Inhibitory Effects of Total Saponin Korean Red Ginseng on Thromboxane A₂ Production and P-Selectin Expression via Suppressing Mitogen-Activated Protein Kinases

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Ginseng has been widely used for traditional medicine in eastern Asia and is known to have inhibitory effects on cardiovascular disease (CVD) such as thrombosis, atherosclerosis, and myocardial infarction. Because, platelet is a crucial mediator of CVD, many studies are focusing on inhibitory mechanism of platelet functions. Among platelet activating molecules, thromboxane A₂ (TXA₂) and P-selectin play a central role in CVD. TXA₂ leads to intracellular signaling cascades and P-selectin plays an important role in platelet-neutrophil and platelet-monocyte interactions leading to the inflammatory response. In this study, we investigated the inhibitory mechanisms of total saponin fraction from Korean red ginseng (KRG-TS) on TXA₂ production and P-selectin expression. Thrombin-elevated TXA₂ production and arachidonic acid release were decreased by KRG-TS dose (25 to 150 μg/mL)-dependently via down regulation of microsomal cyclooxygenase-1 (COX-1), TXA₂ synthase (TXAS) activity and dephosphorylation of cytosolic phospholipase A₂ (cPLA₂). In addition, KRG-TS suppressed thrombin-activated P-selectin expression, an indicator of granule release via dephosphorylation of mitogen-activated protein kinases (MAPK). Taken together, we revealed that KRG-TS is a beneficial novel compound inhibiting TXA₂ production and P-selectin expression, which may prevent platelet aggregation-mediated thrombotic disease.

Key Words: Total saponin fraction, MAPKs. Thromboxane A₂, Arachidonic acid, P-selectin

INTRODUCTION

Platelets are activated at the sites of vascular injury. Upon activation, inositol-1,4,5-trisphosphate (IP₃) is released from plasma membrane (Berridge and Irvine, 1984) and released IP₃ mobilizes Ca²⁺ from endoplasmic reticulum into cytoplasm. Intracellular Ca²⁺ stimulates translocation of cytosolic phospholipase A₂ (cPLA₂) from cytosol to membranes and cPLA₂ is phosphorylated at Ser²⁰⁵ by p38 mitogen-activated protein kinase.
protein kinase (p38 MAPK) for full catalytic activity. The activated cPLA2 hydrolyzes the sn-2 acyl bond of phospholipids and releases arachidonic acid (AA) (Kramer et al., 1996; McNicol and Shibou, 1998). The AA is a precursor of thromboxane A2 (TXA2) (Hamberg et al., 1975) and the TXA2 biosynthesis is triggered by cyclooxygenase-1 (COX-1) and TXA2 synthase (TXAS) (Needleman et al., 1976; Patrignani et al., 1999). TXA2 is released from platelets and interacts with membrane receptor of other platelets in an autacoidal reaction, which acts as a positive promoter on activated platelets and resting platelets simultaneously (Jennings, 2009). It is well known that aspirin and ozagrel have anti-thrombotic effects by inhibiting the TXA2 production. The mechanisms are concerned with COX-1 and TXAS (Patrono, 1994).

Selectins are expressed by activation or inflammatory response on various vascular cells, including platelets, leukocytes and endothelial cells (Kansas, 1996; Ley, 2003). L-selectin is present on leukocytes and E-selectin is present on endothelial cells and platelets. The P-selectin in platelets is released by agonists from α-granule, and is re-expressed to the surface (Zarbock et al., 2007). P-selectin plays an important role in interactions with immune cells (von Hundelshausen and Weber, 2007).

Ginseng, the root of *Panax ginseng* Meyer, is known to have various pharmacological activities (Ernst, 2010; Kim and Park, 2011). Recently, it is reported that KRG-TS has an effect on CVD through reduction of blood pressure (Chung, 2010), anti-coagulation effects (Wee et al., 2010), endothelium relaxation (Jung et al., 2011), and inhibition of hypercholesterolemia-induced platelet aggregation (Hwang et al., 2008). In our previous report, we demonstrated that KRG-TS was involved in increase of cAMP level and subsequent reduction of $\left[\text{Ca}^{2+}\right]_i$ mobilization in thrombin-induced rat platelet aggregation (Lee et al., 2013). However, the inhibitory mechanism by KRG-TS is not fully understood. Thus, we demonstrated that the modulatory mechanism of TXA2 production and P-selectin expression by KRG-TS on human platelets for prevention of CVD.

