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Research Article

Genotoxicity assessment of Copaiba oil and its fractions in Swiss mice

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Abstract

Copaiba oil-resin, extracted from the trunk of *Copaifera*, and traditionally used in folk medicine in the treatment of various disorders, has been shown to be an effective antiinflamatory, antitumor, antitetanus, antiseptic and antiblenorrhagea agent. As, there are few studies evaluating its genotoxicity, this aspect of the commercial oil-resin, and its volatile and resinous fractions, were evaluated in mice by comet assay and micronucleus (MN) test. A single dose of oil resin, volatile or resin fractions (500; 1,000 or 2,000 mg/kg b.w.) was administered by gavage. The chemical compositions of Copaiba oil resin and its fractions was analyzed by gas chromatography. According to comet assaying, treatment with either one did not increase DNA damage, and as to MN testing, there was no alteration in the incidence of micronucleated polychromatic erythrocytes. Chromatographic analysis of the oil-resin itself revealed sesquiterpenes, diterpenic carboxylic acid methyl esters and high levels of β-caryophyllene. Thus, it can be assumed that the oil resin and volatile and resinous fractions from the commercial product are not genotoxic or mutagenic.

Key words: Copaiba oilresin, genotoxicity, comet assay, micronucleus.

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Introduction

In recent years, the use of innumerable medicinal plants has received extensive investigation for therapeutic purposes. They are used worldwide as self-prescribed home medicines, especially in developing countries, as an aid in basic health care in 60%-80% of the population (Agra *et al.*, 2008). Of late, they have even become a source of compounds for the pharmaceutical industry (Tsuboy *et al.*, 2010; Rispler and Sara, 2011). As bioactive compounds from plants have many biological activities, the appreciable biodiversity among Brazilian plants presents great potential in the development of new drugs for application in the treatment of human diseases (Balbani *et al.*, 2009). The identification of any possible toxicological activity in plant bioactive substances through preclinical tests should precede their use in preventive health strategies.

The genus *Copaifera*, classified in the family Leguminosae, Caesalpinoideae, tribe Detarieae (Lewis, 2003), is distributed throughout Africa (four species), Central America (four species), South America (37 species), and probably Asia (one species). Nine occur in the Brazilian Amazon: *C. reticulata* (Ducke), *C. duckei* (Dwyer), *C. glycycarpa* (Ducke), *C. martti* (Hayne), *C. guyanensis* (Desf.), *C. multijuga* (Hayne), *C. piresii* (Ducke), *C. publiflora* (Benth) and *C. paupera* (Herzog). The species most frequently used to obtain oil are *C. reticulata* (70%), *C. guyanensis* (10%) and *C. multijuga* (10%) (Lawrence, 1988).

Since the 16th century, Copaiba oil-resins extracted from the trunk of *Copaifera* have been applied in folk medicine by the natives of north and northeastern Brazil, in the treatment of various diseases. Studies have shown that the beneficial effects of Copaiba are due to its antiinflamatory, antitumor, antitetanus, antiseptic and antiblenorrhagea properties. Usually, the resin is administered orally *in natura,* or applied in ointment form (Paiva *et al.*, 2002; Biavatti *et al.*, 2006; Silva *et al.*, 2009). By reason of its traditional and widespread use, commercialization of Copaiba as an oil or in capsule form has become intense, to the point of being exported to other countries, such as France, Ger-

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many and the United States (Veiga and Pinto, 2002; Veiga *et al.*, 2001).

Copaiba oil has great social and economic representation in the Amazon region, since it represents approximately 95% of the entire oil-resin production countrywise. Annual production is estimated to be 500 tons/year (Medeiros and Vieira, 2008; Brazil, 2011). Even considering its wide use in folk medicine, and in various pharmacological forms, it has not been officially registered as a phytochemical drug. Hence, an assessment of the cytotoxic and mutagenic potential of the resin becomes fundamental to so ensure its safe usage, prior to phytochemical drug development.

