Arch Toxicol (2009) 83:95–99
DOI 10.1007/s00204-008-0324-8

REPRODUCTIVE TOXICOLOGY

Screening for in vivo (anti)estrogenic activity of ephedrine and *p*-synephrine and their natural sources *Ephedra sinica* Stapf. (Ephedraceae) and *Citrus aurantium* L. (Rutaceae) in rats

Marcelo Dutra Arbo · Márcia Toniolo Franco · Elisa Rupp Larentis · Solange Cristina Garcia · Viviane Cristina Sebben · Mirna Bainy Leal · Eliane Dallegrave · Renata Pereira Limberger

Received: 19 March 2008 / Accepted: 26 May 2008 / Published online: 24 July 2008

Abstract Formulations containing *Ephedra sinica* Stapf. (Ephedraceae) and *Citrus aurantium* L. (Rutaceae) are consumed worldwide for body weight control. Considering the related adverse effects and the risk potential, the aim of this study is to evaluate the effects of the thermogenic compounds ephedrine, *p*-sinephrine, *E. sinica* and *C. aurantium* in the female reproductive system through the uterotrophic assay in immature female rats. The animals (n = 6–7) received *E. sinica* 85.5 and 855.0 mg/kg/day, *C. aurantium* 25.0 and 50.0 mg/kg/day, ephedrine 5.0 mg/kg/day and *p*-synephrine 50.0 mg/kg/day for three consecutive days by oral gavage. For detection of antiestrogenicity, tamoxifen 20.0 mg/kg/day, *E. sinica* 855.0 mg/kg/day, *C. aurantium* 50.0 mg/kg/day, ephedrine 5.0 mg/kg/day and *p*-synephrine 50.0 mg/kg/day were administered to estrogen-treated females. Macroscopical alterations were evaluated in liver, kidneys, adrenals and uterus. All analyzed substances showed an antiestrogenic potential, but only ephedrine at 0.5 mg/kg/day presented a significative antiestrogenic effect ($P < 0.01$). Adrenals relative mass were reduced ($P < 0.01$) in all tested compounds when compared to the control, which seems to be related to the alfa-1-adrenoceptor agonist activity, which promote a vasoconstriction and reduction of the liquid in the organ. The endocrine system is highly complex and there are a number of ways in which a chemical may interfere with it, other in vivo and in vitro assays are being necessary to support this mechanism of action.

Keywords Ephedra · Citrus · Ephedrine · *p*-Synephrine · Uterotrophic assay · Immature rats

M. D. Arbo · E. R. Larentis · R. P. Limberger (✉)
Laboratório de Análises e Pesquisas Toxicológicas,
Departamento de Análises, Faculdade de Farmácia,
Universidade Federal do Rio Grande do Sul,
Av. Ipiranga, 2752, Porto Alegre, RS 90610-000, Brazil
e-mail: renata@farmacia.ufrgs.br

M. D. Arbo
e-mail: m.arbo@terra.com.br

M. T. Franco · M. B. Leal
Laboratório de Farmacologia e Toxicologia de Produtos Naturais,
Departamento de Farmacologia, Instituto de Ciências Básicas
da Saúde, Universidade Federal do Rio Grande do Sul,
Rua Sarmento Leite, 500/202, Porto Alegre,
RS 90610-170, Brazil

S. C. Garcia
Laboratório de Toxicologia,
Departamento de Análises Clínicas e Toxicológicas,
Universidade Federal de Santa Maria C P. 5061,
Campus Universitário, Santa Maria, RS 97110-970, Brazil

V. C. Sebben · E. Dallegrave
Centro de Informações Toxicológicas do Rio Grande do Sul,
Fundação Estadual de Produção e Pesquisa em Saúde,
Rua Domingos Crescêncio, 8° andar, Porto Alegre,
RS 90650-090, Brazil

Introduction

Extracts of *Citrus aurantium* L. (Rutaceae) unripe fruits (syn.: zhi shi, green orange, sour orange and bitter orange) have been used in traditional Chinese medicine to stimulate overall gastrointestinal function and recently, have gained significant popularity for the treatment of obesity, as an alternative to ephedra alkaloids, which have been banned from dietary supplements by the United States Food and Drug Administration (FDA) in April 2004 because of an association with serious adverse health effects (Fugh-Bergman and Myers 2004; Andraws et al. 2005). The new products have been marketed as "ephedra free" and usually

contain *C. aurantium* extracts standardized from 3 to 6% of *p*-synephrine (De Smet 2004).

