Journal of Medicinal Plants Research Vol. 5(14), pp. 3277-3285, 18 July, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Reduction of DNA-damage by *Croton cajucara* methanolic extract but not the testicular alterations induced by doxorubicin

Breno Henrique Caneguim[1]*, Juliana Mara Serpeloni[1], Maria Aparecida Medeiros Maciel[2], Ilce Mara de Syllos Cólus[1] and Suzana de Fátima Paccola Mesquita[1]

[1]Departamento de Biologia Geral, Centro de Ciências Biológicas, Universidade Estadual de Londrina (UEL), Londrina, PR, Brazil.
[2]Departamento de Química, Centro de Ciências Exatas, Universidade Federal do Rio Grande do Norte (UFRN), Natal, RN, Brazil.

Accepted 21 April, 2011

Many tests have been done to ameliorate the side effects caused by doxorubicin (DXR) chemotherapy. *Croton cajucara* is a plant that presents several biological beneficial properties. The aim of this study was to verify whether *C. cajucara* methanolic extract (EMeOH) prevents the DXR-induced testicular alterations and whether presents mutagenic or antimutagenic activities. Mice received DXR combined or not with EMeOH (312.5, 625 or 1250 mg/Kg) for 5 weeks. The fertility index and the micronucleous assay were realized in the male mice. The testes were removed and embedding in paraffin for the stereological and morphometric analyses. There were significant reductions in all parameters analyzed in the mice treated with DXR alone or with EMeOH. The plant extract doses evaluated presented no mutagenicity, antimutagenic properties and reduction of the DNA damage. Thus, EMeOH did not ameliorate the DXR-testicular damage, but the mutagenicity tests indicate a possible chemoprotective action of this plant extract.

Key words: Croton, testis, seminiferous tubules, spermatogenesis, chemotherapy, doxorubicin, mutagenicity tests.

INTRODUCTION

The chemotherapic doxorubicin (DXR), an anthracycline antibiotic commonly used to treat a wide variety of cancers (Quiles et al., 2002), is a highly toxic neoplasic agent that interferes on the cellular division, due to its feature of intercalating with DNA causing irreversible lesion (Suter et al., 1997). The high frequency of cellular divisions that occurs in the cells of the seminiferous epithelium, makes the testis a highly vulnerable organ to the chemotherapeutical drugs (Russel and Russel, 1991). Moreover, several studies have reported alterations induced by DXR, like reduction of testicular weight, alterations in the germ cell lineage, as well as in the spermatozoa production and morphology (Lu and Meistrich, 1979; Suter et al., 1997; Vendramini et al., 2010). In order to protect spermatogenesis from toxicant exposure, many clinical and experimental trials have been tested (Endo et al., 2003; Ateşşahin et al., 2006; Yeh et al., 2007), but if these therapies could prevent or reverse doxorubicin testicular toxicity remains unclear. However, it is known that superior plants present an infinite variety of secondary metabolites and that represent a diversity of mutagenic, bioantimutagenic and desmutagenic substances. Thus, it is possible that some antimutagenic compounds of plants have some therapeutic use (Wall et al., 1988). *Croton cajucar* Benth. is an arboreal or bushy plant of the Euphorbiaceae family, that can be found in the Amazon region in the northern part of Brazil, and that is locally known as "sacaca" (Di Stasi et al., 1989). It is widely used in the

*Corresponding author. E-mail: brenohc@gmail.com. Tel: +55 16 3301-6493. Fax: +55 16 3301-6433.

Abbreviations: DXR, doxorubicin; **EMeOH,** *Croton cajucara* methanolic extract; **DCTN,** clerodane diterpene trans-dehydrocrotonin; **DMSO,** dimetyl sulfoxide solution.

Table 1. Groups of treatment and their respective treatments and routes of administration.

Groups	Treatments	Routes
Controls		
GI	Negative: water	GA
GII	Solvent: 2 DMSO:1 water	GA
GIII	Positive: DXR (16 mg/Kg b.w.)	IP
EMeOH (mg/Kg b.w.)		
GIV	312.5	GA
GV	625	GA
GVI	1250	GA
EMeOH (mg/Kg b.w.) + DXR (16 mg/Kg b.w.)		
GVII	312.5 + DXR	GA/IP
GVIII	625 + DXR	GA/IP
GIX	1250 + DXR	GA/IP
Solvent + DXR (16 mg/Kg b.w.)		
GX	2 DMSO:1 water + DXR	GA/IP

All solutions were administrated at 0.1 ml/10 g b.w.; GA: gavage; IP: intraperitoneal.

popular medicine to the treatment of various diseases like diabetes, diarrhea and fever as well as stomach, hepatic and kidney problems and it is also used to control high cholesterol levels (Costa et al., 2007). Maciel et al. (1998) reported that the majority compound isolated from stem-bark extract of adult *C. cajucara* plants is the clerodane diterpene *trans*-dehydrocrotonin (DCTN). As revised by Costa et al. (2007), DCTN has been reported to present antiulcer, anti-inflammatory, hypoglycemic, antimutagenic, and antitumoral properties. Thus, considering that the DXR is a mutagenic agent and induces impairment of spermatogenesis and that the *C. cajucara* extract has been shown to have antimutagenic activities, the aim of this study was to evaluate the effects of *C. cajucara* methanolic extract (EMeOH) in preventing the testicular damage and to assess the antimutagenic properties in mice submitted to subchronic treatment with DXR.

