SCIENZE BIOLOGICHE
BIOLOGIA APPLICATA ALLA SALUTE DELL'UOMO

CYCLE_XXIV

SYNTHESIS AND STRUCTURE/ANTIOXIDANT ACTIVITY RELATIONSHIP OF LIPOPHILIC HYDROXYTYROSYL ESTERS AND THEIR ANALOGUES

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Riassunto

L'albero d'ulivo (*Olea Europea*) è ampiamente sfruttato a scopo alimentare: i suoi frutti e l'olio sono componenti essenziali della dieta mediterranea di gran parte della popolazione europea, d'altra parte sia i frutti che le foglie contengono importanti metaboliti secondari, di cui sono note le proprietà antiossidanti. Queste proprietà sono principalmente associate alla frazione fenolica presente. L'idrossitirosolo (3,4-DHE) è una piccola molecola idrosolubile (log P = 0.08) presente nelle foglie, nei frutti e in piccola parte anche nell'olio d'oliva, in un range di concentrazione tra 113.7 e 381.2 mg/kg. Il 3,4-DHE è un efficace scavenger di radicali perossidici, protegge l'olio d'oliva da eventi ossidativi che portano all'irrancidimento prolungandone quindi la conservazione.

Studi di letteratura hanno evidenziato diverse e importanti proprietà biologiche dell'idrossitirosolo, tra cui la sua biodisponibilità nell'uomo, l'assenza di tossicità *in vivo*, e una serie di funzioni legate ad una diminuzione nello sviluppo e nel decorso di malattie cardiovascolari. Ad esempio è noto che il 3,4-DHE previene l'ossidazione delle LDL, riducendo così il rischio di formazione di placche aterogeniche e quindi l'avanzamento dell'aterosclerosi. Allo stesso modo, il 3,4-DHE come anti-infiammatorio riduce il fenomeno di adesione piastrinica, che in una situazione incontrollata di "gain of function" può portare alla formazione di trombi. Lo studio dei processi metabolici in cui è coinvolto il 3,4-DHE ha rivelato che la sua attività si esplica principalmente attraverso interazione con le componenti sensibili all'omeostasi redox della cellula, quali per esempio l'inattivazione del fattore di trascrizione nucleare NF-κB.

Allo stato attuale delle conoscenze, il 3,4-DHE risulta un composto sicuro, e potrebbe essere utilizzato come conservante alimentare o in campo cosmetico. Tuttavia, l'impiego di questo antiossidante su larga scala è frenato dalla sua elevata idrofilicità che ne ostacola l'estrazione dalla sua fonte naturale (le acque reflue di frantoio), ne rende difficile l'incorporazione in grassi e oli (la quasi totalità delle preparazioni industriali), e ne abbassa la concentrazione disponibile in matrici lipofile, con conseguente riduzione della sua efficacia.

Attualmente, la lipofilizzazione del 3,4-DHE mediante legame estereo con catene di acidi grassi sull'ossidrile alcolico si è rivelata un buon modo per aggirare questi ostacoli, promuovendo le proprietà antiossidanti dei derivati del 3,4-DHE anche in un differente mezzo di dispersione, grazie all'incrementata solubilità in matrici lipofile. Gli esteri lipofili dell'idrossitirosolo, da dati di letteratura, mantengono una buona attività antiossidante, comparabile o superiore al 3,4-DHE stesso. Nonostante questo, non c'è traccia di uno studio sistematico della relazione tra struttura e attività (SAR) di questi nuovi

composti, che invece è fondamentale per la scelta di una adeguata lipofilicità che ottimizzi le proprietà antiossidanti.

Un'accurata analisi del modo in cui modifiche strutturali del 3,4-DHE influenzino la sua attività antiossidante potrebbe in generale arricchire la conoscenza e l'utilizzo di antiossidanti fenolici lipofili, con implicazioni positive nella ricerca di nuovi additivi industriali.

L'obiettivo della presente tesi è stato perciò condurre uno studio della relazione tra struttura e attività antiossidante di quattro serie complete di derivati lipofili dell'idrossitirosolo, ottenuti mediante:

- esterificazione della funzione alcolica con acidi grassi a catena crescente;
- piccole modifiche strutturali della molecola 3,4-DHE naturale.

Il progetto è suddiviso in tre parti:

1. Preparazione di (Figura 1):
   - una serie completa di idrossitirosil esteri di acidi grassi (C2-C18);
   - un omologo superiore dell'idrossitirosolo (chiamato 3,4-DHP) con catena alcolica propilica e la serie completa dei suoi esteri corrispondenti (C2-C18);
   - un isomero dell'idrossitirosolo (chiamato 2,3-DHE) con la funzione catecolica in 2,3 (*ortho-meta* rispetto alla catena alcolica) e la serie completa dei suoi esteri corrispondenti (C2-C18);
   - un catecolo caratterizzato dalla combinazione delle due precedenti modifiche strutturali (chiamato 2,3-DHP) e la serie completa dei suoi esteri corrispondenti (C2-C18).
2. Misura dell'attività antiossidante delle quattro serie di composti preparati, mediante saggio *in vitro* dell'ABTS e in coltura cellulare di mioblasti da ratto mediante dosaggio delle ROS con H_2DCF.
3. Studio dell'interazione tra alcuni idrossitirosil esteri e la membrana fosfolipidica, utilizzando i liposomi come modello di membrana semplice.

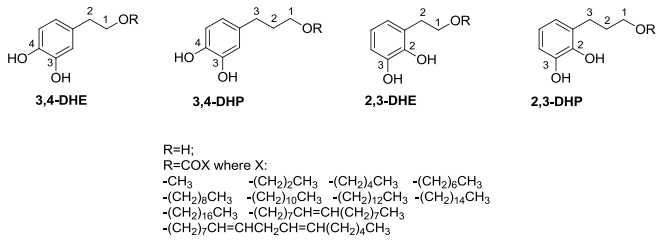


Figure 1. Molecole oggetto di studio nella presente tesi

I risultati ottenuti indicano che la lipofilizzazione di catecoli idrofili, mediante esterificazione regio-selettiva della catena alcolica, effettivamente mantiene o perfino promuove le proprietà antiossidanti. La lipofilicità può essere modulata, scegliendo una adeguata catena alchilica, per poter cooperare con l'attività antiossidante. I risultati mostrano una relazione non-lineare tra lipofilicità e capacità antiossidante, caratterizzata infatti da una evidente riduzione di abilità antiossidante per gli esteri a catena più lunga. Nel saggio ABTS, eseguito in etanolo, studi di dinamica molecolare hanno dimostrato che il 15% delle catene più lunghe possono ripiegarsi su sé stesse; tale arrotolamento (*folding*) scherma gli ossidrili fenolici, interferendo così con l'attività antiossidante. Dalle analisi comparative delle quattro serie di composti si è evinto che la traslazione della funzione catecolica in posizione *ortho-meta* rispetto alla catena alcolica può influenzare positivamente l'attività antiossidante di catecoli lipofili, permettendo la formazione di un legame idrogeno intramolecolare che nei composti con ossidrili in *meta-para* non è consentito. La realizzazione di questo nuovo legame è ulteriormente facilitata dall'allungamento della catena alcolica da etilica a propilica.

Per quanto riguarda i saggi biologici, trattandosi di composti nuovi è stata innanzitutto verificata la eventuale tossicità nei confronti del modello cellulare dei mioblasti. Una vola validata la non tossicità, come peraltro già riportato in letteratura per l'idrossitirosolo, si è effettuato il dosaggio della produzione di ROS che ha mostrato, allo stesso modo nelle quattro serie di composti (indipendentemente dalle modifiche strutturali attuate nel 3,4-DHE), un andamento sigmoide evidentemente influenzato dalla sola lunghezza della catena alchilica ossia dall'aumento di lipofilicità dell'antiossidante. Come già precedentemente rilevato nel saggio ABTS, i composti a catena medio-corta hanno registrato le migliori attività (ovvero una produzione di ROS più contenuta nelle cellule trattate con antiossidante e successivamente indotte a stress-ossidativo da cumene idroperossido), con marcata riduzione della capacità di radical scavenging per i composti con catene da C12 in poi.

La determinazione delle concentrazioni di aggregazione critica, dipendenti ovviamente dalla grandezza di ciascuna molecola, ha mostrato che questi composti, anfifilici, si auto-associano in acqua seguendo le leggi dei classici surfactanti; inoltre, si è determinato che ben il 50% dell'idrossitirosil stearato risulta aggregato, in soluzione acquosa alle stesse concentrazioni di analisi delle ROS (10 μM). Questo potrebbe in parte spiegare la minore attività dei composti a catena lunga. D'altra parte, nell'emulsione cellulare gli esteri non interagiscono solo con sé stessi e con le molecole d'acqua, ma si ritrovano anche a poter interagire con la membrana cellulare. Gli studi condotti con i liposomi hanno in parte chiarito la dinamica di questi derivati antiossidanti nell'ambiente cellulare. Infatti, sebbene sia stato confermato che i composti a catena medio-lunga (C8-C18) non forniscono protezione adeguata ai liposomi, nel caso in cui si utilizzi un saggio con sonda intracellulare, si è anche

verificato che quegli stessi esteri (C8-C18) interagiscono e si legano fortemente alla membrana delle vescicole, proteggendola efficacemente da stress ossidativo, come evidenziato dai saggi di attività effettuati con la sonda di membrana DPH.

In conclusione, è stato dimostrato che, in antiossidanti polifenolici, è possibile modulare la lunghezza della catena alchilica, in modo di ottimizzare la protezione da stress ossidativo nell'ambiente intravescicolare o nel doppio strato fosfolipidico (e per analogia potremmo pensare che lo stesso possa accadere nelle cellule). Inoltre, la razionalizzazione delle modifiche strutturali dell'idrossitirosolo ha introdotto nuove informazioni che possono migliorarne l'efficienza antiossidante e che possono, quindi, risultare utili nella ricerca di nuovi conservanti specialmente in campo alimentare.

I risultati pubblicati su riviste del lavoro condotto dalla candidata durante il dottorato, anche in altri progetti non menzionati nella presente tesi, sono qui riportati:

- Tofani D., Balducci V., Gasperi T., Incerpi S. and Gambacorta A. (2010)**.** Fatty acid hydroxytyrosyl esters: structure/antioxidant activity relationship by ABTS and in cell-culture DCF assays**.** *J. Agric. FoodChem. 58*, 5292-5299.
- Trapani L., Segatto M., Simeoni V., Balducci V., Dhawan A., Parmar V.S., Prasad A.K., Saso L., Incerpi S. and Pallottini V. (2011). Short- and long-term regulation of 3 hydroxy 3methylglutaryl coenzyme A reductase by a 4-methylcoumarine. *Biochimie 93*, 1165-1171.
- De Vito P., Balducci V., Leone S., Percario Z., Mangino G., Davis P.J., Davis F.B., Affabris E., Luly P., Pedersen J.Z. and Incerpi S. (2012). Nongenomic effects of thyroid hormones on the immune system cells: new targets, odl players. *Steroids 77*, 988-995.
- Bernini R., Crisante F., Barontini M., Tofani D., Balducci V. and Gambacorta A. (2012). Synthesis and Structure/Antioxidant Activity Relationship of Novel Catecholic Antioxidant Structural Analogues to Hydroxytyrosol and its Lipophilic Esters. *J. Agric. Food Chem. 60*, 7408-7416.
- Lombardo E., Sabellico C., Hajek J., Stankova V., Balducci V., De Vito P., Leone S., Bavavea E., Proietti Silvestri I., Righi G., Luly P., Saso L., Bovicelli P., Pedersen J.Z. and Incerpi S. Protection of Cells Against Oxidative Stress by Nanomolar Levels of Hydroxyflavones. *Submitted to PlOS*.

Abstract

The olive tree (*Olea Europea*) is widely studied for its alimentary use: the fruits and the oil are essential components in the mediterranean diet of a large part of european's population, whereas fruits and leaves are important for their secondary metabolites, known for their protective (antioxidant) properties. These properties are attributed to the phenolic compounds of olive tree. Hydroxytyrosol (here named 3,4-DHE) is a small hydrosoluble molecule (log P = 0.08), which is present in the leaves, fruits and, in small amount, in olive oil (113.7 ~ 381.2 mg/kg). It acts as an efficient scavenger of peroxyl radicals, preventing oil oxidation and therefore contributing to improve its shelf life.

Previous studies on hydroxytyrosol's biological properties gave information about its bioavailability in humans, its nontoxicity *in vivo*, its antiatherogenic properties (mainly by protecting LDL from oxidation damage), its antiplatelet aggregation ability and its anti-inflammatory effects. All these properties can be related to the ability of 3,4-DHE to interact with redox sensitive cellular elements, such as by inactivating NF-κB pathways.

As for the radical scavenging and biological properties mentioned above, 3,4-DHE seems to be a good antioxidant, safe enough for industry preparation. Nevertheless, easy incorporation of 3,4-DHE as an additive in food and cosmetics, as oil matrices, is still a challenge. The hydrophilic character stands as a serious disadvantage, first of all for the difficulties in extracting it from aqueous solutions, then for the reduction of its effectiveness in stabilizing fats and oils. Therefore, more lipophilic 3,4-DHE derivatives could promote hydroxytyrosol's properties with respect to the dispersion medium. Derivatization of 3,4-DHE demonstrated to be a promising tool to increase its solubility in lipophilic preparations. Several studies have highlighted that 3,4-DHE derivatives possess antioxidant properties. However a systematic study of the structure-activity relationships, required for a fine tuning of the lipophilicity that maximizes its antioxidant properties, is still lacking.

An accurate analysis of the influence of structural rearrangements on the antioxidant activity of 3,4-DHE may help to better understand the basic aspects of the behavior of lipophilic phenolic antioxidants. Furthermore, it may bring worthy information in order to build up efficient antioxidants for industrial employment.

The aim of the present PhD project has been to explore the structure-activity relationship of four complete series of lipophifilic hydroxytyrosyl derivatives, obtained by:

- esterification of the alcoholic moiety with fatty acids of increasing acyl chain length
- small structural modifications of hydroxytyrosol main scaffold.

The project was divided into three parts:

- Preparation of (Figure 1):

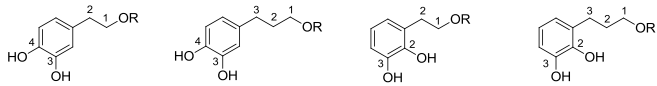
1. hydroxytyrosyl esters of fatty acids by chemical synthesis;

2. a new hydroxytyrosol superior homologue (named 3,4-DHP) having a propyl alcoholic chain and the complete series its fatty acid esters (C2-C18);

3. a new hydroxytyrosol isomer (named 2,3-DHE) having the catecholic hydroxyls in 2,3 position with respect to the alcoholic chain and the complete series of its fatty acid esters (C2-C18);

4. a new catechol obtained by pooling together the two previous rearrangements (named 2,3-DHP) and the complete series of its fatty acid esters (C2-C18).

- Evaluation of antioxidant activity of all the compounds obtained, by *in vitro* ABTS assay and by H_2DCF assay in cultured L6 myoblasts.
- Study of the interaction between a representative series of hydroxytyrosyl esters derivatives and lipid bilayer by using lipid vesicles (liposomes), representing the simplest membrane model.



