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Antithrombotic effect of PMC, a potent α-tocopherol analogue on platelet plug formation *in vivo*

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Summary. Platelet thrombi formation was induced by irradiation of mesenteric venules with filtered light in mice pretreated intravenously with fluorescein sodium. PMC (2, 2, 5, 7, 8-pentamethyl-6-hydroxychromane; 20 μg/g, i.v.) significantly prolonged the latent period of inducing platelet plug formation in mesenteric venules. When fluorescein sodium was given at 10 μg/kg, PMC (20 μg/g) delayed occlusion time by about 1·7-fold. Furthermore, aspirin (250 μg/g) also showed similar activity in delaying the occlusion time. On a molar basis, PMC was about 14-fold more potent than aspirin at delaying the occlusion time. PMC was also effective in reducing the mortality of ADP-induced acute pulmonary thromboembolism in mice when administered intravenously at doses of 5 and 10 μg/g. In addition, intravenous injection of PMC (5 μg/g) significantly prolonged bleeding time by about 1·6-fold compared with normal saline in severed mesenteric arteries of rats. Continuous infusion of PMC (1 μg/g/min) significantly increased the bleeding time by about 1·6-fold and the bleeding time was also significantly prolonged for up to 90 min after cessation of PMC infusion. These results suggest that PMC has an effective antiplatelet effect *in vivo* and may be a potential therapeutic agent for arterial thrombosis, but must be assessed further for toxicity.

Keywords: PMC, fluorescein sodium, occlusion time, platelet plug, arterial thrombosis.

α-Tocopherol (vitamin E) is well known for its antioxidant properties in biological membranes, where it acts to prevent the peroxidation of membrane lipids (Burton *et al*, 1986). It is also known to effectively inhibit the activation of cytokine-induced nuclear factor-κB (NF-κB) (Suzuki & Packer, 1993). α-Tocopherol is also known to inhibit platelet aggregation *in vitro* (Steiner & Anastasi, 1976; Agradi *et al*, 1981), where a concentration of about 1 mmol/l inhibits platelet aggregation (Steiner & Anastasi, 1976). These findings are of scientific interest as they suggest a possible role of α-tocopherol in the modulation of platelet function; however, they are of no clinical value because a concentration of 1 mmol/l α-tocopherol cannot be achieved in human blood by α-tocopherol supplementation (Vatassery *et al*, 1983).

The mechanism of action of α-tocopherol has proved difficult to define, owing to its highly lipophilic nature. Therefore, finding a more hydrophilic analogue is important for exploring the mechanisms of α-tocopherol's antiplatelet aggregation activity. Of the α-tocopherol analogues studied, PMC (2, 2, 5, 7, 8-pentamethyl-6-hydroxychromane), in which the phytyl chain is replaced by a methyl group (Fig 1), is the most potent derivative of α-tocopherol in antioxidation (Suzuki & Packer, 1993). PMC is more hydrophilic than other α-tocopherol derivatives and has potent radical scavenging activity (Suzuki & Packer, 1993).

While researching the antiplatelet activity of PMC, it was found that PMC inhibited the aggregation of human platelets in a dose-dependent manner (Sheu *et al*, 1999a,b). PMC (5–25 μmol/l) inhibited platelet aggregation stimulated by a variety of agonists (i.e. collagen and ADP) (Sheu *et al*, 1999a). We concluded that PMC inhibition of aggregation of human platelets is mediated through inhibition of cyclooxygenase, which leads to reduced prostaglandin formation; this, in turn, is followed by a reduction of thromboxane A_2 formation and finally inhibition of $[Ca^{+2}]_i$ mobilization (Sheu *et al*, 1999a).

