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New insights into phenol and polyphenol composition of *Stevia rebaudiana* leaves

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ABSTRACT

The diversity in phenols and polyphenols of stevia leaf has been simplified applying sequential fractionation techniques on its ethanol extract through ultrasound assisted maceration. Two of the fractions obtained by reverse-phase column chromatography resulted differently active in an extensive antioxidant and cytotoxic screening. Both fractions were chemically profiled by ultra-performance liquid chromatography (UHPLC) coupled with electrospray ionization (ESI) quadrupole/time-of-flight (QqTOF) mass spectrometry (MS). One of the fractions was composed mainly of chlorogenic acids and flavonol triglycosides, whereas the other was rich in flavonoids mono- and diglycosides and in their hydroxycinnamoyl derivatives. Among the fifty compounds identified, non-phenol metabolites, such as benzyl primeveroside and roseoside, as well as a lignan polyphenol (**5**′), are reported for the first time as constituents of the *Stevia* leaf.

1. Introduction

Plants have an innate ability to synthesize and accumulate secondary metabolites to perform their normal physiological functions, to protect themselves from microbial pathogens and animal herbivores, and/or to respond to environmental stress conditions. Among these compounds, phenols and polyphenols appear to act as non-enzymatic antioxidants [1]. Their intrinsic ability to neutralize, deactivate or suppress free radical species by donating an electron or hydrogen atom or, directly, to act as inhibitors of lipoperoxidation chain reactions, allows them to play important roles in ROS metabolism and to avoid uncontrolled oxidation of essential biomolecules. Antioxidant phenol and polyphenols are found ubiquitously in plants, and they are common constituents of edible plants, whose intake has been associated with a lower risk of chronic and degenerative disease onset [2]. For this reason, polyphenols are defined as dietary antioxidants. In this context, the use of plant extracts containing antioxidant compounds, as additives or nutritional supplements, and the use of pure isolated antioxidants are of great interest [3]. Indeed, evaluating the nutraceutical value of some pure (poly)phenols and/or their herbal/food complex on intracellular redox state, it was found that they could exert both anti- and pro-oxidant activities. As pro-oxidants, polyphenols are capable of generating radicals and may exhibit antiproliferative activities towards cancer cells. The relationships between natural polyphenols, apoptosis and cancer were also identified by studies on the ability of these compounds to act as cancer chemopreventive and/or chemotherapeutic agents [4].

Indeed, a full understanding of the phenolic and polyphenolic composition of a plant species is an important challenge, which today can be favorably pursued, thanks to an analytical approach that combines techniques involved in phytochemical analysis with those advanced for mixture analysis. LC–MS-methods are coming more and more into focus, since they allow reliable identification of analytes with a substantially improved sensitivity. The extraction phase is always the key step, and the chosen method can massively influence the qualitative-quantitative phytochemical composition of the plant extracts and thus their bioactivity. With this in mind and with the aim of obtaining functional and bioactive (poly)phenol

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fractions from leaves of *Stevia rebaudiana* experimentally cultivated in the Campania Region (Italy), an extraction/fractionation strategy was applied in order to deeply unravel the metabolic complex of the leaf ethanol extract, obtained through ultrasound accelerated maceration. Two main (poly)phenol fractions (CAsF -Chlorogenic Acids rich Fraction, and FlsF – Flavonoids rich Fraction) were achieved and profiled through UHPLC-ESI-Q*q*TOF-MS/MS techniques. Although leaves of *Stevia rebaudiana*, a species with known sweetening properties [5], are already known to contain potentially bioactive polyphenolic components [6–8], the employment of simple extractive and chromatographic techniques allowed us to discover new *Stevia* chemical entities. Furthermore, the bioactivity screening of the fractions obtained stressed a fundamental, but too often underestimated, concept in natural products chemistry, i.e, the bioactivity of a plant species is always in the chemistry of its derived extracts.

2. Materials and methods

2.1. Reagents and chemicals

All the solvents used for extraction and fractionation purposes, acetonitrile (LC–MS grade), formic acid (98%, for mass spectrometry) and pure reference compounds chlorogenic acid (5-*O*-CQA), neochlorogenic acid (3-*O*-CQA), cryptochlorogenic acid (4-*O*-CQA), quercetin 3-*O*-glucoside and rutin were purchased from Sigma-Aldrich (Buchs, Switzerland), as well as reagents used for assessing antioxidant screening, except for ABTS [2,2′-azinobis-(3-ethylbenzothiazolin-6-sulfonic acid)], which was from Roche Diagnostics (Roche Diagnostics,Mannheim, Germany). Cell culture media and reagents for cytotoxicity testing were purchased from Invitrogen (Paisley, Scotland, UK), except MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] and INT [(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride)], which were from Sigma-Aldrich.

