bioNMR-based identification of natural anti-Aβ compounds in *Peucedanum ostruthium*

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**A R T I C L E   I N F O**

**Keywords:** Alzheimer’s disease, Anti-amyloidogenic compounds, Chlorogenic acids, Furanocoumarins, NMR spectroscopy, *Peucedanum ostruthium*, UPLC-HR-MS

**A B S T R A C T**

The growing interest in medicinal plants for the identification of new bioactive compounds and the formulation of new nutraceuticals and drugs prompted us to develop a powerful experimental approach allowing the detailed metabolic profiling of complex plant extracts, the identification of ligands of macromolecular targets of biomedical relevance and a preliminary characterization of their biological activity. To this end, we selected *Peucedanum ostruthium*, a plant traditionally employed in Austria and Italy for its several potential therapeutic applications, as case study. We combined the use of NMR and UPLC-HR-MS for the identification of the metabolites present in its leaves and rhizome extracts. Due to the significant content of polyphenols, particularly chlorogenic acids, recently identified as anti-amyloidogenic compounds, polyphenols-enriched fractions were prepared and tested for their ability to prevent Aβ1-42 peptide aggregation and neurotoxicity in a neuronal human cell line. STD-NMR experiments allowed the detailed identification of Aβ oligomers' ligands responsible for the anti-amyloidogenic activity. These data provide experimental protocols and structural information suitable for the development of innovative molecular tools for prevention, therapy and diagnosis of Alzheimer’s disease.

**1. Introduction**

The biological activity of medicinal plants is mainly due to the presence of secondary metabolites, compounds with no apparent function in the primary metabolism of the organism, but with a remarkable importance in defense, competition and adaptation to the environment. These molecules often have specific functions and many of them present biological activities that can be useful for the treatment of human diseases, therefore providing new active ingredients for pharmacological research. Due to the growing interest in medicinal plants for the discovery and rational design of new drugs, we decided to investigate the metabolic content and the potential biological activities of the species *Peucedanum ostruthium* (vernacular name “Imperatoria”). This plant is traditionally employed in Austria and Italy. The parts of *Peucedanum ostruthium* with documented uses are the leaves and the rhizome, commonly used for the preparation of tisanes, liqueurs and bitters, or as powders applied on the skin to treat gastro-intestinal, cardiovascular and respiratory diseases [1]. The alcohol extract of the rhizome (*Radix imperatoriae*) has been used as a stimulant, a stomachic, a diuretic for chronic indigestion, as well as a therapeutic for typhoid, intermittent fever, paralytic conditions, and in delirium treatment [2]. Furthermore, its use as anti-inflammatory agent is also documented [3].

Coumarins are the most peculiar secondary metabolites found in this species. In particular, the presence of imperatorin, ostruthol, oxy-peucedanin hydrate, oxypeucedanin, isoimperatorin (all furanocoumarins) and ostruthin and osthole is reported [1]. Coumarins are related to a wide range of biological activities such as antiinflammatory and antioxidant [4], anticancer [5], antifungal and antimicrobial [6] activity. In addition, ostruthol acts as inhibitor of acetylcholinesterase, which could imply a strong potential for the treatment of Alzheimer’s disease (AD) [7].

The many therapeutic applications of Imperatoria prompted us to further deepen its secondary metabolite content by combining NMR spectroscopy and UPLC coupled with high resolution mass spectrometry (HR-MS).
Interestingly, a first metabolic profiling of different kinds of aqueous and hydro-alcoholic extracts revealed the significant presence of chlorogenic acids (CGAs). By dissecting the potential neuroprotective activity of both roasted and green coffee extracts, we recently demonstrated that CGAs show interesting anti-amyloidogenic properties [8]. Briefly, CGAs are Aβ oligomer ligands able to inhibit Aβ1-42 peptide on-pathway aggregation and Aβ-induced toxicity in SH-SY5Y human neuroblastoma cell line, suggesting the potential application of coffee extracts for AD prevention [8]. AD is the most common form of dementia in older population and the number of people affected is expected to increase dramatically in coming years [9]. Since symptom treatments occur in a stage of the disease in which neuronal damage is irreversible, there is a strong need to identify a primary prevention treatment for AD, and Aβ oligomers are a promising target for this purpose [9]. Thus, the interest in natural compounds capable of interfering with toxic oligomers of Aβ peptides is growing.

