Antioxidant and Other Biological Activities of Olive Mill Waste Waters

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During olive oil production, large volumes of water are generated and subsequently discarded. Olives contain a variety of bioactive components, and some of them, according to their partition coefficients, end up in the water phase. The current investigation aimed at comparing different methods for the extraction of biologically active components of the olive mill waste waters (OMWW) and evaluating the in vitro antioxidant and anti-inflammatory activities of the resulting extracts. The results indicate that OMWW extracts are able to inhibit human LDL oxidation (a process involved in the pathogenesis of atherosclerosis) and to scavenge superoxide anions and hypochlorous acid at concentrations as low as 20 ppm. Finally, two of the three extracts also inhibited the production of leukotrienes by human neutrophils. The potency of the extracts depended on their degree of refinement: extracts containing only low molecular weight phenols were the most effective.

Keywords: *Antioxidants; waste water; olive oil; free radicals; polyphenols*

INTRODUCTION

There is growing interest in novel sources of natural antioxidants, due to the recognized involvement of reactive oxygen species in the onset of several human diseases (Aruoma, 1998) and in the oxidative degradation of food, animal feed, and other goods such as cosmetics (Aruoma, 1997).

Olives and olive oil contain phenolic compounds (Tsimidou, 1992, 1998) that, in vitro, have been shown to exert potent biological activities including, but not limited to, antioxidant actions (Visioli and Galli, 1998a,b). It is noteworthy that, during olive oil production, a considerable amount of water is employed during the malaxation process, that is, the continuous washing of the olive paste with warm water prior to the procedure of separation of the oil from the paste (Boskou, 1996; Kiritsakis, 1991). This water, in addition to that endogenously contained in the olives, makes up to 50% of the total yield of the olive paste (olive oil amounts to ∼15%) and is named "waste water". In fact, due to the failure to develop a suitable end-of-pipe treatment technology, olive mill waste waters (OMWW) are currently discarded by olive oil manufacturers and represent a significant burden to the mills' economy. Previous work carried out in our laboratory has shown that OMWW contain a considerable amount of phenolic compounds endowed with potent antioxidant properties (Visioli et al., 1998). Thus, in view of the current need for upgrading byproducts at all stages of the olive oil industry (Demicheli and Bontoux, 1996), a project supported by the European Community was undertaken to investigate different procedures for the recovery of the active components of OMWW and to compare the antioxidant and other biological activities of the various extracts.

EXPERIMENTAL PROCEDURES

Preparation of OMWW Extracts. Olives were collected from different locations in Italy, Spain, and France and were frozen and shipped to the University of Florence. OMWW were obtained by employing a benchtop mill, and the resulting samples were fractionated by applying a liquid−solid extraction (LSE) and further processed in three different ways to obtain extracts with increasing degrees of purity that were analyzed by reverse-phase HPLC. EEC regulations prohibit the full description of the methodology, which is currently under patent application but a brief description follows.

The first extract (hereinafter referred to as extract 1) was obtained by a fractionation of lyophilized OMWW on an XAD 1180 resin column and by subsequent elutions with water and ethanol. The ethanolic fraction was then lyophilized and employed in biological tests. The second extract (hereinafter referred to as extract 2) was obtained by a liquid−liquid extraction (LLE) of the OMWW. Briefly, after a defatting with *n*-hexane, LLE extractions with ethyl acetate were performed, and the extracts were brought to dryness, resuspended in ethanol, and employed in biological tests. The ethyl acetate LLE is selective for low and medium molecular weight phenols; it does not extract heavier molecules that remain in the water phase. The third extract (hereinafter referred to as extract 3) was obtained following a fractionation of extract 2 on a Sephadex LH-20 column. It was then brought to dryness, analyzed, and employed in biological tests.

