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Anticoagulant Activity of Ilexoside D, a Triterpenoid Saponin from *Ilex pubescens*

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The anti-coagulant activity of ilexoside D isolated from the roots of *Ilex pubescens* Hook. et Arn. was investigated in *in vivo* and *in vitro* models of blood coagulation in rats. On oral administration, ilexoside D prolonged the bleeding time and the whole blood recalcified clotting time, but not the plasma recalcified clotting time. *In vitro*, ilexoside D did not affect the recalcified clotting times of whole blood, platelet-rich plasma(PRP), and platelet-poor plasma(PPP), while in the presence of tissue factor the compound prolonged the reduced prothrombin times of whole blood, PRP and PPP in the dose-dependent manner. These results indicate that ilexoside D has the anti-tissue factor activity as well as the antithrombotic activity.

Key words: Ilexoside D, *Ilex pubescens* Hook. et Arn., Anti-coagulant agent, Tissue factor inhibitor

INTRODUCTION

Ilexoside D, 3-O-β-D-glucopyranosyl (1→2)-β-D-xylopyranosyl-20-epipomolic acid was isolated from the roots of *Ilex pubescens* Hook. et Arn. (Aquifoliaceae) as an antithrombotic principle (Han *et al.*, 1987). Studies on its anti-platelet activity in rats revealed that *in vitro* it inhibited more effectively platelet aggregation by ADP and thrombin than by collagen as cpmpared with aspirin, and that *ex vivo* it also inhibited platelet aggregation induced by ADP and collagen, but not by thrombin, and the inhibitory action of ilexoside D was more stronger than that of aspirin (Lee *et al.*, 1991).

However, the effect of ilexoside D on blood plasma coagulation was not fully evaluated until now, though it was known for a methanol extract of *Ilex pubescens* to prolong bleeding time in rats (Han *et al.*, 1987a). In this report, we study the effect of ilexoside D on bleeding time, blood clotting time, and tissue factor (tissue thromboplastin or coagulation Factor III).

MATERIALS AND METHODS

Materials

The roots of *Ilex pubescens* were purchased from a market of Hong Kong in 1990. Ilexoside D was isolated from the roots by the method of Han *et al.* (1987c). All calcium chloride solutions were dissolved in 50mM Tris-HCl buffer, pH 7.5.

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Bleeding and whole blood clotting times

The experiments were performed with male Sprague-Dawley rats weighing 180-200 g, which were individually housed and had free access to food and water. Nine rats per one group were orally administered five ml of the 1% CMC suspension of ilexoside D per kg body weight, twice with a 12hr interval. Thirteen rats of control group were given 1% CMC.

For bleeding time measurement, rats were anesthetized through intraperitoneal injection of sodium pentobarbital (40 mg/kg), two hr after the second administration of the test sample. The tail was transsected at 5 mm from the tip, and the distal 5 cm of the tail was immersed vertically in saline (0.9%) at 37.5℃. The period between transsection and the moment of bleeding stopped was taken as the bleeding time (Hornstra, *et al.*, 1981).

Four hr 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). The whole blood clotting time was measured at room temperature by mixing one ml of blood with 0.2 ml of 1.7% calcium chloride solution in a glass tube.

Preparation of platelet-rich plasma(PRP) and platelet-poor plasma(PPP)

Whole blood samples were collected from the abdominal aorta of rats(200-230g) anesthetized with ether into a plastic injector containing 3.8% sodium citrate to provide a one in ten dilution by blood. Platelet-

rich plasma was obtained by centrifugation at 200g for 30 min at 10℃ to give platelet-poor plasma(Gerrard, 1982).

Recalcified clotting times of PRP and PPP

PRP or PPP (0.1 ml) and 0.15 M saline (0.1 ml) were mixed and warmed to 37℃. One-tenth ml of 25 mM $CaCl_2$ was then added and the time required for a clot to form was determined (Williams, 1964).

