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Stability and biological activity of Merlot (*Vitis vinifera*) grape pomace phytochemicals after simulated *in vitro* gastrointestinal digestion and colonic fermentation

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ABSTRACT

Grape pomace is an abundant/accessible food industry by-product that contains a wide range of phenolic compounds, which have been related to several health benefits and bioactivities. The aim of this study was to mimic the gastrointestinal digestion and the colonic fermentation of Merlot grape pomace, in order to unravel possible phytochemical contents reductions and the processes associated with them, as a tentative to relate the phenolic compound profiles of the extracts with their biological properties. LC-DAD-ESI/MS suggested that the *in vitro* digestion process promoted drastic qualitative and quantitative reductions in the phenolic compounds profile of the Merlot grape pomace crude extract. Such alterations could be related to the decreases of some bioactivities of the extract, which seems to be the case of antioxidant and antibacterial properties, although not in a directly proportional manner. However, the simulated colonic fermentation seems to have a positive effect over the extract's antiproliferative potential.

1. Introduction

Winemaking is currently one of the most relevant agro-industrial activities in the world. Undoubtedly, grapes are an abundant fruit crop worldwide, being *Vitis vinifera* the species most frequently cultivated for wine production (Otero-Pareja, Casas, Fernández-Ponce, Mantell, & Ossa, 2015; Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016).

The co-products generated by the vitiviniculture sector activities, such as pomace, rachis, and lees, corresponds to incredible 30% of the total amount of vinified grapes (Makris, Boskou, & Andrikopoulos, 2007), most of them still underexplored and commonly discarded without adequate treatment, which leads to environmental impact (Melo et al., 2015). Several studies have already proved that these winemaking co-products constitute an interesting source of natural antioxidants, especially phenolic compounds. Grape pomace, the major winery sub-product, consists of the waste seeds, skins and stems that remain after the grape pressing process, characterized by an expressive content of phenolic compounds due to the incomplete extraction that occurs during the winemaking process. (Jara-Palacios et al., 2015; Otero-Pareja et al., 2015).

In recent years, there has been an increasing interest in the exploitation of polyphenol-rich winery sub-products to produce novel extracts and health promoting products (Fontana, Antoniolli, & Bottini, 2013). As such, the recovery of phytochemicals from industrial food co-products represents a sustainable and cost effective source of high-value bioactives, which could be recycled and return to the food chain as functional food ingredients and nutraceuticals (Corrêa et al., 2016).

Although in the past decade a number of researches have addressed the extraction, chemical characterization and antioxidant capacity of grape pomace extracts from diverse varieties (Makris et al., 2007; Amico, Chillemi, Mangiafico, Spatafora, &

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Tringali, 2008; Rockenbach et al., 2011; Sagdic et al., 2011; Fontana et al., 2013; Doshi, Adsule, Banerjee, & Oulkar, 2015; Iora et al., 2015; Otero-Pareja et al., 2015; Ribeiro et al., 2015), only a few studies have explored their antibacterial (Tseng & Zhao, 2012; Oliveira et al., 2013) and anti-inflammatory properties (Melo et al., 2015). However, there are even fewer researches over the antiproliferative potential of grape pomace extracts (Jara-Palacios et al., 2015).

More recently, the influence of the gastrointestinal digestion step on the food phytochemicals contents, and on their bioactivities (mainly antioxidant capacity), have attracted special attention, which is evidenced by the great number of publications dedicated to this theme (Gumienna, Lasik, & Czarnecki, 2011; Tavares et al., 2012; Correa-Betanzo et al., 2014; Pavan, Sancho, & Pastore, 2014; Podswdek et al., 2014; Mosele, Macià, Romero, Motilva, & Rubió, 2015; Wu, Teng, Huang, Xia, & Wei, 2015; Del Pino-García, González-SanJosé, Rivero-Pérez, García-Lomillo, & Muñiz, 2016; Kaulmann, Legay, Schneider, Hoffmann, & Bohn, 2016; López-Barrera, Vázquez-Sánchez, Loarca-Piña, & Campos-Vega, 2016). Nonetheless, except for the recent work of Gil-Sánchez et al. (2017), reports on the effects of *in vitro* digestion and simulated colonic fermentation processes on grape pomace phenolic compounds as well as on its bioactive properties, such as antiproliferative effects and hepatotoxicity, are lacking.

In view of the above, the aim of this study was to mimic the gastrointestinal digestion and the colonic fermentation of Merlot grape pomace, in order to unravel possible phytochemical contents reductions and the processes associated with them, as a tentative to relate the phenolic compound profiles of the extracts with the herein assessed bioactivities. For this purpose, the three grape pomace extracts obtained, namely crude, digested and fermented extracts, were characterized in terms of non-anthocyanin and anthocyanin compounds. The antioxidant, antibacterial and antiproliferative potentials of the grape pomace extracts were also evaluated and compared, and the hepatotoxicity was assessed in a primary cell culture of porcine liver cells.

