Pro-oxidant Activity of Oleuropein Determined in Vitro by Electron Spin Resonance Spin-Trapping Methodology

Armando Mazziotti,[†] Fabio Mazzotti,[†] Manuela Pantusa,[‡]
Luigi Sportelli,[‡] and Giovanni Sindona*,[†]

Dipartimento di Chimica, via P. Bucci, Cubo12/C, and Dipartimento di Fisica and UdR CNISM,
via P. Bucci, Cubo 30/C, Università della Calabria, I-87036 Arcavacata di Rende (CS), Italy

In this work, the pro-oxidant behavior of oleuropein (OLP, **1**) is characterized in a Fenton-like experiment by means of ESR spectroscopy using the spin trap system DMSO and 4-(pyridyl-1-oxide)-*N-tert*-butyl nitrone (POBN) in phosphate buffer (PB) solution. Ferrous ions in the absence of hydrogen peroxide cause the formation of the stable nitroxide species **4** and **5** through the intermediate perferryl species. OLP displays its antioxidant activity in vitro blocking the oxidation path that leads to methoxyl radicals hence to the formation of the stable radical species **5**. The role of the catechol moiety was proved when the perferryl experiments were repeated in the presence of the dimethylated oleuropein homologue (OLP-Met$_2$, **2**). The dual behavior of oleuropein, similar to that ascertained for other catechol and non-catechol natural active species, should provide warnings for its use as nutraceutical or as drug with manifold healing effects.

KEYWORDS: Oleuropein; pro-oxidant activity; ESR; spin trapping; catechols; iron complexes

INTRODUCTION

Fresh vegetables and, generally speaking, agrifoods are the source of many pharmacologically and biologically active principles used as integrators (nutraceuticals) or cosmetic ingredients (nutracosmeceuticals).

The healing effects produced by active ingredients of natural foodstuffs have been so amplified in scientific and daily papers that notice on the anti-inflammatory properties (*1*) of a well-known molecule (*2, 3*) present in commercial olive oil has reached the front page of daily papers in less than 1 day (*4*).

The main biological activity of these nutraceuticals is associated with their action as antioxidants to prevent damage caused by oxygen reactive species (ROS) in vivo. A widely distributed class of such compounds within the plant kingdom is represented by the so-called polyphenols and, among them, by those bearing a catecholic (CE) moiety (*5*). Nevertheless, abiotic stress, causing the accumulation of phenolics in plants, promotes the production of ROS within the cells (*6*). Moreover, it has been demonstrated that the same harmful active oxygens are formed even during regular plant metabolism. Furthermore, CEs, formed in vivo in the oxidation of estrogens, take the 1-electron oxidation path that leads to the formation of quinone derivatives which interact with DNA either forming covalent adducts or causing depurination. Such modifications in critical genes can induce mutations that lead to carcinogenesis (*7−9*). The dual behavior of catechols in vivo may represent a drawback when overdoses of nutraceuticals are assumed with diet or nutracosmeceuticals are used in cosmetics.
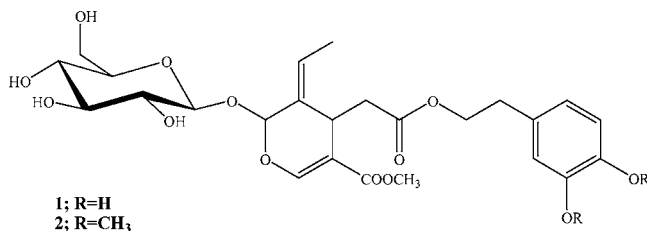
A number of studies have recognized that a diet rich in olive oil, particularly unrefined oils, provides a healthy prevention of artery wall thickening as a consequence of low-density lipoprotein (LDL) oxidation process. This beneficial effect has been associated with the presence of oleic and linoleic mono-unsaturated fatty acids and with the action of potent antioxidants such as tocopherols and the "polyphenols" (*10*). Oleuropein (OLP, **1**), a secondary metabolite of terpenoid origin, is the main iridoid of the "phenolic pool" (*11, 12*) of *Olea europaea*, whose activity is likely associated with its *o*-dihydroxybenzene (catechol) moiety. The same moiety is shared by hydroxytyrosol which is formed by enzymatic degradation of the intact secoiridoid **1** and exhibits similar redox activity. Other phenolic compounds, such as tyrosol, caffeic acid, etc., account for the radical scavenger effect of virgin olive oil (*13*); however, attention has been paid to the actual content of oleuropein in foodstuffs due to its therapeutic action (*14*). The presence of **1** in commercial unrefined olive oils is in the order of hundreds of ppb (*15*), whereas it goes up to thousands of ppm in leaves and drupes (*14*). Dietary supplements rich in the active principle **1**, available in the free-market of developed and developing countries, are regularly administered for their antioxidant properties.

