Evaluation of the antioxidant activity and mutagenicity of Brazil nut (*Bertholletia excelsa* Bonpl.).

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Abstract

This study evaluated the antioxidant capacity and the genotoxic and antigenotoxic effects of the fixed oil of B. excelsa Bonpl. in peripheral blood of Swiss mice. For the antioxidant capacity, were used the β-carotene/linoleic acid and DPPH methods. In the genotoxicity assay, were used healthy, 6-7 week old male Swiss mice, and there were six animals per group. For the genotoxic test, animals were treated with different concentrations of B. excelsa (500, 1.000 and 2.000 mg/kg body weight bw) in 0.5 mL orally. For the antigenotoxic test, animals were treated with predetermined concentrations, followed by intraperitoneal injection of doxorubicin (DXR 15 mg/kg bw) in 0.3 ml, in addition to the negative group (water) and dimethylsulfoxide (200 µL). Peripheral blood samples were collected 24 and 48 hours after the treatments. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) was obtained from

the analysis of 2.000 MNPCEs/animal. After data analysis, the conclusion was that the fixed oil of B. excelsa showed excellent protective activity by the β-carotene/linoleic acid method, demonstrated absence of genotoxic effect, and significant antigenotoxic effect according to the protocols and treatments performed in this study.

Keywords: Mutagenesis; Micronucleus test; *Bertholletia excelsa*; DPPH; β-carotene/linoleic acid method.

1. INTRODUCTION

The use of bioderivatives started thousands of years ago by populations from several countries in order to treat various diseases. Populations used these substances as an alternative or complementary form to synthetic drugs (Veiga-Junior & Mello, 2008) through popular knowledge transmitted from generation to generation over time.

Brazil is known for its rich biodiversity and for having the largest part of the Amazon rainforest that also includes nine other Latin American countries (Da Silva et al., 2016). The forest has a wide variety of plant species that have not been fully studied yet. Nuts are energy-rich foods, mainly due to their high content of proteins (15%), carbohydrates (9%) and lipids (71%) (Yang, 2009; Da Costa et al., 2010; Stockler-Pinto et al.,2015; Cardoso et al., 2017), and this has attracted researchers' attention regarding their chemical composition and use as functional foods. Among nuts, *Bertholletia excelsa* Bonpl. 1807 is a native Amazonian species of the family Lecythidaceae. It is popularly known as Brazil nut, and distributed geographically in countries such as Venezuela, Bolivia, Peru, Colombia, Guianas and Brazil, in Brazilian states of Maranhão, Mato Grosso, Pará, Acre, Rondônia, Amapá, Roraima and Amazonas (Pacheco and Scussel 2006, 2011; Shepard and Ramirez, 2011; Manfio et al., 2012a, 2012b). Harvest of the Brazil nut is one of the main sources of income for Amazonian riverside families, besides moving the international market with its export (Ribeiro et al., 2014; IndexBox, 2016).

The chemical composition of the Brazil nut includes considerable amounts of fibers, folate, vitamin E, vitamin B6, calcium, iron, potassium, zinc, copper, arginine, flavonoids and minerals that can act as antioxidants (Chunhieng et al., 2004; Kornsteiner et al., 2006; Ros et al., 2010). Selenium is one of its minerals (Se, its safe intake range is 50 to 400 μg/day for adults) (Roman et al., 2014), and it was considered toxic, but is now classified as an essential element by its antioxidant action of preventing the body from diseases related to age, such as cancer and cardiovascular diseases, as well as risk factors related to oxidative stress and diabetes (Risher, 2003; Lopez-Uriarte et al., 2009; Ros, 2015; Stockler-Pinto et al., 2015; Mozaffarian, 2016; Asghari et al., 2017).

Natural products have been in evidence for being a natural source of bioactive molecules. Their broad list of biological properties benefits humans because of their biocompatibility and easy metabolization by the body. However, toxicological studies are needed to support the use of these products, because they are a natural source with excellent preventive activity of oxidative processes in the metabolism. In view of the aforementioned biological properties, the aim of the present study was to evaluate the antioxidant capacity and the genotoxic and antigenotoxic effects of the fixed oil of *Bertholletia excelsa* Bonpl. (Brazil nut).

