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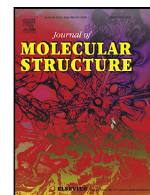

7,8-Dihydroxycoumarin derivatives: *In silico* molecular docking and *in vitro* anticholinesterase activity

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ABSTRACT

In this study, acetylcholinesterase enzyme (AChE) inhibition potential and antioxidant activity of eight different coumarin derivatives together with two (**5** and **8**) newly synthesized coumarins were investigated. The results showed that all compounds exhibited inhibitory activity on AChE. Compounds **1** (96.83%), **3** (96.72%), and **2** (95.48%) showed the highest inhibitory activity and the results were more significant than that of galantamine (93.14%). Compound **7** displayed the most potent inhibition of AChE (92.12%), close to galantamine. Molecular docking studies of AChE were carried out to support *in vitro* testing. In addition, the antioxidant activities of coumarins were performed with DPPH, FRAP, and CUPRAC methods. Among them, compound **7** had the highest results in all the assays. The pharmacokinetic properties of compounds were determined using ADMET estimates; target coumarins may be drug candidates for Alzheimer's disease, especially compound **7** may be used as an antioxidant agent in the future after detailed analysis.

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1. Introduction

Natural bioactive compounds play a potential role in the treatment of dysmetabolic and neurodegenerative diseases [1–5]. Coumarins are secondary metabolites of plants and represent an important class in organic chemistry [6]. While various modifications of the main coumarin structure are found in plants, they can also be obtained through synthesis [7–9]. Coumarins play an important role in medical fields due to their physiological, antibacterial, antioxidant, anticoagulant, and antitumor activities [10–13]. Its toxic effect in medical uses is negligible and they are highly privileged organic compounds for the development of new drugs due to their biodiversity [14,15]. Wedelolactone, a coumarin derivative, was investigated for the treatment of jaundice [16]. Dicumarol, warfarin (Coumadin), acenocoumarol (acenocoumarol), ethyl biscoumacetate (tromexan), and marcoumar drugs are widely used as anticoagulant agents (vitamin K antagonists) [17–19]. As an antimicrobial agent, the effects of novobiocin, clorobiocin, and coumermycin A1 drugs isolated from *Streptomyces* species have also been proven by studies [20,21]. The antibiotic coumarins bound to DNA gyrase block adenosine triphosphatase (ATPase) activity. Coumarin and its derivatives have proven benefits as drugs in medical fields and function as potential inhibitors in cancer cell lines as an anticarcinogen [22–24]. Even natural and synthetic coumarins have been investigated in the literature in many *in silico* screening studies with the aim of inhibiting the SARS-CoV-2 virus [25–27]. As a result, coumarin derivatives are thought to exhibit low toxicity and high selectivity as they are secondary metabolites of plants. It has an active hydroxyl and unsaturated lactone group, contains two benzyl groups, and has intra-structure conjugation, so it will also have a high level of free radical scavenging property.

The aim of this study is to design coumarins with high pharmacokinetic properties so that they can be used in the treatment of Alzheimer's disease. Considering the structure-activity relationship, new polyphenolic coumarins with high inhibition value against acetylcholinesterase enzyme and at the same time effective against free radicals in the body may be the starting point according to the data in this study.

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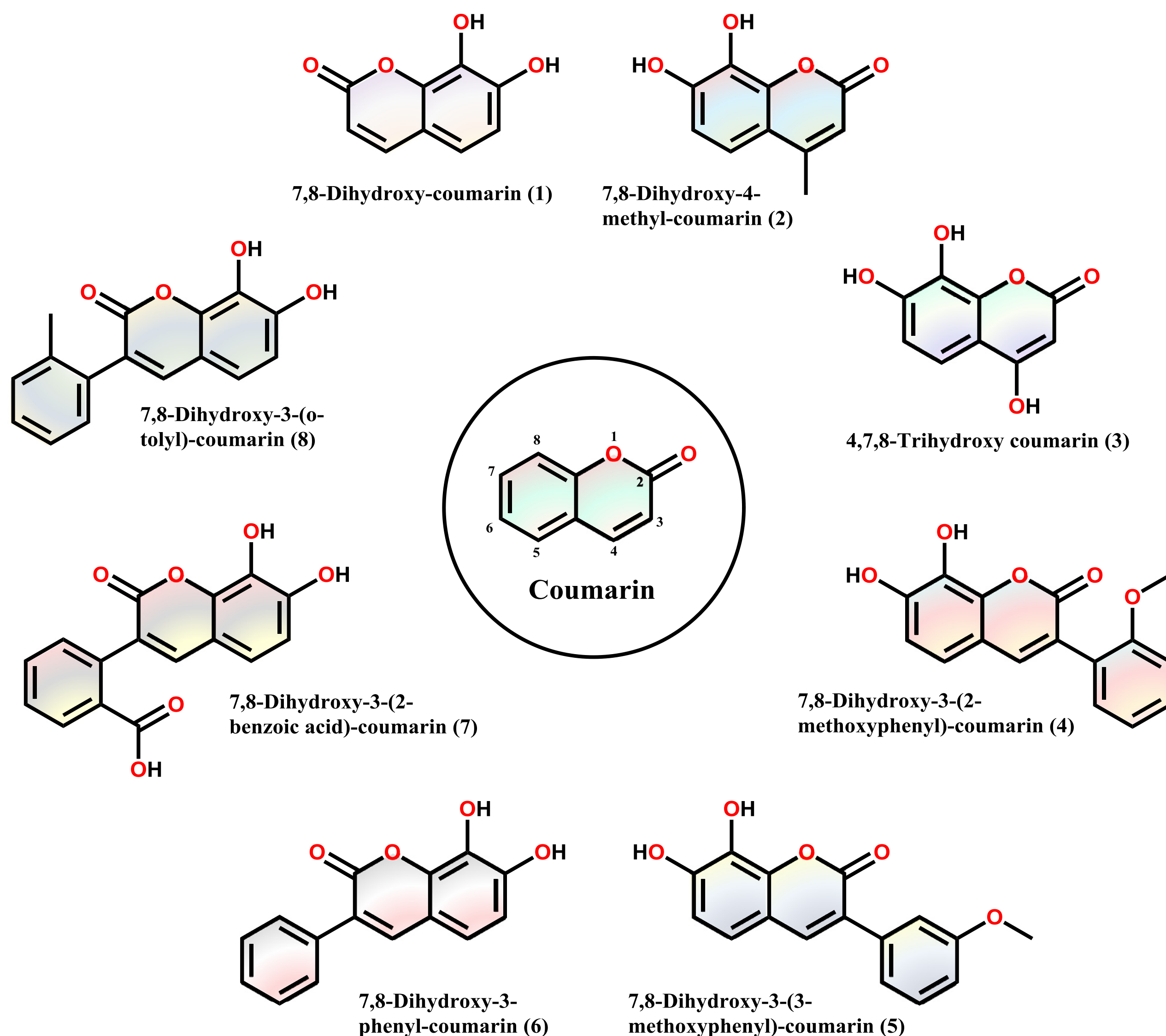


Fig. 1. The chemical structures of 7,8-dihydroxycoumarin derivatives (**1-8**) in this study.

2. Materials and methods

2.1. Materials and equipment

7,8-Dihydroxy-coumarin (**1**), [28] 7,8-dihydroxy-4-methyl-coumarin (**2**), [29] 4,7,8-trihydroxy coumarin (**3**), [30] 7,8-dihydroxy-3-(2-methoxyphenyl)-coumarin (**4**), [31] 7,8-dihydroxy-3-phenyl-coumarin (**6**), [31] 7,8-dihydroxy-3-(2-benzoic acid)-coumarin (**7**) [32] were synthesized according to literature (Fig. 1).