MATERIALS AND METHODS

Animals

Fifty male albino Swiss mice (*Mus musculus*) and one hundred female albino Swiss mice, aged five to six week-old and weighing 30 g, were obtained from the mouse breeding colony at State University of Londrina (Paraná – Brazil) and distributed in polypropylene cages. The animals were maintained under 23±2°C and standard light conditions (12 h light/12 h dark). Water and food were provided *ad libitum*. All the protocols for animal care and experimental procedures were conducted according to the Canadian Council on Animal Care (Olfert et al., 1993).

Plant extract

The EMeOH obtained from the bark of a native *C. cajucara* Bent., collected in Jacunda (Pará, Brazil), was used. The plant was identified by Nelson A. Rosa, and a voucher specimen (n. 247) was deposited in the Emilio Goeld Paraense Museum (Belém, Pará, Brazil). The methanolic extraction was carried out on ground bark (in powder) through Soxhelt, for 48 h. 6 kg of powder and 46 L of methanol were used in the extraction, resulting in 202 g of the methanol extract. Then, this extract was diluted in a dimethyl sulfoxide solution (DMSO) plus water at a ratio 2:1 and administrated to the animals, through gavage, at doses of 312.5, 625 or 1250 mg/Kg of body weight (b.w.). The evaluated doses were chosen according to previous studies of Santos et al. (2006, 2008).

Doxorubicin

Doxorubicin (Adriblastina®, Pharmacia and Upjohn, Italy) was utilized as positive-control in a dose of 16 mg/Kg b.w. and administrated to the mice intraperitoneally (Guevara et al., 1996; Resende et al., 2006).

Treatment of the animals

The mice were distributed into 10 groups, with 5 male mice in each group, according to Table 1. A negative, a positive and a solvent control group were established for the treatment of the animals with distilled water, doxorubicin (16 mg/Kg b.w.) and 2 DMSO:1 H_2O, respectively. The testicular alterations and the mutagenicity of the extracts were assessed in three different doses: 312.5; 625 and 1250 mg/Kg b.w., through gavage. The same doses of EMeOH were used to assess whether the testicular damage caused by DXR were softened, as well as to evaluate the antimutagenic activity. Doxorubicin was administrated in a single dose, intraperitoneally,

one hour after the extract was administered through gavage. All the animals received 0.1 ml/10 g b.w. of each solution once a week for 5 weeks.

Fertility index

To assess the fertility index, each male-treated animal was mated with two untreated virgin females one week before the end of the treatment. The presence of vaginal plug was considered such as early pregnancy. The female mice were euthanized at 18th day of pregnancy by cervical dislocation and their uterine horns were removed. The numbers of live fetuses and reabsorptions were recorded.

Histological procedures

After 24 h of the last dose administration, the male mice were anesthetized with an intraperitoneal injection of 0.08 ml/100 g b.w. of 10% hydrochloride ketamine (Syntec do Brasil Ltda.) and 0.02 ml/ 100 g b.w. of 2% hydrochloride xylazine (Syntec do Brasil Ltda.). The testes were removed, weighed and the animals were euthanized by an overdose of anesthesia, intraperitoneally. Then, the testes were fixed in Bouin´s liquid, dehydrated in crescent alcoholic solution and embedded in Paraplast®. The sections (5 µm) were stained by H.E. and submitted to stereologic and morphometric analyses.

Stereological and morphometric analyses

After testes removal from the scrota, they were weighted and their total volumes were obtained by Scherle´s method (Sherle, 1970). The volume densities (Vv) of seminiferous tubules (VvSt) and interstitial tissue (VvIt) were obtained. For this purpose, 30 random fields from three non-serial testicular sections per animal were analyzed using a 100-point integrating eyepiece attached to a light microscope at x400 magnification. So, the number of points upon seminiferous tubules and interstitial tissue was accounted, totalizing 3,000 points per animal (Weibel, 1963). Then, the percentages values of VvSt and VvIt were determined. The average of seminiferous tubules diameter was measured using an ocular micrometer attached to a light microscope at x100. For this, 50 random round tubular cross-sections per animal were analyzed from three non-serial sections. In this sense, to determinate the length of the seminiferous tubules (LSt) in these tubules, the following formula was used:

$$LSt = VvSt/\pi R^2$$

Where VvSt = volume density of seminiferous tubules and R = tubular diameter/2 (Attal and Courot, 1963; Dorst and Sajonski, 1974).

Micronucleus test

To evaluate the mutagenic and antimutagenic effects of EMeOH of *C. cajucara*, the micronucleus test on peripheral blood mice cells was performed according to Hayashi et al. (1990). The blood was obtained by perforating the caudal vein of the mice with a needle once a week before each treatment (T0, T1, T2, T3 and T4) and 24 h after the last treatment (T5). The collected blood was placed at the centre of pre-stained glass slides with 10 µl of acridine orange (1 mg/ml). These slides were maintained in the dark at -20°C, for a minimum of 24 h, prior to cytological examination of blood cells. The cellular preparations were examined under a fluorescence microscope, with a blue (488 nm) excitation filter and yellow (515 nm) emission (barrier filter), using an immersion objective. One thousand reticulocytes per each treated animal were analyzed and the proportion of micronucleated cells (MNRET) was determined. The EMeOH capacity of reducing damage caused by DXR was calculated by the formula:

$$\% R = A-B/A-C \times 100$$

Where %*R* is the reduction percentage; *A* is the MNRET frequency after treatment with DXR; *B* is the MNRET frequency after treatment with EMeOH of *C. cajucara* and DXR; and *C* is the MNRET frequency after treatment with water (Waters et al., 1990).

Statistical analysis

To assess the stereologic and morphometric data, frequency of micronucleated cells, as well as the fertility index, the parameters were submitted to Analyses of Variance (ANOVA) and the Tukey-test at 95% of significance.