Analyses of the Extracts. HPLC/DAD analyses were performed with an HP 1090L liquid chromatograph equipped with an HP 1040A DAD detector (Hewlett-Packard, Palo Alto, CA). The column was a 6.6 × 250 mm LiChrosorb RP18, 5 μm (Merck), and its temperature was maintained at 26 °C. The mobile phase was H_2O (adjusted to pH 3.2 with H_3PO_4) and CH_3CN. A four-step linear solvent gradient was employed, starting from 100% H_2O to 100% CH_3CN over a 106 min period, at a flow rate of 1 mL min^{-1}, as previously described (Romani et al., 1996).

Quantitation of the Phenolic Components of OMWW Extracts. Quantitation of individual phenolic compounds was

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carried out by using four-point regression curves ($r^2 = 0.99$) obtained by employing authentic standards (Extrasynthese S.A., Lyon, France). Tyrosol and hydroxytyrosol and oleuropein and its derivatives were quantified at 280 nm. Elenolic acid was quantified at 254 nm by employing oleuropein as the reference compound. The appropriate corrections based on the individual molecular weights were applied to correctly quantify individual compounds.

Evaluation of OMWW Antioxidant Capacity. Human low-density lipoproteins (LDL, $d = 1.021-1.063$) were isolated by sequential ultracentrifugation from plasma obtained from healthy, normolipidemic volunteers. Before initiation of the experiments, LDL samples were desalted by size exclusion chromatography and their protein contents determined according to the method of Lowry et al. (1951). LDL samples were diluted with PBS to 200 μg/mL, and oxidation was started by the addition of either 5 μM copper sulfate or the free radical generator 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) at concentrations of 5 and 10 mM. Control samples were added with 5 μL of ethanol, which was the vehicle employed to dissolve OMWW extracts. Incubations were carried out at 37 °C in a shaking bath, and aliquots were withdrawn at different times for the analysis of various oxidation markers (Visioli and Galli, 1997). Lipid hydroperoxide and malondialdehyde formations were evaluated according to the procedure of Balla et al. (1991), and the results are expressed as nanomoles of either LOOH or TBARS per milligram of LDL.

Superoxide anion was generated by the xantine/xantine oxidase system, and its rate of formation was evaluated by spectrophotometry at 550−540 nm (Visioli et al., 1998) and calculated after the addition of superoxide dismutase.

Reactions with hypochlorous acid were evaluated by assessing the protective activity of OMWW extracts versus HOCl-induced inactivation of catalase, as described by Marshall et al. (1996) and Aruoma and Halliwell (1987).

The DPPH-scavenging properties of OMWW were evaluated as described in Visioli and Galli (1998b), and EC_{50} values were calculated by employing MacALLFIT software.

Evaluation of Leukotriene B_4 (LTB_4) Production by Human Neutrophils. Human neutrophils were isolated from blood according to the method of Sala et al. (1996). Samples of 5×10^6 cells were incubated at 37 °C in a shaking bath in the presence or absence to the indicated extracts. Cellular activation was triggered by the addition of 5 μM calcium ionophore A23187 (final concentration). After 10 min of incubation, the reaction was stopped by the addition of an equal volume of chilled methanol. Leukotrienes were separated by HPLC (Sala et al., 1996) and quantified by employing PGB_2 as internal standard.

RESULTS

Three final extracts were obtained from waste waters prepared as described above. Figure 1 reports the chromatographic profile of the three extracts. Extract 1 exhibits a complex chromatographic profile, due to the presence of polymers that are responsible for the high background seen at 254 nm, that is, the wavelength in which phenolic polymers yield their maximum absorption. Extract 2 mainly contains low and medium molecular weight phenols (elenolic acid is the principal constituent), and extract 3 is made of hydroxytyrosol, tyrosol, and a still unidentified derivative of the former only. A quantitation of the extracts is given in Table 1.

A solution of the stable free radical DPPH can be decolorized according to the scavenging potency of antioxidant molecules. Low amounts of OMWW extracts were able to remove DPPH from the ethanolic solution, with EC_{50} values of 9.42, 3.12, and 1.83 ppm for extracts 1, 2, and 3, respectively (Table 2).

