UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Avaliação toxicológica de *p*-sinefrina e extrato de *Citrus aurantium* L. (Rutaceae)

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Porto Alegre, 2008

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Avaliação toxicológica de *p*-sinefrina e extrato de *Citrus aurantium* L. (Rutaceae)

Dissertação apresentado por **Marcelo Dutra Arbo** para obtenção do GRAU DE MESTRE em Ciências Farmacêuticas

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Dissertação apresentada ao Programa de Pós Graduação em Ciências Farmacêuticas, em nível de Mestrado – da Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul e aprovada em 16.5.2008, pela Banca Examinadora constituída por:

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A666a Arbo, Marcelo Dutra
Avaliação toxicológica de *p*-sinefrina e extrato de *Citrus aurantium* L. (Rutaceae) / Marcelo Dutra Arbo – Porto Alegre : UFRGS, 2008 - xv, 107 p.: il ., gráf., tab.

Dissertação (mestrado). UFRGS. Faculdade de Farmácia. Programa de Pós-graduação em Ciências Farmacêuticas.

1. *Citrus aurantium*. 2. Rutaceae. 3. *p*-Sinefrina. 4. Avaliação toxicológica. I. Limberger, Renata Pereira. II. Leal, Mirna Bainy. III. Título.

CDU: 615.9

Bibliotecária responsável:
Heloísa do Canto Canabarro, CRB10/1036
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Este trabalho foi desenvolvido, sob a orientação da Prof[a]. Dr. Renata Pereira Limberger e co-orientação da Prof[a]. Dr. Mirna Bainy Leal, nos laboratórios de Toxicologia, da Faculdade de Farmácia, e Farmacologia e Toxicologia de Produtos Naturais, do Instituto de Ciências Básicas da Saúde, ambos da Universidade Federal do Rio Grande do Sul. O doseamento dos marcadores bioquímicos e do estresse oxidativo foi realizado no Laboratório de Toxicologia do Centro de Ciências da Saúde da Universidade Federal de Santa Maria, sob a coordenação da Prof[a]. Dr. Solange Cristina Garcia. A pesquisa foi financiada pelo CNPq (Processo 478054/2006-8). Marcelo Dutra Arbo recebeu bolsa de mestrado do CNPq.

“Todas as substâncias são venenos, não existe nenhuma que não o seja. A dose correta diferencia o veneno do remédio”.

Paracelsus

AGRADECIMENTOS

À Prof[a]. Dr. Renata Pereira Limberger pela amizade, apoio, confiança e oportunidade de realizar este trabalho.

À Prof[a]. Dr. Mirna Bainy Leal pela acolhida carinhosa, amizade, apoio, dedicação e, principalmente, confiança em todas as minhas idéias.

À Prof[a]. Dr. Solange Garcia pela parceria iniciada, amizade e apoio na realização desta dissertação.

À Prof[a]. Dr. Eliane Dallegrave pela amizade, dedicação, incentivo e por ser a mentora da Sacrifícios S/A.

À Prof[a]. Dr. Vera Steffen por ter me aberto às portas para o mundo da Toxicologia e por toda a amizade.

À Elisa, Mariana e Ana Laura pela responsabilidade, por toda a dedicação prestada durante a execução dos experimentos e pela amizade.

À Vivi por estar sempre disponível a ajudar, em todos os momentos.

Aos amigos do Laboratório de Toxicologia Viviane, Gabi, Adri, Rô, Bea, Elo, Paulinha e Lu, por todos os cafés, discussões científicas, risadas e bons momentos vividos.

Aos amigos do Laboratório de Etnofarmacologia Adri, Micheli, Ângelo, Berna, Anita, Greice e Jeni, por terem me recebido da melhor maneira possível, pela ajuda prestada e por todas as terças-felizes.

Aos amigos do Laboratório de Toxicologia da UFSM Rachel, Ju loira, Ju morena, Gianine, Miguel, Ângela, Mariele, Raquelzinha e Ferdi pela ajuda nas análises, discussões e amizade.

Aos colegas de pós Rafa, Carol, Raquel, Dudu, Cris e Simoni pela amizade dentro e fora das aulas.

À Fabi, por toda a confiança, amizade e por ter acreditado em mim nos meus bons e maus momentos.

Ao Guilherme pela amizade e hospedagem, que possibilitou realizar parte deste trabalho em Santa Maria.

Aos meus amigos Lilica, Elyara, Aletéia, Alexandre, Larissa e Marceli, que estavam lá quando tudo começou.

À minha família pelo apoio incondicional.

A todos que de alguma forma contribuíram para a realização deste trabalho.

SUMÁRIO

LISTA DE ABREVIATURAS

ANVISA	Agência Nacional de Vigilância Sanitária
CAT	Catalase
CK	Creatina Quinase
CLAE/UV	Cromatografia Líquida de Alta Eficiência / Ultravioleta
DL50	Dose Letal para 50% da População
EROs	Espécies Reativas de Oxigênio
FDA	Food and Drug Administration
FEEPS	Fundação Estadual de Produção e Pesquisa em Saúde
GSH	Glutationa Reduzida
GSSG	Glutationa Oxidada
GPx	Glutationa Peroxidase
GR	Glutationa Redutase
MDA	Malonildialdeído
MDMA	Metilenodioximetanfetamina
SOD	Superóxido Dismutase

RESUMO

O uso descontrolado de suplementos alimentares indicados para emagrecer e alcançar maior definição muscular desencadeou uma grande preocupação. Dentre estes produtos, destacam-se aqueles contendo efedrina (de *Ephedra sinica*). Devido à associação com problemas cardíacos, isquemias e hipertensão, alguns países proibiram a venda de suplementos contendo este alcalóide, o qual tem sido substituído por *p*-sinefrina (de *Citrus aurantium*). Assim, o objetivo deste trabalho foi avaliar o perfil toxicológico de extratos de *C. aurantium* e do padrão racêmico de *p*-sinefrina. Foi validada uma metodologia analítica por CLAE/UV para a quantificação de *p*-sinefrina nos extratos. Para a avaliação do perfil toxicológico os extratos (300, 500, 1000, 2500, 3500 e 5000 mg/kg) e a *p*-sinefrina (150, 300, 450, 600, 800, 1000 e 2000 mg/kg) foram submetidos ao teste de toxicidade aguda em camundongos, onde foram observados sinais como piloereção, ofego, sialorréia, exoftalmia e diminuição da atividade locomotora em todos os tratamentos, porém, não foram observadas mortes. A diminuição da locomoção foi confirmada através da avaliação em caixas de atividade locomotora. No teste de toxicidade subcrônica, camundongos foram tratados por 28 dias com *C. aurantium* 400, 2000 e 4000 mg/kg e *p*-sinefrina 30 e 300 mg/kg, foram realizadas análises hematológicas, bioquímicas e de marcadores do estresse oxidativo. Pode-se observar aumento dos níveis de GSH nas doses de *C. aurantium* 4000 mg/kg e *p*-sinefrina 30 e 300 mg/kg, e inibição da enzima GPx nos animais tratados com *C. aurantium* 400 e 2000 mg/kg e *p*-sinefrina 30 e 300 mg/kg. Devido ao alto consumo destes produtos por mulheres em idade reprodutiva, os efeitos de *C. aurantium* e *p*-sinefrina foram avaliados, juntamente com *E. sinica* e efedrina, no sistema reprodutor feminino através do ensaio uterotrófico em ratas imaturas. Foi detectada uma ação antiestrogênica para a efedrina na dose de 5 mg/kg, e houve diminuição do peso relativo das adrenais em todos os tratamentos. Assim, apesar da baixa toxicidade apresentada, suplementos contendo *C. aurantium*/*p*-sinefrina devem ser usados com cautela, pois estão geralmente associados a estimulantes, que podem potencializar seus efeitos.

Palavras-chave: *Citrus aurantium*, *p*-sinefrina, avaliação toxicológica

ABSTRACT

The uncontrolled use of dietary supplements to lose weight and reach a higher muscular definition is concerning. Among these products, are the ones containing ephedrine (from *Ephedra sinica*). Due to the association with cardiac problems, ischemia and hypertension, some countries banned the commercialization of supplements containing this alkaloid, which has been substituted by *p*-synephrine (from *Citrus aurantium*). The aim of this study was to investigate the toxicological profile of *C. aurantium* extracts and the racemic standard of *p*-synephrine. An analytical method using HPLC/UV was validated and optimized for quantification of *p*-synephrine in the extracts. To toxicological profile evaluation extracts (300, 500, 1000, 2500, 3500 or 5000 mg/kg) and *p*-synephrine (150, 300, 450, 600, 800, 1000 or 2000 mg/kg) where submitted to acute toxicology test in mice, being observed signs such as: piloerection, gasping, salivation, exophtalmy and decrease in locomotor activity in all treatments, however deaths not occurred. Decrease in locomotion was confirmed in spontaneous locomotor activity test. In subchronic toxicity, mice were treated for 28 days with *C. aurantium* 400, 2000 or 4000 mg/kg and *p*-synephrine 30 or 300 mg/kg, it were analyzed hematological and biochemical parameters, and oxidative stress biomarkers. It was noted an increase in GSH levels in *C. aurantium* 4000 mg/kg and *p*-sinefrina 30 and 300 mg/kg and inhibition of the GPx activity in animals treated with *C. aurantium* 400 and 2000 mg/kg and *p*-sinefrina 30 and 300 mg/kg. Due to the high consume of these products by young women, the effects of *C. aurantium* and *p*-synephrine, so as *E. sinica* and ephedrine, were evaluated in female reproductive system by means of uterotrophic assay in immature female rats. An antiestrogenic activity was detected in ephedrine 5 mg/kg treated-rats, and there were a decrease in adrenals relative weight in all treated groups. In spite of low toxicity presented, dietary supplements containing *C. aurantium*/*p*-synephrine should be used with concern, since they are frequently associated to stimulants, which can potentializes his action.

Keywords: *Citrus aurantium*, *p*-synephrine, toxicologic evaluation.

1. INTRODUÇÃO

Atualmente, a obesidade é um dos mais graves problemas de saúde pública. Sua prevalência vem crescendo nas últimas décadas, onde se estima um aumento de 33% ao ano (KALMAN *et al*., 2000). O Brasil possui 38,8 milhões de indivíduos acima do peso, o que corresponde a 40,6% da população adulta do país (IBGE, 2005). Esta realidade, associada ao culto a magreza, gera uma busca por fórmulas para emagrecer. Apesar desse grande impacto social, o tratamento da obesidade continua produzindo resultados insatisfatórios, em grande parte por estratégias equivocadas e pelo mau uso dos recursos terapêuticos disponíveis (FLASO, 1999; LANG e FROELICHER, 2006). Muitas vezes, no desejo de obter a forma física ideal, as pessoas acabam fazendo uso de terapias sem comprovação de segurança e eficácia e sem acompanhamento médico (De SMET, 2004). Dentre os tratamentos farmacológicos usualmente aplicados à obesidade, três grupos podem ser distinguidos: (a) aqueles que reduzem a ingestão de alimentos; (b) os que alteram o metabolismo; e (c) aqueles que aumentam a termogênese (BRAY e RYAN, 1997).

Nesse contexto, a fitoterapia é freqüentemente defendida como meio de controlar o apetite e/ou aumentar a termogênese. Esse fato aliado à idéia de que “o que é natural não faz mal”, e que substâncias naturais podem ajudar a queimar calorias sem causar alteração no organismo, acende a procura por produtos para emagrecer a base de plantas (MORO e BASILE, 2000). Produtos “emagrecedores”, muitas vezes utilizados sem qualquer respaldo científico, médico ou legal, estão disponíveis no mercado na forma de suplementos alimentares, fitofármacos, fitoterápicos, nutracêuticos ou alimentos funcionais.

Dentre os recursos naturais empregados no tratamento da obesidade, destacam-se os produtos à base de efedrina, de *Ephedra sinica*. Entretanto, em abril de 2004, o Food and Drug Administration (FDA) proibiu a venda de suplementos alimentares contendo os alcalóides da efedra nos Estados Unidos (BENT *et al*., 2004), devido à associação clínica do seu uso com problemas cardíacos, psiquiátricos, derrames cerebrais e hipertensão (HALLER e BENOWITZ, 2000; SAMENUK *et al*., 2002; FUGH-BERMAN e MYERS, 2004; BOUCHARD *et al*., 2005). Estima-se que na época da proibição, 2 milhões de adultos nos Estados Unidos utilizavam diariamente produtos contendo efedra (BENT *et al*., 2004).

