African Journal of Biotechnology Vol. 12(16), pp. 2105-2109, 17 April, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2798
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

Study of reproductive toxicity of *Combretum leprosum* Mart and Eicher in female Wistar rats

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Accepted 26 September, 2012

Most plants culturally used in Brazil for medicine do not have pre-clinical studies of reproductive toxicity, therefore risks of using such products on the reproductive system are unknown. The aim of the study was to evaluate possible reproductive toxicity of ethanolic extract of *Combretum leprosum* Mart and Eicher (EECL) in female Wistar rats. The animals, weighing between 180 to 250 g, were maintained in controlled environmental conditions of temperature, humidity, light/dark cycle of 12/12 h, water *ad libitum* and fed with commercial diet for rats. To verify the estrogenic activity of EECL, four groups of ovariectomized rats were used: saline + corn oil; saline + estradiol; EECL (500 mg/kg) + corn oil; EECL (500 mg/kg) + estradiol. To study the reproductive toxicity during the fecundation and implantation phases of the embryos and also during the organogenesis phase, two groups were used for each experiment, saline and EECL (500 mg/Kg). No estrogenic or anti-estrogenic activities were observed in the EECL. The ingestion of EECL did not cause modification in the number of implantation sites, which indicates a lack of toxicity during this phase. The EECL administered orally in the dose of 500 mg/kg did not produce adverse effects on the reproductive system of the female rats.

Key words: Estrogenic activity, organogenesis, teratogens.

INTRODUCTION

Many drugs or chemical products that can cause congenital malformations are called teratogens. Drugs vary considerably in their teratogenic capacity. Some may cause severe morphological alterations if administered during the organogenic period; others may produce mental and growth retardations as well as minor malformations when used in excess during development (Moore, 1990; Seip, 2008).

Toxic agents present in food and specific nutrient imbalance in the diet can alter the maternal hormonal concentration and affect the composition of the secretion from the oviduct and ovary, which results in a decrease of embryonary survival or, in less severe circumstances, alterations in development and growth (McEvoy et al., 2001). With the increasing use of phytotherapics, many studies have evaluated the potential reproductive toxicity of some plants used in popular medicine. Montanari and Bevilacqua (2002) observed estrogenic activity and loss of embryos during the pre-implantation period in female murine treated with an extract of *Maytenus ilicifolia* Mart. Fetal reabsorption and an anti-implantation activity without an estrogenic activity in rats treated with extract of *Coutarea hexandra* Schum (Rao et al., 1988; Almeida et al., 1990) have been observed. *Peumus boldus* and the isolated substance boldina caused significant number of reabsorptions and some malformations when administered to pregnant rats (Almeida et al., 2000). Phytoestrogens and estrogenic mycotoxins are widely present in nature. They

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may cause toxic effects on the reproductive system of mammals exposed to natural conditions and, recently, their effects as endocrine disruptors had been studied (Kelce and Gray, 1997).

The estrogenic and anti-estrogenic activity of the phyto-estrogens depends upon their concentration, the endogenous sexual steroids and the specific target organ. These variations in the effects can be explained by the existence of two types of estrogen receptors (ER): α and β (Harris et al., 2005). The α-receptors (ER-α) are the main receptors found in the breast and the uterus, and the β-receptors (ER-β) are predominant in the bones and in the cardiovascular system. The 17- β-estradiol has an affinity for both receptors, while the isoflavones are more selective for the ER-β. Among the isoflavones, the Genistein has, compared to estradiol (100%), 87% of affinity with the ER- β and 4% for the ER- α, while the Daidzein has 0.5% for ER- β, 0.1% for the ER-α (Kuiper et al., 1998). Therefore, the effect level of the isoflavones over a specific tissue depends on the type of receptor that is predominant.

In this context, there are still several species that requires more information concerning their effects on the reproductive system. Among these are the species of the genus *Combretum*, which contain several confirmed pharmacological properties such as: anti-inflammatory, antiulcerogenic and antifungal (Asuzu and Adimorah, 1998; Martini and Elloff, 1998; Baba-Mossa et al., 1999). Among the species that have already been isolated are the combrestatins with potential for cancer treatment (Tozer et al., 1995; Rodrigues, 2006).

