Natural Product Sciences
7(2) : 53~59 (2001)

Anti-thrombosis of Chungpesagan-tang is Activated by Human Intestinal Bacteria

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Abstract – The possibility of Chungpesagan-tang, which has been recommended on the stroke patients with constipation in Korean traditional clinic, and its ingredients as a novel antithrombotic agent was evaluated. Most of its ingredients except Puerariae Radix exhibited *in vitro* antiplatelet aggregation activity. However, Puerariae Radix was significantly effective on *ex vivo* anti-platelet aggregation activity, whereas Angelicae Tenuissimae Radix, Raphani Semen and Angelicae Dahuricae Radix was not effective. Plasma recalcification was potently inhibited only by Puerariae Radix and Rhei Rhizoma treated with intestinal bacteria. Urokinase was also activated only by Chungpesagan-tang, Angelicae Tenuissimae Radix and Puerariae Radix treated with intestinal bacteria. Chungpesagan-tang exhibited the potent anti-thromboembolic activity activity *in vitro*. These results suggest that anti-thrombotic activity of Chungpesagan-tang should be activated by intestinal bacteria and may be important in the prevention of thrombosis and cardiovascular diseases, such as myocardial infraction stroke and arteriosclerosis.

Key words – Chungpesagan-tang, antithrombotic agent, human intestinal bacteria.

Introduction

Platelets play an important role in the pathogenesis of thrombosis. The interactions between platelets and blood vessel walls are important in the development of thrombosis and cardiovascular diseases such as myocardial infraction stroke, and arteriosclerosis (Mustard and Packham, 1975; Mustard *et al.*, 1990; Dinerman *et al.*, 1990). Once 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 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 Sharpe, 1991). Therefore, the inhibition of platelet function represents a promising approach for the prevention of thrombosis. A number of anti-platelet 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 biologically active anti-stroke agents from the medicinal resources, we investigated Chungpesagan-tang (Table 1), because it has been frequently used for patients who suffer from stroke in Korea. This Chungpesagan-tang was developed from Yuldahanso-tang, which also uses for stroke patients, by Jae-Ma Lee (1996). It has been recommended on the stroke patients with constipation more than Yuldahanso-tang (Bae *et al.*, 1987). In the present study, we examined the role of human intestinal bacteria on anti-thrombotic activity of Chungpesagan-tang and its ingredients.

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Materials and Methods

Materials – Adenosine 5'-diphosphate (ADP), epinephrine, collagen, bovine serum albumin, prothrombin, thromboplastin, thrombin and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (USA). The other chemicals were of analytical reagent grade.

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 1 week at a temperature of 22±1°C and a humidity of 55±5% with free access to a commercial pellet

Table 1. The Constituent Herbs of Chungpesagan-tang

Constituent Herbs	Weight (g)
Puerariae Radix (Root of *Pueraria thunbergiana* Bentham)	16
Angelicae Tenuissimae Radix (Root of *Angelica tenuissima* Nakai)	8
Scutellariae Radix (Root of *Scutellaria baicalensis* Georgi)	8
Platycodi Radix (Root of *Platycodon grandiflorum* A. De Candolle)	4
Raphani Semen (Seed of *Raphanus sativus* Linne)	4
Cimicifugae Rhizoma (Rhizome of *Cimicifuga heracleifolia* Komarov)	4
Angelicae Dahuricae Radix (Root of *Angelica dahurica* Bentham et Hooker)	4
Rhei Rhizoma (Rhizome of *Rheum palmatum* Linne)	4
Total amount	52

diet obtained from Samyang Co. (Korea) and drinking water before the experiments. Animal experiments were carried out in accordance with international guidelines.

Herbal medicine extracts and its metabolism by human intestinal microflora – Chungpesagan-tang (208 g) and its ingredients (200 g) were extracted with 1 liter of water, filtrated, evaporated on a rotary vacuum evaporator and then finally lyophilized with a freezing dryer. Their water extracts (500 mg) were suspended in 50 ml of diluted anaerobic medium, anaerobically incubated for 24 h with and without human intestinal bacteria, then extracted twice with ethylacetate, and evaporated on a rotary vacuum evaporator.

