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HPLC-PDA-MS and NMR Characterization of a Hydroalcoholic Extract of *Citrus aurantium* L. var. *amara* Peel with Antiedematogenic Activity

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ABSTRACT: The phytochemical profile of a hydroalcoholic extract of *Citrus aurantium* var. *amara* L. peel, used as herbal medicine, was characterized by HPLC-PDA-MS. Two di-*C*-glycosyl flavones (vincenin II and diosmetin 6,8-di-*C*-glucoside), a series of flavones (luteolin 7-*O*-neohesperidoside, rhoifolin, and neodiosmin), and flavanone (neoeriocitrin, naringin, and neohesperidin) 7-*O*-neohesperidosides and two methoxyflavones (nobiletin and tangeretin), commonly present in *Citrus*, were identified. Furthermore, brutieridin and melitidin, two 3-hydroxy-3-methylglutaryl flavanone glycosides, were also characterized along with rhoifolin 4′-glucoside and three coumarins (8,3′-*β*-D-glucopyranosyloxy-2′-hydroxy-3′-methylbutyl-7-methoxycoumarin, merazin hydrate, and isomerazin). A preparative isolation procedure followed by NMR spectroscopy confirmed the proposed structures of the major flavonoids and identified the coumarins. The phenolic content was found to be 14.8 mg mL^{-1}, and naringin and neohesperidin were the compounds present in the highest concentration (3.6 and 2.6 mg mL^{-1}). The extract of *C. aurantium* peel inhibited significantly ($p < 0.05$) both histamine- and dextran-induced edema in rats in a concentration-dependent manner (IC_{50} = 119.6 and 118.3 mg kg^{-1}, respectively), providing evidence for the therapeutic use of *C. aurantium* var. *amara* peel.

KEYWORDS: *Citrus aurantium var. amara peel, sour orange, HPLC-PDA-MS, flavonoids, coumarins, platelet aggregation, antiedematogenic activity*

INTRODUCTION

Citrus fruits are well-known as sources of flavonoids with potential health-promoting properties related to their antioxidant, anti-inflammatory, anticancer, signaling, and, recently studied, neuroprotective activities.[1,2] Citrus flavonoids have been also suggested for the treatment of vascular diseases,[2,3] inhibiting human platelet aggregation and decreasing capillary fragility and permeability. Moreover, polymethoxylated citrus flavonoids have a positive effect on lipid metabolism, lowering blood cholesterol and triglyceride levels by suppressing hepatic apoB secretion.[4]

Flavonoids reported to be present in *Citrus* include flavanone, flavone, and polymethoxyflavone aglycones, flavanone- and flavone-*O*-glycosides, and flavone-*C*-glycosides.[5] Numerous studies have been undertaken on the analysis of polyphenolic compounds in *Citrus* using HPLC coupled to PDA and/or MS detectors and, recently, HPLC-DAD-MS/MS methods were used to determine the profiles of 83 *Citrus* juices to identify juices from different species and adulterations[6] and to identify and quantify the flavonoids in some tropical species (*C. microcarpa*, *C. hystrix*, *C. medica*, and *C. suhuiensis*).[7]

As a part of our research program on Cuban traditional herbal remedies for the prevention of vascular diseases, in a previous paper we reported the chemical composition and biological activity of lime (*Citrus aurantifolia* Christm.) leaves tincture, developed at the National Institute of Angiology and Vascular Surgey of Cuba.[8,9] In this study, a hydroalcoholic extract obtained from the peel of *Citrus aurantium* L. var. *amara* fruits, used as a herbal medicine and dietary supplement in Cuba for chronic venous insufficiency care,[10] has been studied. *C. aurantium* L. var. *amara*, commonly known as sour orange (SO) or Naranja agria, is widely cultivated in tropical and subtropical regions. The fruit's juice is used as a beverage in weight-loss plans;[11] peel and pulp are used to make marmalades; and decoctions of the leaves and barks are used for their antipyretic, antispasmodic, and tonic actions.[10]

In the present study, we assessed the qualitative and quantitative polyphenolic composition of the hydroalcoholic extract of sour orange peel (SOP) by high-performance liquid chromatography paired with UV photodiode array and electrospray ionization tandem mass spectrometry detectors (HPLC-PDA-ESI-MS^n). The proposed structures of the major constituents were confirmed by nuclear magnetic resonance (NMR) spectroscopy after isolation procedure. The effect of SOP extract on capillary permeability in rat paw was examined

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Table 1. Retention Times and UV and ESI-MS Data of Compounds (1−16) of SOP Extract

