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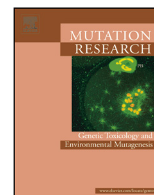

Effect of Brazilian propolis (AF-08) on genotoxicity, cytotoxicity and clonogenic death of Chinese hamster ovary (CHO-K1) cells irradiated with ^{60}Co gamma-radiation

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ABSTRACT

The present study was conducted in order to evaluate the effect of Brazilian propolis (AF-08; 5, 10, 15, 30, 50, 100, and 200 μg/mL) in protecting CHO-K1 cells against genotoxic and cytotoxic damage and clonogenic death induced by ^{60}Co gamma-radiation (1.0, 2.0, 4.0, and 6.0 Gy). For this purpose, three interlinked endpoints were analyzed: induction of DNA damage by use of the micronucleus (MN) test (genotoxic damage), cell viability by means of the MTS assay, and differential staining (cytotoxic damage) and clonogenic death via the colony-formation test (cytotoxic damage). The MN test revealed that propolis alone (5–100 μg/mL) was not genotoxic up to 100 μg/mL and that 30 μg/mL of propolis reduced the radiation-induced DNA damage (~56% reduction, $p < 0.05$), exhibiting a radio-protective effect on irradiated CHO-K1 cells. On the other hand, analysis of cytotoxicity showed that a concentration of 50 μg/mL presented a significant proliferative effect ($p < 0.001$) when associated with radiation, decreasing the percentage of necrotic cells ($p < 0.01$). No mediated cytotoxic effect was found, but the concentration of 200 μg/mL was toxic when analyzed at 24 and 48 h via the differential staining technique, but not at 72 h after irradiation, analyzed with the MTS assay. Differential staining also showed that necrosis was the main death modality in irradiated cells and that apoptosis was induced only at the toxic concentration of propolis (200 μg/mL). Concerning the clonogenic capacity, a concentration of 50 μg/mL also exhibited a significant stimulating effect on cell proliferation ($p < 0.001$), in agreement with the data from differential staining. Taken together, these data suggest that the use of propolis AF-08 for the prevention of the adverse effects of ionizing radiation is promising. Nevertheless, additional investigations are necessary for a better understanding of potential applications of propolis to improve human health.

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1. Introduction

Ionizing radiation is a physical agent known to affect somatic and germ cells by inducing mutation, cell death, malformation, and cancer. The intensity of damage will depend, among other factors, on the absorbed dose, the type of radiation, the irradiation conditions, and the intrinsic radio-sensitivity of the cells [1]. Ionizing radiation induces different types of molecular lesion in mammalian cells, either by direct ionization or indirectly through generation of free radicals and reactive oxygen species (ROS), such as the hydroxyl radical, the superoxide anion, singlet oxygen, nitric oxide, hydrogen peroxide, and peroxyl radicals [2,3]. These free radicals are highly reactive oxidizing species that can attack all cell constituents such as DNA, lipids, protein, and carbohydrates. Among the various radio-induced lesions in DNA (single- or double-strand breaks (DSB), base damage, and DNA–DNA or DNA–protein cross-links), DSBs have been considered to be the critical events after exposure to ionizing radiation, and have been found to be the main cause of cell killing [3]. As a result of the interaction of free radicals

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with the cellular genome, a cascade of events is initiated, leading to diverse cellular responses such as cell-cycle delay, chromosomal rearrangements, micronucleus (MN) formation, mutations, and cell death, eventually resulting in cancer and hereditary disease [4]. Therefore, attempts to minimize the radio-toxicity induced in cells and tissues in cases of occupational radiation exposure have stimulated studies concerning the identification of compounds that are able to provide protection against the harmful effects of ionizing radiation.

Although more than 4000 chemical compounds with radio-protective efficacy have been synthesized since the 1950s, these typically exhibit inherent toxicity and are expensive [5,6]. Therefore, there is a clear need to identify natural, effective, non-toxic, and inexpensive substances that can offer better protection against the effects of genotoxic exposure. As a consequence, plant-derived polyphenolic compounds such as flavonoids, tannins, curcumin, and stilbene resveratrol [2,7–9] have recently been the subject of considerable scientific and therapeutic interest. Nevertheless, their use as radio-protectors requires further scientific evaluation and validation. In this context, propolis, a resinous natural product collected and elaborated by honeybees (*Apis mellifera*) from many plant sources, is of particular interest [10]. It is known to contain a high concentration of flavonoids: approximately 25–30% of its dry weight [11]. A typical sample of propolis contains, in fact, about 25 different flavonoids at significant concentrations, which suggests that propolis extracts retain the majority of the biochemical properties associated with flavonoids [12]. The composition of propolis is very complex, with mainly phenolic constituents, attributable to its plant origin [13]. Typical components include flavonoids, phenolic acids and esters, enzymes, vitamins and minerals, terpenoids, aromatic aldehydes, amino acids, coumarins, and steroids [14,15]. Propolis has been reported to exhibit therapeutic properties, as well as anti-microbial, anti-inflammatory, anti-oxidant, free-radical scavenging, immunomodulatory, and anti-carcinogenic activities [10,11,13,16], thus showing the possibilities of application in the pharmaceutical industry and health-food sectors. More recently, propolis has received special attention in the area of oncology research as a source for cancer prevention and as a co-adjuvant in standard cancer therapy [17,18]. Accordingly, a number of compounds possessing anticancer activity, such as caffeic acid phenylethyl ester (CAPE) [16,19], artepillin C [15,20,21], and propolins [22,23] have been isolated from propolis and characterized.

Despite the wide spectrum of activities of propolis and flavonoids and their recognized importance to human health, few studies on their effects in irradiated cells are available. Benkovic et al. [4,11], Montoro et al. [24,26], and Rithidech et al. [25] analyzed the effects of propolis/flavonoids on *in vitro* irradiated human lymphocytes by use of chromosome aberration, MN, and comet assays. The present study has, as its main objective, the assessment of the effect of Brazilian propolis (AF-08) against genotoxic and cytotoxic damage and clonogenic death of Chinese hamster ovary cells (CHO-K1) induced by ^{60}Co gamma-radiation, analyzed by use of various interlinked parameters. The genotoxic evaluation was performed at the chromosomal level by analysis of MN induction resulting from irreparable and potentially carcinogenic DNA damage. The cytotoxic evaluation was carried out with the viability test based on the MTS assay and the differential staining technique in order to analyze the modality of interphase cell death, either through apoptosis or necrosis [27]. Another cell-death modality, reproductive death, was evaluated via the clonogenic potential employing the classical colony-formation test. The choice of these parameters is justified by their biological importance, in addition to the fact that they are observable and measurable in irradiated cells. The choice of CHO-K1 cells as the object of this study is justified because this cell line is widely utilized as a reference for genotoxicity and cytotoxicity tests and by the fact that these cells present many inherently advantageous characteristics: they belong to a genetically stable cell line, have the ability to form colonies, and have a relatively rapid growth rate with a stable karyotype of 22 ± 2 chromosomes [28].

