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Molecular Binding Signatures of Morelloflavone and Its Naturally Occurring Derivatives on HMG-COA Reductase

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Abstract

Morelloflavone and its derivatives are plant-derived biflavonoids with diverse pharmacological activities including anti-hypercholesterolemia. As natural compounds, researches are still aimed at elucidating possible mechanism of their known biological activities. These compounds may serve as sources of cheap pharmaceutical agents with less or no toxic effects. Previously, using *in vitro* analysis, the inhibitory effect of morelloflavone on 3-hydroxy-3-methylglutaryl-CoA (HMG-COA) reductase was reported. In the current study, the precise interaction as well as the binding capacity of morelloflavone and its naturally occurring derivatives were determined with the aid of *in silico* experimental approach and compared with known statin as control. The results obtained showed that morelloflavone displayed the best binding capacity among the compounds and occupied HMG-binding pocket on HMG-COA reductase thereby blocking access of substrates to the enzyme active site. The molecular analysis clearly revealed that morelloflavone has the highest affinity to the enzyme compared with its derivatives but lower affinity than the control ligand, atorvastatin. The luteolin subunit of morelloflavone was found buried within the shallow hydrophobic pocket where hydrogen bond interactions were seen with residues Asn755, Glu665, Ser684, Cys561, Asp690 and Ala856. The binding pose and precise interaction of morelloflavone with the enzyme was comparable to that of statin. Considering the bulky hydrophobic structure of the biflavonoids, the contribution of van der waals interactions to the binding strength observed between HMG-COA reductase and morelloflavone is predictable. The binding affinity of morelloflavone, as seen clearly in this work, is in agreement with previous reports obtained from *in vitro* experiments. Hence, this work provides insight on the direct interaction and inhibitory potential of these plant-derived biflavonoids on HMG-COA reductase as an anti-hypercholesterolemia target.

1. Introduction

3-hydroxy-3-methylglutaryl-CoA (HMG-COA) reductase is a validated clinical target in management of hyperlipidemia; a known high risk for atherosclerosis. This enzyme is

positioned at the committed step in cholesterol biosynthetic pathway where the entire process can be regulated via feedback inhibition. HMG-COA reductase is Nicotinamide adenine dinucleotide phosphate, reduced (NADPH)-dependent and catalyzes HMG-COA conversion to mevalonic acid [1]. The competitive inhibitors of this enzyme (statins) have been in use for decades in lowering blood cholesterol level in people at the risk of heart-related diseases. However, their use is said to be associated with unwanted side effects [2]. Over the years, there has been an enhanced effort by researchers to identify plant-derived bioactive agents with potentials to block the enzymatic activity of HMG-COA reductase as a means of regulating cholesterol metabolism [3], [4]. These research activities were influenced by the fact that natural product has been a promising source of chemical scaffolds, majority of which have contributed to drug design and development in recent years. The phytochemicals which are present in numerous medicinal plants across the globe remained to be fully explored for their pharmacological efficacies. However, there has been robust research activities geared towards isolation, structural determination and biological evaluation of bioactive ingredients from valuable and commonly used medicinal plants [3], [5]. Although these plant-derived compounds have been reported for one or more biological activities, identification of possible molecular target and the underlying mechanism for such bioactivities or pharmacological effects remained to be unraveled.

One of such bioactive constituents are the biflavonoids which have been described as phytochemicals comprising two monoflavonoids, covalently joined together to form various chemical structures. In other words, they are dimers of two flavonoids and are found in relatively few plant families. Only about a few above hundred biflavonoids have been structurally classified till date [6]. Morelloflavone belongs to the biflavonoids class of phyochemicals. Structural feature has revealed that it is composed of luteolin and apigenin as subunits. Morelloflavone was first isolated from *Garcinia Morella* in 1967 by Karanjgaokar and co-workers [7]. Since this discovery, other researchers have also isolated this compound from different parts of other Garcinia species such as the leaves of *Garcina dulcis*, *Garcinia subelliptica*, *Garcinia livingstonei*, *Garcinia multiflora* as well as the nuts of *Garcinia kola* [8-11] and the heartwood of *Garcinia brasiliensis* [12]. Generally, the members of Garcinia plant family are known for their potent pharmacological activities. The naturally occurring morelloflavone derivatives include the Garcinia GB1, GB2 and kolaflavanone [13]. These compounds have been reported in the nut, leaves and seeds of *Garcinia kola*, *Garcinia preussii*, *Garcinia volkensii* and *Garcinia multiflora* respectively. The GBs are known for their bioactivities such as anti-bacterial, anti-hepatotoxic (hepatoprotective), anti-diabetic and wound healing activities in diabetic rats [14-16]. They were also reported for their moderate *in vitro* anti-HIV activities while their potential to inhibit acetylcholinesterase has been suggested [13].