Para suprir essa demanda, as formulações de suplementos alimentares foram alteradas e comercializadas como “ephedra-free”, ou seja, produtos cuja fórmula não contém mais efedra, a qual muitas vezes é substituída por *Citrus aurantium* L. (laranja-amarga) ou seu principal componente ativo, a *p*-sinefrina (BENT *et al*., 2004). Em estudo recente realizado nos Estados Unidos, aproximadamente ¾ dos consumidores de suplementos alimentares admitiu usar produtos contendo laranja-amarga/*p*-sinefrina e outros estimulantes como cafeína, guaraná, noz-de-cola, chá-verde, teofilina e teobromina (BLANCK *et al.*, 2007).

No Brasil, as formulações a base de efedra ou efedrina não foram proibidas, mas fazem parte da lista de substâncias precursoras de entorpecentes e/ou psicotrópicas e sua comercialização é sujeita à controle especial (BRASIL, 1998). Entretanto, seguindo a tendência mundial, tem-se observado um grande número de formulações no mercado brasileiro, inclusive suplementos alimentares importados, contendo *C. aurantium* ou *p*-sinefrina. Atualmente, tem sido publicado em revistas o uso de *C. aurantium* como auxiliar no emagrecimento, referindo-se a esse como um “produto para agitar o metabolismo” e que, ao contrário da efedra, não causa qualquer efeito no sistema cardiovascular, mesmo afirmando que ele aumenta a liberação de adrenalina. Entretanto, a literatura científica traz relatos recentes de efeitos tóxicos causados em pacientes que faziam uso de suplementos contendo extrato de *C. aurantium* padronizado com altas quantidades de *p*-sinefrina (BENT *et al*., 2004; BOUCHARD *et al*., 2005; FIRENZOULI *et al*., 2005).

Considerando o uso indiscriminado de produtos a base de *C. aurantium* e/ou *p*-sinefrina, o fato de que os dados presentes na literatura sobre a toxicidade são conflitantes e que não existe dados na literatura sobre a DL50 de *p*-sinefrina, o objetivo deste estudo é investigar o perfil toxicológico de extratos padronizados de *C. aurantium*/sinefrina, já que estes são encontrados a venda no mercado brasileiro em farmácias de manipulação, drogarias e supermercados (LINCK *et al.*, 2006; BRAUM, 2006).

2. OBJETIVOS

2.1. Objetivo geral

Validar metodologia por CLAE/UV para quantificação de *p*-sinefrina em *C. aurantium* e avaliar o perfil toxicológico de extratos de *C. aurantium* e de padrão racêmico de *p*-sinefrina.

2.2. Objetivos específicos

- Estabelecer e validar metodologia para determinação do teor de *p*-sinefrina em frutos e folhas de *C. aurantium*, de ocorrência em diversas localidades no Rio Grande do Sul, por cromatografia líquida de alta eficiência (CLAE/UV).

- Preparar extratos de frutos imaturos de *C. aurantium* para realização de ensaios de toxicidade.

- Avaliar a toxicidade aguda em camundongos tratados com extrato de *C. aurantium* e *p*-sinefrina (padrão racêmico), segundo metodologia proposta pela ANVISA para avaliação toxicológica de fitoterápicos.

- Determinar o efeito do extrato de *C. aurantium* e da *p*-sinefrina (padrão racêmico) sobre a atividade locomotora de camundongos, através da avaliação da atividade locomotora espontânea.

- Determinar a DL50 do extrato de *C. aurantium* e da *p*-sinefrina (padrão racêmico) em camundongos.

- Avaliar a toxicidade subcrônica de extratos comerciais de *C. aurantium* e do padrão racêmico de *p*-sinefrina.

- Avaliar, no sangue, o efeito dos extratos comerciais e da *p*-sinefrina (padrão racêmico) sobre os marcadores do estresse oxidativo malonildialdeído (MDA), glutationa reduzida (GSH), e enzimas antioxidantes superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx), após tratamento subcrônico.

- Verificar o efeito estrogênico/antiestrogênico de extratos comerciais de *C. aurantium* e do padrão racêmico de *p*-sinefrina sobre o sistema reprodutor feminino, através do ensaio uterotrófico em ratas fêmeas imaturas.

3. REVISÃO DA LITERATURA

3.1. *Citrus aurantium* e sinefrina

O gênero *Citrus* (Rutaceae) compreende árvores frutíferas de origem Oriental. Existem inúmeras espécies, variedades e híbridos, além de alguns gêneros afins, como *Fortunella*. Os cítricos são muito utilizados principalmente devido ao seu teor de óleo volátil, além de serem fontes de flavonóides, pectinas e cumarinas (KUSTER E ROCHA, 2003; ZUANAZZI e MONTANHA, 2003).

Citrus aurantium é conhecida popularmente como laranjeira-amarga, laranjeira-azeda, laranjeira-cavalo e laranjeira de Sevilha. Seus frutos, flores e folhas têm sido usados na medicina popular para o tratamento de ansiedade, insônia e como anticonvulsivante (CARVALHO-FREITAS e COSTA, 2002; PULTRINI *et al*., 2005). Na região do Mediterrâneo, *C. aurantium* é usada desde os tempos medievais como estimulante cardíaco e vascular, digestivo, estomáquico, sedativo, tranqüilizante, colagogo, estimulante do apetite e tônico geral, além de antídoto contra venenos (ARIAS e RAMÓN-LACA, 2005). Na medicina tradicional chinesa, a laranja-amarga, que é conhecida como “zhi shi”, é usada como estimulante da função gastrintestinal e tônico geral (BOUCHARD *et al*., 2005).

Atualmente, o interesse pelos frutos verdes de *C. aurantium* tem crescido devido ao seu uso em produtos emagrecedores de origem vegetal. O fruto seco imaturo de *C. aurantium* contém aproximadamente 10% de flavonóides e inúmeras feniletilaminas, que incluem metiltiramina, octopamina e, sobretudo, *p*-sinefrina (0,2%) (HAAZ *et al*., 2006). Sinefrina é uma amina quiral presente na natureza apenas na forma (*R*)-(-)-*p*-sinefrina (ou *l*-sinefrina) (ARAI *et al*., 1997). Devido ao seu interesse farmacológico, sinefrina também é comercializada como fármaco sintético, desenvolvido como agente simpatomimético (é um agonista α-adrenérgico com algumas propriedades β-adrenérgicas) sob o nome de oxedrina (HAAZ *et al*., 2006), com atividades vasoconstritora e relaxante da musculatura bronquial (KUSU *et al*., 1996; MATTOLI *et al*., 2005). Também tem sido usada como descongestionante nasal e na forma de colírio (REYNOLDS, 1993). Algumas vezes é usada em doses maiores, de maneira análoga ao uso de altas doses de efedrina para crises asmáticas, via intravenosa (LIMING, 1993). A literatura científica também traz relatos da atividade antidepressiva da *p*-sinefrina em modelos animais (SONG *et al*., 1996; KIM *et al*., 2001).

Quimicamente, *p*-sinefrina é muito similar a outras aminas simpatomiméticas como efedrina e fenilefrina. Apenas duas características químicas distinguem a efedrina da sinefrina: um dos carbonos do anel é hidroxilado (OH substitui H) e um dos grupos metila (CH_3) da cadeia lateral é substituído por hidrogênio (H). Fenilefrina é o isômero meta da sinefrina (Figura 3.1).

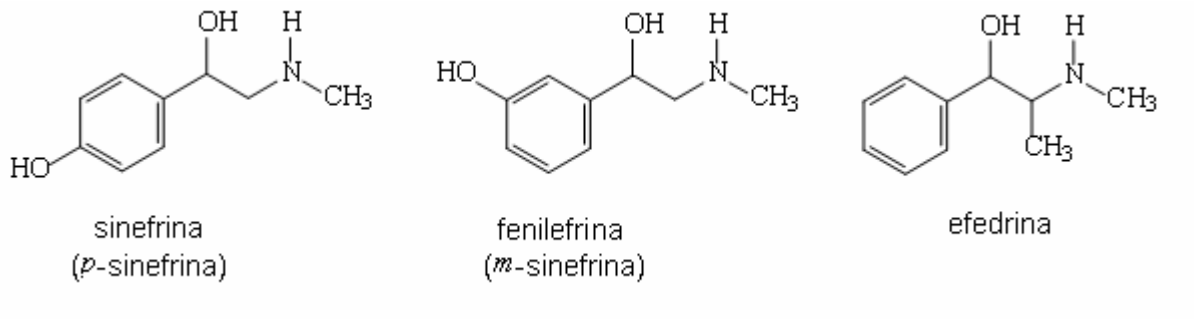

Figura 3.1: Estutura química da sinefrina, fenilefrina e efedrina.

Na natureza, a *p*-sinefrina ocorre em todos os produtos derivados de cítricos (*Citrus* sp., Rutaceae), como frutas e pericarpo (CHEN *et al*., 2002), flores (HUANG, 2001), folhas (WHEATON e STEWART, 1970), inclusive em sucos (CANCALON, 1999; PENZAK *et al*., 2001), sendo consumida em pequenas quantidades se derivados cítricos estiverem incluídos na dieta (KUSU et al., 1996).

As doses de *p*-sinefrina utilizadas na Medicina Tradicional Chinesa são similares, em miligramas, às doses de efedrina e correspondem de 20 a 60 g de *C. aurantium*, o que representa, teoricamente, de 60 a 180 mg de *p*-sinefrina (CHANG e BUT, 1986). Segundo dados da literatura, a quantidade de *p*-sinefrina presente no fruto imaturo de origem chinesa fica em torno de 0,25 % (CHANG e BUT, 1986), o que é equivalente ao conteúdo relatado para frutos secos utilizados na medicina japonesa (HOSODA *et al*., 1991, OHTA *et al*., 1994). Porém, alguns materiais vegetais analisados na China têm apresentado variados teores de *p*-sinefrina, de 0,1 a 2,0 % (TANG e EISENBRAND, 1992). Em sucos de *C. aurantium* a concentração de *p*-sinefrina tem sido estimada entre 5 e 6 % (PENZAK *et al*., 2001).

3.1.1. Aspectos toxicológicos do uso de *C. aurantium* e/ou *p*-sinefrina

Recentemente, os riscos associados ao consumo de extratos de frutos imaturos de *C. aurantium* têm causado preocupação devido aos seus possíveis efeitos no aumento da pressão arterial, o que acarretaria em alterações no sistema cardiovascular (FOUGH-BERMAN e MYERS, 2004; MATTOLI *et al*., 2005).

Em ratos, foi observado uma redução no consumo de alimento e aumento da mortalidade nos grupos tratados com extrato de *C. aurantium*. Também foram observadas alterações no eletrocardiograma, como arritmias ventriculares com alargamento do complexo QRS (CALAPAI *et al*., 1999).

Estudos em humanos têm demonstrado que preparações comerciais contendo *C. aurantium* e/ou *p*-sinefrina provocam aumento da pressão arterial no grupo tratado quando comparado com o controle (placebo) (HALLER *et al*., 2005; BUI *et al*., 2006). Além disso, um caso de infarto do miocárdio, em um paciente sem história de doença cardíaca, foi associado ao uso de produtos contendo *p*-sinefrina (NYKAMP *et al*., 2004).

Entre 1998 e 2004, no Canadá, foi registrado um caso de suspeita do envolvimento do uso de um produto contendo *C. aurantium* com o desenvolvimento de efeitos adversos cardiovasculares, e mais 15 casos onde produtos contendo *C. aurantium* associado a outros estimulantes seriam suspeitos de estarem envolvidos em sintomas como taquicardia, fibrilação ventricular, entre outras alterações cardiovasculares (JORDAN *et al*., 2004).

BOUCHARD e colaboradores (2005) relataram o caso de um paciente de 38 anos que apresentou isquemia cerebral associada ao uso diário (após curto período) de um produto livre de efedra/efedrina contendo *p*-sinefrina. O paciente não tinha história prévia de doença cardiovascular nem outros fatores de risco.

