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Antioxidant Effect of Natural Table Olives Phenolic Extract Against Oxidative Stress and Membrane Damage in Enterocyte-Like Cells.

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ABSTRACT

The phenolic fraction of a naturally fermented cultivar of table olives, “Tonda di Cagliari”, was investigated for the ability to protect Caco-2 cells against oxidative stress and membrane damage induced by tert-butyl hydroperoxyde (TBH). TBH exposure resulted in an alteration of cellular redox status, with an increase of ROS and a decrease of GSH level. A loss of the epithelial integrity, as indicated by the decrease of the transepithelial electrical resistance (TEER) value, was also observed over time, together with an intense lipid peroxidation process. The olives phenolic extract significantly counteracted ROS generation and subsequent alteration of monolayer integrity and membrane oxidative damage. The protective action of the extract is likely due to the scavenging ability of its main components, as hydroxytyrosol, oleuropein and verbascoside among the secoiridoids and derivatives. Since olives phenolic compounds concentrate in the intestinal lumen, they may be an useful tool in the prevention of intestinal disorders related to oxidative damage.

Keywords: Tonda di Cagliari, Natural table olives, Oxidative stress, Caco-2 cells, Phenols

Practical Application: Naturally fermented table olives of the variety “Tonda di Cagliari” have been found to be rich in hydroxytyrosol, oleuropein and verbascoside. The olive phenolic fraction as a whole protected intestinal cells against oxidative damage, highlighting an added nutraceutical value. Likewise olive oil, they may be considered a good source of active phenolic compounds that may contribute to the maintenance of intestinal mucosal integrity.

Introduction

Olives are a foundamental part of the Mediterranean diet, and a column of the Mediterranean social, cultural, and gastronomic heritage. They are widely acknowledged as an important source of health-protective nutrients, such as the oleic monounsaturated fatty acid, α-tocopherol and many antioxidant polyphenols (Lanza 2012). Table olives phenolic fraction ranges from 1 to 3% of the fresh pulp weight, and can include more than 36 different compounds; its composition can vary both in quality and quantity depending upon the processing method, the cultivar, the agronomic practices, and the degree of olives maturation (Charoenprasert and Mitchell 2012). The main classes are represented by phenolic acids, phenolic alcohols, flavonoids and secoiridoids. Oleuropein is the most abundant phenolic compound found in green olives, and is the main responsible for the strong bitter taste of the fruit, making it unpalatable before processing. Unfortunately the technological process can lead to an important loss of phenolic compounds (Ben Othman and others 2009). The "Greek-style" or "natural method" involves a spontaneous fermentation, mainly driven by yeasts and lactic acid bacteria, which provides higher and appreciable amounts of phenolic compounds (Charoenprasert and Mitchell 2012). Thus, processed natural table olives still contain significant amount of phenolic compounds, such as the phenolic alcohol hydroxytyrosol, derived from the hydrolysis of oleuropein, tyrosol, verbascoside, oleuropein, and flavonoids as apigenin, luteolin and its glycosilated form luteolin-7-glucoside (Boskou and others 2006; Marsilio and others 2005). The high content of hydroxytyrosol may confer important properties to table olives, since to this compound are ascribed several pharmacological activities, mainly related to its antioxidant properties (Hu and others 2014). The antioxidant capacity of the phenolic fraction of olives from different cultivars has been demonstrated in chemical experimental trials, as the hypoxanthine/xanthine oxidase assay (Owen and others 2003), the reducing power assay (Pereira and others 2006; Malheiro and others 2011) and DPPH (2,2-diphenyl-1-picrylhydrazyl) method

(Malheiro and others 2014; Campus and others 2015; Boskou and others 2006). However, no studies, until now, have investigated the antioxidant properties of this fraction in cell cultures.

The Caco-2 cell line is a widely used model for the study of epithelial barrier and tight junctions (TJ) integrity (Peterson and Mooseker 1993). After confluence, these cells differentiate spontaneously, both structurally and functionally, into mature enterocytes (Pinto and others 1983), therefore artificially oxidative stress allows the evaluation of the protective capacity of target compounds and extracts against oxidative damage. Artificial oxidative cell injury can be carried out using TBH (tert-butyl hydroperoxide) that catalyzes the peroxidation of membrane lipids (Chamulitrat 1998), and structural changes such as the opening of TJ (Tomita and others 2002), generating free radicals. The effect of TBH upon Caco-2 cells well simulates the damaging action of dietary lipid hydroperoxides on the intestinal mucosa. The redox equilibrium alteration within the intestinal mucosa is deeply related to the onset and progression of the most common degenerative diseases (Biasi and others 2013). It has been reported that one of the most important sites of olive oil polyphenols action is the intestinal lumen (Halliwell and others 2005). Polyphenols concentrate in this district before absorption (Corona and others 2009a), contributing to the preservation of the intestinal mucosa integrity against oxidative damage.

