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Evaluation of the genotoxicity/mutagenicity and antigenotoxicity/antimutagenicity induced by propolis and *Baccharis dracunculifolia*, by in vitro study with HTC cells

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ABSTRACT

The ethanolic extract of propolis, especially the Brazilian green type, is widely and mainly used for therapeutic purposes despite the lack of knowledge about its effects and its cellular mode of action. This type of propolis, derived from *Baccharis dracunculifolia* (alecrim-do-campo), has been extensively commercialized and the consumers use it to enhance health. This work aimed to assess the genotoxic/mutagenic and antigenotoxic/antimutagenic potentials of the ethanolic extracts of Brazilian green propolis and of *B. dracunculifolia*, on mammalian cells. It was not observed genotoxic and mutagenic effects by both extracts. After evaluate the exposure of the cells to each extract with a recognized mutagen, simultaneously, the results showed a significant reduction on DNA damage. The experiment carried out with a pre-incubation period was more effective than without incubation test, showing that the tested extracts were able to inactivate the mutagen before it could react with the DNA.

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1. Introduction

Propolis is a resinous substance elaborated by honeybees from a variety of plant organs as leaves, buds, gems and floral buttons (Marcucci, 1995), from which they extract secretions, generally, lipophilic materials (Crane, 1988). To this material, bees add enzyme-rich saliva and beewax, using this compound to construct the hive (Marcucci, 1995).

The different types of propolis have variable and complex chemical composition, which is inwardly related to the flora from each region visited by bees. In general, propolis is composed by resins and vegetable oil, wax, essence and aromatic oils, pollen and other substances, including unknown organic fragments (Burdock, 1998).

Until now, approximately, more than 300 components were identified from propolis samples, mainly phenolic compounds. According to Simões et al. (2004), a majority of these compounds belongs to flavonoids, phenolic acids and esters, whose concentration could vary in dependence of the flora collected. Based on this composition, propolis is used by bees as a defensive against pathogenic microorganisms, and also by humans as antibiotics (Bankova, 2005a, 2005b). Nowadays, propolis is used in food and beverages, and in folk medicine in some countries, due to the wide biological properties exerted by it (Banskota et al., 2000).

Some authors report that propolis possesses several biological and pharmacological properties, from antibiotic to antioxidant activities (Salatino et al., 2005; Pereira et al., 2002; Menezes, 2005). Among the

Abbreviations: CBMN, Cytokinesis-blockage micronucleus test; CHO, Chinese hamster ovary cells; EEBD, Ethanolic extract of *Baccharis dracunculifolia*; EEGP, Ethanolic extract of green propolis; FBS, Foetal bovine serum; HTC, Hepatoma tissue culture cells; MMS, Methyl methanesulfonate; MN, Micronucleus; MNPCE, Micronucleated polychromatic erythrocytes; NC, Negative control; PBS, Phosphate-buffer solution; PC, Positive control; SC, Solvent control; SD, Standard deviation; TLC, Thin layer chromatography; TSS, Total soluble solids.

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biological activities exerted by propolis, the antimutagenic action is poorly known. Varanda et al. (1999), by tests performed with *Salmonella typhimurium*, observed that propolis induced a significant reduction on the damages caused by daunomycin, benzo[a]pirene and aflatoxin B1. According to these authors, the antimutagenic activity is directly related to the antioxidant mechanism.

According to Bankova et al. (2000), the chemical composition of propolis from tropical and temperate regions is different. Samples from temperate regions have a chemical profile mainly based on flavonoids, while samples from tropical regions have other compounds, like diterpenes, prenylated compounds, lignans, and other phenolic compounds, as phenolic acids. The tropical propolis still possesses inorganic elements, like Cu, Mn, Fe, Ca, Al, Si and V (Marcucci, 1996). Also, some genetic factors of bees can alter the composition of propolis (Park et al., 1998).

After verifying the high relationship between propolis chemical variability and the botanical source used by bees, several researchers are trying to identify the main botanical source to each type of propolis. Park et al. (2000), for example, have classified Brazilian propolis samples among 12 different types, according to their chemical profiles and collection regions.

The Brazilian green propolis is produced by bees located at the south of the State of Minas Gerais and at the north of the State of São Paulo, identified as the number 12 of the classification (Park et al., 2000; Park et al., 2004). This type of propolis has the exudate of leaves from *Baccharis dracunculifolia* DC (Asteraceae) species as the botanical source, commonly known as "alecrim-do-campo" (Kumazawa et al., 2003; Park et al., 2004; Teixeira et al., 2005).

