Hydroxytyrosol: from laboratory investigations to future clinical trials

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Mediterranean countries have lower rates of mortality from cardiovascular disease and cancer than Northern European or other Western countries. This has been attributed, at least in part, to the so-called Mediterranean diet, which is composed of specific local foods, including olive oil. Traditionally, many beneficial properties associated with this oil have been ascribed to its high oleic acid content. Today, it is clear that many of the beneficial effects of ingesting virgin olive oil are due to its minor compounds. This review summarizes the existing knowledge concerning the chemistry, pharmacokinetics, and toxicology of hydroxytyrosol, a minor compound of virgin olive oil, as well as this compound's importance for health. The main findings in terms of its beneficial effects in cardiovascular disease and cancer, including its properties against inflammation and platelet aggregation, are emphasized. New evidence and strategies regarding the use of hydroxytyrosol as a natural drug for the prevention and treatment of diseases with high incidences in Western countries are also presented.

INTRODUCTION

In comparison with Northern European or other Western countries, Mediterranean countries have lower rates of mortality from cardiovascular disease and cancer, and this is attributed, at least in part, to the so-called Mediterranean diet.[1] According to Mataix and Barbancho,[2] nourishment in the Mediterranean region has followed a stable pattern that has changed in its composition only in the last three or four decades of the 20th century. A typical diet is based on products derived from wheat, olive, and grape, which constitute the Mediterranean triad, i.e., bread, oil, and wine, although, in the words of Professor Mataix, "this triad is perhaps incomplete, as legumes have had a great weight and thus could be called the Mediterranean tetrad." Without a doubt, the most characteristic element of the Mediterranean diet is olive oil, as expressed by the well-known phrase of Georges Duhamel: "where the olive withdraws, the Mediterranean ends."[3]

A tree of the Mediterranean area, the olive tree (*Olea europaea L.*), is native to the dry subtropical climatic zone and is well adapted to extreme environmental conditions, but it requires high-intensity light and aerated soil. The olive tree, a polymorphous, medium-sized tree (maximum 10 m) with a furrowed trunk, has fusiform coriaceous grayish-green leaves (generally about 5–6 cm long and about 1–1.5 cm wide at mid-leaf) with smooth edges and a short peduncle.[4] The olive fruit is a drupe composed of the epicarp or skin, the mesocarp or pulp, and the endocarp or pit (which is a woody shell holding the seed).[5] Although the olive is not morphologically

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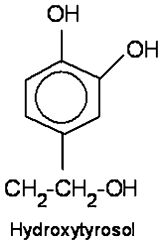

Figure 1 **Chemical structure of hydroxytyrosol.**

unusual, its chemical composition and organoleptic qualities are unique and considered of potential health benefit by cardiologists and nutritionists.

Oil from the olive is known to have been used for food since prehistory. A transparent, yellowish, and aromatic liquid is extracted from the olive by simple pressure; it is the only vegetal fat that is obtained from the fruit, whereas the rest are extracted from seeds.[6] Different processing methods produce virgin, ordinary, or pomace olive oil.[7] Virgin olive oil is produced by direct pressing or centrifugation of the olives. When the acidity of virgin olive oil exceeds 3.0 degrees, this oil is submitted to a refining process in which some components, mainly phenolic compounds and to a lesser degree squalene, are lost.[8] By mixing virgin and refined olive oil, an ordinary olive oil is produced and marketed.[9] After virgin-olive-oil production, the rest of the olive drupe and seed is processed and submitted to a refining process, resulting in pomace olive oil, to which a certain quantity of virgin olive oil is added before marketing.[10]

Much has been written about the health benefits of olive oil.[1] However, traditionally, many of the beneficial properties associated with this oil have been ascribed to its high oleic acid content. Other diets are high in oleic acid, for example, oleic acid is present in pork, but these fail to promote the low cardiovascular disease profiles or other beneficial trends associated with the Mediterranean diet. It is becoming increasingly clear that many of the benefits associated with the ingestion of virgin olive oil are due to its minor compounds. This review presents the current knowledge concerning the main biological properties attributed to hydroxytyrosol (HT), one of the minor compounds found in virgin olive oil (Figure 1); although this compound of olive oil has perhaps received the bulk of research attention, this review represents the first summarization of the existing research.

HYDROXYTYROSOL IN THE CHEMICAL COMPOSITION OF OLIVE OIL

From the standpoint of chemical composition, olive oil is divided into major and minor fractions (Figure 2).[11] The major components make up the saponificable fraction, which comprises 98–99% of the total weight of the oil, and is formed mainly by triacylglycerides. Oleic acid (18:1*n*-9) is the main component (68–81.5%) of the saponificable fraction, accounting for a greater portion than the other acids such as palmitic, stearic, linoleic, and α-linolenic.[12] The minor components of virgin olive oil are classified into two types: the unsaponificable fraction, defined as the fraction extracted with solvents after saponification of the oil, and the soluble fraction, which includes the phenolic compounds.[10] These minor compounds represent around 2% of the total weight of the oil, although more than 230 chemical compounds are included. The minor unsaponificable compounds include the non-glyceride esters, and many fatty acids are esterified with glycerol. Nevertheless, a small portion form esters with alcohols and sterols, such as β-sitosterol, campesterol, or stigmasterol, and with triterpene alcohols, such as trycycloartenol and 24-methylcycloartenol.[13] The total amount of non-glyceride esters ranges from 100 to 250 mg/kg. Waxes are esters of long-chain aliphatic alcohols (C_{27}-C_{32}) and contain up to 58 carbon atoms that affect their physical properties, such as high molecular weights and melting points higher than 70°C. Present in the skin of the olive to avoid water loss, waxes are abundant in pomace oils and lampante oils and are quickly increased in acidic oils by the esterification of aliphatic alcohols with free fatty acids.[14] Also present as minor components in olive oil are aliphatic compounds, which are fundamentally long-chain saturated alcohols, at amounts varying between 60 and 200 mg/kg.[15] Notable among triterpene alcohols, at levels of 500–3,000 mg/kg, are erythrodiol and uvaol.[13] In addition, there are sterols, tetracyclic compounds biosynthesized from squalene. These include β-sitosterol, campesterol, stigmasterol, and others, which are present at levels ranging from 1,800 to 4,939 mg/kg.[16] Among existing hydrocarbons, the main one in olive oil is squalene, which is found in a range of 1250–7500 mg/kg. Other volatile hydrocarbons present in olive oil include phenanthrene, pyrene, fluoranthrene, 1,2-benzanthracene, chrysene, and perilene.[17] Carotenoids are also hydrocarbons present as a minor fraction in olive oil. The predominant carotenoids in olive oil are β-carotene and lycopene, which are responsible for olive oil's color. In virgin olive oils produced from mature olives, the concentrations of β-carotene have been reported to vary from 0.33 to 3.69 mg/kg,[18] but can reach 10 mg/kg, depending on certain factors.[15]

