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Platelet anti-aggregatory and blood anti-coagulant effects of compounds isolated from *Paeonia lactiflora* and *Paeonia suffruticosa*

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The roots of two Paeoniaceae family members have long been used as traditional medicines in Korea, China, and Japan. Dry roots of *Paeonia lactiflora* and dry root bark of *P. suffruticosa* are used under the traditional names of Paeoniae Radix and Moutan Cortex, respectively. Both Paeoniae Radix and Moutan Cortex have been used as remedies for cardiovascular diseases, for improving blood circulation, or for other uses. It was postulated that both plants may contain common active constituents that contribute to inhibiting blood coagulation and/or platelet aggregation. Eighteen compounds, which have been reported to be present in both plant medicines, were evaluated for their effects on platelet aggregation and blood coagulation. Paeonol (**5**), paeoniflorin (**9**), benzoylpaeoniflorin (**11**), and benzoyloxypaeoniflorin (**12**) were found to be the major common active constituents and they would collectively contribute to improving blood circulation through their inhibitory effects on both platelet aggregation and blood coagulation. In addition, methylgallate (**4**), (+)-catechin (**7**), paeoniflorigenone (**8**), galloylpaeoniflorin (**13**), and daucosterol (**16**) may also take part in improving blood circulation by inhibiting ether platelet aggregation and/or blood coagulation.

1. Introduction

Platelets play a key role in the normal hemostatic process caused by injury of blood vessels, as well as in the pathogenesis of thrombosis resulting from improperly regulated hemostatic stimuli in blood flow following the formation of a platelet or hemostatic plug (Packham 1994). Blood coagulation is also an important part of hemostasis whereby a damaged blood vessel wall is covered by a fibrin-clot to stop hemorrhage and repair of the damaged vessel (Gopinath 2008; Tanaka et al. 2009). Activated platelets provide a surface on which coagulation factors can assemble and initiate the clotting cascade leading to thrombin production (Dupont et al. 2009; Konkle et al. 2005). Thus, anti-platelet and anti-coagulant compounds can be therapeutic for various thrombotic circulatory diseases. However, current anti-platelet and anti-coagulant drugs with their associated side effects can have considerable limitations (Ni and Freeman 2003; Barrett et al. 2008; Bird et al. 2008).

Roots of two members of the Paeoniaceae family have long been used in Korea, China, and Japan as sources of traditional medicines. The dried roots of *Paeonia lactiflora* Pall. and the dry root bark of *P. suffruticosa* Andrews are used under the traditional names of Paeoniae Radix and Moutan Cortex, respectively. These are one of the few examples of plants belonging to one plant genus used under two different traditional names for different traditional purposes. Paeoniae Radix is used as an analgesic and antipyretic (World Health Organization 1999) while Moutan Cortex is used as a sedative, anti-inflammatory

agent, and as a remedy for various female disorders (Wu and Gu 2009). In addition, both Paeoniae Radix and Moutan Cortex are used as remedies for cardiovascular diseases and for improving blood circulation. The chemical constituents of Paeoniae Radix and Moutan Cortex have been investigated and found to comprise simple phenols, monoterpenes, and triterpenoid compounds (Chu et al. 2004; Ding et al. 2000; Hu et al. 2006; Kim JS et al. 2008a, 2008b; Kim et al. 2008; Lin et al. 1996, 1998; Shon and Nam 2004; Tanaka et al. 2000; Wang et al. 2005, 2006; Wu et al. 2002; Xu et al. 2006; Yean et al. 2008), which are thought to be responsible for the reported biological activities of these traditional medicines. Since both Paeoniae Radix and Moutan Cortex have been used for improving blood circulation, it was postulated that both might contain common active constituents which contribute to the inhibition of blood coagulation and/or platelet aggregation. However, most of the pharmacological investigations relating anti-thrombotic effects have been limited on the effects of the extract and the major components paeonol (Lv and Liu 2005; Doble et al. 2005; Ghayur et al. 2008; Hsieh et al. 2006; Lin et al. 1999; Shi et al. 1988) and paeoniflorin (Son and Park 2002). Moreover, the reported inhibitory effect of paeonol on ADP- or collagen-induced platelet aggregation was rather very mild (Hirai et al. 1983; Akamanchi et al. 1999), although paeonol have been known to be the major active component of Paeoniae Radix and Moutan Cortex. The present investigation was carried out to identify the common active components which contribute to improve blood circulation. Our survey of the literature revealed that 34 compounds have been previously

reported to be contained in both plant medicines. Of those 34 compounds, 18 compounds were evaluated to determine their effects on platelet aggregation and blood coagulation.

