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Cellular Antioxidant Effects and Bioavailability of Food Supplements Rich in Hydroxytyrosol

Cecilia Bender *, Sarah Straßmann and Pola Heidrich

Institut Kurz GmbH, Stöckheimer Weg 1, 50829 Köln, Germany; s.strassmann@institut-kurz.de (S.S.); p.heidrich@institut-kurz.de (P.H.)

* Correspondence: c.bender@kurz-italia.com

Abstract: The present study evaluates the effect of olive (*Olea europaea* L.) vegetation water on human cells regarding its antioxidant properties and radical scavenger bioactivities. To this aim, two food supplements containing concentrated olive water in combination with 6% lemon juice or 70% grape juice, respectively, were assessed in different oxidation assays. From the investigated polyphenols, hydroxytyrosol, present in olives and in a lesser extent in grapes, was found to be the most abundant in both formulations, followed by tyrosol and oleuropein for the olive-derived concentrate with lemon juice, and by proanthocyanidins and tyrosol for the olive concentrate with grape juice. Cellular studies suggest that both formulations are effective antioxidants. In particular, the combination of olive and grape extracts showed a remarkable superoxides-, hydroxyl radicals-, and hydrogen peroxides-scavenging activity, while the formulation containing 94% olive concentrate wasmore potent in protecting the cells against lipoxidation. Both products showed a significant and similar effect in preventing advanced glycation end products' (AGEs) formation. In addition, preliminary data indicate that hydroxytyrosol is absorbed into the human body when administered via these hydrophilic matrices, as confirmed by the urinary excretion of free hydroxytyrosol. Since the availability of phytochemicals largely depends on the vehicle in which they are solved, these findings are of relevance and contribute to supporting the healthful effects here assessed in a cellular environment.

Keywords: hydroxytyrosol; olive extract; olive polyphenols; grape extract; oleuropein; antioxidant capacity; F2-isoprostanes; AGEs

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1. Introduction

Many consumers associate the fruit of the olive tree (*Olea europaea* L.) mainly with the resulting oil, the precious olive oil that is considered particularly healthy compared to other oils. The raw olive fruit contains several types of phenols, thecontents of which vary with the olive cultivar, mainly tyrosol and its derivatives, phenolic acids, and flavonoids [1]. However, many of these substances that the olive has to offer from a health perspective are water-soluble, and thus remain largely in the residue of the olive pressing and the oil contains only a small part. This is the case of hydroxytyrosol, a polar phenol slightly soluble in fats, which can be found in olives as a simple phenol, or either esterified with elenolic acid to form oleuropein aglycone, and which is naturally present in significantly higher concentrations in the olive fruit's aqueous fraction.

The vegetation water, resulting from the pressing of the olive fruits duringthe production of the olive oil, is rich in bioactive compounds, particularly polar phenols, and typically contains 98% of the total phenols of the olive fruit [2].

The positive health effects of olive polyphenols are already known; in particular, hydroxytyrosol has potential antioxidant, anti-inflammatory, and health benefits mainly related with cardiovascular diseases [3–6].

Bioavailability and pharmacokinetic analyses, which were mainly reported with pure hydroxytyrosol and with olive oil, suggest that hydroxytyrosol can be rapidly absorbed

from blood and distributed in the human body [7], metabolized, and quickly eliminated in urine mainly as glucuronide and sulfate [8]. Currently, hydroxytyrosol from different sources is available on the market. Its absorption and subsequent urine excretion may be dependent on the vehicle of administration [9]. Thus, the bioavailability of hydroxytyrosol and its precursors (oleuropein and tyrosol) from those specific sources would be a prerequisite for its health effects in humans.

The present study addresses the bioactivity of hydroxytyrosol-rich extracts, obtained from the vegetation water resulting from olive oil production. Herein, hydroxytyrosol is present as both a simple phenol and as oleuropein aglycone.

For the study, olive-derived concentrates combined with 6% lemon juice or 70% grape juice and marketed as liquid supplements were characterized. Bioactivities, mainly related to the antioxidant potential, were evaluated in cultured cells by means of the antioxidant capacity (cellular antioxidant activity assay, superoxide dismutase and catalase activities), the protection against lipoxidation (inhibition of F2-isoprostanes formation) and glycation (inhibition of AGEs formation). In addition, preliminary data on the bioavailability and urinary recovery of free hydroxytyrosol through acute administration of the food supplements are presented from an open-label cross-over study with four volunteers.

Despite the difference in the composition of both formulations, the main phytochemical in the ones that were investigated was hydroxytyrosol, present in both olive fruit and to a lesser extent in grapes. The treatment of the cells with the supplements gave positive results, although these were slightly different in magnitude, through antioxidant actions. The high bioactivity observed suggests a possible application in the maintenance of the cellular redox state and for related health benefits.

