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The Inhibitory Effects of *Protaetia brevitarsis seulensis* Larvae Extract on Human Platelet Aggregation and Glycoprotein IIb/IIIa Expression

Hyuk-Woo Kwon[1,2], Man Hee Rhee[3,4], and Jung-Hae Shin[3,4]

[1]Department of Biomedical Laboratory Science and [2]Microbiological Resource Research Institute, Far East University, Chungbuk 27601, Korea
[3]Department of Veterinary Medicine, College of Veterinary Medicine, Kyungpook National University, Daegu 41566, Korea
[4]Cardiovascular Research Institute, School of Medicine, Kyungpook National University, Daegu 41944, Korea

ABSTRACT: The white-spotted flower chafer, *Protaetia brevitarsis seulensis*, is used as a traditional remedy against liver cirrhosis, hepatitis, and hepatic cancer. In this study, we investigated if *P. brevitarsis* extract (PBE) inhibited platelet aggregation via integrin $\alpha IIb/\beta_3$ regulation. We observed that PBE inhibited $\alpha IIb/\beta_3$ activation by regulating the cyclic nucleotides, cyclic adenosine monophosphate and cyclic guanosine monophosphate. Additionally, PBE affected phosphatidylinositol-3 kinase, Akt, SYK, glycogen synthase kinase-3α/β, cytosolic phospholipase A_2, and p38 expression, which are signal transduction molecules expressed by platelets, and consequently suppressed $\alpha IIb\beta_3$ activity and thromboxane A_2 generation. Taken together, PBE showed strong antiplatelet effects and may be used to block thrombosis- and platelet-mediated cardiovascular diseases.

Keywords: cyclic nucleotides, glycoprotein IIb/IIIa, platelet aggregation, thrombosis

INTRODUCTION

Platelets form blood clots to maintain cellular hemostasis. Therefore, correct platelet regulation is required to suppress harmful events during cardiovascular disease, thus antithrombosis therapies are required to specifically target platelet inhibition pathways (Jackson, 2011). However, contrary to expectations, many antiplatelet agents do not ameliorate cardiovascular disease mortality rates (Lee et al., 2021), which often occur due to sudden symptom onset. Therefore, researchers must focus on prevention rather than cure, and identify natural materials which prevent these diseases. Generally, natural materials elicit few side effects, and those products with antiplatelet effects can prevent thrombosis and cardiovascular disease via regular administration (Irfan et al., 2020; Sharifi-Rad et al., 2020). Therefore, in an attempt to identify new antithrombosis drugs, we focused on the antiplatelet activity of *Protaetia brevitarsis seulensis* extract (PBE) from larvae. *P. brevitarsis* larvae are used in ethnomedicine to combat liver cirrhosis, hepatitis, and hepatic cancer (Yoo et al., 2022). In Korea, *P. brevitarsis* is registered as a food material by the Ministry of Food and Drug Safety, therefore, we evaluated PBE effects on platelet activity.

Damaged blood vessels contain exposed collagen fibers which bind to integrins on platelets (Moroi and Jung, 2004), initiates platelet activation, and elevates calcium (Ca^{2+}) concentrations in the cytosol. The first mechanism involved in calcium regulation is Ca^{2+} mobilization. Platelet activation thus releases calcium from the endoplasmic reticulum into the cytosol (Varga-Szabo et al., 2009). Next, a deficiency of stored Ca^{2+} in the endoplasmic reticulum can facilitate extracellular Ca^{2+} influx. Elevated intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) activate Ca^{2+}-dependent kinases which trigger granule release (Farndale, 2006). This agonist-induced signaling cascade or "inside-out signaling" activates glycoprotein IIb/IIIa (integrin $\alpha IIb/\beta_3$), which then binds to other platelets via adhesive proteins (fibrinogen and fibronectin), and $\alpha IIb/\beta_3$-mediated signaling triggers platelet aggregation (Phillips et al., 2001). Therefore, in this study, we examined the inhibitory effects of PBE on platelet aggregation.