2. Investigations, results and discussion

Seven simple phenolic or flavonoid compounds [benzoic acid (**1**), *p*-hydroxybenzoic acid (**2**), gallic acid (**3**), methylgallate (**4**), paeonol (**5**), paeonoside (**6**), and (+)-catechin (**7**)], six monoterpenoids [paeoniflorigenone (**8**), paeoniflorin (**9**), oxypaeoniflorin (**10**), benzoylpaeoniflorin (**11**), benzoyloxypaeoniflorin (**12**), and galloylpaeoniflorin (**13**)], and five steroids [betulinic acid (**14**), β -sitosterol (**15**), daucosterol (**16**), hederagenin (**17**), and oleanolic acid (**18**)], could be isolated from *Paoniae Radix* and *Moutan Cortex* in amounts that were sufficient for this study's biological tests. The 18 compounds were evaluated for their inhibitory effects on shear stress-induced and agonist (collagen, ADP, AA or

U46619)-induced platelet aggregation and on blood coagulation [prothrombin Time (PT), thrombin Time (TT), and activated partial thromboplastin time (aPTT)].

Compounds **1** and **9**, compared with cilostazol (a positive control), showed moderate inhibitory effects ($\geq 20\%$ vs. $>60\%$) on shear stress induced aggregation while **5**, **8**, **10**, **11**, and **16** show mild ($<20\%$) effects. On the other hand, compound **14** augmented shear stress-induced aggregation (Fig. 2). The agonist-induced platelet anti-aggregation effects were compared with the effects of ASA, a well-known inhibitor of platelet aggregation (Table 1). Inhibitory potencies of compounds **8**, **11**, **12**, **13**, and **16** were approximately twice that of ASA in collagen-induced aggregation. Compounds **4**, **5**, **11**, and **16** exhibited inhibitory effects on AA-induced platelet aggregation that were comparable to that of ASA. Furthermore, compounds **11**, **12**, **15**, and **16** showed 5~8 fold greater inhibitory effects than that of ASA to U46619-induced platelet aggregation. All tested compounds including ASA, at 300 μM , produced only relatively

