JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Cuban and Brazilian Red Propolis: Botanical Origin and Comparative Analysis by High-Performance Liquid Chromatography−Photodiode Array Detection/Electrospray Ionization Tandem Mass Spectrometry

Anna Lisa Piccinelli,[†] Cinzia Lotti,[†] Luca Campone,[†] Osmany Cuesta-Rubio,[‡] Mercedes Campo Fernandez,[‡] and Luca Rastrelli[†,*]

[†]Dipartimento di Scienze Farmaceutiche e Biomediche, Università di Salerno, Via Ponte Don Melillo, 84084, Fisciano, Salerno, Italy

[‡]Instituto de Farmacia y Alimentos (IFAL), Universidad de La Habana, Ave. 23, 21425, Lisa, La Habana, CP. 13600, Cuba

Supporting Information

ABSTRACT: Chemical composition of propolis depends on the specificity of the local flora at the site of collection and thus on the geographic and climatic characteristics of this place. This paper describes a comparative analysis of Cuban red propolis (CRP), Brazilian red propolis (BRP), and *Dalbergia ecastophyllum* exudates (DEE) by high-performance liquid chromatography with diode-array detection and tandem mass spectrometry. The aim of this study was to investigate the overall chemical profile and the botanical origin of red propolis and to suggest similarities and differences between samples collected in different tropical regions. Isoliquiritigenin (**1**), liquiritigenin and naringenin (**2** and **17**), isoflavones (**3**−**4** and **16**), isoflavans (**5**−**7** and **18**), and pterocarpans (**8**−**13**) were detected in CRP, BRP, and DEE, whereas polyisoprenylated benzophenones (PPBs) guttiferone E/xanthochymol (**14a**,**b**) and oblongifolin A (**15**) were detected only in BRP. Pigments responsible for the red color of DEE and red propolis were also identified as two C30 isoflavans, the new retusapurpurin B (**19**) and retusapurpurin A (**20**). PPBs and pigments were isolated and unambiguously characterized by 1D and 2D NMR analysis. These results show that red propolis samples from different tropical zones have a similar chemical composition. DEE is the main red propolis source, but the presence of PPBs in BRP suggests the contribution of different botanical sources for Brazilian samples. This chemical information is important for quality control of red propolis and its commercial products and for biological study.

KEYWORDS: red propolis, *Dalbergia ecastophyllum*, tandem mass spectrometry, retusapurpurin A and B, isoflavonoids, polyisoprenylated benzophenones

INTRODUCTION

Propolis is a resinous hive product containing beeswax, sugar, and plant exudates collected by honeybees from various plant sources. Bees use propolis for diverse purposes, among them to seal openings in the hive and as a chemical weapon against microorganisms and insects. This natural role of defense gives to the propolis a broad spectrum of biological activities, which validate its wide use as remedies for the treatment of various conditions, including inflammations, wounds, or burns, sore throat, viral diseases, and ulcers. Propolis is actually marketed by the pharmaceutical industry and health food stores for its claimed beneficial and preventive effects on human health.[1−4]

In the past decade, knowledge concerning propolis chemistry has radically changed. The analysis of numerous samples of propolis revealed that the composition of propolis is extremely variable and the vegetation at the site of collection determines its chemical diversity. This fact results in the striking diversity of propolis chemical composition, especially of propolis originating from tropical regions. In addition, differences are also found among tropical samples. Brazilian propolis was classified in 12 types according to geographical origin, chemical composition, and source plant.[5] The most popular is the green or Alecrim propolis, which originates from *Baccharis dracunculifolia*, and artepillin C was recognized as characteristic constituent of this propolis type.[6] Three main types of Cuban propolis (brown, yellow, and red) were characterized based on their secondary metabolite classes.[7] Brown-type is rich in polyisoprenylated benzophenones (PPBs) and originates from floral resin of *Clusia rosea*, yellow-type contains triterpenoids belonging to oleanane, lupane, ursane, and lanostane skeletons,[8] while chalcones, pterocarpans, isoflavans, and isoflavones were the main constituents of red propolis samples.[9] The majority of the compounds of Cuban red propolis (CRP) type have been reported from the plant belonging to genus *Dalbergia*.[10−16] Also, Nepalese propolis, which originates from *Dalbergia* resin, mainly contains neoflavonoids, chalcones, and pterocarpans.[17] Thus, the possible source of CRP might be plants belonging to species of this genus.

Recently, red propolis samples were collected in the North regions of Brazil and classified as a new type of Brazilian propolis.[5] The botanical origin of Brazilian red propolis (BRP) was identified as resinous exudates of *Dalbergia ecastophyllum*.[18,19] This type of Brazilian propolis demonstrated a notable antimicrobial activity against the microorganisms *Staphylococcus aureus* and *mutans*[20] and a 100% preferential cytotoxicity against human pancreatic PANC-1 cancer cell line in nutrient-deprived medium

Received: March 31, 2011
Accepted: May 20, 2011
Revised: May 18, 2011
Published: May 20, 2011

ACS Publications © 2011 American Chemical Society

dx.doi.org/10.1021/jf201280z | J. Agric. Food Chem. 2011, 59, 6484–6491

at the concentration of 10 μg/mL.[21] Isolated compounds from BRP collected in Northeastern Brazil comprised mainly flavonoids, isoflavonoids, neoflavonoids, chalcones, and PPBs.[21,22] These chemical data suggested a similar composition to that of Cuban red propolis but also some differences and a common plant source for CRP and BRP.

In this study, a comparative analysis by HPLC-DAD-MS of CRP, BRP, and *D. ecastophyllum* exudates (DEE) was performed to investigate the chemistry and botanical origin of red propolis originating from different tropical regions. According the noted differences, PPBs of BRP were isolated and characterized such as the pigments responsible for the characteristic color of DEE and red propolis (CRP and BRP).

■ MATERIALS AND METHODS

Chemicals. Naringenin was purchased from Sigma-Aldrich. HPLC-grade acetonitrile (AcCN), water, and methanol (MeOH) were purchased from Romil Ltd. (Cambridge, U.K.). Dichloromethane, trifluoroacetic acid (TFA), formic acid, and silica gel (0.04−0.63 mm and 0.015−0.025 mm) were purchased from Sigma-Aldrich (Milano, Italy).

