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Protective Effects of Synthetic Hydroxytyrosol Acetyl Derivatives against Oxidative Stress in Human Cells

CATERINA MANNA,*,† VALENTINA MIGLIARDI,† FILOMENA SANNINO,§ ANTONIO DE MARTINO,§ AND RENATO CAPASSO§

Dipartimento di Biochimica e Biofisica "F. Cedrangolo", Facoltà di Medicina e Chirurgia, Seconda Università degli Studi di Napoli, Via De Crecchio 7, 80138 Naples, Italy, and Dipartimento di Scienze del Suolo, della Pianta e dell'Ambiente, Università degli Studi di Napoli "Federico II", Via Università 100, 80055 Portici (NA), Italy

Chemically stable di- and triacetyl derivatives of the natural *o*-diphenol antioxidant hydroxytyrosol were synthesized, and their chemical and biological antioxidant activities were assessed in comparison with that of the native synthetic compound. The chemical antioxidant activity of the selected compounds was evaluated by measuring the ferric reducing antioxidant power (FRAP). The data clearly indicate that, as expected, the hydroxytyrosol analogues, modified in the *o*-diphenolic ring, are devoid of any chemical antioxidant activity. On the contrary, both acetyl derivatives, at micromolar concentrations, equally protect against *t*ert-butylhydroperoxide-induced oxidative damages in Caco-2 cells and human erythrocytes. This paper for the first time reports that chemically stable hydroxytyrosol acetyl derivatives, although devoid of chemical antioxidant activity, are as effective as the parent compound in protecting human cells from oxidative stress-induced cytotoxicity, after metabolization by esterases at the intestinal level, suggesting their possible utilization in either nutritional (functional food), cosmetic, or pharmaceutical preparations.

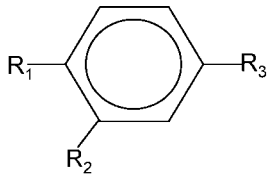
KEYWORDS: Mediterranean diet; antioxidant; polyphenol; oxidative stress; functional food; cardiovascular diseases; acetyl hydroxytyrosol

INTRODUCTION

Antioxidant compounds play a key role in human nutrition as well as in industry. Dietary intake of both vitamin and nonvitamin antioxidants, indeed, is known to decrease the incidence of several pathologies (*1*−*3*), including cardiovascular diseases (CVD). In addition, natural and synthetic antioxidants are widely used in cosmetic as well as nutritional preparations.

In recent years, several studies have been devoted to the exploration of the biological effects of nonvitamin phenolic antioxidants (*4*, *5*), which occur widely in the vegetal kingdom and therefore in plant-derived food, including olive oil, which is the typical lipidic source in the Mediterranean diet. The beneficial health effects of this dietary habit have been partially attributed to the high content of antioxidant compounds, including polyphenols (*6*).

Among the different phenolic compounds, particular attention has focused on hydroxytyrosol [4-(2-(hydroxyethyl)-1,2-benzenediol, hdrx, **1a**] (**Figure 1**) (*7*), naturally occurring in olive oil (*8*), in olive mill solid−liquid wastes (OMW), from two-phase olive oil processing (*9*), and in olive mill wastewaters (OMWW), from traditional and industrial three-phase plants (*10*, *11*). It derives from the hydrolysis of oleuropein, present in olives up to 6.5 g/kg of the fresh weight in unripe olive (*12*).



ANALOG	R_1	R_2	R_3
Hdrx (1a)	OH	OH	CH_2-CH_2OH
Triachdrx (1b)	$OCOCH_3$	$OCOCH_3$	CH_2-CH_2OCOCH_3
Diachdrx (1c)	$OCOCH_3$	$OCOCH_3$	CH_2-CH_2OH

Figure 1. Structures of hydroxytyrosol (hdrx, **1a**) and its acetyl derivatives (triachdrx, **1b**; and diachdrx, **1c**).

* Corresponding author (telephone +39-081-5667523; fax +39-081-5667608; e-mail caterina.manna@unina2.it).
† Seconda Università degli Studi di Napoli.
§ Università degli Studi di Napoli "Federico II".

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This *o*-diphenol has been proved to be a potent scavenger of superoxide anion as well as hydroxyl radical (*13*, *14*), and it is more active than antioxidant vitamins (*15*) as well as the synthetic antioxidants (*16*).