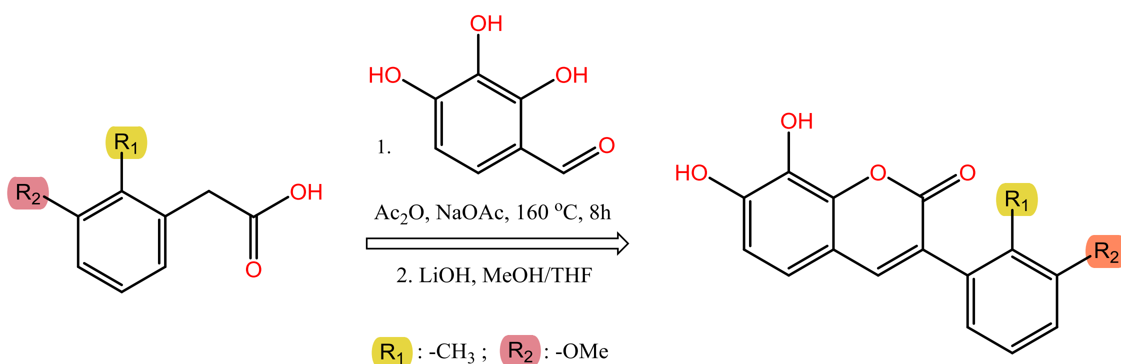
All chemicals and solvents were purchased from Sigma-Aldrich. All reactions were carried out under an argon atmosphere. The purity of the products was checked by the thin-layer chromatography (TLC) technique. FT-IR spectra were recorded on a Shimadzu FT-IR 8300 spectrometer with an ATR accessory. ^{1}H-NMR and ^{13}C-NMR for coumarin derivatives were recorded on VARIAN UNITY INOVA 500 MHz spectrometer. Mass spectra were recorded on a BRUKER Autoflex mass spectrometer using the MALDI-TOF technique. Elemental analyses carried out using a LECO CHN 932 were performed by the Instrumental Analysis Laboratory of the TUBITAK Marmara Research Center.

2.2. Synthesis

A mixture of 2,3,4-trihydroxybenzaldehyde (1.0 g, 7.2 mmol), *o*-(tolyl) acetic acid (1.08 g, 7.2 mmol), or *m*-(methoxyphenyl) acetic acid (1.19 g, 7.2 mmol), anhydrous sodium acetate (0.88 g, 10.8 mmol) and 15 mL acetic anhydride was heated at 160-170°C with stirring under nitrogen atmosphere for 8 h. After the removal of acetic acid by distillation, the resulting solid was dissolved in a 90 mL THF: methanol (3:1) mixture, and then lithium hydroxide (1.0 g, 42.0 mmol) in 5 mL water was added to the suspension. About 2 h later, the reaction mixtures were poured into ice water and added 1mL 10% HCl, and the precipitate was collected by filtration. The dried products were purified by recrystallization from methanol (Scheme 1).

2.2.1. 7,8-Dihydroxy-3-(m-methoxyphenyl) coumarin (5)

Yield 1.4 g (72.5%), Mp; 230–232°C, Anal. Calcd. for $C_{16}H_{12}O_4$: C, 71.64; H, 4.51%; Found: C,71.61; H, 4.49%, FT-IR (ATR, υ_{max}, cm^{-1}): 3352 (–OH), 3085–3034 (Ar–CH), 2966-2832 (Aliphatic–CH), 1687 (lactone–C=O), 1580-1493 (Ar–C=C), ^{1}H-NMR (500 MHz, $CDCl_3$, ppm); 10.13 (bs, 1H, –OH), 9.40 (bs, 1H, -OH), 8.15 (s,



Scheme 1. Synthesis of novel coumarin derivatives (**5** and **8**).

1H, lactone–H), 7.36-7.34 (m, 1H, Ar–H), 7.28 (bd, 1H, Ar–H), 7.12 (d, 1H, *J*= 8.44 Hz, Ar–H), 6.97 (d, 1H, *J*= 8.23 Hz, Ar–H), 6.83 (d, 1H, Ar–H, *J*= 8.33 Hz, Ar–H), 3.81 (s, 3H, –OCH_3), ^{13}C-NMR (125 MHz, $CDCl_3$, ppm); 160.4, 159.5, 150.1, 143.6, 142.3, 136.5, 132.3, 129.7, 122.1, 121.1, 119.7, 113.9, 113.3, 113.2, 55.6, MS(MALDI-TOF) m/z: calcd. 284.27, found 284.59 $[M]^+$.

2.2.2. 7,8-Dihydroxy-3-(o-tolyl) coumarin (8)

Yield 1.3 g (71.8%), Mp; 228–230°C. Anal. Calcd. for $C_{16}H_{12}O_3$: C, 76.18; H, 4.79%; Found: C,76.24; H, 4.77%, FT-IR (ATR, υ_{max}, cm^{-1}): 3350 (–OH), 3055-3023 (Ar–CH), 2957-2830 (Aliphatic–CH), 1681 (lactone–C=O), 1575-1496 (Ar–C=C), ^{1}H-NMR (500 MHz, $CDCl_3$, ppm); 10.14 (bs, 1H, –OH), 9.41 (bs, 1H, –OH), 8.10 (s, 1H, lactone–H), 7.54-752 (m, 2H, Ar–H), 7.33 (d, 1H, *J*= 7.60 Hz, Ar–H), 7.21 (d, 1H, *J*= 7.65 Hz, Ar–H), 7.10 (d, 1H, *J*= 8.15 Hz, Ar–H), 6.85 (d, 1H, Ar–H, *J*= 8.14 Hz, Ar–H), 2.36 (s, 3H, –CH_3), ^{13}C-NMR (125 MHz, $CDCl_3$, ppm); 160.5, 150.0, 143.6, 142.0, 137.7, 135.6, 132.3, 129.3, 129.0, 128.5, 125.9, 122.5, 119.6, 113.2, 21.5, MS(MALDI-TOF) m/z: calcd. 268.27, found 268.32 $[M]^+$.

2.3. In vitro studies

2.3.1. FRAP assay

The FRAP assay was conducted according to Benzie and Strain method [33]. FRAP values of the compounds were given as µM Fe^{2+}/mM analyte.

2.3.2. DPPH assay

The free radical scavenging capacity of compounds was determined by using the previously described method [34]. The data gained from the investigation were given as IC_{50} µM.

2.3.3. Cupric ion reducing/antioxidant power (CUPRAC) assay

In brief, 60 µL Cu(II) × $2H_2O$, 60 µL neocuproine, and 60 µL, NH_4Ac (1M) were mixed. Then 60 µL of the compound and 10 µL of ethanol were added to the mixture. After the duration time of 60 min, the mixture absorbance was spectrophotometrically measured at 450 nm. CUPRAC values of the compounds were given as mM Trolox/mM analyte [35].

2.3.4. Cupric ion reducing/antioxidant power (CUPRAC) assay

The inhibition of acetylcholinesterase of compounds was evaluated according to the Ellman method using a 96-well microplate reader [36] The findings from this study were given as percent acetylcholinesterase enzyme inhibition.

2.4. Molecular docking and DFT studies

Molecular docking studies were conducted to provide a theoretical perspective for possible molecular interactions of compounds with the target protein. Molecular docking calculations were carried out by using the Autodock Vina software [37]. Preparation of 7,8-dihydroxycoumarin derivative compounds (**1**-**8**) for molecular docking was performed with MarvinSketch software [38]. The MMFF94 force field parameter was used for energy minimization and the lowest energy conformer forming of the compounds. The X-ray crystal structure of acetylcholinesterase-AChE (PDB: 4M0E) [39] and nicotinamide adenine dinucleotide phosphate oxidase-NADPH (PDB: 2CDU) [40], human cytochrome P450-CYPs (PDB: 1OG5) [41], human myeloperoxidase (PDB: 1DNU) [42], lipoxygenase (PDB: 1N8Q) [43] and xanthine oxidase (PDB: 3NRZ) [44] were obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (http://www.rcsb.org/) [45]. The water molecules in the crystal package were removed to avoid steric hindrance. Missing polar hydrogens and Kollman charges in the crystal structure of proteins were added. Lamarckian Genetic Algorithm [46] was chosen as the insertion engine. Inhibitors with the lowest binding energy score were selected from ten conformations as a result of the calculation. To increase the reliability of the molecular docking calculations, the same ten repetitions were docked, and the binding affinity values were calculated by averaging with the standard deviation. UCSF Chimera, version 1.16 [47] and Discovery Studio Visualizer v21.1.0.20298 [48] were used to visualize molecular insertion results.

To confirm the molecular docking results, Density Functional Theory (DFT) [49] calculations were performed with the amino acids with which coumarins interact in the active site of the AChE enzyme. Coumarin and its surrounding amino acids in the active site were optimized by Gaussian16 [50] and visualized by GaussView 6.0 [51] and IQMol software. The B3LYP functional [52–54] was used for DFT with the 6-31G(d,p) basis set.

