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Anticlastogenic potential and antioxidant effects of an aqueous extract of pulp from the pequi tree (*Caryocar brasiliense* Camb)

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Abstract

The effectiveness of an aqueous extract of *Caryocar brasiliense* (Caryocaraceae) Camb pulp, popularly known in Brazil as pequi, against clastogenicity induced by cyclophosphamide and bleomycin was evaluated using an *in vivo* mouse bone marrow cell micronuclei test, an *in vitro* Chinese hamster ovary cell (CHO-K1) chromosome aberration test and an *in vitro* antioxidant assay based on the oxidative damage to 2-deoxy-D-ribose (2-DR) induced by hydroxyl radicals ($\cdot\text{OH}$) generated by the reaction between ascorbic acid and (Fe III)-EDTA. In mouse bone marrow cells the extract showed a protective effect against micronuclei induced by cyclophosphamide and bleomycin but did not interfere with polychromatic bone marrow erythrocyte proliferation, except when the mice had been treated with the highest dose of cyclophosphamide. When CHO-K1 cells were pretreated by adding 0.01, 0.05 or 0.1 mL of extract per mL of cell culture medium 24 or 48 h before bleomycin or cyclophosphamide there was a protective effect against chromosome breaks and a significant decrease in the mitotic index (a measure of cytotoxicity) of the CHO-K1 cells. The extract also had a protective effect against oxidative hydroxyl radical damage to 2-DR. This study suggests that *C. brasiliense* pulp aqueous extract has anticlastogenic potential, possibly due to its antioxidative properties.

Key words: *Caryocar brasiliense*, anticlastogenicity, antioxidant, micronucleus.

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Introduction

Medicinal plants often represent the only therapeutic resource for many communities and ethnic groups. In the Brazilian savannah (the cerrado), *Caryocar brasiliense* Camb., popularly known as pequi, is a common tree frequently used in folk medicine to treat many types of ailment, such as influenza, asthma and other respiratory diseases. The pulp of the fruit of this tree is often used in the regional cooking in typical dishes such as “rice with pequi” and contains many compounds with antioxidant properties (Facioli and Gonçalves, 1998; Azevedo-Meleiro and Rodrigues-Amaya, 2004)

Due to the high biodiversity of tropical and subtropical areas Latin America has many plant foods rich in carotenes. However, particularly in Brazil, there is a serious threat that carotenoid-rich indigenous species may be supplanted by introduced crops with a lower carotenoid con-

tent and disappear (Rodrigues-Amaya, 1999). Natural antioxidant compounds in the human diet may prevent the genetic effects of mutagens and carcinogens and many studies have shown that an increased intake of fruits or vegetables rich in antioxidants decreases the level of oxidative DNA damage (Duthie *et al.* 1996; Collins *et al.* 2001; Dusinská *et al.* 2003). Extracts of the plant *Toxicodendron quercifolium* (poison-ivy, eastern poison-oak) and the mushroom *Agaricus blazei* (the Brazilian sun-mushroom) have shown antigenotoxic activity when examined using the *in vivo* mouse bone marrow micronucleus assay and the *in vitro* Chinese hamster ovary cell (CHO-K1) chromosome aberration assay (Mersch-Sundermann *et al.* 2004; Guterres *et al.* 2005; Bellini *et al.* 2006). Edenharter *et al.* (2002) used various test-systems for the evaluation of genotoxicity to demonstrate the protective effects of plant beverages, fruits, vegetables and spices against genotoxicity induced by 2-acetylaminofluorene or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and Edenharter *et al.* (2003) reported that homogenates of spinach, artichoke, peaches and black grapes reduced micronuclei (MN) induction in mouse bone marrow treated with benzo[a]pyrene.

