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HPLC-PDA-MS and NMR Characterization of *C*-Glycosyl Flavones in a Hydroalcoholic Extract of *Citrus aurantifolia* Leaves with Antiplatelet Activity

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A hydroalcholic extract of lime (*Citrus aurantifolia*) leaves has been developed in Cuba to be used as a nutritional supplement and phytomedicine in the form of tincture (TLL). A HPLC-PDA-ESI/MS/MS method has been used for the comprehensive analysis of *C*-glycosyl flavones in TLL. Six *C*-glycosyl flavones were characterized and, to confirm the proposed structures and to elucidate the nature of the sugar units, a preparative procedure was applied, and isolated compounds were characterized by NMR. Apigenin-6,8-di-*C*-β-D-glucopyranoside (vicenin II) (**1**), diosmetin-6,8-di-*C*-β-D-glucopyranoside (**2**), apigenin-8-*C*-β-D-glucopyranoside (vitexin) (**3**), apigenin-8-*C*-[α-L-arabinopyranosyl-(1→6)]-*O*-β-D-glucopyranoside (**4**), apigenin-6-*C*-[α-L-arabinopyranosyl-(1→6)]-*O*-β-D-glucopyranoside (**5**). and apigenin-6-*C*-β-D-glucopyranoside (isovitexin) (**6**) were identified in TLL and quantified by HPLC-PDA. Compounds **4** and **5** were two new arabinosyl derivatives of vitexin and isovitexin. Inhibitor effect of TLL on platelet aggregation induced by physiological agonists of platelets was evaluated in human plasma. TLL inhibited significantly ADP and epinephrine-induced platelet aggregation in a concentration-dependent manner (IC_{50} = 0.40 and 0.32 mg/mL, respectively).

KEYWORDS: *Citrus aurantifolia* Ch.; tincture; phytomedicine; antiplatelet activity; *C*-glycosyl flavones; LC-PDA-ESI-MS; 1D and 2D NMR

INTRODUCTION

Lime (*Citrus aurantifolia* Christm.), which belongs to the family Rutaceae, is widely found in Cuba and in many other tropical and subtropical regions, and it is one of the most popular edible fruits in the world. Lime is used for the extraction of juice, preparation of squash, concentrates, beverages, and byproducts, such as citric acid and pectin (*1*). Lime leaves have been traditionally used for the treatment of skin diseases and as anti-inflammatory agent. The leaf decoction is used in Cuba as eye drops and to bathe a feverish patient and also as a mouthwash and gargle in cases of sore throat and thrush (*2*).

In the past few years there has been an increased interest in the study of *Citrus* plants because their fruits and leaves accumulate large amounts of flavonoids that occur as either *O*- or *C*-glycosides (*3*). Flavonoids constitute one of the most numerous and ubiquitous groups of plant metabolites and are an integral part of both human and animal diets. However, recent interest in food phenolics has increased greatly, owing to their antioxidant capacity and their possible beneficial implications in human and mammalian health, such as in the treatment and prevention of cancer, cardiovascular diseases, and other pathologies (*4*). Flavonoids also affect capillary permeability and fragility and inhibit human platelet aggregation (*5*). The effects of flavonoids derived from *Citrus* peels on collagen-induced platelet aggregation has been reported (*6*), as well as the influence of *Citrus* fruit extracts on platelet arachidonic acid metabolism (*7*), suggesting that *Citrus* plant extracts may have antiplatelet activity.

Cuba has developed a relatively sophisticated pharmaceutical sector, originally to provide medicinal products for her own population and, more recently, to earn hard currency through exports. The importance of this sector is particularly seen with respect to the strong changes that have taken place in Cuba in recent decades and which still carry on. As a part of the research activities that have been done with the aim of characterizing the utility of natural products for the prevention of vascular diseases, a tincture of lime leaves (TLL) has been developed in the laboratories of the National Institute of Angiology and Vascular Surgery, Cuba.

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In the present study, we assessed the qualitative and quantitative phenolic profile of TLL and evaluated its antiplatelet activity. The phenolic compounds of TLL were investigated by high-performance liquid chromatography paired with UV photodiode array and electrospray ionization tandem mass spectrometry detectors (HPLC-PDA-ESI/MS), and their proposed structures were further confirmed by nuclear magnetic resonance (NMR) spectroscopy. The antiplatelet activity of TLL was measured in vitro by turbidimetric method to evaluate inhibition of platelet aggregation induced by ADP, epinephrine, collagen, and arachidonic acid (physiological aggregation inducers) and to compare the capacity with that of acetylsalicylic acid (ASA) usually used in antiplatelet therapy.

MATERIALS AND METHODS

Chemicals. HPLC-grade acetonitrile (CH_3CN), methanol (MeOH), and acid acetic (AcOH) were purchased from J. T. Baker (Baker Mallinckrodt, Phillipssburg, NJ). HPLC-grade water (18 mΩ) was prepared by a Milli-Q^{50} purification system (Millipore Corp., Bedford, MA). Vitexin and isovitexin standards were obtained from Sigma (Milano, Italy).

Plant Material. The leaves of *C. aurantifolia* (Christm.) (Rutaceae) were collected from plants grown in a fruit farm (Finca de los Monos, Cerro, July 2001) without affecting the ecosystem. A voucher sample (no. 8045) is deposited at the Herbario of the Jardín Botánico Nacional "Dr Johannes Bisse", Havana, Cuba. Leaves were collected free of microbial contamination, were dried in a stove at 30 ± 2 °C for 5 days (in the dark) with free circulation of air, and subsequently pulverized.

General Experimental Procedure. Optical rotations were determined on a Jasco DIP-1000 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. UV spectra were obtained with a Beckman DU 670 spectrophotometer in MeOH (*c* 1) and IR spectra with a Bruker IFS-48. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^1H and at 150.86 MHz for ^{13}C, using 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. Distortionless enhancement by polarization transfer (DEPT) ^{13}C, $^1H-^1H$ double quantum filtered corrrelation spectroscopy (DQF-COSY), $^1H-^{13}C$ heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond coherence (HMBC), and ROESY (rotating-frame Overhauser enhancement spectroscopy) were obtained by employing the conventional pulse sequences. The selective excitation spectra, 1D TOCSY, were acquired using waveform generator-based gauss-shaped pulses, mixing time ranging from 100 to 120 ms and a MLEV-17 spin−lock field of 10 kHz preceded by 2.5 ms trim pulse NMR experiments. HPLC analyses were performed using a Surveyor LC pump, a Surveyor autosampler, coupled with a Surveyor PDA detector, and an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with Xcalibur 3.1 software. Exact masses were measured by a Q-TOF premier (Waters, Manifold, MA, USA) instrument. Chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Preparative HPLC with isocratic elution was carried out using a Waters 590 series pumping system equipped with a Waters R401 refractive index detector. TLC analyses were performed with Macherey-Nagel precoated silica gel 60 F_{254} plates.

