Saccharides from *Arctium lappa* L. root reduce platelet activation and thrombus formation in a laser injury thrombosis mouse model

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Abstract. *Arctium lappa* L., also known as burdock, is a popular medicinal plant in traditional Chinese medicine due to its potential therapeutic properties. Saccharides from *Arctium lappa* L. root (ALR-S) have been extensively studied for their anti-inflammatory and anti-diabetes effects. Platelets play a pivotal role in thrombosis. The present study describes the effects of ALR-S on platelet activation and thrombosis using a laser injury thrombosis *in vivo* model. The study also measured the effects of ALR-S on platelet activation by analysing aggregation, ATP release, platelet spreading, adhesion and clot retraction *in vitro*. Specifically, the effects were ALR-S concentration-dependent inhibition of platelet aggregation and ATP release. Activated platelets pretreated with ALR-S showed diminished CD62P expression levels and fibrinogen binding, as measured by flow cytometry. ALR-S inhibited platelet spreading on fibrinogen and adhesion on collagen under shear. ALR-S attenuated platelet activation by decreasing oxidative stress and thrombus formation. These results demonstrated the antiplatelet effects of ALR-S, suggesting the antithrombotic and cardiovascular protective activities of ALR-S as a functional food.

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Introduction

Platelets are the smallest blood cells and function as 'bricks' in the development of arterial thrombosis. When the integrity of the vascular system is disrupted, circulating platelets are activated by the exposure to subendothelial matrix proteins [*e.g.*, von Willebrand factor (vWF) and collagen)]. Glycoprotein Ib-IX-V complex on the platelet surface plays an important role in slowing down and recruiting circulating platelets to the injured sites by binding to vWF (1). ADP and thromboxane A2, released by activated platelets, further amplify the recruitment process (2). Thrombin is an important end product of the coagulation cascade, and aside from directly activating the platelets, it cleaves fibrinogen to promote platelet aggregation (3). Beside the critical role in physiological hemostasis, platelet hyperactivity during arterial thrombosis caused by plaque rapture, immune response infection or metastasis may cause life threatening myocardial infarction and stroke (2). Antiplatelet agents, such as clopidogrel, prasugrel, ticagrelor and acetylsalicylic acid, are widely used in preventing cardiovascular events in clinical practice (4,5). However, the application of antithrombotic agents in patients with cardiovascular disease tends to increase the bleeding risk. Thus, there is an increasing interest in searching for natural food products and biologically active ingredients for the prevention and treatment of thrombosis (6).

Arctium lappa L. has been traditionally used as a healthy and nutritive food (7). Extracts from *Arctium lappa* L. root have been shown to reduce inflammation (8), fight against infection (9,10) and prevent high glucose levels in diabetes (11,12). Saccharides from *Arctium lappa* L. root (ALR-S) is a high-purity fructosaccharide. The antioxidant effects of ALR-S have been well documented and ALR-S has been shown to actively destroy free radicals (13). Recently, it was demonstrated that ALR-S reduced thrombosis in an ferric chloride ($FeCl_3$)-induced mouse arterial thrombosis model by rebalancing the expression of thrombotic and antithrombotic

factors in endothelial cells (6). However, the exact effects of ALR-S on platelet activation *in vitro* and *in vivo* remain elusive.

Reactive oxygen species (ROS) that are produced during vascular injury are key mediators of platelet function. Although multiple ROS sources have been proposed following vascular injury, including superoxide anion, hydroxyl radicals and hydrogen peroxide (14,15), it has previously been identified that NADPH oxidase (NOX)-dependent ROS generation is the critical radical source regulating platelet function (16,17). NOX-dependent ROS generation promotes platelet activation via p38 and ERK1/2 phosphorylation (15-17), and specific deletion of p38α in platelets impairs thrombosis and hemostasis by disturbing the p38α/MAPK-activated protein kinase 2/heat shock protein 27 (HSP27) signaling pathway (18).

The present study investigated the antiplatelet effects of ALR-S using platelets isolated from healthy subjects and a laser-induced mouse arterial thrombosis model. The results may help to determine whether ALR-S could be used as a beneficial food and beverage resource for reducing thrombotic risk.

Materials and methods

Materials. Collagen, thrombin, ADP and luciferin/luciferase were provided by the Chrono-Log Corporation. FITC-phalloidin, H_2O_2 and mepacrine were purchased from Sigma-Aldrich; Merck KGaA. Antibodies against phospho-ERK1/2 (catalog no. 4370S), phospho-p38 (catalog no. 4511S), phospho-HSP27 (catalog no. 2401S), total-p38 (catalog no. 8690S), total-ERK (catalog no. 4695S) and HSP27 (catalog no. 95357S) were purchased from Cell Signaling Technology Inc. β-actin monoclonal antibody (catalog no. 66009-1-Ig) was obtained from ProteinTech Group, Inc. The antibody for integrin β3 (D-11) (catalog no. sc-365679) was obtained from Santa Cruz Biotechnology Inc. PAC-1 antibodies (catalog no. MA5-28564) were from Invitrogen; Thermo Fisher Scientific, Inc., and CD62P antibodies (catalog no. 555524) were from BD Biosciences.

Preparation of ALR-S. Crude extract of *Arctium lappa* L. root (catalog no. wkq-08912) was obtained from Sichuan Weikeqi Biological Technology Co., Ltd. The crude extract was refluxed with anhydrous alcohol and subsequent acetone, and then it was evaporated under reduced pressure. Briefly, Sephadex G-50 gel (MilliporeSigma) in a chromatographic column (25x400 mm) was used for gel filtration chromatography. The gel was washed with 20 ml sterile distilled water, and then quantified with 10 mM Tris HCl buffer (pH 7.4). Samples (5 g) were dissolved into distilled water at 5% (w/v), and loaded into columns at a flow rate of 1 ml/min for 2 h at 40˚C. The saccharide fractions were collected and concentrated using a freeze-dried evaporator (SCIENTZ-10N; SCIENTZ; Ningbo Xinzhi Freeze Drying Equipment Co., Ltd.). ALR-S purity was measured by a Shimadzu Prominence gel permeation chromatography (GPC) system (Shim-pack GPC 803C column; Shimadzu UK Ltd.) at 40˚C using chloroform as the eluent, and the purity was >95%.

