# *Antioxidant and Other Biological Activities of Phenols from Olives and Olive Oil*

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***Abstract:*** Olive oil is the principal source of fat in the Mediterranean diet, which has been associated with a lower incidence of coronary heart disease and certain cancers. Phenolic compounds, e.g., hydroxytyrosol and oleuropein, in extra-virgin olive oil are responsible for its peculiar pungent taste and for its high stability. Recent findings demonstrate that olive oil phenolics are powerful antioxidants, both *in vitro* and *in vivo*, and possess other potent biological activities that could partially account for the observed healthful effects of the Mediterranean diet.
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**Key words:** olive oil; antioxidants; atherosclerosis; Mediterranean diet; phenolics

## *1. INTRODUCTION*

The growing popularity of the Mediterranean diet is due to the large body of epidemiological studies that show how the incidence of coronary heart disease (CHD) and certain cancers, e.g., breast and colon cancers, is lowest in the Mediterranean basin.[1] It has been suggested that this is largely due to the relatively safe and even protective dietary habits of this area.[1,2] In the past, in addition to the low consumption of meat, major emphasis was put on the low saturated fat content—and the concomitant high proportion of monounsaturated fat—of the Mediterranean diet but, more recently, evidence has underlined the importance of plant foods (including carbohydrate and non-digestible fiber) and of a regular use of olive oil. Growing evidence indicates that classic risk factors for CHD such as serum cholesterol and blood pressure are not much different in the populations of the Mediterranean basin when compared to other North-European and Western countries, suggesting that other unexplored risk factors, in addition to the classical ones, may be favorably affected by this diet.[3] Indeed, there are several observations that do not completely link CHD incidence and fat intake and absorption.[3] This evidence has led to the formulation of an antioxidant/atherosclerosis

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hypothesis, which has stimulated experimental and epidemiological studies on the possible role of antioxidants including, but not limited to, vitamins in the relative protection from CHD observed in the Mediterranean area.

## 2. OLIVE OIL

Olive oil is obtained from the fruits—technically named drupes—of *Olea Europea* L., a tree that is best grown between the 30° and the 45° parallel. Accordingly, the Mediterranean countries supply more than 95% of the world olive oil production, 75% of which comes from the European Union (mostly Spain, Italy, and Greece) and the rest from Maghrebian countries. Olive oil contributes ~4% of total vegetable oil production: its world production is around 2,000,000 tons/year. Due to the increasing popularity of the Mediterranean diet, in which olive oil is the major fat component, its production is now expanding to non-traditional producers such as The United States, Canada, Australia, South America, and Japan.

Depending on its chemical properties and its degree of acidity, olive oil is classified into different grades[4] that also serve as guidelines for the consumer in the choice of the preferred kind of oil. From this classification, it can be concluded that the most valuable kind of olive oil is the extra-virgin one, obtained from intact olives that are quickly processed and cold-pressed. In this way, activation of cellular lipases and degradation of the triglycerides is minimized (see below). One of the purposes of this article is to propose the phenolic fraction—responsible for the stability and flavor of olive oil and endowed with ''pharmacological'' properties—as an additional, valuable marker of olive oil quality.

Consumption of olive oil is increasing in non-Mediterranean areas such as The United States (olive oil imports exceed 100,000 metric tons/year), Canada, former Soviet Union, Australia, and Japan, due to the growing interest in the Mediterranean diet and its healthful properties.[5]

Peculiar to olive oil is the abundance of oleic acid, a monounsaturated fatty acid (18:1n-9), which ranges from 56 to 84% of total fatty acids, while linoleic acid (18:2n-6), the major essential fatty acid and the most abundant polyunsaturate in our diet, is present in concentrations between 3 and 21% (usually 7–10%).[6] The effects of monounsaturated fatty acids (MFAs) on circulating lipids and lipoprotein are still somewhat controversial: while the major effects of high monounsaturated fatty acid intakes on serum cholesterol are generally attributed to the associated replacement of saturated fatty acids,[7,8] some studies reviewed by Mensink and Katan[9] attributed a direct, although modest, cholesterol-lowering effect to MFA alone, when they equicalorically replace carbohydrates. Furthermore, MFAs increase the levels of the protective high-density lipoprotein (HDL) more than polyunsaturates (PUFAs) when these two classes of fatty acids replace carbohydrates in the diet.[9] However, other studies suggested a neutral effect of MFA or even a total- and low-density lipoprotein-cholesterol lowering activity. It should also be noted that oleic acid is one of the predominant fatty acids in largely-consumed animal foods, such as poultry and pork; thus, the percentage of oleic acid in the Mediterranean diet is only slightly higher than that of other kinds of Western diets, for example the American one,[10,11] and it is therefore unlikely that oleic acid content is the primary responsible agent for the healthful properties of olive oil. It is also noteworthy that several kinds of seed oils, obtained through genetic selection, are rich in monounsaturates and are now available in the market; examples include sunflower, soybean, and rapeseed oils.

