Arch. Pharm. Res. **14**(4), 352-356 (1991)

Antiplatelet Action of Ilexoside D, a Triterpenoid Saponin from *Ilex pubescens*

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(Received November 15, 1991)

Abstract □ The anti-platelet activity of ilexoside D isolated from the roots of *Ilex pubescens* Hook. et Arn. was investigated in *in vitro* and *ex vivo* models of platelet aggregation induced by ADP, thrombin or collagen in rats. *In vitro* ilexoside D inhibited more effectively platelet aggregation induced by ADP and thrombin than by collagen as compared with aspirin. *Ex vivo* ilexoside D also inhibited platelet aggregation induced by ADP and collagen, but not by thrombin, and the inhibitory action of ilexoside D was more effective than that of aspirin. However, *in vitro* ilexoside D inhibited very poorly the generation of malonyldialdehyde, which is known to be concomitantly released with thromboxane A_2 during platelet aggregation. These results suggest that the anti-platelet activity of ilexoside D may not be responsible for prostaglandin synthesis in platelets.

Keywords □ *Ilex pubescens*, Aquifoliaceae, ilexoside D, anti-platelet action, antithrombotic.

The root of *Ilex pubescens* Hook. et Arn. (Aquifoliaceae)(毛冬青) is widely used in China for the treatment of cerebral thrombosis, thromboangiitis obliterans, coronary disease, *etc.*[1] Recently, we isolated several anti-thrombotic saponins, named ilexosides, from the roots as active principles[2]. Among them, ilexoside D, 3-*O*-β-D-glucopyranosyl (1→2)-β-D-xylopyranosyl-20-epipomolic acid, was found to be the most active component of the saponins[2].

However, the effect of ilexoside D on platelet aggregation was not fully evaluated until now. The purpose of the present investigation was to compare the anti-platelet activity with that of aspirin in order to clarify the anti-thrombotic action mechanism of ilexoside D.

EXPERIMENTAL METHODS

Material and reagents

The roots of *Ilex pubescens* were purchased from a market of Hong Kong in 1988. Ilexoside D was isolated from the roots by the method of Han, *et al.*[2c] The sources of materials were as follows: adenosine diphosphate·2Na, acetylsalicylic acid and bovine thrombin (Sigma Chemical Co., USA), and collagen (Chrono Log Co., USA).

Animal

Male Sprague-Dawely rats weighing 250-300 g and 180-200 g were used for the experiments involving malonyldialdehyde (MDA) formation and platelet aggregation, respectively.

Preparation of platelets[3]

Whole blood samples were collected from the abdominal aorta of rats anesthetized with ether into a plastic injector containing 3.8% sodium citrate to provide a 1 in 10 dilution by blood. Platelet rich plasma (PRP) was obtained by centrifugation at 200 g for 10 min at 10℃. Remaining red cell precipitate of the blood samples was further centrifuged at 1300 g and 10℃ for 30 min to give platelet poor plasma (PPP). To obtain washed platelet suspension, PRP was recentrifuged at 600 g and 10℃ for 20 min, and platelets were suspended in 0.01 M phosphate buffe-

Table I. Effects of ilexoside D and aspirin on rat platelet aggregation induced by ADP, collagen and thrombin *in vitro*

Inducer	Concentration (mM)		Aggregation[c] (%)	Inhibition (%)	IC_{33} (mM)
ADP[a]	Vehicle		46.8± 3.0	–	–
	Ilexoside D	0.5	40.2± 2.2	14.1	1.23
		1.0	34.4± 3.1	26.5	
		1.5	28.3± 6.3	39.5	
		2.0	17.2± 3.4	63.2	
	Aspirin	0.5	40.8± 2.0	12.5	1.53
		1.0	35.2± 4.5	24.8	
		1.5	31.8± 1.3	32.1	
		2.0	25.8± 3.6	44.9	
Collagen[a]	Vehicle		71.3± 5.0	–	–
	Ilexoside D	0.5	64.4± 1.9	9.7	2.21
		1.0	59.8± 3.5	16.1	
		1.5	56.0± 0.3	21.4	
		2.0	49.4± 8.1	30.7	
	Aspirin	0.5	53.8± 11.3	24.5	0.98
		1.0	47.3± 6.0	33.7	
		2.0	28.5± 14.7	60.0	
Thombin[b]	Vehicle		87.9± 1.8	–	
	Ilexoside D	0.5	88.0± 3.4	0	1.25
		1.0	79.0± 2.5	10.1	
		1.5	35.1± 1.6	60.1	
		1.6	20.8± 1.4	76.3	
		2.0	0	100	
	Aspirin	1.0	79.1± 7.5	10.0	2.60
		2.0	65.9± 10.1	25.0	

