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Cytotoxic effect of *cuban* propolis extracts on normal cells and *in-vitro* basal toxicity assay to estimate acute oral toxicity

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Abstract
In-vitro toxicity tests are recognized as alternative methods to animal acute toxicity testing. The aim of this study was to assess toxicity 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 Balb/c 3T3 and Vero cells. Cells were treated at different concentrations (12.5; 25; 50 and 100 µg/mL) of propolis for 72 h and the IC_{50} value was determined. Furthermore we employed *in-vitro* cytotoxicity test described by Spielmann *et al.*, red uptake (NRU) assay to estimate acute oral toxicity. Propolis showed differential cytotoxicity toward normal cells in a dose and tissue-dependence. RCP was the most interesting group because they did not affect normal cells evaluated. In Balb/c 3T3-A31 NRU cytotoxicity assay, after incubation for 48 h, IC_{50} values to RCP-45 and RCP-37 were 86.8 ± 1.14 and 96.1 ± 1.02 µg/mL, respectively. The LD_{50} values of these extracts were 558 and 579 mg/kg, respectively. For both samples the start dose for acute oral toxicity studies is 550 mg/kg in the case of using the Up-and-Down Procedure (UDP) and 300 mg/kg when Fixed Dose Procedure (FDP) and Acute Toxic Class Methods (ATC) were used. Results presented in this work can contribute to understand the toxicological profile of *Cuban* propolis and reduce the number of animals required in subsequent pharmacological / toxicological studies.

Keywords: *Cuban* propolis, cytotoxicity, normal Cells, NRU assay.

1. Introduction
Natural products constitute one of the most promising sources to obtain biologically active compounds. During the last decade, propolis has been found to exhibit a broad spectrum of therapeutic effects, including antibacterial, antifungal, antitumor, antioxidant, immunomodulatory and other beneficial properties [1, 2]. This bee product is derived from various plant resins and widely employed in traditional medicine.
Cuban propolis has been classified according to its composition: brown *Cuban* propolis type (BCP type), rich in polyisoprenylated benzophenones [3], 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 [4]. Recently, extracts of BCP, RCP and YCP have shown potential activity against tumor cells, using murine breast carcinoma, human breast adenocarcinoma and human lung adenocarcinoma [5], and antiprotozoal properties against *Leishmania amazonensis*, *Trichomonas vaginalis* and *Plasmodium falciparum* [6,7]. In this sense, extensive cytotoxic studies should be carried out with *Cuban* propolis.
Therefore, is reasonable to use, at the primary screening stage, *in-vitro* toxicity assays on normal cells to select the less toxic compounds among the most active ones. The toxicological potential of a compound is analyzed through the affectation of vital functions of normal and tumor cell lines from different tissues and different species [8]. Moreover, the fulfilling of this criterion and also the combination with the estimation of the initial dose for acute toxicity tests in rodents, using *in-vitro* data, facilitate the selection of the most promising compounds while the reduction of laboratory animals is accomplished [9].
Basal cytotoxicity is considered the starting point for an integrated assessment of potential *in-vivo* acute systemic toxicity [10, 11].

It provides information about the effect of unknown chemical compounds on cellular lethal endpoints and thus may serve as suitable predictors for LD_{50} of the test material [12]. This procedure was based on the method developed by Spielmann *et al.* [13], where acute toxicity testing in rodents is estimated using *in-vitro* data. Assessment of starting doses is very important in OECD Guidelines for the Testing of Chemicals, approved methods for acute toxicity testing such as Acute Toxic Class (ATC), Up and Down Procedure and Fixed Dose Procedure (UDP, FDP) [14-17].

In the present paper, we investigated the cytotoxic effect of *Cuban* propolis extracts against cultured normal cell lines and estimated the oral acute toxicity based on the *in-vitro* test developed by Spielmann *et al.* [13].

2. Materials and Methods

2.1 Propolis Samples

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 [7]. The origin and classification of *Cuban* propolis samples used in this study are also reported in Table 1 and previously described [4]. The extracts were dissolved in dimethylsulfoxide (DMSO, Riedel-de Haën, Germany) at 40 mg/mL and stored at 4 °C until used.

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)

BCP: brown *Cuban* propolis RCP: red *Cuban* propolis YCP: yellow *Cuban* propolis.

2.2 Cell Lines and Cultures

The cell lines used in this study were mouse fibroblasts Balb/c 3T3-A31 cells (*Mus musculus* murine embryo ATCC CCL-163TM) and Vero (normal African green monkey kidney ATCC CRL-1586™). Cell lines were cultured in a humidified atmosphere at 37 °C in 5% CO_2. Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, SIGMA, USA) supplemented with 2 mM of glutamine, 10%, with heat-inactivated fetal bovine serum (FBS), SIGMA, USA), 10%, penicillin (100 U/mL), and streptomycin (100 µg/mL). When the cells were confluent, they were routinely subcultured using 0.25% trypsin-EDTA solution (Sigma-Aldrich).

