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Investigation of HMG-CoA reductase inhibitory and antioxidant effects of various hydroxycoumarin derivatives

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Abstract

Cardiovascular diseases are one of the primary causes of deaths worldwide, and the development of atherosclerosis is closely related to hypercholesterolemia. As the reduction of the low-density lipoprotein cholesterol level is critical for treating these diseases, the inhibition of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, which is essentially responsible for cholesterol biosynthesis, stands out as a key solution to lower plasma cholesterol levels. In this study, we synthesized several dihydroxycoumarins and investigated their antioxidant and in vitro HMG-CoA reductase inhibitory effects. Furthermore, we carried out in silico studies and examined the quantum-chemical properties of the coumarin derivatives. We also performed molecular docking experiments and analyzed the binding strength of each coumarin derivative. Our results revealed that compound **IV** displayed the highest HMG-CoA reductase inhibitory activity (IC_{50} = 42.0 μM) in vitro. Cupric-reducing antioxidant capacity and ferric-reducing antioxidant power assays demonstrated that coumarin derivatives exhibit potent antioxidant activities. Additionally, a close relationship was found between the lowest unoccupied molecular orbital energy levels and the antioxidant activities.

KEYWORDS

antioxidant, coumarin, HMG-CoA reductase, lipid lowering, radical scavenging

1 | INTRODUCTION

Cardiovascular diseases are the major cause of deaths worldwide, and they are expected to remain a leading cause of mortality in the near future.[1] The majority of coronary artery diseases leading to myocardial infarction are caused due to atherosclerosis (AS), which is mainly characterized by the deposition of lipids in the artery wall and the infiltration of inflammatory cells.[2] Hypercholesterolemia and other dyslipidemias are major factors for the development of AS, and the reduction of low-density lipoprotein (LDL) cholesterol level is the primary target of therapy. For this reason, the inhibition of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase (HMGR), which is the key enzyme for cholesterol biosynthesis, is crucial for lowering plasma cholesterol levels.[3] Besides the deposition of lipids in the artery wall, oxidative stress may also be involved in the formation and development of AS. In the case of oxidative stress, vascular wall cells produce excessive reactive oxygen species (ROS), which can cause peroxidation of lipids and irreversible damage of cell membranes, proteins, and DNA.[2]

Statins have been proved to be remarkably successful HMGR inhibitors in clinical use. They bear HMG-like moieties and bind covalently and competitively to the catalytic site of HMGR.[4] Statins are also known to possess anti-inflammatory and antioxidant effects. Besides, they reduce oxidized LDLs and inhibit their uptake by macrophages.

Despite all the abovementioned benefits, statins are also known to display a number of side effects. The most common adverse side effects are elevated liver enzymes and myalgia, which is a muscle ache and can easily be more severe with higher doses, even leading

to rhabdomyolysis. Therefore, the design of HMGR inhibitors with fewer side effects is receiving increasing attention. Coumarin and its derivatives that were shown to have a lipid-lowering potential[5] have attracted intense interest due to their diverse pharmacological properties with low toxicity.[6]

Human HMGR comprises 888 amino acids, making up to three domains. The catalytic domain (residues 459–888) of HMGR is a tetrameric structure and the active sites are located at the hydrophobic interface of the two monomers of a dimer (Figure 1).[7] HMGR accommodates an N-terminal transmembrane domain (residues 1–340) that is bound to the endoplasmic reticulum, and it is usually not incorporated in the X-ray crystal structures. Statins occupy the area where the HMG portion of HMG-CoA binds and do not spread to the proximity of NADPH.[8] They interact with both monomers of a dimer.[9]

In this study, we have investigated antioxidant and in vitro HMGR inhibitory effects of dihydroxycoumarins. Previously, our group has reported the synthesis and in vivo lipid-lowering activity of 7,8-dihydroxy-3-(4-methylphenyl)coumarin in rats.[5] Therefore, to evaluate the possible role of HMGR inhibition in the lipid-lowering activity, we have synthesized structurally analogous dihydroxycoumarin derivatives and investigated their antioxidant and HMGR inhibitory effects in vitro. Later, we pursued in silico studies to gain insight into the varying inhibition intensities. Toward this end, we calculated several quantum mechanical (QM) properties of the dihydroxycoumarin derivatives and pravastatin, a known statin inhibitor of HMGR. Subsequently, molecular docking simulations were performed. Selected hits were then analyzed and their binding energies were evaluated.

2 | RESULTS AND DISCUSSION

Human HMGR, the rate-limiting enzyme in cholesterol biosynthesis, is tightly related to the ROS content. Inhibitors of HMGR, known as statins, exhibit intrinsic antioxidant activities with both antihydroxyl and peroxyl radical activity.[10,11] The suggestion that some coumarin derivatives with the antioxidant activity would be an inhibitor of HMGR on the basis of structural similarities with statins attracted our attention. These similarities depend on the presence of two phenyl rings and the presence of hydroxyl groups in different positions on these rings. In our initial study, we reported that compound **IV** [7,8-dihydroxy-3-(4-methylphenyl)coumarin] has a considerable lipid-lowering and antioxidant activity,[5] which suggests that synthetic *ortho*-6,7-dihydroxy-4-methyl coumarins might be able to scavenge ROS and modulate HMGR activity.