R=H;
R=COX where X:
-CH_3 -$(CH_2)_2CH_3$ -$(CH_2)_4CH_3$ -$(CH_2)_6CH_3$
-$(CH_2)_8CH_3$ -$(CH_2)_{10}CH_3$ -$(CH_2)_{12}CH_3$ -$(CH_2)_{14}CH_3$
-$(CH_2)_{16}CH_3$ -$(CH_2)_7CH{=}CH(CH_2)_7CH_3$
-$(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

Figure1. Molecules under study in this thesis

The results obtained indicate that the derivatization of hydrophilic catechols, by regio-selective esterification with linear fatty acid on the alkylic alcohol, can effectively maintain or even enhance their antioxidant properties. Lipophilicity can be modulated, by the choice of an adequate acyl chain length, to cooperate with antioxidant activity. It emerged that the dependence between hydrophobicity and the antioxidant capacity is nonlinear, but characterized by a clear cut-off effect for longer chain esters. We investigated the reasons of the decreased activity for longer alkyl chains.

In ethanol (used for ABTS assay), it was found that esters having chains longer than C12-C14 can in part assume folded structures, thus interfering with the catechol moiety's radical scavenging ability. Comparative analysis of the four sets of molecules under study suggested that shifting the hydroxyl to *ortho-meta* position with respect to the alcoholic chain can successfully affect the stability and the antioxidant activity of lipophilic phenols, by creating an intramolecular hydrogen bond that in compounds with *meta-para* catechols is

not allowed. The formation of this new bond is particularly facilitated by elongating the alcoholic side chain from C2 to C3.

Viability assessment of 24h-preincubated myoblasts with all the compounds confirmed the nontoxicity of these substances, as already reported in literature for hydroxytyrosol.

ROS production assay in cells described a similar activity pattern for the four sets of compounds (higher antioxidant activities for short-medium size esters, and a drop for longer chains), demonstrating that in a biological environment rearrangements of hydroxytyrosol structure have minor influence in the activity, instead, the only involved parameter is lipophilicity (that is related to the length of the ester chain).

The determination of the size-dependence on the critical aggregation concentrations reveled that these amphiphilic catechols self-associate in aqueous solutions, like classical surfactants, and - interestingly – that the 50% of 10 μM stearate ester (the concentration used for our experiments) forms supramolecular structures in aqueous solution.

However, this could only partially explain the drop of activity for long chain esters in cells. Activity assays performed with liposomes clarified that medium-long esters are unable to protect the intravesicular environment from induced oxidation giving low values of DCF assay.

Notwithstanding, medium-long esters strongly interact with the membrane where they are soluble, and they can efficiently protect it from oxidative damage as shown by measures of antioxidant activity with DPH probe.

In conclusion, the performed studies demonstrate that it is possible to modulate the length of the acyl chain in order to maximizes the defense from oxidative injure either in the intravesicular environment or in the bilayer, supposing for analogy, that the results obtained with liposomes could fit for cells. Moreover, rational design of hydroxytyrosol structure brought new information about improving its efficiency, which may be useful in the market of food preservants.

INTRODUCTION

1. The good and bad of antioxidants

Antioxidants have a long history of knowledge and use in the scientific community and food industry. According to last decade's literature, the traditional understanding has been that antioxidant chemicals promote health by removing reactive radical species that may otherwise exert harmful effects.[1]

Harman first postulated in 1956 the "free radical hypothesis of aging",[2] and in 1989 Steinberg et al. suggested the "oxidation-stress hypothesis of atherosclerosis".[3] Following these hypothesis, oxidative stress, *id est* the formation of radical species due to oxygen reactions, has been associated with the pathogenesis of many other diseases including diabetes mellitus, atherosclerosis, neurodegenerative deseases, different malignant tumors and virus infections, including AIDS.[4]

In particular, membrane phospholipids are the first molecules involved in structural and functional modifications when oxidative stress occurs. Peroxidation of lipids starts a chain reaction leading to fragmentations of proteins and nucleic acids, and finally, if not counteracted, can bring significant tissue damage.

According to this view, most of the free radicals and reactive oxygen species (ROS) were considered to be harmful[2] implying that maximizing antioxidant concentrations could minimize the risk for chronic disease.

Many dietary compounds, showing antioxidant properties in the commonly used *in vitro* assays, have been employed to evaluate their ability to support protection against ROS damage and reduce the risk of chronic disease in epidemiological research.[5,6] The promising results drove industry to market substances exerting antioxidant benefits both as food additives and nutraceuticals sold in medicinal form.[1]

Recently, many scientists have put forward the theory of "the failure of the antioxidant benefits hypothesis",[7,8,9] due to new major scale randomized clinical trials yielding disappointing results on the effects of some well-known antioxidants on mortality and morbidity. Specifically, Dotan et al.[7] analyzed the results of many large prospective randomized trials on the supplementation of vitamin E, concluding that the only indications of any benefit from administration of high doses of it were registered in specific groups of individuals (es. Male smokers, atherosclerosis patients, hemodialysis patients). Moreover, several trials indicated that vitamin E supplementation may be harmful and increase overall mortality rates.

Similar results were obtained analyzing clinical trials on beta-carotene, vitamins A, and C.[8,9]

These recent studies have invalidated the general attitude towards natural molecules that is, if antioxidants do not help, at least they will not harm. Few years before, Jones[10] moved the focus from the "antioxidant" to the "redox

balance", asserting that changing the balance toward either oxidation or reduction may be deleterious. "Theory of hormesis"[11] strictly addresses to this point. This theory, first pronounced by Stebbing in 1982, claims that biological systems respond with a bell-shaped curve to exposure to chemicals, toxins and radiations. In particular, focalizing on the first tract of the bell-shaped curve, it has been observed that low doses of potentially harmful substances lead to beneficial effects. Increasing evidence suggest that hormesis, whose mechanisms are largely unknown, may operate in higher animals as well. Recently, Radak et al.[12] suggested to extend the hormesis to exercise-induced ROS production, as it plays a role in antioxidants induction, DNA repair and protein degrading enzymes, resulting in decrease of the incidence of oxidative-stress related diseases and retardation of the aging process. From this point of view, an excessive intake of dietary antioxidants could bring damage by unbalancing the equilibrium of the redox system, thus impairing the signals of ROS physiological pathways.

Supporters[13] of the lack of side-effects of antioxidants think that this last issue is not completely true. Niki[13] distinguishes between specific and random lipid oxidation, the former being mediated by enzymes (cyclooxygenase, lipoxygenase and cytochrome P450). The characteristic feature of enzymatic oxidation is specificity: each enzyme oxidizes specific substrate to give region-, stereo- and enantio-specific products. Furthermore, the enzymatic oxidation is, in general, tightly programmed and regulated. On the other hand, free radical mediated peroxidation proceeds randomly to give diverse products. Moreover, free radicals attack proteins and nucleic acids nonspecifically as well as lipids.

There is evidence that chemically stable lipid peroxidation products act as inducers of adaptative response (protective feedback system of hormesis).[13] The enzymatic-generated species (H_2O_2, $O_2^{-\cdot}$) have a half-life long enough to survive inside the cell, if only for a very short time at very low concentrations, in spite of all the antioxidant mechanisms involved in preventing or counteracting the oxidative damage.[14] In order to function as physiologically essential signaling messenger the formation, the reaction and metabolism of ROS should be strictly controlled. This may be possible for enzymatic reactions, but it is difficult to direct time, site and amount of formation of free radicals and also to regulate the reaction pathway. The lack of regulation and specificity in free radical formation and reactions makes them not good candidates to act as physiologically essential signaling messenger. In this context, following the author's reasoning, the role of dietary antioxidants is not to inhibit the enzymatic lipid peroxidation and block the beneficial actions of physiological signaling processes. Low molecular weight dietary antioxidants such as vitamin C and E do not scavenge important signaling ROS such as hydrogen peroxide and superoxide, nor do they inhibit the enzymatic lipid oxidation, but they scavenge extremely reactive free radicals, thus inhibiting

the free radical mediated lipid peroxidation. Antioxidants do not finally impair physiologically essential signaling pathways.

What is certain, after the introduction to these two divergent hypotheses, is that the benefits appear to be realized by consuming "antioxidant rich" foods, whereas interventions with specific antioxidants have not proven to be beneficial.[1,15] An interesting point is that these molecules, having high antioxidant value *in vitro*, are in general reasonable predictors of the stabilization of food stuff and drugs but not of their effects on human health.[4] This apparent inconsistency may be attributed to the dependence of peroxidation on the state of aggregation of lipid substrate. Specifically, most of the commonly used assays occur in solutions, while in biological systems amphiphilic phospholipids reside either in membranes or in emulsion particles and peroxidation therefore occurs at the lipid-water interface. Theoretically, to be relevant to the oxidative stress in human, a reasonable assay has to be based on the effect of externally added antioxidant on the peroxidation of poly-unsaturated fatty acids, thus involving aggregated substrates.[4]

2. Hydroxytyrosol: state of art

2.1. A bit of history

The olive tree (*Olea Europea*, Oleaceae) is a traditional symbol of abundance, glory, wisdom and peace. Its leafy branches were historically used to crown the victorious in friendly games and bloody wars. The olive fruit, its oil and the leaves of olive tree have a rich history of nutritional, medicinal and ceremonial uses. The introduction of olive cultivation coincided with the expansion of the Mediterranean civilizations and the olive leaves have been used widely in traditional remedies in European Mediterranean islands and countries such as Spain, Italy, France, Greece, Israel, Morocco, Tunisia and Turkey.[16] Oil from the olive is known to have been used for food since prehistory.[17]

Olea Europea is widely studied for its alimentary use: the fruits and the oil are important components in the mediterranean diet of a large part of the european's population, whereas the leaves are important for their secondary metabolites such as the secoiridoid compounds oleacein and oleuropein. Olive tree leaves are well known for their beneficial effects on metabolism when used as a traditional herbal drug. These properties are attributed to the phenolic compounds of olive leaves.[16]

Reactivity to pathogen attack, response to insect injuries or to environmental stressors[17] (such as ultraviolet radiation and relatively high temperatures, common in the Mediterranean basin) stimulate the secondary metabolism in the olive tree (Figure 1). This leads to enhanced production of phenolic compounds

with protective (antioxidant) properties. The two main sources of olive polyphenols are olive leaves and the waste from the olive oil industry. This discard product, known as alperujo, is a cheap source of natural antioxidants containing concentrations up to 100-fold higher than those found in olive oil. Olive leaves have the highest antioxidant and scavenging power among the different parts of olive tree. For instance, the concentration of oleuropein, the predominant secoiridoid in olive tree, is between 0.005-0.012% in olive oil, 0.87% in alperujo and between 1-14% in the leaves, respectively.[16]

Figure 1. Main secondary metabolites of olive oil. The picture shows the temple of Athena, as the olive plant was sacred to the goddess (picture from Rafehi et al., 2012).[18]

2.2. Hydroxytyrosol in olive tree

Oleuropein was discovered in 1908 by Bourquelot and Vintilesco. Structurally it is the heterosidic ester of elenolic acid and 3,4 dihydroxyphenylethanol. This latter, more commonly known as hydroxytyrosol (3,4-DHE), is the principal degradation product of oleuropein. So, oleuropein is present in high amounts in unprocessed fruit and leaves being responsible for the bitter taste of immature drupe, while hydroxytyrosol is more abundant in the processed olive fruit and olive oil. The decrease in concentration of oleuropein and the increase of hydroxytyrosol occur due to chemical and enzymatic hydrolysis that take place during maturation of the fruit or as a result of olive processing, such as storage of the oil and preparation of table olives. These processes give rise to oleuropein aglycone, hydroxytyrosol and elenolic

acid, these being responsible, in part, for the complex and varied flavor of the oil and olive.

As a consequence of their polar character, phenolic compounds are found in major quantities in the remains from oil processing, such as pomace olive oil, olive-mill waste water. For this reason, byproducts from olive oil production constitute a major source of hydroxytyrosol. Nevertheless, hydroxytyrosol has also amphipathic behavior, and the molecule is therefore found in olive oil, where its amount ranges between 113.7 and 381.2 mg/kg.[17]

Mediterranean countries have a long tradition of olive cultivation and olive oil production, with both the fruit and the oil being habitual components of diet. It has been estimated[19] that the dietary intake of olive oil polyphenols in these countries is around 9 mg per day with a daily intake of 25-50 mL of olive oil; at least 1 mg is derived from free hydroxytyrosol and tyrosol, while 8 mg are related to their elenolic esters (as oleuropein aglycon).

Brandt[20] et al., screening for health-promoting compounds from edible plants, define three criteria to establish an expertise base to perform initial and more advanced investigations on natural compounds:

1) Presence of chemically reactive functional groups or other chemical properties indicating likely interaction with cell components;

2) Known effects on some physiological functions in humans;

3) Presence in foods which are widely consumed, preferably foods epidemiologically linked with decreased risk of disease, indicating absence of direct toxicity at the concentrations normally found in food and indicating good prospects that new knowledge can and will be utilized to improve diets.

Hydroxytyrosol satisfies these three points:

1) It's a polyphenol. Phenols, which are very common in plants, are the most studied and employed antioxidants in industry. They are very stable in the radicalic form, too, due to the many resonance structures of the benzenic ring. In particular, compounds bearing two phenolic hydroxyls near each other (*ortho* position) are called catechols. The stability, reactivity and hydrophylicity in catechols are higher than in simple phenols. Ortho-diphenolic moiety is very reactive towards oxidant radical species able to extract a hydrogen atom from a phenolic hydroxyl. The remaining electron is stabilized by the hydrogen bond between the hydroxyl and the radicalic oxygen. This intermediate facilitates another hydrogen extraction final leading to stable o-quinone. *In vitro* studies suggest that the antioxidant activity of 3,4-DHE is strictly related to the integrity of orthodiphenolic moiety. Methylation of the phenolic hydroxyl group results in the loss of antioxidant activity. Moreover, the presence of orthodiphenolic group is again a structural requirement for biological activity. In a study, it was demonstrated that 3,4-DHE induces apoptosis in white blood cells, while tyrosol, the structural analogue which presents only one hydroxyl group on the phenyl ring, has no biological activity.[21]

2) 3,4-DHE has been widely used for studies in the context of cardiovascular disease research and antitumoral effect. These points will be discussed below.

*3)*Dietary sources of hydroxytyrosol are the pulps of olives and oil. These two foods are an inherent part of the Mediterranean cultivation and diet, hence the low incidence of cardiovascular disease in this area has been attributed to their consumption.[22] Today, there is no doubt concerning the important effects of the different types of dietary fat ingested, particularly the benefits of olive oil. 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. Olive oil has been demonstrated to generate membranes more resistant to lipid peroxidation and more functional in comparison with those generated by polyunsaturated fat sources such as sunflower oil.[23]

Olive oil also reduces hypertension, improves thrombogenic conditions, prevents or improves diabetes, reduces inflammatory processes and alleviates intestinal inflammatory illness. 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.[17]

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 activity, such as 3,4-DHE.[24]

2.3. Hydroxytyrosol bioavailability

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. The absorption of hydroxytyrosol takes place in the small intestine and colon: it has been suggested that transport through the intestinal epithelium can occur by passive bidirectional diffusion. The absorption of this compound is rapid, with maximum plasma concentration being reached 5-10 minutes after ingestion, followed by a rapid decline.[17] The absorption of hydroxytyrosol differs according to the vehicle in which it is carried. For example, Tuck et al.[25] demonstrated that rats absorbed 75% of 3,4-DHE when it is administered in an aqueous solution and 90% when it is administered in an oily vehicle. The absorption also varies according to the animal species; for instance, different rates are found in rats compared with humans[26] because of the absence of a gall bladder in these rodents. Finally, after postprandial absorption, 3,4-DHE binds to circulating human lipoproteins (as other phenols from olive oil). Whether in plasma or when excreted in urine, 3,4-DHE is found as itself, or as its 3-methoxy-derivativ (homovanillil alcohol), or as glucuronide derivatives.[17]17

A study of the radioactive 3,4-DHE tissue distribution after intravenous administration in rats has demonstrated that its half-life in blood is 1-2 minutes and that, at 5 minutes after injection, most of the marked 3,4-DHE is found in the kidney, where tenfold more radioactive 3,4-DHE accumulates than in other organs, such as skeletal muscle, liver, lungs or heart, which all present very similar levels. It has been shown that 3,4-DHE can cross the blood-brain barrier to appear in the brain (even though its brain uptake is lower when compared with other organs), although it must be considered that 3,4-DHE may be generated endogenously from dihydroxyphenylacetic acid through dihydroxyphenylacetic reductase of the brain. It can also be generated from dopamine. De La Torre[19] suggests that the understanding of 3,4-DHE is hampered by the fact that the 3,4-DHE 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. At 5 hours after injection, only 0,1% of the radioactive 3,4-DHE remains in animal organs. In fact, 3,4-DHE is metabolized rapidly, first in the enterocytes and afterwards in the liver, with metabolites found in blood 5 minutes after intravenous injection. D'Angelo et al.[27] propose three metabolic pathways for 3,4-DHE: 1) oxidation through the enzymes alcohol dehydrogenase and aldehyde dehydrogenase, giving rise to dihydroxyphenylaceticacid; 2) methylation by the enzyme catechol-*O*-methyltransferase, rendering homovanillyl alcohol; 3) methylation-oxidation, a reaction that occur to form homovanillic acid.

