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Antithrombotic Activities of Saponins from *Ilex pubescens*

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Abstract □ Methanol extract of *Ilex pubescens* roots prolonged bleeding time threefold, and inhibitied the generation of malondialdehyde released during platelet aggregation induced by thrombin. Through several purification procedures, its saponin, named ilexoside, was proved to be responsible for the antithrombotic activities of the plant. Ilexosides A, -D and -J, and 24-carboxypomolic acid showed strong inhibitory activities on platelet aggregation induced by thrombin.

Keywords □ *Ilex pubescens*, Aquifoliaceae, saponin, triterpene, antithrombotic activity, ilexoside, ziyu-glycoside, 2C-epipomolic acid, 24-carboxypomolic acid, pomolic acid, ilexolic acid, ginsenoside

The measurement of malondialdehyde (MDA) in platelets can be as an indicator for the generation of thromboxane A_2 (TXA_2), which is known to stimulate platelet aggregation, and it is simpler than the measurement of TXA_2.[1,2] MDA is concomitantly released with 12-L-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT) and TXA_2[1]. MDA generation from platelets is known to be prevented by the inhibitors of cyclooxygenase and thromboxane synthase such as non-steroidal antiinflammatory agents and imidazole derivatives.[3]

We have screened the antithrombotic activities of several crude drugs, utilizing the inhibition of MDA generation in rat platelets together with the measurement of bleeding and clotting times.[4-6] Among them, the root of *Ilex pubescens* Hook. et Arn. (Aquifoliaceae) was shown to prolong the bleeding time three times as compared to control group. And also a buthanol extract of the root strongly inhibited the MDA generation, which was induced by thrombin. Saponin components were found to be the active principles. Seven saponins, named ilexosides A, -D, -E, -H, -J, -K and -O, were purely isolated from the butanol extract of the plant. In China, the root of *Ilex pubescens* (毛冬青) is widely used for the treatment of cerebral thrombosis, thromboangiitis obliterans, cardiovascular diseases, *etc.*[7] Chinese workers reported the active principles to be 3,4-dihydroxyacetophenone, flavonoid glycosides[7,8] and ilexolide A (3-O-β-D-xylofuranosyl ilexolic acid).[9]

The present paper is also concerned with the antithrombotic activities of ilexosides and some structure-related compounds in addition to several ginsenosides.

EXPERIMENTAL METHODS

Material and reagents

The roots of *Ilex pubescens* were purchased from a market of Hong Kong in 1983. Bovine thrombin and Sephadex LH-20 were purchased from Sigma Co.

Preparation of test samples

Dried roots (1 kg) were crushed and extracted with 70% methanol (5*l* × 4) and the MeOH extracts were concentrated to dryness (260g). Twenty six g of the extract was suspended in 1% CMC solution to make 100 m*l*, of which concentration corresponded to 1g crude drug per m*l*.

To test the inhibition of MDA generation, each fraction or purely isolated sample was dissolved or suspended in 0.01 M phosphate buffered solution (PBS, pH 7.4).

Bleeding and clotting times

The experiments were performed with male Sprague-Dawley rats (body weight 180-200g), which were individually housed and had free access to food and water. Ten rats were orally administered 5 m*l* of the suspension of the MeOH extract per kg body weight, once a day for 10 days, together with

vitamin K (3 mg/kg). Ten rats of the control group were given a daily supplement of vitamin K (3 mg/kg).

For bleeding time measurement, rats were anaesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally). The tail was transsected at 5mm from the tip, and the distal 5cm of the tail was immersed vertically in saline (0.9%) at 37.5 °C. The period between transsection and the moment bleeding stopped was taken as the bleeding time.[10]

One day after bleeding time measurement, the ether-anesthetized rats were bled by pucturing the heart. Blood (0.9 volume) was collected in a plastic tube containing 3.13% sodium citrate solution (0.1 volume). After centrifugation, the citrated plasma was used for plasma recalcification time measurement.[4]

MDA generation during thrombin-induced aggregation of rats platelets[2]

Male Sprague-Dawley rats (250-300 g) were anaesthetized with ether and blood was collected by heart puncture into a plastic injector containing 3.13% sodium citrate to provide a 1 in 10 dilution by blood. Platelet rich plasma (PRP) was obtained by centrifugation at 140*g* and 10 °C for 10 min. PRP was recentrifuged at 600*g* and 10 °C for 20 min. Platelets were suspended in PBS (pH 7.4). Platelets prepared from nine rats were pooled and the platelet number was adjusted to 1×10^9 cells per m*l* (TOA Automatic Platelet Counter type PL-100).