The *Combretum leprosum* species that belongs to the combretaceae family, is popularly known as Cipoaba, Mofumbo or Mufumbo (Matos, 2003). It is amply distributed in the northern region of Brazil and is used commonly as a haemostatic, sedative and for healing wounds (Silveira, 2003). The chemical study of the species revealed the presence of triterpenes and flavonoids (Facundo et al., 1993). In pharmacological trials performed with the ethanolic extract of the trunk's bark, analgesic and anti-inflammatory activities were observed (Lira et al., 2002), and also a dose-related antinociception (Pietrovski, 2006). Since the *C. leprosum* presents therapeutic potentials and there is no data related to the reproductive toxicity of this plant in the literature, the goal of this research was to evaluate the effects of an ethanolic extract of *C. leprosum* on estrogenic activity and reproductive toxicity during the fecundation, implantation and organogenesis phases of the embryo.

MATERIALS AND METHODS

Preparation of the extract

Barks from the trunk of *C. leprosum* Mart and Eicher were collected in Teresina, state of Piaui, Brazil, during the month of September 2009. The plant was identified and and deposited in the form of excicata in the Graziela Barroso Herbarium, at the Center of Natural Sciences at the Federal University of Piaui, under number 10557.

The barks were dried in an evaporator at 40 ± 1°C and then grounded in an electrical mill. The material was submitted to a maceration process with ethanol at 70% during three successive extractions and then concentrated in a rotavap at 50°C, and later put in amber glass bottles and kept in the fridge.

Animals

In all of the protocols, immature female Wistar rats weighing 60 to 70 g or adults weighing between 180 to 250 g, were raised and kept at the experimental Vivarium at the Morphofisiological Veterinary Department –CCA, in a light/dark cycle of 12/12 h, maintained in controlled temperature conditions at 22 ± 2ºC. Standard pellet food (FRI-LAB Rat – Fri-Ribe, Ribeirão Preto, SP, Brazil) and drinking water were available *ad libitum.*

All animal studies were carried out in accordance with the requirements of the Committee on Ethics in Animal Experimentation as adopted by the Federal University of Piauí (Protocol number: 33/2009). The experimental protocols were elaborated and developed based on the principle of the three R's (refine, reduce and redesign). That is, the lowest number of animals possible was used in order to determine statistical differences; the protocols developed do not overlap regarding the objective of the study. Moreover, the animals were handled by trained researchers in our laboratory only when necessary. They were not exposed to any kind of pain or stress caused by noise, lack of food, water, or variation in temperature.

Experiment 1: Estrogenic activity assay

This protocol was based on Almeida et al. (1990). Immature 32 female Wistar rats were used, being eight animals per group. The animals were separated in groups that received the following treatments:

Group I: Saline 1 mL/100 g orally and corn oil (0.1 mL/100 g) intramuscular (im)
Group II: Saline 1 mL/100g orally and estradiol (1 μg/100 g) im
Group III: Ethanolic extract of *C. leprosum* (EECL) in the dose of 500 mg/kg orally and corn oil (0,1 mL/100 g) im.
Group IV: EECL (500 mg/kg) orally + estradiol (1 μg/100 g) im.

The dose of EECL was chosen based on preliminary studies performed by the authors, where the ethanolic extract of *C. leprosum* was toxic in high doses. The LD50 (the median lethal dose) value for p.o. administration was 4722 mg/kg (Lira et al., 2002). Thus the dose of 500 mg/kg is safe for use in this protocol.

Treatment was given during three days and on the fourth day, the rats were euthanized by excess inhalation of halothane. The removal, cleaning and weighing of the uterus were done. The weights found were converted to 100 g of body weight and then analyzed statistically.

