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Recovery of polyphenols from red grape pomace and assessment of their antioxidant and anti-cholesterol activities

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The present work aimed at the recovery and characterization of polyphenolic compounds extracted from red grape pomace (*Vitis vinifera* L.), a winemaking by-product. Polyphenolic compounds of wet (WP) and dried (DP) red pomace were recovered by enzymatic digestions and ethanol-based extractions. Fungamyl and Celluclast enzymes were found to be the most effective in enhancing polyphenol release from WP. WP samples showed the highest capacity of releasing polyphenols with 2 h control 24°C and 2 h 1% Celluclast resulting as the best treatments. A significantly lower amount of polyphenols was recovered from DP most probably as a consequence of the pomace drying. The best extracts contained high amounts of total polyphenols, flavonoids, tannins and anthocyanins and exerted antioxidant and cholesterol-lowering activities. The results support the possibility of exploiting the extracts coming from grape processing by-products as ingredients for functional and innovative products in the nutraceutical, pharmaceutical or cosmetic fields.

Introduction

The food processing industry annually produces large quantities of both liquid and solid waste and by-products. These by-products constitute a rich but yet underutilised source of valuable compounds, which may find an application in the food, feed, cosmetic and pharmaceutical industries. Grape (*Vitis* sp.) is the world's largest fruit crop mostly used in wine making, a process during which approximately 20–30% of the weight of processed grapes ends up as pomace, its primary by-product [1]. Pomace mainly consists of pressed skins, seeds and stems. These large amounts of by-products constitute a serious environmental and disposal problem for wineries. However, they also represent a rich source of various high-value molecules, such as phytochemicals with high antioxidant activity [1–4]. Grape pomace is characterized by a high content of polyphenol compounds that are only partially extracted during the winemaking process and whose range and extractability mainly depends on the technological parameters applied during vinification. Flavonoids (such as anthocyanins and catechins), phenolic acids and stilbenes are among the main constituents of grape pomace [2,4]. The beneficial influence on human health of grape and wine phenols has been increasingly investigated with evidences provided for their protective effects against chronic diseases such as cancer, neurodegeneration and cardiovascular pathologies [5]. Grape polyphenols have also been shown to have anti-inflammatory and anti-microbial properties [4,6,7]. Due to their biological and chemical properties, grape pomace extractable components may have many applications: as ingredients of functional foods and feeds, cosmetics and nutraceuticals; as natural colorants and preservatives of foods [2–4]. However, after ingestion dietary phenols are modified

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and degraded in the gastrointestinal tract and appear in the circulatory system at low concentration and in different chemical forms [5]. Comparison of several recovery methodologies showed that solvent (mainly water, ethanol and methanol) and supercritical fluid extractions are the most efficient for grape pomace extraction [3,8,9]. Enzymatic digestion using cell wall polysaccharide degrading enzyme mixtures was also shown to enhance the release of grape phenolics still present in pomace [10,11].

In the present work, the recovery of polyphenolic compounds was studied on wet pomace (WP) and dried pomace (DP) from red grapes (*Vitis vinifera* L., mixture of Sangiovese and Montepulciano cultivars), by means of enzymatic digestions and ethanol-based extractions. The extracts with the highest amount of compounds were tested for their biological activities in view of their possible future application in cosmetic, nutraceutical and pharmaceutical industries.

Materials and methods

Materials

Red wine pomace derived from a mix of *Vitis vinifera* cv. Sangiovese and Montepulciano, harvested in the year 2011, was supplied by the Cantine Moncaro wineries (Jesi, Ancona, Italy). The same day of wine production, pomace was either frozen (wet pomace, WP) or dried (dried pomace, DP) in an industrial vented oven (60°C for 24 h) and stored at −20°C until used for analyses. Both types of pomace contained berry skins, seeds, petioles and stalks.

