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# EFFECT OF ETHANOLIC EXTRACT OF PROPOLIS ON CELL VIABILITY OF CHINESE HAMSTER OVARY CELLS (CHO-K1) IRRADIATED WITH $^{60}$CO GAMMA-RAYS USING DIFFERENTIAL STAINING TECHNIQUE

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## ABSTRACT

The objective of present study was to assess the effect of Brazilian propolis (AF-08) on CHO-K1 cells irradiated with $^{60}$Co, through the differential staining technique, using acridine orange and ethidium bromide. The cells were pre-incubated with different concentrations of propolis (50, 100 and 200 µg/mL) for 24h and irradiated with 5 Gy, analyzed at 24 and 48h after exposure. This technique is based on the cell capacity to incorporate fluorescent DNA dyes, where the viable (green), apoptotic (orange/yellow) and necrotic (red) cells can be identified through fluorescence microscopy. Digital high-resolution images were acquired from at least 5 visualization fields, and cells were analyzed using ImageJ and Flowing softwares. This approach permitted to analyze a large number of cells/sample with the time reduction, much easier and faster, proportioning more statistical power of the technique. The treatment with propolis only was not cytotoxic at 24 and 48h, except for the higher concentration of 200 µg/mL associated or not with radiation, increasing apoptotic and mainly necrotic cells ($p< 0.001$). The data showed a promising use of propolis as well as technique used, pointing out that 200 µg/mL of propolis was cytotoxic, but at lower one (50 µg/mL) presented a radioprotective effect in irradiated CHO-K1 cells.

## 1. INTRODUCTION

The ionizing radiation effect on living matter is resultant, primarily, of the absorption of radiation energy by the cells and organisms. Ionizing radiation like X-rays and γ-rays, ß and α particles, and neutrons have sufficient energy to knock out an electron and ionize atoms of the medium. Their ability to produce ionization is responsible for biological damage. It is well established that ionizing radiation interact with the biological molecules either by direct ionization or indirectly through generation of free radicals causing cellular damage.

Considering that biological systems contain ~90% water, it is plausible to admit that the predominant effect by which ionizing radiation cause damage to the important biomolecules is through indirect mechanism. In this process, ionizing radiation interacts with water molecules to produce a wide range of reactive oxygen species (ROS) such as superoxide anion ($O2^{\bullet -}$), the hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($O^{\bullet}$), nitric oxide (NO), hydrogen peroxide ($H_2O_2$) and peroxyl radicals [1,2]. The $OH^{\bullet}$ radical is a highly reactive and oxidizing specie that can react with all cell constituents such as DNA, lipids, proteins and

carbohydrates. As a result of the interaction with $OH^{\bullet}$ radicals with the cellular genome, a cascade of events is initiated, leading to diverse cellular responses, for example, cell death, chromosomal rearrangements and mutations, eventually resulting in cancer [3].

The cell death is one of main endpoints used to evaluate the biological effect of radiation and, in general can be apoptotic or necrotic. Apoptosis is a genetically mediated type of death in which internal and external signals stimulate cells to produce enzymes which trigger the death process. This strategy is adopted by multicellular organism to avoid the propagation of undesirable cells carrying mutations in the organism, including those cells that could eventually escape the cell division control [4]. Necrosis, on the other hand, is a passive kind of cell death resulting from external cell damage and characterized by an early loss of membrane permeability with cellular swelling, cytoplasmic vesicle dilatation, and generally associated with the inflammatory process [5]. Both types are a modality of cell death known as interphase death, consisting of loss of physical and metabolic cell integrity.

Various studies have been conducted to detect and quantify necrotic and apoptotic cells in irradiated biological system, using differential staining technique based in morphological characteristics and on membrane permeability due to its simplicity and speed [6]. In this method, cells are stained with a dye mixture containing acridine orange and ethidium bromide and observed through a fluorescence microscope equipped with suitable excitation and emission filters. Acridine orange (AO) stains viable cells in green, and apopotic cells in orange. Ethidium bromide (EtBr) is only permeable to membranes in dead cells that loss the membrane integrity, and thus only detect necrotic cell, stained in red.

