Food & Function

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d6fo00232c

Dihydromyricetin attenuates platelet hyperactivity in HFD/STZ-induced diabetic mice by inhibiting intraplatelet ROS generation

Fenglin Song,†[a,b] Huafang Ding,†[a] Yun Chen,[a] Xiangzhen Ge,[a] Ruixue Guo,[a] Fangfang Wu,[a] Yu Guo,[d] Ren-You Gan*[b,c] and Zhen-Yu Chen *[a,e]

The interaction between polyphenols and platelets is an emerging area in understanding how diet affects heart disease. Platelet hyperactivity is one of the critical drivers of cardiovascular complications in type 2 diabetes mellitus (T2DM). As one of the abundant polyphenols in nature, dihydromyricetin (DHM) has been shown to inhibit platelet activation and aggregation *in vitro*. This study aimed to investigate the potential mechanisms of dietary DHM supplementation and its *in vivo* effects on platelet hyperactivity in diabetic mice. A T2DM mouse model was established by feeding a high-fat diet (HFD) combined with streptozotocin (STZ) injection, followed by dietary supplementation with DHM (500 or 1000 mg kg^{-1} in diets) for eight weeks. Flow cytometric analysis revealed that DHM significantly reduced platelet surface expression of CD62P, CD63, and CD40 ligands, decreased integrin αIIbβ3 activation, and suppressed intraplatelet reactive oxygen species (ROS) production. The exaggerated platelet aggregation and ATP secretion induced by thrombin and collagen were also markedly attenuated. Additionally, DHM reduced plasma levels of *in vivo* platelet activation markers, soluble P-selectin, platelet factor 4, thromboxane B2, and the oxidative stress marker 8-iso-prostaglandin F2α. DHM administration also delayed collagen/epinephrine-induced pulmonary embolism formation without prolonging tail bleeding time in T2DM mice. Molecular docking suggested binding interactions of DHM with NADPH oxidase-2, aldose reductase, and platelet receptors PAR-1, GPVI, as well as COX-1. Collectively, dietary DHM attenuated platelet hyperactivity and thrombus formation in T2DM mice, primarily through suppression of intraplatelet ROS formation. These findings highlight DHM as a promising natural candidate for preventing diabetic cardiovascular complications.

Received 16th January 2026,
Accepted 6th May 2026

DOI: 10.1039/d6fo00232c

rsc.li/food-function

1. Introduction

Dietary flavonoids may be associated with a reduced risk of cardiovascular diseases by down-regulation of platelet activity. Platelets play a pivotal role in both physiological hemostasis and pathological thrombosis formation.[1] Enhanced platelet reactivity as the consequence of some pathological conditions, such as hyperlipidemia, hyperglycemia, obesity, hypertension, and dysbiosis, can lead to pathological thrombus formation, which ultimately contributes to blood vessel stenosis, ischemia, and myocardial infarction.[2,3] Type 2 diabetes mellitus (T2DM) is well established as a critical risk factor for cardiovascular diseases (CVDs).[4] Individuals with T2DM have a 2–4 fold risk of CVDs compared to non-diabetic individuals, and up to 80% of these diabetic subjects will die due to CVDs.[5] Increased responsiveness of platelets (hyperactivity) in T2DM has been recognized as a critical driver for cardiovascular disorders.[6] As a previous study reported, platelets from patients with T2DM exhibited intensified adhesion, activation, and aggregation, along with increased fibrinogen binding and surface expression of CD62P and increased production of TXA2 and the superoxide anion.[7] Additionally, plasma derived from T2DM patients contained elevated concentrations of platelet activation markers, such as soluble CD40L (sCD40L), PF-4, and sP-selectin.[8]

Platelet hyperactivity is not only related to hyperglycemia, but also associated with various metabolic comorbidities commonly found in diabetes mellitus, including insulin resistance,

[a]School of Food Science, Guangdong Pharmaceutical University, Zhongshan, Guangdong Province, 528458, China. E-mail: zhenyuchen@cuhk.edu.hk
[b]Department of Food Science and Nutrition, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong SAR, 999077, China. E-mail: renyou.gan@polyu.edu.hk
[c]Research Institute for Future Food, The Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, 999077, China
[d]Department of Blood Transfusion, The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangzhou, Guangdong Province, 510062, China
[e]School of Life Sciences, The Chinese University of Hong Kong, Shatin, NT, Hong Kong, China
†These authors contributed equally.

dyslipidemia, obesity, systemic inflammation and oxidative stress.[9] The oxidative damage of platelets and activation of pro-oxidant enzymes, such as NADPH oxidase (NOX) and aldose reductase (AR), appear to be central to platelet hyperactivity in diabetes.[10] However, current anti-platelet drugs, including aspirin and clopidogrel, exhibit reduced efficacy in diabetic patients, which is, at least in part, attributed to diabetes-associated oxidative stress.[11,12] Furthermore, antiplatelet drugs can cause some serious side effects, including bleeding episodes, gastrointestinal toxicity, neutropenia, and thrombocytopenia. Therefore, the development of alternative or natural anti-platelet bioactive compounds is urgently needed to reduce CVD risk in T2DM patients.

Many dietary components such as anthocyanins, quercetin, resveratrol, curcumin, sulforaphane and gallic acid have been reported to inhibit platelet functions and thrombus formation through distinct mechanisms.[13–17] Meanwhile, these natural compounds were found to be safe and did not increase bleeding risks. Thus, dietary intake of bioactive compounds with a platelet inhibitory activity should be effective to fight against the continued platelet hyperactivity in response to inappropriate nutritional and lifestyle factors including hyperglycemia and hyperlipidemia.[3] Dihydromyricetin (DHM) is widely abundant in plants such as Chinese vine tea, grapes, mulberries, and *Ginkgo biloba*.[18] DHM has exhibited numerous biological activities, such as antioxidant, anti-inflammatory and cardiovascular protection activities.[19] A previous *in vitro* study also demonstrated that DHM effectively inhibited thrombin-induced platelet activation, aggregation, and spreading.[20] However, the effects of DHM on platelet function *in vivo* have not yet been investigated. Specifically, it remains unclear whether DHM can effectively mitigate the persistent platelet hyperactivity induced by chronic hyperglycemia and systemic metabolic dysregulation.

The present study aimed to investigate the potential mechanisms of DHM and its *in vivo* effects on high platelet reactivity in diabetic mice. First, a diabetic mouse model was induced by feeding a high-fat diet (HFD) combined with intraperitoneal injection of streptozotocin (STZ). Second, the effects of DHM supplementation on platelet hyperactivity in diabetic mice were examined by measuring platelet activation, aggregation, granule secretion, and ROS production, as well as the *in vivo* platelet activation markers in plasma. Lastly, collagen/epinephrine-induced pulmonary embolism was induced in mice, and the alleviating effect of DHM on thrombus formation in T2DM mice was investigated.

2. Materials and methods

2.1 Materials

DHM (purity ≥ 99%) was purchased from Zelang Medical Technology Co., Ltd (Nanjing, China). Streptozotocin (STZ) was purchased from Shanghai Aladdin Biological Technology Co., Ltd (Shanghai, China). The platelet agonist thrombin was purchased from Sigma-Aldrich (St Louis, MO, USA). Collagen and the luciferase–luciferin reagent were from Chrono-log (Havertown, PA, USA). APC-conjugated anti-mouse CD63 antibodies, FITC-conjugated anti-mouse CD62P antibodies and PE-conjugated anti-mouse CD40L antibodies were obtained from BD Biosciences (San Diego, CA). PE-conjugated JON/A was purchased from Emfret Analytics (Eibelstadt, Germany). 5-(and 6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals were of reagent grade.

2.2 Animals and treatment

Six-week-old male C57BL/6 mice were purchased from Zhuhai BesTest Bio-Tech Co. Ltd (Zhuhai, Guangdong, China). All animal procedures were approved by the Animal Care and Protection Committee of Guangdong Pharmaceutical University (permit no. 2023212) and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In general, type 1 diabetes can be induced by a single and high dose of STZ, which causes significant beta-cell loss, leading to absolute insulin deficiency, whereas type 2 diabetes can be induced by low-doses of STZ, often combined with a high-fat diet, which creates insulin resistance and modest hyperglycemia. The overall experimental design and procedures of the study are presented in Fig. S1. Experimental Type 2 diabetic mice were established as previously described.[21] Mice were fed a high-fat diet (HFD) (60% calorie from fat, TP23400, Trophic Animal Feed High-Tech Co. Ltd, Nantong, China), and the detailed diet composition is shown in Table S1. After four weeks, mice were given an intraperitoneal injection of STZ dissolved in citrate buffer (pH 4.5) at a daily dose of 40 mg kg^{-1} b.w. for five consecutive days in the fifth week. Meanwhile, normal control mice (NG) were fed a common diet throughout the entire experimental period and received an intraperitoneal injection with citrate buffer. Fasting blood glucose (FBG) levels were examined in a week after the STZ injection, and mice with FBG levels higher than 11.1 mmol L^{-1} were considered diabetic. After successful model establishment, the diabetic mice were further divided into three groups, including the high glycemia group (HG) in which the mice were fed a HFD, the HG-L group in which the mice were fed a HFD with a low dose of DHM (500 mg kg^{-1} diet), and the HG-H group in which the mice were fed a HFD with a high dose of DHM (1000 mg kg^{-1} diet). Based on average food consumption, the dietary doses of DHM corresponded to approximately 60 and 120 mg kg^{-1} day^{-1} in mice. These doses were selected based on previous *in vivo* studies demonstrating their metabolic efficacy and safety.[22,23] After eight weeks, mice were anaesthetized with 3% isoflurane *via* inhalation, and blood samples were collected for the preparation of gel-filtered platelets and subsequent assays.

