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Extra virgin olive oil phenolic extracts counteract the pro-oxidant effect of dietary oxidized lipids in human intestinal cells.

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Abbreviations: EVOO, extra virgin olive oil; HP, fatty acid hydroperoxides; TBH, *tert*-butyl hydroperoxide; UFAs, unsaturated fatty acids; MDA, malondialdehyde

Manuscript highlights

- Two olive oil phenolic extracts were tested in Caco-2 treated with TBH and oxysterols
- Both TBH and oxysterols induced oxidative imbalance, ROS increase and GSH depletion
- Membrane lipid peroxidation was highlighted by MDA, hydroperoxydes, 7-keto detection
- A significant protective effect of both the extracts was pointed out
- Extracts activity was correlated to the phenolic content and to the oxidizing agent

Abstract

The phenolic fraction of extra virgin olive oil (EVOO) concentrates before absorption in the intestinal lumen, where it may contribute to the modulation of enterocytes response to oxidative and inflammatory stimuli. We evaluated the ability of two monovarietal EVOOs phenolic extracts, Bosana and Nera di Gonnos/Tonda di Cagliari, typical and widespread varieties in Sardinia (Italy), to counteract in enterocytes like Caco-2 cells the pro-oxidant action of oxidized lipids, *tert*-butyl hydroperoxide (TBH) or a mixture of oxysterols of dietary origin. We confirmed that TBH treatment causes a significant increase of ROS production, GSH depletion, increase of MDA, fatty acids hydroperoxides and 7-ketocholesterol, and showed first evidence of oxidative imbalance and cell damage due to oxysterols exposure. Preincubation of cells with the phenolic extracts significantly attenuated oxidative modifications. Bosana extract showed the highest concentration of total phenols, mainly hydroxytyrosol and tyrosol, and was the most active in presence of TBH, where the free radical scavenging activity of these simple phenols seems to be a determining factor. The two extracts were equally effective, in spite of the different composition, in presence of oxysterols, where ROS production probably occurs according to different and more complex mechanisms.

Keywords: olive oil, oxidative stress, Caco-2 cells, oxysterols, phenolic extract

1. Introduction

The phenolic fraction of EVOO is of particular interest as it is greatly responsible for the high oxidative stability of this oil and its peculiar sensory characteristics and nutritional qualities among edible vegetable oils. The phenolic composition of EVOO may vary in quantity (150–700 mg/l) and quality, depending on the olive variety, degree of ripeness, soil composition, climate, processing techniques, and storage (Carrasco-Pancorbo et al., 2005). In general, it contains four major classes of phenolic compounds: flavonoids, lignans, simple phenols, and secoiridoids, which are the major phenols found in EVOO. Dietary intake of EVOO polyphenols has been estimated to be around 9 mg, within 25–50 ml of EVOO per day (de la Torre, 2008), and it has been demonstrated that some complex polyphenols, among the secoiridoids, are relatively stable under gastric conditions and reach the intestine, where they may be directly absorbed or metabolized under absorption (Pinto et al., 2011), others undergo an extensive gastrointestinal biotransformation (de Bock et al., 2013). Ingested polyphenols will be particularly concentrated in the intestinal lumen, mainly in the colon (Corona et al., 2009) and they and their products of bacterial fermentation can exert beneficial effects. It has been suggested that polyphenols might exert direct protective effects in the gastrointestinal tract, by scavenging reactive species and/or preventing their formation (Halliwell et al., 2005). EVOO phenolic compounds have been shown to scavenge both free radicals generated by the fecal matrix (Owen et al., 2000) and those induced in colonic cells by hydrogen peroxide (Manna et al., 2002) and lipid hydroperoxydes (Deiana et al., 2010). The intestinal mucosa is constantly exposed to harmful substances, many of them introduced through the diet, including free radicals and lipid oxidation products. A large body of evidence from epidemiological studies indicates that dietary lipids, such as unsaturated fatty acids (UFAs) or cholesterol oxidation products, and intestinal microflora, are the main responsible for the production of oxidized species in the colon (Whiting et al., 2005).

Excessive UFAs hydroperoxides levels in the gut can contribute to the impairment of mucosal detoxification pathways and enterocytes dysfunction, leading to the development of digestive tract disease conditions such as inflammation (Imai and Nakagawa, 2003) and colon cancer (Udilova et al., 2003). In human intestinal cells UFAs hydroperoxydes may be cytotoxic, induce mild oxidative stress or apoptosis, depending on the concentration considered (Wijeratne and Cuppett, 2006).

Besides UFAs oxidation products, dietary oxysterols, derived from cholesterol degradation and oxidation after prolonged storage or cooking of foods rich in cholesterol, have also been shown to contribute to the onset and further development of oxidative stress and inflammation related intestinal diseases (Biasi et al., 2009). It has been reported that the major oxysterols found in food may contribute to oxidative imbalance of the intestinal epithelium by inducing the generation of reactive oxygen species (ROS) (Biasi et al., 2009; Mascia et al., 2010); they are able to lead differentiated Caco-2 cells to both necrotic and apoptotic death, depending on the experimental conditions (Biasi et al., 2009).

In this contest, this paper was aimed to assess a direct protective action of the phenolic fraction of monovarietal EVOOs, in relation to the phenolic content, against the pro-oxidant effect of hydroperoxides and oxysterols in the human colon adenocarcinoma cell line, Caco-2. After confluence, these cells spontaneously undergo full differentiation to enterocytes in vitro, and are a suitable model for evaluating the effect of nutrient components, for both normal dietary constituents and additives, contaminants, toxicants and drugs (Li et al., 2003).

Oxidative cell injury was induced by exposure to *tert*-butyl hydroperoxide (TBH) and oxysterols. TBH is able to generate peroxyl, alkoxyl and methyl radicals (Chamulitrat, 1998), that mimics the pro-oxidant action of dietary UFA hydroperoxydes, whereas an oxysterol mixture is used to represent the most commonly found oxysterols in cholesterol-rich foodstuffs (Plat et al., 2005). The two monovarietal EVOOs used in the study were obtained from the olive varieties Bosana and Nera di Gonnos/Tonda di Cagliari (Nera) (Campus et al., 2013), the most typical and widespread grown in Sardinia. The Bosana cultivar has been shown to have a particularly high content of phenolic compounds (Cerretani et al., 2006).

The protective action of the phenolic extracts was evaluated, by measuring their ability to modify cellular redox status alteration (ROS production and GSH level), and oxidative damage to the membrane lipid fraction, through sensible and precise markers of the peroxidation process of membrane lipids, MDA, fatty acids hydroperoxides (HP) and 7-ketocholesterol (7-keto) production.

2. Materials and methods

2.1. Chemicals

Fatty acids standards, cholesterol, 5-cholesten-3β-ol-7-one (7-keto), *tert*-butyl hydroperoxide (TBH), 2-thiobarbituric acid (TBA), 1,1,3,3,-tetraethoxypropane (TEP), trichloroacetic acid (TCA), 2′,7′-dichlorofluorescein diacetate, 5α,6α-epoxycholesterol (α-epox) and 5β,6β-epoxycholesterol (β-epox), and 85% phosphoric acid were purchased from Sigma–Aldrich (Milano, Italy). Standards of phenolic compounds (purity ≥ 98 %) were obtained from Extrasynthese (Genay Cedex, France). 5-cholesten-3β,7α-diolo (7α-hydroxycholesterol) and 5-cholesten-3β,7β-diolo (7β-hydroxycholesterol) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Desferal (deferoxamine methanesulfonate) was purchased from CIBA-Geigy (Basel, Switzerland). HPLC grade solvents (acetonitrile, methanol, and isohexane) were purchased from J.T. Baker (Baker Mallinckrodt, Phillipsburg, NJ, USA). HPLC grade water (18 MΩ•cm) was prepared using a Millipore (Bedford, MA, USA) Milli-Q purification system. Cell culture materials were purchased from Invitrogen (Milano, Italy).