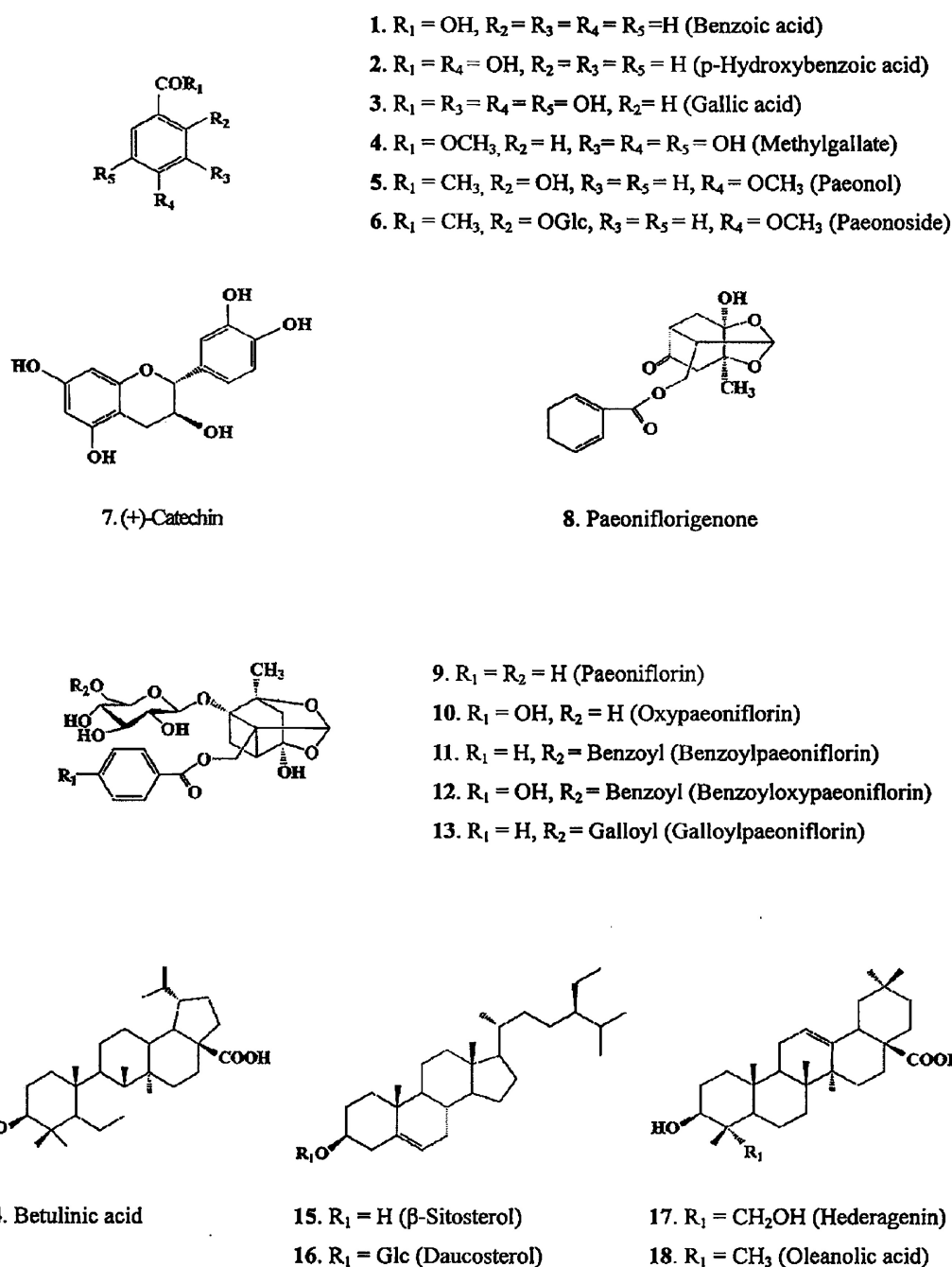


Fig. 1:

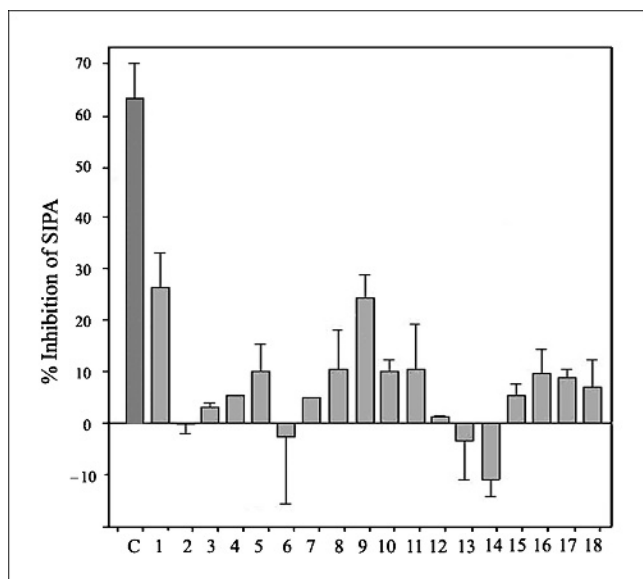


Fig. 2: The effects of the compounds on shear stress-induced platelet aggregation (SIPA). PRP was inflicted for 3 min at the shear rate of 10,800/s. Sample concentration; 25 μ M. Values are means \pm SD of minimum three independent experiments. C: cilostazol (a positive control)

negligible effects, if not at all, on ADP-induced aggregation than on collagen-, AA- or U46619-induced platelet aggregation (data not shown).

