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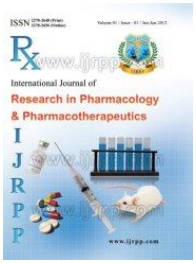

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Computational and molecular designing studies of novel flavonoid analogues as HMG CoA Reductase and cholesterol esterase inhibitors for their Cardioprotective effect using *in Silico* docking studies

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ABSTRACT

The objective of the study was to generate a series of pharmacophores from a parent flavone skeleton and evaluate their *in silico* HMG CoA reductase and cholesterol esterase enzyme inhibitory potential using the software AutoDock 4.2. A total of eighteen flavonoid compounds were generated from flavone structure using the software ChemSketch. The docking studies were carried out for all these compounds using the software AutoDock4.2 with the enzymes HMG CoA reductase and cholesterol esterase. The docking parameters like binding energy, inhibition constant and intermolecular energy were determined. The results obtained were compared with the standard drugs. Rosuvastatin and simvastatin were used as the standards for HMG CoA reductase and cholesterol esterase inhibitory activity respectively. The binding sites of both enzymes for these ligands and their pharmacophores were identified. Based on the docking parameters for the enzyme HMG CoA reductase the binding energy, inhibition constant, intermolecular energy of Rosuvastatin was found to be -7.97 kcalmol^{-1}, 1.44nm was and -11.85 kcalmol^{-1} respectively. The flavonoid compounds showed binding energy ranging between -11.85 to -9.09 to kcalmol^{-1}, inhibition constant ranging from 2.06 nM to 216.45 nM intermolecular energy ranging between -13.94 kcalmol^{-1} to -10.88 kcalmol^{-1}. Among the flavonoid compounds FA5 showed better binding energy -11.85 kcalmol^{-1}, inhibition constant (2.06 nM) and intermolecular energy (-13.94 kcalmol^{-1}) when compared to the standard.The docking parameters of standard Simvastatin to cholesterol esterase exhibited a binding energy -6.72 kcalmol^{-1}, inhibition constant (11.89 nm) and intermolecular energy -9.11 kcalmol^{-1}. The flavonoid compounds showed binding energy ranging between -9.03 kcalmol^{-1} to -7.28 kcalmol^{-1}, inhibition constant ranging from 241.43 nm to 4.71 µM, intermolecular energy ranging between -10.69 kcalmol^{-1} to -9.37 kcalmol^{-1}. From the selected flavonoids FA12 had showed better binding energy (-9.03 kcal/mol), inhibition constant (241.43 nm), when compared to the standard simvastatin (-6.72 kcalmol^{-1}). This proved that FA12 has the potential to inhibit cholesterol esterase. The compound

FA2 exhibited better intermolecular energy -10.69 kcalmol[1]) when compared to the standard and the compound FA12. In conclusion, these results indicate that selected flavonoids, FA5 has better binding sites and interaction for the enzyme HMG CoA reductase and FA12 have better binding sites and interactions with cholesterol esterase enzyme and can be synthesized and screened for their *in vitro* and *in vivo* potential.

Keywords: *In silico*, Atherosclerosis, HMG CoA reductase, cholesterol esterase, AutoDock, Flavonoids

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death for both men and women in the United States and much of the western world and is predicted to be the leading global killer by 2020 [1]. Atherosclerosis, which is caused by hypercholesterolemia, is a major cause of heart diseases such as myocardial infarction. Elevated levels of plasma cholesterol, particularly low-density lipoprotein (LDL) and triglyceride levels, are mainly responsible for hypercholesterolemia, which can also lead to other diseases such as obesity, diabetes, and cancer [2-3]. Relatively high LDL-cholesterol levels are a major risk factor for the development of cardiovascular diseases [4-5].

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is the rate-limiting enzyme in cholesterol biosynthesis that catalyzes the conversion of HMG-CoA to mevalonate. The inhibition of HMG-CoA reductase effectively lowers the level of cholesterol in humans and most animals by the activation of sterol regulatory element-binding protein-2, which upregulates the HMG-CoA reductase and LDL receptor that lead to the reduction of cholesterol levels [6]. HMG-CoA reductase is a unique molecular target in the therapy of hypercholesterolemia, cancer etc. [7-8]. Although statins are well-known HMG-CoA reductase inhibitors, long-term consumption of statins cause severe adverse effects such as myopathy and liver damage, rhabdomyolysis, acute renal failure and potential drug-drug interactions has been reported too [9-10].