Similarly, a wide range of biological activities have been reported for morelloflavone. These include anti-inflammatory effects in experimental animals, anti-HIV effect via inhibition of HIV-1 reverse transcriptase *in vitro* [17]. Morelloflavone also possesses anti-microbial and anti-fungal properties as it is reported to block fungal fatty acid synthase activity [18], [19]. Among the enzymes known to be susceptible to morelloflavone inhibition are the secretory phospholipase A2 [20]. Pereanez and colleagues [21] also claimed that this biflavonoid displayed potent inhibitory activity for both enzymatic and biological functions of snake venom phospholipase A2. Masuda *et al*. [22] reported strong inhibitory property of morelloflavone against tyrosinase, an essential enzyme in skin melanization. This Garcinia biflavonoid also showed *in vitro* inhibitory potential against cysteine proteases (papain and cruzain) and serine peptidase (trypsin) serving as microbial proteolytic apparatus [23]. Anti-oxidant effects of morelloflavone has been reported in diverse experimental systems [20], [24], [25]. Recently, the activation of Rho-GTPases and ERK signaling pathways by morelloflavone as an anti-tumor angiogenesis mechanism has also been observed [26]. The capacity of morelloflavone to prevent restenosis through its ability to block movement of vascular smooth muscle cells and thereby inhibiting injury-induced neointimal hyperplasia was reported by Pinkaew and co-workers [10]. In addition, hypocholesterolemic effect of morelloflavone was observed in experimental animals as this biflavonoid reduced blood cholesterol levels and prevented progression of atherosclerosis in hypercholesterolemic animals fed with high-fat diet [25], [27]. The possible molecular mechanism of this fascinating effect is worthy of elucidation.

Although there is a postulation that morelloflavone may have inhibited the *de novo* synthesis of cholesterol at the rate limiting step catalyzed by HMG-COA reductase, there still exist a dearth of evidence to show the precise HMG-COA reductase-morelloflavone interaction at the molecular level. This work was therefore carried out to investigate the specific binding pattern of morelloflavone and its natural derivatives on this enzyme. In this work, it was shown that the inhibitory mechanism of morelloflavone against HMG-COA reductase is similar to that of known potent inhibitors (statins).

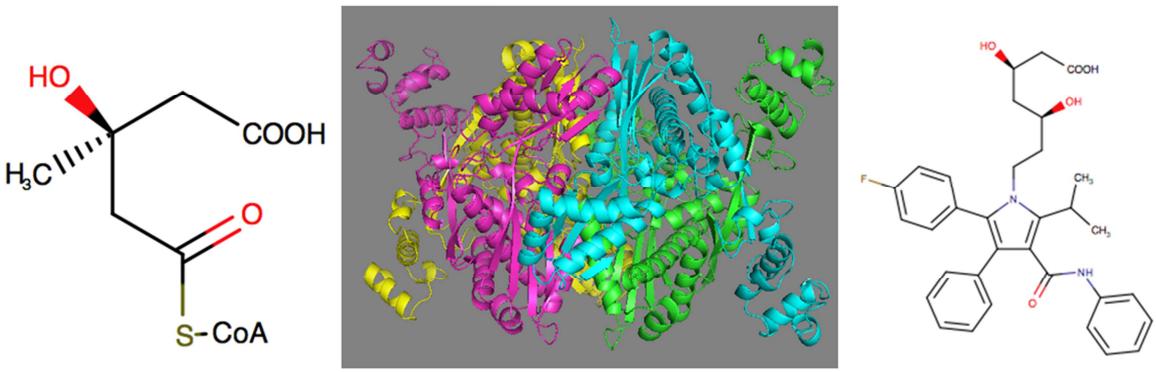


Figure 1. *Structures of HMG-COA, HMG-COA reductase and statin inhibitor (atorvastatin).*

The tetrameric chains of HMG-COA reductase are shown with each chain coloured differently in Figure 1.

