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Research Article

# Antigenotoxic activity and antioxidant properties of organic and aqueous extracts of pequi fruit (*Caryocar brasiliense* Camb.) pulp

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## Abstract

The daily consumption of natural antioxidants protects against oxidative damage caused by reactive oxygen species (ROS), including DNA damage, and can reduce the risk of cancer, atherosclerosis and other degenerative diseases. The pulp of pequi (*Caryocar brasiliense* Camb.) fruit, a tree native to the Brazilian savannah, contains several compounds with antioxidant properties, including carotenoids, vitamin C, phenolic compounds such as flavonoids, saponins and tannins, and essential oils. In this work, we examined the ability of organic and aqueous extracts of pequi fruit pulp to protect against the genotoxicity induced by two antineoplastic drugs, cyclophosphamide (CP) and bleomycin (BLM). Micronucleus tests with mouse bone marrow cells and single-cell gel electrophoresis (comet assay) with peripheral blood leukocytes were used to examine the effects of CP and BLM, respectively. The antioxidant activity of the extracts was assessed by measuring lipid peroxidation with the TBARS method in mouse plasma. The fruit pulp extracts had no clastogenic or genotoxic effects in the cells studied, but both extracts protected against oxidative DNA damage caused by BLM or CP, indicating an ability to inhibit chemical mutagenesis *in vivo*. However, the protective effect against oxidative DNA damage depended on the dose of extract used. At the doses tested, the aqueous extract enhanced lipid peroxidation in mice of both sexes, especially in males. In contrast, the organic extract enhanced lipid peroxidation only in male mice, with no significant effect in females. These results suggest that, with adequate adjustment of the dose, an organic extract of pequi fruit pulp could be a useful dietary supplement with natural antioxidant activity, at least in females.

*Key words:* antigenotoxic, antioxidant, *Caryocar brasiliense*, comet assay, micronucleus, pequi pulp extracts, reactive oxygen species, TBARS assay.

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## Introduction

The pequi tree (*Caryocar brasiliense* Camb.), which belongs to the family Caryocaraceae, is very common in the Brazilian savannah ("cerrado"). Pequi fruit has an internal mesocarp (pequi pulp) that is used in folk medicine to treat a number of respiratory diseases (Almeida and Silva, 1994; Ramos *et al.*, 2001). Each 100 g of pulp contains 20%-27% lipids that constitute the pulp oil, 2.2-6.0% protein, 11.6% fibers, 19.7% total carbohydrates and 225-267.9 kcal (Almeida, 1998; Boletim Informativo UFMG nº 1511, 2005). According to Almeida (1998), the fatty acid composition of pequi pulp oil consists of 51% mono-unsaturated acids, mainly oleic acid, 49% saturated oils (principally palmitic acid) and ~2% polyunsaturated linoleic acid.

Pequi oil is also used in folk medicine for treating ophthalmic problems related to vitamin A deficiency, a use supported by the high content of carotenoids with provitamin A activity (Almeida and Silva, 1994; Almeida, 1998; Ramos *et. al*, 2001; Santos *et al.*, 2005; Oliveira *et al.*, 2006). In addition to β-carotene, pequi pulp contains other carotenoids, such as lycopene (Oliveira *et al.*, 2006), ζ-carotene, cryptoflavin, β-cryptoxanthine, anteraxanthine, zeaxanthine, mutatoxanthine, violanxanthine, lutein and neoxanthine (Ramos *et al.*, 2001; Azevedo-Meleiro and Rodriguez-Amaya, 2004; Oliveira *et al.*, 2006). Pequi pulp is also rich in vitamin C (Barbosa and Amante, 2005). W. Paula-Júnior (MSc Dissertation, Federal University of Paraná, Brazil, 2004) identified phenolic compounds, such as flavonoids, saponins and essential oils in hydroethanolic extracts of the internal mesocarp of pequi pulp, and Almeida (1998) reported a tanin content of 0.17 mg/100 g of pulp. All of these components of pequi pulp have antioxidant properties (Sies, 1993; Tseng *et al.*, 2004).

Natural antioxidants in the human diet can attenuate the effects of mutagens and genotoxic carcinogens. Some antioxidants, such as vitamins A, C and E, minimize the

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side effects of antineoplastic drugs and can improve cancer chemotherapy. An increase in the dietary content of antioxidants through the increased ingestion of fruits and vegetables rich in these compounds can decrease the oxidation of DNA by free radicals, thereby preventing cancer and other degenerative diseases. Oxidative damage to biomolecules caused by stress is one of the major risk factors for atherosclerosis, mainly through the oxidation of low density lipoprotein (LDL) in the blood. Thus, cancer, atherosclerosis and many other degenerative diseases share a common mechanism (Kong and Lillehei, 1998; Santos and Cruz, 2001; Dusinská *et al.*, 2003).

