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Isoflavonoids Isolated from Cuban Propolis

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Chemical investigation of a red-type Cuban propolis sample has led to the isolation of 11 isoflavonoids (2 isoflavones, 3 isoflavans, and 6 pterocarpans), together with gallic acid, isoliquiritigenin, and (−)-liquiritigenin. Structural determination, including the absolute stereochemistry, was accomplished by spectroscopic analysis, particularly CD and 2D NMR techniques. The fragmentation behavior of pterocarpans was studied by electrospray ionization (ESI) tandem mass spectrometry (MS/MS) using an ion-trap analyzer, and a generalized fragmentation pathway, useful in the identification and structural characterization of pterocarpans, is proposed. Isoflavonoids are reported for the first time from propolis samples.

KEYWORDS: Propolis; isoflavonoids; isoflavans; pterocarpans; isoliquiritigenin; (−)-liquiritigenin; 1D and 2D NMR spectroscopy; CD; ESI-MS/MS

INTRODUCTION

Propolis is a resinous hive product collected by honeybees from various plant sources and is used to seal holes in their honeycombs, smooth out the internal walls, and protect the entrance against intruders. Propolis is widely used in traditional medicine and is reported to have a broad spectrum of pharmacological properties (*1*, *2*). Besides its uses in traditional medicine, it has recently gained popularity as a health food supplement and is used extensively in foods and beverages in various parts of the world, where it is claimed to improve health and prevent diseases such as inflammation, heart disease, diabetes, and even cancer (*2*, *3*). Propolis contains sticky plant substances mixed with beeswax and other bee secretions, and its chemical composition is qualitatively and quantitatively variable, depending on the vegetation in the area from which it was collected (*4*). Because of the geographical differences, propolis samples from Europe, South America, and Asia have different chemical compositions. Propolis from Europe and China contains many kinds of flavonoids and phenolic acid esters (*4*). In contrast, the major components in propolis of Brazilian origin are terpenoids and prenylated derivatives of *p*-coumaric acids (*5*−*7*).

In our previous studies, we reported the occurrence of polyprenylated benzophenones from Cuban propolis samples (*8*−*10*). Considering the high percentage of endemic plants present in Cuba and the differences of Cuban flora with respect to that from temperate regions, we decided to continue our research on the chemical composition of Cuban propolis in order to suggest similarities and differences between samples collected in different regions of the country. In this study we conducted a chemical investigation of a red-type Cuban propolis sample, collected in Pinar del Rio Province, that lead to the isolation of 14 phenolic compounds, including isoflavonoids and pterocarpans. This group of natural compounds has a very restricted distribution in the plant kingdom and occurs almost exclusively in legumes (Leguminosae family), with soybeans, chickpeas, and lentils representing the major dietary sources (*11*). Dietary consumption of foods and food additives containing isoflavone phytoestrogens has been associated with a variety of health benefits, including relief from symptoms of menopause (*12*) and reduced risk of hormonal cancer (*13*), osteoporosis, and coronary heart diseases. This appears to be the first report on the occurrence of isoflavonoids in the propolis.

MATERIALS AND METHODS

General Experimental Procedure. CD measurements were carried out on a J-710 dicograph (Jasco, Tokyo, Japan). Optical rotations were determined on a model 192 polarimeter (Perkin-Elmer, Norwalk, CT) equipped with a sodium lamp (589 nm) and a 10 cm microcell. 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, δ H 7.27 and δ C 77.0 for $CDCl_3$; coupling constants, *J*, are in hertz. DEPT, ^{13}C, DQF-COSY, HSQC, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature. Electrospray ionization mass spectrometry (ESIMS) was performed using a Finnigan LCQ Deca instrument from

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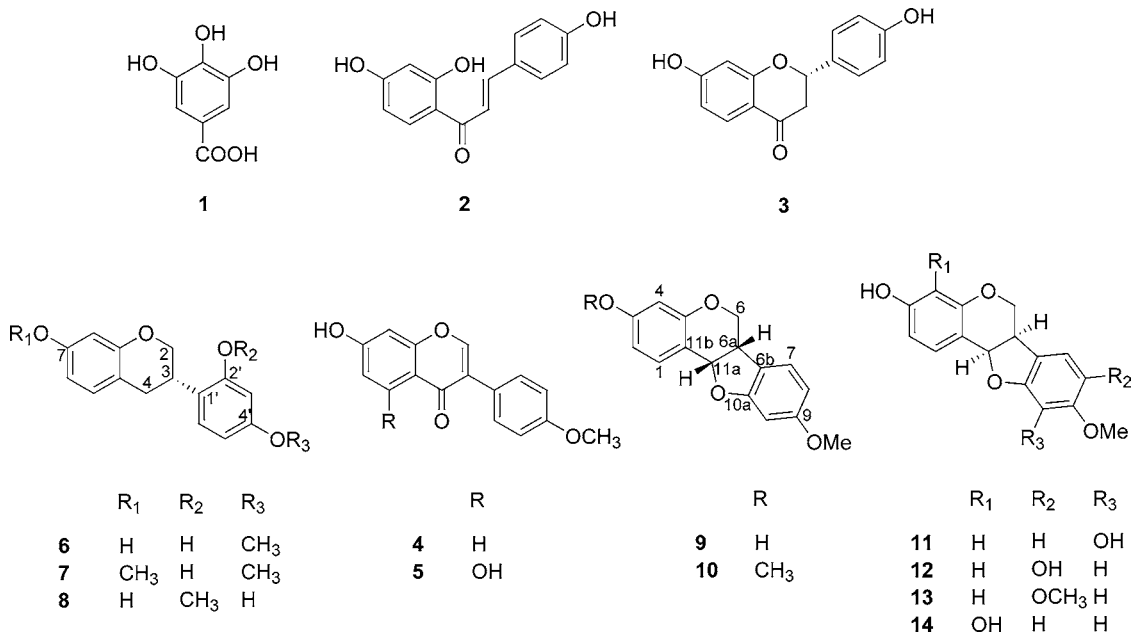


