Indian Journal of Biochemistry & Biophysics
Vol. 62, September 2025, pp. 969-977
DOI: 10.56042/ijbb.v62i9.16046

Assessing the HMG-CoA reductase inhibitory potential of five fatty acids

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Received 27 December 2024; *revised 04 March 2025*

Fatty acids play a key role as building blocks of lipids. Their lipid-lowering properties have been proven in early biochemical investigations. This investigation focuses on assessing the effects of five fatty acids—including oleic acid, linoleic acid, docosahexaenoic acid (DHA), 5,8,11,14-eicosatetraynoic acid (ETYA), and butyric acid—on the direct inhibition of HMG-CoA reductase (HMGCR), the rate-limiting enzyme involved in cholesterol biosynthesis. To the best of our knowledge, this is the first time that the inhibitory effects of these fatty acids on direct HMGCR activity have been investigated. The fatty acids tested displayed varying levels of HMGCR inhibitory potential, with oleic acid exhibiting the highest efficacy, followed by linoleic acid, DHA, ETYA, and butyric acid. *In silico* analysis discovered that these fatty acids make hydrophobic interactions and hydrogen bonds with the catalytic domain of HMGCR. This preliminary investigation provides a foundational basis for evaluating the detailed effects of fatty acids as potential treatment options in combination with cholesterol-lowering drugs such as statins.

Keywords: Cholesterol synthesis inhibition, Enzyme assays, Drug discovery, Lipids, Rate-limiting enzyme

The most publicized lipid, cholesterol, has gained much attention due to its involvement in cardiovascular diseases. Apart from its association with cardiovascular diseases, cholesterol exerts various biochemical and physiological functions in the body[1]. For example, cholesterol plays a key role as a cell membrane component, signaling molecule, pre-cursor for vitamin D, bile acid and steroid hormones. Cholesterol biosynthesis is reported in almost all animal cells through complex enzymatically catalyzed reactions, where the liver serves as the major site for cholesterol synthesis[1]. HMG-CoA reductase (HMGCR) is the rate limiting enzyme of the cholesterol biosynthesis pathway, which catalyzes the reduction of HMG-CoA to mevalonate[2]. Cholesterol-lowering strategies are crucial in the management of cardiovascular diseases and its associated diseases. Statins are a class of cholesterol lowering drugs which inhibit the activity of HMGCR[1,2]. Apart from statins, bempedoic acid, an ATP citrate lyase inhibitor, is an example for a clinically used new cholesterol lowering drug[3].

Fatty acids play an important role as structural components of lipids. These molecules can be categorized into three main types: saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids, depending on the number of double bonds within the hydrocarbon side chain. Fatty acids exert several important functions in the body. They serve as a major source of energy, are essential component of cell membranes, act as a signaling molecule, and play a key role in the production of some steroid hormones[1]. Among the unsaturated fatty acids, there are omega-3, omega-6, omega-7 and omega-9 based on the specific location of their first double bond relative to the end of the carbon chain[4,5].

Butyric acid is a short-chain fatty acid found in dairy products. It is produced by gut bacteria during the fermentation of dietary fiber in the human body. Butyric acid plays a key role in maintaining gut health and shows anti-inflammatory and immunomodulatory effects[6]. Linoleic acid, an essential omega-6 fatty acid, is commonly found in a variety of plant-based oils, seeds, and nuts. It is a precursor to arachidonic acid and arachidonic acid is a precursor to many eicosanoids[7]. Oleic acid is an omega-9 fatty acid that is abundant in plant oils. It is also found in animal fats, such as pork and beef. Oleic acid has been associated with a reduced risk of cardiovascular

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diseases and reported to reduce inflammation[8]. DHA, an omega-3 fatty acid, is mainly found in fatty fish[9]. DHA plays a key role in supporting cognitive function, visual development, memory, and brain health[9]. ETYA, or 5,8,11,14-eicosatetraynoic acid is a competitive analogue of arachidonic acid comprising alkyne bonds[10]. It is a DNA synthesis inhibitor and acts as an antioxidant[11,12]. Early studies suggest ETYA may inhibit fatty acid and cholesterol synthesis[13,14].