2. Materials and methods

2.1. Propolis

Propolis (AF-08), collected in the State of Rio Grande do Sul, Brazil, was supplied by Amazon Food K.K. (Tokyo, Japan). Crude propolis was prepared according to the procedure described by Shimizu et al. [29]. Approximately 100 g of propolis was incubated with 200 mL of 95% (v/v) ethanol for three months at room temperature, covered with aluminum foil and kept in the dark. The mixture was then filtered and the filtrate kept in a freezer for 24 h, followed by a second filtration (to give the ethanolic extract of propolis). Subsequently, the extract was submitted to evaporation, resulting in a resinous extract (AF-08). A stock solution (1 mg/mL) was prepared by adding 10% DMSO + 90% distilled water and maintained at 4 °C. Before use, the resinous extract was filtered through a Millipore membrane filter (0.22 μm). From the stock solution, final concentrations of 5, 10, 15, 30, 50, and 100 μg/mL of propolis were used for the MN test. In the same manner, the extract was tested at 50, 100, and 200 μg/mL with respect to cell viability and differential staining, and at 30, 50, and 100 μg/mL in the clonogenic assay. Park et al. [30,31] and Shimizu et al. [29] showed that quercetin, apigenin, kaempferol, acacetin, melliferone, moronic acid, anwuweizonic acid, betulonic acid, 4-hydroxy-3-methoxy-propiophenone, 4-hydroxy-3-methoxybenzaldehyde, 3-(3,4-dimethoxyphenyl)-2-propenal, and acetoxytremetone were authentic constituents of propolis, but artepillin C and chrysin were not found as major compounds. Moreover, propolis of Brazilian origin was reported not to include CAPE in its chemical composition [15].

2.2. Cell line

CHO-K1 cells, subclones of Chinese hamster (*Cricetulus griseus*) ovary cells, were utilized. The cells were maintained in RPMI 1640 medium (Cultilab) supplemented with 10% fetal calf serum (Gibco), 1% penicillin and streptomycin (Sigma) and incubated at 37 °C in the presence of 5% CO_2.

2.3. Irradiation

After incubation with propolis, CHO-K1 cells in the exponential growth phase were maintained in PBS, trypsinized, kept in Eppendorf tubes, and irradiated at the "Centro de Tecnologia das Radiações" (IPENCNEN/SP) with a ^{60}Co gamma-source, which permitted a homogeneous irradiation (Gammacell 220 Irradiation Unit of the Canadian Atomic Energy Commission, Ltd.) at a dose rate of 2.82 Gy/min with a 90% attenuator, at doses of 1.0, 2.0, 4.0, and 6.0 Gy, at room temperature. After the irradiations, the cells were maintained at 37 °C in the presence of 5% CO_2.

2.4. Genotoxicity assay – micronucleus test

For the genotoxic evaluation, the MN test was carried out according to the cytokinesis block method with cytochalasin B [32] to obtain bi-nucleated cells. Cells in the exponential growth phase were seeded in six-well plates and, after 48 h of adhesion, incubated for 1 h with different concentrations of propolis (5, 10, 15, 30, 50, and 100 μg/mL) and then exposed to ^{60}Co gamma-radiation at doses of 1.0, 2.0, and 4.0 Gy. The incubation time of 1 h before irradiation was based on literature data [26]. After irradiation, cells were seeded on 60-mm-diameter Petri dishes containing RPMI 1640 medium, serum, antibiotics, and cytochalasin B (3 μg/mL), at 37 °C with 5% CO_2. CHO-K1 cells were maintained under these conditions for 48 h. Cells were then trypsinized, transferred to Falcon tubes, treated with isotonic solution (0.85% NaCl), and fixed with acetic acid/methanol (1:3). Finally, the cell suspension was added dropwise to histological slides, fixed at 65 °C in a humid atmosphere, and stained with 10% Giemsa in phosphate buffer, pH 6.8, for 10 min. The micronuclei were identified according to criteria adopted by the International Atomic Energy Agency [33]. The slides were analyzed with a Carl-Zeiss microscope at 400× magnification. For each sample, 500 bi-nucleated cells containing up to five MN were analyzed in at least three independent assays. Cells with more than five MN were not considered in order to avoid possible inclusion of cells that were in the process of nuclear fragmentation [34]. All accompanying mono-nucleated and multi-nucleated cells present in the samples were counted to determine the nuclear division index (NDI). The formula described by Eastmond and Tucker [35] was used: $NDI = (M1 + 2\times M2 + 3\times M3 + 4\times M4)/N$, where M1 to M4 represent the number of cells with 1–4 nuclei, respectively, and N is the total number of viable cells scored. At least 2000 cells were analyzed for each sample.

2.5. Cytotoxicity assay

2.5.1. Cell viability test

To assess cell viability, a colorimetric method based on the MTS assay (Promega Corp., Madison, WI, USA) was adopted. The assay basically determines the number

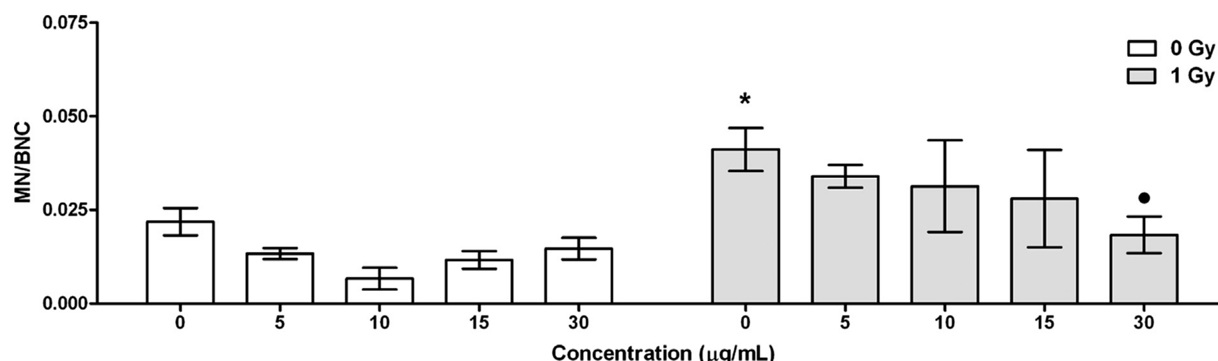

Fig. 1. Frequencies of micronuclei (MN) in bi-nucleated CHO-K1 cells (BNC) treated with different concentrations of propolis (5, 10, 15, and 30 μg/mL) and irradiated with 1 Gy of ^{60}Co gamma-radiation. * $p < 0.05$ vs. 0 Gy (without propolis and without radiation); • $p < 0.05$ vs. 1 Gy (without propolis).