Figure 1. Compounds **1**–**14** isolated from red-type Cuban propolis sample.

Thermo Electron (San Jose, CA) equipped with Xcalibur software. Full mass and collision-induced dissociation (CID) MS/MS spectra were acquired in positive mode. Instrumental parameters were tuned for each investigated compound: capillary voltage was set at 3 V and spray voltage at 5.10 kV; and a capillary temperature of 220 °C was used, and the tube lens offset at −60 V was employed; specific collision energies were chosen at each fragmentation step for all of the investigated compounds, and the value ranged from 15 to 33% of the instrument maximum. Data were acquired in the MS1 scanning mode (*m*/*z* 150−700). All compounds were dissolved in MeOH/H_2O (1:1) and infused in the ESI source by using a syringe pump; the flow rate was 5 μL/min. Chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden) by employing MeOH as solvent. MPLC separations were carried out on a Büchi B-685 borosilicate glass column (230 × 26 mm) using a Buchi 688 pumping system (Büchi, Flawil, Switzerland). Silica gel 60 (0.040−0.063 mm; Carlo Erba) was used as column material. HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a 300 × 7.8 mm i.d. 10 μm μ-Bondapak C18 column (Waters). TLC analysis was performed with Macherey-Nagel precoated silica gel 60 F_{254} plates.

Propolis. The propolis sample was collected in April 2003, in Pinar del Rio, Cuba. Both the sample and the dried methanol extract were stored at 5 °C in the dark.

Extraction and Isolation Procedure of Compounds 1−14. The propolis sample (40 g) was extracted with methanol (100 mL × 6) for 3 h and, after filtration, the methanol extract was taken to dryness under reduced pressure to yield a dark red gum (24.1 g). A portion of this extract (9 g) was fractionated over a Sephadex LH-20 column (100 × 5 cm) using methanol as solvent to furnish nine fractions (1−9). Fraction 2 was purified by column chromatography on silica gel using $CHCl_3$/MeOH gradients and afforded **1** (3 mg) and **2** (9 mg) (**Figure 1**). Fraction 3 produced an insoluble residue that was recrystallized from MeOH to give **4**. Fraction 4 (1.22 g) was subjected to MPLC separations eluting with gradient of hexane/EtOAc (0−100% EtOAc) and EtOAc/MeOH mixtures (0−100%) to give seven fractions (4/1−4/7). Fractions 4/1, 4/2, 4/6, and 4/7 contained **3** (15.6 mg), **6** (250 mg), **7** (4.2 mg), and **9** (170 mg), respectively. Fraction 4/3 was subjected to RP-HPLC (70% MeOH) to afford a main compound that was further purified by preparative TLC ($CHCl_3$/MeOH, 95:5), **14** (9 mg). Fraction 4/4 was purified by HPLC (70% MeOH) to afford **5** (3.6 mg) and **13** (1.2 mg). Fraction 4/5 was purified by RP-HPLC (60% MeOH) to give **12** (2.9 mg). Fraction 5 (930 mg) was subjected to MPLC using a gradient of hexane/$CHCl_3$ (0−100%) and $CHCl_3$/MeOH (0−100%) mixtures to give four fractions (5/1−5/4). Fraction 5/1 contained **10** (1.8 mg). Fractions 5/2−5/4 were purified by RP-HPLC (50% MeOH) to give **11** (2.4 mg) and **8** (2.9 mg), respectively.

*Gallic acid (**1**):* ^{1}H and ^{13}C NMR data were consistent with those previously reported (*14*); ESI-MS (negative mode), *m*/*z* 169 $[M - H]^-$.

*Isoliquiritigenin (**2**):* ^{1}H and ^{13}C NMR data were consistent with those previously reported (*15*).

*(−)-Liquiritigenin (**3**):* $[\alpha]_D$ −37.3 (*c* 0.16, CH_3OH); ^{13}C NMR data were consistent with those previously reported (*16*); ESI-MS (positive mode), *m*/*z* 257.3 $[M + H]^+$; MS/MS, *m*/*z* 239.0, 211.1, 147.0, 137.1.

*Formononetin (**4**):* ^{1}H and ^{13}C NMR data were consistent with those previously reported (*17*); ESI-MS (positive mode), *m*/*z* 269.3 $[M + H]^+$; MS/MS, *m*/*z* 254.2, 237.3, 213.3, 107.1.

*Biochanin A (**5**):* ^{1}H and ^{13}C NMR data were consistent with those previously reported (*17*); ESI-MS (positive mode), *m*/*z* 285.4 $[M + H]^+$; MS/MS, *m*/*z* 270.2, 253.3, 229.2, 179.2, 152.1, 123.1.

