# Decreased Superoxide Anion Production in Cultured Human Promonocyte Cells (THP-1) Due to Polyphenol Mixtures from Olive Oil Processing Wastewaters

Claude L. Léger,* Najib Kadiri-Hassani, and Bernard Descomps

Laboratoire Nutrition Humaine et Athérogénèse, UPRES-EA 2033, Faculté de Médecine, Université Montpellier I, F-34060, Montpellier, France

The purpose of this study was to examine whether human monocytic line THP-1 after differentiation into adherent macrophages, taken as a model of human macrophages implicated in atheroma, is able to produce lower quantities of $O_2^{\bullet-}$ either in the presence of polyphenol-rich olive oil wastewater (OWW) fractions or after OWW preincubation and withdrawal from the medium. In these respective conditions, the purpose was to examine the scavenging activity and the cell action of OWW toward $O_2^{\bullet-}$ production. It was clearly seen that OWW fractions lowered the $O_2^{\bullet-}$ production in both conditions, leading to the conclusion that they were able to scavenge $O_2^{\bullet-}$ and to depress $O_2^{\bullet-}$ production in the cell. Given the role of $O_2^{\bullet-}$ in LDL oxidation and oxidized LDL in atheroma, these results support an antiatherogenic role of OWW and its potential utilization as a food complement.

**Keywords:** *Olive oil wastewaters; polyphenolic compounds; scavenging activity; cell action; antioxidant properties*

## INTRODUCTION

Antioxidants have been reported to play an important role in the prevention of cardiovascular diseases (Rimm et al., 1993; Hodis et al., 1995). This has been first ascribed to LDL protection against oxidation (Parthasarathy et al., 1992). Polyphenols and phenolic acids (or their esterified forms) in olive oil and, in higher amounts, in olive oil wastewaters (OWW) are already known for their oxidation protecting properties toward LDL (Visioli et al., 1995). Additionally, the free radical scavenging properties of olive oil polyphenols—hydroxytyrosol and oleuropein (Visioli et al., 1998a)—and olive mill wastewaters (Visioli et al., 1999) have recently been shown. Interestingly, specific scavenging properties have been found toward superoxide anion and hypochlorous acid, which are both involved in oxidation-dependent atherogenic processes (Fang et al., 1998; Heinecke, 1997). Phenolic antioxidants have also been shown to have several cell-mediated and gene transcription-regulated actions that could have involved—or resulted in—something more than their antioxidant/scavenging properties (Choi et al., 1993) [for a review see also Sen et al. (1996)]. For example, they have been shown to reduce lipoxygenase activities and LTB4 production in human PMN (Kohyama et al., 1997) and TXB2 in human platelets (Petroni et al., 1995). Oleuropein, which contains a hydroxytyrosol residue, has been found to enhance $^{\bullet}NO$ production in murine macrophages (Visioli et al., 1998b).

The decreased production of $O_2^{\bullet-}$ is of particular relevance because of the well-known effect of this oxygen radical species in the oxidative modification of LDL. The pathological relevance of $O_2^{\bullet-}$ in atherosclerosis has recently been underscored in experiments using Cu/Zn SOD-overexpressing endothelial cells. Co-incubation of these cells with LDL led to a noticeable decrease in indicators of LDL oxidation compared with nontransfected cells (Fang et al., 1998). In fact, the decreased production of $O_2^{\bullet-}$ can be achieved either by scavenging processes as reported above or by a cell regulation of its production. It has been recently found by our group that vitamin E exhibits this antioxidant cell-mediated role in human adherent monocytes (Cachia et al., 1998a,b).

The purpose of this study was to examine both the scavenging activity and the cell action of the OWW polypohenol mixture toward $O_2^{\bullet-}$ production. A transformed monocytic leukemic cell, the THP-1 human monocyte, was used after differentiation into an adherent macrophage as a model of a human macrophage for which the implication in atheroma has been well established. The assessment of $O_2^{\bullet-}$ production was first carried out in the presence of OWW polyphenol mixtures. Second, the assessment was carried out after the polyphenols were withdrawn from the cultured cells, which had been preincubated in the presence of them.

## EXPERIMENTAL PROCEDURES

**Cells and Chemicals.** The THP-1 cell line was a gift from Dr. J. Dornan (University of Montpellier). Retinoic acid, IFN$\gamma$, PMA, lucigenin (10,10′-dimethyl-9,9′-bisacridinium dinitrate), xanthine, and xanthine oxidase grade IV were purchased from Sigma Chemical Co. (St Quentin Fallavier, France). Nutritive media and fetal calf serum were from Gibco Chemical (Glasgow, Scotland). 1$\alpha$,25-Dihydroxycholecalciferol was a gift from F. Hoffmann-La Roche SA (Basel, Switzerland). Other current chemicals were from Merck.

**OWW Fractions.** The fractions to be tested were prepared by the Dipartemento di Scienze Farmaceutiche, Universita degli Studi di Firenze. OWW were collected from three

* Address correspondence to this author at the Laboratoire de Biochimie A, Institut de Biologie, Université Montpellier I, F-34060, France (e-mail leger@sc.univ-montp1.fr; fax +33 4 67660871).

10.1021/jf991349c CCC: $19.00 
Published on Web 09/13/2000

cultivars (Picual, Frantoio, and Salonenque) in three olive mills in Spain, Italy, and France, respectively, and a mixture of the three lyophilized OWW was processed in three different ways, as follows:

*Fraction A.* OWW fractionation on an XAD 1180 resin column was carried out by subsequent elutions with water and ethanol. The ethanolic fraction was evaporated, and the residue was lyophilized. Qualitative analysis was performed by HPLC coupled with a diode array detector (HPLC-DAD) and HPLC coupled with a mass spectrometer (HPLC-MS) as described by Romani et al. (1996) and Baldi et al. (1995). Its quantitative composition in low molecular weight compounds was determined by reverse phase HPLC-DAD (Romani et al., 1999). Tyrosol and hydroxytyrosol were calculated at 280 nm using tyrosol as reference. Oleuropein and oleuropein derivatives were evaluated at 280 nm using oleuropein as standard. Pure standards were used for quantitative evaluation of other components at 280 nm. All standards were from Extrasynthese SA (Lyon, France). The dried fraction contained 12.3% of phenolic components. Results were as follows: 1.56% hydroxytyrosol, 0.85% tyrosol, 4.3% elenolic acid, 0.5% oleuropein derivatives, 0.22% luteolin 7-*O*-glucoside, and 0.13% quercetin.

