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SHORT COMMUNICATION

Hydroxycinnamates from elecampane (*Inula helenium* L.) callus culture

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Abstract Hydroalcoholic extract from *Inula helenium* callus tissue showed remarkable reducing capacity. An HPLC/DAD analysis revealed the presence of numerous hydroxycinnamic acid derivatives, including chlorogenic acid (5-*O*-CQA, 0.1 % dry weight) and 3,5-dicaffeoylquinic acid (3,5-DCQA, 0.3 % dry weight), which were among major constituents of the examined extract. Application of a hyphenated chromatographic method—UHPLC/DAD/MS^n—allowed identification of sixteen compounds, derivatives of caffeic acid. Apart from the compounds commonly found in *Inula* sp., the plant material contained eight conjugates of caffeic acid with aldaric acid. The aldarates constituted over 50 % of the hydroxycinnamate fraction in the examined tissue. This is the first report on the occurrence of the caffeoylaldaric acids in *Inula* sp. and in a plant tissue culture.

Keywords Aldaric acids · Caffeic acid derivatives · Callus culture · Hydroxycinnamates · *Inula helenium*

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Introduction

Hydroxycinnamic acid derivatives are common plant constituents. They are present in plant tissues as free acids (i.e., coumaric, ferulic, caffeic and sinapic acids), their oligomers, and as conjugates with other compounds (most commonly with quinic acid and glucose). Mono-, di-, tri- and tetracaffeoylquinic acids as well as feruloylquinic and coumaroylquinic acids and conjugates of caffeic acid with tartaric acid are frequently found in Asteraceae (Jaiswal et al. 2011). Hydroxycinnamates are well-known radical scavengers and inhibitors of lipid peroxidation (Ohnishi et al. 1994; Olmos et al. 2008; Shahidi and Chandrasekara 2010). The compounds also play a role in plant defense against pathogens and insects (Kodoma et al. 1998; Leiss et al. 2009). They are of potential value in pharmacy, as antiviral, hepatoprotective and neuroprotective agents (Robinson et al. 1996; Kim et al. 2005, 2007), in cosmetics (protection against UV) and in food industry (as bioactive components of the diet and precursors for flavors). *Inula helenium* L., a perennial herb of the Inulae tribe (Asteraceae) is a reputed medicinal plant. The species, native of Middle Asia, widely occurs in Europe, Asia and Northern America. Underground parts of the elecampane plant, collected in the autumn after 2 or 3 years of growth, are traditionally used for medicinal purposes, for example to treat asthma, cough, bronchitis, lung disorders, tuberculosis, skin infections and helminthic diseases (Seca et al. 2014). The herbal product under the name of "Radix Inulae", "Radix Helenii" or "Rhizoma Helenii" is described in several European pharmacopeias (e.g. PF X, Ned 5, BHP). Roots of *I. helenium* contain up to 5 % of essential oil with alantolactone, isoalantolactone (eudesmane-type sesquiterpene lactones) and monoterpenoid thymol derivatives as major constituents. Moreover, sterols,

triterpenes and inulin (up to 44 %) were found in the roots (Blaschek et al. 1998). Literature data concerning hydroxycinnamic acid derivatives present in *I. helenium* are sparse. It is only recently that some reports on analysis of extracts from leaves and roots of the plant using high performance liquid chromatography with mass spectrometry (HPLC/MS) and tentative identification of phenolic compounds have been published (Jaiswal et al. 2011; Spiridon et al. 2013). Callus cultures of *I. helenium* maintained in our laboratory showed high total phenolic content. As far as we were aware, secondary metabolites produced by callus cultures of elecampane were not studied before. Thus, the aim of our work was to identify and quantify phenolics accumulated by the tissue.

Materials and methods

Plant material

Seeds of botanically verified *I. helenium* plants, of known wild origin (No. 1026/2001), were supplied by the Botanical Garden and Botanical Museum Berlin-Dahlem of the Free University in Berlin (Germany). Voucher specimen of the donor plant was deposited in the garden herbarium or in the main herbarium of the Botanical Museum. The seeds, after surface sterilization, were aseptically germinated on a half-strength, solidified (0.8 % agar) MS medium (Murashige and Skoog 1962).

Callus initiation and maintenance

Leaf explants (ca. 1 cm long) were excised from aseptic seedlings and subsequently inoculated onto a solidified MS medium supplemented with 2,4-D (2,4-dichlorophenoxyacetic acid, 4.52 μM l^{-1}) and kinetin (1.39 μM l^{-1}), containing 3 % sucrose (pH 5.8, before autoclaving). The explants were kept at 28 °C, under continuous light (40 μmol m^{-2} s^{-1}, cool white fluorescent tubes), to initiate callus proliferation. The calli were further maintained on the culture medium of the same composition with an unaltered temperature and illumination regime. The tissue was transferred to the fresh nutrient medium every 6 weeks. The subculturing was performed by inoculating ca. 2.0 g FW (fresh weight) of the tissue onto 50 ml of the culture medium. For the phytochemical analysis, the calli were harvested at the end of 6 week culture period.