Preparation of TLL. A hydroalcoholic extract of *C. aurantifolia* leaves was obtained by maceration of ground dried leaves in 50% ethanol in a closed dark bottle during 2 days. The tincture obtained is a homogeneous brown liquid. The concentration of solid material was 136 mg/mL.

HPLC-PDA-ESI-MS Analysis. HPLC separations were accomplished using a 150 mm × 2.0 mm i.d., 5 μm, Hypurity Aquastar column (Thermo Finnigan) protected by a 4 mm × 2.0 mm i.d. guard cartridge and a binary gradient composed of water (solvent A) and methanol (solvent B), both containing 0.1% (v/v) formic acid. The following gradient was adopted: a linear gradient of B from 10 to 30 for 20 min, from 30 to 65% B for 30 min, from 65 to 100% B for 5 min, followed by washing and re-equilibrating of the column. Elution was performed at flow rate of 0.2 mL/min, and the volume of the injection was 20 μL. Detection by diode array was performed simultaneously at two different wavelengths: 278 and 330 nm. The UV spectra were recorded with a 200–600 nm range. The mass analyses were performed with an ESI interface in the positive and negative ion modes. The data were acquired in the full scan and MS/MS scanning modes, the maximum injection time was 100 ms, the number of microscans was three, and for the MS/MS scanning mode the percentage of collision energy was 30%. 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); scan range, *m/z* 200–1000; (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); scan range, *m/z* 200–1500.

Acid Hydrolysis. A portion of TLL was dried under vacuum, and 5 mg of residue was dissolved in 1 N HCl (1 mL). The solution was stirred at 100 °C for 30 min. After cooling, the solution was concentrated by blowing with N_2. The residue was dissolved in MeOH/H_2O 3:7 (1 mL) and analyzed by HPLC-PDA-ESI/MS.

Isolation Procedure of Compounds 1–6. The TLL was taken to dryness under reduced pressure at 40 °C. Part of the extract (3 g) was fractionated on a Sephadex LH 20 column (100 cm × 5.0 cm) using methanol as solvent. Fifty-six fractions of 8 mL each were collected. After TLC analysis (Si-gel, *n*-BuOH/AcOH/H_2O, 65:15:25; $CHCl_3$/MeOH/H_2O, 80:18:2) by UV (254 and 366 nm) and $Ce(SO_4)/H_2SO_4$ detections, fractions with similar R_f values were combined, giving three major fractions (I−III). Fractions II and III were purified by HPLC on the 300 × 7.8 mm i.d., 10 μm μ-Bondapak C18 column (flow rate = 3.0 mL/min) using MeOH/H_2O (45:55) as the eluent, to yield pure compounds **1**–**6**.

*Apigenin-6,8-di-C-β-D-glucopyranoside (Vicenin II) (***1***).* **1** was obtained as a yellow amorphous powder; ^1H and ^{13}C NMR data were consistent with those previously reported (*8*); for ESI-MS data, see **Table 1**.

*Diosmetin-6,8-di-C-β-D-glucopyranoside (***2***).* **2** was obtained as a yellow amorphous powder; ^1H and ^{13}C NMR data were consistent with those previously reported (*9*); for ESI-MS data see **Table 1**.

*Apigenin-8-C-β-D-glucopyranoside (vitexin) (***3***).* **3** was obtained as a yellow amorphous powder; ^1H and ^{13}C NMR data were consistent with those previously reported (*8*); for ESI-MS data see **Table 1**.

*Apigenin-8-C-[α-L-arabinopyranosyl-(1→6)]-O-β-D-glucopyranoside (***4***).* **4** was obtained as an amorphous powder: $[\alpha]^{24}_D$ –34.5 (*c* 0.35, MeOH); IR (KBr) λ_{max} 3450, 1515, 1262, 1220, 1070 cm^{-1}; ^1H NMR and ^{13}C NMR (CD_3OD, 600 MHz), see **Table 2**; (+)-HRESI-MS, *m/z* 565.1560 $[M + H]^+$, calcd for $C_{26}H_{29}O_{14}$, 565.1557; for ESI-MS data see **Table 1**.

*Apigenin-6-C-[α-L-arabinopyranosyl-(1→6)]-O-β-D-glucopyranoside (***5***).* **5** was obtained as an amorphous powder: $[\alpha]^{24}_D$ –23.9 (*c* 0.34, MeOH); IR (KBr) λ_{max} 3450, 1515, 1262, 1220, 1070 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz), see **Table 2**; ^{13}C NMR (CD_3OD, 600 MHz), see **Table 2**; (+)-HRESI-MS, *m/z* 565.1553 $[M + H]^+$, calcd for $C_{26}H_{29}O_{14}$, 565.1557; for ESI-MS data see **Table 1**.

*Apigenin-6-C-β-D-glucopyranoside (Isovitexin) (***6***).* **6** was obtained as a yellow amorphous powder; ^1H and ^{13}C NMR data were consistent with those previously reported (*10*); for ESI-MS data, see **Table 1**.

Quantitative Analysis. The HPLC equipment and conditions were the same as those used for qualitative analysis. The chromatograms were recorded at 330 nm for quantification. Isovitexin was selected as standard of calibration for *C*-glycosyl flavones **1**–**6**, and an external standard method was utilized for their quantification. The quantitative determination was performed using a four-point regression curve in the range of 5–100 μg/mL (5, 25, 50, and 100 μg/mL), and triplicate injections were made for each level. UV peak areas of the external standard (at each concentration) were plotted against the corresponding standard concentrations (μg/mL) using weighed linear regression to generate standard curves. For the linear regression of the external standard, R^2 was 0.9995. TLL was diluted 10 times with water, and 20

Table 1. Retention Times and UV and ESI Mass Data of TLL Flavonoids

compd	t_R (min)	λ_{max} (nm)	$[M + H]^+$ (*m*/*z*)	MS^n (*m*/*z*, rel abundance)[a]	$[M - H]^-$ (*m*/*z*)	MS^n (*m*/*z*, rel abundance)[a]
1	29.9	270, 330	595	577 (100), 559 (28), 541 (10), 529 (20), 523 (7), 511 (17), 499 (8), 481 (4), 475 (16), 457 (43), 409 (2)	593	593 (100), 575 (7), 503 (19), 473 (67), 383 (3), 353 (6)
2	32.3	270, 345	625	607 (100), 589 (31), 571 (11), 559 (16), 541 (16), 529 (6), 505 (14), 487 (44), 439 (2)	623	623 (78), 605 (2), 533 (10), 503 (100), 413 (2), 383 (9)
3	33.5	270, 335	433	415 (100), 397 (30), 379 (7), 367 (25), 337 (7), 313 (13) **MS^3 (433→313):**[b] 289 (3), 284 (100), 243 (4), 121 (3)	431	431 (7), 341 (5), 311 (100)
4	34.5	270, 335	565	445 (4), 433 (100), 415 (22), 397 (5), 367 (3), 313 (8)	563	413 (100)
5	35.5	270, 335	565	433 (100), 415 (16), 367 (5), 313 (4)	563	563 (5), 443 (9), 413 (100), 341 (2), 293 (12)
6	36.1	270, 335	433	415 (100), 397 (29), 379 (17), 367 (74), 337 (32), 313 (22) **MS^3 (433→313):**[b] 295 (100), 284 (25), 270 (7), 267 (72), 239 (6), 229 (4), 195 (2), 181 (1), 121 (1)	431	431 (24), 413 (9), 341 (36), 311 (100)

[a] Collision energy = 30%. [b] Collision energy = 50%.