*Fraction B.* OWW was first defatted with *n*-hexane and ethyl acetate, successively, by liquid−liquid extraction, and the ethyl acetate fraction was evaporated to dryness. The residue was then redissolved in ethanol. This ethanolic fraction contained polyphenols with low molecular weight. The total concentration was 47 mmol/L expressed as tyrosol, and the following composition was found: 6.2% hydroxytyrosol, 2.68% tyrosol, 3.62% elenolic acid, 1.17% luteolin 7-*O*-glucoside, and 2.0% quercetin.

*Fraction C.* The ethanolic aforementioned fraction (fraction B) was fractionated and purified using a Sephadex LH20 column and reverse phase HPLC. The ethanolic fraction obtained contained only tyrosol and hydroxytyrosol in the same ratio (HT/T = 2.3) as in fraction B. It was then evaporated to dryness.

*Fraction D.* An ethanolic solution (80:20, ethanol/water, v/v) was carried out from the ethanolic fraction resulting from the process of fraction C. Therefore, fraction D is another form of fraction C. The total concentration (tyrosol plus hydroxytyrosol) was estimated as 24.9 mM expressed as tyrosol.

**Superoxide Anion Production and Measurement.** The THP-1 cell line was used after a 72 h differentiation time period, which led to major cell alterations (such as the adhesion capacity and the capacity at producing $O_2^{\bullet-}$). Differentiation was performed in the presence of $10^{-6}$ M retinoic acid, $10^{-7}$ M 1α,25-dihydroxycholecalciferol, and 100 units/mL IFNγ (Brillant et al., 1999). Once differentiated, cells were submitted to two protocols.

*First Protocol.* Cells were placed at the density of $10^6$ cells/mL in the RPMI medium and incubated in the presence of the indicated increasing concentrations of any of the four fractions for 0, 4, and 24 h. PMA was then added. Lucigenin-enhanced $O_2^{\bullet-}$ bioluminescence was assessed by means of an LKB Wallac 1251 luminometer (Wallac Co.) at 37 °C. The luminescence signal was counted for 10 s every 7 min until 90 min after PMA addition to obtain complete information on the profile of the luminescence production rate. The viability of cells (>95%) was determined by trypan blue exclusion. Results were reported as the total amount of the bioluminescence produced during the first hour of incubation in the presence of PMA.

*Second Protocol.* Cells were preincubated in the presence of the fractions to be tested at indicated concentrations for 24 h; they were then counted and centrifuged, and cell pellets were recovered in the RPMI medium at the density of $10^6$ cells/mL. PMA was then added as previously mentioned. Measures were carried out and results were expressed as explained above.

The 100% value in the histograms corresponded to the control conditions, that is, the $O_2^{\bullet-}$ bioluminescence production in the presence of PMA alone (without adding any polyphenol fraction). The control medium contained the solvent vehicle (alcohol) in quantities that were equivalent to those added with the polyphenol fraction to be tested. It has previously been verified that superoxide dismutase was able to completely depress the luminescence signal in order to establish that $O_2^{\bullet-}$ was responsible for the recorded luminescence. The concentrations reported on the horizontal axis were expressed as tyrosol molar equivalents. Conditions for lucigenin bioluminescence measurement were those previously reported (Cachia et al., 1998; Kadri-Hassani et al., 1995).

The xanthine/xanthine oxidase $O_2^{\bullet-}$-producing, cell-free system was also used (Cachia et al., 1998). Briefly, xanthine (final concentration = 0.25 μM) was prepared in the buffer consisting of 0.1 M glycin, 1 mM EDTA, and 1 mM sodium salicylate adjusted at pH 9.0. Xanthine oxidase (final activity = 0.25 unit/L) was added in the same buffer with 0.1 mM lucigenin.

## RESULTS

Figure 1 shows the typical time course of the production of $O_2^{\bullet-}$ lucigenin bioluminescence by PMA-stimulated THP-1 in the presence of OWW fraction A. The production appears to be linear throughout the co-incubation period at all concentrations of fraction used.

Figure 2 reports the total bioluminescence produced during the 60 min co-incubation time period in the presence of increasing concentrations of each fraction without preincubation. It clearly shows a direct dose−response relationship between the fraction concentration and the decrease in $O_2^{\bullet-}$ lucigenin bioluminescence. The more pronounced inhibitory effect was obtained with 50 μM tyrosol equivalent (60−70% inhibition) without significant differences between fractions. However, intermediate concentrations of fraction D had an inhibition potency significantly higher than that of fraction A. Figure 3 shows the decreased production of total bioluminescence in the presence of growing concentrations of fractions after 4 h (Figure 3A) and 24 h (Figure 3B) preincubation times. No different inhibition potency took place between fractions after 4 h of incubation, whereas intermediate concentrations (10 and 25 μM) of fractions A and B were significantly different with regard to inhibitory activity after 24 h of incubation (given that fractions B−D appeared to have similar effects, only fraction B was tested after 24 h of incubation). For comparison purposes, results in the absence of preincubation time and with 4 and 24 h preincubation times were reported together for each four fractions in Figure 4. Generally speaking, the 4 h preincubation time resulted in an inhibition potency significantly higher than without preincubation. Surprisingly, inhibition was similar without preincubation and after 24 h preincubation for fractions A and B.

