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α-Glucosidase inhibition and antioxidant activity of an oenological commercial tannin. Extraction, fractionation and analysis by HPLC/ESI-MS/MS and ^{1}H NMR

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ABSTRACT

Two batches of the oenological tannin Tan'Activ R, (toasted oak wood – *Quercus robur*), were extracted with ethanol. A fractionation on XAD-16 afforded four fractions for each extract. Extracts and fractions were evaluated for antioxidant activity (DPPH), polyphenol content (GAE) and yeast α-glucosidase inhibitory activity. Comparable results were obtained for both columns, fractions X1B and X2B showing the highest antioxidant activity. Fractions X1C and X2C notably inhibited α-glucosidase, with IC_{50} = 9.89 and 8.05 μg/mL, respectively. Fractions were subjected to HPLC/ESI-MS/MS and ^{1}H NMR analysis. The main phenolic constituents of both X1B and X2B were a monogalloylglucose isomer (**1**), a HHDP-glucose isomer (**2**), castalin (**3**) gallic acid (**4**), vescalagin (**5**), and grandinin (or its isomer roburin E, **6**). X1C and X2C showed a complex composition, including non-phenolic constituents. Fractionation of X2C gave a subfraction, with enhanced α-glucosidase inhibitory activity (IC_{50} = 6.15 μg/mL), with castalagin (**7**) as the main constituent.

1. Introduction

Plant polyphenols have been attracting renewed attention in recent years, and a growing number of studies deals with the beneficial health properties of phenolic compounds found in food and beverages. In parallel, polyphenols are exploited in the fields of agro-food, and in the cosmetic and over-the-counter (OTC) drug industry (Quideau, Deffieux, Douat-Casassus, & Pouysegu, 2011). Originally, the 'plant polyphenols' were substantially equivalent to 'vegetable tannins', with reference to the tanning action of some plant extracts that had been employed for centuries in the leather-making process. Oenological tannins are widely used in wine making and ageing to improve the organoleptic properties of wine (Obreque-Slier, Pena-Neira, Lopez-Solis, Ramirez-Escudero, & Zamora-Marin, 2009). Tannins have other industrial uses such as in dyestuffs and the food industry (Khanbabaee & van Ree, 2001). Further interest towards vegetable tannins comes from other promising and probably underestimated properties of these natural polyphenols. Tannins in medicinal and food plants have various biological properties, among which the inhibition of lipid-peroxidation, antitumor, antimutagenic, antiviral, antibacterial and haemostatic activity (Martinez et al., 2015; Okuda & Ito, 2011). Hydrolyzable tannins are frequently cited for their antioxidant (Chen et al., 2014) or antimicrobial (Martinez et al., 2015) activity as well as for chemoprevention of degenerative diseases (Cerda, Tomas-Barberan, & Espin, 2005). Indeed, there is a well-known relationship between antioxidant activity and chemopreventive activity (Pandey & Rizvi, 2009; Quideau et al., 2011). Recently, plant tannins have attracted attention due to their multifunctional properties that are beneficial to human health, and in particular for managing of diabetes (Kumari & Jain, 2012), a pathology that has reached epidemic proportions in the last decade. A number of hydrolyzable tannins have been reported as inhibitors of α-glucosidase, a key enzyme in the modulation of glucose absorption and consequently a promising target for development of anti-diabetic drugs (Yang, Lian, & Yu, 2015).

However, commercial tannins are complex mixtures and their chemical composition is not always clearly defined. We therefore planned to examine some commercial samples of hydrolyzable tannins that are widely exploited for commercial purposes. To this end we firstly considered tannins produced by Silvateam Spa (http://en.silvateam.com/), a company operating in more than 60 countries with products used for oenology, leather tanning, and other industrial applications including food ingredients. More specifically, the Tan'Activ® series (for oenology applications),

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includes various polyphenol-based powdered products, among which we selected Tan'Activ R (toasted oak wood), because *Quercus* spp. and *Q. robur* in particular are widely reported in the literature as a source of polyphenols with promising biological activities (Fernandes et al., 2009). In addition, it is well-known that oak heartwood used in barrel ageing is a source of tannins in wine (Chatonnet & Dubourdieu, 1998). Their occurrence is also related to the 'toasting' process normally applied to the oak wood for barrels (Doussot, De Jeso, Quideau, & Pardon, 2002). It is also worth noting that only oak and chestnut are approved by the International Organization of Vine and Wine (OIV) for wine ageing (Fernandez de Simon et al., 2014). Oak-derived tannins have been found in wine or whiskey as the product of oxidation processes or complex chemical reactions between wine flavonoids and ellagitannins (Fujieda, Tanaka, Suwa, Koshimizu, & Kouno, 2008; Petit et al., 2013). The presence of oak wood tannins in these beverages and their use in wine production suggests the absence of significant toxicity for these polyphenols, and this is a favorable property for possible applications in agro-food or the food supplement industry.

On the basis of the above, we decided to carry out a simple fractionation method of the ethanol extract of the commercial Tan'Activ R to obtain fractions with enhanced antioxidant activity (scavenging of DPPH radical), for agro-food or nutraceutical applications. At the same time, considering the present active search for new anti-diabetic agents, we planned to evaluate extracts and fractions for inhibition of yeast α-glucosidase. The main constituents in each extract/fraction were identified mainly by means of HPLC/ESI-MS/MS, supported by literature search, as discussed in detail in the following. However, ^{1}H NMR spectra were also run to corroborate some MS-based identifications, as well as to gain further information about possible non-ionizable constituents.

