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Developmental toxicity of copaiba tree (*Copaifera reticulata* Ducke, Fabaceae) oleoresin in rat

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ABSTRACT

The oleoresin of the copaiba tree (*Copaifera* sp., Fabaceae) is traditionally used in Brazilian herbal medicine to treat a variety of illnesses and symptoms. This study, conducted according to the OECD Guideline 414, provides data on the developmental toxicity of oleoresin from *C. reticulata* (COPA-R) in rats. Pregnant Wistar rats (25 per dose group) were treated by gavage with COPA-R (0, 500, 1000 and 1250 mg/kg bw/day) on gestation days (GD) 6–19 and Caesarean sections performed on GD20. Implantations, living and dead fetuses and resorptions were recorded. Half of the fetuses from each litter were examined for visceral abnormalities and the remaining were cleared and stained for skeleton evaluation. COPA-R was maternally toxic (reduced food intake and weight gain) and embryotoxic (lower fetal body weight and increased occurrence of fetal skeleton variations) at the two highest doses, but did not cause embryo deaths or fetal malformations at any dose level. The study derived an oral no-observed-adverse-effect-level (NOAEL) for maternal and developmental toxicity induced by COPA-R of 500 mg/kg bw/day. The results suggest that copaiba oleoresin does not pose a health risk to pregnant women when used according to the recommended doses (up to five drops, three times a day).

1. Introduction

Copaiba trees (*Copaifera* sp., Fabaceae) are native of tropical South America, where they grow mainly in the Amazon rain forest and Central Brazilian savannas, and of western Africa. Sixteen *Copaifera* species are found in Brazil, including *C. reticulata* Ducke, *Copaifera multijuga* Hayne, *Copaifera langsdorffii* Desf. and *Copaifera cearensis* Huber ex Ducke (Veiga Júnior and Pinto, 2002). The copaiba oleoresins are obtained by making holes in the tree trunk to collect the resin that drips. The chemical composition of the oleoresin varies to some extent depending mainly on the copaiba tree species, the season and the geographic and climatic characteristics of the region where the tree grows (Lameira et al., 2009). The oleoresin is basically a mixture of sesquiterpenes (essential oil fraction), mainly β-caryophyllene, and diterpenes (Cascon and Gilbert, 2000; Veiga Junior et al., 2007).

The inhabitants of the Amazon region have long used the oleoresin of copaiba trees in traditional medicine to treat a variety of diseases and symptoms, such as respiratory and urinary tract disorders, stomach ulcers, aching throats, tonsillitis, and infectious diseases. This widespread use has led to the introduction of copaiba phytotherapeutic and cosmetic products in both the Brazilian and international markets (Veiga Júnior and Pinto, 2002). A number of studies have found pharmacological activities of copaiba oleoresin that support some of its uses, including as anti-inflammatory (Carvalho et al., 2005; Veiga Junior et al., 2007), antimicrobial (Tincusi et al., 2002), antinociceptive (Gomes et al., 2007), antioxidant (Lima Silva et al., 2009) and antiparasitic (Santos et al., 2008).

Toxicological studies of the copaiba oleoresin, however, are relatively scarce. The acute oral toxicity of the oleoresin seems to be low, and LD50 determined in mice and rats were higher than 2000 mg/kg bw (Gomes et al., 2007; Sachetti et al., 2009). Maistro et al. (2005) found no evidence of genotoxicity for *Copaifera duckei* oleoresin (at 10%, 25% and 50% of the LD50 given for three consecutive days) in the rat bone marrow micronucleus assay. Nonetheless, kaurenoic acid (30 and 60 μg/mL), a diterpene found in *C. langsdorffii*, showed DNA-damaging activity in hamster fibroblast V79 cells (Cavalcanti et al., 2006). A study by Cunha et al. (2003) demonstrated that kaurenoic acid has uterine relaxant effects in rats that apparently result from two distinct actions, a calcium channel blockade and the opening of ATP-sensitive potassium channels.

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Although women of childbearing age are orally exposed to copaiba oleoresin via traditional medicine portions or phytotherapeutic products, as far as the authors are aware, the developmental toxicity of copaiba oleoresin has so far not been studied. This study was conducted to investigate the developmental toxicity of copaiba oleoresin obtained from *Copaifera reticulata*. The oleoresin obtained from this species is the one most widely used in copaiba phytotherapeutic products marketed in Brazil.

2. Materials and methods

2.1. Animals

Male and nulliparous female Wistar rats (90–120 days old) from the University of Brasilia (UnB) Animal House breeding stock were used. The rats were housed individually in standard plastic cages with stainless-steel covers and wood shavings as bedding, and kept under controlled temperature (23 ± 2 °C), relative humidity (maximum 70%) and a 12:12 h photoperiod, with lights turned on at 09:00 a.m. A standard commercial diet for laboratory rats (Labina, Purina® Evialis Group, Paulinia, SP, Brazil) and tap water were provided *ad libitum*. The animals were acclimatized for 10 days before starting the experiment. The research protocol was approved by the University of Brasilia Ethics Committee on the Use of Laboratory Animals.

2.2. Plant material

The copaiba oleoresin from *C. reticulata* Ducke, Fabaceae was supplied by Embrapa Eastern Amazon. It is a pool of oleoresins (COPA-R) collected in October 2003 and March–July 2004 from one copaiba tree grown in the Mojú Field Research Unit located in the state of Pará, Brazil. The oleoresins were analyzed by GC/MS HP6890/HP5973 system equipped with a HP-5MS fused capillary column (30 m × 0.25 mm; 0.25 mm film thickness). Helium (1 mL/min) was used as carrier gas; the oven temperature program was 60–300 °C at 3 °C/min; 2 μL of the oil solution in hexane (0.2%) was injected. Injector and detector temperatures were 240 °C; ion source was at 180 °C, with a EIMS electron energy of 70 eV. Identification of the compounds was done using the MS library data, with further confirmation with authentic reference compounds. Quantification was performed by GC/FID (Lameira et al., 2009).

