



Schizonepeta tenuifolia inhibits collagen stimulated platelet function *via* suppressing MAPK and Akt signaling

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Abstract

The prevalence of cardiovascular diseases (CVDs) is increasing at a rapid pace in developed countries, and CVDs are the leading cause of morbidity and mortality. Natural products and ethnomedicine have been shown to reduce the risk of CVDs. *Schizonepeta* (*S.*) *tenuifolia* is a medicinal plant widely used in China, Korea, and Japan and is known to exhibit anti-inflammatory, antioxidant, and immunomodulatory activities. We hypothesized that given herbal plant exhibit pharmacological activities against CVDs, we specifically explored its effects on platelet function. Platelet aggregation was evaluated using standard light transmission aggregometry. Intracellular calcium mobilization was assessed using Fura-2/AM, and granule secretion (ATP release) was measured in a luminometer. Fibrinogen binding to integrin $\alpha_{IIb}\beta_3$, was assessed using flow cytometry. Phosphorylation of mitogen-activated protein kinase (MAPK) signaling molecules and activation of the protein kinase B (Akt) was assessed using Western blot assays. *S. tenuifolia* extract potently and significantly inhibited platelet aggregation, calcium mobilization, granule secretion, and fibrinogen binding to integrin $\alpha_{IIb}\beta_3$. Moreover, all extracts significantly inhibited MAPK and Akt phosphorylation. *S. tenuifolia* extract inhibited platelet aggregation and granule secretion, and attenuated collagen mediated GPVI downstream signaling, indicating the potential therapeutic effects of these plant extracts on the cardiovascular system and platelet function. We suggest that *S. tenuifolia* extract may be a potent candidate to treat platelet-related CVDs and to be used as an antiplatelet and antithrombotic agent.

Keywords: *S. tenuifolia*, platelet, MAPK, Akt, natural antithrombotic agent

Introduction

Currently, cardiovascular diseases (CVDs) are the main cause of morbidity and mortality in developed countries^[1]. There are multiple risk factors, but

platelets being a main etiological factor, plays a central role in CVDs. Platelet aggregation is a key step in the development and progression of atherosclerotic plaques, which causes narrowing of the blood vessels that can ultimately lead to stroke and heart attack^[2].

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Hyperactive platelets contribute to thrombosis and are important mediators of atherogenesis. Moreover, intravascular thrombosis is a factor that causes various CVDs. Pharmacological suppression of platelet function has shown great success in reducing thrombotic events, and a number of clinically approved anti-platelet drugs are available to treat cardiovascular ailments. However, these drugs can have serious complications (such as gastric bleeding) and are ineffective in some patients^[3], necessitating the need to develop effective and safer approaches to treat and prevent CVDs. One approach may include the use of natural products, like plant extracts, as antithrombotics and anticoagulants^[4]. Currently, ethnomedicine and natural products are gaining interest as remedies for CVDs^[5], as a number of dietary and herbal compounds have been shown to reduce the risk of CVDs^[6].

Schizonepeta tenuifolia Briq. is a medicinal plant widely found in China, Korea, and Japan and is commonly used for headaches, colds, allergies, and eczema. Studies have shown that this herb has several pharmacological properties, such as anti-inflammatory^[7–9], immunomodulatory^[10–11], antioxidant^[9], and antipruritic^[12] activities. However, cardiovascular effects of this herb have yet to be explored. To date, there is no report on the anti-platelet activity of this medicinal plant. In this study, we evaluated the effects of given plant extract on the cardiovascular system, especially on platelet function, and explored the mechanistic aspects of their anti-platelet and antithrombotic activities.

Materials and methods

Chemicals and reagents

Collagen, ADP and thrombin were purchased from Chrono-log Corp. (Havertown, PA, USA). Fura-2/AM was obtained from Sigma-Aldrich (St. Louis, MO, USA). Fibrinogen Alexa Fluor® 488 conjugate was purchased from Molecular Probes (Eugene, OR, USA), and the ATP assay kit was obtained from Biomedical Research Service Center (Buffalo, NY, USA). Antibodies against phospho-p44/42 (phospho-extracellular signal-regulated kinase (ERK), p44/42 (ERK), MEK, phospho-MEK, stress-activated protein kinase (SAPK)/ c-Jun N-terminal protein kinase (JNK), phospho-SAPK/ JNK, phospho-Akt, and Akt were acquired from Cell Signaling Technology (Beverly, MA, USA). Ultrapure water was obtained from J. T. Baker (Phillipsburg, NJ, USA). All chemicals were reagent grade.

