



Pimpinellin Inhibits Collagen-induced Platelet Aggregation and Activation Through Inhibiting Granule Secretion and PI3K/Akt Pathway

Gang Liu^{1,2,3†}, Zhaowei Yuan^{1†}, Xiaoyun Tian¹, Xiuqin Xiong¹, Fang Guo¹, Zihan Lin² and Zhen Qin^{1*}

¹School of Basic Medical Sciences, Guizhou Medical University, Guiyang, China, ²Guizhou Provincial Engineering Technology Research Center for Chemical Drug R&D, Guizhou Medical University, Guiyang, China, ³Guizhou Provincial Key Laboratory of Pathogenesis and Drug Research on Common Chronic Diseases, Guizhou Medical University, Guiyang, China

OPEN ACCESS

Edited by:

Andres Trostchansky,
Universidad de la República, Uruguay

Reviewed by:

Antonio Marcus de Andrade Paes,
Federal University of Maranhão, Brazil
Renato Simões Gaspar,
University of Reading, United Kingdom
Eduardo Fuentes,
University of Talca, Chile

*Correspondence:

Zhen Qin
75871083@qq.com

[†]These authors have contributed
equally to this work and share first
authorship

Specialty section:

This article was submitted to
Experimental Pharmacology and Drug
Discovery,
a section of the journal
Frontiers in Pharmacology

Received: 10 May 2021

Accepted: 07 July 2021

Published: 22 July 2021

Citation:

Liu G, Yuan Z, Tian X, Xiong X, Guo F,
Lin Z and Qin Z (2021) Pimpinellin
Inhibits Collagen-induced Platelet
Aggregation and Activation Through
Inhibiting Granule Secretion and PI3K/
Akt Pathway.
Front. Pharmacol. 12:706363.
doi: 10.3389/fphar.2021.706363

Pimpinellin is a coumarin-like compound extracted from the root of *Toddalia asiatica*. Its effects on platelet function has not been investigated. This study found that pimpinellin pretreatment effectively inhibited collagen-induced platelet aggregation, but did not alter ADP- and thrombin-induced aggregation. Platelets pretreated with pimpinellin showed reduced α granule (CD62) level and secretion of dense granule (ATP release). Pimpinellin-treated platelets also exhibited decreased clot reaction and TxB2 production. Pimpinellin pretreatment suppressed adhesion and spreading of human platelets on the fibrinogen coated surface. Analysis of tail bleeding time of mice administered with pimpinellin (40 mg/kg) revealed that pimpinellin did not change tail bleeding time significantly, number of blood cells, and APTT and PT levels. Pimpinellin inhibited collagen-induced *ex vivo* aggregation of mice platelets. Immunoblotting results showed that pimpinellin suppressed collagen-induced phosphorylation of PI3K-Akt-Gsk3 β and PKC/MAPK in platelets.

Keywords: pimpinellin, platelet, granule secretion, hemostasis, PI3K/AKT signalling

INTRODUCTION

Platelets play a central role in thrombosis and hemostasis. In addition, they are the smallest biologically active blood cells and are the core regulators of the important balance between thrombosis and bleeding in the blood circulation (Koupenova et al., 2017a). Platelets also play an important role in many important pathophysiological processes, such as atherosclerosis (Davi and Patrono, 2007), immune response (Rondina et al., 2013), infection, tumor progression, and metastasis (Jorgensen, 2006; Lam et al., 2017). Thrombus formation is caused by several dysregulated factors such as endothelial cells (ECs) activation, lipoprotein oxidation, and immune response (Faber et al., 2009). Platelet activation is initiated by the binding of various ligands to their surface receptors, which triggers an intracellular signaling cascade. This leads to platelets' change in shape, the release of particle content, synthesis and release of thromboxane A₂, and thrombus formation (Gibbins, 2004). An important pathological basis of thromboembolic diseases is the abnormal activation of platelets (Koupenova et al., 2017b), and antiplatelet therapy is an important way to prevent and treat thrombotic diseases.

There are three classes of antithrombotic drugs: anticoagulant drugs, thrombolytic drugs, and antiplatelet drugs (Gallus and Hirsh, 1976). Despite significant advances in understanding the nature of the thrombotic process and the use of drugs such as urokinase, aspirin, or clopidogrel, the therapeutic efficacy and prognosis remain limited (Michelson, 2008). For example, aspirin or clopidogrel may cause several serious side effects, including liver damage, renal insufficiency, and gastric bleeding. Most of the currently known coumarins are reported to have strong antiplatelet activity and anti-fungal efficacy (Zaragoza et al., 2016; Hu et al., 2017). Warfarin inhibits platelet aggregation and 5-hydroxytryptamine (5-HT) secretion in a concentration-dependent manner (Nilsson et al., 2019); Isofraxidin is a natural coumarin that significantly inhibits ADP and AA-induced platelet aggregation (Jin et al., 2020); Columbianadin is the main coumarin isolated from *Angelica pubescens* Maxim. *f. biserrata* Shan et Yuan, and it effectively inhibits collagen-induced platelet aggregation (Hou et al., 2020). *Toddalia asiatica* Lam roots functions in dispelling wind and pain, dispersing blood stasis, stopping bleeding, hemostasis and anti-inflammatory activities (Kariuki et al., 2013). Besides, it is also used anti-cancer, antimicrobial, and antidiabetic (Stephen et al., 2012; Li et al., 2018).

Pimpinellin is a major coumarin-like compound obtained from *Toddalia asiatica* Lam (Liu et al., 2014). However, the antiplatelet and antithrombotic effects of pimpinellin have not been reported. Therefore, in this study, we investigated the antiplatelet and antithrombotic effects of pimpinellin. We investigated the effect of pimpinellin on platelet function in response to various agonists, as well as its influence on intracellular signaling pathways. In the present study, we demonstrated that pimpinellin is a potent inhibitor of platelet function with potential as an antiplatelet agent for the prevention of thrombotic disease.

MATERIALS AND METHODS

Materials

Pimpinellin (HPLC \geq 98%) was purchased from Shanghai Macklin Technology Corp (Macklin, Shanghai, China). Luciferin and collagen were purchased from the Chrono-Log Corp (Havertown, PA, United States). Thrombin, ADP, and FITC-Phalloidin were purchased from Sigma (St. Louis, MO, United States). PAC-1 and CD62P (P-selectin) antibody were purchased from BD Biosciences (San Jose, CA, United States). Antibody against total-p85, total-Akt, total-GSK3 β , total-p38, total-ERK, total-JNK, total-Syk, total-SLP76, total-PLC γ 2, and phospho-Akt (Ser473) were from Santa Cruz Biotechnology (Santa Cruz, CA, United States). Antibody for phospho-PI3K (p85/p55), phospho-GSK3 β , phospho-p38, phospho-ERK, phospho-JNK, phospho-SLP76, phospho-PLC γ 2, and phospho-PKC substrates was from Cell Signaling Technology (Beverly, MA, United States). The phospho-Syk antibody was obtained from GeneTex International Corp (GeneTex, Taiwan, China). GAPDH was purchased from Proteintech Group, Inc (Proteintech, IL, United States). The thromboxane B2 and cyclic adenosine monophosphate (AMP) enzyme immunoassay (EIA) kits

were from Cayman (Ann Arbor, MI, United States); ECL Western blotting detection reagent was obtained from Millipore Corp (Millipore, MA, United States). Pimpinellin was dissolved in DMSO and stored at -20°C .

Animal

C57BL/6 mice were maintained at the Animal Experiment Center of Guizhou Medical University. All mice were handled, monitored, and executed in accordance with the approved guidelines. Besides, they were housed in a controlled environment (humidity 40%–60%, $24 \pm 2^{\circ}\text{C}$) with free access to water and food, and domesticated for 1 wk before experimental manipulation. All animal experiments were approved by the Animal Care Welfare Committee of Guizhou Medical University.

Mice were randomly divided into four groups, blank group, aspirin group (100 mg/kg), and pimpinellin group (40 and 100 mg/kg). For mice, the dose was administered by gavage for 1 wk, followed by tail bleeding time and *ex vivo* platelet aggregation tests.

Platelet Preparation

Human blood was obtained from healthy volunteers in accordance with the Declaration of Helsinki guidelines and the Ethics Committee of Guizhou Medical University. Human platelet-rich plasma (PRP) and washed human platelets were prepared as previously described (Vaipayuri et al., 2015). Blood was collected from the elbow vein of a healthy volunteer, then the whole blood was centrifuged at $200 \times g$ for 10 min, the supernatant was aspirated and diluted with $1 \times$ Tyrodes Buffer; PGE1 (50 ng/ml) and Apyrase (0.1 U/ml) were added and centrifuged at $700 \times g$ for 10 min to obtain platelet clumps, which were diluted with $1 \times$ Tyrodes Buffer and repeated 2 times. The platelets were resuspended in $1 \times$ Tyrodes buffer to a final concentration of $3.0 \times 10^8/\text{ml}$.

Platelet Aggregation and ATP Release

An aggregometer was used to measure platelet aggregation and secretion. The washed human platelets were adjusted to $3.0 \times 10^8/\text{ml}$ with modified Tyrodes buffer and stimulated with ADP, collagen, and thrombin. Luciferase reagent was added to the platelet suspension to monitor platelet aggregation and adenosine triphosphate (ATP) secretion. Before stimulation, platelets were incubated with pimpinellin (10 and 20 μM) at 37°C for 10 min.

Determination of Lactate Dehydrogenase (LDH)

Cytotoxic effects were examined by measuring the level of lactate dehydrogenase (LDH). Washed platelets were pre-incubated with pimpinellin (10 and 20 μM) at 37°C for 10 min. Finally, an aliquot (20 μl) of the supernatant was assessed using the LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The maximal value of LDH was observed in Triton-treated platelets.

