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# Biological Activity of High Molecular Weight Phenolics from Olive Mill Wastewater

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Olive oil production generates large amounts of recalcitrant compounds, the olive oil mill wastewater (OMWW), which represent one of the most contaminating effluents among those produced by the agrofood industries. Nowadays, this view has changed to one that recognizes the waste as a low-cost starting material rich in bioactive compounds, particularly biophenols, that can be extracted and applied as natural antioxidants for the food and pharmaceutical industries. The data reported in this paper indicate that the OMWW extracts, besides low molecular weight antioxidant phenolics such as tyrosol and hydroxytyrosol, also contain phenolics with a molecular weight in the range of 600−5000 Da, which exhibit efficient scavenging activities against hydroxyl and peroxyl radicals. This group of phenolics includes, besides verbascoside, isoverbascoside, and an oxidized form of verbascoside, a number of higher molecular weight phenolics arising from oxidative polymerization of hydroxytyrosol and caffeic acid. Overall, these higher molecular weight phenolics prove to be, in some in vitro tests, more efficient scavengers of hydrophilic hydroxyl radicals than hydroxytyrosol, which could be used for industrial applications as natural nontoxic antioxidants.

**KEYWORDS: α-Amylase inhibitors; isoverbascoside; olive mill wastewater phenolics; oxidative stress inhibitors; verbascoside**

## INTRODUCTION

Olives and olive oil are an inherent part of Mediterranean cultivation and diet, and hence the low incidence of cardiovascular disease in this area has been attributed to their consumption. These effects have been ascribed to the high content of oleic acid in olive oil, but could be also due to its antioxidant composition, namely, tocopherols and phenolic compounds (*1*, *2*).

There are presently 800 million productive olive trees (*Olea europaea* L., Oleaceae) on the planet, which occupy a surface area of 9.2 million hectares. The worldwide production of edible olives and olive oil has been calculated at 17.4 and 2.9 million tonnes, respectively. The Mediterranean area alone provides 98% of the total surface for olive tree cultivation and total productive trees and 97% of the total olive production. Olive tree cultivation is especially important in Spain, Italy, Greece, and Tunisia, Italy being second with regard to total culture surface (1,164,211 ha) and production quantity (3,481,379 tonnes) (*3*).

Olive oil production generates large amounts of recalcitrant compounds. Olive extraction is mainly carried out by the traditional discontinuous press process or by the continuous centrifugation of a mixture of milled olives and water. In both systems, three phases are produced: (i) olive oil; (ii) solid residue; and (iii) aqueous liquor, the olive oil mill wastewater (OMWW). Huge amounts (6−7 million tons/year) of OMWW, a complex medium containing polyphenols of different molecular masses, are produced in the Mediterranean countries cultivating the olive tree. In Italy, approximately 2 million cubic meters of wastewater is produced each year by the olive oil industry (*3*). This waste is claimed to be one of the most polluting effluents among those produced by the agrofood industries because of its high polluting load and high toxicity to plants, bacteria, and aquatic organisms, owing to its contents (14−15%) of organic substances and phenols (up to 10 g/L). These latter compounds, characterized by high specific chemical oxygen demand (COD) and resistance to biodegradation, are responsible for its black color, depending on their state of degradation and the olives they come from, and its phytotoxic and antibacterial properties (*4*).

For long time, OMWW has been regarded as a hazardous waste with negative impact on the environment and an economic burden on the olive oil industry. However, this view has changed to recognize OMWW as a potential low-cost starting material rich in bioactive compounds, particularly phenolics, that can be extracted and applied as natural antioxidants for the food and pharmaceutical industries.

There is growing evidence that free radical mediated events are involved in several human diseases, because free radicals attack biomolecules such as lipids, protein, DNA, and biomembranes, and play major roles in the oxidative degradation of food, animal feed, and cosmetics. Therefore, the extraction of biologically

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active phenolics from OMWW constitutes a viable alternative for valorizing this problematic agroindustrial waste (*4*−*11*). The scientific interest in this material derives from the fact that its phenolic fraction possesses interesting biological activities. 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 and as such are potentially capable of preventing passive smoke-induced oxidative stress, reducing thromboxane $B_2$ production by whole blood, and ameliorating symptoms of inflammatory diseases such as osteoarthritis (*12*−*16*). The antioxidant efficiency of olive phenols has been assessed in various tests (*17*). It has been also observed that the administration of OMWW extract fractions as well as purified hydroxytyrosol in diabetic rats caused a decrease in the glucose level in plasma (*18*). In this connection α-amylase has been targeted as a potential avenue for modulation of postprandial hyperglycemia through mild inhibition of the enzymatic breakdown of complex carbohydrates to decrease meal-derived glucose absorption (*19*). In addition, bioavailability studies have shown that olive phenols can be absorbed from the intestine and enter the blood circulation as conjugates (*20*, *21*).

