



## Effects of *Maytenus ilicifolia* on reproduction and embryo-fetal development in Wistar rats

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**ABSTRACT.** *Maytenus ilicifolia* (Celastraceae), popularly known as espinheira-santa, is a native plant from the Atlantic forest and is commonly used in popular medicine to treat inflammation and as an abortifacient. To evaluate the effects of *M. ilicifolia* on pregnant rats during the organogenic period (T1) or throughout the gestational period (T2), an extract obtained using an acetone-water mixture at a 70:30 ratio was administered via gavage at a dose of 15.11 mg·kg<sup>-1</sup>·day<sup>-1</sup> over 2 treatment periods (T1 and T2). No clinical signs of maternal toxicity were observed. Term fetuses did not present malformations or anomalies as the number of implantations, reabsorptions, live, and dead fetuses were similar to the control group. In conclusion, *M. ilicifolia*

hydroacetic extract is non-toxic to pregnant rats and appears to not interfere with the progress of embryo-fetal development.

**Key words:** *Maytenus ilicifolia*; Toxicology; Reproduction; Embryo-fetal development

## INTRODUCTION

*Maytenus ilicifolia* Mart. (Celastraceae) is a plant native to the Atlantic forest (Jorge et al., 2004); its leaves are popularly known as espinheira-santa and are used to treat dyspepsia and gastric ulcers (Carlini, 1988; Leite et al., 2010). In southwestern Brazil, the roots of *M. ilicifolia*, known as cancorosa, are added to alcoholic drinks and terere, a common local beverage with a bitter taste (Nunes et al., 2003). The presence of phenolic metabolites, such as tannins, flavonoids, and triterpenes, has been observed in the leaves (Vilegas et al., 1995; Queiroga et al., 2000; Jorge et al., 2004; Radomski et al., 2004; Leite et al., 2010) and its gastroprotective effect has been associated with the presence of tri- and tetra-glycoside flavonoid derivatives (Leite et al., 2010). Previous studies performed by Vargas et al. (1991) and Horn and Vargas (2003) showed that the aqueous extract of *M. ilicifolia* did not have genotoxicity, but did have a significant antimutagenic effect in *Salmonella*/microsome assays. Moreover, *M. ilicifolia* is used as a contraceptive and abortifacient by women in Paraguay, Argentina, and Southern Brazil (Hnatyszyn et al., 1974; Arenas and Azorero, 1977). In addition, Montanari and Bevilacqua (2002) demonstrated that *M. ilicifolia* has estrogenic activity in pregnant mice and exhibited an uterotrophic effect, suggesting that the compound interferes with uterine receptivity to an embryo (Montanari and Bevilacqua, 2002). Despite its widespread popular usage, toxicological information for *M. ilicifolia* on reproductive performance remains limited. The possible toxic effects resulting from the interaction of *M. ilicifolia* extract with organs involved in cell proliferation as well as in embryonic tissues should be determined.

Medicinal plants play an important role in public health, particularly in developing countries, where it is thought that the utilization of plants with therapeutic action is safe (Mossi et al., 2009). However, it is well-established in the literature that several plant drugs commonly used as folk medicine have adverse effects on fertility in rats and show teratogenic effects. Thus, we investigated the effect of *M. ilicifolia* on reproduction and embryo-fetal development. Pregnant rats were exposed to 2 periods of treatment: during the organogenic period (from days 6-15 of pregnancy) and throughout the gestational period (from days 1-20 of pregnancy). The *M. ilicifolia* single dose used in this experiment was based on the average consumption in the population. An acetone-water ratio of 70:30 was used for extraction of the compounds present in the leaves. This extraction method showed a higher rate of yield (w/w) when compared to hydroethanolic extraction. Moreover, the thin layer chromatography profile showed higher enrichment of flavonoid derivatives (Wagner and Bladt, 2001).

## MATERIAL AND METHODS

### Extract preparation

The leaves of *M. ilicifolia* were collected in Dourados (MS, Brazil), and identified

by E. Jacomassi. A voucher specimen was deposited in the DDMS Herbarium (registration number 901). Dried leaves were percolated in 70:30 acetone: H<sub>2</sub>O (v/v), and the solvent was eliminated under reduced pressure. The resulting brown, amorphous residue, which was the crude extract, weighed 9.1 g (22.8%, w/w yield). This extract was stored in amber glass at room temperature in a desiccator. A similar extraction method was employed with a 70:30 ratio of ethanol: H<sub>2</sub>O and yielded a 7.6% w/w extract.

