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Yahima Frión-Herrera
Food and Pharmacy Institute, Havana University, Havana, Cuba.

Alexis Díaz-García*
Laboratories of Biofarmaceuticals and Chemistries Productions (LABIOFAM), Havana, Cuba.

Hermis Rodríguez-Sánchez
Tropical Medicine Institute "Pedro Kouri" Havana, Cuba

Jenny Ruiz-Fuentes
Tropical Medicine Institute "Pedro Kouri" Havana, Cuba

Lianet Monzote-Fidalgo
Tropical Medicine Institute "Pedro Kouri" Havana, Cuba.

Luis Morier-Díaz
Tropical Medicine Institute "Pedro Kouri" Havana, Cuba.

William N. Setzer
Department of Chemistry, University of Alabama in Huntsville, Alabama 35899, USA

Correspondence:
Alexis Díaz-García*
Laboratories of Biofarmaceuticals and Chemistries Productions (LABIOFAM), Havana, Cuba.
E-Mail: alexisdg@infomed.sld.cu

Cytotoxic effect of Cuban propolis extracts against tumor cells lines

Yahima Frion-Herrera, Alexis Diaz-Garcia*, Hermis Rodríguez-Sanchez, Jenny Ruiz-Fuentes, Lianet Monzote-Fidalgo, Luis Morier-Diaz, and William N. Setzer

ABSTRACT

Propolis is a resin formed by a complex mixture of chemical substances that bees collect from plants. Since ancient times, propolis has been used in folk medicine due to its biological properties that include antimicrobial, antioxidant, anti-inflammatory, and antitumoral activities. The aim of this study was to compare the *in-vitro* cytotoxic activities of 16 Cuban propolis extracts of different chemical types: brown (1, 4, 5, 16, and 17 BCP), red (9, 29, 35, 37, 45 and 72 RCP) and yellow (18, 39, 41, 50 and 60 YCP) against murine breast carcinoma F3II, human breast adenocarcinoma MDA-MB-231, human lung adenocarcinoma A549, and human fibroblast MRC-5. These cells were incubated with different concentrations of propolis and 72 hours after, the IC_{50} was calculated for each cell. The results showed the antiproliferative effect of Cuban propolis against all cell lines tested. RCP was the most interesting group because they did not affect normal cells. RCP-45 extract showed the highest selectivity index (SI>2) toward A549 compared with MRC-5.In addition, this extract induced necrosis and caused an increase in mRNA levels of Xiap in A549 cell after 8 h of treatment. These results could be related tothe classification of Cuban propolis, which is highly dependent onchemical composition. Our results indicate that Cuban propolis represents an interesting natural source for anticancer treatment.

Keywords: Cuban Propolis, Cytotoxicity, Cancer Cells.

1. Introduction

Propolis, are a natural resinous product collected by honeybees from the buds and exudates of various plant sources and has been used empirically as a traditional remedy in folk medicine for centuries [1]. It is well known for potential health benefit and is reported to possess valuable biological activities such as antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory, and anticancer activities [2, 3].

Chemical composition of propolis is complex and varies in accordance with the phytogeographical diversity of the areas from which it is collected [4]. Tropical samples of propolis show unusual compositions including terpenoids, prenylated organic acid derivatives, lignans and flavonoids [5, 6]. Cuba has a great diversity and rich flora and due to this, extensive chemical studies have identified three main types of propolis with significant differences in its chemical composition. They are classified in: brown Cuban propolis type (BCP type), rich in polyisoprenylated benzophenones [7], red type (RCP type), containing isoflavonoids as the main constituents, and yellow type (YCP type) with a variety of triterpenoids as the major chemical components [6]. In Cuba, propolis has displayed therapeutic potential as antipsoriatic, anti-inflammatory, analgesic [8] and antibacterial [7]. Other studies have been carried out against different diseases, such as acute cervicitis [9], parasitic vaginal infections [10], dental plaque [11], facial septic injury [12] and giardiasis [13].

Several researchers have reported the antitumor property of BCP propolis *in vitro* [14]. However, the antitumor activity from all three Cuban propolis groups has not been explored and compared. In the present study, we analyzed the cytotoxic effects of BCP, RCP and YCP extracts collected in different geographic areas, against murine breast carcinoma F3II, human breast adenocarcinoma MDA-MB-231, human lung adenocarcinoma A549, and human fibroblast MRC-5.

