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Verbascoside, Isoverbascoside, and Their Derivatives Recovered from Olive Mill Wastewater as Possible Food Antioxidants

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ABSTRACT: Olive oil processing industries generate substantial quantities of phenolic-rich byproducts, which could be valuable natural sources of antioxidants. This work is focused on the recovery and structural characterization of antioxidant compounds from olive mill wastewater (OMWW), a polluting byproduct of the olive oil production process. Phenolics were extracted from the waste material using a membrane technology coupled to low-pressure gel filtration chromatography on a Sephadex LH-20. The LH-20 fraction was, in turn, characterized for its phenolic composition by HPLC-DAD-MS/MS analyses. Verbascoside, isoverbascoside, β-hydroxyverbascoside, β-hydroxyisoverbascoside, and various oxidized phenolics were identified. Uptake of verbascoside, purified from the LH-20 fraction, by HT-29 cells, an established model system for studying drug transport properties, was also assayed. Finally, the antioxidant activities of the LH-20 fraction and verbascoside were characterized by two different techniques. Individual verbascoside was more active as a scavenger of reactive oxygen species and as a chemopreventive agent protecting low-density lipoproteins from oxidative damage than the LH-20 fraction.

KEYWORDS: *olive mill wastewater, verbascoside, isoverbascoside, β-hydroxyverbascoside, β-hydroxyisoverbascoside, HPLC-DAD-MS/MS, uptake, bioactivity*

INTRODUCTION

Olives (*Olea europaea* L.) have attracted considerable attention during the past few years as sources of biophenols useful for the food and pharmaceutical industries. Two different processes are used for the extraction of olive oil: a two-phase and a three-phase system. The three-phase mill uses large volumes of water to aid the separation of oil, and in this case, the waste formed is mainly what is known as olive mill wastewater (OMWW), a phytotoxic material that must be discarded. The phenolic fraction of olive oil comprises only 2% of the total phenolic content of the olive fruits, with the remainder being lost in olive mill waste, in the form of a solid waste (olive pomace) and an aqueous liquid (OMWW). Thus, OMWW is potentially a rich source of a diverse range of biophenols with a wide array of biological activities, such as antioxidant and antimicrobial activities. In addition, the phenolic profile of OMWW is complex, yet this complexity has not been fully exploited in the recycling of the waste.[1−4]

Several in vitro and in vivo studies have shown that phenols found in olives, olive oil, and OMWW exert potent biological activities including, but not limited to, antioxidant and free radical scavenging actions.[5,6] The most studied compound found in OMWW is hydroxytyrosol, a potent inhibitor of copper- and peroxyl radical-induced oxidation of low-density lipoprotein (LDL), which is one of the initial steps in the onset of atherosclerosis. Hydroxytyrosol is also able to scavenge free radicals and to modulate several enzymatic activities linked to cardiovascular disease.[7−10] Another potentially important compound present in OMWW is verbascoside. Several in vivo and ex vivo systems have documented its antioxidant activity.[5,11−13] Verbascoside has demonstrated antioxidant protective effects on phospholipid membranes and the modulation of plasma antioxidant activity in vivo.[14,15]

Recently, studies have used membrane technology, with the dual aims of reducing the organic load of OMWW and recovering polyphenols from OMWW. In fact, the membrane operations were microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), and reverse osmosis (RO), and the permeate fraction can be discharged into aquatic systems in compliance with national or European Union (EU) regulations or be used for irrigation. The RO retentate, containing enriched and purified low molecular weight polyphenols (such as tyrosol and hydroxytyrosol), has been proposed for the food, pharmaceutical, and cosmetic industries, whereas MF and UF retentates can be used as fertilizers or in the production of biogas in anaerobic reactors.[16]

The current investigation aimed at evaluating the in vitro antioxidant properties of phenolics showing a molecular weight ranging from 300 to 700 Da, extracted from the waste material, using a membrane technology, to investigate their biological significance. Nowadays several methods have been developed for measuring the antioxidant properties of biophenols; these assays differ in their chemistry (generation of different radicals and/or target molecules) and in the way end points are measured. Because different antioxidant compounds may act in vivo through different mechanisms, no single method can fully evaluate the antioxidant capacity of nutraceuticals.

