



Absence of mutagenicity in somatic and germ cells of mice submitted to subchronic treatment with an extract of *Croton cajucara* Benth. (Euphorbiaceae)

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Abstract

The plant *Croton cajucara* Benth. (Euphorbiaceae) is a medicinal plant from the Brazilian Amazon where it is commonly known as *sacaca*. The principal compound isolated from *C. cajucara* stem-bark extracts is the clerodane-type diterpene *trans*-dehydrocrotonin (DCTN) which presents several biological activities, including antiulcerogenic, anti-inflammatory, hypoglycemic, antimutagenic and antitumoral activity. However, few studies have been carried out to evaluate the therapeutic potential of raw *C. cajucara* extracts. We studied mutagenicity and antimutagenicity effects of *C. cajucara* methanol extract using the micronucleus assay in bone marrow cells and the dominant lethal assay in mice submitted to subchronic treatments. The blood testosterone levels of the mice were also measured to assess the effects of the methanol extract on testes function. Statistical analysis of the data obtained in this study showed no statistically significant mutagenicity attributable to *C. cajucara* stem-bark extracts, nor did such extracts show antimutagenic activity at the concentrations assessed. The testosterone concentration was normal in all the mice studied.

Key words: antimutagenicity, mutagenicity, dominant lethal, micronucleus, *Croton cajucara*, medicinal plant.

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Introduction

The medicinal use of plants has been important in medical history and contributed to the development of modern pharmacotherapy. The secondary metabolism of higher plants has been shown to be an almost inexhaustible source of compounds with possible biological activity. However, even widely used plants that apparently present no risk to health should only be used in short treatment periods since their use in disease prevention may create new infirmities caused by active secondary metabolites that do not have a known function but accumulate in the plants. Brazilian medicinal plants are being commercialized on an ever increasing scale to treat and cure various diseases and it is therefore important to assess the safety of these plants.

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The plant *Croton cajucara* Benth. (Euphorbiaceae) is an arboreal or bushy medicinal plant from the south of the state of Pará in the Brazilian Amazon where it is commonly known in Portuguese as *sacaca* and is commonly used in traditional medicine to treat diabetes, diarrhea and fever as well as stomach, hepatic and kidney problems and is also used to control high cholesterol levels (Maciel *et al.*, 1998a). The clerodane diterpene *trans*-dehydrocrotonin (DCTN) is present in high concentrations in stem-bark extracts of adult *C. cajucara* plants more than three years old (Maciel *et al.*, 1998a, b). This compound has shown direct correlation with the therapeutic effects popularly attributed to *C. cajucara*, such as antiulcerogenic (Brito *et al.*, 1998), anti-inflammatory (Carvalho *et al.*, 1996) and hypoglycemic effects (Farias *et al.*, 1997). In addition, DCTN has been shown to also have antimutagenic (Agner *et al.*, 2001) and antitumoral (Grynberg *et al.*, 1999) activities.

Although there is much published work on DCTN there are few pharmacological studies on the biological effects of raw extracts obtained from the bark or leaves of *C. cajucara*, this plant being widely commercialized on the Brazilian phytotherapeutic market where excessive consumption of concentrated *C. cajucara* leaf or bark infusions have been implicated in cases of toxic hepatitis (Maciel *et al.*, 1998a), because of which a broad study is needed involving simple extracts of this plant.

The study described in this paper used the bone marrow micronucleus assay and the lethal dominant assay to assess mutagenic and antimutagenic activity in mice submitted to subchronic treatment with *C. cajucara* methanol stem-bark extracts.

Material and Methods

Plant extract

The methanol plant extract obtained from the bark of a native *Croton cajucara* tree, collected in Jacunda, Pará state (Amazon region) was used. The plant was identified by Nelson A. Rosa, and a voucher specimen (n. 247) was deposited in the Emilio Goeldi Paraense Museum (Belém, Brazil).

