) according to the method of Born and Cross (1963). Briefly, rat PRP (300 μl, 5×10^8 platelets/ml)) was incubated at 37°C for 2 min in the aggregometer with stirring at 1200 rpm, added test agents (150 μl) and then stimulated with ADP (50 μl, 5-8 μM) or collagen (50 μl, 50-80 μM). The herbal medicines or aspirin (as a reference agent) were incubated with PRP for 3 min, followed by addition of the aggregation agents. Any changes in light transmission were recorded for 10 min after stimulation with these agents. Each inhibition rate was obtained from the maximal aggregation induced by the respective agonist at the concentration derived from Equation 1, and then the values of IC_{50} were calculated from the data using a probit method.

Equation 1: Inhibition rate (%) = (1-maximal aggregation rate of sample treated PRP) / (maximal aggregation rate of vehicle treated PRP)×100

***Ex vivo* antiplatelet aggregation** – Male SD rats were used after overnight fasting. 1 g/kg of herbal medicine extract (or 50 mg/kg of aspirin) was administered orally to the rats as a vehicle for three days. The blood was collected 3 h after the final sample treatment, and the PRP was previously described. Platelet aggregation was induced by 80 μg/ml of collagen or 8 μM of ADP. The antiplatelet activities of the sample were investigated according to the method of Kimmura *et al.* (1985).

***In vivo* anti-thrombotic activity** – The anti-thrombotic effects of herbal medicines were investigated by the mouse thromboembolism test according to the method of DiMinno and Silver (1983). Male ICR mice were used after overnight fasting. The herbal medicines (1 g/kg), or aspirin (50 mg/kg) as a positive control, suspended in 0.5% CMC solution were administered orally. A mixture solution of collagen (110 μg) and epinephrine (13 μg) was injected into the mouse tail vein 90 min after the sample treatment, and pulmonary thrombosis was induced 3 h after oral administration of the samples. The number of dead or paralyzed mice was recorded for up to 15 min, and the percentage of protection was calculated as follows: [1 – (dead + paralyzed) / total]×100.

Tail bleeding time in conscious mice – The bleeding time was measured as described by Hornstra *et al.* (1981). The bleeding time is designed to determine the blood's ability to form a hemostatic plug, in which the platelet, plasma factor, and blood vessel wall are involved. In short, the herbal medicine (1 g/kg) or aspirin (50 mg/kg) suspended in 0.5% CMC solution were orally administered once a day for three days. 3 h after the oral administration of the samples, the tail of the male ICR mouse was transected 2 mm from the tip, and 1.5 cm of the distal portion was vertically immersed in saline at 37°C.

***In vitro* coagulation parameters** – The plasma clotting

times, activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) were measured by a modification of Hara's method (1994). The PPP was incubated with the samples for 7 min at 37°C, and the coagulation was started with by adding $CaCl_2$, 100 μl of thromboplastin, and 100 μl of bovine thrombin into the 100 μl of incubated plasma for the APTT, PT, and TT assays, respectively

Results

***In vitro* antiplatelet aggregation effect** – The *in vitro* inhibitory activity of Chungpesagantang and its ingredients on platelet aggregation was measured (Table 2). Chungpesagantang potently inhibited ADP-induced rat platelet aggregation *in vitro* in a dose-dependent manner, with an IC_{50} value of 1.5 mg/ml. Rhei Rhizoma and Scutellariae Radix also potently inhibited ADP-induced rat platelet aggregation *in vitro*, with IC_{50} values of 0.5 and 0.8 mg/ml, respectively. Chungpesagantang, Rhei Rhizoma and Scutellariae Radix inhibited collagen-induced rat platelet aggregation *in vitro*, with IC_{50} values of 1.5, 0.5 and 1.5 mg/ml, respectively. However, Puerariae Radix and Cimicifugae Radix did not inhibit both ADP and collagen-induced rat platelet aggregations. Aspirin, a reference drug that is widely used as an antiplatelet drug in clinical practice, potently inhibited collagen and ADP-induced platelet aggregation, with IC_{50} values of 1.1 and 1.2 mg/ml, respectively. The antiplatelet aggregation activities of Rhei Rhizoma and Scutellariae Radix were more potent than those of aspirin.