Ephedrine and *p*-synephrine are related substances, with sympathomimetic activity which has been associated to a raise in metabolic rates and oxidation of fats through an increase in thermogenesis and stimulated lipolysis, presumably by means of adrenergic β-3-receptors. However, it has been demonstrated that *p*-synephrine acts not only in β-3-receptors, but also in β1, β2 and α-receptors, presenting similar ephedra side effects, such as frequency and cardiac debit increase, peripheric vasoconstriction, broncodilatation and CNS stimulation (Fugh-Bergman and Myers 2004).

Considering the indiscriminate use of these formulations especially by young women and that their use could interfere hormonal routes and, consequently, during reproductive age, alter the menstrual cycle, fertility and even the embrionary development. So, the aim of this study is to evaluate the effects of ephedrine, *p*-sinephrine and its natural sources *Ephedra sinica* Stapf. (Ephedraceae) and *C. aurantium*, in the female reproductive system by means of *Organization for Economic Cooperation and Development* (OECD) uterotrophic assay in immature female rats. This is considered an initial step analysis, a short-time test, which represents an exposition to xenobiotics in sensitive step of reproductive tract development.

Materials and methods

Chemicals and reagents

Ephedrine (CAS 299-42-3, purity 99%) was purchased from Sigma Chemical Co. (St. Louis, USA) and *p*-synephrine (CAS 94-07-5, purity 99%) from Aldrich (St. Louis, USA). Tamoxifen (CAS 10540-29-1) was supplied by Galena (Campinas, SP, Brazil) and estradiol cypionate (CAS 313-06-04) by Pfizer (Paulínia, SP, Brazil). Acetonitrile HPLC grade was obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, Bedford, USA). Methanol (Merck), cyclehexanone (Merck) and trifluoroacetic acid (TFA; Vetec, Rio de Janeiro, Brazil) were from analytical grade.

Animals

Immature female Wistar rats (21 days) weighing 42.3 ± 1.0 g obtained from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS, Porto Alegre, RS, Brazil) were used. They were housed in polyethylene cages under standard conditions of temperature (22 ± 2 C), controlled humidity and 12 h-light/dark cycle. Standard pellet food and tap water were available ad libitum. The experiments were performed after approval of the protocol by the FEPPS and UFRGS Ethics Committees (numbers 04/2007 and 2007784) and were carried out in accordance with current guidelines for the care of laboratory animals.

Plant material

Commercial samples of *E. sinica* were donated by local pharmacies. The material was triturated and submitted to 15 min dynamic maceration with acetone (Schaneberg et al. 2003). The solvent was evaporated and the residue dissolved in water. For GC/MS analysis the residue was derivatized with 100 µl cyclehexanone at 100 C for 30 min.

Unripe fruits of *C. aurantium* were collected from known populations in Porto Alegre (RS, Brazil) in January 2007 and was triturated and submitted to maceration with 80% methanol. After 24 h the extract was filtered, concentrated in rotatory evaporator and lyophilized.

Determination of total ephedrines content

The content of total ephedrines was determined by a GC-MS chromatographic system (VARIAN®, Palo Alto, CA, USA) equipped with a GC 3800 VARIAN® chromatograph, 1079 split/splitless injector with a 8200 VARIAN® autosampler and a SATURN GC/MS/MS 2000 VARIAN® mass detector. Ultrapure helium was used as carrier gas at a constant flow-rate of 1 ml/min. The chromatographic separation was achieved using a CP-SIL 8CB LOW BLEED/MS capilar column (30 m × 0.25 mm × 0.25 µm polydimethyldiphenylsiloxane). The column temperature was programed for 80 C for 2 min, 50 C/min to 250 C for 2 min, and 100 C/min to 280 C for 1 min. The injector and liner temperatures were adjusted to 220 and 260 C, respectively. The injection volume was 1 µl in split (1:10) mode. The analysis was performed in the electronic impact mode with 70 eV ionization energy. The amount of total ephedrines was calculated through external calibration curves.

