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Citrus aurantium (bitter orange) extract: Safety assessment by acute and 14-day oral toxicity studies in rats and the Ames Test for mutagenicity

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ABSTRACT

The primary active constituent in bitter orange extract (BOE) is *p*-synephrine. This study assessed the safety of a BOE standardized to 50% *p*-synephrine following short-term exposure to rats and by the Ames Test. Following 5000 mg/kg of the extract orally to female rats all animals survived. Administration at 2000 mg/kg to female rats for four days yielded no signs of toxicity. Five male and five female rats were administered the BOE at 0, 250, 500, 1000 and 2000 mg/kg/day for 14 days. No significant effects were observed at any dose with respect to body weights, food intake, absolute and relative organ weights, hematology, clinical chemistry, and pathology. Two male rats died after 2000 mg/kg with gastrointestinal impaction at necropsy. During week two of 1000 mg/kg and 2000 mg/kg/day, rats exhibited transient signs of repetitive burrowing of heads in the bedding material (hypoactivity) for about 15 and 45 min, respectively. The no-observed-effect-level (NOEL) was 500 mg/kg/day. The mutagenic potential was assessed at and up to the limit dose of 5000 μg/plate in a *Salmonella typhimurium* reverse mutation (Ames) test, performed in duplicate as a pre-incubation assay in the presence and absence of metabolic activation (S9). The BOE did not induce an increase in the frequency of revertant colonies at any dose in the five tester strains, and was therefore non-mutagenic.

1. Introduction

Standardized aqueous-alcoholic extracts of the immature fruits of *Citrus aurantium* (bitter orange) are widely consumed in dietary supplements for appetite control, weight management, sports performance and energy, and bitter orange products are extensively consumed in the form of foods as juices and marmalades (Stohs and Shara, 2013; Ratamess et al., 2015; Stohs, 2017). The primary active ingredient in bitter orange extract is the phenylethylamine protoalkaloid *p*-synephrine which comprises about 90% or more of the total protoalkaloids (Pellati and Benvenuti, 2007), and to which products as the patented Advantra Z® are standardized (Stohs and Preuss, 2011a; Stohs et al., 2011a, 2012; Stohs and Shara, 2013).

p-Synephrine has a hydroxyl group in the *para* position on the benzene ring of the molecule (Fig. 1). *p*-Synephrine exists in the *l*- or [R-(−)] enantiomeric form, whereas synthetic *p*-synephrine is a racemic mixture of the *l*- and *d*-enantiomeric forms and contains only about half of the pharmacological activity of the naturally occurring *p*-synephrine because the *d*-enantiomer exhibits about one-hundredth the adrenergic receptor binding activity of the *l*-enantiomer (Stohs and Preuss, 2011b; Stohs et al., 2011b).

Relatively few animal studies have assessed the safety of bitter orange extracts, particularly at high doses, primarily because bitter orange has been used extensively in Traditional Chinese Medicine (Chen and Chen, 2004; Fang et al., 2009; Stohs and Shara, 2013) and is used in food products as marmalades, flavoring agents and juices (Stohs and Preuss, 2011a; Stohs, 2017). Arbo et al. (2008) examined the acute toxicity of a bitter orange extract and *p*-synephrine in mice. The acute oral administration of 300–2000 mg/kg of *p*-synephrine produced reduced locomotion, piloerection, salivation, gasping, and exophthalmia. All effects were reversible and resolved in 3–4 h. The authors concluded that these effects were due to adrenergic agonist activity. *p*-Synephrine is a poor adrenergic agonist, as a consequence high doses are required to produce these effects which may result from direct and/or indirect adrenergic

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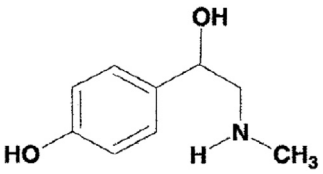

Fig. 1. Structure of *p*-Synephrine.

activity (Stohs et al., 2011b; Stohs and Badmaev, 2016).

In a study in mice, bitter orange extract (7.5% *p*-synephrine) at doses of 400, 2000 or 4000 mg/kg (corresponding to 30, 150 and 300 mg p-synephrine/kg) or *p*-synephrine at 30 mg or 300 mg/kg were administered orally per day for 28 days (Arbo et al., 2009). A reduction in body weight gain was observed at both doses of *p*-synephrine relative to controls. No adverse effects were observed regarding organ weights, biochemical parameters, blood pressure or heart rate in the treated mice at any of the doses.

In addition, both doses (30 and 300 mg/kg) of *p*-synephrine and the high dose (4000 mg/kg) of the bitter orange extract which contained 300 mg *p*-synephrine resulted in increases in the antioxidant and tissue protectant reduced glutathione (GSH), while the bitter orange extract decreased malondialdehyde content (an indicator of lipid peroxidation and lipid damage), and *p*-synephrine increased catalase which neutralizes hydrogen peroxide. The two doses of *p*-synephrine as well as 400 mg/kg and 2000 mg/kg of the bitter orange extract which contained 30 and 150 mg of *p*-synephrine, respectively, also significantly inhibited glutathione peroxidase activity (Arbo et al., 2009). The results indicated a beneficial effect with respect to weight loss without adverse effects while also providing an antioxidant and tissue protective effect.