The color of olive oil is due to several pigments, primarily chlorophylls. Chlorophylls a and b and their oxidation products, pheophytins a and b, are naturally present in olive oil and are responsible for the greenish color of the oils. In virgin olive oil from mature olives, the chlorophyll levels vary from about 1 to 10 mg/kg, while

MAJOR COMPONENTS (Saponificable fraction):

- Oleic acid (18:1*n*-9)
- Palmitic acid (16:0)
- Linoleic acid (18:2*n*-6)
- Stearic acid (18:0)
- Palmitoleic acid (16:1*n*-9)
- Linolenic acid (18:3*n*-3)
- Miristic acid (14:0)

MINOR COMPONENTS (Unsaponificable fraction):

1) Non-glyceride esters and waxes
2) Aliphatic alcohols
3) Triterpene alcohols: erythrodiol and uvaol
4) Sterols: β-sitosterol, campesterol, stigmasterol, etc.
5) Hydrocarbons: squalene, volatile hydrocarbons (phenanthrene, pyrene, fluoranthrene, etc.), carotenoids (β-carotene and lycopene)
6) Pigments: chlorophylls and pheophytins (a and b)
7) Volatile compounds
8) Phenolic compounds:
   a) Lipophilic: tocopherols and tocotrienols (α, β, γ, δ)
   b) Hydrophilic:
      Phenolic acids:
         - Benzoic series: gallic, vanillic, benzoic acids, etc.
         - Cinnamic series: cinnamic, caffeic, coumaric acids, etc.
      Phenolic alcohols: hydroxytyrosol, tyrosol and their glucosides
      Secoiridoids: oleuropein and its aglycon, ligstroside aglycon, dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol and tyrosol
      Lignans: (+)-1-pinoresinol and (+)-1-acetoxypinoresinol
      Flavonoids: apigenin, luteolin

Figure 2 **Fractionation of the major and minor components in olive oil and their subconstituents.**

those of pheophytins are in the range of 0.2–24 mg/kg.[18] Volatile compounds are retained by virgin olive oils during the milling process. Volatile compounds, stimulating the olfactory receptors, are responsible for the aroma of virgin olive oil.[19] Many compounds, mainly carbonyl compounds, alcohols, esters, and hydrocarbons, have been identified in the volatile fraction of virgin olive oil.[13,17]

A group of compounds present in the minor fraction that deserves special mention is the phenolic compounds. Environmental stressors, such as ultraviolet radiation and relatively high temperatures (common in the Mediterranean basin), stimulate the secondary metabolism in fruits and vegetables, including olives and grapes. This leads to enhanced production of phenolic compounds with protective (antioxidant) properties.[20] The group of phenolic compounds includes lipophilic phenols, such as topherols, tocorienols, of which α-tocopherol is the most important, ranging from 12 to 400 mg/kg.[11] Furthermore, there are hydrophilic phenolic compounds (40–1,000 mg/kg), which can be divided into various classes. 1) Phenolic acids: these are acids of the benzoic series (gallic, vanillic, benzoic acids, etc.) and those of the cinnamic series (cinnamic, p- and o- coumaric, caffeic acids, etc.).[21] 2) Phenolic alcohols: this group includes HT ((3,4-dihydroxyphenyl)ethanol), tyrosol (*p*-hydroxyphenyl)ethanol and HT glucoside.[11] According to Owen et al.[22] the concentrations of HT and tyrosol in virgin olive oil are 14.4 and 27.5 mg/kg, respectively, values differing from those estimated for Spanish olive oils, which have a HT content of 113.7–381.2 mg/kg, the range being due to factors such as the origin of the olive, degree of maturity, milling process, etc.[23,24] Servili et al.[21] studied 210 virgin-olive oil samples, extracted by industrial mills from different areas of Mediterranean countries and reported median values of 1.8 (lower quintile, 1; upper quintile 3.6) and 1.9 (lower quintile 0.6; upper quintile 5.0) mg/kg of HT and tyrosol, respectively. 3) Secoiridoids: These contain either elenolic acid or elenolic acid derivatives in their molecular structure. The most important secoiridoids present in virgin olive oil are the dialdehydic form of decarboxymethyl elenolic acid linked to HT, the dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol, oleuropein aglycon, ligstroside aglycon, oleuropein, the dialdehydic form of oleuropein aglycon, and the dialdehydic form of ligstroside aglycon. Together with lignans, secoiridoids are the most abundant phenolics present in virgin olive oil. Owen et al.[25] reported 27.72 mg/kg of total secoiridoids in virgin olive oil. 4) Lignans: These compounds were first detected in virgin olive oil by Owen et al.[22] who found a mean of 41.53 mg/kg of total lignans in virgin olive oil, with some samples reaching 100 mg/kg. Two compounds from the lignan family, termed (+)-1-pinoresinol and (+)-1-acetoxypinoresinol, were specifically identified. Brenes et al.[26] reported that Spanish olive oils contain (+)-1-pinoresinol in a range of 20–45 mg/kg, and (+)-1-acetoxypinoresinol was found in a range of 2–95 mg/kg. 5) Flavonoids: These include apigenin or luteolin.[21]

HYDROXYTYROSOL

Hydroxytyrosol comes from the hydrolysis of oleuropein, which originates during the maturation of the olives, storage of the oil, and preparation of table olives. These processes give rise to oleuropein aglycone, HT, and elenolic acid, these being responsible, in part, for the complex and varied flavor of the oil and olive[20] (Figure 3).

