Title: A phenolic extract from grape byproducts and its main hydroxybenzoic acids protect Caco-2 cells against pro-oxidant induced toxicity

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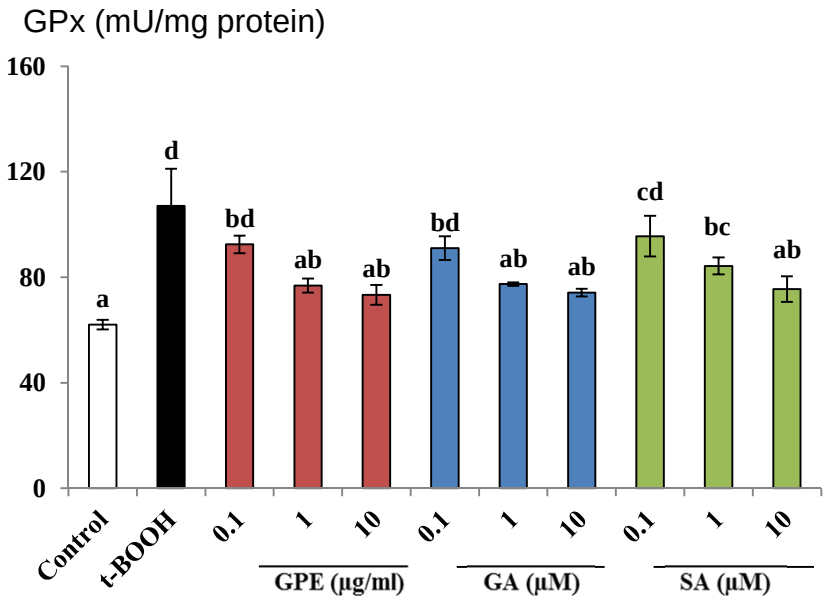
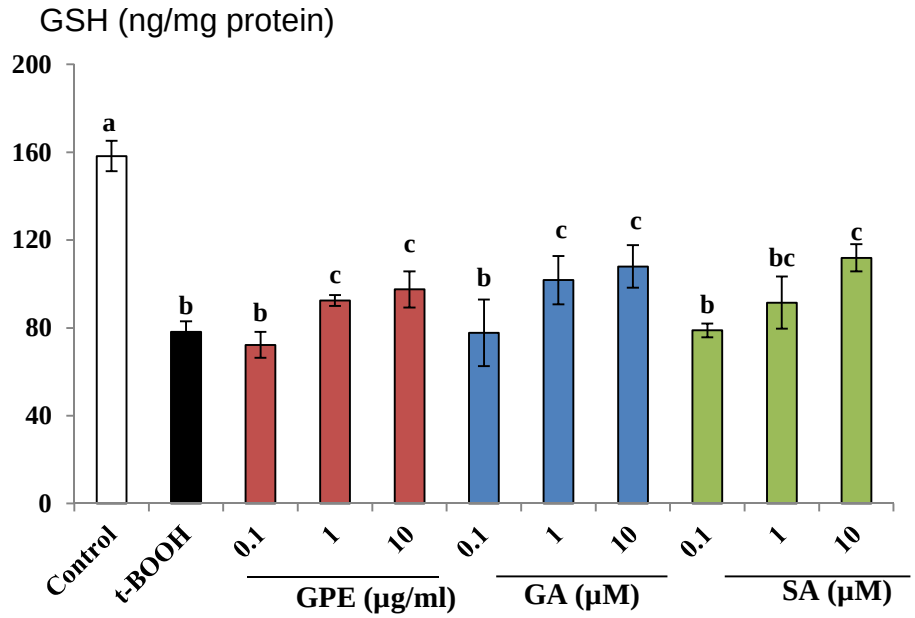
Protective effects of pre-treatment with a grape by-products phenolic extract (GPE), gallic acid (GA) or syringic acid (SA) to exposure of pro-oxidant t-BOOH



Enzymatic defences: Glutathione peroxidase (GPX)



Non-enzymatic defences: Glutathione reductase (GSH)

Abstract

Grape/wine industry produces large amounts of by-products, however knowledge on their health-promoting qualities is limited. This study investigated the effects of a grape phenolic extract (GPE) and its phenolic compounds, gallic acid (GA) and syringic acid (SA) on human intestinal Caco-2 cells, directly or after cytotoxicity induced by tert-butylhydroperoxide (t-BOOH). Direct treatment with 0.1-10 μg/mL GPE, or 0.1-10 μM GA and SA produced no major cytotoxic effect, either changes in antioxidant defenses (glutathione content, glutathione peroxidase and reductase activities) or protein damage (carbonyl groups). However, 10 μg/mL GPE, 1 and 10 μM GA and 10 μM SA decreased reactive oxygen species (ROS) production.

Pre-treatment with GPE, SA and GA at the same concentrations for 20 h showed that 10 μg/mL GPE and 10 μM GA or SA significantly counteracted ROS increase induced by t-BOOH. 10 μg/mL GPE and 1-10 μM GA or 10 μM of SA significantly reduced prooxidant-induced cytotoxicity. 1-10 μg/mL GPE, 1-10 μM GA and 10 μM SA significantly recovered both depleted glutathione and enhanced glutathione reductase and peroxidase activities, and reduced protein oxidative damage. Therefore, treatment with realistic concentrations of GPE and its main hydroxybenzoic acids protected Caco-2 cells against induced oxidative stress.

Keywords: antioxidant, Caco-2 cells, gallic acid, grape by-product, oxidative stress, syringic acid.

1. Introduction

Grape (*Vitis vinifera* L.), wine and winery by-products have great economic value. Italy, France, Spain, and the United States are among the most important producers in the world. Approximately 80% of the grapes are used in winemaking and ~20% of the weight of processed grapes remains as by-products annually generating about 10 million tons of waste from wineries (Kammerer et al., 2004). Use of by-products from wine industry is becoming a hot topic; they are employed in juices, jam, yogurts, jelly and other foods as colorants and a source of dietary fiber. In the last years, grape by-products have also been commercialised as nutraceuticals or dietary supplements (Georgiev et al., 2014). The composition of these products includes phenolic compounds such as phenolic acids, flavonoids, procyanidins and stilbenes (Yu and Ahmedna, 2013). Numerous studies have demonstrated that foods rich in phenolic compounds exert beneficial health effects as it has been reviewed (Bravo, 1998; Tomás-Barberán and Andrés-Lacueva, 2012), being the antioxidant activity one of their most important biological properties. The reduction of oxidative stress by grape by-products has been reported in animal testing (Choi et al., 2012). Ten day pretreatment of rats with a grape seed proanthocyanidin extract decreased DNA oxidative damage in isolated colonocytes (Giovanelli et al., 2000). Due to the "French paradox" the majority of the studies have been focused on flavonoids, being hydroxybenzoic acids (also a major component of grape wine by-products) much less studied or known.

Intestinal epithelium is exposed to toxicity induced by luminal oxidants from ingested foods which can cause oxidative damage to macromolecules and tissues, and as a consequence bowel discomfort. This fact supports the interest of studying

oxidative stress-induced toxicity and the effect of dietary antioxidants in the intestinal epithelium. Human Caco-2 cell line, derived from human colon cancer cells, is widely used for biochemical and nutritional studies as a cell culture model of human colonocytes since they retain their morphology and most of their function in culture (Rodríguez-Ramiro et al., 2011). Under certain culture conditions, Caco-2 cells can form spontaneously polarized microvillus, closely connected with differentiation characteristics mimicking intestinal epithelium (Artursson and Karlsson, 1991, Yamashita et al., 2000). For this reason, Caco-2 cells have been used to study the intestinal absorption of food components such as peptides (Amigo-Benavent et al., 2014) and phenolic compounds (Konishi and Kobayashi, 2004; Mateos et al., 2011), among others. This cell model has also been previously employed in our group to test the protective effect of cocoa flavanols against the damage of food toxic acrylamide (Rodriguez-Ramiro et al., 2011) and the modulation of oxidative status by dietary flavanols (Ramos et al., 2011). Bearing this in mind, the aim of this article was to investigate the protective effect of a grape phenolic extract (GPE) derived from wine by-products and its main hydroxybenzoic compounds against toxicity induced by prooxidant t-BOOH in Caco-2 cells.

2. Materials and Methods

2.1. Reagents

Grape by-product was purchased in a local market in Madrid (Spain) as a powdered grape product. Gallic acid (GA), syringic acid (SA), tert-butylhydroperoxide (t-BOOH), dichlorofluorescein (DCFH), *o*-phthaldialdehyde (OPT), glutathione reductase

(GR), reduced (GSH) and oxidized glutathione, nicotine adenine dinucleotide (reduced) (NADH), nicotine adenine dinucleotide phosphate reduced salt (NADPH), 2',7'-dichlorofluorescin diacetate (DCFH-DA), 2,4-dinitrophenylhydrazone (DNPH), dimethyl sulfoxide (DMSO), gentamycin, penicillin G, streptomycin, β-mercaptoethanol and EDTA were purchased from Sigma, Spain. The Bradford reagent was from BioRad Laboratories Madrid, Spain. The WST-1 reagents were purchased from Roche, Spain. DMEM-F12 culture media and fatal bovine serum (FBS) were from Lonza-Cultek (Madrid, Spain). Caco-2 cells were from the Cell Bank of the Scientific Instrumentation Center at the University of Granada (Spain). All other reagents were of analytical quality. Stock standard solutions (10 mM) of gallic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, coumaric acid, ellagic acid, rutin, quercetin and myricetin were chromatographic grade for the calibration curve.