Table 2. ^1H NMR and ^{13}C NMR Data of Compounds **4** and **5** in CD_3OD at 600 MHz[a]

	4			**5**	
position	^1H	^{13}C	position	^1H	^{13}C
2		165.90	l2		165.90
3	6.55, s	101.71	3	6.61, s	103.73
4		183.65	4		183.83
5		162.54	5		162.94
6	6.21, s	99.94	6		105.05
7		164.50	7		164.92
8		105.41	8	6.51, s	95.94
9		158.11	9		158.81
10		105.81	10		104.75
1′		122.91	1′		123.03
2′	7.96, br d (8.7)	129.19	2′	7.86, d (8.7)	129.35
3′	6.94, d (8.7)	115.95	3′	6.95, d (8.7)	117.06
4′		162.14	4′	6.95, d (8.7)	162.02
5′	6.94, d (8.7)	115.95	5′	7.86, d (8.7)	117.06
6′	7.96, br d (8.7)	129.19	6′		129.42
Gluc8			Gluc 6		
1	5.03, d (9.5)	74.63	1	4.93, d (9.5)	75.34
2	4.15, t (9.5)	72.43	2	4.21, t (9.5)	72.52
3	3.55, m	78.91	3	3.50, m	80.20
4	3.65, t (9.5)	71.23	4	3.51, t (9.5)	71.73
5	3.51, m	80.21	5	3.45, m	81.20
6	3.92, dd (12.0, 5.3) 3.82, dd (12.0, 3.3)	68.16	6	3.77, (12.0, 5.3) 3.90, (12.0,3.3)	69.27
Ara			Ara		
1	5.12, d (5.2)	105.30	1	5.14, d (5.2)	104.88
2	3.84, dd (5.2, 8.7)	74.40	2	3.89, dd (5.2, 8.7)	74.26
3	3.81, dd (8.7, 3.0)	72.41	3	3.87, dd (8.7, 3.0)	72.13
4	3.80, m	69.56	4	3.84, m	69.40
5	3.48, dd (12.0, 3.0) 3.96, dd (12.0, 2.0)	65.67	5	3.49, dd (12.0, 3.0) 4.02, dd (12.0, 2.0)	66.18

[a] Chemical shifts are in ppm from TMS, and *J* values in Hz are presented in parentheses. All signals were assigned by 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments.

μL of this solution was injected for analysis. Triplicate injections were made. The amount of the compounds was finally expressed as milligrams per milliliter of hydroalcoholic extract (TLL). Data are reported as mean ± standard deviation (SD) of triplicate determinations.

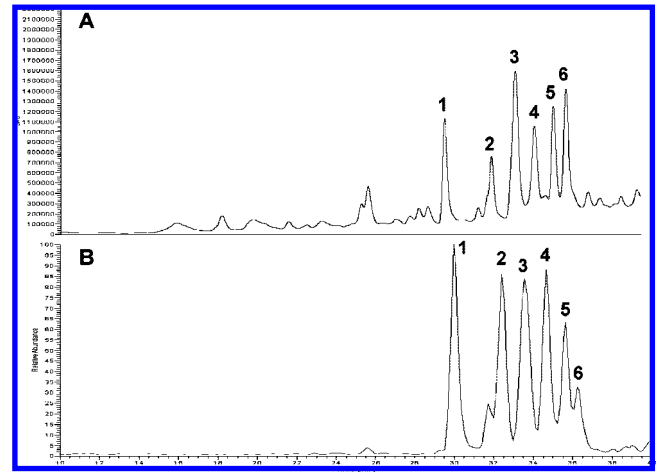

Figure 1. HPLC profile of TLL: (**A**) UV chromatogram (278 nm); (**B**) (+)-ESI-MS chromatogram.

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. Human 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*g* for 10 min at room temperature. After isolation of supernatant (platelet-rich plasma; PRP), the remaining plasma was recentrifuged at 1000*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). The *Citrus* extract was diluted with a phosphate-buffered saline (PBS) solution at pH 7.4 to assess its effect on platelet aggregation. A solution of acetylsalycilic acid (ASA) in 1% sodium bicarbonate was used to assess the effect of the drug (1 mg/mL in the incubation media) in the same conditions used for the lime extract. 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 (*11*) was applied to measure platelet aggregation, using a CLOT 2S Aggregometer (Seac and Radim Group, Rome, Italy). Five

