Introduction

Platelets are responsible for the formation of pathogenic thrombi that cause the acute manifestations of atherothrombotic cardiovascular diseases. Furthermore, increased platelet aggregation is one of the essential contributors to the onset and development of cardiovascular diseases such as ischemic heart disease and stroke, which remain leading causes of death (1). Therefore, much research has been carried out to develop antplatelet agents with improved efficacy to prevent and/or treat platelet hyperactivity–related cardiovascular diseases (2, 3).

Investigations of traditional medicinal plants have resulted in discoveries of a number of modern medicines. Besides expanding the herbal therapeutic and preventive armamentarium, traditional medicines also offer new avenues to identify new pharmacophores and novel drug targets (4). Rhubarb, the rhizome of Rheum species including Rheum palmatum, Rheum tanguticum, Rheum undulatum, and Rheum officinale, is one of the oldest traditional medicines and is most commonly used as a purgative (5). In addition, rhubarb has antibacterial (6), analgesic (7), and anti-inflammatory activities (8). Furthermore, recent studies have reported that rhubarb possesses anticoagulant and antplatelet activities (9) and have determined that these are primarily associated with stilbene derivatives such as resveratrol, rhaponticin, and...
piceatannol (9, 10). Although anthraquinone derivatives are principal components of rhubarb (11, 12), the effects of anthraquinone derivatives on platelet activity are not thoroughly understood.

In the present study, we investigated the effects of four anthraquinones isolated from rhubarb on platelet activity in vitro and in vivo, and we found that chrysophanol-8-O-glucoside (CP-8-O-glc) significantly exerted antiplatelet and anticoagulant effects.

**Materials and Methods**

**Materials**

Cultivated rhubarb was purchased in July 2006 from the Yuseong herbal drug market (Daejeon, Republic of Korea) and identified by Dr. KiHwan Bae (College of Pharmacy, Chungnam National University, Daejeon, Republic of Korea). A voucher specimen (CNU-1345) has been deposited at the herbarium of the College of Pharmacy, Chungnam National University.

Collagen, ADP, thrombin, and arachidonic acid (AA) were purchased from Chrono-Log Co. (Harvertown, PA, USA). Dimethylsulfoxide (DMSO), polyethylene glycol (PEG), Tween-80, bovine serum albumin (BSA), β-nicotinamide adenine dinucleotide (reduced disodium β-NADH), and pyruvic acid were purchased from Sigma (St. Louis, MO, USA). Thromboxane (TX) B2 enzyme immunoassay (EIA) kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Fluorescein-isothiocyanate (FITC)-labeled annexin V (annexin V-FITC) was purchased from BD Biosciences (San Jose, CA, USA).

**Animals**

Sprague-Dawley (SD) rats and ICR mice were purchased from the Samtako Laboratory Animal Center (Republic of Korea) and housed in a conventional animal facility with free access to food and water in a temperature and relative humidity monitored and controlled environment under artificial lighting (12 h of light per day). Animals were allowed to acclimatize for at least 7 days before experiments. All animal-related study protocols were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Committee on Animal Research at Ajou Medical Center, Ajou University.

**Extraction and isolation**

The dried and milled rhizomes of rhubarb (4.7 kg) were extracted with 20 L ethanol, three times. The combined ethanol extract was concentrated to yield a residue (650 g, rhubarb extract). From the rhubarb extract, chrysophanol (CP), CP-8-O-glc, emodin, and physcion were isolated and structurally identified, essentially following the procedures described previously (13). Briefly, rhubarb extract was suspended in water and then successively partitioned with hexane, ethyl acetate (EtOAc), and butanol (BuOH) to afford 32.7 g hexane-, 308 g EtOAc-, and 108 g BuOH-soluble fractions, respectively. The hexane-soluble fraction was subjected to silica gel column chromatography, eluted with hexane-EtOAc (60:1 – 0:1) to give five fractions (H1 – H5). The fraction H1 was rechromatographed on a silica gel column, which was eluted with hexane-EtOAc (40:1) to obtain CP (1500 mg). Physcion (500 mg) was yielded from the H2 by using silica gel column chromatography with hexane-EtOAc (30:1). The EtOAc-soluble fraction was diluted with acetone and then filtered through filter paper to give the acetone fraction (E2, 226.1 g). The E2 (100 g) was subjected to silica gel column chromatography with the mixtures of CHCl3 and MeOH in increasing polarity (80:1 to 0:1) to yield six fractions (E2.1 – E2.6). Emodin (220 mg) were obtained from the subfraction E2.2 by using an ODS column with MeOH-H2O (1:1). The subfraction E2.3 was rechromatographed on a silica gel column with a stepwise gradient of CHCl3 and MeOH (4:1 to 0:1) to give CP-8-O-glc (613 mg).