[a&b]Platelet aggregation induced by the agents was measured with PRP[a] and washed platelet[b], respectively.
[c]Each value represents the mean± S.D. of three experiments.

red saline (pH 7.4, PBS). The platelet numbers of PRP and washed platelet suspension were adjusted to 1×10^9 cells per m*l* (TOA Automatic Platelet Counter, type PL-100), with PPP and PBS, respectively.

In vitro platelet aggregation test

The platelet aggregation test described by Gerrard[3] was performed with ADP (10 μM), collagen (50 μg/m*l*) and thrombin (5 NIH units/m*l*) as aggregating agents. A 220 μ*l* aliquot of PRP was placed in an aggregation cuvette and the content was stirred at 1200 rpm for 3 min at 37℃, then a 5 μ*l* of an ilexoside D solution (dissolved in dimethylsulfoxide) was added. After 3 min, 25 μ*l* of an aggregating agent was added to the reaction mixture. Changes in the light transmittance of the reaction mixture were continuously recorded for 5 min with a Chrono Log platelet aggregometer (USA). Platelet aggregation was expressed as the percent increase in the transmittance taking the transmittance of a control mixture containing no test solution as zero. An anti-platelet aggregating agent, aspirin, was used as a standard drug.

Ex vivo platelet aggregation test

An ilexoside D suspension (0.5-1 m*l*) with 0.5% CMC was intraperitoneally or orally administered

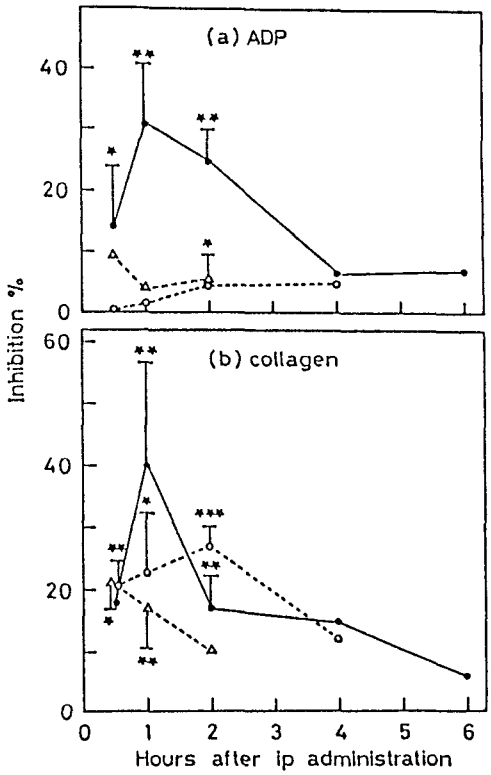

Fig. 1. Time-response curves for the anti-platelet activities of ilexoside D and aspirin *ex vivo*.
A suspension of each drug with 0.5% CMC was intraperitoneally administered to each group of six rats. After a single administration, platelet rich plasma was prepared to test platelet aggregation induced by ADP (a) and collagen (b). Dose: ilexoside D, 0.2 (—●—); aspirin, 0.1 (···△···), 0.5 (···○···) m mole/kg. *p<0.01, **p< 0.005, ***p<0.001.

to rats. After 0.5, 1, 2, 4 and 6 hr, whole blood samples were taken from the treated rats, then PRP and PPP were prepared and platelet aggregation responses were recorded as described above.