2.3. Study design and preliminary pilot experiment

The study was conducted as recommended by the OECD guideline 414, Prenatal Developmental Toxicity Study (OECD, 2001). The dose range tested in the main study was chosen taking into account results from a pilot experiment, where five pregnant rats were treated by gavage with COPA-R at 1500 mg/kg bw/day dose on gestation days (GD) 6–19. Two treated dams died on GD14 and one on GD18. Owing to this high mortality, the upper limit of the dose range tested in the main study was kept at 1250 mg/kg (bw)/day, i.e., the highest dose of COPA-R that caused no maternal death or severe suffering.

2.4. Mating procedure

Mating was carried out by placing three females into the cage of one male for 3 h (6:00–9:00 a.m.) and confirmed by the presence of sperm in the vaginal smear. The day on which spermatozoa were detected in the smear was designated as day 0 of pregnancy (GD0). Pregnant rats were assigned randomly to control and treatment groups.

2.5. Treatment

A freshly prepared emulsion of COPA-R in 2% Tween 80, in water was administered by gavage to pregnant rats on GD6–19 at doses of 0, 500, 1000 and 1250 mg/kg bw/day. The administered volume was 10 mL/kg bw/day and the control group received only the vehicle. Twenty-five pregnant rats were treated per dose group. All females were examined daily for signs of toxicity, while body weight and feed intake were recorded every 3 days. At the Caesarean section (C-section), abnormalities of maternal organs were recorded, and the livers, kidneys and brains of 10 animals per group, chosen at random, were also examined microscopically for histopathological changes.

2.6. Caesarean section

On GD20, rats were killed by CO_2 inhalation. After death, gravid uteri were removed and weighed with their contents. Ovaries were removed and corpora lutea graviditatis were counted. Live and dead fetuses and resorptions were counted as well. Implantation sites were determined by the Salewski method (Salewski, 1964). Opened uteri were placed in 10% ammonium sulfate for 10 min, and subsequently rinsed and immersed in a 2% potassium ferricyanide and 1% hydrochloric acid (1:1) solution for 10 min. Placentas and live fetuses were weighed and the fetuses examined for externally visible abnormalities. Half of the fetuses of each litter, chosen at random, were fixed in Bodian's solution for further evaluation of visceral abnormalities using a micro-sectioning technique described by Miranda et al. (2006). Heart, thymus, liver, spleen, kidneys and lungs of fixed fetuses were also weighed. The remaining fetuses were fixed in a 5% formalin solution, macerated in potassium hydroxide, cleared with glycerin–KOH solutions and stained with Alizarin Red S for skeletal evaluation (Dawson, 1926). Fetal abnormalities were identified and classified according to internationally-agreed terminology and classification scheme (Solecki et al., 2001, 2003; Makris et al., 2009).

Table 1
Composition (%) of the oleoresin volatiles of a *Copaifera reticulata* Ducke, Fabaceae tree collected at different collection times.

Constituents[a]	Time of collection						
	Oct/03	March/04	Apr/04	May/04	June/04	July/04	Mean (pool)
δ-Elemene	0.2	0.2	0.2	0.1	0.2	0.2	0.18
Cyclosativene	0.3	1.6	1.3	0.9	0.9	0.9	0.98
α-Copaene	0.2	0.8	0.6	0.5	0.5	0.5	0.52
β-Elemene	3.0	4.2	3.3	2.5	3.2	3.5	3.3
α-Gurjunene	0.2	0.5	0.8	0.6	0.7	0.7	0.6
β-Caryophyllene	25.1	50.2	41.6	31.5	37.3	39.7	37.6
trans-α-Bergamotene	12.0	9.8	8.1	6.4	9.0	10.3	9.3
Aromadendrene	0.8	1.2	1.0	0.8	0.9	0.9	0.9
Epi-β-santalene	0.1	0.1	0.1	0.1	0.1	0.1	0.1
α-Humulene + (E)-β-farnesene	5.4	5.8	5.3	4.1	5.4	5.8	5.3
β-Chamigrene	0.4	1.2	1.3	0.8	1.0	0.9	0.9
Γ-Gurjunene	0.4	0.9	0.7	0.5	0.6	0.6	0.62
Γ-Curcumene	1.4	0.2	0.3	0.2	0.6	0.7	0.6
β-Selinene	1.8	6.7	6.6	4.7	4.8	4.6	4.9
α-Selinene	1.2	4.2	4.1	2.9	3.0	2.9	3.0
(Z)-α-bisabolene	0.7	2.5	2.3	1.7	1.8	1.7	1.8
α-Bulnesene	3.1	1.8	1.8	1.4	2.2	2.3	2.1
β-Bisabolene	12.0	5.2	6.5	6.1	14.5	17.4	10.3
β-Curcumene	0.3	0.4	0.5	0.3	0.4	0.4	0.38
β-Sesquiphellandrene	2.3	0.6	0.7	0.6	1.2	1.3	1.1
(E)-γ-bisabolene	3.3	0.4	0.6	0.6	1.4	1.6	1.3
Caryophyllene oxide	0.3	0.1	0.1	0.1	0.1	0.3	0.16
Epi-β-bisabolol	0.2	–	0.1	0.1	0.1	0.1	0.12
β-Bisabolol	0.5	–	0.1	0.1	0.2	0.1	0.2

[a] Listed in sequence of their retention indices.