**Preparation of samples**

For in vitro experiments, the four components isolated from rhubarb were dissolved in DMSO. The final concentration of DMSO as vehicle in the platelet suspensions never exceeded 1%. DMSO was without effect on any of the parameters assayed. For ex vivo and in vivo experiments, CP-8-O-glc was suspended in 70% PEG. The ex vivo and in vivo vehicle groups were treated by 70% PEG only. All agents were prepared immediately prior to use.

**Preparation of platelets**

Platelet-rich plasma (PRP) was prepared as described previously (14). Briefly, SD rats, weighing 200 – 250 g, were lightly anesthetized with diethyl ether. Blood was then collected from the abdominal aorta using a syringe containing 3.8% sodium citrate (1:9, V/V) and centrifuged at 150 × g for 10 min at room temperature. The supernatant (PRP) was used in the aggregation study. Platelets in PRP were counted using a hemocytometer after 10 μL PRP was diluted by adding 500 μL Tyrode solution (pH 7.4, 134 mM NaCl, 3 mM KCl, 2 mM MgCl2, 0.3 mM NaH2PO4, 12 mM NaHCO3, 12 mM glucose, 3.5 mg/mL BSA) to get the platelet density low enough for counting; the cell count of the platelet suspension used in the experiments was then adjusted to 2 × 10^8
cells/mL with Tyrode solution.

Washed platelets were prepared as described previously (15). PRP was further centrifuged at 500 × g for 15 min to obtain the platelet pellet. The platelet pellet was suspended in Tyrode solution and centrifuged again at 500 × g for 15 min. The washed platelets were then finally suspended in Tyrode solution to a final cell count of about 2 × 10^8 cells/mL. Just before starting the experiment, CaCl₂ (1 mM) was added to the washed platelet suspension.

In vitro platelet aggregation study
Platelet aggregation studies were performed under the experimental setting described in previous studies (14, 15) using the turbidimetric method described by Mustard et al. (16). Briefly, PRP was stimulated with different aggregating agents at the following final concentrations: collagen, 2 µg/mL; thrombin, 0.4 U/mL; AA, 100 µM; or ADP, 10 µM. Platelet aggregation was recorded for 5 min after agonist addition. Aggregations were measured by a Lumi-aggregometer (Chrono-Log Co., Havertown, PA, USA) connected to a computer and expressed as percent changes in light transmission, taking the value of a blank sample (buffer without platelets) to be 100%. For in vitro studies, PRP was preincubated with different concentrations of the four anthraquinone derivatives for 5 min in the cuvette of the aggregometer before being stimulated with the aggregating agents described above.

Determination of cytotoxicity
The cytotoxic effects of samples on platelets were determined by measuring lactate dehydrogenase (LDH) leakage from platelets, as described previously (14). Rat PRP was incubated at 37°C for 5 min with vehicle or samples and centrifuged at room temperature for 1 min at 10,000 × g. Aliquots of supernatant (25 µL) were then placed into a 96-well plate and mixed with 100 µL of NADH solution (0.03% β-NADH in phosphate buffer) and 25 µL of pyruvate solution (22.7 mM pyruvic acid in phosphate buffer) at room temperature. Reductions in absorbance at 340 nm due to the conversion of NADH to NAD⁺ were used as measures of LDH activity. LDH leakages were expressed as percentages of total enzyme activity measured in platelets completely lysed with 0.2% Triton X-100.

