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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A B S T R A C T

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.

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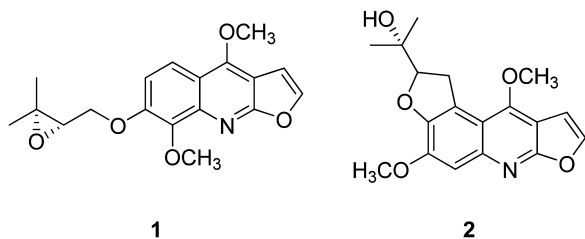
In this study, a high-speed countercurrent chromatography (HSCCC) method was developed for the fractionation of the ethanol

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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.

1   **2**

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 *Choisya ternata* Kunth, the hybrid *C.* Aztec-Pearl and *C. ternata* var. *sundance* , Rutaceae, 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 poly-tetrafluoroethylene 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

2 ml/min, while the apparatus was rotated at 860 rpm, at a constant temperature of 25 °C. The sample solution (*ca.* 500 mg of each *Choisya* extract in 5 ml biphasic system) was injected into the column after hydrodynamic equilibrium was reached. The stationary phase retention Sf for the solvent system in each fractionation was around 84–87%. Fractions of 4 ml were collected in a total of 80 in each run.

Determination of the antioxidant activity

The antioxidant activity was performed on the ethanol extracts of leaves of *Choisya* Aztec-Pearl, *C. ternata* and *C. ternata* var. *sundance*. The antioxidant activity was investigated using four different assays: DPPH free radical scavenging activity micro assay using a 96-well plate, quantification of total phenolics and determination of total flavonoid contents and finally the production of reactive oxygen species.

Antioxidant activity, use of 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The radical scavenging effect of the plant extracts was determined using the DPPH methodology. Here, the hydrogen free radicals donation ability of the plant extracts was measured from the bleaching of the purple colored ethanol solution of DPPH. The assay was based on the study done by Mensor et al. (2001).
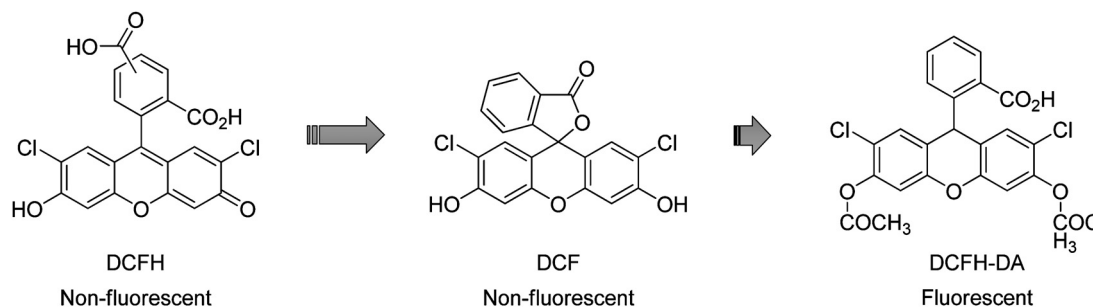
Assay for total phenolics

The total phenolic content of the extracts was determined following the Folin–Ciocalteu method carried out by Gursoy et al. (2009), only adapted to a microscale (Medina-Remon et al., 2009).

Assay for total flavonoids

The determination of the total flavonoid content of the extracts was done based on the methodology adapted by Gursoy et al. (2009).

Determination of the production of reactive oxygen species (ROS)

Leukocytes were collected from a subcutaneous air pouch created in the dorsal area of a mouse for the model of cell migration induced by carrageenan and stored in vials (10^6 cells) in a volume of 1 ml. Cells were further incubated at 37 °C and 5% CO_2 for 1 h. Crude ethanol extracts of the three *Choisya* species or the isolated compounds were then added to the cells at a concentration of 1, 10 and 30 μg/ml and incubated for 30 min more using the same conditions. Cells were then treated with 10 nM phorbol myristate acetate (PMA) and re-incubated at the same conditions for another 45 min. Two microliters of a solution of 2′-7′-dichlorodihydrofluorescein diacetate (DCF-DA) was added, followed by another incubation period of 30 min at the same conditions. The DCF-DA permeated rapidly through the cell membrane. Once in the intracellular cavity it is hydrolysed by the stearases being converted into 2′-7′-dichlorofluorescin (DCFH), which is a non-fluorescent compound impermeable to the cell membrane. In the presence of any ROS, DCFH is oxidized inside the cell forming a fluorescent compound: 2′-7′-dichlorofluorescin (DCF), which stays in the intracellular cavity (Srivastava and Gonugunta, 2009). Fluorescence was measured by flow cytometry in the FL-1 channel.

