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Identification and quantitative determination of the polar constituents in *Helichrysum italicum* flowers and derived food supplements

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ABSTRACT

Helichrysum italicum is widely used in traditional medicine, in cosmetic, in food and pharmaceutical field. In spite of this, very little is known about the chemical composition of its polar extracts. Therefore this study was addressed to the determination of the metabolite profile of the methanol extract of *H. italicum* flowers, by using LC–ESI(IT)MSMS. This approach oriented the isolation of 14 compounds, whose structures were unambiguously elucidated by NMR as belonging to flavonoid, phenylpropanoid and acylbenzofuran classes. In addition, one novel drimane sesquiterpene was identified.

The quantitative determination of the main compounds occurring in the methanol extract of *H. italicum* flowers was carried out and their content was compared with that of three selected commercial food supplements based on *H. italicum*, by using LC–ESI(QqQ)MS.

In conclusion the wide occurrence, in high amounts, of quinic acid derivatives in all the analyzed samples was highlighted, showing these compounds as chemical markers of the species for standardization procedures.

1. Introduction

Helichrysum italicum (Roth) G. Don is one of the best known medicinal plants from the Mediterranean area. The strong and persistent smell of *H. italicum* is reminiscent of that of curry and combines with the long-lasting bright yellow color of its flower heads to make the plant a veritable icon of the Mediterranean environment. The flowers, commonly known as gold-everlasting or eternal flowers, were used in antiquity to make the wreaths to crown idols.

H. italicum is widely used in traditional medicine due to its anti-inflammatory [1,2], antibacterial [3] and antioxidant [4] properties, and it finds large application also in cosmetic and pharmaceutical field [5]. In fact it shows dermofunctional, antiallergic and antieczematic activities and for all these properties it appears in many topical formulations [6]. Furthermore, the preventive effects of *H. italicum* extracts on microcirculation diseases are well known [1,7]. In addition, *H. italicum* is often used as a decoction to soothe cough, to help bronchial mucus expectoration, and to soothe allergies caused by inflammation of mucus membrane of nose.

Moreover, this species is widely utilized in food field [8] to enhance fruit flavors in food or as herbal tea [9].

In spite of these considerations, the greatest part of analytical studies on *H. italicum* were focused on the essential oils, highlighting a large amount of monoterpenoids like neryl acetate, neryl propanoate and α-pinene [10,11], whereas very little is known about the chemical composition of *H. italicum* polar extracts.

Concerning this, Albayrak et al. [12], ascertained the occurrence of phenolic acids and flavonoids in the methanolic extract of some Turkish *Helichrysum* species exhibiting antioxidant and antimicrobic activity by using high-performance liquid chromatography (HPLC) coupled with a diode array detector (DAD). In particular, chlorogenic acid resulted the major compound, followed by apigenin 7-*O*-glucoside and its aglycone apigenin [12].

Recently Taglialatela-Scafati et al. isolated from *H. italicum* flowers minor new phenolics as well as an unusual class of lipids named santinols [13].

Therefore, considering the occurrence of few reports on the metabolite composition of the polar extracts of *H. italicum*, and the high commercial value of products like hydroalcoholic extracts and mother tinctures based on *H. italicum*, this study was oriented to the determination of the metabolite profile of the methanol extract of *H. italicum* flowers, by using high-performance liquid chromatography coupled to electrospray negative ionization tandem ion trap mass spectrometry (LC–ESI(IT)MSMS). By this way, the qualitative analysis of the LC–MS profile and of the mass fragmentation pathways obtained for each chromatographic peak by application of tandem mass spectrometry experiments (LC–MSMS) [14–16]

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guided the isolation of 14 compounds, whose structures were unambiguously elucidated by NMR spectroscopic data as belonging to flavonoid, phenylpropanoid and acylbenzofuran classes.