RESULTS

Stereologic analyses

According to Table 2, the testicular volume of DXR-untreated animals did not present significant difference between them. However, this stereologic parameter was statistically reduced when the animals which received only DXR, DXR+EMeOH or DMSO+DXR, were compared with the groups of DXR-untreated animals. It is important to emphasize that the testicular volume of animals treated with DXR plus EMeOH, independently on the concentration, showed significant reduction when compared to their respective control groups. In respect to volume densities, the same statistical results were found in VvSt; in other words, there were statistical differences between DXR-treated mice and DXR-untreated-animals. In VvIt results, DMSO-treated group and mice treated with 312.5 and 625 mg/Kg b.w. of EMeOH resulted in a significant increase in this parameter when compared to negative (GI) and positive-controls (GIII). It is also important to note that between the animals which received doxorubicin plus EMeOH, only GVII and GVIII showed significant reduction compared to their control animals which were treated with EMeOH of *C. cajucara* in 312.5 and 625 mg/Kg, respectively (Table 2).

Light microscopy

All animals that received different dosages of EMeOH of *Croton cajucara*, as well as those treated only with DMSO showed normal morphology of seminiferous tubules like negative group (Figures 1a to e). In the seminiferous tubules of these animals was observed that the seminiferous epithelium was constituted by concentric layers of germ cells and that the tubular lumen was empty (Figures 1a and b). In the epithelium was observed

Table 2. Testicular volume, volume densities of seminiferous tubules (VvSt) and interstitial tissue (VvIt) of animals from different groups of treatment.

Groups	Testicular volume (ml)	VvSt (ml)	VvIt (ml)
GI	0.116±0.009[a]	0.107±0.000[a]	0.009±0.007[a]
GII	0.114±0.011[a]	0.068±0.007[a]	0.046±0.005[b]
GIII	0.042±0.025[b]	0.019±0.001[b]	0.011±0.021[a]
GIV	0.118±0.008[a]	0.090±0.008[a]	0.030±0.005[b]
GV	0.098±0.004[a]	0.064±0.008[a]	0.034±0.008[b]
GVI	0.108±0.016[a]	0.090±0.017[a]	0.018±0.004[a]
GVII	0.044±0.008[b]	0.026±0.055[b]	0.017±0.005[a]
GVIII	0.042±0.022[b]	0.023±0.012[b]	0.018±0.004[a]
GIX	0.048±0.025[b]	0.023±0.012[b]	0.011±0.005[a]
GX	0.040±0.008[b]	0.027±0.005[b]	0.010±0.006[a]

M±SD followed by the same letter represent statistically identical values in the columns (p≤0.05).

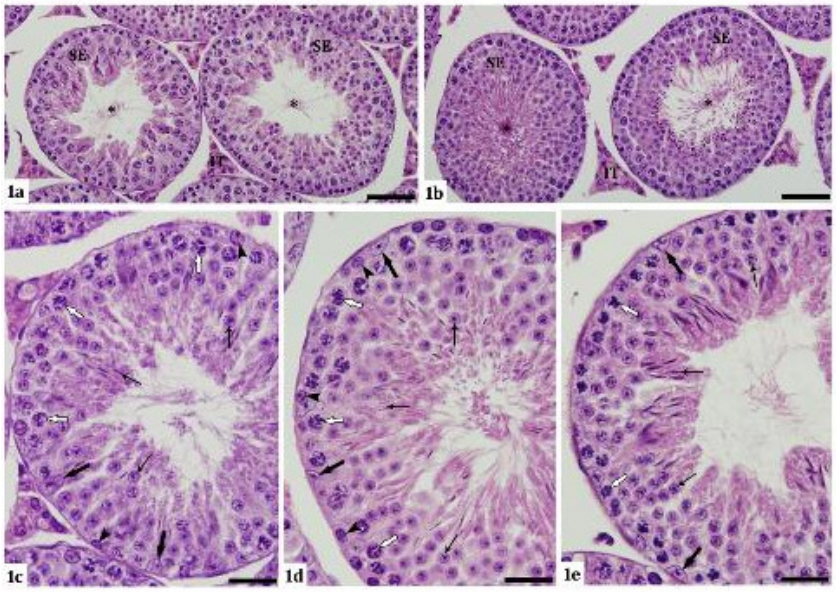

Figure 1. Photomicrographs of seminiferous tubule sections of mice from GI (a), GII (b), GIV (c), GV (d) and GVI (e) groups stained by H.E. In 1a and b, the seminiferous tubules with normal aspect show germ cells organized in concentric layers constituting the seminiferous epithelium (SE). Tubular lumen (asterisks). Interstitial tissue (IT). 1c, d and e: All the animals treated with different dosages of EMeOH of *Croton cajucara* (312.5 mg/Kg – 1c; 625 mg/Kg – 1d; 1250 mg/Kg – 1e) showed organized seminiferous epithelium with normal morphology like GI and GII groups. The Sertoli cell nuclei (black arrows) are positioned adjacent to peritubular tissue, in the basal compartment. Spermatogonia (arrowheads), primary spermatocytes (white arrows) and round/elongated spermatids (thin arrows) are observed in concentric layers on the seminiferous epithelium. Bars: Figures 1a and b: 55 µm; Figures 1c to e: 30 µm.

that typical triangle/ovoid Sertoli cell nuclei were adjacently positioned to the peritubular tissue in the basal compartment. Moreover, all the different cells of Res. germinative lineage, that is, spermatogonia, spermatocytes and round/ elongated spermatids were observed, in concentric layers, in all the seminiferous tubules (Figures 1c to e). The seminiferous tubules of the DXR-group (Figure 2a) and DXR plus DMSO-treated animals (Figure 2b) showed intense epithelial depletion and extreme disorganization of seminiferous epithelium. The same alterations were found in the animals which received the chemotherapeutical agent with EMeOH,

