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Effect of essential oil from *Citrus aurantium* in maternal reproductive outcome and fetal anomaly frequency in rats

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ABSTRACT

Citrus aurantium L., commonly known as bitter orange, is widely used in folk medicine, but there is little data in the literature about the effects on pregnancy. The aim of the present study was to evaluate the influence of essential oil obtained from fruits of *Citrus aurantium* on the maternal reproductive outcome and fetal anomaly incidence in rats. Pregnant Wistar rats were randomized into four groups (n minimum = 12 animals/group): G1 = control, G2 to G4 = treated with essential oil from *C. aurantium* at dose 125, 250 and 500 mg/kg, respectively. Rats were orally treated, by gavage, with plant essential oil or vehicle during pre-implantation and organogenic period (gestational day 0-14). On gestational day 20 the rats were anaesthetized and the gravid uterus was weighed with its contents and the fetuses were analyzed. Results showed that the treated group with 500 mg/kg presented decreased placental weights and placental index, although the treatment with bitter orange essential oil did not show any alteration in maternal reproductive performance, toxicological effect, changes in ossification sites, and malformation index. In conclusion, the treatment of *Citrus aurantium* essential oil was not teratogenic and did not alter the maternal reproductive outcome.

Key words: *Citrus aurantium*, essential oil, limonene, malformation, pregnancy, reproductive outcome.

INTRODUCTION

A large number of plants have been used in folk medicine for centuries. It is common to assume that medicinal plants of traditional use have already been tested and ratified by its prolonged use in the human species. Consequently, they are considered effective medicines and it is presumed that they present no side effects and do not need evaluation. However, medicinal plants do not differ from any another synthetic chemical substance and its use

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must be based on several experimental evidences, confirming that the possible injury cause by its usage is supplanted by the benefits (Lapa et al. 2004).

Fruits of *Citrus aurantium* L. (Rutaceae family), commonly known as bitter orange, sour orange or Seville orange, are used as food, but this popular plant is more commonly used as a medicinal or dietary supplement (Fugh-Berman and Myers 2004). Similar to ephedra, it contains alkaloids that are adrenergic agonists and is often incorporated into supplements designed to aid in weight loss (Haaz et al. 2006). It was confirmed that its essential oil has an antibiotic effect against *Helicobacter pylori* (Bergonzelli et al. 2003). Novel triterpenoid from *C. aurantium* L. also possesses chemopreventive properties against human colon cancer cells (Gougeon et al. 2005). Given that limonene and essential oil of *C. aurantium* are excellent flavoring agents and also present gastroprotective actions, they are being considered promising for the development of a new drug for the prevention of gastric damage (Moraes et al. 2009). However, safety information is extremely limited, and because *Citrus aurantium* contains the sympathomimetic drug m-synephrine (phenylephrine), consumption of the herb may lead to increased blood pressure and risk of adverse cardiovascular events (Bent et al. 2004, Hansen et al. 2011). Hansen et al. (2012) verified that synephrine, a component in the bitter orange extract, did not produce developmental toxicity in rats when administered in lower dose. Arbo et al. (2009) indicated a low subchronic toxicity and possible alteration in the oxidative metabolism of the *C. aurantium* and synephrine in mice.

A great number of experimental studies had indicated that the prenatal administration of plants can result in structural and functional suppression of the growth, fetal anomaly and death (Lemonica and Alvarenga 1994, Damasceno and Lemonica 1999, Damasceno et al. 2002a, Mello et al. 2005, Dallaqua et al. 2013). However, several plants had already been studied during pregnancy, and showed no deleterious effects (Monteiro et al. 2001, Rudge et al. 2007, Volpato et al. 2008, Volpato et al. 2011).

In spite of its popular use, there is little data in the literature about the effects of *Citrus aurantium* on pregnancy. Therefore, the objective of the current study was to evaluate the effect of essential oil from *C. aurantium* on the maternal reproductive outcome and fetal anomaly incidence in rats.

