JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

IN-SILICO IDENTIFICATION OF FLAVONOIDS BASED INHIBITORS AGAINST SORTASE-A FROM *ENTEROCOCCUS FAECALIS* (*Ef*)

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https://doi.org/10.55251/jmbfs.11256

ARTICLE INFO

Received 20. 4. 2024
Revised 10. 9. 2024
Accepted 19. 9. 2024
Published 1. 10. 2024

Regular article

OPEN ACCESS

ABSTRACT

The enzyme known as SortaseA (SrtA) is widespread present in gram-positive bacteria. SrtA is responsible for anchoring a large number of surface protein virulence factors to the cell wall. This enzyme is not involved in bacterial growth and is also present on the cell membrane, which makes it more accessible for the design of inhibitors. In the case of *Enterococcus faecalis* (*Ef*), which is responsible for a wide range of nosocomial infections, SrtA from this organism promotes development of biofilm, which in turn makes bacteria resistant to antibiotics. Numerous inhibitors have been identified and characterized against *Ef*-SrtA; however, none have been clinically approved till now. Natural compounds and their derivatives showed inhibition against *Ef*-SrtA. In this work, a flavanoids-based in-house natural library was screened against *Ef*-SrtA to see how these molecules bind to active site of *Ef*-SrtA. Furthermore, the absorption, distribution, metabolism and excretion (ADME) properties of the top five compounds, Abyssinones lii, Apigenin, Rutin, Fisetin and Kaemferol, were calculated, Additionally, the correlation between the structure and function of these top five compounds was analyzed using Density Functional Theory (DFT) studies.

Keywords: Sortase A, Flavonoids, Docking, ADME, DFT

INTRODUCTION

The pathogenicity and physiology of microorganisms are significantly influenced by the proteins on their surface (**Wilson *et al.*, 2002**). Bacterial surface proteins play a crucial role in the development of antibiotic resistance **(Ghai, 2023)**. Bacterial surface proteins like SrtA anchor surface proteins to the cell wall envelope, promoting adhesion mechanisms and biofilm development, offering a novel approach to bacterial infections and an ideal target for anti-virulence drugs (**Cascioferro *et al*., 2014; Selvaraj *et al*., 2018; Raimondi *et al*., 2019**). Biofilm formation by SrtA may control processes like host cell entry and immune response evasion, making it a target for inhibiting bacterial virulence (Figure 1) (**Oniga *et al*., 2017**). Inhibition of SrtA that is help to attach surface protein can disrupt biofilm formation, helping in combating drug resistance, pathogenesis and infectivity (**Ilangovan *et al*., 2001; Nitulescu *et al*., 2016**). *Enterococci* are opportunistic gram-positive bacteria and in recent years, *Enterococci* strains have caused a lot of worry because they can become immune to many antibiotics. In the case of *Enterococci*, SrtA enzyme plays a significant role in the formation of biofilm (**Lanka *et al.*, 2024**). Several alternative approaches have been proposed to identify and characterise sortase inhibitors. In recent years, there has been a rise in interest in natural anti-sortase agents (**Nitulescu *et al.*, 2017; Thappeta *et al.*, 2020**). Many natural molecules and plant products showed inhibition against SrtA. Flavonoids are an important class of natural products, belonging to a class of plant secondary metabolites **(Kumar *et al.*, 2018; Zandavar *et al.*, 2023**). These are associated with a broad spectrum of health-promoting effects and are gaining popularity because of their unique structures and aromatase inhibitory properties. Many flavonoids showed inhibition properties against SrtA **(Kang *et al.*, 2006; Wang *et al*., 2019).** Rutin isolated from dried flowers of *Sophora japonica* were found to inhibit *Streptococcus mutans* (*S. mutans*) (an oral bacterium responsible for human dental caries) SrtA(**Yang *et al.*, 2016**). It is also a potent inhibitor against *Streptococcus agalactiae* SrtA activity and can significantly increase the survival rate of freshwater fish Oreochromis *niloticus* infected with *Streptococcus agalactiae* (**Khunrang *et al.*,2023**). Luteolin, a tetrahydroxyflavone, is known to increase oxygen production while decreasing hydrogen peroxide levels in lung cancer cells by inhibiting the manganese superoxide dismutase (MnSOD) enzyme function (**Lin *et al.*, 2008**). Many other flavonoids, which are natural substances found in a variety of plants, have been identified as inhibitors of SrtA. For example, Taxifolin, a flavonoid derived from Chinese herbs, has demonstrated the ability to reversibly inhibit SrtA **(Wang *et al*., 2021)**.
Similarly, other flavonoids including Quercetin, Epigallocatechin gallate (a common component of green tea), and Formononetin have been reported to have inhibitory effects on SrtA. Morin and Myricetin have also shown potent inhibitory activities against SrtA **(Olla *et al.*, 2023; Song *et al.*, 2017; Huang *et al.*, 2014;Silva *et al.*, 2017)**. Furthermore, the flavonoid 7-Hydroxy-6-methoxyflavanone has displayed significant inhibitory activity against *S. mutans* SrtA **(Park *et al.*, 2017)**. These studies indicate that flavonoids may serve as potential therapeutic agents in the management of infections caused by gram-positive bacteria.In this work, we have used in-house flavonoid based library (Total fifty flavanoids compounds) and docked on area of the active site of SrtA active site His 120 (79), Cys184 (141), and Arg 197 (149) from *Ef*-SrtA (**Ilangovan *et al.*, 2001; Abujubara *et al.*, 2023; Guiton *et al.*, 2009)**. In this study, three dimensional (3D) model structure of *Ef*-SrtA protein has been developed and the best top ten inhibitors were identified using the PyRx virtual screening program. Further top ten best inhbitors redock and best five flavonoids have been slected for best inhibitor cateogory. ADME properties of all the best five flavanoids have been calculated and correlation between the structure and function of these top five compounds was analyzed using DFT.