Both extracts were dissolved in methanol at a 1:1 ratio, and then 20- $\mu$ L samples were compared using thin layer chromatography covered with silica gel; the ratio of the glycoside flavonoids ethyl acetate: formic acid: acetic acid: H<sub>2</sub>O eluent was 100:11:11:26. A natural product/polyethylene glycol reagent was followed by ultraviolet measurement at 365 nm (Wagner and Bladt, 2001).

## Animals

Female rats were mated with male rats and gestational day 0 (GD0) was determined if spermatozoa were present in the vagina. Animals were housed in a standard animal facility at a controlled temperature (22°C) and photoperiod (12 h light, 12 h dark) with access to water and rodent food *ad libitum*. All procedures and protocols followed approved guidelines for the ethical treatment of animals according to the Ethics Committee in Animal Experimentation of the Universidade Federal de Mato Grosso do Sul (Protocol #115/2006).

## Experimental procedure

Mated females were randomly assigned to 2 experimental groups and exposed to *M. ilicifolia* in different windows of treatment time: during the organogenic period (from days 6-15 of pregnancy, T1, N = 10) or throughout the gestational period (from days 1-20 of pregnancy, T2, N = 10). The dose used in this experiment corresponded to that commonly used in folk medicine (Balbach, 1986). Females in the *M. ilicifolia*-treated group received 15.11 mg·kg<sup>-1</sup>·day<sup>-1</sup> of the extract suspended in 0.5 mL distilled water via gavage during treatment (T1 and T2). The control group (N = 10) received only 0.5 mL/kg vehicle.

Females were weighed on gestational days (GD) 1, 6, 15, and 20. To evaluate maternal toxicity, water and food intake were recorded during treatments. These values were calculated as the difference between the amount of food or water on the morning of one day and the amount of food or water remaining in the morning of the following day. On GD20, females were sacrificed by CO<sub>2</sub> inhalation. To examine the reproductive capacity of female rats, laparotomy was performed and uterine horns were removed. The number of implants, resorptions, and dead and live fetuses was recorded. The ovaries were also observed and the corpora lutea were counted. The rate of preimplantation loss was calculated as: number of corpora lutea - number of implantations x 100/number of corpora lutea. Postimplantation loss rate was calculated as: number of implantation - number of live fetuses x 100/number of implantations. The maternal kidney, spleen, and liver were also weighed.

The offspring group was randomly divided into 2 subgroups, each consisting of half of the litter. The first was fixed in Bodian's solution for visceral examination, which was performed using the incisions/microdissection method proposed by Barrow and Taylor (1969) for the thorax and abdomen and using the strategic incisions proposed by Wilson (1965) for the

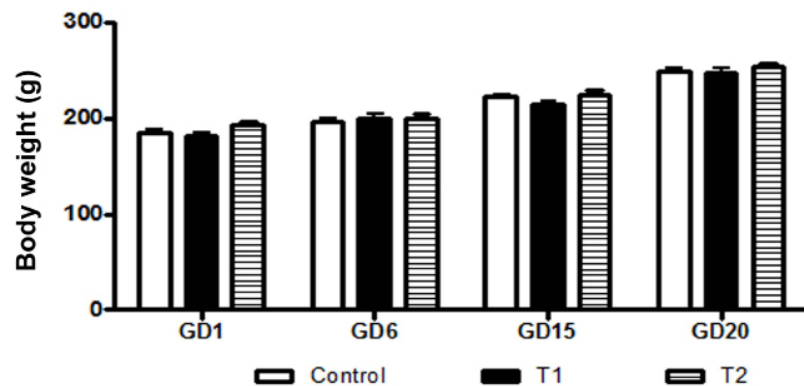
study of the head. Classification of visceral alterations was based primarily on the methods of Taylor (1986) and Manson and Kang (1994) and alterations proposed by Oliveira et al. (2009). The second subgroup was reserved for skeletal examination using the Alizarin Red technique described by Staples and Schnell (1964). The degree of ossification was evaluated using the parameters proposed by Aliverti et al. (1979). Examination of visceral and skeletal fetuses was performed using a dissecting stereomicroscope. For qualitative data and frequencies, the litter was considered the unit basis as recommended in the literature (Haseman and Hogan, 1975; Manson et al., 1982). However, to quantify visceral and skeletal malformations, the fetus was used as the basic unit according to Moreira et al. (2005).

