Chloroform fraction of *Euphorbia maculata* has antiplatelet activity via suppressing thromboxane B_2 formation

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Abstract. *Euphorbia maculata* (EM) is a traditionally used antidiarrheal, antibacterial, antifungal and antioxidant agent. However, the effects of EM on platelet activity remain to be elucidated. Therefore, the present study investigated the antiplatelet effect of various EM extract fractions on platelet aggregation in rats. The antiplatelet activity of the EM fractions on collagen or adenosine diphosphate (ADP)-induced platelet aggregation was evaluated *in vitro* and *ex vivo*. Thromboxane B_2 (TXB_2) formation, rat-tail bleeding time and coagulation time were also measured. Among the fractions, the chloroform fraction of EM (CFEM) significantly inhibited ADP-induced platelet aggregation *in vitro*. Furthermore, oral administration of 50 mg/kg CFEM to rats significantly reduced ADP-induced platelet aggregation without increasing the tail bleeding time or coagulation time. In addition, EM significantly inhibited the level of TXB_2 formation in a dose-dependent manner. These results suggest that CFEM exhibits antiplatelet activity, without causing bleeding, via the suppression of TXB_2 formation. CFEM may be a type of food which has the potential for preventing cardiovascular disease.

Introduction

Platelets are critical in hemostasis and thrombus formation (1,2). When blood vessels are damaged, diverse types of agonists, including collagen, adenosine diphosphate (ADP), arachidonic acid (AA) and thrombin, are promptly released and induce platelet aggregation (3,4). However, abnormal platelet activation may cause cardiovascular diseases (CVDs), including ischemic heart disease, thrombosis, stroke, myocardial infarction and atherosclerosis, which are major diseases responsible for human mortality worldwide (5,6). Thus, reducing platelet aggregation is important for limiting atherosclerosis and CVD (7). Consequently, numerous types of drugs, including antiplatelet, anticoagulant and fibrinolytic agents, have been developed to moderate or prevent thrombosis (8).

A number of anti-thrombotic drugs have been developed and used clinically. Acetylsalicylic acid (ASA), which is a direct cyclooxygenase (COX) inhibitor, is generally used to suppress platelet aggregation (9). However, continuous administration of ASA can induce resistance or side effects, including headache, vomiting, gastric ulceration and abdominal pain in numerous patients (10-13). Therefore, studies have been investigating naturally derived antiplatelet agents with a reduced risk of side effects (14-19).

Euphorbia maculata (EM) is an annual plant, which belongs to the family Euphorbiaceae. EM is grown worldwide, including in East Africa, South America, Korea, Japan and China (20). The antidiarrheal, antibacterial, antifungal and antioxidant activities of this plant have been reported, however, to the best of our knowledge, studies on the antiplatelet effect of EM have not yet been conducted (20-23).

In the present study, the antiplatelet activities of EM fractions were investigated *in vitro* and *ex vivo*.

Materials and methods

Materials. Euphorbia maculata (spotted spurge; EM) was provided by Wonkwang Herb Inc. (Jeonbuk, Korea). Collagen, adenosine diphosphate (ADP), AA and thrombin were obtained from Chrono-Log (Havertown, PA, USA). Trisodium citrate tubes were purchased from Greiner Bio-One (Frickenhausen, Germany). ASA, ketamine HCl, xylazine HCl, β-nicotinamide adenine dinucleotide (β-NADH), ethylenediaminetetraacetic acid (EDTA), indomethacin, pyruvic acid and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). A

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thromboxane B_2 (TXB_2) enzyme immunoassay (EIA) kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Animals. Male Sprague Dawley (SD) rats weighing 250-280 g were obtained from Samtaco Co., Ltd. (Osan, Korea). All the animals were maintained under standard laboratory conditions and allowed free access to food and water *ad libitum*. The specific pathogen-free animal room was maintained at a temperature of 23±2˚C with a humidity of 60±5% in a 12 h light/dark cycle. All animal experimentation protocols used in the present study were approved by the Animal Experiments Committee of Wonkwang University (Iksan, Korea).