The present study aimed to evaluate the protective effect of the phenolic fraction of a naturally fermented table olives of “Tonda di Cagliari” cultivar , against the oxidative stress and membrane damage in Caco-2 cells. The protective action of the olives phenolic extract was evaluated as the ability to modify cellular redox status alteration (ROS production and GSH level), and to counteract the disruption of epithelial integrity, measured as transepithelial electrical resistance (TEER). The protective action against the oxidative damage of the membrane lipid fraction, was evaluated measuring the production of MDA, fatty acids hydroperoxides (HP), and 7-ketocholesterol (7-keto), together with the decrease of the levels of the membrane antioxidant α-tocopherol.

Materials and methods

Chemicals

All analytical standards were at 95% certified purity (Sigma Aldrich). Methanol, acetonitrile, and hexane were of residue analysis grade, purchased from Carlo Erba (Milan, Italy), H_3PO_4 and other chemicals were of analytical grade (Carlo Erba). Phenol extract working solutions were obtained by appropriate dilutions of dried extract, obtained from homogenized olive pulp, to reach the amounts of 10, 25, 50, and 100 μg/mL in the working media. Bradford protein assay and 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCF-DA) were from Sigma-Aldrich. Cell culture materials were from Invitrogen (Milan, Italy) and transwell inserts from Corning Costar Corp. (New York, USA).

Olive samples and fermentation procedure

"Tonda di Cagliari" olives were harvested mechanically at the green-yellow ripe stage, from an irrigated olive orchard, located in the south of Sardinia, Italy (39°23'18.7"N 8°51'46.8"E). Fruits were sorted, discarding injured and defective ones, and graded (18-20 mm diameter), then washed under continuous stirring in chlorinated water. Olives were left to dry at ambient temperature, placed in sanitized HDPE vats (60 kg of olives), and filled with 40 L of brine at 7% NaCl. Salt percentage was kept constant throughout the whole process, checking its concentration with the Mohr method. The vats were kept at 27 °C in a thermostatic storage cell. The experimental design consisted of 3 trials with 3 repetitions (n=9). When reaching a steady pH state the temperature inside the cell was kept constant at 24°C. After 9 months of debittering at final pH of 3.8, and 3 months of simulated shelf life at 24° C, samples (1 Kg of olives for each repetition) were brought to the laboratory for the processing step.

Preparation of the phenolic fraction

Olives were randomly selected out of the batches and the kernel was removed. The resulting olive pulp was cut with a mincing knife into small pieces, homogenized with a blender, weighted in a 40

mL test tube, and extracted with a methanol/water (80/20) solution in a 1/2 ratio. The tube was stirred 2 min in vortex and agitated 15 min in a rotary shaker, thereafter hexane (1/5 of the methanol / water solution volume) was added, and the resulting mixture was agitated for 10 min in a rotary shaker. The extraction mixture was then transferred in centrifuge falcon tubes (clarified polypropylene) of 15 mL and centrifuged at 3200 g for 10 min at 10 °C. The organic fraction was discarded and the methanolic/water solution collected, 1 mL was evaporated to dryness under nitrogenous stream at ambient temperature and recovered with 0.5 mL of syringic acid (15 mg/L) H_3PO_4 0.22 M solution, for HPLC analysis. Total phenolic content of the extract was determined with the Folin Ciocalteau method (Campus and others 2015).

Characterization of the phenolic extract

The characterisation and quantification of the main components was carried out by HPLC-DAD analysis, using an Agilent 1100 HPLC (Agilent Technologies, Milan, Italy) coupled with a DAD detector (UV 6000, Thermo Finnigan, Milan, Italy), and a Kinetex (5 μm, 100 Å, 250 mm x 4.6 mm, Phenomenex, USA) column. The analysis were carried out according to Campus et al. (Campus and others 2015) at 280 and 360 nm. Compounds identification was made by comparison with authentic analytical standards. Quantitative analysis were made according to the International Olive Council method ((IOC) 2009), using siringic acid as internal standard.

Cell culture

Caco-2 cells (ECACC, Salisbury, UK) were cultured in monolayers at 37 °C in a humidified atmosphere at 5% CO_2 (Incani and others 2016). At passage 45–60, cells were plated at a density of about $1x10^5$/mL and used when fully differentiated (21 days post seeding). The Bradford assay was used to determine cell protein concentration.