The methanol extraction was carried out on ground bark (in powder) via Soxhlet, for 48 h. Six kilograms of powder and 46 liters of methanol were used in the extraction, resulting in 202 g of the methanol extract. The plant extract was diluted in a solution of DMSO (Dimethyl sulfoxide) + water at a ratio of 2:1 (v/v) and administered to the animals via gavage in doses of 312.5, 625 or 1,250 mg kg⁻¹ body weight (bw). These doses were chosen with base in the LD₅₀ of the DCTN via gavage (555 mg kg⁻¹ bw), determined by Carvalho *et al.* (1996).

Cyclophosphamide (CP)

Cyclophosphamide (Sigma - CAS: 50-18-0) was diluted in distilled water and used as positive control and as damage inducing agent in the antimutagenicity tests at a dose of 150 mg kg⁻¹ bw and was administered intraperitoneally. The choice of this dose was based on the works of Velez de la Calle *et al.* (1989) and Glode *et al.* (1981).

Animals

Five to six-week old albino Swiss mice (*Mus musculus*), weighing approximately 30 g, from the Central Animal Facility of the State University of Londrina (Parana, Brazil) were kept individually in polypropylene cages following the conditions for animal care recommended by the Canadian Council on Animal Care (Olfert *et al.*, 1993).

Animal treatment

The animals were distributed into ten treatment groups to assess the mutagenic and antimutagenic potential of the *Croton cajucara* bark methanol extract. Each group was composed of ten animals, five males and five females

Mutagenicity test: to assess the mutagenic potential of the *Croton cajucara* extract, the animals of the groups 1, 2 and 3 were treated with doses of 312.5, 625 and 1250 mg kg⁻¹ bw via gavage, once a week for 28 days. In addition to these groups, three other groups were set up: a positive control group (group 4 - cyclophosphamide 150 mg kg⁻¹ bw), a negative control group (group 5 - distilled water) and a solvent control group (group 6 - DMSO + water).

Antimutagenicity test: simultaneous treatment of the extract with the damage-inducing agent was performed to assess the antimutagenic activity in *Croton cajucara*. Cyclophosphamide was administered in a single intraperitoneal dose, one hour after the plant extract was administered via gavage at doses of 312.5, 625 and 1250 mg kg⁻¹ bw for groups 7, 8 and 9, respectively, every seven days for 28 days.

The time difference of one hour between the two treatments allowed the simultaneous entry of cyclophosphamide and the plant extract components in the bloodstream, as the latter were subject to digestive transport. In the solvent-control group 10 cyclophosphamide was administered one hour after animals had received DMSO + water (2:1, v/v) via gavage. To carry out the lethal dominant test male treated animals were mated with untreated virgin females on the 21st day. All treated animals were sacrificed 24 h after the last dose administration; bone marrow was removed and micronucleus test was performed. About 18 days after mating with treated males, nontreated females were sacrificed to assess intra-uterine contents.

Micronucleus assay

All treated mice were humanely sacrificed by cervical dislocation (females) or by decapitation (males) 24 h after the last treatment (on day 29) and bone marrow collected for the micronucleus assay (modified from Schmid, 1975) by washing the femurs with 1 mL of fetal calf serum (Cultilab-Brazil) in a centrifuge tube containing an additional 1 mL of serum, homogenizing the cell suspension and centrifuging it at 800 rpm for 10 min, after which the supernatant was partially discarded to leave about 0.3 mL of fetal calf serum in which the cell pellet was resuspended and then smeared on clean and dry slides which were dried at ambient temperature for 24 h, fixed with absolute methanol for 10 min and stained for 8 min with 5% (v/v) Giemsa stain diluted in phosphate buffer (Na₂HPO₄ 0.06 M and KH₂PO₄ 0.06 M, pH 6.8). One thousand polychromatic erythrocytes (PCEs) were analyzed per animal to ascertain the frequency of micronuclei and micronucleated cells in mice exposed to the different treatments. Cells were scored blind according to the established criteria (Huber *et al.*,

1983; Titenko-Holland *et al.*, 1997) using a Nikon binocular optical microscope fitted with a 100x objective lens.

Dominant lethal assay

On the 21st day after starting treatment each of the five male mice in each test group was mated with two untreated virgin females who had not received any treatment, mating being confirmed by the presence of a vaginal plug. The mated females were sacrificed in mid-pregnancy (18 days) and their intra-uterine content examined to establish the number of pregnant females and embryos (implanted, live and dead) from which male fertility could be calculated.