*(3S)-Vestitol (**6**):* $[\alpha]_D$ +5.02 (*c* 1.06, CH_3OH); CD $[\theta]_{239}$ 3090.3, $[\theta]_{289}$ −1639.4 (*c* 6.7 × 10^4 M, CH_3OH); ^{1}H NMR (CD_3OD) δ 6.84 (H-6′), 6.79 (H-5), 6.38 (H-3′), 6.32 (H-6), 6.28 (H-5′), 6.25 (H-8), 4.17 (H-2b), 3.83 (H-2a), 3.62 (MeO-4′), 3.40 (H-3), 2.81 (H-4a), 2.66 (H-4b); ^{13}C NMR (CD_3OD) δ 160.4 (C-4′), 156.9 (C-7), 156.7 (C-2′), 156.0 (C-9), 131.6 (C-5), 128.7 (C-6′), 121.5 (C-1′), 115.4 (C-10), 109.2 (C-6), 105.7 (C-5′), 103.6 (C-8), 101.7 (C-3′), 70.9 (C-2), 55.1 (MeO-4′), 32.7 (C-3), 31.0 (C-4); ESI-MS (positive mode), *m*/*z* 273.2 $[M + H]^+$; MS/MS, *m*/*z* 163.1, 137.1, 123.1.

*(3S)-7-O-Methylvestitol (**7**):* $[\alpha]_D$ 7.5 (*c* 0.51, CH_3OH); CD $[\theta]_{241}$ 3133.3, $[\theta]_{288}$ −1760.8 (*c* 5.9 × 10^{-4} M, CH_3OH); ^{1}H NMR (CD_3OD) δ 6.90 (H-5), 6.85 (H-6′), 6.46 (H-6), 6.40 (H-3′), 6.38 (H-8), 6.27 (H-5′), 4.22 (H-2b), 3.85 (H-2a), 3.67 (MeO-4′), 3.41 (H-3), 2.86 (H-4a), 2.70 (H-4b); ^{13}C NMR (CD_3OD) δ 160.8 (C-7), 160.5 (C-4′), 157.3 (C-9), 156.5 (C-2′), 131.2 (C-5), 128.8 (C-6′), 121.3 (C-1′), 116.0 (C-10), 107.9 (C-6), 105.8 (C-5′), 102.5 (C-8), 102.3 (C-3′), 71.2 (C-2), 55.6 (MeO-4′, MeO-7), 33.1 (C-3), 31.3 (C-4); ESI-MS (positive mode), *m*/*z* 287.2 $[M + H]^+$; MS/MS, *m*/*z* 163.1, 137.1.

*(3S)-7,4′-dihydroxy-2′-methoxyisoflavan (**8**):* $[\alpha]_D$ −24.8 (*c* 0.07, CH_3OH); CD $[\theta]_{246}$ 1206.2, $[\theta]_{286}$ −1148.3 (*c* 8.3 × 10^{-4} M, CH_3OH); ^{1}H NMR (CD_3OD) δ 6.98 (H-5), 6.90 (H-6′), 6.45 (H-6), 6.37 (H-8), 6.34 (H-3′), 6.28 (H-5′), 4.26 (H-2b), 3.98 (H-2a), 3.76 (MeO-7), 3.49 (H-3), 3.00 (H-4a), 2.82 (H-4b); ^{13}C NMR (CD_3OD) δ 160.4 (C-7), 158.0 (C-2′), 157.3 (C-4′), 156.5 (C-9), 131.2 (C-5), 128.8 (C-6′), 120.0 (C-1′), 116.1 (C-10), 107.8 (C-6), 107.6 (C-5′), 103.6 (C-3′), 102.3 (C-8), 71.3 (C-2), 55.6 (MeO-7), 33.1 (C-3), 31.4 (C-4); ESI-MS (positive mode), *m*/*z* 273.3 $[M + H]^+$; MS/MS, *m*/*z* 255.3, 137.1, 123.1.

*(6aS,11aS)-Medicarpin (**9**):* $[\alpha]_D$ 173.0 (*c* 1.25, CH_3OH); CD $[\theta]_{236}$ 48.6, $[\theta]_{287}$ −18.8 (*c* 5.8 × 10^{-5} M, CH_3OH); ^{1}H NMR (CD_3OD) δ

7.31 (H-1), 7.18 (H-7), 6.52 (H-2), 6.46 (H-8), 6.40 (H-10), 6.33 (H-4), 5.48 (H-11a), 4.23 (Hα-6), 3.76 (MeO-9), 3.59 (Hβ-6), 3.57 (H-6a); ^{13}C NMR (CD_3OD) δ 162.6 (C-9), 162.0 (C-10a), 160.1 (C-3), 158.0 (C-4a), 133.2 (C-1), 126.0 (C-7), 120.9 (C-6b), 112.9 (C-11b), 110.7 (C-2), 107.3 (C-8), 104.1 (C-4), 97.6 (C-10), 80.1 (C-11a), 67.6 (C-6), 55.9 (MeO-9), 40.9 (C-6a); ESI-MS (positive mode), *m/z* 271.2 $[M + H]^+$; MS/MS, *m/z* (relative abundance) 253.2 (1.6), 243.2 (15.1), 239.3 (2.8), 177.1 (3.4), 163.1 (21.2), 161.1 (78.9), 147.2 (27.4), 137.2 (100), 123.1 (20.6).