of viable cells through their capacity to convert MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] in the presence of phenazine methosulfate (PMS; Sigma, St. Louis, MO, USA), via dehydrogenase enzymes present in the mitochondria of metabolically active cells, into a purple formazan compound that is soluble in the culture medium. The quantity of formazan was determined by measurement of absorbance at 490 nm; it is proportional to the number of viable cells in the culture. Cells were incubated with different concentrations of propolis (50, 100, and 200 μg/mL) for 24 h before treatment with 5 Gy of ^{60}Co radiation. The choice of the incubation period of 24 h was based on literature data [22,36]. Cells were then seeded in 96-well plates at 2.5×10^3 cells/well, and maintained for 72 h at 37 °C with 5% CO_2. The cell density was determined by adding 20 μl/well of MTS (2 mg/mL in PBS) and PMS (0.92 mg/mL in PBS) in a 20:1 ratio (vol/vol). One hour after the addition of the mixture, the plates were read in a microplate reader (Dynatech, Model MR4000, Chantilly, VA, USA). Each concentration of the propolis extract was tested in quadruplicate in three independent assays. Results were expressed as the percentage of viable cells, with 100% referring to control cells, without propolis and without irradiation.

2.5.2. Differential staining technique

To assess the modality of cell death in the analysis of the cytotoxic effect of propolis (50, 100, and 200 μg/mL, 24 h of preincubation) in irradiated (5 Gy) CHO-K1 cells, the differential staining method described by Gorman et al. [37] was used. The method is based on the capacity of the cell to incorporate fluorescent DNA dyes. Briefly, stock solutions of acridine orange (100 μg/mL) and ethidium bromide (100 μg/mL) were mixed (vol/vol, 1:1) and 1 μL of the final solution was added to 25 μL of cell suspension. After 2 min at room temperature, 50 μL of this cell suspension was dropped on slides, covered with coverslips and observed with a fluorescence microscope (Nikon Eclipse 80i) through a proper filter set (excitation filter of 450–490 nm; emission filter of 515 nm). The viable and apoptotic cells were stained green and yellow/orange, respectively, both with chromatin condensation, whereas necrotic cells were stained red with organized chromatin. The green cells resulted from the penetration of acridine orange through the plasma membrane but not ethidium bromide, while orange/red cells were permeable to acridine orange and ethidium bromide as a consequence of the loss of membrane integrity. High-resolution RGB (Red–Green–Blue 16-bit colored images) images were acquired from at least five different microscope fields at 10× magnification. The images were analyzed with ImageJ Software (http://http://rsb.info.nih.gov/ij/). The original files were submitted to background-intensity correction (rolling ball radius: 50 pixels) and, after binarization, cell locations were determined by use of the "Analyze Particles" plug-in. These regions of interest were superimposed on images corresponding to red and green channels and pixel intensities of green and red signals inside each cell were determined. A ratio between green and red intensities for each event was calculated (green signal/red signal) and ratio values plotted in a distribution histogram in the Flowing Software (Turku Centre for Biotechnology, Finland). Control samples (without radiation and without propolis) showed specific ratio values (mean ± CV%) for viable (2.93 ± 10.25), apoptotic (1.45 ± 25.0), and necrotic (0.71 ± 9.21) cells, providing stable cutoff values that could be employed to determine how many cells (in % of the total) in a sample could be scored as viable, apoptotic or necrotic on the basis of the ratio intensities. Data were collected from three independent experiments and the evaluations were done 24 and 48 h after irradiation.

2.5.3. Clonogenic assay – survival curve

To evaluate the clonogenic potential, the procedure described by Guo et al. [38] and Murakami et al. [27] was used in the elaboration of survival curves. CHO-K1 cells were incubated with different concentrations of propolis (30, 50, and 100 μg/mL) for 24 h at 37 °C with 5% CO_2. The cells were then irradiated at doses of 1, 2, 4, and 6 Gy of ^{60}Co radiation, seeded into 60-mm culture dishes (200–300 cells/dish) and maintained at 37 °C. Each sample was analyzed in triplicate for each dose and concentration of propolis. After seven days, the medium was removed and the cells were washed with PBS, fixed with 10% formol for 30 min and stained with 20% Giemsa solution in phosphate buffer, pH 6.8, for 30 min. The colonies formed in each dish were analyzed with a CP600 colony counter (Phoenix). Only colonies containing at least 50 cells were considered to have survived the treatments and to be clonogenic [1,39]. The results are expressed as plating efficiency (PE) and survival fraction (SF), as described by Hall [1]:

$$PE = \left(\text{number of counted colonies/number of seeded cells}\right) \times 100$$

$$SF = \left(\text{number of counted colonies in treated culture/number of seeded cells}\right) \times \left(\text{PE in control culture}/100\right)$$

2.6. Statistical analysis

All analyses were performed with the GraphPad Prism program (version 5.0) for elaboration of the figures and tables. To compare the data resulting from different treatments, two-way ANOVA and the Bonferroni post-test were used. A p-value < 0.05 was taken as the minimum basis for assigning significance. The survival curves for CHO-K1 cells treated with propolis and irradiated were fitted to a quadratic exponential model, $Y = e{-}(\alpha D + \beta D^2)$, where Y is the survival fraction, α and β are the model constants, and D is the absorbed dose in Gy.

