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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.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Dunnett's post hoc test. Proportions were evaluated by the chi-square test. In any case, differences were considered significant when $P < 0.05$ (two-sided test). Statistical calculations were performed using the SPSS statistical software version 17.0, or GraphPad Prism software version 2.01.

3. Results

3.1. Chemical characterization of COPA-R

Table 1 shows the volatile compounds of oleoresins collected from a *C. reticulata* tree in 2003 and 2004, listed in sequence according to their retention index. The volatile fractions are composed mainly of β-caryophyllene (25.1–50.2%), β-bisabolene (5.2–17.4%) and *trans*-α-bergamotene (6.4–12%). The composition of the volatile fraction of the *C. reticulata* oleoresin pool used in the study (COPA-R) was estimated as the mean percentage of each constituent from all collection times. The pool has 37.6% of β-caryophyllene, 10.3% of β-bisabolene and 9.3% of trans-α-bergamotene (Table 1). Copaiba oleoresins have been shown to be stable for a period of at least 1½ years when stored at room temperature (data not published).

3.2. Maternal toxicity

All pregnant rats survived to scheduled sacrifice and no behavioral changes and/ or clinical signs of toxicity were observed during the study. As shown in Table 2, percentages of pregnant females (i.e., with implantation sites detected at the C-section) did not differ among groups. At the two highest doses, COPA-R reduced maternal food consumption (per kg body weight) at the beginning of the exposure period, as shown by the data on GD9 ($P < 0.01$) and on GD15, but this reduction was not significant towards the end of the gestational period (on GD18-20). Treatment with COPA-R decreased maternal weight gain between GD6 and GD20, a weight gain deficit that was also detected when the gravid uterus weight was subtracted from the weight gain during the last two weeks of pregnancy (Table 2). The foregoing findings indicated that COPA-R was maternally toxic (i.e., it decreased food intake and pregnancy weight gain) at oral doses equal to or higher than 1000 mg/kg bw/day. Maternal organs, however, did not show any macroscopically visible abnormality in the treated groups. Similarly, no treatment-related finding was noted in the liver, kidney and brain tissue slides (from 10 rats per group) examined for histopathological changes.

Table 2
Effects of copaíba (*C. reticulata*) oleoresin administered orally to rats on gestation days 6–19 on maternal weight gain and food consumption.

Treatment	COPA-R (mg/kg bw/day)			
	0	500	1000	1250
Treated females (*N*)	25	25	25	25
Pregnant rats (%)[a]	88	92	88	96
Maternal body weight (g)				
GD0	250.7 ± 21.9	257.9 ± 15.6	254.7 ± 18.6	237.8 ± 20.0
GD20	362.6 ± 20.2	375.3 ± 22.6	333.3 ± 31.8**	327.0 ± 31.4**
Maternal weight gain (Δg)				
GD0-6	20.9 ± 9.2	22.7 ± 8.4	22.6 ± 8.2	23.6 ± 6.2
GD6-20	91.0 ± 12.3	94.7 ± 19.4	56.0 ± 26.2**	65.5 ± 20.6**
Gravid uterus weight (g)	67.1 ± 9.7	75.8 ± 11.8	60.5 ± 12.3	60.4 ± 12.6
[GD6-20] – gravid uterus wt	23.9 ± 11.6	18.8 ± 15.3	-4.5 ± 21.7**	5.1 ± 15.2**
Daily food consumption (g/kg bw/day)[b]				
GD3	199.2 ± 39.8	211.4 ± 41.7	206.6 ± 49.7	198.2 ± 32.3
GD6	215.5 ± 38.2	224.3 ± 64.2	195.6 ± 40.7	192.2 ± 39.8
GD9	205.9 ± 31.1	190.8 ± 47.4	155.3 ± 53.6**	147.7 ± 47.0**
GD12	202.4 ± 27.2	189.4 ± 53.7	168.3 ± 57.8	170.5 ± 59.4
GD15	212.4 ± 35.4	195.6 ± 34.5	177.5 ± 59.2*	178.4 ± 33.7*
GD18	199.4 ± 28.8	205.0 ± 23.6	185.6 ± 46.1	185.5 ± 34.8
GD20	126.2 ± 16.0	127.8 ± 17.4	115.1 ± 47.8	109.9 ± 24.0

Data are shown as mean ± SD.
[a] Pregnant females (%) = [(Females with implantation sites at the C-section)/(Treated Females)] × 100.
[b] Calculated from the amount of food consumed in the last three days divided by the body weight of that day. Proportions were evaluated by the chi-square test while other data were analyzed by ANOVA followed by the Dunnett (two-sided) test.
* Differences from the vehicle control group are indicated by asterisks: $P < 0.05$.
** Differences from the vehicle control group are indicated by asterisks: $P < 0.01$.

