J. Fd Hyg. Safety 19(3), 167–170 (2004)

Inhibitory Effects of *Moutan Cortex Radicis* Extracts and Paeonol on Rabbit Platelet Aggregation

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ABSTRACT – The present study was undertaken to investigate the effects of *Moutan Cortex Radicis* extracts and paeonol, a major component, on rabbit platelet aggregation and thromboxane (TX) B_2 formation. *Moutan Cortex Radicis* methanol and butanol layers (100 μg/mL) showed the weak inhibitions, and water layer (100 μg/mL) had no effect on the collagen-induced platelet aggregation. Whereas, hexane and EtOAc layers potently inhibited the collagen (3 μg/mL)-induced platelet aggregation with the IC_{50} values of 10.9 ± 1.0 and 31.5 ± 0.8 μg/mL, respectively. Paeonol isolated from the hexane-acetone layer specifically inhibited the collagen-induced platelet aggregation with the IC_{50} value of 113.1 ± 0.9 μM, whereas it had little effects on the other agonists such as AA-, thrombin-, A23187- and thapsigargin-induced platelet aggregations. Paeonol also potently inhibited the collagen-induced TXB_2 formation in rabbit platelet in a concentration-dependent manner. These results suggest that paeonol may inhibit rabbit platelet aggregation by interfering with an essential step in collagen-induced platelet activation and TXA_2 formation. Paeonol may be a promising candidate for an antiplatelet agent.

Key words: *Moutan Cortex Radicis*, Paeonol, Platelet aggregation, Thromboxane A_2

INTRODUTION

Platelets not only play a critical role in normal haemostasis, but also are important contributors to thrombotic disorders, especially cerebral vascular diseases such as transient ischemic attack (Sherman and Hart, 1986), ischemic heart diseases such as myocardial infarction (Hjemdahl-Monsen *et al.,* 1986), and peripheral vascular diseases (Genton *et al.,* 1986). Disruption of the endothelium by trauma, or by a disease such as atherosclerosis, allows platelets to come in contact with and adhere to exposed subendothelial structures, where they become activated. Upon activation, adherent platelets recruit additional platelets into the growing thrombus. Clinical evidence has clearly proven that antiplatelet therapy is a useful means of preventing acute thromboembolic artery occlusions in cardiovascular diseases. Among various antiplatelet agents, aspirin has been used widely in preventing unstable angina, myocardial infarction, transient ischemic attack, stroke and atherosclerosis, either alone or in combination with other platelet inhibitors (Hennekens *et al.,* 1989), in spite of its weak antiplatelet effect and adverse effects which involve the gastrointestinal tract and epigastric discomfort, acute mucosal erosion with gastrointestinal bleeding and chronic peptic ulceration (Schror, 1995). Hence, the more effective and safe antiplatelet drugs with little side effects are still in need. *Moutan Cortex Radicis* is a traditional Chinese Herb as the root of *Paeonia suffructicosa* Andrews, which has been commonly used to treat liver diseases in China, Japan and Korea for centuries. It is also traditionally used for the nourishment of blood, activation of circulation, alleviation of pain and regulation of menstruation (Harada and Yamashita, 1969). The scavenging activity of oxygen radicals was observed in this crude drug (Yoshikawa *et al.,* 1992). In addition, various constituents isolated from *Moutan Cortex Radicis* have diverse biological activities. The methanol extract of *Moutan Cortex Radicis* showed the effects of reducing urea-nitrogen in rat serum (Shibutani *et al.,* 1981) and vasodilator effects (Goto *et al.,* 1996). Paeonol, a major component of *Moutan Cortex Radicis*

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has sedative and anti-inflammatory effects (Harada and Yamashita 1969; Harada *et al.*, 1972) and shows protective effects on cultured neonatal rat heart cells, inhibiting Ca^{2+} influx (Tang and Shi, 1991). The present study was designed to investigate the inhibitory effects of *Moutan Cortex Radicis* extracts and paeonol, a major component, on rabbit platelet aggregation.

MATERIALS AND METHODS

Materials

Collagen and arachidonic acid (AA) were obtained from Chrono-Log Co. (Havertowm, PA, USA). Thrombin, thapsigargin and calcium ionphore, A23187 were from Sigma Chemical Co. (St Louis, MO, USA). TXB_2 enzyme immunoassay (EIA) kit was purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Other chemicals were of analytical grade.

Animals

Male New Zealand white rabbits (3~4 kg) were purchased from Samtako Bio Korea Inc. (Osan, Gyunggi, Korea), and acclimated for at least one week a temperature of 24 ± 1°C and a humidity of 55 ± 5% with free access to a commercial pellet diet obtained from Samyang Co. (Wonju, Kangwon, Korea) and drinking water before experiments. Animal experiments were carried out in accordance with Guidelines for the Care and Use of Laboratory Animals (Chungbuk National University, Korea).

Extraction of Moutan Cortex Radicis and isolation of paeonol

The sample (2 kg) was cut into the small size and extracted with methanol in water bath. The methanol extract was evaporated to be dry. The residue (500 g) was suspended with water and partitioned with hexane. The hexane solution was evaporated to be dry. The hexane fraction (67 g) was fractionated on silica gel (60-230 mesh) column chromatography using a gradient of hexane-acetone (100:1 → 1:1) afforded three fractions (Fr. 1 ~ 3). The Fr. 1 afforded crude crystal and it was recrystallized in ethyl acetate to give white crystal (21 g) as paeonol (Fig. 1).

Identification of paeonol

Mp 48-50 °C, $FeCl_3$ test: positive, UV λ_{max}^{EtOH} nm (log) 314 (3.4), 274 (3.7), 230 (3.6), EI-MS m/z (rel. int.) 166 $[M]^+$ (54), 151 $[M\text{-}CH_3]^+$ (100), 123 $[M\text{-}COCH_3]^+$ (3), ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.84 (1H, d, J = 8.7 Hz, H-3), 6.53 (1H, dd, J = 8.7 Hz and 2.7 Hz, H-4), 6.47 (1H, d, J = 2.7 Hz, H-6), 3.82 (3H, s, OCH_3), 2.56 (3H, s, CH_3). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 113.7 (C-1), 165.7 (C-2), 100.7 (C-3), 164.1 (C-4), 107.3 (C-5), 133.3 (C-6), 55.7 (OCH_3), 203.1 (C=O), 26.6 (CH_3).

