2009 International Nuclear Atlantic Conference - INAC 2009
Rio de Janeiro,RJ, Brazil, September27 to October 2, 2009
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
ISBN: **978-85-99141-03-8**

PROTECTIVE EFFECT OF PROPOLIS ON RADIATION-INDUCED CHROMOSOMAL DAMAGE ON CHINESE HAMSTER OVARY CELLS (CHO-K1)

Geyza Spigoti[1], Shiguetoshi Tsutsumi[2], Paolo Bartolini[1] and Kayo Okazaki[1]

[1] Instituto de Pesquisas Energéticas e Nucleares (IPEN/CNEN-SP)
Av. Professor Lineu Prestes, 2242
05508-000 – São Paulo, SP
kokazaki@ipen.br

[2] Amazon Food Ltda
Setagaya-Ku Ohara, 2-24-28-102
156-0041 –Tokyo, Japan
fwip5138@mb.infoweb.ne.jp

ABSTRACT

In the last years, particular interest has been given to investigations concerning natural, effective and nontoxic compounds with radioprotective capacity in concert with increasing utilization of different types of ionizing radiation for various applications. Among them, propolis, a resinous mixture of substances collected by honey bees (*Apis mellifera*) has been considered promising since it presents several advantageous characteristics, i.e., antiinflammatory, anticarcinogenic, antimicrobial and free radical scavenging action. It is, therefore, a direct antioxidant that protects cells and organisms from the adverse effects of ionizing radiation. These relevant biological activities are mainly mediated by the flavonoids, present at relatively high concentrations in the propolis. Considering that the chemical composition and, consequently, the biological activity of propolis is variable according to the environmental plant ecology, the present study was conducted in order to evaluate the radioprotective capacity of Brazilian propolis, collected in the State of Rio Grande do Sul, against genotoxic damages induced by ^{60}Co γ-radiation in Chinese hamster ovary cells (CHO-K1). for this purpose, micronucleus induction was analyzed concerning irreparable damage, specifically related to DNA double-strand breaks, that are potentially carcinogenic. CHO-K1 cells were submitted to different concentrations of propolis (3 – 33 μg/ml), 1 h before irradiation, with 1 Gy of γ radiation (0.722 Gy/min). The data obtained showed a decreasing tendency in the quantity of radioinduced damage on cells previously treated with propolis. The radioprotective effect was more prominent at higher propolis concentration. The treatment with propolis alone did not induce genotoxic effects on CHO-K1 cells. Beside that, the treatment with propolis, associated or not with radiation, did not influence the kinetics of cellular proliferation.

1. INTRODUCTION

Ionizing radiation is a physical agent known to affect somatic and germinative cells, leading to mutation, cell death, malformation and cancer. Interactions of ionizing radiation with living cells causes a variety of changes, whose damage intensity depend, fundamentally from the absorbed dose, type of radiation, conditions of irradiation and intrinsic radiosensitivity of cell.

It is well-established that the radiation damage is resulting from the energy deposition on irradiated cell by the direct interaction with the target-molecules of the cell (direct effect) or indirectly through the formation of free radicals decurrent of the water radiolysis and of the reactive oxygen species ($OH^{\bullet}$, $O_2^{\bullet -}$, H_2O_2) (indirect effect). These interactions lead to various DNA lesions, as for example damage to bases and sugars, single- or double-strand breaks and DNA-DNA and DNA-protein cross-links [1].

At the organism level, the exposure of the whole body to ionizing radiation will unchain a variety of complex metabolic and physiologic changes as nausea, vomiting, diarrhea, fatigue, blood alterations, lethargic, convulsions and death. This complex of characteristic symptoms is known as acute radiation syndrome and they are didacticly classified as hematopoietic, gastrointestinal and central nervous system syndromes, depending fundamentally of the absorbed dose and of the time leading to death [1].

