Chemical Biology & Drug Design

WILEY

RESEARCH ARTICLE

Imino and Thioureidic Derivatives as New Tools for Alzheimer's Disease: Preliminary Studies

Anna Caruso[1,2] | Giuseppina Tommonaro[3] | Antonio Vassallo[1] | Debora Paris[3] | Magnus Monné[1] | Alessia Catalano[4] | Maria Stefania Sinicropi[2] | Carmela Saturnino[1]

[1]Department of Health Sciences, University of Basilicata, Potenza, Italy | [2]Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Arcavacata di Rende, Italy | [3]National Council of Researches of Italy-Institute of Biomolecular Chemistry (ICB), Pozzuoli, Italy | [4]Department of Pharmacy-Drug Sciences, University of Bari "Aldo Moro", Bari, Italy

Correspondence: Anna Caruso (anna.caruso@unibas.it; anna.caruso@unical.it)

Received: 29 October 2024 | **Revised:** 27 November 2024 | **Accepted:** 4 January 2025

Funding: The authors received no specific funding for this work.

Keywords: AChE | Alzheimer's disease | antioxidant | imino derivatives | inhibit AChE | neurodegenerative disease | thioureidic derivatives | Trolox

ABSTRACT

Alzheimer's disease is a neurodegenerative chronic disease with a severe social and economic impact in the societies, which still lacks an efficient therapy. Several pathophysiological events (β-amyloid [Aβ] deposits, τ-protein aggregation, loss of cholinergic activity, and oxidative stress) occurs in the progression of the disease. Therefore, the search for efficient multi-targeted agents for the treatment of Alzheimer's disease becomes indispensable. In this paper we evaluated the AChE inhibition by Ellman's method and antioxidant activity by DPPH assay of nine synthetic compounds: two hydroxy-benzene derivatives (**1** and **2**), three bis-thioureidic derivatives (**3–5**), two imidazole derivatives (**6** and **7**), and two phenylacetamide derivatives (**8** and **9**). The compound **2**, (3s,5s,7s)-adamantan-1-yl 4-(((*E*)-2,5-dihydroxybenzylidene)amino)benzoate, exhibited the best antioxidant activity ($30.00 \pm 1.05\,\mu$M eq Trolox) and compound **4** showed the highest AChE inhibition value (IC_{50} [μM] 8.40 ± 0.32). In the search for a compound showing combined activities (antioxidant and AChE inhibition), the compound **4**, octane-1,8-diyl-bis-*S*-amidinothiourea dihydrobromide, ($19.02 \pm 1.52\,\mu$M eq Trolox; IC_{50} [μM] 8.40 ± 0.32) was chosen to carry out a molecular docking study. The results showed that compound **4** has the ability to bind the active site of acetylcholinesterase with considerable affinity (estimated binding energies of -8.5 kcal/mol). All data indicate that compound **4** has the potential to be further investigated as a possible candidate in the Alzheimer's disease treatment.

1 | Introduction

Alzheimer disease (AD) is a pathological condition characterized by several cognitive impairments. Its pathophysiology is characterized by distinguishing features such as β-amyloid (Aβ) deposits, τ-protein aggregation, and oxidative stress (Long and Holtzman 2019; Caruso et al. 2009). Oxidative stress plays a critical role in AD. The mitochondria are cell organelles much susceptible to oxidative stress as the site of the electron transport chain for adenosine triphosphate (ATP) production and the main source of reactive oxygen species (ROS). ROS are metabolic byproducts that are necessary for physiological function but can be toxic at high levels. Levels of ROS increase with aging, impairing mitochondrial function and damaging above all the central nervous system. It is now known that accumulated oxidative stress is one of the key mechanisms causing cognitive aging and neurodegenerative diseases such as AD (Chen and Zhong 2014).

Moreover, age-associated loss of mitochondrial function affects the expression and processing of amyloid precursor protein (APP), producing Aβ oligomers that accumulate into plaques in Alzheimer's disease. Aβ is itself an effective source of oxidative

stress and the oxidative stress caused by this is likely due to the complexes it forms with metals, as copper, zinc, and iron. These bind to Aβ promoting its aggregation into plaques. In particular, copper forms the most stable bond, and this complex has been shown to produce superoxide and hydrogen peroxide. The oxidative stress caused by metal–amyloid complexes lead to excitotoxicity, promotes membrane depolarization, and impairs mitochondrial function. Recent studies have shown that the damaging effects of mitochondrial dysfunction can be mitigated, attenuating the pathogenesis of AD (Ionescu-Tucker and Cotman 2021). In addition, recent findings suggest the role of toxic aldehydes in the onset of AD (Catalano et al. 2024).

One of the main causes of neurodegeneration in AD is the loss of cortical cholinergic neurotransmission. Therefore, several drugs behave as inhibitors of AChE, the essential enzyme in the serine hydrolases family in cholinergic synapses that plays a crucial role in this disease (Zhang et al. 2023). Moreover, alteration in the activity of the neurotransmitter acetylcholine is observed. The treatment and management of AD are not only based on drugs that inhibit the activity of AChE and butylcholinesterase (BChE), which are key enzymes in the breakdown of this neurotransmitter (Pope and Brimijoin 2018; Saturnino et al. 2014), but also on drugs, as memantine, that block the flow of current through *N*-methyl-D-aspartate (NMDA) receptor channels, a subfamily of glutamate receptors involved in brain functions (Pichardo-Rojas et al. 2023; Rogawski and Wenk 2003).

Other classes of drugs for AD have been developed in recent years. In particular, several monoclonal antibodies (mAbs) targeting different Aβ species implicated in pathogenesis of AD. Second-generation mAbs, such as lecanemab, a humanized gamma immunoglobulin 1 (IgG1) against soluble and insoluble aggregated forms of the amyloid-β peptide, have shown promising clinical results (Cummings et al. 2023). This neurodegenerative disease is also related to oxidative stress (Collins, Saleh, and Kalisch 2022).

Among the first drugs used to restore acetylcholine levels, we remind tacrine (**I**) and afterward, donepezil (**II**), rivastigmine (**III**), and galantamine (**IV**) (Figure 1). Today, cholinesterase inhibitors and NMDA antagonists, including memantine (**V**), are used for AD (Pichardo-Rojas et al. 2023). AChE inhibitors prevent the hydrolysis of acetylcholine and memantine modulates NMDA receptor activity, leading to a reduction in excitatory glutamate signals. However, they cause several side effects (Zhang et al. 2019; Khan et al. 2020).

As is known, oxidative stress contributes to mental decline, neurodegeneration, and even dysfunctional pathways associated with brain aging (Ionescu-Tucker and Cotman 2021; Franzoni et al. 2021).

Oxidative stress often occurs in AD, promoting neurodegeneration. It has been shown that there are alterations in the phosphorylation of proteins, such as heme oxygenase-1 and biliverdin reductase A, thus intervening in the signaling of the most critical antioxidant pathways (Khan et al. 2020). These effects cause mitochondrial damage that can promote the increase in levels of reactive oxygen species (ROS) (Jomova et al. 2023; Caruso et al. 2020).

