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Phytochemistry and activity against digestive pathogens of grape (*Vitis vinifera* L.) stem's (poly)phenolic extracts

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ABSTRACT

Winemaking industry produces huge amounts of organic residues including grape pomace, grape stems and wine lees, with deleterious impact on the environment and concerning the sustainability of this socio-economic activity, being grape stem the less explored material. The content in phytochemicals of grape and vinification residues as well as the antimicrobial efficiency described for the identified compounds, prompted us to the evaluate the capacity of grape stem's extracts to inhibit the growth of digestive pathogens, and therefore, their potential as a functional ingredient that could prevent microbial disturbance. The phytochemical composition was determined by HPLC-DAD-ESI-MS^n and was correlated with the antimicrobial activity *in vitro*, against Gram positive (*Listeria monocytogenes*, *Staphylococcus aureus* and *Enterococcus faecalis*) and Gram negative (*Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*) digestive pathogens. Antimicrobial activity was determined by the assessment of the Minimum Inhibitory Concentration and the capacity of grape stem's extracts to inhibit microbial growth. The correlation analyses performed pointed out kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside and caftaric acid as compounds responsible for the antimicrobial effect. The confirmation of the inhibitory effect grape stem's extracts on digestive pathogens *in vitro* encourages further evaluations *in vivo* on their capacity to prevent the disturbance of intestinal homeostasis.

1. Introduction

The interest in identifying natural sources of bioactive compounds has enhanced during the last decade, aimed in developing valuable products that contribute to the prevention of degenerative diseases. This interest has promoted, over recent years, to the evaluation of numerous plant materials on their content in bioactive compounds (Galanakis, 2012).

The functional properties evaluated concerning newly identified phytochemicals include antioxidant, anti-inflammatory and antimicrobial activities, among others, which support their rational application by pharmaceutic and cosmetic industries (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). The search for new sources of natural bioactive compounds is of special relevance when considering the valorization of agro-food wastes with direct environmental impact that reduce the sustainability and viability of agro-food socio-economic activities (Rondeau, Gambier, Jolibert, & Brosse, 2013). In this sense, in Southern Europe, wine-making activity is stressed regarding the production of huge amounts of organic residues composed by skins, seeds and stems (Pinelo, Arnous, & Meyer, 2006).

So far, the evaluation of the phytochemical composition and biological activity of vinification residues has been focused in grape seeds, peels and pulp considered as independent materials or making part of grape pomace. The information recorded concerning these materials has encouraged the evaluation of additional residues as a source of valuable antimicrobial compounds, among other biological activities, that might be applied on food protection (Ahmad et al., 2014; Cheng, Bekhit, McConnell, Mros, & Zhao, 2012; Han, 2007; Jayaprakasha, Tamil, & Sakariah, 2003; Oliveira et al., 2013). In this sense, information available on the phytochemical

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composition and biological activity of grape stems continue being scarce because of this material was not considered a direct residue of the industrial process (vinification) but a side material of the wine-making activity (Teixeira et al., 2014).

Data available on the phytochemical composition of grape (*Vitis vinifera* L.) stem have stressed its content in flavan-3-*ols* (Jayaprakasha et al., 2003), being the concentration of flavonols, hydroxycinnamic acids and stilbenes of minor relevance (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012; Barros et al., 2014; Souquet, Labarbe, Le Guerneve, Cheynier, & Moutounet, 2000; Vivas et al., 2004).

To the best of our knowledge, to date, no studies have been done on the antimicrobial properties of grape stems, even though literature on their phytochemical content points out a promising antibacterial potential and encourage the evaluation of their potential as microbial growth inhibitor. In this sense, in the digestive tract these bioactive compounds may act selectively on the growth of pathological microbiota, and provide a valuable contribution to the microbial homeostasis (Cueva et al., 2010).

The aim of this study was to assess the phytochemical profile of (poly)phenolic extracts of grape stem of seven Portuguese varieties as well as evaluate the correlation of the phenolic composition recorded with the antimicrobial capacity against Gram positive (*Listeria monocytogenes*, *Staphylococcus aureus* and *Enterococcus faecalis*) and Gram negative (*Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*) of gastrointestinal pathogens *in vitro*.

2. Material and methods

2.1. Plant material

Grape (*V. vinifera* L.) stems from the red varieties 'Sousão', 'Touriga Nacional', 'Tinta Barroca' and 'Tinta Amarela', and the white cultivars 'Fernão Pires', 'Viosinho' and 'Rabigato' were cultivated in the Spring-Autumn season (2013) under Northern Portugal continental agro-climatic conditions. They were obtained from Quinta da Cavadinha ('Sousão', 'Touriga Nacional', 'Tinta Barroca' and 'Tinta Amarela'). Grape stems from the varieties 'Fernão Pires' and 'Rabigato' were donated by Quinta do Sol and 'Viosinho' was provided by Quinta de Nossa Senhora da Luz, Symington Family Estates.