Testosterone dosage and histological analyses

Serum was separated from blood and frozen (-20 °C) until assayed quantitatively for testosterone using a chemiluminescent immunoassay system (IMMULITE, Diagnostic Products Corporation). Fifty untreated control male mice were also tested to determine the normal range of testosterone for mice. The testes of all the male mice were removed, weighed, fixed with Bouin's fixative, embedded in paraffin and sectioned to produce 6µm sections which were stained with hematoxylin and eosin (HE) and examined by optical microscopy.

Data analysis

The micronucleus and Dominant Lethal assay raw data were transformed according to the equation $y = (x+1/2)^{1/2}$ and testosterone data using the equation $y = \log_{10} y$. For the micronucleus assay the mean frequencies of micronucleated cells and the standard deviations were calculated for one thousand cells for each treatment

group and the Student t-test ($p < 0.05$) used to test for significance.

The Student t-test ($p < 0.05$) was also used to test the dominant lethal data for significance between the different groups regarding the proportion of pregnant and non-pregnant females, the proportion of dead implanted embryos, and the mean embryo implantation per pregnant female. The percentage dominant lethal frequency (%LDF) was calculated from the mean number of live implants (LI) per pregnant female in the experimental (exp) and negative control (con) groups using the formula $\%LDF = [1 - (LI_{exp} \times 1 - LI_{con}^{-1})] \times 100$ (Haseman and Soares, 1976), which takes into consideration the rates of spontaneous lethal dominants present in the negative control group.

Analysis of variance (ANOVA) and the Tukey test were used to test for significance in the testosterone data.

Results

The frequency of micronucleated cells in mice treated with a methanol extract of *Croton cajucara* bark at doses of 312.5, 625 and 1250 mg kg⁻¹ did not differ significantly (student t-test, $p = 0.05$) from negative control mice administered water in place of the methanol extract (Table 1).

The mouse dominant lethal assay which used male mice treated with the different doses of the methanol extract mated with female mice which did not receive any treatment also showed no statistically significant (student t-test, $p = 0.05$) mutagenic activity for the *sacaca* at the doses tested, there being no significant difference between the mean number of live implants and late embryo reabsorptions per untreated pregnant female mated with males that received doses of the vegetal extract or water (Table 2). Of the five males treated with 150 mg kg⁻¹ bw

Table 1 - Micronucleus per cell, micronucleus total number and micronucleated cells frequency for a thousand polynucleated erythrocytes (PCEs) after the sub-chronic treatment with methanol extract of *Croton cajucara* Benth.

Treatments (mg kg ⁻¹ bw)	Number of mice treated	Number of cells assessed	Micronuclei per cell			Total number of micronuclei	Micronucleated cells per 1000 cells*
			1	2	3		
Negative control	10	10,000	12			12	1.2 ± 0.8 ^a
DMSO	10	10,000	14			14	1.4 ± 0.8 ^a
312.5	10	10,000	14			14	1.4 ± 0.8 ^a
625	9	9,000	10			10	1.11 ± 0.6 ^a
1250	10	10,000	12			12	1.2 ± 0.9 ^a
CP	10	10,000	281	29	1	342	31.1 ± 5.7 ^b
DMSO + CP	10	10,000	269	39		347	30.8 ± 4.9 ^b
312.5 + CP	8	8,000	207	42		291	31.1 ± 3.9 ^b
625 + CP	8	8,000	193	25		243	27.25 ± 6.9 ^b
1250 + CP	10	10,000	295	11		317	30.6 ± 3.8 ^b

*Values following by the same letter do not differ statistically (Student t-test, $p < 0.05$).

CP = Cyclophosphamide (150 mg kg⁻¹ bw).

DMSO group received DMSO + water 2:1 (v/v); DMSO + CP group received DMSO + water 2:1 (v/v) and CP at 150 mg kg⁻¹ bw.

cyclophosphamide (positive control) only one succeeded in producing a pregnant female but no live embryos were produced, this result being statistically significant compared to males who had received extract or water only. Mice treated simultaneously with methanol extract of *C. cajucara* and cyclophosphamide showed no significant reduction in cyclophosphamide mutagenicity (Tables 1 and 2).