Preparation of EM fractions. The aerial parts of *Euphorbia maculata* (100 g) were reflux extracted with ethanol at 80˚C for 2 h. Following filtration, the residue was re-extracted twice as described above. The combined filtrate was evaporated and lyophilized to obtain the crude extract. The crude extract was further fractionated using chloroform, ethyl acetate, butanol and distilled water. The fractionation scheme is shown in Fig. 1.

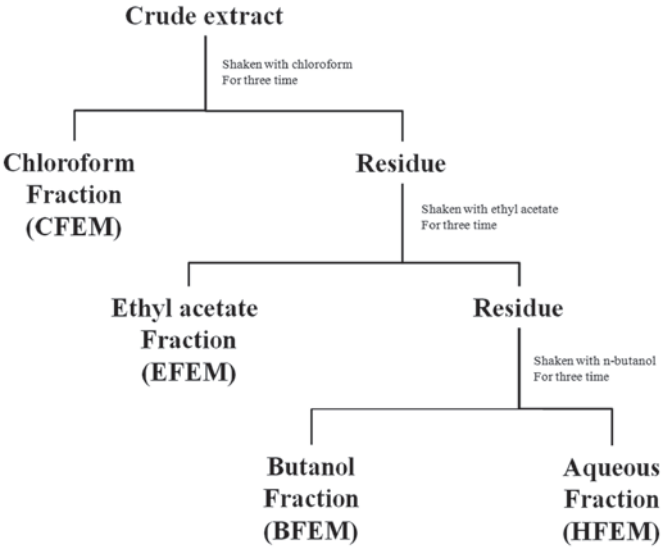

Figure 1. Fractionation scheme of *Euphorbia maculata* extracts.

Lactate dehydrogenase (LDH) release assay. The cytotoxic activity of the EM fractions was determined by measuring LDH release from the platelets, as described previously with minor modifications (24,25). Briefly, male SD rats were anesthetized by intraperitoneal injection with a mixture of ketamine HCl (60 mg/kg) and xylazine HCl (10 mg/kg) and blood was collected from the abdominal aorta using a trisodium citrate tube (3.8%, 1:9, v/v). The collected blood was centrifuged at 120 x g for 10 min at 37˚C to prepare rat platelet rich plasma (PRP) and the remaining blood was further centrifuged at 200 x g for 10 min at 37˚C to obtain platelet-poor plasma (PPP). The platelet count was adjusted to $3x10^8$ platelets/ml using PPP as a diluent for use in the LDH release assay.

PRP was incubated at 37˚C for 10 min in the presence or absence of the EM fractions and centrifuged at 12,000 x g for 2 min. To measure LDH leakage, 50 μl aliquots of PRP were collected and mixed with 200 μl of β-NADH solution (0.03% β-NADH in phosphate buffer) and 50 μl of pyruvate solution (22.7 mM) at room temperature. The reductions in absorbance due to the conversion of NADH to NAD^+ were measured using a spectrophotometer (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 340 nm. LDH release was measured in platelets lysed with 0.2% Triton X-100 and expressed as a percentage of the total enzyme activity.

In vitro platelet aggregation assay. Male SD rats were anesthetized by intraperitoneal injection with a mixture of ketamine HCl and xylazine HCl and blood was collected from the abdominal aortas using trisodium citrate tubes (3.8%, 1:9, v/v). The collected blood was centrifuged at 120 x g for 10 min to obtain PRP and PPP. The platelet count was adjusted to $3x10^8$ platelets/ml for the platelet aggregation assay.

Platelet aggregation was measured using turbidimetric methods with minor modifications (26,27). Briefly, PRP was preincubated with or without various concentrations of the EM fractions for 5 min at 37˚C. Platelet aggregation was induced by adding 5 μM ADP and 3 μg/ml collagen. The quantity of light transmitted through the sample, as a measure of platelet aggregation, was observed for a further 5 min at 37˚C using an aggregometer (Model 700; Chrono-log).

