

ANTIPLATELET ACTIVITY OF HYDROALCOHOLIC EXTRACTS OF *CITRUS SINENSIS* PEELS

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Abstract

The major flavonoids contained in sweet orange fruit peels (SOP), hesperidin and rutin, are able to inhibit platelet aggregation, a biological activity that could provide the products derived from this part of the plant with an antithrombotic potential. This work was aimed to assess the possible antiplatelet activity of SOP extracts in 50 %- and 70 % ethanol (SOPE₅₀ and SOPE₇₀, respectively). The turbidimetric method of platelet aggregation (PA) was used to evaluate the in vitro inhibition of human platelet-rich plasma (PRP) response to ADP, collagen, epinephrine and arachidonic acid (AA), as well as the ex vivo reduction of the reactivity of rat PRP to ADP with acetylsalicylic acid (ASA) as a reference drug. SOPE₅₀ significantly inhibited ADP-, collagen- and epinephrine-induced PA and was ineffective on platelet reactivity to AA. SOPE₇₀ partially inhibited ADP-induced PA and was ineffective against collagen. Platelet reactivity to ADP was significantly inhibited after a single intraperitoneal and a seven-day intragastric but not a single intragastric administration of SOPE₅₀ to rats. ASA significantly inhibited collagen- and arachidonic acid-induced PA, whereas ADP- and epinephrine-induced aggregations were unaffected in vitro. Its ex vivo antiplatelet activity was demonstrated after a seven-day period of treatment to rats. The results of this study suggest that a hydro-alcohol extract of *Citrus sinensis* L. fruit peels could be a candidate for the development of a natural antiplatelet product.

Keywords: *Citrus sinensis*, Sweet orange, medicinal plant, platelet aggregation, flavonoid, antiplatelet drug

Introduction

Citrus sinensis (L.) Osbeck (*C. sinensis*; Sweet orange) from the Rutaceae family, is among the most popular edible fruits worldwide. Moreover, it is used in traditional medicine to treat a variety of diseases [1,2]. It is known that the major flavonoids contained in sweet orange fruit peels (SOP), hesperidin and rutin [3,4], are able to inhibit platelet aggregation [5-7], a biological activity that could provide the products derived from this part of the plant with an antithrombotic potential [8].

Mixtures of ethanol and water have been shown to be effective for the extraction of flavonoids, but optimal results have been found with ethanol concentrations between 50 and 75 % [9-11]. Since SOP extracts in 50 %- and 70 % ethanol are the active ingredients for the manufacture of some tinctures and syrups in Cuban local pharmaceutical laboratories [12], this work was aimed to assess the possible antiplatelet activity of these hydro-alcohol preparations. Thus, the *in vitro* and *ex vivo* effects on platelet aggregation in human and rat plasmas, respectively, were determined for this purpose.

Methods

Plant Material

C. sinensis fruit peels were provided by the Agriculture Ministry in February 2006. A voucher sample (No. 85699) was deposited at the Herbarium of the National Botanic Garden of Havana, Cuba. Peels were collected free of microbial contamination and dried in a stove at 30 ± 2 °C (in the dark) for 5 days.

Preparation of Extracts

The extraction procedure was developed in "Saul Delgado" Pharmaceutical Laboratory. Briefly, plant material (500 g) was grounded and macerated with 50 and 70 % (v/v) ethanol (1.0 L) in closed dark bottles, at room conditions during 7 days followed by paper filtration. Rota-evaporation (at 40 °C and 27 mm Hg reduced pressure) of the homogeneous liquid obtained was performed for ethanol elimination from it in order to avoid possible bias of the results of the pharmacological evaluations. The remaining liquids were stored in closed dark bottles at 4 to 8 °C and identified as follows:

- Sweet orange peel extract in 50% ethanol: SOPE50
 - Sweet orange peel extract in 70 % ethanol: SOPE70
- The total soluble solids (TSS) content of the extracts was gravimetrically determined in dried 1 mL aliquots (N=5) by using a Sartorius MA40 balance, being 17,5 and 15,3 g/100 mL for SOPE₅₀ and SOPE₇₀, respectively.

The flavonoid content was spectrophotometrically determined [13] and expressed as hesperidin equivalents, according to a calibration curve that was plotted using standard operating. It was 38, 1 and 33, 9 mg/100 mL for SOPE₅₀ and SOPE₇₀, respectively corresponding to 0,22 % TSS in both cases.

Assessment of the *in vitro* Effects of *C. sinensis* Peel Extracts on Platelet Aggregation in Human Plasma

Preparation of Human Platelet Suspensions

Human blood samples were taken from adult subjects of both genders who were participating in a routine health study at the National Institute of Angiology and Vascular Surgery and gave their informed consent. All of them denied having consumed any drug with known antiplatelet activity for at least 2 weeks before the phlebotomy. Venous blood was obtained from an antecubital vein using a syringe with a 19 gauge needle and a tourniquet. The collected blood was anticoagulated with 3.8% (w/v) trisodium citrate (1 volume to 11 volumes of blood) and centrifuged at 150 x *g* for 10 min at room temperature. After supernatant (platelet-rich plasma; PRP) separation, the remaining blood was re-centrifuged at 1000 x *g* for 20 min to obtain platelet-poor plasma (PPP). The platelet count of PRP was adjusted to about 2.5×10^8 platelets / mL by dilution with autologous PPP. Both PRP and PPP were used within 3 h after preparation.

