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Full Length Research Paper

Preventive effect of polydatin against thrombosis: and its mechanism

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This study aimed to investigate the effect of polydatin against thrombosis and its possible mechanisms. The methods of injection of arachidonic acid into mouse tail vein electrically stimulated carotid thrombosis in rats, and the rats' inferior vena ligation were used to evaluate the antithrombotic effects of polydatin. Platelet aggregation was tested by use of Born's method, and platelet cytosolic calcium was determined by use of Fura-2/AM. Thromboxane B_2 and 6-keto-prostaglandin $F_{1\alpha}$ level was monitored by the immuno-assay, while Rosette assay and Born's method were used to observe platelet-neutrophil interactions. The results show that polydatin had evident antithrombotic effects in the multiple-thrombosis models; the mechanisms may be closely related to its anti-platelet aggregation, which results in decrease of platelet cytosolic calcium and plasma thromboxane B_2, while increasing plasma 6-keto-prostaglandin $F_{1\alpha}$ Level and suppressing of platelet-neutrophil interactions.

Key words: Polydatin, thromboxane A_2, prostacyclin, platelet, neutrophil, cytosolic calcium.

INTRODUCTION

Modern researches show that the process of thrombosis is closely related with interactions of platelets, leukocytes and other cells. Activated platelets can release some active substances including platelet activating factor (PAF) and thromboxane. Such active substances activate polymorphonuclear leukocytes (PMN), and activated PMNs accelerate the expression of platelet selectin so that adhesion between PMNs and platelets occurs while producing more thromboxane and promoting the mobilization of platelet intracellular calcium (Armstrong et al., 2008; Chlopicki et al., 2003). Therefore, an obvious platelet aggregation is induced, which results in acceleration of the process of thrombosis eventually.

Thromboembolic diseases are great threats to human's life and health and prevention and treatment of thrombosis is a focus of modern medical research. One of the new hopes in this field of disease may be found in high effective drugs from natural products. Polydatin (3,4'-5-trihydroxystilbene-3-beta-D-glucopyranoside, Figure 1), a glucoside of resveratrol, is widely distributed in many plants especially in *Polygonum cuspidatum* and shows protective effects against hyperlipemia and ischemia/reperfusion injury (Xing et al., 2009; Zhang et al., 2008; Huang et al., 2003; Du et al., 2009). Hence, its pharmacological effects in cardiovascular fields are to attract broad attention in recent years.

Platelet hyperfunction and abnormality in thromboxane A_2 (TXA_2) and prostacyclin equilibrium is closely related to thrombosis and TXA_2 is a mediator of the sudden death induced by intravenous arachidonate in mice. AA, as a substrate of cyclooxygenase (COX), is transformed to two unstable intermediate products - prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2). PGG_2 and PGH_2 can form TXA_2 through the action of thromboxane synthetase and an increase of TXA_2 will result in the adhesion, aggregation and release of platelets. PGG_2 and PGH_2 can also form prostacyclin I_2 (PGI_2) through the action of prostacyclin synthetase and the PGI_2 is able to inhibit platelet aggregation. Accordingly, drugs' inhibition on the metabolism of AA is likely to inhibit the activity of COX or affect the activities of thromboxane synthetase or

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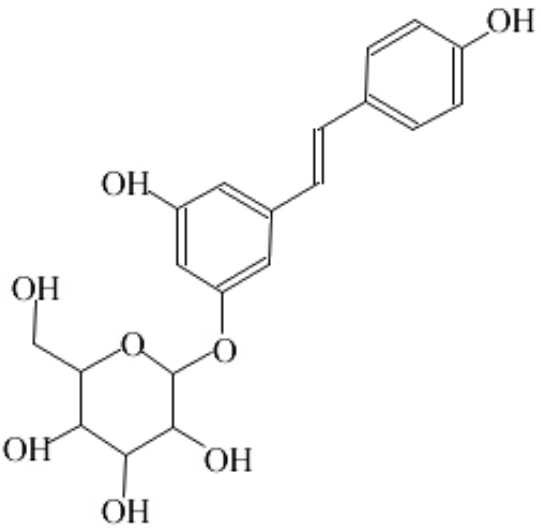

Figure 1. Chemical structural formula of polydatin.

prostacyclin synthetase (Gross and Moore, 2004). The biological half-life of TXA_2 and PGI_2 are about 30 s and 3 min, respectively, then they turn into TXB_2 and 6-keto-$PGF_{1\alpha}$ rapidly. Thus, the determination of TXB_2 and 6-keto-$PGF_{1\alpha}$ is used as a chief means of estimating the levels of TXA_2 and PGI_2 (Terashita et al., 1995).