The aim of this work was to evaluate the genotoxic/mutagenic and also the antigenotoxic/antimutagenic potentials of ethanolic extracts of Brazilian green propolis (EEGP) and of *B. dracunculifolia* (EEBD). In order to assess these properties, were performed, in HTC cell culture, the comet assay and the micronucleus test with cytokinesis blockage.

2. Material and methods

2.1. Samples of propolis and B. dracunculifolia DC (Asteraceae)

The samples of crude Brazilian green propolis and of young leaves and buds of *B. dracunculifolia* (alecrim-do-campo) were gently donated by the Centro de Estudos de Insetos Sociais (CEIS-UNESP), which were collected in the summer of 2007 (warm and wet season), in the municipality of Carvalhópolis (21°46′42″S/45°50′30″W), in the south of the State of Minas Gerais, Brazil. The samples of crude Brazilian green propolis were characterized as solid-flexible material, with a brown-greenish coloration, and a pleasing odour. The samples of alecrim-do-campo were based on young leaves and buds collected from adult plants, located near from the beehives. All samples were conditioned in a closed recipient covered by an aluminium foil until processing.

2.2. Preparation of the ethanolic extracts

To prepare the ethanolic extract of Brazilian green propolis (EEGP), 30 g of crude propolis was macerated and ethanol 70% was added until the volume of 100 mL was complete. After, this mixture was transferred to a flat-bottomed flask, which was submitted to a procedure of hot extraction under simple reflux for 6 h. Next, the extract was filtered while still warm, chilled, then transferred to a 100 mL graduated cylinder to decant the wax in suspension. After this, the extract was filtered again to remove the wax, and the volume of 100 mL was completed with ethanol 70%.

In order to prepare the extract of alecrim-do-campo (EEBD), 30 g of dried young leaves and buds was macerated and used in the extraction procedure, which was the same used for propolis.

According to Burdock (1998), the ethanol 70% is the most used solvent in the preparation of propolis extracts consumed by humans. According to Sforcin and Bankova (2011), ethanol is the most used solvent due its great extraction capacity.

2.3. Phytochemical characterization

The extract of Brazilian green propolis and the extract of alecrim-do-campo were chemically analysed, regarding total flavonoids, flavonoids quality, phenolic compounds and total soluble solids.

To assess the total flavonoids composition, the methodology described by Woisky and Salatino (1998) was used, which is based on the standard of quercetin, with slight modifications. This verification was performed using a test tube with 5 mL of ethanol P.A. and 5 μL of each sample, separately, and 100 μL of the $AlCl_3$ reagent, at 2% methanol concentration. The solution passed through a fast agitation and a rest period of 30 min, protected from light. Then, the solutions were read by a spectrophotometer at $\lambda = 425$ nm.

In addition to this verification, chromatographic profiles were determined by a thin-layer chromatography (TLC). The TLC was performed to identify which type of simple phenolic compounds and flavonoids were present in both ethanolic extracts (Wagner and Bladt, 2001).

To determine the total of soluble solids (TSS) present in the ethanolic extracts, an Abbe's refractometer was used.

2.4. Culture of mammal cells (HTC)

To evaluate the biological effects of the extracts, the rat hepatoma cell line (HTC) was used, kindly provided by Dr. Mário Sérgio Mantovani (Univ. Estadual de Londrina, Paraná, Brazil). The HTC cell line expresses phase I and phase II drug metabolizing enzymes and is recommended to assess drug effects and metabolism. The HTC cells were cultured in 5 mL of D-MEM/F-12 medium (Cultilab, Campinas, Brazil) supplemented with 10% of foetal bovine serum (FBS – Gibco®), and were maintained in flasks with 25 cm^2, with a starting concentration of 1×10^6 cells per flask, in an incubator at 37 °C, for 24 h.

The experiments were followed by a negative control (NC) and a positive control (PC). In the NC, the cells received 50 μL of phosphate-buffer solution (PBS), while in the PC the cells received 50 μL of a methyl methanesulfonate (MMS, CAS No 66-27-3, Sigma-Aldrich) (stock solution: 3.33 μL of MMS in 1 mL of PBS; 4×10^{-2} M after adding 50 μL into the culture flasks containing 5 mL of medium). Thus, a solvent control (SC) was used to certify the lack of genotoxicity/mutagenicity by the ethanol present in the extracts, as stated by Phillips and Jenkinson (2001). In this SC, 50 μL of ethanol 70% solution was added into the flasks, at the same concentration of the extracts (0.042 μL/mL after the dilution in 5 mL of culture medium).