Preparation of platelets – Blood from rats was collected by cardiac puncture into a plastic flask containing 2.2% sodium citrate (1:9 v/v). Platelet rich plasma (PRP) was prepared by centrifugation of the blood at 120×g for 15 min and further centrifuged at 850×g for 10 min to prepare platelet poor plasma (PPP) (Teng and Ko, 1988). The supernatant was pooled and centrifuged at 600×g for 15 min at room temperature. The platelet pellets were washed with modified Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 2 mM EGTA, 5.6 mM glucose, 10 mM HEPESs, 0.35% BSA, pH 7.4) and centrifuged at 600×g for 15 min. Then, platelet pellets were gently resuspended in Tyrode-HEPES buffer and then used in experiment.

Assay of *in vitro* antiplatelet aggregation – The platelet aggregation was measured by turbimetry using a dual channel Whole Lumini-Ionized Calcium Aggregometer (Chrono-Log Co., Ltd, Havertown, PA, USA) according to the method of Born and Cross (1963). Briefly, rat PRP (300 μl) was incubated at 37°C for 2 min in the aggregometer with stirring at 1200 rpm and then stimulated with ADP, collagen and thrombin. Herbal medicines or aspirin, reference agent, was incubated with PRP for 3 min, followed by addition of the aggregation agents. 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 respective agonist at the concentration using Equation 1, and then the values of IC_{50} were calculated from the data using a probit method.

Equation 1:

$$\text{Inhibition rate (\%)} = \frac{\text{1 - maximal aggregation rate of sample treated PRP}}{\text{maximal aggregation rate of vehicle treated PRP}} \times 100$$

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

al. (1985).

Assay of *in vivo* anti-thrombotic activity – The anti-thrombotic effect of herbal medicines were investigated by the mouse thromboembolism test according to the method of DiMinno *et al.* (1983). Male ICR mice were used after overnight fasting. Herbal medicines (1 g/kg), aspirin (50 mg/kg) as a positive control and 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 sample treatment and pulmonary thrombosis was induced 3 h after oral administration of the samples. The number of dead or paralyzed mice was recorded up to 15 min and the percentage of protection was calculated as follows: [1-(dead+paralyzed)/total]×100

Fibrinolytic activity assay – Fibrinolytic activity assay was measured as described by Terasawa *et al.* (1983). Fibrin plate was prepared by adding fibrinogen and thrombin in a Petri dish. The disc (0.5 cm) containing mg of herbal medicine and unit of urokinase was loaded on this fibrin plate and the size of its fibrinolytic haloes was measured.

Plasma recalcification assay – The plasma clotting times was measured by the modification of Hara's method (1994).

Results

In vitro and *ex vivo* inhibitory activities of Chungpesagan-tang and its ingredients on ADP- and collagen-induced platelet aggregation were measured (Table 2). Chungpesagan-tang potently inhibited ADP-induced rat platelet aggregation *in vitro*. Platycodi Radix, Rhei Rhizoma and Scutellariae Raidx, which are ingredients of Chungpesagan-tang, also potently inhibited ADP-induced rat platelet aggregation *in vitro*. However, on *ex vivo* ADP-induced rat platelet aggregation, these herbal medicines did not show potent inhibitory activity. Puerariae Radix did not inhibit *in vitro* ADP-induced platelet aggregations. However, on *ex vivo* ADP-induced rat platelet aggregation, it showed the inhibitory activity. When this Puerariae Radix with each ingredient of Chungpesagan-tang were combined and extracted, they synergistically inhibited the platelet aggregations. Among those ingredients tested, Angelicae Tenuissimae Radix and Cimicifugae Rhizoma were most potent, followed by Platycodi Radix and Rhei Rhizoma. Aspirin, a reference drug which is a widely used anti-platelet drug in clinical practice, potently inhibited *in vitro* and *ex vivo* platelet aggregation. The anti-platelet aggregation activities of Rhei Rhizoma and Scutellariae Radix were more potent than that of aspirin.

In vitro inhibitory activity of Chungpesagan-tang and its ingredients on thrombin-induced platelet aggregation was measured (Table 3). Chungpesagan-tang and its ingredients did not have inhibitory activity. However, when Chungpesagan-tang and its ingredients were metabolized by human intestinal bacteria, they

Table 2. The yield rate on water extract of Chungpesagan-tang and its ingredients