The anti-coagulation effects were evaluated by their effects on aPTT for the intrinsic pathway, PT for the extrinsic pathway, and TT for the common pathway (Table 2). Heparin, as a positive control, showed very strong anti-coagulant effects with marked elongation of PT, TT, and aPTT. Since strong anti-coagulant effects tend to be associated with bleeding disorders, mild anti-coagulant effects are beneficial for long-term treatment. Compounds 3, 5, 7, 9, and 12 showed relatively mild

Table 1: Effects of the compounds on agonist induced platelet aggregation

Compounds	IC ₅₀ (μ M)		
	Collagen ^{a)}	AA ^{b),d)}	U46619 ^{c),d)}
ASA (acetyl salicylic acid)	340.0	63.0	501.0
1 Benzoic acid	N	N	N
2 p-Hydroxybenzoic acid ^{e)}	N	≥ 300	N
3 Gallic acid	N	N	279.6
4 Methylgallate	≥ 300	49.4	N
5 Paeonol	≥ 300	67.1	N
6 Paeonoside	N	N	N
7 (+)-Catechin	≥ 300	N	N
8 Paeoniflorigenone	115.3	≥ 300	≥ 300
9 Paeoniflorin	N	N	N
10 Oxypaeoniflorin	N	N	N
11 Benzoylpaeoniflorin	124.1	42.2	169.2
12 Benzoyloxypaeoniflorin	158.0	≥ 300	106.1
13 Galloylpaeoniflorin	147.4	128.5	≥ 300
14 Betulinic acid	N	≥ 300	≥ 300
15 β -Sitosterol ^{f)}	195.0	145.0	170.0
16 Daucosterol ^{g)}	114.0	66.5	56.1
17 Hederagenin ^{g)}	N	N	N
18 Oleanolic acid ^{h)}	N	N	N

a) collagen: 2–5 μ g/mL, b) AA: 40–80 μ M, c) U46619: 2–5 μ M

d) In the presence of threshold concentration of collagen (1–1.8 μ g/mL)

e) Cited from Pyo et al., 2002, f) Cited from Jin et al., 2004b, g) Cited from Jin et al., 2004a

*N; no effect

Table 2: Effects of the compounds on blood coagulation

Compounds ^{a)}	Clotting time (s)		
	PT	TT	aPTT
Control	12.9 \pm 0.1	18.8 \pm 0.4	32.3 \pm 1.2
Heparin ^{b)}	37.7 \pm 1.5	46.4 \pm 2.2	76.3 \pm 2.0
1 Benzoic acid	13.9 \pm 0.0	20.5 \pm 0.4	32.3 \pm 3.5
2 p-Hydroxybenzoic acid	12.4 \pm 0.8	18.5 \pm 0.3	32.0 \pm 2.3
3 Gallic acid	13.2 \pm 0.2	22.0 \pm 0.2**	32.1 \pm 0.2
4 Methylgallate	12.7 \pm 0.0	19.5 \pm 0.8	33.2 \pm 0.4
5 Paeonol	14.0 \pm 0.1	20.8 \pm 0.2**	35.1 \pm 1.5
6 Paeonoside	13.0 \pm 0.5	17.5 \pm 0.6	26.2 \pm 1.3
7 (+)-Catechin	14.0 \pm 0.1	21.6 \pm 0.3**	37.4 \pm 0.3**
8 Paeoniflorigenone	12.8 \pm 0.2	20.6 \pm 0.3	32.7 \pm 0.3
9 Paeoniflorin	12.4 \pm 0.2	20.7 \pm 0.0**	32.7 \pm 0.7
10 Oxypaeoniflorin	14.9 \pm 0.2**	17.2 \pm 0.6	32.4 \pm 0.8
11 Benzoylpaeoniflorin	12.3 \pm 0.3	19.6 \pm 0.4	35.8 \pm 0.8*
12 Benzoyloxypaeoniflorin	12.5 \pm 0.0	21.9 \pm 0.0**	32.0 \pm 0.2
13 Galloylpaeoniflorin	13.2 \pm 0.2	17.4 \pm 0.8	34.4 \pm 1.8
14 Betulinic acid	12.7 \pm 0.3	17.0 \pm 0.8	31.8 \pm 0.7
15 β -Sitosterol	12.8 \pm 0.4	19.3 \pm 0.9	31.1 \pm 0.2
16 Daucosterol	12.5 \pm 0.3	19.1 \pm 0.0	32.6 \pm 0.7
17 Hederagenin	13.0 \pm 0.8	18.1 \pm 0.4	33.3 \pm 1.7
18 Oleanolic acid	12.0 \pm 0.6	19.9 \pm 1.4	33.6 \pm 0.4