To assess the genotoxicity/antigenotoxicity and the mutagenicity/antimutagenicity of the extracts, the protocol of exposure described by Oliveira et al. (2006) and Oliveira et al. (2007) was used.

Before the exposure, the crude extracts were diluted in serum-free medium, and then it was filtered using a syringe membrane with pores of 22 μm of diameter, to avoid contamination by microorganisms. The concentration of the extracts used in this study was chosen based on the results obtained in the work of Roberto et al. (in press), which tested the cellular protective effects of these extracts over the *Allium cepa* test-system.

In order to evaluate the possible genotoxic and mutagenic effects induced by the extracts, a first test was performed with the exposure time of 24 h (an entire cell cycle for HTC cells). Both extracts were used at the same concentration (stock solution: 6.06 μL of pure extract in 1 mL of medium; 0.06 μL/mL after adding 50 μL into the culture flasks already containing 5 mL of medium), in triplicate. It was chosen to test the same concentration of the extracts (Brazilian green propolis and *B. dracunculifolia*), independently of their flavonoid's content, to check if the DNA protection was associated with the flavonoids or with the total combination of chemical compounds of each extract considered.

The antigenotoxicity and antimutagenicity tests were based on simultaneous exposure, where the cells received a mixture of the ethanolic extracts with MMS, at a 1:1 proportion. In order to obtain the same concentration used in the genotoxicity/mutagenicity assay, the stock solutions for both extracts and for the MMS were prepared twice more concentrated (12.12 μL of pure extract in 1 mL of medium and 6.66 μL of MMS in 1 mL of medium, respectively). In the moment of exposure, the addition of 25 μL of each stock solution (extract and MMS) in 5 mL of medium of each culture flask allowed a proper dilution and the concentrations obtained were 0.06 μL/mL and 4×10^{-2} M, respectively. Then, two forms of exposure were done in the experiment: 1) with a pre-incubation period of the mixture for 1 h, at 37 °C in an incubator, followed by the exposure period of 3 h; and 2) without this pre-incubation time, in which the mixture was made in the moment of starting the exposure of 3 h. In order to evaluate the protective properties of the extracts, 50 μL of each mixture was added into the culture flasks with D-MEM/F-12 medium. The assay was developed in triplicate, by using three different and independent flasks to each treatment, being this procedure very common on genotoxicity/mutagenicity evaluation with cell culture and validated by several authors (Oliveira et al., 2006, 2007; Scolastici et al., 2008; Hoshina et al., 2013; Mazzeo et al., 2013; Hoshina and Marin-Morales, 2014; Bianchi et al., 2015; Araújo et al., 2015).

2.5. Comet assay

The comet assay was performed according to the protocol described by Tice et al. (2000), with some modifications.

All exposures were done at the same conditions, as elucidated in item Section 2.4. After the treatment, the cells were released from the wall of the flasks, using 500 μL of Trypsin-EDTA (0.025%). After this, the process was interrupted by adding 5 mL of complete medium. The cells were transferred to centrifuge tubes and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and only 0.5 mL of this solution was reserved, in which the cells were re-suspended.

To continue the comet assay, cellular viability tests were performed for all treatments. Thus, 20 μL of cell suspension of each treatment replica was mixed with 20 μL of Trypan Blue 0.4% (Gibco®) and transferred to a Neubauer chamber. The number of viable cells (non-stained) and the number of nonviable cells (blue stained) were counted in all four quadrants, and those results were used to calculate the percentage of cellular viability, by which the minimum limit was defined as 80% to proceed with the experiment.

The slides covered by normal agarose have received 20 μL of cell suspension mixed with 120 μL of low melting point agarose, at 37 °C. After, a coverslip was put over the material to spread it on the slide, and then they were placed into a refrigerator, at 4 °C for 15 min. After the solidification of the gel, the coverslips were removed and the slides were transferred to cuvettes with cold lysis solution (1 mL Triton X-100, 10 mL of DMSO and 89 mL of lysis stock solution — NaCl 2.5 M, EDTA 100 mM, Tris 10 mM and ~8.0 g of NaOH, pH 10.0), maintained at 4 °C, in the dark, for 1 h. After lysis, the slides were displaced into an electrophoresis vat and covered with alkaline buffer (NaOH 300 mM + EDTA 1 mM, pH >13.0), for 20 min for stabilization. In sequence, the electrophoresis started at 39 V (1.0 V/cm) and 300 mA, for 20 min. After electrophoresis, the slides were neutralized in Tris buffer (0.4 M Trizma Hydrochloride, pH = 7.5) and fixed for 10 min in absolute ethanol. To preserve the slides, they were maintained at 4 °C until the analysis.