New therapies, targeting oxidative stress, tend to improve cognition and lower ROS levels. The use of antioxidants may have different effects not only on cognition in patients with AD but also in elderly patients; therefore, dosage, timing, combinations of antioxidants and diet must be carefully evaluated to obtain benefits (Ionescu-Tucker and Cotman 2021). Several studies have shown that molecules such as resveratrol (Buglio et al. 2022; Chimento et al. 2016) (**VI**) (Figure 2) reduce neuronal oxidative stress and cognitive decline in studies of patients with Mild Cognitive Impairment (MCI) and AD. Antioxidants are a powerful tool for reducing oxidative stress and improving cognition in the aging brain. The success of studies on antioxidants in reducing cognitive aging has stimulated interest in their potential as therapies for AD. Indeed, vitamin E (**VII**) (Figure 2) and targeted mitochondrial antioxidants reduced oxidative stress in cultured neurons and prevented amyloid beta toxicity (Butterfield and Halliwell 2019). Furthermore, high levels of vitamin E in blood plasma are related to a lower risk of AD (Mecocci et al. 2018). In fact, one study found that daily intake of alpha-tocopherol slows functional decline in AD patients (Dysken et al. 2014).

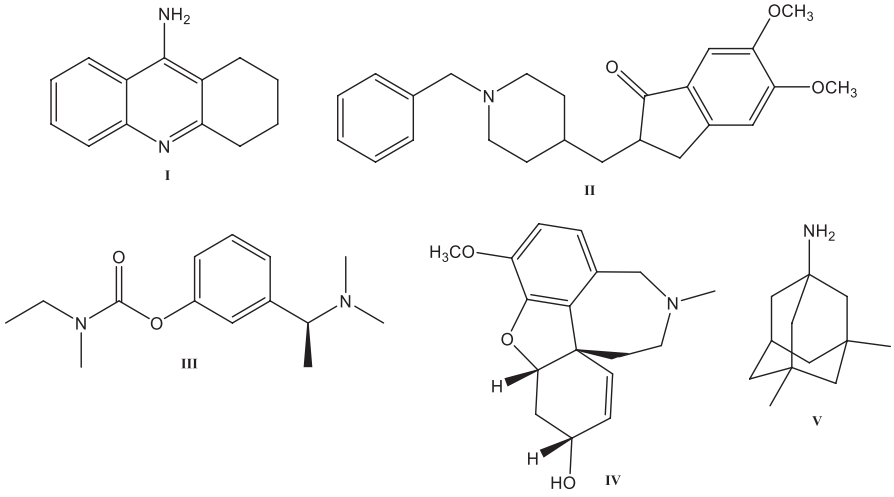


FIGURE 1 | Structures of Tacrine (**I**), Donepezil (**II**), Rivastigmine (**III**), Galantamine (**IV**), and Memantine (**V**).

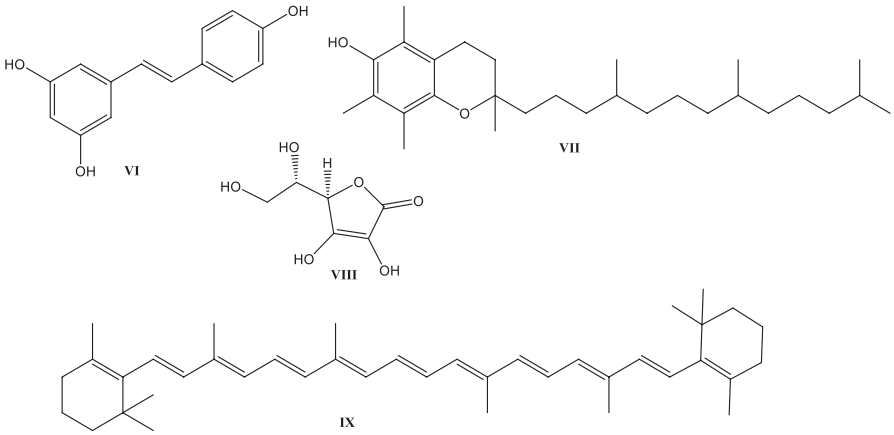


FIGURE 2 | Structures of Resveratrol (**VI**), Vitamin E (**VII**), L(+)-ascorbic acid (**VIII**), and β-carotene (**IX**).

The importance and role of high levels of homocysteine in cognitive decline in multiple forms of degenerative dementia, like Alzheimer's disease, have been renewed by a recent consensus statement (Smith et al. 2018). A clinical study in AD patients already showed that 8 months of polyphenol drinking lowered homocysteine levels (Morillas-Ruiz et al. 2010). Moreover, a recent study showed that intake of flavonols significantly reduced the incidence of Alzheimer's disease (Holland et al. 2020). Therefore, there are many studies that indicate a direct correlation between AD and serum total homocysteine (tHcy). Blood levels of homocysteine may be increased in AD and tHcy concentration may be influenced by administration of polyphenols (Smith et al. 2018; Morillas-Ruiz et al. 2010; Holland et al. 2020).

Different enzymatic systems protect the human body from oxidative stress; unfortunately, their functioning decreases with aging, and for this reason, it is increasingly necessary to introduce antioxidants through the diet or from supplements (Dizdar et al. 2018). Many compounds, present in food supplements, such as phenolic compounds as resveratrol (**VI**), vitamin E (**VII**), L-(+)-ascorbic acid (**VIII**), and carotenoids as β-carotene (**IX**) (Figure 2), show antioxidant activity (Dizdar et al. 2018; Iacopetta et al. 2017).

Numerous studies are available molecules designed with the aim to improve the AChE inhibition activity together with antioxidant activity. For example, a small library of benzoic-based amide nitrones with alkyl linker was synthesized and screened toward cholinesterase enzymes. In particular, the presence as well as the spacer length, was found to be an important contributor for AChEI modulation potency. From the studies carried out, none of the compounds showed BChE inhibitory activity (Oliveira et al. 2019).

For this reason, in this study, we evaluated the AChE inhibition and antioxidant activity of nine in-house compounds: two hydroxy-benzene derivatives (**1** and **2**) structurally similar to VI, three bis-thioureidic derivatives (**3–5**), long-chain compounds with the presence of double bonds as IX, two imidazole derivatives (**6** and **7**) with aliphatic chain as VII, and two phenylacetamide derivatives (**8** and **9**), compounds with aromatic rings separated by a chain as II and VI (Figure 3).

Compound **4**, which showed the highest AChE inhibition activity and moderate antioxidant activity, was chosen to carry out a molecular docking study in order to estimate the binding positions and affinity of the complex target/ligand.

2 | Results and Discussion

2.1 | Chemistry

Bis-thioureidic derivatives **3** and **5** were synthesized by one-pot reaction as reported in Scheme 1.

