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Maternal exposure to aqueous extract of *Mentha pulegium* L. inducing toxicity to embryo development in rats

Marli Gerenutti*, Lívia Modesto, Vanessa Alessandra Carrara, Stefani Alves Magalhães, Nobel Penteado de Freitas and Magali Glauzer Silva

Laboratory for the Development and Evaluation of Bioactive Substances, Laboratory for the Toxicological Research (Lapetox), University of Sorocaba (UNISO) , Cidade Universitária, Rod. Raposo Tavares km 91, CEP 18023-000 Sorocaba, São Paulo, Brasil.

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The aim of this study was to observe the effect of orally administrated aqueous extract of *Mentha pulegium* L. on female rats pregnancy and on the physical development of fetuses. It was administered daily doses of 1.0, 2.5 and 5.0 g per kg of body weight or alternates doses of 2.0 and 4.0 g/kg of the *M. pulegium* L. aqueous extract, to sixty pregnant rats from the 4th day to the 20th day of pregnancy. On the 21th day of pregnancy, the intact rat fetuses were isolated. The aqueous extract of *M. pulegium* L. induced several changes in the reproductive performance of female rats and significant alterations in the skeletal development of fetuses.

Key words: *Mentha pulegium*, medicinal plants, abortifacient agents, reproduction, teratology.

INTRODUCTION

In Brazil, the National Program of Medicinal Plants and Phytoterapics (PNPMF) aims to ensure the population safe access and rational use of medicinal plants and phytoterapics and promote the sustainable biodiversity use, the productive chain and the national industry development. Some of the PNPMF guiding principles are the sustainable use of Brazilian biodiversity; the valuation and the preservation of the knowledge of the traditional and indigenous communities and the strengthening family agriculture (Brazil, 2007).

The Secretariat of Science, Technology and Strategic Inputs, through the Department of Pharmaceutical Care and Strategic Inputs (DAF/SCTIE/MS), in 2005, drew up a list of plant species that supported in 2008, the definition of RENISUS (National List of Medicinal Plants of Interest to Brazilian Health System). *Mentha pulegium* L. (Lamiaceae) commonly known as “pennyroyal” or “poejo” is a plant native to Europe and Western Asia that was one of the selected to compose the RENISUS.

The *M. pulegium* L. leaf infusion is commonly used as aromatic stimulant, expectorant, antitussive, carminative, emmenagogue and for treating sinusitis and bronchitis (Mahboubi and Haghi, 2008). The flowering aerial parts of *M. pulegium* L. has been traditionally used for its antiseptic properties for treatment of infectious diseases (Mahboubi and Haghi, 2008). The effects of the essential

*Corresponding author. E-mail: marli.gerenutti@prof.uniso.br. Tel: + 55 (15) 2101-7197. Fax: + 55 (15) 2101 7000.

Figure 1. *Mentha pulegium* L., the medicinal plants flower bed of the University of Sorocaba (Sao Paulo, Brazil).

oil of *M. pulegium* L. are well known in folk medicine as an abortifacient, due to its characteristic as an uterine musculature stimulant (Chitturi and Farrel, 2000; Conover, 2003). The monoterpene pulegone is the major component of the *M. pulegium* L. essential oil (Aghel et al., 2004; Petrakis et al., 2009; Koutroumanidou et al., 2013).

Despite the fact of *M. pulegium* L widely used in folk medicine, there are no records of embryo/fetotoxicity studies with its extract. This study seeks to correlate through biological assay *in vivo*, pharmacological and toxicological data of the extract of *M. pulegium* L. (Mp), taking into account its medicinal potential, focused in the safety of its aqueous extract on the reproductive performance of female rats and over some morphological parameters of fetuses.

MATERIALS AND METHODS

The plant samples

M. pulegium L. (Figure 1) was grown in the medicinal plants flower bed of University of Sorocaba – UNISO (São Paulo, Brasil). A voucher specimen has been deposited on the herbarium at University of Sorocaba and subsequent identification was carried out by the Sao Paulo Botanical Institute (IBt), authenticated by Dr. Sérgio Romaniuc Neto (PqC VI).

