Influence of in vitro digestion process on polyphenolic profile of skin grape (*cv Italia*) and on antioxidant activity in basal or stressed conditions of human intestinal cell line (HT-29)

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Abstract

Table grape *cv Italia* is a typical food of Mediterranean diet and one of the sources of phenolic compounds, particularly present in the skin portion. The aim of this study was to characterize the polyphenolic profile of skin grape and to assess their stability after *in vitro* digestion process. Further, the modulation of ROS and GSH levels was assessed in basal and in stressed conditions of human intestinal cells (HT-29). The main phenolic compounds identified by HPLC-DAD analysis in skin grape extract were: procyanidin B_1, caftaric acid, catechin, coutaric acid, quercetin 3-glucuronide and quercetin 3-glucoside. All compounds showed a good stability after *in vitro* digestion (from 43 to 80%). On intestinal cells, the biological effect of skin grape polyphenols was influenced by *in vitro* digestion process and was related to their concentration and to the intracellular redox status of cells. In basal conditions of intestinal cells, higher polyphenol concentrations showed pro-oxidant effect (recorded as high ROS level and low GSH content) due to their oxidation in cell culture condition, with consequent hydrogen peroxide production. Otherwise, in stressed conditions, grape polyphenols exerted antioxidant effects up to low levels ($1.3x10^{-6}$ µg/gr) and restored the stress-related GSH reduction. The digestion process influenced the chemical stability of polyphenols and attenuated their biological effects. In conclusion, grape skin

polyphenols showed different activity in relation to their concentrations and to the cellular redox state.

Highlights:

- Grape skin polyphenols pattern did not change after *in vitro* digestion process
- Redox status of intestinal cells modulated bioactivity of polyphenols skin grape
- Production of hydrogen peroxide by polyphenols in neutral environment

Keywords: grape, polyphenols, digestion process, intestinal cell model, antioxidant activity, intracellular ROS, intracellular GSH

Abbreviations

CAA: cellular antioxidant activity.CMHP: cumene hydroperoxide. DCFH-DA: 2′,7′-dichlorofluorescein diacetate. FW: fresh weight. GI: gastro-intestinal. GSH: reduced glutathione. MCB: monochlorobimane. ROS: reactive oxygen species.TP: total polyphenols.

1. Introduction

In gastrointestinal (GI) tract, the control of the intestinal redox environment is central for the nutrient digestion and absorption and for the barrier function (Circu & Aw, 2012) and it is assured by a complex dynamic system, involving antioxidant enzymes as well as non-enzymatic molecules (Spanou et al., 2011). High concentration of Reactive Oxygen Species (ROS) plays a key role in the pathogenesis of numerous chronic inflammatory intestinal diseases (Nawaz et al., 2016; Kim, Kim, & Hahm, 2012). Dietary antioxidants are known to prevent the oxidative damage of macromolecules and to maintain redox homeostasis (Spanou et al., 2011) by quenching excessive ROS and protecting or reinforcing endogenous antioxidative defense systems (Pervin et al., 2014). The most abundant antioxidants in the diet are polyphenols and table grapes represent one of the excellent sources of polyphenolic compounds (Giordano et al., 2016) differently distributed in grape seeds (60-70%), skin (28–35%) and pulp (10% or less) (Nawaz, Shi, Mittal, & Kakuda, 2006). In grape seed and skin the main phenolic compounds are proanthocyanidins, followed by anthocyanins, flavonols, flavanols, stilbens and phenolic acids (Xia, Deng, Guo, & Li, 2010).

Growing interests on phenolic compounds from grapes have focused on their beneficial effects on human health, such as antioxidant, cardioprotective, antiinflammation, antiaging and hypoglycemic properties (Lavelli, Sri Harsha, Ferranti, Scarafoni, & Iametti, 2016; Panico et al., 2006; Xia, Deng, Guo, & Li, 2010).

The present work was addressed to characterize the polyphenol profile of table grape skin *cv Italia* and to assess their stability after *in vitro* digestion process, because the skin, after seeds, contained the main amount of polyphenols. In addition, some markers of redox status were evaluated in order to assess the effect of polyphenols on human intestinal cell line both in basal and in stressed conditions, by measuring the modulation of ROS and GSH levels. Further, the polyphenols oxidation process in neutral cell culture conditions (Long & Halliwell, 2012; Odiatou, Skaltsounis, & Constantinou, 2013) was verified and its influence on antioxidant activity was tested.

2. Materials and methods

2.1 Materials

2′,7′- dichlorofluorescein diacetate (DCFH-DA), cumene hydroperoxide (CMHP), monochlorobimane (MCB) and all enzymes used for *in vitro* digestion were obtained from Sigma–Aldrich (Milan, Italy). The polyphenol standards used in this study were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). HT-29 intestinal cell line was purchased from ECACC (Sigma-Aldrich).