Determination of intracellular ROS production

ROS production in Caco-2 cells was determined using the fluorescent probe H_2-DCF-DA, according to Atzeri et al. (Atzeri and others 2016), with minor modifications. Cells, grown and

differentiated in 96-well plates, were incubated with 10 µM of H_2-DCF-DA in 100 µL of PBS for 30 min. H_2-DCF-DA was then replaced by the PBS solution containing the phenolic extract (10, 25 and 50 µg/mL), 30 min prior to adding tert-butyl hydroperoxide (TBH) 2.5 mM and incubated for 30 min. The increase in cell fluorescence was determined using an Infinite F200 (Tecan, Salzburg, Austria) auto microplate reader at 485 and 530 nm (excitation and emission wavelengths, respectively). Moreover samples were analyzed with the ZOE^{TM} Fluorescent Cell Imager (Bio-Rad Laboratories, Inc.), using the green channel with a blue LED (excitation: 480/17 nm; emission: 517/23 nm) to the size of 100 µm.

Determination of MDA level

MDA concentrations, reported as percentage of the control samples, were determined in the supernatants as nmol of MDA equivalents produced per mg of protein, and were determined with the TBARS test and HPLC-DAD quantification (Agilent Technologies) (Deiana and others 2012).

Determination of intracellular reduced glutathione (GSH) level

GSH level was determined in Caco-2 cells, grown and differentiated in 6-well plates. Cells were treated with different amounts of the extract (10, 25, and 50 µg/mL in PBS), for 30 min. Thereafter TBH 2.5 mM was added and cells were incubated for 30 min. Cells were then scraped into 200 µL of ice-cold PBS and centrifuged at 10000 g for 20 min at 4 °C. The pellet was used to determine GSH level through EC-HPLC quantification (Agilent 1260 infinity coupled with an electrochemical detector DECADE II Antec, Leyden, Netherlands) (Atzeri and others 2016).

Determination of α-tocopherol, fatty acids and cholesterol oxidation products

α-tocopherol, 7-ketocholesterol (7-keto) and fatty acid hydroperoxides (HP) were obtained from the cell pellet by mild saponification of the lipid fraction (Deiana and others 2008). Separation and detection of 7-keto (at 245 nm) and HP(at 234 nm), was performed with an HPLC–DAD system (Agilent Technologies), while α-tocopherol was determined by HPLC-electrochemical detection (DECADE II, Antec) set at an oxidizing potential of 0.6 V (Deiana and others 2010).

169 *Determination of transepithelial electrical resistance (TEER)*

170 Caco-2 cells were seeded in 12 mm i.d. Transwell inserts (polycarbonate membrane, 0.4 µm pore
171 size) in 12-well plates. Before each experiment the monolayer integrity was checked by measuring
172 the TEER value (Millicell-ERS system, Millipore, Bedford, MA). Cells in inserts with TEER values
173 >300 Ω/cm^2 were treated with the phenolic extract dissolved in MeOH (10, 25 and 50 µg/mL) and
174 with TBH (2.5 mM) and incubated for 2 h (Deiana and others 2012). TEER was measured every 30
175 min and reported as percentage of the corresponding TEER value at time zero (T=0).

176 *Statistical analyses*

177 Results are expressed as means ± standard deviations of triplicate values obtained in three
178 independent experiments (n = 9). The statistical significance of parametric differences among sets
179 of experimental data was evaluated by the one-way ANOVA test associated with Bonferroni's
180 multiple comparison post test using GraphPad InStat (GraphPad Software, San Diego, CA, USA).

Results

Characterization of the olives phenolic extract

The most abundant compound in the olives phenolic fraction was hydroxytyrosol, with a concentration of 410.2 ± 15.8 mg/Kg fresh weight (Table 1), while tyrosol showed a concentration ten times lower. Among the secoiridoids and their derivatives, oleuropein and verbascoside showed amounts of 85.2 ± 11.2 and 47.8 ± 10.0 mg/Kg respectively. A substantial amount of the flavonoids luteolin and luteolin 7-glucoside was also detected.

