



In-vitro and *in-silico* determinations of HMG-CoA reductase inhibition potential of caffeic acid for therapeutics of hypercholesterolemia

Heera Ram^{1*} , Chandra Kala¹, Karishma Sen¹, Anita Sakarwal¹, Jaykaran Charan², Paras Sharma³ , Rajsekhar Roy⁴, Surajit Ghosh⁴

¹Department of Zoology, Jai Narain Vyas University, Jodhpur, India.

²Department of Pharmacology, All India Institute of Medical Sciences, Jodhpur, India.

³Department of Pharmacognosy, BVM College of Pharmacy, Gwalior, India.

⁴Department of Bioscience Bioengineering, Indian Institute of Technology Jodhpur, Jodhpur, India.

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ABSTRACT

The recent study was aimed to investigate *in-vitro* and *in-silico* determinations of the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase [HMG-CoA reductase (HMGCR)] inhibition potential of the caffeic acid. The *in-vitro* assay shown the IC₅₀ values of caffeic acid and pravastatin by 10.162 μM and 40.6 nM which performed up to 83.29% and 85.83% inhibition of HMGCR, respectively. Consequently, the kinetics of inhibition of HMGCR showed significant values of K_m and V_{max} of the caffeic acid (0.360198 ± 0.04251 ; 11.8% and 91.0863 ± 1.65 ; 1.811%) and pravastatin (10.325 ± 0.9372) [9.077%; 94.2661 ± 2.458 (2.607%)]. Consequently, the molecular docking revealed significant binding energy, bond length, and H-bonding of caffeic acid with target enzyme of HMGCR. Accordingly, the interactions of protein–ligand complexes under cytosolic conditions were validated through root mean square fluctuation of molecular dynamics. Subsequently, the gastrointestinal absorption authenticated by the BOILED egg prediction is further validated by Absorption, Distribution, Metabolism, Excretion, And Toxicity (ADMET) assays and iLogP value. The drug likeness values of caffeic acid and pravastatin were found suitable as per the five rules of the Lipinski. Supportively, the toxicity profiles of the caffeic acid and pravastatin was made by the ProTox-II web server. Hence, it can be concluded that caffeic acid has the capabilities to inhibit HMGCR which provides the hypercholesterolemic potential.

INTRODUCTION

Voluminous data of the literature illustrated that the diet ingredients of fruit, vegetables, and plant parts having capabilities to manage hypercholesterolemia by the existing potent bioactive phytochemicals such as polyphenol, terpenoids, flavonoids, and alkaloids (Rastogi *et al.*, 2016). In a similar context, caffeic acid is a metabolite of hydroxycinnamate of the non-flavonoids phenolic acid mostly obtained from edible fruits, vegetables,

herbs, and stress climatic plants which are used in therapeutics of numerous ailments. This also exhibits numerous processed dietary sources because of antioxidant properties due to their free radical scavenging capabilities as well as potent ligand can interact with several metabolic targeted protein and enzymes (Agunloye and Oboh, 2018; Laranjinha and Cadenas, 1999). In plant metabolism, caffeic acid or caffeic acid-like compounds furnish a series of hydroxycinnamic acids (C6–C3) that vary from an individual by the quantity of hydroxy and methoxy groups on their phenyl unit (Park, 2009). These kinds of monophenol carboxylic acids are repeatedly found to be esterified to polyols. Accordingly, 3,4-dihydroxycinnamic acid (caffeic acid) is combatted in average-sized polyester branches of the tetraoicquinic acid, i.e., 3,5-di-O-caffeoylquinic acid, that occurs in coffee beans. Along with

*Corresponding Author

Heera Ram, Department of Zoology, Jai Narain Vyas University, Jodhpur, India. E-mail: hr:zo@jnvu.edu.in

this, there are numerous reports on the biomedical uses, such as antidiabetic, antioxidants, antihyperlipidemic, neuroprotective, anti-inflammatory, antioncogenic, and endocrinal booster, of caffeic acid (de Alencar Silva *et al.*, 2020; Tsai *et al.*, 2011). Consequently, the derivatives of caffeic acid also have significant therapeutic applications. Besides this, the active pharmaceutical agent needed suitable bioavailability in accordance with the five rules of the Lipinski (Koehn and Carter, 2005; Kumar, 2016). Thus, most of the polyphenols are considered active pharmaceutical agents because of their ideal profile of the bioavailability and capability to cross the blood–brain barrier (BBB). Therefore, the design of ideal polyphenol-based or polyphenol-stirred drugs against the protein targets may appropriately be a promising direction for future research on polyphenols. Based on the above fact, the current study was planned to investigate the HMG-CoA reductase (HMGCR) inhibition capabilities of caffeic acid by using *in-vitro* and *in-silico* examinations.