2.2 Polyphenols extraction from grape skin

Organic table grape *cv Italia* was provided by local farm (Tarulli O.P., Noicattaro, Bari, Italy). For the polyphenols extraction, 40 grape berries were manually peeled and the skin (about 8% of the whole berry weight) was recovered. The skin (15 g) was subjected to the extraction using 100 mL of boiling methanol/H_2O (50:50) twice for 1 hr. .The boiling methanol was used in order to have the best extraction yield without affecting the polyphenol stability (Perva-Uzunalić et al., 2006). After

filtration, the extracts were concentrated and brought to the final volume of 25 mL. Finally, the extracts were filtered and stored at -20°C for further analyses.

2.3 In vitro digestion process of skin grape extract

Skin grape extract from *Italia cv* underwent *in vitro* GI digestion following the method described by Cardinali et al., (2011). After digestion, the aqueous small intestinal digesta was frozen at −80 °C until HPLC-DAD analysis for assessment of polyphenols stability.

The percentage of polyphenols resulted stable to the GI conditions, was calculate as follows:

$$\text{Stability (\%)} = CF/CI \times 100$$

where CF is the polyphenol concentration in digesta fraction and CI is the initial polyphenols concentration in undigested grape skins extract.

2.4 Polyphenols characterization of skin grape before and after *in vitro* digestion.

HPLC analysis was performed using Agilent 1260 Infinity System equipped with a 1260 binary pump, 1260 HiP Degasser, 1260 TCC Thermostat, 1260 Diode Array Detector and Agilent Open Lab Chem Station Rev C.01.05 software. For the chromatographic separation, the column Luna C-18 (5 μm; 4.6 × 250 mm) (Phenomenex Torrance, California, USA) was used. The polyphenols detection was carried out using the characteristic wavelengths of each compound: 280 nm for catechin and procyanidin B_1, 325 nm for hydroxycinnamic acid and tartaric acid esters, and 360 nm for flavonoids. For the elution, the method reported by Lattanzio (1982) was followed and for the identification and quantification, the external commercial standards were used when available. Instead, caffeic acid and coumaric acid were used respectively for the quantification of caftaric acid and the coutaric acid.

2.5 Intestinal cell line

The human intestinal cell line HT-29 was grown following the procedures described by Minervini et al., (2014). With exception of cellular antioxidant activity assay, for the other assays HT-29 were exposed to skin grape extract and chyme samples at different total polyphenols (TP) concentrations:

from 0.23 to 130 μg TP/gr of fresh weight of grape skin (FW) and from 0.23 to 70 μg TP/gr FW, respectively.

2.6 Basal ROS quantification

In order to verify if grape skin polyphenols induced a modulation on basal oxidative status of human intestinal cells, ROS quantification was performed on HT-29 cells following the protocol described by Garbetta et al., (2014) with some modifications. After the staining phase with DCFH-DA probe, cells were treated for 30 min with skin grape extract and chyme samples. The fluorescence emission at 530 nm was measured with excitation at 485 nm after 1 h at 37°C into a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific). The level of intracellular ROS was expressed as percentage respect to control of DCF fluorescence.

2.7 Cellular antioxidant activity assay.

The antioxidant activity induced by polyphenols on induced ROS levels before and after *in vitro* digestion of grape skin extract *cv Italia*, was determined using Cellular Antioxidant Activity (CAA) assay according to Wolfe & Liu (2007) with some modifications. HT-29 cells were seeded at 5 x 10^4 cells/well on a 96 well white flat-bottom plate and incubated at 37°C for 24 h. Cells were stained with 5μM DCFH-DA and incubated for 30 min. Then, cells were treated for 30 min with methanolic extract from 6 x 10^{-7} to 60 mg of FW skin grape/mL (corresponding to a range of TP from 1.3 x10^{-6} to 130 μg/gr FW) or with chyme samples ranged from 0.05 to 12 mg of FW skin grape /mL (corresponding to a range of TP–from 0.23 to 70 μg/gr FW). Finally, cells were treated with CMHP (12.5 μM) for the last 10 minutes and the fluorescence was measured every 5 min for 1 h at 37°C into a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific) at emission wavelength of 530 nm and excitation wavelength of 485 nm. The quantification of CAA units and the median effective dose (EC_{50}) were performed following the protocols proposed by Wolfe & Liu (2007). The cellular antioxidant activity (CAA) values were expressed as micromoles of catechin equivalent per 100 gr of FW. The catechin was used as standard compound with high antioxidant activity in order to normalize the antioxidant activity.