The lipid-lowering properties of fatty acids have been well-documented in research dating back to earlier times[15]. In general butyric acid and one of its derivatives, 4-phenylbutyric acid display favorable pre-clinical and clinical outcomes against lipid disorders[16]. Caco-2 cells exposed to butyric acid (5mM) demonstrated down-regulated HMGCR gene expression[17]. In another investigation, butyric acid was shown to reduce intracellular HMGCR activity in Caco-2 cells[18]. Butyrate has been reported to lowerplasma cholesterol levels in low-density-lipoprotein receptor knockout (LDLr-/-) mouse model fed with a high-fat diet[19].

It has been shown that moderate consumption of linoleic acid is associated with reduced serum cholesterol levels and low-density lipoprotein (LDL)-cholesterol concentrations[20]. α-linolenic acid demonstrated hypocholesterolemic effects through suppression of the expression of HMGCR mRNA and the inhibition of the activity of HMGCR isolated from the rat liver[21]. A spray-dried milk comprising α-linolenic acid, eicosapentaenoic acid and DHA, reduced HMGCR activity and enhanced the secretion of bile constituents [22]. Rats fed a diet rich in omega-3 polyunsaturated fatty acids (n-3 PUFA) had significantly lower HMGCR activity in their liver microsomes compared to those fed a diet rich in omega-6 polyunsaturated fatty acids (n-6 PUFA)[23]. Eicosapentaenoic acid and DHA were identified as safe secondary pharmacological treatments following a myocardial infarction[24]. When rabbits consumed fish oil rich in omega-3 acids, there was a reduction in HMGCR activity and a rise in Acyl-CoA:cholesterol acyltransferase (ACAT) activity within their liver and intestinal microsomes[25]. MCF-7 breast cancer cells and HepG2 liver cancer cells exposed to eicosapentaenoic acid and DHA demonstrated reduced activity and expression of HMGCR[26,27]. In a hamster species with cholesterol ester transfer protein (CETP), a high-fat diet supplemented with omega-3 fatty acids showed greater fecal cholesterol excretion and bile acid excretion, highlighting the potential role of omega-3 fatty acids in the efflux of cholesterol[28]. DHA was found to reduce the levels of cholesterol anabolites and catabolites[29].

Two studies examined the effect of oleic acid on the enzyme HMGCR activity in C6 glioma cells. One study found that oleic acid (25 μM) inhibited HMGCR activity by 29%[30], while another study demonstrated a stronger inhibition of 45% with a higher concentration of 100 μM oleic acid[31]. Dietary linoleic acid consumption is inversely linked with CHD risk[32]. Linoleic acid was found to reduce serum cholesterol levels in hypercholesterolemic rats[33]. In apoE-deficient mice, dietary linoleic acid was found to reduce serum cholesterol levels[34]. Linoleic acid extracted from Monascus-fermented rice showed moderate HMGCR inhibitory potential[35].

Encouraged by previous investigations indicating several fatty acids lower serum cholesterol levels and reduce the risk of atherosclerosis disease *in vitro* and *in-vivo*, it was hypothesized that fatty acids may have a direct inhibitory effect on the activity of HMGCR. The hypothesis was tested using a selected panel of fatty acids comprising butyric acid, oleic acid (omega-9), DHA (omega-3), linoleic acid (omega-6) and 5,8,11,14-eicosatetraynoic acid (ETYA), picked based on variations in carbon chain length and saturation, assuming these chemical features might influence their inhibitory effects on HMGCR. Figure 1 shows the chemical structures of fatty acids used in this study.

Materials and Methods

Chemicals and kits

The fatty acids butyric acid (Cat. No. B103500), linoleic acid (Cat. No. L1376), oleic acid (Cat. No. O1383), docosahexaenoic acid (Cat. No D2534) and ETYA (5,8,11,14-eicosatetraynoic acid) (Cat. No. E1768) and the HMG-CoA Reductase assay kit (CS1090) were purchased from Sigma-Aldrich, USA. Stock solutions of fatty acids were prepared in ethanol and diluted when conducting enzyme assays.

