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Original Article

Isolation of quinoline alkaloids from three *Choisya* species by high-speed countercurrent chromatography and the determination of their antioxidant capacity

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ABSTRACT

Choisya ternata Kunth, *C. ternata* var. *sundance* Kunth and the hybrid *Choisya* 'Aztec-Pearl' are three related species belonging to the Rutaceae family. Ethanol extracts were prepared from the leaves of these three species and evaluated in relation to their antioxidant activity using *in vitro* and *ex vivo* models. The ethanol extracts belonging to the three species produced a very high antioxidant profile as evidenced by the DPPH radical scavenging activity, the determination of total phenolics and flavonoid equivalent. The generation of reactive species of oxygen in leukocytes stimulated with LPS was dramatically reduced when the three ethanol extracts were used. The alkaloids anhydroevoxine and choisyine were isolated from the ethanol extract of *C. ternata* using HEMWat (4:6:5:5) as the solvent system by means of high-speed countercurrent chromatography. This was the first time quinoline alkaloids were isolated from this species using HSCCC. These compounds were also assayed for their capacity to inhibit the generation of ROS in leukocytes stimulated by LPS and the results also suggested that they are reactive oxygenase inhibitors.

Introduction

Choisya ternata Kunth, known as Mexican orange or Mexican orange blossom, is widely cultivated in Britain since 1825 (Muller, 1940; Benson, 1943). There is also the golden leafy variety, which is called *C. ternata* var. *sundance*. *Choisya dumosa* var. *arizonica* is also known as Mexican-orange or starleaf and to Mexicans as "sorilla" or "zorillo". These two species are considered toxic to the livestock, bearing toxic compounds (Dayton, 1931) that were not yet identified. *C. ternata* and *C. dumosa* var. *arizonica* were artificially crossed in 1982 (Lancaster, 1991) to generate the hybrid *Choisya* 'Aztec-Pearl', which has bigger flowers than its parents but is not as robust in stature as them. Previous phytochemical studies on *C. ternata* have indicated the importance of its non-volatile quinoline alkaloids. There are seven main alkaloids that are widespread in the Rutaceae family and that were found in species of *Choisya*: skimmianine, kokusaginine, 7-isopentenyloxy-*c*-fagarine, evoxine, choisyine, platydesminium methosalt and balfourodinium methosalt (Johns et al., 1967; Grundon et al., 1974; Boyd et al., 2002). Boyd et al. (2007) have isolated seventeen quinoline alkaloids from *C. ternata*, including several levels of oxidation during their biosynthesis.

Different species of Rutaceae have already been used as tonic, febrifuge, against inflammatory and microbial processes and in the treatment of malaria (Cruz, 1995; De Moura et al., 1997; Gonzaga et al., 2003; Moreira and Guarim-Neto, 2009; Jullian et al., 2006). Several chemical compounds isolated from different species of this family could account for the pharmacological properties observed for the extracts but one class is prominent. The quinoline alkaloids mentioned before can be responsible for some of the identified activities.

Previously our group has shown that extracts obtained from *C. ternata* and two of their quinoline alkaloids as well as compounds present in its essential oil possess antinociceptive activity (Radulović et al., 2011; Pinheiro et al., 2014). Yowtak et al. (2011) has shown a relationship between spinal neuropathic pain and oxidative stress.

In this study, a high-speed countercurrent chromatography (HSCCC) method was developed for the fractionation of the ethanol

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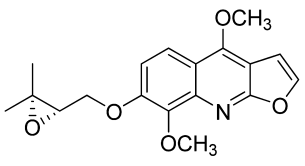
extract of leaves of three species of *Choisya*; *C.* Aztec-Pearl, *C. ternata* and *C. ternata* var. *sundance*. Countercurrent chromatography (CCC) is a form of liquid-liquid partition chromatography where the stationary phase is held inside the column without the use of a solid support (Conway, 1990). In modern CCC equipment, the column can rotate in one axis, generating hydrostatic equilibrium of the two immiscible liquid phases (the stationary phase and the mobile phase), or it can rotate in a planetary motion (two rotation axes), generating a hydrodynamic equilibrium of the two phases. HSCCC machines have hydrodynamic equilibrium of the two liquid phases and have been largely used for the fractionation and purification of natural products (Leitão et al., 2012; Friesen et al., 2015). Many different solvent systems have been used for the purification of alkaloids using modern hydrostatic or hydrodynamic equipment. Ionizable compounds like alkaloids can be purified using a technique called pH-zone refining CCC, developed by Ito in the 1990s (Ito and Ma, 1996), a modification of the CCC technique that uses acids or bases as retainers and/or eluters. In a study from Fang et al. (2011) a compilation of several solvent systems for the purification of alkaloids from herbs, by both HSCCC and pH-zone-refining CCC is presented. In that review it is reported the separation of 94 alkaloids from more than thirty different plant sources by conventional HSCCC using thirteen different solvent systems. The authors report that more than 67% of the alkaloids were purified with hexane–ethyl acetate–methanol–water (the so-called HEMWat system) and $CHCl_3$–MeOH–H_2O. In fact, these solvent systems represent two versatile families that can be used in countercurrent chromatography. Moreover, quinoline alkaloids have not yet been isolated from *C. ternata* by means of countercurrent chromatography.