As a consequence of their polar character, which includes HT, phenolic compounds are found in great quantities in the remains from oil processing, such as pomace olive oil, olive-mill waste water, or the rinse waters. For this reason, byproducts from olive oil production constitute a major source of HT. Nevertheless, HT also has amphipathic behavior, and the molecule is therefore found in olive oil, where its amount ranges between 113.7 and 381.2 mg/kg.[23,27] In addition to being found in the olive, HT is also located in the olive leaf, although it is accompanied by other phenolic compounds such as oleuropein.[28]

Spain, together with other Mediterranean countries such as Italy and Greece, has a long tradition of olive cultivation and olive oil production, with both the fruit and the oil being habitual components of the Spanish diet. Dietary intake of olive oil polyphenols in these countries has been estimated to be around 9 mg per day with a daily intake of 25–50 mL of olive oil; at least 1 mg is derived from free HT and tyrosol, while 8 mg are related to their elenolic esters and to oleuropein- and ligstroside-aglycons.[29]

Pharmacokinetics

In general, hydrophilic phenolic compounds of olive oil are absorbed in a dose-dependent manner in animals and humans, and are excreted in the urine mainly as glucuronide conjugates.[20] The absorption of HT takes place in the small intestine and colon[30]: It has been suggested that transport through the intestinal epithelium can occur by passive bidirectional diffusion.[31] The absorption of this compound is rapid, with maximum plasma concentration being reached 5–10 min after ingestion, followed by a rapid decline.[32] The absorption of HT differs according to the vehicle in which it is carried. For example, Tuck et al.[33] demonstrated that rats absorbed 75% of the HT when it was administered in an aqueous solution and 90% when it was administered in an oily vehicle. The absorption also

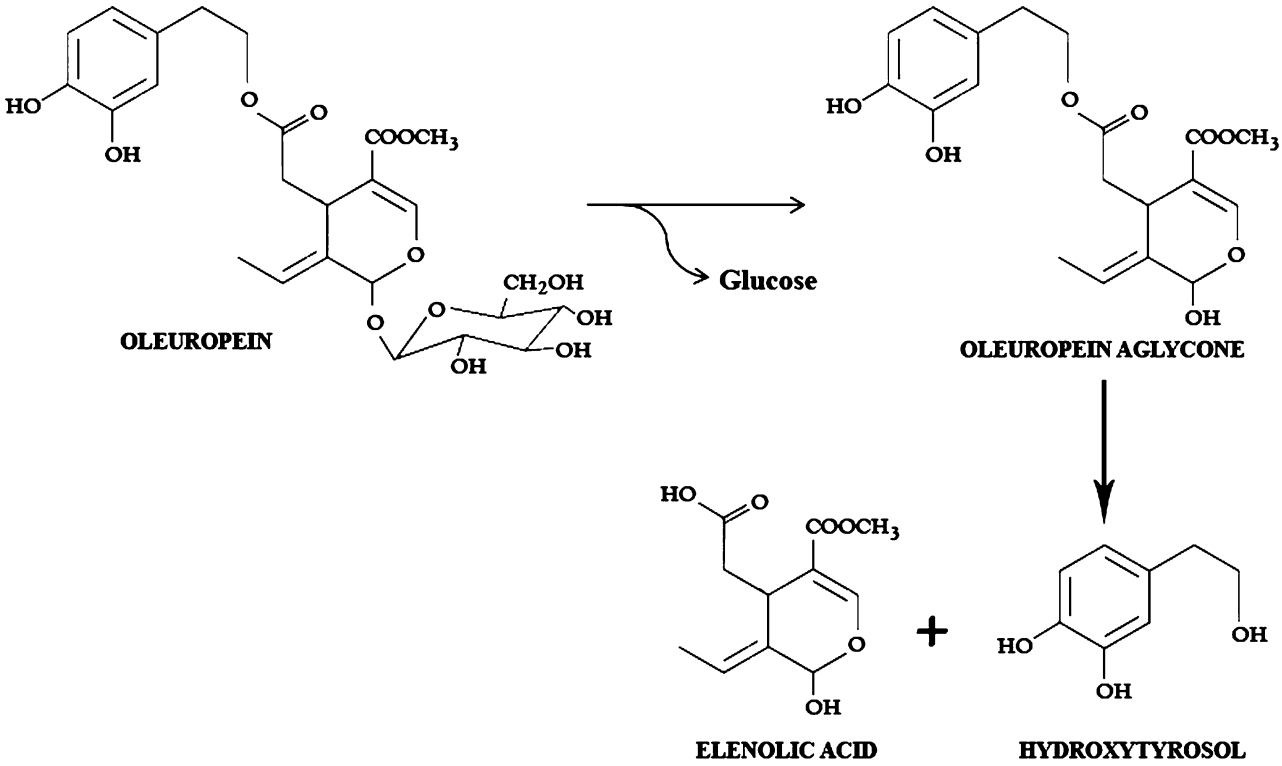

Figure 3 **Origin of hydroxytyrosol from oleuropein.** After the hydrolysis of oleuropein (resulting in oleuropein aglycone), which originates during the maturation of the olives, storage of the oil, and preparation of table olives, hydroxytyrosol is given rise together with elenolic acid. These are responsible, in part, for the complex and varied flavor of the oil and the olive.

varies according to the animal species; for instance, different rates are found in rats compared with humans because of the absence of a gallbladder in these rodents.[34]

Finally, after postprandial absorption, HT bonds to circulating human lipoproteins (as do other phenols from olive oil).[35] Whether in plasma or when excreted in urine, HT is found alone, as are its *ortho*-methylic derivatives (homovanillic alcohol), glucuronide derivatives,[36] and its glutathionyl conjugates.[37]

A study of the tissue distribution after intravenous administration of radioactive HT in rats has demonstrated that its half-life in blood is 1–2 min and that, at 5 min after injection, most of the marked HT is found in the kidney, where tenfold more radioactive HT accumulates than in other organs, such as skeletal muscle, liver, lungs, or heart, which all present very similar levels.[38] It has been shown that HT can cross the blood-brain barrier to appear in the brain, although it must be taken into account that HT may be generated endogenously from dihydroxyphenylacetic acid through dihydroxyphenylacetic reductase of the brain.[39] It can also be generated from dopamine.[40] De la Torre[29] has suggested that our understanding of the function of HT is hampered by the fact that HT is a metabolite of dopamine and that the HT concentration in body fluids is a combination of the exogenous and endogenous sources. This is the main reason it is not possible to minimize its concentration in biological fluids, hindering the evaluation of its availability in the organism, whether after a period without food intake or after strict dietary control.[10] In addition, although HT is well absorbed in the gastrointestinal tract, its bioavailability is poor both in the gut and the liver, leading to the formation of sulfate and glucuronide conjugates, to the extent that concentrations of its free form in body fluids are almost undetectable.[29]