In the 50 untreated male mice tested, testosterone concentrations were between 20 to 1200 ng dL⁻¹, and there was no significant difference between these levels and those found in mice administered water only (negative control) or treated with the methanol extract, cyclophosphamide or both (Table 2). This indicates that testosterone levels were high enough to permit normal spermatogenesis.

Discussion

According to Agner *et al.* (2001) and Maciel *et al.* (2000) the population in the north of Brazil (Amazon region) uses extracts from *Croton cajucara* leaves and bark on a large scale to treat various diseases, and the medicinal use of this plant spread to the southeast of Brazil at the end of the 1990s. Similarly to *Croton cajucara*, other medicinal plants are intensely used by the world population without the proper knowledge of the risk that these treatments can incur. Cases of toxic hepatitis have been reported in several hospitals in Belém (state of Pará, Brazil) because of excessive consumption of extremely strong *Croton cajucara* tea (Maciel *et al.*, 1998a,b; Maciel *et al.*, 2000). According to the "Institute of Chinese Materia Medica" (*apud* Chan and Critchley, 1996) only about 230 of the thousands of species of medicinal plants used in China have been studied phar-

macologically and clinically, and only ten were considered toxic. This low incidence of side effects is a factor encouraging the low-income population to ingest medicinal plant.

In this study, the animals were submitted to sub-chronic treatment every seven days for 28 days via gavage of the methanol extract solutions. This treatment regimen and the administration method were considered the most suitable because they were closer to the form that treatments are conducted and *Croton cajucara* products (teas and tablets) are ingested by the population.

Statistical analysis of our micronucleus assay results (Table 1) revealed no significant difference between the frequency of micronucleated cells in mice treated with 312.5, 625 or 1250 mg kg⁻¹ bw of the extract and those that received distilled water, indicating that subchronic administration of the methanol extract of *C. cajucara* is not mutagenic in mice. Similarly, the DMSO solvent also showed no statistically significant mutagenicity by the micronucleus assay. Our results agree with those of Agner *et al.* (1999) who subjected mice to acute treatment with DCTN (the major ingredient of *C. cajucara*) and found no significant genotoxic activity for this compound. In our experiments, the absence of genotoxicity may have been due to the absence of forbol diterpene esters which are present in many toxic *Croton* species and are known to cause various toxic effects and to be carcinogenic (Hecker and Schmidt, 1974; Weber and Hecker, 1978).

The stem bark of *C. cajucara* is a rich source of clerodane-type diterpenes such as DCTN (Maciel *et al.*, 2000) that present many therapeutic properties (Farias *et al.*, 1997; Maciel *et al.*, 2002). Agner *et al.* (2001) showed that acute treatment of mice with DCTN administered by

Table 2 - Lethal Dominant Assay and testosterone concentration in Swiss albino mice submitted to sub-chronic treatment with the methanol extract of *Croton cajucara* for the assessment of mutagenicity and antimutagenicity.

Treatments given to the male mice (mg kg ⁻¹ bw)	Fertile males/mated males	Pregnant females/mated females	Average of implantations per pregnant female	Average of alive embryos per pregnant female	Average of embryos died per pregnant female	Percentage of later reabsortion	Lethal dominant frequency LDF (%)	Testosterone (ng dL ⁻¹)
Water	5/5	9/10	10.44	9.8	0.67	6.38	0.00	610.0
DMSO	4/4	8/8	10.25	8.87	1.37	13.41	9.48	601.2
312.5	5/5	9/10	8.77	8.4	1.3	15.18	14.28	694.6
625	4/4	8/8	11.75	10.37	1.37	11.7	-5.0	720.1
1250	3/5	5/10	11.4	8.4	3.0	26.3	14.28	735.2
CP*	1/5	1/10	10*	0	10*	100*	100*	368.2
DMSO + CP	3/5	4/10	2.75 ^a	1.5 ^a	1.25	45.45	84.69	338.6
312.5 + CP	3/5	4/10	3.25 ^a	1.0 ^a	2.25 ^a	69.23	89.79	1114.6 ^b
625 + CP	2/4	3/8	5.0 ^a	0 ^a	5.0 ^a	100	100	977.4 ^b
1250 + CP	2/5	3/10	2.0 ^a	0 ^a	2.0 ^a	100	100	1173.6 ^b

CP - cyclophosphamide (150 mg kg⁻¹ bw).