Electrical stimulation on rat carotid artery can injure vas endodermis, activates platelets and neutrophils, induce platelet aggregation and finally leads to a mixed thrombus. This experiment is to investigate antithrombotic effect of polydatin by the use of multiple arterial and venous thrombosis models and to explore its antithrombotic mechanisms by detecting the effects of polydatin on platelets, neutrophils, thromboxane B_2 (TXB_2), 6-keto-prostaglandin $F_{1\alpha}$, and platelet cytosolic calcium, in view of providing more detailed bases for curing thrombotic diseases with polydatin.

MATERIALS AND METHODS

Drugs and reagent

The rhizomatic parts of *P. cuspidatum* were obtained in Yunnan Province of China. The plant was identified by Prof. Renwei Zhang (Yunnan Institute of Materia Medica) and a voucher specimen is deposited in the herbarium collection of the Institute (Yun. No. 025618). The medicinal materials were extracted with alcohol and the filtrates were extracted by ethyl acetate. The extracts were concentrated and separated on macroporous resin and Al_2O_3 column. High performance liquid chromatography (HPLC) was used for quantitative analysis of the obtained polydatin (molecular formula $C_{20}H_{22}O_8$, molecular weight 390.40, purity 99%).

Polydatin was dissolved in 0.9% saline (pH 7.0) before use. Aspirin (Sigma) was dissolved with 100 mmol/L Na_2CO_3 before use, pH7.0. Arachidonic acid (AA) (Sigma) was dissolved in absolute alcohol and also diluted with 100 mmol/L Na_2CO_3. Adenosine diphosphate (ADP) (Fluka) was dissolved in 0.9% saline, pH 7.0. Platelet activating factor (PAF) (Sigma) was dissolved in Tris-NaCl buffer solution, which involved 0.25% bovine serum albumin (BSA), pH7.5. Thrombin (Sigma) was dissolved in 0.9% saline, pH 7.0. N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma) was dissolved in dimethyl sulfoxide and was diluted with distilled water before use. Fura-2/AM was from Sigma. The radioimmunoassay kits of TXB_2 and 6-keto-$PGF_{1\alpha}$ were purchased from ShenKe Biological Technology Co. LTD, Shanghai, China.

Animals

Healthy rabbits weighing 2.0 to 3.0 kg, male Sprague-Dawley (SD) rats weighing 250 to 300 g and male ICR mice weighing 22 to 26 g, were offered by the Experimental Animal Center of Yunnan Key Laboratory of Pharmacology for Natural Products. The protocol of this study was approved by the Ethics Committee of Kunming Medical University (SCXK2009008).

AA caused mouse's death

Male ICR mice were randomly divided into 5 groups of 15 mice each. Group A: 0.9 % saline, group B: 10 mg/kg aspirin, and groups C to E: 5, 10 and 20 mg/kg polydatin. These 75 male ICR mice were no-eating overnight. Morrow, the mice were injected the above substances into tail veins. 0.5 h after administration, 80 mg/kg AA was intravenously injected into mice's tails at 50 µL / 10 g body weight. Myers's method was modified (Myers et al., 1986). After 15 min, the number of surviving animals in the treatment and control groups was counted.

Electrically stimulated rat carotid artery thrombosis

SD rats were randomly divided into 5 groups of 10 rats each. Group A: 0.9% saline, group B: 10 mg/kg aspirin, and groups C to E: 5, 10, and 20 mg/kg polydatin respectively. The rats were anaesthetized by 30 mg/kg of sodium amobarbital, and were injected in femoral vein with the aforementioned substance. 15 min after administration, Charlton's method was modified (Charlton et al., 1996). The artery was electrically stimulated (1.5 mA for 7 min) using an electronic stimulator (model SEN-7203, Nihon Kohden Corporation, Japan). The time to the formation of an occlusive thrombus (zero arterial flow) was recorded with a Direction Volume Meter (model DVM-4200, Hayashi Denki Corporation, Japan). If the vessels were still patent at the end of this observation period, the duration of 60 min was ascribed to allow statistical analysis of the data.