Preparation of washed platelets. Washed platelets were obtained as previously described (19). Briefly, blood was acquired from healthy individuals (12 female and 9 male) who had not taken any medication for at least 1 week. Platelet rich plasma (PRP) was obtained by centrifugation of whole blood samples at 150 x g for 20 min at room temperature. Platelets were resuspended in modified Tyrode's buffer (138 mmol/l NaCl, 5 mmol/l D-glucose, 5 mmol/l HEPES, 1 mmol/l $MgCl_2$, 12 mmol/l $NaHCO_3$, 400 mmol/l Na_2HPO_4 and 2.7 mmol/l KCl) after centrifugation at 800 x g for 10 min at room temperature. Platelet concentration was measured by a hemocytometer (BC-2800Vet; Mindray Medical International Ltd.) and the washed platelet concentration was adjusted to a final concentration of $3x10^8$/ml, equivalent to the platelet concentration in a healthy individual. Platelets were stimulated by the addition of $CaCl_2$ at a final concentration of 1 mmol/l. All experiments were approved by the Ethics Committee of Zhengzhou University for the Use of Human Subjects (approval no. 2020-KY-122).

Platelet aggregation and ATP release assay. Platelet aggregation and ATP release were carried out by optical aggregometry as described previously (19), using a lumi-aggregometer model 700 (Chrono-log Corporation), under continuous stirring at 1,200 rpm. Washed platelets were treated with ALR-S (20, 60 and 200 μg/ml) or a vehicle at 37˚C for 10 min. Luciferin/luciferase (10 μl) were added, and then stimulation was performed with collagen (1 μg/ml), thrombin (0.025 U/ml) or ADP (10 μM), respectively. Traces for aggregation and ATP release were recorded for 10 min at 37˚C. For H_2O_2 enhanced platelet aggregation, washed platelets were treated with ALR-S or a vehicle at 37˚C for 10 min, then the agonists were immediately joined into platelets after H_2O_2 (20 μM) was added.

Flow cytometry. Activated platelet integrin αIIbβ3 and α-granule secretion were measured by flow cytometric analysis as previously described (19). Briefly, washed platelets were adjusted to a concentration of $5x10^7$ cells/ml, and 100 μl ($5x10^6$ cells) were stained with PE-CD62P or FITC-PAC-1, respectively, for 30 min at room temperature. Stained platelets were then incubated with ALR-S or saline vehicle for 10 min at room temperature. Reactions were started with collagen-related peptide (CRP, 2 μg/ml), thrombin (0.025 U/ml) and ADP (10 μM) for 5 min at room temperature, respectively. The reactions were stopped by addition of 500 μl PBS. A total number of 10,000 events per tube were collected in an Accuri™ C6 flow cytometer (BD Biosciences) and analyzed by FlowJo (version 10.0; Tree Star, Inc.).

Platelet spreading. Washed platelets were adjusted to $2x10^7$ cells/ml, which is a proper concentration to avoid platelet-platelet overlap on coverslip. Washed platelets were preloaded with ALR-S (20, 60 and 200 μg/ml) or saline and then were allowed to spread on fibrinogen-coated slides for 1 h at 37˚C. Slides were washed with PBS 3 times for 10 sec, fixed with 1% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 20 min and stained with FITC-phalloidin (catalog no. P5282; MilliporeSigma) for 1 h at room temperature. Images of spreading platelets were captured by a fluorescence microscope (BX53; Olympus) with a charge-coupled device (CCD) camera (DP74; Olympus Corporation). Platelet spreading

area was evaluated using ImageJ software (version 1.4; National Institutes of Health).

Clot retraction. PRP ($3x10^8$ cells/ml) was preincubated with ALR-S (20, 60 and 200 μg/ml) or saline for 5 min. Addition of thrombin (0.4 U/ml) at room temperature started clot retraction; images were captured at 0, 20, 40 and 60 min, and evaluated by ImageJ software (version 1.4).

Measurement of intracellular ROS. As previously reported (17), washed platelets ($1x10^8$ cells/ml) were incubated with fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; 50 μmol/l) (catalog no. CA1410; Beijing Solarbio Science & Technology Co., Ltd.) for 15 min at 37˚C in darkness, followed by stimulation with CRP (2 μg/ml), thrombin (0.025 U/ml), or ADP (10 μM) for 5 min at 37˚C in darkness. Samples were then diluted with 10-fold HEPES/Tyrode's buffer containing 50 μmol/l H_2DCFDA and analyzed by flow cytometry immediately as aforementioned.

Immunoblotting analysis. After aggregation of platelets in an aggregometer at 1,200 rpm, samples were lysed with 2X lysis buffer (50 mmol/l Tris and 150 mmol/l NaCl; pH 7.4) containing 2X protease inhibitor (catalog no. P1011; Beyotime Institute of Biotechnology) and 2X phosphatase inhibitor (catalog no. P1081; Beyotime Institute of Biotechnology). Proteins (20 μl) were separated by SDS-PAGE (10% gels) and transferred onto PVDF membranes, and then blocked with 5% BSA in Tris-buffered saline with Tween (TBST) (0.5%) for 1 h at room temperature. The membranes were incubated with the indicated antibodies at 4˚C overnight. Membranes were washed with TBST 3 times, then incubated with the corresponding anti-mouse (catalog no. SA00001-1) or anti-rabbit (catalog no. SA00001-2) secondary antibodies (ProteinTech Group, Inc.) (at a dilution of 0.5%) for 1 h at room temperature. After washing with TBST 3 times, proteins were visualized by Tanon 4800 (Tanon Science and Technology Co., Ltd.) with ECL western blotting detection reagent (MilliporeSigma).

Platelet adhesion to fibrillar collagen under shear. Platelet adhesion was evaluated using a Bioflux-200 system (Fluxion Biosciences) as previously described (17). Briefly, bioflux plates were coated with fibrillar collagen (50 μg/ml) overnight at 4˚C, and then blocked with 0.5% BSA at room temperature for 30 min. Heparin-treated (10 U/ml; catalog no. H3149; MilliporeSigma) human whole blood was incubated with mepacrine (100 μM; catalog no. Q3251; MilliporeSigma) for 30 min at 37˚C. Mepacrine-labeled blood was allowed to adhere to a fibrillar collagen-coated plate under a shear rate of 40 dynes/cm^2 for 5 min using a Bioflux-200 system (Fluxion Biosciences). Images of adherent thrombi were viewed and captured using an inverted fluorescence microscope (IX73; Olympus) with a CCD camera (DP74; Olympus). Platelet-covered area was calculated using Bioflux software (version 2.0.5.8; Fluxion Biosciences).

