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Pre-clinical validation of a vaginal cream containing copaiba oil (reproductive toxicology study)

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ABSTRACT

The aims of this study was to evaluate the effects of oil-resin of Copaiba (*Copaifera duckei* Dwyer), aired in vaginal cream on the reproductive performance of female rats (*Rattus norvegicus*). To determine the components of the *C. duckei* oleoresin, gas chromatography coupled with mass spectrometry (CG–MS) was used, and considering the *trans*-caryophyllene sesquiterpene as a phytochemical marker in the oleoresin. Due to the extensive use of copaiba oleoresin in the suppository form for gynecological infections, an evaluation was carried out on the effects of copaiba oleoresin (*Copaifera duckei* Dwyer), delivered in a vaginal cream, on the reproductive performance of female Wistar rats. For this purpose, three groups ($n = 5–6$/group) of female rats were treated as follows: 1 – vaginal cream of copaiba oleoresin (28.6 mg/kg), 2 – base vaginal cream and 3 – control (physiological saline 0.9%), administered intravaginally, for 30 days before pregnancy, and from day zero to day 20 during pregnancy. Laparotomy was performed on the 21st day of pregnancy, followed by the determination of reproductive variables: number of live and dead fetuses, mass of the fetuses and placentas, number of implantations and resorptions, number of *corpora lutea*, pre- and post-implantation loss, and analyses of the fetuses with regard to external and internal anomalies and/or malformations (skeletal and visceral). The *trans*-caryophyllene present in the sample is suggested as a phytochemical marker and the results of this study demonstrate an absence of maternal toxicity and foetotoxicity embryofoetotoxicity at the dose administered, corresponding to ten times the recommended dose for use in humans. Accordingly, no significant statistical difference was observed between the treated and control groups, for the variables analyzed.

Thus, it is concluded that the vaginal cream containing 2.5% copaiba oleoresin is safe during gestation, in female rats (*Rattus norvegicus*) of the Wistar strain.

Introduction

Nowadays, traditional knowledge, particularly concerning the products of Amazonian biodiversity, has become an important tool in the development of new pharmaceutical products. Copaiba oil is an important natural product used in traditional medicine.

Pharmacological studies with the copaiba oleoresin show that its use is fully justified by the Indians. Tests *in vivo* and *in vitro* have shown that the oils of several species of Copaifera possess anti-inflammatory, antiseptic, wound healing, antitumor and antibacterial.

Various pharmacological applications of oleoresin of *Copaifera* spp. have been described in literature, including antimicrobial and antibacterial (Opdyke 1976; Miranda et al. 2000; Tincusi et al. 2002; Santos et al. 2008), anti-helmintic (Pellegrino 1967; Gilbert et al. 1972), analgesic (Fernandes et al. 1992; Carvalho et al. 2005), anti-inflammatory (Basile et al. 1988; Carvalho et al. 2005; Fernandes et al. 1992; Veiga et al. 2001), healing (Brito et al. 1999), gastroprotective (Paiva et al. 1998), antitumoral (Ohsaki et al. 1994; Lima et al. 2003) and tripanomicide activities (Cascon et al. 1998), cervicitis and leukorrhea (Le Cointe 1934).

It is commonly indicated as an anti-inflammatory, antiseptic and healing product, mainly of the upper air and urinary tracts (Le Cointe 1934; Basile et al. 1988; Carvalho and Cascon 2003; Carvalho et al. 2005).

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Copaiba oleoresin is one of the most commonly used natural products among the population of the Brazilian Amazon region, where it has considerable economic and social significance, as it is a native product, and several communities depend on its extraction for their livelihoods (Santos et al. 2001). Despite the existence of various vaginal creams on the market, the copaiba suppository has always been used by the population. Its application in gynecology is not recent, and copaiba suppositories have been sold in pharmacies and natural product stores throughout Brazil for many years.

The anti-inflammatory and antimicrobial activities are the most commonly investigated. Studies on rats using inflammatory process models, such as carrageenan-induced edema, induction of granulomatous tissue and bradykinin, indicate that copaiba oleoresin presents anti-inflammatory activity and low toxicity (Basile et al. 1988). Lima et al. (2003) demonstrate that the oil of *C. multijuga* presents tumoricide activity in melanoma cells, both *in vivo* and *in vitro*. The analgesic and anti-inflammatory activity of *C. duckei* showed significant results, when used via the topical route in the carrageenan-induced paw edema, granuloma, and croton oil-induced dermatitis tests (Carvalho et al. 2005). In another experimental model involving acetic acid-induced colitis in rats, it was observed that kaurenoic acid, applied together with the acetic acid, decreased the inflammatory cell infiltrate and edema of the intestinal mucosa, suggesting its anti-inflammatory effect (Paiva et al. 2003).

Santos et al. (2008) tested the antimicrobial activity of eight species of the genus *copaifera*. The species *C. Martii*, *C. officinalis* and *C. reticulata* exhibited good antibacterial activity against gram-positive bacteria, including MRSA (Methicillin-Resistant *Staphylococcus aureus*) strains. By contrast, all the oils tested were inactive against gram-negative bacteria. Antifungal activity in the species *C. paupera* and *C. lucens, C. cearensis, C. langsdorffi* and *C. multijuga* proved to be moderate.

The copaíba oleoresin (*Copaifera duckei*) showed antimicrobial activity against strains of microorganisms studied (*Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, *Bacillus cereus*, *Enterococcus faecalis*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Streptococcus salivarius* and *Micrococcus luteus*). The formation of inhibition halos was observed in all of the dosages of oleoresin. The serial dilution test in broth showed correlation with the diffusion in agar test. It was concluded that the oleoresin of *C. duckei* Dwyer, in the conditions of this assay, presented bacteriostatic and bactericidal selective activity against gram-positive and fungi microorganisms in different concentrations, but it was ineffective against gram-negatives (Cabral 2008).

The pregnancy affects the vaginal environment by modifying the immune and hormone balance, disappearance of vaginal discharge, abnormal vaginal discharge and increased pH (Ghione and De Palo 1996). These changes may lead pregnant women to a picture of vulvovaginitis. The vulvovaginitis is bacterial polymicrobial infection that causes vaginal discharge without clinical signs of inflammation. It occurs in 10% to 26% in pregnancy (Biswass 1993). Its importance arises in pregnancy may lead to premature labor and premature rupture of membranes (Sullivan and Smith 1993). The term bacterial vaginosis is attributed to a syndrome in which there is reduced amount of *Lactobacillus* sp. concomitant increase in anaerobic organisms, like *Gardnerella vaginalis*, *Mobiluncus* and *Bacteroides*, among others (Linhares et al. 1994).

Although the occurrence and diagnosis of vulvovaginitis in pregnant women are similar to nonpregnant women, the management of various infections vulvar and vaginal during pregnancy is often a dilemma. First, because some medications may be teratogenic to the fetus, and second, some infections can affect the neonate directly or trigger preterm labor, and finally, the qualitative and quantitative changes in the microflora during pregnancy, some infections may be more resistant to therapy (Sullivan and Smith 1993). Given this, it becomes necessary to introduce new vaginal creams in the pharmaceutical market that are safe and effective.

In this context it is important to emphasize the importance of herbal preparations, because although the pharmaceutical industry employ the most different techniques, such as molecular biology, combinatorial chemistry and computational chemistry, natural products are synthesized by a unique process of combinatorial chemistry, the biological evolution and natural selection, which provides a structural diversity of different molecules to biological targets (Mcchesney et al. 2007).

Popular and even traditional use, are not sufficient to ethically validate natural products as effective and safe. The recommendation or official authorization of their medicinal use should be backed by supporting experimental evidence, and the safety of their use should also be proven, including during the gestational period (Simões 2000).

It was believed for some time that the uterus was impermeable to external agents, and that the placenta constituted a true barrier between the maternal and fetal organisms. The "placental barrier" concept was disproven, at the beginning of the 1960s, by the "Thalidomide Tragedy", when significant fetal defects were caused with the ingestion of a single dose of the drug during gestation (Smithells and Newman 1992).

The appraisal of reproductive performance consists of the administration of the substance to be evaluated, in pregnant females, from implantation to the end of gestation, which corresponds to one day before the birth. Consequently, knowledge of the stages of embryonic development is essential, as some stages of development are more vulnerable than others (Rogers and Kavlock 2001).