Pomace enzymatic digestion

WP was ground in a kitchen blender with the addition of distilled water (1:5 g/mL), while DP was ground directly and rehydrated with distilled water (1:5 g/mL for 1 h) just before enzymatic digestion. To determine the percentage of dry weight (DW), 10 g fresh weight (FW) of WP were placed at 80°C for 48 h and weighed (DW was about 54% of FW). Enzymatic digestions of ground WP and DP pomace suspension (20 mL aliquots) were carried out by adding different concentrations (0.5, 1 or 2% enzyme volume/pomace DW) of Pectinex 3XL (pectinase from *Aspergillus niger*, 3595 U/mL), Pectinex Ultra SPL (pectinase from *Aspergillus aculeatus*, 4218 U/mL), Termamyl (α-amylase from *Bacillus licheniformis*, 605 U/mL), Fungamyl (α-amylase from *Aspergillus oryzae*, 881 U/mL), Pentopan 500BG (xylanase from *Thermomyces lanuginosus*, 2.75 U/g), or Celluclast (cellulase from *Trichoderma reesei* ATCC 26921, 790 U/mL), all purchased from Sigma–Aldrich (Milano, Italy). The different enzymatic treatments were incubated on an orbital shaker (150 rpm) at different incubation times and at the enzyme optimal working temperature according to the Sigma working certificate (Pentopan at 30°C, Celluclast at 37°C, all the other enzymes at 24°C). Controls performed without the addition of enzymes were incubated under the same conditions as the treated samples. After incubation, the samples were centrifuged at 5000 rpm for 10 min at room temperature (Eppendorf centrifuge 5804 R, rotor A 4-44, Hamburg, Germany). The supernatant was removed and filtered under vacuum through Whatman GF/B filters using a Millipore funnel apparatus, and stored at −20°C until further analyses. After having selected the best treatment conditions for both WP and DP, the water enzymatic treatments were repeated and after removing the supernatant the residual pellet was incubated with 30 mL of 95% (v/v) ethanol at 24°C overnight on an orbital shaker (150 rpm). Subsequently, the ethanol supernatant was separated from the pellet by centrifugation for 10 min at room temperature at 5000 rpm and filtered through a Whatman GF/B filter. Both aqueous and ethanol supernatants were stored at −20°C until further analyses.

Spectrophotometric and HPLC-DAD analyses

Total polyphenol and total flavonoid content of aqueous and ethanol pomace extracts was quantified by using the Folin–Ciocalteu assay and the Zhishen *et al*. [12] methods with minor modifications [13]. The results were, respectively, expressed as gallic acid (GA) and catechin (CAT) equivalents by means of calibration curves. The amount of tannins was analysed using the method of Porter *et al*. [14] with minor modifications. Anthocyanin content was determined as in Ferri *et al*. [15] while color density was determined as in Mazza *et al*. [16]. Phenolic compounds were extracted both from water supernatants and ethanol pomace extracts [15] before being directly injected into the HPLC-DAD system (column Gemini C18, 5 μm particles 250 mm × 4.6 mm, pre-column SecurityGuard Ea, Phenomenex, Torrence CA, USA) equipped with an on-line diode array detector (MD-2010, Plus, Jasco Instruments, Großumstad, Germany). The adopted HPLC-DAD separation procedure allowed for the simultaneous analysis and identification of 28 different compounds among stilbenes, phenolic acids and flavonoids by direct comparison both of retention time and absorbance spectra to the related compound standard [15].

Determination of biological activities

In vitro antioxidant activities were measured using the 2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method with minor modifications [13]. The results were expressed as ascorbic acid (AA) equivalents by means of a dose–response calibration curve. Cell-based assays were performed to evaluate the effect of pomace extracts with the highest polyphenol amount on transcriptional regulation of cholesterol 7α-hydroxylase (cyp7a1) and sterol 27-hydroxylase (cyp27a1). This dual-color reporter assay allowed to preliminary investigation the cholesterol-lowering activity of the samples. Human hepatocarcinoma HepG2 cells overexpressing the human farnesoid X receptor (FXR) (a generous gift from Prof. N. Carulli from the University of Modena, Italy) were used and cultured and assays were performed as previously reported [17].

Statistical analyses

All the treatments were performed two times independently and the two extracts were analysed in two technical replicate each. The results are expressed as the mean of four data ± SD. Cell-based assays were performed three times and each data point had at least three technical replicates. The results are expressed as the mean of nine data ± SD. Statistically significant differences between data sets were analysed using the Student's *t* test ($p < 0.05$) (Statistica 6 programme, Statasoft Inc., USA).

Results and discussion

Red pomace enzymatic digestions

Enzymatic digestions of both WP and DP were carried out by adding different concentrations (0.5, 1 or 2% enzyme volume/pomace DW)

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