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Anti-platelet Activity of Tissue-cultured Mountain Ginseng Adventitious Roots in Human Whole Blood

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Abstract Present study investigated the effects of the 70% ethanol extracts of tissue-cultured mountain ginseng (TCMG), Korean red ginseng (KRG), and *Panax ginseng* (PG) on agonist-induced platelet aggregation and activation in human whole blood. The IC_{50} values for TCMG, KRG, and PG were 1.159, 3.695, and 4.978 mg/mL for collagen-induced aggregation, 0.820, 2.030, and 4.743 mg/mL for arachidonic acid-induced aggregation, and 1.070, 2.617, and 2.954 mg/mL for ADP-induced aggregation, respectively. Also, this study assessed the effects of the most active extract, TCMG, on markers of platelet activation by determining receptor expression on platelet membranes in healthy subjects, including expression of GPIIb/IIIa-like (PAC-1) and P-selectin (CD62), by flow cytometry. A significant decrease in PAC-1 expression (p=0.018) was observed in the presence of TCMG. These results show that TCMG has potent anti-platelet activity.

Keywords: tissue-cultured mountain ginseng, Korean red ginseng, *Panax ginseng*, platelet aggregation, GPIIb/IIIa-like expression

Introduction

Ginseng Radix, the root of *Panax ginseng* C.A. Meyer, has been used in eastern Asia for 2,000 years as a tonic and restorative, promoting health, and longevity. Indeed, mountain *P. ginseng* C.A. Meyer in Korea is widely known as a miraculous medicine. This herb grows wild in cool, shady forests from Korea and north eastern China to far eastern Siberia (1-3). It is traditionally accepted in Korea that mountain ginseng is more active than cultivated ginseng (4). Nowadays, mountain ginseng has become extremely scarce and the ginseng supply depends almost exclusively on field cultivation, which is a time-consuming and labor-intensive process. The demand for the plant in the international market makes bioreactor technology a useful tool for large-scale production of root biomass. Therefore, suspension culture of ginseng roots in bioreactors is viewed as the primary alternative method for large-scale production, and a protocol has been developed for culturing *P. ginseng in vitro* (5-7). Recently, tissue culture techniques for mountain *P. ginseng* were also developed, which has made its mass production possible. The Korea Food & Drug Administration (KFDA) has approved tissue-cultured mountain ginseng (TCMG) adventitious root as food material in health supplements produced by 4 companies in Korea (8,9). The TCMG used in this study was produced from 100-year-old wild mountain *P. ginseng* root calluses derived from adventitious roots in commercial scale bioreactors, using the methods described by Sivakumar *et al.* (10,11). TCMG was produced to contain higher concentrations of bioactive ginsenosides or biophenols than cultivated ginseng; these compounds are believed to be beneficial to human health (12).

Recent research findings prove that TCMG helps prevent or treat spermatogenic disorders (13,14), erectile dysfunction (15), symptoms of hyperlipidemia (16), learning and memory impairment (17), and has anti-fibrotic and antioxidant activity (18,19). In addition, it stimulates immune cells and inhibits cancer cell growth (20).

Based on the reported biological effects, we hypothesized that TCMG would influence physiological hemostatic and pathological thrombotic processes. Platelets play a critical role in normal hemostatic processes and in pathological conditions, such as thrombosis, vascular remodeling, inflammation, atherosclerosis, acute coronary syndromes, and wound repair (21-23). When platelets come into contact with an abnormal blood vessel or are exposed to extravascular tissue after injury to a blood vessel, they undergo a variety of changes, collectively known as platelet activation. Activation stimuli include tissue compounds such as collagen, shear force, epinephrine, and locally released mediators, such as adenosine-5'-diphosphate (ADP), serotonin, and thromboxane A2 (24). Platelet activation and aggregation are controlled by the activity of platelet membrane receptors such as glycoprotein IIb/IIIa and P-selectin. Specific receptors for many agonists on the platelet surface, such as those for thrombin, ADP, collagen, arachidonic acid (AA), and epinephrine, have the ability to induce platelet aggregation and activation. Many of the receptor-agonist complexes interact with coupling protein in the platelet membrane to trigger a biochemical reaction. These biochemical reactions, resulting from the stimulatory agonists, lead to platelet activation and aggregation (22,

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25,26). The modulation of platelet activity by specific pharmacological agents has proven to be a successful strategy for preventing thrombosis (27). Platelets have multiple activation signaling mechanisms. Therefore, agents with anti-platelet and anti-thrombotic effects may have wide therapeutic potential for circulatory diseases (23,28,29).

