Inhibitory effects of luteolin-4'-*O*-β-D-glucopyranoside on $P2Y_{12}$ and thromboxane A_2 receptor-mediated amplification of platelet activation *in vitro*

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Abstract. Platelet activation and subsequent accumulation at sites of vascular injury are central to thrombus formation, which is considered to be a trigger of several cardiovascular diseases. *Callicarpa nudiflora* (*C. nudiflora*) Hook is a traditional Chinese medicinal herb for promoting blood circulation by removing blood stasis. In our previous study, several compounds extracted from this herb, including luteolin-4'-*O*-β-D-glucopyranoside (LGP), were revealed to exert inhibitory effects on adenosine diphosphate (ADP)-induced platelet aggregation. The aim of present study was to confirm these antiplatelet effects and elucidate the potential mechanisms. Using a platelet-aggregation assay, it was revealed that LGP significantly inhibited platelet aggregation induced by ADP, U46619 and arachidonic acid. It was also found that LGP exhibited marked inhibitory effects on the activation of $\alpha_{IIb}\beta_3$ integrin, the secretion of serotonin from granules, and the synthesis of thromboxane A_2. In addition, the results showed that LGP suppressed Ras homolog family member A and phosphoinositide 3-kinase/Akt/glycogen synthase kinase 3β signal transduction. Data from a radio-labeled ligand-binding assay indicated that LGP exhibited apparent competing effects on thromboxane receptor (TP) and $P2Y_{12}$ receptors. In conclusion, the data presented here demonstrated that LGP, a natural compound from *C. nudiflora* Hook, inhibited the development of platelet aggregation and amplification of platelet activation. These inhibitory effects may be associated with its dual-receptor inhibition on $P2Y_{12}$ and TP receptors.

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Introduction

Platelets are small, a nucleate blood cells, the major role of which is in hemostasis and thrombosis owing to their capacity to adhere to damaged blood vessels and to accumulate at sites of injury (1). However, platelets are also important contributors to thrombotic disorders, including atherothrombosis, which are the final events complicating cardiovascular diseases (2-4). Upon vascular injury, platelets are exposed to the subendothelium, and several agonists, including adenosine diphosphate (ADP) and thrombin, are generated at the injury site, which can stimulate platelet adhesion, activation and aggregation. Adherent, activated platelets recruit additional platelets to the growing thrombus (5,6). The uncontrolled progression of these processes through a series of self-sustaining amplification loops can initiate unrestrained platelet activation and aggregation, and eventually lead to thromboembolic events (7,8). It has been demonstrated clinically that the use of antiplatelet agents to prevent and/or reverse platelet aggregation is a successful strategy for the prevention of thrombosis (7,8). However, due to their disturbance of the thromboregulatory balance, existing antiplatelet drugs can cause severe side effects, the majority of which limit the efficacy and safetyof these drugs. Uncontrolled hemorrhage is the most frequent side effect of antithrombotics/antiplatelets (9,10). Therefore, understanding the molecular mechanisms of platelet activation and identifying novel techniques for platelet inhibition remain critically important.

Natural products remain a major resource and have become increasingly important for novel drug identification. The *Callicarpa* genus includes ~190 extant species (11). Among these, there are >10 medical herbs, and the majority of these have hemostasis-associated usage (11). *Callicarpa nudiflora* (*C. nudiflora*) Hook, which is one of the medical herbs of *Callicarpa* with a long history, is used for eliminating stasis in order to subdue swelling and hemostasis (11). According

to traditional Chinese medicine theory, eliminating stasis to subdue swelling is similar to the antithrombotic effect. Led by these concepts, the present study hypothesized that antiplatelet activities may contribute to the traditional usage of this plant. In our previous study, hundreds of constituents were screened, including several derivatives of luteolin. It was found that two novel triterpenoids, extracted from the leaves of *C. nudiflora*, showed inhibitory effects on ADP-induced platelet activation (12,13). In the present study, it was shown that one of the derivatives of luteolin, 1,6-di-*O*-caffeoyl-β-D-glucopyranoside (LGP) exhibited potent inhibitory effects on platelet activation, and it was demonstrated that the effects of this natural compound may be mediated by dual receptor antagonism on $P2Y_{12}$ receptor and thromboxane A_2 (TXA_2) receptor (TP).

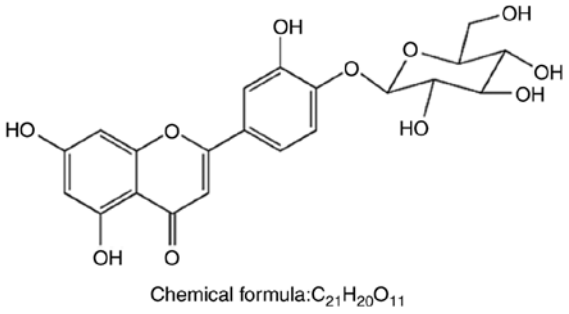

Figure 1. Molecular structure of luteolin-4'-*O*-β-D-glucopyranoside.

Materials and methods

Drugs and chemicals. Luteolin-4'-O-β-D-glucopyranoside (LGP) was isolated from the leaves of *C. nudiflora* Hook and its molecular structure is shown in Fig. 1. The purity of LGP was ≥95%, as determined by high-performance liquid chromatography. A stock solution was prepared by dissolving the LGP in 100% dimethyl sulfoxide (DMSO) and was used throughout the investigation. The final DMSO concentration did not exceed 0.1%. ADP, arachidonic acid (AA), ticagrelor (an antagonist of the $P2Y_{12}$ receptor) and BM-531 (an antagonist of the TP receptor), were purchased from Sigma-Aldrich; EMD Millipore (Billerica, MA, USA). U46619 was the product of Tocris Bioscience (Bristol, UK). [^{3}H]-2-methylthioadenosine diphosphate ([^{3}H]-2-MeS-ADP) and [^{3}H] SQ-29548 were purchased from GE Healthcare Life Sciences (Chalfont, UK) and PerkinElmer, Inc. (Waltham, MA, USA), respectively.

Animals. A total of 80 female Sprague-Dawley rats (aged 6-8 weeks old and weighing 180-220 g) were obtained from Vital River Laboratories (Beijing, China) and maintained under pathogen-free conditions in the Animal Center of Jiangxi University of Traditional Chinese Medicine (Nanchang, China). All the animals were maintained in a 12 h light/dark cycle at room temperature (25±2˚C) in 60% humidity. The animals were allowed water *ad libitum* and were fed a standard laboratory diet. The protocol used for animal experiments (JZAEC-2016-0031) was approved by the Animal Ethics Committees of Jiangxi University of Traditional Chinese Medicine, and all animal experiments were performed in strict accordance with the requirements of this protocol.

