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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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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. Pre-incubation 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.

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

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

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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), 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 ml/min, mixed in linear gradients as follows: t = 0 A:B (85:15, v/v), reaching 60:40 (v/