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VIRTUAL SCREENING OF FLAVONOIDS AS INHIBITORY AGENTS OF P-GLYCOPROTEIN

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ABSTRACT : Flavonoids are constituents of fruits, vegetables, and plant derived beverages, as well as components in herbal dietary supplements. The objective of this investigation was to characterize and determine the effect of the Flavonoids on P-glycoprotein (P-gp) which is an important protein involved in multidrug resistance (MDR). Homology modeling of P-glycoprotein (Human) has been performed based on the crystal structure of the 2HYD (Chain A; Structure of a bacterial multidrug ABC transporter) by using Modeller software. With the aid of the molecular mechanics and molecular dynamics methods, the final model is obtained and is further assessed by procheck and verify 3D graph programs, which showed that the final refined model is reliable. With this model, a flexible docking study of P-glycoprotein with a group of Flavonoids which were selected from the previous publications was performed. The results indicated that GLN- 47, TYR -53, SER -83, ILE- 87, GLY -100, ARG -154 in P- glycoprotein are important determinant residues in binding as they have strong hydrogen bonding with Flavonoids. These hydrogen binding interactions play an important role for stability of the complex. Among the 13 Flavonoids docked, Acetylgenistin showed best docking result with P-glycoprotein. Our results may be helpful for further experimental investigations.

Key Words: Acetylgenistin, Flavonoids, Homology modeling, P-glycoprotein (P-gp), Molecular dynamics.

INTRODUCTION

Flavonoids are the most abundant polyphenolic compounds present in fruits, vegetables, and plant-derived beverages such as tea and red wine. Flavonoids have long been associated with a variety of biochemical and pharmacological properties, including antioxidant, antiviral, anticarcinogenic, and anti-inflammatory activities believed to be beneficial to human health(Middleton *et al*., 2000). Cancer chemotherapy has been of limited success because of the intrinsic or acquired resistance of cancer cells to a broad range of chemically and functionally distinct anticancer agents, a phenomenon termed multidrug resistance (MDR). The classical form of MDR involves the overexpression of drug efflux transporters such as P-glycoprotein (P-gp) (Juliano and Ling, 1976; Kartner *et al*., 1983) and multidrug resistance-associated protein 1 (MRP1) (Cole *et al*., 1992) in the cell membrane, which pump anticancer drugs out of the cells, resulting in low intracellular drug concentrations. P-gp is a 170- to 180-kDa plasma membrane protein encoded by the human MDR1 and MDR3 genes and the murine mdr1a, mdr1b, and mdr2 genes.

P-gp belongs to the ATP-binding cassette transporter superfamily and is responsible for the efflux of a broad array of hydrophobic compounds, including a number of important chemotherapeutic agents such as vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxol (Germann, 1996). Structure analysis based on the P-gp sequence predicts that P-gp consists of two homologous halves, each containing six transmembrane domains and a cytoplasmic nucleotide-binding domain (Gottesman and Pastan, 1993). P-gp acts as an efflux pump by exporting its substrate from the membrane or cell cytosol to the exterior of the cells, with ATP hydrolysis as the driving force (Gottesman and Pastan, 1993). Overexpression of this efflux protein has been associated with the clinical MDR phenotype and poor prognosis for many human cancers (Nooter and Sonneveld, 1994; List, 1996). Since the report that the calcium channel blocker Verapamil could reverse resistance by inhibiting P-gp-mediated drug efflux (Tsuruo *et al*., 1981), a wide panel of substances has been tested both in vitro and in clinical trials for their ability to reverse MDR. Although a variety of agents, including calcium channel blockers, calmodulin antagonists, cyclosporins, quinolines, and their analogs have been found to be potent P-gp inhibitors in MDR cancer cells, the clinical results have been disappointing, due to the toxicities resulting from administration of the P-gp modulators and resulting from the pharmacokinetic interactions between the modulators and cytotoxic drugs (Volm, 1998). Besides its role in conferring MDR, P-gp has also been found in many normal tissues, including the intestinal epithelium, blood-brain barrier, hepatocytes, and renal tubular cells, suggesting its important role in drug absorption, elimination, and distribution (Yu, 1999). It has been shown that the oral absorption and brain penetration of P-gp substrates are significantly lower in normal mice compared with mdr1a (-/-) mice (Schinkel, 1998), and oral bioavailability and brain penetration of P-gp substrates can be significantly enhanced by simultaneous administration of P-gp modulators (Mayer *et al*., 1997; Fromm *et al*., 1999). Clearance of a P-gp substrate due to biliary excretion and renal secretion can be also significantly decreased in the presence of a P-gp inhibitor (Song *et al*., 1999; Kiso *et al*., 2000). The objective of this investigation was to determine the inhibitory effects of some naturally occurring Flavonoids, on P-gp- insilico.