One m*l* of the suspended platelet was warmed to 37 °C in a small polypropylene tube and 100 m*l* of inhibitor suspension was added. The mixture was preincubated at 37 °C for 30 min and then 50 m*l* (10 U) of bovine thrombin was added. After further incubation at 37 °C for 30 min. one m*l* of 0.3% thiobarbituric acid plus 0.4% sodium dodecyl sulfate in 7.5% acetic acid buffer (pH 4.0) was added to the reaction mixture, and the tube was heated in a boling water bath for 1 hr. After cooling to room temperature, extraction with butanol (2m*l*) was carried out. Optical density of butanol layer was determined at 534 nm in a UV/visible spectrometer (Gilford, 2600).

RESULTS AND DISCUSSION

Antithrombotic activity of Ilex pubescens

Effects of the methanol extract of *Ilex pubescens* on bleeding and clotting times were investigated. Since the plant is known to contain some coumarins[11], vitamin K was administered to rats together with the sample in order to protect the vitamin K-dependent plasma coagulation factors from their damages induced by coumarin.[12] As shown in Table I, bleeding time increased by an average of 288% and clotting time did not change, suggesting that the prolongation of bleeding time was responsible for the inhibition of rats platelets aggregation

Malondialdehyde (MDA) production is known to be a good measure of the production of thromboxane A_2, thrombogenic agent in platelet aggregation.[2,10] MDA generation during thrombin-induced aggregation of rats platelets was strongly inhibited by the MeOH extract, of which IC_{50} was calculated as 2.54 mg/10^9 platelets cells (Table II). The data including the bleeding time increase exhibited the roots of *Ilex pubescens* to possess strong antithrombotic activity.

For the purpose of identifying the active principles, the MeOH extract of *Ilex pubescens* was first partitioned with BuOH and H_2O. The inhibitory activity against MDA generation from rats platelets was completely extracted into BuOH (Table II). IC_{50} of the BuOH extract was determined as 1.33 mg/10^9 cells. The BuOH extract was chromatographed on a column of Sephadex LH-20 in order to separate flavonoid glycosides from other constituents, since in China a flavonoid glycosides preparation has been used clinically for the treatment of cardiovascular disease.[7] The eluates were divided

Table I. Effects of methanol extract of *Ilex pubescens* on bleeding and clotting times in rats

Bleeding T.(Sec.)		Clotting T. (Sec.)	
Control*	Sample**	Control*	Sample**
24	120	146	112
20	85	200	150
20	166	364	248
90	210	390	255
85	117	235	162
28	159	300	262
20	159	210	206
60	23	230	264
29	89	162	390
50	155	208	208
42.6 ± 25.8	122.5 ± 49.9	244.5 ± 77.4	225.7 ± 73.9

*Orally administered vitamin K 3 mg/kg, once a day for 10 days.

**Orally administered methanol-extract of 5g crude drug/kg plus vitamin K 3 mg/kg, once a day for 10 days.

Table II. Inhibition of malondialdehyde (MDA) generation by fractions obtained by solvent fractionation of methanol extracts of *Ilex pubescens*

Fractions	Concentration in incubation mixture* (mg/1.15 m*l*)	Inhibition** (%)
Methanol	60	91.5
Butanol	20.5	97.8
Water	30	0

*One m*l* washed rat platelets(1×10^9 cells/m*l*) was preincubated with 0.1 m*l* of inhibitor solution in 0.01M phosphate buffered saline(PH 7.4, PBS) at 37°C for 30 min and then 50μl(10 units) of bovine thrombin was added to aggregate platelets.