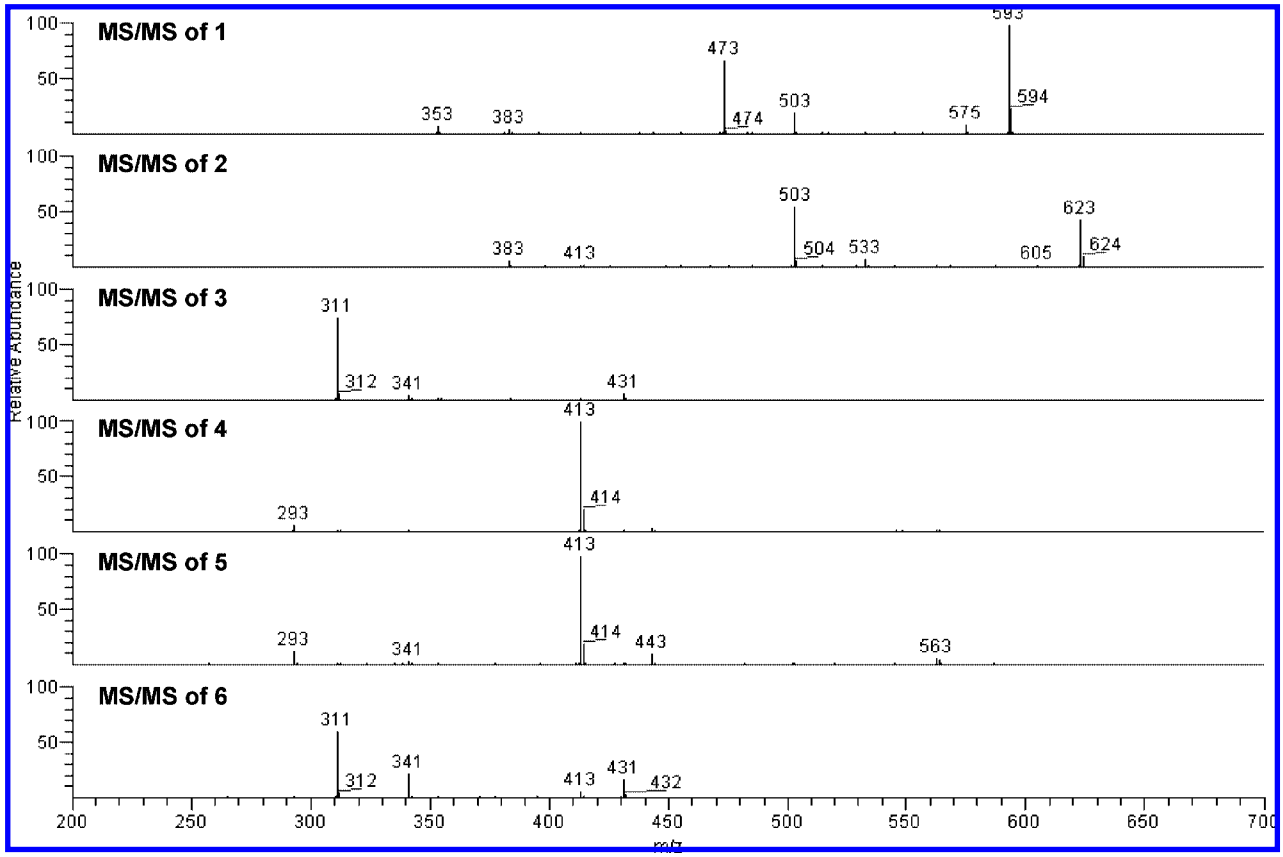

Figure 2. (−)-ESI-MS/MS spectra of peaks 1–6.

microliters of different dilutions of *Citrus* extract in PBS (0.1, 0.3, and 1 mg/mL) or PBS (control) was added to 280 μL aliquots of PRP in aggregometer cuvettes. Successively, 15 μL of ADP, epinephrine, collagen, or arachidonic acid (final concentrations in the incubation media being 5 μmol/L, 5 μmol/L, 2 μmol/mL, and 0.5 mmol/L, respectively) were added after 2 min of preincubation 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 − (platelet aggregation of sample/platelet aggregation of control)] × 100%. Each sample was measured in triplicate. IC_{50} values (the concentration necessary to reduce the induced platelet aggregation by 50% with respect to control) were obtained from concentration−effect curves.

Statistical Analysis. Experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared with those for controls by the Student's *t* test, and *p* values of <0.05 were considered to be statistically significant. For HPLC quantitative analysis data are reported as mean ± SD of triplicate determinations. The statistical analysis was carried out using the Microsoft Excel software package (Microsoft Corp.).

RESULTS AND DISCUSSION

HPLC-PDA-ESI-MS Analysis. The TLL was analyzed by HPLC-PDA-ESI-MS^n to investigate the flavonoid profile. **Figure 1** shows the simultaneous HPLC-ESI-MS and HPLC-UV chromatograms of the lime extract. Six major peaks (1–6) were detected for TLL and their retention times, UV λ_{max} values, pseudomolecular ions, and MS/MS fragments are listed in **Table 1**.

All compounds showed similar UV spectra with their absorption maxima (λ_{max}) between 330 and 345 nm (band I associated with absorption of the B ring cinnamoyl system) and at 270 nm (band II due to the A ring benzoyl system). These UV spectra are typical of flavone derivatives (*12*).

To evaluate the information generated online by LC-ESI-MS/MS analysis of TLL, both positive and negative ionization modes have been performed. The ESI-MS/MS fragmentation patterns of peaks 1–3 and 6, in negative and positive modes, are in agreement with those of *C*-glycoside derivatives. The characteristic fragment ions of the *C*-glycoside flavones, obtained by selecting the $[M + H]^+$ as a precursor ion, were $^{0.4}X^+ - 2H_2O$ $[M + H - 96]^+$, $^{0.3}X^+$ $[M + H - 90]^+$, $^{0.2}X^+$ $[M + H - 120]^+$, and $^{0.1}X^+$ $[M + H - 150]^+$ (*13*). Instead, in negative mode, the ESI-MS/MS spectra of *C*-glycoside derivatives show fragments generated by typical losses of 18 (E_1^-), 90 ($^{0.3}X^-$), and 120 ($^{0.2}X$) mass units from the $[M - H]^-$ ion (*13*).

Peak 1 presented a UV spectrum with λ_{max} at 270 and 330 nm, indicating that this compound was a flavone derivative with one hydroxyl in ring B (*1*). The (+) and (−)-ESI-MS spectra showed pseudomolecular ions at *m/z* 595 ($[M + H]^+$) and 593 ($[M - H]^-$), respectively. MS/MS analysis of these ions provided a typical fragmentation of 6,8-di-*C*-hexosyl flavones, particularly, the fragment ions in (−)-MS/MS spectrum at *m/z* 503 ($[M - H - 90]^-$), 473 ($[M - H - 120]^-$), 383 ($[M - H - 90 - 120]^-$), and 353 ($[M - H - 2 \times 120]^-$) (*1, 14*) (**Figure 2**). The ions at *m/z* 353 ([aglycone + 83]$^-$) and 383 ([aglycone + 113]$^-$) indicated the aglycone as a trihydroxyflavone. These mass and UV data indicated for peak 1 the structure of 6,8-di-*C*-hexosyltrihydroxyflavone, and they are superimposable with those reported for 6,8-di-*C*-glucosylapigenin (*1, 9, 14, 15*).