2.1 | Antioxidant activity assays

Table 1 shows the antioxidant activities of coumarins obtained from DPPH (1,1-diphenyl-2-picrylhydrazyl), CUPRAC (cupric-reducing antioxidant capacity), FRAP (ferric-reducing antioxidant power), and metal chelating assays. Among the five compounds examined, compound **II** exhibited the strongest efficiency, followed by **I**, **III**, and **IV**, whereas compound **V** rendered the weakest effect. As seen in Table 1, $TEAC_{FRAP}$ and $TEAC_{CUPRAC}$ values of all compounds decreased in the following order: **II** > **I** > **III** > **IV** > **V**. Ferrous ion chelating activity of coumarins is expressed as EC_{50} (mM), and compounds **I**, **II**, **III**, and **IV** displayed nearly the same metal chelating activity when compared with standard antioxidants, but compound **V** did not show any activity.

Coumarins recognized as possessing a potent antioxidant activity are also strong scavengers of DPPH, a relatively stable nitrogen-centered free radical.[12] Our results demonstrated that the free radical scavenging activity of the coumarin compounds is concentration dependent. As the concentration of the test compounds increases, the radical scavenging activity increases, and a lower EC_{50} value reflects a better protective action. The antioxidant activity of these coumarin derivatives could be attributed to the electron-donating

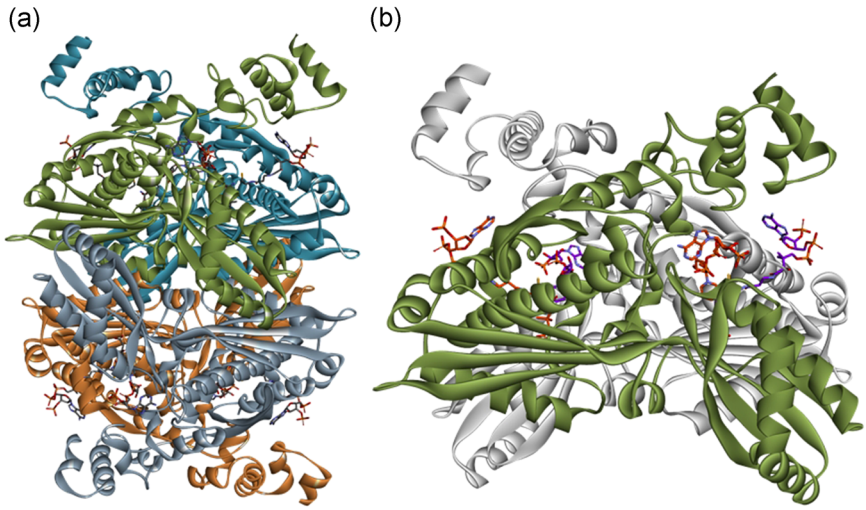


FIGURE 1 (a) The homotetrameric structure of human 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR) enzyme. (b) Dimer of HMGR, including chains A and B. One monomer is colored for clarity. The binding region of HMG-CoA is mainly on one monomer, neighboring the NADPH-binding region of another monomer. Image obtained by using Discovery Studio 4.5

TABLE 1 Radical scavenging and reducing power of tested coumarin derivatives

Compound	DPPH[a]	FRAP[b]	CUPRAC[b]	Metal chelating[c]
I	74.70 ± 0.057	2.28 ± 0.091	2.44 ± 0.016	0.782 ± 0.011
II	64.27 ± 0.109	3.61 ± 0.107	2.82 ± 0.108	0.728 ± 0.016
III	92.64 ± 0.841	2.06 ± 0.058	2.25 ± 0.139	0.734 ± 0.027
IV	94.85 ± 0.917	2.00 ± 0.101	2.24 ± 0.121	0.746 ± 0.009
V	6,604.92 ± 2.157	1.22 ± 0.055	1.18 ± 0.182	2.702 ± 0.101
Ascorbic acid	7.24 ± 0.089	1.16 ± 0.017	1.04 ± 0.089	0.905 ± 0.089
BHA	331.42 ± 1.899	1.22 ± 0.056	1.43 ± .009	0.984 ± 0.057
BHT	1,077.10 ± 2.114	0.39 ± 0.008	1.04 ± 0.057	0.963 ± 0.007
Trolox	93.19 ± 1.089	1.00 ± 0.051	1.00 ± 0.037	1.191 ± 0.039

