Food Research International 87 (2016) 92–102

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Oxidative stress prevention and anti-apoptosis activity of grape (*Vitis vinifera* L.) stems in human keratinocytes

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ARTICLE INFO

Article history:
Received 27 April 2016
Received in revised form 22 June 2016
Accepted 30 June 2016
Available online 4 July 2016

Keywords:
Grape stems
Phenolic compounds
HPLC–PDA-ESi-MSn
Scavenging capacity
Glutathione
Lipid peroxidation
ROS
Apoptotic markers

ABSTRACT

To date, grape stems have been partially assessed on their content in phenolics and their radical scavenging activity, whilst the potential to modulate oxidative stress in biological models remains underexplored. In the present work, the effect of grape stems' phenolics on redox unbalance was evaluated in human keratinocytes (HaCaT cells). Grape stems' extracts were assessed on their phenolic composition by high performance liquid chromatography coupled with photodiode array detection and electrospray ionization-mass spectrometry (HPLC–PAD-ESi-MSn), besides on radical scavenging capacity (ABTS and DPPH). In addition, their protective effect against oxidative stress induced by H_2O_2 by the determination of the level of glutathione, reactive oxygen species, lipid peroxidation, and overall oxidative stress in HaCaT cells by flow cytometry was evaluated. This characterization allowed to identify five flavonols, one cinnamic acid, and one stilbene. A close correlation between the concentration of these phenolics and the capacity to scavenge free radicals and with the potential to modulate the redox balance in vitro was observed. From the analysis of correlation, the activity of malvidin-3-*O*-glucoside, malvidin-3-*O*-(6-*O*-caffeoyl)-glucoside, and malvidin-3-*O*-rutinoside with respect to the prevention of basal oxidative stress and the capacity of isorhamnetin-3-*O*-(6-*O*-feruloyl)-glucoside and kaempferol-3-*O*-rutinoside to prevent H_2O_2-induced redox unbalance were stated. Furthermore, grape stems' phenolics also showed an efficient capacity to modulate apoptosis in HaCaT cells, reducing the frequency of annexin V/PI double positive apoptotic cells by up to 99.5% relative to controls, which was further confirmed by the determination of the appearance of the occurrence of apoptotic bodies and the expression of activated (cleaved) caspase-3 by flow cytometry and western-blot, respectively. These results supported the potential of individual phenolics from grape stems to modulate oxidative stress, allowing to envisage dedicated combinations of single compounds for the development of efficient formulations efficient against oxidative stress.

1. Introduction

Grape fruit (*Vitis vinifera* L.) is widely produced in Europe due to its nutritional and sensory characteristics and the commercial uses foreseen. Classes of grapes produced include table grapes, wine grapes (used in viniculture), and raisin grapes. In addition to its physicochemical features, this fruit contains a myriad of bioactive compounds represented by nutrients and non-nutrients, among which have been highlighted polyphenols (En-Qin, Gui-Fang, Ya-Jun, & Hua-Bin, 2010). Hence, this agronomical production has an unquestionable economic relevance within the agro-food industry, as it indicates the high amounts processed annually (>60 mT), which corresponds to the 39% of European production of grapes (FAOSTAT, 2016).

Concerning the manufacturing applications of grapes, a high proportion of grapes produced annually are addressed to the winery industry. As a result of this use, a great volume of residue is generated, which is constituted by seeds, peels, stems, and residual pulp, representing around 30% of the fruit weight (Gonzalez-Centeno et al., 2012). From these by-products, grape stems make almost 25% of the total by-products, being the less characterized and valorized material (Bustamante et al., 2008). Hence, although grape stems have focused growing attention as a source of bioactive phytochemicals (Anastasiadi, Pratsinis, Kletsas, Skaltsounis, & Haroutounian, 2012; Barros et al., 2014; Karvela, Makris, Kalogeropoulos, & Karathanos, 2009), the available literature reporting the biological potential of the bioactive compounds present in these by-products is restricted to radical scavenging and antimicrobial activities (Barros et al., 2014; Dias et al., 2015). In this regard, the evaluation of the biological power of grape stems' phenolics by using more informative experimental

Abbreviations: AB, Alamar Blue®; CE, catechin equivalents; Fl, flavonoids; GAE, gallic acid equivalents; GSH, glutathione; LP, lipid peroxidation; MFI, mean fluorescence intensity; odP, *ortho*-diphenols; PI, propidium iodide; ROS, reactive oxygen species; RT, room temperature; TP, total phenols.