2. Materials and methods

2.1. Standards and reagents

Acetonitrile (99.9%) was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Phenolic standards were from Extrasynthèse (Genay, France). Salivary alpha-amylase (6.66 U/mL), pancreatin (100 U/mL), pepsin A (1923 U/mL), bile extract, Sulforhodamine B, trypan blue, trichloroacetic acid (TCA), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), formic acid and Tris were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) were purchased from Gibco Invitrogen Life Technologies (California, Massachusetts, USA). The tumor cell lines were provided by DSMZ (Braunschweig, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other general laboratory reagents were purchased from Panreac Química S.L.U. (Barcelona, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Carrollton, TX, USA).

2.2. Grape pomace

The fresh Merlot (*Vitis vinifera*) grape pomace was donated by a winemaking company located in the State of Paraná, Brazil. Immediately after its obtainment, in order to prevent microbiological contamination and fermentation processes, the grape pomace was dried in an air circulation oven at 80 °C during 36 h. The dried mass was therefore milled to a fine powder (40 mesh), transferred to polyethylene film bags under vacuum packing and kept at −20 °C until analysis (Ribeiro et al., 2015). This material constituted the matrix used to obtain three different extracts, according to the process shown in the diagram of Fig. 1.

2.3. Crude extract preparation

The grape pomace crude extract was obtained according to the procedure previously described by Ribeiro et al. (2015). The extractions were performed in the ratio 1:50 (m/v—solute/solvent) with ethanol and distilled water (40:60, v/v), respectively. The mixture was stirred for 24 h on a shaker at 25 °C. The tubes containing the solutions were centrifuged at 5000 rpm for 25 min and the supernatant was separated. The obtained filtrate was concentrated with a rotary vacuum evaporator at 40 °C in order to eliminate the solvent, posteriorly freeze-dried and stored at −20 °C until use.

2.4. In vitro digestion

The *in vitro* gastrointestinal digestion was simulated according to methodology described by Koehnlein et al. (2016). Briefly, 13 g of the lyophilized grape pomace hydroethanol extract was mixed

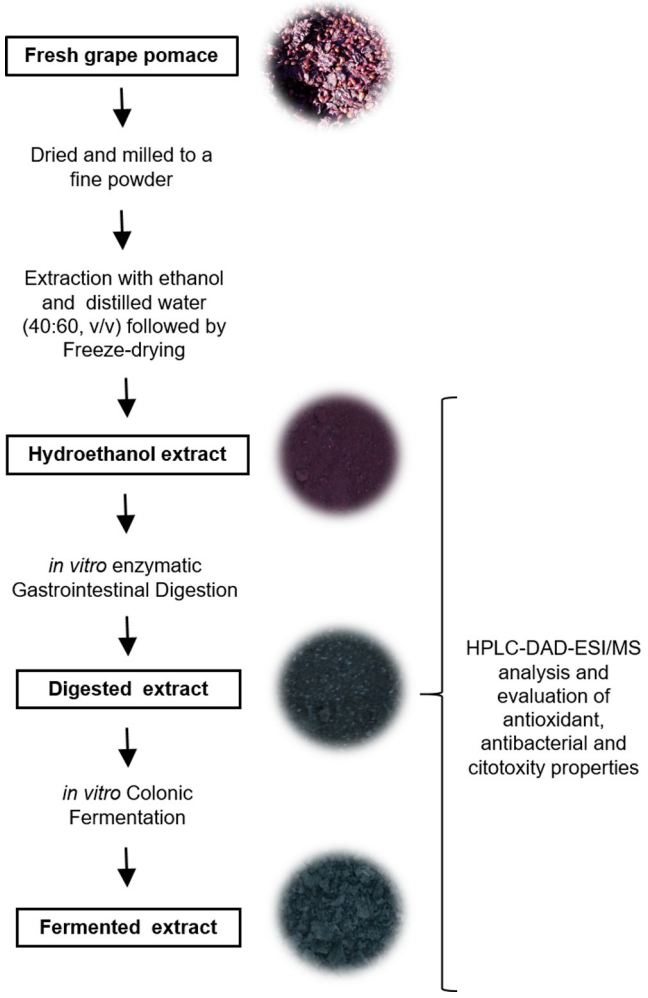

Fig. 1. Diagram of the main steps performed in the obtainment of the Merlot grape pomace extracts and comparative performed assays.

with 39 mL of artificial saliva solution (2.38 g Na_2HPO_4, 0.19 g KH_2PO_4, 8 g NaCl in 1 L of distilled water). The pH was regulated to 6.75, at the temperature of 37 °C and α-amylase was added to produce an enzyme activity of 200 U. This blend was shaken at 150 rpm during 10 min. In sequence, the pH was adjusted to 1.2 and 39 mL of artificial gastric fluid (0.32 g pepsin in 100 mL of 0.03 M NaCl, pH 1.2) was included. The mixture was then incubated on a shaker at 37 °C for 120 min, under agitation of 150 rpm. Lastly, the pH was adjusted back to 6.0 following the addition of 6.5 mL of NaCl (120 mM), 6.5 mL of KCl (5 mM) and 39 mL of artificial intestinal fluid (0.15 g of pancreatin and 0.9 g of bile extract in 100 mL of 0.1 M $NaHCO_3$). The mixture was incubated at 37 °C for 60 min, at 150 rpm. Thereon the obtained digested extract was freeze-dried and stored at −20 °C.