Studies on the medicinal plants in northern Brazil have been well documented in the medical literature, especially as regards Copaiba oil-resin (Tappin *et al.*, 2004; Comelli Júnior *et al.*, 2010), and its manifold therapeutic properties – antiinflammatory, antitumoral, antimelanoma, antiulcerogenic, antilipoperoxidation and antioxidant (Ohsaki *et al.*, 1994; Paiva *et al.*, 2002; Gomes *et al.*, 2007; Silva *et al.*, 2009; dos Santos *et al.*, 2010). Furthermore, new lines of research have been developed, with the aim of analyzing the chemical components involved. There is, for example, evidence of healing and antiinflammatory properties in certain fractions of the diterpenes, sesquiterpenes, and kaurenoic and polyaltic acids, present in the pure oil (Tappin *et al.*, 2004; Comelli Júnior *et al.*, 2010).

Thus, considering that, although the Copaiba oil-resin, widely used in folk medicine, is now commercially produced, with ample demonstration of its therapeutic potential, its possible effects in DNA damage have not yet been appraised. Hence, the aim was to evaluate the cytotoxic and genotoxic potential of oil-resin, and its volatile and resinous fractions in the liver, blood and bone-marrow cells of Swiss mice using the comet assay and micronucleus (MN) test.

Materials and Methods

Chemical agents

The oil-resins, donated through a producer cooperative, came from bulk raw material originally from the Tarauacá region, Acre State, Brazil. After receipt, the samples were transferred to and stored in amber glass bottles at 20-22 °C.

Doxorubicin (DXR, Rubidox®, CAS: 25316-40-9), purchased from the Laboratório Químico Farmacêutico Bergamo Ltda (São Paulo, Brazil), and methyl methanesulfonate (MMS, CAS: 66-27-3), obtained from Sigma-Aldrich (St Louis, MO, USA), were dissolved in distilled water, just before each experiment, all of which took place in minimal, indirect light. Ethidium bromide (CAS 1239-45-8), trypan blue (CAS 72-57-1), phosphate buffered saline (PBS), Tween® 20 (CAS 9005-64-5), Giemsa (CAS 51811-82-6) and acridine orange (CAS 65-61-2), were all purchased from Sigma – Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS), low melting point agarose (CAS 9012-36-6) and normal melting point agarose (CAS 9012-36-6), were purchased from Invitrogen (California, CA, USA). All the other chemicals were analytical grade products.

Oil-resin partitioning and derivation

Commercial oil-resin was submitted to exhaustive 2-day hydrodistillation (8-12 h), in a modified Clevenger apparatus, thereby generating a colorless volatile fraction and a viscous residue. The resinous fraction was decanted and allowed to dry at room temperature. Aliquots (10-20 mg) of the original oil-resin and resinous fraction were dissolved in dichloromethane (2 mL) and treated with diazomethane. The methylated sample and volatile fraction were then analyzed through gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS).

Gas chromatographic analysis

GC-FID analysis was performed on a gas chromatograph (HP 6890N Network CG System), fitted with a 30 m 0.32 mm 0.25 μm film thickness HP-5 capillary column, operating in split mode at a ratio of 1:50 (split/splitless injector). Helium was used as the carrier gas (flow 2.5 mL/min; inlet pressure 26.06 psi). The initial oven temperature was kept at 110 °C for 2 min, raised to 140 °C at 5 °C/min and then raised to 290 °C at 20 °C/min, where it remained for 10 min. Sample injection volume was 1 μL from a 3 mg/mL solution in CH_2Cl_2. The relative abundance of oil constituents was obtained from electronic integration measurements, using flame ionization detection (FID, 270 °C. Subsequent GC-MS analysis was performed in an HP 6890N equipment fitted with a HP-5 MS capillary column (30 m 0.32 mm 0.25 μm film thickness) and processed using MSD Productivity ChemStation Software (Hewlett Packard, Palo Alto CA, USA). Chromatographic conditions were the same as above. The mass analyzer operated at an ion source temperature of 280 °C, electron impact ionization energy of 70 eV, and an acquisition mass range of 40 to 500 *m*/*z* (3.66 scan/s). Individual sesquiterpene constituents in the oils were identified by calculating their GC retention indices, determined in reference to a homologous series of normal C_{10}-C_{30} alkanes, and by comparing their fragmentation patterns in mass spectra with those from Wiley Library Software 59943B (Hewlett Packard, Palo Alto CA, USA) and data from the literature (Adams, 2007). Individual diterpenes were identified by comparing their mass fragmentation with data from the literature.