2. Materials and Methods

2.1. Standards and Reagents

2,2′-azobis [2-methylpropionamide] dihydrochloride (AAPH), quercetin dihydrate, and 2′-7′-dichlorodihydrofluorescin diacetate (DCFH2-DA) were purchased from Sigma-Aldrich (Milan, Italy). Hydroxytyrosol and oleuropein were procured from Cayman Chemical (Ann Arbor, MI, USA). Resveratrol was purchased from Sigma-Aldrich (Steinheim, Germany). Dulbecco's modified Eagle's medium (DMEM) high-glucose culture media, L-glutamine, trypan blue solution, and trypsin-EDTA solution 10X were culture grade and purchased from Merck (Milan, Italy). Fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (PBS) without Mg^{2+} and Ca^{2+}, and Hank's balanced salts solution (HBSS) were culture grade and purchased from Euroclone SpA (Milan, Italy). Water, acetonitrile, formic acid, and methanol (all LC-MS-grade) were purchased from VWR Chemicals (Darmstadt, Germany). All other chemicals were analytical grade and purchased from common sources.

2.2. Sample Material

The food supplements analyzed are derived from olive fruit (*Olea europaea* L.) vegetation water subjected to filtration and concentration, and were supplied by Fattoria La Vialla (Castiglion Fibocchi, Arezzo, Italy). The commercial brands are Oliphenolia bitter™ and Oliphenolia™, hereinafter referred to as P-1 and P-2, respectively. P-1 consists of 94% concentrated olive aqueous fraction and 6% lemon juice (*Citrus limon* L. fructus); while P-2 is characterized by 30% concentrated olive extract and 70% grape juice (*Vitis vinifera* L. fructus).

2.3. Analysis of Polyphenols

The samples were diluted with methanol (50:50 v/v), ultrasonicated, centrifuged, and filtrated through 0.45 µm regenerated cellulose filters prior to measurement by UHPLC-MS, with an Acquity UPLC I-Class system coupled to a XEVO-TQS micro mass spectrometer (bothWaters, Milford, MA, USA). The instrument consisted of a sample manager cooled at 10 °C, a binary pump, a column oven, and a diode array detector measuring at 280 nm.

The column oven temperature was set at 40 °C. The gradient started with 2% A and raised linearly to 15% within 5.5 min, then to 100% A within 1 min before holding for 1.5 min as a washing step; it then decreased back to 2% B within 1 min and was equilibrated for 2 min. Eluent B was water with 0.1% formic acid, eluent A was acetonitrile with 0.1% formic acid, the flow was 0.4 mL/min on an HSS T3 RP column (150 mm x 2.1 mm, 1.7 µm particle size) combined with a precolumn (Acquity UPLC HSS T3 VanGuard, 100 Å, 2.1 mm × 5 mm, 1.8 µm), both from Waters (Milford, MA, USA). The injection volume was 2 µL.

The peaks were identified by MS/MS (MRM 153 > 123 for hydroxytyrosol, 539 > 377 for oleuropein and SIR 137 for tyrosol in negative mode and MRM 229 > 135 for resveratrol operating in positive ion mode). The source voltage was kept at 1.5 kV, and the cone voltage was 20 V. The source temperature was set at 150 °C and the desolvation temperature at 350 °C with a desolvation gas flow of 650 L/h and a cone gas flow of 50 L/h. Standard substances were used as reference.

Proanthocyanidin monomers were determined according to Kelm et al. [10].

Data were acquired and processed using MassLynx (Waters, Milford, MA, USA).

2.4. Cell Cultures

Human hepatocellular carcinoma (HepG2) and human keratinocytes (HaCat) cell lines were obtained from CLS (Cell Lines Service GmbH, Germany) and cultured at 37 °C under a humidified atmosphere of 5% CO_2 in DMEM containing 2 mM L-Glutamine, 4.5 g/L glucose, and 10% of heat-inactivated FBS. Experiments were performed with DMEM low-glucose (Lonza Ltd., Morristown, NJ, USA) supplemented with 2 mM L-Glutamine without FBS in either, 6-well culture plates for AGEs, catalase, and superoxide dismutase (SOD), 12-well plates for F2-isoprostanes, or 96-well black plates for the cellular antioxidant activity (CAA) and vitality assays. For each cell-based test, P-1 and P-2 samples were centrifuged, sterile-filtered and directly diluted into culture media before testing.