Some dietary antioxidants can act as adjuvants in cancer therapy because of their ability to induce apoptosis *in vitro*. Antioxidants also show promise in cancer therapy because of their palliative action in reducing painful side effects associated with chemotherapy (Borek, 2004). Khouri *et al.* (2007) showed that an aqueous extract of pequi pulp can prevent mouse bone marrow cells against chromosomal aberrations induced by bleomycin. Experiments with cultured CHO-K1 cells also showed that this aqueous extract protected cells against the clastogenic effects of cyclophosphamide.

In this study, the anticlastogenic potential of an organic extract of pequi was evaluated in mice bone marrow cells *in vivo* by using the micronucleus assay. The antigenotoxic effects of aqueous (AEP) and organic (OEP) extracts of pequi pulp were also evaluated by single-cell gel electrophoresis (*SCGE* - comet assay) in mouse peripheral blood leukocytes. The antioxidant activity of AEP and OEP on lipid peroxidation in mouse blood was assessed by the thiobarbituric acid-reactive substances (TBARS) assay.

## Materials and Methods

### Chemicals

Bleomycin (BLM) (CAS 011056-06-7) was obtained as Bleoxane sulphate from Bristol-Myers (São Paulo, Brazil) and cyclophosphamide (CP) (CAS 6055-19-2), sold a Genuxal, was from ASTA Medica Laboratory (São Paulo, Brazil).

### Plant material

Pequi fruit was obtained *in natura* from vendors in Brasília, DF (Brazil) and the surrounding region. The internal mesocarp was peeled or grated to obtain the pulp, which was then packed in a covered pot and frozen to -86 °C.

### Extraction procedure

Pequi pulp was extracted by the soxhlet procedure under an argonium atmosphere using chloroform (organic extract of pequi pulp - OEP) or distilled water (aqueous extract of pequi pulp - AEP) as the solvent. After extraction, the extracts were submitted to evaporation under reduced pressure to remove the solvents, dried at high vacuum and immediately frozen at -86 °C.

### Animals

Swiss white mice of both sexes (60 days old, 30 ± 2 g) obtained from the Central Animal Facility of the University of Brasília were housed in plastic cages (6 or 8/cage) at room temperature (20 ± 2 °C) on a 12 h light/dark cycle with lights on at 6 a.m. and free access to food and water. The experimental protocols described here were approved by the institutional Ethics Committee for Animal Research (Institute of Biological Science, University of Brasília).

### Anticlastogenic and antigenotoxic assays

#### *Micronucleus (MN) test*

For the MN test, only the OEP was used since the AEP has already been studied by Khouri *et al.* (2007). Mice of both sexes were randomly allocated to groups M1 to M12 (n = 8 mice/group; Table 1). OEP (0.5 mL $kg^{-1}$ or 1 mL $kg^{-1}$ of body weight, respectively, corresponding to 15 µL and 30 µL of extract per mouse) was administered orally (*per os*, p.o.) by gavage daily for 10 days either alone or in combination with a single intraperitoneal (i.p.) injection of bleomycin diluted in 0.9% NaCl or aqueous cyclophosphamide (see Table 1 for concentrations). Control mice received filtered water in place of extract and no clastogen was administered. When required, the mice were killed by cervical dislocation and slides of bone marrow cells were prepared by a standard method (Schmid, 1975). Two thousand erythrocytes were counted per mouse (1000 normochromatic erythrocytes, NCE, and 1000 polychromatic erythrocytes, PCE) and the frequency of micronuclei (MN) in PCE and NCE, and the percentage of polychromatic erythrocytes (%PCE) were calculated.

#### *Single-cell gel electrophoresis (SCGE) assay (comet assay)*

The SCGE assay was done essentially as for the MN test, using mice of both sexes randomly allocated to groups C1 to C9 (n = 8 mice/group; Table 2). The comet assay was done after the MN test, using OEP (0.5 mL $hg^{-1}$) and AEP (1 mL $kg^{-1}$) administered p.o. daily for 10 days, either alone or in combination with a single injection of bleomycin (25 mg $kg^{-1}$, i.p.) or two injections of cyclophosphamide (at 30 mg $kg^{-1}$, i.p. 48 h and 24 h before the mice were killed) (Table 2).