2.2. Olive oil samples

The two tested monovarietal EVOOs were obtained from an olive orchard located in South Sardinia (Villasor, Cagliari, Italy). Olive trees were grown under the same agronomic and agro-technical conditions. The olive trees (18 year-old) were planted according to a 5.5 × 4 m planting pattern. Olives were drip irrigated with 800-1000 m^3 per hectare. Five batches of 200 kg of olive fruits were collected from each variety and harvested at the same maturity index under the same agro-climatic conditions. Batches were processed separately in an industrial oil extraction plant (Rapanelli ECO 3750, ex Rapanelli RCM S.p.A. Foligno, Italy) within 24 h from harvesting. The fruits were crushed with a hammer crusher and olive pastes were malaxed at 26 ± 1 °C, for 35 min, in a olive paste mixer. The olive oil was separated by centrifugation through a two phase decanter (Decanter Rapanelli RAMEF 3750/ECO), without addition of warm water. Before and after the processing of each olive oil batch, the extraction plant was carefully cleaned. After extraction, oils were transferred in glass bottles and stored at 15 °C in the dark, until the time of analyses.

2.3 Preparation of the phenolic extracts

One litre of oil was divided into 4 fractions of 250 ml. Each fraction was put in a 500 ml separating funnel and extracted by vigorous shaking with 100 ml of MeOH/H_2O 80:20 (v/v). After 20 min incubation, the oil phase was separated from the aqueous one, put in a round-bottom flask and concentrated in a Rotavapor (30 °C, minimum pressure 30 mbar). The oil phase was extracted again with 100 ml of MeOH/H_2O 80:20, repeating the procedures previously described. The extraction of the oil phase was repeated two more times for a total of four extractions on each 250 ml oil fraction. The aqueous phases concentrated in the round-bottom flask were put in a separating funnel with 100 ml of isohexane. After shaking, the concentrated aqueous phase was collected and dried in a Rotavapor. The residue was dissolved in MeOH, filtered through a cellulose acetate syringe Whatman GD/X 0.45 μm, diam. 25 mm, and dried in a Rotavapor.

2.4. LC-DAD Characterisation of the phenolic extracts

The quali-quantitative determination of phenolic compounds and secoiridoids contained in the hydrophilic fraction was performed using a LC-DAD method (Sarolic et al., 2015). A ProStar HPLC system (Varian Inc., Walnut Creek, CA, USA) was employed, fitted with a pump module 230, an autosampler module 410, and a ThermoSeparation diode array detector SpectroSystem UV 6000lp (Thermo Separation, San Jose, CA, USA). Separation was obtained with a Gemini C18 column (150 × 4.60 mm, 3 μm, Phenomenex, Casalecchio di Reno, BO, Italy) using 0.2 M H_3PO_4 (solvent A), and acetonitrile (solvent B) at a constant flow rate of 1.0 ml/min, mixed in linear gradients as follows: t = 0 A:B (85:15, v/v), reaching 60:40 (v/v) in 30 min, then 40:60 (v/v) in 10 min, and finally at 100% B until 50 min. Before each injection the LC system was stabilized for 10 min with the initial A/B ratio (85:15, v/v). The injection volume was 10 μl. According to the optimal detection wavelength, the phenols analysis was performed at: 280 nm (hydroxytyrosol, tyrosol, vanillic acid, pinoresinol, oleuropein, and ligstroside derivatives), 313 nm (p-coumaric acid), and 360 nm (luteolin and apigenin). Oleuropein and ligstroside derivatives were tentatively identified by comparison with literature data (Capriotti et al., 2014; Dierkes et al., 2012). Chromatograms and spectra were elaborated with a ChromQuest V. 2.51 data system (ThermoQuest, Rodano, Milan, Italy). Stock standard solutions were prepared in methanol and working solutions in methanol-water (80:20 v/v). The method was validated in agreement with the International Conference on Harmonisation of Technical Requirements for Registration of

Pharmaceuticals for Human Use (ICH) guidance note (ICH Topic Q2 (R1)) by determining linearity, limits of detection (LOD) and limits of quantification (LOQ). The LODs and LOQs were used to establish the sensitivity of the method and were determined according to the equation LOD = 3.3σ/S and LOQ = 10σ/S, respectively (where σ = standard deviation of the blank, and S = slope of the calibration curve). The linearity was evaluated by preparing a standard mixture at six different concentrations, and analysing them by LC-DAD. The analyte peak areas were plotted against the corresponding concentrations, and the calibration curves were constructed by means of the least-squares method. All compounds were dosed using the calibration curve built with the respective standard, except oleuropein and ligstroside derivatives that were dosed using the oleuropein calibration curve. The correlation values were comprised between 0.9993 and 0.9999.

2.5. Cell cultures

Caco-2 cells (ECACC Salisbury, Wiltshire UK) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated bovine serum, 2 mM L-glutamine, 1% non-essential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin, in monolayers at 37 °C in a humidified atmosphere of 5% CO_2. For experimental studies Caco-2 cells, at passage 45-60, were plated and used 18-21 days post seeding, when fully differentiated. Cell protein concentration was determined by the Bradford protein assay (Sigma).

2.6. Cytotoxic activity

The cytotoxic effect caused by the exposure to increasing concentrations of the oxysterol mixture or TBH was assessed on Caco-2 cells, seeded in 24-well plates (1×10^5 cells /ml in 500µl), by the Neutral Red method (Fautz et al., 1991). Cells were exposed to the oxidizing agents in complete medium and incubated for 2 h (TBH) and 24 h (oxysterol mixture). After incubation, the medium was removed; a Neutral Red Solution (Sigma Aldrich) (0.033% in medium) was then added to the wells to determine cell viability. After 30 min of incubation, the Neutral red solution was carefully removed and cells quickly rinsed with PBS. The incorporated dye was then solubilised in Neutral Red Solubilisation Solution, acetic acid/ethanol /water (5/45/50, v/v/v), and the absorbance was measured at 540 nm.

2.7. Determination of intracellular ROS production

Intracellular ROS production was monitored in differentiated Caco-2 seeded in 96-well plates (1×10^5 cells /ml in 100µl). The old medium was removed, cells were washed with 200 µl of PBS and incubated for 30 min with 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 10 µM, according to Dinicola et al. (Dinicola et al., 2013). DCFH-DA was then removed and cells were added with the oxysterol mixture (150 µM; 7-ketocholesterol 42.96%, 7α-hydroxycholesterol 4.26%, 7β-hydroxycholestrol 14.71%, 5α,6α-epoxycholesterol 32.3% and 5β,6β-hydroxycholesterol 5.76%) or TBH (2.5 mM). ROS production was monitored by reading the fluorescence emitted, taking readings at intervals of 5 min for 120 min (TBH) and 180 min (oxysterols), using a micro plate reader (Infinite 200, Tecan, Salzburg, Austria) at a controlled temperature of 37 °C. The reading was performed using an excitation of 490 nm and an emission of 520 nm. To assess the effect of the phenolic extracts cells were pre-treated with different concentrations (5-100 µg/ml) of the extracts for 30 min prior to incubation with the oxidizing agent.

2.8. Determination of intracellular glutathione (GSH) level

GSH level was determined in treated cells with EC-HPLC quantification, using the method described by Khan et al. (Khan et al., 2011), with few modifications. Different concentrations of phenolic extracts (5-100 µg/ml) in MeOH solutions, or an equivalent volume of MeOH for the controls, were added in PBS to cells, grown and differentiated in 6-well plates (1×10^5 cells /ml in 2ml). After 30 min of incubation, cells were treated with the oxysterol mixture 150 µM or TBH 2.5 mM for different times (15-120 min). After treatment cells were scraped into 500 µl of PBS and centrifuged at 10000 × g for 20 min at 4 ºC; pellet were used to determine GSH level. 150 µl of freshly prepared 10% meta-phosphoric acid solution were added to the samples and the mixture was vortexed for 2 min. Then 150 µl of 0.05% TFA solution was added and centrifuged for 10 min at 10000 × g at 4 °C. After centrifugation the clear supernatant was injected into the HPLC system. GSH amount was measured by electrochemical detection, using an HPLC (Agilent 1260 infinity, Agilent Technologies, Palo Alto, USA) equipped with an electrochemical detector (DECADE II Antec, Leyden, The Netherlands) and an Agilent interface 35900E. A C-18 Phenomenex Luna C18 column, 5µm particle size, 150 x 4.5 mm, was used with a mobile phase of 99% water with 0.05% TFA (v/v) and 1% MeOH at a flow rate of 1 ml/min. Electrochemical detector was set at an oxidizing potential of 0.74 V. Data were collected and analyzed using the Agilent Chemstation A.10.02.

software, and expressed as percentage of the control samples (GSH control value: 4.39±0.66 nmol/mg of protein).