Platelet Aggregation (PA) in Human PRP

C. sinensis extracts were diluted with a phosphate-buffered saline (PBS) solution at pH 7.4 to assess its effect on platelet aggregation. A solution of acetylsalicylic acid (ASA) in 1% sodium bicarbonate was used as a reference drug at 1mg/mL in the incubation media in the same conditions used for the extracts. Adenosine diphosphate (ADP), collagen, epinephrine, and arachidonic acid were obtained from CPM (CPM S.A.S, Rome, Italy) and prepared according to the instructions provided. The turbidimetric method [14] was applied to measure platelet aggregation, using a CLOT 2S Aggregometer (SEAC and Radim Group, Rome, Italy). Five microliters of different dilutions of *Citrus* extract (0,1; 0,3 or 1,0 mg TSS /mL in the incubation media), PBS (control) or ASA was added to 280 μ L aliquots of PRP in Aggregometer cuvettes. Successively, 15 μ L of ADP, epinephrine, collagen, or arachidonic acid (final concentrations in the incubation media being 5 μ mol/L; 5 μ mol/L; 2 μ mol/mL; and 0.5 mmol/L, respectively) were added after 2 min of pre-incubation at 37 °C. Platelet aggregation was monitored for 5 min.

The results are expressed as percentages of aggregation as provided by the instrument. The percentage inhibition of platelet aggregation was calculated as follows: percentage inhibition $[1 - (\text{platelet aggregation of sample}/\text{platelet aggregation of control})] \times 100\%$. Five experiments in duplicate were performed.

Assessment of the ex vivo Effect of a *C. sinensis* Peel Extract on Platelet Aggregation in Rat Plasma Rat Treatment and PRP Preparation

Male Wistar rats (250–300 g) were provided by the National Center for Laboratory Animals (Havana, Cuba). The animals were housed in a controlled environment, and free access to feed and water was allowed. Ten rats were randomly included in each treatment group.

SOPE₅₀ (1 mL/100 g b.w.) (600 mg TS/ kg b.w) was administered to rats at single intraperitoneal, as well as acute and repeated (once daily x 7 days) oral doses (600 mg TS/ kg b.w).

ASA was suspended with acacia 10 mg/mL and given to rats (1 mL/100 g b.w.) in single and daily oral doses for 7 days (600 mg/kg b.w.). Extract and ASA vehicles were administered to the respective control groups. The animals were randomly assigned to the treatment groups (10/group).

The animals were anesthetized by ether inhalation two hours after single treatments and 24 hours after the last administration of the daily dosing. Blood (9 volumes) was withdrawn from the cava vein using a 20 G x 1 ½ gauge needle couple to a syringe containing 1 volume of 3.8% (w/v) trisodium citrate. Anticoagulated blood was poured into a plastic tube and centrifuged at 100 x g for 5 min at room temperature. After PRP separation, the remaining blood was re-centrifuged at 1000 x g for 20 min to obtain the PPP.

Platelet Aggregation in rat PRP

Twenty six microliters of ADP (5 µmol/L in the incubation medium) were added to 400 µL duplicate aliquots of PRP in Aggregometer cuvettes and PA was followed by five minutes. Results were obtained and expressed as explained above. In addition percentage inhibition of platelet aggregation in PRP of treated animals compared with the control groups were calculated by the following equation: percentage inhibition (%) = $(1 - T/C) \times 100$, where T represents PA after administration of drugs (extract or ASA) to the rats and C PA in the negative control groups animals.

Statistical Analysis

Experimental results are expressed as the means ± S.E.M. The data from *in vitro* experiments were statistically analyzed by ANOVA followed by the Student's *t* test. The last test was also used for the analysis of the results of *ex vivo* evaluations. The differences with *p* values of <0,05 were considered to be statistically significant.

Ethics

All procedures described were carried out using a protocol approved by the Institutional Research Ethics Committee of the National Institute of Angiology and Vascular Surgery, according to the national and international guidelines for the human use of laboratory animals.

Results

***In vitro* Effects of *C. sinensis* Peel Extracts on Platelet Aggregation in Human Plasma**

The Table 1 shows that SOPE₅₀ significantly inhibited ADP-, collagen- and epinephrine-induced platelet aggregation in a concentration-dependent manner. The IC50 values were 0,69; 0,72 and 0,32 mg/mL, respectively. However, the concentration 1,0 mg/mL was ineffective on platelet reactivity to AA (71,7 ± 4,4 vs. 65,0 ± 2,8 in control; *p* = 0.19). As expected, ASA significantly inhibited collagen- and arachidonic acid-induced platelet aggregation, whereas ADP- and epinephrine-induced aggregations were unaffected. However, the highest concentration of SOPE₇₀ (1 mg/mL) inhibited ADP-induced PA (26,7 ± 4,6 vs 49,6 ± 3,7% in control; *p* = 0,02 ; % of inhibition 47,0 ± 5,9), but it was ineffective against collagen-induced PA (50,8 ± 5,8 vs. 68,3 ± 10,1 in control; *p* = 0,13).