The effect of each fraction was examined when the $O_2^{\bullet-}$ cell generation was replaced by an $O_2^{\bullet-}$ cell-free generation system, the xanthine/xanthine oxidase system (Figure 5). Respective concentrations of substrate and enzyme were carefully chosen to bring about a similar $O_2^{\bullet-}$ production rate compared to the THP-1 generation system. A similar inhibition potency was observed for all four fractions. Generally speaking, the data of Figures 2 and 5 indicate that the dose−response relationships obtained with each of the two $O_2^{\bullet-}$ generation methods were similar, although the inhibitory effect for each concentration was more marked with the cell-free system.

Figure 6 shows a typical $O_2^{\bullet-}$ production time course of PMA-stimulated THP-1 cells after 24 h of preincubation with, and then after withdrawal of, fraction A. It was done to assess the effect of the cell preincubation

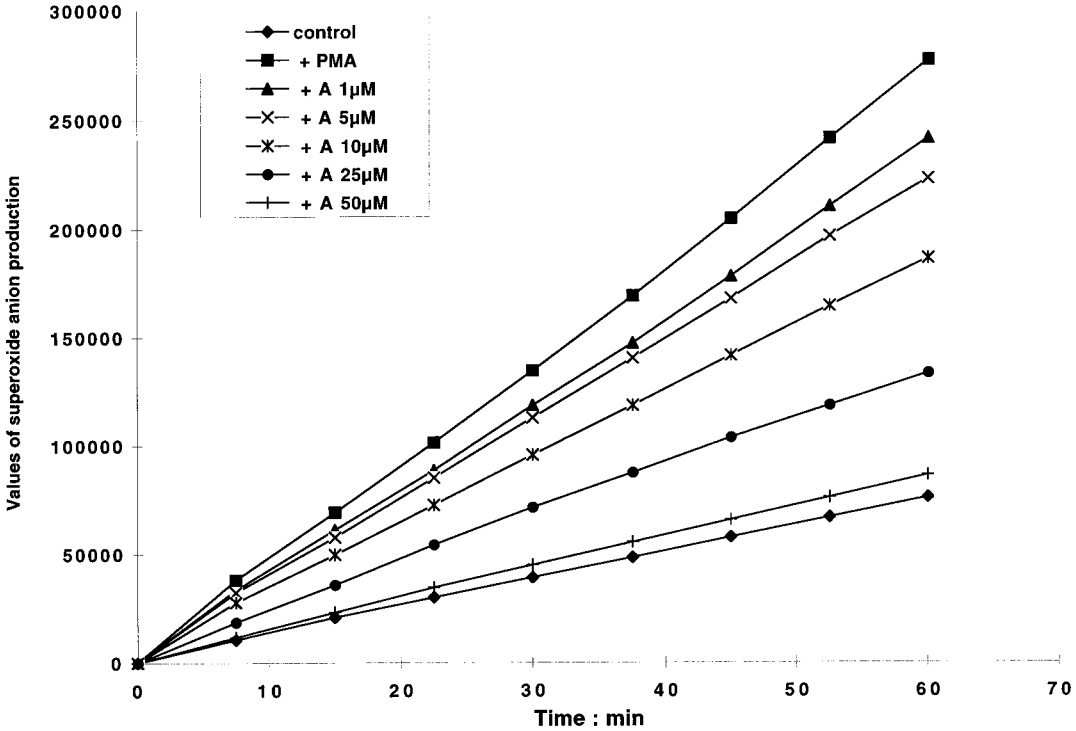


**Figure 1.** Typical time course of the production of $O_2^{\bullet-}$ lucigenin bioluminescence by 0.1 $\mu$M PMA-stimulated THP-1 in the presence of increasing concentrations of fraction A expressed as tyrosol molar equivalents.

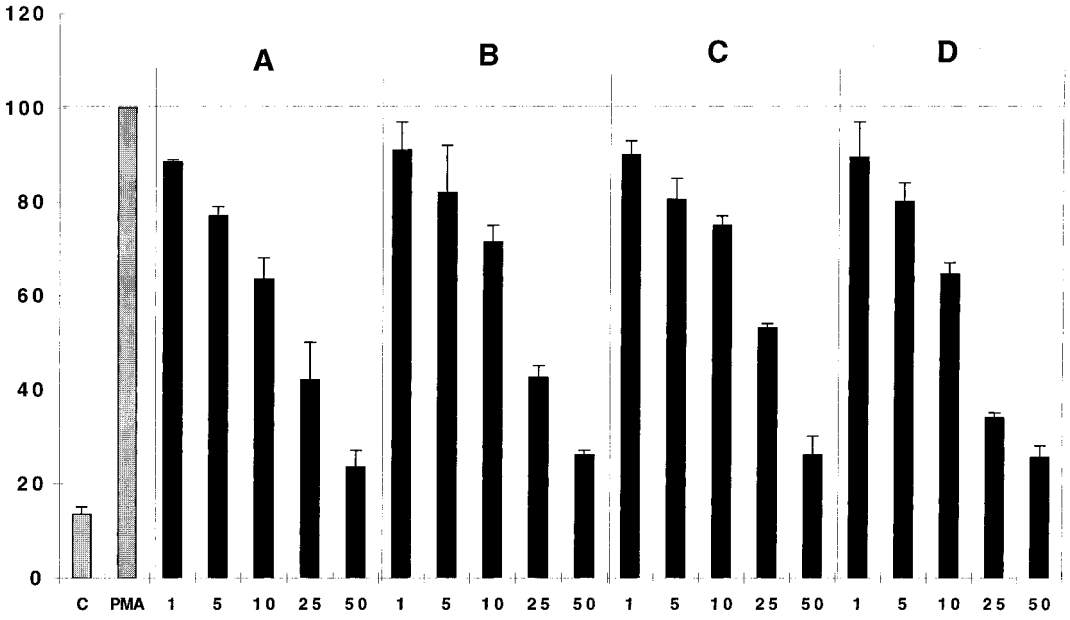


**Figure 2.** Production of $O_2^{\bullet-}$ lucigenin bioluminescence by 0.1 $\mu$M PMA-stimulated THP-1 ($10^6$ cells/mL) in the presence of increasing concentrations of fraction A, B, C, or D (expressed as tyrosol molar equivalents) in conditions when there was no OWW preincubation time; that is, PMA was added immediately after the addition of the OWW fraction. The 100% value (with PMA and in the absence of polyphenols) corresponded to a bioluminescence of 275000 arbitrary units.