Preparation of *S. tenuifolia* extract

Aerial part of *S. tenuifolia* was collected from Rural Development Administration (RDA), Suwon 441-100, South Korea, in 2005. The powder (100 g) of *S. tenuifolia* was extracted with methanol in accelerated solvent extraction system (Dionex, USA) at 50 °C, and evaporated in rotary evaporator (N-1000, Eyela, Japan). Finally, extract (11 g) from the powder of *S. tenuifolia* was obtained and stored at –30 °C. Powder was dissolved in DMSO for further use and vehicle concentration was kept at less than 0.1%.

Animals

Male Sprague-Dawley (SD) rats (240–260 g) were purchased from Orient Co. (Seoul, Korea) and were acclimatized for one week before conducting the experiments in a special air conditioned animal room with 12/12 hours light/dark cycle at a temperature and humidity of (23±2) °C and (50±10)%, respectively. All animal-related studies were carried out following the Institutional Animal Care and Use Committee (IACUC) guidelines, and the protocols were approved by the Ethics Committee of the College of Veterinary Medicine, Kyungpook National University, Daegu, Korea (Permit number: KNU2013-001). Three to four animals were used for each experiment (two replicates per treatment) with $n = 1$ on each different day.

Platelet preparation

Blood was collected from rats via heart puncture and transferred to a tube containing the anticoagulant, acid citrate dextrose (ACD) solution. Blood was centrifuged at 170 g for seven minutes to obtain platelet-rich plasma (PRP). The PRP was further centrifuged at 350 g for seven minutes to isolate platelets. The concentration of platelets was adjusted to (3×10^8) cells/mL using Tyrode's buffer without calcium (137 mmol/L NaCl, 12 mmol/L NaHCO₃, 5.5 mmol/L glucose, 2 mmol/L KCl, 1 mmol/L MgCl₂, and 1 mmol/L NaHPO₄, pH 7.4), and these platelets were used for aggregation assays. All platelet preparation procedures were performed at room temperature [(23±2) °C].

Platelet aggregation assay and scanning electron microscopy analysis

Platelet aggregation was performed using a standard technique, light-transmission aggregometry (Chronolog Corp., Havertown, PA, USA), as previously described^[13]. Briefly, washed platelets were preincubated with various concentrations of either *S. tenuifolia* extract or vehicle for two minutes at 37 °C in the presence of 1 mmol/L CaCl₂, followed by

stimulation with the agonist, collagen, ADP or thrombin. The mixture was incubated for five minutes with continuous stirring.

A field emission scanning electron microscope (SU8220, Hitachi) was used to assess aggregation ultrastructure at the Center for Scientific Instrument, Kyungpook National University, Daegu, Korea. Briefly, following the collagen-induced platelet aggregation assay, the platelet mixture was fixed with 0.5% paraformaldehyde (first fixation) and osmium tetroxide (second fixation), dehydrated with various concentrations of ethanol, then freeze-dried and scanned.

Intracellular calcium ion concentration ($[Ca^{2+}]_i$) measurements

The $[Ca^{2+}]_i$ was assessed using Fura-2/AM as previously described^[14]. Briefly, platelets were preincubated with 5 μ mol/L Fura-2/AM for one hour at 37 °C. Following incubation, the platelets were washed and treated with *S. tenuifolia* extract for one minute in the presence of 1 mmol/L $CaCl_2$ at 37 °C, followed by stimulation with collagen for two minutes. Fluorescence was recorded using a spectrofluorometer (F-2500, Hitachi, Japan) and $[Ca^{2+}]_i$ was calculated with Schaeffer and Blaustein's method^[15], using the following formula: $[Ca^{2+}]_i$ in cytosol = $224 \text{ nmol/L} \times (F - F_{\min}) / (F_{\max} - F)$, where 224 nmol/L is the dissociation constant of the Fura-2- Ca^{2+} complex and $F_{\min}$ and $F_{\max}$ represent the fluorescence intensity levels at very low and very high Ca^{2+} concentrations, respectively.