			(+)-MS^n data		(−)-MS^n data	
	t_R (min)	λ_{max} (nm)	parent ions	product ions, m/z (RA > 5)	parent ions	product ions, m/z (RA > 5)
1	34.9	280; 340	595, $[M + H]^+$	577 (100); 559 (28); 541 (10); 529 (20); 523 (7); 511 (17); 499 (8); 481 (4); 475 (16); 457 (43); 409 (2)	593, $[M - H]^-$	593 (100); 575 (7); 503 (19); 473 (67); 383 (3); 353 (6)
2	36.6	280; 350	625, $[M + H]^+$	607 (100); 589 (31); 571 (11); 559 (16); 541 (16); 529 (6); 505 (14); 487 (44); 439 (2)	623, $[M - H]^-$	623 (78); 605 (2); 533 (10); 503 (100); 413 (2); 383 (9)
3	37.6	280; 330	741, $[M + H]^+$	271 (39); 433 (100); 595 (22) MS^3 (741→271): 145 (20); 153 (100); 173 (5); 201 (5); 203 (14); 225 (57); 229 (52); 243 (5); 271 (25)		
4	39.6	285	597, $[M + H]^+$	289 (36); 331 (32); 355 (7); 397 (5); 399 (10); 415 (5); 417 (22); 433 (83); 435 (71); 451 (100); 475 (25); 477 (16); 543 (15); 561 (32); 579 (30) MS^3 (597→289): 145 (26); 153 (27); 163 (100); 179 (22); 247 (14); 253 (18); 271 (26)	595, $[M - H]^-$	287 (6); 459 (100)
5	43.1	285	581, $[M + H]^+$	273 (33); 301 (16); 302 (13); 315 (28); 383 (11); 401 (22); 417 (84); 419 (100); 435 (97); 443 (7); 459 (29); 461 (14); 509 (5); 527 (21); 545 (44); 563 (33) MS^3 (581→273): 147 (90); 153 (100); 179 (12); 189 (5); 231 (10)	579, $[M - H]^-$	193 (4); 235 (26); 271 (40); 213 (18) 257 (7); 441 (5); 459 (100)
6	43.4	290, 350	595, $[M + H]^+$	287 (100); 449 (14) MS^3 (595→287): 135 (11); 137 (11); 153 (82); 161 (35); 203 (9); 213 (5); 219 (24); 241 (47); 245 (31); 263 (5); 269 (9)	593, $[M - H]^-$	284 (6); 285 (80); 327 (7); 447 (100)
7	44.8	285	611, $[M + H]^+$	303 (27); 345 (29); 369 (5); 413 (9); 429 (7); 431 (12); 447 (100); 449 (50); 465 (94); 473 (7); 489 (36) ; 491 (14); 539 (6); 557 (22); 575 (54); 593 (37) MS^3 (611→303):145 (9); 153 (35); 177 (100); 179 (86); 193 (9); 201 (6); 243 (9)	609, $[M - H]^-$	301 (100); 343 (99); 489 (8)
8	45.7	330	463, $[M + Na]^+$	283 (100); 301 (51); 463 (7);		
9	46.4	275, 340	579, $[M + H]^+$	271 (100); 433 (14); MS^3 (579→271): 119 (8); 121 (12); 145 (32); 153 (100); 185 (5); 203 (43); 225 (73); 229 (55); 243 (8); 271 (31);	577, $[M - H]^-$	269 (100); 463 (3)
10	47.2	275, 350	609, $[M + H]^+$	301 (100); 463 (12) MS^3 (609→301): 286 (100)	607, $[M - H]^-$	284 (17); 299 (100); 341 (6)
11	48.3	330	261, $[M + H - w]^+$	177 (6); 189 (45); 243 (100)		
12	49.6	285			723, $[M - H]^-$	579 (100); 621 (20); 661 (5) MS^3 (723→579): 271 (22); 313 (9); 459 (100)
13	57.7	285			753, $[M - H]^-$	609 (100); 651 (29); 691 (9) MS^3 (753→609): 301 (100); 325 (7); 343 (20); 489 (16)
14	54.1	330	261, $[M + H]^+$	177 (5); 189 (42); 243 (100)		
15	63.3	280, 340	403, $[M + H]^+$	342 (6); 355 (5); 373 (31); 388 (100)		
16	64.8	285, 335	373, $[M + H]^+$	312 (6); 325 (3); 343 (26); 358 (100)		

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by measuring spectrophotometrically Evans blue dye accumulation at the site of injection of histamine or dextran (phlogistic agents). Finally, the inhibitory activity of SOP on platelet aggregation induced by ADP, epinephrine, collagen, and arachidonic acid was evaluated in human plasma.

MATERIALS AND METHODS

Chemicals. Analytical grade acetic acid (HOAc), ethanol (EtOH), chloroform ($CHCl_3$), methanol (MeOH), and *n*-butanol (*n*-BuOH) were obtained from Carlo Erba (Milan, Italy). Water and MeOH employed for the HPLC-PDA-MS analyses were of HPLC super gradient quality (Romil Ltd., Cambridge, UK). Histamine chlorhydrate was obtained from BIOCEN (Havana, Cuba). Grade A dextran molecular weight 200,000−275,000, Evans Blue dye, and coumarin as a standard were purchased from Sigma Chemical (St. Louis, MO, USA). Adenosine diphosphate (ADP), collagen, epinephrine, and arachidonic acid were obtained from CMP (CMP S.A.S., Rome, Italy).

Plant Material. Fruits of *C. aurantium* L. var. *amara* were obtained from trees grown on a fruit farm of the Cuban Ministry of Agriculture (Finca de los Monos, Cerro, July 2008). A voucher sample (no. 804002) is deposited at the Herbario of the Jardín Botánico Nacional "Dr Johannes Bisse", Havana, Cuba. The peel was collected free of microbial contamination, dried in an oven at 30 ± 2 °C (in the dark) with free circulation of air, and subsequently powdered.