Determination of antioxidant activity

The protective action of the phenolic extract was evaluated at non toxic concentrations in differentiated Caco-2 cells, exposed to the oxidizing action of TBH. After 30 min of incubation, TBH determined in the treated cells a significant production of intracellular ROS, with levels more than twice with respect to the controls, as indicated by the increase of fluorescence (Figure 1). Moreover a significant reduction of cellular GSH, around 50% of the initial value, was detected (Figure 2). Pretreatment with the phenolic extract, from 25 μg/mL, counteracted TBH induced alteration of cellular redox status, with a significant and dose dependent decrease of ROS level and increase of GSH concentration. Cellular redox imbalance caused a significant alteration of the monolayer integrity with time (Figure 3), starting from 30 min of incubation, when the registered TEER value was 80% of the basal level. TEER values measured in monolayers treated with amounts from 25 μg/mL of the extract were significantly higher than in those incubated without the extract at the same time points (60, 90 and 120 min; Figure 3). After 120 min of incubation TBH caused the complete loss of membrane integrity with TEER value close to zero (Figure 3). Under these experimental conditions (TBH 2.5 mM/120 min), an oxidative stress-dependent lipid peroxidation process determined a significant increase of oxidated products, such as fatty acids hydroperoxides (HP) and 7-ketocholesterol (7-keto), compared to controls (Figure 4). A two-fold increase of MDA level was also observed in TBH-treated samples (Figure 4). The lipid

206 peroxidation process also led to a significant decrease of α-tocopherol, with a reduction up to 30%
207 after 120 min of TBH exposure (Figure 5). Pretreatment with the phenolic extract significantly
208 inhibited membrane oxidative damage. In presence of 25 μg/mL of the extract, the production of
209 HP and 7-keto was significantly reduced compared with oxidized samples, whereas MDA
210 formation was inhibited at all tested concentrations. In addition the level of α-tocopherol was
211 completely preserved in samples pretreated with 25 and 50 μg/mL of the extract (Figure 5).
212
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Discussion

Table olives phenolic compounds contribute to the color, taste and texture of the product (Marsilio and others 2001), as well as to the antioxidant capacity and related health benefits. The phenolic profile and its amount in table olives is related to several factors, among which the cultivar, the maturity state and the processing method play a major role. All processing methods may reduce the phenolic content of table olives by different ways. The Greek-style like method, where olives are directly put into brine and left to ferment, results in a final product that retains a good quantity of polyphenols (Charoenprasert and Mitchell 2012).

The cultivar "Tonda di Cagliari" is a well-known olive used for the preparation of table olives. The chemical characterization of the extract revealed the presence of a high quantity of hydroxytyrosol, oleuropein and verbascoside, among the secoiridoids and derivatives, and luteolin, and luteolin 7-glucoside among the flavonoids. It has been reported that hydroxytyrosol, the most concentrated phenolic compound in the extract, is one of the strongest antioxidant in nature (Granados-Principal and others 2010), able to scavenge reactive species, both in the reaction medium and near the membrane surface (Paiva-Martins and others 2003), and lipid peroxyl radicals generated from membrane UFA and cholesterol (Deiana and others 2010). It is the only phenol that has been recognized by the European Food Safety Authority (EFSA) with a protective activity on blood lipids from oxidative damage, when consumed daily within a balanced diet (EFSA 2011). A protective role of hydroxytyrosol against alteration of redox status and membrane oxidative damage in Caco-2 cells has been reported (Atzeri and others 2016; Deiana and others 2010). Hydroxytyrosol may permeate Caco-2 membranes (Corona and others 2006), thus its protective role may be exerted both inside and outside the cell.

Oleuropein, present in significant amount in the extract, may also contribute to the overall antioxidant activity. Several in vitro and in vivo studies, recently summarized by Hassen et al. (Hassen and others 2015), have demonstrated its strong activity as a free radical scavenger and

metal-chelating agent, related to the presence of phenolic hydroxyls and other active molecular sites (Hassanzadeh and others 2014). It has been shown that oleuropein and oleuropein rich extracts have gastroprotective effects, due to their ability to maintain cell membrane integrity and to strengthen the mucosal barrier, inhibiting lipid peroxidation and potentiating the activity of key cellular antioxidant enzymes (Alirezaei and others 2012). A substantial portion of oleuropein reach unmodified the lower gastrointestinal tract, where it is mainly metabolized in hydroxytyrosol by the colonic microflora (Corona and others 2006; Corona and others 2009b). Thus, oleuropein and hydroxytyrosol can have more significant direct antioxidant effects in the gastrointestinal tract than in other sites within the body.