MATERIALS AND METHODS

Preparation of Essential Oil

A plant sample of the specie *Citrus aurantium* was collected and the exsiccates deposited in the Herbarium "Irina D. Gemtchujnicov" - BOTU, Department of Botany at UNESP – Univ Estadual Paulista (BOTU 23123). Fruit peels were cut manually into small fragments (approximately 2 mm×10 mm) for better vapor penetration to yield, and in turn to better access to essential oil, which was extracted by hydro distillation in a Moritz apparatus, using approximately 200 g of peels, for 75 min. Extractions were performed in triplicate (Rozza et al. 2011).

Experimental Animals

Female and male Wistar rats (210-230 g) were obtained from the UNESP breeding center, and were maintained under standard laboratory conditions (22 ± 3 °C, humidity 50±10%,12-h light/dark cycle), with pelleted food (Purina rat chow, Purina®, São Paulo, SP, Brazil) and tap water *ad libitum*. The animals were cared for in accordance with the Principles of the Guide for Care and Use of Experimental Animals. The local Committee of Ethics in Animal Experimentation approved all experimental procedures of this study (49/08-CEEA).

Pregnancy Diagnostics and Experimental Groups

After acclimation period, the female rats were mated overnight. The morning on which sperm were found in the vaginal smear was designated as gestational day (GD) 0.

The rats were distributed into four experimental groups (n minimum = 12 animals/group): G1 = control (vehicle), G2 to G4 = treated with essential oil from *C. aurantium* at dose 125, 250 and 500 mg/kg, respectively. Rats were orally treated, by gavage, with essential oil from *C. aurantium* or vehicle (Tween® 80 at 8%, maximum volume of 0.20 mL) during pre-implantation and organogenic period (from GD 0 to GD 14). Previous studies verified that lowest effective dose of oil was 250 mg/kg (Moraes et al. 2013, Polo et al. 2012). The US Environmental Protection Agency suggests that doses be scaled from rats to humans on the basis of the ratio of human body weight to rat body weight raised to the 0.667 power, as follows: Dose human =Dose rat x (body weight human/ body weight rat) 0.667 (Bachmann et al. 1996). Thus, the high dose used to treat rats is approximately 19 g/kg for humans.

Course of Pregnancy

Maternal weight, food intake and water consumption were measured about every 7 days up to the end of pregnancy, at approximately 9 a.m. On GD 20 the rats were anesthetized by sodium pentobarbital and sacrificed by inducing pneumothorax. The gravid uterus was weighed and dissected to count dead and live fetuses, resorption, implantation sites, and corpora lutea numbers. The number of implantation sites was determined by the Salewski method (Salewski 1964). The rate of pre-implantation loss was calculated as: No. of corpora lutea – No. of implantations x 100/ No. of corpora lutea, and post- implantation loss rate was calculated as: No. of implantations – No. of live fetuses x 100/ No. of implantations (Damasceno et al. 2008). The fetuses and placentas were weighed to calculate the placental index: placental weight/fetal weight. The mean birth weight of the control pups (G1) was 4.2 ± 0.4 g. Newborns in the experimental groups whose birth weights did not diverge more than ± 1.0 standard deviation (SD) from the G1 mean (i.e., those that were within the 3.8- to 4.6- g range) were classified as appropriate for pregnancy age (APA). Those whose weights were at least 1.0 SD greater than the G1 mean birth weight were classified as large for pregnancy age (LPA). Those whose birth weights were at least 1.0 SD lower than the G1 mean birth weight were classified as small for pregnancy age (SPA) (Volpato et al. 2009). The fetuses were evaluated on a microscope with respect to incidence of external anomaly. After external analysis, half the fetuses were fixed in Bouin's fluid and serial sections were prepared as described by Wilson (1965) for visceral examination. The remaining fetuses were prepared for examination of the skeletons by the staining procedure of Staples and Schnell (1964). The ossification degree was evaluated using the parameters proposed by Aliverti et al. (1979).