Taking into account the harmful effect of ionizing radiation, efforts are underway to develop the radioprotective compounds since many decades. A wide variety of compounds have been tested for radioprotective activity, including various synthetic compounds. Unfortunately, most of chemical radioprotectors have shown toxic effects that limit their use in the practice. In view of this fact, the search for natural, less toxic and effective compounds with radioprotective capacity is justified. More recently, various studies have showed that various natural products protect DNA or cells against radiation-induced oxidative damage.

Flavonoids are compounds available in a diversity of natural sources, and belong to a class of substances that had been the object of study in the past years [7]. Some authors describe this group of substances as antioxidant and free radical chelating agents [8]. One of the most prominent natural source of flavonoids is propolis, a resin produced by honey bees (*Apis mellifera*) from many plant sources. The main polyphenols constituents of the propolis are flavonoids that comprises about 20-30% of its dry weight [9]. Propolis and flavonoids possess a variety of biological properties namely antioxidant, antitumor, anti-inflammatory, immunostimulative and antibacterial, besides being frequently cited as a radioprotective compound [10].

The present study aimed to evaluate the effects of ethanolic extract of Brazilian propolis (EEP) (AF-08) on Chinese hamster ovary cells (CHO-K1) irradiated with 5 Gy of gamma radiation ($^{60}Co$), using a differential staining technique (acridine orange and ethidium bromide) associated with ImageJ and Flowing softwares to analyze viable, apoptotic and necrotic cells.

## 2. MATERIALS AND METHODS

### 2.1. Cell Culture and Plating

Chinese hamster (*Cricetulus griseus*) ovary cells (CHO-K1, ATCC CCL-61) were maintained in 25cm$^2$ culture flasks in RPMI 1640 medium (pH 7,4; 10% fetal bovine serum; 1% penicilin/streptomicin), and kept on incubation chamber (5% $CO_2$, 37ºC) until 60-70% of confluence. Medium was replaced every 48 hours. After reaching confluence, cells were trypsinized and plated in triplicates on 60mm-diameter Petri dishes at a 6 x $10^3$ cells/mL (3 mL per plate).

### 2.2. Ethanolic extract of propolis (EEP)

Pieces of crude Brazilian propolis (AF 08) collected at Rio Grande do Sul state (Brazil) were purchased from Amazon Food K.K. (Tokyo, Japan). Propolis pieces (100g) were incubated with 95% ethanol at room temperature and away from light exposures for three months. Primary extract was passed through paper filter and kept at -20ºC for 24 hours. After cold incubation, extract was again filtered and concentrated by evaporation. Final concentration of EEP (1 mg/mL) was prepared dissolving 10% DMSO + 90% culture medium and maintained at $4^0$C. Before use, the ethanolic extract was filtered for sterilization (0.22μm).

### 2.3. EEP treatment

CHO-K1 cells were incubated for 24 h with different concentrations of EEP (50, 100 and 200 μg/mL) and analyzed 24 and 48h after irradiation. The assays were carried out in triplicate.

