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Research Article

Sappanchalcone Attenuates Platelet Activation and Clot Retraction Through Coordinated Modulation of Cyclic Nucleotides and PI3K/MAPK Signaling Pathways

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Abstract

Background: Aberrant platelet activation is a major contributor to thrombotic disorders, prompting interest in naturally derived candidates that can safely regulate platelet function. Sappanchalcone, a chalcone-type flavonoid derived from *Caesalpinia sappan* L., has diverse biological activities; however, its effects on platelet activation and clot retraction remain unclear. This study evaluated the antiplatelet potential of sappanchalcone and its underlying mechanisms in human platelets. **Methods:** Washed human platelets and platelet-rich plasma were used to assess platelet activation and clot retraction, respectively. Samples were pretreated with sappanchalcone (16–128 μM, depending on the assay) and stimulated with collagen or thrombin. Platelet aggregation and cytotoxicity were assessed by light transmission aggregometry and lactate dehydrogenase (LDH) leakage assay. cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), and thromboxane B_2 (TXB_2) levels were quantified using enzyme-linked immunosorbent assay (ELISA) kits, and intracellular Ca^{2+} mobilization was measured by Fura-2/AM-based fluorescence analysis. Protein phosphorylation was examined by Western blotting, whereas integrin αIIbβ$_3$–fibrinogen binding was analyzed by flow cytometry. Dense granule secretion was evaluated using adenosine triphosphate (ATP) luminescence and serotonin ELISA assays. Thrombin-induced clot retraction was quantified by ImageJ (v1.54k, U.S. National Institutes of Health, Bethesda, MD, USA)-based residual clot area analysis. Data are presented as mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer post hoc test (n = 4; $p < 0.05$). **Results:** Sappanchalcone concentration-dependently inhibited collagen-induced platelet aggregation, reducing aggregation from 83.0% to 10.7% at 128 μM, with a half-maximal inhibitory concentration (IC_{50}) of 57.16 μM, without cytotoxicity. At 128 μM, sappanchalcone increased cAMP from 7.02 ± 0.85 to 11.96 ± 0.57 pmol/10^8 platelets and reduced Ca^{2+} mobilization from 711.97 ± 47.29 to 66.11 ± 6.83 nM. Vasodilator-stimulated phosphoprotein (VASP) Ser^{157} and Ser^{239} phosphorylation increased by 2.97- and 2.07-fold, respectively, whereas fibrinogen binding decreased from 74.43 ± 5.67% to 21.90 ± 1.25%. Phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 phosphorylation was reduced to 0.69-, 0.21-, 1.81-, 0.27-, and 0.49-fold, respectively. ATP secretion, serotonin release, TXB_2 levels reflecting thromboxane A_2 (TXA_2) generation, cytosolic phospholipase A_2 ($cPLA_2$) phosphorylation, and thrombin-induced clot retraction were also suppressed. **Conclusions:** Sappanchalcone limits platelet activation, aggregation, and clot retraction by enhancing cyclic nucleotide-mediated inhibitory signaling and suppressing PI3K/Akt and mitogen-activated protein kinase (MAPK) phosphorylation. These findings provide mechanistic evidence supporting sappanchalcone as a potential naturally derived antiplatelet candidate.

Keywords: platelet activation; sappanchalcone; cyclic AMP; cyclic GMP; mitogen-activated protein kinases; phosphatidylinositol 3-kinases; proto-oncogene proteins c-akt; clot retraction

1. Introduction

Thrombosis is a pathological condition in which blood abnormally coagulates within blood vessels, leading to vascular occlusion, and is a major cause of various cardiovascular diseases. According to the World Health Organization, cardiovascular diseases account for approximately 32% of global deaths, with an estimated 17.9 million people dying from these disorders each year [1]. A substantial proportion of ischemic heart disease and stroke is directly associated with thrombus formation, highlighting the clinical importance of regulating thrombotic processes [2]. In normal hemostasis, platelets rapidly respond to vascular injury and mediate primary hemostasis; however, excessive platelet activation can lead to pathological thrombosis and serious complications [3]. Therefore, preventive modulation of platelet activation at the early stage may be more effective than therapeutic interventions after thrombus formation [4,5].

Platelet activation is initiated by vascular injury–associated stimuli, such as collagen and von Willebrand factor (vWF), as well as by soluble agonists including U46619, adenosine diphosphate (ADP), and thrombin. These agonists activate intracellular signaling cascades involving phospholipase C (PLC)–dependent Ca^{2+} mobilization and

downstream pathways, including mitogen-activated protein kinases (MAPKs) [6,7]. Activation of MAPKs and cytosolic phospholipase A_2 ($cPLA_2$) promotes granule secretion and thromboxane A_2 (TXA_2) generation, thereby amplifying platelet activation [8,9]. In parallel, signaling through the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway facilitates glycoprotein IIb/IIIa (integrin $\alpha IIb\beta_3$) activation, enabling fibrinogen binding, platelet aggregation, and subsequent clot retraction that stabilizes the developing thrombus [10,11].

However, excessive clot retraction can further narrow the vascular lumen and worsen blood flow obstruction, making this process another potential target for thrombosis regulation [11,12]. Conversely, inhibitory signaling pathways such as cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) and cyclic guanosine monophosphate/protein kinase G (cGMP/PKG) suppress platelet hyperactivation through regulation of inositol 1,4,5-trisphosphate receptor (IP_3R) and vasodilator-stimulated phosphoprotein (VASP) phosphorylation [13,14]. A delicate balance between activating and inhibitory pathways is essential, and disruption of this balance significantly increases the risk of thrombosis.

Recently, concerns regarding adverse effects and resistance associated with long-term use of conventional antiplatelet agents have highlighted the importance of identifying safer, natural product–derived antithrombotic candidates. *Caesalpinia sappan* L. (sappan wood), traditionally used as a medicinal and dye plant across Southeast Asia, contains a variety of flavonoids and phenolic compounds in its heartwood, and numerous studies have reported its anti-inflammatory, antioxidant, and immunomodulatory properties [15,16]. Among its constituents, sappanchalcone is a representative chalcone-type flavonoid isolated from the heartwood, and previous studies have demonstrated its anti-inflammatory and anticancer effects [17,18]. However, the effects of sappanchalcone on platelet activation or thrombus formation have not yet been fully elucidated. Structurally related chalcones, including cardamonin and licochalcone A, have demonstrated antiplatelet activity. Cardamonin has been reported to inhibit arachidonic acid-induced platelet aggregation in human whole blood, whereas licochalcone A suppresses platelet activation and thrombus formation through inhibition of PLCγ2-PKC, Akt, and MAPK signaling pathways [19,20]. Based on these structural and mechanistic similarities, we hypothesized that sappanchalcone may also exert antiplatelet activity.

Accordingly, this study investigated the inhibitory actions of sappanchalcone on platelet activation, aggregation, and clot retraction, with the aim of assessing its potential as a novel antiplatelet and antithrombotic agent. These results are anticipated to offer mechanistic insight into the biological actions of sappanchalcone and to support the development of natural product–derived approaches for the prevention of thrombotic disorders.

2. Materials and Methods

2.1 Preparation of Washed Platelet Suspensions

Platelet-rich plasma (PRP) was supplied by the Korean Red Cross Blood Center (Gyeonggi Red Cross Blood Center, Suwon, Korea). PRP was obtained from eligible healthy volunteer donors who met the blood donation criteria of the Korean Red Cross Blood Center and had not taken any antiplatelet medications prior to blood donation. All experiments involving human platelets were approved by the Institutional Review Board of Namseoul University (IRB No. BR-202310-003-01).