MATERIAL AND METHODS

In vitro HMGCR inhibition assay

The HMGCR inhibition activity of the caffeic acid was assessed by using the commercially available assay kit (Sigma-Aldrich) based on spectrophotometric measurement through 96 well plate (Liang *et al.*, 2015; Saeedi Saravi *et al.*, 2017). The HMGCR concentration was taken as a stock solution (0.50–0.70 mg/ml). The stock solution of caffeic acid compound was prepared at 0.02 mg/ml. The 1 μ l volumes of the test samples were mixed with the reaction mixture containing 184 μ l assay buffer, Nicotinamide Adenine Dinucleotide Phosphate (NADPH) (4 μ l), HMG-CoA substrate (12 μ l), and HMGR (2 μ l). Pravastatin (Sigma Aldrich co.) and distilled water were used as the positive and negative controls, respectively. The IC₅₀ values of caffeic acid and pravastatin were calculated by the online calculator of AAT Bioquest (Anwar *et al.*, 2020).

Inhibition kinetics of HMGCR

The inhibition kinetics of caffeic acid against HMGCR was determined by following standard method (Sjögren *et al.*, 2009; Wang *et al.*, 2015). A particular quantity of HMGCR was incubated with increasing concentrations of the caffeic acid at an optimum temperature of 37°C for 15 minutes, in the absence or presence of samples (concentration alike to IC₅₀). Reactions were completed and absorption was estimated and transformed to the reaction by the Lineweaver–Burk plot.

Ligand preparation

The three-dimensional conformation chemical structure of caffeic acid was downloaded from PubChem (pubchem.ncbi.nlm.nih.gov) web interface of the National Institute of Health. The PubChem CID of the caffeic acid was 689043. The compound was further processed by using PyMol and Autodock tools for the analysis of molecular docking (Madhavi Sastry *et al.*, 2013).

Target protein preparation

The molecular three-dimensional structure of the HMGCR protein [Protein Data Bank (PDB) ID 1DQA] was retrieved from a Protein Data Bank (www.rcsb.org) in PDB

format. Pre-processing of protein was done by deleting the water molecules, and the addition of hydrogen atoms and further processes were performed with the help of Autodock tools (Madhavi Sastry *et al.*, 2013; Rizvi *et al.*, 2013).

Molecular docking

Molecular docking was performed by using the Autodock to investigate the interaction between the target protein (HMGCR) and ligands (Caffeic acid and pravastatin) (Iheagwam *et al.*, 2019; Rizvi *et al.*, 2013). The molecular docking was carried out based on ligand binding to a receptor protein using scoring functions. The default grid dimension predicted by the Autodock tools depending on the pocket region of the protein was considered for further analysis. As a result of molecular docking, binding energy in kcal/mol was generated. The molecular interaction of the protein and ligand in three dimensions was analyzed by using the PyMol. The number of hydrogen bonds, bond length, and the interacting residues of the protein and ligand were acquired from Lig plot.

Molecular dynamics (MD) simulation

Caffeic acid was obtained for the MD simulation in a complex with the enzyme HMGCR. Simulation studies were carried out with Biovia discovery studio client 2020 by following the standard protocol (Brooks *et al.*, 2021; Noha *et al.*, 2017). Protein was prepared and any prebound ligand or unnecessary molecules were removed. Any missing stretch or residues were prefixed. The structure of the ligand i.e., caffeic acid was retrieved from Pubchem database as mentioned earlier. The protein–ligand complex was put in a solvated environment of water. CHARMM 36 forcefield was used to detect the constraints, allowing the MD simulation to run through an environment of periodic boundary to avoid unnecessary surface artifacts.

The solvated system was then put through 1,000 steps of steepest descent minimization and 1,000 steps of conjugate gradient minimization using the forcefield. Next, the entire system was then heated at 60 to 300 K in 6 ps, for a runtime of 200 ps. The initial confirmation was taken as a reference to determine the root mean square fluctuation (RMSF) of the entire complex.