Determination of *p*-synephrine content

The content of *p*-synephrine in *C. aurantium* unripe fruits extracts was performed by HPLC/UV. The dry extract was dissolved 1:12 in water, filtered through 0.45 mm membrane pore (Millipore, Bedford, USA) and injected in the chromatographic system (Knauer, Berlin, Germany) equipped with a K 1001 pump, K 5004 online degasser, manual injector with 20 µl loop furthermore a K 2501 UV/VIS detector with a EUROCHROM 2000 SOFTWARE®, 2.05 for Windows (Knauer, Berlin, Germany). The chromatographic separation was realized in a C18 Eurospher-100® (1.50 × 4 mm × 5 µm) column with a Eurospher-100® (5 × 4 mm × 5 µm) pre-column. The analyte was

detected at 220 nm. The mobile phase consisted of acetonitrile–water–TFA (5:95:0.01, v/v/v) as solvent A and pure acetonitrile as solvent B, using a gradient elution in 0–8 min with 100–59% A, 8–9 min with 59–0% of A and 9–12 min 100% of A, at a flow-rate of 0.6 ml/min. The injection volume was 20 µl, in a 12 min run-time. The amount of *p*-synephrine was calculated through external calibration curves.

Uterotrophic assay

The test compounds were given daily for three consecutive days by oral gavage (po) to the immature female rats (6–7 animals/group) according the experimental design (Table 1). Two dose levels of each standardized extracts, *E. sinica* 85.5 and 855.0 mg/kg/day and *C. aurantium* 25.0 and 50.0 mg/kg/day, and their main constituents ephedrine 5.0 mg/kg/day and *p*-synephrine 50.0 mg/kg/day were used to assess possible estrogenic activity. For detection of anti-estrogenicity, tamoxifen 20.0 mg/kg/day, *E. sinica* 855.0 mg/kg/day, *C. aurantium* 50.0 mg/kg/day, ephedrine 5.0 mg/kg/day and *p*-synephrine 50.0 mg/kg/day were administered to estrogen-treated females (estradiol cypionate 0.4 mg/kg/day). The vehicle (corn oil 10 ml/kg/day) was administered as a negative control while estradiol cypionate (0.4 mg/kg/day) was used as a positive control for estrogenicity and tamoxifen (20.0 mg/kg/day) as a positive control for antiestrogenicity. The dosing volume for all solutions was 10 ml/kg body. Animals were weighed everyday and killed by cervical dislocation 24 h after the final dose. After the sacrifice, the rats were necropsied and analyzed for macroscopical alterations in liver, kidneys, adrenals and uterus. The uterus was excised, trimmed free of fat, pierced, and blotted to remove fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries (Odum et al. 1997). The weight of the organs were determined and expressed as relative weight (organ mass/body weight × 100).

Table 1 Experimental design

Treatment groups	Control	Ephedra	Ephedrine	Citrus	*p*-Synephrine
Negative control	Corn oil 10 ml/kg				
Estrogenicity	Estradiol cypionate 0.4 mg/kg	*E. sinica* 85.5 mg/kg	Ephedrine 5.0 mg/kg	*C. aurantium* 25.0 mg/kg	*p*-Synephrine 50 mg/kg
		E. sinica 855.0 mg/kg		*C. aurantium* 50.0 mg/kg	
Antiestrogenicity	Tamoxifen 20.0 mg/kg + estradiol cypionate 0.4 mg/kg	*E. sinica* 855.0 mg/kg + estradiol cypionate 0.4 mg/kg	Ephedrine 5.0 mg/kg + estradiol cypionate 0.4 mg/kg	*C. aurantium* 50.0 mg/kg + estradiol cypionate 0.4 mg/kg	*p*-Synephrine 50.0 mg/kg + estradiol cypionate 0.4 mg/kg

Statistical analysis

Data were analyzed by analysis of variance (ANOVA). Differences between groups were determined by Bonferroni's post hoc, the significant level was set at 1% ($P < 0.01$).

