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Ageing impact on the antioxidant and antiproliferative properties of Port wines

Hélder Oliveira, Iva Fernandes, Victor de Freitas, Nuno Mateus *

Chemistry Investigation Centre (CIQ), Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, 4169-007 Porto, Portugal

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ABSTRACT

This study is focused on the impact of Port wine ageing on some antioxidant features and antiproliferative properties towards human cancer cells. For this, two types of Port wines with different ageing stages were used: a young Vintage and a 20-year-old Tawny. The wines were dealcoholized and two wine phenolic fractions were also characterized and tested. The radical scavenging capacity was similar amongst the wines tested but the reducing capacity was significantly reduced for both Port wine extracts. The results from the FRAP assay, but not DPPH, seem to be positively correlated with the amounts of phenolics with lower structural complexity.
MKN-28 (stomach) was found to be the most susceptible cell line to the antiproliferative effect of the young Vintage Port. However, for all wines, Caco-2 (colon) was the cell line that showed the lowest IC_{50}. Interestingly, the old Tawny Port was found to maintain some antiproliferative activity.

1. Introduction

Port wine is a type of fortified wine from the Douro Demarcated Region in Northern Portugal commercialized worldwide with a growing market. There are different types of Port wine, being this classification based on the storage and ageing process. All Port wines undergo a couple of years of ageing in wood barrels and then some of them may be bottled (e.g. Vintage Ports) whilst others are left to age in large oak barrels (e.g. Tawny Ports) (Moreira & Guedes de Pinho, 2011). The ageing conditions in bottle and oak lead to several chemical reactions involving all phenolic compounds present in Port wines and also other wine components such as aldehydes, alcohols and organic acids. Like other red wines, red Ports are rich in phenolic compounds such as flavanols (e.g. procyanidins) and anthocyanins. These compounds have been associated with the prevention of several illnesses including age-related diseases and some types of cancer (Hou, 2003; Hou et al., 2004; Meiers et al., 2001). Their antioxidant and biological properties have been extensively studied and even explored over the last decades (Rice-Evans, Miller, & Paganga, 1997; Scalbert, Johnson, & Saltmarsh, 2005; Visioli, Bellomo, & Galli, 1998).

During red wine ageing, phenolic compounds undergo structural transformations yielding new stable structures that contribute both to colour and flavour changes in the wine (Mateus, Oliveira, Haettich-Motta, & de Freitas, 2004). These compounds include polymeric structures of catechins and anthocyanins, and newly formed polyphenolic classes such as pyranoanthocyanins or polyphenols arising from the reaction with wood aldehydes (de Freitas & Mateus, 2011). All these chemical reactions that change the polyphenolic profile of the wines are also likely to influence their antioxidant and biological properties. There are several reports in the literature dealing with the antioxidant and biological properties mainly attributed to the phenolic compounds typically found in red wines.

In addition, it should be noted that data in the literature dealing with the evolution of antioxidant activity during red wine ageing are still very conflicting. The changes in the antioxidant activity during ageing of red wines, for example, have been reported to show a significant increase, decrease or no significant change comparing to their respective young wines (Alén-Ruiz, García-Falcón, Pérez-Lamela, Martínez-Carballo, & Simal-Gándara, 2009; Rivero-Pérez, González-Sanjosé, Ortega-Herás, & Muñiz, 2008; Zafrilla et al., 2003).

The antioxidant and biological features of these compounds have been positively related with some of the health-related properties associated with the ingestion of red wine (Kim et al., 2006; Leifert & Abeywardena, 2008; Oi et al., 2010; Sharif et al., 2010). However, there is few data available on the effect of red wine ageing on their antioxidant and biological features. The formation of new polyphenolic compounds displaying a higher structural complexity may impair such properties or perhaps lead to new ones.