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Correspondence to Jung-Hae Shin, E-mail: mlsjshin@naver.com

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MATERIALS AND METHODS

Materials

P. brevitarsis larvae were obtained from Chungbuk Agricultural Research and Extension Services. Dried larvae were pulverized in a grinder and powdered material successively extracted in 70% ethanol (350 mL) using a Soxhlet apparatus at 150°C for 1 h. The PBE was concentrated in a vacuum evaporator, lyophilized, redissolved in dimethyl sulfoxide, and adjusted to 100 mg/mL. Human platelets were obtained from the Korean Red Cross Blood Center. Phospho-IP_3R, Phospho-vasodilator-stimulated phosphoprotein (VASP) (Ser^{157} and Ser^{239}), Phospho-PI_3K, Phospho-Akt (Ser^{473} and Thr^{308}), Phospho-GSK-3α/β, β-actin, phosphor-SYK, phosphor-p38, phosphor-ERK (1/2), and phosphor-cytosolic phospholipase A_2 ($cPLA_2$) antibodies were purchased from Cell Signaling Technology. Alexa Fluor 488-conjugated fibrinogen and fura 2-acetoxymethyl (Fura-2 AM) antibodies were purchased from Invitrogen. Platelet agonists, collagen, and thrombin were bought from Chrono-Log Corp.. Cyclic nucleotides [cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)] assay kits and thapsigargin were purchased from Cayman Chemical.

Platelet aggregation analysis

Platelets were separated and washed in washing buffer (pH 6.5) and adjusted in suspension buffer (pH 6.9) to 10^8/mL. PBE was poorly soluble in water, therefore it was dissolved in dimethyl sulfoxide (0.1%). Platelets (10^8/mL) were preincubated with different PBE concentrations (75, 100, 150, and 200 μM) at 37°C while stirring, and collagen was added for full platelet aggregation using an aggregometer (Chrono-Log Corp.).

Cytotoxicity analysis

We investigated if PBE concentrations affected lactate dehydrogenase (LDH) levels in platelets. Platelets (10^8/mL) were preincubated with different PBE concentrations for 15 min at 37°C while stirring. After centrifugation at 12,000 *g*, supernatants were separated and LDH levels analyzed using an enzyme-linked immunosorbent assay (ELISA) kit and ELISA plate reader (TECAN).

cAMP and cGMP analysis

Platelets (10^8/mL) were preincubated with different PBE concentrations (75, 100, 150, and 200 μM) for 5 min at 37°C. After platelet aggregation was stopped by ethanol (80%), platelets were centrifuged at 500 *g*, and separated supernatants used to determine cyclic nucleotide (cAMP and cGMP) levels using cAMP- and cGMP-ELISA kits and an ELISA plate reader.

Ca^{2+} mobilization and influx analysis

To measure $[Ca^{2+}]_i$, the Grynkiewicz method (Grynkiewicz et al., 1985) was used. Platelets were incubated with Fura-2 AM for 20 min, washed, and platelet concentrations adjusted to 10^8/mL using suspension buffer. Platelets (10^8/mL) were incubated with different PBE concentrations (75, 100, 150, and 200 μM) at 37°C for 5 min and then stimulated with collagen (2.5 μg/mL). To detect Ca^{2+} influx, platelets were stimulated with 1 μM thapsigargin in the presence of 100 μM EGTA, and 2 mM calcium was added at 3 min. Ca^{2+} concentrations were analyzed using a fluorescence spectrophotometer (F-2700, Hitachi).

Western blotting

To investigate phosphorylation events, platelet aggregation was performed and platelet lysates quantified. Proteins were separated by electrophoresis and then transferred to polyvinylidene fluoride membranes. Primary antibodies were incubated with membranes overnight at 4°C, and after washing (Tris-buffered saline plus 0.1% Tween 20), a secondary antibody was added and incubated with membranes at room temperature for 2 h. Then, protein signals were developed in a darkroom. Western blotting results were calculated using the Quantity One program (Bio-Rad Laboratories).

Analyzing $\alpha IIb/\beta_3$ binding to fibrinogen

To examine fibrinogen binding, fibrinogen dye (Alexa Fluor 488) was used in platelet aggregation experiments. During platelet aggregation, artificial fibrinogen binds to activated platelet integrin $\alpha IIb/\beta_3$ and induces strong aggregation. Platelet binding to fibrinogen dye increases fluorescence and if $\alpha IIb/\beta_3$ activity is inhibited by PBE, fluorescence is reduced. We tested platelet aggregation using different PBE concentrations (75, 100, 150, and 200 μM) for 5 min. Then, platelet and fibrinogen dye binding was fixed in paraformaldehyde, transferred to flow cytometry tubes, and binding forces analyzed using a BD Biosciences flow cytometer.