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Phenolic antioxidants can prevent reactive oxygen species (ROS) related damages in different ways, such as interfering with the initial reactions that generate ROS, scavenging for the free oxygen molecules required to begin the production of ROS, or chelating metals that speed oxidative processes. The imbalance between ROS and antioxidant capacity of the organism leads to a condition of oxidative stress.[17] Studies in humans and laboratory animals have reported that oxidative stress is related to some common degenerative diseases, such as cancer and cardiovascular pathologies.[18] Oxidative stress has also been identified as a causative agent for diseases, such as the decline of immune function and atherosclerosis.[19] In particular, reactive oxygen metabolites such as superoxide (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$) have been reported to act as cytotoxic agents and damage unsaturated lipids in membranes.[20]

Furthermore, in the pathogenesis of atherosclerosis, evidence is increasing that different factors play key roles at various times in the evolution of the atherosclerotic plaque. It has been also suggested that oxidation of LDL by cells in the artery wall leads to a proatherogenic particle that may help initiate early lesion formation. Hence, the effect of compounds with antioxidant properties on LDL oxidation assumes great significance in the protection of LDL against oxidative modification with the potential to prevent atherosclerotic lesions. Accordingly, there is a growing body of data from epidemiological and controlled studies that correlates a high intake of antioxidants, including olive phenolics, with a lower incidence of coronary heart disease.[21−25] Many reports are available on the antioxidant effects of biophenols from OMWW, whereas the antioxidant properties of the phenolic fractions purified from OMWW after membrane technologies and chromatographic procedures have been poorly investigated. The aim of this study was to characterize the polyphenolic fraction, obtained by membrane process and chromatographic purification on Sephadex LH-20, of OMWW. In addition, the inhibitory effects of verbascoside, the main phenolic component in this fraction, against oxidative stress in cultures of HT-29 human colon carcinoma cells as well as its capacity to inhibit LDL oxidative modification induced by metal catalysts were also investigated. Finally, uptake of verbascoside on the HT-29 cell model, an established model system for intestinal drug permeability, was analyzed.

MATERIALS AND METHODS

Chemicals. Extraction and chromatography solvents, methanol (MeOH), glacial acetic acid (AcOH), ethanol (EtOH), and ethyl acetate (EtOAc), were of certified high-performance liquid chromatography (HPLC) grade, and pure standard of verbascoside was obtained from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Sephadex LH-20 was obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

Preparation of High Molecular Weight Phenolic Fraction. Fresh OMWW samples, obtained from the Coratina cultivar, were collected from an olive oil manufacturer (Andria, Italy). The raw OMWW (10 L) was processed with a laboratory-scale system (Permeare s.r.l., Milano, Italy) consisting of a series of membranes at different porosities (0.1, 0.05, and 0.005 μm) to give in turn three types of permeated fractions: MF (clear OMWW), UF (from 5000 to 200 Da), and NF (<200 Da). The UF fraction (from 5000 to 200 Da) was separated by low-pressure gel filtration chromatography on a Sephadex LH-20 column (40 cm × 1.6 cm; Pharmacia) equilibrated and eluted with 30% ethanol, pH 5.3. This LH-20 fraction was further eluted on a Sephadex LH-20, thus separating an almost pure verbascoside. The LH-20 fraction and verbascoside, stored at −20 °C, were successively characterized by HPLC analysis.

Determination of Total Phenolic Content. The total phenolic content of the OMWW and fractions was determined using a modified Folin−Ciocalteu spectrophotometric method.[26] Results were expressed as micrograms per milliliter (μg/mL) of verbascoside equivalents.

HPLC Analysis. HPLC-DAD-MS/MS analyses were performed on a capillary HPLC 1100 series system, equipped with a degasser, quaternary pump, thermostated column compartment, diode array detector, and MSD Trap XCT Plus in a series configuration (Agilent Technologies, Palo Alto, CA). The optimum chromatographic separation was obtained with a Zorbax SB-C18 column (2.1 × 50 mm i.d., 1.8 μm packing), using a binary gradient composed of (solvent A) water containing 5% formic acid and (solvent B) acetonitrile. The gradient was run as follows: (1) from 0 to 10% B in 2 min, (2) from 10 to 20% B in 6 min, (3) from 20 to 50% B in 10 min, (4) from 50 to 75% B in 12 min, followed by washing and re-equilibration of the column. A capillary pump was used in normal flow mode: the flow rate was 0.2 mL/min, and the injection volume was 1 μL.

The UV−visible absorption chromatogram was detected at 325 nm. Spectra were recorded from 220 to 600 nm. MSD Trap XCT Plus was an ion trap mass spectrometer equipped with an ESI source. Voltages for the skimmer and the capillary were, respectively, −40 and 3500 V. Other MS conditions were as follows: nebulizer gas (N_2), 30 psi; drying gas (N_2), 8 L/min; dry temperature, 350 °C. MS analysis was as follows: first, an MS full-scan acquisition (m/z 400−700) in a negative mode, at ultrascan resolution (26000 m/z s^{-1}), was performed to obtain preliminary information on the predominant m/z ratios observed during the elution. The information obtained in this first analysis was confirmed by extract ion chromatogram (EIC) generation. Then, an MS/MS full-scan acquisition was performed: ions with m/z ratio corresponding to the chosen peaks were isolated in the ion trap and fragmented. The collision energy was set to "smart frag" mode to ensure the generation of useful product ion spectra from all species detected. All data were acquired and processed using ChemStation software (Agilent Technologies). Compound identification was achieved by combining different types of information, elution pattern, UV−vis and MS spectra, MS/MS fragmentation patterns, and with the help of structural models already hypothesized in the literature.