2.2. Characterization of phenolic compounds in GPE by HPLC–ESI-QToF analysis

GPE was prepared by the conventional organic solvent extraction (0.8% HCl in methanol/water, 50/50, v/v and acetone/water, 70/30, v/v) described by Bravo and Saura-Calixto (1998). The extracts were evaporated to eliminate methanol, acetone and HCl, lyophilized and dissolved in 10% DMSO in deionized water. For HPLC analysis, GPE was resuspended in 1% (v/v) formic acid and filtered through a PVDF 0.45 µm filter prior to injection on the HPLC system. The chromatography was performed on an Agilent 1200 series LC system coupled to an Agilent 6530A accurate-mass quadrupole time-of-flight (Q-ToF) with ESI-Jet Stream Technology (Agilent Technologies). Separation was performed on a Superspher 100 RP18 column (250 mm × 4.6 mm i.d., 4 µm, Agilent Technologies) preceded by an ODS RP18 guard column thermostatic oven

at 30°C. Each sample (20 µL) was injected and separated by using a mobile phase consisting of water and acetonitrile, both containing 1% formic acid, at a flow rate of 1 mL/min. The solvent gradient changed from 10% to 20% solvent B over 5 min, 20% to 25% solvent B over 20 min, 25% to 35% solvent B over 10 min, isocratically for 25 min, returning to the initial conditions over 10 min. Chromatograms were recorded at 280, 360 and 520 nm according to the expected maxima spectra of phenolic compounds. The Q-ToF acquisition conditions were as follows: 2 GHz, mass range between 100 and 1000 m/z, drying gas volume and temperature 10 L/min and 350 °C, sheath gas volume and temperature 11 L/min and 350°C, nebulizer pressure 45 psi, cap voltage 3500 V for negative polarity and 4000 V for positive polarity, nozzle voltage 1000 V, and fragmentor voltage 75 V. Negative polarity was used to identify phenolic acids and flavonols while positive polarity to characterize anthocyanins. Data acquisition and qualitative analysis were performed by using MassHunterWorkstation Software.

2.3. Quantification of phenolic compounds in GPE by HPLC-DAD

Phenolic composition of GPE was analyzed using an Agilent 1200 liquid chromatographic system equipped with an autosampler, quaternary pump and diode-array (DAD) detector. Chromatographic conditions (eluents, column, flow rate, gradient, etc.) were as described above. Chromatograms were acquired at 280 nm, 360 nm and 520 nm to quantify phenolic acids, flavonols and anthocyanins, respectively. Quantitative analysis used the external standard method. Pure standard compounds (except ellagic acid, which was dissolved in 0.1 M NaOH) were dissolved in 10% DMSO in deionized water and injected onto the HPLC system. Extractions were performed in triplicate and the results are expressed as the mean value.

2.4. Cell Culture and treatment

For cell experiments a GPE stock solution of 10 mg/mL in 10% DMSO was prepared and stored at -20°C. The day of the experiment, the stock solution was diluted with FBS-free DMEM-F12 medium to prepare 0.1, 1 and 10 µg/mL solutions (final concentration of DMSO in the cell culture was 0.1%). GA and SA stock solutions (10 mM) were dissolved in 10% DMSO and diluted with FBS free medium to prepare fresh working solutions at concentrations of 0.1, 1 and 10 µM (final concentration of DMSO in the cell culture was 0.1%). Caco-2 cells were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37°C and grown in DMEM F-12 medium supplemented with 10% FBS and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin (Rodriguez-Ramiro et al., 2011). The culture medium was changed every other day in order to remove the non-adhered and dead cells. Cells were usually split 1:3 when they reached confluence. The day before the experiments, cells were changed to FBS-free medium to avoid potential FBS interference with the assays.

For direct effect assays, cells were incubated with GPE (0.1, 1 and 10 µg/mL in 0.1% DMSO), GA and SA (0.1, 1 and 10 µM in 0.1% DMSO) for 20 h. For protection effect assays, cells were pre-treated with the same concentrations of the GPE, GA or SA for 20 h, then the medium was discarded, the cells were washed with PBS and FBS-free medium containing 400 ∝M t-BOOH was added for 3 h except in the ROS assay (see determination of ROS production), after which the cell cultures were processed as detailed below for each assay. A minimum of four culture plates were set with each of the conditions studied.

2.5. Evaluation of cell viability and cytotoxicity

The colorimetric WST-1 reagent assay was used to test cell viability with GPE, GA and SA following manufacturer's instructions. This method is based on the reduction by viable cells of WST-1 reagent by NAD-dependent dehydrogenase activity to its water-soluble colored form formazan. The amount of dye formed directly correlates to the number of viable cells. Cells were seeded (100 µL, $4x10^3$ cells/ well) in 96-well plates. After 24 h of incubation, the medium was removed and cells were treated with 100 µL of the different concentrations of GPE, GA and SA for 20 h. Then, 10 µL of WST-1 reagent were added into the wells and the plates were incubated for 1 h. The absorbance at 440 nm was measured using a microplate reader (Bio-Tek, Madrid, Spain). Background control was also prepared by adding culture medium or sample plus WST-1 reactive in the absence of cells. Results are expressed as percentage of cell viability referred to the absorbance measured in control cells.

Cytotoxicity was determined by the lactate dehydrogenase (LDH) assay (Welder & Acosta, 1994). Cells (1.5×10^6) were seeded in 60 mm diameter plates and treated with 3 mL of the different concentrations of GPE, GA and SA. After treatment, the culture medium was collected and separately the cells were scraped in 3 mL of PBS. Cells were sonicated to ensure the breakdown of the cell membrane and release of the total amount of LDH and centrifuged at 2000 rpm at 4°C for 10 min. The absorbance at 340 nm in the culture medium and in the cell content was determined in a microplate reader after employing 40 µL of cell culture or supernatant and adding 200 µL of mixture of 1.35 M Tris, 0.08 M pyruvate and 2 mg/mL NADH. The percentage of LDH

leakage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content.

2.6. Determination of ROS production

Cellular ROS were quantified by the DCF assay using a microplate reader to screen the antioxidant effect of the different concentrations of each of the compounds studied (Alia et al., 2006). Briefly, the cells were seeded in 24-well plates (2 $\times 10^5$ cells per well) in medium containing FBS and replaced with the FBS-free medium the following day. After 20 h, 5 µM DCFH-DA was added to the wells at 37°C for 30 min, cells were then washed with PBS, placed in fresh FBS-free medium with the different concentrations of GPE, GA and SA and ROS production was monitored for 60 min. For the protection assay, cells were seeded and left overnight before treating them with GPE, GA and SA in FBS-free medium for further 20 h. The following steps were the same as indicated above except that in addition 400 µM t-BOOH was added to every well but controls with further incubation for 1 h. Control cells without t-BOOH treatment were used as negative control. Using a fluorescent microplate reader, fluorescence readings were taken at excitation and emission wavelengths of 485 and 528 nm, respectively. Results are expressed as percent of fluorescence units.

2.7. Determination of GSH content

GSH content was quantitated by the fluorometric assay of Hissin and Hilf (1976). The method takes advantage of the reaction of reduced glutathione with OPT at pH 8.0. Caco-2 cells were plated in 60 mm diameter plates at a concentration of 1.5 x 10^6 cells/plate. Cells were treated with the different conditions of GPE, GA and SA for

20 h, collected by scraping in 1.5 mL of PBS and centrifuged (1500 rpm, 4°C, 5 min). Cells were lysed by adding 110 µL of 5% (w/v) trichloroacetic acid containing 2 mM EDTA and protein concentration was measured. Following centrifugation of cells at 7500 rpm and 4°C for 30 min, 50 µL of the clear supernatant were transferred to wells in a 96-well plate. Then, 15 µL of 1 M NaOH was added to neutralize the sample and 175 µL of 0.1 M sodium phosphate buffer, pH 8.0, containing 5 mM EDTA and finally 10 µL per well of a stock solution of OPT (10 mg/mL) in methanol were added. After 15 min at room temperature in the dark, fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The results of the treatments were referred to those of a standard calibration curve of GSH (5–1000 ng).