DMSO group received DMSO + water 2:1 (v/v); DMSO + CP group received DMSO + water 2:1 (v/v) and CP at 150 mg kg⁻¹ bw.

*In this group only one female was pregnant and all of the embryos died.

^aIn the same column values with this superscript were statistically different from the negative control group (Student t-test, p < 0.05).

^bIn the same column values with this superscript were statistically different from the positive control group (Student t-test, p < 0.05).

gavage protects their bone marrow cells against cyclophosphamide-induced damage. Our studies with 312.5, 625 and 1250 mg kg⁻¹ bw of the methanol extract administered subchronically to mice did not demonstrate any protective activity in the bone marrow cells against cyclophosphamide-induced damage. There was no significant reduction at any of the plant extract doses tested in the frequency of micronucleated cells in the treated groups that suffered cyclophosphamide-induced damage, possibly because of the low concentration of DCTN (1.4%) in *C. cajucara* extracts (Maciel *et al.*, 1998a) as compared with the doses (138.75, 277.50 and 416.25 mg kg⁻¹ bw) of pure DCTN used by Agner *et al.* (2001). Another explanation for the difference between the results published by Agner *et al.* (2001) and our results is that Agner's study used single doses of both pure DCTN and cyclophosphamide while our study involved subchronic treatment. Another possible reason why extract failed to show a protector effect in our study is that we used a higher concentration of cyclophosphamide (150 mg kg⁻¹ bw) than the 10 mg kg⁻¹ bw used by Agner *et al.* (2001). We used a high cyclophosphamide concentration to enable this mutagenic chemotherapeutic alkylating agent to cross the testicular barrier and induce damage in testicular tissue which resulted in lethal dominants. However, the increased toxicity resulting from the high dose of cyclophosphamide may have masked any protector effect of the methanol extract. In spite of the high cyclophosphamide dose used in our experiments the mice showed no toxic effects (*e.g.* mortality, diarrhea, prostration or behavioral alterations), supporting the work of Velez de la Calle *et al.* (1989) who found no toxic effects in adult rats injected with 100 mg kg⁻¹ bw in a treatment regime similar to that used in our study. In summary, our micronucleus assay indicate that the methanol extract of *Croton cajucara* did not cause genetic alterations in mice bone marrow erythrocytes but was not capable of reducing the genotoxic effects of cyclophosphamide administered to the mice intraperitoneally at a dose of 150 mg kg⁻¹ bw. Our results for the dominant lethal assay (Table 2) showed no statistically significant evidence for the extract analyzed exerting mutagenic activity on male Swiss albino mice germ cells. The frequencies of live implantations and late reabsorptions per pregnant female were not altered in groups 1, 2 and 3 treated with the vegetal extract as compared to males in the negative control (distilled water). There was no statistically significant difference in fertility between the male mice in groups 1, 2 and 3 which had been treated with extract and the negative control group.

Cyclophosphamide was fairly effective in inducing lethal dominants as shown by the fact that 80% of males treated with doses of 150 mg kg⁻¹ cyclophosphamide were infertile after treatment with this alkylating agent and only one of the ten females mated presented implanted embryos, which were all dead (Table 2). The Dominant Lethal Assay protocol used (Generoso, 1978, *apud* Leber, 1988) was expected to induce mutational events in testicular spermatid

or sperm cells but our assay results suggest that cyclophosphamide-induced mutations occurred in sperm located in the epididymus, with histological analysis of the testes showing changes in the seminiferous tissue and a few sperm cells in the seminiferous tubules (Figure 1).