Animals and treatments

Healthy, male albino Swiss mice (*Mus musculus*), 7|8-weeks-old and weighing approximately 35 g, were ob-

tained from the Animal Center of Coordenadoria do Campus de Ribeirão Preto, Universidade de São Paulo (Brazil). The experimental protocols for this study were approved by the Local Ethics Committee for Animal Use from the Campus of Ribeirão Preto, USP, Brazil (register No. 140/2011). Procedures involving animals and their care were in accordance with the Canadian Council on Animal Care (Olfert *et al.*, 1993). The mice were housed in polycarbonate cages with steel wire tops (four animals per cage) under standard room temperature (22 ± 2 °C), humidity (55 ± 10%), and 12 h light/dark cycle. They received standard food and fresh water *ad libitum*, and were divided into 11 groups of six animals per treatment. The micronucleus test and the comet assay were performed in the same animals.

The doses of oil-resin, and volatile and resinous fractions from commercial Copaiba oil-resin, were 500, 1,000 and 2,000 mg/kg b.w. The agents were administered in a single dose by gavage 24 h before euthanasia. The negative control (water), solvent control (Polyoxyethylenesorbitan monolaurate – Tween® 20), and positive controls (DXR, 16 mg/kg i.p. or MMS, 50 mg/kg i.p.), were also evaluated. MMS was the positive control in comet assays and DXR in micronucleus tests. The dose of DXR (16 mg/kg b.w.) was selected based on its effectiveness in inducing chromosomal damage in rodents (Ribeiro *et al.*, 2010). 24 h after treatment, the mice were anesthetized and peripheral blood was collected from the caudal vein to perform the MN tests. Immediately thereafter, the animals were euthanized by cervical dislocation, the femurs and liver were freed from adherent tissues and dissected out.

Comet assay

Liver samples were collected after euthanasia. The preparation of a single-cell suspension from liver was according to Tice *et al.* (2000). 0.2 g from each liver in 1 mL of chilled Hank's solution was placed in a Petri dish, and then sliced into fragments with scissors. Immediately prior to comet assaying, the viability of liver cells was defined by the trypan dye exclusion method. In a viable cell, trypan blue is not absorbed, but traverses that of cells with a compromised membrane.

Comet assays were under alkaline conditions (pH < 13), according to Singh *et al.* (1988), and guidelines for using this assay in genetic toxicology (Tice *et al.*, 2000). The liver cell suspension (80 μL), first mixed with 240 μL of low melting point agarose dissolved in phosphate buffered saline, was then spread onto microscope slides precoated with 1.5% normal melting point agarose. These were then covered with a coverslip and kept at a temperature of 4 °C for 15 min, prior to immersion in a freshly prepared lysis solution consisting of 2.5 M of NaCl, 100 mM of EDTA, 1% Triton X-100, and 10 mM of Tris, pH 10, for 24 h at 4 °C. After lysis, the slides were placed in a horizontal electrophoresis unit containing 300 mM of NaOH and 1 mM of EDTA, pH < 13, for 20 min at an electric field strength of 1 V/cm (25 V and 300 mA) to allow the DNA to unwind and express alkali-labile sites and DNA breaks. This was followed by washing in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min.

After drying at ambient temperature, the slides were fixed in absolute ethanol for 2 min and stored until analysis. Each slide was stained with 30 μL ethidium bromide (20 μL/mL) and immediately analyzed. All took place in the dark or under dim light. Comet observation, at 400x magnification, was with a fluorescence microscope (Zeiss, Axiostarplus®) equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. The comets were analyzed using public-domain Comet Image Analysis System software (CometScore software; TriTek, Sumerduck, VA). Data were based on 100 randomly selected nucleoids (50 nucleoids from each replicate slide). DNA damage was assessed by the percentage of DNA in the tail (%DNA tail) and Olive moment.