Results and discussion

Countercurrent chromatography (CCC), a liquid–liquid partition chromatography technique with no solid support, is ideal for the separation of complex natural product mixtures and its use in this area of interest keeps growing each day (Friesen et al., 2015). The major challenge of the technique is the selection of the correct solvent system, which can account for 90% of the time spent on the analysis (Ito, 2005). Several strategies have been proposed in the last years for a fast solvent system selection (Friesen and Pauli, 2007; Costa and Leitão, 2010; Yin et al., 2010; Leitão et al., 2012; Liang et al., 2015) Literature reports hexane–ethyl acetate–methanol–water, usually referred to as HEMWat, as the most used solvent system in CCC separations (Leitão et al., 2012). This is because it covers a large range of polarities, being useful in the purification of either low to medium polarity compounds as well as medium to high polarity ones. So, this solvent system was our choice for the purification of the three extracts of *Choisya: C.* Aztec-Pearl, *C. ternata* and *C. ternata* var. *sundance*. Several ratios of the HEMWat solvent system were tested for the fractionation of the extracts: HEMWat 10:5:5:1, 1:1:1:1, 6:4:6:4 and 4:6:5:5, being the latter the one that gave the best results, affording *K* values of target compounds (the alkaloids) near 1. Two alkaloids, anhydroevoxine, **1** (0.5% yield) and choisyine, **2** (1.1% yield), were isolated in a single HSCCC step from *C. ternata* leaves. Choisyine was also isolated from *C.* Aztec-Pearl and *C. ternata* var. *sundance*. However, anhydroevoxine could not be isolated as pure compounds in one step from these two species. This could be due to differences in the complexity of the matrix (the ethanol extract), which was previously observed by our group when optimizing the isolation of verbascoside from *Lantana* and *Lippia* species (Leitão et al., 2015) These two alkaloids were further identified by HPLC in the ethanol extract of the other two species (data not shown).

Anhydroevoxine (**1**) was isolated as a white crystal and its structure was undoubtedly established by means of MS and NMR analysis and comparison with the literature data (Akhmedzhanova et al., 1975).

White crystalline solid; R_f: 0.52 (chloroform:methanol:water; 9:1:1); mp: 88–92 °C; $[\alpha]_D^{20}$ 0.0 (*c* 0.009, CH_2Cl_2); EIMS for $C_{18}H_{19}NO_5$ *m/z*; 330 [M+H]$^+$ and 352 [M+Na]$^+$; ^{1}H NMR (CDCl$_3$, 400 MHz) δ: 3.32 (1H, t, *J* = 10.92, H-2′), 4.11 (3H, s, OCH$_3$–C-8), 4.29 (1H, m, H-1′), 4.41 (3H, s, OCH$_3$–C-4), 7.02 (1H, d, *J* = 2.4, H-9), 7.22 (1H, d, *J* = 9.0, H-6), 7.57 (1H, d, *J* = 2.4, H-10), 7.96 (1H, d, *J* = 9.0, H-5); ^{13}C NMR (CDCl$_3$, 100 MHz) δ: 18.9 (CH$_3$, C-4′), 24.6 (CH$_3$, C-5′), 58.3 (CH, C-3′), 59.0 (OCH$_3$, C-4), 61.6 (OCH$_3$, C-8), 61.7 (CH, C-2′), 69.4 (CH$_2$, C-1′), 102.3 (CH, C-9), 104.6 (C, C-3), 114.8 (CH, C-6), 118.1 (CH, C-5), 141.6 (C, C-8a), 143.1 (CH, C-10), 143.2 (C, C-8), 151.1 (C, C-7), 155.5 (C-4a), 157.1 (CH, C-4), 164.3 (C-2).

Choisyine (**2**) was isolated as an amorphous yellowish powder and its structure was undoubtedly established by means of MS and NMR analysis and comparison with the literature data (Frolova and Kuzovkov, 1963).

Yellowish amorphous solid; R_f: 0.44 (chloroform:methanol:water; 9:1:1); mp: 88–92 °C; $[\alpha]_D^{20}$ 0.0 (*c* 0.009,

DCFH
Non-fluorescent

DCF
Non-fluorescent

DCFH-DA
Fluorescent

Table 1
Correlation between studied parameters DPPH antioxidant activity with total phenolic and flavonoid equivalent of each ethanol extract.

Plant species	DPPH (EC_{50}) (μg/ml)	Phenolics (μg GAEs/mg extract)	Flavonoids (μg GAEs/mg extract)
Choisya Aztec-Pearl	25.84 ± 0.005	338.33 ± 0.002	0.59 ± 0.003
Choisya ternata 'Sundance'	[a]	713.33 ± 0.012	182.77 ± 0.005
Choisya ternata	9.32 ± 0.002	655.00 ± 0.014	37.42 ± 0.003

[a] EC_{50} value is undefined. Concentration of EC_{50} value too low to allow for interpretation but is indicative of high potency. EC_{50} for *Ginkgo biloba* in the DPPH assay was 38 ± 0.005. Each assay was performed in triplicate.