Based on these evidences, we decided to perform a preliminary investigation of the nutraceutical potential of *Peucedanum ostruthium* extracts for the prevention of AD. To this end, the chemical analysis done through NMR and UPLC-HR-MS was complemented with biochemical, biophysical and biological assays, the last carried out on SH-SY5Y human neuroblastoma cell line.

2. Experimental section

All chemicals and solvents were purchased from Sigma-Aldrich or Fisher scientific and used without further purification.

2.1. Preparation of plant extracts

Samples of *Peucedanum ostruthium* (rhizome and leaves) were field-collected (Barmasc, Rì Courtod, Ayas, AO, Italy, 1900 m above sea level) on August 13, 2016 and identified by a local ethnobotanical expert (Dr. Fiorenza Cout). Samples were frozen in liquid nitrogen and grounded into a fine powder. 1 g of each sample was introduced into a cellulose thimble and extracted in a Soxhlet apparatus (100 mL) with a hydroalcoholic mixture (80% EtOH) during 10 cycles. Finally, the organic phase was removed under reduced pressure at 40 °C and the aqueous residue was freeze-dried. The lyophilized extracts were stored at −20 °C.

2.2. Preparation of polyphenol-enriched fractions

2.2.1. Solid-phase extraction

Solid-phase extraction (SPE) was performed with Amberlite® XAD®-4. A SPE cartridge loaded with 1 g of resin was washed with EtOH (3x 10 mL) and equilibrated with H2Oaq. A solution of extract (120 mg in 2 mL of H2Oaq) was loaded and the column was stirred for 12 h. Then, non-ad sorbed fraction (fract.1 - N.A.), washing fraction (2 mL of H2O, fract. 2 - W) and elution fraction (4 mL of EtOH, fract. 3 – Ext) were collected, the organic solvent was removed under reduced pressure and aqueous residues were freeze-dried obtaining fraction 1 – 3 as solid residues.

2.2.2. Preparative reverse-phase column chromatography

Automated flash chromatography was performed on a Biotage® Isolera™ Prime system equipped with Spectra package. A solution of extract sample (200 mg in 1.5 air-dried overnight. Column chromatography was performed on SNAP KP-C18-HS (12 g) cartridge using water (solvent A) and methanol (solvent B) as eluent solvents. A linear elution gradient was applied (2% B for 2 CV, 2-100% of B in 15 CV and 100% B for 3 CV) at flow rate of 12 mL/min. The eluate was automatically collected in fraction based on photodiode array detector signal (range 200–400 nm). Fractions were pooled in homogenous groups, organic solvent was removed under reduced pressure and residues were freeze-dried obtaining fractions A-E.

2.3. Chemical characterization

2.3.1 NMR spectroscopy

Freeze-dried samples were suspended in D2O at a final concentration of 25 mg/mL, sonicated (37 kHz, 20 min, Elmasonic P 30H, Elma Schmidbauer GmbH, Singen, Germany) and centrifuged (14,000 rpm, 5 min, 20 °C, ScanSpeed 1730R Labogene, Lyne, Sweden). 4.4-Dimethyl-4-silapentane-1-sulfonic acid (DSS, final concentration 0.5 mM) was added to the supernatant as an internal reference for concentrations and chemical shift. The pH of each sample was verified with a microelectrode (Mettril Toledo, Columbus, OH, USA) for 5 mm NMR tubes and adjusted to 7.4 with NaOD or DCl. All pH values were corrected for the isotope effect. The acquisition temperature was 25 °C. All spectra were acquired on an Avance III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a QCI (1H, 13C, 15N/31P and 2H lock) cryogenic probe. 1H NMR spectra were recorded with ppmgr1d, nosygpppr1d, ledhpqppr2s1d pulse sequences (Bruker library) and 256 scans, spectral width 20 ppm, relaxation delay 5 s. They were processed with 0.3 Hz line broadening, automatically phased and baseline corrected. Chemical shifts were internally calibrated to the DSS peak at 0.0 ppm. Compound identification and assignment were done with the support of 2D NMR experiments, comparison with reported assignments and the SMA analysis tool integrated in MestreNova Software. The 1H, 1H-TOCSY (Total Correlation Spectroscopy) spectra were acquired with 48 scans and 512 increments, a mixing time of 80 ms and relaxation delay of 2 s. 1H, 13C-HSQC (Heteronuclear Single Quantum Coherence) spectra were acquired with 64 scans and 512 increments, relaxation delay 2 s.

