Spinach Saponin-Enriched Fraction Inhibits Platelet Aggregation in cAMP- and cGMP-Dependent Manner by Decreasing TXA₂ Production and Blood Coagulation

Hyun-Jeong Cho^{1,a}, Sun-A Choi^{2,a,b}, Chun-Gyu Kim³, Tae-Sung Jung⁴, Jeong-Hwa Hong⁵, Man-Hee Rhee⁶, Hye-Jin Park⁷ and Hwa-Jin Park^{2,*}

¹Department of Biomedical Laboratory Science, College of Medical Science, Konyang University, Daejeon 302-718,

²Department of Biomedical Laboratory Science, College of Biomedical Science, Inje University,

³Department of Pharmaceutical Engineering, College of Engineering, Inje University, Gimhae 621-749,

⁴Department of Pharmacy, Kungsung University, Busan 608-736,

⁵School of Food and Life Science, College of Biomedical Science and Engineering, Inje University, Gimhae 621-749,

⁶College of Veterinary Medicine, Kyungpook National University, Daegu 702-701,

⁷Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea

Abstract

In this study, we investigated the effect of spinach saponin-enriched fraction (SSEF) on collagen (10 µg/ml)-stimulated platelet aggregation. SSEF inhibited collagen-induced platelet aggregation, and which was involved in the inhibition of thromboxane A₂ (TXA₂) production, an intracellular Ca²⁺-agonist as an aggregation-inducing autacoidal molecule. In addition, SSEF significantly increased the formation of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), intracellular Ca²⁺-antagonists as aggregation-inhibiting molecules, in collagen-stimulated platelets. These results suggest that SSEF might inhibit Ca²⁺-elevation and TXA₂ formation by increasing the production of Ca²⁺-antagonistic molecules cAMP and cGMP. These mean that SSEF is a potent inhibitor of collagen-stimulated platelet aggregation. On the other hand, prothrombin time (PT) and activated partial thromboplastin time (APTT) were potently prolonged by SSEF. These findings suggest that SSEF prolongs the internal time between the conversion of fibrinogen to fibrin. Accordingly, our data demonstrate that SSEF may be a crucial tool for a negative regulator during platelet activation and blood coagulation on thrombotic diseases.

Key Words: Spinach saponin-enriched fraction (SSEF), Platelet aggregation, Blood coagulation, Thrombotic diseases

INTRODUCTION

Spinach (*Spinacia oleracea*) is an outstanding food source of folic acid (Prinz-Langenohl *et al.*, 1999), vitamin A (Edwards *et al.*, 2001), and so on. It is known that aqueous extracts from spinach have the effects of anti-oxidation (Bergman *et al.*, 2001; Lomnitski *et al.*, 2003) and anti-carcinogenesis (Sani *et al.*, 2004). However, it is unknown whether spinach has an inhibitory effect on platelet aggregation-induced thrombosis.

Platelet aggregation is essential to the formation of a hemostatic plug when normal blood vessels are injured. However, the interactions between platelets and collagen can also cause circulatory disorders, such as thrombosis, atheroscle-

rosis, and myocardial infarction (Schwartz *et al.*, 1990). Inhibition of the platelet-collagen interaction might be a promising approach to the prevention of thrombosis. An important role in the mechanism by which collagen induces platelet aggregation is played by thromboxane A₂ (TXA₂) formation (Cattaneo *et al.*, 1991), which also contributes to an increase in cytosolic free Ca²⁺ level ([Ca²⁺]_i) in collagen-activated platelets. An increase in [Ca²⁺]_i activates both the Ca²⁺/calmodulin-dependent phosphorylation of myosin light chain (MLC) and the diacylglycerol (DG)-dependent phosphorylation of cytosolic pleckstrin to induce platelet aggregation (Nishikawa *et al.*, 1980). On the other hand, both cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), as

www.biomolther.org

Open Access DOI: 10.4062/biomolther.2011.19.2.218

pISSN: 1976-9148 eISSN: 2005-4483

Copyright © 2011 The Korean Society of Applied Pharmacology

Received Sep 27, 2010 Revised Dec 6, 2010 Accepted Dec 29, 2010

*Corresponding Author

E-mail: mlsjpark@inje.ac.kr

Tel: +82-55-320-3538, Fax: +82-55-334-3426

^aThese two authors equally contributed to this work.