Analyzing $\alpha IIb/\beta_3$ adhesion to fibronectin

Fibronectin is a plasma protein and functions as an adhesive protein to bind platelet integrin $\alpha IIb/\beta_3$. Therefore, we analyzed $\alpha IIb/\beta_3$ activity in fibronectin-coated wells. Platelets and different PBE concentrations (75, 100, 150, and 200 μM) were added to fibronectin-coated wells and stimulated by collagen. In normal reactions, platelets adhere to fibronectin-coated wells to form thin films. After reactions, wells were washed twice in buffer, and platelet layers stained using cell staining solution. After this, extract solution was added to extract stained platelet layers and absorbances analyzed using an ELISA plate reader to determine platelet adhesion.

Analyzing thromboxane A_2 (TXA_2)

Activated platelets synthesize TXA_2 via an "inside-out signaling cascade." TXA_2 acts as a strong agonist and is quickly converted to thromboxane B_2 (TXB_2), which was measured. After collagen-induced platelet aggregation with PBE, indomethacin was added to stop reactions and mixtures centrifuged briefly to generate TXB_2-containing supernatants, which were analyzed using an ELISA plate reader.

Data analysis

Results were expressed as the mean±standard deviation; the number of observations varied between different groups. To determine significant differences between groups, we used one way analysis of variance, and the Tukey–Kramer method was used for *posthoc* comparisons in IBM SPSS Statistics 21 software (IBM Corp.). A $P<0.05$ value was considered statistically significant.

RESULTS

Platelet aggregation and cytotoxicity

We used collagen (2.5 μg/mL) to promote full platelet aggregation; platelets (10^8/mL) were stirred for 2 min with different PBE concentrations, reacted for 5 min, and then collagen added. As shown Fig. 1A, platelets stimulated with collagen were strongly aggregated, but were dose-dependently inhibited by PBE; the half maximal inhibitory concentration was 119.5 μg/mL (Fig. 1B). To confirm PBE cytotoxic effects toward platelets, LDH release after platelet incubation with PBE was analyzed. As shown Fig. 1C, PBE did not affect cytotoxicity.

Variations in cAMP and cGMP levels

The most well-known inhibitory molecules secreted form platelets are cyclic nucleotides (cAMP and cGMP), which are synthesized from nitric oxide and prostacyclin in endothelial cells (Haslam et al., 1978). In platelets, inositol 1,4,5-triphosphate receptor (IP_3R), Rap1b, glycoprotein Ibβ, phosphodiesterase 3, and VASP are major cAMP and cGMP substrates. These signaling molecules can affect $[Ca^{2+}]_i$ mobilization and $\alpha IIb/\beta_3$ activity (Schwarz et al., 2001). As shown Fig. 2, PBE increased cAMP and cGMP concentrations.

IP_3R-, ERK-, and p38-phosphorylation

We examined calcium concentrations and the phosphorylation of Ca^{2+}-related signaling molecules. We first focused on Ca^{2+} mobilization. As shown Fig. 3A, collagen addition increased Ca^{2+} mobilization but was dose-dependently suppressed by PBE. Ca^{2+} regulation also occurs via Ca^{2+} influx, therefore, we investigated if PBE affected this. As shown Fig. 3B, thapsigargin-elevated Ca^{2+} influx was dose-dependently decreased by PBE.

Next, we investigated if PBE could control IP_3R phosphorylation and ERK dephosphorylation. IP_3R is located on the surface of the endoplasmic reticulum and cAMP and cGMP are negative regulators of Ca^{2+} mobilization. IP_3R phosphorylation by cAMP/cGMP-dependent kinases can block Ca^{2+} mobilization. In addition, the depletion of stored Ca^{2+} can initiate Ca^{2+} influx, and ERK is an impor-