Flow Cytometry

CD62P expression in platelets and PAC-1 binding to activated $\alpha\text{IIb}\beta_3$ was determined using the FACScan flow cytometer. Unstirred washed

platelets ($5.0 \times 10^7/\text{ml}$) were incubated with $20 \mu\text{M}$ pimpinellin or vehicle at 37°C for 10 min. Then, platelets were activated with agonist CRP ($1 \mu\text{g}/\text{ml}$) for 5 min. The washed platelets were pre-incubated with fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody, FITC-conjugated CD62P antibody, or isotype control antibody. Flow cytometry was performed after 15 min of treatment in a dark room at room temperature.

Platelet Spreading on Fibrinogen

The spreading of platelets on the fibrinogen-coated surface was carried out as described previously (Yue et al., 2016). The adhered platelets were observed with a fluorescence microscope. The image was acquired with a Nikon camera. ImageJ software was used to analyze the number of adherent platelets and the average spreading area.

Clot Retraction

Washed platelets were incubated with vehicle or pimpinellin (10 , 20 and $40 \mu\text{M}$) at 37°C for 10 min and the reaction mixture was transferred to a glass tube for observation. Trigger clot retraction by adding thrombin ($0.4 \text{ U}/\text{ml}$) and proceed at 37°C . Take pictures after 30 min to observe the phenomenon.

Thromboxane B_2 (TxB_2) Assay and Measurement of Cyclic AMP (cAMP) Levels

Pre-incubate platelets ($3 \times 10^8/\text{ml}$) with vehicle or pimpinellin (10 and $20 \mu\text{M}$) for 10 min, and then treated with collagen. Ethylenediaminetetraacetic acid (EDTA, 2 mM) was added to the platelet suspension. The TxB_2 and circulating AMP levels were measured for each specimen using a Cayman ELISA kit, centrifugation at $12,000 \text{ rpm}$ for 10 min.

Immunoblotting

Washed platelets were incubated with pimpinellin for 10 min, and stimulated with or without an agonist for 3 min. A similar volume of $2 \times$ Lysis Buffer was added to the reaction to lyse the aggregated platelets. The lysate was prepared, analyzed by SDS-PAGE, electro-transferred to PVDF membrane. Then it was blocked with 5% (w/v) BSA and detected with a primary antibody. The membrane was rinsed with TBST and incubated with appropriate secondary antibodies. Western blotting was carried out as described previously (Liu et al., 2016).

Tail Bleeding Assay

The mice were randomly divided into four groups; aspirin group ($100 \text{ mg}/\text{kg}$), pimpinellin group (40 and $100 \text{ mg}/\text{kg}$), and control group (0.5% CMC-Na). To calculate the amount of gavage, mice were weighed and dose administration was performed by gavage daily for 1 wk. Mice were anesthetized before the tail was transected 5 mm from the tip using a sterile scalpel. A bleeding assay was performed by excision of a tail from the tip, and the time taken for cessation of bleeding was recorded.

Hematology Assessment

Two hours after administration of the last gavage, mice blood was taken after anesthesia, and centrifuged at 1200 rpm for 10 min, and

the supernatant was used to detect activated partial thromboplastin time (APTT) and prothrombin time (PT). Using standard mice parameters, complete hematology analysis was performed using an automatic cell counter (Mindray; BC-5130).

Ex vivo Platelet Aggregation

One week after the last gavage, washed platelets ($3.0 \times 10^8/\text{ml}$) were prepared and stimulated with collagen. The platelet aggregation rate is measured with an aggregator, and the result is recorded by the aggregolink software.

Statistical Analysis

All the data were generated from at least three independent experiments, with data presented as mean \pm standard error of the mean (SEM). All data were tested for significance using one-way ANOVA with Bonferroni's *post hoc* test, $p < 0.05$ was considered statistically significant.

RESULTS

Pimpinellin Inhibits Platelet Aggregation and ATP Secretion Induced by Collagen

We examined the effect of pimpinellin on platelet aggregation induced by different agonists. Pimpinellin was found to inhibit platelet aggregation induced by collagen (Figure 1A). Collagen ($2 \mu\text{g}/\text{ml}$) in stimulated platelet aggregation rate was reduced by pimpinellin (10 and $20 \mu\text{M}$) from $74 \pm 8\%$ to $36 \pm 5\%$ and $5 \pm 1\%$ ($n = 3$, $p < 0.001$). The 50% inhibitory concentration (IC₅₀) of pimpinellin on collagen ($2 \mu\text{g}/\text{ml}$)-induced platelet aggregation was $13.6 \mu\text{M}$. Pimpinellin exhibited a inhibition of collagen-stimulated platelet aggregation but it had no effect on thrombin (0.04 and $0.08 \text{ U}/\text{ml}$), ADP (3 and $5 \mu\text{M}$)-induced platelet aggregation. Meanwhile, pimpinellin did not affect ADP-induced platelet aggregation in PRP (Supplementary Figure S1). The anti-platelet effect of pimpinellin can also be confirmed by microscopic observation that pimpinellin reduced the formation of platelet aggregates (Figure 1B).

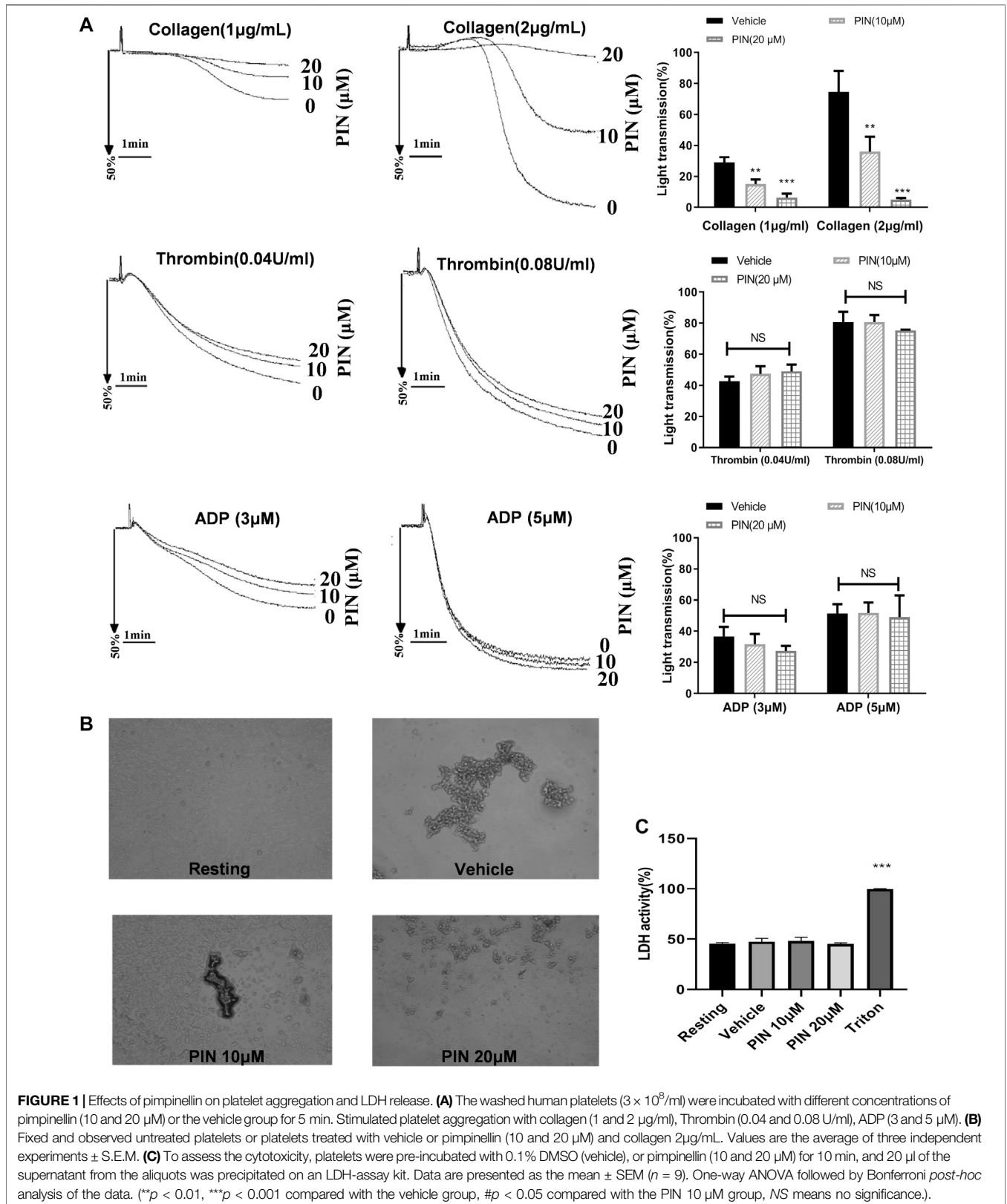
A similar inhibitory effect on collagen-induced ATP release was also observed (Figure 2A). The data indicate that $20 \mu\text{M}$ pimpinellin can effectively inhibit the release of ATP. These results strongly suggested that pimpinellin suppressed platelet aggregation and ATP release.