The use of any drug during gestation, even natural products, should always take in to consideration the risk-benefit ratio. Reproductive toxicology studies of natural products are still in the early stages in Brazil, but they are necessary to enable medical professionals to prescribe the use of these natural products (Calixto 2005).

Lyra et al. (2005) evaluate the toxic effect of oil of neem seed (*Azadiracha indica* A. JUSS), used in popular medicine as a repellent, in pregnant rats. This study did not demonstrate maternal toxicity and anti-implantation effect; however, there was reduction of fetal body mass, which might indicate possible embryofoetotoxicity. Based on the reports presented, this study seeks to evaluate the reproductive performance, at pre-clinical level, of animals treated with vaginal cream containing copaiba oleoresin.

Material and methods

Obtaining the copaiba oleoresin

The copaiba oil used in the study was obtained from the company Beraca Ltda, located in Ananindeua, in the state of Pará, Brazil. According to the company's report, the oil was obtained from the species *Copaifera duckei* Dwyer.

Analysis of the copaiba oleoresin by gas chromatography

To determine the components of the *C. duckei* oleoresin, gas chromatography coupled with mass spectrometry (CG–MS) was used, following the method described by Adams (1995) and considering the *trans*-caryophyllene sesquiterpene as a phytochemical marker in the oleoresin.

The Gas Chromatograph Agilent® 6850 was used, coupled with a 5973 (CG-EM) Mass Spectrometer, and Helium carrier gas, with flow of 1.5 ml/min, injector temperature of 270 °C, oven at 120 °C, and detector at 300 °C. Capillary column DB −1.25 m – 0.32 mm, 0.25 μm. Injection volume of 1.0 μl, split mode 20:1. Heating ramp:

120 °C for 2 min, heating of 3 °C per min up to 160 °C, and 8 °C per min up to 290 °C, maintained for 5 min. The sample was read in scan mode, with 40–600 AMU.

The sample was prepared according to the sterification method described by the IUPAC (1987), with adaptations described in Method 2 of the Manual de Métodos Analíticos (manual of analytical methods) of the Instituto Adolfo Lutz (2005).

The "external standard" technique was adopted to quantity the copaiba oleoresin, using the *trans*-caryophyllene standard (Sigma–Aldrich Co.) as a marker. The quantification was performed by the external standardization method, which consisted of constructing a calibration curve with the standard of the compound present in the sample, thereby determining the concentration of the given compound.

Obtaining the copaiba vaginal cream

The copaiba vaginal cream and the base cream were obtained from the Laboratório Farmacêutico Almeida Prado Ltda, in the city and state of São Paulo, Brazil, at a concentration of 2.5%, supplied in polyethylene bottles.

Reproductive toxicology study

Animals

Before the start of the activities, this project was submitted for approval by the Research Ethics Committee of the Federal University of Amapá, under registration number 003A/2007.

Male and female and albino rats (*Rattus norvegicus*) of the Wistar strain were used, weighing around 250 g, supplied by the Multidisciplinary Center for Biological Investigation in the Area of Science in Laboratory Animals (CEMIB) of the University of Campinas/Unicamp, São Paulo.

The female rats were acclimatized in the Drug Research Laboratory of the Federal University of Amapá, for seven days. They were kept in polyethylene boxes with maximum capacity for five animals each, under a constant temperature of 23 °C ± 2 °C and light/dark cycle of 12 h. After the acclimatization period, the female rats were randomly drawn, to form the three groups: UG, TBVC and TCVC.

Experimental groups

The females were randomly distributed in three experimental groups: the Control or Untreated Group (UG): Untreated pregnant rats, which received 0.5 ml of physiological saline intravaginally for 30 days and throughout their pregnancy ($n = 5$); the Group Treated with Base Vaginal Cream (TBVC): Pregnant rats, which received only the base vaginal cream, intravaginally, in the same quantity as that used in the group treated with copaiba vaginal cream, for 30 days and throughout pregnancy ($n = 5$); and the Group Treated with Copaiba Vaginal Cream (TCVC): Pregnant rats, which received a dose of 28.6 mg/kg of copaiba vaginal cream, intravaginally, corresponding to 10 times the dose that would be used in humans, for 30 days and throughout the pregnancy ($n = 6$).

Experimental sequence

Mating period

After the acclimatization period, and on reaching sexual maturity, the female rats were treated for 30 days, according to their experimental group. The mating phase started after this period, lasting for approximately 20 days. For mating purposes, the phase of the estral cycle of each female rat was observed in the morning by means of a vaginal smear, classifying the phase as proestrus (12 h), estrus (14 h, ovulation occurs spontaneously half way through the dark cycle during this phase.), metaestrous (21 h), or diestrus (57 h).

After detecting the proestrus phase, the female rats were distributed in pairs, in polyethylene cages, with appropriate bedding, in the presence of a male rat at the end of the afternoon. The following morning, the males were removed and the vaginal smears were collected by introducing a 10 μl pipette and aspirating with 0.9% physiological saline solution (Marcondes et al. 2008). The presence of spermatozoids was considered indicative of pregnancy, associated with the diagnosis of the estrous phase of the estral cycle. This phase is characterized as the maximum estrogenic phase. The day on which pregnancy was confirmed was considered day zero (Calderon 1988).

Pregnancy period

The three different groups of pregnant rats, Control, TCVC and TBVC, received, intravaginally, 0.5 ml physiologic saline, 28.6 mg/kg of copaiba vaginal cream, and the base vaginal cream, respectively, until the 20th day. The treatment was always administered in the morning, adjusting the doses every five days, according to the weight of the rats.

During pregnancy, the females were kept in individual cages for 21 days, weighed on days 0, 5, 14 and 21, and observed in relation to behavior and water and food ingestion.

Cesarian procedure

On the 21st day of pregnancy, the rats were anesthetized with 50 mg/kg of sodium thiopental (Thiopentax®, Cristalia Ltda.) to perform the laparotomy, exposing the uterine horns to observe the resorption nodules, and removing of the ovaries to count the *corpora lutea*. The fetuses and placentas were immediately removed. After this procedure, the mothers were submitted to euthanasia by volemia depletion. The newborns were immediately analyzed and then weighed and measured (Calderon 1988).

Evaluation methods

Maternal weight

A set of Gehaka BG4000 scales was used for the daily weight evaluation, and the weight gain was calculated by the differences in body weight observed on days zero, 5, 14 and 21 of pregnancy.

Maternal reproductive performance

The implantation sites, resorption nodules and numbers of live and dead fetuses were observed and counted. The ovaries were also observed, in order to count the *corpora lutea*, with the aid of a Nikow® magnifying glass, model SMZ 645 (C-W 10x A/22).

The embryo loss rate in the period preceding implantation was denominated pre-implantation loss percentage, and was calculated by the following formula:

$$\frac{Nr.\ of\ corpora\ lutea - Nr.\ of\ implantation}{Nr.\ of\ corpora\ lutea} \times 100$$

The death of the embryos after their implantation process (percentage of post-implementation loss) was calculated by the following formula:

$$\frac{Nr.\ of\ implantations - Nr.\ of\ live\ fetuses}{Nr.\ of\ implantation} \times 100$$

Test to confirmation the implantation and of resorption sites

In cases of absence of fetal development or visible implantation sites, the uterus was placed in a 0.2% NaOH solution to reveal whether there was any implantation site in that uterus. If the 0.2% NaOH solution revealed the presence of an invisible implantation site, it was then considered NaOH positive (+).

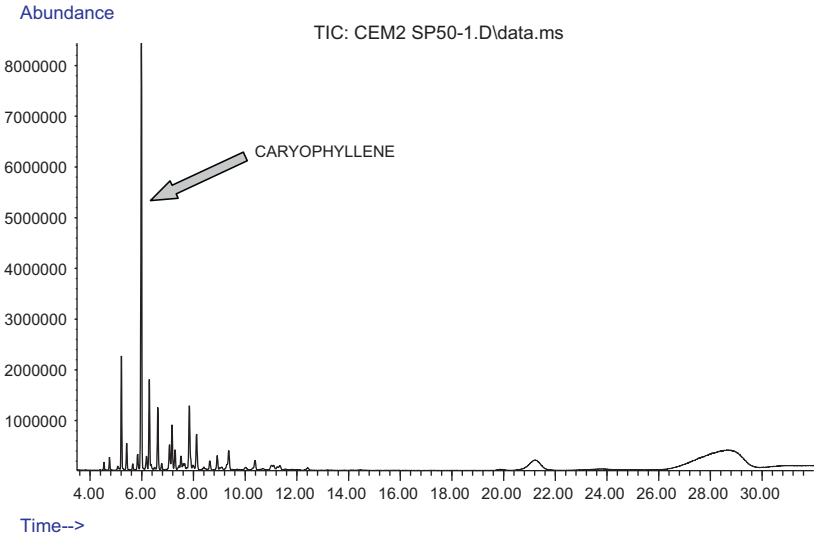

Fig. 1. Chromatogram of copaiba oleoresin (RF 3340) sterified by the IUPAC method, obtained in a 6850 (Agilent®) Gas Chromatograph coupled with 5973 (CG-EM) Mass Spectrometer.