2. Materials and methods

2.1 Propolis Sample

Sixteen samples of Cuban propolis were provided by ¨La Estación Experimental Apícola¨, Havana, Cuba. Samples were collected in 9 provinces of Cuba including Eastern, Central and Western regions (Table 1). Propolis samples were obtained as previously described [15]. The origin and classification of Cuban propolis samples used in this study are also reported in Table 1. The extracts were dissolved in dimethylsulfoxide (DMSO), Riedel-de Haën, Germany) at 40 mg/mL and stored at 4 °C until analysis.

Table 1: Cuban propolis samples used in this study, classification and origin.

Samples	Province (municipality)	Samples	Province (municipality)
BCP-1	La Habana (Jardín Botánico)	RCP-37	Pinar del Rio (Güanes)
BCP-4	Gramma (Buey Arriba)	RCP-45	Matanzas (Jagüey Grande)
BCP-5	Guantánamo (Imías)	RCP-72	Ciego de Ávila
BCP-16	Las Tunas (Puerto Padre)	YCP-18	La Habana (Jardín Botánico)
BCP-17	Guantánamo (Salvador)	YCP-39	Pinar del Rio (Candelaria)
RCP-9	Pinar del Rio (Cabo de S. Antonio)	YCP-41	Pinar del Rio (Bahía Honda)
RCP-29	Villa Clara (Manicaragua)	YCP-50	Matanzas (Unión de Reyes)
RCP-35	Pinar del Rio (La Coloma)	YCP-60	Holguín (Bagüanos)

2.2 Cell Lines and Cell Culture

The cell lines used in this study were the murine breast carcinoma F3II (kindly donated by The Molecular Inmunology Center, Cuba), human breast adenocarcinoma MDA-MB-231, human lung adenocarcinoma A549, and human fibroblast MRC-5. The cells were provided by ATCC culture collection and cultured in a humidified atmosphere at 37 °C in 5% CO_2. The cells MRC-5, A549 and F3II were maintained in Dulbecco's modified Eagle's medium (DMEM, SIGMA, EUA) supplemented with 2 mM of glutamine, 10%, with heat-inactivated fetal bovine serum (FBS, SIGMA, EUA), 10%, penicillin (100 U/mL), and streptomycin (100 µg/mL). The MDA-MB-231 cell line was maintained in essential minimum medium (MEM) supplemented with 2 mM of glutamine, 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL). When the cells were confluent, they were routinely sub-cultured using 0.25% trypsin-EDTA solution (Sigma-Aldrich). After trypsin detachment, cells were counted, subcultured at 2×10^5 cells per milliliter on 96-well plates, and incubated for 24 h at 37 °C with 5% CO_2 environment to allow for cell attachment.

2.3 Cell Viability by MTT Assay

Cultured cells were treated with different propolis extracts (triplicate wells per condition) by the addition of 50 µL of serial dilutions dissolved in DMEM or MEM (depending of cell line) to give a final concentration of 12.5; 25; 50; 100 and 200 µg/mL. Untreated cells were used as a control and in all cases DMSO was below 0.1%. The cells were then cultured as above for another 72 h prior to the addition of 10 µL of 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) into each well. The incubation was continued for another 3 h before the media was removed. DMSO (150 µL) was added to each well and mixed to ensure cell lysis and dissolving of the formazan crystals. Absorbance (A) was read in a UV–visible spectrophotometer multiplate reader (MRX Revelation Dynex Technologies, Germany) at 570 nm. Absorbance from untreated cells was consider as 100% of growth and used for viability calculation. The effect of propolis extract on the viability for cell lines panel was expressed as the % viability, using the formula: %viability = A_{570} of treated cells / A_{570} of control cells × 100%. The median inhibitory concentration (IC_{50}) values from line cells were determined.

2.4 Morphological Assessment and Measurement of Apoptotic or Necrotic Cells

To investigate the cell death event, A549 cells (2×10^5 cell/well) were grown in 24-well culture plates overnight and treated for 8 h and 16 h with IC_{10} from RCP-45 extracts. At this time, treated cells and untreated cell (used as control) were incubated with a mixture of acridine orange (AO) and ethidium bromide (EB) in PBS (100 µg/mL AO/100 µg/mL EB). The nuclei of living cells were stained by the membrane-permanent dye AO, while necrotic cells were stained by the highly fluorescent ET. Following the addition of fluorochromes, 200 cells were analyzed and counted in each of three independent experiments using fluorescence microscopy IX-71 (Olympus, Japan) with 480 and 520 nm filters, using a magnification of 400X.

