Olive Oil Phenols as Promising Multi-targeting Agents Against Alzheimer's Disease

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Stefania Rigacci

Abstract

Amyloid diseases are characterized by the deposition of typically aggregated proteins/peptides in tissues, associated with degeneration and progressive functional impairment. Alzheimer's disease is one of the most studied neurodegenerative amyloid diseases and, in Western countries, a significant cause of dementia in the elderly. The so-called "Mediterranean diet" has been considered for long as the healthier dietary regimen, characterised by a great abundance in vegetables and fruits, extra virgin olive oil as the main source of fat, a moderate consumption of red wine and a reduced intake of proteins from red meat. Recent epidemiological studies support the efficacy of the Mediterranean diet not only against cardiovascular and cancer diseases (as previously demonstrated) but also against the cognitive decline associated with ageing, and several data are highlighting the role played by natural phenols, of which red wine and extra virgin olive oil are rich, in such context. In the meantime, studies conducted both *in vivo* and *in vitro* have started to reveal the great potential of the phenolic component of extra virgin olive oil (mainly oleuropein aglycone and oleocanthal) in counteracting amyloid aggregation and toxicity, with a particular emphasis on the pathways involved in the onset and progression of Alzheimer's disease: amyloid precursor protein processing, amyloid-beta (Aβ) peptide and tau aggregation, autophagy impairment, neuroinflammation. The aim of this review is to summarize the results of such research efforts,

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showing how the action of these phenols goes far beyond their renowned antioxidant activity and revealing their potential as multi-targeting agents against Alzheimer's disease.

Keywords
Alzheimer's disease • Mediterranean diet • Polyphenols • Extra virgin olive oil • Oleuropein aglycone

1.1 Introduction

Age-associated cognitive decline, in the relatively benign form of mild cognitive impairment (MCI) and in the more severe one of Alzheimer's disease (AD), has become a considerable social and clinical problem during the last decades, particularly in Western countries. With the important exception of familial AD, which is associated with one of several genetic mutations favouring the early onset of the disease, such a condition develops over a considerable period of time, maybe even decades. Moreover, in spite of the great efforts spent by researchers to identify early and accessible markers of this disease, AD is still diagnosed very late, when the neurological symptoms appear and the neuropathology is already in an advanced stage. For these reasons (life-long development – late diagnosis – neuronal loss) two important branches of the research in this field are early diagnosis and prevention.

With regards to prevention, a lot of attention has been placed on the role played by different lifestyles in favouring this disease. Epidemiological evidence points to a lower incidence of MCI and AD in populations adhering to the Mediterranean diet (MD), a dietary regimen that has already been strongly associated with a reduced risk for cardiovascular diseases and cancer (Martinez-Gonzalez et al. 2011; Sofi et al. 2010; Benetou et al. 2008; Lopez-Miranda et al. 2010). MD is characterized by extra virgin olive oil (EVOO), high intake of plant-based foods, relevant consumption of seafood, low-to moderate intake of dairy products, low intake of meat and a regular but moderate intake of red wine. In spite of regional and cultural variations (for example, fish or cheese can be more-or-less abundant), one ingredient should never be absent from the Mediterranean table: EVOO, probably the most typical component of MD. EVOO consists of 98 % glycerides, mainly monounsaturated fatty acids (MUFA). In any case, it is the remaining 2 %, including various so-called "minor compounds", that deserves much of our attention in this context. These consist of α-tocopherol and of several specific phenolic compounds including phenolic acids (caffeic, vanillic, syringic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic, *p*-hydroxybenzoic and gallic), phenolic alcohols (tyrosol and hydroxytyrosol), lignans (acetoxypinoresinol and pinoresinol), flavones (apigenin and luteolin) and, last but not least, secoiridoids (oleuropein aglycone, oleochantal and their derivatives). The latter are the most abundant and typical phenolic components of EVOO; hence investigations aimed at identifying the active principles responsible for the specific healthy benefits of EVOO have been mainly focused on them (Servili et al. 2009).

The concentration of these substances in oil is highly variable, depending on a number of different factors: olive cultivar, ripening stage at harvesting, geographic origin of olives, olive trees irrigation, operative conditions applied during crushing, malaxation and oil separation, oil storage modalities. The last two factors are particularly relevant. In fact, the highest phenolic content is present in oil immediately after its cold mechanical extraction from olives (a mandatory procedure to obtain an EVOO) and progressively declines with oil ageing, particularly if it is exposed to air and light that promote phenols oxidation and degradation. With some notable exceptions, total phenols in olive oil generally

range between 130 and 350 mg/kg, with EVOO at the highest end of this concentration interval.

Regarding the protection provided by EVOO consumption against age-associated cognitive decline, the results provided by the studies on the PREDIMED (PREvención con DIeta MEDiterránea) cohort are particularly interesting. The aim of these clinical trials was to test the efficacy of MD in counteracting cardiovascular disease events in asymptomatic people at high cardiovascular risk. In one of these studies, 578 subjects reasonably conforming to the traditional MD (except for a higher intake of meat and diary products) were evaluated for their cognitive performance and total urinary polyphenol excretion (Valls-Pedret et al. 2012). Results showed that higher intake of both total olive oil and its virgin variety, coffee, walnuts, and wine were associated with better memory function and global cognition. Interestingly, the consumption of different foods and beverages was found significantly associated with the improvement in specific cognitive capabilities. Thus, EVOO and coffee were found to associate with better delayed verbal memory, walnuts with improved working memory, and red wine with higher Mini-Mental State Examination scores. Urinary phenols excretion was dose-dependently associated with improved memory, supporting the hypothesis that the common denominator of all those different foods, that is their high phenolic content, was the main factor responsible for the observed benefits.

The more recent PREDIMED-Navarra randomized trial included a cohort of 268 subjects (74.1 ± 5.7 years old, 44.8 % men with no cardiovascular disease but at high vascular risk because affected by type 2 diabetes mellitus or by three or more vascular risk factors) which were randomly assigned to three groups receiving for 6.5 years a low-fat diet (control), or a typical MD containing either EVOO (1 L/week) or mixed nuts (30 g/day) (Martinez-Lapiscina et al. 2013). The intervention with the EVOO-diet was associated with better cognitive performances, especially across fluency and memory tasks, and less MCI as compared to controls, while no significant differences were associated with the nuts-diet. Moreover, those who received the EVOO supplementation had significantly better performances on both visual and verbal memory domains compared to those who received nuts supplementation. These results support an inverse association between the consumption of EVOO and amnestic cognitive impairment, the deficit most commonly associated with the risk for AD. These data further suggest that, in contrast to what was postulated to justify the reduced cardiovascular disease risk associated with MD, it was not the unsaturated lipid component of EVOO that was the major determinant factor for its beneficial effect in this context; indeed, nuts are a valuable source of polyunsaturated fatty acids too. These and previous data strongly indicate that EVOO phenolic content is the main ingredient responsible for the reduced risk for age-related cognitive impairment (Jacomelli et al. 2010).