Table 3
Caesarean section data of rats treated orally with copaiba (*C. reticulata*) oleoresin on days 6–19 of pregnancy.

Treatment	COPA-R (mg/kg bw/day)			
	0	500	1000	1250
Corpora lutea (mean ± SD)	14.14 ± 1.25	15.41 ± 1.74	14.48 ± 1.69	14.43 ± 1.83
Implantation sites				
Per litter (*N*, mean ± SD)	12.8 ± 1.5	13.9 ± 1.7	12.8 ± 2.3	12.4 ± 3.1
Resorptions				
Early/late (*N*)	16/0	7/0	15/1	19/1
Per litter (*N*, mean ± SD)[a]	0.73 ± 0.98	0.32 ± 0.78	0.71 ± 0.84	0.78 ± 0.99
Dead fetuses	0	1	0	0
Live fetuses				
Total (*N*)	266	300	252	266
Sex ratio (F/M)	1.05	1.08	1.13	1.06
Fetuses per litter (*N*, mean ± SD)	12.1 ± 1.7	13.6 ± 1.7	12.0 ± 2.4	11.6 ± 2.7
Fetal body weight (g, mean ± SD)	3.75 ± 0.25	3.78 ± 0.47	3.35 ± 0.5*	3.48 ± 0.34*
Placenta weight (g, mean ± SD)	0.49 ± 0.03	0.49 ± 0.04	0.47 ± 0.06	0.48 ± 0.06

N (number).
[a] Total (early + late). Resorptions were classified as "early" when there were implantation sites with no corresponding fetuses or fetal/placental remnants; and "late" when fetal and placental remnants were found.
* Significant differences in comparisons with a control group, using ANOVA followed by the Dunnett (two-sided) test. Differences from the vehicle control group are indicated by asterisks: $P < 0.05$. The litter was the unit of statistical analysis.

Table 4
Occurrence of externally visible and visceral abnormalities in the offspring of rats treated orally with copaiba (*C. reticulata*) oleoresin on days 6–19 of pregnancy.

Treatment	COPA-R (mg/kg bw/day)			
	0	500	1000	1250
Externally-visible abnormalities				
Fetuses/litters examined	266/22	300/22	252/21	266/23
Findings (fetuses/litters)				
Hemorrhage		0		
Anal atresia	1/1	0	0	0
Short tail	0	0	1/1	0
Malrotated paw	0	0	1/1	0
Visceral abnormalities				
Fetuses/litters examined	128/21	141/21	115/20	121/21
Dilated cerebral ventricle	1/1	0	0	0
Supernumerary liver lobe	8/5	5/4	8/7	10/6
Malpositioned left kidney	0	0	3/3	2/2
Dilated left ureter	0	4/3	1/1	4/3
Convoluted left ureter	0	3/3	1/1	3/2
Dilated right ureter	0	8/5	1/1	5/4
Convoluted right ureter	0	7/4	1/1	2/1
Malpositioned testis	2/2	3/3	4/3	3/3

Proportions were compared by the chi-square test ($P < 0.05$)

Table 5
Occurrence of skeleton abnormalities in the offspring of rats treated orally with copaiba tree (*C. reticulata*) oleoresin on days 6–19 of pregnancy.