The antioxidant activity of the polyphenolic pool is exerted by scavenging the causative agent of the oxidation of lipids,

* Corresponding author. Mailing address: Dipartimento di Chimica, Università della Calabria, via P. Bucci, Cubo 12/C, I-87036 Arcavacata di Rende (CS), Italy. Tel: +39-0984-492046. Fax: +39-0984-493307. E-mail: sindona@unical.it.
† Dipartimento di Chimica.
‡ Dipartimento di Fisica and UdR CNISM.

Pro-oxidant Activity of Oleuropein Determined in Vitro

J. Agric. Food Chem., Vol. 54, No. 20, 2006   **7445**


1; R=H
2; R=CH₃

proteins, carbohydrates, and DNA. They may also chelate those transition metals, such as iron and copper, involved in Fenton reactions (eq 1) which produce the extremely reactive $^{\bullet}$OH radical (*16−18*).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \qquad (1)$$
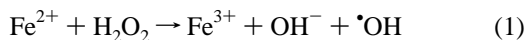
The radical scavenging capacity is enhanced when a catechol moiety is present (*19*), whereas it is decreased in the case of partial methylation of the hydroxyl groups (*20*).

Fenton-like reactions have also been invoked in the apparently paradoxical behavior of some antioxidant agents acting as pro-oxidants (*21−24*). In vivo studies have, in fact, shown that α-tocopherols, well-known as scavengers of free radicals in a hydrophobic milieu, display both anti-and pro-oxidant activities in isolated LDL. A similar behavior is attributed to ascorbic acid and β-carotene.

The $^{\bullet}$OH dependent mechanism, briefly outlined above (eq 1), has been questioned and ruled out in many biological oxidation processes, on the grounds of the number of observations related to the availability of hydrogen peroxide at cellular level and to the small rate constant of the Fenton reaction (*25*). An alternative biological oxidant is represented by the perferryl/ferryl ion system (eq 2) verified in many environments (*26*) which support the observations that H_2O_2 cannot be essential for initiating biological oxidation processes:

$$Fe^{2+} + O_2 \rightleftharpoons [Fe-O-O^{\bullet}]^{2+} \rightleftharpoons Fe^{3+} + O_2^{\bullet -} \qquad (2)$$
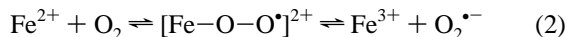
On the grounds of the dual behavior in vivo of many CE molecules, considering the widespread use of pharmaceutical formulations based on oleuropein extract from olive leaves, we have undertaken an electron spin resonance (ESR) investigation using the spin trap method of the redox properties of **1** and **2**, to ascertain the dual action in vitro of oleuropein, if any.

MATERIALS AND METHODS

Sample Preparation. Oleuropein (OLP, **1**) was extracted from olive leaves as described (*27*) and purified by HPLC.

Dimethyloleuropein (OLP-Met₂, **2**) was obtained by diazomethane treatment of **1** in ethyl ether followed by HPLC purification. FeCl₂· 6H₂O, salts for phosphate buffer solution, and H₂O₂ were Merck reagent grade. Bidistilled water was used throughout.

The standard reaction mixture contained 0.1 mM FeCl₂, 0.2 mM OLP, and 20 mM H₂O₂ (when needed) in 20 mM phosphate buffer (PB) solution at pH 7.2. In some reaction mixtures the FeCl₂ concentration was varied as indicated hereafter. All samples were incubated for 15 min at 37 °C in a thermostated bath Haake D8 before the ESR run. Measurements were carried out at room temperature.

ESR Measurements. Free radical ESR spectra were recorded with a 10 GHz, X-band Bruker spectrometer, model ESP 300, and digitized with the spectrometer's built-in microcomputer using OS-9 compatible ESP 1600 spectral acquisition and data handling software. Such data handling routines were used to carry out spectral subtraction (see text) to identify the free radical adduct species and to evaluate their relative ratio. ESR spectra simulation was also performed with the Bruker WIN-EPR SimFonia simulation software package.

Sample capillaries were inserted in a standard 4 mm (i.d.) ESR quartz tube and centered in a TE₁₀₂ standard rectangular cavity (ER 4201, Bruker). The temperature was controlled with the ER 4111 VT Eurotherm variable temperature control system (accuracy ±0.1 °C). The free radical ESR spectra were acquired with the following experimental setup: 10 mW microwave power, 0.1 G peak-to-peak magnetic field modulation, 100 kHz magnetic field modulation frequency for phase sensitive detection, 100 G magnetic field scan width. All spectra were displayed as first derivative in-phase absorption signal.