For analytical purposes, grape stems were washed in tap water, chopped into small pieces and mixed thoroughly to be bulked again into three well-mixed replicates per variety ($n = 3$). Samples were then dried in oven at 40 °C for 72-h (Memmert, Schwabach, Germany), ground to a fine powder and stored protected from light for further analysis.

2.2. Qualitative and quantitative analysis of individual phenolic compounds by HPLC-DAD-ESI/MS^n

For phenolic extractions, each sample (100 mg) was mixed with 1 mL of methanol/formic acid/water (50:2:48, v/v/v), vortexed and sonicated in an ultrasonic bath. A centrifugation step (10,000 × g, 5 min) was used to separate the supernatant from the solid residue. Supernatants were filtered through a 0.45-μm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA). Chromatographic analyses for the identification were carried out on a Luna C18 column (250 × 4.6 mm, 5 μm particle size; Phenomenex, Macclesfield, UK). Water/formic acid (99:1, v/v) (solvent A) and acetonitrile/formic acid (99:1, v/v) (solvent B) were used for chromatographic separation in an Agilent HPLC 1100 series equipped with a photodiode array detector and a mass detector in series (Agilent Technologies, Waldbronn, Germany). The flow rate was 1 mL min^{-1} using the linear gradient scheme (*t* in min; %B): (0; 5%), (15; 15%), (30; 30%), (40; 50%), (45, 95%), and (50; 5%). The equipment consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A) and a photodiode array detector (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, version 08.03). Flavonols were quantified as quercetin-3-*O*-glucoside at 360 nm, hydroxycinnamic acids as 5-*O*-caffeoylquinic acid at 320 nm, anthocyanins as cyanidin-3-*O*-glucoside at 520 nm, and stilbenes as resveratrol at 320 nm.

For proanthocyanidins extraction, 1.6 g of each dried and powdered material was homogenized one round for 1 min with 25 mL of acetone/water/acetic acid (70:29.5:0.5, v/v/v) using an Ultraturrax® (Ika, Staufen, Germany), sonicated for 15 min and centrifuged (JP Selected Centronic Centrifuge, Barcelona, Spain) at 10,000 × g for 10 min. Supernatants were concentrated in a rotary evaporator at 35 °C, reconstituted in ultrapure-water (1 mL) and filtered through a C18 Sep-Pak cartridge (Waters Associates, Milford, MA). Retained phenolic compounds were eluted with 8 mL of methanol. The methanol was evaporated in a rotary evaporator at 35 °C. The dried residues were dissolved in 1 mL of acetonitrile/acetic acid (98:2, v/v) and filtered through a 0.22 μm PDVF filter (Millex HV13, Millipore, Bedford, MA). Each sample (3 μL) was injected into LCMS/MS for identification and quantification of proanthocyanidins.

Chromatographic separation of target compounds was carried out on a Develosil 100 Å normal phase column (250 × 0.5 mm, 5 μm particle size) (Phenomenex, Seto, Japan). The mobile phases employed were acetonitrile/acetic acid (98:2, v/v) (solvent A) and methanol/water/acetic acid (95:3:2, v/v/v) (solvent B). The flow rate was 10 μL min^{-1} using the linear gradient scheme (*t* in min; %B): (0; 0%), (40; 40%) and (50; 80%). Identification of compounds was made in a 1200 series micro-HPLC–DAD system (Agilent Technologies, Waldbronn, Germany) equipped with a degasser (model G1379B), a thermostatted autosampler (model G1377A), a capillary pump (model G1376A) and a photodiode array detector (model G1315D). The HPLC system was coupled to an ion trap mass spectrometer (ultra HCT Bruker, Bremen, Germany) equipped with electrospray ionization (ESI) and operated in negative ion mode. Data acquisition and processing were performed using software B.01.03-SR2 for ChemStation for an LC-3D system from Agilent Technologies. The capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan and daughter spectra were measured from m/z 100 to 1500. Collision-induced fragmentation experiments were realized in the ion trap using helium as the collision gas, with the collision energy set at 50%. Proanthocyanidins were quantified as (−)-epigallocatechin at 280 nm.

2.3. Bacterial isolates

Gram positive (*S. aureus* and *E. faecalis*) and Gram negative (*E. coli* and *Klebsiella pneumoniae*) bacterial isolates were collected from human patients, isolated and identified by the Hospital Center of Vila Real, North of Portugal. Isolates identification was performed by means of standard biochemical classification techniques (Murray, Boron, Pfaller, Tenover, & Yolken, 1999) using API 20E, API 20NE, API Staphy and API Step (BioMerieux), according to the procedure previously described (Jorgensen, Turnidge, & Washington, 2009), followed by genetic identification through 16S rRNA sequencing. *P. aeruginosa* and *L. monocytogenes* strains were obtained from American Type Culture Collection (ATCC) (Table 1).

2.4. Antimicrobial activity

2.4.1. Minimum inhibitory concentration

For the Minimum Inhibitory Concentration (MIC) assay a modified resazurin microtitre-plate assay was used as reported