2.3 Tail vein bleeding assay

As a measurement of the hemostatic function, tail bleeding time was determined at the end of the treatment period. As previously described,[24] mice were anesthetized and a 3 mm

segment of the distal tail tip was amputated with a sharp scalpel. The tail was immediately immersed in normal saline that has been pre-warmed to 37 °C. The time from incision to cessation of bleeding was recorded as the bleeding time. If bleeding did not stop spontaneously, it was stopped by cauterization at 600 seconds.

2.4 Hematological study and plasma lipid determinations

After the determination of tail bleeding time, blood samples were collected and the anticoagulated whole blood was analyzed using an automatic multi-parameter blood cell counter, SysmexXP-300 (Sysmex Corporation, Kobe, Japan). Hematological parameters, including red blood cell (RBC) count, hematocrit (HCT), hemoglobin (Hb) concentration, white blood cells (WBCs), platelets (PLTs), mean corpuscular volume (MCV), and mean platelet volume (MPV), were analyzed using standard methods. Plasma levels of total cholesterol (TC), total triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured spectrophotometrically following the kit protocol.

2.5 Gel-filtered platelet preparation

The whole blood of mice was collected into a tube containing acid–citrate–dextrose (ACD, 1/9, v/v), and was incubated at 37 °C for 10 min. Platelet-rich plasma (PRP) was obtained from blood samples by centrifugation at 300*g* for 7 min at room temperature. Gel-filtered platelets were isolated from PRP using a Sepharose 2B column in PIPES buffer (5 mmol L^{-1} PIPES, 1.37 mmol L^{-1} NaCl, 4 mmol L^{-1} KCl, 0.1% (w/v) glucose, pH 7.0) according to our previously described method.[25] Due to the limited blood volume obtained from individual mice and the requirement for concomitant hematological analysis and plasma preparation, gel-filtered platelets were randomly pooled from two mice within the same group to obtain sufficient gel-filtered platelets. Each pooled sample was treated as a single independent biological replicate.

2. 6 Platelet aggregation and ATP release measurement

Platelet aggregation and ATP release were simultaneously measured using a Chrono-log aggregometer (Chrono-Log, Havertown, PA, USA). Briefly, the gel-filtered platelets were pooled from two mice of each group and adjusted to 3×10^8 cells per mL. The luciferin–luciferase reagent was added directly to platelet suspensions, followed by the stimulation with 0.1 U mL^{-1} thrombin or 1 μg mL^{-1} collagen in the presence of 1 mmol L^{-1} Ca^{2+}. Then, real-time platelet aggregation and ATP secretion were recorded over a period of 6 min.

2.7 Flow cytometry

Gel-filtered platelets were adjusted to 1×10^6 cells per mL and were preincubated with FITC-conjugated anti-mouse CD62P, PE-conjugated anti-mouse CD40L, PE-conjugated JON/A, or FITC-conjugated anti-mouse CD63 for 15 min at room temperature in the dark. The labeled platelets were then activated with thrombin or collagen for 5 min and fixed by adding 1% paraformaldehyde. All samples were analyzed by flow cytometry using a CytoFLEX flow cytometer (Beckman Coulter Inc., CA, USA). Ten thousand platelet events were acquired per sample and the mean fluorescence intensity of positive platelets was analyzed.

For reactive oxygen species (ROS) determination, gel-filtered platelets were incubated with 10 μM CM-H_2DCFDA, a ROS-sensitive fluorescent probe, for 30 min at 37 °C in the dark. The excessive dye was removed and platelets were resuspended in PIEPS buffer. Then, 0.5U mL^{-1} thrombin or 2 μg mL^{-1} collagen was added to stimulate platelets for 30 min, and the intracellular ROS generation was immediately detected by flow cytometry. These agonist concentrations were specifically optimized to ensure a detectable oxidative burst. In contrast, submaximal concentrations were used in platelet aggregation and activation assays to prevent saturated responses and allow accurate assessment of the inhibitory potential of DHM.

2.8 Determination of sP-selectin, PF4, TXB2, and 8-iso-PGF2α

Commercial enzyme-linked immunosorbent assay kits were used to measure plasma levels of sP-selectin (R&D Systems, Minneapolis, MN, USA) and PF4 (eBioscience, San Diego, CA, USA). The contents of TXB2 and 8-iso-PGF2α in plasma were also determined using TXB2 quantikine and 8-iso-PGF2α quantikine ELISA kits from ENZO Life Science (New York, USA), respectively, in accordance with the manufacturer's protocols.

2.9 Molecular docking analysis

To investigate the interactions between DHM and key receptors and catalytic enzyme involved in platelet ROS production and platelet hyperactivity, molecular docking analysis was performed as previously described.[26] Briefly, the 3-dimensional chemical structure of DHM was obtained from the PubChem database, while the crystal structures of PAR-1(PDB:3VW7), GPVI (PDB:2GI7), COX-1 (PDB:6Y3C), NOX-2 (PDB:8GZ3) and aldose reductase (AR, PDB:1US0) were downloaded from the RCSB Protein Data Bank (https://www.rcsb.org/). Protein structures were preprocessed by removing all crystallographic water molecules using PyMOL 3.0 and the polar hydrogen atoms were added using AutoDockTools 1.5.7. Then, molecular docking simulations were performed using AutoDock Vina (version 1.1.2) to calculate the binding affinities between DHM and target proteins. The grid box for each target protein was defined to encompass the active binding site based on the location of co-crystallized ligands or reported key residues. The grid box center coordinates and dimensions for each protein are provided in Table S3. The exhaustiveness parameter was set to 30 to ensure sufficient conformational sampling. For each docking simulation, the top 15 binding poses were generated, and the best-ranked pose based on binding affinity was selected for further analysis. Additionally, the 3D interaction diagrams were generated using PyMOL 3.0 and post-docking analyses were performed using LigPlot+ to identify and quantify the intermolecular bonding interactions between DHM

and the amino acid residues within the active pockets of target proteins.

2.10 Acute pulmonary thromboembolism in mice

To investigate the effect of DHM on thrombosis *in vivo*, a collagen- and epinephrine-induced acute pulmonary thromboembolism experiment was performed as previously described.[27] Briefly, mice were challenged with a mixture of collagen (430 μg kg^{-1}) and epinephrine (20 μg kg^{-1}) by smooth injection into one of the tail veins. The survival time of mice was determined by monitoring chest palpations, and death was determined when spontaneous respiratory chest expansions ceased for 1 min. The lungs of mice were then removed and immediately fixed in 4% paraformaldehyde for hematoxylin–eosin (HE) staining and observed under a light microscope.

2.11 Statistical analysis

Data are expressed as mean ± standard deviation (SD). The specific sample size (n) for each individual experiment is indicated in the corresponding figure legends. Differences between groups were analyzed by one-way ANOVA followed by the Student–Newman–Keuls multiple comparison test. For survival analyses as represented by Kaplan–Meier curves, a log-rank (Mantel–Cox) test was performed. All data were analyzed using SPSS software. P-value <0.05 was defined as statistically significant.

3. Results

3.1 Effects of DHM on the physiological changes in diabetic mice

The initial body weights of mice for all groups were similar (Table 1). At the end of the study, diabetic mice in the HG group had a significant increase in body weight ($p < 0.05$) and liver index ($p < 0.001$) compared with the NG group. As shown in Table 2, significant increases of the fasting blood glucose and dramatic decreases of HDL-C levels were also found in the HG group. After DHM (1000 mg kg^{-1}) treatment for 8 weeks, the fasting blood glucose significantly decreased in mice of the HG-H group ($p < 0.01$). The TG/HDL-C ratio, a highly sensitive surrogate marker for insulin resistance,[28] was significantly elevated in the HG group compared to the NG group ($P < 0.05$), confirming the development of insulin resistance in our model. Notably, DHM supplementation significantly attenuated this ratio in the HG-H group compared to the HG group ($P < 0.05$), suggesting an improvement in insulin sensitivity. No statistically significant differences were observed in hematological parameters among the four groups (Table S1).