Preparation of washed rabbit platelet

The preparation of washed rabbit platelet was carried out as previously described (Son *et al.*, 2004). In brief, rabbit blood was withdrawn from the ear artery vessel and collected directly into 0.15 (v/v) of ACD solution (0.8% citric acid, 2.2% trisodium citrate and 2% dextrose (w/v)) as anti-coagulant. Platelet rich plasma (PRP) was prepared by centrifugation at 230×g for 10 min at room temperature. Platelets were sedimented by centrifugation of PRP at 2,100×g for 10 min, and then washed twice with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 0.35% bovine serum albumin and 3.8 mM HEPES, pH 6.5) containing 0.4 mM EGTA. After centrifugation, the pellets were resuspended in HEPES buffer (pH 7.4). The platelet concentration was counted using a Coulter Counter (Coulter Electronics, Hialeah, FL, USA) and adjusted to 4×10^8 platelets/mL.

Measurement of platelet aggregation

Platelet aggregation was measured using an aggregometer (Chrono-Log Co., Havertown, PA, USA) as previously described (Son *et al.*, 2004). Briefly, washed platelet suspensions were incubated at 37°C in the aggregometer with stirring at 1,000 rpm. Platelet aggregation was induced by the addition of collagen (3 μg/mL), arachidonic acid (100 μM), thrombin (0.05 U/mL), A23187 (5 μM) or thapsigargin (1 μM). The resulting

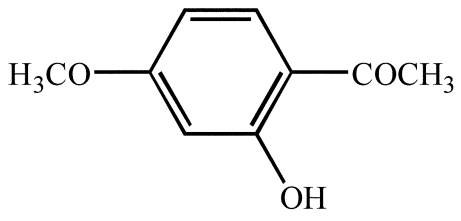

Fig. 1. Structure of paeonol (2-hydroxy-4-methoxyacetophenone)

aggregation measured as the change in light transmission, was recorded for 10 min. The inhibition of platelet aggregation was expressed as % inhibition (X) using the following equation:

$$X = [(A - B)/A] \times 100$$

A = maximal aggregation of control
B = maximal aggregation of sample-treated platelet

Measurement of thromboxane B_2 formation

The formation of TXA_2 in platelets was measured by determining TXB_2, the stable metabolite of TXA_2. After the incubation of washed platelets with paeonol at 37°C for 3 min, the agonist was added and then the reaction was terminated by the addition of 50 μM indomethacin and 5 mM EDTA. The amount of TXB_2 in the supernatant was determined using TXB_2 EIA kit (Amersham Pharmacia Biotech) according to the procedure described by the manufacturer.

Statistical analysis

The experimental results were expressed as the mean ± S.D. A one-way analysis of variance (ANOVA) was used for multiple comparisons followed by Dunnetts test, and the data were considered significant with a probability less than 0.05.

RESULTS AND DISCUSSION

Antiplatelet therapy has become a useful means of preventing acute thromboembolic artery occlusions in cardiovascular diseases, because platelet activation and thrombus formation are important in the pathophysiology of ischaemic events in the heart, brain, and peripheral arterial territories. Antiplatelet compounds such as aspirin, ticlopidine, and dipyridamole are in clinical use, but show several side effects (Schror, 1995). In the present study, the effects of *Moutan Cortex Radicis* extracts and its major component paeonol on rabbit platelet aggregation were examined. To study the effect on platelet aggregation, washed platelet was pretreated with *Moutan Cortex Radicis* extracts (100 μg/mL), and then the platelet aggregation was induced by collagen (3 μg/mL). As shown in Table 1, *Moutan Cortex Radicis* methanol and buthanol layers showed the weak inhibitions, and water layer had no effect on the collagen-induced platelet aggregation. However, hexane and EtOAc layers concentration-dependently inhibited the collagen-induced platelet aggregations with the IC_{50} values of 10.9 ± 1.0 and 31.5 ± 0.8 μg/mL, respectively. Paeonol isolated from hexane and acetone layer specifically inhibited the collagen-induced platelet aggregation with the IC_{50} value of 113.1 ± 0.9 μM, whereas it had little effect on the platelet aggregation induced by other agonists such as arachidonic acid (AA), thrombin, A23187 and thapsigargin at the concentration of 200 μM (Table 2). It is well known that TXA_2 is an important mediator of the release reaction and aggregation of platelets (FitzGerald *et al.*, 1991). Collagen stimulates tyrosine kinase via collagen receptors, intracellular calcium then increases, followed by activation of phospholipase (PL) A_2. PLA_2 activation then leads to arachidonic acid release from membrane phospholipids,

Table 1. Effect of *Moutan Cortex Radicis* extracts on rabbit platelet aggregation induced by collagen

Sample (100 μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
MeOH extract	39.0 ± 0.4	–
H_2O layer	–	–
BuOH layer	42.4 ± 1.1	–
Hexane layer	95.0 ± 2.1	10.9 ± 1.0
EtOAc layer	92.9 ± 1.8	31.5 ± 0.8

Washed rabbit platelet suspension was incubated at 37? in an aggregometer with stirring at 1,000 rpm and then *Moutan Cortex Radicis* extracts were added. After 3 min preincubation, the platelet aggregation was induced by addition of collagen (3 μg/ml). The resulting aggregation was measured as the change in light transmission.

Table 2. Effect of paeonol on rabbit platelet aggregation induced by various agonists

Sample	Inducer	Inhibition (%)	IC_{50} (μM)
Paeonol (200 μM)	Collagen	92.0 ± 0.2	–
	Arachidonic acid	22.5 ± 1.9	–
	Thrombin	2.8 ± 3.1	113.1 ± 0.9
	A23187	5.3 ± 2.7	–
	Thapsigargin	2.9 ± 2.9	–

Washed rabbit platelet suspension was incubated at 37°C in an aggregometer with stirring at 1,000 rpm and then 200 μM paeonol was added. After 3 min preincubation, the platelet aggregation was induced by addition of collagen (3 μg/ml), arachidonic acid (100 μM), thrombin (0.05 u/ml), calcium ionophore A23187 (5 μM) or thapsigargin (1 μM). The resulting aggregation was measured as the change in light transmission.