2. MATERIAL AND METHODS

2.1 Fixed oil of Bertholletia excelsa

The fixed oil of *Bertholletia excelsa* was kindly provided by the Laboratory of the Nucleus of Food Science and Technology (Portuguese acronym: NUCTECNAL) of the Institute of Scientific and Technological Research of the State of Amapá (Portuguese acronym: IEPA), where the nuts were manually broken and their exposed almonds were later dehydrated in a laboratory stove (Quimis®) at 50°C for ten hours for processing of the fixed oil. The almonds were pressed to exhaustion in a 72 hours period by using a hydraulic press (Marconi®). The fixed oil was stored in an amber bottle at 4°C for subsequent analyzes.

2.2 Preparation of extracts for antioxidants assays

This method was adapted from the technique described by Larrauri et al., (1997). For preparation of the extracts, were weighed 5g of the oil in a 100 mL beaker, 20 mL of 50% methanol were added, homogenized and allowed to stand for 60 minutes at room temperature. Then, centrifuged at 15.000 rpm for 15 minutes, and the supernatant was transferred to a 50 mL volumetric flask. From the first extraction residue, 20 ml of 70% acetone was added, homogenized and allowed to stand for 60 minutes at room temperature. Centrifuged again at 15.000 rpm for 15 minutes, and the supernatant was transferred to the volumetric flask containing the first supernatant. The volume was completed to 50 mL with distilled water.

2.3 β-carotene/linoleic acid method

The technique used was that described by Rufino et al. (2007a) with modifications. From the extracts obtained, at least three different dilutions in triplicate were prepared in test tubes as follows: 0.4 mL of each dilution of extracts was mixed with 5 mL of the system solution (β-carotene/linoleic acid). A mixture of 0.4 ml of the Trolox solution (synthetic antioxidant) with 5 ml of the system solution was used as control. Test tubes were homogenized on a shaker and kept in water bath at 40°C. Concentrations used in this assay were 10.000 and 15.000 ppm. The first reading was performed (470 nm) after 2 minutes of mixing, and then at 15 minutes intervals until completing 120 minutes. The spectrophotometer was previously calibrated with distilled water.

Results were expressed as percent inhibition of oxidation. The absorbance reduction of the system without antioxidant (Eq. 1) was considered as 100% oxidation.

$$\text{Absorbance reduction} = \text{Abs}_{\text{initial}} - \text{Abs}_{\text{final}} \quad \text{(Eq.1)}$$

The decrease of the absorbance reading of the samples is correlated with the system and establishes the oxidation percentage (Eq.2) by subtracting the oxidation percentage of each sample of 100 (Eq.3). The antioxidant action of the sample (*B. excelsa*) was verified by comparing it with the activity of the synthetic antioxidant (Trolox).

$$\% \text{ Oxidation} = \frac{[(\text{Abs reduction})_{\text{sample}} \times 100]}{(\text{Abs reduction})_{\text{system}}} \quad \text{(Eq.2)}$$

$$\% \text{ Protection} = 100 - (\% \text{ Oxidation}) \quad \text{(Eq.3)}$$

2.4 DPPH method (2,2-diphenyl-1-picrylhydrazyl)

The technique used was that described by Brand-Williams et al., (1995) and Rufino et al., (2007b) with modifications. From extracts obtained, at least three different dilutions in triplicate were prepared in test tubes as follows: in a dark environment, a 0.1 mL aliquot of each extract dilution was transferred into test tubes with 3.9 mL of the DPPH radical (0.06 mM) and homogenized on a tube shaker. A mixture of 0.1 mL of the control solution (methyl alcohol, acetone and water) with 3.9 mL of the DPPH radical was used and homogenized. Three concentrations were used, namely: 50.000, 70.000 and 100.000 ppm, and methyl alcohol was used as blank in order to calibrate the spectrophotometer. The readings (515 nm) were monitored every minute, and absorbance reduction was observed until its stabilization. The final absorbance reading for the calculation of inhibitory concentration 50 (IC_{50}) was performed after absorbance stabilization (IC_{50} time).

