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Free Radical-Scavenging Properties of Olive Oil Polyphenols

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Plants in the Mediterranean basin, such as vine and olive trees, have developed an array of antioxidant defences to protect themselves from environmental stress. Accordingly, the incidence of coronary heart disease and certain cancers is lower in the Mediterranean area, where olive oil is the dietary fat of choice. As opposed to other vegetable oils, extra virgin olive oil, which is obtained by physical pressure from a whole fruit, is rich in phenolic components that are responsible for the particular stability of the oil. We have investigated the scavenging actions of some olive oil phenolics, namely hydroxytyrosol and oleuropein, with respect to superoxide anion generation, neutrophils respiratory burst, and hypochlorous acid. The low EC_{50}s indicate that both compounds are potent scavengers of superoxide radicals and inhibitors of neutrophils respiratory burst: whenever demonstrated *in vivo,* these properties may partially explain the observed lower incidence of CHD and cancer associated with the Mediterranean diet.

Key Words: **olive oil; oleuropein; hydroxytyrosol; antioxidants; Mediterranean diet; polyphenols; coronary heart disease.**

The distinctive climate of the Mediterranean basin, characterized by warm weather and prolonged sunlight irradiation, has allowed the development of plants such as olive trees and vine whose fruits require a high proportion of antioxidant molecules [1, 2]. The synthesis of pigments such as anthocyanins, flavonoids and polyphenols, in fact, is activated by white light irradiation [3] and result in dark-colored fruits that, in this way, protect themselves from the noxious effects of prolonged exposure to sunlight. A diet rich in fresh fruits and vegetables and in olive oil, such as the Mediterranean diet, grants an elevated intake of such "nonnutrients", that may transpose their biological activities from the fruit in which they have developed to the human body.

Epidemiological evidence shows that the Mediterranean diet is associated with a lower incidence of coronary heart disease (CHD) [4, 5] and certain tumors (prostate and colon cancers) [6, 7], diseases for which the involvement of an uncontrolled free radical production has been hypothesized. Olive oil is the fat of choice of the Mediterranean area. It is primarily composed of triglycerols and of up to 2-3% of non-saponifiable components [8] . The concentrations and the relative proportions of olive oil "minor components" depend on several factors, including the cultivar, the soil, the climate, and the way the oil is produced and stored. The polyphenolic fraction, in particular, amounts to up to 800 mg/Kg and provides the typical pungent taste and aroma of extra-virgin olive oil, i.e. the kind of oil that is obtained by the simple physical separation of the oil from the olive paste and whose free acidity (expressed as free oleic acid) must not exceed 1%, according to the current regulations.

During the past few years, we have investigated the biological properties of phenols, extracted and purified from olive oil [9-11]. Oleuropein and hydroxytyrosol, both sharing an ortho-diphenolic residue, but not tyrosol were shown to exert potent antioxidant activities in *in vitro* models of LDL oxidation [9-11]. *In vivo* LDL oxidation, a process which is thought to enhance the atherogenicity of these lipoproteins [12], is likely to be mediated by an excessive free radical generation by cells such as macrophages and/or through other processes such as the release of transition metals into the bloodstream.

We have therefore investigated the free radical-scavenging properties of olive oil phenolics by employing several tests that included both cell-free environments and activated human neutrophils.

MATERIALS AND METHODS

Materials. Oleuropein was from Extrasynthese (Genay, France). Pure hydroxytyrosol was a generous gift of Prof. GF Montedoro. All other reagents were from Sigma (St. Louis, MO).

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TABLE 1

DPPH Test

Compound	EC_{50}
Vitamin C	1.31×10^{-5}
Vitamin E	5.04×10^{-6}
BHT	1.05×10^{-4}
Hydroxytyrosol	2.60×10^{-7}
Oleuropein	3.63×10^{-5}

Human neutrophils isolation. Venous blood was collected by venipuncture from healthy volunteers and human neutrophils were isolated from plasma as described in Sala et al. [13].