2.9. Determination of oxidative damage

Cell oxidative damage was induced by TBH (2.5 mM) or the oxysterol mixture (150 μM) on Caco-2 grown and differentiated in Petri dishes (1×10^5 cells /ml in 10 ml). Different concentrations of phenolic extracts (5-100 μg/ml) in MeOH solutions, or an equivalent volume of MeOH for the controls, were added to cells in PBS. After 30 min of incubation, TBH in aqueous solution was added and incubation was continued for 2 h more. In the samples treated with the oxysterol mixture, incubation was continued for 24 h. After incubation, cells were scraped on ice and centrifuged at 12000 x g for 5 min at 4 °C. After centrifugation, pellets were separated from supernatants: the pellet was used for lipid analyses, while MDA quantification was performed in the supernatant.

2.9.1. Determination of MDA level

MDA levels in the supernatants from treated cells were measured with the TBARS test with HPLC quantification, using the method described by Templar et al. (Templar et al., 1999) with few modifications. Briefly, 100 μl of TCA 10% were added to 400 μl of supernatant, samples were mixed and left at room temperature. After 20 min, 200 μl of TBA (0.6%) were added; sample were incubated at 90 °C for 45 min and then centrifuged at 5000 x g for 15 min at 4 °C. Aliquots of the supernatant were injected into an Agilent 1100 HPLC system equipped with a diode-array detector (HPLC-DAD) and analysis were carried out using a Varian (Middelburg, The Netherlands) column, Inertsil 5 ODS-2, 150×4.6 mm; the mobile phase was a mixture of KH_2PO_4 50 mM pH 7/MeOH (65/35, v/v) at a flow rate of 1ml/min. The adduct MDA-TBA was revealed at 532 nm. A standard curve was prepared using a 1,1,3,3,-tetraethoxypropane (TEP) solution in PBS (0.05–10 μM). MDA levels were measured as nmol of MDA equivalents produced per mg of protein and expressed as percentage of the control samples (MDA control value: 22.14±1.97 pmol/mg of protein), as previously described (Deiana et al., 2012).

2.9.2. Lipid extraction and determination of fatty acids and cholesterol oxidation products

Lipids were extracted from the cell pellet and separation of 7-keto and HP was obtained by mild saponification and HPLC analyses, as previously reported (Deiana et al., 2010). Data are reported as

percentage of the control samples (7-keto control value: 334.02±65.8 pmol/mg of protein, HP control value: 509.91±110.98 pmol/mg of protein).

2.10. Statistical analyses

Biological data are presented as mean ± standard deviation of triplicate values obtained in three independent experiments. Statistical significance within sets of data was determined by one-way analysis of variance ANOVA using the Graph Pad INSTAT Software (GraphPad Software, San Diego, CA, USA) and the Bonferroni post test. Statistical significance among data regarding chemical characterization of the extracts was determined by Tukey's test.

3. Results

3.1. Chemical characterisation of the phenolic extracts

The extracts obtained from the two monovarietal EVOOs were analyzed by LC-DAD to quantify the main phenolic compounds and the results are shown in Table 1. Targeted analysis showed that the most abundant secoiridoids of the samples were the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol (*p*-HPEA) respectively assigned as 3,4-DHPEA-EDA, 3,4-HPEA-EA, *p*-HPEA-EDA and *p*-HPEA-EDA. Bosana extract showed the highest amount of the phenolic compounds dosed (142.2 ± 4,7 mg/g), and was characterized by the statistically highest amount of 3,4-HPEA-EA and 3,4-DHPEA-EDA (30.8 ± 1,5 and 27.9 ± 1,1 mg/kg, respectively). Flavones, such as apigenin and luteolin, showed in total the highest value in the Nera extract. Lignans, such as pinoresinol and 1-acetoxypinoresinol, were estimated in both phenolic extracts, with Nera showing a 3-5 times lower amount than Bosana.

3.2. Biological activity of the phenolic extracts

To investigate the protective activity of the extracts against the pro-oxidant effect of TBH and oxysterols, Caco-2 cells were treated with TBH 2.5 mM and oxysterols 150 μM, the highest concentrations able to induce a significant oxidative damage but not cell death (Figure 1) in the used experimental conditions; ROS formation and GSH level alteration were then monitored with time. Treatment with TBH resulted in a significant increase of ROS production starting from 15 min of incubation (Figure 2A) and proceeding with a steep and time-dependent increase. ROS production was associated with a significant reduction of Caco-2 cellular GSH, around 30% of the initial value at 15 min of incubation, followed by a recovery over time, as shown in Figure 2B. After 60 min of incubation a significant production of ROS, in comparison with the control, was also observed in the cells treated with the oxysterol mixture 150 μM (Figure 3A); the level of GSH was already decreased after 30 min of incubation and slightly recovered over time (Figure 3B).

Pretreatment with the phenolic extracts counteracted TBH induced alteration of cellular redox status; the Bosana extract was the most effective in inhibiting ROS production and GSH decrease, whereas the Nera extract was active only at the highest concentration tested (Figure 4A,B), not showing however a dose-dependent effect in the tested concentrations range. Oxidative damage to the Caco-2 membrane lipid fraction was also evaluated as a consequence of TBH induced ROS production; MDA level was measured

together with HP and 7-keto, major oxidation products of membrane UFAs and cholesterol. As previously reported in Caco-2 cells (Deiana et al., 2010; Deiana et al., 2012), TBH treatment determined after 2 h of incubation a significant increase of oxidation products: a two fold increase of 7-keto level compared with control was detected (Figure 5A) together with an approximately 3 fold increase of HP and MDA (Figure 5B,C). Pre-incubation of cells with the phenolic extracts prevented membrane lipid degradation; the Bosana extract was active at all tested concentrations, while the Nera extract counteracted the formation of 7-keto and HP from 50 μg/ml, and of MDA at the highest concentration used (Figure 5A,B,C).

A similar protective activity was observed against the pro-oxidant action of the oxysterol mixture. Notably, both the extracts completely prevented ROS formation (Figure 6A) at all tested concentrations and GSH level decrease (Figure 6B) from 10 μg/ml. Since oxysterols treatment determined a significant production of ROS, we investigated whether there was an oxidative damage to the cellular lipid fraction, by measuring HP and MDA production. Oxysterols exposure for 24 h resulted in a significant increase of HP and MDA level in the treated samples compared with controls, as shown in Figure 7A,B, indicating an ongoing oxidative process. Both the extracts were equally effective in protecting cells from oxidation, as the level of HP and MDA was significantly lower in all pre-treated samples, from 5μM, with respect to the oxidized ones (Figure 7A,B); in the presence of 10 and 25 μg/ml of each extract, the production of HP and MDA was completely inhibited.

4. Discussion

The gastrointestinal tract is constantly exposed to dietary oxidized lipids, produced in the reactions that occur during processing and storage of foods, including lipid hydroperoxides and oxysterols. Oxidized lipids can impact intestinal integrity, inducing a strong cytotoxic effect on enterocytes, and can alter the redox status, triggering inflammatory reactions and oxidative damage (Rezaie et al., 2007). Several experimental studies suggest that phenolic compounds ingested with the diet can exert antioxidant actions in the gut, are able to counteract the oxidative stress and inflammatory response and inhibit the onset of gastrointestinal diseases, such as inflammatory bowel disease (IBD) (Biasi et al., 2011; Romier et al., 2009). EVOO, the primary source of fat in the Mediterranean diet, is a good source of bioactive compounds, such as phenols. Studies in murine models of IBD have suggested that EVOO supplementation improves colitis, probably alleviating oxidative events and the related pro-inflammatory process, and these effects are related to the presence of phenolic compounds (Cardeno et al., 2014a). Luminal concentrations of phenols might be much higher than serum concentration; because of incomplete absorption, sufficient amounts of unabsorbed polyphenols can reach the colon (Corona et al., 2006), where they might exert health benefits, interacting directly with microbiota, mucosal cells, and dendritic cell projections in the intestinal lumen (Martin and Bolling, 2015). Being ROS generation a key event in the pathogenesis of intestinal mucosal diseases (Bhattacharyya et al., 2014), it is likely that EVOO polyphenols may also act protecting intestinal mucosa from oxidative damage by scavenging reactive species and/or preventing their formation.