HMG-CoA reductase (HMGCR) assay

The HMG-CoA reductase assay kit measures the activity of HMGCR. This colorimetric assay, carried out at 340 nm, uses the conversion of HMG-CoA to mevalonate by HMGCR, where decrease in the absorbance reveals the oxidation of NADPH by HMGCR when its substrate, HMG-CoA, is available. In the assay, 100 μM of each fatty acid

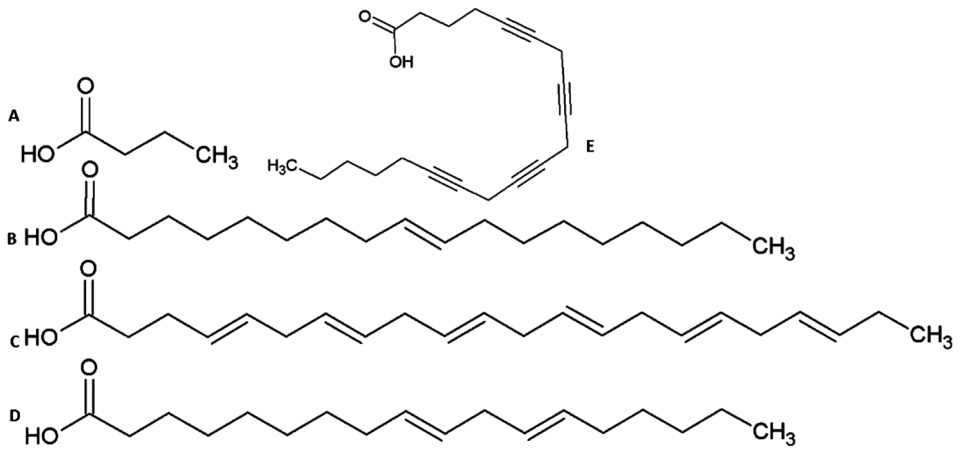

Fig. 1 — The chemical structures of fatty acids used in the present study. (A) Butyric acid; ($C_4H_8O_2$); (B) Oleic acid ($C_{18}H_{34}O_2$); (C) DHA ($C_{22}H_{32}O_2$); (D) Linoleic acid ($C_{18}H_{32}O_2$); and (E) ETYA ($C_{20}H_{24}O_2$)

was subjected to the enzyme assay. For inhibition assays in 96-well plates, the reaction mixtures comprised of 2 µL of HMGCR, 12 µL HMG-CoA, 181 µL of 1X assay buffer, 4 µL of NADPH and 1 µL of fatty acids (200 µL total). Negative controls lacked fatty acids. Pravastatin was used as the positive control. The absorbance was recorded for 15 min at 30 sec time intervals with vigorous shaking according to manufacturer's instructions. The percentage enzyme inhibition was calculated using the following equation (absorbance decline from 540 to 900 sec):

Percentage enzyme inhibition = (ΔA340 nm/ΔT (enzyme control)- ΔA340 nm/ΔT (enzyme + fatty acids)/ ΔA340 nm/ΔT (enzyme control) x 100 [36]

Receptor preparation

The crystal structure of the protein HMGCR was obtained from RCSB Protein Data Bank (PDB ID: 1HWJ). AutoDockTools v.1.5.6 was used for molecular docking analysis with necessary configurations[37]. The chain A of HMGCR was selected for the docking process following removal of water molecules and heteroatoms and the missing atoms were repaired to ensure the completeness of the protein. To advance the accuracy of the docking methods, polar hydrogens were added to the prepared 1HWJ structure and Gasteiger charges were assigned to the atoms. The prepared 1HWJ structure was saved as a pdbqt file for molecular docking with the respective fatty acids used in the study.

Ligand preparation

The chemical structures of fatty acids used in the study were obtained from PubChem and the 3D conformer of each fatty acid was downloaded as SDF files. The conversion of the ligand file to pdbqt was conducted using Open Babel v.2.4.1 which is compatible with AutoDockTools. The ligand file was subject to Gasteiger charge assignment to maintain accurate representation with the utilization of the 'Torsion Tree' feature to define the binding site and improve the docking process. The 'Detect Root' option was applied to establish the suitable starting configuration for the ligand.