due to types of action other than those depending only on the scavenging effect of polyphenols, which we designate the cell-mediated effect. To evaluate this cell effect, and because fractions A and B were previously tested after 24 h of preincubation but in the presence of fractions (see Figure 4A,B), we chose to assess $O_2^{\bullet-}$ production after 24 h of preincubation and withdrawal of the same fractions. Figure 7 clearly shows that cells responded in a dose-dependent manner to the increasing concentrations of OWW fractions and regardless of the type of fraction. It can be concluded that both fractions were able to depress $O_2^{\bullet-}$ cell production in a very similar manner (fraction C was omitted because of very few available amounts, whereas fraction D, not shown here, exhibited the same action as fractions A and B).

## DISCUSSION

Cell response to PMA was studied to assess the scavenging and cell properties of OWW fractions, so THP-1 differentiation had to proceed through signal pathways that do not use the usual exposure to PMA. It is indeed well-known that PMA differentiation causes

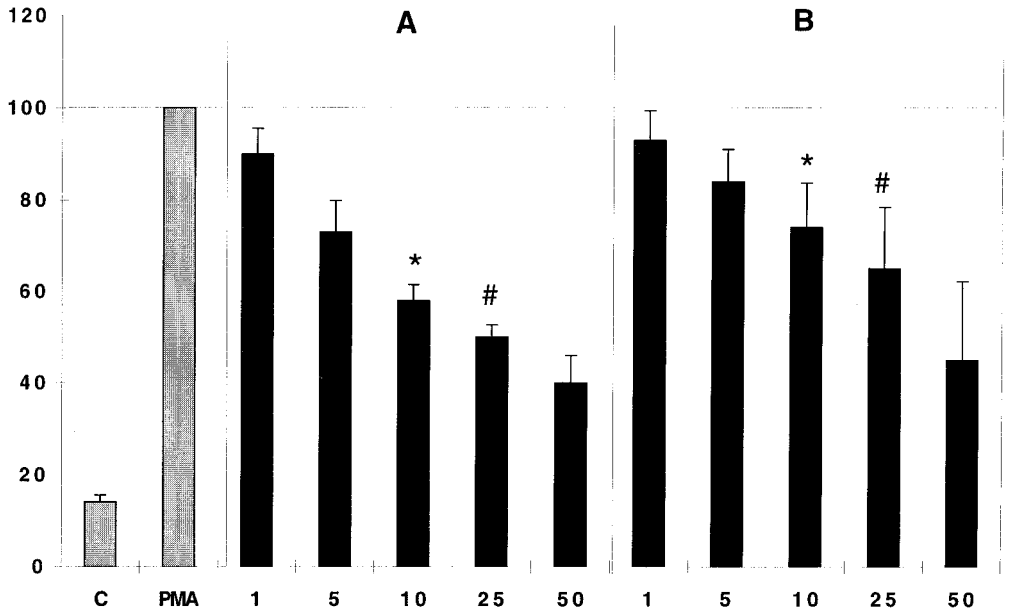


**Figure 3.** Production of $O_2^{\bullet-}$ lucigenin bioluminescence by 0.1 $\mu$M PMA-stimulated THP-1 in the same conditions as in Figure 2, but PMA was added 4 (A) or 24 h (B) after the addition of the OWW fraction. Bars with the same symbol (*, #) are significantly ($p \leq 0.05$) different.