3.2 DHM decreased platelet aggregation in diabetic mice

To evaluate whether DHM affects platelet hyperactivity in diabetic mice, platelet aggregation was measured after

Table 1 Effects of DHM administration on the body weight, average food intake, and organ index in T2DM mice

Groups	NG	HG	HG-L	HG-H
Initial body weight (g)	20.70 ± 1.41	20.99 ± 1.21	20.21 ± 1.23	20.52 ± 1.18
Final body weight (g)	29.37 ± 2.43^{a}	32.80 ± 3.58^{b}	32.61 ± 2.76^{b}	30.36 ± 2.56^{a}
Food intake (g d^{-1})	3.64 ± 0.19	3.27 ± 0.28	3.10 ± 0.27	3.21 ± 0.23
Organ index (%)				
Heart	0.57 ± 0.11	0.56 ± 0.07	0.51 ± 0.09	0.56 ± 0.07
Liver	4.43 ± 0.42^{a}	5.21 ± 0.73^{b}	4.93 ± 0.50^{b}	4.88 ± 0.50^{b}
Spleen	0.33 ± 0,07	0.35 ± 0.08	0.31 ± 0.09	0.31 ± 0.08
Lungs	0.69 ± 0.18	0.66 ± 0.12	0.68 ± 0.06	0.69 ± 0.12
Kidneys	1.32 ± 0.13^{a}	1.31 ± 0.11^{a}	1.29 ± 0.11^{b}	1.33 ± 0.13^{a}

NG, normal control mice; HG, high glycemia model group; HG-L, high glycemia group with a low dose of dihydromyricetin (DHM) (500 mg kg^{-1}); HG-H, high glycemia group with a high dose of DHM (1000 mg kg^{-1}). The organ index was calculated as the organ-to-body weight ratio (relative weight, percentage). Data are presented as the mean ± SD (n = 20). Different lowercase letters within the same row indicate significant differences between groups ($p < 0.05$).

Table 2 Effects of DHM supplementation on fasting blood glucose and the plasma lipid profile in T2DM mice

Groups	NG	HG	HG-L	HG-H
FBG (mmol L^{-1})	8.56 ± 1.01^{a}	21.15 ± 3.67^{b}	18.01 ± 7.35^{b}	16.51 ± 3.76^{c}
TC (mmol L^{-1})	1.75 ± 0.21^{a}	1.60 ± 0.20^{ab}	1.60 ± 0.19^{ab}	1.58 ± 0.22^{b}
TG (mmol L^{-1})	1.08 ± 0.24^{a}	1.35 ± 0.33^{a}	1.38 ± 0.40^{b}	1.11 ± 0.35^{a}
HDL (mmol L^{-1})	1.14 ± 0.16^{a}	0.97 ± 0.10^{b}	1.00 ± 0.10^{b}	0.97 ± 0.12^{b}
LDL (mmol L^{-1})	0.53 ± 0.19	0.55 ± 0.16	0.53 ± 0.12	0.53 ± 0.16
TG/HDL-C	0.95 ± 0.19^{a}	1.40 ± 0.35^{b}	1.41 ± 0.48^{b}	1.13 ± 0.36^{a}

NG, normal control mice; HG, high glycemia model group; HG-L, high glycemia group with a low dose of dihydromyricetin (DHM) (500 mg kg^{-1}); HG-H, high glycemia group with a high dose of DHM (1000 mg kg^{-1}). Data are presented as the mean ± SD (n = 14). Different lowercase letters within the same row indicate significant differences between groups ($p < 0.05$).

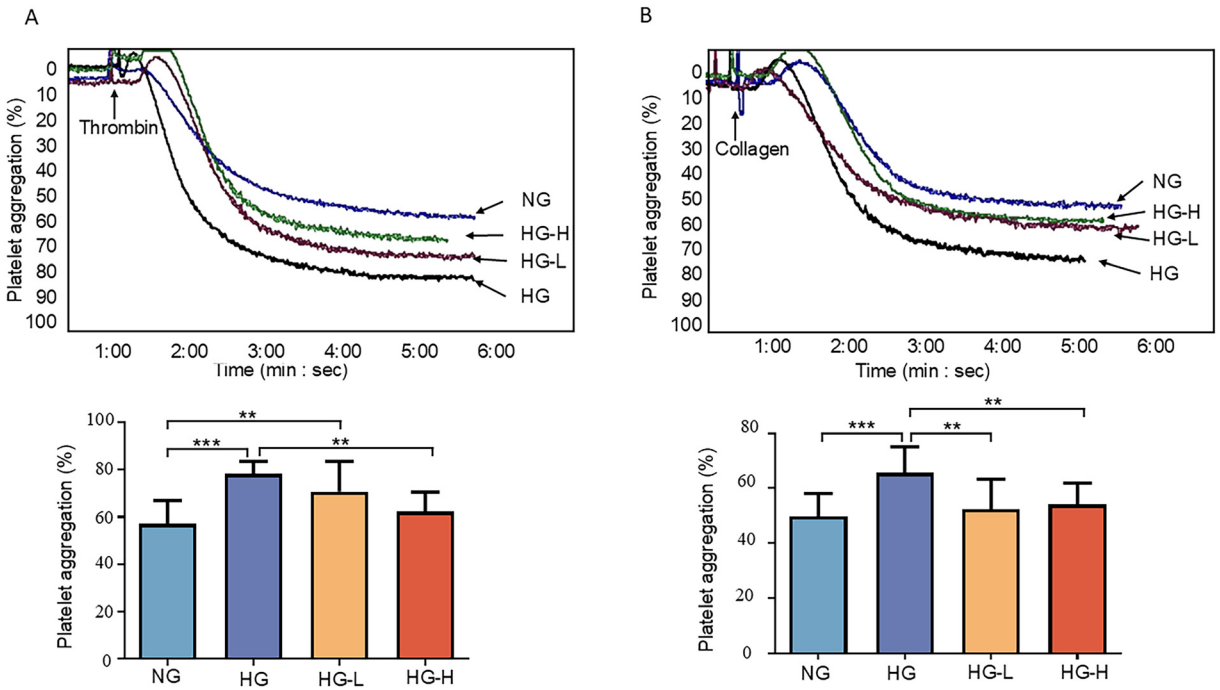

Fig. 1 Effects of DHM administration on diabetic platelet aggregation. Gel-filtered platelets were prepared from the normal control and T2DM mice. Platelet aggregation responses were induced by 0.1 U mL^{-1} thrombin (A) or 1 μg mL^{-1} collagen (B) using an aggregometer. Aggregation was turbidimetrically assessed and expressed as percent change in light transmission. NG, normal control mice; HG, high glycemia model group; HG-L, high glycemia group with a low dose of dihydromyricetin (DHM) (500 mg kg^{-1}); HG-H, high glycemia group with a high dose of DHM (1000 mg kg^{-1}). Data are presented as the mean ± SD (n = 7 independent biological replicates, each pooled from two mice per group). *p < 0.05, **p < 0.01, and ***p < 0.001.

stimulation with either thrombin or collagen. As shown in Fig. 1A, the platelet aggregation rate induced by thrombin was 78.30 ± 5.23% in the HG group mice, which was significantly higher than that in the NG control mice (57.11 ± 9.88%, p < 0.001). The addition of 1000 mg kg^{-1} DHM normalized thrombin-induced platelet hyperaggregability to the level of the NG group. Consistently, platelet aggregation induced by collagen was also significantly greater in gel-filtered platelets separated from the HG group compared with the NG group (65.64 ± 9.44% and 49.73 ± 8.40%, respectively). Similarly, the treatment of DHM in HG-L and HG-H groups remarkedly inhibited platelet aggregation induced by collagen (Fig. 1B).

3.3 DHM reduced platelet ATP secretion in diabetic mice

To further determine the effects of DHM supplementation on platelet dense granule secretion in diabetic mice, ATP secretion from gel-filtered platelets was detected. When compared to the NG group, a significant increase in ATP secretion induced by thrombin was observed in the HG group (p < 0.001, Fig. 2A). DHM treatment dramatically decreased platelet ATP secretion in both HG-L and HG-H groups (1.12 ± 0.13 and 1.04 ± 0.28 nmol mL^{-1}, respectively). Similar trends among these groups were also observed in collagen-induced platelet ATP secretion (Fig. 2B). DHM treatment at the dose of 1000 mg kg^{-1} significantly normalized collagen-induced platelet ATP secretion to levels of the NG group.