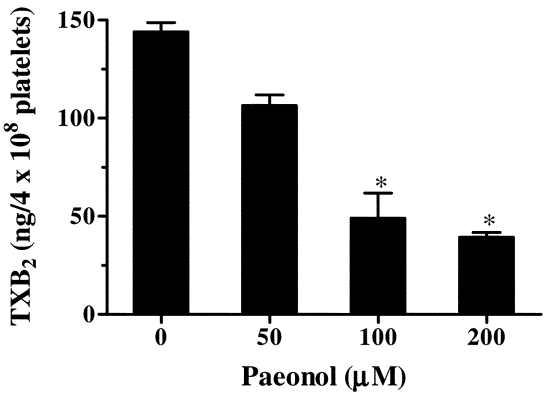

Fig. 2. Effect of paeonol on the collagen-induced TXB_2 formation. Washed rabbit platelet suspension was incubated with indicated concentrations of paeonol for 3 min at 37°C. And then collagen (1 g/mL) was added in washed platelet suspension. The reaction was terminated by supplement of EGTA and indomethacin (50 μM). TXB_2 level in medium was determined using TXB_2 EIA kit according to the described procedure by the manufacturer. The values are expressed as mean ± S.D. (n=3). *P<0.05.

followed by TXA_2 generation. We examined the ability of paeonol to inhibit rabbit platelet activation by interfering with the collagen-induced TXB_2 formation. TXB_2 level in resting platelets was less than 1.5 ng/4×10^8 cells. Our results show that paeonol also potently inhibits the formation of TXB_2 induced by collagen in a concentration-dependent manner (Fig. 2). These results suggest that paeonol can inhibit rabbit platelet aggregation by interfering with an essential step in collagen-induced platelet activation and TXA_2 formation. Paeonol may be a promising candidate for an antiplatelet agent.

Acknowledgement

This study was supported by the research fund from Ministry of Health and Welfare, Republic of Korea (2003-2004, HMP 00-CD-02-0004).

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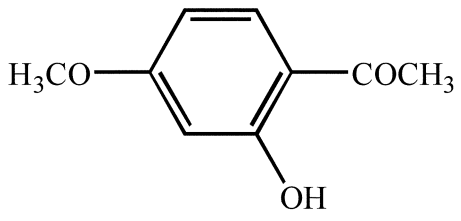

Fig. 1. Structure of paeonol (2-hydroxy-4-methoxyacetophenone)

aggregation measured as the change in light transmission, was recorded for 10 min. The inhibition of platelet aggregation was expressed as % inhibition (X) using the following equation:

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The experimental results were expressed as the mean ± S.D. A one-way analysis of variance (ANOVA) was used for multiple comparisons followed by Dunnetts test, and the data were considered significant with a probability less than 0.05.

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Table 2. Effect of paeonol on rabbit platelet aggregation induced by various agonists

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Paeonol (200 μM)	Collagen	92.0 ± 0.2	–
	Arachidonic acid	22.5 ± 1.9	–
	Thrombin	2.8 ± 3.1	113.1 ± 0.9
	A23187	5.3 ± 2.7	–
	Thapsigargin	2.9 ± 2.9	–

Washed rabbit platelet suspension was incubated at 37°C in an aggregometer with stirring at 1,000 rpm and then 200 μM paeonol was added. After 3 min preincubation, the platelet aggregation was induced by addition of collagen (3 μg/ml), arachidonic acid (100 μM), thrombin (0.05 u/ml), calcium ionophore A23187 (5 μM) or thapsigargin (1 μM). The resulting aggregation was measured as the change in light transmission.

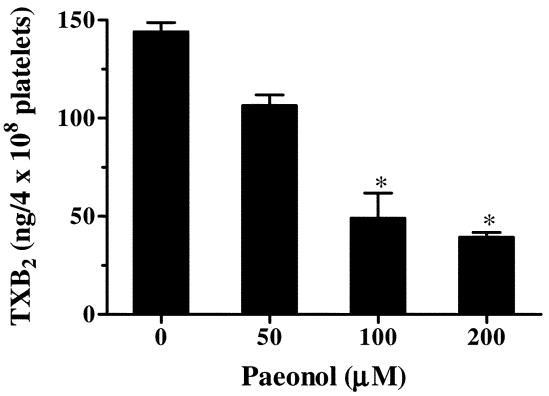

Fig. 2. Effect of paeonol on the collagen-induced TXB_2 formation. Washed rabbit platelet suspension was incubated with indicated concentrations of paeonol for 3 min at 37°C. And then collagen (1 g/mL) was added in washed platelet suspension. The reaction was terminated by supplement of EGTA and indomethacin (50 μM). TXB_2 level in medium was determined using TXB_2 EIA kit according to the described procedure by the manufacturer. The values are expressed as mean ± S.D. (n=3). *P<0.05.

followed by TXA_2 generation. We examined the ability of paeonol to inhibit rabbit platelet activation by interfering with the collagen-induced TXB_2 formation. TXB_2 level in resting platelets was less than 1.5 ng/4×10^8 cells. Our results show that paeonol also potently inhibits the formation of TXB_2 induced by collagen in a concentration-dependent manner (Fig. 2). These results suggest that paeonol can inhibit rabbit platelet aggregation by interfering with an essential step in collagen-induced platelet activation and TXA_2 formation. Paeonol may be a promising candidate for an antiplatelet agent.

Acknowledgement

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J. Fd Hyg. Safety 19(3), 167–170 (2004)

Inhibitory Effects of *Moutan Cortex Radicis* Extracts and Paeonol on Rabbit Platelet Aggregation

Kyung-Sup Lee, Ki-Wan Oh, KiHwan Bae*, Young-Ho Kim*, Mi-Yea Lee, Mi-Ra Cho, Yong-Ri Jin, and Yeo-Pyo Yun†**

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ABSTRACT – The present study was undertaken to investigate the effects of *Moutan Cortex Radicis* extracts and paeonol, a major component, on rabbit platelet aggregation and thromboxane (TX) B_2 formation. *Moutan Cortex Radicis* methanol and butanol layers (100 μg/mL) showed the weak inhibitions, and water layer (100 μg/mL) had no effect on the collagen-induced platelet aggregation. Whereas, hexane and EtOAc layers potently inhibited the collagen (3 μg/mL)-induced platelet aggregation with the IC_{50} values of 10.9 ± 1.0 and 31.5 ± 0.8 μg/mL, respectively. Paeonol isolated from the hexane-acetone layer specifically inhibited the collagen-induced platelet aggregation with the IC_{50} value of 113.1 ± 0.9 μM, whereas it had little effects on the other agonists such as AA-, thrombin-, A23187- and thapsigargin-induced platelet aggregations. Paeonol also potently inhibited the collagen-induced TXB_2 formation in rabbit platelet in a concentration-dependent manner. These results suggest that paeonol may inhibit rabbit platelet aggregation by interfering with an essential step in collagen-induced platelet activation and TXA_2 formation. Paeonol may be a promising candidate for an antiplatelet agent.