Clear epidemiological and biochemical evidence indicates that **1a** is endowed with significant antithrombotic, antiatherogenic, and anti-inflammatory activities (*17*−*19*). Mechanisms underlying these biological effects include inhibition of platelet aggregation (*20*) and lipoxygenase and cycloxygenase activities (*21*); this phenol also prevents copper sulfate-induced lipoprotein oxidation (*22*). It should be stressed in this respect that oxidized lipoproteins have been shown to be more atherogenic than the native one. Finally, a recent finding reveals new molecular mechanisms of **1a** antiatherogenic activity: this phenol, indeed, is able to inhibit leukocyte adhesion to vascular endothelial cells, which represents a key step in the formation of atherosclerotic plaque (*23*).

As far as **1a** transport is concerned, we demonstrated that it permeates cell membranes via a passive diffusion mechanism (*24*) and that it rapidly distributes in all organs and tissues when intravenously injected in rats (*25*); moreover, its high bioavailability in humans has been reported (*26*). **1a** is metabolized in vivo both in the aromatic moiety as well as in the lateral chain, yielding metabolites that still retain the antioxidant power of the native molecule (*25*). Therefore, **1a** represents a good candidate for a potential utilization as antioxidant for either pharmaceutical and cosmetic preparations; furthermore, **1a** could be successfully utilized as an ingredient in the development of the so-called "functional foods" (FF), which claim to improve health and/or to prevent diseases.

In this respect, however, a major problem is that **1a** is chemically unstable, unless preserved dried in the absence of air and in the dark (unpublished data). Therefore, the efficiency of this molecule added in its native form to biological matrices as a protective agent against reactive oxygen species (ROS) in human cells could not be guaranteed. In addition, it cannot be added to lipophilic preparations because of its relative polarity. On the basis of these considerations, it would be useful to conveniently produce **1a** in chemically more stable derivatives able to be biochemically converted in vivo into its original active form. Considering that the acetyl group is a ubiquitous substrate in the biochemical processes and that the acetylating agents are very common and manageable, we have planned and succeeded in preparing hydroxytyrosol acetyl derivatives (*10*).

The aim of this paper is to investigate the antioxidant activity of synthetic triacetylhydroxytyrosol [4-(acetoxyethyl)-1,2-diacetoxybenzene, triachdrx, **1b**] and diacetylhydroxytyrosol [4-(hydroxyethyl)-1,2-diacetoxybenzene, diachdrx, **1c**] (**Figure 1**), compared with the parent synthetic **1a**.

Their ferric reducing capacity has been evaluated by the ferric reducing antioxidant power (FRAP) assay (*27*). Moreover, the chemical antioxidant properties of the tested compounds have been compared with their protective effect against oxidative stress-induced molecular alterations in intestinal Caco-2 cells (*28*) and human erythrocytes (RBC) (*29*).

MATERIALS AND METHODS

Materials. Solvents of HPLC grade were provided by Carlo Erba (Milan, Italy), and reactants of analytical grade for the synthesis of **1a**, **1b**, and **1c** were supplied by Fluka (Sigma-Aldrich, Milan, Italy). All of the compounds utilized for the antioxidant and biological tests, unless otherwise specified, were obtained from Sigma-Aldrich. High-glucose Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, nonessential amino acids, *N*-2-(hydroxyethyl(piperazine-*N*′-2-ethanesulfonic acid (HEPES), glutamine, penicillin, streptomycin, and PBS tablets were purchased from Gibco, Life Science Technologies (S. Giuliano Milanese, MI, Italy).

Analytical HPLC. The HPLC analyses were performed using an Agilent 1100 series liquid chromatograph equipped with a DAD array. The wavelength 264 nm was used for **1b** and **1c** and 280 nm for **1a**. A Nucleosil 100-5 C18 column (stainless steel, 250 × 4 mm) was utilized for all of the compounds. An isocratic elution at a flow rate of 1.0 mL min^{-1} with acetonitrile/water (45:55, v/v) as mobile phase was used for **1b** and **1c**, and a flow rate of 1.2 mL min^{-1} with acetonitrile/water (containing 0.35% acetic acid) (15:85, v/v) was used as mobile phase for **1a**. A sample volume of 20 μL was used for the injection.

Synthesis of Hdrx (1a), Triachdrx (1b), and Diachdrx (1c). All of the considered compounds were synthesized following our previously reported method (*10*, *30*). Their purity grade was assessed by HPLC analysis.