### A. Olive Oil Minor Constituents

Fruits and vegetables—including olives and grapes—are continuously exposed to environmental stress, including UV radiation and relatively high temperatures (common in the Mediterranean basin), and thus need a variety of compounds, for example antioxidants, to preserve their integrity.[12,13] While most vegetable oils are *extracted* from seeds by solvents (with the exception of

rice bran oil), olive oil is obtained from the whole fruit by means of physical pressure, without the use of chemicals. As a result, prevalently lipophylic components of the drupe are transferred to the oil, which in turn retains the organoleptic properties of olives.

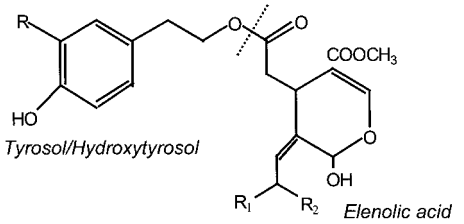
Among the several minor constituents of virgin olive oil, there are vitamins such as α- and γ-tocopherols (around 200 ppm) and β-carotene (which, together with chlorophylls is responsible for the oil color), phytosterols, pigments, terpenic acids, flavonoids such as luteolin and quercetin, squalene, and phenolic compounds, usually and incorrectly termed *poly*phenols.[6]

### *1. Olive Oil Phenols*

The amount of phenolic compounds in olive oil depends on several factors, including cultivar (olives from the Coratina variety are the richest in phenolics), degree of maturation, possible infestation by the olive fly *Dacus Olea*, and climate.[6] It usually decreases with over-maturation of olives, although there are some exceptions to this rule: for instance, olives grown in warmer climates, in spite of a more rapid maturation, yield oils that are richer in phenols. On the other hand, intact olives that are hand-picked at the right moment (when the skin color changes from pale green to dark brown), that are immediately brought to the mill, that are processed right away in a clean plant, and that are crushed and pressed at temperatures lower than 25–30°C yield a high-quality oil that is also rich in phenolic constituents (Fig. 1).

The influence of the elaboration process (see Ref. [6] for an exhaustive discussion of the various methods) on the phenolic content is yet to be fully elucidated. It appears, for instance, that oils that have been obtained by centrifugation have a lower phenols content,[14] possibly because this process involves the use of large quantities of warm water, with which the olive paste is continuously hosed during the milling. This is named "waste water" and is produced in extremely large quantities (~800,000 tons/year in Italy). Despite the fact that a considerable amount of phenols, according to their partition coefficient, end up in the waste water, the latter is currently disposed of. A series of experiments performed by Visioli et al.,[15,16] demonstrated that waste water extracts have powerful (in the ppm range) antioxidant activity and might therefore be recovered and employed in preservative chemistry as a cheap, as yet unused, source of natural antioxidants (see below).