Propolis and Plant Material. CRP sample (Pinar del Rio, Cuba) was provided by "La Estación Experimental Apicola" and characterized by spectroscopic techniques.[7] Brazilian red propolis (BRP) sample (Alagoas, Brazil) and *D. ecastophyllum* exudates (DEE), collected near beehives producing BRP, were supplied by Biosline SpA (Padova, Italy). Raw materials of CRP, BRP, and DEE were frozen at −20 °C overnight and then rapidly ground in a mortar to obtain homogeneous powders. Ethanol extracts of CRP, BRP, and DEE were obtained by maceration of ground sample (10 g) with ethanol (100 mL) in a closed dark bottle during one day. Ethanol extracts were evaporated at 40 °C under reduced pressure to obtain dry extracts. For HPLC analysis, ethanol solution of each extract (3 mg mL^{-1}) was prepared and acidified with 0.1% TFA. Compounds **1**−**13** were purified as reported elsewhere from CRP sample.[9]

General Experimental Procedures. HPLC-DAD-MS analyses were performed with a HPLC system (ThermoFinnigan, San Jose, CA) including a Surveyor LC pump, Surveyor autosampler, Surveyor PDA detector, and LCQ Advantage ion trap mass spectrometer equipped with Xcalibur 3.1 software. Preparative HPLC was performed on an Agilent 1100 series system (Agilent, Palo Alto, CA) consisting of a G-1312 binary pump, a G-1328A Rheodyne injector (100 μL loop), a G-1322A degasser, and a G-1315A photodiode array detector (PDA) using Luna C8 (250 mm × 10 mm i.d., 10 μm) or Kromasil C18 (250 mm × 10 mm i.d., 10 μm) columns from Phenomenex (Torrance, CA, USA). A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H 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. TLC analyses were performed with Macherey−Nagel precoated silica gel 60 F_{254} plates (Delchimica, Napoli, Italy).

HPLC-PDA-ESI-MS Analysis. HPLC separations were performed on a Luna C18 column (150 mm × 2.0 mm, 5 μm, Phenomenex) protected by its Guard Cartridge (4 mm × 2.0 mm i.d.) with a gradient of water (solvent A) and AcCN (solvent B), both containing 1% (v/v) formic acid. The following gradient was adopted: 0−6 min, isocratic on 20% B; 6−10 min, linear gradient from 20% to 30% B; 10−40 min, linear gradient from 30% to 40% B; 40−60 min, linear gradient from 40% to 60% B; 60−80 min, linear gradient from 60% to 98% B; 80−90 min, isocratic on 98% B. Elution was performed at a flow rate of 200 μL min^{-1}, and the volume of the injection was 5 μL. Detection by diode array was performed simultaneously at three different wavelengths: 278, 350, and 482 nm. The UV spectra were recorded with a 200−600 nm range. The mass analyses were performed with an ESI interface in the positive modes. The data were acquired in the full scan (range of *m*/*z* 200−1000) and tandem mass scanning modes, the maximum injection time was 100 ms, and the number of microscans was three. For the tandem mass scanning mode, the percentage of collision energy range was 32−45%. The optimized instrumental parameters were as follows: capillary temperature 300 °C; capillary voltage 3.0 V; spray voltage 5.10 kV; sheath gas flow rate 30 (nitrogen, arbitrary units); auxiliary gas flow rate 20 (arbitrary units).

Isolation Procedures for Compounds 14a,b, 15, and 16. A portion of BRP dry extract (3 g) was fractionated over a Sephadex LH-20 column (100 cm ×5.0 cm) using methanol as solvent. After TLC analysis, fractions with similar R_f values were combined in five major fractions (I−V) and analyzed by HPLC-DAD-MS to guide the isolation of compounds **14a**,**b**, **15**, and **16**. Fraction II, containing PPBs, was purified by HPLC-UV (280 nm) with a C8 column using isocratic elution (AcCN/H_2O 80:20, v/v, flow rate of 2.5 mL min^{-1}) to yield inseparable mixture guttiferone E/xanthochymol (**14a**,**b**) and oblongifolin A (**15**). Fraction IV was fractionated over a silica gel column using as solvent CH_2Cl_2/MeOH gradient (0−60% MeOH), and the fraction containing compound **16** was subjected to HPLC-UV (280 nm) on a C18 column using isocratic elution (MeOH/H_2O 55:45, v/v, flow rate of 3.0 mL min^{-1}) to yield pure compound 7,3′-dihydroxy-5′-methoxyisoflavone.

Isolation Procedure of Red Pigments, 19,20. A portion of DEE ethanol extract (2 g) was fractionated by vacuum liquid chromatography on a silica gel column with an $CHCl_3$/MeOH stepwise gradient from 100:0 to 70:30 (v/v) to afford six fractions. The fractions rich in pigments were further fractionated by preparative HPLC-UV (482 nm) on a C18 column with isocratic elution (AcCN/H_2O 45:55, v/v, with 0.1% TFA, flow rate of 2.5 mL min^{-1}) to yield pure compounds retusapurpurin A (**20**) and B (**19**).

Spectroscopic Data of Retusapurpurin B (19). $[\alpha]_D$ 112.96 (*c* 0.11, CH_3OH); (+)-HRESI-MS, *m*/*z* 523.1732 $[M]^+$, calcd for $C_{32}H_{27}O_7$ 523.1757; ^{1}H NMR (CD_3OD with TFA, 600 MHz) δ 4.32 (H-2, m), 3.78 (H-3, m), 3.23 (H-4, dd, 16.2, 6.0), 2.84 (H-4, dd, 16.2, 5.6), 6.92 (H-5, d, 8.0), 6.47 (H-6, dd, 8.0, 2.0), 6.27 (H-8, d, 2.0), 8.13 (H-3′, s), 7.72 (H-5′, s), 7.49 (H-8′, s), 8.29 (H-2″ and H-6″, d, 7.5), 7.08 (H-3″ and H-5″, d, 7.5), 6.63 (H-3‴, d, 1.5), 6.52 (H-5‴, dd, 8.0, 1.5), 7.07 (H-6‴, d, 8.0), 3.76 (7-OCH_3, s), 4.78 (2‴-OCH_3, s); ^{13}C NMR (CD_3OD with TFA, 600 MHz) δ 69.1 (C-2), 32.2 (C-3), 29.9 (C-4), 114.0 (C-4a), 131.1 (C-5), 108.4 (C-6), 160.6 (C-7), 102.5 (C-8), 156.7 (C-8a), 169.9 (C-2′), 114.6 (C-3′), 164.7 (C-4′), 118.5 (C-4′a), 130.2 (C-5′), 135.4 (C-6′), 159.8 (C-7′), 102.7 (C-8′), 167.2 (C-8′a), 121.2 (C-1″), 133.3 (C-2″), 118.3 (C-3″), 166.4 (C-4″), 118.3 (C-5″), 133.3 (C-6″), 115.1 (C-1‴), 160.6 (C-2‴), 100.7 (C-3‴), 164.7 (C-4‴), 109.9 (C-5‴), 134.1 (C-6‴), 55.8 (7-OCH_3), 56.1 (2‴-OCH_3).