^bPresent address: Pusan National University Hospital, 305 Gudeok-Ro, Seo-Gu, Busan, 602-739, Republic of Korea

anti-platelet regulators, decrease the $[Ca^{2+}]_i$, an essential factor for platelet aggregation.

Blood coagulation systems, an another common region in generation of thrombosis, have intrinsic blood coagulation system (activated partial thromboplastin time, APTT) from the injured blood vessel, and extrinsic blood coagulation system (prothrombin time, PT) derived from tissue. The prolongation of blood coagulation time such as PT and APTT means the prolonged time of forming fibrin clot and is the mark of inhibited blood coagulation. Extended APTT and PT hint that thrombin is produced with delay (Davie and Ratnoff, 1964; Furie and Furie, 1988; Akoum *et al.*, 1990; Sano *et al.*, 2003).

As described above, there is no report showing its anti-thrombotic activity with spinach saponin. In order to clarify the mode of anti-thrombotic action of SSEF, we investigated the effect of SSEF on various parameters associated to collagen-induced platelet aggregation and blood coagulation.

MATERIALS AND METHODS

Materials

Collagen was obtained from the Chrono-Log Corporation (Havertown, PA, U.S.A.), and other reagents were obtained Sigma Chemical Corporation (St. Louis, U.S.A.). cAMP-, cGMP-, and TXB₂-enzymeimmunoassay (EIA) kits were purchased from Amersham Bioscience (Buckinghamshire, U.K.). Ginseng total saponin was donated from Central Institutes of KT&G, Daejeon, Korea.

Preparation of SSEF

We used fresh spinach from a local market. Spinach roots were broken and dried. A total 1 kg of dry spinach roots was denatured by heating at 95°C for 6 h with 5 L distilled water, and then 0.6 L of resultants was extracted by a dianion HP-20 column (Supelco, Bellefonte, PA, USA) with 40% ethanol. SSEF in 40% ethanol was evaporated, frozen, and dried. SSEF was dissolved with distilled water to investigate the effects on platelet aggregation and blood coagulation.

Determination of saponin concentration in SSEF

The SSEF 0.1 ml was added in 8% vanillin-ethanol 0.3 ml and 72% sulfuric acid 4 ml, and then heated at 60°C for 10 min. The concentration of saponin in SSEF was measured at 545 nm of UV/visible spectrophotometer (Optizen 2120 UV, Mecasys, daejeon, Korea), and was calculated by using the calibration curve of ginsenoside-Rc (G-Rc). The total saponin content was expressed as %.

Preparation of washed rat platelets

Blood was collected from Sprague-Dawley rats (6-7 weeks, male), and anti-coagulated with ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose). Platelet-rich plasma was centrifuged at 125×g for 10 min to remove the red blood cells, and the platelets were washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 5.5 mM glucose, and 1 mM EDTA, pH 7.4). The washed platelets were then resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 0.49 mM MgCl₂, 5.5 mM glucose, 0.25% gelatin, pH 7.4) to a final concentration of 5×10⁸/ml. All of the above procedures were carried out at 25°C to avoid platelet aggregation

on cooling. The Ethical Committees for Animal Experiments of Inje University approved this study.

Measurement of platelet aggregation

Washed platelets (10⁸/ml) were preincubated for 3 min at 37°C in the presence of 2 mM exogenous CaCl₂ with or without SSEF, and then stimulated with collagen (10 µg/ml) for 5 min. Aggregation was monitored using an aggregometer (Chrono-Log, Corp., Havertown, PA, USA) at a constant stirring speed of 1,000 rpm. Each aggregation rate was evaluated as an increase in light transmission. The suspension buffer was used as the reference. SSEF was reconstituted with distilled water.

Measurement of TXB₂

Washed platelets (10⁸/ml) were preincubated with or without SSEF for 3 min in the presence of 2 mM CaCl₂, and activated for 5 min with collagen (10 µg/ml). The reactions were terminated by the addition of ice-cold EDTA (5 mM) and indomethacin (0.2 mM). The amount of TXB₂, a stable metabolite of TXA₂, was determined with Synergy HT Multi-Model Microplate Reader (Bio Teck Instrument, Winooski, U.S.A.) using a TXB₂ EIA kit. SSEF was reconstituted with DW.