Animal model of laser injury thrombosis. C57BL/6 mice were provided by the Experimental Animal Center of Zhengzhou University. The animal research protocol was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (approval no. 2021-KY-256). A total of 14 male mice (aged 8-12 weeks; weight, 20-25 g) were kept at room temperature and atmosphere in a normal light/dark cycle after acquisition. Groups of 4-5 mice were maintained in a single cage and had free access to food and fresh water. Mice were anesthetized with 40 mg/kg intraperitoneal sodium pentobarbital (1%) and then 0.05 mg/kg intraperitoneal FITC-labeled antibody for glycoprotein VI (GPVI; catalog no. M011-1; Emfret) was injected. Mice were preloaded with different concentrations of ALR-S (0.2, 0.6 and 2 mg/kg) by intraperitoneal injection for 30 min. The mouse cremaster muscle was exposed, and the connective tissue was cleaned and maintained under a constant flow of saline at 37˚C. Arterioles with diameter of 30-50 mm were visualized using a BX61WI microscope (Olympus Corporation) with a 40X (0.9 NA) water-immersion objective lens. When arterioles were injured with an SRS NL100 pulsed nitrogen dye laser (440 nm), images were captured using a Photometrics Cool Snap HQ CCD camera (Teledyne Photometrics). A total of 4, 4, 3 and 3 mice were used for the vehicle group, and the 0.2, 0.6 and 2 mg/kg groups, respectively. A total of six arterioles for each group were captured, with a duration of 400 sec at an interval of 8 sec. Mice were sacrificed by sodium pentobarbital injection (90 mg/kg) followed by incubation in an Isoflurane Vaporizer box (Matrx™ VIP 300) with 3% isoflurane after the experiments.

Statistical analysis. All data were analyzed by GraphPad Prism (version 8.0.2; GraphPad Inc.) using one-way analysis of variance (ANOVA) to compare normally distributed variables and Dunnett's post hoc test for pairwise comparison between vehicle (saline) and ALR-S treated groups. All data are expressed as the mean ± SEM. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ALR-S decreases agonist-induced platelet aggregation and ATP release. The effects of ALR-S on agonist-induced platelet aggregation and ATP release were measured using a Chrono-log aggregometer. It has been previously reported that ALR-S (200 μg/ml) could significantly reduce ROS generation in $FeCl_3$-treated aortic endothelial cells, and rebalance thrombotic and antithrombotic factor expression and secretion in endothelial cells (6). In the present study, the experiments were performed using ALR-S at concentrations of 20, 60 and 200 μg/ml (Fig. 1). The data revealed that ALR-S (60 and 200 μg/ml) inhibited platelet aggregation induced by collagen, thrombin and ADP (Fig. 1A and C). Meanwhile, ATP release induced by thrombin and collagen and measured using a Luciferin/Luciferase system showed that ALR-S (200 and 60 μg/ml) significantly attenuated ATP release from dense granules of washed platelets (Fig. 1B and D).

ALR-S attenuates agonist-induced platelet αIIbβ3 activation and CD62P expression. It is notable that αIIbβ3 (also known as CD41/CD61) is an important integrin on the platelet surface (20), while CD62P (also known as P-selectin) is located in the inner surface of resting platelet α-granules (21). Upon activation, αIIbβ3 on the platelet surface is transformed