General Experimental Procedures. A Bruker DRX-600 NMR spectrometer was used, operating at 599.2 MHz for ^1H and at 150.9 MHz for ^{13}C, and the UXNMR software package was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD; coupling constants, *J*, are in hertz; $^1H-^1H$ DQF-COSY, $^1H-^{13}C$ HSQC, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature.[12,13] Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and thin-layer chromatography (TLC) on precoated Kieselgel 60 F_{254} plates (E. Merck, Darmstadt, Germany), visualized with the spray reagents cerium sulfate (saturated solution in dilute H_2SO_4). HPLC analyses were performed using a Surveyor LC pump, and a Surveyor autosampler, coupled with a Surveyor PDA detector and an LCQ Advantage ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA) equipped with Xcalibur 3.1 software. Semipreparative HPLC, with isocratic elution, was performed with a Waters 590 series pumping system equipped with a Waters R401 refractive index detector, a μ-Bondapak C_{18} column (300 × 7.8 mm i.d.), and a Rheodyne injector (100 μL loop). For the platelet aggregation a CLOT 2S aggregometer (Seac and Radim Group, Rome, Italy) was used.

Preparation of the Extract. The extraction procedure was developed in the laboratories of the National Institute of Angiology and Vascular Surgery, Cuba. Ground dried peel (500 g) of *C. aurantium* was extracted with 70% EtOH (2.5 L) in a closed dark bottle, at room conditions during 7 days. The obtained extract was a homogeneous yellow liquid. The concentration of solid material was 64 mg mL^{-1}.

HPLC-PDA-ESI-MS Analysis. The HPLC conditions were the same as those used previously for the lime tincture analysis.[9] Detection by diode array was performed simultaneously at two different wavelengths: 280 and 330 nm. The UV spectra were recorded over a 200−600 nm range. The mass analyses were performed with an ESI interface in the positive and negative ionization modes. The data were acquired in the full scan (range of *m*/*z* 200−1200) and MS tandem modes, the maximum injection time was 100 ms, and the number of microscans was one. The optimized instrumental parameters were as follows: (positive mode) capillary temperature, 250 °C; capillary voltage, 18 V; spray voltage, 5.10 kV; sheath gas flow rate, 30 (nitrogen, arbitrary units); auxiliary gas flow rate, 10 (arbitrary units); collision energy, 20−30%; (negative mode) capillary temperature, 300 °C; capillary voltage, −46 V; spray voltage, 4.00 kV; sheath gas flow rate, 30 (nitrogen, arbitrary units); auxiliary gas flow rate, 10 (arbitrary units); collision energy, 20−30%.

Isolation and Identification of Compounds. The hydroalcoholic extract (0.25 L) was dried under vacuum, giving 14.0 g of residue. This was partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH soluble portion (5.0 g). An aliquot (2.5 g) of the *n*-BuOH extract was fractionated over a Sephadex LH20 column (100 cm × 3 cm i.d.) using MeOH as eluent. Fractions (8 mL each) were collected and checked by TLC (Si-gel, *n*-BuOH/OHAc/H_2O, 60:15:25; $CHCl_3$/MeOH/H_2O, 70:30:3). Fractions with similar R_f values were joined, giving four major fractions (I−IV). Each fraction was further purified by semipreparative RP-HPLC. Fraction I (474 mg) was separated with MeOH/H_2O 4:6 (v/v) as mobile phase (flow rate of 2 mL min^{-1}) to yield pure compounds 8,3′-β-D-glucopyranosyloxy-2′-hydroxy-3′-methylbutyl-7-methoxycoumarin (**8**) (1.5 mg) and meranzin hydrate (**11**) (2.0 mg). Fraction II (780 mg) was separated with MeOH/H_2O 7:3 (v/v) as the eluent to afford isomeranzin (**14**) (1.1 mg), nobiletin (**15**) (1.2 mg), and tangeretin (**16**) (1.0 mg). Fractions III and IV were purified using MeOH/H_2O 35:65 (v/v) as mobile phase. Fraction III (753 mg) yielded pure compounds neoeriocitrin (**4**) (11.9 mg), naringin (**5**) (24.8 mg), and neohesperidin (**7**) (14.8 mg); fraction IV (43 mg) gave apigenin-6,8-di-*C*-β-D-glucopyranoside (**1**) (3.0 mg), luteolin 7-*O*-neohesperidose (**6**) (2.0 mg), rhoifolin (**9**) (6.0 mg), and neodiosmin (**10**) (1.5 mg).

NMR data of compounds **4**−**11**[14−21] and **14**−**16**[22,23] were consistent with those reported in the literature, and their ESI-MS data are reported in Table 1.

Quantitative HPLC Analysis of the Extract of *C. aurantium* Peel. HPLC equipment and conditions were the same as used for qualitative analysis. The UV chromatograms were recorded at 280 nm for the quantification of flavanone derivatives and at 330 nm for flavone derivatives and coumarins, whereas the extracted ion chromatogram at *m*/*z* 595 was used to quantify luteolin 7-*O*-neohesperidoside. Naringin, rhoifolin, luteolin 7-*O*-neohesperidoside, and coumarin were selected as external standards. The calibration curve of naringin was used to quantify the flavanone derivatives, whereas the flavone derivatives were quantified using the curve of rhoifolin. Coumarins were quantified with the calibration curve of coumarin. Standard calibration curves were obtained in a concentration range of 1.0−50.0 μg mL^{-1} with four concentration levels (1.0, 5.0, 10.0, and 50.0 μg mL^{-1}) and triplicate injections for each level. UV peak areas of the external standard (at each concentration) were plotted against the corresponding standard concentrations (μg mL^{-1}) using weighed linear regression to generate standard curves. For the linear regression of external standards, R^2 values were >0.990. SOP hydroalcoholic extract was diluted 10 and 20 times with water, and 20 μL was injected for analysis. The amount of the compounds was finally expressed as micrograms per milliliter of the hydroalcoholic extract, as the mean of triplicate determinations.

