JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Constituents of Hondurian Propolis with Inhibitory Effects on *Saccharomyces cerevisiae* Multidrug Resistance Protein Pdr5p

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ABSTRACT: Chemical investigation of a propolis sample collected in Honduras has led to the isolation of the new (*E*,*Z*)-cinnamyl cinnamate (**2**) together with 14 known compounds: 6 cinnamic ester derivatives, 2 flavanones, 1 chalcone, 2 triterpenes, and 3 aromatic acids. Structural determination was accomplished by spectroscopic analysis, particularly two-dimensional (2D) nuclear magnetic resonance (NMR) and electrospray ionization−tandem mass spectrometry (ESI−MS/MS) techniques. Futhermore, we checked the ability of the propolis extract and the most representative compounds of each class (**1**, **5**, **8**, and **10**) to inhibit the activity of Pdr5p, a protein responsible for a multidrug resistance phenotype in yeast. The present study appears to be the first report on Honduras propolis. Isolated cinnamic ester derivatives indicated the possible relation between Honduras propolis and the genus *Liquidambar*.

KEYWORDS: *Honduras propolis, cinnamic ester derivatives, aromatic compounds, triterpenes, 1D and 2D NMR spectroscopy, ESI−MS/MS, Pdr5p, multidrug resistance*

INTRODUCTION

Propolis is a resinous composite material collected by honeybees from the buds, sap flows, and barks of certain plants and trees, and this material is thought to serve as a defense substance for bee's hives.[1] Propolis releases a pleasant aromatic odor and presents from a yellow−green, red, to dark brown color depending upon its resource and age.

Propolis is well-known for its potential health benefit and is reported to possess valuable biological activities, such as antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory, and anticancer activities.[1,2] Recently, it has been extensively marketed by the pharmaceutical industries as an alternative medicine and a health food in various parts of the world.

Propolis chemical composition is qualitatively and quantitatively variable, depending upon the season, the species of bee and vegetation, and the area of collection.[3,4] In our previous studies, we reported the occurrence of polyprenylated benzophenones, isoflavonoids, and triterpenes from Cuban propolis samples,[4−6] isoflavonoids and polyisoprenylated benzophenones from Brazilian propolis,[7] and 1,3-diarylpropane derivatives, flavonoids, and isoflavonoids from Mexican propolis.[8] Considering the high percentage of endemic plants present in Central America, we decided to continue our research about chemical composition of Mesoamerican propolis to suggest similarities and differences between samples collected in different countries. In this paper, we reported the chemical investigation of a Honduras propolis sample that lead to isolation of seven cinnamic ester derivatives, among them one new natural product, two flavanones, one chalcone, two triterpenes, and three aromatic acids. Cinnamic ester derivatives are usually identified by gas chromatography (GC) and GC/mass spectrometry (MS). In this paper, we investigated the constituents of Honduras propolis using MS and nuclear magnetic resonance (NMR) techniques after isolation of each compound, providing useful NMR assignments for these structures. Four purified compounds from this propolis sample were tested to verify the ability to inhibit the Pdr5p from yeast plasma membrane, a protein that is responsible to confer a multidrug resistance phenotype in fungi. We observed that all compounds tested were very effective and inhibited the ATPase activity of Pdr5p with a low IC_{50} value. The present study appears to be the first report on a propolis sample collected in Honduras and shows the biological activity of compounds purified from it.

MATERIALS AND METHODS

Chemicals. Analytical-grade chloroform ($CHCl_3$) and methanol (MeOH) and high-performance liquid chromatography (HPLC)-grade MeOH were obtained from Carlo Erba (Milan, Italy). Water and MeOH employed for the electrospray ionization (ESI)−MS analyses were of HPLC supergradient quality (Romil Ltd., Cambridge, U.K.).

General Experimental Procedure. Optical rotations were determined on a model DIP-1000 polarimeter (Jasco, Easton, MD)