a) sample concentration: 300 μ M

b) concentrations for PT: 2.0 U/mL, TT: 0.1 U/mL, aPTT: 0.05 U/mL. Values are means \pm SD (standard deviation) of minimum three independent experiments * p < 0.05, ** p < 0.001

anti-coagulant effects by elongating TT by about 10~17% compared with that of the control. In addition, aPTT was prolonged by about 10~15% from the control time by compounds 7 and 11 while PT was elongated by approximately 15% compared to that of the control by compound 10.

In summary, paeonol (5), paeoniflorin (9), benzoylpaeoniflorin (11), and benzoyloxypaeoniflorin (12) are suggested to be the major common active constituents in Radix Paeoniae Radix and Moutan Cortex. Those compounds would collectively contribute to improvements in blood circulation through their inhibitory effects on both platelet aggregation (either shear stress induced or agonist induced) and blood coagulation. In addition, methylgallate (4), (+)-catechin (7), paeoniflorigenone (8), galloylpaeoniflorin (13), and daucosterol (16) also affect platelet aggregation and/or coagulation and may also take parts in improving.

3. Experimental

3.1. Materials and instruments

The 18 compounds were separated from either Paeoniae Radix and Moutan Cortex according to previously reported methods and were identified by direct comparison of their ¹H- and ¹³C NMR and other spectroscopic data with the previously reported data: 1, 2, 3, 4, 5 (Kim et al. 2008a), 6 (Yu et al. 1986), 7 (Kim et al. 2008a), 8 (Lin et al. 1998), 9, 10, 11 (Yean et al. 2008), 12, 13 (Xu et al. 2006), 14, 15, 16, 17 and 18 (Kim et al. 2008b). Platelet counts were determined on an ExcellTM 18 Hematology Analyzer (Drew Scientific Inc., Dallas, TX, U.S.A.) and a hemocytometer (Neubauer, Superior Marienfeld, Lauda-Königshofen, Germany). Platelet aggregation was measured on a platelet aggregometer (490X, Chrono-Log Corp., Havertown, PA, U.S.A.) interfaced with a personal computer. Blood coagulation times were established using a fibrometer (BBL[®] Fibrosystem[®], Fisher Scientific, Philadelphia, PA, U.S.A.). Shear stress-induced aggregation was determined using a con-plate viscometer (RotoVisco 1, Haake, Karlsruhe, Germany), a thermomixer (DE/thermomixer compact, Eppendorf, Hamburg, Germany) and a microscope (CX41, Olympus Corp., Tokyo, Japan). Collagen was purchased from Chrono-Log Corp. and Beckman Coulter Inc. (Fullerton, CA, USA), respectively. Glutaraldehyde, ADP (adenosine 5'-diphosphate), sodium arachidonate (AA), U46619 (9,11-dideoxy-11 α ,9 α -epoxymethanoprostaglandin F2 α , TX₂ mimetic), and ASA (acetyl

salicylic acid) were obtained from Sigma Chem. Co. (St. Louis, MO, U.S.A.). aPTT (STA®-PTT A ⑤), PT (Neoplastine® CI plus), TT (STA®-Thrombin) test kits, STA®-Coag Control (N)+ (P), and STA®-CaCl₂ were purchased from Diagnostica-STAGO, Asnieres, France.

3.2. Blood sample collection from humans and rats

Venous blood from normal, healthy males, who had not taken any medications for the previous 14 days, was collected using a 21G needle into a vacutainer (Becton-Dickinson, Franklin Lakes, NJ, U.S.A.), containing 0.1 volume of 3.2% sodium citrate. Volunteers were informed of the purpose of the study before providing written consent according to guidelines from the Ethics Committee of Seoul National University Hospital.