2.3.2 Ultra-performance liquid chromatography/electrospray ionization-high resolution mass spectrometry (UPLC/ESI-HR-MS)

The UPLC/ESI-HR-MS analysis was carried by coupling an Acquity UPLC separation module (Waters, Milford, MA, USA) with in-line photodiode array (FDA) detector (Waters) to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer and an HESI-II probe for electrospray ionization (Thermo Scientific, San Jose, CA, USA). The ion source and interface conditions were: spray voltage +3.0/−2.5 kV, sheath gas flow 60, auxiliary gas flow 20 and temperature 300 °C, capillary temperature 350 °C. Positive mass calibration was performed with Pierce LTQ ESI Positive Ion Calibration Solution (Thermo Scientific Pierce, Rockford, IL, USA), containing caffeine, the tetrapeptide MRFA and Ultramark 1621. Negative mass calibration was performed with Pierce ESI Negative Ion Calibration Solution (Thermo Scientific Pierce), containing sodium dodecyl sulfate, sodium taur ocholate and Ultramark 1621. Four μL of sample (2 mg/mL in water) were separated using a Waters Acquity BEH C18 column (150 × 2.1 mm, 1.7 μm, 130 Å) (Waters, Milford, MA, USA) kept at 40 °C, and using 0.1 mL 100 mL−1 of formic acid in H2O Milli-Q-treated water (solvent A) and 0.1 mL 100 mL−1 formic acid in acetonitrile (solvent B). For the UPLC separation, a linear elution gradient was applied (isosotic 5% B for 5 min then 5–50% of solvent B in 20 min) at a flow rate of 0.2 mL min−1. The LC eluate was analyzed by Full MS and data dependent tandem MS analysis (dd-MS2) of five the most intense ions (Top 5). The resolution was set at 70,000 and the AGC targets were 1 × 106 and 1 × 105 for Full MS and dd-MS2 scan types, respectively. The maximum ion injection times were 50 ms. The MS data were processed using Xcalibur software (Thermo Scientific) and Mnsa MS (Mestrelab). Metabolites were determined according to their calculated exact mass and absorption spectra. Their structures were confirmed by high resolution tandem MS (HR-MS/MS) by comparison to reported assignments in literature or databases.

2.4. In vitro antioxidant activity

Antioxidant activity was evaluated by mean in vitro spectrophotometric assays reported below. Absorbance measurements were...
performed with Varian Cary 50 Scan UV–Visible Spectrophotometer using disposable polymethyl methacrylate (PMMA) semimicro 10 mm-cuvettes relative to a blank solution. Extract samples were diluted to 0.2 mg/mL, and standard solutions (0–200 μg/mL) of 5-CQA were used for calibration (linear fitting $R^2 = 0.9985$, $n = 7$). Results were expressed as μg of Chlorogenic acid Equivalent (CQA eq)/mg of freeze-dried extract. Data were reported as means (±SD) of triplicate measures of three independent experiments.

2.4.1 Total reducing capacity (TRC) - Folin-Ciocalteu assay

Total reducing capacity was measured by Folin-Ciocalteu’s phenol assay prior described by Singleton, Orthofer, and Lamuela-Raventós (1999) [24]. Briefly, 80 μL of diluted samples (or standards/blank) and 40 μL of Folin’s reagent were dispensed in a cuvette containing 400 μL of $H_2O$; then 480 μL of 10.75% (w/v) Na$_2$CO$_3$ solution was added and the solution was quickly mixed. The absorbance at 760 nm was read after 30 min of incubation at room temperature.

2.4.2 ABTS radical scavenging

ABTS assay is based on the scavenging ability of antioxidants to the long-life intense colored radical cation 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). A 7 mM stock solution of ABTS$^+$ was produced by mixing an equal amount of a 14 mM ABTS solution and a 4.9 mM K$_2$S$_2$O$_8$ solution in $H_2O_{aq}$ (final concentration 7.00 mM and 2.45 mM, respectively). The mixture was left at room temperature in dark for at least 12–16 h before use and stored at 4°C for 7 days. A working solution of ABTS$^+$ was daily prepared by diluting the stock solution (1:50) reaching an absorbance of 0.70 ± 0.05 at 734 nm. Fifty μL of sample (or standards) were added in a cuvette containing 950 μL of ABTS$^+$ solution, and the absorbance at 734 nm was read after 30 min of incubation at room temperature.