The reaction mixtures were further incubated at 37°C for 30 min.

**Amounts of MDA were determined by TBA method. Each was dupulicate data.

into 12 fractions, and the inhibitory activity of each fraction was assayed (Table III). Flavonoid-like substances could be detected in the fractions L6 to L9, but the inhibitory activities were found in the fractions L2, L3 and L5, which showed the presence of saponins (Table III and Fig. 1).

The BuOH extract was again chromatographed over a silica gel column to be divided into six fractions (Table IV). The inhibitory activities were found in fractions S2 to S5, in which saponins were detected as shown in Fig. 2. Further chromatography over silica gel of fractions L2, L3 and L5 showed the similar results to those described above (data not shown).

The observations suggested saponin constituents of *Ilex pubescens* to be the active principles.

There are at least 23 kinds of saponins in the

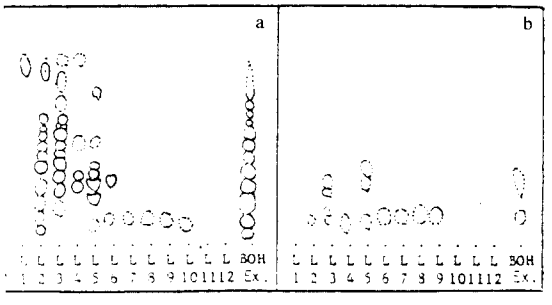


Fig. 1. Thin layer chromatogram of fractions obtained during Sephadex LH-20 column chromatography of butanol extract of *Ilex pubescens*.
Solvent: $CHCl_3/MeOH/H_2O$ (70:30:4)
Detection: a. 10% H_2SO_4, b. $FeCl_3$

Table III. Inhibition of MDA generation by fractions obtained during Sephadex LH-20 column chromatography of butanol extract of *Ilex pubescens*

Fraction	Tube No. (21 m*l*/tube)	Dried Weight* (g)	Inhibition of MDA generation** (%)
Void Vol.	260m*l*	0	0
L-1	1- 6	0.20	0
L-2	7-16	1.91	89.2
L-3	17-22	1.45	84.5
L-4	23-26	0.31	0
L-5	27-36	0.31	83.6
L-6	37-43	0.18	0
L-7	44-45	0.06	0
L-8	46-51	0.06	0
L-9	52-57	0.04	0
L-10	58-62	0.01	0
L-11	63-66	0.02	0
L-12	67-70	0.003	0

*Butanol extract (4.1g) was chromatographed by Sephadex LH-20 column (size, 3.8 × 66cm) eluting with methanol.

**Each fraction was dissolved in 10m*l* of methanol and 0.5m*l* of the solution was taken and fred from solvent. The residue was dissolved in 1m*l* of 0.01M PBS (pH 7.4) and an aliquot (0.1m*l*) was incubated with 1m*l* of rats platelets.

Table IV. Inhibition of MDA generation by fractions obtained during silica gel column chromatography of butanol extract of *Ilex pubescens*

Fraction	Dried weight* (g)	Inhibition of MDA generation** (%)
S-1	1.64	0
S-2	3.54	70.44
S-3	2.29	19.50
S-4	3.68	63.52
S-5	0.83	23.27
S-6	0.30	0

*Butanol extract (12.5g) was chromatographed by silica gel column eluting with chloroform/methanol/water.

**Each fraction was dissolved in 30m*l* of methanol and 0.3m*l* of the solution was taken and fred from solvent. The residue was dissolved in 1m*l* of 0.01M PBS (pH 7.4) and then 0.1m*l* of the sample solution was incubated with 1m*l* of rats platelets.