This study investigated the effect of 70% ethanol extracts of TCMG on agonist-induced platelet aggregation and activation in human whole blood. Based on the results, TCMG had the most effective anti-platelet activity compared with Korean red ginseng (KRG) or P. ginseng (PG) commercial products.

Materials and Methods

Reagents and materials Catechin was purchased from Wako Co. (Osaka, Japan). Adenosine-5'-diphosphate (ADP), arachidonic acid (AA), and collagen were obtained from Chrono-Log Co. (Havertown, PA, USA). Fluorescent-labeled monoclonal antibodies, including CyChrom-labeled CD41a, fluorescein isothiocyanate (FITC)-labeled PAC-1, and phycoerythrin (PE)-labeled CD62P were purchased from Becton Dickinson (San Diego, CA, USA). The root of tissue-cultured mountain ginseng (TCMG, 100 years) was supplied by CBN Biotech in Ochang, Chungbuk, Korea, in 2007. Korean red ginseng (KRG, 4 years) and *P. ginseng* (PG, 4 years) were purchased from a commercial supplier in Daejeon, Korea, in 2006 and were identified by the Herbal Quality Control Team and deposited at the herbarium of the Department of Herbal Resources Research, Korea Institute of Oriental Medicine (KIOM).

Preparation of extract Dried roots of TCMG, KRG, and PG were extracted 2 times with 70% ethanol for 72 hr at room temperature and then concentrated under vacuum using a rotary evaporator. All extracts were freeze-dried to yield extract powders. The yields of TCMG, KRG, and PG were 32.54, 34.28, and 25.57%, respectively.

Measurement of crude saponin content The method for measuring crude saponin contents has been described in detail (8). Briefly, TCMG, KRG, and PG extracts (weight of sample, *S*, g) were each dissolved in 50 mL of distilled water and washed twice in a separating funnel with 50 mL of diethyl ether. The aqueous layer was extracted 3 times with 50 mL of water-saturated *n*-butanol. The resulting butanolic solution was washed twice with 20 mL of distilled water. The remaining butanol solution was transferred to a round bottom flask (*W1*, g) for evaporation under vacuum at 55°C using rotary evaporator. The flask with the evaporated residue was dried under vacuum at −40°C to constant weight (*W2*, g). The crude saponin contents of the sample was calculated according to the formula:

$$\text{Contents } (\%)=[(W2-W1)/S]\times 100$$

Volunteers A total of 18 healthy volunteers (age range: 20-45 years; 7 females, 11 males) entered the study. All subjects had no history of bleeding disorders or cardiovascular disease, and refrained from any pharmacological therapy for at least 2 weeks before enrollment. None of the subjects smoked or exhibited hypertension, diabetes, or abnormal hematocrit. All subjects gave written informed consent before participation. The study was approved by the Institutional Review Board, The Oriental Hospital of Daejeon University, Korea.

Platelet aggregation assay Platelet aggregation studies were performed with a 500VS Chrono-Log aggregometer (Chrono Log) by the impedance method (30,31). Blood was drawn intravenously from healthy volunteers into vacuum tubes (Becton Dickinson) with 3.8% sodium citrate; the blood was quickly diluted with 1 volume of 0.9% saline and used directly. After determining the amounts of the agonists, collagen (2 μg/mL), AA (0.5 mM), and ADP (15 μM), necessary to cause a suitable impedance (Ω) full-scale deflection, 50 μL aliquots of different doses of samples (TCMG, KRG, or PG extract) were added to 900 μL of diluted human whole blood in a polystyrene cuvette (Chrono-Log) containing magnetic stir bar and were incubated 5 min prior to addition of the agonist (collagen, AA, or ADP) and stirred at 200×g. The deflection observed at 8 min after addition of agonists was used for the calculation. Aggregation was quantified as the change in impedance. Percent inhibition of platelet aggregation was calculated as [(control response−inhibited response)/control response]×100. Several concentrations of TCMG, KRG, and PG extract were used so that a half-maximal inhibitory dose (IC_{50}) value could be determined from a plot of concentration vs. percentage inhibition. Catechin was used as a reference inhibitor for all batches of human whole blood.