Many studies with Brazilian plants used in folk medicine have shown antimutagenic properties due to a range of substances that act as antioxidants. Nascimento *et al.* (2006) reported that leaf extracts of *Turnera ulmifolia*, used in Brazilian folk medicine for their anti-inflammatory activity, reduce spontaneous lipid peroxidation in rat brain homogenates as measured using the thiobarbituric acid reactive substrates (TBARS – method, Janero 1990). Delmanto *et al.*, (2001) demonstrated that the edible mushroom *A. blazei*, consumed as a food or tea, has anticlastogenic activity against *in vivo* clastogenicity induced by cyclophosphamide. Nogueira *et al.* (2006) reported that the flower extract of *Melampodium divaricatum* (known in Brazil as the false-Calendula, or false-marigold) is used in Brazil as an anti-inflammatory agent and possess chemopreventive properties when tested in different strains of *Salmonella typhimurium*. Bleomycin sulfate is a glycopeptide antibiotic used in cancer chemotherapy and has been shown to be a potent radiomimetic antibiotic which induces micronuclei in humans, rats and mice through the induction of oxidative DNA damage (Erexson *et al.*, 1995). The active mechanism of bleomycin has been related to the activation of molecular oxygen in the presence of ferrous iron, producing oxygen radicals such as superoxide anions which are toxic and mutagenic in a variety of *in vitro* and *in vivo* test systems (Sausville *et al.* 1978; Albertini and Gurnier-Suillerot 1984; Sikic 1986). Cyclophosphamide is an alkylating agent used to treat a wide range of neoplastic diseases and also as an immunosuppressive agent prior to organ transplantation (Anderson *et al.* 1995). Hepatic mixed function oxidases activate cyclophosphamide, the major anti-neoplastic metabolite of cyclophosphamide degradation being phosphoramidate mustard which binds to DNA forming labile covalent DNA adducts and cross-links (Anderson *et al.* 1995). Cyclophosphamide has been widely used as a positive control in the single-dose mouse micronuclei assay because it induces a variety of changes in genetic material (IARC 1981; Krishna *et al.* 1987).

During the study described in this paper we assessed the anticlastogenic potential of *C. brasiliense* using the two known clastogens bleomycin and cyclophosphamide and the *in vivo* mouse bone marrow micronucleus assay and the *in vitro* Chinese hamster ovary cell chromosome aberration assay. We also assessed antioxidant properties using the degradation of 2-deoxyribose in the Fenton reaction assay.

Material and Methods

Materials

Fresh *Caryocar brasiliense* Camb (Caryocaraceae) fruits was purchased from a local market in January/February Brasilia and aqueous extracts obtained by extracting 100 g (wet weight) of the pulp in 1000 mL of distilled water using Soxhlet extraction for 10 h under an argon atmosphere. After extraction the aqueous extract (henceforth

called simply 'the extract') was immediately frozen at -85 °C and stored until needed. Bleomycin (Chemical Abstracts Service (CAS) registry number 011056-06-7) was obtained as Bleoxane sulfate (Bristol-Myers, USA) and cyclophosphamide (CAS 6055-19-2) as Genuxal (ASTA Medica Laboratory, Brazil). This project was approved by the Animal Ethics Committee of Institute of Biological Sciences of the University of Brasilia.

Swiss mouse micronucleus test

Swiss albino mice of both sexes (60 days old and weighing 30 ± 2 g) were obtained from the Central Animal Facility of the University of Brasilia and randomly allocated to groups M1 to M10 as shown in Table 1, each group consisting of eight mice. Extract was administered to the mice orally, either alone or in combination with single intraperitoneal injections of bleomycin diluted in NaCl 0.9% or aqueous cyclophosphamide, see Table 1 for dosage. A control group (M1) was given water in place of extract and no clastogens were administered. The animals were sacrificed by cervical dislocation and slides of bone marrow cells were prepared according to a standard method (Schmid, 1975). The frequency of micronuclei (MN) in polychromatic erythrocytes (PCE) and the ratio of polychromatic erythrocytes to total erythrocytes (%PCE) were estimated by scoring 1000 PCE per animal. Results were analyzed using the Mann-Whitney *U* test with $\alpha = 0.05$.

The 2-deoxyribose degradation assay

The formation of hydroxyl radicals ($\cdot\text{OH}$) by Fenton reagents was quantified based on the oxidative degradation of 2-deoxyribose (2-DR) and the quantification of its degradation product (malonaldehyde) by condensation with 2-thiobarbituric acid – TBARS method (Janero, 1990). The typical reaction was started by adding Fe(II) at a final concentration of 6 μM to a 0.5 mL final volume of 20 mM phosphate buffer, 5 mM of 2-deoxyribose, Cu(II) (5 μM) (pH = 7.2) and 100 μM H_2O_2 with and without 10 μM of ascorbate as an iron chelator. Reactions were carried out for 10 min at $25 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ and were stopped by adding of 0.5 mL of 50 mM NaOH containing 4% (w/v) phosphoric acid. After boiling for 15 min, the absorbance of the solution containing the 2-DR oxidation products was measured at 532 nm using a Hitachi U-2001 spectrophotometer. Assays were conducted by adding 10, 20, 30, 40 50, 100 or 200 μL of extract to the mixture described above (without ascorbate), pre-incubating for 30 min at $25 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ and then adding 100 μM ascorbate, incubating for 12 min and stopping the reaction and measuring the absorbance as described above. Spectra of the extract complexed with Fe(II) or Fe(III) were obtained by pre-incubating the complexes in the phosphate buffer described above for one hour and measuring the absorbance at 532 nm in the absence of iron against blanks of buffer and buffer containing the same