Indeed, it has already been shown for grape seed procyanidins that their antioxidant and antiproliferative properties towards tumour cells decrease with the increase of their structural complexity (Faria, Calhau, de Freitas, & Mateus, 2006). This outcome could be explained by the impairment due to some steric hindrances exhibited by the more complex structures.

The aim of the present work was to study some antioxidant features and the potential antiproliferative properties towards cancer cell lines of two different types of Port wines: a young Vintage Port and a typical

* Corresponding author. Tel.: +351 220402562.
E-mail address: nbmateus@fc.up.pt (N. Mateus).

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old-aged Tawny Port. A young red table wine was also included in this work in order to allow comparison with the data already reported in the literature which involves mainly regular table wines.

2. Materials and methods

2.1. Reagents

TSK Toyopearl HW-40 (S), reverse phase C-18, Folin–Ciocalteu reagent, tanic acid, gallic acid and DMSO were provided by Fluka, Switzerland. Foetal bovine serum, trypsin–EDTA 0.25%, phosphate buffered saline solution, RPMI-1640 AQMedia and DPPH were provided by Sigma-Aldrich-Madrid, Spain. TPTZ 10 mM in 40 mM of HCl; iron chloride (III) solution 20 mM. Glacial acetic acid was provided by Carlo Erba, Italy.

2.2. Wine samples

All wines were provided by Sogevinus Fine Wines, S.A.: a 2010 Vintage Port, a 20-year-old Tawny Port and a 2012 table red wine (3 bottles of each wine were used). The grape varieties used were the traditional ones used to produce Port wines (Touriga Nacional, Touriga Franca, Tinta Barroca, Tinto Cão and Tinta Roriz). The vinification procedure is the traditional one used for Port wine production. These wines represent the more typical commercialized Port wines. 200 mL of each wine sample was used and ethanol was eliminated through rotative evaporation at 30 °C under vacuum. The wine samples were purified by reverse phase C-18 silica gel to eliminate all sugars and recover the wine phenolics. The eluents were acidic water (HCl 2% (v/v)) for conditioning of stationary phase and acidic methanol (HCl 2% (v/v)) for eluting. A new evaporation was made to remove methanol from the wine samples. Each wine sample was then frozen, freeze-dried and stored at −20 °C until use.

2.2.1. Wine fractions

Wine sample extracts were applied on a 110 × 11 mm i.d. Toyopearl HW-40 (S) gel column. The conditioning was established with methanol and flux was adjusted to 0.8 mL/min. 0.190 g of each extract was dissolved in a minimum volume of methanol. The eluent used was distilled methanol with an elution time for fraction I (FI) of 30 min and then 45 min for fraction II (FII). For fraction 0 (F0), the sample was used in the original conditions, without any separation. All fractions were then purified by chromatography as described in 2.2 and concentrated in a rotative evaporator at 30 °C under vacuum. The fractions were then frozen, freeze-dried and stored at −20 °C until use.

2.3. High pressure liquid chromatography (HPLC)

Two HPLC-DAD methods were used one to detect catechins and procyanidins and other to detect anthocyanins and their derivatives (Fernandes et al., 2009; Mateus, Pinto, Ruão, & de Freitas, 2004). The analysis of catechins and procyanidins was realized with two reverse phase C18 LiChrospher® 100Å columns (250 × 4.6 mm, 5 μm) (Merck) at 25 °C. The solvents used were: solvent A — H_2O/CH_3COOH (97.5:2.5) and solvent B — CH_3CN/solvent A (80:20). The solvent gradient was 93% of solvent A and 7% of solvent B isocratic for 5 min and then gradient changed to final conditions of 80% of solvent A and 20% of solvent B after 110 min. The washing process was realized with 100% of solvent B, for 20 min. All the chromatograms of each wine fraction were recorded at 280 nm with a DAD L-7420 detector (Merck-Hitachi).