Measurement of cAMP and cGMP

Washed platelets (10⁸/ml) were preincubated for 3 min at 37°C with various concentrations of SSEF in the presence of 2 mM CaCl₂, and then stimulated with collagen (10 µg/ml) for 5 min for platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. cAMP and cGMP were measured with Synergy HT Multi-Model Microplate Reader (Bio-Teck Instrument, Winooski, U.S.A.) using cAMP and cGMP EIA kits. SSEF was reconstituted with DW.

Measurement of PT and APTT

Citrated platelet-poor plasma (PPP) was prepared by centrifuging the blood remaining after the removal of PRP at 1,300 ×g for 10 min. The PPP (0.1 ml) was preincubated in a two-channel coagulator (KG Behnk Elektronik GMBH & Co., Germany) cup (BioMérieux, 95-662) with gentle stirring for 1 min at 37°C. PT was determined as the time interval between the addition of PT reagent (0.1 ml) to the PPP and the formation of a fibrin clot. After preincubation for APTT measurement, 0.1 ml of APTT reagent was added to the PPP and incubated for 3 min at 37°C. Following incubation, 0.1 ml of 25 mM CaCl₂ was rapidly added to the PPP solution containing APTT reagent. APTT was determined as the time required to form a fibrin clot. SSEF was reconstituted with distilled water.

Statistical analysis

The experimental results are expressed as the means ± S.E.M. and are accompanied by the number of observations. Data were with 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 less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Saponin concentration of SSEF

The saponin level in SSEF, as shown in Fig. 1, was 10% when was calculated by calibration curve of G-Rc. It is known

that the total saponin contents in leafy vegetables such as *Chenopodium* (*C*) *album*, *Sonchus* (*S*) *asper*, *Solanum* (*S*) *nigrum*, and *Urtica* (*U*) *urens* as well as *Spinach oleracea* are from 3.25 to 5.25% (Afolayan and Jimoh, 2009). Any could know that total saponin from the roots of spinach, *Spinach oleracea*, is higher than that of leafy vegetables such as *C. album*, *S. asper*, *S. nigrum*, and *U. urens*. In the present, we could not know what kinds of saponins such as steroid saponins or triterpenoid saponins are contained in SSEF. SSEF was extracted with ethanol, which was used to extract saponin fraction from the medicinal herbs such as *Gynostema pentaphyllum* or *Platycodon grandiflorum* (Moon *et al.*, 2010; Tsai *et al.*, 2010). Tsai *et al.* (2010) and Moon *et al.* (2010) reported that saponin-containing ethanol extract has the effect of anti-amnesia and anti-hepatoma. As described in "Introduction", aqueous extracts from spinach have known to inhibit oxidation and carcinogenesis (Bergman *et al.*, 2001; Lomnitski *et al.*, 2003). From these previous reports, we could know that polar-solvent extracts have lots of biological activities. However, it is unknown whether polar-solvent (i.e. water and ethanol) extracts have anti-platelet aggregation. Therefore, here, we investigated the effects of SSEF, ethanol extract containing saponin, on platelet aggregation.

Inhibitory effects of SSEF on collagen-induced platelet aggregation

Platelet aggregation results from exposure to collagen from vascular endothelium damaged by hypertension, diabetic mellitus, atherosclerosis, etc. Thus, collagen is the first major platelet aggregatory factor (the primary step in the hemostatic system) *via* damaged endothelial cells. Thus, we used collagen as an inducer to investigate the effect of SSEF on anti-platelet function. The concentration of collagen used was 10 $\mu\text{g/ml}$, which was the threshold concentration to induce maximal aggregation (Fig. 2A) (Cho *et al.*, 2004). Since $[\text{Ca}^{2+}]_i$ is a critical regulator of platelet response, 2 mM CaCl_2 as exogenous Ca^{2+} resource was used to examine the effects of SSEF in collagen-induced platelet aggregation. When washed platelets ($10^8/\text{ml}$) were activated with 10 μg of collagen/ml in the presence of 2 mM CaCl_2 , the aggregation rate was $87 \pm 4\%$. However, various concentrations of SSEF (100 to 500 μg /

