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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 9–10 mice received for 28 consecutive days a commercial *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 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 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, and was no alteration in malondialdehyde (MDA) levels. Thus, the results indicate a low subchronic toxicity of the tested materials in mice and a possible alteration in the oxidative metabolism. However, further tests are required to better elucidate the effects of these compounds in the antioxidant system.

1. 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. Recently, they have gained significant popularity for the treatment of obesity, as an alternative to ephedrine 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 and 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* spp., 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 lipolysis stimulation, 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 and 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 and 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 and a transitory toxicity (Arbo et al., 2008) 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

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attenuates the dopaminergic deficit inducted by amphetamine (Yamamoto and Zhu, 1998; Shankaran et al., 2001; Brown and Yamamoto, 2003). Evidences indicate that reactive oxygen species (ROS) direct or indirectly contribute to amphetamines mechanism of toxicity (Kovacic and 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 et al., 2006). As part of the endogenous antioxidant system, there are the enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). The SOD enzyme catalyzes the dismutation of the superoxide anion (O_2^-) in oxygen (O_2) and hydrogen peroxide (H_2O_2). The H_2O_2 is not a free radical, but in high concentrations it can reacts with superoxide or iron (Fe^{2+}) producing the highly reactive hydroxyl radical (OH^-). The conversion of H_2O_2 to water (H_2O) can be realized by CAT or GPx (Michiels et al., 1994). Indeed, GPx also catalyzes the reduction phospholipids-hydroperoxide and other organic hydroxyperoxides by glutathione (GSH), yielding oxidized glutathione (GSSG) which, in turn, is reduced back to GSH in a NADPH-dependent reaction catalyzed by glutathione reductase (GR). In this cycle, GSH acts as an immediate donor of electrons to neutralize the reactive species (Gul et al., 2000). Malondialdehyde (MDA) is one of the better-known secondary products of lipid peroxidation, and it can be used as an indicator of cell membrane injury (Esterbauer et al., 1991).

Due to the wide use of *C. aurantium*/*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.

2. Materials and methods

2.1. 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). *C. aurantium* dried extract was obtained 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.

2.2. 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 47 × 34 × 18 cm polyethylene cages (eight animals per cage) under standard conditions of temperature (22 ± 2 °C), controlled humidity and 12 h-light/dark cycle. Standard pellet food and tap water were available *ad libitum*. The experiments were performed after approval of the protocol by the UFRGS Ethics Committee (number 2006641) and were carried out in accordance with current guidelines for the care of laboratory animals.

2.3. 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 min, samples were centrifuged at 3000 rpm for 15 min and the supernatant was filtered through Whattman no. 2 filter paper under vacuum. The remanescent material was re-extracted more two 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 of EUROCHROM 2000 SOFTWARE®, 2.05 for Windows (Knauer, Berlin, Germany). The chromatographic separation was realized in a C18 Eurospher-100® (15.0 × 4 mm × 5 μm) column with a Eurospher-100® (5 × 4 mm × 5 μm) pre-column. The analyte was detected at 220 nm. The mobile phase consisted of acetonitrile–water–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.

2.4. 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, 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 and hematological evaluation and selected organs (heart, liver, brain, spleen, kidneys and adrenals) were collected, observed for macroscopic evaluation and weight. Biochemical and hematological parameters evaluated in plasma include alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, creatine kinase MB fraction (CK-MB), total proteins, and in whole blood, hemoglobin and hematocrit, all were realized using commercial kits.

2.5. Oxidative stress biomarkers

Part of the blood collected with anticoagulant were centrifuged at 1500*g* for 10 min at 4 °C, plasma was used to quantify malondialdehyde (MDA) and the erythrocytes were used for reduced glutathione (GSH) measurement, these procedures were realized immediately. The whole blood was stored at −20 °C until analysis for antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity.

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 and 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 and Valentine, 1967). All the spectrophotometrical analyses were realized in an UV–Vis Hitachi spectrophotometer model U-1800® (Tokyo, Japan).

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

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.