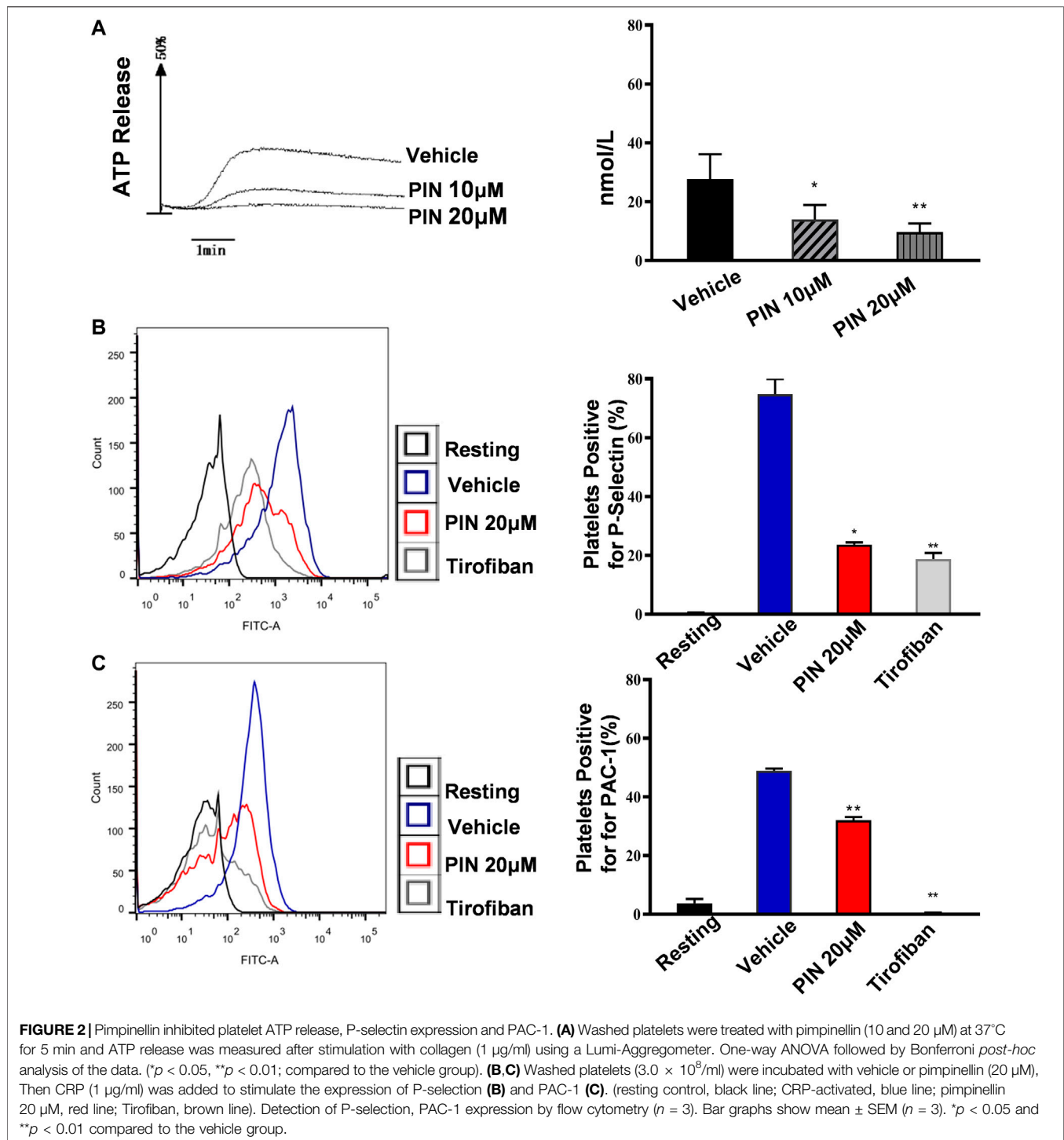
Effects of Pimpinellin on LDH Release

Pimpinellin (10 and $20 \mu\text{M}$) did not significantly ($p > 0.05$) increase LDH activity (Figure 1C). This indicated that pimpinellin did not affect platelets permeability or induce platelet cytolysis.

Pimpinellin Inhibits Platelet Activation

To further demonstrate that pimpinellin inhibits the release of platelet granules content, we examined the expression of CRP ($1 \mu\text{g}/\text{ml}$)-induced platelet surface P-selectin expression. Compared with the control group without pimpinellin, pimpinellin evidently reduced CRP-stimulated P-selectin expression (Figure 2B).

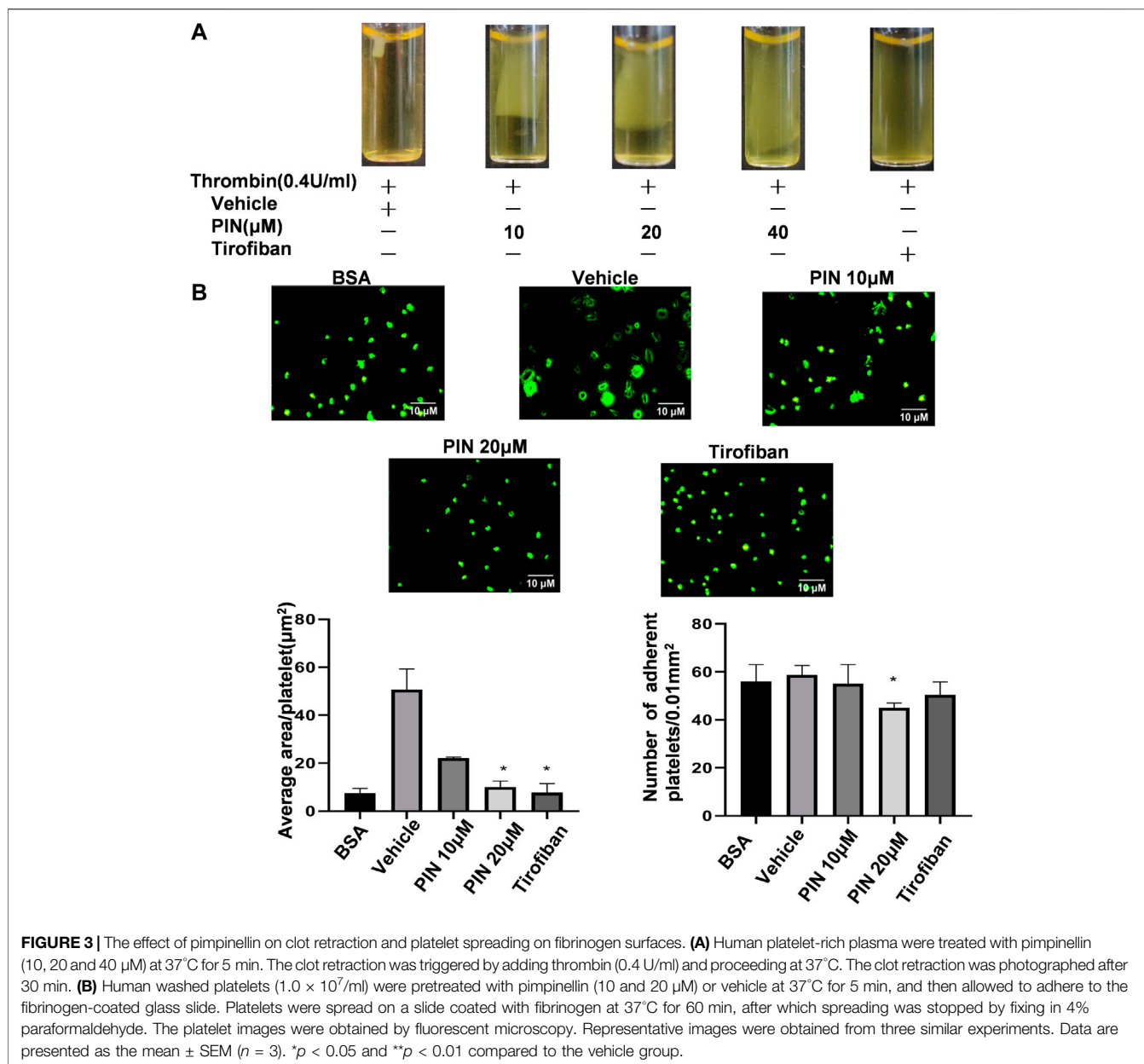




PAC-1 is used to detect the activation level of human platelet integrin α IIb β 3. To determine whether pimpinellin could interfere with integrin α IIb β 3 activation, flow cytometry was used to assess PAC-1 binding. In the presence of pimpinellin (20 μ M), PAC-1 binding was significantly reduced (p < 0.01) (**Figure 2C**).

Pimpinellin Negatively Regulates Integrin α IIb β 3-Mediated Outside-in Signaling

As shown in **Figure 3A**, clot retraction was reduced significantly in the presence of pimpinellin (10, 20, and 40 μ M) at 30 min compared with the vehicle group. We selected the integrin α IIb β 3 antagonist tirofiban as a positive control (Huang et al., 2019)



and found that tirofiban could significantly inhibited clot retraction.

Next, we examined the spreading of platelets on the surface of fibrinogen in the presence of pimpinellin at different concentrations (10 and 20 μM). As shown in **Figure 3B**, pimpinellin (20 μM) significantly inhibited platelet spreading and reduced single platelet surface coverage area from 50.0 ± 8.6 μm² to 9.89 ± 0.64 μm², respectively. Compared to the vehicle group, pimpinellin (20 μM) inhibited platelet adhesion from 55 ± 8 platelets/0.01 mm² to 38 ± 1 platelets/0.01 mm².

These data suggested that the outside-in signaling pathway that regulates the coordinated process of clot retraction and platelet spreading on fibrinogen *via* integrin αIIbβ3 was influenced by pimpinellin.

Effects of Pimpinellin on Collagen-induced Platelet TxB₂ Production and cAMP Levels

To investigate the possible mechanism of the anti-platelet effect of pimpinellin, the effects on TxB₂ formation were evaluated. Compared with collagen-activated platelets, resting platelets produce less TxB₂. Pimpinellin (10 and 20 μM) significantly (*p* < 0.001) inhibited the formation of platelet TxB₂ stimulated by collagen (1 μg/ml) (**Figure 4A**).

We determined the cAMP levels in pimpinellin-pretreated platelets interacting with different reagents. Our findings showed that pimpinellin does not affect cAMP production compared with resting washed platelets (**Figure 4B**).