Ligated rat inferior vena cava thrombosis

The rats were randomly divided into 5 groups of 10 rats each. Group A: 0.9% saline, group B: 10 mg/kg aspirin and groups C to E: 5, 10 and 20 mg/kg polydatin respectively. The rats were anaesthetized by 30 mg/kg of sodium amobarbital. Vein thrombosis model were duplicated according to the method described by CX Chen (Chen et al., 1992). The rat's abdomen was opened and the inferior vena cava was isolated and ligated below the left renal vein level, and then the abdomen was closed. After 1 h, the rats were injected in femoral vein with the above substance in a volume of 0.1 mL/ 100 g body weight, respectively. Two hours later, the abdomen was reopened. The thrombus in the inferior vena cava was collected into a glass dish for measurement of wet weight. It was

then placed in a drying oven at 60℃ for 20 h before measuring the dry weight.

Preparation of platelet (Shen et al., 2003)

Healthy rabbits were used. Blood from rabbit's carotid artery was mixed with one-sixth volume of acid citrate dextrose (sodium citrate 25, citric acid 15, and D-glucose 20 g/L, for the measurement of platelet cytosolic calcium level). This blood was then spun to obtain platelet-rich plasma (PRP) at 300 × *g* for 15 min. PRP was further spun at 1000 × *g* for 10 min to pellet the platelets. Platelet pellets were gently suspended in prewarmed Tyrode-HEPES buffer (containing NaCl 134, KCl 2.9, $NaHCO_3$ 12, NaH_2PO_4 0.36, $MgCl_2$ 1, glucose 5, HEPES 5 mmol/L and bovine serum albumin 0.35%, pH 7.2). After washed twice, the cells were resuspended in the above buffer solution. The platelet count was adjusted to about 5 × 10^8 cell / L.

Blood from rabbit carotid artery was collected into plastic tubes, anticoagulated with 2.7% of ethylenediaminetetraacetic acid (EDTA) (for the binding of platelets to neutrophils) or 3.8% sodium citrate acid (for aggregation). This sample was spun for 10 min at 180 × *g* to obtain PRP. PRP was further spun to pellet platelets at 1000 × *g* for 10 min. Platelet pellets were washed three times and resuspended in phosphate buffer solution (PBS, containing 1.0% bovine serum albumin, 1.4 mmol/L EDTA, and 1 mmol/L $CaCl_2$). Cell viability by Typan blue exclusion was above 95 % and cell counter was adjusted to 10^8 cell/mL.

Preparation of neutrophils (Shen et al., 2003)

Neutrophils were isolated from the blood (coagulated by 2.7% edetic acid, from which platelet-rich plasma was removed). The cell pellet was resuspended in an erythrocyte lysis buffer composed of 155 mmol/L NH_4Cl, 2.96 mmol/L $KHCO_3$, and 3.72 mmol/L acetic acid. The tube was gently inverted, then after 5 min the suspension was centrifuged at 350 × *g* for 10 min, and the cell pellet was washed in PBS lacking calcium; then resuspended in Hanks' solution (containing 1 mmol/L $CaCl_2$ or 5 mmol/L egtazic acid in vehicle, reflecting the situation with or without external calcium). Cells were adjusted to a count of 2 × 10^8 cell/mL (for adhesion) or 0.5 ×10^6 cell/mL (for platelet aggregation). Cells prepared in this manner contained 98% neutrophils and were 96% viable.

Platelet aggregation *in vitro*

Platelets aggregation was determined by Born's methods (Born, 1962). PRP was further spun at 3000 × *g* for 15 min to obtain platelet-poor plasma (PPP). The maximal aggregation was monitored in a Lumiaggregometer (Kemao Limited Company, Beijing, China). The final concentrations of ADP, AA and PAF were 3 μmol/L, 0.35 mmol/L and 7.2 nmol/L, respectively. Percentage inhibition by drugs was calculated by use of the following equation:

Inhibition of aggregation (%) = (A - B) / A ×100

Where, A is the maximum change of turbidity when the control (saline) is added and B is the maximum change of turbidity when the drug (different concentrations of polydatin or aspirin) is added.

Platelet aggregation *ex vivo*

The animals were randomly divided into 5 groups of 6 rabbits. Group A: 0.9% saline, group B: 10 mg/kg aspirin and groups C to E: 5, 10, and 20 mg/kg polydatin respectively. Blood sample from rabbit carotid artery was collected before administration. PRP and PPP were prepared before administration and at 0.5, 1, 1.5, 2, 3 and 4 h after administration, respectively. Platelet aggregation induced by ADP, AA or PAF was monitored as the *in vitro* test, respectively.