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http://dx.doi.org/10.1016/j.foodres.2016.06.030
0963-9969/

approaches would allow to take advantage of this material, contributing to the sustainability of the entire productive chain.

In vivo, over 95% of all the oxygen breath is processed by mitochondrial cytochrome oxidase, which is responsible for the production of the high energy containing molecules required for cells to function. These reactions, along with those catalyzed by monoamine oxidase, entail the generation of reactive oxygen species (ROS) (Cadenas & Davies, 2000), which display an important role in several biochemical processes such as signal transduction and gene expression (Ji, 2007). However, a disordered augment of ROS due to disturbances of the pro-oxidant/antioxidant balance influences the onset and severity of diverse pathologies (Halliwell, 2005). To revert this situation cells account with non-enzymatic and enzymatic mechanisms (Ergin, Hariry, & Karasu, 2013). Although the determination of the radical scavenging power by distinct methods provides very valuable information on the antioxidant potential, further characterizations using biological systems are still required. Thus, the use of biological models (in vitro and in vivo) would allow to monitor the oxidative stress reactions in human keratinocytes, providing information on the actual capacity of grape stems' phenolics to modulate oxidative stress at concentrations compatible with in vivo administrations. In this sense, the evaluation of the oxidative events in human keratinocytes (HaCaT cells) exposed to grape stems' phenolics with and without the concurrence of an oxidative stimulus (H_2O_2) constitutes an interesting approach because of the topological situation in the outermost layer of the skin, more exposed to external oxidative stress-inducing factors (Bito & Nishigori, 2012). This approach would allow to gain a rational understanding on the extent in which individual phenolics and the radical scavenging capacity may provide proper information on the activity against free radicals produced by cells and so act on the molecular pathways responsible for diverse pathophysiological situations.

To date, the group responsible for the present work has developed passionate work to evaluate the potential of grape stems as a source of phytochemicals with valuable biological activities, including proanthocyanidins, cinnamic acids, flavonols, and anthocyanins (Barros et al., 2014; Dias et al., 2015). Going forward from these previous works, the aim of the present study was to evaluate for first time the effect of grape (*V. vinifera* L.) individual phenolics on the modulation of H_2O_2-dependent oxidative stress in biological systems (human keratinocyte cell line, HaCaT), as well as to investigate their protective effect against oxidative stress and related apoptosis. Thus, the results presented in the present work will contribute to shed some light on the biological value of the underexploited residues obtained from winery industry.

2. Material and methods

2.1. Chemicals

The compounds 2,2-diphenyl-1-picrylhidracyl radical (DPPH•), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt ($ABTS^{\bullet+}$), 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ), mono and dibasic sodium phosphate, trizma® hydrochloride, potassium phosphate, annexin V-FITC, Mercury Orange, 2′,7′-dichlorofuorescein diacetate (DCFDA), and propidium iodide (PI) were obtained from Sigma-Aldrich (Steinheim, Germany). Meanwhile, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and magnesium chloride hexahydrate were purchased from Fluka Chemika (Neu-Ulm, Switzerland). Ultrapure water was produced using a Millipore water purification system (Millipore, Bedford, MA, USA). The Alamar Blue® (AB) reagent was purchased from Invitrogen (Life-Technologies, Oporto, Portugal). Trypsin-EDTA, Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), penicillin/streptomycin, glycine, sodium pyruvate, and (*N*-(fluorescein-5-thiocarbamoyl)-1.2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) (DHPE-FITC) were purchased from Gibco (Life-Technologies, Oporto, Portugal). The flat bottom 96-well plates were from Corning (New York, USA). The monoclonal antibody mouse anti-human β-actin (C4; sc-47778) was from Santa Cruz Biotechnology, Inc. (Frilabo, Portugal). Monoclonal antibody rabbit anti-human cleaved caspase-3 (Asp175; 5A1E) was purchased from Cell Signaling Technology (Izaza, Portugal). Polyclonal antibodies goat anti-mouse (RPN 5781) and goat anti-rabbit (RPN 5783), and the Amersham ECF Western Blotting Reagent Packs were from VWR (Portugal). Salts and chemicals for preparation of buffers for western blot were from Sigma Aldrich (Steinheim, Germany).