The comet assay (alkali method) proposed by Singh *et al.* (1988) and improved by Tice (1995) was used. Microscope slides were dipped briefly into 1.5% hot (60 °C) normal melting agarose prepared in phosphate-buffered saline (PBS). The slides were dried overnight at room temperature and then stored at 4 °C until used. Subsequently, freshly collected, heparinized peripheral blood (20 µL) was suspended in 120 µL of 0.5% low melting point agarose in

**Table 1** - Micronuclear evaluation of bone marrow cells from Swiss white mice treated orally with an organic extract of pequi (*Caryocar brasiliense*) fruit pulp (OEP; 0.5 mL kg$^{-1}$ or 1 mL kg$^{-1}$) alone or with a single intraperitoneal injection of bleomycin (BLM) or cyclophosphamide (CP).

| Group | Treatment | Polychromatic erythrocytes (PCE) | | Normochromatic erythrocytes (NCE) |
|---|---|---|---|---|
| | | Cellular proliferation index (%PCE) | MN-PCE | MN-NCE |
| M1 | Control group (filtered water) | 59.22 ± 3.34 | 1.38 ± 1.06 | 0.75 ± 0.88 |
| M2 | CP (30 mg kg$^{-1}$, i.p., 24 h before sacrifice) | 58.23 ± 6.81 | 11.5 ± 6.71$^{g}$ | 0.5 ± 0.92 |
| M3 | OEP (1 mL kg$^{-1}$, p.o., daily for 10 days) | 67.26 ± 6.33$^{a}$ | 1.0 ± 0.75 | 0.5 ± 1.06 |
| M4 | OEP (1 mL kg$^{-1}$, p.o., daily for 10 days) + CP (30 mg kg$^{-1}$, i.p., 24 h before sacrifice) | 63 ± 10.10 | 22.63 ± 13.23$^{h, i}$ | 1.25 ± 1.48 |
| M5 | BLM (50 mg kg$^{-1}$, i.p., 24 h before sacrifice) | 0* | 0* | 0* |
| M6 | OEP (1 mL kg$^{-1}$, p.o., daily for 10 days) + BLM (50 mg kg$^{-1}$, i.p., 24 h before sacrifice) | 53.65 ± 8.38$^{b}$ | 5.37 ± 2.82$^{j, k}$ | 2.0 ± 1.41 |
| M7 | BLM (25 mg kg$^{-1}$, i.p., 24 h before sacrifice) | 49.99 ± 11.88 | 2.25 ± 1.38 | 1.75 ± 1.03 |
| M8 | OEP (0.5 mL kg$^{-1}$, p.o., daily for 10 days) | 67.62 ± 9.66$^{c}$ | 1.0 ± 1.41 | 0.5 ± 0.75 |
| M9 | OEP (0.5 mL kg$^{-1}$, p.o., daily for 10 days) + CP (30 mg kg$^{-1}$, i.p., 24 h before sacrifice) | 57.84 ± 7.31 | 9.25 ± 4.71 | 1.87 ± 1.95 |
| M10 | OEP (0.5 mL kg$^{-1}$, p.o., daily for 10 days) + BLM (25 mg kg$^{-1}$, i.p., 24 h before sacrifice) | 51.01 ± 6.56$^{d}$ | 2.75 ± 0.88$^{l}$ | 0.63 ± 0.74$^{o}$ |
| M11 | BLM (10 mg kg$^{-1}$, i.p., 24 h before sacrifice) | 59.07 ± 2.28 | 8.25 ± 3.24$^{m}$ | 1.37 ± 1.18 |
| M12 | OEP (0.5 mL kg$^{-1}$, p.o., daily for 10 days) + BLM (10 mg kg$^{-1}$, i.p., 24 h before sacrifice) | 65.37 ± 6.77$^{e, f}$ | 5.37 ± 1.50$^{n}$ | 1.62 ± 1.59 |

MN-PCE and MN-NCE - micronucleus test results for PCE and NCE, respectively. The results are expressed as the mean±SD. *Extensive cell lysis was seen in bone marrow, with few cells and no reticulocytes. $^{a}$p = 0.0117 compared to M1. $^{b}$p = 0.0003 compared to M5. $^{c}$p = 0.0460 compared to M1. $^{d}$p = 0.01003 compared to M1. $^{e}$p = 0.0357 compared to M11. $^{f}$p = 0.0357 compared to M1. $^{g}$p = 0.0006 compared to M1. $^{h}$p = 0.0309 compared to M2. $^{i}$p = 0.0006 compared to M1. $^{j}$p = 0.0003 compared to group M5. $^{k}$p = 0.0009 compared to M1. $^{l}$p = 0.0172 compared to M1. $^{m}$p = 0.0006 compared to M1. $^{n}$p = 0.0009 compared to M1. $^{o}$p = 0.0332 compared to M7.