To analyse the material, the slides were stained with 50 μL of GelRed® solution (15 μL of GelRed® 10,000× in water, 5 mL of NaCl at 1 M, and 45 mL of distilled water) and immediately analysed. The analysis was performed under an epifluorescence microscope (Olympus), filter B–34 (excitation: λ = 420–490 nm, barrier: λ = 520 nm), at 400× magnification. The nucleoids were visually classified in classes 0, 1, 2 and 3, according to fragments migration, as follows: class 0, no tail; class 1, small tail with size smaller than the head diameter; class 2, medium tail with size equal to or up to two times larger than the head diameter; and class 3, large tail with size two times larger than the head diameter (Rigonato et al., 2005). Examples of the nucleoids are presented in Fig. 1.

For each sample, a total of 300 nucleoids were analysed (50 comets/slide, 2 slides/flask, 3 flasks/treatment) and the total score was calculated. To obtain the score, the number of nucleoids in each class was multiplied by the class damage, according to the formula: total score = $(0 \times n_1) + (1 \times n_2) + (2 \times n_3) + (3 \times n_3)$, where n is the number of nucleoids in each class analysed. Therefore, the total score has a minimum of 0 and a maximum of 300. After verifying the normality distribution by D'Agostino & Pearson test ($p < 0.05$), the most suitable statistical comparison test was chosen. The statistical analysis was performed by the Kruskal–Wallis nonparametric test, followed by the Dunn's comparison test ($p < 0.05$), for the genotoxicity experiment, and by the One-Way ANOVA parametric test, followed by the Holm–Šídák's comparison test ($p < 0.05$), for the antigenotoxicity experiment.

2.6. Cytokinesis-blockage micronucleus test (CBMN)

The cytokinesis-blockage micronucleus test (CBMN) was performed according to Natarajan and Darroudi (1991), with some modifications. After the cells exposure by the several treatments proposed, the culture medium was discharged and the cells were washed with 5 mL of PBS, twice. The excess of PBS was removed and 5 mL of fresh medium with FBS + 50 μL of cytochalasin B (3 μg/mL — Sigma Aldrich). The culture flasks were maintained in the incubator for 30 h, to certify that the cells could complete another cell cycle of 24 h.

To collect the cells, the medium was transferred to centrifuge tubes and reserved. Next, the cells were washed again by two consecutive baths with 5 mL of PBS and released from the flask walls using 500 μL of Trypsin-EDTA (0.025%). After this, the trypsin was neutralized by adding the medium previously reserved, and the flasks content was homogenised and returned to the centrifuge tubes, with a drop of 40% formaldehyde. Then, the cellular suspension was centrifuged at 1000 rpm for 5 min, and the supernatant was discarded, leaving only 1.5 mL of the medium, in which was added another 1.5 mL of hypotonic solution (sodium citrate 1%). The pellet was re-suspended again and centrifuged as earlier. At the end, all of the content was discarded and 5 mL of methanol-acetic acid solution (3:1) was added to fix the cells. After another re-suspension of the cells, all tubes were stored at 4 °C. Prior to analysis, the slides were stained with Giemsa 5% for 8 min.

For the analysis of micronuclei frequency, a total of 6000 binucleated cells per treatment (1000 cells/slide, 2 slides/flask, 3 flasks/treatment) were counted, using a microscope objective of 100× of magnification and following the criteria established by Fenech (2000). After performing the D'Agostino & Pearson normality test ($p < 0.05$), the significance analysis of the results was verified by the Kruskal–Wallis nonparametric test, followed by the Dunn's comparison test ($p < 0.05$).

2.7. Determination of the antigenotoxic and antimutagenic effect

The antigenotoxic and antimutagenic activities were assessed by the analysis of the reduction of damages induced by each treatment conducted with the ethanolic extracts. To calculate the percentage of reduction, the following formula was used: reduction (%) = $[(a - b) / (a - c)] \times 100$ (where: a = number of damages induced by the PC; b = number of damages induced by each treatment; c = number of damages induced by the NC).

To assess the antigenotoxicity, the total score was used in the formula, while to assess the antimutagenicity the number of micronuclei was used.