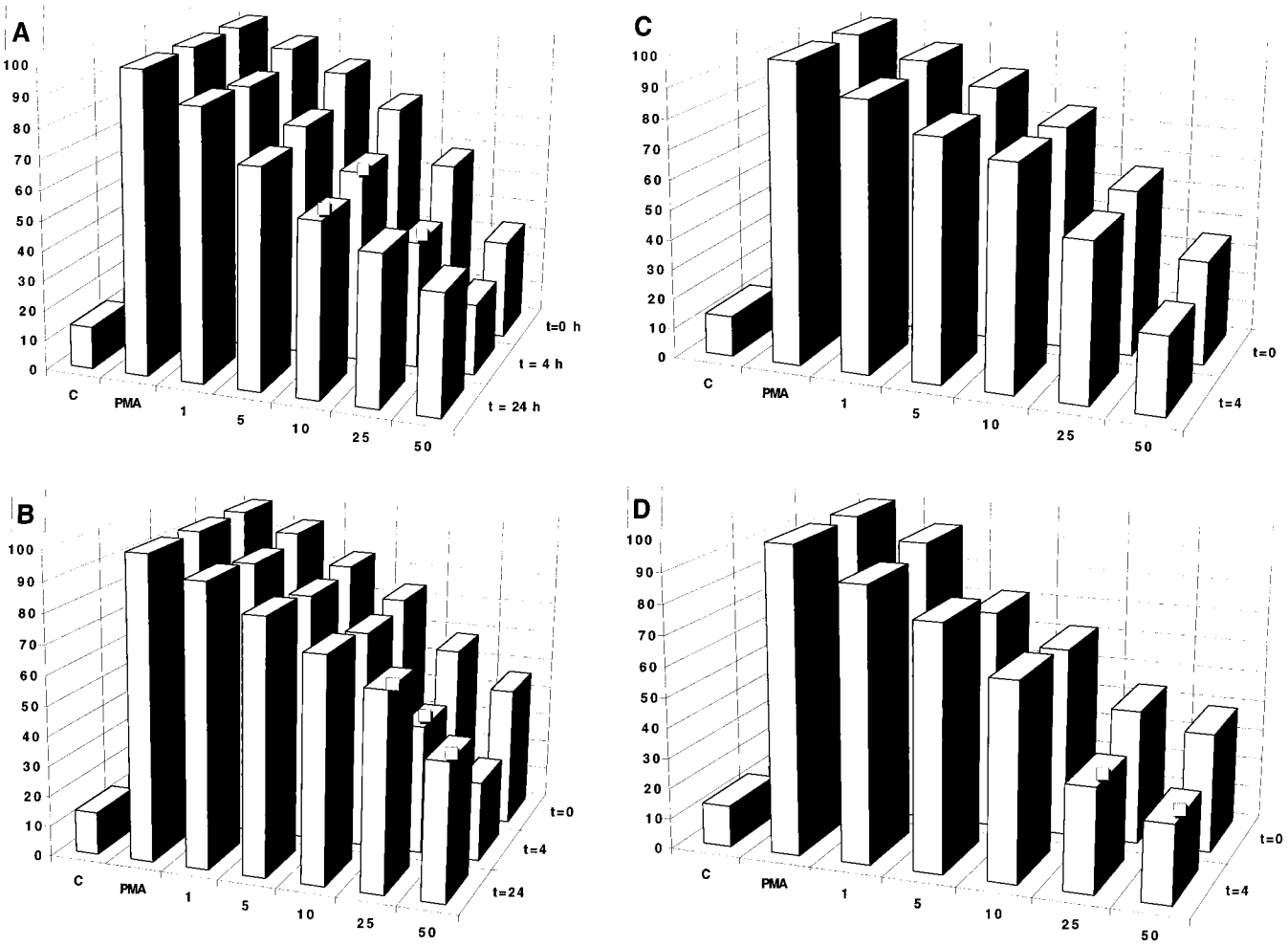


**Figure 4.** Comparison of the effects of each four fractions (A, B, C, and D) for each of three preincubation times (0, 4, and 24 h). Results were separately shown in the Figures 2 and 3. □ specifies that a significant difference ($p \leq 0.05$) exists with the condition where there was no preincubation period ($t = 0$) before luminescence measurement.

a translocation of protein kinase C (Schwende et al., 1996), which possibly leads to down-regulation of the enzyme (Johnson et al., 1995; Watson et al., 1994) and would impair the PKC-dependent $O_2^{\bullet-}$ production (Cachia et al., 1998a).

Cell differentiation was achieved according to a

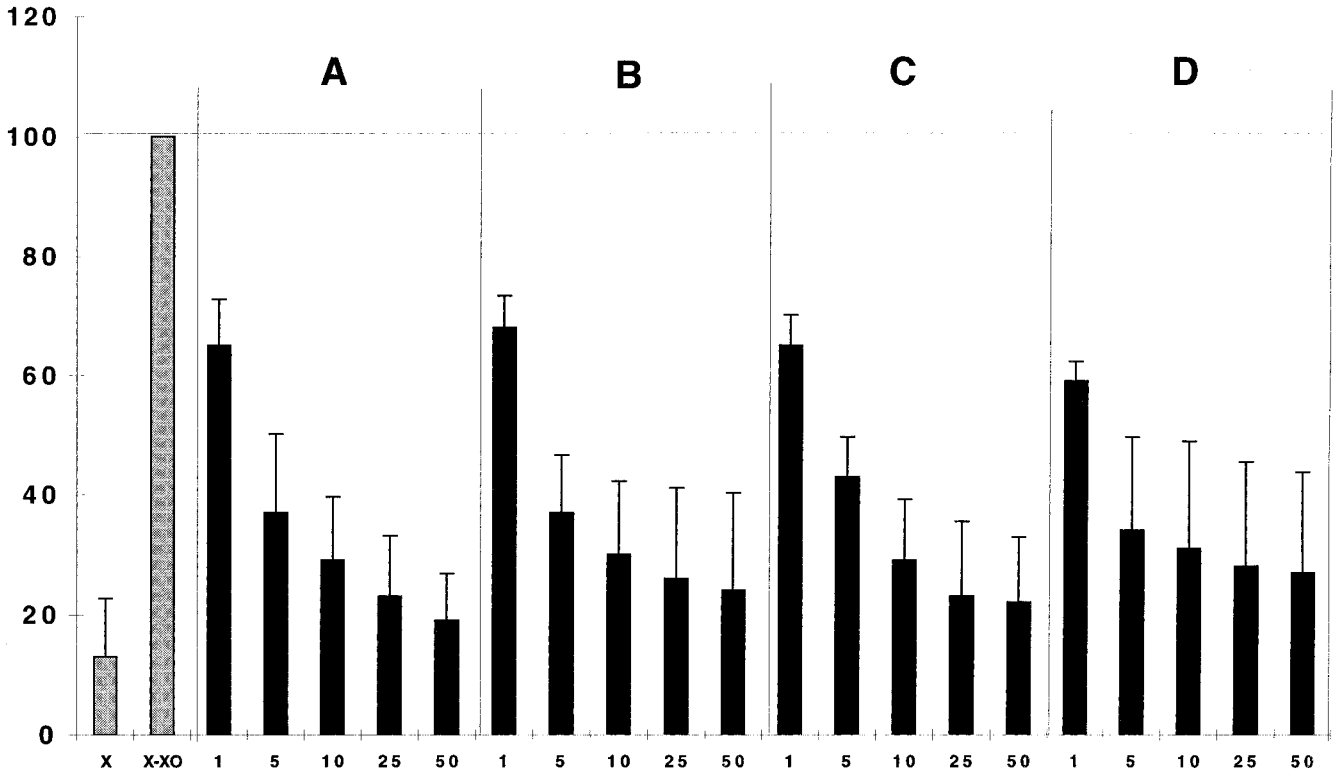


**Figure 5.** Effect of increasing concentrations of the OWW fractions A−D (expressed as tyrosol molar equivalents) on the production of $O_2^{\bullet-}$ lucigenin bioluminescence supplied by the cell-free xanthine/xanthine oxidase system (0.25 $\mu$M xanthine, 0.25 unit/L of xanthine oxidase, pH 9.0). The system conditions were chosen to produce a bioluminescence similar to that obtained in Figure 2 (229000 arbitrary units after 60 min of incubation time). The 100% value corresponded to the system without polyphenols.