ADMET assessment

ADMET analysis was carried out using Swiss ADME (<https://www.swissadme.ch>) web tool predictor to evaluate the absorption, distribution, metabolism, and excretion (ADME) properties of the compound. The access of physicochemical properties, lipophilicity, water solubility, pharmacokinetics profile, drug likeness, and medicinal chemistry properties of the phytocompounds can be predicted for the development of drug (Daina *et al.*, 2017). Along with that the interpretation of iLOGP and Bioavailability Radar can also be done by using Swiss ADME. The evaluation of caffeic acid can be done based on their ability to pass the BBB and follow the Lipinski rule of five. Lipinski's "Rule of Five" interprets whether the compound is orally well absorbed or not as a drug molecule. The bioavailability of the drug molecule can be predicted based on total polar surface area (TPSA) as per Weber's rule. $TPSA \leq 140 \text{ \AA}$ indicates good oral bioavailability. An ideal compound following these criteria would be able to pass the BBB if the number of hydrogen bonds is between 8 and 10 and no acidic groups are present in the molecule.

Gastrointestinal absorption—a Brain Or IntestinaL Estimated (BOILED-Egg) model

The BOILED-Egg permeation method is a predictive model for the estimation of two pharmacokinetic behavior, i.e., gastrointestinal passive absorption and the permeability of the blood–brain penetration barrier. It works with speed, accuracy, and conceptual ease by computing the polarity and lipophilicity of chemicals and generates clear graphical outputs (Daina and Zoete, 2016). Along with the pharmacokinetics, bioavailability also plays a major role in drug development.

Toxicity prediction

ProTox-II (https://tox-new.charite.de/protox_ii) web server is computational freely available in silico toxicity prediction tool consisting of 33 models to design the development of drug process. It incorporates the prediction of oral toxicities of chemicals which can be measured in different levels of toxicity mainly qualitatively in terms of endpoints as binary output (active or inactive) along with confidence score such as acute toxicity, hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity, cytotoxicity, adverse outcomes (Tox21) pathways, and toxicity targets. It can be further measured quantitatively as lethal dose (LD_{50}) values in mg/kg body weight. Toxicity classes (Class I–VI) can also be predicted based on their toxic doses. Prediction accuracy, average similarity along with LD_{50} , and toxicity class were generated instantly for the prediction of acute toxicity and toxicity targets.

Statistical analysis

Data of the *in-vitro* determinations were performed through devoted AAT Bioquest, Inc. “Quest Graph™ IC50

Calculator.” Retrieved from <https://www.aatbio.com/tools/ic50-calculator> by following the standard statistical approaches of enzymatic kinetics.

RESULTS

In-vitro assay

The *in-vitro* assay performed inhibition by the increasing concentrations of caffeic acid and the target enzyme of the HMGCR at optimum temperature and specific time intervals which showed 83.29% and 85.83%, respectively. The IC50 values of caffeic acid and pravastatin were calculated by using the online calculator of AAT Bioquest shown 40.6 nM and 10.162 μ M, respectively (Fig. 1A and B).

Inhibition kinetics of enzyme assay

The values of K_m and V_{max} of the caffeic acid were found to be 0.360198 ± 0.04251 (11.8%) and 91.0863 ± 1.65 (1.811%), respectively. The values of K_m and V_{max} of test compound were further compared with the positive control i.e., Pravastatin had K_m value as 10.325 ± 0.9372 (9.077%) and V_{max} value as 94.2661 ± 2.458 (2.607%) (Fig. 2A and B).

Molecular docking

Molecular docking result analysis of binding interactions of the ligand with HMG CoA reductase docked against PDB 1DQA in comparison to standard drug. As a result of Autodock Vina, the binding energy of the caffeic acid compound was found to be -6.9 kcal/mol. Further processing of the protein and ligand was done by Lig plot to find the number of hydrogen bonds, bond length, and interacting residues (Table 1). The interacting residues