Received: July 2, 2012
Revised: September 20, 2012
Accepted: September 25, 2012
Published: September 25, 2012

equipped with a sodium lamp (589 nm) and a 10 cm microcell. Infrared (IR) spectra were determined on an IR-230 (Jasco, Easton, MD). A Bruker DRX-600 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany), 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 δH 3.34 and δC 49.0 for CD_3OD and δH 7.26 and δC 77.0 for $CDCl_3$. Coupling constants, J, are in Hertz. Distortionless enhancement by polarization transfer (DEPT), ^{13}C, double-quantum-filtered correlation spectroscopy (DQF-COSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC) NMR experiments were carried out using the conventional pulse sequences, as described in the literature. ESI−MS was performed using a Finnigan LCQ Advantage instrument from Thermo Fisher Scientific (San Jose, CA) equipped with Xcalibur 3.1 software. Full mass and collision-induced dissociation (CID) MS/MS spectra were acquired in both positive and negative modes. Instrumental parameters were tuned for each investigated compound, and specific collision energies were chosen at each fragmentation step for all of the investigated compounds, with the value ranging from 15 to 33% of the instrument maximum. Data were acquired in the MS1 scanning mode (m/z 100−700). All compounds were dissolved in 1:1 (v/v) MeOH/H_2O at a concentration of 5 μg mL^{-1} and infused in the ESI source with a flow rate of 5 μL min^{-1}. Exact masses were measured by a Q-TOF Premier (Waters, Milford, MA) instrument. Chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden), employing MeOH as the solvent. Column chromatography was carried out employing Silica gel 60 (0.040−0.063 mm; Carlo Erba) and $CHCl_3$/MeOH gradients. HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector. The column used was a 250 × 10 mm inner diameter, 10 μm, Kromasil RP-18 (Phenomenex, Torrance, CA). Thin-layer chromatography (TLC) analysis was performed with Macherey−Nagel precoated silica gel 60 F_{254} plates.

Propolis. The propolis sample (Marcala, Honduras) was provided by "Estacion Apicola La Mora" (August 2010). Both the sample and dried methanol extract were stored at 5 °C in the dark.

Extraction and Isolation Procedure of Compounds 1−15. The propolis sample (20 g) was extracted with methanol (100 mL × 6) for a total of 3 h. After filtration, the methanol extract was taken to dryness under reduced pressure to yield a dark red gum (16.4 g). A portion of this extract (2.5 g) was fractionated over a Sephadex LH-20 column (100 × 5 cm) using methanol as the solvent to furnish five fractions (1−5). Fraction 1 (160.1 mg) was purified by reversed-phase (RP)-HPLC [9:1 (v/v) MeOH/H_2O] to give triterpenes **8** (38.5 mg) and **9** (25.0 mg). Fractions 2 and 3 (144.1 and 55 mg, respectively) were purified by RP-HPLC [85:15 (v/v) MeOH/H_2O] to give the cinnamic ester derivatives **1** (78.5 mg), **2** (8.5 mg), **3** (5.5 mg), and **4** (18.0 mg). Fraction 4 (140.7 mg) was purified by column chromatography on silica gel using $CHCl_3$/MeOH gradients and afforded pure cinnamic ester derivatives **5** (34.1 mg), **6** (20.9 mg), and **7** (12.0 mg). Fraction 5 (394.9 mg) was purified by RP-HPLC [7:3 (v/v) MeOH/H_2O] to give flavonoids **10** (10.5 mg), **11** (12.4 mg), and **12** (6.5 mg) and aromatic acids **13** (15.4 mg), **14** (12.7 mg), and **15** (20.0 mg).

*(E)-Cinnamyl-(E)-cinnamate (**1**).* Yellow oil. IR (KBr) ν_{max} (cm^{-1}): 3140, 1720, 1180. High-resolution (HR)-ESI−MS (m/z): [M + H]$^+$ calcd for $C_{18}H_{16}O_2$, 265.2990; found, 265.3184. ^{1}H and ^{13}C NMR: see Table 1.

*(E)-Cinnamyl-(Z)-cinnamate (**2**).* Yellow oil. IR (KBr) ν_{max} (cm^{-1}): 3150, 1740, 1180. HR-ESI−MS (m/z): calcd for $C_{18}H_{16}O_2$ [M + H]$^+$, 265.3040; found, 265.3184. ^{1}H and ^{13}C NMR: see Table 1.

*Hydrocinnamyl-(E)-cinnamate (**3**).* Yellow oil. ^{1}H NMR ($CDCl_3$) δ: 7.71 (1H, d, J = 16.0 Hz, H-3), 7.57 (2H, dd, J = 1.5 and 7.5 Hz, H-5, H-9), 7.44 (2H, t, J = 1.5 and 7.5 Hz, H-6, H-8), 7.43 (1H, m, H-7), 7.42 (1H, m, H-7′), 7.34 (2H, t, J = 1.5 and 7.0 Hz, H-6′, H-8′), 7.25 (2H, dd, J = 1.5 and 7.0 Hz, H-5′, H-9′), 6.49 (1H, d, J = 16.0 Hz, H-2), 4.27 (2H, t, J = 6.5 Hz, H-1′), 2.79 (2H, t, J = 7.5 Hz, H-3′), 2.09 (2H, m, H-2′). ^{13}C NMR ($CDCl_3$) δ: 167.40 (C-1), 144.22 (C-3), 141.40 (C-4′), 134.40 (C-4), 129.40 (C-6, C-8), 128.95 (C-7), 128.90 (C-7′), 128.60 (C-5′, C-9′), 128.44 (C-6′, C-8′), 128.13 (C-5, C-9), 117.98 (C-2), 63.92 (C-1′), 32.05 (C-3′), 30.17 (C-2′). ESI−MS (positive mode) (m/z): [M + H]$^+$, 267.

