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Antioxidant and Biological Activities of Hydroxytyrosol and Homovanillic Alcohol Obtained from Olive Mill Wastewaters of Extra-Virgin Olive Oil Production

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ABSTRACT: Some constituents of the Mediterranean diet, such as extra-virgin olive oil (EVOO) contain substances such as hydroxytyrosol (HT) and its metabolite homovanillic alcohol (HA). HT has aroused much interest due to its antioxidant activity as a radical scavenger, whereas only a few studies have been made on the HA molecule. Both chemical synthesis and extraction techniques have been developed to obtain these molecules, with each method having its advantages and drawbacks. In this study, we report the use of tyrosol from olive mill wastewaters as a starting molecule to synthesize HT and HA, using a sustainable procedure characterized by high efficiency and low cost. The effects of HT and HA were evaluated on two cell lines, THP-1 human leukemic monocytes and L-6 myoblasts from rat skeletal muscle, after treating the cells with a radical generator. Both HT and HA efficiently inhibited ROS production. In particular, HT inhibited the proliferation of the THP-1 leukemic monocytes, while HA protected L-6 myoblasts from cytotoxicity.

KEYWORDS: *antioxidant, sustainable, olive mill wastewaters, tyrosol, hydroxytyrosol, homovanillic alcohol*

1. INTRODUCTION

Many studies have focused on the beneficial health effects of the Mediterranean diet and, in particular, some of its constituents such as extra-virgin olive oil (EVOO) that has been reported to prevent cardiovascular and neurodegenerative diseases when consumed daily within the context of a balanced diet.[1,2] Among the variety of bioactive components found in olives, several phenolic compounds such as hydroxytyrosol (HT) 4-(2-hydroxyethyl)-1,2-benzenediol seem to have key roles. These phenols are powerful hydrogen-donating antioxidants and scavengers of reactive oxygen and nitrogen species. Several reports have demonstrated a role for specific phenolic compounds through their selective interactions within signaling cascades, such as tyrosine kinase, PI3-kinase/Akt, as well as the protein kinase C and mitogen-activated protein kinase pathways, which regulate cell survival following exposure to oxidative stress.[3,4]

The concentrations of phenolic compounds such as oleuropein (OLE) and HT differ widely among olive products, depending on factors such as the cultivar, soil composition, the climate, and the degree of ripeness of the olives.[5] OLE is the main phenolic compound present in *Olea europaea* fruits and leaves; it is a hydroxytyrosol ester with an α-glucosylated elenolic acid and it is responsible for the bitter taste of unripened olives.[6]

The structure of HT contains a catechol moiety, and HT has been investigated and successfully tested for its free-radical scavenging activity.[7−9] This is mainly due to the presence of the two adjacent hydroxyl groups, to the conjugation and the resonance effects, and also to the ability to improve the stability of the corresponding phenoxyl radicals formed by the hydrogen-transfer reaction.[10−13] The role of HT in promoting health benefits by lowering oxidative stress in biological systems such as human cells and plasma has also been investigated.[14−16] Recently, the ability of HT and of some of its analogues to fit into the catalytic domain of COX-2 regulating the anti-inflammatory response has been reported.[17]

HT can undergo further metabolization in the organism; its major identified metabolite in humans is the methylated derivative homovanillic alcohol (HA), 4-(2-hydroxyethyl)-2-methoxyphenol, produced by the activity of catechol-*O*-methyltransferase that is also naturally present in EVOO.[18]

Despite the scarcity of studies on HA, those conducted have shown that this molecule exerts an antioxidant activity comparable to that of HT in simple chemical systems. In fact, HT and HA both inhibit H_2O_2-induced oxidative damage in kidney cells[18] and can protect other cells in culture against peroxide-induced renal epithelial injury.[17,19−21]

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The results of the research on the characteristic properties of these compounds have led to a strong interest in their production in a pure form. For this purpose, both neosyntheses and strategies based on extraction have been developed. Among the first group, the method published by Deffieux et al.[22] for HT is elegant and convenient; however, the starting substrate, eugenol, is rather expensive and in itself displays interesting biological properties.[23] Also, the biosynthetic methods reported for HT usually require expensive substrates and/or have a low efficiency.[24,25]

Other synthetic strategies, such as the one described in Capasso et al.[26] have been applied, starting from compounds that are not present in the olives. Few studies have been published describing the synthesis of HA; to the best of our knowledge, these studies report only the biocatalyzed synthesis of the monoglucuronide form of HA.[27]

The HT extraction has been based on olive mill wastewater (OMW) because it is very rich in this substance. Moreover, OMW is not only inexpensive but also constitutes a polluting material, the disposal of which is a subject to the EU regulations for waste management and is becoming significantly costly.[28] However, the extraction of HT from OMW involves drawbacks such as the need to use large amounts of organic solvents. Diethyl ether, methyl isobutyl ketone, methyl ethyl ketone, and ethyl acetate are the solvents most frequently used.[29] Finally, the high reactivity of HT makes its extraction extremely difficult. OMW is also rich in tyrosol (TY),[14] a precursor of HT deprived of the catechol moiety, which unlike HT has low reactivity and can be easily obtained from OMW by decantation.

In this study, we used TY as a starting molecule to synthesize HT and HA. We evaluated the toxic effects of HT and HA on two cell lines, L-6 myoblasts and THP-1 human leukemic monocytes, and treated the cells with a radical generator, cumene hydroperoxide (CH). The results show that both HT and HA from OMW could be obtained by simple and environmental friendly synthesis pathways with high yields (>42% for HT and >80% for HA), and they had significant roles as antioxidants, similar to that of commercially available compounds.

2. MATERIALS AND METHODS

2.1. Materials and Cell Lines. Organic solvents and reagents, thin-layer chromatography (TLC) plates 0.25 mm F254, and silica gel (F230-400 mesh) were purchased from Merck/Sigma-Aldrich (Darmstadt, Germany).

Roswell Park Memorial Institute medium (RPMI-1640), Dulbecco's modified Eagle medium (DMEM), streptomycin (100 mg/mL), penicillin (100 U/mL), D-glucose, cumene hydroperoxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and phosphate-buffered saline (PBS) one tablet/L buffer without calcium and magnesium were provided from Sigma-Aldrich (St. Louis, MO). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was obtained from Molecular Probes (Eugene, OR). Sterile plasticware for cell culture was obtained from Falcon Brand (San Diego, CA); fetal bovine serum was from GIBCO (Grand Island, NY).

2.2. Organic Synthesis. An amount of 3 g of tyrosol (**1**) was dissolved in 86.9 mL of methanol (21 mmol) and then 4.6 g of *N*-bromosuccinimide (NBS) was added to the mixture. The reaction was proceeded at room temperature (RT) until the bromuration was completed (about 3 h). The monitoring of the reaction was accomplished by TLC using *n*-hexane/ethyl acetate 1:1 v/v as a mobile phase. Once the bromuration was complete, the solvent was eliminated at low pressure, and then 20 mL of diethyl ether was added to obtain a partial purification by decantation. The mixture was evaporated at low pressure and the purification by diethyl ether was repeated. The product was constituted by 2-bromo-4- (2-hydroxyethyl)-phenol (4.49 g, 20 mmol, 98%) as a yellow oil (**2**).

An amount of 2.157 g of 2-bromo-4-(2-hydroxyethyl)-phenol (9.9 mmol) (**2**) was dissolved in 14.6 mL of dimethyl carbonate (DMC). The mixture was put under stirring while heating at 100 °C, the copper complex (0.867 g CuBr, 6 mmol) together with 55.63 mL of CH_3ONa/CH_3OH 25% w/v were added, and then the mixture was allowed to reflux for 15 h. The monitoring of the reaction was accomplished by TLC using *n*-hexane/ethyl acetate 1:1 v/v as a mobile phase. Once the methoxylation was complete, the mixture was initially cooled to RT and then placed in an ice bath. HCl 6 N was added to obtain a weakly acidic pH, and then the solvent was eliminated at low pressure. The aqueous residue was extracted using diethyl ether, the extract was washed by a saturated NaCl solution, and then the organic extract was dried over anhydrous sodium sulfate. The solvent was removed at low pressure obtaining a pale-yellow oil constituted by a mixture of 4-(2-hydroxyethyl)-2-methoxyphenol (**3**) and its methyl carbonate derivative (**4**).