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128 **2.8 GSH quantification**

129 The effect of polyphenols on redox status of HT-29 cells was studied assessing the intracellular
130 reduced Glutathione (GSH) by using MCB as specific probe (Loikkanen, Naarala, & Savolainen,
131 1998) in the presence and in the absence of stress inducer CMHP. Briefly, HT-29 were seeded in 96
132 well white flat-bottom plate and, after overnight incubation, were supplemented with skin grape
133 methanolic extract or with chyme samples for 1 hr. The cells were loaded with MCB (40µM/well)
134 for 15 min at room temperature in the dark. Thereafter, the formed fluorescent MCB-GSH complex
135 was measured at an excitation wavelength of 395 nm and an emission wavelength of 460 nm with
136 Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific).

137 **2.9 Hydrogen peroxide determination**

138 The hydrogen peroxide (H_2O_2) production, consequent to the oxidation of polyphenols in a neutral
139 conditions, was previously reported by other authors (Long & Halliwell, 2012; Odiatou,
140 Skaltsounis, & Constantinou, 2013). In order to evaluate the possible production of H_2O_2 by grape
141 skin polyphenols in neutral conditions, the quantification of H_2O_2 in the culture medium was
142 performed by using Life Technologies's Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit
143 with Varioskan Flash Spectral Scanning Multimode Reader (Thermo Fisher Scientific) at excitation
144 and emission maxima of approximately 571 nm and 585 nm. Hydrogen peroxide amount was
145 calculated by a standard curve from 0 to 5 µM and assessed in the presence or in the absence of HT-
146 29 intestinal cell line after 1 hr exposure to methanolic extract or chyme samples.

147 **2.10 Statistical Analysis**

148 Statistical analysis was performed by using SigmaPlotTM software v.12 (Systat Software, Inc.,
149 SigmaPlot for Windows). All pairwise Multiple Comparisons Dunn's method was used to evaluate
150 significant differences between cells treated with methanolic grape skin extract or chyme from *in*
151 *vitro* digestion of grape skin and control. Data were expressed as mean ± standard deviation of 9
152 values from 3 independent experiments. Values of $P < 0.05$ were considered statistically different.

3, Results

3.1 Polyphenols characterization of skin grape before and after *in vitro* digestion

Table 1 shows the phenolic characterization of grape skin extract from white grape (*Italia cv).* The quali-quantitative composition of non-anthocyanic polyphenols is very similar between white and red grape varieties, although the polyphenols pattern may be also influenced by the degree of ripeness, climate, berry size and grapevine variety (Giovinazzo & Grieco, 2015; Kammerer, Claus, Carle, & Schieber, 2004). The main phenolic compounds identified were: procyanidin B_1, caftaric acid, catechin, coutaric acid, quercetin 3-glucuronide and quercetin 3-glucoside. Traces of fertaric and caffeic acids were also found, but they cannot be quantified (data not shown). The main hydroxycinnamic acid, esterified with tartaric acid, was caftaric acid (658.02 μg/gr FW) followed by coutaric acid (96.87 μg/gr FW) although the latter, during grape maturation, undergoes to a reduction. The main flavanols present in the grape skin extract were procyanidin B_1 (360.33 μg/gr FW) and catechin (87.02 μg/gr FW). Finally, the presence of two flavonols, such as quercetin 3-glucuronide and 3-glucoside (100.95 μg/gr FW and 32.83 μg/gr FW, respectively), was noteworthy. After *in vitro* digestion, a good stability of the main polyphenols identified (from 43 to 80 %) was found with a recovery of 66%, of the total amount in grape extract. The figure 1 showed the chromatographic profile of grape skin extract (Fig.1 A) and after GI digestion (Fig.1 B), Interesting to underline that all the identified peaks were detected after digestion process and the formation of new compounds after digestion process was not evident.

3.2 Basal ROS quantification

The effect of grape skin polyphenols on basal oxidative status of intestinal cell line was showed in Fig 2. The higher TP concentrations (up to 30 μg TP/gr FW) of methanolic extract (Fig 2A) induced a significant and remarkable ($P<0.001$) increase in ROS production by HT-29 cells. The concentrations ranging from 0.23 to 15 μg TP/gr FW of grape skin did not induce any significant modification of basal oxidative status. Concerning chyme samples (Fig 2B) of grape skin, only the

highest concentration (70 μg TP/gr FW) significantly ($P<0.001$) increased the physiological levels of intracellular ROS.

3.3 Antioxidant activity against ROS induction by using CAA assay

As shown in Table 2, the methanolic extract resulted significantly more antioxidant (10 times) respect to the chyme sample. The EC_{50} values, reported in Table 2, corresponded to TP levels of 0.03 ± 0.006 μg/gr FW for methanolic extract and 0.27 ± 0.04 μg/gr FW for chyme sample.