Platelet Aggregation (PA). A turbidimetric method was applied to measure platelet aggregation using a CLOT 2S Aggregometer as previously reported.[9] Briefly, 5 μL of different dilutions of SOP extract in phosphate saline buffer (PBS) (0.1, 0.3, and 1.0 mg mL^{-1}), PBS (negative control), or acetylsalycilic acid (ASA, reference drug, 1 mg mL^{-1}) were added to 280 μL of platelet-rich plasma (PRP) in aggrometer cuvettes. Successively, 15 μL of platelet aggregation inducers, adenosine diphosphate (ADP), epinephrine, collagen, or arachidonic acid, was added after 2 min of pre-incubation at 37 °C. Platelet aggregation was monitored for 5 min. The results were expressed as percentages of aggregation as provided by the instrument. The percentage inhibition of platelet aggregation was calculated as follows: percentage inhibition (%) = (1 − platelet aggregation of sample/platelet aggregation of control) × 100. IC_{50} values (the concentration necessary to reduce the induced platelet aggregation by 50% with respect to control) were obtained from concentration−effect curves.

Animals. Male Wistar rats (250−300 g) were obtained from 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. 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

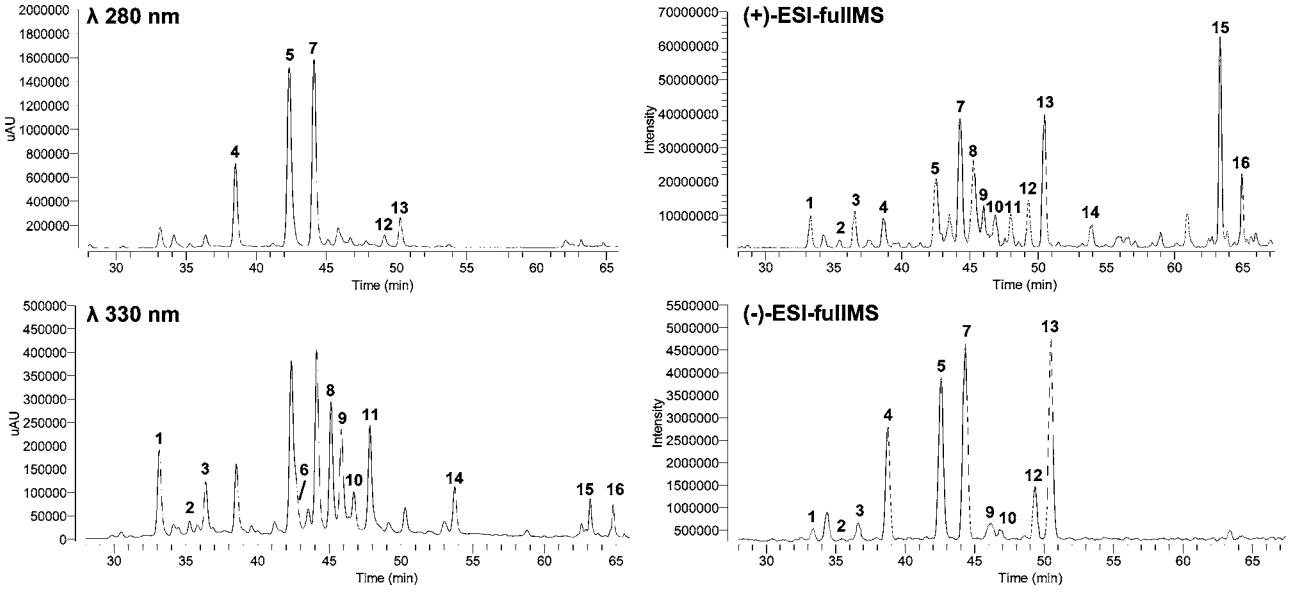

Figure 1. HPLC chromatograms of SOP (hydroalcoholic extract of sour orange peel): (A) 280 nm; (B) 330 nm; (C) (+)-ESI-MS-TIC; (D) (−)-ESI-MS-TIC.