PBS (Gibco BRL) at 37 °C and pipetted onto a microscope slide pre-coated with a layer of normal melting point agarose. This mixture was allowed to set at 4 °C for 10 min and the slides then immersed in a freshly prepared cold (4 °C) lysis solution (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris and 1% lauroyl sarcosine, and the pH adjusted to pH 10.0-10.5 with NaOH; 1% Triton X-100 and 10% dimethyl sulfoxide were added immediately before use) at 4 °C for 1 h. After lysis, the slides were placed in an electrophoretic tank containing 300 mM NaOH and 1 mM EDTA, pH > 13.0 for 30 min, and electrophoresis was then done at 25 V, 350 mA for 40 min. Soon after neutralization (3 x 5 min in 0.4 M Tris, pH 7.5 at 4 °C), the slides were stained with ethidium-bromide (20 µg/mL), fixed in 100% ethanol for 5 min and analyzed with a Zeiss Axioskop 2 fluorescence microscope (filter 510-560 nm, barrier filter 590 nm) with a final magnification of 400x. All of the slides were prepared and analyzed in duplicate.

SCGE analysis: One hundred comets on each slide were scored visually and assigned to classes 0-4, as proposed by Collins (1995). Nucleoids with bright heads and no apparent tails were assigned to category 0, whereas comets with very small heads and long, diffuse tails were assigned to category 4. Comets with intermediate features between classes 0 and 4 were assigned to classes 1, 2 and 3. The number of comets in each category was counted and the average DNA damage (DD, expressed in arbitrary units, a.u.) was calculated according to Jalonszynski *et al.* (1997), as follows:

$$DD = \frac{n_1 + 2n_2 + 3n_3 + 4n_4}{\frac{\sum}{100}}$$

where $n_1$- $n_4$ = the number of comets in categories 1-4, and $\Sigma$ = the sum of all comets, including category 0.

## *In vivo* antioxidant assay: thiobarbituric acid-reactive substances (TBARS) test

For the TBARS test, six groups (T1-T6) of eight mice each (4 males, 4 females) were used, as follows:

T1: Female control group (filtered water only, by gavage, daily, for 10 days)

T2: Male control group (filtered water only, by gavage, daily, for 10 days)

T3: Females treated with OEP (0.5 mL kg$^{-1}$, by gavage, daily for 10 days)

T4: Males treated with OEP (0.5 mL kg$^{-1}$, by gavage, daily for 10 days)

T5: Females treated with AEP (1.0 mL kg$^{-1}$, by gavage, daily for 10 days)

T6: Males treated with AEP (1.0 mL kg$^{-1}$, by gavage, daily for 10 days)

The TBARS assay was done according to Wasowicz *et al.* (1993), with slight modification. The TBA solution

**Table 2** - Comet assay of peripheral leukocytes from Swiss white mice treated orally with an organic (OEP, 0.5 mL $kg^{-1}$) or aqueous (AEP, 1 mL $kg^{-1}$) extract of pequi (*Caryocar brasiliense*) fruit pulp, alone or with bleomycin (BLM, single injection, i.p.) or cyclophosphamide (CP, two injections, i.p.).