2.5 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis in A549 Cells

A549 cells (2×10^5 cell/well) were cultured in 24-well plates and incubated at 37 °C in a humidified air atmosphere enriched with 5% (v/v) CO_2 for 24 h. Total RNA was isolated from cells treated with IC_{10} of RCP-45 extracts for 8 h and 16 h using a TRIzol reagent (LS Reagent, Sigma, EUA) as recommended by the manufacturer. Total RNA concentration in the final elutes was determined using a Biophotometer plus (Eppendorf, Germany). Each sample of isolated RNA (1 µg) was reverse transcribed by M-MLV reverse transcriptase system (Promega Inc. USA) in a 50-µL volume reaction. Each PCR was carried out in a master mix containing 1X Green Go Taq Flexi Buffer, 2 mM $MgCl_2$, 10 mM dNTPs and 1.25 U Go Taq DNA polymerase (Promega Inc. US) with 0.2 mM of respective forward and reverse primers and 5 µL of DNA_C in 25 µL reaction mix. The PCR amplification was carried out in a thermal cycler Nahita (AUXILAB, S.L., Spain). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) amplification was performed as a housekeeping gene. PCR conditions for GAPDH were 30 cycles at 94 °C for 30 sec, at 54 °C for 30 sec at 72 °C for 1 min. PCR conditions for p53 were 30 cycles at 94 °C for 30 sec, at 56 °C for 30 sec at 72 °C for 1 min. PCR conditions for Xiap were 35 cycles at 94 °C for 45 sec, at 58°C for 45 sec at 72 °C for 1 min. PCR products were analyzed in agarose gel electrophoresis and stained with EB and a 50 bp DNA ladder molecular marker (Promega Inc. US) was used. The results were confirmed by repeating experiments. Size of the amplified PCR products were 120 bp for GAPDH, 130 bp for p53, and 368 bp for Xiap.

2.6 Statistical Analysis

The IC_{50} and IC_{10} values were determined by interpolation of tendency line from linear regression curve. For all analyses, we used the GraphPad Prism version 5.03 for Windows, (GraphPad Software, San Diego California, USA). Data were statistically analyzed using the Kruskal-Wallis and Dunns post-test. Significance was accepted at the $P<0.05$ level.

3. Results & Discussion

In this study, we found the growth inhibitory activity of each group of extracts: BCP type, RCP type and YCP type, against tumor cell lines evaluated during 72 h, was dose-dependent. In F3II and MDA-MB-231 cell lines, BCP extracts caused a significant growth inhibition with IC_{50} values in the range of 19-35 µg/mL and 8-64 µg/mL respectively (Table 2). These cell lines were more sensitive to BCP extracts compared to A549 ($P<0.05$). Our results agree with Popolo *et al*.[14] and Diaz-Carballo *et al*. [16] who reported antiproliferation activity of BCP and nemorosone in breast tumor cells. Nemorosone, as the main component [17], represents more than 10% in our BCP extracts [18]. Therefore, differences in IC_{50} values could be explained by the proportion of nemorosone in each sample of BCP.

Table 2: IC_{50} values of Cuban propolis extracts on A-549, MDA-MB-231, and F3II cell lines.