In the leaves and fruits of *O. europaea* L. (Oleaceae) have been identified different monomeric and polymeric phenolic compounds. All of these compounds can be found in OMWW (*1*, *11*, *22*−*24*). A typical phenolic substance identified in olive fruit is oleuropein, a secoiridoid glucoside that is absent in OMWW due to enzymatic hydrolysis during olive oil extraction resulting in the formation of side products such as hydroxytyrosol and elenolic acid. Other phenolics identified in OMWW are verbascoside, tyrosol, catechol, 4-methylcatechol, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, and gallic acid (*4*, *8*).

This paper deals with the antioxidant and α-amylase inhibitory activities of OMWW phenol fractions and extracts that might, eventually, be recovered from the matrix and employed as potentially very important natural antioxidants for the food and pharmaceutical industries. The ability of these phenols to act as natural antioxidants was examined by a group of bioassays with different molecular targets. The inhibitory effect of test compounds against oxidative stress was also investigated on cancer cell lines. Besides low molecular weight phenolics, such as tyrosol and hydroxytyrosol, widely considered by previous papers (*5*, *8*, *25*, *26*), the biological activities of higher molecular weight phenolic compounds, extracted from the waste material (*27*), have been also considered to investigate the biological significance of these oligomeric/oxidized phenolics.

## MATERIALS AND METHODS

**Reagents.** Methanol, ethanol, and acetic acid were of HPLC grade and purchased from Sigma-Aldrich (Milano, Italy). HT-29 cells (human colon adenocarcinoma grade II cells), McCoy 5A medium, fetal calf serum, L-glutammine, and antimicotic and antibiotic solutions were obtained from Sigma-Aldrich. Amicon YM 30 membrane was purchased from Millipore Corp. (Billerica, MA), Sephadex LH20 was purchased from GE Heathcare Bio-Science (Uppsala, Sweden), and reference compounds (tyrosol, hydroxytyrosol, verbascoside, and isoverbascoside) were purchased from PhytoLab (Vestenbergsgreuth, Germany).

**Preparation OMWW Extracts.** Fresh OMWW samples, obtained from the Coratina cultivar, were collected from an olive oil manufacturer (Andria, Italy). Samples were acidified (pH 2.2) with acetic acid and kept refrigerated at −20 °C in tightly closed PVC vessels to store the samples before analysis and to guarantee sample stability over time. Before chemical and biological characterization of the phenolic fraction, raw OMWW was first filtered on gauze, centrifuged at 24000*g* (RCF) at 10 °C for 20 min, and then filtered successively through a 0.45 μm and a 0.20 μm cellulose acetate filter (clear OMWW). This crude extract was then submitted to ultrafiltration on Amicon YM30 membrane. The filtrate (OMWW YM30 fraction) was successively separated into two fractions by ultrafiltration on Amicon YM3 membrane: the ultrafiltrate containing monomeric and oligomeric phenolics (OMWW MW < 3000 Da) and the retentate (OMWW MW > 3000 Da) containing polymeric phenolics.

**Preparation of Higher Molecular Weight Phenolic Fraction.** 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 (from 0.1 to 0.005 μm) to give three types of permeated fractions: microfiltrate (MF, above 5000 Da), ultrafiltrate (UF, from 5000 to 200 Da), and nanofiltrate (NF, below 200 Da). The UF fraction (from 5000 to 200 Da) was separated by gel filtration low-pressure chromatography on Sephadex LH-20 equilibrated and eluted with 30% ethanol, pH 5.3. The fractions of interest were characterized for the phenolic composition 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 (*28*). Results were expressed as parts per million of catechol equivalents.