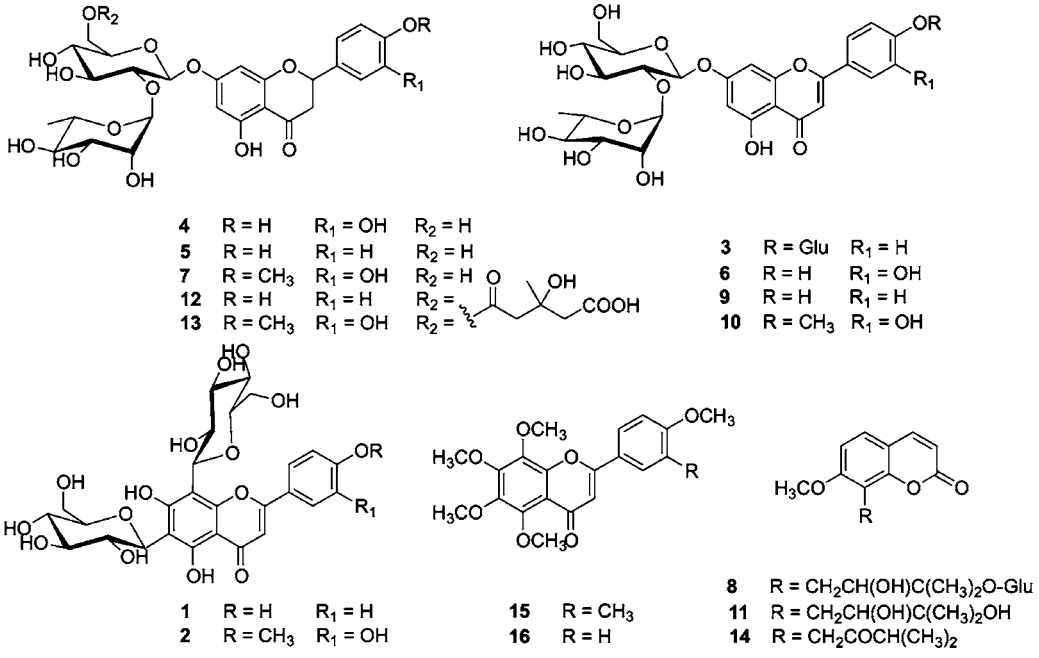

Figure 2. Structures of 16 compounds identified in SOP.

the national and international guidelines for the humane use of laboratory animals.

Increase of the Vascular Permeability Induced by Histamine in Rat Paw. The animals were divided into five groups (six rats each). For in vivo experiments, SOP aqueous extract was prepared by evaporation of ethanol followed by reconstitution to the initial volume with distilled water. This form of liquid SOP extract was administered at different dose levels (75, 150, and 200 mg kg^{-1} bw) intragastrically to the different groups via a gavage needle for 14 days. Cyproheptadine (10 mg kg^{-1} bw) and vehicle alone (water) were administered as positive and negative control, respectively. The following day, the rats were anesthetized with 1.5 mL of a mixture of atropine, diazepam, and ketamine. Thereafter, 100 μL of histamine (40 μg mL^{-1}) was injected intradermally into the preshaved and peeled rat paws. Ten minutes later, the rats received an intravenous injection of 1 mL of Evans Blue solution (10 mg mL^{-1} dissolved in saline). After an additional 10 min, the animals were sacrificed by opening the thoracic cavity, and the area of skin that included the injection site was removed. Evans Blue dye was extracted from the skin by incubation with formamide for 48 h at 37 °C. The material was centrifuged at 1000*g* for 20 min, and the dye concentration was determined in supernatant, spectrophotometrically at 620 nm. The dye extravation was quantified from a standard curve and expressed as milligrams per milliliter. The results were reported as percentage inhibition of the vascular permeability in treated animals compared with the control group calculated by the following equation: percentage inhibition (%) = $(1 - D/C) \times 100$, where D represents the concentration of Evans Blue dye after administration of drugs (extract or cyproheptadine) to the rats and C the dye concentration in the negative control group animals. IC_{50} values (the concentration necessary to reduce the induced increase of vascular permeability by 50% with respect to control) were obtained from concentration−effect curves.

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Increase of the Vascular Permeability Induced by Dextran in Rat Paw. The animals were treated in a manner similar to that of histamine-induced paw edema model; dextran (100 μL, 200 μg mL^{-1} in normal saline) was used in place of histamine.

Statistical Analysis. The experimental results were expressed as the mean ± SEM. Data were assessed by ANOVA followed by Student's t test. A p value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

HPLC-PDA-ESI-MS Analysis. A HPLC-PDA-ESI-MS^n method previously developed to analyze a lime (*C. aurantifolia* Christm.) leaves tincture[9] was applied to the SOP extract to investigate its polyphenol profile. HPLC-MS/MS analyses were performed in positive and negative ionization modes to obtain complementary information useful to characterize SOP constituents. Figure 1 shows the HPLC-UV and HPLC-ESI-MS profiles, in both positive and negative ionization modes, of SOP hydroalcoholic extract. Sixteen (**1**−**16**) major peaks were detected, and their retention times, λ_{max} values, and MS data are listed in Table 1. Analysis of UV and MS spectra led to the identification of two flavone di-*C*-glycosydes (**1** and **2**), a flavone triglycoside (**3**), three flavanone *O*-diglycosides (**4**, **5**, and **7**), three flavone *O*-diglycosides (**6**, **9**, and **10**), two 3-hydroxy-3-methylglutaryl (HMG) conjugates of flavanone *O*-diglycosides (**12** and **13**), three coumarins (**8**, **11**, and **14**), and two methoxyflavones (**15** and **16**) (Figure 2).

Compounds **1** and **2** showed UV spectra suggesting the structure of flavone derivatives, and in (+)- and (−)-MS/MS they yielded product ions (Table 1) typical of di-*C*-hexosyl flavones.[24] MS data of **1** and **2** were superimposable to those of apigenin 6,8-di-*C*-glucoside and diosmetin 6,8-di-*C*-glucoside, respectively, previously identified in *C. aurantifolia* leaves.[9] Their identities were confirmed by comparison with the pure compounds.