Washed platelet suspensions were prepared according to a previously established protocol, with no modification, as described in our earlier study [21]. Briefly, PRP was subjected to sequential centrifugation to obtain washed platelets, which were resuspended in suspending buffer containing 138 mM NaCl (Cat. No. 7548-4105, Daejung Chemicals & Metals Co., Ltd., Siheung, Gyeonggi-do, Republic of Korea), 2.7 mM KCl (Cat. No. P112143, Aladdin Industrial Corporation, Shanghai, China), 12 mM $NaHCO_3$ (Cat. No. S112338, Aladdin Industrial Corporation, Shanghai, China), 0.36 mM NaH_2PO_4 (Cat. No. S102320, Aladdin Industrial Corporation, Shanghai, China), 0.49 mM $MgCl_2$ (Cat. No. M140955, Aladdin Industrial Corporation, Shanghai, China), and 5.5 mM glucose (Cat. No. G107849, Aladdin Industrial Corporation, Shanghai, China), pH 7.4 and adjusted to a final concentration of 1×10^8 platelets/mL for subsequent experiments. Unless otherwise stated, washed platelet suspensions were maintained at 25 °C or room temperature before use.

2.2 Platelet Aggregation

Platelet aggregation was assessed using washed platelets according to a previously established protocol [21]. Briefly, platelet suspensions (1×10^8 platelets/mL) were pretreated with sappanchalcone at concentrations ranging from 16 to 128 μM in the presence of 2 mM $CaCl_2$ (C3881-500G, Sigma-Aldrich, St. Louis, MO, USA) and subsequently stimulated with collagen (Cat. No. 385, Chrono-Log Corporation, Havertown, PA, USA; 2.5 μg/mL) under continuous stirring. Platelets were preincubated with sappanchalcone for 1 min at 37 °C before agonist stimulation. Aggregation was then monitored for 5 min at 37 °C with continuous stirring at 1000 rpm using a light transmission aggregometer (Chrono-Log Corporation, Havertown, PA, USA). Aggregation responses were continuously monitored, and maximal aggregation (%) was quantified. Vehicle-treated samples containing 0.1% dimethyl sulfoxide (DMSO) were used as controls.

For additional agonist experiments, platelet aggregation was induced by U46619 (Item No. 16450, Cayman Chemical, Ann Arbor, MI, USA; 0.5 μM) or thrombin (Cat. No. 386, Chrono-Log Corporation, Havertown, PA, USA; 0.05 U/mL) under the same assay conditions, unless otherwise indicated.

2.3 Cytotoxicity Assay

Membrane integrity of platelets was evaluated by measuring lactate dehydrogenase (LDH) leakage into the extracellular medium [22]. Washed human platelets were exposed to sappanchalcone (16–128 μM) at 37 °C for 5 min, after which the samples were centrifuged at 10,000 ×g for 2 min and the supernatants were harvested for analysis. LDH enzymatic activity was determined using a commercially available Quanti-LDH PLUS™ kit (BCT-LDHP500; Biomax, Republic of Korea) according to the manufacturer's instructions. Absorbance was measured at 490 nm using a SpectraMax ABS Plus microplate reader operated with SoftMax Pro software (v7.1.0, Molecular Devices, San Jose, CA, USA). The extent of cytotoxicity was expressed as a percentage of total LDH release obtained by detergent-induced platelet disruption, while cell viability was calculated as the complementary value (100 − cytotoxicity). Platelets treated with vehicle alone were used as the reference control.

2.4 Measurement of Cyclic Nucleotides

Intracellular cAMP and cGMP levels were measured following platelet aggregation using an established protocol, as previously described [22]. Briefly, washed platelet suspensions (1×10^8 platelets/mL) were preincubated with sappanchalcone at concentrations ranging from 32 to 128 μM in the presence of 2 mM $CaCl_2$ for 1 min at 37 °C. Platelets were then stimulated with collagen (2.5 μg/mL) for 5 min. Thus, samples for cyclic nucleotide measurement were collected at 5 min after collagen stimulation, corresponding to the endpoint of the aggregation reaction.

Aggregation reactions were immediately terminated by ethanol extraction under ice-cold conditions through the addition of 80% ethanol. After centrifugation at 10,000 ×g for 10 min, the supernatants were dried using a vacuum centrifugal concentrator (NB-503CIR, N-BIOTEK, Bucheon, Korea). at 55 °C under vacuum with the tube lids open and then reconstituted in assay buffer. Cyclic nucleotide levels were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits for cAMP and cGMP (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Absorbance was measured at 410 nm using a SpectraMax ABS Plus microplate reader operated with SoftMax Pro software (v7.1.0, Molecular Devices, San Jose, CA, USA). Concentrations of cAMP and cGMP were calculated from standard curves generated using the standards provided with each kit.

2.5 Measurement of Intracellular Calcium

Intracellular calcium mobilization was measured using a Fura-2/AM–based fluorescence assay, as previously described [23]. Briefly, PRP was loaded with Fura-2/AM (Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA; 5 μM) for 60 min at 37 °C and then washed. Platelets loaded with Fura-2/AM were adjusted to a final concentration of 1×10^8 platelets/mL and treated with sappanchalcone at concentrations ranging from 32 to 128 μM in the presence of 2 mM $CaCl_2$, followed by stimulation with collagen (2.5 μg/mL) for 5 min. Changes in intracellular Ca^{2+} levels were monitored by fluorescence spectrophotometry using a fluorescence spectrofluorometer (Hitachi F-7000, Tokyo, Japan).

2.6 Western Blotting

Immunoblotting was conducted according to a previously reported protocol [23]. In brief, platelet aggregation was induced in the presence of sappanchalcone at concentrations of 32–128 μM, followed by cell lysis and preparation of total protein extracts. Unless otherwise indicated, washed platelets were preincubated with sappanchalcone for 1 min at 37 °C in the presence of 2 mM $CaCl_2$ and then stimulated with collagen (2.5 μg/mL) for 5 min before lysis. Reactions were terminated by adding 1× cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA). Equivalent amounts of protein (15 μg per lane) were resolved by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham™ Hybond™ P 0.45, Cytiva, Marlborough, MA, USA) Membranes were blocked with 3% bovine serum albumin (BSA) in Tris-Buffered Saline with Tween-20 (TBST) for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies (1:1000, Cell Signaling Technology, Danvers, MA, USA). An anti-rabbit IgG, horseradish peroxidase (HRP)-linked secondary antibody (1:2000, Cat. No. 7074, Cell Signaling Technology, Danvers, MA, USA) was applied for 1 h at room temperature. Immunoreactive signals were detected using a chemiluminescent detection system (WesternBright ECL, Advansta, San Jose, CA, USA), and band intensities were determined by densitometric analysis using Quantity One software (v4.5, BioRad, Hercules, CA, USA). Phosphorylated protein levels were normalized to the corresponding total protein levels, and total protein levels were normalized to β-actin or total protein loading control, as appropriate.

2.7 Fibrinogen Binding Assay

The interaction between fibrinogen and platelet surface integrins was evaluated using a flow cytometric approach, adapted from a previously established method [24]. Washed platelets were pretreated with sappanchalcone (16–128 μM) under calcium-containing conditions and subsequently activated by collagen in the presence of fluorescently labeled fibrinogen. Briefly, platelet suspensions (1×10^8 platelets/mL) were incubated with sappanchalcone and 2 mM $CaCl_2$ for 1 min at 37 °C, followed by stimulation with collagen (2.5 μg/mL) in the presence of Alexa Fluor 488-conjugated fibrinogen (30 μg/mL) for 5 min.

After fixation, samples were analyzed by flow cytometry to determine fibrinogen association with inte-

grin αIIbβ$_3$. After stimulation, an equal volume of 0.5% paraformaldehyde was added to fix the platelets, and samples were protected from light throughout the staining, stimulation, fixation, and acquisition steps. Fixed samples were diluted with 1× PBS before acquisition to maintain an appropriate event rate.

Approximately 1 × 10^4 platelet events were acquired per condition using a flow cytometer (FACSCanto II, BD Biosciences, San Jose, CA, USA). Platelets were first identified by forward scatter (FSC) and side scatter (SSC) characteristics, and debris and platelet aggregates were excluded by appropriate gating. Fluorescence-positive events were defined using unstimulated platelets and fibrinogen-negative samples as negative controls. Fibrinogen binding was quantified as the percentage of Alexa Fluor 488-positive platelet events within the parent platelet gate (%Parent) using BD FACSDiva software (v8.0.3, BD Biosciences, San Jose, CA, USA).