Table 1 - The number of polychromatic erythrocytes as a percentage of the total number of erythrocytes (the proliferation index) and the mean number of micronuclei in polychromatic and normochromatic erythrocytes of Swiss albino mice of both sexes treated orally with 0.03 mL of *Caryocar brasiliense* (pequi) aqueous extract, equivalent to 1 mL kg⁻¹ body weight (bw), alone or with a single intraperitoneal bleomycin or cyclophosphamide injection administered 20 h before sacrifice for bleomycin and 24 h before sacrifice for cyclophosphamide. Each group contained eight mice. Significant differences by the Mann-Whitney *U* test are shown for the polychromatic erythrocytes.

Group	Treatment	Polychromatic erythrocytes		Normochromatic erythrocytes
		Proliferation index	Mean number of micronuclei	Mean number of micronuclei
M1	Control (distilled water)	43.6	1.3 ± 0.3	1.3 ± 0.2
M2	Extract ¹ , 0.03 mL orally for 10 days	51.4	1.4 ± 0.4	2.1 ± 0.5
M3	Bleomycin, 50 mg kg ⁻¹ bw 20 h before sacrifice	61.2	3.4 ± 0.6*	1.1 ± 0.3
M4	Extract as for M2 then bleomycin as in M3	53.5	1.7 ± 0.3 [‡]	0.4 ± 0.2
M5	Bleomycin, 100 mg kg ⁻¹ bw. 20 h before sacrifice	55.2	9.0 ± 4.6*	3.0 ± 1.3
M6	Extract as for M2 then bleomycin as in M5	38.3	3.7 ± 3.2 [‡]	2.1 ± 0.8
M7	Cyclophosphamide 33 mg kg ⁻¹ bw 24 h before sacrifice	28.5*	28.4 ± 13.5*	4.6 ± 2.2
M8	Extract as for M2 then cyclophosphamide as in M7	38.7	18.2 ± 9.1 [#]	4.4 ± 3.6
M9	Cyclophosphamide 66 mg kg ⁻¹ bw 24 h before sacrifice	25.1*	54.7 ± 27.4*	5.8 ± 4.1
M10	Extract as for M2 then cyclophosphamide as in M9	16.7	39.7 ± 21.4	3.5 ± 3.0

¹Ratio of polychromatic erythrocytes to total erythrocytes. *significant at $p < 0.05$ compared to group M1. [‡]significant at $p < 0.05$ compared to group M3. [#]significant at $p < 0.05$ compared to groups M2 and M5. [†]significant at $p < 0.05$ compared to group M7.

concentration of extract. (Gutteridge, 1981; Halliwell *et al.*, 1987).

Chinese hamster ovary cell chromosome aberration assay

Chinese hamster ovary cells strain K1 (CHO-K1) were seeded in petri plates containing 10 mL of fresh complete HAM-F12 medium (Gibco) containing 10% (v/v) fetal calf serum (Gibco) and grown to 70% confluence before being subjected to the treatments listed in Table 2 as groups H1 to H9. A control group (H1) was mounted without extract or added clastogens. [See notes above.] Colchicine (Sigma) was added during the last 2 h of incubation and all the experiments were duplicated. Cultures were harvested and by incubating at 37 °C for 10 min in hypotonic 0.075 M KCl and then fixed in methanol/acetic acid (3:1 v/v). Slides were stained for 10 min with 10% (v/v) Giemsa. One hundred metaphases were analyzed for structural chromosome aberrations (Savage, 1976) and the results were statistically evaluated using the Mann-Whitney *U* test ($\alpha = 0.05$).