Thus, attempts to minimize radiotoxicity induced to tissues and organs in cases of accidental and occupational exposure, have supported the study of substances that can provide protection against the damaging effects of ionizing radiation. Because radiation-induced cellular damage is atributed mostly to the noxious effects of free radicals, molecules with radical scavenging properties are particularly promising as radioprotectors [2]. However, many synthetic compounds tested that have been (e.g., AET, WR2721 or amifostine, WR1065), produced serious side effects as nausea and vomiting and are considered toxic at the necessary doses. This fact limits their use in medical practice [3].

Taking into account the limited successes of these chemical compounds, the need to identify naturals, effective and nontoxic substances of easy disponibility and with radioprotector abilities is justified. This makes possible to foresee promising strategies for protecting accidentally, occupationally or even therapeutically exposed individuals against ionizing radiation damage.

In this context, flavonoids, that represent a class of plant pigments mostly derived from benzo-γ-pyrone, have been the subject of considerable scientific and therapeutic interest in the last years [4]. Much attention given to the flavonoids is decurrent of the epidemiologic study that has shown a direct association between fruits and vegetables consumption and a decreased risk of cancer, including breast, colon, larynx, pancreas and prostate cancer [5, 6]. The possible protective effects of flavonoids have been observed both *in vivo* [7, 8] and *in vitro* [2, 9] and have gained a public interest concerning flavonoids consumption for their potential benefits to health.

Since the natural concentration of flavonoids in vegetables is generally low and their extraction is laborious, an attractive alternative has been the use of propolis which contains a high concentration of flavonoids [9, 10]. A tipical sample of propolis contains in fact about 25 different flavonoids at significant concentrations, what suggests that the

propolis extract retain the majority of biochemical properties associated with flavonoids [4].

Propolis is a yellow-brownish resinous substance collected and elaborated by bees (*Apis mellifera*) from various plant sources. It is used by the bees to seal holes in their honeycombs and to eliminate outside intruders. Among various apicultural products as honey, royal jelly and polen, propolis has exhibited therapeutic properties as well as antimicrobial, antiinflammatory, antioxidant and free radical scavenging, immunostimulative and anticarcinogenic activities [9, 11, 12]. However, factors such as the plant ecosystem where propolis is collected and even the genetic variability of bees, influenced the chemical composition of propolis and, consequently, their biological activity [13, 14]. Since the chemical composition of propolis changes according to the local ecology, there is a need for qualitatively and quantitatively characterize its chemical composition, also evaluating their biological effectiveness on irradiated cells.

In spite of the considerable quantity of work published about propolis and flavonoids, little information is avalilable about the cytogenetic evaluation of propolis as protector agent on irradiated cells. In this context Montoro et al. (2005) [15], Rithidech et al. (2005) [6], Benkovic et al. (2008b) [9] and Benkovic et al. (2009) [2], who analyzed the effects of propolis/flavonoids on *in vitro* irradiated human lymphocytes, must be cited.

The present study is proposing to evaluate the radioprotector capacity of ethanolic extracts of propolis (EEP), proceeding from Rio Grande do Sul (Brazil), against genotoxic damages induced by ^{60}Co γ-rays on Chinese hamster ovary (CHO-K1) cells. For this, different concentrations of propolis before cell irradiation with 1 Gy of ^{60}Co were analyzed. The genotoxic evaluation of these treatments was assessed at the chromosomic level by analysing micronucleus induction, originating from irreparable damage, specially DNA double-strand breaks, that are potentially carcinogenic.

2. MATERIALS AND METHODS

2.1. Propolis

Propolis collected by *Apis mellifera*, obtained directly from beehive proceeding from the State of Rio Grande do Sul, Brazil, was utilized.

2.2. Cell line

CHO-K1 cells, a subclones of Chinese hamster ovary cells (*Cricetulus griseus*) were maintained in RPMI 1640 medium (Cultilab), supplemented with 10% fetal calf serum

(Gibco), 1% penicillin and streptomycin (Sigma) and incubated at 37^{o}C in the presence of 5% CO_2.