Table 2. Effect of Chungpesagantang and its ingredients on *in vitro* antiplatelet aggregation (aggregation inducer, ADP)

Herbal medicine	IC_{50}[a](mg/ml)	
	ADP	Collagen
Puerariae Radix	>15	>15
Scutellariae Radix	0.8	1.5
Angelicae Tenuissimae Radix	9.8	4.5
Raphani Semen	6	3.9
Platycodi Radix	>15	9
Cimicifugae Rhizoma	>15	>15
Angelicae Dahuricae Radix	5	4.5
Rhei Rhizoma	0.5	0.5
Chungpesagantang	1.5	1.5
Acetyl salicylic acid	1.2	1.1

[a]50% inhibitory concentration (IC_{50}) was calculated as follows: (control aggregation (%)-herbal medicine-treated aggregation (%))/control aggregation (%)×100 = inhibition (%)

***In vitro* anticoagulation effect** – The effect of herbal medicines on plasma clotting time was evaluated by APTT, PT and TT assays using human PPP (Table 3). Chungpesagantang and most of its ingredients did not affect the tested plasma clotting times. However, Rhei Rhizoma protected plasma clotting at a concentration of more than 0.5 mg/ml.

***Ex vivo* antiplatelet aggregation effect** – The *ex vivo* inhibitory activities of Chungpesagantang and its ingredients on platelet aggregation were measured, after they were orally administered once a day for three days into SD rats (Table 4). The inhibition of ADP or collagen-induced platelet aggregation in the group treated with Chungpesagantang was statistically significant from that of the control group. Chungpesagantang inhibited ADP-induced platelet aggregation more potently than aspirin (50 mg/kg). Among its ingredients, Puerariae Radix and Rhei Rhizoma exhibited the most potent inhibitory activity of ADP-induced platelet aggregation, followed by Scutellariae Radix. Collagen-induced platelet aggregation was potently inhibited by Angelicae Dahuricae Radix and

Table 3. Effect of Chungpesagantang and its ingredients on human plasma coagulation time

Herbal medicine	Concentration	Coagulation time (s)		
		APTT	PT	TT
Control		36.1 ± 1.2	40.8 ± 2.6	34.1 ± 2.4
Puerariae Radix	3 mg/ml	35.4 ± 2.1	27.6 ± 1.8	30.1 ± 1.1
Scutellariae Radix	3 mg/ml	30.8 ± 2.2	35.8 ± 1.7	33.2 ± 1.1
Angelicae Tenuissimae Radix	3 mg/ml	35.1 ± 0.7	37.1 ± 0.3	32.1 ± 2.5
Raphani Semen	3 mg/ml	35.3 ± 0.7	40.9 ± 1.8	35.1 ± 2.4
Platycodi Radix	3 mg/ml	38.1 ± 3.3	41.3 ± 0.7	30.8 ± 1.4
Cimicifugae Rhizoma	3 mg/ml	38.8 ± 3.5	26.8 ± 0.7	40.1 ± 1.8
Angelicae Dahuricae Radix	3 mg/ml	37.7 ± 3.9	38.1 ± 1.1	33.6 ± 0.4
Rhei Rhizoma	3 mg/ml	>500*	>500*	>500*
Rhei Rhizoma	1.5 mg/ml	105.3 ± 9.6*	>500*	>500*
Rhei Rhizoma	0.3 mg/ml	35.8 ± 1.2	46.1 ± 0.4*	31.8 ± 0.7
Chungpesagantang	10 mg/ml	45.6 ± 1.1*	33.9 ± 2.9	35.9 ± 1.4
Heparin	3 μg/ml	174.8 ± 22.4*	57.0 ± 7.7*	>500*
Heparin	0.3 μg/ml	40.6 ± 1.0*	48.2 ± 4. 9*	45.9 ± 0.6*

The results were expressed as mean ± standard deviation (n=5).
*Significantly different to control ($p<0.05$).

Table 4. Effect of Chungpesagantang and its ingredients on *ex vivo* antiplatelet aggregation

Herbal medicine	Platelet aggregation[a] (%)	
	ADP	Collagen
Control	60.5±0.7	56.0±1.4
Acetyl salicylic acid	42.5±10.6*	39.5±7.8*
Puerariae Radix	43.5±9.2*	53.5±0.7*
Scutellariae Radix	49.7±3.5*	48.7±5.7*
Angelicae Tenuissimae Radix	50.0±2.1*	42.0±11.3*
Raphani Semen	51.0±2.1*	56.5±4.9
Platycodi Radix	61.0±4.2	52.0±0.1*
Cimicifugae Rhizoma	52.0±15.6	39.0±8.5*
Angelicae Dahuricae Radix	59.5±6.4	30.7±.1.4*
Rhei Rhizoma	42.7±7.1*	59.0±6.1
Chungpesagantang	38.3±10.8*	44.6±16.2*

Samples were orally administered once a day for three days before the test.
The results were expressed as mean± standard deviation (n=5).
*Significantly different to control ($p<0.05$).
[a]Platelet aggregation was induced by 80 g/ml of collagen or 8M of ADP.