Docking with AutoDock-GPU

The docking input files for AutoDock was prepared using AutoDockTools. The dimensions of the grid box were modified to ensure maximum coverage of the protein and ligand complex. The grid was adjusted to contain 126 points in each direction with a spacing of 0.5, and the center dimensions were changed to house the protein and ligand complex. To create the necessary grid maps for the docking calculations AutoGrid was used. The docking process was carried out using a Graphical Processing Unit (GPU) and involved the Lamarckian Genetic Algorithm (LGA) and ADADELTA gradient-based local search methods for conformation search algorithms[38]. The quality of each binding pose was assessed using an energy-based scoring function. The docking parameters consisted of fifty genetic algorithm (GA) runs and was conducted with a population size of 300 individuals, totalling 2,500,000 energy evaluations and 27,000 generations per Lamarckian genetic algorithm run (LGA). Following the completion of the docking runs, the resulting DLG file was analyzed in AutoDockTools. The Root mean square deviation (RMSD) was studied, and the conformation with the

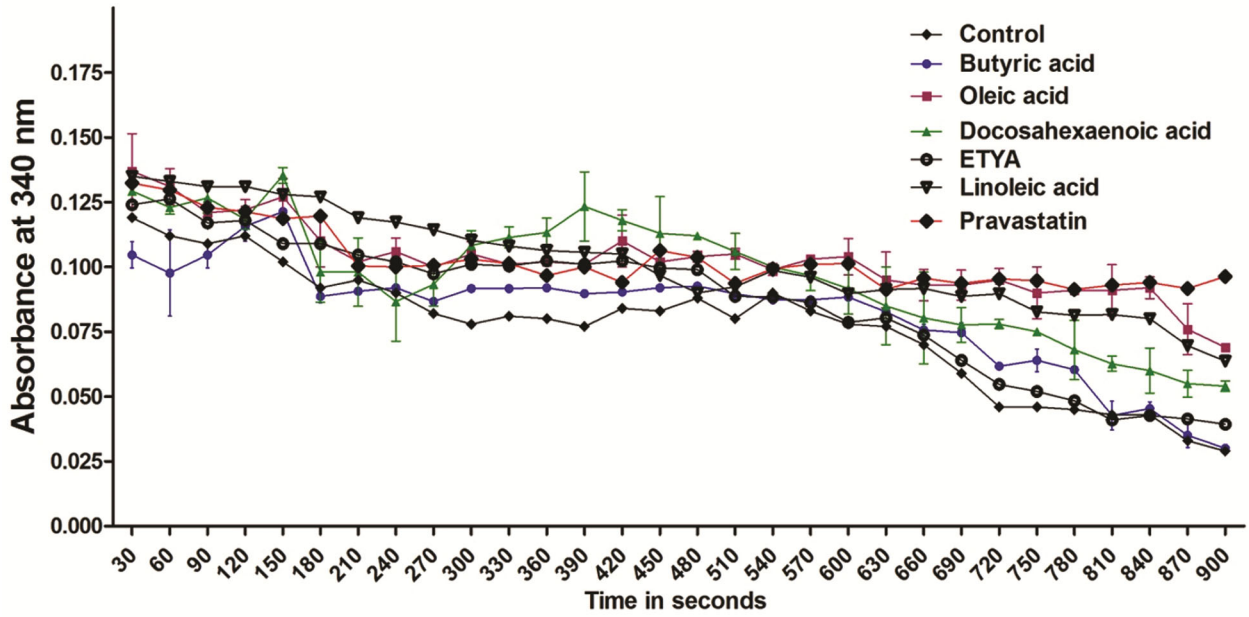

Fig. 2 — The spectrophotometric time scans showed the reaction progress catalyzed by HMGCR with and without the presence of 100 µM of each fatty acid (butyric acid, oleic acid, DHA, ETYA, and linoleic acid). The enzyme kinetic assays were conducted in 96-well plates using a microplate reader, and absorbance was measured at 340 nm every 30 sec for 15 min. Bar mean ± SD (n = 3) was used for data representation. Negative controls were treated with ethanol only

most negative binding energy (indicating strong binding) was selected. Open babel was used to convert the pdbqt file of the complex into a pdb format, which was then visualized using PLIP (Protein-Ligand Interaction Profiler)[39]. The resulting complex pse file was downloaded from PLIP and further visualized to create three-dimensional (3D) images using PyMOL 2.5 software.