2.8 Measurement of ATP and Serotonin Release

Secretion from platelet dense granules was assessed by quantifying extracellular adenosine triphosphate (ATP) and serotonin following platelet activation, based on an established procedure [24]. Washed platelet suspensions (1 × 10^8 platelets/mL) were preincubated with sappanchalcone (32–128 µM) and 2 mM $CaCl_2$ for 1 min at 37 °C and then stimulated with collagen (2.5 µg/mL) for 5 min. Platelet responses were halted by chelating extracellular calcium with EDTA (2 mM), after which the supernatants were isolated for subsequent measurements by centrifugation at 10,000 × g for 10 min. ATP content was determined using an ATP Detection Assay Kit—Luminescence (Cat. No. 700410, Cayman Chemical Company, Ann Arbor, MI, USA) and a luminescent image analyzer (ImageQuant LAS 4000 mini, GE Healthcare, Tokyo, Japan), while serotonin levels were analyzed concurrently using a Serotonin ELISA Fast Track kit (Cat. No. BA E-8900R, Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany), in accordance with the respective assay protocols.

2.9 Measurement of Thromboxane A_2 Production

Thromboxane A_2 production was assessed by measuring its stable metabolite thromboxane B_2 (TXB_2), as previously described [24]. Briefly, platelet suspensions (1 × 10^8 platelets/mL) were treated with sappanchalcone at the indicated concentrations in the presence of $CaCl_2$, and reactions were terminated to prevent further enzymatic activity. Specifically, platelets were preincubated with sappanchalcone (32–128 µM) and 2 mM $CaCl_2$ for 1 min at 37 °C and then stimulated with collagen (2.5 µg/mL) for 5 min. Reactions were immediately stopped by adding ice-cold ethanol to inhibit further enzymatic conversion.

TXB_2 levels were quantified using a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Absorbance was measured at 410 nm using a SpectraMax ABS Plus microplate reader operated with SoftMax Pro software (v7.1.0, Molecular Devices, San Jose, CA, USA). TXB_2 concentrations were calculated from a standard curve generated using the kit-provided standards.

2.10 Thrombin-Induced Clot Retraction

The inhibitory effect of sappanchalcone on thrombin-induced clot retraction was assessed according to a previously described method [24]. PRP was pre-treated with sappanchalcone (32 to 128 µM) and $CaCl_2$ before the addition of thrombin to trigger fibrin clot formation. Briefly, PRP (400 µL) was transferred into polypropylene tubes and mixed with sappanchalcone (32–128 µM) and $CaCl_2$ (2 mM). Clot formation and retraction were initiated by adding thrombin (0.05 U/mL).

The subsequent retraction process was monitored under stationary conditions. Samples were incubated at 37 °C for 20 min under static conditions without agitation. At the end of incubation, clot images were acquired immediately using a digital camera.

To evaluate the degree of retraction, digital images of the clots were captured, and the residual clot area was determined through ImageJ software (v1.54k, National Institutes of Health, Bethesda, MD, USA). For quantification, the clot boundary was manually outlined using the same region-of-interest procedure for all images. The residual clot area was measured using ImageJ software and expressed as mm^2. A larger residual clot area was interpreted as reduced clot retraction.

2.11 Data Processing and Statistical Evaluation

Results are presented as the mean ± standard deviation (SD). Platelet preparations were obtained from at least three donors. Unless otherwise indicated, n = 4 represents four independent biological experiments conducted using separately prepared platelet samples. These independent biological experiments were used to evaluate the reproducibility and consistency of the assay results. Statistical differences among experimental groups were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer post hoc test for multiple comparisons. All analyses were performed using SPSS software (v21.0, IBM Corp. or SPSS Inc., USA), with a *p*-value of less than 0.05 being the threshold for statistical significance.

3. Results

3.1 Inhibition of Collagen-Induced Platelet Aggregation by Sappanchalcone and Preservation of Cell Viability

Collagen (2.5 µg/mL) stimulation increased platelet aggregation up to 83% (Fig. 1A,B). However, aggregation was reduced to 79.7% with 16 µM sappanchalcone, 73.7% with 32 µM, 36.7% with 64 µM, and 10.7% with 128 µM (Fig. 1A,B). In addition, representative real-time light transmission aggregometry traces showed that sappan-

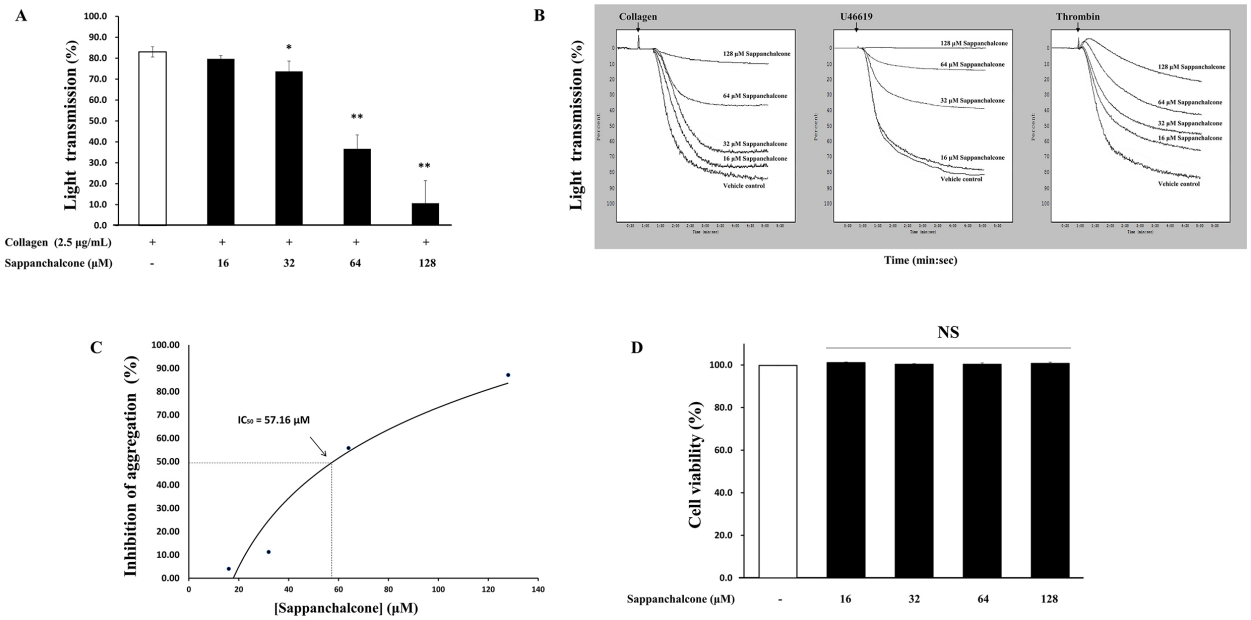

Fig. 1. Effect of sappanchalcone on agonist-induced platelet aggregation and platelet viability. (A) Quantitative analysis of collagen-induced platelet aggregation measured by light transmission aggregometry in the absence or presence of sappanchalcone. (B) Representative real-time light transmission aggregometry traces recorded during platelet aggregation induced by collagen (2.5 μg/mL), U46619 (0.5 μM), or thrombin (0.05 U/mL) in vehicle-treated and sappanchalcone-treated platelets. (C) Half-maximal inhibitory concentration (IC_{50}) value of sappanchalcone against collagen-induced platelet aggregation. (D) Effect of sappanchalcone on the viability of suspended human platelets. NS indicates no significant difference compared with untreated platelets. Data are presented as mean ± standard deviation (SD) from four independent biological experiments (n = 4). Statistical significance is indicated by $*p < 0.05$, $**p < 0.01$ compared with collagen-stimulated vehicle control platelets. NS, not significant.

chalcone also attenuated platelet aggregation responses induced by other agonists, including U46619 (0.5 μM) and thrombin (0.05 U/mL) (Fig. 1B). Based on these data, the half-maximal inhibitory concentration (IC_{50}) value of sappanchalcone was calculated to be 57.16 μM (Fig. 1C). Cell viability did not decrease at any of the concentrations tested, confirming that sappanchalcone exhibited no cytotoxicity toward platelets (Fig. 1D).