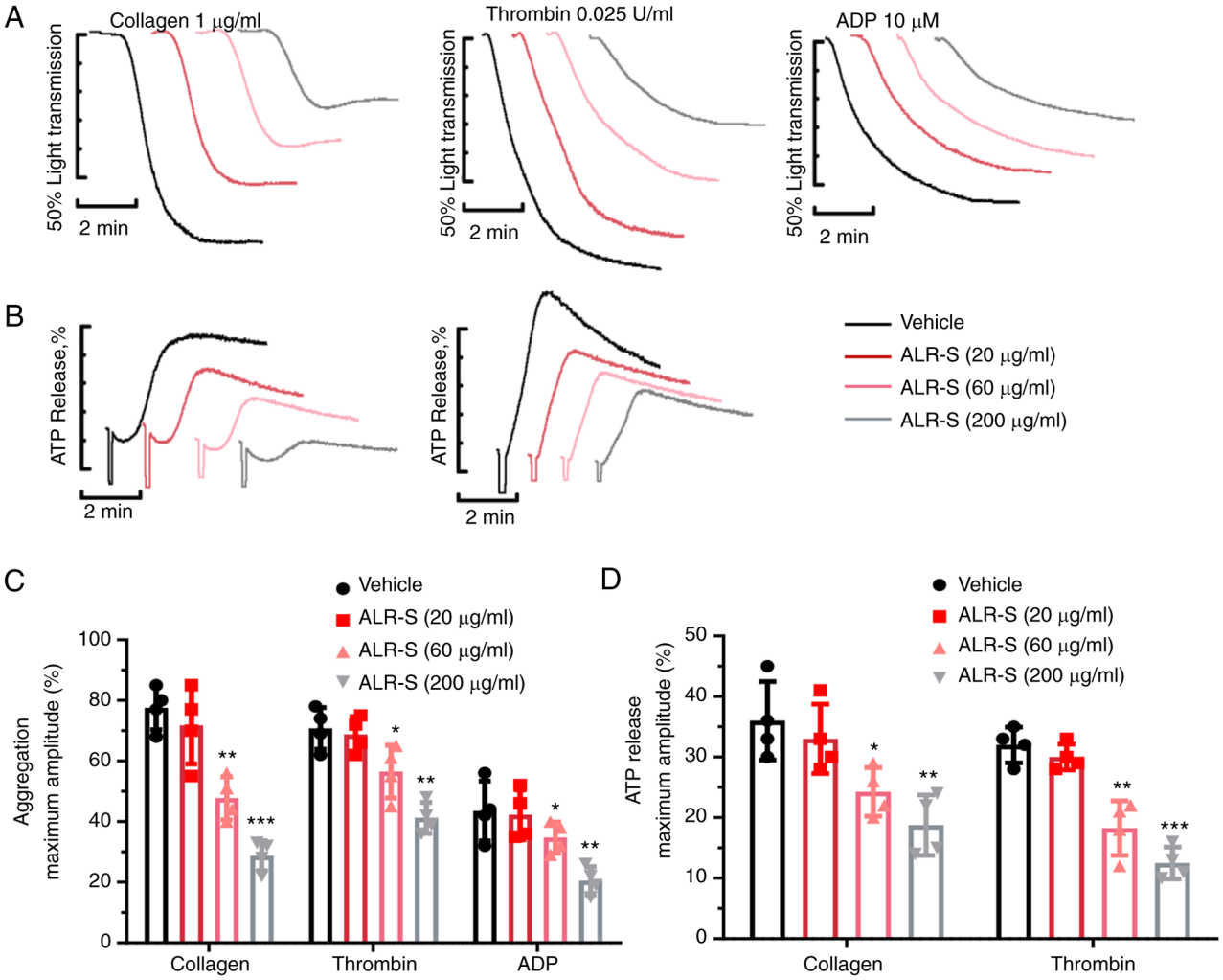

Figure 1. ALR-S reduces platelet aggregation and ATP release in a concentration-dependent manner. (A and B) Representative (A) aggregation and (B) ATP release traces of washed platelets from healthy donors, in response to collagen, thrombin or ADP. Aggregation and ATP release were assessed with a Chrono-log aggregometer under stirring. The y-axis represents the relative percentage change of light transmission, when the light transmission of Tyrode's buffer (without platelet) is defined as 100%, and washed platelets (resting) were adjusted to 0% before the aggregation was started. (C and D) Quantification of (C) platelet aggregation and (D) ATP release. *P<0.05, **P<0.01 and ***P<0.001 vs. control (vehicle). ALR-S, saccharides from *Arctium lappa* L. root.

into an active state to bind fibrinogen, which subsequently mediates the 'outside-in' signaling and regulates platelet aggregation, while CD62P translocates to the platelet surface. PE-conjugated CD62P antibody (binding to released CD62P on the platelet surface) and FITC-conjugated PAC-1 antibody (binding to an activation-induced conformational epitope PAC-1 on αIIbβ3) were applied to investigate the effects of ALR-S on single-platelet activation by flow cytometry. CRP was used to substitute collagen in the flow cytometry analysis. ALR-S (200 and 60 μg/ml) showed a potent inhibitory effect on CRP, thrombin and ADP-induced αIIbβ3 activation and CD62P expression (Fig. 2A-C). Notably, ALR-S (20 μg/ml) was less effective in inhibiting agonist-induced platelet activation compared with the high concentrations.

ALR-S inhibits platelet spreading on immobilized fibrinogen and delays clot retraction. Integrin αIIbβ3-initiated 'outside-in' signaling could promote platelet spreading on immobilized fibrinogen. ALR-S was applied to determine whether it could affect 'outside-in' signaling. The average surface coverage for 100 spread platelets was 192±19 μm^2 in the absence of ALR-S. ALR-S at a concentration of 60 μg/ml inhibited platelet spreading and the average area of 100 spread platelets was reduced to 152±14 μm^2 (Fig. 3A and B). Consistently, clot retraction in PRP requires platelet integrin αIIbβ3-mediated tight interactions between the membrane and cytoskeleton. In ALR-S-treated platelets, clot retraction was delayed. The maximum clot retraction occurred at 60 min after stimulation with thrombin (0.4 U/ml). By contrast, platelets treated with ALR-S (60 and 200 μg/ml) failed to form tight clots at 40 min and only partial clots were observed at 60 min (Fig. 3C and D).

ALR-S decreases ROS generation during platelet activation. The antioxidant effects of ALR-S on platelets were examined by flow cytometry. H_2DCFDA-loaded platelets (50 μmol/l) were challenged with agonist in the absence or presence of ALR-S. ALR-S repressed ROS generation during platelet activation in a concentration-dependent manner (Figs. 4A and S1). Furthermore, ALR-S reduced ROS induced intracellular signaling activation, and the phosphorylation of p38 and ERK 1/2 was reduced by ALR-S (Fig. 4B). HSP27 could be activated