Ingredient	Water Ex (%)	Ethylacetate Ex (mg)	
		Non-treated	Treated
Puerariae Radix	29.1	30.4	57.0
Scutellariae Radix	40.0	36.1	68.5
Angelicae Tenuissimae Radix	25.0	8.8	15.7
Raphani Semen	13.2	0.8	2.4
Platycodi Radix	38.2	1.2	9.0
Cimicifugae Rhizoma	11.8	19.7	25.0
Angelicae Dahuricae Radix	31.6	5.8	14.2
Rhei Rhizoma	19.4	72.9	149.5
Puerariae Radix : Scutellariae Radix 2:1	29.9	23.7	67.7
Puerariae Radix : Angelicae Tenuissimae Radix 2:1	22.5	28.5	51.7
Puerariae Radix : Raphani Semen 4:1	26.0	26.4	45.4
Puerariae Radix : Platycodi Radix 4:1	32.7	26.9	55.3
Puerariae Radix : Cimicifugae Rhizoma 4:1	23.9	30.6	61.6
Puerariae Radix : Angelicae Dahuricae Radix 4:1	27.7	31.5	59.4
Puerariae Radix : Rhei Rhizoma 4:1	32.5	45.5	77.9
Puerariae Radix : Scutllariae Radix : Angelicae Tenuissimae Radix 2:1:1	22.0	17.4	53.9
Chungpesagan-tang	36.0	25.6	60.7

Table 3. Effect of water extract of Chungpesagan-tang and its ingredients on antiplatelet aggregation activity (inducer, 8 μM ADP)

Ingredient[a)]	Aggregation (%)		
	In vitro	*Ex vivo*	
	ADP	ADP	collagen
Control	52.5±3.5	60.5±0.7	56±1.4
Puerariae Radix	100	43.5±9.2*	53.5±0.7*
Scutellariae Radix	25.0±7.1	52±3.5*	52.3±5.7
Angelicae Tenuissimae Radix	47.5±3.5	52.5±3.5*	421±1.3*
Raphani Semen	40.0±7.1	50.3±2.1*	56.5±4.9
Platycodi Radix	20.0±7.1	61±4.2	52±0.1*
Cimicifugae Rhizoma	42.5±3.5	53±15.6	46±8.5*
Angelicae Dahuricae Radix	42.5±3.5	59.5±6.4	46.0±1.4*
Rhei Rhizoma	37.5±3.5	47.0±2.8*	45.5±4.9*
Puerariae Radix : Scutellariae Radix 2:1	100	36±1.4*	15±0*
Puerariae Radix : Angelicae Tenuissimae Radix 2:1	50±3.5	19.5±12.0*	0*
Puerariae Radix : Raphani Semen 4:1	100	32±7.1*	14±8.5*
Puerariae Radix : Platycodi Radix 4:1	100	27.5±16.3*	0*
Puerariae Radix : Cimicifugae Rhizoma 4:1	100	28±7.1*	0*
Puerariae Radix : Angelicae Dahuricae Radix 4:1	100	40.5±4.9*	24.5±24.7*
Puerariae Radix : Rhei Rhizoma 4:1	100	32±18.4*	13.5±19.1*
Puerariae Radix : Scutellariae Radix : Angelicae Tenuissimae Radix 2:1:1	37.5±3.5	30±2.8*	11±15.6*
Yuldahanso-tang	46.5±2.1	47.1±5.8*	56.5±8.0
Chungpesagan-tang	40.0±1.4	44.7±13.9*	46.0±8.0*

[a)]Final concentration of each herbal medicine or each polyprescription was 3 mg/ml.
*Significantly different from control group ($p<0.05$).

Table 4. Antiplatelet aggregation activity of Chungpesagan-tang (thrombin, 4 unit/ml)

Ingredient[a)]	Aggregation (%)		
	Water Ex[b)]	Treated[c)]	Non-treated[d)]
Control (D.W)		68.8	
Puerariae Radix	60	36	60
Scutellariae Radix	65	23	30
Angelicae Tenuissimae Radix	60	39	45
Raphani Semen	52	24	61
Platycodi Radix	60	0	44
Cimicifugae Rhizoma	60	45	52
Angelicae Dahuricae Radix	60	25	49
Rhei Rhizoma	68	0	0
Puerariae Radix : Scutellariae Radix 2:1	60	0	64
Puerariae Radix : Angelicae Tenuissimae Radix 2:1	55	47	61
Puerariae Radix : Raphani Semen 4:1	60	55	69
Puerariae Radix : Platycodi Radix 4:1	55	12	63
Puerariae Radix : Cimicifugae Rhizoma 4:1	65	10	48
Puerariae Radix : Angelicae DahuricaeRadix 4:1	60	0	53
Puerariae Radix : Rhei Rhizoma 4:1	60	0	24
Puerariae Radix : Scutellariae Radix : Angelicae Tenuissimae Radix 2:1:1	62	0	60
Chungpesagan-tang	55	0	17
Blank (intestinal bacterial strains)		63	68