■ RESULTS AND DISCUSSION

Existing chemical data on red propolis samples collected in different regions of Northeastern Brazil showed evidence of a similar composition to that of CRP. An exhaustive investigation of a BRP sample collected from the South coast of Paraiba led to the isolation of 43 compounds including isoflavonoids, flavonoids, neoflavonoids, chalcones, and lignans.[21] All compounds found in CRP[9] were also identified in this Brazilian propolis sample, and the major constituents of CRP (medicarpin, vestitol, liquiritigenin, isoliquiritigenin, formononetin, and biochanin A) were also observed in other BRP samples produced in other regions of Northeastern Brazil.[18,20,22] However, the presence of the PPBs in a BRP sample from Alagoas State was reported,[22] suggesting a possible variation of chemistry always linked to the

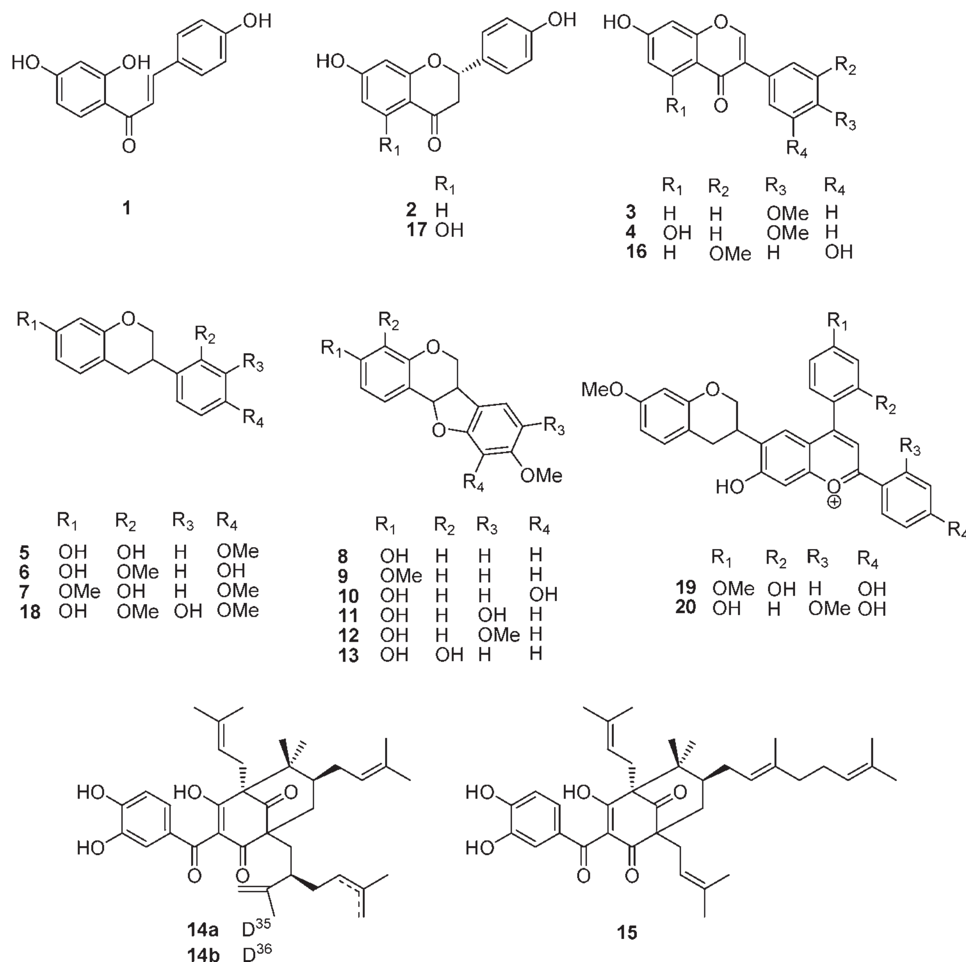

Figure 1. Structures of compounds **1**−**20** detected in red propolis samples (CRP and BRP) and *D. ecastophyllum* exudates (DEE).

botanical origin and thus to the site to collect the resin from which originates the propolis.

In order to investigate the similarities and differences between the chemical compositions of red propolis from different tropical regions, ethanol extracts of Cuban (CRP) and Brazilian (BRP) red propolis were preliminarily analyzed by our HPLC-MS classification method, previously used to study Cuban propolis.[7] In the same way, DEE was analyzed to assess the botanical origin of this tropical propolis type. Red propolis from Alagoas was chosen as a representative sample of BRP because available literature data[22] suggested some differences with CRP. Regarding Cuban propolis, we selected a red sample from Pinar del Rio Province, which contains exclusively isoflavonoids.[9]

Preliminary Comparative Analysis by HPLC-MS. To compare preliminarily the red propolis samples and their supposed botanical source, two specific HPLC-ESI/MS methods for the detection of flavonoids of CRP and PPBs, respectively, were applied to the extracts of BRP, CRP, and DEE.[7]

Tandem mass spectrometry experiments in the selected reaction monitoring (SRM) mode were performed to evaluate the occurrence of marker compounds of CRP[9] in BRP and DEE. The compounds isoliquiritigenin (**1**), liquiritigenin (**2**), formononetin (**3**), biochanin A (**4**), vestitol (**5**), neovestitol (**6**), 7-*O*-methylvestitol (**7**), medicarpin (**8**), homopterocarpin (**9**), vesticarpan (**10**), 3,8-dihydroxy-9-methoxypterocarpan (**11**), 3,4-dihydroxy-9-methoxypterocarpan (**12**), and 3-hydroxy-8,9-dimethoxypterocarpan (**13**) (Figure 1) were detected with the selected precursor ion/fragment ion transitions previously reported.[7] All these compounds were detected in BRP and DEE extracts (Figure 2), demonstrating that red propolis from different tropical zones have a similar chemical composition. Furthermore, these results confirm that the botanical source of red propolis is *D. ecastophyllum* exudate.