2.8. Determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities

For the assay of the GPx and GR activities the protocols described by Alía et al. (2005) were followed. Briefly, Caco-2 cells were seeded in 100-mm diameter plates ($2x10^6$ cells/plate) and after treatment, were scraped in 1.5 mL of PBS and centrifuged at 1500 rpm, 4°C for 10 min. Cell pellets were resuspended in 200 µL of 50 mM Tris containing 5 mM EDTA and 0.5 mM β-mercaptoethanol, sonicated and centrifuged at 1248 g, 4°C for 15 min. Enzyme activities were measured in the supernatants. Determination of GPx activity was based on the oxidation of GSH by GPx, using t-BOOH as a substrate, coupled to the disappearance rate of NADPH by GR. GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione. Protein concentrations in the samples were measured by the Bradford reagent following provider's protocol.

2.9. Determination of protein damage

Carbonyl groups were determined as a biomarker of protein damage in Caco-2 cells following the protocol described by Levine et al. (1990) and modified by Martín et al. (2014). Cells were seeded in 100-mm diameter plates (2×10^6 cells/plate) and subjected to the different treatments. Culture medium was collected and cells scrapped and collected in PBS. Then, cells were centrifuged (2000 rpm, 4°C, 10 min), resuspended in 0.25 M Tris buffer pH 7.4 containing 0.2 M sucrose and 5 mM DTT (dithiothreitol), and sonicated. The cytoplasmic content obtained by centrifugation was incubated with 4 volumes of 10 mM DNPH, another with the same volume were incubated in 2 N HCl as the control. All the tubes were kept in darkness for 1 h at room temperature, mixed several times during incubation and protein content was precipitated with the same volume of 10% trichloroacetic acid. After centrifuging and washing twice with 1 mL ethyl acetate:ethanol (1:1, v/v), the pellet was resuspended in 500 µL 6 M guanidine and the absorbance was measured at 360 nm. Results are expressed as nmol of carbonyl groups per mg protein.

2.10. Statistics

SPSS version 19.0 program (IL, USA) was used for the statistical analysis of data. Homogeneity of variances was checked by the test of Levene. Multiple comparisons within the different treatments were carried out using One-way ANOVA followed by Bonferroni test. The level of significance was $p < 0.05$.

3. Results

3.1 Characterisation and quantification of phenolic compounds in GPE

Figure 1 shows HPLC profiles of GPE at 280 (A), 360 (B) and 520 (C) nm. The peaks were identified by HPLC-ESI-QToF analysis and comparison of retention time and UV spectrum with commercial standards of phenolic compounds. Up to 28 different phenolic compounds were detected in GPE. Table 1 shows the list of compounds along with their retention time, UV characteristics of the chromatographic peaks, molecular ion, fragment ions and molecular formula (M.F.), in addition to the quantitative analysis. According to the chromatographic analysis, the total phenolic content in GPE was 2211.6 μg/g dry matter, mostly phenolic acids (1877.1 μg/g) with lower content of flavonols (305.5 μg/g) and anthocyanins (29.0 μg/g). The major phenolic acids were GA and ellagic acid. Among flavonols, quercetin was the most abundant. Due to the difficult solubilisation of ellagic acid, GA was chosen to carry out the cell culture experiments. SA (4-hydroxy-3,5-dimethoxybenzoic acid) was also selected on the basis of its abundance and to evaluate the biological effect that its double methyl substituents presents, in comparison with GA (3,4,5-trihydroxybenzoic acid). Although the quercetin content was similar to that of SA, it was not selected as this compound has been extensively studied in our group and has shown to strongly protect HepG2 cells against oxidation induced by tert-butyl hydroperoxide (Alía et al., 2005; Alía et al., 2006 among others).

3.2. Viability, cytotoxicity and antioxidant effects of GPE, GA and SA on Caco-2 cells in basal conditions

Table 2 shows the results obtained in basal conditions after direct treatment of Caco-2 cells with the GPE and the two specified compounds. No statistically significant effects were found in cell viability determined by WST-1 reagent, except for 10 µM of GA which showed a percentage of viability of 89%. The percentage of LDH when cells were incubated with 0.1-10 µg/mL GPE and 0.1-10 µM GA or SA was similar to control cells. High LDH values indicate a cytotoxic effect; therefore, it can be assumed that the range of concentrations finally selected (0.1-10 µM) does not induce cytotoxic effects in Caco-2 cells. This was an essential point to test before looking into the potential nutraceutical and/or therapeutical properties of these compounds.

Likewise, there were no statistically significant changes in cell antioxidant defenses (GSH content, GPx and GR activities) and protein oxidation after 20 h of treatment with these samples. Thus, it can be assumed that this range of concentration can be safely used to study the protective effect of GPE, GA and SA against a condition of oxidative stress on cells. Moreover, incubation of Caco-2 cells with 1-10 µM SA and 10 µg/mL GPE or 10 µM of GA for 1 h statistically decreased ROS production (Fig. 2).

3.3. Protective effect of GPE, GA and SA on Caco-2 cells in a condition of oxidative stress

The protective effect of pre-treatment with 0.1, 1 and 10 µg/mL GPE, or 0.1, 1 and 10 µM GA and SA for 20 h in Caco-2 cells against oxidative stress induced by t-BOOH treatment was studied. In order to generate a condition of cellular oxidative stress, cells were treated with 400 µM t-BOOH, a strong pro-oxidant, and ROS were evaluated for 1 h of t-BOOH exposure, and cytotoxicity and antioxidant defences after

3 h of oxidative insult. In these experiments, a negative control with t-BOOH-untreated cells and a positive control of oxidative stress in cells with the same amount of t-BOOH and for the same incubation time were included.

3.3.1. Cell toxicity

Treatment with 400 µM t-BOOH significantly enhanced (3.5 times compared with negative control) leakage of LDH, indicating prominent cell damage in Caco-2 cells at 3 h (Fig. 3). However, when cells were pre-treated with 10 µg/mL GPE, 1-10 µM GA and 10 µM SA a significant decrease in cytotoxicity induced by t-BOOH was observed compared with the stressed control.

3.3.2. ROS generation

Caco-2 cells treated with t-BOOH for 1 h showed a two-fold increase in intracellular ROS generation as compared to non-stressed controls. Treatment of cells with 10 µg/mL GPE and 1-10 µM GA or SA significantly decreased ROS production compared to that of stressed cells (Fig. 4). Lower concentrations of the tested compounds did not show statistical differences compared with t-BOOH control cells.

3.3.3. Antioxidant defences

Non-enzymatic defences: Addition of 400 µM t-BOOH to Caco-2 cells for 3 h evoked a dramatic decrease in the cytoplasmic GSH (ng/mg protein) to 50% of non-stressed cells (Fig. 5). Pre-treatment with 1-10 µg/mL GPE, 1-10 µM GA and 10 µM SA partly recovered GSH levels.

Enzymatic defences: Oxidative insult with 400 µM t-BOOH in Caco-2 cell culture induced a ~2-fold increase in the enzyme activities of GPx and GR (Fig. 6). When cells were pre-treated with 1-10 µg/mL GPE and 1-10 µM GA or SA for 20 h, the chemically-induced rise in GPx activity was prevented or reduced compared with stressed cells. GR over-activation was also restricted compared with t-BOOH treated cells after pre-incubation with 1-10 µg/mL GPE and 1-10 µM of either phenolic compound. Lower concentrations of GPE, GA and SA did not significantly decrease GPx and GR activities.

3.3.4. Biomarker of protein oxidative damage

Caco-2 cells treated with 400 µM t-BOOH for 3 h showed a significant increase in the cellular concentration of carbonyl groups, indicating extensive oxidative damage to cell proteins (Fig. 7). Pre-treatment of Caco-2 cells with 1-10 µg/mL GPE, 0.1-10 µM GA and SA for 20 h significantly prevented the increase of carbonyl groups induced by t-BOOH, indicating a reduced level of protein oxidation in response to the stressor.

4. Discussion

In this article, the phenolic composition of a wine by-product has been investigated together with the protective antioxidant effects of its phenolic extract and its major hydroxybenzoic acids, GA and SA, in Caco-2 cells.

The HPLC analysis showed that hydroxybenzoic acids were the most abundant phenolic compounds in GPE followed by flavonols and hydroxycinnamic acids, with lower amounts of anthocyanins. The presence of vanillic, gallic and protocatechuic acids agreed with Obreque-Slier et al. (2010) and Rodríguez-Montealegre *et al.* (2006) for grape pomace. Flavonol content was similar to the concentration described in red

grape pomace (Kammerer et al., 2004) and dietetic supplements derived from grape skin and stalks (Monagas et al., 2006).