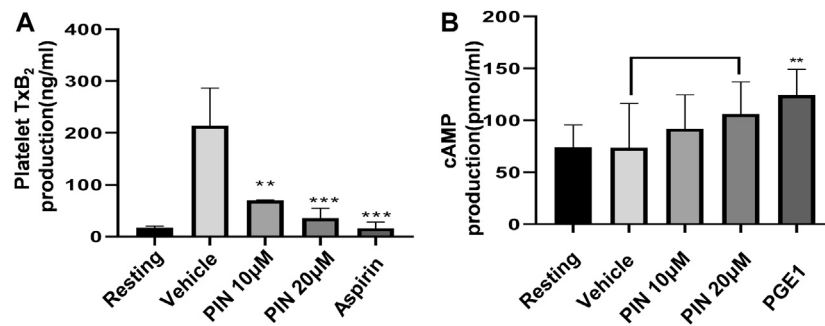


FIGURE 4 | Effects of pimpinellin on cAMP levels, and TxB₂ production. **(A)** We pre-incubated washed human platelets (3×10^8 /ml) with vehicle, different concentrations of pimpinellin, or aspirin (100 µM) for 10 min, after which the platelets were stimulated with collagen (1 µg/ml). TxB₂ production levels were evaluated by measuring the OD values. Results were expressed as TxB₂ production (ng/ml). **(B)** Washed human platelets (3×10^8 /ml) were pre-incubated with the vehicle, different concentrations of pimpinellin or PGE1 (50 ng/ml) for 10 min, after which they were stimulated with collagen. After 5 min, the supernatant was obtained by centrifugation. The effect of pimpinellin on cAMP levels was determined by detecting OD value. Data are mean \pm SEM values ($n = 4$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the vehicle group.

Pimpinellin Inhibits Signaling Pathways of Platelet Activation Induced by Collagen

To explore the molecular mechanism of pimpinellin inhibition of platelet aggregation, platelet lysates were obtained from platelets pre-incubated with pimpinellin (10 and 20 µM) for 5 min, they were then lysed for 5 min after collagen stimulated. Western blot analysis showed that Syk, SLP76, PLCγ2, PI3K (p85/p55), Akt and GSK3β phosphorylation were inhibited by pimpinellin. We concluded that pimpinellin may negatively regulated of Syk-SLP76-PLCγ2-PI3K-Akt-GSK3β signaling (Figure 5).

We evaluated the MAPK signaling molecules including p38 MAPK, JNKs, and ERKs. Our findings showed that pimpinellin inhibited the phosphorylation of p38, but not ERK or JNK. Therefore, the inhibition of p38 signaling may be involved in the mechanism of pimpinellin-mediated inhibition (Figure 5).

Stimulation of platelets with collagen induces the activation of protein kinase C (PKC) and subsequent phosphorylation of p47 proteins. When collagen was added to human platelets, 47 kDa (p47) was predominately phosphorylated compared to resting platelets. Data shows the inhibition of PKC activation in platelets following incubation with different concentrations of pimpinellin (Figure 5).

Exogenous ADP Normalizes Pimpinellin Inhibition of Platelet Aggregation

We supplemented pimpinellin-incubated platelets with exogenous ADP to verify whether it could restore the aggregation rate of pimpinellin-inhibited platelets to normal levels (Figure 6A). ADP (1 µM) is not enough to induce platelet aggregation, but reversed the inhibitory effect of pimpinellin-incubated platelets aggregation stimulated by collagen (1 µg/ml). And ADP restored the decreased phosphorylation of Akt (Figure 6B).

Effect of LY294002 on Pimpinellin-treated Platelet Aggregation

PI3K/Akt signaling plays a central role in platelet activation and granule secretion. We previously found that pimpinellin significantly

inhibited the phosphorylation of PI3K (p85/p55) and Akt. Besides, pimpinellin combined with the PI3K-specific inhibitor LY294002 significantly inhibited collagen-activated platelet aggregation and Akt phosphorylation (Figures 6C,D). These findings confirmed that the mechanism of pimpinellin inhibition of platelet activation involved the PI3K/Akt signaling pathway.

Effect of Pimpinellin on Coagulation Function and Platelet Aggregation *ex vivo*

Compared with the control group, pimpinellin (40 mg/kg) did not affect the APTT levels, an indicator of endogenous coagulation pathway. However, pimpinellin (100 mg/kg) significantly prolonged the APTT in mice ($p < 0.001$) (Figure 7A), PT is mainly used to evaluate the exogenous coagulation pathway, pimpinellin did not affect the PT levels (Figure 7B). In this study, pimpinellin did not affect exogenous coagulation pathway, suggesting that the anticoagulation mechanism of high dose pimpinellin was mainly mediated by the endogenous coagulation system. In addition, pimpinellin (40 mg/kg) did not influence platelet count (PLT), mean platelet volume (MPV), white blood cell count (WBC) and red blood cell count (RBC) (Table 1).

To evaluate the ability of pimpinellin as an antiplatelet agent, platelet aggregation *ex vivo* was assessed. Pimpinellin produced a concentration-related inhibition of collagen-induced *ex vivo* aggregation in platelets, which was consistent with the inhibitory effect of pimpinellin on platelet aggregation *in vitro* (Figure 7C). These results suggested that pimpinellin inhibited platelet function under normal conditions *in vivo*.

We further evaluated the role of pimpinellin in hemostasis by measuring the tail bleeding time. We found that tail bleeding time was not affected in mice gavaged with low doses of pimpinellin (40 mg/kg) relative to the controls, while high doses of pimpinellin (100 mg/kg) prolonged the tail bleeding time (Figure 7D).

DISCUSSION

In recent years, traditional medicine has become more popular, and long-term clinical practice and scientific studies have shown

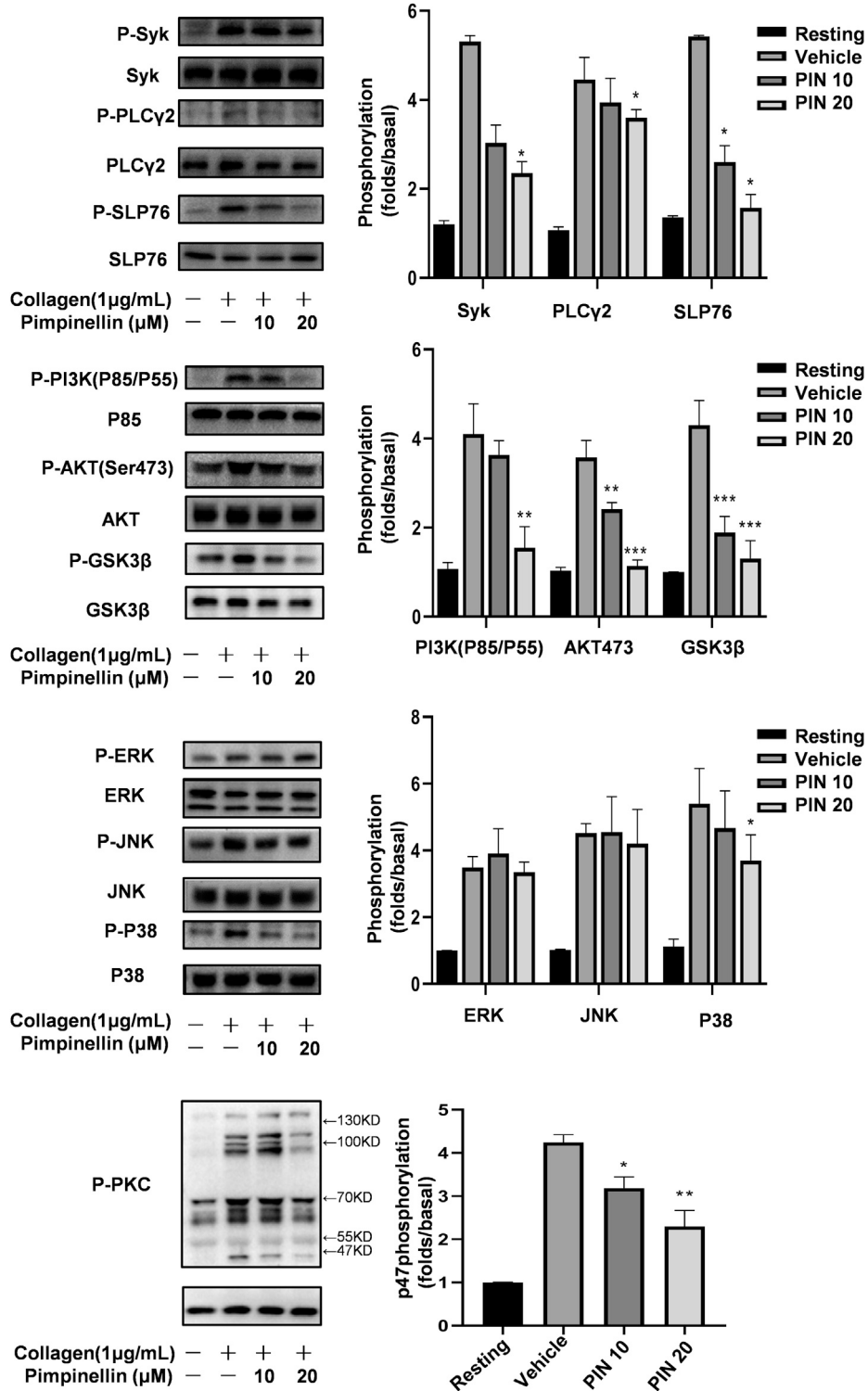


FIGURE 5 | Effect of pimpinellin on collagen-induced signal transduction. Washed platelets were stimulated with 2 μg/ml collagen (3.0×10^8 /ml) and then lysed with cell lysis buffer. Syk, PLCγ2, SLP76, JNK, ERK, P-38, p85, Akt, GSK3β, and PKC phosphorylation levels were detected with relevant antibodies. Band density was analyzed using the ImageJ software. Data are presented as the mean ± SEM ($n = 3$). * $p < 0.05$ and ** $p < 0.01$ compared to the vehicle group; NS means no significance.