BRP and DEE extracts were also examined by HPLC-DAD-MS method for analysis of PPBs.[7] The DEE chromatogram, obtained by using a C8 column, did not reveal the presence of peaks in the retention time range of PPBs. In contrast, in the BRP chromatogram, two main peaks, **14** and **15**, were detected (Figure 3). Both peaks showed a protonated molecule $[M + H]^+$ at m/z 603, and their product ion spectra revealed fragment ions corresponding to successive eliminations of the alkylic chains from the bicyclic core of PPBs (Figure 3).[23] The main fragmentation was the loss of a chain at C5 ($[M + H - 136]^+$ at m/z 467 for **14** and $[M + H - 68]^+$ at m/z 535 for **15**, due to the elimination of geranyl and prenyl groups, respectively) followed by the elimination of the chain at C7 occurring through the opening of the bicyclic system ($[M + H - 136 - 124]^+$ and $[M + H - 68 - 192]^+$ at m/z 343 for **14** and **15**, respectively). According to the fragmentation pattern proposed for the PPB nemorosone,[23] peaks **14** and **15** have prenyl and geranyl chains at C7, respectively. Moreover, UV data of **14** and **15** (λ_{max} at 250 and 355 nm) suggested the presence of a dihydroxy-benzoyl moiety conjugated to the 1,3-diketone system of PPBs,[24] and

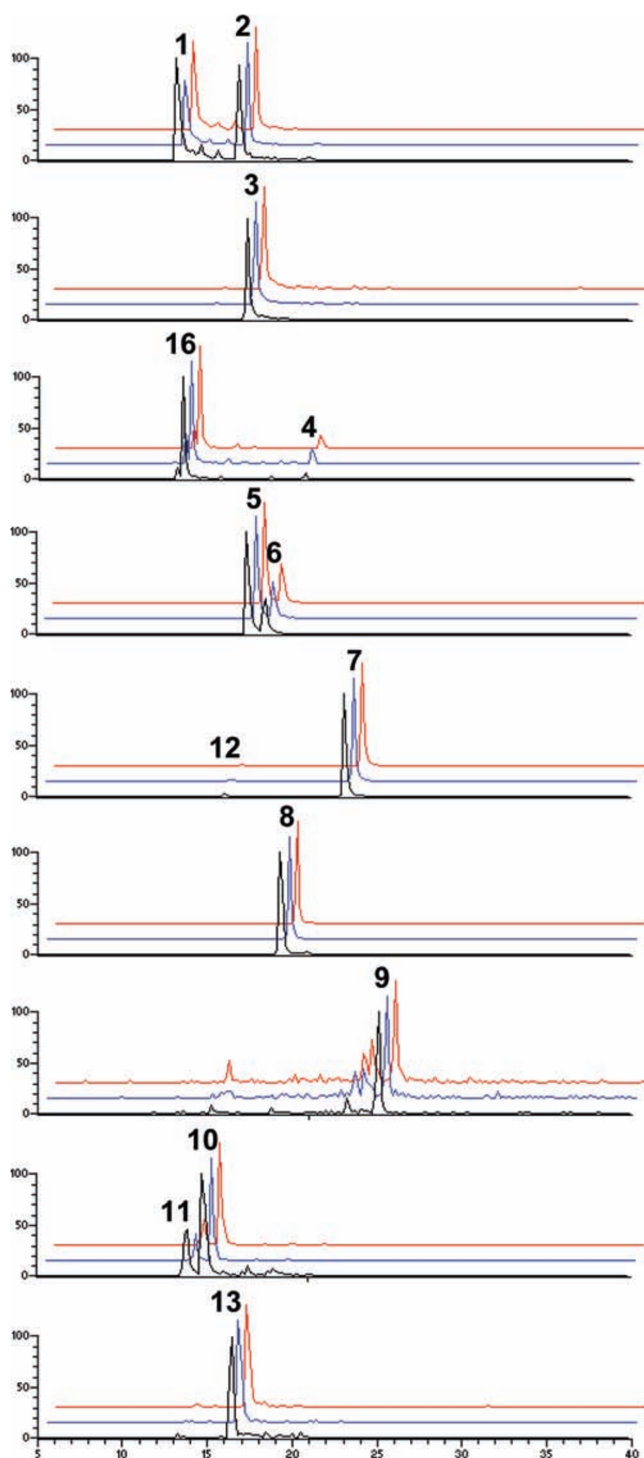

Figure 2. HPLC−tandem mass spectrometry analysis in SRM mode performed to evaluate the occurrence of CRP marker compounds **1**−**13** in CRP (black), BRP (blue), and DEE (red);[9] **1**, **2** m/z 257→239; **3** m/z 269→254; **4** m/z 285→270; **5**, **6** m/z 273→123; **7** m/z 287→137; **8** m/z 271→161; **9** m/z 285→161, **10**, **11** m/z 287→153; **12** m/z 287→137; **13** m/z 301→191.

therefore the peaks **14** and **15** detected in BRP are probably PPBs of type B[25] with a dihydroxybenzoyl, one geranyl, and two prenyl groups as substituents of the bicyclic ring system. To confirm the presence of PPBs in BRP and characterize these compounds, peaks **14** and **15** were isolated by preparative chromatographic techniques from the ethanol extract of the BRP sample (see Materials and Methods). The structural elucidation was carried out by spectroscopic techniques, mainly NMR, and the peaks **14** and **15** were identified as the isomeric mixture of guttiferone E/xanthochymol and oblongifolin A (Figure 1), respectively, by comparison with data reported in the literature.[26,27] According to MS and UV data, compounds **14** and **15** are PPBs with a 3,4-dihydroxybenzoyl group (C3) and a geranyl chain (C5 in **14** and C7 in **15**).

Comparative HPLC-DAD-MS Analysis. Based on the composition differences highlighted by the preliminary comparative analysis, an approach based on HPLC combined with online PDA detection and tandem mass spectrometry was used to determine the overall chemical profile of red propolis samples (CRP and BRP) and their botanical source (DEE). In order to obtain LC chromatograms with good resolution of adjacent peaks and the elution of a wide range of compounds of varying polarity, mobile phase compositions were screened, and a gradient elution, with AcCN and 0.1% aqueous formic acid, was found the most suitable eluting solvent system for the separation of most constituents of extracts. The monitoring wavelengths chosen for detection of most isoflavonoids and BBPs were 280 and 320 nm. In addition, a wavelength at 490 nm was selected by preliminary UV analysis of crude extracts to identify the compounds responsible for the characteristic color of red propolis samples and DEE. For MS analysis, the positive ion mode of ESI was selected because it provided extensive information on fragmentations. Under the optimized HPLC and tandem mass spectrometry conditions, compounds **1**−**15** were unequivocally identified by comparing their product ion spectra and retention times with pure compounds isolated from red propolis samples (Table 1).

The CRP, BRP, and DEE chromatographic profiles at 280 and 490 nm are shown in Figure 4. The HPLC chromatogram of DEE was exactly the same as those of CRP extract and differed from the BRP profile, which exhibited additional peaks eluting with retention times more than 80 min. Compounds **1**−**13** were detected in all three extracts (CRP, BRP, and DEE), whereas the PPBs **14** and **15** were only in BRP extract, fully in agreement with the results above. The UV chromatograms of CRP, BRP, and DEE also showed three other common peaks (**16**−**18**) at 15.7, 18.8, and 22.8 min with protonated molecules $[M + H]^+$ at m/z of 285, 273, and 303, respectively.