*(6aS,11aS)-Homopterocarpin (**10**):* $[\alpha]_D$ 54.5 (*c* 0.18, CH_3OH); CD $[\theta]_{236}$ 21291.2, $[\theta]_{287}$ −6213.63 (*c* 6.0×10^{-5} M, CH_3OH); ^{1}H NMR (CD_3OD) δ 7.41 (H-1), 7.20 (H-7), 6.66 (H-2), 6.48 (H-8), 6.41 (H-10), 6.46 (H-4), 5.50 (H-11a), 4.26 (Hα-6), 3.79 (MeO-3), 3.77 (MeO-9), 3.59 (Hβ-6), 3.57 (H-6a); ^{13}C NMR (CD_3OD) δ 162.6 (C-9), 162.5 (C-3), 162.0 (C-10a), 158.0 (C-4a), 133.1 (C-1), 126.0 (C-7), 120.7 (C-6b), 114.0 (C-11b), 109.8 (C-2), 107.3 (C-8), 102.4 (C-4), 97.6 (C-10), 79.9 (C-11a), 67.6 (C-6), 55.9 (MeO-9), 55.7 (MeO-3), 40.9 (C-6a); ESI-MS (positive mode), *m/z* 285.2 $[M + H]^+$; MS/MS, *m/z* (relative abundance) 270.2 (1.5), 257.2 (5.0), 253.1 (1.5), 181.1 (1.1), 177.1 (7.6), 161.2 (51.4), 137.1 (100), 109.1.

*(6aR,11aR)-Vesticarpan (**11**):* $[\alpha]_D$ 29.6 (*c* 0.03, CH_3OH); CD $[\theta]_{236}$ −571.9, $[\theta]_{282}$ 806.1 (*c* 5.0×10^{-5} M, CH_3OH); ^{1}H NMR (CD_3OD) δ 7.37 (H-1), 6.76 (H-7), 6.53 (H-8), 6.51 (H-2), 6.33 (H-4), 5.50 (H-11a), 4.25 (Hα-6), 3.84 (MeO-9), 3.57 (Hβ-6), 3.54 (H-6a); ^{13}C NMR (CD_3OD) δ 160.0 (C-3), 157.5 (C-4a), 150.0 (C-9), 147.8 (C-10a), 133.1 (C-1), 132.2 (C-10), 122.3 (C-6b), 115.3 (C-7), 112.6 (C-11b), 110.4 (C-2), 105.6 (C-8), 103.8 (C-4), 80.1 (C-11a), 67.3 (C-6), 56.6 (MeO-9), 41.3 (C-6a); ESI-MS (positive mode), *m/z* 287.2 $[M + H]^+$; MS/MS, *m/z* (relative abundance) 269.2 (3.9), 259.2 (4.9), 255.2 (19.0), 193.1 (4.0), 177.1 (20.1), 153.1 (100), 147.1 (11.1).

*(6aR,11aR)-3,8-Dihydroxy-9-methoxypterocarpan (**12**):* $[\alpha]_D$ −266 (*c* 0.1, CH_3OH); CD $[\theta]_{236}$ −12472.4, $[\theta]_{294}$ 3954.9 (*c* 5.5×10^{-5} M); ^{1}H NMR (CD_3OD) δ 7.30 (H-1), 6.81 (H-7), 6.53 (H-2), 6.51 (H-10), 6.33 (H-4), 5.43 (H-11a), 4.24 (Hα-6), 3.83 (MeO-9), 3.59 (Hβ-6), 3.57 (H-6a); ^{13}C NMR (CD_3OD) δ 159.8 (C-3), 157.8 (C-4a), 153.8 (C-10a), 149.1 (C-9), 141.5 (C-8), 133.0 (C-1), 119.2 (C-6b), 112.8 (C-11b), 112.2 (C-7), 110.4 (C-2), 103.9 (C-4), 96.0 (C-10), 97.6 (C-10), 79.3 (C-11a), 67.3 (C-6), 56.4 (MeO-9), 41.4 (C-6a); ESI-MS (positive mode), *m/z* 287.4 $[M + H]^+$; MS/MS, *m/z* (relative abundance) 272.3 (27.0), 269.2 (35.7), 259.1 (16.3), 255.2 (40.4), 193.1 (1.1), 177.2 (100), 161.1 (44.1), 153.1 (59.4), 147.1 (16.5), 123.1 (51.4).

*(6aR,11aR)-3-Hydroxy-8,9-dimethoxypterocarpan (**13**):* $[\alpha]_D$ −67.9 (*c* 0.1, CH_3OH); CD $[\theta]_{237}$ −21086.9, $[\theta]_{292}$ 10023.3 (*c* 3.1×10^{-5} M, CH_3OH); ^{1}H NMR (CD_3OD) δ 7.31 (H-1), 7.02 (H-7), 6.55 (H-2), 6.44 (H-10), 6.35 (H-4), 5.46 (H-11a), 4.28 (Hα-6), 3.76 (MeO-9), 3.75 (MeO-8), 3.63 (Hβ-6), 3.57 (H-6a); ^{13}C NMR (CD_3OD) δ 159.7 (C-3), 157.6 (C-4a), 154.9 (C-10a), 151.6 (C-9), 144.8 (C-8), 133.0 (C-1), 118.3 (C-6b), 113.3 (C-7), 113.0 (C-11b), 110.4 (C-2), 103.8 (C-4), 96.6 (C-10), 78.9 (C11a), 67.0 (C-6), 57.3 (MeO-9), 56.2 (MeO-8), 41.2 (C-6a); ESI-MS (positive mode), *m/z* 301.1 $[M + H]^+$; MS/MS, *m/z* (relative abundance) 283.1 (7.6), 269.2 (11.7), 241.1 (4.1), 207.1 (0.5), 191.1 (100), 179.1 (3.9), 167.1 (86.9), 163.1 (6.8), 153.1 (13.1).

