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ANTIEDEMAGENIC ACTIVITY OF HYDROALCOHOLIC EXTRACTS OF CITRUS PLANT FRUIT PEELS AND LEAVES IN RAT SKIN

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Abstract

Citrus flavonoids have been suggested to be useful for the treatment of vascular diseases by inhibiting human platelet aggregation and decreasing capillary fragility and permeability. The present work was aimed to evaluate the effects of hydroalcoholic extracts of *Citrus aurantium* L. and *Citrus sinensis* (L.) Osbeck fruit peels, as well as *Citrus aurantifolia* Ch. leaves on vascular hyper-permeability induced by histamine and dextran in rat skin, as compared with the micronized purified flavonoid fraction (MPFF), a reference drug for the treatment of chronic venous insufficiency. The extracts (50; 100 and 200 mg/kg b.w) and MPFF (200 mg/kgb.w.) were intragastrically administered to the animals once daily for 14 days (N=10/treatment group). The antiedemagenic activity was determined by measuring the area of the blue spots due to Evans blue dye accumulation at the site of intradermic injection of histamine or dextran into the rat back skin. There was not significant difference among the treatment groups with respect to the percentage of inhibition of histamine and dextran- induced vascular hyper-permeability (p = 0, 15 and 0,17, respectively by ANOVA), demonstrating a similarity between *Citrus* preparations at the three dose levels and the reference drug, suggesting that the four examined products could be useful for developing venotonic formulations. Nevertheless, further pharmacological investigation should be done to characterize the effects of these *Citrus* plant preparations that could make them resemble MPFF and determine their therapeutic potentials.

Key words*: Citrus aurantifolia , Citrus aurantium, Citrus sinensis,* lime, sour orange, sweet orange, flavonoid, edema, chronic venous insufficiency, histamine, dextran

Introduction

Citrus flavonoids have been suggested to be useful for the treatment of vascular diseases by inhibiting human platelet aggregation and decreasing capillary fragility and permeability (1, 2), the crucial pharmacological targets for antithrombotic therapy (3) and the treatment of chronic venous insufficiency (CVI) (4,5) respectively. For instance, diosmin and rutin have a demonstrated activity as venotonic agents and are present in several pharmaceutical products like the micronized purified flavonoid fraction (MPFF) (6-8). On the other hand, it has been demonstrated the anti-inflammatory activity of Citrus flavonoids is mediated by the inhibition of the synthesis of arachidonic acid metabolites, including thromboxane A2 (2), an arachidonic acid metabolite with potent platelet activating activity (9) Consequently, our research interest has been focused on assessing the possibility that extracts from different parts of Citrus species grown in Cuba show antiplatelet and antiedemagenic abilities that could provide a scientific basis for developing natural therapeutic options for contributing to the management of vascular diseases in Cuba.

In this regard pre-clinical assays has revealed that hydro-alcohol extracts of *Citrus aurantifolia* Ch. (lime) leaves and *Citrus sinensis* (L.) Osbeck (sweet orange) fruit peels were able to inhibit human platelet aggregation *in vitro* stimulated by different agonists, showing a variable inhibitory profile but a *Citrus aurantium* L. (sour orange) fruit peels extract was ineffective, thus suggesting that this effect depends on the plant species and the alcohol concentration used for the preparation of the extracts (10-12). On the other hand the preparation of *C aurantium* fruit peels prevented the increase of vascular permeability induced by histamine and dextran in rat paw like cyproheptadine, a histamine H1 antagonist drug (12). However, it is unknown whether its effect is comparable with that of MPFF, a reference phlebotonic drug and whether *C sinensis* fruit peels and *C aurantifolia* leaves extracts also possess antiedemagenic capacities. Therefore, the present work was aimed to evaluate the effects of these Citrus plant products on vascular hyper-permeability induced by histamine and dextran in rat skin as compared with MPFF.

Materials and Methods

Histamine chlorhydrate from BIOCEN (Havana, Cuba). Grade A dextran molecular weigh 200.000-275.000 and Evans Blue dye from Sigma Chemical (St. Louis, MO, USA). MPFF from Kern Pharma, S. L. (Barcelona, Spain)

Plant Material

C. aurantifolia leaves, C. sinensis and C aurantium fruit peels were provided by the Agriculture Ministry. Voucher samples were deposited at the Herbarium of the National Botanic Garden of Havana, Cuba. They were washed and dried in a stove at 30 ± 2 °C (in the dark) for 5 days with free circulation of air, and subsequently powdered.

Preparation of plant extracts

As described earlier (10-12), the extracts were obtained by maceration of dried plant materials with hydro-alcohol solutions in closed dark bottles, at room conditions as follows:

C. aurantifolia leaves 300 g/L of 50 % ethanol during 2 days (*Citrus aurantifolia* leaves extract).

C. aurantium fruit peels 200 g/ L of 70 % ethanol during 7 days (*Citrus aurantium* fruit peels extract).

C. sinensis fruit peels 500 g/L of 50 and 70 % ethanol during 7 days (*Citrus sinensis* peels extract 50 and 70 %, respectively).

The macerates were paper filtered. Afterwards, rota-evaporation (at 40 ° C and 27 mm Hg reduced pressure) of the homogeneous liquids obtained was performed for ethanol elimination from them 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 ^{0}C until used. 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 141; 175; 153 and 136 mg/1mL for CAM, CS_{50}, CS_{70} and CAF, respectively. The flavonoid content was spectrophotometrically determined (13) and expressed as hesperidin equivalents, according to a calibration curve that was plotted using standard operating. The data were 62,0; 38, 1; 33, 9 and 41,3 mg/mL for CAM, CS_{50}, CS_{70} and CAF, respectively, corresponding to 44; 22; 22 and 30 % of TSS , respectively.