Table 5. Effect of Chungpesagantang and its ingredients on the mouse tail bleeding time

Herbal medicine	Dose (mg/Kg)	Tail bleeding time
Control	–	68±12.5
Acetyl salicylic acid	50	258±8.5*
Puerariae Radix	1000	>600*
Scutellariae Radix	1000	177±16.1*
Angelicae Tenuissimae Radix	1000	94±18.2*
Raphani Semen	1000	76±15.7
Platycodi Radix	1000	85±14.4
Cimicifugae Rhizoma	1000	81±15.7
Angelicae Dahuricae Radix	1000	62±8.2
Rhei Rhizoma	1000	>600*
Chungpesagantang	1000	108±19.6*

The results were expressed as mean ± standard deviation (n=5).
*Significantly different to control ($p<0.05$).

Cimicifugae Rhizoma.

Effect on the tail bleeding time of mice – The effect of herbal medicines on bleeding time was studied using the mouse tail bleeding system. As shown in Table 5, the tail bleeding time of the control mice was 68±10.5 s, while Puerariae Radix and Rhei Rhizoma markedly prolonged the mouse tail bleeding time (>600 s) compared to the control ($p>0.001$) and was more potent than aspirin at a dose of 50 mg/kg.

***In vivo* antithrombotic effect** – The *in vivo* antithrombotic activity of Chungpesagantang and its ingredients was measured (Table 6). Chungpesagantang showed siginificant protection from death due to pulmonary thrombosis in mice. Among their ingredients, Scutellariae Radix, Angelicae Tenuissimae Radix and Raphani Semen had more potent antithrombotic activity than Chungpesagantang, and exhibited a more potent antithrombotic activity than aspirin at a dose of 50 mg/kg.

Table 6. Antithrombosis activity of Chungpesagantang and its ingredients

Herbal medicine	No. dead or paralyzed / No. tested	Protection (%)
Control	20/25	20
Aspirin (25 mg/kg)	6/10*	40
Aspirin (50 mg/kg)	3/10*	70
Puerariae Radix (1 g/kg)	4/10*	60
Scutellariae Radix (1 g/kg)	2/10*	80
Scutellariae Radix (500 mg/kg)	4/10*	60
Angelicae Tenuissimae Radix (1 g/kg)	2/10	80
Angelicae Tenuissimae Radix (500 mg/kg)	7/10*	30
Raphani Semen (1 g/kg)	2/10*	80
Raphani Semen (500 mg/kg)	6/10*	40
Planticodi Radix (1g/kg)	6/10*	40
Cimicifugae Rhizoma (1g/kg)	8/10	20
Angelicae Dahuricae Radix (1g/kg)	8/10	20
Rhei Rhizoma (1g/kg)	4/10*	60
Chungpesagantang (1g/kg)	4/10*	60

The samples were orally administered 90 min before tail vein injection of epinephrine and collagen.
The χ^2 Test was used to examine the difference between vehicle- and sample-treated groups (*, $p<0.01$).

Discussion

Chungpesagantang, Sunghyangjunggisan and Yankyuksanwhatang have been used frequently in Korean Sasang Constitutional Clinics for patients who suffer from stroke (Bae *et al.*, 1987; Lee, 1996). Therefore, we investigated these formulas as part of our continuing search for biologically active anti-stroke agents from medicinal resources and reported that Chungpesagantang potently showed antiplatelet aggregation actvity. Therefore, in the present study we investigated *in vivo* and *in vitro* antithrombosis its ingredients of Chungpesagantang. Chungpesagantang exhibited *in vitro* and *ex vivo* antiplatelet aggregation activity as previously reported. Among its ingredients, Puerariae Radix significantly inhibited *ex vivo* antiplatelet activity, but did not inhibited *in vitro* platelet aggregation activity. Angelicae Tenuissimae Radix, Raphani Semen, and Angelicae Dahuricae Radix did not exhibit *ex vivo* anti-platelet aggregation activity, although these herbal medicines had *in vitro* antiplatelet aggregation activity. These results suggest that the components of herbal medicines could be transformed to the active or inactive components for antiplatelet aggregation by human intestinal bacteria. For example, puerarin, a main component of Puerariae Radix, could be transformed to daidzin or calycosin by human intestinal bacteria (Kim *et*