Platelet membrane-receptor expression For both platelet preparation and staining, we referred to the modified Becton Dickinson Procedure for flow cytometric analysis for platelets (32). The surface expression of platelet receptor was determined by flow cytometry using the anti-CD41a (GPIIb/IIIa), PAC-1, or P-selectin monoclonal antibodies. PAC-1 binding and P-selectin (CD62) expressions were used as markers of activated platelets, as previously described (33). Within 20 min of blood collection from healthy volunteers, 90 μL of whole blood was incubated for 5 min at room temperature in the presence or absence of 10 μL of diluted TCMG extract (final concentration: 5 mg/mL). The platelet activator ADP (final concentration: 10 μM) was added to the whole blood mixtures and then incubated for 2 min. Ten μL aliquots from whole blood mixtures with ADP and TCMG extract were transferred to tubes containing 20 μL of anti-CD41a (GPIIb/IIIa) and PAC-1 or of anti-CD41a and P-selectin monoclonal antibodies. The samples were incubated for 20 min at room temperature in the dark, fixed with 500 μL of 2% buffered paraformaldehyde to inhibit further activation, and then analyzed. Platelets were identified on the basis of CD41a expression. Activated platelets were defined as CD41a-positive events binding PAC-1 or expressing P-selectin. Samples were analyzed on a FACsCalibur (Becton Dickinson) flow cytometry set-up to measure fluorescent light scattering. A total of 5,000 events were measured for each sample. Acquisition and data processing were performed using CellQuest Prosoftware (Becton Dickinson).

Table 1. Comparison of the crude saponin contents from TCMG, KRG, and PG extracts[1)]

Extract	TCMG	KRG	PG
Crude saponin (%)	13.4	9.4	8.6

[1)]TCMG, Tissue-cultured mountain ginseng; KRG, Korean red ginseng; PG, *P. ginseng*.

Statistical analysis IC_{50} values were calculated using the Regression Wizard from Sigma Plot. The results are presented as mean±standard deviation (SD). Groups were compared by independent-samples *t*-test using SPSS 11.0 for Windows (SPSS, Chicago, IL, USA). Values of $p<0.05$ were considered statistically significant.

Results and Discussion

Influence on platelet aggregation in human whole blood The roots of *P. ginseng* C.A. Meyer have long been used to treat various diseases including diabetes mellitus, thrombosis, hyperlipidemia, and arteriosclerosis. In the literature, interest in the pharmacological action of *P. ginseng* has been focused on the principal active components of ginseng, i.e., triterpenoid saponins known as ginsenosides (34-36). Ginsenosides are attributed with cardio-protective, immuno-stimulatory, anti-fatigue, and hepato-protective physiological and pharmacological effects (37). According to some studies, ginsenosides isolated from *P. ginseng* are the main components affecting the hemostatic and thrombotic processes (34,35). However, some reports describe the anti-platelet activity of ginsenosides (38-40). The anti-platelet effects of the ginsenosides are diverse in the reports from different research groups. From these reports, the potencies of ginsenosides are very weak and those actions can not explain the anti-platelet activity of ginseng. Therefore, in this study, we confirmed the different effects of diverse commercial ginseng products, such as TCMG, KRG, and PG, on anti-platelet activity in human whole blood aggregation.

First, to standardize the extracts, this study measured the crude saponin contents in 70% ethanol extracts from TCMG, KRG, and PG commercial products. The total crude saponin contents of TCMG, KRG, and PG extracts were 13.4, 9.4, and 8.6%, respectively. The crude saponin content of TCMG was 1.56-times higher than PG (Table 1). In this study, control aggregation in saline was systematically induced by collagen or ADP or AA at the beginning of each experiment to verify the physiological status of the platelets. Figure 1, 2, and 3 show the inhibitory effects of TCMG, KRG, and PG on human platelet aggregation in human whole blood induced by ADP, AA, and collagen. Table 2 shows the half-maximal inhibitory dose (IC_{50}) of each extract for ADP-, AA-, and collagen-induced platelet aggregation. The IC_{50} values for TGMG, KRG, and PG calculated from the concentration inhibition curves in Fig. 4, 5, and 6 were 1.070, 2.617, and 2.954 mg/mL for ADP-induced aggregation, 0.820, 2.030, and 4.743 mg/mL for AA-induced aggregation, and 1.159, 3.695, and 4.978 mg/mL for collagen-induced aggregation, respectively. TCMG extract showed higher anti-platelet activity compared to KRG and PG, as listed in Table 2.