Ninety percent of the administered radioactivity incorporated in 3,4-DHE is detected in urine collected up to 5 hours in rats[26] (in humans 3,4-DHE and its metabolites are found in urine after 4 hours),[28] indicating that renal excretion represents the preferential route for the depletion of 3,4-DHE and its metabolites. Again, the elimination (as already seen for the absorption) is lower in rats than in humans.

2.4. Hydroxytyrosol toxicity

Although toxicity studies on 3,4-DHE are scarce, some conclusions can be outlined on the basis of the few data available. D'Angelo et al.[27] administered a single dose of 2g/kg of body weight in rats and found an absence of toxic effects or macroscopic alterations of internal organs; the only clinical sign observed was piloerection, which started 2 hours after treatment and disappeared within 48 hours. Body weight did not vary. The absence of adverse effects at so high concentrations did not allow the calculation of the LD_{50} value.

Diverse toxicity studies were performed using aqueous olive-pulp extracts in which 3,4-DHE content ranged between 50-70% of phenols total quantity.[29] In another study, oral administration to rats of a single dose of solid olive-pulp extract from 0 to 2 g/kg caused no adverse effect , except for soft feces.[30] At a dosage of 2 g/kg×day of this extract, an absence of acute toxicity was found,

without either teratogenic or mutagenic effects. High doses of this extract (5 g/kg×day for 29 consecutive days) did not induce mortality or other clinical signs, suggesting that the extract is practically non-toxic.

2.5. Hydroxytyrosol and cardiovascular desease

2.5.1. Antiatherogenic capacity and cardioprotective effects

Oxidative modification of Low Density Lipoproteins (LDL) plays a key role in the development of atherosclerosis, so that they are considered as biomarker of cardiovascular deseases. In numerous studies 3,4-DHE demonstrated to have antiatherogenic properties with strong antioxidant power, scavenging free radicals and chelating metals as iron. *In vitro* 3,4-DHE prevents oxidation of LDL by macrophages, since it increases the antioxidant capacity of these cells to low glutathione levels.[31] Postprandial lipemia is a risk factor for atherosclerosis and it has been reported that 3,4-DHE promotes hypocholesterolemia *in vivo*, lowering the plasma levels of LDL and total cholesterol in rats fed on lipid-rich diet. Furthermore, it enables a lowering of triglycerides while raising high-density lipoprotein levels and the antioxidant capacity, thereby reducing LDL oxidation.[32]

The antiatherogenic effect of 3,4-DHE is also associated to the lowering of expression of cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in *in vitro* endothelial cells.[33] This reduction is due to the inactivation by 3,4-DHE of NF-κB, activator protein-1 (Figure 2a).[34]

Again, in vascular endothelial cells, 3,4-DHE enhances cell proliferation and confers cytoprotection from H_2O_2-induced oxidative stress dependent on Nrf2, a nuclear factor regulating the antioxidant enzyme expression.[35]

Despite this studies, a work[36] in which APO-E knock-out mice (a mice model spontaneously developing atherosclerosis) were fed with a regular low-fat/ low-cholesterol diet and an aqueous solution of 5 mg/kg 3,4-DHE, demonstrates an increase in atherosclerotic lesion development. The authors sustain that natural compounds, out of the original matrix, could have detrimental effects.

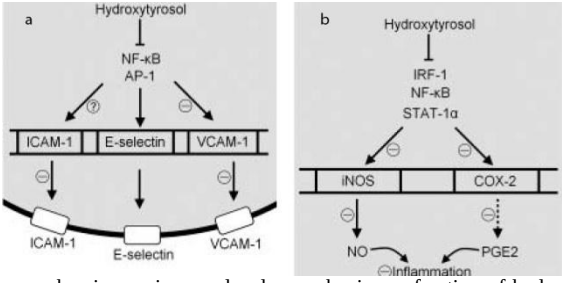


Figure 2. Pathways showing various molecular mechanisms of action of hydroxytyrosol. a) It has been shown that treatment of vascular endothelial cells with hydroxytyrosol can result in the decreased expression of the adhesion molecules ICAM-1 and VCAM-1. In the case of VCAM-1, it has been shown that the decreased mRNA levels are a result of the action of hydroxytyrosol on the transcription factors NF-*κ*B and AP-1. This actions prevent the adhesion of macrophages to the vascular endothelium. b) The treatment of macrophages with hydroxytyrosol has been shown to prevent inflammatory processes. This occurs as a result of the suppression of the transcription factors IRF-1, NF-*κ*B, and STAT-1*α*, which leads to reduced expression of iNOS and COX-2. Image from Rafehi et al. 2012[18]

2.5.2. Anti-platelet aggregation and anti-inflammatory effects

Platelet aggregation often accompanies and aggravates cardiovascular disease, and its tight regulation is an essential requisite for intact vessel physiology. Platelet function is regulated by a number of physiological activators (thromboxane A2, vasopressin, ADP, thrombin, serotonin) and inhibitors (endothelium-derived relaxing factor, prostaglandin-inhibitor-2). Platelet antagonists inhibit platelet function by increasing intracellular level of cyclic nucleotides cAMP and cGMP through the activation of the respective cyclases. Cyclic nucleotide levels are down regulated by degradation through phosphodiesterases (PDE). Therefore platelet PDE inhibition is reasonably considered a therapeutic tool to vascular disease. Indeed PDE inhibitors are currently used as anti-aggregating agents.[37]

Many literature articles can be found concerning the reduction of platelet aggregation *in vitro* by olive oil phenols, including hydroxytyrosol. It's reported that 3,4-DHE diminishes the synthesis of thromboxane A2 and its metabolite thromboxane B2, by inhibition of the enzyme cyclooxygenase.[38] More, it's indicated that 3,4-DHE inhibits the PDE.[17]

During inflammation process, large amounts of pro-inflammatory mediators such as nitric oxide and prostaglandin E_2 are generated by iNOS and COX-2. Hydroxytyrosol has been shown to display anti-inflammatory properties *in vitro*. In various studies using macrophages, treatement with hydroxytyrosol resulted in the inhibition of expression of two pro-inflammatory genes, inducible nitric oxide synthase (iNOS) and COX-2. This was found to be due to the prevention of activation of the transcription factors NF-κB, STAT-1α and IRF-1 (Figure 2b)[39,40,41,42] Moreover, 3,4-DHE encourages the action of

immune system cells protecting neutrophils from H_2O_2 induced oxidative-stress. It protects DNA of mononuclear blood cells and peripheral monocytes of Alzheimer's patients from genotoxic damage caused by free radicals.[17]

In rats 3,4-DHE reduces the degree of inflammation associated to post-menopause osteoporosis; again, in rats treated with carrageenan (acute inflammation inducer), the administration of a preparation constituted by 3,4-DHE 22%, polyphenol 4%, saccharide 67% and other components, significantly inhibit both the acute inflammation and the pain due to carrageenan.[43]

2.6. Hydroxytyrosol and cancer

The antitumor effect of 3,4-DHE has been studied as a result of its capacity to inhibit proliferation and promote apoptosis in several malignant cell lines by diverse mechanisms, in addition to its ability to be chemopreventive with its high antioxidant activity.

3,4-DHE shows antiproliferative effects on promyelocytic leukemia cells (HL60) and human peripheral blood mononuclear cells (PBMC) by reducing H_2O_2-induced DNA damage.[44] On HL60, human breast cancer MCF-7,[45] colon cancer cells CaCo2[46] and HT29,[47] treatment with 3,4-DHE resulted in the reduction in cell viability as a result of blockage of the cellular cycle in the G_0/G_1 phase and increased apoptosis.

3,4-DHE also shows an inhibitory action on the damage that ultraviolet radiation inflicts on both melanoma cells and HepG2.[48]

Importantly, all these effect have been reported in tumor cell lines, whereas in normal cells as vascular endothelial cells, limphocytes and polymorphonuclear cells 3,4-DHE shows no toxicity or a proliferative effect.[35]

Anyway, all the data collected comes from *in vitro* studies, *in vivo* studies are still not presented.

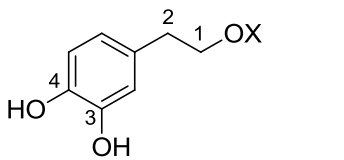
3. Hydroxytyrosyl esters

As for the radical scavenging and biological properties above, hydroxytyrosol seems to be an antioxidant safe enough for industry preparation, and in effect it has already been used as an additive in tomato juice and fish products.[49]

Nevertheless, easy incorporation of hydroxutyrosol as an additive in food and cosmetics, as oil matrices, is still a challenge. The hydrophilic character stands as a serious disadvantage, first of all for the difficulties in extracting it

from aqueous solutions, then for the reduction of its effectiveness in stabilizing fats and oils. Therefore, more lipophilic hydroxytyrosol derivatives could promote hydroxytyrosol's properties with respect to the dispersion medium. A lipophilic antioxidant could locate in a different area (respect to 3,4-DHE) of the matrix in which it is added, and consequently its effect on lipid oxidation may result in being increased trough a major accumulation at the oxidative sensible interfaces.[50] A common working hypothesis for antioxidant activity is the "antioxidant polar paradox" formulated by Porter[51] in 1989: hydrophilic antioxidants are more effective in bulk oils, whereas lipophilic antioxidants are more effective in systems of high surface/volume ratio, such as emulsions, micelles, or membranes. This behavior is explained by the concept of interfacial oxidation. In oil-in-water emulsions, lipophilic antioxidants would have more affinity for the oil-water interface and, therefore, would inhibit lipid oxidation more efficiently. In contrast, polar antioxidants would readily partition into the aqueous phase decreasing their concentration in the lipid part and thus lowering their capability to prevent oxidation. In bulk oils polar antioxidants would concentrate into the air-oil interface and would be more efficient in this type of matrix. However, the effectiveness of antioxidants in oil-in-water emulsions could also be dependent on their surface activity and ability to accumulate at the oil-water interface where oxidative reactions are prevalent.[52] By this way, a good antioxidant in oil-in-water emulsions should be, at the same time, an excellent radical scavenger and an effective surfactant.[53]

These considerations trigger off the development of a suitable esterification approach to obtain high-yielding lipophilic hydroxytyrosyl derivatives. In the last decade many chemicals[54,55,56,57] or enzymatic[52,58,59] methods to synthesize esters and ethers have been reported (Figure 2).



esters X=COR or eters X= R

Figure 2. Hydroxytyrosyl lipophilic derivatives

These derivatives have been evaluated as for their antioxidant activity. The first thing to notice, is that any derivatization (methylation, esterification) of the catecholic moiety leads to not active scavengers, confirming the hypothesis that the orthodiphenolic group is fundamental for the antioxidant activity, due to its

role in stabilization of the phenolic radical through hydrogen bond. The suitable place for derivatization is the primary alcohol group.

The hydroxytyrosyl fatty acids esters, accordingly with the polar paradox theory, registered the greatest activities in oil-in-water emulsions, rather than in bulk oils. They resulted even more active than the commonly used food antioxidants, such as butylated hydroxytoluene (BHT) and ascorbyl palmitate.[60]

As a matter of fact, hydroxytyrosyl fatty acid derivatives have an amphiphilic nature due to the presence of the orthodiphenolic moiety on one side and the alkyl chain on the other side and they behave as surfactants. As stated above, surface-activity impacts positively on antioxidant efficacy, in particular for new potential applications in food, pharmaceutical or personal-care industries.

Biological evaluation of these compounds is still scarce. Hydroxytyrosyl esters were studied for their capacity to protect biomolecules against oxidation damage. Proteins and lipids[54]54 damage against cumene hydroperoxide was established using brain homogenate as an *ex vivo* model particularly vulnerable to oxidation. In both cases the most protective esters resulted the longest chain ones, hydroxytyrosyl oleate and linoleate. On the other hand, assessment of DNA damage on whole blood cells[59] indicated that short chain esters (acetate to butyrate) were more effective in protecting cells against hydrogen peroxide insult.

More recent work has been conducted on hydroxytyrosyl ethers. Short chain ethers uptake and metabolism have been followed on HepG2 cells (as model system of human liver) within 18 hours.[61] The authors found a slow cellular uptake and metabolism, with a direct relationship between the lipophilic nature of each compound and the higher level of metabolization. The pathway suggested includes primarily methylation together with glucuronidation, as seen for 3,4-DHE. On the other hand, no traces of free 3,4-DHE were found, indicating that HepG2 enzymes did not hydrolyze the ether bond. The same authors verified the HepG2 protection against oxidative stress in presence of hydroxytyrosyl ethers.[62]

In human volunteers plasma, hydroxytyrosyl linoleyl ether showed an activity similar to its parental hydroxytyrosol in protecting LDL against oxidation by cupric sulfate, and it presented an acute food intake modulation profile (comparable to that of oleoylethanolamine) on 24h food-deprived rats (hypophagic effect).[63]

On the basis of the collected data, bearing in mind the necessity to find new more suitable food antioxidants lacking of side effects, it's clear that hydroxytyrosyl derivatives are good candidates as for their chemical properties, but still few information is present about their biological effects. In one of the laboratories where I've carried out my Ph. D. project, a convenient chemical way to synthesize hydroxytyrosyl esters has been developed.[64] This method permits the regio-specific esterification of the primary alcohol group by

preventively protecting the orthodiphenolic moiety as methyl orthoester. Then the easy reaction of esterification occurs. Protected hydroxytyrosyl esters are stable to air and light and can be stored for months. Afterwards, a mild diphenolic group deprotection is achieved in order to obtain the free antioxidant derivatives (Results, Figure 1 and 2).

AIM OF THE WORK

Derivatization of hydroxytyrosol demonstrated to be a fine tool to increase its solubility in lipophilic preparations. Several studies have highlighted that hydroxytyrosol and its derivatives possess antioxidant properties. Despite the potentiality due to the lipophilization of hydroxytyrosol, a systematic study of the structure-activity relationship is still lacking. An accurate analysis of the influence of structural modifications on the antioxidant activity of this small molecule may help to better understand the basic aspects of the behavior of lipophilic phenolic antioxidants. It may bring worthy information in order to build up efficient antioxidants for industrial employment. For this reason the aim of this present PhD project is to explore the structure-activity relationship of four complete series of lipophifilic hydroxytyrosyl derivatives. The project is divided into three parts.