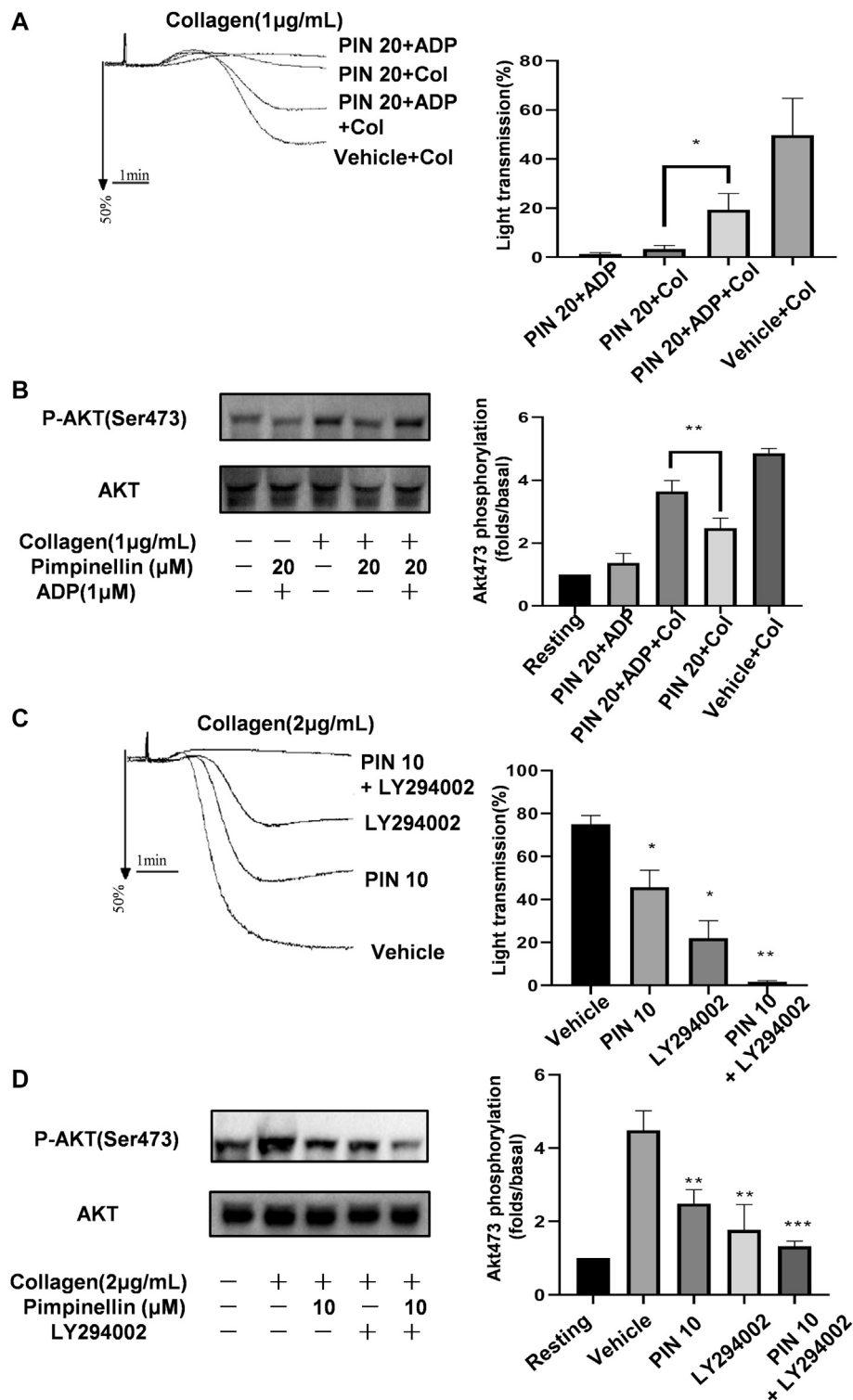


FIGURE 6 | Effects of pimpinellin on platelet aggregation in the presence of ADP or LY294002. **(A)** Washed platelets (3×10^8 /ml) were pretreated with 20 μ M pimpinellin, 1 μ M ADP or both for 10 min after which they were stimulated with collagen (1 μ g/ml). Platelet aggregation rates were calculated in at least three independent experiments and the results are shown as mean \pm SEM (* p < 0.05 compared with the PIN 20+Col group, PIN 20 means pimpinellin 20 μ M, COL means collagen). **(B)** Lysates from each group were analyzed by immunoblotting to determine the Akt phosphorylation levels. **(C)** Washed platelets (3×10^9 /ml) were pre-incubated with 10 μ M pimpinellin, 2 μ M LY294002 or both for 10 min, after which they were stimulated with 2 μ g/ml collagen. (* p < 0.05 and ** p < 0.01 compared to the vehicle group, PIN 10 means pimpinellin 10 μ M). **(D)** Lysates in each group were analyzed by immunoblotting to determine the Akt phosphorylation levels. Band density was calculated by the ImageJ software. Bar graphs represent the mean \pm SEM ($n = 3$).

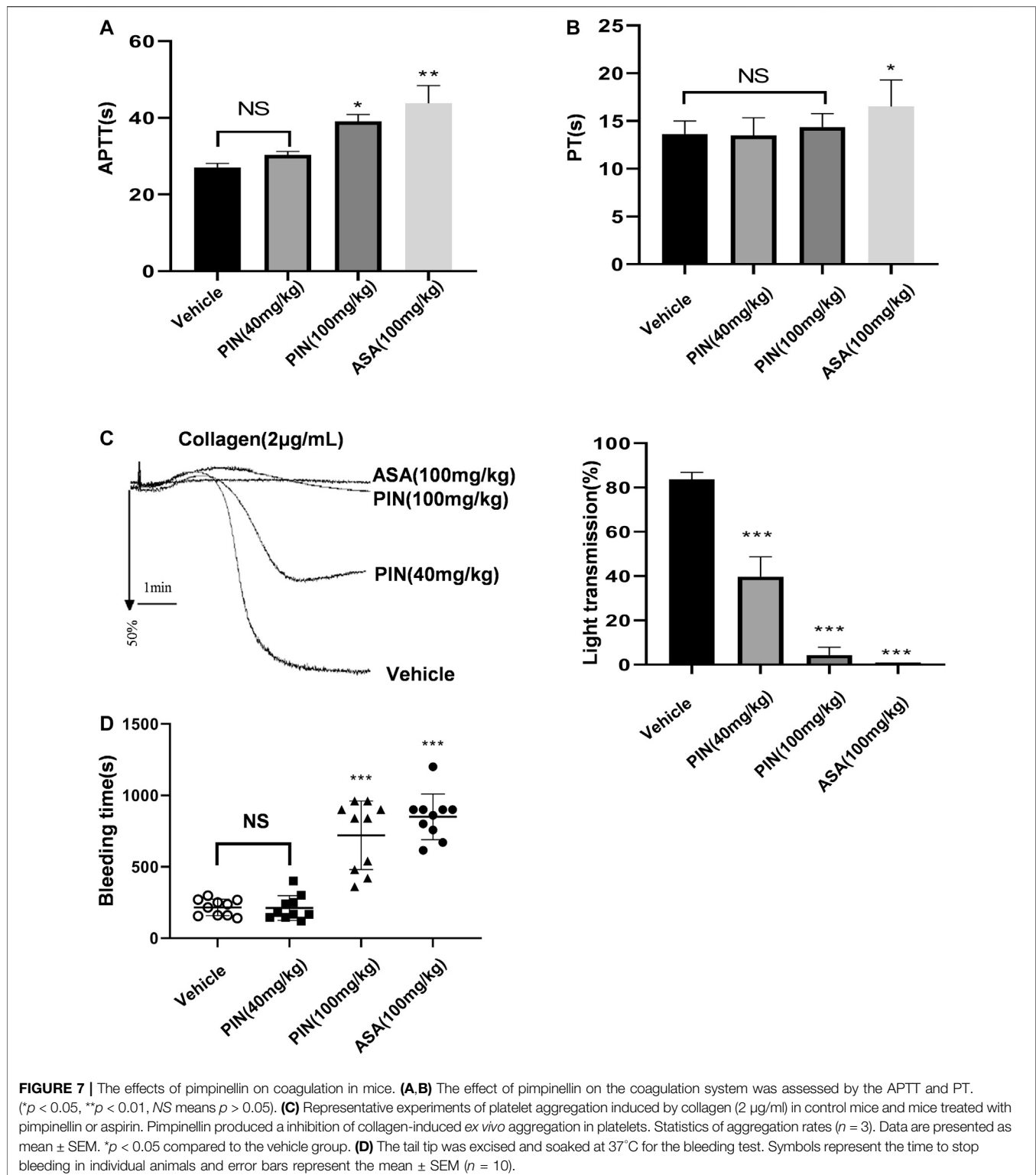


FIGURE 7 | The effects of pimpinellin on coagulation in mice. **(A,B)** The effect of pimpinellin on the coagulation system was assessed by the APTT and PT. (* $p < 0.05$, ** $p < 0.01$, NS means $p > 0.05$). **(C)** Representative experiments of platelet aggregation induced by collagen (2 $\mu\text{g}/\text{mL}$) in control mice and mice treated with pimpinellin or aspirin. Pimpinellin produced a inhibition of collagen-induced *ex vivo* aggregation in platelets. Statistics of aggregation rates ($n = 3$). Data are presented as mean \pm SEM. * $p < 0.05$ compared to the vehicle group. **(D)** The tail tip was excised and soaked at 37°C for the bleeding test. Symbols represent the time to stop bleeding in individual animals and error bars represent the mean \pm SEM ($n = 10$).

that some herbs and their extracts have good antithrombotic and antiplatelet functions (Jin et al., 2007; Mousa, 2010; Lee et al., 2013; Gao et al., 2019). The active ingredient or drug monomer of a natural product is an important component for the development of new compounds. *Toddalia asiatica* Lam has a

long history of medicinal use in China due to its various biological effects such as dispelling wind and pain, relieving bleeding, hemostasis and anti-inflammatory activities (Zhang et al., 2017; Alagaraj and Muthukrishnan, 2020). Pimpinellin is a coumarin-like compound extracted from *Toddalia asiatica*

TABLE 1 | Effect of pimpinellin on coagulation index in mice.