Data analysis and statistics

GraphPad Prism version 5 (GraphPad Software, Inc., San Diego, CA, USA) software was used to analyses the data and generate graphs for the enzyme assays. Experiments were carried out in triplicate and mean ± SD was considered when generating graphs for the enzyme inhibition assays. One-way ANOVA with Tukey's test was used to determine significant differences among the control group and the fatty acid experimental dose (100 µM).

Results

This investigation attempted to evaluate how a selected group of fatty acids—including oleic acid, linoleic acid, docosahexaenoic acid (DHA), ETYA (5,8,11,14-eicosatetraynoic acid), and butyric acid—affects the direct inhibition of HMGCR. The HMGCR enzyme inhibition assay displayed results as shown in (Fig. 2). From the plots, by considering the absorbance

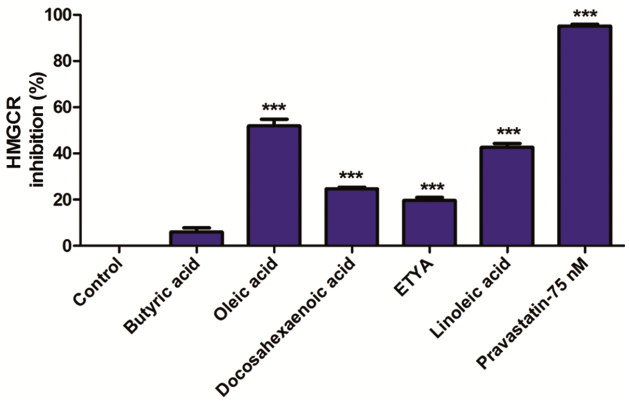

Fig. 3 — The percentages of HMGCR inhibition by butyric acid (100 µM), oleic acid (100 µM), DHA (100 µM), ETYA (100 µM), linoleic acid (100 µM), and pravastatin (75 nM) were determined. Data are presented as mean ± SD. Statistical analysis comparing the control group to different doses of fatty acids was performed using one-way ANOVA with Tukey test, with a significance level set at $P< 0.05$

decline from 540 to 900 sec, the HMGCR enzyme inhibitory potential of fatty acids were calculated. According to the assay results (Fig. 3), fatty acids at tested doses demonstrated enzyme inhibitory potential in the following order: oleic acid > linoleic acid > DHA > ETYA > butyric acid. The corresponding inhibition percentages were as follows: 51.87% ± 5.04, 42.61% ± 2.86, 24.59% ± 1.42, 19.63% ± 2.26, and 5.96% ± 3.21, respectively. Except for the butyric acid,