[a)]Final concentration of each herbal medicine was 1.5 mg/ml.
[b)]Water Ex. is the extract of polyprescription with water according to the Material and Methods.
[c)]Each water extract of polyprescription was treated with human intestinal bacteria and then the reaction mixture extracted at pH 2 with the ethylacetate.
[d)]Each water extract of polyprescription was not treated with human intestinal bacteria and then the reaction mixture was extracted at pH 2 with the ethylacetate.

showed potent inhibitory activity. Chungpesagan-tang was most potent, followed by Rhei Rhizoma. When this Puerariae Radix with each ingredient of Chungpesagan-tang were combined and extracted, most ingredients synergistically inhibited the platelet aggregations. However, among the ingredients tested, Angelicae Tenuissimae Radix and Raphani Semen interfered antagonistically the inhibitory action of Puerariae Radix on thrombin-induced platelet aggregation.

In vitro inhibitory activities of Chungpesagan-tang and its ingredients on plasma recalcification were measured (Table 4). Chungpesagan-tang and its ingredients except Scutellariae Radix and Cimicifugae Radix did not have inhibitory activity. However, when Chungpesagan-tang and its ingredients were metabolized by human intestinal bacteria, most ingredients showed the potent inhibitory activity. Rhei Rhizoma was best, followed by Puerariae Radix. When this Puerariae Radix with each ingredient of Chungpesagan-tang were combined and extracted, the inhibitory activity of Puerariae Radix was not affected.

We also measured whether Chungpesagan-tang and its ingredients activate urokinase, which hydrolyzes fibrin, or not (Table 5). Chungpesagan-tang and its ingredients could not activate urokinase. However, when Chungpesagan-tang and its ingredients were metabolized by human intestinal bacteria, Angelicase Dahuricae Radix, Angelicae Tenuissimae Radix and Platycodi Radix showed potent inhibitory activity. When this Puerariae Radix with each ingredient of Chungpesagan-tang were combined and extracted, Platycodi Radix activate the fibrinolytic activity of Puerariae Radix.

In vivo antithrombotic activity of Chungpesagan-tang and Puerariae Radix were measured (Table 6). Chungpesagan-tang and Puerariae Radix showed significant protection from death due to pulmonary thrombosis in mice. These protections were similar to that of aspirin at the dose of 50 mg/kg.

Discussion

Chungpesagan-tang has been frequently used for patients who suffer from stroke in Korean traditional clinics. However, its anti-stroke activities have not been evaluated. Therefore, we investigated this Chungpesagan-

Table 5. Effect of Chungpesagan-tang and its ingredients on plasma recalcification time

Ingredient[a]	Recalcification time (sec)		
	Water Ex[b]	Treated[c]	Non-treated[d]
Distilled water		189.7±15.6	
Puerariae Radix	224.3±14.3	840.0±0	375.0±77.8
Scutellariae Radix	382.2± 5.9	317.5±22.2	517.5±73.7
Angelicae Tenuissimae Radix	226.7±10.4	840.0±0	460.0±0
Raphani Semen	240.7±17.3	647.5±60.1	262.5±10.6
Platycodi Radix	226.1±21.1	290.0±0	200.0±0
Cimicifugae Rhizoma	306.6±29.7	515.0±176.8	690.0±130.8
Angelicae Dahuricae Radix	210.5±25.3	720.0±169.7	640.0±282.8
Rhei Rhizoma	273.0± 5.9	>1000	590.0±44.2
Puerariae Radix : Scutellariae Radix 2:1	256.2± 5.9	356.7±72.3	435.0±33.2
Puerariae Radix : Angelicae Tenuissimae Radix 2:1	203.0±23.3	494.3±40.1	398.8±130.7
Puerariae Radix : Raphani Semen 4:1	239.3±11.8	280.0±22.9	315.0±127.7
Puerariae Radix : Platycodi Radix 4:1	244.3±16.8	388.3±23.6	383.8±74.5
Puerariae Radix : Cimicifugae Rhizoma 4:1	270.2±15.5	246.7±20.8	435.0±63.8
Puerariae Radix : Angelicae Dahuricae Radix 4:1	243.8±26.0	290.0±0	640.0±122.9
Puerariae Radix : Rhei Rhizoma 4:1	271.9± 8.7	275.0±39.7	381.3±43.3
Puerariae Radix : Scutellariae Radix : Angelicae Tenuissimae Radix 2:1:1	228.5±28.3	386.7±47.3	366.3±81.4
Chungpesagan-tang	252.9±23.8	335.0±31.2	376.3±101.3
Blank (intestinal bacterial strains)	–	212.5±74.2	165.0±0