2.4.3 DPPH radical scavenging

DPPH assay is based on the scavenging of the stable free-radical 2,2'-diphenyl-1-picrylhydrazyl, according to literature [25]. Briefly, 950 μL of a diluted solution of DPPH in buffered MeOH (100 μM in a mixture of 60% MeOH and 40% acetate buffer pH 4.5, Abs 0.70 ± 0.05) and 50 μL of a diluted sample (or standard) were added into a cuvette, the absorbance at 517 nm was read after 30 min of incubation at room temperature.

2.5. Biological activity

2.5.1 Peptide synthesis and sample preparation

Aβ1-42 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGIAGLMVGGVIA) was prepared by Solid Phase Peptide Synthesis (SPPS) on a Syro I synthesizer (Biotage, Uppsala, SE) using Fmoc-protected L-amino acid derivatives, NOVASYN-TGA resin and a 0.1 mM scale [26]. Peptide was cleaved from the resin and was treated to obtained monomers and oligomers as previously described [27]. The peptide was purified by RP-HPLC on a semi-preparative Jupiter C4 column (5 μM, 300 Å, 250 x 10 mm, Phenomenex) with a mobile phase of 0.1% TFA in water (elucent A) and 0.08% TFA in acetonitrile (elucent B), using a linear gradient.
from 5 to 50% of eluent B in 40 min. Peptide identity and purity was confirmed by MALDI-TOF analysis (model Reflex III, Bruker). The purity of peptide was always above 95%.

2.5.2 Thioflavin T binding

Aβ1–42 was dissolved in 10 mM NaOH, H₂O and PBS (1:1:2) to 2.5 μM with or without Imperatoria extracts (25 μg/mL) and were incubated at 37 °C with 20 μM ThT (Sigma, PB 50 mM, pH 7.4) in 96-well black plates (Isoplate, Perkin Elmer). ThT fluorescence was monitored for 24 h by a plate reader (Infinite F500 Tecan: excitation 448 nm, emission 485 nm, 37 °C). Data were expressed as the mean of three replicates, calculated by subtracting the relative control solutions (extracts alone) and were expressed as the percentage reduction of Aβ1–42 aggregation.

2.5.3 Aβ-induced cytotoxicity

Human neuroblastoma SH-SYSY cell line was grown in Dulbecco’s Modified Eagle’s medium (DMEM, Lonza) supplemented with l-glutamine (5 mM, Gibco), antibiotics (penicillin/streptomycin 10,000 U, Lonza) and 10% heat-inactivated fetal calf serum (FCS, Gibco). The SH-SYSY cell line was seeded in 96-well plates (105 cell/mL) and incubated overnight (37 °C, in a humidified 5% CO₂ atmosphere). After completing planting the medium was replaced with 1% of FCS in DMEM, to reduce cell growth.

Aβ1–42 was dissolved in 10 mM NaOH, H₂O and PBS (1:1:2) and added to Imperatoria fractions (100 μg/mL in 0.5% DMSO) to obtain a final concentration of 10 μM for Aβ1–42 in the well. Cytotoxicity was evaluated after 24 h incubation, using the MTT reduction assay.

Fig. 2. Structures of the main compounds identified in the crude Soxhlet extract obtained from Imperatoria rhizome.
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Tetrazolium solution (20μL of 5mg/mL, Sigma Aldrich) was added to each well and incubated for 4 h. The medium was replaced with acidified isopropanol (0.04 M HCl) to dissolve the purple precipitate and the absorbance intensity was measured at 570 nm, using a plate reader (Infinite M200, Tecan). Data were expressed as percentages of controls (vehicle) for three separate replicates.

2.5.4 Statistical analysis
All data were shown as mean ± standard deviation (SD). Statistical analysis was done using GraphPad Prism 4.0. One-way ANOVA, followed by Dunnett’s multiple comparison test, was used to assess the significance of differences between groups.

2.6. Atomic force microscopy (AFM)

Aβ1-42 was dissolved in 10mM NaOH, H2O and PB 50mM (1:1:2) to 2.5μM with or without Imperatoria fractions (25μg/mL) and were incubated at 37°C for 24 h. At different time points, 20μL of samples were spotted onto a freshly cleaved Muscovite mica disk and incubated for 5 min. The disk was then washed with 5 mL of H2O and dried under a gentle nitrogen stream. Samples were mounted onto a Multimode AFM with a NanoScope V system (Veeco/Digital Instruments) operating in Tapping Mode using standard antimony(n)-doped Si probes (T: 3.5–4.5mm, L: 115–135mm, W: 30–40mm, f 0: 313–370kHz, k: 20–80N/m) (Bruker). Samples were analyzed with the Scanning Probe Image Processor (SPIP Version 5.1.6 (released April 13, 2011) data analysis package. SPIP software was used to analyze the distribution of the molecular assemblies of the different populations in terms of height and diameter profiles, as previously described [28].