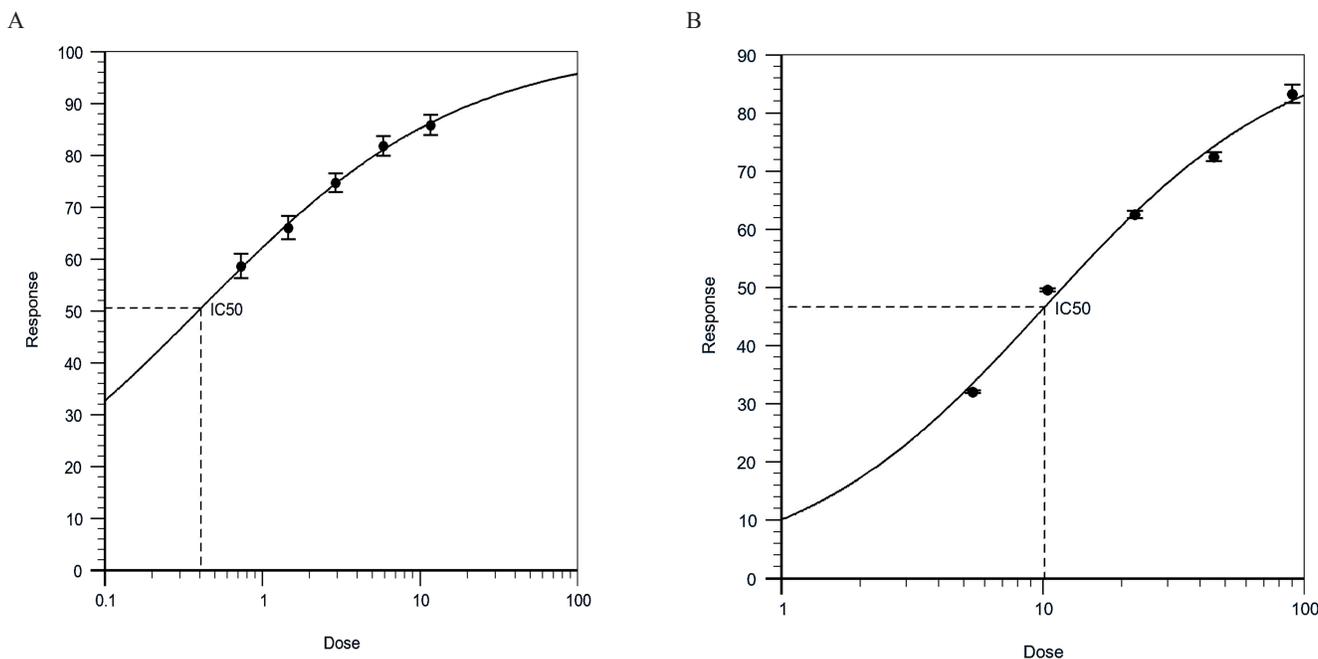


Figure 1. A) *In-vitro* inhibitory activity of Pravastatin as positive control against HMGCR enzyme. (IC50 = 40.6 nM). B) *In-vitro* inhibitory activity of caffeic acid compound against HMGCR enzyme. (IC50 = 10.162 μ M).