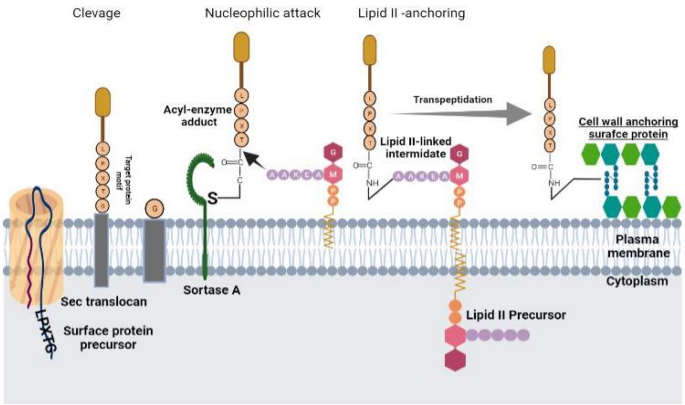


Figure 1 Role of SrtA in anchoring the surface proteins to the cell membrane of bacteria

MATERIAL AND METHODS

Preparation and optimization *Ef*-SrtA (Homology Modeling)

The protein *Ef*-SrtA with a length of 242 amino acids was obtained from uniprot (https://www.uniprot. org/ uniprotkb /A0A855UI50/entry) to develop model three-dimensional (3D) structure of *Ef*-SrtA using PHYRE2 Protein Fold software. Quality of model was checked by using the proSA-web tool, which indicated a Z-Score of -6.2 and showed that 91.6% of residues were located within the favorable region in the Ramachandran plot. Further refinement and energy minimization has been done using Yet Another Scientific Artificial Reality Application (YASARA) software. Furthermore, the active site in *Ef*-SrtA was predicted utilizing the DoGSiteScorer (https://bio.tools/dogsitescorer).