The ESR runs were repeated after 24, 48, and 72 h of incubation at 25 °C. The shape of the ESR spectrum does not change with time. Only a small increase of the signal intensity is observed after 24 h incubation. The intensity remains constant at 48 and 72 h incubation (spectra not shown). In the following, only spectra recorded after 24 h will be presented and discussed. As spin trap, the system DMSO (2%) and α-4-(pyridyl-1-oxide)-*N-tert*-butyl nitrone (POBN) 25 mM in 20 mM phosphate buffer (PB) solution was used.

RESULTS AND DISCUSSION

The bioactivity of oleuropein, often associated with the catechol system of its hydroxytyrosol moiety, could be affected by the partial methylation of the hydroxyl groups and/or the presence in the environment of transition metal ions. In particular, the typical tris(catecholate) ferrate(III) structure formed by catechol with iron at alkaline pH seems to be replaced (*28*) by the 1:2 complex at pH 7.2. It can be assumed, therefore, that, at physiological conditions, a 1:2 complex (**3**) could be formed by OLP and Fe³⁺.


R=
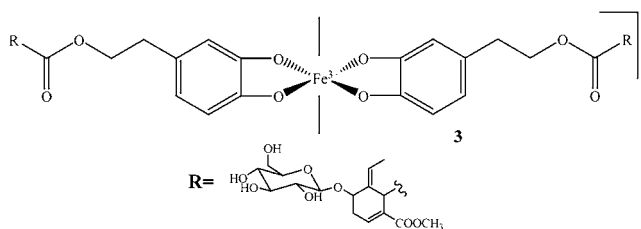
$^{\bullet}$OH dependent or independent oxidation environments have been simulated in vitro to investigate, by ESR, to what extent **1** behaves as an antioxidant of the polyphenolic pool. Its involvement in redox processes was evaluated by the detection of the free radical species formed in the presence of the spin trapping system, dimethylsulfoxide/α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (DMSO/POBN).

The chemistry of Fe²⁺ in the presence of DMSO/POBN was examined by ESR in a phosphate buffer (PB) at pH 7.2, after incubation of the reaction mixture at 37 °C for 15 min (**Figure 1**). The blank, obtained in the absence of OLP (**Figure 1A**), is a complex ESR spectrum which results from the overlapping of two signals with small different magnetic properties, each one resulting from a triplet of doublet of resonance lines (see **Figure 1B**), due to the formation of the POBN adducts **4** (51%) and **5** (49%), as reported in similar experimental conditions (*26*).

These "stable" radical species should be formed at the burst of the reaction by the action of perferryl ions on DMSO (**Scheme 2**) thus causing the formation of methyl, $^{\bullet}$CH₃, radicals further oxidized to methoxyl, CH₃O$^{\bullet}$, radicals in aerobic conditions (*29*) (eq 2 and **Scheme 2**).