2.5. ADMET predictions

In drug design, the estimation of the pharmacophore properties of the target molecules saves time. ADMET is an acronym for absorption, distribution, metabolism, excretion, and toxicity, and a pharmacophore is an approach that offers parameters for the placement of a compound into a living organism. All these five criteria determine the pharmacological activity of the compounds. pkCSM, a free online web server (http://structure.bioc.cam.ac.uk/pkcsm) [55] was used to predict the ADMET properties of the coumarin derivatives (**1**-**8**).

2.6. Purity analysis of coumarin compounds

Purity analysis of coumarin compounds (**1**-**8**) was carried out using an HPLC-DAD instrument (Agilent 1260 Infinity). In this study, a C18 reverse-phase Nova-Pak (3.9 mm × 150 mm inner diameter, 5 µm) analytical column was used for separation. The

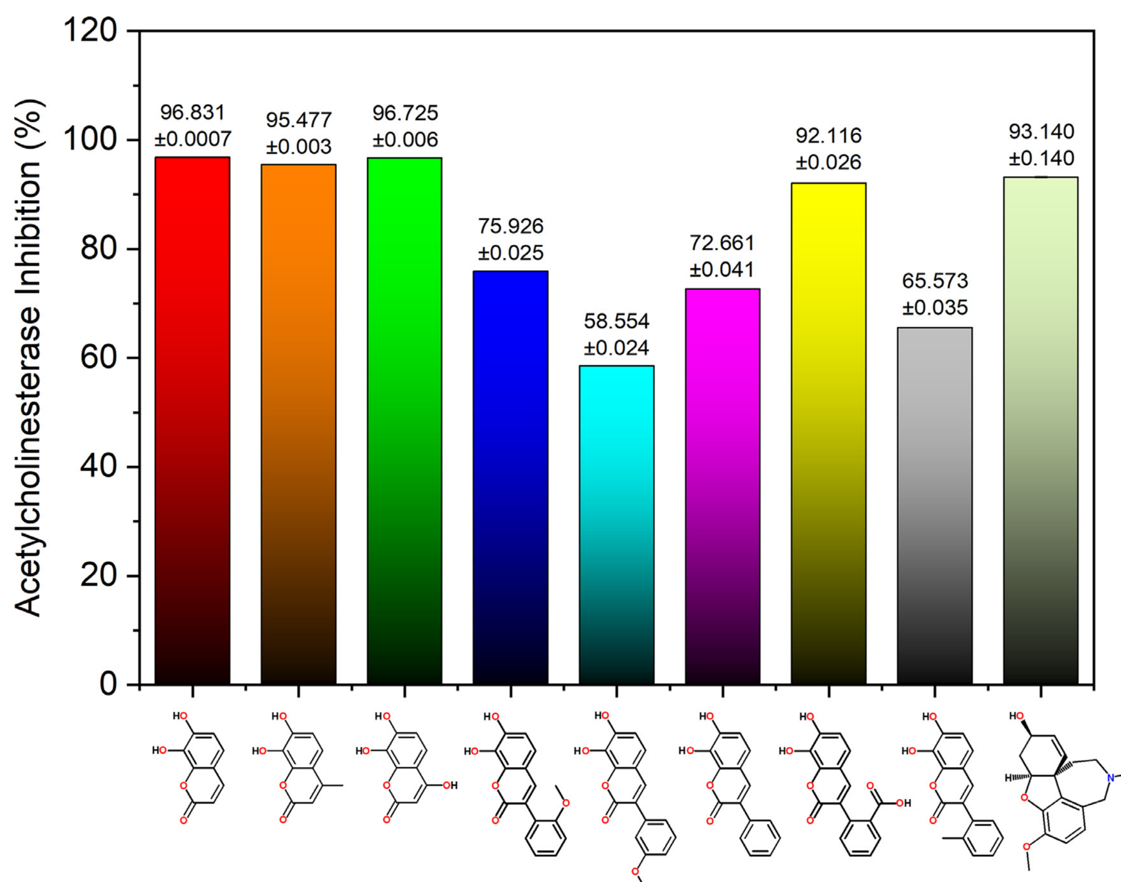


Fig. 2. Acetylcholinesterase inhibition (%) results in 7,8-dihydroxycoumarin derivatives (**1**-**8**). Galantamine positive control for acetylcholinesterase inhibition assay value is mean of triplicate determination (n = 3) ± standard deviation; [a]P < 0.05 compared with the positive control, [b]P < 0.001 compared with positive control.

column temperature was kept at 30°C. The chromatography mobile phases were water (0.05% acetic acid) and (B) acetonitrile. The gradient elution step was used: the mobile phase B was increased from 0% to 20% in 5 min, 40% in 10.00 min, 50% in 20.00 min, 60% in 30.00 min, 90% B in 40.00 min and 45.00 min, 20%. Re-equilibration of the column was made within 10 min, which enabled column equilibration. Before injecting all compounds, a 0.22 µm injector tip was filtered through the filter and 20 µL of it was injected into the HPLC system.

3. Results and discussion

3.1. Characterization

The FT-IR spectrum of novel 7,8-dihydroxy-3-(*o*-tolyl) coumarin (**8**) and 7,8-dihydroxy-3-(*m*-methoxyphenyl) coumarin (**5**) shows characteristic peaks at the range of 3350-3352 cm^{-1} (for aromatic–OH), 3085–3023 cm^{-1} (for aromatic–CH), 1680-1690 cm^{-1} (for lactone–C=O), 1580-1493 (for aromatic–C=C). The ^{1}H-NMR spectrum of coumarin derivatives (**8** and **5**) showed resonance bands at 7.52-6.83 ppm for aromatic protons as doublets, the characteristic lactone-vinylic proton was assigned at 8.10 and 8.15 ppm for **8** and **5**, respectively. Another characteristic proton for dihydroxyl groups was also assigned at 10.14 and 9.41 for **8**, 10.13, and 9.40 for **5**. In the high-field range of the ^{1}H-NMR spectra, the $-CH_3$ group peak was observed at 2.36 ppm for **8**, $-OCH_3$ group peak was observed at 3.81 ppm for **5**. The ^{13}C-NMR spectra also confirm the structure of both novel coumarin **8** and **5**. Finally, the MALDI-TOF mass spectra of compounds **8** and **5** showed *m/z* peaks at 268.32 and 284.59 as $[M]^+$. The drug-level purity of all compounds was determined by the high-performance liquid chromatography (HPLC) method (Figs. S3–S10).

3.2. ADMET properties

The pharmacokinetics of compounds were predicted by the parameters of absorption, distribution, metabolism, excretion, and toxicity as shown in Table S1-8. The Caco-2 permeability values (log Papp>0.90) of 7,8-dihydroxy-coumarin (**1**), 7,8-dihydroxy-3-phenyl-coumarin (**6**), 7,8-dihydroxy-3-(*o*-tolyl)-coumarin (**8**), 7,8-dihydroxy-3-(2-methoxyphenyl)-coumarin (**4**) and 7,8-dihydroxy-3-(3-methoxyphenyl)-coumarin (**5**) were predicted to be high. All the compounds were predicted to have high absorbed intestinal absorption (human) (A>30%) and were predicted to be permeable skin (log Kp>-2.5). All the compounds were predicted to have no P-glycoprotein I and II inhibitory effects. 7,8-dihydroxy-coumarin (**1**), 7,8-dihydroxy-3-(2-methoxyphenyl)-coumarin (**4**), 7,8-dihydroxy-3-(2-benzoic acid)-coumarin (**7**), 7,8-dihydroxy-3-(3-methoxyphenyl)-coumarin (**5**) had low the volume of distribution (VDss) while 7,8-dihydroxy-3-phenyl-coumarin (**6**) had high VDss (human) values. 7,8-Dihydroxy-3-(2-benzoic acid)-coumarin (**7**), 7,8-dihydroxy-3-(3-methoxyphenyl)-coumarin (**5**) compounds were predicted to be poorly distributed to the Blood-brain barrier. 7,8-dihydroxy-4-methyl-coumarin (**2**) and 4,7,8-trihydroxy coumarin (**3**) were predicted to be unable to penetrate the Central Nervous System (CNS), but 7,8-dihydroxy-3-phenyl-coumarin (**6**), 7,8-dihydroxy-3-(*o*-tolyl)-coumarin (**8**), 7,8-dihydroxy-3-(2-methoxyphenyl)-coumarin (**4**) were predicted to penetrate the CNS. All compounds do not show the feature of substrates to the CYP2D6 enzyme. Synthesized coumarin

