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Review

Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods

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Abstract

The olive fruit, its oil and the leaves of the olive tree have a rich history of nutritional, medicinal and ceremonial uses. Olive oil, table olives and olive products are an important part of the Mediterranean diet, the greatest value of which may be due to olive polyphenols that contribute to the modulation of the oxidative balance in vivo. The objective of this review is to examine the available safety/toxicity literature on olive polyphenols, particularly hydroxytyrosol, to determine the safety-in-use of a standardized aqueous olive pulp extract (HIDROX®). Among the polyphenols found in the extract, the major constituent of biological significance is hydroxytyrosol (50–70%). In oral bioavailability studies, urinary excretion of hydroxytyrosol and its glucuronide was found to be associated with the intake of hydroxytyrosol. Oral bioavailability of hydroxytyrosol in olive oil and in an aqueous solution was reported as 99% and 75%, respectively. In comparative studies, urinary excretion of hydroxytyrosol was greater in humans than in rats. The LD_{50} of the extract and hydroxytyrosol was reported to be greater than 2000 mg/kg. In a subchronic study, the no observed adverse effect level (NOAEL) of the extract in rats was found to be 2000 mg/kg/day. In developmental and reproductive toxicity studies, HIDROX® did not cause toxicity at levels up to 2000 mg/kg/day. In an in vivo micronucleus assay, oral exposure of rats to HIDROX® at dose levels up to 5000 mg/kg/day for 29 days did not induce increases in polychromatic erythrocytes in bone marrow. Based on the available studies of the extract and polyphenols, and a history of exposure and use of components of the extract through table olives, olive products and olive oil, the consumption of HIDROX® is considered safe at levels up to 20 mg/kg/day.
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Keywords: Olive; Polyphenols; Hydroxytyrosol; HIDROX®; Antioxidant; Antimicrobial; Food safety

Contents

1. Introduction 904
  1.1. Historical perspective 904
    1.1.1. History 904
    1.1.2. Description 905
    1.1.3. Phenols in olives 905

Abbreviations: CAS, chemical abstract service; CFU, colony forming units; FDA, US Food and Drug Administration; MIC, minimum inhibitory concentrations; NOAEL, no observed adverse effect level; ORAC, oxygen radical absorbance capacity; RBC, red blood cells; USDA, United States Department of Agriculture; WBC, white blood cells.

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1.1.4. Specifications . . . 906
1.1.5. Economic uses . . . 906
1.1.6. Exposure reports . . . 907
2. Biological data . . . 907
2.1. Absorption, metabolism and excretion . . . 907
2.1.1. Absorption . . . 907
2.1.2. Metabolism . . . 908
2.1.3. Excretion . . . 909
2.2. Biochemical/pharmacological effects . . . 909
2.3. Toxicological studies . . . 910
2.3.1. Acute toxicity studies . . . 910
2.3.2. Subchronic toxicity studies . . . 910
2.3.3. Developmental (embryo-fetal toxicity/teratogenicity)/reproduction toxicity . . . 911
2.3.4. Genotoxicity . . . 911
2.3.5. Antimicrobial activity . . . 912
2.3.6. Antioxidant activity . . . 913
3. Discussion . . . 913
Authors' disclosure of potential conflict of interest . . . 914
References . . . 914

1. Introduction

The olive is the fruit of an evergreen olive tree that grows in the temperate climate of the Mediterranean region (Gruenwald, 1998; Kiple and Ornelas, 2000). The term 'olive' refers to the fruit, which is ellipsoid and drupaceous in character. The fleshy, indehiscent fruit (or drupe with one to two seeds), initially green then red and blue-black when ripe, surrounds a very hard stone (or pit), which contains oblong compact seeds with plentiful endosperm (Gruenwald, 1998). The content of phenolic compounds in olives and olive oil depends on the cultivars and the ripeness of the fruit at the time of harvest. The oil contained in olives is normally extracted by a multi-stage process, which involves crushing the whole olive (including the pits), kneading the resulting paste, collection of the free flow oil, pressure separation and collection of residual oil in the grinds, and separation of solids and vegetation water from the olive oil. The "vegetation water" is rich in phenolic compounds. Although there are many governing bodies that attempt to define olive oil, generally virgin olive oil has a free acidity, expressed as oleic acid, of not more than 2% as well as the other characteristics that correspond to those fixed for this category in this standard. Extra virgin oil has free acidity of not more than 0.8%.

Phenolic substances are common to many plants, and have evolved as an antioxidant defense to environmental stress resulting from a variety of oxidizing and potentially harmful free radicals. These antioxidant components in olives are also responsible for the stability of olive oil (Visioli et al., 1998). Because of the strong antioxidant properties of the olive phenols, several investigators have attempted to isolate phenols from the vegetation water, but because the entire olive is macerated, the vegetable water contains some undesirable substances from the crushed pits. The proprietary process developed by CreAgri Inc., removes the pits prior to maceration, resulting in vegetation water with a different composition and a high percentage of hydroxytyrosol. Several independent animal and human studies on the safety and efficacy of olive polyphenols have appeared in the published literature. However, no systematic review on the safety of olive polyphenols based on the published reports has appeared. In view of the increasing popularity of olive polyphenols as antioxidants in humans, the objective of the present review is to evaluate information on the safety of olive phenols, particularly hydroxytyrosol, to determine the safety-in-use of a standardized aqueous olive pulp extract (HIDROX®).

1.1. Historical perspective

1.1.1. History

Historical records indicate that the olive tree was cultivated in Crete as early as 3500 BC. It has always represented a symbol of abundance, glory and peace, and its leafy branches were used to crown the victorious in friendly games and bloody war. The oil of its fruit has anointed the noblest of heads throughout history. The Egyptian ruler during 1300 and 1200 BC, Ramses II, used olive oil for nearly every ailment. Shortly after the Iron Age began (1100–750 BC), Greece became a large producer of olives/olive oil, spurred in the sixth century BC by the prohibition of Solon (an Athenian lawmaker) of export of any agricultural produce other than olive oil. In addition, throughout the Roman Empire, olive oil became a popular staple in the diet. At present, approximately 90% of the world's olives are used in the production of oil, with Spain, Italy, Greece and Portugal representing the main producers (Kiple and Ornelas, 2000).

Olives selected for consumption, as opposed to oil production, are picked green and unripe. However, the fruit

cannot be consumed unless further prepared/processed. Preparation of edible olives involves pickling in a solution of lye to remove the bitter taste (rendered by oleuropein), and this practice has been in use since Roman times. The traditional way of processing olives, which is still a standard practice, involves three steps: blanching, salting and drying of mature olives (Borzillo et al., 2000). The black color of table olives is obtained by exposure to air after lye extraction and has nothing to do with ripeness. Olives, used in the extraction of oil, are allowed to ripen on the tree until after the time of the first frost.