Increase of the vascular permeability induced by histamine and dextran in the rat skin

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 food and water was allowed. Since the presence of flavonoids in preparations of Citrus plants was the basis of the hypothesis leading to this research and the extracts to be evaluated were different with respect to the

proportion of total flavonoid in total solids, dose levels to be administered to the animals were calculated as mg flavonoids/kg in order to compare each other and with the reference drug (a mixture of these active chemical compounds) under homogeneous conditions The extracts (1 mL/100 g b.w.) were intragastrically administered to rats (N=10/ treatment group) once daily equivalent to 50; 100 and 200 mg / kg b.w x day during 14 days.

MPFF tablets were powdered and suspended in acacia 10 mg/mL and given to rats by using the same administration scheme. The dose was calculated with respect to the content of active principles declared by the pharmaceutical information of the drug. Preliminary experiments showed the highest pharmacological effect at 200 mg active principles/kg b.w., therefore, this dose level was given to the animals of the positive control group.. Negative control groups (N = 10 each) of extracts and MPFF treatments received water and acacia 10 mg/mL, respectively.

Twenty four hours after the last administration the animals were anesthetized with 1.5 mL of a mixture of atropine, diazepam and ketamine. Thereafter, histamine (400 µg/ mL saline solution) and dextran (2000 µg/ mL saline solution) were administered by intradermic injection of 100 µL into the pre-shaved back of each rat. It was done in duplicate (both sides). Ten minutes later, the animals received an intravenous injection of 1 mL of Evans Blue solution (10 mg/ mL in saline) into the penis vein. After additional ten minutes, they were sacrificed by opening the thoracic cavity, the mean diameters of the blue spots at the injection sites of the phlogistic agents were measured and the spot areas calculated as mm^2. Results were reported as percentage inhibition of the vascular permeability in treated animals compared with the control groups calculated by the following equation: percentage inhibition (%) = (1-E/C) x 100, where E represents the area of the blue spot after the treatment with plant extracts or MPFF and C the area of the blue spot in the respective negative control groups.

Statistical analysis

The experimental results were expressed as the mean S.E.M. Data were assessed by the method of analysis of ANOVA followed by Student´s t test. A *p* value < 0,05 was 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

Mean values of the areas of edema induced by histamine and dextran in rats skin were statistically reduced in the groups of animals treated with the plant extracts (50; 100 and 200 mg/kg daily) and MPFF (200 mg/kg) during 14 days with respect to the corresponding negative control groups ($p < 0,05$ by Studentñ´s t test). As the Table 1 shows, there was no significant difference between the mean percentages of inhibition of edema formation after the treatments, with extracts and the reference drug (p = 0, 15 and 0, 17 for histamine and dextran respectively by ANOVA).

Discussion

This study provided additional evidence on the antiedemagenic effect of a *C. aurantium* fruit peels extract in 70 % alcohol and demonstrated its comparable effect with MPFF, a plebotonic agent with clinical effectiveness (6-8). Furthermore, this research has demonstrated similar results with *C. sinensis* fruit peels extracts in 50 and 70 % alcohol and *C aurantifolia* leaves extract in 50% alcohol, thus suggesting that, contrary to the experience taken from the evaluation of the antiplatelet activity, the four examined preparations could be useful for developing venotonic formulations, though, the lack of dose-response relationships for each product recommends assessing doses lower than 50 mg/kg b.w. in order to determine their corresponding ED50 values. The use of two phlogistic stimuli acting through different mechanisms allows hypothesizing about the possible mechanism of the antiedemagenic action of these extracts. Histamine activates H1 receptors at the vascular wall and dextran induces histamine and serotonin release from mast cells (14- 16). It has been reported the inhibition of histamine release by flavonoids (17-19), thus, a decrease of dextran but not histamine-induced edemagenic reaction would be expected if mas cells were the main pharmacological targets of extracts activity, however, it seems that specific or unspecific antagonisms of muscle receptors could better explain these results. Nevertheless, further pharmacological investigation should be done to characterize the effects of these *Citrus* plant preparations that could make them resemble MPFF and determine their therapeutic potentials.

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Table 1. Inhibition of histamine and dextran-induced vascular hyperpermeability in rat skin by C aurantium and C sinensis fruit peels and C. aurantifolia leaves extracts Results are expressed as the mean ± SEM; N = 10; [1] p =0,15 and [2] p = 0,17 by ANOVA.

	Phlogistic agent	
Dose	**Histamine[1]**	**Dextran[2]**
***Citrus aurantium* peels extract**		
50	62,6 ± 6,8	63,3 ± 18,3
100	76,0 ± 7,02	73,3 ± 10,8
200	67,1 ± 7,9	67,1 ± 11,0
***Citrus sinensis* peels extract 50 %**		
50	63,5 ± 6,8	50,2 ± 24,7
100	64,4 ± 5,2	70,1 ± 7,6
200	68,5 ± 5,1	56,9 ± 14,1
***Citrus sinensis* peels extract 70 %**		
50	61,5 ± 11,7	58,3 ± 14,2
100	81,2 ± 7,8	75,5 ± 4,3
200	71,9 ± 3,3	68,5 ± 24,8
***Citrus aurantifolia* leaves extract**		
50	72,1 ± 7,4	74,4 ± 9,1
100	68,7 ± 6,5	78,8± 8,3
200	70,7± 9,5	74,2 ± 9,0
MPFF 200 mg/kg	64,4 ± 8,9	79,8 ± 7,1