Compounds **1–9** were tested for their AChE inhibition and antioxidant activities, by means of Ellman's method and DPPH assay, respectively, at a concentration of 0.5 μM.

2.2 | Acetylcholinesterase Inhibition

From the screening for AChE inhibition activity, samples were assayed at a concentration of 5 μM up to 100 μM with the aim to calculate the IC_{50} value (Figure 4). Samples **4** and **9**, that were not chemically related, displayed significant values of IC_{50} (Table 1), 8.40 ± 0.32 and 10.63 ± 1.20 μM, respectively. In particular, compound **4** showed the best AChE inhibition activity ($IC_{50} = 8.40$ μM). Compounds **6** ($IC_{50} = 65.02 \pm 3.40$) and **7** ($IC_{50} = 15.66 \pm 1.28$) although chemically related, as well as compounds **8** ($IC_{50} = 24.19 \pm 1.87$) and **9** ($IC_{50} = 10.63 \pm 1.20$), exhibited very different AChE inhibition activity as evidenced by the IC_{50} values. In contrast, compounds **1** and **2** show no AChE inhibition activity.

2.3 | Antioxidant Activity

Among all samples, **1**, **2**, and **4** showed the most interesting antioxidant activity compared with Trolox (Table 2). Indeed, the three samples exhibited values of percentage of DPPH inhibition ranging between 49.46% and 77.88% at a concentration of 0.5 μM. The same values were registered for Trolox, but at higher concentration (between 19.02 and 30.00 μM) (Figure 5). Instead,

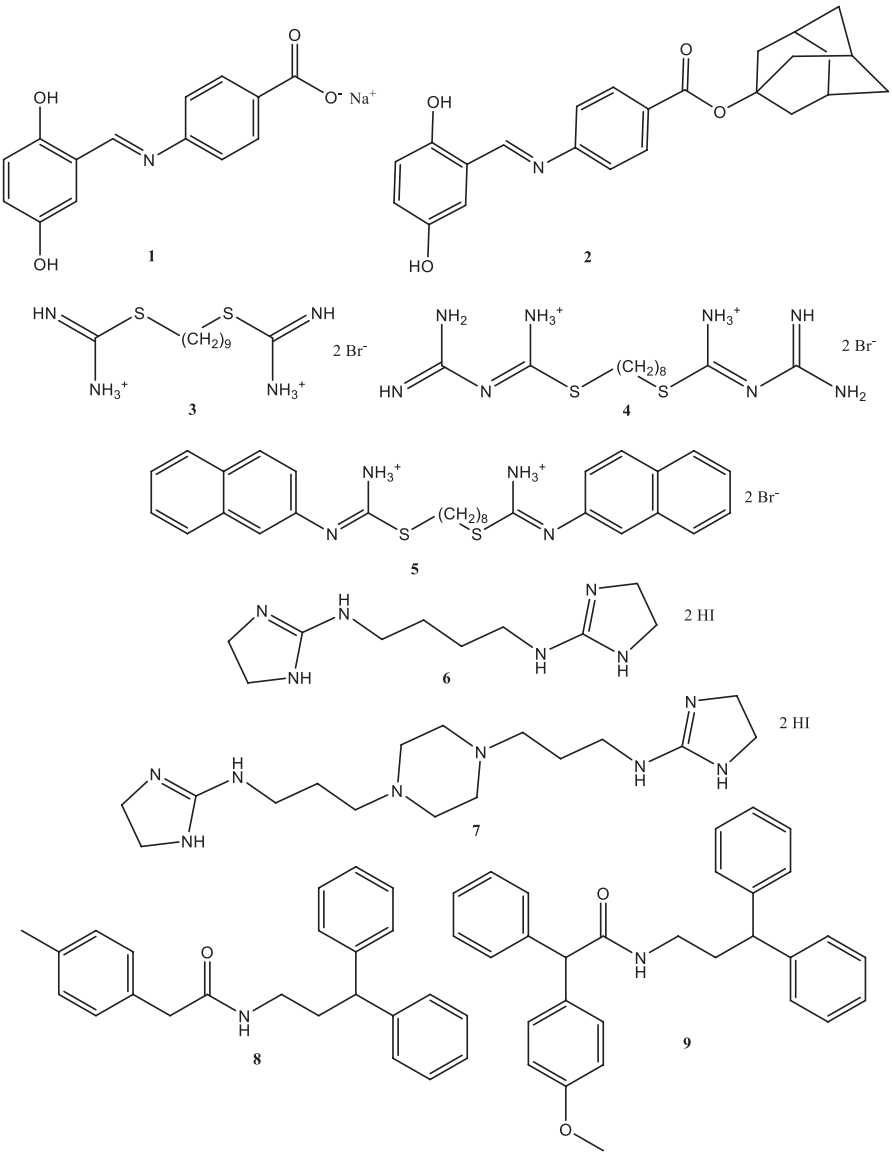
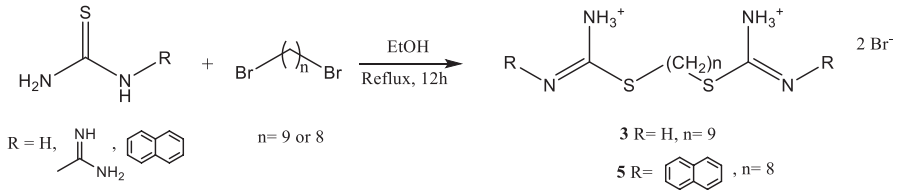


FIGURE 3 | Structures of: (*E*)-4-((2,5-Dihydroxybenzylidene))imino-sodium benzoate (**1**); (3s,5s,7s)-Adamantan-1-yl 4-(((*E*)-2,5-dihydroxybenzylidene)amino)benzoate (**2**); 2,2′-(Nonane-1,9-diyl)diisothiouronium-dibromide (**3**); Octane-1,8-diyl-bis-*S*-amidinothiourea-dihydrobromide (**4**); 2,2′-(Octane-1,8-diyl)bis(1-(naphthalen-2-yl))isothiouronium-dibromide (**5**); N^1,N^4-Bis(4,5-dihydro-1*H*-imidazol-2-yl)butane-1,4-diamine-dihydroiodide (**6**); 1,4-Piperazine-bis(propanamine-*N*-(4,5-dihydro-1*H*-imidazol-2-yl))dihydroiodide (**7**); *N*-(3,3-diphenylpropyl)-2-(p-tolyl)acetamide (**8**); and *N*-(3,3-diphenylpropyl)-2-(4-methoxyphenyl)-2-phenylacetamide (**9**).

compound **8** showed a significantly lower antioxidant activity (0.34 ± 0.06 corresponding to $0.13 \pm 0.05\,\mu$M eq Trolox).