But what exactly is the mechanism by which EVOO phenols exert their protection? These molecules have for long been considered just for their antioxidant activity (Tasset et al. 2011; Pierno et al. 2014; Farr et al. 2012) but accumulated data now suggest that other properties play an even more important role when we look at the maintenance of a good cognitive performance. For example, recent research conducted on C57Bl/6 J mice fed with an EVOO rich in phenols (10 % EVOO weight/weight of dry diet for a daily total polyphenol dose of 6 mg/kg), showed improved contextual memory and reduced age-related impairment in motor coordination with respect to controls. While the latter effect was associated with reduced lipid peroxidation in the cerebellum, the former did not correlate with oxidation or inflammation parameters, suggesting the involvement of other mechanisms not relying on increased antioxidant protection (Pitozzi et al. 2012). In support of this, are studies on the determination of the antioxidant activity of the main phenolic derivatives in human biological fluids after dietary EVOO intake, i.e. hydroxytyrosol (HT), tyrosol and homovanillyl alcohol glucuronide derivatives, at concentrations that covered all biologically relevant ranges (0.01–0.1 μM). The results of such assays show that glucuronidation rapidly decreases the protective activity of

EVOO phenols against Cu-mediated low-density lipoprotein (LDL) oxidation and in 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, confirming that the biological activity of a phenol-rich olive oil cannot merely be an antioxidant one (Khymenets et al. 2010).

Actually, the investigation on individual EVOO phenolic compounds has uncovered a lot of specific biochemical activities in different biological contexts. Here I will review the most relevant ones for what concerns AD counteraction, but first of all I will provide a brief description of this disease, particularly for those that are not completely familiar with its biochemical traits.

1.2 General Traits of Alzheimer's Disease

AD is characterised by a progressive decline of several cognitive domains including memory, visuospatial skills and executive function (Sa et al. 2012). The histopathological analysis of affected brains reveals selective neuronal degeneration and synaptic loss, particularly in the hippocampus, amygdala and temporal neocortex (Serrano-Pozo et al. 2011), accompanied by extracellular senile plaques and intraneuronal neurofibrillary tangles. The main constituent of plaques is a fibrillar network of polymeric unbranched fibrils made of Aβ peptides, fragments of different length; the most represented are Aβ(1–40), Aβ(1–42) and the highly aggregation prone N-terminally truncated Aβ(3–42) and Aβ(11–42) pyroglutamylated-species. Aβ fragments originate from the sequential activity of two proteases, γ-secretase and β-secretase (BACE) that act on the extracellular N-terminal domain of the transmembrane amyloid precursor protein (APP). These natively unfolded peptides aggregate into increasingly ordered structures (oligomers – protofibrils – fibrils) that become insoluble and resistant to the clearing activities of cells and tissues (Mohamed et al. 2011), thanks to the acquisition of a peculiar β-sheet rich fold called "amyloid structure" (Fig. 1.1) (Serpell et al. 2000).

Neurofibrillary tangles are amyloid in nature too, being mainly formed by the cytoskeletal protein tau, a member of the microtubule-associated protein family normally concurring to the assembly and stabilization of microtubules, physiologically involved in axonal transport and neurite outgrowth (Maccioni et al. 2001). Tau exists in six different isoforms (45–65 kDa) originating from alternative splicing; it is a hydrophilic cationic protein, unfolded under native conditions (Jeganathan et al. 2008), whose normal function is regulated by phosphorylation, glycosylation, ubiquitination, truncation, and nitration (Farias et al. 2011). In AD, abnormal phosphorylation occurs on specific tau residues (Ser202, Thr205, Ser235, and Ser404) (Alvarez et al. 1999). These post-translational modifications are catalyzed by two main protein kinases: the Cyclin-dependent kinase (Cdk)5/p35 system and glycogen synthase kinase-3 beta (GSK-3β) (Farias et al. 2011), and are thought to promote tau aggregation.

More than two decades ago, Hardy and Higgins (1992) put forward the "amyloid cascade hypothesis" of AD, posing the insoluble Aβ fibrils as the primary toxic species. More recently, widespread support has been provided for the "toxic oligomer hypothesis" which proposes that pre-fibrillar intermediates are the toxic determinant of several amyloid diseases, including AD (Benilova et al. 2012; Brorsson et al. 2010; Walsh et al. 1999, 2002; Walsh and Selkoe 2004; Cleary et al. 2005; Billings et al. 2005; Koffie et al. 2009). Accordingly, such oligomers have been retrieved in diseased tissues both from animal models and humans, and *in vitro* assays have repeatedly confirmed their toxicity to cells. Several mechanisms determine oligomer cytotoxicity: membrane destabilization and derangement of ion homeostasis, particularly of Ca^{2+}; oxidative damage; overload and dysfunction of the pathways devoted to protein quality control (proteasome, unfolded protein response, autophagy). With regards to tau, its oligomers are also now considered to be the most toxic species, possibly inducing neurodegeneration by affecting mitochondrial and synaptic function, both of which are early hallmarks of AD