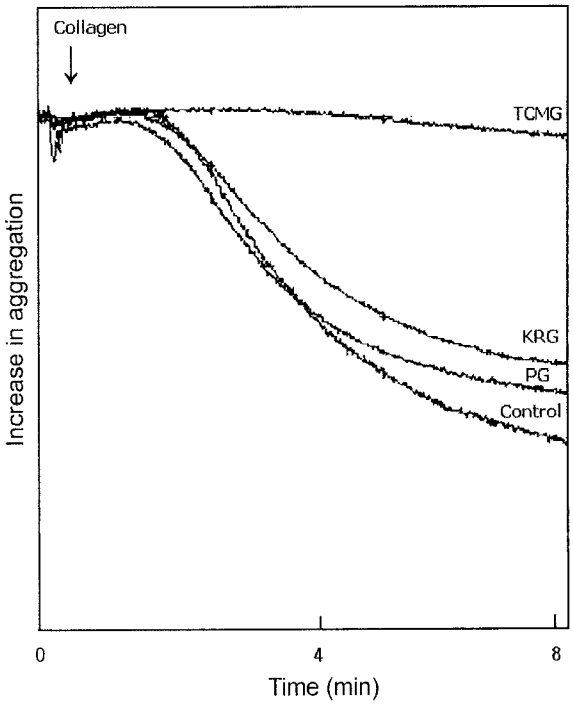

Fig. 1. Typical curves of collagen-induced platelet aggregation obtained with saline (control) or samples of tissue-cultured mountain ginseng (TCMG), Korean red ginseng (KRG), and *P. ginseng* (PG) at final concentration 2.5 mg/mL.

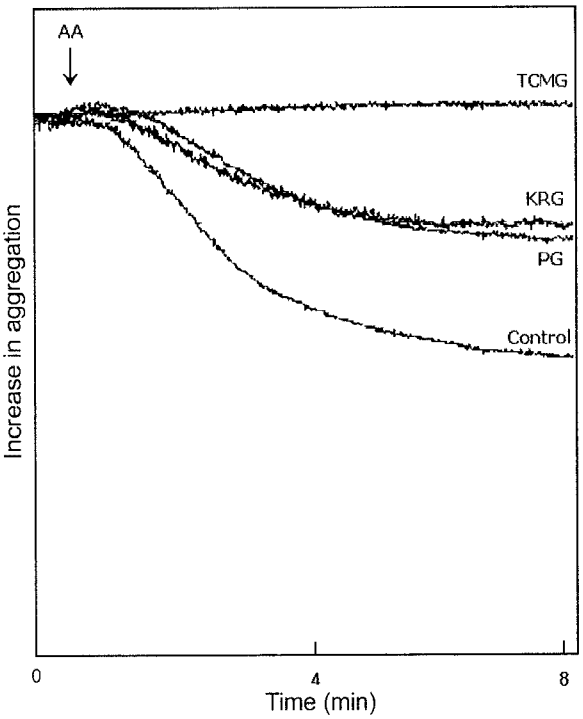

Fig. 2. Typical curves of AA-induced platelet aggregation obtained with saline (control) or samples of tissue-cultured mountain ginseng (TCMG), Korean red ginseng (KRG), and *P. ginseng* (PG) at final concentration 2.5 mg/mL.