Several animal studies have been conducted by the National Center for Toxicological Research in conjunction with the US FDA regarding the safety of bitter orange extract and *p*-synephrine (Hansen et al., 2011, 2012; 2013). In a study which examined the developmental toxicity of *Citrus aurantium* in rats, the authors concluded that doses of up to 100 mg p-synephrine/kg body weight did not induce developmental toxicity (Hansen et al., 2011). At this dose there were no adverse effects with respect to embryo lethality, fetal weight, or incidence of gross, skeletal or visceral abnormalities. No developmental effects were observed under the dosing conditions used in this study.

The physiological effects were examined after administering *p*-synephrine in the form of bitter orange extract as well as isolated *p*-synephrine to rats for 28 days at doses of up to 50 mg/kg with and without caffeine at 25 mg/kg (Hansen et al., 2012). Minimal, clinically insignificant effects were produced by these high doses of *p*-synephrine with respect to heart rate and blood pressure. As expected, caffeine alone and in combination with *p*-synephrine produced more pronounced but small increases in heart rate and blood pressure.

The potential cardiovascular effects of bitter orange extract and *p*-synephrine were also examined in exercised rats given up to 50 mg/kg *p*-synephrine in the presence and absence of 25 mg/kg caffeine for 28 days (Hansen et al., 2013). Small increases in heart rate and body temperature were reported due to caffeine, while *p*-synephrine exhibited small, clinically insignificant effects on blood pressure at 50 mg/kg.

The purpose of the current study was the assessment of the safety of a bitter orange extract standardized to 50% *p*-synephrine administered to rats via oral gavage as an acute dose and also when administered daily for 14 days at doses up to 2000 mg/kg (1000 mg/kg *p*-synephrine). The study also assessed the potential mutagenicity of the extract in a bacterial reverse mutation assay (Ames Test) using five tester strains of *Salmonella typhimurium*. In addition, the study aimed at identifying markers for any toxicity which could be assessed in future studies on this extract.

2. Materials and methods

2.1. Test material

Bitter orange extract (Advantra Z®) standardized to 50.1% *p*-synephrine was provided by Novel Ingredients, East Hanover, NJ 07936 USA. The dried, powdered immature fruits are soaked in water, and 0.5% HCl is added to convert *p*-synephrine to the soluble hydrochloride. The aqueous acidified fraction is collected by filtration and absorbed onto a cation exchange resin. The *p*-synephrine binds to the resin, and impurities are removed by washing the resin with purified water. The *p*-synephrine base is eluted using dilute 5% aqueous ammonia, and the resulting solution is concentrated by evaporation which results in a crude *p*-synephrine product. This product is further purified by crystallization using food grade ethanol.

The amount of *p*-synephrine (50.1%) present in the extract was confirmed by an independent testing laboratory (Intertek Laboratories, Champaign, IL) by HPLC with UV detection using a Hewlett Packard 1100 system with an Agilent 1200 gradient series, agreeing with the original HPLC analytical results of the manufacturer. Further chemical analysis indicated that the remainder of the extract was composed of approximately 45% carbohydrate and 5% ash (minerals) (Eurofins Scientific, Inc., Petaluma, CA USA). The same batch of test material was used throughout the study. The extract (test item) was suspended in analytical grade water which contained 0.50% w/v carboxymethyl cellulose as a suspending agent to prepare dosing formulations with the desired test concentrations. The visually homogenous suspension was prepared freshly daily, shortly prior to dose administration, and kept under constant stirring throughout dosing administration by gavage. This is a standard practice for non-clinical studies, and ensures homogeneity of distribution of suspended test materials. Control animals received the vehicle composed of 0.50% carboxymethyl cellulose in water.

2.2. Animals

Male and female Sprague Dawley rats (NTac:SD) were obtained from Taconic Biosciences, Inc., USA through its vendor Vivo Bio Tech Ltd., Telangana, India. Female rats were nulliparous and non-pregnant. The animals were subjected to veterinary examination prior to experimentation to ensure that the rats were in good health. Only female animals at 11 weeks of age were used for the acute toxicity study while the rats used for the 14-day study were between the ages of 6–7 weeks at the beginning of treatment. Animals were housed singly for the acute study and for the 14-day study they were housed in groups of two or three of similar sex per cage in sterilized, solid bottom, suspended, polypropylene cages with stainless steel grill tops and clean and sterilized corn-cob bedding.

The animal facility was supplied with 100% fresh, filtered air with 10–15 air changes per hour. The room was maintained at a temperature between 19 and 25 °C with the relative humidity between 30 and 70%, and a 12 h light/dark cycle. The animals were fed *ad libitum* Altromin brand extruded pelleted rat chow manufactured by M/s Altromin Spezialfutter GmbH & Co. KG, Germany. Drinking water which had been passed through an Aquaguard water filter and subjected to ultra violet irradiation was provided *ad libitum*. The animals were allowed to acclimate for at least five days prior to experimentation, assigned an individual tail number, weighed and randomized.