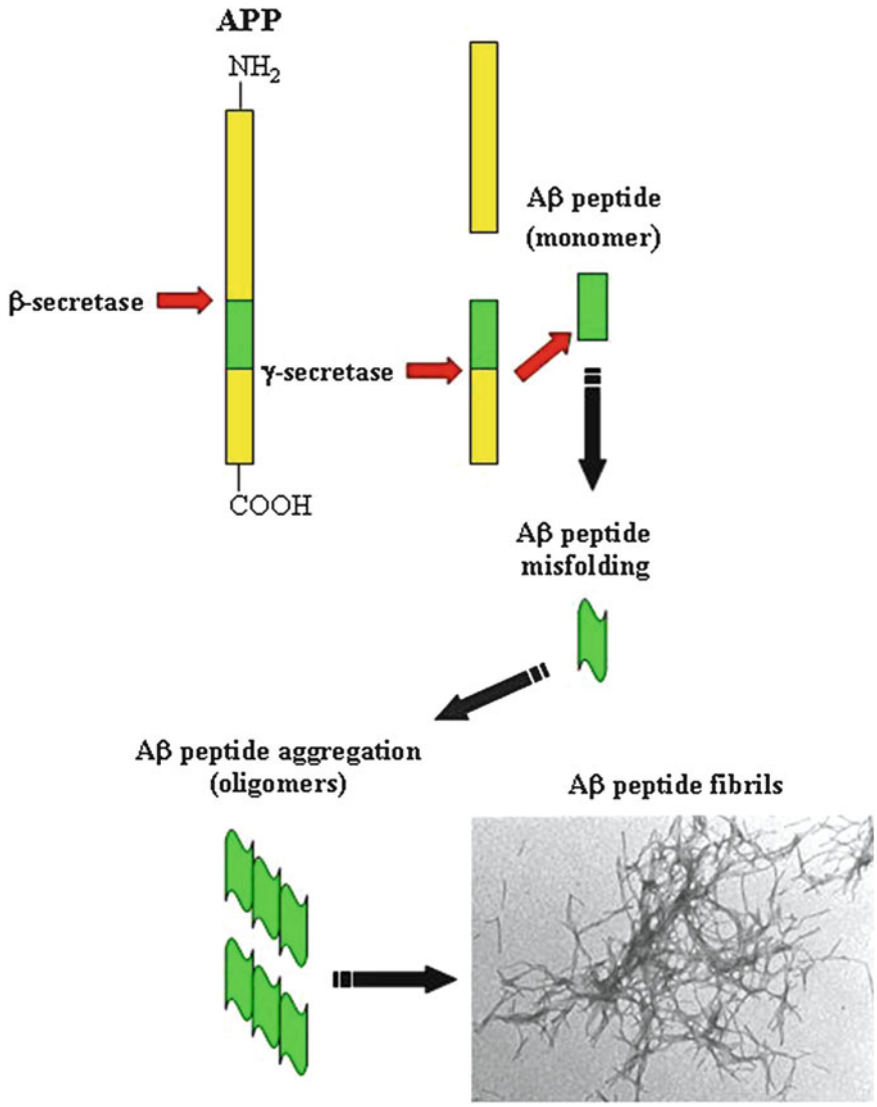

Fig. 1.1 Aβ peptide generation from APP by γ-secretase and β-secretase, followed by aggregation into oligomers and fibrils

and other tauopathies (Lasagna-Reeves et al. 2011; Guzman-Martinez et al. 2013).

Recently, the complexity of the amyloid scenario has been further increased by the discovery of different species of oligomers, varying in their structural features as well as in their toxicity (the so-called "amyloid polymorphism") (Lee et al. 2007; Meinhardt et al. 2009; Stefani 2012). Moreover, a reappraisal of the role of fibrils in amyloid toxicity is taking place in recent years, particularly concerning AD, supporting a role for Aβ fibrils not only as a possible reservoir of toxic oligomers but also as a neurotoxic species in their own right (Pan et al. 2011; Gilbert 2013).

As efficaciously summarized in a recent review, inflammation seems to play an important role in AD pathogenesis (Meraz-Rios et al. 2013). While initial activation of microglia and particularly astrocytes may be a positive response in the attempt to clear protein aggregates through phagocytosis and intracellular degradation, this process soon becomes dysfunctional leading to a

worsening of the pathology through the production of nitric oxide (NO), reactive oxygen species (ROS), pro-inflammatory cytokines (tumor necrosis factor-α, interleukin-1β and interleukin-6), and prostaglandin-E2, which eventually promote neuronal death. Moreover, pro-inflammatory cytokines mediate an increase in tau phosphorylation, APP synthesis and Aβ generation through BACE-1 transcription (Lyman et al. 2014; Chen et al. 2012; Krstic et al. 2012).

This brief description of AD, which does not pretend to be exhaustive, can give an idea of the complexity of this pathology; accordingly, the search for possible therapeutic interventions must face such a highly multiform scenario. The investigation of EVOO phenols in the context of amyloid diseases has started relatively later with respect to other extensively-studied polyphenols like curcumin, epigallocatechin-gallate (EGCG) and resveratrol. Nevertheless, the results obtained with oleuropein aglycone (OLE) and oleocanthal (OLC) are promising. Here, I will critically review published reports, placing an emphasis on the multi-targeting potential of such compounds which, in my opinion, constitutes their main strength.

1.3 Oleuropein Aglycone

OLE or 3,4-dixydroxyphenylethanol elenolic acid (3,4-DHPEA-EA) (Fig. 1.2), is a secoiridoid derived from oleuropein by the activity of a β-glucosidase released from olive fruits during crushing (Brenes et al. 1999). Its content in EVOO is highly variable, depending on several factors: olive cultivar (Romani et al. 1999; Franconi et al. 2006), ripening (Brenes et al. 1999), method of oil production (Tripoli et al. 2005) and storage conditions, with oxygen and light being the most detrimental factors by promoting oxidation (Cicerale et al. 2009). Moreover, the method of extraction of OLE from oil samples and the procedure followed to determine its concentration seem to considerably affect the reported values. To give a general idea, I will refer in this review to the work of Servili *et al.* who analyzed 263 EVOO samples and found

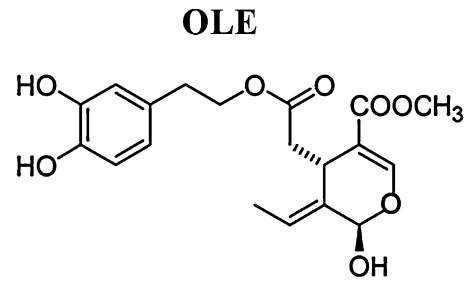

Fig. 1.2 Oleuropein aglycone (OLE)

a median value of 137 mg/kg for OLE and of 308 mg/kg for its dialdehydic form 3,4-DHPEA-EDA, also called Oleacein (Servili et al. 2009). Multiple beneficial effects were demonstrated for OLE, ranging from anti-atherogenic and anti-hypertensive to anti-cancer, anti-microbial and anti-inflammatory (Rigacci and Berti 2009) and in the last years promising anti-amyloid, neuroprotective and gerosuppressant activities have started to emerge for this secoiridoid.