Results

Standardized *Ephedra sinica* extract

The extract was submitted to qualitative and quantitative analysis of ephedrines content through a previously

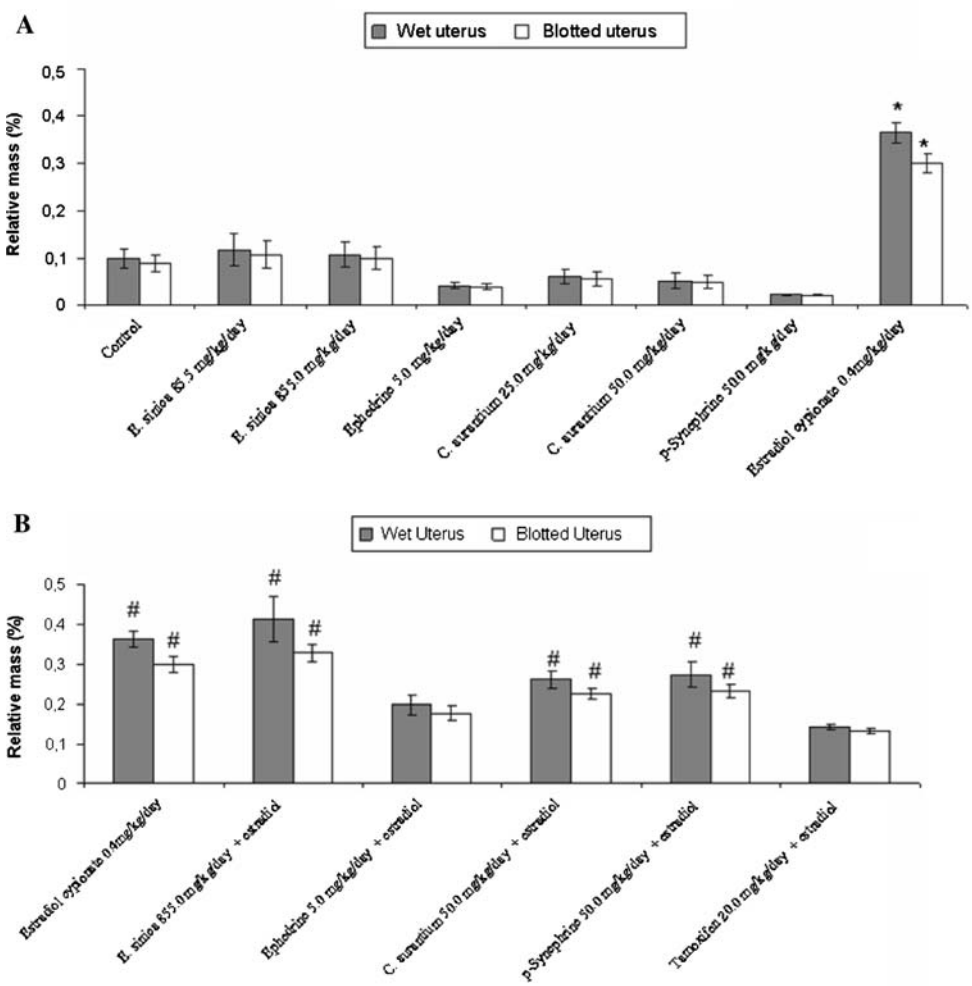

Fig. 1 Estrogenic (**a**) and antiestrogenic (**b**) effect of *Ephedra sinica* and *Citrus aurantium* extracts, ephedrine and *p*-synephrine in the wet and blotted uterus relative mass. Values represent mean ± SE of the relative uterus weight ($n = 6–7$). *Significantly different from control and #significantly different from tamoxifen (ANOVA/Bonferroni; $P < 0.01$)

validated GC/MS method, total alkaloids were 0.65%, while ephedrine was 0.41%.

Standardized *Citrus aurantium* extract

The extract was submitted to qualitative and quantitative analysis through a previously validated HPLC/UV method for the content of *p*-synephrine, which was 3.0%.