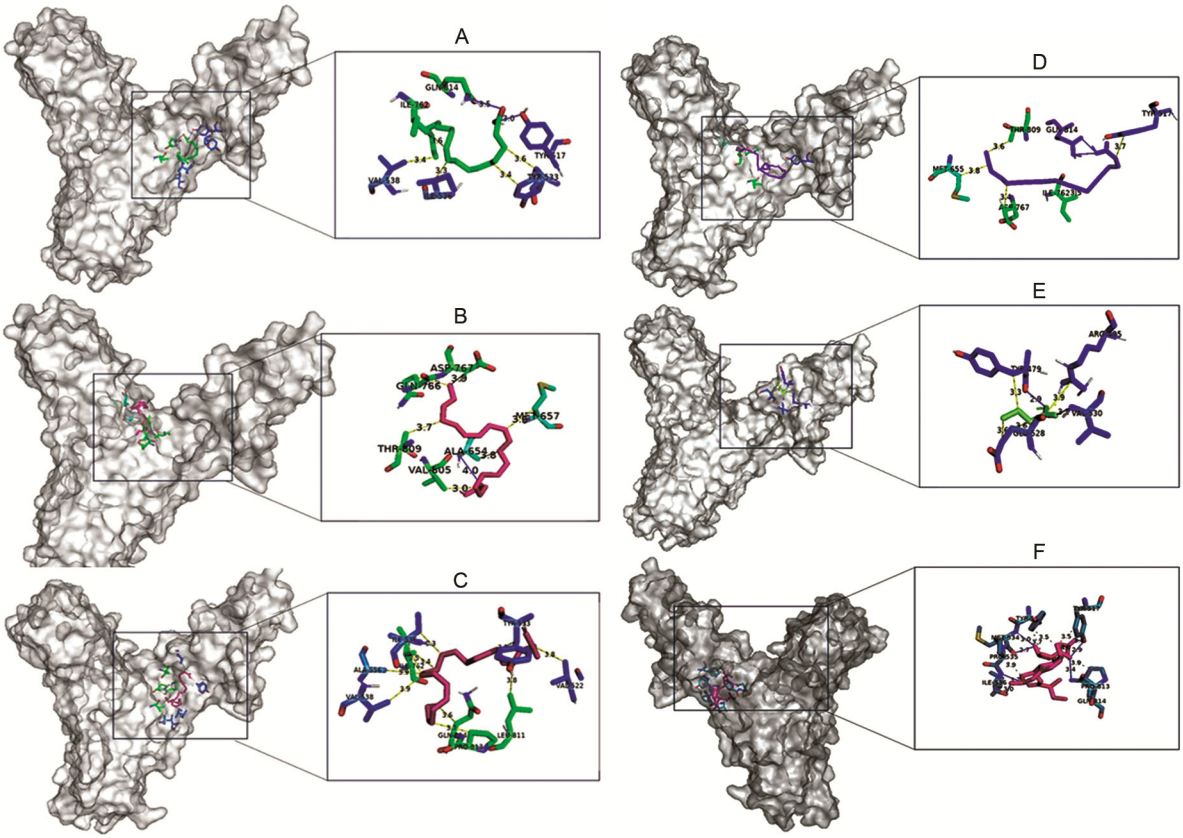

Fig. 4 — The docking of the HMGCR receptor (PDB ID: 1HWJ) with the (A) oleic acid (B) linoleic acid (C) DHA (D) ETYA (E) butyric acid and (F) pravastatin is depicted through visualization. Hydrogen bonds are represented by dark blue lines, while hydrophobic interactions are indicated by yellow lines

other fatty acids showed a significant inhibition compared to the control. The positive control, pravastatin demonstrated 95.11% ± 1.47 inhibition at 75 nM (Fig. 3).

The results of the molecular docking study of fatty acids bound to HMGCR are shown in (Fig. 4A-F). Based on the results of molecular docking, oleic acid, linoleic acid, DHA, ETYA, and butyric acid showed a binding energy of -4.10 kcal/mol, -4.30 kcal/mol, -4.69 kcal/mol, -4.73 kcal/mol and -3.26 kcal/mol, respectively. PLIP analysis revealed that oleic acid formed two hydrogen bonds between Tyr517 and Gln814 residues and four residues, Tyr533, Ile536, Val538 and Ile762, formed hydrophobic interactions with HMGCR (Figure 4). Linoleic acid formed one hydrogen bond with the Ala654 residue of HMGCR and six hydrophobic interactions were formed between Ala654, Met657, Gln766, Asp767, Val805 and Thr809 amino acid residues (Fig. 4B). DHA formed multiple hydrophobic interactions with Val522, Tyr533, Ile536, Val538, Ala556, Ile762, Leu811, Pro813 and Gln814 residues of HMGCR. No hydrogen bonds were formed (Fig. 4C). ETYA formed two hydrogen bonds with the Gln814 amino acid residue of HMGCR. Several hydrophobic interactions were observed between Tyr517, Met655, Ile762, Asp767 and Thr809 amino acid residues (Fig. 4D). Butyric acid formed two hydrogen bonds with Tyr479 and Val530. Two hydrophobic interactions were formed between Tyr479 and Glu528 (Fig. 4E). Pravastatin, the positive control showed a binding energy of -5.36 kcal/mol and formed hydrogen bonds with Tyr517, Met534, Ile536, Gln814, where it formed hydrophobic interactions with Tyr517, Tyr533, Pro535 and Pro813 (Fig. 4F). Table 1 shows a summary of binding energies and amino acids involved in hydrogen bonds, hydrophobic interactions and salt bridges formed between HMGCR subunit A.