Evaluation of superoxide anion production. Superoxide anion production was evaluated *in vitro* as follows: cytochrome C (from bovine heart) 1 mg/ml, xantine 200 μM in phosphate-buffered saline 50 mM, and the compound to be tested were mixed in a quartz cuvette and placed in a spectrophotometer. The reaction was started by the addition of 10 μU of xantine oxidase and the rate of reduction of cytochrome C was continuously followed at 550-540 nm. Inhibition of the rate of superoxide formation due to the compound under examination was calculated after the addition of 50 ng/ml of superoxide dismutase.

Ex vivo experiments were performed as follows: a suspension of 2×10^6 neutrophils in phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$ 1 mM, and glucose 5.5 mM was added with cytochrome C 1 mg/ml and phorbol 12-myristate 13-acetate (PMA) 620 ng/ml. Rates of cytochrome C reduction were calculated as above.

DPPH scavenging test. A 15 μM ethanolic solution of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was added with the compounds to be tested. After a 15 min incubation, absorbance was read at 517 nm and compared to control samples. EC_{50} was calculated by employing MacALLFIT as software.

Measurement of neutrophil oxidative burst. The oxidative burst of human neutrophils and its inhibition by olive oil phenolics was evaluated by a slight modification of the method described by Braga et al. [14] A suspension of 1×10^6 cells was added with 3-aminophthalhydrazide (luminol) 10^{-5} M and placed in a luminometer (Lumat LB 9501, Berthold, Germany). PMA 620 ng/ml was then added and the increase in chemiluminescence recorded for 5′.

Reactions with hypochlorous acid. The activity of olive oil phenolics versus HOCl were tested by evaluating their protective effect in a model of hypochlorous acid-mediated inactivation of catalase [15, 16] . A solution of 75 μM bovine catalase in phosphate-buffered saline was incubated with 80 μM HOCl at 37 °C for 15 min. The absorption spectrum was then measured. Also, the ability of olive oil polyphenols to inhibit HOCl-mediated oxidation of 5-thio-2-nitrobenzoic acid (TNB) to 5,5′ dithiobis (2-nitrobenzoic acid) (DTNB) was evaluated according to Aruoma and coworkers [16, 17], except that HOCl concentration in this set of experiments was 200 μM.

RESULTS

The reduction of the stable radical DPPH by antioxidants can be monitored by following the disappearance of the absorption at 515 nm. As shown in Table 1, both hydroxytyrosol and oleuropein exhibited a strong radical-scavenging activity, with EC_{50}s that were 2.6×10^{-7} M and 3.63×10^{-5} M, respectively. Both EC_{50}s were comparable to or higher than those of established water- and lipid-soluble antioxidants such as ascorbate, BHT, and α tocopherol.

Olive oil phenolics have also been tested for a direct scavenging effect on superoxide anion production *in vitro* by employing both cell-free and neutrophil-based generation methodologies. The former adopts the xantine/xantine oxidase combination, that concomitantly generates urate and superoxide, while the latter is based on the production of superoxide by PMA-challenged human neutrophils (an example is given in Fig. 1). In both systems, a potent scavenging capacity of hydroxytyrosol and oleuropein was clearly evident (Table 2). It is noteworthy that Vitamin E and BHT had no effect on $O_2^{\bullet-}$ production (data not shown), while ascorbate and Trolox could not be tested because *per se* they potently reduced cytochrome C.

The free radical production due to the respiratory burst of neutrophils, triggered by the addition of PMA, was evaluated by continuous monitoring of luminol oxidation and consequent development of chemiluminescence. Addition of PMA 620 μM to a neutrophil suspension lead to a steep increase in chemiluminescence (Fig. 2, rate of formation: 23891 CLU/min). A dose-dependent inhibition of the rate of chemiluminescence formation was observed following co-incubation of cell samples with either oleuropein or hydroxytyrosol.