Peak 2 showed two λ_{max} at 270 and 345 nm, suggesting the presence of a flavone disubstituted in ring B (*1*). The protonated and deprotonated molecules in (+) and (−)-ESI-MS spectra of **2** were at *m/z* 625 and 623, respectively, which were 30 mass

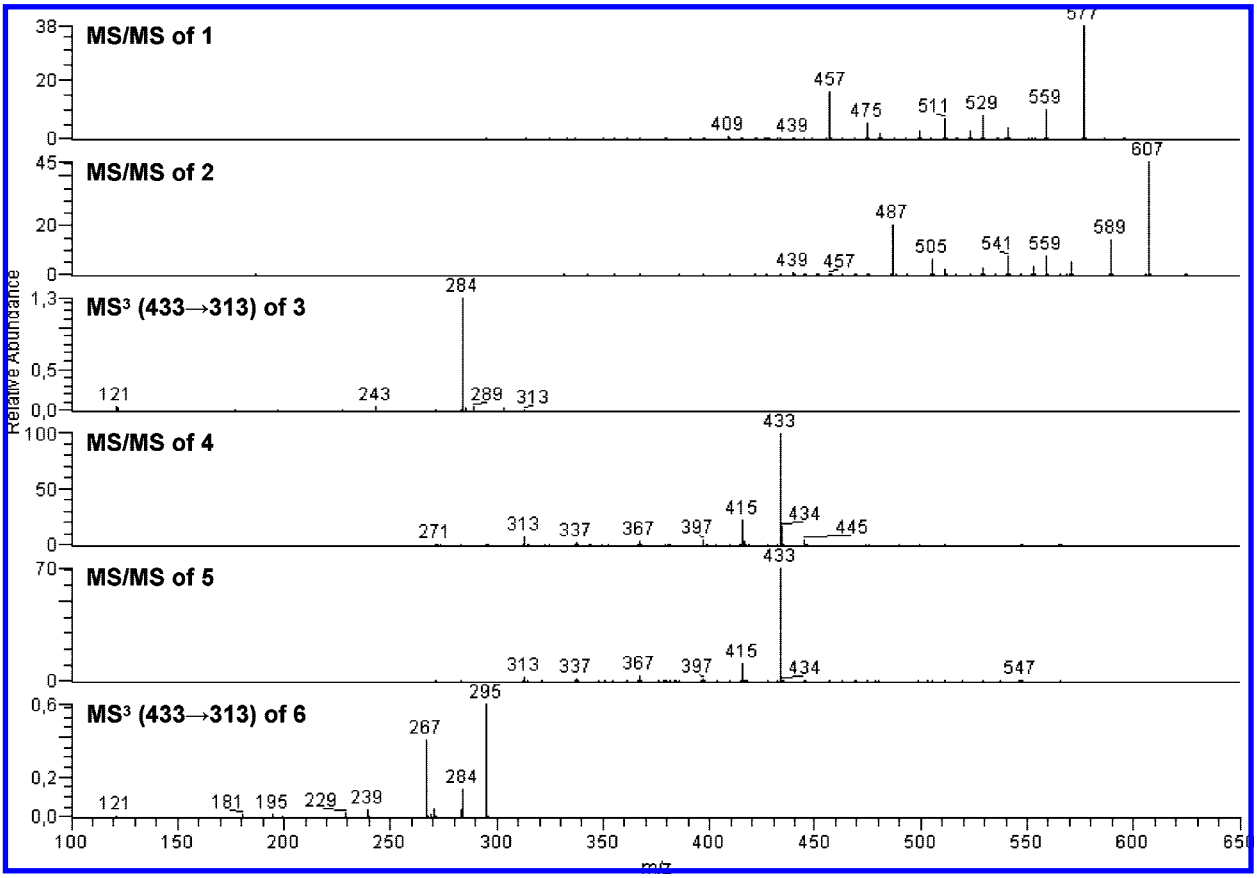

Figure 3. (+)-ESI-MS^n spectra of peaks 1–6.

units higher than that of **1**, revealing the presence of an extra methoxyl group. The MS/MS spectra of peak 1 and 2 showed the same type of fragmentation ($[M - H - 90]^-$, $[M - H - 120]^-$, $[M - H - 90 - 120]^-$, and $[M - H - 2 \times 120]^-$) (**Figure 2**), indicating that the two compounds had the same glycosylation. On the basis of these data, peak 2 was a 6,8-di-*C*-hexosyltrihydromethoxyflavone, and comparison with UV and MS data reported in the literature suggested that **2** could be 6,8-di-*C*-glucosyldiosmetin or 6,8-di-*C*-glucosylchrysoeriol (*1, 9, 14, 15*).

As for peak 1, UV spectra of peaks 3 and 6 indicated that these compounds were flavone derivatives with one hydroxyl in ring B (*1*). The MS spectra for peaks 3 and 6 showed a pseudomolecular ion $[M + H]^+$ at *m/z* 433, in positive mode, and a $[M - H]^-$ ion at *m/z* 431, in negative mode. Moreover, they exhibited the same MS/MS fragments in both positive and negative modes. These data suggested that peaks 3 and 6 were *C*-glycosyl isomers of apigenin. In (+)- and (−)-MS/MS spectra of peaks 3 and 6, no diagnostic fragment ions were observed; therefore, the MS/MS spectra of fragment ions $^{0,2}X^+$ $[M + H - 120]^+$ of **3** and **6** were recorded in the MS^3 mode (433 → 313), using the multiple-stage MS/MS experiments, in order to obtain MS/MS spectra that exhibited differences between 6-*C* and 8-*C* isomers (*13*). As shown in **Figure 3** the MS^3 spectrum of **6** showed ions at *m/z* 195 and 121, but **3** exhibited the ion only at *m/z* 121. Taking into account this difference, it was possible to discriminate between 6-*C* and 8-*C* glycosidic flavonoids as previously reported in the literature (*13*) and characterize peak 3 as 8-*C*-glucosylapigenin (vitexin) and peak 6 as 6-*C*-glucosylapigenin (isovitexin). The proposed structures of peaks 3 and 6 were confirmed by comparison of retention time, UV spectra, and MS data with vitexin and isovitexin standards.

The analysis of UV spectra of peaks 4 and 5 indicated, as for peaks 1, 3, and 6, the presence of a flavone moiety with one hydroxyl in ring B. Peaks 4 and 5 showed the same (+)- and (−)-ESI-MS spectra ($[M + H]^+$ at *m/z* 565 and $[M - H]^-$ ion at *m/z* 563), suggesting that the compounds were isomers. MS/MS spectra of the $[M + H]^+$ ion showed a fragment ion at *m/z* 433 for both peaks (**Figure 3**), showing a loss of 132 mass units, being characteristic of a pentose sugar linking by an *O*-glycosidic linkage. The presence of an ion at *m/z* 433 in the MS/MS spectra of peaks 4 and 5 suggested that these compounds were *O*-glycosyl derivatives of vitexin (**3**) and/or isovitexin (**6**). Multiple-stage MS/MS experiments performed on **4** and **5** gave results similar to those obtained for **3** and **6**, respectively. The MS^3 spectra (565 → 433) of the ion at *m/z* 433 were similar to the MS^2 spectrum of vitexin (**3**) for **4** and the MS^2 spectrum of isovitexin (**6**) for **5**. The MS^4 spectra (433 → 313) of $^{0,2}X^+$ $[M + H - 132 - 120]^+$ gave the same diagnostic ions as those observed in the MS^3 spectra of **3** and **6**, proving that C-glycosylation occurred at position 8 in **4** and at position 6 in **5**. These pieces of evidence suggested for peaks 4 and 5 the structures of *O*-pentosylvitexin and *O*-pentosylisovitexin, respectively.