2.5. In vitro colonic fermentation

The fermentation medium, prepared according to methodology described by Karppinen, Liukkonen, Aura, Forssell & Poutanen (2000) with modifications, was a carbonate-phosphate buffer. The mineral medium was regulated to pH 7.0 and glucose was added to a final concentration of 0.8%. The mixture was purged with nitrogen until the anaerobic indicator (methylene blue) turned colorless. The inoculum was obtained from fresh feces collected from the entire large intestines of male Wistar rats (75-days old animals, average 250 g) immediately after euthanasia. A fecal pool of 5 animals was made. Immediately after collecting, the material was homogenized with the culture medium at a ratio of 1:10 (w/v). The bottles were bubbled over again with nitrogen and closed airtight. Afterwards, the bottles were incubated at 37 °C for 24 h under agitation of 50 rpm, in order to simulate the condition in the colonic lumen. The initial pH was 7.0 and the final pH was around 5.0. A control with the culture medium and inoculum was prepared. In order to verify the absence of phenolic in the diet, the control sample was submitted to the Folin-Ciocalteu assay, with negative results. Subsequently, the material was ultra-centrifuged at 31,000 rpm during 30 min, sterilized by filtration, and freeze-dried. As phenolic compounds and antioxidant activity were not detected in the control, it was not considered for the antioxidant and bioactive assays.

2.6. Phenolic compounds analysis

The phenolic profile was determined by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA). The lyophilized extracts were re-dissolved at a concentration of 5 mg/mL with an ethanol:water (40:60, v/v) mixture.

The non-anthocyanin compounds were separated and identified as previously described by Bessada, Barreira, Barros, Ferreira, and Oliveira (2016) and the detection was carried out in a DAD (280 and 370 nm as preferred wavelengths) and in a mass spectrometer (MS), operating in negative mode.

The anthocyanin compounds were separated and identified as previously described by Gonçalves et al. (2017) and detection was carried out in DAD (520 nm the preferred wavelength) and in a mass spectrometer (MS), operating in positive mode. For both non-anthocyanin and anthocyanin compounds the MS detection was performed using a Linear Ion Trap LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source.

The identification of the phenolic compounds (non-anthocyanin compounds and anthocyanin compounds) were performed using standard compounds, when available, by comparing their retention times, UV–vis and mass spectra; and also, comparing the obtained information with available data reported in the literature giving a tentative identification. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard, such as for compounds 1 and 3–8 were quantified (+)-catechin ($y = 84950x - 23200$; $R^2 = 0.999$), compound 2 using gallic acid ($y = 208604x + 173056$; $R^2 = 0.999$), compound 9 with myricetin ($y = 23287x - 581708$; $R^2 = 0.999$), compounds 10–11 with quercetin-3-*O*-glucoside ($y = 34843x - 160173$; $R^2 = 0.999$) and compounds 12–16 with peonidin-3-*O*-glucoside ($y = 122417x - 447974$; $R^2 = 0.999$). The results were expressed as mg/g of extract.

2.7. Bioactive in vitro assays

2.7.1. Antioxidant activity evaluation

The lyophilized extracts were re-dissolved in ethanol:water (40:60, v/v) mixture to obtain a stock solution of 1 mg/mL, which were further diluted to obtain a range of concentrations for antioxidant activity evaluation by DPPH radical-scavenging, reducing power, inhibition of β-carotene bleaching and TBARS inhibition assays (Corrêa et al., 2015). The results were expressed as EC_{50} values (mg/mL), sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Trolox was used as positive control while the negative control was water.

2.7.2. Evaluation of cytotoxic properties

The aqueous extracts were dissolved in water in order to obtain a final concentration of 8 mg mL^{-1}. The final solution was further diluted to different concentrations (400–1.5 μg mL^{-1}) to be subjected to *in vitro* cytotoxicity evaluation. Four human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma), being all cell lines mycoplasma free. The cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) (MCF-7 and NCI-H460) and 2 mM glutamine or in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, 100 U per mL penicillin and 100 mg per mL streptomycin (HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density [1.0 × 104 cells (10,000 cells) per well] in 96-well plates and allowed to attach for 24 h. The cells were then treated for 48 h with the different diluted sample solutions. Following this incubation period, the adherent cells were fixed by adding cold 10% TCA (100 μL) and incubated for 60 min at 4 °C. Plates were then washed with deionized water and dried; Sulforodamina B (SRB) solution (0.1% in 1% acetic acid, 100 μL) was then added to each plate-well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, the bound SRB was solubilised with 10 mM Tris (200 μL, pH 7.4) and the absorbance was measured at 540 nm in the microplate reader mentioned above. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). For the negative control, cells were cultured with the same culture medium; however, the samples volumes were replaced by water (carrier solvent). Ellipticine was used as positive control.