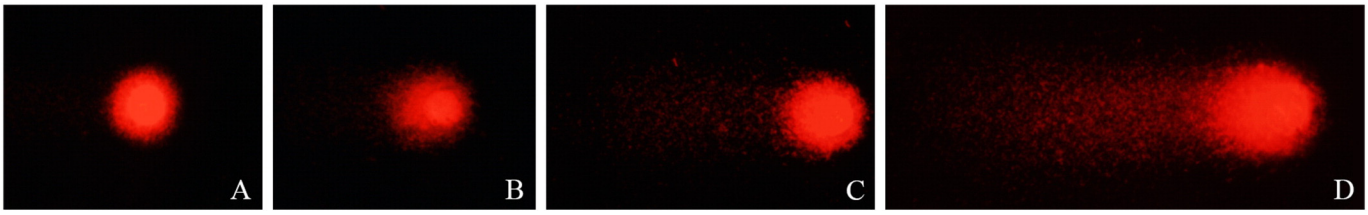


Fig. 1. Nucleoids examples according to Rigonato et al. (2005) classification. A: class 0; B: class 1; C: class 2; D: class 3.

3. Results

3.1. Phytochemical characterization

The analysis of total flavonoids showed the EEGP as a rich-flavonoid extract, with 3.65% (w/w) of its content, as the EEBD showed a lower concentration of flavonoids, with only 0.67% (w/w). On the other hand, the analysis made by TLC showed a small difference in their composition, both presented ferulic acid, caffeic acid, rutin and quercetin, but only the EEBD showed kaempferol as an exclusive substance.

The total soluble solids demonstrated a high quantity of solids on the EEGP, with 35°Bx, while the EEBD showed a lower concentration of solids, 25°Bx. The Ministério da Agricultura e Abastecimento from Brazil states a minimum of 35°Bx of total soluble solids for the ethanolic extract of propolis (Brasil, 2001). Thus, the EEGP used in this work respects the law and this verification shows the equality of it when compared to a commercial extract.

3.2. Comet assay

The analysis of cell viability, prior the comet assay, showed numbers higher than 98%, as shown in Table 1. These results showed that the cells presented the required viability to perform the comet assay, being the concentration of the EEGP and the EEBD not cytotoxic to HTC cells.

Based on the results presented in Table 1, only the PC showed genotoxic potential, as expected, because the MMS is a clastogenic agent. Neither the SC nor the ethanolic extracts showed genotoxicity, which shows that both did not induced DNA damages on the HTC cells after 24 h exposure. Regarding the antigenotoxicity experiment, only the PC showed statistically significant results. The ethanolic extracts protected the cells and reduced the DNA damage promoted by MMS, as follows: EEGP (59.91%), EEGP incubated (62.39%), EEBD (65.32%) and EEBD incubated (57.43%).

3.3. Cytokinesis-blockage micronucleus test (CBMN)

After the treatments with the ethanolic extracts, HTC binucleated cells were counted. The results of micronuclei frequency, variation, average and standard deviation, and the percentage of reduction of DNA damages are presented in Table 2.

The statistical analyses showed that the SC and the ethanolic extracts did not induced mutagenicity after 24 h exposure. The antimutagenicity experiment showed only the PC and the EEGP + MMS statistically different in relation to the NC. Besides EEGP + MMS showed a significant result, it still showed the lowest reduction on the DNA damage promoted by the MMS. On the other hand, when the exposure was done with the mixture EEGP + MMS incubated, the percentage raised to 86.67% of reduction. For the EEBD, the mixture EEBD + MMS showed a reduction of 61.67%, while the same mixture, but incubated, also showed a higher reduction, of 85.00%.

4. Discussion

The genotoxic, mutagenic, antigenotoxic and antimutagenic potentials of two ethanolic extracts were assessed in the present work. To evaluate these effects, the HTC cell line was used as a bioindicator to perform the cytokinesis-blockage micronucleus test and the comet assay. The ethanolic extract of Brazilian green propolis (EEGP) and the extract of its main botanical source, *B. dracunculifolia* or alecrim-do-campo (EEBD), showed a high ability to protect the cells against the clastogenicity induced by methyl methanesulfonate.

Only one concentration was tested based on a preliminary work performed by our research group. The work done by Roberto et al. (in press) tested other concentrations of both extracts over *Allium cepa* test system and the most effective concentration was chosen for tests with HTC cells. In this study the same concentration of the ethanolic extract of propolis and of *B. dracunculifolia* was used, even knowing about the difference on flavonoids content between them. This method was

Table 1
Cellular viability, distribution of comets among the different classes of damages and total score obtained by comet assay with HTC cells.