Ex vivo platelet aggregation study and anticoagulation assay
Two hours after the oral administration of CP-8-O-glucoside (10, 30, or 100 mg/kg) or aspirin (50 mg/kg), rat blood samples were collected and platelet aggregation monitored as described above. Heparin (2,000 U) was intraperitoneally administered once at 1 h before the experiments.

The anticoagulation activity of CP-8-O-glucoside was evaluated by measuring plasma clotting times as previously described (17). Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured according to the manufacturer’s instructions using an Automated Coagulation Laboratory 100 Instrument (Instrumentation Laboratory Company, Milan, Italy). The plasma from CP-8-O-glucoside (10, 30, or 100 mg/kg)-, aspirin (50 mg/kg)-, or heparin (2,000 U)-administered rats were collected. A 100-µL aliquot of plasma was mixed with 50 µL of cephaline, and coagulation was initiated with the addition of 0.025 M CaCl₂ or 100 µL of thromboplastin into 100 µL of plasma for aPTT and PT, respectively. The PT and aPTT were expressed in seconds.

Measurement of TXA₂ formation
PRP (2 × 10⁸ cells/mL) was preincubated for 5 min in the presence or absence of CP-8-O-glucoside (10, 30, or 100 µM), and then collagen (2 µg/mL) or thrombin (0.4 U/mL) was added. After incubation at 37°C for 6 min with stirring, 10 mM EDTA was added to stop TXA₂ formation. The amount of TXA₂ produced was assessed using TXB₂ (the stable metabolite of TXA₂) as a proxy. After centrifugation at 12,000 × g for 3 min, the amount of TXB₂ in the supernatant was measured using a TXB₂ EIA kit (Cayman).

In vivo mouse tail bleeding times
Bleeding times were determined as previously described (18). Male ICR mice, weighing 35 – 40 g, were fasted overnight before the experiments. Two hours after administering CP-8-O-glucoside (3, 10, or 30 mg/kg) or aspirin (50 mg/kg) orally, mice were anesthetized with sodium pentobarbital (75 mg/kg), and individually placed on a hotplate to control body temperature at 37°C. Tails were transected 3 mm from the tips with a razor blade, and then immersed in a 15-mL clear conical tube containing normal saline prewarmed to 37°C. Times to blood flow cessation (defined as no bleeding for 15 s) were measured.

Measurement of platelet phosphatidylserine (PS) exposure
Platelet PS exposure was measured as previously described (19). Washed platelets (2 × 10⁸ cells/mL) were preincubated with CP-8-O-glucoside (100 µM) for 5 min and stimulated with collagen (2 µg/mL) at 37°C for 8 min. After the reaction was terminated by adding 10 mM EDTA, the suspension was centrifuged at 10,000 × g for 10 s. The platelet pellet obtained was suspended in 100 µL binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl,
2.5 mM CaCl₂), stained with annexin V-FITC for 20 min at room temperature in the dark, and then added with additional binding buffer (400 μL). The suspension was analyzed with a FACS Aria III cytofluorometer (BD Biosciences) equipped with an argon laser (488 nm). Data from 10,000 events were collected and analyzed using WinMDI2 software.

Statistical analyses
Results are expressed as means ± S.E.M. of at least four different experiments. The Student t-test and/or one way ANOVA followed by Dunnett’s test were used for the analysis. P-values of < 0.05 were considered statistically significant.