Platelet cytosolic calcium level

Intracellular Ca^{2+} concentration of platelets was measured by using Fura-2-AM with a spectrofluorophotometer (Model RF-5000, Shimazhu, Japan) at 37℃ and magnetically stirred. The cells were treated with 0.1% Triton X-100 followed by the addition of egtazic acid 10 mmol/L to obtain the maximal and minimal fluorescence, respectively. The ratio of the measured fluorescence values at 340 and 380 nm excitation was calculated. $[Ca^{2+}]_i$ was calculated by the method of Grynkiewicz G (Grynkiewicz et al., 1985). AA (200 μmol/L)-induced rise in $[Ca^{2+}]_i$ was measured in the presence of 1 mmol/L $CaCl_2$ or 1 mmol/L egtazic acid, respectively.

Determination of TXB_2 and 6-keto-$PGF_{1\alpha}$

Before injection and at 30, 60, 120 min after injection of the drugs, rabbit blood from carotid artery was anticoagulated with heparin (3.5 mg/mL) and spun at 1000 × *g* at 0℃ for 10 min to obtain plasma. TXB_2 and 6-keto-$PGF_{1\alpha}$ in plasma were extracted from the supernatant and assayed by use of radioimmunoassay kits.

Rosette assay (Shen et al., 2004)

The method of Rosette assay was modified. 50 μL of platelets and 0.2 U/mL thrombin were mixed and incubated for 15 min at 37℃, 50 μL of drugs were then added and incubated for another 15 min. Then 100 μL of neutrophils was added to the platelet suspension. One hundred neutrophils were scored for the presence (two or more platelets per neutrophil) or absence (zero or one platelet per neutrophil) of platelets. Neutrophils bearing two or more platelets were thus defined as rosettes. For each assay done in triplicate, the rosetting score was assessed by two different observers.

Inhibition of platelet aggregation induced by the suspension of activated neutrophils

100 μL of platelets (10^8 cell/mL) were mixed with 100 μL of neutrophils (0.5 ×10^7 cell/mL) and stirred for 2 min at 37℃. 0.9% saline or drug solution was added and incubated for 5 min and then 2 μmol/L of fMLP was added. Platelet aggregation was observed using the earlier described method (Born, 1962).

Statistical analysis

The data were analyzed by the software bag of SPSS (Version 13, SPSS Inc., USA). All enumeration data were expressed as percentages and analyzed by use of Chi-square test. All measurement data were expressed as mean ± SD, and analyzed by one way ANOVA and least-significant difference (LSD) test, respectively. Differences were considered statistically significant when $P < 0.05$.

Table 1. Preventive effect of polydatin against mouse sudden death caused by injection of arachidonic acid.

Group	Dose (mg/kg)	Died / Total	Mortality (%)
Saline	-	13 / 15	87.0
Aspirin	10	4 / 15	26.6**
Polydatin	5	6 / 15	40.0**
	10	3 / 15	20.0**
	20	2 / 15	13.3**

Died: the number of animals that died 15 min after injection of arachidonic acid; Total: the number of animals that were used in the study. The mortality was estimated 15 min after injection of AA (80 mg/kg) into the mouse tail vein. ** $P < 0.01$, compared with saline group.

Table 2. Effect of polydatin on electrically stimulated carotid arterial thrombosis in rats.

Group	Dose (mg/kg)	Occlusion time (min)
Saline	-	17.6 ± 1.1
Polydatin	5	29.8 ± 4.7**
	10	31.9 ± 4.7**
	20	37.6 ± 4.1**
Aspirin	10	27.1 ± 2.0**

Values are means ± SD, $n = 10$, ** $P < 0.01$, compared with saline. All the substances were administered intragastrically. 15 min after administration, the carotid artery was electrically stimulated at 1.5 mA for 7 min. The experiment was terminated 60 min after stimulation.

RESULTS

Preventive effect of polydatin against mouse sudden death caused by injection of AA

Polydatin at 5, 10 and 20 mg/kg had a potent inhibitory effect on mouse death as a result of pulmonary thrombi induced by AA injection into the tail vein (Table 1).

Effect of polydatin on electrically stimulated arterial thrombosis in rats

Polydatin at 5, 10 and 20 mg/kg significantly prolonged the occlusion time in a dose-dependent manner. The reference compound aspirin also delayed the occlusion time (Table 2).