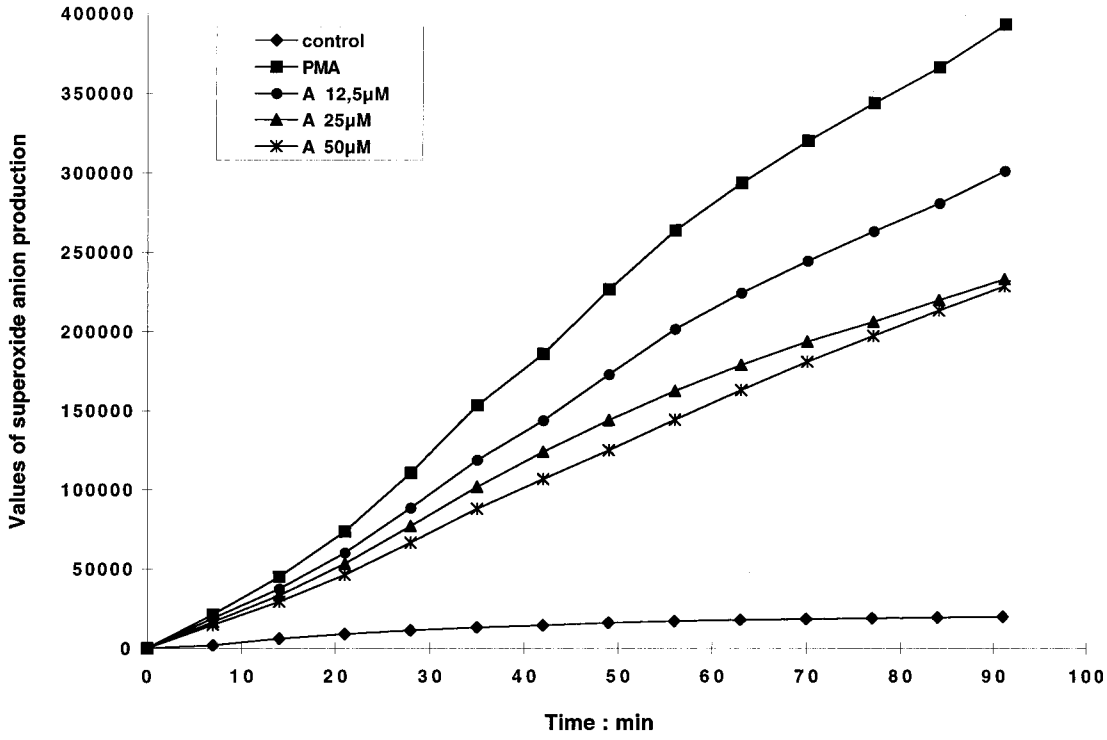


**Figure 6.** Typical time course of the production of $O_2^{\bullet-}$−lucigenin bioluminescence by 0.1 $\mu$M PMA-stimulated THP-1 ($10^6$ cells/mL) without polyphenols. Cells were preincubated for 24 h in the presence of increasing concentrations of fraction A, and then the OWW-containing medium was removed by centrifugation and replaced by OWW-free medium with PMA for bioluminescence assay.

procedure already reported in cell line U937, a line blocked at a stage of the myelomonocytic lineage which takes place earlier than in THP-1 (Taimi et al., 1991). Moreover, PMA-stimulated THP-1 has long been known to be equipped with receptor(s) allowing modified LDL degradation (Hara et al., 1987) and adherence (Schwende et al., 1996). Its differentiation into adherent macrophages cells is well established. The present