3.4 GSH quantification

Concerning the effect of grape skin polyphenols on basal level of GSH, as shown in Fig 3, only the highest concentration of methanolic extract induced a significant ($P< 0.001$) decrease in GSH-bimane fluorescence respect to the control, as a consequence of increased basal ROS production. No effect on basal level of GSH was observed after cell exposure to grape skin chyme samples (data not shown).

After ROS induction in HT-29 cells with CMHP, a significant ($P< 0.001$) decrease in GSH-bimane fluorescence was observed in control samples, as a defensive response of cells to the oxidative stress. In presence of skin grape methanolic extract, significant ($P<0.05$) increased levels of GSH (about 10% respect to the stimulated control) was found at all tested skin grape concentrations. On the contrary, in presence of chyme samples of skin grape, the level of GSH fluorescence remained lower and unchanged respect to control samples stimulated by CMHP (data not shown).

3.5 Hydrogen peroxide determination

After incubation in the cellular media, all the tested samples (extract and chyme) generated a high level of H_2O_2, in a concentration-dependent manner (both in absence and in presence of cells) as a consequence of polyphenols oxidation. In Table 3 are showed the H_2O_2 quantifiable concentrations and the percentage of fluorescence increment relative to the H_2O_2 production respect to the control. In particular, without intestinal cells, the skin grape sample incubated in the culture media at concentration up to 7.5 μg TP/gr FW, produced an off-scale level of H_2O_2 (> 5 μM). Moreover, at

all other tested concentrations, the H_2O_2 levels were significant ($P<0.001$) higher than the control sample in dose-dependent manner. Also the chyme samples, up to 30 μg TP/gr FW, induced a not quantifiable H_2O_2 levels (> 5 μM). Furthermore, when the polyphenol concentrations were lower (up to 0.94 μg TP/gr FW of grape skin), the H_2O_2 production was significantly ($P<0.05$) higher than the control sample, but with less extend.

Moreover, the presence of cells reduced the H_2O_2 levels in medium both in control and in treated (methanolic and chyme) samples. In particular, with methanolic extract, apart the concentrations up to 15 μg TP/gr FW, a quantifiable H_2O_2 level was recovered. At concentrations ranged from 0.94 to 7.5 μg TP/gr FW, H_2O_2 levels were significantly ($P<0.05$) higher respect to the control. The lowest polyphenol concentrations of methanolic extract produced H_2O_2 levels comparable to the control sample (Table 3).Intestinal cell exposure to grape skin chyme samples induced a quantifiable H_2O_2 amount at the all tested polyphenol concentrations. A significant ($P<0.05$) difference respect to the control was found up to 15 μg TP/gr FW.

4. Discussion and conclusions

In the current paper, the stability of table grapes skin phenolics to the GI conditions and their effect toward cellular antioxidant response , were investigated. According to Rodrìguez- Montealegre et al., (2006), the caftaric acid was the main hydroxycinnamic acid present in grape skin followed by procyanidin B1, quercetin 3-glucuronide, 3-glucoside and catechin. The caftaric acid was the main hydroxycinnamic acid present in grape, responsible of the astringent taste in grape and wine, albeit during winemaking for the enzymatic oxidation, its amount decreased (Singleton, Salgues, Zaya, & Trousdale, 1985). Furthermore, all the identified compounds showed a good stability to the *in vitro* GI conditions (about 66%) giving some preliminary insight on the potential for bioaccessibility. The here showed results are in agreement with those reported by other authors on table grape polyphenols bioaccessibility (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010), and also on different matrices, such as grape juices and grape seeds (Moser et al., 2016; Laurent, Besancon, & Caporiccio, 2007).

Further, the influence of grape polyphenols on intestinal cells, in relation to their redox status, was also considered. In cell-free condition, the skin grape polyphenols (extract and chyme samples) produced a higher H_2O_2 amount respect to the control samples in a dose-dependent manner, in agreement to oxidation process toward polyphenols reported by authors (Long & Halliwell, 2012; Odiatou, Skaltsounis, & Constantinou, 2013). The digestion process negatively influenced the polyphenol oxidation in cell-free condition, reducing H_2O_2 production. The presence of HT-29 cells reduced the H_2O_2 levels in the medium of both controls and treated (methanolic extract and chyme) samples, probably due to cellular uptake/decomposition of H_2O_2 (Bellion et al., 2009).