3. Results

3.1. Genotoxicity assay – micronucleus test

Figs. 1 and 2 show the cytogenetic data obtained for CHO-K1 cells treated with propolis and then exposed to radiation. Propolis *per se* did not induce any genotoxic effect up to 100 μg/mL ($p > 0.05$) in CHO-K1 cells. When cells were irradiated, we observed an increment of genotoxic damage (increase in MN frequency) whose values were 1.9-fold (control vs. 1 Gy), 3.9-fold (control vs. 2 Gy), and 6.3-fold higher (control vs. 4 Gy) in comparison with the control (Fig. 2). When submitted to the combined treatment of propolis plus radiation, the cells showed less genotoxic damage than cells that were only irradiated. This was observed for

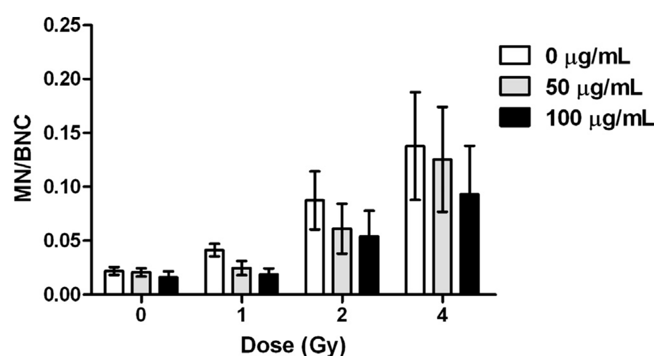

Fig. 2. Frequencies of micronuclei (MN) in bi-nucleated CHO-K1 cells (BNC) treated with 50 and 100 μg/mL of propolis and irradiated with 1, 2, and 4 Gy of ^{60}Co gamma-radiation.

lower concentrations of propolis (5, 10, 15, and 30 μg/mL) and a radiation dose of 1 Gy of ^{60}Co (Fig. 1) and for higher concentrations of propolis (50 and 100 μg/mL) and the higher radiation doses of 2 and 4 Gy (Fig. 2). In all analyzed samples, the prevalence of bi-nucleated cells with one MN was verified. There was a tendency for the quantity of DNA damage to decrease as a function of increasing propolis concentration in irradiated cells. The propolis concentration that showed the highest efficiency in reducing DNA damage was 30 μg/mL ($p < 0.05$) when cells were irradiated with 1 Gy, resulting in ~56% maximum reduction in the frequency of MN (Fig. 1). Although the values are still lower than those of the respective controls for higher propolis concentrations (50 and 100 μg/mL) and for higher radiation doses (2 and 4 Gy) (Fig. 2), the statistical analyses showed no significant differences between the treatments ($p > 0.05$). The NDI values obtained from the frequency of mono-nucleated, bi-nucleated, and multi-nucleated cells were 1.95 ± 0.12, 1.96 ± 0.12, 1.89 ± 0.18, and 1.87 ± 0.13 for the control sample (without radiation and without propolis), and the irradiated samples (1 Gy, 2 Gy, 4 Gy), respectively. For samples treated with different concentrations of propolis alone (5, 10, 15, 30, 50, and 100 μg/mL), the values varied from 2.00 ± 0.09 to 1.83 ± 0.08 and for samples treated with propolis and radiation, the NDI values varied from 1.95 ± 0.02 to 1.87 ± 0.19. Statistical analysis showed no significant difference ($p > 0.05$) between the treated samples and the respective controls.

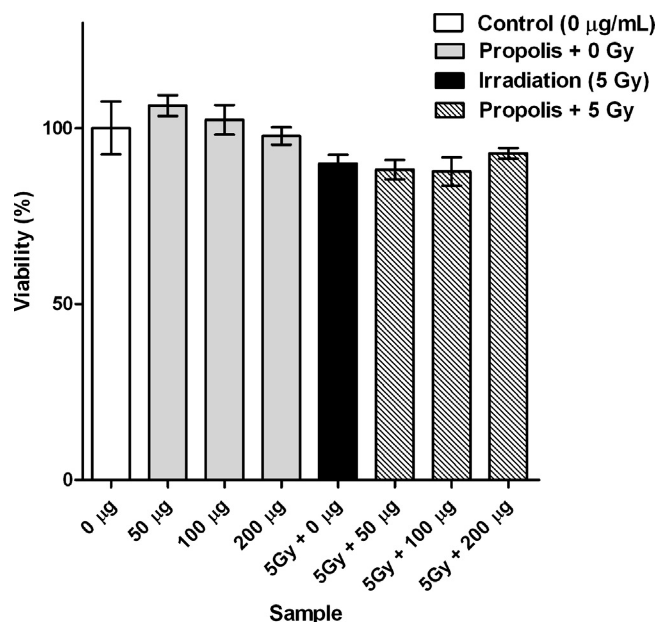

Fig. 3. Effect of different concentrations of propolis (50, 100, and 200 μg/mL) on the viability of CHO-K1 cells, irradiated with 5 Gy of ^{60}Co gamma-radiation, evaluated at 72 h by use of the MTS assay.