| Group | Treatment | Comet classes (mean±SD) | | | | | Mean±SD of DD |
|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | |
| C1 | Control group (filtered water) | 11.62 ± 5.34 | 61.25 ± 18.7 | 22 ± 10.4 | 4.75 ± 5.6 | 0.38 ± 0.52 | 121 ± 20.89 |
| C2 | CP (30 mg $kg^{-1}$, i.p., 48 and 24 h before sacrifice) | 3.63 ± 2.88 | 6.63 ± 4.84 | 10.38 ± 6.80 | 31.88 ± 12.45 | 47.63 ± 13.75 | 313.5 ± 26.91[a] |
| C3 | OEP (0.5 mL $kg^{-1}$, p.o., daily for 10 days) | 12 ± 7.98 | 46 ± 20.78 | 26.29 ± 8.03 | 5.57 ± 8.55 | 9.57 ± 18.61 | 171.13 ± 68.18 |
| C4 | OEP (0.5 mL $kg^{-1}$, p.o., daily for 10 days) + CP (30 mg $kg^{-1}$, i.p., 48 and 24 h before sacrifice) | 21 ± 10.40 | 41.5 ± 24.57 | 14.88 ± 9.73 | 14.5 ± 15.67 | 7.5 ± 9.75 | 144.75 ± 57.22[b] |
| C5 | BLM (25 mg $kg^{-1}$, i.p., 24 h before sacrifice) | 4.29 ± 2.81 | 36.5 ± 12.99 | 47 ± 13.53 | 13.75 ± 10.36 | 2.13 ± 3.44 | 180.25 ± 39.96[c] |
| C6 | OEP (0.5 mL $kg^{-1}$, p.o., daily for 10 days) + BLM (25 mg $kg^{-1}$, i.p., 24 h before sacrifice) | 8.38 ± 6.74 | 39 ± 13.42 | 41.5 ± 15.48 | 9.25 ± 6.78 | 2.0 ± 2.98 | 157.75 ± 27.03[d] |
| C7 | AEP (1 mL $kg^{-1}$, p.o., daily for 10 days) | 9.75 ± 7.06 | 39.88 ± 13.91 | 29.88 ± 14.09 | 12.38 ± 14.94 | 8.13 ± 7.07 | 169.25 ± 33.13 |
| C8 | AEP (1 mL $kg^{-1}$, p.o., daily for 10 days) +CP (30 mg $kg^{-1}$, i.p., 48 and 24 h before sacrifice) | 2.0 ± 1.85 | 68 ± 10.81 | 21.88 ± 9.49 | 5.13 ± 3.76 | 3.0 ± 3.38 | 139.13 ± 16.57[e] |
| C9 | AEP (1 mL $kg^{-1}$, p.o., daily for 10 days) + BLM (25 mg $kg^{-1}$, i.p., 24 h before sacrifice) | 6.13 ± 6.10 | 62.5 ± 16.48 | 27.63 ± 13.64 | 1.63 ± 2.26 | 2.13 ± 3.09 | 131.13 ± 14.29[f, g] |

[a]$p = 0.0008$ compared to C1. [b]$p = 0.0008$ compared to C2. [c]$p = 0.0016$ compared to C1. [d]$p = 0.0134$ compared to C1. [e]$p = 0.0008$ compared to C2. [f]$p = 0.0273$ compared to C6. [g]$p = 0.0023$ compared to C5.

was prepared by dissolving TBA (Merck; final concentration 29 mmol/L) in acetic acid (8.75 mol/L; Merck). The stock standard solution of MDA was prepared by dissolving 480 µL of 1,1,3,3-tetraethoxypropane (Sigma) in 100 mL of ethanol (Merck). Immediately before use, the solution was diluted in Milli-Q water to yield a working solution of 10 µmol/L. The other reagents for the assay were purchased from Sigma (n-butanol) or Merck (EDTA, glutathione - GSH, and hydrochloric acid). Working aqueous solutions of EDTA (67.3 mmol/L) and GSH (32.5 mmol/L) were prepared freshly immediately before use (to avoid GSH oxidation).

Blood was collected by cardiac puncture using na insulin syringe containing EDTA as anticoagulant. After centrifugation (1500 x *g*, 10 min, 4 °C), the plasma was carefully removed and EDTA and GSH were added to final concentrations of 1.34 mmol/L and 0.65 mmol/L, respectively. The samples were then quickly frozen in liquid nitrogen and stored at -80 °C until used.

For the TBARS test, 50 µL of plasma or an equal volume of MDA working standard was added to 10 mL glass tubes containing 1 mL of Milli-Q water, followed by 1 mL of solution containing TBA (29 mmol/L) in acetic acid (pH of the reaction mixture, 2.4-2.6), mixing, and heating in a water bath for 1 h at 95° -100 °C. The samples were then cooled and 25 µL of 5 mol/L HC1 was added (final pH 1.6-1.7), followed by extraction with 3.0 mL of n-butanol and vortex mixing for 30 s. The butanol phase was separated by centrifugation (1500 x g, 10 min) and its fluorescence was measured with a Jasco FP-777 spectrofluorometer (excitation: 525 nm, emission: 547 nm). A standard curve was prepared with MBA (0-0.15 µmol/L).

### Statistical analysis

The results were expressed as the mean ± SD. Statistical comparisons were done by using the Mann-Whitney U-test, with $p < 0.05$ indicating significance.