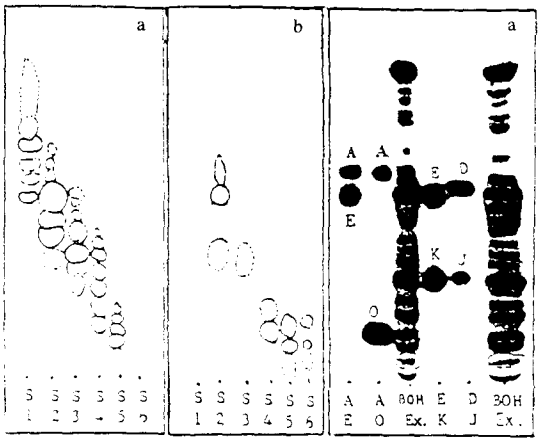


Fig. 2. Thin layer chromatogram of fractions obtained during silica gel column chromatography of butanol extract of *Ilex pubescens*.
Solvent: $CHCl_3/MeOH/H_2O$ (60:40:4)
Detection: a. 10% H_2SO_4, b. $FeCl_3$
A,D,E,J,K, and O: ilexoside A,-D,-E,-J,-K and -O

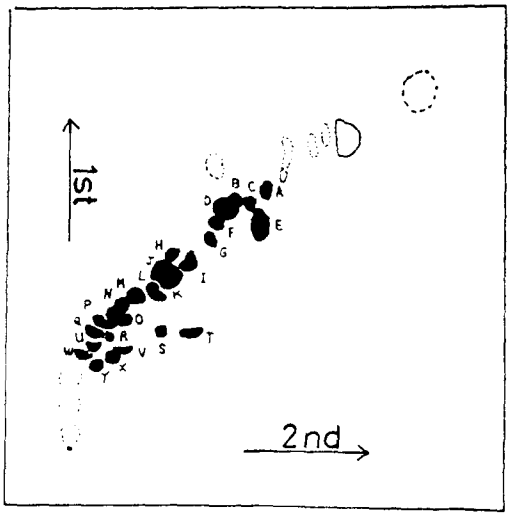


Fig. 3. Thin layer chromatogram of methanol extract of *Ilex pubescens* on a silica gel plate.
Solvent; first, $CHCl_3/MeOH/H_2O$ (60:40:10); Second, EtOAc/MeOH/H_2O (50:10:4)
Detection: 10% H_2SO_4

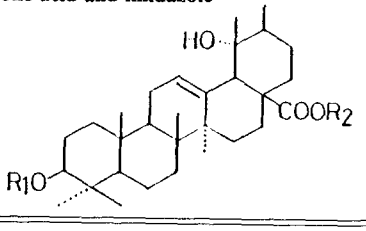
roots of *Ilex pubescens* (Fig. 3). Among them, we purely isolated seven saponins, named ilexosides A, -D, -E, -H, -J, -K and -O. Determination of their chemical structures was reported in separate articles.[13,14] To put them briefly, the aglycone of ilexosides A, -D, -J, -K and -O is 20-epipomolic acid, named pubescenolic acid, that of ilexoside E is 24-carboxy-pomolic acid, named pubescenic acid, and that of ilexoside H is ilexolic acid. Prosapogenins A and -H were obtained from alkaline hydrolysates of ilexosides A and -H. Their structures are seen in Table V, VI and VII.

Antithrombotic activities of ilexosides and their structure- related compounds

The inhibitory activities against MDA generation during thrombin-induced platelet aggregation were assayed on ilexosides, prosapogenins, ziyuglycosides I and -II, and their genins. Ziyuglycosides I and -II are known to possess pomolic acid as their aglycones, and ziyu-glycoside II is the prosapogenin of ziyu-glycoside I.[15]

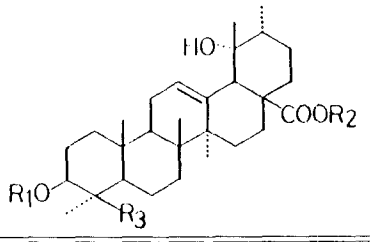
Ilexosides A, -D and -J, and ziyu-glycoside I strongly inhibited MDA generation, and their IC_{50} values were measured as 0.22, 0.17, 0.68 and 0.26, respectively, when IC_{50} of imidazole, a known thromboxane synthase inhibitor, was 0.21 mg/10^9 platelet cells/1.15 ml (Table V and VI). Other ilexosides, prosapogenins and ziyu-glycoside II did not show any activities (Table V-VII). However, ilexosides K and -O can be converted into active forms,