Our data support the hypothesis of a direct antioxidant action of EVOO phenolic compounds at intestinal level. The tested phenolic extracts were able to protect Caco-2 cell monolayers against TBH and oxysterols oxidative injury.

TBH exposure resulted in a significant increase of ROS production, in accordance with the reported ability of this hydroperoxyde to generate reactive species (Chamulitrat, 1998); in our oxidative stress model ROS production was inversely correlated to a decrease of GSH level, that slightly recovered over time. The initial GSH depletion is likely due to the direct oxidizing action of ROS to its oxidized form (GSSG) (Goya et al., 2007), whereas GSH rise may be the result of the reduction of GSSG back to GSH

by GSSG reductase or the induction in GSH synthesis stimulated by the pro oxidant agent itself, as already shown in hepatocytes (Lu, 1999; Scarf et al., 2003).

In cells pretreated with the phenolic extracts, we observed an inhibition in the formation of ROS and also prevention of GSH level decrease. After 2 h of incubation with TBH 2.5 mM, we observed a significant increase of the level of MDA, that matched the pattern of formation of more specific markers of lipid peroxidation, HP and 7-keto, indicating an intense oxidative process, as previously shown (Deiana et al., 2012). The two extracts attenuated TBH-induced oxidative damage in the cell monolayers, being the Bosana extract the most active.

The tested phenolic extracts, obtained through liquid–liquid extraction with MeOH/H_2O, contain all the main phenolic compounds and secoiridoids that characterize EVOO phenolic hydrophilic fraction. The comparison with the data reported in literature is not easy because usually values are referred to the oil and not to the extract, and sometimes data are referred to the total amount of the detected phenols (Cardeno et al., 2014b). Extracts prepared from olives of the cultivar Moraiolo, characterized using a similar procedure, contain a higher amount of dosed phenolic compounds than the Bosana and Nera varieties, but with amounts of hydroxytyrosol and tyrosol just slightly higher (ca. two times), and without the detectable presence of flavonols (Hashim et al., 2014).

The LC-DAD analysis showed a higher amount of phenolic compounds in the Bosana extract with respect to the Nera variety. Taking into account that the mechanical extraction process (grinding, malaxing, separation) was the same for both the oils, differences in phenolic compounds amount, although possibly affected by peculiar atmospheric conditions, agronomic practices, and maturation grade, can be primarily due to the cultivar, thus confirming that the oil obtained from the cultivar Bosana is one of the richest in polyphenols (Cerretani et al., 2006).

The activity of a complex mixture of phenols may be influenced by the possible synergy or antagonism among the different classes of phenols; however, our data suggest that some individual phenolic compounds may contribute more than others to the overall effect of the extract, due to their molecular structures. The Bosana extract showed the highest concentration of secoiridoids, hydroxytyrosol and tyrosol. Preliminary work done by our group showed that these two simple phenols were able to protect

Caco-2 cells against TBH-induced oxidative damage, probably acting as scavengers of reactive species (Deiana et al., 2010).

In order to evaluate the protective action of the selected extracts against the harmful effects of oxysterols, cells were treated with a biologically compatible mixture of the most widely oxysterols present in cholesterol-rich foods (Kanner, 2007). Their concentrations in foods have been quantified in the 10-100 μM range, but the consumption of different foods containing oxysterols could lead to luminal concentrations of these products much higher than 100 μM (Kanner, 2007). In the present study, we investigated the toxic effects of a concentration of oxysterols compatible with a high fat diet (150 μM) in terms of alteration of cellular redox status and oxidative damage. The incubation with the oxysterol mixture induced a significant increase of intracellular ROS production starting from 60 min, in accordance with studies carried out by Biasi and colleagues using an equivalent oxysterol mixture (Biasi et al., 2013a; Biasi et al., 2013b; Biasi et al., 2009). In our experimental conditions ROS production was inversely correlated to a decrease of GSH level. Other studies carried out in different cell types showed that individual oxysterols, such as 7-ketocholesterol or 7β-hydroxycholesterol, may cause the formation of ROS and GSH depletion, inducing cell death (Han et al., 2007; O'Callaghan et al., 2002). Individual oxysterols-induced ROS production has also been correlated to enhanced lipid peroxidation in U937 cells (Miguet-Alfonsi et al., 2002), in vascular smooth muscle cells (Tang et al., 2005), and in endothelial cells (Wu and Huang, 2006). Our data show for the first time that an oxysterol mixture, matching what present in foods and intestinal lumen, is able to trigger in Caco-2 cells oxidative unbalance and cell damage; in oxysterols treated cells we observed a significant increase of MDA and HP production with time, indicating an advanced oxidative process of cellular macromolecules. Both phenolic extracts counteracted this pro-oxidant effect, showing a significant, but not dose-dependent, protective activity at all tested concentrations. The activity of Bosana and Nera extracts was comparable, in spite of the different composition. The efficacy of the Nera extract in presence of oxysterols may rely on the mechanism that underlies their pro-oxidant action; ROS production led to cell damage, but through an oxidative process that appeared to be much slower and less pronounced then in presence of TBH. Thus, the compounds that play a crucial role in the protective activity against TBH, quickly responding to ROS production as free

radical scavengers (e.g. HT), may not be so crucial in a system where ROS production probably occurs according to different and more complex mechanisms.

At present little is known about a protective action of phenolic compounds against oxidative stress and inflammation triggered by oxysterols at intestinal level. Wine phenols have been reported to counteract the induction of intestinal inflammation by dietary oxysterols (Biasi et al., 2013b; Guina et al., 2015), acting as modulators at different sites of pro-oxidant and pro-inflammatory cell signals. Oxysterols-induced ROS overproduction in Caco-2 cells treated with specific oxysterols has been reported to be due at least in part to the modulation of cell signaling pathways that result in the activation of NADPH oxidase (Biasi et al., 2009).

Thus, EVOO phenols present in the extracts may act through their simple direct antioxidant effect as free-radical scavengers, but also indirectly interfering with specific signaling proteins, modulated in response to oxidative stress and inflammation, as reported for several classes of phenolic compounds (Fraga et al., 2010).

5. Conclusions

Oxidized lipids, both hydroperoxides (TBH) and oxysterols, at a dosage compatible with a high fat diet, are able to induce oxidative damage at intestinal level. The total phenolic compounds extracted from EVOOs produced from Bosana and Nera varieties exert an effective protective action, correlated to the quantity and quality of the phenolic content but also to the oxidizing agent present in the reaction environment. As suggested for single EVOO phenols in intestinal cells, phenolic extracts activity may be due to their immediate function as scavengers of ROS production, thus partially preventing pro-oxidant insult in presence of both TBH and oxysterols. However, the mechanism of their action may be more complex than a simple direct scavenging activity and will need to be further elucidated.

The presence of EVOO phenols in the intestinal lumen may inhibit the onset of intestinal disorders related to inflammation and oxidation process, modulating pro-inflammatory mediators, but also preventing the production of diet oxidative compounds or directly neutralizing the toxic effect of diet oxidants, thus preserving the integrity of intestinal mucosa against oxidative damage.

Acknowledgements

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Figure Captions

Fig. 1. Cell viability, expressed as % of the control samples, measured in Caco-2 cells after incubation with different concentrations of TBH (A) for 2 h and oxysterol mixture (B) for 24 h. Values (means ± SD, n=9 per group) not sharing a superscript letter are significantly different ($p<0.05$).