Samples	IC_{50} (µg/mL) ± SD		
	A-549	MDA-MB-231	F3II
BCP-1	63.4 ± 0.3^{b}	8.9 ± 1.1^{a}	19.1 ± 1.1^{a}
BCP-4	83.1 ± 1.1^{bc}	57.1 ± 1.4^{b}	45.7 ± 0.9^{b}
BCP-5	76.7 ± 0.1^{bc}	44.8 ± 0.9^{b}	34.9 ± 0.6^{ab}
BCP-16	96.5 ± 0.4^{c}	63.1 ± 1.7^{b}	59.7 ± 1.2^{b}
BCP-17	93.5 ± 0.2^{c}	64.1 ± 1.4^{b}	34.3 ± 0.6^{ab}
RCP-9	93.4 ± 0.9^{c}	101.3 ± 1.1^{c}	75.9 ± 0.9^{bc}
RCP-29	99.5 ± 0.8^{c}	97.4 ± 0.6^{c}	78.1 ± 0.3^{bc}
RCP-35	57.9 ± 1.0^{b}	175.9 ± 0.4^{d}	55.8 ± 1.3^{b}
RCP-37	49.2 ± 0.6^{b}	111.4 ± 0.6^{c}	74.2 ± 0.8^{bc}
RCP-45	$35,48\pm0,6^{ab}$	172.1 ± 0.1^{d}	60.7 ± 1.4^{b}
RCP-72	95.1 ± 1.4^{c}	124.0 ± 1.8^{cd}	42.5 ± 0.8^{b}
YCP-18	61.9 ± 0.3^{b}	26.9 ± 1.5^{a}	14.9 ± 2.5^{a}
YCP-39	79.8 ± 0.6^{bc}	103.3 ± 0.6^{c}	78.8 ± 0.5^{bc}
YCP-41	78.19 ± 0.6^{b}	100.9 ± 0.6^{bc}	79.3 ± 1.2^{bc}
YCP-50	86.2 ± 0.4^{bc}	90.7 ± 2.1^{bc}	76.6 ± 1.1^{bc}
YCP-60	83.3 ± 1.2^{bc}	111.5 ± 0.4^{c}	83.9 ± 2.0^{bc}

Data are shown as the mean ±1 SD derived from three independent repeats after a 72-h exposure of cells to extracts. Different lower case superscript letters mean significantly different ($P<0.05$).

RCP extracts showed mainly cytotoxic activity against A459 and F3II. IC_{50} values were in the range of 35-99 µg/mL and 42-78 µg/mL respectively (Table 1). These cells lines were more sensitive to growth inhibition by the RCP extracts compared to the MDA-MB-231 cell line ($p < 0.05$). Feng Li *et al.* reported cytotoxic activity from a variety of Brazilian red propolis constituents, like pterocarpans and medicarpin, against a panel of different cancer cell lines including A549 [19]. These findings may explain the cytotoxic effects of RCP extracts because of our samples contain more than 3% of pterocarpans and more than 20% of medicarpin [18].

MDA-MB-231 is an estrogen-negative cell line (ER-) and previously has been reported the estrogenic potential of RCP extracts [20, 21]. Additionally, studies conducted by Le Bail *et al*., demonstrated that the cytotoxic effects of different compounds like medicarpin and isosativan on MDA-MB-231 depends on their concentrations [20]. Isosativan is also present in our RCP samples (more than 5%) [18]. These results suggest that higher concentration of BCP extracts are required to induce a significant decrease in MDA-MB-231 viability.

In the current study, YCP extracts showed significant cell viability reduction in A549 and F3II with IC_{50} values in the range of 61-86 µg/mL and 14-84 µg/mL, respectively (Table 2). However MDA-MB-231 showed a marginal growth inhibition, with IC_{50} values in the range of 26-111 µg/mL. For the first time, the cytotoxic effects of YCP are reported. These results clearly indicate that some components contained in our samples [18] may inhibit tumor cell growth.

Selectivity is also an important issue in cancer prevention and therapy. Lung cell carcinoma is considered one of the most lethal cancers worldwide, including Cuba [22]. Therefore, it is interesting to identify selectivity among propolis extracts tested on lung cancer cell line A549 with MRC-5 human normal lung cellsby comparison of IC_{50} values. Table 3 shows the effects of BCP, RCP, YCP types on the viability of MRC-5. The effect of RCP extracts were significantly lower on MRC-5 ($P<0.05$) compared with BCP and YCP. Meanwhile, BCP and YCP extracts significantly reduced the MRC-5 viability, except BCP-17 (IC_{50}: 136.4±2.7 µg/mL) and YCP-39 (IC_{50}: 132.8±0.5 µg/mL) (Table 3). BCP and YCP extracts inhibited the proliferation of MRC-5 in a concentration-dependent manner. The differential result obtained among BCP, RCP, YCP types is likely to be explained by the compounds present in each propolis. Certainly, Diaz-Carballo *et al*. reported a cytotoxic effect of nemorosone on MRC-5 [16]. Additionally, this effect could be attributed to some synergistic effect between the main constituents of our samples.