The major shortcoming of the qualitative/quantitative evaluation of olive oil phenolic compounds lies in the current lack of an appropriate methodology. At present, the most widely employed methods for evaluating the polyphenolic content of olive oil are the Folin–Ciocalteau colorimetric assay[16] and high performance liquid chromatography.[17] The former is simple and




| R | $R_1$ | $R_2$ | |
|---|---|---|---|
| OH | H | H | Oleuropein |
| H | H | H | Ligstroside |
| H | OH | OH | 10-hydroxyligstroside |
| OH | OH | OH | 10-hydroxyoleuropein |

***Figure 1.*** Structures of the most representative olive oil phenolics. Hydroxytyrosol derives from oleuropein by hydrolysis where indicated.

economical to perform but is limited by the low specificity of the reagent toward phenolic compounds; further, it does not provide qualitative information of single phenolics. Conversely, HPLC is very sensitive and specific, but it is time-consuming (one run lasts for about 1 hr) and does not provide information on phenolic molecules for which reference standards are unavailable. An enzymatic assay for the quantitative determination of olive oil phenolics has been set-up by Mosca et al.[18] This method is rapid and easy to perform and is more sensitive and specific for phenolic compounds than the Folin–Ciocalteau method. Alas, it only provides quantitative information and does not detect important ''minor constituents'' such as cinnamic and vanillic acids. Finally, a rapid and sensitive method to evaluate the phenolic components of olive oil by atmospheric pressure chemical ionization-mass spectrometry (APCI-MS), has been described by Caruso et al.,[19] and allows for analyses of the crude methanolic extract of olive oil without extensive analytical workup and for the quantitation of oleuropein aglycone.

## *2. Phenolics and Olive Oil Taste*

The secoiridoid oleuropein (CAS RN Number 32619-42-4) is the bitter principle of olives and is found in olive oil as such and in its aglycone form. It was first named and studied by Bourquelot and Vintilesco[20] and investigated in humans by Panizzi et al.,[21] who reported on the hypotensive properties of this complex phenol. Oleuropein amounts to up to 14% of the dry weight in unripe olives[22] but, during maturation, undergoes hydrolysis and yields several simpler molecules (simple phenols) that build up the well-known olive oil complex taste. Moreover, oxidative modifications of phenolic compounds are sometimes advantageous, as they enhance the aroma and flavor of foods, including olive oils.[23] It is noteworthy that olives contain complex phenols as glycosides, that is, they are present in a rather polar and hydrophylic form, whereas the oil contains their aglyconic form, that is the more lipid-soluble residue of their molecule.

The phenolic constituents confer a bitter and pungent taste to the oil. The effect of bitterness and pungency is the result of complex interactions between the ''minor constituents'' and the taste buds, including inactivation of ptyalin. In particular, phenolic acids such as phenol and cinnamic acid are responsible for the bitter sensation that can be felt on the lateral and posterior areas of the tongue, while secoiridoids confer the peculiar pungency. As a result, the better tasting oils—rich in phenols—remind of artichoke, pepper or chili peppers. This is confirmed by panel tests—adopted to evaluate the organoleptic quality of the oil and to assess its flaws—in which oils produced from greener olives usually obtain higher scores[24] because of their ''fruity'' and complex aroma, provided by their high phenols content. Conversely, ''sweet'' oils are almost devoid of phenols. It should however be noted that a very high load of phenols may result in an excessive and unpleasant bitterness and is not synonymous with quality. Accordingly, the continuous system (in which the oil is separated from the paste by centrifugation after the addition of lukewarm water) employed to extract the oil sampled in a panel test reported by Cimato et al.,[24] prevented the development of off-flavors that can derive from dirty fiber mats or molds. Thus, in spite of removing a portion of phenols, this system lowers the percentage of defective oils. In turn, high phenol levels in virgin olive oils are very likely to exhibit a high stability and a strong, fruity flavor, indicating a high, but not necessarily the most preferred, organoleptic quality of the oil.

## *3. Olive Oil Phenolics and Human Health*

### IN VITRO STUDIES

*Antioxidant activities.* The epidemiological evidence of a lower incidence of CHD in the Mediterranean area[1] led to the hypothesis of a protective effect of some olive oil phenolics, with

respect to chemically induced oxidation of human LDL, i.e., one of the key steps in the initiation of atherosclerosis.[25] The recent availability of pure compounds, namely hydroxytyrosol (2 (3,4 dihydroxyphenyl)ethanol) and oleuropein, stimulated research in this field.