2.4 | Molecular Docking of Compound 4 in the Active Site of Acetylcholinesterase

Docking studies were performed with a structural homology model of acetylcholinesterase to get further insight into the inhibitory effects of compound **4** on the activity of this enzyme. The docking procedure provides a search in conformational space leaving all possible rotamers of the ligand and binding pocket side chains flexible. The highest ranking docking solution indicated that compound **4** has the ability to bind the active site of acetylcholinesterase with considerable affinity (estimated binding energies of −8.5 kcal/mol). The results showed that compound **4** binds with one of the terminal amidinothiourea groups deeply buried into the binding pocket of acetylcholinesterase, the hydrocarbon chain linkage approximately where galantamine is located in the structure and the other terminal amidinothiourea is pointing towards the entrance of the pocket (Figure 6). The more penetrating amidinothiourea moiety forms hydrogen bonds with the side chains of Y155 as well as the backbone of W108 and G142. The outer amidinothiourea moiety forms several hydrogen bonds with the side chains of Q93, D96, N109, Y146 and S147, as well as the backbone of W108. Besides the hydrogen bonds there are several additional interactions between compound **4** and acetylcholinesterase (Figure 6). This top pose of compound **4** in the binding pocket of acetylcholinesterase would mean that it blocks substrate entry similarly to galantamine, which also binds close to the catalytic triad.



SCHEME 1 | One-pot reaction for the preparation of compounds **3** and **5**.

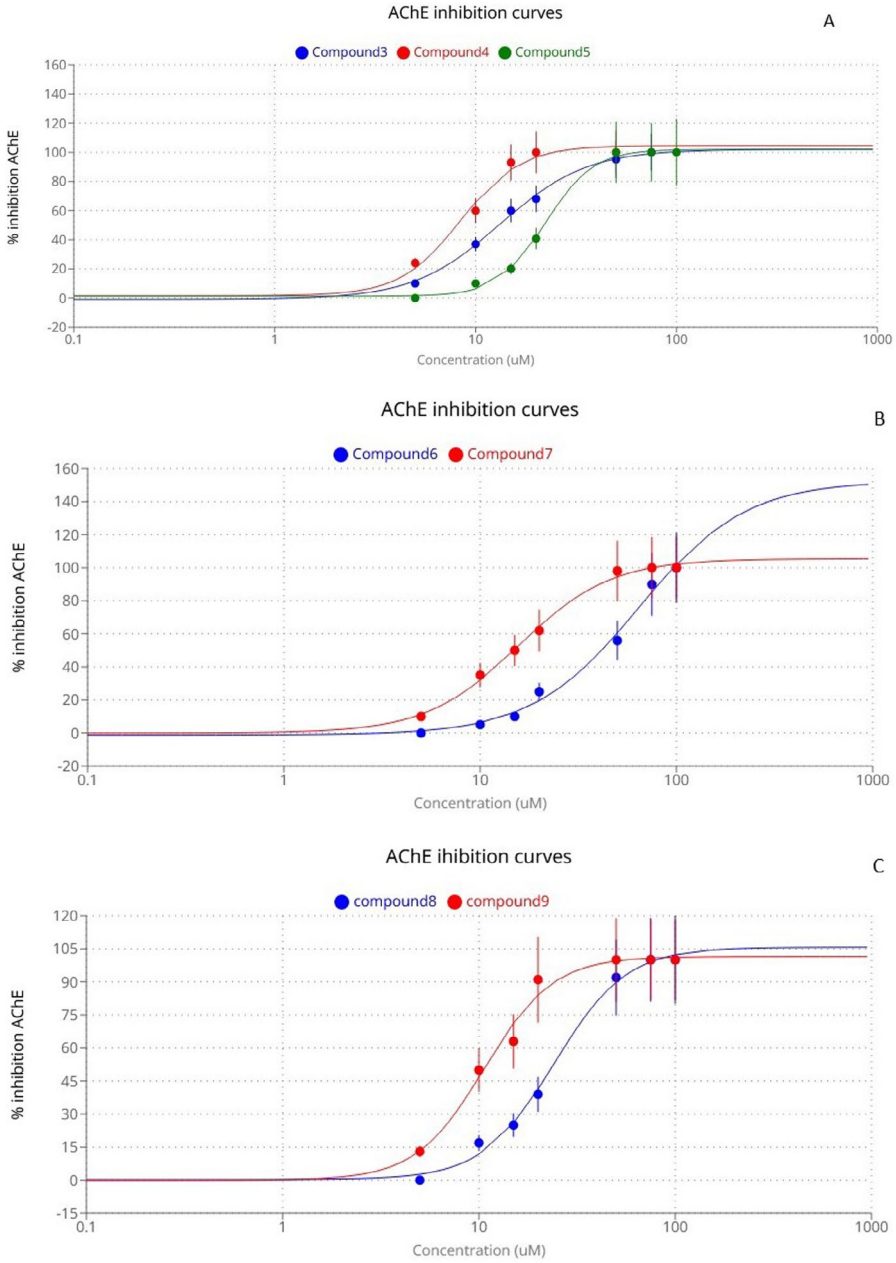


FIGURE 4 | AChE inhibition curve of compounds: (A) **3**, **4**, and **5**; (B) **6** and **7**; (C) **8** and **9**. AChE inhibition values were measured in triplicate for each compound. Data are expressed as mean value ± standard deviation.

In summary, for this study, we chose to test compounds **1–9** as they have structural similarities with known anti-Alzheimer and antioxidant drugs (**I–IX**). In particular, compounds **1** and **2** have a structure similar to resveratrol (**VI**) with two aromatic rings linked by a two-membered chain with a double bond. Furthermore, compound **2** presents the adamantane core as memantine (**V**). Instead, compounds **3**, **4**, and **5** were chosen as they have a long chain like carotene (**IX**). It is known that, carotenoids (carotene, astaxanthin, lycopene, lutein, fucoxanthin, crocin, and others) are reported to be used in the treatment of several diseases, including AD (Su et al. 2023). Carotenoid astaxanthin showed efficacy in the reducing of

TABLE 1 | AChE inhibition activity of samples expressed as IC_{50} (μM).

Samples	IC_{50} (μM) ± SD
1	N.A.
2	N.A.
3	13.20 ± 1.80
4	8.40 ± 0.32
5	22.26 ± 1.99
6	65.02 ± 3.40
7	15.66 ± 1.28
8	24.19 ± 1.87
9	10.63 ± 1.20
Galantamine	0.65 ± 0.05

Note: Data are expressed as mean value of three measurements ± standard deviation. Galantamine was used as standard.
Abbreviation: N.A., no activity.

TABLE 2 | Antioxidant activity of samples evaluated by means of DPPH assay compared with Trolox (standard).