Weight and classifying the newborns

After the laparotomy, the newborns were weighed using a set of Marte scales. The newborns were classified as AAP (Adequate for Age of Pregnancy), SAP (Small for Age of Pregnancy) and LAP (Large for Age of Pregnancy), according to their body weights, in accordance with the parameters established by Calderon (1988): AAP: body weight between the mean weight of the control group, plus or minus the standard deviation; SAP: body weight below the mean weight of the control group, minus the standard deviation and LAP: body weight above the mean weight of the control group, plus the standard deviation.

Weight of the placentas and placental index

The placentas, without the membrane and umbilical cord, were weighed on a set of Marte scales. The placental index (PI) was determined by the ratio between the placental weight (PW) and the fetal weight (FW) (Calderon 1988).

$$PI = \frac{PW}{FW}$$

Analysis of external malformations

After the weighing, the newborns were examined externally, meticulously analyzing the eyes, mouth, ear implantation, cranial shape, fore and rear limbs, anal perforation and tail (Wilson 1965).

Analysis of visceral anomalies and malformations

Immediately after the external examination, half of the newborns from each litter were placed in Bouin solution for four days, for fixing of the visceral structures and decalcification of the bones. They were then placed for two days in 80% hydroalcohol solution, which was later replaced by 90% hydroalcohol solution, up until the day of analysis. The serial sectioning method was used to observe visceral anomalies and/or malformations, according to the descriptions proposed by Wilson (1965). The analyses were carried out with the help of a Nikon® microscope, model SMZ 645 (C-W 10x A/22).

Analysis of skeletal anomalies and/or malformations and count of ossification points

The method described by Staples and Schnell (1964) was used to analyze the skeletal anomalies and/or malformations. Half of the newborns from each litter were placed in 70% hydroalcohol solution for 12 h. The newborns were then placed in acetone P.A for 24 h, eviscerated, diaphanized and stained with alizarin red dye. The analyses were conducted according to the method described by Aliverti et al. (1979). Ossification points were observed and counted in the litter processed for the analysis of skeletal malformations. The ossification points were evaluated in the following places: sternum, anterior and posterior phalanges, metacarpals, metatarsuses and cervical and caudal vertebrae.

Statistical analysis

Analysis of variance (ANOVA) was used to compare the body weight values, followed by Tukey's Test. The Kruskal–Wallis nonparametric test (Siegel 1970) was used to compare the mean values of the parameters of reproductive performance, fetal and placental weights, and placental index of the experimental groups. The Chi-squared Test (Berquó and Souza 1981) was used to compare the percentages of SAP, AAP and LAP. The Goodman test (Goodman 1964) was used to evaluate the incidence of malformations or of fetal anomalies in the untreated and treated groups. Results with $p < 0.05$ were considered statistically significant.

Results

Analysis of copaiba oleoresin by gas chromatography

Fig. 1 and Chart 1 represent the chromatogram of the sample sterified by the IUPAC method, and unsterified, presenting the most abundant and well-resolved peak, caryophyllene, and retention times of the components. The copaiba oleoresin samples exhibited a caryophyllene content of around 500 ng/ml (0.5%), which represents an adequate range for quantification by the proposed method.

Maternal weight

During the 30-day treatment period and gestation period (from day zero to day 21) no visible clinical signs of maternal toxicity, such as weight loss or reduced feed intake, were recorded.

The mean weight of the female rats of the Control group, TBVC and TCVC presented very similar values, with no significant sta-

Oleoresin – IUPAC		Oleoresin – Unsterified	
RT	**COMPOUND**	**RT**	**COMPOUND**
4,544	Cyclohexene	4,556	Cyclohexene
4,754	A-Cubebene	4,766	α.-Cubebene
5,218	α.-Copaene	5,231	α.-Copaene
5,422	Cyclohexane	5,434	Cyclohexane/ β-elemene
5,651	3H-3a,7-Methanoazulene,	5,670	α-Gurjunene
5,842	*trans*-Caryophyllene	5,861	*trans*-Caryophyllene
5,988	Caryophyllene	6,007	Caryophyllene
6,185	1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-bicyclogermacrene	6,204	γ-Elemene
6,293	*trans*-α-Bergamotene	6,312	*trans*-.α.-Bergamotene
6,624	alpha.-Caryophyllene	6,643	α.-Humulene
6,777	1H-Cycloprop[e]azulene	6,789	1H-Cycloprop[e]azulene
7,076	Naphthalene	7,095	Naphthalene
7,171	Germacrene D	7,197	Germacrene-D
7,286	α-Selinene	7,311	1H-Cycloprop[e]azulene, decahydro- 1,1,7-trimethyl-4-methylene
7,521	4,7-Methanoazulene	7,540	4,5-dimethyl-11-methylenetricyclo[7.2.1.0(4.9)]dodecane
7,846	β-Bisabolene	7,629	α-Muurolene
8,126	delta.-Cadinene	7,871	beta.-Bisabolene
8,635	cis-α-bisabolene	8,151	γ-Cadinene
8,921	Germacrene B	8,666	cis-α-bisabolene
9,366	Caryophyllene oxide	8,953	Germacrene B
10,384	-	9,398	Caryophyllene oxide
21,225	Palmitic acid-methyl Ester	10,422	Fonenol
28,687	Linoleic acid, methyl Ester		

Chart 1. Composition of *C. duckei* oleoresin and the respective retention times (RT) of the sterified and unsterified sample.

tistical difference observed among these groups from day zero to day 21 of pregnancy. Treatment with the copaiba oleoresin vaginal cream did not affect maternal weight in any period of pregnancy (Table 1).

The development of maternal weight gain was progressive during the pregnancy period for all the groups studied. Some female rats of the TBVC and TCVC groups attained a weight gain above 80 g at the end of pregnancy. In the Control group this increase was smaller, with values below 75 g (Fig. 2). No significant difference was observed among the three groups.

Table 1
Weights (g) of the female rats of the Control, Base Vaginal Cream and Copaiba Vaginal Cream groups, expressed in mean ± standard deviation, according to the day of pregnancy.

	Control	TBVC	TCVC
Day zero	251.4 ± 23.3	252.8 ± 15.6	252.1 ± 19.8
Day 5	266.6 ± 20.6	270.6 ± 17.3	267.8 ± 16.1
Day 14	293.2 ± 20.2	296.8 ± 17.2	294.5 ± 19.4
Day 21	344.4 ± 22.0	363.4 ± 18.3	346.0 ± 37.2

n = 5–6/group. The values do not differ from one another at a level of 5%. ANOVA, followed by Tukey's test.

Maternal reproductive performance

Of the five and six female rats mated from the Control and TCVC groups, respectively, all had a full term pregnancy (100%). How-

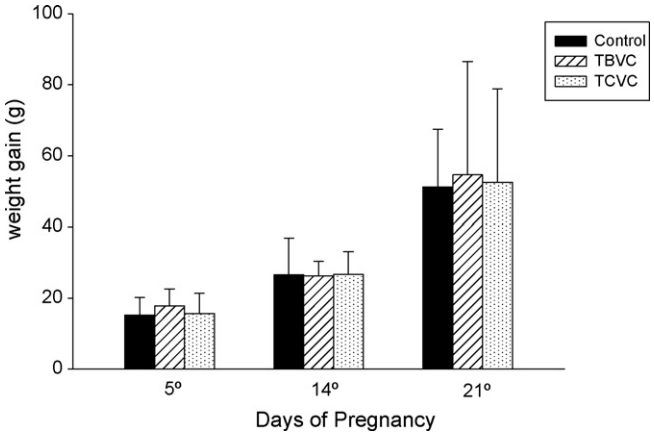

Fig. 2. Evolution of weight gain of the female rats, expressed in grams (g), of the Control, TBVC and TCVC Groups, according to the day of pregnancy. The columns represent the mean values and standard deviation for the variable weight of n = 5–6/group.