At 5 h after injection, only 0.1% of the radioactive HT remains in animal organs. In fact, HT is metabolized rapidly, first in the enterocytes and afterwards in the liver, with metabolites being found in blood 5 min after intravenous injection.[38] As indicated above, in plasma, HT alone, *ortho*-methyl derivatives (homovanillic alcohol), glucuronide derivatives, and their glutathionyl conjugates appear, indicating that perhaps the derivatives of glucuronic acid and glutathionyl conjugates occur in the enterocytes and liver, while homovanillic alcohol appears after HT metabolism in the liver.[37] D'Angelo et al.[38] propose three metabolic pathways for HT: 1) oxidation: through the enzymes alcohol dehydrogenase and aldehyde dehydrogenase, giving rise to dihydroxyphenylacetic acid; 2) methylation: by the enzyme catechol-O-methyltransferase, rendering homovanillic alcohol; 3) methylation-oxidation: a reaction that occurs to form homovanillic acid.

Also, from HT and from the metabolites mentioned above, a high number of conjugates with glucuronide and sulfate are generated, which are detectable both in the plasma and in the urine.[41] The time required for HT and its metabolites to be eliminated from the urine is roughly 5 h in rats[38] and 4 h in humans.[42] The endogenous production of HT, related to dopaminergic metabolism, could be the reason it tends to accumulate in the urine, independent of the dosage administered, as occurs with tyrosol.[10,36,43] As with absorption, the subsequent excretion of HT and its metabolites differ according to the vehicle used in the administration of the compound. Thus, Visioli et al.[34] observed that in humans HT elimination through the urine is greater when administered in the form of olive oil than when provided in low-fat yogurt or when this oil is enriched in phenolic compounds. Again, as occurred with absorption, the excretion of HT and its metabolites in urine differed between rats and humans. This implies that absorption and elimination is lower in rats than in humans, and this should be taken into account when extrapolating the results of absorption/excretion from experiments in rats.[20,34]

Toxicological studies

Toxicity studies conducted with HT are scarce. To study acute toxicity, however, D'Angelo et al.[38] administered a single dose of 2 g/kg of body weight in rats and found an absence of toxic effects or macroscopic alterations in organs; only the appearance of piloerection was indicated at 2 h after administration, and this disappeared in less than 48 h. In addition to this phenomenon, diverse toxicity studies using aqueous olive-pulp extracts have been performed in which the HT content ranged from 50 to 70% of the total quantity of phenols.[44] In another study, oral administration to Sprague-Dawley rats of a single gavage dose of solid olive-pulp extract at levels of 0, 1,000, 1,500, or 2,000 mg/kg caused no adverse effects, except for soft or liquid feces.[45] At a dosage of 2,000 mg/kg/day of this extract, an absence of acute toxicity was found, without either teratogenic or mutagenic effects, suggesting that the LD50 of the extract is greater than 2,000 mg/kg, which would be similar to 1,000–1,400 mg of HT. As part of a micronucleus assay, Sprague-Dawley rats (five/sex) were administered a single gavage dose of 5,000 mg olive-pulp extract/kg and observed for 6 days, after which the 5,000 mg/kg dose was given for 29 consecutive days. No mortality or clinical signs of toxicity were noted. For the authors, this study demonstrated that the LD50 of the solid olive-pulp extract was greater than 5 g/kg (2.5–3.5 g/kg of HT), suggesting that the extract is practically non-toxic.[45]

With respect to studies on subchronic toxicity, only relative evidence is available on the above-mentioned extract. The results indicate that no adverse effect was observed for the same compound at 2,000 mg/kg/day, which was the maximum dosage administered[44,45]

BRIEF HISTORY OF THE RESEARCH ON OLIVE OIL AND HEALTH: EVIDENCE OF HYDROXYTYROSOL'S IMPORTANCE FOR HEALTH

For years, olive oil has been considered a product with many healthy properties. Early studies showed its bactericidal capacity[46] and effectiveness against rickets,[47] as well as its possible vitamin-E content.[48] Research then began to focus on the alterations of the lipid profile that olive oil prompted in experimental animals.[49] Beneficial effects began to be described for this oil and its components such as oleic acid and squalene in the gastric secretion and mobilization of cholesterol.[50,51]

In 1962, Gounelle et al.[52] began to look for the compound in olive oil that was responsible for lowering cholesterol, since this effect could not be due only to the lipids ingested through this oil. It was found that non-Mediterranean countries did not consume equal or larger quantities of certain fatty acids found in the Mediterranean diet. From this time on, oleic acid began to be studied for its possible beneficial effects on such processes as atherosclerosis[53] and thrombosis.[54]

For decades, research on the health-promoting effects of olive oil has been primarily focused on cardiovascular disease. Thus, the seven-country study concluded that the consumption of monounsaturated fatty acids (MUFAs) and olive oil is a key factor in the cardioprotection found in Mediterranean countries.[55–57] In general, though, epidemiological studies have not been consistent. In combination with case-control studies, as well as metabolic and biochemical studies, the emphasis has been on the health-promoting effects of olive oil consumption, mainly in association with the Mediterranean diet.[58] This special capacity of olive oil has been related to its MUFA content with a sufficient degree of certainty that the American Health Association states that the consumption of a diet moderately rich in MUFA (15% of the calories ingested) and relatively low in polyunsaturated fatty acids (PUFAs) (8% of caloric ingestion) benefits cardiovascular health.[59]