The mixture of 4-(2-hydroxyethyl)-2-methoxyphenol (**3**) and its methyl carbonate derivative (**4**) was subjected to basic hydrolysis by adding 100 mL of K_2CO_3/CH_3OH 1% w/v and stirring at RT for 12 h. The monitoring of the reaction was accomplished by TLC using *n*-hexane/ethyl acetate 1:1 v/v as a mobile phase. Once the reaction was complete, the solvent was eliminated at low pressure and the aqueous residue was extracted using cold ethyl acetate. The extract was washed using a saturated NaCl solution, the organic extract was then dried over anhydrous Na_2SO_4, and the solvent was eliminated at low pressure. Any remaining dibrominated residue was removed by column chromatography (n-hexane: ethyl acetate 1:1 v/v). The product obtained (**3**) was homovanillic alcohol (1.43 g, 8.46 mmol, 86%).

An amount of 0.3 g (1.79 mmol) of homovanillic alcohol (**3**) was heated for 12 h at 50 °C in 1 mL of acetic anhydride containing 0.1 mL of acetic acid. The monitoring of the reaction was accomplished by TLC using n-hexane/ethyl acetate 1:1 v/v as a mobile phase. Once the acetylation reaction was complete, the mixture was diluted by 1 mL of cold distilled water and extracted (four times) using 0.5 mL of ethyl acetate. The organic extract was washed three times using 0.15 mL of a saturated $NaHCO_3$ solution, then twice by 0.2 mL of a saturated NaCl solution, and then dried over anhydrous Na_2SO_4. The organic solvent was eliminated at low pressure at 40 °C, and the product obtained was 4-(2-acetoxyethyl)-3-methoxy-1-acetoxy-benzene (**5**), (0.306 g, 1.20 mmol, 67%).

An amount of 0.239 g 4-(2-acetoxyethyl)-1,2-dihydroxybenzene (**5**) was dissolved in 1 mL of CH_2Cl_2 at −20 °C in an inert atmosphere and 0.2 mL of BBr_3 was added dropwise. The reaction was monitored by TLC using n-hexane/ethyl acetate 1:1 v/v as a mobile phase. Once the reaction of demethylation/deacetylation was complete, quenching was obtained by adding crushed ice directly into the solution.

The reaction mixture was extracted by ethyl acetate five times, and then the organic phase was washed by a saturated NaCl solution. After checking that the pH was less than 4, the solution was dried over anhydrous Na_2SO_4. The organic solvent was eliminated at low pressure at 40 °C, and the product obtained was 4-(2-acetoxyethyl)-1,2-dihydroxybenzene (**6**), (0.186 g, 0.94 mmol, 78%).

An amount of 0.186 g of 4-(2-acetoxyethyl)-1,2-dihydroxybenzene (**6**) was dissolved in 0.95 mL of CH_2Cl_2 and added to 2.85 mL of HCl 2 M. The reaction was allowed to continue at RT for 24 h under magnetic stirring.

The monitoring of the reaction was accomplished by TLC using n-hexane/ethyl acetate 1:1 v/v as a mobile phase. Once the deacetylation reaction was complete, the organic solvent was eliminated at low pressure at 40 °C, and the product obtained was hydroxytyrosol (**7**), (0.140 g, 0.90 mmol, 96%).

2.3. Cells in Culture. L-6 myoblasts and human leukemic monocytes THP-1 were obtained from American Type Culture Collection (Rockville, MD). L-6 were seeded in 75 mL tissue culture flasks and grown in Dulbecco's modified Eagle's medium containing

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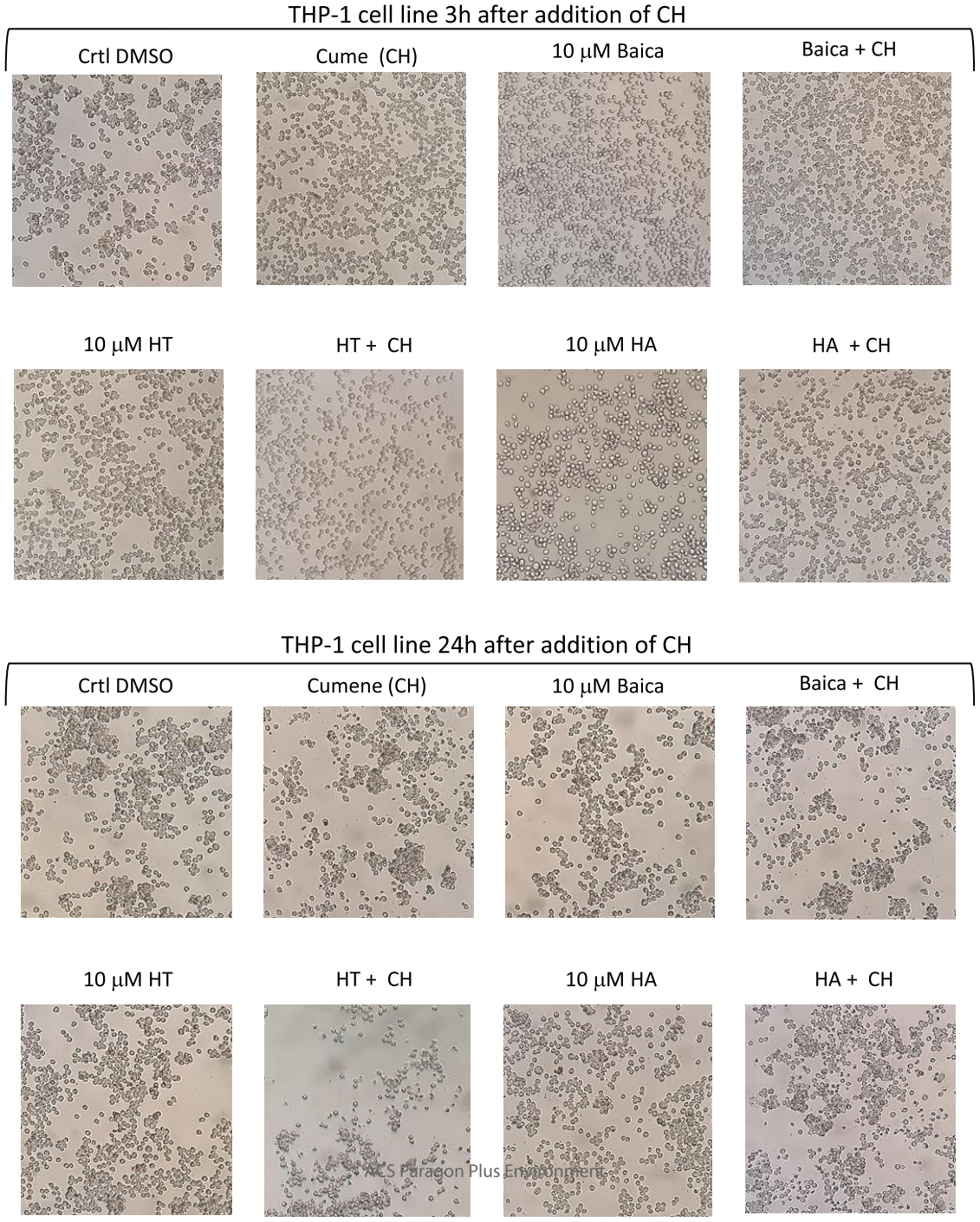

Figure 1. THP-1 cell images after pretreatment with baicalein, hydroxytyrosol, homovanillic alcohol, and the radical generator cumene hydroperoxide. Magnification 400×.

4.5 g/L glucose, supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin in a humidified atmosphere with 5% CO_2 at 37 °C. Cells reached confluency after 5 days (approximately 6×10^6 cells/flask) and were kept in culture as myoblasts by continuously passaging at preconfluent stages.[1]

Human leukemic monocytes THP-1 were grown in suspension in an RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin in a humidified atmosphere with 5% CO_2 at 37 °C.[1,30] THP-1 monocytes were passaged twice a week by 1:4 dilutions and reseeded in 25 mL tissue culture flasks.