Note: Results are given as the mean value ± standard deviation ($n = 3$); (Tukey's test, $p < .05$).
Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CUPRAC, cupric-reducing antioxidant capacity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FRAP, ferric-reducing antioxidant power.
[a]Antioxidant activity was measured by using the DPPH radical assay and data are expressed as EC_{50} (μM).
[b]FRAP and CUPRAC results were expressed as trolox equivalent antioxidant capacity (mM).
[c]The metal chelating activity was determined by inhibiting ferrous–ferrozine complex formation and data are expressed as EC_{50} (mM).

nature of substituents like –OH, $–CH_3$, and also C═C on a coumarin scaffold, as they play a key role in reducing free radicals such as DPPH, which can consequently prevent oxidative damage to cells. Although compound **V** has a similar structure with other coumarins, it displayed the lowest DPPH activity. This low activity may be attributed to the *para* position of 5-OH and 4-phenyl groups instead of *ortho* position when compared with other compounds. As shown in Figure 2, the results of the time-dependent free radical scavenging activities of the studied compounds and the standard antioxidant trolox have showed that all coumarin compounds have a very fast initial reaction with DPPH when compared with Trolox.

Coumarins possess antioxidant activities probably due to their structural analogy with flavonoids, and it has been reported that they could bind to transition metal ions.[13] The abilities of the coumarin

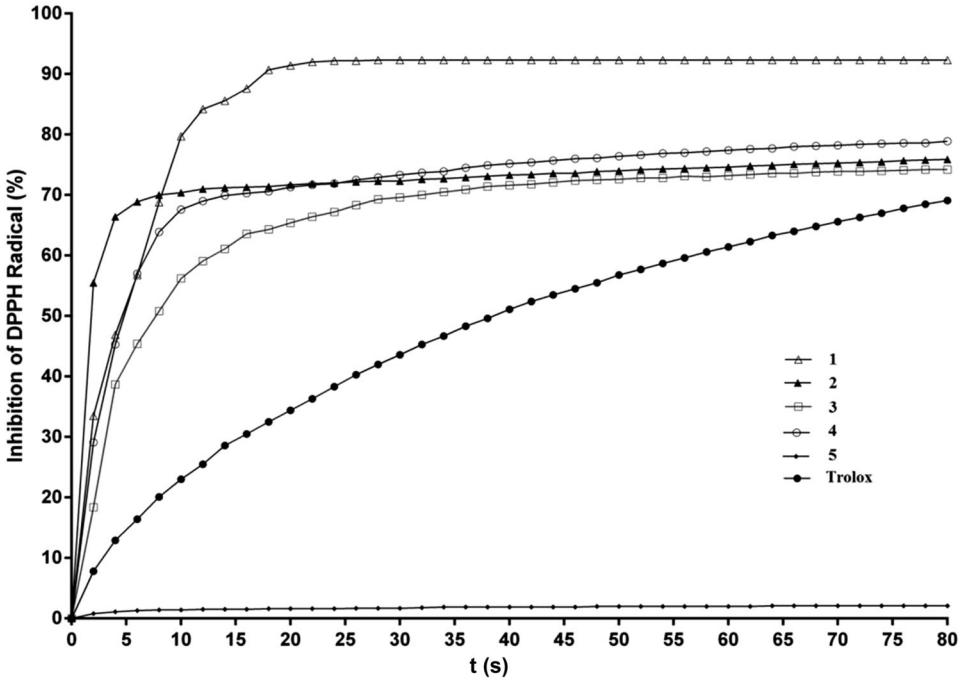


FIGURE 2 DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging capacity (% inhibition) of coumarin derivatives as a function of the reaction time (s)

compounds to reduce ferric ions (FRAP), cupric ions (CUPRAC), and their ferrous ion chelating activities were compared with that of Trolox, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and ascorbic acid, and it was found that coumarin derivatives, except compound **V**, have more potent antioxidant activities than the standard antioxidants that were used in the study. The FRAP and CUPRAC assays offer accepted methods for the evaluation of the antioxidant activity. It is clear from the results that the CUPRAC and FRAP activities of compound **II** are the highest among all the species studied. The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions, thus inhibiting hydroxyl radical and hydrogen peroxide formation produced by Fenton's reactions. Our results revealed that compound **II** also exhibits a mild chelating activity in vitro; therefore, it could have therapeutic potential in the treatment of oxidative stress-related diseases.