### Statistical analysis

Values are reported as means  $\pm$  SE and data were analyzed using one-way analysis of variance followed by Tukey's *post-hoc* test using the GraphPad Prism (version 5; Graph-Pad Software Inc.; San Diego, CA, USA). The significance level was set at  $P < 0.05$ .

## RESULTS

No difference was observed between the body weights of control and treated animals for the 2 pregnancy periods during which the *M. ilicifolia* hydroacetic extract was administered (Figure 1). In addition, there was no significant weight gain or loss observed during the experiment (Table 1). Moreover, no maternal deaths, locomotor alterations, diarrhea, or piloerection, which are clinical signs of maternal toxicity, were observed in any of the groups investigated. However, other criteria indicative of maternal toxicity were evident with respect to changes in water and food consumption. Compared with controls, the T1 group showed significantly reduced ( $P < 0.05$ ) water consumption from GD8-14 (Figure 2A), which corresponds to the period of *M. ilicifolia* extract exposure. Food consumption was significantly reduced ( $P < 0.05$ ) at GD16 in the T1 group and at GD2, GD5, GD6, GD7, and GD11 in the T2 group compared with controls (Figure 2B).

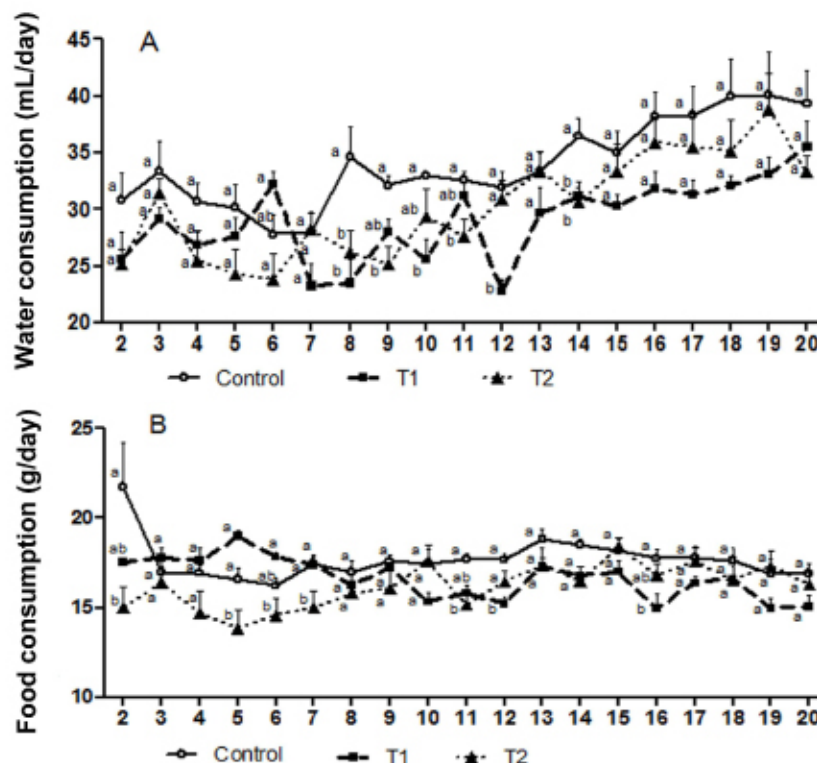


**Figure 1.** Maternal body weight of pregnant Wistar rats treated with extract of *Maytenus ilicifolia* from early gestation (GD1) through to late gestation (GD20) in the different treatment windows (T1: days 6-15 of pregnancy and T2: days 1-20 of pregnancy).  $P > 0.05$ .

**Table 1.** Maternal toxicity variables after treatment with extract of *Maytenus ilicifolia* in Wistar rats from 6-15 (T1) or 1-20 (T2) days of pregnancy.