1. Chemical synthesis of the four series of compounds, obtained by:
    - esterifying the alcoholic moiety in 3,4-DHE with a complete series of fatty acids of increasing length of the acyl chain (from C2 to C18 linear chains, and the unsaturated C18Δ9 oleate)
    - preparing the same series of fatty acids esters for an 3,4-DHE isomer obtained by shifting the position of the catecholic hydroxyls in the aromatic ring from 3,4 (*ortho-meta*) to 2,3 (*meta-para*)
    - preparing the same series of fatty acids esters for the superior homologue of hydroxytyrosol obtained by modifying the length of the hydroxytyrosol side alcoholic chain from C2 to C3.
    - pooling together the two previous points, to achieve a new cathecol analogue to hydroxytyrosol and, consequently, its esters.
2. Determination of structure-activity relationships of these novel catechols, with the aim to study the influence of the ester chain length and of the structural modifications of 3,4-DHE on the ability to scavenge free radicals. Antioxidant activity can be measured both by *in vitro* ABTS assay and by in *in vitro* cultured cells H_2DCF assay. To avoid misleading results due to different solubility of the tested compounds, oxidation studies by ABTS chemical assay are performed in ethanol. As for the evidence of antioxidant activity of hydroxytyrosol in *in vitro* cultured cells and *in vivo*, all derivatives are evaluated for their antioxidant properties in a rat muscle model by fluorescent probe H_2DCF.
3. Study the influence of the presence of a bilayer on antioxidant activity by a simple model membrane.

RESULTS

1. Chemical synthesis of hydroxytyrosol and its esters.

The synthesis of hydroxytyrosol, protected as methylorthoformiate, started from commercial 3,4-dihydroxyphenylacetic acid. The protection pathway (Figure 1) has a fundamental role for the subsequent selective esterification. In fact, if the catechol moiety were free, the esterification would involve the alcohol group as well as the catechol moiety. The intermediate substrates and protected hydroxytyrosol were obtained in high yields. We choose to protect the phenolic hydroxyls as orthoesters due to the easy removal of this kind of protection under very mild conditions.

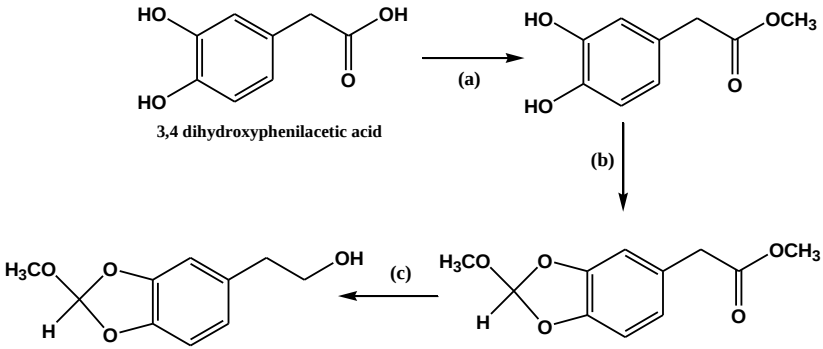


Figure 1. Synthesis of protected hydroxytyrosol from 3,4-dihydroxyphenylacetic acid. (a): substrate: 3,4-dihydroxyphenylacetic acid; reagent: H_2SO_4; solvent: methanol (98 % yield). (b): substrate: 3,4-dihydroxyphenylacetic methyl ester; reagent: trimetylorthoformiate; catalyst: amberlyst 15; solvent: benzene(70 % yield after purification). (c): substrate: 3,4-dihydroxyphenylacetic methyl esterorthoformiate; reagent: $LiAlH_4$; solvent:tetrahydrofuran (97 % yield).

Protected hydroxytyrosol is a stable compound, that can be stored for long periods at -20°C. It permits the selective esterification of the primary alcohol (Figure 2a), followed by a mild deprotection at buffered pH (Figure 2b). This pH condition (about 7.2) allows the detachment of the methylorthoformiate and prevents the undesirable hydrolysis of the fatty chain ester. Eleven hydroxytyrosyl esters, with C2, C4, C6, C7, C8, C9, C10, C12, C16, C18 and C18Δ9 aliphatic chain, have been synthesised by this protocol (Table 1) . Even with the free catechol moiety, the esters result more stable to air and light than hydroxytyrosol itself and, at -20°C, they can be stored for a few weeks without degradation.

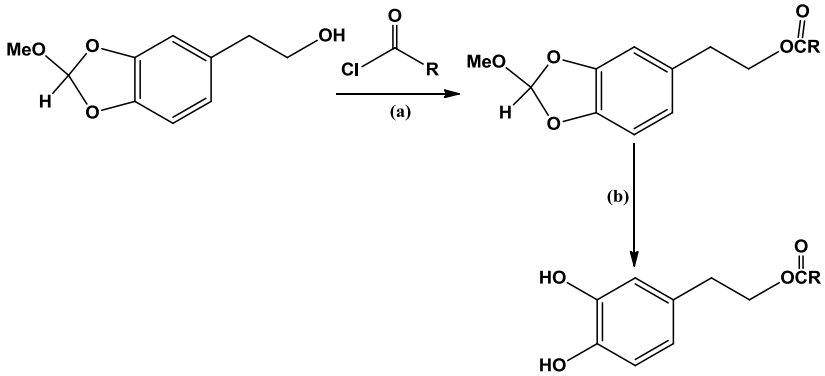


Figure 2. Synthesis of hydroxytyrosyl esters. (a): substrate: trimetylorthoformiate hydroxytyrosol; reagents: acyl chorides of fatty acids, pyridine; solvent: tetrahydrofuran. The yields of the substrates range between 92 and 97 %, and there is no necessity of purification. (b): substrate: fatty acid esters of hydroxytyrosol trimethyl orthoformiate; reagent: methanol; catalyst: amberlyst 15 in phosphate buffer; solvent: tetrahydrofuran. The yields of the substrates range between 82 and 95 % after purification. R (the alkyl substituent) and its correspondent name of the ester are indicated in Table 1.

Table 1. Alkil chain of the synthesized esters.

R	compound	R	compound	R	compound
CH_3	acetate	C_7H_{15}	octanoate	$C_{15}H_{31}$	palmitate
C_3H_7	butyrate	C_8H_{17}	nonanoate	$C_{17}H_{35}$	stearate
C_5H_{11}	hexanoate	C_9H_{19}	decanoate	$C_{17}H_{33}$	oleate
C_6H_{13}	heptanoate	$C_{11}H_{23}$	laurate		

2. ***Chemical structure of hydroxytyrosol and hydroxytyrosyl esters homologues and analogues.***

The part of chemical synthesis that follows is planned to study how small intrinsic modifications of the hydroxytyrosol structure can affect its activity. For this reason, three hydroxytyrosol homologues or analogues have been synthesized in collaboration with the chemistry laboratory of La Tuscia University, Viterbo (Figure 3). Namely, with respect to hydroxytyrosol and its esters (3,4-DHE), the 3-(3,4-dihydroxyphenyl)propanol (3,4-DHP) derivatives are higher homologues; 2-(2,3-dihydroxyphenyl)ethanol (2,3-DHE) derivatives show a different substitution pattern of the catechol moiety and 3-(2,3-dihydroxyphenyl)propanol (2,3-DHP) derivatives exhibit a combination of the two previous structural modifications.[65]

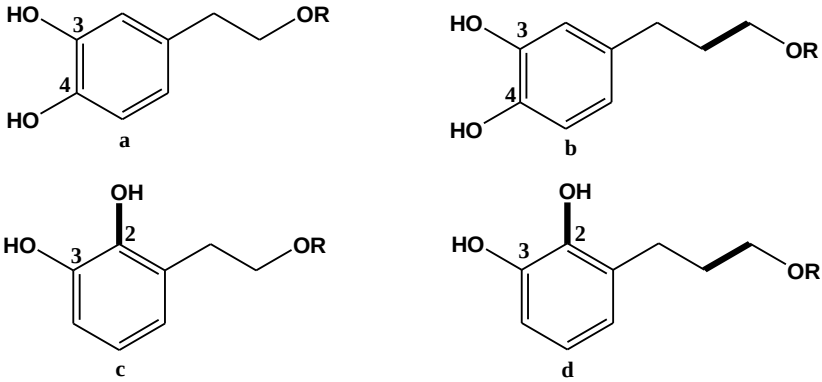


Figure 3. . Hydroxytyrosol and its sintesized homologues and analogues.
(a) R = H, Hydroxytyrosol 2-(3,4-dihydroxyphenyl)ethanol, here named 3,4-DHE.
R = COR', 3,4-DHE esters.
(b) R = H, 3(3,4-dihydroxyphenyl)propanol, here named 3,4-DHP.
R = COR', 3,4-DHP esters.
(c) R = H, 2-(2,3-dihydroxyphenyl)ethanol, here named 2,3-DHE.
R = COR', 2,3-DHE esters.
(d) R = H, 3-(2,3-dihydroxyphenyl)propanol, here named 2,3-DHP.
R = COR', 2,3-DHP esters.

3. Antioxidant activity of 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3-DHP and their esters by ABTS assay

The antioxidant capacities of the four sets of compounds were measured by a widespread chemical assay, namely 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS assay.[66] The analyses were performed in alcoholic (0.2% of water) environment and, only for 3,4 DHE and its esters, in EtOH/refined olive oil (2% of olive oil), measuring the variation of absorbance of $ABTS^{\cdot+}$ radical cation at 734 nm by a spectrophotometer.

All the compounds were used directly after their deprotection and purification to avoid oxidation of the samples. The reaction between the antioxidant and the radical $ABTS^{\cdot+}$ is very fast, both in ethanol and in EtOH/refined oil. After two minutes it reaches the equilibrium (Figure 4).

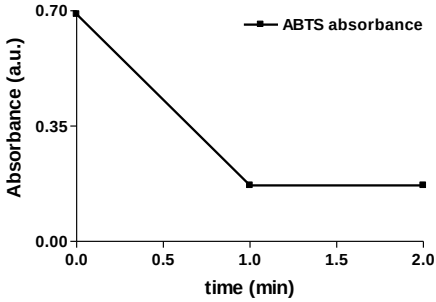


Figure 4. Representative graph of the reaction between antioxidant and $ABTS^{\cdot+}$: variation of $ABTS^{\cdot+}$ absorbance; t=0: $ABTS^{\cdot+}$ ~ 20 μM aqueous solution + 3,4-DHE-octanoate 10 μM absorbance; t=1 min: $ABTS^{\cdot+}$ absorbance after 60 seconds reaction with antioxidant. The absorbance decreases within the first minute, after which the measure stabilizes. All the antioxidants behaved the same way.

Each compound has been tested for its ability to quench ABTS radical cation at four concentrations, in order to get a linear fit for its antioxidant capacity (Figure 5 and Table 2). 6-Hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox) was used as reference compound (it is the hydrophilic analogue of vitamin E), and the measured antioxidant capacities, either in ethanol and in ethanol/refined olive oil solution, were reported as Trolox Equivalent Antioxidant Capacity (TEAC) defined as the concentration (mmol/L) of trolox having the equivalent antioxidant activity to a 1mmol/L solution of the substance under investigation (Figure 6).

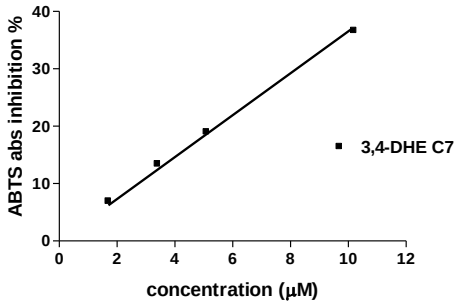


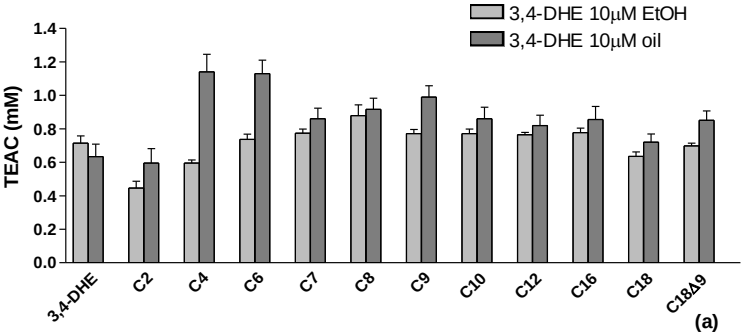
Figure 5. Linear fit of Hydroxytyrosyl eptanoate. The antioxidant capacity is represented as percentage of absorbance decrease (% of $ABTS^{\cdot+}$ inhibition) versus substrate concentrations (in the range of 0-15 μM). Each value represents the mean ± S.D. of 4 different measurements at 4 concentrations in 4 independent experiments. Collected data showed S.D.s always below 3%. Linear regression was elaborated for trolox, 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3-DHP and all their esters. Linear regression was elaborated using Microcal Origin 5.0 software.

Table 2. Slopes elaborated by linear regression of ABTS assay results for trolox, 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3-DHP and all their esters.

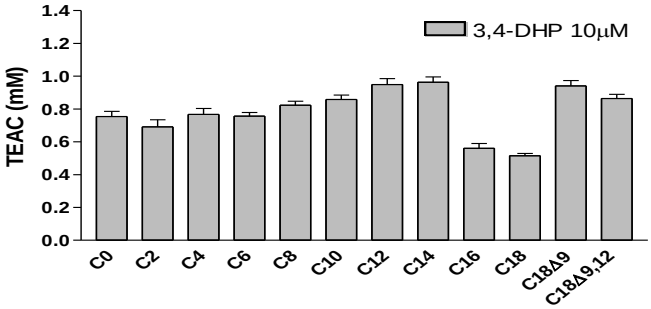
EtOH			EtOH/oil		
compound	**slope**	**R^2**	**compound**	**slope**	**R^2**
trolox	5.04±0.07	0.995	**trolox**	5.00±0.25	0.977
3,4- DHE	3.60±0.17	0.984	**3,4-DHE**	3.17±0.21	0.962
C2	2.25±0.17	0.957	**C2**	2.98±0.28	0.921
C4	3.00±0.06	0.996	**C4**	5.70±0.24	0.986
C6	3.71±0.11	0.992	**C6**	5.45±0.12	0.996
C7	3.90±0.07	0.996	**C7**	4.50±0.10	0.992
C8	4.43±0.26	0.950	**C8**	4.58±0.11	0.978
C9	3.89±0.07	0.991	**C9**	4.95±0.09	0.997
C10	3.89±.026	0.993	**C10**	4.30±0.13	0.987
C12	3.95±0.02	0.999	**C12**	4.10±0.10	0.995
C16	3.92±0.11	0.995	**C16**	4.28±0.18	0.986
C18	3.20±0.09	0.994	**C18**	3.60±0.06	0.998
C18Δ9	3.52±0.03	0.999	**C18Δ9**	4.26±0.07	0.999

EtOH			EtOH		
compound	**slope**	**R^2**	**compound**	**Slope**	**R^2**
3,4-DHP	3.80±0.11	0.998	**2,3-DHE**	2.91±0.13	0.984
C2	3.48±0.17	0.971	**C2**	3.50±0.11	0.983
C4	3.87±0.13	0.983	**C4**	2.72±0.08	0.986
C6	3.81±0.06	0.997	**C6**	2.49±0.03	0.999
C8	4.15±0.06	0.999	**C8**	7.05±0.23	0.997
C10	4.32±0.08	0.998	**C10**	4.68±0.10	0.994
C12	4.78±0.11	0.998	**C12**	4.30±0.12	0.995
C14	4.85±0.10	0.999	**C14**	3.92±0.07	0.998
C16	2.83±0.11	0.964	**C16**	3.34±0.19	0.957
C18	2.59±0.03	0.998	**C18**	2.90±0.11	1.000
C18 Δ9	4.74±0.10	0.996	**C18 Δ9**	3.31±0.10	0.000
C18Δ9,12	4.35±0.06	0.998	**C18Δ9,12**	3.20±0.06	0.994