When the antioxidant protection against damage by HOCl was evaluated, both oleuropein and hydroxytyrosol exhibited a protective activity that was comparable to, or greater than, that of reference antioxidant compounds, i.e. vitamins E and C. Protection of catalase heme group was in fact suggested by the absorption spectrum of the enzyme following incubation with HOCl (Fig. 3). The effects of Vitamin C and Vitamin E were superimposable to those of oleuropein and hydroxytyrosol, respectively. HOCl-mediated formation of DTNB was also reduced by olive oil phenolics (Table 3), although at quite high concentrations (10^{-4} to 10^{-5} M, higher than those of vitamins C and E), confirming the hypochlorite-scavenging properties of several phenolic compounds, including flavonoids [17].

DISCUSSION

This paper presents strong evidence of a free radical-scavenging activity of some olive oil polyphenols, namely hydroxytyrosol and oleuropein. The latter has been previously shown to increase nitric oxide (NO) production from LPS-challenged mouse macrophages [18]. In that report it was suggested that, together with a direct tonic effect of oleuropein toward the inducible form of macrophagic nitric oxide synthase (iNOS), a concomitant removal of superoxide anion might contribute to the observed net increase in NO levels. The data presented here support that hypothesis and confirms a potent scavenging activity of oleuropein and

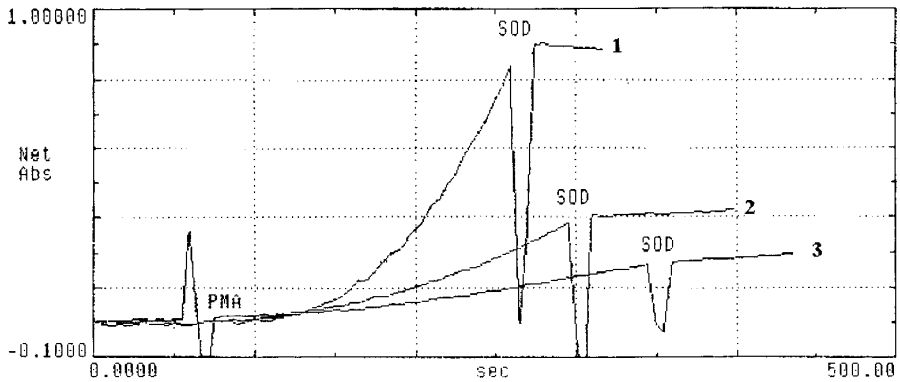

FIG. 1. PMA-induced superoxide production by human neutrophils. Samples were as follows: 1) Control; 2) Hydroxytyrosol 10^{-5} M; 3) Hydroxytyrosol 10^{-4} M. SOD, superoxide dismutase 50 ng/ml.

hydroxytyrosol both in *in vitro* and in *ex vivo* models of superoxide formation.

The use of the free radical DPPH has been discussed by Brand-Williams et al. [19], who described how the disappearance of the absorption at 515 nm was correlated with the reduction of DPPH by antioxidants. As shown in Table 1, both oleuropein and hydroxytyrosol reduced DPPH with very low EC50s, indicating a potent scavenging effect toward this radical species.

Superoxide anion was produced by either the xantine/xantine oxidase system (Table 2) or by PMA-challenged human neutrophils (Fig. 1). As indicated by the EC50s, which are in the low micromolar range, olive oil phenolics potently removed superoxide from the reaction medium. Since oleuropein was previously shown to increase nitric oxide (NO) [18] production by murine macrophages *via* an augmented iNOS expression, we speculate that the concomitant removal of superoxide could contribute to the observed net increase in NO and prevent formation of the powerful oxidant peroxinitrite [20].