2.5. Cellular Viability Assay

To determine the optimal growth conditions of cells following 4 h treatment with the sample material, five serial dilutions were evaluated for P-1 and P-2 (range 1:150 to 1:750 and 1:250 to 1:1250 for HaCat and HepG2 cells, respectively), and the metabolic activity was monitored using the resazurin toxicity assay according to the manufacturer's instructions (Tox-8 kit, Sigma-Aldrich, Italy). Fluorescence was read at 37 °C (Em. 590 nm/Ex. 540 nm) in a multiwell fluorescence reader (Fluostar Optima, BMG LabTech, Germany). Data were processed using Mars 2.0 Optima Data Analysis software (BMG LabTech GmbH, Germany).

2.6. Cellular Antixidant Activity (CAA)

The intracellular reactive oxygen species (ROS) formation was detected with the CAA method by spectrofluorimetry using the cell-permeable probe DCFH2-DA, as previously described [11,12]. Briefly, HepG2 cells were cultured until confluence and pre-incubated for an hour with DCFH2-DA and increasing concentrations of the sample dilutions (1:750 to 1:250 v/v) or the quercetin standard. After the addition of AAPH, the absorbed probe was oxidized to a high fluorescent molecule within the cytoplasm, which was measured at 37 °C for an hour at excitation 485 nm and emission 540 nm (Fluostar Optima, BMG LabTech, Germany). Raw data were analyzed using MARS 2.0 Optima Data Analysis software (BMG LabTech, Germany). Results are expressed as µmol of quercetin equivalency (QE) per mL of product and as the mean of five independent measurements ± standard deviation.

2.7. Cellular Extract Preparation

At the indicated time points, the cells were harvested in ice-cold PBS and collected in 2 mL centrifuge tubes before homogenization for the AGEs, catalase and SOD experiments. For whole lysates preparation, samples were homogenized using the Cell Disruptor Genie® (Scientific Industries Inc., Bohemia, NY, USA) with 0.5 mm glass beads, according to the manufacturer's instructions. Whole protein lysates were obtained by centrifugation for

10 min at 10,000× *g*, clear supernatants were transferred to clean tubes and their total protein content was determined according to the Bradford method [13], and they were then preservedat −80 °C for further analysis.

2.8. Catalase Activity Assay

HepG2 cells were cultured without (untreated control) or with the specific sample material (dilution 1:750 *v/v*) for 72 h. After treatment cells were harvested and lysed, the catalase activity was immediately measured by fluorescence using a commercial assay (Arbor Assays Ltd., Ann Arbor, MI, USA, Cat. No: K033-F1) according to manufacturer's instructions. Raw data were analyzed using Mars 2.0 Optima Data Analysis software (BMG LabTech GmbH, Germany), and the results were normalized with the total protein content, expressed as the mean of two experiments and as units of catalase activity per mg of protein ± standard deviation, and then compared with the untreated control.

2.9. Superoxide Dismutase (SOD)

HepG2 cells were incubated for 72 h with 1:750 *v/v* dilution of the samples.After treatment cells were harvested and lysed for further absolute quantification of SOD activity using a commercial kit (Sigma-Aldrich, Italy; SOD assay, Cat. No: 19160) following the manufacturer's recommendations. In the presence of oxygen, xanthine oxidase generates $O_2^{\bullet -}$, which in turn converts a colorless substrate into a yellow product. Samples with increasing levels of SOD cause a decrease in the $O_2^{\bullet -}$ concentration, reducing the yellow color, which is read at 450 nm. Raw data were analyzed using Mars 2.0 Optima Data Analysis software (BMG LabTech GmbH, Germany), and the results were normalized, expressed as mean of three experiments in terms of units of SOD activity per mg of protein ± standard deviation, and compared with the untreated cells.

2.10. Endogenous F2-Isoprostanes Measurement

HaCat cells were seeded in 12-well plates (500,000 cells/mL) and pre-incubated overnight without (untreated control) or with diluted samples (1:750 v/v). After replacing the culture media, lipoxidation was provoked by incubating with AAPH 1 mM for 2.5 h. Supernatants were then removed, centrifuged and immediately investigated for 8-epi PGF2α concentrations using a commercial ELISA kit (item n. 516360, Cayman Chemical, USA) following manufacturer's protocol. Results, expressed as the mean of three experiments ± standard deviation, were determined using Mars 2.0 Optima Data Analysis software (BMG LabTech GmbH, Germany).