Peak **16** showed the same (+)-MS^2 spectral behavior (Table 1 and Supporting Information Figure 1) of methoxylated isoflavones, formononetin (**3**) and biochanin A (**4**), with a base peak at 270 corresponding to the loss of CH_3 radical from the $[M + H]^+$ precursor ion.[28] The presence of further diagnostic fragments confirmed the isoflavone core ($[M + H - 56]^+$ ion at m/z 229 is a characteristic feature for differentiation of isomeric flavone/isoflavone) and indicated the kind of substituents of aromatic rings ($^{1,3}A^+$ ion at m/z 137).[28] Using the MS^2 information, peak **16** can be characterized as an isoflavone with a hydroxyl group on ring A and one hydroxyl and one methoxyl substituent of ring B. For unambiguous characterization, peak **16** was isolated from BRP extract (see Materials and Methods) and identified by 1D and 2D NMR analysis as 7,5′-dihydroxy-3′-methoxyisoflavone, previously reported from *D. odorifera* heartwood.[29]

Using the tandem mass spectrometry information (Table 1 and Supporting Information Figure 2), we characterized peak **17** as a flavanone.[30] The retro-Diels−Alder fragment $^{1,3}A^+$, $^{1,3}B^+$

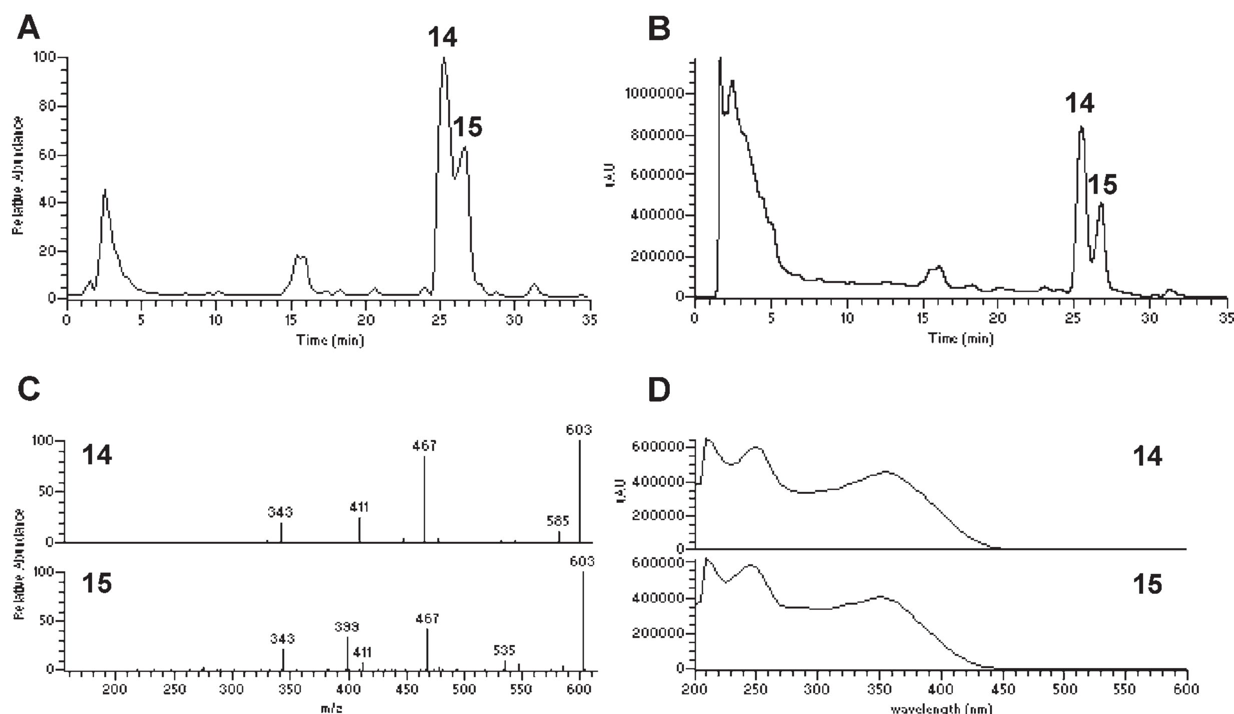

Figure 3. HPLC-DAD-MS profile of BRP crude extract on C8 column:[7] (A) (+)-MS chromatogram, scan range of m/z 200−1000; (B) UV chromatogram, 280 nm; (C) MS^2 and (D) UV spectra of peaks **14** and **15** detected in BRP.

Table 1. Retention Times (t_R) and MS Data for the Compounds Detected in CRP, BRP, and DEE

compound	t_R (min)	$[M + H]^+$	Tandem Mass Spectrometry (m/z, relative abundance)[a]
isoliquiritigenin (**1**)	14.1	257	137 (69), 147 (78), 163 (9), 171 (2), 187 (3), 211 (22), 215 (3), 229 (3), 239 (100), 242 (34)
liquiritigenin (**2**)	23.9	257	137 (62), 147 (72), 163 (12), 187 (2), 211 (19), 215 (5), 229 (3), 239 (100), 242 (36)
formononetin (**3**)	24.6	269	137 (2), 154 (2), 163 (2), 199 (2), 213 (35), 237 (39), 241 (10), 254 (100)
biochanin A (**4**)	36.6	285	123 (12), 149 (7), 152 (9), 170 (4), 179 (5), 229 (19), 253 (22), 257 (11), 270 (51)
vestitol (**5**)	25.3	273	123 (74), 137 (100), 149 (3),151 (12), 163 (20)
neovestitol (**6**)	28.8	273	123 (70), 137 (100), 149 (6),151 (11), 163 (5)
7-*O*-metilvestitol (**7**)	48.1	287	137 (100), 163 (10)
medicarpin (**8**)	30.7	271	123 (9), 137 (100), 147 (7), 149 (3), 161 (44), 163 (2), 177 (2), 243 (3)
homopterocarpin (**9**)	53.9	285	137 (100), 161 (51), 177 (8), 253 (2), 257 (5), 270 (2)
vesticarpan (**10**)	15.9[b]	287	123 (2), 147 (5), 153 (100), 169 (2), 177 (19), 193 (2), 255 (7), 259 (2), 269 (3)
3,8-dihydroxy-9-methoxy-pterocarpan (**11**)	18.1	287	123 (51), 147 (17), 153 (59), 161 (44), 177 (100), 255 (40), 259 (16), 269 (36), 272 (27)
3,4-dihydroxy-9-methoxy-pterocarpan (**12**)	20.8	287	137 (55), 139 (100), 161 (23), 163 (4), 259 (4), 269(4)
3-hydroxy-8,9-dimethoxy-pterocarpan (**13**)	21.6	301	153 (13), 163 (7), 167 (87), 179 (4), 191 (100), 241 (4), 269 (12), 283 (8)
guttiferone E/xanthochymol (**14**)	84.0	603	343 (21), 411 (25), 449 (4), 467 (85), 479 (4), 535 (2), 547 (3), 585 (12)
oblongifolin A (**15**)	84.5	603	343 (24), 399 (32), 411 (8), 467 (41), 479 (2), 535 (9), 547 (5), 585 (4)
7,3′-dihydroxy-5′-methoxy-isoflavone (**16**)	15.7	285	137 (7), 181 (2), 225 (18), 229 (8), 253 (55), 257 (5), 270 (100)
naringenin (**17**)	18.8	273	121 (3), 147 (99), 153 (100), 171 (6), 179 (7), 189 (3), 207 (6), 231 (8), 255 (2)
mucronulatol (**18**)	22.8	303	123 (23), 149 (19), 167 (100), 181 (9), 193 (12)
retusapurpurin B (**19**)	26.9[c]	523	371 (2), 373 (9), 385 (53), 387 (100), 399 (61), 507 (2), 508 (4)
retusapurpurin A (**20**)	30.1[c]	523	371 (3), 373 (7), 385 (59), 387 (100), 399 (69), 493 (3), 507 (2)