Statistical Analyses

For comparison of the mean values between the experimental groups, analysis of variance followed by Tukey's test were used. The proportions were calculated by the Fisher Exact test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Body Weight Gain, Water Intake, and Food Consumption

As shown in Table I, the treatment with essential oil from *Citrus aurantium* did not interfere with body weight gain, water intake, and food consumption. These parameters were similar to control group during the entire experimental period (Table I).

Maternal Reproductive Outcome

The treatment with essential oil did not alter the parameters of maternal reproductive outcome (number of corpora lutea, implantation, resorptions, live and dead fetuses, maternal weight gain, gravid uterus weight, fetal weight, and sex ratio) compared to control group. The G4 group presented decreased placental weights and placental index compared to G1 group (Table II).

TABLE I
Body weight, water intake and food consumption of rats treated or not with *Citrus aurantium* essential oil, from day 0 to 14 of pregnancy.

	Groups			
	G1 (Control)	*G2 (125 mg/kg)*	*G3 (250 mg/kg)*	*G4 (500 mg/kg)*
Body weight (g)				
Day 0	249.7± 24.0	252.3± 21.6	241.9± 16.2	248.4± 21.8
Day 7	265.8± 27.1	269.1± 25.2	259.1± 21.4	258.9± 25.3
Day 14	293.7± 27.9	296.2± 29.8	285.7± 23.9	284.8± 31.8
Day 20	356.4± 32.5	361.2± 42.3	351.9± 25.2	355.7± 46.2
Water intake (mL)				
Day 0	28.7± 6.7	29.3± 5.2	30.1± 7.2	27.0± 4.6
Day 7	28.2± 4.7	30.4± 4.9	29.2± 5.3	31.4± 9.6
Day 14	36.2± 6.7	35.9± 3.8	38.2± 4.7	37.5± 8.0
Day 20	38.9± 7.1	40.2± 7.4	43.8± 11.1	44.3± 10.0
Food consumption (g)				
Day 0	16.8± 1.5	14.9± 1.9	15.8± 3.1	15.4± 2.1
Day 7	18.7± 2.6	16.8± 2.8	17.9± 2.9	17.2± 3.6
Day 14	21.3± 1.9	19.6± 3.5	20.4± 1.6	20.5± 2.9
Day 20	24.5± 3.2	23.8± 3.9	25.1± 3.3	24.8± 3.7

Data shown as mean ± standard deviation (SD). *$p > 0.05$ – non-significant statistically difference (ANOVA followed by Tukey's test).*

Ossification Sites

As shown in Table III, treated groups presented the number of forepaw and hindpaw phalanx, metatarsus, metacarpus, caudal vertebra, sternebrae, and total ossification sites similar to G1 group (Table III).

Anomaly Frequency

Data on the occurrence of external, skeletal and visceral anomalies indicated that the treatment with essential oil did not modify the total number of fetuses with alterations (Table IV).

DISCUSSION

Citrus aurantium L. (Rutaceae family), popularly known in Brazil as bitter orange, is among the species most frequently used for medicinal purposes. Phytochemical analyses of the essential oil in the fresh fruit peel of *C.aurantium* revealed the presence of limonene, a monoterpene as the major constituent that is commonly used as a flavoring in foods and drinks (Moraes et al. 2009, Sun 2007), for which it is classified in the U.S. Code of Federal Regulation as safe. Similarly, chromatogram revealed the monoterpene limonene to be the major substance, with a relative abundance of 70.75% and the molecular formula $C_{10}H_{16}$. The monoterpene*β*-Pinene, whose molecular formula is also $C_{10}H_{16}$, appears to rank second in relative abundance at 13.19% (Rozza et al. 2011). In this study, the plants used were of the same origin as those determined by Moraes et al. (2009) and Rozza et al. (2011), and therefore could have similar proportions of the active substances.