***Ex vivo* Effect of a *C. sinensis* Peel Extract on Platelet aggregation in Rat Plasma**

SOPE₇₀ was a weak inhibitor of PA in human PRP *in vitro*. Therefore, the *ex vivo* effect of SOPE₅₀ but not SOPE₇₀ on PA in rat plasma was assessed. Platelet reactivity to ADP was significantly inhibited after a single intraperitoneal and a seven-day intragastric but not a single intragastric administration of SOPE₅₀ to rats. ASA antiplatelet activity was demonstrated after a seven- day period of treatment (Table 2).

Discussion

Physiological agents like ADP, epinephrine, collagen and arachidonic acid stimulate circulating platelets onto a damaged blood vessel wall leading to coagulation or thrombosis. A distinctive characteristic of platelet activators, additional to the specific receptors they bind to on the cell membrane,

is the relative importance of TXA₂ formation in the final cell response. Thus, it mediates to arachidonic acid mechanism, ADP second irreversible aggregation wave and is essential for collagen effect. On the other hand, it seems to act in some extent through the liberation of ADP from platelet dense granules during the release reaction. Epinephrine is considered a weak stimulus that acts as an amplifier of the other agonists via the increase of intracellular [Ca²⁺] and reduction of cyclic AMP synthesis. In general, a COX-1 inhibitor like ASA, mainly prevents collagen- and AA-induced platelet aggregation, while ADP and epinephrine mechanisms are less affected [15].

This investigation has demonstrated an unexpected difference of antiplatelet activity between 50 % and a 70%-ethanol extracts of SOP that a qualitative chemical characterization would aid to explain.

The *in vitro* inhibition of COX-1 activity by albedo and flavedo extracts from *C sinensis* fruit peels has been reported [16]. Consequently, we hypothesized that SOPE₅₀ had an ASA-like mechanism of antiplatelet action. Nevertheless, its inhibitory profile (positive against ADP, collagen and epinephrine but negative against AA) does not support it. Additional platelet targets could be involved.

The results of *ex vivo* experiments suggest that pharmacologically active components of the extract could influence on platelet reactivity into the circulating blood. They and /or their metabolites could show a cumulative- pharmacokinetics after oral administration of the product.

We have previously demonstrated the *in vitro* antiplatelet effect of a *Citrus aurantifolia* Ch. leaves extract [17]. The information presented here suggests that a hydro-alcohol extract of *Citrus sinensis* L. fruit peels could be another candidate for the development of a natural antiplatelet product.

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Table 1. Inhibition of ADP-, epinephrine-, collagen-, and arachidonic acid-induced human platelet aggregation by SOPE₅₀. Results are expressed as the mean \pm SEM; N = 5; * Statistical difference between SOPE₅₀ 1 mg/mL and ASA by Students t test ($p < 0, 05$) N.S.= not significant difference.

SOPE ₅₀ Concentration (mg/mL)	ADP μ mol/L	Collagen	Epinephrine	Arachidonic acid
0,1	5,1 \pm 1,9	8,2 \pm 4,4	27,1 \pm 3,7	-
0,3	3,8 \pm 2,5	10,0 \pm 5,4	58,2 \pm 2,4	-
1,0	92,7 \pm 3,2 *	80,3 \pm 3,3 N.S.	89,4 \pm 2,8 *	-
ASA 1 mg/mL	8,1 \pm 4,2	90,1 \pm 2,8	26,2 \pm 5,8	87,3 \pm 3,4

Table 2. *Ex vivo* effects of SOPE₅₀ 600 mg. kg b.w. and Aspirin on ADP-induced rat platelet aggregation after acute and 7 days of intragastric administration. Results are expressed as the mean \pm SEM; N=5; Statistical difference between SOPE₅₀ and Control* and between SOPE₅₀ and ASA[‡] by Students' t test ($p < 0, 05$).

Treatment	Platelet aggregation (%)		% Inhibition
	Control	Extract	
SOPE ₅₀ 600 mg/kg i.p. (Single dose)	67,3 \pm 5,2	11,3 \pm 6,3*	80,9 \pm 9,7
SOPE ₅₀ 600 mg/kg oral (Single dose)	44,1 \pm 7,4	37,5 \pm 12,8 (N.S.)	-
SOPE ₅₀ 600 mg/kg oral (Once/day x 7-days)	54,1 \pm 7,4	22,5 \pm 10,3*	49,0 \pm 3,4 ‡
ASA 600 mg/kg oral (Single dose)	54,2 \pm 7,1	46,0 \pm 4,8 (N.S.)	-
ASA 600 mg/kg oral (Once/day x 7-days)	62,5 \pm 4,7	5,6 \pm 5,5*	87 \pm 8,1