Preliminary Mass Spectrometry (MS) analysis performed on its glycoside, oleuropein, revealed that it can associate the monomeric form of $A\beta 1-40$ and the oxidized form $A\beta Met^{35}(O)$ with a 1:1–2:1 oleuropein:peptide stoichiometric ratio. Such interaction is non-covalent but possesses a remarkable binding energy, since the complexes are still observable when an orifice potential of 100 V is applied to the Electrospray ionization (ESI)-MS apparatus (Bazoti et al. 2006, 2008). Through enzymatic cleavage of the Aβ:oleuropein complex prior to ESI-MS analysis, three peptide regions were identified as being implicated in the interaction between the two and, of these, the hydrophobic (17–21) one was the best candidate for the association with the non-polar moiety of oleuropein and hence also with its aglycone derivative OLE (Galanakis et al. 2011). The importance of this peptide region for the interaction of Aβ with oleuropein has been further confirmed using NMR (Benaki et al. 2009; Kallberg et al. 2001). Interestingly, the Aβ sequence critical for peptide fibrillization overlaps the putative OLE-binding region (Kallberg et al. 2001; Tjernberg et al. 1996, 1999);

thus, an interference of such secoiridoid with peptide aggregation could somehow be anticipated.

Actually, most of the studies on oleuropein as an aggregation inhibitor were performed on its aglycone OLE, which was shown to interfere with both Aβ42 and human islet amyloid polypeptide (hIAPP) aggregation *in vitro* (Rigacci et al. 2010, 2011). Through a combination of structural analysis by Thioflavin-T (ThT) and Anilinonaphthalene-8-sulfonate (ANS) binding, Circular Dichroism (CD) analysis, Atomic Force Microscopy (AFM), Transmission Electron Microscopy (TEM), and toxicity assays on cultured cells, it was demonstrated that OLE is capable of redirecting the aggregation pathway, thereby avoiding the formation of toxic oligomers and triggering peptide precipitation into amorphous aggregates from which non-harmful protofibrils eventually evolve. Concerning hIAPP, the amorphous aggregates that originate during the first phases of incubation in the presence of OLE are unable to interact with, and to damage, the cell membrane (Rigacci et al. 2010). Pre-existing Aβ fibrils can also be remodeled by the addition of OLE, with a reduction in the density of the fibrillar deposit and, most importantly, no release of toxic fragments (Rigacci et al. 2011).

Such preliminary *in vitro* results paved the way to experimentation on model organisms, which have corroborated the idea that OLE can be protective against amyloid aggregation and aggregate toxicity not only in cultured cells but also *in vivo*. In fact, administration of OLE to the CL2600 *Caenorhabditis elegans* (*C. elegans*) strain, constitutively expressing Aβ(3–42), resulted in significantly lower plaque deposition and toxic oligomer formation, with a reduction in the extent of worm paralysis and an increase in survival (Diomede et al. 2013). When Aβ(1–42) was aggregated in the presence of OLE and then injected in the *nucleus basalis magnocellularis* of adult male Wistar rats, it was not toxic to cholinergic neurons and did not raise an inflammatory reaction. This is in contrast to what happened when Aβ aggregates, grown under the same conditions but in the absence of OLE, were injected. The much lower amount of soluble toxic oligomers (recognized by the conformation-specific A11 antibody) in the injected rat brain suggested that OLE stably hinders the formation/release of toxic species, also when the aggregated peptide is introduced in a complex tissue environment (Luccarini et al. 2014).

A significant step forward in the *in vivo* research on the anti-amyloid potential of OLE was made by using the TgCRND8 transgenic mouse model of AD, encoding a double-mutant form of APP and showing cognitive impairment and amyloid plaque deposition from the age of 3 months. A robust improvement in cognitive performance and a remarkable reduction in Aβ plaque number, size and compactness was evident in mice fed for 8 weeks with an OLE-supplemented diet (50 mg/kg of diet). Improvement occurred even when the treatment was started at 4 months, when amyloid deposits are already present, hence suggesting that OLE can not only prevent amyloid deposition but also disaggregate preformed plaques. In this model, other relevant biological effects of this secoiridoid were uncovered: microglia migration to the plaques for phagocytosis was increased, astrocyte reaction was reduced, hippocampal neurogenesis was stimulated and, most interestingly, an intense and functional (i.e. leading to substrates degradation following vacuoles fusion with lysosomes) autophagy induction was elicited. Indeed, data obtained with cultured N2a mouse neuroblastoma cells confirmed that OLE dose-dependently activates autophagy acting on the "classical" pathways which involves mTOR inhibition (Grossi et al. 2013, 2014).

Notably, OLE antioxidant activity does not seem to be relevant in these systems. In fact, OLE does not significantly reduce either lipid peroxidation in the cortex of TgCRND8 mice (Grossi et al. 2013), nor intracellular superoxide level in the transgenic CL2600 *C. elegans* strain (Diomede et al. 2013). Rather, OLE acts both as an inhibitor of amyloidogenic peptide aggregation (possibly through direct binding, as suggested by the previously cited MS and NMR studies) and as a signaling molecule promoting cellular protective responses like autophagy stimulation and inflammation reduction. The latter property sounds particularly attractive since