Verbascoside Uptake in HT-29 Human Colon Carcinoma Cell Lines. The experiments were carried out using a protocol described by Cardinali et al.[27] with some modifications. Briefly, HT-29 cells (passages 40−41) were seeded at a density of 2.5 × 10^5 in 6-well polystyrene plates (Costar) and cultured with McCoy's medium (Sigma, Milan, Italy) containing 10% fetal bovine serum, 2 mM L-glutamine, and 1% antibiotic−antimycotic stabilized solution (Sigma) in a humidified atmosphere containing 5% of CO_2. When confluence was reached (after 3−4 days of incubation), monolayers were first washed with 2 mL of PBS, after which 2 mL of verbascoside fraction in PBS was added. The chosen verbascoside concentrations ranged from 62.4 to 312 μg/mL. Cells were then incubated at 37 °C for 5, 15, 30, and 60 min of exposure. Following incubation, medium was aspirated and monolayers were washed first with PBS and then with 2 mL of 0.1% fatty acid free bovine albumin (w/v) in PBS. Finally, cells were scraped from the plate into 1 mL of cold PBS, collected, and stored at −80 °C under N_2 until analysis. Protein content was determined according to the Bradford method.[28] Verbascoside was extracted from sonicated HT-29 cells with 3 mL of EtOAc. Extraction was repeated a total of three times, and ethyl acetate layers were pooled, dried under vacuum, and resolubilized in 200 μL of mobile phase for HPLC analysis.

Verbascoside uptake in HT-29 human colon carcinoma cell lines was measured with a Thermo Scientific HPLC Spectra System equipped with a P2000 gradient pump, an SCM1000 vacuum membrane degasser, a UV6000LP diode array UV−vis detector, an AS3000 autosampler, and ThermoQuest software. The ChromQuest Chromatography data system 4.1 version was used for spectra and data processing. For the HPLC analysis, an analytical Phenomenex (Torrance, CA) Luna C18 (5 μm) column (4.6 × 250 mm) was used throughout this work. The solvent system consisted of (A)

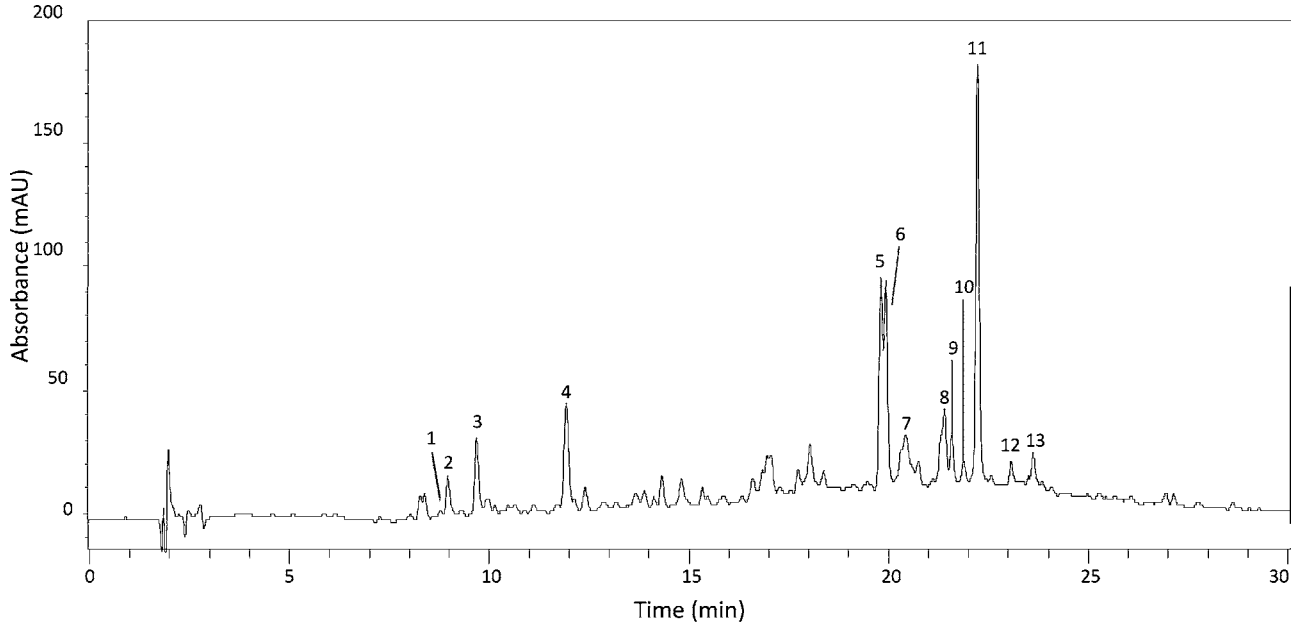

Figure 1. HPLC profile of OMWW LH-20 fraction at 325 nm.