These findings demonstrate that sappanchalcone effectively inhibits collagen-induced platelet aggregation while maintaining platelet viability, suggesting that it might be a safe compound for potential therapeutic use.

3.2 Sappanchalcone Increases Cyclic Nucleotides and Enhances IP_3R Phosphorylation, Leading to Inhibition of Intracellular Calcium Mobilization

Compared to the collagen-only stimulated group, sappanchalcone treatment led to a dose-dependent elevation in intracellular cAMP levels (Fig. 2A). Specifically, at a concentration of 128 μM, sappanchalcone increased the cAMP content to 11.96 ± 0.57 pmol/10^8 platelets, representing a 1.70-fold rise from the baseline of 7.02 ± 0.85 pmol/10^8 platelets observed in the absence of the compound (Fig. 2A). A similar upward trend in cGMP levels was also recorded in a dose-responsive manner following sappanchalcone administration (Fig. 2B).

Conversely, sappanchalcone significantly attenuated cytosolic Ca^{2+} mobilization induced by collagen in a concentration-dependent manner (Fig. 2C). Collagen-induced calcium mobilization, which was 711.97 ± 47.29 nM, was strongly inhibited by 128 μM sappanchalcone treatment to 66.11 ± 6.83 nM (Fig. 2C). While the total expression of inositol 1,4,5-trisphosphate receptor (IP_3R) remained unaffected, its phosphorylated form (p-IP_3R) exhibited a progressive increase at sappanchalcone concentrations of 32, 64, and 128 μM (Fig. 2D).

Taken together, these findings suggest that sappanchalcone enhances the levels of cyclic nucleotides (cAMP and cGMP) under collagen-stimulated conditions. This subsequently promotes the phosphorylation of IP_3R, which inhibits the release of calcium into the cytosol, thereby exerting a potent inhibitory effect on platelet activation.

3.3 Sappanchalcone Enhances VASP Phosphorylation and Reduces Fibrinogen Binding

As illustrated in Fig. 3A, sappanchalcone treatment markedly enhanced the phosphorylation of VASP at both Ser^{157} and Ser^{239}, which are recognized as PKA- and PKG-dependent phosphorylation sites, respectively. At a concentration of 128 μM, sappanchalcone induced a 2.97-fold increase in VASP Ser^{157} phosphorylation and a 2.07-fold increase in VASP Ser^{239} phosphorylation (Fig. 3A). These el-

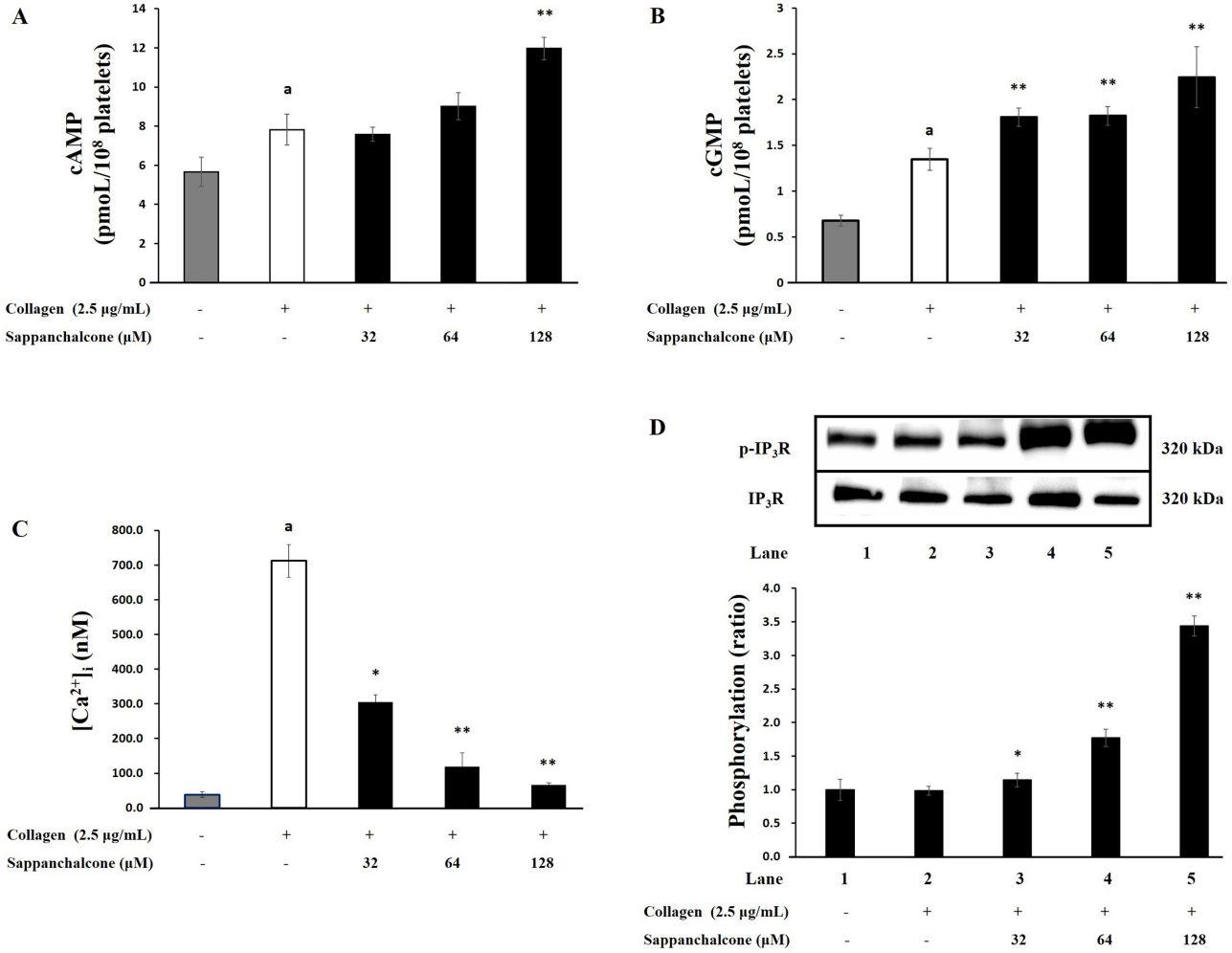

Fig. 2. Effect of sappanchalcone on cyclic nucleotide levels, inositol 1,4,5-trisphosphate receptor (IP_3R) phosphorylation, and intracellular Ca^{2+} mobilization. (A) Enhancement of cyclic adenosine monophosphate (cAMP) production by sappanchalcone under collagen-stimulated conditions. (B) Enhancement of cyclic guanosine monophosphate (cGMP) production by sappanchalcone under collagen-stimulated conditions. (C) Inhibitory effect of sappanchalcone on collagen-induced intracellular calcium ($[Ca^{2+}]_i$) mobilization. (D) Representative immunoblots and densitometric quantification showing increased IP_3R phosphorylation following sappanchalcone treatment in collagen-stimulated platelets. Data are presented as mean ± SD (n = 4). Statistical significance is indicated by $^a p < 0.05$ compared with non-stimulated platelets, and $^*p < 0.05$, $^{**}p < 0.01$ compared with collagen-stimulated platelets. p-IP_3R, phosphorylated inositol 1,4,5-trisphosphate receptor.

evations in VASP phosphorylation align with the previously observed dose-responsive increases in intracellular cAMP and cGMP levels (Fig. 2A,B).