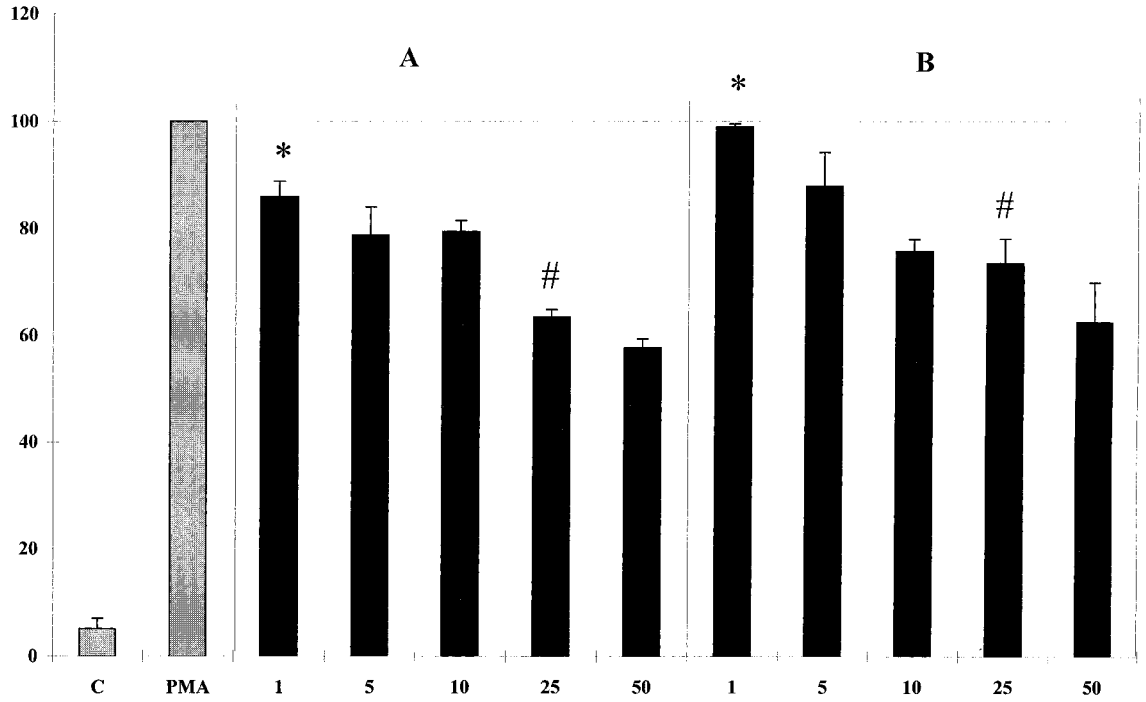


**Figure 7.** Production of $O_2^{\bullet-}$ lucigenin bioluminescence by 0.1 $\mu$M PMA-stimulated THP-1 ($10^6$ cells/mL) without polyphenols, after preincubation in the presence of the indicated concentrations of fractions A and B (expressed as $\mu$M tyrosol molar equivalents) as indicated in Figure 6. The 100% value as defined in Figure 2 corresponded to a bioluminescence of 290000 arbitrary units. Bars with the same symbol (*, #) are significantly ($p \leq 0.05$) different.

results showed that all four mixtures were able to depress the $O_2^{\bullet-}$ production no matter what type of reactive oxygen species generation used. Additionally, it can be concluded that the fractions were not only able to scavenge released $O_2^{\bullet-}$ but also to down-regulate $O_2^{\bullet-}$ cell production, as shown when polyphenols were removed from the medium.

It has been reported that olive polyphenols were able to depress free radical production in human neutrophils (Visioli et al., 1998a) using luminol as a luminescence probe. In a model of human macrophage-differentiated monocyte and using a luminescence probe more specific to $O_2^{\bullet-}$, we presently found that the $O_2^{\bullet-}$ production of cells is down-regulated by OWW fractions A and B. Given the role played by this cell type in the vessel wall in the early stage of and all along the atherogenic process (Stary et al., 1994, 1995), the implication of $O_2^{\bullet-}$ in LDL oxidation (Fang et al., 1998) and the well-established, crucial role played by oxidized LDL in atherosclerosis (Jialal and Devaraj, 1996; Parthasarathy and Rankin, 1992), the present results support an antiatherogenic role of OWW leading to a potential use as a food complement.

Interestingly, there were no marked differences between fractions. This may result from the fact that all concentrations of fractions were calculated on the basis of molar concentration expressed as tyrosol equivalent. Besides, this would suggest that the matrix providing tyrosol plus hydroxytyrosol (i.e., the rest of the processed material) present in the fractions was inactive in the tests presently performed. Therefore, considering the action of fractions in the whole, it is likely that hydroxytyrosol is the superoxide- and cell-active component present in the preparations.

These results also raise the question of the type of cell action such products are able to exert, having as the final target the $O_2^{\bullet-}$-releasing NADPH oxidase enzyme complex. Expression and phosphorylation of key proteins for assembling the NADPH oxidase complex (rac1, p47*phox*; Kim et al., 1998; Cachia et al., 1998a), translocation and/or activity of protein kinase C (Cachia et al., 1998a), or more generally alteration of the redox-sensitive transduction cascades (AP-1, NF$\kappa$B), which are important in mediating cellular responses to environmental stimuli (Sen et al., 1996; Engelhardt, 1996), are now routes to explore a better understanding of the effects of polyphenols from food origin in atherosclerosis and inflammatory processes (Liao et al., 1994).

## ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; IFN$\gamma$, interferon $\gamma$; LDL, low-density lipoprotein; LTB4, leukotriene B4; $O_2^{\bullet-}$, superoxide anion; OWW, olive oil wastewaters; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophils; SOD, superoxide dismutase; $TXB_2$, thromboxane $B_2$; AP-1, activator protein 1; NF$\kappa$B, nuclear factor $\kappa$B; rac1, a member of the rho subfamily of GTPases; RPMI, Roswell Park Memorial Institute medium.

## ACKNOWLEDGMENT

We are grateful to the Vincieri group for supplying the OWW fractions.

## LITERATURE CITED

Baldi, A.; Romani, A.; Mulinacci, N.; Vincieri, F. F.; Casetta, B. HPLC/MS application to anthocyanins of *Vitis vinifera* L. *J. Agric. Food Chem.* **1995**, *43*, 2104.

Brillant, L.; Léger, C. L.; Descomps, B. Vitamin E inhibition of $O_2^{\bullet-}$-production in the promonocyte cell line THP-1 is essentially due to RRR-$\delta$-tocopherol. *Lipids* **1999**, *34* (Suppl.), S293.

Cachia, O.; El Benna, J.; Pedruzzi, E.; Descomps, B.; Gougerot-Pocidalo, M. A.; Leger, C. L. α-tocopherol inhibits the respiratory burst in human monocytes attenuation of p47*phox* membrane translocation and phosphorylation. *J. Biol. Chem.* **1998a**, *49*, 32801−32805.

Cachia, O.; Léger, C. L.; Descomps, B. Monocyte superoxide production is inversely related to normal content of α-tocopherol in low-density lipoprotein. *Atherosclerosis* **1998b**, *138*, 263−269.

Choi, H. S.; Moore, D. D. Induction of c-fos and c-jun gene expression by phenolic antioxidants. *Mol. Endocrinol.* **1993**, *7*, 1596−1602.