methanol and (B) acetic acid/water (5:95, v/v). The elution profile was 0−25 min 15−40% A in B; 25−30 min 40% A in B (isocratic); 30−45 min 40−63% A in B; 45−47 min 63% A in B (isocratic); 47−51 min 63−99% A in B. The flow rate was 1 mL/min. Samples of 25 μL were applied to the column by means of a 25 μL loop valve.[29]

Antioxidant Bioassays. The ability of verbascoside to act as a natural antioxidant was examined using two different bioassays: a cell-free system based on in vitro Cu^{2+}-induced LDL peroxidation and a method assessing the inhibitory activity of verbascoside on ROS production in cultures of HT-29 human colon carcinoma cells. Antioxidant activity was expressed as IC_{50} values [i.e., verbascoside or LH-20 total phenolic concentration (μg/mL) that gives 50% inhibition of the model reaction as interpolated by dose−response curves].

Detection of Intracellular ROS: 2′,7′-Dichlorodihydrofluorescein Diacetate (DCFH-DA) Test. The HT-29 intestinal cell line (ECACC, Sigma-Aldrich code 91072201) was cultured in 25 cm^2 flasks using McCoy's 5A medium, with 10% fetal bovine serum, 2 mM L-glutamine, and 1% antibiotic−antimycotic stabilized solution at 37 °C under 5% CO_2 humidified air. Cells were passaged at 70−75% confluence using a solution of 0.025% trypsin and 0.01% EDTA. For ROS determination, the cellular suspension was used between the 18th and 48th passages. Intracellular ROS were measured fluorometrically using the membrane-permeable dye DCFH-DA. The assay, carried out as described previously by Stockmann-Juvala et al.[30] with slight modifications, consists of the oxidation of nonfluorescent 2′,7′-dichlorodihydrofluorescein (DCFH) (after transport of DCFH-DA across the cell membrane and deacetylation by esterases) to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF) by oxygen reactive species, the fluorescence of which can in turn be measured. Oxidative stress in cells was induced by applying hydrogen peroxide (H_2O_2) as an extracellular free radical generator. For the assay, 180 μL/well of cellular suspension (1×10^6 cells/mL) in PBS were treated with 20 μL of verbascoside (20 μL of PBS as blanks for background fluorescence) at different concentrations (from 0.01 to 5 μg/mL for verbascoside and from 0.024 to 12.5 μg/mL for the LH-20 fraction). Cells were stained with 5 μM DCFH-DA and incubated for 10, 30, 60, and 120 min. The cells, except for the control, were further treated with a free radical generator (100 μM H_2O_2), for 5 min. The control and each concentration were tested in six wells. Then, plates were centrifuged (300*g* for 10 min), the medium was removed, and the pellets were resuspended with 200 μL/well of PBS. The fluorescence of the probe was read with a Perkin-Elmer LS55 fluorescence spectrophotometer set at 485 nm (excitation) and 530 nm (emission).

LDL Antioxidant Assay. The antioxidant assay with LDL as substrate is based on measuring hexanal, one of the major end-products formed by Cu^{2+}-catalyzed oxidation of LDL. The production of hexanal was monitored by headspace gas chromatography (GC) following the method of Frankel et al.[31] with some modifications. Briefly, 45 μL of LDL samples (1 mg/mL), 300 μL of $CuSO_4$ (80 μM), and PBS up to a final volume of 4 mL were sealed in 10 mL vials and incubated at 37 °C. At the same time, various concentrations of polyphenols from OMWW were added to vials to determine the inhibition of copper-catalyzed LDL oxidation. The degree of inhibition of hexanal formation from LDL oxidation in the presence of antioxidant competitors was determined by headspace GC (Varian CP3800 equipped with a flame ionization detector). Hexanal was separated by a ZB-Wax-Plus fused silica capillary column (30 m × 0.32 mm i.d., 0.5 μm film thickness, Zebron Phenomenex Inc., Torrance, CA), and helium was the carrier gas. GC conditions were as follows: injector temperature, 180 °C; detector temperature, 200 °C; oven program, held at 40 °C for 2 min, increased at 20 °C/min to 140 °C, and then held for 1 min. Hexanal formation from LDL oxidation was measured at 0, 1, 2, and 3 h, whereas antioxidant activity was determined at 2 h (propagation phase) with five different concentrations (from 0.062 to 0.624 μg/mL for verbascoside and from 1.56 to 156 μg/mL for LH-20 fraction). The results, obtained after replicate analyses, were expressed as percent of relative inhibition

$$(\%\ \mathrm{In}) = [(C - S)/C] \times 100$$

where C was the amount of hexanal formed in the control and S was the amount of hexanal formed in the sample.

Statistics. Differences in antioxidant activity were tested statistically by one-way ANOVA followed by Dunn's multiple-comparison method (SigmaPlot software). A p value of <0.05 was considered to be statistically significant. Concerning the assessment of IC_{50} values on antioxidant activity in cell-free and HT-29 cell systems, interpolation of the Hill slope was carried out using SigmaPlot software.