**MATERIALS AND METHODS**

**Materials**

KRG-TS was obtained from Korea Ginseng Corporation (Daejeon, Korea). Thrombin was purchased from ChronoLog Corporation (Havertown, PA, USA). Aspirin was purchased from Sigma Chemical Corporation (St. Louis, MO, USA). TXB2 EIA kit, COX-1 fluorescent activity assay kit, ozagrel, and Prostaglandin H2 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-phosphor-cPLA2 (Ser505), anti-phosphor-p38, anti-p38, anti-phosphor-ERK1/2, anti-ERK1/2, anti-phosphor-JNK1, and anti-rabbit IgG-horseradish peroxidase conjugate (HRP) and lysis buffer were purchased from Cell Signaling (Beverly, MA, USA). Anti-β-actin, anti-COX-1 and anti-TXAS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PVDF membrane and ECL solution were purchased from GE Healthcare (Piscataway, NJ, USA). Human arachidonic release kit was purchased from (Cusabio, Wuhan, Hubei, China). CD62P (P-selectin) antibody was purchased from Biolegend (San Diego, CA, USA).

**Preparation of washed human platelets**

Human platelet-rich plasma (PRP) with ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) was supplied from Korean Red Cross Blood Center (Changwon, Korea). To remove red blood cells, it was centrifuged for 10 min at 250 g, then centrifuged for 10 min at 1,300 g. The platelets were washed using washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 0.36 mM NaH2PO4, 5.5 mM glucose, and 0.25% gelatin, pH 6.9) was 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 6.9). The human platelets in suspension buffer was adjusted to initial concentration of $5 \times 10^9$/mL and aforementioned procedures were performed at 25°C to maintain platelet activity. The approval (PIRB12-072) for these experiments was received from National Institute for Bioethics Policy Public Institutional Review Board (Seoul, Korea).
Determination of platelet aggregation

Human Platelets (10^8/mL) were preincubated with or without KRG-TS in the presence of 2 mM of CaCl₂ for 2 min at 37°C, then, stimulated with thrombin (0.05 U/mL). The platelet aggregation rate was determined as an increase in light transmission for 5 min using an aggregometer (Chrono-Log Corporation, Haverton, PA, USA).

Measurement of TXB₂

TXA₂ is unstable and quickly converted to thromboxane B₂ (TXB₂) in platelets. Thus, the amounts of TXA₂ were evaluated by measuring TXB₂ concentration (Hamberg et al., 1975). Thrombin-induced platelet aggregation was terminated by adding both ice-cold 5 mM EDTA and 0.2 mM indomethacin to inhibit subsequent metabolism of arachidonic acid to TXA₂. The amounts of TXB₂ were determined using a TXB₂ EIA kit according to the procedure described by the manufacturer (Cayman Chemical Co, Ann Arbor, MI, USA).

Isolation of microsomal fraction

Human platelets (10^8/mL) containing suspending buffer (pH 7.4) with 1% protease inhibitor was sonicated at sensitivity 100% for 20 sec, 1 cycles, and 10 times on ice to obtain platelet homogenates using a sonicator (Bandelin, HD2070, Germany). And then, homogenates were ultracentrifuged at 105,000 g for 1 h at 4°C to obtain microsomal fraction containing endoplasmic reticulum (ER) membrane (Lagarde et al., 1981).

COX-1 activity assay

For the measurement of COX-1 activity, the microsomal fraction of platelets was pre-incubated with a positive control, aspirin (500 μM), and with various concentrations of KRG-TS at 37°C for 30 min. COX-1 activity was assayed with COX-1 fluorescent assay kit (Cayman Chemical Co, Ann Arbor, MI, USA).