Results
In vitro antiplatelet effects of the four anthraquinone derivatives
The antiplatelet effects of rhubarb extract and the four anthraquinone derivatives were examined using rat PRP. As shown Fig. 1, rhubarb extract at 300 μg/mL reduced collagen (2 μg/mL)-induced platelet aggregation to 45.5% ± 5.7% (% of inhibition). At 100 μM, all four anthraquinone derivatives maximally inhibited collagen (2 μg/mL)-induced platelet aggregation (CP by 42.9% ± 6.0%, CP-8-O-gluc by 98.2% ± 1.8%, emodin by 77.1% ± 2.4%, and physcion by 29.7% ± 4.3%; % of inhibition). The IC₅₀ values of the anthraquinone derivatives with respect to collagen (2 μg/mL)- and thrombin (0.4 U/mL)-induced aggregation are shown in Table 1. Of the four anthraquinone derivatives tested, CP-8-O-gluc inhibited platelet aggregation most and more potently than aspirin.

To determine whether cytotoxicities of these anthraquinone derivatives might influence their antiplatelet effects, we examined their effects on LDH release (an index of cellular injury) from platelets. However, the amount of LDH released was not significantly altered by exposure to any of four anthraquinone derivatives for 5 min at the highest concentrations examined (Fig. 2), whereas digitonin (40 μM, a positive control) significantly increased LDH release. These findings show that the four anthraquinone derivatives did not affect cell membrane integrity.

In vitro antiplatelet effect of CP-8-O-gluc
We evaluated further the antiplatelet effects of CP-8-O-gluc on thrombin (0.4 U/mL)-, AA (100 μM)-, and ADP (10 μM)-induced platelet aggregation. CP-8-O-gluc was found to inhibit the platelet aggregation induced by all

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Collagen (μM)</th>
<th>Thrombin (μM)</th>
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<tbody>
<tr>
<td>CP</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>CP-8-O-gluc</td>
<td>33.4 ± 0.2</td>
<td>25.9 ± 0.2</td>
</tr>
<tr>
<td>Emodin</td>
<td>84.2 ± 0.5</td>
<td>61.7 ± 0.4</td>
</tr>
<tr>
<td>Physcion</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>Aspirin</td>
<td>130.0 ± 14.1</td>
<td>1810.2 ± 12.0</td>
</tr>
</tbody>
</table>

Values are presented as the mean ± S.E.M. CP: chrysophanol, CP-8-O-gluc: chrysophanol-8-O-glucoside.
Antiplatelet Effect of CP-8-O-glc

three agonists significantly and concentration-dependently (Fig. 3).

Ex vivo platelet response to CP-8-O-glc administration

The inhibitory effect of CP-8-O-glc on ex vivo platelet aggregation is shown in Fig. 4. PRP from rats administered CP-8-O-glc (10, 30, or 100 mg/kg, orally) showed a great tendency to inhibit collagen (2 μg/mL)-, thrombin (0.4 U/mL)-, or AA (100 μM)-induced platelet aggregation, which concurred with the results obtained when in vitro rat PRP was directly incubated with CP-8-O-glc. Aspirin (50 mg/kg) also significantly inhibited ex vivo platelet aggregation.

Effect of CP-8-O-glc on TXA2 formation

The effect of CP-8-O-glc on TXA2 formation was evaluated. TXA2 is unstable and quickly converted to TXB2, the stable metabolite of TXA2, and thus, a TXB2 EIA kit was used to determine TXA2 concentrations. As shown in Fig. 5, the TXB2 level in the control group was 1416.7 ± 45.8 pg/mL and this increased to 3922.4 ± 25.5 or 3928.8 ± 41.6 pg/mL after stimulation with collagen (2 μg/mL) or thrombin (0.4 U/mL), respectively. However, CP-8-O-glc significantly inhibited the increase of TXB2 formation in a manner reminiscent of its suppression of platelet aggregation. Aspirin (1 mg/mL) as a positive control completely blocked TXB2 formation.

Effect of CP-8-O-glc on bleeding times

We also determined bleeding times in ICR mice administered CP-8-O-glc to evaluate its in vivo antiplatelet effect. CP-8-O-glc significantly prolonged bleeding times as compared with vehicle alone. Furthermore, mice administered CP-8-O-glc at 10 mg/kg had bleeding times similar to those of mice administered aspirin at 50 mg/kg (Fig. 6).