To confirm the pentose link to glucose of vitexin and isovitexin, through an O-glycosidic linkage, the TLL was submitted to acid hydrolysis. The HPLC chromatogram (**Figure 4**) after acid hydrolysis showed the disappearance of peaks 4 and 5 and an increase in intensity for peaks 3 and 6, in agreement with the proposed structures of *O*-pentosylvitexin

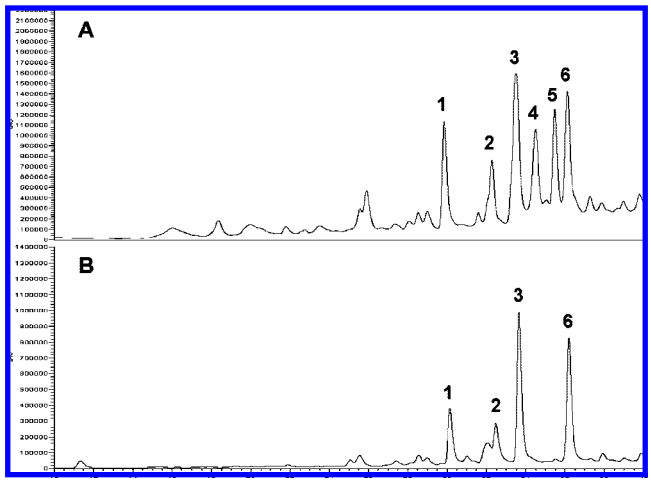

Figure 4. HPLC chromatogram at 278 nm of TLL before (**A**) and after acid hydrolysis (**B**).

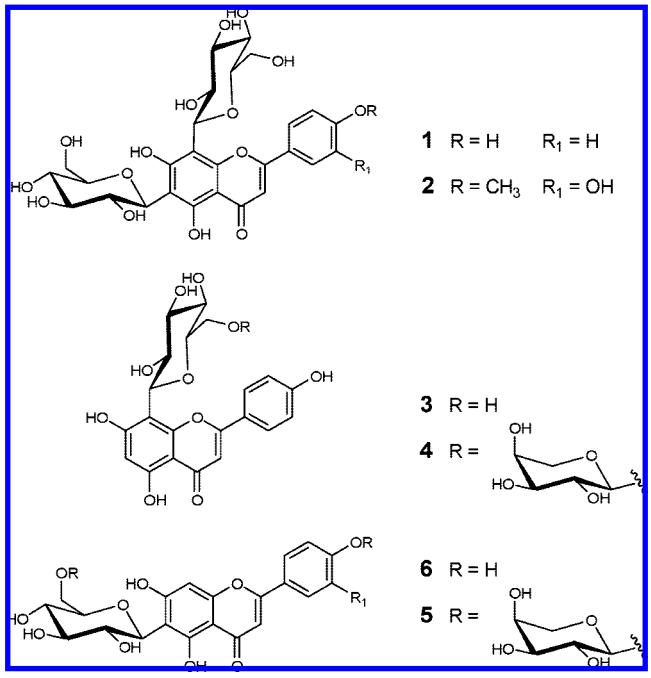

Figure 5. Structures of compounds **1–6** identified in TLL.

for **4** and *O*-pentosylisovitexin for **5**. In addition, the results of acid hydrolysis confirmed the C-glycosidic linkage in peaks 1–3 and 6 rather than the O-glycosidic linkage.

Isolation and Structure Determination of Compounds 1–6. To confirm the proposed structures of compounds **1** and **2** and identify the type and position of sugar residues of compounds **4** and **5**, the isolation procedure of TLL was undertaken. The lime extract was fractionated preliminarily on Sephadex LH-20. Repeated column chromatography of the LH-20 fractions on reversed-phase HPLC yielded two new compounds, **4** and **5**, along with four known compounds, **1–3** and **6** (**Figure 5**).

The molecular formula of compounds **4** and **5** was determined to be $C_{26}H_{28}O_{14}$ by HRESI-MS and ^{13}C NMR spectra analyses. The ^{13}C NMR spectrum showed 26 signals, 15 of them corresponding to the aglycone moiety (**Table 2**). ^{1}H NMR analysis of the aglycone moieties revealed resonances at δ 7.96 (2H, br d, $J = 8.7$ Hz) and 6.94 (2H, d, $J = 8.7$ Hz) for **4** and at δ 7.86 (2H, d, $J = 8.7$ Hz) and 6.95 (2H, d, $J = 8.7$ Hz), for **5**, corresponding to their para-substituted flavonoid B-rings, at δ 6.55 (1H, s) and 6.61 (1H, s), respectively, which are typical of flavonoid position 3 vinyl protons (*16*), and at δ 6.21 (1H, s, H-6) and 6.51 (1H, s, H-8), respectively, in accordance with an 8-C and 6-C-substituted apigenin. Furthermore, resonances of anomeric protons were observed in the ^{1}H NMR spectra at δ 5.03 (1H, d, $J = 9.5$ Hz) and 5.12 (1H, d, $J = 5.2$ Hz) for **4** and at δ 4.93 (1H, d, $J = 9.5$ Hz) and 5.14 (1H, d, $J = 5.2$ Hz) for **5**. 1D-TOCSY, DQF-COSY, and HSQC NMR experiments showed the presence of one α-L-arabinopyranosyl unit (δ 5.12 and 5.14) and one β-D-glucopyranosyl unit (δ 5.03 and 4.93), respectively, in the structures of **4** and **5** (**Table 2**). The HSQC spectra also showed glycosylation shifts for the C-6″ (ca. 6.0 ppm) of the glucopyranosyl units, suggesting that α-L-arabinopyranosyl was a terminal unit linked at this position of both compounds.

The C−C linkages of the 8-*C*- and 6-*C*-glucosyl were confirmed by the HMBC cross-peaks at δ 6.21/74.63 (H-6/C-1″) and δ 5.03/164.50 (H-1″/C-7) for **4** and at δ 6.51/75.34 (H-8/C-1″) and δ 4.93/164.92 (H-1″C-7) for **5**. The C-6″ substitution of the glucopyranosyl moieties with an arabinopyranosyl unit for both compounds was confirmed by the HMBC cross-peak observed between the anomeric proton of the arabinose and the C-6″ of the glucose (δ 5.12/68.16 for **4** and δ 5.14/69.27 for **5**). The β-configuration at the anomeric position for the glucopyranosyl units ($J_{H1-H2} = 9.5$ Hz) was easily seen from their relatively large $^{3}J_{H1-H2}$ coupling constants, whereas the α-configuration for the arabinopyranosyl unit ($J_{H1-H2} = 5.2$ Hz) was established by the results of ROESY experiments as previously reported (*17*). On the basis of this evidence, the new compounds **4** and **5** were established as apigenin 8-*C*-[α-L-arabinopyranosyl-(1→6)]-*O*-β-D-glucopyranoside and apigenin 6-*C*-[α-L-arabinopyranosyl-(1→6)]-*O*-β-D-glucopyranoside, respectively.