Micronucleus test in mouse bone marrow and peripheral blood cells

Bone-marrow smearing and staining were according to the MacGregor *et al.* (1987) method. Briefly, before centrifuging both femurs were removed, and the respective bone marrow flushed out into a centrifuge tube with fetal calf serum. The resulting pellet was resuspended in 0.3 mL of supernatant. A drop of this suspension was then smeared onto a clean slide, air dried, and fixed in absolute methanol for 10 min, ready for staining the following day with Giemsa (diluted with phosphate buffer, pH 6.8). Two thousand polychromatic erythrocytes (PCEs, immature erythrocytes) were analyzed, and the number of micronucleated PCE (MNPCE) recorded. The PCE/NCE (normochromatic erythrocytes) ratio among 500 erythrocytes (PCEs + NCEs) was determined for the same sample, to so evaluate the cytotoxic effect of any of the treatments.

Micronucleus testing of peripheral blood cells was performed according to the procedure described by Hayashi *et al.* (1990), which uses slides pre-stained with acridine orange. Blood samples were obtained by mouse caudal vein perforation, thereby collecting 5 μL (one drop). Each sample was placed in the center of a pre-stained slide and covered with a cover-slip (24 x 50 mm). The slides were then kept in the dark at -20 °C for a minimum of 24 h before cytological examination. The cell preparations were examined under a fluorescence microscope (Zeiss) with a blue (488 nm) excitation filter and a yellow (515 nm) emission (barrier) filter, using an oil immersion objective. 1,000 PCE per treated animal were analyzed and the proportion of micronucleated cells (MNPCE) counted.

Statistical analysis

Results are expressed as mean ± standard deviation (n = 6/group). The data were submitted to multiple analysis of one way-ANOVA and *post-hoc* Tukey tests using

GraphPad Prism 2.01 software program (GraphPad Software Inc., San Diego, USA). A value of $p < 0.05$ was considered statistically significant for all the parameters evaluated.

Results

Data from comet analysis of liver cells appear in Table 1. No increase in %DNA tail, in mice treated orally with various doses of commercial oil-resin, and its volatile and resinous fractions, signifies the absence of genotoxic effects. Furthermore, there was no significant difference between solvent and negative controls, demonstrating that the solvent did not interfere with the results. As expected, compared to negative control and solvent groups, animals treated with MMS as positive control revealed a higher level of DNA damage ($p < 0.05$). The viability of liver cells was > 80% for each cell suspension, both in the control and treated groups (data not shown).

The results obtained from micronucleus assaying of all the treatments appear in Table 2. Bone-marrow cytotoxicity was evaluated by counting the PCE/NCE ratio in 500 erythrocytes, whereby it was shown that the commercial oil-resin and its volatile and resinous fractions, as well as positive control treatments, induced no change in PCE/NCE ratio, when compared to negative or solvent control groups. There were no significant differences ($p > 0.05$) between the negative or solvent controls and the commercial-oil-resin treated groups in MN frequency in either bone

Table 1 - Percentage of DNA in the tail (%DNA tail) and Olive moment obtained from liver cells of mice treated with different doses of oil-resin, and volatile and resinous fractions (500, 1,000 or 2,000 mg/kg b.w.) from commercial Copaiba oil-resin and respective negative, positive (methyl methanesulphonate – MMS, 50 mg/kg b.w., i.p.) and solvent (Tween 20) controls.

Treatments (mg/kg)	% DNA tail Mean ± SD	Olive moment Mean ± SD
Negative control	5.84 ± 0.58	4.03 ± 0.95
Solvent control	8.37 ± 3.85	7.05 ± 4.70
Positive control	28.15 ± 0.75[a, b]	26.03 ± 3.19[a, b]
Oil-resin		
500	4.05 ± 1.31	4.41 ± 3.75
1000	4.16 ± 1.63	2.05 ± 1.46
2000	5.21 ± 1.80	3.09 ± 1.51
Volatile fraction		
500	11.42 ± 4.40	10.24 ± 5.26
1000	9.59 ± 4.73	8.16 ± 5.72
2000	6.88 ± 2.19	4.33 ± 2.03
Resin		
500	10.09 ± 4.66	7.42 ± 4.17
1000	8.94 ± 3.01	7.11 ± 3.39

100 nucleoids were analyzed per animal, with six animals per group. [a]$p < 0.05$ compared to negative control. [b]$p < 0.05$ compared to solvent control. One way-ANOVA and post-hoc Tukey test.