Cholesterol esterase initially secreted from pancreatic acinar cells and lactating glands of higher mammals including humans and released into small intestine [11]. Pancreatic cholesterol esterase (CEase), also known as bile salt-activated lipase, is responsible for the hydrolysis of dietary cholesterol esters, fat soluble vitamin esters, phospholipids, and triglycerides [12]. Inhibition of CEase has attracted much attention in the last decades as a potential approach to treat hypocholesterolemia and atherosclerosis by limiting the bioavailability of dietary cholesterol [13]. Inhibition of cholesterol esterase is expected to limit the absorption of dietary cholesterol, resulting in delayed cholesterol absorption [14]. The role of CEase in atherogenesis and the relationship of this enzyme to various pathological conditions are not completely established so far [15].

A number of conventional drugs were available for treatment of hyperlipidemia and atherosclerosis, but all of these drugs exhibited serious adverse effects[16]. Flavonoids, naturally occurring compounds, are widely distributed in nature and are ubiquitous in plants, fruits, seeds, vegetables. Flavonoids can be defined as a group of biologically active compounds that possess a cardioprotective effect. Recent reports suggest that phosphorylated flavones displayed excellent Cholesterol esterase inhibitory activities with IC_{50} values in the nanomolar range [17]. They also possess antithrombotic anticancerogenic, antiviral, antioxidant, hepatoprotective, anti-inflammatory, and other biological effect [18-19]. Significant attention during the recent period has been devoted to the studies on the mechanisms of cardiovascular disorders resulting in hypertension, atherosclerosis, myocardial infarction, and ischemia/reoxygenation injury. It was reported that the intake of flavonoids inversely correlates with the plasma total cholesterol and low density lipoprotein cholesterol concentrations, the occurrence of ischemic heart disease, and the mortality from a coronary heart disease [20-24]. Several flavonoids were found to possess a vasodilatatory effect [25-26] and to reduce the aggregation of erythrocytes [27].

Molecular docking is a computational technique that predicts the noncovalent interaction between two macromolecules or more repeatedly in a macro and small molecules. Drug discovery, molecular docking, and virtual screening, offering multi-user capability, enhanced accuracy [28].

AutoDock 4.2 is the most recent version which has been widely used for virtual screening, due to its enhanced docking speed [29]. Its default search function is based on Lamarckian Genetic Algorithm (LGA), a hybrid genetic algorithm with local optimization that uses a parameterized free-energy scoring function to estimate the binding energy. Each docking is comprised of multiple independent executions of LGA and a potential way to increase its performance is to parallelize the aspects for execution [30].

The stereochemistry of flavonoids binding on HMG CoA reductase and CEase has not been characterized. In this study, the search of flavonoids in the molecular basis for binding to active site of HMG CoA reductase and CEase is revealed by computer aided docking analysis.

MATERIALS AND METHODS

Molecular modeling is a very much investigated technique for recognizing the potent compound without putting excessively exertion and investment in research. AutoDock4.2 software is used by us to investigate the activity in terms of binding affinity and there after the outcomes is compared in binding affinity score for best-docked conformation [31].

Softwares required

Python 2.7 - language was downloaded from www.python.com, Cygwin (a data storage) c:\program and Python 2.5 were simultaneously downloaded from www.cygwin.com, Molecular graphics laboratory (MGL) tools and AutoDock4.2 was downloaded from www.scripps.edu, Discovery studio visualizer 2.5.5 was downloaded from www.accelerys.com, Molecular orbital package (MOPAC), Chemsketch was downloaded from www.acdlabs.com. Online smiles translatory notation was carried out using cactus.nci.nih.gov/translate.

Preparation of ligand data set and rule of five screening

The subset of 18 compounds were drawn using ACD labs Chemsketch v 12.0 and their SMILES notations were generated.

An extended PDB format, termed as PDBQT file was used for coordinate files which includes atomic partial charges. AutoDock Tools was used for creating PDBQT files from traditional PDB files. Crystal structure of HMG CoA Reductase (PDB ID: 1HWL) and cholesterol esterase (PDB ID: 1CLE) was downloaded from the Brookhaeven protein data bank and refined with the help of Accelrys studio viewer. The flavone skeleton used in the designing of flavonoid compounds is given in Fig 1. The flavonoid ligands were built using Chemsketch and optimized using "Prepare Ligands" in the AutoDock 4.2 for docking studies. The substituent groups at R and R_1 position of flavone skeleton for all the compounds included in the study are represented in **Table 1**.