Prothrombin time

A rat lung microsomal fraction was used as tissue factor source (Williams, 1964, 1966). One gram of rat lung was ground for 1 min in a stainless-blade grinder. The ground lung was mixed with 9ml of saline and was then disintegrated in a teflon homogenizer for 1 min. The resultant mixture was allowed to centrifuge at 2000 rpm for 20 min. The sediment was discarded, and the supernatant was subjected to centrifuge at 105,000g for 1 hr. The microsomal fraction was suspended into 10ml of saline to make a tissue factor preparation.

For determining prothrombin time, 0.1 ml of tissue factor solution and 0.1 ml of 25 mM $CaCl_2$ were mixed and warmed to 37℃. One-tenth ml of PPP or PRP was then added and the time required for a clot to form was determined. The undiluted tissue factor preparation clotted normal rat plasma in 18 sec; when diluted 100-fold, the preparation gave about 50% acceleration of normal plasma clotting time.

Inhibition of coagulant activity by ilexoside D

Ilexoside D (2.3 mg) was dissolved in 0.03 ml of dimethylsulfoxide. The mixture was diluted with 0.97 ml of 50 mM Tris-HCl buffer (pH 7.5) to make 3 mM ilexoside D solution. An aliquot of 3 mM ilexoside D and an aliquot of tissue factor (or saline) were mixed, diluted with saline to make the 0.1 ml of total volume, and then one-tenth ml of 25 mM $CaCl_2$ was added. The mixture was warmed to 37℃, and then 0.1 ml of PPP or PRP was added for determing the recalcified clotting time (without tissue factor) and the prothrombin time (with tissue factor).

RESULTS

Effects of oral administration of ilexoside D on bleeding and clotting times

The effect of ilexoside D was firstly investigated on bleeding time, which can be as an indicator for the formation of platelet plug *in vivo*. Ilexoside D was orally administered to rats with wide ranges of 0.3 to 30 mg/kg, twice at an interval of 12 hr, and 2 hr after the second administration bleeding time was measured under pentobarbital anesthesia. Fig. 1 shows the comparison of bleeding time of the ilexoside D-treated rats with that of the aspirin-treated rats. Aspirin prolonged about three times the bleeding time at the dose of 30 mg/kg than the control which received vehicle (1% CMC), and a dose-dependency was found within the usual doses of one to 30 mg/kg. Ilexoside D prolonged two times the bleeding time without dose-dependency within 0.5 mg to 30 mg/kg, but its dose-dependency was found below the dose of 0.5 mg/kg. When both compounds were compared at their minimum doses which gave the two-fold prolongation of the bleeding time, ilexoside D was about ten-fold stronger than aspirin.

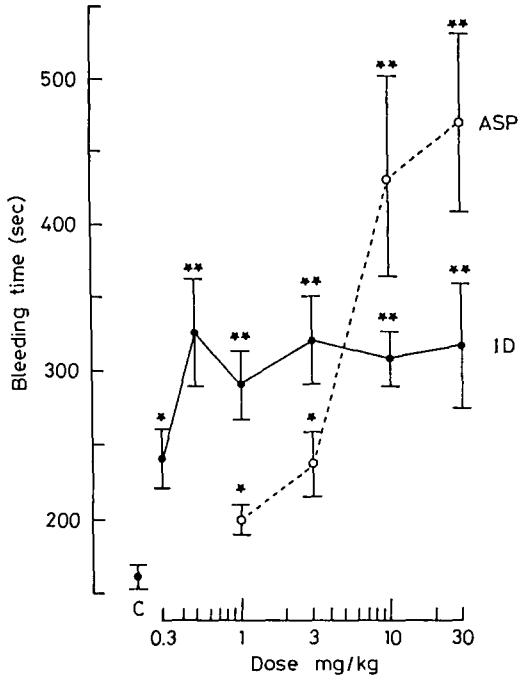

Fig. 1. The effects of oral administrations of ilexoside D and aspirin on the bleeding time in rats. The compounds were orally administered twice with a 12 hr interval, and then the bleeding time was measured two hr after the second administration. The standard deviation of the mean was calculated for all points, and the statistical significance was calculated vs control values, *$p<0.01$, **$p<0.001$. The number of rats in each group is 13 for control (c), and 9 for ilexoside D (ID) and aspirin (ASP).