Results

The *in vivo* micronucleus test showed that when administered orally the extract (group M2) resulted in no statistically significant ($p = 0.9369$) increase in the number of micronuclei compared with the M1 water control group, indicating that the extract produced no clastogenic effect in respect to micronuclei frequency (Table 1).

There was also no statistically significant difference ($p = 0.1$) in the number of mouse polychromatic erythrocytes as a percentage of the total number of erythrocytes

(the proliferation index) between the M1 and the M2 groups (Table 1) but the proliferation index decreased in groups treated with extract plus 100 mg kg⁻¹ bw bleomycin (group M6, compared to groups M2 and M5) and extract plus cyclophosphamide 66 mg kg⁻¹ bw (group M10), indicating a cytotoxic effect on bone marrow cells (Table 1).

As expected, the groups administered clastogens without extract showed statistically significant increases in micronucleus frequency when compared to the M1 group and hence clastogenic effects (bleomycin: M3, $p = 0.0001$; M5, $p = 0.0093$. cyclophosphamide: M7, $p = 0.0001$; M9, $p = 0.009$.) and there was a marked increase in the number of micronuclei in group M5 cells, treated with 100 mg kg⁻¹ bw bleomycin, as compared to group M3 treated with 50 mg kg⁻¹ bw bleomycin (Table 1).

For the groups administered extract followed by bleomycin (groups M4 and M6) there was a significant decrease in the micronucleus frequency compared to their respective bleomycin-only controls ($p = 0.009$ for M4 compared to M3; $p = 0.0001$ compared to M2 and M5), indicating that the extract was anticlastogenic against bleomycin-induced chromosome damage (Table 1). The M8 group administered extract followed by 33 mg kg⁻¹ cyclophosphamide showed a statistically significant decrease ($p = 0.009$) in the micronuclei frequency compared to the M7 control group. However, the extract showed no statistically significant protective effect ($p = 0.0007$) for the M10 group, administered extract plus 66 mg kg⁻¹ bw cyclophosphamide, compared to the M9 matching cyclophosphamide control group (Table 1). The micronucleus frequency induced by cyclophosphamide in the M8 and M10 groups pre-treated with extract remained high, while the proliferation index was

Table 2 - Number of chromosome aberrations and percentage mitotic index (MI) recorded in Chinese hamster ovary cells (CHO-K1) subjected to different *in vitro* treatments and treatment times with *Caryocar brasiliense* (pequi) aqueous extract, with or without the clastogens cyclophosphamide and bleomycin. Aliquots of the extracts were sterilized using a Millipore 0.2 mm nitrocellulose membrane before addition to the medium. Significant differences by the Mann-Whitney *U* test are shown for the total aberrations and the percentage MI. Aberration key: F = fragment; D = dicentric; B' = chromatid break; B'' = chromosome break; CB = centromere break; and G' = chromatid gap.

Group	Treatments	Chromosome aberrations						Total	MI (%)
		F	D	B'	B''	CB	G'		
H1	Control (distilled water), assessed after 48 h	08	01	04	02	00	01	16	2.8
H2	Extract 0.05 mL mL ⁻¹ added 24 h before assessment	05	02	00	01	01	02	11	1.2 [#]
H3	Extract 0.05 mL mL ⁻¹ added 48 h before assessment	08	00	01	02	05	04	20	1.1 [#]
H4	Extract 0.01 mL mL ⁻¹ added 48 h before assessment	16	00	01	00	00	00	17	1.9 [#]
H5	Extract 0.1 mL mL ⁻¹ added 24 h before assessment	13	00	02	00	00	04	19	1.4 [#]
H6	Bleomycin 10 µg mL ⁻¹ added 20 h before assessment	22	01	00	10	00	03	36	1.3 [#]
H7	Extract 0.1 mL mL ⁻¹ added 48 h before assessment plus bleomycin 10 µg mL ⁻¹ added 20 h before assessment	17	01	01	02	00	03	24 [†]	1.0 [#]
H8	Cyclophosphamide 6 µg mL ⁻¹ added 24 h before assessment	25	00	00	01	07	00	33	1.4 [#]
H9	Extract 0.1 mL mL ⁻¹ added 48 h before assessment plus cyclophosphamide 6 µg mL ⁻¹ added 24 h before assessment	05	01	01	00	03	01	11 [‡]	0.8 [#]

[†]significant at $p < 0.05$ compared to group H6. [‡]significant at $p < 0.05$ compared to group H8. [#]significant at $p < 0.05$ compared to group H1.

lower for both these groups than for the M1 water controls (Table 1).