*Benzyl-(E)-cinnamate (**4**).* ^{1}H NMR (CD_3OD) δ: 7.73 (1H, d, J = 16.0, H-3), 7.52 (2H, dd, J = 1.5 and 7.5 Hz, H-5, H-9), 7.41 (2H, dd, J = 1.5 and 7.5, H-3′, H-7′), 7.40 (2H, t, J = 1.5 and 7.5 Hz, H-6, H-8), 7.39 (2H, t, J = 1.5 and 7.0 Hz, H-4′, H-6′), 7.38 (1H, m, H-7), 7.35 (1H, m, H-5′), 6.49 (1H, d, J = 16.0, H-2), 5.26 (2H, s, H-1′). ^{13}C NMR (CD_3OD) δ: 167.33 (C-1), 145.78 (C-3), 136.64 (C-1′), 134.95 (C-4), 131.06 (C-4′, C-6′), 130.28 (C-7), 129.50 (C-6, C-8), 128.87 (C-3′, C-7′), 128.86 (C-5′), 128.70 (C-5, C-9), 118.47 (C-2), 66.95 (C-1′). ESI−MS (positive mode) (m/z): [M + H]$^+$, 239.

*(E)-Cinnamyl-(E)-p-coumarate (**5**).* ^{1}H NMR ($CDCl_3$) δ: 7.70 (1H, d, J = 15.9 Hz, H-3), 7.44 (2H, dd, J = 2.5 and 8.0 Hz, H-5, H-9), 7.43 (2H, dd, J = 1.8 and 7.2 Hz, H-5′, H-9′), 7.35 (2H, t, J = 1.8 and 7.2 Hz, H-6′, H-8′), 7.28 (1H, m, H-7′), 6.88 (2H, dd, J = 2.5 and 8.0 Hz, H-6, H-8), 6.73 (1H, d, J = 15.8 Hz, H-3′), 6.39 (1H, dt, 6.1, 15.8 H-2′), 6.36 (1H, d, J = 15.8 Hz, H-2), 4.98 (1H, d, J = 6.1 Hz, H-1′). ^{13}C NMR ($CDCl_3$) δ: 168.0 (C-1), 158.67 (C-7), 145.65 (C-3), 136.67 (C-4′), 134.74 (C-3′), 130.60 (C-5, C-9), 129.15 (C-6′, C-8′), 128.62 (C-7′), 127.46 (C-4), 127.17 (C-5′, C-9′), 123.87 (C-2′), 116.41 (C-2), 116.40 (C-6, C-8), 65.69 (C-1′). ESI−MS (positive mode) (m/z): [M + H]$^+$, 281.

*(E)-Cinnamyl-(Z)-p-coumarate (**6**).* ^{1}H NMR ($CDCl_3$) δ: 7.68 (2H, dd, J = 2.5 and 8.0 Hz, H-5, H-9), 7.41 (2H, dd, J = 1.8 and 7.4 Hz, H-5′, H-9′), 7.35 (2H, t, J = 1.8 and 7.4 Hz, H-6′, H-8′), 7.28 (1H, m, H-7′), 6.90 (1H, d, J = 12.7 Hz, H-3), 6.82 (2H, dd, J = 2.5 and 8.0 Hz, H-6, H-8), 6.67 (1H, d, J = 15.9 Hz, H-3′), 6.32 (1H, dt, J = 6.1 and 15.8 Hz, H-2′), 5.90 (1H, d, J = 12.7 Hz, H-2), 4.82 (2H, d, J = 5.6 Hz, H-1′). ^{13}C NMR ($CDCl_3$) δ: 167.51 (C-1), 157.92 (C-7), 143.54 (C-3), 137.48 (C-4′), 134.03 (C-3′), 132.27 (C-5, C-9), 129.22 (C-4′), 128.50 (C-6′, C-8′), 127.89 (C-7′), 126.40 (C-5′, C-9′), 123.10 (C-2′), 116.80 (C-2), 114.85 (C-6, C-8), 64.90 (C-1′). ESI−MS (positive mode) (m/z): [M + H]$^+$, 281.