Four hr after measuring the bleeding time, blood samples were taken to test whole blood and plasma clotting times. Fig. 2 shows the comparison of the whole blood clotting time of the ilexoside D-treated rats with that of the aspirin-treated rats. Ilexoside D also prolonged the whole blood clotting time within the doses of 0.3 to 10 mg/kg in the dose-dependent manner, but aspirin did not affect the time. Both the compounds tend to shortening the time at the high dose of 30 mg/kg.

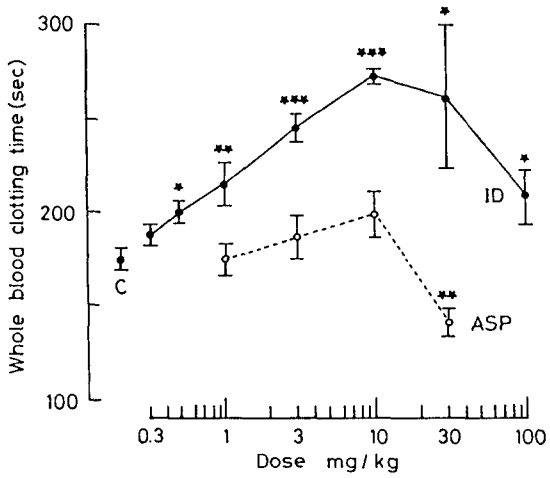

Fig. 2. The effects of oral administrations of ilexoside D and aspirin on the recalcified clotting time of rat whole blood. The time was measured four hr after the bleeding time (Fig. 1). *p<0.05, **p<0.01, ***p<0.001.

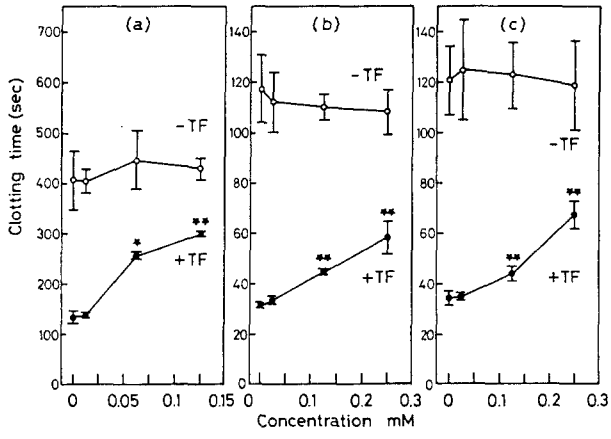

Fig. 3. The effect of ilexoside D on the prothrombin times of (a) whole blood, (b) platelet-rich plasma, and (c) platelet-poor plasma in a plastic test tube. A microsomal fraction of rat lungs was used as tissue factor (TF). The vertical bars indicate S.D. from the mean of six experiments. *p<0.005, **p<0.001.

After centrifugation of the blood samples, plasma was obtained, and the plasma recalcified clotting time was measured in a glass tube. Both the compounds did not affect the clotting time (data not shown; refer to Fig. 3c and 4c).

Effect of ilexoside D on tissue factor *in vitro*

The influence of ilexoside D on tissue factor was investigated in both plastic and glass test tubes as shown in Fig. 3 and Fig. 4, respectively. A rat lung microsomal fraction was used as tissue factor source, and its coagulant activity was assessed by measuring the prothrombin time of PPP.

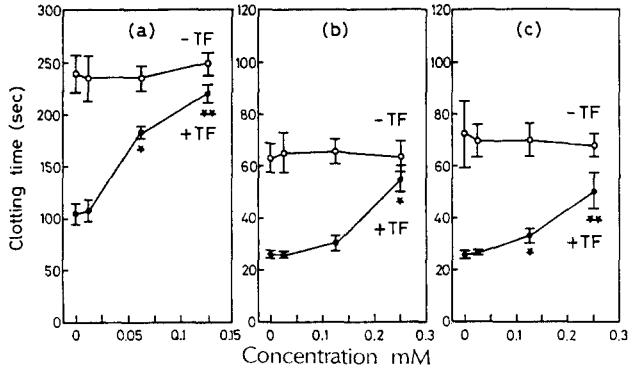

Fig. 4. The effect of ilexoside D on the prothrombin times of (a) whole blood, (b) platelet-rich plasma, and (c) platelet-poor plasma in a glass test tube. The vertical bars indicate S.D. from the mean of six experiments. *p<0.005, **p<0.001.