Maternal variables	Experimental groups		
	Control (N = 10)	T1 (N = 10)	T2 (N = 10)
Body weight gain (g)	64 ± 4.3	65.6 ± 5.4	59.5 ± 3.7
Liver: absolute weight (g)	11.6 ± 0.3	10.4 ± 0.3	10.6 ± 0.3
Relative weight (g)	4.2 ± 0.001	4.2 ± 0.001	4.6 ± 0.001
Kidney*: absolute weight (g)	0.76 ± 0.03	0.8 ± 0.02	0.75 ± 0.02
Relative weight (g)	0.31 ± 0.0001	0.3 ± 0.0001	0.32 ± 0.0001
Spleen absolute weight (g)	0.52 ± 0.01	0.46 ± 0.02	0.5 ± 0.02
Relative weight (g)	0.2 ± 0.0001	0.2 ± 0.0001	0.2 ± 0.0001
Uterine weight (g)	3.9 ± 0.3	4.2 ± 0.2	3.9 ± 0.3
Ovary weight (mg)**	82.3 ± 0.003	83.4 ± 0.003	82.3 ± 0.005
Corpora lutea (N)	10.7 ± 0.7	11.8 ± 0.4	11.3 ± 0.4
Implantation sites	10.5 ± 0.7	11.8 ± 0.4	10.6 ± 0.09
Reabsorptions	2.3 ± 0.8	1.5 ± 0.2	1.8 ± 0.7
Implantation rate (%)	98.13 (105/107)	100 (118/118)	93.8 (106/113)
Preimplantation loss (%)	1.87	0	6.2
Postimplantation loss (%)	21.9 ± 8.4	13 ± 2	15.2 ± 6
Birth rate (%)	78 ± 8	87 ± 2	85 ± 2

Results are reported as means ± standard error. P > 0.05. \*Left and right kidney average weight. \*\*Left and right ovary average weight.



**Figure 2.** Water (A) and food (B) consumption by pregnant Wistar rats treated with extract of *Maytenus ilicifolia* (T1 = days 6-15 of pregnancy and T2 = days 1-20 of pregnancy). Different letters for the same day indicate significant differences (P < 0.05).

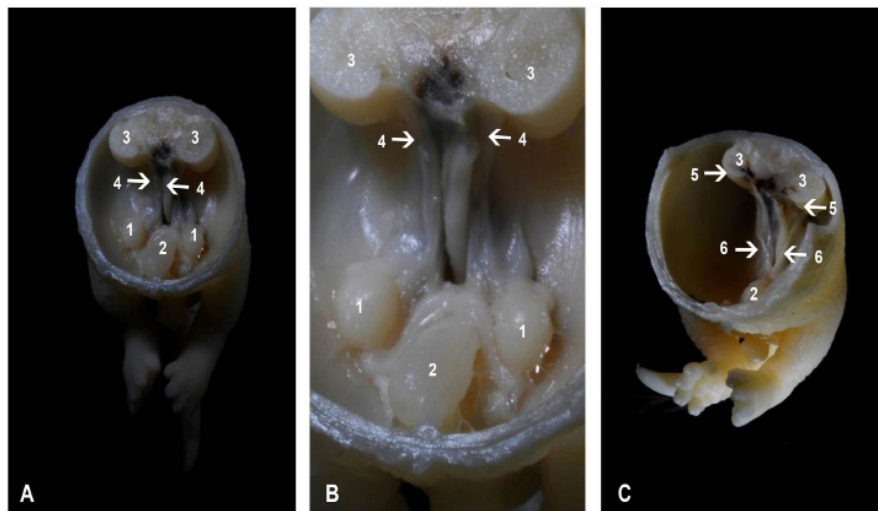
Table 1 shows the maternal toxicity variables of the control, T1, and T2 *M. ilicifolia*-exposed groups. The absolute and relative weights of the liver, kidneys, spleen, and ovary in both experimental groups investigated (T1 and T2) were similar ( $P > 0.05$ ). In addition, macroscopic analysis of these organs revealed no morphological changes, such as color, size, texture, or cysts. No significant alterations were observed in the number of corpora lutea, implantation sites, resorptions, pre- and post-implantation losses, and birth rate for all groups investigated. Thus, *M. ilicifolia* gestational exposure did not alter physiological and intrauterine conditions related to reproduction and fetal development.

Table 2 summarizes the fetal variables after gestational treatments with *M. ilicifolia* hydroacetic extract. The number of live and dead fetuses per group as well as average size and weight of the fetuses were similar ( $P > 0.05$ ) between control and *M. ilicifolia*-exposed animals. No significant visceral abnormalities were observed in the offspring of different experimental groups. Macroscopic analysis of the fetuses and the urogenital system was normal in all groups investigated (Figure 3). In all animals examined, there were no skeletal abnormalities in the fetuses exposed to *M. ilicifolia* (Figure 4).