2.7.3. Antibacterial activity evaluation

The lyophilized samples were dissolved in water at 100 mg/mL and then submitted to further dilutions. The microorganisms used were clinical isolates from patients hospitalized in various departments of the Local Health Unit of Bragança and Hospital Center of Trás-os-Montes and Alto-Douro Vila Real, Northeast of Portugal. Six Gram-negative bacteria (*Escherichia* coli 1, *E. coli* 2, *Klebsiella pneumoniae* 1, *K. pneumoniae* 2, *Morganella morganii* and

Pseudomonas aeruginosa isolated from urine and expectoration) and four Gram-positive bacteria (*MRSA*- methicillin-resistant *Staphylococcus aureus*, *MSSA*- methicillin-susceptible *Staphylococcus aureus*, *Listeria monocytogenes* and *Enterococcus faecalis*) were used to screen the antibacterial activity of the lyophilized extract. MIC determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay following the methodology suggested by Kuete, Ango, et al. (2011) & Kuete, Justin, et al. (2011) with some modifications. MIC was defined as the lowest extract concentration that prevented this change and exhibited inhibition of bacterial growth. Three negative controls were prepared (one with MHB/TSB, another one with the extract, and the third one with medium and antibiotic). One positive control was prepared with MHB and each inoculum. For the Gram-negative bacteria, antibiotics, such as amikacin, tobramycin, amoxicillin/clavulanic acid and gentamicin were used. For the Gram-positive bacteria, ampicillin and vancomycin were selected. The antibiotics concentrations applied are presented in the Supplementary Material. The antibiotic susceptibility profile of gram negative and gram positive bacteria has been already described by Dias et al. (2016).

2.8. Statistical analysis

Three repetitions of the samples were used and triplicates for each concentration reading were carried out in all the assays. The results were expressed as mean values ± standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $p = 0.05$. For every parameter with only two available values a Student's *t*-test was applied to determine the significant difference among the corresponding samples, with $p = 0.05$. When the p value was lower than 0.05, significant differences between samples were considered. Analyses were carried out using IBM SPSS Statistics for Windows, version 23.0. (IBM Corp., Armonk, New York, USA).

3. Results and discussion

Although aware that the use of rat feces instead of human feces presents limitations, mainly due to the differences in microbiota (Becker, Kunath, Loh, & Blaut, 2011), this experimental model was chosen because both the anti-inflammatory and antioxidant effects of the hydroethanolic grape pomace extract have been assessed in a rat model in a recent work of our group (Gonçalves et al. 2017). Therefore, our attempt was to evaluate what would be the real molecules absorbed by the rats, reason why we made the option for the experimental model with animal feces.

3.1. Phenolic compounds analysis

The samples were submitted to hydroethanolic extractions in order to obtain the largest possible number of compounds classes, reason why our samples were not acidified for anthocyanin stabilization purposes.

Retention time, maximum absorption wavelengths in the visible region, mass spectral data and tentative identification of the Merlot grape pomace hydroethanol, digested and fermented extracts are show in Table 1. The phenolic profile of Merlot grape pomace hydroalcohlic extract was previously described by the authors, where the effect on the oxidative and inflammatory states of adjuvant-induced arthritic rats was investigated (Gonçalves et al., 2017). The most abundant phenolic compounds found in the three herein tested extracts were B-type (epi)catechin dimer (compound **2**), (+)-catechin (compound **5**) and (−)-epicatechin (compound **9**) (Table 2). In the hydroethanolic extract, 20 non-anthocyanin compounds were identified (with a total content of 66 mg/g of extract), a number that was significantly reduced to 11 compounds after the *in vitro* digestion. Apparently, compounds **7**, **8**, **11**, **14**, **16**, **17**, **19** and **20** were degraded during the digestion step, and remained absent from the extract after the simulated colonic fermentation. During the gastrointestinal digestion compound **2** had a reduction of almost 18-fold, while both compounds **5** and **9** suffered reductions around 4-fold. However, pronounced reductions in the contents of all phenolic molecules was apparent, being that the decreases were much more drastic after the *in vitro* digestion step, than after *in vitro* colonic fermentation. Correa-Betanzo et al. (2014), in their investigation over the stability and bioactivities of blueberry phenolic compounds during their passage through *in vitro* gastrointestinal digestion, also reported significant compound losses. The authors found that simulated intestinal digestion decreased both polyphenol and anthocyanin contents (by 49% and 15%, respectively) in comparison with the non-digested samples. During the simulated colonic fermentation (chemostat fermentation), some phenolic compound constituents suffered degradation (e.g., syringic, cinnamic, caffeic, and protocatechuic acids). According to the authors, the colonic fermentation also produced negative alterations in both antioxidant and antiproliferative potentials of blueberry phenolic compounds.