Today, there is no doubt concerning the important effects of the different types of dietary fat ingested, particularly the benefits of olive oil, and the significant influence these fats have on diverse aspects of health.[1,60] For example, it is known that saturated fatty acids raise the plasma levels of low-density lipoproteins (LDL), whereas oleic acid elevates the plasma levels of high-density lipoproteins while slightly lowering those of LDL.[61,62] Changes in biochemical parameters also occur at the level of biological membranes, such as in the mitochondria[63,64]

or erythrocytes.[65] In this sense, it is known that the dietary-lipid profile is capable of altering the fatty-acid composition of biological membranes, as well as different structural and functional aspects of the mitochondrial electron-transport system and susceptibility of the mitochondrial membrane to oxidation.[66,67] Olive oil, in particular, has been demonstrated to generate membranes that are more resistant to lipid peroxidation and more functional in comparison with those generated by polyunsaturated fat sources such as sunflower oil.[68]

Studies concerning the antioxidant capacity of olive oil extend to several fields. For example, an increase in plasma antioxidants from volunteers fed with olive oil has been described.[69] In reference to the oxidation of LDL, it was observed that omega-3 PUFAs increased the susceptibility of LDL to oxidation in comparison to omega-6 PUFAs and MUFAs.[70] However, the oxidative alterations of LDL caused by the high percentage of omega-3 PUFAs can be reduced with the simultaneous administration of olive oil.[71] These effects of MUFAs on circulating lipids and lipoproteins still generate controversy, despite the fact that consumption of oleic acid has been related to healthy effects.[20]

Olive oil also reduces hypertension,[69] improves thrombogenic conditions,[72,73] prevents or improves diabetes,[74] reduces inflammatory processes,[75,76] and alleviates intestinal inflammatory illness,[77] in addition to promoting effects related to the MUFA (oleic acid) content. Moreover, diets with olive oil have been shown to lower the exocrine pancreatic secretion in dogs, while enhancing utilization of certain nutrients, such as proteins.[78,79] This effect is related to the action of oleic acid with respect to the stimulation-inhibition balance of pancreatic secretion,[80] and to other benefits at the gastrointestinal level.[81]

The health-promoting effects of olive oil consumption have been related traditionally to its high oleic-acid content. However, it should be noted that other foods such as pork and chicken are also rich in this fatty acid. Since the ingestion of these foods is somewhat higher in the United States than in Mediterranean countries, for example, the oleic-acid levels are quite similar in these countries. Furthermore, it has been noted that the female population in Malmö, Sweden, presents higher levels of oleic acid than women in Granada, Spain. Despite these similar levels, and even higher ones, the Mediterranean population presents fewer cases of cardiovascular disease. This argument suggests that oleic acid is not the only component of olive oil that is responsible for cardiovascular protection.[10,20,82]

Given that some component of olive oil other than oleic acid could be related to its healthy properties, many studies conducted on this subject in recent years can be looked at for clues regarding its identity. For example, Ochoa et al.[83] described the relative importance of the saponificable and unsaponificable fractions of virgin olive oil for lipid peroxidation in mitochondria of rabbit heart. In the same year, Ramirez-Tortosa et al.[84] found that oleic acid was not the only compound responsible for the antioxidant effect of olive oil in relation to the formation of atheroma plaques. Other studies have emphasized the positive influence of the minor components in virgin olive oil. Thus, it has been reported that refined olive oil (free of phenols) lacks the antioxidant effects present in virgin olive oil.[67,85] These authors later noted that the phenolic fraction of olive oil considerably improves the lipid peroxidation of LDL lipoproteins, which is related to an increase in antioxidant capacity.[66,86]

At present, it is well established that the health benefits of olive oil are not concentrated solely in their fatty-acid content. Other sources are currently thought to be the minor bioactive compounds, primarily phenols with high antioxidant capacity, such as HT.[87,88]

HYDROXYTYROSOL IN THE CONTEXT OF CARDIOVASCULAR DISEASE RESEARCH

Antiatherogenic capacity and cardioprotective effects

Hydroxytyrosol has been demonstrated in numerous studies to have antiatherogenic properties with strong antioxidant power. It acts as a powerful scavenger of free radicals with the superoxide anion, hydrogen peroxide, hypochlorous acid, etc. In addition, it has a strong chelating effect on metals such as iron, and thus diminishes the appearance of reactive oxygen species derived from reactions associated with this metal.[20,89] This characteristic is the main factor responsible for the effect in atherosclerosis, characterized by the entrance of LDL particles oxidized in the interior of the arterial intima. Oxidation is probably due to the action of macrophages as well as endothelial and smooth-muscle cells. Hydroxytyrosol is capable of preventing oxidation of these lipoproteins by macrophages, since it increases the antioxidant capacity of these cells related to lowered glutathione levels.[90]

The antioxidant capacity of HT on LDL has been suspected for decades. In fact, in 1995 Salami et al.[91] reported lower levels of isoprostanes and thiobarbituric acid-reactive substances, and therefore lipid peroxidation, in LDL due to HT. Subsequent studies confirmed the protective effect of this antioxidant on LDL lipoproteins, suggesting a modulation of atherosclerosis, working with in vitro models as well as with animals.[20,90,92–94] It has also been confirmed that the incubation of plasma with phenolic compounds of olive oil (among them HT) reduces and prevents LDL oxidation.[95] More recently, it has been demonstrated that olive phenols and their metabolites are much more efficient inhibitors of lipid and protein oxidations compared to vitamins C and E.[96]

Postprandial lipemia is a risk factor for atherosclerosis[10] and, in this sense, it has been reported that HT promotes hypocholesterolemia, lowering the plasma levels of LDL and total cholesterol in rats fed on lipid-rich diets. Furthermore, it enables a lowering of triglycerides while raising high-density lipoprotein levels and the antioxidant capacity, thereby reducing LDL oxidation.[97,98]

The antiatherogenic effect of HT appears to not only be related to reducing the oxidation of LDL particles, it has also been reported to effect a decrease in the expression of the adhesion molecules vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in in vitro endothelial cells; this has been related to the inactivation of NF-kβ (nuclear factor-kappa beta), activator protein-1, GATA (a transcription factor that regulates T lymphocyte differentiation and maturation), and nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase.[87,99–101] Furthermore, this antioxidant improves oxidative stress, which depresses the levels of nitric oxide, thereby promoting aortic relaxation and enhancing protection of the vascular endothelium.[102] However, HT has not been found to increase the production of nitric oxide in endothelial cells; thus, it may not exert its action directly on endothelial nitric oxide synthase.[103] This finding contrasts with the observations of González-Correa et al.[104] in rats, which indicated that HT raised the plasma and aortic levels of nitric oxide.