Engelhardt, J. F. Redox-mediated gene therapies for environmental injury: approaches and concepts. *Antiox. Redox Signal.* **1999**, *1*, 5−27.

Fang, X.; Weintraub, N. L.; Rios, C. D.; Chappell, D. A.; Zwacka, R. M.; Engelhardt, J. F.; et al. Overexpression of human superoxide dismutase inhibits oxidation of low-density lipoprotein by endothelial cells. *Circ. Res.* **1998**, *29*, 1289−1297.

Hara, H.; Tanishita, H.; Yokoyama, S.; Tajima, S.; Yamamoto, A. Induction of acetylated low-density lipoprotein receptor and suppression of low-density lipoprotein receptor on the cells of human monocytic leukemia cell line (THP-1 cell). *Biochem. Biophys. Res. Commun.* **1987**, *146*, 802−808.

Heinecke, J. W. Mechanisms of oxidative damage of low-density lipoprotein in human atherosclerosis. *Curr. Opinion Lipidol.* **1997**, *8*, 268−274.

Hodis, H. N.; Mack, W. J.; LaBree, L.; Cashin-Hemphill, L.; Sevanian, A.; Johnson, R.; et al. Serial coronary angiographic evidence that antioxidant vitamin intake reduces progression of coronary artery atherosclerosis. *JAMA* **1995**, *273*, 1849−1854.

Jialal, I.; Devaraj, S. The role of oxidized low-density lipoprotein in atherogenesis. *J. Nutr.* **1996**, *126*, 1053S−1057S.

Johnson, J. A.; Adak, S.; Mochly-Rosen, D. Prolonged phorbol ester treatment down-regulates protein kinase C isozymes and increases contraction rate in neonatal cardiac-myocytes. *Life Sci.* **1995**, *57*, 1027−1038.

Kadri-Hassani, N.; Léger, C. L.; Descomps, B. The fatty acid bimodal action on superoxide anion production by human adherent monocytes under phorbol 12-myristate 13-acetate or diacylglycerol activation can be explaind by the modulation of protein kinase C and p47*phox* translocation. *J. Biol. Chem.* **1995**, *270*, 15111−15118.

Kim, K. S.; Takeda, K.; Sethli, R.; Pracyk, J. B.; Tanaka, K.; Zhou, Y. F.; et al. Protection from reoxygenation injury by inhibition of rac1. *J. Clin. Invest.* **1998**, *101*, 1821−1826.

Kohyama, N.; Nagata, T.; Fujimoto, S.; Sekiya, K. Inhibition of arachidonate lipoxygenase activities by 2-(3,4-dihydroxyphenyl) ethanol, a phenolic compound from olives. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 347−350.

Liao, F.; Andalibi, A.; Qiao, J.-H.; Allayee, H.; Fogelman, A. M.; Lusis, A. J. Genetic evidence for a common pathway mediating oxidative stress, inflammatory gene induction, and aortic fatty streak formation in mice. *J. Clin. Invest.* **1994**, *94*, 877−884.

Parthasarathy, S.; Rankin, S. M. Role of oxidized low-density lipoprotein in atherogenesis. *Prog. Lipid Res.* **1992**, *31*, 127−143.

Petroni, A.; Blasevich, M.; Salami, M.; et al. Inhibition of platelet aggregation and eicosanoid production by phenolic components of olive oil. *Thromb. Res.* **1995**, *78*, 151−160.

Rimm, E. B.; Stampfer, M. J.; Ascherio, A.; Giovanucci, E.; Colditz, G. A.; Willett, W. C. Vitamin E consumption and the risk of coronary heart disease in men. *N. Engl. J. Med.* **1993**, *328*, 1450−1456.

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

Schewende, H.; Fitzke E.; Ambs, P.; Dieter, P. Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *J. Leukoc. Biol.* **1996**, *59*, 555−561.

Sen, C. K.; Packer, L. Antioxidant and redox regulation of gene transcription. *FASEB J.* **1996**, *10*, 709−720.

Stary, H. C.; Chandler, A. B.; Glagov, S.; Guyton, J. R.; Insull, W.; Rosenfeld, M. E.; et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. *Arterioscler. Thromb.* **1994**, *14*, 840−856.

Stary, H. C.; Chandler, A. B.; Dinsmore, R. E.; Fuster, V.; Glagov, S.; Insull, W.; et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **1995**, *15*, 1512−1531.

Taimi, M.; Chateau, M. T.; Cabane, S.; Marti, J. Synergistic effect of retinoic acid and 1,25-dihydroxyvitamin D3 on the differentiation of the human monocytic cell line U937. *Leukemia Res.* **1991**, *15*, 1145−1152.

Visioli, F.; Bellomo, G.; Montedoro, G. F.; Galli, C. Low-density lipoprotein oxidation is inhibited in vitro by olive oil constituents. *Atherosclerosis* **1995**, 117, 25−32.

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

Visioli, F.; Bellosta, S.; Galli, C. Oleuropein, the bitter principle of olives, enhances nitric oxide production by mouse macrophages. *Life Sci.* **1998b**, *62*, 541−546.

Visioli, F.; Romani, A.; Mulinacci, N.; Zarini, S.; Conte, D.; Vincieri, F. F.; Galli, C. Antioxidant and other biological activities of olive mill wastewaters. *J. Agric. Food Chem.* **1999**, *47*, 3397−3401.

Watson D. G.; Wainer B. H.; Lenox R. H. Phorbol ester- and retinoic acid-induced regulation of the protein kinase C substrate MARCKS in immortalized hyppocampal cells. *J. Neurochem.* **1994**, *63*, 1666−1674.

Received for review December 10, 1999. Accepted July 17, 2000. This study is a part of the EU Project FAIR CT 97-3039, C. Galli, coordinator.

JF991349C