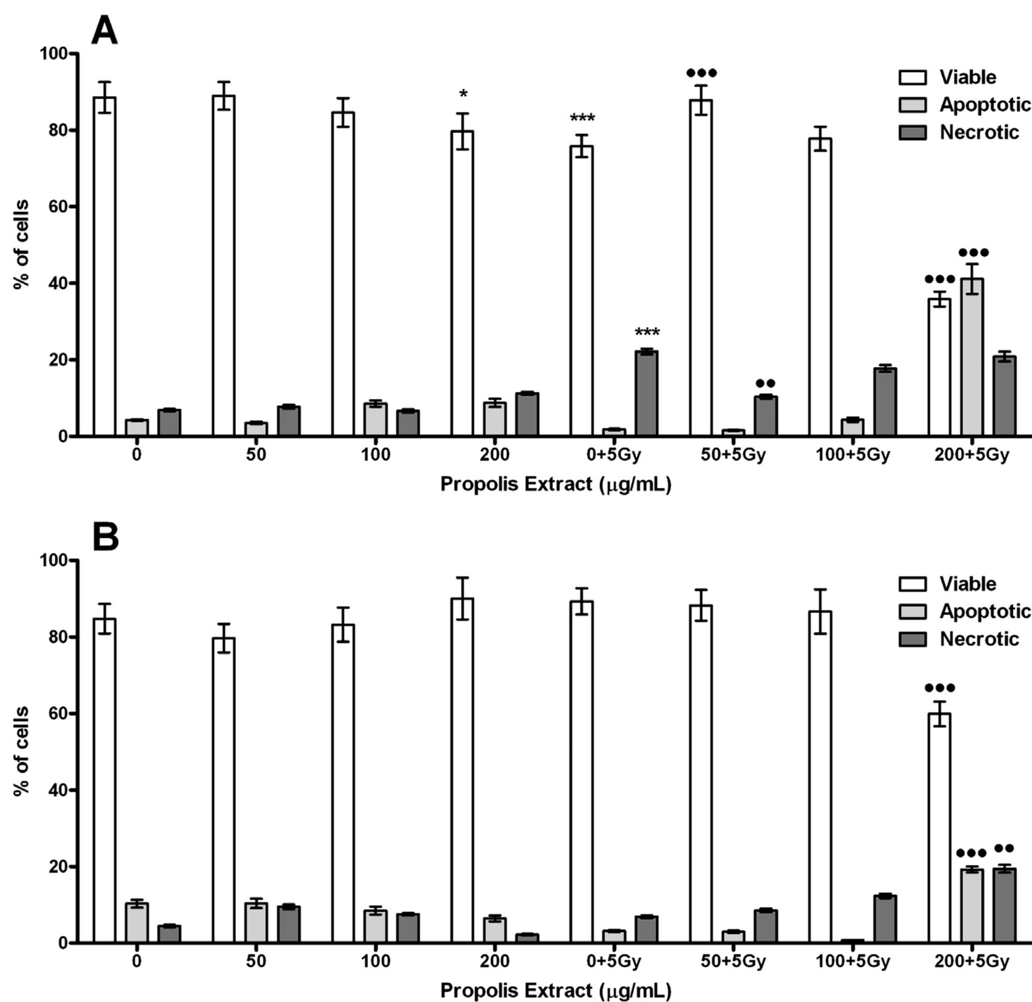

Fig. 4. Frequencies of viable, necrotic, and apoptotic cells in CHO-K1 cells pretreated with different concentrations of propolis (50, 100, and 200 μg/mL), evaluated at 24 h (A) and 48 h (B) after exposure to 5 Gy of ^{60}Co gamma-radiation by differential staining technique. * $p < 0.05$, *** $p < 0.001$ vs. the corresponding controls without radiation and without propolis; •• $p < 0.01$, ••• $p < 0.001$ vs. the corresponding controls only irradiated.

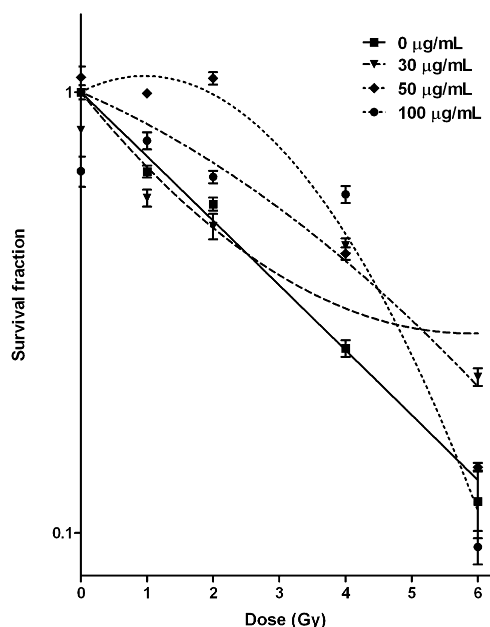

Fig. 5. Survival curves, adjusted according to the model $Y = e^{-(\alpha D+\beta D^2)}$, for CHO-K1 cells treated with different concentrations of propolis (30, 50, and 100 μg/mL) as a function of radiation dose. Points represent the means with the corresponding error bars.

3.2. Cytotoxicity assay

3.2.1. Cell viability test

The cytotoxicity data obtained with the MTS assay for the effect of propolis on CHO-K1 cells incubated for 24 h with different propolis concentrations before irradiation, evaluated 72 h after exposure, are shown in Fig. 3. The treatments with propolis alone or propolis associated with radiation apparently did not influence CHO-K1 cell growth, that is, propolis was not cytotoxic to these cells.

3.2.2. Differential staining technique

In the same manner, Fig. 4 presents the data at 24 h (Fig. 4A) and 48 h (Fig. 4B) after irradiation, showing the percentages of viable, necrotic, and apoptotic cells obtained by the differential staining technique. Treatment with propolis alone did not cause differences in the viability of CHO-K1 cells ($p > 0.05$) in relation to the control at 24 h and 48 h after irradiation, except for the propolis concentration of 200 μg/mL ($p < 0.05$) at 24 h. A radiation dose of 5 Gy induced a significant decrease in cell viability ($p < 0.001$), with a concomitant increase in the proportion of necrotic cells ($p < 0.001$) at 24 h after exposure compared with the control, but not at 48 h. However, the treatment with propolis at 50 μg/mL and, to a lesser extent, with 100 μg/mL induced a decrease in necrotic cells ($p < 0.01$) with an increase in viable cells ($p < 0.001$) compared with the respective controls for irradiated cells, but this was without statistical significance for 100 μg/mL. Again, the concentration of 200 μg/mL combined with irradiation showed a cytotoxic effect, decreasing viable cells and increasing apoptotic cells ($p < 0.001$). At 48 h after irradiation, on the other hand, the treatment with radiation alone or propolis combined with radiation apparently did not influence CHO-K1 cell viability, except at the highest propolis concentration of 200 μg/mL, which was again cytotoxic to irradiated cells.

3.2.3. Clonogenic assay – survival curve

The data obtained for the clonogenic survival of CHO-K1 cells treated with different concentrations of propolis (30, 50, and 100 μg/mL) and/or different radiation doses (1, 2, 4, and 6 Gy) are shown in Fig. 5. The control sample (without radiation and without propolis) was also analyzed. The CHO-K1 cells showed a relatively high PE before irradiation (92.6 ± 3.4%). The survival curves obtained from the colony-formation test were best fitted by a quadratic exponential model. Pretreatment with propolis had a stimulating effect on the reproductive capacity of the cells at concentrations of 50 (F test, $p < 0.001$), and 100 μg/mL (F test, $p > 0.05$) when compared with the control survival curve, but the increase was statistically significant only at 50 μg/mL. As shown in Table 1, the values of the coefficients of the quadratic model for each dose–response curve indicate that the data adequately fitted an exponential quadratic mathematical model. There was a predominance of the linear component (the coefficient α) for all the survival curves.