RESULTS

The investigation reported in this paper, as part of a wider investigation concerning fractionation of raw OMWW and structural characterization of the isolated compounds by HPLC-DAD and ESI-MS/MS analysis, was undertaken to characterize and to compare the biological activities of OMWW extracts that were subjected to different steps of purification

Table 1. Main Ions Identified by HPLC-DAD-MS^n in the OMWW LH-20 Fraction and Their Proposed Structures

peak	t_R (min)	UV λ_{max} (nm)	$[M - H]^-$	MS/MS fragments	MS^3 fragments	suggested structure
1	8.8		477	459, 367, 161	np[a]	verbascoside residue
2	9.0	275sh, 325	477	459, 367, 161	np	isoverbascoside residue
3	9.7	300, 370	365	np		unknown
4	11.9	298sh, 312, 380	355	np		oxidized dimeric caffeic acid
5	19.8		621	459, 487, 179	np	oxidized verbascoside
6	19.9		639	621, 529, 487, 179	**621**, 459, 397, 179, 135	β-hydroxyverbascoside diastereoisomer
7	20.4	295, 367	377	np		unknown
8	21.4	247sh, 288sh, 332	639	621, 529, 487, 179	**621**, 459, 397, 179, 135	β-hydroxyverbascoside diastereoisomer
9	21.6	247sh, 288sh, 332	639	621, 529, 487, 179	**621**, 459, 397, 179, 135	β-hydroxyisoverbascoside diastereoisomer
10	21.9	247sh, 288sh, 332	639	621, 529, 487, 179	**621**, 459, 397, 179, 135	β-hydroxyisoverbascoside diastereoisomer
11	22.2	250, 290sh, 331	623	461, 315, 179	**461**, 315, 297, 161	verbascoside
12	23.1	288sh, 332, 390sh	621	459, 487, 179	np	oxidized isoverbascoside
13	23.6	250, 290sh, 328	623	461, 315, 179	**461**, 315, 297, 161	isoverbascoside

[a]np, not performed.

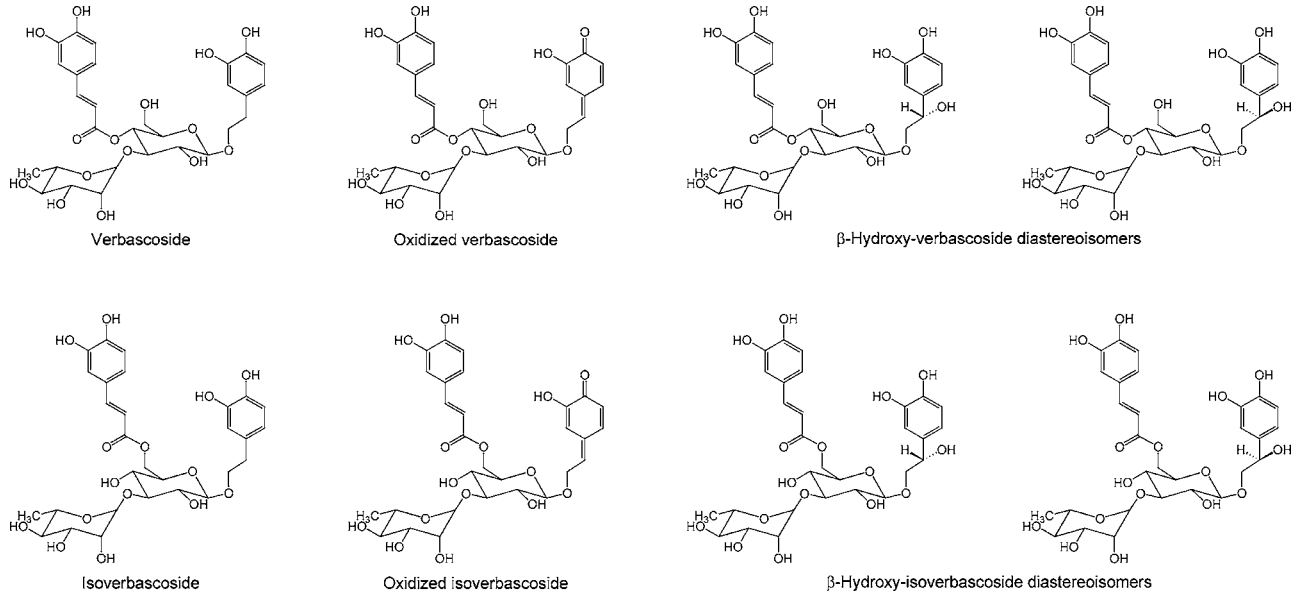

Figure 2. Chemical structures of verbascoside derivatives identified in the OMWW LH-20 fraction by HPLC-DAD-MS^n (see Table 1 for peak identification).

coupling a membrane technology and a low-pressure gel filtration chromatography on a Sephadex LH-20.[1] High molecular weight phenolics from clear OMWW were obtained using a membrane filtration technology. An ultrafiltrate fraction, containing phenolic compounds with a molecular weight in the range of 200−5000 Da (about 52% of total phenolic content of clear OMWW), was recovered. The ultrafiltrate fraction was further submitted to low-pressure chromatographic separation on a Sephadex LH-20 to yield one OMWW extract (LH-20 fraction) free of tyrosol and hydroxytyrosol, which has been extensively investigated for its oxidation protecting properties toward low-density lipoproteins as well as for its free radical-scavenging properties.