In the *in vitro* Chinese hamster cell (CHO) experiments, there was also no evidence of chromosome aberrations indicative of clastogenicity in the H2, H3, H4 and H5 groups treated with extract as compared to the H1 control (Table 2) and the mitotic index of groups H2 to H5 was significantly lower ($p < 0.05$) than that of the H1 control, indicating that the extract possessed antiproliferative activity (Table 2).

Our results indicate that bleomycin and cyclophosphamide were effective as positive DNA damage controls for the CHO-K1 method. The H7 group treated with extract followed by bleomycin showed a significantly lower total number of chromosome aberrations as compared to the H6 group treated with bleomycin only and, similarly, the H9 group treated with extract followed by cyclophosphamide showed a significantly lower total number of chromosome aberrations compared to the H8 group treated with cyclophosphamide only (Table 2). The mitotic index of the cells in groups H2 to H9 were all significantly lower than the H1 control (Table 2)

We also found that the extract inhibited the Fenton reaction, decreased the formation of hydroxyl radicals and reduced the oxidative degradation of 2-DR (Figure 1), probably by removing Fe(III) from EDTA by the formation of a complex with iron which is then unavailable for participation in the Fenton reaction.

Discussion

The *C. brasiliense* pulp was not analyzed by us but the main antioxidant, carotenoid and mineral composition of *C. brasiliense* has been investigated qualitatively and

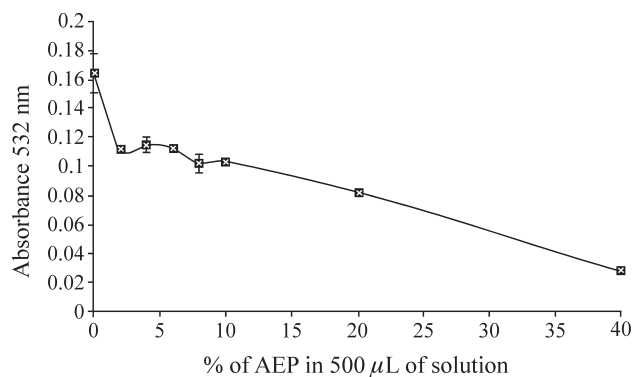


Figure 1 - Kinetics of the oxidation inhibition of 2-deoxyribose by hydrogen peroxide (H_2O_2). Changes in absorbance ($\lambda = 532$ nm) mean trapping action of AEP on hydroxyl radicals. Absorbencies were read every 5 min for 40 min.

quantitatively using high performance liquid chromatography (Azevedo-Meleiro and Rodriguez-Amaya 2004) and the fat and vitamin composition described using gas chromatography (Facioli and Gonçalves 1998), this data being summarized Table 3.

We found that *C. brasiliense* aqueous extract showed anticlastogenic activity and was able to inhibit bleomycin-induced DNA damage in mice (Table 1). Bleomycin causes various types of oxidative DNA lesions by generating reactive oxygen species (ROS) (Povirk, 1996), which have been shown to cause DNA damage, gene mutation, micro-nuclei formation and chromosome aberrations (Imlay and Linn, 1988; Gutteridge, 1993; Schiffel *et al* 1997; Cooke *et al.*, 2003).

We found that the extract decreased the formation of hydroxyl radicals and reduced the oxidative degradation of

Table 3 - Main antioxidant compounds, minerals and vitamins present in *Caryocar brasiliense* (pequi) pulp.

Fatty acids (grams per 100g pulp)		Minerals (mg 100 g ⁻¹ pulp)		Vitamins* (mg per 100g of pulp)		Carotenoids (unquantified)
Oleic acid	53.9	Fe	800	Vitamin C	12	β-carotene
Palmitic acid	40.2	Ca	50	Riboflavin	0.46	β-cryptoxanthin
Stearic acid	2.3	Na	2.1	Niacin	0.39	Lutein
Linoleic acid	1.5	Mn	0.6	Thiamin	0.03	Neoxanthin
Palmitoleic acid	1.4	Zn	0.5	Vitamin A	20000 U*	Violaxanthin
Linolenic acid	0.7	Cu	0.4			Zeaxanthin
Araquidic acid	0.2	K	0.02			
		P	0.006			
		Mg	0.005			

*Except for vitamin A, which is in units per 100 g. Source: fatty acids from Facioli and Gonçalves (1998); minerals, vitamins and carotenoids from Azevedo-Meleiro and Rodrigues-Amaya (2004).