## Results

### Anticlastogenic and antigenotoxic assays

#### *Micronucleus (MN) test*

Neither of the doses of OEP (1 mL $kg^{-1}$ - group M3 or 0.5 mL $kg^{-1}$ - group M8) had any clastogenic effect on mouse bone marrow cells when compared with the control group (M1) (Table 1). However, the cellular proliferation index in mouse bone marrow cells, expressed as the percentage of PCE, increased in groups M3 and M8 ($p = 0.0117$ and $p = 0.0460$, respectively). There was no significant difference in the frequencies of MN-PCE ($p = 0.5780$), MN-NCE ($p = 0.7004$) and the cellular proliferation index ($p = 0.9163$) between groups M3 and M8. Cyclophosphamide (30 mg $kg^{-1}$, group M2) increased the frequency of MN-PCE, indicating this drugs clastogenic effect. Concomitant treatment with OEP (0.5 mL $kg^{-1}$, group M9) did not significantly affect the percentage of PCE compared to M2. In contrast, concomitant treatment with OEP (1 mL $kg^{-1}$, group M4) increased the frequency of MN-PCE to a greater extent than seen with CP or OEP alone ($p = 0.0309$).

Bleomycin (50 mg $kg^{-1}$; group M5) was clearly cytotoxic and caused cellular lysis (no erythroblasts were seen). At a dose of 25 mg $kg^{-1}$, bleomycin did not significantly alter the frequency of MN-PCE (group M7 *vs.* M1), but caused significant induction at 10 mg $kg^{-1}$ (group M11), indicating a clastogenic effect on these cells. Comparison of groups M5 and M6 showed that OEP (1 mL $kg^{-1}$) clearly protected bone marrow cells against bleomycin (50 mg $kg^{-1}$)-induced lysis ($p = 0.0003$ for MN-PCE, $p = 0.0011$ for MN-NCE and $p = 0.0003$ for %PCE). On the other hand, the combination of OEP (0.5 mL $kg^{-1}$) + bleomycin (25 mg $kg^{-1}$) (group M10) did not significantly affect MN-PCE, but reduced MN-NCE when compared with group M7 ($p = 0.0332$). Although the combination OEP (0.5 mL $kg^{-1}$) + bleomycin (10 mg $kg^{-1}$) (group M12) did not significantly decrease MN-PCE when compared with group M11, it significantly increased ($p = 0.0357$) the %PCE relative to the latter group.

### *Single-cell gel electrophoresis assay (SCGE - comet assay)*

Table 2 shows the results of the comet assay in peripheral blood leukocytes of mice treated with OEP (0.5 mL $kg^{-1}$; group C3) and AEP (1 mL $kg^{-1}$; group C7). Neither OEP (C3) nor AEP (C7) significantly affected the DNA damage (DD) compared to group C1 (negative control), indicating that the extracts had no genotoxic effect in these cells. Likewise, there was no significant difference between C3 and C7. CP (30 mg $kg^{-1}$; C2) and BLM (25 mg $kg^{-1}$; C5) were significantly genotoxic when compared with C1 ($p = 0.0008$ and $p = 0.0016$, respectively). However, there was no significant difference between C1 and C4 (OEP 0.5 mL $kg^{-1}$ + CP 30 mg $kg^{-1}$) or C8 (AEP 1 mL $kg^{-1}$ + CP 30 mg $kg^{-1}$), indicating that at the doses tested OEP and AEP were efficient in protecting cells against the genotoxicity of CP (30 mg $kg^{-1}$). Comparison of C2 with C4 and C8 corroborated this protective effect ($p = 0.0008$ in both cases), although there were no significant differences between C4 and C8. When group C1 was compared with C6 (OEP 0.5 mL $kg^{-1}$ + BLM 25 mg $kg^{-1}$) or C9 (AEP 1 mL $kg^{-1}$ + BLM 25 mg $kg^{-1}$), only AEP showed significant protection ($p = 0.0134$). Similar protection was seen in the comparison of C6 with C9 ($p = 0.0273$) and C5 with C9 ($p = 0.0023$), indicating that AEP provided greater protection than OEP against bleomycin (25 mg $kg^{-1}$)-induced DNA damage; there was no difference between C5 (BLM 25 mg $kg^{-1}$) and C6.

### Antioxidant activity (TBARS assay)

There was no significant difference in the TBARS levels of female (group T1) and male (group T2) mice (Table 3). There were also no significant differences between T1 and T3, T3 and T5 or T4 and T6. However, mice treated with AEP showed significant lipid peroxidation ($p = 0.0248$ for T1 *vs.* T5, and $p = 0.0001$ for T2 *vs.* T6), indicating that AEP (1 mL $kg^{-1}$) enhanced oxidative stress in both sexes, especially in males. A similar result was observed between control males and males treated with OEP (0.5 mL $kg^{-1}$) (T2 *vs.* T4, $p = 0.00002$). Thus, at the doses used, neither of the extracts protected males against lipid peroxidation.