No ESR signals were detected in the absence of DMSO in the reaction mixture, when Fe²⁺ or Cu²⁺, another element known to be involved in oxidation processes, was present. It cannot be excluded that the superoxide anions (eq 2) and later hydrogen peroxide (**Scheme 5**) could behave as oxidants along the available reaction paths.
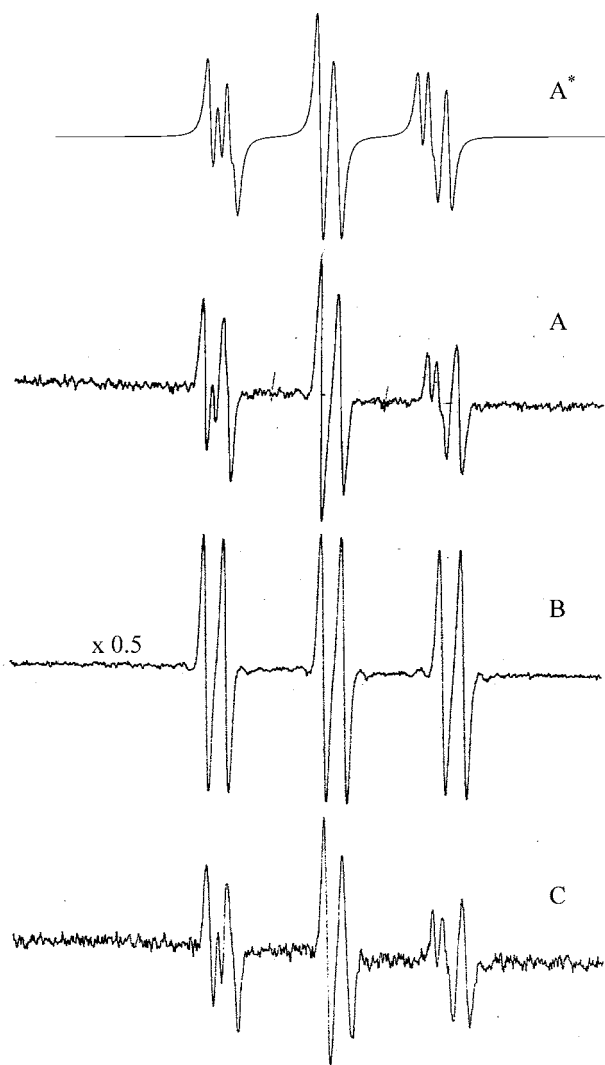
Figure 1. ESR spectra of the reaction mixtures incubated in aerobic conditions for 15 min at 37 °C: (**A**) Fe^{2+} (0.1 mM), DMSO (2%), POBN (25 mM) in PB (20 mM); (**B**) Fe^{2+} (0.1 mM), DMSO (2%), POBN (25 mM), OLP (0.2 mM) in PB (20 mM); (**C**) Fe^{2+} (0.1 mM), DMSO (2%), POBN (25 mM), OLP-Met$_2$ (0.2 mM) in PB (20 mM); (**A***) computer simulation of the ESR spectrum **1A**.
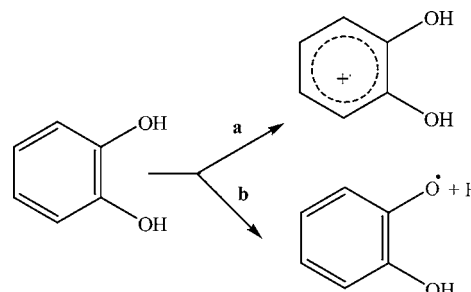
Scheme 1
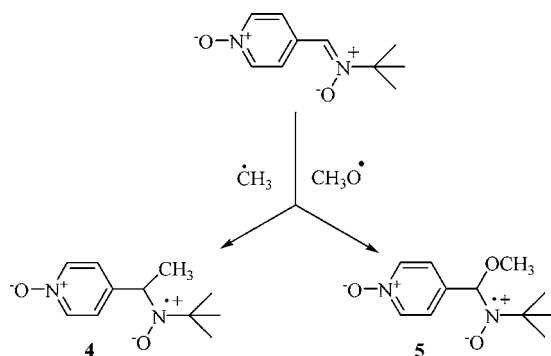
The spin-trapping species POBN provides the spectrum of **Figure 1B** when OLP is incubated in the DMSO/POBN/FeCl$_2$ mixture, in the same experimental conditions as for **Figure 1A**. The hyperfine splitting constant values $a^N = 16.02$ G and $a^H = 2.73$ G of this spectrum are in agreement with the presence of the *N*-oxide radical species **4** (*26*), POBN/$^\bullet$CH$_3$, only (**Scheme 1**).
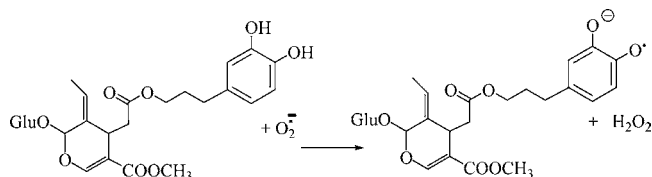
Scheme 2
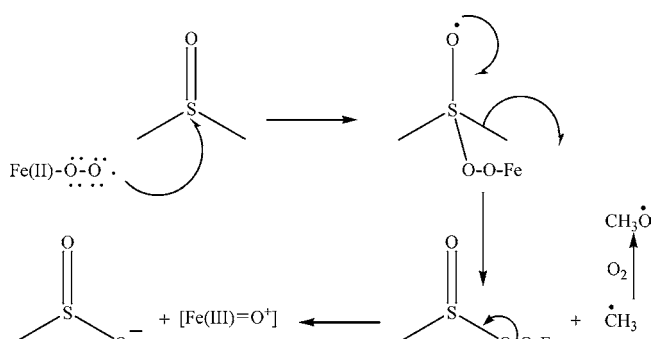
Scheme 3