At a cellular level, the polyphenol concentrations and the cell redox status (in not stressed and in stressed conditions) of cells influenced the bioactivity of skin grape. In basal condition, the pro-oxidant effect (recorded as high intracellular ROS) after exposure to higher TP concentrations was probably due to the diffusion of H_2O_2 through the plasma membrane mediated by aquaporins (Miller, Dickinson, & Chang, 2010). As a consequence, a reduced GSH level was observed in basal conditions. In fact Wang et al., (2016), using lower polyphenols concentrations (from 0.1 to 10 μg/ml), reported no influence on GSH level in basal condition of Caco-2 cells.

The effect of polyphenols changed after stress induction. A dose-dependent reduction of induced ROS levels was observed following exposure to skin grape extract in agreement with Wang et al., (2016) on Caco-2 cells and on rat colon explants reported by Giordano et al., (2016). Concerning GSH levels, a dramatic depletion of intracellular GSH was observed in control samples exposed to oxidative stress inducer partly prevented by the presence of polyphenols. Similar results on partial restored GSH content were found by Wang et al., (2016) when Caco-2 cells were exposed to grape polyphenolextract. These results supported that the antioxidant activity of dietary polyphenols is due both to ROS scavenger and to the reinforcement of endogenous GSH level, one of the antioxidant defense system (Pervin et al., 2014).

The digestion process decreased the polyphenols levels with consequent reduction of H_2O_2 production in culture medium (with and without cells) and it did not influence the basal redox

intracellular markers (ROS and GSH). The digestion process significantly reduced the antioxidant activity towards induced ROS levels without modifications of GSH level. Similarly Tagliazzucchi et al., (2010) reported, during digestion, modifications of antioxidant activity (assessed by FRAP method), correlated to the variations in whole grape polyphenol content as well as to the pH intestinal conditions, as also found for other fruits and vegetables (Huang et al., 2017).

In conclusion, antioxidant polyphenols present in skin grape extract may have pro-oxidant activity under certain conditions, such as high concentrations, not stressed cell status and/or neutral environment leading to cellular dysfunctions. In stressed cellular condition, skin grape polyphenols showed a high antioxidant activity. For this reason, as the digestion process could attenuate both effects, adequate amount of antioxidant food should be consumed, especially in oxidative stress-related diseases.

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References

Bellion, P., Olk, M., Will, F., Dietrich, H., Baum, M., Eisenbrand, G., & Janzowski, C. (2009). Formation of hydrogen peroxide in cell culture media by apple polyphenols and its effect on antioxidant biomarkers in the colon cell line HT-29. *Molecular Nutrition & Food Research*, 53, 1226–1236.

280 Cardinali, A., Linsalata, V., Lattanzio, V., & Ferruzzi, M. G. (2011). Verbascosides from olive mill
281 waste water: assessment of their bioaccessibility and intestinal uptake using an in vitro
282 digestion/Caco-2 model system. *Journal of Food Science*, 76, H48-54.
283 Circu, M. L., & Aw T. Y. (2012). Intestinal redox biology and oxidative stress. *Seminars in Cell &*
284 *Developmental Biology*, 23, 729–737.
285 Garbetta, A., Capotorto, I., Cardinali, A., D'Antuono, I., Linsalata, V., Pizzi, F., & Minervini, F.
286 (2014). Antioxidant activity induced by main polyphenols present in edible artichoke heads:
287 influence of in vitro gastro-intestinal digestion. *Journal of Functional Foods*, 10, 456-464.
288 Giordano, M. E., Ingrosso, I., Schettino, T., Caricato, R., Giovinazzo, G., & Lionetto, M. G. (2016).
289 Intracellular Antioxidant Activity of Grape Skin Polyphenolic Extracts in Rat Superficial
290 Colonocytes: In situ Detection by Confocal Fluorescence Microscopy. *Frontiers in Physiology*, 27,
291 7-177.
292 Giovinazzo, G, & Grieco, F. (2015). Functional properties of grape and wine polyphenols. *Plants*
293 *Foods for Human Nutrition*, 70, 454-462.
294 Huang, W., Mao, S., Zhang, L., Zheng, L., Zhou, F., Zhao, Y., & Li, M. (2017). Phenolic
295 compounds, antioxidant potential and antiproliferative potential of 10 common edible flowers from
296 China assessed using a simulated in vitro digestion-dialysis process combined with cellular assays.
297 *Journal of the Science of Food and Agriculture*, 97, 4760–4769.
298 Kammerer, D., Claus, A., Carle, R., & Schieber, A. (2004). Polyphenol Screening of Pomace from
299 Red and White Grape Varieties (Vitis vinifera L.) by HPLC-DAD-MS/MS. *Journal of Agricultural*
300 *and Food Chemistry*, 52, 4360-4367.
301 Kim, Y. J., Kim, E. H., & Hahm, K. B. (2012). Oxidative stress in inflammation-based
302 gastrointestinal tract diseases: challenges and opportunities. *Journal of Gastroenterology and*
303 *Hepatology*, 27:1004–1010.
304 Lattanzio, V. (1982). High performance reversed phase liquid chromatography of free and bound
305 phenolic acids in eggplant (Solanum melongena L.). *Journal of Chromatography*, 250, 143–148.