Table 3: IC_{50} values of Cuban propolis extracts on the MRC-5 cell line.

Samples	IC_{50} (µg/mL) ± SD	Samples	IC_{50} (µg/mL) ± SD
BCP-1	50.2±1,0[a]	RCP-37	126.4±0.6[bc]
BCP-4	65.33±1.2[a]	RCP-45	114.1±0.7[bc]
BCP-5	62.5±0,5[a]	RCP-72	95.1±0.4[bc]
BCP-16	61.8±0.7[a]	YCP-18	20.9±0.4[a]
BCP-17	136.4±2.7[c]	YCP-39	132.8±0.5[c]
RCP-9	95.4±0.5[b]	YCP-41	68.1±0.4[ab]
RCP-29	>200	YCP-50	89.4±0.3[b]
RCP-35	104.5±0.5[bc]	YCP-60	67.3±0.6[ab]

Data are shown as the mean ± 1 SD derived from three independent experiments after a 72-h exposure. Different lower case superscript letters mean significantly different (P<0.05).

The selectivity index for A549 tumor cells was obtained for all propolis extracts. Figure 1 shows that RCP types are the most selective toward A549 compared to MRC-5. RCP-45 extract showed the highest selectivity index (SI: 3.2). In contrast BCP and YCP type exhibited lower selective indices. High selectivity of RCP extracts toward A549 cells could be explained by the presence and proportion of their components [18]. Several articles report that flavonoids and derivatives thereof, present in our RCP, possess anticarcinogenic activity and exert a low cytotoxicity in normal cells depending on their concentration [19, 20, 23, 24].

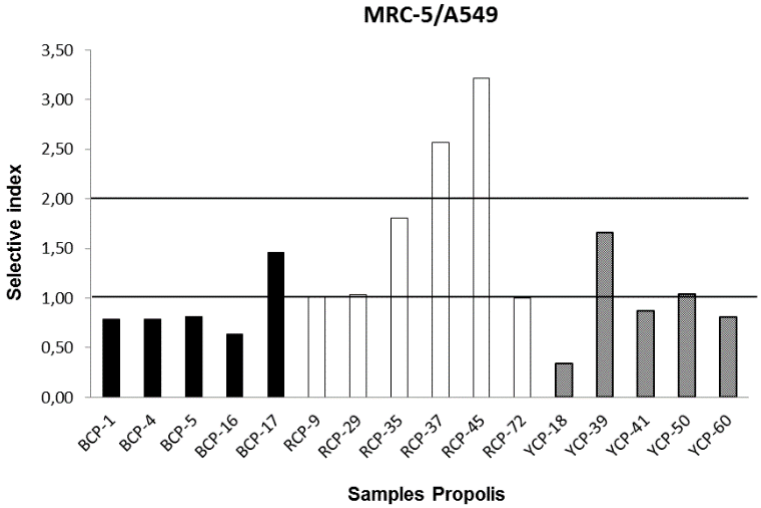

Fig 1: Selectivity index (MRC-5IC_{50}/A549-IC_{50}) of Cuban propolis extracts.

In addition, we have examined the effect of RCP-45 extract in the cell death event on A549 cells. Morphological assay of cell death was investigated using a mixture of acridine orange and ethidium bromide (AO/EB). A549 cells were exposed to RCP-45 for 8 h and 16 h and analyzed for different morphological features. Control cells were seen to be uniformly green with normal nuclear morphology (Figure 2A). In contrast, treated cells with RCP-45 displayed a high number of necrotic cells after 8 h of incubation (Figure 2B). We observed similar effects after 16 h exposure to RCP-45 (data not shown). These results suggest that RCP-45 is able to induce necrosis in A549. Despite different reports that show apoptosis induction by propolis [25-27], our results indicate additional cancer cell death induction is probably due to differential composition and proportion [28].

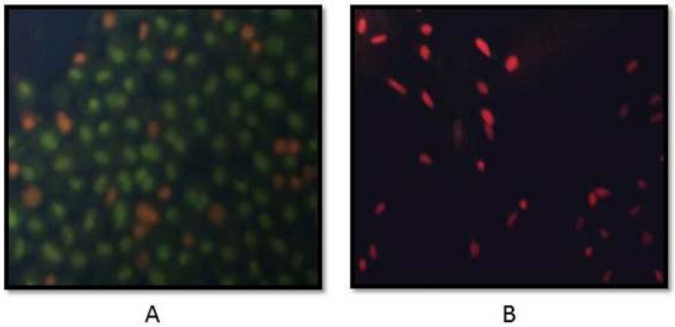

Fig 2: AO/EB staining of A549 cancer cells. **A**: nucleus from untreated control cells (green). **B**: nucleus from (IC_{10}) RCP-45-treated cells (red) after 8 h of incubation showed evidence of necrotic cell death. Images are magnified at 400X.