Key words: *Moutan Cortex Radicis*, Paeonol, Platelet aggregation, Thromboxane A_2

INTRODUTION

Platelets not only play a critical role in normal haemostasis, but also are important contributors to thrombotic disorders, especially cerebral vascular diseases such as transient ischemic attack (Sherman and Hart, 1986), ischemic heart diseases such as myocardial infarction (Hjemdahl-Monsen *et al.,* 1986), and peripheral vascular diseases (Genton *et al.,* 1986). Disruption of the endothelium by trauma, or by a disease such as atherosclerosis, allows platelets to come in contact with and adhere to exposed subendothelial structures, where they become activated. Upon activation, adherent platelets recruit additional platelets into the growing thrombus. Clinical evidence has clearly proven that antiplatelet therapy is a useful means of preventing acute thromboembolic artery occlusions in cardiovascular diseases. Among various antiplatelet agents, aspirin has been used widely in preventing unstable angina, myocardial infarction, transient ischemic attack, stroke and atherosclerosis, either alone or in combination with other platelet inhibitors (Hennekens *et al.,* 1989), in spite of its weak antiplatelet effect and adverse effects which involve the gastrointestinal tract and epigastric discomfort, acute mucosal erosion with gastrointestinal bleeding and chronic peptic ulceration (Schror, 1995). Hence, the more effective and safe antiplatelet drugs with little side effects are still in need. *Moutan Cortex Radicis* is a traditional Chinese Herb as the root of *Paeonia suffructicosa* Andrews, which has been commonly used to treat liver diseases in China, Japan and Korea for centuries. It is also traditionally used for the nourishment of blood, activation of circulation, alleviation of pain and regulation of menstruation (Harada and Yamashita, 1969). The scavenging activity of oxygen radicals was observed in this crude drug (Yoshikawa *et al.,* 1992). In addition, various constituents isolated from *Moutan Cortex Radicis* have diverse biological activities. The methanol extract of *Moutan Cortex Radicis* showed the effects of reducing urea-nitrogen in rat serum (Shibutani *et al.,* 1981) and vasodilator effects (Goto *et al.,* 1996). Paeonol, a major component of *Moutan Cortex Radicis*

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has sedative and anti-inflammatory effects (Harada and Yamashita 1969; Harada *et al.*, 1972) and shows protective effects on cultured neonatal rat heart cells, inhibiting Ca^{2+} influx (Tang and Shi, 1991). The present study was designed to investigate the inhibitory effects of *Moutan Cortex Radicis* extracts and paeonol, a major component, on rabbit platelet aggregation.

MATERIALS AND METHODS

Materials

Collagen and arachidonic acid (AA) were obtained from Chrono-Log Co. (Havertowm, PA, USA). Thrombin, thapsigargin and calcium ionphore, A23187 were from Sigma Chemical Co. (St Louis, MO, USA). TXB_2 enzyme immunoassay (EIA) kit was purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Other chemicals were of analytical grade.

Animals

Male New Zealand white rabbits (3~4 kg) were purchased from Samtako Bio Korea Inc. (Osan, Gyunggi, Korea), and acclimated for at least one week a temperature of 24 ± 1°C and a humidity of 55 ± 5% with free access to a commercial pellet diet obtained from Samyang Co. (Wonju, Kangwon, Korea) and drinking water before experiments. Animal experiments were carried out in accordance with Guidelines for the Care and Use of Laboratory Animals (Chungbuk National University, Korea).

Extraction of Moutan Cortex Radicis and isolation of paeonol

The sample (2 kg) was cut into the small size and extracted with methanol in water bath. The methanol extract was evaporated to be dry. The residue (500 g) was suspended with water and partitioned with hexane. The hexane solution was evaporated to be dry. The hexane fraction (67 g) was fractionated on silica gel (60-230 mesh) column chromatography using a gradient of hexane-acetone (100:1 → 1:1) afforded three fractions (Fr. 1 ~ 3). The Fr. 1 afforded crude crystal and it was recrystallized in ethyl acetate to give white crystal (21 g) as paeonol (Fig. 1).

Identification of paeonol

Mp 48-50 °C, $FeCl_3$ test: positive, UV λ_{max}^{EtOH} nm (log) 314 (3.4), 274 (3.7), 230 (3.6), EI-MS m/z (rel. int.) 166 $[M]^+$ (54), 151 $[M\text{-}CH_3]^+$ (100), 123 $[M\text{-}COCH_3]^+$ (3), ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.84 (1H, d, J = 8.7 Hz, H-3), 6.53 (1H, dd, J = 8.7 Hz and 2.7 Hz, H-4), 6.47 (1H, d, J = 2.7 Hz, H-6), 3.82 (3H, s, OCH_3), 2.56 (3H, s, CH_3). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 113.7 (C-1), 165.7 (C-2), 100.7 (C-3), 164.1 (C-4), 107.3 (C-5), 133.3 (C-6), 55.7 (OCH_3), 203.1 (C=O), 26.6 (CH_3).

Preparation of washed rabbit platelet

The preparation of washed rabbit platelet was carried out as previously described (Son *et al.*, 2004). In brief, rabbit blood was withdrawn from the ear artery vessel and collected directly into 0.15 (v/v) of ACD solution (0.8% citric acid, 2.2% trisodium citrate and 2% dextrose (w/v)) as anti-coagulant. Platelet rich plasma (PRP) was prepared by centrifugation at 230×g for 10 min at room temperature. Platelets were sedimented by centrifugation of PRP at 2,100×g for 10 min, and then washed twice with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 0.35% bovine serum albumin and 3.8 mM HEPES, pH 6.5) containing 0.4 mM EGTA. After centrifugation, the pellets were resuspended in HEPES buffer (pH 7.4). The platelet concentration was counted using a Coulter Counter (Coulter Electronics, Hialeah, FL, USA) and adjusted to 4×10^8 platelets/mL.

Measurement of platelet aggregation

Platelet aggregation was measured using an aggregometer (Chrono-Log Co., Havertown, PA, USA) as previously described (Son *et al.*, 2004). Briefly, washed platelet suspensions were incubated at 37°C in the aggregometer with stirring at 1,000 rpm. Platelet aggregation was induced by the addition of collagen (3 μg/mL), arachidonic acid (100 μM), thrombin (0.05 U/mL), A23187 (5 μM) or thapsigargin (1 μM). The resulting

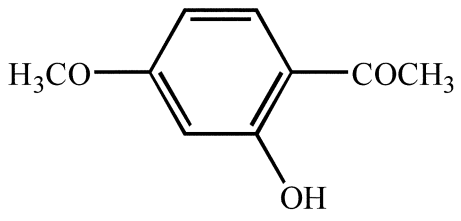

Fig. 1. Structure of paeonol (2-hydroxy-4-methoxyacetophenone)

aggregation measured as the change in light transmission, was recorded for 10 min. The inhibition of platelet aggregation was expressed as % inhibition (X) using the following equation:

$$X = [(A - B)/A] \times 100$$

A = maximal aggregation of control
B = maximal aggregation of sample-treated platelet

Measurement of thromboxane B_2 formation

The formation of TXA_2 in platelets was measured by determining TXB_2, the stable metabolite of TXA_2. After the incubation of washed platelets with paeonol at 37°C for 3 min, the agonist was added and then the reaction was terminated by the addition of 50 μM indomethacin and 5 mM EDTA. The amount of TXB_2 in the supernatant was determined using TXB_2 EIA kit (Amersham Pharmacia Biotech) according to the procedure described by the manufacturer.

