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Original Research Article

Effects of a tincture of *Citrus aurantium* on isolated vascular tissues from rats

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Abstract

Citrus aurantium (bitter orange) is used as medicinal herb in many disorders. The peel of *C. aurantium* fruit tincture-induced vascular effects were investigated in endothelium-denuded rat aortic rings precontracted with 60 mmol/L of KCl. This tincture at low concentrations did not produce significant vascular effects, but from 10 to 100 mg/mL, it has produced a vasorelaxing effect. The maximal relaxant effect of *C. aurantium* peel tincture was 87.52 ± 3.51% at a concentration of 100 mg/mL ($p < 0.05$; $n = 4$). The concentration of this tincture necessary to reduce KCl-induced contractions of the endothelium-denuded aorta by 50% was 18.36 ± 0.72 mg/mL.

Keywords: aortic rings; bitter orange; *Citrus aurantium L.*; herbal medicine; vasorelaxation.

1 Introduction

Citrus aurantium L., the bitter orange tree, is a citrus tree of the Rutaceae family. Many varieties of bitter orange are used for their essential oil for perfumes, flavorings, and as medicine. The fruit is also known by the names of sour orange, bigarade orange, Andalusian orange, Seville orange, cashier orange, puppy orange [1, 2].

Citrus aurantium fruits play a prominent role as they constitute a natural source of secondary metabolites such as essential oils, flavonoids and coumarins. The main metabolites with biological activity in the citrus genus are the flavonoids to which anti-inflammatory, antioxidant, protective of blood vessels, antiplatelet and antiatherogenic activity is attributed [3, 4, 5]. These biological properties indicate the citrus's flavonoids as possible options for the treatment of vascular diseases. Also *C. aurantium*´s fruits contain phenylethylamine alkaloids with p-synephrine being the most abundant [1]. The essential oils of *C. aurantium* are rich in monoterpenes with the major component being d-limonene (65.3–95.9%); also contain linalyl acetate, linalool and β-myrcene [1, 2, 5].

Citrus aurantium is used in folk medicine to treat anxiety, insomnia, as an anticonvulsant [6], as a protector of small blood vessels [7]. Also *C. aurantium* has been traditionally used for treatment of obesity, indigestion, chest congestion, bellyache, nausea, and vomiting [1].

Several studies have revealed that flowers, fruits, essential oils and phytoconstituents of this plant exerted biological effects including antimicrobial, antioxidant, cytotoxic, anxiolytic, antidiabetic, antiobesity, heart protection, anti-allergic and anti-inflammatory actions [1, 5]. A cardiovascular study indicated that inhalation of the essential oil of *Citrus aurantium* L. var. amara (neroli oil) helped relieve menopausal symptoms, increased sexual desire, and reduced blood pressure in postmenopausal women [8]. A case report showed that neroli causes bradycardia and hypotension in adolescent patients [9], and limonene, the major component of neroli essential oil, has been reported to decrease blood pressure and heart rate in rats fed a high-fat diet [10]. Neroli is an endothelium- and smooth-muscle-dependent vasodilator that can alleviate cardiovascular symptoms [11].

Despite the therapeutic potential for vascular diseases that citrus plants have in Cuba, no studies have been carried out to evaluate these pharmacological actions, which is an indispensable requirement to determine their possible usefulness for the treatment of vascular diseases.

The objective of this study was to evaluate the effects of a tincture of peel of *Citrus aurantium* (TCA) on vascular smooth muscle.

2 Materials and methods

2.1 Plant extract

The 20% tincture of dried peel of fruits of *Citrus aurantium* L. (TCA) here employed (Lot number 1901002) is a raw

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material for the production of over-the-counter phytotherapy commercialized in Cuba. This tincture was obtained from the dried peel of the plant and was made with 70% ethanol. Quality control certification was provided by The Provincial Pharmacy Enterprise of Havana, governed by the branch regulations [12].

2.2 Animals

Male adult (7-8 weeks) Wistar rats were obtained from the National Center for Laboratory Animal Reproduction (CENPALAB; La Habana). Prior to experiment, animals were adapted for seven days to laboratory conditions (controlled temperature 25 ± 2°C, relative humidity 60 ± 10% and 12 h light/dark cycles). Tap water and standard diet for rodents supplied by CENPALAB were freely provided. All procedures were also conducted according to the European Commission guide-lines for the use and care of laboratory animals and approved by the Ethic Committee from the Institute of Cardiology and Cardiovascular Surgery (No. 02-2018, folio 3, book 01, 2018). The minimum number of animals and duration of observation required to obtain consistent data were employed.