This LH-20 fraction was, in turn, characterized by HPLC-DAD-MS/MS (Figure 1). HPLC-DAD and HPLC-MS/MS analyses of the LH-20 fraction revealed the presence, besides verbascoside (peak 11) and isoverbascoside (peak 13), of various phenolic compounds, some of which probably arise from oxidative reactions, showing molecular masses in the range of 300−700 Da. Information on peaks observed during HPLC-UV-MS/MS analyses, that is, peak labels, retention times, m/z ratios for the base peaks (i.e., the major ions observed in the ESI-MS spectra averaged under each HPLC peak), and the corresponding MS/MS and MS^3 fragments are reported in Table 1.

Peaks 1 and 2 showed similar MS spectra, with a molecular ion at m/z ratio 477, and similar MS/MS spectra, thus suggesting the presence of two stereoisomers that were not distinguishable by mass spectrometry. The fragmentation pattern provided useful information to identify the compounds as verbascoside residues lacking the rhamnose moiety. Indeed, fragmentation revealed three main fragment ions at the following m/z ratios: 459, corresponding to water loss; 367, generated from the loss of the *o*-diphenol (catechol) unit; and 161, assigned to the dehydrated ion of the caffeic acid unit. The two isomers were attributed to verbascoside and isoverbascoside residues, respectively.

Peak 4, exhibiting a molecular ion at m/z 355 and caffeic acid type UV spectrum, was tentatively identified, according to data in the literature, as a dimeric oxidized caffeic acid, arising from an oxidative dimerization reaction.[32,33]

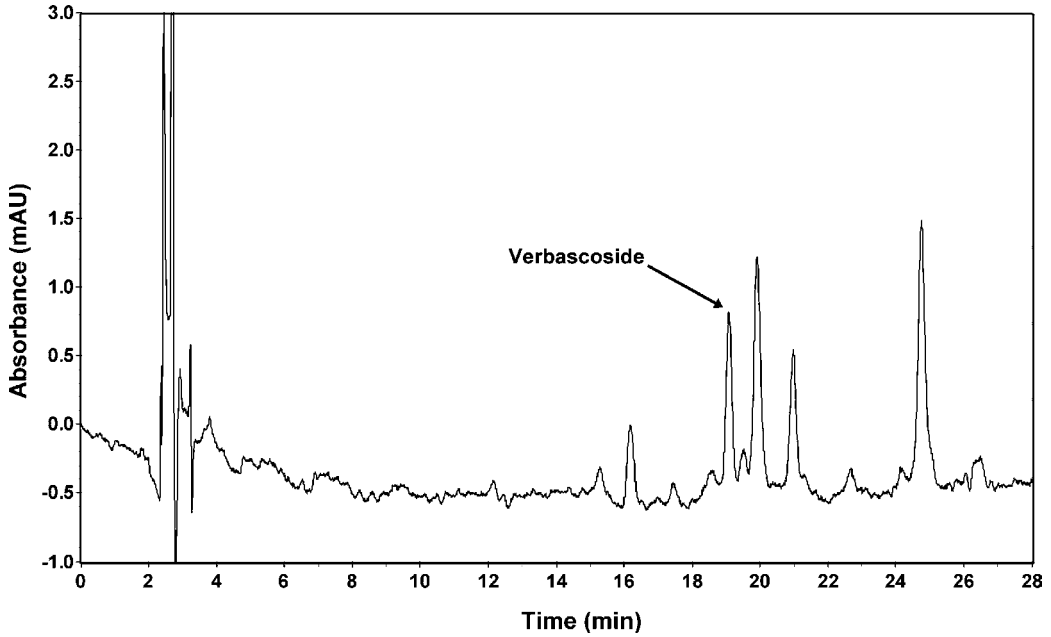

Figure 3. HPLC analysis of verbascoside absorbed by HT-29 colon carcinoma cell lines.