Preparation of the extract and pharmacognosy assays

Whole aerial flowering parts of the plant (3.60 kg) were dried for 48 h in a forced air incubator at 40°C. The material was then ground in a mill (MA 340®, Marconi, Brazil), macerated for three days (1120.00 g) in 70% ethanol (11.2 L). The suspension, protected from light, was percolated at 20 drops/min resulting in a 38% (w/v) hydro alcoholic extract as described by Gerenutti et al. (2008). The obtained extract was concentrated in a rotary evaporator (TE-210®, Tecnal, Brazil) and lyophilized (Multi-Tasking Freeze Drying S, SNL216V-115, Thermo Fisher Scientific, USA), resulting in 352 g. Dry extract polyphenols (Y = 0.1318, X + 0.0473, r = 0.9984, 720 nm, calculated as pyrogallol) and total flavonoids (Y = 0.0279, X + 0.0104, r = 0.99979, 420 nm, calculated as rutin) were measured (Woisky and Salatino, 1998). The volatile oily fraction of the lyophilized extract was reassembled with 5% v/w oil obtained by hydro distillation (Wasicky 1989) for each dose of lyophilized extract employed in biological assays. The oil was characterized by thin layer chromatography (TLC) (stationary phase: silica gel - mobile phase: mixed solvent of toluene with ethyl acetate - 93:7 - visualization reagent: p-anisaldehyde–sulfuric acid).

Animals

This study was approved by the UNISO Institutional Committee for Ethics in Research and the experiments were carried out according to the guidelines of Brazilian College for Animal Experimentation – COBEA, “The Guide for the Care and Use of Laboratory Animal” (National Research Council 1996) and “European Community guidelines” (EEC Directive of 1986; 86/609/EEC). All animals, supplied by UNICAMP's vivarium and kept in UNISO’s vivarium, were maintained in groups of 5 rats per cage, housed in the laboratory conditions a week before the experiments starts, at 25 ± 3°C on a 12 h light/dark cycle with access to food and water *ad libitum* during all the period.

Acute toxicity assay

Fifty Swiss mice (50% of each gender) were distributed into 5 groups (1 control and 4 experimental) composed of five animals of each gender. Experimental groups received 0.5, 1.0, 2.5 and 5.0 g/kg of body weight of Mp aqueous extract (w/w). Control group received the vehicle (deionized water). After dosing, each group was observed for 30, 60, 120, 240 and 360 min every day for fourteen days. The parameters used for acute toxicity assay studies were: general activity, contortion, tremors, convulsions, straub tail, hypnosis, ptosis, urination, defecation, piloerection and hypothermia

