Hypochlorous Acid Scavenging Properties of Local Mediterranean Plant Foods

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ABSTRACT: Oxidative modification of low density lipoprotein (LDL) is involved in the pathogenesis of atherosclerosis and coronary heart disease, which are low in the Mediterranean area possibly due to a high dietary proportion of plant foods. Ethanolic extracts were prepared from more than 120 Mediterranean edible plants collected in remote areas (which maintain their traditional diet) and their antioxidant potential was studied. Extracts derived from Agaricus campestris, Cynara cardunculus, Thymus pulegioides, and Vicia faba were subjected to further analysis in this study. The extracts' potential to scavenge the DPPH radical, (2,2-diphenyl-1-picrylhydrazyl radical) and hypochlorous acid (HOCl), as well as their antioxidant capacity, was comparable to the those obtained for standard antioxidants (e.g., quercetin, Trolox). Myeloperoxidase (MPO) catalyzes the production of the highly chlorinating and oxidizing agent HOCl, which reacts with the LDL apoprotein moiety, leading to the derivatization of its aminoacidic residues. Coincubation with extracts significantly prevented HOCl-induced modification of the LDL residue tryptophan, whereas higher concentrations were required to retard lysozyme damage. Moreover, the extracts inhibited MPO-catalyzed guaiacol oxidation in a concentration-dependent manner in a cell-free assay but, in contrast, did not affect MPO activity in isolated human neutrophils. MPO is also known to facilitate nitric oxide oxidation. The formation of 3-nitrotyrosine was significantly lower in bovine endothelial aortic cells incubated with C. cardunculus or T. pulegioides. In synthesis, our study shows that local Mediterranean plant foods prevent HOCl toxicity in vitro and, thus, suggests further mechanisms responsible for the reported health-beneficial effect of the Mediterranean diet.


Oxidative modification of low density lipoprotein (LDL) plays a role in atherogenesis and, consequently, in coronary heart disease (CHD) mortality (1). Reactive oxygen and nitrogen species (ROS and RNS) have been hypothesized to be the main cause of LDL oxidation. Accordingly, a series of clinical trials have been undertaken to evaluate the effects of vitamin E, a liposoluble antioxidant, on CHD mortality. However, the majority of such trials failed to demonstrate any protective effect of vitamin E supplementation, in part questioning the true contribution of lipid peroxidation to the formation of atherosclerotic plaques (2,3). The enzyme myeloperoxidase (MPO; EC 1.11.1.7) catalyzes the formation of the chlorinating and oxidizing agent hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions (4). This enzyme is activated, mostly in leukocytes, by inflammatory stimuli, which are known to strongly contribute to atherogenesis (5). Unlike “classic” reactions of lipid peroxidation, for example, those that are metal-dependent, HOCl reacts directly with the apoprotein moiety, derivatizing its aminoacidic residues (6). In addition, HOCl avidly reacts with other macromolecules such as thiols and nucleotides, enhancing tissue damage during inflammation, when HOCl concentrations can reach the high micromolar to low millimolar range (7). As vitamin E is unable to prevent HOCl-mediated lipoprotein modification (8), other antioxidants such as vitamin C and polyphenols are to be tested for their potential antiatherosclerotic effects, especially if MPO and HOCl are involved in atherogenesis and endothelial dysfunction, as indeed suggested by many investigators (9,10).

The incidence of atherosclerosis and CHD is low in the Mediterranean area, where plant foods contribute the majority of caloric intake (11). Vegetables, fruits, legumes, wine, and extra virgin olive oil, in particular, provide antioxidants both vitaminic and nonvitaminic in nature, which have been proposed to exert antiatherosclerotic and cardioprotective effects (12).

Within the EU-funded project “Local Food-Nutraceuticals” (www.biozentrum.uni-frankfurt.de/Pharmakologie/EU-Web/index.html) (13), local wild plant foods of the Mediterranean basin, namely Southern Spain and Southern Italy, were selected and tested for their in vitro ability to scavenge HOCl and, consequently, their potential protective role toward CHD and other degenerative and inflammatory diseases. Table 1 summarizes some of the characteristics of the plants used in this study.
MATERIALS AND METHODS

Materials. Bovine aortic endothelial cells (BAEC) were purchased from Cambrex (Milan, Italy). The reagents for Western blot analysis were from Bio-Rad Laboratories (Hercules, CA) and the enhanced chemiluminescence (ECL) kit for Western blot analysis was from Amersham Biosciences (Milan, Italy). Mouse monoclonal anti-3-nitrotyrosine antibody was purchased from Calbiochem (Darmstadt, Germany); the secondary goat anti-mouse horseradish peroxidase-conjugate antibody was obtained from Bio-Rad (Hercules, CA); and the secondary fluorescent antibody, Alexa 594-conjugated goat anti-mouse antibody, was purchased from Molecular Probes (Eugene, OR). A dialysis membrane (20 × 32 mm) was purchased from MAGV (Rabanau-Londorf, Germany). Myeloperoxidase was obtained from Calbiochem (Darmstadt, Germany), Sephadex G-25 from Amersham Biosciences (Uppsala, Sweden), and hemoglobin from ICN (Eschwege, Germany). All other reagents were of the highest purity available and were purchased from Sigma Chemical (Milan, Italy, and Munich, Germany) or Merck (Darmstadt, Germany).