After reading, the value corresponding to half of the initial absorbance of the control was replaced (Eq. 4) by the y of the DPPH curve equation in order to find the DPPH consumption in µM.

Equivalence of control and DPPH:

$$Y = ax - b \qquad \text{(Eq.4)}$$

Where:

Y = Initial control absorbance/2 (item determining total antioxidant capacity).

x = result in µM DPPH.

Note: convert to g DPPH by transformation: g DPPH = (µM DPPH/1.000.000) *394.3 (molecular weight of DPPH).

From absorbance values obtained from the different dilutions of extracts, absorbance was plotted on the Y-axis and dilution (mg/L) on the X-axis, and the equation of the line (Eq. 5) was determined. In order to calculate the total antioxidant capacity, the absorbance equivalent to 50% of the DPPH concentration was replaced by y (Eq. 5) and was found the result corresponding to the sample needed to decrease the initial concentration of the DPPH radical (IC_{50}) by 50%.

Calculation of IC_{50}

$$Y = - ax + b \qquad \text{(Eq.5)}$$

Where:

Y = Initial control absorbance/2 (item determining total antioxidant capacity).

x = IC_{50} (mg/L).

The result (mg/L) found in equation 5 was divided by 1.000 to find the value in g. Then, it was divided by the value found in g DPPH (Eq. 4) in order to obtain the final result (Eq. 6) that is expressed in g sample/g DPPH.

IC_{50} expressed in g sample/g DPPH:

$$\text{g sample/g DPPH} = (IC_{50}\ (mg/L)/\ 1.000*1)/\text{g DPPH} \qquad \text{(Eq.6)}$$

2.5 Chemical agent inducing DNA damage

The chemotherapy medication 50 mg doxorubicin (98% purity) was purchased from Sare Drogarias (DXR, Rubidox®, São Paulo, Brazil). It is used as micronuclei inducer in erythrocyte (positive control). The inducer was dissolved in distilled water and administered once intraperitoneally (0.3 mL/animal). The concentration of DXR (15 mg/kg body weight, bw) was determined according to the literature (Franke et al., 2005; Venkatesh et al., 2007; Carneiro et al., 2017).

2.6 Animals and treatments

For the experiments, were used Swiss male mice aged 6-7 weeks, of approximately 25 g of body weight (bw), from the Biothermium of the Multidisciplinary Center for Biological Research in the Laboratory Animal Science Area (Portuguese acronym: CEMIB) of the University of Campinas (UNICAMP, São Paulo). The study was conducted in accordance with internationally accepted protocols for the use and care of laboratory animals. Animals were kept in polypropylene boxes measuring 37x25x16 cm in an experimental room under controlled conditions of temperature (22±2ºC), humidity (50±10%), 12 hours of light-dark cycle, ad libitum access to feed and water (15 days before the start of experiment for acclimatization). Treatment protocols performed in this study were submitted to the Ethics Committee on Animal Use (Portuguese acronym: CEUA) of the Federal University of Amapá (UNIFAP), and accepted under protocol number 020/2015 - CEUA/UNIFAP, of October 27, 2015.

2.7 Experimental design

Mice were divided into ten experimental groups containing six animals each. The doses of fixed oil of *B. excelsa* for the micronucleus test in mammalian erythrocytes were (500, 1.000 and 2.000 mg/kg bw) for genotoxicity, and (500 + DXR, 1.000 + DXR, and 2.000 + DXR mg/kg bw) for antigenotoxicity, selected according to Guidelines (MacGregor et al., 1980; ANVISA, 2013; OECD 474, 2016), and administered once. Negative control groups (water), positive control groups (DXR 15 mg/kg bw) and two solvent groups (dimethylsulfoxide; DMSO, Sigma-Aldrich, St Louis, MO, USA; 0.085 g/kg bw) DMSO and DMSO + DXR were also included. The different concentrations of *B. excelsa* were prepared from a stock solution of 300 mg of fixed oil and administered by gavage (0.5 mL/animal). The solvent group was treated by gavage (0.5 mL/animal) at the same DMSO concentration used to dissolve the group treated with 2.000 mg/kg bw of *B. excelsa* (200 µL) and DXR (0.3 mL/animal, intraperitoneally, i.p.). Peripheral blood samples were collected 24 and 48 hours after the treatments to make the smears and perform subsequent analysis of the slides (which were fixed for 5 min in methanol and stained for 20 min with giemsa).