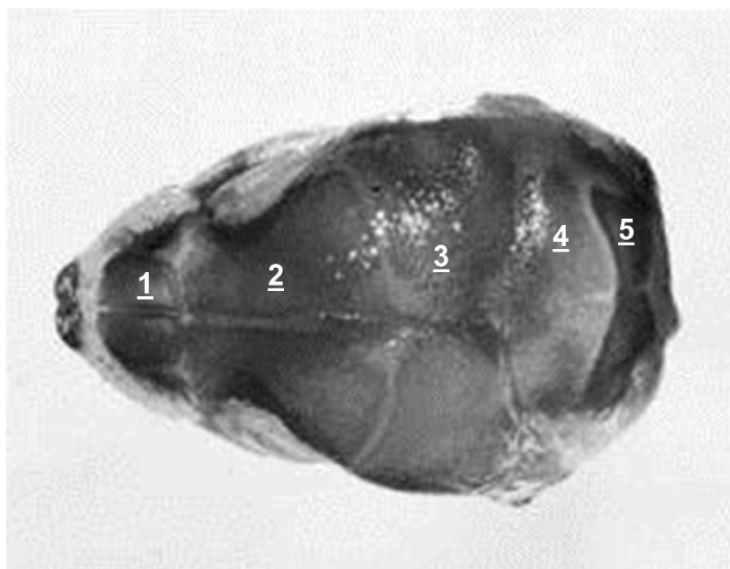
**Table 2.** Fetal variables after treatment with extract of *Maytenus ilicifolia* in Wistar rats from 6-15 (T1) or 1-20 (T2) days of pregnancy.

Fetal variables	Experimental groups		
	Control	T1	T2
Live fetuses (N)	8.2 ± 1	10.3 ± 0.4	8.8 ± 1
Dead fetuses (N)	2.5 ± 1	1.5 ± 0.2	2.5 ± 1
Fetuses size (cm)	3.5 ± 0.1	3.4 ± 0.1	3.6 ± 0.08
Fetuses weight (g)	2.7 ± 0.1	2.5 ± 0.02	2.7 ± 0.1

Results are reported as means ± standard error.  $P > 0.05$ .



**Figure 3.** Pelvic incisions of male (A and B) and female (C) fetuses exposed to *Maytenus ilicifolia* from 1-20 days of pregnancy (T2). Normal morphology of the urogenital system was observed: 1 = testes, 2 = bladder, 3 = kidney, 4 = ureter, 5 = ovary, 6 = uterine horn.



**Figure 4.** Skeletal analyses of the head of fetuses exposed to *Maytenus ilicifolia* from 1-20 days of pregnancy. Normal ossification process in the offspring was observed. Lane 1 = nasal; lane 2 = frontal; lane 3 = parietal; lane 4 = interparietal; lane 5 = supraoccipital.

## DISCUSSION

The leaves of *M. ilicifolia* are used in traditional medicine to treat dyspepsia and gastric ulcers; antiulcerogenic effects have also been reported. However, the mechanism of action remains unknown (Carlini, 1988; Leite et al., 2001, 2010; Baggio et al., 2012). The leaves of *M. ilicifolia* contain triterpenes (Itokawa et al., 1991), flavonoid glycosides and catechin derivatives (Leite et al., 2010). In addition, an *M. ilicifolia* lyophilized aqueous infusion was used for antiulcerogenic evaluation in several biological models. According to phytochemical analyses, flavonoids, glycosides, and catechins were the main compounds observed in the infusion (Leite et al., 2001, 2010).

During pregnancy, multiple agents can interfere with normal development of the embryo, leading to functional and/or morphological alterations. The intensity of the teratogenic effects depends on several factors, including the maternal-fetal genotype, stage of embryonic development, time of exposure to the substance (Spritzer et al., 2001), and mutagenic events such as chromosomal aberrations and non-disjunction (Oliveira et al., 2009). In this study, we evaluated the effects of *M. ilicifolia* hydroacetic extract on female reproductive performance and embryo-fetal development. Because an infusion prepared from *M. ilicifolia* leaves is popularly used by South American women to provoke abortion (Hnatyszyn et al., 1974; Arenas and Azorero, 1977), we investigated *M. ilicifolia* exposure in pregnant Wistar rats at a dose similar to that used by women.