In addition, the known stabilizing activity on extra virgin olive oil of its non-saponifiable components [21, 22] can be possibly explained by the removal of free radicals, including superoxide, by hydroxytyrosol and other simple and hydrolyzable phenols. Since other lipid-soluble antioxidants such as tocopherols have no effect toward superoxide (see Results), this can explain the observed higher stability of olive oil, as compared to other vegetable oils, despite the fact that tocopherols levels in the latter are ~10 times higher (Dr. A. Cimato, from IPSL, Italy, personal communication).

TABLE 2

Inhibition of Superoxide Anion Formation Rates by Olive Oil Phenolics

	Superoxide-producing system	
	Xantine-xantine oxidase	PMN ± PMA
Hydroxytyrosol (EC_{50})	9.1 μM	3.2 μM
Oleuropein (EC_{50})	14.3 μM	29.3 μM

Note. Details on superoxide anion production are given in the Methods section. EC_{50}s were calculated by employing MacALLFIT as software. PMN, human polymorphonuclear neutrophils; PMA, phorbol-12-myristate-13 acetate.

The respiratory burst of PMN is involved in asthmatic reactions and is associated with an abnormal production of superoxide anion, hydrogen peroxide, and hypochlorous acid [14]. As a consequence, reactive oxygen species (ROS) overproduction has been reported in asthmatic patients [23, 24] and may aggravate tissue damage by direct bronchoconstriction [25, 26] and/or by initiating the arachidonic acid cascade [27]. As shown in Fig. 2, oleuropein and hydroxytyrosol inhibited the PMA-elicited respiratory burst of human PMN, as assessed by chemiluminescence, in a dose-dependent manner. These data add further evidence [28] suggestive of the *in vitro* anti-inflammatory properties of olive oil phenolics: by removing the above mentioned oxidant species, these compounds compensate for the over-production of important mediators of inflammation.

The production of HOCl by myeloperoxidase of activated neutrophils at the site of inflammation can contribute to tissue damage and have important effects toward proteins and enzymes. For instance, HOCl may rapidly inactivate α_1-antiproteinase, activate collagenase and gelatinase, deplete antioxidant vitamins such as ascorbic acid, and inactivate antioxidant enzymes [15]. The antioxidant activity of micromolar concentrations of hydroxytyrosol and oleuropein has been tested as their ability to prevent HOCl-mediated inactivation of catalase at physiologically feasible concentrations of hypochlorous acid [29]. The physiological significance of catalase protection is clear if one considers its ability to scavenge H_2O_2, which is co-produced by activated neutrophils at the site of inflammation. Preservation of catalase activity, together with other anti-inflammatory properties shown for olive oil phenolics, may