Thromboxane A synthase activity

For the measurement of TXAS, microsomal fraction was preincubated with a positive control, ozagrel (11 nM, IC₅₀), and with various concentrations of KRG-TS at 37°C for 5 min. The reaction is initiated by adding prostaglandin H₂, and incubated at 37°C for 1 min. The reactions are terminated by the addition of 1 M citric acid and neutralized by 1 N NaOH, the amount of TXB₂ was determined by using TXB₂ EIA kit according to the procedure described by manufacturer (Cayman Chemical Co, Ann Arbor, MI, USA).

Arachidonic release

Thrombin-induced platelet aggregation was terminated, and centrifuged with 200 g at 4°C for 10 min, and supernatants were used for the assay of AA release EIA kit (Cusabio, Wuhan, Hubei, China). AA release was measured at 450 nm using a Synergy HT multi-Model Microplate Reader (BioTek Instruments, Winoosku, VT, USA).

Western blot for analysis of COX-1, TXAS, cPLA₂-, and MAPKs-phosphorylation

The platelet aggregation was terminated by adding a lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM ATPase, 1 mM Na₃VO₄, 1 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride) then, platelet protein and microsomal fraction were measured using a BCA protein assay kit (Pierce Biotechnology, IL, USA). Protein (15 μg) and microsomal fraction (30 μg) were separated by SDS-PAGE (6%, 1.5 mm), then PVDF membrane was used for protein transfer. The dilutions for 1st antibody and 2nd antibody were 1:1,000 and 1:10,000, respectively. The membranes were visualized using ECL solution. The degrees of phosphorylation were analyzed using the Quantity One, Ver. 4.5 (BioRad, Hercules, CA, USA).

Determination of P-selectin release

After platelet aggregation, the platelets were resuspended by ice-cold PBS (pH 7.4) and cells were incubated with Alexa Fluor 488 anti-human CD62P for 60 min at 4°C under the dark condition. Next, platelets were washed three times by ice-cold PBS to reduce unbounded antibody and fixed using 0.5% paraformaldehyde. Alexa Fluor 488 anti-human CD62P binding to platelets were determined using...
flow cytometry (BD Biosciences, San Diego, CA, USA) and data were analyzed using CellQuest software.

**Statistical analyses**

The experimental results are presented as the mean ± standard deviation accompanied by the number of observations. Data were determined by analysis of variance (ANOVA). If this analysis showed significant differences among the group means, then each group was compared by the Newman-Keuls method. Statistical analysis was carried out according to the SPSS 21.0.0.0 (SPSS, Chicago, IL, USA). *P*<0.05 was considered to be statistically significant.

**RESULTS**

**Effects of KRG-TS on thrombin-induced human platelet aggregation**

The concentration of thrombin-elevated maximal human platelet aggregation was approximately 0.05 U/mL (Fig. 1A). Thus, thrombin (0.05 U/mL) was used as an agonist in this study. Thrombin increased human platelet aggregation rate was 87.8 ± 5.7%. However, KRG-TS strongly reduced thrombin-induced platelet aggregation dose-dependently (Fig. 1B). In our previous report showed that various concentration of KRG-TS (20, 50, 100, 150 μg/mL) did not influence on unstimulated human platelet (Kwon et al., 2016).

**Effects of KRG-TS on TXA2 production and COX-1, TXAS activity**

Next, we investigated TXA2 production. The amount of TXA2 was markedly increased by thrombin from 1.1 ± 0.1, resting platelets, to 55.2 ± 2.2 ng/10^8 platelets. However, KRG-TS reduced the TXA2 production (Fig. 2). To determine whether KRG-TS involves in inhibition of COX-1 and TXAS, we needed enzyme sources having COX-1 or TXAS. We determined which fraction in platelets expresses COX-1 and TXAS in homogenates fraction, microsome fraction,
and cytosols fraction. As the result, high expressed COX-1 (70 kDa) and TXAS (58 kDa) were observed in microsomal fraction (Fig. 3A). Thus, we used to determine the activity of COX-1 and TXAS using microsomal fraction. As shown in Fig. 3B, COX-1 activity of microsomal fraction in the absence of KRG-TS (control) was 2.3 ± 0.04 nmol/protein-mg/min. However, 50, 100, and 150 μg/mL of KRG-TS inhibited COX-1 activity to 1.8 ± 0.10, 1.5 ± 0.11, and 1.4 ± 0.14 ng/protein-mg/min respectively. 500 μM of aspirin, a positive control, inhibited COX-1 activity to 1.3 ± 0.05 nmol/protein-mg/min (Fig. 3B).