Previous studies have demonstrated that wine by-products may have beneficial effects on diabetes, cancer and other diseases mediated by oxidative stress (Shrikhande, 2000). Their use as antioxidants against rancidity and bacterial pathogens has also been reported (García-Lomillo et al., 2014). The biological actions of main phenolic compounds in wine by-products such as hydroxybenzoic acids have been ascribed to their antioxidant capacity, i.e. free radical scavenging and chelation of redox active metal ions (Tomás-Barberán and Andrés-Lacueva, 2012). These phenolic compounds may have potent antioxidant effects *in vitro* and *in vivo*, both in cell cultures and in animals (Chen et al., 2007; Lakshmi et al., 2014; Tung et al., 2009; Yonguc et al., 2015). SA has also shown protective effects in mice with CCl_4-induced liver injury (Itoh et al., 2010). However, the possible chemo-protective effects of hydroxybenzoic acids in the colon have been scarcely studied. Therefore, the present work evaluated the effects of GPE, a wine by-product and two of its major hydroxybenzoic acids in cultured colon-derived cells. In an attempt to understand the cellular mechanisms involved in the chemo-protection, as well as their potential role as regulators of antioxidant defences, as a primary approach we investigating the biochemical mechanisms underlying the antioxidant defence response which will safeguard the cell in a severe condition of oxidative stress. Biochemical pathways such as reduced glutathione stores and antioxidant enzyme activity are the primary response and represent the first in-deep regulatory mechanism.

Elevated doses of these dietary compounds can also act as pro-oxidants in cell culture systems inducing cellular damage (Forester and Waterhouse, 2010; Halliwell, 2014). Previous studies have shown that high doses of GA (>100 µM) in the cell media could convert O_2 to hydrogen peroxide, which increases ROS and induces selective cytotoxicity for cancer cells but present much lower toxicity for normal cells (Forester and Waterhouse, 2010; Inoue et al., 1995; Isuzugawa et al., 2001). Therefore, it was necessary to ensure that no direct cell damage was caused by concentrations within the physiological range of the tested antioxidant before investigating its protective effect (Sarriá et al., 2012). Doses of 10-40 µM hydroxycinnamic acids and 10-50 µg/mL of green coffee polyphenolic extract have shown protection against oxidative conditions in HepG2 cells (Baeza et al., 2014). The concentration range used for this study is not far from realistic in order to evaluate the effect at the biological level. A concentration of 4 µM of GA in human plasma has been observed after consumption of 50 mg of pure gallic acid (Manach et al., 2005). Likewise, 239 µM of GA has been reported in rat plasma after the consumption of 100 mg/kg of grape seed polyphenol extract (Ferruzzi et al., 2009). In the present study, only the highest GA concentration evoked a slight decrease in cell viability after 20 h and most interestingly, 10 µg/mL GPE, 10 µM GA and 1-10 µM SA induced a significant reduction in ROS generation. Moreover, antioxidant defences such as GSH, GPx and GR were not altered by any treatment. Therefore, cells treated with GPE, GA or SA seem to be in a favourable condition to face an oxidative challenge.

T-BOOH has been extensively used to induce cytotoxicity by oxidative stress in cell culture experiments. It can induce epithelial cell damage, mainly by inhibiting cell viability, inducing cell apoptosis, increasing protein peroxidation and inflammatory

response, reducing cellular antioxidant capacity, and increasing intracellular ROS levels (Alia et al., 2006; Baeza et al., 2014; Martín et al., 2010). Before starting the study, several concentrations of t-BOOH were tested to assure that toxicity by oxidative stress on Caco-2 cells was produced. Results show that concentrations over 400 µM evoke significant cell damage in the medium and over 40% cell death measured by the crystal violet assay (data not shown). Cells treated with t-BOOH also showed remarkably high LDH leakage, but those pre-treated with 10 µg/mL GPE, 10 µM SA or 1-10 µM GA exhibited a significant decrease in t-BOOH cytotoxicity. This result indicated that the integrity of the challenged cells was remarkably protected against the potent oxidative insult.

ROS production is a good indicator of the oxidative status in cells (Alía et al., 2005) being induced by t-BOOH when added to Caco-2 cells. Pre-treatment of cells with 10 µg/mL GPE and 1-10 µM GA or SA significantly reduced ROS over-production induced by t-BOOH. This result shows that the high levels of ROS generated were significantly quenched by the antioxidant compounds in cells pre-treated for 20 h. This ROS-quenching effect by GPE, GA and SA could be a first explanation for the reduced oxidative stress and subsequent cell protection.

GSH is a substrate in glutathione peroxidase-catalysed detoxification of organic peroxides, which repairs free radical induced damage through electron-transfer reactions. A dramatic depletion of GSH intracellular levels was observed when 400 µM t-BOOH was added to Caco-2 cells. Pre-treatment with 1-10 µg/mL GPE, 1-10 µM GA or 10 µM SA partly prevented the depletion of GSH induced by t-BOOH. Other studies have reported similar results with a cocoa phenolic extract and its main flavanols in Caco-2 cells submitted to acrylamide-induced oxidative toxicity (Ramiro-Rodriguez et

al., 2011). Moreover, it should be highlighted that the loss of cellular GSH seems to play an important role in apoptotic signaling. Flavanols have recently shown to provide parallel protection by enhancing the activity of a number of protective GSH dependent enzymes (Ramos et al., 2011), which are essential in cancer prevention (Rodriguez-Ramiro et al., 2011).

The enzymatic constituents of antioxidant defence system play a crucial role against oxidative stress; thus, the significant increase in the activity of GPx and GR observed after 3 h treatment with 400 µM t-BOOH clearly indicates a positive response in the cell defence system to face the intense ROS production in the presence of the prooxidant and overcome the oxidative insult (Alía et al., 2006). However, a rapid return of the antioxidant enzyme activities to basal values once the challenge has been surmounted will position the cell in a favorable condition to deal with a new challenge (Alía et al., 2006; Baeza et al., 2014; Sarriá et al., 2012). Accordingly, we have previously reported that realistic concentrations of a cocoa (Martín et al., 2010) and green coffee (Baeza et al., 2014) extracts averted cell damage by preventing the permanently increased activity of GPx and GR induced by t-BOOH. The present study has shown that pre-treatment of cells with 1-10 µg/mL GPE and 1-10 µM GA or SA can efficiently return GPx and GR activities to basal values preparing cells to further oxidative insults. These results, together with those of GSH, indicated that the prevention or delay of appearance of conditions causing oxidative stress in the cell may also reflect the ability of a compound to modulate the cellular antioxidant defences.

Oxidative stress often leads to loss in specific protein or enzymatic function due to macromolecular damage. Carbonyl groups are considered as consistent biomarkers

of oxidative damage to proteins, because of its relatively early formation and relative chemical stability of oxidised proteins and a crucial event in the development of cellular toxicity (Dalle-Donne et al., 2003). Pre-treatment of Caco-2 with 1-10 µg/mL GPE and 0.1-10 µM GA or SA decreased protein oxidation in response to the stressful situation. Chemo-protective effects on oxidative markers have also been reported with a cocoa phenolic extract and its main flavonoids (Rodríguez-Ramiro et al., 2011) in the same cell line, and with derivatives from olive oil hydroxytyrosol (Pereira-Caro et al., 2011) and green coffee phenolic compounds (Baeza et al., 2014) in a liver-derived cell line.

Attending to the outcome above, the smallest concentrations used in the present study did not show a significant protection against t-BOOH. These results are in agreement with Sun et al. (2014) who described no prevention of ROS generation or lipid peroxidation when cells were pre-incubated with 0.1 µM GA for 24 h before $Na_2S_2O_4$ exposure. GA is well absorbed in humans compared with other polyphenols and is found in plasma and urine, reaching concentrations up to 4 µM (Lu et al., 2006; Shahrzad and Bitsch, 1998). However, dietary phenolic compounds are limited by their absorption and metabolization rate (Deiana et al., 2012; Manach et al., 2005). *In vivo* experiments reported that GA is metabolized into methylation (unconjugated and conjugated 4-O-methylgallic acid, 2-O-methylgallic acid), decarboxylation (unconjugated and conjugated pyrogallol, 4-O-methylpyrogallol), and dehydroxylation (resorcinol) (Lu et al., 2006). Such high metabolism of hydroxybenzoic acids favours their disappearance as pure compounds from circulation and rapid excretion, explaining the need for doses in the micromolar range to effectively regulate antioxidant responses and exhibit the chemo-protective capacity. Nevertheless,

activity of phenolic compounds metabolized by intestinal microbiota should not be discarded since microbial-derived metabolites from cocoa flavonoids have shown anti-diabetic properties in cultured beta cells (Fernández-Millán et al., 2014).

5. Conclusions

GPE is a wine by-product rich in hydroxybenzoic acids, flavonols and hydroxycinnamic acids. The treatment of human Caco-2 cells in culture with physiological doses of GPE and two of its major phenolic compounds, GA and SA, provides cells with significant protection against toxicity by oxidative stress. This chemo-protection prepares Caco-2 cells, and potentially enterocytes, to face oxidative damage induced by dietary toxins. Further studies in animals and humans are needed to assess this biological activity of GPE and confirm its potential as a nutraceutical.

6. Acknowledgements

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7. Figure captions

Figure 1. HPLC chromatogram profiles recorded at 280 (A), 360 (B) and 520 (C) nm of the grape phenolic extract. Peaks were identified by HPLC–ESI-QToF analysis and quantified by HPLC-DAD.