Peaks 5 and 6, which were poorly resolved by HPLC, showed different molecular ions, at m/z 621 and 639, respectively, and UV spectra similar to the verbascoside one. Other peaks (peaks 8−10), showing a molecular ion at m/z 639 and the same fragmentation pattern as peak 6, were also detected in the investigated fraction. MS/MS fragmentation of molecular ions at m/z 639 yielded to the main daughter ion at m/z 621, corresponding to the water loss, and three minor fragments at m/z 529, corresponding to the loss of a catechol unit, m/z 487, corresponding to the loss of a hydroxytyrosol unit, and m/z 179, assigned to caffeic acid ion. In the MS^3 spectra, fragmentation of the main fragment (m/z 621) led to the loss of caffeoyl group or rhamnose to give an ion at m/z 459 and a fragment at m/z 135 consistent with the hydroxytyrosol moiety after the loss of water. These MS^2 and MS^3 spectra are consistent with diastereoisomeric structures of the two β-hydroxy derivatives of verbascoside and isoverbascoside, showing a hydroxyl group in the β-position of the hydroxytyrosol moiety (Figure 2). Peaks 5 and 12, with a molecular ion at m/z 621, were attributed to oxidized verbascoside and isoverbascoside, respectively. Fragmentation of oxidized (iso)verbascoside originated the ions at 459, 487, and 179, the first two arising from the losses of rhamnose and of a fragment corresponding to a quinonic moiety of hydroxytyrosol (m/z 134) and the third one corresponding to the caffeic acid.

Finally, peaks 11 and 13, showing the same MS^2 and MS^3 spectra, were identified as verbascoside and isoverbascoside (m/z 623), respectively, by direct comparison of their retention times and UV spectra with those of the standard and by using their MS, MS^2, and MS^3 spectra. The fragments found at m/z 461 and 315 corresponded to the loss of the caffeoyl moiety and the subsequent loss of dehydrated rhamnose, respectively. The fragment at m/z 179 was assigned to the caffeic acid ion. In the MS^3 spectra, fragments at 315, 297, and 161 were observed, arising from fragmentation of the m/z 461 ion. Fragments at m/z 315 and 297 corresponded to the loss of the dehydrated rhamnose moiety followed by water elimination, respectively. The fragment at m/z 161 was assigned to a dehydrated caffeic acid ion according to the findings of Ryan et al.[34]

Verbascoside (22 μg/mL) is the most abundant compound in this LH-20 fraction (550 μg/mL of total phenols). Therefore, a further purification of this fraction was carried out to obtain a verbascoside with a high degree of purity, as confirmed by HPLC analysis in comparison with a pure standard.

Before the antioxidant properties of verbascoside were analyzed in a cell-free system and in an HT-29 (colon carcinoma cell line) cell system, cellular uptake of verbascoside incubated with HT-29 cells, an established model system for studying drug transport properties, was analyzed (Figure 3).[35] This is because one of the prerequisites for assessing the in vivo physiologic significance of OMWW phenolics is to assess their potential bioavailability. Many papers[36−38] have demonstrated that olive oil phenolics are absorbed by humans in a time- and dose-dependent manner with the phenolic content of the olive oil. Uptake of verbascoside was found to be time-dependent and reached a maximum at 5 min after start of exposure. At 62.4 μg/mL, cells accumulated 267 pmol of verbascoside/mg of cellular protein. This represents approximately 0.12% of absorption of the tested verbascoside. This is in good agreement with previous data obtained by Cardinali et al.[27] showing that intestinal uptake of verbascoside from the LH-20 fraction using Caco-2 cells was linear between 6.24 and 62.4 μg/mL with an accumulation efficiency of about 0.1%.

Thereafter, the relationship between uptake of verbascoside and the response of HT-29 human colon carcinoma cells exposed to oxidative stress induced by hydrogen peroxide (H_2O_2) was examined. In this cell system verbascoside exhibited good antioxidant activity. Data also show a dose- and time-dependent inhibitory activity of both LH-20 fraction and verbascoside on ROS production. A significant ($p < 0.05$) reduction in ROS levels, ranging from 25 to 32% compared to the control, was observed after just 10 min of exposure to verbascoside. More pronounced reduction in ROS (almost 65%) was observed after 30 and 60 min of exposure. In particular, after 30 min of incubation, the percentage of ROS

reduction ranged from 60 to 74% compared to the control. After 60 min of exposure, a ROS reduction of 55% was observed. After 120 min of exposure, no significant antioxidant effect was observed at any verbascoside concentrations. The IC_{50} value obtained by interpolation of ROS reduction was 0.7 μg/mL after 30 min of exposure. In the same conditions the IC_{50} found for the LH-20 fraction was 1.56 μg/mL.

In vitro inhibition of Cu^{2+}-induced LDL peroxidation by verbascoside has been also assayed. In vitro oxidation of human LDL likely does not translate in similar in vivo actions. However this model is still widely accepted as proxy of human pathology. Today it can represent a useful substrate to test lipid peroxidation in a complex lipidic and proteic environment such as that of human LDL.[25] In the control sample, the LDL was oxidized with 80 μM copper in the propagation phase for 2 h following hexanal formation. The inhibition of hexanal production by verbascoside was determined in the propagation phase. LDL peroxidation measured by hexanal formation was inhibited by 20−99% by the addition of verbascoside in the considered concentration range. The IC_{50} value for verbascoside was 0.44 μg/mL. The IC_{50} found for the LH-20 fraction was 4.8 μg/mL.