### 2.4. Irradiation

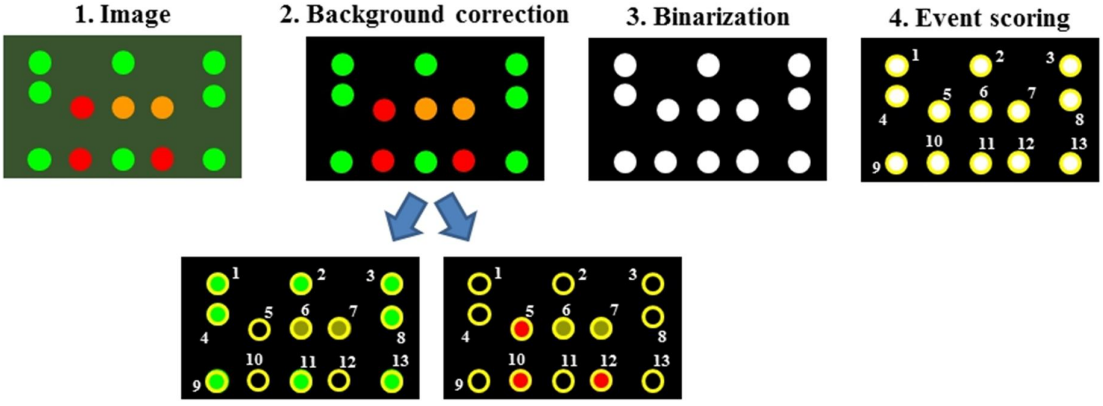
After treatment, cells were washed in PBS and received fresh medium (3 mL). Sealed Petri dishes were gamma-irradiated at room temperature in a $^{60}$Co source (Gammacell 220 Irradiation Unit of the Canadian Atomic Energy Commission, Ltd) on Centro de Tecnologia das Radiações of IPEN/CNEN-SP. Plates were irradiated with 5Gy using a 90% attenuator to reach a 140Gy/h medium dose rate. After irradiation, plates were maintained again in incubator until the use.

### 2.5. Differential staining viability assay

Cultures were incubated for 24 and 48h after irradiation. After that, cells were trypsinized and washed on cold (4ºC) PBS by 5 min at 1500 rpm. Cell pellets were suspended in 50 μL sterile staining solution, containing acridine orange and ethidium bromide (1:1) (10 μg/mL each) in PBS and incubated on ice for 5 minutes. After incubation, 25 μL of stained cell suspension were analyzed for fluorescence microscopy (Nikon 80i) at 10X magnification, using proper filter (Ex.: 450-490nm; Em.: 515nm). In this assay, viable cells are shown to emit green fluorescence, apoptotic cells in orange, and necrotic cells in red color.

### 2.6. Semi-automated viability assessment

High resolution images of at least five microscopy fields (at 10X magnification) were acquired from samples previously prepared. Images containing viable (green), apoptotic (orange) and necrotic (red) cells were analyzed using ImageJ software. For the analysis, the images were submitted to background correction (rolling ball radius: 50 pixels) to remove non-specific fluorescence. After that, corrected images were binarized, and cells were counted individually, creating regions of interest. These regions were used to quantify green and red fluorescence on green and red image channels, respectively. Area values (in pixels or μm) from every region of interest were also scored. Values of integrated density of pixels inside regions of interest in both channels were used to calculate ratios between green and red fluorescence (G/R ratio). A brief scheme of this data acquisition is represented in Figure 1. Area and ratio values were organized in a spreadsheet, and analyzed using Flowing software. Histograms were used to determine high (viable), low (necrotic) or intermediate (apoptotic) fluorescence ratio events. An example of this quantification is represented in Figure 2. Results were expressed as percentages of total number of cells. Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests were used to assess statistically significant differences between percentages of viable, necrotic and apoptotic cells in relation to irradiated or treated cultures. Graph plotting and statistical analysis were performed using GraphPad Prism 5.0 software.