3.4 DHM attenuated platelet activation in diabetic mice

Platelet activation was measured by the detection of CD62P, CD63, and CD40L, which were localized to internal granules and expressed on platelet surface upon activation.[29] In the HG group, the mean fluorescence intensity (MFI) of CD62P, CD63, and CD40L on platelets activated by thrombin and collagen was significantly increased compared to the NG group (Fig. 3A–C). The hyperactivation of platelets associated with diabetes was ameliorated by treating with DHM, as evidenced by inhibiting the expression of CD62P, CD63, and CD40L on platelets. Representative flow cytometry histograms are presented in Fig. S2. Additionally, the expression levels of CD62P and CD63 on resting platelets isolated from mice in the HG-H group were also significantly lower than those in the HG group (Fig. S3).

The integrin αIIbβ3, the most highly expressed integrin on platelets, is maintained in a low-affinity state in resting platelets. During platelet activation, αIIbβ3 changes its conformation to a state capable of high-affinity ligand binding.[30] Therefore, we further investigated the effects of DHM on platelet αIIbβ3 activation by using JON/A, a monoclonal antibody that only binds to the activated form of integrin αIIbβ3. As shown in Fig. 3D, JON/A binding induced by thrombin and collagen was significantly increased in gel-filtered platelets obtained from the HG group compared with the NG group. Treatment with DHM in HG-L and HG-H groups significantly reduced platelet αIIbβ3 activation compared to the HG group.

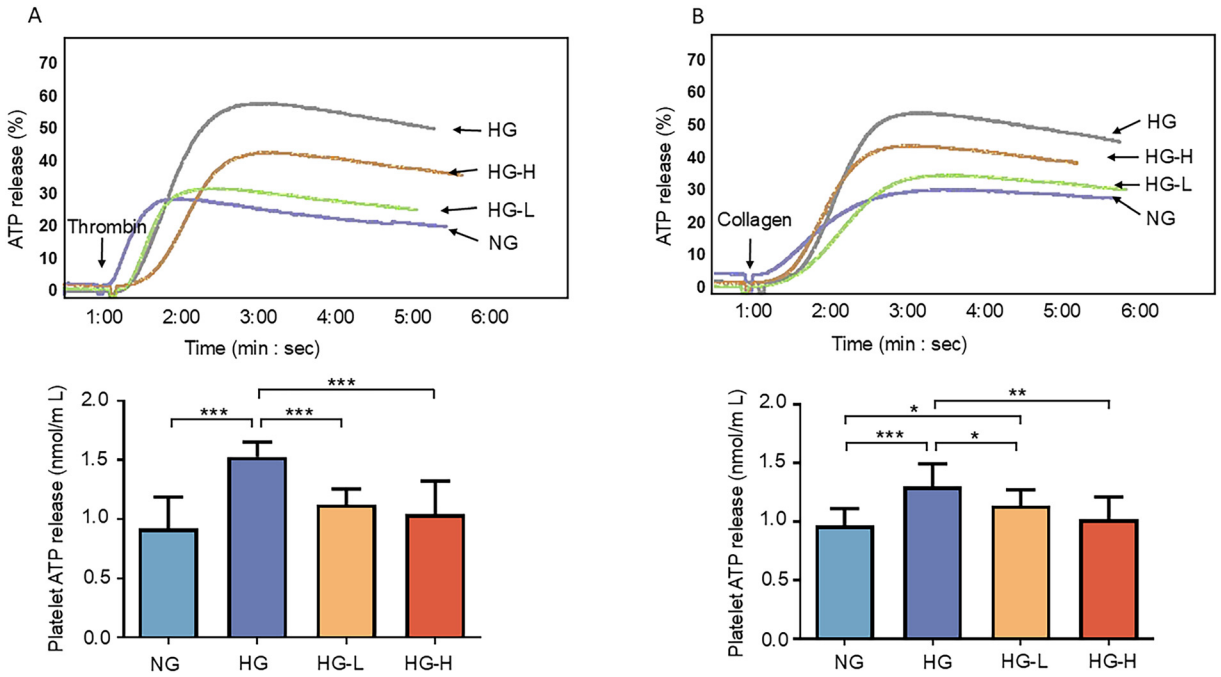

Fig. 2 Effects of DHM supplementation on diabetic platelet ATP secretion. Gel-filtered platelets from normal control and T2DM mice were incubated with the luciferin–luciferase reagent before being stimulated with 0.1 U mL^{-1} thrombin (A) or 1 μg mL^{-1} collagen (B). ATP secretion from platelet dense granules was determined using a Chrono-log lumi-aggregometer and expressed as nmol mL^{-1}. NG, normal control mice; HG, high glycemia model group; HG-L, high glycemia group with a low dose of dihydromyricetin (DHM) (500 mg kg^{-1}); HG-H, high glycemia group with a high dose of DHM (1000 mg kg^{-1}). Data are presented as the mean ± SD (n = 7 independent biological replicates, each pooled from two mice per group). *p < 0.05, **p < 0.01, and ***p < 0.001.

3.5 DHM reduced platelet ROS generation *via* potential interactions with NOX-2 and AR active sites

Considering the crucial role of ROS in platelet hyperresponsiveness associated with diabetes, we further examined the potential mechanism of DHM relative to the reduction of ROS generation in platelets. As shown in Fig. 4A and B, thrombin- and collagen-stimulated ROS production in platelets was significantly enhanced in the HG group compared to the NG group (p < 0.001). As expected, treatment with DHM inhibited platelet intracellular ROS production. Thrombin- and collagen-induced ROS generation in platelets was significantly lower in the HG-H group than in the HG group (p < 0.001 and p < 0.01, respectively).

To explore the potential molecular mechanism by which DHM modulates platelet ROS generation, molecular docking was employed to examine whether DHM can interact with NOX-2 and AR. The results indicated that DHM could be docked into the NADPH-binding cavity of NOX-2 with a binding energy of −8.5 kcal mol^{-1}. As shown in Fig. 4C, DHM interacts with NOX-2 through hydrogen bonds with His119, Val228, and Arg54, a carbon hydrogen bond with His115, Pi–cation interactions with Arg54, and Pi–alkyl contacts with Ala57. These interactions, supported by extensive van der Waals contacts, suggest that DHM likely occupies a region critical for NADPH binding or electron transfer. Similarly, DHM also showed a stable binding potential within the catalytic pocket of aldose reductase (AR), with a binding energy of −7.7 kcal mol^{-1}. The key stabilizing interactions include a conventional hydrogen bond with the catalytic residue His110 and a carbon hydrogen bond with Tyr48. Additional van der Waals and Pi–alkyl contacts were observed with residues such as Trp219, Trp20, Ala299, Cys 303, Trp79, Cys298, Leu300 and Phe115 (Fig. 4D). Taken together, these docking predictions suggested that DHM might interact with the amino acid residues of NOX-2 and AR active sites and thus inhibit their activities, which led to the reduction of intraplatelet ROS production.

3.6 DHM reduced plasma levels of sP-selectin and PF4 in diabetic mice

Plasma levels of sP-selectin and PF4 have been proposed as useful markers of *in vivo* platelet activation.[31] To further explore the effects of DHM on *in vivo* platelet activation in diabetic mice, we measured plasma levels of sP-selectin and PF4 by the quantitative ELISA. As shown in Fig. 5A, plasma levels of sP-selectin in diabetic mice from the HG group (198.97 ± 105.54 ng mL^{-1}) were markedly elevated compared to those of control mice in the NG group (109.63 ± 30.49 ng mL^{-1}, p < 0.001). Dietary supplementation with DHM significantly attenuated the increase in both the HG-L group (139.04 ± 42.95 ng mL^{-1}) and the HG-H group (107.66 ± 19.01 ng mL^{-1}). Similarly, with DHM supplementation in diabetic mice, the

