Antioxidant activities of hydroxytyrosol main metabolites do not contribute to beneficial health effects after olive oil ingestion

Olha Khymenets, Montserat Fitó, Sonia Touriño, Daniel Muñoz-Aguayo, Mitona Pujadas, Josep Lluis Torres, Jesús Joglar, Magí Farré, Maria-Isabel Covas, Rafael de la Torre

Human Pharmacology and Clinical Neurosciences Research Group, Institut Municipal d’Investigació Mèdica (IMIM-Hospital del Mar), Barcelona, Spain (OKh, MP, MFa, RdIT)
CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Santiago de Compostela, Spain (OKh, MFi, MP, MIC, RdIT)
Cardiovascular Risk and Nutrition Research Group, IMIM-Hospital del Mar, Barcelona, Spain (MFi, DMA, MIC)
Instituto de Química Avanzada de Cataluña, Consejo Superior de Investigaciones Científicas (IQAC-CSIC), Barcelona, Spain (ST, JLT, JJ)
Universitat Autónoma de Barcelona (UAB), Barcelona, Spain (MFa)
Universitat Pompeu Fabra (CEXS-UPF), Barcelona, Spain (OKh, RdIT)
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Correspondence should be addressed to:
Rafael de la Torre, PharmD, PhD,
Tel.: +34 93 316 04 84; fax: +34 93 316 04 60,
e-mail: rtorre@imim.es

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List of Abbreviations: ACN, acetonitrile; ARC, antiradical capacity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; HOPhPr, 3-(4-hydroxyphenyl)propanol; HOTYR, hydroxytyrosol; HVA, homovanillic acid; HVAIc, homovanillyl alcohol; I.S., internal Standard; LDL, low density lipoproteins; MeOH, methanol; 4´-O-Gluc-HOTYR, 4´-O-hydroxytyrosol glucuronide; 3´-O-Gluc-HOTYR, 3´-O-hydroxytyrosol glucuronide; 4´-O-Gluc-TYR, 4´-O-tyrosol glucuronide; 4´-O-Gluc-HVAlc, 4´-O-homovanillyl alcohol glucuronide; 4´-O-Gluc-HOPhPr, 4´-O-hydroxyphenylpropanol glucuronide; OR oxidation rate; TYR, tyrosol; UDPGT, uridine diphosphate glucuronyltransferase; VOO, virgin olive oil.
Abstract

Hydroxytyrosol (HOTYR) and tyrosol (TYR), main phenolic compounds of olive oil have been reported to contribute to the prevention of cardiovascular diseases due to their antioxidant activities, e.g. protection of LDL oxidation. Their bioavailability in humans is poor and they are found in biological fluids mainly as conjugated metabolites. Free phenols concentrations are low and unlikely to explain biological activities seen in humans after olive oil intake. In this context antioxidant activities of conjugated metabolites in a range of concentrations compatible with their dietary consumption were evaluated. Concentrations of metabolites and their core compounds were estimated in an intervention study with 11 healthy volunteers supplemented with 50 mL virgin olive oil, using a high performance liquid chromatography coupled to mass spectrometry (UPLC-MS) method for the simultaneous analysis of 3´-O- and 4´-O-HOTYR-glucuronides, and 4´-O-glucuronides of TYR and HVAlc (homovanillyl alcohol) in human urine. Glucuronides and core compounds were tested for their chemical (hydrogen donation by DPPH test) and in vitro biological (inhibition of Cu-mediated LDL oxidation) antioxidant activities at the concentration ranges observed in human biological fluids (range 0.01-10 µM) after dietary olive oil consumption. None of the glucuronides displayed significant antioxidant activities at concentrations tested.
Introduction