MDA formation

MDA formation during thrombin-induced platelet aggregation was tested according to the method of Han *et al.*[2a]

RESULTS

Effect on platelet aggregation in vitro

The effect of ilexoside D was investigated on rat platelet aggregation induced by ADP, collagen and thrombin *in vitro*. PRP was used for ADP-or collagen-induced aggregation, and the wash platelet suspension took the place of PRP for thrombin-induced aggregation. Table I shows the comparison of aggregation of the ilexoside D-treated platelet with that of the aspirin-treated platelet. Ilexoside D inhibited more effectively the aggregation induced by ADP and thrombin than aspirin. The collagen-induced aggregation was less effectively prevented by ilexoside D than by aspirin.

Effect on platelet aggregation ex vivo

For *ex vivo* studies, ilexoside D was intraperitoneally administered to rats at the doses of 0.1 to 0.25 m mole/kg. One hr later blood samples were taken, and then PRP or washed platelet suspension was prepared to test platelet aggregation induced by ADP, collagen and thrombin. As shown in Table II, ilexoside D showed more stronger inhibition on the platelet aggregation induced by ADP and collagen than aspirin. The anti-platelet activity of ilexoside D was exhibited at a relatively high dose [IC_{33}, about 0.19 m mole (=145 mg)/kg *i.p.*]. On inducing by thrombin, both the compounds were not effective (data not shown).

In order to examine duration of the action of ilexoside D, platelet aggregation induced by ADP and collagen was tested with PRP's from the rats 0.5, 1, 2, 4 and 6 hr after a single *i.p.* administration at the dose of 0.2 m mole/kg. As shown in Fig. 1, the anti-aggregatory action of ilexoside D reached a peak at 1 hr after injection and then gradually decreased to 6 hr. On inducing by ADP, ilexoside D showed more stronger anti-aggregatory action than aspirin (Fig. 1a).

In an *ex vivo* study after oral administration, ilexoside D and aspirin did not inhibit platelet aggregation induced by the three agregating agents under the doses described in Table II (data not shown).

Effect on malonyldialdehyde formation by platelets

The effect of ilexoside D was studied on MDA produced from washed rat platelets in response to thrombin. As shown in Table III, ilexoside D (IC_{50}, 193 μM) was for less potent than aspirin (IC_{50}, 3.3 μM), indicating that ilexoside D inhibited very weakly prostaglandin synthesis.

Table II. Effects of ilexoside D and aspirin on rat platelet aggregation induced by ADP and collagen *ex vivo*

Inducer	Dose (m mole/kg)		No. of rats	Aggregation[a] (%)	Inhibition(%)	ED_{33}
ADP	Control		5	56.9± 2.9	−	
	Ilexoside D	0.10	10	50.2± 4.4*	11.8	0.19
		1.15	4	46.5± 6.1*	18.3	
		0.20	9	39.3± 5.9**	30.9	
		0.25	4	23.8± 3.4***	58.2	
	Aspirin	0.10	6	54.8± 3.5	3.7	>5.0
		0.25	5	56.1± 6.6	1.4	
		0.50	5	56.0± 2.7	1.6	
Collagen	Control		5	69.7± 1.3	−	
	Ilexoside D	0.10	10	69.0± 3.0	1.0	0.19
		0.15	4	60.9± 9.2	12.6	
		0.20	9	41.9± 12.8**	39.9	
		0.25	4	9.4± 1.9***	86.5	
	Aspirin	0.10	6	58.0± 4.7**	16.8	>5.0
		0.25	5	60.3± 4.3*	13.5	
		0.50	5	53.8± 6.9	22.8	

[a]Platelet aggregation was measured with PRP prepared from rat blood samples one hour after ip administration of the drugs. Each value represents the mean± S.D.
Significationtly different from the control, *$p<0.01$, **$p<0.005$, ***$p<0.0005$.

Table III. IC_{50} values of ilexoside D and aspirin on malonyldialdehyde generation during thrombin-induced platelet aggregation of rats

Compounds	IC_{50}	
	μg/1.15 m*l*	μM
Ilexoside D	170	193
Aspirin	0.72	3.3

*One m*l* of washed rat platelets (1×10^9 cells/m*l*) was preincubated with 0.1 m*l* of inhibitor solution in 0.01 M phosphate buffered saline (pH 7.4) at 37℃ for 30 min and then 50 μ*l* (10 U) of bovine thrombin was added to aggregate platelets. The reaction mixture was further incubated at 37℃ for 30 min. Amounts of MDA released were determined by 2-thiobarbituric acid method[4].