All product ion mass spectra obtained for the protonated molecules of the *O*-diglycosyl flavonoids (peaks 4−7, 9, and 10, $[M + H]^+$ at m/z 597, 581, 595, 611, 579, and 609, respectively) showed Y_1^+ (m/z 451, 435, 449, 465, 433, and 463 for compounds **4**−**7**, **9**, and **10**) and Y_0^+ (m/z 289, 273, 287, 303, 271, and 301 for compounds **4**−**7**, **9**, and **10**) product ions due to the cleavage of two glycosidic linkages. The neutral losses of −146 and −308 amu, producing the above ions, allowed the disaccharide sequence to be determined as deoxyhexose−hexose−flavonoid. These data suggested that compounds **4**−**7**, **9**, and **10** are rutinoside (rhamnosyl 1→6 glucoside) or neohesperidoside (rhamnosyl 1→2 glucoside) derivatives, which are the most common disaccharides linked to flavonoids and reported in *Citrus* spp.[5] UV spectra of compounds **6**, **9**, and **10** provided two maxima at 330−350 nm (band I) and 275−290 nm (band II), coincident with flavones, whereas compounds **4**, **5**, and **7** showed UV spectra (maxima at 285 nm and shoulder at 330−335 nm) characteristic of flavanones.[25] Identification of aglycone moieties of compounds **4**−**7**, **9**, and **10** was achieved by analyses of MS^3 spectra of the protonated aglycones (Y_0^+). On the basis of the m/z values of the diagnostic fragment ions $^{1,4}B^+$, $^{1,3}A^+$, and $[M + H - B\text{-ring}]^+$ present in MS^3 spectra of the flavanone *O*-diglycosides **4** (m/z 163, 153, and 179), **5** (m/z 147, 153, and 179), and **7** (m/z 177, 153, and 179), eriodictyol, naringenin, and hesperetin were identified as the aglycones, respectively. The structurally informative $^{1,3}B^+$, $^{1,3}A^+$, $^{0,2}B^+$, $^{0,4}B^+$, and $^{0,4}B^+ - H_2O$ ions, instead, allowed luteolin and apigenin to be established as the aglycones of flavones *O*-diglycosides **6** (m/z 135, 153, 137, 179, and 161) and **9** (m/z 119, 153, 121, 145, and 163), respectively. In the MS^3 spectrum (609 → 301) of compound **10** only the fragment ion at m/z 286, related to the loss of a methyl radical, was detected. Therefore, the structure of diosmetin was proposed on the basis of occurrence data in *Citrus* genus.

In the case of flavone *O*-diglycosides, the relative abundance of Y_1^+ (m/z 449, 433, and 463 for **6**, **9**, and **10**) and Y_0^+ (m/z 287, 271, and 301 for **6**, **9**, and **10**) product ion spectra allowed a 1→2 interglycosidic linkage to be established between the hexose and deoxyhexose residues ($Y_1^+ > Y_0^+$).[9] On the basis of this evidence, the structures of luteolin-7-*O*-neohesperidose, apigenin-7-*O*-neohesperidose (rhoifolin), and diosmetin-7-*O*-neohesperidose (neodiosmin) were proposed for compounds **6**, **9**, and **10**, respectively.

Otherwise, the interglycosidic linkage type in the glycan moiety of flavanones **4**, **5**, and **7** cannot be inferred on the basis of the absolute peak heights of Y_0^+ and Y_1^+ because the relative abundance of Y_1^+ (m/z 451, 435, and 465 for **4**, **5**, and **7**) always exceeds that of Y_0^+ (m/z 289, 273, and 303 for **4**, **5**, and **7**).[26] In this particular case, product ion mass spectra of the deprotonated molecules were evaluated to distinguish the disaccharide residues. In negative ion mode, compounds **4**, **5**, and **7** showed a pronounced fragmentation and characteristic fragment ions of flavonoid *O*-neohesperidosides.[26] Particularly, the base peaks in the (−)-MS/MS spectra of **4** and **5** were the ion $[M - H - {}^{1,3}B]$ at m/z 459 produced by a retro-Diels−Alder reaction in ring C.[27] This ion was not observed for **7** due to the absence of a hydroxyl group at the 4′-position of ring B.[27] From the above results, compounds **4**, **5**, and **7** were tentatively identified as eriodictyol 7-*O*-neohesperidoside (neoeriocitrin), naringenin 7-*O*-neohesperidoside (naringin), and hesperetin 7-*O*-neohesperidoside (neohesperidin), respectively.

Compound **3** showed a band I hypsochromic shift compatible with a 4′ substitution on the flavone skeleton.[25] Its (+)-MS-MS spectrum ($[M + H]^+$ at m/z 741) suggested a tri-*O*-glycosyl flavonoid structure. According to previous papers,[26,28] the relative intensities of product ions at m/z 595 ($[M + H - \text{deoxyhexose}]^+$), 433 ($[M + H - \text{deoxyhexose} - \text{hexose}]^+$), and 271 (aglycone) indicated the presence of a hexose unit along with a disaccharide (neohesperidoside) on a flavone skeleton. In line with the literature on the *Citrus* species, the disaccharide and hexose substitutions were assigned to positions 7 and 4′, respectively. Apigenin was inferred as the aglycone of **3** by MS^3 (741 → 271) analysis. Therefore, compound **3** was identified as rhoifolin-4′-*O*-glucoside.