Preparation of rat platelets. Blood was collected from the abdominal aorta of anesthetized rats into a vacuum blood collection tube, which allowed 10% blood volume with 3.8% sodium citrate as anticoagulant. The citrated blood was then centrifuged (Allegra™ X-12R centrifuge; Beckman Coulter, Inc., Brea, CA, USA) at 110 x g for 15 min at 4˚C to obtain platelet-rich plasma (PRP), and the quantity of platelets in the PRP was determined using the automatic blood cell analyzer (HEMAVET 950FS; Drew Scientific, Miami Lakes, FL, USA). Platelet-poor plasma (PPP) was obtained by a second centrifugation of the remaining blood (1,000 x g, 10 min, 4˚C). The PRP was adjusted to a platelet count of $400x10^9$ platelets/l by diluting in PPP.

Platelet aggregation. Platelet aggregation in 96-well plates was measured using a modified light transmission method (14,15). Briefly, the PRP ($400x10^9$ platelets/l) was incubated with 40, 80 and 160 μM of LGP, antagonists (positive controls) or dissolvent for 15 min at 37˚C, respectively. The optical density (OD) was then determined at 595 nm and marked as OD_1. Platelet aggregation was induced by the following agonists: ADP (10 μM), U46619 (1 μM) or AA (600 μM), and OD (OD_2) was determined again at 595 nm every 30 sec for 15 min, with 15 sec incubation and 15 sec shaking between readings. In addition, the OD value at 595 nm was determined for the same volume of PPP, and was marked as OD_3. All experiments were performed at least three times. The percentage of aggregation was calculated using the absorbance of PRP without agonist as 0% aggregation and the absorbance of PPP as 100% aggregation. The relative aggregation was expressed using the following formula: Relative aggregation (%)=$[(OD_1-OD_2)/(OD_1-OD_3)]$ x100.

Activated $\alpha_{IIb}\beta_3$ integrin abundance. $\alpha_{IIb}\beta_3$ integrin is expressed on the surface of platelets, which reflects platelet activation or secretion from platelet granules. This was determined by the measurement of fluorescent agent-labeled antibody binding, as described previously (16,17). For measurement of the expression of $\alpha_{IIb}\beta_3$ integrin, briefly, 50 μl activated platelets ($400x10^9$ platelets/l) were pretreated for 15 min with DMSO or the indicated concentrations (20, 40, 80 and 160 μM) of LGP, and then fixed for 30 min in 0.5% paraformaldehyde. Following washing once in incubation buffer, the fixed platelets were added into 96-well plates, and incubated with Oregon Green-labeled fibrinogen (Molecular Probes; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 15 min at room temperature without shaking. The total fluorescence was determined by measuring the fluorescence of the plate on a multi-label counter (VICTOR3™; PerkinElmer, Inc.). Subsequently, the plates were centrifuged at 1,000 x g for 10 min at 4˚C to remove platelets from the supernatant. The supernatants were transferred to separate plates and the fluorescence was determined (background or nonspecific). Specific Oregon Green binding was determined by subtracting the background fluorescence from the total fluorescence.

Measurement of serotonin (5-HT) secretion. 5-HT secretion was determined using a Serotonin ELISA kit (Abcam, Cambridge, MA, USA; cat. no. ab133053) according to the manufacturer's protocol. The assay procedure is based on the competition between an alkaline phosphatase-conjugated 5-HT (supplied) and a non-labeled antigen (5-HT extracted from PRP) for a fixed number of antibody binding sites on the micro-titer plate. First, a curve of the OD_{405} of 5-HT, compared with its concentration in the standard wells, was plotted. Subsequently, 50 μl platelets ($400x10^9$ platelets/l) were treated with DMSO or the indicated concentrations (20, 40, 80 and 160 μM) LGP for 15 min, followed by 3 min incubation with agonists (10 μM ADP or 1 μM U46619). The reaction was terminated by snap freezing. Following thawing at room temperature, the samples were centrifuged at 3,000 x g for 10 min at 4˚C. The supernatants were used for the measurement of 5-HT release. By comparing the absorbance of the samples with the standard curve, the 5-HT concentration in the unknown samples was determined, with data representative of at least five independent experiments.

Measurement of TXA_2 synthesis. In the present study, TXB_2, the stable metabolite of TXA_2, was measured to reflect the level of TXA_2. PRP ($400x10^9$ platelets/l) was pretreated with DMSO or various concentrations of LGP (20, 40, 80 and 160 μM) for 15 min at 37˚C, and was stimulated with ADP (10 μM) or U46619 (1 μM) at 37˚C for 3 min whilst stirring. The reaction was also terminated by snap freezing. Following thawing at room temperature and centrifuging at 3,000 x g for 10 min at 4˚C, the supernatants were diluted (1:20) with the assay buffer in the TXB_2 ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA). TXB_2 was measured according to the manufacturer's protocol.

Western blot analysis. PRP ($400x10^9$ platelets/l), pretreated with DMSO or various concentrations (40, 80 and 160 μM) LGP for 15 min, were stimulated for 3 min with agonists, and the reaction was terminated by rapid freezing of the sample in a dry ice-ethanol bath. Following thawing at room temperature, the samples were centrifuged at 3,000 x g for 10 min at 4˚C. The platelets were rinsed twice with PBS, and total proteins were extracted with lysis buffer. Aliquots of each platelet lysate containing equal quantities of protein (ranging between 500 and 750 μg between experiments) were added to SDS-PAGE gels (ranging between 8 and 12%), and then transferred onto hybond nitroblotting membranes and subjected to western blot analysis. Membranes were blocked using 5% non-fat dried milk for 1 h at room temperature and subsequently incubated with primary antibodies overnight at 4˚C. Following washing with 0.5% TBST three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The immunoreactive bands were detected using an enhanced chemiluminescence kit (EMD Millipore). β-actin (1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat. no. SC-130656) served as an internal control. The signal intensities of the bands of interest were quantified and normalized to β-actin using the Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The primary antibodies used in the present study were as follows: Anti-Rho guanine nucleotide exchange factor 1 (ARHGEF1) antibody (1:1,000; Abcam, cat. no. ab220892), anti-Ras homolog family member A (RhoA) antibody (1:1,000; Abcam; cat. no. ab54835), anti-RhoA antibody (1:1,000; phospho S188; Abcam; cat. no. ab41435), anti-Rho-associated kinase 1 (ROCK1) antibody (1:1,000; Abcam; cat. no. ab45171), anti-ROCK1 (phospho T455+S456) antibody (1:1,000; Abcam; cat. no. ab203273), anti-phosphoinositide 3-kinase (PI3K) p85 (phospho Y607) antibody (1:1,000; Abcam; cat. no. ab182651), anti-pan-AKT antibody (1:1,000; Abcam; cat. no. ab8805), anti-pan-AKT (phospho T308) antibody (1:1,000; Abcam; cat. no. ab38449), anti-AKT1 (phospho S473) antibody (1:1,000; Abcam; cat. no. ab81283) and anti-glycogen synthase kinase 3β (GSK3β) antibody (1:1,000; phospho S9; Abcam; cat. no. ab75814). The secondary antibodies used in the present study were as follows: Goat polyclonal secondary antibody to mouse IgG (1:5,000; Abcam; cat. no. ab6789) and goat anti-rabbit IgG (1:5,000; Abcam; cat. no. ab6721).