	Treatment	Cellular viability (%)	Classes				Total score	Reduction (%)
			0	1	2	3		
Genotoxicity	NC	99.32 ± 0.07	80.00 ± 1.00	18.33 ± 0.58	1.67 ± 0.58	0.00 ± 0.00	21.67 ± 1.53	–
	PC	99.56 ± 0.17	2.00 ± 2.00	68.00 ± 12.12	28.33 ± 10.21	1.67 ± 1.53	**129.67 ± 12.50***	–
	SC	98.77 ± 0.29	83.00 ± 7.21	15.33 ± 5.51	1.33 ± 2.31	0.33 ± 0.58	19.00 ± 10.00	–
	EEGP	98.27 ± 0.34	74.67 ± 4.51	23.33 ± 4.51	2.00 ± 0.00	0.00 ± 0.00	27.33 ± 4.51	–
	EEBD	99.00 ± 0.36	75.33 ± 8.14	23.67 ± 6.43	1.00 ± 1.73	0.00 ± 0.00	25.67 ± 9.87	–
Antigenotoxicity	NC	99.43 ± 0.24	78.00 ± 1.00	22.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00	22.00 ± 1.00	–
	PC	95.73 ± 5.61	2.00 ± 2.65	37.67 ± 30.44	48.67 ± 29.37	11.67 ± 4.73	**170.00 ± 39.66**+	–
	EEGP + MMS	98.74 ± 0.70	21.67 ± 24.01	75.33 ± 21.08	3.00 ± 4.36	0.00 ± 0.00	81.33 ± 27.32	59.91%
	EEGP + MMS (Inc.)	98.55 ± 1.14	24.00 ± 14.42	74.33 ± 13.61	1.67 ± 1.15	0.00 ± 0.00	77.67 ± 15.28	62.39%
	EEBD + MMS	98.61 ± 0.67	29.67 ± 16.92	67.33 ± 13.05	3.00 ± 4.36	0.00 ± 0.00	73.33 ± 20.98	65.32%
	EEBD + MMS (Inc.)	99.42 ± 0.28	22.00 ± 14.80	71.33 ± 6.81	6.33 ± 7.51	0.33 ± 0.58	85.00 ± 23.43	57.43%

NC: negative control; PC: positive control; SC: solvent control; EEGP: ethanolic extract of Brazilian green propolis; EEBD: ethanolic extract of *Baccharis dracunculifolia*; Inc.: Incubated. Class 0: no damage; Class 1: small damage; Class 2: medium damage; Class 3: high damage. 300 nucleoids were analysed (50 comets/slide, 2 slides/flask, 3 flasks/treatment).

* statistically significant when compared to the negative control (Kruskal–Wallis/Dunn, $p < 0.05$);

\+ Statistically significant when compared to the negative control (ANOVA One-Way/Holm–Šídák, $p < 0.05$).

Table 2
Frequency of micronuclei, variation, average, standard deviation and percentage of reduction obtained by CBMN with HTC binucleated cells.

	Treatment		MN and SD frequencies (%)	Variation*	Average and SD	Reduction (%)
Mutagenicity	NC	24 h	0.28 ± 0.08	2–4	2.83 ± 0.75	–
	PC		**1.65 ± 0.36***	12–21	16.50 ± 3.62	–
	SC		0.33 ± 0.12	2–5	3.50 ± 1.22	–
	EEGP		0.28 ± 0.10	1–4	2.83 ± 1.47	–
	EEBD		0.32 ± 0.12	2–5	3.17 ± 1.17	–
Antimutagenicity	NC	3 h	0.25 ± 0.24	0–5	2.50 ± 2.43	–
	PC		**1.25 ± 0.23***	10–17	12.50 ± 2.35	–
	EEGP + MMS		**0.73 ± 0.12***	6–9	7.33 ± 1.21	51.67%
	EEGP + MMS (Inc.)		0.38 ± 0.13	3–6	3.83 ± 1.33	86.67%
	EEBD + MMS		0.63 ± 0.10	5–8	6.33 ± 1.03	61.67%
	EEBD + MMS (Inc.)		0.40 ± 0.14	2–5	4.00 ± 1.41	85.00%

MN: micronucleus; NC: negative control; PC: positive control; SC: solvent control; EEGP: ethanolic extracts of Brazilian green propolis; EEBD: ethanolic extract of *Baccharis dracunculifolia*; Inc.: incubated; MMS: methyl methanesulfonate (4×10^{-2} M); SD: standard deviation.

★ Minimum and maximum of MN observed in 6000 binucleated cells (1000 cells/slide, 2 slides/flask, 3 flasks/treatment).

* Statistically significant when compared to the negative control (Kruskal–Wallis/Dunn, $p < 0.05$).

chosen to evaluate if only the flavonoids could exert protective activity or if the total combination of chemical compounds in each extract is considered.