Group	PLT (10^6 ml^{-1})	MPV (μm^3)	WBC (10^6 ml)	RBC (10^9 ml^{-1})
CMC-Na (0.5%)	587.800 \pm 22.522	5.630 \pm 0.096	1.815 \pm 0.236	5.556 \pm 0.206
Aspirin (100 mg/kg)	562.100 \pm 35.652	5.440 \pm 0.048	1.536 \pm 0.178	5.699 \pm 0.202
PIN(40 mg/kg)	570.100 \pm 33.581	5.460 \pm 0.06	1.985 \pm 0.239	5.498 \pm 0.418
PIN(100 mg/kg)	721.400 \pm 61.666 ^a	5.670 \pm 0.073	1.279 \pm 0.135	5.492 \pm 0.134

Data are presented as the mean \pm SEM (n = 10). Mice were orally administered with CMC-Na, pimpinellin (40 and 100 mg/kg), and ASA for a week. Effect of pimpinellin on mouse platelet count (PLT), mean platelet volume (MPV), white blood cell count (WBC) and red blood cell count (RBC) ex vivo.

^ap < 0.05 compared with the CMC-Na group.

Lam. An understanding of the effects of pimpinellin and platelet function may help to develop improved strategies for the treatment of cardiovascular diseases. In this study, the antiplatelet effects of pimpinellin were analyzed.

We for the first time investigated the effects of pimpinellin on platelet aggregation, release, and spreading. The results indicated that pimpinellin inhibited collagen-induced platelet aggregation and reduced both dense and α granule secretion, and inhibited spreading and adhesion of human platelets. Besides, we further investigated the potential antiplatelet mechanism of pimpinellin, by focusing on the collagen/GPVI-mediated signaling pathway. The results demonstrated that pimpinellin may exert antiplatelet effects by inhibiting the PKC pathway as well as the PI3K-Akt-GSK3 β /p38 MAPK pathway.

Pimpinellin significantly inhibited collagen-induced platelet aggregation, but did not affect thrombin- and ADP-induced platelet aggregation, suggesting that pimpinellin may have a specific inhibitory effect on collagen. We also found that pimpinellin was not toxic to platelets, and pimpinellin had no effect on LDH at concentrations that inhibited platelet aggregation.

Upon activation, platelets release the contents of three main types of granules: dense granules, alpha granules, and lysosomal granules (Blair and Flaumenhaft, 2009), which contain a variety of active substances. Dense granules and alpha granules play an important role in platelet aggregation and activation (Binsker et al., 2018). Besides, platelet dense granules contain ADP and polyphosphate, which contribute to hemostasis and coagulation. Alpha granules contain various growth factors and adhesion molecules P-selectin and CD63 (Fuentes and Palomo, 2013). Pimpinellin was found to inhibit collagen-induced ATP release and P-selectin expression. Exogenous supplementation of ADP partially reversed the inhibitory effect of pimpinellin on platelet aggregation. Pimpinellin exerted its inhibitory effect on platelet aggregation and activation by inhibiting the platelet release function.

Thromboxane A₂ is produced by activated platelets during hemostasis and has procoagulant properties such as platelet activation and increased platelet aggregation (Fontana et al., 2014). However, TxA₂ has a short half-life and TxB₂ is produced by the non-enzymatic hydration of TxA₂. Therefore, we measured the concentration of TxB₂ to determine the level of TxA₂ formation. Pimpinellin inhibited the formation of TxB₂, suggesting that pimpinellin exerts its antiplatelet effect by inhibiting the formation of TxB₂. cAMP is an important regulator of platelet activity and inhibits platelet aggregation induced by a variety of stimulants (Raslan and Naseem, 2014).

Besides, cAMP is also involved in regulatory functions, such as calcium mobilization, adhesion, and skeletal rearrangement in platelets (Margarucci et al., 2011; Raslan et al., 2015). Our data showed that although pimpinellin tended to elevate cAMP levels, there was no significant (*p* > 0.05) difference compared to the control group.

When blood vessels are damaged, the exposed collagen substrate drives platelet activation and the formation of a hemostatic thrombus. The formation of the hemostatic thrombus results from continuous platelet adhesion, activation, and aggregation (Jurk and Kehrel, 2005; Mackman et al., 2007; Jurk and Kehrel, 2010). Platelet spreading is a process by which adherent platelets increase platelet contact with the damaged site after skeletal rearrangement at the site of thrombus damage, and this process is considered to be a critical step in hemostasis and thrombosis (Koupenova et al., 2018). In our study, we found that pimpinellin significantly (*p* < 0.05) reduced the number of platelet adhesions and average platelet spreading area. These results suggest that pimpinellin inhibits the adhesion and spreading function of platelets.

The platelet activation signals induced by specific receptors on platelets eventually converge into a common signaling event that induces an “inside-out” signaling process that activates integrin α IIb β 3 (Ginsberg, 2014). This leads to platelet spreading, secretion of additional granules, stabilization of platelet adhesion and aggregation, and clot retraction. Platelet adhesion to immobilized fibrinogen and mediated clot retraction involves an “outside-in” signaling and cytoskeletal reorganization of integrin α IIb β 3 (Hynes, 2002; Durrant et al., 2017). Clot retraction is an important process of vascular injury healing, and it also reflects the “outside-in” signal transduction process of platelets. Abnormal clot retraction indicates that the “outside-in” signal of platelets is damaged, and eventually leads to prolonged bleeding time and abnormal thrombus formation. We found that pimpinellin inhibited the binding of PAC-1 to activated integrin α IIb β 3, suggesting that pimpinellin inhibited the “inside-out” signaling. Pimpinellin also inhibited both platelets spreading area and clot retraction, suggesting that pimpinellin also inhibited “outside-in” signaling.

Many compounds or extracts of plant origin inhibited platelet function and mediated the collagen-induced GPVI signalling pathway. Glaucoalyxin A incubation reduced phosphorylation of SYK, LAT and PLC γ 2 (Li et al., 2013); Tussilagone decreased the phosphorylation of Syk/PLC γ 2-PKC/MAPK signaling pathways (Zhou et al., 2020); Salidroside inhibits platelet function through AKT/GSK3 β signaling pathway (Wei et al., 2020). In the present

study, we also demonstrated that pimpinellin inhibited multiple key protein phosphorylation related to collagen-induced signaling pathways in platelets. Activation of collagen/GPVI receptors leads to tyrosine phosphorylation of Fyn and Lyn, followed by Syk activation and LAT tyrosine phosphorylation (Poole et al., 1997), which in turn recruits PI3K and PLC γ 2. PLC γ 2 then catalyzes the release of DAG and IP3 from phosphatidylinositol for subsequent PKC activation and Ca²⁺ movement (Harper and Poole, 2010), ultimately leading to platelet secretion, aggregation, and thrombus formation. We found that pimpinellin inhibited Src416 (Supplementary Figure S3), Syk, PI3K, PLC γ 2, and PKC phosphorylation. Therefore, our results suggest that pimpinellin inhibits collagen-stimulated platelet activation and aggregation by negatively regulating GPVI signaling.

We are familiar with the GPVI signals often involved in SYK, PLC γ 2, SLP76 and PI3K (Watson et al., 2005). And phosphoinositide 3-kinase (PI3K) plays an important role in GPVI-mediated platelet activation (Chen et al., 2004; Gibbins, 2004; Kim et al., 2009). MAPKs, including ERK, p38 MAPK, and JNK, are also involved in collagen-induced platelet activation (Fan et al., 2018; Cargnello and Roux, 2011). p38 MAPK is reported to be a key signal for collagen-induced aggregation (Kuliopulos et al., 2004; Hanai et al., 2009). The present study showed that pimpinellin-mediated inhibition of collagen-stimulated platelet activation involves p38 MAPK activation but not ERK and JNK activation. It was demonstrated that PKC α not only affects the release of platelet alpha particles and dense granules (Konopatskaya et al., 2009), but also mediates the GPVI signaling pathway (Pula et al., 2005). Our data suggest that pimpinellin can effectively inhibit the phosphorylation of PKC. The PI3K/Akt signaling pathway regulates a variety of cellular processes such as inflammation, mitosis, and cell migration in several cell types (Fresno et al., 2004). In platelets, PI3K/Akt is an important regulator of platelet function such as granule secretion and activation of integrin α IIb β 3 (Niu et al., 2012). In this study, we found that pimpinellin inhibited collagen-induced phosphorylation of P85, Akt, and GSK3 β (a recognized downstream effector protein of Akt). LY294002 is an inhibitor of the PI3K/Akt pathway and effectively reduces collagen-induced platelet aggregation and phosphorylation of Akt. Besides, we also found that a combination of pimpinellin and PI3K-specific inhibitor LY294002 (Vlahos et al., 1994), enhanced the inhibition of platelet aggregation by pimpinellin and Akt phosphorylation, suggesting that pimpinellin inhibition of platelet activation and function by inhibiting downstream of collagen-induced GPVI signaling downstream. We found that pimpinellin can affect the phosphorylation of SRC and Syk, thus suggesting that pimpinellin can affect GPVI proximal signaling.