Samples	% Inhibition at 0.5 μM (±SD)	μM eq Trolox (±SD)
1	55.80 ± 3.21	21.46 ± 0.74
2	77.88 ± 4.53	30.00 ± 1.05
3	11.53 ± 2.06	4.43 ± 0.20
4	49.46 ± 2.61	19.02 ± 1.52
5	5.12 ± 0.51	1.97 ± 0.52
6	22.84 ± 1.22	8.78 ± 0.87
7	14.70 ± 1.32	5.65 ± 0.12
8	0.34 ± 0.06	0.13 ± 0.05
9	8.03 ± 0.73	3.09 ± 0.47

Note: Values were measured in triplicate for each compound. Data are expressed as mean value ± standard deviation.

neurotoxicity induced by the Aβ fragments in cell culture models of AD (Lobos et al. 2016; Wang et al. 2010). In vitro and in vivo studies demonstrated that pretreatment with lycopene reduced Aβ-induced cellular damage and prevented Aβ 1–42-stimulated cellular apoptosis via the inhibition of ROS production (Ratto et al. 2022; Dias et al. 2014). However, numerous reports are available on synthetic or semisynthetic compounds containing long alkyl chains linked to nitrogen or sulfur together with moieties/heterocycles or positively charged groups like compounds **3**, **4**, and **5**. These compounds were designed with the aim to improve the AChE inhibition activity together with antioxidant activity. The simple structure of tacrine (**I**) make it the scaffold for developing new molecules with additional chemical moieties (long alkyl chains, charged groups) showing significant cognitive improvements as well as their antioxidant activity and reduced hepatotoxicity (Bubley et al. 2023). In fact, compound **5** has an aromatic bicyclic system and amine functionality as in tacrine (**I**).

Compounds **6** and **7** contains the imidazole ring, analogous to dihydrofuran (they have isoster groups, NH in the imidazole O in the dihydrofuran) which is essential for the antioxidant activity of L(+)-ascorbic acid (**VIII**), and for the aliphatic chain, also present in vitamin E (**VII**). Furthermore, derivative **7** presents the piperazine moiety, similar to the piperidine moiety present in donepezil (**II**). Compounds **8** and **9** have similar chemical properties to AChE inhibitors as donepezil (**II**) and rivastigmine (**III**); in particular, aromatic rings at the ends of the molecule, diphenyl and *p*-tolyl for **8** and methoxyphenyl-phenyl and diphenyl for **9** (in donepezil (**II**), phenyl and indanone group, portions involved in interactions with the active site of the AChE (Sugimoto et al. 2000)), linked by a chain containing an amide group as in rivastigmine (**III**).

Among all tested compounds, three of them exhibited the most significant AChE inhibition: derivatives **3**, **4**, and **9** with IC_{50} values of 13.20, 8.40, and 10.63 μM, respectively. These results suggested that two main chemical features influence the enzyme inhibition: the presence of charged functional groups and the stereochemistry of molecules. Compounds **3** and **4** have a linear structure with two positively charged residues of thiourea. These residues could interact with anionic subsite of AChE by electrostatic interactions. Compound **9** possesses four aromatic rings symmetrically arranged that, likely, could be responsible of π-stacking interactions with aminoacidic residues of AChE at peripheral site.

The compound **4** showed a moderate antioxidant activity and the highest AChE inhibition activity. Furthermore, the results of our docking study suggest that **4** is compatible with acetylcholinesterase binding and provide some insights into potential interactions formed between the inhibitor and the active site residues.

3 | Materials and Methods

3.1 | Chemistry

Commercial reagents were purchased from Aldrich and Alfa Aesar and used without additional purification. Melting points were determined on a Kofler melting point apparatus. ESI-MS spectra were performed on a Waters Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray ion source. ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) were recorded on a Bruker Avance 300 MHz spectrometer. Chemical shifts were expressed in parts per million downfield from tetramethylsilane as an internal standard.

Compounds **1** and **2** (Figure 3) were obtained as reported in the literature (Longo et al. 2019) using *p*-hydroxy-benzene derivative as starting material. Bis-thioureidic derivatives **3**–**5** (Figure 3) were synthesized by one-pot reaction (Saturnino et al. 2003; Ceramella et al. 2020) using thiourea or suitable thiourea and dibromoalkane. Compound **4** was synthesized

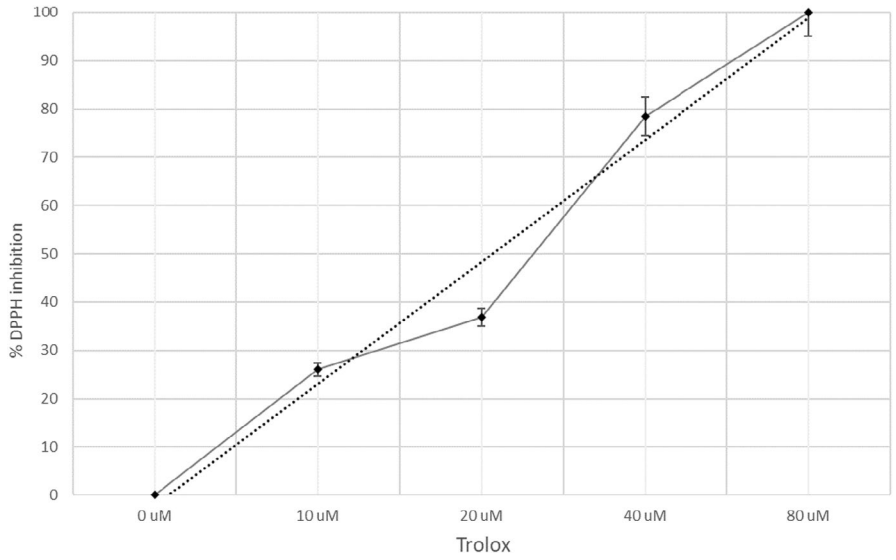


FIGURE 5 | DPPH inhibition curve of Trolox (standard). DPPH inhibition values were measured in triplicate. Data are expressed as mean value ± standard deviation.

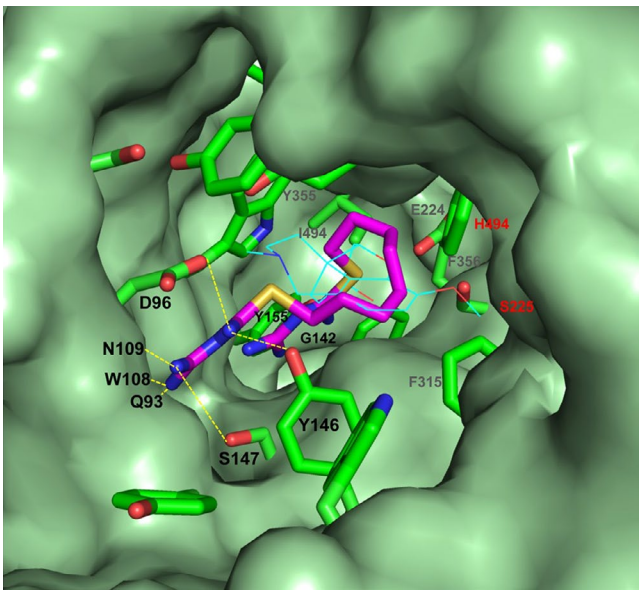


FIGURE 6 | Docking result of compound **4** and the original position of galantamine in the homology model of *Electrophorus electricus* acetylcholinesterase. Compound **4** is shown with sticks and carbons in magenta, and galantamine with lines and carbons in cyan. Flexible residues are displayed with sticks and carbons in green in the active site of acetylcholinesterase, and residues forming hydrogen bonds with compound **4** are indicated in black, other interactions in grey, and the catalytic triad in red.

as previously described (Ceramella et al. 2020). Bis-imidazole derivatives **6** and **7** (Figure 3) were prepared using suitable diamines which were reacted with 2-methylthio-2-imidazoline hydroiodide (molar ratio 1:2) (Ceramella et al. 2020). Phenylacetamide derivatives **8–9** were obtained by direct condensation (Urbani et al. 2008) of *p*-tolylacetic acid (for **8**) and 2-(4-methoxyphenyl)-2-phenylacetic acid (for **9**) and appropriate amines using *n*-propylphosphonic acid anhydride (PPAA) as catalyst (Figure 3).