[a] Relative abundance > 2. [b] t_R obtained from LC-MS chromatogram. [c] Corresponding to $[M]^+$.

and $[^{1,4}B - H_2 + H]^+$ ions at m/z 153, 121, and 147, respectively, provided information on the number and type of substituents in the A and B rings, suggesting the structure of naringenin. The proposed structure was confirmed by comparison of retention time, UV spectra, and MS data with reference standard.

Tandem mass spectrometry data of peak **18** (Table 1 and Supporting Information Figure 3) revealed the same fragmentation pattern observed for isoflavans **5**−**7**. In Figure 5 are shown the various retrocyclization fragments caused by cleavage of C-ring bonds of isoflavans. The cleavage of two and three bonds of the C ring is the most important fragmentation observed for

Figure 4. HPLC-UV profiles of CRP (A), BRP (B), and DEE (C). Peak numbers refer to compounds in Table 1. Black line corresponds to channel 280 nm and red line to 490 nm.

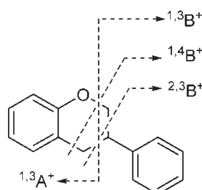

Figure 5. Retrocyclization cleavage of C-ring bonds observed for isoflavans **5**−**7** and **18**.

isoflavans. The C-ring cleavage product ions can be used to determine the number and nature of the substituents on the A and B rings. For example, the product ion spectra of vestitol (**5**) and 7-*O*-methylvestitol (**7**) showed the same $^{1,4}B^+$ and $^{2,3}B^+$ fragment ions (m/z 163 and 137, respectively) whereas the corresponding $^{1,3}A^+$ ions were found at m/z 123 and 137, respectively. This indicates that the two compounds differ in the substitution of the A ring. Similarly, the fragment ions containing the B ring of **18** ($^{1,3}B^+$, $^{1,4}B^+$, and $^{2,3}B^+$ at m/z 181, 193, and 167, respectively) have a difference of 30 Da from those in **5**−**7**, indicating the presence of an additional methoxyl group on the B ring of **18**. These MS data suggested for peak **18** the structure of the isoflavan named mucronulatol, which was unambiguously confirmed by comparison with pure compound isolated previously from Mexican propolis.[31]

Regarding the pigments that give the typical red color to *D. ecastophyllum* exudates, the DEE chromatogram at 490 nm (Figure 4) showed two main peaks at 26.9 and 30.1 min, **19** and **20**, which were also detected in crude extracts of BRP and

CRP (Figure 4). *Dalbergia* species are known for their intensely colored heartwood pigments. These pigments are extracted to dye fabrics and could be used as colorants for foods and cosmetic. The *Dalbergia* pigments belong to a relatively small group of natural products: isoflavans condensed with a chalcone unit.[32−34] In candenatone and neocandenatone, the A ring of the isoflavan nucleus is coupled to the chalcone unit by a $6 \rightarrow \beta$ linkage,[32,34] whereas retusapurpurin A, a related isomer, formally derived from a $5' \rightarrow \alpha$ linkage.[33] The quinonemethide structures of these purple pigments are converted into pyrylium ions by protonation in an acidic buffer, which causes a change of the color solution (from red to orange) due to a hypsochromic effect.[32−34] Also the color of DEE ethanol extract was pH sensitive. The UV−vis spectra of ethanol solution displayed absorption maxima at 520 nm. Acidification with 0.1% TFA caused a strong hypsochromic shift of 525 nm bands, and a single absorption maximum at 482 nm, suggesting that the pigments of DEE must have the same chromophore of candenatone, neocandenatone, and retusapurpurin A. UV chromatogram (520 and 482 nm) of the acidified DEE extract showed a better peak resolution and a greater reproducibility of retention times than the ethanol solutions. Thus, DEE, BRP, and CRP extracts were acidified before HPLC analysis, using 490 nm as characteristic wavelength for a great absorbance.

Full MS spectra of **19** and **20** (Table 1 and Supporting Information Figure 4 and 5) showed a base peak $[M]^+$ at *m/z* 523, indicating the same molecular mass of known *Dalbergia* pigments. Collision-induced fragmentation of **19** and **20** produced three ions due to C-ring cleavages of the isoflavan unit at *m/z* 373 ($^{2,3}B^+$), 387 ($^{1,3}B^+$), and 399 ($^{1,4}B^+$). These diagnostic fragment ions indicated that the chalcone unit is condensed to the B-ring of an isoflavan with the same A-ring substitution of 7-*O*-methylvestitol (**7**). As a result of the analysis of UV and MS data, peaks **19** and **20** can be tentatively identified as retusapurpurin A or its isomers. In order to confirm this hypothesis, the DEE pigments were isolated by preparative chromatographic techniques (see Materials and Methods) and their structures were characterized on the basis of 1D and 2D NMR experiments, as protonated 2,4-diarylbenzopyrylium structures. Both NMR spectra of **19** and **20** showed signals of the heterocyclic C-ring of isoflavan moiety in addition to 13 aromatic protons and two aromatic methoxy signals. Pigment **20** was identified as retusapurpurin A.[33] Comparison of NMR data of retusapurpurin A with those of **19** confirmed the isomeric relationship between them. The ^1H and ^{13}C chemical shifts (see Materials and Methods) of the two compounds were almost identical for the isoflavan substructure, while for the chalcone moiety, **19** showed two major differences. First, the 1,2,4-trisubstituted aromatic ring in **20** was replaced by a para-substituted aromatic ring (H-2″ and H-6″ δ 8.29, H-3″ and H-5″ δ 7.08). The chemical shift assigned to H-6″ is consistent only with the A ring of the chalcone; in fact this proton, as well as H-3′ (δ 8.13), is known to undergo downfield shifts as the pyrylium character of the ring increases.[35] H-6″ showed HMBC correlations to C-2′ (δ 169.9) and C-4″ (δ 160.4), whereas no correlation was observed between with the carbon bearing the methoxy group. The second change was in the ring B. The proton signals at δ 6.52 (H-5‴), 6.63 (H-3‴), and 7.07 (H-6‴) and COSY correlations suggested a 1,2,4-trisubstituted ring in **19**. The methoxy protons (δ 3.78) showed HMBC correlations to C-4‴ (δ 164.7) which in turn showed a three-bond correlation with H-6‴. The latter proton also showed a cross peak with the C-2‴ (δ 160.6). The NOE effect between H-3 and methyl protons was not observed confirming that the methoxy group is in the para position in the 1,2,4-trisubstituted aromatic ring B. On the basis of this evidence, the new compound **19** was established as retusapurpurin B, a novel C30 isoflavan whose formation can be rationalized to occur via oxidative coupling of the isoflavan neovestitol to 4,2′-dihydroxy-4′-methoxychalcone.