ATP release assay

Washed platelets were pre-incubated with *S. tenuifolia* extract in the presence of 1 mmol/L $CaCl_2$ for two minutes at 37 °C, and then were stimulated with collagen for five minutes. The aggregation reaction was terminated and the platelet mixture centrifuged. The supernatant was used to measure ATP secretion with a luminometer (GloMax 20/20, Promega, Madison, WI, USA), using an ATP assay kit (Biomedical Research Service Center).

Flow cytometry

The fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ was quantified using flow cytometry. Briefly, washed platelets were pre-incubated with *S. tenuifolia* extract for two minutes in the presence of 0.1 mmol/L $CaCl_2$, followed by stimulation with collagen for five minutes. The stimulated platelets were treated with fibrinogen Alexa Fluor® 488 conjugate (20 μ g/mL) for five minutes at room temperature $[(23 \pm 2)^\circ C]$, and

were then fixed with 0.5% paraformaldehyde for thirty minutes at 4 °C. Following fixation, platelets were washed thrice in phosphate-buffered saline (PBS) and suspended in 400 μ L PBS. Fluorescence was recorded with a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA), and data were analyzed using CellQuest™ software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Immunoblotting

Washed platelets were pre-incubated with *S. tenuifolia* extract in the presence of 1 mmol/L $CaCl_2$ for two minutes at 37 °C, and then were stimulated with collagen for five minutes. Platelet aggregation was terminated by the addition of lysis buffer (PRO-PREP; iNtRON Biotechnology, Seoul, Korea) to the mixture, followed by the estimation of protein concentration using the BCS assay (PRO-MEASURE; iNtRON Biotechnology, Seoul, Korea). Total platelet proteins were separated using 10% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% skim milk, probed with respective antibodies, and visualized using enhanced chemiluminescence (Advansta, CA, USA) and quantified using Image J software (NIH, Maryland, USA).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's posthoc tests to measure statistical significance among and between measurements (SAS Institute Inc., Cary, NC, USA). All data are presented as the means $\pm$ standard error of the mean (SEM). A *P*-value of 0.05 or less was considered statistically significant.

Results

S. tenuifolia inhibits collagen-induced platelet aggregation

In vitro effects of *S. tenuifolia* extract on platelet aggregation were assessed using light transmission aggregometry. We tested *S. tenuifolia* extract against different ligands (collagen, ADP, and thrombin) and found collagen-induced platelet aggregation to be the most affected by it (**Fig. 1A**). *S. tenuifolia* extract noticeably inhibited collagen-induced platelet aggregation in a concentration-dependent manner (**Fig. 1B**). Activation of platelet receptors (e.g., GPVI or P2Y12) triggers downstream signaling events which causes granule secretion, platelet shape change, fibrin formation and subsequently leading to platelet aggregation. The continuous change in shape from the