DISCUSSION

In order to isolate anti-thrombotic principles from medicinal plants, we have utilized both *in vivo* and *in vitro* screening methods as follows: the *in vivo* method was the measurement of bleeding and clotting times after oral administration of methanol extracts of medicinal plants for screening anti-thrombotic activity, and the *in vitro* method was the measurement of prostaglandin synthesis (MDA formation) in platelets for isolating active principles from the extracts showing prolongation of bleeding time by the *in vivo* method[2a].

The methanol extract of *I. pubescens* roots prolonged bleeding time, but not plasma recalcified clotting time by the former method, and inhibited MDA formation during thrombin-induced platelet aggregation by the latter method[2a]. Through the activity-guided purification, several anti-thrombotic saponins named ilexoside were isolated and characterized[2c]. Ilexoside D was found to be the most active component of the saponins, being evaluated by the inhibitory activity on MDA formation[2a]. Thus, the anti-thrombotic activity of ilexoside D may be due to the inhibition of platelet aggregation.

The present paper is concerned with the anti-platelet activity of ilexoside D. *In vitro*, ilexoside D inhibited more effectively platelet aggregation induced by ADP and thrombin, and less effectively platelet aggregation induced by collagen than aspirin (Table I). However, both the compounds showed

the reletively high IC_{33} values, and did not affect platelet aggregation induced by the three agents when tested at concentrations below 0.5 mM.

Ex vivo after intraperitoneal administration to rats, ilexoside D strongly inhibited platelet aggregation induced by ADP and collagen, but aspirin showed a little effect. However, the anti-platelet activity of ilexoside D was shown at a relatively high dose, IC_{33}=0.19 m mole (145 mg)/kg, *i.p.* (Table II). On inducing by thrombin, ilexoside D and aspirin did not affect platelet aggregation (data not shown). Furthermore, an *ex vivo* study after oral administration to rats at various doses showed that both the compounds did not inhibit platelet aggregation induced by the three agents (data not shown).

The measurement of malonyldialdehyde (MDA) which is released during platelet aggregation induced by thrombin can be used as an indicator of prostaglandin synthesis in platelets, and be a useful test in the study of platelet function[4]. MDA generation from platelets is known to be prevented by some acidic anti-inflammatory drugs such as aspirin and indomethacin, and by some basic thromboxane A synthese inhibitors[5]. The effect of ilexoside D was studied on MDA generation from rat platelets in response to thrombin. The IC_{50} value of ilexoside D (193 μM) was more high than that of aspirin (3.3 μM (Table III), suggesting that the anti-platelet activity of ilexoside D may not be responsible for prostaglandin synthesis in platelets.

LITERATURE CITED

1. "*Directory of Chinese Materia Medica*" (Zhong Yao Da Ci Dian or 中藥大辭典), ed. by Jiangsu New Medical College, Shanghai Scientific and Technological Publisher, Shanghai, p. 441 (1977).
2. Han, Y. N., Baik, S. K., Kim, T. H. and Han, B. H.: (a) Antithrombotic activities of saponins from *Ilex pubescens*, *Arch. Pharm. Res.* **10**, 115 (1987); (b) Triterpenoids of *Ilex pubescens*, *ibid*, **10**, 121 (1987); (c) New triterpenoid saponins from *Ilex pubescens*, *ibid.*, **10**, 132 (1987).
3. Gerrard, J. M.: Platelet aggregation and the influence of prostaglandins, *Methods in Enzymology*, **86**, 642 (1982).
4. Smith, J. B., Ingerman, C. M. and Silver, M. J.: Malondialdehyde formation as an indicator of prostaglandin production of human platelets, *J. Lab. Clin. Med.*, **88**, 167 (1976).
5. Flower, R. J. and Vane, J. R.: Inhibition of prostaglandin biosynthesis, *Biochem. Pharmacol.* **23**, 1439 (1974).