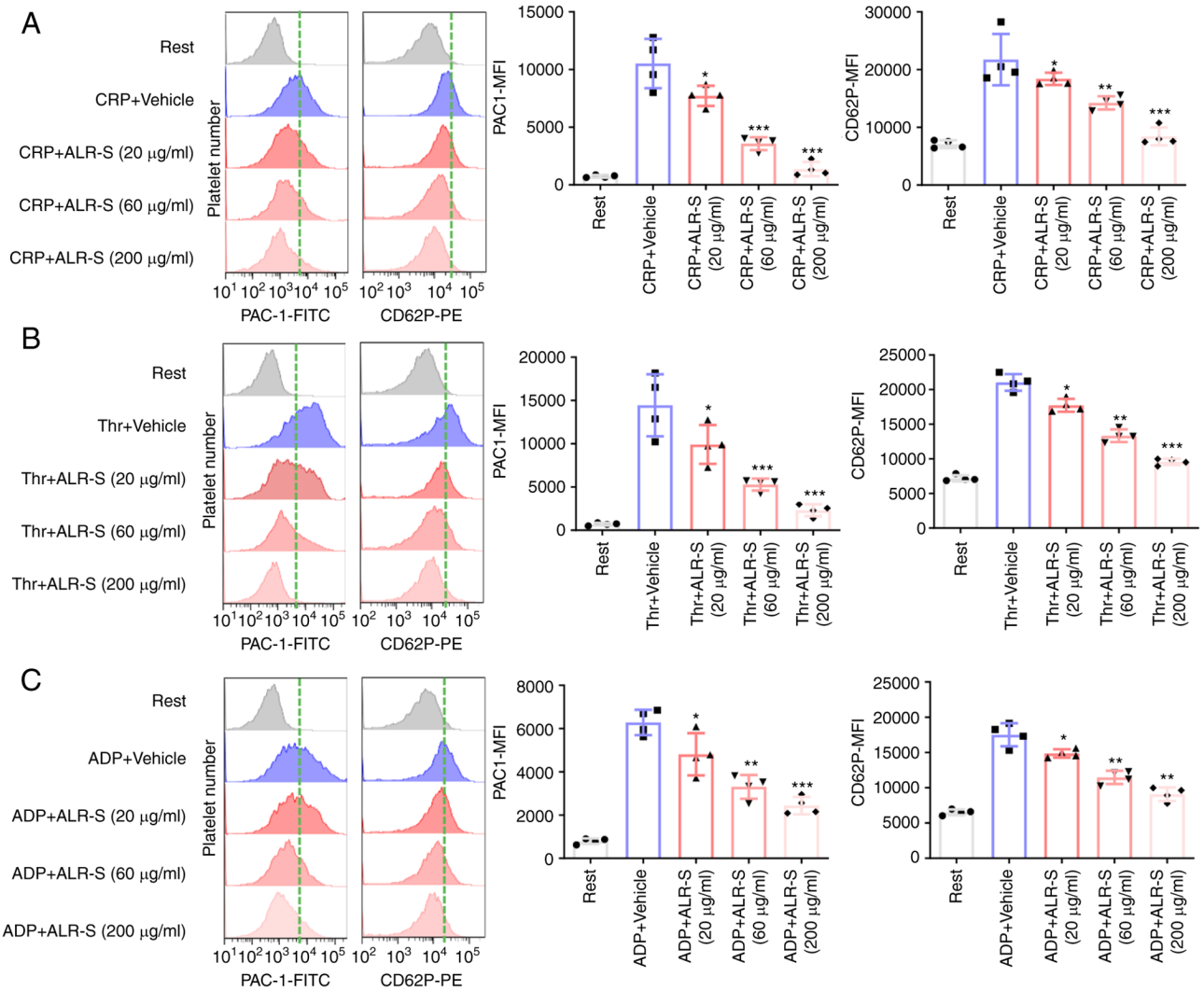

Figure 2. ALR-S attenuates platelet αIIbβ3 activation and CD62P expression. Representative histograms and summaries of αIIbβ3 activation and CD62P expression induced by (A) CRP (2 μg/ml), (B) thrombin (0.025 U/ml) and (C) ADP (10 μM) are presented. Dashed lines are used to gate a subpopulation of activated platelets. *P<0.05, **P<0.01 and ***P<0.001 vs. vehicle group. MFI, mean fluorescence intensity; ALR-S, saccharides from *Arctium lappa* L. root; CRP, collagen-related peptide.

by p38 phosphorylation (18). Consistent with this previous report, HSP27 phosphorylation during platelet activation was reduced in the presence of ALR-S in response to various agonists in the present study (Fig. 4B). To further testify the antioxidant effects of ALR-S on exogenous ROS, H_2O_2 (20 μM) was added to platelets before aggregation. Administration of exogenous H_2O_2 increased platelet aggregation in response to stimuli (Fig. 4C), while preincubation with ALR-S showed decreased H_2O_2-mediated platelet aggregation (Fig. 4C).

ALR-S inhibits thrombosis both in vivo and ex vivo. Once endothelium cells are injured *in vivo*, platelets are recruited to form stable adhesion on the initially exposed collagen surface (22). To clarify the role of ALR-S in platelet adhesion under shear, a whole-blood microfluidic perfusion system was applied under an arterial flow condition (40 dynes/cm^2). ALR-S (60 and 200 μg/ml) significantly inhibited platelet adhesion over collagen-coated surfaces as shown by the reduced area coverage of adherent platelets (P=0.0063 and P<0.0001, respectively) (Fig. 5A and B). These results confirmed that ALR-S could reduce thrombosis by inhibiting platelet adhesion onto exposed collagen. Using a laser injury thrombosis model, the effects of ALR-S on thrombosis were examined and characterized by real-time imaging. FITC-conjugated anti-GPVI antibody was intravenously injected in the tail to label circulating platelets in mice. ALR-S (0.6 and 2 mg/kg) pretreated mice showed delayed and smaller thrombi (Fig. 5C and D) as determined by fluorescence intensity at the injured site. Additionally, the time for thrombi to reach maximal fluorescence intensity was reduced by ALR-S treatment of the same concentrations (0.6 and 2 mg/kg) (Fig. 5E).

Discussion

Medicinal plants are considered important sources of functional foods to treat and prevent multiple diseases. *Arctium lappa* L. is among the most popular plants in the traditional Chinese pharmacopoeia. Extracts from *Arctium lappa* L. exhibit a wide range of pharmacological effects on various diseases, including hypertension, gout,