2.3. Ethanolic extract of propolis (EEP)

Ethanolic extract of propolis was prepared as proposed by Li et al. (2007) [16] with some modifications. Briefly, collected propolis samples (100g) were crushed into small pieces and extracted with 200 ml of 95% (v/v) ethanol for 6 months. After extraction, the mixture was filtered and maintained in the freezer during 24h, followed by a second filtration. Subsequently, the extract obtained was submitted to evaporation, resulting in a resinous substance (ethanolic extracts of propolis). The final concentration of 1 mg/ml (10% DMSO + 90% water) was used and kept at 4 ^{o}C. Before use, EEP was filtered through Millipore with 0.22 μm of porosity.

2.4. Irradiation condition

CHO-K1 cells were maintained in Eppendorf tubes and were exposed to the ^{60}Co gamma radiation of the panoramic type (Yoshizawa Kiko, Japan) (0.72 Gy/min), at a dose of 1 Gy, at room temperature. The source was calibrated using a Fricke/Alanine dosimeter (Merk) and read in a spectrophotometer (Hitachi 100-40).

2.5. Experimental model

CHO-K1 cells in the exponential growth phase were trypsinized and seeded in Petri dishes of 60 mm. After 48h of culture at 37 ^{o}C with 5% CO_2, cells were submitted to the different concentrations of propolis (3 – 33 μg/ml) for 1h before irradiation with ^{60}Co. Cells were then trypsinized, irradiated and processed for the micronucleus test.

2.6. Micronucleus (MN) test

Cytokinesis block method using cytochalasin B [17] was adopted according to the procedure described by Murakami et al. (2004) [18] for obtaining binucleated cells. After irradiation, cells were seeded on Petri dishes containing medium, serum, antibiotic and cytochalasin B (3 μg/ml). After 48h of incubation, cells were trypsinized, transferred to Falcon tubes, treated with isotonic solution (0.85% NaCl) and fixed with acetic acid - methanol (1:3). The cell suspensions were added drop-wise to histological slides, fixed at 65 ^{o}C in a humid atmosphere and stained with 10% Giemsa in phosphate buffer, pH 6.8 for 10 min. Micronuclei were identified according to criteria adopted by AIEA (2001) [19]: micronuclei were counted only when present in preserved cell cytoplasm, on the

same focal plane and with no contact with the main nucleus with a diameter of less than one-third of the main nucleus and with a similar staining pattern. The slides were analysed with a Carl Zeiss microscope at X400 magnification. For each sample, 500 binucleated cells were examined in 3 independent assays. The proliferation index (PI), that evaluates the kinetics of cell proliferation, was determined, considering all mononucleated and multinucleated cells until completing a total of 500 binucleated cells, according the formula reported by Erexson et al. (1989) [20]:

PI= [(number of mononucleated cells) + 2 (number of binucleated cells) + 3 (number of multinucleated cells)] /total counted cells

For estimating the % damage reduction, the formula of Favy et al. (1999) [21] was used:

Damage reduction (%)= (obtained value – basal value)/ (basal value) X 100

2.7. Statistical analysis

Statistical analysis was performed with the GraphPad Prism program (version 2.0) for graphs and tables elaboration. For comparing the data obtained from different treatments a Student's t-test was used. A value of $p < 0.05$ was considered significant. The correlation of the dose-response curve relating propolis concentration versus frequency of MN, was also determined.