Furthermore, the quantitative determination of the main compounds occurring in the methanol extract of *H. italicum* flowers was performed and their content was compared to that displayed by three selected commercial food supplements by an analytical approach based on liquid chromatography coupled to mass spectrometry with ESI source and triple quadrupole mass analyzer (LC–ESI(QqQ)MS), using a very sensitive and selective tandem mass spectrometry experiment called multiple reaction monitoring (MRM).

2. Materials and methods

2.1. General experimental procedures

Optical rotations were measured on a JASCO DIP 1000 polarimeter. IR measurements were obtained on a Bruker IFS-48 spectrometer. NMR experiments were performed on a Bruker DRX-600 spectrometer (Bruker BioSpinGmBH, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI CryoProbe at 300 K. All 2D-NMR spectra were acquired in CD_3OD (99.95%, Sigma–Aldrich) and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, HMBC and ROESY spectra. The NMR data were processed using UXNMR software. HRESIMS was carried out by an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) operated in negative ion mode. The Orbitrap mass analyzer was calibrated according to the manufacturer's directions using a mixture of caffeine, methionine-arginine-phenylalanine-alanine-acetate (MRFA), sodium dodecyl sulfate, sodium taurocholate and Ultramark 1621. Data were collected and analyzed using the software provided by the manufacturer.

2.2. Plant material

The air dried flowers of *H. italicum* (Roth) G. Don were collected in Acciaroli (Salerno) in May 2012 and identified by Prof. V. De Feo, Dept. of Pharmacy, University of Salerno. The three mother tinctures A, B, and C obtained from fresh flowers ranging from 25% (A and B) to 30% (C) in ethanol (45%) were purchased from three different herbal companies.

2.3. ESI(IT)MS and LC–ESI(IT)MS analyses

The ESI source parameters of a LCQ Deca ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) were tuned by using a standard solution of chlorogenic acid in methanol (1 μg/ml) infused at a flow rate of 10 μl/min with a syringe pump. Mass spectra were recorded from $m/z = 50$ to 1500 in negative ionization mode in the following operative conditions: capillary voltage −47 V, spray voltage 4.9 kV, tube lens offset −40 V, capillary temperature 280 °C, sheath gas (nitrogen) flow rate 10 (arbitrary units). To optimize the $ESIMS^2$ conditions, the collision energy percentage was increased up to 35 to produce a whole fragmentation pattern showing an array of all possible fragments. The MS and MS^2 spectra were acquired and elaborated using the software provided by the manufacturer.

Qualitative LC–ESI(IT)MS analysis of the methanol extract of *H. italicum* flowers (400 ng/μl) was performed using a Thermo Finnigan SpectraSystem HPLC equipped with a Synergy Polar column (250 mm × 3 mm, 4 μm, Phenomenex, Torrance, CA, USA) and coupled with the LCQ Deca ion trap mass spectrometer. A mobile phase consisting of water acidified with 0.1% acetic acid (solvent A) and acetonitrile (solvent B) was used to perform the following linear elution gradient: from 30% B to 100% B in 25 min, hold at 100% B for 10 min. The mobile phase was supplied at a flow rate of 200 μl/min, keeping the column at room temperature; the column effluent was injected directly into the ESI source. The operative conditions were the same described for ESI(IT)MS analysis. The column effluent was analyzed by ESIMS and $ESIMS^2$ in negative ion mode according to the optimized parameters indicated for the direct injection with the auxiliary gas (nitrogen) supplied at a flow rate of 10 (arbitrary units). Data were acquired in MS^1 and MS^2 scanning modes and processed using the software provided by the manufacturer.

2.4. Extraction and isolation procedures

The air-dried, powdered flowers of *H. italicum* (500 g) were extracted with petroleum ether (2 l) three times. After filtration the raw material was extracted for three times with chloroform (2 l) and for three times with methanol (2 l). The combined methanol extracts were purified by size exclusion chromatography using Sephadex LH-20 as stationary phase and methanol as mobile phase, by obtaining 21 fractions. On the basis of the results obtained from the LC–ESI(IT)MS analyses, Sephadex fractions A, B, C, D, and E were submitted to semipreparative HPLC separations by using a Phenomenex C18 Synergi-Hydro-RP (250 mm × 10 mm, 10 μm) column.