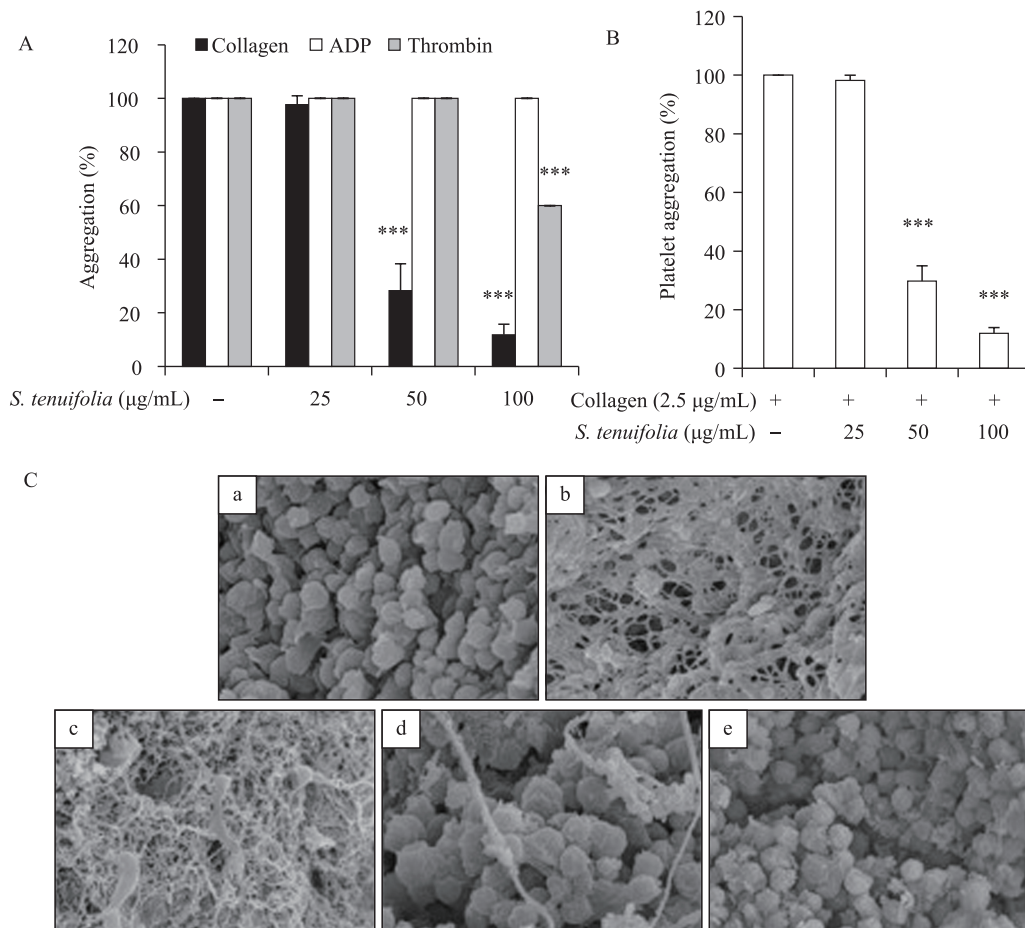


Fig. 1 *Schizonepeta tenuifolia* extract inhibits collagen-stimulated platelet aggregation. A–C: Washed platelets were pretreated with *S. tenuifolia* extract or vehicle for two minutes in the presence of 1 mmol/L CaCl_2 , and then stimulated with collagen (2.5 μg/mL), or ADP (10 μmol/L) or thrombin (0.1 U/mL) for five minutes. C: Scanning electron microscopy was performed in collagen (2.5 μg/mL) treated platelets. Representative scanning electron microscopy images of platelets treated with (a) Resting (b) Vehicle, (c) 25 μg/mL *S. tenuifolia*, (d) 50 μg/mL *S. tenuifolia*, and (e) 100 μg/mL *S. tenuifolia*. Graph represents the mean±SEM of experiments performed on four independent days with $n=1$ on each day. *** $P<0.001$ vs. control.

un-activated to the fully activated platelet is best seen on scanning electron microscopy. We confirmed these results using scanning electron microscopy to show inhibition of platelet shape change and platelet aggregation. A clear inhibition in platelet shape change can be seen in collagen stimulated platelets pre-treated with increasing concentration of *S. tenuifolia* as compared with collagen stimulated alone platelets (Fig. 1C).

S. tenuifolia attenuates $[\text{Ca}^{2+}]_i$ elevation and inhibits ATP secretion

Calcium mobilization is considered a key step in platelet activation and degranulation^[16]. We, therefore, evaluated the effect of *S. tenuifolia* extract on $[\text{Ca}^{2+}]_i$ mobilization. Extract treatment significantly inhibited collagen-induced $[\text{Ca}^{2+}]_i$ elevation in a concentration-dependent manner (Fig. 2A), which suggests that inhibition of platelet aggregation may be possible. The rapid release of ATP occurs in early phases of platelet

activation^[17], which contributes to platelet aggregation. Therefore, we assessed the effect of *S. tenuifolia* extract on collagen-induced ATP secretion. As shown in Fig. 2B, *S. tenuifolia* extract remarkably inhibited ATP release from dense granules in agonist-induced platelets.

Effect of *S. tenuifolia* on fibrinogen binding to integrin $\alpha_{IIb}\beta_3$

Collagen-induced platelet activation induces a conformational change in integrin $\alpha_{IIb}\beta_3$ and eases the interaction between fibrinogen and integrin $\alpha_{IIb}\beta_3$ (a marker for $\alpha_{IIb}\beta_3$ inside-out signaling), which is an important step for platelet aggregation^[18]. We assessed fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ in the presence of various concentrations of *S. tenuifolia* extract in agonist-stimulated platelets. Fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ was significantly inhibited in the presence of the extract in a concentration-dependent manner (Fig. 3).