## Discussion

Micronuclei in interphase cells result from chromosomal breaks or chromosomal lagging (Schmid, 1975; Natarajan and Obe, 1982; FDA, 2000), and DNA damage to eukaryotic organisms or individual cells is frequently assessed with the comet assay (Henderson *et al.*, 1998; Garcia *et al.*, 2004). In the present study, the MN test and comet assay showed that OEP (0.5 mL $kg^{-1}$ or 1 mL $kg^{-1}$) had no clastogenic or genotoxic effect on mouse bone marrow cells or peripheral blood lymphocytes, a finding that corroborated the MN test results reported by Khouri *et al.* (2007).

Bleomycin sulphate (BLM), a radiomimetic glycopeptide routinely used in cancer chemotherapy, produces MN in human, rat and mouse cells, mainly through oxidative damage to DNA (Erexson *et al.*, 1995). Bleomycin-mediated DNA degradation requires the presence of a redox-active metal ion such as $Fe^{2+}$ or $Cu^{2+,}$ as well as molecular oxygen, to produce reactive oxygen species (ROS) that are toxic and mutagenic in a variety of biological models *in vitro* and *in vivo* (Hoffmann *et al.*, 1993; Anderson *et al.*, 1995; Erexson *et al.*, 1995; Hecht, 2000).

**Table 3** - Lipid peroxidation (TBARS levels) in plasma of Swiss white mice treated orally an organic (OEP, 0.5 mL $kg^{-1}$) or aqueous (AEP, 1 mL $kg^{-1}$) extract of pequi (*Caryocar brasiliense*) fruit pulp.

| Group | Treatment | Fluorescence (mean±SD) | MDA (μmol/L) (mean±SD) |
|---|---|---|---|
| T1 | Female control group (filtered water) | 311.16 ± 41.26 | 0.023 ± 0.003 |
| T2 | Male control group (filtered water) | 307.9 ± 18.17 | 0.023 ± 0.001 |
| T3 | Females treated with OEP (0.5 mL $kg^{-1}$, p.o., daily for 10 days) | 337.31 ± 39.59 | 0.025 ± 0.003 |
| T4 | Males treated with OEP (0.5 mL $kg^{-1}$, p.o., daily for 10 days) | 421.36 ± 46.29[a] | 0.031 ± 0.003 |
| T5 | Females treated with AEP (1 mL $kg^{-1}$, p.o., daily for 10 days) | 345.0 ± 28.42[b] | 0.026 ± 0.002 |
| T6 | Males treated with AEP (1 mL $kg^{-1}$, p.o., daily for 10 days) | 392.2 ± 39.95[c] | 0.029 ± 0.003 |

[a]$p = 0.00002$ compared to T2. [b]$p = 0.0248$ compared to T1. [c]$p = 0.0001$ compared to T2.

As shown here, the prior administration of OEP (1 mL $kg^{-1}$) clearly protected mouse bone marrow cells against lysis induced by BLM (50 mg $kg^{-1}$); a lower dose of OEP (0.5 mL $kg^{-1}$) also protected against the natural clastogenic effects, although the decrease in MN-NCE was not significant. Hence, both of the OEP doses used offered some protection against the clastogenicity of BLM. This finding was corroborated by the comet assay. AEP (1 mL $kg^{-1}$) offered greater protection against BLM (25 mg $kg^{-1}$)-induced DNA damage than OEP (0.5 mL $kg^{-1}$); the reduction in DNA damage by the latter was not significant.

Since the cytotoxicity of BLM involves the production of ROS (Hoffmann *et al.*, 1993; Anderson *et al.*, 1995; Erexson *et al.*, 1995; Hecht, 2000), our results suggest that the protection against BLM-induced oxidative damage to DNA was mediated by antioxidant components in the *C. brasiliense* extracts. Indeed, previous studies have indicated that compounds with antioxidant activities may modulate BLM-induced genotoxicity (Povirk and Austin, 1991; Povirk, 1996; Khouri *et al.*, 2007). The results of the comet assay corroborated those of the MN test and showed that the ability of the two extracts to protect against BLM was limited by the dose of extract used. The greater protection seen with AEP probably reflected its higher content of antioxidants compared to that of OEP at the doses used.

Chemotherapy with CP can cause secondary tumors in humans by activating hepatic mixed function oxidases. Phosphoramide mustard, the major antineoplastic metabolite of CP, is an alkylating agent that induces a variety of changes in DNA (IARC, 1981; Krishna *et al.*, 1987) through its ability to form labile covalent DNA adducts and cross-linkages (Anderson *et al.*, 1995).