Figure 2. Direct effect on Caco-2 cell intracellular ROS generation after treatment with GPE (A), SA (B) and GA (C) over 60 min. Results are expressed as % of fluorescence arbitrary units against time (n=4–6). Different letters denote statistically significant differences within the same time period ($p<0.05$).

Figure 3. Protective effect of pre-treatment with GPE, GA and SA for 20 h on cell toxicity (LDH %) against oxidative stress induced by t-BOOH for 3 h. Results are means ± SD (n= 4–8). Different letters denote statistically significant differences ($p<0.05$).

Figure 4. Protective effect on Caco-2 cell intracellular ROS production pre-treated with the noted concentrations of GPE (A), SA (B) and GA (C), and after t-BOOH exposure for 60 min. Results are expressed as % of fluorescence arbitrary units against time (n=4–6). Different letters within the same time denote statistically significant differences ($p<0.05$).

Figure 5. Protective effect of pre-treatment with GPE, GA, SA for 20 h, and after t-BOOH exposure for 3 h on GSH content. Results are means ± SD (n= 4–8). Different letters denote statistically significant differences ($p<0.05$).

Figure 6. Protective effect of pre-treatment with GPE, GA and SA for 20 h, and after t-BOOH oxidative insult for 3 h on Caco-2 cell antioxidant enzymes GPx (A) and GR (B). Results are means ± SD (n=4–8). Different letters denote statistically significant differences ($p<0.05$).

Figure 7. Protective effect of pre-treatment with GPE, GA and SA against oxidative stress induced by t-BOOH for 3h on the Caco-2 cell protein-oxidation (carbonyls groups). Results are means ± SD (n=4–8). Different letters denote statistically significant differences ($p<0.05$).

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Table 1. Content of phenolic compounds identified in GPE by HPLC-DAD in combination with LC-QTOF analysis. Results expressed as mean ± standard deviation (n=3). DM means dry matter.

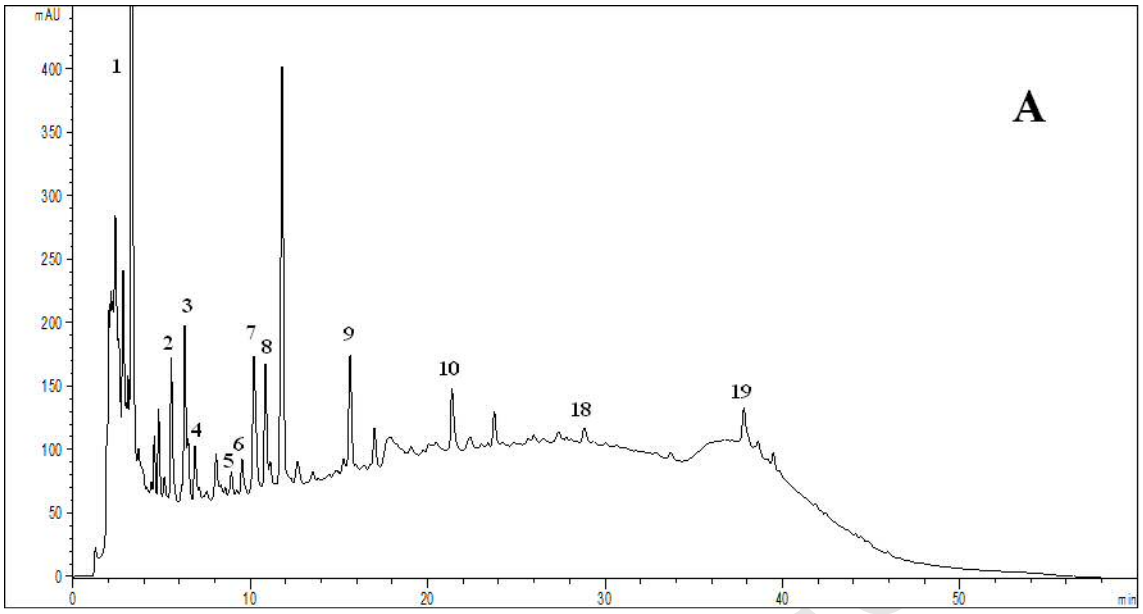
Chr. Peak	Compound	RT (min)	λmax	Molecular Ion	Fragment ions	M.F.	㎍/g DM
Phenolic acids (280 nm)							
1	Gallic acid	3.3	272	169.0142	125	$C_7H_6O_5$	627.3 ± 63.1
2	Protocatechuic acid	5.5	260, 292	153.0193	109	$C_7H_6O_4$	231.0 ± 19.7
3	Caftaric acid	6.3	296, 324	311.0409	179	$C_{13}H_{12}O_9$	109.9 ± 7.7
4	*p*-hydroxybenzoic acid	6.5	285	137.0244	93	$C_7H_6O_3$	60.6 ± 2.3
5	*Cis*-coutaric acid	9.5	294, 309	295.0459	163	$C_{13}H_{12}O_8$	11.7 ± 1.2
6	*Trans*-coutaric acid	9.8	295, 314	295.0459	163	$C_{13}H_{12}O_8$	20.4 ± 2.3
7	Fertaric acid	10.2	297, 331	325.0565	193	$C_{14}H_{14}O_9$	162.3 ± 10.7
8	Vanillic acid	10.8	260	167.0350	123	$C_8H_8O_4$	126.7 ± 20.2
9	Syringic acid	15.6	279	197.0455	153, 182	$C_9H_{10}O_5$	102.5 ± 4.1
10	Ellagic acid	21.3	254, 368	300.9990		$C_{14}H_6O_8$	424.6 ± 35.6
Total phenolic acids							**1877.1 ± 166.9**
Flavonols (360 nm)							
11	Rutin	19.8	256, 354	609.1461	301	$C_{27}H_{30}O_{16}$	9.0 ± 0.8
12	Quercetin-3-*O*-glucoside	22.1	256, 354	463.0882	301	$C_{21}H_{20}O_{12}$	21.2 ± 2.1
13	Quercetin-3-*O*-glucuronide	22.2	254, 353	477.0675	301	$C_{21}H_{18}O_{13}$	25.1 ± 0.8
14	Miricetin-3-*O*-glucoside	22.9	258, 358	479.0831	317	$C_{21}H_{20}O_{13}$	1.7 ± 0.1
15	Isoramnetin-3-*O*-glucoside	23.1	254, 354	477.1038	315	$C_{22}H_{22}O_{12}$	8.4 ± 1.1
16	Kampherol-3-*O*-glucoside	25.8	264, 348	447.0933	285	$C_{21}H_{20}O_{11}$	21.8 ± 2.3
17	Siringetin-3-*O*-glucoside	27.8	252, 360	507.1144	345	$C_{23}H_{24}O_{13}$	17.1 ± 2.0
18 (18)	Miricetin	28.7	254, 368	317.0303		$C_{15}H_{10}O_8$	34.8 ± 3.3
19 (19)	Quercetin	38.1	255, 370	301.0354		$C_{15}H_{10}O_7$	107.8 ± 6.2
20	Laricetrin	38.7	250, 372	331.0459		$C_{16}H_{12}O_8$	16.5 ± 1.4
21	Kaempherol	44.8	264, 363	285.0405		$C_{15}H_{10}O_6$	19.3 ± 6.4
22	Isoramnetin	45.4	256, 368	315.0510		$C_{16}H_{12}O_7$	11.5 ± 1.0
23	Siringetin	46.0	254, 374	345.0616		$C_{17}H_{14}O_8$	11.0 ± 0.5
Total flavonols							**305.5 ± 27.9**
Anthocyanins (520 nm)							
24	Delphinidin-3-*O*-glucoside	10.7	520	465.1028	303	$C_{21}H_{20}O_{12}$	4.1 ± 0.2
25	Cyanidin-3-*O*-glucoside	14.2	510	449.1078	287	$C_{21}H_{20}O_{11}$	2.0 ± 0.2
26	Petunidin-3-*O*-glucoside	14.9	528	479.1184	317	$C_{22}H_{23}O_{12}$	4.0 ± 0.2
27	Peonidin-3-*O*-glucoside	18.1	520	463.1235	301	$C_{22}H_{23}O_{11}$	2.3 ± 0.2
28	Malvidin-3-*O*-glucoside	19.0	522	493.1341	331	$C_{23}H_{25}O_{12}$	16.6 ± 1.1
Total Anthocyanins							**29.0 ± 1.9**
Total Phenols							**2211.6 ± 196.7**