To determine whether KRG-TS is involved in TXAS, cell-free enzyme assay method with microsomal fraction of platelets was also used. In microsomal fraction (control), TXAS activity was 200.1 ± 1.8 ng/protein-mg/min (Fig. 3C). However, 50, 100, and 150 μg/mL of KRG-TS inhibited TXAS activity to 182.0 ± 2.2, 166.6 ± 2.1, and 150.2 ± 1.8 ng/protein-mg/min respectively. In addition, 11 nM of ozagrel as a positive control was used, which inhibited TXAS activity to 130.2 ± 1.1 ng/protein-mg/min (Fig. 3C).

Effects of KRG-TS on Arachidonic acid release and cPLA2 phosphorylation

In order to verify the inhibitory mechanism of KRG-TS on TXA2 production, we investigated AA release. Thrombin-induced AA release increased to 1925.5 ± 22.2, but KRG-TS dose (25, 50, 100, 150)-dependently inhibited the AA release (Fig. 4A). Moreover, because cPLA2 acts as a key mediator to regulate the AA release in human platelets, we investigated whether KRG-TS inhibited the phosphorylation of cPLA2. As shown in Fig. 4B, the cPLA2 was strongly phosphorylated by thrombin (Fig. 4B, lane 2), but KRG-TS inhibited the cPLA2-phosphorylation dose-dependently (Fig. 4B, lanes 3 to 5). These results indicated that KRG-TS inhibited TXA2 production was due to the down regulation of cPLA2/AA release pathway.

Effects of KRG-TS on MAPKs phosphorylation

The MAPKs are divided into three subgroups: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK. It is reported that ERK2, JNK1 and p38 MAPK detected in human platelet and phosphorylated by thrombin (Bugaud et al., 1990; Kramer et al., 1995; Fadal-Wollbold et al., 2002). It is well known that the enzyme activity of cPLA2 is achieved by its phosphory-
translation and p38 MAPK phosphorylates cPLA2 (Ser505). The p38 MAPK is also activated by its phosphorylation. Thus, we evaluated that KRG-TS has inhibitory effect on p38 MAPK phosphorylation. As shown in Fig. 5A, KRG-TS inhibited thrombin-elevated the phosphorylation of p38 MAPK dose (50 to 150 μg/mL)-dependently.

It has been revealed that ERK2 is involved in the TXA2 production (Yacoub et al., 2006; Garcia et al., 2007). The specific inhibitors of ERK1/2 and p38 MAPK, PD98059 and SB203580, blocked COX-1 directly, and SB203580 inhibited the conversion of PGH2 to TXA2 (Börsch-Haubold et al., 1998), which means that MAPKs may influence on endo-

Fig. 5. Effects of KRG-TS on MAPKs-phosphorylation. (A) Effects of KRG-TS on p38 MAPK phosphorylation. (B) Effects of KRG-TS on ERK1/2 phosphorylation. (C) Effects of KRG-TS on JNK1 phosphorylation. Western blot was determined as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=3). *P<0.05 versus the thrombin-stimulated human platelets, **P<0.01 versus the thrombin-stimulated human platelets.
genous enzyme activity leading to TXA₂ production.