**HPLC Analysis.** Analytical-scale HPLC analyses of the OMWW and fractions were performed with an Agilent Technologies series 1100 liquid chromatograph (Waldbronn, Germany) equipped with a binary gradient pump G1312A, a G1315A spectrophotometric photodiode array detector, and a G1316A column thermostat set at 45 °C. Chem Station for LC3D (Rev. A. 10.02) software was used for spectra and data processing. 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) methanol and (B) acetic acid/water (5:95, v/v). For low molecular weight phenolics the elution profile and chromatographic conditions were as reported by Lattanzio (*29*). For oligomeric phenolics the elution profile was 0−10 min, 0−10% A; 10−30 min, 10−20% A; 30−50 min, 20−50% A; 50−55 min, 50−75% A; 55−60 min, 75−100% A. The flow rate was 1 mL/min. Samples of 25 μL were applied to the column by means of a 25 μL loop valve.

HPLC-MS/MS analyses were performed on a QTrap MS/MS system (Applied Biosystems, Foster City, CA), equipped with an ESI interface and a 1100 series micro-LC system comprising a binary pump and a microautosampler from Agilent Technologies (Waldbronn, Germany). The ESI interface was used in positive ion mode, with the following settings: temperature (TEM), 350 °C; curtain gas (CUR), nitrogen, 30 psi; nebulizer gas (GS1), air, 10 psi; heater gas (GS2), air, 30 psi; ion spray voltage, +4500 V. Full-scan chromatograms were acquired in the mass range of 100−800 amu, and MS/MS chromatograms were acquired at a collision energy of 20 V. LC conditions were as for HPLC-DAD analyses.

**Antioxidant Bioassays.** The ability of OMWW phenolics to act as natural antioxidants was examined by using different bioassays: the β-carotene/linoleate assay to determine their inhibitory ability of β-carotene bleaching, the deoxyribose assay to determine their reactivity in Fenton systems, and the metmyoglobin assay to rank their ability to react with active oxygen species represented by the stabile radical cation $ABTS^{\bullet+}$. The antioxidant activity of OMWW phenolics was also investigated on cancer cell lines by using the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay. The antioxidant activity was calculated as percent of inhibition of the control reaction rate and expressed as $IC_{50}$, as interpolated by the dose−response curves. $IC_{50}$ is the amount of phenolic antioxidants (micrograms) that caused 50% inhibition of control reaction, in the reaction volume, under the conditions described.

*β-Carotene Bleaching Test*. The antioxidant activity of OMWW phenolics against peroxyl radicals was determined in a β-carotene/linoleate model system according to the method of Lee et al. (*30*). In this method, antioxidant activity is measured by the ability of a compound to minimize loss of β-carotene during the coupled oxidation of linoleic acid and β-carotene in an emulsified aqueous system.

*Deoxyribose Assay*. The protective effect of OMWW phenolics in the control of oxidative damage caused by hydroxyl radicals, as measured by deoxyribose degradation assay, was determined by using the method of Aruoma (*31*) as modified by Hagerman et al. (*32*).

*Metmyoglobin Assay*. Iron chelates, such as myoglobin, can form ferryl species in Fenton systems. In addition, incubation of heme protein with an excess of $H_2O_2$ can cause heme breakdown to release iron ions. Both ferryl formation and the release of iron ions result in hydroxyl radical formation.

**Table 1.** Total Phenolic Content and Antioxidant Activity of Olive Oil Mill Wastewater

| | | antioxidant activity ($IC_{50}$) | | |
|---|---|---|---|---|
| sample | phenolic content (ppm of catechol) | $\beta$-carotene/linoleate assay | deoxyribose assay | metmyoglobin assay |
| clear OMWW | 6756 | 15.3 | 13.9 | 19.1 |
| ultrafiltrate fraction (MW < 3000 Da) | 4428 | 13.4 | 15.2 | 17.6 |
| polymeric fraction (MW > 3000 Da) | 2817 | 14.7 | 13.5 | 36.1 |

The antioxidant activity of OMWW phenolics against these radicals was determined by a slight modification of the method described by Miller et al. (*33*). This spectrophotometric assay is based on the reduction of the blue-green $ABTS^{\bullet+}$ (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical by hydrogen-donating antioxidants, which is measured by the suppression of its long-wave absorption spectrum.