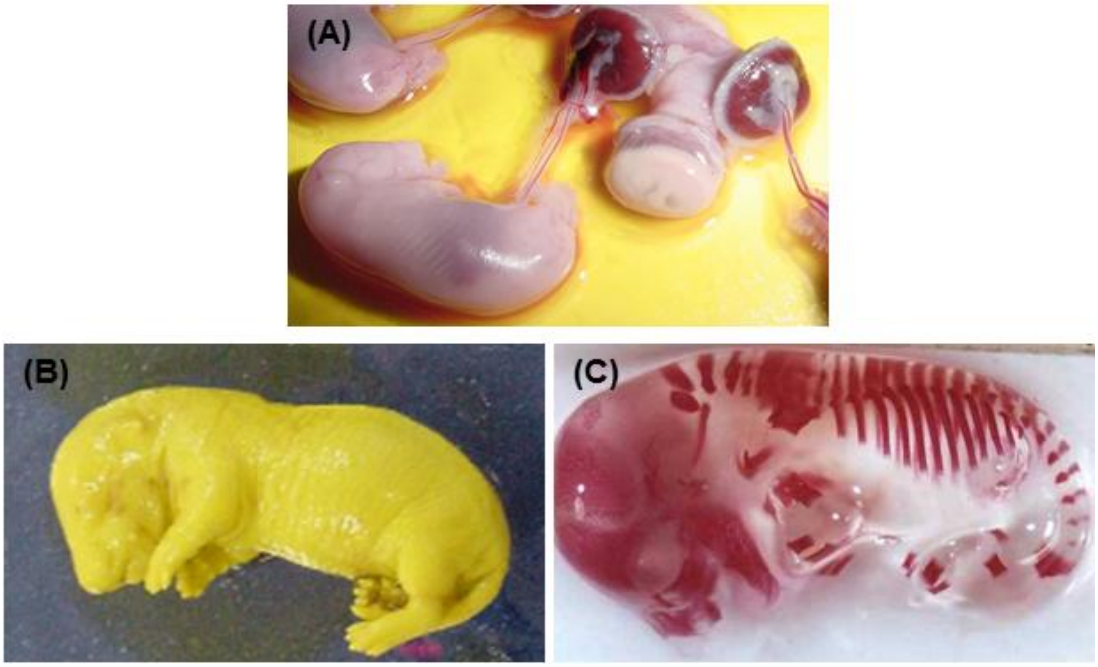

Figure 2. Embryo-lethal lesions resulting in resorption (A), fetus fixaded in Bouin's solution (B) and diaphanizated fetus for skeletal analysis (C).

(Amos et al., 2001).

Embryo- and feto-toxicity assay

The method of reproductive evaluation previously applied elsewhere (Esteves-Pedro et al., 2012), consists in 60 sexually naive female rats mated with males (Rattus norvegicus albinus, Wistar), 5 females per cage with 2 males, weighing between 160 and 200 g. Pregnancy was confirmed by the presence of sperm in vaginal-washing rubbing observed in microscopic analysis. Presence of spermatozoids was considered the first day of pregnancy (Vickery and Bennett, 1970). Pregnant females were kept in separate cages. Water and food were supplied *ad libitum* during the experiment and the consumption was daily monitored. For reproductive evaluation, each group of 10 females received from the 4th day till the 20th day of pregnancy: 1.0 g/kg/day; 2.5 g/kg/day and 5.0 g/kg/day of *Mentha pulegium* L. aqueous extract or deionized water (Control A), by gavage. After the results analysis, we started a second test with doses of 2.0 and 4.0 g/kg aqueous extract of *M. pulegium* or deionized water (Control B) on alternate days, from the 4th day till the 20th day of pregnancy. In both studies, to evaluate teratogenicity, mothers were anesthetized with halothane (Halotano®, Cristalia, Brazil), killed and submitted to a rapid excision of their uterus. The following macroscopic parameters were evaluated in order to observe the rats' reproductive performance (Randazzo-Moura et al., 2011): (1) mothers' weight gain (g); (2) post-implantation loss (%) = implantation no. - alive fetus no. / implantation no. × 100; (3) offspring vitality (%) (Figure 2A). To study the fetuses development they and the placentas were weighed (g); after that, the offspring was anesthetized, killed and fixed in Bouin's solution (Figure 2B) for 24 to 48 h, replacing it by a 70% hydro alcoholic solution to measure the following parameters (cm): anteroposterior and lateral-lateral of the skull; anteroposterior and lateral-lateral of the thorax; cranio-caudal and tail. Other offspring group was anesthetized, killed, eviscerated and diaphanized for posterior skeletal examination (Figure 2C). Fetuses selected were fixed in ethanol, then "cleared" and stained by koh-alizarin red method (Damasceno and Kempinas, 2008). The examination included: enumeration of the vertebra, ribs and other bone structures; degree of ossification and any fusions or abnormalities in bone's shape or position (Keller, 2001).