Table 2
Number of female rats mated, full term pregnancy and loss rates (%) before and after implantation of the blastocyst in the pregnant rats of the Control, TBVC and TCVC groups.

	Control	TBVC	TCVC
Mated female rats	5	6	6
Full term pregnancy	5	5	6
Rate of full term pregnancy (%)	100	83.33	100
Pre loss (%)	30.76	6.66	45.53
Post loss (%)	22.22	0	3.33

n = 5–6/group. The values do not differ from one another at the level of 5%. Kruskal–Wallis Test.

Table 3
Weight of the rat pups (fetal weight), expressed as mean and standard deviation, of the female rats of the Control, TBVC and TCVC groups.

	Control	TBVC	TCVC
Minimum	2.93	3.85	3.98
Maximum	6.24	5.42	7.25
Median	4.77	4.85	4.78
p25	4.63	4.60	4.54
p75	5.00	5.03	4.97
Arithmetic mean	4.82	4.81	4.82
Standard deviation	0.52	0.35	0.56

n/groups: Control (n = 34); TBVC (n = 51) and TCVC (n = 43). The values do not differ from one another at a level of 5%. Kruskal–Wallis Test.

ever, of the six female rats mated from the TBVC group, only five reached the end of their pregnancy (83.33%). This difference was not significant when compared with the Control and TCVC groups (Table 2).

The pre-implantation loss was greater in the TCVC group, and the post-implantation loss was greater in the control group, however, there was no statistically significant difference among the three groups analyzed (Table 2).

Weight of the fetuses and placentas, and placental index

The presence of stillborns was not verified among the three groups after the laparotomy. In the group treated with copaiba oleoresin vaginal cream (TCVC) the mean fetal weight was 4.82 g and the placental weight was around 0.5 g, determining a mean placental index of 0.08 g. No significant difference was observed among these parameters in relation to treatment with the base vaginal cream (TBVC), which contained only the components of the formulation, or the control group (UG), which received only physiological solution at 0.9%. These evaluation parameters are shown in Tables 3–5.

Test to confirmation the implantation and resorption sites

In immersing the uterus in the solution containing sodium hydroxide at 0.2%, the invisible resorption nodules were revealed and after the observation, were identified as positive, NaOH (+). This revelation occurred in the uterus of three rats (3/6) treated with the copaiba oleoresin vaginal cream (TCVC) and in the uterus of all the rats (5/5) of the control group. Although the resorptions were high in the control group (Fig. 3), there was no significant difference among the three groups.

Table 4
Weight of the placentas (placental weight) of the female rats from the Control, TBVC and TCVC groups, expressed as mean and standard deviation.

	Control	TBVC	TCVC
Minimum	0.31	0.28	0.28
Maximum	0.61	0.6	0.94
Median	0.40	0.38	0.38
p25	0.36	0.35	0.35
p75	0.43	0.42	0.43
Arithmetic mean	0.40	0.39	0.41
Standard deviation	0.07	0.06	0.12

n/groups: Control (n = 34); TBVC (n = 51) and TCVC (n = 43). The values do not differ from one another at a level of 5%. Kruskal–Wallis Test.

Table 5
Placental index (PI), obtained by the ratio between each placental weight and respective fetal weight of the female rats of the Control, TBVC and TCVC groups.

	Control	TBVC	TCVC
Minimum	0.06	0	0.05
Maximum	0.13	0.11	0.16
Median	0.08	0.08	0.08
p25	0.07	0.07	0.07
p75	0.09	0.08	0.08
Arithmetic mean	0.08	0.07	0.08
Standard deviation	0.01	0.01	0.02

n/groups: Control (n = 34); TBVC (n = 51) and TCVC (n = 43). The values do not differ from one another at a level of 5%. Kruskal–Wallis Test.

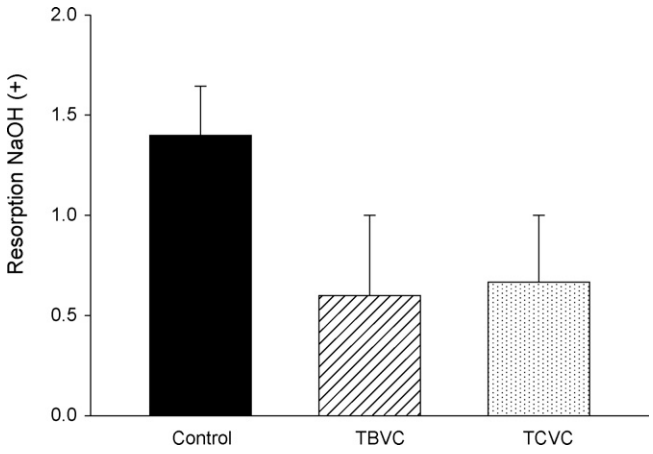

Fig. 3. The columns represent the means ± standard error of the mean resorption variable for the female rats of the Control group, TBVC and TCVC of n = 5–6/group. It was not significant for $p < 0.05$.

Weight and classification of the newborns

The majority of newborn pups of female rats of the TCVC and TBVC groups presented weights which were classified as Adequate for the Age of Pregnancy (AAP), of 72.1% and 72.5%, respectively. The percentage of newborns classified as Small for Age of Pregnancy (SAP) was higher in the TCVC group; nevertheless, this difference was not statistically significant, at a level of 5%, when compared with the other groups, Control and TBVC (Table 6).

The predominance of AAP newborns, with equal numbers for LAP and SAP, was not related to the use of the copaiba oleoresin vaginal cream, although it was observed that the percentage of small pups (SAP) was lower in the control group, no significant statistical difference was observed (Fig. 4).

Table 6
Percentage (%) of newborns classified as small (SAP), adequate (AAP) and large (LAP) for Age of pregnancy, from female rats treated with Copaiba Vaginal Cream (TCVC), Base Cream (TBVC) and Control.

	Control	TBVC	TCVC
SAP	2.9%	9.8%	14.0%
AAP	82.4%	72.5%	72.1%
LAP	14.7%	17.6%	14.0%

The values do not differ from one another at the level of 5%. Chi-squared Test.

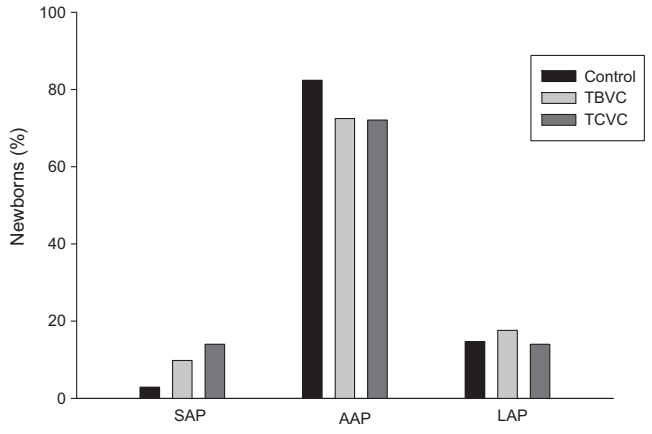

Fig. 4. Percentage (%) of newborns classified small (SAP), adequate (AAP) and large (LAP) for age of pregnancy, from female rats treated with copaiba vaginal cream (TCVC), base cream (TBVC) and control.

Count of ossification points

The total count for ossification points was, on average, 42.3, 39.6 and 39.9, respectively, for the Control, TBVC and TCVC groups. Treatment with copaiba oleoresin vaginal cream did not evidence a significant difference in the total or differential count of these ossification points, when compared with the TBVC and Control groups (Table 7).

Analysis of external and internal anomalies and malformations (skeletal and visceral)

No external malformations or anomalies were diagnosed in the Control group. In spite of the occurrence of anomalies and/or malformations detected in the TBVC and TCVC groups, this difference was not considered significant. In relation to skeletal and visceral malformations and/or anomalies, all the groups were affected, though with no significant statistical difference at a level of 5%. Neither did the number of litters affected present any significant difference among the groups. Accordingly, the topical treatment with copaiba oleoresin did not increase the frequency of skeletal and visceral malformations and anomalies (Table 8).