2.4. Proliferation Studies. L-6 cells were seeded in 60 × 15 mm^2 Petri dishes in 4 mL of the complete growth medium at a density of approximately 5×10^4 cells/dish as specified under Materials and Methods section. The following day, the cells were treated with HT and HA at a final concentration of 1 μM and 10 μM. Cell counts using a Neubauer chamber were made every 24 h up to confluence after mild trypsinization.[31]

THP-1 cells were seeded in 12-well plates (1×10^5 cells/well) in 1 mL of the growth medium supplemented as reported in Materials and Methods section. After 24 h, the cells were treated with HT and HA at the same concentrations specified in the previous paragraph and counted up to 96 h from seeding using a Neubauer chamber.

2.5. Intracellular ROS Determination. The method used was a standard assay based on the intracellular fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA).[30] THP-1 mono-

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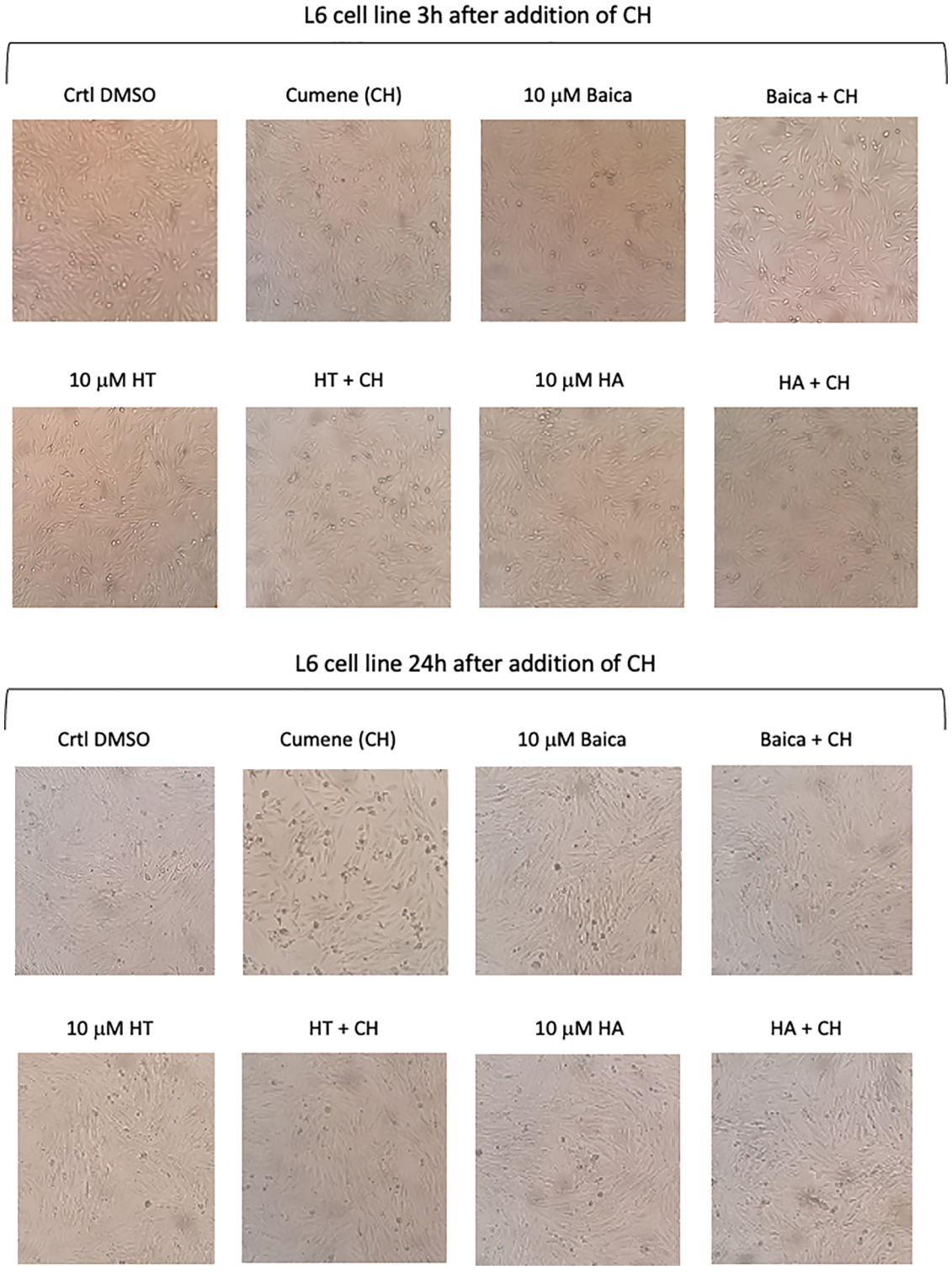

Figure 2. L-6 cell images after pretreatment with baicalein, hydroxytyrosol, homovanillic alcohol, and the radical generator cumene hydroperoxide. Magnification 400×.

cytes were resuspended and centrifuged at 180*g* for 10 min; the supernatant was discarded and cells were washed twice with 5 mL of PBS−glucose at 37 °C to remove the serum that might affect the action of the fluorescent probe. After the final centrifugation, the pellet was resuspended in PBS−glucose and H_2DCF-DA was added at 10 μM final concentration (from a stock solution of 10 mM in dimethyl sulfoxide (DMSO)). The cells were then left to incorporate the fluorescent probe for 30 min in the dark at 37 °C. At the end of the incubation, cells were washed twice, centrifuged at 180*g* for 5 min, and the final cell pellet was resuspended in PBS−glucose. Before the experiments, cells recovered at RT for 1 h. ROS production was measured in a Perkin-Elmer Multilabel Counter Victor3V Wallac 1420 by measuring the intracellular DCF fluorescence at the excitation and emission wavelengths of the probe (498 and 530 nm, respectively). We measured the ability of HT and HA to buffer ROS production in cells exposed to the radical generator cumene hydroperoxide (CH; 200 μM) at different times (30 min, 1, 3, and 24 h). Before addition of CH, the cells were preincubated with

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different concentrations of HT or HA (0.01, 0.1, 1, and 10 μM) at 37 °C for 10 min.

2.6. Cell Images after Pretreatment with Baicalein, Hydroxytyrosol, Homovanillic Alcohol, and the Radical Generator Cumene Hydroperoxide. The protocol applied was the same as that used for ROS determination and the images were obtained after 3 and 24 h because these are the times when the effect of the treatments is best appreciated on the cell morphology (Figures 1 and 2).

L-6 myoblasts and THP-1 monocytes were seeded in 24-multiwell plates, 10^5 cells/mL or well and 4×10^5/mL or well, respectively, in a complete medium. After 24 h, the cells were pretreated with either baicalein or hydroxytyrosol or homovanillic alcohol at 10 μM for 10 min, and then cumene hydroperoxide was given. For both the cell types, the images were taken at 3 and 24 h at magnification 400×.

2.7. MTT Assay. Cell viability and the potential cytotoxic effect of HT and HA were assessed by the MTT assay. L-6 cells were seeded in 96-wells plates at 1×10^4 cells/well in 200 μL of DMEM containing 10% serum. The day after seeding, the medium was discarded and 100 μL of the new medium containing cumene hydroperoxide (27.5 μM)[30] with or without HT and HA at different concentrations was added to each well, depending on the specific experimental condition, as described above. Then, an MTT solution (5 mg/mL in PBS) was added at a final concentration of 10% with respect to the total volume, and incubation was carried out at 37 °C for 3−4 h in the dark. During the incubation, there was a conversion of the yellow MTT to purple formazan by the mitochondrial succinate dehydrogenase of living cells. Then, a lysis buffer (DMSO containing ammonia)[32] was added and further incubation at 37 °C for 30 min in the dark was carried out. Cells were then resuspended, and the optical density was read with an ELISA-reader at 550−570 nm. Results are reported as mean ± standard deviation (SD) of two experiments carried out in quintuplicate.