*Dichlorofluorescein Assay for Oxidative Stress*. Oxidative stress in cells has been also evaluated by using the DCFH-DA assay (*34*−*36*). In this study the assessment of oxidative stress in cells, induced by applying hydrogen peroxide ($H_2O_2$) as free radical generators extracellular, was carried out by using DCFH-DA as the probe. A simultaneous staining with propidiun iodide (PI) was used to evaluate the cellular viability. Assessments of oxidative stress and cellular viability were carried out by using flow cytometry FACSCalibur (BD).

*Cell Cultures*. HT-29 cells (human colon adenocarcinoma grade II cells) were grown in McCoy 5A modified medium containing L-glutamine (1%), heat-inactivated fetal bovine serum (10%), and antibiotic−antimicotic solution (1%).

*Oxidative Stress Measurement*. A cellular suspension of $1 \times 10^6$ cells/mL in PBS was plated into 24-well plates (900 $\mu$L/well) and 100 $\mu$L of OMWW phenolics, at different concentrations, was added. After 1.5 h of incubation in 5% $CO_2$ at 37 °C, cells were stained with 5 $\mu$M DCFH-DA and incubated for 30 min. The cells were further treated with a free radical generator, such as $H_2O_2$ (10 $\mu$M), for 10 min. The plate was centrifuged, the supernatant was removed, and 1 mL of PI solution (2 $\mu$g/mL) was added and further incubated for 30 min. Each sample was analyzed by flow cytometry FACSCalibur (BD) after acquisition of 20000 total events at a flow rate of ∼200 cells/s. Reactive oxygen species (ROS) production was read with 530/30 nm bandpass emission filter (FL-1) by using a logarithmic histogram. PI was read with 650/13 nm bandpass emission filter (FL-3) by using a logarithmic histogram. Viable cells with intact membrane (PI-negative) were observed in the second decade, whereas membrane-damaged cells (PI-positive) were visible in the fourth decade.

**α-Amylase Inhibitor Assay.** The inhibitory activity assay was performed by adding different amounts of phenolics sampled from OMWW fractions or different amounts of hydroxytyrosol to 5 $\mu$L of α-amylase (human salivary) preparation diluted 1:2000 in a total volume of 1.2 mL of barbital buffer solution, pH 5.4. The mixture was incubated at 20 °C for 10 min before the addition of 0.2 mL of substrate solution (0.1% potato starch solution in water). After incubation at 20 °C for 10 min, the reaction was stopped with 0.2 mL of 3 N HCl. The undigested starch was determined by adding 0.4 mL of an I/KI solution (1.2 and 1.8 mM, respectively) and measuring the change in absorbance at 620 nm. Controls without inhibitors were included to determine amylase activity of each preparation (expressed as amylase unit, that is, the amount of enzyme that gave 50% hydrolysis of the added starch) (*37*). The α-amylase inhibitory activity was expressed as a relative α-amylase activity without preincubation with the OMWW extract.

## RESULTS

The total phenolic content and antioxidant activity of "clear OMWW", the ultrafiltrate containing monomeric and oligomeric phenolics (MW < 3000 Da), and the fraction (MW > 3000 Da) containing polymeric phenolics are given in **Table 1**. Clear OMWW shows a very high phenolic content (about 7 g/L). It must be emphasized that this value is largely dependent on cultivar, harvesting time, and processing conditions (*38*). About 60% of these phenolics have been found in the ultrafiltrate fraction (MW < 3000 Da), whereas the remaining 40% of the total phenolic content was made by polymeric phenolics with a molecular weight higher than 3000 Da.

The extracts were subjected to three antioxidant assays, representing three different antioxidant mechanisms. The $\beta$-carotene bleaching assay provides a simple way to evaluate the antioxidant action against peroxyl radicals. The metmyoglobin and the deoxyribose assays provide convenient methods to determine the antioxidant action against hydroxyl radicals. In addition, the deoxyribose assays allow one to assess the abilities of phenolic compounds to exert pro-oxidant action and to chelate metal iron (*39*). It must also be stressed that antioxidants may not protect all substrates subject to oxidation. This means that the analytical and biological significance of the method utilized for determining antioxidant activity must be taken into consideration as well as the target molecule and the composition of the reaction medium. Clear OMWW, the ultrafiltrate fraction, and the polymeric fraction exhibited elevated antioxidant activities in the control of oxidative damage caused by peroxyl ($\beta$-carotene bleaching test) or hydroxyl radicals (deoxyribose and metmyoglobin assays). This biological activity is attributable to the complex phenolic content of different samples analyzed. The overall correlation between total phenol content and antioxidant capacity was found to be insignificant, except the lower ability of polymeric fraction in the control of oxidative damage caused to protein target by hydroxyl radicals.