Chemicals and solvents

5-*O*-caffeoylquinic acid (chlorogenic acid, 5-*O*-CQA, purity >97 % by HPLC) and a standard sample of cynarin (1,3-dicaffeoylquinic acid, 1,3-DCQA, purity >99 % by HPLC) were bought from Roth (Karlsruhe, Germany). 3,5-DCQA was isolated from *L. virosa* hairy root culture (Stojakowska et al. 2012). The compound was of 90.0 % purity (by HPLC). Folin-Ciocalteu reagent was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Methanol (MeOH) of analytical grade was purchased from POCh S.A. (Gliwice, Poland). Water for chromatography was purified by a Mili-Q system (Milipore Corp., Bedford, MA, USA). Acetonitrile (MeCN) and MeOH of gradient grade for liquid chromatography as well as glacial acetic acid and formic acid (HCOOH) of analytical grade were bought from Merck (Darmstadt, Germany).

Determination of total phenolic content (TPC)

The "total phenolic content" which reflects reducing capacity of the plant material (Huang et al. 2005) was analyzed using the Folin-Ciocalteu colorimetric method as described previously (Stojakowska et al. 2013). Results (means of three measurements) were expressed as gallic acid equivalents.

Quantification of hydroxycinnamates

The dry, pulverized plant tissue (100 mg) was extracted twice (for 3 h) with 10 ml of 70 % MeOH, at room temperature, on a rotary shaker (100 r.p.m.). The combined methanol extracts were evaporated in vacuo to give a dry residue which was redissolved in 1 ml of 70 % MeOH and subsequently centrifuged (11,340*g*, 5 min) just before chromatographic analysis. Analytical HPLC separations of the samples were carried out according to the method described by Malarz et al. (2013). Quantification was performed using an external standard method. The calibration curves were calculated using four concentration levels (0.001, 0.01, 0.1 and 1.0 mg ml^{-1}) of 5-*O*-CQA and 1,3-DCQA. Peak areas (at 325 nm) were referred to the corresponding calibration curves. Caffeic acid derivatives were quantified as equivalents of 5-*O*-CQA (monocaffeoyl derivatives) and 1,3-DCQA (di- and tricaffeoyl derivatives).

Identification of caffeic acid derivatives by ultra-high performance liquid chromatography with photodiode array detection and ion trap mass spectrometry (UHPLC-DAD-MS^n)

The dry, powdered tissue (500 mg) was extracted with 10 ml of 0.1 % HCOOH/MeOH (8/2) in an ultrasonic bath (40 °C), for 30 min. The obtained extract was filtered through paper filter, diluted 1:2 with 0.1 % HCOOH and than filtered through 0.45 μm Chromafil membrane (Machery-Nagel, Duren, Germany).

UHPLC/DAD/MS^n analysis was carried out on a UHPLC-3000 RS system (Dionex, Germany) with DAD detection and an AmaZon SL ion trap mass spectrometer with ESI interface (Bruker Daltonik GmbH, Germany). The chromatographic separation was achieved on a Zorbax SB C18 column (150 × 2.1 mm, 1.9 µm) (Agilent, USA), at 25 °C. The mobile phase was a mixture of H_2O/MeCN/HCOOH 95/5/0.1 (solvent A) and MeCN/HCOOH 100/0.1 (solvent B). Gradient elution conditions applied were as follows: 0–45 min 5–40 % B, at a flow rate of 0.2 ml min^{-1}. The column was equilibrated for 8 min between injections. The chromatograms were assessed at 325 nm and UV spectra of the compounds were recorded over a range of 200–450 nm. The column eluate was introduced directly, without splitting, into the ESI interface. The nebuliser pressure was 40 psi; dry temperature 300 °C; dry gas flow 9 l min^{-1}; and capillary voltage 4.5 kV. The mass spectral analysis was performed using scan from 90 to 2200 *m/z*, in a negative ion mode. The MS^2 fragmentation spectra were acquired for the most abundant ions at a given time.