Our results showed that treatment of rats with *Citrus aurantium* essential oil did not interfere in maternal weight gain since the dams presented a progressive weight gain during pregnancy. In contrast, many studies suggest that *C. aurantium* induces weight loss effects (Cherniack 2008). In the present study, the essential oil of the plant was

TABLE II
Maternal reproductive outcome of rats treated or not with *Citrus aurantium* essential oil, from day 0 to 14 of pregnancy.

	Groups			
	G1 (Control)	*G2 (125 mg/kg)*	*G3 (250 mg/kg)*	*G4 (500 mg/kg)*
Pregnant females (N)	12	12	13	12
Corpora lutea				
Total (N)	159	161	177	168
Mean± SD	13.2 ± 1.7	12.8 ± 1.9	13.8 ± 2.3	14.0 ± 2.0
Implantation sites				
Total (N)	136	144	152	147
Mean ± SD	11.2 ± 3.8	12.1 ± 3.1	11.8 ± 3.5	12.2 ± 3.4
Live fetuses				
Total (N)	125	136	145	142
Mean ± SD	10.4 ± 3.8	11.1 ± 3.8	10.9 ± 3.8	11.8 ± 3.5
Dead fetuses				
Total (N)	1	1	3	1
Mean ± SD	0.1 ± 0.3	0.1 ± 0.2	0.3 ± 0.6	0.1 ± 0.3
Resorptions				
Total (N)	10	7	4	4
Mean ± SD	0.8 ± 1.2	0.4 ± 0.5	0.2 ± 0.4	0.3 ± 0.6
Pre-implantation loss (%)	14.5	10.6	14.1	12.5
Post-implantation loss (%)	8.1	5.6	4.6	3.4
Maternal weight gain (g) - MWG	106.6 ± 22.6	109.6 ± 24.8	110.6 ± 21.1	106.7 ± 27.6
Gravid uterus weight (g) - GUW	62.0 ± 23.0	69.3 ± 25.1	72.1 ± 28.2	68.5 ± 19.8
MWG minus GUW (g)	43.5 ± 9.9	39.5 ± 8.6	38.1 ± 15.4	40.2 ± 12.6
Fetal weight (g)				
Mean ± SD	4.2 ± 0.4	4.1 ± 0.3	4.3 ± 0.4	4.2 ± 0.3
SPA Fetuses (%)	11.2	13.4	9.3	10.6
APA Fetuses (%)	72.8	71.4	79.7	82.4
LPA Fetuses (%)	16.0	15.2	11.0	7.0
Placental weight (g) x 10				
Mean ± SD	5.3 ± 0.8	5.1 ± 1.0	5.4 ± 0.8	4.6 ± 0.9*
Placental Index (g) x 100				
Mean ± SD	12.6 ± 2.1	12.2 ± 1.2	12.9 ± 0.9	11.1 ± 0.2*
Sex ratio (M/F)	60/65	65/71	74/71	68/74

N= number. Data shown as mean ± standard deviation (SD) and proportions (%). * *$p<0.05$ – significant statistically difference (ANOVA followed by Tukey's test).*

used, which contains no compounds responsible for weight loss. The substances that reduce body weight are similar to ephedra, a component of the fruit, that contains alkaloids that are adrenergic agonists and is often incorporated to supplements designed to aid in weight loss (Preuss et al. 2002). In some reports, *Citrus aurantium* products were combined with other agents, making it unclear which caused the benefit (Haaz et al. 2006).

In general, maternal deaths, reduction in body weight gain, and food and water intake are simple and sensitive indices of toxicity after exposure to toxic substances (Raza et al. 2002, Teo et al. 2002). In this study, the maternal weight gain did not differ. Furthermore, no alterations of daily food and water intake were observed, suggesting that the *C. aurantium* administration did not provoke maternal toxicity.

TABLE III
Ossification sites of rats treated or not with *Citrus aurantium* essential oil, from day 0 to 14 of pregnancy.