Table 2 - Frequencies of micronucleated polychromatic erythrocytes (MNPCE) in bone marrow and peripheral blood of mice treated with different doses of oil-resin, and volatile and resinous fractions (500, 1,000 or 2,000 mg/kg b.w.) from commercial Copaiba oil-resin, and respective negative, positive (Doxorubicin, 16 mg/kg b.w., i.p.) and solvent (Tween 20) controls.

Treatments (mg/kg)	Bone marrow erythrocytes*		Peripheral blood erythrocytes**
	MNPCE Mean ± SD	PCE/NCE	MNPCE Mean ± SD
Negative control	3.83 ± 1.78	1.47 ± 0.44	1.50 ± 1.04
Solvent control	2.0 ± 1.41	1.42 ± 0.27	1.50 ± 1.22
Positive control	31.16 ± 7.90[a, b]	1.31 ± 0.14	14.16 ± 5.11[a, b]
Oil-resin			
500	2.83 ± 1.72	1.26 ± 0.17	1.66 ± 1.50
1000	2.83 ± 0.75	1.74 ± 0.68	1.16 ± 0.75
2000	2.66 ± 0.81	1.48 ± 0.19	1.33 ± 1.03
Volatile fraction			
500	2.16 ± 1.16	1.51 ± 0.21	1.33 ± 1.03
1000	2.66 ± 0.91	1.41 ± 0,21	0.83 ± 0.70
2000	4.16 ± 1.72	1.27 ± 0.33	1.33 ± 1.03
Resin fraction			
500	4.66 ± 1.03	1.32 ± 0.27	2.16 ± 1.16
1000	2.50 ± 1.51	1.28 ± 0.12	1.83 ± 1.04

*2,000 PCE were analyzed per animal, with six animals per group. **1,000 PCE were analyzed per animal. [a]$p < 0.05$ compared to negative control. [b]$p < 0.05$ compared to solvent control. One way-ANOVA and post-hoc Tukey test.

marrow or peripheral blood cells, thereby demonstrating the absence of mutagenicity. In the positive control (DXR group), there was a significant increase in micronucleus frequency, in both bone marrow and peripheral blood erythrocytes, when compared to negative and solvent controls.

Chromatographic analysis of methylated oil-resin indicated sesquiterpenes (hydrocarbons and alcohol) and diterpenic carboxylic acid methyl esters. The composition of methylated oil-resin, volatile fractions, and resin with the retention indices used to identify sesquiterpenes, are presented in Table 3.

Table 3 - Relative abundance (%) of the constituents of commercial Copaiba oil-resin.

RI_{lit} [a]	RI [b]	Constituent[b]	Relative peak area (%)		
			Oil resin	Volatile fraction	Resin
1338	1340	δ-Elemene	0.38	0.65	
1348	1350	α-Cubebene	0.22	0.47	
1376	1379	α-Copaene	2.46	4.46	
1390	1391	β-Elemene	1.02	1.53	
1398	1403	Cyperene	0.36	0.60	
1419	1423	β-Caryophyllene (*trans*)	51.78	70.01	
1434	1439	α-Bergamotene (*trans*)	1.07	1.58	
1436	1441	γ-Elemene	0.92	1.19	
1454	1465	α-Humulene	8.57	10.91	
1479	1483	γ-Muurolene	0.23	0.56	
1484	1485	α-Amorphene	1.14	1.35	
1485	1488	Germacrene D	1.20	1.00	
1490	1493	β-Selinene	0.56	0.65	
1498	1499	α-Selinene	0.53	0.30	
1505	1509	β-Bisabolene	1.48	1.4	
1523	1522	γ-Cadinene	1.40	1.27	
1561	1565	Germacrene B	0.48	0.26	
1572	1578	Caryophyllenyl alchool	0.63	0.27	
1582	1589	Caryophyllene oxide	0.32	1.42	
1640	1636	epi-α-Cadinol	0.15		
1644	1642	epi-α-Muurolol	0.43		
1654	1651	α-Cadinol	0.22		
		Eperuic acid	0.46		0.67
		Copalic acid	4.70		32.07
		Kovalenic acid	0.30		0.71
		Danielic acid	2.58		16.70
		Pinifolic acid	0.58		1.11
		Agathic acid	3.35		11.95
		Hydroxy-copalic acid	5.08		6.19
		Acetoxy-copalic acid	4.02		26.98
		Sesquiterpenes hydrocarbons	78.80	98.19	
		Sesquiterpenes oxygenated	1.75	1.69	
		Total sesquiterpenes	75.55	99.88	
		Diterpenes	21.07		96.38
		Total identified	96.62	99.88	96.38

[a]RI lit = Retention Index. See Adams (2007).
[b]Diterpene acids were analyzed as methyl esters derivatives.