Our results show that cyclophosphamide causes testicular dysfunction in male mice without reducing blood testosterone levels (Table 2), indicating that cyclophosphamide did not alter Leydig cell function. Our results support those of Velez de la Calle *et al.* (1989), who obtained similar results in adult rats treated for five consecutive weeks with cyclophosphamide. According to Vigil and Bustos-Obregon (1991) cyclophosphamide interferes with late spermatogenesis, possibly by damaging germ cell DNA or its products. Our observation that cyclophosphamide reduced fertility but did not affect serum testosterone levels agrees with the suggestion by Howell and Shalet (2002) that germinal epithelium is more sensitive than Leydig cells to the effects of cytotoxic drugs. In our study, although the testosterone levels of the cyclophosphamide-treated male mice were within the normal range they were lower than those seen for male mice in groups 1 to 5 which received no cyclophosphamide.

The increase in testosterone production after co-administration of methanol extract and cyclophosphamide to male mice in groups 7, 8 and 9 suggests that the extract protected against cyclophosphamide-induced testicular gametogenic and androgenic dysfunction, possibly because of the restoration of testicular androgenesis given that androgen is a prime regulator of gametogenesis (Huang *et al.*, 1987).

The simultaneous administration of extract with cyclophosphamide to males in groups 7, 8 and 9 did not alleviate the effects of cyclophosphamide because in females mated to these males the frequency of live implantations and the total number of implantations were very close to the values observed for females mated with males from the

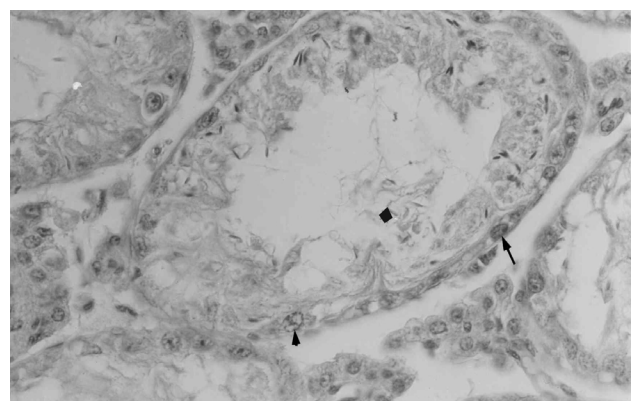


Figure 1 - Photomicrography of the seminiferous tubules of the testis of Swiss albino mice treated orally with *C. cajucara* extract (1250 mg kg⁻¹ body weight) and injected intraperitoneally with cyclophosphamide (150 mg kg⁻¹ bw). Note sertolization in the epithelium (arrow), sparse spermatogonia (arrow head) and rare spermatozooids (♦). Hematoxylin-eosin, 250x.

positive control group and much less than those observed for females mated with males belonging to the negative control group. The percentage of late reabsorption was also very high in females mated to group 7, 8 and 9 males and was, in fact, similar to the values observed for females mated with males from the positive control. Table 2 shows that the late reabsorption frequency in females mated to group 7, 8 and 9 males was much lower than in females mated with cyclophosphamide-treated males from the positive control group. However, the results for the positive control group males should be treated with caution because only one male in this group succeeded in making a female pregnant and all the embryos in this female were dead, creating a controversial value for the implantation means. Shukla and Taneja (2001) showed that the dominant lethal assay could be useful for detecting the antimutagenic potential of substances, but our study showed no reduction in cyclophosphamide-induced dominant lethal frequency group 7, 8 and 9 mice.

To clarify the situation involving the interaction between cyclophosphamide and the *C. cajucara* methanol extract more work needs to be carried out involving the use of higher doses of extract association with cyclophosphamide or the use of a new protocol with continuous administration of extract and a single weekly dose of cyclophosphamide.

Overall, our results show no statistically significant mutagenicity caused by application of different doses of *Croton cajucara* stem-bark methanol extract to Swiss albino mice under the conditions used. These results support the data from studies concerning the biological activities of DCTN, the active principal of *C. cajucara* bark extract, which indicate that consumption of this phytotherapeutic agent by the population may be safe, and hence may serve as a stimulus to the pharmaceutical industry for development of *Croton cajucara*-derived products.

However, in spite of the positive data for antimutagenic activity of DCTN (Agner *et al.*, 2001) our results indicate that it is still early to recommend the use of alcoholic extracts of stem-bark in the prevention of diseases such as cancer or to protect healthy cells during chemotherapy with cyclophosphamide.

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