2.8. Statistical Analysis. All data obtained from cultured cells were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's multiple comparison test. In some cases, Student's *t*-test was applied. Analyses were carried out using the Prism 4.0 statistics program (GraphPad, San Diego, CA). Differences were considered significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

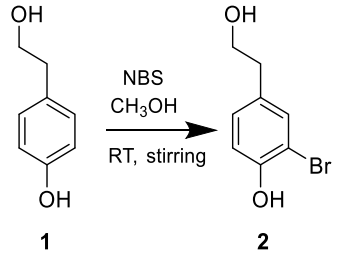
In the course of this study, the synthesis of homovanillic alcohol (HA) and hydroxytyrosol (HT) from tyrosol (TY), a byproduct from the production of olive oil, has been examined.[33] The effect of HA and HT on ROS production and on cell proliferation in THP-1 and L-6 cell lines was also evaluated. THP-1 cells are leukemic monocytes from peripheral blood; the monocytes defend the body against infections by killing foreign cells through the production of large amounts of ROS.

L-6 cells from rat skeletal muscle are able to differentiate from myoblasts in myotubes when they reach confluency. The contraction of skeletal muscles requires low levels of ROS, but the presence of ROS at high levels may result in contractile dysfunction and fatigue.[34−36]

The synthesis of HA and HT was accomplished using a sequence of simple reactions of bromination and alkoxylation from TY. The approach is to oxyfunctionalize the aromatic rings of natural substances to increase their biological activity; in particular, the strategy adopted involves the methoxylation of aromatic bromides.

3.1. HA Synthesis. NBS allows TY (**1**) bromination in the ortho-phenolic position (Scheme 1). To avoid the further bromination of TY (**1**) in the second activated position of the ring, the addition of NBS is carried out in three aliquots to keep the reagent as a limiting factor. Given that the second bromination is slower, this procedure allows the obtainment

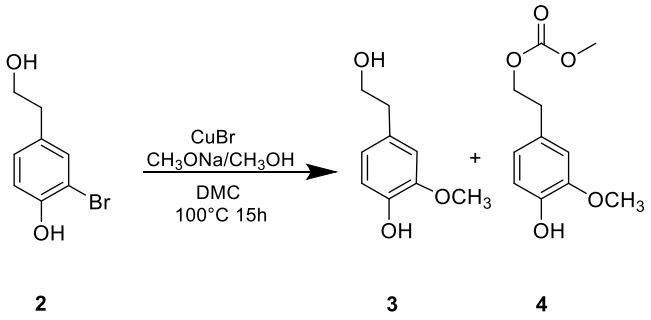
Scheme 1. Synthesis of 2-Bromo-4-(2-hydroxyethyl)-phenol (2)



of only the monohalogenated product through an electrophilic aromatic substitution. The conversion of TY to 2-bromotyrosol (**2**) under these reaction conditions reaches yields higher than 95%.

The brominated TY (**2**) is the substrate of the methoxylation reaction that is conducted using DMC as a solvent (Scheme 2). The use of DMC as a solvent is a safe

Scheme 2. Synthesis of 4-(2-Hydroxyethyl)-2-methoxyphenol (HA) (3)



and environmentally friendly replacement for dimethylformamide since it does not produce inorganic salts in either acylation or alkylation reactions. Moreover, DMC is synthesized through a clean process.[37]

However, in these conditions, the reaction led not only to the formation of HA (**3**) but also to its side-chain methyl carbonate (**4**). This compound is obtained through the reaction between the alkoxylated HA and dimethyl carbonate (DMC). The reaction is a nucleophilic acyl substitution via a base-catalyzed alkyl cleavage bimolecular mechanism[37] (Scheme 2).

Scheme 7 represents the proposed reaction mechanism of a coupling reaction mediated by copper(I) salt.

The mechanism involves the oxidative addition of copper(I) to the Ar−Br bond, the coordination of the methoxide, and the formation of a copper complex (III). The reaction ends with a reductive elimination, which provides the product of coupling Ar−OCH_3 and brings the copper to the previous state of oxidation (+1). Other methods for the etherification of aryl bromides are reported in the literature, such as through the catalysis using an expensive palladium complex.

To transform the HA side-chain methyl carbonate (**4**) in HA (**3**), the mixture of these compounds was subjected to a basic hydrolysis reaction by a solution of K_2CO_3 (Scheme 3). HA (**3**) was used both as such in the subsequent biological tests on THP-1 cells and as an intermediate for the synthesis of HT (**7**), which, in turn, was used in the biological tests.

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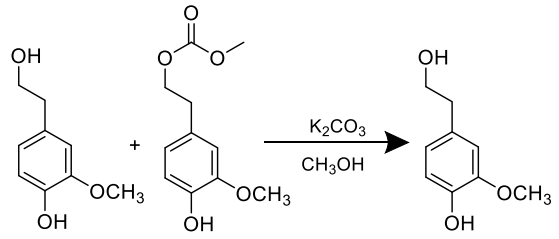
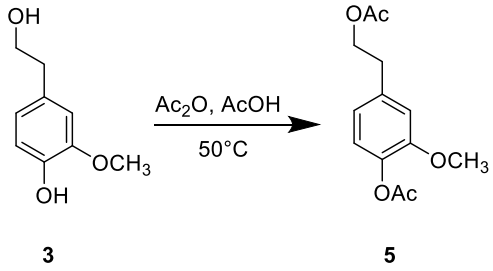
Scheme 3. Hydrolysis of Carbonate Derivative (4)



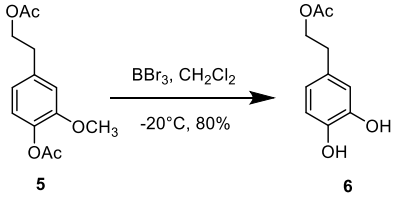
3.2. HT Synthesis. The transformation of HA (**3**) into HT (**7**) requires the protection of hydroxyl groups through acetylation. The reaction was accomplished using acetic anhydride and acetic acid, which do not require tedious work-up (Scheme 4).

Scheme 4. Synthesis of 4-(2-Acetoxyethyl)-3-methoxy-1-acetoxy-benzene (5)



The acetylated product (**5**) was subjected to a demethylation reaction obtaining the product (**6**) shown in Scheme 5,

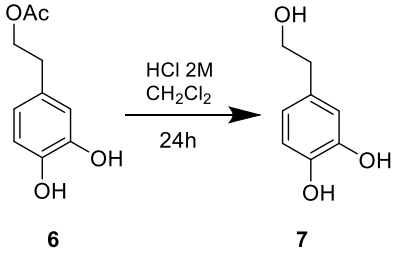
Scheme 5. Synthesis of 4-(2-Acetoxyethyl)-1,2-dihydroxybenzene (6)



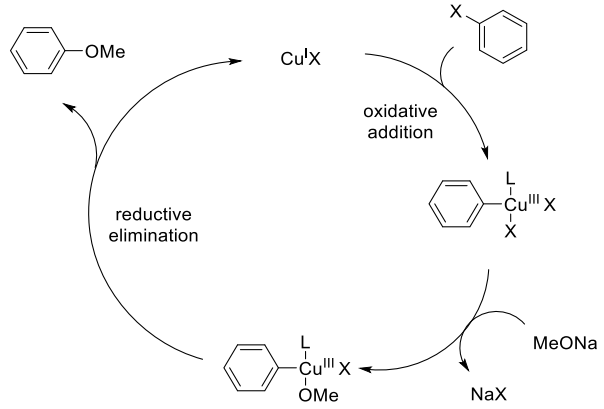
thanks to simultaneous selective deacetylation of the acetoxy group on the aromatic ring. The hydrolysis on the product (**6**) was accomplished using 2 M HCl in CH_2Cl_2, leading to the formation of hydroxytyrosol (**7**) (Schemes 6 and 7).

3.3. Effect of HT and HA on ROS Production in THP-1 Human Leukemic Monocytes. The THP-1 human

Scheme 6. Synthesis of Hydroxytyrosol (HT) (7)



Scheme 7. Reaction Mechanism of a Coupling Reaction Mediated by Copper(I) Salt



leukemic monocytes are very sensitive to ROS due to their nature of cells responsible for the immune defense against infections, thus representing the killing machine of the body. HT and HA were tested in a wide concentration range (0.01−10 μM) to investigate their effect on ROS production. The experiments were carried out at a time course of 30 min, 1, 3, and 24 h (Figures 3 and 4), either alone or in combination with CH, a known radical generator. Baicalein (BAI), a very effective antioxidant compound, was used to compare its scavenger action with respect to HT and HA.[30,38]

In THP-1 cells, HT (0.1 μM) was able to inhibit ROS generation in the presence of CH (Figure 3) at 30 min ($p < 0.05$), although the most significant effect was at 10 μM at all time points, being more effective at 24 h ($p < 0.001$). The HT molecule is strongly reactive toward ROS, and therefore capable of a very prompt action; the more effective concentrations were 1 and 10 μM. On the other hand, because of its high reactivity, this molecule could undergo a lowering of the active concentration in contexts where there is a high presence of ROS, so with the prolongation of the incubation period, a greater HT concentration is necessary to obtain a significant effect.