Statistical analysis

The experimental results were expressed as the mean ± S.D. A one-way analysis of variance (ANOVA) was used for multiple comparisons followed by Dunnetts test, and the data were considered significant with a probability less than 0.05.

RESULTS AND DISCUSSION

Antiplatelet therapy has become a useful means of preventing acute thromboembolic artery occlusions in cardiovascular diseases, because platelet activation and thrombus formation are important in the pathophysiology of ischaemic events in the heart, brain, and peripheral arterial territories. Antiplatelet compounds such as aspirin, ticlopidine, and dipyridamole are in clinical use, but show several side effects (Schror, 1995). In the present study, the effects of *Moutan Cortex Radicis* extracts and its major component paeonol on rabbit platelet aggregation were examined. To study the effect on platelet aggregation, washed platelet was pretreated with *Moutan Cortex Radicis* extracts (100 μg/mL), and then the platelet aggregation was induced by collagen (3 μg/mL). As shown in Table 1, *Moutan Cortex Radicis* methanol and buthanol layers showed the weak inhibitions, and water layer had no effect on the collagen-induced platelet aggregation. However, hexane and EtOAc layers concentration-dependently inhibited the collagen-induced platelet aggregations with the IC_{50} values of 10.9 ± 1.0 and 31.5 ± 0.8 μg/mL, respectively. Paeonol isolated from hexane and acetone layer specifically inhibited the collagen-induced platelet aggregation with the IC_{50} value of 113.1 ± 0.9 μM, whereas it had little effect on the platelet aggregation induced by other agonists such as arachidonic acid (AA), thrombin, A23187 and thapsigargin at the concentration of 200 μM (Table 2). It is well known that TXA_2 is an important mediator of the release reaction and aggregation of platelets (FitzGerald *et al.*, 1991). Collagen stimulates tyrosine kinase via collagen receptors, intracellular calcium then increases, followed by activation of phospholipase (PL) A_2. PLA_2 activation then leads to arachidonic acid release from membrane phospholipids,

Table 1. Effect of *Moutan Cortex Radicis* extracts on rabbit platelet aggregation induced by collagen

Sample (100 μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
MeOH extract	39.0 ± 0.4	–
H_2O layer	–	–
BuOH layer	42.4 ± 1.1	–
Hexane layer	95.0 ± 2.1	10.9 ± 1.0
EtOAc layer	92.9 ± 1.8	31.5 ± 0.8

Washed rabbit platelet suspension was incubated at 37? in an aggregometer with stirring at 1,000 rpm and then *Moutan Cortex Radicis* extracts were added. After 3 min preincubation, the platelet aggregation was induced by addition of collagen (3 μg/ml). The resulting aggregation was measured as the change in light transmission.

Table 2. Effect of paeonol on rabbit platelet aggregation induced by various agonists

Sample	Inducer	Inhibition (%)	IC_{50} (μM)
Paeonol (200 μM)	Collagen	92.0 ± 0.2	–
	Arachidonic acid	22.5 ± 1.9	–
	Thrombin	2.8 ± 3.1	113.1 ± 0.9
	A23187	5.3 ± 2.7	–
	Thapsigargin	2.9 ± 2.9	–

Washed rabbit platelet suspension was incubated at 37°C in an aggregometer with stirring at 1,000 rpm and then 200 μM paeonol was added. After 3 min preincubation, the platelet aggregation was induced by addition of collagen (3 μg/ml), arachidonic acid (100 μM), thrombin (0.05 u/ml), calcium ionophore A23187 (5 μM) or thapsigargin (1 μM). The resulting aggregation was measured as the change in light transmission.

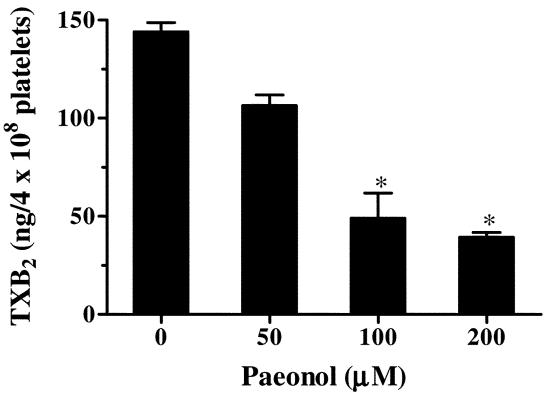

Fig. 2. Effect of paeonol on the collagen-induced TXB_2 formation. Washed rabbit platelet suspension was incubated with indicated concentrations of paeonol for 3 min at 37°C. And then collagen (1 g/mL) was added in washed platelet suspension. The reaction was terminated by supplement of EGTA and indomethacin (50 μM). TXB_2 level in medium was determined using TXB_2 EIA kit according to the described procedure by the manufacturer. The values are expressed as mean ± S.D. (n=3). *P<0.05.

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Preparation of washed rabbit platelet

The preparation of washed rabbit platelet was carried out as previously described (Son *et al.*, 2004). In brief, rabbit blood was withdrawn from the ear artery vessel and collected directly into 0.15 (v/v) of ACD solution (0.8% citric acid, 2.2% trisodium citrate and 2% dextrose (w/v)) as anti-coagulant. Platelet rich plasma (PRP) was prepared by centrifugation at 230×g for 10 min at room temperature. Platelets were sedimented by centrifugation of PRP at 2,100×g for 10 min, and then washed twice with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 0.35% bovine serum albumin and 3.8 mM HEPES, pH 6.5) containing 0.4 mM EGTA. After centrifugation, the pellets were resuspended in HEPES buffer (pH 7.4). The platelet concentration was counted using a Coulter Counter (Coulter Electronics, Hialeah, FL, USA) and adjusted to 4×10^8 platelets/mL.