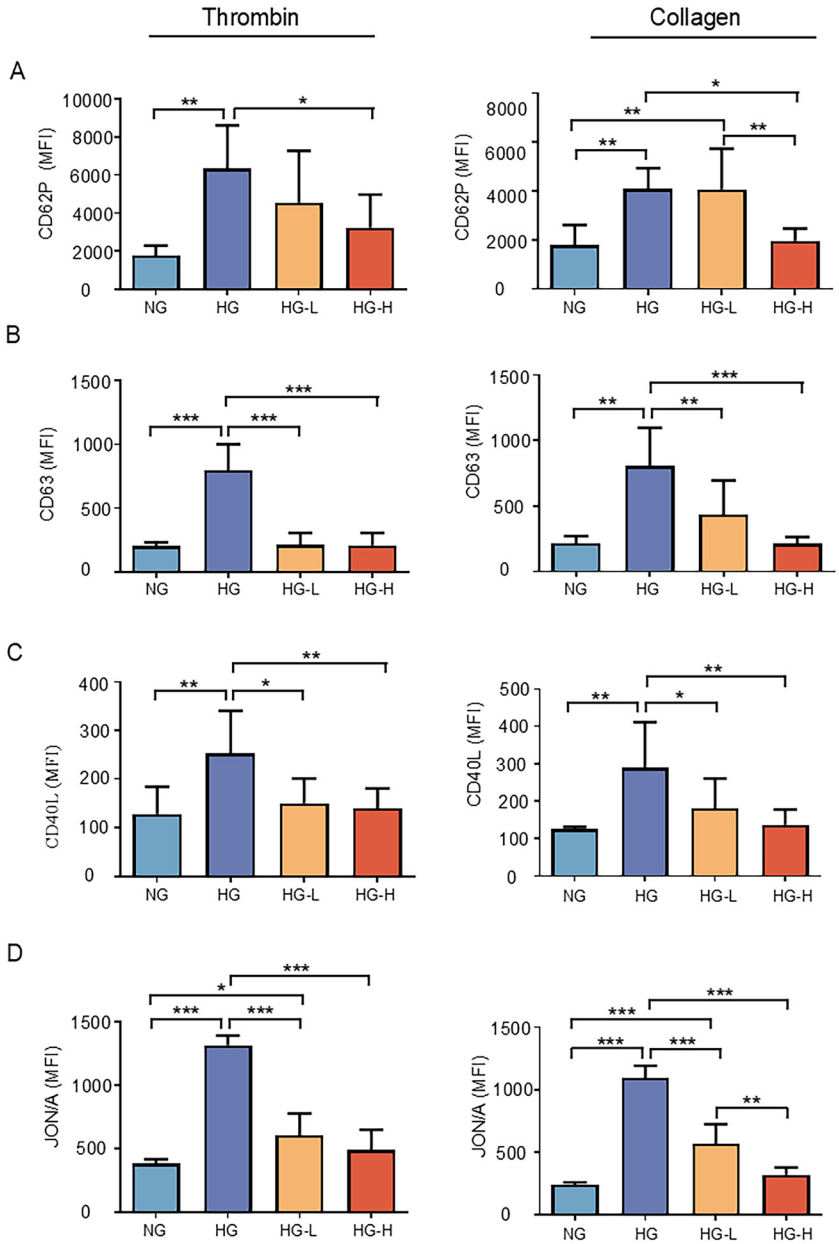

Fig. 3 Effects of DHM on the diabetic platelet surface expression of P-selectin, CD63, CD40L, and activated integrin αIIbβ3. Platelet activation markers were analyzed by flow cytometry after stimulation by thrombin (0.1 U mL^{-1}) or collagen (1 μg mL^{-1}). (A) P-selectin expression on platelet in response to thrombin or collagen, (B) surface expression of CD63, (C) surface expression of CD40L, and (D) binding of PE-labeled JON/A antibodies to platelets, which represents integrin αIIbβ3 activation. NG, normal control mice; HG, high glycemia model group; HG-L, high glycemia group with a low dose of dihydromyricetin (DHM) (500 mg kg^{-1}); HG-H, high glycemia group with a high dose of DHM (1000 mg kg^{-1}). Data are presented as the mean ± SD (n = 7 independent biological replicates, each pooled from two mice per group). *p < 0.05, **p < 0.01, and ***p < 0.001.

increase of PF4 levels was dramatically attenuated but was not fully reversed to the normal level of the NG group (Fig. 5B).

3.7 DHM decreased plasma TXB2 and 8-iso-PGF2α in diabetic mice

TXB2, the stable metabolite of thromboxane A2, is markedly elevated in diabetes mellitus and leads to platelet hyperactivation.[32] In the present study, plasma levels of TXB2 were significantly higher in the HG group than in the NG group, and were normalized to the control levels after DHM treatment. As shown in Fig. 5C, there were no significant differences in TXB2 levels among HG-L (87.24 ± 21.78 ng mL^{-1}), HG-H (82.99 ± 16.74 ng mL^{-1}), and NG (67.78 ± 27.46 ng mL^{-1}) groups.

We also detected plasma levels of 8-iso-PGF2α, a stable isoprostane and reliable marker of oxidative stress, *in vivo*.[33] As shown in Fig. 5D, plasma levels of 8-iso-PGF2α (13.83 ± 5.16 ng mL^{-1}) were significantly higher in the HG group than in the NG group (3.19 ± 1.62 ng mL^{-1}, p < 0.05), and were normal-

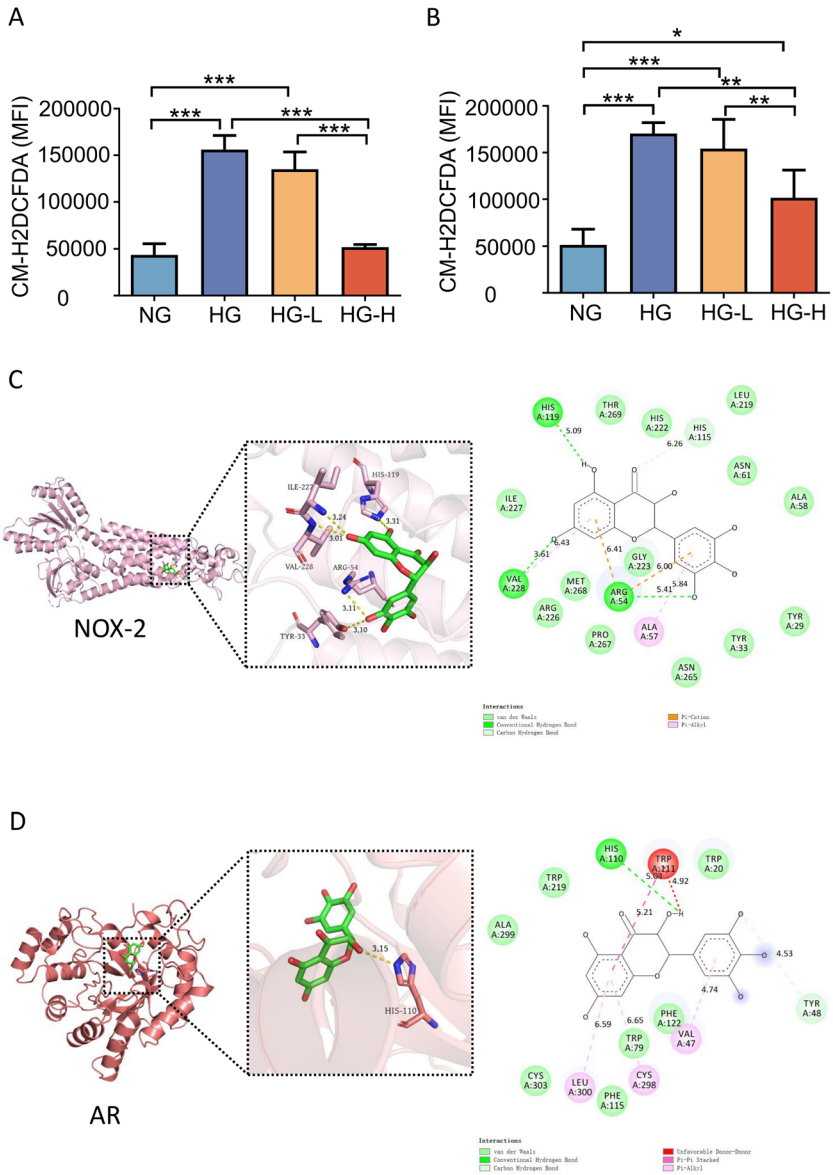

Fig. 4 Effects of DHM on intraplatelet ROS formation and molecular docking analysis with NOX-1 and AR. Generation of ROS was quantified by flow cytometry using 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). Gel-filtered platelets separated from mice were loaded with CM-H2DCFDA dye and stimulated with (A) 0.5 U mL^{-1} thrombin or (B) 2 μg mL^{-1} collagen. (C) Molecular interaction diagram of DHM with NOX-2. (D) Molecular interaction diagram of DHM with AR. NG, normal control mice; HG, high glycemia model group; HG-L, high glycemia group with a low dose of dihydromyricetin (DHM) (500 mg kg^{-1}); HG-H, high glycemia group with a high dose of DHM (1000 mg kg^{-1}). Data are presented as the mean ± SD (n = 7). *p < 0.05, **p < 0.01, and ***p < 0.001.

ized to the control levels by DHM treatment in the HG-H group (3.73 ± 2.03 ng mL^{-1}).

3.8 DHM ameliorated thrombus formation in DM mice

To investigate the effects of DHM on thrombus formation in diabetic mice, we subjected mice to collagen/epinephrine-induced pulmonary embolism. In this model, collagen/epinephrine injection caused platelet activation that led to the formation of thrombi in the lungs and resulted in the mortality of mice. Platelet thrombi were observed in mouse lung sections. Mice from the HG group showed severe pulmonary embolization, whereas DHM exerted substantial protective effects (Fig. 6A). As shown in Fig. 6B, mice in the HG group showed significantly reduced length of survival relative to mice in the NG group. Intriguingly, mice in the HG-H group exhibited significantly prolonged survival in contrast to the DM mice (p < 0.05).