Despite all these studies suggesting that HT is related to declines in atheroma plaque, some studies have reported that HT administration enhances atherosclerotic lesion development in apo E-deficient mice.[105]

The cardioprotective effects of HT have been supported in a study conducted with cardiomyocytes extracted from Sprague-Dawley rats treated with HT (2.5 mg/kg), tyrosol (2.5 mg/kg), resveratrol (2.5 mg/kg), white wine, and red wine. The results show that HT reduces the expression of proteins related to ageing in cardiac cells (sirtuins, Forkhead box subfamily O [FoxO] proteins, and pre-B cell colony-enhancing factor) to a greater degree than red wine and to a lesser degree than white wine, resveratrol, and tyrosol. However, the cardioprotection exerted by HT in terms of the size of the infarcted region and apoptosis of the cardiomyocytes was exceeded only by resveratrol. These results highlight that the ability to induce signals of survival and/or longevity does not depend on the number of hydroxyl groups of a molecule.[106]

Anti-inflammatory and antiplatelet aggregation effects

One of the factors involved in thrombotic processes is platelet aggregation. Hydroxytyrosol can be considered antithrombotic, since it significantly reduces platelet aggregation.[107] Petroni et al.[108] reported that HT diminished the synthesis of thromboxane B_2 in an in vitro model of platelet-rich plasma, probably as a result of reduced production of eicosanoids derived from arachidonic acid (such as 12-hydroxyeicosatetranoic acid). Meanwhile, González-Correa et al.[104] found a reduction in the synthesis of thromboxane A_2 measured by the reduction of its metabolite, thromboxane B_2. This decline was due mainly to the inhibition of activity of the enzyme cyclooxygenase. For these authors, these antithrombotic effects would be helped by the decline in the production of vascular prostacyclin, effects similar to those presented by acetyl salicylic acid.

Other authors have noted that HT reduced the production of leukotriene B_4 of leukocytes.[109,110] Later, Dell'Agli et al.[111] indicated that the reduction of platelet aggregation was due not only to the above but also to the reduction that HT caused in cAMP and cGMP platelet phosphodiesterase. Meanwhile, other authors have proposed, as a possible mechanism related to the inhibition of platelet aggregation, the selective inhibition of eicosanoid synthesis by enzymes 5- and 12-lipooxygenase in leukocytes.[110,112]

The lower expression of adhesion molecules such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 is also related to the anti-inflammatory and antiaggregate properties of HT.[100,101,113] Moreover, HT impedes the synthesis of prostaglandin E_2 by indirectly blocking the enzymes inducible nitric oxide synthase and cyclooxygenase-2 (COX-2). This effect arises from the prevention of the transcriptional activation of NF-kβ, interferon regulatory factor-1, and transducer and activator of transcription 1α, which prevent the activation of mouse macrophages J774.[114] It is known that HT is capable of bringing about arylating/alquilant adducts in the residues of NF-kβ cysteine. The action of HT on this factor blocks the transcription of the enzymes COX-2 and 5-lipooxygenase, reducing the prostaglandin E_2 synthesis and, thus, the chronic influence associated with diseases such as cancer.[115] Moreover, Zhang et al.[116] concluded that HT likely exerts its anti-inflammatory activity by suppressing COX-2 and inducible nitric oxide synthase expression in human monocytic cells.

The anti-inflammatory capacity of HT has also been noted with rats in a model of post-menopause osteoporosis and senility caused by estrogen deficiency.[117] In this model, a reduction was found in the degree of inflammation associated with these pathologies, and osteopenia was prevented by the augmentation of bone formation. Moreover, HT also encouraged the action of cells of the immune system, with its high antioxidant capacity protecting neutrophils against oxidation mediated by hydrogen peroxide.[118] In addition, HT effectively protects the

DNA of mononuclear blood cells[119] and peripheral monocytes of patients with Alzheimer's disease.[120] In this sense, diverse studies have shown that HT protects against the genotoxic damage caused by free radicals in the DNA of multiple human cell lines[119,121–123] and red blood cells.[124] Finally, HT augments the cytosolic levels of Ca^{2+}, activating T and B lymphocytes.[125,126]

To test the anti-inflammatory effect of erythrodiol, beta-sitosterol, and squalene, as well as of oleuropein, tyrosol, HT, and caffeic acid, De la Puerta et al.[127] studied a mouse model with inflammation induced in the ear by arachidonic acid and/or phorbol esters. These researchers found that the topical application of erythrodiol and beta-sitosterol inhibited the edematous tissue by 61.4 and 82.1%, respectively, while the four phenolics shrank the swelling by 33–45%, with the latter reductions related to the lower infiltration of neutrophils, as measured by the inhibition of myeloperoxidase. More recently, Gong et al.[128] studied Sprague-Dawley rats with acute inflammation induced by intravenous injection of carrageenan at 2% (w/v). The rodents received different dosages (100, 250, and 500 mg/kg of body weight) by gavage of a preparation in which the main ingredients were HT (22%), polyphenol (4%), saccharide (67%), lipid (2%), ignition residue (4%), and moisture (1%). This preparation, called hydroxytyrosol-20 (HT-20), significantly inhibited both the acute inflammation as well as the pain associated with carrageenan administration. The analgesic action of HT-20 was not dose-dependent, and levels of mRNA of the inflammatory cytokines interleukin-1β and tumor necrosis factor alpha (TNF-α) lowered without raising those of the anti-inflammatory cytokine interleukin-10.