TCMG displayed 4.30-, 5.78- and 2.76-fold stronger inhibition of platelet aggregation induced by collagen, AA, and ADP, respectively, than PG. These results indicate that

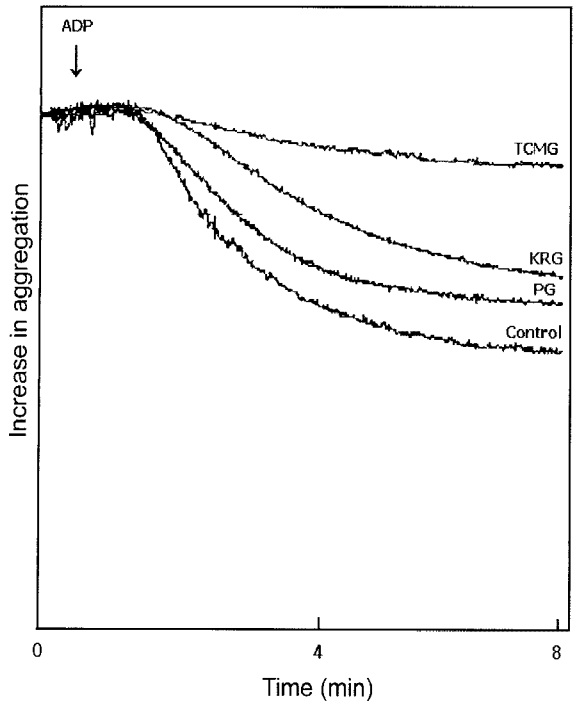

Fig. 3. Typical curves of ADP-induced platelet aggregation obtained with saline (control) or samples of tissue-cultured mountain ginseng (TCMG), Korean red ginseng (KRG), and *P. ginseng* (PG) at final concentration 2.5 mg/mL.

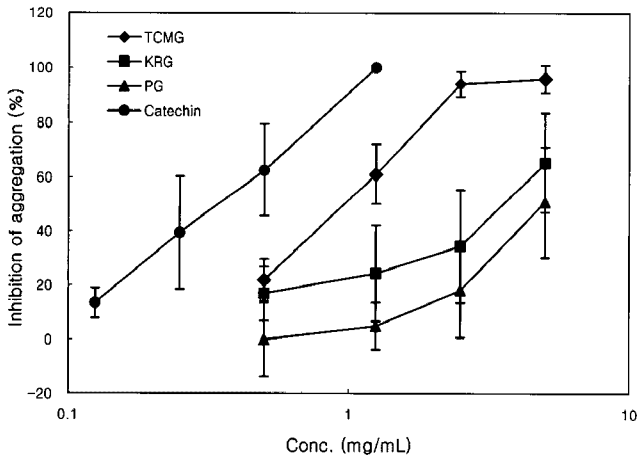

Fig. 4. Inhibitory effects of tissue-cultured mountain ginseng (TCMG), Korean red ginseng (KRG), and *P. ginseng* (PG) extracts on collagen-induced platelet aggregation.

TCMG was the most effective extract in inhibition of platelet aggregation induced by collagen, ADP, or AA. In contrast, KRG and PG displayed only mild anti-platelet effects in agonist-induced aggregation.

Influence on platelet-membrane receptor activation

Platelet function in whole blood can be comprehensively evaluated by flow cytometry. This method has attracted much attention recently, with particular interest in the flow cytometric assessment of platelet activation (41). The present study verified the inhibition of platelet activation by TCMG by measuring the binding of PAC-1 and P-selectin monoclonal antibodies by flow cytometry. Laboratory

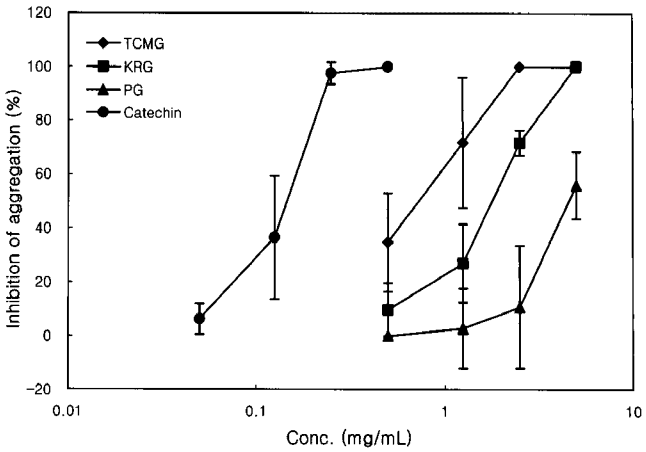

Fig. 5. Inhibitory effects of tissue-cultured mountain ginseng (TCMG), Korean red ginseng (KRG), and *P. ginseng* (PG) extracts on AA-induced platelet aggregation.