2.7. NMR binding studies

NMR spectra were recorded on a Bruker Avance III 600MHz equipped with a QCI cryo-probe (Bruker, Billerica, MA, USA). Preparation of a sample containing Aβ oligomers: lyophilized Aβ1–42 was dissolved in 10mM NaOD (280µL) then diluted with 20mM phosphate buffer (140µL) and the pH adjusted to 7.4. After 1h incubation at 37°C, extract (final concentration 15mg/mL) or Fraction C sample (final concentration 15mg/mL) dissolved in 140µL of 20mM phosphate buffer (pH 7.4) was added. The pH was measured with a Microelectrode (Mettler Toledo) for 5mm NMR tubes and adjusted to pH 7.4 with NaOD and/or DCl. Total sample volume was 560µL.

A basic sequence from Bruker library was employed for STD experiment. For STD, a train of Gaussian-shaped pulses of 50ms each was employed to selectively saturate the protein envelope; the total saturation time of the protein envelope was adjusted by the number of shaped pulses and set at 3s. The on- and off-resonance spectra were acquired in an interleaved mode with the same number of scans. The STD NMR spectrum was obtained by subtracting the on-resonance spectrum from the off-resonance spectrum. A reference experiment acquired on a sample containing only the crude extract was run under the same experimental conditions to verify true ligand binding. The effects observed in the presence of the peptide oligomers were due to true saturation transfer because there were no signals in the STD NMR spectra obtained in the reference experiments.

3. Results and discussion

3.1. Preparation and characterization of Imperatoria extracts and fractions enriched in polyphenolic compounds

As already mentioned, the traditional local medicine uses *Peucedanum ostruthium* for the preparation of medicative tisanes or liqueurs. Thus, in order to optimize the extraction of bioactive metabolites from plant leaves and rhizomes, we tested three different experimental procedures: (a) aqueous extraction at 100°C, (b) hydroalcoholic
(20% ethanol) ultrasound-assisted extraction, (c) hydroalcoholic (80% ethanol) Soxhlet extraction.

Preliminary analysis of extracts, i.e. UV–Vis, total polyphenol content and $^1$H NMR metabolic profiling (Supplementary material – Figs. S1–S3) highlighted that hydroalcoholic Soxhlet extraction of rhizome afforded the best results in terms of overall yield of polar aromatic metabolites, allowing to obtain 43% w/w yield (430 ± 32 mg out of 1 g of dried sample, n = 3).

Metabolites contained in the crude rhizome extract were identified by a combined analytical approach based on NMR spectroscopy and UPLC-HR-MS. In particular, UPLC separation was monitored through a PDA detector to reveal the characteristic polyphenol absorbances at 280 and 320 nm. NMR spectroscopy data were complementary used for metabolites identification and quantification, including non-ionizable compounds. Fig. 1 depicts the chromatographic UV trace extracted at 320 nm (1A) and the $^1$H NMR spectrum (1B) of crude Soxhlet extract obtained from Imperatoria rhizome. Structures and detailed spectroscopic data for each identified compound are reported in Fig. 2 and Table 1 respectively.

Data analyses allowed the identification of 27 polyphenolic compounds in Peucedanum ostruthium crude extracts, including phenolic acids, simple coumarins, furanocoumarins and flavonoids glycosides.

CGAs, a group of esters formed between quinic acid and trans-hydroxycinnamic acids, mainly caffeic, $p$-coumaric and ferulic acids, were found as the most abundant polyphenols. Among them we identified O-caffeoylquinic acids (3-CQA, 4-CQA and 5-CQA isomers), 5-O-feruloylquinic acid (5-FQA) and di-O-caffeoylquinic acids (3,4-diCQA, 3,5-diCQA, 4,5-diCQA isomers) as the most representative; furthermore, di-O-feruloyl-caffeoylquinic acids (3F,4-CQA, 4F,5-CQA and 4C,5-FQA isomers) and 3,4,5-tri-O-caffeoylquinic acid (3,4,5-CQA)
were detected as minor components. Structures and isoforms of CGAs were determined on the basis of tandem MS(-) (data not shown), in agreement with literature data [10]. In addition, the main CGAs were identified also by 1H NMR with the support of 1H,1H-TOCSY and 1H, 13C-HSQC and through comparison with reported assignments [11].