The commercial oleoresin showed high yields (v/v) of sesquiterpene hydrocarbons (78.8%) and low ones for oxigenated sesquiterpenes (1.75%). β-caryophyllene was the most abundant component (51.78%). Furthermore, the number of caryophyllene-type compounds increased by the presence of 8.57%, 0.63% and 0.32% of α-humulene, caryophyllene alcohol and caryophyllene oxide, respectively.

Discussion

The aim was to evaluate the acute genotoxicity and mutagenicity of the commercial oil-resin, and its volatile and resinous fractions, with a view to its application as a herbal therapeutic product. Comet assays and micronucleus tests in liver cells, peripheral blood and bone-marrow cells in mice were performed to determine Copaiba effects concerning DNA damage.

An important aspect in genetic toxicology is the choice of doses. In cases where the toxicity of the agent tested is unknown, it is recommended to evaluate doses either up to 2,000 mg/kg, or up to the maximum solubility of the agent (Tice *et al.*, 2000). In the present case, there were no available data on toxicity, cytotoxicity or genotoxicity of the oil-resin studied. Nonetheless, Gomes *et al.* (2007) showed that mice treated with Copaiba oil (500 mg/kg b.w.) presented neither acute toxicity, nor alterations in behavior, lesions or stomach bleeding. LD_{50} (Lethal Dose) values were 3.9 and 4.3 g/kg for *Copaifera reticulata* and *Copaifera multijuga*, respectively. Hence, in the present study, the doses chosen for the oil-resin, and volatile and resinous fractions were 500, 1,000 and 2,000 mg/kg, respectively. Worthy of note: oil-resin presented problems with solubility above 1,000 mg/kg.

The comet assay is a rapid and sensitive method for measuring DNA damage, through detecting DNA strand breaks, such as alkali-labile sites and incomplete excision repair events, in individual cells (Tice *et al.*, 2000; Collins, 2004; Moller, 2006). Hence, an important factor in DNA damage assessed by this procedure, is DNA repair capacity, which, besides depending on the activity of several enzymes, is influenced by both the cell cycle phase and the rate of cell proliferation (Bonassi *et al.*, 2007; Knudsen and Hansen, 2007). Furthermore, several tissues can be analyzed simultaneously for DNA damage. Even so, the liver is the most widely used, through being the main site in the metabolism of many drugs (Rothfuss *et al.*, 2011).

The micronucleus test provides a simple and rapid form for indirectly measuring induced structural and numerical chromosome aberrations. Moreover, it is scientifically and regulatorily accepted by international agencies, principally supranational authorities, such as the Organization for Economic Cooperation and Development (OECD), International Conference on Harmonization (ICH) and European Union (EU) (Mavournin *et al.*, 1990). In Brazil, the Agência Nacional de Vigilância Sanitária (ANVISA) has recommended that the mutagenicity of herbal plants be evaluated (Brazil, 1996,).

The PCE/NCE ratio is another parameter that can be evaluated by micronucleus testing. The progression of cells from erythroblasts through the PCE stage to NCE, is an indicator of acceleration or inhibition in erythropoiesis, and thus, a decrease in this ratio indicates cytotoxicity (Venkatesh *et al.*, 2007). The results of the present study showed that oilresin, volatile and resinous fractions from commercial Copaiba oilresin and the positive control treatments did not decrease the PCE/NCE ratio when compared with the negative or solvent control groups, indicating that treatment with Copaiba did not induce cytotoxicity in the bone marrow.