Compounds **12** and **13** were identified as flavanone derivatives by UV spectral analysis. Their negative product ion spectra ($[M - H]^-$ at m/z 723 and 753) showed $[M - H - CO_2 - H_2O]^-$ (m/z 661 and 691 for **12** and **13**), $[M - H - C_4H_6O_3]^-$ (m/z 621 and 651 for **12** and **13**), and $[M - H - C_6H_8O_4]^-$ (m/z 579 and 609 for **12** and **13**) ions, which indicate the presence of a 3-hydroxy-3-methylglutaryl substituent.[29] The product ions at m/z 579 (**12**) and 609 (**13**) and their MS^3 spectra, strictly close to (−)-MS/MS spectra of **5** and **7**, respectively, led to the assumption that **12** and **13** were HMG conjugates of naringin and neohesperidin, namely, melitidin and brutieridin.[29]

Less polar compounds **15** and **16** were identified as polymethoxylated flavones by UV and (+)-MS spectra (Table 1). Their protonated molecules ($[M + H]^+$ at m/z 403 and 373 for **15** and **16**, respectively) dissociated predominantly via loss

of methyl radicals, producing the $[M + H - CH_3^{\bullet}]^+$ ion (m/z 388 and 358 for **15** and **16**) as base peak and other main fragments corresponding to the loss of 30 ($[M + H - 2CH_3^{\bullet}]^+$ at m/z 373 and 343 for **15** and **16**), 61 ($[M + H - CH_3^{\bullet} - CO - H_2O]^+$ at m/z 342 and 312 for **15** and **16**), and 48 amu ($[M + H - 2CH_3^{\bullet} - H_2O]^+$ at m/z 355 and 325 for **15** and **16**). MS data indicated the presence of six and five methoxyl groups in **15** and **16**, respectively, but not did not allow their positions to be established. In any case, according to the literature data[30,31] **15** and **16** were tentatively identified as nobiletin and tangeretin.

With regard to compounds **8**, **11**, and **14**, characteristic UV spectra of coumarins (λ_{max} at 330 nm)[30] were observed. The presence of tropilium ion at m/z 189 in the (+)-MS/MS spectra of **11** and **14** suggested the structure of 7,8-disubstituted coumarins as merazin, isomerazin, or merazin hydrate.[31] For **11**, instead, two fragment ions at m/z 301 and 283 due to the loss of a hexose residue indicated a glycosyl coumarin structure.

Isolation and Structure Determination of Compounds. To confirm the structures proposed by HPLC-DAD-MS and to identify coumarins **8**, **11**, and **14**, a purification procedure of the SOP extract was undertaken. A portion of dry residue of the extract was partitioned between *n*-BuOH, and the *n*-BuOH fraction was subjected to purification by gel permeation chromatography and semipreparative HPLC. All isolated pure compounds were analyzed by 1D- and 2D-NMR experiments, confirming the proposed structure of flavone (**6**,[16] **9**,[19] and **10**[20]) and flavanone (**4**,[14] **5**,[15] and **7**[17]) 7-*O*-neohesperidosides. Polymethoxyflavones **15** and **16** were identified as nobiletin[23] and tangeretin,[23] respectively, two flavonoids with fully methoxylated A-rings recently reported as potent inhibithors of apoB secretion.[4] Moreover, coumarins **8**, **11**, and **14** were identified as 8,3′-*β*-D-glucopyranosyloxy-2′-hydroxy-3′-methylbutyl-7-methoxycoumarin,[18] merazin hydrate,[21] and isomerazin,[22] respectively. Coumarin glycoside **8** was previously reported only in the aqueous extracts of bitter orange, grapefruit, and *C. maxima* flavedo together with meranzin, meranzin (**11**) hydrate, and isomeranzin (**14**).[18] The latter coumarins, instead, were characteristic of bitter orange and grapefruit essential oil.[30,31]

The adopted purification procedure did not allow melitidin (**12**) and brutieridin (**13**) to be obtained due to their low content in the extract. These acylated conjugates of naringin and neohesperidin have been recently reported as components of *C. bergamia*,[29] *C. aurantium*,[32] and *C. myrtifolia*[33] fruits. They are structural analogues of statins, inhibitors of the 3-hydroxy-3-methylglutaryl-CoA reductase enzyme, used as cholesterol concentration lowering drugs.[34]

Quantitative Analysis. The concentrations of compounds **1**−**16** in the SOP hydroalcoholic extract were obtained by HPLC-DAD-MS analysis. The calibration curves of naringin (λ 280 nm), rhoifolin (λ 330 nm), and coumarin (λ 330 nm) were used to quantify flavanone derivatives (**4**, **5**, **7**, **12**, and **13**), flavone derivatives (**1**−**3**, **6**, **9**, **10**, **15**, and **16**), and coumarins (**8**, **11**, and **14**), respectively. Because of poor UV chromatographic resolution of luteolin 7-*O*-neohesperidoside (**6**), it was necessary to quantify this compound by HPLC-MS (extracted ion chromatogram at m/z 595). Concentrations of the phenolic compounds and coumarins in SOP hydroalcoholic extract are summarized in Table 2. The overall phenolic content was 14.8 mg mL^{-1}. Flavanone *O*-diglycosides were the most abundant compounds of the extract, with naringin (**5**) and neohesperetin (**7**) showing the highest concentrations, followed by flavone derivatives. Coumarin **8**, **11**, and **14** contents were below 0.4 mg mL^{-1}.