Oxidation of LDL can be investigated *in vitro* by incubating isolated LDL with a variety of oxidative agents, including chemicals such as transition metal ions and azo compounds, cultured cells such as macrophages and endothelial cells, or by physical means such as UV light radiation.[26] Several markers of oxidative stress must be taken into account, as they provide information on the oxidative modifications of lipids and apolipoproteins.[27]

Both hydroxytyrosol (HT) and oleuropein (OE) potently inhibit copper sulfate-induced oxidation of LDL in a dose-dependent manner, at concentrations of $10^{-6}$ to $10^{-4}$ M.[28,29] The protective effects of HT and OE are demonstrated through the assessment of various markers, such as a reduced formation of short-chain aldehydes (evaluated as thiobarbituric acid-reacting substances, TBARS) and of lipid peroxides, by a higher vitamin E content in the residual LDL (indicating sparing of endogenous antioxidants), and by a reduced formation of malondialdehyde-lysine and 4-hydroxynonenal-lysine adducts, indicating protection of the apoprotein layer.[28]

The antioxidant activities of hydroxytyrosol and oleuropein, which have been proven to be more effective than BHT or vitamin E, were further confirmed, by the use of metal-independent oxidative systems[28,30] and stable free radicals, such as DPPH,[31] in a series of experiments that demonstrated both a strong metal-chelation and a free-radical scavenging action. In particular, both HT and OE scavenged superoxide anions generated by either human polymorphonuclear cells or by the xanthine/xanthine oxidase system,[32] it is noteworthy that, in these experimental set ups, both vitamin E and BHT were found to be inactive. Furthermore, a scavenging effect of hydroxytyrosol and oleuropein was demonstrated with respect to hypochlorous acid,[32] a potent oxidant produced *in vivo* at the site of inflammation[33] and a major component of chlorine-based bleaches that can often come into contact with food during manufacturing. The HOCl-scavenging property of hydroxytyrosol may bear important consequences in terms of protection from atherosclerosis: the formation of chloramines via the myeloperoxidase-catalyzed formation of HOCl and subsequent chlorination of apoB-100 has been identified as an initiating agent in LDL lipid peroxidation.[34]

Additional evidence of the antioxidant properties of hydroxytyrosol was provided by Manna et al.,[35] who demonstrated an antioxidant effect of hydroxytyrosol (but not of tyrosol) in a model of oxidative stress induced in intestinal epithelial cells. In this experimental model tyrosol, which lacks the ortho-diphenolic structure, was found to be ineffective, as it was in the models of LDL oxidation described above (Visioli et al., unpublished data). The same group described a protective effect of hydroxytyrosol toward hydrogen peroxide-induced damage to human erythrocytes.[36]

It is well-known that the antioxidant properties of *o*-diphenols are related to hydrogen-donation, i.e., their ability to improve radical stability by forming an intramolecular hydrogen bond between the free hydrogens of their hydroxyl group and their phenoxyl radicals.[37] In fact, although specific investigations on the structure–activity relationship of olive oil phenols are yet to be carried out, similar studies have been performed on flavonoids and have indicated that the degree of antioxidant activity is correlated with the number of hydroxyl substitutions.[38] Particularly, the ortho-diOH substitution confers a high antioxidant capacity, whereas single hydroxyl substitutions, as in the case of tyrosol, provide no activity.

The activities of hydroxytyrosol toward chemically-induced DNA and amino acid modifications have been investigated by Aruoma, et al., [30] and Deiana et al.[39] Low concentrations of hydroxytyrosol, i.e., 50 μM, were able to scavenge peroxynitrite and therefore to prevent $ONOO^-$-dependent DNA damage and tyrosine nitration; also, in a model of copper-induced DNA damage, the prooxidant activities of hydroxytyrosol (which were due to its copper-reducing properties) were evident at non-physiological concentrations ($> 500$ μM) and were 40-fold weaker than those of ascorbate.[39]

*Interference with enzymes.* Olive oil phenolics are amphiphilic and they partition between the lipid (oil) and water (waste water) phases; their activities on enzymes potentially sensitive to phenolic compounds were tested in a variety of cellular models, i.e., platelets, leukocytes, macrophages. Indeed, lipid-soluble antioxidants such as tocopherols are unable to affect enzymes such as cyclo- and lipoxygenases, NAD(P)H oxidase, and nitric oxide synthase that are involved in key functions of those cells.