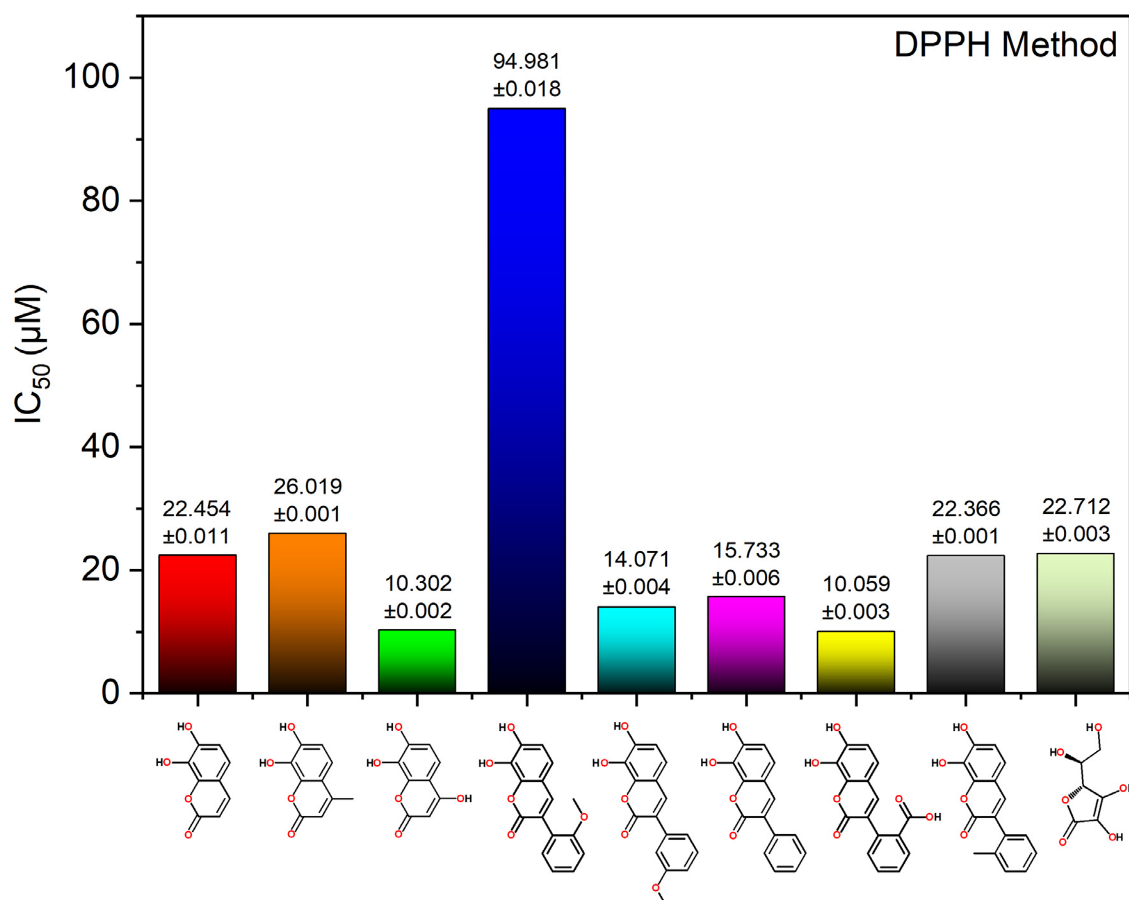


Fig. 3. Antioxidant activity results in 7,8-dihydroxycoumarin derivatives (**1**-**8**) using the DPPH method. Positive control for DPPH assay values is mean of triplicate determination (n = 3) ± standard deviation; [a]P < 0.05 compared with the positive control, [b]P < 0.001 compared with positive control.

molecules (**1**-**8**) were predicted to not show the feature of substrates to the CYP3A4 enzyme except 7,8-dihydroxy-3-phenyl-coumarin (**6**). It has been predicted that all compounds have an inhibitory effect on CYP1A2. It was predicted that while 7,8-dihydroxy-3-phenyl-coumarin (**6**), 7,8-dihydroxy-3-(*o*-tolyl)-coumarin (**8**), 7,8-dihydroxy-3-(2-methoxyphenyl)-coumarin (**4**), 7,8-dihydroxy-3-(3-methoxyphenyl)-coumarin (**5**) had the potential to inhibit the CYP2C19 CYP2C9 CYP3A4 enzymes, but 7,8-dihydroxy-coumarin (**1**), 7,8-dihydroxy-4-methyl-coumarin (**2**), 7,8-dihydroxy-3-(2-benzoic acid)-coumarin (**7**), 4,7,8-trihydroxy coumarin (**3**) were not able to inhibit the enzyme involved.

It is estimated that no compound synthesized is a substrate for organic cation transport protein 2. Among all the compounds synthesized 7,8-dihydroxy-3-phenyl-coumarin (**6**), 7,8-dihydroxy-3-(*o*-tolyl)-coumarin (**8**), 7,8-dihydroxy-3-(2-methoxyphenyl)-coumarin (**4**) and 7,8-dihydroxy-3-(3-methoxyphenyl)-coumarin (**5**) were predicted to show AMES toxicity. None of the synthesized coumarin molecules were predicted to have the potential to inhibit hERG I. However, 7,8-dihydroxy-3-phenyl-coumarin (**6**), 7,8-dihydroxy-3-(*o*-tolyl)-coumarin (**8**), 7,8-dihydroxy-3-(2-methoxyphenyl)-coumarin (**4**) and 7,8-dihydroxy-3-(3-methoxyphenyl)-coumarin (**5**) have hERG II inhibitory effects. All synthesized compounds were predicted to not have a Hepatotoxicity effect, skin sensitization, and Minnow toxicity.

When the log P values of all compounds except 7,8-dihydroxy-coumarin (**1**) and 4,7,8-trihydroxy coumarin (**3**) are examined, it is estimated that they are lipophilic. The most lipophilic compound is 7,8-dihydroxy-3-(*o*-tolyl)-coumarin (**8**). When analyzing the results of ADME studies of all synthesized compounds in terms of the 5 rule of Lipinski [56], which is used to estimate the oral bioavailability of a drug, it is found that the molar mass of all compounds is less than 500 g / mol, log P values less than 5, the number of hydrogen bond acceptors less than 10 and the donor less than 5, the polarized surface area is less than 140 $Å^2$, and finally the number of rotatable bonds it was found to be less than 10. (Table S1-8). According to our present knowledge, no studies have been reported regarding the ADMET profiling of these coumarin compounds. ADMET properties of these compounds were examined in detail for the first time. ADMET estimates of the compounds show that these compounds are easily absorbed and distributed. Not all compounds analyzed are predicted to be hepatotoxic but compounds **2, 3, 5**, and **8** are mutagenic. However, compound **7**, which is the most biologically active, is promisingly non-mutagenic. Hence, the good activity and ADMET results of compound **7** reveal that this compound can be used as an antioxidant and anticholinesterase agent in the future.