*(E)-Cinnamyl-benzoate (**7**).* ^{1}H NMR ($CDCl_3$) δ: 8.08 (2H, dd, J = 1.5 and 7.5 Hz, H-3, H-7), 7.57 (2H, t, J = 1.5 and 7.5 Hz, H-4, H-6), 7.45 (1H, m, H-5), 7.42 (2H, dd, J = 1.5 and 8.0 Hz, H-5′, H-9′), 7.32 (2H, t, J = 1.5 and 8.0 Hz, H-6′, H-8′), 7.25 (1H, m, H-7′), 6.75 (1H, d, J = 15.9 Hz, H-3′), 6.41 (1H, dt, J = 6.2 and 15.8 Hz, H-2′), 4.99 (1H, d, J = 6.2 Hz, H-1′). ^{13}C NMR ($CDCl_3$) δ: 165.92 (C-1), 136.47 (C-4′), 134.01 (C-3′), 133.10 (C-2), 132.60 (C-4, C-6), 129.32 (C-3, C-7), 128.28 (C-6′, C-8′), 128.07 (C-5), 127.76 (C-7′), 126.35 (C-5′,

Table 1. ^{1}H and ^{13}C NMR (600 MHz) Data for Compounds 1 and 2 in $CDCl_3$[a]

	compound 1		compound 2	
position	δ (^{1}H) (J_{HH})	δ (^{13}C)	δ (^{1}H) (J_{HH})	δ (^{13}C)
1		167.3		167.3
2	6.52 (d, 16.0)	118.5	6.02 (d, 12.5)	121.0
3	7.75 (d, 16.0)	145.7	7.02 (d, 12.5)	144.9
4		134.9		135.8
5, 9	7.55 (dd, 1.5; 7.5)	128.7	7.35 (dd, 1.5; 7.5)	130.1
6, 8	7.41 (t, 1.5; 7.5)	129.5	7.37 (t, 1.5; 7.5)	129.5
7	7.40 (m)	130.9	7.62 (m)	130.8
1′	4.90 (d, 6.1)	65.7	4.80 (d, 6.1)	65.9
2′	6.39 (dt, 6.1; 15.8)	123.9	6.31 (dt, 6.1; 15.5)	124.2
3′	6.74 (d, 15.8)	134.8	6.64 (d, 15.5)	135.4
4′		136.8		137.0
5′, 9′	7.44 (dd, 1.4; 7.3)	127.2	7.39 (dd, 1.5; 7.5)	127.8
6′, 8′	7.36 (t, 1.4; 7.3)	129.2	7.36 (t, 1.5; 7.5)	129.4
7′	7.30 (m)	128.6	7.32 (m)	128.6

[a]Chemical shift values are in ppm from tetramethylsilane (TMS), and J values in Hz are presented in parentheses. All signals were assigned by DQF-COSY, HSQC, and HMBC experiments.