MATERIALS AND METHODS

3D model building

The initial model of P-glycoprotein (P08183) was built by using homology-modeling methods and the MODELLER software; a program for comparative protein structure modeling optimally satisfying spatial restraints derived from the alignment and expressed as probability density functions (pdfs) for the features restrained. The pdfs restrain C^{α}- C^{α} distances, main-chain N-O distances, main-chain and side-chain dihedral angles. The 3D model of a protein is obtained by optimization of the molecular pdf such that the model violates the input restraints as little as possible. The molecular pdf is derived as a combination of pdfs restraining individual spatial features of the whole molecule. The optimization procedure is a variable target function method that applies the conjugate gradients algorithm to positions of all non-hydrogen atoms (Sali, *et al*, 1991). The query sequence from Homo sapiens was submitted to SBASE server for domain prediction. The predicted domain (1035-1273) was searched to find out the related protein structure to be used as a template by the BLAST (Basic Local Alignment Search Tool) (Altschul, *et al*, 1990;1997) program against PDB (Protein Databank). Sequence that showed maximum identity with high score and less e-value was aligned and used as a reference structure to build a 3D model for P-glycoprotein. The sequence of P-glycoprotein (Accession Number: P08183) was obtained from NCBI. The co-ordinates for the structurally conserved regions (SCRs) for P-glycoprotein were assigned from the template using multiple sequence alignment, based on the Needleman-Wunsch algorithm (Needleman and wunch, 1970).

The structure having the least modeller objective function, obtained from the modeller was improved by molecular dynamics and equilibration methods using NAMD 2.5 software (Kale, *et al*, 1999) using CHARMM27 force field for lipids and proteins (Schlenkrich, *et al*, 1999) along with the TIP3P model for water (Jorgensen, *et al*, 1983). The energy of the structure was minimized with 10,000 steps. A cutoff of 12 Å (switching function starting at 10 Å) for van der Waals interactions was assumed. No periodic boundary conditions were included in this study. An integration time step of 2 fs was used, permitting a multiple time-stepping algorithm (Grubmuller, *et al*, 1991) to be employed in which interactions involving covalent bonds were computed every time step and short-range non bonded interactions were computed every two time steps, and long-range electrostatic forces were computed every four time steps. The pair list of the non bonded interaction was recalculated every ten time steps with a pair list distance of 13.5 A°. The short-range non bonded interactions were defined as van der Waals and electrostatic interactions between particles within 12 A°. A smoothing function was employed for the van der Waals interactions at a distance of 10 A°. CHARMM27 force-field parameters were used in all simulations in this study. The equilibrated system was simulated for 1 ps with a 500 kcal/mol/Å2 restraint on the protein backbone under 1 atm constant pressure and 310 K constant temperature (NPT) and the Langevin damping coefficient was set to 5 ps unless otherwise stated (Mackkerelle, *et al*, 1992). Finally, the structure having the least energy with low RMSD (Root Mean Square Deviation) was used for further studies. In this step, the quality of the initial model was improved. The final structure obtained was analyzed by Ramachandran's map using PROCHECK (Programs to check the Stereo chemical Quality of Protein Structures) (Brunger, 1992) and environment profile using ERRAT graph (Structure Evaluation server) (Laskoswki, *et al*, 1993). This model was used for the identification of active site and for docking of the substrate with the protein.