4. Discussion

In the present study, the effect of Brazilian propolis (AF-08), harvested in the State of Rio Grande do Sul (southern Brazil), was investigated in CHO-K1 cells irradiated with ^{60}Co gamma-rays. To our knowledge, only few studies of propolis from this region are available in the literature. Ito et al. [40] identified new anti-AIDS activities of AF-08 propolis in H9 lymphocytes. Similarly, Shimizu et al. [41] showed an anti-influenza virus activity in a murine infection model, but not in an *in vitro* system [29]. The propolis samples used in this study were previously characterized with respect to chemical composition by Park et al. [30,31] and more recently by Shimizu et al. [29] through HPLC analysis. It is known that the chemical composition of propolis and its biological activity vary depending on several factors such as environment, time of collection, geographic differences, and local vegetation [30,31]. The genotoxicity of ionizing radiation was analyzed by use of the MN test, capable of detecting the effect of irreparable damage such as DNA DSB. Double-strand breaks are in fact considered to be the primary lesion leading to radiation-induced mutagenesis, carcinogenesis and cell death [3,5], for which repair is known to be intrinsically more difficult than that for other types of DNA damage [42]. The MN count gives an indirect measure of cytogenetic damage induced by any genotoxic agent and the inhibition of radiation-induced MN formation is indicative of radio-protective potential of any test agent [2]. The data obtained indicate that propolis alone was not genotoxic to CHO-K1 cells, even at concentrations higher than 100 μg/mL, without induction of any additional DNA damage. Moreover, in irradiated cells (1 Gy), the MN test indicated that, at the concentrations tested (5, 10, 15, 30, 50, and 100 μg/mL), propolis had a radio-protective effect, decreasing the frequency of radiation-induced MN in comparison with the control without propolis; however, the difference was statistically significant ($p < 0.05$) only for 30 μg/mL (Fig. 1), which showed a ∼56% reduction in DNA damage. Other groups have reported a similar radio-protective effect against ionizing radiation by propolis or its flavonoid constituents for a different cell type, that is, human peripheral blood lymphocytes [4,11,24–26]. The NDI values obtained showed no significant alteration in the cell-proliferation kinetics as a function of the treatment. The exact mechanism of action of propolis in radio-protection has not yet been totally elucidated due to the complex chemical composition of propolis and the wide variety of possible interactions with living matter [4]. Nevertheless, the results from several studies indicate that the antioxidant properties of propolis can, among other factors, lead to a direct scavenging of free radicals, particularly of ROS [2]. It is well known that radiation induces toxicity that is mediated mainly through the generation of free radicals and ROS (OH•, O_2•, H_2O_2, H•, NO, singlet oxygen) [5]. Considering that biological systems contain 75–90% water, the predominant effect by which ionizing radiation causes damage to important bio-molecules is through an indirect

Table 1
Values of the α and β coefficients of the linear–quadratic model $Y = e^{-(\alpha D + \beta D^2)}$, with the respective standard errors (SE), for CHO-K1 cells treated with three concentrations of propolis (30, 50, 100 μg/mL) and irradiated with 1–6 Gy of ^{60}Co gamma-radiation.

Cell line	Concentration of propolis (μg/mL)	α (±SE) × 10^{-2}	β (±SE) × 10^{-2}	r^2
CHO	0	33.480 ± 3.439	0.047 ± 0.931	0.9778
	30	42.730 ± 7.860	3.620 ± 1.654	0.6523
	50	17.290 ± 4.170	8.949 ± 1.321	0.9514
	100	14.880 ± 10.470	1.788 ± 2.622	0.3956

effect, where ionizing radiation interacts with water molecules to produce a wide range of ROS [3]. An estimated 60–70% of tissue damage induced by ionizing radiation is believed to be caused by OH• radicals [5]. Thus, radio-protectors or antioxidants can avoid free-radical damage by donating an electron without the resultant generation of a damaging free radical or by preventing the oxidation of critical cellular bio-molecules [2]. One of the arguments in support of the radical-scavenger hypothesis is that the radio-protector must be present before or during the irradiation in order to avoid the harmful effects of free radicals produced by the indirect effect [5,24–26]. Montoro et al. [26] reported that treatment with propolis after irradiation produced no significant difference in the frequency of chromosome damage in human blood lymphocytes compared with untreated samples. Therefore, in order to understand the mode of action of an antioxidant as a radio-protector, it is important to investigate its effects on radio-induced genotoxicity or cytotoxicity. Since propolis was not genotoxic up to 100 μg/mL and irradiated cells also showed less genotoxic damage (decrease in the frequency of MN) when pretreated with propolis, a cytotoxicity assay was carried out with a higher radiation dose (5 Gy) and a higher concentration of propolis (200 μg/mL). A dose of 5 Gy did not cause significant differences in the viability of CHO-K1 cells at 72 h after irradiation, as assessed by the MTS assay (Fig. 3). Nevertheless, the data showed no significant decrease in the viability of cells treated only with propolis or in combination with radiation. Considering these observations, the differential staining technique was used to evaluate cell viability at 24 and 48 h after irradiation, instead of at 72 h, taking into account the mode of interphase cell death (Fig. 4). A concentration of 50 μg/mL showed a significant protective effect in irradiated CHO-K1 cells, decreasing the percentage of necrotic cells. A decrease was also observed with 100 μg/mL, but this was without statistical significance. Again, no propolis-mediated cytotoxic effect was found in CHO-K1 cells, except for a propolis concentration of 200 μg/mL, associated or not with irradiation, when analyzed at 24 and 48 h after irradiation. This fact may be due to the ability of flavonoids to intercalate into DNA molecules at higher concentrations [25] and potentiated by radiation. Thus, propolis extracts were toxic to irradiated CHO-K1 cells only at the highest concentration of 200 μg/mL. The data indicate that necrosis was the main mode of cell death in these irradiated cells and that apoptosis could be induced only at a cytotoxic concentration of propolis. The results of the differential staining technique at 48 h after irradiation were comparable, at least in part, to those obtained by the MTS assay at 72 h after exposure. The gradual increase in viability with time (24, 48, and 72 h after irradiation) in cells treated with 200 μg/mL may be the result of various interlinked biological mechanisms: elimination of dead cells, repair mechanisms, and normal turnover of CHO-K1 cells, considering a relatively rapid cell cycle of 12–14 h [28]. These data thus demonstrate the need to use several parameters for a better understanding of the cellular response to the action of xenobiotic agents as radio-protectors. The results of the colony formation test confirm in part those obtained by another cytotoxic test, the differential staining technique, where the combined treatment with propolis (50 μg/mL) and radiation showed a slight stimulatory effect on cellular proliferation in the clinically relevant dose range. The radio-protective activity of propolis at the DNA level was statistically significant for 30 μg/mL whereas at the cellular level, 50 μg/mL of propolis showed proliferative activity. These data thus suggest the occurrence of an adequate/optimal concentration of propolis for either genotoxicity or cytotoxicity. However, the potential mechanisms involved have not been fully elucidated. The beneficial activity of propolis is probably not attributable to a single mechanism, but rather to several mechanisms [3]. It has been reported that, besides scavenging free radicals, propolis/flavonoids can also increase the antioxidant status by stimulating the function of endogenous antioxidant enzymatic systems (superoxide dismutase, catalase, glutathione peroxidase) and the production of DNA-repair enzymes (glycosylase, endonuclease, DNA polymerase beta) and of their mRNA, as well as by reducing lipid peroxidation and elevating sulphydryl groups in irradiated systems [2,3,5,10,25].