Fig. 1. Calibration curve of G-Rc and concentration of SSEF from spinach roots. Analytical methods were described in "MATERIALS AND METHODS", the calibration curve was determined by measuring the absorbance of each dose of G-Rc, which was used to determine the concentration of saponin in SSEF. The concentration of saponin is indicated in small Table of this Fig. and is expressed as %. SSEF, spinach saponin-enriched fraction.

ml) significantly reduced the collagen (10 $\mu\text{g/ml}$)-stimulated platelet aggregation (Fig. 2B). On the other hands, we used ginseng saponin (GS) as a positive control, which has report-

Fig. 2. Effects of SSEF on collagen-induced platelet aggregation. (A) The concentration threshold of collagen on platelet aggregation. Washed platelets ($10^8/\text{ml}$) were stimulated with various concentrations of collagen for 5 min in the presence of 2 mM CaCl_2 at 37°C . Platelet aggregation (%) was recorded as an increase in light transmission. (B) Effects of SSEF on collagen-induced platelet aggregation. (C) Comparison of SSEF and ginseng saponin (GS) on collagen-induced platelet aggregation. Washed platelets ($10^8/\text{ml}$) were preincubated with various concentrations of SSEF or GS in the presence of 2 mM CaCl_2 for 3 min at 37°C , and then stimulated with collagen for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. Inhibition by SSEF or GS was recorded as percentage of that of control. Data are expressed as means $\pm$ S.E.M. ($n = 4$). * $p < 0.05$. ** $p < 0.001$. SSEF: spinach saponin-enriched fraction, GS: ginseng saponin.

ed that it has anti-thrombotic effects (Kimura *et al.*, 1988). The inhibition rates of SSEF (500 $\mu\text{g/ml}$) and GS (500 $\mu\text{g/ml}$) were $50.0 \pm 5.2\%$ and $24.6 \pm 2.3\%$ as compared with that of control, respectively (Fig. 2C). As the results, the inhibitory effect of SSEF on collagen-induced platelet aggregation is stronger than that of GS.

Inhibitory effects of SSEF on TXA_2 production

Among several aggregation-inducing molecules, TXA_2 is known to be essential for platelet aggregation (Charo *et al.*, 1997), and is also a vasoconstrictor and a bronchoconstrictor (Hamberg *et al.*, 1975; Moncada and Vane, 1978). Therefore, we next examined whether SSEF blocks the production of TXA_2 under collagen exposure (Fig. 3A). However, TXA_2 has a half life of only 37 sec under physiological conditions, and TXB_2 is produced by the non-enzymatic hydration of TXA_2 . So, we measured the TXB_2 concentration in order to determine TXA_2 formation level. TXA_2 (determined as TXB_2) level in intact platelets was $1.4 \pm 0.1 \text{ ng}/10^8$ platelets, and this was markedly increased to $3.4 \pm 0.2 \text{ ng}/10^8$ platelets in the collagen-stimulated platelets (Fig. 3A). However, SSEF significantly reduced the production of TXA_2 by $2.8 \pm 0.2 \text{ ng}/10^8$ platelets at a concentration of 500 $\mu\text{g/ml}$ (Fig. 3A). These results suggest that SSEF inhibits the collagen-induced platelet aggregation by down-regulating the TXA_2 production. Given the wide implications of TXA_2 in several pathologies, various studies have been undertaken to find therapeutic agents able to counteract the negative effects of TXA_2 . TXA_2 receptor antagonist sulotroban (Stegmeier *et al.*, 1984), TXA_2 synthase inhibitor furegrelate (Gorman *et al.*, 1983), and drugs combining the properties of both TXA_2 receptor antagonist and TXA_2 synthase inhibitor (i. e. ridogrel and terbogrel) have been developed. Dual compounds proved to be more interesting in therapeutics and promising anti-thrombotic agents (Dogné *et al.*, 2004; Hanson *et al.*, 2005).