Hydroxytyrosol has been therefore tested for activities in addition to its antioxidant properties, such as the *in vitro* effect on platelet function, where the compound was proven to inhibit the chemically induced aggregation, the accumulation of the pro-aggregant agent thromboxane in human serum, the production of the pro-inflammatory molecules leukotrienes by activated human leukocytes, and the inhibition of arachidonate lipoxygenase.[40–43]

The potent ($EC_{50}$s in the $10^{-5}$ M range) inhibitory effect of hydroxytyrosol toward all these parameters reveals unpredicted biological activities of olive oil phenolics that go beyond their antioxidant properties. For instance, when added to murine macrophages together with a bacterial lipopolysaccharide, oleuropein increases the functional activity of these immune-competent cells, as evaluated by a significant increase ($58.7 \pm 4.6\%$) in the production of the bactericidal and cytostatic factor nitric oxide.[44] This increase was due to a direct tonic effect of oleuropein on the inducible form of the enzyme nitric oxide synthase (iNOS), as demonstrated by Western blot analysis of cell sonicates and by the coincubation of LPS-challenged cells with the iNOS inhibitor L-nitromethylarginine methylester.[44] Macrophage-derived nitric oxide during acute sepsis and inflammation represents an adaptive response of the organism that reacts to the endotoxin challenge by increasing the production of this mediator. In fact, nitric oxide inhibits platelet aggregation and adherence and it maintains a proper perfusion rate through increased vasorelaxation; accordingly, inhibition of nitric oxide synthesis during sepsis increases cellular damage and animal mortality.[45] Finally, macrophagic nitric oxide exerts a protective role in preventing oxidative LDL modification.[46]

Finally, the hypothesis was tested that both oleuropein and hydroxytyrosol possess estrogenic or androgenic activities but both compounds were found to be devoid of such actions (Visioli, Galli, and Poletti, unpublished data).

The potency of olive phenols as antioxidants suggests that they could be fruitfully employed as prophylactic agents; this might be particularly true in the case of olive mill waste waters, from where highly purified extracts that contain a high proportion of hydroxytyrosol can be obtained.[15] What renders hydroxytyrosol particularly interesting as an antioxidant preservative is that it is both hydro- and liposoluble, thus useful in emulsions or systems that contain both water and oil phases.[47]

## IN VIVO STUDIES

In addition to the large body of epidemiological data, experimental evidence that phenolic compounds are uptaken from the diet is accumulating. Experiments with laboratory animals, for example rats or rabbits, have demonstrated a higher resistance to oxidation of LDL obtained from animals fed virgin olive oil, as compared to animals that were only given a triglyceride preparation with an equivalent amount of oleic acid, i.e., triolein,[48] or ''plain'' olive oil.[49] Visioli et al., demonstrated that olive oil phenolics are dose-dependently absorbed in humans and that they are excreted in the urine as glucuronide conjugates; interestingly, increasing amounts of phenolics administered with olive oil stimulated the rate of conjugation with glucuronic acid.[50] Finally, the postprandial absorption of olive oil phenolics and their incorporation into human lipoproteins has been reported by Bonanome et al.[51] These data add to the experimental evidence indicating absorption and disposition of some flavonoids in humans.[52] It is worth speculating that the low bioavailability at times attributed to various phenols and flavonoids such as quercetin, luteolin, and apigenin after ingestion of diverse plant foods[53] might be attributed to their strong interactions with the food matrix, as in the case with artichokes, carrots, etc. This is not the case of olive oil phenolics,

***Table I.*** Biological Activities of Olive Oil Phenolics

| *Activity* | *Reference(s)* |
|---|---|
| Inhibition of LDL oxidation, both *in vitro* and *ex vivo* | 28–30, 48, 49 |
| Inhibition of apoprotein derivatization | 28 |
| Inhibition of platelet aggregation | 40, 41 |
| Reduced $TXB_2$ and $LTB_4$ production by activated leukocytes | 40, 42, 43 |
| Scavenging of superoxide and other ROS | 32, 35, 36, 59–63 |
| Inhibition of peroxynitrite-induced DNA damage | 39 |
| Inhibition of peroxynitrite-induced tyrosine nitration | 39 |
| Scavenging of hypochlorous acid | 32 |
| Increased nitric oxide production by LPS-challenged macrophages | 44 |
| Inhibition of neutrophil respiratory burst | 32 |
| Inhibition of bacterial growth and activity | 64, 65 |
| Cytostasis | 66 |
| Hypotensive action | 21 |
| Decreased isoprostane excretion in humans and in sidestream smoke-exposed rats | 67, 68 |
| Increased plasma antioxidant capacity | 54 |

which are easily transferred to the oil by the pressing of the drupe and then are simply dissolved in olive oil.