Outro caso, reportado por FIRENZUOLI e colaboradores (2005), consiste em uma paciente de 52 anos que fazia uso de tiroxina e ao utilizar um produto contendo *C. aurantium* (500mg, 6% de sinefrina) apresentou episódio de taquicardia e arritmia cardíaca, confirmando os mesmos efeitos adversos esperados para efedrina.

GANGE e colaboradores (2006) descreveram o caso de um homem de 57 anos que apresentou um quadro de angina após 35 dias de consumo de um produto "ephedra-free", contendo *p*-sinefrina.

A literatura também alerta para os perigos do uso de produtos contendo *C. aurantium*/sinefrina por pacientes com distúrbios alimentares (anorexia nervosa), pois podem mascarar sinais de hipotensão e bradicardia (GRAY e WOOLF, 2005).

3.2. Estresse Oxidativo

O estresse oxidativo é caracterizado como uma condição na qual a produção de radicais livres excede a capacidade antioxidante dos sistemas presentes no organismo. Muitas pesquisas indicam que a geração de radicais livres, resultante do estresse oxidativo, ocupa um importante papel na patogênese da lesão isquêmica cerebral (KOSSI e ZAKHARY, 2002), aterosclerose (HALLIWEL, 1993) e doenças cardiovasculares (LEICHTWEIS e JI, 2001).

3.2.1. Anfetaminas e estresse oxidativo

Segundo divulgado pelo jornal Zero Hora em 18 de março de 2006, o Brasil é responsável por 83% do consumo mundial de anfetaminas. O termo anfetamina refere-se ao grupo de estimulantes que incluem, entre outros, anfetamina, metanfetamina, êxtase (nome popular do 3,4-metilenodioximetanfetamina – MDMA) e efedrina (Figura 3.3). Estes fármacos básicos de baixo peso molecular são aminas simpatomiméticas, caracterizadas por sua semelhança estrutural às catecolaminas, derivadas da feniletilamina e possuem atividade estimulante central e periférica (DIAS *et al*., 2001). Embora os efeitos tóxicos causados pelo uso abusivo destas substâncias sejam conhecidos, os fatores moleculares que contribuem para estes efeitos ainda não estão completamente esclarecidos (YAMAMOTO e ZHU, 1998; BROWN e YAMAMOTO, 2003; FREY *et al*., 2006).

Há alguns anos, estudos têm demonstrado que a administração de altas doses de anfetamina e análogos aumenta a produção de radicais livres e o pré-

tratamento com antioxidantes atenua o déficit dopaminérgico induzido por anfetamina (YAMAMOTO e ZHU, 1998; SHANKARAN *et al*., 2001; BROWN e YAMAMOTO, 2003). Assim, evidências indicam que espécies reativas de oxigênio (EROs) contribuem direta ou indiretamente no mecanismo de toxicidade das anfetaminas (KOVACIC e COOKSY, 2005).

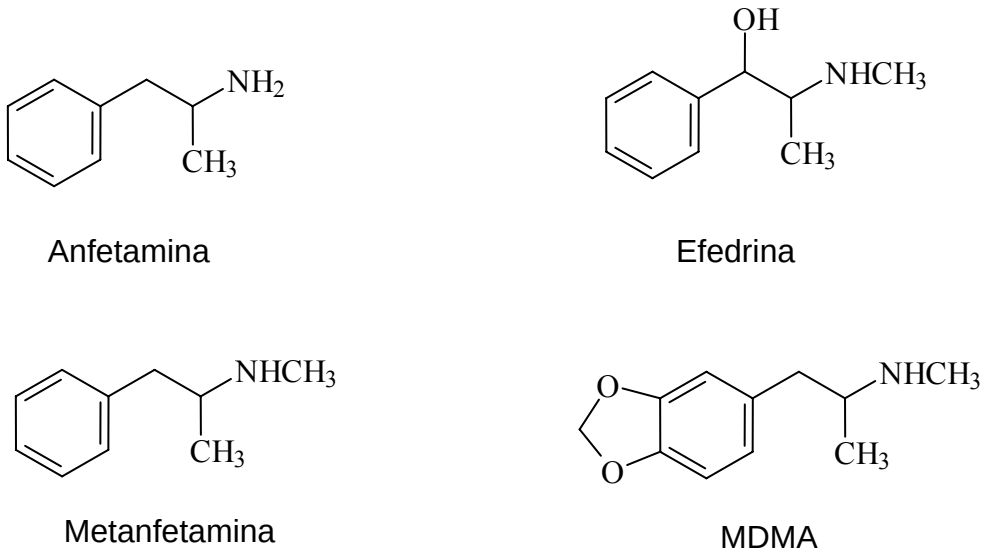

Figura 3.2: Estrutura química de algumas anfetaminas.

Sob condições fisiológicas normais, a mitocôndria é a fonte intracelular primária de EROs. A produção de EROs pela mitocôndria é originada a partir da cadeia de transporte de elétrons, localizada na parte interna da membrana mitocondrial, e da atividade da MAO, presente no exterior da membrana. Desse modo, anfetaminas podem aumentar a formação de EROs promovendo estas funções mitocondriais basais. Outro mecanismo possível seria através da estimulação dopaminérgica produzida pelas anfetaminas, pois a autoxidação da dopamina via reação de Fenton, utilizando ferro como co-fator, é outra fonte de EROs. Além disso, o aumento intracelular de dopamina poderia levar a formação de dopamina quinona reativa, que pode também formar EROs (BROWN e YAMAMOTO, 2003; FREY *et al*., 2006).

Estudos em ratos demonstraram que a exposição repetida a *d*-anfetamina aumentou a produção de ânions superóxido e substâncias reativas ao ácido tiobarbitúrico (TBA-RS) em partículas submitocondriais do córtex pré-frontal (FREY *et al*., 2006). A tiramina, através do metabolismo da MAO, também aumentou a produção de EROs em estriado de camundongos (SCHMIDT e FERGER, 2004). Em humanos, foi observado um aumento da lipoperoxidação e uma diminuição das atividades da CAT e SOD em eritrócitos de usuários de MDMA (ZHOU *et al*., 2003).

4. MANUSCRITO I:

“Concentrations of *p*-synephrine in fruits and leaves of *Citrus* species (Rutaceae) and the acute toxicity testing of *Citrus aurantium* extract and *p*-synephrine.”

Aceito para publicação na revista *Food and Chemical Toxicology* (doi:10.1016/j.fct.2008.04.037)

O presente trabalho descreve a validação de um método analítico por CLAE/UV para a quantificação de *p*-sinefrina em folhas e frutos verdes imaturos de *C. aurantium*. Este método foi aplicado a folhas e frutos de outras espécies do gênero *Citrus* de ocorrência no sul do Brasil, como *C. sinensis* Osbeck, *C. deliciosa* Ten, *C. limon* Burm e *C. limonia* Osbeck, a fim de comparar o teor de *p*-sinefrina nas espécies cítricas mais consumidas pela população. A toxicidade aguda do extrato preparado a partir de frutos imaturos de *C. aurantium* e do padrão racêmico de *p*-sinefrina também foi avaliada.

O método proposto se mostrou simples, seletivo, exato e robusto. As espécies *C. sinensis* e *C. deliciosa* apresentaram os maiores teores de *p*-sinefrina em frutos e folhas. O extrato de *C. aurantium* e a *p*-sinefrina apresentaram toxicidade transitória, pois os efeitos observados após a administração oral, como piloereção, ofego, exoftalmia, sialorréia e diminuição da atividade locomotora, foram reversíveis após 3 a 4 horas após o tratamento.

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Concentrations of *p*-synephrine in fruits and leaves of *Citrus* species (Rutaceae) and the acute toxicity testing of *Citrus aurantium* extract and *p*-synephrine.

Running title: Synephrine in *Citrus* and acute toxicity.

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Abstract

Dietary supplements containing bitter orange unripe fruit extract/*p*-synephrine are consumed worldwide for lose weight. This study were conducted to determine the concentration of *p*-synephrine in unripe fruits and leaves from *Citrus aurantium* Lin, *C. sinensis* Osbeck, *C. deliciosa* Ten, *C. limon* Burm and *C. limonia* Osbeck, collected in Southern Brazil, and to evaluate the acute toxicity of *C. aurantium* extract and *p*-synephrine. A high performance liquid chromatographic method with diode array detector (HPLC-DAD) was optimized and validated for determination of *p*-synephrine. The results indicate that all of analyzed samples present *p*-synephrine in amounts that range from 0.012 to 0.197% in the unripe fruits and 0.006 to 0.087 % in the leaves. Acute oral administration of *C. aurantium* extracts (2.5% *p*-synephrine, 300-5000 mg/kg) in mice produced reduction of locomotor activity, *p*-synephrine (150-2000 mg/kg) produced piloerection, gasping, salivation, exophtalmia and reduction in locomotor activity, which was confirmed in spontaneous locomotor activity test. All the effects were reversible and persisted for 3-4 hours. The toxic effects observed seem to be related with adrenergic stimulation and should alert for possible side effects of *p*-synephrine and *C. aurantium*.

***Keywords*:** synephrine, citrus, Rutaceae, acute toxicity.

1. Introduction

p-Synephrine (Fig. 1) is an amine also referred as oxedrine and worldwide used in the treatment of hypotensive states and as an ocular descongestant (Reynolds, 1993). It is the most abundant active component of extracts from *Citrus aurantium* L. unripe fruits (bitter orange or zhi shi in Traditional Chinese Medicine, Rutaceae). Structurally is related to endogenous neurotransmitters (epinephrine and norepinephrine) and has gained significant popularity for the treatment of obesity (Fugh-Bergman & Myers, 2004) 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 (Bouchard et al., 2005; Avula et al., 2005). The new dietary supplements produced have been marketed as "ephedra free" weight-loss and usually contain *C. aurantium* extracts standardized from 3% to 6% of *p*-synephrine (De Smet, 2004). Among past year supplement users, almost 75% used a product containing bitter orange/*p*-synephrine and other stimulants such as caffeine, guarana, kola nut, green tea, theophylline and theobromine (Blanck et al., 2007).

<Insert figure 1 here>

Chemically similar to ephedrine (from *Ephedra sp.*- Ephedraceae), *p*-synephrine also presents sympathomimetic activity which has been associated to oxidation of fats through an increase in thermogenesis and stimulated lipolysis, presumably by means of β_3-adrenoceptors. While *p*-synephrine anti-obesity effectiveness is widely debated, it has been demonstrated that this amine acts not only in β_3-adrenoceptors, but also in β_1, β_2 and α-adrenoceptors, presenting ephedra-like side effects (Fugh-Bergman & Myers, 2004). There are numerous reports of cardiovascular problems (e.g. hypertension, tachyarrhythmia, etc) associated with the use of synephrine-containing products in animals (Calapai et al., 1999) and humans

(Bouchard et al., 2005; Jordan et al., 2004; Nykamp et al., 2004; Firenzuoli et al., 2005; Haller & Benowitz, 2000; Bui et al., 2006; Gange et al., 2006).

Unlike ephedrine, *p*-synephrine can not be considered as alkaloid, once it occurs naturally in the human body in small quantities, being called elusive or trace amine (D'Andrea et al., 2003; Haaz et al., 2006). These amines occur in trace amounts in the central nervous system (Baud et al., 1985; Haaz et al., 2006) and are considered to be "false transmitters", which displace active biogenic amines from their stores, and are also believed to act on transporters in an amphetamine-like manner, thus increasing the extracellular concentrations of dopamine (Baud et al., 1985; Geracitano et al., 2004). They can affect uptake or release of catecholamines, or serotonin activity, at nerve endings, and might also act as neuromodulators through direct actions on receptors for monoamines (Branchek & Blackburn, 2003). Trace amines have received considerable attention because many of their behavioral effects resemble those of amphetamine (Lindemann & Hoener, 2005). The physiological role of these amines in the brain is still not resolved, but their levels are altered in human disorders such as schizophrenia, depression, attention deficit/hyperactive disorder, parkinsonism, Rett syndrome, migraine, phenylketonuria, hepatic encephalopathy and hypertension (Shimazu & Miklya, 2004).