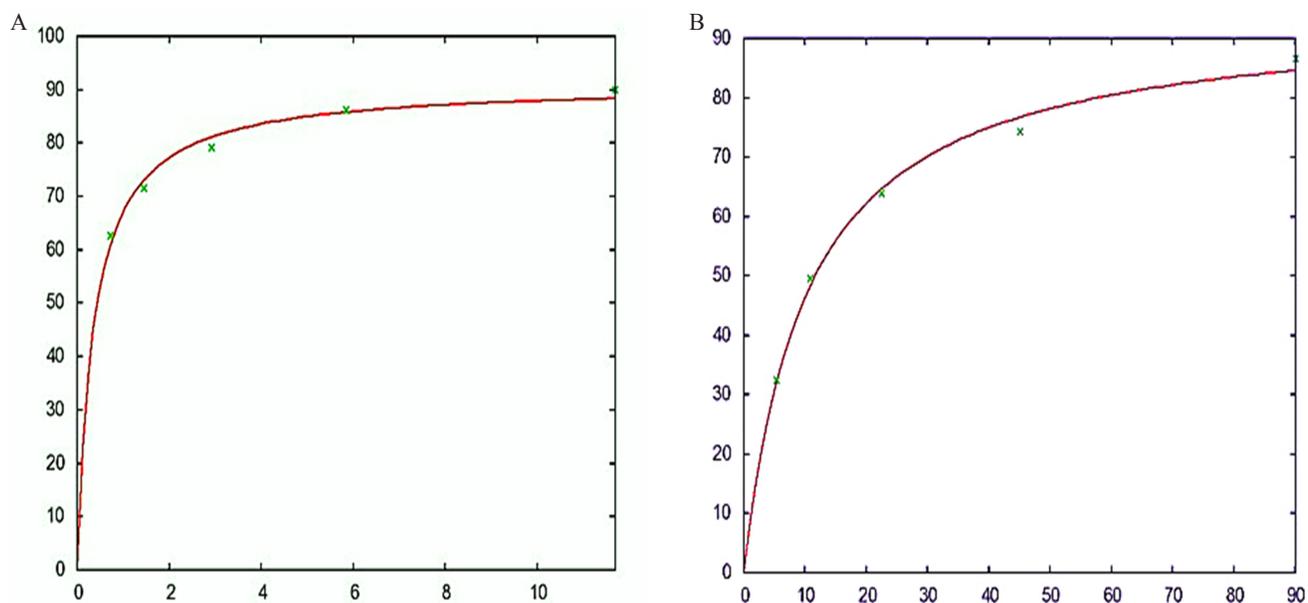


Figure 2. A) K_m and V_{max} of Pravastatin as positive control against HMGR enzyme was found to be 10.325 ± 0.9372 (9.077%) and 94.2661 ± 2.458 (2.607%) respectively. B) K_m and V_{max} of caffeic acid compound against HMGR enzyme was found to be 0.360198 ± 0.04251 (11.8%) and 91.0863 ± 1.65 (1.811%) respectively.

Table 1. Molecular interaction studies of the caffeic acid against the target enzyme (HMG CoA reductase)

Ligand	Caffeic acid	Pravastatin
Binding energy (kcal/mol)	-6.9	-6.6
No. of H-bonds	5	5
Bond length (Å)	2.95, 2.83, 2.80, (3.21, 2.82)	3.27, (2.91, 3.27), 2.76, 2.84
Interacting residues	Arg641, Ser794, Ala639, Asn642	Lys633, Lys606, Lys636, Ile 699

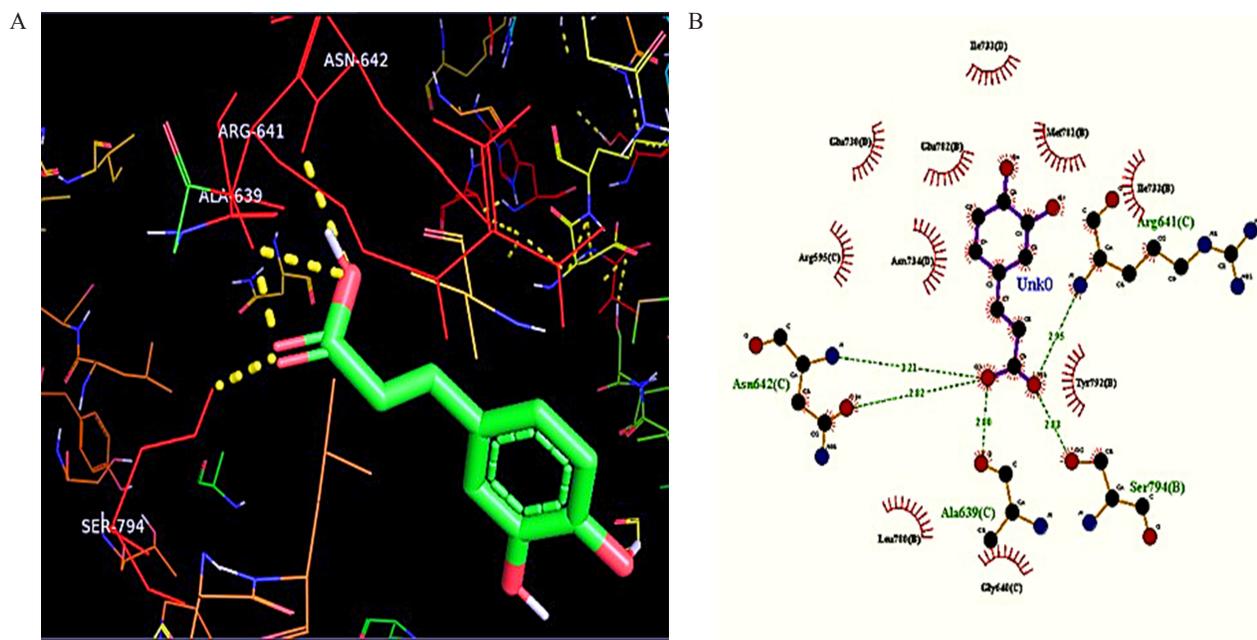


Figure 3. A) Molecular interaction studies of ligand caffeic acid against HMGR by docking analysis. B) Ligplot demonstrating the interactions of ligand-protein. Diagrams interpret as follows: the residues involved in ligand bonds shown in thick lines (purple), residues involved in hydrogen binding with the ligand (black) with thin orange bonds along with their hydrogen bonds with the length in middle (olive green), and residues involved in non-bonded interactions or hydrophobic contacts between protein and ligand (red spikes).