2.2 | In vitro HMGR inhibitory activity

Human HMGR, the rate-limiting enzyme in cholesterol biosynthesis, is a transmembrane glycoprotein that catalyzes the NADPH-dependent four-electron reduction of HMG-CoA to CoA and mevalonate. Inhibitors of HMGR, such as statins, are effective agents used to reduce serum cholesterol levels and prevent coronary artery diseases.[5] For known pitavastatin analogs, a phenyl group is connected at position 4 in the quinoline, and it is located in the *ortho* position to the pharmacophore group at position 3. This substitution pattern is also found in other artificial HMGR inhibitors such as fluvastatin, atorvastatin, and rosuvastatin, which belong to the indole, pyrrole, and pyrimidine derivatives, respectively.[14]

According to our results, compound **IV** displayed the highest HMGR inhibitory activity (IC_{50} = 42.0 μM) when compared with other derivatives, whereas compound **V** shows the lowest activity (Figure 3). However, the inhibitory activities of the studied coumarin derivatives on HMGR are lower than that of standard inhibitor, pravastatin (IC_{50} = 6.9 μM). In our previous study, we investigated in vivo antioxidative and lipid-lowering effects of 7,8-dihydroxy-3-(4-methylphenyl)coumarin (compound **IV**) in hyperlipidemic rats and reported that this compound displayed potent antioxidant and lipid-lowering effects.[5] Our results suggest that the apparent in vivo lipid-lowering activities of coumarin derivatives could be caused by the inhibition of the HMGR activity.

2.3 | QM descriptors

QM studies were carried out to derive theoretical data. Electron densities of frontier molecular orbitals provide insights into donor–acceptor interactions that play a key role in several pharmacological mechanisms.[15] In addition to this, the frontier orbitals have a strong correlation with the antioxidative activity.[16] The energy of the highest occupied molecular orbitals (HOMO) is directly proportional to the electron-donating ability of a compound.[17,18] Figure S1 shows that electron-donating groups increase the HOMO energy, whereas electron-withdrawing groups (EWGs) such as $-NO_2$, as in compound **II**, reduces the HOMO energy, as well as the lowest unoccupied molecular orbital (LUMO) energy. It was also observed that HOMO orbitals are localized in the coumarin ring, whereas LUMO orbitals are delocalized in the whole molecule.

The energy of the HOMO orbitals is proportional to the tendency of the molecule toward an electrophile attack. In contrast, the

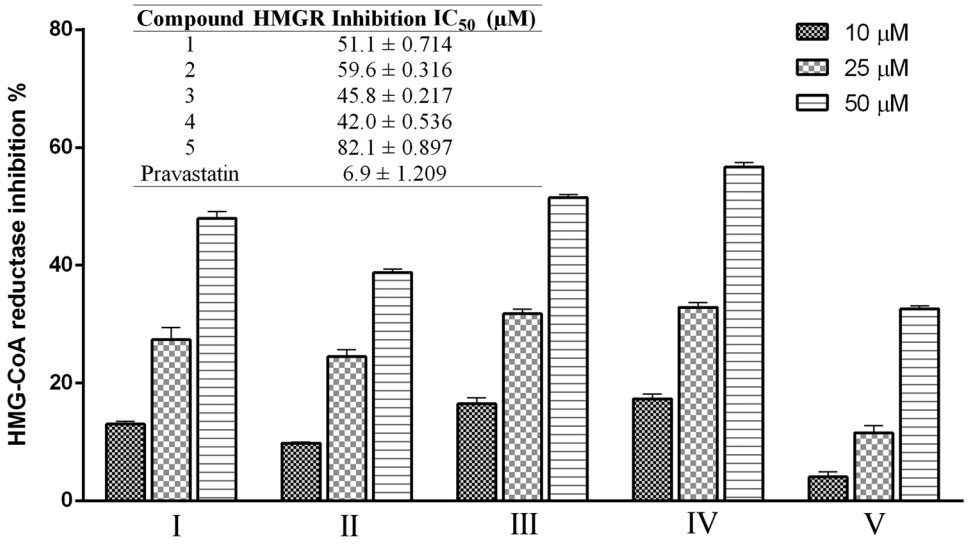


FIGURE 3 HMG-CoA (3-hydroxy-3-methyl-glutaryl coenzyme A) reductase (HMGR) inhibitory activities (%) and IC_{50} values of the coumarins investigated

TABLE 2 Energies of HOMO and LUMO orbitals calculated by the M062X method

Coumarin	Frontier orbital	Energy in gas phase (eV)	Energy in water phase (eV)
I	HOMO	−7.073	−6.813
	LUMO	−1.004	−0.744
II	HOMO	−7.481	−6.978
	LUMO	−1.726	−1.463
III	HOMO	−7.036	−6.784
	LUMO	−0.966	−0.713
IV	HOMO	−6.973	−6.733
	LUMO	−0.955	−0.705
V	HOMO	−7.365	−7.157
	LUMO	−0.885	−0.713

Abbreviations: HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital.

energy of the LUMO levels reflects the susceptibility of the molecule to a nucleophilic attack.[15] We analyzed HOMO and LUMO energies and evaluated correlations between inhibition capacity and antioxidant activity of the coumarin derivatives. According to Table 2, HOMO energy values of coumarin derivatives that are obtained from M062X calculations in the water phase are strongly correlated with their inhibitory activities. The HOMO energies are ordered as **IV** > **III** > **I** > **II** > **V**, which is in accordance with the experimental data. However, further QM research is required to illuminate the inhibition mechanism that involves possibly multiple reactions in the active site of the HMGR enzyme.[19]

Furthermore, according to the DPPH assay, there is a strong correlation between LUMO energy levels and the antioxidant activity of studied compounds. Compound **II**, which was found to have the highest antioxidant activity, also has the lowest LUMO energy level, both in gas and water phase. The low LUMO energy level increases the susceptibility of the molecule to a nucleophilic attack, therefore its potential to reduce DPPH, FRAP, and CUPRAC.