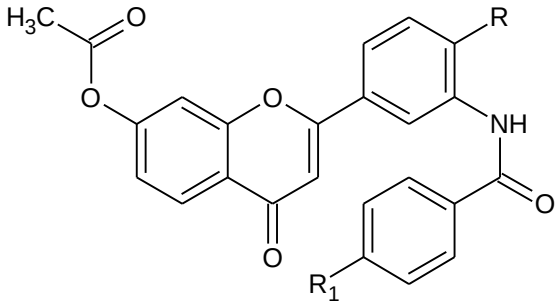

Fig.1 Flavone skeleton

Table 1: Compound code and substitution at R and R_1 position of flavone skeleton

Sl.No	Compound Code	Substitution at R	Substitution at R_1
1.	FA1	-OH	- Cl
2.	FA2	-OH	- Br
3.	FA3	-OH	- F
4.	FA4	-OH	- CH_3

5.	FA5	-OH	- NO_2
6.	FA6	-OH	- OCH_3
7.	FA7	-OCH_3	- Cl
8.	FA8	-OCH_3	- Br
9.	FA9	-OCH_3	- F
10.	FA10	-OCH_3	- CH_3
11.	FA11	-OCH_3	- NO_2
12.	FA12	-OCH_3	- OCH_3
13.	FA13	-CH_3	- Cl
14.	FA14	-CH_3	- Br
15.	FA15	-CH_3	- F
16.	FA16	-CH_3	- CH_3
17.	FA17	-CH_3	- NO_2
18.	FA18	-CH_3	- OCH_3

Molecular Docking Studies

Lamarckian genetic algorithm (LGA) for ligand conformational searching, which is a hybrid of a genetic algorithm and a local search algorithm was employed in present study. This algorithm first builds a population of individuals (genes), each being a different random conformation of the docked molecule. Each individual is then mutated to acquire a slightly different translation and rotation and the local search algorithm then performs energy minimizations on a user-specified proportion of the population of individuals. The individuals with the low resulting energy are transferred to the next generation and the process is then repeated. The algorithm is called Lamarckian because every new generation of individuals is allowed to inherit the local search adaptations of their parents.

Grid generation and molecular docking

The optimized ligand molecules were docked into refined CoA Reductase and cholesterol esterase model using "LigandFit" in the AutoDock 4.2 [32]. AutoDock is docking software structured in a way to predict the binding of a substrate to a receptor molecule and virtual screening is a technique used for identifying assuring compounds to bind to a target molecule with a known structure [33]. The preparation of the target protein 1HWL and 1CLE with the AutoDock Tools software involved adding all hydrogen atoms to the macromolecule, which is a step necessary for correct calculation of partial atomic charges. Gasteiger charges are calculated for each atom of the macromolecule in AutoDock 4.2 instead of Kollman charges which were used in the previous versions of this program. Three-dimensional affinity grids of size 277 × 277 × 277 Å with 0.6 Å spacing were centered on the geometric center of the target protein and were calculated for each of the following atom types: HD, C, A, N, OA, and SA, representing all possible atom types in a protein. Additionally, an electrostatic map and a desolvation map were also calculated [34].

Rapid energy evaluation was achieved by precalculating atomic affinity potentials for each atom in the ligand molecule. In the AutoGrid procedure, the target enzyme was embedded on a three dimensional grid point [35]. The energy of interaction of each atom in the ligand was encountered. We have selected important docking parameters for the LGA as follows: population size of 150 individuals, 2.5 million energy evaluations, maximum of 27000 generations, number of top individuals to automatically survive to next generation of 1, mutation rate of 0.02, crossover rate of 0.8, 10 docking runs, and random initial positions and conformations. The probability of performing local search on an individual in the population was set to 0.06. AutoDock was run several times to get various docked conformations, and used to analyze the predicted docking energy. The binding sites for these molecules were selected based on the ligand-binding pocket of the templates. AutoDock Tools provide various methods to analyze the results of docking simulations such as, conformational similarity, visualizing the binding site and its energy and other parameters like intermolecular energy and inhibition constant. For each ligand, ten best poses were generated and scored using AutoDock 4.2 scoring functions.