Uterotrophic assay

No abnormal clinical findings or body weight changes were detected in the rats given the test compounds. The wet and blotted relative weights of the uterus of rats given estradiol cypionate (0.4 mg/kg/day) were significantly increased compared to the vehicle (Fig. 1a). Tamoxifen 20.0 mg/kg/day and ephedrine 5.0 mg/kg/day significantly reverted the estradiol effect. The relative mass of uterus of *C. aurantium* 50.0 mg/kd/day and *p*-synephrine 50.0 mg/kg/day plus estradiol cypionate 0.4 mg/kg/day treated rats were significantly higher than control and tamoxifen 20.0 mg/kg/day (Fig. 1b).

A significant reduction was observed in the adrenals relative mass of animals treated with ephedrine, *p*-synephrine and both doses of ephedra and citrus extracts and an elevation in the kidneys relative mass in the *p*-synephrine plus estradiol cypionate treated group. There were no macroscopic alterations observed in the other analyzed organs (Table 2).

Discussion

Herbal medicines have become a popular form of therapy in many countries. Even though they are often prompted as natural and therefore harmless, medicinal plants are by no means free from toxicity. The present study was undertaken to evaluate the female reproductive toxicity of *E. sinica*, *C. aurantium*, ephedrine and *p*-synephrine, which are thermogenic herbal remedies used in weight loss formulations and dietary supplements. In this study, we used the uterotrophic assay, one of the most widely used short-term screening assays designed to detect (anti)estrogenic activity of chemical substances or mixtures (Baker 2001; Andrade et al. 2002; Dalsenter et al. 2004).

Table 2 Relative body weight (RBW) and relative mass of immature female Wistar rats organs

Experimental groups	RBW	Liver	Right kidney	Left kidney	Right adrenal	Left adrenal
Control	120.6 ± 2.7	3.87 ± 0.16	0.523 ± 0.018	0.500 ± 0.012	0.0191 ± 0.0011	0.0200 ± 0.0019
E. sinica 85.5 mg/kg/day	113.9 ± 1.7	3.50 ± 0.07	0.510 ± 0.022	0.473 ± 0.011	0.0087 ± 0.0007*	0.0089 ± 0.0011*
E. sinica 855.0 mg/kg/day	116.8 ± 1.2	3.85 ± 0.13	0.461 ± 0.008	0.451 ± 0.006	0.0076 ± 0.0011*	0.0080 ± 0.0014*
Ephedrine 5.0 mg/kg/day	124.8 ± 3.9	4.08 ± 0.12	0.452 ± 0.006	0.450 ± 0.011	0.0070 ± 0.0009*	0.0085 ± 0.0016*
C. aurantium 25.0 mg/kg/day	117.7 ± 3.2	3.71 ± 0.12	0.438 ± 0.009	0.425 ± 0.008	0.0088 ± 0.0007*	0.0090 ± 0.0010*
C. aurantium 50.0 mg/kg/day	114.0 ± 3.1	3.61 ± 0.08	0.453 ± 0.010	0.420 ± 0.011	0.0073 ± 0.0013*	0.0095 ± 0.0010*
p-Synephrine 50.0 mg/kg/day	116.6 ± 1.2	4.06 ± 0.12	0.459 ± 0.008	0.449 ± 0.011	0.0066 ± 0.0009*	0.0087 ± 0.0017*
Estradiol cypionate 0.4 mg/kg/day	114.5 ± 1.5	4.04 ± 0.09	0.530 ± 0.022	0.526 ± 0.015	0.0146 ± 0.0012	0.0177 ± 0.0021
E. sinica 855.0 mg/kg/day + estradiol	114.3 ± 1.2	4.04 ± 0.09	0.561 ± 0.013	0.549 ± 0.005	0.0218 ± 0.0025	0.0211 ± 0.0015
Ephedrine 5.0 mg/kg/day + estradiol	115.6 ± 3.2	3.94 ± 0.16	0.540 ± 0.011	0.548 ± 0.020	0.0183 ± 0.0013	0.0215 ± 0.0007
C. aurantium 50.0 mg/kg/day + estradiol	120.6 ± 1.8	4.20 ± 0.17	0.618 ± 0.007	0.583 ± 0.013	0.0198 ± 0.0013	0.0210 ± 0.0014
p-Synephrine 50.0 mg/kg/day + estradiol	122.7 ± 2.6	4.43 ± 0.09	0.652 ± 0.034*	0.620 ± 0.034*	0.0185 ± 0.0013	0.0191 ± 0.0019
Tamoxifen 20.0 mg/kg/day + estradiol	108.7 ± 2.4	4.25 ± 0.18	0.564 ± 0.016	0.567 ± 0.013	0.0224 ± 0.0011	0.0243 ± 0.0013

