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# Hydroxytyrosol, as a Component of Olive Mill Waste Water, is Dose- Dependently Absorbed and Increases the Antioxidant Capacity of Rat Plasma

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Hydroxytyrosol is the most potent phenolic antioxidant of olive oil and olive mill waste water (OMWW) and its biological activities have stimulated research on its potential role in cardiovascular protection. However, evidence of the absorption of OMWW phenolics and on their possible *in vivo* activity has, until now, never been provided. Three groups male Sprague-Dawley rats were administered 1, 5, or 10 mg/Kg of the OMWW extract, respectively, providing 41.4, 207, and 414 μg/Kg of hydroxytyrosol, respectively. Urine was collected for 24 h and the urinary levels of hydroxytyrosol were quantified by mass spectrometry. Hydroxytyrosol was dose-dependently ($R^2$= 0.95) absorbed and excreted in the urines mostly as a glucuronide conjugate. Further, the administration of an hydroxytyrosol-rich OMWW extract (10 mg/kg) to the rats was also associated with an increase of their plasma antioxidant capacity. Future experiments will eventually further clarify its metabolic fate and its *in vivo* actions.

*Keywords:* hydroxytyrosol, antioxidants, olive oil, waste waters, polyphenols, atherosclerosis

Olive mill waste water (OMWW), a by-product of olive oil production which is currently disposed of, is rich in phenolic antioxidants whose *in vitro* biological activities have been recently described[1,2]. Hydroxytyrosol (2 (3,4) dihydroxyphenyl ethanol), in particular, is the most potent antioxidant of OMWW; due to its amphiphilic properties, its antioxidant and additional biological activities have attracted considerable attention and stimulated research on its potential role in cardiovascular protection[3,4] and as a preservative in foods or cosmetics[5–7]. However, evidence of the absorption of OMWW phenolics and on their possible *in vivo* activity has, until now, never been provided.

This investigation aimed at elucidating the bioavailability and the antioxidant activity of hydroxytyrosol, provided as a component of an OMWW extract that was administered to rats by gastric gavage.

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## MATERIALS AND METHODS

### Preparation and Characterization of an OMWW Extract

An olive mill waste water extract was prepared from freshly ground olives as previously described[1].

HPLC analysis[8] reveals that the OMWW extract used in the current investigation contains 26.7% (by weight) of phenolic compounds. The individual components were separated and collected and their antioxidant capacity was evaluated in a model of copper sulphate-oxidized low-density lipoproteins (LDL)[3]. The results (not shown) demonstrated that, of all the phenolics in the extract, only hydroxytyrosol was able to inhibit LDL oxidation, whereas the other compounds were inactive.

The extract was analyzed by gas chromatography-mass spectrometry (GC-MS/MS, see below) in order to identify and quantify HT, the most representative simple phenol and the most potent antioxidant of OMWW[1].

### Evaluation of the Dose-Dependent Absorption of OMWW Phenolics

This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Three groups (n= 3) of three male Sprague-Dawley (200–225 g, Charles River, Calco, Italy) rats each were housed in the local animal care facility and were given free access to food and water. On the day of the experiment, the rats were administered 1, 5, or 10 mg/Kg of the OMWW extract, respectively. These doses provided 41.4, 207, and 414 μg/Kg of hydroxytyrosol, respectively. The animals were then placed into metabolic cages and urine was collected for 24 h. For the quantitation of total hydroxytyrosol in urine, 360 U of β-glucuronidase (Sigma, St. Louis, MO) were added to one ml of urine and, after an overnight incubation at 37°C and pH 5, the sample was extracted twice with three volumes of ethyl acetate; the organic phase was evaporated to dryness under nitrogen. The residue was derivatized with a mixture of bis-trimethylsyl-trifluoro acetamide:pyridine (4:1, by vol.) for 30 min at 60°C. For the quantitative determinations, calibration curves were prepared using one-ml urine samples from control animals: these samples did not contain the compounds under investigation, as shown by preliminary GC-MS/MS analysis. The samples were spiked with α-naphthol (one μg/ml) and increasing amounts, *i.e.* from 10 to 2000 ng/ml, of authentic HT and then they were extracted and analyzed. GC-MS/MS analyses were performed on a BP1 fused silica capillary column (SGE s.r.l., Italy), connected with a GCQ mass spectrometer (ThermoQuest, USA), monitoring ions at m/z 201 and 267 derived from the collision of the $[M\text{-}H]^-$ ions of α-naphthol (m/z 216) and HT (m/z 370), respectively. These ions were selected on the bases of mass spectra of pure standards.