*(6aR,11aR)-3,4-Dihydroxy-9-methoxypterocarpan (**14**):* $[\alpha]_D$ −105.7 (*c*, CH_3OH); CD $[\theta]_{235}$ −10437.8, $[\theta]_{285}$ 4934.1 (*c* 6.9×10^{-5} M, CH_3OH); ^{1}H NMR (CD_3OD) δ 7.18 (H-7), 6.86 (H-1), 6.56 (H-2), 6.46 (H-8), 6.33 (H-10), 5.50 (H-11a), 4.32 (Hα-6), 3.75 (MeO-9), 3.59 (Hβ-6), 3.57 (H-6a); ^{13}C NMR (CD_3OD) δ 162.6 (C-9), 162.0 (C-10a), 147.1 (C-3), 145.8 (C-4a), 134.3 (C-4), 126.0 (C-7), 122.1 (C-1), 120.7 (C-6b), 113.8 (C-11b), 110.4 (C-2), 107.3 (C-8), 97.5 (C-10), 80.3 (C-11a), 67.9 (C-6), 55.9 (MeO-9), 41.0 (C-6a); ESI-MS (positive mode), *m/z* 287.4 $[M + H]^+$; MS/MS *m/z* (relative abundance) 272.1 (1.1), 269.1(3.9), 259.1 (3.8), 179.1 (1.2), 177.1 (0.6), 163.1 (4.4), 161.1 (22.8), 139.1 (100), 137.1 (55.1).

RESULTS AND DISCUSSION

The MeOH soluble fraction of one red-type propolis sample was fractionated by Sephadex LH-20 to investigate its chemical constituents. Fractions were further purified by MPLC and reversed-phase HPLC to yield 14 phenolic compounds including two isoflavones (**4** and **5**), three isoflavans (**6**−**8**), and six pterocarpans (**9**−**14**), which were identified using spectroscopic methods (**Figure 1**).

Compounds **1**−**3** were identified as gallic acid, isoliquiritigenin, and (−)-liquiritigenin, respectively, for comparison of their spectroscopic data with those reported in the literature (*14*−*16*). Both (−)-liquiritigenin (**2**) and isoliquiritigenin (**3**) are considered to be characteristic constituents of the Leguminosae family (*18*) and have exhibited interesting biological activities. (−)-Liquiritigenin and isoliquiritigenin have been shown to inhibit of xanthine oxidase activity in vitro (*19*) and have dose-related antiallergic activities (*20*). In addition, isoliquiritigenin was found to have effects in inhibiting the proliferation of the human non-small cell lung cancer A549 cell line, inducing apoptosis and locking cell cycle progression in the G_1 phase (*21*), suppressing azoxymethane (AOM)-induced colon carcinogenesis in ddY mice (*22*), and inhibiting the growth of prostate cancer (*23*). It has been suggested that isoliquiritigenin merits investigation as a potential cancer-chemopreventive agent in humans.

Compounds **4** and **5** showed the characteristic signals of isoflavones: H-2 in the ^{1}H NMR spectrum (δ 8.30 in **4** e δ 8.36 in **5**) and C-2, C-3, and C-4 in the ^{13}C NMR spectrum (δ 152.9, 124.3, and 174.5 in **4** and δ 154.2, 122.9, and 180.1 in **5**, respectively). These compounds were identified as formononetin (7-hydroxy-4′-methoxyisoflavone) (**4**) and biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) (**5**), respectively, by comparison of their spectroscopic data (UV, IR, ^{1}H and ^{13}C NMR, and ESIMS) with those reported in the literature (*17*). Formononetin (**4**) and biochanin A (**5**), the principal metabolites of red clover, are among naturally occurring isoflavones that have shown estrogenic activity. After mammals consume these isoflavones, they can be metabolized to daidzein and genistein, respectively, the main phytoestrogens derived from the diet (*24*).

The ^{1}H NMR spectra of **6**−**8** showed signals at 3.83−3.98 (1H, t, J = 10, 10 Hz, H-2ax), 4.17−4.26 (1H, dd, J = 10, 3 Hz, H-2eq), 3.40−3.49 (1H, m, H-3ax), 2.81−3.00 (1H, dd, J = 15.7, 10.5 Hz, H-4ax), and 2.66−2.82 (1H, dd, J = 15.7, 5.1 Hz, H-4eq), assigned to the heterocyclic protons of isoflavan moiety and confirmed also by signals in ^{13}C NMR spectrum (δ 70.9−71.3 for C-2, δ 32.7−33.1 for C-3, and δ 31.0−31.4 for C-4). Isoflavans **6**−**8** were identified by detailed analysis of 1D and 2D NMR experiments (DQF-COSY, HSQC, and HMBC), as the widely known vestitol (*25*) and as 7-*O*-methylvestitol (*26*) and 7,4′-dihydroxy-2′-methoxyisoflavan (*27*), respectively.