The approach based on HPLC combined with online PDA detection and tandem mass spectrometry was successfully used in determination of the compounds of Cuban and Brazilian red propolis samples (CRP and BRP) and of *D. ecastophyllum* exudates (DEE). Twenty compounds were characterized by tandem mass spectrometry, and the proposed structures were confirmed by comparison with reference standards or after isolation and NMR characterization. Flavonoids (**1**−**2** and **17**) and isoflavonoids (**3**−**13**, **16**, and **18**) seem to be common in both red propolis samples and DEE. This evidence supports the hypothesis that the chemistry of the studied red propolis samples could arise from the resin collected by bees from *D. ecastophyllum.* Considering the close similarity between the propolis composition and DEE and the abundance of this plant in the area where beehives are placed, we suggest that *D. ecastophyllum* exudates are the botanical origin of both Cuban and Brazilian red propolis. On the other hand, the presence of polyisoprenylated benzophenones (**14**, **15**) was recognized only in BRP suggesting for the Brazilian sample the contribution of different vegetal sources (belonging to *Clusiaceae* family) present at the site of collection. Polyisoprenylated benzophenones were also documented in 38 propolis samples produced in Venezuela suggesting that *Clusia* spp. is the main source for propolis also in tropical Venezuela.[36] The pigments responsible for the red color of DEE and red propolis were characterized by extensive MS and NMR analysis as rutusapurpurin A (**20**) and B (**19**). The latter is a novel pigment belonging to an unusual natural product group, the C30 isoflavans, closed to *Dalbergia* species. The chemical information obtained in this study is important not only for the correct understanding of similarities and differences between samples collected in different tropical regions, but also for the future validation of these compounds as markers for the assessment of red propolis and for the quality control of its commercial products.

■ ASSOCIATED CONTENT

Supporting Information. MS/MS spectra (Figures 1−5) of compounds **16**−**20** and UV spectra (Figures 6−11) of characteristic compounds (**8**, **16**−**20**) of red propolis. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 0039 89969766. Fax: 0039 89 969602. E-mail: rastrelli@unisa.it.

■ REFERENCES

(1) Ghisalberti, E. L. Propolis: A review. *Bee World* **1979**, *60*, 59–84.

(2) Marcucci, M. C. Propolis: Chemical composition, biological properties and therapeutic activity. *Apidologie* **1995**, *26*, 83–99.

(3) Burdock, G. A. Review of the biological properties and toxicity of bee propolis (propolis). *Food Chem. Toxicol.* **1998**, *36*, 347–363.