HA is able to inhibit ROS generation in THP-1 human leukemic monocytes (Figure 4); the inhibition started after 30 min of incubation ($p < 0.05$ with HA 1 μM; $p < 0.001$ with HA 10 μM) and was more efficient at 24 h in cells treated with HA at 10 μM ($p < 0.001$). The efficacy of HT and HA in inhibiting the generation of ROS is similar to that shown by baicalein at the same concentration (10 μM). A lower inhibition of ROS production shown by HA compared to HT could be due to the presence of a methoxy group, which confers rigidity and lowers the reactivity to the HA molecule. In addition, the HA molecule is hydrolyzed in the cells, giving rise to HT, thus the antioxidant effect observed following the treatment with HA is, at least in part, due to the HT formed by the demethylation of HA.[39]

3.4. Effect of HT and HA on ROS Generation in L-6 Cells. L-6 myoblasts require low levels of ROS for the contraction of skeletal muscles but the presence of ROS at high levels results in contractile dysfunction.[35,36,40]

As shown in Figure 5, HT significantly inhibits the generation of ROS in L-6 cells stimulated with cumene hydroperoxide for 1 h ($p < 0.05$ with HT 10 μM) and up to 24 h ($p < 0.001$ with HT at 1 and 10 μM).

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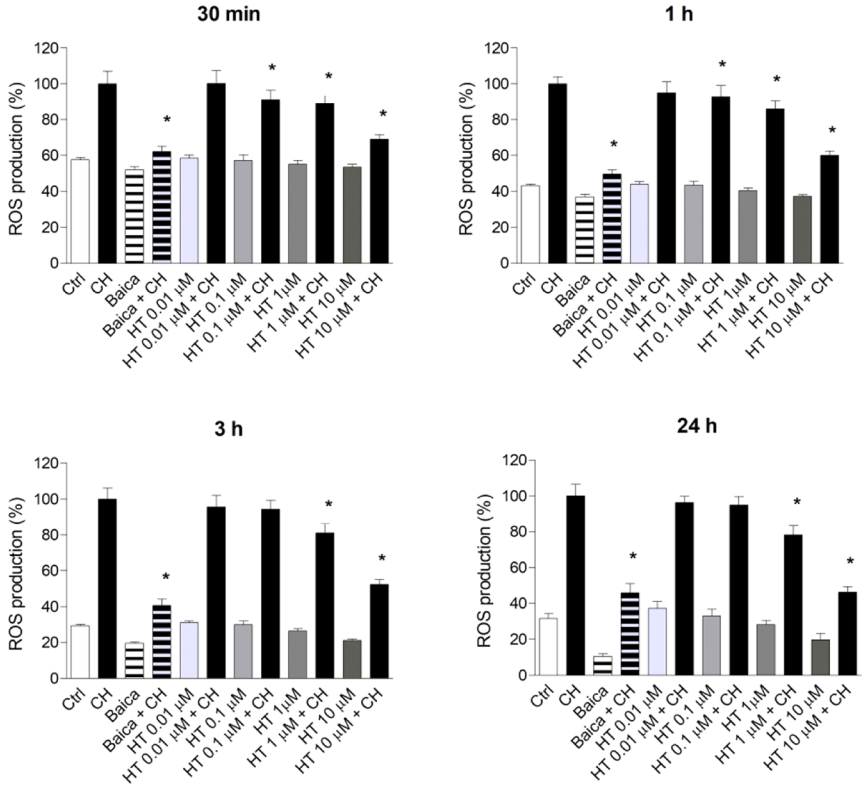

Figure 3. Dose-response effect of hydroxytyrosol (HT) on ROS production in THP-1 cells after 30 min, 1, 3, and 24 h from stimulation with cumene hydroperoxide (CH). The results are mean ± SD of four to five different experiments carried out in quadruplicate. *$p < 0.01$, at least vs CH.

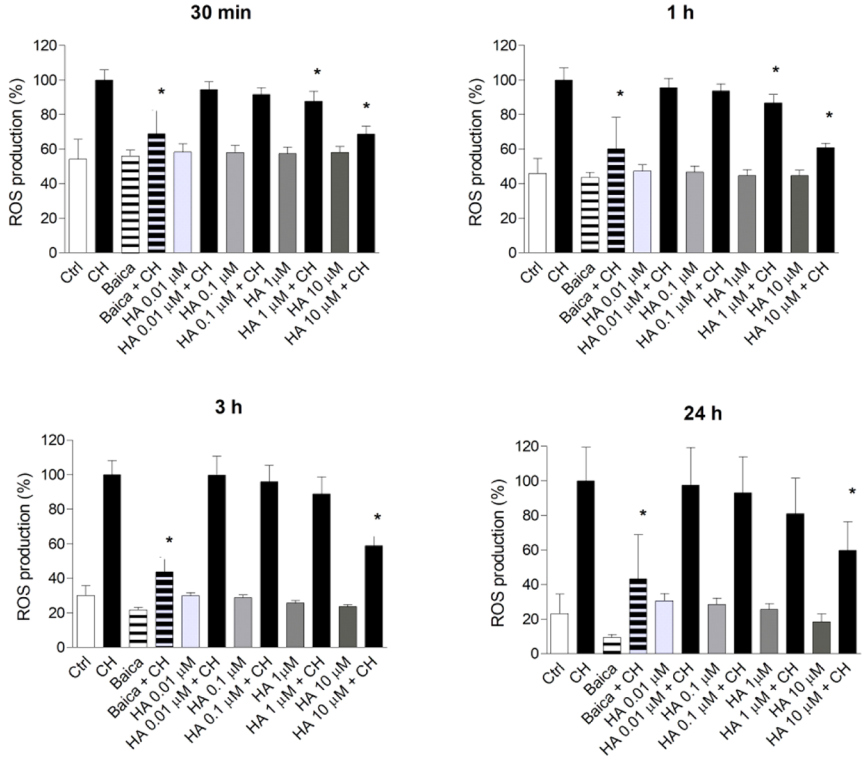

Figure 4. Dose-response effect of homovanillic alcohol (HA) on ROS generation in THP-1 cells after 30 min, 1, 3, and 24 h from stimulation with cumene hydroperoxide (CH). The data are mean ± SD of two different experiments carried out in quadruplicate. *$p < 0.05$, at least vs CH.

The inhibition by HT at 10 μM of the production of ROS is similar to that of baicalein at the same concentration in cells stimulated with CH. In the untreated cells, baicalein showed an effect by itself on basal ROS production; a similar effect