In addition, we evaluated the effects of DHM on bleeding time *via* tail transection. The bleeding times of the four groups were 175.07 ± 103.59 s (NG group), 145.00 ± 48.84 s (HG group), 149.21 ± 70.98 s (HG-L group), and 178.29 ± 82.09 s (HG-H group). No significant differences were observed in the

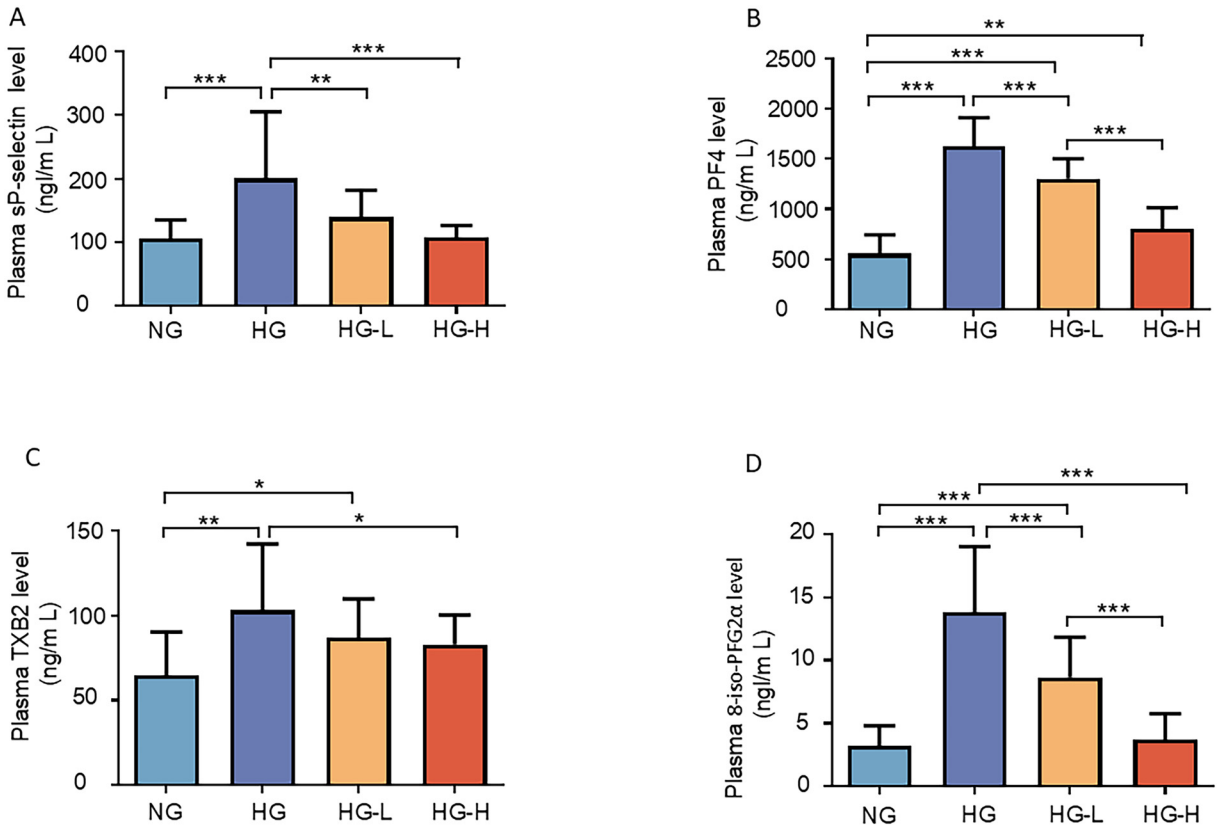

Fig. 5 Effects of DHM on plasma levels of sP-selectin, PF4, TXB2, and 8-iso-PGF2α in T2DM mice. The plasma levels of sP-selectin (A), PF4 (B), TXB2 (C), and 8-iso-PGF2α (D) were measured by ELISA. NG, normal control mice; HG, high glycemia model group; HG-L, high glycemia group with a low dose of dihydromyricetin (DHM) (500 mg kg^{-1}); HG-H, high glycemia group with a high dose of DHM (1000 mg kg^{-1}). Data are presented as the mean ± SD (n = 14). *p < 0.05, **p < 0.01, and ***p < 0.001.

four groups (Fig. 6C). The results suggested that dietary intake of DHM reduced platelet plug formation without significantly increasing bleeding risk in diabetic mice.

3.9 DHM potentially interacts with GPVI, PAR-1 and COX-1

Molecular docking was employed to predict the binding interactions of DHM with platelet receptors PAR-1, GPVI, as well as COX-1, all of which play important roles in platelet hyperactivation in diabetes. As shown in Fig. 7A, DHM showed stable binding potential within the active site of platelet GPVI. DHM formed conventional hydrogen bonds with Glu84, Ser16 and Glu138, which anchored DHM to the polar region of the pocket. Moreover, a Pi–anion interaction was observed between the aromatic ring of DHM and Glu84, further enhancing electrostatic attraction. Several surrounding residues, including Leu83, Val86, Arg142, Trp171, and Ser15, were involved in van der Waals interactions, which further reinforced the overall binding affinity. The binding energies obtained from computational docking analyses revealed that DHM exerted even stronger binding affinity with PAR-1 (−9.5 kcal mol^{-1}) compared to GPVI (−5.9 kcal mol^{-1}). DHM interacted with multiple surrounding amino acid residues in the binding pocket of PAR-1 through a combination of hydrogen bonds and van der Waals forces. Hydrogen bonds were observed between DHM and Thr261, Glu260, Tyr337, Tyr353, and Ser344, which contributed to the initial anchoring of DHM in the pocket. Additionally, DHM formed a carbon hydrogen bond with His255. van der Waals interactions were also prominent between DHM and various amino acids, such as Leu262, Leu332, Leu333, Ala349, Ser259, Tyr350, and His255, which collectively contributed to the binding stability (Fig. 7B). These findings provide a structural basis for its inhibitory effects on platelet function activated by both collagen and thrombin.

Furthermore, COX-1, a key enzyme mediating TXA2 formation in platelets, was also predicted to bind stably with DHM, with a binding energy of −8.5 kcal mol^{-1}. Multiple conventional hydrogen bonds were formed between DHM and Val119, Thr76, Arg79, Tyr64, and Arg83, indicating that hydrogen bonding plays a critical role in stabilizing. In addition, carbon hydrogen bonds with Gly471 contributed to ligand anchoring within the binding pocket. Extensive van der Waals interactions with His43, Gln44, Asn80 and Asn122 supplemented the overall binding stability (Fig. 7C). These interactions might prevent access of arachidonic acid to the COX-1 catalytic site, leading to enzyme inactivation, which was in accordance with the reduction of plasma levels of TXB2 in DM mice. These docking results indicate potential structural bases