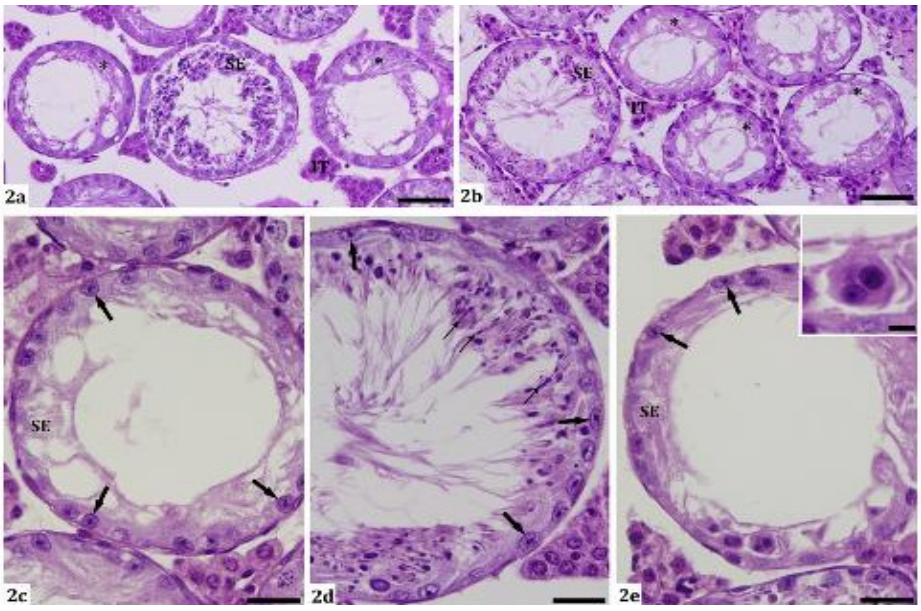

Figure 2. Photomicrographs of seminiferous tubule sections of mice from GIII (a), GX (b), GVII (c), GVIII (d) and GIX (e) groups stained by H.E. The doxorubicin (2a) and doxorubicin plus DSMO (2b) treated animals showed tubules with disorganized seminiferous epithelium (SE) or accentuated epithelial depletion (asterisks). The animals treated with doxorubicin plus the different dosages of EMeOH of *Croton cajucara* (312.5 mg/Kg – 2c; 625 mg/Kg – 2d; 1250 mg/Kg – 2e) presented the same alterations found in the GIII and GX groups. In these animals, the seminiferous tubules were formed by only Sertoli cells (arrows) and none germ cells were observed in the seminiferous epithelium (SE) (Figures 2c and e). Some tubules with discontinuous and disorganized epithelium with disarranged spermatids (thin arrows) also were found (Figure 2d). In all animals was common to find multinucleated formation (Figure 2e). Bars: Figures 2a and b: 55 µm; Figures 2c to e: 30 and 5 µm.

independently on the dosages (Figures 2c to e). Two different morphological aspects of seminiferous tubules were seen in these animals. The majority of seminiferous tubules presented accentuated cellular depletion in the epithelium in which none germ cells were observed; only Sertoli cell nuclei were found, feature of typical Sertolization (Figures 2c and e). The other tubules showed discontinuous and disorganized epithelium, in which only round and elongated spermatids were noted in a disarranged manner (Figure 2d). Moreover, in all DXR-treated animals, multinucleated formation of round spermatids were the most common alteration observed (Figures 2e).

Morphometric analyses

In Table 3, the measurement of the diameter of seminiferous tubules revealed that the animals from DXR group and those treated with different dosages of EMeOH of *C. cajucara* plus doxorubicin showed significant reduction in relation to the animals from negative-control and the animals treated only with EMeOH.

Considering the tubular length, the animals that received doxorubicin plus EMeOH, independently of the dosages, presented a significant reduction when compared to negative control group. However, only the GIX group animals showed significant difference when compared to their respective control group (GVI).

Fertility index

In Figure 3, we can observe that all the animals were fertile. However, the animals who received DXR, in association or not with EMeOH or DMSO, presented reduction in the number of fetuses as well as increase in the number of reabsorptions in comparison to the negative control group.

Micronucleus test

According to Table 4, the mean of MNRET frequencies observed after using three different concentrations of EMeOH of *C. cajucara* in mice were statistically similar to those obtained in the negative control group (GI); that is, they presented no mutagenic effect. However, the animals treated with all the concentrations of EMeOH

Table 3. Diameter and length of seminiferous tubules (DSt and LSt) of animals from different groups of treatment.

Groups	DSt (µm)	LSt (µm)
GI	196±9.089[a]	3.52±0.259[a]
GII	173±26.310[a]	3.00±0.818[a]
GIII	153.5±31.06[b]	1.26±0.123[b]
GIV	220±13.084[a]	2.34±0.356[a]
GV	175±10.300[a]	2.43±0.398[a]
GVI	176±9.298[a]	3.64±0.503[a]
GVII	136±12.595[b]	1.90±0.636[b]
GVIII	137±9.216[b]	1.40±0.291[b]
GIX	135±8.400[b]	2.14±1.402[b]
GX	137±6.65[b]	1.8760.546[b]

M±SD followed by the same letter represent statistically identical values in the columns (p≤0.05).