Receptor-binding assay. The effects of LGP on $P2Y_{12}$ ADP receptor binding were determined by the binding of [^{3}H]-2-MeS-ADP to rat platelets with a filter technique to separate the free from bound [^{3}H]-2-MeS-ADP. [^{3}H]SQ-29548 (PerkinElmer, Inc.) was also used in to assess the effects of LGP on TXA_2 receptor binding activities. Briefly, PRP ($1x10^9$ platelets/ml) was incubated with [^{3}H]SQ-29548 (40 nM final concentration) in a total of 400 μl Tyrode's buffer (pH 7.2) for 30 min at room temperature. Subsequently, indicated concentrations of LGP were added and incubated for 40 min at room temperature to compete binding between agonists and their receptors. The binding assays were terminated by rapid filtration on Packard GF-B filters (Packard Instrument Co., Inc., Meriden, CT, USA). The filters were then placed in plastic scintillation vials containing an emulsion-type scintillation mixture (4 ml) and the radioactivity, representing the binding of [^{3}H]SQ-29548 to TP receptor (B), was detected by Tri-Carb® Liquid Scintillation (PerkinElmer, Inc.). The radioactivity of [^{3}H]SQ-29548 (40 nM final concentration) in DMSO-treated platelets served as the total binding (Bt) of [^{3}H]SQ-29548 to the TP receptor. Non-specific binding (Bns) was defined as the total radioactivity measured in the presence of 100 μM (final concentration) unlabeled SQ-29548. The specific binding rate (Bs) of [^{3}H]SQ-29548 to the TP receptor was calculated using the following formula: Bs = (B - Bns)/Bt x 100.

For the $P2Y_{12}$ ADP receptor binding assay, a similar procedure to the TP receptor binding assay was used. PRP ($1x10^9$ platelets/ml) was prepared, as previously described, and incubated with [^{3}H]-2-MeS-ADP (5 nM final concentration) in a total of 400 μl Tyrode's buffer (pH 7.2) for 30 min at room temperature. Subsequently, indicated concentrations of LGP, DMSO or 2-MeS-ADP (5 μM, final concentration) were added for an additional 40 min at room temperature. Following filtration on Packard GF-B filters, radioactivity was detected by Tri-Carb® Liquid Scintillation (PerkinElmer, Inc.) and the specific binding rate of [^{3}H]-2-MeS-ADP to the $P2Y_{12}$ receptor was calculated.

Data presentation and statistical analysis. Data are presented as the mean ± standard error of the mean; n represents the number of independent experiments. Statistical significance was determined using one-way analysis of variance.

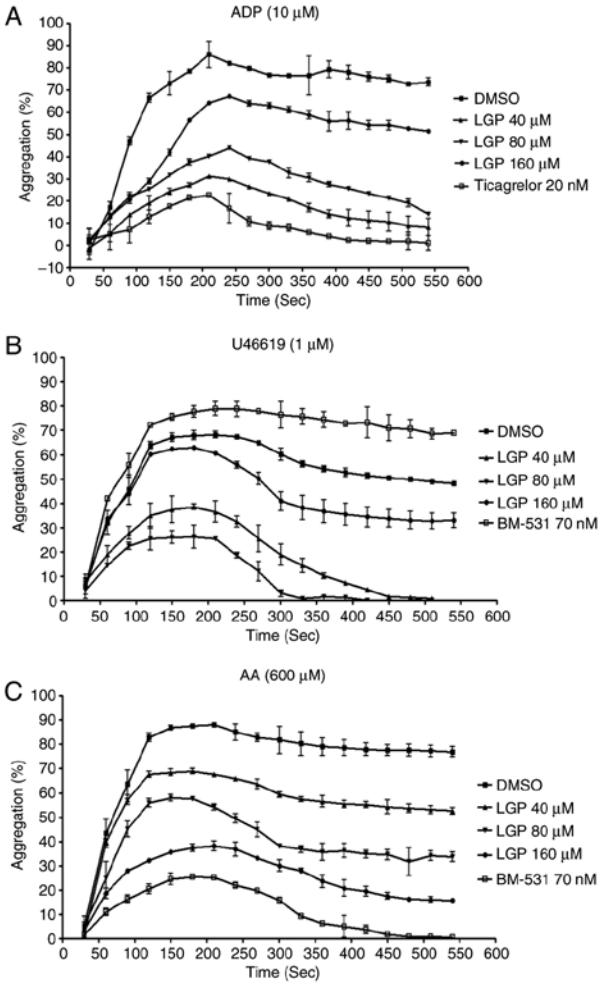

Figure 2. Effects of LGP on platelet aggregation induced by several agonists. (A) Effects of LGP on ADP-induced platelet aggregation. (B) Effects of LGP on U46619-induced platelet aggregation. (C) Effects of LGP on AA-induced platelet aggregation. Each assay was performed in triplicate. Vehicle (DMSO) was used as a control, and ticagrelor and BM-531 served as the positive controls. LGP, luteolin-4'-*O*-β-D-glucopyranoside; ADP, adenosine diphosphate; AA, arachidonic acid.