	Groups			
	G1 (Control)	G2 (125 mg/kg)	G3 (250 mg/kg)	G4 (500 mg/kg)
Forepaw phalanx	1.7 ± 0.6	1.6 ± 0.4	1.6 ± 0.5	1.4 ± 0.5
Metacarpus	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0
Hindpaw phalanx	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Metatarsus	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.0
Caudal vertebra	3.3 ± 0.6	3.2 ± 0.5	3.5 ± 0.6	3.3 ± 0.3
Sternebra	6.0 ± 0.0	5.9 ± 0.1	6.0 ± 0.0	5.9 ± 0.1
Total	19.0 ± 1.2	18.7 ± 0.9	19.1 ± 0.7	18.7 ± 0.6

Data shown as mean ± standard deviation (SD). *p>0.05 – non-significant statistically difference (ANOVA followed by Tukey's test).*

TABLE IV
Anomaly frequency of rats treated or not with *Citrus aurantium* essential oil (500 mg/kg body weight/day), by gavage, from day 0 to 14 of pregnancy.

	Groups			
	G1 (Control)	*G2 (125 mg/kg)*	*G3 (250 mg/kg)*	*G4 (500 mg/kg)*
External anomalies				
Number fetuses examined (litter)	126 (12)	137 (12)	148 (13)	143 (12)
Total number of fetuses (%) with alteration	0 (0.0)	0 (0.0)	1 (0.7)	0 (0.0)
Mean % fetuses with alteration per litter (mean ± SD)	0.00	0.00	1.31	0.00
Gastrosquises	0 *(0.0 %)*	0 *(0.0 %)*	1 *(0.7 %)*	5 *(6.9 %)*
Skeletal anomalies				
Number fetuses examined (litter)	64 (12)	80 (12)	85 (13)	72 (12)
Total number of fetuses (%) with alteration	21 (32.8)	35 (43.7)	32 (37.6)	34 (47.2)
% fetuses with alteration per litter	33.51	41.43	36.21	45.54
Craniofenestria	0 *(0.0 %)*	2 *(2.5 %)*	2 *(2.4 %)*	5 *(6.9 %)*
Dumbbell ossific.of vertebral centrum	0 *(0.0 %)*	0 *(0.0 %)*	1 *(1.2 %)*	2 *(2.8 %)*
Supernumerary rib	8 *(12.5 %)*	9 *(11.2 %)*	5 *(5.9 %)*	16 *(22.2 %)*
Sternebra agenesis	0 *(0.0 %)*	2 *(2.5 %)*	0 *(0.0 %)*	2 *(2.8 %)*
Unossified sternebra	6 *(9.4 %)*	7 *(8.7 %)*	10 *(11.8 %)*	7 *(9.5 %)*
Incomplete ossification of sternebra	3 *(4.7 %)*	2 *(2.5 %)*	4 *(4.8 %)*	1 *(1.4 %)*
Abnormally shaped sternebra	14 *(21.9 %)*	16 *(20.0 %)*	13 *(15.3 %)*	10 *(13.9 %)*
Visceral anomalies				
Number fetuses examined (litter)	62 (12)	77 (12)	83 (13)	71 (12)
Total number of fetuses (%) with alteration	25 (40.32)	28 (36.36)	28 (32.94)	22 (30.99)
% fetuses with alteration per litter	39.07	32.35	34.89	31.84
Dilated trachea	2 *(3.1 %)*	2 *(2.6 %)*	3 *(3.6 %)*	0 *(0.0 %)*
Ectopic testis	0 *(0.0 %)*	1 *(1.3 %)*	0 *(0.0 %)*	1 *(1.4 %)*
Dilated renal pelvis	0 *(0.0 %)*	2 *(2.6 %)*	4 *(4.8 %)*	1 *(1.4 %)*
Hydroureter	22 *(35.4 %)*	25 *(32.5 %)*	19 *(22.9 %)*	20 *(28.2 %)*
Hydronephrosis	2 *(3.1 %)*	1 *(1.3 %)*	3 *(3.6 %)*	0 *(0.0 %)*