1.1.2. Description

HIDROX®, an aqueous olive pulp extract, is a standardized freeze-dried powder prepared as a byproduct during the processing of the pulp of olives (*Olea europaea* L.) for oil extraction. The powder has an odor of processed olives and a characteristic aromatic sour/olive flavor. General descriptive parameters and properties of HIDROX® are summarized in Table 1. The powder is composed of 98–99% dry solids, including 1–2% citric acid and 6% polyphenols. Other constituents of the extract include protein, fat and carbohydrate. The biologically important constituents of HIDROX® are polyphenols. Among the phenolics, the major constituent of the pulp extract is hydroxytyrosol (50–70%), while other polyphenols present include oleuropein (5–10%), tyrosol (0.3%), oleuropein aglycone and gallic acid. All of the polyphenols are also found in olive oil and are thus commonly consumed. Because of potent antioxidant and anti-inflammatory properties, olive polyphenols, including hydroxytyrosol, have been the subject of several clinical and preclinical studies addressing their imputed benefits. HIDROX®, which contains olive polyphenols, lends itself as a novel ingredient to a broad use in dietary supplements, functional foods and natural cosmetics.

1.1.2.1. Hydroxytyrosol. Hydroxytyrosol, also known as 3,4-dihydroxytyrosol or 3,4-dihydroxyphenylethanol (CAS No.: 10597-60-1; Fig. 1), is the major component of the phenolic fraction of olive extract and olive oil; the presence of hydroxytyrosol has also been identified and quantified in wines (Di Tommaso et al., 1998). Hydroxytyrosol is present

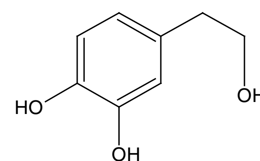

Fig. 1. Chemical structure of hydroxytyrosol.

in olive oil either as simple phenol or esterified with elenolic acid to form oleuropein aglycone. Pure hydroxytyrosol is a clear, colorless, tasteless liquid and can be hydrosoluble or liposoluble. Although hydroxytyrosol represents a minor constituent of the aqueous olive pulp extract, as well as of the olive oil phenolic fraction, it is considered the most potent (as measured by ORAC) phenolic antioxidant of HIDROX® and of olive oil. It greatly contributes to the shelf life of olive oil, preventing its autooxidation. The chemical formula of hydroxytyrosol is $C_8H_{10}O_3$ and molecular weight is 154.

1.1.2.2. Oleuropein. Oleuropein is a phenolic secoiridoid glycoside found in the bark, leaves and fruit of the olive tree, as well as in some other genera of the *Oleaceae*. The most abundant phenolic substance in the drupe is oleuropein, a bitter glycoside that constitutes up to 14% of the fruit's dry weight. Oleuropein (Fig. 2) (CAS No.: 32619-42-4) has the chemical formula $C_{25}H_{32}O_{13}$ and a molecular weight of 541.

1.1.2.3. Tyrosol. Tyrosol (CAS No.: 501-94-0), a minor component of HIDROX®, has a faint sweet fruity-floral odor and a sweet but very weak taste. Tyrosol (Fig. 3) has the chemical formula $C_8H_{10}O_2$ and a molecular weight of 138.

1.1.3. Phenols in olives

Olive fruit is known to contain simple, as well as complex phenolic substances. The phenolic content and the specific composition of these phenols in whole olives

Table 1
General descriptive characteristics of HIDROX®

Botanical source (order)	Oleales
Botanical family	Oleaceae
Botanical name	*Olea europaea* Linné. s.l.; *Olea sativa* Hoffsmeg et Link
Other names of botanical source	Olive tree; Oliver; Olivenbaum; Oliva; *Olea europea* oil; *Olea europea* extract
Physical state	Powder or liquid
Color	Purple/brownish
Chemical family	Polyphenols (~6%)
Odor	Processed olives
Taste	Sour/olives
Packing and storage	Preserve in tight containers and prevent exposure to excessive heat

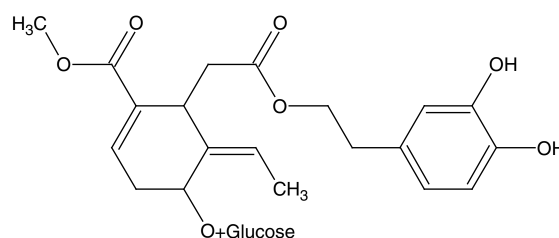

Fig. 2. Chemical structure of oleuropein.

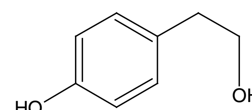

Fig. 3. Chemical structure of tyrosol.

depend on the altitude where the olive trees are grown, the harvesting time and the processing conditions. Similarly, the levels of phenolics in olive oil depend upon several factors (cultivar, climate, ripeness of olives, preparation and storage of the oil). These phenolics are responsible for the stability of the oil from oxidation and for the organoleptic properties (Visioli and Galli, 2001). In olive oil, phenols are present at levels up to 1% by weight, both as simple (hydroxytyrosol and tyrosol) and complex compounds (hydroxytyrosol or tyrosol esterified to elenolic acid, in the form of oleuropein and ligstroside, respectively) (hydroxytyrosol + elenolic acid → oleuropein and tyrosol + elenolic acid → ligstroside), (Fig. 4). In the intact olive, oleuropein and ligstroside are present in the glycosidic, relatively polar form. Hydroxytyrosol and tyrosol, as well as the lipid soluble oleuropein and ligstroside aglycones, are partially released (5–10% of the total in olives) from olives into the oil during production (crushing), while a substantial proportion remains in the water phase (vegetation water).

Owen et al. (2003) investigated the phenolic content of two brined olive drupe types (black and green) (Table 2). The green type contained predominantly hydroxytyrosol, while the black olives contained tyrosol, hydroxytyrosol, dihydrocaffeic acid, dihydro-*p*-coumaric acid (phloretic acid), acetoside (a disaccharide linked to hydroxytyrosol and caffeic acid), acetoside isomer and the flavonoids apigenin and luteolin. These investigators also reported that consumption of approximately 50 g of black olive pericarp would provide about 400 mg of phenolic substances to the daily dietary intake, while a similar quantity of extra virgin olive oil (produced with conventional methods) provides about 12 mg. The *percent* of wet weight for phenolics in black and green olives was reported as 0.082 and 0.118, respectively. In a recent analysis carried out on 48 olive samples, Romero et al. (2004) reported that the 'turning color olives' in brine had the highest concentration of polyphenols (~0.12%).

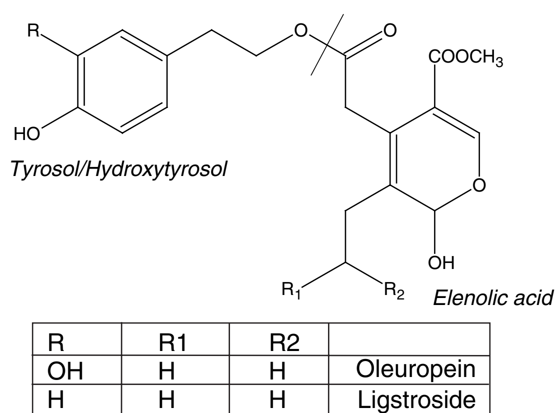

Fig. 4. Most relevant phenolics found in olive and olive products. Hydroxytyrosol is formed by cleavage where indicated (adapted from Visioli and Galli, 2003).