Scheme 4
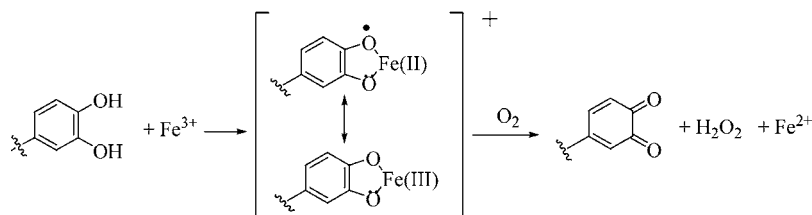

The spectral subtraction, carried out with the data handling routines available in the spectrometer, of the pure ESR signal of POBN/$^\bullet$CH$_3$ (**Figure 1B**) from the complex pattern of **Figure 1A** supports the observation that the POBN/$^\bullet$OCH$_3$ ($a^N = 14.63$ G; $a^H = 2.23$ G) adduct species was present in the spectrum reported in **Figure 1A** and that it disappears when OLP is added (**Figure 1B**). Moreover, in this condition a 2-fold increasing of the signal intensity due to the radical species **4** is observed. The ESR spectrum in **Figure 1A** results from the overlapping of 57% of the POBN/$^\bullet$CH$_3$ ESR signal and 43% of the POBN/$^\bullet$OCH$_3$ one. Computer simulation of the ESR signal in **Figure 1A** is reported in **Figure 1A***. It was obtained using in the simulation the same percentage composition as obtained by spectral subtraction. The simulated spectrum is, in the main features, completely similar to the experimental one, so that in the following only results coming from spectral subtraction will be reported.

In a recent report on the iron induced off-color development of foodstuffs containing polyphenols, the necessary involvement of ferric iron in the oxidation of catechols was clearly demonstrated. For ferrous species to participate in the process, a previous conversion into the ferric state by oxygen was, in fact, required (*30*). Furthermore, the complexity of the mechanism of the radical scavenging action of phenols depends on, among other factors, the energetics (*31*) associated with the ionization of the molecule (**Scheme 3**, path **a**) and with the hydroxyl bond dissociation (BDE, **Scheme 3**, path **b**).

The number of applications that have appeared so far (*31*) allow the assumption that path **b** (**Scheme 3**) should be the driving force in the case of catechol oxidation.

Pro-oxidant Activity of Oleuropein Determined in Vitro

J. Agric. Food Chem., Vol. 54, No. 20, 2006   **7447**

Scheme 5

When OLP is added, therefore, to the mixture which causes the formation of the species displayed in **Figure 1A**, it can be assumed that the oxidation of its catechol moiety (**Scheme 4**) competes with the oxidation of methyl radical and thus with the formation of the radical species **5** (**Scheme 1**).

Moreover, the hydrogen peroxide formed by oxidation of OLP could induce a concomitant Fenton oxidation process of DMSO which enhances the formation of methyl radicals, hence the concentration of species **4**.

For a better understanding of the role of the catechol moiety of OLP, and of its competing oxidation to *o*-quinone, likely in competition with the oxidation of methyl to methoxyl radical, the experiments were repeated in the presence of dimethyl-oleuropein (OLP-Met$_2$, **2**), obtained by methylation of the phenolic hydroxyl groups. Previous studies have reported on the specific role of the catecholic moiety (*32−34*) and on the inhibition of activity after methylation of the hydroxyl groups of the aromatic ring (*19*).

When the experiment of **Figure 1B** was repeated by replacing OLP with OLP-Met$_2$, the spectrum **1C** was obtained. The ESR signal is now again complex and extremely similar to that of **Figure 1A**, and it comes out from the summation of about the same percentage of ESR spectra of species **4** and **5**. The methylated species **2** does not, likely, interfere with the process which brings about the formation of the radical species **4** and **5** (**Scheme 1**).

The same results were obtained when the perferryl ions were replaced by copper Cu^{2+} species (*35*), i.e., ESR spectra similar to those in **Figure 1B** and **Figure 1C** were obtained in the presence of OLP or OLP-Met$_2$, respectively (data not shown).

The experiments carried out in the presence of methylated oleuropein (**2**) allows us to rule out, at least in this particular case, the production of alkyl and alkoxide radicals from the glucose moiety of oleuropein, as observed in similar experiments dealing with the oxidation of saccharides in an oxidizing xanthine oxidase/hypoxanthine system (*36*). The spectrum of **Figure 1C**, in fact, does correspond to the sum of the POBN derivatives **4** and **5** (**Scheme 1**), and no other POBN/radical adducts are shown.

The experiments discussed above have been repeated in typical Fenton aerobic conditions.

It has already been mentioned that (i) ascorbic acid is usually added to Fenton reactions to regenerate (*16*) Fe^{2+} from Fe^{3+} and (ii) other species such as β-carotenoids and α-tocopherols can behave similarly. It was, therefore, considered a possibility that oleuropein could behave similarly.