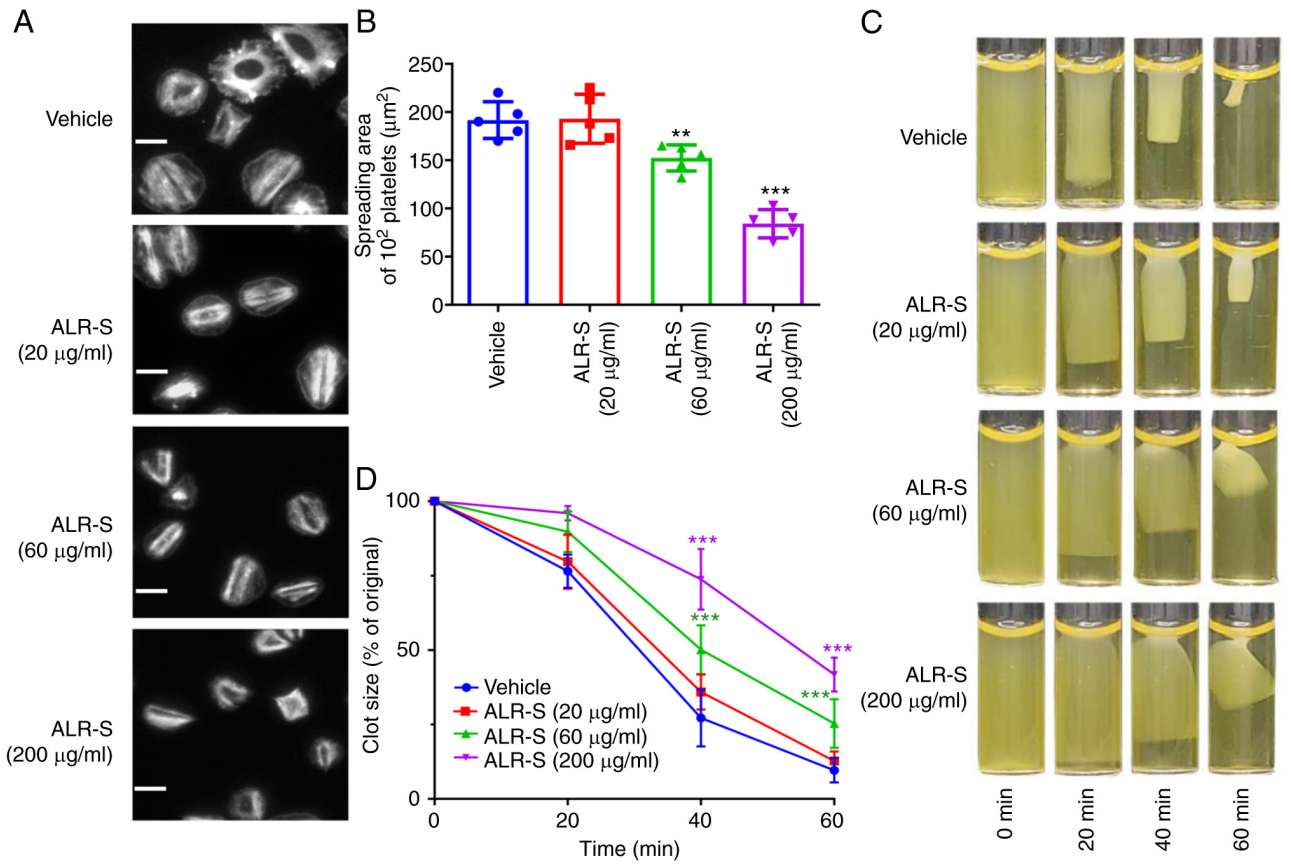

Figure 3. ALR-S inhibits platelet spreading and delays clot retraction. (A) Representative images and (B) summary of platelet spreading. Original magnification, x100. Scale bars, 2 μm. (C) Representative results and (D) summaries of clot retraction. After incubation with ALR-S or vehicle, platelet-rich plasma ($3x10^8$/ml) was stimulated with thrombin (0.4 U/ml) and images were captured at designated time points. **P<0.01 and ***P<0.001 vs. vehicle group. ALR-S, saccharides from *Arctium lappa* L. root.

arteriosclerosis and other inflammatory disorders (7,23). These effects originate from the biological activities of its components, such as caffeoylquinic acid derivatives, lignans and various flavonoids (24). Water-soluble polysaccharide is an important ingredient in the root extracts of *Arctium lappa* L. Polysaccharide extracts from *Arctium lappa* L. root could increase production of short chain fatty acids and improve the gut microbiota environment in mice (25,26), and administration of water-soluble saccharide could significantly enhance activities of antioxidant enzyme (13). var. Herkules, a low-molecular-weight fructofuranan from the roots of *Arctium lappa* L. has exhibited significant bioactivity in treating coughs (27). In terms of hemostasis, Qiu *et al* (6) showed that ALR-S could be protective against arterial thrombosis risk in a mouse model of $FeCl_3$-induced mesenteric arterial injury by interfering with the endothelial thrombotic/antithrombotic factor expression and secretion. In addition to injured endothelial cells, multiple events (*e.g.*, platelet reactivity, inflammation, coagulation and fibrinolysis) could participate in the complex hemostasis, in which platelets play a vital role. The present study described the direct effects of ALR-S on platelet activation. More specifically, ALR-S directly inhibited platelet activation stimulated by exogenous agonist, via aggregation, ATP secretion, CD62P expression and PAC-1 binding. Presence of ALR-S induced a decrease in platelet spreading and coverage areas on immobilized fibrinogen, and adhesion on collagen under shear, respectively. In a laser injury thrombosis model, exogenous uptake of ALR-S significantly inhibited thrombosis. Reduced ROS generation and subsequent MAPK phosphorylation during platelet activation were responsible for hampered thrombus formation.

Platelet surface receptors are glycosylated (28), and both N- and O-glycosylation of platelets play critical roles in the hemostatic system, including receptor expression, platelet clearance and signal transduction (29). Defective platelet surface glycosylation has been reported to be significantly associated with coronary heart disease and type 2 diabetes mellitus (30,31). Notably, there are several lectin-like receptors linking platelet activation with sulfated polysaccharides belonging to the dextran and fucoidan families, such as C-type lectin-like type II (32), platelet endothelial aggregation receptor-1 (PEAR1) (33), galectin 1 (34), and galectin 8 (35). Heterogeneous fucose-containing sulfated polysaccharides show complex and controversial effects on hemostasis. Fucosylated glycosaminoglycan, saccharides that were initially found in the body wall of echinoderms, may lead to platelet aggregation, possibly depending on the structural interaction with platelet αIIbβ3 (36). Synthetic glycopolymers and natural fucoidans promote platelet aggregation via PEAR1 and glycoprotein Ibα (33). In line with the previous report from Qiu *et al* (6), the present study showed that water-soluble saccharides from *Arctium lappa* L. root significantly decreased platelet aggregation in response to various stimuli accompanied by reduced ROS generation. The effects