dx.doi.org/10.1021/jf302578r | J. Agric. Food Chem. 2012, 60, 10540−10545

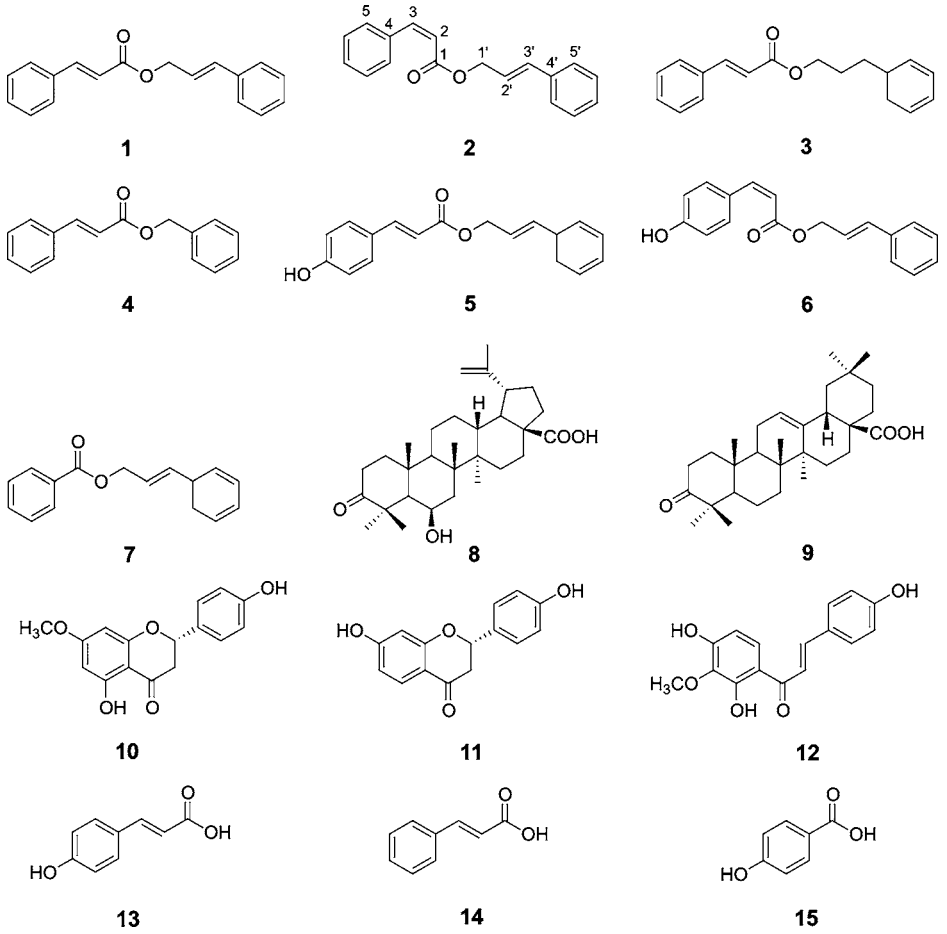

Figure 1. Compounds (**1**–**15**) identified from Honduras propolis.

C-9′), 122.91 (C-2′), 65.42 (C-1′). ESI–MS (positive mode) (*m*/*z*): $[M + H]^+$, 239.

*6β-Hydroxy-3-oxo-lup-20(29)-en-28-oic Acid (**8**).* ^1H and ^{13}C NMR data were consistent with those previously reported.[9] ESI–MS (positive mode) (*m*/*z*): $[M + H]^+$, 471.

*3-Oxo-oleanoic Acid (**9**).* ^1H and ^{13}C NMR data were consistent with those previously reported.[10] ESI–MS (positive mode) (*m*/*z*): $[M + H]^+$, 455.

*(−)-Sakuranetin (**10**).* $[\alpha]_D$ −11.2 (*c* 0.16, CH_3OH). ^1H and ^{13}C NMR data were consistent with those previously reported.[11] ESI–MS (positive mode) (*m*/*z*): $[M + H]^+$, 287.

*(−)-Liquiritigenin (**11**).* $[\alpha]_D$ −37.3 (*c* 0.16, CH_3OH). ^1H and ^{13}C NMR data were consistent with those previously reported.[4] ESI–MS (positive mode) (*m*/*z*): $[M + H]^+$, 257.3. MS/MS (*m*/*z*): 239.0, 211.1, 147.0, 137.1.

*Kukulkanin B (**12**).* ^1H and ^{13}C NMR data were consistent with those previously reported.[12] ESI–MS (positive mode) (*m*/*z*): $[M + H]^+$, 287.

*(E)-p-Coumaric Acid (**13**).* ^1H and ^{13}C NMR data were identical with those of authentic samples. ESI–MS (negative mode) (*m*/*z*): $[M - H]^-$, 163.

*(E)-Cinnamic Acid (**14**).* ^1H and ^{13}C NMR data were identical with those of authentic samples. ESI–MS (negative mode) (*m*/*z*): $[M - H]^-$, 147.

*p-Hydroxybenzoic Acid (**15**).* ^1H and ^{13}C NMR data were identical with those of authentic samples. ESI–MS (negative mode) (*m*/*z*): $[M - H]^-$, 137.

Preparation of Plasma Membrane. Plasma membrane fractions were obtained from yeast *Saccharomyces cerevisiae* mutants AD124567 and AD1234567,[13] as previously reported by Rangel et al.[14] The plasma membranes highly enriched in Pdr5p were stored in liquid nitrogen.