ANTIMICROBIAL ACTIVITY

Years ago, olive oil and extracts from olive leaves were identified as antimicrobial agents with activity against *Escherichia coli, Candida albicans, Kluyveromyces marxianus, Clostridium perfringens, Streptococcus mutans, Shigella sonnei, Salmonella enterica*, and others.[129] It appears that the main components of olives and olive leaves responsible for the antimicrobial effect are the dialdehyde and decarboxymethyl forms of elenolic acid together with HT.[130]

In addition, it has been demonstrated in vitro that HT also possesses antimicrobial properties against infectious agents of the respiratory and gastrointestinal tracts such as *Vibrio parahaemolyticus, Vibrio cholerae, Salmonella typhi, Haemophilus influenzae, Staphylococcus aureus*, or *Moraxella catarrhalis*, at low inhibitory concentrations. These concentrations are even lower than those of certain antibiotics, such as ampicillin,[131] and are also effective against mycoplasms such as *Mycoplasma pneumoniae*.[132]

CANCER STUDIES

The antitumor effect of HT has been studied as a result of its capacity to inhibit proliferation and promote apoptosis in several tumor-cell lines by diverse mechanisms, in addition to its ability to be chemopreventive with its high antioxidant activity. Della Ragione et al.[133] demonstrated that HT is capable of stopping the cell cycle, reducing growth and proliferation, and inducing apoptosis in HL60 cells (promyelocytic leukemia) and HT29 (colon adenocarcinoma). This effect depends on the phenotype, since, according to this study, it does not affect the Caco2 colon cells but does affect lymphocytes. However, these studies state that HT does not cause apoptosis in non-carcinogenic lymphocytes.[134] This discrepancy could be due to the different apoptosis assays used by the research groups. Della Ragione et al.[133] indicated that the apoptosis induced by HT depends on the release of cytochrome c, which activates the effector caspase 3; these authors further observed that this excludes the death receptor pathway FAS (TNF receptor superfamily, member 6), since the release of cytochrome c precedes caspase 8 activation for the part of any death receptor, including the FAS receptor. The release of cytochrome c does not completely rule out FAS activation, however, since a bonding nexus has been found between apoptosis by death receptors (FAS/CD95, tumor-necrosis factor [ligand] superfamily, member 10, TNF) and the mitochondrial pathway,, i.e., between the extrinsic and intrinsic pathways of apoptosis.[135]

It was later clarified that one of the possible pathways by which HT triggers apoptosis is the activation of c-jun by c-jun NH2-terminal kinase, which under certain circumstances causes cell death and inactivates the antiapoptotic protein B-cell lymphoma protein 2.[136]

As mentioned above, Fabiani et al.[134] also studied HL60 cells, demonstrating that HT inhibits cell proliferation, blocking the G1 phase of the cycle, with a proportional increase of cells in the G0/G1 phase and a concomitant decline in the S and G2/M phases. The mechanisms they proposed are as follows: 1) a direct blockage of HT on cyclin-dependent kinases; 2) an induction of the cyclin-dependent kinase inhibitors; 3) blockage of messengers involved in cell proliferation, such as reactive oxygen species, causing apoptosis and favoring differentiation of these HL60 cells.[137] Recently, the same authors reported that HT (100 μM) causes increased expression of $p21^{WAF/Cip1}$ (cyclin-dependent kinase inhibitor 1A) and $p27^{Kip1}$ (cyclin-dependent kinase inhibitor 1B) and inhibits cyclin-dependent kinase-6 in the same type of cells,

arresting the cell-cycle phases G2/M and G0/G1; they also reported that HT promotes apoptosis in cells that are in the S phase.[138]

Recently, Han et al.[139] showed in MCF-7 breast-cancer cells that both HT (25 μg/mL) and oleuropein (100 μg/mL) exhibited a G1 to S phase transition blockade, manifested by an increase in the number of cells in the G0/G1 phase. Concomitantly, these substances decreased the number of cells in the G2/M cell-cycle phase.

Guichard et al.[140] indicated that HT in HT29 cells stops the cell cycle at phases S and G2/M, but the concentration studied was far greater (400 μM) than in the previous work (100 μM) and the cells used were different. Furthermore, they demonstrated that HT induced apoptosis-dependent stress in the endoplasmic reticulum. This leads to activation of the proapoptotic pathway endoplasmic reticulum to nucleus signaling 1 (Ire1)/c-jun NH2-terminal kinase/c-jun/activator protein-1/NADPH oxidase 4 (Nox4), a result that agrees at least partly with those of Della Ragione et al.[133] In addition, an inhibition of the pro-survival pathway phosphoinositol 3 kinase/v-akt murine thymoma viral oncogene/PKB resulted (probably by activation of the phosphatase serine/treonine PP2A, which inactivates v-akt murine thymoma viral oncogene). This inhibition prevented the activation of TNF-α-dependent NF-kβ.[140]

In invasive colon adenocarcinoma cells (HT115), HT has not been demonstrated to have any significant reducing action on the invasive capacity of these cells.[141] According to Corona et al.,[142] HT exerts its antiproliferative activity in colon Caco2 cancer cells by inhibiting the activity of p38 and cAMP response element binding protein, leading to a subsequent reduction of COX-2 expression.

Apart from studying the possible mechanisms by which HT exerts its antiproliferative characteristics, D'Angelo et al.[143] have shown an inhibitory action of HT on the damage that ultraviolet radiation inflicts on melanoma cells as well as in hepatocarcinoma cells (HepG2) subjected to tert-butylhydroperoxide.[144] Hydroxytyrosol can inhibit HER2 (neuro/glioblastoma-derived oncogene homolog) expression in breast-cancer cells resistant to trastuzumab (Herceptin®, Roche), which overexpress this oncogene (SKBR3); in addition, it can cause cytotoxic activity on these SKBR3, MCF7 cells (breast-cancer cells with natural HER2 expression) and MCF7/HER2 (HER2-induced breast-cancer cells). However, in these studies the best results have been found for oleuropein aglycone, which, in addition to amplifying these effects, also triggers apoptosis in cancer cells.[145] Together with these results, a subsequent study performed with the same types of cells reflects that, in general, simple phenols (e.g., HT, tyrosol) have lower antiproliferative and proapoptotic effects than do compounds such as lignan (+)-1-acetoxypinoresinol or the secoiridoid lingstroside aglycone.[146] In addition, both HT and oleuropein have proven to be powerful inhibitors of the fatty-acid enzyme synthase, a key enzyme in converting carbohydrates into fats and one that is deeply involved in carcinogenesis in SKBR3 and MCF7 cells, which overexpress the receptor HER2 and thereby confer strong chemopreventive power.[147] A recent study by Menendez et al.[148] suggested that simple phenols of extra-virgin olive oil, such as HT and tyrosol, do not inhibit the HER2 tyrosine kinase domain. In this sense, it is probable that a more complex structure is required (e.g., two or more phenolic rings) to effectively block HER2 tyrosine kinase activity.