2. Materials and Methods

2.1. Preparation of Ligands

A total of five (5) ligands used in this docking study were selected from the literature. Out of these compounds, four (4) were biflavonoids isolated from Garcinia species while atorvastatin was used as control ligand. The chemical structures of these compounds: morelloflavone (CID: 5464454), Garcinia GB1 (CID: 161087), Garcinia GB2 (CID: 161259), kolaflavanone (CID: 155169) were obtained from NCBI PubChem compound database and prepared using Marvinsketch. 2D-coordinates of the ligands were sketched using ChemAxon software. Using the Conformers suit of Marvin-Sketch, the 2D structures were converted to 3D geometry and saved as sdf files. The Merck molecular force field (MMFF94) was employed. This format of the compounds was docked into the HMG-COA reductase model using the AutoDock 4.2.

2.2. Selection and Preparation of Protein Structure Through Homology Modelling

The starting coordinate of HMG-COA reductase employed in this study was retrieved from the Brookhaven protein data bank with PDB ID: 1HWK having resolution of 2.22 Å. The crystal structure was deposited by Istvan and Deisenhofer in 2001 [28]. The macromolecule was co-crystallized with atorvastatin and ADP. The "FASTA" files (Accession: IHWK_A GI: 14277864) for the protein was also retrieved from www.pubmed.org and used in homology modeling for HMG-COA reductase as done on the Swiss-Model Server. The active site was identified with reference to the co-crystallized ligand (atorvastatin). All co-crystallized ligands and crystallographic water molecules were deleted from the protein before molecular docking procedures.

2.3. Molecular Docking and Scoring

For ligand docking and binding site analysis, Autodock vina suite on PYMOL was used [29]. First, based on the already present co-crystallized ligand in the pdb file, the inhibitor binding site was defined with grid parameters set at x=100, y=100 and z=100 while the coordinate of origin was set at 18.31, 8.38 and 15.17 (x, y and z) to include all the amino acid residues at the active site. The spacing between grid points was maintained at 0.375 angstroms. All optimized ligands were docked to the active site of HMG-COA reductase. While the rotatable bonds of the ligands were set to be free, the protein molecule was treated as a rigid structure [30]. Throughout this *in silico* experiment, ten (10) docking runs were performed for each ligand with the number of modes set to 10 so as to achieve more accurate and reliable results.

2.4. Validation of Molecular Docking Procedure

One of the major ways of validating docking procedure is to accurately regenerate both the pose and the molecular interaction of the co-crystallized ligand on the crystallographically-determined protein structure [31]. The ligand found at the binding site of the experimentally-determined HMG-COA reductase was deleted. The structure of the ligand (sdf format) was separately prepared using Marvin sketch as described above and re-docked into HMG-COA reductase active site. The molecular interaction, majorly hydrogen bond in this case, was compared to that of the x-ray diffraction crystal structure.

2.5. Data Analysis

The protein-ligand complexes as well as the molecular interaction were all visualized using PYMOL and snapshots were taken.

3. Results and Discussion

In addition to life style changes, increased physical activity and weight management as one of the strategies aimed at reducing the risk of hypercholesterolemia, pharmaceutical agents which can help to modify lipid metabolism in the body are also useful. Among the bioactive compounds which can be helpful in this regard is morelloflavone; a biflavonoid isolated from *Garcina dulcis*. In this study, the interaction footprint and inhibitory potential of morelloflavone against HMG-COA reductase was investigated. Since the Garcinia

GB compounds and kolaflavanone are structurally similar to morelloflavone, their potential to bind and inhibit HMG-COA reductase was also evaluated. The chemical structure and properties of these compounds are presented in Table 1. They are flavone-flavone dimers, linked at the position (C-3'→C-8"). They contribute immensely to the biological effects of the Garcinia species containing them [6]. Hence, they may be useful as sources of bioactive scaffolds in drug development.