Table 2
Some basic characteristics and phenolics in black vs. green-brined olives (Owen et al., 2003)

Component	Olive type	
	Black	Green
Pericarp		
Total g wet wt.	71.78	111.6
Total g dry wt.	35.89	29.3
G dry wt. per drupe	1.794	1.465
Water (% of wet wt.)	50	73.7
Phenolics in pericarp		
Mg per drupe	29.43	6.56
% of wet wt.	0.82	0.118
% of dry wt.	1.64	0.448
Oil		
Total g	5.52	18.22
% of wet wt.	7.69	16.3
% of dry wt.	15.4	62.2

Values for 20 drupes of each olive type.

Vegetation water is a good source of phenolic antioxidants (1–1.8% w/v), as about 90% of the phenols in olives are transferred to the water phase during the pressing of the drupes. Approximately 10–20% of the total phenol content can be recovered; the only bioactive catechol recovered is hydroxytyrosol (Visioli and Galli, 2003). In another study, Fernandez-Bolanos et al. (2002) reported extraction of 3 kg of hydroxytyrosol (90–95% purity) from 1000 kg of olives during liquid–solid waste of two-phase (conventional) olive oil processing. Recently, CreAgri Inc., has been granted two patents for the recovery of hydroxytyrosol from olive mill water (US Patent numbers 6,416,808 and 6,197,308).

1.1.4. Specifications

Physical characteristics and specifications of HIDROX® are summarized in Table 3.

The chemical composition of standardized aqueous olive pulp extract along with analysis of the phenol fraction is summarized in Table 4. The phenolic content of the extract is 6% (60 mg/g).

1.1.5. Economic uses

The olive fruit, its oil and the leaves of the olive tree have a myriad of medicinal and other uses. Primarily, olives are used for their oil or as table olives and are an important part of the Mediterranean diet. Because of their organoleptic characteristics, olives require processing prior to consumption (Brenes-Balbuena et al., 1992a; Brenes-Balbuena et al., 1992b; Brenes-Balbuena et al., 1995; Goupy et al., 1991; Robards et al., 1999; Sciancalepore and Longone, 1984). In both fruit and oil, the phenolics constitute a complex mixture, although there are some notable differences in composition between the two that are attributed to a series of chemical or enzymatic alterations of some phenols during oil extraction. These modifications include hydrolysis of glycosides by glucosidases

Table 3
Specifications of aqueous olive pulp extract

Description	Characteristic/specification
Appearance	Purple brownish powder
Dry solids	98–99%[a]
Citric acid	1–2%
Polyphenols	6%
Moisture–vacuum oven (%wt./wt.)	8–10%
Ash (%wt./wt.)	8–10%
Solubility in water	>95%
pH (range)	2–4
Heavy metals (as Pb)	<10 mg/kg
Lead	<1 mg/kg
Free flow density	0.2–0.3 g/ml
Tap density	0.3–0.45 g/ml
Granulations	
Sieve size +25 (%wt./wt.)	6–8%
Sieve size −25 + 30 (%wt./wt.)	21–23%
Sieve size −30 + 45 (%wt./wt.)	53–56%
Sieve −45 (pan)	13–18% retained on pan
Microbial standards	
Escherichia coli and coliforms	<3 MPN/g
Salmonella (TECRA)	Negative
Yeast	<10 CFU/g
Mold	<10 CFU/g
Staphylococcus aureus	<3 MPN/g
Pseudomonas	Negative
Standard plate count	<10 CFU/g
ORAC value[b]	1450–2000 μmole TE/g

MPN = Most probable number; CFU = Colony forming units.
[a] The chemical composition of standardized HIDROX® dry solids is given in Table 4.
[b] ORAC = Oxygen radical absorbance capacity. ORAC is performed to measure the antioxidant capacity of HIDROX® and the value is expressed as μmole Trolox equivalent/g.

Table 4
Chemical composition of aqueous olive pulp extract

Chemical analysis	Value g per 100 g with range
Moisture	2.8 (1–3)
Ash	9.6 (6–10)
Protein	6.7 (4–7)
Fat	27.0 (20–30)
Carbohydrate (total)	53.5 (40–55)
Phenolics (total)	6.0
Analysis of phenol fraction	**% of total phenol**
Total polyphenol	100
Hydroxytyrosol	50–70
Tyrosol	0.3
Oleuropein	5–10
Other polyphenols	~20[a]

[a] The other polyphenols detected in the extract include gallic acid and oleuropein aglycone.

(Montedoro et al., 1993; Angerosa et al., 1996), oxidation of phenolic compounds by polyphenol oxidases and, the polymerization of free phenols (Ryan et al., 1999). The quality of virgin oil is affected by the presence of phenolic compounds in olive fruits, as these compounds are partly responsible for the stability and sensory characteristics.

1.1.6. Exposure reports

The current primary source of exposure to the constituents (phenolics) of aqueous olive pulp extract (HIDROX®) is via consumer use of olives and their products. Blekas et al. (2002) reported hydroxytyrosol (unbound) content of table olives as 250–750 mg/kg (~0.5 mg/g) in two cultivars. Based on this information and United States Department of Agriculture (USDA) eaters-only data, per capita consumption of hydroxytyrosol can be determined (CSFII, 2000). Eaters-only data describe the amount of substance naturally present in food consumed only by those individuals that actually consume the particular food. The eaters-only per capita mean and 90th percentile consumption of olives in the US is 20.15 and 40.50 g/day, respectively. As table olives contain approximately 0.5 mg hydroxytyrosol/g of olive, the mean (20 g) and 90th percentile (40 g) consumption of table olives may result in a daily intake of 10 or 20 mg of hydroxytyrosol.

Another study reported that consumption of 50 g of black olive pericarp provides approximately 400 mg of phenolic substances, while a similar quantity of extra virgin olive oil provides about 12 mg. Of the phenolic compounds found in olives, approximately 10% was reported as hydroxytyrosol (Marsilio et al., 2001). As olives contain approximately 0.8 mg hydroxytyrosol/g of olive, the mean (20 g) and 90th percentile (40 g) consumption of olives will result in a daily intake of 16 or 32 mg of hydroxytyrosol.

2. Biological data

2.1. Absorption, metabolism and excretion

2.1.1. Absorption

Bai et al. (1998) reported that oral administration of hydroxytyrosol to rats resulted in rapid appearance of hydroxytyrosol in the blood, with maximal concentrations obtained in 5–10 min. Hydroxytyrosol was almost completely eliminated and/or metabolized within 180 min of administration. The amount of hydroxytyrosol found in plasma/blood fluctuated widely and was low, compared to the dose administered. In another study, Sprague Dawley rats (6/sex/group) were administered HIDROX® (1000, 1500 and 2000 mg/kg/day; corresponding to hydroxytyrosol at 24, 36 and 48 mg/kg/day, respectively) by oral gavage for 90 days (Christian et al., 2004). Blood samples were collected on Day 90, prior to dosing and at 0.5, 1, 2, 4 and 8 h post-dose. Pre-dose plasma samples contained no measurable mean concentrations of hydroxytyrosol, suggesting minimal carry-over of hydroxytyrosol from prior doses. The results of this study suggest that hydroxytyrosol was rapidly absorbed, and mean concentrations were measurable through 1–4 h at 1000 and 1500 mg/kg and through 8 h at 2000 mg/kg. Despite differences between this and the Bai et al. (1998) study, both studies suggest a rapid absorption and excretion of hydroxytyrosol.