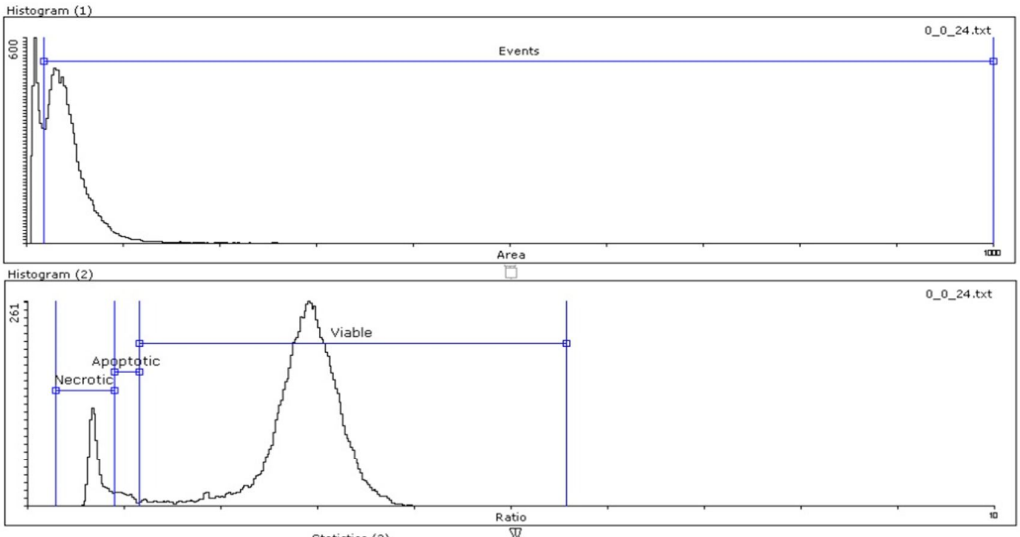



**6. Ratio calculation**

| Event # | Area | IntDen – Green channel | IntDen – Red channel | Ratio (IntDen G / IntDen R) |
|---|---|---|---|---|
| 1 | 100 | 3 | 1 | 3 |
| 6 | 102 | 2 | 2 | 1 |
| 10 | 98 | 1 | 3 | 0.3 |

**Figure 1: Diagram representing fluorescence quantification and cell viability assessment by analysis of digital images. After background correction, images were binarized and cell regions were determined. Color channels (green and red) from**

**background corrected images were used to quantify mean fluorescence in pixel units of cell areas. Values were used on calculations of G/R ratios. Item 6 show hypothetical integrated density (IntDen) values from viable (Event 1), apoptotic (Event 6) and necrotic (Event 10) cells.**




Statistics (3)

| | Events | % of Vis | Mean |
|---|---|---|---|
| All events | 10,008 | 100.00 | 2.68 |
| Viable | 9,046 | 90.39 | 2.88 |
| Apoptotic | 176 | 1.76 | 1.01 |
| Necrotic | 786 | 7.85 | 0.72 |

C

**Figure 2: Quantification of viable, apoptotic and necrotic cell fractions from a control experiment (CHO cells non-treated and non-irradiated). Based on event areas, artifacts such as cell debris were excluded from analysis using a cutoff region on histogram (A). Histogram plotting of relevant events acquired in (A) are classified according to its fluorescence G/R ratio as viable, apoptotic or necrotic cells, setting this regions directly on histogram (B). Calculations done by Flowing Software with number and percentages of specific cell fractions (C).**

## 3. RESULTS AND DISCUSSION

### 3.1. Frequencies of viable, apoptotic and necrotic cells

The data obtained for the viability of CHO-K1 cells treated with EEP and irradiated are presented in Table 1. The differential staining method associated to semi-automated scoring allowed to analyze a large number of cells in smaller time period and also improving the accuracy of the analysis.

**Table 1: Percentages of viable, apoptotic and necrotic CHO-K1 cells, irradiated and treated with different concentrations of ethanolic extract of propois (EEP)**

| Dose | Time after irradiation | EEP concentration | Total of analyzed cells | % of total (Mean± SD) | | |
|---|---|---|---|---|---|---|
| *(Gy)* | *(h)* | *(µg/mL)* | | Viable cells | Apoptotic cells | Necrotic cells |
| 0 | 24 | 0 | 6545 | 85.06±8.20 | 1.47±0.91 | 13.45±8.38 |
| | | 50 | 7376 | 75.21±5.68 | 1.68±1.50 | 23.09±5.93 |
| | | 100 | 6924 | 87.28±3.68 | 2.09±1.66 | 10.62±4.18 |
| | | 200 | 3475 | 64.31±22.76 | 12.79±11.86 | 22.88±17.84 |
| | 48 | 0 | 6716 | 84.35±5.74 | 1.17±0.53 | 14.47±5.71 |
| | | 50 | 6564 | 85.46±4.02 | 1.37±0.94 | 13.22±4.26 |
| | | 100 | 7041 | 81.60±9.59 | 1.57±1.08 | 17.29±9.30 |
| | | 200 | 2930 | 65.39±13.80 | 2.77±3.25 | 15.93±11.31 |
| 5 | 24 | 0 | 4890 | 81.49±4.85 | 2.00±1.91 | 16.49±5.59 |
| | | 50 | 4659 | 85.22±3.06 | 1.76±1.05 | 13.01±3.06 |
| | | 100 | 4558 | 85.26±5.52 | 1.49±0.37 | 13.23±5.47 |
| | | 200 | 4347 | 53.99±7.86 | 11.11±4.83 | 34.89±11.18 |
| | 48 | 0 | 4520 | 92.66±1.19 | 1.79±0.96 | 5.53±1.87 |
| | | 50 | 5056 | 87.01±5.75 | 1.88±1.05 | 11.10±5.63 |
| | | 100 | 5061 | 84.30±8.17 | 1.87±1.24 | 13.81±9.15 |
| | | 200 | 2340 | 56.74±26.16 | 5.73±4.70 | 37.52±21.54 |