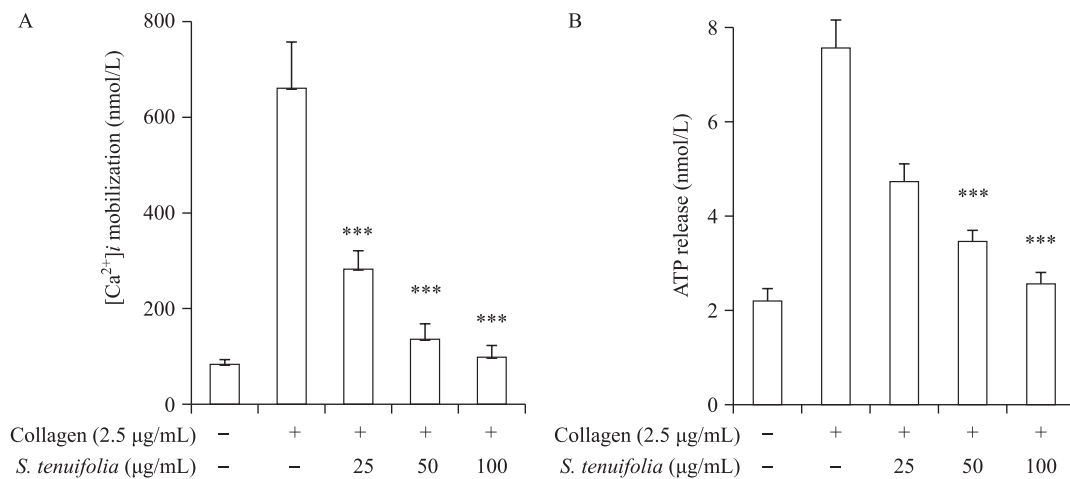


Fig. 2 The inhibitory effect of *Schizonepeta tenuifolia* extract on collagen-stimulated intracellular calcium concentration ([Ca²⁺]_i) elevation and ATP secretion. A: Washed platelets were loaded with a calcium fluorophore (5 µmol/L Fura-2/AM) for one hour. Fura 2/AM-loaded platelets were pretreated with *S. tenuifolia* extract for two minutes at 37 °C and then stimulated with collagen (2.5 µg/mL). B: After platelet aggregation was terminated, the concentration of ATP was assessed in collagen-stimulated platelets treated with *S. tenuifolia*, using a luminometer. Results represent the mean±SEM of experiments performed on four independent days with *n*=1 on each day. ****P*<0.001 vs. control.