the pathogenetic role of neuroinflammation in AD seems relevant – perhaps, even more so than the role of amyloid toxicity (McGeer and McGeer 2013). Though this position is debatable, it is a fact that AD progression is accompanied by a widespread neuroinflammation. Accordingly, incidence-based, population-based and case-control studies in humans suggest that some non-steroidal anti-inflammatory drugs (NSAIDs) could provide a degree of protection against AD (Vlad et al. 2008; in t' Veld et al. 2001). In any event, the ineffectiveness of different NSAIDs in several clinical trials leads us to hypothesise that, maybe, they work as preventative agents only if their administration is started early on – at least 2 years before the clinical diagnosis of AD (McGeer and McGeer 2013). This, together with the adverse gastrointestinal and cardiovascular effects that were recorded in some clinical trials, strongly reduce the potential of NSAIDs as anti-AD drugs (Scharf et al. 1999; Group 2006). Also for these reasons, research on the anti-inflammatory properties of natural compounds is particularly active and has led to substantiate at molecular level the already documented efficacy of EVOO and of its phenolic component in this respect. For example, significant reductions in serum leukotriene B4 and thromboxane B2 concentrations at 2 and 6 h after consumption of EVOO, but not after consumption of either olive oil or corn oil (both poor in phenols) were observed (Bogani et al. 2007); a 3-month intervention with a MD characterised by EVOO as the main source of fats determined a decrease in the expression of inflammation related genes (INF-γ, Rho GTPase-activating protein-15 and interleukin-7 receptor) higher than that coming from the adoption of a MD containing an olive oil poor in phenols (Konstantinidou et al. 2010); 50 μM OLE (a concentration that was supposed to be reached in plasma by individuals consuming an EVOO-rich diet) inhibited tumour necrosis factor-α (TNF-α) induced matrix metalloproteinase 9 (MMP-9) expression and secretion in THP-1 human monocytic leukaemia cells by impairing NF-κB -mediated genes transcription (Dell'Agli et al. 2010); similar oleuropein and HT concentrations inhibited PMA-stimulated COX-2 and MMP-9 expression and activities thus reducing inflammatory angiogenesis in cultured endothelial cells (Scoditti et al. 2012); in this case too, a reduction in the ROS-sensitive NF-κB transactivation was observed. However, the opposite result was obtained in a different cell type (embryonic kidney HEK293 cells stably expressing APP) where oleuropein seemed to increase MMP-9 gelatinolytic activity in the culture medium (Kostomoiri et al. 2013). The authors observed that treatment with oleuropein modified APP processing, increasing the formation of the non-amyloidogenic and neuroprotective sAPPα fragment, and they attributed this result to an increase in the α-secretase activity of MMP-9. The discrepancy between the two studies could derive from the different conditions employed (different cell lines, PMA-stimulated or basal MMP-9 secretion) and the different approaches to MMP-9 evaluation (in the first case MMP-9 mRNA level, protein expression and enzymatic activity were evaluated and found to be decreased (Scoditti et al. 2012)) while in the second study only MMP-9 gelatinolytic activity was determined (Kostomoiri et al. 2013). OLE dose-dependently inhibited the production of the pro-inflammatory chemokine CCL2 by human endothelial cells too, thus reducing endothelium inflammation (Sindona et al. 2012), and attenuated the inflammatory response in a carrageenan-challenged mouse model of inflammation (Impellizzeri et al. 2011).

The potential of OLE in the context of AD is corroborated also by its ability to inhibit the fibrillization of tau (both wild-type and carrying the P301L mutation, a mutation that increases its aggregation propensity and leads to frontotemporal dementia and parkinsonism in carriers) leading to reduced deposition of fibrillar material in the form of short rods (TEM analysis) (Daccache et al. 2011). The IC50 of OLE for the inhibition of P301L tau aggregation was 1.3 μM, as determined by the Thioflavin-S (ThS) fluorescence assay; in contrast, HT was poorly effective, inducing the growth of fibrils that were less dense but structurally similar to those originated in the absence of any inhibitor, thus confirming the

superiority of the secoiridoid as an anti-amyloid agent. Data concerning the toxicity of tau aggregates obtained in the absence or in the presence of OLE are still lacking.

Olive oil phenols are renown for their antioxidant activity. Nevertheless, the relevance of this property following ingestion is questionable because of phenols modification (this point will be more thoroughly discussed in Sect. 1.4). Nevertheless, an increase in cellular antioxidant defense can be observed following exposure to these molecules: a 6-month oleuropein administration to aged rats (50 mg/kg body weight/day) significantly increased superoxide dismutase, catalase and glutathione peroxidase activities in the brain, thus reducing the lipoperoxidative damage. Interestingly, a concomitant increase in dopaminergic neurons in *substantia nigra* was also observed. This could derive from the increase in enzymatic antioxidant defense, since these neurons are particularly sensitive to oxidative stress because of their abundance in iron ions and in free radicals, generated during dopamine metabolism (Sarbishegi et al. 2014).

With regards to the molecular mechanisms by which OLE produces its cellular effects, these are far from being clearly defined. A step forward in this direction was made by investigating the whole-genome transcription profile of human breast cancer cells exposed for 6 h to an EVOO extract containing 25 % OLE and 49 % its dialdehydic form 3,4-DHPEA-EDA (Menendez et al. 2013). This extract induced a marked increase in the expression of several genes; bioinformatic analysis of the global transcriptomic profiles revealed the induction of the endoplasmic reticulum (ER) stress chaperones and of the unfolded protein response (UPR), both relevant against the accumulation of protein aggregates. Age-related changes in cell size, morphology and senescence associated β-galactosidase (SA-β-gal) staining were significantly counteracted by the EVOO extract, and these effects correlated with the increase in the expression of the histone deacetylase Sirtuin1 and the activation of the AMP-activated protein kinase (AMPK). These two proteins regulate a multitude of signaling pathways in cells and one of them, significantly involved in the maintenance of cellular homeostasis, is autophagy. AMPK can activate autophagy by both positively regulating the Atg1/(ULK1) complex and inhibiting mTOR (Cai et al. 2012), while Sirtuin1 can deacetylate and activate the pro-autophagic Atg5, 7, 8 proteins and the LKB1 kinase, which in turn can activate AMPK (Chung et al. 2010). AMPK activity deregulation in AD seems to be involved in the perturbed brain energy metabolism, in Aβ generation and accumulation and in altered tau phosphorylation (Cai et al. 2012). Although the precise mechanism by which OLE activates autophagy still needs to be defined, these pieces of evidence reporting AMPK activation by a phenol-rich EVOO extract support the hypothesis of an involvement of this pathway.

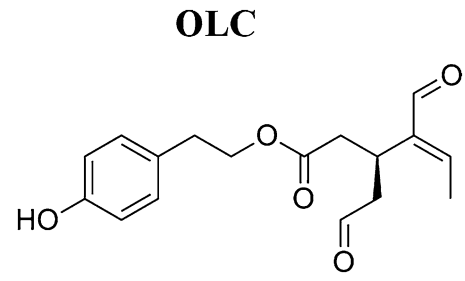

Fig. 1.3 Oleocanthal (OLC)

1.4 Oleocanthal

OLC (3,4-HPEA-EDA) is the dialdehydic form of the decarboxymethyl derivative of ligstroside aglycone 3,4-hydroxyphenylethanol elenolic acid (3,4-HPEA-EA) (Servili et al. 1999) (Fig. 1.3). The median value of its concentration in EVOO, according to Servili et al. (2009), is 85 mg/kg depending on several variables (Brenes et al. 2000, 2001; Tovar et al. 2001), as already mentioned for OLE.