Although the five anthocyanins identified in the hydroethanolic extract remained present in both digested and fermented extracts, after the simulated digestion process significant reductions occurred in their contents: compound **21** decreased 3-fold, compound **22** decreased in almost 7-fold, compound **23** decreased in more than 10-fold, and both compounds **24** and **25** presented reductions of almost 4-fold.

On its turn, the *in vitro* colonic fermentation process apparently produced no significant losses of the anthocyanin compounds, once their contents remained practically unchanged after this last stage.

Malvidin-3-*O*-glucoside (compound **23**) was the most abundant anthocyanin in all three assessed extracts, followed by peonidin-3-*O*-glucoside (compound **22**). Ribeiro et al. (2015) identified thirteen anthocyanins in a hydroethanolic extract of Merlot grape pomace, within which all the five anthocyanins (compounds **21**–**25**) found in the present study were also included. The total anthocyanin content in the hydroethanolic extract (6.988 mg/g) was similar (8.280 mg/g) to the value reported by Rockenbach et al. (2011) for a Merlot grape pomace methanolic extract.

Even though previous studies reported the presence of phenolic acids and valerolactones derivatives (Lingua, Fabani, Wunderlin, & Baroni 2016; Martins, Roberto, Blumberg, Chen, & Macedo, 2016; Gil-Sanches et al., 2017) in grape pomace samples, no peaks were found in the our extracts, whose UV spectra could be associated with phenolic acids, such as hydroxycinnamic acids or their tartaric or quinic esters (i.e., chlorogenic acids) or valerolactones derived from the digestion process. Further, no detection of those compounds could be made when the full mass chromatograms of the samples were screened for their molecular ions. Thus, no other phenolic compounds were identified in this extract, being characterized by the presence of flavonoids, mainly flavan-3-ols derivatives, galloyl derivatives, flavonols derivatives and five anthocyanins derivatives.

3.2. Evaluation of bioactive properties

The *in vitro* antioxidant, antiproliferative, cytotoxicity and antibacterial properties of the Merlot grape pomace extracts, submitted or not to *in vitro* digestion and colonic fermentation were evaluated, and the results are presented in Table 3.

For all four antioxidant activity evaluation assays, the hydroethanolic extract remained practically unchanged after the

Table 1
Retention time (R_t), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectrometric data, and tentative identification of phenolic compounds of crude grape pomace, grape pomace submitted to *in vitro* digestion and grape pomace subjected to simulated colonic fermentation.

Compounds	R_t (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	4.68	280	325	169(100), 125(8)	Galloylshikimic acid
2	5.6	280	577	451(23), 425(100), 407(22), 289(12), 287(10)	B-type (epi)catechin dimer
3	5.74	267	495	343(100), 191(8), 169(3)	Digalloylquinic acid
4	6.3	280	577	451(23), 425(100), 407(22), 289(12), 287(10)	B-type (epi)catechin dimer
5	7.16	280	289	245(100), 203(50), 187(10), 161(9), 137(3)	(+)-Catechin
6	7.62	280	577	451(23), 425(100), 407(22), 289(12), 287(10)	B-type (epi)catechin dimer
7	7.94	280	577	451(23), 425(100), 407(22), 289(12), 287(10)	B-type (epi)catechin dimer
8	8.57	278	477	325(100), 169(3), 125(2)	Digalloylshikimic acid
9	9.65	280	289	245(100), 203(35), 187(6), 161(8), 137(3)	(−)-Epicatechin
10	11.06	279	865	739(78), 713(47), 695(100), 577(62), 575(42), 425(12), 407(9), 289(6), 287(11)	B-type (epi)catechin trimer
11	11.1	279	865	739(78), 713(47), 695(100), 577(62), 575(42), 425(12), 407(9), 289(6), 287(11)	B-type (epi)catechin trimer
12	12.3	280	1153	865(25), 739(78), 713(47), 695(100), 577(62), 575(42), 425(12), 407(9), 289(6), 287(11)	B-type (epi)catechin tretramer
13	15.61	350	479	317(100)	Myricetin-*O*-hexoside
14	18.4	350	477	301(100)	Quercetin-3-*O*-glucuronide
15	19.3	350	463	301(100)	Quercetin-3-*O*-glucoside
16	19.6	350	493	331(100)	Laricitrin-*O*-hexoside
17	21.56	349	433	301(100)	Quercetin-*O*-pentoside
18	22.52	348	447	301(100)	Quercetin-*O*-rhamnoside
19	23.49	350	477	315(100)	Isorhamnetin-3-*O*-glucoside
20	24.36	351	655	509(15), 501(49), 475(63), 347(20), 329(100), 314(13)	Methylisorhamnetin derivative
Compounds	R_t (min)	λ_{max} (nm)	$[M+H]^+$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
21	40.1	520	479	317(100)	Petunidin-3-*O*-glucoside
22	43.2	520	463	301(100)	Peonidin-3-*O*-glucoside
23	44.2	520	493	331(100)	Malvidin-3-*O*-glucoside
24	53.3	520	505	301(100)	Peonidin-3-*O*-acetylglucoside
25	53.8	520	535	331(100)	Malvidin-3-*O*-acetylglucoside