The exposure performed by treating the HTC cells only with the ethanolic extracts (concentration of 0.06 μL/mL) was important to certify the absence of genotoxicity and mutagenicity induced by these compounds. None of the extracts were able to induce statistically significant fragmentation of the DNA or significant frequencies of MN. The results obtained by comet assay with HTC cells regarding the genotoxicity of the extracts, are in agreement with the work of Munari et al. (2010), which showed the lack of genotoxic potential by ethyl acetate extracts of alecrim-do-campo, in V79 cells, using the same investigation technique. Rodrigues et al. (2009) also tested the genotoxicity and the mutagenicity of an aqueous extract of alecrim-do-campo on female mice (CF-1). These authors checked that the extract did not induce DNA damages on blood and hepatic cells when used in low concentration. Regarding the extract of Brazilian green propolis, our results did not show genotoxic potential, being similar to the result obtained by Tavares et al. (2006) using CHO (Chinese hamster ovary cells) cell line.

Regarding the mutagenic activity, our results corroborate the data obtained by Tavares et al. (2006), which also indicated the absence of mutagenicity by the extract of propolis when used at low concentrations over CHO cells, for 3 h. Our work was based on a concentration similar to those recommended by producers of commercial extracts of propolis, which was lower than the lowest concentration used by Tavares et al. (2006). Thus, the results about the mutagenicity of EEBD also confirm the results obtained by Resende et al. (2007), which did not verify significant frequencies of micronucleated polychromatic erythrocytes (MNPCEs) on Wistar rats treated with ethyl acetate extract of alecrim-do-campo, for 24 and 48 h.

Our results showed both extracts as antigenotoxic and antimutagenic compounds. Besides a slight raise on the scores of comets after the treatments performed, none of them was statistically significant when compared to the NC. The formula applied to verify the reduction of DNA damage showed that all treatments were able to reduce the clastogenicity induced by the MMS. For the EEGP + MMS treatment, the pre-incubated mixture was more effective in reducing the damage (62.39% for incubated vs 59.91% for non-incubated), while for the EEBD + MMS treatment, the non-incubated mixture had higher efficacy (57.43% for incubated vs 65.32% for non-incubated). Munari et al. (2010) also verified the antigenotoxic potential of the extract prepared with alecrim-do-campo, mainly for the lowest concentration used at the simultaneous experiment executed in their work, which reduced 64.00% of the MMS-induced DNA damages. These authors did not perform the incubated procedure. Tavares et al. (2006) suggested an antigenotoxic potential for the ethanolic extract of Brazilian green propolis against the oxidative damages induced by doxorubicin, a well-studied chemotherapy drug. They have shown that the reduction of damage was more obvious for the lowest concentration, also by carrying out an experiment with simultaneous treatment (64.28% of reduction).

The results of micronucleus analysis were more consistent, in which the incubated mixtures were more effective when compared to the non-incubated mixtures. The EEGP + MMS treatment, without incubation, was not effective in reducing the mutagenicity induced by MMS, but when compared to the PC, it still showed some decrease of damages (51.67%). This same mixture presented a higher rate of reduction (86.67%) when submitted to a pre-incubation period. About the alecrim-do-campo extract, the EEBD + MMS treatment without previous incubation showed a reduction of 61.67%, i.e. more effective if compared to the EEGP + MMS treatment (also non-incubated). The EEBD + MMS treatment (incubated) had a better effectiveness (85.00%) when compared to the non-incubated mixture, but with results similar to those found for EEGP + MMS treatment (incubated).

Based on the results obtained by the CBMN test and supported by the improved effectiveness exhibited by those mixtures submitted to the pre-incubation period, we can suggest the EEGP and the EEBD as compounds capable to inactivate almost all clastogenicity exerted by the alkylating agent and, consequently, reducing the frequency of MN on HTC cells. It is possible that these compounds start to neutralize the MMS action before it could reacts with the DNA, avoiding the cell damages.

Nevertheless, it was observed that even without the pre-incubation period the extracts reduced the MN frequency. Possibly, the other factor responsible for this difference was the time of interaction of the MMS with each extract, as the incubation provided one extra hour (1 h of incubation + 3 h of exposure, while the other had only 3 h of exposure).

Varanda et al. (1999) used the *Salmonella typhimurium* assay to test the ethanolic extract of propolis and observed an antimutagenic activity. The ethanolic extract of propolis was also studied by Jeng et al. (2000), and the authors showed the extract as capable to inhibit the mutagenicity of four mutagens by the *S. typhimurium* assay, with liver S9 mix. This study showed that the ethanolic extract of propolis did not induce cytotoxicity and mutagenicity, when tested in TA98 and TA100 strains of *S. typhimurium*. Resende et al. (2007) have proved the efficacy of the ethanolic extract of *B. dracunculifolia* in order to reduce the MN frequency in rats. These authors related the observed effects to the presence of phenolic compounds in the extract.