Effect of CP-8-O-glc on coagulation activities

To determine if the coagulation process is impaired by CP-8-O-glc, PT and aPTT were evaluated at 2 h after orally administering CP-8-O-glc at 10, 30, or 100 mg/kg. As shown in Table 2, PT and aPTT values in the vehicle-
treated mice plasma were 17.5 ± 0.3 and 34.9 ± 1.5 s, respectively. PT was not altered by CP-8-O-gluc at any tested dose, whereas aPTT was significantly prolonged by 100 mg/kg CP-8-O-gluc to 50.5 ± 4.5 s. In order to evaluate whether the observed prolongation of aPTT was due to the effect on the intrinsic pathway, we investigated the effects of CP-8-O-gluc on activities of intrinsic factors and on platelet PS exposure. Collagen stimulation gradually enhanced platelet PS exposure (38.9%). CP-8-O-gluc significantly reduced the level of platelet PS exposure to 18.8% (Fig. 7). However, 100 mg/kg CP-8-O-gluc had no effect on factor VIII, IX, XI, and XII under the same conditions in which prolonged aPTT was observed (data not shown).

Discussion

Herbal medicines offer a rich potential source of novel antiplatelet agents, and many of these medicines have excellent pharmacological effects. Accordingly, much effort has been focused on the identification of plant-based materials with antiplatelet potential (20, 21). The present study demonstrates that CP-8-O-gluc was the most potent of the four compounds examined, in terms of in-
hbiting platelet aggregation, and suggests that it has therapeutic potential for the prevention of platelet hyperactivity–related cardiovascular diseases. In addition, in agreement with a previous study (22), emodin was found to have antiplatelet activity in the present study.

Anthrachinone derivatives are considered to be the important constituent in this plant. However, the pharmacological potencies are different for each component. For instance, emodin shows high anti-inflammation activity (23), physcion exhibits a hepatoprotective effect (24), and CP has anticancer activity (25), whilst CP-8-O-gluc promotes glucose transport (26). The differences in pharmacological activities of these compounds may be related to their structures. For example, a recent study on the structure–activity relationships of anthraquinone derivatives and their pharmacological activities indicated that emodin displayed stronger anti-angiogenic activity than CP (27). CP, physcion, and emodin only differ from one another at the C-6 position. In the case of CP-8-O-gluc, a glucoside moiety is added to CP. Physcion and CP showed very weak antiplatelet activity in an in vitro study, while emodin and CP-8-O-gluc showed high activity. Comparing the structural characteristics of these four anthraquinones, the methyl or hydroxyl group substitution at the C-6 position or a glucoside moiety at the C-8 position may contribute to the antiplatelet potency. In terms of glycosylation, many studies have reported that the aglycon and glycosylated forms of natural compounds have different potencies. Our results agree with those of another study (28), in which it was suggested that the glycosylation of polyphenols helps protect them from enzymatic oxidation, thus extending their half-lives in the cell and maintaining their antioxidant capacity. However, the effect of the glycosylation on biological activity is still unclear. For example, it has been reported that secoiridoids, such as ligustroside and oleuropein, show greater anticancer potency in the aglycon form (29). Different results on the effects of glycosylation may be explained by different bioavailabilities and/or stabilities (30). Indeed, it has been reported that genistin, a glycosylated form of genistein, has greater bioavailability and greater potency in antitumor effect than its aglycon (30, 31).

In this study, we found that CP-8-O-gluc exerted similar inhibitory effects on collagen-, thrombin-, and AA-induced platelet aggregation, but had a relatively weak inhibitory effect on ADP-induced platelet aggregation. Besides, when platelets were stimulated by collagen and thrombin, CP-8-O-gluc significantly inhibited TXB₂, a stable form of TXA₂, formation. TXA₂ plays a crucial role by mediating the effects of collagen and thrombin to elicit aggregation of platelets (32). However, in terms of ADP-induced platelet aggregation, the change in the levels of cAMP plays major roles in responses to activation by Gαi-linked P₂Y₁₂, one of two ADP receptors (33). Based on these results, it is likely that CP-8-O-gluc has a limited effect on the cAMP signaling pathway, and thus, a weak inhibitory effect on ADP-induced platelet aggregation. However, further studies are required to elucidate whether the antiplatelet effect of CP-8-O-gluc is associated with the change in the levels of cAMP.