Ex vivo platelet aggregation assay. Male SD rats were divided randomly into three groups of 10 rats each and orally administered water (normal), aspirin (20 mg/kg) or CFEM (2, 10 and 50 mg/kg) 2 h prior to sacrifice. Rats were anesthetized with a mixture of ketamine HCl and xylazine HCl by intraperitoneal injection, and blood was collected from the abdominal aortas using trisodium citrate tubes. The collected blood was then centrifuged to obtain PRP and PPP. The platelet aggregation assay was performed as described above.

Ex vivo anticoagulant assay. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) was measured as previously described (27). The coagulation assay was performed using PPP from CFEM-treated rats and measured using a coagulometer (Coatron M4; Teco Medical Instruments GmbH, Neufahrn, Germany). Determination of aPTT and PT were performed according to the manufacturer's instructions. For the PT assay, 25 μl of PPP was incubated for 1.5 min at 37˚C. The assay reaction was started by adding 50 μl of PT reagent and observed for 3 min. For the aPTT assay, 25 μl of PPP was preincubated with 25 μl of the aPTT reagent for 5 min at 37˚C. Subsequently, the assay reaction was initiated by adding 25 μl of 25 mM $CaCl_2$ and observed for 5 min at 37˚C. The clotting time was determined as being the time required to achieve a 50% change in the scattered light transmission as measured at 660 nm.

Tail bleeding time. Bleeding time was measured as described with minor modifications (28). Rats were anesthetized with ketamine and xylazine. The tail was transected 5 mm from the tip and promptly immersed in 0.9% saline at 37˚C. Bleeding time was defined as the time required for the blood flow to stop for at least 30 sec. If bleeding continued for >30 min, the assay was halted and the bleeding time recorded as 30 min for statistical analysis.

Measurement of TXB_2 formation. PRP ($3x10^8$ platelets/ml) was preincubated with various concentrations of CFEM for 5 min and 5 μM ADP was added. Subsequently, 50 mM indomethacin and 2 mM EDTA were mixed to stop the reaction. The aliquots were centrifuged at 12,000 x g for 10 min and the supernatant was collected. TXB_2 formation was measured using an EIA kit according to the manufacturer's instructions.

Statistical analysis. The results are presented as the mean ± standard deviation. Comparisons between groups were performed using Student's t-test and all statistical analyses were performed using SPSS 22.0 software (International Business Machines, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of EM fractions on LDH release. The cytotoxicity of the EM fractions on rat platelets was investigated by measuring LDH release. As shown in Fig. 2, up to 100 μg/ml of EM extract did not induce LDH release from the platelets. This suggested that the EM fractions did not affect platelet viability or induce platelet lysis in rat plasma.

Effect of EM fractions on platelet aggregation. As shown in Fig. 3, none of the EM fractions affected collagen-induced platelet aggregation. However, CFEM significantly reduced ADP-induced platelet aggregation. When PRP was stimulated by ADP, the aggregation rate was significantly increased from 1.4±0.71 to 68.5±2.12%. Furthermore, CFEM decreased ADP-induced platelet aggregation by 66.4±3.1, 57.0±4.7 and 50.2±1.4% at 10, 30 and 100 μg/ml (Fig. 4).

Effect of CFEM on platelet aggregation in rats. Since CFEM was an effective antiplatelet agent against ADP-induced platelet aggregation *in vitro*, the effects of CFEM were assessed in an animal model of platelet aggregation induced by ADP. As shown in Fig. 5, the aggregation rate of the untreated rats was 71.73±4.65%, but was reduced by CFEM treatment. In rats treated with 2, 10 and 50 mg/kg of CFEM, ADP-induced platelet aggregation was decreased to 67.33±5.20, 61.50±2.59 and 51.25±1.13%, respectively.

Effect of CFEM on tail bleeding time in rats. Fig. 6 shows the effect of CFEM on bleeding time in rats. The bleeding time of untreated rats was 824±120.32 sec and was significantly increased to 1557±165.61 sec in aspirin-treated rats. The tail bleeding time of rats treated with 2, 10 and 50 mg/kg CFEM was 901±57.56, 1020±120.79 and 1044±86.53 sec, respectively.