HPLC analysis of clear OMWW shows that the ultrafiltrate fraction contains tyrosol (0.223 g/L) and hydroxytyrosol (1.112 g/L). These two monomeric phenolics represent about 30% of the total phenolic content of the ultrafiltrate fraction and about 20% of the total phenolic content of the clear OMWW. Tyrosol and hydroxytyrosol are absent in the polymeric fraction. These data, together with data concerning antioxidant activities of OMWW extracts, on the one hand justify the great interest raised by hydroxytyrosol as a natural antioxidant, which has been extensively investigated for its oxidation protecting properties toward low-density lipoproteins as well as for its free radical scavenging properties (*5*, *8*, *40*). On the other hand, these data suggest that the antioxidant activity of OMWW is not completely attributable to monomeric phenolics and that oligomeric and polymeric phenolics, still now totally neglected, must be also considered.

Therefore, it was decided to recover oligomeric phenolics from clear OMWW to evaluate their biological activity. Thus, clear OMWW was submitted to an additional separation procedure by 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. HPLC analyses show that this ultrafiltrate fraction, besides verbascoside and isoverbascoside, also contains tyrosol and hydroxytyrosol. Therefore, the ultrafiltrate fraction was further submitted to low-pressure chromatographic separation on Sephadex LH-20 to yield one OMWW extract free of tyrosol and hydroxytyrosol (LH-20 fraction), which, in turn, has been characterized by HPLC-MS/MS. HPLC-DAD and HPLC-MS/MS analyses of the LH-20 fraction reveal the presence in this fraction of, besides verbascoside and isoverbascoside, many phenolic compounds absorbing at 278−283, 313−330, and 375−390 nm and showing molecular masses in

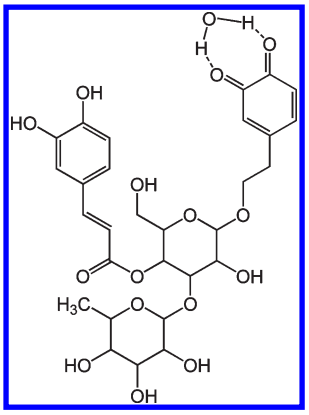

**Figure 1.** Oxidized verbascoside identified in LH-20 fraction.

**Table 2.** Total Phenolic Content and Biological Activity of Olive Oil Mill Wastewater Fractions

| | | biological activity ($IC_{50}$) | |
|---|---|---|---|
| sample | phenolic content (ppm of catechol) | antioxidant activity | α-amylase inhibitor activity |
| ultrafiltrate fraction (MW < 5000 Da) | 1692 | 0.88 | 147 |
| LH-20 fraction | 550 | 0.62 | 926 |
| hydroxytyrosol (std) | 50 | 7.02 | 11.4 |

the range of 600−1000 Da. As the main aim of this study was to screen OMWW extracts for biological activities, a detailed characterization of individual peaks was not attempted; however, data are consistent with the existence in this LH-20 fraction of an oxidized form of verbascoside (**Figure 1**) and different compounds perhaps produced by oligomerization reactions of hydroxytyrosol on the occasion of its oxidation (*17*, *41*).

