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Chemical profile and anti-leishmanial activity of three Ecuadorian propolis samples from Quito, Guayaquil and Cotacachi regions

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ABSTRACT

Three propolis samples were collected from different regions of Ecuador (Quito, Guayaquil and Cotacachi) and their methanolic extracts were prepared. Preliminary information supplied by TLC and NMR data, allowed us to define two main types of propolis: Cotacachi propoli sample (CPS), rich in flavonoids and Quito and Guayaquil samples (QPS and GPS) containing triterpenic alcohols and acetyl triterpenes as the main constituents. Two different approaches based on RP-HPLC preparative procedure and NMR structural determination (CPS) and GC–MS analysis (QPS and GPS) were successfully used for the chemical characterization of their major compounds. All three propolis extracts were able to inhibit *Leishmania amazonensis* growth but propolis sample rich in flavonoids was the most active (IC_{50} = 17.1 ± 1.7 μg/mL). In the literature this is the first study on propolis from Ecuador.

1. Introduction

Propolis is a mixture of substances produced by bees employing exudates or parts of the plants, which join with beeswax and secretions from the salivary glands of the worker bee rich in enzymes. It can exhibit a variety of colours, depending on the species from which the resinous material is collected [1]. Propolis is not only a building material, it is the most important "chemical weapon" used by bees to maintain an aseptic internal environment in beehives. Its potential as an antimicrobial agent has justified its medicinal use for centuries [2,3]. Propolis has been used in traditional medicine since antiquity and several studies have demonstrated its advantage as antiinflammatory, antiulcer, antitumour, immunostimulant, hepatoprotective, antibacterial, antifungal, and potential against protozoa [3–5]. Nowadays, it is as an active ingredient in dietary supplements and cosmetic formulations.

Despite all its benefits, propolis has the disadvantage of having a highly variable chemical composition, which depends on the vegetation around the hive and seasonality [6]. Numerous studies led in recent years to the idea that different propolis samples could be completely dissimilar with respect to their chemical components and biological potential. Due to this situation, many researchers have focused their attention on propolis from unexplored regions of the world in order to provide new valuable natural products.

In our previous studies, we have investigated both chemical composition and biological potentialities of propolis samples collected in different countries including Cuba, Brazil, Mexico [1,7–10]. Flavonoids containing different chemical features were identified as the main constituents in some propolis samples. However, triterpenoids, prenylated benzophenones and organic acids have been also recognized as major components in the other ones. Many others researchers have also evidenced the presence of these compounds or detected other organic compounds including lignans, diterpenoids and so on [11]. Many articles reported in the literature characterize the main constituents of propolis using HPLC-PDA and HPLC-ESI/MS methods, whereas the GC–MS technique has been shown to be useful for the chemical determination of apolar and aliphatic compounds in some propolis

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samples [12], this analytical techniques need accurate sample preparation and are time-consuming because matrix complexity and the presence of compounds with different polarity. Therefore, the use of faster analytical techniques, consenting a rapid screening of constituents of propolis is strongly suggested. We reported previously 1D NMR spectroscopy to be a useful method to differentiate among Cuban propolis samples [1] as well as TLC analysis as easy alternative tool to screen commercial formulation of propolis [13].

Although there are a large number of publications related to chemical and biological studies, many propolis samples remain unexplored yet. Considering the great biological diversity and climate zones that exist in Ecuador, we decided to conduct a research on the chemical composition of Ecuadorian propolis. In Ecuador, propolis alcoholic extract is popular as a homemade remedy and some preparations are offered in the local market in 10–20% ethanolic tinctures or powder mixed with honey that have antioxidant, anti-inflammatory and antimicrobial activities. Despite its use there are no studies in the literature on its chemical composition, phytochemical origins, and phytotherapic properties.

We reported here for the first time the chemical investigation of three propolis samples collected in Quito, Guayaquil and Cotacachi provinces from Ecuador. Antiprotozoal activity against *Leishmania amazonensis* was also evaluated. To our knowledge, this is the first study on chemical composition and antileishmanial activity of propolis samples from Ecuador.

2. Material and methods

2.1. Chemicals

Methanol, ethanol, hexane, ethyl acetate, chloroform, sulfuric acid and pyridine (C_5H_5N) were purchased from J.T. Baker (Baker Mallinckrodt, Phillipsburg, NJ). The derivatization reagent, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Supelco (Supelco Park, PA). α-Amyrin, β-amyrin and lupeol were from Sigma-Aldrich (Milan, Italy), lanosterol was purchased from Merck (Darmstadt, Germany), and β-amyrone, 24-methylene-9,19-ciclolanostan-3β-ol, lupeol acetate and lanosta-9(11)-24-dien acetate were obtained from Centro de Química Farmacéutica (Havana, Cuba).