Active site Identification

Active site of P-glycoprotein (P08183) was identified using CASTP server (Carpena, et *al*, 2003). A new program, CAST, for automatically locating and measuring protein pockets and cavities, is based on precise computational geometry methods, including alpha shape and discrete flow theory. CAST identifies and measures pockets and pocket mouth openings, as well as cavities. The program specifies the atoms lining pockets, pocket openings, and buried cavities; the volume and area of pockets and cavities; and the area and circumference of mouth openings.

Docking method

In order to elucidate possible interactions of the protein and Flavonoids performed docking studies using FRED (OpenEye Scientific Software, Santa Fe, NM). The relevant stereo isomers of the compounds were minimized with the MMFF force field in the Openeye package. Conformation and minimization of the compounds was performed using Omega (OpenEye Scientific Software, Santa Fe, NM). Fred requires a set of input conformers for each ligand. The conformers were generated by Omega and stored in a single binary file. After this the output file is used for docking.

Docking calculations were performed with FRED version 1.1 for efficient handling of large compound databases. The first stage in docking is a shape fitting process. The crude docking solutions are further tested against a pharmacophore. Various options are available for optimization with respect to the built-in scoring functions: optimization of hydroxyl group rotamers, rigid body optimization, torsion optimization, and reduction of the number of poses that are passed on to the next scoring function. Available scoring functions in FRED are Chemgauses, Chemscore, PLP, Screenscore and Shapegauses.

RESULTS

Homology Modelling of P-glycoprotein Domain

A high level of sequence identity should guarantee more accurate alignment between the target sequence and template structure. In the results of BLAST search against PDB, only one-reference protein, including 2HYD (Chain A; Structure of a bacterial multidrug ABC transporter) with P-gp have a high level of sequence identity and the identity of the reference protein with the P-glycoprotein domain is 50%. Structurally conserved regions (SCRs) for the model and the template were determined by superimposition of the two structures and multiple sequence alignment (fig. 1). In the following study, we have chosen 2HYD as a reference structure for modeling P-glycoprotein (P08183). In our study, the model is made up of residues 1035-1273 because the active domain region was identified in between these residues by the SBASE server. Coordinates from the reference protein (2HYD) to the SCRs, structurally variable regions (SVRs), N-termini and C-termini were assigned to the target sequence based on the satisfaction of spatial restraints. All side chains of the model protein were set by rotamers. The initial model was thus generated with the above procedure. The options used for running the modeller are shown in Tab. 1. This model was refined by molecular dynamics method and the final stable structure of the P-glycoprotein obtained is shown in fig 2.

From fig. 2 it is evident that this protein has 9 helices and 11 sheets. The final structure was further checked by VERIFY-3D graph and the results are shown in fig 3. The compatibility score above zero in the VERIFY-3D program showed except four, all residues are reasonable which makes us to believe that the structure of the P-glycoprotein is reliable.

Tab. 1: Options used in MODELLER program

INCLUDE	#include the predefined TOP routines
SET ALNFILE	= ‘alignment.ali’
SET KNOWNS	= ‘1SHU’
SET SEQUENCE	= ‘query’
SET HETATM_IO	= on
SET WATR_IO	= off
SET HYDROGEN	= off
SET STARTING_MODEL	= 1
SET ENDING_MODEL	= 20
CALL ROUTINE	= ‘model’