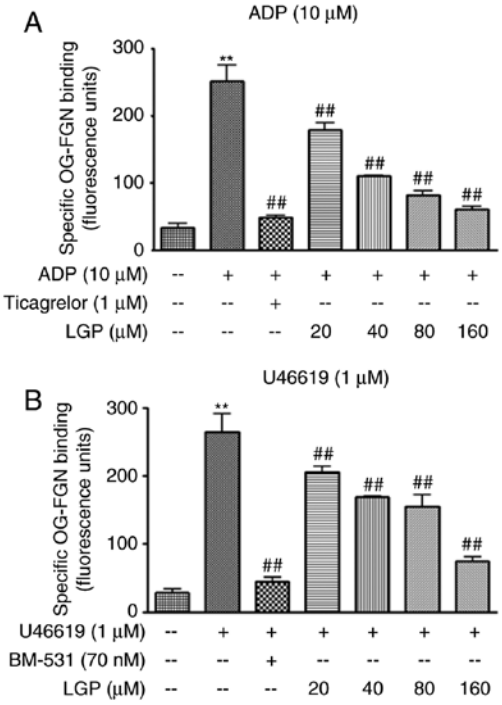

Figure 3. Effects of LGP on ADP- and U46619-mediated $\alpha_{IIb}\beta_3$ integrin activation. (A) Effects of LGP on ADP-induced $\alpha_{IIb}\beta_3$ integrin activation. (B) Effects of LGP on U46619-induced $\alpha_{IIb}\beta_3$ integrin activation. Each assay was performed in triplicate. **P<0.01, compared with control; $^{\#\#}$P<0.01, compared with ADP or U46619. Vehicle (DMSO) was used as a control, and ticagrelor and BM-531 served as the positive controls. LGP, luteolin-4'-*O*-β-D-glucopyranoside; ADP, adenosine diphosphate; OG-FGN, Oregon Green-labeled fibrinogen.

Dose-response curves were generated using GraphPad Prism software (version 4.0; GraphPad Software, Inc., La Jolla, CA, USA). The IC_{50} value for each agent was determined from three different concentrations of the agent using Schild analysis using GraphPad Prism software. P<0.05 was considered to indicate a statistically significant difference.

Results

LGP inhibits ADP-, U46619- and AA-induced platelet aggregation. The initial experiment defined the effects of LGP on platelet aggregation induced by several agonists. Rat platelets were isolated and platelet aggregation was observed. The platelets were pretreated with LGP for 15 min and were incubated with 10 μM ADP. As shown in Fig. 2A, 10 μM ADP (to PRP) produced typical aggregation curves. Ticagrelor and LGP significantly inhibited aggregation in a dose-dependent manner, and the IC_{50} value of LGP at 540 sec was 74.9±1.6 μM. Similar results were obtained when platelet aggregation was induced by U46619 (1 μM) and AA (600 μM). LGP exhibited concentration-dependent inhibition of aggregation induced by U46619 and AA with IC_{50} values of 61.7±1.2 and 81.7±1.1 μM at 540 sec, respectively (Fig. 2B and C).

LGP suppresses ADP and U46619-mediated $\alpha_{IIb}\beta_3$ integrin activation in rat platelets. It is widely accepted that integrin $\alpha_{IIb}\beta_3$-mediated outside-in signaling is the most important amplifier of platelet activation. To confirm the effects of LGP on outside-in signal transduction, the active integrin $\alpha_{IIb}\beta_3$ on the platelet surface was assessed by the measurement of fibrinogen binding. As shown in Fig. 3A, the level of integrin $\alpha_{IIb}\beta_3$ was negligible at the surface of resting platelets. There was a sharp increase in the level of integrin $\alpha_{IIb}\beta_3$ following ADP (10 μM) treatment, and a significant attenuation in the presence of LGP and the positive control ticagrelor. Similarly, the level of integrin $\alpha_{IIb}\beta_3$ was significantly increased by treatment with U46619 (1 μM). Again, the effect was significantly inhibited in the presence of LGP and BM-531 (Fig. 3B). These data indicated that LGP inhibited the activation of integrin $\alpha_{IIb}\beta_3$ in a concentration-dependent manner when the platelets were stimulated by ADP or U46619. These results are compatible with the results of the aggregation assay.

LGP inhibits5-HTrelease stimulated by ADP and U46619. The platelets pretreated by LGP were incubated with agonists to activate platelets, and the content of 5-HT in supernatants was measured using a Serotonin ELISA kit. As shown in Fig. 4A, the level of serotonin was low when the platelets were

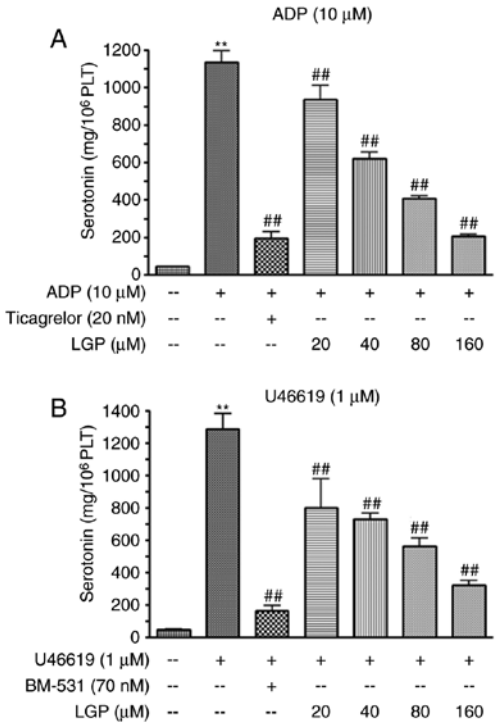

Figure 4. Effects of LGP on ADP- and U46619-mediated secretion of 5-HT. (A) Effects of LGP on ADP-induced 5-HT release. (B) Effects of LGP on U46619-induced 5-HT release. Each assay was performed in triplicate. **P<0.01, compared with control; ##P<0.01, compared with ADP or U46619. Vehicle (DMSO) was used as a control, and ticagrelor and BM-531 served as the positive controls. LGP, luteolin-4'-*O*-β-D-glucopyranoside; ADP, adenosine diphosphate; 5-HT, serotonin.