ATPase Activity Assay. The ATPase activity assay was performed incubating Pdr5p-enriched membranes (0.013 mg mL^{-1} final concentration) in a 96-well plate at 37 °C for 60 min in a reaction medium containing 100 mM Tris-HCl at pH 7.5, 4 mM $MgCl_2$, 75 mM KNO_3, 7.5 mM NaN_3, 0.3 mM ammonium molybdate, and 3 mM ATP. The compounds **1**, **5**, **8**, and **10** were added from stock solution in dimethylsulfoxide up to 5% (v/v) final concentration. After the incubation period, the reaction was stopped with 1% sodium dodecyl sulfate (SDS), as described previously.[15] The released inorganic phosphate (P_i) was measured using the method from Fiske and Subbarow.[16] Control plasma membranes prepared from the PDR5-deleted strain AD1234567 were used.

RESULTS AND DISCUSSION

Propolis obtained from Honduras was extracted with methanol, and the extract was subjected to column chromatographic separation to yield 15 compounds: 7 cinnamic ester derivatives (**1**–**7**), 2 triterpenes (**8** and **9**), 2 flavanones (**10** and **11**), 1 chalcone (**12**), and 3 aromatic acids (**13**–**15**). The compounds were characterized by 1D and 2D NMR and MS experiments as (*E*)-cinnamyl-(*E*)-cinnamate (**1**), (*E*)-cinnamyl-(*Z*)-cinnamate (**2**), hydrocinnamyl-(*E*)-cinnamate (**3**), benzyl-(*E*)-cinnamate (**4**), (*E*)-cinnamyl-(*E*)-*p*-coumarate (**5**), (*E*)-cinnamyl-(*Z*)-*p*-coumarate (**6**), (*E*)-cinnamyl-benzoate (**7**), 6*β*-hydroxy-3-oxo-lup-20(29)-en-28-oic acid (**8**), 3-oxo-oleanoic acid (**9**), sakuranetin (**10**), liquiritigenin (**11**), kukulkanin B (**12**), (*E*)-*p*-coumaric acid (**13**), (*E*)-cinnamic acid (**14**), and *p*-hydroxybenzoic acid (**15**). Compound **2** was a new natural product. Figure 1 shows the structures of isolated compounds from Honduras propolis.

dx.doi.org/10.1021/jf302578r | *J. Agric. Food Chem.* 2012, 60, 10540–10545

The ^{1}H NMR of compound **1** showed, in the olefinic region, six proton signals attributable to those of two monosubstituted aromatic rings at δ 7.30−7.55 and two sets of signals because of the olefin hydrogens. ^{1}H NMR also showed a doublet for a methylene group at δ 4.90 (2H, d, J = 6.1 Hz). These data, together with those derived from the COSY experiment, suggested the presence of cinnamyl acid and cinnamyl alcohol moieties, and the coupling constant value of the propenylic and propanoid chains indicated an *E* geometry for the two double bonds. ^{13}C NMR confirmed the hypothesis, showing six protonated aromatic carbon signals and two tetrasubstituted carbons, besides the olefinic methynes at δ 118.5 and 145.7 (H-2 and H-3) and at δ 123.8 and 134.8 (H-2′ and H-3′), a methylene, and a carbonyl group. The three mutually coupled protons of the propenylic chains were readily assigned to H-3′, H-2′, and H-1′ from the HMBC correlations. The olefinic proton signals (H-2′ and H-3′) were observed to correlate with the aromatic ring B (H-2′/C-4′ and H-3′/C-1′, C-5′, and C-9′). The carbinol protons (H-1′) were correlated with both the olefinic carbons (C-2′ and C-3′) and the carbonyl carbon (C-1), which showed a cross-peak with the protons at 7.75 (H-3). The HMBC experiment also showed correlations between the aromatic carbons of ring A (C-4, C-5, and C-9) and the protons at δ 6.52 and 7.75 of the cinnamyl acid moiety. These data suggested the structure of (*E*)-cinnamyl-(*E*)-cinnamate for compound **1**.

Compound **2** was also obtained as a yellow oil. The molecular formula of compound **2** was determined to be $C_{18}H_{16}O_2$ by HR-ESI−MS; therefore, compound **2** must be an isomer of compound **1** with an identical mass. The IR spectrum of compound **1** indicated the presence of aromatic groups and carbonyl functions. Compound **2** showed 1D and 2D NMR features similar to those of compound **1**. The main differences were the AA′BB′ system of the 4-monosubstituted aromatic ring shifted at δ 7.35 (H-5 and H-9) and 7.37 (H-6 and H-8), and the propanoid chain signals present as doublets at δ 6.02 and 7.02 and coupled between them in a *cis* relationship (J = 12.5 Hz). The observed $\Delta\delta$H and $\Delta\delta$C values for 2 and 3 positions were consistent for a *Z* geometry of the propanoid chain (Table 1). The HMBC experiment demonstrated the following correlations: H-2 with C-4, H-3 with C-1, C-5, and C-9, H-1′ with C-1 and C3′, H-2′ with C-4′, and H-3′ with C-1′, C-5′, and C-9′. From all of these data, the structure of compound **2** was concluded to be the novel (*E*)-cinnamyl-(*Z*)-cinnamate.