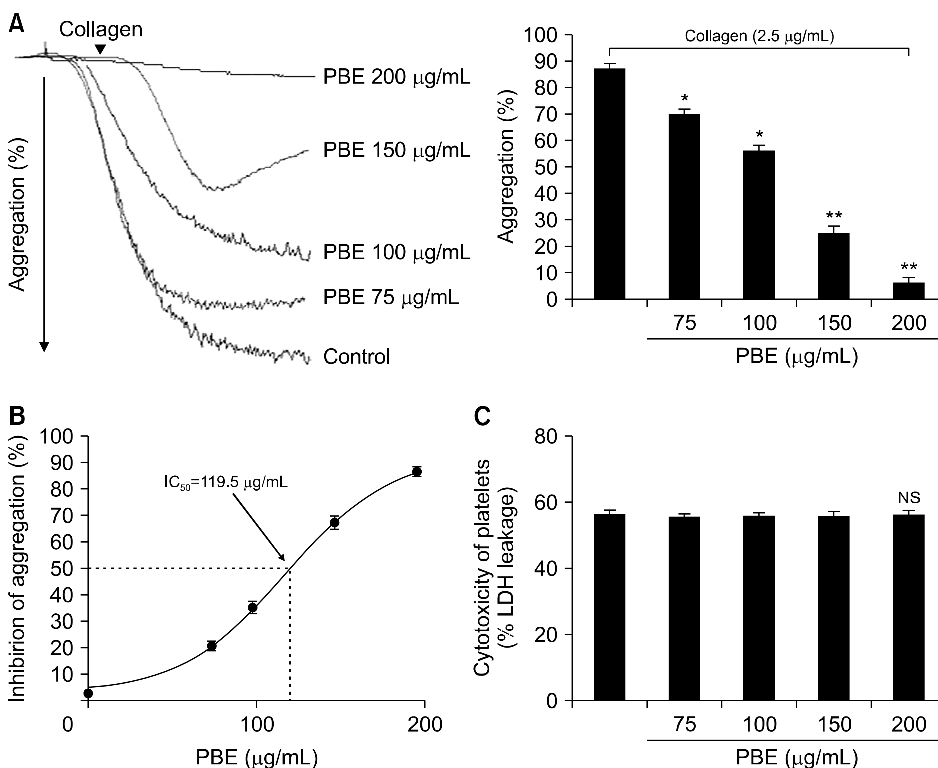

Fig. 1. *Protaetia brevitarsis seulensis* extract (PBE) effects on platelet aggregation. (A) PBE effects on collagen-induced human platelet aggregation. (B) Half maximal inhibitory concentration (IC_{50}) value of PBE on collagen-induced human platelet aggregation. (C) PBE effects on cytotoxicity. Data are presented as the mean±SD (n=4). *P<0.05, **P<0.01 vs. collagen-stimulated human platelets. NS, not significant.

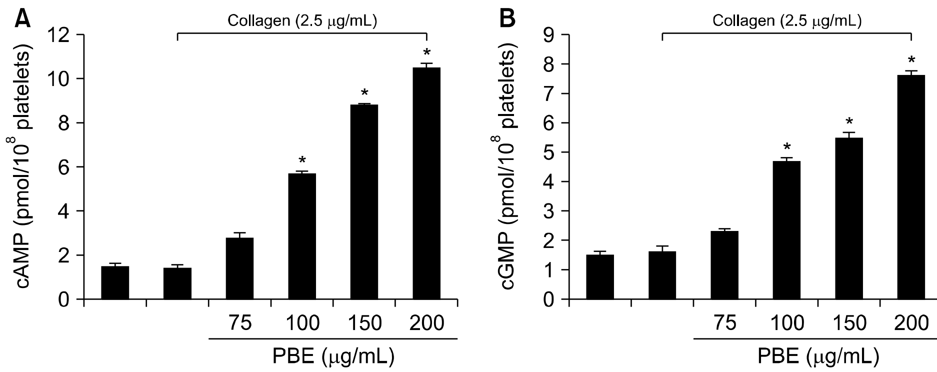

Fig. 2. *Protaetia brevitarsis seulensis* extract (PBE) effects on cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) production. (A) PBE effects on collagen-induced cAMP production. (B) PBE effects on collagen-induced cGMP production. Data are presented as the mean±SD (n=4). *P<0.05 vs. collagen-stimulated human platelets.