In summary, on the basis of these observations, it can be suggested that the Brazilian propolis (AF-08) used in the present study presents a radio-protective effect in the range of 30–50 μg/mL in irradiated CHO-K1 cells, pointing to its potential application in radio-medicine for the prevention of the adverse effects of ionizing radiation. Nevertheless, additional investigations with different methodologies are necessary for a better understanding of the mechanisms of action of propolis in irradiated cells. Studies using tumor and normal cell lines are ongoing to evaluate whether there is variability in the effect of propolis on different cell lines, or if there is a difference in their mode of action.

Conflict of interests

The authors declare that they have no conflict of interests.

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References

[1] E.J. Hall, Radiobiology for the Radiologist, 5th edn., Lippincott Williams, New York, 2000.
[2] B.S.S. Rao, R. Shanbhoge, D. Upadhya, G.C. Jagetia, S.K. Adiaga, P. Kumar, K. Guruprasad, P. Gayathri, Antioxidant, anticlastogenic and radioprotective effect of Coleus aromaticus on Chinese hamster fibroblast cells (V79) exposed to gamma radiation, Mutagenesis 21 (4) (2006) 237–242.
[3] G.C. Jagetia, Radioprotection and radiosensitization by curcumin, Adv. Exp. Med. Biol. 595 (2007) 301–320.
[4] V. Benkovic, A.H. Knezevic, N. Orsolic, I. Basic, S. Ramic, T. Viculin, F. Knezevic, N. Kopjar, Evaluation of radioprotective effects of propolis and its flavonoid constituents: in vitro study on human white blood cells, Phytother. Res. (2009), doi: 10. 1002/ptr. 2774.
[5] R.J. Vijayalaxmi, D.X. Reiter, T.S. Tan, C.R. Herman, Thomas, Melatonin as a radioprotective agent: A review, Int. J. Radiat. Oncol. Biol. Phys. 59 (3) (2004) 639–653.
[6] A. Shirazi, G. Ghobadi, M. Ghazi-Khansari, A radiobiological review on melatonin: A novel radioprotector, J. Radiat. Res. 48 (4) (2007) 263–272.