Experiment 2: Evaluation of reproductive toxicity during the fecundation and implantation phases

The protocol was carried out using 16 female adult rats, divided in two groups of eight animals each, one being the control and the other treated with EECL. The protocol executed was according to Garg et al. (1970). The rats were examined daily to identify the phase of the estrous cycle, by fresh vaginal smear. Those detected to be in proestrus were mated with a fertile male and the presence of spermatozoids in the smear the following morning of the mating

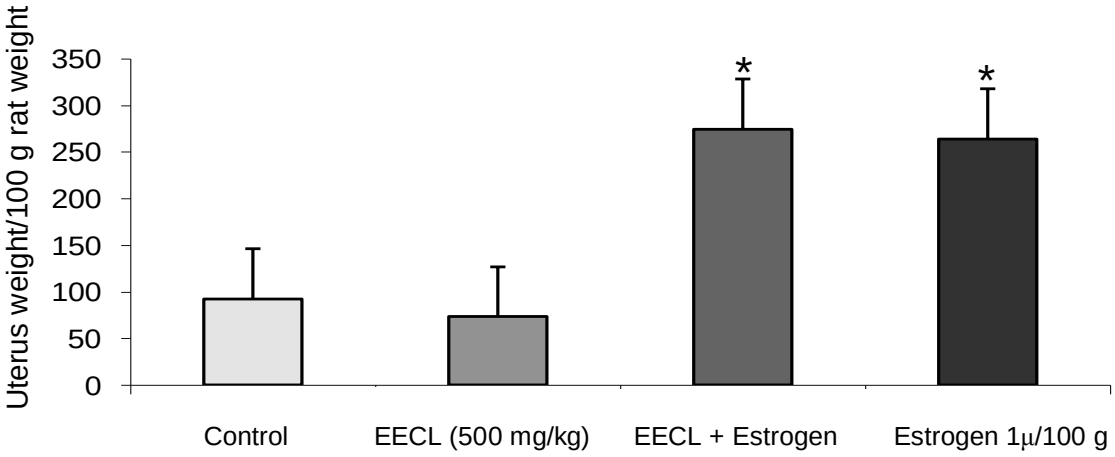

Figure 1. Effect of treatment with ethanolic extract of *C.* leprosum in the uterine weight of the prepubertal rats, with and without reposition of estradiol. The values are mean ± S.E.M; n = 8; *significant difference estatítica $p < 0.05$.

was indicative of pregnancy (day 1). The control group received 1 mL/100 g of body weight of saline orally, from the first to the seventh day of gestation. The test group received EECL 500 mg/mL, with a dose of 1 mL/100g of body weight orally, during the same period. During the eighth day, the animals were sacrificed by excessive inhalation of halothane and the uteri were removed and weighed. Then the number of corpus luteus and the sites of embryonary implantation in each uterus were verified.

Experiment 3: Evaluation of reproductive toxicity during organogenesis

The protocol was done according to Garg et al. (1970), using 16 female adult rats, divided into two groups of eight animals, one being the control group and the other the experimental group. The rats were examined and mated according to the previous protocol. They also received saline (1 mL/100 g) or EECL 500 mg/mL orally, from the sixth to the fifteenth day of gestation. After treatment, the animals were kept in observation until delivery. The number of offspring, the weight and the presence of congenital defects, macroscopically, were observed for each animal.

The parametric data was submitted to the analysis of variance (ANOVA test) and the means compared through the *Student-Newman-Keuls* test and the nonparametric data was evaluated by the Kruskal-Wallis test. Data were expressed as mean ± standard error for mean with $p<0.05$.

RESULTS AND DISCUSSION

EECL treated animals showed no change in uterine weight when compared to the saline group rats (Figure 1). This demonstrates an absence of estrogenic activity. Estrogenic activity of a specific substance is understood as the capacity it possesses in linking itself to estrogen receptors promoting effects similar to their own hormone. In recent years, the scientific community has turned its attention to a series of compounds known collectively as phytoestrogens. Such molecules have a structural similarity with the 17-β-estradiol and synthetic antiestrogens, such as tamoxifen and its mechanism of action are studied to identify estrogenic and antiestrogenic activities (Mathieson and Kitts,1980; Peterson and Barnes, 1996; Morito, 2001; Müller et al., 2009), aiming at possible therapeutic applications or toxic effects.