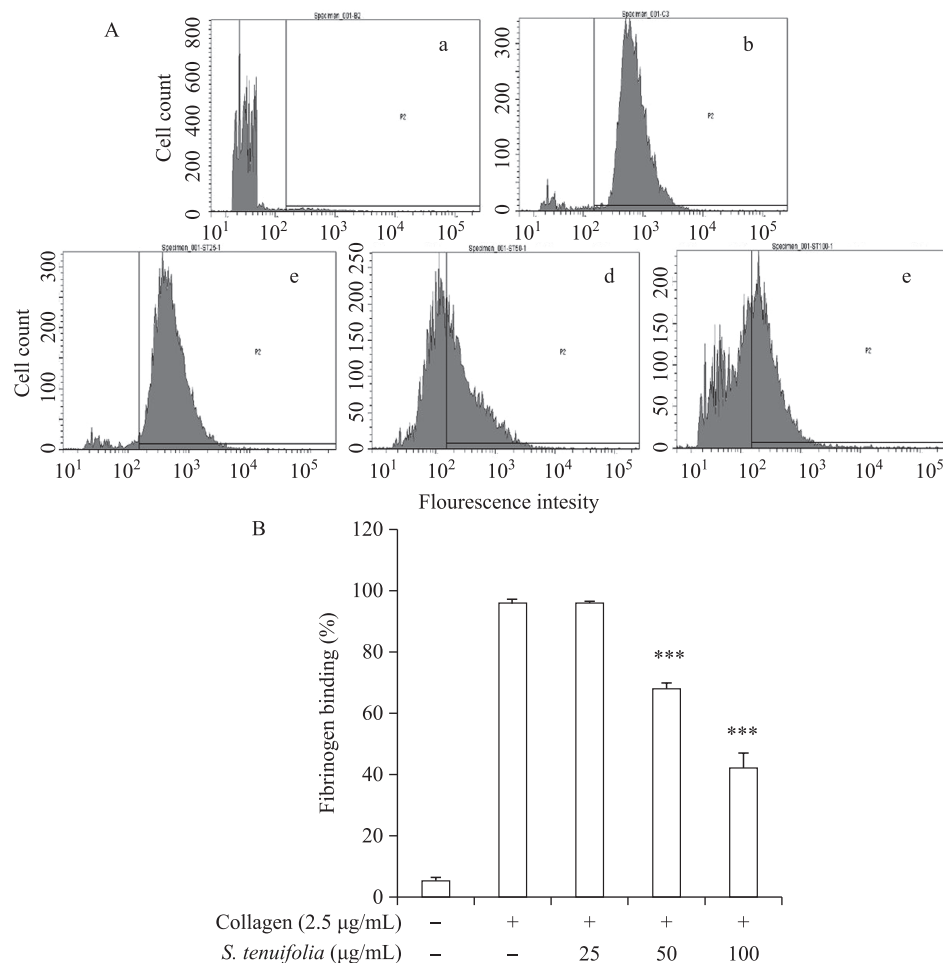


Fig. 3 *Schizonepeta tenuifolia* blocks fibrinogen binding to integrin α_{IIb}β₃ in collagen-induced platelets. A: Flow cytometry was used to measure fibrinogen binding to collagen (2.5 µg/mL)-stimulated platelets after treatment with (a) Resting, (b) Vehicle, (c) 25 µg/mL *S. tenuifolia*, (d) 50 µg/mL *S. tenuifolia*, and (e) 100 µg/mL *S. tenuifolia*. B: Bar graph summarizing the inhibitory effect of *S. tenuifolia* extract on fibrinogen binding to integrin α_{IIb}β₃. Results represent the mean±SEM of experiments performed on four independent days with *n*=1 on each day. ****P*<0.001 vs. control.

S. tenuifolia attenuates mitogen-activated protein kinase (MAPK), MAPK kinase (MEK), and Akt phosphorylation

MAPKs, such as ERK, JNK, and p38, are known to be expressed in platelets and become activated by stimulation with several agonists^[19]. In order to explain the core mechanism of the effects of *S. tenuifolia* extract on agonist-induced platelet activation, we investigated the modulation of the MAPK signaling pathways. *S. tenuifolia* extract reduced the phosphorylation of the MAPK family members, ERK and JNK, in a concentration-dependent manner (Fig. 4A). In addition, *S. tenuifolia* extract inhibited the phosphorylation of MEK (Fig. 4A), an upstream activator of ERK in the MAPK pathway^[20]. Moreover, the PI3K/Akt pathway plays a very important role in platelet function, dense granule secretion, and aggregation^[21]. We found that *S. tenuifolia* extract markedly blocked Akt phosphorylation in a concentration-dependent manner (Fig. 4B).

Discussion

Platelets are small fragments derived from

megakaryocytes that help to maintain hemostasis and prevent blood loss by inducing fibrin clot formation at the site of vascular injury. However, hyperactivation of platelets may prove fatal and contributes to the formation of atherosclerotic plaques within blood vessels, which progressively restricts blood flow, leading to hypoxia and ischemic injury. Plaques can also rupture from vessels making emboli, leading to stroke and myocardial infarction^[22]. A number of antiplatelet drugs are available and have proven to be beneficial in reducing the risk of thrombotic events. However, these drugs can produce undesirable side effects and complications, and they are ineffective in some patients^[23], which necessitates the development of safer approaches to treat CVDs. Natural compounds have been shown to reduce CVDs^[6], and, in our effort to discover alternative compounds, we found *S. tenuifolia*. This medicinal plant has been used in China, Korea, and Japan in traditional remedies^[24–25].