Discussion

Fatty acids demonstrate statin-like effects on cholesterol. Fatty acids have been reported to lower plasma cholesterol levels, reduce cholesterol

Table 1 — Binding energies and amino acid residues involved in hydrogen bonds, hydrophobic interactions and salt bridges formed between HMGCR subunit A

Fatty acid	Binding energies(kcal/mol)	Residues involved in hydrogen bonds as analyzed by PLIP	Residues involved in hydrophobic interactions as analyzed by PLIP	Salt bridges as analyzed by PLIP
Butyric acid	-3.26	Tyr479, Val530	Tyr479 and Glu528	Arg495
Oleic acid	-4.10	Gln 814, Tyr517	Tyr533, Ile536, Val538 and Ile762.	-
DHA	-4.69	-	Val522, Tyr533, Ile536, Val538, Ala556, Ile762, Leu811, Pro813 and Gln814	-
ETYA	-4.73	Gln814	Tyr517, Met655, Ile762, Asp767 and Thr809.	-
Linoleic acid	-4.30	Ala654	Ala654, Met657, Gln766, Asp767, Val805 and Thr809.	-
Pravastatin	-5.36	Tyr517, Met534, Ile536, Gln814.	Tyr517, Tyr533, Pro535, Pro813.	-

synthesis, cholesterol absorption, increase cholesterol excretion, enhance cholesterol transport from plasma to tissues, reduce the cholesterol content of lipoproteins and increase the catabolism of cholesterol, suggesting the potential use of fatty acids in the prevention of atherosclerotic diseases[15,40].

The human HMGCR protein comprises 888 amino acids and three main domains, namely the membrane anchor domain (residues 1–339), which is found in the endoplasmic reticulum membrane, the cytoplasmic catalytic domain (residues 460–888) and the linker region (340–459) which links the above two domains[41-43]. In the catalytic domain of HMGCR, three subdomains, N, L, and S, are present[41-43]. The N subdomain (residues 460–527) links the L domain and the linker region of HMGCR. The amino acid residues 528–590 and 694–872 in the L-subdomain interacts with HMG-CoA, while the amino acid residues 591–682 in the S-subdomain interacts with NADPH[41-43]. The cis-loop (residues 682–694), which is present only in human, links the L-domain and with the S-domains, is important for the HMG and NADPH-binding regions[41-43]. The core active site of HMGCR is found in the cis-loop having amino acid residues Lys691, Glu559, Asp767 and His866[42].

The catalytic regions of HMGCR is a tetramer comprising four identical monomers, where the active sites are located at the point which connects two monomers, making a homodimer, which is the functional unit of the enzyme. The tetrameric structure of HMGCR does not influence substrate binding. The active site of HMGCR has three binding sites, namely HMG, -CoA, and NADPH binding sites. The residues Ser565, Asn567, Arg568, Lys722, Ser865, His866, and Tyr479 are involved in the CoA binding pocket. NADPH binds to the S-domain of the opposing subunit in the active site of HMGCR, while the binding pocket for HMG-CoA is located. The residues Ser626, Arg627, Phe628, Asp653, Met655, Gly656, Met657, Asn658, Val805, as well as Asn870 and Arg871 are involved in NADPH binding[41-43]. The HMG binding site is formed by residues from two subunits. One subunit provides Ser684, Asp690, Lys691, Lys692, and Asp767, while the other subunit provides Glu559, Lys735, Asn755, Leu853, and His866. Potent HMGCR inhibitors have been reported to bind to the active site of the enzyme through hydrogen bonding and hydrophobic interactions[44,45].