The occurrence of this possible reaction path has been checked by evaluating the effect of **1** in an aerobic Fenton system in the presence of excess H_2O_2, at ferrous iron concentrations identical (**Figure 2A**) to that used in the experiments reported in **Figure 1**.

The ESR spectrum (**Figure 2A**) thus obtained was similar to that reported in the literature (*25*) and to that obtained by us in the absence of hydrogen peroxide (**Figure 1A**), with the only difference being that the signal due to species **5**, POBN/•OCH$_3$,

is enhanced (80%) with respect to that of species **4**, POBN/•CH$_3$ (20%) (**Scheme 1**). It could be assumed that the oxidation power of the Fenton reaction is more effective than those caused by perferryl ions, when hydrogen peroxide is absent. In this case, in fact, it can be expected that a higher production of methoxyl radicals, a secondary oxidation product of the initially formed methyl radical, takes place.
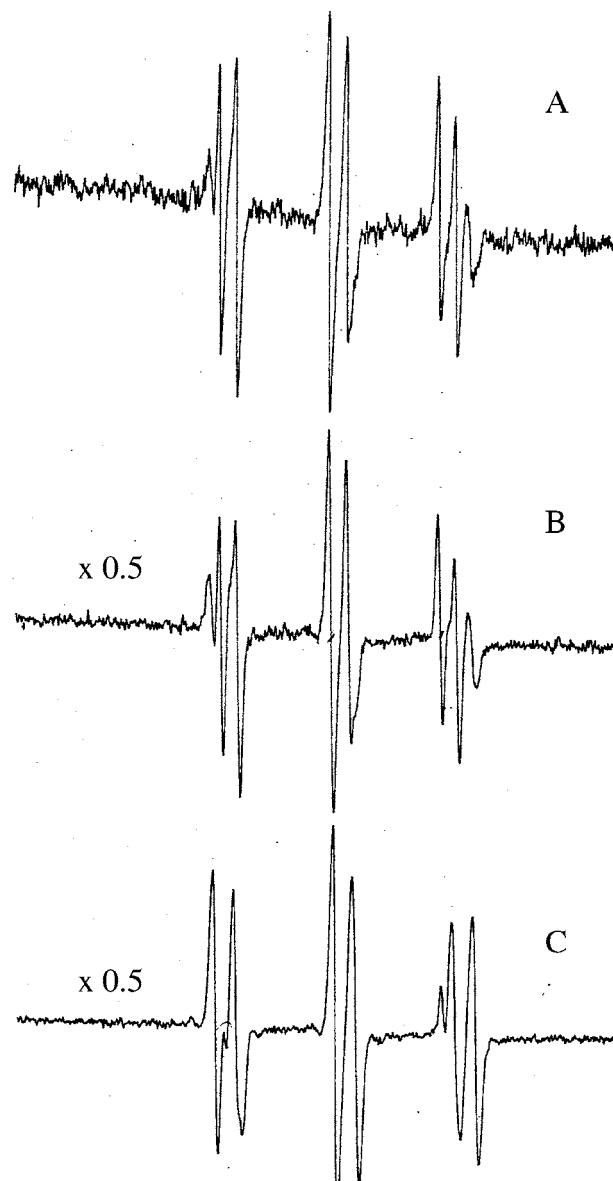
Figure 2. ESR spectra of the reaction mixtures incubated for 15 min at 37 °C: (**A**) Fe^{2+} (0.1 mM) + H_2O_2 (20 mM) + DMSO (2%) + POBN (25 mM) in PB (20 mM); (**B**) Fe^{2+} (0.08 mM) + H_2O_2 (20 mM) + DMSO (2%) + POBN (25 mM) + OLP (0.2 mM) in PB (20 mM); (**C**) Fe^{2+} (0.8 mM) + H_2O_2 (20 mM) + DMSO (2%) + POBN (25 mM) + OLP (0.2 mM) in PB (20 mM).

7448 *J. Agric. Food Chem.,* Vol. 54, No. 20, 2006

Mazziotti et al.

When compound **1** is present at a molar concentration more than double with respect to Fe^{2+} ions (**Figure 2B**), while the concentration of hydrogen peroxide is kept constant, the ESR signal shows the same spectral characteristics as that observed in the spectrum reported in **Figure 2A** but the signal intensity is increased by a factor of 2. This could be an indication of a more efficient oxidation system which could be due to the ability of oleuropein to recycle ferric ions according to **Scheme 5**.

The availability of a higher concentration of methoxyl radicals should be an indication that, in Fenton conditions, the kinetics of the pro-oxidation path taken by **1** does not compete with its oxygen scavenger activity exerted in the redox process induced by perferryl ions (**Figure 1B**).