Among 3-phenyl-substituted coumarins, compound **II** stands out as the only compound that bears an EWG on its phenyl ring. Compound **I** does not possess any substituent on the phenyl ring. This shows that EWG renders the compound an easier target for an electron transfer, due to their lowering effect on LUMO orbital energies (Table 2). In contrast, compounds **III** and **IV** bear the same electron-donating group at adjacent positions. However, due to this difference in position, the molecule has a slight change in HOMO energy in both gas and water phase. There is an increase in the HOMO energy of the methyl group at 4-position (**IV**), compared with one that bears the same group at 3-position (**III**). Compound **V**, with the lowest antioxidant and inhibitory activity, has OH− substituents at the 5- and 7-positions, unlike others that have substituents at the 7- and 8-positions and also bear the phenyl ring at the 4-position instead of the 3-position. This provided the molecule with more localized HOMO orbitals, located in the coumarin scaffold. Figure S1 displays that LUMO orbitals are more delocalized in compound **II**. This is in accordance with the order of LUMO energies (Table 2). This arises from the fact that EWGs lower the energy of LUMO orbitals, rendering them more susceptible to a nucleophilic attack.

TABLE 3 Docking scores of coumarin derivatives and pravastatin

Compounds	Lowest binding energy (kcal/mol)	Mean binding energy (kcal/mol)
I	−7.36	−7.31
II	−9.52	−8.34
III	−7.4	−7.28
IV	−7.39	−7.36
V	−6.45	−5.09
Pravastatin	−8.15	−7.81

2.4 | Docking scores

Molecular docking studies were carried out to see the affinities of coumarin derivatives toward HMGR. Scores are given in Table 3.

According to the docking scores, compound **II** has the highest affinity to the HMGR enzyme. This may be attributed to more delocalized LUMO orbitals on the coumarin ring of compound **II** (Figure S1), which enables a nucleophilic attack, particularly on the coumarin ring. Furthermore, being the only coumarin that bears an EWG on its phenyl ring, compound **II** has the highest electron density on its phenyl ring. Additional electrostatic interactions that are formed with **II** contribute to strong affinity of **II** toward HMGR, as the catalytic site of HMGR has a positive electrostatic potential.[15] Pravastatin, a known good inhibitor of HMGR, follows compound II in binding affinity. Compound IV, of which lipid-lowering activity on rats has been validated earlier, comes after compound II.[5]

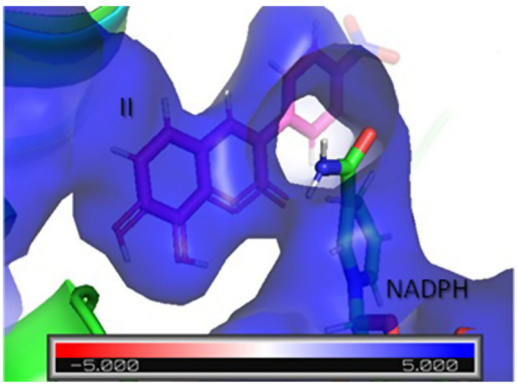


FIGURE 4 Coumarin **II** bound to positively charged pocket in HMGR (3-hydroxy-3-methyl-glutaryl coenzyme A reductase). Surface visualization was performed using the APBS (Adaptive Poisson–Boltzmann Solver) Electrostatics plugin for PyMOL (http://www.pymol.org)