In the present study, we evaluated the *in vivo* antiplatelet activity of PMC in three *in vivo* models: irradiation of the mesenteric microvessels in fluorescein sodium-pretreated mice, ADP-induced acute pulmonary thrombosis in mice,

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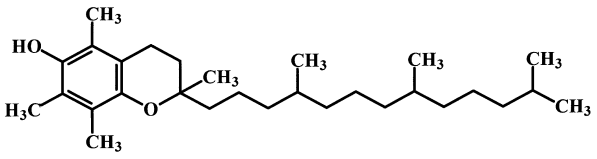
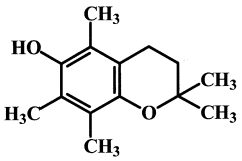


Alpha-tocopherol



PMC

Fig 1. Chemical structures of PMC and α-tocopherol.

and haemostatic bleeding time in rat mesenteric arteries. It has been reported that platelet thrombi were induced by irradiation with filtered light in the microvasculature of mice pretreated with fluorescein sodium (Sato & Ohshima, 1984, 1986), and the platelet thrombi thus obtained were localized to the irradiated region in arteriolar or venular walls (Sheu *et al*, 1994). Therefore, we used this model to evaluate the *in vivo* antiplatelet activity of PMC. Concurrently, we also compared the relative activities of PMC with other antiplatelet agents such as aspirin.

MATERIALS AND METHODS

Materials. PMC was purchased from Wako Pure Chemical Co. (Osaka, Japan). It was dissolved in dimethyl sulphoxide (DMSO) and stored at −4°C. Aspirin and fluorescein sodium were purchased from Sigma Chemical (St. Louis, MO, USA). Rats (Sprague–Dawley strain) and mice (ICR strain) were anaesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal injection.

ADP-induced acute pulmonary thrombosis in mice. Acute pulmonary thromboembolism was induced according to a previously described method (Nordoy & Chandler, 1964). Various doses of PMC and aspirin (all in a 50 μl volume) were administered by injection into the tail vein of mice (20–25 g). Four minutes later, ADP (0·7 mg/g) was injected into the contralateral vein. Mortality of mice in each group after injection was determined within 10 min.

Fluorescein sodium-induced platelet thrombus in mesenteric microvessels of mice. A method modified from previous reports (Sato & Ohshima, 1984, 1986; Sheu *et al*, 1994) was used. Mice were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). After a tracheotomy was performed, an external jugular vein was cannulated with polyethylene tubing (PE-10) for administration of the dye and drug (i.v. bolus), while additional tubing was cannulated through the femoral artery for monitoring blood pressure. A segment of the small intestine with its mesentery attached was loosely exteriorized through a midline incision on the abdominal wall and was placed onto a transparent culture dish for microscopic observation. Frequent rinsing of the mesentery with warm saline solution kept at 37 ± 0·5°C was performed to prevent the mesentery from drying out. Microvessels in the mesentery were observed under transillumination from a halogen lamp. Venules with diameters of 30–40 μm were selected for irradiation to produce a microthrombus. In the epi-illumination system, light from a 100 W mercury lamp was filtered (B-2 A, Nikon, Tokyo, Japan) with a dichromic mirror (DM 510, Nikon). This filtered light, which eliminates wavelengths below 520 nm, irradiated a microvessel (the area of irradiation was about 100 μm in diameter on the focal plane) through an objective lens (×20). Doses of fluorescein sodium used were 10 and 20 μg/kg. The injected volume of test solution or normal saline (control) was smaller than 50 μl. Five minutes after the administration of dye, irradiation by filtered light and the timer were started simultaneously, and platelet aggregation was observed on a monitor. The time lapse for inducing thrombus formation leading to cessation of blood flow was measured and the image of the microvascular bed recorded by a video recorder. The elapsed time for inducing platelet plug formation was measured repeatedly every 5 min with irradiation of venules.

Measurement of bleeding time in mesenteric arteries of rats. The bleeding time of severed mesenteric arteries was measured according to modifications of a method of Zawilska *et al* (1982). We performed these experiments on rats (150–200 g of body weight) that were maintained on food and water ad libitum before investigation. After administration of sodium pentobarbital (50 mg/kg, i.p.), the rats were shaved in preparation for surgery. The trachea was cannulated with PE-100 (polyethylene tubing, Intramedic, Becton Dickinson San Jose, CA, USA) to facilitate spontaneous breathing. Both femoral artery and vein were cannulated with PE-50 tubing to monitor blood pressure and drug administration respectively. The body temperature was maintained at 37·5°C with a heating pad and monitored with a rectal thermometer. Blood pressure was measured with a pressure transducer (Statham P23D) via a polyethylene cannula placed in the right femoral artery, and data were recorded on a polygraph (Grass model 7B).