In a human study, Visioli et al. (2000) investigated the absorption of olive oil phenolics. Six male human

volunteers (ages 27–33) were given 50 ml of four olive oil samples spiked with hydroxytyrosol, and the first 24 h urine was analyzed. The concentrations of total phenol, hydroxytyrosol and tyrosol in the four oils were 488/20/36, 975/44/72, 1463/66/110 and 1950/84/140 ppm, respectively. The urinary excretion of tyrosol and hydroxytyrosol for the four individual oils was 21/29, 28/64, 21/35 and 24/40 (as *percent* of administered dose). The authors reported that the ratio of tyrosol/hydroxytyrosol found in urine was similar to that present in the oil (~1.7). If the ratio as reported in the article is considered correct, then the amount of urinary excretion of tyrosol and hydroxytyrosol cited in the article appears to be switched. The proportions of total tyrosol and hydroxytyrosol excreted were in the range of 20–22% for tyrosol and 30–60% for hydroxytyrosol. It was unclear as to the fate of the remaining amounts. The data suggest that simple olive oil phenols such as tyrosol and hydroxytyrosol are absorbed after administration and are excreted as glucuronide conjugates. In a subsequent study, analysis of urine revealed two more metabolites of hydroxytyrosol, homovanillic acid (4-hydroxy-3-methoxy phenylacetic acid) and homovanillyl alcohol (Caruso et al., 2001).

In a bioavailability study, Tuck et al. (2001) investigated the absorption and elimination of radiolabeled hydroxytyrosol and tyrosol, in Sprague Dawley male rats following intravenous (in saline) and oral (in oil- and water-based solutions) administration. For both compounds, the intravenously and orally administered oil-based dosing resulted in significantly greater elimination of the phenolics in urine within 24 h, than for the oral aqueous dosing method. For both tyrosol and hydroxytyrosol, there was no significant difference in the amount eliminated in urine between the intravenous and the oral oil-based dosing methods. Urine samples revealed the presence of hydroxytyrosol and five metabolites. Oral bioavailability estimates of hydroxytyrosol, when administered in an olive oil solution and in an aqueous solution, were 99% and 75%, respectively, while the bioavailability estimates for tyrosol were 98% and 71%, respectively. The differences between this and above-described human study are presented in Section 3.

To further explore the differences in the elimination of hydroxytyrosol, Visioli et al. (2003) compared metabolism and urinary excretion of hydroxytyrosol between rats and humans. These investigators also compared the human excretion of hydroxytyrosol, when consumed as a natural component of extra virgin olive oil, when added to refined olive oil or when added to yogurt. The urinary excretion of hydroxytyrosol was greater in humans compared to rats, a species with a high basal excretion of hydroxytyrosol and its metabolites. Administration of hydroxytyrosol to humans resulted in increased 24-h urinary excretion of hydroxytyrosol and homovanillyl alcohol compared to background levels. The recovery of these two substances in the urine was 44% of the total administered hydroxytyrosol and 234% of free hydroxytyrosol administered. In humans, the high excretion of free hydroxytyrosol (234%) suggests that hydrolysis of oleuropein (present in olive oil) may be responsible. These data also suggest differences in the metabolism of hydroxytyrosol in rats and humans. In comparative studies among different vehicles, hydroxytyrosol excretion in humans was much higher after its administration as a natural component of olive oil (44.2% of hydroxytyrosol) than after its administration in refined olive oil (23% of hydroxytyrosol) or yogurt (5.8% of dose or ~13% of that recorded after virgin olive oil intake).

In another study, eight healthy volunteers received an oral fat load consisting of 100 g of extra virgin olive oil, and blood was collected at different time points. The investigators concluded that phenolic compounds in olive oil were absorbed from the intestine, though not through a pathway dependent on chylomicron formation, and may exert a significant antioxidant effect in vivo, probably in the post-prandial phase (Bonanome et al., 2000). In another study of extra virgin olive oil phenol absorption in ileostomy subjects, Vissers et al. (2001) reported that phenols from olive oil are primarily absorbed from the small intestine. Absorption was confirmed by the excretion of tyrosol and hydroxytyrosol in urine. It was estimated that the apparent absorption of phenols was at least 55–66% of the ingested dose. In ileostomy subjects, 12 mol/100 mol, and in subjects with a colon, 6 mol/100 mol of the phenols from the non-polar supplement were recovered in urine as tyrosol or hydroxytyrosol. In a study to validate the quantitative estimation method for hydroxytyrosol levels in blood, Ruiz-Gutierrez et al. (2000) reported that administration of 20 mg/kg of hydroxytyrosol to rats resulted in plasma concentrations of 1.22 and 1.91 μg/ml of hydroxytyrosol at 5 and 10 min, respectively.

Manna et al. (2000) investigated the molecular mechanism of intestinal transport of hydroxytyrosol, using a differentiated Caco-2 cell monolayer as the model system of the human intestinal epithelium. The kinetic data suggest that [^{14}C]-hydroxytyrosol transport occurs via a passive diffusion mechanism and is bi-directional. The calculated apparent permeability coefficient suggests that hydroxytyrosol is quantitatively absorbed at the intestinal level. Based on the results of this study, it is likely that hydroxytyrosol will be completely (100%) absorbed in humans. The metabolism data suggest that the only labeled metabolite detectable in the culture medium was 3-hydroxy-4-methoxyphenylethanol (10% conversion), the product of catechol-*o*-methyltransferase. In an in vitro hydroxytyrosol assay with the purified enzyme from porcine liver in the presence of the methyl donor, *S*-adenosinemethionine, a *K*m of 40 μM was calculated. This value is significantly lower than that of the endogenous substrates, including dopamine, suggesting that hydroxytyrosol could be a preferential substrate for this enzyme in vivo. These data provide the evidence that hydroxytyrosol is highly bioavailable.

2.1.2. Metabolism

Following intravenous administration of [^{14}C]-hydroxytyrosol to rats, less than 8% of the administered radioactiv-

ity was present in the blood stream 5 min after injection (6% associated with plasma and 2% with cell fraction). Only 0.1% of the administered hydroxytyrosol dose was detectable in the blood 5 h after administration. Approximately 90% of the administered radioactivity was detected in urine within 5 h, while about 5% was detected in feces and the gastrointestinal content. [^{14}C]-hydroxytyrosol was enzymatically converted to four oxidized and/or methylated derivatives. A significant fraction of total radioactivity was associated with sulfo-conjugated forms, which also represented the major urinary excretion products. Based on these results, the investigators proposed a metabolic pathway of exogenously administered hydroxytyrosol involving catechol-*o*-methyltransferase, alcohol dehydrogenase, aldehyde dehydrogenase and phenolsulfotransferase (D'Angelo et al., 2001).