In addition, we studied the effect of pimpinellin on the coagulation system. PT usually reflects the extrinsic coagulation system, while APTT is mainly used as an endogenous coagulation system (Liu et al., 2018). Pimpinellin (40 mg/kg) showed no effect on APTT and PT, suggesting that low doses of pimpinellin did not affect coagulation function. However, high doses of pimpinellin (100 mg/kg) significantly ($p < 0.01$) affected APTT, suggesting that the anticoagulation mechanism at high dose was mainly mediated by the endogenous coagulation system. The mice platelet *ex-vivo*

assay also confirmed that pimpinellin (40 and 100 mg/kg) inhibited collagen-induced platelet aggregation. We also found that pimpinellin (40 mg/kg) did not affect tail bleeding time in mice, pimpinellin (100 mg/kg) prolonged tail bleeding in mice, suggesting that low doses of pimpinellin does not cause serious bleeding side effects at concentrations that inhibit platelet aggregation.

In conclusion, pimpinellin has significant anti-platelet aggregation, release, adhesion, and spreading effects and inhibits clot retraction. Besides, its anti-platelet effects are mainly mediated by key molecules in the collagen/GPVI signaling pathway, which involves Syk-SLP76-PLC γ 2-PKC-p38 MAPK and PI3K/Akt signaling pathways. Our study shows that pimpinellin has anti-platelet activity in addition to its other well-known properties. This indicates that pimpinellin may be a potential agent for effectively preventing platelet-related thromboembolic diseases, and it may also be an ideal choice for preventing atherosclerotic diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of GuiZhou Medical University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The Animal Care Welfare Committee of Guizhou Medical University.

AUTHOR CONTRIBUTIONS

ZQ performed the study design. GL and ZY analyzed data and wrote the manuscript. XT, XX, FG, and ZL collected and analyzed data. All authors contributed to manuscript revision and read and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China (No 81760653), Education department of Guizhou province young science and technology talent development project, Grant/Award Number: (KY (2018)185), Scientific Research Starting Foundation for Doctor Talent of Guizhou Medical University ((2017)019).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2021.706363/full#supplementary-material>

REFERENCES

- Alagaraj, P., and Muthukrishnan, S. (2020). Toddalia Asiatica L. - A Rich Source of Phytoconstituents with Potential Pharmacological Actions, an Appropriate Plant for Recent Global Arena. *Chamc* 18 (2), 104–110. doi:10.2174/1871525718666200212095756
- Binsker, U., Palankar, R., Wesche, J., Kohler, T., Prucha, J., Burchhardt, G., et al. (2018). Secreted Immunomodulatory Proteins of *Staphylococcus aureus* Activate Platelets and Induce Platelet Aggregation. *Thromb. Haemost.* 47 (4), 745–757. doi:10.1055/s-0038-1637735
- Blair, P., and Flaumenhaft, R. (2009). Platelet α -granules: Basic Biology and Clinical Correlates. *Blood Rev.* 23 (4), 177–189. doi:10.1016/j.blre.2009.04.001
- Cargnello, M., and Roux, P. P. (2011). Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. *Microbiol. Mol. Biol. Rev.* 75 (1), 50–83. doi:10.1128/MMBR.00031-10
- Chen, J., De, S., Damron, D. S., Chen, W. S., Hay, N., and Byzova, T. V. (2004). Impaired Platelet Responses to Thrombin and Collagen in AKT-1-Deficient Mice. *Blood* 104 (6), 1703–1710. doi:10.1182/blood-2003-10-3428
- Davi, G., and Patrono, C. (2007). Platelet Activation and Atherothrombosis. *N. Engl. J. Med.* 357 (24), 2482–2494. doi:10.1056/NEJMra071014
- Durrant, T. N., van den Bosch, M. T., and Hers, I. (2017). Integrin α IIb β 3 Outside-In Signaling. *Blood* 130 (14), 1607–1619. doi:10.1182/blood-2017-03-773614
- Faber, D. R., de Groot, P. G., and Visseren, F. L. J. (2009). Role of Adipose Tissue in Haemostasis, Coagulation and Fibrinolysis. *Obes. Rev.* 10 (5), 554–563. doi:10.1111/j.1467-789X.2009.00593.x
- Fan, X., Wang, C., Shi, P., Gao, W., Gu, J., Geng, Y., et al. (2018). Platelet MEK3 Regulates Arterial Thrombosis and Myocardial Infarct Expansion in Mice. *Blood Adv.* 2 (12), 1439–1448. doi:10.1182/bloodadvances.2017015149
- Fontana, P., Zufferey, A., Daali, Y., and Reny, J.-L. (2014). Antiplatelet Therapy: Targeting the TxA2 Pathway. *J. Cardiovasc. Trans. Res.* 7 (1), 29–38. doi:10.1007/s12265-013-9529-1
- Fresno Vara, J. A., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., and González-Barón, M. (2004). PI3K/Akt Signalling Pathway and Cancer. *Cancer Treat. Rev.* 30 (2), 193–204. doi:10.1016/j.ctrv.2003.07.007
- Fuentes, E., and Palomo, I. (2013). Relationship Between Platelet PPARs, cAMP Levels, and P-Selectin Expression: Antiplatelet Activity of Natural Products. *Evidence-Based Complement. Altern. Med.* 2013, 1–10. doi:10.1155/2013/861786
- Gallus, A. S., and Hirsh, J. (1976). Antithrombotic Drugs. *Drugs* 12 (1), 41–68. doi:10.2165/00003495-197612010-00002
- Gao, P., Li, S., Liu, K., Sun, C., Song, S., and Li, L. (2019). Antiplatelet Aggregation and Antithrombotic Benefits of Terpenes and Flavones from Hawthorn Leaf Extract Isolated Using the Activity-Guided Method. *Food Funct.* 10 (2), 859–866. doi:10.1039/c8fo01862f
- Gibbins, J. M. (2004). Platelet Adhesion Signalling and the Regulation of Thrombus Formation. *J. Cel Sci.* 117 (Pt 16), 3415–3425. doi:10.1242/jcs.01325
- Ginsberg, M. H. (2014). Integrin Activation. *BMB Rep.* 47 (12), 655–659. doi:10.5483/bmbrep.2014.47.12.241
- Hanai, Y., Adachi, S., Yasuda, I., Takai, S., Matsushima-Nishiwaki, R., Kato, H., et al. (2009). Collagen-induced P38 MAP Kinase Activation Is a Biomarker of Platelet Hyper-Aggregation in Patients with Diabetes Mellitus. *Life Sci.* 85 (9–10), 386–394. doi:10.1016/j.lfs.2009.07.003
- Harper, M. T., and Poole, A. W. (2010). Diverse Functions of Protein Kinase C Isoforms in Platelet Activation and Thrombus Formation. *J. Thromb. Haemost.* 8 (3), 454–462. doi:10.1111/j.1538-7836.2009.03722.x
- Hou, S.-M., Hsia, C.-W., Tsai, C.-L., Hsia, C.-H., Jayakumar, T., Velusamy, M., et al. (2020). Modulation of Human Platelet Activation and In Vivo Vascular Thrombosis by Columbianadin: Regulation by Integrin α IIb β 3 Inside-Out but Not Outside-In Signals. *J. Biomed. Sci.* 27 (1), 60. doi:10.1186/s12929-020-0619-5
- Hu, X.-L., Xu, Z., Liu, M.-L., Feng, L.-S., and Zhang, G.-D. (2018). Recent Developments of Coumarin Hybrids as Anti-fungal Agents. *Curr. Top. Med. Chem.* 17 (29), 3219–3231. doi:10.2174/1568026618666171215100326
- Huang, J., Li, X., Shi, X., Zhu, M., Wang, J., Huang, S., et al. (2019). Platelet Integrin α IIb β 3: Signal Transduction, Regulation, and its Therapeutic Targeting. *J. Hematol. Oncol.* 12 (1), 26. doi:10.1186/s13045-019-0709-6
- Hynes, R. O. (2002). Integrins. *Cell* 110 (6), 673–687. doi:10.1016/s0092-8674(02)00971-6
- Jin, L., Ying, Z.-H., Yu, C.-H., Zhang, H.-H., Yu, W.-Y., and Wu, X.-N. (2020). Isofraxidin Ameliorated Influenza Viral Inflammation in Rodents via Inhibiting Platelet Aggregation. *Int. Immunopharmacology* 84, 106521. doi:10.1016/j.intimp.2020.106521
- Jin, Y.-R., Yu, J. Y., Lee, J.-J., You, S.-H., Chung, J.-H., Noh, J.-Y., et al. (2007). Antithrombotic and Antiplatelet Activities of Korean Red Ginseng Extract. *Basic Clin. Pharmacol. Toxicol.* 100 (3), 170–175. doi:10.1111/j.1742-7843.2006.00033.x
- Jorgensen, L. (2006). The Role of Platelets in the Initial Stages of Atherosclerosis. *J. Thromb. Haemost.* 4 (7), 1443–1449. doi:10.1111/j.1538-7836.2006.02006.x
- Jurk, K., and Kehrel, B. E. (2010). Pathophysiologie und Biochemie der Thrombozyten. *Internist* 51 (9), 1086–1094. doi:10.1007/s00108-010-2595-4
- Jurk, K., and Kehrel, B. E. (2005). Platelets: Physiology and Biochemistry. *Semin. Thromb. Hemost.* 31 (4), 381–392. doi:10.1055/s-2005-916671
- Kariuki, H., Kanui, T., Yenesew, A., Patel, N., and Mbugua, P. (2013). Antinociceptive and Anti-inflammatory Effects of Toddalia Asiatica (L) Lam. (Rutaceae) Root Extract in Swiss Albino Mice. *Pan Afr. Med. J.* 14, 133. doi:10.11604/pamj.2013.14.133.2130
- Kim, S., Mangin, P., Dangelmaier, C., Lillian, R., Jackson, S. P., Daniel, J. L., et al. (2009). Role of Phosphoinositide 3-Kinase β in Glycoprotein VI-mediated Akt Activation in Platelets. *J. Biol. Chem.* 284 (49), 33763–33772. doi:10.1074/jbc.M109.048553
- Konopatskaya, O., Gilio, K., Harper, M. T., Zhao, Y., Cosemans, J. M. E. M., Karim, Z. A., et al. (2009). PKC α Regulates Platelet Granule Secretion and Thrombus Formation in Mice. *J. Clin. Invest.* 119 (2), 399–407. doi:10.1172/JCI34665
- Koupenova, M., Clancy, L., Corkrey, H. A., and Freedman, J. E. (2018). Circulating Platelets as Mediators of Immunity, Inflammation, and Thrombosis. *Circ. Res.* 122 (2), 337–351. doi:10.1161/CIRCRESAHA.117.310795
- Koupenova, M., Kehrel, B. E., Corkrey, H. A., and Freedman, J. E. (2017a). Thrombosis and Platelets: An Update. *Eur. Heart J.* 38 (11), 785–791. doi:10.1093/eurheartj/ehw550
- Koupenova, M., Kehrel, B. E., Corkrey, H. A., and Freedman, J. E. (2017b). Thrombosis and Platelets: An Update. *Eur. Heart J.* 38 (11), 785–791. doi:10.1093/eurheartj/ehw550
- Kuliopulos, A., Mohanlal, R., and Covic, L. (2004). Effect of Selective Inhibition of the P38 MAP Kinase Pathway on Platelet Aggregation. *Thromb. Haemost.* 92 (6), 1387–1393. doi:10.1160/TH04-03-0187
- Lam, M., Roszik, J., Kanikarla-Marie, P., Davis, J. S., Morris, J., Kopetz, S., et al. (2017). The Potential Role of Platelets in the Consensus Molecular Subtypes of Colorectal Cancer. *Cancer Metastasis Rev.* 36 (2), 273–288. doi:10.1007/s10555-017-9678-9
- Lee, J.-J., Kim, T., Cho, W.-K., and Ma, J. Y. (2013). Antithrombotic and Antiplatelet Activities of Soshiho-Tang Extract. *BMC Complement. Altern. Med.* 13, 137. doi:10.1186/1472-6882-13-137
- Li, W., Tang, X., Yi, W., Li, Q., Ren, L., Liu, X., et al. (2013). Glaucoalyxin A Inhibits Platelet Activation and Thrombus Formation Preferentially via GPVI Signaling Pathway. *PLoS One* 8 (12), e85120. doi:10.1371/journal.pone.0085120
- Li, X., Qiu, Z., Jin, Q., Chen, G., and Guo, M. (2018). Cell Cycle Arrest and Apoptosis in HT-29 Cells Induced by Dichloromethane Fraction from Toddalia Asiatica (L) Lam. *Front. Pharmacol.* 9, 629. doi:10.3389/fphar.2018.00629
- Liu, G., Xie, W., He, A.-D., Da, X.-W., Liang, M.-L., Yao, G.-q., et al. (2016). Antiplatelet Activity of Chrysin via Inhibiting Platelet α IIb β 3-mediated Signaling Pathway. *Mol. Nutr. Food Res.* 60 (9), 1984–1993. doi:10.1002/mnfr.201500801
- Liu, J., Xu, D., Xia, N., Hou, K., Chen, S., Wang, Y., et al. (2018). Anticoagulant Activities of Indobufen, An Antiplatelet Drug. *Molecules* 23 (6), 1452. doi:10.3390/molecules23061452
- Liu, Z. G., Wang, X. Y., Mao, B. P., and Xie, X. L. (2014). [Study on Chemical Constituents of Toddalia Asiatica]. *Zhong Yao Cai* 37 (9), 1600–1603. doi:10.13863/j.issn1001-4454.2014.09.024
- Mackman, N., Tilley, R. E., and Key, N. S. (2007). Role of the Extrinsic Pathway of Blood Coagulation in Hemostasis and Thrombosis. *Arterioscler Thromb. Vasc. Biol.* 27 (8), 1687–1693. doi:10.1161/ATVBAHA.107.141911
- Margarucci, L., Roest, M., Preisinger, C., Bleijerveld, O. B., van Holten, T. C., Heck, A. J. R., et al. (2011). Collagen Stimulation of Platelets Induces a Rapid Spatial Response of cAMP and cGMP Signaling Scaffolds. *Mol. Biosyst.* 7 (7), 2311–2319. doi:10.1039/c1mb05145h