Ligand retrieval

We obtained a list of natural 50flavonoid compounds, from the PubChem database **(Kumar*et al.*, 2023)**. These compounds 3D conformers were downloaded in Structured Data File (sdf) format. The compounds were underwent energy minimization, prior to the virtual screening and all the compounds were converted to the Protein Data Bank, Partial Charge (Q) and Atom Type (T) (pdbqt) format using PyRx before screening with modeled *Ef*-SrtA Virtual Screening Tool 0.8 PyRx https://pyrx.sourceforge.io/ **(Dallakyan *et al*., 2015)**.

Virtual screening

PyRx, an open-source software, was used in this study for the virtual screening of in-house flavonoid-based natural inhibitors. In order to conduct virtual screening, it is necessary to first build a grid in which the ligand is expected to bind successfully **(Kumar*et al.*, 2023**). In close proximity to the binding site of *Ef*-SrtA, grid boxes were generated at the center of the protein with XYZ coordinates of 8.178579 Å, 29.398211 Å, and -14.835526 Å, respectively. For all the ligands same grid box size was used for virtual screening. An exhaustiveness value of 8 was utilized to thoroughly explore the natural compounds library. To get potential inhibitor against *Ef*-SrtA among the 50 flavonoids we have made binding energy cuttoffof -7.0 kcal/mol.

Molecular docking with ADME profiling

The screening results were validated and recalculated by molecular docking experiments carried out by using Autodock tool 1.5.7 **(Morris *et al.*, 2009; Allouche, 2012; Sharma *et al.*, 2022)**. Top ten compounds on the basis of binding energy redock using a flexible docking approach reported by with minor modification **(Trott *et al.*, 2010)**. Prior to docking Kollman charges were assigned to the *Ef*-SrtA molecule and the docking simulations employed the Lamarckian genetic algorithm with 10 Genetic Algorithm (GA) runs to enhance accuracy and reliability. The grid box on the protein was set at XYZ coordinates of 8.178579Å, 29.398211Å,and -14.835526Å, respectively, with a spacing of 0.375 Å. After forming complexes between the compounds and *Ef*-SrtA, a comprehensive analysis was conducted to evaluate binding energies and molecular interactions. The protein-ligand complex was selected based on the lowest docking energy. Subsequently, the interactions between the protein and ligand were further analyzed using visualization tools, including Discovery Studio Visualizer 20.1.0.19295, PyMOL, and Chimera. Additionally, the drug-likeness properties of the compounds were assessed using Swiss ADME requiring the generation of SMILES notation for each compound **(Gupta *et al.*, 2022)**. Furthermore, the toxicity profiling of the compounds were carried out using the ProTox-2 tool to provide supplementary insights into their safety profiles.

DFT analysis of top five hit compounds

The correlation between the structure and function of top five hit compounds were analyzed using DFT studies **(Kumar *et al.*, 2023)**. We have used the GAUSSIAN 16 software package for all theoretical calculations and GAUSS-VIEW 6.1 for visualization **(Becke, 1993; Raghavachari, 2000)**.To optimize the top hits, DFT with Becke's three-parameter method for exchange interaction and Lee-Yang-Parr for correlation functional (B3LYP) was utilized, along with the 6-31+G(d,p) basis sets (**Scott *et al.*, 1996**). Initial molecular structures were sketched using GAUSS-VIEW 6.1 (**Ricca, 1995**). For the compounds studied, significant parameters including total energies, dipole moments, Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO)energy levels and band gap energy were determined (**Thomas *et al.*, 2023**).

RESULTS AND DISCUSSION

Exploring potential flavonoid inhibitors for the development of *Ef*-SrtA inhibitors using virtual screening

Virtual screening is an effective technique for identifying optimal lead molecules for drug discovery from large libraries of small molecules. By screening small molecules databases, it can predict which molecule will interact best with a specific target (*Ef*-SrtA) to form a stable complex. The present study demonstrated the use of 50 flavonoids (natural compounds) based from PubChem to conduct virtual screening against the *Ef*-SrtA, to identify putative optimal hits. The top ten molecules were selected based on lowest binding energy ranging from −6.9 kcal/mol to −7.5 kcal/mol. Additionally, the orientation of the molecules and the existence of various interactions, including hydrogen bond and hydrophobic interactions, were also taken into consideration.