Fig. 2. ROS (A) and GSH (B) levels, expressed as % of the control samples (0 min), in Caco-2 cells treated with TBH 2.5 mM. Values (means ± SD, n=9 per group) not sharing a superscript letter are significantly different ($p<0.05$).

Fig. 3. ROS (A) and GSH (B) levels, expressed as % of the control samples (0 min), in Caco-2 cells treated with the oxysterol mixture 150 μM. Values (means ± SD, n=9 per group) not sharing a superscript letter are significantly different ($p<0.05$).

Fig. 4. ROS (A) and GSH (B) levels, expressed as % of the control samples (non oxidized nor pre-treated samples) in Caco-2 after 15 min incubation, the shortest time where a significant change in ROS and GSH level was observed, with TBH 2.5 mM and pre-treated with the phenolic extracts (30 min). Values (means ± SD, n=9 per group) not sharing a superscript letter are significantly different ($p<0.05$).

Fig. 5. Values of 7-ketocholesterol (7-keto, A), fatty acids hydroperoxides (HP, B) and MDA (C) measured in Caco-2 cells after 2 h incubation, when a significant oxidative damage but not cell death was observed, with TBH 2.5 mM and pre-treated with the phenolic extracts (30 min). Values, expressed as % of the control samples (non oxidized nor pre-treated samples), not sharing a superscript letter are significantly different ($p<0.05$).

Fig. 6. ROS (A) and GSH (B) level, expressed as % of the control samples (non oxidized nor pre-treated samples), in Caco-2 after 60 min incubation, the shortest time where a significant change in ROS and GSH level was observed, with the oxysterol mixture 150 μM and pre-treated with the phenolic extracts (30 min). Values (means ± SD, n=9 per group) not sharing a superscript letter are significantly different ($p<0.05$).

Fig. 7. Values of fatty acids hydroperoxides (HP, A) and MDA (B) measured in Caco-2 cells after 24 h incubation, when a significant oxidative damage but not cell death was observed, with the

oxysterol mixture 150 μM and pre-treated with the phenolic extracts (30 min). Values, expressed as % of the control samples (non oxidized nor pre-treated samples), not sharing a superscript letter are significantly different ($p<0.05$).

Table 1. Phenolic compounds from EVOOs extracts determined by LC-DAD.

Compounds	**RT**	**LOD (μg/kg)**	**LOQ (μg/kg)**	***Bosana* (mg/g)** Mean ± SD	***Nera* (mg/g)** Mean ± SD
Hydroxytyrosol [a]	10.7	0.3	1.0	10.7 ± 0.1**a**	5.2 ± 0.2**b**
Tyrosol [a]	13.2	0.4	1.2	19.0 ± 0.7**a**	7.0 ± 0.3**b**
Vanillic acid [a]	15.6	0.5	1.6	0.8 ± 0.1**a**	0.5 ± 0.3**a**
p-Coumaric acid [a]	18.9	0.7	2.1	1.4 ± 0.0**a**	0.4 ± 0.0**b**
3,4-DHPEA-EDA [b]	22.4	0.9	2.9	27.9 ± 1.1**a**	7.1 ± 0.2**b**
Pinoresinol [a]	25.1	0.2	0.5	9.0 ± 0.3**a**	2.7 ± 0.1**b**
p-HPEA-EDA [b]	25.6	0.9	2.9	19.6 ± 0.8**a**	17.0 ± 0.9**b**
Luteolin [a]	26.2	0.5	1.4	3.5 ± 0.1**a**	3.9 ± 0.1**b**
Acetoxypinoresinol [c]	26.5	0.2	0.5	3.2 ± 0.1**a**	0.6 ± 0.0**b**
3,4-HPEA-EA [b]	28.5	0.9	2.9	30.8 ± 1.5**a**	24.9 ± 1.3**b**
Apigenin [a]	28.2	0.4	1.1	0.9 ± 0.0**a**	1.7 ± 0.0**b**
p-HPEA-EA [b]	29.8	0.9	2.9	15.4 ± 1.7 **a**	18.6 ± 0.9**b**

RT = retention time; LOD = limit of detection; LOQ = limit of quantification; mean = average value; SD = standard deviation; nd = not detected (<LOD); [a] Identification based on RT and UV-Vis spectra of pure compounds; [b] Tentative identification by UV-Vis spectra and comparison of retention times with the literature data, and compounds dosed using the oleuropein calibration curve; [c] Tentative identification by UV-Vis spectra and comparison of retention times with the literature data, and dosed using the oleuropein calibration curve; 3,4-DHPEA-EDA, dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (decarboxymethyl oleuropein aglycon); p-HPEA-EDA, dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol (decarboxymethyl ligstroside aglycon); 3,4-DHPEA-EA, oleuropein aglycon; the mean values within each row labeled with different letters are significantly different ($p<0.05$)

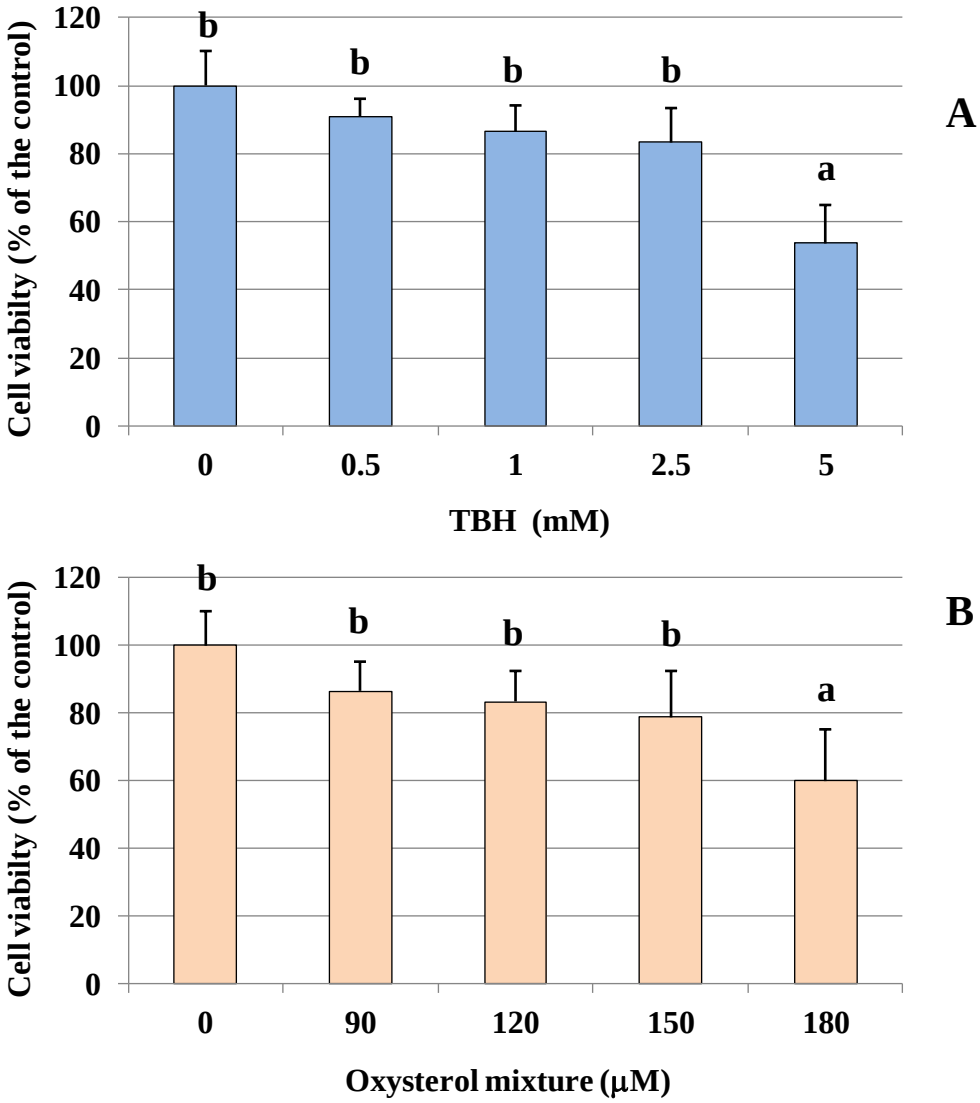
A
Cell viabilty (% of the control)
120
100
80
60
40
20
0
b
b
b
b
a
0
0.5
1
2.5
5
TBH (mM)
B
Cell viabilty (% of the control)
120
100
80
60
40
20
0
b
b
b
b
a
0
90
120
150
180
Oxysterol mixture (µM)