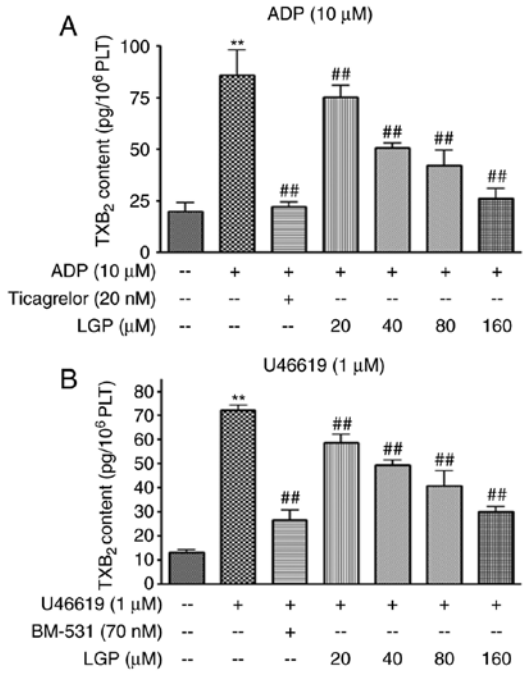

Figure 5. Effects of LGP on ADP- and U46619-mediated TXA_2 formation. (A) Effects of LGP on ADP-induced TXA_2 formation. (B) Effects of LGP on U46619-induced TXA_2 formation. As TXA_2 is unstable and is rapidly converted into a stable non-enzymatic hydration product TXA_2. A TXB_2 content assay was performed. Each assay was performed in triplicate. **P<0.01, compared with control; ##P<0.01, compared with ADP or U46619. Vehicle (DMSO) was used as a control, and ticagrelor and BM-531 served as the positive controls. LGP, luteolin-4'-*O*-β-D-glucopyranoside; ADP, adenosine diphosphate; TX, thromboxane.

treated with vehicle, whereas ADP (10 μM) treatment induced a sharp increase in the level of 5-HT. The positive control (ticagrelor) significantly suppressed the increase induced by ADP. LGP also caused a significant reduction in the release of serotonin in a concentration-dependent manner, with the inhibitory percentages of 22.55, 38.04, 47.71 and 65.95%, respectively. Similarly, LGP decreased the release of 5-HT stimulated by U46619 (1 μM) in a concentration-dependent manner (Fig. 4B).

LGP inhibits ADP and U46619-induced TXA_2 synthesis. TXA_2, which is produced by activated platelets, serves to promote further platelet activation by binding to TP receptor. The present study examined the effects of LGP on the content ofTXB_2 in platelets treated with ADP and U46619. As shown in Fig. 5A, ADP markedly stimulated TXB_2 release, whereas LGP and ticagrelor caused a significant inhibition in the formation of TXB_2 in a dose-dependent manner. Similar results were obtained with platelet activatorU46619 (Fig. 5B).

Effects of LGP on RhoA signaling induced by U46619. One previous study with inhibitors and/or genetic manipulations has demonstrated that RhoA signaling contributes to TXA_2-induced platelet activation by binding the TP receptor (18). In order to determine whether TP-mediated signal transduction is involved in the inhibitory effects of LGP on platelet activation, the present study examined the effects of LGP on the activation of RhoA signaling transducers. As shown in Fig. 6A and B, LGP significantly reduced the expression of phospho-RhoA in the presence of 1 μM U46619, however, there was no significant change in the expression of RhoA. Furthermore, it was found that the expression of ARHGEF1 (p115RhoGEF), an activator of RhoA, and phospho-ROCK1, an effecter of RhoA, were decreased by LGP in a dose-dependent manner.

Effects of LGP on regulating PI3K/Akt/GSK3β signal transduction stimulated by ADP. Subsequently, the present study examined the effects of LGP on PI3K/Akt/GSK3β signal transduction stimulated by ADP. Western blot analysis with platelet lysates revealed that pre-incubation of the platelets with LGP (40, 80 and 160 μM) attenuated the expression of phospo-PI3K in the presence of ADP. Consistently, the phosphorylation of Akt and GSK3β were also suppressed by LGP (40, 80 and 160 μM; Fig. 7A and B).

Effects of LGP on platelet $P2Y_{12}$ and TP receptor binding of [^{3}H]-2-MeS-ADP and [^{3}H] SQ-29548. In order to further define whether the LGP-mediated inhibitory effects on platelet activation are due to antagonism at $P2Y_{12}$ and TP receptors, the present study performed a radiolabeled ligand binding assay using [^{3}H]-2-MeS-ADP and [^{3}H]SQ-29548. LGP inhibited the binding of [^{3}H]-2-MeS-ADP to rat platelet membranes with K_i=0.8317 mM as shown in Fig. 8A. LGP also exhibited apparent competing effects on the TP receptor, which displaced [^{3}H] SQ-29548, a high affinity ligand of TP receptor from rat