Data shown as proportions (%). *p>0.05 – non-significant statistically difference (Fisher Exact test).*

Effects of chemicals or drugs during pregnancy may manifest as abortions, anomalies or retarded development (Sullivan 1993). Our results showed non-significant differences in the number of corpora lutea, implantation sites, resorptions, live and dead fetuses, gravid uterus weight, fetal weight, and sex ratio after treatment of the dams with *C. aurantium* essential oil. However, litters from the rats treated with high dose presented impaired placental development, as verified by reduced placental weights. This finding could be related to limonene presence in the plant essential oil because this monoterpene presents antitumoral action (Chen et al. 2006), suppressing cellular proliferation in the placenta, which could have led to the reduced placental weight. The placental index is used for verification of placental function in relation to maternal-fetal exchange (Kingdom and Kaufmann 1999). In treated group with 500 mg/kg, the placental index was decreased, which can cause possible disturbances in the exchange of oxygen and nutrients from the mother to the fetuses contributing to intrauterine growth restriction, however this was not observed.

It is notable that the treated groups did not present signs of delayed ossification in the specific ossification sites (forelimbs, metatarsus, metacarpus, sternebra, and caudal vertebrae), and in total ossification sites. Chahoud and Paumgartten (2005) verified strong correlation between body weight and degree of ossification. In the present study, ossification sites and fetal weight did not alter, indicating that *C. aurantium* did not cause prenatal growth retardation and impaired fetal somatic development.

Prenatal administration of plants during pregnancy can result in fetal anomalies (Lemonica and Alvarenga 1994, Damasceno and Lemonica 1999, Mello et al. 2005). In our investigation, *Citrus aurantium* treatment did not alter the incidence of external, skeletal, and visceral anomalies. No external anomalies were found in control or treated groups. The most common skeletal anomalies in fetuses were supernumerary rib, unossified and abnormally shaped sternebra. In relation to visceral anomalies, the control and treated group presented high incidence of hydroureter, which is easily recognized because of the "S" form and complete transparency, as seen in this study. Moreover, hydroureter may or may not be associated with congenital hydronephrosis, it might possibly be a transitory variation in rodents (Taylor 1986). According to the literature, approximately 20% of newborns of normal rats have this kind of visceral anomaly (Damasceno et al. 2002b, 2011a, b).

Thus, we concluded that essential oil from *Citrus aurantium*, administered orally to rats on GD 0 to 14, caused no teratogenic effect and did not alter maternal reproductive outcome in the doses tested.

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RESUMO

Citrus aurantium L., conhecida popularmente como laranja amarga, é amplamente utilizada na medicina popular, mas há poucos dados na literatura sobre seus efeitos na gestação. O objetivo do presente estudo foi avaliar a influencia do óleo essencial obtido das frutas de *Citrus aurantium* no desempenho reprodutivo materno e na incidência de anomalias fetais em ratos. Ratas Wistar prenhes foram randomizadas em quarto grupos (n mínimo = 12 animais/grupo): G1 = controle, G2 à G4 = tratados com óleo essencial de *C. aurantium* nas doses de 125, 250 e 500 mg/kg, respectivamente. Ratas foram tratadas oralmente, por gavage, com óleo essencial da planta ou veículo durante os períodos de pré-implantação e organogênese (dias de prenhez 0-14). No dia 20 de prenhez as ratas foram anestesiadas e o útero foi pesado

com seu conteúdo e os fetos foram analisados. Resultados mostraram que o grupo tratado com 500 mg/kg apresentou diminuição do peso e índice placentário, embora o tratamento com óleo essencial de laranja amarga não mostrou nenhuma alteração no desempenho reprodutivo materno, efeito tóxico, mudanças na ossificação e nas taxas de malformações. Concluindo, o tratamento com óleo essencial de *Citrus aurantium* não foi teratogênico e não alterou o desempenho reprodutivo materno.

Palavras-chave: *Citrus aurantium*, óleo essencial, limoneno, malformação, prenhez, desempenho reprodutivo.

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