Analysis of anthocyanins and their derivatives was made with one reverse phase C18 LiChrospher® 100 Å column (250 × 4.6 mm, 5 μm) (Merck). The solvents used were: solvent A — H_2O/HCOOH (90:10) and solvent B — H_2O/CH_3CN/HCOOH (60:30:10). The solvent gradient was from 80% of solvent A and 20% of solvent B to 15% of solvent A and 85% of solvent B after 70 min. The washing process was realized with 100% of solvent B, for 20 min. All the chromatograms of each wine fraction were recorded at 520 nm with a DAD L-7420 detector (Merck-Hitachi).

2.4. Total phenolic content determination

The total phenolic content of the extracts was determined following the Folin–Ciocalteu method adjusted to a microscale (Arnous, Makris, & Kefalas, 2001). For the analysis of total phenolic content of the wine fractions, 15 μL of each fraction (original concentration: 40 mg/mL with 5% DMSO) were diluted to 0.5 mg/mL. Then 75 μL of Folin–Ciocalteu reagent and 500 μL of water (H_2O) were added to the reaction mixture. After stirring vortex in all samples 300 μL of 20% aqueous sodium carbonate and 610 μL H_2O were added to the mixture and the samples were incubated for 30 min.

After the incubation period 350 μL of each sample were added (0.5 mg/mL) to the wells of a microplate (with quadruplicates of each bottle) and absorbance was read in a microplate reader at 760 nm. To determine the concentration of phenolics present in each fraction a calibration standard curve with a range of concentrations of gallic acid (0.05 to 0.4 mg/mL) was used. The results are shown in total phenolic concentration (mg/mL).

2.5. Radical scavenging capacity assay (DPPH)

Following the method described in the literature (Bondet, Brand-Williams, & Berset, 1997) with some modifications, radical activities were determined by using DPPH (2,2-diphenyl-1-picrylhydrazyl) as a free radical. The tested samples reacted with DPPH and the decrease in the absorbance at 515 nm was measured, which indicated the scavenging potential of the samples. As all samples tested absorbed at 515 nm, previous control assays were performed with all the wine fractions in order to subtract their contribution. The reaction for scavenging DPPH radicals was performed in a microplate reader (quadruplicates of each wine bottle) (Biotek Powerwave XS with software KC4). The reaction was carried out on the plate wells with a temperature of 25 °C. A solution of 60 μM DPPH was prepared in methanol. 270 μL of this solution was added in each well together with 30 μL of wine sample fraction. The samples tested were at the final concentration of 2.5 μg/mL. The decrease in absorbance was measured at 515 nm, at t = 0 and t = 20 min. Antiradical activity was expressed as μM Trolox equivalents. The antiradical activity was calculated from linear regression after plotting known solutions of Trolox with different concentrations.

2.6. Ferric reducing antioxidant power assay (FRAP)

The FRAP assay was performed according to the procedure described in the literature (Benzie & Strain, 1996) with some modifications. The reaction was performed in a microplate reader of 96 well plates (quadruplicates of each wine bottle) (Biotek Powerwave XS with software KC4). The reaction was carried out on the plate wells with a temperature of 37 °C. Briefly, FRAP reagent (10 vol of 300 mM acetate buffer, pH 3.6 + 1 vol of 10 mM TPTZ in 40 mM HCl + 1 vol of 20 mM $FeCl_3$) was diluted to one-third with acetate buffer. 270 μL of this solution was added to each well together with 30 μL of sample to be tested. The blank assay was performed using 270 μL of FRAP reagent and 30 μL of methanol. The samples to be tested were dissolved in methanol and used at the final concentration of 5 μg/mL. The absorbance was measured at 593 nm in time 0 and 4 min. The results were expressed as μM Trolox equivalents.

2.7. Cell culture conditions

Three human cancer cell lines (MKN-28 (stomach), Caco-2 and HT-29 (colon)) were grown as monolayer. For routine maintenance, cells were cultured in 25 cm^2 plates as monolayer and maintained in