2. Materials and methods

2.1. Samples and chemicals

As we know from a personal communication by Silvateam SpA, Tan'Activ R was obtained from oak wood (*Quercus robur*). The wood logs were selected in the forest of Piemonte (Italy), stored for few months and subsequently the bark was removed. The wood material was milled and extracted with hot water. The water extract was then concentrated and extracted with food grade organic solvents, normally ethanol/water mixtures. The solvents were then completely stripped away under vacuum. The concentrated solution was spray-dried in order to obtain a powdered product. Two different batches of the commercial tannin Tan'Activ R were supplied by Silvateam (S. Michele Mondovì, CN, Italy). For convenience, in the following these batches are named QRT1 (batch 1: N 01121) and QRT2 (batch 2: N 03113). HPLC-grade acetonitrile (ACN) and water, methanol (MeOH), ethanol (EtOH) and Folin-Ciocalteau reagent were purchased from Merck; 2,2-diphenylpicrylhydrazyl radical (DPPH·) and formic acid (FA) were acquired from Fluka; gallic acid, quercetin, fast red B dye, methanol-d_4 (CD_3OD), Amberlite XAD-16 and Sephadex LH-20 were purchased from Sigma Aldrich. Cerium (IV) sulphate and ammonium molybdate were purchased from Carlo Erba. α-Glucosidase from *Saccharomyces cerevisiae* (Type I, lyophilized powder, ⩾10 units/mg protein) and the substrate *p*-nitrophenyl-α-D-glucopyranoside (*p*-NP-α-Glc) were purchased from Sigma Aldrich. TLC was carried out using pre-coated silica gel F254 plates (Merck); spot visualization was done under UV light at wavelengths of 254 and 366 nm, or by staining with a solution of cerium sulphate and phosphomolybdic acid followed by heating; or with DPPH or fast red B solutions.

2.2. HPLC/ESI-MS/MS analysis

Mass spectrometric analyses were performed using a Surveyor MS Pump and a Surveyor autosampler (Thermo Scientific) maintained at 4 °C. 20 μL of each sample (10 μg/μL) were loaded onto a reversed phase Waters Symmetry C18 column (150 mm × 1 mm i.d., 100 Å, 3.5 μm). Separation was conducted at 25 °C with a linear gradient of H_2O + 1% FA (solvent A) and ACN + 1% FA (solvent B) at 50 μL/min. Chromatographic separation was performed in 55 min with B: from 5% to 15% in 25 min, 25% in 40 min, 30% in 45 min and 55% in 55 min. The HPLC system was interfaced with a Thermo Scientific LCQ-DECA ion trap mass spectrometer equipped with an ESI ion source operating in MS/MS negative ion mode under the following conditions: capillary temperature 220 °C, sheath gas 30 a.u.; source voltage −3.5 kV and capillary voltage −18 V. Repetitive mass spectra were acquired in negative ion mode in the *m*/*z* range 100–2000. Analysis was performed by the data dependent method as follows: (i) full-scan MS in the *m*/*z* range 100–2000; (ii) zoom scan of the five most intense ions (isolation width: 2); (iii) MS/MS analysis of with normalized collision energy: 30 a.u. and activation Q: 0.250. Mass calibration was made using a standard mixture of caffeine (Mr. 194.1 Da), MRFA peptide (Mr. 524.6 Da) and Ultramark (Mr. 1621 Da). Data acquisition and data analyses were performed using the Xcalibur v. 1.3 Software.

2.3. NMR analysis

^{1}H NMR spectra of fractions were run on a Varian 500 V NMR-S spectrometer operating at 499.86 MHz (^{1}H). Samples (0.0100 g) were dissolved in CD_3OD and chemical shifts (δ) where indirectly referred to TMS using residual solvent signal as reference. All ^{1}H NMR and COSY experiments were acquired at constant temperature (300 K) and performed using software supplied by the manufacturers.

2.4. Preparation of Tan'Activ R extracts

Powdered samples of QRT1 (10.0875 g) and QRT2 (10.0620 g) were extracted with EtOH for 2 h (2 × 100 mL) and subsequently for 20 h (100 mL) with stirring at room temperature (25 °C). After filtration and subsequently evaporation of the solvent, a residue (QR1E) of 6.6209 g (65.6%) was obtained from QRT1 extraction and a residue (QR2E) of 7.5617 g (75.2%) was recovered from QRT2 extraction. In Table 1 (top) we report the percentage yields for the two ethanolic extracts QR1E and QR2E, expressed as g extract/100 g powdered QRT1 and QRT2.

2.5. Amberlite fractionation

2.5.1. QR1E Amberlite fractionation

An aliquot of ethanolic extract QR1E (1.1071 g) was solubilised in MeOH and subjected to column chromatography (25 cm × 2.5 cm) on Amberlite XAD-16 resin previously packed with methanol and equilibrated with deionised water. The column was eluted with deionised H_2O (300 mL) followed by EtOH (300 mL). The eluate was split into four fractions: X1A (200 mL), X1B (100 mL), X1C (100 mL) and X1D (200 mL). The four fractions were evaporated under vacuum, weighed and stored for further analyses: X1A (0.2299 g), X1B (0.0377 g), X1C (0.7518 g) and X1D (0.0757 g) with a total weight of 1.0951 g (98.9% of QR1E recovered).

2.5.2. QR2E Amberlite fractionation

A sample of ethanolic extract QR2E (1.2043 g) was fractioned on Amberlite XAD-16 column (25 cm × 2.5 cm) eluting with H_2O (300 mL) and subsequently with EtOH (300 mL). Also in this case