Verbascoside has shown to possess beneficial activities for human health, including antioxidant, anti-inflammatory and antineoplastic properties (Alipieva and others 2014). Thanks to its proton-donating capacity, verbascoside is able to act as radicals scavenger (Wang and others 1996; Georgiev and others 2011), even if its antioxidant activity in biological systems seems to be mainly related to its capacity to enhance the activities and induce gene transcription of major cellular antioxidant enzymes (Alipieva and others 2014). Although there are no data on the bioavailability of verbascoside in humans, animal studies suggest that it can reach the intestine and can be absorbed in its intact form or after metabolization to simple phenols as hydroxytyrosol (Quirantes-Pine and others 2013). Verbascoside may be rapidly incorporated in Caco-2 cells (Cardinali and others 2011) and HT 29 cells, where it has been shown to inhibit H_2O_2 induced oxidative stress, acting as free radicals scavenger (Cardinali and others 2012). Verbascoside also showed the capacity to inhibit lipid peroxidation and malondialdehyde generation in liposomal systems (Funes and others 2009). Being localized in some regions of the bilayer, it may act as a radicals scavenger in the surroundings of the membrane, thus preventing the lipid peroxidation process (Funes and others 2010).

263 The “Tonda di Cagliari” phenolic extract significantly counteracted the alteration of cellular
264 redox status, inhibiting ROS generation and GSH level decrease and subsequent membrane
265 oxidative damage. The protective action of the olives phenolic extract is likely due to the ability of
266 its main components to scavenge TBH generated radicals or intermediate of reaction metabolites, as
267 shown by the fluorescence data. The scavenging activity of the phenolic compounds present in the
268 extract led to the blockade of the oxidation process in the early stage, thus maintaining barrier
269 integrity, as shown by the higher TEER values measured in samples pretreated with the phenolic
270 extract. Our data confirm that the structural changes of the TJ in Caco-2 monolayers are mainly due
271 to the reactive species generated by TBH (Tomita and others 2002). These reactive species caused
272 oxidative injury to enterocytes membrane, as shown by the increase of MDA and lipid peroxidation
273 products. Pretreatment with the phenolic extract significantly inhibited membrane disruption and
274 preserved α-tocopherol level.
275 The protective action of the phenolic extract has a great biological relevance. Changes in
276 membrane fluidity and structure is a key event in the initiation and progression of mucosal barrier
277 dysfunction, correlated to the onset of inflammatory and degenerative intestinal diseases (Biasi and
278 others 2013). This is especially true in the colon, whose antioxidant capacity is low, in contrast to
279 the high level of oxidizing species (Blau and others 1999). Therefore polyphenols together with
280 their metabolites, may give a contribution to the redox environment, thus maintaining cell integrity
281 and function. The consumption of naturally fermented table olives, in combination with olive oil,
282 within a balanced Mediterranean diet, provide a large amount of important antioxidants. About 5-10
283 table olives might cover the daily intake of polyphenols (Boskou and others 2006), providing an
284 amount of sodium (around 5% in commercial products) (Lopez-Lopez and others 2004) that is not
285 in contrast with dietary recommendation (Lanza 2012), except in the presence of hypertensive
286 pathologies.
287

Conclusions

The data reported showed that table olives “Tonda di Cagliari” processed with the “Greek style” or “natural method” yields a final processed product with peculiar antioxidant properties due to its phenolic composition, being rich in hydroxytyrosol, oleuropein and verbascoside, and luteolin among flavonoids. The olives phenolic extract significantly prevented oxidative stress and membrane damage in Caco-2 cells treated with TBH, suggesting a protective role in the intestinal lumen, where they concentrate before absorption.

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ABBREVIATIONS: HP, fatty acid hydroperoxides; 7-Keto, 7-ketocholesterol; UFA, unsaturated fatty acids; TBH, tert-butyl hydroperoxide; MDA, malondialdehyde; TEER, transepithelial electrical resistance; TJ, tight junctions.

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420 **Table 1**. Total phenols and main phenolic compounds identified in the Tonda di Cagliari pulp.

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	Tonda di Cagliari
Compunds	*mg/Kg fresh pulp*
Total phenols	1507.80 ± 27.33
Hydroxytyrosol	410.2 ± 15.8
Tyrosol	44.0 ± 3.6
Verbascoside	47.8 ± 10.0
Oleuropein	85.2 ± 11.2
Luteolin	105.9 ± 15.3
Luteolin-7-glucoside	27.8 ± 15.5
Apigenin	23.4 ± 3.3