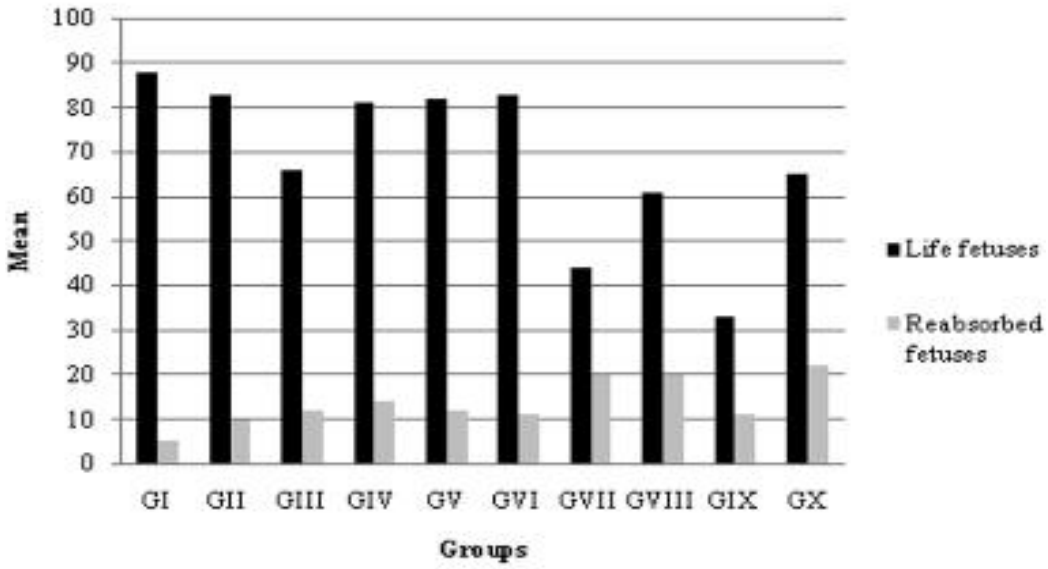

Figure 3. Representative histogram of fertility index.

plus DXR presented antimutagenic effect, since the mean MNRET frequencies were statistically different from those obtained in the positive-control group (GIII). The percentage reductions in DNA damage obtained in the micronucleus test after treatments with different concentrations of EMeOH of *C. cajucara* were calculated and are presented in Table 5. The reductions in micronucleated cells were more significant in the groups treated with the 625 or 1250 mg /Kg b.w. concentration of the extract of *C. cajucara* and less significant for the 312.5 mg/Kg b.w. concentration of the extract.

DISCUSSION

Early experimental evidences suggest that the occurrence of testicular damage and, consequently, permanent or transitory sterility in animals of different ages were results of treatment with different doses of anthracyclinic antibiotics (Au and Hsu, 1980; Ward et al., 1988; Meistrich et al., 1990). Between them, DXR, the most common antineoplasic drug used in the treatment of many solid tumors, causes several adverse effects in male fertility (Meistrich et al., 1982). Tsunenari et al. (2000) described that the testicular toxicity of DXR could be detected 2 weeks after a single intravenous administration, including decrease of the testicular weight without causing toxicity on normal tissue. Based on this and histopathological disorders. A perfect chemotherapeutic treatment would selectively attack tumor cells concept, a great amount of studies have been done to decrease the cytotoxicity of these drugs (Vaisheva et al., 2007; Vendramini et al., 2010), including the actions of plant extracts (Yeh et al., 2009). The alteration in the measurements of stereological and morphometrical testicular data indicates that there was injury in the

Table 4. Frequency of micronucleated reticulocytes of the peripheral blood from mice of different treatment groups.

Groups	Time sampling					
	T0	T1	T2	T3	T4	T5
GI	1.2±0.92[a]	1.0±0.94[a]	0.9±0.74[a]	1.1±0.74[a]	1.1±0.74[a]	0.9±0.74[a]
GII	1.4±0.84[a]	1.3±0.95[a]	1.0±0.94[a]	1.2±0.94[a]	1.6±1.13[a]	1.3±1.0[a]
GIII	3.0±1.56[b*]	22.9±5.53[b]	13.5±2.22[b]	16.1±1.79[b]	20.9±4.04[b]	23.6±9.05[b]
GIV	1.2±0.9[a]	1.5±1.3[a]	0.9±0.7[a]	1.2±0.8[a]	1.3±0.9[a]	1.3±0.9[a]
GV	1.0±0.82[a]	0.7±0.68[a]	0.9±0.99[a]	1.4±0.73[a]	1.3±0.71[a]	1.6±1.19[a]
GVI	0.9±0.74[a]	0.9±0.74[a]	1.0±0.82[a]	1.4±0.73[a]	1.0±1.0[a]	1.4±0.73[a]
GVII	1.9±1.27[a*]	12.1±2.09[c*]	8.3±2.24[c]	7.9±2.23[c]	8.4±2.01[c]	9.4±1.13[c]
GVIII	0.8±0.83[a*]	5.6±2.77[c]	4.0±1.41[d#]	5.1±1.45[d]	7.2±1.93[c]	7.9±2.08[c]
GIX	1.9±1.1[a*]	7.3±1.83[c]	6.8±1.55[c]	7.0±1.31[c]	8.4±1.67[c]	7.7±2.12[c]
GX	1.0±0.94[a*]	11.7±2.2[c]	6.9±1.52[c]	8.6±1.42[c]	9.3±1.16[c]	9.4±1.33[c]

M±SD followed by the same letter represent statistically identical values in the columns (p≤0.05). * Different values of others in the same line (p≤0.05). **#**Value different from that of the times T4 and T5 of the same line (p≤0.05).

Table 5. Percentage of damage reduction to DNA (% R) in groups with EMeOH showing antimutagenicity.