Regarding OLC effects on amyloid aggregation, currently available data support its ability to convert peptide monomers and oligomers (but not fibrils) into high-molecular-weight (HMW) species. Regarding Aβ(1–42), the latter's immunoreactivity to the conformation-specific an-

tibody NU1 (recognizing oligomers) as well as to the sequence-specific M89, 4G8, and 6E10 antibodies, increased when it was incubated in aggregation conditions together with OLC. This suggests the adoption of a different conformation, while no effect was evident when OLC was added to pre-formed fibrils (Pitt et al. 2009). Such an outcome could be beneficial only if the newly-formed HMW species lost the potential to induce cytotoxic effects. The ability of OLC-modified aggregates to bind the synapses of hippocampal neurons was not significantly altered (a reduction was observed, but it was not statistically relevant); however, unfortunately, cytotoxicity of the OLC-modified aggregates was not assessed by the authors. For such reasons, these data do not, as yet, convincingly support a beneficial effect of OLC in redirecting Aβ aggregation. However, something different occurs if neurons are pre-treated with OLC before coming in contact with toxic Aβ oligomers: in such situation, a reduced binding of oligomers to the cell membrane and a protection against synaptic deterioration was observed (Pitt et al. 2009). Moreover OLC, once present in the culture medium, increases the immunoreactivity of subsequently added toxic oligomers, making them more prone to clearance by NU1 antibodies.

Collectively, these data suggest a complex interaction of OLC with amyloid peptides, with structural remodeling (and subsequently altered immunoreactivity) varying depending on the preliminary aggregation state of the amyloid species. Such preliminary pieces of evidence lead us to hypothesize that OLC could work as a preventative agent since its presence in the cellular environment prior to the appearance of amyloid deposits seems particularly efficient in inhibiting the toxic outcome. An *in vivo* experimentation is required to assess OLC actual potency in reducing toxic amyloid deposition in the brain.

Conversion of monomers and oligomers into non-fibrillar HMW aggregates was also shown for tau in the presence of OLC (Li et al. 2009). An interesting comparison of the different chemical behavior of OLE and OLC as tau aggregation inhibitors was made by Daccache et al. (2011) also on the basis of other data (Li et al. 2009; Monti et al. 2011): a structure–activity relationship study based on a series of derivatives of OLC pointed to an anti-fibrillization pharmacophore comprising both the saturated and unsaturated aldehyde moieties. Such a dialdehyde was proposed to cross-link two lysine residues within the third repeat of tau that would initiate the fibrillization process, rapidly followed by the modification of a single lysine, thus producing a cyclic adduct that evolves towards a more stable pyridinium-like complex by rearrangement of the skeleton. This would lock tau into a random coil conformation, preventing the transition to the β-sheet rich amyloid form and thus favoring the formation of non-amyloid HMW aggregates (Li et al. 2009; Monti et al. 2011). On the contrary, the methoxycarbonyl group of OLE increases the acidity of the hydrogen on the adjacent carbon thus inducing an intramolecular rearrangement leading to the dihydropyran form as the main isomer of OLE in solution. This form is in equilibrium with a monoaldheydic one that, when reacting with a lysine residue, would yield an aliphatic Schiff base that is unstable in aqueous media. OLE, therefore, does not work as a stable crosslinker and it does not promote the precipitation of tau into amorphous macro-aggregates; rather it favours the maintenance of a soluble form of tau so that the amount of fibrillar species is highly reduced (Daccache et al. 2011).

Although OLC does not seem to significantly affect the ability of tau to promote microtubule assembly in an *in vitro* tubulin polymerization assay (Li et al. 2009), some concerns still remain about retention of this important tau physiological function following OLC crosslinking, and an *in vivo* confirm is warranted.

Apart from its interference during amyloid aggregation, OLC usefulness against AD was recently supported by additional findings. A study conducted on cultured bEnd3 mice brain endothelial cells and C57BL/6 mice suggests that intraperitoneal administration of OLC to mice can increase the clearance of ^{125}I-Aβ(1–40) from the brain by up-regulating both Aβ degrading enzymes (Neprilysin and Insulin degrading enzyme) and Aβ transporters (P-glycoprotein and LDL receptor related protein-1) at the Blood

Brain Barrier (BBB) (Abuznait et al. 2013). These effects are highly relevant because the expression of such transporters declines with ageing and in AD, possibly contributing to amyloid accumulation in the brain parenchyma (Silverberg et al. 2010; Vogelgesang et al. 2004). OLC was also found to activate AMPK (Khanal et al. 2011). As already mentioned in the previous section, this kinase not only represents a node in the complex network regulating energy supply to the cell, but also participates in the quality control of proteins and organelles *via* autophagy. Autophagy is particularly beneficial and overexploited during neurodegenerative diseases, where protein amyloid deposition eventually overwhelms the cellular buffering capacity. The possibility that OLC activates autophagy, and the relevance of this in the context of AD models, has not been directly investigated yet. Last but not least, OLC possesses a striking ibuprofen-like activity: it dose-dependently inhibits COX-1 and COX-2 but has no effect on lipoxygenase *in vitro* (Beauchamp et al. 2005), and it decreases lipopolysaccharide-induced nitric oxide synthase in chondrocytes (Iacono et al. 2010). This strong anti-inflammatory activity is probably, at the moment, one of the most appealing properties of OLC, potentially useful also in counteracting AD-associated neuroinflammation.

1.5 Bioavailability

Bioavailability of phenolic compounds represents a critical issue that has to be addressed by answering three fundamental questions: (1) to what extent are these compounds absorbed following ingestion? (2) How are they modified following ingestion? (3) How do they (or their modification products.) distribute in tissues? Unfortunately these topics have been to date investigated with very different and non-standardized approaches, as it has been outlined in a recent review (D'Archivio et al. 2010), so that it is not easy to derive conclusive answers.

Concerning the phenolic compounds OLE and OLC, the main variables are represented by the form in which they are ingested (pure compounds, extracts containing different percentages of the compounds, whole EVOOs with different phenolic composition), dosing, duration of the treatment, and association with different foods. Moreover, the great majority of the studies have been performed employing oleuropein, which can be extracted from olive leafs or olive mill wastewater and so is more convenient (and commercial) than OLE, which is enriched in EVOO.