Evaluation of the Chemical Antioxidant Activity of 1a and Its Derivatives: FRAP Assay. The ferric reducing ability of **1a** and its acetyl derivatives was measured using the FRAP assay, a colorimetric method based on the reduction of a ferric−tripyridyltriazine (ferric−TPTZ) complex to its ferrous form (*27*). Increasing amounts of tested antioxidants were added to 1 mL of working solution, prepared by mixing 25 mL of 300 mM acetate buffer (pH 3.6), 2.5 mL of TPTZ solution (10 mM TPTZ in 40 mM HCl), and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$; after incubation for 6 min at room temperature (23−25 °C), the absorbance was read at 593 nm.

Biological Tests for the Antioxidant Activity of 1a and Its Derivatives in Caco-2 Cells. Human colon carcinoma (Caco-2) cells were grown in DMEM, supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (50 units/mL), streptomycin (50 units/mL), and 1% nonessential amino acids, in a humidified incubator with a 5% CO_2/95% air atmosphere (*28*). Cells were seeded at a density of 90000 cells/cm^2 in multiwell dishes, and 12−14 days after confluence cells were treated with *tert*-butyl hydroperoxide (*t*-BHP) to a final concentration of 4 mM. At the end of 6 h of incubation, both cell viability and lipoperoxidation extent were evaluated as described below. To assay the antioxidant protective effect on Caco-2 cells from oxidative injury, the cells were pretreated for 30 min, in the absence or presence of increasing concentrations of each selected antioxidant (50−100 μM) before the induction of oxidative stress. Phenolic antioxidants, indeed, exert their biological activities at micromolar concentrations.

Evaluation of Caco-2 Cell Viability (MTT Assay). The *t*-BHP-induced cytotoxicity on Caco-2 cells was measured by evaluating cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (*31*). This colorimetric method is based on the reduction of the tetrazolium ring of MTT by mitochondrial dehydrogenases, yielding a blue formazan product, which can be measured spectrophotometrically; the amount of formazan produced is proportional to the number of viable cells. After the oxidative treatment, the medium was removed and MTT (5 mg/mL in DMEM without phenol red) was added to the cells (10% final concentration). After 4 h of incubation, the medium was aspirated and the insoluble formazan produced was dissolved in 2-propanol; the optical densities were measured at 570 nm.

Evaluation of Lipoperoxidation. After the removal of the medium, cells were solubilized and lysates centrifuged at 12000*g* for 5 min; the obtained supernatants were assayed for lipoperoxidation products (*28*). Aliquots of supernatants were added to thiobarbituric acid (TBA) in 0.05 N NaOH (0.2% w/v final concentration) and heated in a boiling water bath for 10 min. The absorbance of the developed pink chromophore was determined at 532 nm.

Biological Tests for the Antioxidant Activity of 1a and Its Derivatives in Human Erythrocytes. The preparation of human RBC was performed by employing heparinized fresh human blood from healthy donors, as previously reported (*29*). The RBC suspensions (2% hematocrit) were treated with *t*-BHP (500 μM final concentration). At the end of 2 h of incubation, the extents of both hemolysis and lipoperoxidation were evaluated as described below. To assay the antioxidant protective effect on RBC from oxidative injury, the cells were pretreated for 15 min, in the absence or presence of increasing concentrations (10−50 μM) of each selected antioxidant, before the induction of oxidative stress.

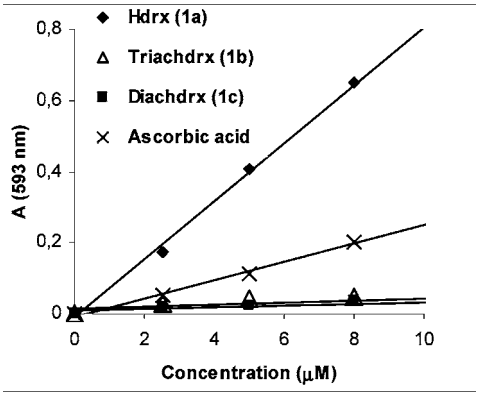

Figure 2. Ferric reducing abilities of hydroxytyrosol (hdrx, **1a**) and its acetyl derivatives (triachdrx, **1b**; and diachdrx, **1c**). Ferric reducing abilities were determined as reported under Materials and Methods.

Evaluation of RBC Hemolysis. The extent of hemolysis was measured spectrophotometrically, as previously described (*29*). After the oxidative treatment, samples were centrifuged at 1500*g* for 10 min, and the absorption (*A*) of the supernatant (S_1) at 540 nm was measured. The precipitates **(**packed RBC) were then hemolyzed with 40 volumes of ice-cold distilled water and centrifuged at 1500*g* for 10 min. The supernatant (S_2) was then added to S1 and the absorption (*B*) of the combined supernatants ($S_1 + S_2$) was measured at 540 nm; the percentage of hemolysis was calculated from the ratio of the readings (A:B) $\times$ 100.