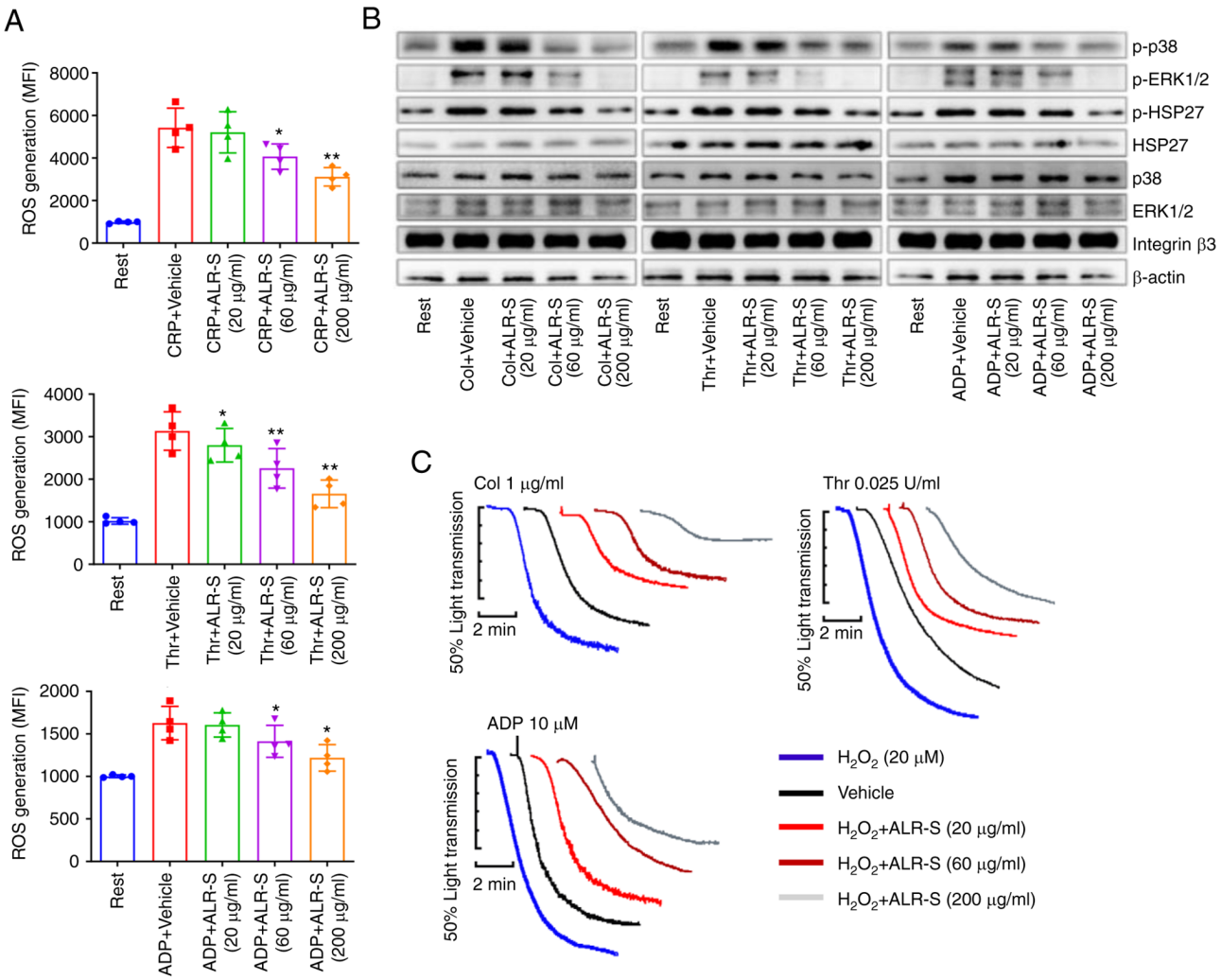

Figure 4. ALR-S decreases ROS generation during platelet activation. (A) Platelet intracellular ROS level was determined using flow cytometry. Platelets loaded with H_2DCFDA (50 μmol/l) were incubated with ALR-S or vehicle for 10 min and then stimulated with CRP (2 μg/ml), thrombin (0.025 U/ml), or ADP (10 μM) for 5 min. *P<0.05 and **P<0.01 vs. control. (B) Western blotting was performed to analyze the effects of ALR-S on phosphorylated ERK1/2, p38 and HSP27 during platelet activation in an aggregometer. (C) Representative aggregation traces of washed platelets in response to collagen, thrombin or ADP. Washed platelets were preincubated with ALR-S (0, 20, 60 and 200 μg/ml), respectively. H_2O_2 (20 μM) was added before aggregation was started, and traces were recorded by a Chrono-log aggregometer under stirring. ALR-S, saccharides from *Arctium lappa* L. root; p38, p38 mitogen-activated protein kinase; HSP27, heat shock protein 27; Col, collagen; Thr, thrombin; MFI, mean fluorescence intensity; ROS, reactive oxygen species; CRP, collagen-related peptide.

of saccharides on platelet activation vary due to chain length, branching, and degree of sulfation (33,37,38).

The radical-scavenging activity of polysaccharides may depend on the saccharide spectrum and molecular weight (39). It has been reported that mannose and glucose, rather than galactose content are highly associated with the antioxidant activity of polysaccharide from *Parthenocissus tricuspidata* (40). Considering the results of previous reports (39,41), the antioxidant activity of ALR-S could be attributed to its high mannose content or suitable ratios of different monosaccharides.

Elderly individuals are vulnerable to undesired thrombosis, including myocardial infarction, cerebral ischemia and venous thrombosis, representing the common causes of morbidity and mortality for that age group (42). There is increasing evidence suggesting that the aging-related prothrombotic state is derived from increased oxidative stress (43). Oxidative stress triggers platelet hyperreactivity and thrombotic susceptibility by decreasing nitric oxide bioavailability (42). A gain in platelet function during aging increases the thrombotic risk, and modulating oxidative stress in platelets is likely a beneficial approach to counterbalance the hypercoagulation state in the elderly. In the present study, it was shown that the exogenous uptake of ALR-S significantly inhibited platelet activation, with decreased ROS generation, indicating that applying ALR-S as a beverage in daily life may improve vascular behavior and reduce thrombotic risk.