Nonetheless, in the work of Bazo et al. (2002) the authors used the comet assay in blood cells of rats treated with ethanolic extract of propolis, with pre, simultaneous and post administration. The consumption of the extract was not enough to reduce the 1,2 dimethylhydrazine-induced DNA damage in the pre- and simultaneous exposures. Despite the effects obtained, when it was given to the rats after the carcinogen injection, the researchers observed a significant reduction in the number of aberrant crypt foci in the colon of the rats.

It is known that propolis is a phenolic compounds-rich substance, due to the presence of these molecules in the plants used by bees as raw material. Among the most common phenolic compounds found in propolis, the majority is flavonoids (Park et al., 2002). Therefore, several researchers have been trying to establish a relationship between the biological activity promoted by propolis samples and its content of phenolic compounds. Rao et al. (1992) verified the relationship between some biological properties exerted by propolis and the presence of caffeic acid esters in its composition. These authors tested the mutagenicity of this class of substances and they did not detect a damage induction for *S. typhimurium*. On the other hand, the researchers observed an antimutagenic effect, as the esters were able to neutralize the effects of a mutagen when kept in contact with it. According to Christov et al. (2006), the caffeic acid is well known as an antioxidant, used as a positive control in techniques designed to evaluate the activity of free radical scavengers. Furthermore, these researchers confirmed the antioxidant activity of propolis samples and they related such activity with the presence of phenolic compounds and other aromatic acids, e.g. ferulic acid. In the samples used in the present work was identified the presence of caffeic and ferulic acids, further the presence of other three flavonoids, which could represent a possible antioxidant activity by EEGP and EEBD.

Choi et al. (1994) evaluated the properties of flavonoids and concluded that could exist an activity difference among the diverse classes of flavonoids, but, in general, all of them were effective to reduce the mutagenicity induced by aflatoxin B1. Beudot et al. (1998) also showed the mutagenic and antimutagenic effects of some flavonoids against different mutagens.

Several authors report the antigenotoxic and antimutagenic properties exhibited by extracts of propolis as a consequence of their antioxidant potential. Park et al. (2005) demonstrated the antioxidant effects of ethanolic extracts of Brazilian green propolis and of *B. dracunculifolia*, using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. The authors concluded that both extracts possess antioxidant activity, but propolis extract had a little higher effectiveness when compared with the plant extract. According to the authors, this variation might be the result of the difference in the content of phenolic compounds from the extracts.

The flavonoids were reported by Scheller et al. (1990) as the most abundant and effective antioxidant substances present in ethanolic extracts of propolis. As our chemical characterization showed EEGP and EEBD chemically similar, as already confirmed by other studies (Park et al., 2002), we believe that the EEBD should be capable to promote an antioxidant effect similar to the effect of EEGP, i.e. the antimutagenic effects of both extracts could be related with the capacity of accepting free radicals.

De Flora (1998) suggests the antimutagenic activity of flavonoids mainly as a result of their ability to scavenge free radicals, which is one of the most important mechanisms of antimutagenesis and anticarcinogenesis. Others, as Russo et al. (2006), demonstrated the antimutagenicity of propolis and suggested the DNA protective effect as a consequence, at least in part, of the antioxidant properties of its main components.

Banskota et al. (2000) showed that the aqueous extract of Brazilian propolis was capable to exert an expressive antioxidant activity. The authors associated this effect due the presence of flavonoids and cinnamic acids. At the same context, Nieva Moreno et al. (2005) indicated the Argentinian propolis as a powerful antioxidant. The authors considered substantial for their results the relationship between its flavonoids content and the antioxidant activity, but they emphasized that other factors, still unknown, could be involved in this property.

5. Conclusion

The results obtained in this work verified the absence of genotoxicity and mutagenicity for ethanolic extracts of Brazilian green propolis and of *B. dracunculifolia* (alecrim-do-campo). Further, a protective effect was observed by both extracts against the MMS-induced DNA damages over HTC cells. The MMS is an alkylating agent that possesses a direct action and, probably, induces damages to the DNA by inserting methyl groups on the nitrogenous bases, e.g. by the DNA methylation. Thereby, as free radicals scavengers, the phenolic compounds and the flavonoids present in the extracts tested were able to react with the MMS and capture the free radicals that could damage the DNA. This process may have occurred during the incubation period of the extracts mixed with MMS, which suggests that the simple simultaneous exposure seems to not have been enough to promote a better scavenging of the free radicals before it reacts with the DNA strands.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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