2-DR, so it is reasonable to assume that the anticlastogenic effect of the extract against bleomycin was due to its antioxidant properties. This is supported by the fact that the micronuclei induced by bleomycin are mainly due to the generation of ROS, so compounds with antioxidative activities could presumably modulate bleomycin-induced genotoxicity (Povirk and Austin, 1991; Povirk, 1996).

Cyclophosphamide clearly induced a dose-related bone marrow micronucleus response in mouse polychromatic erythrocytes and bone marrow toxicity and the *in vivo* treatment with 66 mg kg⁻¹ bw cyclophosphamide, either alone or in combination with the equivalent of 1 mL kg⁻¹ bw of the extract, was toxic, as shown by the decreased proliferation index (Table 1). In genotoxicity evaluations as well as antimutagenic and/or anticlastogenic studies of chemicals, the treatments should not be carried out at levels that are toxic to the cells or animals, to avoid cell-cycle inhibition. However, if no suitable data are available, a range finding is performed because dose levels should cover a range from the maximum to little or not toxicity (US-EPA, 1998). Since cyclophosphamide is an alkylating agent that is metabolized to phosphoramidate mustard which damages DNA by forming labile covalent DNA adducts and cross-links while bleomycin produces hydroxy radicals the fact that in this study the extract was a more efficient anticlastogen against bleomycin than cyclophosphamide supports the hypothesis that antioxidants in the extract inhibited chemical mutagenesis *in vivo*. These results are in agreement with many other studies showing that ingredients present in plant extracts have antioxidant properties which can protect DNA against damage caused by free radicals (Owen *et al.*, 2003; Cano *et al.*, 2003; Lee *et al.*, 2003). In the CHO-k1 cell experiments the reduced mitotic index indicated that bleomycin, cyclophosphamide and extract induced a substantial delay in the cell-cycle, while the pretreatment of cells with extract before the addition of bleomycin or cyclophosphamide depressed the mitotic index even further. This interference with the cell-

cycle would be expected to result in a lower yield of chromosome aberrations. The indirectly-acting mutagen cyclophosphamide must be metabolically transformed to interact with DNA but in our system the CHO-k1 cells were sensitive to cyclophosphamide even without the S-9 mix activation system. This data supports the observations of some workers who unexpectedly reported that cyclophosphamide exhibited *in vitro* clastogenic activity in cultured rat, rabbit and human lymphocytes without exogenous metabolic activation (Ikeuchi and Sasaki, 1981; Abe *et al.*, 1983; Dearfiled *et al.*, 1983), raising the question of whether or not the observed clastogenic effects were due to the spontaneous hydrolysis of cyclophosphamide known to occur at temperatures above 30 °C (Mohn and Ellenberger, 1976; Anderson 1995). In our study, *in vitro* anticlastogenicity was observed in conjunction with specific cytotoxicity as shown by the significant reduction in the mitotic index as compared to the H1 untreated control (Table 2). However, our *in vivo* study indicated more clearly that the extract had a protective effect against bleomycin-induced oxidative damage. According to Liu *et al.* (2003) and Berger (2005), an antioxidant dietary supplement can reduce the level of DNA oxidative damage and protect normal cells against the adverse side-effects of some cancer chemotherapy protocols.

The 2-deoxyribose degradation assay supports the data (Figure 1) on the *in vivo* and *in vitro* antioxidant effect of the extract and its ability to scavenge hydroxyl radicals. The fact that the extract was able to reduce Fe(III) to Fe(II) suggests that redox cycling of Fe(III) formed by the Fenton reaction could have occurred and that the extract was able to prevent the generation of the hydroxy radicals and 2-deoxyribose oxidative damage induced by Fenton reagents. The kinetics of the inhibition of 2-deoxyribose oxidation by H₂O₂ are shown in Figure 1, which shows the decrease in absorbance and hence the mean trapping action of the extract on hydroxyl radicals. This mechanism has been used for the quantification of antioxidant properties of many

compounds present in fruits and vegetables (Lopes, 1999). Our observations on the antioxidant activity of *C. brasiliense* aqueous extract may in part explain the anti-clastogenic potential of this compound.

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