Effect of CFEM on coagulation time in rats. As shown in Table I, CFEM did not extend the coagulation time in rats. The PT of untreated rats was 24.4±0.45 sec and the coagulation times of rats treated with 2, 10 and 50 mg/kg of CFEM were 24.5±0.34, 25.0±0.44 and 24.9±0.52 sec, respectively. In addition, the aPTT of CFEM-treated rats was 18.8±1.07, 19.3±0.74 and 18.6±0.52 sec, which was not significantly different compared with the untreated rats (18.9±0.63 sec).

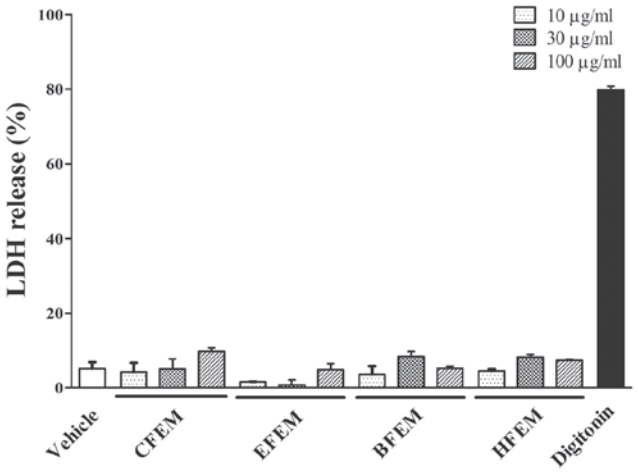

Figure 2. Effects of EM fractions on LDH release. PRP ($3x10^8$ platelets/ml) was incubated with or without various doses of the EM fractions for 10 min at 37˚C and centrifuged at 12,000 x g for 2 min. Released LDH was measured using a spectrophotometer. Data are expressed as the mean ± standard deviation. LDH, lactate dehydrogenase; EM, *Euphorbia maculata*; PRP, platelet rich plasma; CFEM, chloroform fraction of EM; EFEM, ethyl acetate fraction of EM; BFEM, butanol fraction of EM; HFEM, aqueous fraction of EM.

Effect of CFEM on TXB_2 formation. Fig. 7 shows the effect of CFEM on TXB_2 formation. ADP increased the level of generated TXB_2 to approximately three times that of the control and was reduced in a dose-dependent manner by CFEM. ADP-induced PRP produced 24.64±0.319 ng/ml of TXB_2 and 10, 30 and 100 μg/ml of CFEM decreased TXB_2 to 23.80±0.647, 21.48±1.337 and 13.73±0.432 ng/ml, respectively.

Discussion

In the present study, the inhibitory effect of our EM fractions on collagen- and ADP-induced platelet aggregation in rat plasma was evaluated.

Platelet activation and aggregation have pivotal roles in the pathogenesis of thrombotic complications, including atherosclerosis, myocardial infarction, thromboembolism and other vascular diseases (2,29). Thus, reducing platelet hyperactivation and aggregation is directly connected to the treatment of thrombotic disease in the clinic (30). Anti-coagulating agents are widely used to effectively inhibit platelet aggregation and prevent cardiovascular events. However, continuous use of these agents is prohibited due to adverse effects, including an increased likelihood of a bleeding event (9,13). For this reason, there has been several attempts to overcome these limitations in atherothrombosis treatment and substitute antiplatelet agents, including ADP-receptor agonists, are recommended, as has been demonstrated in numerous clinical trials (31).

In the resting state, platelets freely flow through blood vessels. However, when platelets adhere to damaged vessels, platelet activation and aggregation promptly occur. This process is stimulated by endogenous agonists, including ADP, thrombin, collagen, serotonin, TXA_2 and AA (32). Although weak, ADP is the most important mediator among these, as it causes alterations in platelet shape, aggregation and TXA_2 secretion (33). ADP, along with adenosine triphosphate and serotonin, is stored in dense granules inside of platelets and is secreted at sites of injury by platelet activation. The released ADP binds to the puri-