Cinnamic ester derivatives (**3**−**7**) were also isolated. Such compounds are usually identified by GC and GC/MS analysis, and many authors did not isolate the compound and did not determine the full structure on the basis of NMR data. We isolated and identified these compounds, and full assignment of all ^{1}H and ^{13}C resonances were carried out by extensive analysis of their NMR spectra (see the Materials and Methods).

Comparison of ^{1}H and ^{13}C NMR data of hydrocinnamyl cinnamate (**3**) to those of compound **1** suggested that the double bound at C-2′−C-3′ was missing.

For compound **4**, the ^{1}H NMR and COSY spectra revealed two sets of 4-monosubstituted rings, a disubstituted *trans*-double bond, and a methylene. The ^{13}C NMR spectrum showed 16 carbon signals. In a HMBC experiment, the following correlations were observed: H-2/C-4, H-3/C-5, and H-1′/C-3′ and C-7′. Thus, the structure benzyl-(*E*)-cinnamate was established for compound **4**.

The spectroscopic data of compounds **5** and **6** revealed that the only difference between them and compounds **1** and **2**, respectively, was the level of substitution of the aromatic ring attached to C-3. In compounds **5** and **6**, the 4-monosubstituted benzene ring was replaced by an AA′BB′ spin system. Thus, the structures of compounds **5** and **6** were established to be (*E*)-cinnamyl-(*E*)-*p*-coumarate (**5**) and (*E*)-cinnamyl-(*Z*)-*p*-coumarate (**6**).

The ^{1}H NMR and COSY spectra of compound **7** revealed two 4-monosubstituted benzene rings, mutually coupled protons of a *trans* double bond, and a methylene of a propenylic chain. The chemical shift assigned to H-3−H-5 (δ 8.08) is attributable to the aromatic proton vicinal to the carbony group; in fact, this proton is known to undergo downfield shifts because of the carbonyl anisotropy and electric field. The ^{13}C NMR spectrum showed 16 carbon signals. In a HMBC experiment, the following correlations were observed: H-3 and H-5/C-1, H-1′/C-5 and C-3′, H-2′/C-4′, and H-3′/C-1′, C-5′, and C-9′. Thus, the structure (*E*)-cinnamyl-benzoate was established for compound **7**.

Compounds **8**−**15** were characterized as triterpenes 6β-hydroxy-3-oxo-lup-20(29)-en-28-oic acid (**8**) and 3-oxo-oleanoic acid (**9**), flavanones sakuranetin (**10**) and liquiritigenin (**11**), chalcone kukulkanin B (**12**), and aromatic acids (*E*)-*p*-coumaric (**13**), (*E*)-cinnamic (**14**), and *p*-hydroxybenzoic (**15**). The structures of the isolated compounds were established by ^{1}H and ^{13}C NMR data and confirmed by a comparison to those reported in the literature.

6β-Hydroxy-3-oxo-lup-20(29)-en-28-oic acid (**8**) was the most abundant triterpene constituent in the cones of *Liquidambar styraciflua* L. (Hamamelidaceae).[9] Its cytotoxicity against human cancer cell lines was reported.[10] 3-Oxo-oleanolic acid (**9**) exists widely in the plant kingdom and possesses various pharmacological activities. In recent years, it was found that it had marked antitumor and anti-inflammatory effects.[17,18]

Sakuratenin (**10**), known as phytoalexin in rice plant,[11] induces adipogenesis of 3T3-L1 cells through enhanced expression of PPARγ2.[19] (−)-Liquiritigenin (**11**), detected in Brazilian and Cuban propolis samples, is considered to be a characteristic constituent of the Leguminosae family and has exhibited interesting biological activities.[5,7] For methoxychalcone kukulkanin B (**12**) the presence of the methoxyl group and its location at the C position of ring A were established on the basis of the ^{13}C NMR spectra and HMBC data with respect to an unmethoxylated model. Compounds **13**−**15** showed spectroscopic data and chromatographic behavior identical with those of (*E*)-*p*-coumaric acid (**13**), (*E*)-cinnamic acid (**14**), and *p*-hydroxybenzoic acid (**15**).