Treatment	COPA-R (mg/kg bw/day)			
	0	500	1000	1250
Fetuses/litters examined (N)	106/20	104/17	128/21	115/21
Fetuses/Litters (%) showing abnormalities in:				
Skull				
Frontal (incomplete ossification)	0/0	0.96/5.9	9.4*/14.3	4.3*/14.3
Frontal (hole)	0/0	0/0	0/0	0.9/4.8
Frontal (unossified)	0/0	0/0	0.8/4.8	0/0
Hyoid (unossified)	0/0	0/0	3.9*/9.5	0/0
Interparietal (bipartite)	12.3/40.0	7.7/41.2	8.6/33.3	9.6/28.6
Interparietal (incomplete ossification)	20.8/40.0	21.2/52.9	28.1/61.9	13.0/47.6
Interparietal (unossified)	0/0	0/0	2.3/9.5	0/0
Palate (split)	0/0	0/0	0.8/4.8	0.9/4.8
Parietal (incomplete ossification)	6.6/20.0	5.8/29.4	12.5/23.8	6.1/23.8
Parietal (unossified)	0/0	0/0	0.8/4.8	0/0
Squamosal (incomplete ossification)	0/0	0/0	2.3/14.3	0/0
Squamosal (misshapen)	3.8/10	3.8/17.6	7.8/28.6	7.8/28.6
Squamosal (holes)	17.0/55.0	14.4/58.8	24.2/76.2	28.7*/90.5*
Squamosal (unossified)	0/0	0/0	0.8/4.8	0/0
Supraoccipital (bipartite)	0/0	0/0	1.6/9.5	2.6/9.5
Supraoccipital (incomplete ossification)	6.6/25.0	16.3*/58.8*	11.7/47.6	13.0/47.6
Supraoccipital (misshapen)	9.4/35.0	11.5/52.9	14.8/57.1	4.3/19.0
Supraoccipital (unossified)	0/0	0/0	47*/9.5	0.9/4.8
Lacrimal (unossified)	0/0	0/0	0.8/4.8	0/0
Maxilla (incomplete ossification)	0/0	0.96/5.9	0.8/4.8	0/0
Nasal (incomplete ossification)	0/0	0/0	0.8/4.8	0/0
Tympanic annulus (incomplete ossification)	0/0	0/0	1.6/4.8	0.9/4.8
Tympanic annulus (unossified)	0/0	0/0	0.8/4.8	0/0
Zygomatic (incomplete ossification)	0/0	0/0	0.8/4.8	0/0
Zygomatic (unossified)	0/0	0/0	0.8/4.8	0/0
Forelimbs				
Metacarpal (incomplete ossification)	11.3/25.0	8.7/41.2	15.6/61.9*	15.7/42.9
Metacarpal (unossified)	9.4/35.0	26.9*/76.5*	33.6*/71.4*	60.9*/76.2*
Forepaw phalanx (incomplete ossification)	15.1/45.0	18.3/58.8	14.8/47.6	20.9/47.6
Forepaw phalanx (unossified)	12.3/25.0	15.4/64.7	10.2/33.3	7.8/28.6
Ulna (incomplete ossification)	0/0	0/0	0.8/4.8	0/0
Radius (incomplete ossification)	0/0	0/0	0.8/4.8	0/0
Clavicle				
Clavicle (unossified)	0/0	0/0	0.8/4.8	0/0
Hindlimbs				
Femur (incomplete ossification)	0/0	0/0	0.8/4.8	0/0
Tibia (unossified)	0/0	0/0	0.8/4.8	0/0
Fibula (unossified)	0/0	0/0	0.8/4.8	0/0
Metatarsal (incomplete ossification)	0.9/5.0	0.96/5.9	2.3/14.3	2.6/9.5
Metatarsal (unossified)	0/0	0/0	6.3*/9.5	3.5*/14.3
Hindpaw Phalanx (incomplete ossification)	29.2/65.0	34.6/82.4	23.4/71.4	17.4*/66.7
Hindpaw Phalanx (unossified)	20.8/45.0	22.1/76.5	19.5/57.1	27.0/47.6
Pelvic girdle				
Ilium (incomplete ossification)	0/0	0/0	0.8/4.8	0/0
Ilium (unossified)	0/0	0/0	1.6/4.8	0.9/4.8
Ischium (unossified)	0/0	0/0	4.7*/4.8	2.6/9.5
Pubis (unossified)	0/0	0/0	3.1/4.8	1.7/4.8
Sternebrae				
Sternebra (bipartite)	0.9/5.0	1.9/11.8	0/0	0.9/4.8
Sternebra (incomplete ossification)	24.5/75.0	22.1/64.7	29.7/85.7	30.4/76.2
Sternebra (mishappen)	44.3/95.0	49.0/88.2	27.3/66.6	37.4/90.5
Sternebra (unossified)	9.4/35.0	9.6/41.2	18.0/57.1	9.6/33.3
Rib				
Lumbar rib (extra, rudimentary)	33.0/70.0	42.3/82.35	42.9/90.4	65.2*/100*
Vertebrae				
Sacral centrum (unossified)	0/0	0/0	0.8/4.8	0/0
Sacral arch (unossified)	0/0	0/0	0.8/4.8	2.6/9.5
Sacral arch (incomplete ossification)	64.2/100	64.4/94.1	38.3*/76.2	48.7*/90.5
Thoracic centrum (dumbbell)	2.8/10.0	0.8/5.9	1.6/9.5	1.7/4.8
Thoracic centrum (incomplete ossification)	3.8/20.0	27.9*/64.7*	32.0*/85.7*	28.7*/71.4*
Thoracic centrum (bipartite)	0/0	0/0	0/0	2.6/9.5
Thoracic centrum (unossified)	0/0	0/0	2.3/4.8	0/0
Caudal arch (unossified)	11.3/45.0	6.7/17.6	16.4/42.9	13.9/47.6
Caudal centrum (unossified)	1.9/5.0	0/0	0.8/4.8	0/0
Lumbar arch (unossified)	0/0	0/0	0.8/4.8	0/0
Lumbar centrum (incomplete ossification)	0/0	0/0	1.6/4.8	0/0
Lumbar centrum (unossified)	0/0	0/0	2.3/9.5	0/0
Lumbar vertebra (supernumerary)	0.9/5.0	1.9/11.8	0/0	0.9/4.8