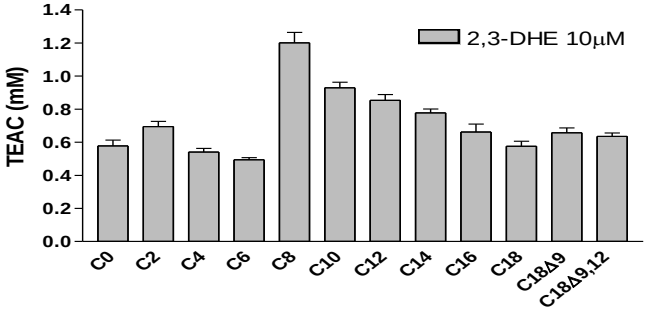
EtOH			EtOH		
compound	**slope**	**R^2**	**compound**	**slope**	**R^2**
2,3-DHP	4.11±0.17	0.985	**C12**	3.67±0.04	0.998
C2	5.54±0.19	0.985	**C14**	4.68±0.10	0.991
C4	4.21±0.06	0.999	**C16**	2.91±0.12	1.000
C6	4.39±0.12	0.985	**C18**	3.21±0.09	0.984
C8	4.76±0.13	0.989	**C18 Δ9**	3.59±0.05	0.999
C10	4.76±0.12	0.995	**C18Δ9,12**	3.26±0.13	1.000


3,4-DHE 10μM EtOH
3,4-DHE 10μM oil
TEAC (mM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3,4-DHE
C2
C4
C6
C7
C8
C9
C10
C12
C16
C18
C18Δ9

(a)


3,4-DHP 10μM
TEAC (mM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C0
C2
C4
C6
C8
C10
C12
C14
C16
C18
C18Δ9
C18Δ9,12

(b)


2,3-DHE 10μM
TEAC (mM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C0
C2
C4
C6
C8
C10
C12
C14
C16
C18
C18Δ9
C18Δ9,12

(c)

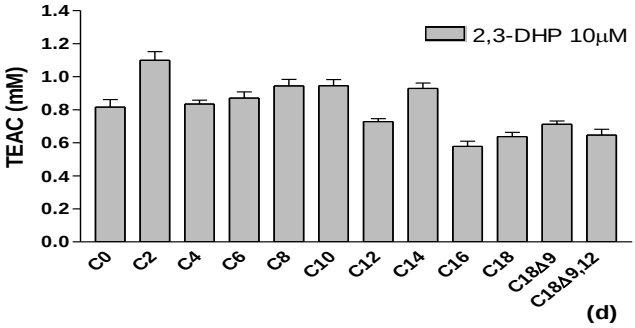


Figure 6. TEAC (Trolox Equivalent Antioxidant Capacity) of the ABTS results for 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3-DHP and their esters. Antioxidant capacity is reported as mmoles of trolox equivalents.
(a): TEAC of 3,4-DHE series either in EtOH and in EtOH/refined oil solution.
(b): TEAC of 3,4-DHP series in EtOH.
(c): TEAC of 2,3-DHE series in EtOH.
(d): TEAC of 2,3-DHP series in EtOH.

All tested esters show a nearly constant antioxidant capacity, comparable or higher than that of hydroxytyrosol itself. In particular, all hydroxytyrosyl esters show a slightly better antioxidant capacity when analyzed in presence of very small amounts (around 2%) of refined olive oil in the ABTS solution. The better values are shown by medium sized acyl chains both in ethanol solution andin EtOH/refined olive oil. Log P for the four sets of tested molecules was calculated, and it was used to analyze the obtained data. This attempt to correlate the increase of lipophilicity, expressed as log P (Table 3), with their antioxidant capacity, failed.

Table 3. Partition coefficient values (LogP) of 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3-DHP and their esters.

Catechol	Log P	Catechol	Log P
3,4- DHE and 2,3-DHE	0.794	**3,4-DHP and 2,3-DHP**	1.250
C2	1.387	**C2**	1.843
C4	2.299	**C4**	2.755
C6	3.211	**C6**	3.667
C7	3.617		
C8	4.123	**C8**	4.579
C9	4.680		
C10	5.035	**C10**	5.491
C12	5.947	**C12**	6.017
C14	6.434	**C14**	6.851
C16	7.269	**C16**	7.686
C18	8.103	**C18**	8.520
C18Δ9	7.784	**C18Δ9**	8.201
C18Δ9,12	7.465	**C18Δ9,12**	7.882

Partition Coefficient Values (Log P) were calculated by ACD Chem Sketch Software V12.01 (© 1994-2009 ACD/Lab)

Trying to find an explanation for the reduction of activity in long chain esters, 3,4-DHE stearate (C_{18}) was chosen as an example of long extension ester and analysed by molecular dynamics. The GROMACS method, which utilizes an RMSD cut-off to count number of neighbours in a cluster, was applied. A RMSD cut-off of 0.3 nm was used. Six clusters of conformations were obtained with the first two most representative clusters containing 2,373 and 463 structures, respectively[67] (Figure 7).

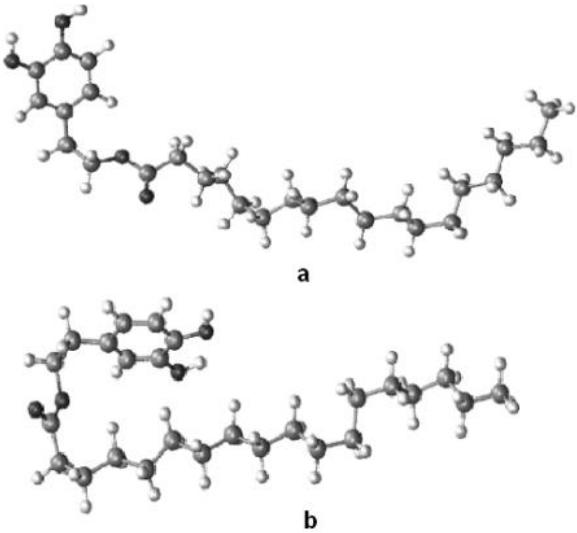


Figure 7. Molecular dynamics of 3,4-DHE stearate in EtOH solution. Six clusters of conformations were obtained. Here the first two most representative clusters containing 2,373 (a) and 463 (b) structures are represented.

The calculations were done supposing the molecule was in an ethanol solution, thus reproducing the environment of the ABTS assay.

Comparisons among the four sets of compounds, which will be argued in the discussions, where facilitated by the analysis of the possible formation of a hydrogen bond between the acyl oxygen and the nearest catechol hydroxyl: these distances were calculated after MM2 minimizations using CS Chem3D Ultra, 2001 Cambridge Soft Corp., in the four acetate esters, chosen as representative of each series (Figure 8).

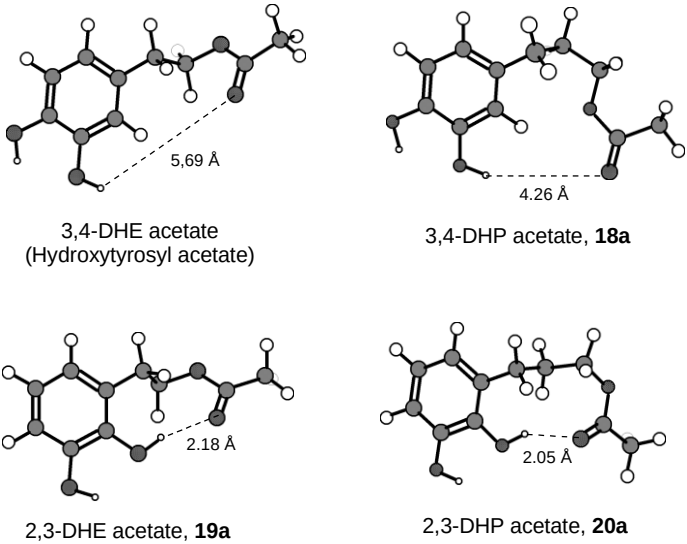


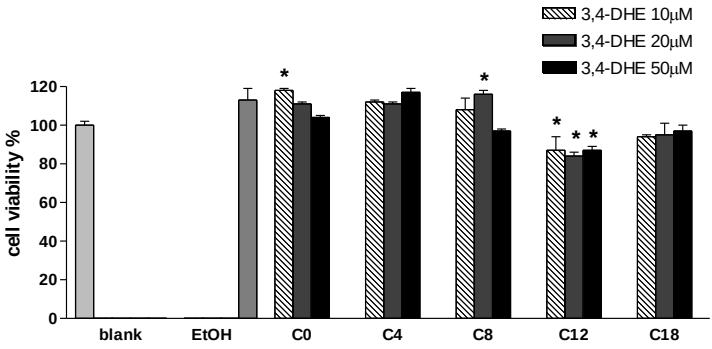
Figure 8. Calculated distances between the acyl oxygen and the nearest catechol hydroxyl after MM2 minimizations in 3,4-DHE acetate, 3,4-DHP acetate, 2,3-DHE acetate, and 2,3-DHE acetate. The distances between the acyl oxygen and the nearest catechol hydroxyl in hydroxytyrosyl acetate and acetates 3,4-DHP, 2,3-DHE and 2,3-DHP were calculated after MM2 minimizations using *CS* Chem3D Ultra®, ©2001 Cambridge Soft Corporation.[65]

The molecular mechanics minimizations permit to verify the possibility of intramolecular hydrogen bond formation, which is important to characterize both the molecular stability and its radical scavenger activity.[65]

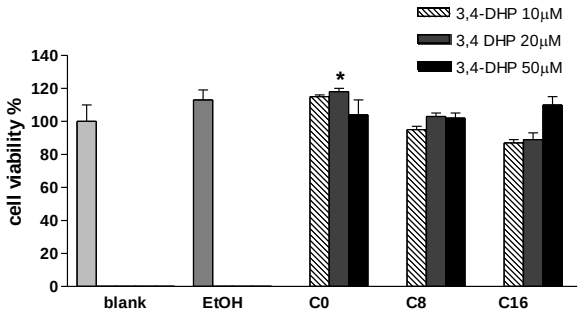
The antioxidant ability of the compounds was tested by biological assay, too. Cultured cells from rat skeletal muscle where chosen, and primarily the cytotoxicity of the molecules against these cells was estimated.

4. Cytotoxicity and antioxidant activity of 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3 DHP and their esters on L6 cells.

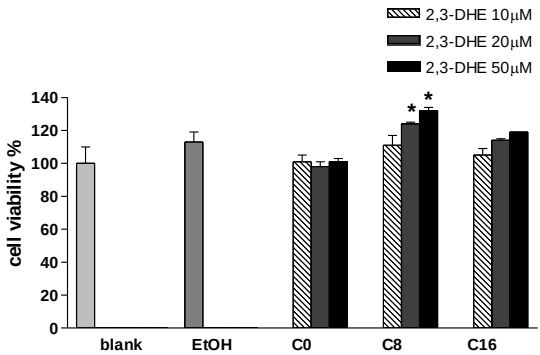
To examine the cytotoxicity of the four compounds sets, some of them were chosen as representative for cell survival assessment by MTT assay.[68] For this reason, 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3 DHP (the parental molecules) and some of their esters, covering all the range of chain lengths, have been tested for their risk to affect L6 cells from rat skeletal muscle survival (Figure 9).[56]


3,4-DHE 10μM
3,4-DHE 20μM
3,4-DHE 50μM
cell viability %
0
20
40
60
80
100
120
blank
EtOH
C0
C4
C8
C12
C18
*
*
*
*
*

(a)


3,4-DHP 10μM
3,4 DHP 20μM
3,4-DHP 50μM
cell viability %
0
20
40
60
80
100
120
140
blank
EtOH
C0
C8
C16
*

(b)


2,3-DHE 10μM
2,3-DHE 20μM
2,3-DHE 50μM
cell viability %
0
20
40
60
80
100
120
140
blank
EtOH
C0
C8
C16
*
*

(c)

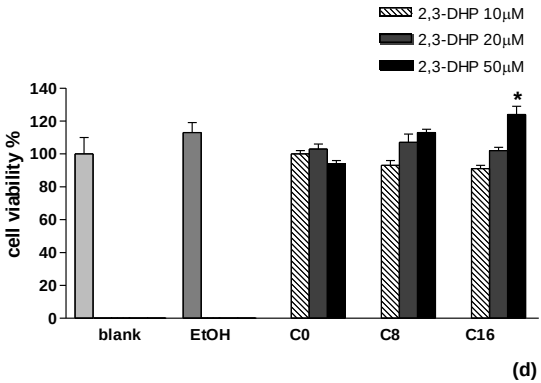
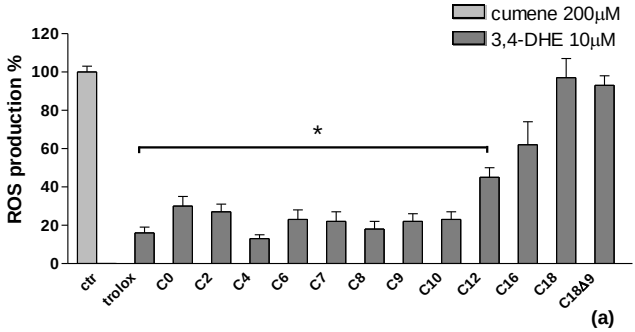


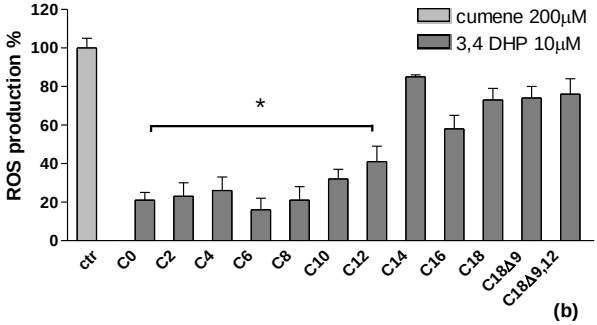
Figure 9. Cell survival assay of 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3-DHP and some of their esters on L6 cells. Effect of (a) hydroxytyrosol and its esters butyrate, octanoate, dodecanoate and stearate on cell L6 survival. Effect of (b) 3,4-DHP, (c) 2,3-DHE, (d) 2,3-DHP and their esters octanoate and palmitate on cell L6 survival. Cells were plated in 96-well for 24 h with nothing or ethanol (controls) or 10-20-50 μM antioxidants. At the end of the treatment, cells were subjected to MTT assay. Results are reported as mean ± S.D. of at least n = 3 independent experiments. *$p<0.05$ as from Bonferroni t-test with respect to the blank considered as 100%; in all the other cases data were not statistically different from the blank.

The compounds result not toxic for cells, even used at high concentrations (more than 80% of cell viability in all the tested compounds).