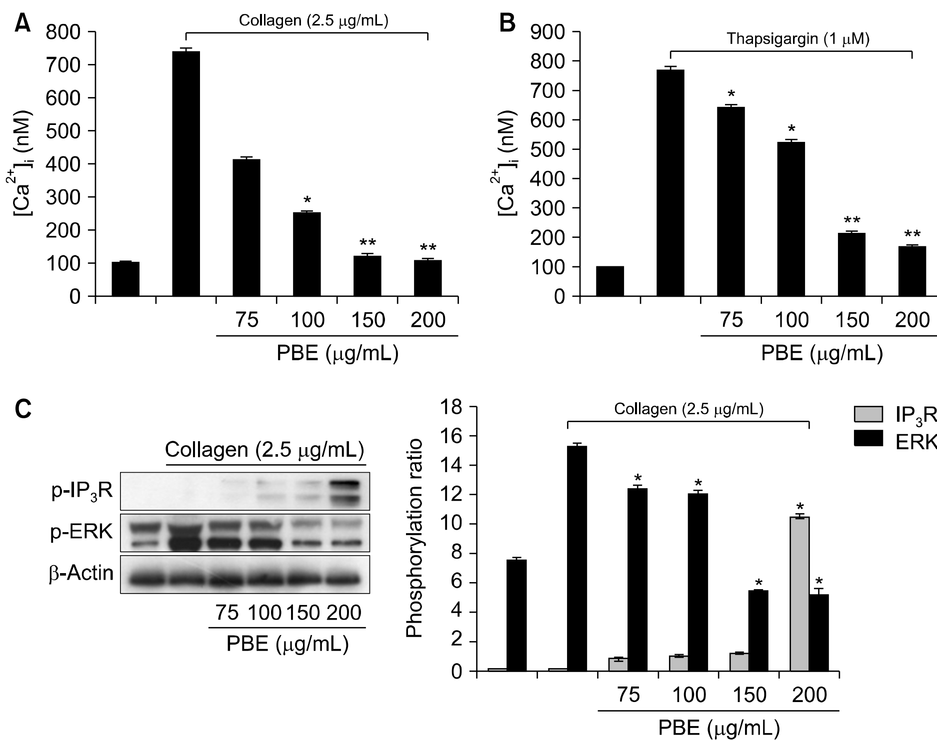

Fig. 3. *Protaetia brevitarsis seulensis* extract (PBE) effects on $[Ca^{2+}]_i$ mobilization and IP_3R/ERK/p38 phosphorylation. (A) PBE effects on collagen-induced $[Ca^{2+}]_i$ mobilization. (B) PBE effects on thapsigargin-induced Ca^{2+} influx. (C) PBE effects on collagen-induced IP_3R and ERK phosphorylation. Data are presented as the mean±SD (n=4). *P<0.05, **P<0.01 vs. agonist (collagen or thapsigargin-stimulated human platelets).

tant factor controlling this (Rosado and Sage, 2001). We observed that PBE increased IP_3R phosphorylation and decreased ERK phosphorylation when induced by collagen (Fig. 3C).

TXA_2, $cPLA_2$-, and p38-phosphorylation

TXA_2 acts as an agonist which stimulates platelet activation (Needleman et al., 1976). As shown Fig. 4A, TXA_2 was dose-dependently inhibited by PBE. It is accepted

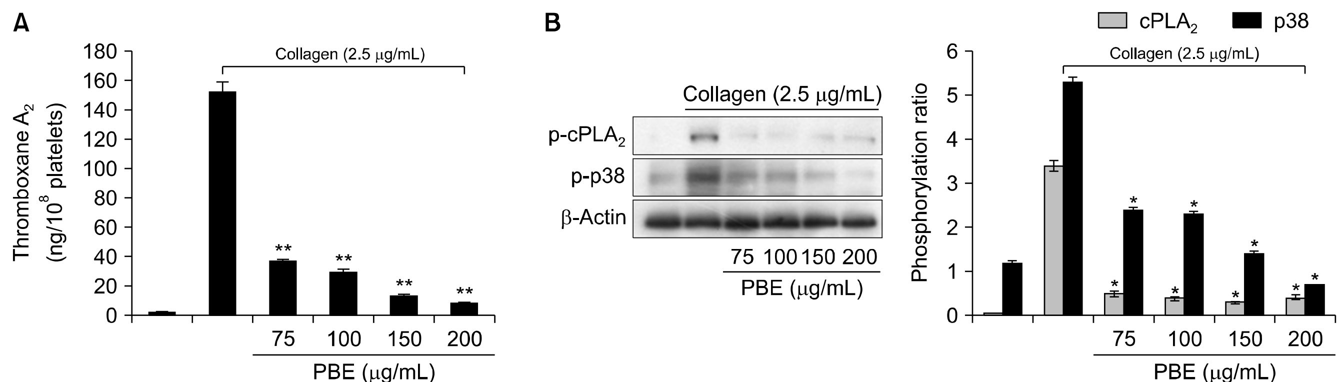

Fig. 4. *Protaetia brevitarsis seulensis* extract (PBE) effects on thromboxane A_2 generation and $cPLA_2$/p38 phosphorylation. (A) PBE effects on collagen-induced thromboxane A_2 generation. (B) PBE effects on collagen-induced $cPLA_2$/p38 phosphorylation. Data are presented as the mean±SD (n=4). *P<0.05, **P<0.01 vs. collagen-stimulated human platelets.

that two signaling molecules affect TXA_2 synthesis; $cPLA_2$ and mitogen-activated protein kinase p38 (p38) are TXA_2 regulators (Kramer et al., 1996). As shown Fig. 4B, collagen-elevated $cPLA_2$ and p38 phosphorylation was inhibited by PBE.