Effect of polydatin on inferior venous thrombosis in rats

In saline group, the wet and dry thrombus weights were 10.3 ± 1.6 and 4.8 ± 0.8 mg, respectively. Polydatin at 5, 10 and 20 mg/kg evidently reduced the wet and dry thrombus weight (Table 3).

Effects of polydatin on rabbit platelet aggregation *in vitro*

Polydatin concentration-dependently suppressed platelet aggregation induced by AA and ADP, but not by PAF. The IC_{50} values were 5.13 for AA and 10.07 µmol/L for ADP, respectively (Table 4).

Effects of polydatin on rabbit platelet aggregation *ex vivo*

Polydatin at 5, 10 and 20 mg/kg also showed potent inhibitory effects on AA or ADP- induced platelet aggregation, but had no inhibitory effect on PAF- induced platelet aggregation (Table 5).

Effect of polydatin on platelet cytosolic calcium level

In the presence of 1 mmol/L $CaCl_2$, the resting cytosolic

Table 3. Effect of polydatin on inferior venous thrombosis in rats.

Group	Dose (mg/kg)	Thrombus weight (mg)	
		Wet	Dry
Saline	-	10.3 ± 1.6	4.8 ± 0.8
Polydatin	5	5.4 ± 1.0**	1.8 ± 0.7**
	10	3.6 ± 1.1**	1.1 ± 0.4**
	20	1.6 ± 0.9**	0.7 ± 0.2**
Aspirin	10	2.3 ± 0.5**	1.3 ± 0.5**

Values are means ± SD, n = 10, ** P < 0.01, compared with saline. The inferior vena cava was isolated and ligated below the left renal vein level. 1 h after ligation, the substances were administered through the femoral vein in a volume of 0.1 mL/100 g body weight, respectively. 2 h later, the thrombus in the inferior vena cava was collected into a glass dish for measurement of wet weight. It was then placed in a drying oven at 60℃ for 20 h before measuring the dry weight.

Table 4. Effects of polydatin on rabbit platelet aggregation induced by AA, ADP and PAF *in vitro.*

Group	Dose (μmol/L)	Inhibition of platelet aggregation (%)		
		AA	ADP	PAF
Polydatin	4.7	49.8 ± 5.9**	36.5 ± 3.6**	1.3 ± 1.0
	9.4	63.8 ± 8.9**	42.8 ± 4.8**	1.5 ± 0.9
	18.8	73.2 ± 6.9**	59.8 ± 6.7**	1.5 ± 1.0
	37.5	90.3 ± 2.0**	79.8 ± 5.6**	2.0 ± 0.9
	75	93.7 ± 2.9**	87.0 ± 4.1**	2.3 ± 1.2
	150	96.3 ± 1.6**	85.3 ± 5.2**	1.8 ± 1.5
Aspirin	37.5	55.5 ± 2.9**	1.4 ± 0.9	1.6 ± 1.1
	75	70.3 ± 4.0**	2.1 ± 1.2	1.9 ± 1.5
	150	96.5 ± 3.9**	1.8 ± 0.7	2.2 ± 1.0

The maximal aggregation was recorded (final concentrations of AA 0.35 mmol/L, ADP 3 μmol/L and PAF 7.2 nmol/L). Percentage inhibition by drugs was calculated by use of the following equation: Inhibition of aggregation = (A - B) / A ×100. (A is the platelet aggregation rate of drugs and B is the platelet aggregation rate of NS control. Values are means ± SD, n = 6, ** P < 0.01, compared with saline (platelet aggregation rate of saline: AA 78.0 ± 4.7, ADP 67.5 ± 3.1 and PAF 59.0 ± 2.8). AA, Arachidonic acid; ADP, adenosine diphosphate; PAF, platelet-activating factor.

free calcium ($[Ca^{2+}]_i$) was (95 ± 13) nmol/L; the $[Ca^{2+}]_i$ activated by AA was (313 ± 26) nmol/L. Pretreated with polydatin at 75, 150 and 300 μmol/L, the resting $[Ca^{2+}]_i$ were (93 ± 19), (91 ± 18) and (88 ± 15) nmol/L, respectively. In the presence of EGTA, the resting $[Ca^{2+}]_i$ was (73 ± 14) nmol/L, and AA-stimulated $[Ca^{2+}]_i$ increase was (121 ± 12) nmol/L. AA-elevated $[Ca^{2+}]_i$ either in the presence of $CaCl_2$ or EGTA was markedly suppressed by polydatin. Verapamil significantly lowered $[Ca^{2+}]_i$ elevation, but it had no influence on the resting $[Ca^{2+}]_i$ increase (Table 6).