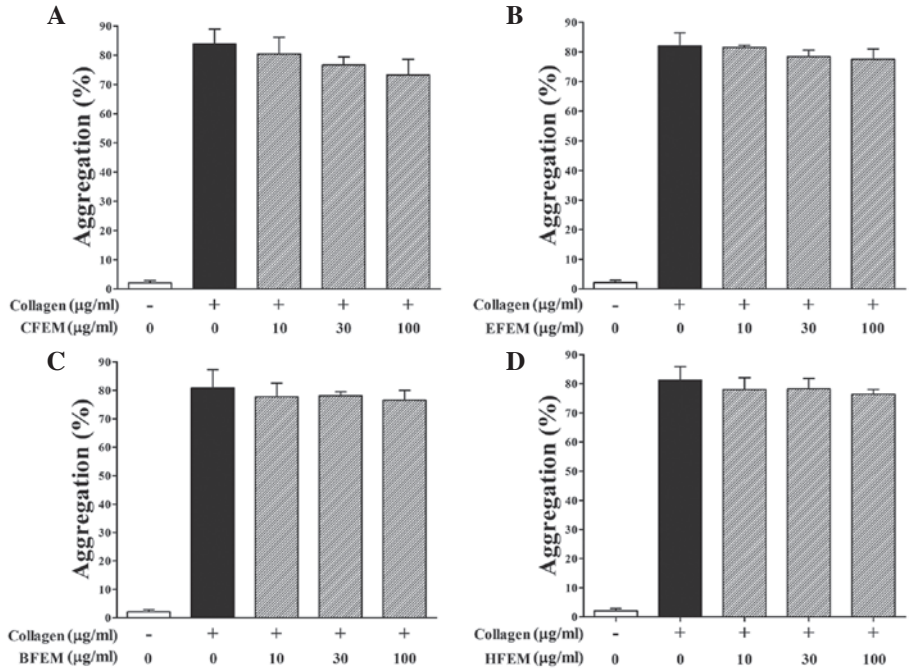

Figure 3. Effects of EM fractions on collagen-induced platelet aggregation *in vitro*. PRP ($3x10^8$ platelets/ml) was preincubated with various concentrations of the EM fractions at 37˚C for 5 min. Collagen (3 μg/ml) was then added to induce platelet aggregation. (A) CFEM; (B) EFEM; (C) BFEM; (D) HFEM. Data are expressed as the mean ± standard deviation of three experiments. *P<0.05, compared with the control. EM, *Euphorbia maculata*; PRP, platelet rich plasma; CFEM, chloroform fraction of EM; EFEM, ethyl acetate fraction of EM; BFEM, butanol fraction of EM; HFEM, aqueous fraction of EM.