3.3. Biological activity

3.3.1. Acetylcholinesterase inhibition

Alzheimer's disease (AD) is a degenerative brain condition that most frequently affects older people. In the parts of the brain responsible for learning, memory, behavior, and emotional reactions, there are lower acetylcholine levels in people with Alzheimer's disease. Acetylcholine (ACh) synaptic availability is decreased when basal forebrain cholinergic cells are lost. The most noticeable clinical finding in Alzheimer's disease is a decrease in the levels of acetylcholine neurotransmitters, which are in charge of trans-

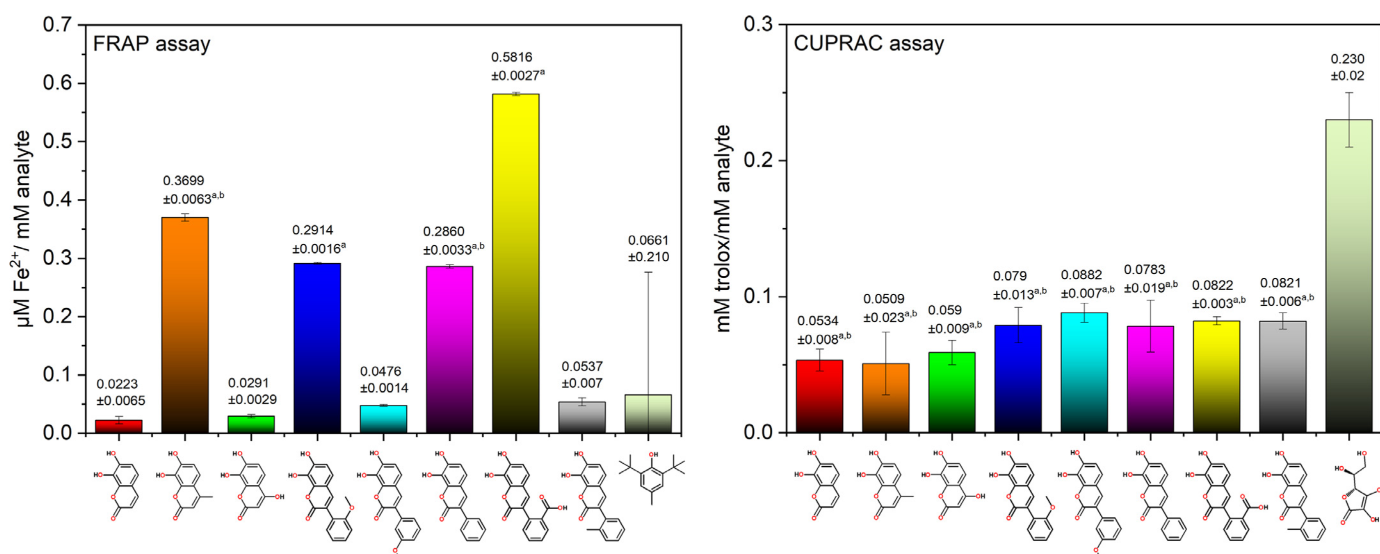


Fig. 4. FRAP and CUPRAC assays results of 7,8-dihydroxycoumarin derivatives (**1-8**) and standard compounds. Positive control for FRAP assay; ascorbic acid positive control for CUPRAC assay values are mean of triplicate determination (n = 3) ± standard deviation; [a]P < 0.05 compared with the positive control, [b]P < 0.001 compared with positive control.

mitting electrical impulses from one nerve cell to another nerve cell due to their rapid breakdown by acetylcholinesterase (AChE). According to all these findings, acetylcholinesterase (AChE), an enzyme that reduces and hydrolyzes acetylcholine, needs to be inhibited to enhance the synaptic levels of acetylcholine in the brain.

Anticholinesterase activities of 7,8-dihydroxycoumarin derivatives (**1-8**) and galantamine at 500 μg/mL concentration were decreased as: **1** (96.831%) > **3** (96.725%) > **2** (95.477%) > galantamine (93.14%) > **7** (92.116%) > **4** (75.926%) > **6** (72.661%) > **8** (65.573%) > **5** (58.554%). The results showed that all compounds had the ability to inhibit the potent cholinesterase enzyme. The ability to inhibit the cholinesterase enzyme of coumarins **1, 2**, and **3** were found to be stronger than the standard galantamine compound. The enzyme inhibition power of **7** appears to be very close to galantamine (Fig. 3).

When methyl and hydroxy groups were added to the 7,8-dihydroxycoumarin compound (**1**) from the C-4 position, it almost did not affect inhibition. While the methyl group decreased slightly, the hydroxy group did not change. When the phenyl ring was added to the coumarin skeleton from the C-3 position, the inhibition effect was considerably reduced. When coumarins containing a phenyl ring (**4-8**) were evaluated among themselves, the methyl group attached to the phenyl ring from the ortho position decreased the inhibition as in compound **2**. While the methoxy group attached from the ortho position slightly increased the inhibition, the methoxy group at the meta position had the opposite effect and decreased it considerably. In coumarin **7**, the carboxylic acid group ortho-linked to the phenyl ring increases inhibition considerably due to its effective hydrogen bonding capacity. Another disadvantage that occurs when the phenyl ring is attached to coumarins is that it disrupts the rigidity of the structure and makes the coumarin structure more flexible and bulkier.

3.3.2. Antioxidant assay

DPPH was used as a synthetic radical to evaluate the antiradical activity of the compounds. The IC_{50} values of the compounds and standard for DPPH radical scavenging were found as follows, respectively; compound **7** > **3** > **5** > **6** > **8** > **1** > ascorbic acid > **2** > **4** (Fig. 4). In the DPPH assay, it was determined that many molecules exhibited stronger effects than standard ascorbic acid. In this study, the DPPH scavenging activity of compound **7** was found to be the strongest (IC_{50} of 10.06 μM).

If the antioxidant activity against DPPH is evaluated, the 7,8-dihydroxycoumarin structure exhibited almost the same activity as ascorbic acid. The IC_{50} value increased slightly when methyl was attached to the coumarin structure from the C-4 position but decreased considerably when another hydroxy was added to the structure instead of methyl. Again, it continued to decrease when phenyl and phenyl derivatives were added from the C-3 position. Interestingly, the IC_{50} value in the 2-methoxyphenyl structure is 14.071±0.006, while it is 94.981±0.018 in the 1-methoxyphenyl structure. Similarly, coumarin carrying the 1-methylphenyl group has a higher IC_{50} value than other derivatives. Coumarin, which carries the 1-carboxybenzene group, has the lowest IC_{50} value, unlike the others. The inhibition effect is much better than the others, probably because the tendency to hydrogen bond is very high.

FRAP and CUPRAC tests were used to evaluate the ability of the compounds to reduce Ferric ions (Fe^{3+}) ferrous ions (Fe^{2+}) and Cu^{2+}- Cu^{+}, respectively. Fe^{3+} reducing powers of the compounds and the standard antioxidant (BHT) decreased in following orders: compounds **7** > **2** > **4** > **6** > BHT > **8** > **5** > **3** > **1**. The results demonstrated that both compounds **2** and **7** had potent Fe^{3+} reducing ability. Cu^{2+} reducing powers of the compounds and standards were decreased as; ascorbic acid > **5** > **7** > **8** > **4** > **6** > **3** > **2** > **1**. The results demonstrated that all compounds had moderate Cu^{2+} reducing ability. The Cu^{2+} reducing power of all compounds was found to be lower than the standard ascorbic acid compound.

Xia *et al.* tested the 7,8-dihydroxycoumarin compound with the DPPH method and measured its antioxidant activity as 46.20±1.45 μM [57]. Beillerot et al. tested the compound 7,8-dihydroxy-4-methylcoumarin with the FRAP method and found a value of 4.52±0.13 μM [58]. Xia et al. tested it with the DPPH method and found 8.43±0.973 μM and 85.63±2.13 μM, respectively [57,59]. The 7,8-dihydroxy-3-phenylcoumarin compound was described by Xia et al. and Musa *et al.*, by the DPPH method, Ozalp *et al.* and tested with DPPH, FRAP, and CUPRAC methods [57,60,61]. Xia found 43.67±2.08 μM, while Musa found 9.70±0.31 μM. Ozalp recorded the antioxidant activity values of the 7,8-dihydroxy-3-phenylcoumarin compound as 74.70±0.057 μM for DPPH, 2.28±0.091 mM for FRAP and 2.44±0.016 mM for CUPRAC. When Xia *et al.* bind the phenyl group to the C-4 posi-