Figure 1

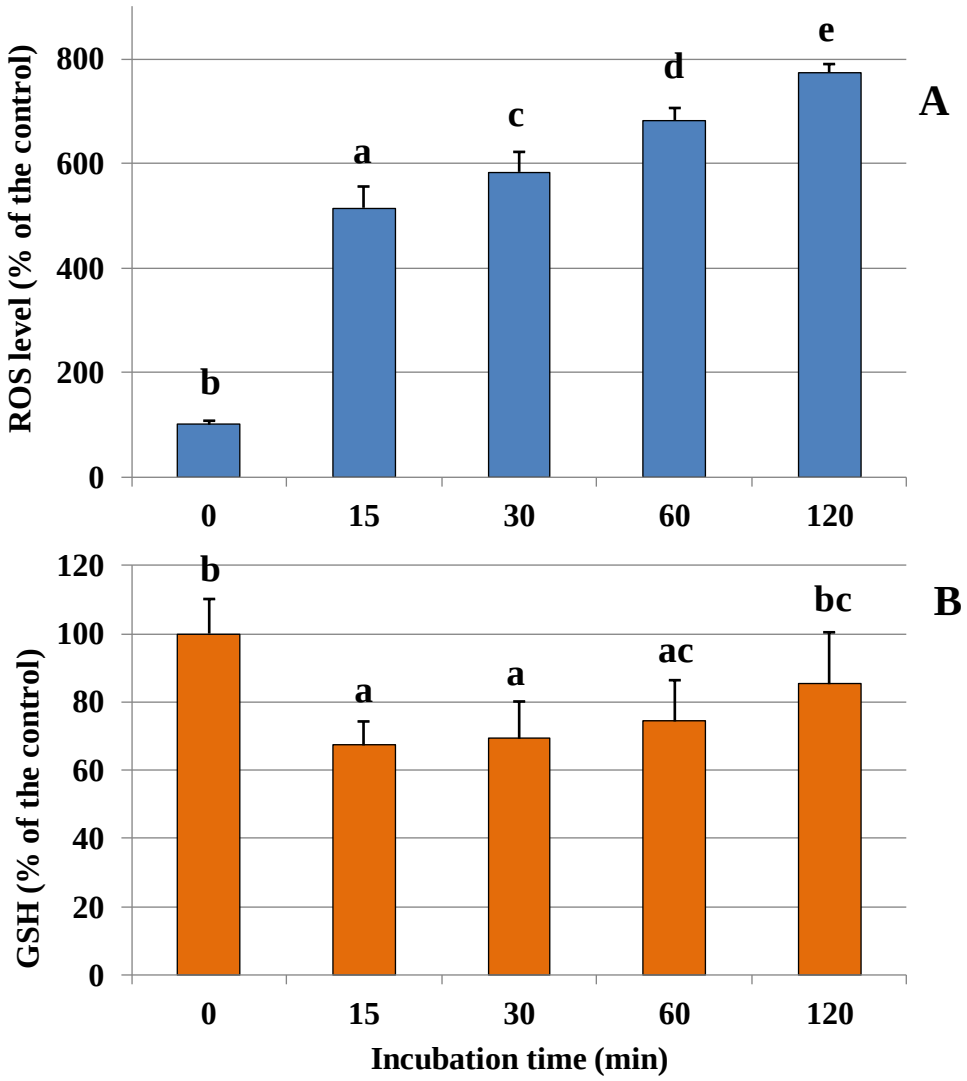
ROS level (% of the control)
800
600
400
200
0
b
a
c
d
e
A
0
15
30
60
120
GSH (% of the control)
120
100
80
60
40
20
0
b
a
a
ac
bc
B
0
15
30
60
120
Incubation time (min)

Figure 2

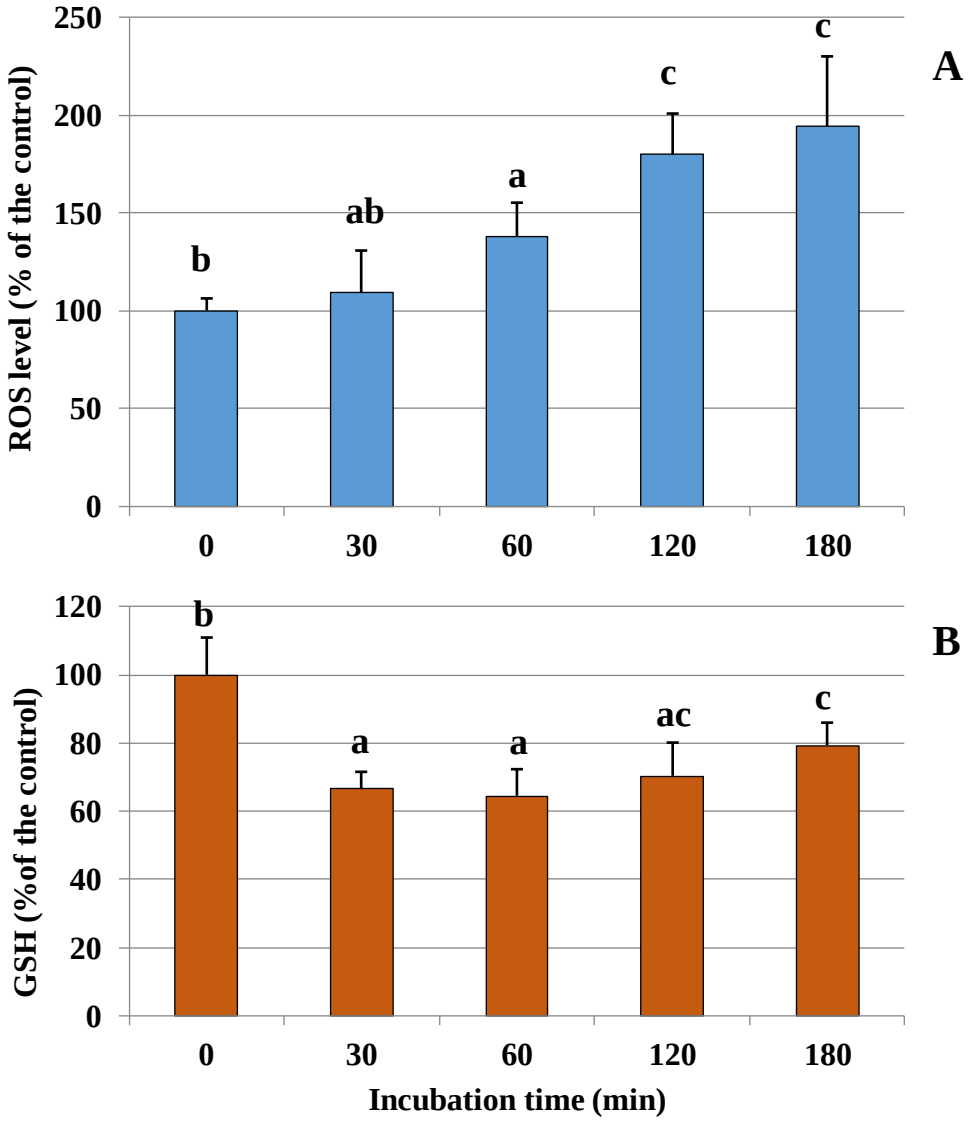
A
ROS level (% of the control)
250
200
150
100
50
0
b
ab
a
c
c
0
30
60
120
180
B
GSH (%of the control)
120
100
80
60
40
20
0
b
a
a
ac
c
0
30
60
120
180
Incubation time (min)