- Michelson, A. D. (2008). P2Y12 Antagonism. *Arterioscler Thromb. Vasc. Biol.* 28 (3), s33–8. doi:10.1161/ATVBAHA.107.160689
- Mousa, S. A. (2010). Antithrombotic Effects of Naturally Derived Products on Coagulation and Platelet Function. *Methods Mol. Biol.* 663, 229–240. doi:10.1007/978-1-60761-803-4_9
- Nilsson, B., Back, V., Wei, R., Plane, F., Jurasz, P., and Bungard, T. J. (2019). Potential Antimigraine Effects of Warfarin: An Exploration of Biological Mechanism with Survey of Patients. *TH Open* 03 (2), e180–e189. doi:10.1055/s-0039-1692989
- Niu, H., Chen, X., Gruppo, R. A., Li, D., Wang, Y., Zhang, L., et al. (2012). Integrin α IIb-Mediated PI3K/Akt Activation in Platelets. *PLoS One* 7 (10), e47356. doi:10.1371/journal.pone.0047356
- Poole, A., Gibbins, J. M., Turner, M., van Vugt, M. J., van de Winkel, J. G. J., Saito, T., et al. (1997). The Fc Receptor γ -chain and the Tyrosine Kinase Syk Are Essential for Activation of Mouse Platelets by Collagen. *EMBO J.* 16 (9), 2333–2341. doi:10.1093/emboj/16.9.2333
- Pula, G., Crosby, D., Baker, J., and Poole, A. W. (2005). Functional Interaction of Protein Kinase Ca with the Tyrosine Kinases Syk and Src in Human Platelets. *J. Biol. Chem.* 280 (8), 7194–7205. doi:10.1074/jbc.M409212200
- Raslan, Z., Aburima, A., and Naseem, K. M. (2015). The Spatiotemporal Regulation of cAMP Signaling in Blood Platelets—Old Friends and New Players. *Front. Pharmacol.* 6, 266. doi:10.3389/fphar.2015.00266
- Raslan, Z., and Naseem, K. M. (2014). The Control of Blood Platelets by cAMP Signalling. *Biochem. Soc. Trans.* 42 (2), 289–294. doi:10.1042/BST20130278
- Rondina, M. T., Weyrich, A. S., and Zimmerman, G. A. (2013). Platelets as Cellular Effectors of Inflammation in Vascular Diseases. *Circ. Res.* 112 (11), 1506–1519. doi:10.1161/CIRCRESAHA.113.300512
- Stephen Irudayaraj, S., Sunil, C., Duraipandiyan, V., and Ignacimuthu, S. (2012). Antidiabetic and Antioxidant Activities of *Toddalia Asiatica* (L.) Lam. Leaves in Streptozotocin Induced Diabetic Rats. *J. Ethnopharmacol.* 143 (2), 515–523. doi:10.1016/j.jep.2012.07.006
- Vaiyapuri, S., Roweth, H., Ali, M. S., Unsworth, A. J., Stainer, A. R., Flora, G. D., et al. (2015). Pharmacological Actions of Nobiletin in the Modulation of Platelet Function. *Br. J. Pharmacol.* 172 (16), 4133–4145. doi:10.1111/bph.13191
- Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994). A Specific Inhibitor of Phosphatidylinositol 3-kinase, 2-(4-Morpholinyl)-8-Phenyl-4H-1-Benzopyran-4-One (LY294002). *J. Biol. Chem.* 269 (7), 5241–5248. doi:10.1016/s0021-9258(17)37680-9
- Watson, S. P., Auger, J. M., Mccarty, O. J. T., and Pearce, A. C. (2005). GPVI and Integrin α IIb β 3 Signaling in Platelets. *J. Thromb. Haemost.* 3 (8), 1752–1762. doi:10.1111/j.1538-7836.2005.01429.x
- Wei, G., Xu, X., Tong, H., Wang, X., Chen, Y., Ding, Y., et al. (2020). Salidroside Inhibits Platelet Function and Thrombus Formation Through AKT/GSK3 β Signaling Pathway. *Aging* 12 (9), 8151–8166. doi:10.18632/aging.103131
- Yue, M., Luo, D., Yu, S., Liu, P., Zhou, Q., Hu, M., et al. (2016). Misshapen/NIK-related Kinase (MINK1) Is Involved in Platelet Function, Hemostasis, and Thrombus Formation. *Blood* 127 (7), 927–937. doi:10.1182/blood-2015-07-659185
- Zaragoza, C., Monserrat, J., Mantecón, C., Villaescusa, L., Zaragoza, F., and Álvarez-Mon, M. (2016). Antiplatelet Activity of Flavonoid and Coumarin Drugs. *Vasc. Pharmacol.* 87, 139–149. doi:10.1016/j.vph.2016.09.002
- Zhang, X., Sun, W., Yang, Z., Liang, Y., Zhou, W., and Tang, L. (2017). Hemostatic Chemical Constituents from Natural Medicine *Toddalia Asiatica* Root Bark by LC-ESI Q-TOF MSE. *Chem. Cent. J.* 11 (1), 55. doi:10.1186/s13065-017-0283-3
- Zhou, J., Yang, R.-P., Song, W., Xu, H.-M., and Wang, Y.-H. (2020). Antiplatelet Activity of Tussilagone via Inhibition of the GPVI Downstream Signaling Pathway in Platelets. *Front. Med.* 7, 380. doi:10.3389/fmed.2020.00380

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Liu, Yuan, Tian, Xiong, Guo, Lin and Qin. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.