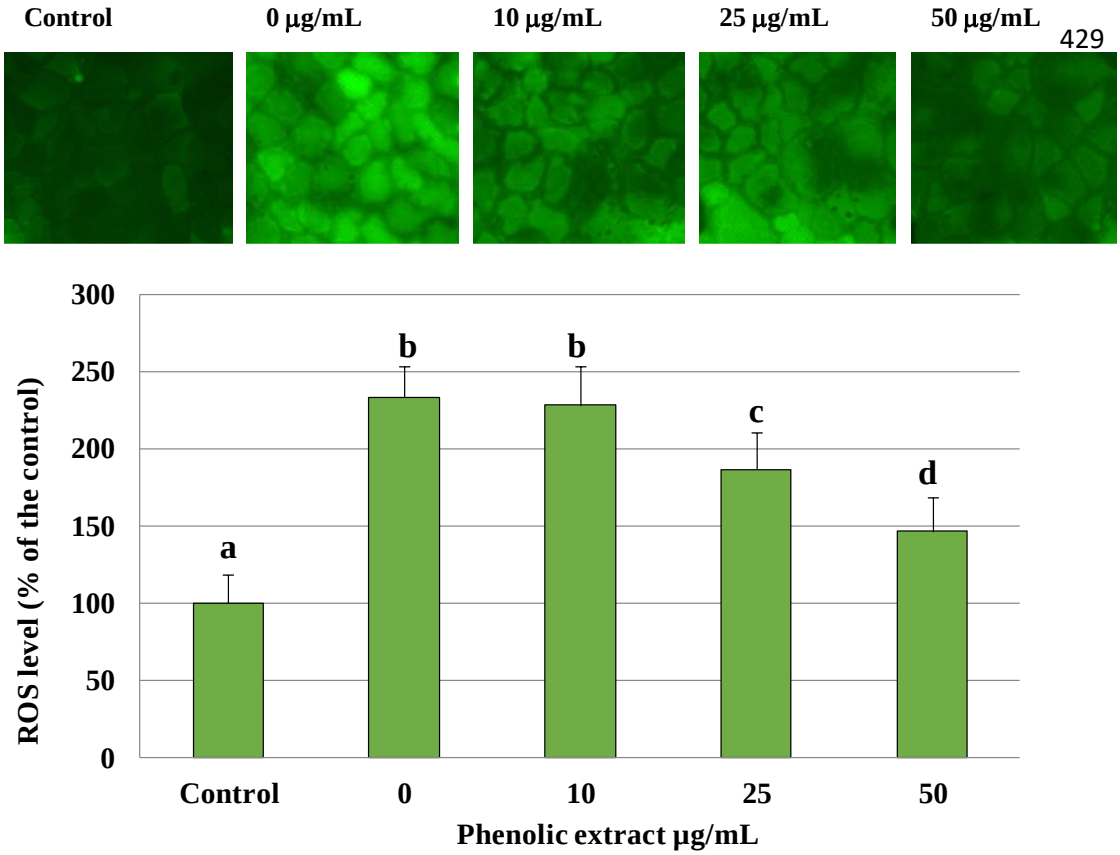

Figure 1- ROS level, visualized as H_2-DCF-DA fluorescence and expressed as % of the control samples (non oxidized nor pre-treated samples), in Caco-2 after 30 min incubation with TBH 2.5 mM and pre-treated with the phenolic extract (30 min). Values not sharing a superscript letter are significantly different ($p < 0.05$).