Table V. Inhibition of MDA generation by ilexoside A, -D, -J, -K, and -O, prosapogenin A, pubescenolic acid and imidazole



	R_1	R_2	IC_{50}
Pubescenolic acid	H	H	0
Ilexoside A	β-D-Xyl	β-D-Glu	0.22
Ilexoside D	β-D-Glu(1→2)-β-D-Xyl	H	0.17
Ilexoside J	α-L-Rha(1→2)-β-D-Glu(1→2)-β-D-Xyl	H	0.68
Ilexoside K	β-D-Glu(1→2)-β-D-Xyl	β-D-Glu	0
Ilexoside O	α-L-Rha(1→2)-β-D-Glu(1→2)-β-D-Xyl	β-D-Glu	0
Prosapogenin A	β-D-Xyl	H	0
Imidazole			0.21

Table VI. Inhibition of MDA generation by ilexoside E, ziyu-glycosides I and II, pubescenic acid and pomolic acid



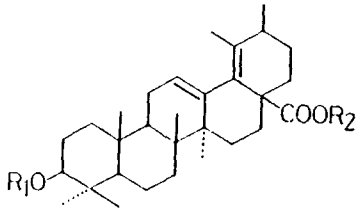
	R_1	R_2	R_3	IC_{50}
Pubescenic acid	H	H	COOH	0.41
Ilexoside E	H	β-D-Glu	COOH	0
Pomolic acid	H	H	CH_3	0
Ziyu-glycoside I	α-L-Ara	β-D-Glu	CH_3	0.26
Ziyu-glycoside II	α-L-Ara	H	CH_3	0

ilexosides D and -J by simple alkaline treatment. Among the genins, pubescenic acid only exhibited the activity (IC_{50}=0.48) (Table VI).

Recently, Matsuda H. *et al.*[16] reported that ginsenosides Rg_1 and -Rg_2 showed inhibitory activities on platelet aggregation induced by endotoxin, collagen and arachidonic acid, and ginsenoside Rb_1 was effective against endotoxin and collagen. Thus, using our experimental method, five ginsenosides were assayed. They showed relatively weak activities (Table VIII). We already reported that some diterpene acids showed the inhibitory activities against MDA generation induced by thrombin.[5]

All the saponins did not exhibit inhibitory activities on MDA generation, suggesting not due to amphipathic properties of saponins. Some structural features-activity relations are found out as follows: i) Saponin consisting of two sugars moieties at C-3 and free acid at C-28 in aglycone exhibited strong activity (ilexoside D). ii) Saponin consisting of one sugar moiety at C-3 and the other sugar moiety at C-28 in aglycone exhibited activity (ilexoside A and ziyu-glycoside I). iii) Triterpene acid having an additional carboxylic acid in A-ring together with carboxylic acid at C-28 as like pubescenic acid showed activity. iv) Triterpene acids such as pubescenolic acid, pomolic acid and ilexolic acid did not exhibit the activities, while all diterpene acids tested showed the activities. Further studies should be continued to establish complete structure-activity relations. Effects of ilexosides, ziyu-glycosides and other saponins on platelet aggregation induced by some aggregating agents are under investigation.

Table VII. Effects of ilexoside H, its prosapogenin and ilexolic acid on MDA generation during platelet aggregation induced by thrombin.



	R_1	R_2	IC_{50}
Ilexolic acid	H	H	0
Prosapogenin H	β-D-Glu(1→2)-β-D-Xyl	H	0
Ilexoside H	β-D-Glu(1→2)-β-D-Xyl	Glu	0

Table VIII. Inhibition of MDA generation by ginsenosides Rb_1, -Rb_2, -Rc, -Re and -Rg_1

	Concentration in reaction mixture (mg/1.15 ml)	Inhibition (%)
Ginsenoside Rb_1	0.5	25.6
Ginsenoside Rb_2	1.0	45.1
Ginsenoside Rc	0.5	7.3
Ginsenoside Re	1.0	14.6
Ginsenoside Rg_1	1.0	34.8

ACKNOWLEGEMENTS

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