RESULTS AND DISCUSSION

Nowadays, the use of computers to predict the binding of libraries of small molecules to known target structures is an increasingly important component of the drug discovery process [36-37]. *In silico* approaches contribute significantly to early pharmaceutical research and are especially important in target discovery and lead discovery [38]. *In silico* docking study, was carried out to identify the inhibiting potential of selected flavonoids against HMG CoA reductase and cholesterol esterase enzymes. Lead optimization of the selected compounds was done by computation of druglikeness properties. The druglikeness scores of the compounds were evaluated with the help of Lipinski's rule. The docking studies were performed by the use of AutoDock4.2. In the docking studies, if a compound shows lesser binding energy compared to the standard it proves that the compound has higher activity. Binding energy of the individual compounds were calculated using the following formula, **Binding energy = A+B+C-D** where**,** A denotes final intermolecular energy + van der Walls energy (vdW) + hydrogen bonds + desolvation energy + electrostatic energy ($kcalmol^{-1}$), B denotes final total internal energy ($kcalmol^{-1}$), C denotes torsional free energy ($kcalmol^{-1}$), D denotes unbound system's energy ($kcal/mol^{-1}$).

Docking results of flavonoid compounds and Rosuvastatin for HMG CoA reductase enzyme

The binding energy, inhibition constant and intermolecular energy of simvastatin for HMG CoA reductase and flavonoid compounds are tabulated in **Table 2**. The binding energy of Rosuvastatin for the HMG CoA reductase was found to be -7.97 $kcalmol^{-1}$. The binding energy of flavonoid compounds were ranging between -11.85 to -9.09 to $kcalmol^{-1}$. From the selected flavonoid compounds FA5 showed better binding energy -11.85 $kcalmol^{-1}$ when compared to the standard. This indicate that FA5 possess potential HMG CoA reductase inhibitory binding site when compared to the standard.

Table 3: Docking parameters of flavonoid compounds and Rosuvastatin with the enzyme HMG CoA reductase

Compound code	Binding energy ($kcalmol^{-1}$)	Inhibition constant (nm)	Intermolecular energy (($kcalmol^{-1}$)
FA1	-11.57	3.28	-13.36
FA2	-11.69	2.69	-13.48
FA3	-11.11	7.12	-12.9
FA4	-11.5	3.74	-13.29
FA5	-11.85	2.06	-13.94
FA6	-11.12	7.04	-13.21
FA7	-9.68	80.58	-11.47
FA8	-9.84	61.35	-11.63
FA9	-9.09	216.45	-10.88
FA10	-9.62	88.79	-11.41
FA11	-9.24	168.79	-11.33
FA12	-9.18	185.16	-11.27
FA13	-10.02	45.47	-11.51
FA14	-10.12	38.32	-11.61
FA15	-9.68	80.53	-11.17
FA16	-9.94	51.99	-11.43
FA17	-10.15	36.04	-11.94
FA18	-10.26	29.96	-12.05
Standard drug-Rosuvastatin	-7.97	1.44	-11.85

In addition, two other parameters like inhibition constant (kI) and intermolecular energy were also determined. Inhibition constant is directly proportional to binding energy. Theoretical IC_{50} was calculated with the help of AutoDock 4.2. Flavonoids showed inhibition constant ranging from 2.06 nM to 216.45 nM (**Table 2**). FA5 had better inhibition constant (2.06 nM) when compared to the standard (1.44 µM). This implies that the flavonoid compound FA5 was found to be higher activity against HMG CoA reductase enzyme when compared to Rosuvastatin. We found a decrease in inhibition constant of all the selected flavonoids with a simultaneous decrease in the binding energy.

Intermolecular energy is also directly proportional to binding energy. We found a decrease in intermolecular energy of all the selected compounds with a simultaneous decrease in the binding energy. Flavonoids showed intermolecular energy ranging between -13.94 $kcalmol^{-1}$ to -10.88 $kcalmol^{-1}$. (Table.3) FA5 had better intermolecular energy (-13.94 $kcalmol^{-1}$) when compared to the standard (-11.85 $kcalmol^{-1}$). This result further proved that FA5 consist of better HMG CoA reductase inhibitory activity when compared to the standard.