Measurement of platelet aggregation

Platelet aggregation was measured using an aggregometer (Chrono-Log Co., Havertown, PA, USA) as previously described (Son *et al.*, 2004). Briefly, washed platelet suspensions were incubated at 37°C in the aggregometer with stirring at 1,000 rpm. Platelet aggregation was induced by the addition of collagen (3 μg/mL), arachidonic acid (100 μM), thrombin (0.05 U/mL), A23187 (5 μM) or thapsigargin (1 μM). The resulting

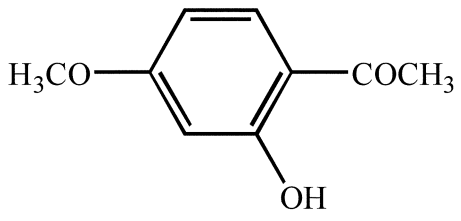

Fig. 1. Structure of paeonol (2-hydroxy-4-methoxyacetophenone)

aggregation measured as the change in light transmission, was recorded for 10 min. The inhibition of platelet aggregation was expressed as % inhibition (X) using the following equation:

$$X = [(A - B)/A] \times 100$$

A = maximal aggregation of control
B = maximal aggregation of sample-treated platelet

Measurement of thromboxane B_2 formation

The formation of TXA_2 in platelets was measured by determining TXB_2, the stable metabolite of TXA_2. After the incubation of washed platelets with paeonol at 37°C for 3 min, the agonist was added and then the reaction was terminated by the addition of 50 μM indomethacin and 5 mM EDTA. The amount of TXB_2 in the supernatant was determined using TXB_2 EIA kit (Amersham Pharmacia Biotech) according to the procedure described by the manufacturer.

Statistical analysis

The experimental results were expressed as the mean ± S.D. A one-way analysis of variance (ANOVA) was used for multiple comparisons followed by Dunnetts test, and the data were considered significant with a probability less than 0.05.

RESULTS AND DISCUSSION

Antiplatelet therapy has become a useful means of preventing acute thromboembolic artery occlusions in cardiovascular diseases, because platelet activation and thrombus formation are important in the pathophysiology of ischaemic events in the heart, brain, and peripheral arterial territories. Antiplatelet compounds such as aspirin, ticlopidine, and dipyridamole are in clinical use, but show several side effects (Schror, 1995). In the present study, the effects of *Moutan Cortex Radicis* extracts and its major component paeonol on rabbit platelet aggregation were examined. To study the effect on platelet aggregation, washed platelet was pretreated with *Moutan Cortex Radicis* extracts (100 μg/mL), and then the platelet aggregation was induced by collagen (3 μg/mL). As shown in Table 1, *Moutan Cortex Radicis* methanol and buthanol layers showed the weak inhibitions, and water layer had no effect on the collagen-induced platelet aggregation. However, hexane and EtOAc layers concentration-dependently inhibited the collagen-induced platelet aggregations with the IC_{50} values of 10.9 ± 1.0 and 31.5 ± 0.8 μg/mL, respectively. Paeonol isolated from hexane and acetone layer specifically inhibited the collagen-induced platelet aggregation with the IC_{50} value of 113.1 ± 0.9 μM, whereas it had little effect on the platelet aggregation induced by other agonists such as arachidonic acid (AA), thrombin, A23187 and thapsigargin at the concentration of 200 μM (Table 2). It is well known that TXA_2 is an important mediator of the release reaction and aggregation of platelets (FitzGerald *et al.*, 1991). Collagen stimulates tyrosine kinase via collagen receptors, intracellular calcium then increases, followed by activation of phospholipase (PL) A_2. PLA_2 activation then leads to arachidonic acid release from membrane phospholipids,

Table 1. Effect of *Moutan Cortex Radicis* extracts on rabbit platelet aggregation induced by collagen

Sample (100 μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
MeOH extract	39.0 ± 0.4	–
H_2O layer	–	–
BuOH layer	42.4 ± 1.1	–
Hexane layer	95.0 ± 2.1	10.9 ± 1.0
EtOAc layer	92.9 ± 1.8	31.5 ± 0.8

Washed rabbit platelet suspension was incubated at 37? in an aggregometer with stirring at 1,000 rpm and then *Moutan Cortex Radicis* extracts were added. After 3 min preincubation, the platelet aggregation was induced by addition of collagen (3 μg/ml). The resulting aggregation was measured as the change in light transmission.

Table 2. Effect of paeonol on rabbit platelet aggregation induced by various agonists

Sample	Inducer	Inhibition (%)	IC_{50} (μM)
Paeonol (200 μM)	Collagen	92.0 ± 0.2	–
	Arachidonic acid	22.5 ± 1.9	–
	Thrombin	2.8 ± 3.1	113.1 ± 0.9
	A23187	5.3 ± 2.7	–
	Thapsigargin	2.9 ± 2.9	–

Washed rabbit platelet suspension was incubated at 37°C in an aggregometer with stirring at 1,000 rpm and then 200 μM paeonol was added. After 3 min preincubation, the platelet aggregation was induced by addition of collagen (3 μg/ml), arachidonic acid (100 μM), thrombin (0.05 u/ml), calcium ionophore A23187 (5 μM) or thapsigargin (1 μM). The resulting aggregation was measured as the change in light transmission.

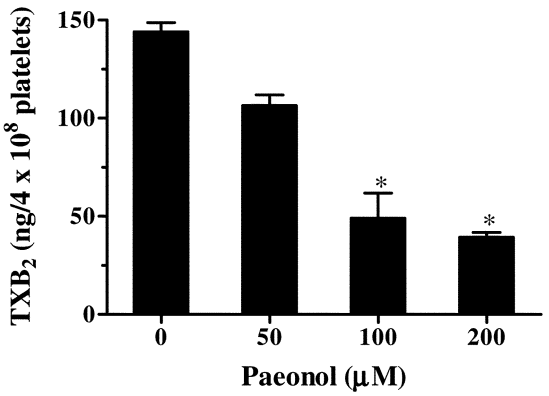

Fig. 2. Effect of paeonol on the collagen-induced TXB_2 formation. Washed rabbit platelet suspension was incubated with indicated concentrations of paeonol for 3 min at 37°C. And then collagen (1 g/mL) was added in washed platelet suspension. The reaction was terminated by supplement of EGTA and indomethacin (50 μM). TXB_2 level in medium was determined using TXB_2 EIA kit according to the described procedure by the manufacturer. The values are expressed as mean ± S.D. (n=3). *P<0.05.

followed by TXA_2 generation. We examined the ability of paeonol to inhibit rabbit platelet activation by interfering with the collagen-induced TXB_2 formation. TXB_2 level in resting platelets was less than 1.5 ng/4×10^8 cells. Our results show that paeonol also potently inhibits the formation of TXB_2 induced by collagen in a concentration-dependent manner (Fig. 2). These results suggest that paeonol can inhibit rabbit platelet aggregation by interfering with an essential step in collagen-induced platelet activation and TXA_2 formation. Paeonol may be a promising candidate for an antiplatelet agent.