For each experiment, after trypsin detachment, cells were counted, seeded in 96-well plates, and incubated for 24 h at 37 °C with 5% CO_2 to allow cell attachment. Cell density in culture plates depended on the type of test to be performed.

2.3 MTT Assay

Balb/c 3T3-A31, MRC-5 and Vero cells (2×10^4 cells/well) were treated with different propolis extracts (triplicate wells per condition) by the addition of 50 µL of serial dilutions dissolved in DMEM to give a final concentration of 12.5; 25; 50 and 100 µg/mL to complete 100 µL of final volume. 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 additional 3 h before the medium was removed. DMSO (150 µL) was added to each well and mixed to ensure cell lysis and dissolving of the formazan crystals. Optical density 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 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 cell lines were determined.

2.4 Neutral Red Uptake Assay (NRU Assay)

The NRU assay was performed according to the standard protocol of Borenfreund and Puerner [18] modified by NICEATM [9]. Balb/c 3T3-A31 cells (10^4 cells / well) were treated with different propolis extracts (triplicate wells per condition) by the addition of 50 µL of serial dilutions dissolved in DMEM to give a final concentration of 10, 21.5, 46.4 and 100 µg/mL and incubated for 48 h. Untreated cells were used as a control and in all cases DMSO was below 0.1 %. After 48 h, the solutions were removed from all plates and the cells washed twice with 200 µL of phosphate buffered saline (PBS) for well. Then, cells received 250 µL of neutral red solution (Sigma, USA) (50 mg/mL NR in DMEM 24 h pre-incubated at 37 °C and then filtered before use through 0.22 µm syringe filter. Plates were incubated for 3 h and then cells were washed three times with PBS. The dye within viable cells was released by extraction with a mixture of acetic acid (Merck, Germany), ethanol (Merck, Germany) and water (1:50:49). Absorbance of neutral red was measured using UV–visible spectrophotometer multiplate reader (MRX Revelation Dynex Technologies, Germany) at 540 nm. The optical density (OD) was calculated using the formula DO (treated cells)*100/DO (control cells). The IC_{50} values from line cells were determined.

2.5 Estimation of LD_{50} from IC_{50} Values

To obtain the predicted LD_{50} values from the IC_{50} values (µg/mL) obtained in the NRU assay were using the regression formula: Log LD_{50} (mg/kg) = 0.372 log IC_{50} (µg/mL) + 2.024 [17].

2.6 Statistical Analysis

The IC_{50} 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 and Discussion

3.1 MTT Assay

Table 2 shows the growth inhibitory activity (IC_{50}) on Balb/c 3T3-A31 and Vero cells, exposed to each propolis for 72 h. BCP and RCP showed no effects on Balb/c 3T3-A31; while among YCP extracts only YCP-18 showed a significant cytotoxicity compared to untreated control with an IC_{50} value of 54.6 ± 1.7 µg/mL (Table 2). In case of Vero cells, some BCP extracts were slightly toxic, and RCP was the only extract nontoxic towards the cell lines tested.

Table 2: The *in-vitro* cytotoxic IC_{50} values on selected normal cell lines.

Samples	IC_{50} (µg/mL) ± SD	
	Balb/c 3T3-A31	Vero
BCP-1	>100	>100
BCP-4	>100	>100
BCP-5	>100	>100
BCP-16	>100	88.1 ± 0.1[a]
BCP-17	>100	82.0 ± 1.7[a]
RCP-9	>100	>100
RCP-29	>100	>100
RCP-35	>100	>100
RCP-37	>100	>100
RCP-45	>100	>100
RCP-72	93.5 ± 1.6[b]	>100
YCP-18	54.6 ± 1.7[a]	>100
YCP-39	>100	>100
YCP-41	>100	>100
YCP-50	>100	>100
YCP-60	>100	90.9 ± 0.4 [a]

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

3.2 Neutral Red Uptake Assay

To assess the potential toxicity of *Cuban* propolis, 3T3 NRU assay was used. Taking into consideration previous studies [5], different concentrations of RCP-45 and RCP-37 were exposed on Balb/c3T3-A31 for 48 h and the results are shown in Fig. 1. The IC_{50} values found for RCP-45 and RCP-37 were 86.8 ± 1.14 and 96.1 ±1.02 µg/mL, respectively, which did not show significant differences ($P>0.05$).

The IC_{50} values obtained were used to predict the starting doses for acute oral toxicity test using animals, based on the regression equation (RE). The computed LD_{50} values were 558 mg/kg and 579 mg/kg for RCP-45 and RCP-37, respectively.

