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The safe use of *Doliocarpus dentatus* in the gestational period: Absence of changes in maternal reproductive performance, embryo-fetal development and DNA integrity

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ABSTRACT

Ethnopharmacological relevance: *Doliocarpus dentatus* (Dilleniaceae) is commonly used in Brazil for the treatment of inflammatory process pain and urinary retention. Previous studies of our group have demonstrated the anti-inflammatory and antimycobacterial action of the ethanolic extract of *Doliocarpus dentatus* (EEDd) as well as the safety of its use.

Aim of the study: we investigated the effects of EEDd on reproductive performance, fetal development and DNA integrity in pregnant female *Swiss* mice.

Material and methods: thirty female *Swiss* mice were divided into three experimental groups (n = 10): control group treated with 1% tween-80 and EEDd1 and EEDd2 groups treated with EEDd at doses of 100 and 1000 mg/kg, respectively. The treatment occurred by oral gavage throughout the gestational period. At the end of pregnancy, parameters related to reproductive performance, embryofoetal development and DNA integrity was evaluated.

Results: both doses of the extract tested did not alter the reproductive parameters, did not present significant differences in the embryofetal development when compared to the control group and also did not induce the formation of micronuclei.

Conclusion: the EEDd do not alter the reproductive parameters, embryofetal development and DNA integrity, ensuring its safe use during pregnancy.

1. Introduction

Doliocarpus dentatus, popularly known as “Cipó de fogo”, “Cipó Mata Sede” or “Cipó Vermelho” is a plant of the Dilleniaceae family with moderate growth, resistant to the drought and low temperatures. It is found in the tropical forests of Mexico, Peru and Bolivia and in Brazilian

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ecosystems such as the Atlantic Forest, the Amazon and the Cerrado (Aponte et al., 2008; Bianki Filho et al., 2015). Its aqueous sap is popularly used to relieve thirst, its barks are used in infusions to treat leishmaniasis, and its leaves and roots to treat cystitis, pain induced by inflammation and urinary retention (Rodrigues, 2007; Jagessar and Persid, 2014).

A previous research from our group determined the presence of phenols (204.04 mg/g), flavonoids (89.17 mg/g) and tannins (12.05 mg/g) as well as sitosterol-3-O-D-glucopyranoside, kaempferol 3-O-L-aminopyranoside, betulinic acid and betulin in the ethanolic extract of *D. dentatus* (EEDd) (Ishikawa et al., 2017). Another phytochemical study has also shown that this plant contains butyric acid, steroids, lactones, anthracensides, betulinic acid, tannins, flavones and trigonellin (Jagessar et al., 2013).

In vitro studies on the biologic activity of *D. dentatus* demonstrated anti-Leishmania action against amastigotes of *Leishmania (L.) amazonenses* (Sauvain et al., 1996), antimicrobial against strains of *Escherichia coli, Klebsiela pneumoniae* and *Staphylococcus aureus* (Jagessar and Persid, 2014; Ishikawa et al., 2017), anti-inflammatory (Ishikawa et al., 2017) and cytotoxic in leukemic cells of the K562 lineage (Aponte et al., 2008). In addition, our research group has recently demonstrated that EEDd does not cause genomic and chromosomal damage which suggests safety of use (Ishikawa et al., 2017). However, no additional data on the effects of EEDd in the gestational period were found in the literature. Hence, this study is a pioneer and aimed to evaluate the effects of EEDd on the reproductive performance, embryofoetal development and DNA integrity of pregnant female *Swiss* mice.

2. Materials and methods

2.1. Ethanolic extract of Doliocarpus dentatus

The EEDd employed in this research has previously been studied and described by Ishikawa et al. (2017). Briefly, the young and mature leaves of *Doliocarpus dentatus* were collected at the Universidade Federal de Mato Grosso do (UFMS), in the city of Campo Grande - MS. The botanical identification was performed by Professor Dr. Arnildo Pott and a sample was deposited in the UFMS herbarium under the number # 49860.

The collected leaves were dried at 40 °C, ground to powder and extracted with ethanol (95%) by the maceration for 7 consecutive days. The extracts were collected, dried in an evaporator and then lyophilized to obtain the crude dried extract. For administration to the animals the extract was diluted in 1% tween-80 and saline and the doses used were 100 and 1000 mg/kg body weight (b.w.) via oral gavage.