Furthermore, sappanchalcone demonstrated a potent inhibitory effect on fibrinogen binding to the platelet surface in a concentration-dependent manner (16, 32, 64, and 128 μM). Specifically, the percentage of fibrinogen-binding was significantly reduced from 74.43 ± 5.67% in the collagen-stimulated control to 21.90 ± 1.25% following treatment with 128 μM sappanchalcone (Fig. 3B,C).

Collectively, these results indicate that under collagen-stimulated conditions, sappanchalcone facilitates the dual phosphorylation of VASP at Ser^{157} and Ser^{239}. This signaling event leads to the suppression of integrin $\alpha IIb\beta_3$ activation, which ultimately contributes to the compound's potent anti-aggregatory activity in platelets.

3.4 Sappanchalcone Suppresses PI3K/Akt and MAPK Phosphorylation

To investigate the involvement of the PI3K/Akt pathway, we analyzed the phosphorylation status of PI3K and Akt following sappanchalcone treatment (Fig. 4A). Sappanchalcone significantly attenuated the phosphorylation of both PI3K and Akt in a concentration-dependent manner. Notably, at 128 μM, sappanchalcone markedly suppressed PI3K phosphorylation to 0.69-fold, effectively reversing the 1.43-fold increase induced by collagen. Similarly, the 4.19-fold elevation in Akt phosphorylation stimu-

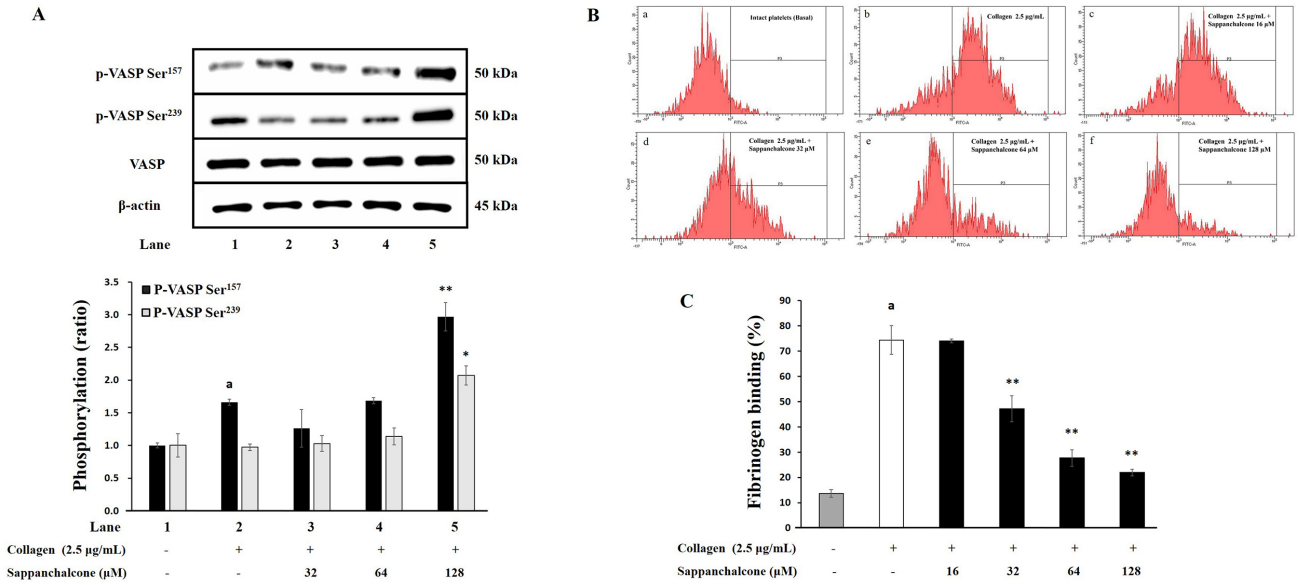

Fig. 3. Effect of sappanchalcone on VASP phosphorylation and fibrinogen binding. (A) Representative immunoblots and densitometric quantification of phosphorylated VASP at Ser^{157} and Ser^{239} normalized to total VASP, showing enhanced VASP phosphorylation following sappanchalcone treatment in collagen-stimulated platelets. (B) Representative histograms from flow cytometry analysis of fibrinogen binding. a: Base (intact platelets), b: collagen (2.5 μg/mL), c: collagen (2.5 μg/mL) + sappanchalcone (16 μM), d: collagen (2.5 μg/mL) + sappanchalcone (32 μM), e: collagen (2.5 μg/mL) + sappanchalcone (64 μM), f: collagen (2.5 μg/mL) + sappanchalcone (128 μM). (C) Quantitative analysis of the inhibitory effect of sappanchalcone on collagen-induced fibrinogen binding. Data are presented as mean ± SD (n = 4). Statistical significance is indicated by $^{a}p < 0.05$ compared with non-stimulated platelets, and $*p < 0.05$, $**p < 0.01$ compared with collagen-stimulated platelets. p-VASP, phosphorylated vasodilator-stimulated phosphoprotein; FITC-A, fluorescein isothiocyanate-area.

lated by collagen was strongly reduced to 0.21-fold by 128 μM sappanchalcone (Fig. 4A). These results suggest that the inhibition of the PI3K/Akt signaling axis is a key mechanism underlying the antiplatelet effects of sappanchalcone.

In parallel, we evaluated the impact of sappanchalcone on the MAPK signaling pathways, which are critical regulators of platelet activation. Under collagen-stimulated conditions, sappanchalcone treatment resulted in a striking, dose-dependent reduction in the phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (Fig. 4B). Specifically, at a concentration of 128 μM, sappanchalcone drastically lowered ERK phosphorylation to 1.81-fold from the 10.12-fold increase seen in the collagen-only group. Furthermore, JNK and p38 phosphorylation levels, which were increased to 2.22-fold and 2.20-fold by collagen, were significantly diminished to 0.27-fold and 0.49-fold, respectively, following treatment with 128 μM sappanchalcone (Fig. 4B).

Collectively, these findings demonstrate that sappanchalcone exerts its inhibitory effect on collagen-induced platelet activation by concurrently targeting and suppressing the protein phosphorylation within both the PI3K/Akt and MAPK signaling cascades.

3.5 Sappanchalcone Reduces Dense Granule Release (ATP and Serotonin) and Suppresses TXA_2 Synthesis Via Inhibition of $cPLA_2$ Phosphorylation

Under collagen-stimulated conditions (2.5 μg/mL), sappanchalcone treatment (32, 64, and 128 μM) resulted in a concentration-dependent reduction in the release of dense granule constituents, specifically ATP and serotonin (Fig. 5A,B). Collagen-induced ATP secretion, which measured 5.58 ± 0.35 μM, was potently suppressed to 2.19 ± 0.06 μM following treatment with 128 μM sappanchalcone (Fig. 5A). Similarly, the release of serotonin was markedly diminished from 39.28 ± 3.20 ng/10^8 platelets in the collagen-only group to 17.97 ± 1.19 ng/10^8 platelets in the 128 μM sappanchalcone-treated group (Fig. 5B).

In addition, TXA_2 synthesis was inhibited in a dose-responsive manner, which was accompanied by a significant decrease in the phosphorylation of $cPLA_2$ (Fig. 5C,D). Collagen-triggered TXB_2 levels, used as an index of TXA_2 generation, reached 120.09 ± 11.97 ng/10^8 platelets but was strikingly reduced to 27.27 ± 3.13 ng/10^8 platelets by 128 μM sappanchalcone (Fig. 5C). Furthermore, at the same concentration, sappanchalcone effectively reversed the collagen-induced 2.48-fold increase in $cPLA_2$ phosphorylation, lowering it to a mere 0.25-fold level (Fig. 5D).