306 Laurent, C.; Besancon, P., & Caporiccio, B. (2007). Flavonoids from a grape seed extract interact
307 with digestive secretions and intestinal cells as assessed in an in vitro digestion/Caco-2 cell culture
308 model. *Food Chemistry*, 100, 1704–1712.
309 Lavelli, V., Sri Harsha, P. S. C., Ferranti, P., Scarafoni, A., & Iametti, S. (2016). Grape skin
310 phenolics as inhibitors of mammalian α-glucosidase and α-amylase – effect of food matrix and
311 processing on efficacy. *Food and Function*, 7, 1655–1663.
312 Loikkanen, J. J., Naarala, J., & Savolainen, K. M. (1998). Modification of glutamate-induced
313 oxidative stress by lead: the role of extracellular calcium. *Free Radical Biology and Medicine*, 24,
314 377-384.
315 Long, L. H., & Halliwell, B. (2012). The effects of oxaloacetate on hydrogen peroxide generation
316 from ascorbate and epigallocatechin gallate in cell culture media: Potential for altering cell
317 metabolism. *Biochemical and Biophysical Research Communications*, 417, 446–450.
318 Miller, E. W., Dickinson, B. C., & Chang, C. J. (2010). Aquaporin-3 mediates hydrogen peroxide
319 uptake to regulate downstream intracellular signaling. *Proceedings of the National Academy of*
320 *Sciences of the United States of America*, 107, 1568-15686.
321 Minervini, F., Garbetta, A., D'Antuono, I., Cardinali, A., Martino, N. A., Debellis, L., & Visconti,
322 A. (2014). Toxic mechanisms induced by fumonisin b1 mycotoxin on human intestinal cell line.
323 *Archives of environmental contamination and toxicology*, 67, 115-123.
324 Moser, S., Lim, J., Chegeni, M., Wightman, J. D., Hamaker, B. R., & Ferruzzi, M. G. (2016).
325 Concord and niagara grape juice and their phenolics modify intestinal glucose transport in a coupled
326 in vitro digestion/Caco-2 human intestinal model. *Nutrients*, 8, 414.
327 Nawaz, H., Shi, J., Mittal, G. S., & Kakuda, Y. (2006). Extraction of polyphenols from grape seeds
328 and concentration by ultrafiltration. *Separation and Purification Technology*, 48, 176–181.
329 Odiatou, E. M., Skaltsounis, A. L., & Constantinou, A. I. (2013). Identification of the factors
330 responsible for the *in vitro* pro-oxidant and cytotoxic activities of the olive polyphenols oleuropein
331 and hydroxytyrosol. *Cancer Letters*, 330, 113–121.

Panico, A. M., Cardile, V., Avondo, S., Garufi, F., Gentile, B., Puglia, C., Bonina, F., Santagati, N. A., & Ronsisvalle, G. (2006). The *in vitro* effect of a lyophilized extract of wine obtained from Jacquez grapes on human chondrocytes. *Phytomedicine*, 13, 522–526.

Perva-Uzunalić, A., Škerget, M., Knez, Ž., Weinreich, B., Otto, F., & Grüner, S. (2006). Extraction of active ingredients from green tea (Camellia sinensis): Extraction efficiency of major catechins and caffeine. *Food Chemistry*, 96, 597-605.

Pervin, M., Hasnat, M. A., Lee, Y. M., Kim, D. H., Jo, J. E., & Lim, B. O. (2014). Antioxidant activity and acetylcholinesterase inhibition of grape skin anthocyanin. *Molecules*, 19, 9403-9418.

Rodrìguez-Montealegre, R., Romero-Peces, R., Chacón-Vozmediano, J. L., Martínez-Gascueña, J., & García-Romero, E. (2006). Phenolic compounds in skins and seeds of ten grape *Vitis vinifera* varieties grown in a warm climate. *Journal of Food Composition and Analysis*, 19, 687–693.

Singleton, V. L., Salgues, M., Zaya, J., & Trousdale, E. (1985). Caftaric Acid Disappearance and Conversion to Products of Enzymic Oxidation in Grape Must and Wine. *American Journal of Enology and Viticulture*, 36, 50-56.

Spanou, C., Veskoukis, A. S., Stagos, D., Liadaki K., Anastasiadi, M., Haroutounian, S. A., Tsouka, M., Tzanakouli, E., & Kouretas, D. (2011). Effects of grape extracts on the in vitro activity of enzymes involved in oxidative stress regulation. *In vivo*, 25, 657-662.