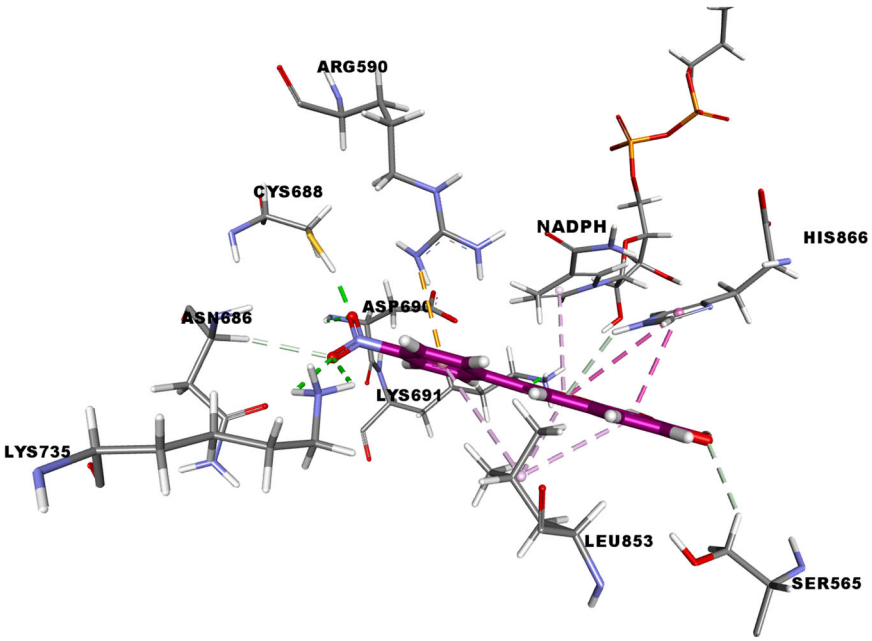


FIGURE 5 The binding mode of compound **II** in the active site of HMGR (3-hydroxy-3-methyl-glutaryl coenzyme A reductase). Image obtained by Discovery Studio 4.5

ADME properties	Pravastatin	I	II	III	IV	V
Molecular weight (Da)	424.53	254.24	299.24	268	268.26	254.24
The number of H bond acceptors	7	4	6	4	4	4
The number of H bond donors	4	2	2	2	2	2
Log *P*	2.34	2.5	1.85	2.82	2.82	2.43

Abbreviation: ADME, absorption, distribution, metabolism, and excretion.

TABLE 4 ADME properties concerning Lipinski's rule of five

2.5 | Binding site of coumarins and electrostatic potential

The coumarin scaffolds of the studied compounds are expected to be rich in electron density, as they bear electron-donating groups such as –OH. This is supported by the localized HOMO orbitals (Figure S1). Considering the positive electrostatic potential in the catalytic site in HMGR (Figure 4), it can be understood that these electron-rich compounds' binding is favored in the pocket.

Figure 5 shows the binding site of **II** in the active site. Hydrogen bonds and π electron-originated interactions are found to be dominant in the stabilization of **II**. Hydrogen bonds are formed with Asn686, Cys688, Asp690, Lys691, and Lys735 with distances of 2.72, 1.76, 2.23, 1.61, and 2.47 Å, respectively. Cation–π interactions were observed with Arg590 (3.75 Å). Alkyl–π interactions were observed with Leu853 and NADPH (4.44, 5.27 Å). His866 contributes to π–π stacked interactions, with distances of 3, 3.81, and 4.87 Å.

TABLE 5 Toxicity prediction of synthesized compounds and pravastatin

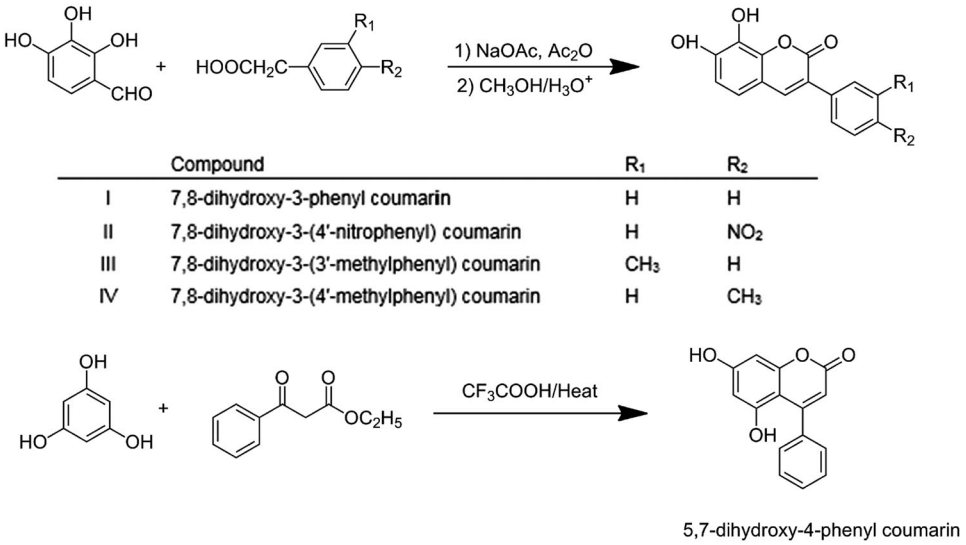
	Pravastatin	I	II	III	IV	V
LD_{50} (mg/kg)	8,939	350	600	350	250	3,200
Hepatotoxicity (%)	83	71	61	71	71	68
Cytotoxicity (%)	61	96	71	95	95	86

2.6 | ADME (absorption, distribution, metabolism, and excretion) properties

For all coumarin derivatives and pravastatin, Lipinski's rule of five parameters, which are important to evaluate the druglikeness of drug lead compounds, was applied using the web server (http://www.swissadme.ch/).[20] Briefly, the rule states that the molecular weight of an oral drug should be lower than 500 Da, the number of hydrogen bond acceptors should be at most 10, the number of hydrogen bond donors should be at most 5, and log *P* should be lower than 5. As seen in Table 4, none of the coumarin derivatives violate the "Rule of Five" parameters.[21]