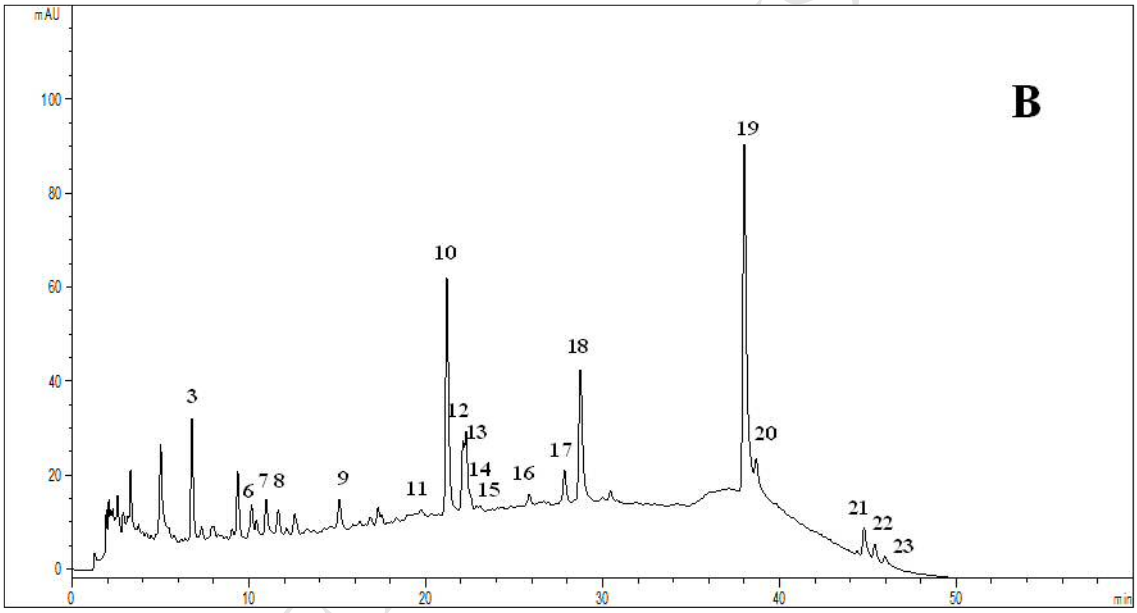
Chr. Peak: chromatographic peak; RT (min): retention time (min); M.F.: molecular formula.

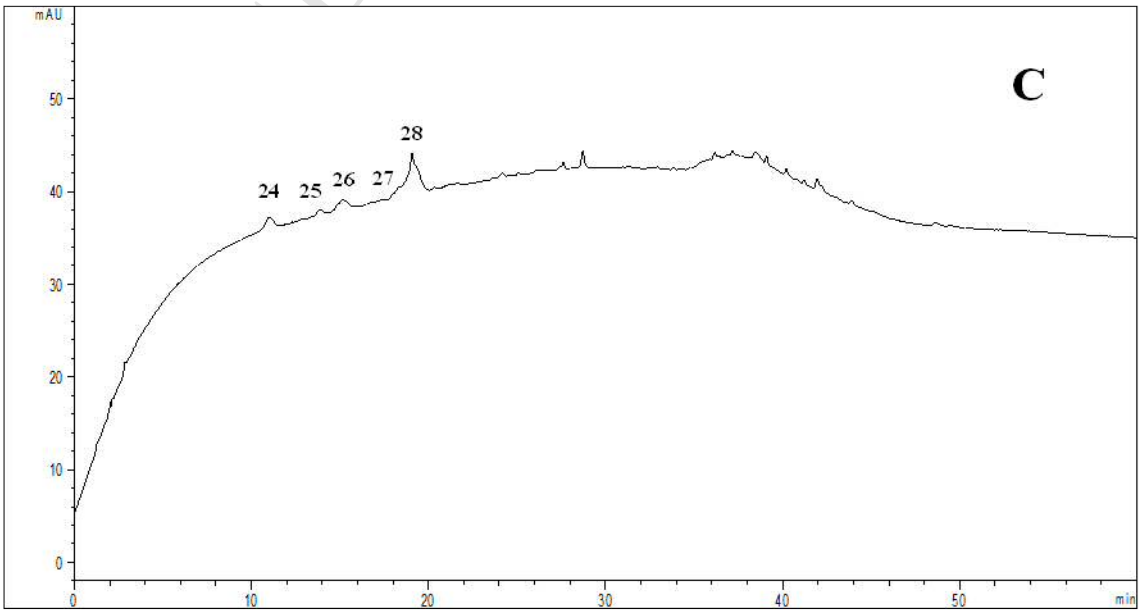
Table 2. Direct effect of GPE, GA and SA on Caco-2 cell viability, antioxidant defenses (GSH, GPx and GR activities) and protein damage (carbonyls). Results expressed as mean ± standard deviation (n = 4-8). Different letters within a column denote statistically significant differences ($p<0.05$).

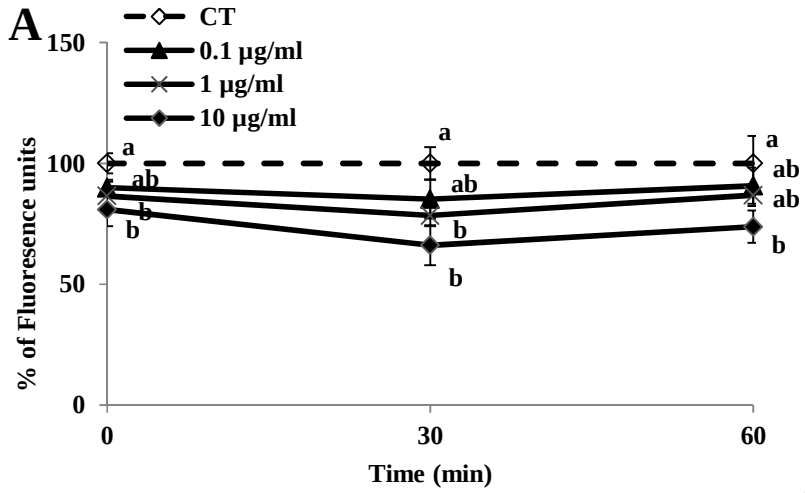
	Cell viability %	LDH %	GSH ng/mg protein	GPx mU/mg protein	GR mU/mg protein	Carbonyls nmol/mg protein
Control	100.0±3.9^{a}	17.9±1.5^{a}	244.3±14.5^{a}	104.1±12.5^{a}	2.8±0.2^{a}	2.1±0.4^{a}
GPE						
0.1µg/mL	93.4±6.1^{ab}	18.5±1.0^{a}	240.8±10.6^{a}	100.2±9.4^{a}	2.8±0.2^{a}	1.9±0.3^{a}
1 µg/mL	91.6±4.3^{ab}	17.9±0.8^{a}	233.8±6.4^{a}	104.6±5.4^{a}	2.7±0.1^{a}	2.3±0.3^{a}
10 µg/mL	91.8±4.3^{ab}	16.9±1.7^{a}	248.6±14.2^{a}	89.1±4.2^{a}	2.6±0.3^{a}	2.3±0.2^{a}
GA						
0.1µM	100.2±3.5^{a}	16.2±1.2^{ab}	241.0±16.4^{a}	101.8±5.5^{a}	2.9±0.2^{a}	1.8±0.2^{a}
1 µM	96.6±5.7^{ab}	17.1±0.6^{a}	248.7±20.3^{a}	108.6±2.5^{a}	2.6±0.3^{a}	2.0±0.2^{a}
10 µM	88.9±2.6^{b}	15.9±0.6^{ba}	236.9±2.5^{a}	102.6±2.6^{a}	2.5±0.3^{a}	2.0±0.1^{a}
SA						
0.1 µM	93.6±4.8^{ab}	16.2±1.2^{ab}	228.7±17.4^{a}	95.0±8.1^{a}	2.6±0.1^{a}	2.0±0.2^{a}
1 µM	94.1±7.1^{ab}	16.2±2.0^{ab}	221.1±17.9^{a}	98±1.1^{a}	2.8±0.1^{a}	2.0±0.1^{a}
10 µM	92.0±4.3^{ab}	16.4±1.7^{ab}	229.4±13.4^{a}	90.0±8.5^{a}	2.7±0.1^{a}	2.2±0.1^{a}

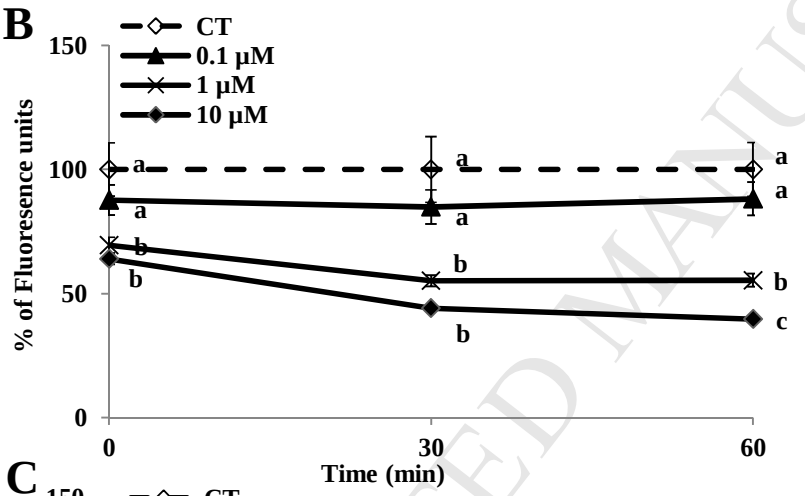
Figure 1.