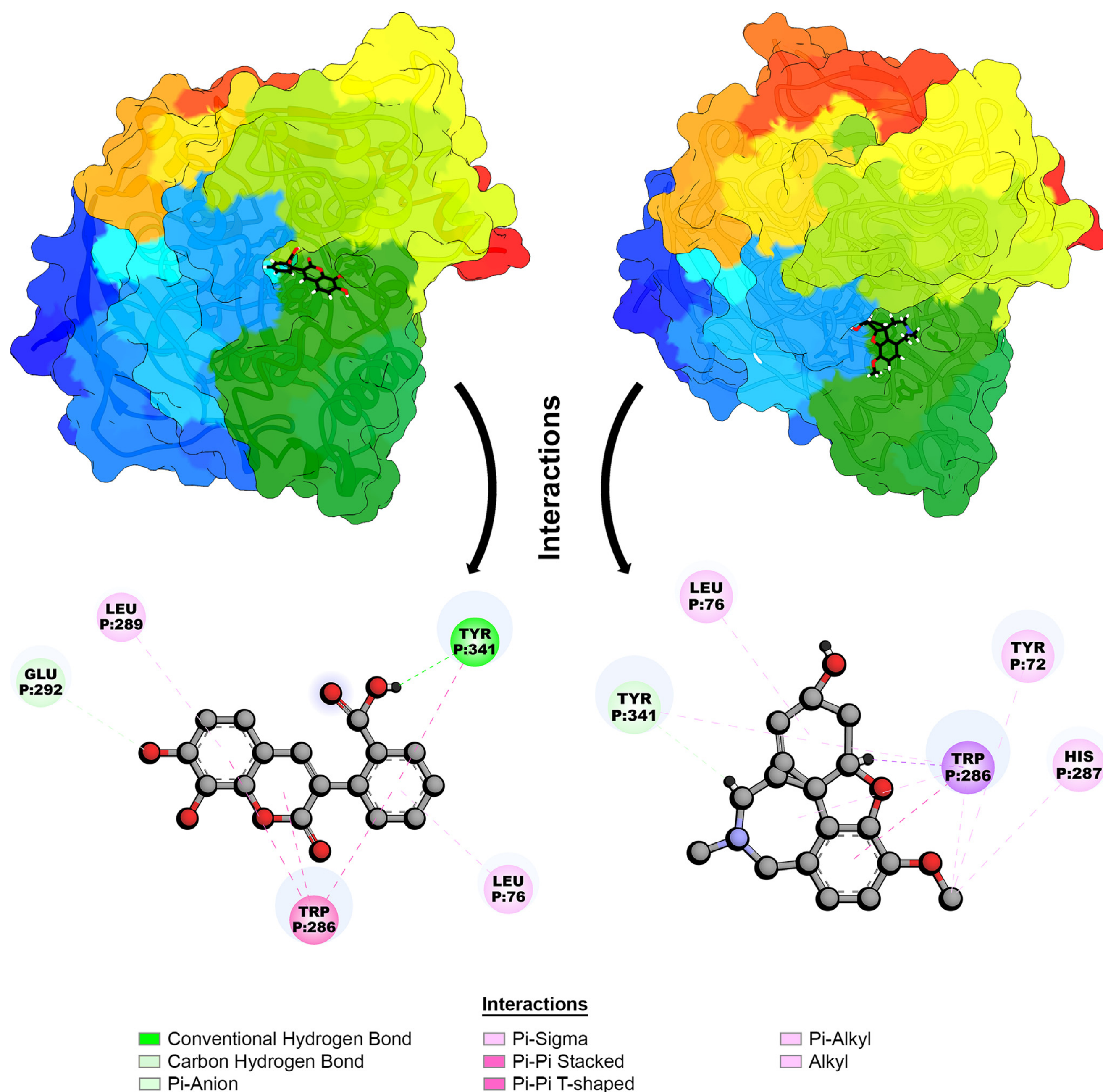


Fig. 5. The placement of 7,8-dihydroxy-3-(2-benzoic acid)-coumarin (**7**) and galantamine compounds on acetylcholinesterase enzyme and ligand-amino acid interactions in the catalytic region of the AChE.

tion instead of the C-3 position (7,8-dihydroxy-4-phenylcoumarin), its value is 55.60±0.78 µM according to the DPPH method and its antioxidant activity decreases. Musa tested the compound 7,8-dihydroxy-3-(p-methoxybenzene)-coumarin, similar to coumarins **4** and **5** in this study, according to DPPH, and found 10.8±0.45 µM. In addition, the activity of the 7,8-dihydroxy-3-(2,3,4-trimethoxybenzene)-coumarin compound was slightly increased with 9.70±0.52 µM by multiplying the methoxy groups. Similar to coumarin **8** in this study, Ozalp tested the 7,8-dihydroxy-3-tolylcoumarins from the meta and para positions against DPPH, FRAP, and CUPRAC. The antioxidant activities of 7,8-dihydroxy-3-(m-tolyl)-coumarin compound were 92.64±0.841 µM for DPPH, 2.06±0.058 mM for FRAP and 2.25±0.139 mM for CUPRAC. The antioxidant activities of 7,8-dihydroxy-3-(p-tolyl)-coumarin compound were 94.85±0.917 µM for DPPH, 2.00±0.101 mM for FRAP and 2.24±0.121 mM for CUPRAC. As seen in Xia's study, the activity of the 7,8-dihydroxy-4-methylcoumarin structure can be changed by modifications. The antioxidant activity of the 7,8-dihydroxy-4-methylcoumarin which was 85.63±2.13 µM with the DPPH method was increased by adding phenyl, *tert*-butyl, trifluoromethyl, chlorine, hydroxy, azide, and carboxyl groups to the structure instead of methyl. The increase in activity was mostly with the addition of the carboxyl group, and this is also seen in Sersen's study. When the carboxyl group turns into an ester group, the activity decreases again. All these tests performed in the literature are in correlation with the results of this study.

3.3. Molecular docking and DFT study

Docking analyzes were performed to understand the molecular interaction mechanisms between coumarins **1**-**8** and enzymes. In this study, 7,8-dihydroxycoumarin derivatives were docked into

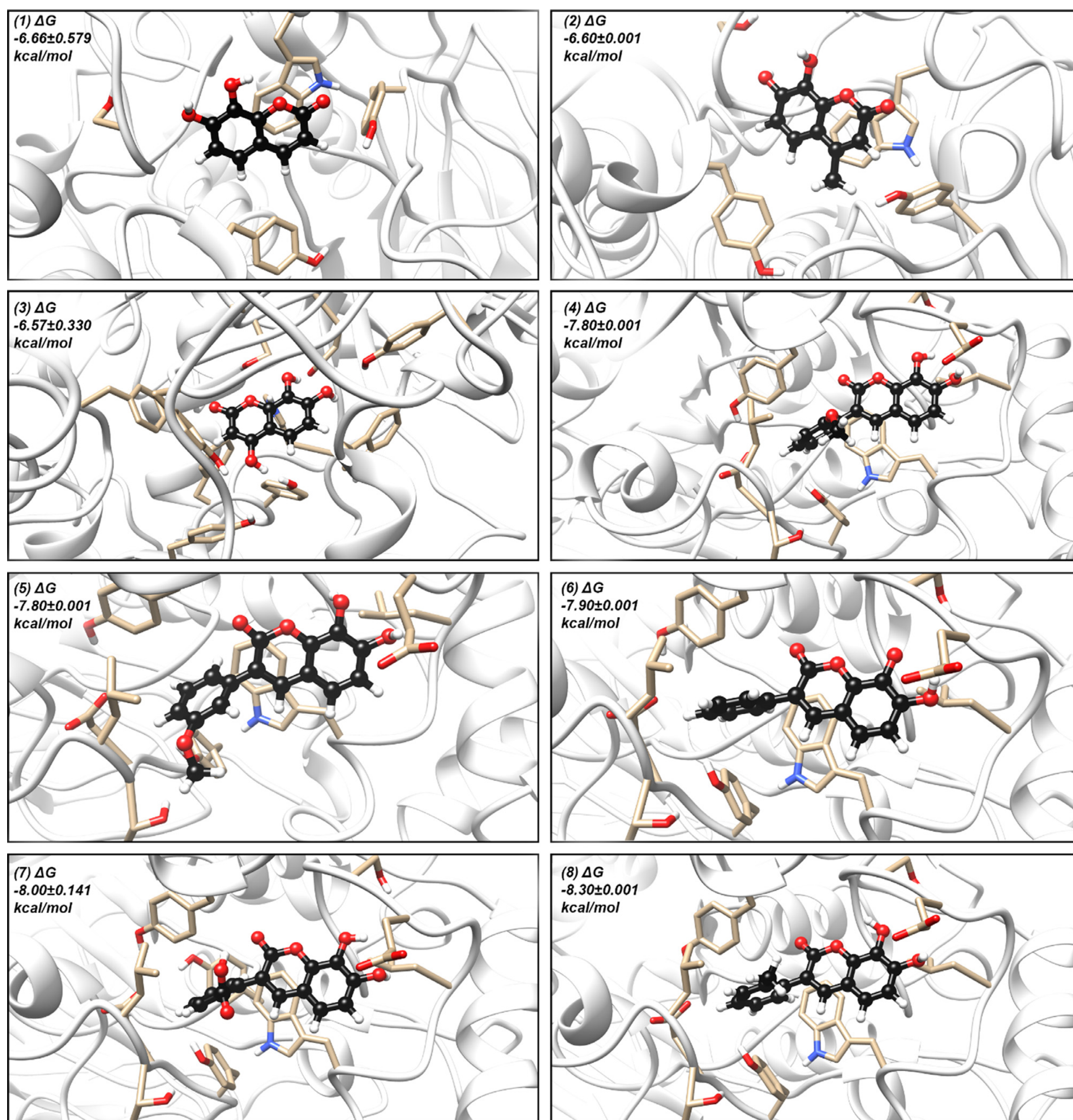


Fig. 6. Docking scores of coumarin derivatives (**1**-**8**) on the AChE enzyme.

the ligand-binding site of AChE and NADPH by using the Autodock Vina module to explore the molecular mechanisms for inhibition of AChE and NADPH. The docking results of the complexes were given in Figs. 5–7.