3.1.1 | General Procedure for the Synthesis of Compounds 3 and 5

Thiourea or amidinothiourea or (naphthalen-2-yl)thiourea (1 equiv.) and 1,9-dibromopentane or 1,8-dibromopentane (0.5 equiv.) were refluxed in ethanol 95% for 12 h. The reaction mixture was evaporated to dryness under vacuum. The residue was washed three times with diethyl ether and then taken up with hot CH_3CN and crystallized giving **3–5** (70%–75% yield) as a white solid.

2,2′-(Nonane-1,9-diyl)diisothiouroniumdibromide (**3**): Mp 202°C–203°C (CH_3CN); ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.90–7.90 (bs, 8H, 2 NH, 2 NH_3^+); 2.98–2.89 (m, 4H, 2 SCH_2); 1.82–1.26 (m, 14H, 7 CH_2); ^{13}CNMR (75 MHz, DMSO-d_6): δ 165.15, 31.32, 30.14, 28.52, 27.35, and 26.97. MS (ESI, CH_3OH) $m/z = 278.15$ $[C_{11}H_{26}N_4S_2]^{2+}$.

2,2′-(Octane-1,8-diyl)bis(1-(naphthalen-2-yl))isothiouronium-dibromide (**5**): Mp 187°C–188°C (CH_3CN); ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.20–8.00 (m, 8H, Ar); 7.60–7.40 (m, 6H, Ar); 7.32 (s, 6H, 2 NH_3^+); 3.42–2.98 (m, 4H, 2 SCH_2); 1.87–1.34 (m, 12H, 6 CH_2); ^{13}CNMR (75 MHz, DMSO-d_6): δ 164.19, 147.32, 135.32, 132.21, 128.32, 127.43, 126.67, 120.54, 119.65, 30.38, 28.32, 28.29, and 26.54. MS (ESI, CH_3OH) $m/z = 516.23$ $[C_{30}H_{36}N_4S_2]^{2+}$.

3.2 | AChE Inhibition Activity

AChE inhibition activity was evaluated by Ellman's method (Ellman et al. 1961; Tommonaro et al. 2016) with some modifications. Briefly, the reaction was performed in multi-well Petri dishes of 48 wells in a final volume of 500 mL 0.036 U/mL of EeAChE (Sigma Aldrich, Milan, Italy) in 0.1 M pH = 8 phosphate buffer was incubated for 15 min at different sample concentrations (from 5 μM up to 100 μM) at 37°C. Afterward, the reaction was triggered by the addition of 0.35 mM acetylthiocholineiodide (ATChI) and 0.35 mM of 5,5′-dithiobis-2-(nitrobenzoic

acid) (DTNB). Changes in absorbance were measured at 410 nm in a BiotekPowerWaveXS spectrophotometer microplate reader. IC_{50} values were calculated as the concentration of the compound yielding 50% AChE activity inhibition. Galantamine was used as standard.

3.3 | Antioxidant Activity-DPPH Assay

Antioxidant activity of samples **1–9** was evaluated both as percentage inhibition of DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical and as μM equivalent of Trolox, the water-soluble analog of vitamin E which shows several advantages (Lúcio et al. 2009). It was used as a standard chemical for comparing free radical scavenging activity expressed as Trolox equivalent antioxidant capacity (TEAC) (Frangu et al. 2020). Specifically, antioxidant activity of samples was determined by means of DPPH method (Blois 1958; Tommonaro et al. 2021); therefore, 50 μL of sample solution (concentration of 0.5 μM) was added to 0.7 mL of DPPH in methanol (6 mg/50 mL; 0.1 mM final concentration) and adjusted to a final volume of 2 mL with methanol. The absorbance was determined after 30 min at $\lambda = 517$ nm at room temperature, and the percentage of free radical inhibition was calculated.

3.4 | Molecular Docking Studies

In the docking studies, an energy minimized structural homology model of *Electrophorus electricus* acetylcholinesterase (as used in the inhibition assay), generated by Swiss-model based on the structures of its counterpart from *Tetronarce californica* (Faraone et al. 2020), was used. In silico molecular docking of conformationally flexible **4** into the semi-rigid homology model of acetylcholinesterase was performed with AutoDock Vina (Trott and Olson 2010). Selected flexible residues were Q93, Y94, D96, S98, W108, W139, Y146, S147, Y155, E224, S225, S251, W258, W304, F313, F315, E352, Y355, F356, Y359, H494, Y496, E497, and I498. Ligand interactions were analyzed with PyMOL and PISA (Kissinel and Henrick 2007).

4 | Conclusions

In the present study, we investigated for ability to inhibit the AChE by a spectrophotometrically assay based on an Ellman's method and antioxidant activity of nine compounds selected in a chemical library. Their efficiency as radical scavengers was evaluated by their reactivity toward the stable free radical DPPH (Paesano et al. 2005) and as μM equivalent of Trolox. Among all tested molecules, compound **4** showed a good antioxidant activity and the highest AChE inhibition activity. Also, for **4**, molecular docking studies of AChE were done to estimate the binding positions and affinity of the complex target/ligand. These results suggested that octane-1,8-diyl-bis-*S*-amidinothiourea dihydrobromide (**4**) meets the requirements to be considered a good antioxidant and an AChE inhibitor, which suggests that this compound could be used as a potential active ingredient in some medicines.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

References

Blois, M. 1958. "Antioxidant Determinations by the Use of a Stable Free Radical." *Nature* 181: 1199–1200.

Bubley, A., A. Erofeev, P. Gorelkin, E. Beloglazkina, A. Majouga, and O. Krasnovskaya. 2023. "Tacrine-Based Hybrids: Past, Present, and Future." *International Journal of Molecular Sciences* 24: 1717. https://doi.org/10.3390/ijms24021717.

Buglio, D. S., L. T. Marton, L. F. Laurindo, et al. 2022. "The Role of Resveratrol in Mild Cognitive Impairment and Alzheimer's Disease: A Systematic Review." *Journal of Medicinal Food* 25: 797–806. https://doi.org/10.1089/jmf.2021.0084.