The abdomen was opened using a midline incision and a portion of the small intestine was brought out to display the mesenteric artery. The mesentery was draped over a plastic plate, and exposed tissue was kept moist by continuous superfusion or was rinsed by means of a dropper with warmed normal saline. Experimental solutions were infused into the right femoral vein at 0·2 ml/min for a 10 min period or were given as a bolus injection. An arterial vessel (external diameter 125–200 mm) located at the junction of the small intestinal wall and the mesentery was incised at 4 min after the start of the 10 min infusion or immediately after the bolus injection. Blood was flushed away by the superfusion system. Bleeding was observed through a dissecting microscope (×100), and bleeding time was recorded from the start of incision until the bleeding was arrested by haemostatic plug formation. Each animal

was used as its own control with bleeding time determined during the infusion of both saline and the selected experimental drug. Repeated measurements were made by selecting sequential vessels of the same diameter along the small intestine mesentery. To ensure similar blood flow characteristics for each test, once a vessel had been severed and a plug had formed, it was not used for additional determination of bleeding time. Five rats were evaluated with a normal saline infusion (0·2 ml/min for 10 min) to ensure that repeated measurements did not influence the subsequent bleeding time response. In other experiments, immediately before and at the end of the infusion, blood (1 ml) was collected from the femoral artery of rats and mixed with heparin (0·2 U/ml) for determination of platelet counts and basic haematological parameters (i.e. red blood cell, white blood cell, platelet, haematocrit and haemoglobin) with an automatic cell counter (Coulter, A^c T).

Statistical analysis. Each experiment was repeated several times as indicated (n) using different rats and mice, and a mean and standard error mean (SEM) were thus obtained. Mean blood pressure was expressed as [(systolic pressure–diastolic pressure)/3 + diastolic pressure]. Data were assessed by analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman–Keuls method. A P value $< 0{\cdot}05$ was considered statistically significant.

RESULTS

Changes of blood pressure with various doses of fluorescein sodium in anaesthetized mice

In anaesthetized mice, changes of blood pressure during the intravenous injection of fluorescein sodium alone or in combination with PMC and aspirin were recorded. The mean arterial pressure (MAP) for the control mice (normal saline) was 92 ± 7 mmHg. The base-line blood pressure was not significantly changed by pretreatment of fluorescein sodium (10 and 20 μg/kg) or the combination of fluorescein sodium (20 μg/kg) with PMC (40 μg/g) and aspirin (250 μg/g) within 2 h (data not shown). Therefore, in subsequent experiments, we used appropriate doses of fluorescein sodium (i.e. 10 and 20 μg/kg) to investigate the effect of PMC in this model.

Effect of PMC on thrombosis formation in the microvessels of fluorescein sodium-pretreated mice

The latent period of inducing platelet plug formation was shortened as the administered dose of fluorescein sodium increased. When fluorescein sodium was given at 10 μg/kg and 20 μg/kg, the occlusion time required was 115 ± 24 s and 62 ± 9 s respectively (Fig 2). PMC is a potent inhibitor of platelet aggregation (Sheu *et al*, 1999a,b). We examined its effect on the formation of platelet-rich thrombi in this model. When PMC was administered at 20 μg/g in mice pretreated with 10 μg/kg of fluorescein sodium (Fig 2), the occlusion time was significantly delayed; however, even increasing the doses of PMC (40 μg/g) did not further delay the occlusion time (Fig 2). In contrast, PMC (20 μg/g) did not significantly delay the occlusion time until it was administered at 40 μg/g in fluorescein sodium (20 μg/g)-induced platelet plug formation (Fig 2). PMC also exhibited an antithrombotic effect in arterioles (data not shown). However, arterioles sometimes showed slight vasoconstriction while fluorescein sodium was irradiated (Sato & Ohshima, 1984), thus venules were chosen for induction of platelet plug formation in this study. In addition, the solvent control of PMC (0·5% DMSO) did not affect occlusion time in this study (data not shown).