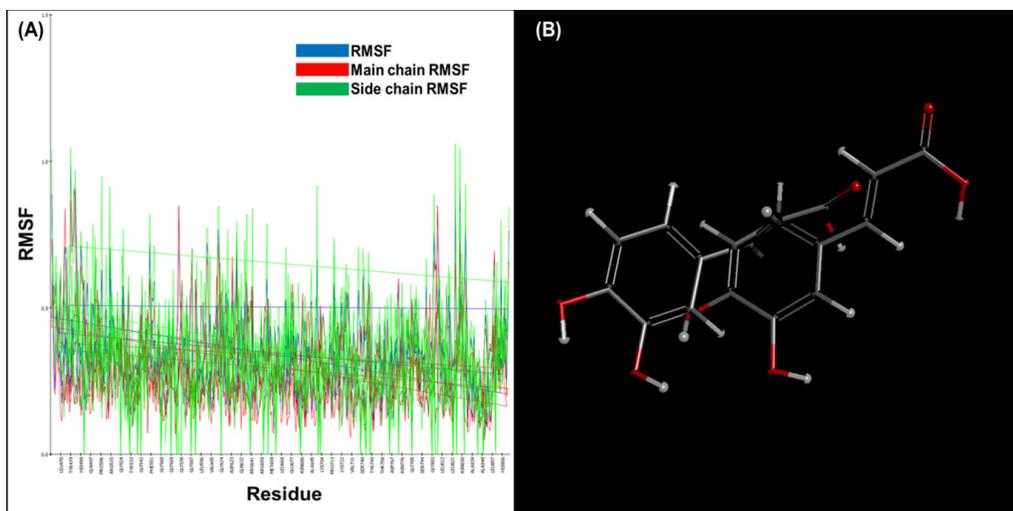
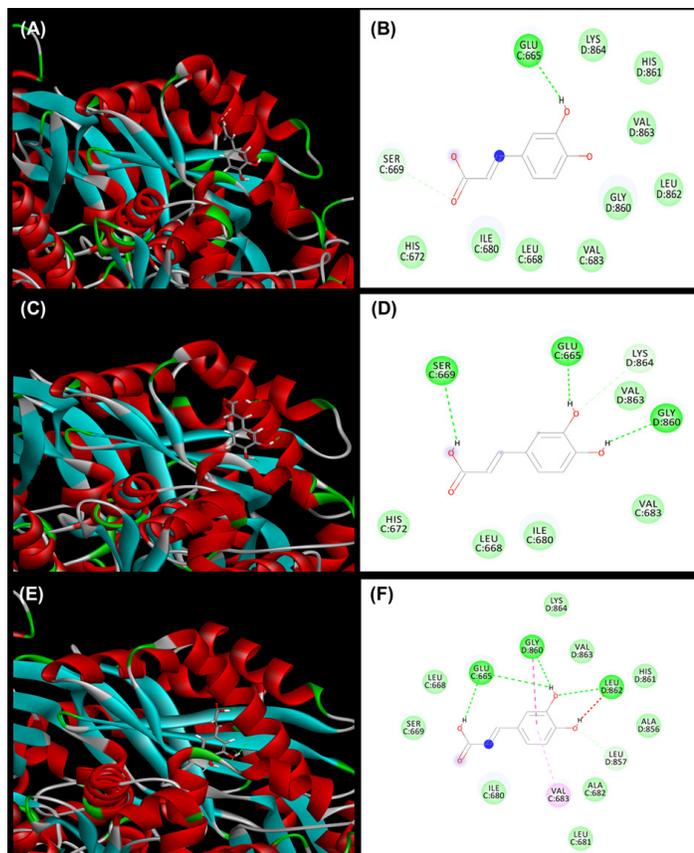


Figure 4. A) MD simulation of caffeic acid with HMGCR over a period of 200ps showing no unwanted projection overall. (A,B) Screenshot and interactive residues at 60 ps. (C, D) Screenshot and interactive residues at 120 ps. (E, F) Screenshot and interactive residues at 200 ps. (B) RMSF value along with of main chain and side chain RMSF values with respect to the amino acid residues of protein. (B)Molecular overlay of initial and final conformation after the simulation.

Arg641, Ser794, Ala639, and Asn642 were present in the catalytic groove of the protein (Fig. 3A and B).

MDs simulation

MD simulation was performed using HMGCRC-caffeic acid complex for around 200 ps (Fig. 4). The temperature constraints up to 300 K denotes a stable character of the complex.