L6 rat skeletal muscle cells have been employed to evaluate the capacity of 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3-DHP and their linear esters to penetrate cell membrane and act as radical scavengers inside the cells by classical H_2DCF assay[69] (Figure 10).


cumene 200μM
3,4-DHE 10μM
ROS production %
120
100
80
60
40
20
0
*
ctr
trolox
C0
C2
C4
C6
C7
C8
C9
C10
C12
C16
C18
C18Δ9

(a)


cumene 200μM
3,4 DHP 10μM
ROS production %
120
100
80
60
40
20
0
*
ctr
C0
C2
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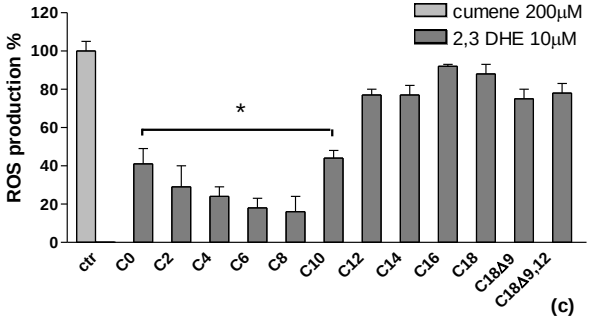
C6
C8
C10
C12
C14
C16
C18
C18Δ9
C18Δ9,12

(b)


cumene 200μM
2,3 DHE 10μM
ROS production %
120
100
80
60
40
20
0
*
ctr
C0
C2
C4
C6
C8
C10
C12
C14
C16
C18
C18Δ9
C18Δ9,12

(c)

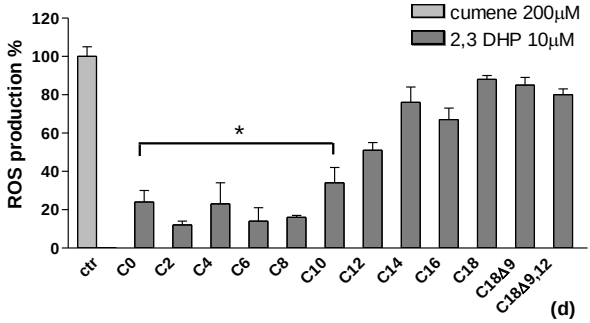


Figure 10. ROS production inhibition of 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3-DHP and their esters on L6 cells. ROS assay was carried out *via* fluorescent probe DCF. Cumene hydroperoxide 200 μM was used as radical generator to produce oxidative stress inside the L6 rat muscle. The control was untreated cells plus 200 μM cumene. The figure shows the ROS production induced by 200 μM cumene hydroperoxide in untreated (which antioxidants were not given) and 15 min 10 μM antioxidants pre-treated cells. Fluorescence was measured for the subsequent 10 min since cumene addiction. Results are reported as mean ± S.D. of at least $n = 3$ independent experiments. *$p < 0.005$ as from a Student's *t*-test with respect to cumeme hydroperoxide considered as 100%. a) cells pre-incubated with 3,4-DHE and its esters, then cumene hydroperoxide was added; b) cells pre-incubated with 3,4-DHP and its esters, then cumene hydroperoxide was added; c) cells pre-incubated with 2,3-DHE and its esters, then cumene hydroperoxide was added; d) cells pre-incubated with 2,3-DHP and its esters, then cumene hydroperoxide was added;

The results in Figure 10 show that the four series of compounds display a similar pattern of activity, irrespective of both alcohol chain length and the phenol hydroxyls position. Medium length chains (C2-C10) have antioxidant activity similar or higher than their parental compounds. On the other hand, a constant activity drop is observed for esters carrying C12 to C18 acyl chains.[56,57]

For the short-medium length esters, showing to be more efficient in contrasting oxidative stress, dose-response studies (Figure 11) and cytoprotective assay (Figure 12) were performed, choosing the set of 3,4-DHE esters.

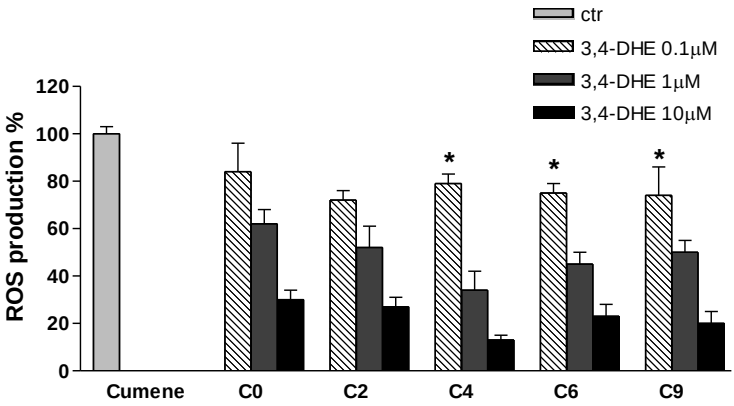


Figure 11. Dose-response experiments of short-medium chain 3,4-DHE esters on L6 cells. ROS assay was carried out via fluorescent probe DCF. Cumene hydroperoxide 200 μM was used as radical generator to produce oxidative stress inside the L6 rat muscle. The control was untreated cells plus 200 μM cumene. The figure shows the ROS production induced by 200 μM cumene hydroperoxide in untreated and 15 min 0.1–1–10μM antioxidants pre-treated cells. Fluorescence was measured for the subsequent 10 min since cumene addiction. Results are reported as mean ± S.D. of at least n = 3 independent experiments. *$p<0.05$ as from a Student's t-test with respect to cumeme hydroperoxide considered as 100%. All the tested compounds, at concentrations 1-10 μM, where significantly different from the control with $p<0.001$.

Results from dose-response experiments illustrate that 3,4-DHE and all tested esters maintain a good antioxidant activity at 1.0 μM concentration with a decrease of ROS production, with respect to cumene, ranging from 62% of hydroxytyrosol itself to 34% of 3,4-DHE butyrate. Even at 0.1 μM concentration the antioxidant activity of esters against cumene hydroperoxide is not negligible and better than 3,4-DHE itself.

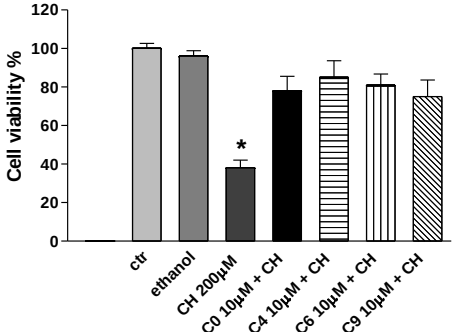


Figure 12. Evaluation of the cytoprotective effect of 3,4-DHE and its esters against oxidative stress induced by cumene hydroperoxide in L6 cells. Cytoprotective effect of 3,4-DHE and its esters butyrate, hexanoate and nonanoate from cumene hydroperoxide (CH) induced toxicity. Cells where pre-incubated with nothing (ctr), ethanol or 10 μM antioxidant for 10 minutes, then 200 μM cumene hydroperoxide was added and left for 10 minutes. The medium was discarded, fresh medium was added and cells where subjected to MTT assay. Results are reported as mean ± S.D. of at least *n*=3 independent experiments. *p<0.001 as from Bonferroni *t*-test with respect to the control considered as 100%; in all the other cases data were not statistically different from the control

As from figure 12, it results that short-medium sized esters effectively protect cells from cumene hydroperoxide induced toxicity.

5. ***Behavior of the antioxidants in aqueous solution***

As shown in the previous section (DCF assay on cultured cells), the antioxidant activity of the compounds under study strongly depends from their chain length. In particular, the relationship between acyl chain length and antioxidant activity in cells (Figure 10) follows a general and distinctive sigmoid curve, irrespective of both the alcohol side chain length and the phenol hydroxyls position. Short-chain antioxidants resulted to be more active than long-chain ones. With the aim of understanding the reason of this trend, four 3,4-DHE esters (butyrate, octanoate, laurate and stearate esters) were chosen as representative compounds and employed for further studies in aqueous environment.

Firstly, it was confirmed, by spectroscopic analysis, that the above mentioned esters are completely soluble in ethanol, as revealed by the absence of turbidity in their absorption spectra (data not shown). On the contrary, a turbidity signal is present when they are solubilized in phosphate buffered saline (PBS), indicating – not unexpectedly – that these hydroxytyrosyl derivatives self-associate to form supramolecular structures. In order to investigate the concentration dependency on the formation of aggregates, it was carried out the classical diphenylhexatriene (DPH) assay,[70] that allows the determination of the critical aggregation concentration (CAC), defined as the threshold concentration that must be overcome for the formation of aggregates. As shown in Figure 13 for some selected 3,4-DHE esters and as expected on the bases of the higher hydrophobicity of long polymethylene chains, the CAC values for the assessed compounds decreases as their chain length increases as well. By plotting the log CAC *vs* the number of carbon atoms of the ester chain it is obtained a straight line (Figure 14). This is in agreement with the general theory of surfactants aggregation in aqueous solutions as reported.[71] Thank to this linear dependence, we tried to estimate the CAC of butyrate and stearate

esters by an extrapolation procedure (for these two compounds it was not possible to directly measure their CAC). It turns out that plausible CAC values for such compounds are, respectively, 1500 and 5.5 μM.

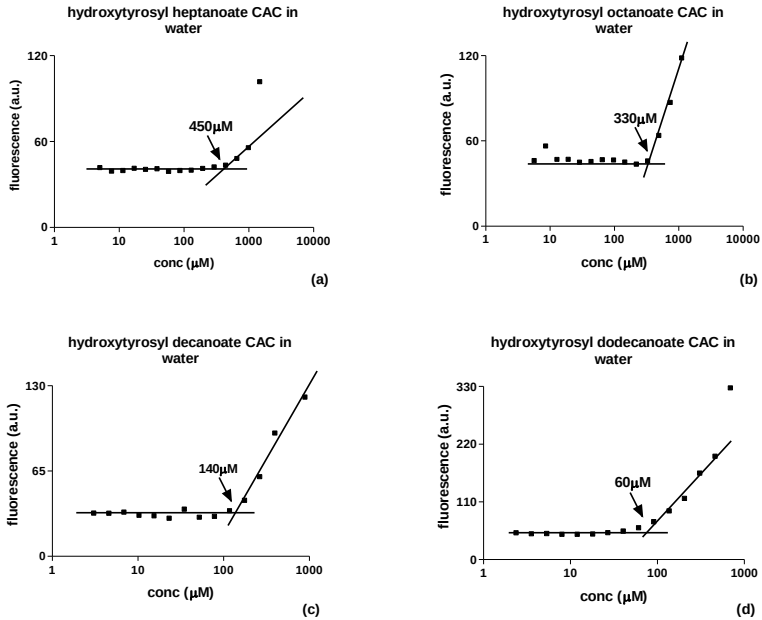


Figure 13. Determination of 3,4-DHE esters CAC by the DPH method: 3,4-DHE heptanoate (a), octanoate (b), decanoate (c) and dodecanoate (d). Samples were prepared by successive dilutions of an initial ~ 1mM concentrated solution of each compound in 1 μM DPH aqueous solution. Prior to each measurement, samples were left 30 min in repose to attain equilibrium. DPH fluorescence was measured at $\lambda_{emission}$=460 nm. DPH fluorescence is negligible in aqueous solution and markedly increases when DPH can bind to the hydrophobic aggregates formed by the antioxidants above their CAC.

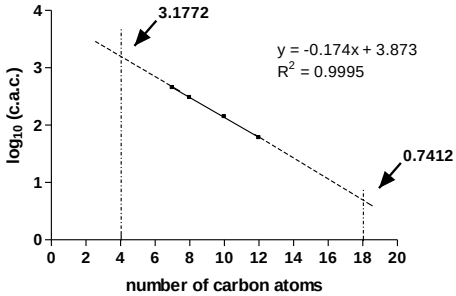


Figure 14. Log CAC *vs* number of carbon atoms of the antioxidant acyl chain. The four experimental points follows a straight line, which was extrapolated to estimate the butyrate and stearate esters CACs, namely log (3.1772) = 1503μM, and log (0.7412) = 5.5μM, respectively.

6. ***Studies with liposomal model system***

Phospholipid vesicles (liposomes) constituted of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) were used (*i*) for investigating the interaction of the 3,4-DHE esters with lipid membranes and (*ii*) as simplified cell models, to assess the dynamic of the antioxidant function on a reconstructed cell-like system.

6.1 Binding assay in liposomes.

Due to their hydrophobic tail, it is expected that 3,4-DHE esters interact with lipid membranes. The physical stability of POPC liposomes was firstly assessed.

Dynamic Light Scattering (DLS) analysis indicates that the liposome size (and therefore, their structure) is not affected in the presence of antioxidants (Figure 15).

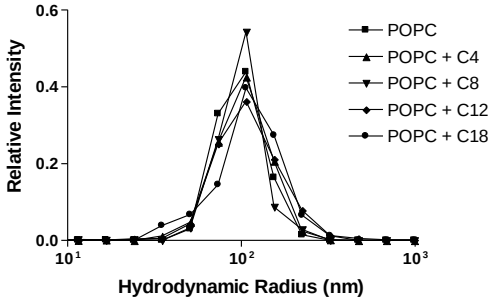
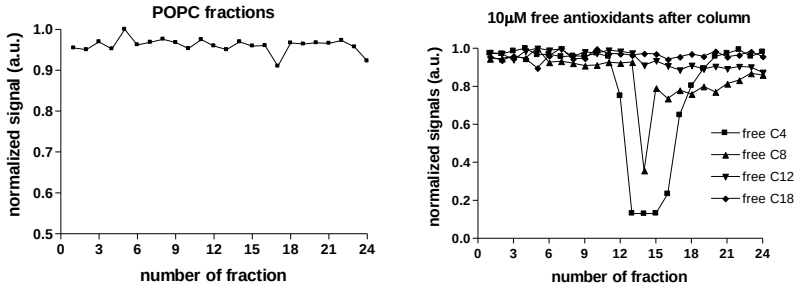


Figure 15. Size distribution, determined by DLS (90°) of 5mM POPC VET 100 (vesicles of 100 nm in diameter, prepared by the extrusion technique) in the presence of 10μM of 3,4-DHE esters.

Binding studies between vesicles and 3,4-DHE esters were carried out as it follows. An ethanol solution containing POPC and an antioxidant was used as a precursor to form liposomes by the "ethanol injection method". It is expected that the esters will bind (partially or quantitatively) to the lipid membrane, depending on their chain length. Free, unbound antioxidants (as monomers or as small aggregates) can be separated from antioxidant-containing liposomes by size exclusion chromatography (SEC), run on a Sepharose 4B minicolumn. Liposomes (size ca. 100 nm) are eluted first, small aggregates or free molecules later. The amount of antioxidant in each chromatographic fraction can be estimated by the ABTS classical assay, allowing the determination of the liposome-bound antioxidants (Figure 16).



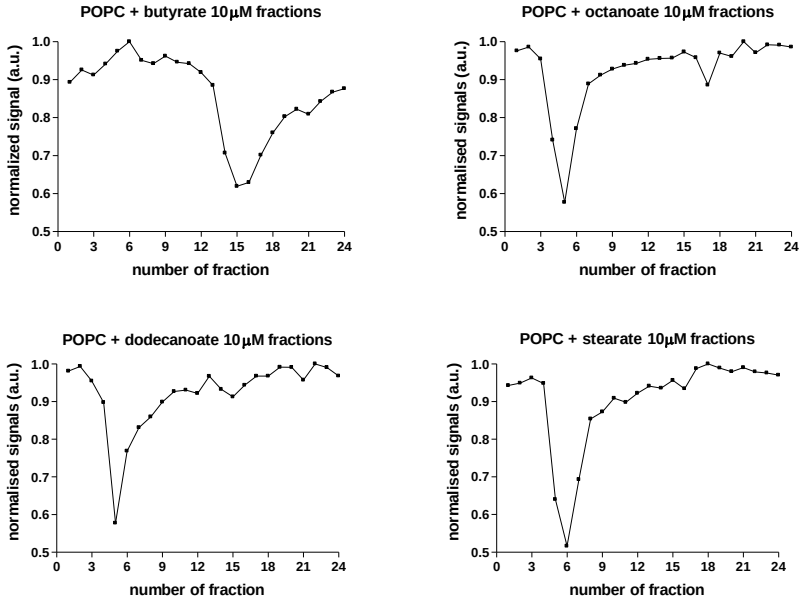


Figure 16. SEC profiles of POPC liposomes prepared in the presence of antioxidants. Mixed liposomes, composed of 5mM POPC and 10μM of each tested compound were prepared by the injection method. Liposomes were then separated through a Sepharose 4B gel column in PBS, and 24 fractions were collected. An aliquot of each fraction was added to an aqueous solution of 100 μM ABTS, used as a detector for antioxidant presence, and absorbance was read at 405 nm. POPC alone (a) and of 3,4-DHE esters alone (b) were used as control samples. (c), (d), (e) and (f) represent the fractions of POPC preincubated with butyrate, octanoate, dodecanoate and stearate esters, respectively. ABTS absorbance is expressed in arbitrary units.