Tagliazucchi, D., Verzelloni, E., Bertolini, D., & Conte, A. (2010). *In vitro* bio-accessibility and antioxidant activity of grape polyphenols. *Food Chemistry*, 120, 599–606.

Wang, S., Mateos, R., Goya, L., Amigo-Benavent, M., Sarrià, B., & Bravo, L. (2016). A phenolic extract from grape by-products and its main hydroxybenzoic acids protect Caco-2 cells against pro-oxidant induced toxicity. *Food and Chemical Toxicology*, 88, 65-74.

Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry*, 55, 8896–8907.

Xia, E. Q., Deng, G. F., Guo, Y. J., & Li, H. B. (2010). Biological activities of polyphenols from grapes. *International Journal of Molecular Sciences*, 11, 622-646.

Figures captions

Fig. 1 Effect of cv *Italia* white grape table skin polyphenols on basal ROS production in HT-29 intestinal cell line before (a) and after (b) *in vitro* digestion. (Mean ± SD n=9. ** P< 0.001 respect to the control).

Fig. 2 Effect of grape skin methanolic extract on intracellular GSH level in HT-29 intestinal cell line. (Mean ± SD n=9. ** P< 0.001 respect to basal control; # P<0.05 respect to stimulated control).

Table 1 Polyphenolic compounds in skins of cv *Italia* white grape table: hydroalcoholic extract, digested, and stability after *in vitro* digestion.

Polyphenolic compounds	***Extract (µg/g fw)***	***Digested (µg/g fw)***	***Stability (%)***
Procyanidin B1	360.33 ± 9.0	170.46 ± 5.1	47.31
Caftaric acid	658.02 ± 18.4	527.77 ± 15.8	80.21
Catechin	87.02 ± 2.6	46.83 ± 1.4	53.82
Coutaric acid	96.87 ± 3.4	64.68 ± 2.3	66.77
Quercetin 3-glucuronide	100.95 ± 3.8	52.28 ± 2.0	51.79
Quercetin 3-glucoside	32.83 ± 1.3	14.22 ± 0.5	43.31
TOTAL	**1336.02 ± 46.8**	**876.24 ± 33.3**	**65.59**

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Table 2 Comparison of antioxidant activity of cv *Italia* white grape table skin after and before *in vitro* digestion. (Mean ± SD n=9. * values for methanolic sample and chyme sample are significantly different P< 0.05)

	EC_{50} value (mg of grape skin/ml)	***Cellular Antioxidant Activity (CAA) (µmol CAT equivalent/100 gr FW)***
Grape skin methanolic extract	**0.07 ± 0.04**	**4.39 ± 0.93**
Grape skin chyme sample	**0.7 ± 0.03 ***	**0.43 ± 0.03***

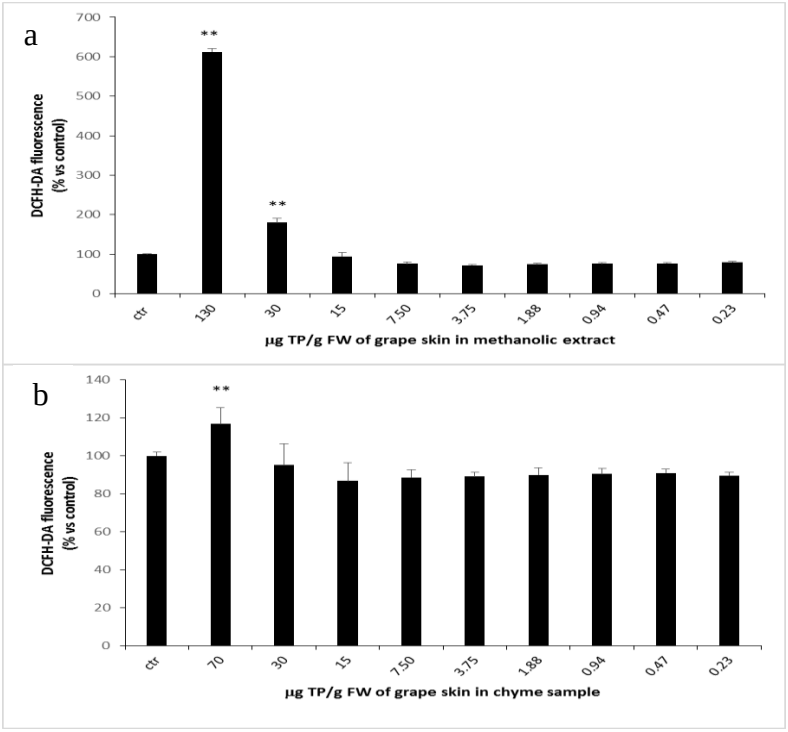
373
374
375

Table 3 Effect of grape skin polyphenols before and after *in vitro* digestion on H_2O_2 production.