Evaluation of Lipoperoxidation. After the oxidative treatment, RBC samples were mixed with trichloroacetic acid (10% w/v final concentration) and centrifuged at 5000*g* for 15 min (*29*). The obtained supernatants were finally assayed for lipoperoxidation products as reported above.

Statistical Analysis. Results are reported as means $\pm$ SD; $n = 4$. Student's t test was routinely utilized.

RESULTS

To obtain chemically stable forms of **1a**, two acetyl analogues (**1b** and **1c**) (**Figure 1**) were synthesized and spectroscopically characterized according to the methods previously reported (*10*, *30*). All of the synthesized compounds showed a high purity grade, as evidenced in the respective HPLC chromatograms by a single and sharp peak corresponding to $t_R = 3.13$ min for **1a**, $t_R = 7.62$ min for **1b**, and $t_R = 3.61$ min for **1c**. Their antioxidant properties were then evaluated.

The chemical antioxidant power was tested by means of the FRAP assay (*27*). **Figure 2** reports the data on the ferric reducing activity of **1a** as well as its acetyl derivatives **1b** and **1c**. In agreement with our previous paper, the results indicate that **1a** is endowed with a strong ferric reducing efficacy, stronger than that of vitamin C (*15*). In contrast, both acetyl derivatives **1b** and **1c** are completely inefficient as hydrogen donors, indicating that they are devoid of any chemical antioxidant activity. This finding confirms that the *o*-diphenolic structure of **1a** is particularly crucial for its antioxidant activity.

To investigate the biological antioxidant activity of the synthetic compounds in cellular systems, Caco-2 cells, originally derived from a human colon carcinoma, were selected as a model of the intestinal epithelium. These cells, able to differentiate in culture, are considered to be a suitable model to mimic the food−intestinal tract interactions in vitro (*32*). Caco-2 cells were preincubated for 30 min at the same antioxidant concentration and then exposed to *t*-BHP. As shown in **Figure 3A**, the hydroperoxide induces a 38% decrease in cell viability, as assessed by MTT assay; moreover, a remarkable protection against ROS-induced cytotoxicity was observable in antioxidant-

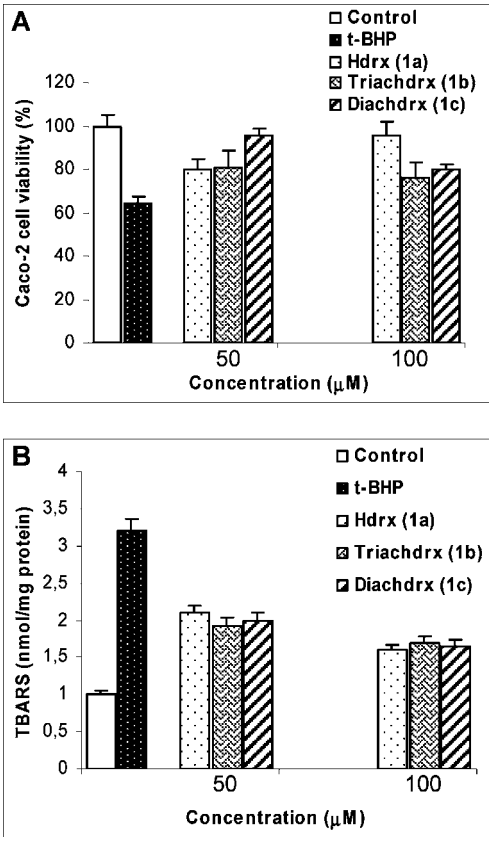

Figure 3. Effect of hydroxytyrosol (hdrx, **1a**) and its acetyl derivatives (triachdrx, **1b**; and diachdrx, **1c**) on *t*-BHP-induced oxidative stress in Caco-2 cells. Cell viability (**A**) and TBARS concentration (**B**) were measured as described under Materials and Methods. Values are means $\pm$ SD; $n = 4$.

treated Caco-2 cells, the protective effect of both **1b** and **1c** being of the some order of magnitude as the native phenol: all of the tested compounds, indeed, equally prevent Caco-2 cytotoxicity at a micromolar concentration range.