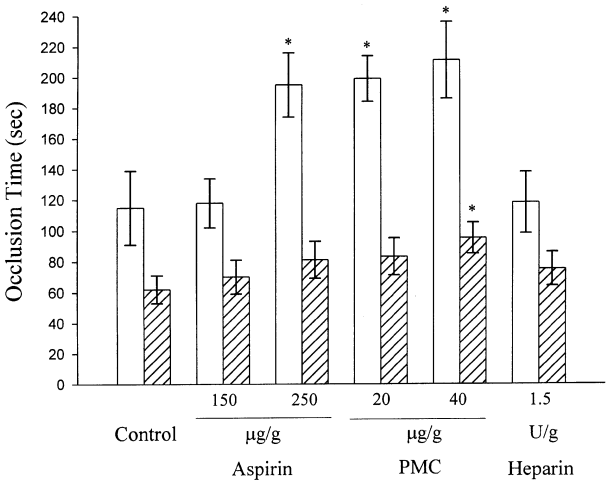


Fig 2. Effect of PMC (20 and 40 μg/g) and aspirin (150 and 250 μg/g) on occlusion time for inducing thrombus formation upon light irradiation of mesenteric venules of mice pretreated with fluorescein sodium (10 μg/kg, open bars; 20 μg/kg, hatched bars). Data are presented as occlusion time (s) of platelet plug formation, means ± SEM ($n = 5$). *$P < 0{\cdot}05$ compared with the individual control group.

Aspirin also exhibited similar inhibitory activity in this experiment. When aspirin was administered at 150 μg/g, occlusion time was not significantly prolonged until 250 μg/g was administered in fluorescein sodium (10μg/kg)-inducing platelet plug formation (Fig 2). However, aspirin (150 and 250 μg/g) did not significantly delay occlusion time when 20 μg/kg of dye was used for pretreatment (Fig 2). When mice were pretreated with 10 μg/kg of fluorescein sodium, PMC was about 14-fold more potent than aspirin at prolonging the occlusion time in microvessels on a molar basis. On the other hand, heparin (1·5 U/g) showed no significant effect on occlusion time (Fig 2).

Effect of PMC on ADP-induced acute pulmonary thrombosis in mice

In this study, we further demonstrated the effect of PMC in preventing fatal acute pulmonary embolism in mice. The results summarized in Table I show that PMC significantly lowered mortality and reversed platelet numbers in blood in mice challenged with ADP (0·7 mg/g). PMC (5 and 10 μg/g) at both concentrations reduced mortality from 75% to 30%. Furthermore, aspirin (20 and 50 μg/g) also reduced mortality and reversed platelet numbers in this experiment

Table I. Effect of aspirin and PMC on mortality and platelet count of acute pulmonary thrombosis caused by intravenous injection of ADP in experimental mice.

	Number of death	Total number	Mortality (%)	Platelet count (10^9/l)
Control	0	6	0	241 ± 26 (6)
ADP (0·7 mg/g)	15	20	75	157 ± 20* (20)
+ Aspirin (μg/g)				
20	7	20	23·3	181 ± 27 (20)
50	7	20	23·3	175 ± 22 (20)
+ PMC (μg/g)				
5	6	20	30	186 ± 28 (20)
10	6	20	30	181 ± 23 (20)

*$P < 0·05$ compared with control group (normal saline). Platelet count is presented as mean ± SEM (n).

(Table I). These results indicate that PMC effectively prevents ADP-induced acute pulmonary thrombosis in mice.

Effect of PMC on bleeding time in mesenteric arteries in rats

The reproducibility of bleeding time was verified in control experiments. In control rats, normal saline was injected into the circulation and bleeding time as measured in mesenteric arteries was about 4·1 ± 0·6 min. Table II shows that PMC administered as bolus to rats markedly increased bleeding time in a dose-dependent manner. At 5 and 10 μg/g, PMC dose-dependently increased bleeding time by about 1·6-fold and 1·7-fold compared with the control (4·1 ± 0·6 *vs* 6·5 ± 0·4 and 7·1 ± 0·8 min) respectively. Bleeding time showed no further increase even at a higher dose of PMC (20 μg/g) (data not shown). The solvent control (0·5% DMSO) of PMC did not affect bleeding time in this study (data not shown). A comparison of the effect of PMC with aspirin on bleeding time is shown in Table II. This result indicated that PMC was over 10-fold more potent than aspirin at prolonging the bleeding time in mesenteric arteries of rats on a molar basis. The effect of the continuous infusion of PMC (1 μg/g/min) on the prolongation of bleeding time is shown in Fig 3. This result demonstrated that the bleeding time of the severed mesenteric arteries was prolonged by about 1·6-fold (6·9 ± 0·7 *vs* 4·2 ± 0·5 min)

Table II. Comparison of the effects of aspirin and PMC on bleeding time in mesenteric arteries of rats.