in vitro digestion. However, the simulated colonic digestion step promoted a significant decrease in the antioxidant capacities assessed by DPPH scavenging activity (6-fold reduction), β-carotene beaching inhibition (more than 2-fold reduction) and TBARS inhibition (6-fold reduction). Nonetheless, the antioxidant capacity data contained in Table 3, especially those regarding the hydroethanolic and digested extracts (EC_{50} values of 0.023 mg/mL and 0.029 mg/mL, respectively) assessed by TBARS formation inhibition, evidence a significant antioxidant potential of all Merlot grape pomace extracts tested herein, even after the *in vitro* colonic fermentation.

Interestingly, only in the reducing power assay, it was observed a clear improvement of the antioxidant capacity (of almost 5-fold) after the colonic fermentation step. This result can be corroborated by the study of Del Pino-García et al. (2016), in which the authors verified that both *in vitro* gastrointestinal digestion and colonic fermentation promoted significant positive outcomes on the total antioxidant capacities of seasonings produced from red wine pomace. According to Pavan et al. (2014), the increment in the antioxidant activity of digested fruit extracts can be a result of phenolic compounds release after the *in vitro* digestion.

Amico et al. (2008) found higher DPPH scavenging activity (EC_{50} = 0.01 mg/mL) for a Sicilian grape pomace hydroethanol extract than the values presented herein, while Otero-Pareja et al. (2015) reported an average EC_{50} of 0.008 mg/mL for grape pomace extracts from different varieties (including Merlot) obtained by pressurized liquid extraction using ethanol as solvent. On the other hand, Iora et al. (2015) reported lower antioxidant capacity for hydroethanol extracts of Merlot, Cabernet Sauvignon and Tanat grape pomaces, with DPPH˙ assay values ranging from 5.05 to 6.54 mg/mL. Although the DPPH method is a unanimous choice in the evaluation of the antioxidant capacity of grape pomace (Fontana et al., 2013), the other methods used in the present study have not been much explored for this purpose. Hence, to the best of our knowledge, this is the first research work on the antioxidant capacity of grape pomace using the herein set of antioxidant capacity methods.

Results regarding the antiproliferative effects of the assayed extracts of Merlot grape pomace on the inhibition growth of four human tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2) are shown in Table 3, expressed as concentrations that promoted 50% of the cell growth inhibition (GI_{50}). In general, both hydroethanolic and digested extracts did not present antiproliferative activity against the tested tumor cell lines, except for the expressive cytotoxic activity (GI_{50}=15 μg/mL) found for the grape pomace hydroethanol extract against the HeLa line (cervical carcinoma). However, the fermented grape pomace extract showed antiproliferative effects against all four tumor cell lines, with GI_{50} values ranging from 227 μg/mL (against HepG2) to 251 μg/mL (against HeLa). Jara-Palacios et al. (2015) found that a purified methanolic extract obtained from white grape pomace (Zalema variety), significantly inhibited adenocarcinoma cell proliferation (GI_{50} = 100 μg/mL), and also suggested that phenolic compounds contained in the extract (such as catechin and quercetin) were the mediating components of both anti proliferative action and direct initiation of cell death.

Apparently, the *in vitro* colonic fermentation step promoted significant transformations that increased the bioactivity of the herewith tested Merlot grape pomace extract. Considering that both non-anthocyanin and anthocyanin compounds underwent significant degradation during the simulated digestion and fermentation steps, it can be inferred that the bioactive components responsible for the antiproliferative effects of the grape pomace are not the phenolic compounds shown in Table 1. In fact, other components of grape pomace extract, other than phenolic compounds, may be responsible for its observed bioactivities.

All Merlot grape pomace extracts presented no toxicity in liver primary culture PLP2, being all the obtained GI_{50} values higher than the highest concentration tested (400 μg/mL) (Table 3). Ellipticine, the positive control, presented a GI_{50} of 2.29 μg/mL. The

Table 2
Quantification of the identified phenolic compounds (mg/g extract) of crude grape pomace, grape pomace submitted to *in vitro* digestion and grape pomace subjected to simulated colonic fermentation.