In the ^{1}H spectra of compounds **9**−**14**, a characteristic set of four protons [δ_H 3.57−3.59 (m, H-6β), 4.23−4.32 (dd, J = 10.4, 4.6 Hz, H-6α), 3.57−3.59, (m, H-6a), 5.43−5.50 (d, J = 6.5 Hz, H-11a)] due to hydrogens at C-6 (δ_C 67.3−67.9, CH_2), C-6a (δ_C 40.9−41.4), and C-11a (δ_C 79.9−80.3), respectively, suggested compounds **9**−**14** have a pterocarpan skeleton. The structures of **9**−**14** were deduced from detailed analysis of the ^{1}H and ^{13}C NMR data aided by 2D NMR experiments (DQF-COSY, HSQC, and HMBC) and identified as medicarpin (**9**), homopterocarpin (**10**), vesticarpan (**11**), 3,8-dihydroxy-9-methoxypterocarpan (**12**), 3-hydroxy-8,9-dimethoxypterocarpan (**13**), and 3,4-dihydroxy-9-methoxypterocarpan (**14**) (*25*, *28*−*31*). The presence of hydroxyl and methoxyl groups and their locations were established on the basis of the ^{13}C NMR spectra and HMBC data. Pterocarpans possessing a *cis*-6a,11a-dihydro-6*H*-benzofuro[3,2-*c*]chromene skeleton constitute the second largest group of natural isoflavonoids, and they have been gaining considerable interest due to their wide range of biological effects.

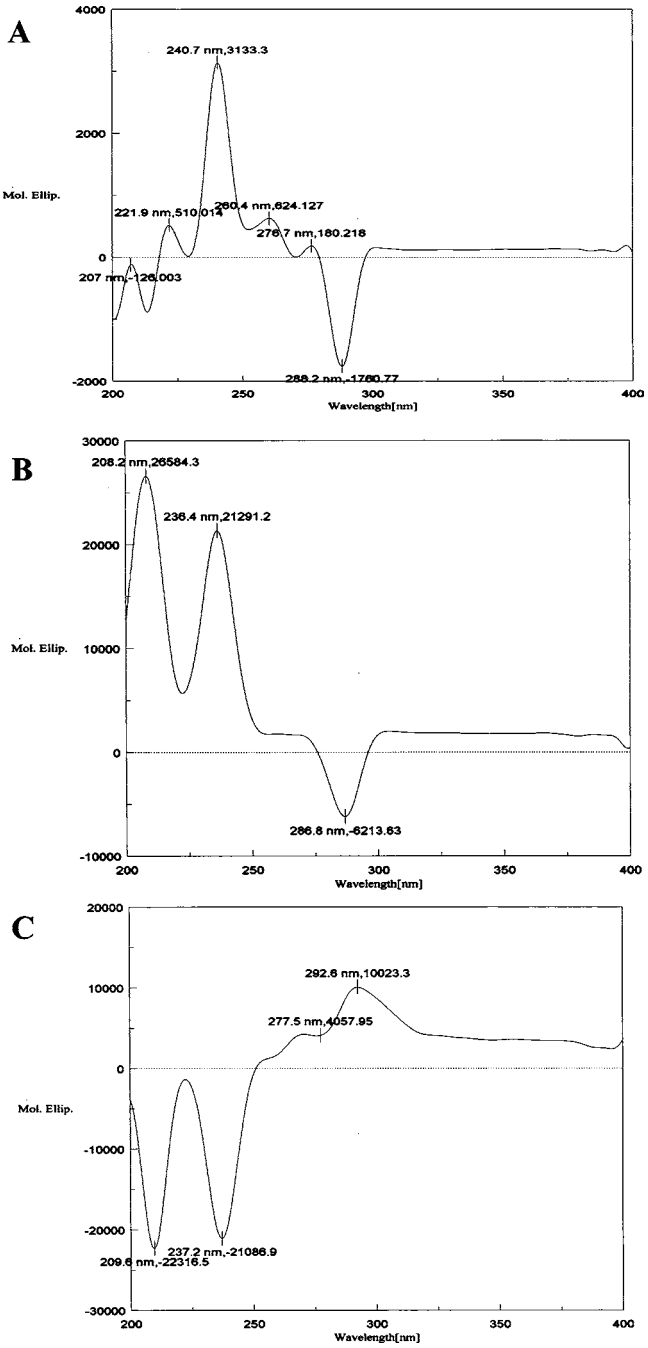


Figure 2. CD spectra of (3*S*)-isoflavan and (6a*S*,11a*S*)- and (6a*R*,11a*R*)-pterocarpans: (**A**) (3*S*)-7-*O*-methylvestitol (**7**); (**B**) (6a*S*,11a*S*)-homopterocarpin (**10**); (**C**) (6a*R*,11a*R*)-3-hydroxy-8,9-dimethoxypterocarpan (**13**).