Groups	Time sampling				
	T1	T2	T3	T4	T5
GVII	49.32	41.27	54.67	63.13	62.56
GVIII	79	75.4	73.33	69.19	69.16
GIX	71.23	53.18	60.67	63.13	70.04

gonads of the animals submitted to DXR. Decrease in parameters such as testicular volume, volume density of seminiferous tubules, as well as, in the diameter and length of these tubules can also give information about the testicular damage degree as a consequence of germ cells death (Ateşşain et al., 2006; Vendramini et al., 2010). The histopathological analysis of the seminiferous tubules of animals treated with DXR showed tubules with epithelial disorganization, in which multinucleated formation and round and elongated spermatids in disarranged manner were the most common alterations observed. Moreover, the majority of the seminiferous tubules presented accentuated epithelial depletion, presenting only Sertoli cell nuclei, feature of typical Sertolization. It is important to emphasize that the testicular volume represents the sum of testicular parenchyma and interstitial volumes. These same results were demonstrated by Tsunenari et al. (2000) and Vendramini et al. (2010).

In the present study the testicular parenchyma volume was the parameter more affected by chemoterapeutical treatment, since the interstitial volume did not present significant change. Thus, this result suggests that the drug cytotoxic effect occurs, essentially, upon seminiferous tubules. Therefore, considering the morphemetrical and histophatological aspects early described, it is probable that the decrease in the morphometric data was a consequence of the germ cells loss (França and Russel, 1998) induced by doxorubicin treatment. The transitory exposure of a male to an agent may result in death of specific types of germ cell (Amann, 1986). It has been shown that doxorubicin-induced toxicity is mainly concentrated in early spermatogenic cells, like type A and intermediate spermatogonia, which undergo rapid proliferation and differentiation (Shinoda et al., 1999).

Some experiments have indicated that upon microscopic evaluation, type A1-4, $A_{isolated}$, A_{paird}, and $A_{aligned}$ spermatogonia were the most vulnerable cells to doxorubicin (Lui et al., 1986; Matsui et al., 1993; Suter et al., 1997). The intercalation of doxorubicin in the germ cell DNA during division is considered the main cause of cellular death induction in the seminiferous epithelium. In addition, it is widely accepted that oxidative stress and the production of free radicals are main causes for DXR-induced cytotoxicity (Quiles et al., 2002). Oxidative stress could play a critical role in the induction of sperm abnormalities, namely a higher susceptibility of sperm DNA to denaturation and fragmentation (Sikka, 1996). In the current study we demonstrated that animals from all groups, including those which received DXR, were fertile. This was probably due to fact to the exposure to DXR did not prevent that more mature germ cells than those affected proceeded through spermatogenesis and, consequently, the male mice were able to fertilize the females. However, the results showed that there was a reduction and an increase in the number of alive and reabsorbed fetuses, respectively, in all the animals that received DXR. This result is in agreement with another

study of our group that assessed the lethal dominant frequency in cyclophosphamide-treated mice plus EMeOH of *C. cajucara* (Santos et al., 2006). Thus, the treatment with EMeOH of *C. cajucara* did not prevent the decrease on the number reabsorbed fetuses in mice treated with two different agents inductors of DNA damage.

DXR is responsible for inducing oligozoospermia (Sawada et al., 1994) and long or short-term male infertility (da Cunha et al., 1983; Meistrich, 1993) since this drug causes male germ cell apoptosis (Shinoda et al., 1999). In addition, the cells that survived to DXR action may have developed genetic alterations, like sister-chromatid exchanges (Abraham and Fränz, 1983). In this study, the treatment with EMeOH of *C. cajucara*, independently of used dosage, that is, 312.5, 625 or 1250 mg/Kg, did not induce any testicular alteration. The assessment of histopathological and morphometric parameters were normal in comparison to the animals from control groups, suggesting that the plant extract does not have cytotoxic effects upon germ cells. By the way, the pre-treatment with EMeOH, considering the three different doses, did not protect the testicular damage induced by DXR. All the morphometric parameters analyzed and the histopathological findings are in consonant with the results found in the testes of DXR-treated animals. Tieppo et al. (2006) demonstrated that the extract obtained from *C. cajucara* leaves presents free radicals scavenging capacity and, so, it is able to reduce the effects of oxidative stress caused by Paraquat. These authors suggest that the use of this plant extract is a beneficial source of natural antioxidant agent. However, considering that DXR induces free radicals forming and oxidative stress, and that the treatment with *C. cajucara* plus DXR showed the same results observed in the animals which received only the chemotherapeutical agent, it is possible to conclude that the plant extract did not prevent the testicular damage caused by DXR. In the current study, the results obtained from mutagenicity assessment of *C. cajucara* EMeOH indicated absence of mutagenicity of all extract dosages as well as the solvent (DMSO) used in this experimental protocol. These results confirm the data obtained by Santos et al. (2006, 2008), which utilized the same EMeOH dosages to evaluate its effects upon bone marrow cells of mice.