Collectively, these findings demonstrate that sappanchalcone attenuates platelet activation by limiting the gen-

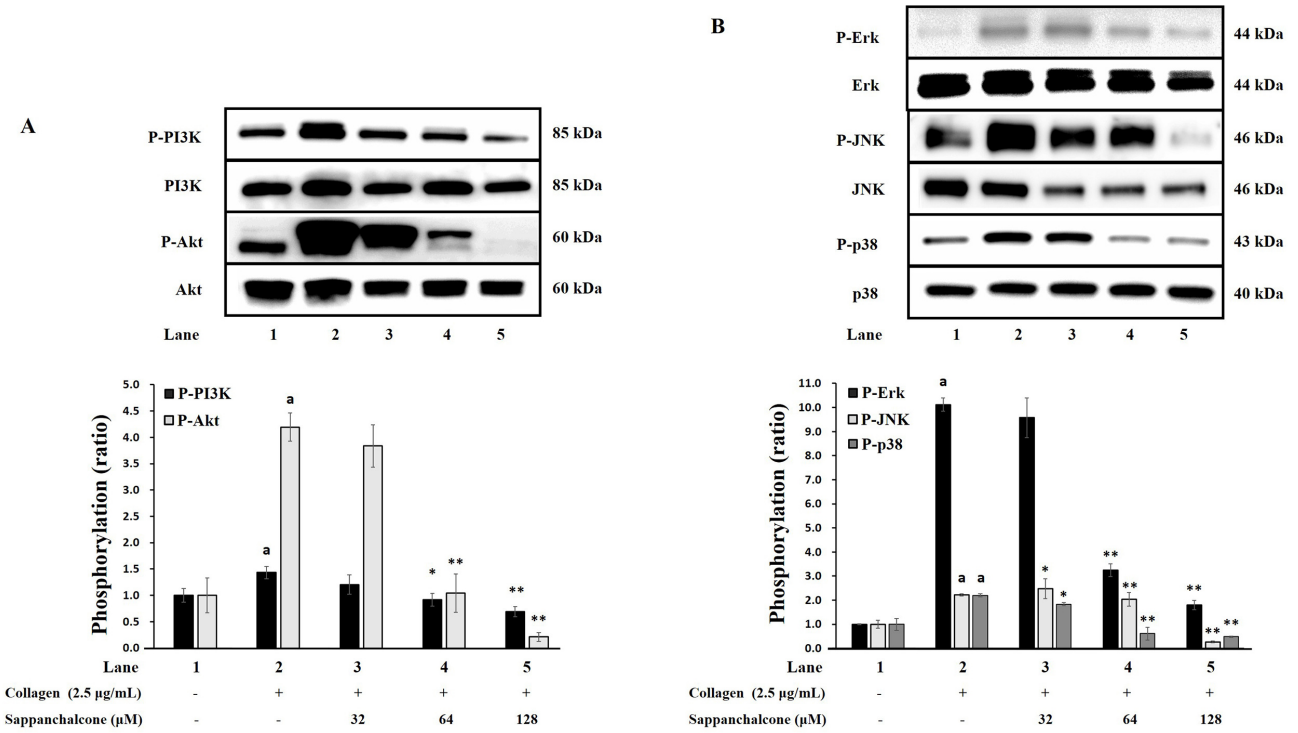

Fig. 4. Inhibitory effect of sappanchalcone on phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) phosphorylation. (A) Representative immunoblots and densitometric quantification of phosphorylated PI3K and Akt normalized to their respective total protein levels, showing inhibition of collagen-induced PI3K and Akt phosphorylation following sappanchalcone treatment. (B) Representative immunoblots and densitometric quantification of phosphorylated ERK, JNK, and p38 normalized to their respective total protein levels, showing inhibition of collagen-induced MAPK phosphorylation following sappanchalcone treatment. Data are presented as mean ± SD (n = 4). Statistical significance is indicated by $^{a}p < 0.05$ compared with non-stimulated platelets, and $*p < 0.05$, $**p < 0.01$ compared with collagen-stimulated platelets. p-PI3K, phosphorylated phosphoinositide 3-kinase; p-Akt, phosphorylated protein kinase B; p-ERK, phosphorylated extracellular signal-regulated kinase; p-JNK, phosphorylated c-Jun N-terminal kinase.

eration and liberation of secondary mediators, including ATP, serotonin, and TXA_2, under collagen-stimulated conditions. This inhibitory profile is consistent with the upstream suppression of the MAPK and PI3K/Akt signaling pathways observed previously (Fig. 4).

3.6 Sappanchalcone Decreases Thrombin-Induced Clot Retraction

Platelet activation triggers cytoskeletal reorganization and facilitates platelet-fibrin interactions, which subsequently leads to the contraction of the fibrin clot and stabilization of the developing thrombus. To determine whether sappanchalcone influences these platelet-dependent physiological processes, we monitored thrombin-stimulated clot retraction. As expected, thrombin alone induced a robust and extensive contraction of the fibrin clot. In contrast, treatment with sappanchalcone significantly suppressed the extent of clot retraction in a concentration-dependent manner (Fig. 6). Specifically, the presence of 128 μM sappanchalcone resulted in a substantial inhibition of the process, with the residual clot area remaining at 60.9 mm^2 compared to the initial size, indicating a marked reduction in the contractile force (Fig. 6).

These findings suggest that sappanchalcone interferes with thrombin-mediated platelet contractile activity, thereby reducing clot retraction and limiting fibrin clot consolidation.

4. Discussion

Sappanchalcone is a chalcone-type flavonoid derived from the heartwood of *Caesalpinia sappan* L., and previous studies have mainly reported its anti-inflammatory and anticancer activities [17,18]. However, the regulatory effects of sappanchalcone on platelet activation and thrombus formation have not yet been fully elucidated. Among structurally related chalcones, cardamonin has been reported to inhibit arachidonic acid-induced platelet aggregation in human whole blood, whereas licochalcone A has been shown to suppress platelet activation and thrombus formation through inhibition of the PLCγ2–PKC, Akt, and MAPK signaling pathways [19,20]. Based on these structural similarities and previous findings, we hypothe-