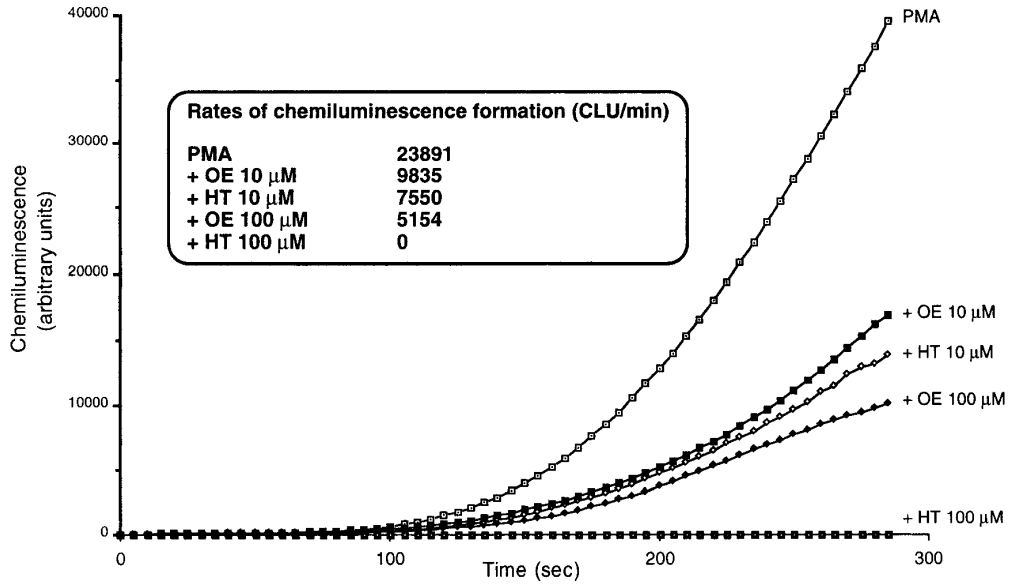

FIG. 2. PMA-induced respiratory burst in human macrophages. Conditions were as in Method section.

therefore play a role in tissue protection during inflammation [15, 30].

The ability of oleuropein and hydroxytyrosol to scavenge high concentrations of HOCl, i.e. 200 μM, as evaluated by a reduced oxidation of TNB (Table 3), may be somehow relevant to food that comes into contact with chlorine-based bleaches: it is noteworthy that several spice extracts, many of which phenolic in nature, are currently employed as additives by the food industry [31, 32]. Hence, the preservative potential of antioxidants derived from olive oil production, many of them are currently discarded [33], may be worth further investigation.

Finally, HOCl can oxidize the proteic portion of LDL: the data presented here add more evidence to the described protective effects of olive oil phenolics toward LDL oxidation.

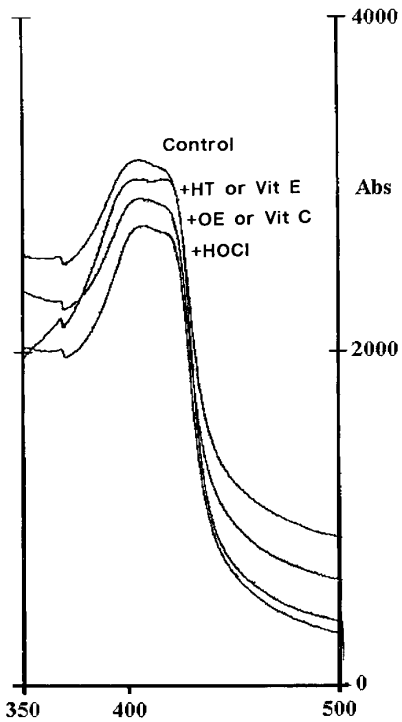

FIG. 3. Absorption spectra of catalase incubated with HOCl and the compounds under investigation. Conditions and concentrations were as in Method section.

TABLE 3

Inhibitory Effects of Antioxidants on HOCl-Mediated Oxidation of TNB

Compound	Abs_{412}
None	0.707
Plus HOCl	0.085
Vit C 10^{-4} M	0.226
Vit C 10^{-5} M	0.187
Vit C 10^{-6} M	0.138
Vit E 10^{-4} M	0.217
Vit E 10^{-5} M	0.098
Vit E 10^{-6} M	0.013
OE 10^{-4} M	0.143
OE 10^{-5} M	0.099
OE 10^{-6} M	0.035
HT 10^{-4} M	0.152
HT 10^{-5} M	0.112
HT 10^{-6} M	0.086

Note. Data are means of duplicate experiments that did not differ by more than 5% [17].

Evidence of the absorption and disposition of flavonoids and polyphenols in humans is still scarce. However, the available data suggest that part of the ingested compounds is degraded by the intestinal flora while others are absorbed intact [34, 35]. Furthermore, laboratory animals fed extra-virgin olive oil showed increased resistance of their LDL to oxidation [36, 37]. Future availability of pure compounds and development of appropriate analytical techniques will clarify this important issue.

In conclusion, these data add to the current opinion that part of the beneficial effects of the Mediterranean diet may be due to the presence of "minor components", including olive oil-derived ortho-diphenols, that exhibit biologically relevant activities.

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