Fibrinogen binding and fibronectin adhesion

Next, we examined $\alpha IIb/\beta_3$ function, which can affect platelet aggregation and adhesion. As shown Fig. 5A and 5B, PBE suppressed collagen-elevated binding forces. To confirm PBE effects on $\alpha IIb/\beta_3$ activity, we analyzed its activity using fibronectin. As shown Fig. 5C, PBE strongly suppressed platelet adhesion and reduced $\alpha IIb/\beta_3$ activity. Thus, PBE inhibited $\alpha IIb/\beta_3$ structural changes.

PI_3K-, Akt-, GSK-3-, SYK-phosphorylation, and VASP-dephosphorylation

We next analyzed the phosphorylation of $\alpha IIb/\beta_3$-related signaling molecules (PI_3K/Akt/GSK-3/SYK/VASP), which are essential regulators of the $\alpha IIb/\beta_3$-mediated signaling cascade (Sudo et al., 2003; Guidetti et al., 2015). We confirmed that PBE significantly reduced PI_3K/Akt/GSK-3/SYK-phosphorylation and elevated VASP (Ser^{157}, Ser^{239}) phosphorylation (Fig. 5).

DISCUSSION

In normal circulation, vascular endothelial cells release prostaglandin I_2 and nitric oxide to promote cAMP and cGMP nucleotide production, which are the most important second messengers involved in the negative feedback of platelet actions (Smolenski, 2012) and are regulated by adenylate, guanylate cyclase, and phosphodiesterases (Haslam et al., 1999; Gresele et al., 2011). The cAMP/cGMP-dependent protein kinase phosphorylates various substrates such as actin binding protein, heat shock protein 27, G-protein α_{13} subunit, glycoprotein Ibβ subunit, inositol 1,4,5-trisphosphate receptor, Rap1b, caldesmon, VASP, and phosphodiesterase 3 (Schwarz et al., 2001). Therefore, we investigated if PBE affected cAMP and cGMP production and showed that it increased these levels (Fig. 5B).

Next, we examined if PBE regulated $[Ca^{2+}]_i$ mobilization and Ca^{2+} influx levels via phosphorylation and dephosphorylation mechanisms, as shown Fig. 3A, PBE inhibited $[Ca^{2+}]_i$ mobilization. Another Ca^{2+} regulation pathway occurs via influx, therefore, we evaluated thapsigargin-induced Ca^{2+} influx effects by PBE. As shown Fig. 3B, thapsigargin-induced Ca^{2+} influx was suppressed by PBE. Additionally, we confirmed that PBE regulated Ca^{2+} signaling by regulating IP_3R phosphorylation and ERK dephosphorylation (Fig. 3C).

TXA_2 is a platelet activator, therefore we examined if PBE could regulate TXA_2 concentrations and associated signaling molecules ($cPLA_2$ and p38). p38 activates $cPLA_2$ via phosphorylation (Kramer et al., 1996). Activated $cPLA_2$ hydrolyzes polyunsaturated fatty acids in mem-