Table 1 Drug likeness properties of top ten flavonoids after virtual screening

Compounds (Name and CID)	MW (g/mol)	Log P	Hb Donar	Hb Acceptor	Topological polar surface (Å)	Solubility	Lipinski	Pains	Veber
Abyssinones lii (10408069)	390.47 g/mol	3.65	1	4	55.76	Moderately soluble	Yes; 0 violation	0 alert	Yes
Apigenin (5280443)	270.24	1.89	1	5	90.90	soluble	Yes; 0 violation	0 alert	Yes
Rutin (5280805)	610.52	0.46	10	16	269.43	soluble	No; 3 Violation	1 alert:	No; 1 violation: TPSA>140
Fisetin (5281614)	286.24	1.50	4	6	111.13	soluble	Yes; 0 violation	0 alert	Yes
Kaemferol (5280863)	286.24	1.70	4	6	111.13	soluble	Yes; 0 violation	0 alert	Yes
Dalbergin (442768)	268.26	2.63	1	4	59.67	soluble	Yes; 0 violation	0 alert	Yes
Apigenidin (441647)	290.70	-5.51	3	4	94.06	Moderately soluble	Yes; 0 violation	0 alert	Yes
Pelargonidin (440832)	271.24	-2.44	4	5	94.06	soluble	Yes; 0 violation	0 alert	Yes
Abyssinone II (10064832)	324.37	2.83	2	4	66.76	Moderately soluble	Yes; 0 violation	0 alert	Yes
Colophyllolide (5281392)	416.47	3.68	0	5	65.74	Moderately soluble	Yes; 0 violation	0 alert	Yes

Molecular docking, interaction studies and drug-likeness analysis

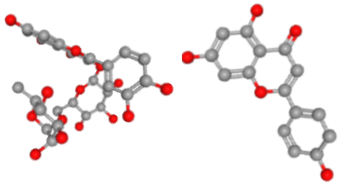
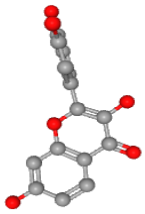
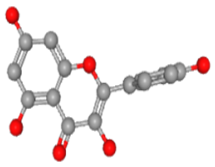
After virtual screening with PyRx, the top ten flavonoid-based inhibitors were re-docked. All complexes were visualized on PYMOL, and molecular interactions were analysed. Drug likeness and physo-chemical properties of best ten molecules were calculated (Table1) using Swiss-ADME (http://www.swissadme.ch/). In addition, it provides safety and effectiveness of the molecules. In general, a chemical is considered a potential medication, if it fits the requirements of Lipinski's Rule of Five. These requirements include a molecular weight (MW) of less than 500 g/mol, fewer than 10 hydrogen bond acceptors, fewer than 5

hydrogen bond donors and a LogP value of less than 5. All ten best compounds also passed the VEBER rule where the rotatable bond should <=10 and TPSA <=140Pan-assay interference compounds, also known as PAINS, are a group of chemical compounds notorious for their propensity to yield false-positive results in high-throughput screening procedures. Rather than interacting with a single, specific biological target, these compounds are recognized for their indiscriminate reactions with multiple targets **(Baell*et al.*, 2018)**. This broad reactivity is often attributed to the existence of certain disruptive functional groups that are prevalent among PAINS. In the PAINS analysis, all of the top 10 compounds were successful. Keeping all the above things in consideration we choose the five best flavonoids Abyssinones lii, Apigenin, Rutin, Fisetin and Kamferol **(**Table 2**).** All the listed compounds have zero violation of Lipinski's rule except Rutin, whose MW was greater the 500 gm/mol (Table 1).