Differential analysis of malformations and anomalies did not evidence a significant difference from the effect of the treatment with copaiba oleoresin, delivered in vaginal cream. Of the skeletal malformations detected, the most visible in the three groups was phalangeal agenesis (Fig. 5) and of the anomalies, the most common one was "butterfly" sternebrae (Fig. 6). Nevertheless, the differences were not considered significant among the three groups (Table 9).

Table 7
Mean and standard deviation of the ossification points for newborn pups of female rats of the Control, TBVC and TCVC groups.

	Control	TBVC	TCVC
Anterior phalanges	6.0 ± 2.4	4.9 ± 2.2	3.0 ± 1.7
Metacarpals	7.8 ± 0.4	8.0 ± 0.0	7.8 ± 0.5
Posterior phalanges	6.6 ± 2.3	5.3 ± 3.2	5.7 ± 2.9
Metatarsusals	9.7 ± 0.6	10.0 ± 0.0	10.0 ± 0.0
Caudal vertebra	3.9 ± 1.0	3.8 ± 1.1	3.8 ± 1.4
Sternebrae	3.9 ± 1.0	3.8 ± 1.1	3.8 ± 1.4
Total ossification	42.3 ± 9.8	39.6 ± 7.7	39.9 ± 5.5

n/group: Control ($n = 18$), TBVC ($n = 26$) and TCVC ($n = 23$). The values do not differ from one another at a level of 5%. Kruskal–Wallis Test.

Table 8
Frequencies of external, skeletal and visceral anomalies and malformations in litters and newborn pups from female rats of the Control, TBVC and TCVC groups.

	Control	TBVC	TCVC
External malformations			
Newborns affected	0/36	1/51	2/43
Litters affected	0/5	1/5	2/6
External anomalies			
Newborns affected	0/36	0/51	1/43
Litters affected	0/5	0/5	1/6
Skeletal malformations			
Newborns affected	2/18	4/26	7/23
Litters affected	1/5	3/5	3/6
Skeletal anomalies			
Newborns affected	8/18	15/26	12/23
Litters affected	3/5	5/5	3/6
Visceral malformations			
Newborns affected	8/18	5/25	7/21
Litters affected	4/4	3/5	5/5
Visceral anomalies			
Newborns affected	1/18	4/25	4/21
Litters affected	1/4	3/5	3/5

The values do not differ from one another at a level of 5%. Goodman Test.

Observing the details of visceral anomalies and malformations, it is noted that the percentage of cryptorchidism in the control group was higher (22.2%) than in the TCVC group (14.3%), though this difference was not significant among the three groups analyzed. The TBVC group exhibited a higher percentage of visceral anomaly, located in the lateral ventricle (Fig. 7), though this difference was not significant (Table 10).

Discussion

Several studies report the use of the GC-EM technique for qualitative and quantitative analysis of copaiba oleoresin (Veiga et al. 1997; Rigamonte-Azevedo et al. 2004; Tappin et al. 2004; Biavatti et al. 2006). This chromatographic technique was effective in this study as it enabled the separation of the sample components, permitting the identification of constituents present in the *C. duckei* Dwyer oleoresin used in the production of copaiba oil vaginal cream (Fig. 1). The phytochemical marker *trans*-caryophyllene is available on the market, and is used as standard in various copaiba oleoresin quantification studies. It is pinpointed as the factor responsible for several pharmacological actions described in copaiba oleoresin (Tappin et al. 2004; Veiga and Pinto 2002). Although the study by Lameira et al. (2009) showed a seasonal variation in the chemical composition of the volatile fraction of this species, this compound is

Table 9
Percentage (%) of external and skeletal malformations and anomalies of newborn pups from female rats of the Control, TBVC and TCVC Groups.

	Control	TBVC	TCVC
External malformations and/or anomalies (%)			
Poorly positioned eyes	0	2.0	0
Malformed tail	0	0	2.3
Skeletal malformations (%)			
Metacarpal	5.6	0	8.7
Metatarsus	11.1	0	0.0
Phalangeal agenesis	27.8	30.8	30.4
Rib agenesis	0.0	3.8	0.0
Rib hypoplasia	0.0	3.8	0.0
Skeletal anomalies (%)			
Atrophied sternebrae	5.6	0	4.3
Rudimentary sternebrae	5.6	3.8	17.4
Irregularly shaped sternebrae	0.0	0.0	4.3
Butterfly-shaped sternebrae	22.2	11.5	8.7
Skull – reduced ossification	0.0	3.8	4.3

The values do not differ from one another at the level of 5%. Goodman Test.

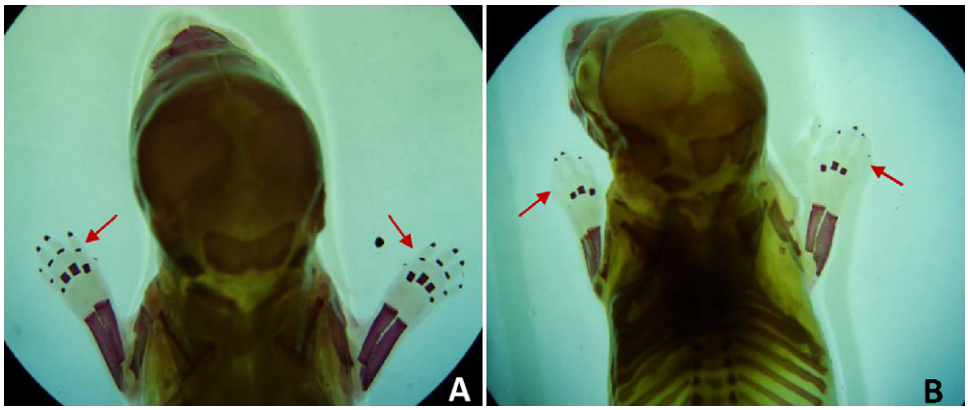

Fig. 5. Newborn from the TBVC group with normal phalanges (A) and with phalangeal agenesis (B) (observe the tip of the arrow).

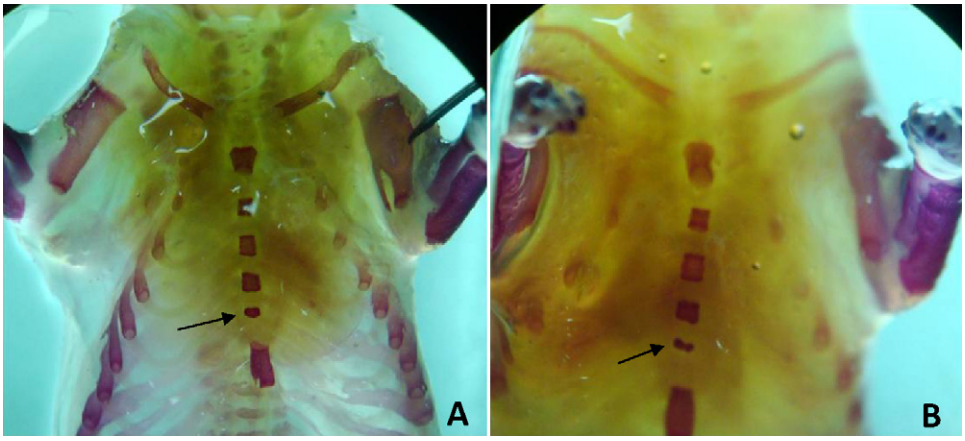

Fig. 6. Newborn from the Control group with normal sternebrae (A) and with the butterfly-shaped 5th sternebra (B) (observe the tip of the arrow).

present in all the species of *copaifera*, and was consequently chosen as a quality marker of the product studied here.

A study on the chemical composition of the oleoresin of several species of common occurrence in Brazilian Amazon was performed by Cascon and Gilbert (2000), with *C. guianensis*, *C. duckei* and *C. multijuga*, and all these species shows that significant chemical variation. All the samples analyzed only have in common the sesquiterpenes β-caryophyllene and α-bergamotene which are not restricted to the *Copaifera* genus. Copalic acid previously considered a characteristic diterpene of the *Copaifera* genus is, however, not a reliable marker because it occurs only in traces, if at all in *C. duckei* of all the samples analyzed and *C. multijuga* oleoresin is characterized by the great predominance of β-caryophyllene.