Elevating effects of SSEF on cAMP and cGMP levels

The platelet-activating reagent-elevated TXA_2 level is lowered by either cAMP or cGMP. This is associated with the activation of cAMP-dependent protein kinase (A-kinase) or cGMP-dependent protein kinase (G-kinase), since these enzymes blockade Ca^{2+} -mobilization from Ca^{2+} -pool (i.e. dense tubular system) to cytosol by phosphorylating inositol 1, 4, 5-trisphosphate receptor to inhibit TXA_2 production (Jang *et al.*, 2002). Therefore, we examined whether SSEF affected the production of cAMP and cGMP in intact platelets or collagen-induced platelets. As shown in Fig. 3B, collagen decreased intracellular cAMP level from $3.83 \pm 0.23 \text{ pmol}/10^8$ platelets (basal level) to $1.75 \pm 0.05 \text{ pmol}/10^8$ platelets in the washed platelets. When the platelets, however, were incubated in the presence of both SSEF and collagen, SSEF (100 to 500 $\mu\text{g/ml}$) significantly increased the cAMP level in a dose-dependent manner. SSEF (500 $\mu\text{g/ml}$) increased the cAMP level by $10.35 \pm 0.05 \text{ pmol}/10^8$ platelets in comparison with that ($3.83 \pm 0.23 \text{ pmol}/10^8$ platelets) of basal in resting platelets on collagen-induced platelet aggregation (Fig. 3B). It is interesting to note that SSEF modulated the production of cAMP in collagen-stimulated platelets. cGMP also has an anti-platelet function as well as cAMP. Therefore, we investigated whether SSEF increases the cGMP production in collagen-stimulated platelets. As shown in Fig. 3C, collagen decreased intracellular cGMP level from $0.27 \pm 0.01 \text{ pmol}/10^8$ platelets (basal level)

Fig. 3. Anti-platelet effects of SSEF. (A) Effects of SSEF on TXB_2 production on collagen-induced platelet aggregation. Washed platelets ($10^8/\text{ml}$) were preincubated for 3 min in the presence of 2 mM CaCl_2 , and then stimulated with collagen (10 $\mu\text{g/ml}$). TXB_2 was experimentally as described in "Materials and Methods". The content of TXB_2 was measured using a TXB_2 EIA kit. (B) Effects of SSEF on cAMP production on collagen-induced platelet aggregation. (C) Effects of SSEF on cGMP production on collagen-induced platelet aggregation. Washed platelets ($10^8/\text{ml}$) were preincubated in the presence of 2 mM CaCl_2 and then stimulated with collagen (10 $\mu\text{g/ml}$) for 5 min at 37°C . The reactions were terminated by adding 80% ice-cold ethanol. cAMP and cGMP contents were measured using EIA kits. Data are expressed as means $\pm$ S.E.M. ($n = 4$). * $p < 0.05$. ** $p < 0.001$. SSEF: spinach saponin-enriched fraction.

to 0.2 ± 0.01 pmol/ 10^8 platelets in the washed platelets. When the platelets, however, were incubated in the presence of both SSEF and collagen, SSEF (100 to 500 μ g/ml) significantly increased the cGMP level in a dose-dependent manner. SSEF (500 μ g/ml) increased the cGMP level by 0.64 ± 0.01 pmol/ 10^8 platelets in comparison with that (0.27 ± 0.01 pmol/ 10^8 platelets) of basal in resting platelets on collagen-stimulated platelets (Fig. 3C). These results suggest that SSEF modulated the production of cGMP in collagen-stimulated platelets. The levels of cAMP and cGMP in platelets are regulated by adenylate cyclase producing cAMP from ATP, cGMP-stimulated phosphodiesterase (PDE2) hydrolyzing both cAMP and cGMP, cGMP-inhibited phosphodiesterase (PDE3) hydrolyzing cAMP rather than cGMP, and cGMP-binding cGMP-specific phosphodiesterase (PDE5) hydrolyzing cGMP alone (Schwarz *et al.*, 2001). In the present study, because we did not determine the activities of PDE2, PDE3 and PDE5, we have not an obvious evidence for regulation of PDE2, PDE3, and PDE5, which remain to be studied. It is known that downstream pathways of both cAMP/A-Kinase and cGMP/G-kinase are involved in the inhibition of platelet aggregation by inhibiting both the Ca^{2+} /calmodulin-dependent phosphorylation of MLC and Ca^{2+} /DG-dependent phosphorylation of pleckstrin (Schwarz *et al.*, 2001). Schwarz *et al.* (2001) reported that platelet aggregation-associated phosphoproteins such as MLC and pleckstrin are inhibited by Ca^{2+} -antagonistic intracellular cAMP and cGMP. Accordingly, it is thought that anti-platelet effect of SSEF might be also involved in inhibition of Ca^{2+} -dependent phosphoproteins such as MLC and pleckstrin by producing cAMP and cGMP on collagen-induced platelet aggregation. In this present study, although we did not determine the intracellular Ca^{2+} level, SSEF increased both the levels of cAMP and cGMP (Fig. 3B, C), and Ca^{2+} -antagonistic intracellular cAMP is known to inhibit platelet aggregation by phosphorylating vasodilator-stimulated phosphoprotein (VASP) via activating A-kinase (Sudo *et al.*, 2003), one could also speculate that SSEF-elevated cAMP (Fig. 3B) may involve in the inhibition of Ca^{2+} /calmodulin-dependent phosphorylation of MLC and Ca^{2+} /DG-dependent phosphorylation of pleckstrin by decreasing intracellular Ca^{2+} level via phosphorylation of VASP.