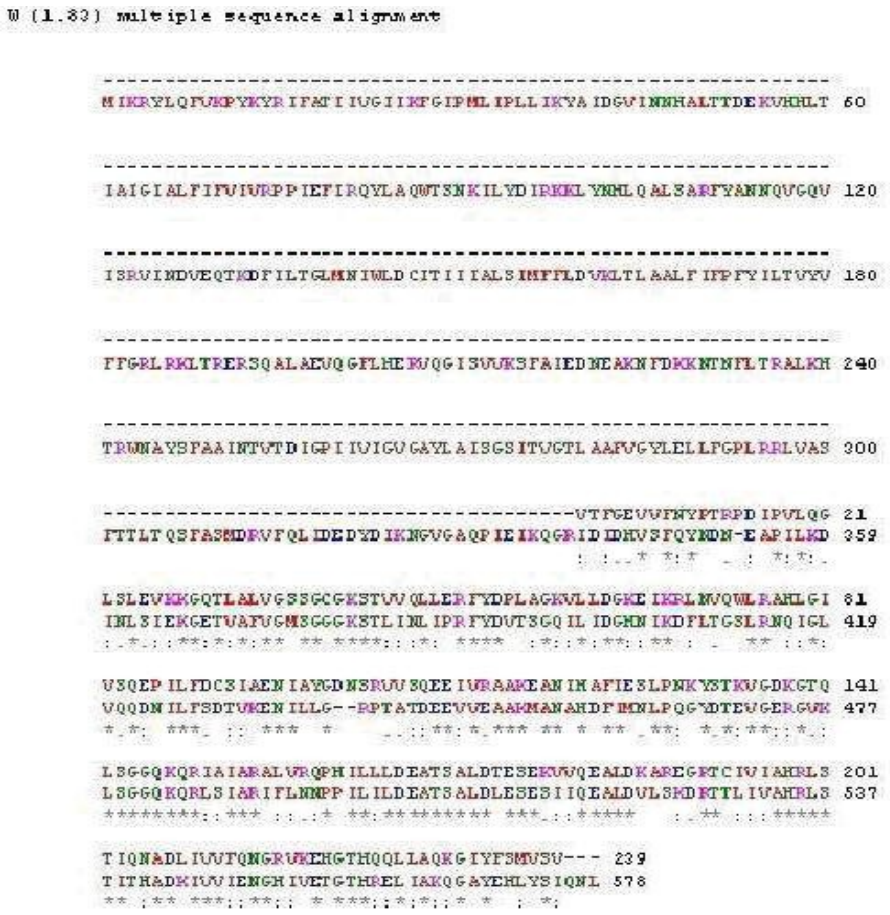

Fig I: sequence alignment of P-glycoprotein (P-gp) from human with Structure of a bacterial multidrug ABC transporter (PDB code 2hyd) done using clustalw server that was subsequently submitted to MODELLER. The conserved regions are indicated by '*'.

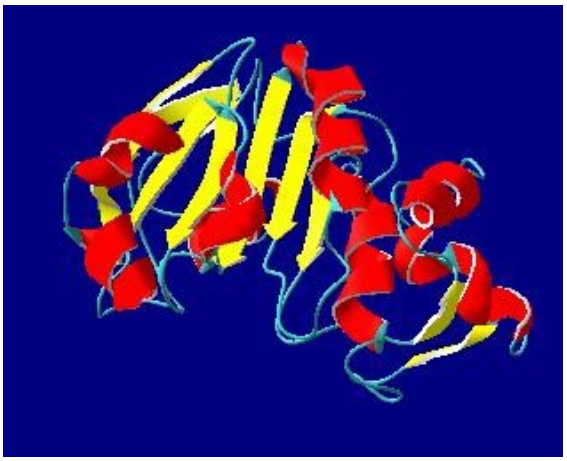

Fig II: The final 3D structure of P-glycoprotein. The structure is obtained by energy minimizing the average conformation over the last 1000 femto seconds of molecular dynamics simulation. The α-helix is represented by red cylinders and β-sheet by yellow arrows.

Validation of P-glycoprotein Domain

After the refinement process, validation of the model was carried out using Ramachandran plot calculations computed with the PROCHECK program. The φ and ψ distributions of the Ramachandran plots of non-glycine, non-proline residues are summarized in fig. 4 and Tab. 2. Altogether 99.5% of the residues of P-glycoprotein (P08183) was in favored and allowed regions. The overall PROCHECK G-factor of P-glycoprotein (P08183) was – 2.32 and VERIFY 3D environment profile was good.