2.11. Endogenous AGEs Measurement

HaCat cells were plated and incubated overnight in complete culture medium. For the experiments the culture medium was replaced with serum-free medium without (untreated control) or with diluted samples (1:750 v/v). After an hour of incubation, the medium was replaced with an AGEs-inducer solution containing glyoxal and/or S-p-bromobenzylglutathione cyclopentyl diester at increasing concentrations and incubated for 4 h. Whole protein lysates were processed for quantitative determination of AGEs with a commercial ELISA kit (Cusabio Ltd., Wilmington, DE, USA, Cat. No: CSB-E09412h) according to the manufacturer's recommendations. Spectrophotometric measurements were recorded with a multiwell reader (Fluostar Optima, BMG Labtech, Germany), and the raw data were analyzed using Mars 2.0 Optima Data Analysis software (BMG LabTech GmbH, Germany) and expressed as the mean of two experiments ± standard deviation and as AGEs concentrations relative to protein content.

2.12. Bioavailability

A pilot, open-label, single-dose, two-period, cross-over design study was conducted in our laboratory to test the urinary excretion of free hydroxytyrosol and trans-resveratrol in self-reported healthy volunteers. In the investigation, two males and two females received,

after an overnight fast, a single dose (50 mL) of one food supplement with 200 mL of water separated by one week wash-out period before administration of a single dose of the second food supplement. Urine samples were collected immediately before intake (baseline) and after 30 min of intake. The samples were centrifuged and filtered before being measured by LC-MS/MS, as described above. Freshly prepared urine-blank samples spiked with standards were used for the hydroxytyrosol calibration.

3. Results

3.1. Characterization of the Food Supplements

Table 1 shows the chemical characterization of a representative batch of P-1 and P-2. Of the selected phytochemicals identified, hydroxytyrosol is the main phenolic compound in both samples, as shown by the LC-MS analysis.

Table 1. Chemical analysis of selected phytochemicals of olive-derived food supplements. Values are expressed as mean ± standard deviation of 2 determinations. [a] single determination. * manufacture's data; – not determined.

	P-1	P-2
Parameters	**Content (mg/L)**	
Hydroxytyrosol	1196 ± 35	1399 ± 45
Oleuropein [a]	18.1	11.9
Tyrosol	27.0 ± 0.4	43.9 ± 3.5
Trans-resveratrol	–	3.34 ± 0.04
Total phenolic content [a]*	11,220	11,371
Proanthocyanidin monomers	–	165 ± 4

A comparison of the average amount of total phenolics gives similar results for both samples. Despite the ratio of olive water being lower in P-2, this compound contains higher amounts of hydroxytyrosol and derivatives. This result is consistent with P-2′s combination of grape juice and further concentrated olive vegetation water, as both would contribute to the hydroxytyrosol content. In addition, P-2 comprises trans-resveratrol and proanthocyanidin monomers from grapes, phytonutrients that have considerable antioxidant properties, and that are also said to have positive effects on health [14–18]. It is very likely that P-2 contains additional polyphenols from grapes, and that the two products may also differ in the content of vitamins, or the phytocomplex they contain, though they will not be described in detail here, as hydroxytyrosol is the main polyphenol from the olive on which we are focused.

3.2. Bioassays

Since the aqueous olive extracts show a high content of natural phenols, we examined their possible connection with the prevention of oxidative stress. To this end, sample dilutions found to be non-toxic to the HepG2 and HaCat cell lines were further investigated for their antioxidant effects.

3.2.1. CAA

The CAA measures the ability of an antioxidant sample to inhibit the formation of induced reactive oxygen species (ROS) within cells [11]. While antioxidant samples inhibit the formation of free radicals in a dose-dependent manner, an increment in intracellular fluorescence denotes an increment in ROS formation [19]. Both purified extracts exert a strong antioxidant activity in the CAA assay (Table 2 and Figure 1) by inhibiting the production of peroxides at the intracellular level.

Table 2. CAA results. ₮ HepG2 cells' viability > 90% in the dilution range tested; ** QE = quercetin equivalency; * $p < 0.05$ according to *t* test.

Treatment	Dilution Range ₮ (*v*/*v*)	μmol QE/mL **
Sample P-1	1:250- 1:750	6.77 ± 0.77 *
Sample P-2	1:250- 1:750	9.20 ± 1.57 *

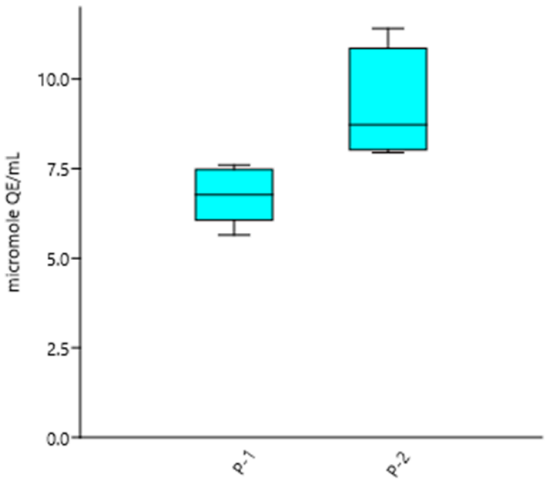

Figure 1. CAA of P-1 and P-2. Data are represented as box plots, showing median, 25–75% quartiles, standard error, and total range of values from 5 experiments. QE = quercetin equivalency.