Table 1. *Structure and properties of morelloflavone and its derivatives used in this study.*

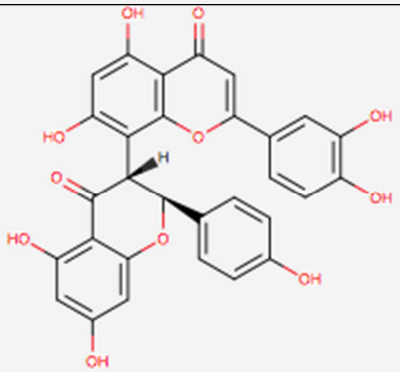
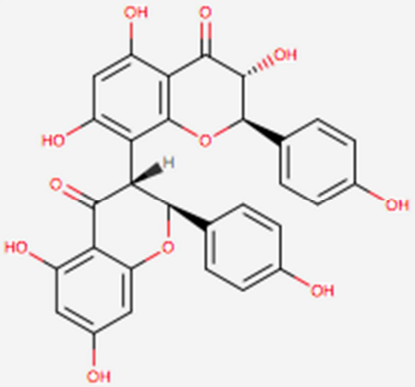
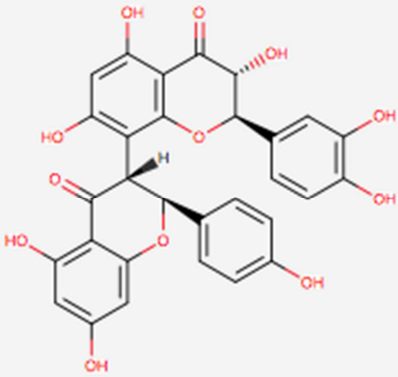
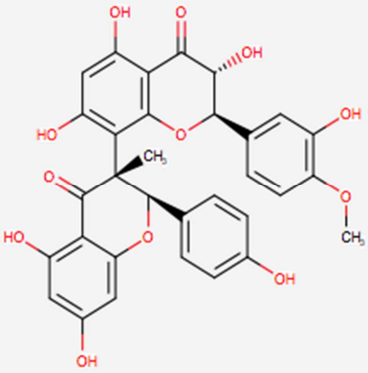
S/No	Compound name	Chemical formular	Molecular weight	2D Structure
1	Morelloflavone	$C_{30}H_{20}O_{11}$	556.101	
2	Garcinia GB1	$C_{30}H_{22}O_{11}$	558.495	
3	Garcinia GB2	$C_{30}H_{22}O_{12}$	574.494	
4	Kolaflavanone	$C_{31}H_{24}O_{12}$	588.521	

Table 2. *Binding energy and hydrogen bond interaction of morelloflavone and its derivatives.*

S/No	Compound name	Energy value (kcal/mol)	Amino acid residues involved in Hydrogen bond
1	Morelloflavone	-9.0	Cys561, Asp690, Asn755, Glu665, Ser684, Ala856
2	Garcinia GB1	-8.7	Arg568, Ser565, Asn755, Arg590, Glu665
3	Garcinia GB2	-8.7	Arg568, Ser565, Asn755, Arg590, Glu665
4	Kolaflavanone	-8.6	Ser565, Arg568, Glu665, Ser852, Arg590, Ser661
5	Atorvastatin	-9.5	Asn755, Arg590, Ser684, Lys691, Glu559, Ser565, Lys735, Lys692