3. RESULTS

The preliminary cytogenetic analysis by MN assay showed a decreasing tendency in the extent of radioinduced damage/cell (Fig. 1A), as well as in the % of cells containing MN (Fig. 1B) in the samples previously treated with propolis in relation with the sample only irradiated. In spite of the apparent difference observed between treatments, a t-test showed no statistical difference ($p > 0.05$) in the two parameters analyzed, except for the sample treated with 33 μg/ml of propolis ($p < 0.05$). Table 1 shows the distribuition and frequency of radioinduced MN and proliferation index obtained in CHO-K1 cells submitted to different treatments. In all analyzed samples, the prevalence of BN cells with one MN was verified. The treatment with the propolis only did not cause genotoxic effects on CHO-K1 cells. The proliferation index apparently showed that the treatment with propolis associated or not with radiation did not influence cell proliferation kinetics.

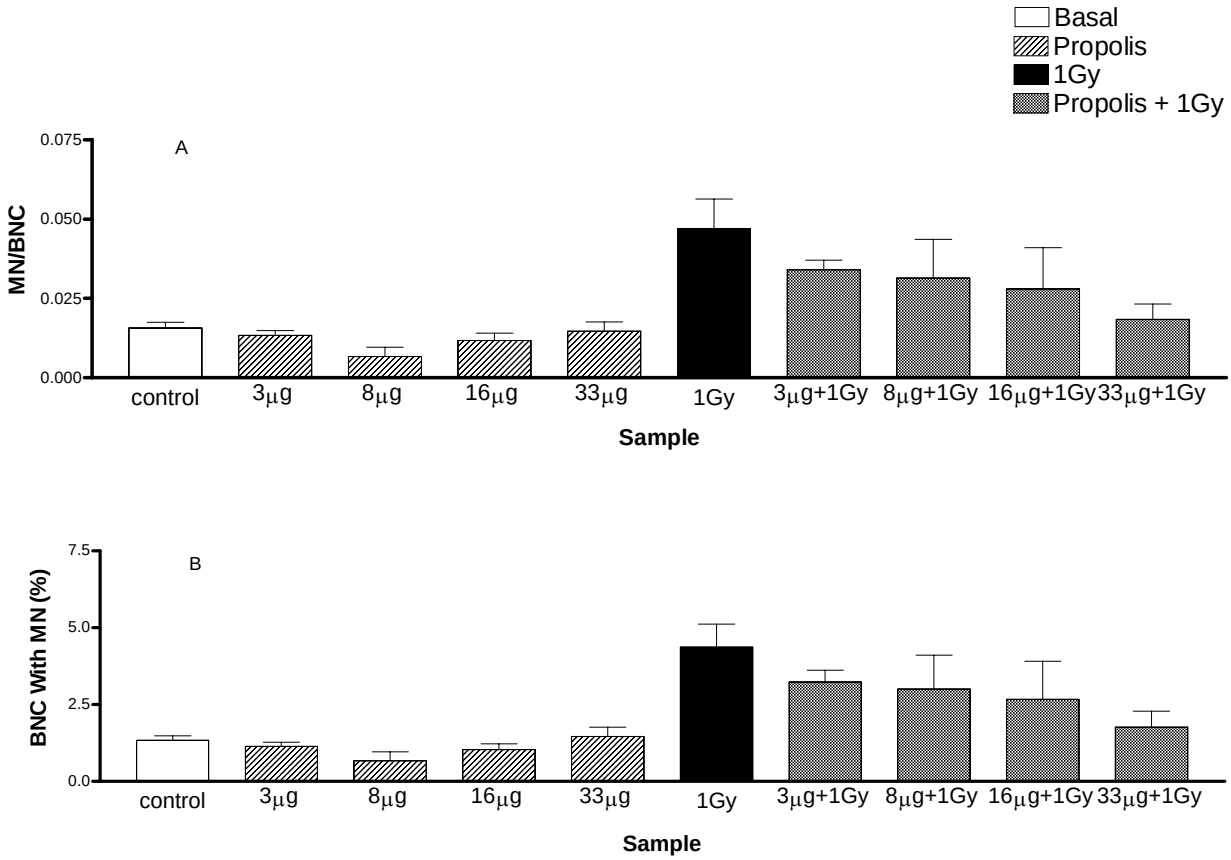

Figure 1: Frequencies of micronuclei (MN) observed in binucleated CHO-KI cells (BNC) after different treatments. A) Number of micronuclei per binucleated cell. B)Percentage of binucleated cells with MN.