Similarly, Veiga et al. (2007) also studied the chemical and anti-inflammatory activity investigations from the Copaiba oils obtained from *C. multijuga*, *C. cearensis* and *C. reticulate*, confirming the findings of Cascon and Gilbert (2000), since chromatographic studies showed that the main compound among sesquiterpenes β-caryophyllene was (57.5, 19.7 and 40.9%, respectively), α-humulene Followed by, α-copaene, α-bergamotene, δ-cadinene, with different amounts in each oleoresin. However, species have proved that, although similar, these oleoresins possess varied composition and anti-inflammatory activity. In this study β-caryophyllene was considered as a pharmacological and phytochemical marker.

Mendonça and Onofre (2008) showed that the Copaiba oil (*C. multijuga* Hayne) was able to inhibit the growth of three bacteria,

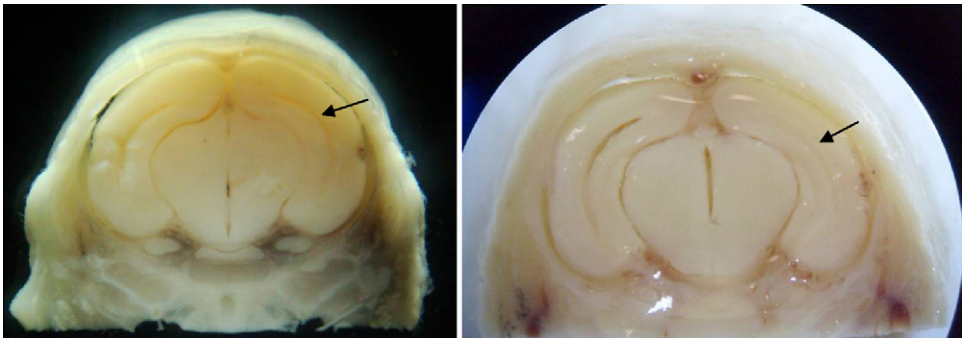

Fig. 7. Section of the head, evidencing two normal lateral ventricles, from a newborn pup from the Control group (A), and one from the TBVC group, with closed lateral ventricle (B) (observe the tip of arrow).

Table 10
Percentage of visceral malformations and anomalies in newborns from female rats of the Control, Base Vaginal Cream (TCVC) and Copaiba Vaginal Cream (TCVC) groups.

	Control	TBVC	TCVC
Visceral malformations (%)			
Cryptorchidism	22.2	4	14.3
Hypoplastic Kidney	5.6	0	0.0
Supranumerary kidney	5.6	0	0.0
Microphthalmia	5.6	4	0.0
Anaphthalmia	0.0	0	4.8
Alteration in the nasal septum	5.6	0	9.5
Lung inversion	0.0	8	0.0
Aortic defect	0.0	4	0.0
Dextrocardia	0.0	4	0.0
Alteration in the interventricular septum	0.0	0	4.8
Visceral anomalies (%)			
Lateral ventricle	5.6	12	4.8
Retinal alteration	0.0	4	4.8
Poorly positioned eyes	0.0	4	9.5
Cerebral hemorrhage	5.6	0	0.0
Diaphragmatic hernia	5.6	0	0.0

The values do not differ from one another at a level of 5%. Goodman Test.

presenting a minimum inhibitory concentration of 1.56, 3.12 and 12.5% for *E. coli*, *S. aureus* and *P. aeruginosa*, respectively.

Maternal exposure to chemical agents during the gestation period can result in alterations in the fetal development. These alterations depend on factors inherent to the maternal organism, placental functionality, or a direct action on the embryo-fetal organism itself, which in turn, can lead to the death of the fetus, malformations and/or anomalies, or impairment of the physical and/or behavioral development of the newborn pup (Calliari-Martin et al. 2002).

The use of medicinal plants during gestation, even those with topical use, can result in different alterations in embryo-fetal development, due to their effect on embryonary implantation, resulting in abortive (embryolethality) or embryofoetoxicity effects (Almeida and Lemonica 2000; Lyra et al. 2005). Maternal reproductive performance was evaluated in this study through the observation of certain parameters, such as maternal weight, number of live fetuses and percentage of pre- and post-implantation loss.

Taking into consideration the greater likelihood of absorption of the drug during the gestational period, particular attention should be paid, when performing the toxicological assays, to the evident clinical signs of maternal toxicity that have resulted in death or a reduction in body weight gain (Calliari-Martin 1998).

It is important to emphasize that weight evaluation of pregnant rats is one of the most relevant parameters, because it indirectly evaluates the degree of maternal and fetal impairment. Thus, we can say that loss of body mass is one of the main indicators of maternal toxicity (Lyra et al. 2005), as insufficient weight gain may result in restriction of intrauterine growth (Schwarez et al. 1996). In this study, there was no significant difference in weight between the female rats of the three groups; control, treated with base vaginal cream, and treated with the vaginal cream containing copaiba oleoresin, both in the pre-implantation period and in the total gestation period, which suggests the absence of maternal toxicity (Fig. 2).

In the pre-implantation period, the embryo has undifferentiated cells in mitotic division. The reduction in the number of cells in the embryo, during this phase, can induce a delay in the formation of the blastocyst (Kola and Folb 1986) and consequently, an increase in pre- and post-implantation losses (Lemônica et al. 1996). According to Almeida and Lemonica (2000), the rate of pre-implantation loss establishes a correlation between the number of ova released and those that, after being fecundated, manage to be implanted in the uterus. Post-implantation loss, on the other hand, refers to the ratio between the number of blastocysts implanted and those that did not manage to develop. The implanted blastocyst, which did not manage to develop, is given the name "resorption" and indicates a fault in embryonary development.

The results of this study indicate that the copaiba oleoresin delivered in a vaginal cream at 2.5% does not interfere in the pre- and post-implantation phases, in the animal species studied and at the dose tested here, which corresponded to ten times the dose recommended for use in humans (Fig. 3, Tables 2–5).

The significant reduction in mass of the fetuses in the groups treated may indicate a possible fetotoxic effect (Lyra et al. 2005). No statistically significant difference among the three groups was observed in this study, in relation to the mass of the rat pups. Therefore, the copaiba oil delivered in the vaginal cream, in the concentration employed, did not determine the appearance of fetotoxicity, even though the number of pups classified as small for gestational age (SAP) was higher in this group (Fig. 4, Table 6).

In the organogenesis period, there is intense proliferation and migration of cells, remodeling of the tissues, and formation of the rudimentary organs (Brent 1993). This period is characterized as the most susceptible to teratogenic agents, with a higher probability of occurrence of fetal malformations. The type of malformation will depend on the embryonary stage and on the affinity of the agent for a particular embryonary tissue (Chang et al. 2002). This study did not evidence any relation between the frequency of external and internal anomalies and malformations (skeletal and visceral analyses) with the use of the vaginal cream containing copaiba oleoresin. The use of *C. duckei* Dwyer oleoresin delivered in a vaginal cream at 2.5%, administered in pregnant rats of the Wistar strain during the pre-implantation and organogenesis period, proved to be safe in this species for this variable (Tables 7–9).

Nonetheless, despite the differences, these results on the toxic effects of copaiba oleoresin, corroborated the study by Lourenço et al. (2008a) in relation to animal species (mouse, Swiss) and administration route (gavage). But it showed that mice from the negative control group and groups treated with three different doses of the copaiba oil did not produce any malformation. According to the results of Lourenço et al. (2008b), copaiba oil does not present any toxic potential, and it appears to have a protective effect against hydrocephalus and vertebral malformation.

Despite the clinical use of many medications, there is no sufficiently reliable information available on the teratogenic potential of many of them, and some are even controversial. Rosa (1996) analyzed seventy pregnant women exposed to a single dose of itraconazole, during the first trimester, and did not find any evidence of teratogenic effects. In another study, a retrospective cohort trial, with pregnant women exposed to itraconazole during the first trimester of pregnancy, the prevalence was 13% (Bar-Oz et al. 1999).

In the context of topical antimicrobial medications, it is necessary to consider the potential risk and the benefit, both for the mother and for the fetus, if the infection is treated or not. In this case, it is important to consider the treatment of these infections in the genital tract during gestation.