Statistical analysis

The results were submitted for statistical analyses, considering a significance level of 5%. Tukey-Kramer test was used to compare experimental and control groups, considering body-weight gain, placenta's weight, fetuses' weight and offspring's morphological parameters. Chi-square test was used to evaluate changes (%) in osseous development parameters, post-implantation losses and fetuses' vitality.

RESULTS AND DISCUSSION

Qualitative phytochemical constituents of the hydro alcoholic extract of the *Mentha pulegium* L.

Briefly, the obtained lyophilized extract containing: 13.04% of polyphenols, 5.22% of flavonoids totals, 10.85% ± 0.0064 of total ashes, 0.96% ± 0.0081 of insoluble ashes, 0.4% by hydrodestilation of volatile oil content. The thin layer chromatography (TLC) indicated 4 spots: Rf 0.98 (brown-orange); Rf 0.72 (orange); Rf 0.49 (nut-brown, majority, probably pulegone) and Rf 0.28

Table 1. Effects of *Mentha pulegium* L. aqueous extracton the reproductive performance of female rats.

Experimental groups	Weigt gain of pregnant rats (g)	Offspring vitality (%)	Weight of placenta (g)	Weight of fetus (g)	Fetus/pregnancy rats	Implantationlosses Post (%)
I	115.00 ± 2.17	100	0.51 ± 0.04	2.91 ± 0.27	10.0 ± 1.41	0.99
II	102.1 ± 2.14*	91.81*	0.53 ± 0.09	2.44 ± 0.22	10.1 ± 1.10	7.27*
III	97.1 ± 1.69*	87.25*	0.52 ± 0.08	2.53 ± 0.19	9.90 ± 1.4	11.76*
IV	85.4 ± 3.46*	100	0.46 ± 0.05	1.58 ± 0.29	9.0 ± 0.31	5.26*
V	74.2 ± 4.78*	90.14*	0.46 ± 0.06	1.56 ± 0.20	10.67 ± 2,06	10.29*
VI	47.9 ± 3.87*	73.13*	0.41 ± 0.06	1.47 ± 0,29	9.8 ± 2.58	26.86*

Groups: I:Control; II: *Mp* 2.0 g/kg/alternate day;; III: *Mp* 4.0 g/kg/alternate day;; IV: *Mp* L 1.0 g/kg/day; V: *Mp* L 2.5 g/kg/day; VI: *Mp* 5.0 g/kg/day.N: 10 animals per group, * p<0.05 (Chi-square test or Tukey-Kramer test).

(pink).

The acute toxicity of hydro alcoholic extract of the *Mentha pulegium* L.

Sztajnkrycer et al. (2003) suggested that pullegone intake is associated with severe hepatotoxicity and death. However our results of the acute toxicity assay showed that, despite the fact that with the Mp 5.0 g/kg dose the general activity of rats was slightly reduced but no death observed with any of the tested doses.

Effects of varying doses of hydro alcoholic extract of the *Mentha pulegium* L. in the embryo development in rats

Toxicology related to reproduction and development is an area that has achieved great scientific advances in recent decades. Studying the actions of toxic agents on the different stages of the reproductive process and development allows us to evaluate its effects on: fertility, transport and implantation of the egg, embryogenesis and fetal stage, birth, the newborn, lactation, weaning and care for the brood, delayed postnatal development, sexual behavior, estrus cycles or rhythms of design and placental and uterine functions (Oliveira et al., 2010; Tanaka et al., 2012; Behl et al., 2013; Repo et al., 2014).