A correlation analysis of the dose-response curve showed a significant correlation ($p < 0.05$) indicating a linearity of the response at the analyzed concentration range of propolis. This considering the number of MN/cell (Y) versus concentration (X): $Y = -0.00072 X + 0.040$, $r = -0.9120$ as well as the % of cells with MN (Y) versus concentration (X): $Y = -0.0654 X + 3.806$, $r = -0.9229$.

The data concerning the radioprotective effect of propolis, expressed as % of damage reduction in relation with the irradiated sample are shown in Table 2. The radioprotective effect increased considerably as a function of propolis concentration, on CHO-K1 cells irradiated with 1 Gy of ^{60}Co.

Table 1 : Frequencies, distribution of micronucleus (MN) and proliferation index in CHO-K1 binucleated cells (BNC) after different treatments.

Sample	Total of BNC analyzed	BNC with MN		MN/BNC (mean ± SEM)	% BNC with MN (mean ± SEM)	Proliferation index (mean ± SEM)
		1	2			
Basal	1559	17	3	20(0.016±0.003)	23(1.33±0.25)	2.02±0.09
3 μg	1737	17	2	19(0.013±0.025)	21(1.13±0.25)	2.00±0.09
8 μg	1413	10		10(0.006±0.050)	10(0.66±0.50)	1.94±0.15
16 μg	1630	15	2	17(0.012±0.040)	19(1.03±0.32)	1.98±0.04
33 μg	1586	23	5	28(0.014±0.005)	33(1.46±0.50)	1.97±0.12
1 Gy	1638	67	5	72(0.047±0.016)	77(4.36±1.30)	2.02±0.07
3 μg + 1 Gy	1551	47	3	50(0.034±0.005)	53(3.23±0.66)	1.95±0.04
8 μg + 1 Gy	1592	46	2	48(0.031±0.021)	50(3.00±1.90)	1.96±0.07
16 μg + 1 Gy	1558	39	2	41(0.028±0.023)	43(2.66±2.15)	2.00±0.02
33 μg + 1 Gy	1541	26	1	27(0.018±0.008)	28(1.76±0.89)	1.95±0.05

Table 2 : Percentage of damage reduction observed in CHO-K1 bunucleated cells (BNC), after exposure to ^{60}Co, with prior treatment with different concentrations of propolis (μg/mL).

Reduction of damage (%)		
Sample	MN/BNC	BNC with MN
3 μg + 1 Gy	27.6	25.9
8 μg + 1 Gy	34.0	31.2
16 μg + 1 Gy	40.4	39.0
33 μg + 1 Gy	61.7	59.6

4. DISCUSSION

In the present study, the potential radioprotective properties of the ethanolic extract of Brazilian propolis on CHO-K1 cells were investigated. Ionizing radiation is a classic mutagen and the DNA of exposed cells undergoes various types of lesions, among them, single- or double-strand breaks and damage to bases and sugars, ultimately leading to consequences of great biological significance. Genotoxic effects of ionizing radiation are also mediated through formation of free radicals and reactive oxygen species that additionally harm DNA [3]. All these lesions significantly contribute to increased levels of primary DNA damage that could be detected by chromosomal aberrations and

micronucleus assays in proliferating cells. Micronucleus test is amply employed in many biological systems because of its sensitivity, versatility and practicability as a good biomarker of radioinduced damage.

The results obtained indicate a radioprotective effect of Brazilian propolis (Table 2) with a decreased number of micronuclei in irradiated cells (Table 1), a decreased extent of radioinduced damage (number of MN/cell) (Fig. 1A) as well as a lower incidence of damaged cells (% of binucleated cells with MN) (Fig. 1B). They also indicated a dose-dependent radioprotective effect. Apparently, the ethanolic extract of propolis in it self did not cause any genotoxic effect and did not influence the kinectics of cell proliferation.