Known compounds **1**, **2**, **3**, and **6** were characterized as apigenin-6,8-di-*C*-β-D-glucopyranoside (vicenin II) (**1**), diosmetin-6,8-di-*C*-β-D-glucopyranoside (**2**), apigenin-8-*C*-β-D-glucopyranoside (vitexin) (**3**), and apigenin-6-*C*-β-D-glucopyranoside (isovitexin) (**6**), respectively. The structures of the isolated compounds were established by ^{1}H and ^{13}C NMR data and confirmed by comparison with those reported in the literature (*8–10*). For compound **2** the presence of the methoxyl group and its location at the C-4′ position of ring B were established on the basis of the ^{13}C NMR spectra and HMBC data with respect to an unmethoxylated model. A reference methanol/water solution of pure compounds **1–6** (0.1 mg/mL, each) was prepared, and each solution was subjected to LC-PDA-MS to determine the retention times (t_R), UV, and MS data of the isolated compounds and to compare with the peaks present in the chromatograms of TLL. In this way, peaks 1–6 were identified as compounds **1–6**.

Quantitative Analysis. The quantitative analysis of the *C*-glycosyl flavones of lime hydroalcoholic extract was performed by HPLC-PDA. The concentrations of each compound in the extract, calculated from the experimental peak areas by interpolation to standard calibration curves, were 0.32 ± 0.002, 0.22 ± 0.002, 0.56 ± 0.005, 0.32 ± 0.002, 0.26 ± 0.001, and 0.35 ± 0.004 mg/mL of TLL for compounds **1–6**, respectively. Relative standard deviations were in the range of 3.8–11.4% calculated as the mean of three replications, whereas those for retention times were <0.3%.

C-Glycosyl flavone intake (2.05 mg/mL) of TLL appears to be very remarkable in view of their well-known biological activity. In the literature on *Citrus* flavonoids, a broad spectrum of biological activity including anticarcinogenic and antitumor activities has been discussed (*18, 19*); moreover, the antiplatelet

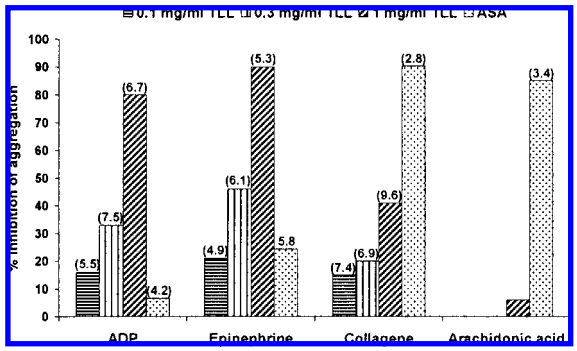

Figure 6. Inhibition of ADP-, epinephrine-, collagen-, and arachidonic acid-induced platelet aggregation by TLL at three concentration levels. Results are expressed as the mean, SEM in parentheses; $N = 5$.

in vitro effect of flavonoids from citric plants has been demonstrated (*4*), suggesting the biological potential of extracts prepared from different parts of these plants. The proposed HPLC-PDA-MS method may be considered as suitable for routine analysis of either the plant material or its derived products and, thus, to be the starting point of quality control protocols

Antiplatelet Activity of TLL. PA was induced in human PRP by platelet aggregation inducers (ADP, epinephrine, collagen, and arachidonic acid). Different dilutions of *Citrus* extract (0.1, 0.3, and 1 mg/mL) or PBS (control) were added to the PRP before the addition of aggregation-inducing agents. Aggregation was determined by platelet aggregometer and expressed as percentage of inhibition. The antiplatelet activity of TLL was compared with that of ASA, usually used in antiplatelet therapy.

As shown in **Figure 6**, the hydroalcholic extract of lime leaves (TLL) inhibited significantly ADP- and epinephrine-induced platelet aggregation in a concentration-dependent manner. The IC_{50} values were 0.4 and 0.325 mg/mL, respectively. However, platelet aggregation induced by collagen was not statistically modified; the percentage of inhibition attained with the maximal TLL concentration assessed (1 mg/mL) as lower than 50% (**Figure 6**). Arachidonic acid-induced platelet aggregation was unaltered by TLL at the concentration of 1 mg/mL (53.5 ± 7.6 vs 57.0 ± 7.9% in control, % inhibition of PA = 6, $p = 0.21$). As expected, ASA significantly inhibited collagen- and arachidonic acid-induced platelet aggregation, whereas ADP- and epinephrine-induced aggregations were unaffected (**Figure 6**).

Platelet aggregation induced by ADP is a two-phase event; the first one is related with the activation of the purinergic receptors P2Y1 and P2Y12 and the second one is due to TXA_2 formed during platelet release reaction; therefore, an agent may act separately on both phases of aggregation. TLL prevented ADP- and epinephrine-induced platelet aggregation in a similar way. Both agonists stimulate different receptors on the platelet membrane (*20–22*); however, these mechanisms of platelet activation are associated with the inhibition of the adenylcyclase activity (*23*) and the release of TXA_2 and ADP from platelets (*24–26*). The lack of effect against arachidonic acid as well as the insignificant reduction of collagen-induced platelet aggregation makes it improbable that the extract is functioning through an aspirin-like mechanism of action (*27*). Antiplatelet therapy is recommended in patients with a high risk of atherosclerotic diseases. There is still the need of new antiplatelet options with inhibitory effect of a wide range of stimuli of platelet aggregation and without undesirable side effects. The antiplatelet in vitro effects of flavonoids from *Citrus* plants have been demonstrated, suggesting the antiplatelet potential of extracts prepared from different parts of these plants.

This research has provided evidence about the chemical composition and antiplatelet effects of TLL; therefore, *C. aurantifolia* leaves possibly will become in the near future a plant of interest for pharmaceutical and dietary supplement companies. Chemical and biological information obtained could be significant not only for understanding folk utilization of lime leaves but also for the future validation of isolated compounds as markers for the quality control of this botanical product.

LITERATURE CITED

(1) Gil-Izquierdo, A.; Riquelme, M. T.; Porras, I.; Ferreres, F. Effect of the rootstock and interstock grafted in lemon tree (*Citrus limon* (L.) Burm.) on the flavonoid content of lemon juice. *J. Agric. Food Chem.* **2004**, *52*, 324–331.