The docking protocols used in this study was first validated to ensure the quality and reliability of docking results obtained. HMG-COA reductase crystal structure with PDB ID: 1HWK from the protein data bank was selected as the starting coordinate for the target protein. To date, more than ten (10) crystal structures of HMG-COA reductase, co-crystallized with different inhibitors, have been deposited in the protein database. The docking program successfully regenerated the ligand binding conformational geometry in a comparable pattern to the experimental results (Figure 2). Both the poses and molecular interactions found in the crystallographic HMG-COA reductase structure were accurately reproduced indicating the acceptability of the docking protocols [31], [32]. Docking analysis has been vigorously carried out on HMG-COA reductase in the search for natural compounds as possible replacement for the statins in the management of hyperlipidemia. Recently, *in silico* approached was used to identify inhibitors of HMG-COA reductase from natural products [4]. In the current study, all selected ligands were docked with the target and the best results in terms of binding energy and accurate protein-ligand conformations were selected for further analysis. Serendipitously, when the generated HMG-COA reductase-morelloflavone complex was examined, morelloflavone was found embedded within the active site of the enzyme indicating that morelloflavone exerts its inhibition via competitive mechanism. The pose was similar to that of the control ligand (statin) (Figure 3) and accordingly, the HMG of HMG-COA [29]. Morelloflavone was buried within the binding site, inserting the luteolin subunit into the shallow non-polar groove and established hydrogen bond interactions with amino acid residues Asn755, Ser648, Cys561, Asp690, Ala856 and Glu665 (Figure 4) while the apigenin subunit was found at the solvent-accessible areas and enjoyed hydrophobic interactions with Glu860 and Leu857 at the active site. Specifically, it was noted that morelloflavone subunits did not interfere with the nicotinamide-binding site. This observation is in agreement with previous wet experiment reports [33]. Interestingly, this pattern of interaction is similar to that of control ligand indicating that morelloflavone inhibits HMG-COA reductase in a comparable manner with the statins. The statin inhibitors such as atorvastatin, pravastatin, fluvastatin, etc. are known for their potent inhibitory effects on the protein. Hence, they are used as drugs in the treatment of hyperlipidemia aimed at lowering of the blood cholesterol level. Atorvastatin was used as the reference ligand in the current study due to its relatively large size (just as the biflavonoids) and high inhibitory activity with IC_{50} of 8nM [28]. The amino acid residues Asp767, Lys691 and Glu559 of HMG-COA reductase have been reported to establish hydrogen bond-linked network which can enhance interaction with the carbonyl group of HMG-CoA during catalytic steps of the enzyme. It has been observed that all statins can interact with residues Asp690, Arg590 and Lys691 of the enzyme. As earlier reported by Barira *et al.* [34] the rings of phenolic compounds such as flavonoids can be accommodated in the shallow and mobile hydrophobic core of the HMG-COA reductase formed by the terminal –COOH amino acids. However, it is worth noting that monoflavonoids may not completely occupy the active site on the enzyme because of their rigid structure, hence, they may not act in full capacity of competitive inhibitor of HMG-COA reductase. Conversely, morelloflavone as a dimeric flavonoid molecule, gained a unique capacity since one of its monoflavonoid subunits can easily occupy the hydrophobic groove while the other can stay at the solvent-exposed region and, competitively inhibit HMG-COA reductase.

The estimated binding energy obtained for morelloflavone was -9.0 kcal/mol compared to -9.7 kcal/mol obtained for atorvastatin (Table 2). It is evident, from these results, that morelloflavone has relatively lower inhibitory activity than the control ligand. However, compared to the derivatives, morelloflavone possesses higher affinity and thus, higher inhibitory potential on HMG-COA reductase. These observations are compatible with the previous results obtained in *in vitro* experiment [28].

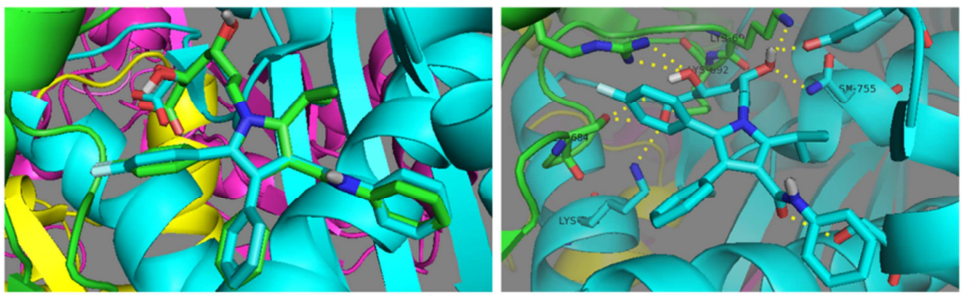


Figure 2. *Validation of docking poses. Co-crystallized ligand (green) versus docked ligand (cyan). The hydrogen bond interactions were maintained after docking.*