Graphical representations of the relationship between viable, apoptotic, necrotic cells and different concentrations of EEP, 24 and 48h after irradiation are shown in Figs 3, 4 and 5, respectively.

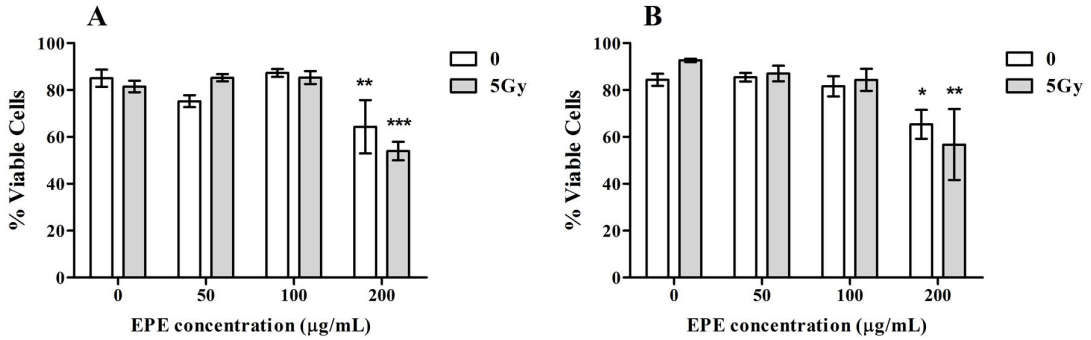



**Figure 3: Percentages of viable cells after 24 (A) and 48h (B) after irradiation with 5Gy. Error bars represent standard error means (SEM). Statistically significant differences comparing to non-treated controls (Bonferroni post-hoc test) are represented as (*)p<0.05; (**)p<0.01; (***)p<0.001.**

The treatment with 50 and 100 µg/mL of EEP alone or associated with radiation not reduced the frequency of viable cells when compared to respective controls, 24 or 48h after exposure, but not with 200 µg/mL (Fig. 3). In the same manner, a dose of 5 Gy did not induced

statistically significant difference ($p > 0.05$) in cell viability in relation to non-irradiated cells, except for cells treated with 200 µg/mL of propolis.

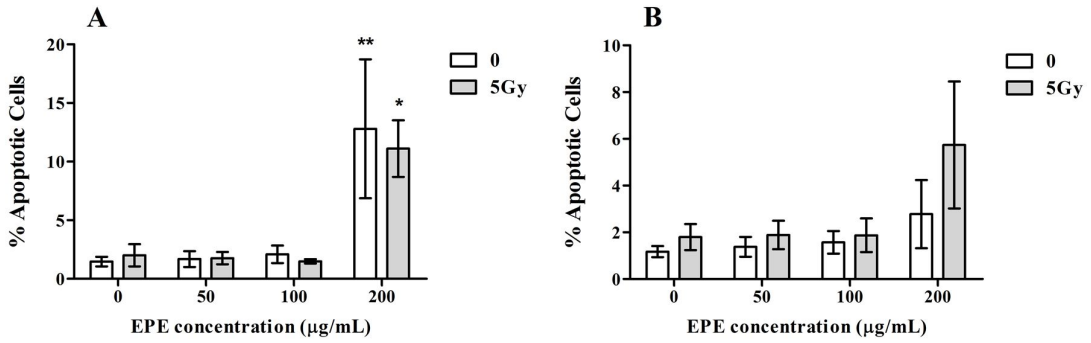



**Figure 4: Percentages of apoptotic cells after 24 (A) or 48 (B) after irradiation (5Gy). Error bars represent standard error means (SEM). Statistically significant differences comparing to non-treated controls (Bonferroni post-hoc test) are represented as (*)p<0.05; (**)p<0.01.**