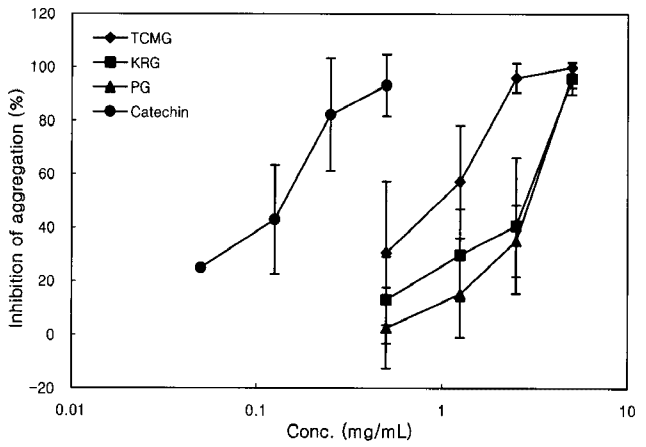

Fig. 6. Inhibitory effects of tissue-cultured mountain ginseng (TCMG), Korean red ginseng (KRG), and *P. ginseng* (PG) extracts on ADP-induced platelet aggregation.

Table 2. IC_{50} values for 70% ethanol extracts of TCMG, KRG, and PG on human platelet aggregation induced by various agonists[1)]

Agonist	IC_{50} value (mg/mL)			
	Catechin	TCMG	KRG	PG
Collagen (2 μg/mL)	0.384	1.159	3.695	4.978
AA (0.5 mM)	0.149	0.820	2.030	4.743
ADP (15 μM)	0.141	1.070	2.617	2.954

[1)]IC_{50}, Half-maximal inhibitory dose; TCMG, tissue-cultured mountain ginseng; KRG, Korean red ginseng; PG, *P. ginseng*.

markers of platelet activation include activation-dependent changes in the glycoprotein GPIIb/IIIa complex, exposure of granular membrane proteins, binding of secreted platelet proteins, and development of a pro-coagulant surface. The two most widely studied types of activation-dependent monoclonal antibody are those directed against conformational changes in the GPIIb/IIIa complex, such as PAC-1, and those directed against granular membrane proteins, such as P-selectin (42). To assess the inhibitory efficacy of TCMG extracts on 2 markers of platelet activation, we performed

Table 3. Effects of TCMG, KRG, and PG extract on the expression of platelet membrane receptors after stimulation with 10 μM ADP[1)]

Platelet receptor	Control	TCMG (5 mg/mL)	KRG (5 mg/mL)	PG (5 mg/mL)
	% gated			
PAC-1	98.95±0.54[2)]	78.61±0.34 (p=0.018)	86.51±1.57	84.58±6.24
P-Selectin	81.96±9.73	71.28±6.33	78.41±1.33	76.80±5.28

[1)]TCMG, Tissue-cultured mountain ginseng; KRG, Korean red ginseng; PG, *P. ginseng*; ADP, adenosine-5'-diphosphate.
[2)]Mean±SD (n=3).

a platelet activation assay by measuring receptor expression on the platelet membrane in healthy subjects by flow cytometry. All data are represented as % gated of the platelets staining positive for a particular antibody. In the present study, TCMG had significant effects on the conformational change of GPIIb/IIIa (PAC-1) and low effects on the activation of P-selectin (Table 3). The results presented in this study reveal that TCMG can potently inhibit ADP-induced platelet aggregation in human whole blood according to a membrane receptor assay system. Therefore, we suggest that TCMG extract inhibits the platelet activation pathway in ADP-, AA-, and collagen-induced aggregation and suppresses platelet membrane receptor expression.

Platelets induce thrombosis at damaged blood vessels, since thrombus formation occurs through the activation and aggregation of platelets. Thus, platelet aggregation is the major pathogenic mechanism in thrombosis. Considering the importance of thrombosis in cardiovascular disorders, natural products, such as *Ginkgo biloba*, garlic, etc, have been successfully screened for better anti-platelet drugs (43). In conclusion, the results of the present study provide pharmacological evidence supporting the traditional use of *P. ginseng* and documenting that TCMG exerts anti-platelet activity. Therefore, TCMG extract could be a suitable anti-platelet agent. TCMG may help to control conformational changes in receptor activation and to inhibit platelet aggregation. Further studies are needed to identify the active components in TCMG related to the anti-platelet activity.

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