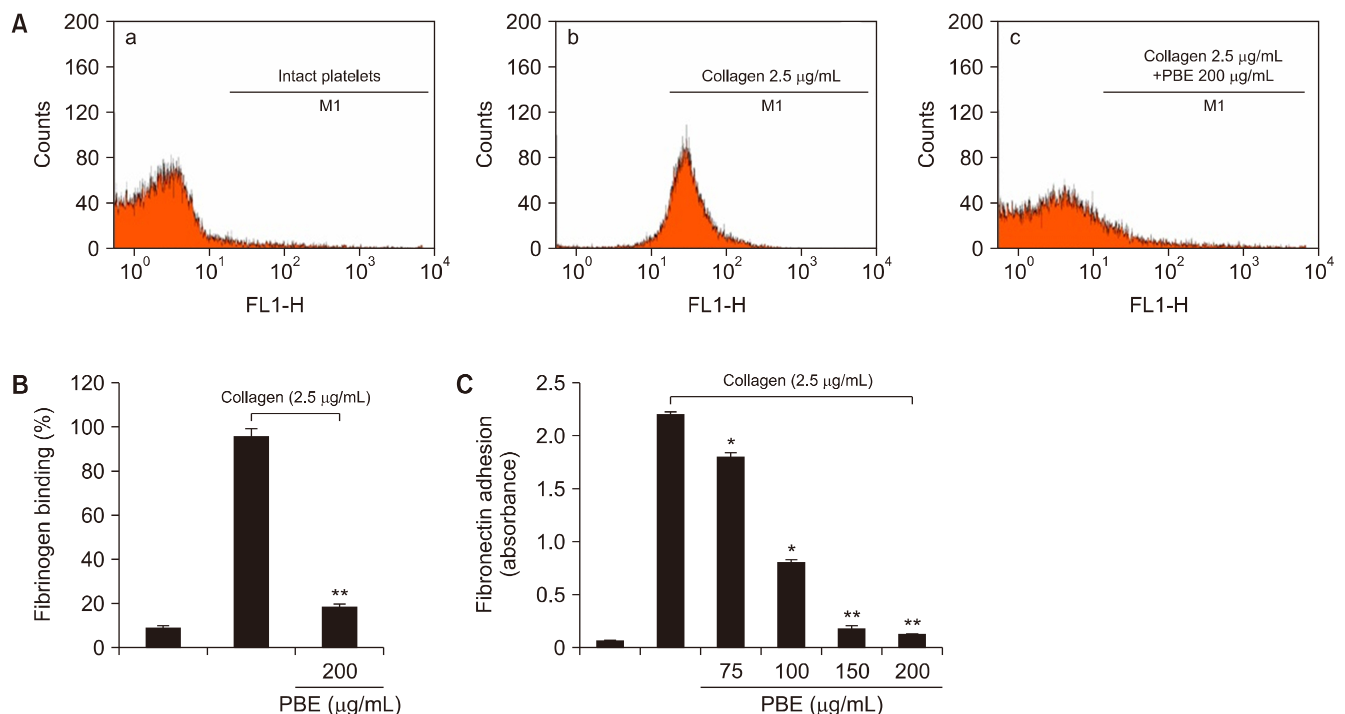

Fig. 5. *Protaetia brevitarsis seulensis* extract (PBE) effects on fibrinogen binding to $\alpha IIb/\beta_3$ and fibronectin adhesion. (A) Flow cytometry histograms show fibrinogen binding. (B) PBE effects on collagen-induced fibrinogen binding (%). (C) PBE effects on collagen-induced fibronectin adhesion. Data are presented as the mean±SD (n=4). $^*P<0.05$, $^{**}P<0.01$ vs. collagen-stimulated human platelets.

brane phospholipids, with arachidonic acid release. Next, cyclooxygenase-1 and TXA_2 synthase generate TXA_2, which is released from the platelet cytoplasm and activates other platelets (FitzGerald, 1991). We confirmed that PBE suppressed TXA_2 levels via the dephosphorylation of signaling molecules (Fig. 4).

The $\alpha IIb/\beta_3$-mediated signaling cascade is important in hemostasis. Therefore, we investigated $\alpha IIb/\beta_3$ activity and related signaling molecules. As shown Fig. 5, PBE suppressed fibrinogen binding and fibronectin adhesion. We next evaluated if PBE could inhibit $\alpha IIb/\beta_3$-related signaling molecules. PI_3K/Akt/GSK-3 is a signaling molecule that facilitates $\alpha IIb/\beta_3$ activation (Chen et al., 2004; Moroi and Watson, 2015; Valet et al., 2016; Moore et al., 2021). SYK is a 72 kDa tyrosine kinase that is stimulated and phosphorylated by platelet agonists adenosine diphosphate, thrombin, and collagen, and also functions after $\alpha IIb/\beta_3$ activation and promotes platelet aggregation (Clark et al., 1994; Keely and Parise, 1996). VASP helps regulate actin filament dynamics and platelet shape, but its phosphorylation can inhibit platelets. We investigated PBE effects on PI_3K/Akt/GSK-3 phosphorylation and showed (Fig. 6) that PBE inhibited collagen-elevated PI_3K, Akt (Ser^{473} and Thr^{308}), and GSK-3α/β phosphorylation. Additionally, PBE decreased collagen-induced SYK and elevated VASP (Ser^{157}, Ser^{239}) phosphorylation. Therefore, PBE inhibited $\alpha IIb/\beta_3$ activation via $\alpha IIb/\beta_3$-related signaling molecules.