We found that P-2, containing grape and olive concentrates, displayed a more potent cellular antioxidant potential compared to P-1 containing 94% of the olive extract ($p < 0.05$). In a preventive treatment, the CAA values were 9.20 and 6.77 μmoles quercetin equivalents per mL for P-2 and P-1, respectively.

Interestingly, the results for both purified extracts of olive water are even superior to those of extracts known to be highly antioxidant, such as pure chokeberry juice, which in the CAA test yields an average of 5.27 μmol QE/mL (unpublished results).

Similarly, data reported elsewhere have showed that polyphenols extracted from olive vegetation water are able to inhibit ROS production in human neutrophils and in endothelial cells exposed in vitro [20,21].

3.2.2. SOD and Catalase Activities

To minimize the harmful effects of excess ROS, aerobic organisms have developed several lines of antioxidant defense, which are employed in addition to the direct action of antioxidant molecules [22]. Among these, the enzymatic defense plays a key role in oxidative damage prevention [23]. Catalase and superoxide dismutase (SOD) directly scavenge hydrogen peroxide and superoxide radicals, respectively, converting them into less reactive species.

Given the performance of P-1 and P-2 samples in the CAA test, we evaluated the endogenous SOD and catalase defense in HepG2 human cells after treatment with both formulations, and then compared these to the untreated cells (baseline activity). Under the conditions tested, both supplements showed an increased SOD activity ($p < 0.01$) in liver cells compared to the untreated control (Table 3). A similar effect on catalase activity was found with the P-2 sample, but not with the P-1 sample.

Table 3. Antioxidant enzyme activity measured in cell-lysates after 72 h of treatment with the sample material. Average results are expressed in terms of activity units per mg of protein ± standard deviation. UTC: untreated control cells. * $p < 0.01$ according to *t*-test.

Treatment	U SOD/mg Protein	U Catalase/mg Protein
UTC	6.87 ± 0.44 *	5.52 ± 0.04
P-1	7.93 ± 0.13 *	5.51 ± 0.03
P-2	9.23 ± 0.24 *	7.39 ± 0.18

Similar results supporting the increase in catalase and SOD after treatment with phenolic-rich olive water were obtained by others through a series of cellular tests [20], and in vivo in rats' liver [24].

In accordance with the results obtained in the CAA dosage, the P-2 formulation containing grape and olive extracts exerts a stronger antioxidant effect than the formulation without grape extract. This suggests a potent synergism of olive water polyphenols in the presence of grape extract, and could be explained by the different phenolic compositions, which include, among other things, grape-derived phenolic compounds, such as trans-resveratrol, anthocyanins and proanthocyanidins, as well as a further concentration of the olive vegetation water, which results in a higher content of hydroxytyrosol and tyrosol.

3.2.3. Cellular Peroxidation

Isoprostanes are a family of eicosanoids produced by the random oxidation of tissue phospholipids by oxygen radicals. Several studies have shown that the content of F2-isoprostanes in the human body (i.e., measured in vivo) is directly related to oxidative damage to lipids. In particular, the 8-epi-prostaglandin F2-alpha isomer (8-isoprostane), an end product of the lipid peroxidation chain reaction, has been proposed as a reliable signaling molecule for antioxidant deficiency and oxidative stress [25–28]. The degradation of lipids by lipoxidation occurs as a result of oxidative damage, and consequently the levels of isoprostanes increase, contributing in turn to the development of many diseases related to oxidative stress.

To evaluate the potential of olive-derived supplements in protecting cellular lipids from oxidative damage, we measured the levels of free 8-isoprostanes in cultured cells. Modest amounts of 8-isoprostanes were present in the culture media under normal culture conditions (untreated control), which were increased by oxidative stress after induction by AAPH treatment, but these decreased after incubation with olive-derived extracts or quercetin treatments (Figure 2 and Table 4); compared with untreated cells (baseline level), treatment with P-1 caused a significantly ($p < 0.05$) greater reduction in the content of free 8-isoprostanes (~29% reduction), while P-2 showed a moderate yet not significant effect on lipid oxidation protection (~6% reduction).