Hesperetin-7-rutinoside (Hesperidin), whose presence in *Peucedanum ostruthium* rhizome had been previously described [12], was identified as the major glycosylated flavonoid in the mixture. The coumarin ostruthin (6-geranyl-7-hydroxycoumarin) and the linear furanocoumarins oxypeucedanin, oxypeucedanin hydrate, imperatorin and isoimperatorin were unequivocally identified as mean of monoisotopic mass and their MS(+) fragmentation pattern, in comparison with published data [1]. The presence of an angelicin-type dihydroxyfuranocoumarin glycoside (Apterin) was detected according to what observed in other *Peucedanum* species [13].

Furthermore, compounds eluting at 14.38 and 14.80 min retention time gave an identical mass spectrum corresponding to a molecular formula of C22H26O11 and the neutral loss of 162 amu in MS2 spectra indicating the presence of an O-hexosyl group and a common C16H16O6 fragment, representing a psolaren-type 7-(2,3-dihydroxy-3-methylbutoxy) coumarin aglycone. Esters or glycosides of furanocoumarins containing hydroxyl groups in the prenyl chain are seldom found in nature [14]. However, some linear furanocoumarin glycosides were found in the rhizomes of *Apiaceae* family [15]. Thus, we tentatively identify these compounds with oxypeucedanin hydrate 3′-O-glucopyranoside [15c] and oxypeucedanin hydrate 2′-O-glucopyranoside.

Compounds eluting with a retention time of 16.47 and 17.12 min showed mass data corresponding to a molecular formula of C24H28O12 and C27H30O5 respectively and a common aglycone fragment relative to a molecular formula of C18H19O7, clearly identified as oxypeucedanin hydrate 3′-acetate, as previously found in *Peucedanum ostriuthium* [16]. The first one showed a neutral loss of 162 amu (anhydromalonylglucose) and (less abundant) loss of 248 amu (malonylglucose), resulting in the identification of oxypeucedanin hydrate 3′-acetate-2′-(6″-malonyl) glucopyranoside. Detailed MS data and a fragmentation pathway for the identification of compounds 21 and 22 are available in Supplementary material – Figs. S4 and S5.

Moreover, a mass peak corresponding to a molecular formula of C16H24O7 and with a retention time of 9.78 min was observed. Upon fragmentation, it gave a neutral loss of 162 amu, generically identified as phenylpropanoid glycoside. Compounds eluting at a retention time of 15.02 and 15.52 min showed a mass peak of 585.2175 [M+H+] resulting in a molecular formula of C27H36O14 and gave fragment ions in positive mode at 261.1123 (87%) and 205.0501 as base peak. Neutral losses of 324 amu and 56 amu indicated the presence of a
gentiobioside moiety and a prenyl substituent respectively, thus sug-
gestng a prenylated aglycone with a molecular formula of C_{15}H_{16}O_{4},
tentatively identified as peucenin. To the best of our knowledge, no
peucenin glycoside was reported in literature, so we tentatively iden-
tified these compounds as chromone gentiobioside isomers, presenting
peucenin as aglycone moiety.

Finally, compound eluting with 11.33 min of retention time showed a mass peak at 501.1607 [M+H^+]. Upon fragmentation, ions at 355.1026 (6%) and 193.0497 as base peak, with a neutral loss of 148 and 308 amu, were observed, suggesting the presence of a rutinoside moiety, and a C_{10}H_{8}O_{4} aglycone, resulting in a tentative identification of scopoletin-7-O-rutinoside, also found in Asteraceae species [17].

In addition, both NMR and MS analysis allowed the identification of other metabolites and nutrients, such as choline, malic acid, γ-aminobutyric acid (GABA), l-alanine, d-glucose and sucrose.

Starting from a portion of crude rhizome extract (Fig. 3A), a fraction enriched in polyphenolic compounds was prepared by solid phase extraction (SPE) on Amberlite XAD-4 resin. A retained XAD fraction eluted with ethanol was achieved with a 10.3% yield. Its 1H NMR profile is reported in Fig. 3A-1. As can be deduced from the comparison with the 1H NMR spectra of the crude extract (Fig. 3A-1), the not retained fraction (Fig. 3A-2) and the fraction eluted with water (Fig. 3A-3), XAD-retained fraction (from here named as “fraction XAD”) (Fig. 3A-4) showed the major content of aromatic metabolites.