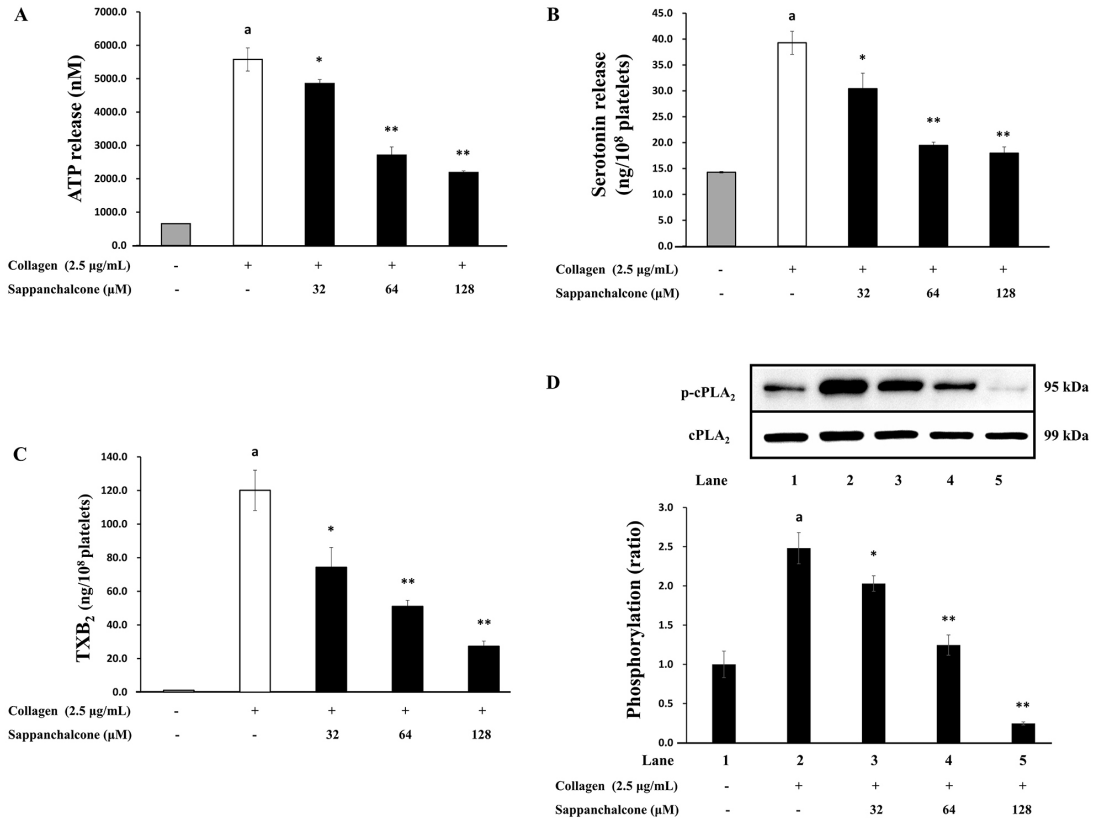

Fig. 5. Effect of sappanchalcone on dense granule (ATP and serotonin) release, cytosolic phospholipase A_2 ($cPLA_2$) phosphorylation and thromboxane A_2 (TXA_2) production in platelets. (A) Inhibitory effect of sappanchalcone on ATP secretion from dense granules under collagen-stimulated conditions. (B) Inhibitory effect of sappanchalcone on serotonin secretion from dense granules under collagen-stimulated conditions. (C) Inhibitory effect of sappanchalcone on collagen-induced thromboxane B_2 (TXB_2) production, measured as an index of TXA_2 generation. (D) Representative immunoblots and densitometric quantification of phosphorylated $cPLA_2$ normalized to total $cPLA_2$, showing inhibition of collagen-induced $cPLA_2$ phosphorylation following sappanchalcone treatment. Data are presented as mean ± SD (n = 4). Statistical significance is indicated by $^a p < 0.05$ compared with non-stimulated platelets, and $*p < 0.05$, $**p < 0.01$ compared with collagen-stimulated platelets. ATP, adenosine triphosphate; p-$cPLA_2$, phosphorylated cytosolic phospholipase A2.

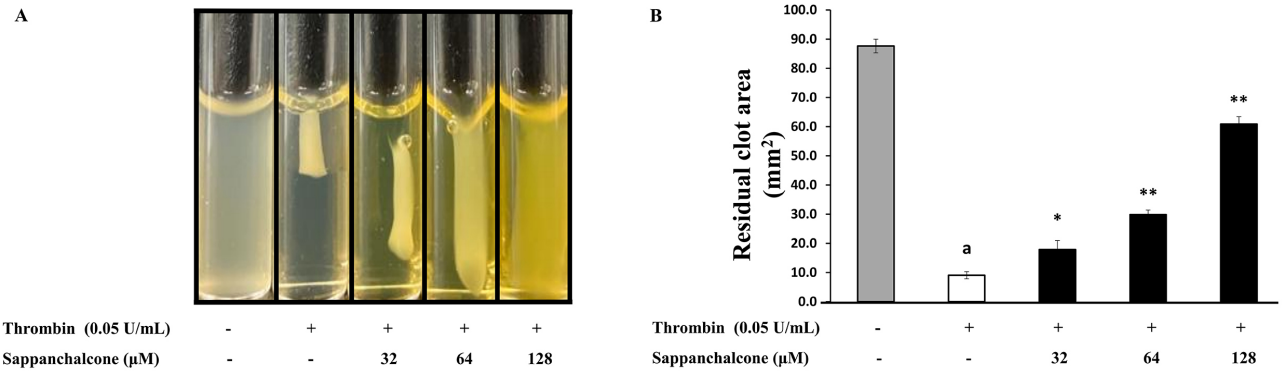

Fig. 6. Effect of sappanchalcone on platelet-mediated clot retraction. (A) Representative images showing the inhibitory effect of sappanchalcone on thrombin-induced fibrin clot retraction. (B) Quantitative analysis of residual clot area. Data are presented as mean ± SD (n = 4). Statistical significance is indicated by $^a p < 0.05$ compared with non-stimulated platelets, and $*p < 0.05$, $**p < 0.01$ compared with thrombin-stimulated platelets.

sized that sappanchalcone may also inhibit platelet activation and aggregation. In the present study, sappanchalcone concentration-dependently reduced collagen-induced platelet aggregation, with an IC_{50} value of 57.16 μM, under conditions that did not induce cytotoxicity. These findings indicate that sappanchalcone suppresses platelet aggregation without causing overt platelet damage, providing a basis for further investigation of its effects on platelet activation signaling. In addition, representative real-time aggregation traces showed that sappanchalcone also attenuated platelet aggregation induced by U46619 and thrombin, suggesting that its inhibitory effect may not be limited to collagen-induced platelet activation. However, because the present study focused mainly on collagen-stimulated platelet activation, the mechanistic findings should be interpreted primarily in the context of collagen/glycoprotein VI (GPVI)-dependent signaling.

Collagen is exposed at sites of vascular injury and activates the platelet GPVI–spleen tyrosine kinase (Syk)–phospholipase C gamma 2 (PLCγ2) pathway, leading to the generation of IP_3, which induces intracellular Ca^{2+} mobilization through IP_3R [25]. Increased cytosolic Ca^{2+} in platelets is a key early activation signal that leads to shape change, granule secretion, integrin activation, and platelet aggregation [25]. In contrast, the cAMP/PKA and cGMP/PKG pathways are representative inhibitory signaling pathways in platelets and limit excessive platelet activation by suppressing Ca^{2+} signaling and integrin activation [26]. Increases in platelet cAMP and cGMP may be associated with inhibition of phosphodiesterase (PDE) or regulation of adenylate cyclase (AC) and guanylate cyclase (GC) activity; however, further validation is required to determine how each natural compound regulates these enzyme systems [26,27].

In the present study, sappanchalcone increased cAMP and cGMP production under collagen stimulation, indicating enhancement of PKA/PKG-dependent inhibitory signaling. This increase in cyclic nucleotides was accompanied by increased IP_3R phosphorylation, and collagen-induced cytosolic Ca^{2+} mobilization was consequently markedly suppressed. Therefore, sappanchalcone appears to regulate IP_3R through increased cAMP/cGMP levels and thereby effectively inhibit Ca^{2+}-dependent activation at an early stage of platelet activation. These findings are important in that they indicate that sappanchalcone enhances inhibitory signaling at an upstream regulatory stage of platelet activation.

Cyclic nucleotide signaling affects not only IP_3R-mediated Ca^{2+} regulation but also integrin activation through VASP phosphorylation. VASP Ser^{157} is mainly phosphorylated by cAMP-dependent PKA, whereas Ser^{239} is phosphorylated by cGMP-dependent PKG, and phosphorylation at these sites is an important indicator reflecting platelet inhibitory signaling activation [28]. In this study, sappanchalcone increased both VASP Ser^{157} and Ser^{239} phosphorylation, which was consistent with the increased production of cAMP and cGMP. Increased VASP phosphorylation can act to restrict integrin $\alpha IIb\beta_3$ activation and thereby reduce fibrinogen binding [29,30]. Indeed, sappanchalcone markedly decreased collagen-induced integrin $\alpha IIb\beta_3$–fibrinogen binding. Therefore, sappanchalcone is considered to inhibit integrin $\alpha IIb\beta_3$–fibrinogen interaction, a direct execution step of platelet aggregation, through the cAMP/cGMP–VASP axis.

In addition, sappanchalcone appears to further strengthen the inhibition of fibrinogen binding by reducing PI3K/Akt phosphorylation. The PI3K/Akt pathway is involved in inside-out signaling of integrin $\alpha IIb\beta_3$, converting integrin into a high-affinity state and promoting fibrinogen binding and platelet aggregation [10,29]. In this study, sappanchalcone concentration-dependently reduced collagen-induced PI3K and Akt phosphorylation. This indicates that sappanchalcone dually restricts integrin $\alpha IIb\beta_3$ activation by increasing VASP phosphorylation while simultaneously reducing PI3K/Akt phosphorylation [11]. Therefore, the decrease in integrin $\alpha IIb\beta_3$–fibrinogen binding caused by sappanchalcone can be interpreted as the combined result of enhanced cAMP/cGMP–VASP inhibitory signaling and suppressed PI3K/Akt activation signaling.