Many of them are phytoalexins possessing high antifungal (*32*) and antibacterial activity (*33*), and several of them have been reported to inhibit HIV-1 reverse transcriptase and the cytopathic effect of HIV-1 in cell cultures (*34*, *35*), as well as antisnake venom activity (*36*). Recently, indigocarpan showed cyclooxygenase-1 (COX-2) inhibitory and in vivo anti-inflammatory activities (*37*).

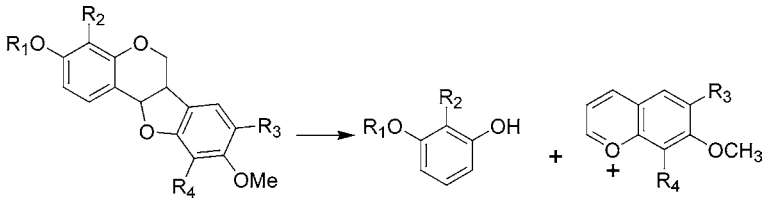
In the nonplanar isoflavonoids (isoflavans, isoflavanones, isoflavan-4-ols, pterocarpans, and rotenoids) chirality is confined to two of the carbon atoms of the chroman heterocycle. The CD data provide a powerful probe to unequivocally establish the absolute configuration of the naturally occurring compounds. The CD spectra of all isoflavans isolated (**6**−**8**) exhibit positive and negative Cotton effects in the 240−246 and 283−289 nm regions (**Figure 2A**), respectively, indicating the *S*-configuration at C-3 (*38*). Also, the absolute stereochemistry at C-6a and C-11a of pterocarpans **9**−**14** was established from CD spectra. Although there had been only empirical correlations between the CD data of pterocarpans and their absolute configuration, CD spectroscopy was widely utilized for their configurational assignments (*36*, *39*−*41*), which exploited the ^1L_b and ^1L_a CD bands of the chromane and the 2,3-dihydrobenzo[*b*]furan chromophores being cis annulated in natural pterocarpans. Compounds **9** and **10** showed positive and negative Cotton effects in the 236−238 and 287−294 nm regions (**Figure 2B**), respectively, in contrast to the CD curves of **11**−**14**, which exhibited opposite Cotton effects in these regions (**Figure 2C**). On the basis of the well-established 6a*R*,11a*R* configuration of (−)-maackiain (*42*) and the same positive−negative sign patterns of the major ^1L_b and ^1L_a band CDs (*43*), we conclude that the pterocarpans **9** and **10** and **11**−**14** have, respectively, the 6a*S*,11a*S* and 6a*R*,11a*R* configuration.

Both vestitol (**6**) and medicarpin (**9**) have been isolated from plants of the family Leguminosae frequently. However, some isoflavans (**7** and **8**) and pterocarpans (**11**−**14**) are less common, and their spectroscopic data were reported many years ago. In this work ^{13}C and ^1H NMR data of compounds **7**, **8**, and **10**−**13** have been assigned on the basis of modern 2D NMR techniques. Absolute stereochemistry of isoflavans and pterocarpans was confirmed or determined for the first time on the basis of CD measurements. ^{13}C NMR data of vesticarpan (**11**) and the absolute stereochemistry of (6a*R*,11a*R*)-3-hydroxy-8,9-dimethoxypterocarpan (**13**) are also reported herein for the first time.

There are only a few papers in the literature dealing with electron impact (EI) fragmentation mass spectrometry of pterocarpans (*44*, *45*). Pelter and co-workers (*44*) have reported the EIMS of pterocarpan derivatives, and they found that the retro-Diels−Alder (RDA) rearrangement, which is characteristic of the chromanone or chromone derivatives (such as flavanone, flavone, and isoflavone) was completely absent in the spectra of pterocarpan derivatives. They found that O-5 and C-6, as well as C-6 and C-6a, cleavages were the favored processes yielding highly stable benzofuran-type heterocyclic ions. In the present study we report for the first time the ESI-MS/MS fragmentation data of pterocarpans. ESI-MS spectra of **9**−**14** were acquired in both positive and negative modes, the former giving the best results both in full-scan MS and MS/MS experiments; consequently, only positive ion mass spectra are discussed.

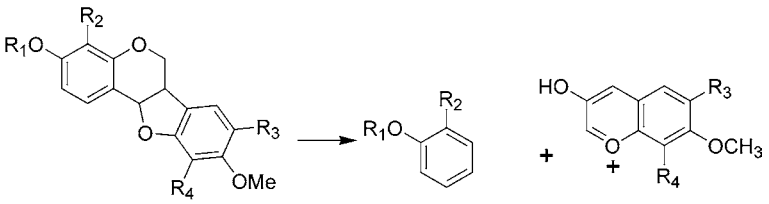
The full-scan MS spectra of **9**−**14** showed pseudomolecular ions $[M + H]^+$ (*m*/*z* 271, 285, 287, 287, 301, 287, respectively) and $[M + Na]^+$ (*m*/*z* 293, 307, 309, 309, 323, 309, respectively). The MS/MS spectrum of the $[M + H]^+$ precursor ion of **9** showed as main fragment ions **9a** (*m*/*z* 161, $[M + H - 110]^+$), corresponding to the loss of a *m*-hydroxyphenolic neutral fragment; **9b** (*m*/*z* 177, $[M + H - 94]^+$), corresponding to the loss of a phenol unit; and **9c** (*m*/*z* 137, $[M + H - 134]^+$), due to the loss of benzofuran derivative neutral fragment (**Figure 3**). The MS/MS spectra of the $[M + H]^+$ precursor ions of the other pterocarpans (**10**−**14**) showed the same fragmentation pathways as those described for compound **9**. The proposed structures of the most characteristic fragment ions in the MS/MS spectra of the pterocarpans **9**−**14** are given in **Figure 3**. In all cases the base peaks of MS/MS spectra are the ions **9c**−**14c** due to the neutral loss of benzofuran derivative. The structures of these fragment ions were proposed as showed in **Figure 3c** on the basis of the presence of an intense fragment ion corresponding to the loss of CO in their MS^3 spectra. Another