We also compared the protective effects of the selected compounds against *t*-BHP-mediated oxidation of polyunsaturated fatty acids, by measuring the formation of lipoperoxidation degradation products able to react with TBA. The oxidative treatment results in ~3-fold increase in TBARS concentration compared with control cells; also in this case, pretreatment of Caco-2 cells with either **1a**, **1b**, or **1c** significantly decreases TBARS formation: at both 50 and 100 μM, indeed, all of the tested compounds equally protect against lipid peroxidation (**Figure 3B**).

The finding that both acetyl compounds show a biological antioxidant activity compared with that of the parent compound suggests that both **1b** and **1c** are metabolized at intestinal level by cellular esterases, to yield **1a**, which is the effective antioxidant compound.

To rule out artifact degradation of **1b** and **1c**, they were incubated in the same experimental conditions in the absence of cells. The results clearly indicate that neither compound is chemically degraded in the experimental conditions of the biological assays (data not shown); moreover, they also appear to be stable in the simulated acidic and thermal conditions of the stomach (data not shown).

To further elucidate the biological antioxidant effect of the tested compounds in human cells against *t*-BHP-induced molecular alterations, RBC were selected as a second experimental system. Human RBC have been amply used to study oxidative

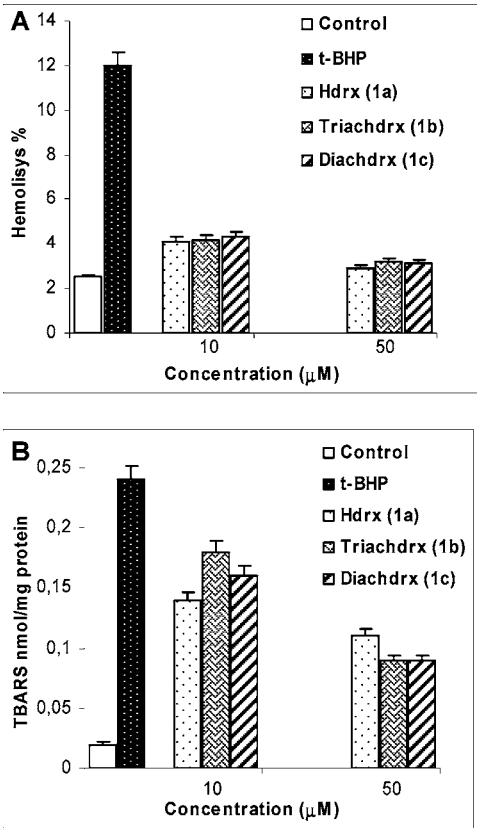

Figure 4. Effect of hydroxytyrosol (hdrx, **1a**) and its acetyl derivatives (triachdrx, **1b**; and diachdrx, **1c**) on *t*-BHP-induced oxidative stress in RBC. Hemolysis percentage (**A**) and TBARS concentration (**B**) were measured as described under Materials and Methods. Values are means ± SD; $n = 4$.

stress-induced cytotoxicity as well as the protective effect played by antioxidants, in both physiological and pathological conditions (*33*−*35*). As a matter of fact, these cells are characterized by a particularly high ROS production, deriving from spontaneous autoxidation of hemoglobin (*36*). A very efficient antioxidant defense system rapidly removes these high reactive molecular species; however, if ROS are overproduced or the antioxidant defenses are impaired, severe oxidative alterations occur, eventually leading to hemolysis.

To investigate the protective effect of the selected compounds against oxidative hemolysis, RBC were treated with 500 μM *t*-BHP. As shown in **Figure 4A**, RBC preincubation in the presence of 50 μM **1a** completely prevents oxidative hemolysis; moreover, a comparable protective effect on ROS-mediated cytotoxicity is observable in RBC samples pretreated with the same concentrations of both **1b** and **1c**.

Finally, the biological antioxidant activity of **1a** and its acetyl analogues against membrane phospholipid alterations was evaluated. Also in this case, all of the tested compounds significantly protect RBC from lipid peroxidation in the same micromolar concentration range (**Figure 4B**).

Altogether the data on the biological tests using RBC as model system suggest that **1b** and **1c** can be completely metabolized to **1a** also when intravenously injected.

DISCUSSION

Polyphenols are widely distributed in the vegetal kingdom and, therefore, are present in very high concentrations in plant-derived food relevant to human nutrition. These compounds, endowed with powerful antioxidant activity, have attracted a great deal of attention in light of their possible key role in the dietary prevention of those pathologies for which their etiology and progression have been related to ROS-mediated tissue injury, including CVD (*1*−*5*).