2.7 | Toxicity

The C-3 and C-4 epoxidation reaction that unsubstituted coumarins undergo in the liver has been shown to cause toxicity. CYP2A6 cytochrome P-450 oxidases generate this reaction, followed by a series



	Compound	R_1	R_2
I	7,8-dihydroxy-3-phenyl coumarin	H	H
II	7,8-dihydroxy-3-(4′-nitrophenyl) coumarin	H	NO_2
III	7,8-dihydroxy-3-(3′-methylphenyl) coumarin	CH_3	H
IV	7,8-dihydroxy-3-(4′-methylphenyl) coumarin	H	CH_3

SCHEME 1 The scheme of synthesis and chemical structures of the coumarin derivatives

of reactions that finally give stable complexes with heavy metals, which are thus toxic to the liver.[22] It was shown that 3,4-double bond has a strong correlation with coumarin toxicity.[23] Ergo, coumarins with cyclic substituents at position 3 are promising, as they are likely to be resistant to C-3 and C-4 epoxidation. Table 5 shows toxicity percentages obtained from web server, http://tox.charite.de/.[24]

3 | CONCLUSION

We investigated the inhibitory activities of several coumarin derivatives against the HMGR enzyme. HMGR is known to be tightly related to the ROS content. Therefore, we also analyzed antioxidant activities of these compounds. The results of in vitro experiments showed that compound **IV** showed the highest inhibitory activity, whereas compound **II** showed the highest antioxidant activity. Docking studies revealed that compound **II** has also the strongest affinity toward HMGR. The results are in accordance with common pharmacophore features of the coumarin derivatives. The presence of an EWG, $-NO_2$, on the phenyl ring has rendered the compound **II** to be a good/valuable antioxidant. Compounds **III** and **IV** show higher inhibition activities than **I**, supported by relatively higher binding affinities, which is related to the presence of $-CH_3$ group instead of –H, providing more interactions with the cavity. The position of phenyl ring at position 4 of compound **V** was found to be responsible for poor inhibition activity, as it yielded an arrangement that is not favorable for binding to the catalytic site. Previously, our group has shown that compound **IV** might be a drug lead for its in vivo lipid-lowering effects.[5] The findings of this study suggest that compound **II** also might have a potential to be a drug lead for oxidative stress-related and atherosclerotic diseases; thus, further in vivo studies are recommended to evaluate its lipid-lowering effects.

4 | EXPERIMENTAL

4.1 | Materials

HMG-CoA Reductase Assay Kit (#CS1090), Trolox, BHT, and BHA were purchased from Sigma-Aldrich Co., St. Louis, MO. Neocuproine was purchased from Merck KGaA, Darmstadt, Germany. All other chemicals used were of analytical grade. For quantum-chemical analysis, Gaussian 09[25] software was used. AutoDock4[26] was used for docking experiments. Discovery Studio 4.5[27] was used for visualization.

The InChI keys of the investigated compounds, together with some biological activity data, are provided as Supporting Information Data.

4.2 | Methods

4.2.1 | Chemistry

3-Phenylcoumarin derivatives (**I**, **II**, **III**, and **IV**) were prepared by the reaction of substituted hydroxybenzaldehydes with the corresponding arylacetic acids under the traditional Perkin conditions.[28–30] 4-Phenylcoumarin derivative (**V**) was obtained by condensation of selected phenol with ethyl-3,4-dimethoxybenzoylacetate according to the Pechmann reaction.[13] Scheme 1 shows the scheme of synthesis and chemical structures of the coumarin derivatives. To the best of the authors' knowledge, compound **III** has only been designed under a patent (WO 9424119, 1994) without proper characterization. Therefore, compound **III** have been characterized with ^1H and ^{13}C nuclear magnetic resonance, and the spectra have been given in Figure S2.

4.2.2 | DPPH radical scavenging activity

The free radical scavenging activities were measured by DPPH using the method of Blois.[31] Briefly, 0.1-mM solution of DPPH in methanol was prepared, and 1 ml of this solution was added to 250 μl of coumarins in methanol containing 5 μl dimethyl sulfoxide (DMSO) at different concentrations (10–100 μg/ml). The mixture was shaken vigorously and allowed to be kept at room temperature for 30 min. Later, the absorbance was spectrophotometrically measured at 517 nm. The radical scavenging activities of the samples were expressed in terms of EC_{50} (concentration required for a 50% decrease in absorbance of DPPH radical). To determine the rate of the reaction, assay was repeated for coumarin derivatives and Trolox at 50 μM final concentration, and the decrease of absorbance was monitored every 2 s for 90 s at 517 nm using the Helios Zeta UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