2.2. General experimental procedure

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, δ H 2.04 and δ_C 29.3 for CD_3COCD_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 Thermo Electron (San Jose, CA) equipped with Xcalibur software. Full mass spectra were acquired in the negative mode. Instrumental parameters were tuned for each investigated compound: capillary voltage was set at 3 V and spray voltage at 5.0 kV; and a capillary temperature of 220 °C was used. 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. Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden) by employing MeOH as solvent. HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a 250 × 10 mm i.d. 10 μm Phenomenex C8 column. TLC analysis was performed with Macherey-Nagel precoated silica gel 60 F_{254} plates employing hexane: ethyl acetate (7:3) as solvent system, and a mixture $Ce(SO_4)_2/H_2SO_4$ as general detection reagent. $FeCl_3$ solution (5%, v/v) was used in order to detect the presence of phenolic derivatives.

2.3. Propolis samples

Propolis samples from Quito (QPS), Guayaquil (GPS) and Cotacachi (CPS) were collected in Ecuador (April 2014). Methanol extracts were obtained by maceration of ground samples (35 g each one) with methanol (6 × 50 mL), for 2 h at room temperature (25–30 °C), using a shaker at 150 rpm. Extracts were evaporated at 40 °C under reduced pressure to obtain dry extracts. Propolis samples and the dried methanol extracts were stored at 5 °C in the dark until required for analysis.

2.4. Sample preparation and GC–MS analysis

About 1 mg of the QPS and GPS propolis extracts were dissolved in 50 μL of piridin and 100 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) in a sealed glass tube for 30 min at 100 °C. An Agilent Model GC6890N Series gas chromatograph coupled with an HP 5973N series mass-selective detector quadrupole mass spectrometer was employed for all analyses. The injector was heated to 310 °C and was on split mode with a split ratio of 1:50, and the injection volume was 1.0 μL. Samples were separated on a 30 m × 0.25 mm i.d., 0.25 μm film thicknesses, HP-5MS column. Oven temperature was programed at 60 °C for 2 min, and then the temperature was raised to 310 °C at a rate of 3 °C min^{-1}, followed by an isothermal period of 20 min. The total run time was 100 min. The mass spectrometer was operated by electron impact (EI) at 70 eV. The MSD was acquiring data in the full scan mode from 35 to 700 amu. Ultrahigh-purity helium with an inline oxygen trap was used as carrier gas at a flow rate of 1 mL/min.

2.5. GC–MS identification

The identified structures were proposed on the basis of their fragmentation patterns. The individual peaks were also compared with the PMW-TOX, NIST 98, and Wiley 275 computer mass libraries. Eight triterpenoids (compounds **7**, **8**, **12**, **15**, **16**, **19**, **20** and **21**) were co-chromatographed to confirm GC retention times and mass spectra. Some components in the chromatograms remained unidentified because of the lack of authentic samples and library spectra of the corresponding compounds.

2.6. Isolation procedure of compounds *1–6*

A portion of Cotacachi dry propolis extract (3 g) was fractionated over Sephadex LH-20 using methanol as solvent (85 fractions of 8 mL each one). After TLC analysis fractions with similar *Rf* values were combined in nine major fractions (1–9). Fractions 4–8 were dissolved (25–35 mg/mL) in acetone or methanol (fraction 4), centrifuged at 4000 rpm × 5 min (Hettich Universal centrifuge) and purified by HPLC using isocratic elution (methanol 70% or 65%), flow rate of 3 mL/min, on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a 250 × 10 mm i.d. 10 μm Phenomenex C8 column. In all the cases the injection volume was 100 μL. Fraction 4 (58.6 mg) yielded compound **4** (5.6 mg), and from fraction 5 (289.1 mg) compound **2** was obtained (12.6 mg). Fraction 6 (120.1 mg) produced compounds **1** (10.2 mg) and **3** (8.3 mg), and fractions 7 (37.6 mg) and 8 (31.1 mg) yielded products **6** (5.2 mg) and **5** (4.8 mg), respectively.

2.7. 5,7,4′-Trihydroxyflavanone (naringenin) (1)

^{1}H and ^{13}C NMR data were consistent with those previously reported [14]; ESI-MS (negative mode), *m/z* 271 $[M-H]^-$. The structure was confirmed by 2D NMR experiments.