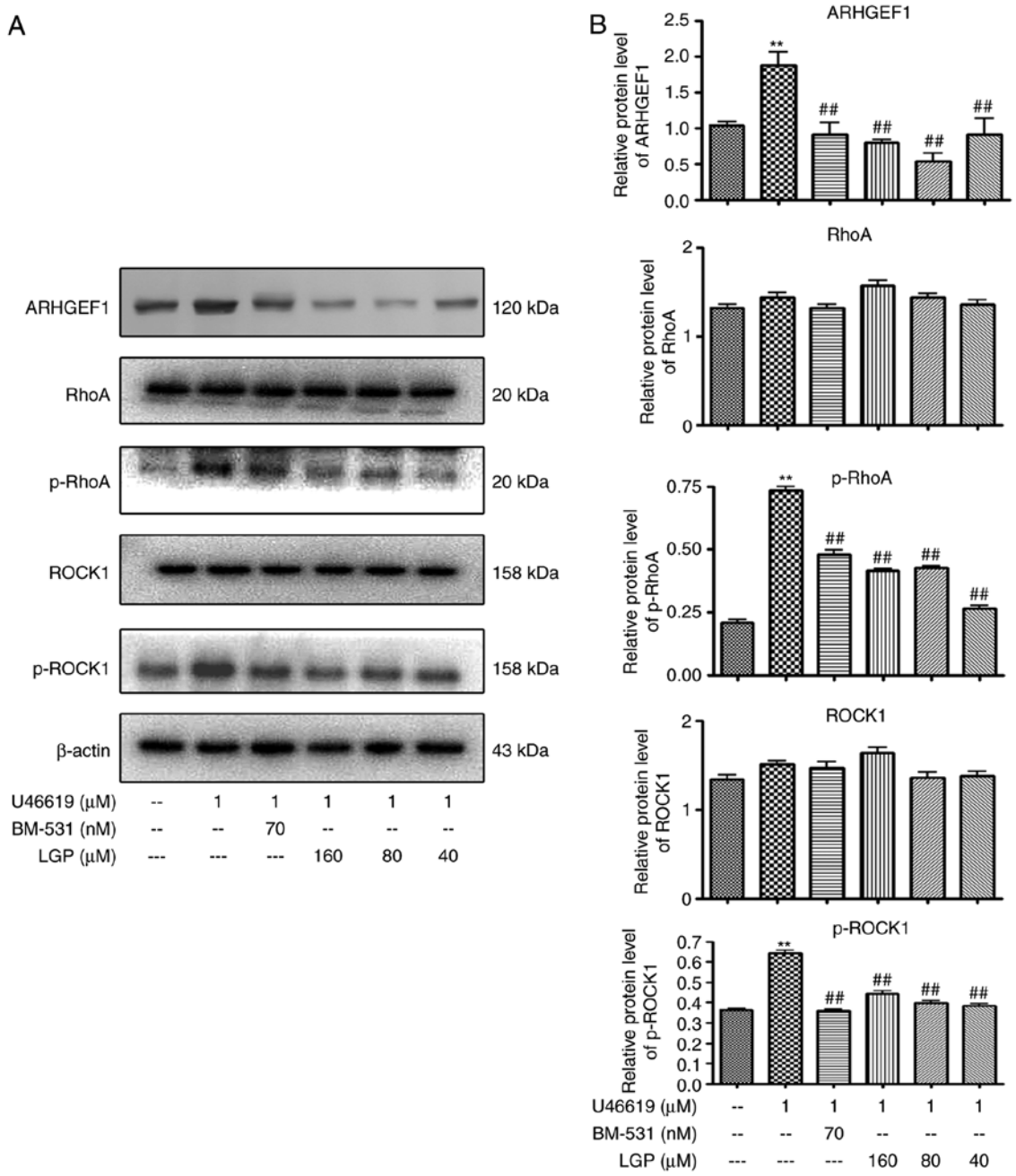

Figure 6. Effects of LGP on U46619-induced RhoA signaling. (A) Western blot analysis of protein lysates with (B) densitometric analysis of electrophoretic bands. Each assay was performed in triplicate. **P<0.01, compared with control; $^{\#\#}$P<0.01, compared with U46619. Vehicle (DMSO) was used as a control, and BM-531 served as the positivecontrol. LGP, luteolin-4'-*O*-β-D-glucopyranoside; ADP, adenosine diphosphate; RhoA, Ras homolog family member A; ROCK1, Rho-associated kinase 1; ARHGEF1, Rho, guanine nucleotide exchange factor 1; p-, phosphorylated.

platelet membranes with Ki=1.520 mM (Fig. 8B). In addition, ticagrelor, a selective $P2Y_{12}$ receptor antagonist, and BM-531, a selective TP receptor antagonist, displaced [^{3}H]-2-MeS-ADP and [^{3}H]SQ-29548 from their respective receptors at concentrations in the nanomolar range (Fig. 8C and D). From these data, the dose-dependent displacement of [^{3}H]-2-MeS-ADP and [^{3}H] SQ-29548 from their receptors by LGP was observed in rat platelets; however, compared with the effects of selective antagonists of these receptors, the K_i values were higher than for the selective antagonists. These data indicated that LGP exhibited weak dual receptor inhibitory effects on $P2Y_{12}$ and TP receptors.

Discussion

Platelets have a major role in thromboembolic diseases, which are the final events complicating cardiovascular diseases and peripheral vascular diseases (19). Therefore, antiplatelet therapy remains crucial for patients with these diseases in treatment and prophylaxis (19). However, the multiple pathways of platelet activation limit the effects of currently available antiplatelet agents, resulting in limited clinical efficacy. The efficacy of existing antiplatelet therapies cannot be dissociated from an increased risk of bleeding (20,21). Previous lessons have demonstrated that, despite the implementation of existing