Citric products are consumed as edible plants, which are part of the normal human diet, and as herbal remedy in folk medicine, furthermore the relevance of its pharmacological and toxicological properties. This research was undertaken to determine the concentrations of *p*-synephrine in leaves and unripe fruits, the most common source of this amine, of *C. aurantium* Lin, *C. sinensis* Osbeck, *C. deliciosa* Ten, *C. limon* Burm and *C. limonia* Osbeck, collected in Southern Brazil, as well as evaluate its acute toxicity, spontaneous locomotor activity and body temperature effect in mice. Although *p*-synephrine has been described in citrus fruits collected in China, Japan and Italy, the presence of this amine has not been

previously reported in Brazilian species neither the quantification in leaves worldwide or its acute toxicity in animals.

2. Materials and methods

2.1. Chemicals and reagents

p-Synephrine (CAS 94-07-5, purity 99%) was purchased from Aldrich (Aldrich, St. Louis, USA). Acetonitrile HPLC grade was obtained from Merck (Germany). Water was purified using a Milli-Q system (Millipore, Bedford, USA). Methanol (Merck) and trifluoroacetic acid (TFA; Vetec) were from analytical grade.

2.2. Animals

Male albino CF1 mice weighing 42.9 ± 3.1g obtained from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS) were used. They were housed in 47x34x18 cm polyethylene cages (8 animals per cage) under standard conditions of temperature (22 ± 2 ºC), controlled humidity and 12h-light/dark cycle. All animals were fasted overnight but provided free access to water and experimental procedures were performed during the light phase of the cycle. The experiments were performed after approval of the protocol by the University Ethics Committee (number 2006641) and were carried out in accordance with current guidelines for the care of laboratory animals (Olfert et al., 1998).

Considering the ethical aspects and the reduction in the number of animals in biological studies, we chose not to use female mice in these tests, since behavioral alterations, which have strong influence of the estral cycle, were evaluated.

2.3. Plant materials

Leaves and unripe fruits of *Citrus* species were collected in different places of Southern Brazil from December 2005 to January 2006 and identified by Professor Otto Carlos Koller from Faculty of Agronomy (UFRGS). The plant material was tritured and 4 g were weighed and submitted to maceration with 4 mL of methanol. After 20 minutes, the samples were centrifuged at 3000 rpm for 15 minutes and the extract was filtered through Whattman no. 2 filter paper under vacuum. The remanescent material was re-extracted following the same parameters. The volume was adjusted to 10.0 mL with water and the extracts were stored at 4 °C until chromatographic analysis. The different species were processed separately.

For biological tests, unripe fruits of *C. aurantium* were triturated and submitted to maceration with 50% methanol. After 24h the extract was filtered, concentrated in rotatory evaporator and analyzed by HPLC.

2.4. Standard stocks

Standard stocks for analytical chemistry were prepared by solving 10 mg of *p*-synephrine in 10 mL of methanol to give a concentration of 1 mg/mL. This solution was diluted to give a final concentration of 100 μg/mL. Both were stored at 4 °C prior to further dilutions to obtain working solutions of appropriate concentrations.

2.5. Instrumentation and chromatographic conditions

Analytical experiments were performed on an Alliance 2695 HPLC system equipped with a 996 photodiode array detector (Waters, Milford, USA) and data were analized by Empower Software (Milford, MA, USA). A Nova-Pak® C-18 column (150 x 3.9 mm i.d., 5 μm, Waters, Milford, USA) and a C-18 guard column (10 x 4 mm i.d, 10 μm, Waters, Milford, USA) were used for all separations. 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 - 10 min with 59 - 0% of A, 10 - 12 min 0% of A, 12 - 13 min with 0–100% of A, 13 - 18 min with 100% A, at a flow-rate of 0.6 mL/min. The injection volume was 5 μL and the run time 18 min. *p*-Synephrine was determined by UV detection at 220 nm. Examples of the chromatogram and UV spectra obtained are presented in figure 2.

2.6. Calibration

The content of *p*-synephrine was determined using a calibration graphic established with dilutions of each standard, at concentrations ranging from 1.0 to 4.4 μg/mL. Each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentration of the amount injected. Peak identification was achieved by comparison of both the retention time and UV absorption spectrum with those obtained for standards.

2.7. Assay validation

2.7.1. Selectivity

Selectivity was checked by using synephrine-containing dietary supplements, *Citrus* extracts and available standard optimizing separation and detection. The purity of the peaks was checked by DAD (λ = 200–400 nm) through multivariate analysis.

2.7.2 Linearity, limits of detection and quantification, precision and accuracy

Linearity, or the ability to show that the results are directly proportional to the analyte concentrations in samples within a given range, was assessed by means of linear regression obtained by the calibration graphic in the range of 1.0 to 4.4 μg/mL. From the calibration curve, linear regression analyses were carried out to obtain the correlation coefficient (R^2) and limits of detection (LOD) and quantification (LOQ). The LOD and LOQ were determined by calculation of the signal-to-noise ratio. Signal-to-noise ratios of approximately 3:1 and 10:1 were used for estimating the detection limit and quantification limit, respectively, of the method.

The precision of a method is determined by the extent to which the test results of multiple injections of standards agree. It can be subdivided into repeatability or intra-run precision and intermediate precision or inter-run. Accuracy is the extent to which the results generated approached the real value. Repeatability was expressed as the relative standard deviation (%RSD) and it was obtained through the analysis of nine extracts at the concentration of 100%. Accuracy was determined by analyzing the percentage of recovery of *p*-synephrine in *Citrus* extracts. The samples were spiked with three different amounts of standard compound in triplicate and analyzed under the previously established optimal conditions.

2.7.3 Ruggedness

The ruggedness of the method was tested varying chromatography equipment and several chromatographic parameters, such as mobile phase pH, mobile phase composition, brand and lots of acetonitrile, flow rate, chromatographic columns and analysts.

2.8 Acute toxicity

The experiment were conducted distributing the rodents into groups of eight mice each, that were treated orally (by gavage) with water (control) or 300, 500, 1000, 2500, 3500 or 5000 mg/kg of *C. aurantium* unripe fruits extract (containg 2,5% *p*-synephrine) and 150, 300, 450, 600, 800, 1000 or 2000 mg/kg of *p*-synephrine dissolved both in distilled water and observed for one minute at 5, 15, 30 minutes and 1, 2, 3, 4, 5, 6 hours after dosing. All doses were administered at a constant volume of 10ml/kg. Mice were observed for the presence of respiratory, digestive and neurological alterations. The number of deaths and weight was noted in each 24 hours for 14 days. Animals that died were immediately necropsied and analyzed for macroscopic alterations in heart, liver, kidneys and adrenals. After the 14^{th} day, mice which survived were sacrificed and also necropsied.

In acute toxicologic studies, we used 8 male mice to minimize the biological variability, in 6-7 different dose levels, to guarantee the results observed with the tested compounds and make possible to calculate DL50 level. The C. aurantium doses were calculated based in the concentration of *p*-synephrine in the extract.

2.9 Spontaneous locomotor activity

The method for the spontaneous locomotor activity was adapted from Creese et al. (1976). Activity cages (45 x 25 x 20 cm, Albarsch Electronic Equipment), equipped with three parallel photocells, automatically record the number of crossings. Animals were

individually habituated to an activity cage for 10 minutes and received the following treatments (n = 8-10): water, *p*-synephrine 300 mg/kg and *C. aurantium* extract (3.0% *p*-synephrine) 5000 and 10000 mg/kg by oral gavage. The animals returned to the activity cages 30 minutes after treatments, and the crossings were recorded for 15 minutes.

Doses reported in these and next sections differ from acute toxicologic tests since these tests were done after the acute toxicity test, and the doses were chosen based on it. It was used a *C. aurantium* extract containing 3.0% *p*-synephrine, the doses of extract were calculated to be *C. aurantium* extract 5000 mg/kg corresponding to 150 mg/kg *p*-synephrine, and *C. aurantium* extract 10000 mg/kg corresponding to 300 mg/kg *p*-synephrine. We chose these doses of *p*-synephrine because they presented less toxicity in acute toxicity test. The doses of *C. aurantium* in acute toxicity test did not show so much effect so, we preferred calculate all the doses for the next tests based on *p*-synephrine acute toxicity profile furthermore, the *C. aurantium* extract is used by people based on its concentration of *p*-synephrine. Due to the small amount of the *C. aurantium* extract used in the acute toxicologic study, a new one was prepared to the next tests (spontaneous locomotor activity and body temperature), and occasionally it had a higher concentration of *p*-synephrine.

2.10 Body temperature

Groups of six mice (n=6) were treated by oral gavage with water, *p*-synephrine 300 mg/kg and *C. aurantium* extract (3.0% *p*-synephrine) 5000 and 10000 mg/kg. Body temperature was measured by inserting the sensor probe of a digital thermometer into the rectum (1cm), was recorded before drug treatment (time 0) and 15 and 30 minutes after drug administration (Dallmeier & Carlini, 1981).

2.11 Statistical analysis

All the results were expressed as mean ± S.E.M (standard error of mean). Statistical analysis for body weight gain was evaluated by analysis of variance (ANOVA) of repeated measures and relative organs weight were done by one-way ANOVA, Bonferroni´s post-hoc test for multiple comparison was applied. The data obtained in spontaneous locomotor activity and body temperature were analyzed by one-way ANOVA followed by Student-Newman-Keuls (SNK) post hoc analysis. The significant level was set at $p < 0.05$.

3. Results

3.1. p-Synephrine content in Citrus species

Hydroalcoholic extracts of unripe fruits and leaves of *C. aurantium* (4 different sites of collection), *C. sinensis* (6 different sites of collection), *C. deliciosa* (4 different sites of collection), *C. limon* (2 different sites of collection) and *C. limonia* (3 different sites of collection) were submitted to qualitative and quantitative analysis of *p*-synephrine through a validated HPLC method. The results expressed in Table 1, showed that *p*-synephrine was observed in all analyzed samples, in levels from 0.012 to 0.197% in the fruits and from 0.006 to 0.087% in the leaves.

<Insert table 1 here>

The content of *p*-synephrine in different parts of *C. aurantium* fruits (Table 2) and in fruits of *C. sinensis* with different sizes (Table 3) were also evaluated and varied according to the different parts and the maturation period.

<Insert table 2 here>

For acute toxicity test, an extract of unripe fruits of *C. aurantium* was submitted to quantitative analyses of *p*-synephrine, the concentration of this amine was 2.5%. For spontaneous locomotor activity and body temperature tests another *C. aurantium* extract, whose concentration of p-synephrine was 3.0%, was used. Concentrations of *p*-synephrine were evaluated before and after the tests in order to confirm the maintenance of the amine in the extracts.

3.2. Assay validation

The HPLC-method was validated for parameters such as selectivity, linearity, precision, LOD, LOQ, accuracy and robustness. The selectivity was evaluated and no impurities or co-elutions were observed in the peak of interest, as can be seen in figure 2. Nine different concentration standard solutions of *p*-synephrine, ranging from 1.0 to 4.4 μg/mL, were prepared for the construction of the calibration graphic. The value obtained for the correlation coefficient (R^2) was found to be 0.9999, indicating a good linear relationship between the corresponding peak areas and the concentrations obtained for the analyte.

<Insert figure 2 here>

The precision of the method, determined by the repeatability and expressed as the relative standard deviation (%RSD), was obtained through the analysis of nine extracts at the concentration of 100%. The %RSD was 0.62%, indicating a low variability between the values obtained for each sample. The LOD and LOQ were found to be 1.042 μg/mL and 3.474 μg/mL, respectively. The accuracy values ranged from 98 to 107%. The ruggedness was assessed through modifications of the mobile phase (from 0 to 0.05% of TFA in the solvent B), brand of acetronile (from Merck, Vetec and Fischer), flow rate (from 0.5 to 1.0 mL/min), column package and size (Nova-Pak® C-18, 150 x 3.9 mm, 5 μm, Waters;

Brownlee Analytical C-18, 100 x 4.6 mm, 3 μm, Perkin Elmer and Eurospher 100 C-18, 250 x 4.6 mm, 5 μm, Knauer) and HPLC equipment (Waters Alliance 2695 HPLC-DAD, Perkin Elmer Series 200 UV/Vis and Knauer WellChrom UV/Vis). These changes had no effect on *p*-synephrine peak resolution.