CH_2Cl_2); EIMS for $C_{18}H_{19}NO_5$ m/z; 330 $[M+H]^+$ and 352 $[M+Na]^+$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 1.28 (3H, s, H-4′), 1.41 (3H, s, H-5′), 2.02 (1H, s, H-1′), 3.72 (1H, m, H-3′), 3.95 (3H, s, OCH_3–C-7), 4.33 (3H, s, OCH_3–C-4), 4.78 (1H, t, J = 9.5, H-2′), 6.97 (1H, d, J = 2.4, H-9), 7.18 (1H, s, H-8), 7.51 (1H, d, J = 2.4, H-10); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 24.1 (CH_3, C-4′), 26.1 (CH_3, C-5′), 33.7 ($CH2$, C-1′), 55.7 (OCH_3, C-7), 58.8 (OCH_3, C-4), 71.9 (CH, C-3′), 90.5 (CH, C-2′), 93.08 (C, C-3), 102.7 (C, C-4a), 104.6 (C, C-7), 106.4 (CH, C-8), 118.7 (C, C-5), 142.5 (C, C-8a), 144.5 (C, C-6), 148.3 (C, C-4), 156.5 (CH, C-4), 164.3 (C-2).

Antioxidant activity of the ethanol extract of the three *Choisya* species

DPPH, total phenolics and flavonoid equivalent

The ethanol extracts of leaves of *Choisya* Aztec-Pearl, *C. ternata* var. *sundance* and *C. ternata* were tested for antioxidant activity and then compared (Table 1). The DPPH free radical scavenging activity using UV–vis spectrophotometry was first employed, followed by total phenolics and flavonoid equivalent. It is clear from the values that the ethanol extracts of leaves from the *Choisya* species have much greater antioxidant activity than the ethanol extract of *Ginkgo biloba*, used as positive control. Negative control consisted of only ethanol and DPPH. Therefore, according to this study, all three samples are more potent antioxidants than the *G. biloba* standard.

The antioxidant activity of phenolics and their derivatives depend on the number and position of hydroxyl groups bound to the aromatic ring, the binding sites and the mutual positions of hydroxyl groups in the aromatic ring and the type of substituent (Rico et al., 2013). The highest total phenolic content was seen in the extracts of *C. ternata* Aztec-Pearl (713.33 μg GAE/mg extract). This was followed by the *C. ternata* var. *sundance* extracts and then the *C. ternata* extracts.

Flavonoids are phenolic compounds that could be responsible for the antioxidant capacity of medicinal plants. According to the results in this study, the highest amount of flavonoids, 182.77 ± 0.005 equivalent of quercetin, was found, as expected, for *C. ternata* var. *sundance*, the golden leafy species.

From the analysis of Table 1, it can be seen that there was a distinct correlation between the studied parameters (total phenolic content, flavonoid equivalent and DPPH scavenging activity) for *Choisya* Aztec-Pearl, *C. ternata* and *C. ternata sundance* extracts. The scavenging activity measured by the DPPH showed a very good correlation to the quantity of phenols and flavonoids in each of these extracts.

ROS production

The generation of reactive species of oxygen was measured in leucocytes by the method described previously. It is observed that *C. ternata* and *C. ternata* var. *sundance* similarly protected leucocytes from generating free radicals when stimulated with PMA by 83% both at 10 μg/ml and by 95% and 92% at 30 μg/ml, respectively. However, in this model *C.* Aztec-Pearl confers a slightly higher protection of the cells after stimulation with PMA; at a concentration

of 10 μg/ml the protection is around 98% and at a concentration of 30 μg/ml it goes up to 99%. According to this model, anhydroevoxine protects the cells by 71% at 30 μg/ml while choisyine confers 64% protection at the same concentration. In leukocytes, ROS are mainly generated by NADPH oxidase (Sauer et al., 2001) a target of protein kinase C, which is activated by PMA (Karlsson et al., 2000). The PMA-induced ROS formation was significantly reduced by *Choisya* species at 10 mM, reflecting its known radical-scavenging properties (Lo et al., 2002). In contrast, *Choisya* species did not interfere with PMA-activated ROS generation up to 10 mM, excluding unspecific anti-oxidant properties. These results suggest that *C. ternata* and its alkaloids could inhibit ROS generation or production. This study also showed that *Cyathula prostata* exhibited anti-inflammatory and antinociceptive activities and this was mediated by other pathways but not by a direct action on the oxidative metabolism (Imbrahim et al., 2012). Poeckel et al. (2008) observed that the action of *Rosmarinus officinalis* and *Salvia officinalis*, as well as of their main constituents, the diterpenes carnosol and carnosic acid in relation to their anti-inflammatory activity had a strong component linked to their antioxidant capacity. The evaluation of the generation of reactive oxygen species in leukocytes stimulated by PMA, followed by the use of these diterpenes and the plant extracts showed the strong link between their anti-inflammatory action and the specific antioxidant capacity.

It could thus be concluded that the three species of *Choisya* evaluated possessed a very high antioxidant potential as illustrated by *in vitro* and *ex vivo* assays used in this study. The same goes for the two quinoline alkaloids isolated from *C. ternata.* It was also the first time that quinoline alkaloids were isolated by means of countercurrent chromatography from this species.

Authors' contribution

GGL and JPBP did HSCCC separations and choice of solvent systems. PRC, DRR, PDF and FB did antioxidant experiments. FB also performed HSCCC separations.

Conflicts of interest

The authors declare no conflicts of interest.

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