On the other hand, plants of the genus *Croton* are known for their toxic effects, as irritant and carcinogenic effects, attributed to the presence of phorbol diterpene esters (Schmidt, 1986). However, early studies reported that *C. cajucara* presents clerodane diterpenes like cajucarinolide, isocajucarinolide (Ichihara et al., 1992) and large amount of DCTN, which does not show toxic effects but shows several therapeutic properties (Maciel et al., 1998). Thus, differently of the other plants from its genus, the absence of phorbol ester bioactivity in *C. cajucara* would explain the fact that the plant extract used in this study did not show mutagenic activity in any of the tested concentrations. *C. cajucara* stem bark methanolic extract exhibited antimutagenic properties, protecting the mouse cells from mutagenicity induced by doxorubicin. The methanolic extract was able to decrease the frequency of micronucleated reticulocytes from mice treated with the chemotherapeutic agent and the *C. cajucara* EMeOH independently of the used dosage, that is, 312.5, 625 or 1250 mg/Kg b.w. The high percentage of damage reduction associated with the absence of mutagenic effects indicates the possibility of *C. cajucara* EMeOH chemoprotection action. In early mutagenic studies using cyclophosphamide as mutagenic agent, Agner et al. (2001) and Santos et al. (2008) demonstrated that DCTN and *C. cajucara* stem bark methanolic extract, respectively, presented antimutagenic properties. The main antimutagenic mechanism proposed by both of them was desmutagenesis, indicating that the mutagenic agent used (cyclophosphamide) was inactivated prior the damaging. Despite the many beneficial effects described in the literature considering the derivates from *C. cajucara* (Costa et al., 2007), in the present experimental model no protective effect of stem bark methanolic extract on testicular alterations caused by DXR was detected.

The results from the absence of testicular damage and mutagenic effect by *C. cajucara* EMeOH, in the conditions used in this study, along with those of other studies which described various biological activities for its active principle, the DCTN (Agner et al., 2001; Costa et al., 2007), can ensure the use of this herbal medicine by the population. These observations reinforce the indication of the use of *C. cajucara* extract in the folk medicine in the concentrations evaluated and encourage the development of drugs to be used as co-adjuvant chemotherapy to prevent side effects.

ACKNOWLEDGEMENTS

The authors thank Prof. Dr. Estela Sasso-Cerri and Prof. Dr. Paulo Sérgio Cerri for light microscope assistance, Mr. Carlos Lourenço and Mr. Dário P. Tormena by the helpful laboratorial assistance, and PROPPG/Prodici-UEL and State University of Londrina for funding.

REFERENCES

Abraham SK, Fränz J (1983). Induction of sister-chromatid exchanges by chemotherapeutic drugs in spermatogonia of mice: Effects of procarbanize, adriamycin, cyclophosphamide and mitomycin. Mutat. Res., 108: 373-381.

Agner AR, Maciel MAM, Pinto AC, Cólus IMS (2001). Antigenotoxicity of *trans*-dehydrocrotonin, a clerodanediterpene from *Croton cajucara*. Planta Med., 67: 815-819.

Ateşşahin A, Türk G, Karahan I, Yilmaz S, Çeribaşi AO, Bulmuş Ö (2006). Lycopene prevents adriamycin-induced testicular toxicity in rats. Fertil. Steril., 85 Suppl1: 1216-1222.

Amann RP (1986). Detection of alterations in testicular and epididymal function in laboratory animals. EHP, 70: 149-158.