We have examined the effect of RCP-45 extract on p53 and Xiap mRNA levels in A549. The housekeeping gene GAPDH did not show observable changes after incubation of A549 cells with RCP-45 as depicted in Figure 3A (a,b,c,d). The band intensity of p53 mRNA did not change [Figure 3A (e,f,g,h)]. These results suggest that p53 status is not mediated by RCP-45 treatment. It is well known that up-regulation of p53 induces apoptosis [29]. In contrast, a transient increase in Xiap mRNA expression was seen in cells treated with RCP-45 extract [Figure 3B (a,b,c,d)] after 8 h of exposure. However, no significant changes were seen after 16 h of RCP-45 exposure. Xiap can bind to and inhibit caspases, the key executioners of apoptosis [30]. In our study, the maximum level of expression of Xiap was at 8 h of treatment. Therefore, these findings suggest that the over-expression of Xiap may cooperate with the induction of necrosis and thereby further contribute to RCP-45 necrotic cell death. According to Orsolic [31], propolis and a variety of polyphenolic/flavonoid compounds can influence a number of key intracellular targets that are associated with the cancer process and can serve as regulators of gene expression and/or modulate gene products.

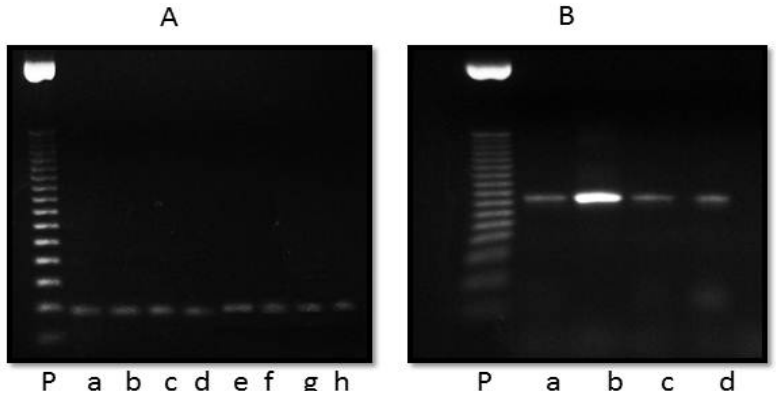

Fig 3: Agarose gel electrophoresis of RT-PCR from mRNA levels expression in A549 cells treated for 8 h and 16 h with RCP-45 (IC_{10}). P: DNA ladder marker. A) a: GAPDH (untreated control cells, 8 h) b: GAPDH (RCP-45 (IC_{10})-treated cells 8h)c:GAPDH (untreated control cells 16h) d:GAPDH (RCP-45 (IC_{10})-treated cells, 16 h). e: p53 (untreated control cells, 8 h). f: p53 (RCP-45 (IC_{10})-treated cells, 8 h). g: p53 (untreated control cells, 16 h). h: p53 (RCP-45 (IC_{10})-treated cells, 16 h). B) a: Xiap (untreated control cells, 8 h). b: Xiap (RCP-45 (IC_{10})-treated cells, 8 h). c: Xiap (untreated control cells, 16 h). d: Xiap (RCP-45 (IC_{10})-treated cells, 16 h).

4. Conclusion

The results of this study represent the first evidence that all types of Cuban propolis display effective cytotoxic activity against murine breast carcinoma F3II, human breast adenocarcinoma MDA-MB-231, and human lung adenocarcinoma A549 cells. All three propolis groups displayed differential cytotoxicity suggesting that the chemical compositions of propolis could influence their biological activities. The study reveals that RCP extracts represent the most interesting propolis extracts and suggests that Cuban propolis should be explored as a promising therapeutic agent in cancer treatment. Further investigations are needed to elucidate the molecular mechanism(s) of the anticancer effects and the study of individual constituents from Cuban propolis.

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