This LH-20 fraction has been, in turn, tested for its biological activities, α-amylase and oxidative stress inhibitory activities. α-Amylase has been targeted as a potential avenue for modulation of postprandial hyperglycemia through mild inhibition of the enzymatic breakdown of complex carbohydrates to decrease meal-derived glucose absorption. Human salivary α-amylase was allowed to react with phenolic-optimized OMWW extracts and a standard solution of hydroxytyrosol. **Table 2** shows that the α-amylase activity was inhibited more in the presence of the ultrafiltrate fraction compared to the LH-20 fraction. This difference is attributable to the presence in the ultrafiltrate fraction of hydroxytyrosol, which was found to possess the strongest inhibitory activity. Hydroxytyrosol is absent in the LH-20 fraction. Finally, oxidative stress, a cellular condition during which the ROS far exceed the antioxidant defenses and which is implicated in various degenerative diseases in aging such as atherosclerosis, cancer, Parkinson's disease, and Alzheimer's disease (*42*, *43*), has been considered. OMWW extracts and standard hydroxytyrosol were assayed on HT29 human colon carcinoma cells by using a DCFDA probe to evaluate its protective effect against an induced oxidative stress ($H_2O_2$) using flow cytometry. Both the ultrafiltrate fraction (MW < 5000 Da) and LH-20 fraction (MW = 600−1000 Da) showed concentration-dependent antioxidant activity. No cytotoxic effect was observed at all concentrations of OMWW phenolic extracts utilized. **Table 2** shows the $IC_{50}$ values of ultrafiltrate fraction and LH-20 fraction (0.88 and 0.62 μg, respectively). These values when compared to the $IC_{50}$ value (7.02 μg) recorded for hydroxytyrosol suggest that high molecular weight phenolics possess a greater capacity to inhibit oxidative stress than low molecular weight phenolics. This hypothesis is confirmed by the fact that the LH-20 fraction, lacking low molecular weight phenolics, exhibited a greater antioxidant activity than the ultrafiltrate fraction, the phenolic composition of which includes tyrosol (112 ppm) and hydroxytyrosol (715 ppm) besides high molecular weight phenolics.

## DISCUSSION

The investigation reported in this paper was undertaken to compare the biological activities of OMWW extracts that were subjected to different extraction and purification procedures. Up to now only hydroxytyrosol, the most exhaustively studied phenolic component of OMWW extracts, has been considered of particular interest because of its amphiphilic nature and because of its ability to scavenge ROS in different model systems (*6*, *22*). In addition, recent data demonstrate that hydroxytyrosol is dose-dependently absorbed by humans and rats and, at very low doses, maintains its antioxidant activities in vivo (*44*).

The data reported in this paper indicate the OMWW extracts, besides low molecular weight antioxidant phenolics such as tyrosol and hydroxytyrosol, also contain potent antioxidant phenolics with molecular weights in the range of 600−5000 Da, which exhibit efficient scavenging activities against hydroxyl and peroxyl radicals, especially against hydroxyl radicals generated in the dichlorofluorescein assay for oxidative stress. This group of phenolics includes, besides verbascoside, isoverbascoside, and an oxidized form of verbascoside, a number of higher molecular weight phenolics arising from oxidative polymerization of hydroxytyrosol and caffeic acid.

It has been, in fact, suggested that phenolic antioxidants generally undergo polymerization reactions on the occasion of their oxidation induced by oil extractive process (*17*, *45*). Such polymerization reactions of polyphenolic antioxidants can reproduce oxidizable −OH moieties in their polymeric products. The unusually large numbers of electrons for their oxidation, that is, their higher radical scavenging activities, may be ascribed to such reproduction of −OH moieties by oxidative polymerization (*46*). This suggestion is consistent with the hypothesis that the ability of polyphenols to scavenge radicals is proportional to the degree of polymerization and that the proximity of many aromatic rings and hydroxyl groups is more important than specific functional groups (*32*).

In conclusion, this study shows that OMWW represents a complex medium containing mainly phenolic compounds of different molecular masses endowed with a wide array of biological activities. For their recovery from OMWW various extraction procedures have been used, but most of them focus on maximizing the recovery of hydroxytyrosol, the major component (up to 15−20% of total phenolic content) in crude OMWW. **Table 2** shows that the recovery of a less refined fraction obtained by using a membrane filtration technology and representing about 50−60% of (ultrafiltrate fraction, MW < 5000 Da), or a more refined fraction (LH-20 fraction, MW = 600−1000 Da), about 17−20% of total phenolic content of crude OMWW, could be regarded as a source of natural, as yet unused, antioxidants. Overall, these two fractions prove to be, in some in vitro tests, more efficient scavengers of hydrophilic hydroxyl radicals than hydroxytyrosol, without cytotoxic effects, that could be used for industrial applications as natural antioxidants. It must be, also, stressed that these biological activities observed in in vitro assays does not necessarily translate into any physiological significance. In any case, some experiments on Caco-2 human intestinal cell model showed that the uptake (< 1%) of verbascoside and isoverbascoside, identified in the LH-20 fraction, was rapid with peak accumulation occurring by 30 min (*47*).

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