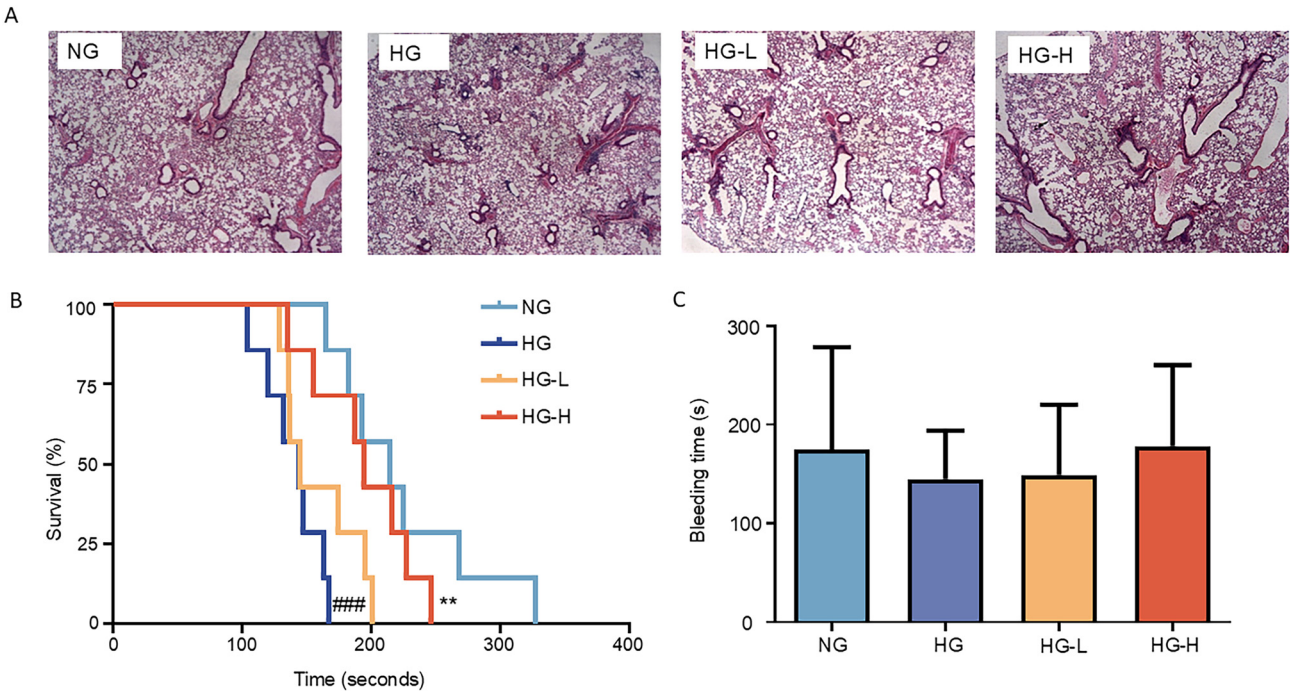

Fig. 6 Effects of DHM consumption on acute pulmonary thrombus formation in T2DM mice. T2DM mice were fed with a high fat diet supplemented with DHM for 8 weeks. Acute pulmonary thromboembolism was then initiated by collagen/epinephrine injection, and the survival times of mice were recorded. (A) Representative hematoxylin and eosin-stained images of lung sections. (B) Survival curves of mice subjected to collagen/epinephrine-induced pulmonary embolism (n = 6 per group). $^{\#\#\#}p < 0.001$ compared to the NG group. $^{**}p < 0.01$ compared to the HG group (log-rank (Mantel–Cox) test). (C) Tail bleeding time measured after amputating 2 mm of the tail tip (n = 14). NG, normal control mice; HG, high glycemia model group; HG-L, high glycemia group with a low dose of dihydromyricetin (DHM) (500 mg kg^{-1}); HG-H, high glycemia group with a high dose of DHM (1000 mg kg^{-1}). Data are presented as the mean ± SD. No significant differences in bleeding times were observed among the four groups.

for the inhibitory effects of DHM on platelet activation mediated by GPVI, PAR-1, and COX-1.

4. Discussion

The present study demonstrated that feeding a high-fat diet to STZ-induced diabetic mice could induce *in vivo* platelet activation and platelet hyperreactivity in response to stimulation of thrombin and collagen. Dietary supplementation of DHM prevented platelet activation, granule secretion and aggregation and decreased plasma levels of *in vivo* platelet activation markers PF4 and sP-selectin, along with oxidative and thrombogenic markers 8-iso-PGF2α and TXB2. Molecular docking analysis suggested that DHM may potentially interact with key platelet surface receptors GPVI and PAR-1, as well as NOX-2, COX-1, and AR, providing a molecular basis for the observed suppression of intraplatelet ROS formation. Furthermore, DHM treatment delayed the collagen/epinephrine-induced pulmonary embolism without prolonging tail bleeding time. Given that intraplatelet ROS can act as a pivotal second messenger that enhances agonist-induced platelet activation and aggregation, we concluded that DHM could attenuate platelet hyperactivity under hyperglycemic conditions, at least in part, through the suppression of ROS generation and the downstream signaling events.

T2DM is a serious global metabolic health problem with high prevalence and morbidity. It is now well accepted that the morbidity and mortality associated with diabetes commonly result from microvascular and macrovascular complications.[34] Although glycemic control is the cornerstone to prevent the microvascular complications of diabetes mellitus, diabetic subjects are at a high risk of macrovascular CVD, as up to 80% of individuals with diabetes mellitus will die from cardiovascular causes.[4,35] The lack of an effect of intensive glucose-lowering on most macrovascular outcomes calls for more comprehensive strategies to manage cardiovascular risk factors alongside the glycemic control.[36] Platelet hyperactivity, characterized by increased activation, adhesion, and aggregation of the platelets in comparison with the normal responses, has been claimed as a major contributor to the development of micro- and macro-angiopathy in patients with T2DM.[37] The inhibition of platelet dysfunction is an approach for preventing and treating these disorders. In the current study, platelets from diabetic mice were found to be hyper-aggregable in response to thrombin and collagen when compared to NC mice, which is in agreement with previous observations.[38] DHM administration resulted in a 20% greater inhibition of platelet aggregation in diabetic mice, eliminating the hyper-aggregability in this condition and resulting in levels similar to those of non-diabetic mice. Markers of platelet activation,

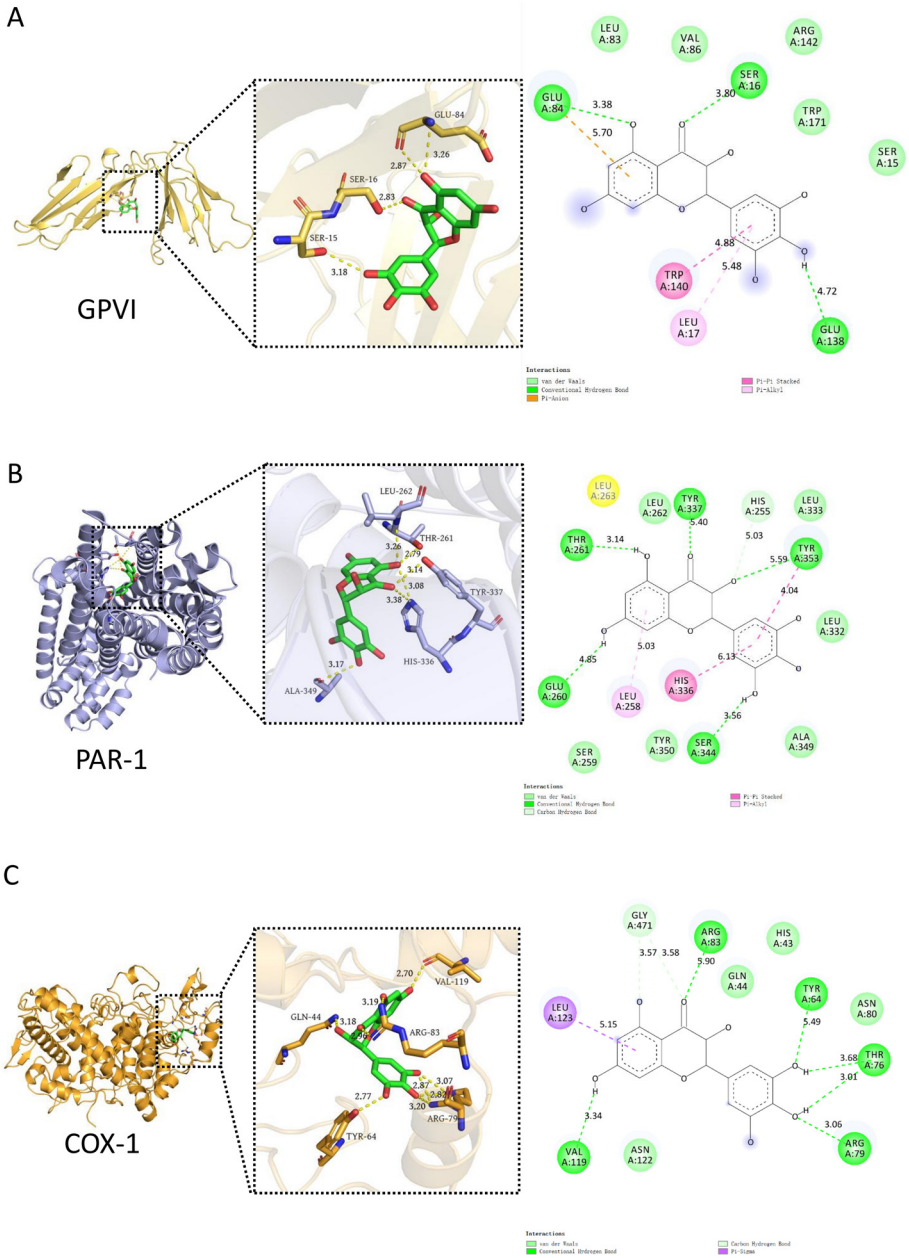

Fig. 7 Molecular docking analysis of DHM with GPVI, PAR-1 and COX-1. Molecular docking analysis showing the 3D and 2D interaction diagrams of DHM with GPVI (A), PAR-1 (B) and COX-1 (C).

such as P-selectin and CD40L, increased in T2DM patients, which reflects a systemic prothrombotic state driven by chronic platelet hyperactivity.[39] In the present study, the expression of platelet surface activation antigens upon stimulation with collagen and thrombin was higher in diabetic mice than in NC mice, confirming the hyperreactivity of diabetic platelets, and it then tended to be normalized after DHM treatment.