al., 1998). These compounds exhibited rat *in vitro* antiplatelet aggregation activity (data not shown). Puerariae Radix and Rhei Rhizoma potently inhibited plasma recalcification, followed by Chungpesagantang and Cimicifugae Rhizoma. Chungpesagantang, Angelicae Tenuissimae Radix and Puerariae Radix also activated urokinase, although they did not display urokinase-like activity (data not shown). When the prolongation activity of Chungpesagantang and its ingredients on bleeding time was investigated using the mouse tail bleeding system, Puerariae Radix, Angelicae Tenuissimae Radix, Rhei Rhizoma and Chungpesagantang were all effective. Scutellariae Radix, Angelicae Tenuissimae Radix and Raphani Semen exhibited the most potent thrombotic activity, followed by Chungpesagantang, Planticodi Radix and Rhei Rhizoma. These results support that the antithrombotic activity of Chungpesagantang can be caused by the activities of urokinase and antiplatelet aggragation originated from Puerariae Radix, Scutellariae Radix and Rhei Rhizoma. The antithrombotic activity of Chungpesagantang and its ingradients Puerariae Radix, Scutellariae and Rhei Rhizoma may be important in the prevention of thrombosis and cardiovascular diseases, such as myocardial infraction stroke and arteriosclerosis. These herbal medicines can prevent the development of thrombosis or its recurrence.

Acknowledgements

This study was supported by the grant of Oriental Medicine R&D Project from Korean Ministry of Health and Welfare (2000).

References

Bae, C.H., Cho, K.H., Lee, W.C., Kim, Y.S., Bae, H.S., Lee, K.S. and Koo, B.H., Clinical analysis of occlusive cerebrovascular disease, Kyung Hee Univ. *Orient. Med. J.*, **10**, 665-687 (1987).

Born, G.V.R. and Cross, M.J., The aggreagation of blood platelet. *J. Physiol.*, **168**, 178-195 (1963).

Dinerman, J.L. and Mehta, J.L., Endothelial, platelet and leukocyte interactions in ischemic heart disease: insights into potential mechanisms and their clinical relevance. *J. Am. Coll. Cardiol.*, **16**, 207-222 (1990).

Di Minno, G. and Silver, M., Mouse antithrombotic assay: a simple method for the evaluation of antithrombotic agents in vivo. Potentiation of antithrombotic activity by ethyl alcohol., *J. Pharmacol. Exp. Ther.*, **225**(1) pp. 57-60 (1983).

Hara, T., Yokoyama, A., Ishihara, H., Yokoyama, Y., Nagahara, T., and Iwanoto, M., DX-9065a, a new synthetic, potent anticoagulant and selective inhibitor for factor Xa. Thromb. Haemost., **71**, 314-9 (1994).

Hornstra, G., Christ-Hazelhof, E., Haddeman, E., Ten, H.F., and Nugsteren, D.H., Fish oil feeding lowers thromboxane- and prostacyclin production by rat platelets and aorta and does not result in the formation of prostaglandin I_3. *Prostaglandins*, **21**, 727-38 (1981).

Kang, J.K., Bae H.S., Kim, Y.S., Cho, K.H., Lee K.S., Park E.K., and Kim, D.-H., Antithrombosis of Chungpesagantang is activated by human intestinal bacteria. *Nat. Prod. Sci.*, **7**, 53-59 (2001).

Kim, D.-H., Yu, K.-U., Bae, E.-A., and Han, M.J., Metabolism of puerarin and daidzin by human intestinal bacteria and their relation to *in vitro* cytotoxicity. **21**, 628-630 (1998).

Kimura, Y., Tani, T., Kanbe, T., and Watanabe, K., Effect of cilostazol on platelet aggregation and experimental thrombosis., Arzneim., Forsch. Drug Res., **35**, 1144-49 (1985).

Lee, J.M., Longegity and life Preservation in Oriental Medicine (translated by S.H. Choi) pp 153-175, Kyung Hee Univ. Press, Seoul (1996).

MacMahon, S. and Sharpe, N., Long-term antiplatelet therapy for the prevention of vascular disease. *Med. J. Aust.*, **154**, 477-80 (1991).

Mustard, J.F., Platelets, thrombosis and drugs. *Drugs* **9**, 19-76 (1975).

Mustard, J.F., Packham, M.A., Kinlough-Rathbone, and R.L., Platelets, blood flow, and the vessel wall. Circulation **81**, 124-7 (1990).

Packham, M.A., Role of platelets in thrombosis and hemostasis. *Can. J. Physiol. Pharmacol.*, **72**, 278-84 (1994).

Park, E.K., Han, Y.O., Kim, J.M., Han, M.J., and Kim, D.-H. Antithrombotic activities of Chungpesagantang, Sunghyangjunggisan and Yankyuksanwhatang. *Nat. Prod. Sci.*, **8**, 173-176 (2002).

Stein, B. and Fuster, V., Role of platelet inhibitor therapy in myocardial infarction. Cardiovasc. *Drugs Ther.*, **3**, 797-813 (1989).

Teng, C.M. and Ko, F.N., Comparison of the platelet aggregation induced by three thrombin-like enzymes of snake venoms and thrombin. *Thromb. Haemost.*, **59**, 304-9 (1988).

(Accepted June 9, 2004)