Table 2 Interacting residues of top flavonoids with *Ef*-SrtA

Compound	Interacting residues (bond distance in Å)		
	Hydrogen bond	Hydrophobic interactions	Electrostatic interactions
Abyssinones lii	Arg149(2.87)	Leu51(5.21), Met63(5.09), Ala77(4.45), Pro121(4.62), Val124(3.91), Ile126(4.92), Leu127(4.73), Ile139(4.83)	Nil
Rutin	His79 (2.41), Cys141(2.32)	Leu56(5.23), Ala77, Val124(5.02), Ile139(5.29), Thr140(2.43),Arg149(5.27)	Nil
Apigenin	Ser78(2.43)	Met63(3.99), Ala77(5.26), Ile127(5.48), Ile139(4.57), Arg149(3.66)	Nil
Fisetin	Ser78(2.63), Cys141(2.63)	Leu56(5.90), His79(5.35), Ala77(3.96), Val124(5.10), Ile139(5.06), Arg149(3.78)	Nil
Kaemferol	Ser78(2.47), Arg123(2.33), Ile127(2.69)	Ala77(4.97), Val124(4.93), Ile139(4.58), Arg149(3.70)	Nil

Table 3 Best five flavonoids molecular weight, binding energy and structure

Flavonoids	Abyssinones lii	Rutin	Apigenin	Fisetin	Kaemferol
MW(g/mol)	390.47	610.52	270.24	286.24	286.24
Binding Energy (kcal/mol)	-7.4	-7.2	-7.0	-7.0	-6.9
Structure					