Butterfield, D. A., and B. Halliwell. 2019. "Oxidative Stress, Dysfunctional Glucose Metabolism and Alzheimer Disease." *Nature Reviews Neuroscience* 20: 148–160. https://doi.org/10.1038/s41583-019-0132-6.

Caruso, A., A. Barbarossa, A. Tassone, et al. 2020. "Pomegranate: Nutraceutical With Promising Benefits on Human Health." *Applied Sciences* 10: 6915–6948. https://doi.org/10.3390/app10196915.

Caruso, A., A. Garofalo, F. Grande, et al. 2009. "Synthesis and Biological Evaluation of 1,3-Idandione Derivatives as Acetylcholinesterase Inhibitors." *Pharmacology* 1: 264–277. https://doi.org/10.1021/jm00024a009.

Catalano, A., A. Mariconda, A. D'Amato, et al. 2024. "Aldehydes: What We Should Know About Them." *Organ* 5: 395–428. https://doi.org/10.3390/org5040021.

Ceramella, J., A. Mariconda, C. Rosano, et al. 2020. "α–ω Alkenyl-Bis-S-Guanidine Thiourea Dihydrobromide Affects HeLa Cell Growth Hampering Tubulin Polymerization." *ChemMedChem* 15: 2306–2316. https://doi.org/10.1002/cmdc.202000544.

Chen, Z., and C. Zhong. 2014. "Oxidative Stress in Alzheimer's Disease." *Neuroscience Bulletin* 30: 271–281. https://doi.org/10.1007/s12264-013-1423-y.

Chimento, A., R. Sirianni, C. Saturnino, A. Caruso, M. S. Sinicropi, and V. Pezzi. 2016. "Resveratrol and Its Analogs as Antitumoral Agents for Breast Cancer Treatment." *Mini Reviews in Medicinal Chemistry* 16: 699–709. https://doi.org/10.2174/1389557516666160321113255.

Collins, A. E., T. M. Saleh, and B. E. Kalisch. 2022. "Naturally Occurring Antioxidant Therapy in Alzheimer's Disease." *Antioxidants* 11: 213–250. https://doi.org/10.3390/antiox11020213.

Cummings, J., L. Apostolova, G. D. Rabinovici, et al. 2023. "Lecanemab: Appropriate Use Recommendations." *Journal of Prevention of Alzheimer's Disease* 10: 362–377. https://doi.org/10.14283/jpad.2023.30.

Dias, I. H. K., M. C. Polidori, L. Li, et al. 2014. "Plasma Levels of HDL and Carotenoids Are Lower in Dementia Patients With Vascular Comorbidities." *Journal of Alzheimer's Disease* 40: 399–408. https://doi.org/10.3233/JAD-131964.

Dizdar, M., D. Vidic, F. Požgan, B. Štefane, and M. Maksimović. 2018. "Acetylcholinesterase Inhibition and Antioxidant Activity of N-Trans-Caffeoyldopamine and N-Trans-Feruloyldopamine." *Scientia Pharmaceutica* 86: 11–22. https://doi.org/10.3390/scipharm86020011.

Dysken, M. W., P. D. Guarino, J. E. Vertrees, et al. 2014. "Vitamin E and Memantine in Alzheimer's Disease: Clinical Trial Methods and Baseline Data." *Alzheimer's & Dementia* 10: 36–44. https://doi.org/10.1016/j.jalz.2013.01.014.

Ellman, G. L., K. D. Courtney, B. J. Andres, and R. M. Feather-Stone. 1961. "A New and Rapidcolorimetric Determination of Acetylcholinesterase Activity." *Biochemical Pharmacology* 7: 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

Faraone, I., D. Russo, L. Chiummiento, et al. 2020. "Phytochemicals of *Minthostachys diffusa* Epling and Their Health-Promoting Bioactivities." *Food* 9: 144–161. https://doi.org/10.3390/foods9020144.

Frangu, A., A. M. Ashrafi, M. Sýs, et al. 2020. "Determination of Trolox Equivalent Antioxidant Capacity in Berries Using Amperometrictyrosinase Biosensor Based on Multi-Walled Carbon Nanotubes." *Applied Sciences* 10: 2497–2509. https://doi.org/10.3390/app10072497.

Franzoni, F., G. Scarfò, S. Guidotti, J. Fusi, M. Asomov, and C. Pruneti. 2021. "Oxidative Stress and Cognitive Decline: The Neuroprotective Role of Natural Antioxidants." *Frontiers in Neuroscience* 15: 729757. https://doi.org/10.3389/fnins.2021.729757.

Holland, T. M., P. Agarwal, Y. Wang, et al. 2020. "Dietary Flavonols and Risk of Alzheimer Dementia." *Neurology* 94: e1749–e1756. https://doi.org/10.1212/WNL.0000000000008981.

Iacopetta, D., F. Grande, A. Caruso, et al. 2017. "New Insights for the Use of Quercetinanalogs in Cancer Treatment." *Future Medicinal Chemistry* 9: 2011–2028. https://doi.org/10.4155/fmc-2017-0118.

Ionescu-Tucker, A., and C. W. Cotman. 2021. "Emerging Roles of Oxidative Stress in Brain Aging and Alzheimer's Disease." *Neurobiology of Aging* 107: 86–95. https://doi.org/10.1016/j.neurobiolaging.2021.07.014.

Jomova, K., R. Raptova, S. Y. Alomar, et al. 2023. "Reactive Oxygen Species, Toxicity, Oxidative Stress, and Antioxidants: Chronic Diseases and Aging." *Archives of Toxicology* 97: 2499–2574. https://doi.org/10.1007/s00204-023-03562-9.

Khan, H., H. Ullah, M. Aschner, W. S. Cheang, and E. K. Akkol. 2020. "Neuroprotective Effects of Quercetin in Alzheimer's Disease." *Biomolecules* 10: 59–78. https://doi.org/10.3390/biom10010059.

Kissinel, E., and K. Henrick. 2007. "Inference of Macromolecular Assemblies From Crystalline State." *Journal of Molecular Biology* 372: 774–797. https://doi.org/10.1016/j.jmb.2007.05.022.

Lobos, P., B. Bruna, A. Cordova, et al. 2016. "Astaxanthin Protects Primary Hippocampal Neurons Against Noxious Effects of Abeta-Oligomers." *Neural Plasticity* 2016: 3456783. https://doi.org/10.1155/2016/3456783.

Long, J. M., and D. M. Holtzman. 2019. "Alzheimer Disease: An Update on Pathobiology and Treatment Strategies." *Cell* 179: 312–339. https://doi.org/10.1016/j.cell.2019.09.001.

Longo, P., C. Saturnino, C. Arra, et al. 2019. "Hydroxybenzene Derivatives Having a N-Aryl Substitute Imino Group and Use Thereof in the Treatment of Solid Tumours." Patent No: EP3558932A1. Publication Date: 2019-10-30.