DISCUSSION

Olive mill wastewaters are a potential source of biophenols that can be extracted and applied as natural antioxidants by the food industry, but they have a complex composition with many unknown phenolics. The data reported in this paper indicate the OMWW extracts, besides low molecular weight antioxidant phenolics such as tyrosol and hydroxytyrosol, also contain antioxidant phenolics with molecular weights in the range of 300−700 Da. This group of phenolics includes verbascoside, isoverbascoside, a number of verbascoside and isoverbascoside derivatives, a dimeric oxidized caffeic acid, and two fragments with an m/z ratio of 477 arising from verbascoside and isoverbascoside lacking the rhamnose moiety. The presence in the OMWW LH-20 fraction of two pairs of diastereoisomeric forms of β-OH-verbascoside and β-OH-isoverbascoside, with the presence of a hydroxyl group in the β-position of the 3,4-dihydroxyphenylethanol (Figure 2), must be emphasized. These compounds have been previously found in *Forsythia viridissima* and in *O. europaea* L.[39,40] In addition, one oxidized form (m/z 621) of verbascoside and another of isoverbascoside were also found.

A number of in vitro studies have shown that OMWW phenolics, especially hydroxytyrosol, exert powerful free radical-scavenging activities, proposed to be potential therapeutic antioxidants. OMWW phenolics have been proposed to act as beneficial agents in a multitude of disease states, including some cancers, cardiovascular diseases, passive smoke-induced oxidative stress, thromboxane B_2 production by whole blood, and inflammatory disease such as osteoarthritis.[41−44] In any case, even though there is much strong in vitro evidence to support this healthy effect of these phenolics, there is little knowledge about their bioavailability. It remains unclear whether they would provide activity similar to that seen in vitro, and studies are needed to show that these agents reach target cells to elicit the activities suggested by the in vitro experiments. The biological effect of these polyphenols and their in vivo circulating metabolites will also depend on the extent to which they associate with cells, either by interactions at the membrane or, more importantly, by their uptake into the cytosol.[45] Therefore, HT-29 cells were used as a model to evaluate the cellular uptake of verbascoside recovered from the OMWW phenolic fraction, because colonic cells are exposed to polyphenols from the diet via the lumen of the gastrointestinal tract. Verbascoside was rapidly incorporated into HT-29 cells in a concentration- and time-dependent manner with an accumulation efficiency of about 0.12%. This low absorption is consistent with poor in vivo verbascoside bioavailability, as reported by Funes et al.[14] following oral administration of lemon verbena extract in rodents.

The glycosylated phenylpropanoid verbascoside has previously been characterized as an effective scavenger of biologically active free radicals and an inhibitor of lipid peroxidation. In this paper verbascoside, purified from an LH-20 fraction, was tested (i) in cultures of HT-29 human colon carcinoma cells for its inhibitory activity on H_2O_2-induced ROS production as well as (ii) in a cell-free systems for its ability to inhibit Cu^{2+}-induced LDL peroxidation.

ROS are essential intermediates in oxidative metabolism. Nonetheless, when generated in excess, ROS can damage cells by peroxidizing lipids and disrupting structural proteins, enzymes, and nucleic acids. The present data indicate that in the cell system, the incorporated verbascoside significantly counteracted the cytotoxicity of ROS in HT-29 cells via scavenging ROS, generated from H_2O_2 prior to attacking biomolecules. Verbascoside is a water-soluble compound acting on ROS that are generated from the water phase. Despite its hydrophilic character, verbascoside shows a significant affinity for negatively charged phospholipid membranes. This property may explain some aspects of its antioxidant properties: the localization of verbascoside in some regions of the bilayer could help to prevent lipid peroxidation.[15]

Verbascoside also protects LDL against copper-catalyzed oxidation. LDL assay provides information on the antioxidant activity of molecules toward lipid substrates and could be considered physiopathologically important and more informative with regard to the induction of atherosclerotic processes. The potent antioxidant activity of verbascoside is probably due to its being a good electron donor, independent of any capacity to act as a Cu^{2+} chelator.[12,46]

In any case, the low IC_{50} values found in the two assays show that verbascoside is a powerful antioxidant both by direct scavenging of biologically active free radicals and by acting as an inhibitor of lipid peroxidation. The antioxidant activity of individual verbascoside is higher than that found for the LH-20 fraction (2−10 times higher, depending on the assay utilized), although additional research is needed to characterize the biological activity of the verbascoside derivatives identified in this fraction.

These findings support the hypothesis that verbascoside, a potent antioxidant biophenol extracted from olive mill wastewater, may be considered a promising nutraceutical compound for the treatment of oxidative stress-related diseases, which might have interesting applications in cosmetics, nutraceuticals, or functional foods.

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