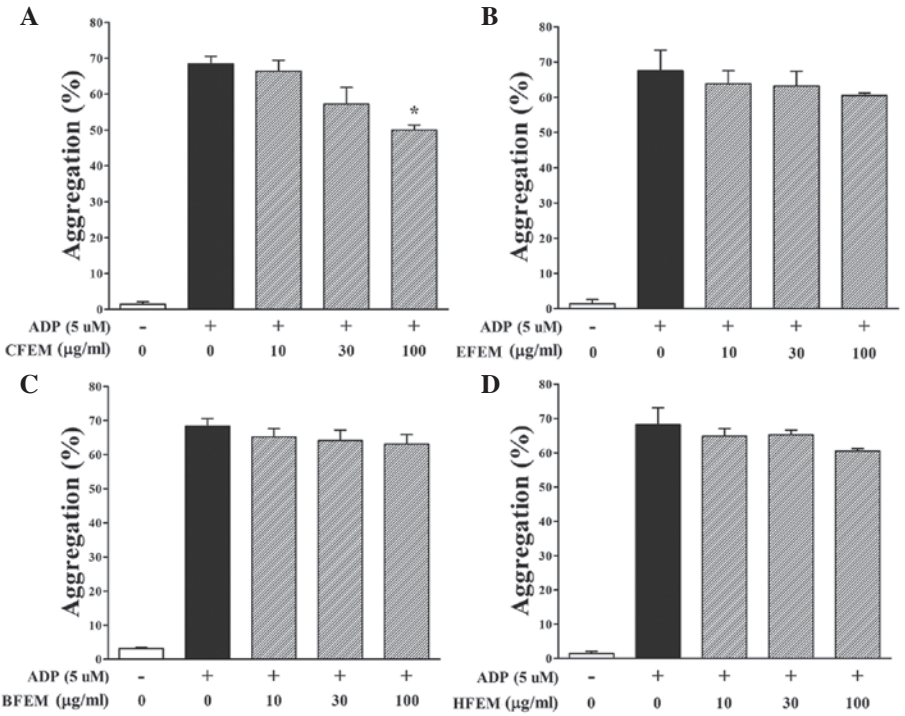

Figure 4. Effects of EM fractions on ADP-induced platelet aggregation *in vitro*. PRP was preincubated with various concentrations of the EM fractions at 37˚C for 5 min. ADP (5 μM) was then added to induce platelet aggregation. (A) CFEM; (B) EFEM; (C) BFEM; (D) HFEM. Data are expressed as the mean ± standard deviation of three experiments. *P<0.05, compared with the control. EM, *Euphorbia maculata*; PRP, platelet rich plasma; CFEM, chloroform fraction of EM; EFEM, ethyl acetate fraction of EM; BFEM, butanol fraction of EM; HFEM, aqueous fraction of EM; PRP, platelet rich plasma; ADP, adenosine diphosphate.

nergic receptors $P2Y_1$ and $P2Y_{12}$. Activation of the $P2Y_1$ receptor pathway triggers intracellular calcium mobilization and alters platelet shape, while $P2Y_{12}$ receptor activation inhibits adenylyl cyclase-dependent cyclic adenosine monophosphate generation and prolongs platelet aggregation (34). The antiplatelet agents currently in use (e.g. clopidogrel, ticagrelor and prasugrel) effectively reduce platelet aggregation by inhibiting ADP-$P2Y_{12}$ receptor binding (35-38). In the present study, CFEM reduced collagen and ADP-induced platelet aggregation *in vitro* and CFEM-administered rats decreased ADP-triggered platelet

Table I. Effect of CFEM on coagulation time in rats.

Group	Dose (mg/kg)	PT (sec)	aPTT (sec)
Normal		24.4±0.45	18.9±0.63
Aspirin	20	25.6±0.92	18.7±0.98
CFEM	2	24.5±0.34	18.8±1.07
	10	25.0±0.44	19.3±0.74
	50	24.9±0.52	18.6±0.52

Data are expressed as the mean ± standard deviation (n=10). CFEM, chloroform fraction of EM; PT, prothrombin time; aPTT, activated partial thromboplastin time.

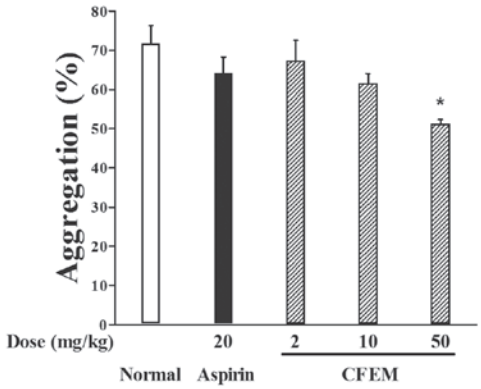

Figure 5. Effects of CFEM on ADP-induced platelet aggregation in rats. Platelet rich plasma was mixed with ADP (5 μM) and platelet aggregation was observed turbidimetrically. Data are expressed as the mean ± standard deviation (n=10). *P<0.05, compared with the normal group. CFEM, chloroform fraction of EM; ADP, adenosine diphosphate.

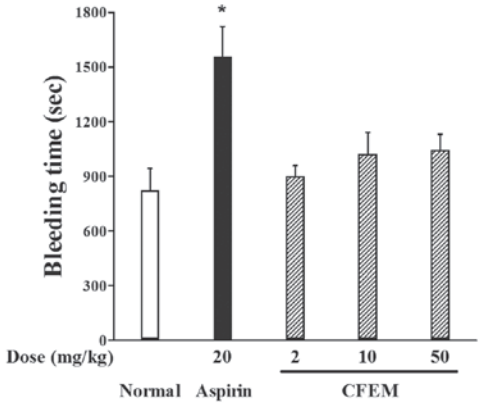

Figure 6. Effects of CFEM on tail bleeding time in rats. Rats were anesthetized using ketamine HCl (60 mg/kg) and xylazine HCl (10 mg/kg). Subsequently, the tail was transected 5 mm from the tip and rapidly submerged in saline at 37˚C. Data are expressed as the mean ± standard deviation (n=10). *P<0.05, compared with the normal group. CFEM, chloroform fraction of EM.

aggregation in a dose-dependent manner *ex vivo*. These results suggest that CFEM exerts antiplatelet activity particularly on ADP-induced platelet aggregation.