Although the data demonstrated in the study by Kazy et al. (2005) are not equivalent, several issues should be considered. A key point is the differences in teratogenic manifestations in humans (with limb abnormalities) and mice (defects of the skeletal extremities) (Tiboni et al. 2008). These phenotypic differences may reflect, as a first hypothesis, a difference in sensitivity among species to teratogen. This is a well-known phenomenon in teratology.

For this reason, the fact that the vaginal cream of copaiba oleoresin does not affect reproductive performance and does not cause teratogenic effects in Wistar rats does not rule out the need for further studies using other doses and other animal species, to evaluate the safety of this product in relation to embryotoxicity and

teratogenicity, even though copaiba oleoresin is traditionally used by women in the vaginal cavity to treat gynecological infections.

Conclusion

Based on the results obtained, the *trans*-caryophyllene present in the sample is suggested as a phytochemical marker, and the treatment with the vaginal cream containing copaiba oleoresin did not affect maternal weight during any phase of the pregnancy. Consequently, the dose used was not capable of causing maternal toxicity. It did not affect loss of the blastocyst before or after implantation, and is therefore not embryofoetotoxic. During pregnancy, it did not prevent the female rats from having a full term pregnancy, neither did it affect the mean weight of the newborns or placentas, or the placental index. Thus it did not interfere in reproductive performance during pregnancy at the dose tested, and did not affect the appearance of external anomalies and/or malformations. The use of the vaginal cream containing 2.5% copaiba oil proved to be safe during the gestation of Wistar rats (*Rattus norvegicus*).

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References

Adams, R.P., 1995. Identification of Essential Oil Components by Gas Chromatography/Ion Trap Mass Spectrometry. Allured Pub, Carol Stream.

Aliverti, V., Bonanomi, L., Giavini, E., Leone, V.G., Mariani, L., 1979. The extent of fetal ossification as an index of delayed development in teratogenic studies on the rat. Teratology 20, 237–242.

Almeida, F.C.G., Lemonica, I.P., 2000. The toxic effects of *Coleus barbatus* B. on the different periods of pregnancy in rats. J. Ethnopharmacol. 73, 53–60.

Bar-Oz, B., Moretti, M.E., Mareels, G., 1999. Reporting bias in retrospective ascertainment of drug induced embryopathy. Lancet 354, 1700–1701.

Basile, A.C., Sertie, J.A., Freitas, P.C.D., Zanini, A.C., 1988. Antiinflammatory activity of oleoresin from Brazilian Copaifera. J. Ethnopharmacol. 22, 101–109.

Berquó, E.S., Souza, J.M.P., 1981. Bioestatística. EPU, São Paulo.

Biavatti, M.W., Dossin, D., Deschamps, F.C., Lima, M.P., 2006. Análise de óleos-resinas de copaíba: contribuição para o seu controle de qualidade. Rev. Bras. Farmacogn. 16, 230–235.

Biswass, M.K., 1993. Bacterial vaginosis. Clin. Obstet. Gynecol. 36, 166–176.

Brent, R.L., 1993. What is the relationship between birth defects and pregnancy bleeding? New perspectives provided by the NICHD workshop dealing with the association of chorionic villous sampling and the occurrence of limb reduction defects. Teratology 48, 93–95.

Brito, N.M.B., Simões, M.J., Gomes, P.O., Pessoa, A.F., Melo, M.C.F., 1999. Aspectos microscópicos da cicatrização de feridas cutâneas abertas tratadas com óleo de copaíba em ratos. Rev. Par. Med. 13, 12–17.

Cabral, D., 2008. Avaliação da atividade antimicrobiana *in vitro* do óleo-resina de copaíba (*Copaifera duckei* Dwyer). Dissertação de Mestrado, Universidade de Alfenas, Alfenas, Minas Gerais, Brazil, 122 pp.

Calderon, I.M.P., 1988. Modelo experimental em ratas para estudo do binômio diabete e gravidez [Dissertação]. Botucatu (SP): Universidade Estadual Paulista Júlio de Mesquita Filho.

Calixto, J.B., 2005. Twenty-five years of research on medicinal plants in Latin America. A personal view. J. Ethnopharmacol. 100, 131–134.

Calliari-Martin, R.T., Dieterich, S., Bortolini, C.E., Cunha, C.T., Lorenceti, G., 2002. Embriotoxicidade da Artemisia Vulgaris Linné em ratas. Rev. Med. HSVP 11, 12–17.

Calliari-Martin, R.T., 1998. Effects of prenatal exposure to restraint stress and monocrotophos on behavioral and physical development in the rat. Genet. Mol. Biol. 21, 171.

Carvalho, J.C.T., Cascon, V., Possebon, L.S., Morimoto, M.S.S., Cardoso, L.G.V., Kaplan, M.A.C., et al., 2005. Topical antiinflamatory and analgesic activities of *Copaifera duckei* Dwyer. Phytother. Res. 19, 946–950.

Carvalho, J.C.T., Cascon, V., 2003. Fitoterápicos: Nova Opção Terapêutica de Antiinflamatórios (Aspectos Químicos, Farmacológicos e Aplicações Terapêuticas). Tecmedd, São Paulo.

Cascon, V., Fernandez-Ferreira, E., Soares, R.O.A., Gibaldi, D., Gilbert, B., Santos, R.R., 1998. Avaliação da composição química e da atividade tripanosomicida in vitro de óleo-resinas de Copaifera spp. In: XV Simpósio de Plantas Medicinais do Brasil 1998. Resumos, Águas de Lindoia, SP, Brasil, p. 199.

Cascon, V., Gilbert, B., 2000. Characterization of the chemical composition of oleoresins of *Copaifera guianensis* Desf., *Copaifera duckei* Dwyer and *Copaifera multijuga* Hayne. Phytochemistry 55, 773–778.

Chang, C.V., Felício, A.C., Reis, J.E.P., Guerra, M.O., Peters, V.M., 2002. Fetal toxicity of *Solanum lycocarpum* (Solanaceae) in rats. J. Etnnopharmacol. 81, 265–269.

Fernandes, R.M., Pereira, N.A., Paulo, L.G., 1992. Anti-inflammatory activity of copaiba balsam (*Copaifera cearensis* Huber). Rev. Bras. Farmacogn. 73, 53–56.

Ghione, M., De Palo, G., 1996. Ecossistema microbiológico vaginal. In: De Palo, G. (Ed.), Colposcopia e Patologia do Trato Genital Inferior, Rio de Janeiro, Medsi, pp. 17–22.

Gilbert, B., Mors, W.B., Baker, P.M., Tomassini, T.C.B., Goulart, E.G., Holanda, J.C., et al., 1972. A atividade anti-helmíntica de óleos essenciais e de seus componentes químicos. Anais da Academia Brasileira de Ciências, 423–428.

Goodman, L.A., 1964. Simultaneous confidence intervals for contrasts among multinomial populations. Ann. Math. Statist. 35, 716–725.

Instituto, A.L., 2005. Métodos Físico-Quimicos para Analise de Alimentos. 4ªed.

International Union of Pure and Applied Chemistry – IUPAC, 1987. Standard Methods for Analysis of Oils, Fats and Derivatives. Method 2.301; Report of IUPAC Working Group WG 2/87. 7 ed. [S.L.]: Blackwell Scientific Publications.

Kazy, Z., Puhó, E., Czeizel, A.E., 2005. The possible association between the combination of vaginal metronidazole and miconazole treatment and polysyndactyly. Population-based case-control teratologic study. Reprod. Toxicol. 20, 89–94.

Kola, I., Folb, P., 1986. Chlorpromazine inhibits the mitotic index, cell number, and the formation of blastocyst, and delays implantation of CBA mouse embryos. J. Reprod. Fertil. 76, 527–536.

Lameira, O.A., Martins-da-Silva, R.V.C., Zoghbi, M.G.B., Oliveira, E.C.P., 2009. Seasonal Variation in the Volatiles of *Copaifera duckei* Dwyer Growing Wild in the State of Pará – Brazil. J. Essen. Oil Res. 21, 105–107.

Le Cointe, P., 1934. Árvores e Plantas Úteis: a Amazônia brasileira (III). Livraria Clássica, Belém, pp. 131–132.

Lemônica, L.P., Damasceno, D.C., Di-Stasi, I.C., 1996. Study the embryotoxic effects of an extract of Rosemary (*Rormarinus officinalis* L.). Braz. J. Med. Biol. Res. 29, 223–227.