The antioxidant activities of OMWW extracts were tested by employing both metal ions, that is, 5 μM copper sulfate and the metal-independent free radical generator AAPH. As shown in Figure 2, the copper sulfate-induced production of thiobarbituric acid-reacting substances (TBARS), mostly made of short-chain aldehydes such as malondialdehyde and 4-hydroxynonenal originating from the oxidative degradation of lipid hydroperoxides, was greatly reduced by the coincubation of LDL samples with OMWW extracts at the concentration of 20 ppm. After 6 h of incubation, extract 1 lost part of its antioxidant capacity, whereas the other two extracts were still able to inhibit lipid peroxidation.

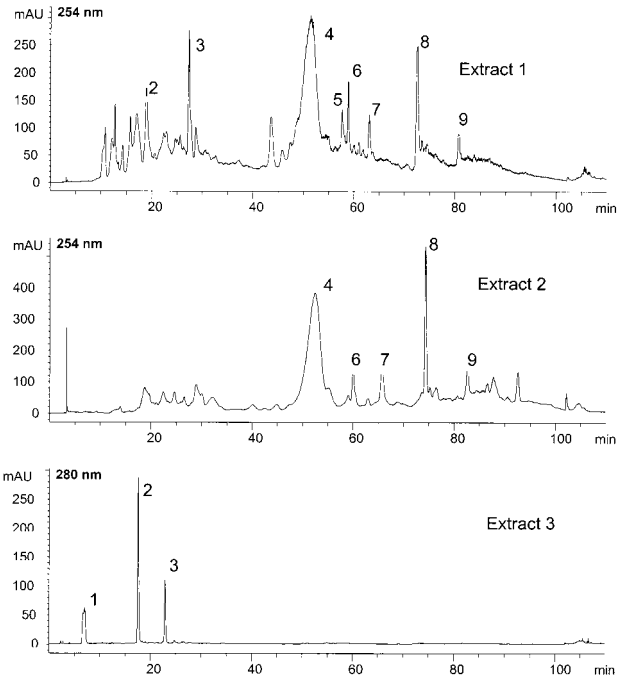

Figure 1. HPLC chromatograms of OMWW extracts. The extracts were obtained as described under Experimental Procedures, and some compounds were identified as follows: (1) hydroxytyrosol derivative; (2) hydroxytyrosol; (3) tyrosol; (4) elenolic acid; (5) oleuropein derivative; (6) luteolin 7-glucoside; (7 and 8) cinnamic acid derivatives; (9) quercetin.

Table 1. HPLC Evaluation of Major Phenolic Compounds Identified in OMWW Extracts (Grams per 100 g of Dry Matter)[a]

compound	extract 1	extract 2	extract 3
hydroxytyrosol	1.56	1.2	9.79
tyrosol	0.85	0.45	4.72
elenolic acid	4.3	11	2.62
oleuropein derivatives	0.5		
luteolin 7-glucoside	0.22	0.67	
quercetin	0.13	0.77	
cinnamic acid derivatives	0.55	1.47	
hydroxytyrosol derivatives			5.65
total polyphenols	8.11	15.56	22.78

[a] Molecular weights employed for calculations are as follows: hydroxytyrosol, 154; tyrosol, 138; elenolic acid, 242; oleuropein derivative, 378; luteolin 7-glucoside, 448; quercetin, 302; cinnamic acid derivatives, 180; hydroxytyrosol derivative, 154.

Table 2. DPPH Test[a]

extract	EC_{50} (ppm)
1	9.42
2	3.12
3	1.83

[a] The methodology employed for this test is described in Visioli and Galli (1998b) and EC_{50} values were calculated by employing MacALLFIT software.