Here, we demonstrated that SSEF might be an anti-platelet agent through inhibition of platelet TXA_2 production and elevation of cAMP/cGMP production.

Effects of SSEF on blood coagulation

Platelet aggregation is dependent on the coagulation factors surrounding the platelets (Sinha *et al.*, 1983). PT is defined as the time interval between the addition of thromboplastin to plasma and the formation of a fibrin clot. PT is dependent on coagulation factors II, V, VII, and X. As shown in Table 1,

PT was prolonged by 16.5 ± 0.5 sec in the presence of 500 μ g SSEF/ml, which was prolonged to 13% as compared with that of control value (14.5 ± 0.3 sec). These results suggest that SSEF prolongs the PT by inhibiting the formation of fibrin clots in the presence of thromboplastin with coagulation factors II, V, VII, and X. APTT is defined as the blood coagulation time in the intrinsic pathway. Both intrinsic and extrinsic thromboplastin convert prothrombin to thrombin to form a fibrin clot. APTT is dependent on coagulation factors VIII and IX. As shown in Table 1, APTT was prolonged by 47.5 ± 0.7 sec in the presence of 500 μ g SSEF/ml, which was prolonged up to 21% as compared with that of control value (39.3 ± 0.9 sec). These results suggest that SSEF prolongs APTT by inhibiting the formation of fibrin clot in the presence of coagulation factors VIII and IX. Thus, the results suggest that SSEF, which inhibits platelet aggregation (Fig. 2), might have an anti-coagulative effect by inhibiting the formation of fibrin clots in the presence of coagulation factors. In this study, it is unknown which saponins or other compounds in SSEF have the effects of both anti-platelet and anti-coagulation. If a saponin in SSEF has the effects of both anti-platelet and anti-coagulation (Fig. 2, 3, and Table I), anyone should consider the cytotoxicity of saponin. The hemolysis of erythrocytes is characteristics of the saponins (Podolak *et al.*, 2010). In another *in vivo* experiment, we investigated the effect of dietary SSEF on the hemolysis of erythrocytes, which was observed with the absorbance of oxyhemoglobin at 430 nm. When 0.4 M sucrose was added into the erythrocyte suspensions from control rats (SPD, male), the absorbance of oxyhemoglobin was increased to 2.13 ± 0.2 ($n=9$, Data not shown). However, 0.4 M sucrose was added into the erythrocyte suspensions from SSEF-treated rats (SPD, male), the absorbance of oxyhemoglobin was decreased to 1.265 ± 0.1 ($n=9$, Data not shown). These results showed that dietary SSEF inhibits 0.4 M sucrose-induced the hemolysis of erythrocytes. We demonstrated that SSEF without hemolytic cytotoxicity could be a negative regulator for thrombus-related vascular diseases via platelet aggregation and blood coagulation.