As expected, POPC liposomes prepared in the absence of antioxidants, were undetectable by the ABTS assay. Turbidity measurements, however, confirm that they are eluted in the 4-7th fractions (data not shown). Free antioxidants, when used as control, were eluted in the 11-18th fractions. Long-chain C12 and C18 esters were not detected in any fraction, possibly due to the formation of large aggregates that cannot run into the SEC column. When liposomes were prepared in the presence of antioxidants, long-chain esters (C8, C12 and C18) bound to liposomes, as revealed by the appearance of an early peak in the chromatogram, whereas the short-chain ester (C4) can be found only as free (unbound) molecule. From the quantitative viewpoint, the comparison of the ABTS signal (peak area) reveal that in the case of long-chain esters the three compounds under study bind approximately with similar affinity to POPC membrane, although in the case of 3,4-DHE octyl ester a small amount (ca.

8%) is eluted as free molecule.

An independent experiment was carried out to confirm these observations. The four compounds were added at 10μM concentration to preformed POPC liposomes, prepared by the extrusion technique (diameter = 100 nm). After incubation, samples were ultrafiltered to separate free, unbound antioxidants from antioxidant-containing liposomes. The spectrophotometric analysis of the filtrates showed the presence of the 3,4-DHE butyric ester, whereas the other compounds were undetectable. Quantitative analysis revealed that 100% of the C4-ester was found in the filtrate (data not shown).

6.2 Antioxidant activity assays of four 3,4-DHE esters in vesicles.

With the aim of gaining information on the antioxidant role of 3,4-DHE esters, their effect on oxidative stress was assessed using liposomes as biomembrane models. The water soluble azocompound 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was used as radical generator. Primary radicals generate reactive oxygen species (ROS), so that the addition of AAPH well simulates the oxidative stress. The antioxidant activity of 3,4-DHE esters was evaluated using two fluorescent probes: hydrophobic DPH as membrane probe, and hydrophilic H_2DCF-DA as internal probe.

6.2.1 Evaluation of antioxidant activity with DPH probe

For the experiments in membrane, DPH-containing vesicles were prepared. DPH remains trapped in the vesicles bilayer and its spectrum can be detected by fluorescence (Figure 17).

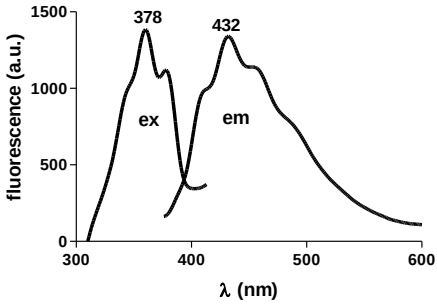


Figure 17. Fluorescence excitation (ex) and emission (em) spectra of DPH-containing vesicles (VET_{200})

The central idea of the assay is that DPH will function as a probe to reveal the oxidative events within the membrane, both due to lipid oxidation as well as DPH oxidation. In both cases it is expected that the DPH fluorescence intensity

will decrease, due to a variation of the membrane polarity and/or to the disruption of DPH fluorophore.[72] It should be mentioned that – in the same conditions – the DPH fluorescence polarization should increases.[73]

Radical generator AAPH was then added to vesicles, and the DPH fluorescence decay monitored in time. Moreover, the DPH fluorescence emission diminishes as AAPH increments, in a dose-dependent manner (Figure 18). AAPH was used at a concentration of 15 mM in next experiments.

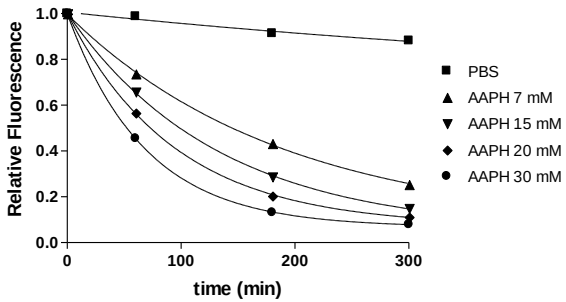


Figure 18. Effect of the radical generator AAPH on DPH fluorescence in DPH-containing POPC. POPC VET 200 containing DPH 0.3 % in buffer solution were subjected to oxidative events by adding AAPH 0-30mM. The DPH fluorescence values are inversely related with AAPH concentration.

To test the antioxidant efficiencies of 3,4-DHE esters, DPH-containing vesicles were pre-incubated with the antioxidants, and then AAPH was added.

Figure 19 shows the decrease of DPH fluorescence. When compared with the negative control (no antioxidants), it is evident that all 3,4-DHE esters restore DPH fluorescence of about 60%, although the butyrate ester seems to be less effective than the longer-chain analogues.

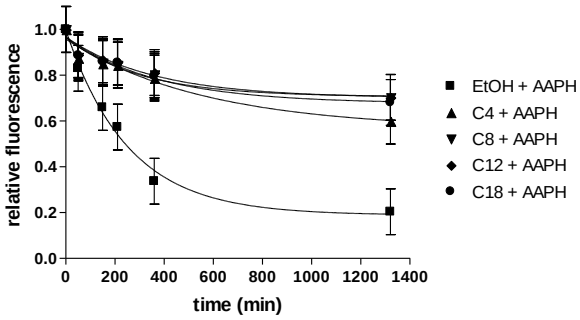


Figure 19. Effect of 3,4-DHE esters on DPH fluorescence decay induced by AAPH in DPH-containing POPC. AAPH (15 mM) was used as radical generator for the oxidation of DPH-containing POPC liposomes. The graph shows the time course of DPH fluorescence for untreated POPC (■) and in 10 μM antioxidant-treated POPC (pre-incubation: 10 minutes).

6.2.2 Evaluation of antioxidant activity with H_2DCF probe

In order to test the efficacy of antioxidants in the liposome inner aqueous compartment, H_2DCF (not fluorescent) was used as a probe. The product of the reaction with oxidants is the fluorescent species DCF. H_2DCF was formed *in situ* starting from its diacetyl ester, H_2DCF-DA, in the presence of a catalytic amount of proteinase K, which acts, in this context, as an esterase.

The oxidation of H_2DCF, induced by AAPH, was first recorded fluorimetrically in buffer solution, in presence or absence of 3,4-DHE esters (Figure 20). Three of the four esters tested resulted in having similar protective activities, while the stearate ester activity could not be detected, probably due to the formation of macroscopic aggregates in polar medium.

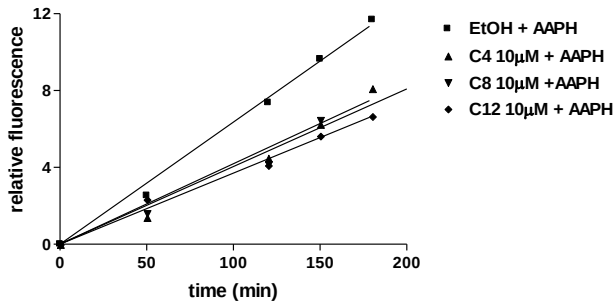


Figure 20. Time course of the DCF fluorescence intensity, after 15 mM AAPH addition at pH 7.4 in buffer, in absence and in presence of 3,4-DHE esters butyrate, octanoate and dodecanoate.

Having tested the antioxidant effectiveness of 3,4-DHE esters in bulk solution, we carried out a similar assay with H_2DCF-containing liposomes. The latter were prepared by encapsulating the H_2DCF-DA/proteinase K premix (which actually contains H_2DCF and proteinase K) inside POPC liposomes prepared by the film hydration method, followed by 200 nm extrusion (VET_{200}). Liposomes were purified from the non-encapsulated solutes via SEC, as described before, and the effectiveness of separation was checked by measuring the turbidity and the fluorescence of each fraction (Figure 21). The turbidity profile reveals the liposome-containing fraction, whereas the fluorescence profile reveals the H_2DCF-containing fractions. Note that this

fluorescence comes from traces of DCF present together with H_2DCF. The encapsulation of H_2DCF is clearly detectable. Liposome fractions (2nd-6th) were pooled and used for the assay.

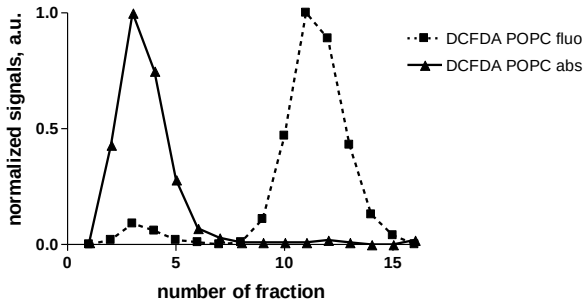


Figure 21. Chromatographic separation of liposomes (fractions 2-6) from free, unentrapped solutes (fractions 9-15). POPC was dried and the resultant lipidic film was hydrated with 1 mL of H_2DCF-DA/proteinase K pre-mix in PBS. The suspension was extruded (200 nm), and liposomes were purified via SEC. In the plot, the turbidity (absorbance at 490 nm) and the fluorescence (520 nm) of each fraction are reported.

H_2DCF-containing liposomes, obtained after SEC, were pre-incubated with 3,4-DHE esters (10 minutes), then AAPH was added (Figure 22). All esters showed an effective antioxidant activity, but with markedly different efficiency. Butyric ester resulted to be the most active in preventing the probe oxidation.

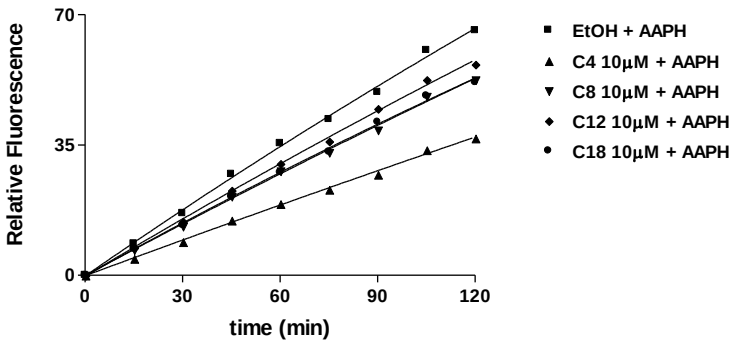


Figure 22. Effect of radical generator AAPH on the fluorescence of H_2DCF-

containing liposomes in presence of 3,4-DHE esters**.** Intravesicle DCF Fluorescence increases in absence and in presence of 3,4-DHE esters butyrate, octanoate, dodecanoate and stearate, as induced by the addition of 15 mM AAPH.

The efficiency of 3,4-DHE esters for contrasting the intravesicle oxidation of H_2DCF was further evaluated by repeating the previous experiment with different antioxidant concentration (Figure 23). Data confirm the superior activity of the 3,4-DHE butyryl esters, when compared with long-chain ones (C8, C12 and C18), even when high ester concentrations were used.

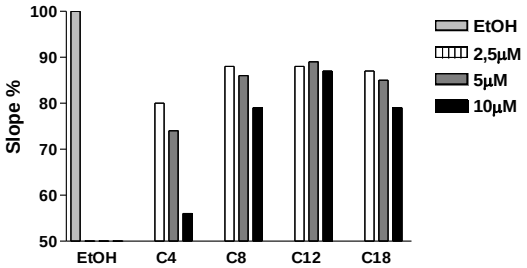


Figure 23. Relative rate (slope) of intravesicle DCF fluorescence increase, as obtained in absence and in presence of different concentrations of 3,4-DHE esters butyrate, octanoate, dodecanoate and stearate (0; 2.5; 5; 10 µM), induced by 15 mM AAPH.

DISCUSSION OF THE RESULTS

It has been proposed that antioxidants are more effective in lipid matrixes of foods if they are able to accumulate at the oil-water interface where oxidative reactions are most prevalent.[74] The efficiency of antioxidants in oil-in-water emulsions could be dependent not only on their ability to accumulate in the adequate position, but also on their surface activity. This latter property can be increased by conjugation of the antioxidants to fatty acid or fatty alcohol chains. Formation of such antioxidant surfactants could be an effective method to increase the concentration of antioxidants at the oil-water interface of emulsion droplets.[52] Among the natural polyphenols, olive oil phenols, and in particular hydroxytyrosol (3,4-DHE), have shown highly potent antioxidant activity in oils and in oil-in-water emulsions, even higher than several commonly used food antioxidants, as α-tocopherol, butylated hydroxytoluene (BHT), or ascorbyl palmitate.[60] Moreover, hydroxytyrosol displays some remarkable biological properties, as seen in the introduction. Having amphiphilic character (log P=0.08), 3,4-DHE has only limited applications in hydrophobic/lipidic media or as preservative in food technology and cosmetic industry. Derivatization of 3,4-DHE, in order to get more lipophilic compounds, could overcome trouble due its hydrophilicity.

The easy protocol (Results, Figure 1 and 2) developed in our laboratory permits to have 3,4-DHE esters in high yield. The orthoformiate protection of the catecholic moiety can be easily removed immediately before the use of the compounds. By this way, 3,4-DHE orthoformiate esters can be stored for long periods reducing the risks of degradation due to oxidation. The choice of esterification rather than etherification (employed by other research groups)[61,63] is related to the easier hydrolysis by intracellular esterases of the ester bond with respect to the ether one. This was supported by studies on uptake and metabolism of 3,4-DHE ethers,[61] in which presence of free hydroxytyrosol, resulting from the hydrolysis of hydroxytyrosyl ethers, was not detected. On the contrary, ester derivatives could be hydrolyzed into free 3,4-DHE and a fatty acid.

The results obtained by comparing the antioxidant capacity of 3,4-DHE in ethanol and in ethanol/refined oil (2%) indicate that all tested esters show a nearly constant antioxidant capacity, comparable or higher than that of 3,4-DHE itself (Results, Figure 4a). All 3,4-DHE esters show a slightly better antioxidant capacity when analyzed in the presence of very small amounts (around 2%) of refined olive oil in the ABTS solution. As previously specified, these data agree with the behavior of amphiphilic compounds in emulsion. The best values are shown by medium sized acyl chains, whereas long sized acyl chain derivatives show a minor radical scavenging capacity. This trend has already been reported for 3,4-DHE derivatives,[49,50] where an increased antioxidant capacity is reported for medium sized (C4-C9) 3,4-DHE esters or ethers, in comparison with 3,4-DHE, while longer acyl chains do not enhance the antioxidant activity. This confirms that antioxidant capacity does not only

depend on lipophilicity. A possible explanation could be related to a competition between two contemporary increasing but opposite effects. As the acyl chain length and lipophilicity increase (Log P data, Table 1), the conformational freedom of the esters chain increases as well and this could result in folded structures where catechol hydroxyls are partially shielded. Molecular dynamics data (Results, Figure 7) of 3,4-DHE stearate (supposed to be in ethanol) confirm that almost 15% of the conformers has a structure in which the ester acyl chains half cover the aromatic ring, so interfering with the scavenging activity of the catechol moiety. This can explain the reduction of activity for long chain esters. Accordingly, the higher antioxidant capacity measured for oleic ester can derive from lowering of the acyl chain conformational freedom as a consequence of the double bonds rigidity.[57] This trend of activity, depending on the length (C2-C18) of the acyl chain, is repeated in and can be equally explained in the case of 3,4-DHE analogues.