A total of 2.000 polychromatic erythrocytes (PCEs) were analyzed by animal in order to determine the frequency of micronucleated polychromatic erythrocytes (MNPCEs). For calculation of the nuclear division index [NDI, PCE/PCE + NCE (normochromic erythrocyte)] in order to determine the cytotoxicity of experimental groups, were analyzed 400 erythrocytes per animal (Mersch-Sundermann et al., 2004). The slides were blind-encoded and read using a light microscope in objective (40x) for detection of good quality fields. After the field identification, was performed a reading in immersion objective (100x) for visualization of micronuclei.

For the evaluation of antigenotoxicity, the percentage reduction in MNPCE frequency was calculated according to (Waters et al., 1990; Delmanto et al., 2001) by using the following formula:

$$\%Reduction = \frac{A - B}{A - C} x100$$

Where **A** - corresponds to the mean value obtained for the treatment with DXR (positive control), **B** – corresponds to the group treated with *B. excelsa* associated to DXR, and **C** – is the group treated with distilled water (negative control).

Data were analyzed statistically by analysis of variance (ANOVA) for completely randomized experiments with calculation of the F statistic and its respective 'p-value'. In cases where P <0.05, the means of treatment were compared by the Tukey method with calculation of the minimum significant difference for α = 0.05.

3. RESULT AND DISCUSSION

Nowadays, emphasis has been given to studies on natural products because their chemical composition is rich in biologically active substances, they play an excellent role as functional products, and are effective in the fight against pathologies, mainly in the prevention of chronic degenerative diseases (Haida et al. 2011; Melo et al. 2011).

Despite the intense consumption of plant species in the most diverse forms of extractive processes, such as teas, bottles, oils or patches, little is still described about the toxicology of some species used (Alonso, 2008; Belcavelo et al., 2012). This fact has drawn attention to toxicological studies with the aim of ensuring the use of these products and proving the absence of harmful effects on exposed organisms.

In order to evaluate the antioxidant activity of phenolic compounds in plants, Melo et al. (2006) have evaluated the antioxidant capacity of fifteen vegetables commercialized in the northeast of Brazil by using the β-carotene/linoleic acid method and the DPPH method. All vegetables analyzed showed antioxidant capacity, and these methodologies were used in the present study. Samples exhibiting protective capacity (oxidation inhibition) above 70% are considered as excellent antioxidants; between 50 and 70% are considered moderate antioxidant action; and below 50% is low protective capacity (De Almeida Melo et al., 2008).

In a study with nuts, Oliveira et al. (2018) used hydroethanolic extracts and fractions rich in flavonoids from leaves of *Anacardium occidentale linn* for the evaluation of the DPPH free radical sequestration activity at the selected concentrations of 50, 150 and 250 μg/mL, and *A. occidentale* showed antioxidant capacity of 14, 44 and 72%, respectively. Silva and De Carvalho Melo, (2015) analyzed the same activity, but of a substance present in the cashew nutshell liquid (*A. occidentale*), the epicatechin. The results for concentrations at 1; 5; 10; 25; 50 and 100 μg/mL were 33.43, 32.72, 48.72, 54.74 and 60.41%, respectively. Faria et al., (2016) analyzed the jabuticaba (*Myrciaria jabuticaba*), an Amazonian species known for the already reported high antioxidant rate. Two solutions were used in the study, namely, methanolic and hydroalcoholic, and were found results in percentage of oxidation inhibition of 97 and 95%, respectively. The present study evaluated the oxidation protection/inhibition activity of the fixed oil of *B.*