It is also known that phosphorylation of ERK is involved in extracellular Ca²⁺ influx (Rosado and Sage, 2001; Rosado and Sage, 2002) and the study in JNK1-deficient mice showed that JNK1 is involved in granule secretion and TXA₂ production (Adam et al., 2010). Thus, we investigated the effect of KRG-TS on dephosphorylation of ERK2 and JNK1. As shown in Fig. 5B, thrombin potently phosphorylated ERK2 (42 kDa) of ERK1 (44 kDa) and ERK2 (42 kDa) (Fig. 5B, lane 2) as compared with those by control, unstimulated platelets (Fig. 5B, lane 1), and phosphorylated JNK1 (46 kDa) (Fig. 5C, lane 2) as compared with those by control

Fig. 6. Effects of KRG-TS on P-selectin expression. (A) The flow cytometry histograms on P-selectin expression. a, Intact platelets (base); b, Thrombin (0.05 U/mL); c, Thrombin (0.05 U/mL) + KRG-TS (25 μg/mL); d, Thrombin (0.05 U/mL) + KRG-TS (50 μg/mL); e, Thrombin (0.05 U/mL) + KRG-TS (100 μg/mL); f, Thrombin (0.05 U/mL) + KRG-TS (150 μg/mL). (B) Effects of KRG-TS on thrombin-induced P-selectin expression (%). Determination of P-selectin expression was carried out as described in “Materials and Methods” section. The data are expressed as the mean ± standard deviation (n=3). *P<0.05 versus the thrombin-stimulated human platelets, **P<0.01 versus the thrombin-stimulated human platelets.
(Fig. 5C, lane 1). However, KRG-TS dose (50 to 150 μg/mL)-dependently inhibited thrombin-induced the phosphorylation of ERK2 and JNK1 (Fig. 5B, lanes 3 to 5), (Fig. 5C lanes 3 to 5).

**Effects of KRG-TS on P-selectin expression**

Compounds in α-granule of platelets are known to involve in inflammation, coagulation and angiogenesis (Broos et al., 2011), these are also Ca²⁺-dependently released by various platelet agonists. In special, P-selectin is involved in inflammation by binding to the P-selectin glycoprotein ligand-1 receptor on monocyte (von Hundelshausen and Weber, 2007; Zarbock et al., 2007). Therefore, we investigated the effect of KRG-TS on P-selectin expression on human platelet surface. Thrombin elevated the expression of P-selectin (Fig. 6A-B) as compared with that by unstimulated platelets (Fig. 6A-a). However, KRG-TS dose (25 to 150 μg/mL)-dependently inhibited thrombin-induced the expression of P-selectin (Fig. 6A-c-f, 6B). The expression of P-selectin is achieved by Ca²⁺ dependent kinase (Nishikawa et al., 1980), and ERK is involved in increase of intracellular Ca²⁺ concentration when platelets are activated (Rosado and Sage, 2002). In addition, JNK1 knockout mouse showed decreased granule release (Adam et al., 2010). Thus, it is thought that KRG-TS suppressed the P-selectin expression is achieved by dephosphorylation of ERK2 and JNK1.

**DISCUSSION**

Aspirin has a clear inhibition of TXA₂ production in platelets through the inactivation of COX-1. Several clinical trials have shown that inhibition of platelet COX-1 activity by aspirin leads the prevention of myocardial infarction and ischemic stroke. Aspirin reduces the risk of serious vascular events, but aspirin has a bleeding problem (Trialists'Collaboration, 2002). Therefore, a compound that can inhibit TXA₂ production has a potential for application as an anti-thrombotic agent.

KRG-TS decreased thrombin-elevated platelet aggregation dose-dependently (Fig. 1B), which is accordance with that KRG-TS inhibited thrombin-induced TXA₂ production (Fig. 2). However, it is not insufficient to understand an inhibitory action of KRG-TS on TXA₂ production. Thus, we tried to explain its inhibitory mechanism by KRG-TS on TXA₂ production by assaying the activities of TXA₂ production-associated COX-1, TXAS, cPLA₂, and p38 MAPK. Because COX-1 and TXAS are localized in endoplasmic reticulum (Patrignani et al., 1999; Needleman et al., 1976), we isolated microsomes from cytosol in platelets, and confirmed the abundant expression of COX-1 (70 kDa) and TXAS (58 kDa) in microsomal fraction (Fig. 3A). We determined effects of KRG-TS on the activities of both enzymes by using microsomal fraction. As the results, KRG-TS inhibited COX-1 and TXAS activity in the presence of KRG-TS (50, 100, 150 μg/mL) directly like aspirin and ozagrel (Fig. 3B, 3C). Next, we focused on thrombin-induced AA release, upstream signaling molecule of TXA₂ and as shown in Fig. 4A, thrombin-induced AA release was decreased by KRG-TS. The AA release-associated signaling molecule, cPLA₂ and p38 MAPK are also diminished by KRG-TS (Fig. 4B, 4C). Comparing the inhibitory ratio of TXA₂ production and its associated enzymes (COX-1, TXAS) by KRG-TS, the inhibitory degree of TXA₂ production by KRG-TS (150 μg/mL) is very high as compared with inhibitory degrees of COX-1 and TXAS by KRG-TS (150 μg/mL). These results mean that the strong inhibition of TXA₂ production by KRG-TS is due to the various inhibitory effects such as inhibition of COX-1, TXAS, AA release and dephosphorylation of cPLA₂ and p38 MAPK.