In AChE docking experiments, binding affinities for small coumarin molecules (**1**-**3**) were relatively low binding scores compared to coumarin derivatives (**4**-**8**) with phenyl rings bonded at the C-3 position. However, when evaluated in terms of ligand efficiency (Ligand efficiency = binding affinity / heavy atoms), it has the highest ligand efficiencies, 0.51 for **1**, 0.47 for **2**, and 0.47 for **3**, respectively. The ligand efficiencies of 7,8-dihydroxy-3-phenylcoumarin derivatives (**4**-**8**) are in the range of 0.36-0.42. Non-covalent π-π stacking interactions were observed between all coumarin derivatives (**1**-**8**), galantamine, and tryptophan (TRP286) in the active site of the AChE enzyme. Since the compounds have a macrocyclic structure, π-π stacking interactions with cyclic amino acids are more dominant. The most non-covalent interactions belonged to coumarin **8**, and it formed π-π stacking interactions with amino acids leucine (LEU289 and LEU76), tryptophan (TRP86), and hydrogen bond interactions with tyrosine (TYR341). Similar interactions were also present in the reference galantamine compound. Coumarin **3** is also one of the most effective inhibitors in AChE inhibition experiments. *In vitro* results for AChE enzyme correlate and confirm with molecular docking results.

The *in silico* results of the radical scavenging properties of 7,8-dihydroxycoumarins on potential enzymes that can generate free radical oxygen, as measured by the *in vitro* DPPH, FRAP, and CUPRAC methods, are given in Fig. 7 and Table S1. NADPH, cytochrome P450, myeloperoxidase, lipoxygenase, and xanthine oxidase were chosen as potential enzymes. The highest docking scores

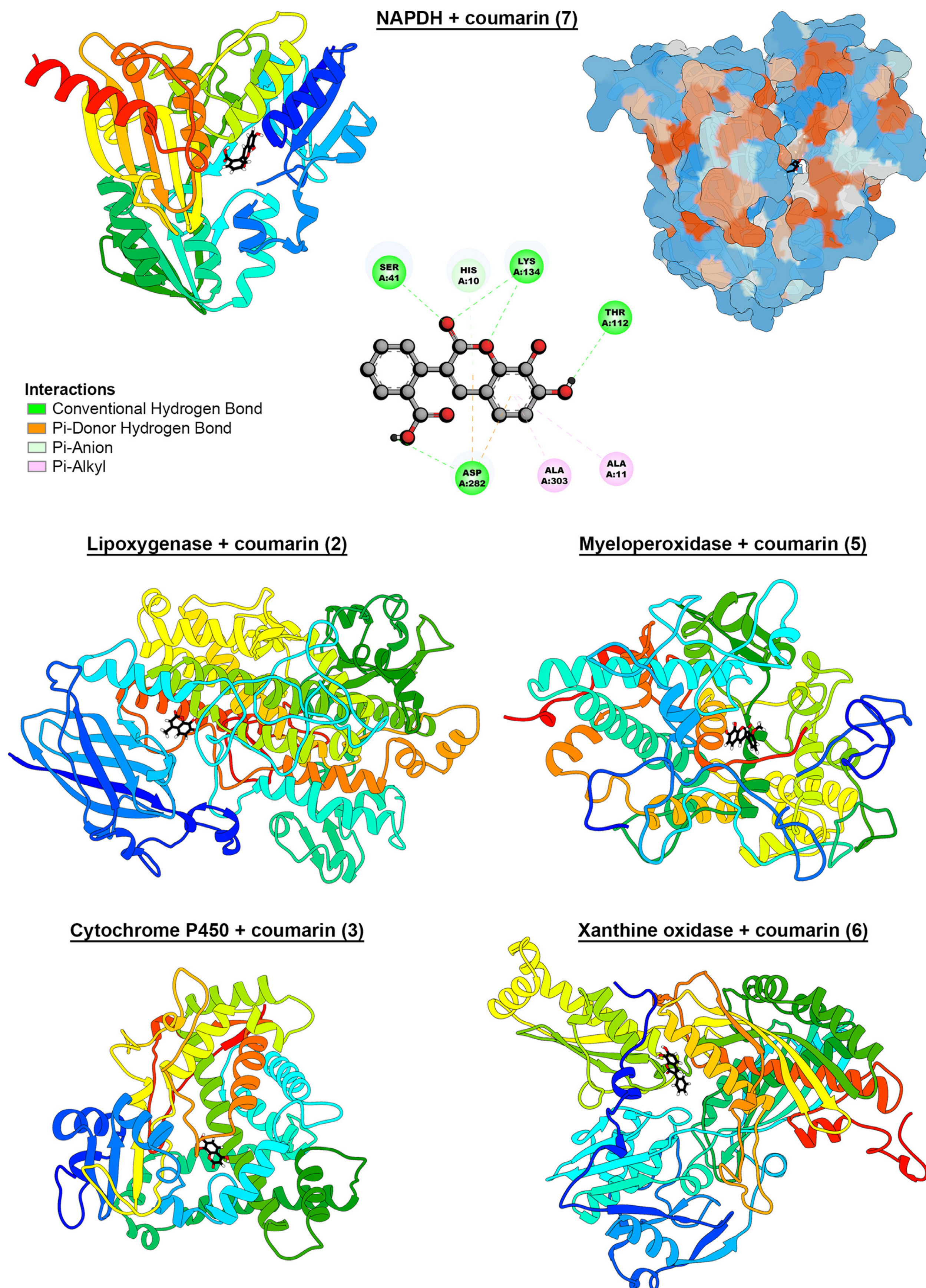


Fig. 7. The molecular docking results of 7,8-dihydroxy-coumarin derivatives on NADPH, cytochrome P450, myeloperoxidase, lipoxygenase, and xanthine oxidase enzymes.