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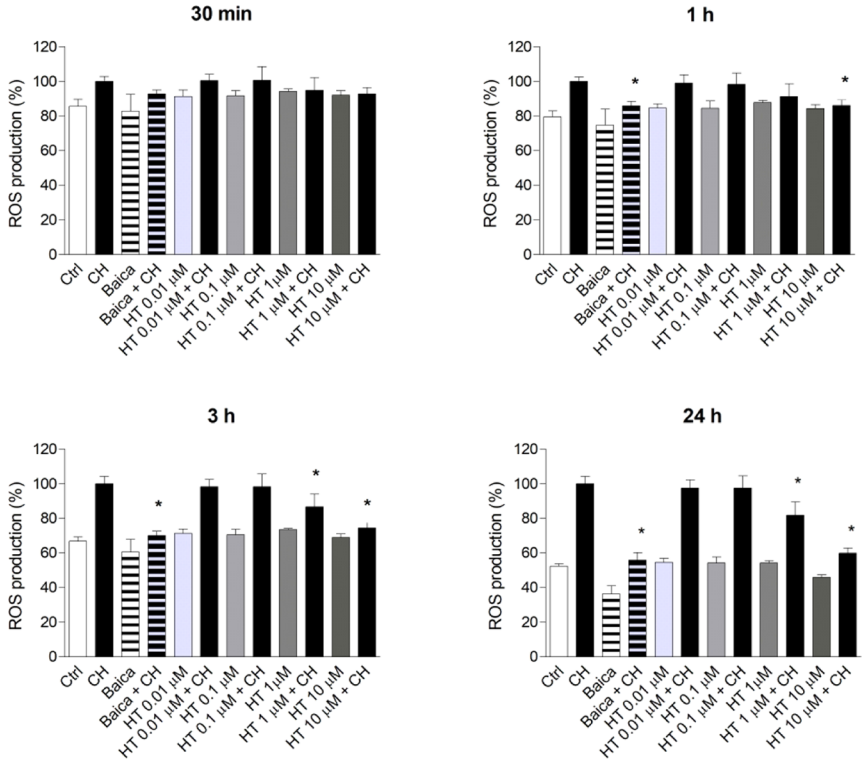

Figure 5. Effect of hydroxytyrosol (HT) at different concentrations on ROS generation in L-6 cells after 30 min, 1, 3, and 24 h from stimulation with cumene hydroperoxide (CH). The data are mean ± SD of two different experiments carried out in quadruplicate. *$p < 0.05$, at least vs CH.

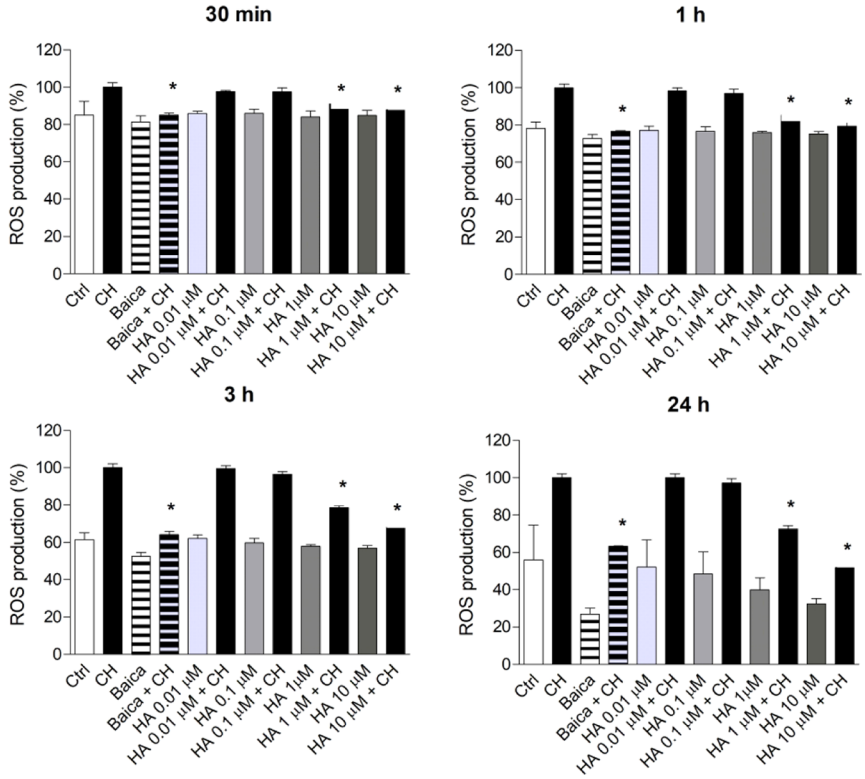

Figure 6. Dose-response effect of homovanillic alcohol (HA) on ROS production in L-6 myoblasts after 30 min, 1, 3, and 24 h from stimulation with cumene hydroperoxide (CH). The data are mean ± SD of two different experiments carried out in quadruplicate. *$p < 0.01$, at least vs CH.

was observed in THP-1 monocytes. We do not know at present why, but it could depend on some stress of the cells due to the experimental procedure or a basal activity of ROS production.

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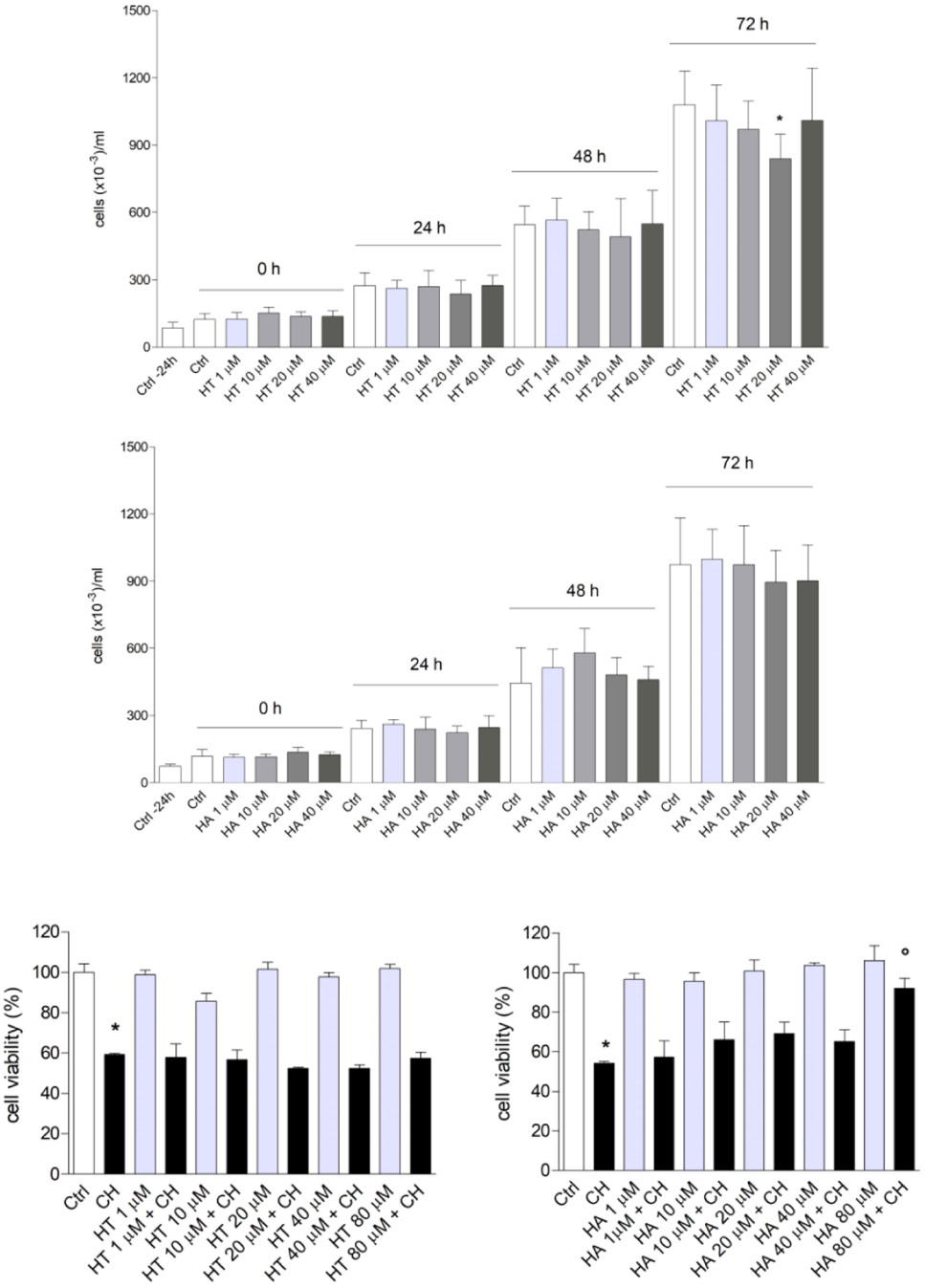

Figure 7. Dose-response effect of HT and HA on the proliferation of THP-1 human leukemic monocytes (upper and center panels). The THP-1 cells growing in suspension were seeded in 24-well plates with 1 mL of the complete medium. Cells were stimulated with either HT or HA 24 h from seeding and then counted every 24 h up to optimal density (96 h). Results are mean ± SD of three different experiments carried out in duplicate. *$p < 0.05$ vs control, as from Student's *t*-test. The cytotoxicity/proliferation (MTT) assay (lower panel) was carried out with either HT or HA in L-6 myoblasts. The concentration of cumene hydroperoxide (CH) was 27.5 μM throughout the experiments. Results are mean ± SD of two different experiments carried out in quintuplicate. *$p < 0.001$ vs control and either HT or HA alone; °$p < 0.001$ vs CH.