Since species of *Choisya* and some of their isolated compounds have already shown activity toward spinal neuropathic pain, we decided to evaluate the ethanol extracts of these three species of *Choisya* in relation to their capacity of protecting against reactive oxygen species. anhydroevoxine (**1**), and choisyine (**2**), isolated from *C. ternata* in one step HSCCC analysis, were also evaluated.



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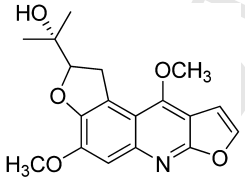

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Materials and methods

Chemicals

Solvents used for plant extraction were of analytical grade and acquired from the Hazard Material Facilities store (HMF-Trinity College Dublin) and those for countercurrent chromatography separations were of HPLC grade purchased from Tedia Brazil (Rio de Janeiro, Brazil).

Plant material

Fresh leaves of *C. ternata* Kunth, the hybrid *C.* Aztec-Pearl and *C. ternata* var. *sundance* were collected in Dublin, Ireland in September 2013 and their voucher specimen (ref. TCD Hodkinson & Ropero 01, 02 and 03) were deposited in the Herbarium of Trinity College, Dublin. The samples were identified by Dr. Trevor Hodkinson from the Department of Botany, Trinity College, Dublin.

Plant extraction

The extraction of leaves (100 g) of *C. ternata*, *C.* Aztec-Pearl and *C. ternata* var. *sundance* was performed using Soxhlet apparatus (80 °C) for 72 h. The solvent used for the Soxhlet extraction was ethanol. Extract (*ca.* 40 g each plant) was reduced to dryness under reduced pressure using a rotary evaporator in a heating bath.

Equipment

HSCCC fractionations were performed on the 98 ml coil of a Quattro HT-Prep counter-current chromatograph (AECS, Bridgend, United Kingdom) equipped with two bobbins containing two polytetrafluoroethylene multi-layer coils each (Bobbin 1 contains the 26 ml and the 224 ml coils, 1.0 mm i.d. and 3.2 mm i.d. respectively; Bobbin 2 contains the 95 ml and the 98 ml coils, both 2.0 mm i.d.). The rotation speed is adjustable up to 865 rpm. The HSCCC systems were connected to a constant flow pump Jasco PU-2089S Plus (Japan Spectroscopic Corporation, Japan). A 5 ml sample loop (low pressure sample injection valve, Model 5020, Rheodyne) was used to inject the sample. Separations were performed at room temperature.

HPLC analysis

The HPLC system consisted of a Waters Alliance Separations module equipped with a temperature programmable auto sampler and Waters 2996 PDA detector. The LC separation was performed on a reversed phase column C18 (250 mm × 4.6 mm, 5 μm) from Thermo Scientific, using mobile phase A (water) and mobile phase B (acetonitrile with 1% formic acid) in a gradient program with a flow of 0.8 ml/min: 0–30 min: 10% B; 30–32 min: 100% B; 32–35 min: 10% B and 35–38 min: 10% B. The volume of a single injection was 10 μl. UV/vis spectra between 190 and 400 nm were recorded.

Choice of the solvent system for CCC fractionations

Small amounts of the ethanol extracts of leaves of each *Choisya* species were dissolved in separate test tubes containing the hexane–ethyl acetate–methanol–water (HEMWat) solvent system in the ratios 10:5:5:1, 1:1:1:1, 6:4:6:4 and 4:6:5:5. Then the test tubes were shaken and the sample was allowed to partition between the two liquid phases. Equal aliquots of each phase were spotted side by side on silica gel TLC plates (Merck, Art. 5554, Germany) developed with the solvent system chloroform:methanol:water (9:1:1). The results were visualized under UV light (254 and 365 nm) followed by spraying TLC plates with vanillin (2% in methanol) and sulfuric acid (1% in methanol).

HSCCC fractionations

Preparation of the two-phase solvent system and samples for injection

The selected solvent system (hexane:ethyl acetate:methanol:water 6:4:5:5) was thoroughly equilibrated in a separation funnel at room temperature. The two phases were separated shortly before use and degassed by sonication for 10 min. The sample solutions were prepared by dissolving the sample (*ca.* 500 mg of each *Choisya* extract) in the solvent mixture of 2.5 ml stationary phase and 2.5 ml mobile phase of the solvent system used for the HSCCC separation.

HSCCC separation procedure

The 95 ml coil was first fully filled with the stationary lower aqueous phase at a flow rate of 5 ml/min. Then the upper organic mobile phase was pumped into the column at a flow rate of

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