Recent intervention clinical trials have provided evidence that the phenolic content of an olive oil contributes to the protection in humans against lipid oxidative damage in a dose dependent manner (Covas et al., 2006a and 2006b). Some of the most representative phenolic compounds in olive oil are hydroxytyrosol (HOTYR) and tyrosol (TYR), and their respective secoiridoid derivatives, oleuropein and ligstroside. One of the first steps in relating biological activities of dietary phenol compounds to health benefits in humans is to demonstrate their bioavailability from diet. Several intervention studies in human and animal models have reported that olive oil phenolic compounds are rapidly absorbed and extensively metabolized (Weinbrenner et al., 2004; Visioli et al., 2000). As a result, olive oil phenols appear in biological fluids mainly as phase II metabolites (e.g. glucuronides and sulfates) of HOTYR, TYR and O-methylconjugate of HOTYR (homovanillyl alcohol, HVAlc), whereas their free forms are present only at very low concentrations (Miró-Casas et al., 2003a and 2001). The transformation of HOTYR and TYR into their phase II metabolites was predicted to negatively influence their activities as antioxidants (Nenadis et al., 2005). However, in a study in rats (Tuck et al., 2002) it was reported that the urinary excreted 3′-O-HOTYR-glucuronide, but not its 3′-O-sulfate conjugate, was a more potent antioxidant (by DPPH test) than its parent compound HOTYR. Therefore, the proper evaluation of the bioactivity of the olive oil phenols metabolites is crucial in order to understand the impact of metabolism on health beneficial effects promoted by dietary consumption of phenol rich olive oils (Covas et al., 2006b; Weinbrenner et al., 2004). In addition, properly estimated concentrations for both phenols and their main metabolites in humans after olive
oil consumption are required in order to establish the concentrations range for their *in vitro* and *in vivo* bioactivities evaluation.

The aim of the present study was to assess the antioxidant capacity of the olive oil phenol glucuronides, within a range of biologically relevant concentrations. The range of concentrations tested was derived from those detected in human urine after a dietary dose of virgin olive oil (VOO).
Methods and Materials

Reagents and chemicals. Hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol) and tyrosol (4-hydroxyphenylethanol) were purchased from Extrasynthese (Extrasynthèse, Lyon, France). Homovanillyl alcohol, 3-(4-hydroxyphenyl)propanol (HOPhPr), 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), and 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) were supplied by Sigma-Aldrich (Sigma-Aldrich Inc., St. Louis, MO). Methanol (MeOH) and acetonitrile (ACN) were of analytical grade from Scharlau (Scharlau Chemie, Barcelona, Spain). Mobile phase was filtered with 0.22 μm nylon membrane (Whatman, England). Sodium bisulfite, acetic acid, ammonium hydroxide, hypochloric and phosphoric acid were supplied by Merck (LiChrosolv®, Barcelona, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Molsheim, France). 4’-O- (4’-O-Gluc-HOTYR) and 3’-O- (3’-O-Gluc-HOTYR) glucuronides of hydroxytyrosol, 4’-O-glucuronides of tyrosol (4’-O-Gluc-TYR), homovanillyl alcohol (4’-O-Gluc-HVAIc), and hydroxyphenylpropanol (4’-O-Gluc-HOPhPr) were synthesized according to the previously described method (Khymenets et al., 2006) (Fig 1). Standard stock solutions of all analytes (olive oil phenols and corresponding glucuronides) and corresponding internal standards (HOPhPr and 4’-O-Gluc-HOPhPr as I.S.2 and I.S.1, respectively) were prepared in MeOH and kept at -20ºC.

Study design and sample collection. Eleven healthy volunteers (six males and five females, aged 22 to 44) were recruited. The institutional ethics’ committee (CEIC-IMAS) approved the protocol and the participants signed an informed consent. Prior to the dietary intervention, volunteers followed a 1-week washout period, during which they followed low-phenolic antioxidant diet and the last three
7 days they were also asked to exclude any phenol rich food from their diet. Sunflower oil was provided as a source of fat for all purposes. On the intervention day, at fasting state, 50 mL (44 g) of VOO were administered in a single dose with bread (200 g). The amount of total HOTYR, HVAlc, and TYR in VOO was determined as previously described (Miró-Casas et al., 2003b).

A spot urine sample was collected at 8 a.m. at fasting state. Urine was collected from 0-6 h and 6-24 h after VOO consumption on the intervention day, preserved with sodium bisulfite (1 mM final concentration) at acidic conditions (0.24 M HCl final concentration) and stored at -20°C prior to use.

**Analysis of free and glucurononoconjugated HOTYR, TYR, and HVAlc in urine samples by SPE-UPLC-MRM.** Samples were spiked with 500 ng/mL of I.S.1 and 1000 ng/mL of I.S.2, diluted 1:1 with 4% H₃PO₄ and applied to pre-conditioned with 2mL of MeOH and equilibrated with 2 mL of water Oasis® HLB 3cc (60 mg) cartridges (Waters Corporation, Ireland). Then they were washed with 2 mL of water, extracted with 3 mL of MeOH, evaporated under nitrogen (25°C, 10-15 psi), reconstituted in 200 µL of solvent [A] of mobile phase and filtered using Spin-X® Centrifuge 2-mL polypropylene tubes with 0.22 µm nylon filter (Corning®, Corning Incorporated, NY, USA) and analyzed by UPLC-MS as described below.