Results expressed as mean ± standard error of the mean (SEM)

* Significantly different from control group ($P < 0.01$) by ANOVA/Bonferroni

The extract obtained with a commercial sample of *E. sinica* was analyzed for the presence of ephedrines, the value found was in accordance with the literature reports (Schaneberg et al. 2003) and was below the one admitted by official institutions (JPXIII 1996). The standardized extract of *C. aurantium* was according to the previous reports (De Smet 2004).

When submitted to the uterotrophic assay, ephedrine 0.5 mg/kg/day reduced the uterus relative mass, indicating an antiestrogenic effect. However, this was a screening assay and it is not sufficient to fully characterize the mechanism of action of ephedrine in the endocrine system. There were found a reduction in the adrenals relative mass in the groups which received ephedrine, *p*-synephrine and both doses of the extracts, this action could be due to the alfa-1-adrenoceptor agonist activity of these substances, which promote vasoconstriction and reduction of the liquid in the organ (AHFS 2005). In the group treated with *p*-synephrine and estradiol cypionate there was an elevation observed in the kidneys relative weight which could indicate a possible interaction between *p*-synephrine and estradiol that should be more investigated.

In conclusion, an antiestrogenic property of ephedrine was detected, however, the endocrine system is highly complex and there are a number of ways in which a chemical may interfere with it. Some of these mechanisms, such as interference with the hypothalamic–pituitary–gonadal axis, may not be detected by short-term screening assays (Andrade et al. 2002), being necessary other in vivo and in vitro assays, such as hormones quantification, to support this mechanism of action. Besides that, the long-term effects were not evaluated, more attention should be given to anti-obesity products and dietary supplements containing ephedrine/*p*-synephrine.

Acknowledgments This work was supported by CNPQ (Processo 478054/2006-8) and FEPPS (animals donation).

References

AHFS - American Hospital Formulary Service Drug Information (2005) American Society of Health-System Pharmacists. Bethesda, USA

Andrade AJM, Araújo S, Santana GM, Ohi M, Dalsenter PR (2002) Screening for in vivo (anti)estrogenic and (anti)androgenic activities of technical and formulated delthamethrin. Regul Toxicol Pharmacol 35:379–382

Andraws R, Chawla P, Brown DL (2005) Cardiovascular effects of ephedra alkaloids: a comprehensive review. Prog Cardiovasc Dis 47:217–225

Baker VA (2001) Endocrine disrupters: testing strategies to assess human hazard. Toxicol In Vitro 15:413–419

Fugh-Bergman A, Myers A (2004) Citrus aurantium, an ingredient of dietary supplements marketed for weight loss: current status of clinical and basic research. Exp Biol Med 299:698–704

Dalsenter PR, Cavalcanti AM, Andrade AJM, Araújo SL, Marques MCA (2004) Reproductive evaluation of aqueous crude extract of *Achillea millefolium* L. (Asteraceae) in Wistar rats. Reprod Toxicol 18:819–823

De Smet PAGM (2004) Health risks of herbal remedies: an update. Clin Pharmacol Ther 76:1–17

JPXIII (1996) The Japanese Pharmacopoeia. The Society of Japanese Pharmacopoeia, Tokyo

Odum J, Lefevre PA, Tittensor S, Paton D, Routledge EJ, Beresford NA, Sumpter JP, Ashby J (1997) The rodent uterotrophic assay: critical protocol features, studies with phenols, and comparison with a yeast estrogenicity assay. Regul Toxicol Pharmacol 25:176–188

Schaneberg BT, Crockett S, Bedir E, Khan IA (2003) The role of chemical fingerprinting: application to Ephedra. Phytochemistry 62:911–918