Moreover, another portion of crude extract was submitted to reverse phase (RP) chromatographic separation on RP-C18 column, collecting the eluate in seven fractions (A-G) (Fig. 3B), based on chromatographic UV-DAD trace, as reported in Supplementary material – Fig. 56. As inferred from 1H NMR profiles (Fig. 3B), fraction C (Fig. 3B-3) showed the highest enrichment in aromatic compounds. In particular, both NMR and UPLC-HR-MS revealed that fractions XAD and C resulted enriched in CGAs with a complete removal of the apolar furanocoumarins comprised from 21.33 and 23.52 min RT in the chro-
mogram of the total extract (Fig. 1A and S7). Fraction XAD resulted enriched in mono-substituted and di-substituted CGAs, being 5-CQA and 3,5-CQA the most abundant (Fig. 58), while fraction C in di-sub-
stituted CGAs, in particular di-cafeoyl and caffeoyl-feruloylquinic acid iso-
mers (dCQA and F, CQA), flavonoids and furanocoumarin glyco-
sides (Fig. 59).

3.2. Effects of polyphenol-enriched fractions on Aβ peptide aggregation and neurotoxicity.

In light of 5-CQA ability to bind Aβ1-42 and prevent its aggregation and neurotoxicity [8], the identification of several isomers of CGAs, including di-cafeoyl and caffeoyl-feruloylquinic acids, as major com-
ponents of polyphenol-enriched fractions of Imperatoria rhizome extract (A, B and C fractions from RP-chromatography and XAD fraction from SPE), prompted us to test their potential anti-amylloidogenic ac-
tivity.

Firstly, their efficacy in inhibiting the aggregation of Aβ1-42 pep-
tide was verified by ThT assay [18] and AFM analysis, employed to evaluate peptide aggregation after 24 h of co-incubation at 37 °C with the tested fractions dissolved at a concentration of 25 μg/mL. According to relative ThT fluorescence values (Fig. 4), all the fractions reduced peptide aggregation. In fact, peptide co-incubation with the tested fractions induced a decrease of the fluorescence emitted by ThT upon binding to Aβ aggregates of 71%, 82.3%, 88.3% and 75.7% for fractions A, B, C and XAD respectively.

The AFM images (Fig. 5) obtained after 24 h of incubation of 2.5 μM Aβ 1–42 peptide with the same enriched fractions of Imperatoria extract at a concentration of 25 μg/mL showed a significant reduction of peptide aggregation, in particular the formation of peptide fibrils and macro-aggregates, with a “potency” following the order XAD < B < C. In fact, comparing the AFM image of the peptide alone with that of peptide co-incubated with fraction C, the disappearance of fibrils and the presence of small globular structures with a diameter of 40 nm ± 15 nm and only a few prefibrillar structures with a length of 60 nm ± 20 nm were evident. The sample obtained from the peptide co-incubation with fraction B showed almost exclusively short pre-fi-
brillary structures of 150 nm ± 20 nm in length, while amorphous material (big globular structures) and few pre-fibrillar structures were present upon peptide co-incubation with XAD fraction. Fraction A induces only a very mild reduction of the peptide aggregation, at variance with ThT assay results (Fig. 4).

Finally, an MTT assay [19] carried out on human neuroblastoma SH-SY5Y cell line [8] allowed the evaluation of the protective effect of Imperatoria fractions against the neurotoxicity induced by Aβ1–42 oligomers (Fig. 6).

Cells were treated with 10 μM Aβ1-42 peptide in oligomeric form and incubated for 24 h with or without 100 μg/mL of different
Imperatoria fractions. The co-treatment with fractions B, C and XAD improved significantly cell survival (+13.5%, +21.3% and +12.5% respectively), while fraction A showed no significant effect.

In addition, in view of the dramatic role of oxidative stress in AD onset [20], we briefly characterized the in vitro antioxidant activity of our samples, measuring the total reducing capacity (Folin-Ciocalteu assay) and the ability to scavenge radical species (ABTS and DPPH assay). Our data indicated that fraction B and C from RP chromatography showed the highest antioxidant capacity, suggesting that this effect correlates with the total amount of CGAs (Supplementary material – Fig. S10).

3.3. NMR-based identification of Aβ oligomer ligands contained in extracts from Imperatoria rhizome

NMR-based ligand-receptor interaction studies were exploited to detect extract components able to bind directly Aβ1-42 oligomers as putative molecules responsible for the biological activities previously investigated.