The dose of 100 mg/kg was chosen due its anti-inflammatory efficacy as proven by Ishikawa et al. (2017). It was also resoluted to test a 10× higher dose owing to the fact that according to the guidelines in the area of reproductive toxicology (OECD, 2010) and genetic toxicology (OECD, 2014; 2012), as well as the National Agency of Sanitary Surveillance (ANVISA, 2013), preclinical trials should be conducted with the doses intended for use in humans and another test with 10× larger dose. Only if the highest dose is free of side effects, the lowest dose may be considered safe.

2.2. Chemical composition

The extract was fractionated by liquid chromatography employing XAD-2 (Supelco, Bellefonte, PA, USA) resin column chromatography (30 cm × 3 cm). The extract (2.45 g) was eluted with 0.5 L of water, followed by elution with 0.5 L of methanol and with 0.4 L of ethyl acetate. An aliquot of 0.67 g of the ethyl acetate fraction was dissolved in 20 ml of methanol, fractionated by liquid chromatography on a Sephadex LH-20 column (80 × 2 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), and eluted with methanol at a rate of 0.3 ml 28 fractions were collected. The fractions were combined according to their chemical profile, as determined by thin layer chromatography. Samples were applied onto silica gel plates using ethyl acetate-methanol (75:25, v/v) as the eluent. Fraction 21–24 gave the isolated compound quercetin (2 mg) and fraction 26–27 Kampferol (1 mg), respectively. Chemical characterization was performed using nuclear magnetic resonance (Bruker DPX-300, 300 MHz for1H and 75 MHz for 13 C) and the chemical structures were confirmed by comparison with literature data (Agrawal, 1989; Harborne, 1996).

2.3. Experimental design

15 male and 30 female mice, *Mus musculus* (*Swiss*), with 35 g average weight, at reproductive age, were obtained from the State Agency of Animal and Plant Health Protection (IAGRO). The present study was approved by the Ethics Committee on the Use of Animals of the Universidade Federal de Mato Grosso do Sul under autorization # 776/2016.

The animals were kept in polypropylene boxes, covered by wood shaving at a controlled temperature of 22 ± 2 °C and relative humidity of (55 ± 10%) Water and food were provided *ad libitum*. The fed was with commercial ration (Nuvital®). The mice were mated overnight in the proportion of 1 male: 2 females. It was checked females for vaginal plugs to determine if pregnancy has occurred. If the plugged female is pregnant, the first day of gestation is considered to be the day after the plug is found (Oliveira et al., 2015). Females were divided into 3 experimental groups (n = 10): control group - animals received 0.1 ml/10 g b.w. of EEDd vehicle per oral gavage throughout gestation (1st to 18th). Gestational Group - animals received EEDd per oral gavage at doses of 100 mg/kg (EEDd1) and 1000 mg/kg (EEDd2) throughout gestation.

2.4. Reproductive performance and teratogenicity testing

On the eighth day of gestation, the mice were submitted to euthanasia, by cervical dislocation, followed by laparotomy, hysterectomy and omphalectomy. Organs, fetuses and placentas were collected and weighed. The fetuses underwent external evaluation to detect possible malformations and were subsequently sexed. The number of implantations, resorptions, live fetuses and dead fetuses were recorded and based on the data, it was obtained the: fetal viability (number of live fetuses × 100/number of implantations), post-implantation loss rate [number of implantations - (number of resorptions × 100/number of implantations)], placental index (placental weight/fetal weight) and sex ratio (number of male fetuses/number of female fetuses). After external analysis, the fetuses were randomly distributed into two subgroups. The first one was aimed at the visceral analysis and, therefore, were fixed in a solution of Bodian's and submitted to microdissection with strategic cuts (Barrow and Taylor, 1969; Wilson, 1965; Oliveira et al., 2009). The visceral changes were classified based on the studies of Taylor (1986), Manson and Kang (1994), Damasceno and Empinas (2008) Oliveira et al. (2009) and Oliveira et al. (2015). For skeletal analysis, the second group was subjected to fixation in acetone and subsequently to KOH and stained with Alizarin Red during the diaphanization process as proposed by Straples and Schenell (1964) and modifications by Oliveira et al. (2015).

2.5. Micronucleus assay in peripheral blood

For the micronucleus assay, 20 µl of peripheral blood was stained vitally by dropping blood on an Acridine Orange-coated slide (1.0 mg/ml) and covering the sample with a coverslip. The material remained in freezer (−20 °C) for a minimum of 7 days and 2.000 cells/animal were analyzed under epifluorescence microscope at 400× magnification with a 420–490 nm excitation and a 520 nm barrier filter, as proposed by Hayashi et al. (1990) and modified by Oliveira et al. (2009).