### Effects of an OMWW Extract on Plasma Antioxidant Capacity

Male Sprague-Dawley rats (200–225 g, Charles River, Calco, Italy; n= 3 for each time period) were administered the OMWW extract (10 mg/kg dissolved in water:ethanol, 9:1, by vol., providing 414 μg/Kg of hydroxytyrosol); control animals were given the same amount of ethanol (50 μl/rat). After 15, 30, 90, and 240 min, the rats were placed under ether anesthesia and a sample of blood was collected from the heart using heparin (1 I.U./ml) as anticoagulant. Plasma was separated by centrifugation at 800 *g* and its antioxidant capacity was evaluated according to a commercially available kit (MED. DIA, Milan, Italy), which is based on the reduction of Cu(II) to Cu(I) by antioxidants, with uric acid as the reference compound. Results are expressed as mEq uric acid.

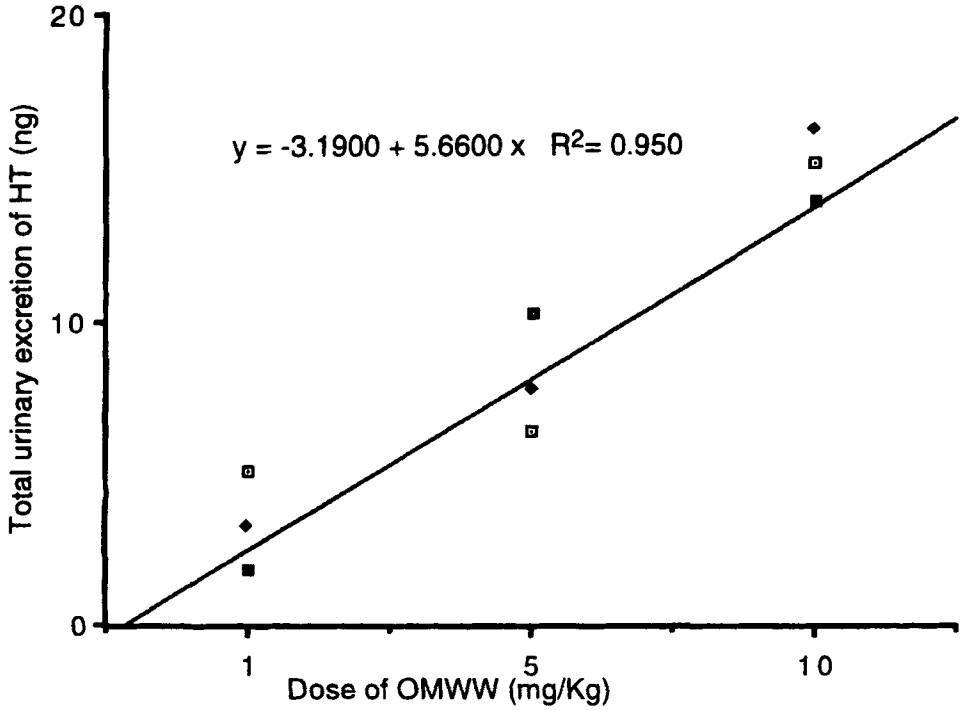


FIGURE 1 Urinary levels of hydroxytyrosol (HT) in rats administered 10 mg/kg of an olive mill waste water extract providing 414 μg/kg of hydroxytyrosol. Urines were collected for 24 h and subsequently extracted as described under Materials and Methods. Data are the means ± S.D. of four determinations for each dose

## RESULTS

Hydroxytyrosol was dose-dependently absorbed and excreted in the urines (Fig. 1), where ~24.5 % of the administered dose was found as total, *i.e.* free and glucuronide conjugated, hydroxytyrosol. Parallel incubation with sulfatase excluded a relevant presence of sulfate conjugates (data not shown).

The administration of an hydroxytyrosol-rich OMWW extract (10 mg/kg) to the rats was associated with an increase of their plasma antioxidant capacity at 15 min (+ 13%, Fig. 2). Then, the antioxidant capacity of plasma declined but increased again at 240 min, when it was found to be significantly higher as compared to basal levels (+ 22.4%, $p < 0.05$).