Three groups of Sprague Dawley male rats were administered (gavage) 1, 5 or 10 mg/kg of vegetation water extract, providing 41.4, 207 or 414 μg/kg of hydroxytyrosol, respectively. Hydroxytyrosol was dose-dependently absorbed and excreted in the urine, mostly as a glucuronide conjugate. Approximately 25% of the administered dose was found as total (i.e. free and glucuronide conjugated) hydroxytyrosol. Additional studies revealed that administration of hydroxytyrosol-rich olive mill water extract (10 mg/kg) to the rats was also associated with an increase of their plasma antioxidant capacity (Visioli et al., 2001).

Caruso et al. (2001) investigated the metabolic fate of hydroxytyrosol after ingestion of virgin olive oil (50 ml, containing 7–23 mg total hydroxytyrosol) enriched with a phenolic extract in six healthy male human volunteers. The results suggested that hydroxytyrosol was metabolized by catechol-*o*-methyltransferase resulting in an enhanced excretion of homovanillyl alcohol. A significant increase in homovanillic acid was also noted, indicating an oxidation of the ethanolic residue of hydroxytyrosol and/or of homovanillyl alcohol in humans. The excretion of both metabolites significantly correlated with the administered hydroxytyrosol in olive oil.

In summary, several investigators reported determination or bioavailability of hydroxytyrosol in humans after oral administration of olive oil. These studies demonstrate the presence of hydroxytyrosol in blood and urine. However, oleuropein, which is also present in olive oil, can be absorbed and hydrolyzed to hydroxytyrosol. In the majority of the studies with olive oil, levels of hydroxytyrosol were not given, and it is difficult to estimate how much hydroxytyrosol is formed as a result of hydrolysis of absorbed oleuropein.

2.1.3. Excretion

Visioli et al. (2003) studied urinary excretion of free and conjugated hydroxytyrosol in rats and humans administered olive oil. The results of this study indicate that within 24 h, humans excrete 31% of the hydroxytyrosol, while rats excrete only 5% of the hydroxytyrosol. In another study, Miro-Casas et al. (2001) determined urinary excretion of hydroxytyrosol and tyrosol in humans (6 men and 5 women) after virgin olive oil intake (50 ml). The amount of hydroxytyrosol recovered in 24-h urine after olive oil ingestion was significantly higher than during washout or basal periods. In a subsequent study, Miro-Casas et al. (2003b) reported an increase in 24-h urine of hydroxytyrosol, following both a single-dose ingestion (50 ml) and short-term consumption (25 ml/day for a week) of virgin olive oil in seven healthy subjects. Miro-Casas et al. (2003a) also reported increases in plasma hydroxytyrosol and 3-*o*-methyl-hydroxytyrosol following ingestion of virgin olive oil (25 ml) in humans, reaching maximum concentrations at 32 and 53 min, respectively. The estimated hydroxytyrosol elimination half-life was 2.43 h, while the Cmax was reported as 26 μg/l. Based on the results of this study, approximately 98% of hydroxytyrosol appears to be present in plasma and urine in conjugated forms, mainly glucuronides, suggesting extensive first-pass intestinal/hepatic metabolism of the ingested hydroxytyrosol.

In a follow up investigation on the urine samples from the absorption study (see section 2.1.1), Tuck et al. (2002) identified three metabolites of hydroxytyrosol by tandem MS/MS as monosulfate conjugate, 3-*o*-glucuronide conjugate and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid). In a review article, Tuck and Hayball (2002) reported that hydroxytyrosol is excreted through the kidney unchanged and also metabolized to the following metabolites: glucuronide conjugate, sulfate conjugate, homovanillic acid, homovanillyl alcohol, 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylacetaldehyde.

2.2. Biochemical/pharmacological effects

It has been suggested that the protective effects of olive oil in lowering the incidence of degenerative pathologies could be ascribed not only to its high oleic acid content but also to the antioxidant properties of its polyphenols (Pellegrini et al., 2001; Poli et al., 2002). Among the polyphenols, in recent years, particular attention has been focused on the specific olive polyphenol, hydroxytyrosol, for its protective properties. Hydroxytyrosol has been shown to prevent in vitro LDL oxidation (Grignaffini et al., 1994; Salami et al., 1995), inhibit platelet aggregation (Petroni et al., 1995), inhibit 5- and 12-lipooxygenases (Kohyama et al., 1997; De la Puerta et al., 1999), effectively counteract the cytotoxic effects of reactive oxygen species in various human cellular systems (Manna et al., 1997; Manna et al., 1999a) and, act as a free radical scavenger (Saija et al., 1998). Hydroxytyrosol has been shown to exert inhibitory effects on peroxynitrite-dependent base modification and tyrosine nitration (Deiana et al., 1999). Hydroxytyrosol has been also shown to exert an antiproliferative effect, inducing apoptosis in HL-60 cells and in resting and activated peripheral blood lymphocytes (Della Ragione et al., 2000; Della Ragione et al., 2002; Fabiani et al., 2002). Some biological effects of hydroxytyrosol are summarized in Table 5.

Table 5
Biological effects and effective concentration of hydroxytyrosol (adapted from Manna et al., 1999a)

Biological effect noted	Concentration (μM)
In vitro inhibition of LDL oxidation	5–10
Inhibition of platelet and leukocyte arachidonate lipoxygenases	10–100
Inhibition of PMA-induced respiratory burst in human neutrophils	10–100
Antiproliferative and differentiating effects; inhibition of apoptosis	50–100
Protection against ROS-mediated cytotoxicity of human intestinal cells and human erythrocytes	50–250
Inhibition of peroxynitrite-dependent tyrosine nitration and DNA damage	50–500
Inhibition of platelet aggregation and eicosanoid production	100–400

2.3. Toxicological studies

2.3.1. Acute toxicity studies

In acute toxicity studies in CRL:CD1 ICR (BR) mice, oral gavage administration or dermal application of a single dose of aqueous olive pulp extract at levels of 500, 1000 or 2000 mg/kg failed to produce any adverse effects. No mortality was noted in any of the treatment groups, suggesting that the LD_{50} of the extract is greater than 2000 mg/kg (Christian et al., 2004). In another study, oral administration of a single gavage dose of solid olive pulp extract at levels of 0, 1000, 1500 or 2000 mg/kg to Crl:CD® Sprague Dawley rats did not cause any adverse effects except soft or liquid feces (Christian et al., 2004). As part of a micronucleus assay study, Crl:CD® Sprague Dawley rats (5/sex) were administered (gavage) a single dose of 5000 mg olive pulp extract/kg, and the rats were observed for six days, after which the 5000 mg/kg dose was given for 29 consecutive days (Christian et al., 2004). No mortality or clinical signs of toxicity were noted. This study demonstrates that the LD_{50} of HIDROX® is greater than 5 g/kg and suggests that HIDROX® is practically non-toxic.

Sprague Dawley male rats ($n = 6$) were administered a single gavage dose of 2000 mg hydroxytyrosol/kg. At the end of the experiment (14 days), the rats were killed, and gross and pathological changes in "main organs" (not specified in the publication) were evaluated. During the study period, no deaths occurred. The only clinical sign observed in the male and female rats was piloerection, which started two hours after treatment and disappeared within 48 h of treatment (D'Angelo et al., 2001).