Positive control compounds in micronucleus tests, other than comet assays, have also been used (de Azevedo Bentes Monteiro Neto *et al.*, 2011; Malini *et al.*, 2010). DXR was selected through being an effective genotoxic and mutagenic agent in *in vivo* and *in vitro* studies (Antunes and Takahashi, 1998; Takeuchi *et al.*, 2008; Dutra *et al.*, 2009; Tan and Porter, 2009; Ribeiro *et al.*, 2010). Apparently, the main mechanisms responsible for DXR genotoxicity are the inhibition of DNA topoisomerase II, the capacitation of DNA intercalating agents, and the generation of free radicals (Ferguson and Denny, 2007; Granados-Principal *et al.*, 2010). Animals of DXR-treated groups received a single intraperitoneal injection of this antitumoral agent, thus evoking marked exposure to the agent tested (Preston *et al.*, 1987). As expected, in the present investigation, DXR induced a significant increase in micronucleus frequency in both bone marrow and peripheral blood erythrocytes, as compared to negative and solvent control groups.

MMS, a potentially carcinogenic alkylating agent, through its capacity to directly break DNA strands, thereby leading to the formation of both DNA monoadducts and crosslinks, causes mutations that involve different base substitutions (Wyatt and Pittman, 2006). MMS causes predominantly methylation in nitrogens of purine rings, which can lead to the formation of apurinic sites (de Azevedo Bentes Monteiro Neto *et al.*, 2011). Our results demonstrated that a single administration of MMS (50 mg/kg i.p.) significantly increased DNA damage in mouse liver cells.

In the present study, all doses of commercial Copaiba oilresin evaluated (500, 1,000 and 2,000 mg/kg) were found not to be genotoxic by the comet assay. In bone marrow and peripheral blood cells, no increase was observed in the frequencies of micronuclei, when receiving acute treatment, as compared to negative controls.

Other essential oils and herbal extracts were not mutagenic in other experimental systems. The toxicity of *L. cubeba* oil, extracted from the fruit, was evaluated with a battery of acute and genetic toxicity tests, thereby indicating that oral LD_{50} was approximately 4,000 mg/kg of body

weight, and genetic toxicity was negative for MN induction in bone marrow (Luo *et al.*, 2005). Infusions prepared from the bark of *Bauhinia variegata* also showed no increase in micronuclei frequency, when tested at doses of 300 to 900 mg/kg b.w. in mice (Agrawal and Pandey, 2009). As revealed by MN tests in bone marrow, treatment of mice with 2,000 mg/kg b.w. of Copaiba oil from *Copaifera martii* showed no mutagenic effects (dos Santos *et al.*, 2011).

Chromatographic analysis of methylated Copaiba oil resin and its volatile fraction revealed a high concentration of β-caryophyllene, described as a main component of several active oils, and with antimicrobial, antiinflammatory and antiallergic activities (Lourens *et al.*, 2004; Sabulal *et al.*, 2006; Passos *et al.*, 2007; Leandro *et al.*, 2012). The diterpene fraction yield (v/v) was 21%. Copalic acid was the predominant diterpene, followed by other, also relevant, oxidized labdane derivatives, such as hydroxycopalic and acethoxycopalic acid (up to 9%). The oil-resin used here is rich in labdane diterpenes, as was the case in *C. multijuga* analyzed by Veiga Junior *et al.* (2007) and Gomes *et al.* (2007), but different from *C. langsdorffi* and *C. reticulata*, which were shown to contain some clerodane diterpens.

Copaiba oil genotoxicity has been little studied. β-caryophyllene, the major constituent of its oil-resin and volatile fractions, besides producing no cytotoxic or genotoxic effects in human lymphocyte cultures, proved to be protective against ethyl methanesulfonate-induced DNA damage (Di Sotto *et al.*, 2010). Nine sesquiterpenic compounds, including caryophyllene-*trans*, was screened in an Ames test and none of the compounds showed mutagenicity (Gonçalves *et al.*, 2011). Cavalcanti *et al.* (2006) reported that low concentrations of kaurenoic acid, a bioactive diterpenoid extracted from *Copaifera langsdorffii*, failed to significantly induce DNA damage or increase micronucleus frequency in V79 cells. Nonetheless, on exposure to higher concentrations (30 or 60 μg/mL), a significant increase in DNA damage became evident.

Concluding, under the experimental conditions employed in this study, the oil-resin itself, and volatile and resinous fractions from commercial Copaiba oil-resin showed no genotoxic or mutagenic effects.

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