3.3. Acute toxicity

In *C. aurantium* treated mice were observed reduction in locomotor activity, which, was evident 15 minutes after administration of 1000 – 5000 mg/kg and persisted for 2 hours. In *p*-synephrine treated groups, the signs observed were reduction in locomotor activity, piloerection, gasping, salivation and exophthalmia. Piloerection and exophthalmia were evident within 15 minutes after administration of 300 - 2000 mg/kg *p*-synephrine and persisted for 2 hours. Reduction of locomotor activity was evident 15 minutes after administration of 300 – 2000 mg/kg p-synephrine and the effect persisted for until 1 hour. Salivation was evident within 15 minutes in all tested doses and persisted for 30 minutes, while gasping was evident at the same time in all tested doses and persisted for 3 - 4 hours (Table 3). The control group did not exhibited any abnormality. Deaths were not observed in any of the tested doses and body weight gain was similar among the groups during the 14 days. In the necropsy, there were no alterations in the removed organs.

<Insert table 3 here>

3.4. Spontaneous locomotor activity

As can be seen in Figure 4, *p*-synephrine 300 mg/kg and *C. aurantium* extract 5000 and 10000 mg/kg significantly decreased spontaneous locomotion ($p < 0.01$; ANOVA/SNK).

<Insert figure 4 here>

3.5 Body temperature

Neither the treatment with *p*-synephrine 300 mg/kg, nor *C. aurantium* extract 5000 and 10000 mg/kg, significantly altered the body temperature in the measured times (data not showed).

4. Discussion

The concentrations of *p*-synephrine in citrus fruits matches with previously published data from fruits collected in China, Japan and Italy, which presented from 0.001 to 0.3% of this amine (Avula et al., 2005; Takei et al., 1999; Pellati et al., 2002; Pellati et al., 2004; Mattoli et al., 2005; Pellati et al., 2005). In fruits and leaves of *C. deliciosa* was observed a higher concentration than the reported for other *Citrus* species. Although the presence of *p*-synephrine in leaves has already been described (Stewart et al., 1964), data about its quantification have not been published until now.

p-Synephrine is distributed in the whole *C. aurantium* fruits; however it is found in higher concentration in the peel than in the pulp or albedo. The study of *C. sinensis* revealed a variation on the content of *p*-synephrine according to the maturation period, indicating that its content is inversely proportional to the size of the fruit. These results are in agreement with previous studies conducted by Hosoda et al. (1991), which report that the concentration of *p*-synephrine in *C. aurantium* decreases with regard to an increase in diameter of the fruits. This profile can be attributed to the higher level of *p*-synephrine in the peel than in pulp of the fruits.

The HPLC method proposed can be regarded as selective, accurate, precise and robust, being considered appropriate for determination of *p*-synephrine in citrus extracts. Its application to juices and synephrine-containing dietary products is also possible.

Acute administration of *C. aurantium* extract (2.5% *p*-synephrine) and *p*-synephrine produced reduction in locomotor activity, which was confirmed in spontaneous locomotor activity test. This effect may be produced by *p*-synephrine adrenergic stimulation, due to the administration of β_2-adrenoceptor agonists and high doses of α_1-adrenoceptor agonists produce the same effect (Consoli et al., 2007; Stone et al., 2007). The presence of gasping in treated animals is an additional indication of *p*-synephrine unspecific action in the adrenergic system, since the administration of hydroalcoholic extract of *Sida cordifolia* leaves, containing ephedrine alkaloids, produced gasping (Almeida et al., 1999; Franco et al., 2005). Another sign induced by *p*-synephrine was exophthalmia, which may be related to an increase in intraocular pressure. Furthermore, the presence of piloerection and salivation, are indicative of effects mediated by α_1-adrenoceptor agonists (Cordioli, 2005). Corroborating to the hypothesis that *p*-synephrine can act not only in β_3-adrenoceptors, the toxicological profile seems to be due to stimulation of other adrenergic receptors.

The extracts used in weight loss products, standardized to contain from 6 to 90% *p*-synephrine (Avula et al., 2005), differs markedly from the obtained from raw citrus plant materials evaluated in this work. This resembles those used in folk medicine and in Traditional Chinese Medicine (typically containing up to 4% *p*-synephrine), which are generally regarded as non-toxic. Moreover, the use of *p*-synephrine is usually associated with other stimulants such as amphetamines, caffeine and/or salicin, which potentiate its action and could cause the cardiovascular problems previously reported (Bouchard et al., 2005; Jordan et al., 2004; Nykamp et al., 2004; Firenzuoli et al., 2005; Haller & Benowitz, 2000; Bui et al., 2006; Gange et al., 2006). Considering that this was an acute toxicity test (only one

administration) and the long-term effects were not evaluated, more attention should be given to anti-obesity products and dietary supplements. In this way, more toxicological studies, such as subchronic and chronic toxicity tests, are recommended to guarantee the safety of these products.

This study indicates that Southern Brazilian *C. aurantium*, *C. sinensis*, *C. deliciosa*, *C. limon* and *C. limonia* present a concentration of *p*-synephrine in fruits and leaves similar to the related for the Eurasian fruits. The lack of content of this amine during the fruits maturation period and the higher concentration in the peel than in the pulp or albedo were also observed, as well as the elevated concentration in the leaves of *C. deliciosa* and *C. limonia*. The HPLC method proposed allows the determination of *p*-synephrine in different citrus extracts and is suggested to be applied to juices and dietary supplements. The main characteristics of the assay are the rapid and simple extraction and sample preparation procedures, free from derivatization steps, the relative quick analysis and the ruggedness. The toxicity effects observed in this study seem to be related with adrenergic stimulation and should alert for possible side effects, indeed more evaluations are necessary for a better comprehension of *p*-synephrine and *C. aurantium* toxicology.

Acknowledgments

The authors would like to thank CNPq for financial support (Processo 478054/2006-8), Prof. Otto Carlos Koller for plant material identification and Ana Paula Herrmann for manuscript revision.

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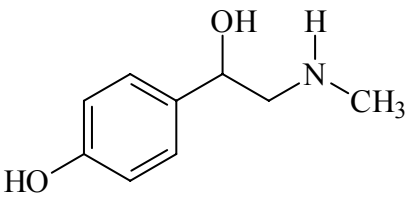

Figure 1: Chemical structure of *p*-synephrine.

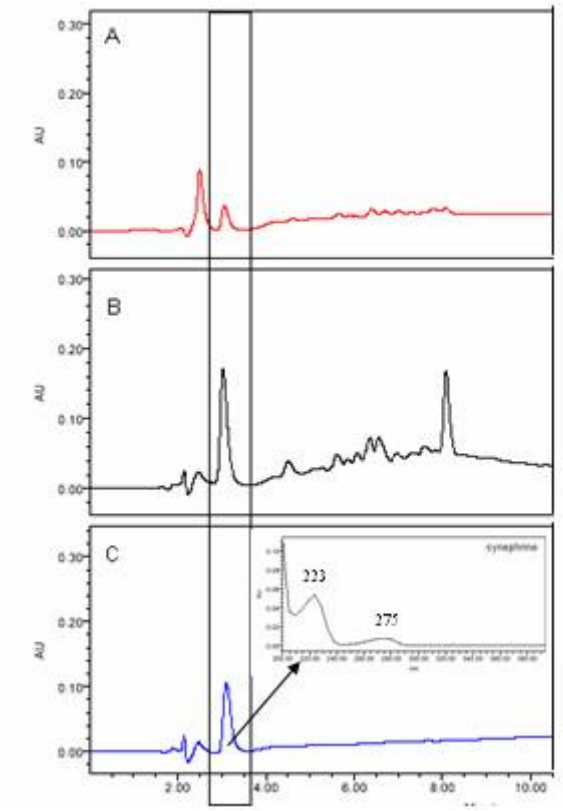

Figure 2: HPLC chromatogram and UV spectra of (A) leaves and (B) fruits extracts of *C. deliciosa* and (C) *p*-synephrine standard.

Table 1: *p*-Synephrine concentrations in fruits and leaves of *Citrus* species collected in Rio Grande do Sul, Brazil.

Species	Local name	Site of collection	*p*-synephrine (%)	
			fruits	leaves
Citrus aurantium Lin	*Laranja-azeda*	Porto Alegre (RS)	0.044	0.006
	Laranja-azeda	Porto Alegre (RS)	0.046	0.006
	Laranja-azeda	Porto Alegre (RS)	0.048	0.006
	Laranja-azeda	Minas do Leão (RS)	0.041	0.007
Citrus sinensis Osbeck	*Laranja-do-céu*	Minas do Leão (RS)	0.069	0.016
	Laranja-de-umbigo	Minas do Leão (RS)	0.062	0.021
	Laranja-de-umbigo	Porto Alegre (RS)	0.091	0.032
	Laranja-de-suco	Minas do Leão (RS)	0.068	0.022
	Laranja-de-suco	Porto Alegre (RS)	0.086	0.025
	Laranja-comum	Minas do Leão (RS)	0.099	0.023
Citrus deliciosa Ten	*Mexerica*	Porto Alegre (RS)	0.197	0.028
	Mexerica	Porto Alegre (RS)	0.103	0.028
	Mexerica	Campos Novos (SC)	0.093	0.071
	Mexerica	Minas Leão (RS)	0.077	0.087
Citrus limon Burm	*Limão*	Porto Alegre (RS)	0.045	0.010
	Limão	Porto Alegre (RS)	0.037	0.010
Citrus limonia Osbeck	*Limão-bergamota*	Campos Novos (SC)	0.012	0.016
	Limão-bergamota	Campos Novos (SC)	0.031	0.016
	Limão-bergamota	Arroio dos Ratos (RS)	0.051	0.025

Table 2: Variation in *p*-synephrine concentration in different parts of *C. aurantium* fruit and in different period of maturation in *C. sinensis* fruit.

Specie		*p*-Synephrine (%)
	Part of fruit	
C. aurantium	peel	0.056
	albedo	0.028
	pulp	0.019
	Diameter of fruit	
C. sinensis	d=2.0 cm	0.148
	d=3.5 cm	0.050
	d=4.5 cm	0.043
	d=5.2 cm	0.032

d=diameter of fruits
n=2 samples of each specie

Table 3: Acute toxicity effects of *Citrus aurantium* extract (containing 2.5% *p*-synephrine) and *p*-synephrine after oral admistration to CF1 mice (n=8 animals/group).

Treatment	Dose (mg/kg)	Toxic signs
C. aurantium	0	None
	100	None
	300	None
	500	None
	1000	Reduction in locomotor activity
	2500	Reduction in locomotor activity
	3500	Reduction in locomotor activity
	5000	Reduction in locomotor activity
p-Synephrine	0	None
	150	Gasping, salivation
	300	Reduction in locomotor activity, piloerection, gasping, salivation, exophthalmia
	450	Reduction in locomotor activity, piloerection, gasping, salivation, exophthalmia
	600	Reduction in locomotor activity, piloerection, gasping, salivation, exophthalmia
	800	Reduction in locomotor activity, piloerection, gasping, salivation, exophthalmia
	1000	Reduction in locomotor activity, piloerection, gasping, salivation, exophthalmia
	2000	Reduction in locomotor activity, piloerection, gasping, salivation, exophthalmia

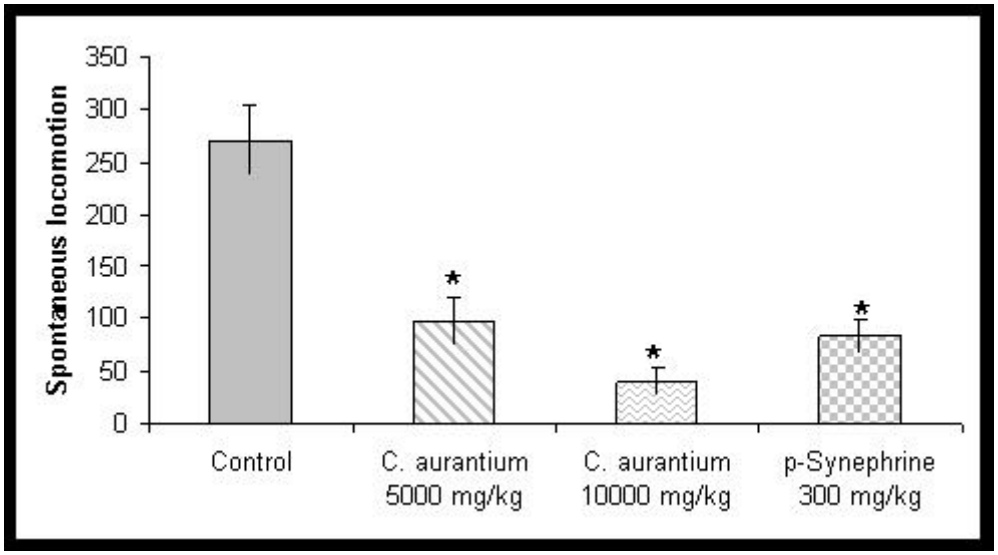

Figure 4: Effect of *Citrus aurantium* extract (containing 3.0% *p*-synephrine) 5000 and 10000 mg/kg, *p*-synephrine 300 mg/kg or water on spontaneous locomotor activity. Each column represents the mean ± standard error of the mean (n = 8-10). * Significantly different from control group ($p < 0.01$) by ANOVA/SNK.