Analysis was performed using a Waters Acquity Ultra Performance LC system (Waters, Milford, MA, USA) coupled to a triple quadrupole (Quattro Premier XE) mass spectrometer with an electrospray interface (ESI) (Waters Associates, Milford, MA, USA). Gradient chromatographic separation of HOTYR, TYR, HVAlc and their glucuronides was performed on Acquity UPLC™ BEH C₁₈ column (100 mm x 2.1 mm, i.d., 1.7 µm particle size) (Waters Corporation®, Ireland) at 40°C and using mobile phase [A] 1 mM ammonium acetate at pH 5 and
phase [B] 100% ACN at flow rate 0.4 mL/min. All compounds were monitored in negative ionization applying the multiple reaction mode (MRM) (for details see Supplementary Table 1).

HOTYR, TYR, and HVAlc were quantified by comparison of their peak area ratios with HOPhPr (as I.S.-2) and their glucuronides with 4’-O-Gluc-HOPhPr (as I.S.-1). The correlation coefficient was >0.99 in all cases, and their linear range was 20 – 2000 ng/mL. Both limits of detection and quantification in all cases were not higher than the lower calibrator (20 ng/mL), except for HOTYR, and recovery was within 89% - 97% for all tested compounds.

**LDL resistance to oxidation assay.** LDL extraction from blood samples and conjugated dienes formation after Cu-mediated LDL oxidation was performed as previously described (Fitó et al., 2000) with some modification for testing in small sample volumes. Briefly, 160 µl of dialyzed in PBS LDL (final concentration 0.05 g of Apo-B/L) were incubated with 10 µL of MeOH in the presence (samples) or absence (control) of the tested compounds. Next, 10 µL of a 100 µM cupric sulfate solution was added to a 96-well half-area flat bottom UV-transparent microplate (Corning®), additionally, 10 µL of mineral oil (Sigma-Aldrich) were layered over the reaction mixture and the plate was covered with an adhesive optical transparent film. Absorbance at 234 nm was continuously monitored at 15 min intervals for 24 h at 36ºC in an Infinite M200 Reader (TECAN IBERICA, Männedorf, Switzerland). Controls and samples at 0.01, 0.1, 1, 5, and 10 µM (for all tested compounds) were evaluated in the same run in duplicate and each experiment was repeated three times. The length of the Lag phase was determined as the intercept of the propagation phase tangent with the extrapolated line for the slow reaction. Lag-time was calculated as ratio between
values observed for each compounds vs. those corresponding to the control LDL oxidation reaction. The interseries CV(%) was 9.4.

**DPPH assay.** The antiradical capacity of all tested compounds was evaluated by the DPPH stable radical method as described earlier (Touriño et al., 2005) with some modifications for testing in small reaction volumes. Briefly, compounds (5 µL) were added to aliquots (250 µL) of a 60 µM DPPH solution, both made up in degassed MeOH, in a 96-well transparent flat-bottom microplate (Greiner Bio-One GmbH, Frickenhausen, Germany), covered by an adhesive ViewSeal transparent film (Greiner Bio One GmbH) and analyzed for absorbance at 517 nm in an Infinite M200 Reader every 2 min up to 1h and subsequently every 30 min over the following 23 h at 25°C in the dark. Each calibration curve and the five different concentrations of the tested compounds (0.062, 0.125, 0.25, 0.5, and 1 mM) were acquired in duplicate and each experiment was repeated three times. The % of remaining DPPH was calculated according to the calibration curves made separately for each experiment and read within the same plate and at the same time points as analyzed samples. The within plate and between plate CV(%) of slopes for calibration curves, as well as intraday and interday performance, did not exceed 10% for the 24-h period of kinetic measurements. Results were expressed as ED$_{50}$ (the concentration able to consume half the initial amount of free radical, expressed as the molar ratio compound to radical) and inverse to its antiradical capacity (ARC). The total stoichiometry of the reaction was evaluated at three points: 30 min, 4 h, and 24 h and expressed as amount of hydrogen atoms transferred from the analyte to the DPPH radical according to the following formula: $n_H = 1/(ED_{50} \times 2)$. 
Data evaluation, quantification, and statistical analysis. Normality of continuous variables was assessed by Kolmogorov–Levene test. A least-squares (\(1/\chi^2\)) regression analysis was used to obtain correlation coefficients and slopes. For data comparison a paired Student t-test was employed. Data from LDL resistance to oxidation test were evaluated using one-way ANOVA. Statistical analyses were performed with SPSS for Windows (version 12.0) and significance was defined as \(P < 0.05\). Data are expressed as mean ± SD.
Results and Discussion