Plant samples were collected in Southern Italy (Castelmuzano) and Southern Spain (Rio Segura Valley, Murcia). Samples provided by the groups of Drs. Diego Rivera (Universidad de Murcia, Spain) and Michael Heinrich (The School of Pharmacy, University of London, UK) were dried and extracted with 90% hot ethanol under reflux. The extraction and characterization procedure will be fully reported elsewhere by those groups. The total polyphenolic content of the extracts was determined by the Folin-Ciocalteau method, using gallic acid as the reference compound (14).

DPPH scavenging test. A 15 µM ethanolic solution of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was added to the compounds under investigation. After 15 min of incubation, the absorbance was read at 517 nm. EC50 was calculated by employing MacAllfit® as the software (15).

Total antioxidant potential. A validated assay (Bioxytech® AOP-490™; OXIS Research, Portland, OR) based upon the reduction of Cu2+ to Cu+ by antioxidants was employed (16,17). The results are shown as micromoles of Cu2+ reduced.

Oxyhemoglobin bleaching assay. Hemoglobin was reduced and oxygen-loaded according to manufacturer’s instructions, with slight modifications. Briefly, a Sephadex G-25 column was equilibrated with phosphate buffer saline (PBS; 20 mM; pH 7.0) containing EDTA (1 mM). Sodium dithionite (200 mg) was added to the column and drained into the gel by adding 2 mL of PBS. After dissolving 100 mg of hemoglobin in 10 mL of PBS, the hemoglobin solution was applied to the column and eluted. The reduced hemoglobin was saturated with oxygen and dialyzed against oxygen-saturated PBS without EDTA to eliminate excess dithionite and to achieve complete conversion to oxyhemoglobin. Oxyhemoglobin (OxyHb) was stored at −20°C. Concentration of HOCl was determined at 292 nm, using a molar extinction coefficient of 142 (18).

Samples and OxyHb (10 µL) were added to 24- or 96-well plates and the bleaching reaction was started by adding a bolus of HOCl (400 µM). Change in absorbance was recorded at 405 nm by using a Wallac Victor2 1420 Multilabel Counter (Perkin-Elmer, Rodgau-Jügesheim, Germany).

Isolation of LDL. Peripheral blood was drawn in evacuated tubes from healthy volunteers and LDL was isolated from plasma by sequential ultracentrifugation.

Total protein was estimated by the Lowry method (19), with bovine serum albumin as the standard. For all experiments, LDL was diluted to a concentration of 0.5 mg of protein/mL in PBS 10 mM.

Quantification of HOCl-mediated amino acid modifications. Freshly isolated LDL diluted in PBS (0.5 mg/mL; pH: 7.4) was incubated with or without the compounds under investigation at 37°C and exposed to 100 µM HOCl.

Tryptophan residues were evaluated directly by fluorescence (E_x 280 nm; E_m 335 nm). Lysine residues were also determined by fluorescence (E_x 390 nm; E_m 475 nm) after the addition of 163 µM fluorescamine (20).

Changes in relative electrophoretic mobility (REM) of LDL were determined by agarose-gel electrophoresis. LDL (100 µg) were loaded onto the gel, which was resolved at 50 V for 1.5 h. The bands were stained with Sudan Black.

Inhibition of MPO-catalyzed guaiacol oxidation. Lyophilized human MPO was reconstituted according to manufacturer’s recommendation and aliquots were stored at −20°C. Compounds, guaiacol (5 mM in 20 mM phosphate buffer, pH 7.0), and MPO (0.2 µg/mL) were added to 24- or 96-well plates, and the reaction was initiated by addition of H2O2 (200 µM). Absorbance was read over time at 485 nm using a Wallac Victor2 1420 Multilabel Counter (PerkinElmer, Germany).

MPO activity in human neutrophils. Human polymorphonuclear leukocytes (PMNL) were obtained from blood drawn from healthy donors using evacuated tubes with ACD (41 mM citric acid • H2O; 100 mM sodium citrate • 2H2O, 136 mM glucose) as the anticoagulant. PMNL were separated by centrifugation on Ficoll cushions (density 1.077 g/mL), as described in Table 1.

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Abbreviation</th>
<th>Parts used for extraction</th>
<th>Polyphenol content (mg/g)</th>
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<tr>
<td>Agaricus campestris</td>
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<td>Cynara cardunculus</td>
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<td>Vicia faba</td>
<td>Vicia f.</td>
<td>Young fruits</td>
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