excelsa by the β-carotene/linoleic acid method. The results obtained were 92 and 87% for concentrations of 10.000 and 15.000 ppm (Table 1), respectively, which is an excellent classification by the method. The Total Antioxidant Capacity (TAC) by capturing DPPH free radical of the extracts obtained from the fixed oil of *B. excelsa* was evaluated at the following three concentrations: 50.000, 70.000, and 100.000 ppm. This way, they could react with the DPPH free radical, and results found were 14.53, 25.57 and 40.33% for sequestration of DPPH (Table 2), respectively. They were classified as having low antioxidant capacity, because it was below the recommended level of 50% decrease of the initial DPPH concentration in the conditions of the present study (Figure 1). This demonstrated a low interaction between extracts obtained from the sample with the free radical by indicating a possible interference in the solubilization of the sample. In another study, were analyzed Brazil nut soluble phenolic extracts and the results of IC_{50} for the three extracts obtained (methanolic, ethanolic and acetonic) were excellent, being 0.921 ± 0.050; 0.904 ± 0.072 and 0.919 ± 0.048 mg / mL, respectively (John and Shahidi, 2010). In addition, the antioxidant capacity of a compound is directly related to the present bioactive components, the chemical structure and concentrations of these phytochemicals in the analyzed sample (Magalhães et al., 2008; Barreira et al., 2010).

Table 1: Antioxidant activity of the fixed oil of *Bertholletia excelsa* through the β-carotene/linoleic acid method.

Concentration (ppm)	BE fixed oil (%)
10.000	92.00
15.000	87.00

Table 2: Capture of the free radical of the fixed oil of *Bertholletia excelsa* by the DPPH method.

Concentration (ppm)	BE fixed oil (%)
50.000	14.53
70.000	25.57
100.000	40.33

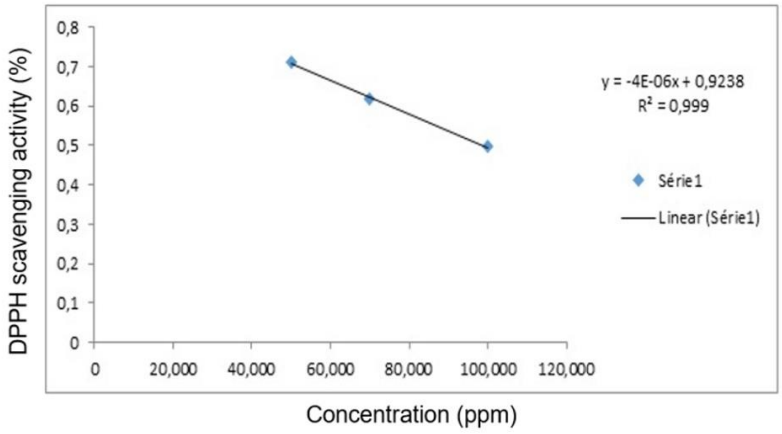

Figure 1: DPPH absorbance at 50.000, 70.000 and 100.000 ppm concentrations.

In the cytogenetic assay, the results obtained for treatments with different concentrations of the fixed oil of *B. excelsa* and/or DXR and their respective controls are shown in Figures 2 and 3. Data showed the differences in the frequencies of MNPCEs between the solvent group and the negative control group in peripheral blood samples were not statistically significant. Furthermore, no significant differences were observed in the frequencies of MNPCEs between animals treated with the three concentrations of fixed oil *B. excelsa* (500, 1.000 and 2.000 mg/kg bw) compared to the negative control and solvent groups, thus demonstrating the absence of genotoxic effect of *B. excelsa* oil at the concentrations used. As expected, the animals treated with DXR showed high frequency of MNPCEs when compared to controls, which is similar to other studies with nuts, such as that of De Araújo et al. (2018). It evaluated the mutagenicity of the *A. occidentale* by using the concentration of 2.000 mg/kg bw of *A. occidentale*, in addition to control groups, negative (deionized water) and positive (cyclophosphamide 50 mg/kg bw). After 24 hours of treatment, peripheral blood was drawn, 2.000 polychromatic erythrocytes were analyzed, and the following results were found: negative control 0.8 ± 0.1, positive control 9.3 ± 1.4, and *A. occidentale* 0.8 ± 0.2, showing no mutagenicity. In a study by Encarnação et al., (2016), were evaluated extracts of *A. occidentale* bark at a concentration of 2.000 mg/kg bw for micronucleus testing. Peripheral blood samples were collected after 48 hours of treatment (negative control, water and positive control cyclophosphamide 50 mg/kg bw), and no genotoxic effect was found.