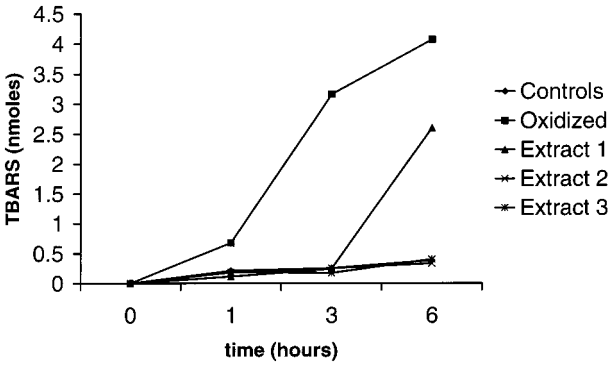

Figure 2. TBARS levels in LDL samples oxidized with 5 μM $CuSO_4$ and coincubated with 20 ppm of OMWW extracts. Control samples were added with the same amount of ethanol employed to dissolve OMWW extracts. Values are means of duplicate analyses that did not differ by more than 5%.

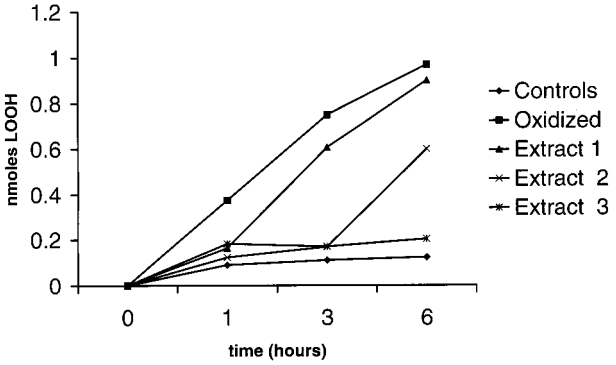

Figure 3. LOOH levels in LDL samples oxidized with 5 μM $CuSO_4$ and coincubated with 20 ppm of OMWW extracts. Control samples were added with the same amount of ethanol employed to dissolve OMWW extracts. Values are means of duplicate analyses that did not differ by more than 5%.

These results were confirmed by the evaluation of lipid peroxide (LOOH) production (Figure 3), which showed that OMWW extracts were able to inhibit LOOH production throughout the experiment.

When AAPH was employed to trigger oxidative stress, a slightly lower antioxidant effect of OMWW extracts was noted, depending on the concentration of AAPH employed. In particular, when AAPH was adopted at the concentration of 5 mM (Figures 4 and 5, upper panels); a significant degree of protection of LDL samples from oxidation was observed until termination of the experiment, whereas when oxidation was induced by 10 mM AAPH (Figures 4 and 5, lower panels), a similar degree of efficacy was noted.

The superoxide-scavenging properties of OMWW extracts were tested in a cell-free system and revealed that, at EC_{50}values <10 ppm, the extracts were able to remove $O_2^{\bullet -}$ from the reaction medium (Table 3), unlike vitamin E and BHT (not shown) that were found to be ineffective.

The antioxidant activities of OMWW extracts were also tested against HOCl, which could be considered as a source of reactive chlorine species. The absorption spectra of a catalase solution were evaluated after the addition of HOCl (Figure 6), which caused a degradation of the heme group of the enzyme and a modification of its absorption spectrum. Coincubation of the catalase solution with OMWW extracts afforded protection of the catalase heme group: extract 1 was less effective than extracts 2 and 3, which were found to be equipotent.

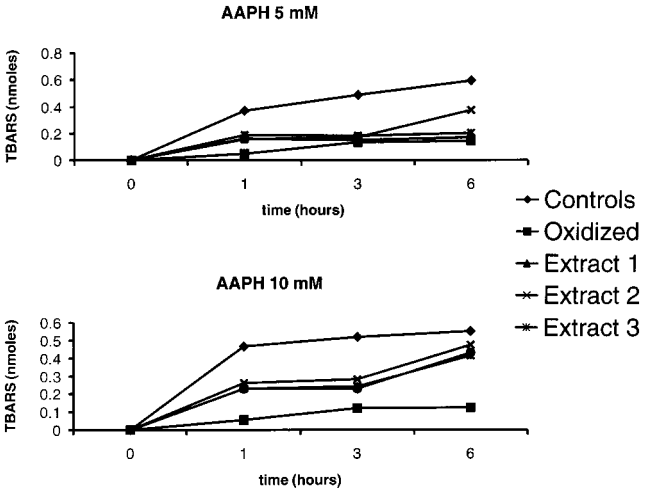

Figure 4. TBARS levels in LDL samples oxidized with 5 or 10 mM AAPH) and coincubated with 20 ppm of OMWW extracts. Control samples were added with the same amount of ethanol employed to dissolve OMWW extracts. Values are means of duplicate analyses that did not differ by more than 5%.