According to *in silico* analysis, fatty acids exhibited multiple hydrophobic bindings and hydrogen bonding in the catalytic portion (residues 426-888) of HMGCR, suggesting a potential blockage of access to the substrate HMG-CoA. This could be attributed to the HMGCR inhibitory effects of the tested fatty acids. However, through *in silico* analysis, fatty acids did not form interactions with the amino acid residues involved in the core active site (Lys691, Glu559, Asp767 and His866) of HMGCR, although some natural compounds have been reported to form strong interactions with these amino acid residues and potent HMGCR inhibitory effects[46-49]. Butyric acid, which has the shortest carbon side chain, displayed the lowest binding affinity (-3.26 kcal/mol) and the lowest HMCR inhibitory potential. The other fatty acids (oleic acid, linoleic acid, DHA, and ETYA), which have longer side chains, showed higher binding affinities and enzyme inhibition than butyric acid. This might indicate the effects of the length of the carbon chain of fatty acids on the enzyme inhibitory effects.

However, we were unable to provide a strong justification for the differences in the enzyme inhibitory effects of oleic acid, linoleic acid ETYA, and DHA. All these fatty acids have long carbon chains with more than fifteen carbons in their side chains [oleic acid ($C_{18}H_{34}O_2$), linoleic acid ($C_{18}H_{32}O_2$), DHA ($C_{22}H_{32}O_2$), and ETYA ($C_{20}H_{24}O_2$)]. Although oleic and linoleic acid have fewer carbon atoms in their side chains compared to DHA and ETYA, they exhibited higher inhibitory potentials with lower binding energies (-4.10 kcal/mol and -4.30 kcal/mol) than DHA and ETYA (-4.69 kcal/mol and -4.73 kcal/mol). This indicates that solely based on the carbon chain length, it is difficult to draw a strong correlation between the chain length and the inhibitory potential of fatty acids on HMGCR with long carbon chains. Moreover, although oleic and linoleic acid have the same number of carbons in their side chains, it was very difficult to identify the exact reason for oleic acid's greater inhibitory effects on HMGCR.

In recent investigations, some natural compounds such as geraniol, geranylgeraniol, phytol, and farnesyl acetate, which structurally mimic the carbon side chain of fatty acids, were reported to exert HMGCR inhibitory effects, supporting our findings[46,49]. Notably, the binding energy serves as a measure of the strength of interactions between molecules, but it is not the sole factor influencing the inhibitory effect. In this case, additional interactions like hydrogen bonding, hydrophobic interactions, and salt bridges may also exert a substantial impact on the inhibition effect. The spatial conformation and flexibility of the inhibitor could also affect the inhibitory potential[50]. Oleic acid and linoleic acid might be better suited to align with the structure of the HMGCR and establish stable interactions[50], an aspect that will be a crucial point in our upcoming investigations with fatty acids.

Conclusion

The fatty acids oleic acid, linoleic acid, docosahexaenoic acid, ETYA, and butyric acid - were found to exhibit HMGCR inhibitory effects *in vitro*, mimicking statin-like activity. Oleic acid demonstrated the highest inhibitory effects among the fatty acids tested. Given that statins have been reported to have adverse health effects following prolonged consumption, this study will provide a solid foundation for evaluating the effects of fatty acids as potential combination treatment options with statins or for developing hybrid molecules combining statins and fatty acids for investigation using *in vitro* and *in vivo* systems. Due to financial constraints and limited budgets for purchasing enzyme assay kits, which are expensive, we were unable to study the detailed inhibitory mechanisms of these fatty acids on HMGCR activity. This represents a limitation of our study.

Acknowledgement

The support given by the Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Colombo is greatly acknowledged. Author MKE is immensely grateful for the funding support provided by UNESCO-TWAS (Grant number 22-140 RG/BIO/AS_I) and the Swedish International Development for young scientists in Sri Lanka. This funding has been crucial in enabling our research in basic sciences, especially during the economic crisis when securing funding was extremely challenging.

Conflicts of Interest

All authors declare no conflicts of interest.

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