The total amounts of HOTYR and TYR consumed with 50 mL of VOO, estimated as a fraction combining free forms of HOTYR and TYR and those resulting from the acidic hydrolysis of their secoiridoid derivatives present in olive oil, were 22.0 ± 1.4 µmoles (3.38 ± 0.22 mg) and 15.2 ± 0.9 µmoles (2.10 ± 0.12 mg), respectively. Despite of a wash-out period, almost all basal urines (0h) contained some traces (under quantification limit of the method applied) of HOTYR, TYR and HVAlc and their glucuronides (4´-O-GlucTYR, 4´-O-GlucHVAlc, 4´- and 3´-O-GlucHOTYR) (data not shown). Concentrations of phenols and their metabolites were calculated in urines collected within 0-6h and 6-24h after VOO consumption. TYR and HOTYR, as well as the methylated metabolite of the later, HVAlc, in their free form were detected at significant but relatively low concentrations (2.38 ± 1.14 µM, 1.83 ± 1.12 µM and 1.64 ± 0.92 µM, respectively) in all urines collected 6h after VOO acute intake. Lower concentrations were observed in urines collected from 6h to 24h (they never exceed 0.1 µM, (data not shown)). Concentrations of O-glucuronide metabolites were substantially higher in 6h postprandial urines when compared to their parent compounds: 6.65 ± 2.91 µM, 2.66 ± 1.18 µM, 3.89 ± 1.69 µM and 2.32 ± 1.31 µM for 4´-O-GlucTYR, 4´-O-GlucHVAlc, 4´-O- and 3´-O-GlucHOTYR, respectively. Glucuronides were also detected in 6-24h urines samples in a 0.1-0.6 µM concentrations range (data not shown). Results show that the fraction of olive oil phenols absorbed is rapidly and extensively metabolized, mainly to glucuronides. On the whole, urinary concentrations of free phenols and their glucuronides do not exceed 10 µM which might represent their highest concentration attained after olive oil dietary intake in human biological fluids.
The impact of glucuronidation on the antioxidant activity of olive oil phenols was evaluated by determining their protective effect against Cu-induced oxidation of LDL. The LDL oxidation test was performed at concentrations, covering all biologically relevant ranges: 0.01 – 0.1 µM (mainly referring to plasmatic postprandial phenols concentrations reported earlier by Miró-Casas *et al.* [Miró-Casas *et al.*, 2003a] and 1 – 5 – 10 µM (the postprandial urinary phenols concentrations estimated within this study) ranges. No data could be generated for HVAlc at concentrations of 5 and 10 µM due to a regular deterioration in the monitored diene conjugate formation, the cause of which was unknown to us. Both HOTYR and HVAlc expressed much stronger protective effects than Trolox against LDL oxidation by extending almost twice the Lag time of dienes formation at the concentration of 1 µM (Fig 2A), which was in agreement with previously reported data (Rietjens *et al.*, 2007; Turner *et al.*, 2005). In contrast, TYR appeared to have a very weak activity in this model even at the highest concentration (10 µM) (Fig 2A), in agreement with an earlier report (Briante *et al.*, 2003). Both glucuronides of HOTYR appeared to maintain some residual activity in protecting LDL from Cu-mediated oxidation (Fig 2A), in particular at the highest concentration tested (5 and 10 µM). In contrast, the glucuronidation of HVAlc entails the loss of activity against lipid oxidation. Not surprisingly, TYR glucuronide was totally inactive. Overall, glucuronidation of phenols decreases to a great extent the protective activities of olive oil phenols against Cu-mediated LDL oxidation.