Concerning the frequency of apoptotic cells, 200 µg/mL of EPE induced statistical significant difference on cells irradiated or not, when compared to respective untreated control, but only at 24 h after irradiation (Fig. 4). Despite of some increase had been observed, apoptotic fractions at 48 h after irradiation were not considered significant ($p>0.05$).

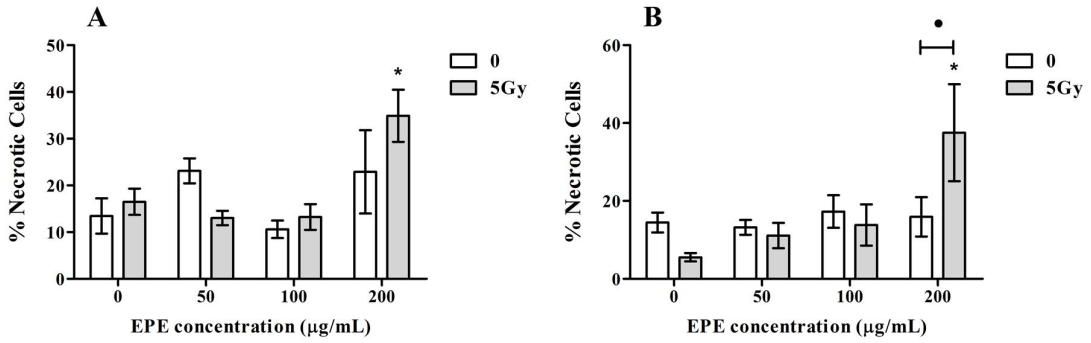



**Figure 5: Percentages of necrotic cells after 24 (A) or 48 (B) after irradiation (5Gy). Error bars represent standard error means (SEM). Statistically significant differences comparing to non-treated controls (Bonferroni post-hoc test) are represented as (*)p<0.05. Compared to non-irradiated controls, differences are represented as (●)p<0.05.**

In relation to the necrotic cell death, the data obtained showed that the necrosis was the main modality of interphase death in irradiated CHO-K1 cells (Fig. 5). The highest concentration of EEP (200 µg/mL) showed an additive cytotoxic effect, increasing the effect of radiation, 24 and 48h after exposure. On the other hand, concentration of 100, and mainly 50 µg/mL,

showed a decrease in the cell death in irradiated cells when compared with cells without EEP, suggesting a radioprotective capacity, although without statistical significance ($p > 0.05$).

## 3. CONCLUSIONS

The data obtained through of the differential staining technique showed that the EEP (50 and 100 µg/mL) was not cytotoxic on CHO-K1 cells, irradiated or not, evaluated 24 and 48h after treatment. However, at a higher concentration of 200 µg/mL, the EEP showed a cytotoxic effect, increasing the frequency of apoptotic and necrotic cells. The necrosis was the main modality of interphase cell death. On the other hand, the concentration of 50 µg/mL presented a radioprotective effect, decreasing the frequency of necrotic death, 24 and 48h after irradiation. These data suggest the occurrence of an optimal concentration of EEP, what can be lead to a discussion about the possible radioprotector potential of propolis on mammalian cells.

The proposed modification of the technique associated to the images analysis showed several advantages over the conventional method. A large number of cells/sample (in order of some thousands) can be scored in shorter time period, permitting to improve the statistical accuracy of the technique. Moreover, faster acquisition of images by digital camera avoided the photobleaching of staining, what can lead to inaccurate results.

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