2.2. Plant material and sample processing

Grape (*Vitis vinifera* L.) stems from varieties 'Tinto Cão', 'Tinta Barroca', 'Malvasia Fina', and 'Moscatel Branco' were cultivated in the Spring–Autumn season (2014) under northern Portugal continental climate and obtained from Quinta da Cavadinha (Pinhão, Portugal). For analytical purposes, plant material was washed in tap water, chopped into small pieces, and mixed thoroughly to be bulked again into 3 replicates per variety ($n = 3$). Then, samples were dried in oven (Memmert, Schwabach, Germany) at 40 °C for 72 h, grounded to a fine powder, and stored protected from light and humidity till analysis.

Each sample (40 mg) was mixed with 1.5 mL of methanol/distilled water (70:30, v/v). Then, samples were vortexed and phenolic compounds were extracted by agitation at room temperature (RT) for 30 min, afterwards were centrifuged at 5000 rpm for 5 min, at 4 °C (Sigma, Steinheim, Germany), and the supernatant was collected. This procedure was repeated three times and the final volume was made up to 5 mL. Supernatants were filtered through a 0.45-μm PVDF filter (Millex HV13, Millipore, Bedford, MA, USA) and stored at 4 °C until analysis.

2.3. HPLC–PAD-ESi-MSn analysis of phenolic content

Chromatographic separation of individual phenolic compounds present in grape stems was carried out on a Luna C18 column (250 × 4.6 mm, 5 μm particle size; ACE, Aberdeen, Scotland), using distilled water/formic acid (99:1, v/v) (solvent A) and acetonitrile/formic acid (99:1, v/v) (solvent B) in the linear gradient scheme (t in min; %B): (0; 5%), (15; 15%), (30; 30%), (40; 50%), (45, 95%), and (50; 5%). The flow rate and injection volume were 1 mL/min and 20 μL, respectively. Chromatograms were recorded at 320, 360, and 520 nm using high performance liquid chromatography coupled with photodiode array detection and electrospray ionization-mass spectrometry (HPLC–PAD-ESi-MSn) in an equipment consisting in a LC pump (SRVYR-LPUMP), an autosampler (SRVYR-AS), and a photodiode array detector (SRVYR-PDA5) in series, controlled by ChemStation software (Agilent, version 08.03). The identification of individual phenolics was carried out resorting electrospray ionization-mass spectrometry analysis using a mass detector, which was an ion trap spectrometer (model LCQ-Advantage-Max) equipped with an electrospray ionization interface, operated in the positive (anthocyanins) and negative (cinnamic acids, flavonols, and stilbenes) modes, and controlled by Tune Plus 1.3 SR1 software (Fisher Scientific, Lisboa, Portugal). The ionization conditions were adjusted at 250 °C and 4.0 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 2.0 bar and 8.0 L/min, respectively. The full scan mass covered the range from *m/z* 100 up to *m/z* 1500. Collision-induced fragmentation experiments were performed using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2.0 V. Flavonoids were quantified as quercetin-3-*O*-glucoside at 360 nm, cinnamic acids and stilbenes as 5-*O*-caffeoylquinic acid and resveratrol, respectively, at 320 nm, and anthocyanins as malvidin-3-*O*-glucoside at 520 nm, and expressed as mg/g of dry weight (mg/g dw).