Results and discussion

After 6 weeks of culture, FW and DW (dry weight) of *I. helenium* calli reached 8.86 ± 1.60 and 0.28 ± 0.06 g per culture vessel, respectively. Longer cultivation led to the growth arrest and a change in tissue color from green to dark brown. TPC in the 6-week-old callus tissue, calculated on a DW basis, reached 6.78 ± 0.37 % (67.8 mg g^{-1}), expressed as gallic acid equivalents. According to literature data (Kähkönen et al. 1999; Malarz et al. 2013), reducing capacities of *Cichorium intybus* leaves and *Aronia melanocarpa* fruits which are known sources of antioxidants were lower than that estimated for the examined tissue. Callus cultures of *Inula crithmoides*, grown on MS medium supplemented with 2,4-D (1.0 mg l^{-1}), contained 4.4–28.3 mg g^{-1} of phenolics. The content depended on the phase of growth (maximum at the stationary phase) and was lower in cultures grown in the dark (Bucchini et al. 2013). TPC estimated in *I. helenium* roots ranged from 3.5 to 5.8 mg g^{-1} (Wang et al. 2013).

Preliminary HPLC/DAD analysis of hydroalcoholic extracts from *I. helenium* calli revealed the presence of numerous compounds with absorption maxima at about 325 nm, which corresponded to those of hydroxycinnamic acid derivatives. Three of the compounds (5-*O*-CQA, 1,3-DCQA and 3,5-DCQA) were identified based on their UV spectra, retention times and co-chromatography with standards. To identify the remaining constituents of the extract, HPLC/DAD/MS^n technique was applied. Based on UV–Vis spectra and MS^n, we were able to identify 16 compounds (Fig. 1). In accordance with Clifford et al. (2005), the negative ion MS^2 and MS^3 fragmentation patterns of compounds **1**–**3** allowed their identification as 3-, 5-, and 4-*O*-CQA (IUPAC numbering system), while compounds **5**, **9**–**11** and **13** were assigned as isomers of DCQA (Table 1). Peaks **4**, **6**–**8**, **12** and **14**–**16** showed a cleavage of two to four caffeoyl $[M - H\text{-}162]^-$ moieties resulting in *m/z* 209 fragment (Table 1), indicating the presence of glucaric acid or galactaric acid, the so-called aldaric acids (Ruiz et al. 2013; Lorentz et al. 2012). However, up to now, only the presence of hydroxycinnamic acid glucarates has been reported (Maas et al. 2009; Lorentz et al. 2012; Ruiz et al. 2014). The presence of this rare type of compounds was not reported in *Inula* sp. before, although the presence of di- tri- and tetracaffeoylglucaric acid derivatives was demonstrated in other genera of Asteroideae subfamily such as *Leontopodium*, *Gnaphalium* and *Eupatorium* (Schwaiger et al. 2005; Maas et al. 2009; Cicek et al. 2012). Jaiswal et al. (2011) identified six hydroxycinnamic acid derivatives in the extract from aerial parts of *I. helenium*, including three monocaffeoylquinic acids (1-*O*-, 3-*O*-, and 5-*O*-CQA), 5-*O*-feruloylquinic acid, 5-*O*-*p*-coumaroylquinic acid, and 3-*O*-caffeoyl-4-*O*-*p*-coumaroylquinic acid. According to Spiridon et al. (2013), roots of the plant contained: caffeic acid, caffeic acid hexose of undetermined structure, 5-*O*-CQA, 5-*O*-feruloylquinic acid, ferulic acid-4-*O*-glucoside, DCQA (structure not determined), 3-*O*-feruloyl-4-*O*-caffeoylquinic acid, and 3,4,5-tri-*O*-caffeoylquinic acid. Our research revealed the presence of 16 caffeic acid derivatives in the examined tissue, whereas derivatives of ferulic and *p*-coumaric acids were absent from the analyzed calli.

Quantification of caffeic acid derivatives in *I. helenium* callus culture was done by HPLC/PDA (results are shown in Table 1). Total content of hydroxycinnamic acid derivatives in the analyzed plant material, calculated as a sum of detected compounds, was about 1.4 % (on a DW basis). Total content of caffeoylquinates (ca. 0.7 %) was slightly lower than that of caffeoyl aldarates (ca. 0.8 %). Five major compounds, i.e., 5-*O*-CQA, 3,5-DCQA, 1,5-DCQA, and tricaffeoyl aldarates (I) and (II) constituted over 70 % of the hydroxycinnamate fraction. In vitro cultures of Asteraceae are good producers of hydroxycinnamates (mainly caffeoylquinates and caffeoyltartrates). The highest total content of the compounds (up to 7 % DW) was found in a hairy root culture of *Cichorium intybus* L. (Malarz et al. 2013). Hairy roots of *Echinacea purpurea* (L.) Moench. (Abbasi et al. 2007), *Lactuca virosa* L. (Stojakowska et al. 2012) and *Rhaponticum carthamoides* (Willd.) Iljin (Skała et al. 2015) also accumulated substantial amounts of the hydroxycinnamic acid derivatives. Caffeic acid derivative contents in callus cultures of Asteraceae were lower than those in the corresponding root