Effect of polydatin on rabbit plasma levels of TXB_2 and 6-keto-$PGF_{1\alpha}$

Polydatin at 5, 10 and 20 mg/kg significantly decreased plasma TXB_2 level and increased plasma 6-keto-prostaglandin $F_{1\alpha}$ level. Aspirin strongly decreased the levels of both TXB_2 and plasma 6-keto-prostaglandin $F_{1\alpha}$ in plasma (Tables 7 and 8).

Effect of polydatin on the binding of thrombin-activated platelets to neutrophils

The percentage of rosettes in saline group was 69.7 or 13.3% in a condition of external 1 mmol / L $CaCl_2$ or 5 mmol/L egtazic acid. Polydatin and aspirin significantly decreased the binding of platelets to neutrophils with 1 mmol/L external Ca^{2+}. The IC_{50} values were 51.9 and 61.8 μmol/L, respectively (Table 9).

Effect of polydatin on platelet aggregation induced by the suspension of activated neutrophils

Polydatin markedly inhibited platelet aggregation induced

Table 5. Effects of polydatin on rabbit platelet aggregation induced by AA, ADP and PAF *ex vivo*.

Group	Dose (mg/kg)	Inhibition of platelet aggregation (%)					
		30 min	60 min	90 min	120 min	180 min	240 min
AA	Polydatin 5	60.0 ± 2.4**	74.5 ± 4.2**	78.5 ± 7.3**	58.2 ± 4.8**	48.3 ± 6.1**	45.5 ± 4.1**
	10	67.2 ± 4.2**	89.7 ± 2.9**	88.2 ± 6.8**	65.5 ± 4.2**	58.8 ± 4.6**	50.8 ± 6.4**
	20	71.5 ± 4.6**	92.2 ± 2.6**	93.2 ± 2.4**	71.8 ± 6.9**	60.5 ± 3.7**	49.0 ± 6.4**
	Aspirin 10	54.8 ± 8.7**	72.2 ± 9.7**	84.2 ± 6.6**	90.5 ± 4.3**	72.5 ± 7.2**	68.8 ± 6.1**
ADP	Polydatin 5	51.3 ± 2.1**	67.3 ± 6.1**	64.0 ± 7.9**	51.3 ± 8.4**	40.2 ± 3.7**	37.5 ± 5.0**
	10	56.7 ± 3.3**	77.3 ± 2.6**	74.5 ± 5.2**	57.3 ± 5.9**	48.8 ± 3.2**	41.7 ± 6.8**
	20	65.3 ± 5.3**	79.5 ± 7.4**	70.3 ± 3.8**	62.5 ± 7.2**	53.7 ± 6.1**	45.0 ± 10.1**
	Aspirin 10	7.7 ± 2.2	11.3 ± 3.3	14.8 ± 3.1	16.5 ± 4.9	12.7 ± 3.3	11.5 ± 3.9
PAF	Polydatin 5	10.0 ± 2.9	8.3 ± 3.1	9.5 ± 1.9	8.5 ± 2.6	7.7 ± 2.2	6.5 ± 1.9
	10	11.8 ± 2.5	9.7 ± 2.5	9.2 ± 3.4	11.5 ± 4.0	8.2 ± 2.8	7.0 ± 1.4
	20	11.5 ± 2.7	12.2 ± 4.0	10.7 ± 3.1	9.7 ± 2.0	7.2 ± 2.6	5.5 ± 1.9
	Aspirin 10	1.8 ± 1.2	3.8 ± 1.5	4.8 ± 2.1	6.2 ± 2.8	4.5 ± 1.9	4.2 ± 2.4

Values are means ± SD, $n = 6$, $^*P < 0.05$, $^{**}P < 0.01$, compared with 0 min. Platelet aggregation rate at 0 min (%): AA 73.7 ± 5.6, ADP 64.3 ± 4.6 and PAF 58.2 ± 5.3.

Table 6. Effect of polydatin on rabbit platelet cytosolic calcium.