STD-NMR experiment [21] is a robust and reliable method to reveal protein ligands in complex compound mixtures [8,22]. In addition, in the last years, several works have demonstrated the suitability of this experiment for the identification of ligands of Aβ oligomers and the characterization of their binding mode [8,22b,22c,23].

STD NMR experiments were carried out on a sample containing a mixture of the total Soxhlet extract of Imperatoria rhizome and Aβ oligomers (nominal peptide concentration of 80 μM) dissolved in deuterated phosphate buffer (pH 7.4, 25 °C) (Fig. 7).

Aβ1-42 peptide was dissolved in aqueous buffer according to the procedure previously reported [23c] that assures its oligomeric state. Brieﬂly, lyophilized Aβ1-42 is dissolved in 10 mM NaOD to allow the disaggregation of some preformed seeds, leading to an Aβ peptide solution containing monomers and some low-molecular-weight oligomers. The sample is then diluted with phosphate buffer and the pH adjusted to 7.4, inducing immediately the peptide aggregation, to afford a sample enrichment in Aβ oligomers. After 1 h of incubation at 37 °C the sample consists mainly of soluble Aβ oligomers, as checked by AFM and NMR diffusion experiments [23c].

Crude extract was added after its dissolution in phosphate buffer, pH 7.4, 25 °C. The selective saturation of some aliphatic protons of Aβ oligomers was achieved by irradiating at −1.00 ppm (on-resonance frequency). If the compounds present in the test mixture are not irradiated directly (verified by blank experiments on a sample containing the mixture only), the detection of their NMR signals in the STD spectrum is a non-ambiguous evidence of their interaction with the receptor. Conversely, any signal from non-binding compounds is deleted in the difference spectrum (STD spectrum). Thus, the presence of resonances belonging to mono- and di-CGAs, furanocoumarins and flavonoids in the STD spectrum (Fig. 7B) was the unequivocal demonstration of their interaction with peptide oligomers. In particular, the compounds showing the highest STD relative intensities were di-substituted CGAs. Notably, also signals in the regions 4.5-3.2 ppm appeared in the STD spectrum. Notably, they are not signals of the free sugars glucose and sucrose, metabolites that are very abundant in the crude extract. In fact, the resonances in this region of the STD spectrum do not belong to these molecules, as can be deduced from the form of the signals by comparing the STD spectrum (Fig. 7B) with the 1H NMR spectrum of the mixture (Fig. 7A). We can infer that they belong to the carbohydrate moieties of glycosylated furanocoumarins and flavonoids identified by UPLC-HR-MS. Thus, being fraction C from RP chromatography the only one containing all these molecular classes (mono- and di-CGAs, furanocoumarins and flavonoid glycosides), STD-NMR binding studies allowed rationalizing the highest biological efficacy of this enriched fraction among those tested.

An additional STD experiment, confirming these findings, was performed on a sample containing fraction C and Aβ oligomers and is reported in Supplementary Material – Fig. S11.

4. Conclusion

In conclusion, we exploited NMR and UPLC-HR-MS to characterize the metabolic profile of Peucedanum ostruthium, a medicinal plant already described for its several beneficial effects on human health.

The detection of a conspicuous content of CGAs tickled us to test the putative anti-amyloidogenic activity of Imperatoria, based on recent findings about the preventive effect of mono-substituted CGAs against Aβ peptides [8].

We employed ThT assay and AFM analysis to monitor Aβ1-42 aggregation and to study aggregates’ morphology in absence and presence of polyphenol-enriched fractions of crude Imperatoria rhizome extract. The sample showing the highest potency in preventing peptide aggregation was also the most effective in preventing Aβ-induced neurotoxicity, according to MTT assays carried out on the SH-SY5Y human cell line.

STD-NMR experiments revealed that di-substituted CQAs are the best ligands of Aβ1-42 oligomers contained in Imperatoria rhizome extract, as they showed relative STD intensities higher than mono-substituted CQAs and thus higher affinities for the target. Our study showed also glycosylated flavonoids and furanocoumarins as able to bind Aβ1-42 oligomers. The co-presentation of these compounds in the same extract allows obtaining a significant biological activity.

All together, these data provide important structural information for the rational design of new molecules with anti-amyloidogenic activity and molecular tools for the specific targeting of amyloid aggregates.

Moreover, our results demonstrate once again the power of NMR spectroscopy in the screening of complex natural extracts for the direct identification of bioactive molecules, bypassing the isolation of the single components.

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Conflicts of interest

Authors declare no conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.10.016.

References