Based on the docking studies, the HMG CoA reductase inhibitory activity of the selected compounds was found to be decreased in the order of FA5, FA2, FA1, FA4, FA6, FA3, FA18, FA17, FA14, FA13, FA16, FA8, FA15, FA7, FA10, FA11, FA12, FA9, Rosuvastatin. All the flavonoids possess binding sites with cholesterol esterase enzyme. But, only the FA12 showed better binding interactions and docking parameters (binding energy, inhibition constant, intermolecular energy) than the other selected flavonoids and the standard.

Docking results of flavonoid compounds and Simvastatin with cholesterol esterase enzyme

Docking parameters of flavonoid compounds and standard drug Simvastatin with the cholesterol esterase enzyme is tabulated in **Table 3**. For Flavonoids showed binding energy ranging between -9.03 $kcalmol^{-1}$ to -7.28 $kcalmol^{-1}$. From the selected flavonoids FA12 had showed better binding energy (-9.03 kcal/mol) when compared to the standard simvastatin (-6.72 $kcalmol^{-1}$). This proves that FA12 consist of potential cholesterol esterase inhibitory binding sites when compared to the standard.

Table 3: Docking parameters of flavonoid compounds and Simvastatin for the enzyme cholesterol esterase

Compound code	Binding energy ($kcalmol^{-1}$)	Inhibition constant	Intermolecular energy ($(kcalmol^{-1})$)
FA1	-8.66	446.98*	-10.45
FA2	-8.9	299.31*	-10.69
FA3	-8.07	1.23**	-9.85
FA4	-8.52	570.83*	-10.31
FA5	-7.91	1.59**	-10.0
FA6	-8.38	725.86*	-10.46
FA7	-8.68	436.69*	-10.47
FA8	-8.79	358.36*	-10.58
FA9	-8.26	884.91*	-10.05
FA10	-7.28	4.61**	-10.11
FA11	-8.26	874.96*	-9.37
FA12	-9.03	241.43*	-10.35
FA13	-8.93	283.82*	-10.52
FA14	-8.97	283.92*	-10.42
FA15	-8.38	720.2*	-9.87
FA16	-8.99	257.81*	-10.48
FA17	-8.3	824.83*	-10.09
FA18	-8.34	771.3*	-10.13
Standard drug-Simvastatin	-6.72	11.89*	-9.11

(nm*, µm)**

In addition, two other parameters like inhibition constant (kI) and intermolecular energy were also determined (**Table 3**). Inhibition constant is directly proportional to binding energy. Theoretical IC_{50} was calculated with the help of AutoDock 4.2. Flavonoids showed inhibition constant ranging from 241.43 nm to 4.71 µM. FA12 had better inhibition constant (241.43 nm) when compared to the standard (6.72 µM). This implies that the FA12 has found to possess higher activity against cholesterol esterase enzyme when compared to Simvastatin. We found a decrease in inhibition constant of all the selected flavonoids with a simultaneous decrease in the binding energy. Intermolecular energy is also directly proportional to binding energy. We found a decrease in intermolecular energy of all the selected compounds with a simultaneous decrease in the binding energy.

The intermolecular energy of standard drug Simvastatin was found to be -9.11 $kcalmol^{-1}$. The flavonoids compound showed intermolecular energy ranging between -10.69 $kcalmol^{-1}$ to -9.37 $kcalmol^{-1}$. FA2 had better intermolecular energy -10.69 $kcalmol^{-1}$) when compared to the standard Simvastatin. This result further proved that FA2 consist of better cholesterol esterase inhibitory activity when compared to the standard and the compound FA12.

Based on the docking studies, the cholesterol esterase inhibitory activity of the selected compounds was found to be decreased in the order of FA12, FA16, FA13, FA14, FA2, FA8, FA7, FA1, FA4, FA15, FA6, FA18, FA17, FA11, FA9, FA3, FA5, FA10, Simvastatin. All the flavonoids possess binding sites with cholesterol esterase enzyme. But, only the FA12 showed better binding interactions and docking parameters (binding energy, inhibition constant, intermolecular energy) than the other selected flavonoids and the standard.

Docked poses of flavonoid compounds and standards

Analysis of the receptor/ligand complex models generated after successful docking of the flavonoids was based on the parameters such as hydrogen bond interactions, Π- Π interactions, binding energy, RMSD of active site residues and orientation of the docked compound within the active site [39]. As a general rule, in most of the potent anti inflammatory compounds, both hydrogen bond and Π- Π hydrophobic interactions between the compound and the active sites of the receptor have been found to be responsible for mediating the biological activity.