The potential energy was calculated on average which was found out to be, $-277,696$ kJ/mol. As shown in Figure 4A, RMSF values for the main chain and for the side chain that came out to be 1.41958 Å. This indicates the active binding of the compound to the protein structure. Even more the motion of the conformation was detected using biovia discovery studio client 2020 visualizer, the result shows a prompt interaction in a stable manner in all three snapshots.

Even the molecular structure overlay also shows to overlap the presimulated and postsimulated conformations portraying no such alteration of position from the active cavity (Fig. 4B).

ADMET pharmacokinetics predictions

Pharmacokinetic (ADMET) profile of the caffeic acid compound was shown in table compared with the positive control such as pravastatin indicated an ideal drug profile. Further confirming the Lipinski rule of five as no violations has been shown by the compound indicating drug-likeness (Table 2). TPSA of the compound was found as 77.76 which characterizes significant cellular plasma membrane permeability.

BOILED-Egg predictions

With the help of graphical classification model, the Egan's BOILED-Egg permeation predictive model diagram has shown that the caffeic acid compound has good potential to cross Human Intestinal Absorption (HIA) (the passive human gastrointestinal absorption) but no BBB permeability. The compound was predicted as non-substrate of the p-glycoprotein (Fig. 5).

Toxicity

The prediction of toxicity of caffeic acid compound based on various properties, such as hepatotoxicity, carcinogenicity,

Table 2. Physicochemical ADMET analysis of the caffeic acid against the Lipinski rule of five and BBB filter by Swiss ADMET web tool.

Compound	Caffeic acid	Pravastatin
MW	180.16	424.53
ilogP	0.97	3.01
ClogP	0.93	2.37
HBA	4	7
HBD	3	4
nHB	7	11
TPSA	77.76	124.29
N violations	0	0
Drug likeness	Yes	Yes
Filter L/B	L	L

MW = molecular weight; ilogP = partition coefficient; ClogP = consensus log P; HBA = hydrogen bond acceptor; HBD = hydrogen bond donor; nHB = number of hydrogen bond; TPSA = total polar surface area; N violations = number of violations; Filter L = Lipinski rule of five and B = blood brain barrier.

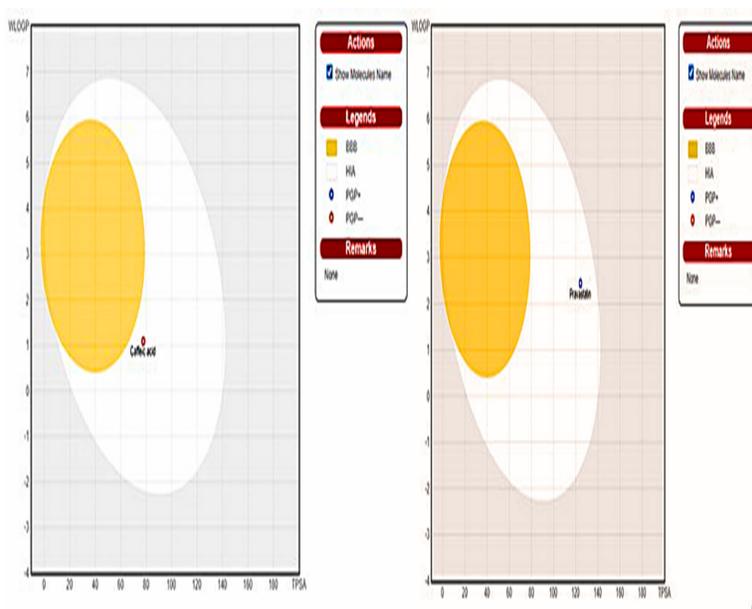


Figure 5. Predicted BOILED-Egg model of caffeic acid and Pravastatin using SwissADME.

Table 3. *In-silico* toxicity prediction of the caffeic acid by ProTox-II server.

Compound	Caffeic acid	Pravastatin
Hepatotoxicity	Inactive	Inactive
Carcinogenicity	Inactive	Active
Immunotoxicity	Active	Active
Mutagenicity	Inactive	Inactive
Cytotoxicity	Inactive	Inactive
Predicted LD50 (mg/kg)	2,000	8,939
Predicted Toxicity class	4	6

immunotoxicity, mutagenicity, cytotoxicity, LD50, and toxicity. The compound belonged to class 4; LD50 was predicted as 2,000 in mg/kg and was inactive as a toxicant but showed immunotoxicity (Table 3).