[a] Final concentration of each herbal medicines was 2.5 mg/ml.
[b] Water Ex. is the extract of polyprescription with water according to the Experimental.
[c] Each water extract of polyprescription was treated with human intestinal bacteria and then the reaction mixture extracted at pH 2 with the ethylacetate.
[d] Each water extract of polyprescription was not treated with human intestinal bacteria and then the reaction mixture was extracted at pH 2 with the ethylacetate.

Table 6. Effect of Chungpesagan-tang and its ingredient herbal medicines on the fibrinolytic activity of urokinase

Ingredient[a]	Diameter (mm)		
	Water Ex[b]	Treated[c]	Non-treated[d]
Distilled water		13	
Puerariae Radix	10	12	10
Scutellariae Radix	12	15	10
Angelicae Tenuissimae Radix	10	16	11
Raphani Semen	10	12	9
Platycodi Radix	10	16	12
Cimicifugae Rhizoma	9	15	18
Angelicae Dahuricae Radix	11	18	15
Rhei Rhizoma	9	9	10
Puerariae Radix : Scutellariae Radix 2:1	9	13	9
Puerariae Radix : Angelicae Tenuissimae Radix 2:1	11	13	9
Puerariae Radix : Raphani Semen 4:1	9	10	10
Puerariae Radix : Platycodi Radix 4:1	8	16	15
Puerariae Radix : Cimicifugae Rhizoma 4:1	10	17	19
Puerariae Radix : Angelicae Dahuricae Radix 4:1	9	19	15
Puerariae Radix : Rhei Rhizoma 4:1	8	11	10
Puerariae Radix : Scutellariae Radix : Angelicae Tenuissimae Radix 2:1:1	9	13	12
Chungpesagan-tang	9	14	8

[a]Loaded amount of each herbal medicines on disk was 0.3 mg.
[b]Water Ex. is the extract of polyprescription with water according to the Material and Methods.
[c]Each water extract of polyprescription was treated with human intestinal bacteria and then the reaction mixture extracted at pH 2 with the ethylacetate.
[d]Each water extract of polyprescription was not treated with human intestinal bacteria and then the reaction mixture was extracted at pH 2 with the ethylacetate.

tang and its ingredients as part of our continuing search for biologically active anti-stroke agents from the herbal medicinal resource. This polyprescription and its ingredients except Puerariae Radix exhibited *in vitro* antiplatelet aggregation activity. However, *ex vivo* anti-platelet aggregation activity of Puerariae Radix was significantly effective, whereas it did not exhibited *in vitro* anti-platelet activty. However, 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* anti-platelet aggregation activity. These results suggest that the components of herbal medicines could be transformed to the active or inactive components for anti-platelet aggregation by human intestinal bacteria. For example, puerarin, the major component of Puerariae Radix, could be transformed to daidzin or calycosin, which have rat antiplatelet aggregation activity, by human intestinal bacteria (Data not shown).

Table 7. Anti-thromboembolic activity of Chungpesagan-tang and Puerariae Radix

Herbal medicine	Dose (g/kg)	No. dead+ No. paralyzed/ No. tested	Protection (%)
Control (Distilled water)		40/50	20
Aspirin	0.025	6/10	40
	0.05	6/15	60
Puerariae Radix	1	4/10	60
Yuldahanso-tang	1	4/10	60
Chungpesagan-tang	1	4/10	60

Puerariae Radix and Rhei Rhizoma potently inhibited plasma recalcification, followed by Chungpesagan-tang and Cimicifugae Rhizoma. Chungpesagan-tang, Angelicae Tenuissimae Radix and Puerariae Radix also activated urokinase, although they did not have urokinase-like activity (Data not shown). Puerariae Radix and Chungpesagan-tang exhibited the potent anti-thromboembolic activity. These inhibitory activities are important in the prevention of thrombosis and cardiovascular diseases such as myocardial infarction stroke, and arteriosclerosis. Based on these findings, Chungpesagan-tang could prevent the development of thrombosis or its recurrence.

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(Accepted June 2, 2001)