Methanolic extract					***Chyme sample***				
	medium		***cells***			***medium***		***cells***	
µgTP/g FW	H_2O_2 (µM)	% Fluorescence increment	H_2O_2 (µM)	% Fluorescence increment	**µg TP/g FW**	H_2O_2 (µM)	% Fluorescence increment	H_2O_2 (µM)	% Fluorescence increment
ctr	1.19 ± 0.16^{a}		0.29 ± 0.04^{a}		ctr	1.17 ± 0.06^{a}		0.34 ± 0.07^{a}	
130	> 5	*5868*	> 5	*6800*	70	> 5	*1543*	1.93 ± 0.10^{b}	*281*
30	> 5	*3026*	> 5	*2207*	30	> 5	*578*	0.95 ± 0.01^{b}	*107*
15	> 5	*1810*	> 5	*1317*	15	3.66 ± 0.04^{b}	*264*	0.51 ± 0.04^{b}	*30*
7.50	> 5	*862*	2.52 ± 0.04^{b}	*437*	7.50	2.25 ± 0.10^{c}	*114*	$0.45 \pm 0.06^{a,b}$	*20*
3.75	5.00 ± 0.20^{b}	*406*	1.16 ± 0.11^{c}	*171*	3.75	1.65 ± 0.05^{d}	*51*	$0.35 \pm 0.03^{a,b}$	*1*
1.88	3.13 ± 0.14^{c}	*202*	0.67 ± 0.05^{d}	*75*	1.88	$1.38 \pm 0.06^{e,f}$	*22*	$0.36 \pm 0.02^{a,b}$	*3*
0.94	2.16 ± 0.11^{d}	*101*	0.42 ± 0.06^{e}	*27*	0.94	1.32 ± 0.05^{f}	*16*	$0.33 \pm 0.06^{a,b}$	*0*
0.47	$1.79 \pm 0.25^{d,e}$	*62*	$0.36 \pm 0.03^{a,e}$	*14*	0.47	$1.23 \pm 0.10^{a,f}$	*6*	$0.28 \pm 0.03^{a,b}$	*0*
0.23	1.44 ± 0.04^{e}	*26*	$0.25 \pm 0.02^{a,f}$	*0*	0.23	$1.17 \pm 0.02^{a,f}$	*0*	$0.26 \pm 0.02^{a,b}$	*0*

(Mean ± SD n=9). Different letters within a column denote significant differences ($P<0.05$)

Fig.1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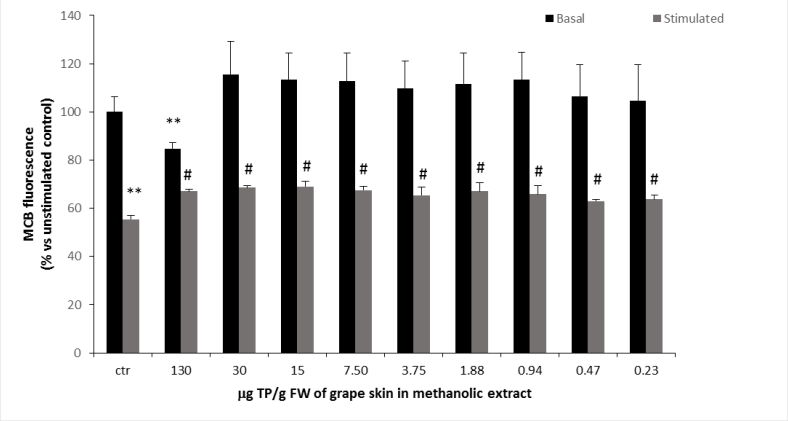
DCFH-DA fluorescence (% vs control)
700
600
500
400
300
200
100
0
**
**
ctr
130
30
15
7.50
3.75
1.88
0.94
0.47
0.23
µg TP/g FW of grape skin in methanolic extract
b
DCFH-DA fluorescence (% vs control)
140
120
100
80
60
40
20
0
**
ctr
70
30
15
7.50
3.75
1.88
0.94
0.47
0.23
µg TP/g FW of grape skin in chyme sample

Fig 2


Basal
Stimulated
MCB fluorescence (% vs unstimulated control)
140
120
100
80
60
40
20
0
**
**
#
#
#
#
#
#
#
#
#
ctr
130
30
15
7.50
3.75
1.88
0.94
0.47
0.23
µg TP/g FW of grape skin in methanolic extract