In plastic test tube, ilexoside D without tissue factor did not affect the recalcified clotting times of whole blood, PRP and PPP under the final concentrations below 0.125 mM (Fig. 3a) or 0.25 mM ilexoside D (Fig. 3b and 3c). However, ilexoside D with tissue factor inhibited the reduced prothrombin times of all the three blood samples. The inhibition of tissue factor activity by ilexoside D was dose-dependent.

In glass test tube, the effects of ilexoside D on the recalcified clotting and prothrombin times were similar as in the plastic tube (Fig. 4). The anti-tissue factor activity of ilexoside D seems to be more potent in the glass tube than in the plastic tube.

DISCUSSION

On oral administration, ilexoside D prolonged the bleeding time even at the dose of 0.5 mg/kg (Fig. 1). In the previous ex *vivo* study using PRP, ilexoside D showed the anti-platelet activity at relatively high doses (ED_{33}=150 mg/kg, ip) (Lee *et al.*, 1991). In view of the dosages, there is a great difference between the effects of ilexoside D on platelet aggregation and bleeding time. This suggests that the prolongation of bleeding time by ilexoside D may not be due to its anti-platelet activity, but may be due to its inhibitory activity on tissue factor.

Tissue factor is associated with endothelial cell membranes, where it functions as a receptor on Factor VII. Tissue factor accelerates the conversion of Factor VII to its activated form (Factor Xa) (Nemerson *et al.*, 1985). The tissue factor/Factor VII(a)/phospholipid complex mediates the cleavage of Factor IX and Factor X, thus activating both the intrinsic and the extrinsic pathway (Φsterud *et al.*, 1977; Nemerson *et al.*, 1964). Therefore, tissue factor is a primary physiologic initiator of blood coagulation. The other trigger known in the blood coagulation system is Factor XII, which initiates

the intrinsic pathway through the reciprocal activation of prekallikrein and high-molecular-weight kininogen in the presence of a negatively charged surface, such as glass, kaolin, dextran sulfate, or sulfatide (Davie, 1981; Jackson *et al.*, 1980; Fujikawa *et al.*, 1981).

Thus, the influence of ilexoside D on tissue factor was investigated in the plastic and glass test tubes (Fig. 3 and 4). Ilexoside D without the addition of tissue factor did not affect the recalcified clotting times of whole blood and PRP as well as PPP, indicating that ilexoside D was not effective to platelets and plasma coagulation factors in both the test tubes. In the presence of tissue factor, ilexoside D inhibited the reduced prothrombin times of all the three blood samples in the dose-dependent manner, and moreover the inhibition curve in PRP was similar to that in PPP, suggesting that ilexoside D acted as the anti-tissue factor agent rather than the anti-platelet agent in the test tubes. On oral administration, however, ilexoside D prolonged the whole blood recalcified clotting time, but did not the plasma recalcified clotting time, indicating the ilexoside D possessed the anti-platelet activity. In the previous studies (Lee *et al.*, 1991), ilexoside D was found to inhibit platelet aggregation induced by ADP *in vitro* and *ex vivo*.

Three kinds of protein inhibitors that are specific for the tissue factor/Factor VIIa complex have been reported: 1) liproprotein-associated coagulation inhibitor (LACI) (Broze *et al.*, 1988), tissue factor inhibitor (Broze *et al.*, 1987), 2) apolipoprotein A-II (Carson, 1987), and 3) placental anticoagulant protein (PAP) (Funakoshi *et al.*, 1987). It is worthy to note that ilexoside D is reported for the first time as a tissue factor inhibitor from the plant kingdom.

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