The docking poses were ranked according to their docking scores and both the ranked list of docked ligands and their corresponding binding poses [40]. The **Fig. 2**, docked pose of HMG CoA reductase with the compound FA5 and cholesterol esterase with the ligand flavonoid compound FA12 **(Fig. 4)**. The docked pose of standards Rosuvastatin **(Fig.3)** and Simvastatin **(Fig.5)** clearly demonstrated the binding positions of the ligand with the enzyme.

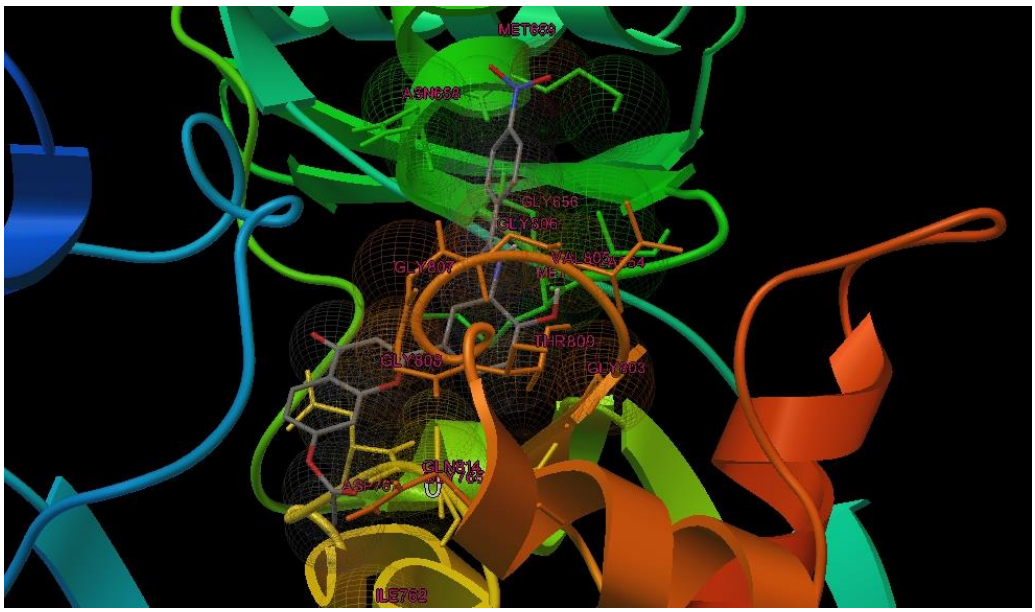

Fig. 2: Docked pose and interactions of FA5 with HMG CoA reductase

Human HMG CoA Reductase enzyme contains 888 amino acids, with the first 339 residues as the membrane anchor domain located in the endoplasmic reticulum. A linker region is located between residues ranging from 340 to 449, while the catalytic domain, from residues ranging from 450 to 888, resides in the cytoplasm. The structure of the catalytic portion of human HMG CoA Reductase consists of a proteinic tetramer containing four actives sites formed by residues of two monomers. This active site is characterized by the so-called *cis*-loop (residues 682–694), which is involved in the reduction of the substrate HMG-CoA [41].

The potential binding sites of the FA5 was found **(Fig.2)** that MET655, GLY806, GLY656, GLY658, MET659, ASP757, ILE762, GLY765, GLY767, GLY803, VAL805, GLY807, GLY808, THR809, GLN814. The binding sites of the Rosuvastatin with HMG CoA reductase was found to be ALA654, MET655, GLY656, GLY658, GLY765, GLN766, ASP767, ALA768, GLY803, VAL805, GLY806, GLY807, GLY808, THR809 **(Fig.3)**. This proves that the effective binding sites are present in the selected flavonoid compound when compared with the standard Rosuvastatin.