To elucidate the antiplatelet effect of CP-8-O-gluc in an animal model, we performed the ex vivo platelet aggregation study in rats and the measurement of in vivo bleeding time in mice. However, there is a difference between the dosage administered to rats and mice. Indeed, it has been reported that the differences in the species of animals used may account for part of the variability in the effective dose and/or results (34, 35). The well-defined reason for these differences between rats and mice is not known. However, one possible explana-
tion is the species differences in the activities of the liver microsomal enzymes. Because their activities reflected the variations in the process and rate of drug metabolism, it is important to know what drug levels are present in plasma and tissues and to determine the proper dose of drug (36). This can explain not only the possibility of a species difference in the effective dose but also the difference in concentration between the in vitro and in vivo situation. In a previous study (37), drug metabolites accumulated in rats, but not in mice, because rat liver microsomes slowly transformed metabolites to other products. In accord with this view, the difference of the effective dose of CP-8-O-glc in mice for the bleeding time and in the rat ex vivo platelet aggregation assay is also explainable. However, the study on the pharmacokinetics of CP-8-O-glc is requisite for clarification of these issues.

Blood coagulation and platelets are complementary, mutually dependent processes in hemostasis and thrombosis (32). Blood coagulation can be initiated via an intrinsic pathway or extrinsic pathway, and both pathways lead to the activation of a final common pathway. The intrinsic pathway involves coagulation factors (XII, XI, IX, and VIII) and co-factors (Ca²⁺ and phospholipids from platelets, erythrocytes, or endothelial cells), and the extrinsic pathway involves tissue factor and coagulation factor VII (38). Meanwhile, platelets play an essential role in the coagulation cascade (39, 40). Although coagulation is the result of a complex process initiated by the intrinsic or extrinsic pathways, it is also highly regulated by interactions between platelets and coagulation factors in plasma. Activated platelets can release some factors (e.g., Ca²⁺, factor V, Factor VIII) that are known to be essential for the intrinsic pathway to function normally (41). Also, activated platelets support and potentiate the intrinsic pathway of coagulation by expressing negatively charged PS on their outer surface. Exposed PS strongly propagates the coagulation process by providing the surface for assembly of factor X with the factors VIIIa and IXa (32, 42). In this context, platelet activation is more related with the intrinsic pathway than the extrinsic pathway. In the present study, CP-8-O-glc was found to significantly prolong bleeding time and aPTT, but not to alter PT. Prolonged aPTT and unchanged PT implies that the intrinsic pathway was only affected, and thus, we further checked the effect of CP-8-O-glc on the activities of intrinsic factors (VIII, IX, XI, and XII) and on platelet PS exposure. However, the CP-8-O-glc only showed an inhibitory effect on PS exposure, suggesting that the anticoagulant effect of CP-8-O-glc is presumably indirect effect though inhibition of platelet PS exposure due to potent antiplatelet activity, but not direct inhibition on coagulation factors in the intrinsic pathway. In a recent study, emodin was also reported to prolong aPTT, but its target or mechanism was not investigated (43).

In conclusion, the present study demonstrates for the first time that CP-8-O-glc has considerable antiplatelet and anticoagulant activities. Specifically, it was found to inhibit platelet aggregation in in vitro and ex vivo and to prolong bleeding time and aPTT. Furthermore, our find-
ings suggest that CP-8-O-glc may be a useful prophylactic treatment for platelet hyperactivity and that it might be therapeutically beneficial for the treatment of cardiovascular diseases associated with platelet hyperactivity.

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References

31 Allred CD, Ju YH, Allred KF, Chang J, Helferich WG. Dietary genistin stimulates growth of estrogen-dependent breast cancer