Lúcio, M., C. Nunes, D. Gaspar, H. Ferreira, J. L. Lima, and S. Reis. 2009. "Antioxidant Activity of Vitamin E and Trolox: Understanding of the Factors That Govern Lipid Peroxidation Studies In Vitro." *Food Biophysics* 4: 312–320. https://doi.org/10.1007/s11483-009-9129-4.

Mecocci, P., V. Boccardi, R. Cecchetti, et al. 2018. "A Long Journey Into Aging, Brain Aging, and Alzheimer's Disease Following the Oxidative Stress Tracks." *Journal of Alzheimer's Disease* 62: 1319–1335. https://doi.org/10.3233/JAD-170732.

Morillas-Ruiz, J. M., J. M. Rubio-Perez, M. D. Albaladejo, P. Zafrilla, S. Parra, and M. L. Vidal-Guevara. 2010. "Effect of an Antioxidant Drink on Homocysteine Levels in Alzheimer's Patients." *Journal of the Neurological Sciences* 299: 175–178. https://doi.org/10.1016/j.jns.2010.08.050.

Oliveira, C., D. Bagetta, F. Cagide, et al. 2019. "Benzoic Acid-Derived Nitrones: A New Class of Potential Acetylcholinesterase Inhibitors and Neuroprotective Agents." *European Journal of Medicinal Chemistry* 174: 116–129. https://doi.org/10.1016/j.ejmech.2019.04.026.

Paesano, N., S. Marzocco, C. Vicidomini, et al. 2005. "Synthesis and Biological Evaluation of 3-Benzyl-1-Methyl- and 1-Methyl-3-Phenyl-Isothioureas as Potential Inhibitors of iNOS." *Bioorganic & Medicinal Chemistry Letters* 15: 539–543. https://doi.org/10.1016/j.bmcl.2004.11.047.

Pichardo-Rojas, D., P. S. Pichardo-Rojas, J. M. Cornejo-Bravo, and A. Serrano-Medina. 2023. "Memantine as a Neuroprotective Agent in Ischemic Stroke: Preclinical and Clinical Analysis." *Frontiers in Neuroscience* 17: 5–14. https://doi.org/10.3389/fnins.2023.1096372.

Pope, C. N., and S. Brimijoin. 2018. "Cholinesterases and the Fine Line Between Poison and Remedy." *Biochemical Pharmacology* 153: 205–216. https://doi.org/10.1016/j.bcp.2018.01.044.

Ratto, F., F. Franchini, M. Musicco, G. Caruso, and S. G. Di Santo. 2022. "A Narrative Review on the Potential of Tomato and Lycopene for the Prevention of Alzheimer's Disease and Other Dementias." *Critical Reviews in Food Science and Nutrition* 62: 4970–4981. https://doi.org/10.1080/10408398.2021.1880363.

Rogawski, M. A., and G. L. Wenk. 2003. "The Neuropharmacological Basis for the Use of Memantine in the Treatment of Alzheimer's Disease." *CNS Drug Reviews* 9: 275–308. https://doi.org/10.1111/j.1527-3458.2003.tb00254.x.

Saturnino, C., M. Buonerba, N. Paesano, J. C. Lancelot, and G. De Martino. 2003. "In Vitro Anti-Acanthamoeba Action by Thioureidic Derivatives." *Il Farmaco* 58: 819–822. https://doi.org/10.1016/S0014-827X(03)00138-1.

Saturnino, C., D. Iacopetta, M. S. Sinicropi, et al. 2014. "N-Alkyl Carbazole Derivatives as New Tools for Alzheimer's Disease: Preliminary Studies." *Molecules* 19: 9307–9317. https://doi.org/10.3390/molecules19079307.

Smith, A. D., H. Refsum, T. Bottiglieri, et al. 2018. "Homocysteine and Dementia: An International Consensus Statement." *Journal of Alzheimer's Disease* 62: 561–570. https://doi.org/10.3233/JAD-171042.

Su, W., W. Xu, E. Liu, W. Su, and N. E. Polyakov. 2023. "Improving the Treatment Effect of Carotenoids on Alzheimer's Disease Through Various Nano-Delivery Systems." *International Journal of Molecular Sciences* 24: 7652. https://doi.org/10.3390/ijms24087652.

Sugimoto, H., Y. Yamanish, Y. Iimura, and Y. Kawakami. 2000. "Donepezil Hydrochloride (E2020) and Other Acetylcholinesterase Inhibitors." *Current Medicinal Chemistry* 7: 303–339. https://doi.org/10.2174/0929867003375191.

Tommonaro, G., G. R. Abbamondi, B. Nicolaus, et al. 2021. "Productivity and Nutritional Trait Improvements of Different Tomatoes Cultivated With Effective Microorganisms Technology." *Agriculture* 11: 112–121. https://doi.org/10.3390/agriculture11020112.

Tommonaro, G., N. García-Font, R. M. Vitale, et al. 2016. "Avarol Derivatives as Competitive AChE-Inhibitors, Non Hepatotoxic and Neuroprotective Agents for Alzheimer's Disease." *European Journal of Medicinal Chemistry* 122: 326–338. https://doi.org/10.1016/j.ejmech.2016.06.036.

Trott, O., and A. J. Olson. 2010. "AutoDockVina: Improving the Speed and Accuracy of Docking With a New Scoring Function, Efficient Optimization, and Multithreading." *Journal of Computational Chemistry* 31: 455–461. https://doi.org/10.1002/jcc.21334.

Urbani, P., A. Ramunno, R. Filosa, et al. 2008. "Antioxidant Activity of Diphenylpropionamide Derivatives: Synthesis, Biological Evaluation and Computational Analysis." *Molecules* 13: 749–761. https://doi.org/10.3390/molecules13040749.

Wang, H.-Q., X.-B. Sun, Y.-X. Xu, H. Zhao, Q.-Y. Zhu, and C.-Q. Zhu. 2010. "Astaxanthin Upregulates Heme Oxygenase-1 Expression

Through ERK1/2 Pathway and Its Protective Effect Against Beta-Amyloid-Induced Cytotoxicity in SH-SY5Y Cells." *Brain Research* 1360: 159–167. https://doi.org/10.1016/j.brainres.2010.08.100.

Zhang, Y., H. Chen, R. Li, K. Sterling, and W. Song. 2023. "Amyloid β-Based Therapy for Alzheimer's Disease: Challenges, Successes and Future." *Signal Transduction and Targeted Therapy* 8: 248–273. https://doi.org/10.1038/s41392-023-01484-7.

Zhang, Y., S. Li, Y. Wang, et al. 2019. "Potential Pharmacokinetic Drug–Drug Interaction Between Harmine, a Cholinesterase Inhibitor, and Memantine, a Non-Competitive N-Methyl-d-Aspartate Receptor Antagonist." *Molecules* 24: 1430–1446. https://doi.org/10.3390/molecules24071430.