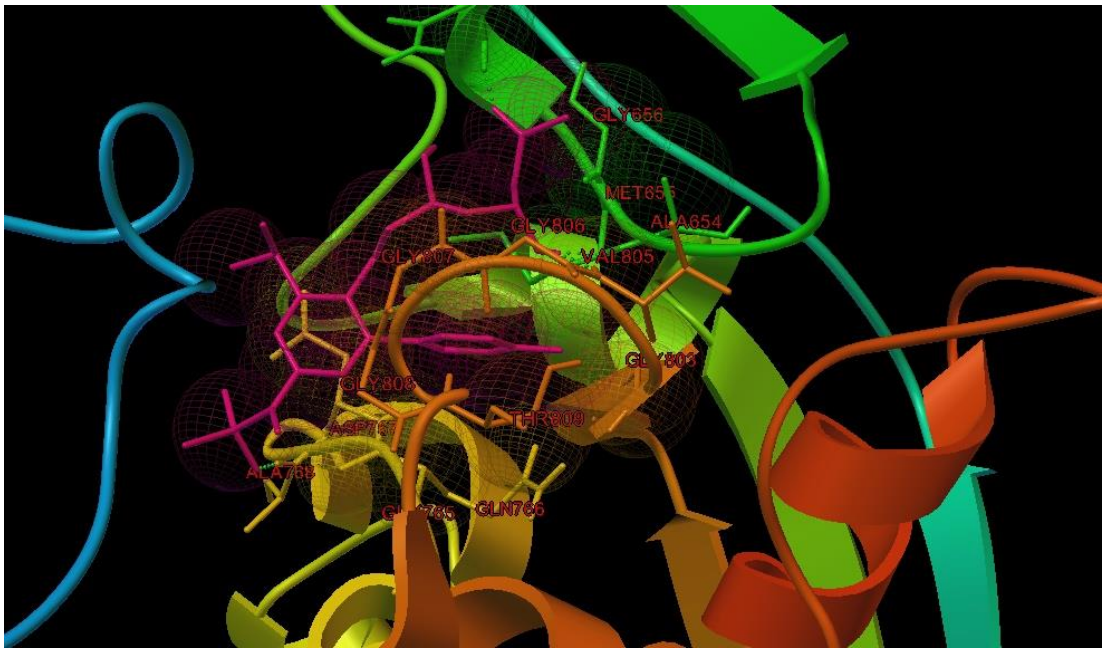

Fig. 3: Docked pose and binding interaction of Rosuvastatin with HMG CoA reductase

Similarly The potential binding sites of the FA12 **(Fig.4)** was found that, GLY123, GLY124, ILE127, GLY128, ILE132, PHE133, GLU208, SER209, MET213, PHE296, LEU302, PHE345, ALA450.

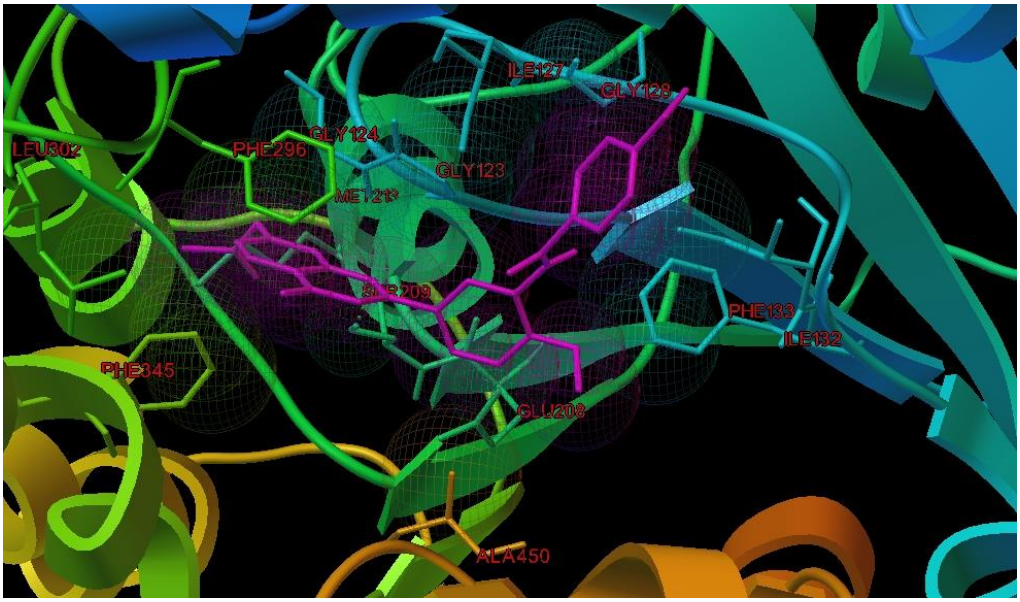

Fig. 4:Docked Pose and binding interactions of FA12 with cholesterol esterase

The binding sites of the standard drug Simvastatin was found to be GLY122, GLY123, GLY124, ILE127, GLY128, ILE132, PHE133, GLU208, SER209, PHE296, LEU302, LEU304, PHE345, HIS449, ALA410 **(Fig.5)**. This proves that the effective binding sites are present in the selected flavonoid compound when compared with the standard Simvastatin.