HA is able to inhibit ROS both in cells stimulated with CH as well as in those not stimulated at 24 h (Figure 6).

In the first group, ROS inhibition is evident after 24 h of incubation ($p < 0.05$) with HT 10 μM. In the cells stimulated with CH, ROS inhibition takes place after 30 min ($p < 0.05$ HA 1 μM; $p < 0.001$ HA 10 μM) and remains at the same level until 24 h.

The lower concentration of ROS in the cellular environment of muscle cells compared to that of leukemic monocytes might explain the lower efficacy of HT on L-6 cells in the first 3 h of incubation. HT demonstrates the same efficacy on THP-1 cells and L-6 cells after 24 h of incubation in the presence of CH, when radical generation is also remarkable in L-6 cells (Figures 5 and 6).

The rigidity and the lower reactivity of the HA with respect to the HT molecule, in this case, are advantageous as they allow a greater resistance and durability. In this way, HA can be available even after long incubation times (24 h in this case).

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3.5. Proliferation Curves and Cell Viability/Cytotoxicity Assay. The effect of HT and HA on the proliferation of THP-1 monocytes and L-6 myoblasts was tested at concentrations from 1 to 40 μM. A significant effect was evident only in THP-1 cells at 20 μM HT at 72 h, so for the sake of simplicity, only THP-1 is reported in the graphic (Figure 7, upper and center panels). The proliferation of L-6 myoblasts was not significantly affected by either HT or HA and it is not shown.

The possible cytotoxic effect of HT and HA as well as their effect on cell viability were assessed by the MTT assay on L-6 myoblasts (Figure 7, lower panel). No cytotoxic effect was found for either HT or HA. HA (80 μM) was able to protect the cells from oxidative stress but the other concentrations did not protect the L-6 myoblasts from cumene hydroperoxide, in good agreement with previously published data.[16]

In this study, tyrosol, a phenol abundant in olives and a polluting waste product from the oil industry,[41] has been transformed into value-added products. In particular, tyrosol was transformed into HA and HT. These phenolic molecules are present in plants and have demonstrated remarkable biological activity in the considered system. Hydroxytyrosol and homovanillic alcohol are metabolites of dopamine and both endogenous and exogenous derived products may be found in the plasma from both sources and modulate cell activities.[42]

The value of tyrosol, a product with low biological activity and high polluting power that must be disposed of according to special procedures as required by law, has thus been enhanced. The procedures used in the synthesis are characterized by high efficiency and low cost or in one word, are sustainable; moreover, they can become part of the circular economy of olive oil production.[43]

The yield was high for both HA and HT, being >80 and >42%, respectively. Our data show that HT and HA derived from tyrosol of OMW have antioxidant and biological activities in cells in culture similar to that of the compounds commercially available or derived from other more conventional sources.

In particular, HA gave full protection from oxidative insult given by cumene hydroperoxide at the MTT assay, whereas HT (20 μM) was capable of killing tumor cell THP-1 monocytes.

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Notes

The authors declare no competing financial interest.

ABBREVIATION USED

ROS, reactive oxygen species; EVOO, extra-virgin olive oil; OLE, oleuropein; HT, hydroxytyrosol; DPPH, 2,2-diphenyl-1-picryhydrazyl; HA, homovanillic alcohol; OMW, olive mill wastewater; TY, tyrosol; DMC, dimethyl carbonate; TLC, thin-layer chromatography; NBS, *N*-bromosuccinimide; CH, cumene hydroperoxide; PBS, phosphate-buffered saline; RT, room temperature

REFERENCES

(1) Rossi, M.; Caruso, F.; Kwok, L.; Lee, G.; Caruso, A.; Gionfra, F.; Candelotti, E.; Belli, S. L.; Molasky, N.; Raley-Susman, K. M.; Leone, S.; Filipsky, T.; Tofani, D.; Pedersen, J.; Incerpi, S. Protection by extra virgin olive oil against oxidative stress *in vitro* and *in vivo*. Chemical and biological studies on the health benefits due to a major component of the Mediterranean diet. *PLoS One* **2017**, *12*, No. e0189341.

(2) Gorzynik-Debicka, M.; Przychodzen, P.; Cappello, F.; Kuban-Jankowska, A.; Marino Gammazza, A.; Knap, N.; Wozniak, M.; Gorska-Ponikowska, M. Potential Health Benefits of Olive Oil and Plant Polyphenols. *Int. J. Mol. Sci.* **2018**, *19*, 686.

(3) Williams, R. J.; Spencer, J. P.; Rice-Evans, C. Flavonoids: antioxidants or signalling molecules? *Free Radical Biol. Med.* **2004**, *36*, 838−849.

(4) Stevenson, D. E.; Hurst, R. D. Polyphenolic phytochemicals–just antioxidants or much more? *Cell. Mol. Life Sci.* **2007**, *64*, 2900−2916.

(5) Briante, R.; Patumi, M.; Limongelli, S.; Febbraio, F.; Vaccaro, C.; Di Salle, A.; La Cara, F.; Nucci, R. , Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of *Olea europaea*. *Plant Sci.* **2002**, *162*, 791−798.

(6) Aruoma, O. I. Antioxidant actions of plant foods: use of oxidative DNA damage as a tool for studying antioxidant efficacy. *Free Radical Res.* **1999**, *30*, 419−427.

(7) Cicerale, S.; Lucas, L.; Keast, R. Biological activities of phenolic compounds present in virgin olive oil. *Int. J. Mol. Sci.* **2010**, *11*, 458−479.

(8) Lee, O. H.; Lee, B. Y.; Lee, J.; Lee, H. B.; Son, J. Y.; Park, C. S.; Shetty, K.; Kim, Y. C. Assessment of phenolics-enriched extract and fractions of olive leaves and their antioxidant activities. *Bioresour. Technol.* **2009**, *100*, 6107−6113.

(9) Hassen, I.; Casabianca, H.; Hosni, K. Biological activities of the natural antioxidant oleuropein: Exceeding the expectation − A mini-review. *J. Funct. Foods* **2015**, *18*, 926−940.

https://dx.doi.org/10.1021/acs.jafc.0c05230
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