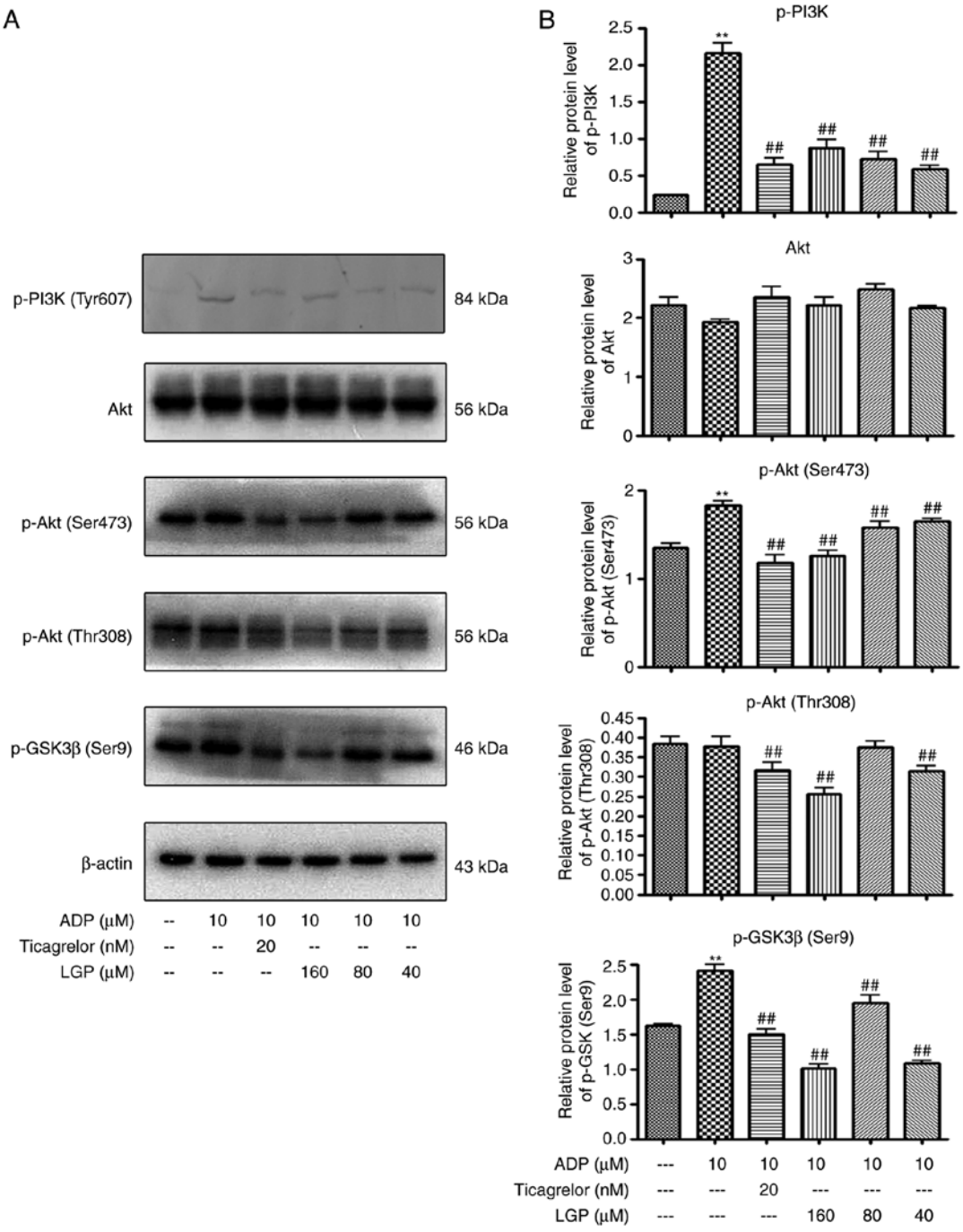

Figure 7. Effects of LGP on ADP-induced PI3K/Akt/GSK3β signal transduction. (A) Western blot analysis of protein lysates with (B) densitometric analysis of electrophoretic bands. Western blot analysis was performed. Each assay was performed in triplicate. **P<0.01, compared with control; $^{\#\#}$P<0.01, compared with ADP. Vehicle (DMSO) was used as a control, and ticagrelor served as the positive control. LGP, luteolin-4'-*O*-β-D-glucopyranoside; ADP, adenosine diphosphate; PI3K, phosphoinositide 3-kinase; GSKβ, glycogen synthase kinase 3β; p-, phosphorylated.

treatments, the incidence of side events remains high (20). Therefore, the development of effective and safe methods to inhibit platelet function remains critically important. Several medicinal herbs exhibit antiplatelet effects, and these herbs are used in traditional Chinese medicine for promoting blood circulation. In our previous study, the effects of several derivatives of luteolin on ADP-induced platelet aggregation were evaluated, and it was found that LGP exhibited significant suppressive effects. The present study reported on the antiplatelet effects of LGP, a flavonoid from *C. nudiflora* which is used as a treatment for promoting blood circulation in China.

Based on the screening results (data not shown), the initial experiments performed in the present study were designed to confirm the effects of LGP on platelet aggregation induced by different agonists. It was found that LGP caused the concentration-dependent inhibition of the platelet aggregation induced by ADP, U46619 and AA. In general, the formation of a stable platelet plug occurs in three distinct steps: Platelet adhesion, platelet activation and platelet aggregation (22). Platelet adhesion to sites of vascular injury is triggered by exposure of the subendothelial extracellular matrix (ECM) following vascular injury, which is mediated by platelet interactions

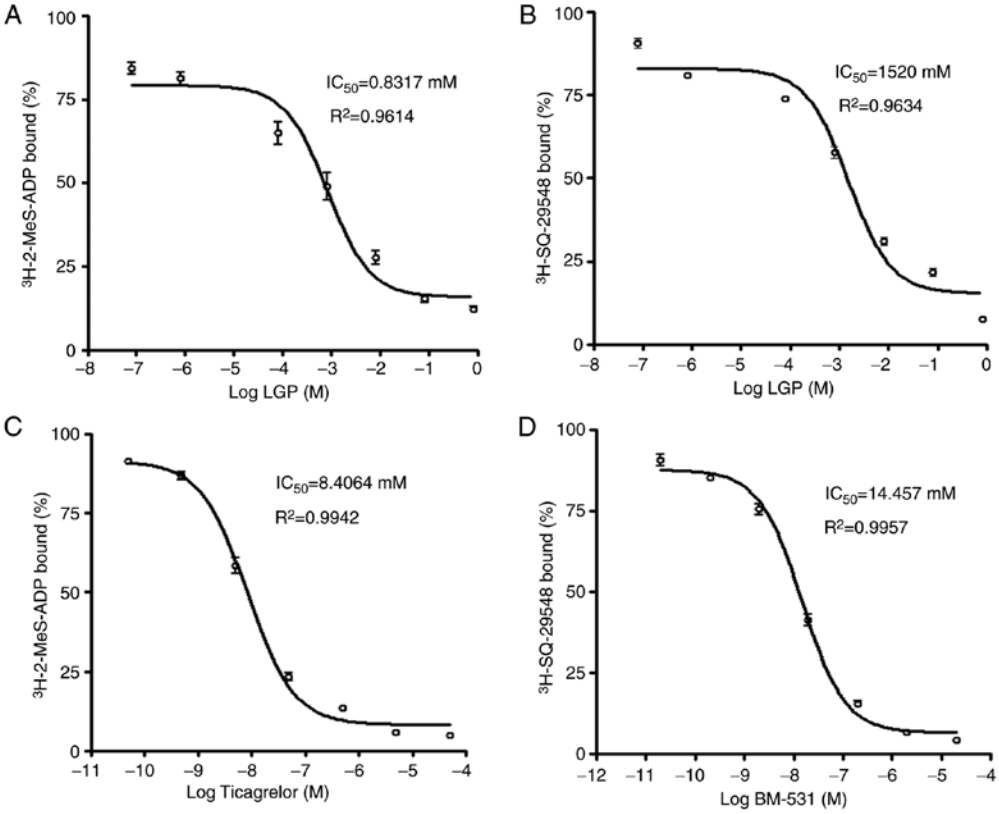

Figure 8. Effects of LGP on platelet $P2Y_{12}$ and TP receptor binding of [^{3}H]-2-MeS-ADP and [^{3}H] SQ-29548. Displacement curves show the specific binding induced by LGP. (A) [^{3}H]-2-MeS-ADP displacement curve of the specific binding induced by LGP. (B) [^{3}H] SQ-29548 displacement curve of the specific binding induced by LGP. (C) [^{3}H]-2-MeS-ADP displacement curve of the specific binding induced by ticagrelor. (D) [^{3}H] SQ-29548 displacement curve of the specific binding induced by BM-531. LGP, luteolin-4'-*O*-β-D-glucopyranoside; ADP, adenosine diphosphate; TP, thromboxane receptor.