Ensuring security in using bioactive compounds sometimes becomes more necessary than the pharmacological use itself, mainly considering the cultural application of certain compounds. In this sense, study models that ensure security in using vegetal drugs during pregnancy are fundamental in developing new drugs. Reproductive toxicity studies reveal possible effects of one or more active substances on reproduction of mammals and therefore investigations and interpretations of the results should always be related to other pharmacological and toxicological data available. Many of the changes observed during birth, growth and development are especially due to the mother´s exposure to chemical agents. Table 1 shows that, despite the statistically significant differences between the groups in some periods, the Mp extract did affect the reproductive capacity of female rats, reducing the weight gain when exposed to daily doses (F: 669 583, $p < 0.0001$) and to alternate doses (F: 404.96, $p < 0.0001$); increased post-implantation loss for all daily doses (Chi-square df: 40.94,3, $p < 0.0001$) and for the alternate dose of 4 g/kg (Chi-square df: 12.25,2, p: 0.0002); beyond reducing offspring's vigor (df Chi-square: 54.55,3, $p < 0.0001$, and df Chi-square: p 10.55,2; 0.0051). There are no statistically significant differences between the number of liveborn pups in the groups (F: 0.058, p: 0.9433).

Developmental abnormalities can be assessed as: embryo-lethal lesions that may culminate in resorptions, miscarriages or stillbirths; teratogenic lesions that can result to structural or functional anomalies; and embryo- and fetotoxic lesions that cause growth retardation or alterations in physiology (Schwarz et al., 2007). Table 2 shows significant changes in external measurements of morphological parameters in fetuses that all Mp doses caused, in addition to promoting a delay period of sternum's ossification, and skull flattening. Pigmentation in liver and kidney was also observed. Nevertheless, other common alterations such as syndactyly, cleft palate and abnormal eyes/ears implantation have not been noted.

Table 2. Offspring external and internal morphological parameters whose mothers were exposed to *Mentha pulegium* L. aqueous extract.

Fetus external morphological parameters (cm)	Group					
	I	II	III	IV	V	VI
Skull antero-posterior	1.42 ± 0.03	1.35 ±0.03*	1.36 ± 0.02*	1.37 ± 0.04*	1.35 ± 0.02*	1.38 ± 0.03*
Skull latero-lateral	1.02 ± 0.04	0.84 ± 0.04*	0.89 ± 0.06*	0.86 ± 0.04*	0.85 ± 0.05*	0.91 ± 0.04*
Thorax antero-posterior	1.09 ± 0.03	1.09 ± 0.07	1.03 ± 0.02*	0.85 ± 0.05*	0.91 ± 0.03*	1.02 ± 0.02*
Thorax latero-lateral	1.12 ± 0.01	0.93 ± 0.08*	1.01 ± 0.08*	0.90 ± 0.07*	0.94 ± 0.10*	1.01 ± 0.09*
Cranio-caudal	4.22 ± 0.03	4.26 ± 0.04	4.09 ± 0.07*	4.23 ± 0.01	4.24 ± 0. 01	4.12 ± 0.01*
Tail	1.08 ± 0.02	0.96 ± 0.03*	1.17 ± 0.03*	0.95 ± 0.02*	0.96 ± 0.01*	1.19 ± 0.01*
Fetus osseous structure and soft tissue (%)	**Group**					
	I	**II**	**III**	**IV**	**V**	**VI**
Renal pigmentation	0	32.43*	37.50*	42.30*	53.33*	31.57*
Liver pigmentation	2.27	35.13*	53.12*	23.07*	26.00*	100*
Flattening (skul soft/bones)	0	0	46 *	0	0	73*
Parietal ossification	100	8.10	100	0	76.66	100
Sternumossification	100	94.59	50*	100	100	2*

Groups: I:Control; II: *Mp* 2.0 g/kg/alternate day; III: *Mp* 4.0 g/kg/alternate day; IV: *Mp* L 1.0 g/kg/day; V: *Mp* L 2.5 g/kg/day; VI: *Mp* 5.0 g/kg/day.N: 60 animals per group, * p<0.05 (Chi-square test or Tukey-Kramer test).

Conclusion

Overall, the results indicate that the oral administration of *M. pulegium* L. aqueous extract in daily doses of 1.0, 2.5 and 5.0 g per kg of body weight and alternates doses of 2.0 and 4.0 g/kg promotes changes in reproductive performance of female rats and induces fetotoxicity, proving to be unsafe.

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