Table 2. Flavonoid and Coumarin Contents in SOP Extract

compound	μg mL^{-1} ± SD[a]
apigenin-6,8-di-*C*-glucoside (**1**)[b]	1412 ± 20
diosmetin-6,8-di-*C*-glucoside (**2**)[b]	711 ± 24
rhoifolin 4′-glucoside (**3**)[b]	834 ± 2
neoeriocitrin (**4**)[c]	1279 ± 14
naringin (**5**)[c]	3606 ± 160
luteolin 7-*O*-neohesperidoside (**6**)	215 ± 5
neohesperidin (**7**)[c]	2649 ± 270
8,3′-*β*-glucosyloxy-2′-hydroxy-3′-methylbutyl-7-methoxycoumarin (**8**)[d]	241 ± 2
rhoifolin (**9**)[b]	1429 ± 54
neodiosmin (**10**)[b]	954 ± 35
meranzin hydrate (**11**)[d]	64 ± 3
melitidin (**12**)[c]	185 ± 3
brutieridin (**13**)[c]	445 ± 9
isomeranzin (**14**)[d]	87 ± 1
nobiletin (**15**)[b]	456 ± 24
tangeretin (**16**)[b]	228 ± 4

[a]SD, standard deviation of three replications. [b]Expressed ad equivalents of rhoifolin. [c]Expressed as equivalents of naringin. [d]Expressed as equivalent of coumarin.

Platelet Aggregation. Effects of SOP extract (0.1, 0.3, and 1.0 mg mL^{-1} in PBS) on human platelet aggregation were determined as previously reported.[9] Platelet aggregation induced by ADP, epinephrine, collagen, and arachidonic acid was not significantly modified by the extract (data not shown). In a previous study[9] we reported that the hydroalcoholic extract of lime (*C. aurantifolia*) leaves (TLL) inhibited ADP and epinephrine-induced platelet aggregation with IC_{50} values of 0.40 and 0.32 mg mL^{-1}. TLL contains as major constituents *C*-glycosyl flavones such as compounds **1** and **3**; in contrast, SOP carries mainly *O*-glycosyl flavones and flavanones as well as coumarins. The different chemical compositions of sour orange peel and lime leaf extracts may justify the observed dissimilar biological activity.

Effect on Increase of the Vascular Permeability Induced by Histamine and Dextran in Rat Paw. Increased vascular permeability is one of the main features of acute inflammation and results in exudation of fluid rich in plasma proteins and cells, with subsequent edema at the injured site. Vascular permeability responses are commonly identified and assessed in the skin of laboratory animals by the accumulation at injured sites of a dye, such as Evans Blue. It binds serum albumin, using this as its transporter molecule, so that the dye accumulation can be used as marker for the plasma protein exudation at the site of phlogosis.[35] In our study, the Evans Blue dye leakage assay was used to evaluate the effect of SOP extract on the vascular hyperpermeability induced by both dextran and histamine in the rat paw. These edema-producing agents stimulate the increase of the vessel wall permeability through different mechanisms. Dextran induces an acute inflammatory response, characterized by extravation and edema formation as a consequence of the liberation of histamine and serotonin from mast cells,[36] whereas histamine, one of the most important inflammation mediators and a potent vasodilatator substance, acts by stimulating its H_1 receptors on the vessel wall.[37] The obtained results shown in

Figure 3 indicate that the pretreatment for 14 days with herbal product SOP inhibited significantly ($p < 0.05$) and in dose-

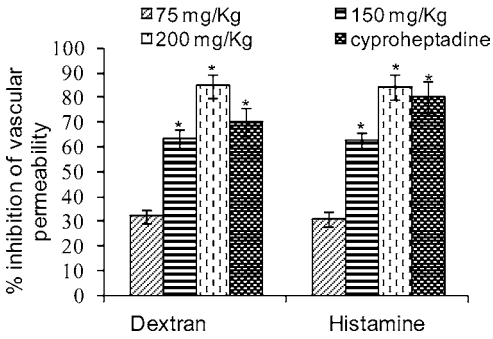

Figure 3. Effect of SOP (aqueous extract 75, 150, and 200 mg kg^{-1}) and cyproheptadine (10 mg kg^{-1}) on dextran- and histamine-induced vascular hyperpermeability in rat paw after 14 days of intragastrical administration. Each column represents the mean ± SEM of $n = 6$. (*) $p < 0.05$, ANOVA followed by Student's t test.

dependent manner (IC_{50} = 119.6 mg kg^{-1} in histamine-induced and 118.3 mg kg^{-1} in dextran-induced edema) the vascular permeability induced by both agents. In the same experiments, the H_1 antagonist synthetic drug cyproheptadine, used as control, showed IC_{50} values of 6.2 and 7.1 mg kg^{-1}, respectively. The observed effect demonstrated that the extract is unable to inhibit the release of preformed histamine and serotonin; however, it may be suggested that the extract acts as a receptor blocking substance, such as cyproheptadine, inhibiting the action of the mediators by antagonism with both histamine and serotonin receptors. As a matter of fact, an inhibitory effect on the histamine release could reduce the dextran-induced vascular hyperpermeability without affecting the edema produced by histamine.

The present research has provided evidence about the chemical composition and antiedematogenic effect of a hydroalcoholic extract of *C. aurantium* peel used in Cuba. Information on the qualitative and quantitative profile could be a support for the future validation of isolated compounds as markers for the quality control of the extract. Moreover, actual chemical and biological studies provide a scientific background to the traditional use of this natural and nontoxic product in the treatment of vascular diseases in Cuba.

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Notes

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