2.3.2. Subchronic toxicity studies

In a subchronic study, Crl:CD® Sprague Dawley rats (20/group/sex) were administered (via gavage) a daily dose of aqueous olive pulp extract (HIDROX®) (in 0.5% methylcellulose) at levels of 0, 1000, 1500 and 2000 mg/kg/day (0, 60, 90 and 120 mg/kg/day of phenolics) for 90 days (Christian et al., 2004). The selection of the gavage route was based on the fact that (1) gavage administration most simulates the method of intake in humans, consumed over a relatively short period of time; and (2) high doses of the extract are not palatable. Morbidity and mortality observations did not reveal any unusual findings. Excess salivation and presence of perioral substance noted in treatment group were probably associated with technical difficulties in administering the relatively thick granular suspensions of the extract. All other clinical observations were considered unrelated to test article. Administration of the extract did not affect body weights, body weight gains or feed consumption. A significant decrease in body weight gain noted for male rats in the 1000 mg/kg/day group on days 71–78 was considered unrelated to test article because the value was not dose-related. HIDROX® administration did not affect the organ weights. Ophthalmologic observations did not reveal any treatment-related lesions.

Hematological analysis (WBC, RBC, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelet counts, prothrombin time and total serum protein) did not reveal any treatment-related differences between the groups, except some incidental findings. Although not significant at two lower doses (1000 and 1500 mg/kg/day), a dose-related increasing trend in the number of RBC was noted in female rats. The increase was significant in the 2000 mg/kg/day dose group. However, the values were within range of historical control values. Increases in RBCs in female rats, in the absence of changes in mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were interpreted as a slight erythropoietic stimulation of the bone marrow without any toxicological consequences. All other hematological parameters in the male and female rats were unaffected.

Plasma levels of liver function enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) showed a negative trend (Table 6). Reduction in these enzymes may be related to decreases in cholesterol levels. Levels of ALT were significantly reduced in both male and female rats in all the extract treated groups. A significant reduction in SDH levels was noted in female rats treated with 1500 and 2000 mg/

Table 6
Effects of aqueous olive pulp extract administration on plasma enzymes (ALT, AST and SDH)

Plasma enzyme	Animal sex	Dose (mg/kg/day)			
		0 (vehicle)	1000	1500	2000
ALT	Male	33 ± 3.5	28 ± 5.3*	26 ± 5.8**	25 ± 7.9**
	Female	30 ± 3.6	25 ± 4.0**	25 ± 3.7**	23 ± 5.9**
AST	Male	74 ± 6.8	71 ± 6.7	69 ± 9.8	67 ± 9.5
	Female	76 ± 9.0	72 ± 10.5	69 ± 7.4	69 ± 12.8
SDH	Male	16 ± 4.0	14 ± 3.5	16 ± 6.8	12 ± 4.2
	Female	15.5 ± 6.5	13.5 ± 8.8	9.5 ± 2.4**	10.7 ± 2.5**

Values are mean ± SD.
* and ** indicate values significantly different from the vehicle control at $p \leqslant 0.05$ and $p \leqslant 0.01$, respectively.

kg/day doses. Increases in plasma levels of ALT, AST and SDH are considered as markers of hepatotoxicity. Decreases in these plasma enzymes (ALT, AST and SDH), observed in most of the extract treated groups of male and female rats, were not considered to be a toxic manifestation. Although negative trends in plasma levels of ALT, AST and SDH were noted, all values were within historical control value ranges. The changes were minor and do not appear to be a toxicologically adverse response. In addition to the above-described changes, some incidental findings were noted. These incidental findings were considered insignificant, as these changes were either not dose-related, of small magnitude or not consistent between sexes.

Histopathological observations revealed minimal or mild focal hyperplasia of the mucosal squamous epithelium of the limiting ridge of the forestomach of male and female rats in the 2000 mg/kg/day dose group. Similar changes with a very low incidence of hyperplasia were noted in animals treated with 1500 mg/kg/day of the extract and in the control group. All other microscopic changes that were noted in the various organs and tissues examined were considered spontaneous in origin, incidental to treatment and not associated with any systemic toxicity of the extract. Although focal, minimal or mild hyperplasia of the mucosal squamous epithelium of the limiting ridge of the forestomach was noted, this type of change is often associated with irritation of this area of the gastric mucosa and was considered to be due to local irritation by the large incubated volume of viscous, granular formulation. Hence, the NOAEL for this study is 2000 mg/kg/day, the highest dose administered.

2.3.3. Developmental (embryo-fetal toxicity/teratogenicity)/reproduction toxicity

In a study designed to provide an overall screening of potential reproductive and developmental toxicities, olive pulp extract (HIDROX®) was administered by oral gavage to Crl:CD® Sprague Dawley rats (Christian et al., 2004). Rats (8/sex/group) were assigned to five dose groups and the extract (0, 500, 1000, 1500 and 2000 mg/kg/day) was administered once daily for 14 days before cohabitation and continued until the day before necropsy (males were euthanized after being administered a total of 49 daily doses of the extract; females were euthanized after completion of the 22-day post-partum period). The details of protocol and results of these studies are described by Christian et al. (2004). All F_0 generation male rats survived to the scheduled euthanasia. Occasional instances of excess salivation and non-dose-related increases in body weight gains were the only findings associated with the HIDROX® administration. In the F1 generation, estrous cycling, mating and reproductive performance of the female rats were not affected by the extract treatment. Small reductions (<10%) in pup body weights on lactation days 7, 14 and 21 in the 1000, 1500 and 2000 mg/kg/day dose groups were noted. All other delivery and litter observations were comparable among the five dose groups. Based on the results of this study, aqueous olive pulp extract does not appear to be a reproductive or developmental toxicant. As no toxic effects were observed in the parental rats during the first two weeks of treatment, doses of 0, 1000, 1500 and 2000 mg/kg/day of HIDROX® were recommended for use in the developmental toxicity study and 90-day toxicity study in rats. The high dose of 2000 mg/kg/day is generally considered the highest dose necessary for studies of this type (Christian et al., 2004).

In a follow-up investigation to the aforementioned study, the developmental toxicity (embryo-fetal toxicity and teratogenic potential) of aqueous olive pulp extract (HIDROX®) was studied in Crl:CD® Sprague Dawley rats (Christian et al., 2004). This study was designed in accordance with the FDA Redbook 2000 (FDA, 2004). Time-mated female rats were randomly divided into four groups (Groups I–IV), 25 rats per group. On days 6–20 of presumed gestation, the extract or the vehicle (0.5% w/v methylcellulose) was administered via gavage once daily at doses of 0, 1000, 1500 and 2000 mg/kg/day. The phenolic content of the extract was 6% (60 mg/g).