In the present study, we evaluated the inhibitory effects of *S. tenuifolia* extract on platelet aggregation, and explored the mechanistic aspects of these extracts on platelet function. Our results showed that *S. tenuifolia* extract significantly inhibited platelet aggregation induced by collagen and thrombin while having no inhibitory effect against ADP; possibly the

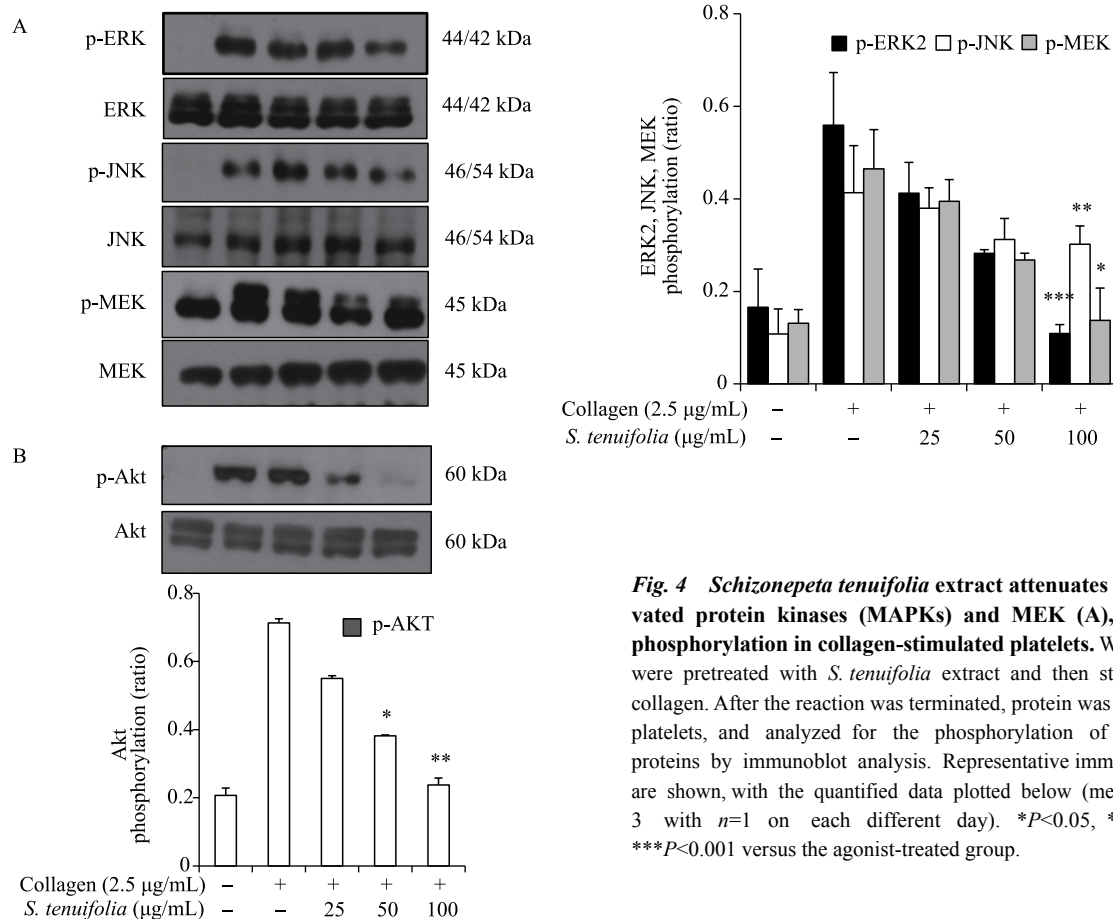


Fig. 4 *Schizonepeta tenuifolia* extract attenuates mitogen-activated protein kinases (MAPKs) and MEK (A), and Akt (B) phosphorylation in collagen-stimulated platelets. Washed platelets were pretreated with *S. tenuifolia* extract and then stimulated with collagen. After the reaction was terminated, protein was extracted from platelets, and analyzed for the phosphorylation of the indicated proteins by immunoblot analysis. Representative immunoblot images are shown, with the quantified data plotted below (mean±SEM of $n=3$ with $n=1$ on each different day). * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ versus the agonist-treated group.

extract exert antiplatelet effects through collagen mediated GPVI signaling and not by P2Y₁₂. Results also showed inhibition of $[Ca^{2+}]_i$ mobilization and dense granule secretion in a concentration-dependent manner. Intracellular calcium plays a critical role in platelet activation, aggregation, and thrombus formation^[26]. Increased $[Ca^{2+}]_i$ triggers granule secretion, which enhances platelet activation, while calcium chelation inhibits ATP release (dense granule secretion). Comparing our results with those in the literature, our data suggests that *S. tenuifolia* extract exerts inhibitory effects on granule secretions to regulate platelet function.