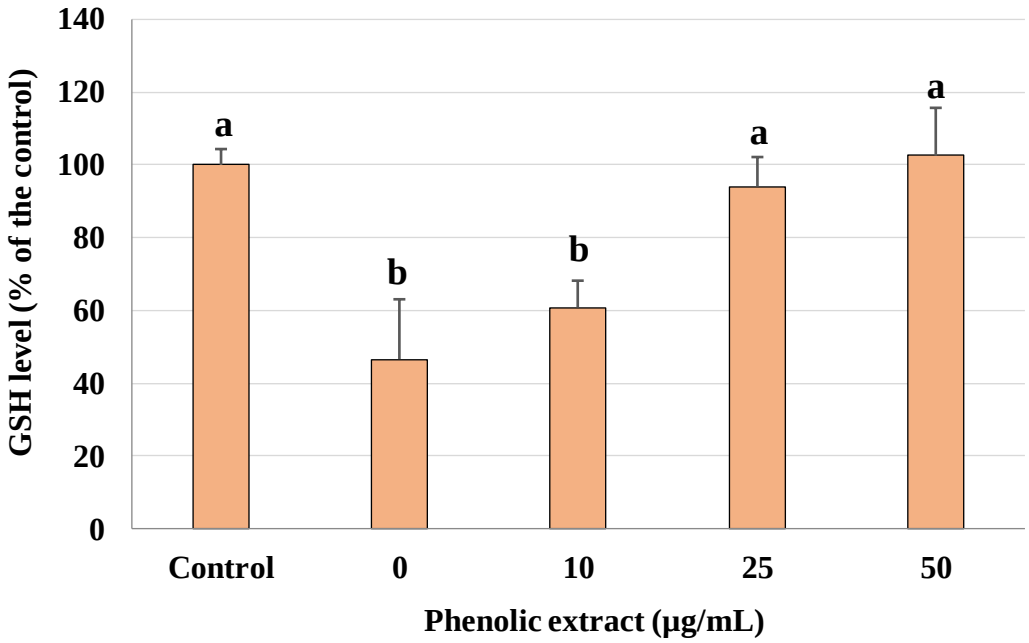

Figure 2 - GSH level, expressed as % of the control samples (non oxidized nor pre-treated samples), in Caco-2 after 30 min incubation with TBH 2.5 mM and pre-treated with the phenolic extract (30 min). Values not sharing a superscript letter are significantly different ($p < 0.05$).

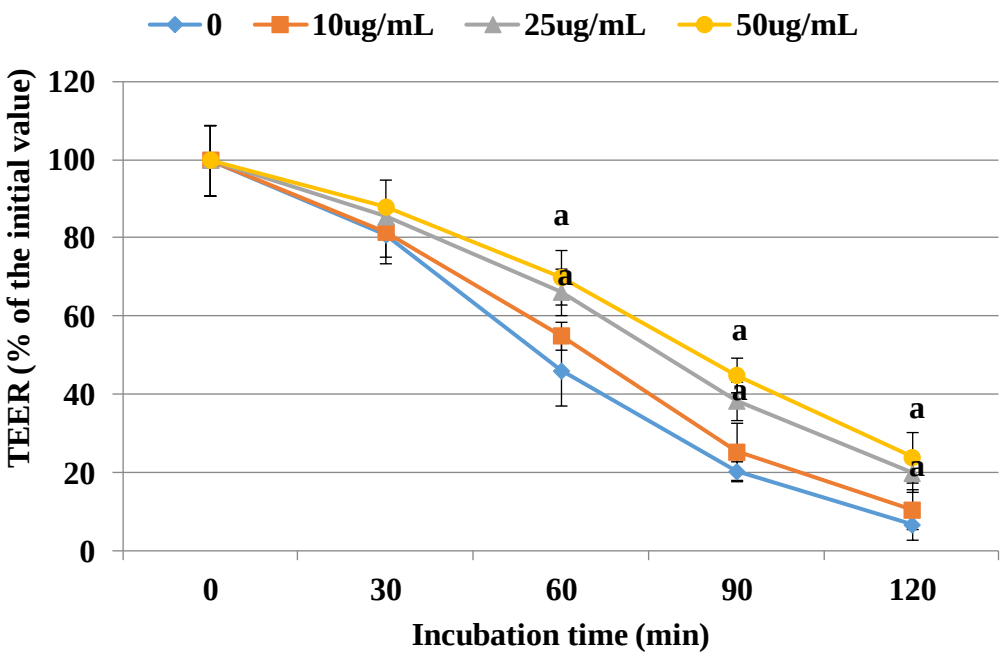

Figure 3 - TEER value in Caco-2 cell monolayers incubated with TBH 2.5 mM and different amounts of olives phenolic extract. All values reporting changes with time for each series are significantly different ($p < 0.05$). a=$p<0.001$ versus oxidized control (0 μg/mL) at the same time.