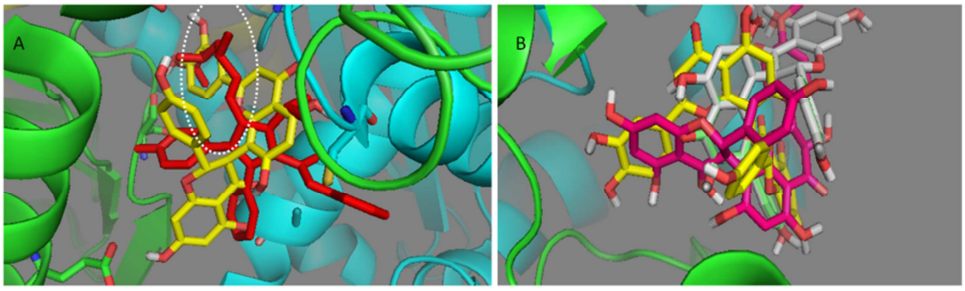


Figure 3. *(a) Superimposition of morelloflavone (yellow) with atorvastatin (red) at HMG-COA active site. The luteolin subunit of MF displayed similar pose compared to the HMG-like portion of atorvastatin. (b) Morelloflavone versus its derivatives on HMG-COA active site (GB1, grey; GB2, green and kolaflavanone, pink).*

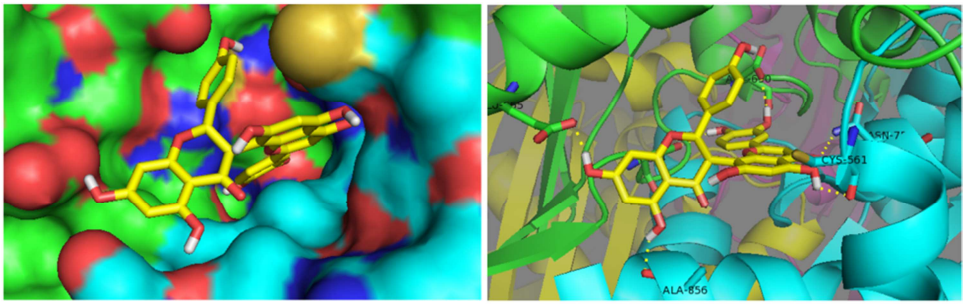


Figure 4. *Binding pose and molecular interaction of morelloflavone with HMG-COA reductase.*

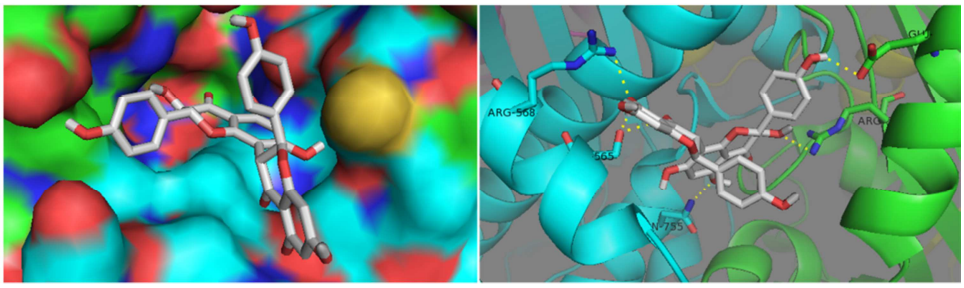


Figure 5. *Binding pose and molecular interaction of Garcinia GB1 with HMG-COA reductase.*

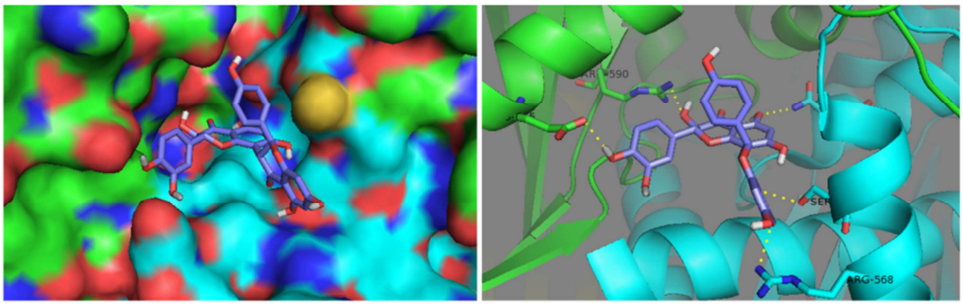


Figure 6. *Binding pose and molecular interaction of Garcinia GB2 with HMG-COA reductase.*