a



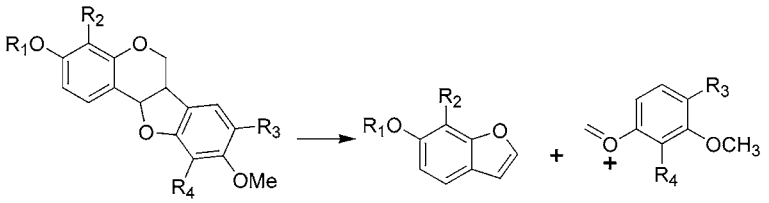
	R_1	R_2	R_3	R_4	$[M+H]^+$	NL*		*m/z*
9	H	H	H	H	*m/z* 271	- 110	**9a**	161
10	CH_3	H	H	H	*m/z* 285	- 124	**10a**	161
11	H	H	H	OH	*m/z* 287	- 110	**11a**	177
12	H	H	OH	H	*m/z* 287	- 110	**12a**	177
13	H	H	OCH_3	H	*m/z* 301	- 110	**13a**	191
14	H	OH	H	H	*m/z* 287	- 126	**14a**	161

b



	R_1	R_2	R_3	R_4	$[M+H]^+$	NL		*m/z*
9	H	H	H	H	*m/z* 271	- 94	**9b**	177
10	CH_3	H	H	H	*m/z* 285	- 108	**10b**	177
11	H	H	H	OH	*m/z* 287	- 94	**11b**	193
12	H	H	OH	H	*m/z* 287	- 94	**12b**	193
13	H	H	OCH_3	H	*m/z* 301	- 94	**13b**	207
14	H	OH	H	H	*m/z* 287	- 110	**14b**	177

c



	R_1	R_2	R_3	R_4	$[M+H]^+$	NL		*m/z*
9	H	H	H	H	*m/z* 271	- 134	**9c**	137
10	CH_3	H	H	H	*m/z* 285	- 148	**10c**	137
11	H	H	H	OH	*m/z* 287	- 134	**11c**	153
12	H	H	OH	H	*m/z* 287	- 134	**12c**	153
13	H	H	OCH_3	H	*m/z* 301	- 134	**13c**	167
14	H	OH	H	H	*m/z* 287	- 150	**14c**	137

Figure 3. Fragmentation pathway proposed for $[M + H]^+$ ions of pterocarpans **9**–**14**. *, NL = neutral loss.

characteristic peak appreciated in all pterocarpans is the benzopyrilum ion **9a**−**14a** (**Figure 3a**). In all cases the hydroxybenzopyrilium ions **9b**−**14b** are produced in addition to the benzopyrilium ions, as indicated in **Figure 3b**.

The fragmentation pathway observed for pterocarpans **9**−**14** confirmed the known structures of these compounds and the pattern of substitution of aromatic rings. These data demonstrated that the nature of substituents did not modify the general fragmentation process; thus, the diagnostic key fragmentations proved to be useful in the structural characterization of pterocarpans. It is worth noting that between the most characteristic fragment ions in the EI mass spectra (70 eV) of eight deuterated pterocarpans there were benzopyran and benzofuran derivatives, and their structures were supported by high-resolution accurate mass measurements (*45*). In this ESI-MS study, benzopyran derivatives are also assumed to be main ions on the basis of the structural relationship among the six isolated pterocarpans. The main difference observed in ESI-MS spectra with respect to those obtained by the EI technique is the absence of benzofuran fragments as positive ions. Benzofuran moieties were deduced as the loss of neutral fragments from the molecular ion.

In our previous studies, we reported the occurrence of polyprenylated benzophenones from two brown-type propolis samples collected in Nuevitas and Guantanamo Provinces of Cuba. The botanical origin of these propolises was mainly *Clusia rosea* resinous exudates, a tree widely distributed in the island. It seems that *Clusia* species are important as propolis sources in tropical regions, together with *Baccharis* species in Brazil. In this study isoflavonoids, but not prenylated benzophenones, were isolated from a red-type propolis sample collected in Pinar del Rio Province, suggesting the presence of at least two groups of propolises in Cuba. Our results suggest the contribution of some Leguminosae species, producing exudates that bees

collected to produce propolis; however, further studies on the chemical compositions of propolis and plants are needed for confirmation. The constituents of Cuban propolis were dissimilar to not only the propolis from Europe and China but also those of propolis from Brazil, whereas some of them were seen in the propolis of Venezuela. The presence of isoflavonoids in Cuban propolis suggests new biological potentialities for this natural product with respect to those propolises rich in nemorosone. This appears to be the first report on the occurrence of isoflavonoids in the propolis, and these compounds could be useful as chemical markers for Cuban red-type propolis.

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