In the last experiment (**Figure 2C**) the ferrous ion concentration was raised by 1 order of magnitude compared to the experiment in **Figure 2B**, keeping constant the concentration of the other analytes. The ESR spectrum thus obtained shows nearly the six typical resonance lines, only, of the methyl POBN adduct **4** (85%) (**Scheme 1**), whereas the POBN/•OCH$_3$ adduct is nearly absent (15%). It might be assumed that in a reducing environment, due to the enormous excess of ferrous species, the reaction path leading to the oxidation of the methyl radical is hardly to be taken.

The very complex matter of gathering unambiguous results on the role of a well-studied natural antioxidant such as oleuropein (**1**) has been tackled by exploiting the action of ferrous ions in aerobic conditions. The POBN spin-trapping system, used to evaluate by ESR spectroscopy the formation of unpaired electron spin species in the presence of DMSO, was widely applied in the identification of redox biochemical pathways.

In the experiments discussed above, the 2-fold activity of (i) dioxygen scavenger, in the presence of perferryl ions, and (ii) a pro-oxidant agent in Fenton conditions seems to be taken by oleuropein (**1**).

In the absence of hydrogen peroxide and then of hydroxyl radicals, at least at the burst of the reaction, OLP acts as a scavenger along the reaction path that leads to the oxidation of methyl to methoxyl radicals. In the Fenton-like approach a cooperative effect of OLP was observed, whereby the recycling of ferric as ferrous species represented a feedback of the oxidation processes, as observed with other natural molecules such as ascorbic acid (*16*), β-carotene, and α-tocopherol (*20–23*).

A clear line cannot be drawn on the effectiveness of the redox procedures described by the equations 1 and 2, along the oxidation pathways available for OLP. When hydrogen peroxide was absent, the reaction proceeded via the formation of superoxide/ferric ion couples (eq 2) that induce, among others, the formation of hydrogen peroxide/ferrous ions, the elements driving the Fenton reaction. Nevertheless, the dual role of oleuropein clearly emerges from the data previously discussed. There is, in fact, the need of an antioxidant in the perferryl driven process to block the formation of methoxyl radicals. Accordingly, the yield enhancement observed in the Fenton driven process when **1** is present can be, reasonably, attributed to behavior of OLP as reducing agent in recycling the excess of the formed ferric ions. Finally, the role of the catechol moiety was unambiguously ascertained when the methylated homologue OLP-Met$_2$ was used.

In conclusion, ESR experiments prove, in agreement with other published data, that oleuropein, a secoiridoid present in olive oil, leaves, and drupes, behaves as an antioxidant in vitro. Moreover, this important active principle, proposed as helpful in many therapies, acts, in vitro, as a pro-oxidant. This new property has a direct impact on the formulation of those drugs freely available in the market whose intake may cause unexpected consequences.

LITERATURE CITED

(1) Beauchamp, G. K.; Keast, R.S. J.; Morel, D.; Lin, J.; Pika, J.; Han, Q.; Lee, C. H.; Smith, I. I., A. B.; Breslin, P. A. S. *Nature* **2005**, *437*, 45–46.

(2) Montedoro, G.; Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A. *J. Agric. Food Chem.* **1993**, *41*, 2228–2234.

(3) De Nino, A.; Mazzotti, F.; Perri, E.; Procopio, A.; Raffaelli, A.; Sindona, G. *J. Mass Spectrom.* **2000**, *35*, 461–467.

(4) Ripamonti, L. *Corriere della Sera* **2005**, Sept 2.

(5) Aruoma, O. I. *Mutat. Res.* **2003**, *523*, 9–20.

(6) Sakihama, Y.; Cohen, M. F.; Grace, S. C.; Yamashi, H. *Toxicology* **2002**, *177*, 67–80.

(7) Zhu, B. T.; Conney, A. H. *Carcinogenesis* **1998**, *19*, 1–27.

(8) Samuni, A. M.; Chuang, E. Y.; Krishna, M. C.; Stein, W.; De Graff, W.; Russo, A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5390–5395.

(9) Cavalieri, E. L.; Stack, D. E.; Devanesan, P. D.; Todorovic, R.; Dwivedy, I.; Higginbotham, S.; Johansson, S. L.; Patil, K. D.; Gross, M.; L.; Gooden, J. K. R.; Ramanathan, R.; Cerny, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10937–10942.

(10) Halliwell, B.; Aeschbach, R.; Loliger, J.; Aruoma, O. I. *Food Chem. Toxicol.* **1995**, *33*, 601.

(11) Servili, M.; Montedoro, G. *Eur. J. Lipid Technol.* **2002**, *104*, 602–613.