Acknowledgement

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J. Fd Hyg. Safety 19(3), 167–170 (2004)

Inhibitory Effects of *Moutan Cortex Radicis* Extracts and Paeonol on Rabbit Platelet Aggregation

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ABSTRACT – The present study was undertaken to investigate the effects of *Moutan Cortex Radicis* extracts and paeonol, a major component, on rabbit platelet aggregation and thromboxane (TX) B_2 formation. *Moutan Cortex Radicis* methanol and butanol layers (100 μg/mL) showed the weak inhibitions, and water layer (100 μg/mL) had no effect on the collagen-induced platelet aggregation. Whereas, hexane and EtOAc layers potently inhibited the collagen (3 μg/mL)-induced platelet aggregation with the IC_{50} values of 10.9 ± 1.0 and 31.5 ± 0.8 μg/mL, respectively. Paeonol isolated from the hexane-acetone layer specifically inhibited the collagen-induced platelet aggregation with the IC_{50} value of 113.1 ± 0.9 μM, whereas it had little effects on the other agonists such as AA-, thrombin-, A23187- and thapsigargin-induced platelet aggregations. Paeonol also potently inhibited the collagen-induced TXB_2 formation in rabbit platelet in a concentration-dependent manner. These results suggest that paeonol may inhibit rabbit platelet aggregation by interfering with an essential step in collagen-induced platelet activation and TXA_2 formation. Paeonol may be a promising candidate for an antiplatelet agent.

Key words: *Moutan Cortex Radicis*, Paeonol, Platelet aggregation, Thromboxane A_2

INTRODUTION

Platelets not only play a critical role in normal haemostasis, but also are important contributors to thrombotic disorders, especially cerebral vascular diseases such as transient ischemic attack (Sherman and Hart, 1986), ischemic heart diseases such as myocardial infarction (Hjemdahl-Monsen *et al.,* 1986), and peripheral vascular diseases (Genton *et al.,* 1986). Disruption of the endothelium by trauma, or by a disease such as atherosclerosis, allows platelets to come in contact with and adhere to exposed subendothelial structures, where they become activated. Upon activation, adherent platelets recruit additional platelets into the growing thrombus. Clinical evidence has clearly proven that antiplatelet therapy is a useful means of preventing acute thromboembolic artery occlusions in cardiovascular diseases. Among various antiplatelet agents, aspirin has been used widely in preventing unstable angina, myocardial infarction, transient ischemic attack, stroke and atherosclerosis, either alone or in combination with other platelet inhibitors (Hennekens *et al.,* 1989), in spite of its weak antiplatelet effect and adverse effects which involve the gastrointestinal tract and epigastric discomfort, acute mucosal erosion with gastrointestinal bleeding and chronic peptic ulceration (Schror, 1995). Hence, the more effective and safe antiplatelet drugs with little side effects are still in need. *Moutan Cortex Radicis* is a traditional Chinese Herb as the root of *Paeonia suffructicosa* Andrews, which has been commonly used to treat liver diseases in China, Japan and Korea for centuries. It is also traditionally used for the nourishment of blood, activation of circulation, alleviation of pain and regulation of menstruation (Harada and Yamashita, 1969). The scavenging activity of oxygen radicals was observed in this crude drug (Yoshikawa *et al.,* 1992). In addition, various constituents isolated from *Moutan Cortex Radicis* have diverse biological activities. The methanol extract of *Moutan Cortex Radicis* showed the effects of reducing urea-nitrogen in rat serum (Shibutani *et al.,* 1981) and vasodilator effects (Goto *et al.,* 1996). Paeonol, a major component of *Moutan Cortex Radicis*

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has sedative and anti-inflammatory effects (Harada and Yamashita 1969; Harada *et al.*, 1972) and shows protective effects on cultured neonatal rat heart cells, inhibiting Ca^{2+} influx (Tang and Shi, 1991). The present study was designed to investigate the inhibitory effects of *Moutan Cortex Radicis* extracts and paeonol, a major component, on rabbit platelet aggregation.

MATERIALS AND METHODS

Materials

Collagen and arachidonic acid (AA) were obtained from Chrono-Log Co. (Havertowm, PA, USA). Thrombin, thapsigargin and calcium ionphore, A23187 were from Sigma Chemical Co. (St Louis, MO, USA). TXB_2 enzyme immunoassay (EIA) kit was purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Other chemicals were of analytical grade.

Animals

Male New Zealand white rabbits (3~4 kg) were purchased from Samtako Bio Korea Inc. (Osan, Gyunggi, Korea), and acclimated for at least one week a temperature of 24 ± 1°C and a humidity of 55 ± 5% with free access to a commercial pellet diet obtained from Samyang Co. (Wonju, Kangwon, Korea) and drinking water before experiments. Animal experiments were carried out in accordance with Guidelines for the Care and Use of Laboratory Animals (Chungbuk National University, Korea).

Extraction of Moutan Cortex Radicis and isolation of paeonol

The sample (2 kg) was cut into the small size and extracted with methanol in water bath. The methanol extract was evaporated to be dry. The residue (500 g) was suspended with water and partitioned with hexane. The hexane solution was evaporated to be dry. The hexane fraction (67 g) was fractionated on silica gel (60-230 mesh) column chromatography using a gradient of hexane-acetone (100:1 → 1:1) afforded three fractions (Fr. 1 ~ 3). The Fr. 1 afforded crude crystal and it was recrystallized in ethyl acetate to give white crystal (21 g) as paeonol (Fig. 1).

Identification of paeonol

Mp 48-50 °C, $FeCl_3$ test: positive, UV λ_{max}^{EtOH} nm (log) 314 (3.4), 274 (3.7), 230 (3.6), EI-MS m/z (rel. int.) 166 $[M]^+$ (54), 151 $[M\text{-}CH_3]^+$ (100), 123 $[M\text{-}COCH_3]^+$ (3), ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.84 (1H, d, J = 8.7 Hz, H-3), 6.53 (1H, dd, J = 8.7 Hz and 2.7 Hz, H-4), 6.47 (1H, d, J = 2.7 Hz, H-6), 3.82 (3H, s, OCH_3), 2.56 (3H, s, CH_3). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 113.7 (C-1), 165.7 (C-2), 100.7 (C-3), 164.1 (C-4), 107.3 (C-5), 133.3 (C-6), 55.7 (OCH_3), 203.1 (C=O), 26.6 (CH_3).