Figure 3

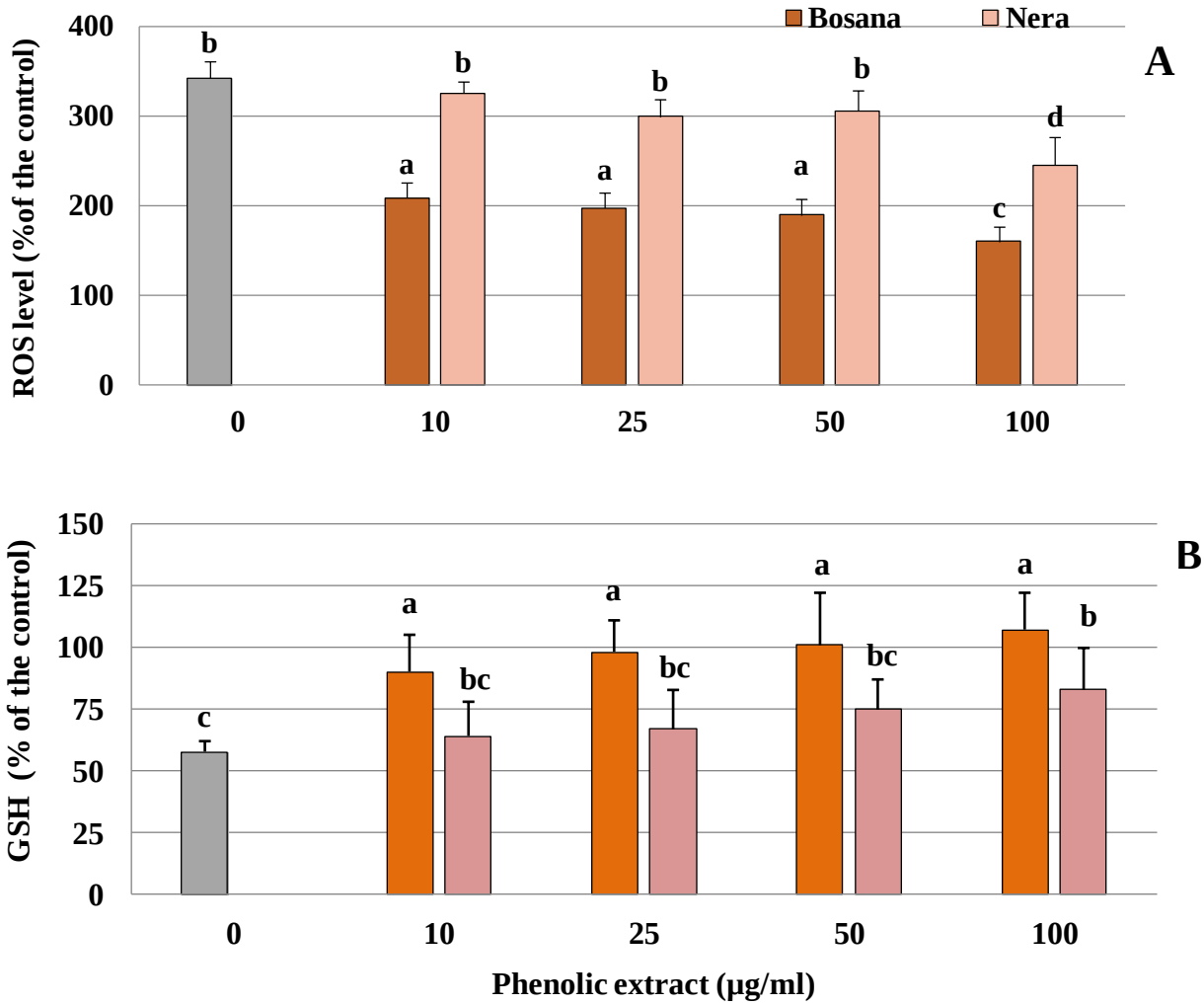
Bosana
Nera
A
ROS level (%of the control)
400
300
200
100
0
b
a
b
a
b
a
b
c
d
0
10
25
50
100
B
GSH (% of the control)
150
125
100
75
50
25
0
c
a
bc
a
bc
a
bc
a
b
0
10
25
50
100
Phenolic extract (µg/ml)

Figure 4

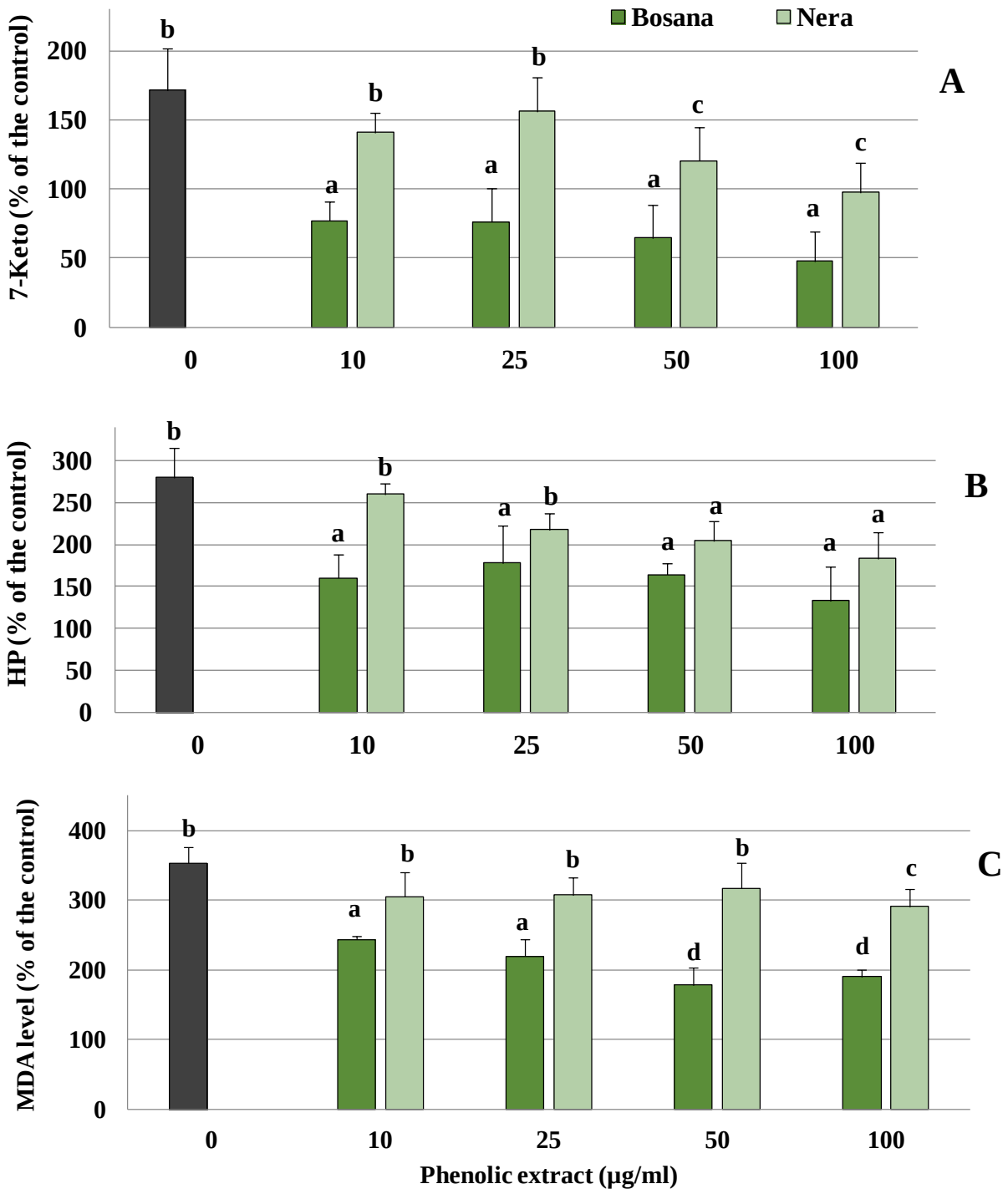
Bosana
Nera
7-Keto (% of the control)
200
150
100
50
0
b
b
b
c
c
a
a
a
a
0
10
25
50
100
A
HP (% of the control)
300
250
200
150
100
50
0
b
b
b
a
a
a
a
a
0
10
25
50
100
B
MDA level (% of the control)
400
300
200
100
0
b
b
b
b
c
a
a
d
d
0
10
25
50
100
Phenolic extract (µg/ml)
C