2.3 Aortic rings

Thoracic aortic rings were dissected from rats under pentobarbital anesthesia. Care was taken to mechanically remove the endothelium with the purpose of verifying the direct actions of the tincture on vascular smooth muscle. They were fixed to a force transducer and placed in bath chamber continuously perfused (10 mL/min) with Tyrode solution of the following composition (mmol/L): 140 NaCl, 2.5 KCl, $MgCl_2$, 2 $CaCl_2$, 10 trishydroxymethylaminomethane, 10 glucose (pH =7.4, gassed with O_2; T = 35°C) and stabilized, under a load of 0.8 g, for 30 min before the beginning of the experiment, according to the procedure of Galán et al. [13] with slight modifications. Contraction was induced by replacing NaCl by KCl (60 mmol/L). After an equilibration period of 30 min, the endothelium removal was confirmed by the administration of acetylcholine (10 µmol/L) to precontracted vascular rings. A set of experiments was conducted using cumulative addition of tincture (1, 3, 10, 30, and 100 mg/mL) at 10-min interval between successive additions. The control rings were similarly treated but the corresponding vehicle (70% ethanol) was added instead.

2.4 Statistical analysis

Results are expressed as means and standard errors of means. Student's t- test evaluated the statistical significance for paired samples, previously checked that the data complied with the premise of normality. Differences were considered statistically significant for $p < 0.05$. The graphics and the statistical processing were done using the software OriginPro 8 SRO v8.0724 (MA, USA).

3 Results

Fig. 1 shows a typical recording of the maximal contractile response induced by 60 mmol/L of KCl in the aorta without endothelium followed by exposure to cumulative concentrations of the tincture of peel of *Citrus aurantium* (TCA). TCA induced a concentration-dependent relaxation.

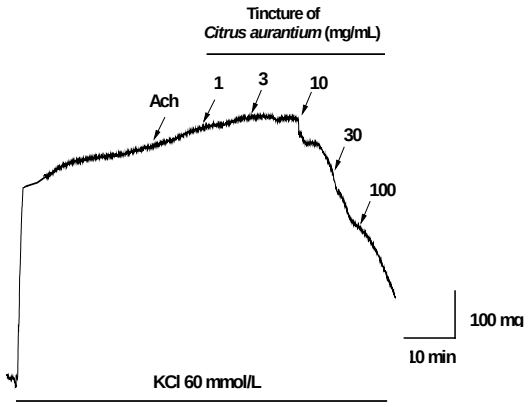

Figure 1. Examples of the vasorelaxant effect of tincture of *Citrus aurantium* at different concentrations in a rat aortic ring pre-contracted with 60 mmol/L of KCl. The endothelium removal was confirmed by the administration of acetylcholine (ACh, 10 µmol/L) to precontracted vascular rings.

TCA at low concentrations (1 to 3 mg/mL) did not produce significant vascular effects compared to vehicle control (70% ethanol), but from 10 to 100 mg/mL, it has produced a vasorelaxing effect. At 10 and 30 mg/mL of TCA, there was a vasodilatation of 28.07 ± 0.65 % and 65.12 ± 8.70%, respectively. The highest concentration studied (100 mg/mL) of TCA caused a vasorelaxation of 87.52 ± 3.51% ($p < 0.05$; $n = 4$) (Fig. 2). The concentration of TCA necessary to reduce 50% of the maximal contraction induced by 60 mmol/L of KCl (IC_{50}) was 18.36 ± 0.72 mg/mL, which was estimated by a concentration-response curve, based on the Hill function and fitted to the experimental data obtained after applying TCA concentrations from 1 to 100 mg/mL.

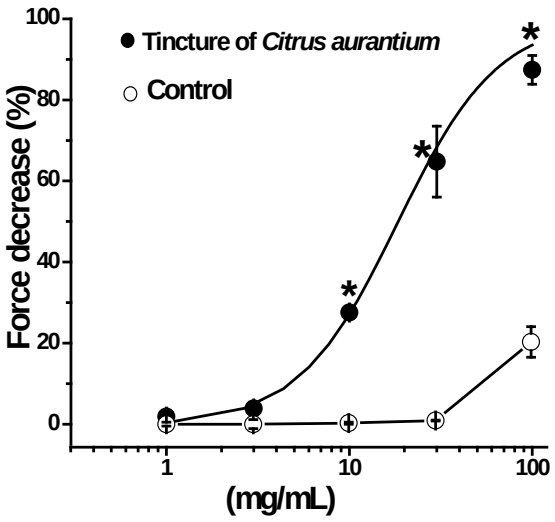

Figure 2. Concentration-response curves for the inhibition of force of contraction by tincture of *Citrus aurantium* and vehicle control on KCl (60 mmol/L) induced contraction in endothelium-denuded rat thoracic aortic rings. Values are shown as means ± standard errors of means with n = 5 rats, $p < 0.05$ compared with the vehicle control. Experimental data were fitted to a Hill function.