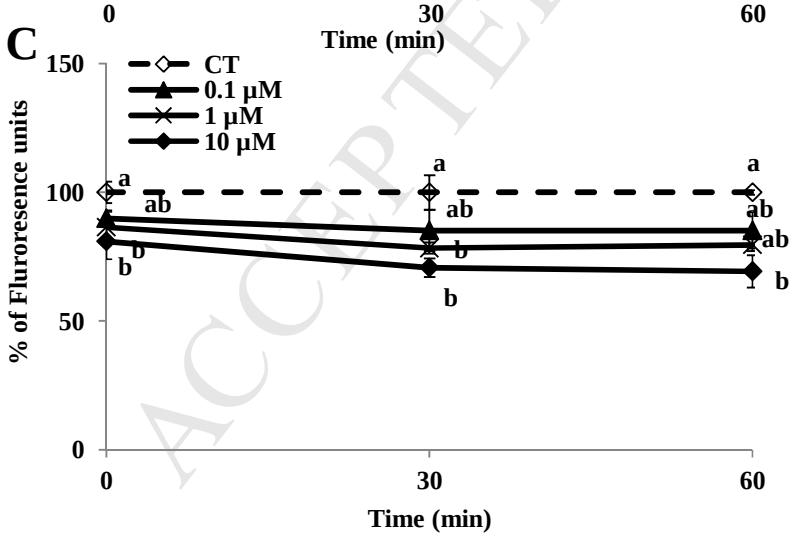












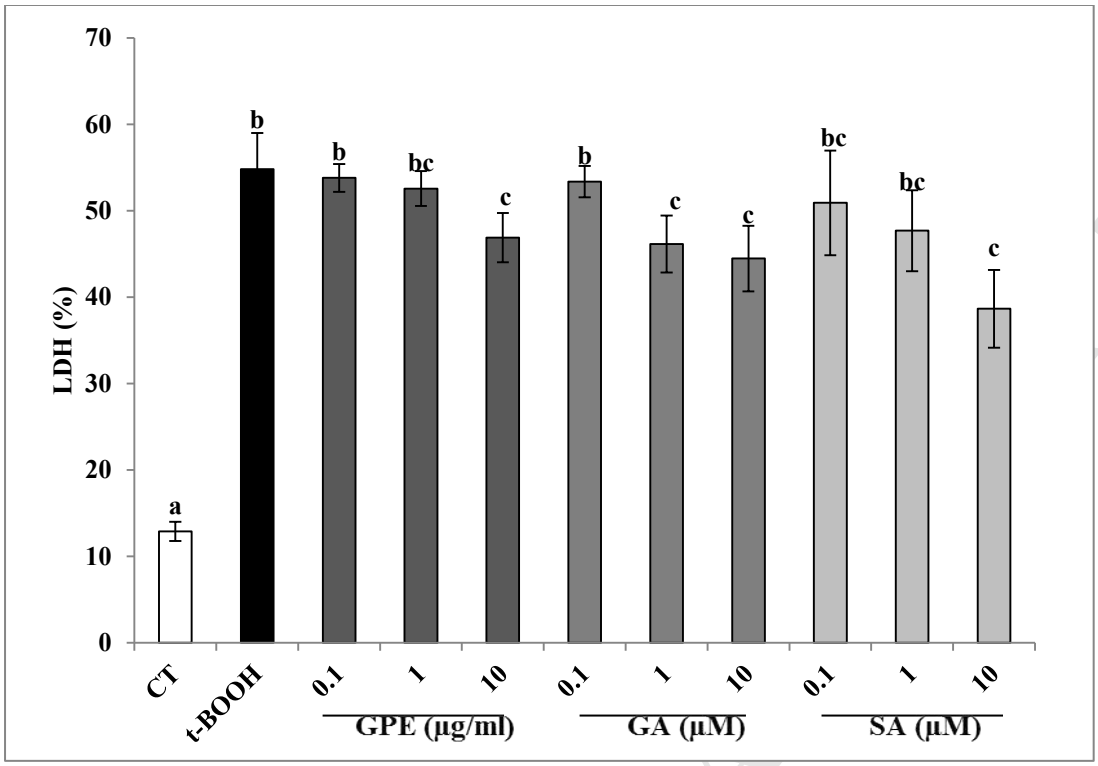
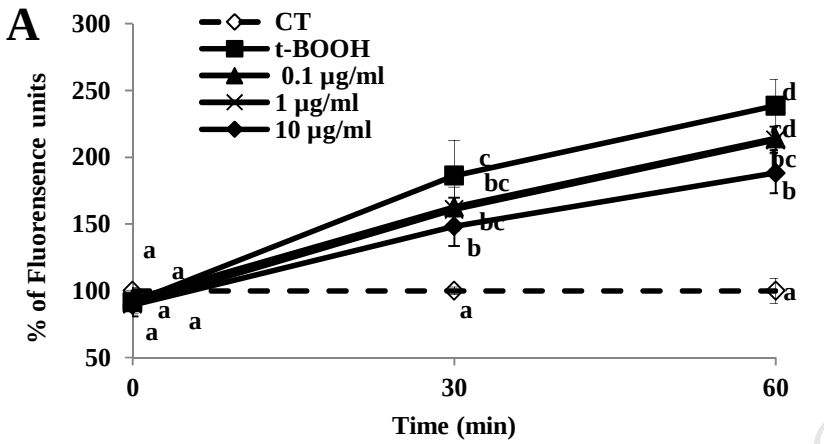
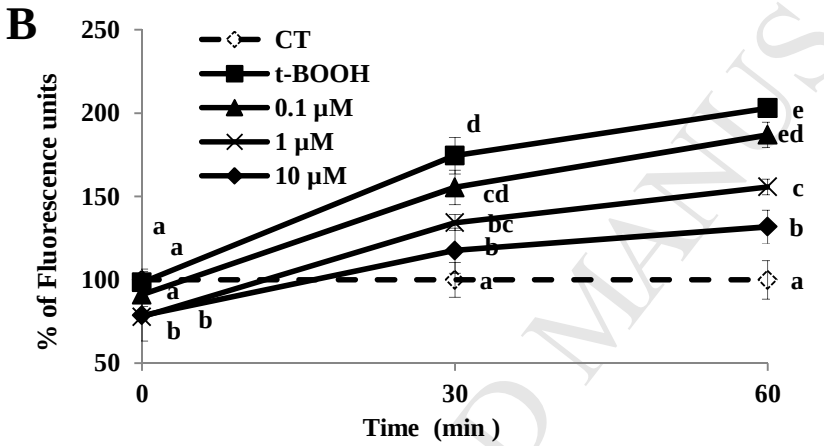
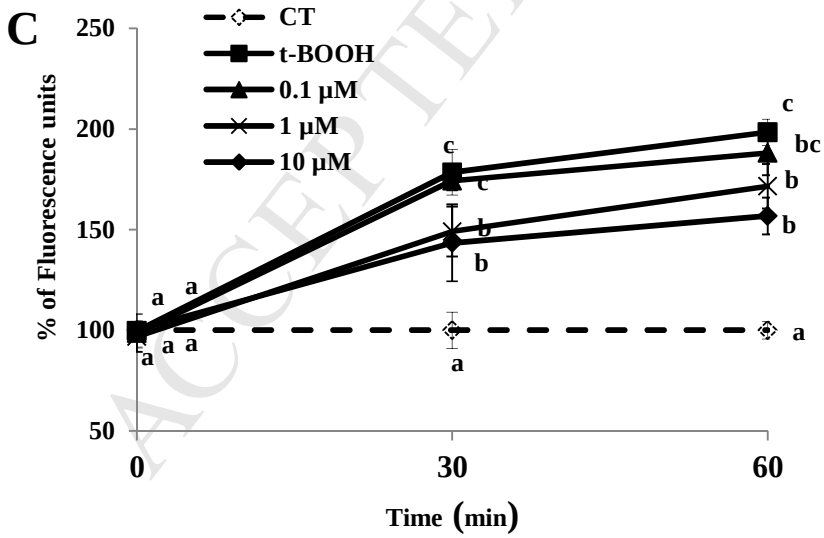