Compounds	Hydroethanol extract*	*In vitro* digestion	Colonic fermentation
Phenolic compounds non-anthocyanins			
Galloylshikimic acid	3.37 ± 0.04	nd	nd
B-type (epi)catechin dimer	10.2 ± 0.2a	0.58 ± 0.02b	0.48 ± 0.01c
Digalloylquinic acid	2.30 ± 0.01a	0.111 ± 0.002b	0.113 ± 0.001b
B-type (epi)catechin dimer	6.7 ± 0.2a	0.79 ± 0.01b	0.85 ± 0.03b
(+)-Catechin	7.27 ± 0.12a	1.77 ± 0.01b	1.44 ± 0.03c
B-type (epi)catechin dimer	3.2 ± 0.1a	0.81 ± 0.01c	0.90 ± 0.03b
B-type (epi)catechin dimer	5.33 ± 0.02	nd	nd
Digalloylshikimic acid	1.88 ± 0.03	nd	nd
(−)-Epicatechin	7.3 ± 0.3a	1.73 ± 0.02b	1.53 ± 0.04c
B-type (epi)catechin trimer	4.9 ± 0.1a	0.56 ± 0.02c	0.89 ± 0.01b
B-type (epi)catechin trimer	3.6 ± 0.1	nd	nd
B-type (epi)catechin tretramer	6.2 ± 0.3a	0.38 ± 0.01c	0.67 ± 0.02b
Myricetin-*O*-hexoside	1.42 ± 0.01a	1.14 ± 0.01b	1.11 ± 0.01c
Quercetin-3-*O*-glucuronide	0.56 ± 0.02	nd	nd
Quercetin-3-*O*-glucoside	0.52 ± 0.01a	0.23 ± 0.01b	0.22 ± 0.01c
Laricitrin-*O*-hexoside	0.36 ± 0.02	nd	nd
Quercetin-*O*-pentoside	0.40 ± 0.01	nd	nd
Quercetin-*O*-rhamnoside	0.38 ± 0.01a	0.24 ± 0.01b	0.23 ± 0.01c
Isorhamnetin-3-*O*-glucoside	0.51 ± 0.01	nd	nd
Methylisorhamnetin derivative	0.31 ± 0.01	nd	nd
Total non-anthocyanin compounds	66.6 ± 0.7a	8.34 ± 0.05b	8.42 ± 0.02b
Phenolic compounds anthocyanins			
21	0.592 ± 0.001a	0.184 ± 0.001b	0.180 ± 0.001c
22	1.555 ± 0.002a	0.227 ± 0.002b	0.216 ± 0.003c
23	3.407 ± 0.001a	0.34 ± 0.01b	0.31 ± 0.01c
24	0.694 ± 0.001a	0.180 ± 0.001b	0.170 ± 0.0001c
25	0.740 ± 0.001a	0.193 ± 0.002b	0.181 ± 0.001c
Total anthocyanin compounds	6.988 ± 0.003a	1.12 ± 0.01b	1.06 ± 0.01c

nd – not detected.
* Results previously published in Gonçalves et al. (2017). In each row different letters mean significant differences ($p < 0.05$).

Table 3
Antioxidant, cytotoxic, hepatotoxicity and antimicrobial activity of Merlot grape pomace hydroethanol extract, *in vitro* digestion and colonic fermentation (mean ± SD).

	Hydroethanol extract	*In vitro* digestion	Colonic fermentation
Antioxidant activity EC_{50} values (μg/mL)[a]			
DPPH scavenging activity	58 ± 2b	60 ± 2b	365 ± 15a
Reducing power	101 ± 1b	158 ± 1a	34 ± 2c
β-carotene bleaching inhibition	215 ± 8c	257 ± 6b	599 ± 7a
TBARS inhibition	23 ± 1c	178 ± 8a	29 ± 1b
Cytotoxic activity GI_{50} values (μg/mL)[b]			
MCF-7 (breast carcinoma)	>400	>400	243 ± 7
NCI-H460 (non-small cell lung carcinoma)	>400	>400	242 ± 8
HeLa (cervical carcinoma)	15 ± 1*	>400	251 ± 9*
HepG2 (hepatocellular carcinoma)	>400	>400	227 ± 6
Hepatotoxicity GI_{50} values (μg/mL)[b]			
PLP2	>400	>400	>400
Antimicrobial activity MIC values (mg/mL)			
Gram negative bacteria			
Escherichia coli ULSNE	20	>20	20
Escherichia coli CHTMAD	>20	>20	>20
Klebsiella pneumoniae ULSNE	>20	>20	>20
Klebsiella pneumoniae CHTMAD	>20	>20	>20
Morganella morganii	20	>20	20
Pseudomonas aeruginosa	20	>20	>20
Gram positive bacteria			
Enterococcus faecalis	5	10	nd
Listeria monocytogenes	5	10	>20
MRSA	10	10	10
MSSA	10	10	20

EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2.
[a] Trolox EC_{50} values: 62.98 μg/mL (DDPH), 45.71 μg/mL (reducing power), 10.25 μg/mL (β-carotene bleaching inhibition).
[b] Ellipticine GI_{50} values: 1.21 μg/mL (MCF-7), 1.03 μg/mL (NCI-H460), 0.91 μg/mL (HeLa), 1.10 μg/mL (HepG2) and 2.29 μg/mL (PLP2). MIC values correspond to the minimal sample concentration that inhibited the bacterial growth. In each row different letters mean significant differences ($p < 0.05$).
* Statistically different values, Student's *t*-test *p*-value < 0.001.

proved absence of cytotoxicity against liver cells, considered a great *in vitro* model for assessing human cytotoxicity (Corrêa et al., 2015), is a crucial requisite for the application of the tested extracts as nutraceuticals or food ingredients. Melo et al. (2015), which investigated the bioactivities of winery by-products, reported the low toxicity of hydroethanol extracts of Chenin Blanc, Petit Verdot and Syrah grape pomaces against mouse macrophage RAW 264.7 cells.

The Merlot grape pomace extracts' minimum inhibitory concentration (MIC) results for Gram-negative and Gram-positive bacteria are presented in Table 3. All three assessed extracts showed higher antibacterial efficiency against Gram-positive bacteria than Gram-negative bacteria, which corroborates the results reported by Tseng and Zhao (2012) in their work regarding the antimicrobial potential of Pinot Noir and Merlot grape pomaces. Our hydroethanolic extracts exhibited highest inhibitory activities against *Enterococcus faecalis* and *Listeria monocytogenes*, with significant reduction of their activities after the *in vitro* digestion (2-fold reduction) and simulated colonic fermentation. Although the digestion and fermentation steps did not affect the inhibitory activity of the grape pomace hydroethanolic extract against MRSA-methicillin-resistant *Staphylococcus aureus* (MIC values remained 10 mg/ml), the *in vitro* fermentation did promote a 2-fold reduction in its inhibitory activity against MSSA-methicillin-susceptible *Staphylococcus aureus*.

Oliveira et al. (2013), when studying the bioactivities of Merlot grape pomace extracts obtained by CO_2 supercritical extraction (SC-CO_2), classified them as: strong inhibitors (MIC below 0.5 mg/mL), moderate inhibitors (MIC between 0.6 and 1.5 mg/mL) and weak inhibitors (MIC above 1.6 mg/mL). Based on their classification parameters, all grape pomace extracts assessed herein could be considered as weak inhibitors. Nevertheless, the bacteria strains used in this study are clinical isolated multiresistant strains (Dias et al., 2016), that present an antibiotic profile resistance much higher than ATCC bacterial strains. Oliveira et al. (2013) reported that the SC-CO_2 Merlot grape pomace extracts were moderate inhibitors of Gram-positive bacteria (mainly *S. aureus*, with MICs ranging from 0.625 to 0.750 mg/mL) and weak inhibitors of Gram-negative bacteria (with MIC values above 1.6 mg/mL against *E. coli* and *P. aeruginosa*).

Regarding the correlation between the bioactivity of the studied extracts and the presence of phenolic compounds, the results obtained showed slight correlation between peak 6 (B-type (epi)-catechin dimer) and the antioxidant activity mearsured using the β-carotene bleaching inhibition and TBARS inhibition (R^2=0.777 and 0.744, respectively) and peak 7 (B-type (epi)catechin dimer) with DPPH scavenging activity (R^2=0.666); no correlation were observed for reducing power assay, cytotoxicity neither for antibacterial activity.

4. Conclusion

Results of the present study showed that the *in vitro* digestion process led to drastic qualitative and quantitative reductions in the phenolic compounds profile of the Merlot grape pomace crude extract. Such alterations can be related to the decreases of some bioactivities of the extract, which seems to be the case of antioxidant and antibacterial properties, although not in a directly proportional manner. However, the *in vitro* digestion step apparently had no effect on the cytotoxic properties of the crude extract, except for the HeLa cell line. Interestingly, the simulated colonic fermentation seems to have a positive effect over the extract's antiproliferative potential. Unquestionably, further *in vivo* studies such as dietary intervention, are necessary with the view to unravel and confirm these results. In order to reduce the losses of grape pomace phenolic compounds and to preserve their bioactivities, the use of traditional and emerging microencapsulation technologies to ensure the delivery of these compounds could be easily performed. Overall, our findings contribute to the still scarce knowledge about the stability of grape pomace phenolic compounds and corresponding bioactivities during gastrointestinal digestion and colonic fermentation process, which could be useful in the development of nutraceutical supplements and functionalized food products.

5. Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.07.030.

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