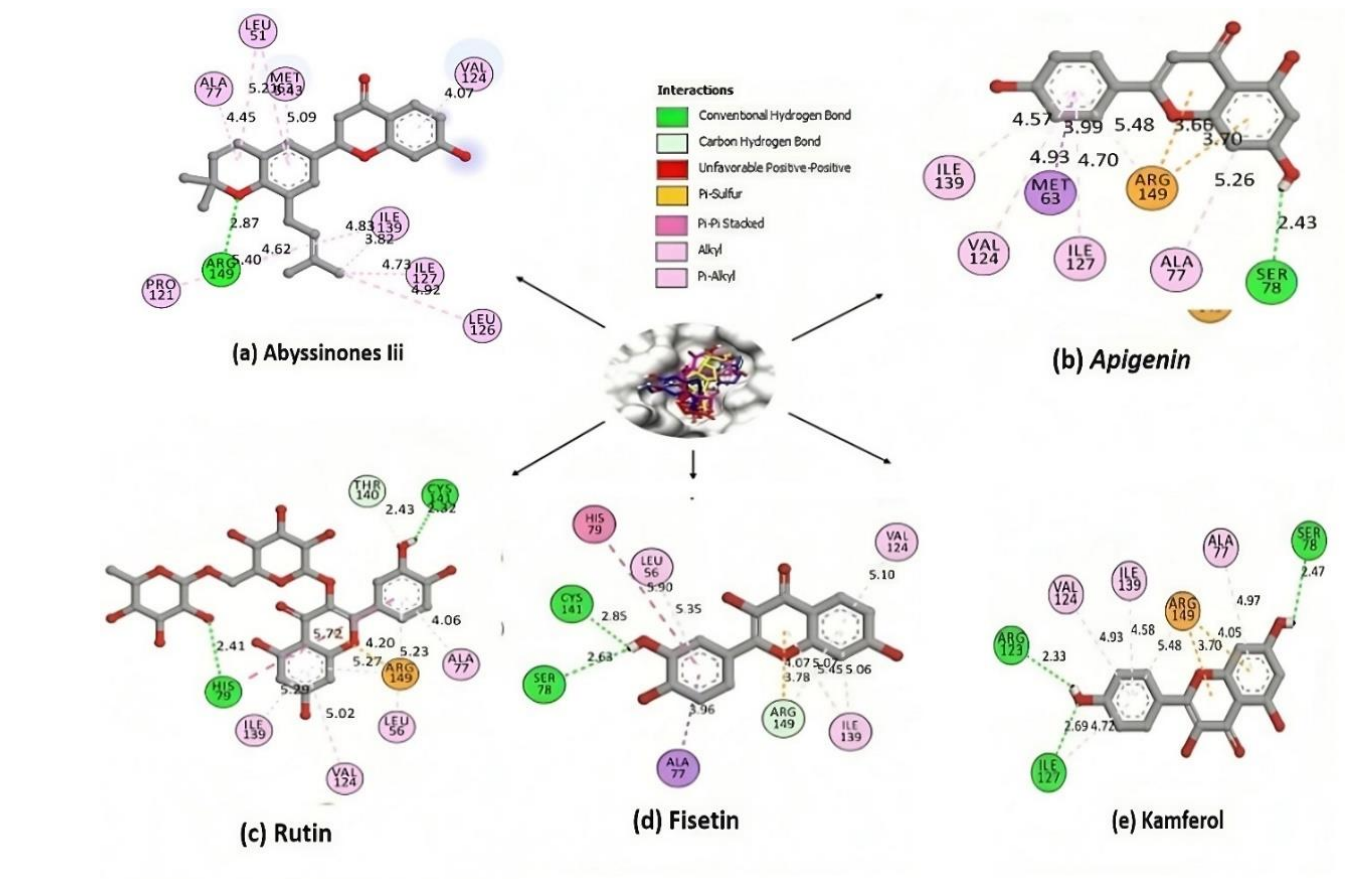


Figure 2 2D illustration was created to depict the binding interactions between the top five screened flavonoids and *Ef*-SrtA.(a) Abyssinones lii, (b) Apigenin, (c) Rutin, (d) Fisetin, (e) Kamferol

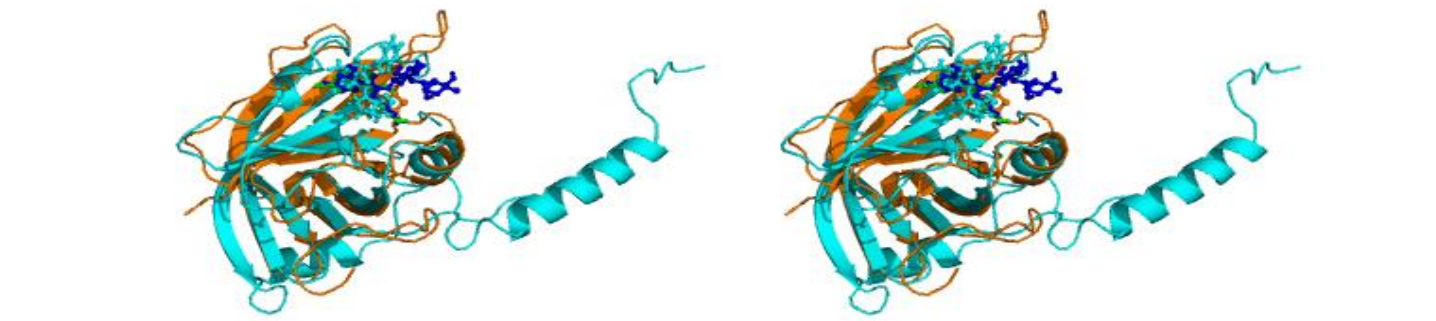


Figure 3 Inhibitor 2-(aminomethyl)-3-hydroxy-4H-pyran-4-one (Cyan colour) (PDB ID;6R1V), LPTA substrate analogue (orange colour) complex (PDB ID; 2KID), Rutin (Blue) and Fisetin (green) *Ef*-SrtA superimposed.