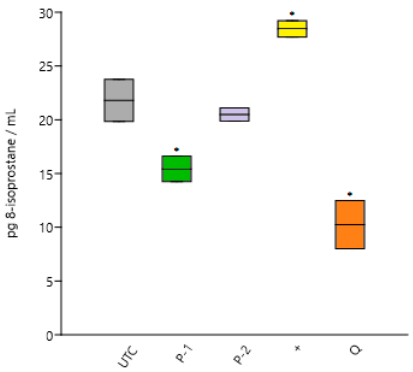

Figure 2. Box plots represent the free isoprostanes (8-epi PGF2α) released in culture, showing median, 25–75% quartiles, standard error, and total range of values from 3 experiments. UTC: untreated control cells; +: lipid peroxidation initiator; Q: quercetin control. * $p < 0.05$ versus UTC.

Table 4. Free isoprostanes released in culture after treatments. The relative values (third column) were normalized to the UTC, which represents the basal amount of isoprostanes under normal culture conditions, and multiplied by 100. UTC: untreated control cells; AAPH: lipid peroxidation initiator; Q: quercetin 100 μM. * $p < 0.05$ vs. UTC.

Treatment	8-epi PGF2α (pg/mL)	Relative Isoprostanes (%)
UTC	21.80 ± 1.95	100.0
AAPH	28.45 ± 0.76 *	130.5 *
P-1	15.42 ± 1.18 *	70.7 *
P-2	20.49 ± 0.60	93.9
Q	10.24 ± 2.23 *	47.0 *

The products derived from olives appear to cut down the number of oxidative attacks on the cell lipid membrane; in fact, the basal oxidative stress is effectively quenched compared to the untreated control cells, thus promoting cellular health. Similarly, in humans, oils rich in olive polyphenols have been shown to reduce the urinary excretion of F2-isoprostanes [29].

3.2.4. Antiglycation Activity

Advanced glycation end products (AGEs) are a heterogeneous group of substances that are formed in the human body during non-enzymatic glycosylation between the carbonyl group of a reducing sugar and a free amino group of a protein [30,31]. AGEs play an important, albeit complicated, role in cellular aging processes, in which they are produced in large quantities, causing oxidative stress, inflammatory reactions, and chronic diseases such as diabetes and cardiovascular disease [32,33]. Hyperglycemia, the accumulation of triosephosphates and ketone bodies, lipid peroxidation, and oxidative stress can increase AGEs formation [34]. The irreversible glycation of proteins in turn results in structural alterations and the accumulation of defective proteins in cells, which impactnormal physiological functions [35].

Since increased intracellular and extracellular stress is a source of AGEs accumulation in vivo, and the antioxidant activities of natural phenolics may inhibit the AGEs production [36,37], we investigated cellular AGEs to further support the potential of P-1 and P-2 in preventing the cellular stress.

We have found that both products reduce the formation of new AGEs (Table 5).

Table 5. AGEs content in cell lysates after treatment with the extract products and an AGEs-inducer mix. UTC: untreated control cells; +: AGEs inducer solution. Relative AGEs values are normalized to the UTC, which represents the basal amount of AGEs in normal culture conditions, multiplied by 100.

Treatment	μg AGEs/mg Protein	Relative AGEs (%)
UTC	1.02 ± 0.006	100
+	2.64 ± 0.042	258
P-1	0.52 ± 0.008	50.9
P-2	0.47 ± 0.007	46.0

Relative values greater than 100 indicate cellular accumulation of AGEs (e.g., treatment with the AGE-inducer alone), while lower values indicate a potential for the sample to reduce the AGEs formation. Pre-incubation of the cells with each product, followed by the induction of AGEs formation, effectively cleanses the cellular AGEs, with respect to both the AGEs-inducer treatment alone and with the cells under normal culture conditions. The strong positive influence is similar for both pre-treatments with each olive-derived concentrates, and both show a nearly 50% reduction in AGEs formation in vitro.

Our results are consistent with data from a recent study conducted with an olive leaf extract concentrated in hydroxytyrosol (54.5 mg/g), in which a reduction in AGEs produc-

tion was demonstrated in HepG2 cells subjected to carbonyls-induced stress [38].These authors reported that, likely due to a synergistic effect of hydroxytyrosol and other minor compounds with similar polarities, the olive leaf extract exerts a wide antiglycative activity.

3.3. Bioavailability

Several cellular effects have been evaluated in vitro to test the antioxidant potential of olive water concentrates. Although, the bioavailability of such components is a prerequisite to any health claim made based on cellular tests. To investigate whether the hydroxytyrosol and trans-resveratrol consumed with the food supplements here studied are bioavailable, we conducted a pilot internal study with four volunteers (two male, two female). Volunteers in a fasted state received a single dose of 50 mL of P-2 in period 1 and 50 mL of P-1 in period 2, separated by a one-week wash-out period. Trans-resveratrol and hydroxytyrosol in its free form were undetectable in urinary samples collected immediately before intake. Free hydroxytyrosol, but not free trans-resveratrol, was detectable in urinary samples collected 30 min after the intake of both supplements and in all the volunteers (Figure 3).