Rat (Sprague-Dawley, male, 220 ± 20 g) blood was collected from the heart after surgery using a syringe containing 0.1 volume of 2.2% sodium citrate. The animal experiments were conducted under animal ordinance license issued by the Institute of Laboratory Animal Resources Seoul National University and according to guideline of the Seoul National University Institutional Animal Care and Use Committee, approval No. SNU050502-13.

3.3. Preparation of test sample solution

The test samples were dissolved in DMSO and then directly applied to the test system to give 1% of the final DMSO concentration.

3.4. Shear stress-induced platelet aggregation (SIPA)

The citrated whole human blood was centrifuged at 150 × g for 15 min at room temperature to obtain supernatant PRP. Subsequently, platelet poor plasma (PPP) was obtained from the residue by centrifugation at 2000 × g for 20 min. With the aid of the hemocytometer, the supernatant PRP was diluted with PPP to adjust the number of platelets to 300 × 10⁶ platelets/mL (i.e., adjusted PRP). After 3 min pre-incubation (37 °C) of the adjusted PRP in the thermomixer, the test sample (25 µM) or vehicle was added and incubation then continued for 10 additional minutes. The reaction mixture was subjected to a regular shear rate of 10,800/s on the cone-plate viscometer for 3 min. Immediately, after exposure to shear stress, 20 µL of the PRP aliquots were fixed with 280 µL of Isoton II buffer containing 0.5% glutaraldehyde. Single platelets were counted using the hemocytometer and microscope (1000 × magnification). SIPA, as a percentage, was quantified based on the decrease in the number of single platelets (Huang and Hellums 1993; Jen and McIntire 1984). The data are expressed as the percentage inhibition compared to the control (vehicle).

3.5. Platelet aggregation tested by the turbidimetric method

Citrated whole rat blood was centrifuged at 200 × g for 10 min at room temperature to obtain supernatant PRP. PPP was obtained from the residue by centrifugation at 1500 × g for 15 min. With the aid of the hematology analyzer, the supernatant PRP was diluted with PPP and saline to adjust the number of platelets to 400–450 × 10⁶ platelets/mL. The degree of platelet aggregation, measured with the platelet aggregometer, was determined following addition of an aggregating agent and was standardized by assuming that PPP represented 100% light transmission and PRP represented 0% light transmission (McNicol 1996). The test sample (5 µL) or vehicle was added to the adjusted rat PRP (500 µL), after 3 min pre-incubation at 37 °C, and an aggregation inducing agent [ADP (2–5 µM) or collagen (2–5 µg/mL)] was added at 1 min after sample or vehicle addition. AA- or U46619-induced aggregations were measured in the presence of a threshold concentration of collagen using a previously described method (Yun-Choi et al. 2000). Briefly, a test sample was added to PRP 30 s before addition of the threshold concentration of collagen (1.0–1.8 µg/mL), i.e., the concentration at which platelet shape change was induced but aggregation did not occur. AA (40–80 µM) or U46619 (2–5 µM) was added 30 s after the addition of the threshold concentration of collagen. The concentrations causing 50% inhibitory effects (IC₅₀) were determined using the Regression Wizard from the SigmaPlot equation library (Systat Software Inc., Chicago, IL, U.S.A.).

3.6. Measurement of coagulation time

Human plasma from the Blood Bank, Seoul National University Hospital, was stored in a freezer at –80 °C. The appropriate amount of plasma was thawed at room temperature and immediately used in aPTT, PT and TT assays. The plasma (200 µL) was pre-incubated with 3 µL of test sample or vehicle at 37 °C for 2 min. Pre-warmed Neoplastine® CI plus (PT-reagent) or STA®-Thrombin (TT-reagent) was added and the time until coagulation was measured by the fibrometer. STA®-PTT automate 5 (aPTT reagent) was added to the pre-incubated human plasma and incubation was continued at 37 °C for 3 additional minutes. The time to coagulation was measured by the fibrometer after the addition of CaCl₂ (0.025 M). All data were standardized to the clotting time of control plasma.

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