Interaction studies of best five flavonoids with *Ef*-SrtA

The primary residues interacting with top hits were Ser78, His79, Cys141 and Arg149. All the best five molecules bound to active site of *Ef*-SrtA (Table 2). *Ef*-SrtA-*Abyssinones* lii complex has a docking score ΔG-7.4 kcal/mol. Arg149 formed week hydrogen bond with O3 of *Abyssinones* lii. Hydrophobic interactions with Abyssinones lii are formed by Leu51, Met63, Ala77, Pro121, Ile126, Leu127 and Ile139. Ef-SrtA-Apigenin complex has a docking score ΔG-7.0 kcal/mol. Ser78formes hydrogen bonds with apigenin. Hydrophobic interactions with Apigeninare formed by Met63, Ala77, Ile127, Ile139 and Arg149. Arg149 also formed Pi-Cation interactions with Apigenin. The *Ef*-SrtA-Rutin complex has a docking score ΔG-7.2 kcal/mol.Ser78, His79 and Cys141 formed hydrogen bonds with Rutin. Leu56, Ala77, Val124, Ile139, Thr140 and Arg149 formed hydrophobic interactions with rutin. *Ef*-SrtA-Finestin complex has a docking score ΔG-7.0 kcal/mol.Ser78 and Cys141 formed hydrogen bond finestin. Hydrophobic interactions with finestin are formed by Leu56, His79, Ala77(3.96), Val124, Ile139 and Arg149.*Ef*-SrtA-Kaemferolcomplex has a docking score ΔG-6.9kcal/mol. Ser78 and Cys141 formed hydrogen bond Finestin. Hydrophobic interactions with Finestin are formed by Ala77, Val124, Ile139 and Arg149 (Figure 2). On the basis of binding energy and mode of interaction Fisetin and Rutin showed potential inhibitors against *Ef*-SrtA (Table 3). Comparison with the LPTA substrate analogue complex (PDB ID; 2KID) and 2-(aminomethyl)-3-hydroxy-4H-pyran-4-one based prodrug-SortaseA complex (6R1V) showed both Fisetin and Rutin bound near to substrate binding site of *Ef*-SrtA (Figure 3).

Table 4 Different energy of natural compounds after the DFT analysis

Ligands	Total energy (eV)	HOMO energy (eV)	LUMO energy (eV)	Band gap (eV)	Dipole moment
Abyssinones lii	-34538.368	-0.21121	-0.05902	0.1638	3.006
Apigenin	-25820.963	-0.20799	-0.19309	0.0147	1.184
Rutin	-60875.008	-0.19955	-018034	0.0192	7.663
Fisetin	-27885.401	-0.24384	-0.22136	0.0224	5.009
Kaemferol	-27886.163	-0.24107	-0.21772	0.0233	2.246