Lima, S.R.M., Veiga-Jr, V.F., Christo, H.B., Pinto, A.C., Fernandes, P.D., 2003. *In vivo* and *in vitro* studies on the anticancer activity of *Copaifera multijuga* Hayne and its fractions. Phytother. Res. 17, 1048–1053.

Linhares, I.M., Bagnoli, V.R., Halbe, H.W., 1994. Vaginose bacteriana, candidose e tricomoníase. In: Halbe, H.W. (Ed.), Tratado de Ginecologia. , 2nd ed. Rocca, São Paulo, pp. 875–880.

Lourenço, A.C.S., Miguel, L.K., Faria, M.J.S.S., 2008a. Efeitos do óleo de copaíba na teratogenicidade causada pela exposição à ciclofosfamida em camundongos. I Congresso de Toxicologia Clínico-Laboratorial; 2008, Porto Alegre, Rio Grande do Sul, Brasil.

Lourenço, A.C.S., Miguel, L.K., Faria, M.J.S.S., 2008b. Toxicidade da ciclofosfamida no desenvolvimento intra-uterino de camundongos e efeito protetor do óleo de copaíba. I Congresso de Toxicologia Clínico-Laboratorial; 2008, Porto Alegre, Rio Grande do Sul, Brasil.

Lyra, M.M.A., Costa-Silva, J.H., Lima, C.R., Arruda, V.M., Araújo, A.V., Ribeiro e Ribeiro, A., et al., 2005. Estudo toxicológico reprodutivo da *Azadirachta indica* A JUSS. (Neem). Rev. Fitos 1, 53–57.

Marcondes, F.K., Bianchi, F.J., Tanno, A.P., 2008. Determination of the estrous cycle phases of rats: some helpful considerations. Braz. J. Biol. 62, 609–614.

Mcchesney, J.D., Venkataraman, S.K., Henri, J.T., 2007. Plant natural products: Back to the future or into extinction? Phytochemistry 68, 2015–2022.

Mendonça, D.E., Onofre, S.B., 2008. Atividade antimicrobiana do óleo-resina produzido pela copaiba – *Copaifera multijuga* Hayne (Leguminosae). Braz. J. Pharmacog. 19, 577–581.

Miranda, R.C.M., Wanderley, T.K.V., Moura, W., Araújo, J.M., 2000. Atividade antimicrobiana do óleo de copaíba (*Copaifera spp.*) de diferentes procedências. In *XVI Simpósio de Plantas Medicinais do Brasil*. Recife, Brasil 2000: Anais do 16 Simpósio de Plantas Medicinais do Brasil; Recife, 223.

Ohsaki, A., Yan, L.T., Shigeru, I., Edatsugi, H., Iwata, D., Komoda, Y., 1994. The isolation and in vivo potent antitumor activity of clerodane diterpenoid from the oleoresin of the Brazilian medicinal plant, *Copaifera langsdorfii* Desfon. Biol. Med. Chem. Lett. 4, 2889–2892.

Opdyke, D.L., 1976. Inhibition of sensitization reactions induced by certain aldehydes. Food Cosmet. Toxicol. 14, 197–198.

Paiva, L.A.F., Gurgel, L.A., Silva, R.M., Tome, A.R., Gramosa, N.V., Silveira, E.R., et al., 2003. Anti-inflammatory effect of kaurenoic acid, a diterpene from *Copaifera langsdorffii* on acetic acid-induced colitis in rats. Vasc. Pharmacol. 39, 303–307.

Paiva, L.A.F., Rao, V.S.N., Gramosa, N.V., Silveira, E.R., 1998. Gastroprotective effect of *Copaifera langsdorffii* oleoresin on experimental gastric ulcer models in rats. J. Ethnopharmacol. 62, 73–78.

Pellegrino, J., 1967. Protection against human *Schistosome cercariae*. J. Exp. Parasitol. 21, 12.

Rigamonte-Azevedo, O.C., Wadt, P.G.S., Wadt, P.H.O., Veiga Jr., V.F., Pinto, A.C., Regiani, A.M., 2004. Variabilidade química e física do óleo-resina de *copaifera* spp. no sudoeste da amazônia brasileira. Rev. Bras. Ol. Fibros. 8, 851–861.

Rogers, J.M., Kavlock, R.J., 2001. Developmental toxicology. In: Klaasen, C.D. (Ed.), Cassarett and Doulls's Toxicology: The Basic Science Of Poisons. , 6th ed. Mc Graw-Hill, New York, pp. 107–132.

Rosa, F., 1996. Azole fungicide pregnancy risks. In: Proceedings on the Ninth International Conference of The Organization of Teratology Information Services , pp. 2–4.

Santos, A.O., Ueda-Nakamura, T., Dias Filho, B.P., Veiga-Jr, V.F., Pinto, A.C., Nakamura, C.V., 2008. Antimicrobial activity of Brazilian copaiba oils obtained from different species of the *Copaifera* genus. Mem. Inst. Oswaldo Cruz 103, 277–281.

Santos, J.C., Leite, A.C.P., Wadt, L.H.O., Borges, K.H., Andrade, F.G., Menezes, R.S., et al., 2001. Demandas Tecnológicas para o Sistema Produtivo do Óleo de Copaíba (*Copaifera* spp) no Estado do Acre. EMBRAPA, Acre, Brasil (Documentos, 69).

Schwarez, R.L., Duverges, C.A., Diaz, A.G., Fescina, R.H., 1996. Relato Del crecimiento intrauterino. In: El Ateneo (Ed.), Obstetrícia. Buenos Aires, pp. 232–244.

Siegel, S., 1970. Estatística não paramétrica (para ciência do comportamento). Mac Graw-Hill, São Paulo, p. 350.

Simões, C.M., 2000. O. Farmacognosia: da planta ao medicamento, 2nd ed. Universidade/UFRGS/UFSC, Porto Alegre/Florianópolis.

Smithells, R.W., Newman, C.G.H., 1992. Recognition of thalidomide defects. J. Med. Genet. 29, 716–723.

Staples, R.E., Schnell, V.L., 1964. Refinements in rapid clearing technique in the KOH-alizarin red s method for fetal bone. Stain Technol. 39, 61–63.

Sullivan, C., Smith, L.G., 1993. Management of vulvovaginitis in pregnancy. Clin. Obstet. Gynecol. 36, 195–205.

Tappin, M.R.R., Pereira, J.F.G., Lima, L.A., Siani, A.C., Mazzei, J.L., Ramos, M.F.S., 2004. Análise química quantitativa para a padronização do óleo de copaíba por cromatografia em fase gasosa de alta resolução. Quim. Nova 27, 236–240.

Tiboni, G.M., Marotta, F., Castigliego, A.P., 2008. Teratogenic effects in mouse fetuses subjected to the concurrent in uterine exposure to miconazole and metronidazole. Reprod. Toxicol. 26, 254–261.

Tincusi, B.M., Jiménez, I.A., Bazzocchi, I.L., Moujir, L.M., Mamani, Z.A., Barroso, J.P., et al., 2002. Antimicrobial terpenoids from the oleoresin of the Peruvian medicinal plant *Copaifera paupera*. Plant Med. 68, 808–812.

Veiga Jr., V.F., Pinto, A.C., 2002. O gênero *Copaifera* L. Quím. Nova 25, 273–286.

Veiga Jr., V.F., Patitucci, M.L., Pinto, A.C., 1997. Controle de autenticidade de óleos de copaíba comerciais por cromatografia gasosa de alta resolução. Quim. Nova 20, 612–615.

Veiga Jr., V.F., Zunino, L., Calixto, J.B., Patituti, M.L., Pinto, A.C., 2001. Phytochemical and antioedematogenic studies of commercial copaiba oils available in Brazil. Phytother. Res. 15, 476–480.

Veiga Jr., V.F., Rosas, E.C., Carvalho, M.V., Henriques, M.G., Pinto, A.C., 2007. Chemical composition and anti-inflammatory activity of copaiba oils from *Copaifera cearensis*, *C. reticulata* and *C. multijuga* – a comparative study. J. Ethnopharmacol. 13, 248–254.

Wilson, J.C., 1965. Methods for administering agents and detecting malformations in experimental animal. In: Wilson, J.C., Warkany, J. (Eds.), Teratology: Principles and Techniques. Univ. of Chicago Press, Chicago, pp. 262–277.