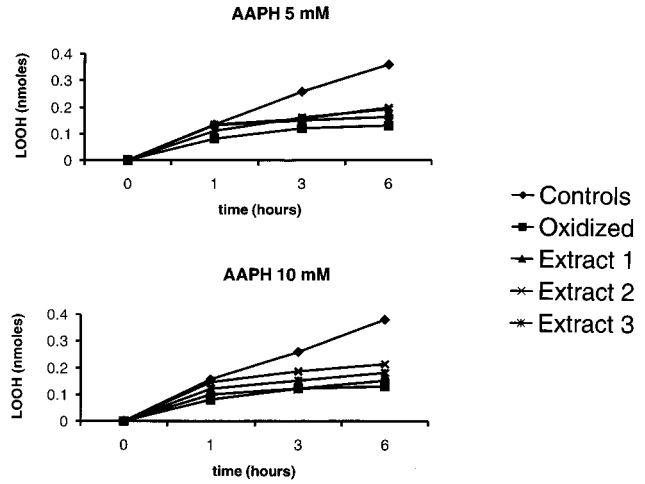

Figure 5. LOOH levels in LDL samples oxidized with 5 or 10 mM AAPH and coincubated with 20 ppm of OMWW extracts. Control samples were added with the same amount of ethanol employed to dissolve OMWW extracts. Values are means of duplicate analyses that did not differ by more than 5%.

Table 3. Scavenging Effects of OMWW Extracts on the Rate of Formation of Superoxide Anion[a]

compound	EC_{50} (ppm)
extract 1	8.46
extract 2	5.32
extract 3	2.87

[a] The rate of formation of superoxide anion (Δabsorbance/min) was calculated after the addition of an appropriate amount of superoxide dismutase, as described in Visioli et al. (1998).

LTB_4 production by activated human neutrophils (Figure 7) was potently inhibited by extracts 2 and 3, whereas extract 1 was found to be ineffective. The amounts of LTB_4 that were formed by neutrophils were calculated by comparison with the internal standard prostaglandin B_2, and the resulting EC_{50} values indicate that the inhibition of cellular activation by extracts 2 and 3 takes place at very low concentrations (Table 4).