Figure 3







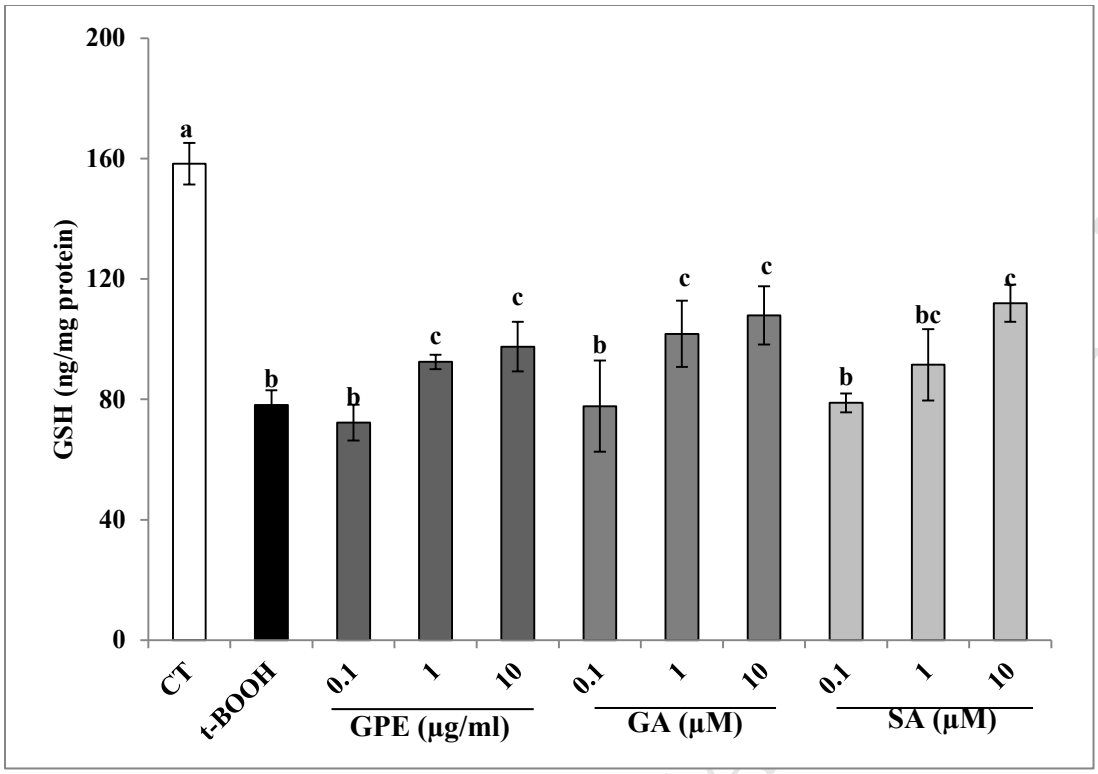
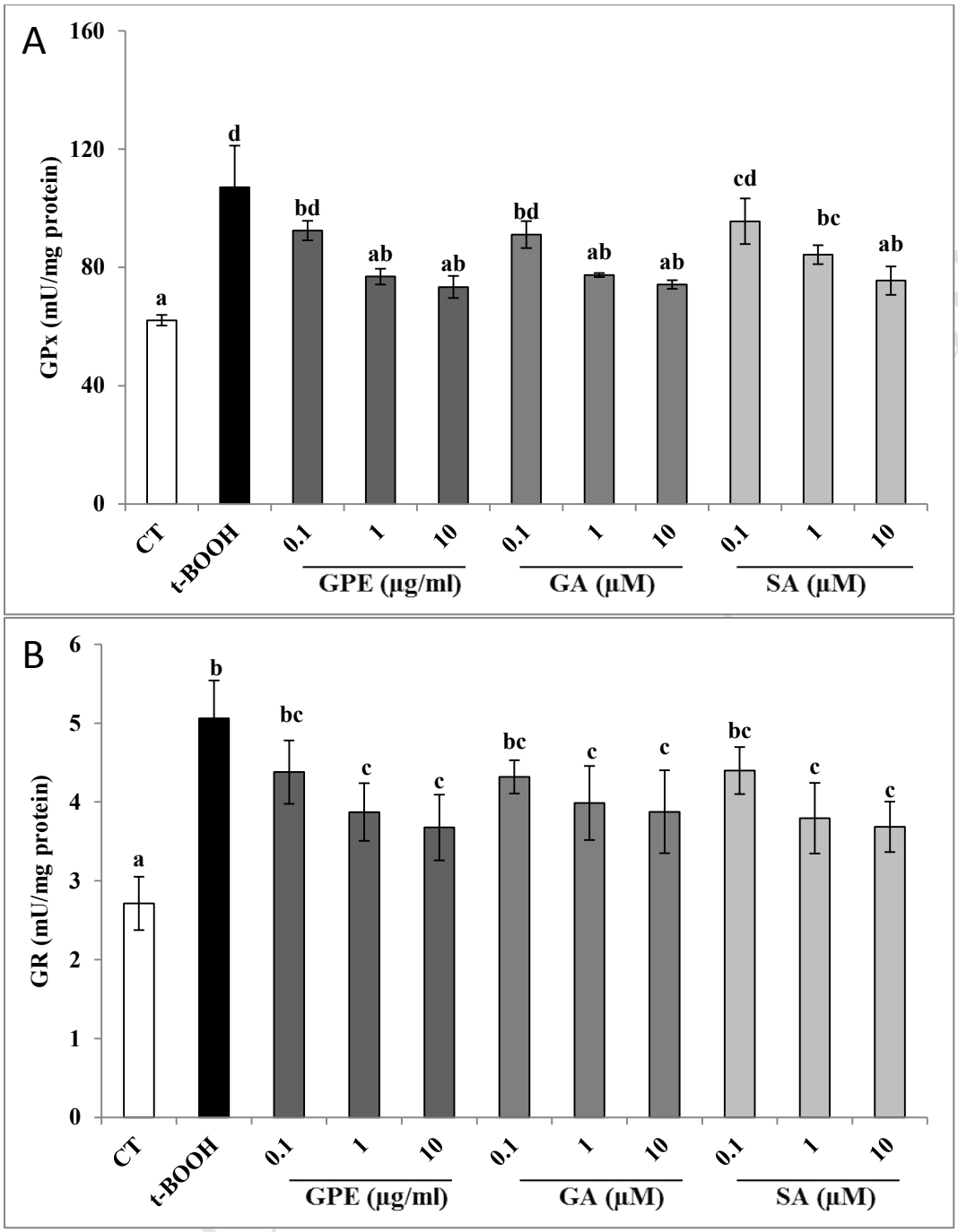


Figure 5


A
GPx (mU/mg protein)
160
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40
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CT
t-BOOH
0.1
1
10
GPE (µg/ml)
GA (µM)
SA (µM)
B
GR (mU/mg protein)
6
5
4
3
2
1
0
a
b
bc
c
c
bc
c
c
bc
c
c

Figure 6

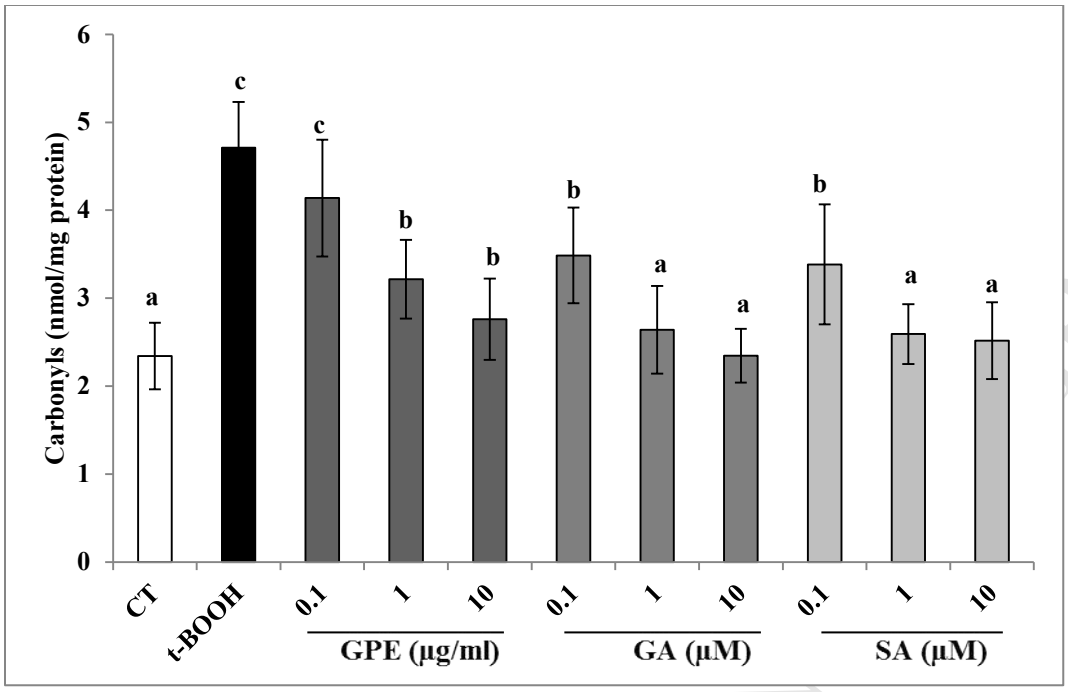

Figure 7

HIGHLIGHTS

- GPE, gallic and syringic acid treatment reduced induced ROS production in Caco-2 cells
- GPE, gallic and syringic acid treatment reduced induced cytotoxicity in Caco-2 cells
- GPE, gallic and syringic acid treatment recovered enzymatic and non-enzymatic defences in Caco-2 cells
- GPE, gallic and syringic acid treatment reduced oxidative damage to proteins in Caco-2 cells
- Grape phenolic extract (GPE) and its components gallic and syringic acid protect against induced oxidative stress in Caco-2