The results reported in this paper represent the first evidence that **1b** and **1c**, chemically stable acetyl analogues of **1a**, are as effective as the native compound in preventing ROS-mediated molecular oxidative alterations and cytotoxicity in human cells, despite the fact that they are completely devoid of chemical antioxidant activity.

This finding is particularly important in light of their potential utilization for human purposes. The acetyl compounds, indeed, could be successfully utilized in the development of new FF for cardiovascular protection (*37*−*39*). As discussed in the Introduction, the majority of the studies on the beneficial health effects of polyphenols clearly indicate that **1a** is able to counteract the progression of atherosclerosis by interfering in different stages of the mechanism of plaque formation. Moreover, we have recently demonstrated that oleuropein (**1a** precursor) exerts a strong direct cardioprotective effect. It is able, indeed, to significantly reduce myocardial injury induced by ischemia and reperfusion, using isolated rat heart as model system (*40*).

Polyphenols exert their biological activities in vitro at micromolar concentrations within the concentration range expected after the nutritional intake of plant-derived food. Furthermore, using human cells in culture, a complete protection against oxidative hemolysis was observed in RBC pretreated with as little as total 5 μg of *o*-diphenols, corresponding to ∼300 μL of virgin olive oil containing 100 mg/kg *o*-diphenols (*15*). In the past few years, indeed, increasing data have indicated that olive oil polyphenols exert their beneficial action also in vivo: the oxidative stability of lipoproteins isolated from animals fed virgin olive oil is significantly increased (*41*, *42*); moreover, oral administration of polyphenol-rich olive oil decreases isoprostane excretion (*43*) and modulates oxidative/antioxidative status in humans (*44*). Therefore, the daily intake of antioxidant fortified FF could be useful to design dietary strategies for the prevention of CVD. Moreover, these high-quality FF should be promoted to increase the endogenous defense in those countries whose populations have dietary habits that are particularly low in fruits and vegetables and therefore lacking in antioxidants. The interest of the food industry in this area, indeed, has increased tremendously in the past few years, and scientists are deeply involved in the identification of new "functional" molecules, showing health-promoting properties.

It should also be stressed, in this respect, that because of their different polarities **1b** and **1c** could be utilized as versatile additives for a broad spectrum of biological matrices. In particular, as previously reported by TLC analysis using direct and reverse phase chromatography (*45*), **1a** is the most polar (hydrophilic) compound, **1b** being the most lipophilic one; **1c** shows intermediate properties.

Finally, it is noteworthy that **1b** and **1c**, which release in vivo **1a** and acetate, can be regarded as nontoxic compounds: we previously demonstrated, indeed, that treatment of rats with up to 2 g/kg **1a** does not induce any significant organ damage (*25*) and that acetate is rapidly metabolized.

Besides their chemical synthesis, **1b** and **1c** could also be obtained from natural sources. Among the different types of olive oils, virgin olive oil is produced by purely mechanical means without refining and, consequently, retains a significant amount of polyphenols originally present in olives. Also in this case, however, the majority of the phenolic compounds are lost

in the water fraction during the separation of the oil from the must. As discussed in the Introduction, both OMW and OMWW contain large amounts of phenolic antioxidants, **1a** being the most abundant (*45*, *46*). Experiments are under way by our group to design a protocol for the recovery of **1a** from olive oil processing byproducts by direct chemical treatment of OMWW to obtain the chemically stable and biologically active **1b** and **1c**. It should be emphasized, in this respect, that the presence of high concentrations of phenolic compounds makes both OMWW and OMW disposal an unsolved problem, because of their strong antimicrobial and antibacterial activities (*47*), and therefore the olive oil farm should be encouraged in recovering this highly valuable waste product.

ABBREVIATIONS USED

*t*BHP, *tert*-butylhydroperoxide; CVD, cardiovascular diseases; diachdrx (**1c**), diacetylhydroxytyrosol [4-(hydroxyethyl)-1,2-diacetoxybenzene]; FF, functional food; FRAP, ferric reducing antioxidant power; hdrx (**1a**), hydroxytyrosol [4-(2-hydroxyethyl)-1,2-benzenediol]; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OMW, olive mill waste; OMWW, olive mill wastewater; RBC, red blood cells; ROS, reactive oxygen species; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TPTZ, tripyridyltriazine; triachdrx (**1b**), triacetylhydroxytyrosol [4-(acetoxyethyl)-1,2-diacetoxybenzene].

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