5. MANUSCRITO II:

“Subchronic toxicity of *Citrus aurantium* L. (Rutaceae) extract and *p*-synephrine in mice”.

A ser submetido à revista *Regulatory Toxicology and Pharmacology*.

Dando continuidade a avaliação toxicológica de *C. aurantium* e *p*-sinefrina e considerando que estes produtos são utilizados por um longo período de tempo, este trabalho avaliou a toxicidade sub-crônica (28 dias) de um extrato comercial de *C. aurantium* e do padrão racêmico de *p*-sinefrina.

Não foram encontradas alterações no peso nem nos parâmetros bioquímicos e hematológicos avaliados. Entretanto, foram observadas alterações nos marcadores de estresse oxidativo analisados, como GPX e GSH. Foi detectada uma inibição da enzima GPx plasmática e um conseqüente aumento dos níveis de GSH eritrocitária.

Subchronic toxicity of *Citrus aurantium* L. (Rutaceae) extract and *p*-synephrine in mice

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Abstract

Extracts of *Citrus aurantium* L. (Rutaceae) unripe fruits have gained popularity for the treatment of obesity. Due to the wide use of *C. aurantium*/*p*-synephrine containing products, this research was undertaken to evaluate its subchronic toxicity in mice and their actions in oxidative stress biomarkers. Groups of ten mice received for 28 consecutive days *C. aurantium* dried extract (containing 7.5% *p*-synephrine) 400, 2000 or 4000 mg/kg and *p*-synephrine 30 or 300 mg/kg by oral gavage. There was observed a reduction in body weight gain of animals treated with both doses of *p*-synephrine. Organs relative weight, biochemical and hematological parameters were not altered in all treated mice. There was found an increase in reduced glutathione (GSH) concentration in groups treated with *C. aurantium* 4000 mg/kg and *p*-synephrine 30 and 300 mg/kg. In glutathione peroxidase (GPx), there were an inhibition of the activity in *C. aurantium* 400 and 2000 mg/kg and *p*-synephrine 30 and 300 mg/kg treated animals, respectively. Molecularly, *p*-synephrine could act as an inhibitor of GPx reaction which would lead to an increase of GSH. However, further tests are required to better elucidate the effects of these compounds in the antioxidant system.

Key Words: *Citrus aurantium*, *p*-synephrine, subchronic toxicity, oxidative stress

INTRODUCTION

Extracts of *Citrus aurantium* L. (Rutaceae) unripe fruits (syn.: zhi shi, green orange, sour orange and bitter orange) have been used for centuries in traditional Chinese medicine 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 due to an association with serious adverse health effects (Fugh-Bergman & Myers, 2004). 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).

Chemically similar to ephedrine (from *Ephedra* sp. - Ephedraceae) and amphetamine, *p*-synephrine also presents 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 β_3-adrenoceptors. However, it has been demonstrated that this amine acts not only in β_3-adrenoceptors, but also in β_1, β_2 and α-adrenoceptors (Fugh-Bergman & Myers, 2004). Consequently, several cardiovascular problems (e.g. hypertension, tachyarrhythmia, etc) associated with the use of synephrine-containing products have been reported in animals (Calapai et al., 1999) and humans (Bouchard et al., 2005; Jordan et al., 2004; Nykamp et al., 2004; Firenzuoli et al., 2005; Haller & Benowitz, 2000; Bui et al., 2006; Gange et al., 2006). Previous studies of our group evaluated the acute toxicity of *C. aurantium* extract and *p*-synephrine, demonstrating an unspecific adrenergic stimulation however, these products are consumed for a long period being important a subchronic evaluation.

On the other hand, amphetamines and analogues increase the production of free radicals and the pre-treatment with antioxidants attenuates the dopaminergic deficit inducted by amphetamine (Yamamoto & Zhu, 1998; Shankaran et al., 2001; Brown & Yamamoto,

2003). So, evidences indicate that reactive oxygen species (ROS) direct or indirectly contribute to amphetamines mechanism of toxicity (Kovacic & Cooksy, 2005), and although the toxic effects caused by the abusive consumption of these substances have been known, the molecular factors that contribute to these effects are not completely understood (Frey at al., 2006).

Due to the wide use of *C. aurantium* and *p*-synephrine containing products and the relevance of its pharmacological and toxicological properties this research was undertaken to evaluate its subchronic toxicity in mice and their actions in oxidative stress biomarkers.

MATERIALS AND METHODS

Chemicals

p-Synephrine (purity 99%) was purchased from M.P. Biomedical (Solon, Ohio, USA), 5-5'-dithio-*bis*(2-nitrobenzoic) acid was obtained from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile, methanol and *n*-butanol were supplied by Tedia Company (Fairfield, USA). *Citrus aurantium* dried extract was purchased from Galena (Campinas, SP, Brazil). Water was purified using a Milli-Q system (Millipore, Bedford, USA). All the other chemicals used were of analytical grade.

Animals

Male albino CF1 mice weighting 42.57 ± 0.60 g obtained from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS) were used. They were housed in 47x34x18 cm polyethylene cages (8 animals per cage) under standard conditions of temperature (22 ± 2 ºC), controlled humidity and 12h-light/dark cycle. Standard pellet food and tap water were available *ad libitum*. The experiments were performed after approval of the protocol by the

University Ethics Committee (number 2006641) and were carried out in accordance with current guidelines for the care of laboratory animals.

Determination of p-synephrine content

The content of *p*-synephrine in commercial *C. aurantium* dried extracts was determined by HPLC/UV. The dried powder was weighed and 4.0 g were submitted to maceration with methanol, after 20 minutes, samples were centrifuged at 3000 rpm for 15 minutes and the supernatant was filtered through Whattman no 2 filter paper under vacuum. The remanescent material was re-extracted more 2 times following the same parameters. The solvent was evaporated and the extract was dissolved in 10 ml water, filtered through 0.22 μm 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. The data acquisition was realized through a EUROCHROM 2000 SOFTWARE®, 2.05 for Windows (Knauer, Berlin, Germany). The chromatographic separation was realized in a C18 Eurospher-100® (15.0 x 4 mm x 5 μm) column with a Eurospher-100® (5 x 4 mm x 5 μm) pre-column. The analyte was detected at 220 nm. The mobile phase consisted of acetonitrile - water – trifluoroacetic acid (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.

28-Day toxicity test

Adult male mice (n=9-10) were treated for 28 consecutive days with *C. aurantium* methanolic extract 400, 2000 and 4000 mg/kg and *p*-synephrine 30 and 300 mg/kg by oral gavage. Animals were observed twice daily for signs of toxicity, morbidity and mortality and body weight was measured daily. At the end of the study the mice were sacrificed and subjected to full necropsy. Blood were collected, using heparin as anticoagulant, from caudal vein for biochemical evaluation and selected organs (heart, liver, brain, spleen, kidneys and adrenals) were observed, colleted and weight for macroscopic evaluation. Biochemical and hematological parameters evaluated in plasma include alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, creatine kinase MB fraction (CK-MB), total proteins, hemoglobin and hematocrit, using commercial kits.

Oxidative stress biomarkers

Part of the blood collected with heparin as anticoagulant were centrifuged at 1500 g for 10 min at 4 °C, plasma was used to determine malondialdehyde (MDA) and the erythrocytes were used for gluthatione (GSH) measurement. The whole blood was stored at – 20 ºC until analysis for antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity. Plasma MDA and GSH in erythrocytes were all processed immediately.

Lipid peroxidation was evaluated through the MDA analysis by HPLC with VIS detection according to Grotto et al. (2007). This method quantifies MDA levels after alkaline hydrolysis and extraction with *n*-butanol at 532nm.

The levels of reduced GSH were measured as non-protein thiols based on the protocol develop by Ellman (1959) with modifications. Aliquots (0.3 ml) of erythrocytes were added

to a phosphate buffer 0.3 mol/l (0.85 ml), pH 7.4 and the reaction was read in spectrophotometer at 412 nm after addiction of 10 mM 5-5'-dithio-*bis*(2-nitrobenzoic) acid (DTNB) (0.05 ml). The results were expressed as μmol/ml erythrocytes.

CAT activity was determined using H_2O_2 as substrate, according to Aebi (1984). SOD activity was determined based on its ability to inhibit the autoxidation of adrenaline to adrenochrome at an alkaline pH, according to McCord & Fridovich (1969). GPx activity was measured using glutathione reductase and NADPH. The method is based on the oxidation of NADPH, which is indicated by a decrease in absorbance at 340 nm (Paglia & Valentine, 1967). All the spectrophotometrical analyses were realized in an UV-VIS Hitachi spectrophotometer model U-1800® (Tokio, Japan).

Statistical analysis

Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni´s (body and organs relative weight) or Dunnet´s (biochemical and oxidative stress biomarkers) post-hoc. Significance level was set at 5% ($p<0.05$).

RESULTS

The commercial extract of *C. aurantium* was submitted to qualitative and quantitative analyses through a previously validated HPLC/UV method. *p*-Synephrine content was 7.5 ± 0.06%.

In the 28-day toxicity test, any clinical signs of toxicity were observed. Body weight gain was lower in groups treated with both doses of *p*-synephrine (figure 1). There were no changes in organs relative weight of treated mice. It was observed a decrease in hematocrit of mice treated

with *C. aurantium* 400 mg/kg and a decrease in total proteins of *p*-synephrine 30 mg/kg treated group (Table 1). All the other analyzed parameters were normal.

There was found a significant increase in GSH concentration in groups treated with *C. aurantium* 4000 mg/kg and *p*-synephrine 30 and 300 mg/kg (Table 2). In GPx, there were a 72.6%, 60.9%, 59.7% and 64.1% inhibition of the activity in *C. aurantium* 400 and 2000 mg/kg and *p*-synephrine 30 and 300 mg/kg treated animals, respectively (Table 2). MDA decreased in *C. aurantium* 400 mg/kg treated group and CAT activity increased in group treated with *p*-synephrine 30 mg/kg. All the other evaluated oxidative stress biomarkers were normal.

DISCUSSION

The content of *p*-synephrine in commercial *C. aurantium* extract analyzed was according to the marketed by the manufacturer, which should contain at least 6.0% *p*-synephrine. Usually, commercial extracts are standardized in 3% to 6% *p*-synephrine, however some products could contain until 90% *p*-synephrine, and they are called special extracts (Avula et al., 2005).

Repeated-dose oral toxicity study of *C. aurantium* extract and *p*-synephrine in mice did not show any sign of toxicity, suggesting low subchronic toxicity. In body weight gain, *p*-synephrine demonstrated a reduction in body weight gain, which can be attributed to the β_3-adrenoceptor agonist activity confirming their use. In biochemical evaluations, there were observed slight changes in total plasmatic proteins and hematocrit, but they did not show a linear profile. According to Lewis et al. (2002), some factors can be useful in differentiating a significant change from control values, from a treatment-related effect. This difference is less likely to be an effect of treatment if: there is no obvious dose response; it is due to finding(s) in one or more animals that could be considered outlier(s) and/or it is within normal biological variation (within the range of reference value).