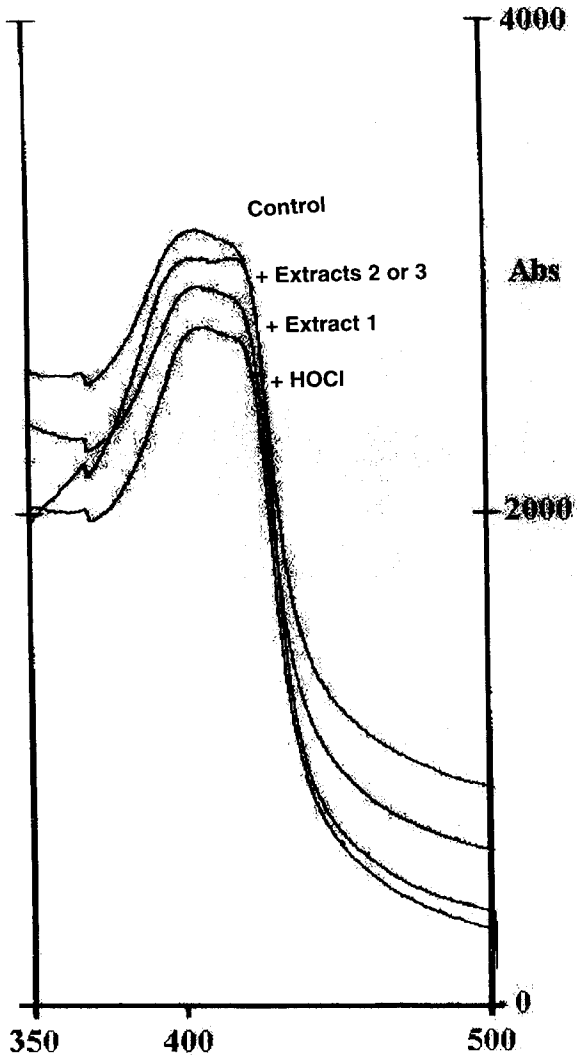

Figure 6. Representative absorption profile of a catalase solution incubated with 80 μM HOCl and with OMWW extracts as described under Experimental Procedures.

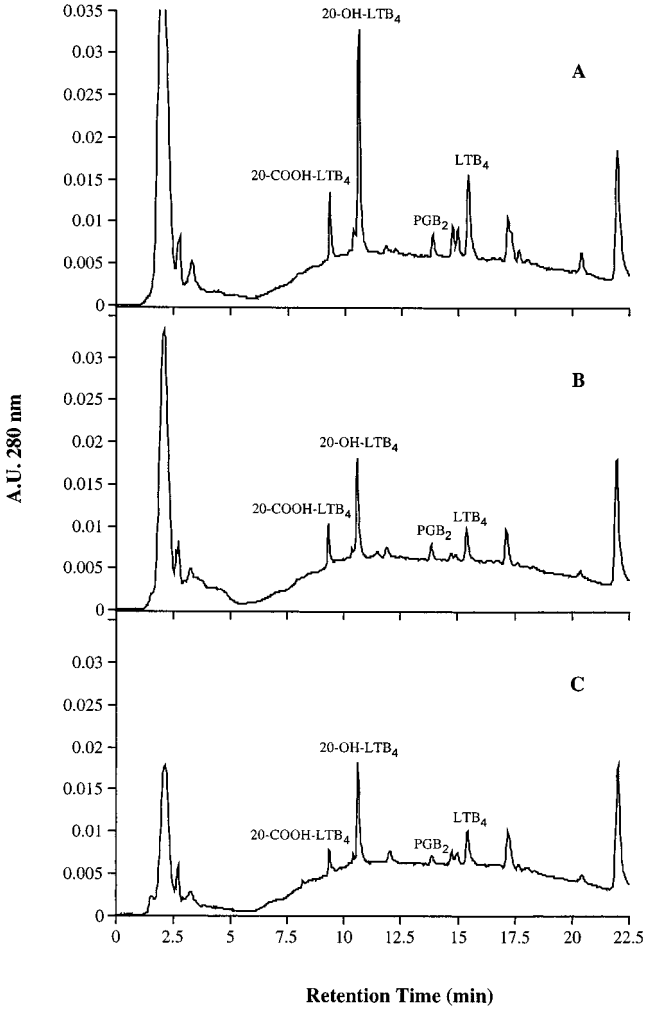

Figure 7. HPLC profiles showing the production of LTB_4 and its metabolites by human neutrophils challenged with the 5 μM calcium ionophore A23187 for 10 min and coincubated with 1 ppm of extract 2 (B) or 3 (C). Prostaglandin B_2 (PGB_2) was employed as internal standard to calculate the relative EC_{50} values (see Table 3).

DISCUSSION

The investigation reported in this paper was undertaken to compare the biological activities of OMWW extracts that were subjected to different extraction and purification methods. OMWW are the major byproduct of olive oil production and are generated in large amounts (~800000 tons/year in Italy alone) at olive mills that, at present, must discard them, thus increasing the costs of waste disposition and raising ecological issues due to the hypothesized soil contamination. Analysis of OMWW shows that their content in phenolic compounds fluctuates from 0.5 to 1.8% of phenolics (Table 5). Other components such as flavonoids, anthocyanins, and tannins are of potential biological interest due to their antioxidant activities (Vinson and Hontz, 1995; Vinson et al., 1995).