Although the antioxidant activity of ALR-S has been indicated in previous studies (8,39,44), another molecular basis may underlie the antithrombotic ability of ALR-S. For instance, glycosaminoglycan has been indicated to non-selectively inhibit the coagulation cascade and platelet activation (45,46). Heparin, a negatively charged sulfated glycosaminoglycan, is the most widely used anticoagulant saccharide in clinical practice (47). The anticoagulant ability of heparin derives from its affinity to several serine proteases of the coagulation cascade in plasma, especially thrombin, factor Xa and factor IXa. Notably, in addition to the classic anticoagulant properties, heparin also modulates vascular cell behavior by binding to angiogenic growth factors, including

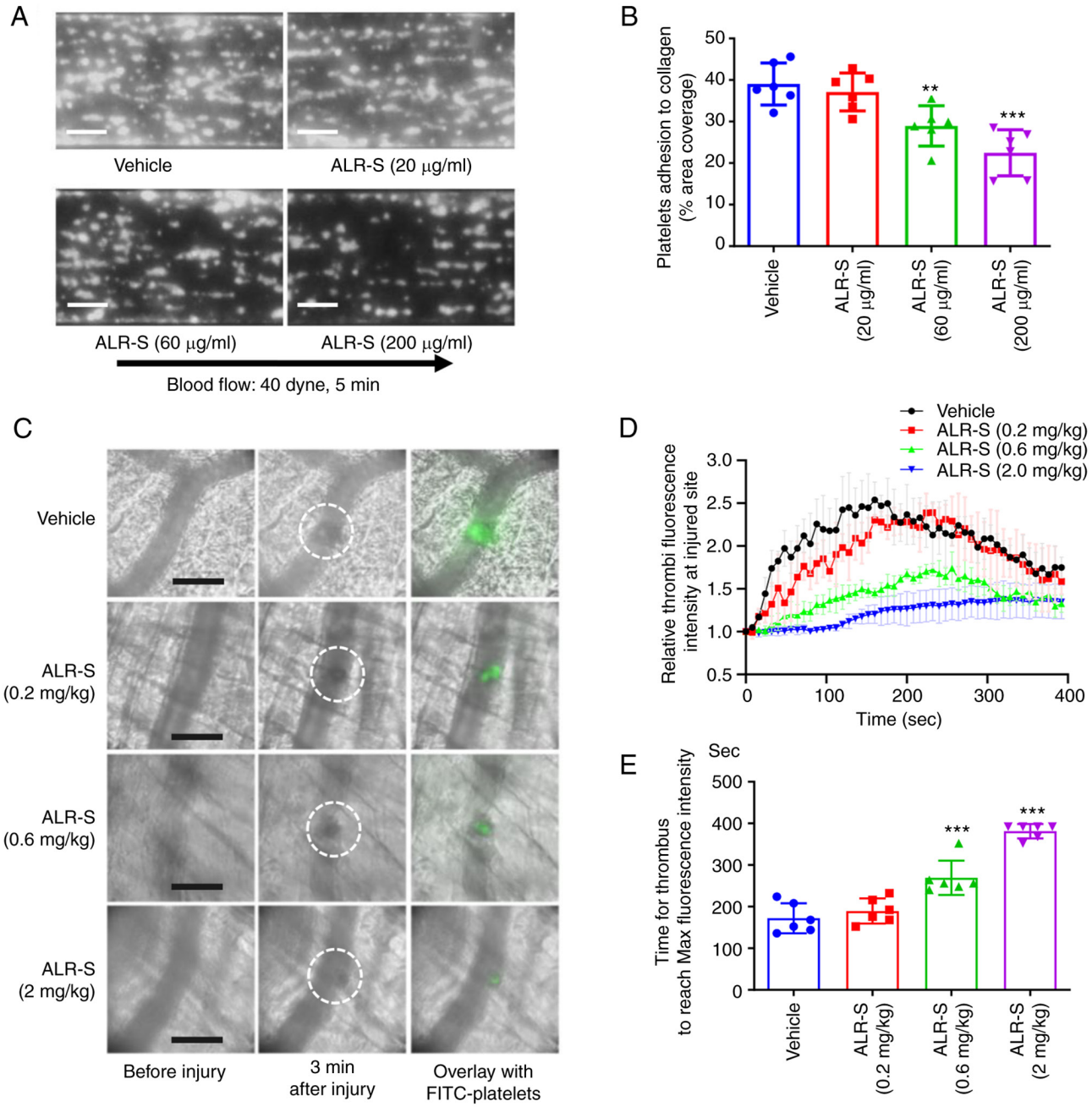

Figure 5. ALR-S inhibits platelet adhesion onto fibrillar collagen under shear and thrombosis formation *in vivo*. (A) Representative images and (B) coverage area quantification of platelet adhesion on fibrillar collagen in a microfluidic perfusion system. Original magnification, x20. Scale bar, 100 μm. (C) Representative images of mouse cremaster arteriole thrombosis induced by laser injury (white circle), and thrombi (green) observed using anti-GPVI-labeled platelets. Original magnification, x40. Scale bars, 50 μm. (D) Time course of relative thrombi fluorescence intensity at laser-injured site. The fluorescence intensity of injured site after 8 sec was normalized to 1. (E) Summary of the time for thrombus reaching maximal fluorescence intensity after laser injury. **P<0.01 and ***P<0.001 vs. control (vehicle). ALR-S, saccharides from *Arctium lappa* L. root; GPVI, glycoprotein VI.

VEGF and basic fibroblast growth factor, through their heparin binding sites (48). Thus, the structural basis for antithrombotic activity and other beneficial effects of ALR-S for cardiovascular disease should be further investigated.

Taken together, the results of the present study described the antithrombotic effects of ALR-S *in vitro* and *in vivo*. These findings indicate that water-soluble ALR-S may serve as a useful antithrombotic agent via its antioxidant activity. With possible use as an accessible beverage in daily life, further follow-up studies may be required to dissect the exact effects of ALR-S on cardiovascular health for elderly individuals.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YR designed and performed experiments, and analyzed data. YD conducted flow cytometry. MW and ML helped to maintain the C57BL mice and performed animal experiments. LW contributed to the western blotting. XL, CZ and JX helped with the chromatographic gel filtration of ALR-S. YL and JD designed the research and wrote the manuscript. LH, XZ and ZD helped to design the animal experiments, and provided critical advice to optimize the experiment protocol in the laser induced thrombosis injury. All the authors read and approved the final manuscript. YR and YL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Written informed consent was provided before blood sampling from human volunteers who claimed no underlying disease. The blood collection procedure was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University for Use of Human Subjects (approval no. 2020-KY-122). The animal research protocol was carried out in compliance with the guidelines of the International Society on Thrombosis and Hemostasis and was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (approval no. 2021-KY-256).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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