Group	Dose (μmol/L)	Cytosolic free calcium $[Ca^{2+}]_i$ (nmol/L)	Ca^{2+} release (nmol/L)	Ca^{2+} influx (nmol/L)
Saline	-	313 ± 26	121 ± 12	192 ± 14
Polydatin	75	247 ± 32**	101 ± 10*	136 ± 22**
	150	206 ± 19**	87 ± 14**	119 ± 5**
	300	188 ± 24**	71 ± 11**	107 ± 13**
Verapamil	80	177 ± 20**	119 ± 8	58 ± 12**

Values are means ± SD, $n = 6$, $^*P < 0.05$, $^{**}P < 0.01$, compared with saline. AA (200 μmol / L) -stimulated rise in $[Ca^{2+}]_i$ was measured separately in the presence of 1 mmol/L $CaCl_2$ or egtazic acid. The rise in $[Ca^{2+}]_i$ in the presence of $CaCl_2$ 1 mmol/L represents a combination of Ca^{2+} release and influx of Ca^{2+}. The rise in the presence of egtazic acid 1 mmol/L reflects Ca^{2+} release. The difference between these two measurements reflects the influx of external Ca^{2+}.

by the suspension of fMLP-activated neutrophils. The IC_{50} was 307.6 μmmol/L (Table 10).

DISCUSSION

Injection of AA caused platelet aggregation in lung, a lesser circulation and brought about a decline in the breath and blood circulation which eventually induce sanimal's death (Dogné et al., 2004; De Leval et al., 2004). Polydatin obviously reduced the mortality of mice in this model. It is suggested that polydatin showed potent inhibition on the activity of AA and TXA_2. Electrical stimulation thrombosis model is similar to human artery thrombosis and has little determinate error (Schumacher et al., 1996). Polydatin at 5 mg/kg could significantly prolong the occlusion time. It is suggested that polydatin showed the activity of anti-artery thrombosis.

In rat inferior vena ligation model, polydatin at 5, 10 and 20 mg/kg evidently reduced the wet and dry thrombus weight. It is suggested that polydatin prevented against the formation of venous thrombosis. In these thrombotic models, polydatin showed dose-dependently anti-thrombotic effects. To confirm its possible mechanisms, a

Table 7. Effect of Polydatin on rabbit plasma levels of TXB_2 (μg/L).

Group	Dose (mg/kg)	0 min	30 min	60 min	120 min
Saline	-	1.69 ± 0.38	1.65 ± 0.32	1.69 ± 0.35	1.61 ± 0.43
Aspirin	10	1.55 ± 0.40	1.06 ± 0.33*	0.61 ± 0.19**	0.54 ± 0.10**
Polydatin	5	1.70 ± 0.31	1.46 ± 0.22	1.10 ± 0.27*	0.93 ± 0.09**
	10	1.66 ± 0.27	1.12 ± 0.15*	0.74 ± 0.13**	0.64 ± 0.14**
	20	1.58 ± 0.30	0.93 ± 0.18**	0.58 ± 0.23**	0.41 ± 0.20**

Values are means ± SD, $n = 6$, *$P < 0.05$, **$P < 0.01$, compared with saline.

Table 8. Effect of Polydatin on rabbit plasma levels of 6-keto-$PGF_{1\alpha}$ (μg/L).

Group	Dose (mg/kg)	0 min	30 min	60 min	120 min
Saline	-	0.59 ± 0.18	0.51 ± 0.20	0.52 ± 0.15	0.57 ± 0.12
Aspirin	10	0.65 ± 0.14	0.31 ± 0.07**	0.26 ± 0.10**	0.22 ± 0.08**
Polydatin	5	0.66 ± 0.16	0.65 ± 0.24	1.07 ± 0.29*	1.62 ± 0.25**
	10	0.58 ± 0.26	2.46 ± 0.35**	3.27 ± 0.43**	3.03 ± 0.42**
	20	0.61 ± 0.19	3.17 ± 0.14**	4.05 ± 0.56**	3.62 ± 0.36**

Values are means ± SD, $n = 6$, *$P < 0.05$, **$P < 0.01$, compared with saline.

Table 9. Effect of polydatin on the binding of thrombin stimulated platelets to neutrophils in rats.

Dose (μmol/L)	Adhesion rate (%)	
	Polydatin	Aspirin
0	67.1 ± 5.8	66.1 ± 7.9
18.8	61.6 ± 4.4	59.7 ± 3.1
37.5	41.3 ± 4.1**	41.9 ± 3.8**
75	28.6 ± 5.2**	33.1 ± 3.4**
150	23.1 ± 3.4**	22.4 ± 4.6**
300	15.7 ± 3.8**	13.1 ± 2.4**

Values are means ± SD, $n = 6$, *$P < 0.05$, **$P < 0.01$, compared with saline. The percentage of saline group was 69.7 in a condition of external 1 mmol/L $CaCl_2$.