On gestation day 21, one dam in the 2000 mg/kg/day group began to prematurely deliver its litter before scheduled Caesarean-sectioning and was euthanized. No abnormal findings were noted for this dam or its litter. All other rats survived until scheduled Caesarean-sectioning. No adverse clinical or necropsy observations or significant differences in maternal body weights, body weight gains, gravid uterine weights, corrected maternal body weights or body weight gains or absolute or relative feed consumption values were noted between the groups. Caesarean-sectioning observations were based on 23, 22, 22 and 24 pregnant rats with one or more live fetuses in the four respective groups. The extract treatment did not affect litter parameters at any of the doses. No treatment-related increases in gross external, soft tissue and skeletal fetal alterations (malformations or variations) were noted. A significantly increased mean number of corpora lutea of the 2000 mg/kg dose was well within the historical range of 14.5–20.1 per litter and was attributed to two females that had 27 or 30 corpora lutea. The maternal and developmental no observed adverse effect level (NOAEL) of the extract was greater than 2000 mg/kg/day, the highest dose administered.

2.3.4. Genotoxicity

2.3.4.1. In vitro studies. In an in vitro mutagenicity study, olive pulp extract (HIDROX®) was evaluated in a bacterial reverse mutation assay employing *Salmonella typhimurium* strains TA97, TA98, TA100 and TA1535 and *Escherichia coli* strain WP2 *uvr*A (328), in the presence and absence of S9. The extract was tested at concentrations of 0, 5, 10, 50, 100, 500, 1000, 2500 and 5000 μg/plate. Concentrations of 50, 100, 500, 1000 and 2500 μg/plate were used in the more sensitive confirmatory preincubation assay. A result was classified as positive (i.e., mutagenic) if the

average number of revertants in any strain at any test article concentration was at least two times greater than the average number of revertants in the concurrent vehicle control and/or there was a concentration-related increase in the mean revertants/plate in that same strain. In the Ames assay, at concentrations of 100 μg/plate or above of the extract, precipitates were observed. As determined by a concentration-related reduction in the mean number of revertants/plate and/or the reduction of the microcolony background lawns, toxicity was observed at concentrations of 500 μg/plate or above. Evidence of mutagenic activity was only detected in strains TA98 and TA100 at doses of 1000 and 2500 μg/plate (in the presence of S9 activation for both the strains). The number of revertants per plate at concentrations of 0, 1000 and 2500 μg/plate for TA 98 were reported as 23, 52 and 133, respectively. Similarly, the number of revertants per plate for the strain TA100 was reported as 157, 372 and 1051, respectively. In experiments with *E. coli*, no mutagenicity was noted at any of the concentrations tested, except for a twofold increase in mean number of revertants at concentration of 2500 μg/plate, in the absence of S9. The positive results were confirmed in the more sensitive preincubation test, but only with metabolic activation. Some inconsistencies between the regular and repeat trials were noticed. The antibacterial properties of the test article, and observation of positive findings only at one or two concentrations, where precipitates and toxicity occurred, tended to complicate the interpretation of these mutagenic findings. The investigators concluded that under the conditions of the study, equivocal evidence of mutagenic activity of the extract was detected in *S. typhimurium* strains TA98 and TA100 (Christian et al., 2004).

In another in vitro genotoxicity assay, the effects of solid olive pulp extract (HIDROX®) on chromosome aberrations in Chinese hamster ovary cells were investigated, in the presence and absence of an exogenous metabolic activation system (S9) (Christian et al., 2004). The cell cultures were treated with 0, 10, 50, 100, 300, 600 and 1000 μg of the extract/ml; positive and negative (vehicle, dimethyl sulfoxide) controls were also included. Cultures were incubated with the extract for approximately three hours, after which the treatment medium was washed and replaced with a fresh culture medium. Cells were sampled at a time approximately 20 h from the beginning of treatment. Approximately 2 h prior to harvest, Colcemid® was added to arrest cells in metaphase. Test article concentrations of 100, 300 and 1000 μg/ml were assessed for effects on mitotic index, polypoid cells and aberrations (chromatid and chromosome breaks/exchanges). No clear evidence of test article-associated toxicity, as evidenced by the confluence rate or mitotic index, was observed at any concentration level of the extract. The extract elicited a significant increase in the *percentage* of aberrant cells at 1000 μg/ml in the presence of S9. At this concentration, slight increases in the numbers of polyploid and/or endoreduplicated cells (numerical chromosome changes) were also noted. The positive response was associated with the presence of test article precipitate during treatment. Based on the results of this study, the investigators concluded that the extract was positive for the induction of chromosome aberrations (Christian et al., 2004).

2.3.4.2. In vivo studies. In the micronucleus assay, adult Crl:CD® Sprague Dawley IGS BR male and female rats (20/sex/group, except 5/sex for 5000 mg/kg group) were administered 0, 1000, 1500, 2000 or 5000 mg/kg/day olive pulp extract (HIDROX®) by gavage for 28 days. Approximately 24 h after the last dose, the rats were euthanized, and bone marrow samples from the femur of each rat were collected for further analysis. Additional experiments were performed with single doses of the extract at 1000, 1500 or 2000 mg/kg. These rats were euthanized at 24 or 48 h after the treatment, and bone marrow samples were subsequently collected. In both of these experiments, groups of 20, 12, 12, 21 and 5 rats/sex were assigned to the vehicle control, the three dose groups and a positive control group, respectively. A minimum of 2000 polychromatic erythrocytes was scored for micronuclei. The number of polychromatic erythrocytes among 500 total erythrocytes (expressed as polychromatic erythrocytes) was determined for each animal. The number of micronucleated normochromatic erythrocytes was also recorded. The extract did not produce adverse clinical or necropsy observations or affect absolute or relative feed consumption values. Body weight gains for the male and female rats in the highest dose group were reduced at the third and fourth weeks of daily dose, as compared with previous weeks. The numbers of micronucleated polymorphic erythrocytes were not significantly increased in any of the extract treated groups, as compared to the control. The ratio of polychromatic erythrocytes to normochromatic erythrocytes was not affected by the administration of HIDROX®. The results of this study demonstrate that the extract was negative in the micronucleus assay at 24 and 48 h after a single dose of 1000, 1500 or 2000 mg/kg and also at 24 h after 28 daily doses of 0, 1000, 1500, 2000 or 5000 mg/kg.

2.3.5. Antimicrobial activity

Hydroxytyrosol has been shown to inhibit or delay the rate of growth of a range of bacteria and microfungi and pathogenic bacteria in humans (human pathogens). Olive oil vegetation water has been reported to be toxic to both phytopathogenic *Pseudomonas syringae* (Gram-negative) and *Corynebacterium michiganense* (Gram-positive) bacteria. Capasso et al. (1995) reported that among the main polyphenols of the vegetation water, methylcatechol proved to be the most active against *P. syringae* 10^{-4} mol/l, and also demonstrated bactericidal activity, while against *C. michiganense* it was only slightly active. Other polyphenols, such as catechol and hydroxytyrosol, were less active on *P. syringae*, and inactive on *C. michiganense*.