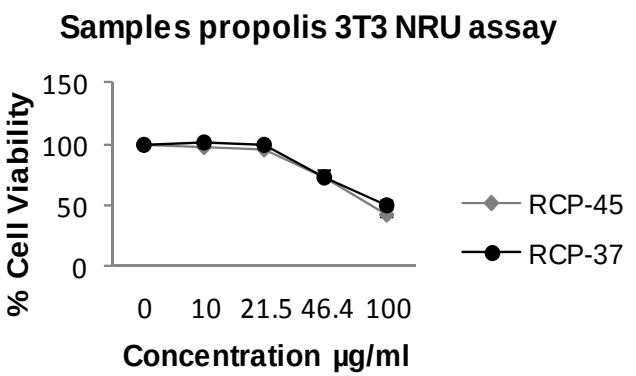

Fig 1: Cell viability from NRU cytotoxicity assay after 48h exposure of RCP-45 and RCP-37 to Balb/c 3T3-A31. Each point represents media ± SD of 3 independent experiments.

4. Discussion

The toxicological potential of a compound is analyzed through the affectation of vital functions of normal and tumor cell lines from different tissues and different species. It is important that such selection could be done at the very beginning of developmental process, at the stage of *in-vitro* studies [8]. Balb/c 3T3-A31 and Vero cell lines are usually employed in prospective studies to determine the cytotoxic effect of different natural products [19-21]. In this work, sensitivity analysis of these cells allowed the characterization of cytotoxicity profiles of *Cuban* propolis considering BCP, RCP and YCP extracts. In general, toxicity from each group depends on individual propolis and tissue origin.

We have already reported that BCP and YCP were significantly toxic towards MRC-5 cells [5]. Our study confirm that human lung tissue is significantly more sensitive to BCP and YCP groups that the other two normal tissues, the murine embryo and monkey kidney tissues. Diaz-Carballo *et al*. reported the cytotoxic effect of nemorosone on embryonic human kidney cells (HEK293WT), MRC-5 and Balb/c 3T3-A31 [22]. Similar to us, they find that MRC-5 was the most responsive cell line to nemorosone compared to the HEK293WT and Balb/c 3T3-A31 cell lines [22]. Nemorosone is the main component of BCP extracts and is the responsible of the *in-vitro* antitumor effects of these extracts [23]. Propolis usually contains a proportion of components with cytotoxic effects on normal cells [6]. In the case of RCP extracts, they were the least toxic among the three groups. Different reports corroborate that flavonoids and derivatives, the main components of *Cuban* red propolis, possess low cytotoxicity on Balb/c 3T3-A31 and MRC-5 cell lines. Besides, they are responsible for the biological activities of this kind of propolis [19, 20, 24, 25].

The progress of regulatory implementation of *in-vitro* methods to estimate acute oral toxicity is supported by the success of other established alternative methods [12]. Additionally, currently available *in-vitro* methods that could reduce the number of animals for experiments, and consequently their cost, are usually welcome [26, 27].

The approach for predicting toxicity presented in this paper is based on the regression model developed by Spielmann *et al.* [13]. Taking into consideration previous studies where RCP-45 and RCP-37 were the extracts least toxic and the most

selective toward tumor cells, we determined for this propolis the IC_{50} and LD_{50} values. These values indicated that acute oral toxicity studies using the UDP method, where the range of concentration established is: 1.75, 5.5, 17.5, 55, 175, 550, and 2000 mg/kg (upper limit of 2000 mg/kg) or 1.75, 5.5, 17.5, 55, 175, 550, 1750, and 5000 mg/kg (upper limit of 5000 mg/kg), can be initiated with the starting dose of 550 mg/kg for both propolis extract. If ATC and FDP methods are used where the concentration range is 5, 50, 300, and 2000 mg/kg (upper limit of 2000 mg/kg) 5, 50, 300, 2000, and 5000 mg/kg (upper limit of 5000 mg/kg), the starting dose is 300 mg/kg for both propolis extracts.

Studies conducted by Pilar Prieto *et al.*, have shown that if a chemical compound is tested with the 3T3 NRU test method and the LD_{50} estimated using the RE is higher than 2000 mg/kg, then it is likely that this product would not require a hazard label for acute oral toxicity [28]. Nevertheless LD_{50} value estimated by 3T3 NRU test is determined by the chemical characteristics of the compound [17]. Several toxicity studies in animal models showed that propolis are generally well tolerated [29]. Previous studies carried out to determinate different biological effects of RCP extract, showed satisfactory results in the range of 10 -100 mg/kg [30-32]. Therefore, these findings suggest that at doses lower than 300 mg/kg, the RCP-37 and RCP-45 propolis should be safe.

5. Conclusions

Previous studies have shown that propolis has important biological activities for the development of new therapeutic agents. The results of this study allowed characterizing the cytotoxicity profile of *Cuban* propolis on normal cells of different histological types. We suggest that toxicity towards non-cancerous cells should be also included inside the battery to develop an antitumor drug. Moreover LD_{50} values determined for estimating starting doses for acute toxicity, reduce the number of animals required and consequently cutting down on the cost. Indeed, data on biological effects are crucial for the interpretation of pharmacological and toxicological results. In any case, data derived from the 3T3 NRU should be supplemented with other available information on propolis, such as physicochemical properties, mechanism(s) of action and its biokinetic profile, much of which could be generated by using non-animal methods.

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