Table 10. Effect of polydatin on platelet aggregation stimulated by the suspension of fMLP–activated neutrophils in rats.

Dose (μmol/L)	Platelet aggregation rate (%)	
	Polydatin	Aspirin
0	72.3 ± 6.5	70.9 ± 8.2
37.5	60.1 ± 5.2*	69.7 ± 3.9
75	51.4 ± 3.5**	65.8 ± 7.2
150	42.2 ± 8.1**	63.0 ± 10.3
300	36.4 ± 5.8**	66.2 ± 5.4
600	29.3 ± 6.9**	64.5 ± 7.5

Values are means ± SD, $n = 10$, *$P < 0.05$, **$P < 0.01$, compared with the platelet aggregation rate before drugs were added.

series of studies were conducted. Activation and aggregation of platelets is an important reason in the process of thrombosis. Multiple pathways contribute to platelet activation and AA, ADP and PAF are considered as the main activating agents in this process (Vargaftig et al., 1980). This study confirms that polydatin significantly inhibited AA- and ADP-induced platelet aggregation *in vitro* and *ex vivo*. It is suggested that polydatin may selectively affect ADP receptors and cyclooxygenase pathway, but do not act on PAF receptors. These results indicate that the antithrombotic effects of polydatin may be related with its antiplatelet aggregation.

Activation of blood platelets (including aggregation and release) occurs always through an elevation of $[Ca^{2+}]_i$ (Nesbitt et al., 2003). Influence of polydatin on the increase of $[Ca^{2+}]_i$ stimulated by AA was measured in this investigation. The AA-induced $[Ca^{2+}]_i$ increase in the presence of $CaCl_2$ was more than that in the absence of extracellular Ca^{2+}, suggesting that the major component of the AA-induced increase in $[Ca^{2+}]_i$ is caused by the influx of Ca^{2+} ions across the plasma membrane. Polydatin concentration-dependently lowered the $[Ca^{2+}]_i$ increase stimulated by AA in the presence of $CaCl_2$ or EGTA, respectively. This demonstrated that polydatin inhibited both the influx of extracellular calcium and the mobilization of calcium from intracellular stores.

This study shows that polydatin significantly elevated plasma level of 6-keto-$PGF_{1\alpha}$ while markedly reducing that of TXB_2. It is suggested that the mechanism of polydatin on inhibiting the metabolism of AA do not be restrain of the activity of COX, but due to its modulation on PGI_2 and TXA_2. AA can cause rapid formation of TXA_2 in platelets and TXA_2 can stimulate $[Ca^{2+}]_i$ level and result in platelet aggregation. Polydatin at the dose range that inhibited platelet aggregation and TXB_2 formation, could significantly suppress $[Ca^{2+}]_i$ increase stimulated by AA. It is suggested that the antiplatelet effect of polydatin may be due to its ability to inhibit Ca^{2+} elevation. Polydatin, therefore, is different from aspirin both in potency and quality of antiplatelet and antithrombosis.

Neutrophils and platelets, but not platelets alone, are closely associated with thromboembolic disorders. Interactions of these two kinds of blood cells are involved in the process of thrombomodulation (Zhao et al., 2008). Platelet-derived products are able to promote neutrophil chemotaxsis, enzyme release and phagocytosis, and to inhibit oxidative burst. On the other hand, neutrophil-derived products can enhance platelet aggregation (Caron et al., 2002). Thus, it is more advantageous to develop an antithrombotic drug especially at an angle of influencing multiple cellular interactions. Thrombus formation is mediated by the platelet-neutrophil interactions including cell binding and platelet aggregation (Wetterö et al., 2003). Clinical studies showed that platelet-neutrophil rosette in patients with thrombotic disease significantly increased (Papalambros et al., 2004). Polydatin showed significant inhibition on rosetting between thrombin-activated platelets and neutrophils. Polydatin also exerted a concentration-dependent inhibitory effect on platelet aggregation induced by the supernatant from fMLP-activated neutrophils. It is suggested that polydatin showed potent antithrombotic activity also due to its influence on platelet-neutrophil interactions. Taken together, these results suggest that polydatin has the potential to become a useful antiplatelet and antithrombotic drug.

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