Bisignano et al. (1999) studied the in vitro susceptibility of several human intestinal or respiratory tract pathogens

to hydroxytyrosol and oleuropein. The pathogens studied included, five ATCC standard bacterial strains (*Haemophilus influenzae* ATCC 9006; *Moraxella catarrhalis* ATCC 8176; *Salmonella typhi* ATCC 6539; *Vibrio parahaemolyticus* ATCC 17802 and *Staphylococcus aureus* ATCC 25923) and 44 fresh clinical isolates (*Haemophilus influenzae*, eight strains; *Moraxella catarrhalis*, six strains; *Salmonella* species, 15 strains; *Vibrio cholerae*, one strain; *Vibrio alginolyticus*, two strains; *Vibrio parahaemolyticus*, one strain; *Staphylococcus aureus*; five penicillin-susceptible strains and six penicillin-resistant strains). The minimum inhibitory concentrations (MICs) reported in this study suggested the broad antimicrobial activity of hydroxytyrosol against these bacterial strains (MIC values between 0.24 and 7.85 μg/ml for ATCC strains and between 0.97 and 31.25 μg/ml for clinically isolated strains). The investigators suggested hydroxytyrosol may be useful in an antimicrobial treatment of intestinal or respiratory tract infections in man.

2.3.6. Antioxidant activity

Aeschbach et al. (1994) investigated in vitro antioxidant and prooxidant properties of several naturally occurring food constituents, including hydroxytyrosol. The ability of hydroxytyrosol to inhibit peroxidation of membrane lipids from ox brain phospholipid liposomes was studied. Hydroxytyrosol dose-dependently inhibited lipid peroxidation of phospholipid liposomes in the presence of iron (III) and ascorbate. Hydroxytyrosol promoted deoxyribose and DNA damage in the deoxyribose assay and in the bleomycin-Fe(III) system, respectively. This promotion was strongly inhibited in the deoxyribose assay by the addition of bovine serum albumin to the reaction mixture. The in vitro prooxidant properties of hydroxytyrosol in two of these assays are difficult to explain, but in in vivo conditions and other in vitro assays, hydroxytyrosol has been shown to be a strong antioxidant. Secondly, the slight in vitro prooxidant activity of hydroxytyrosol on DNA was noted at non-physiological concentrations (0.065–0.65 mM). In contrast to the prooxidant properties, several studies show cytoprotective effects of hydroxytyrosol (Manna et al., 1999b, 2002).

3. Discussion

Comparative urinary excretion studies of hydroxytyrosol in rats and humans suggest a high basal excretion of hydroxytyrosol and its metabolite in rats. When given in extra virgin olive oil, absorption of hydroxytyrosol was higher in humans, compared to rats. Hydroxytyrosol is excreted in urine as the unchanged parent compound, in the form of metabolites, including homovanillic acid, homovanillic alcohol, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetaldehyde, and as its glucuronide and sulfate conjugates. The differences in findings of Tuck et al. (2001), from animal studies, and those of Visioli et al. (2000), from human studies, on hydroxytyrosol elimination are difficult to explain. Two reasonable hypotheses are as follows: First, hydroxytyrosol and tyrosol could be handled differently in humans than in rats. Alternatively, the analytical method employed by Tuck et al. (2001) may be a more accurate method for assessing the absorption and excretion of hydroxytyrosol and tyrosol, because the presence of numerous labeled conjugates of hydroxytyrosol and tyrosol could be detected, not just those hydrolyzed from the parent compound in β-glucuronidase-hydrolyzed urine. Additional comparative studies of metabolism of hydroxytyrosol between rats and humans by Visioli et al. (2003) also revealed differences. Visioli et al. (2003) suggested that the difference in absorption and/or excretion of hydroxytyrosol in rats and humans might be due to the absence of a gall bladder in rats, which results in the presentation of lipid soluble or amphiphilic molecules such as hydroxytyrosol to the intestinal flora.

In a subchronic study in rats, the gavage administration of aqueous pulp extract at doses up to 2000 mg/kg/day for a period of 90 days did not reveal any signs of toxicity. Markers of liver function tests, such as levels of ALT, AST and SDH, trended downward. The reasons for decreased activity may be associated with the large quantities of olive polyphenols that have to be excreted in the bile. The biliary excretion of large doses of the extract may also account for the slightly decreased serum cholesterol levels in both male and female rats (significantly decreased in females at 2000 mg/kg/day), because primary bile acids are synthesized by the liver from cholesterol (Tennant, 1999). Although negative trends in plasma levels of ALT, AST and SDH were noted, all values were within historical control ranges. The changes were minor and do not appear to be a toxicologically adverse response. Secondly, other biomarkers of liver function (serum bilirubin, alkaline phosphatase, protein levels and histopathology) were unaffected by the extract. Histological investigations of the major tissues did not reveal any treatment-related pathological changes except for very low to mild focal hyperplasia of the mucosal squamous epithelium of the limiting ridge of the forestomach. These changes appeared to be related to the slightly irritating effect on this area of the gastric mucosa resulting from gavage administration of the extract and the granularity and high viscosity of the suspended extract. Based on the data from this study, the no observed adverse effect level (NOAEL) of the extract for rats is 2000 mg/kg/day, the highest dose tested.

In a developmental toxicity study in rats, olive pulp extract (HIDROX®) did not cause maternal or developmental toxicity at levels up to 2000 mg/kg/day (highest dose tested). In an oral dose-range reproduction study in rats, doses of the extract ranging from 500 to 2000 mg/kg/day did not adversely affect any of the parental reproductive performance parameters (estrous cycling, mating, fertility, parturition, lactation, maternal behavior) investigated or the viability, growth or development of the offspring through one week post-partum. In an in vitro mutagenicity study, aqueous olive pulp extract was not

mutagenic in the presence or absence of metabolic activation in *S. typhimurium* strains TA97 and TA 1535, while the results in strains TA98 and TA100 were equivocal. In genotoxicity assays with *E. coli*, no mutagenicity was noted. In chromosome aberration studies in Chinese hamster ovary cells, the extract elicited increases in aberrant cells at 1000 μg/ml in the presence of metabolic activation. In contrast to the in vitro positive results, in an in vivo micronucleus assay, exposure of rats to the extract did not induce increases in polychromatic erythrocytes in bone marrow.

There is sufficient qualitative and quantitative scientific evidence to determine the safety-in-use, i.e., the acceptable daily intake (ADI) for aqueous pulp extract (HIDROX®). Ordinarily, ADIs are derived by applying an uncertainty factor to the no effect level in an animal study. Based on the NOAEL of 2000 mg/kg/day from a 13-week study in rats and also from a maternal and developmental toxicity study, and applying uncertainty factors of 10 for intraspecies differences and 10 for interspecies differences, an ADI for ingestion of HIDROX® by humans can be determined. The overall uncertainty factor of 100 was judged appropriate, based on the considerations of animal studies and the fact that constituents of the extract are frequently consumed from food. Application of the uncertainty factor of 100 to the NOAEL of 2000 mg/kg/day yields a safe intake for humans of 20 mg of HIDROX®/kg per day or 1200 mg/day (for an adult weighing 60 kg).

In summary, based on a critical evaluation of the available human, animal, analytical, and other scientific studies, and a history of exposure and use of components of HIDROX® through table olives, olive products and olive oil, the consumption of the extract is considered safe at levels up to 1200 mg/day.

Authors' disclosure of potential conflict of interest

The following authors or their immediate family members have indicated a financial interest. Employment: C.M. Bitler and R. Crea. HIDROX® is manufactured and marketed by CreAgri Inc. Preparation of this review article was supported by Burdock Group and CreAgri Inc.

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