Activation of platelets leads to conformational changes in the structure of integrin $\alpha_{IIb}\beta_3$, which enhances platelet aggregation^[27]. Therefore, integrin inactivation is of great interest in the development of antiplatelet therapy. Altered fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ in response to these conformational changes is also known as inside-out signaling, and later steps involve further signal transduction that is necessary for complete platelet aggregation^[28]. Our results show that fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ was significantly inhibited by pretreatment of platelets with *S. tenuifolia* extract, which indicates that pretreatment of platelets with these extracts may attenuate the conformational changes (*i.e.*, inside-out signaling) and impair integrin $\alpha_{IIb}\beta_3$ activation.

Many studies have shown that platelets are continuously exposed to several factors that cause their activation and aggregation, such as collagen, ADP, thrombin, fibrinogen, von Willebrand factor (vWF), and thromboxane; some factors are also inhibitory, such as prostacyclin (PGI₂) and ADPase^[29]. Any imbalance with these opposing factors may cause impairment in hemostasis, thus, a strong equilibrium is necessary for normal platelet function. Our results indicate that pretreatment of platelets with *S. tenuifolia* extract may contribute to maintaining this balance and hemostasis.

Platelets express MAPK, which includes ERK, JNK, and p38, are activated by several agonists (*e.g.*, collagen, ADP, and thrombin)^[19]. ERK2 has been shown to enhance collagen-stimulated platelet secretion, while p38 is involved in platelet spreading and adhesion. ERK2 and p38 inhibitors have been shown to suppress platelet activation^[30]. Moreover, *S. tenuifolia* Briq has been shown to possess anti-inflammatory properties *via* suppressing MAPK pathway^[7–8]. The involvement of PI3K/Akt pathway in cardiac protection by inducing anti-apoptotic effects and reducing myocardial ischemia reperfusion injury (MI/RI) has been reported^[31] while PI3K/Akt pathway also plays an important role in anti-inflammatory

activities. Therefore, we sought to determine the effect of *S. tenuifolia* on MAPK and PI3K/Akt pathways. Our results show that *S. tenuifolia* extract strongly inhibited PI3K/Akt pathway in a dose dependent manner while having less but inhibitory tendency toward MAPK pathway; possibly due to both are independent pathways in their mode of action. Present data indicates that PI3K/ Akt pathway may be involved in the antiplatelet mechanism of *S. tenuifolia* extract. Furthermore, our findings suggest that *S. tenuifolia* extract inhibit PI3K/ Akt signaling, and its involvement in modulating the reperfusion injury salvage kinase (RISK) pathway.

Chun *et al*^[32] have analyzed the compounds present in *S. tenuifolia* Briq. The GC/MS analysis allowed the tentative identification of 21 compounds, with similarities higher than 85%, in accordance with the NIST/ Wiley mass spectral library. Major components such as (+)-menthone (14.32%), (-)-pulegone (47.73%), 2-hydroxy-2-isopropenyl-5-methylcyclohexane (5.97%), cis-pulegone oxide (4.12%), and schizonal (5.36%) were identified by comparison of retention time and mass spectral data of standards isolated from *S. tenuifolia* Briq. The above mentioned compounds may be possibly involved in anti-platelet effects of *S. tenuifolia* Briq extract.

In conclusions, Our results show that *S. tenuifolia* extract is a potent inhibitor of collagen-induced platelet aggregation, granule secretion, and fibrinogen binding to integrin $\alpha_{IIb}\beta_3$. Moreover, given extract modulate platelet function via impaired MAPK phosphorylation and inactivation of PI3K/Akt pathway, suggesting their antiplatelet and antithrombotic potential. We suggest that given plant extract is a potent antithrombotic candidate that can be used to treat platelet-related cardiovascular disorders.

Acknowledgments

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