In spite of the broad spectrum of propolis activities and its recognized effectiveness in many biological systems, the exact mechanisms of protection against radioinduced damage is little known, due to a complex chemical composition and a variety of possible interactions with living matter [2]. Nevertheless, various studies suggest possible immunostimulatory and immunomodulatory activities of propolis/flavonoids, influencing also different endogenous enzyme systems of the cells [8, 22, 23].

Propolis is also well-known for presenting antioxidative properties based on the ability to directly scavenge free radicals or to stabilize reactive oxygen species, thus preventing oxidative damage to DNA [2, 24]. Many analyses have identified at least 200 compounds in propolis extracts, including large number of flavonoids, phenolic acids, esters, enzymes, aromatic aldehydes, terpenes, vitamins and minerals [25]. It is believed that many of the properties of propolis are due to its relatively high flavonoids content, which makes up approximately 25-30% of its dry weigh [10].

Considering that micronuclei originate both from clastogenic and aneugenic events caused by ionizing radiation, the results obtained suggest a radioprotective action of the ethanolic extract of propolis both at the DNA level and mitotic spindle on *in vitro* irradiated CHO-K1 cells.

5. CONCLUSION

The present cytogenetic study realized on *in vitro* irradiated CHO-K1 cells, showed that the ethanolic extract of Brasilian propolis, at the analyzed concentration range, offers a protection against the DNA damage produced by ionizing radiation. The data obtained also indicate a potential promising use of propolis as natural, non-toxic and protective substance. Additional investigations are necessary, however, for a better understanding of the radioprotective mechanism of propolis and of its flavonoids constituents.

REFERENCES

1. E.J. Hall, "Radiobiology for the radiologist", 5th ed. J.B. Lippincott, Philadelphia (2000).
2. 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", *Phytotherapy Research*, **DOI** 10.1002/ptr.2774, (2009).
3. 3 A. Shirazi, G. Ghobadi, M. Ghazi-Khansari, "A radiobiological review on melatonin: a novel radioprotector, *Journal of Radiation Research*, **Vol.48**, pp. 263-272 (2007).
4. B.H. Havsteen, "The biochemistry and medical significance of the flavonoids", *Pharmacology & Therapeutics*, **Vol. 96**, pp. 67-202 (2002).
5. J.A. Ross, C.M. Kasum, "Dietary flavonoids: biovailability, metabolic effects, and safety", *Annual Review of Nutrition*, **Vol.22**, pp. 19-34 (2002).
6. K.N. Rithidech, M. Tungjai, E.B. Whorton, "Protective effect of apigenin radiation-induced chromosomal damage in human lymphocytes", *Mutation Research,* **Vol.585**, pp. 96-104, (2005).
7. N. Orsolic, V.Benkovic, A.Horvat-Knezevic, N. Kopjar, I. Kosalec, M. Bakmaz, Z. Mihaljevic, K. Bendeija, I. Basic, "Assessment of survival analysis of the radioprotective properties of propolis and its polyphenolic compounds", *Biological & Pharmaceutical Bulletin*, **Vol.30 n5**, pp. 946-951 (2007).
8. V.Benkovic, N.Orselic, 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", *Biological & Pharmaceutical Bull*etin, **Vol. 31 n1**, pp.167-172 (2008a).
9. V.Benkovic, N.Kopjar, A.H. Knezevic, D. Dikic, I.Basic, S. Ramic, T. V. F. Knezevic, N. Orsolic, "Evaluation of radioprotective effects of propolis and quercetin on human white blood cells in vitro", *Biological & Pharmaceutical Bull*etin, **Vol. 31 n9**, pp.1778-1785 (2008b).
10. W. Krol, Z. Czuba, S. Scheller, "Anti-oxidant property of ethanolic extract of propolis (EEP) as evaluated by inhibiting the chemiluminescence oxidation of luminol", *Biochemistry International*., **Vol.21**, pp. 593-597 (1990).
11. 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 Taiwenese propolis", *Cancer Letters*, **Vol.245**, pp. 218-231 (2007).
12. A.Daugsch, C.S. Moraes, P. Fort, Y.K. Park, "Brazilian red propolis-chemical composition and botanical origin, *Evidence-Based Complementary and Alternative Medicine, Advance Access*, pp. 1-7 (2007).
13. Y.K. Park, S.M. Alencar, C.L. Aguiar, "Botanical origin and chemical composition of Brazilian propolis", *Journal of Agricultural and Food Chemistry*, **Vol.50**, pp. 2502-2506 (2002).
14. 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", *Journal of Agricultural and Food Chemistry,* **Vol.52**, pp.1100-1103 (2004).
15. 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 propolis extract", *Radiation Protection Dosimetry*, **Vol.225 n1-4**, pp. 461-464 (2005).