A total of 15 compounds were characterized by NMR and MS. Benzoic or cinnamic acid derivatives (**1**−**7** and **13**−**15**) are reported for two samples of green propolis from the south of the Minas Gerais state[20] and from Uruguayan propolis.[21] Liquiritigenin (**11**) was previously reported as a chemical constituent of red Brazilian and Cuban propolis.

Cinnamic ester derivatives (**1**−**7**) were previously reported as chemical constituents of exudate obtained from the trunk of *L. styraciflua*, (Honduras styrax, Hamamelidaceae). Chalchat et al. reported that the composition of the volatile fraction of Honduras styrax consists of compounds **1** (38%), **3** (32.3%), and **14** (4.8%).[22] Compound **4** was successively reported in the composition of the volatile fraction from Honduras styrax by Fernandez et al.,[23] whereas compound **8** was one of the triterpenes at the highest concentration in the cones of *L.*

styraciflua.[9] This evidence indicated the close relation between the Hondurian propolis and Honduras styrax. Considering the abundance of this plant in the area where beehives are placed, we suggest that *L. styraciflua* exudates are the botanical origin of Hondurian propolis.

Recently, we demonstrated that purified compounds from Brazilian red propolis were able to inhibit the activity of Pdr5p, an ATP-binding cassette (ABC) transporter from yeast plasma membrane, that confers high resistance to xenobiotics when it is overexpressed in the cell.[24] The ABC transporters use the energy of ATP hydrolysis to pump out the xenobiotics from intracellular space.[24] In this way, we checked the effect of the most abundant compounds from Hondurian propolis on the ATPase activity of Pdr5p. As observed in Figure 2, the crude

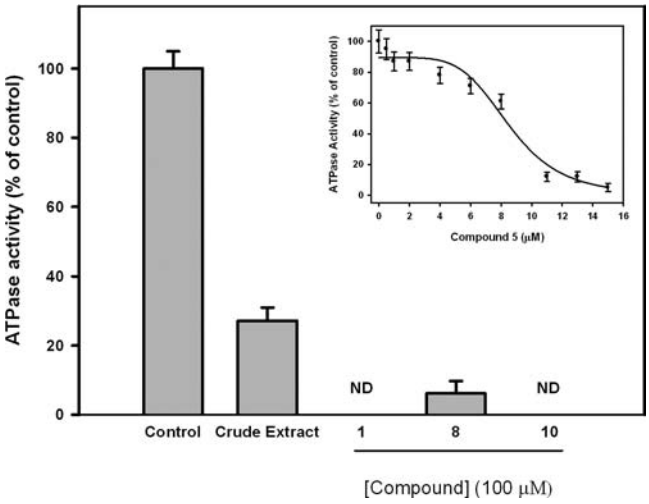

Figure 2. Effects of Honduras propolis compounds on the ATPase activity of Pdr5p from yeast plasma membrane. The enzymatic assays were performed as described in the Materials and Methods, in the absence (control bar) or in the presence of 100 μg mL^{-1} crude extract or 100 μM purified compounds (**1**, **8**, and **10**). (Inset) Dose−response curve of compound **5** purified from the HP extract. All of these data represent means of three independent experiments. ND = not detected.

extract (HP) was able to inhibit around 70% of ATPase activity at a final concentration of 100 μM, while compounds **1**, **5**, **8**, and **10** abolished the enzymatic activity at the same concentration, except compound **5** that was able to inhibit the enzyme at 15 μM (inset of Figure 2). When dose-dependent curves were performed, using concentrations of compounds below 100 μg mL^{-1}, it can observed that those compounds are very powerful in relation to inhibition of ATPase activity of Pdr5p because all of them demonstrated low values of IC_{50} (Table 2). This behavior is promising and

Table 2. IC_{50}[a] Values of Purified Compounds from Honduras Propolis for ATPase Activity Inhibition

compounds	IC_{50} (μM)
1	2.58
5	1.54
8	1.03
10	1.20

[a]These values were obtained from dose-dependent curves using the software SigmaPlot, version 11 (Systat Software, Inc.).

encouraging because they have a natural origin that is now returning as a medical scenario as an alternative to treatment or an assistant as an adjuvant of known antifungals in fungal infections that display a multidrug resistance phenotype. In conclusion, we demonstrated that these purified compounds tested here represent nice tools to block ABC transports, as Pdr5p, and could be a good way to decrease or eliminate high resistance displayed by fungi or bacteria. Therefore, new experiments must be performed, including those using clinical strains instead of *S. cerevisiae* as a model.

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Notes

The authors declare no competing financial interest.

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