Fraction A (110.5 g) was separated by Reverse Phase-HPLC-IR on a Waters 590 system equipped with a Waters R401 refractive index detector and an injector U6K, using a MeOH–H_2O (38:62, v:v) solution and a flow rate of 2.0 ml/min. By these conditions compounds **1** (1.2 mg Rt = 7.20 min), **2** (7.1 mg, Rt = 3 min), and **4** (1.2 mg, Rt = 8.50 min) were isolated.

Fractions B, C, D, and E were analyzed by a RP-HPLC-UV system on an Agilent 1260 Infinity system (Agilent Technologies, Palo Alto, CA, USA), equipped with a binary pump (G-1312C), and an UV detector (G-1314B). Elution gradient was executed by using 0.5% acetic acid (A) and 0.5% acetic acid CH_3CN (B) as mobile phases, at a flow rate of 2 ml/min and monitoring the analytes at 3 wavelengths (210, 254 and 310 nm). The gradient profiles are given below.

Fractions B (410.5 g), C (532.1.5 g) and D (257.0 g) were analyzed by using the same elution conditions: after a 10 min hold at 8% B, from 8% B to 20% B in 16 min, hold at 20% B for 11 min, from 20% B to 21% B in 16 min, to 25% B in 18 min, hold at 25% B for 20 min, from 25% B to 30% B in 20 min, and to 100% B in 15 min, followed by 10 min at 100% B. By using these chromatographic conditions it was possible to isolate compounds **3** (2.9 mg, Rt = 42.81 min), **5** (0.8 mg Rt = 54.90 min), **6** (2.6 mg, Rt = 57.35 min), and **7** (5.6 mg, Rt = 48.62 min) from fraction B; compounds **8** (0.9 mg, Rt = 57.10 min), **10** (1.6 mg, Rt = 73.92 min), **11** (1.3 mg, Rt = 84.74 min), and **12** (0.8 mg, Rt = 106.3 min) from fraction C, and compound **9** (1.2 mg, Rt = 63.24 min) from fraction D.

For the chromatographic separation of fraction E (89.4 mg) a different gradient was employed: after a 5 min hold at 5% B, from 5% B to 20% B in 13 min, to 25% B in 12 min, to 28% B in 10 min, to 30% B in 5 min, hold at 30% B for 20 min, from 30% B to 45% B in 5 min, to 100% B in 10 min, followed by 10 min at 100% B. By these conditions compounds **13** (16.3 mg, Rt = 53.73 min) and **14** (7 mg, Rt = 34.38 min) were isolated.

2.5. 6,11-Diacetyl-9,11-dihydroxydrimane (***10***)

Amorphous white solid; $C_{19}H_{32}O_5$; $[\alpha]^{25}_D$ −53.7 (*c* 0.1 MeOH); IR ν^{KBr}_{max} cm^{-1}: 3450 (OH), 2940 (CH), 1650 (C=O), 1250 and 1045 (C—O—C); ^1H NMR spectroscopic data (CD_3OD, 600 MHz): δ (ppm) 5.41 (brs, H-6), 4.90 (2H, s, H-11), 2.20 (m, H-8), 2.05 (s, $COCH_3$), 1.93 (s, $COCH_3$), 1.77 (d, J = 1.7 Hz, H-5), 1.72 (m, H-7), 1.71 (m, H-2), 1.69 (m, H-1), 1.53 (m, H-2), 1.51 (m, H-7), 1.49 (td, J = 12.4, 3.2 Hz, H-1), 1.36 (m, H-3), 1.33 (s, Me-13), 1.24 (m, H-3), 1.06 (s, Me-14),