	Dose (μg/g)	Bleeding time (min)	n
Control	–	4·1 ± 0·6	6
Aspirin	20	5·1 ± 0·6	6
	50	6·2 ± 0·5*	6
PMC	5	6·5 ± 0·4*	6
	10	7·1 ± 0·8*	6

*$P < 0·05$ compared with control group (normal saline). Data are presented as mean ± SEM (n).

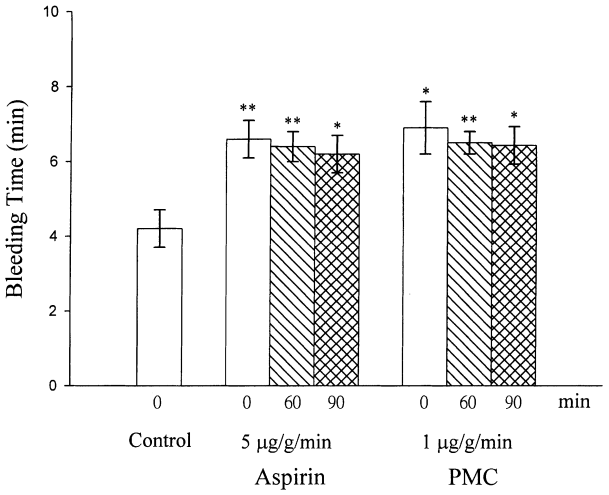


Fig 3. Effect of continuous infusion of PMC (1 μg/g/min) and aspirin (5 μg/g/min) for 10 min on bleeding time of rat mesenteric arteries. Bleeding time was immediately measured after termination of 10 min normal saline, PMC, and aspirin infusion (open bars); bleeding time was measured 60 min (hatched bars) and 90 min (cross-hatched bars) after termination of 10 min drugs infusion respectively. Data are presented as means ± SEM ($n = 5$). *$P < 0·05$ and **$P < 0·01$ compared with control group.

after termination of 10 min infusion of PMC. The bleeding time was also significantly prolonged by about 1·5-fold (6·4 ± 0·5 *vs* 4·2 ± 0·5 min) for up to 90 min after termination of PMC infusion. On the other hand, bleeding time was also prolonged by about 1·5-fold (6·2 ± 0·5 *vs* 4·2 ± 0·5 min) for up to 90 min after termination of aspirin infusion (Fig 3). Therefore, the prolongation of bleeding time by aspirin was similar to PMC in severed mesenteric arteries of rats (Fig 3).

Effect of PMC on blood pressure and haematological parameters in rats

The blood pressure response to intravenous injection of PMC in anaesthetized rats is depicted in Table III. The baseline blood pressure was not significantly altered during the infusion of aspirin (5 μg/g/min) (91·6 ± 19·5 mmHg) or PMC (1 μg/g/min) (85·6 ± 8·6 mmHg) (Table III). There were also no significant differences in the haematological parameters, including haemoglobin, haematocrit, red blood cell, platelet and white blood cell counts, between the control (normal saline treatment) and post-aspirin or post-PMC values.

DISCUSSION

Intravascular thrombosis is one of the generators of a wide variety of cardiovascular diseases. Initiation of intraluminal thrombosis is believed to involve platelet adherence and aggregation. Thus, platelet aggregation may play a crucial role in atherothrombotic processes. Indeed, antiplatelet agents (e.g. ticlopidine and aspirin) have been shown to reduce the incidence of stroke in high-risk patients (Gent *et al*, 1989; Hass *et al*, 1989). For this

Table III. Blood pressure and haematological parameters after 10 min i.v. infusion of normal saline (control) or aspirin (5 μg/g/min), and PMC (1 μg/g/min) in rats.