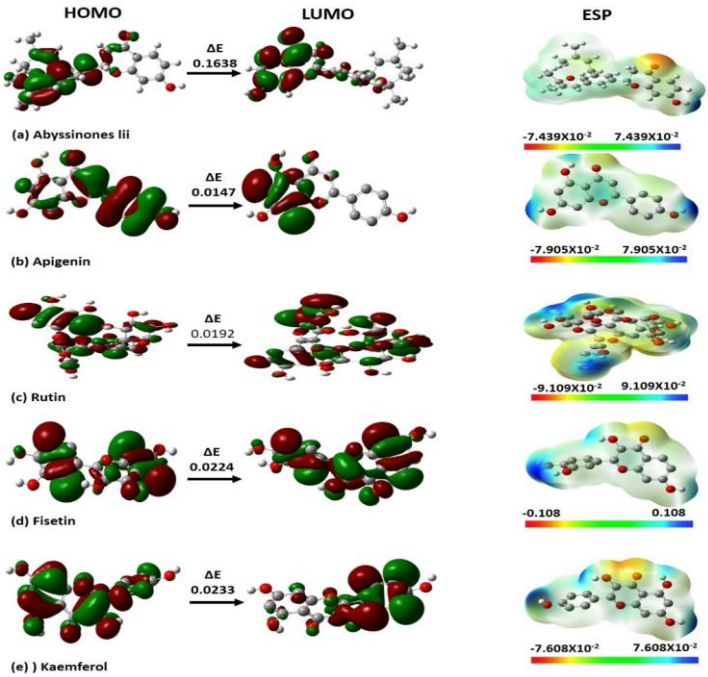


Figure 4 The diagrams of the Highest Occupied Molecular Orbital (HOMO) and Lowest Unoccupied Molecular Orbital (LUMO), as well as the Electrostatic Potential (ESP) maps of the best-docked compounds are presented. These compounds include **(a)** Abyssinones lii, **(b)** Apigenin, **(c)** Rutin, **(d)** Fisetin, and **(e)** Kamferol The molecular orbital wave function displays positive (red) and negative (green) phase distributions. The HOMO, which represents electron donor regions, determines the ionization potentials, while the LUMO, representing electron acceptor regions, determines the electron affinity. The ESP illustrates the electron-rich (red) and electron-poor (blue) regions

Electronic structure of best five flavonoids using DFT

The DFT is supported by quantum mechanics, which offers a precise representation of the electronic and structural characteristics of a compound. In order to determine the electronic distribution of five naturally occurring compounds, we have implemented orbital energy calculations. This comprehension of electronic distribution could illuminate the interactions between ligands and proteins and facilitate the investigation of the binding patterns of these compounds. Electrostatic potentials provide a more detailed perspective on regions with differing electron densities. The distributions of red and green in the molecular orbital wave function denote positive and negative phases, respectively. In a ligand, the HOMO and LUMO positions are crucial, as they regulate the interaction with a potential receptor. The HOMO of the ligand and the LUMO of the receptor are in reciprocal interaction. Therefore, an increase in the ligand's HOMO energy narrows the energy gap with the receptor's LUMO, potentially intensifying binding. Similarly, a decrease in the ligand's LUMO energy is anticipated to enhance binding. An Electrostatic Potential (ESP) map presents a comprehensive view of a ligand's polarity **(Azarhazin*et al.*, 2018)**. Comparing the total energy for all five compounds it was inferred that the least negative energy was shown by the Abyssinones lii -3458.368 (eV) while the highest negative energy of -60875.008 (eV) was shown by Rutin. The compounds that exhibited a smaller energy gap (E_{HOMO}-$E_{LUMO)}$ showed more reactivity and less stability like in the case of Apigenin which is 0.0147 (eV) and with the highest energy band gap of 0.1638eV in Abyssinianslii. While negative nature of the HOMO and LUMO for all compounds shows the stable nature of the top natural compounds with *Ef*-SrtA. DFT also calculated the dipole moment which shows the hydrogen bonding ability of the listed compound. Apigenin (1.184) shows the lowest dipole moment which is a very good agreement with the docking result while Rutin (7.663) shows the highest value (**Table 4)**. The computations of the color-coded Molecular Electrostatic Potential (MEP) offer a deeper understanding of the electrostatic

potential of the compounds. The red color represents the electronegative region, which serves as a hydrogen bond acceptor. Conversely, the blue color denotes the electropositive region, acting as a hydrogen bond donor. The neutral regions, which can engage inhydrophobic interactions, are depicted by colors that span from yellow to green (**Figure4).**

CONCLUSION

Plants contain an abundance of naturally occurring compounds known as flavonoids. It has been suggested in numerous reports that flavonoids have the potential to inhibit the activity of SrtA. In order to identify flavonoid-based compounds that may function as potential inhibitors of SrtA, in silico studies were carried out. After conducting virtual screening, five flavanoids (Rutin, Apigenin, Fisetin, Kaemferol and Abyssinones lii) were selected for docking experiments out of a total of fifty flavanoids. All the best five flavonoids compounds bound to active sites of SrtA. All of the five flavonoids that are considered to be the best are bound near to the active sites of SrtA. Fisetin and Rutin demonstrated potential inhibitory effects against *Ef*-SrtA based on their binding energy and mode of interaction. Fisetin and Rutin were observed to be bound in close proximity to the substrate binding site of *Ef*-SrtA.

Acknowledgments: Author Suraj Singhthanks the Indian Council of Medical Research (**ICMR-SRF. File no BMI/11(23)/2022**) for research funding. S.K thanks to ICMR for research funding.

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