The progression of atherosclerosis is triggered by inflammatory cell such as monocytes, neutrophils, and macrophages (Phillips et al., 2005). Thus, P-selectin is an important molecule for inflammatory response leading atherosclerosis. Although KRG-TS has antplatelet effects through inhibition of TXA₂ production, if KRG-TS does not inhibit inflammation, atherosclerosis lesion would be generated at injury site of vascular wall. ATP and serotonin released from dense body are known to involve in amplification of platelet aggregation (Mustard and Packham, 1970; Holmsen and Day, 1970) and P-selectin released from α-granule is known to involve in causing of inflammation (von Hundelshausen and Weber, 2007; Zarbock et al., 2007). Because KRG-TS inhibited the P-selectin expression, it is thought that KRG-TS may inhibit aggregation-amplification and inflammation.
In real, many studies about anti-inflammatory activity by KRG-TS have been reported (Byeon et al., 2009; Park and Cho, 2009; Lee et al., 2014).

About dietary of a ginseng extract, it has reported that long-term (4 to 5 years) intake of red ginseng products (i.e. water extract, tea, drink) inhibit platelet aggregation, blood coagulation and hyperlipidemia, an index of atherosclerosis, and their effects were well also sustained in the subjects who have obesity, hyperlipidemia, and hypertension (Lee and Park, 1998; Park et al., 2000). Moreover, it has reported that dietary water-extract of Korean red ginseng (KRG-WE) suppressed rabbit platelet aggregation under hypercholesterolemia (Hwang et al., 2008) and oral administration (250 to 500 μg/kg-BW) of KRG-WE significantly inhibited platelet aggregation ex vivo, and KRG-WE (300, 500 μg/mL) inhibited washed rabbit platelet aggregation in vitro (Yang et al., 2015). With regard to the effects of ginsenosides on platelet aggregation, it is reported that ginsenoside Rg3 has inhibitory effect on collagen-induced blood platelet aggregation (Matsuda et al., 1985) and thromboxane A2 production, ATP release and [Ca2+]i mobilization (Lee et al., 1997). Furthermore, ginsenoside Rg3-enriched red ginseng extract showed inhibitory effects on ERK 1/2, JNK and p38 MAPK (Jeong et al., 2017). In our previous study, we also checked the inhibitory effects of G-Rg3 (20S, 20R) in thrombin induced human platelet (Shin et al., 2015). Moreover, we revealed the inhibitory effects of ginsenoside Ro on vasodilator-stimulated phosphoprotein and clot retraction (Shin et al., 2016). These reports suggest that inhibitory effects of KRG-TS are achieved by ginsenoside Rg3 and ginsenoside Ro. However, the inhibitory mechanism of ginsenosides is not fully understood.

In conclusion, we have revealed that anti-platelet effects of KRG-TS on TXA2 production and P-selectin expression. KRG-TS suppressed the phosphorylation of cPLA2, and p38 MAPK leading TXA2 production. Simultaneously, KRG-TS decreased the phosphorylation of ERK2 and JNK1 leading P-selectin expression. These results show that the anti-platelet effects by KRG-TS are achieved through down regulation of MAPKs phosphorylation, which is useful to understand the anti-platelet effects of ginseng saponin we eat. Therefore, it is thought that KRG-TS would be a great potentiality as a functional food in the therapy and prevention of CVD associated with platelet aggregation.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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