with ECM components, particularly Von Willebrand factor, collagen, fibronectin, thrombospondinand laminin. The adhesion between platelets and ECM leads to the deceleration of flowing platelets and capture of circulating platelets to the vessel wall. At least two separate receptors on platelet cell membranes, GpIb-V-IX complex and GpVI, function to tether the platelet and initiate cellular activation, simultaneously. Once the platelets have adhered to the damaged vascular endothelium, they recruit additional platelets from the circulation to augment the fragile platelet monolayer and eventually form a stable plug (23). Following platelet activation, a coordinated series of events is triggered, including a rapid conformational change and the secretion of α,δ-granules and other intracellular vesicles, including lysosomes, which provide a positive feedback signal during platelet activation (19). Led by these concepts, the present study examined the effects of LGP on the content of platelet granule components to further confirm the effects of LGP on platelet activation. It was found that the secretion of 5-HT, an agonist of platelet activation stored in δ-granules, was significantly decreased by LGP. TXA_2, a labile prostanoid synthesized by activated platelets, is referred to as a second wave mediator of platelet activation (24). The synthesis of TXA_2 is mediated by a cascade of enzymes, including cyclooxygenase-1; this enzyme is activated by elevated Ca^{2+}, which induces translocation to the plasma membrane and phosphorylation by the stress kinase P38 and extracellular signal-regulated kinase 1/2. Once synthesized, it diffuses across the platelet membrane and causes conformational change, phosphoinositide hydrolysis, Ca^{2+} mobilization, protein phosphorylation and secretion, further amplifying the activation signaling (24,25). It was also observed in the present study that LGP caused a significant decrease in the production of TXA_2. LGP also inhibited the activation of $\alpha_{IIb}\beta_3$ integrin in a dose-dependent manner. The activation of integrin, particularly $\alpha_{IIb}\beta_3$ integrin, is considered the most important step in platelet aggregation. Specific interactions of agonists with their receptors generate inside-out signaling, leading to the conformational activation of integrins, particularly $\alpha_{IIb}\beta_3$ integrin, increasing their ligand affinity. The binding of $\alpha_{IIb}\beta_3$ integrin to its ligands, mainly fibrinogens, supports processes including the close contact between aggregated platelets, and eventually promotes platelet activation and aggregation. The present study demonstrated that ADP, U46619 and AA induced platelet aggregation, and α,δ-granule release and TXA_2 synthesis were inhibited by LGP in a dose-dependent manner. These data suggested that the inhibitory effects of LGP on aggregation may be associated with its suppression of platelet activation.

ADP is a critical mediator of platelet activation. By binding to its receptor, this agonist leads to full activation events, including platelet conformational change, Ca^{2+} influx, TXA_2 synthesis and granule secretion. Additionally, ADP is released from the δ-granules of activated platelets and amplifies its own effects (26). Therefore, ADP, in addition to TXA_2 and 5-HY, are termed second wave mediators, which are released from platelets and amplify effects of platelet

activation (26). There are two distinct G-protein-coupled ADP receptors expressed on the surface of human platelets, $P2Y_1$ and $P2Y_{12}$. Several studies have suggested that $P2Y_{12}$ is the major receptor in the amplification of platelet activation, and the PI3K/PDK1/Akt/GSK3β pathway, particularly p110β and p110γ PI3K isoforms, has emerged as a major signaling axis regulating $P2Y_{12}$-mediated platelet activation (27-29). As mentioned above, TXA_2 is another stimulator amplifying platelet activation, which is synthesized by activated platelets. Once synthesized and diffused from platelets, TXA_2 activates and recruits platelets to the growing platelet aggregation via the G_q- and $G_{12/13}$-coupled thromboxane-prostanoid receptors TPα and TPβ (30). It is accepted that Rho GTPase signaling is involved in TP-mediated platelet activation by causing platelet conformational change and regulating platelet secretion (31). Huang *et al* found that RhoA was activated by ARHGEF1 when platelets were stimulated by U46619, a mimetic of TXA_2 (32). Therefore, in order to determine whether the inhibitory effects of LGP on ADP-induced platelet aggregation were due to $P2Y_{12}$-mediated signaling inhibition, the effects of LGP on the activities of PI3K/PDK1/Akt/GSK3β were examined. LGP led to a dose-dependent decrease in the expression of p-PI3K (Tyr607), p-AktSer473, Thr308) and p-GSK3β (Ser9) (Fig. 7). Similarly, it was found that LGP inhibited U46619-induced RhoA signaling (Fig. 6). These data indicated that the signal transduction mediated by $P2Y_{12}$ and TP receptors was involved in the LGP-induced platelet inhibition. To further assess this possibility, the effects of LGP on $P2Y_{12}$ and TP receptors were evaluated by a radiolabeled ligand binding assay. As shown in Fig. 8, the dose-dependent displacement of [^{3}H]-2-MeS-ADP and [^{3}H] SQ-29548 from their receptors was caused by LGP in rat platelets, however, the K_i values were higher compared with the selective antagonists. These data confirmed the effects of LGP on the activities of $P2Y_{12}$ and TP receptors and downstream signal transduction. However, how LGP affects the $P2Y_{12}$ and TP receptors remains to be fully elucidated. Future investigations will focus on the association between the chemical structure of LGP and the $P2Y_{12}$ and TP receptors, to elucidate why and how this compound affects $P2Y_{12}$ and TP receptors.

In conclusion, the data presented in the present study demonstrated that LGP, a natural compound from *C. nudiflora* Hook, inhibited the development of platelet aggregation and amplification of platelet activation. These inhibitory effects may be associated with its dual-receptor inhibition on $P2Y_{12}$ and TP receptors.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author upon reasonable request.

Authors' contributions

HX performed the platelet aggregation assay, western blot analysis and the receptor-binding assay. XZ contributed to platelet preparation and integrain assay. WW was responsible for 5-HT and TXA_2 measurement. ZZ participated in the receptor-binding assay. HF participated in the western blot analysis. SM and YL were responsible for LGP preparation and wrote the manuscript. JF took responsibility for the design of this project, analysis and interpretation of data. All authors read, edited and approved the final manuscript.

Ethics approval and consent to participate

The protocol used for animal experiments (JZAEC-2016-0031) was approved by the Animal Ethics Committees of Jiangxi University of Traditional Chinese Medicine, and all animal experiments were performed in strict accordance with the requirements of this protocol

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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