Compounds with antioxidant properties, such as vitamin C (present in AEP) and β-carotene (present in OEP) can have antioxidant and oxidant effects that are dose-dependent (Antunes and Takahashi, 1999; Paolini *et al.*, 2003). This two-sided or Janus effect reflects the dual nature of certain chemical substances such as antioxidants and drugs used to treat cancer. As shown here, OEP had a Janus effect: at 0.5 mL $kg^{-1}$ OEP effectively protected cells against the genotoxicity of CP (comet assay) without significantly protecting against chromosomal aberrations (MN test) whereas at 1 mL $kg^{-1}$ OEP increased the frequency of MN in the presence of CP (30 mg $kg^{-1}$). Similar results were reported by Khouri *et al.* (2007) who studied the combination of AEP (1 mL $kg^{-1}$) plus CP (33 mg $kg^{-1}$ or 66 mg $kg^{-1}$).

With the treatment schedule used here, it was not possible to determine whether the protection offered by the extracts was related to a reduction in the efficacy of BLM or CP (by affecting the pharmacokinetics of these compounds), or to a direct effect on the cells themselves. However, previous studies have shown that natural antioxidants can prevent the oxidation of biomolecules, including DNA, without decreasing the effectiveness of chemotherapeutic compounds. According to Liu *et al.* (2003) and Berger (2005), an antioxidant dietary supplement can reduce the level of oxidative damage to DNA and protect normal cells against the adverse side-effects of some chemotherapeutic agents. Nutritional therapy with antioxidants concomitant with chemotherapy reduces the frequency and severity of adverse effects associated with many drugs in cancer patients, thereby allowing the treatment to be continued (the toxicity of anti-cancer drugs is a frequent limitation to their extended use) (Borek, 2004). As shown here, OEP and AEP protected against oxidative DNA damage caused by BLM or CP, which agrees with the ability of antioxidants to inhibit chemical mutagenesis *in vivo* and with studies showing that plant extracts contain antioxidants that can protect DNA against damage by ROS (Cano *et al.*, 2003; Lee *et al.*, 2003; Owen *et al.*, 2003; Khouri *et al.*, 2007). However, our results indicate that this protection is related to the dose of extract used.

Organisms are constantly exposed to the action of ROS and the presence of antioxidants in the diet can protect against oxidative processes (Ferreira and Matsubara, 1997; Degáspari and Waszczynskyj, 2004). In contrast, the use of nutritional supplements with antioxidant properties is controversial. Antunes and Takahashi (1999) showed that vitamin C had antimutagenic activity only at certain concentrations; at high concentrations this compound did not protect against mutations but was cytotoxic to lymphocytes. Paolini *et al.* (2003) reported that β-carotene prevented oxidative lesions in DNA but stimulated cytochrome P450 enzymes, leading to the bioactivation of pro-carcinogenic agents such as those found in cigarette smoke. In agreement with these studies, the OEP of pequi fruit may contain high concentrations of many antioxidant compounds capable of exerting a pro-oxidative effect and increasing DNA lesions when combined with CP or BLM.

The TBARS assay is a simple, reliable and reproducible fluorometric method for assessing lipid peroxidation in serum, and is based on the reaction between malondialdehyde (MDA), a byproduct of lipid peroxidation, and thiobarbituric acid. Oxidative attack of cellular components by ROS is a common phenomenon in the pathogenesis of several human diseases, including cardiovascular diseases (*e.g.*, atherosclerosis), diabetes, various liver disorders, and inflammatory rheumatic diseases. For this reason, there is considerable interest in the quantification of MDA in body fluids or cells (Wasowicz *et al.*, 1993; Jentzsch *et al.*, 1996). The TBARS results obtained here indicate that high doses of plant extracts rich in antioxidant compounds can produce unexpected effects. Since the 1980s, there has been a considerable increase in the search for natural antioxidants that can be included in the diet as a substitute for synthetic antioxidants (Degáspari and Waszczynskyj, 2004). The TBARS results obtained here in female mice suggest that the antioxidant properties of OEP

make this product a potentially useful dietary supplement, in addition to its other nutritional properties.

The anticlastogenic and antigenotoxic activities of OEP seen in this study were attributable to its content of antioxidants. However, the comet assay and MN test results obtained here and by Khouri *et al.* (2007) indicate that, at the doses used, AEP was more effective than OEP in protecting DNA against damage by clastogenic agents. However, the ability of OEP and AEP to protect against BLM- or CP-induced oxidative damage to DNA depended on the dose of extract used.

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