In the oxidative stress biomarkers evaluation, there was found an increase in GSH levels and inhibition in GPx activity. One of the most important roles of GSH antioxidant system is the detoxification of endogenously generated peroxides. Indeed, GPx catalyzes the reduction of hydrogen peroxide, phospholipids-hydroperoxide and other organic hydroxyperoxides by GSH, yielding oxidized glutathione (GSSG) which, in turn, is reduced back to GSH in a NADPH-dependent reaction catalyzed by glutathione reductase (GR) (Gul et al., 2000). Molecularly, *p*-synephrine, alone or contained in the extract, could be an inhibitor of GPx reaction which would lead to an increase of GSH. Production of GSH is considered to be the first line of defense against oxidative damage and free radical generation, where GSH functions as a scavenger and co-factor in metabolic detoxification. The increase of the GSH levels could be contributing to the absence of lipoperoxidation, since there are no alterations in MDA levels. This could be an adaptive mechanism to slight oxidative stress, however, a severe oxidative stress may suppress GSH levels due to the loss of adaptive mechanisms and the oxidation of GSH to GSSG (Chater et al., 2006). Alterations in MDA and CAT in *C. aurantium* 400 mg/kg and *p*-synephrine 30 mg/kg respectively, should not be considered, since they did not show a linear profile (Lewis et al., 2002).

CONCLUSIONS

In this experimental work we evaluate the subchronic toxicity of *C. aurantium* extract and *p*-synephrine, which have been used in weight loss formulations. The results confirmed its lypolitic activity and indicate a low toxicity of the tested compounds, however it was demonstrated that they can alter the oxidative metabolism. The effects of *C. aurantium*/*p*-synephrine in the antioxidant system should be better elucidated, long-term studies, such as 90-days toxicity, and the evaluation of other oxidative stress biomarkers are recommended. Moreover, the use of *C. aurantium*/*p*-synephrine is usually associated with other stimulants such

as amphetamines, caffeine and/or salicin, which potentiate its action and could be related to the cardiovascular problems previously reported, justifying the toxicologic evaluation of this association.

ACKNOWLEDGMENTS

We would like to thank Farmácia Calêndula for *C. auratium* commercial extract donation and the technical assistance of Msc. Viviane de Moura Linck and Ana Paula Herrmann. This work was supported by CNPQ (Processo 478054/2006-8).

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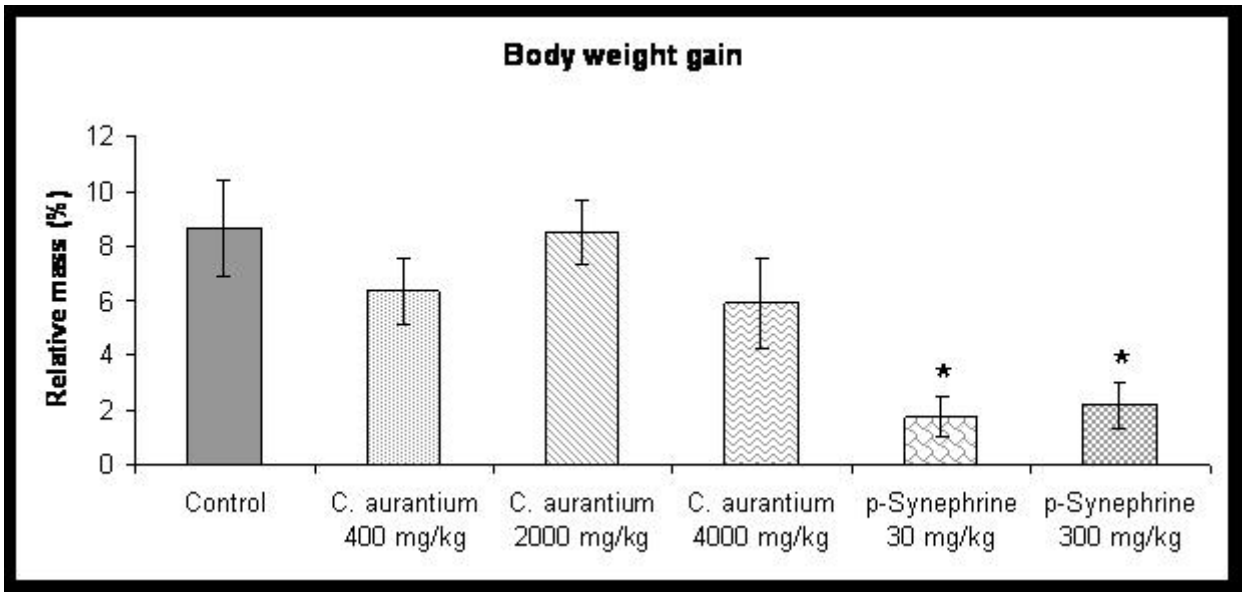

Figure 1. Relative body weight gain in mice after 28 days treatment with *C. aurantium* extract (containing 7.5% *p*-synephrine) 400, 2000 or 4000 mg/kg and *p*-synephrine 30 or 300 mg/kg. Each column represents the mean ± standard error of the mean (n = 8-10). * Significantly different from control group ($p < 0.05$) by ANOVA/Dunnet.

Table 1. Clinical parameters evaluated in CF1 mice after 28 days of treatment.

Clinical Parameters	Control	*C. aurantium* 400 mg/kg	*C. aurantium* 2000 mg/kg	*C. aurantium* 4000 mg/kg	*p*-Synephrine 30 mg/kg	*p*-Synephrine 300 mg/kg
Hematocrit (%)	44.20 ± 2.26	34.25 ± 1.33*	38.00 ± 4.34	44.25 ± 0.62	41.22 ± 1.63	41.63 ± 1.76
Hemoglobin (g/dl)	16.34 ± 1.40	13.80 ± 1.11	15.17 ± 1.94	14.81 ± 0.44	13.65 ± 0.68	14.14 ± 0.63
MCHC (%)	37.03 ± 2.83	40.09 ± 2.44	40.03 ± 2.46	33.55 ± 0.80	33.03 ± 0.68	34.05 ± 1.08
Total proteins (g/l)	61.2 ± 1.7	55.0 ±1.7	60.0 ± 0.1	57.0 ± 1.2	54.0 ± 1.4*	56.5 ± 1.0
AST (U/ml)	151.66 ± 24.99	197.94 ± 19.60	168.11 ± 21.82	81.26 ± 9.33	77.69 ± 9.20	134.55 ± 29.39
ALT (U/ml)	82.23 ± 18.34	130.30 ± 20.97	82.93 ± 24.34	39.56 ± 4.91	39.70 ± 5.48	47.55 ± 7.23
Creatinine (mg/dl)	0.42 ± 0.10	0.37 ± 0.09	0.75 ± 0.32	0.49 ± 0.06	0.41 ± 0.07	0.67 ± 0.11
CK-MB (U/ml)	67.05 ± 7.15	66.38 ± 7.10	54.07 ± 7.98	63.00 ± 6.11	72.12 ± 5.56	76.57 ± 3.22

Results expressed as mean ± standard error of the mean (SEM), n=5.

* Significantly different from control group ($p<0.05$) by ANOVA/Dunnet.

Table 2. Oxidative stress biomarkers evaluated in CF1 mice after 28 days of treatment.

Biomarkers	Control	*C. aurantium* 400 mg/kg	*C. aurantium* 2000 mg/kg	*C. aurantium* 4000 mg/kg	*p*-Synephrine 30 mg/kg	*p*-Synephrine 300 mg/kg
CAT (U/ml)	18.65 ± 1.36	7.41 ± 0.68	9.08 ± 0.37	10.07 ± 0.93	110.08 ± 36.50*	32.75 ± 15.41
SOD (U/ml)	1.17 ± 0.20	0.89 ± 0.05	1.15 ± 0.18	1.07 ± 0.12	1.27 ± 0.24	0.99 ± 0.10
GPx (nmol NAD/min/ml)	23.35 ± 1.45	16.95 ± 2.04*	14.22 ± 0.95*	19.13 ± 0.56	13.94 ± 0.79*	14.98 ± 1.49*
MDA (mM)	17.61 ± 1.48	9.58 ±1.33*	12.44 ± 2.33	12.64 ± 1.21	13.90 ± 1.58	15.75 ± 0.39
GSH (mM)	1.08 ± 0.09	1.52 ± 0.10	1.79 ± 0.32	2.57 ± 0.03*	2.44 ± 0.18*	2.30 ± 0.37*

Results expressed as mean ± standard error of the mean (SEM), n=4.

* Significantly different from control group ($p<0.05$) by ANOVA/Dunnet.

6. MANUSCRITO III:

“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”.

Aceito para publicação na revista *Archives of Toxicology* (DOI: 10.1007/s00204-008-0324-8).

Considerando que produtos emagrecedores são utilizados principalmente por mulheres jovens, em idade reprodutiva, e que compostos químicos podem interferir em rotas hormonais e alterar o ciclo menstrual, a fertilidade e o desenvolvimento embrionário, este trabalho teve o intuito de avaliar o efeito de *C. aurantium* e *p*-sinefrina e compará-los ao efeito de *E. sinica* e efedrina, sobre o sistema reprodutor feminino.

Foi possível detectar um efeito antiestrogênico para a efedrina a partir do ensaio uterotrófico em ratas fêmeas imaturas, porém o mesmo não foi observado com a *p*-sinefrina. Por outro lado, foi encontrada uma diminuição na massa relativa das adrenais nos grupos tratados com *C. aurantium*, *p*-sinefrina, *E. sinica* e efedrina.

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

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Abstract

Formulations containing *Ephedra sinica* Stapf. (Ephedraceae) and *Citrus aurantium* L. (Rutaceae) are consumed worldwide for body weight control. Considering the adverse effects related 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 for 3 consecutive days by oral gavage *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 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, being necessary other in vivo and in vitro assays to support this mechanism of action.

Keywords Ephedra – Citrus – Ephedrine – p-Synephrine – Uterotrophic assay – Immature rats

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, based in a short-time test, which represents a 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, Darmstadt, Germany), cyclehexanone (Merck, Darmstadt, Germany) 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 ºC), 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 ºC 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 24h 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 x 0.25 mm x 0.25 µm polydimethyldiphenylsiloxane). The column temperature was programmed for 80 ºC for 2 min, 50 ºC/min to 250 ºC for 2 min, and 100 ºC/min to 280 ºC for 1 min. The injector and liner temperatures were adjusted to 220 ºC and 260 ºC, 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 x 4 mm x 5 µm) column with a Eurospher-100® (5 x 4 mm x 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 3 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 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 (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 x 100).

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 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 (Figure 1A). Tamoxifen 20.0 mg/kg/day and ephedrine 5.0 mg/kg/day significantly reverted the estradiol effect. The uterus relative mass 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 (Figure 1B).

There was observed a significant reduction 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 observed no macroscopic alterations 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).

The extract obtained with a comercial sample of *E. sinica* was analyzed for the presence of ephedrines, the value found was according with literature reports (Schaneberg et al., 2003) and below the admitted by official institutions (JP XIII, 1996). The standardized extract of *C. aurantium* was according 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 2000). In the group treated with *p*-synephrine and estradiol cypionate there was observed an elevation 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, so 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).