N (Number).
Fetuses were removed on GD20. Proportions were analyzed by the chi-square test.
* Different from the vehicle-control group, $P < 0.05$.

3.3. Embryotoxicity

As shown in Table 3, the average number of corpora lutea graviditatis, implantation sites and resorptions per female did not differ between the vehicle-control group and treated groups. The average number of live fetuses per litter and sex ratios did not differ among groups either (Table 3). COPA-R did not induce embryo deaths at the tested doses. The average fetal body weight per litter was decreased ($P < 0.05$) by treatment with the two highest doses (1000 and 1250 mg/kg bw/day), but no reduction of body weight was observed among fetuses from the lowest dose group. The average placental weight per litter, however, was unaffected by treatment with COPA-R (Table 3). The weights of fetal heart, thymus, liver, spleen, kidneys and lungs were not affected by treatment either (data not shown).

The occurrence of externally visible abnormalities and visceral abnormalities were not consistently altered by treatment with COPA-R (Table 4). Malpositioned left kidney was recorded in 3 and 2 fetuses from the intermediate and highest dose groups, respectively. Although being found only in treated groups, ureter abnormalities were of minor severity, being of low incidence and unrelated to the dose (Table 4).

The incidences of fetal skeleton abnormalities in the vehicle-control group and in the treated groups are shown in Table 5. Exposure to the two highest doses of COPA-R on GD6-19 enhanced the occurrence of incompletely ossified and or unossified skull bones (frontal, hyoid and supraoccipital), metacarpal bones, metatarsal bones, hindpaw phalanx, ischium, sacral arches and thoracic vertebrae centra. An increased frequency of squamosal bone holes was noted in fetuses from the group treated with the highest dose, but this frequency was relatively high in fetuses from the vehicle control group as well. The occurrence of extra rudimentary lumbar ribs was higher in the group treated with the highest dose (Table 5). In summary, the results indicate that COPA-R at the two highest doses (1000 and 1250 mg/kg bw/day) increased the occurrence of incompletely ossified and unossified bone, squamosal bone holes and extra rudimentary lumbar ribs. It is noteworthy that, in most instances, increases in the incidence of skeleton anomalies induced by COPA-R were unrelated to the dose.

4. Discussion

The results from this study indicated that the two highest doses of COPA-R (1000 and 1250 mg/kg bw/day) were toxic to mothers because they reduced maternal food consumption and weight gain during pregnancy. No evidence of maternal toxicity was noted at the lowest dose tested (500 mg/kg bw/day). The lowest dose of COPA-R was not embryotoxic either. It did not cause any enhancement of embryolethality, nor did it produce any embryofetal growth retardation or any increase in the occurrence of fetal malformations.