In conclusion, SSEF is a potent up-regulator of cAMP/cGMP, and inhibits collagen-stimulated calcium signaling, TXB_2 production and platelet aggregation. SSEF also was prolonged the PT and APTT on blood coagulation system. In conclusion, these results suggest that SSEF may have an inhibitory effect on platelet aggregation- and blood coagulation-mediated thrombotic diseases such as cardiovascular disease by reacting as physiologically an effective negative regulator during platelet aggregation and blood coagulation.

Table 1. Effects of SSEF on PT and APTT *in vitro*

		PT (sec)	PT prolongation rate of control (%)	APTT (sec)	APTT prolongation rate of control (%)
Control		14.5 ± 0.3	0	39.3 ± 0.9	0
SSEF	100 μ g/ml	14.6 ± 0.2	0.68	39.3 ± 0.7	0
	500 μ g/ml	$16.5 \pm 0.5^*$	13.8 ^a	47.5 ± 0.7^b	21 ^b

PT and APTT were determined as described in "Materials and Methods". Data are expressed as mean $\pm$ S.E.M ($n=9$). ^a $p<0.05$, ^b $p<0.001$. PT: prothrombin time, APTT: activated partial thromboplastin time, SSEF: spinach saponin-enriched fraction.

ACKNOWLEDGMENTS

This study was supported (in part) by a grant from the Ministry of Commerce, Industry and Energy (MOCIE) and Korean Institute of Industrial Technology Evaluation & Planning (ITEP) through the Biohealth Products Research Center (BPRC) of Inje University, Korea.