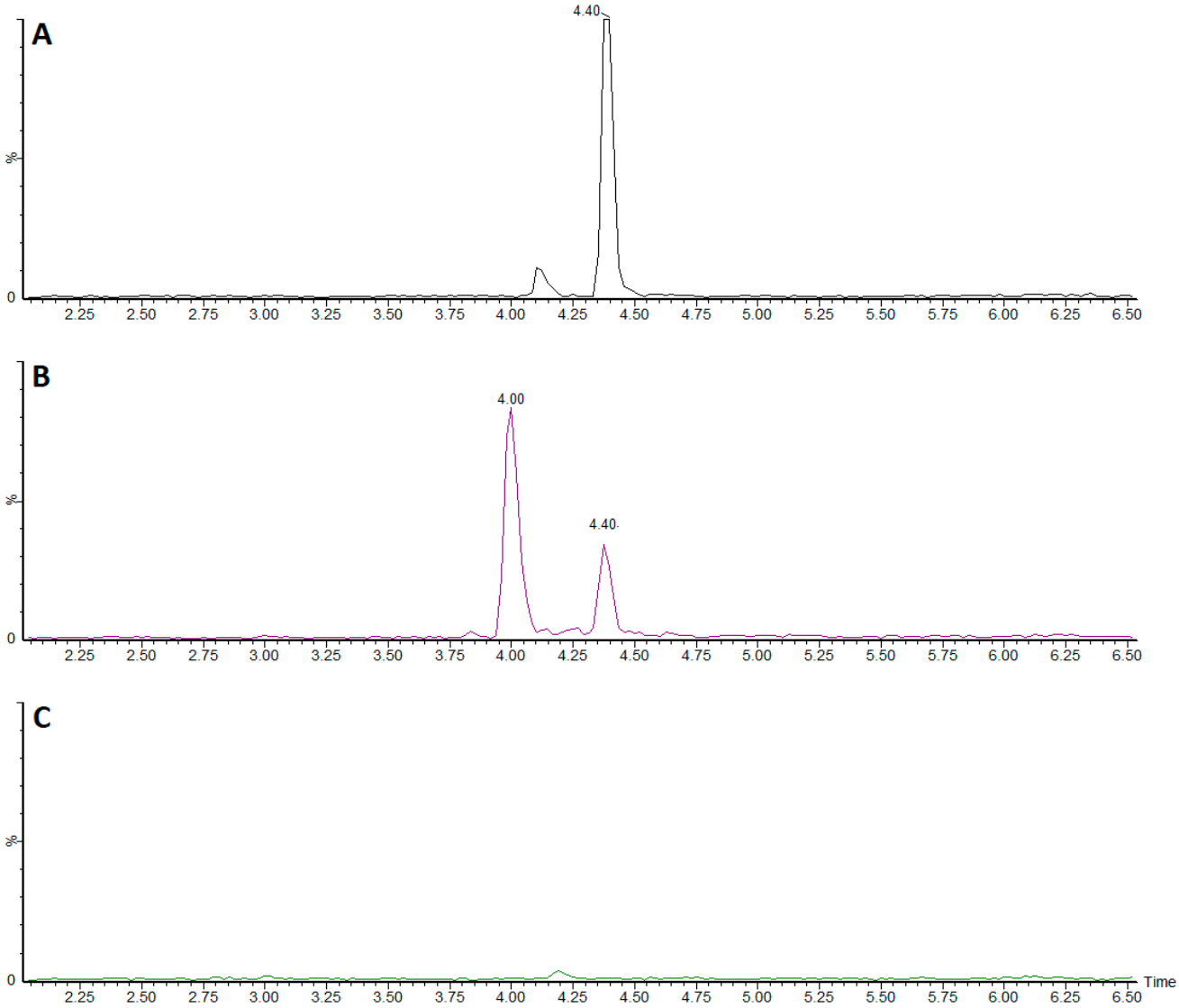

Figure 3. UHPLC-MS/MS chromatograms of the MRM 153 > 123. (**A**) hydroxytyrosol standard in urine; (**B**) representative urine sample taken 30 min after ingestion; (**C**) representative urine sample before intake of the supplement/product.