4.2.3 | FRAP assay

Ferric ion (Fe^{3+}) reducing power was evaluated according to the protocol of Oyaizu[32] with adjustments. BHT, BHA, and Trolox were used as positive controls. 100 μl of samples (dissolved in phosphate buffer 50 μM, pH 6.6, containing 5 μl DMSO) were mixed with phosphate buffer (200 μl, 50 μM, pH 6.6) and $K_3Fe(CN)_6$ (200 μl, 1%). The mixture was incubated at 50°C for 20 min. A portion (250 μl) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 10,000*g* for 10 min. The upper layer of the solution (700 μl) was mixed with $FeCl_3$ (150 μl, 0.1%) and the absorbance was measured at 700 nm. BHT, BHA, and Trolox were used as positive controls. Results were expressed as the Trolox equivalent antioxidant capacity (TEAC), which is defined as the millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0-mM solution of the substance under investigation.

4.2.4 | CUPRAC assay

CUPRAC was determined as described by Apak et al.[33] with slight changes. Briefly, 800 μl of coumarin derivatives were dissolved in NH_4Ac buffer, pH 7, containing 5 μl DMSO, and mixed with 160 μl NH_4Ac buffer, pH 7, 160 μl $CuCl_2$ (10 mM), and 160 μl neocuproine (7.5 mM). After 30 min, absorbance was measured at 450 nm. Results were expressed as the TEAC, which is defined as the millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0-mM solution of the compound under investigation.

4.2.5 | Metal chelation

Ferrous ion (Fe^{2+}) chelating activity was determined by inhibiting ferrous–ferrozine complex formation after treatment of test material with ferrous ion (Fe^{2+}). 20–100 μg/ml concentrations of compounds in 500 μl methanol were added to a solution of 2 mM $FeCl_2$ (500 μl). The reaction was initiated by the addition of 5 mM ferrozine (200 μl) in methanol. Then, the mixture was shaken vigorously at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_s] \times 100$, where A_0 is the absorbance of the control and A_s is the absorbance of the compound/standard. The ferrous ion chelating effects of the compounds were expressed in terms of EC_{50} (concentration required for chelating 50% of ferrous ions).

4.2.6 | HMGR inhibition assay

Coumarin derivatives were screened for their ability to inhibit the catalytic activity of human recombinant HMGR in vitro. The assay is based on the spectrophotometric measurement of the decrease in absorbance at 340 nm, which represents the oxidation of NADPH by the catalytic subunit of HMGR in the presence of the substrate, HMG-CoA. The HMGR assay was performed using the manufacturer's protocol. Briefly, about 6 μg of the enzyme was incubated at 37°C with 400 μM NADPH, 0.3 mg/ml HMG-CoA, and different concentrations (10–25–50 μM) of coumarin derivatives in a 96-well plate, and their absorbance change was monitored at 37°C using Epoch microplate spectrophotometer (BioTek, Winooski, VT). The concentration of an inhibitor required to inhibit 50% of the HMGR under the assay conditions was defined as IC_{50}. Pravastatin was utilized as a control for positive inhibition.

4.2.7 | Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA) software in triplicate, and the difference of parameters was statistically tested with unpaired Student's *t*-test. The level of significance was defined as $p < .05$.

4.2.8 | QM Calculations

Pravastatin and coumarin derivatives (compounds **I**, **II**, **III**, **IV**, and **V**) were optimized with M062X functional and 6–31 G(d,p) basis set by using Gaussian 09.[25] Temperature was set to 310 K. HOMO–LUMO orbitals were produced and the energies of HOMO and LUMO levels were calculated in gas and water phase.

4.2.9 | Minimization of HMGR enzyme and docking calculations

The crystal structure of HMGR was obtained from the Brookhaven Protein Data Bank (PDB ID: 1DQA). To eliminate any steric clashes in the crystal structure, the dimer was first subjected to a minimization

procedure by using the steepest descent algorithm with GROMACS 5.1.2 package.[34] Minimization was complete as the maximum force of 10 kJ/mol was reached. Water, CoA, and HMG were removed from the minimized structure for molecular docking simulations. NADPH was kept intact in the protein, as statins are not known to be interfering with NADPH binding.[35] Before docking, HMG was removed and redocked into the enzyme by using AutoDock4.[26] A root mean square deviation of 0.941 Å confirmed the reliability of the docking protocol. Optimized structures of compounds **I**, **II**, **III**, **IV**, **V**, and pravastatin were docked into the dimeric structure. The calculations were confined within a grid box with dimensions of 60 × 60 × 60 Å and centered at a sulfur atom of CoA, which was large enough to include the binding site of HMG-CoA. The Lamarckian genetic algorithm[36] was employed, which is implemented in AutoDock4. The maximum number of energy evaluations was set to 2,500,000 and 100 runs were performed for each ligand. All calculations were performed on Linux platform. Hits with the lowest Gibbs binding energy (ΔG) of each compound were analyzed.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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