(2) Fuentes, V.; Granda, M. Estudio sobre la medicina tradicional en Cuba. *Rev. Cub. Farm.* **1988**, *22*, 77–90.

(3) Bocco, A.; Cuvelier, M.-E.; Richard, H.; Berset, C. Antioxidant activity and phenolic composition of *Citrus* peel and seed extracts. *J. Agric. Food Chem.* **1998**, *46*, 2123–2129.

(4) Cook, N. C.; Samman, S. Flavonoids—chemistry, metabolism, cardioprotective effects, and dietary sources. *J. Nutr. Biochem.* **1996**, *7*, 66–76.

(5) Benavente-Garcia, O.; Castillo, J.; Marin, F. R.; Ortuno, A.; Del Rio, J. A. Uses and properties of *Citrus* flavonoids. *J. Agric. Food Chem.* **1997**, *45*, 4505–4515.

(6) Bocco, A.; Cuvelier, M.-E.; Richard, H.; Berset, C. Antioxidant activity and phenolic composition of *Citrus* peel and seed extracts. *J. Agric. Food Chem.* **1998**, *46*, 2123–2129.

(7) Nogata, Y.; Yoza, K.-I.; Kusumoto, K.-I.; Kohyama, N.; Sekiya, K.; Ohta, H. Screening for inhibitory activity of *Citrus* fruit extracts against platelet cyclooxygenase and lipoxygenase. *J. Agric. Food Chem.* **1996**, *44*, 725–729.

(8) Siciliano, T.; De Tommasi, N.; Morelli, I.; Braca, A. Study of *Sechium edule* (Jacq) Swartz (Cucurbitaceae) different edible organs by liquid chromatography photodiode array mass spectrometry. *J. Agric. Food Chem.* **2004**, *52*, 6510–6515.

(9) Caristi, C.; Bellocco, E.; Panzera, V.; Toscano, G.; Vadala, R.; Leuzzi, U. Flavonoids detection by HPLC-DAD-MS-MS in lemon juices from Sicilian cultivars. *J. Agric. Food Chem.* **2003**, *51*, 3528–3534.

(10) Peng, J.; Fan, G.; Hong, Z.; Chai, Y.; Wu, Y. Preparative separation of isovitexin and isoorientin from *Patrinia villosa* Juss by high-speed counter-current chromatography. *J. Chromatogr., A* **2005**, *1074*, 111–115.

(11) Born, G. V. R.; Cross, M. J. J. The aggregation of blood platelets. *J. Physiol.* **1968**, *108*, 178–183.

(12) Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The Systematic Identification of Flavonoids*; Springer-Verlag: New York, 1970.

(13) Waridel, P.; Wolfender, J.; Ndjoko, K.; Hobby, K. R.; Major, H. J.; Hostettmann, K. Evaluation of quadrupole time-of-flight tandem mass spectrometry and ion-trap multiple-stage mass spectrometry for the differentiation of *C*-glicosidic flavonoids isomers. *J. Chromatogr., A* **2001**, *926*, 29–41.

(14) Ferreres, F.; Silva, B. M.; Andrade, P. B.; Seabra, R. M.; Ferreira, M. A. Approach to the study of *C*-glycosyl flavones by ion trap HPLC-PDA-ESI/MS/MS: Application to seeds of quince (*Cydonia oblonga*). *Phytochem. Anal.* **2003**, *14*, 352–359.

(15) Caristi, C.; Bellocco, E.; Gargiulli, C.; Toscano, G.; Leuzzi, U. Flavone-di-*C*-glycosides in *Citrus* juices from southern Italy. *Food Chem.* **2006**, *95*, 431–437.

(16) Harborne, J. B.; Mabry, T. J. *The Flavonoids: Advances in Research*; Chapman and Hall: New York, 1982.

(17) Rastrelli, L.; Aquino, R.; Abdo, S.; Proto, M.; De Simone, F.; De Tommasi, N. Studies on the constituents of *Amaranthus caudatus* leaves. Isolation and characterization of new triterpene

saponins and ionol glycosides. *J. Agric. Food Chem.* **1998**, *46*, 1797–1804.

(18) Tripoli, E.; La Guardia, M.; Giammanco, S.; DiMajo, D.; Giammanco, M. *Citrus* flavonoids: molecular structure, biological activity and nutritional properties: a review. *Food Chem.* **2007**, *104*, 466–479.

(19) Manthey, J. A.; Guthrie, N. Antiproliferative activities of *Citrus* flavonoids against six human cancer cell lines. *J. Agric. Food Chem.* **2002**, *50*, 5837–5843.

(20) Mihta, J.; Mihta, P.; Krap, Y.; Lausen, D. The primary wave of epinephrine-induced platelet aggregation represents α2-adrenoceptor status. *Thromb. Res.* **1988**, *49*, 531–537.

(21) Gachet, C. L.; Hechler, B.; Leon, C.; Vial, C.; Leray, C.; Ohlman, P. ADP receptor and platelet function. *Thromb. Haemost.* **1997**, *78*, 271–275.

(22) Spalding, A.; Vaitkevicius, H.; Dill, S.; MacKenzie, S.; Schmaier, A.; Lockette, W. Mechanism of epinephrine-induced platelet aggregation. *Hypertension* **1998**, *31*, 603–607.

(23) Ware, J. A.; Smith, M.; Salzman, E. W. Synergism of platelet-aggregating agents. Role of elevation of cytoplasmic calcium. *J. Clin. Invest.* **1987**, *80*, 267–73.

(24) Levy-Toledano, S.; Gallet, C.; Nadel, F.; Bryckaert, M.; Maclout, J.; Rosa, J. Phosphorylation and dephosphorylation mechanisms in platelet function. A tightly regulated balance. *Thromb. Haemost.* **1997**, *78*, 226–233.

(25) Kahner, B. N.; Shankar, H.; Murugappan, S.; Prasad, G. L.; Kunapuli, S. P. Nucleotide receptor signaling in platelets. *Thromb. Haemost.* **2006**, *4*, 2317–2326.

(26) Figures, W. R.; Scearce, L. M.; Wachtfogel, Y.; Chen, J.; Colman, R. F. Platelet ADP receptor and α2-adrenoreceptor interaction. Evidence for an ADP requirement for epinephrine-induced platelet activation and an influence of epinephrine on ADP binding. *J. Biol. Chem.* **1986**, *261*, 5981–5986.

(27) Parise, L. V.; Venton, D. L.; Le Breton, G. C. Arachidonic acid-induced platelet aggregation is mediated by a thromboxane A2/prostaglandin H2 receptor interaction. *J. Pharmacol. Exp. Ther.* **1984**, *228*, 240244.

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