The MAPK pathway acts as an important amplification signaling pathway in collagen-induced platelet activation. ERK, JNK, and p38 MAPK regulate granule secretion, $cPLA_2$ activation, arachidonic acid metabolism, and cytoskeletal rearrangement, and these processes contribute to amplification of the platelet aggregation response [31]. In the present study, sappanchalcone reduced the collagen-induced phosphorylation of ERK, JNK, and p38. This suggests that sappanchalcone does not selectively inhibit only a specific MAPK axis, but may broadly restrict MAPK-driven signaling events associated with platelet activation and aggregation. These findings are consistent with previous reports showing that cardamonin and licochalcone A, which share the same chalcone skeleton as sappanchalcone, exert antiplatelet effects by suppressing MAPK signaling and regulating Ca^{2+} mobilization in platelets [19,20].

Decreased MAPK phosphorylation is also linked to inhibition of dense granule secretion. ATP and serotonin released from platelet dense granules act as secondary mediators that further activate surrounding platelets and amplify platelet activation and aggregation [32,33]. In this study, sappanchalcone decreased both ATP secretion and serotonin release. These results can be interpreted as indicating that sappanchalcone attenuates amplification signaling during platelet activation by suppressing MAPK-dependent granule secretion.

Sappanchalcone also inhibited $cPLA_2$ phosphorylation and TXA_2 generation. Activation of MAPK signaling induces $cPLA_2$ phosphorylation, and activated $cPLA_2$ promotes arachidonic acid release from the platelet mem-

brane [8]. Subsequently, arachidonic acid is converted to PGH_2 through COX-1 and is ultimately synthesized into TXA_2 [8]. The generated TXA_2 acts as a potent secondary agonist that promotes intracellular Ca^{2+} elevation, granule secretion, and integrin activation again through the thromboxane–prostanoid receptor [34,35]. In this study, sappanchalcone decreased $cPLA_2$ phosphorylation, and TXB_2 levels reflecting TXA_2 generation were also reduced in a concentration-dependent manner. This demonstrates that sappanchalcone limits arachidonic acid release and TXA_2 synthesis through inhibition of $cPLA_2$ phosphorylation and, as a result, effectively blocks amplification signaling during platelet activation.

Recent studies have increasingly suggested that natural-product-derived antiplatelet compounds exert their inhibitory effects by regulating multiple platelet activation pathways rather than acting through a single target. For example, flavonoids, ginsenosides, and other natural substances have been reported to modulate platelet activation-related signaling pathways, including cyclic nucleotide-dependent inhibitory signaling, Ca^{2+} mobilization, PI3K/Akt and MAPK phosphorylation, arachidonic acid/thromboxane generation, and integrin $\alpha IIb\beta_3$ activation [36,37,38,39]. In line with these recent findings, sappanchalcone affected several key regulatory axes of platelet activation, including cAMP/cGMP signaling, IP_3R-mediated Ca^{2+} mobilization, VASP phosphorylation, PI3K/Akt and MAPK phosphorylation, integrin $\alpha IIb\beta_3$–fibrinogen binding, dense granule secretion, and thromboxane generation. These results suggest that sappanchalcone shares the multi-target regulatory features reported for other natural-product-derived antiplatelet compounds and may therefore be considered a candidate compound for further antiplatelet research.

In addition to platelet aggregation, platelet-mediated clot retraction was examined to determine whether sappanchalcone affects the contractile response of platelets within a fibrin clot. Clot retraction is a platelet-driven contraction process that occurs after thrombin-induced fibrin formation and platelet activation, and its kinetics and mechanics are regulated by the molecular and cellular composition of blood, including platelets, fibrin(ogen), thrombin, factor XIIIa, and other blood components [40]. In the present study, sappanchalcone inhibited thrombin-induced clot retraction. These findings indicate that sappanchalcone affects not only collagen-induced platelet aggregation but also platelet-mediated fibrin clot contraction.

5. Limitations

This study has several limitations that should be considered when interpreting the results. First, although sappanchalcone increased cAMP/cGMP levels and regulated the phosphorylation of IP_3R, VASP, PI3K/Akt, MAPKs, and $cPLA_2$, pharmacological inhibitor experiments were not performed to directly determine whether these signaling pathways are required for its antiplatelet effects. Therefore, the present findings should be interpreted as sappanchalcone-associated changes in platelet activation-related signaling pathways rather than definitive evidence of pathway dependency.

Second, the upstream mechanism by which sappanchalcone increases cAMP and cGMP levels remains unclear. Increases in cyclic nucleotide levels may result from the regulation of phosphodiesterases, adenylate cyclase, or guanylate cyclase activity; however, these enzyme systems were not directly examined in this study. This limitation restricts the ability to define the precise molecular target responsible for the cyclic nucleotide-mediated inhibitory effects of sappanchalcone.

Finally, the relatively high concentrations required for marked platelet inhibition should be considered when interpreting the translational relevance of the present findings. Sappanchalcone inhibited collagen-induced platelet aggregation with an IC_{50} of 57.16 μM, and near-complete inhibition was observed at 128 μM. At present, pharmacokinetic parameters, systemic exposure, and oral bioavailability of sappanchalcone have not been fully established; therefore, it cannot be concluded from the present *in vitro* data whether these effective concentrations are achievable *in vivo*. Nevertheless, sappanchalcone has been reported to exert biological activity in an *in vivo* collagen-induced arthritis mouse model [17], and limited oral bioavailability is a recognized issue for several naturally occurring chalcone-related compounds, including licochalcone A [41,42]. In addition, formulation or bioenhancer strategies have been investigated to improve systemic exposure of phytochemical compounds [43]. Therefore, the present study should be interpreted as an initial *in vitro* investigation suggesting the potential of sappanchalcone as a candidate antiplatelet compound and providing mechanistic information for future pharmacokinetic, formulation, safety, and *in vivo* thrombosis studies.

6. Conclusions

Sappanchalcone enhanced cAMP/cGMP-dependent inhibitory signaling, which was associated with increased IP_3R phosphorylation and suppression of cytosolic Ca^{2+} mobilization. In addition, increased VASP phosphorylation at Ser^{157} and Ser^{239}, together with reduced PI3K/Akt phosphorylation, contributed to the inhibition of integrin $\alpha IIb\beta_3$–fibrinogen binding, thereby directly limiting platelet aggregation. Sappanchalcone also attenuated MAPK phosphorylation, leading to reduced ATP and serotonin release from dense granules, decreased $cPLA_2$ phosphorylation, and suppression of TXA_2 generation, thereby blocking amplification signaling during platelet activation. Furthermore, sappanchalcone significantly inhibited thrombin-induced clot retraction, indicating that its inhibitory effects extend beyond platelet aggregation to platelet-mediated fibrin clot contraction.

Taken together, these findings suggest that sappanchalcone modulates key signaling pathways involved in platelet activation and aggregation and also affects platelet-mediated fibrin clot contraction, supporting its potential value as a natural product-derived antiplatelet candidate for further investigation.

Availability of Data and Materials

The datasets used and analysed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

GHL: Conceptualization, methodology and writing-original draft preparation. JPL and CEP: Data curation, methodology and formal analysis. DHL: Conceptualization, formal analysis, interpretation of data, supervision, writing- review and editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Platelet-rich plasma (PRP) used in this study was supplied by the Gyeonggi Red Cross Blood Center (Suwon, Korea), Korean Red Cross Blood Services. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Namseoul University (IRB No. BR-202310-003-01). Blood donation was performed voluntarily through the Korean Red Cross blood donation system, and informed consent was obtained from donors in accordance with the procedures of the Korean Red Cross. The investigators received anonymized PRP samples only and had no access to any personally identifiable information of the blood donors.

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this manuscript, the authors used ChatGPT solely to assist with English language editing, sentence refinement, and readability improvement. The authors reviewed and edited all AI-assisted text and take full responsibility for the final content of the manuscript.

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