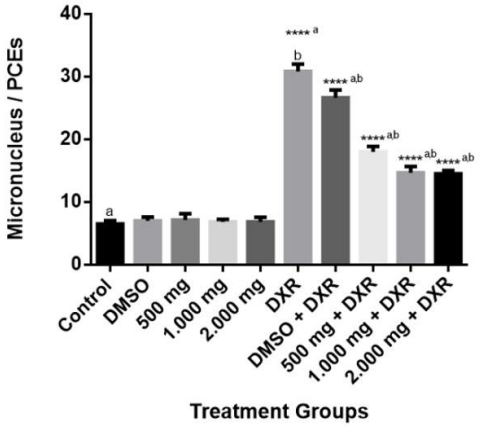

Figure 2: Results of control groups (negative and positive), DMSO and groups treated with different doses of fixed oil of *B. excelsa* after 24h (P <0.05. Groups compared by the Tukey method).

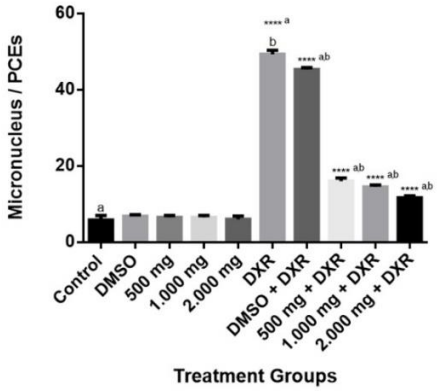

Figure 3: Results of control groups (negative and positive), DMSO and groups treated with different doses of fixed oil of *B. excelsa* after 48h (P <0.05. Groups compared by the Tukey method).

Carvalho et al. (2011) evaluated the genotoxicity of the cashew anacardic acid (*A. occidentale*) by the micronucleus test in bone marrow. Four control groups were used, namely: negative (0.9% saline solution); positive (1 mL/100 g bw of N-methyl-N-nitrosourea, MNU, diluted in 0.9% saline at a concentration of 50 mg/kg bw by intraperitoneal route); test groups with 250 mg/kg bw of anacardic acid dissolved in 100 μL of cashew nut oil; and the fourth group receiving 100 μL of cashew nut oil via oral, and no genotoxicity was found in the test groups.

Simultaneous administration of a single oral dose of each concentration of the fixed oil of *B. excelsa* with DXR injection resulted in a significant reduction. The decrease ranged from 52.08 to 67.36% in samples collected after 24h, and from 76.78 to 86.89% in samples collected after 48h (Figures 2 and 3). Tables 3 and 4 show the NDI for all treatment groups at different sampling times. No significant reduction in the percentage of PCEs was observed in relation to the total number of erythrocytes for any of the treatment groups compared to the negative control group, which demonstrates the absence of cytotoxicity in the different treatments in the experimental conditions present in this study. This is in line with the study by De Carvalho Melo-Cavalcante et al., (2011), in which fresh processed cashew juices (*A. occidentale*) were evaluated. The concentration of 0.15 mL/10 g bw was used in the micronucleus test of cashew juice and cajuína (Brazilian beverage made of cashew apples) in Swiss mice. The genotoxicity test showed no genotoxic effect, and a reduction in the micronucleus frequency of 81.81% and 83.11% for cashew juice and cajuína, respectively, when associated with the positive control (50 mg/kg bw cyclophosphamide).

The MNPCE frequency was lower in animals treated with DMSO associated with DXR than in those treated with DXR alone, but there were no statistically significant differences (Tables 3 and 4). The micronucleus test in peripheral blood is satisfactory for the identification of agents capable of inducing or preventing chromosomal damage hence it can be used in mutagenicity and antimutagenicity assays (Grawe, 2005). The results obtained show that fixed oil of *B. excelsa* did not increase the frequency of micronucleated cells (Figure 4). Therefore, the oil of *B. excelsa* did not present genotoxic effect according to the protocols used in the present study.