Moreover, we have been recently able to demonstrate that hydroxytyrosol, administered to rats as the only bioactive component of an olive mill waste water extract, is able to increase plasma antioxidant capacity.[54] Also, a low dose of hydroxytyrosol, i.e., only 414 μg/rat, are able to inhibit passive smoking-induced oxidative stress in rats, as demonstrated by a reduced urinary excretion of the $F_2$-isoprostane 8-*iso*-$PGF_{2\alpha}$ ($iPF_{2\alpha}$-III).[67]

Finally, a dose-dependent inverse correlation between the rate of 8-*iso*-$PGF_{2\alpha}$ exretion and increasing amounts of phenolics ingested with olive oil was observed in human volunteers;[68] interestingly, the urinary levels of 8-*iso*-$PGF_{2\alpha}$ inversely correlated with those of homovanillyl alcohol, i.e., a catechol-*O*-methyl-transferase (COMT)-derived metabolite of hydroxytyrosol,[36] suggesting that the latter enters into cellular compartments where it exerts its antioxidant activity. To date, these data represent the first, albeit limited, experimental evidence of a healthful effect of olive oil components on human health.

In the future, availability of pure—or even labeled—compounds in adequate quantities and development of appropriate methodologies will further clarify the metabolic fate of phenolic micronutrients, including those of olive oil.

## *3. CONCLUSIONS*

Olive oil currently represents a small share of the whole vegetable oil market, but its use is gaining ground owing to the increasing popularity of the Mediterranean diet. The olive oil industry is actively trying to improve the overall olive oil quality by selecting the appropriate cultivars and by optimizing each production step, from harvesting to extraction, although this process is somewhat limited by the fragmentation of the production chain, i.e., a high number of farmers and mills, each employing its own traditional methods.

The contribution of excessive free radical formation to the onset of certain pathologies such as atherosclerotic heart disease and cancer calls for a higher dietary intake of fruits and vegetables, i.e. food with a substantial proportion of antioxidant vitamins, flavonoids, and polyphenols.

Accordingly, the observation that in the Mediterranean area there is a lower incidence of CHD[1,55] and certain types of cancers[56,57] lead to the hypothesis that a diet rich in grain, legumes, fresh fruits and vegetables, wine in moderate amounts, and olive oil had beneficial effects on human health. Further, while the beneficial effects of the Mediterranean diet on the cardiovascular system have been so far mostly attributed to its unique lipid profile, the contribution of natural antioxidant and other components of the diet, such as fiber, to this effect should also be taken into consideration.

The evidence illustrated in this review (Table I) suggests that choosing a phenols-rich olive oil would contribute to the dietary intake of biologically active compounds, in estimated quantities that have been correlated with a reduced risk of developing CHD.[2,58] Moreover, a phenols-rich, tasty olive oil can be used in small quantities to dress foods, thus reducing the overall caloric density.

Finally, although this review focuses on a specific subfraction of olive oil, i.e. polyphenols, the large body of evidence that indicates a beneficial role of olive oil as a whole should not be overlooked. It is in fact difficult to single out an individual component of such diet and correlate it with the observed lower incidence of CHD and certain cancers. For instance, the choice of olive oil as the fat of choice increases the consumption of fresh, raw vegetables, thus providing an additional, indirect benefit. In fact, epidemiological studies, of which the Seven Countries Study is the most cited one, as well as intervention trials, have reported on the healthful effects of the Mediterranean diet and olive oil: it is noteworthy that in Crete, where fat consumption provides up to 40% of total calories and is almost totally derived from olive oil, the incidence of CHD is lowest. In conclusion, the biologically relevant properties of olive oil phenolics described in this review provide new insights on the mechanisms by which good-quality olive oil may contribute to lower CHD mortality.

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