Parameter	Control	Aspirin	PMC
Mean blood pressure (mmHg)	98·1 ± 12·3 (4)	91·6 ± 19·5 (4)	85·6 ± 8·6 (5)
White blood cell (10^9/l)	8·4 ± 0·9 (4)	8·2 ± 0·6 (4)	8·0 ± 0·7 (5)
Red blood cell (10^{12}/l)	7·7 ± 0·5 (4)	8·0 ± 0·7 (4)	7·6 ± 0·6 (5)
Platelet (10^9/l)	1050 ± 120 (4)	900 ± 60 (4)	930 ± 80 (5)
Haemoglobin (g/dl)	14·8 ± 1·2 (4)	12·5 ± 1·0 (4)	13·5 ± 9·6 (5)
Haematocrit (%)	48·3 ± 7·6 (4)	42·3 ± 4·7 (4)	46·8 ± 5·8 (5)

Data are presented as mean ± SEM (*n*).

reason, we have started searching for new antiplatelet agents. We have previously shown that PMC (5–25 μmol/l) inhibits platelet aggregation stimulated by a variety of agonists, i.e. collagen and ADP, through inhibition of cyclooxygenase, which leads to reduced prostaglandin formation and finally inhibition of $[Ca^{2+}]_i$ mobilization (Sheu *et al*, 1999a).

It has been reported from electron microscopy studies that irradiation of vascular endothelium by means of a laser leads to the formation of platelet thrombi to the damaged vessel wall (Hovig *et al*, 1974; Sheu *et al*, 1994). The degree of endothelial cell damage after irradiation seems to depend on the intensity of irradiation and the amount of fluorescein dye given. Hovig *et al* (1974) have reported that endothelial cell injury induced platelet aggregation and adhesion to the vessel wall in a laser model.

In this study, PMC exhibited a marked antithrombotic effect *in vivo*. It prolonged the occlusion time of thrombus formation induced by irradiation of fluorescein sodium in venules or arterioles. As the light beam covered the entire microscopic field, a simultaneous observation of arterioles and venules was made. Our data revealed that platelet aggregation usually occurs first in the venules rather than in the arterioles. This may be explained by a higher flow velocity in arterioles, resulting in delayed adhesion of platelets to arteriolar endothelial cells (Callahan *et al*, 1960). In this system, the occlusion time is related to the blood flow rate, size of microvessel diameter and dose of fluorescein dye (Sato & Ohshima, 1986). In this study, PMC caused significant prolongation of occlusion time in mice pretreated with fluorescein sodium, mainly through its antiplatelet activity. Furthermore, on a molar basis, PMC exerted more potent antithrombotic activity than the aspirin group in this experiment.

Nordoy & Chandler (1964) have demonstrated that platelet aggregation was intimately involved in experimental acute pulmonary thrombosis. Latour *et al* (1984) also demonstrated that ADP initiated platelet-rich thrombi in the pulmonary vasculature. Furthermore, rabbits given ADP solution developed electrocardiographic changes and histological lesions in the lungs (Mahesree *et al*, 1975). The present study demonstrated that PMC was effective in preventing ADP-induced fatal thromboembolism. Furthermore, it has been shown that other antiplatelet drugs such as aspirin were also effective in preventing platelet accumulation, whereas heparin (1·5 U/g) was unable to delay the occlusion time and reduce the mortality induced by ADP injection (data not shown). These data are consistent with the fact that platelet aggregation rather than fibrin formation is the crucial event causing thromboembolism in both animal models. Furthermore, prolongation of bleeding time was seen in all experimental rats receiving PMC. It has been reported that the platelet glycoprotein IIb/IIIa antagonists in animal studies prolong the bleeding time (Cook *et al*, 1994).

In this study, the mesenteric venules of mice were continuously irradiated by filtered light during the experimental period; nevertheless, a single incision of the mesenteric arteries of rats was made for bleeding-time measurement. In addition, the animal species used were different in haemostatic (rat) and thrombotic platelet plug (mouse) studies. These reasons may partly explain why PMC prolonged the occlusion time of thrombus formation in mice at a dose higher than that used for haemostatic bleeding time in rats. However, other mechanisms may be involved in these two experimental models and they remain to be elucidated. The mechanisms of PMC and aspirin inhibition of thrombotic and haemostatic platelet plug formation in experimental animals are probably through inhibition of platelet aggregation.

In conclusion, platelet aggregation plays a pathophysiological role in a variety of thromboembolic disorders. Therefore, prevention of platelet aggregation by drugs should provide effective prophylactic and/or therapeutic means of treating such diseases. In this study, our work suggests that PMC has promising antithrombotic activity and it may be a potential therapeutic agent for the treatment of arterial thromboembolism, but must to be assessed further for toxicity.

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