(12) Servili, M.; Salvaggini, R.; Esposto, S.; Taticchi, A.; Montedoro, G.; Marozzi, G. *J. Chromatogr. A* **2004**, *1054*, 113–127.

(13) Roche, M.; Dufour, C.; Mora, N.; Dangles, O. *Org. Biomol. Chem.* **2005**, *3*, 423–430.

(14) Lee-Huang, S.; Zhang, L.; Huang, P. L.; Chang, Y. T.; Huang, P. L. *Biochem. Biophys. Res. Commun.* **2003**, *307*, 1029–1037.

(15) De Nino, A.; Di Donna, L.; Mazzotti, F.; Muzzalupo, I.; Perri, E.; Tagarelli, A. *Anal. Chem.* **2005**, *77*, 5961–5964.

(16) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 3rd ed.; Oxford University Press: Oxford, 1999.

(17) Lubec, G. *J. Invest. Med.* **1996**, *44*, 324–346.

(18) (a) Halliwell, B. Gutteridge, J. M. *Methods Enzymol.* **1990**, *186*, 1–85. (b) Koppenol, W. H. *Free Radical Biol. Med.* **1993**, *15*, 645–651.

(19) Fang, J. G.; Lu, M.; Chen, Z. H.; Zhu, H. H.; Li, Y.; Yang, L.; Wu, L. M.; Liu, Z. L. *Chem. Eur. J.* **2002**, *8*, 4191–4198.

(20) Briante, R.; Febbraio, F.; Nucci, R. *J. Agric. Food Chem.* **2003**, *51*, 6975–6981.

(21) Floyd, R. A. *FASEB J.* **1990**, *4*, 2587–2597.

(22) Podmore, I. D.; Griffiths, H. R.; Hebert, K. E.; Mistry, N.; Mistry, P.; Lunec, J. *Nature* **1998**, *392*, 559–559.

(23) Polyarov, N. E.; Leshina, T. V.; Konovalova, T. A.; Kispert, L. D. *Free Radical Biol. Med.* **2001**, *31*, 398–404.

(24) Cao, G.; Sofic, E.; Prior, R. L. *Free Radical Biol. Med.* **1997**, *22*, 749–760.

(25) Qian, S. Y.; Buettner, G. R. *Free Radical Biol. Med.* **1999**, *26*, 1447–1456.

(26) (a) Burkitt, M. J.; Mason, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8440–8444. (b) Guengerich, F. P.; Vaz, A. D. N.; Ranier, G. N.; Pernecky, S. J.; Coon, M. J. *Mol. Pharmacol.* **1997**, *51*, 147–151.

(27) Panizzi, L.; Scarpati, M.; Oriente, E. G. *Gazz. Chim. Ital.* **1960**, *90*, 1449–1485.

(28) (a) Avdeef, A.; Sofen, S. R.; Bregante, T. L.; Raymond, K. N. *J. Am. Chem. Soc.* **1978**, *100*, 5362–5370. (b) Moridiani, M. Y.; O' Brien, P. J. *Biochem. Pharmacol.* **2001**, *62*, 1579–1585.

(29) Veltwisch, D.; Janata, E.; Asmus, K. D. *J. Chem. Soc., Perkin Trans. 2* **1980**, 146–153.

(30) Mellican, R. E. I.; Li, J.; Mehansho, H.; Nielsen, S. S. *J. Agric. Food Chem.* **2003**, *51*, 2304–2316.

Pro-oxidant Activity of Oleuropein Determined in Vitro

J. Agric. Food Chem., Vol. 54, No. 20, 2006 **7449**

(31) Nenadis. N.; Wang, L. F.; Tsimidou, M. Z.; Zhang, H. Y. *J. Agric. Food Chem.* **2005**, *53*, 295−299.

(32) De la Puerta, R.; Ruiz Gutierrez, V.; Hould, J. R. *Biochem. Pharmacol.* **1999**, *57*, 445−449.

(33) Kohyama, N.; Nagata, T.; Fujimoto, S.; Sekiya, K. *Biosci., Biotechnol., Biochem.* **1993**, *61*, 347−350.

(34) Visioli, F.; Bellomo, G.; Galli, C. *Biochem. Biophys. Res. Commun.* **1998**, *247*, 60−64.

(35) Gunther, M. R.; Hanna, P. M.; Mason, R. P.; Cohen, M. S. *Arch. Biochem. Biophys.* **1995**, *316*, 515−522.

(36) Luo, G. M.; Qi, D. H.; Zheng, Y. G.; Mu, Y.; Yan, G. L.; Yang, T. S.; Shen, J. C. *FEBS Lett.* **2001**, *492*, 29−32.