16. H. Li, A. Kapur, J.X. Yang, S. Srivastava, D.G. McLeod, J.F. Paredes-Guzman, A. Daugsch, T.K. Park, J. S. Rhim, "Antiproliferation of human prostate cancer cells by ethanolic extracts of Brazilian propolis and its botanical origin", *International Journal of Oncology*, **Vol.31**,pp. 601-606 (2007).
17. M. Fenech, A.A. Morley, "Measurement of micronuclei in lymphocytes", *Mutation. Research*., **Vol.147 n.1-2**, pp. 29-36 (1985).
18. D. Murakami, M.F. Suzuki, M.S. Dias, K. Okazaki, "Genotoxic and cytotoxic effects of ^{60}Co γ-rays and ^{90}Sr/^{90}Y β-rays on Chinese hamster ovary cells (CHO-K1), *Radiation and Environmental Biophysics*, **Vol.43**, pp. 91-99 (2004).
19. International Atomic Energy Agency. Technical Report Series, "Cytogenetics analysis for radiation dose assessment: A manual", IAEA, Vienna (2001).
20. G.L.Erexson, A.D. Kligerman, E.C. Halperin, G.M. Honore, J.W. Allen, "Micronuclei in binucleated lymphocytes of mice following exposure to gamma radiation", *Environmental and Molecular Mutagenesis*, **Vol.13**, pp.128-132 (1989).
21. D.A. Favy, P. Rio, J.C. Maurizis, C. Hizel, Y.J.Bignon, D.J. Bernard-Gallon. "Prolactin-dependent up-regulation of BRCA1 expression in human breast cancer cell lines", *Biochemical Biophysical Research Communications*, **Vol.258 n4**, pp. 284-291 (1999).
22. S.-M. Jeon, S.-H. Bok, M.-K. Jang, Y.-H. Kim, K.-T. Nam, T.-S. Jeong, Y.B.Park, M.-S. Choi, "Comparison of antioxidant effects of naringin and probucol in cholesterol-fed rabbits", *Clinica Chimica Acta*, **Vol. 317**, pp. 181-190 (2002).
23. N. Orsolic, I. Kosalec, I. Basic, "Synerggystic antitumor effect of polyphenolic components of water soluble derivative of propolis against Ehrlich ascites tumour", *Biological & Pharmaceutical Bulletin*, **Vol.28**, pp. 694-700 (2005).
24. 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", *Evidence-Based Complementary and Alternative Medicine*, **Vol.1 n2**, pp.175-185 (2004).
25. L.R. Ribeiro, M.S. Mantovani, D.A. Ribeiro, D.M.F. Salvadori, "Brazilian natural dietary components (annatto, propolis and mushrooms) protecting against mutation and cancer", *Human &. Experimental Toxicology*, **Vol.25 n5**, pp. 267-272 (2006).