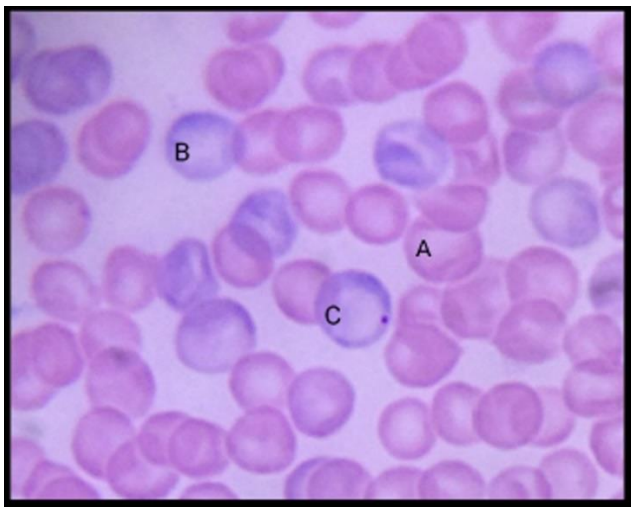

Figure 4: A – Normochromic Erythrocyte (NCE). B – Polychromatic Erythrocyte (PCE). C – Micronucleated Polychromatic Erythrocyte (MNPCE).

On the other hand, the fixed oil of *B. excelsa* caused a significant reduction in the frequency of DXR-induced MNPCEs. The genotoxic activity of the chemotherapeutic agent DXR has been attributed to its ability to produce free radicals (Keizer et al., 1990) that cause different types of cellular damage, including DNA cleavage. The chemical structure of DXR favors the generation of free radicals and the compound can bind to iron and form complexes with DNA, and two-strand breaks are induced (Eliot et al., 1984). The production of free radicals is the main mechanism responsible for their toxicity by resulting in oxidative stress and causing DNA damage, which can be transformed into mutations, and later in the process of carcinogenesis if not repaired (Pan et al., 2008; Monteiro Neto et al., 2011).

The *B. excelsa* oil has a rich chemical composition of lipids, carbohydrates, proteins and selenium. This last compound is found at high levels and responsible for the substance antioxidant activity (Kornsteiner et al., 2006; Santos, 2010), which may have contributed to inhibition of oxidation in the in vivo test. The performance of selenium antioxidant activity is better in association with enzymes such as glutathione peroxidase that is present in the mammalian system (Freitas & Naves, 2010).

Calvo et al. (2002) and Patrick (2004) highlight selenium among the compounds with highest functional recognition. It is considered one of the most important antioxidants, acts on physiological and metabolic changes in the delay or prevention of the organic natural oxidation process. This is corroborated by the study that showed a reduction rate of the micronucleus frequency at the concentrations of the fixed oil of *B. excelsa* used, which were analyzed in 24 and 48 hours (52.08%, 63.88%, 67.36% and 76.78%, 80.46%, 86.89%, respectively). Regarding dose-response, a statistically significant dose-dependent protective effect of the fixed oil of *B. excelsa* administered was observed.

4. CONCLUSION

In conclusion, with use of the β-carotene/linoleic acid method, the fixed oil of *B. excelsa* demonstrated excellent oxidation inhibition activity. For the DPPH method, at the concentrations used, the fixed oil presented below-desirable results that is 50% of DPPH capture, and was classified as having little protective activity.

As for micronucleus testing, the fixed oil of *B. excelsa* did not show a genotoxic effect, but it showed an effective reduction in DXR-induced chromosomal damage in the assay using peripheral blood erythrocytes of Swiss mice. Although the exact mechanism underlying the antigenotoxicity of *B. excelsa* is not fully understood, its oxidation inhibition activity observed in the β-carotene/linoleic acid method may explain its effect on the genotoxicity of DXR. Therefore, the ability of *B. excelsa* for reduction of frequency of DXR-induced MNPCEs is an indication of its promising chemopreventive potential.

5. CONFLICT OF INTEREST

The authors state there is no conflict of interest that may be perceived as prejudicial to the impartiality of the reported study.

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