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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 *E. sinica* 855.0 mg/kg	Ephedrina 5.0 mg/kg	*C. aurantium* 25.0 mg/kg *C. aurantium* 50.0 mg/kg	*p*-Synephrine 50 mg/kg
Antiestrogenicity	Tamoxifen 20.0 mg/kg + estradiol cypionate 0.4mg/kg	*E. sinica* 855.0 mg/kg + estradiol cypionate 0.4mg/kg	Ephedrine 5.0 mg/kg + estradiol cypionate 0.4mg/kg	*C. aurantium* 50.0 mg/kg + estradiol cypionate 0.4mg/kg	*p*-Synephrine 50.0 mg/kg + estradiol cypionate 0.4mg/kg

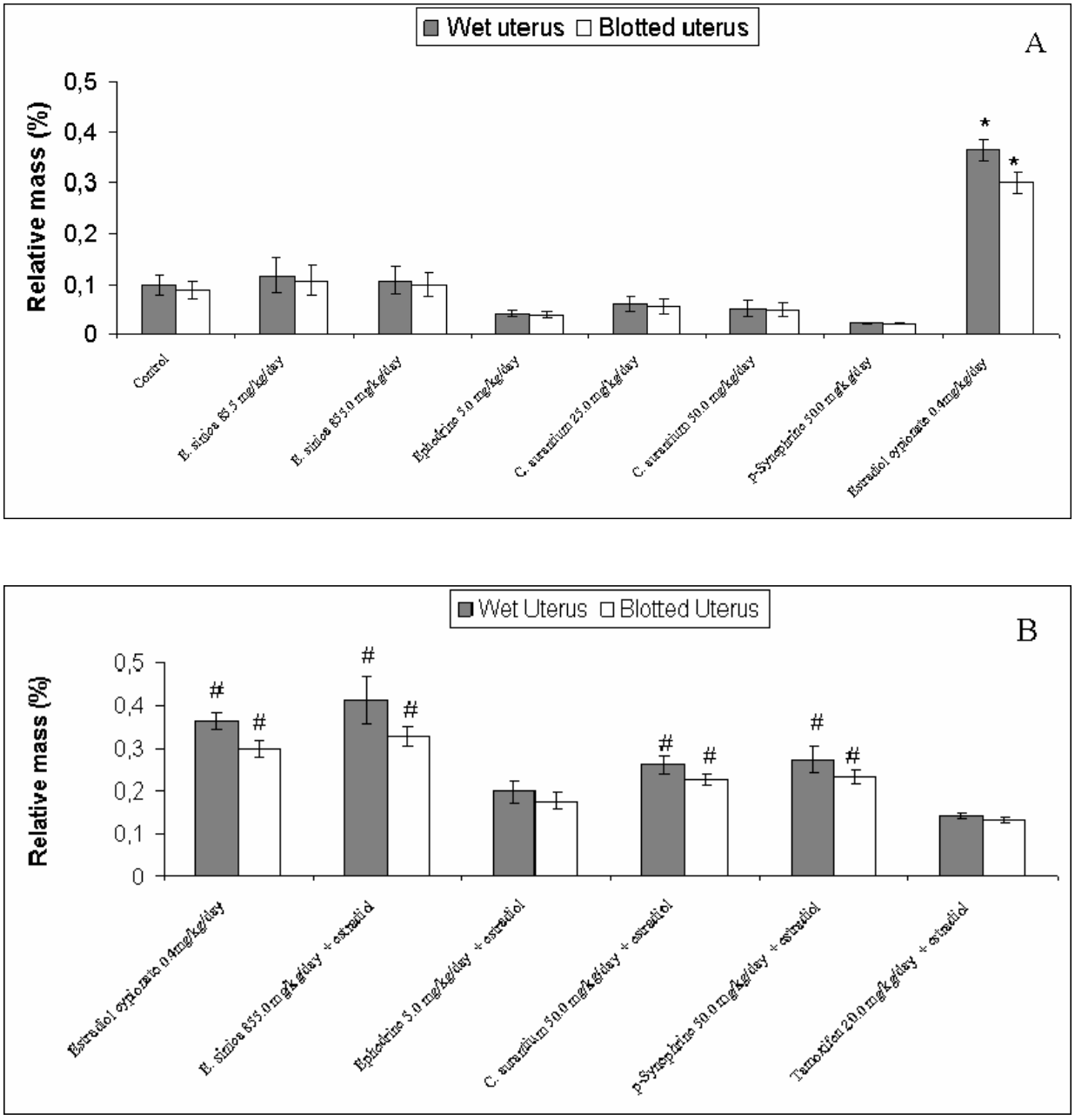

Figure 1. Estrogenic (A) and antiestrogenic (B) effect of *Ephedra sinica* and *Citrus aurantium* extracts, ephedrine and *p*-synephrine in the wet and blotted uterus relative mass. Values represent mean ± SE of the relative uterus weight (n=6-7). * Significantly different from control and # significantly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

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 0.4mg/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.

7. DISCUSSÃO GERAL

A presença de *p*-sinefrina foi detectada em folhas e frutos imaturos de *C. aurantium*, *C. sinensis*, *C. deliciosa*, *C. limon* e *C. limonia*, em quantidades similares a de frutos cultivados na China, Japão e Itália (TAKEI *et al.*, 1999; PELLATI *et al.*, 2002; PELLATI *et al.*, 2004; AVULA *et al.*, 2005; MATTOLI *et al.*, 2005; PELLATI *et al.*, 2005), indicando a ampla distribuição desta amina. Isto pode comprovar a aplicabilidade do método analítico proposto. Além disso, o método se mostrou simples, preciso e robusto.

Embora não tenha sido realizado um ensaio específico de ligação a receptores adrenérgicos, pode-se sugerir que a administração aguda do extrato hidroalcoólico de *C. aurantium* e *p*-sinefrina provocou efeitos relacionados à estimulação adrenérgica.

A diminuição da atividade locomotora, observada após o tratamento com *C. aurantium* 5000 e 10000 mg/kg e *p*-sinefrina 300 mg/kg, também é observada com o uso de agonistas adrenérgicos β_2 e altas doses de agonistas adrenérgicos α_1 (CONSOLI *et al.*, 2007; STONE *et al.*, 2007).

Outro sinal presente após a administração de *p*-sinefrina, o ofego, também foi observado com a administração de extrato de *Sida cordifolia* L. (Malvaceae), espécie vegetal caracterizada pela presença de efedrina (ALMEIDA *et al.*, 1999; FRANCO *et al.*, 2005). Além disso, piloereção e sialorréia também podem ser indicativos de efeitos mediados por receptores α_1 (CORDIOLI, 2005), corroborando com a hipótese de que *p*-sinefrina não atuaria especificamente sobre receptores adrenérgicos β_3.

Devido a não-ocorrência de mortes nas doses testadas, não foi possível calcular a $DL_{50.}$ Alterações anátomo-patológicas macroscópicas também não foram observadas.

O tratamento subcrônico, com duração de 28 dias, não provocou alterações clínicas nos animais, também não foram observadas mortes, nem alterações anátomo-patológicas.

Os tratamentos com *p*-sinefrina promoveram uma diminuição significativa do ganho de peso, confirmando a ação lipolítica da *p*-sinefrina devido à estimulação dos receptores β_3-adrenérgicos.

Nas avaliações bioquímicas e hematológicas foram observadas pequenas diminuições no hematócrito e na concentração de proteínas plasmáticas totais nos grupos tratados com *C. aurantium* 400 mg/kg e *p*-sinefrina 30 mg/kg, respectivamente. Entretanto, estas alterações não demonstraram um perfil linear e não devem ser relacionadas ao tratamento (LEWIS *et al.*, 2002).

Ao serem quantificados alguns biomarcadores do estresse oxidativo, foi detectado um aumento dos níveis de GSH nos grupos tratados com *C. aurantium* 4000 mg/kg e *p*-sinefrina 30 e 300 mg/kg, e uma inibição da atividade da GPx nos animais que receberam *C. aurantium* 400 e 2000 mg/kg e *p*-sinefrina 30 e 300 mg/kg.

O sistema antioxidante da GSH tem um papel fundamental na detoxificação de peróxidos endógenos. Nesse sentido, a GPx cataliza a redução de peróxido de hidrogênio e hidroperóxidos orgânicos através da oxidação da GSH, formando glutationa oxidada (GSSG) que, por sua vez, é reduzida novamente a GSH em uma reação NADPH-dependente catalizada pela glutationa redutase (GR) (GUL *et al.*, 2000). Dessa forma, os resultados obtidos sugerem que a *p*-sinefrina atuaria como um inibidor da atividade da GPx, levando ao aumento de GSH.

A síntese de GSH é considerada a primeira linha de defesa contra a produção de radicais livres. Assim, o aumento dos níveis de GSH poderia estar contribuindo para a ausência de lipoperoxidação, observada devido a não alteração dos níveis de MDA. Este pode ser um mecanismo adaptativo ao estresse oxidativo leve, que pode ser perdido em casos de estresse oxidativo severo (CHATER *et al.*, 2006). Além disso, *p*-sinefrina é um inibidor da NADPH-oxidase, enzima que catalisa a formação de EROs, o que levaria a uma diminuição da formação destes compoestos (KO *et al.*, 2007).

As outras enzimas antioxidantes avaliadas, CAT e SOD, também não apresentaram alterações. Os valores obtidos de MDA e CAT nos animais tratados com *C. aurantium* 400 mg/kg e *p*-sinefrina 30 mg/kg, não podem ser considerados efeitos de tratamento devido ao perfil não-linear (LEWIS *et al.*, 2002).

No ensaio uterotrófico, efedrina demonstrou um efeito antiestrogênico, devido à diminuição da massa relativa do útero. Entretanto, o sistema endócrino é

altamente complexo, sendo difícil propor um mecanismo de ação a partir de um único teste de *screening*, pois ensaios de curta duração, como o uterotrófico, são incapazes de detectar efeitos no eixo hipotálamo-hipófise-adrenal (ANDRADE *et al.*, 2002), justificando o desenvolvimento de estudos posteriores, sobretudo com a quantificação de hormônios específicos.

Também foi observada a diminuição da massa relativa das adrenais nos grupos tratados com *C. aurantium*, *Ephedra sinica*, *p*-sinefrina e efedrina, que pode estar relacionada com a atividade agonista α_1, que leva a vasoconstrição e diminuição de líquido no órgão (AHFS, 2005), mais uma vez corroborando com a hipótese de ação adrenérgica inespecífica.

Apesar dos resultados obtidos neste trabalho indicarem uma baixa toxicidade, sem a ocorrência de mortes ou danos hepáticos, cardíacos ou renais, estudos mais específicos devem ser conduzidos. Enquanto resultados mais conclusivos não estiverem disponíveis na literatura científica, produtos contendo *C. aurantium e*/ou *p*-sinefrina, sobretudo em associações, devem ser consumidos com cautela. Especial atenção deve ser dada a formulações emagrecedoras e suplementos alimentares associados a potencializadores como anfetaminas, cafeína e/ou salicina, que podem aumentar exponencialmente o seu potencial toxicológico.

8. CONCLUSÕES E PERSPECTIVAS

Neste trabalho realizamos a avaliação toxicológica do extrato de *C. aurantium* e seu suposto componente termogênico, a *p*-sinefrina, que estão sendo consumidos mundialmente para a perda de peso. Adicionalmente, validamos um método analítico por CLAE/UV para a quantificação desta amina em extratos vegetais. O método desenvolvido se mostrou simples, robusto, preciso e eficaz na quantificação de *p*-sinefrina em diferentes matrizes.

A avaliação toxicológica indica uma baixa toxicidade do extrato e da *p*-sinefrina, mas sugere uma ação inespecífica desta amina sobre o sistema adrenérgico, contrariando a suposta estimulação específica de receptores β_3-adrenérgicos, conforme justificativa empregada pela indústria nutracêutica para substituição da efedrina por sinefrina em suplementos alimentares.

Além disso, estes suplementos contêm componentes estimulantes como extratos de guaraná, noz-de-cola, chá-verde e erva-mate, que contêm cafeína, e extrato de *Salix* sp., fonte de salicina, associados ao extrato de *C. aurantium* ou a *p*-sinefrina. Com base nos resultados obtidos, podemos supor que os efeitos adversos cardiovasculares freqüentemente relatados, como angina, infarto agudo do miocárdio e isquemias, sejam decorrentes destas associações, justificando novos trabalhos a fim de avaliarem o uso simultâneo destes produtos.

Apesar de verificado um aumento da GSH eritrocitária, e uma inibição da GPx sangüínea, os efeitos de *C. aurantium* e *p*-sinefrina sobre o metabolismo oxidativo não estão totalmente esclarecidos, justificando novas avaliações, uma vez que produtos contendo esta amina vem sendo comercializados no mercado internacional como agente antioxidante.

p-Sinefrina e efedrina, bem como os extratos de *E. sinica* e *C. aurantium,* diminuíram a massa relativa das adrenais de ratas imaturas. Efedrina demonstrou ainda um efeito antiestrogênico. O mecanismo de ação deste efeito não está esclarecido, e estudos de longa duração e com a quantificação de hormônios, deve ajudar a entender a atividade destes compostos sobre o sistema endócrino.

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10. ANEXOS

Comitê de Ética