## DISCUSSION

The data reported in this paper demonstrate that hydroxytyrosol, an OMWW phenolic previously shown to exert potent biological activities *in vitro*, is dose-dependently absorbed by rats after oral administration and increases their plasma antioxidant capacity. In fact, a tendency to the elevation of the antioxidant capacity of rats was noted shortly (15') after the administration of the OMWW extract, although it did not reach statistical significance. This effect is in agreement with the rapid absorption reported for synthetic hydroxytyrosol in rats[9]. After this initial increase in the antioxidant activity of plasma, this parameter tapered off toward pretreatment values until a second, significant increase was

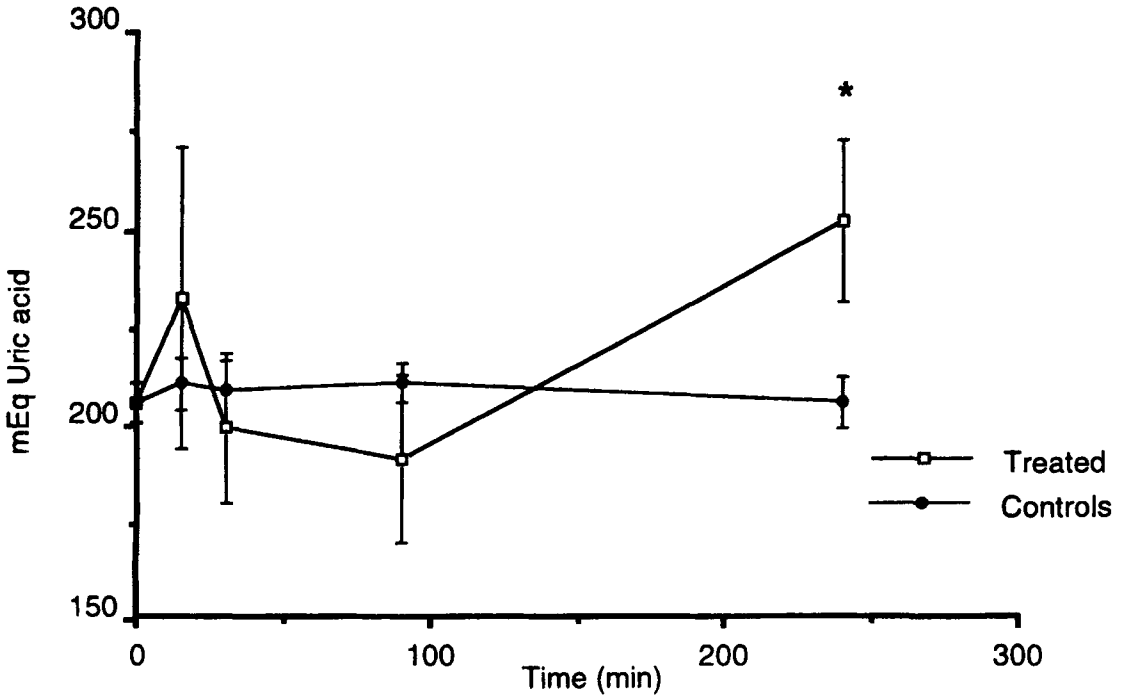


FIGURE 2 Plasma antioxidant capacity of rats (n= 3) administered 10 mg/kg of olive mill waste water containing 8.3% of hydroxytyrosol (by weight, *i.e.* 414 μg/kg). At the indicated time points, blood was drawn from the heart and the antioxidant capacity of plasma was evaluated in terms of its ability to reduce Cu(II) to Cu(II), as described under Material and Methods. Data are the means ± S.D. of three determinations (performed in triplicate) for each time point. *$p< 0.05$ as compared to basal levels

observed 240 min after the administration of the OMWW extract. Possibly, this second increase in antioxidant activity is attributable to the formation of hydroxytyrosol metabolites such as the glucuronide conjugates subsequently found in urines. It is noteworthy that the dose of hydroxytyrosol that was associated with an increase in plasma antioxidant activity observed in this investigation was very low, *i.e.* 414 μg/Kg or ~82.8 μg/rat, confirming this olive oil phenol as a potent antioxidant.

A highly significant ($R^2$= 0.95, Fig. 1) dose-dependent absorption and urinary disposition of hydroxytyrosol was detected, providing new evidence of the uptake of phenolic micronutrients. Moreover, this study corroborates previous reports that conjugation with glucuronide represents a relevant step in the metabolism of phenolics and flavonoids[10,11], as also confirmed by recent results obtained by Visioli *et al.*[12]; future availability of appropriate methodology will eventually further clarify their metabolic fate and their *in vivo* actions.

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