In general, aqueous preparations such as tea are used in folk medicine; however, at pharmacies (galenic pharmacies), more appropriate formulations include hydroethanolic compounds such as tincture. The use of hydroethanolic tincture may not be the most appropriate

medicine. *Ginkgo biloba* is an example of this. Most clinical efficacy studies with *G. biloba* have been conducted using a standardized water acetone extract, while the alcoholic extract is not recommended for consumption because of the presence of undesirable compounds (Sticher, 1993). Mixtures of acetone and water have also been suggested as suitable solvents for extracting phenolic constituents.

This is the first report in which an extract of *M. ilicifolia* obtained from an aqueous acetone mixture has been used in a pregnancy model. This extract yielded more weight than hydroethanolic extract. In addition, the thin layer chromatography profile showed prominent spots with retentions (Rf) comparable to flavonoid glycosides (Wagner and Bladt, 2001). Because there were no maternal deaths, changes in locomotor activity, piloerection, diarrhea, or vaginal blood loss in any group, administration of *M. ilicifolia* extract to pregnant rats is concluded to be not toxic. Changes in water and food consumption are also clinical signs indicating drug toxicity (Manson and Kang, 1994). Although there was a reduction in water (during organogenesis) and food consumption in the T1 group and in food consumption on some days in the T2 group, these effects appeared to be transient since no differences were observed regarding body weight and weight gain in all groups investigated. Another indication of the lack of toxicity was the absence of alterations in organ weights in the treated groups (Damasceno et al., 2002). In addition, this study confirms earlier findings that *M. ilicifolia* does not produce toxic symptoms in mice at doses up to 1000 g/kg (Montanari and Bevilacqua, 2002).

Investigations performed in the present study provide important biometric data related to the ovary weights and the number of corpora lutea, which remained unchanged following *M. ilicifolia* treatment. Thus, pregnant rats may have showed similar hormone production, and thus, the maternal hormonal environment did not differ between treatments. Ovarian weight is largely dependent on the number and volume of corpora lutea because they are the largest structures in this organ (Waynforth, 1971). The corpora lutea are the main source of progesterone secretion (Kato et al., 1979), and an increase in volume during pregnancy and its growth is correlated with increased secretion of progesterone and 20-hydroxy-progesterone (Uchida et al., 1970), hormones that are essential for maintaining pregnancy. In rats, unlike in humans, the corpora lutea remain active throughout the gestational period (Keller, 2006).

Our study showed that in contrast to the hypothesis of Montanari and Bevilacqua (2002), *M. ilicifolia* does not interfere with uterine receptivity to the embryo. Differences in the experimental results may be explained by the different sensitivities between the species and methods of preparing the botanical extracts (Damasceno et al., 2002). Specifically, using *M. ilicifolia*, Radomski et al. (2004) found higher concentrations of phenolic compounds and density of *M. ilicifolia* leaves in plants cultivated with direct sun exposure compared to those grown under controlled light and environmental conditions. In addition, significant variation in tannin concentration observed in different *M. ilicifolia* populations suggests a correlation between environmental parameters and tannin concentration in this species (Mossi et al., 2009). However, our results confirm the findings of Oliveira et al. (1991) indicating that treatment with *M. ilicifolia* does not alter the fertility of female rats during pregnancy and that the potential teratogenic effects were not adverse.

The number of implantation sites did not differ between groups, indicating that *M. ilicifolia* exposure did not affect the implantation process. In this study, no alteration in embryo viability markers was observed, including implants ratio, pre- and post-implantation loss per group ratio, or birth rate. In addition, the dose tested in the present study showed no direct teratogenic effects since the live fetuses showed normal development (Cunha-Laura et al.,



2013) with no visceral or skeletal malformations. The size of the fetus, including body weight and placenta, were similar in all experimental groups, indicating that the treatments did not affect the availability of nutrients needed for maintaining maternal metabolism and fetal development. Our results reveal that the maternal reproductive capacity was not affected by *M. ilicifolia* exposure.

In conclusion, this study provides evidence that *M. ilicifolia* exposure during the organogenic period or throughout gestation is nontoxic and does not interfere with embryo-fetal development or with maternal reproductive parameters.

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