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The cytotoxic effects of brown Cuban propolis depend on the nemorosone content and may be mediated by mitochondrial uncoupling

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ABSTRACT

Three main types of Cuban propolis directly related to their secondary metabolite composition have been identified: brown, red and yellow propolis; the former is majoritarian and is characterized by the presence of nemorosone. In this study, brown Cuban propolis extracts were found cytotoxic against HepG2 cells and primary rat hepatocytes, in close association with the nemorosone contents. In mitochondria isolated from rat liver the extracts displayed uncoupling activity, which was demonstrated by the increase in succinate-supported state 4 respiration rates, dissipation of mitochondrial membrane potential, Ca^{2+} release from Ca^{2+}-loaded mitochondria, and a marked ATP depletion. As in cells, the degree of such mitotoxic events was closely correlated to the nemorosone content. The propolis extracts that do not contain nemorosone were neither cytotoxic nor mitotoxic, except R-29, whose detrimental effect upon cells and mitochondria could be mediated by its isoflavonoids and chalcones components, well known mitochondrial uncouplers. Our results at least partly unravel the cytotoxic mechanism of Cuban propolis, particularly regarding brown propolis, and raise concerns about the toxicological implication of Cuban propolis consumption.

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1. Introduction

Propolis is a complex mixture of beeswax, small amount of sugars, and plant exudates collected by honeybees [1]; in Cuba, propolis alcohol extract is popular as a domestic medicine. The composition of propolis depends on the local and time of collection; a big variety of organic compounds has been identified so far, among which phenolic compounds are the major ones [2]. Because of its range of antibiotic, antifungal, antinflammatory, anticancer, and antioxidant activities, there is renewed interest in the composition and therapeutic application of propolis [3,4].

Our previous studies indicated that the chemical composition of Cuban propolis is both qualitatively and quantitatively unique and variable [5–8]. The presence of polyprenylated benzophenones derivatives in Cuban propolis had been well established; an HPLC study indicated that nemorosone is the major constituent of 18 analyzed samples [7]. Other prenylated benzophenones derivatives identified as minor components of Cuban propolis are propolones A–D, clusianone, hyperibone B, garcinielliptone I, xanthochymol, and guttiferone [5–7]. However, we also reported the occurrence of isoflavonoids as constituents of red Cuban propolis, wherein polyprenylated benzophenones were not detected [8,9]. A comprehensive study using a combination of NMR, HPLC-PDA, and HPLC-ESI/MS techniques allowed the definition of three main types of Cuban propolis, directly related to their secondary metabolite classes: brown Cuban propolis, rich in polyisoprenylated benzophenones (mainly nemorosone), red Cuban propolis, containing isoflavonoids as the main constituents, and yellow Cuban propolis, containing triterpenoids as major constituents [10,11]. Brown Cuban propolis is the major group of Cuban propolis [5,7,10], and nemorosone is its main component [10,12]. We recently reported that nemorosone and its chemical analogues guttiferone A and clusianone presented strong cytotoxic effects mediated by mitochondrial uncoupling [13–15]. Therefore, it can be hypothesize that the

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cytotoxic and antiparasitic effects of the brown Cuban propolis reported elsewhere [16,17] may be related to their nemorosone's content and mediated by a mitotoxic uncoupling effect.

In this context, we addressed in the present study the mitochondrial uncoupling properties of 8 samples of Cuban propolis (with or without nemorosone) and their toxicity for hepatic carcinoma cell (HepG2) and isolated rat hepatocytes and liver mitochondria. The brown Cuban propolis induced cytotoxicity closely reproducing the nemorosone effects, so we proposed that this benzophenone may be the main responsible for the propolis extract's cytotoxicity. Its association with nemorosone is discussed.

2. Materials and methods

2.1. Compounds and propolis samples

All reagents were obtained from Sigma–Aldrich Corp. (St. Louis, MO, USA). All stock solutions were prepared using glass-distilled deionized water. Samples of Cuban propolis (eight) were provided by La Estación Experimental Apícola or collected by one of us. Samples were collected between October 2011 and December 2012 in 7 provinces of Cuba. Cuban propolis samples, their origin, and classification are reported in Table 1. Both propolis samples and extracts were kept at 0–5 °C and protected from light.

2.2. Extraction procedure

All samples were ground prior to extraction. Propolis samples (about 3 g) were extracted by maceration with methanol (10 ml, 3 times) for 1 h at room temperature (25–30 °C) employing agitation sporadically. The combined extracts were filtered on paper filters, and solvent was evaporated at 40 °C under reduced pressure to obtain dry extracts.