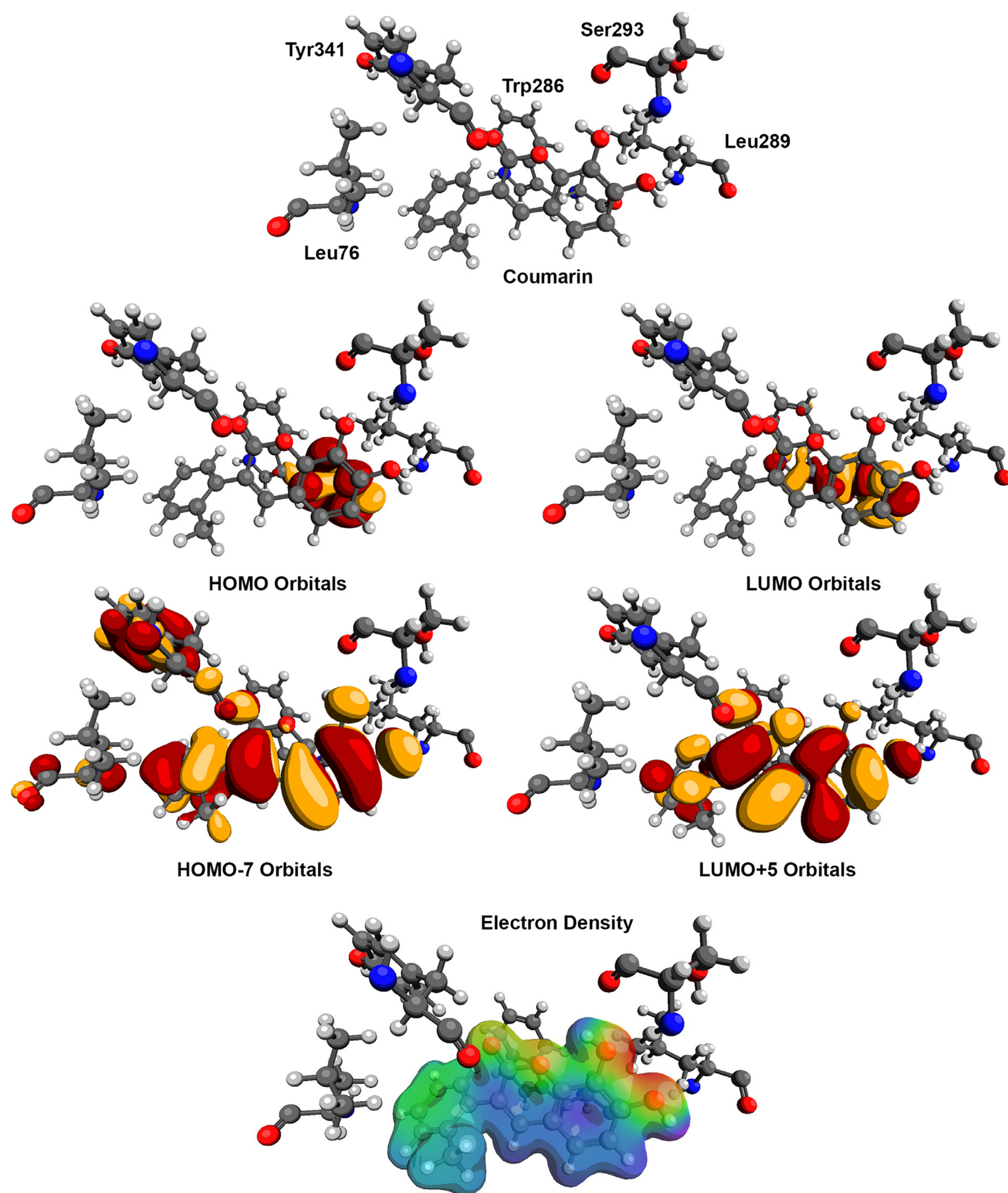


Fig. 8. The molecular orbital contributions and electron density map between coumarin **8** and acetylcholinesterase active site amino acids via DFT calculations.

of coumarin compounds (**1**-**8**) were determined for xanthine oxidase and myeloperoxidase enzymes.

When evaluated in terms of ligand efficiency, compounds **1**-**3** stand out as the most effective molecules. Compound **5** surprisingly exhibited selective inhibitory properties for myeloperoxidase. In addition to the behavior of coumarins to form pi-pi interactions in the acetylcholinesterase enzyme, many hydrogen bond interactions were formed in the active site of the NADPH enzyme. Due to their small and flexible structures, coumarin compounds tend to be located toward the inner parts of the active sites of enzymes and exhibit antagonistic behavior.

It has been observed that the coumarin **8** and the amino acids in the acetylcholinesterase enzyme active site continue to interact with each other stably as a result of DFT calculations. Electrons migrate between the amino acids TYR341, SER293, LEU289, LEU76, and TRP286 located in the active site and coumarin. While the HOMO orbitals are on the TRP286 structure, the LUMO orbitals are also on the TRP286. While the HOMO-1 and LUMO+1 orbitals are

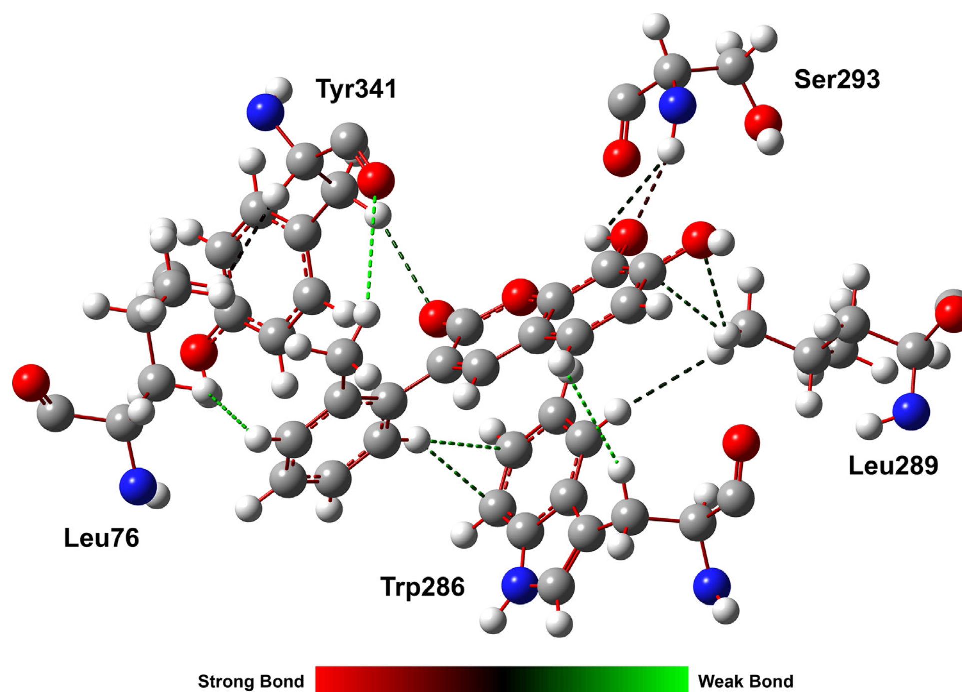


Fig. 9. Inter and intramolecular bond strengths of coumarin **8** and acetylcholinesterase active site amino acids by DFT calculations.

on the amino acids, the contribution of coumarins to the orbitals was realized at the HOMO-7 and LUMO+5 energy levels. LUMO+5 orbitals are on coumarin only, whereas in HOMO-7 the orbitals are on both coumarin and TYR341. These results show that the interactions are stable as electrons migrate over these structures. On the electron density map, the hydroxyl, ether bridge and carbonyl groups of the coumarin compound are nucleophilic (red part), the C-3, C-4, and C5 positions, and the terminal hydrogens attached to the C3-C5 carbons are electrophiles (blue part). Green, on the other hand, is the part that is neutral or close to neutral (Fig. 8). As can be seen, the parts approaching the amino acids are the red and green parts. As a result of DFT, the forces of interactions between coumarin and amino acids increased as the structures approached each other, decreasing as they moved away. While the green bonds are weak, the blacks are of moderate intensity and the reds are quite rigid and stable (Fig. 9). The DFT results comply with *in silico* and *in vitro* experiments.

4. Conclusion

In summary, we have selected to investigate the acetylcholinesterase and antioxidant properties of 7,8-dihydroxycoumarin derivatives. Two of these coumarins (**5** and **8**) were synthesized originally and thoroughly characterized using, FT-IR, ^{1}H-NMR, ^{13}C-NMR, and MALDI-TOF-MS techniques. The biological activities of all compounds were examined. Among the analyzed compounds compound **7** showed potent enzyme inhibitory activity as compared to the standard galantamine. And molecular docking studies of compound **7** have also been carried out to support *in vitro* testing with high molecular docking scores, and compound **7** had the highest results in all the antioxidant assays. According to the ADMET estimation and biological activity results, it is thought that compound **7** can be used as an antioxidant or anticholinesterase agent in the future, after *in vivo* and clinical studies since it does not show toxic effects.

Declaration of Competing Interest

The authors declared no conflict of interest.

CRediT authorship contribution statement

Mücahit Özdemir: Software, Investigation, Data curation, Writing – original draft, Visualization. **Duygu Taşkın:** Methodology, Validation, Writing – original draft. **Deniz Ceyhan:** Software, Data curation. **Baybars Köksoy:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Turgut Taşkın:** Conceptualization, Methodology, Validation, Writing – original draft. **Mustafa Bulut:** Investigation, Writing – review & editing. **Bahattin Yalçın:** Conceptualization, Investigation, Resources, Writing – original draft, Supervision.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.molstruc.2022.134535.

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