Indeed, as part of our research is devoted to the preparation of biologically active compounds inspired to 3,4-DHE, it seemed interesting to explore Structure/Activity Relationship (SAR) in novel catechols. Therefore, we planned a study focused on the synthesis and the evaluation of the antioxidant activities of a large panel of novel catecholic compounds (3,4-DHP; 2,3-DHE; 2,3-DHP) and their lipophilic derivatives (Results, Figure 3). The antioxidant capacity of these three sets of catechols by ABTS assay was performed in ethanol, where all catecholic compounds are soluble in the range of concentrations used, and thus the differences in the antioxidant activity can be ascribed to structural influences. To facilitate the examination of the data, results from Figure 6 are reported below (Figure 1), where the TEAC of each group of compounds is compared with the other two. Comparisons will be discussed by taking into account two parameters: the length (C2 or C3) of the alcohol chain (Discussion, Figure 1a and 1b); the position (2,3- or 3,4-) of the phenolic hydroxyls (Discussion, Figure 1c).

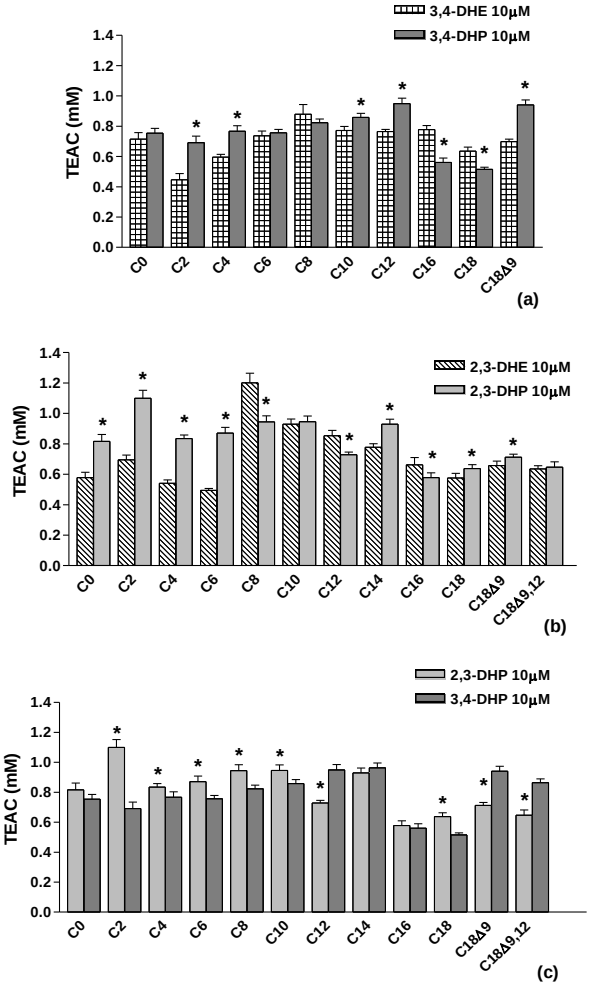


Figure 1. Comparisons between the TEAC results of 3,4-DHE, 3,4-DHP, 2,3-DHE, 2,3-DHP and their esters. (a): comparison between 3,4-DHE and its propilic homologue ester sets having the catechol moiety in *meta-para* position; (b): comparison between 2,3-DHE and its propilic homologue ester sets having the catechol moiety in *ortho-meta* position; (c) comparison between propilic homologues ester sets, differing for the catechol position.*=P< 0.05 at least as from a Student's *t*-test with respect to each correspondent homologue.

As to the first point, elongation of the alcoholic side chain from two to three carbons seems to positively affect the antioxidant capacity. Considering the significant data in which a compound resulted with higher TEAC value than the

correspondent homologue, we can conclude that the propylic sets display better antioxidant capacity.

The position of the phenolic groups seems to positively affect the radical scavenging activity as well. In Figure 1c of Discussion the comparison between the two propylic sets, just differing for the catechol moiety position, is represented. Therefore, both the homologation of the alcoholic chain and the 2,3-position of catecholic hydroxyls produce an increase of TEAC values by acting either alone or together. The increased antioxidant activity in 2,3-dihydroxy derivatives could be related to the easier formation of intramolecular hydrogen bonds between the C(2')-OH and the acyl oxygen of the ester chain (Results, Figure 8). Indeed, molecular mechanics MM2 minimizations showed that only in 2,3-DHE acetate and 2,3-DHP acetate the distance between the C(2')-OH and the acyl oxygen of the ester chain is suitable for intramolecular H-bonding, while, due to the presence of the proton at C(2'), this is impossible in both 3,4-DHE and 3,4-DHP acetates. Therefore, both homologation and hydroxyls position cooperate to make effective intramolecular H-bonding in either 2,3-DHE esters or 2,3-DHP esters. Intramolecular H-bonding could weaken the phenolic O-H bond, thus resulting in both an easier hydrogen extraction and better radical scavenging properties. On the other hand, these positive effects can be reduced by the above mentioned shielding effect of folded conformations. This could account for the lower antioxidant activity observed in longer chain esters (>C14).[65]

A study on *in vitro* cultured rat skeletal muscle L6 cells was performed. These cells are particularly sensitive to ROS, as their generation is one of the most prominent events during contractile activity.[75]

To examine whether the compounds affect cell survival in L6 cells, cultured cells were treated with different doses (10, 20,50 μM) of all prepared alcohols and some of their esters for 24 h. The percentage of cell survival remained high (>80%) for all concentrations (Results, Figure 9). Some compounds (3,4-DHE and its ester octanoate 10 and 20μM, respectively; 3,4-DHP 20μM; 2,3-DHE octanoate 20, 50 μM; 2,3-DHP palmitate 50μM) displayed a slightly proliferative effect. A proliferative activity in non-tumoral cells has already been registered in endothelial cells for hydroxytyrosol.[35]

The antioxidant properties of the four sets were checked (Results, Figure 10). Ten minutes treatment with cumene hydroperoxide 200 μM gave rise to a significant increase in the intracellular ROS production, while 10 min pre-incubation with 10 μM short- medium sized chain esters, before cumene hydroperoxide stimulation, effectively protected the L6 cells from oxidative events, with a cut-off point at C12. The causes of this cut off effect are still matter of study. It is possible to suppose that, at a certain level of lipophilicity, the easy diffusion of esters into the cells could be balanced (C10) or even make unproductive (C12-C18) by the entrapment into the plasma membrane caused by the higher affinity of long acyl chains with the phospholipids or

hydrophobic proteins inside the bilayer. This hypothesis might find a confirmation in the recent experiments on membrane fluidity in daidzein alkoxy derivatives.[74,76] It has been taken into account by our research group, and 3,4-DHE esters were employed for further studies on the behavior of these molecules in membrane.

Among the short-medium chain compounds, 3,4-DHE esters were assessed for their capability to contrast oxidative stress at lower concentration (Results, Figure 11). Therefore, dose–response experiments were carried out also at the concentration of 1.0 and 0.1 μM on 3,4-DHE and its esters acetate, butyrate, hexanoate and nonanoate. All tested compounds maintained a low ROS level production, at 1.0 μM concentration. At 0.1 μM concentration the % of ROS produced was higher, but significantly reduced with respect to the cumene hydroperoxide. MTT assay was again applied to test the cytoprotection of short-medium 3,4-DHE esters from cumene hydroperoxide induced toxicity (Results, Figure 12). Ten minutes antioxidants pre-incubated L6 cells, to which 200 μM cumene hydroperoxide was then given, significantly preserved viability with respect to untreated cells.

The previously discussed results, obtained by the evaluation of the antioxidant properties of the four sets of molecules in cells, showed a marked decrease of the biological activity in long chain esters. One possible motive of this behavior, often noticed in the long chains of a homologues series of surface active substances,[77] could be due to limited aqueous solubility. Consequently, cut-off effect could derive from the decrease of the available compound concentration for limited solubility. Results (Results, Figure 13) from Critical Aggregation Concentration assessment permits to observe an inverse relation between length of the acyl alkyl chain and CAC values. When log CAC is plotted *vs* the number of carbons of the acyl alkyl chain (Results, Figure 14), a continuous linear decrease is obtained. This linearity is found in compounds displaying optimal surfactant properties. Surface active property is recently thought to be strictly related to the efficacy of an antioxidant in oil-in-water emulsion. Indeed, some authors sustain that to improve the ability to counteract lipid oxidation in emulsions and other dispersed lipid systems, an antioxidant should also be an effective surfactant.[53] Extrapolations from the function of log CAC/alkyl-chain allow us to approximate a CAC value for 3,4-DHE butyrate and stearate esters. Actually, it is possible that C4 ester is completely soluble in water, whereas the C18 CAC calculated value is reasonably true and it suggests that, in the experimental conditions in cells (10 μM ester in buffer), half of it could be aggregated. This can in part explain the reduction of activity for long alkyl chain compounds.

Another formulated hypothesis for the drop of bioactivity achieved in derivatives having acyl chain length >C12 is the entrapment into the plasma membrane. The similarity between the liposomal and cellular membranes makes liposomes a very useful tool to investigate how significant the

antioxidant-membrane interactions are for antioxidant activity. For this reason liposomes were chosen as simple biomembrane model. Dynamic light scattering data (Results, Figure 15) advised of a not disruptive interaction between POPC membranes and antioxidants, as in their presence liposomes do not self-aggregate or break. Binding studies (Results, Figure 16) revealed that C4 ester does not bind to the membrane, confirming its high hydrophilic character already depicted by CAC studies. In contrast, C8, C12 and C18 ester result bond almost quantitatively to the lipid bilayer. Further quantitative studies are however necessaries to characterize in detail the partition of each ester in the aqueous and lipidic phase.

Information on the antioxidant role of 3,4-DHE esters was gained by the activity tests performed with H_2DCF and DPH fluorescent probes. These probes have different lipophilic properties, and consequently different locations (aqueous or lipidic, respectively), thus the antioxidant capacities of the molecules employed could be related to their ability to interact with and penetrate the lipid bilayer. DPH fluorescent assay (Results, Figure 18) measures the rate of fluorescence intensity decay due to the susceptibility of the probe. This latter possesses a conjugated double bond structure that reacts with free radical species. The antioxidant efficiency was related to the capacity of compounds to diminish DPH fluorescence decay (Results, Figure 19), and it resulted in about 60% efficiency for all the esters. Butyrate derivative, which was found not to bind with the membrane, was the less effective, as reasonably expected. The induced peroxidation of the hydrophilic probe (H_2DCF) with the peroxyl radical was assessed both in aqueous and liposome media, to check whether the membrane presence could affect the activity of the molecules. In aqueous media (Results, Figure 20), antioxidant activity depends only on the capacity of the substance to scavenge the peroxyl radicals, thus avoiding oxidation of the probe. In these conditions, butyrate, octanoate and dodecanoate esters display a similar activity. Stearate ester activity couldn't be detected, probably because of solubility problems in the aqueous buffer. The repetition of this antioxidant assay in presence of liposomes (Results, Figure 22), outlined that the differences of action, depending on the chain length, can be ascribed to major affinity of long size esters towards the bilayer. Indeed, butyrate ester proved to be the most efficient in preventing the intravesicle probe oxidation. It is possible that the butyrate ester can easily cross the lipid membrane and reach the liposome aqueous core, whereas the other esters remains anchored to the membrane. The antioxidant efficiency of this short chain ester in hydrophilic environment is strengthened by its dose-dependent effect on preventing H_2DCF oxidation (Results, Figure 23), whereas the other compounds (except octanoate ester, proving a slightly concentration dependency, to be confirmed) did not improve their activity by increasing amounts.

Comparing data obtained by antioxidant assays in *in vitro* cultured cells and liposomes, it firstly outcomes that activities in L6 cells result higher than in

membrane model. Considering the most efficient 3,4-DHE butyrate 10 μM in its ability to reduce oxidation of H_2DCF, it exhibits 87% of activity (deduced by the 13% of residual ROS produced) in cells, and 56% in liposomes.

Dissimilar cut-off points have been found in cellular and liposomal hydrophilic probe assay, pointing out C12 as the maximum chain length in cells (after which the activity drops), whereas in POPC liposomes it seems to be at C8. This inconsistency is still subject of study, but the idea is that the different composition of lipidic bilayer between plasma membrane and vesicles can affect the ratio of affinity towards the length of the acyl chain of the homologous series studied. In the case of liposomes the cut-off point has shifted in the direction of shorter chains.

CONCLUSIONS

The systematic study of Structure/Activity Relationship analysis about the antioxidant properties of novel catecholic compounds, analogues to hydroxytyrosol and its lipophilic esters, has been carried out. The results obtained indicate that the derivatization of hydrophilic catechols by esterification of the alcohol group with linear fatty acid chains can effectively maintain or even enhance their antioxidant properties in a different dispersion medium. Lipophilicity can be modulated, by the choice of an adequate alkyl chain length, to cooperate with antioxidant activity. As supported by recent reports, contemporary to our studies,[50,76,77] we found that, both in ABTS and biological assay, the dependence between hydrophobicity and the antioxidant capacity is nonlinear, but characterized by a clear cut-off effect. This nonlinear hypothesis, if confirmed by other methods of antioxidant evaluations, can lead to a much better understanding of the behavior of phenolic antioxidants in dispersed systems, which is a prerequisite to improve the current antioxidant strategies. Therefore, the establishment of a rational rule to design new antioxidants may optimize the protection of nutritional lipids in multiphasic systems.

Getting deeper into this finding, we investigated the reasons of the decreased activity for longer alkyl chains. In ethanol, it was found that esters having chains longer than C12-C14 can in part assume folded structures, thus interfering with the catechol moiety's radical scavenging. Comparative analysis of the four sets of molecules that we synthesized suggested that shifting the hydroxyl to *ortho-meta* position with respect to the alcohol chain can successfully affect the stability and the antioxidant activity of lipophilic phenols, by creating an intramolecular hydrogen bond that in *meta-para* compounds is not allowed. The formation of this new bond is particularly facilitated when a carbon atom is introduced before the acyl oxygen of the ester bond; specifically, in this case, by elongating the alcoholic side chain from C2 to C3. This issue can be taken into account in the design of new, efficient polyphenolic antioxidants.

From the MTT biological assays we could assess the nontoxicity of these substances. Their antioxidant activity was tested on cells, by the classical H_2DCF assay, demonstrating that rearrangements of hydroxytyrosol structure do not influence the activity, instead, the only involved parameter is lipophilicity (that is related to the length of the ester chain). For this reason, the subsequent experiments were carried out by employing just a homologue series, namely hydroxytyrosyl esters (3,4-DHE set). The second aspect marked out by ROS production assay was a nonlinear trend between lipophilicity and activity, following, in general, a sigmoid curve as depicted in ABTS assay. Nevertheless, being in an aqueous buffer in presence of cells, reasons for this cut-off effect should have different explanations respect to the folded structures outlined in ethanol.

The determination of the size-dependence of the Critical Aggregation Concentrations reveled that these amphiphilic catechols pursue the rules of surfactants and, interestingly, that the 50% of 10 μM stearate ester is aggregated in aqueous solution.

The above information could lead to think that long chain esters were not suitable to exert antioxidant activity in cells. On the contrary, activity assays performed with liposomes clarified that medium-long esters strongly interact with the membrane, and can efficiently protect it from oxidative damage. In the future, it would be interesting to verify a similar antioxidant activity in plasma membrane. At variance with the behavior of long chain esters, the butyrate ester does not seem associated to lipid membrane, but functions quite effectively as protective antioxidant in aqueous solution. Thanks to its partial lipophilic character, however, it is expected that it can easily cross the membrane and therefore act in the intracellular core as radical scavenger. Further studies will be devoted to the measurement of butyrate esters permeability through lipid bilayers.

Finally, as for the consistent difference between the percentage of activity in liposomes and in myoblasts, the subsisting hypothesis is that hydroxytyrosyl esters in cells may act by up regulating some antioxidant pathways, as already demonstrated for hydroxytyrosol itself in vascular endothelial cells[35] and in keratinocytes.[18] This could explain the amplified response to oxidative stress registered in cells.

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