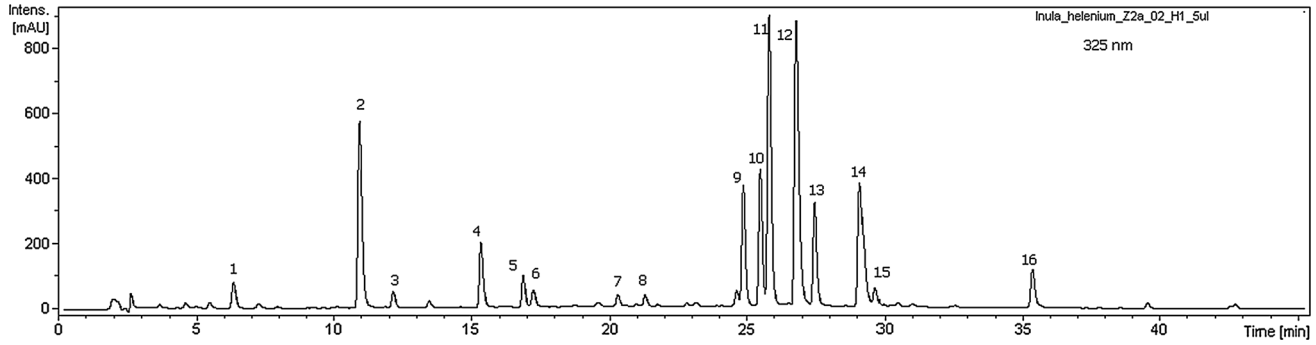

Fig. 1 HPLC-UV chromatogram of *I. helenium* extract at a concentration of 25 mg/ml (injection volume—5 µl), acquired at 325 nm

Table 1 Retention times, UV maxima, MS^n data in the negative ion mode, and contents of caffeic acid derivatives detected in *Inula helenium* callus extract

No	Compound	Rt (min)	UV (nm)	$[M - H]^-$	Product ions main peaks[a]	Content[b] (%DW)
1	3-*O*-caffeoylquinic acid	6.3	216, 324	353	**191**, 179	0.014 ± 0.0005
2	5-*O*-caffeoylquinic acid	10.9	216, 326	353	**191**	0.101 ± 0.0019
3	4-*O*-caffeoylquinic acid	12.1	213, 325	353	191, 179, **173**	0.010 ± 0.0010
4	Dicaffeoyl aldarate (I)	15.3	215, 324	533	**371**, 209	0.052 ± 0.0025
5	1,3-Di-*O*-caffeoylquinic acid	16.8	215, 325	515	**353**, 335, 191, 179	0.018 ± 0.0002
6	Dicaffeoyl aldarate (II)	17.3	212, 326	533	**371**, 209	0.013 ± 0.0020
7	Dicaffeoyl aldarate (III)	20.4	214, 326	533	**371**, 209	0.010 ± 0.0001
8	Dicaffeoyl aldarate (IV)	21.3	214, 326	533	**371**, 209	0.010 ± 0.0005
9	3,4-Di-*O*-caffeoylquinic acid	24.9	216, 326	515	**353**, 335, 299, 255, 203, 191, 179, 173	0.070 ± 0.0003
10	1,5-Di-*O*-caffeoylquinic acid	25.4	215, 328	515	**353**, 335, 191	0.107 ± 0.0049
11	3,5-Di-*O*-caffeoylquinic acid	25.8	215, 327	515	**353**, 191	0.259 ± 0.0088
12	Tricaffeoyl aldarate (I)	26.7	217, 327	695	**533**, **371**, 209	0.400 ± 0.0022
13	4,5-Di-*O*-caffeoylquinic acid	27.4	215, 327	515	**353**, 317, 299, 255, 203, 173	0.075 ± 0.0030
14	Tricaffeoyl aldarate (II)	29.1	216, 328	695	**533**, **371**, 209	0.164 ± 0.0008
15	Tricaffeoyl aldarate (III)	29.7	216, 327	695	**533**, **371**, 209	0.044 ± 0.0008
16	Tetracaffeoyl aldarate	35.3	217, 328	857	**698**, 533, 371, 209	0.077 ± 0.0051

[a] MS^2 ions in bold—most abundant ion peak

[b] Data are means of three measurements ± SD

cultures. Calli of *C. intybus* and *L. virosa* accumulated 1.4 % DW and 0.7 % DW of caffeic acid derivatives, respectively (Stojakowska et al. 2012; Malarz et al. 2013). The present study is, to our knowledge, the first report on the occurrence of caffeoyl aldarates in in vitro-cultured plant tissues.

Author contribution statement Anna K. Kiss performed UHPLC/DAD/MS^n analysis and contributed in writing of the manuscript. Janusz Malarz and Anna Stojakowska have contributed equally to the remaining part of this publication.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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