Normal homeostasis relies on the balance between procoagulation and anticoagulant activities (39). When these activities favor anticoagulant effects or procoagulation effects, continuous bleeding is likely to occur (40). The bleeding time,

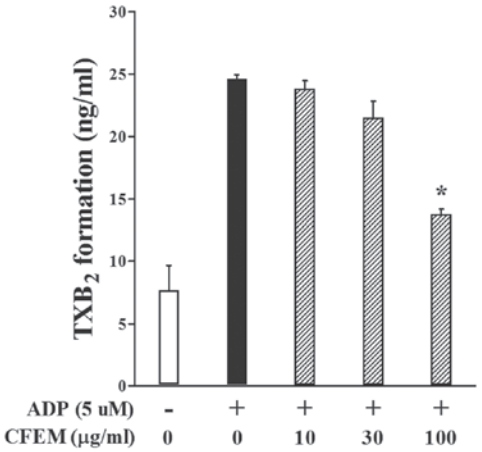

Figure 7. Effects of CFEM on TXB_2 formation in rat PRP. PRP ($3x10^8$ platelets/ml) was preincubated with 10, 30 and 100 μg/ml of CFEM at 37˚C for 5 min and then ADP (5 μM) was added. TXB_2 formation was measured using an enzyme immunoassay kit. Data are expressed as the mean ± standard deviation of triplicate experiments. *P<0.05, compared with the control. CFEM, chloroform fraction of EM; ADP, adenosine diphosphate; PRP, platelet rich plasma; TXB_2, thromboxane B_2.

as determined using the tail-transection assay is a well-recognized index of hemostasis and is used to investigate clinically important adverse effects of anticoagulants (41,42). CFEM did not demonstrate significant alterations in bleeding time, which is the main adverse effect of antiplatelet treatment. Thus, this suggested that CFEM treatment is unlikely to produce uncontrollable bleeding.

Coagulation is the consequence of a complex process that occurs by intrinsic, extrinsic and common pathways. Thus, blood coagulation is highly associated with platelet interactions with coagulation factors and blood vessels (43). When measuring coagulation, the PT assay is used to evaluate the extrinsic pathway and the aPTT assay measures the activation of the intrinsic pathway. These tests are recognized as useful methods to confirm bleeding risk and the potential for thrombotic adverse effects (44,45). The anticoagulant activities of CFEM were examined using the coagulation assays and CFEM did not affect PT and aPTT.

A TXA_2-mediated pathway is also important in antiplatelet treatments. During platelet aggregation, AA is liberated from the platelet plasma membrane and promptly metabolized to TXA_2 via the COX and thromboxane A2 synthase pathway (46). TXA_2 acts to amplify the intracellular signals for platelet aggregation,

vasoconstriction and recruitment of platelets (47). In addition, P2Y receptor activation, in turn, stimulates TXA_2 receptor activation, which triggers ADP secretion (48,49). This suggests that TXA_2 formation is closely associated with ADP-induced platelet aggregation and agents that reduce TXA_2 secretion may be therapeutically useful in platelet-associated CVDs. However, TXA_2 has an extremely short half-life and is unstable; therefore, TXB_2 levels are assessed as a measure of TXA_2 formation (50). CFEM significantly decreased the level of TXB_2 suggesting that suppression of TXB_2 formation is associated, in part, to the inhibitory effect of CFEM on platelet aggregation.

In conclusion, CFEM inhibits ADP-induced platelet aggregation by reducing TXB_2 formation, without affecting bleeding time or the coagulation pathways. These results suggest that CFEM possesses antiplatelet activity and may be a useful therapeutic for thrombotic disease.

Acknowledgements

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