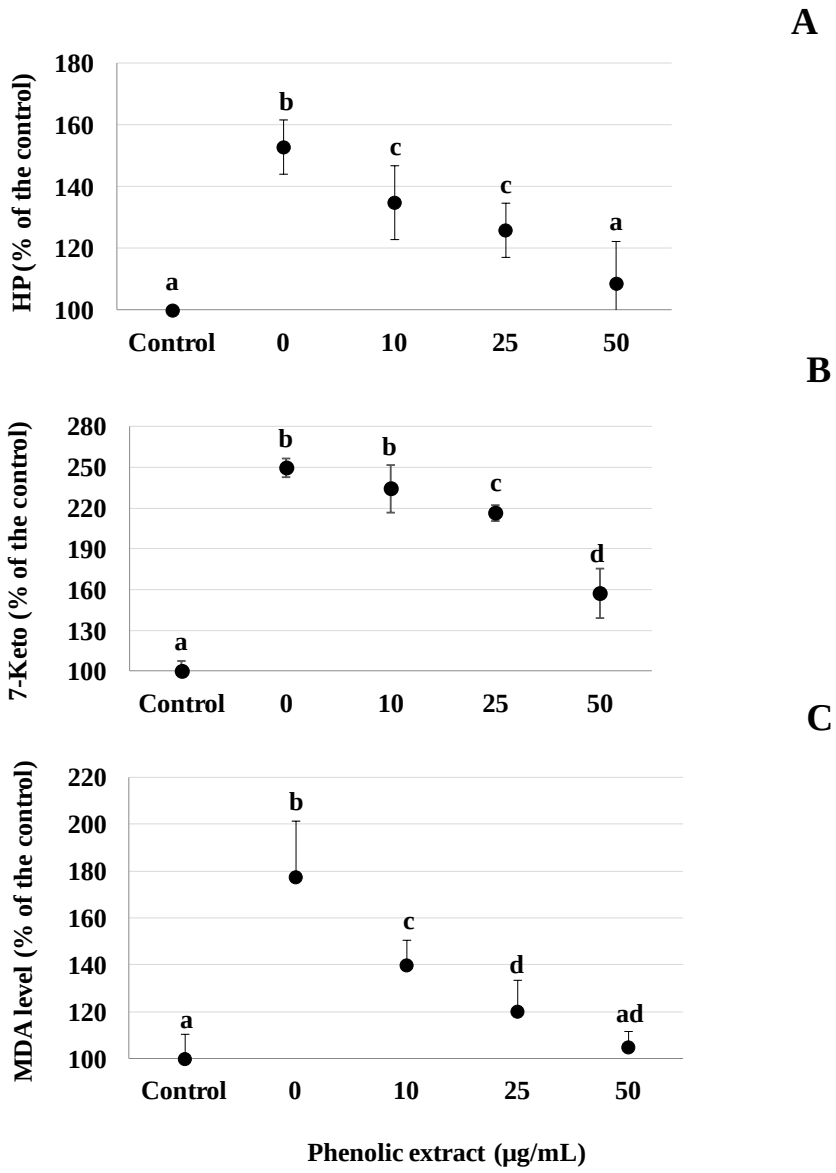

Figure 4 - Values of fatty acids hydroperoxides (HP, A), 7-ketocholesterol (7-keto, B) and MDA (C) measured in Caco-2 cells after 2 h incubation with TBH 2.5 mM and pre-treated with the phenolic extract (30 min). Values not sharing a superscript letter are significantly different ($p < 0.05$).

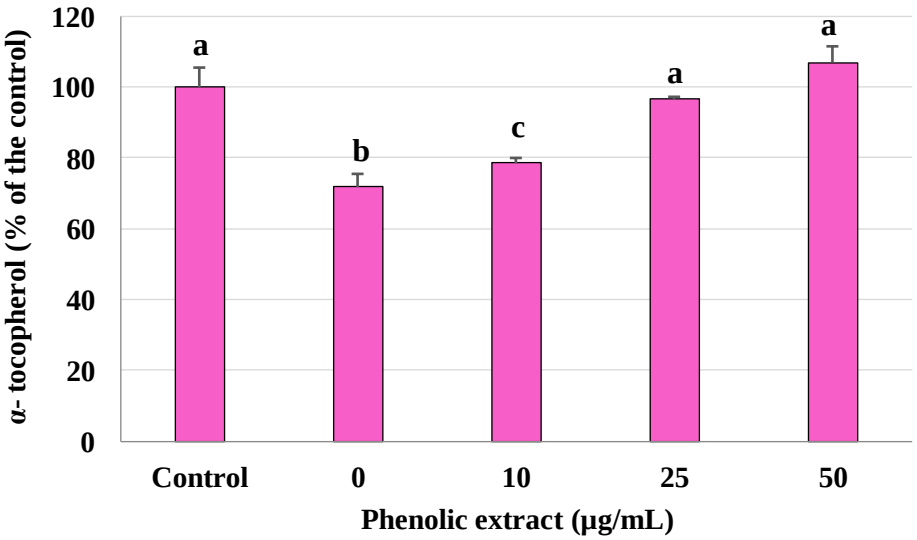

Figure 5 - Values of α-tocopherol, expressed as % of the control samples (non oxidized nor pre-treated samples), in Caco-2 after 2 h incubation with TBH 2.5 mM and pre-treated with the phenolic extract (30 min). Values not sharing a superscript letter are significantly different ($p < 0.05$).