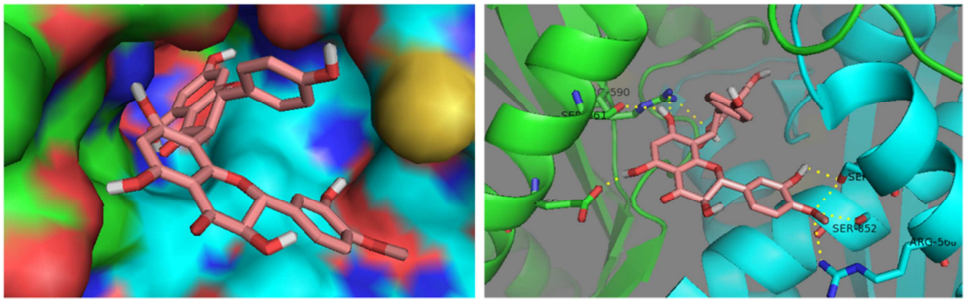


Figure 7. *Binding pose and molecular interaction of kolaflavanone with HMG-COA reductase.*

The docking results for HMG-COA reductase with GB1 revealed a similar mode of interaction and binding configuration as that of GB2 (Figure 5 and 6). In these protein-ligand complexes, the shallow hydrophobic cage remained unoccupied since GB1 and GB2 were found at the active site without any of their subunits binding to the hydrophobic pocket. In a related manner, the binding energy value obtained for both compounds was -8.7 kcal/mol (Table 2) which revealed their lower affinity compared to morelloflavone and atorvastatin. This reduced activity may not be unconnected with the structural modifications seen in GB1 and GB2 compared to morelloflavone. For instance, either loss or gain of hydroxyl, methyl or other functional groups in biflavonoids has been reported to alter the potency of their biological activities [6], [35]. It was observed that residues Arg568, Ser565, Asn755, Arg590 and Glu665 were essential for polar interactions between the protein and these compounds. Kolaflavanone displayed the lowest binding affinity among the ligands selected for this experiment (Table 2). The monoflavonoid subunits of kolaflavanone also failed to penetrate into the hydrophobic core on HMG-COA reductase. In addition to the hydrogen bond established with the amino acid residues similar to GB1 and GB2, kolaflavonone formed polar interactions with residues Ser661 and Ser852 (Figure 7). Hydrogen bond interactions are often found between protein-substrate and protein-inhibitor complexes, and play a key role in enzyme catalysis as well as structural stability of various biological molecules. Hydrogen bond is unique in the sense of its endowed capacity to possess a positive charge at the physiological pH despite being in covalent bond within molecules. Although these compounds bind to the active site on the enzyme, their inability to interact with the shallow non-polar pocket may be responsible for their reduced potential to inhibit HMG-COA reductase. These results suggest that morelloflavone may majorly be responsible for the anti-atherogenic effect of seed and leaf extracts of Gar*cinia spp* that are reported to contain these biflavonoids [25], [27]. Taken together with previous findings, these results corroborate the fact that morelloflavone and it derivatives can interact with HMG-COA reductase with morelloflavone competitively inhibiting the enzyme. Therefore, combination of *in silico* and *in vitro* experiments might be a better option to gain insight into protein-ligand interaction mechanisms.

4. Conclusion

The interaction pattern of morelloflavone, Garcinia GB1, GB2 and kolaflavanone with HMG-COA reductase at molecular level was elucidated in this research. The ability of morelloflavone, a known *Garcina dulcis* biflavonoids, to inhibit enzymatic activity of HMG-COA reductase is because of its binding to the enzyme active site where it competes with the natural substrate (HMG-COA) for accessibility of the catalytic pocket. The luteolin subunit of morelloflavone appeared to move deep into the shallow hydrophobic pocket and interacted with some amino acid residues in the active site via hydrogen bond while the apigenin subunit remained bond at the other part of the active site where it was solvent-exposed. Out of the compounds studied in this research, morelloflavone displayed the highest potential to block HMG-COA reductase catalytic activity compared with the other biflavonoids and, the molecular binding signatures are similar to that of atorvastatin. However, the estimated binding energy value for morelloflavone was higher than that of atorvastatin, implying its moderate inhibitory effect. The results obtained in this study therefore lend a support to previous reports which claimed that morelloflavone is a unique competitive inhibitor of HMG-COA which interact with the enzyme in manners comparable with the statins.

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