One mechanism described recently involves catechol quinones from HT metabolism and from reaction with hydrogen peroxide. These are reactive arylating electrophilic species that produce Michael adducts with cellular thiol nucleophiles in glutathione and proteins, particularly on cysteinyl proteins. In this way, they exert their cytotoxic, anti-inflammatory, and anticarcinogenic properties, very probably due to the inhibition of NF-kβ by these quinones. These bonds to cysteine residues, forming arylating/alquilant adducts, hamper the bonding of this transcription factor with DNA; this impedes the start of transcription of COX-2 and 5-lipooxygenase, reducing the synthesis of prostaglandin E_2 and therefore chronic inflammation. In addition, antitumor activity is expressed.[115] This suppressive capacity over NF-kβ activation has been shown more recently in THP-1 cells and, moreover, the treatment with HT significantly suppressed the specific DNA-binding activities of NF-kβ.[149]

In addition to all these studies related to the anticancer capacity of HT in tumor cell lines, a new area of research has arisen in relation to the potential of HT to assist in the diagnosis of pheochromocytomas of sporadic and familial origin, neuroblastomas, and carcinoids, among others. This new research makes use of the strong antioxidant capacity of HT (at a 5 μM dosage) in order to augment the transporter activity of norepinephrine in pheochromocytoma P12 cells.[131] The increased activity of this transporter suggests that HT therapy in combination with I-iodometaiodobenzylguanidine could improve the effectiveness of the latter in the diagnosis and treatment of pheochromocytomas, neuroblastomas, and carcinoids.[150]

Table 1 summarizes the most important mechanisms of HT described for cancer, microbial, and cardiovascular diseases.[101,103,114,116,118–120,134,137]

From the studies reviewed, it can be concluded that all that is currently known comes from in vitro studies, as no in vivo study has demonstrated that HT is capable of blocking or diminishing tumor proliferation or growth.

Table 1 **Molecular mechanisms of hydroxytyrosol determined in cell culture studies.**

Capacity of hydroxytyrosol	Biochemical effects	Cell-signaling effects
In cardiovascular diseases		
Antiatherogenic effect	Reduction of LDL oxidation (high antioxidant capacity and lipid peroxidation decrease)	Adhesion molecules (VCAM-1 and ICAM-1) reduced expression
	Hypocholesterolemic effect	NF-kβ, AP-1, GATA, and NAD(P)H oxidase inactivation
	Increase in HDL action	
Antithrombotic and anti-inflammatory	Diminishes the synthesis of thromboxane A_2 and B_2, leukotriene B_4, and vascular prostacyclin	VCAM-1 and ICAM-1 reduced expression
	Reduces cAMP and cGMP platelet phosphodiesterase	NF-kβ, IRF-1, and STAT-1α transcriptional activation prevention
	Inhibits 5- and 12-LOX	Inflammatory cytokines (IL-1β and TNF-α) reduced expression
	Lowers synthesis of PGE_2	
	Blocks COX-2 and iNOS	
Vascular endothelium protection	Nitric oxide increase	
Cardioprotective effect	Lowers size of infarcted region and apoptosis of cardiomyocytes	Reduced expression of ageing-related proteins (Sirts, FoxO, and PBEF)
In cancer		
Antiproliferative	Not found	Cell cycle arrest (G0/G1, S, G2/M)
		Increased expression of $p21^{WAF/Cip1}$ and $p27^{Kip1}$
		CDK6 inhibition
		HER2 oncogene inhibition
		Fatty acid synthase inhibition
Proapoptotic	Not found	Cytochrome c release
		Caspase 3 and c-jun activation
		Bcl-2 activation
		Ire1/JNK/c-jun/AP-1/Nox-4 pathway activation
		PI3K/Akt/NF-kβ pathway inhibition
		NF-kβ inhibiton

Abbreviations: Akt, v-akt murine thymoma viral oncogene; AP-1, activator protein-1; Bcl-2, B-cell lymphoma protein 2; CDK, cyclin-dependent kinase; COX-2, cyclooxygenase-2; GATA, a transcription factor that regulates T lymphocyte differentiation and maturation; HDL, high-density lipoproteins; ICAM-1, intercellular adhesion molecule-1; IL-1β, interleukin-1beta; iNOS, inducible nitric oxide synthase; IRF-1, interferon regulatory factor-1; JNK, c-jun NH2-terminal kinase; LDL, low-density lipoproteins; LOX, lipooxygenase; NAD(P)H, nicotinamide adenine dinucleotide phosphate; NF-kβ, nuclear factor-kappa beta; PBEF, pre-B cell colony-enhancing factor; PGE_2, prostaglandin E_2; PI3K, phosphoinositol 3 kinase; Sirts, sirtuins; STAT-1α, transducer and activator of transcription-1alpha; TNF-α, tumor necrosis factor-alpha; VCAM-1, vascular cell adhesion molecule-1.

CONCLUSION

Mediterranean countries have a lower rate of mortality from chronic disorders such as cardiovascular disease and cancer, and this is attributed, at least in part, to the so-called Mediterranean diet. Olive oil is a common component of this diet, exerting different kinds of beneficial effects as discussed above. Such health-promoting properties of olive oil have traditionally been attributed to oleic acid. However, current knowledge indicates components of its minor fraction can fortify health and one of these is the single phenol, HT. Many studies of this compound have demonstrated its powerful antioxidant capacity, as well as its anti-inflammatory and antiplatelet aggregation action, which may help counteract the development of chronic diseases such as cardiovascular disease and cancer.

To learn more about the possible role of HT in the prevention of certain chronic diseases, more extensive studies on the effects of HT are needed. Similar to HT, many phytochemicals such as curcumin, resveratrol, epigallocatechin gallate, caffeic acid, capsaicin, compounds from cruciferous and allium vegetable families, quercetin, ginestein, etc., also present promising chemopreventive and chemotherapeutic properties. As discussed, all of the studies performed to date concerning the anticancer effects of HT have focused on human cancer-cell lines. Thus, more animal studies are needed for better

insight into the antitumor mechanisms of HT. Given its dose-dependent absorption in animals and humans, good bioavailability after oral administration, and apparent low acute and chronic toxicity, HT appears to be a very good candidate for preclinical cancer research in animal models and, perhaps, future clinical trials in humans.

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