COPA-R did not produce embryo deaths at the two highest doses either. Nonetheless, fetal body weight near term (GD20) was slightly to moderately reduced (<10%) in the offspring from dams treated with the intermediate and high doses on GD6-20. The foregoing findings suggested that the two highest doses (1000 and 1250 mg/kg bw/day), which were toxic to mothers, retarded the offspring prenatal growth as well.

No increase in the occurrence of externally visible and visceral abnormalities attributable to treatment with COPA-R was observed. Although some minor abnormalities of ureters (e.g., dilated and convoluted ureters) and malpositioned kidneys were noted only in the offspring from mothers treated with COPA-R, no clear dose-dependent response was evident in these cases. It is noteworthy that convoluted and dilated ureters are regarded as transient/reversible changes and thus are generally classified as variations (Solecki et al., 2003; Carney and Kimmel, 2007).

A higher incidence of incompletely ossified and unossified skull bones, metacarpal bones, metatarsal bones, hindpaw phalanx, ischium, and vertebral column bones was recorded among fetuses from mothers treated with COPA-R. These observations, however, also occur at a relatively high spontaneous frequency in the rat strain used in this study and should be regarded as variations rather than as malformations (Solecki et al., 2001). Incompletely ossified and unossified bones are decreases in the extent to which the bone anlagen were calcified. Since bone mineralization is still in progress on GD19-20, the aforementioned findings are consistent with the view that there was a delayed ossification of these bones in some fetuses exposed to COPA-R. These abnormalities were also found in untreated controls and were considered as being of minor toxicological relevance. It should be pointed out that near GD20 ossification in some skeleton regions progressed quite rapidly and thus a higher proportion of incompletely ossified after incompletely and unossified bones in these regions may actually reflect a very short delay in development.

The occurrence of squamosal bone holes at the highest dose of COPA-R was somewhat higher than that recorded in the control group. The squamosal bone holes found in this study could be classified as a skeleton variation in our rat strain (Solecki et al., 2001). Actually, they were small holes that occurred at a relatively high background incidence in the squamosal bones of control fetuses. Similarly, rudimentary extra lumbar ribs, which are also generally classified as variations (Solecki et al., 2001), were recorded at a relatively high frequency in the offspring from control and treated dams. The elevated incidences of squamosal bone holes and rudimentary extra lumbar ribs in fetuses from the high dose group are consistent with the view that COPA-R, at maternally toxic doses, increased the occurrence of some skeleton variations.

In summary, results from this study showed that COPA-R at maternally toxic doses caused slight to moderate prenatal growth retardation and an increase in the occurrence of some skeleton variations. No evidence of maternal and developmental toxicity was found at the lowest dose tested. No evidence of teratogenicity in rats was noted at any dose level of COPA-R tested in this study. Based on these results, the NOAEL for maternal and developmental toxicities was set at 500 mg/kg bw/day.

The risk of COPA-R to women of childbearing age can be estimated by applying a safety factor of 100 to the NOAEL to derive a safe human exposure level of 5 mg/kg bw/day. This safety factor accounts for experimental animal-to-human extrapolation (10×) and for human sensitivity variation (10×). This approach is similar to that used by national and international bodies to derive human acceptable daily intake or reference dose for chemicals based on animal experimental data (WHO, 1990; EPA, 2002).

In Brazil, not all copaiba oleoresin product labels contain information on how the product should be used. Whenever this information is available, the recommended oral dose is 3–5 drops, up to three times a day, i.e., 0.75 mL/day or approximately 0.0125 mg/kg bw/day, for an adult weighing 60 kg. The estimated safe exposure for maternal and developmental toxicity (5 mg/kg bw/day) is 400 times the recommended daily dose of the phytotherapeutic product.

5. Conclusion

The results from this study indicate that the oleoresin from *C. reticulate* was not teratogenic to rats at any dose level tested. The estimated safe exposure level for humans, based on the NOAEL found in rats for maternal and developmental toxicity, suggests that copaiba oleoresin does not pose a health risk to pregnant women when used according to the recommended dose. However,

additional studies are necessary before this conclusion is generalized to oleoresins from other *Copaifera* species.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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