Not only did platelets from DM patients show hyperresponsiveness when stimulated *in vitro*, but also they were activated *in vivo* as shown by the enhanced expression of surface activation antigens on circulating platelets and secretion of granule contents.[40] In diabetic patients, elevated circulating levels of sP-selectin, sCD40L, β-thromboglobulin (β-TG), and PF4, resulting from platelet degranulation, are regarded as reliable indicators of the *in vivo* platelet activation status.[8,41] PF4 is a pivotal platelet-derived α-granule protein released upon platelet activation, serving as a critical mediator in regulating thrombotic and inflammatory responses.[42] Similarly, P-selectin, also stored in platelet α-granules, translocated to the platelet surface during platelet activation, where it is rapidly cleaved off, resulting in the release of soluble P-selectin. Plasma sP-selectin is thought to arise predominantly from activated platelets, establishing it as a suitable cir-

culating marker of *in vivo* platelet activation.[31] Elevated plasma sP-selectin concentrations are observed in several thrombotic diseases and are associated with the risk of developing a future vascular event.[43] Consistent with prior evidence, elevated plasma levels of PF4 and sP-selectin were observed in T2DM mice, confirming *in vivo* platelet activation and granule secretion in diabetic conditions.[44] Dietary supplementation with DHM significantly ameliorated *in vivo* platelet activation, as evidenced by reduced PF4 and sP-selectin levels, highlighting its therapeutic potential to ameliorate platelet hyperactivity and mitigate thrombotic dysregulation in diabetes.

Platelet hyperactivity in diabetes is a complex phenomenon that encompasses multiple mechanisms, among which oxidative stress seems to play a pivotal role.[2] In platelets, ROS, such as the superoxide anion or hydrogen peroxide, are primarily produced by NOX-2 upon stimulation with common agonists and function as secondary messengers to amplify platelet activation and aggregation.[45] DM is associated with enhanced activities of AR and NOX in platelets, resulting in overproduction of ROS, which in turn promotes lipid peroxidation and free radical-catalyzed conversion of arachidonic acid (AA) into bioactive F2-isoprostanes, such as 8-iso-prostaglandin F2α (8-iso-PGF2α).[46] Elevated F2-isoprostane production may also potentiate platelet hyperactivity by enhancing platelet response to agonist-induced platelet adhesion and aggregation, as well as by amplifying the signaling of platelet receptors.[7] The plasma level of 8-iso-PGF2α is well established to affect platelet functions and it also serves as a gold-standard biomarker of oxidative stress.[47] In previous studies, *N*-acetylcysteine (NAC), a precursor of antioxidant glutathione (GSH), has been shown to attenuate platelet hyperreactivity by scavenging platelet-derived ROS in T2DM patients.[48] Similarly, we found that DHM supplementation in diabetic mice suppressed collagen- and thrombin-induced platelet ROS production, which was accompanied by reduced plasma levels of iso-8-PGF2α. Given the established role of ROS in potentiating platelet hyperactivity, the inhibitory effects of DHM on platelet hyperactivation can be explained, at least in part, by the inhibition of ROS formation. Molecular docking analysis revealed that DHM may interact with NOX-2 and AR, as well as the thrombin receptor PAR-1 and the collagen receptor GPVI, through multiple hydrogen bonds and hydrophobic interactions, which may partially explain the ROS-lowering effects of DHM observed in platelets. Moreover, DHM also exhibited favorable binding conformations with the active site of platelet COX-1, an enzyme that catalyzes AA into pro-inflammatory prostaglandins and pro-thrombotic TXA2, accompanied by production of ROS.[7] Although these docking simulations provide valuable mechanistic insights into how DHM may modulate platelet ROS production and function, further biochemical validation will be essential in future investigations to definitively confirm the functional blockade of these molecular targets by DHM in platelets. Furthermore, ROS are well-established as upstream regulators of classical signaling pathways that drive platelet activation, including the Syk/PLCγ2/calcium signaling axis, as well as PI3K/Akt, MAPK and P53 signaling pathways.[49,50] Together with a previous *in vitro* study that demonstrated that DHM attenuated thrombin-induced calcium mobilization and phosphorylation of ERK1/2 and p38 in platelets,[20] the current findings raise the possibility that under diabetic conditions, the antioxidant capacity of DHM may further attenuate these downstream cascades.

Platelet hyperactivity in diabetes can easily lead to thrombosis and induce adverse cardiovascular events.[51] To determine whether dietary supplementation of DHM can reduce thrombus formation in diabetic mice, we subjected mice to a collagen/epinephrine-induced pulmonary embolism model which has been widely used to evaluate the anti-thrombotic activity of natural and dietary components.[52] The lungs contain many small arteries and capillaries, and intravenous injection of thrombus-initiating factors such as thrombin, ADP, and collagen with epinephrine may cause fatal and acute platelet-rich thrombosis in the vasculature of the lungs within a short period of time.[53] This *in vivo* model is largely driven by platelet activation rather than endothelial changes, which are also prominent in diabetes.[27] Indeed, diabetic mice showed enhanced thrombus formation when compared with non-diabetic mice. DHM exhibited protective effects on pulmonary microcirculation occlusion in diabetic mice, indicated by prolonged survival times and reduced thrombotic occlusions in the lung tissues of mice in the HG-H group. Our findings are consistent with a previous study demonstrating that intravenous injection of DHM delayed $FeCl_3$-induced carotid arterial thrombosis in mice.[20] Importantly, we observed that the bleeding time of DHM-treated mice in the HG-L and HG-H groups remained unchanged and were the same as that of the control mice, suggesting a favorable safety profile without an increased hemorrhagic risk. Additionally, previous clinical evidence has demonstrated that daily supplementation with 10 g of *Ampelopsis grossedentata* (vine tea), which provided approximately 970 mg of DHM, was both safe and effective in improving glycemic control in T2DM patients.[54] The dietary doses of DHM used in this study (500 and 1000 mg kg^{-1} diet) correspond to estimated human equivalent doses of approximately 300 and 600 mg day^{-1} for adults, which are substantially lower than the dosage in dietary supplement interventions. Therefore, the antiplatelet effects observed in this study are physiologically achievable through the consumption of vine tea or functional food supplements. This reinforces the potential application of DHM as a safe and effective dietary strategy for preventing diabetes-related thromboembolic complications.

5. Conclusions

In summary, the present study demonstrated that DHM effectively ameliorated platelet dysfunction in HFD/STZ-induced diabetic mice, which may be associated with the suppression of intraplatelet ROS generation. To the best of our knowledge, this is the first study showing that dietary supplementation of DHM is able to produce beneficial platelet inhibitory effects

under diabetic prothrombotic conditions. However, several limitations should be acknowledged. First, although our findings suggest a central role of ROS inhibition, the present study did not directly investigate key intracellular signaling pathways involved in platelet activation. Given the multifactorial mechanisms underlying platelet hyperreactivity in diabetes, further studies are warranted to determine whether DHM can also reverse the dysfunction of other intracellular pathways affected by diabetes mellitus in platelets. Second, the P2Y12 receptor pathway plays a pivotal role in diabetic platelet hyperactivity, and future studies are needed to determine whether DHM modulates ADP-induced platelet activation and aggregation, thereby providing a more comprehensive evaluation of its antiplatelet effects. Finally, further clinical studies are required to validate the efficacy of DHM supplementation and to determine the optimal doses capable of ameliorating platelet hyperreactivity in humans, which would provide a stronger basis for its clinical application. Given the growing global burden of diabetes and its associated cardiovascular complications, the identification of DHM as a promising natural candidate for managing diabetic thrombotic risk may contribute to the development of preventive nutritional strategies.

Author contributions

Fenglin Song: project administration, resources, investigation, methodology, and writing – original draft. Huafang Ding: investigation, methodology, and writing – review. Yun Chen: investigation and methodology. Xiangzhen Ge: investigation and methodology. Ruixue Guo: methodology and writing – review. Fangfang Wu: methodology and writing – review. Yu Guo: resources and writing – review. Ren-You Gan: supervision and writing – review & editing. Zhen-Yu Chen: supervision, resources, and writing – review & editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that there is no conflict of interests.

Ethics statements

Our study was approved by the ethics committee of Guangdong Pharmaceutical University and conformed to the Helsinki Declaration. All animal procedures were approved by the Animal Care and Protection Committee of Guangdong Pharmaceutical University (permit no. 2023212).

Data availability

The original contributions presented in the study are included in the article/supplementary information (SI). Supplementary information includes supplementary tables, figures, and supporting experimental data related to platelet function and ROS analyses. See DOI: https://doi.org/10.1039/d6fo00232c.

Further information is available by contacting the corresponding author.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81502808), the Key Projects of Social Welfare and Basic Research of Zhongshan (No. 2020B2012), the Scientific Research Project of the Guangdong Provincial Bureau of Traditional Chinese Medicine (No. 20252019), and the Research Group of Functional Foods Development for Specific Populations (No. 2024ZZ12).

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