2.3. Extract preparation and nemorosone content estimation

Stocks solutions of propolis were prepared in absolute ethanol (5, 25 and 50 mg/ml final concentrations) and added to the cells culture or mitochondrial reaction media at 1/1000 (v/v) dilution. Nemorosone was used as control in all experiments at the concentration it occurs in N-17 propolis extract, which contain the highest amount of this compound.

The nemorosone concentration in the propolis extracts was estimated by an UV–VIS procedure using a Model U-2910 Hitachi spectrophotometer (Japan). A standard curve of nemorosone was used. Briefly, nemorosone (10 μM final concentration) was added to the propolis extract solutions (25 μg/ml) in 10 mM TRIS–HCl, pH 7.0. The absorbance at 244 nm for nemorosone (10 μM), and the propolis extracts plus nemorosone were recorded. Then, the nemorosone's absorbance was subtracted from the absorbance of propolis extracts/nemorosone mixture. The resulting values were interpolated into the nemorosone calibration curve. The nemorosone content are displayed in Table 1.

Table 1
Cuban propolis samples, their origin, classification and nemorosone's content.

Sample	Province	Type	Nemorosone concentration (μM)*
N-1	La Habana	Brown	6.96
N-3	Granma	Brown	6.01
N-12	Holguín	Brown	2.36
N-17	Guantánamo	Brown	17.12
N-60	Holguín	Brown	0.51
A-46	Matanzas	Red	NN
B-8	Santiago de Cuba	Red + yellow	NN
R-29	Villa Clara	Red	NN

* Estimated spectrophotometrically for 25 μg/ml of propolis extract. NN: no nemorosone.

2.4. Culture of HepG2 cells

HepG2 cells were obtained from the American Type Culture Collection (ATCC ® HB-8065™). The cell line was cultured in Dulbecco's medium with 10% defined supplement fetal bovine serum plus 100 mg/ml streptomycin, 100 IU/ml penicillin G and 1 μg/ml amphotericin. The cells were seeded into 96-well plates (Nunc, Roskilde, Denmark), with 2×10^4 cells/well in 200 μl of culture medium at 37 °C, flushed with 5% CO_2 in air for 24 h in a humidified incubator (Forma Series II, Thermo Fisher Scientific Inc., MA, USA). After the incubation period, the cells were rinsed with buffered saline solution.

2.5. Isolation of rat hepatocytes

Hepatocytes were isolated from adult male Wistar rats, weighting 150–180 g, by collagenase perfusion of the liver [18]. The hepatocytes were cultured in Dulbecco's medium with 10% defined supplement fetal bovine serum plus 100 mg/ml streptomycin, 100 IU/ml penicillin G, 250 μg/ml amphotericin B and 2 mg/ml ciprofloxacin. The cells were seeded as described for HepG2 cell culture.

2.6. Cell viability assay

The viabilities of HepG2 cells or isolated rat hepatocytes were evaluated using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) colorimetric assay. Both types of cells (2×10^4) were incubated for 8 h in 96-well microtiter cell culture plates, either in the absence (control cells) or presence of propolis extracts or nemorosone, in a 200 μl final volume. After the treatments, cells were incubated for 3 h at 37 °C in culture medium containing 10 μM MTT in phosphate-buffered saline (PBS). The blue MTT formazan precipitate was dissolved in 50 μl of dimethyl sulfoxide, and the absorbance was measured at 570 nm on a multiwall plate reader (Varian Cary 50, Australia). Cell viability is expressed as a percentage of these values in treated cells, compared to the non-treated (control) cells.

2.7. Isolation of rat liver mitochondria

Mitochondria were isolated by standard differential centrifugation [19], as described previously [13,20]. Male Wistar rats weighing approximately 200 g were sacrificed by decapitation; livers (10–15 g) were immediately removed, sliced in medium containing 250 mM sucrose, 1 mM ethyleneglycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)–KOH, pH 7.2, and homogenized with Potter–Elvehjem homogenizer. Homogenates were centrifuged (580 × g, 5 min) and the resulting supernatant was further centrifuged (10,300×g, 10 min). Pellets were suspended in medium containing 250 mM sucrose, 0.3 mM EGTA and 10 mM HEPES–KOH, pH 7.2, and centrifuged (3400×g, 15 min). The final mitochondrial pellet was suspended in medium containing 250 mM sucrose and 10 mM HEPES–KOH, pH 7.2. Mitochondrial protein content was determined by the Biuret reaction.

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