4 Discussion

The present study shows that the tincture of peel of *Citrus aurantium* (TCA) relaxes vascular smooth muscle in concentration-dependent manner and via the endothelium-independent pathway.

In the similar way, Kang *et al*. (2016) [11] indicated that the essential oil neroli of *Citrus aurantium* L. evoked relaxation of precontracted aortic rings in the endothelium-intact or endothelium-denuded condition. Their findings further showed that the endothelial component of the essential oil neroli of *C. aurantium* -induced vasodilatation is partly mediated by the nitric oxide (NO)-soluble guanylyl cyclase pathway, whereas the smooth muscle component involved modulation of intracellular Ca^{2+} concentration through inhibition of cation channel-mediated extracellular Ca^{2+} influx and store- operated Ca^{2+} release mediated by the ryanodine receptor signaling pathway. Consistent with this, a cardiovascular study also reported that neroli essential oil reduced blood pressure in humans [8]. Also neroli caused bradycardia and hypotension in adolescent patients [9]. Limonene, the major component of neroli essential oil, decreased blood pressure and heart rate in rats fed a high-fat diet [10]. Also linalool, another abundant component of neroli, exerted an antagonizing effect on extracellular Ca^{2+}-dependent contractions in intact and in endothelium-denuded rings precontracted with phenylephrine of rat mesenteric arteries, and reduced blood pressure without changing the heart rate in hypertensive rats. These results demonstrated that linalool reduced blood pressure probably due to a direct effect on the vascular smooth muscle leading to vasodilatation [14].

Although further studies are needed to see if the chemical components of TCA has any direct effect on calcium channels, the decrease of force of vascular contraction by this tincture should be at least partly due to an inhibition of voltage- gated calcium channels. This is because the contraction of vascular smooth muscle caused by high-K^+ solution (60 mmol/L) containing calcium is associated with an increased entry of extracellular calcium through voltage-gated calcium channels [15].

On the other hand, anti-edematogenic action of extracts from *Citrus aurantium* L. fruit barks was demonstrated on a vascular hyperpermeability model in rats [7].

A number of substances isolated from plants have already been shown to produce endothelium-independent vasorelaxation. These include polyphenolic compounds such as flavonoids [16, 17]. The fruit of *C. aurantium* contains flavonoids as naringenin, hesperetin, and apigenin [1, 2, 18]. He *et al.* (2018) [19] showed inhibitory effects on isolated jejunum (smooth muscle as well as vascular) contraction of flavonoids naringenin and nobiletin, present in dried fruits of *Citrus aurantium* L. Naringenin induced concentration-dependent relaxation in endothelium-denuded rat aortic rings [17], and hesperetin decreased the KCl‑induced contraction in rat abdominal aortic rings [20]. Qin *et al*. (2016) [21] showed that apigenin and naringenin restored ACh-mediated vasodilatation and increased nitric oxide level in the rat aorta and that apigenin and naringenin protected against high glucose-induced endothelial dysfunction.

Furthermore, the *C. aurantium* may contain low amounts of flavonols, as quercetin [1, 22]. The vasorelaxation effect of quercetin has been demonstrated in several studies. Quercetin induces vasodilatation through both endothelium-dependent [23] and endothelium-independent [17] pathways.

Also *C. aurantium* contains essential oils and compounds such as limonene and linalool [5]. Limonene exhibited relaxation activity on the rat superior mesenteric artery [24]. Linalool induced endothelium-dependent vasorelaxation in mouse thoracic aorta by activating soluble guanylyl cyclase and K^+ channels [25].

Other phytocompounds present in TCA can also influence the response. So, the mixture of components present may have synergistic vasorelaxant actions.

Citrus's essential oils are non-toxic, non-mutagenic, and non-carcinogenic, they are generally safe to use with negligible toxicity to humans [5]. Approximately 30 human studies indicate that p-synephrine and bitter orange extracts do not result in cardiovascular effects and do not act as stimulants at commonly used doses. Because p-synephrine exhibits greater adrenergic receptor binding in rodents than humans, data from animals cannot be directly extrapolated to humans. Bitter orange extract and p-synephrine are safe for use in dietary supplements and foods at the commonly used doses [26].

It is necessary to carry out preclinical and clinical trials to identify the varieties and parts of these plants most useful for the preparation of phytopharmaceuticals in our environment and to characterize the concentrations of the active principles necessary to guarantee pharmacological efficacy, which should be used as quality criterion.

5 Conclusion

The tincture of peel of *Citrus aurantium* induces an endothelium-independent relaxation on the rat aorta, indicating that it could be considered a source of natural bioactive products with vasodilatory activity.

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