To characterize PBE effects on platelet aggregation, component analysis research was conducted and alkaloid components identified (Lee et al., 2017). Among alkaloids, 5-hydroxyindolin-2-one (5-HI) and (1R,3S)-1-methyl-1,2,3,4-tetrahydro-b-carboline-3-carboxylic acid showed antiplatelet and anticoagulation activity and also bleeding time elongation (Lee et al., 2017; Choi et al., 2019). In our previous study, we used 5-HI to evaluate antiplatelet mechanisms in platelets and identified several inhibitory signaling molecules (Kwon et al., 2022). Therefore, antiplatelet PBE effects may be due to indole alkaloid effects, thus these putative PBE components should be characterized and confirmed.

Finally, we showed that PBE decreased platelet aggregation, intracellular Ca concentrations, and $\alpha IIb/\beta_3$ activation via cyclic nucleotides and phosphoprotein regulation. Therefore, *P. brevitarsis* is useful in antithrombosis applications.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

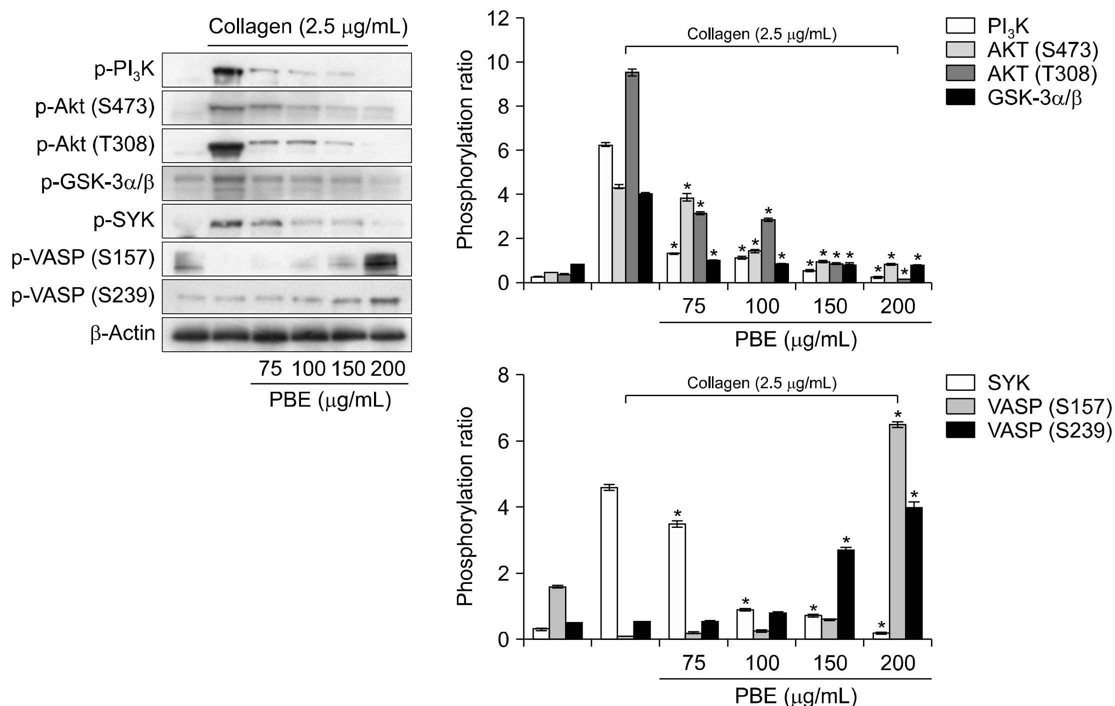

Fig. 6. *Protaetia brevitarsis seulensis* extract (PBE) effects on PI_3K/Akt/GSK-3α/β, SYK, and VASP phosphorylation. Data are presented as the mean±SD (n=4). *P<0.05 vs. collagen-stimulated human platelets.

AUTHOR CONTRIBUTIONS

Concept and design: JHS. Analysis and interpretation: HWK. Data collection: HWK. Writing the article: MHR, JHS. Critical revision of the article: HWK. Final approval of the article: all authors. Statistical analysis: HWK. Obtained funding: JHS. Overall responsibility: JHS.

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