[7] S.M. Hadi, S.F. Asad, S. Singh, A. Ahmad, Putative mechanism for anticancer and apoptosis-inducing properties of plant-derived polyphenolic compounds, Life 50 (2000) 167–171.
[8] S.J. Hosseinimehr, Trends in the development of radioprotective agents, Drug Discov. Today 12 (1920) (2007) 794–805.
[9] P.U. Devi, P.K. Agrawala, Normal tissue protectors against radiation injury, Defence Sci. J. 61 (2) (2011) 105–112.
[10] V. Benkovic, N. Orsolic, A.H. Knezevic, S. Ramic, D. Dikic, I. Basic, N. Kopjar, Evaluation of the radioprotective effects of propolis and flavonoids in gamma-irradiated mice: the alkaline comet assay study, Biol. Pharm. Bull. 31 (1) (2008) 167–172.
[11] V. Benkovic, N. Kopjar, A.H. Knezevic, D. Dikic, I. Basic, S. Ramic, T. Viculin, F. Knezevic, N. Orsolic, Evaluation of radioprotective effects of propolis and quercetin on human white blood cells in vitro, Biol. Pharm. Bull. 31 (9) (2008) 1778–1785.
[12] B.H. Havsteen, The biochemistry and medical significance of the flavonoids, Pharm. Therapeut. 96 (2-3) (2002) 67–202.
[13] G.A. Burdock, Review of the biological properties and toxicity of bee propolis, Food Chem. Toxicol. 36 (1998) 347–363.
[14] V. Benkova, Chemical diversity of propolis and the problem of standardization, J. Ethnopharmacol. 100 (2005) 114–117.
[15] K. Shimizu, S.K. Das, T. Hashimoto, Y. Sowa, T. Yoshida, T. Sakai, Y. Matsuura, K. Kanazawa, Artepillin C in Brazilian propolis induces G0/G1 arrest via stimulation of Cip1/p21 expression in human colon cancer cells, Mol. Carcinogen. 44 (4) (2005) 293–299.
[16] D.C. Tavares, G.R.M. Barcelos, L.F. Silva, C.C.C. Tonin, J.K. Bastos, Propolis-induced genotoxicity and antigenotoxicity in Chinese hamster ovary cells, Toxicol. in Vitro 20 (2006) 1154–1158.
[17] F. Li, S. Awale, Y. Tezuka, S. Kadota, Cytotoxic constituents of propolis from Myanmar and their structure-activity relationship, Biol. Pharm. Bull. 32 (12) (2009) 2075–2078.
[18] N. Orsolic, V. Benkovic, D. Lisicie, D. Dikie, J. Erhardt, A.H. Knezevic, Protective effects of propolis and related polyphenolic/flavonoid compounds against toxicity induced by irinotecan, Med. Oncol. 27 (2010) 1346–1358.
[19] Y.-J. Chen, H.-F. Liao, T.-H. Tsai, S.-Y. Wang, M.-S. Shiao, Caffeic acid phenethyl ester preferentially sensitizes CT26 colorectal, adenocarcinoma to ionizing radiation without affecting bone marrow radioresponse, Int. J. Radiat. Oncol. Biol. Phys. 63 (4) (2005) 1252–1261.
[20] T. Kimoto, M. Aga, K. Hino, S. Koya-Miyata, Y. Yamamoto, M.J. Micallef, T. Hanaya, S. Arai, S. Ikeda, M. Murimoto, Apoptosis of human leukemia cells induced by artepillin C, as active ingredient of Brazilian propolis, Anticancer Res. 21 (2001) 221–228.
[21] Y. Akao, H. Maruyama, K. Matsumoto, K. Ohguchi, K. Nishizawa, T. Sakamoto, Y. Araki, S. Mishima, Y. Nozawa, Cell growth inhibitory effect of cinnamic acid derivatives from propolis on human tumor cell lines, Biol. Pharm. Bull. 26 (7) (2003) 1057–1059.
[22] C.N. Chen, M.S. Weng, C.L. Wu, J.K. Lin, Comparison of radical scavenging activity, cytotoxic effects and apoptosis induction in human melanoma cells by Taiwanese propolis from different sources, eCAM 1 (2) (2004) 175–185.
[23] C.N. Chen, C.L. Wu, J.K. Lin, Apoptosis of human melanoma cells induced by the novel compounds propolin A and propolin B from Taiwanese propolis, Cancer Lett. 245 (1-2) (2007) 218–231.
[24] A. Montoro, M. Almonacid, J. Serrano, M. Saiz, J.F. Barquinero, L. Barrios, G. Verdu, J. Perez, J.I. Villaescusa, Assessment by cytogenetic analysis of the radioprotection properties of própolis extract, Radiat. Prot. Dosim. 115 (1-4) (2005) 461–464.
[25] K.N. Rithidech, M. Tungjai, E.B. Whorton, Protective effect of apigenin on radiation-induced chromosomal damage in human lymphocytes, Mutat. Res. 585 (1–2) (2005) 96–104.
[26] A. Montoro, J.F. Barquinero, M. Almonacid, N. Sebastia, G. Verdu, V. Sahuquillo, J. Serrano, M. Saiz, J.L. Villaescusa, J.M. Soriano, Concentration-dependent protection by ethanol extract of propolis against gamma-ray-induced chromosome damage in human blood lymphocytes, eCAM 2011 (2011) 1–7.
[27] D. Murakami, M.F. Suzuki, M.S. Dias, K. Okazaki, Genotoxic and cytotoxic effects of ^{60}Co gamma-rays and ^{90}Sr/^{90}Y ß-rays on Chinese hamster ovary cells (CHO-K1), Radiat. Environ. Biophys. 43 (2) (2004) 91–99.
[28] B.J. Dean, N. Danford, Assays for the detection of chemically-induced chromosome damage in cultured mammalian cells, in: S. Venitt, J.M. Parry (Eds.), Mutagenicity Testing: a Practical Approach., IRL Press, Oxford, 1984, pp. 187–232.
[29] T. Shimizu, Y. Takeshita, Y. Takamori, H. Kai, R. Sawamura, H. Yoshida, W. Watanabe, A. Tsutsumi, Y.K. Park, K. Yasukawa, K. Matsuno, K. Shiraki, M. Kurokawa, Efficacy of Brazilian propolis against herpes simplex virus type I infection in mice and their modes of antiherpetic efficacies, eCAM 1-9 (2011), doi: 10. 1155/2011/9761 96.
[30] Y.K. Park, S.M. Alencar, C.L. Aguiar, Botanical origin and chemical composition of Brazilian propolis, J. Agr. Food Chem. 50 (9) (2002) 2502–2506.
[31] Y.K. Park, J.F. Pasredes-Guzman, C.L. Aguiar, S.M. Alencar, F.Y. Fujiwara, Chemical constituents in *Baccharis dracunculifolia* as the main botanical origin of southeastern Brazilian propolis, J. Agr. Food Chem. 52 (5) (2004) 1100–1103.
[32] M. Fenech, A.A. Morley, Cytokinesis-block micronucleus method in human lymphocytes: effect of in vivo ageing and low dose X-irradiation, Mutat. Res. 161 (2) (1986) 193–198.
[33] International Atomic Energy Agency, Cytogenetic Analysis for Radiation Dose Assessment, A, Manual, Technical Reports Series N0 405, IAEA, Vienna, 2001.
[34] M. Kirsch-Volders, A. Elhajouji, E. Cundari, P.V. Hummelen, The in vitro micronucleus test: a multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction, Mutat. Res. 392 (1997) 19–30.
[35] D.A. Eastmond, J.D. Tucker, Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody, Environ. Mol. Mutagen. 13 (1989) 34–43.
[36] N.R. Prasard, A. Karthikeyan, S. Karthikeyan, B.V. Reddy, Inhibitory effect of caffeic acid on cancer cell proliferation by oxidative mechanism in human HT-1080 fibrosarcoma cell line, Mol, Cell Biochem. 349 (2011) 11–19.
[37] A. Gorman, J. McCarthy, D. Finucane, W. Reville, T.G. Coster, Morphological assessment of apoptosis, in: T.G. Cotter, S.J. Martin (Eds.), Techniques in Apoptosis: a User's Guide, Portland Press, London, 1996, pp. 2–20.
[38] G.Z. Guo, K. Sasai, N. Oya, T. Takagi, K. Shibuya, M. Hiraoka, Simultaneous evaluation of radiation-induced apoptosis and micronuclei in five cell lines, Int. J. Radiat. Biol. 73 (3) (1998) 297–302.
[39] S.L. Scott, P.H. Gumerlock, L. Beckett, Y. Li, Z. Goldberg, Survival and cell cycle kinetics of human prostate cancer cell lines after single-and multifraction exposures to ionizing radiation, Int. J. Radiat. Oncol. Biol. Phys. 59 (1) (2004) 219–227.
[40] J. Ito, F.R. Chang, H.K. Wang, Y.K. Park, M. Ikegaki, N. Kilgore, K.H. Lee, Anti-AIDS agents, 48. 1 Anti-HIV activity of moronic acid derivatives and the new melliferone-related triterpenoid isolated from Brazilian propolis, J. Nat. Prod. 64 (10) (2001) 1278–1281.
[41] T. Shimizu, A. Hino, A. Tsutsumi, Y.K. Park, W. Watanabe, M. Kurokawa, Anti-influenza virus activity of propolis in vitro and its efficacy against influenza infection in mice, Antivir. Chem. Chemoth. 19 (1) (2008) 7–13.
[42] K.K. Khanna, S.P. Jackson, DNA double-strand breaks: signaling, repair and the cancer connection, Nat. Genet. 27 (3) (2001) 247–254.