From a commercial point of view, the most widely employed antioxidants are those indigenous to foods, the water-soluble ascorbate (E = 300) and the lipid-soluble butylated hydroxytoluene (BHT; E = 321), butylated hydroxyanisole (BHA; E = 320), the esters of 3,4,5-trihydroxybenzoic acids (E = 310, 311, and 312), and vitamin E (mostly *dl*-α-tocopherols, E = 307). Plant extracts are also in use, and their share of the antioxidant market is expected to grow by ~15% by the year 2005 (Krishnakumar and Gordon, 1996). Hydroxytyrosol, the most active component of OMWW extracts, is of particular interest because it is amphiphilic and thus it acts at the oil–water interface and in systems where both oil and water phases are present, such as emulsions (Aruoma, 1997).

The data reported in this paper indicate the OMWW extracts contain potent antioxidants. Due to the different degrees of purity of the three extracts (extract 1 is

Table 4. Inhibition of Leukotriene B_4 and Related Metabolite Formation in Human Neutrophils by OMWW Extracts

compound	EC_{50} (ppm)
extract 1	ineffective
extract 2	1.26
extract 3	8.74

Table 5. Average Composition of OMWW

water (%)	83
minerals (carbonates, phosphates, potassium and sodium salts, etc.) (%)	~2
organic compounds (%)	~15
sugars	~2–8
proteins, pectins, macromolecules, etc.	~1.2–5
polyphenols	~0.5–1.8

the less refinee, whereas extract 3 is the most purified), a diverse degree of activity was noted. The first extract is, in fact, less potent in inhibiting LDL oxidation, whereas the other two extracts, containing only low molecular weight phenols such as hydroxytyrosol, prevented LDL oxidation when it was induced by either metal ions or a metal-independent free radical generator. The slightly lower efficacy of OMWW extracts noted when AAPH was employed as oxidant suggests that the extracts act mostly as ion chelators but are also able to scavenge preformed radicals, as also indicated by the DPPH test, in which the extracts removed the stable free radical at low concentrations.

The ability of OMWW extracts to scavenge superoxide, already reported for hydroxytyrosol and oleuropein (Visioli et al., 1998), is suggestive of a potential use of OMWW extracts in environments in which Fenton and Haber−Weiss reactions take place and in which the concomitant production of superoxide and nitric oxide would yield the powerful oxidant peroxinitrite. It is noteworthy that the established antioxidants vitamin E and BHT do not scavenge superoxide, and thus OMWW extracts may add stability to products exposed to high $O_2^{\bullet -}$ levels.

The protection of hypochlorous acid-induced damage of catalase (Figure 6) is of biological significance due to the well-known protein-damaging activity of HOCl, which is produced, in biological systems, at the site of inflammation by activated neutrophils through the enzyme myeloperoxidase (Aruoma and Halliwell, 1987). Also, because foods often come into contact with chlorine-based bleaches, employed as disinfectants in food plants, the use of HOCl scavengers may provide additional protection against reactive chlorine species.

Finally, the potent inhibition of calcium ionophore-stimulated production of LTB_4 and its metabolites by human neutrophils suggests that OMWW extracts exert biological effects beyond their antioxidant capacities. The activity of several enzymes, including those involved in the production of eicosanoids, for example, phospholipases and oxygenases, is modulated by the intracellular peroxide tone. Thus, by scavenging reactive oxygen species, OMWW extracts could lower the activity of such enzymes and, in turn, decrease the production of pro-inflammatory factors. Additional studies are needed to verify if such anti-inflammatory effects could also take place in vivo and the exact enzymatic target of the bioactive compounds.

In conclusion, these data show that OMWW are rich in antioxidant compounds that could be recovered from the matrix and employed both in preservative chemistry and, following appropriate trials to evaluate their safety and efficacy, as prophylactic agents in the prevention of certain radical-induced human diseases.

ABBREVIATIONS USED

OMWW, olive mill waste waters; AAPH, 2,2′-azobis-(2-amidinopropane) dihydrochloride; LTB_4, leukotriene B_4; PGB_2, prostaglandin B_2; LOOH, lipid hydroperoxides; TBARS, thiobarbituric acid-reacting substances; DPPH, 1,1-diphenyl-2-picrylhydrazyl hydrate.