Attal J, Courot M (1963). Développement testiculaire et établissement

de la spermatogénèse chez le taureaw. Ann. Biol. Anim. Bioch. Biophys., 3: 219-241.
Au WW, Hsu TC (1980). The genotoxic effect of adriamycin in somatic and germinal cells of the mouse. Mutat. Res., 79: 351-361.
Costa MP, Magalhães NSS, Gomes FES, Maciel MAM (2007). A review of the biologic activities of *trans*-dehydrocrotonin, a natural product obtained from *Croton cajucara*. Rev. Bras. Farmacogn., 17: 275-286.
da Cunha MF, Meistrich ML, Ried HL, Gordon LA, Watchmaker G, Wyrobek AJ (1983). Active sperm production after cancer chemotherapy with doxorubicin. J. Urol., 130: 927-930.
Di Stasi LC, Santos EMC, Santos CM, Hiruma-Lima CA (1989). Plantas Medicinais da Amazônia. São Paulo: UNESP.
Dorst VJ, Sajonski H (1974). Morphometricheuntersuchunhen am tubulussytem des schweinehodenswährend der postnatalenentwicklung. Monotsh. Ver. Med., 29: 650-652.
Endo F, Manabe F, Takeshima H, Akaza H (2003). Protecting spermatogonia from apoptosis induced by doxorubicin using the luteinizing hormone-releasing hormone analog leuprorelin. Int. J. Urol., 10: 72-77.
França LR, Russel LD (1998). The testis of domestic animals. In: Regadera J, Martínez-García F (eds) Male reproduction: A multidisciplinary overview. Churchill Communications, Italy, pp. 198-219.
Guevara AP, Amor E, Russell G (1996). Antimutagens from *Plumeria acuminateait*. Mutat. Res., 361: 67-72.
Hayashi M, Morita T, Kodama Y, Sofuni T, Ishidate Jr M (1990). The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. Mutat. Res., 245: 245-249.
Ichihara Y, Takeya K, Hitotsuyanagi Y, Morita H, Okuyama S, Suganuma M, Fujiki H, Motidome M, Itokawa H (1992). Cajucarinolide and isocajucarinolide: Anti-inflammatory diterpenes from *Croton cajucara*. Planta Med., 58: 549-551.
Lu CC, Meistrich ML (1979). Cytotoxic effects of chemotherapeutic drugs on mouse testis cells. Cancer Res., 39: 3575-3582.
Lui RC, Laregina MC, Herbold DR, Johson FE (1986). Testicular cytotoxicity of intravenous doxorubicin in rats. J. Urol., 136: 940-943.
Maciel MAM, Pinto AC, Brabo SN, da Silva MN (1998). Terpenoids from *Croto cajucara*. Phytochemistry, 49: 823-828.
Matsui H, Toyoda K, Shinoda K, Okamiya H, Furukawa F, Kawanishi T, Takahashi M (1993). Quantitative histopathological study on the adriamycin testicular toxicity in rats. Eisei Shikenjo Hokoku, 111: 39-46.
Meistrich ML (1993). Effects of chemotherapy and radiotherapy on spermatogenesis. Eur. Urol., 23: 136-142.
Meistrich ML, Finch M, da Cunha MF, Hacker U, Au WW (1982). Damage effects of fourteen chemotherapeutic-drugs on mouse testis cells. Cancer Res., 42: 122-131.
Meistrich ML, van Beek MEAB, Liang JC, Johnson SL, Lu J (1990). Low levels of chromosomal mutations in germ cells derived doxorubicin-treated stem spermatogonia in the mouse. Cancer Res., 50: 370-374.
Olfert ED, Cross BM, Macwilliam AA (1993). The guide to the care and use of experimental animals. Volume 1. Ontario: Canadian Council on Animal Care.
Quiles JL, Huertas JR, Battino M, Mataix J, Ramírez-Tortosa MC (2002). Antioxidant nutrients and adriamycin toxicity. Toxicology, 180: 75-95.
Resende FA, Barcala CAMA, Faria MCS, Kato FH, Cunha WR, Tavares DC (2006). Antimutagenicity of ursolic acid and oleanolic acid against doxorubicin-induced clastogenesis in Balb/c mice. Life Sci., 79:1268-1273.
Russel LD, Russel JA (1991). Short-term morphological response of the rat testis to administration of five chemotherapeutic agents. Am. J. Anat., 192: 142-168.
Santos FV, Mesquita SFP, Faria MJSS, Poersch A, Maciel MAM, Pinto AC, Morimoto HK, Colus IMS (2006). Absence of mutagenicity and germ cells of mice submitted to subchronic treatment with an extract of *Croton cajucara* Benth (Euphorbiaceae). Genet. Mol. Biol., 29: 159-165.
Santos FV, Santos VJSV, Farias MJ, Mesquita SFP, Maciel MA, Pinto AC, Colus IMC (2008). Mutagenicity and antimutagenicity of *Croton cajucara*. Biologia, 63: 327-331.
Sawada T, Tamada H, Mori J (1994). Secretion of testosterone and epidermal growth factor in mice with oligozoospermia caused by doxorubicin hydrochloride. Andrologia, 26: 151-153.
Scherle W (1970). A simple method for volumetry of organs in quantitative sterology. Mikroskopie, 26: 57-63.
Schmidt RJ (1986). Biosystematic and chemosystematic aspects of the Euphorbiaceae and Thymelaceae. In: Evans FJ (ed.) Naturally Occurring Phorbol Esters. CRC Press, USA, pp. 87-106.
Shinoda K, Mitsumori K, Yasuhara K, Uneyama C, Onodera H, Hirose M, Uehara M (1999). Doxorubicin induces male germ cell apoptosis in rats. Arch. Toxicol., 73: 274-281.
Sikka SC (1996). Oxidative stress and role of antioxidants in normal and abnormal sperm function. Front. Biosci., 1: e78-e86.
Suter L, Bobadilla M, Koch E, Bechter R (1997). Flow cytometric evaluation of the effects of doxorubicin on rat spermatogenesis. Reprod. Toxicol., 11: 521-531.
Tieppo M, Porawiski M, Salvador M, Moreira AJ, Collado PS, Gonzáles-Gallego J, Marroni NP (2006). *Croton cajucara* Benth. leaf extract scavenges the stable free radical DPPH and protects against oxidative stress induced by Paraquat. Biol. Pharm. Bull., 29: 161-165.
Tsunenari I, Kawachi M, Matsumaru T, Katsuki S (2000). Collaborative work to evaluate toxicity on male reproductive organs by repeated dose studies in rats: 10) testicular toxicity of adriamycin observed 2 and 4 weeks after a single intravenous administration. J. Toxicol. Sci., 25: 103-115.
Vaisheva F, Delbes G, Hales BF, Robaire B (2007). Effects of the chemotherapeutic agents for non-Hodgkin lymphoma, cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), on the male rat reproductive system and progeny outcome. J. Androl., 28: 578-587.
Vendramini V, Sasso-Cerri E, Miraglia SM (2010). Amifostine reduces the seminiferous epithelium damage on doxorubicin-treated prepubertal rats without improving the fertility status. Reprod. Biol. Endocrinol., p. 8.
Wall ME, Wani MC, Hughes TJ, Taylor H (1988). Plant antimutagenic effects. General bioassay and isolation procedures. J. Nat. Prod., 51: 866-873.
Ward JA, Bardin W, Knight M, Robinsin J, Gunsalus G, Morris ID (1988). Delayed effects of doxorubicin on spermatogenesis and endocrine function in rats. Reprod. Toxicol., 2: 117-126.
Waters MD, Brady AL, Stack HF, Brackman HE (1990). Antimutagenicity profiles for some model compounds. Mutat. Res., 238: 57-85.
Weibel ER (1963). Principles and methods for the morphometric study of the lung and other organs. Lab. Invest., 12: 131-155.
Yeh YC, Lai HC, Ting CT, Lee WL, Wang LC, Wang KY, Lai HC, Liu TJ (2007). Protection by doxycycline against doxorubicin-induced oxidative stress and apoptosis in mouse testis. Biochem. Pharmacol., 74: 969-980.
Yeh YC, Liu TJ, Wang LC, Lee HW, Ting CT, Lee WL, Hung CJ, Wang KY, Lai HC, Lai HC (2009). A standardized extract of *Ginkgo biloba* suppresses doxorubicin-induced oxidative stress and p53-mediated mitochondrial apoptosis in rat testes. Br. J. Pharmacol., 156: 48-61.