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The preparation of washed rabbit platelet was carried out as previously described (Son *et al.*, 2004). In brief, rabbit blood was withdrawn from the ear artery vessel and collected directly into 0.15 (v/v) of ACD solution (0.8% citric acid, 2.2% trisodium citrate and 2% dextrose (w/v)) as anti-coagulant. Platelet rich plasma (PRP) was prepared by centrifugation at 230×g for 10 min at room temperature. Platelets were sedimented by centrifugation of PRP at 2,100×g for 10 min, and then washed twice with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 0.35% bovine serum albumin and 3.8 mM HEPES, pH 6.5) containing 0.4 mM EGTA. After centrifugation, the pellets were resuspended in HEPES buffer (pH 7.4). The platelet concentration was counted using a Coulter Counter (Coulter Electronics, Hialeah, FL, USA) and adjusted to 4×10^8 platelets/mL.

Measurement of platelet aggregation

Platelet aggregation was measured using an aggregometer (Chrono-Log Co., Havertown, PA, USA) as previously described (Son *et al.*, 2004). Briefly, washed platelet suspensions were incubated at 37°C in the aggregometer with stirring at 1,000 rpm. Platelet aggregation was induced by the addition of collagen (3 μg/mL), arachidonic acid (100 μM), thrombin (0.05 U/mL), A23187 (5 μM) or thapsigargin (1 μM). The resulting

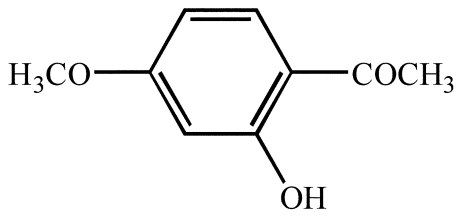

Fig. 1. Structure of paeonol (2-hydroxy-4-methoxyacetophenone)

aggregation measured as the change in light transmission, was recorded for 10 min. The inhibition of platelet aggregation was expressed as % inhibition (X) using the following equation:

$$X = [(A - B)/A] \times 100$$

A = maximal aggregation of control
B = maximal aggregation of sample-treated platelet

Measurement of thromboxane B_2 formation

The formation of TXA_2 in platelets was measured by determining TXB_2, the stable metabolite of TXA_2. After the incubation of washed platelets with paeonol at 37°C for 3 min, the agonist was added and then the reaction was terminated by the addition of 50 μM indomethacin and 5 mM EDTA. The amount of TXB_2 in the supernatant was determined using TXB_2 EIA kit (Amersham Pharmacia Biotech) according to the procedure described by the manufacturer.

Statistical analysis

The experimental results were expressed as the mean ± S.D. A one-way analysis of variance (ANOVA) was used for multiple comparisons followed by Dunnetts test, and the data were considered significant with a probability less than 0.05.

RESULTS AND DISCUSSION

Antiplatelet therapy has become a useful means of preventing acute thromboembolic artery occlusions in cardiovascular diseases, because platelet activation and thrombus formation are important in the pathophysiology of ischaemic events in the heart, brain, and peripheral arterial territories. Antiplatelet compounds such as aspirin, ticlopidine, and dipyridamole are in clinical use, but show several side effects (Schror, 1995). In the present study, the effects of *Moutan Cortex Radicis* extracts and its major component paeonol on rabbit platelet aggregation were examined. To study the effect on platelet aggregation, washed platelet was pretreated with *Moutan Cortex Radicis* extracts (100 μg/mL), and then the platelet aggregation was induced by collagen (3 μg/mL). As shown in Table 1, *Moutan Cortex Radicis* methanol and buthanol layers showed the weak inhibitions, and water layer had no effect on the collagen-induced platelet aggregation. However, hexane and EtOAc layers concentration-dependently inhibited the collagen-induced platelet aggregations with the IC_{50} values of 10.9 ± 1.0 and 31.5 ± 0.8 μg/mL, respectively. Paeonol isolated from hexane and acetone layer specifically inhibited the collagen-induced platelet aggregation with the IC_{50} value of 113.1 ± 0.9 μM, whereas it had little effect on the platelet aggregation induced by other agonists such as arachidonic acid (AA), thrombin, A23187 and thapsigargin at the concentration of 200 μM (Table 2). It is well known that TXA_2 is an important mediator of the release reaction and aggregation of platelets (FitzGerald *et al.*, 1991). Collagen stimulates tyrosine kinase via collagen receptors, intracellular calcium then increases, followed by activation of phospholipase (PL) A_2. PLA_2 activation then leads to arachidonic acid release from membrane phospholipids,

Table 1. Effect of *Moutan Cortex Radicis* extracts on rabbit platelet aggregation induced by collagen

Sample (100 μg/mL)	Inhibition (%)	IC_{50} (μg/mL)
MeOH extract	39.0 ± 0.4	–
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EtOAc layer	92.9 ± 1.8	31.5 ± 0.8

Washed rabbit platelet suspension was incubated at 37? in an aggregometer with stirring at 1,000 rpm and then *Moutan Cortex Radicis* extracts were added. After 3 min preincubation, the platelet aggregation was induced by addition of collagen (3 μg/ml). The resulting aggregation was measured as the change in light transmission.

Table 2. Effect of paeonol on rabbit platelet aggregation induced by various agonists

Sample	Inducer	Inhibition (%)	IC_{50} (μM)
Paeonol (200 μM)	Collagen	92.0 ± 0.2	–
	Arachidonic acid	22.5 ± 1.9	–
	Thrombin	2.8 ± 3.1	113.1 ± 0.9
	A23187	5.3 ± 2.7	–
	Thapsigargin	2.9 ± 2.9	–

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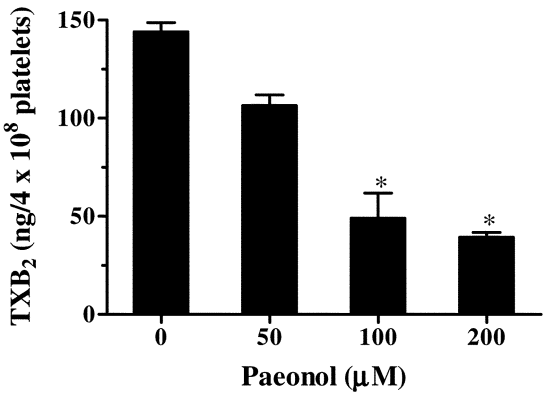

Fig. 2. Effect of paeonol on the collagen-induced TXB_2 formation. Washed rabbit platelet suspension was incubated with indicated concentrations of paeonol for 3 min at 37°C. And then collagen (1 g/mL) was added in washed platelet suspension. The reaction was terminated by supplement of EGTA and indomethacin (50 μM). TXB_2 level in medium was determined using TXB_2 EIA kit according to the described procedure by the manufacturer. The values are expressed as mean ± S.D. (n=3). *P<0.05.

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