There was no statistically significant difference between the group treated with EECL plus estradiol and the group treated with saline plus estradiol during the fecundation and implantation phases (Figure 1). This indicates the lack of antiestrogenic activity in this plant, and this goes to show that it is safe for therapeutic use in women of reproductive age. Plants that exhibit antiestrogenic activity have the capacity of interrupting the gestation of rats and mice, by the inhibition of the estrogen necessary for implantation (Ghandi et al., 1991). In mice and humans, estrogen has an important role in implantation, as it participates in the estrogen/progesterone balance and in the receptivity of the uterus to the embryo (Ements, 1970).

In the experiment of reproductive toxicity, the EECL was administered from the first to the seventh day of gestation, a period that involves the stages before and after implantation. Implantation usually occurs between the fourth and fifth day of gestation in rodents and interference in this period can lead to embryonic losses (Beaudoin, 1980; Hodgen and Itskovit, 1988). The fundamental characteristic of this process is the synchronized development of the embryo to the blastocyst stage and the uterus differentiation to the receptive condition. Later interactions between the activated blastocysts and the uterine epithelium occur to begin implantation (Paria et al., 2000).

The ingestion of EECL did not cause modifications in the number of implantation sites (Figure 2). This indicates the lack of toxicity during this phase and also confirms the lack of estrogenic activity, as the presence of this activity in plant extracts can inhibit 100% of implantation in female rats, as was observed by Jagadish and Rana (2002) for *Calotropis procera*. The receptivity of the uterus

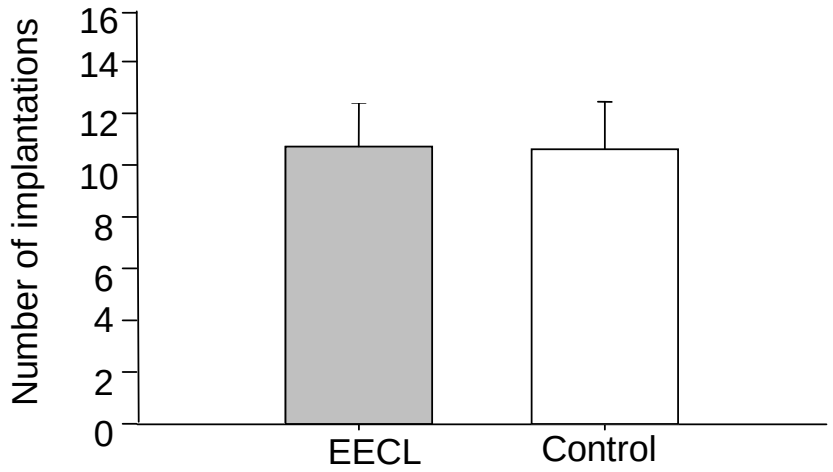

Figure 2. Number of embryonary implantation sites in rats treated with ethanolic extract of *Combretum* leprosum. The values are mean ± S.E.M; n = 8.

to the embryo depends, among other factors, on a high ratio of progesterone: estrogen (Goodman, 1994) and estrogen injections can prevent implantation (McDonald, 1989).

The number of newborn rats per litter was not altered by the treatment of the mothers with EECL (Figure 3). Since there was no fetal mortality after treatment with the extract, it can be suggested that a lack of toxicity for the normal fetus growth depends on a complex interaction between genetic, immunological, endocrinological, nutritional, vascular and environmental factors. Alterations in any of these factors can interrupt the normal growth and development of the embryo/fetus (Chahoud et al., 1999). There was also no alteration in the newborn's weight, which would indicate retardation in fetal growth (Frohberg, 1977). No congenital defects were observed, demonstrating a lack of teratogenic effects in the extract.

In summary, the data indicates that the bark extract from *C. leprosum* did not present neither estrogenic nor antiestrogenic activity. Also it did not present fetal mortality when administered during the gestational period.

ACKNOWLEDGMENTS

We are grateful to the Coordenacão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for their financial support.

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