(4) Banskota, A. H.; Tezuka, Y.; Kadota, S. Recent progress in pharmacological research of propolis. *Phytother. Res.* **2001**, *15*, 561–571.
(5) Park, Y. K.; Alencar, S. M.; Aguiar, C. L. Botanical origin and chemical composition of Brazilian propolis. *J. Agric. Food Chem.* **2002**, *50*, 2502–2506.
(6) Park, Y.; Paredes-Guzman, J.; Aguiar, C.; Alencar, S.; Fujiwara, F. Chemical constituents in *Baccharis dracunculifolia* as the main botanical origin of southeastern Brazilian propolis. *J. Agric. Food Chem.* **2004**, *52*, 1100–1103.
(7) Cuesta-Rubio, O.; Piccinelli, A. L.; Fernandez, M. C.; Hernandez, I. M.; Rosado, A.; Rastrelli, L. Chemical characterization of Cuban propolis by HPLC-PDA, HPLC-MS, and NMR: The brown, red, and yellow Cuban varieties of propolis. *J. Agric. Food Chem.* **2007**, *55*, 7502–7509.
(8) Màrquez, I.; Cuesta-Rubio, O.; Campo, M.; Rosado, A.; Montes de Oca, R.; Piccinelli, A. L.; Rastrelli, L. Studies on the constituents of yellow Cuban propolis: GC-MS determination of triterpenoids and flavonoids. *J. Agric. Food Chem.* **2010**, *58*, 4725–4730.
(9) Piccinelli, A. L.; Campo, M.; Cuesta-Rubio, O.; Marquez, I.; De Simone, F.; Rastrelli, L. Isoflavonoids isolated from Cuban propolis. *J. Agric. Food Chem.* **2005**, *53*, 9010–9016.
(10) Ingham, J. L. Isoflavonoid phytoalexins from leaflets of *Dalbergia sericea*. *Z. Naturforsch. C: J. Biosci.* **1979**, *34*, 630–631.
(11) Matos, F. J. A.; Gottlieb, O. R.A.; Souza, C. H. Flavonoids from *D. ecastophyllum*. *Phytochemistry* **1975**, *14*, 825–826.
(12) Liu, R. X.; Wang, Q.; Guo, H. Z.; Li, L.; Bi, K. S.; Guo, D. Simultaneous determination of 10 major flavonoids in *Dalbergia odorifera* by high performance liquid chromatography. *J. Pharm. Biomed. Anal.* **2005**, *39*, 469–476.
(13) Beldjoudi, N.; Mambu, L.; Labaieed, M.; Grellier, P.; Ramanitrahasimbola, D.; Rasoanaivo, P.; Martin, M. T.; Frappier, F. Flavonoids from *Dalbergia louvelii* and their antiplasmodial activity. *J. Nat. Prod.* **2003**, *66*, 1447–1450.
(14) Hamburger, M. O.; Cordell, G. A.; Tantivatana, P.; Ruangrungsi, N. Traditional medicinal plants of Thailand, VIII. Isoflavonoids of *Dalbergia candenatensis*. *J. Nat. Prod.* **1987**, *50*, 696–699.
(15) Shrestha, S. P.; Amano, Y.; Narukawa, Y.; Takeda, T. Nitric oxide production inhibitory activity of flavonoids contained in trunk exudates of *Dalbergia sissoo*. *J. Nat. Prod.* **2008**, *71*, 98–101.
(16) Umehara, K.; Nemoto, K.; Kimijima, K.; Matsushita, A.; Terada, E.; Monthakantirat, O.; De-Eknamkul, W.; Miyase, T.; Warashina, T.; Degawa, M.; Noguchi, H. Estrogenic constituents of the heartwood of *Dalbergia parviflora*. *Phytochemistry* **2008**, *69*, 546–552.
(17) Awale, S.; Shrestha, S. P.; Tezuka, Y.; Ueda, J. Y.; Matsushige, K.; Kadota, S. Neoflavonoids and related constituents from Nepalese propolis and their nitric oxide production inhibitory activity. *J. Nat. Prod.* **2005**, *68*, 858–864.
(18) Daugsch, A.; Moraes, C. S.; Fort, P.; Park, Y. K. Brazilian red propolis — chemical composition and botanical origin. *Evidence Based Complementary Altern. Med.* **2008**, *5*, 435–441.
(19) Silva, B. B.; Rosalen, P. L.; Cury, J. A.; Ikegaki, M.; Souza, V. C.; Esteves, A.; Alencar, S. M. Chemical composition and botanical origin of red propolis, a new type of Brazilian propolis. *vidence Based Complementary Altern. Med.* **2008**, *5*, 313–316.
(20) Alencar, S. M.; Oldoni, T.; Castro, M.; Cabral, I.; Costa-Neto, C.; Cury, J.; Rosalen, P.; Ikegakid, M. Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis. *J. Ethnopharmacol.* **2007**, *113*, 278.
(21) Awale, S.; Li, F.; Onozuka, H.; Esumi, H.; Tezuka, Y.; Kadota, S. Constituents of Brazilian red propolis and their preferential cytotoxic activity against human pancreatic PANC-1 cancer cell line in nutrient-deprived condition. *Bioorg. Med. Chem.* **2008**, *16*, 181–189.
(22) Trusheva, B.; Popova, M.; Bankova, V.; Simova, S.; Marcucci, M.; Miorin, P.; Rocha Pasin, F.; Tsvetkova, I. Bioactive constituents of Brazilian red propolis. *Evid. Based Complement. Alternat. Med.* **2006**, *3*, 249–254.
(23) Piccinelli, A. L.; Campone, L.; Dal Piaz, F.; Cuesta-Rubio, O.; Rastrelli, L. Fragmentation pathways of polycyclic polyisoprenylated benzophenones and degradation profile of nemorosone by multiple-stage tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 1688–1698.
(24) Krishnamurthy, N.; Lewis, Y. S.; Ravindranath, B. On the structures of garcinol, isogarcinol and cambogiol. *Tetrahedron Lett.* **1981**, *22*, 793–796.
(25) Cuesta-Rubio, O.; Piccinelli, A. L.; Rastrelli, L. Chemistry and biological activity of polyisoprenylated benzophenone derivatives. *Stud. Nat. Prod. Chem.* **2005**, *32* (Bioactive Natural Products, Part L), 671–720.
(26) Roux, D.; Hadi, H. A.; Thoret, S.; Guénard, D.; Thoison, O.; Païs, M.; Sévenet, T. Structure-activity relationship of polyisoprenyl benzophenones from *Garcinia pyrifera* on the tubulin/microtubule system. *J. Nat. Prod.* **2000**, *63*, 1070–1076.
(27) Hamed, W.; Brajeul, S.; Mahuteau-Betzer, F.; Thoison, O.; Mons, S.; Delpech, B.; Van Hung, N.; Sévenet, T.; Marazano, C. Oblongifolins A—D, polyprenylated benzoylphloroglucinol derivatives from *Garcinia oblongifolia*. *J. Nat. Prod.* **2006**, *69*, 774–777.
(28) Kuhn, F.; Oehme, M.; Romero, F.; Abou-Mansour, E.; Tabacchi, R. Differentiation of isomeric flavone/isoflavone aglycones by MS^2 ion trap mass spectrometry and a double neutral loss of CO. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1941–1949.
(29) An, R. B.; Jeong, G. S.; Kim, Y. C. Flavonoids from the heartwood of *Dalbergia odorifera* and their protective effect on glutamate-induced oxidative injury in HT22 cells. *Chem. Pharm. Bull* **2008**, *56*, 1722–1724.
(30) Zhou, D. H.; Xu, Q.; Xue, X. Y.; Zhang, F. F.; Jing, Y.; Liang, X. M. Rapid qualitative and quantitative analyses of flavanone aglycones in *Fructus aurantii* by HPLC ion-trap MS. *J. Sep. Sci.* **2007**, *30*, 858–867.
(31) Lotti, C.; Campo Fernandez, M.; Piccinelli, A. L.; Cuesta-Rubio, O.; Marquez Hernandez, I.; Rastrelli, L. Chemical constituents of red Mexican propolis. *J. Agric. Food Chem.* **2010**, *58*, 2209–2213.
(32) Hamburger, M. O.; Cordell, G. A.; Ruangrungsi, N.; Tantivatana, P. Candenatone, a novel purple pigment from *Dalbergia candenatensis*. *J. Org. Chem.* **1988**, *53*, 4161–4165.
(33) Czakó, M.; Márton, L. A heartwood pigment in *Dalbergia* cell cultures. *Phytochemistry* **2001**, *57*, 1013–1022.
(34) Barragán-Huerta, B. E.; Peralta-Cruza, J.; González-Laredo, R. F.; Karchesy, J. Neocandenatone, an isoflavan-cinnamylphenol quinone methide pigment from *Dalbergia congestiflora*. *Phytochemistry* **2004**, *65*, 925–928.
(35) Millan, L.; Fuentealba, M.; Manzur, C.; Carrillo, D.; Faux, N; Caro, B.; Robin Le-Guen, F.; Sinbandhit, S.; Ledoux-Rak, I.; Hamon, J. R. Dipolar organoiron pyranylideneacetaldehyde hydrazone complexes: synthesis, characterization, crystal structure, linear and nonlinear optical properties. *Eur. J. Inorg. Chem.* **2006**, *6*, 1131–1138.
(36) Tomás-Barberán, F. A.; García-Viguera, C.; Vit-Oliviera, P.; Ferreres, F.; Tomás-Lorente, F. Phytochemical evidence for the botanical origin of tropical propolis from Venezuela. *Phytochemistry* **1993**, *34*, 191–196.