Similarly, previous human studies have showed that plasma and/or urinary hydroxytyrosol increase following the oral administration of hydroxytyrosol, consumed with olive oil [39–41], liquid or encapsulated olive leaf extract [42], and encapsulated extract from oil mill wastewater [8]. In these experiments, the absorption and excretion of orally administered hydroxytyrosol, collected mainly in conjugated forms and in a dose-dependent manner, have been shown. Furthermore, it has been shown that human absorption may differ depending on the composition of the food matrix through which the hydroxytyrosol is dispensed. Besides this, the bioavailability is likely influenced by wide interindividual variability in the absorption and metabolism [43].

Our preliminary results show that hydroxytyrosol consumed together with a hydrophilic vehicle is bioavailable in the human body, independently of the interactions of the combined fruit extracts used. Moreover, hydroxytyrosol can be detected in urine in its free form. This is relevant as the absorption of hydroxytyrosol is dependent on the vehicle of administration [9]. This is likely because the interaction between olive water and grape or lemon juices influences the hydroxytyrosol absorption and recovery yield. Further studies should be done to verify this hypothesis.

4. Discussion

Our study explores the antioxidant potential of two food supplements derived from olive vegetation water, mainly characterized by a high content of hydroxytyrosol. The antioxidant activity was determined by measuring the cellular antioxidant activity, the catalase and SOD activities in the HepG2 cell line, as well as the inhibition of lipid peroxidation and glycoxidation in the HaCat cell line. The tests carried out have shown that both olive-derived products have a strong positive influence on cells; this influence is complex and not one-dimensional. This reflects the complex nature of the sample materials and suggests a powerful synergy of hydroxytyrosol with other olive phenols, which is further potentiated in the presence of the grape phytocomplex. In particular, both supplements are able to reduce oxidative parameters in vitro.

On one hand, P-1, containing 94% olive water concentrate, showed a good capacity in the CAA and SOD assays, but a better performance in preventing isoprostanes formation in vitro when compared to P-2 (Table 6). On the other hand, P-2, containing olive and grape concentrates, showed a greater antioxidant potential for scavenging reactive species, as indicated by its greater potential in removing the hydroxyl radicals and by its higher SOD and catalase activities. Regarding the prevention of AGEs accumulation, both products showed an excellent capacity in vitro. The overall better antioxidant performance of P-2 in vitro could be explained by the higher concentration of olive-derived polyphenols, as well as the presence of grape-derived antioxidants, which include, among others, the trans-resveratrol, anthocyanins and proanthocyanidin monomers.

Table 6 describes the cellular effects measured, and their links to the attributed in vivo effects.

The mechanism of this positive influence needs to be better understood and has led us to further investigations on the mechanisms and dynamics of the effects of food supplements derived from olive vegetation water on human cells. However, understanding the absorption and bioavailability of these key molecules after oral administration remains a prerequisite before any potential health effect can be derived. In this sense, the pilot trial shows that the hydroxytyrosol supplied with ahydrophilic matrix combining olive fruit concentrate and lemon or grape juices is effectively absorbed, and then urinarily excreted as hydroxytyrosol in its free form.

In subsequent studies, the exact excreted fraction will be determined, and further focus will be placed on the metabolites to obtain a broader picture of the entire ADME properties.

Overall, preliminary data obtained in vitro indicate that the aqueous extracts of olives can actually improve the cellular redox status and related markers, and that their main active ingredient is bioavailable to the human body. Aqueous olive concentrates, with or

without grape concentrate, are valid candidates for the prevention of cellular oxidative damage, and thus merit further attention.

Table 6. Results of bioassays on human cell lines and the effects' connections in vivo. Direction of in vitro effect: increased (↑), decreased (↓), or no effect (~). Effect magnitude compares P-1 to P-2.

In Vitro Test	Cellular Effect	Magnitude and Direction of the Effect In Vitro		Associated In Vivo Effect
		P-1	P-2	
CAA	Removal of hydroxyl radicals	↑	↑↑	Hydroxyl radical damage protection. Consequences of hydroxyl radicals are related to artherosclerosis, cancer and neurological disorders [44].
AGEs	Glycation ability	↓	↓	An increase in AGEs is associated with cellular aging processes, oxidative stress, inflammatory reactions, and chronic diseases such as diabetes, cardiovascular disease [32,33], and chronic kidney disease [45].
SOD activity	Superoxide removal	↑	↑↑	Oxidative damage protection. SOD deficiency is associated with diabetes [46].
Catalase activity	Hydrogen peroxide removal	~	↑↑	Oxidative damage protection. Catalase deficiency is associated with diabetes, Alzheimer's disease, Parkinson's disease, and acatalasemia [47].
8-isoprostane	Lipid peroxidation	↓	~	Reliable biomarker of lipoxidation (an increase on F2-isoprostanes is correlated with oxidative stress) [28,48].

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