DISCUSSION

Enzyme inhibitors, analogs, and inactivators include coarsely half of all the prescribed medicines which are using for health concerns (Holdgate *et al.*, 2017). In the 1980s, statins, represented by atorvastatin and lovastatin, developed as inhibitors of 3-hydroxy-3-glutaryl CoA reductase (HMG-CoA reductase or HMGCR) and nowadays it is most prescribing drugs to treat hypercholesterolaemia (Marahatha *et al.*, 2021; McKenney, 2003). Accordingly, it is a well-established fact that phytochemicals have capabilities to ameliorate numerous metabolic disorders by the interactions with particular target protein or enzymes, scavenging of free radicals, and other accelerative activities (Ademosun *et al.*, 2015; Janice *et al.*, 2020). In a similar context, caffeic acid is one of the leading ingredients of edible plant materials and dietary content which contributing to ameliorate numerous metabolic disorders as well as benefiting to the health (de Alencar Silva *et al.*, 2020; Mukherjee *et al.*, 2015; Tsai *et al.*, 2011). The current study revealed that caffeic acid has a significant concentration-dependent HMGCR inhibition capability which relies on the strength of their bond to the enzyme. This kind of inhibition depicted the base structures of the compounds determine how well the molecule fits into the binding pocket of the target enzyme and binds with it. Whereas the pravastatin-like statin is synthetic statin which has an additional fluorinated phenol group and other moieties in the base structure that provide additional sites for binding within the enzyme pocket (Salvamani *et al.*, 2016). Therefore, the test compound i.e., caffeic acid and pravastatin have been shown different inhibition capabilities as well as different IC50 in this study. The IC50 value of the test compound against the enzyme depending upon the concentrations of the target molecule, the inhibitor, and the substrate or ligand along with chemical and physical experimental milieu (Srinivas, 2016; Zeytinlioglu and Zihnioğlu, 2015). Similarly, the significant reaction rates shown by the values of K_m and V_{max} of the *in-vitro* assays of HMGCR inhibition that indicates the capabilities of interactions of reactants and generation of products (Buker *et al.*, 2019; Hasanah *et al.*, 2016). It indicates that in an enzyme catalyzed reaction when there is a large excess of substrate and the enzyme concentration is held constant, if substrate concentration is plotted against velocity or reaction rate, a hyperbolic curve is obtained. It reflects that the affinity of the enzyme for its substrate

and represents a particular enzyme-substrate system. The smaller the value of K_m explained the extra strongly the enzyme binds to the substrate. Kinetic values of enzyme catalyzed reactions are generally considering under the steady-state conditions that described by V_{max} and K_m (Sjögren *et al.*, 2009; Zaharudin *et al.*, 2019). Consequently, the significant interactions shown by molecular docking through the availabilities of numbers of hydrogen bonds, potential binding energy, and interacting residues of the caffeic acid and pravastatin with target protein (HMGCR). Furthermore, the assessments of MDs revealed the active binding of the compound and protein in a stable manner without any position alteration from the active cavity. The several studies reported and suggested that the protein and ligands interactions depend upon the capabilities of functional groups by the numbers of H-bond, binding energy, and related interactions of small molecule phytochemicals and terminal catalytic residues of target protein (Jasmine and Vanaja, 2013; Makegowda *et al.*, 2019). Along with this, caffeic acid and pravastatin performed optimised ranged parameters of ADMET and the drug likeness by following the five rules of the Lipinski. It well established fact that the BBB effectively regulates the selective drive of cells, ions, fluid, nutrients between the blood and the brain which plays a vital role of protecting the brain or vital organs against potential toxins, encephalitogenic cells, and microbial pathogens, to maintain cerebral homeostasis that is required for proper neuronal and synaptic functions (Jia *et al.*, 2020; Tian *et al.*, 2015). The value of iLogP of the caffeic acid and pravastatin showed significant lipophilicity and hydrophobicity which indicate suitable bioavailability as reported by numerous studies (Daina *et al.*, 2014).

CONCLUSION

Based on the determinations of the study, it can be concluded that caffeic acid has the capability to inhibit the HMGCR activity which may be useful for therapeutics of hypercholesterolemia by subsiding the cholesterol biosynthesis. Further validations can be confirmed from the *in-vivo* assessments of biochemistry and molecular biology.

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AUTHOR CONTRIBUTIONS

HR & SG-designed and review the study. KS & AC—Molecular docking and *in-vitro* assessments. CK-first rough draft. RSR-MDs.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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