Figure 5

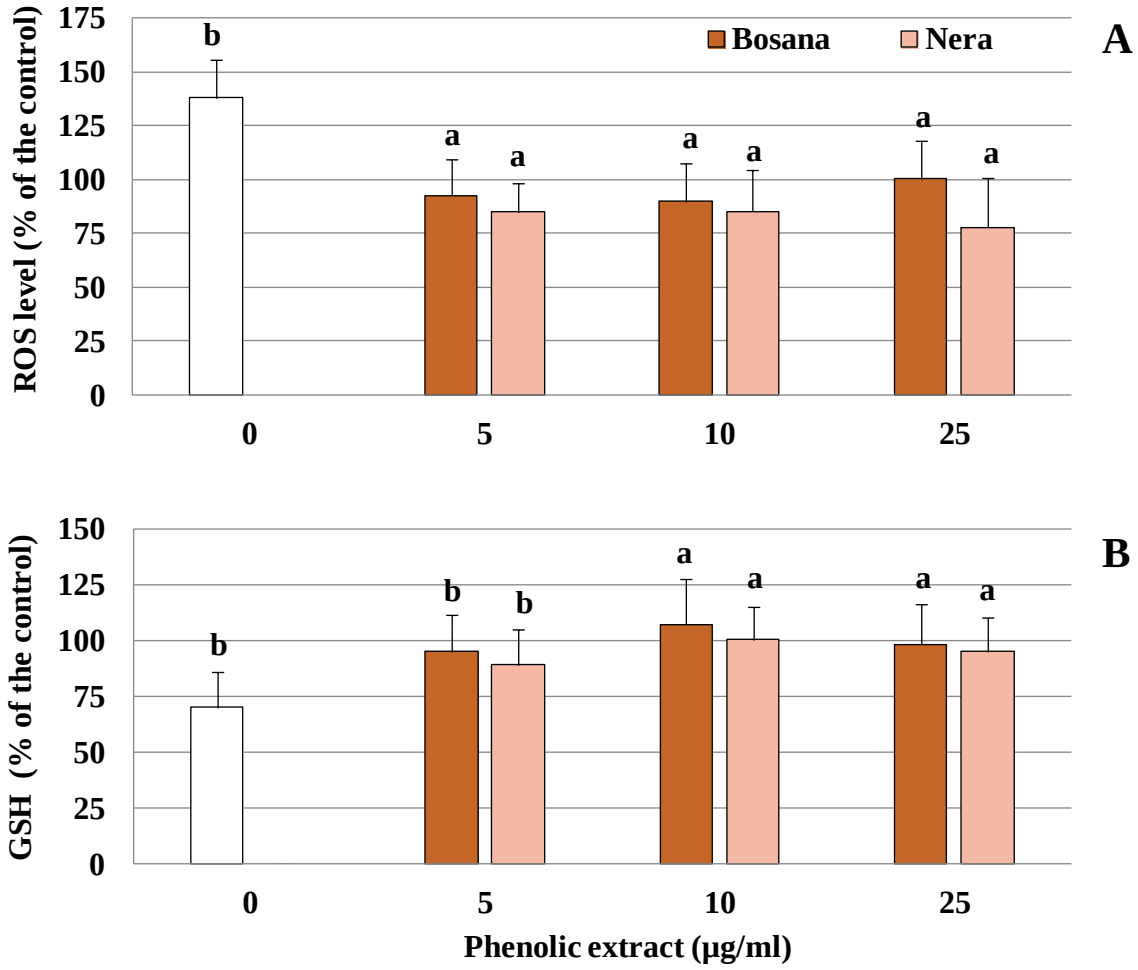
A
ROS level (% of the control)
175
150
125
100
75
50
25
0
Bosana
Nera
b
a
a
a
a
a
a
0
5
10
25
B
GSH (% of the control)
150
125
100
75
50
25
0
b
b
b
a
a
a
a
0
5
10
25
Phenolic extract (µg/ml)

Figure 6

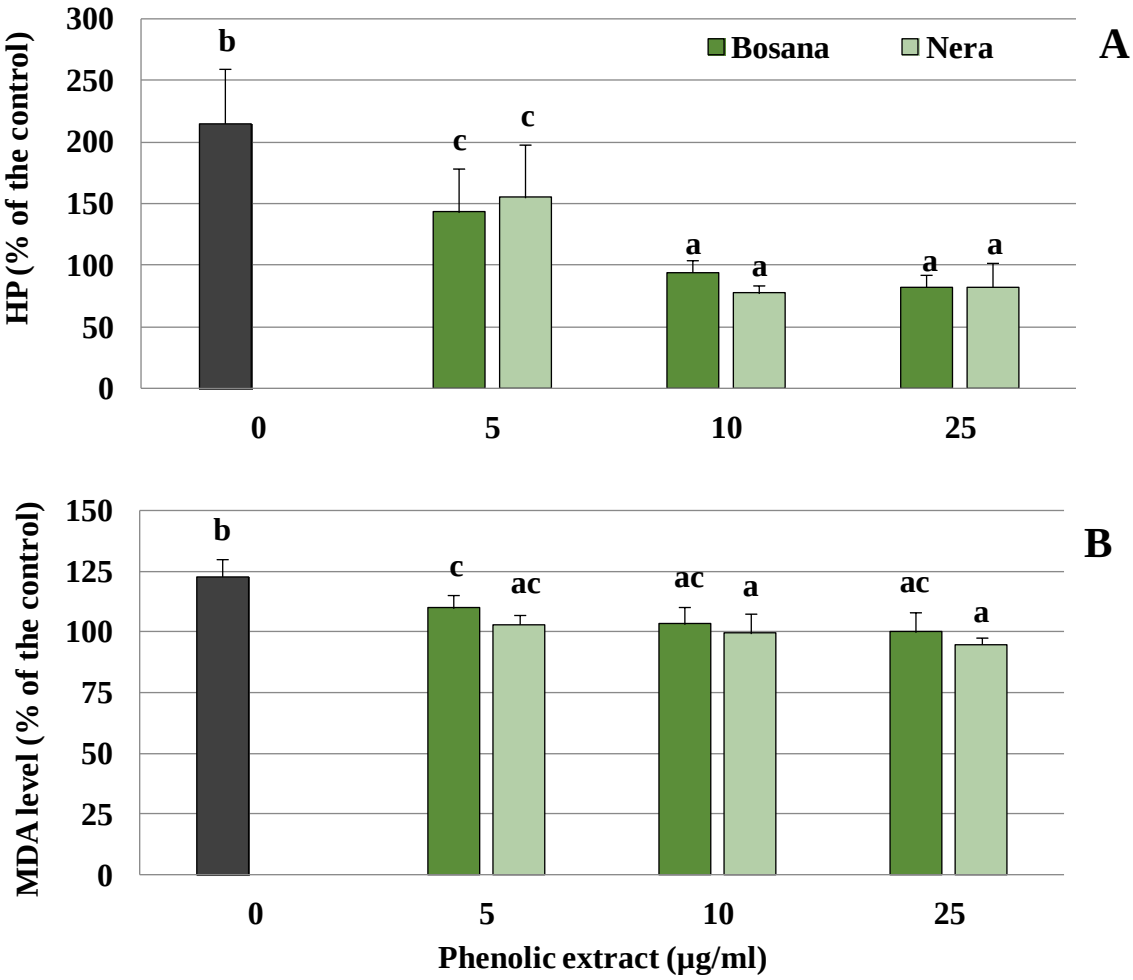
A
HP (% of the control)
300
250
200
150
100
50
0
b
c
c
a
a
a
a
Bosana
Nera
0
5
10
25
B
MDA level (% of the control)
150
125
100
75
50
25
0
b
c
ac
ac
a
ac
a
0
5
10
25
Phenolic extract (µg/ml)

Figure 7