2.2. Plant drug extraction and fractionation

Stevia rebaudiana seedlings, transplanted in nursery beds, were experimentally planted out, in May 2015, in a dedicated field of the Campania region (Italy) near Caserta (latitude 41° 06′ 13.5″ N, longitude 14° 11′ 25.6″ E, 24 m a.s.l). *Stevia rebaudiana* leaves harvested at the beginning of August 2015, were transported in laboratory and freeze-dried (3 days) by using the FTS-System Flex-Dry™ instrument (SP Scientific, Stone Ridge, NY, USA). Cryo-dried leaves were pulverized by a rotating knives homogenizer and a sample (~3.0 g) underwent ultrasound accelerated maceration (Advantage Plus model ES, Darmstadt, Germany), using ethanol as extracting solvent. In order to obtain the complete recovery of the metabolic content from *Stevia* leaves, three sonication cycles were performed (30 min × 40 mL EtOH, each). At the end of each cycle the sample was centrifuged at 5000 × *g* for 5 min at 4 °C in an AvantTM J-25 centrifuge (Beckman Coulter, USA), equipped with a JA-14 rotor. The obtained supernatants were dried using a rotary evaporator (Heidolph Hei-VAP Advantage, Germany). Ethanol extracts were further fractionated by column chromatography (SiO_2 CC; h 10 cm, Ø 2.5 cm), eluting first with $CHCl_3$ (40 mL × 3), and then with MeOH (40 mL × 3). The alcoholic fraction (1.05 g) was then chromatographed by RP-CC 18 (h 124 cm, Ø 1.0 cm), eluting with MeCN:MeOH:H_2O solutions with decreasing polarity. Among the fractions obtained, CAsF and FlsF fractions were eluted with MeCN:MeOH:H_2O (1:1:6) and MeCN:MeOH:H_2O (1:1:4) solutions, respectively.

2.3. UHPLC-ESI-QqTOF MS/MS analyses of Stevia rebaudiana fractions

A Shimadzu NEXERA UHPLC system was used with Phenomenex Luna Omega Polar C-18 and Luna Omega 1.6 μm C18 50 × 2.1 mm columns. Separation was achieved with a gradient of water (A) and acetonitrile (B), both with 0.1% formic acid. Starting with 95% A, a linear gradient was followed to 85% A in 4 min, held at 85% A for other 4 min, then decreasing to 87.5% A at 10 min, and to 55% A at 13 min. The mobile phase composition was maintained at 55% A for another 1.5 min, then returned to the starting conditions and allowed to re-equilibrate for 2 min. The total analysis time was 16.5 min, the flow rate was 0.5 mL min^{-1}, and the injection volume was 2.0 μL.

MS analysis was performed using a hybrid Q-TOF MS instrument, the AB SCIEXTripleTOF®4600 (AB Sciex, Concord, ON, Canada), equipped with a DuoSpray™ ion source (consisting of both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) probes), which was operated in the negative ESI mode. The APCI probe was used for automated mass calibration using the Calibrant Delivery System (CDS). The CDS injects a calibration solution matching the polarity of ionization and calibrates the mass axis of the TripleTOF® system in all scan functions used (MS and/or MS/MS). The Q-TOF HRMS method, which combines TOF-MS and MS/MS with Information Dependent Acquisition (IDA) for identifying non-targeted and unexpected compounds, consisted of a full scan TOF survey (dwell time 100 ms, 150–1500 Da) and a maximum number of eight IDA MS/MS scans (dwell time 50 ms, 80–1300 Da). The MS parameters were as follows: curtain gas (CUR) 35 psi, nebulizer gas (GS 1) 60 psi, heated gas (GS 2) 60 psi, ion spray voltage (ISVF) 4.5 kV, interface heater temperature (TEM) 600 °C, declustering potential (DP) 70 V. Collision Energy (CE) applied was 35 V with a collision energy spread (CES) of 25 V. The instrument was controlled by Analyst® TF 1.7 software, while data processing was carried out using PeakView® software version 2.2.

2.4. Antioxidant screening of Stevia rebaudiana fractions

The assessment of antioxidant and radical scavenging abilities of *Stevia* fractions was carried out by applying three different methods. Each fraction was solubilized in a MeOH:H_2O (1:1, v:v) solution to form a stock solution of 50.0 mg/mL. The activity, estimated for dose levels equal to 5, 10, 25, 50 and 75 μg/mL, was compared to a blank arranged in parallel to the samples. Pure reference compounds 5-*O*-caffeoylquinic acid and rutin, representative of identified metabolites, were also tested. ABTS [2,2′-azinobis-(3-ethylbenzothiazolin-6-sulfonic acid)] radical cation scavenging capacity and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capability were determined as previously reported [9]. Results are the mean ± SD values. ID_{50} values were calculated by non-linear regression analysis (x-axis was logarithm-transformed) by using the GraphPad Software (version 6.0, CA, USA). The total phenol content of the samples was also measured and expressed as mg of the relative reference compound equivalents per 100 g of the matrix.

2.5. Cytotoxicity assessment

Samples of each extract were prepared as stock solutions of 50.0 mg/mL in DMSO and further diluted in FBS-free cell culture medium to appropriate final dose levels (DMSO final concentration was equal to 0.1% (v/v)). Recorded activities were compared to an untreated blank in parallel to the samples. Results are the mean ± SD values. ID_{50} values were calculated by non-linear