In spite of all these variables, a general consensus has emerged regarding the fact that EVOO phenols are in fact absorbed and metabolized by humans, because their degradation and modification products are retrieved in urine following ingestion (Vissers et al. 2002; Weinbrenner et al. 2004; Miro-Casas et al. 2003). Nevertheless, absorption profiles vary depending on the source of such phenols: when they are ingested as an oleuropein-rich olive leaf extract, mainly sulfate and glucuronide derivatives are detected both in plasma and in urine, with HT glucuronide being the most abundant one, hence suggesting extensive degradation of the secoiridoid component (Garcia-Villalba et al. 2014; de Bock et al. 2013). On the other hand, when phenols are introduced with EVOO, OLE, ligstroside aglycone and their Phase II metabolites are mostly abundant in urine (Garcia-Villalba et al. 2010; Suarez et al. 2010). These results support the view that secoiridoids aglycones are better absorbed than their glycated counterparts and suggest that EVOO matrix can contribute to phenols stability in the gastrointestinal tract and favour their absorption. Indeed, when EVOO is mixed with acidified water (pH 2.0) in a 1:1 ratio at 37 °C (a condition simulating the stomach environment), the secoiridoid aglycones present remain stable for 4 h (Romero et al. 2007). Under these conditions, 50 % of total phenols diffuse from EVOO into the simulated gastric juice; this percentage increases as the pH rises, whilst the EVOO:water ratio decreases to 1:2 (the latter two conditions mimic those present in the duodenum).

Oleuropein is far more stable, both in the stomach and in the intestine (over 12 h), if it is

ingested with a meal (Markopoulos et al. 2009). Conversely, oleuropein is strongly hydrolyzed in the upper gastrointestinal tract, with the rapid appearance of HT glucuronide in the plasma, when it is taken as an olive leaf extract under fasting conditions (Garcia-Villalba et al. 2014). Moreover, OLE is absorbed more efficiently than oleuropein, probably because its greater apolarity favours passive transport across the cell membrane. In fact, when rabbits are fed an EVOO containing a 9-fold concentration of OLE *vs* oleuropein, a 60-fold concentration of OLE *vs* oleuropein is reached in the plasma (Coni et al. 2000). A recent report further shows that, after oleuropein administration to rats, OLE is retrieved both in faeces and urine (together with hydrolysis and modification products) (Lin et al. 2013). Particularly relevant in the context of AD is experimental evidence suggesting that, in rat and humans, orally-administered olive oil phenols, including OLE, oleuropein and/or one of its derivatives arising from tissue metabolism, cross the BBB and are found inside brain parenchyma (Serra et al. 2012; Vissers et al. 2002). Finally, OLE and 3,4-DHPEA-EDA seem to associate to membranes as a consequence of their hydrophobicity (Paiva-Martins et al. 2010); this implies that they may accumulate at the cellular level, reaching a local concentration higher than that expected on the basis of their plasma concentration alone.

Considering that HT and tyrosol are the main degradation products of OLE and OLC, respectively, either in oil (increasing with EVOO ageing) or in the organism following ingestion, a few words should be spent on these phenols too. HT penetration into the brain has been demonstrated in a pharmacokinetic study assessing the metabolic fate of intravenously injected [^{14}C]HT (D'Angelo et al. 2001). Anti-atherogenic, anti-inflammatory, anti-microbial, anti-proliferative and pro-apoptotic effects have been attributed to HT (Granados-Principal et al. 2010). The antioxidant potency of HT *in vitro* is very high but its biological importance as a ROS scavenger has been questioned of late because this seems to be greatly affected by HT modification (i.e. glucuronidation) following ingestion (Khymenets et al. 2010). Nonetheless, when mice are fed for 12 days with 100 mg of HT/kg of body weight, basal and Fe^{2+}-induced malondialdehyde (MDA) formation are significantly reduced in explanted brain cells, in spite of no systemic increase in antioxidant capacity. This suggests that, besides direct ROS and iron-scavenging activity of HT, cytoprotection would derive also from different mechanisms (Scharf et al. 1999). Indeed, several experiments have shown that HT increases the expression of antioxidant enzymes, an ability potentially relevant in the context of AD. This happens as a result of a hormetic mechanism by which HT, behaving like a mild pro-oxidant, induces the activation of cellular defenses: in fact, in the presence of peroxidases, HT can undergo a catechol-semiquinone-quinone redox cycling generating superoxide, which in turn increases MnSOD expression. As a result, age-associated mitochondrial ROS accumulation is counteracted and the chronological lifespan of normal human fibroblasts is extended (Sarsour et al. 2012). Hormesis seems to be implied also in the increase in lifespan and stress resistance of tyrosol-fed *C.elegans*, subsequent to the activation of components of the heat shock response and the insulin pathway (Canuelo et al. 2012). The up-regulation of several antioxidant enzymes was also observed in keratinocytes exposed to HT: glutaredoxin, thioredoxin reductase, glutathione peroxidase-3, heme oxygenase-1, biliverdin reductase and ferritin, the latter three participating in the degradation of heme to biliverdin (a potent antioxidant) and in storage of the pro-oxidant free iron which is produced as a heme degradation by-product (Rafehi et al. 2012). Interestingly, in vascular endothelial cells the HT-mediated up-regulation of catalase expression and the associated protection against ROS increase are dependent on the activation of the AMPK-FOXO3a pathway, since they are abrogated when AMPK expression is suppressed by siRNA (Zrelli et al. 2011). On the basis of such HT-mediated AMPK activation we could speculate whether HT is an autophagy activator, too. However, the available data seem suggest the opposite: exposure of prostate cancer PC-

3 cells to 80 μM HT resulted in a significant increase in superoxide production accompanied by a defect in autophagy (Luo et al. 2013), while HT supplementation to rats counteracted autophagy activation induced by intense physical exercise (Feng et al. 2011). In a context closer to AD, HT was found to reduce the cytotoxicity of Aβ(25–35) to N2a neuroblastoma cells by decreasing NF-κB nuclear translocation and cell death, but no effect was observed on Aβ − or H_2O_2-induced decrease in cellular glutathione (GSH) (St-Laurent-Thibault et al. 2011). Limited data are available concerning HT activity as an amyloid aggregation inhibitor: as already mentioned, the *in vitro* aggregation of tau was affected by HT (as demonstrated by the reduction in ThS binding) but amyloid fibrils structurally similar to those grown in the absence of the phenol were eventually formed (Daccache et al. 2011).