LITERATURE CITED

Aruoma, O. I. Extracts as antioxidant prophylactic agents. *Int. News Fats, Oils Relat. Mater.* **1997**, *8*, 1236−1242.

Aruoma, O. I. Free radicals, oxidative stress, and antioxidants in human health and disease. *J. Am. Oil Chem. Soc.* **1998**, *75*, 199−212.

Aruoma, O. I.; Halliwell, B. Action of hypochlorous acid on the antioxidant protective enzymes superoxide dismutase, catalase and glutathione peroxidase. *Biochem. J.* **1987**, *248*, 973−976.

Balla, G.; Jacob, H. S.; Eaton, J. W.; Belcher, J. D.; Vercelloti, G. M. Hemin a possible physiological mediator of low-density lipoprotein oxidation and endothelial injury. *Arterioscl. Thrombosis* **1991**, *11*, 1700−1711.

Boskou, D. *Olive Oil Chemistry and Technology*; AOCS Press: Champaign, IL, 1996.

Demicheli, M.; Bontoux, L. *Survey on Current Activity on the Valorization of Byproducts from the Olive Oil Industry*; Report EUR 16466; 1996.

Kiritsakis, A. *Olive Oil*; American Oil Chemists' Society: Champaign, IL, 1991.

Krishnakumar, V.; Gordon, I. Antioxidants. Trends and developments. *Int. Food Ingred.* **1996**, *5*, 41−44.

Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Marshall, K. A.; Reiter, R. J.; Poeggeler, B.; Aruoma, O. I.; Halliwell, B. Evaluation of the antioxidant activity of melatonin in vitro. *Free Radical Biol. Med.* **1996**, *21*, 397−315.

Romani, A.; Baldi, A.; Mulinacci, N.; Vincieri, F. F.; Tattini, M. Extraction and identification procedures of polyphenolic compounds and carbohydrates in Phillyrea (*Phillyrea angustifolia* L.) leaves. *Chromatographia* **1996**, *42*, 571.

Sala, A.; Bolla, M.; Zarini, S.; Muller-Peddinghaus, R.; Folco, G. Release of LTA_4 versus LTB_4 from human polymorphonuclear leukocytes. *J. Biol. Chem.* **1996**, *271*, 17944−17948.

Tsimidou, M. Polyphenols and quality of virgin olive oil in retrospect. *Ital. J. Food Sci.* **1998**, *10*, 99−116.

Tsimidou, M.; Papadopoulos, G.; Boskou, D. Phenolic compounds and stability of virgin olive oil−Part 1. *Food Chem.* **1992**, *45*, 141−144.

Vinson, J. A.; Hontz, B. A. Phenol antioxidant index: comparative antioxidant effectiveness of red and white wines. *J. Agric. Food Chem.* **1995**, *43*, 401−403.

Vinson, J. A.; Dabbagh, Y. A.; Serry, M. M.; Jang, J. Plant flavonoids, especially tea flavonols, are powerful antioxidants using an *in vitro* oxidation model for heart disease. *J. Agric. Food Chem.* **1995**, *43*, 2800−2802.

Visioli, F. Galli, C. Evaluating oxidation processes in relation to cardiovascular disease: a current review of oxidant/antioxidant methodology. *Nutr. Metab. Cardiovasc. Dis.* **1997**, *7*, 459−466.

Visioli, F.; Galli, C. Olive oil phenols and their potential effects on human health. *J. Agric. Food Chem.* **1998a**, *46*, 4292−4296.

Visioli F.; Galli, C. The effect of minor constituents of olive oil on cardiovascular disease: new findings. *Nutr. Rev.* **1998b**, *56*, 142−147.

Visioli, F.; Bellomo, G.; Galli, C. Free radical-scavenging of olive oil phenols. *Biochem. Biophys. Res. Commun.* **1998**, *247*, 60−64.

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