Because free radical activities play a central role in LDL oxidation, olive oil phenols and their glucuronides were assessed for their radical scavenging capacities (hydrogen donation abilities) using the DPPH assay. The 24-h kinetics
of DPPH radical scavenging (% of remaining DPPH) of parent compounds and glucuronides are shown in Fig 2 (B). Already after the first minutes of reaction, HOTYR appeared to be the strongest antiradical scavenger (Fig 2 (B)). The Trolox reaction reached its plateau within the first 5 minutes of incubation whilst HOTYR induced a first decay in absorbance within the first 3 minutes, followed by a slower slope (up to 60 min) and only afterwards did the reaction reach its plateau. This biphasic kinetic behavior is well known for HOTYR as a catechol antioxidant (Goupyi et al., 2003; Gordon et al., 2001), and can be explained by the degradation products of HOTYR which are involved in the reaction until equilibrium is reached. In comparison to HOTYR, the reaction of HVAlc with DPPH was slower and the equilibrium could be reached only at a time closer to the end of the 24-h incubation period. Nevertheless, at 2 h a more reduced DPPH could be detected in the reaction with HVAlc than with Trolox, and at 24 h it was close to the amount reduced by HOTYR (Fig 2 (B)). Some long term residual activities in DPPH radical scavenging were observed for HOTYR glucuronides, but not for HVAlc and TYR glucuronides. Due to the variation in the kinetic behavior of the tested compounds, their total stoichiometry of reaction was estimated at three time points: 30 min, 4 h, and 24 h (data shown in Supplementary Table 1). Among the parent olive oil phenols, HOTYR displayed excellent radical scavenging activities, in agreement with previous reports (Briante al., 2003; Roche al., 2005), while TYR was found to be practically inactive. HVAlc shows some scavenging activity, but lower than for HOTYR with respect to kinetics and stoichiometry of reaction. Overall results demonstrate that HOTYR and HVAlc glucuronides do not display the antiradical activities present in their parent compounds. Previous observations suggesting a radical scavenging activity of glucuronides higher than for HOTYR
itself do not agree with present results (Tuck et al., 2002). The present study made use of well characterized pure compounds (Khymenets et al., 2006), instead of compounds extracted from urine. We believe that this is relevant to explain discrepancies with the previous report (Tuck et al., 2002). In addition, our results confirm previous theoretical studies where the bond dissociation enthalpy of phenolic hydroxyl groups used to calculate the H-atom-donating capacity of HOTYR and its phase II metabolites glucuronides and sulfates, already predicted the loss of activity of metabolites (Nenadis et al., 2005).

Results of this study show that after a single dose of 50 mL VOO, two glucuronides of HOTYR and one of TYR and of HVAlc, are the main metabolites detected in humans. Whilst olive oil polyphenols with different degrees display radical scavenging (DPPH test) and capacity to protect LDL against oxidation, their glucuronides do not. To the best of our knowledge this is the first time that their antioxidant capacity at concentrations relevant to real-life doses of olive oil, are reported. Overall, the lack of antioxidant activity of glucuronides, compared with that of the parent compounds suggest that they are not chief contributors for explaining the antioxidant effects provided by olive oils rich in phenolic compounds seen in humans. Further mechanisms should be examined to solve this enigma.
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Footnotes

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Figure Legends

Fig 1: General structure of olive oil phenols and their metabolites studied in this study (synthesized and characterized according to Khymenets et al., 2006).

Fig 2: Antioxidant activities of olive oil phenols HOTYR and TYR and their metabolites:

(A) Changes in Lag time ratio of in vitro Cu-induced LDL oxidation in the presence of different HOTYR, TYR, HVAIc and their glucuronides in comparison to Trolox as a reference compound (see Material and Methods for explanation). * - significance at $P < 0.001$ level for linear trend in response.

(B) The 24-h and 1-h kinetics of remaining DPPH (%) in reaction between DPPH radical and tested compounds (see Materials and Methods section for explanations).
Figure 1

Chemical structures and their corresponding abbreviations:

- **R<sup>1</sup>** and **R<sup>2</sup>** denote substituents.
- **n** indicates the number of repeat units.
- **HOTYR**
- **TYR**
- **HVA<sub>1c</sub>**
- **HOPhPr (I.S.2)**
- **3'-O-Gluc-HOTYR**
- **4'-O-Gluc-HOTYR**
- **4'-O-Gluc-TYR**
- **4'-O-Gluc-HVA<sub>1c</sub>**
- **4'-O-Gluc-HOPhPr (I.S.1)**
Figure 2

A

Lag-Time Ratio

Concentration µmol/L

B

%DPH remaining

Time (min)

0 - 60 min

%DPH remaining

Time (min)

0 - 60 min

HOTYR

3'-O-Gluc-HOTYR

4'-O-Gluc-HOTYR

TYR

4'-O-Gluc-TYR

HVAlc

4'-O-Gluc-HVAlc

TROLOX