REFERENCES

- Afolayan, A. J. and Jimoh, F. O. (2009) Nutritional quality of some wild leafy vegetables in South Africa. *Intl. J. Food Sci. Nutr.* **60**, 424-431.
- Akoum, A., Josefovnicz, J. and Sigot, M. (1990). Anticoagulant activity of a bacterial glycopeptide. *Thromb. Res.* **60**, 9-18.
- Bergman, M., Varshavsky, L., Gottlieb, H. E. and Grosman, S. (2001) The antioxidant activity of aqueous spinach extract: chemical identification of activity fractions. *Phytochemistry* **58**, 143-152.
- Cattaneo, M., Tenconi, P. M., Lecchi, A. and Mannucci, P. M. (1991) In vitro effects of picotamide on human platelet aggregation, the release reaction and thromboxane B₂ production. *Thromb. Res.* **62**, 717-724.
- Charo, I. F., Feinman, R. D. and Detwiler, T. C. (1997) Interrelations of platelet aggregation and secretion. *J. Clin. Invest.* **60**, 866-873.
- Cho, H. J., Ham, H. S., Lee, T. K., Jung, Y. J., Choi, S. A., Kang, H. C. and Park, H. J. (2004) Inhibitory effect of cordycepin on human platelet aggregation. *J. Biomed. Lab. Sci.* **10**, 1-8.
- Davie, E. W. and Ratnoff, O. D. (1964) Waterfall sequence for intrinsic blood clotting. *Science* **145**, 1310-1312.
- Dogné, J. M., de Leval, X., Hanson, J., Frederich, M., Lambermont, B., Ghuyssen, A., Casini, A., Masereel, B., Ruan, K. H., Pirotte, B. and Kolh, P. (2004) New developments on thromboxane and prostacyclin modulators part I: thromboxane modulators. *Curr. Med. Chem.* **11**, 1223-1241.
- Edwards, A. J., You, C. S., Swanson, J. E. and Parker, R. S. (2001) A novel extrinsic reference method for assessing the vitamin A of plant foods. *Am. J. Clin. Nutr.* **74**, 348-355.
- Furie, B. and Furie, B. C. (1988) The molecular basis of blood coagulation. *Cell* **53**, 505-518.
- Gorman, R. R., Johnson, R. A., Spilman, C. H. and Aiken, J. W. (1983) Inhibition of platelet thromboxane A₂ synthase activity by sodium 5-(3'-pyridinylmethyl)benzofuran-2-carboxylate. *Prostaglandins* **26**, 325-342.
- Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci.* **72**, 2994-2998.
- Hanson, J., Rolin, S., Reynaud, D., Qiao, N., Kelley, L. P., Reid, H. M., Valentin, F., Tippins, J., Kinsella, B. T., Masereel, B., Pace-Asciak, C., Pirotte, B. and Dogné, J. M. (2005) In vitro and in vivo pharmacological characterization of BM-613 [N-n-pentyl-N'-[2-(4'-methylphenylamino)-5-nitrobenzenesulfonyl] urea], a novel dual thromboxane synthase inhibitor and thromboxane receptor antagonist. *J. Pharmacol. Exp. Ther.* **313**, 293-301.
- Jang, E. K., Azzam, J. E., Cickinson, N. T., Davidson, M. M. and Haslam, R. J. (2002) Roles for both cyclic GMP and cyclic AMP in the inhibition of collagen-induced platelet aggregation by nitroprusside. *Br. J. Haematol.* **117**, 664-675.
- Kimura, Y., Okuda, H. and Arichi, S. (1988) Effects of various ginseng saponins on 5-hydroxytryptamine release and aggregation in human platelets. *J. Pharm. Pharmacol.* **40**, 838-843.
- Lomnitski, L., Bergman, M., Nyska, A., Ben-Shaul, V. and Grosman, S. (2003) Composition, efficacy, and safety of spinach extracts. *Nutr. Cancer.* **46**, 222-231.
- Moncada, S. and Vane, J. R. (1978) Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A₂ and prostacyclin. *Pharmacol. Rev.* **30**, 293-331.
- Moon, M. K., Ahn, J. Y., Kim, S., Ryu, S. Y., Kim, Y. S. and Ha, T. Y. (2010) Ethanol extract and saponin of *Platycodon grandiflorum* ameliorate scopolamine-induced amnesia in mice. *J. Med. Food.* **13**, 584-588.
- Nishikawa, M., Tanaka, T. and Hidaka, H. (1980) Ca²⁺-calmodulin-dependent phosphorylation and platelet secretion. *Nature* **287**, 863-865.
- Podolak, I., Galany, A. and Sobolewska, D. (2010) Saponins as cytotoxic agents: a review. *Phytochem. Rev.* **9**, 425-474.
- Prinz-Langenohl, R., Bronstrup, A., Thorand, B., Hages, M. and Pietrzik, K. (1999) Availability of food folate in humans. *J. Nutr.* **129**, 913-916.
- Sani, H. A., Rahat, A., Ismail, M., Rosli, R. and Endrini, S. (2004) Potential anticancer effect of red spinach (*Amaranthus gangeticus*) extract. *Asia Pac. J. Clin. Nutr.* **13**, 396-400.
- Sano, Y., Sato, K., Uchida, M. and Murata, M. (2003) Blood coagulation fibrinolysis of rats fed fish oil: reduced coagulation factors especially involved in intrinsic pathway and increased activity of plasminogen activator inhibitor. *Biosci. Biotechnol. Biochem.* **67**, 2100-2105.
- Schwartz, S. M., Heinmark, R. L. and Majesky, M. W. (1990) Developmental mechanisms underlying pathology of arteries. *Physiol. Rev.* **70**, 1177-1209.
- Schwarz, U. R., Walter, U. and Eigenthaler, M. (2001) Taming platelets with cyclic nucleotides. *Biochem. Pharmacol.* **62**, 1153-1161.
- Sinha, A. K., Rao, A. K., Willis, J. And Colman, R. W. (1983) Inhibition of thromboxane A₂ synthesis in human platelets by coagulation factor Xa. *Proc. Natl. Acad. Sci. USA.* **80**, 6086-6090.
- Stegmeier, K., Pill, J., Muller-Beckmann, B., Schmidt, F. H., Witte, E. C., Wolff, H. P. and Patscheke, H. (1984) The pharmacological profile of the thromboxane A₂ antagonists BM 13.177. A new antiplatelet and anti-thrombotic drug. *Thromb. Res.* **35**, 379-395.
- Sudo, T., Ito, H. and Kimura, Y. (2003) Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) by the antiplatelet drug, cilostazol, in platelets. *Platelets* **14**, 381-390.
- Tsai, Y. C., Lin, C. L. and Chen, B. H. (2010) Preparative chromatography of flavonoids and saponins in *Gynostemma pentaphyllum* and their antiproliferation effect on hepatoma cell. *Phytomedicine.* **18**, 2-10.