(10) Servili, M.; Taticchi, A.; Esposto, S.; Urbani, S.; Selvaggini, R.; Montedoro, G. Effect of olive stoning on the volatile and phenolic composition of virgin olive oil. *J. Agric. Food Chem.* **2007**, *55*, 7028−7035.
(11) De Leonardis, A.; Macciola, V.; Cuomo, F.; Lopez, F. Evidence of oleuropein degradation by olive leaf protein extract. *Food Chem.* **2015**, *175*, 568−574.
(12) López de las Hazas, M. C.; Piñol, C.; Macià, A.; Romero, M. P.; Pedret, A.; Solà, R.; Rubió, L.; Motilva, M. Differential absorption and metabolism of hydroxytyrosol and its precursors oleuropein and secoiridoids. *J. Funct. Foods* **2016**, *22*, 52−63.
(13) Dávalos, J. Z.; Valderrama, A. C.; Barrios, J. R.; Freitas, V. L. S.; Ribeiro da Silva, M. Energetic and Structural Properties of Two Phenolic Antioxidants: Tyrosol and Hydroxytyrosol. *J. Phys. Chem. A* **2018**, *122*, 4130−4137.
(14) Meschini, R.; D'Eliseo, D.; Filippi, S.; Bertini, L.; Bizzarri, B. M.; Botta, L.; Saladino, R.; Velotti, F. Tyrosinase-Treated Hydroxytyrosol-Enriched Olive Vegetation Waste with Increased Antioxidant Activity Promotes Autophagy and Inhibits the Inflammatory Response in Human THP-1 Monocytes. *J. Agric. Food Chem.* **2018**, *66*, 12274−12284.
(15) Visioli, F.; Colombo, C.; Galli, C. Differential partitioning of antioxidants, including hydroxytyrosol, in human plasma and LDL: implications for their antioxidant activity in vivo. *Food Chem.* **2012**, *132*, 499−501.
(16) Bernini, R.; Barontini, M.; Cis, V.; Carastro, I.; Tofani, D.; Chiodo, R. A.; Lupattelli, P.; Incerpi, S. Synthesis and Evaluation of the Antioxidant Activity of Lipophilic Phenethyl Trifluoroacetate Esters by In Vitro ABTS, DPPH and in Cell-Culture DCF Assays. *Molecules* **2018**, *23*, 208.
(17) Kumar, N.; Gorai, B.; Saurabh, G. S.; Goel, N. Extrapolation of hydroxytyrosol and its analogues as potential anti-inflammatory agents. *J. Biomol. Struct. Dyn.* **2020**, 1−12.
(18) Deiana, M.; Incani, A.; Rosa, A.; Corona, G.; Atzeri, A.; Loru, D.; Melis, P.; Dessi, M. A. Protective effect of hydroxytyrosol and its metabolite homovanillic alcohol on H_2O_2-induced lipid peroxidation in renal tubular epithelial cells. *Food Chem. Toxicol.* **2008**, *46*, 2984−2990.
(19) Incani, A.; Deiana, M.; Corona, G.; Vafeiadou, K.; Vauzour, D.; Dessi, M. A.; Spencer, J. P. Involvement of ERK, Akt and JNK signalling in H_2O_2-induced cell injury and protection by hydroxytyrosol and its metabolite homovanillic alcohol. *Mol. Nutr. Food Res.* **2010**, *54*, 788−796.
(20) Bernini, R.; Mincione, E.; Barontini, M.; Crisante, F. Convenient synthesis of hydroxytyrosol and its lipophilic derivatives from tyrosol or homovanillyl alcohol. *J. Agric. Food Chem.* **2008**, *56*, 8897−8904.
(21) Piersanti, G.; Retini, M.; Espartero, J. L.; Madrona, A.; Zappia, G. An efficient, economical synthesis of hydroxytyrosol and its protected forms via Baeyer−Villiger oxidation. *Tetrahedron Lett.* **2011**, *52*, 4938−4940.
(22) Deffieux, D.; Gossart, P.; Quideau, S. Facile and sustainable synthesis of the natural antioxidant hydroxytyrosol. *Tetrahedron Lett.* **2014**, *55*, 2455−2458.
(23) Nam, H.; Kim, M. M. Eugenol with antioxidant activity inhibit MMP9 related to metastasis in human fibrosarcoma cells. *Food Chem. Toxicol.* **2013**, *55*, 106−112.
(24) Li, X.; Chen, Z.; Wu, Y.; Yan, Y.; Sun, X.; Yuan, Q. Establishing an artificial pathway for efficient biosynthesis of hydroxytyrosol. *ACS Synth. Biol.* **2018**, *7*, 647−654.
(25) Chen, W.; Yao, J.; Meng, J.; Han, W.; Tao, Y.; Chen, Y.; Guo, Y.; Shi, G.; He, Y.; Jin, J. M.; Tang, S. Y. Promiscuous enzymatic activity-aided multiple-pathway network design for metabolic flux rearrangement in hydroxytyrosol biosynthesis. *Nat. Commun.* **2019**, *10*, No. 960.
(26) Capasso, R.; Evidente, A.; Avolio, S.; Solla, F. A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural waste waters. *J. Agric. Food Chem.* **1999**, *47*, 1745−1748.
(27) Khymenets, O.; Joglar, J.; Clapés, P.; Parella, T.; Covas, M.-I.; de la Torre, R. Biocatalyzed synthesis and structural characterization of monoglucuronides of hydroxytyrosol, tyrosol, homovanillic alcohol, and 3-(4′-hydroxyphenyl) propanol. *Adv. Synth. Catal.* **2006**, *348*, 2155−2162.
(28) Soil Science Institute of Athens-ELGO DEMETER. Prosodol Integrated strategy of actions, measures and means suitable for Mediterranean countries, Analysis of national and European legislative frameworks for Oil Olive Waste and Soil Protection; April 2012.
(29) Allouche, N.; Fki, I.; Sayadi, S. Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters. *J. Agric. Food Chem.* **2004**, *52*, 267−273.
(30) Lombardo, E.; Sabellico, C.; Hajek, J.; Stankova, V.; Filipsky, T.; Balducci, V.; De Vito, P.; Leone, S.; Bavavea, E. I.; Silvestri, I. P.; Righi, G.; Luly, P.; Saso, L.; Bovicelli, P.; Pedersen, J. Z.; Incerpi, S. Protection of cells against oxidative stress by nanomolar levels of hydroxyflavones indicates a new type of intracellular antioxidant mechanism. *PLoS One* **2013**, *8*, No. e60796.
(31) Tofani, D.; Balducci, V.; Gasperi, T.; Incerpi, S.; Gambacorta, A. Fatty Acid Hydroxytyrosyl Esters: Structure/Antioxidant Activity Relationship by ABTS and in Cell-Culture DCF Assays. *J. Agric. Food Chem.* **2010**, *58*, 5292−5299.
(32) Wang, H.; Wang, F.; Tao, X.; Cheng, H. Ammonia-containing dimethyl sulfoxide: an improved solvent for the dissolution of formazan crystals in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. *Anal. Biochem.* **2012**, *421*, 324−326.
(33) Yadav, T. C.; Kumar, N.; Raj, U.; Goel, N.; Kumar Vardawaj, P.; Prasad, R.; Pruthi, V. Exploration of interaction mechanism of tyrosol as potent antiinflammatory agent. *J. Biomol. Struct. Dyn.* **2020**, *38*, 382−397.
(34) Reid, M. B. Invited Review: redox modulation of skeletal muscle contraction: what we know and what we don't. *J. Appl. Physiol.* **2001**, *90*, 724−731.
(35) Powers, S. K.; Jackson, M. J. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol. Rev.* **2008**, *88*, 1243−1276.
(36) Powers, S. K.; Ji, L. L.; Kavazis, A. N.; Jackson, M. J. Reactive oxygen species: impact on skeletal muscle. *Compr. Physiol.* **2011**, DOI: 10.1002/cphy.c100054.
(37) Tundo, P.; Musolino, M.; Aricò, F. The reactions of dimethyl carbonate and its derivatives. *Green Chem.* **2018**, *20*, 28−85.
(38) Caioli, S.; Candelotti, E.; Pedersen, J. Z.; Saba, L.; Antonini, A.; Incerpi, S.; Zona, C. Baicalein reverts L-valine-induced persistent sodium current up-modulation in primary cortical neurons. *Biochim. Biophys. Acta, Mol. Basis Dis.* **2016**, *1862*, 566−575.
(39) Caruso, F.; Mendoza, L.; Castro, P.; Cotoras, M.; Aguirre, M.; Matsuhiro, B.; Isaacs, M.; Rossi, M.; Viglianti, A.; Antonioletti, R. Antifungal activity of resveratrol against *Botrytis cinerea* is improved using 2-furyl derivatives. *PLoS One* **2011**, *6*, No. e25421.
(40) Reid, M. B. Free radicals and muscle fatigue: Of ROS, canaries, and the IOC. *Free Radical Biol. Med.* **2008**, *44*, 169−179.
(41) Delisi, R.; Saiano, F.; Pagliaro, M.; Ciriminna, R. Quick assessment of the economic value of olive mill wastewater. *Chem. Cent. J.* **2016**, *10*, No. 63.
(42) de la Torre, R. Bioavailability of olive oil phenolic compounds in humans. *Inflammopharmacology* **2008**, *16*, 245−247.
(43) Abu Tayeh, H. N.; Azaizeh, H.; Gerchman, Y. Circular economy in olive oil production-Olive mill solid waste to ethanol and heavy metal sorbent using microwave pretreatment. *Waste Manage.* **2020**, *113*, 321−328.

https://dx.doi.org/10.1021/acs.jafc.0c05230
J. Agric. Food Chem. XXXX, XXX, XXX−XXX