Conventional drug design techniques are based on trial and error testing using cells or animals. High-throughput screening for chemicals with desired bioactivities requires specialized labs that make the process costly. With a growing number of known experimental structures of target molecules, computational methods have been used successfully to supplement and speed up drug discovery. Computer-based molecular design combines methods of informatics, medicine, and biophysics [42]. There is a wide range of software packages available for the conduct of molecular docking simulations like, AutoDock and DOCK, GOLD, FlexX and ICM [43].

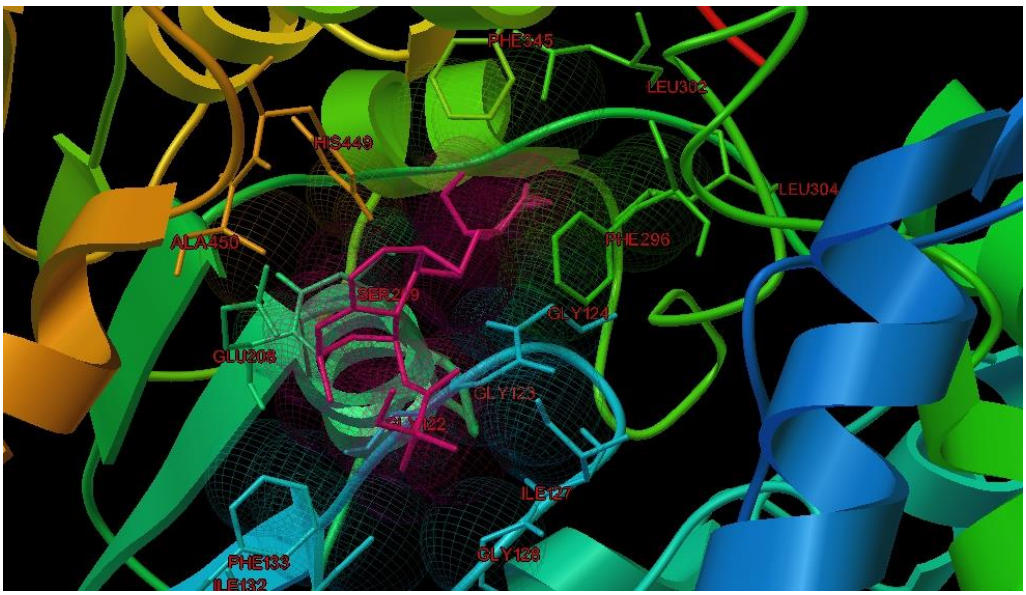

Fig. 5: Docked pose and interactions of Simvastatin with cholesterol esterase

Number of studies has demonstrated that consumption of polyphenols limits the incidence of coronary heart diseases. Atherosclerotic lesions may be present and clinically silent for decades before becoming active and producing pathological conditions such as acute MI, unstable angina or sudden cardiac death [1].

On the basis of the above study FA5, possess potential cholesterol esterase inhibitory binding sites and docking parameters compared to that of the standard. This may be attributed due to the differences in the position of the functional groups at R(-OH) and R_1 (-NO_2) in that compound. Similarly, the flavonoid compound FA12, had exhibited a potential cholesterol esterase inhibitory binding sites and docking parameters compared to that of the standard. This may be attributed due to the differences in the position of the functional groups at R(-OCH_3) and R_1 (-OCH_3) in that compound. This proves that the selected compounds have the ability of inhibiting HMG CoA reductase and cholesterol esterase enzyme.

CONCLUSION

In conclusion, further development of FA5 could be a potential drug candidate as an HMG CoA reductase inhibitor and F12 could be a potential drug candidate for the cholesterol for the cholesterol inhibition in future. These flavonoid compounds can be synthesized and screened for their HMG CoA reductase and cholesterol esterase inhibitory potential.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

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