From these data the superiority of OLC and OLE, with respect to HT, as potential drugs against amyloid diseases seems evident. Accordingly, studies are currently under way to improve the bioavailability and preserve the integrity of such secoiridoids following ingestion. A promising approach is represented by encapsulation: simple olive oil phenols (tyrosol, HT, homovanillic acid, 3,4-dihydroxyphenylacetic acid, and protocatechuic acid) have been successfully enclosed into the hydrophobic cavity of β-cyclodextrin (Rescifina et al. 2010). Oleuropein, too, was found to enter the β-cyclodextrin particle with its phenolic portion, and this seemed to protect it from oxidation and to increase its aqueous solubility (Mourtzinos et al. 2007). Further suggestions come from trials performed with other polyphenols: for instance, promising results were obtained when curcumin was encapsulated in polyethylene glycol (PEG) nanoparticles stabilised with β-cyclodextrin. Such nanoparticles (<80 nm in size) did not tend to aggregate, were fairly stable and performed well both in an *in vitro* cell monolayer permeability assay (mimicking BBB transit) and after oral administration to TgCNRD8 mice, allowing an increase in curcumin penetration into the brain (Cheng et al. 2013).

1.6 Conclusions and Perspectives

The beneficial effects of MD in counteracting human diseases and aging are widely recognised, and multiple evidence points to EVOO as one of the most valuable ingredients of such a dietary regimen. The main differences in the composition of EVOO with respect to other edible oils are the prevalence of oleic acid as the main lipid (up to 85 % of total fatty acids, as compared with 14 to 59 % in sunflower, soybean and peanut oils) and the minor unsaponifiable fraction (0.5–2 %) containing squalene, tocopherol, various sterols and some peculiar phenols which contribute significantly to the unique properties of EVOO, not least oxidative stability and distinguished flavour. Though oleic acid has been endorsed with multiple beneficial activities following its enrichment in the membrane lipid bilayer, as summarised in a recent review (Lopez et al. 2014), it is the minor phenolic constituents which have generated increased attention, and are now considered as predominantly responsible for most of the health-promoting properties of EVOO. Accordingly, the accumulating data on OLC and OLE reviewed in this paper collectively support the hypothesis that such phenols are capable of eliciting multiple biochemical and biological responses, that might be harnessed in counteracting several age-associated diseases and, particularly, cognitive impairment and AD (Table 1.1).

To date, the activities of EVOO phenols potentially useful against AD can be grouped into several main categories: (i) inhibition of the formation of amyloidogenic Aβ fragments from APP, (ii) promotion of Aβ peptide clearance, (iii) inhibition of Aβ and tau toxic amyloid aggregation, (iv) reduction of inflammation, (v) autophagy activation, (vi) neurogenesis stimulation and (vii) antioxidant defence activation. OLE has until now given more unequivocal positive results than OLC with regards to the inhibition of toxic amyloid aggregation and the stimulation of cellular protective mechanisms like autophagy. Moreover, the specificity and selectivity of OLC crosslinking activity still

Table 1.1 Summary of OLE and OLC activities useful in counteracting AD

	Activity	References
OLE and oleuropein	Inhibition of the formation of Aβ toxic oligomers and plaques and remodelling of pre-existing Aβ deposits *in vitro* and *in vivo*	Rigacci et al. (2011), Diomede et al. (2013), Luccarini et al. (2014), and Grossi et al. (2013)
	Improvement in survival, cognitive function and motor performance in AD transgenic models (*C. elegans*, mouse)	Diomede et al. (2013) and Grossi et al. (2013, 2014)
	Inflammation reduction	Luccarini et al. (2014), Grossi et al. (2013, 2014), Dell'Agli et al. (2010), Scoditti et al. (2012), Sindona et al. (2012), and Impellizzeri et al. (2011)
	Autophagy activation	Grossi et al. (2013, 2014)
	Stimulation of hippocampal neurogenesis and increase in dopaminergic neurons	Grossi et al. (2014) and Sarbishegi et al. (2014)
	Inhibition of tau aggregation	Daccache et al. (2011)
	Promotion of sAPPα fragment production	Kostomoiri et al. (2013)
	Antioxidant enzymes activation	Sarbishegi et al. (2014)
OLC	Reduction of Aβ oligomers binding to the membrane, protection against synaptic deterioration	Pitt et al. (2009)
	Conversion of tau monomers and oligomers into non-fibrillar aggregates	Li et al. (2009)
	Up-regulation of Aβ-degrading enzymes and Aβ transporters at the BBB	Abuznait et al. (2013)
	AMPK activation	Khanal et al. (2011)
	Inflammation reduction	Beauchamp et al. (2005) and Iacono et al. (2010)

needs to be confirmed *in vivo*, to exclude negative side effects on functional proteins. Nevertheless, the potent anti-inflammatory activity of OLC and its ability to promote Aβ degradation and clearance are valuable properties.

The molecular mechanism/s by which OLE and OLC evoke those multiple cellular responses must still be clarified. Do they enter cells and directly bind to target molecules or do they mainly interact with membranes thereby inducing a signalling cascade? Data obtained with unilamellar phosphatidylcholine vesicles using fluorescence anisotropy of probes and fluorescence quenching studies led to conclusions that both OLE and OLC remained at the surface of the lipid bilayer (Paiva-Martins et al. 2003). Yet experimental evidence provided more recently by the same authors using erythrocytes suggested that OLE not only interacts with the cellular membrane but may also penetrate into the bilayer to reach the radicals formed *in situ* (Paiva-Martins et al. 2010). One explanation for this discrepancy could be that biological membranes present microdomains of peculiar lipid composition that may facilitate phenols uptake. Accordingly, OLE (as well as oleuropein and other phenols and derivatives) was recently found inside the cytoplasm of human breast carcinoma cells exposed to an olive leaf extract (Quirantes-Pine et al. 2013) and of colon cancer cells exposed to EVOO extracts (Fernandez-Arroyo et al. 2012). These results leave the possibility of a direct intracellular signalling activity by EVOO phenols open to discussion.

Finally, in spite of the documented low bioavailability of these compounds, the positive results already obtained following *in vivo* administration confirm their efficacy, at least in model organisms. It is hoped that OLE and OLC will soon enter clinical trials to

rigorously assess their efficacy against AD during the different phases of disease progression, especially preclinical stages. Investigations employing different encapsulation strategies aimed at increasing the stability, bioavailability and brain targeting of these phenols are very promising and critical in this phase of the research.

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