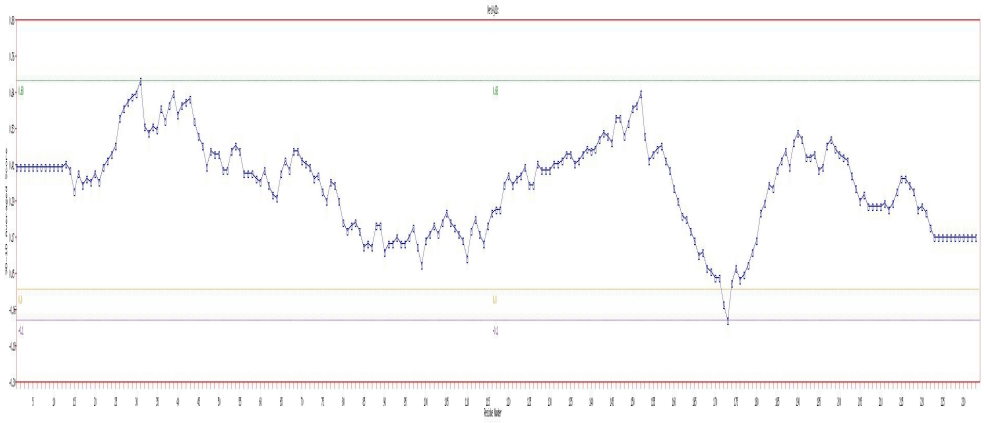

Fig III: The 3D profiles verified results of P-glycoprotein model, residues with positive compatibility score are reasonably folded.

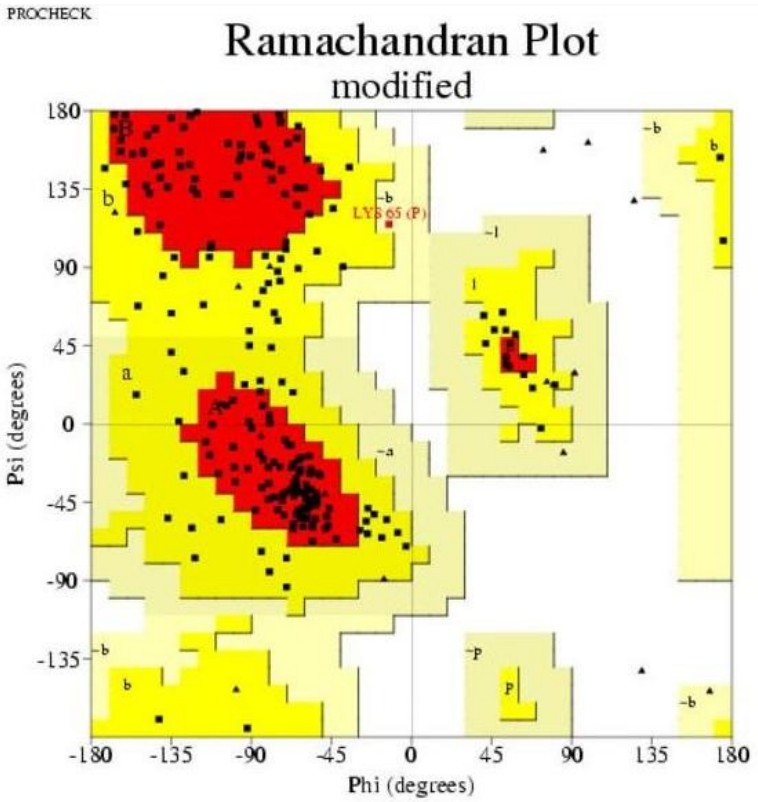

Fig IV: Ramachandran's map of P-glycoprotein built using MODELLER software. The plot calculations on the 3D model of P-glycoprotein were computed with the PROCHECK program.

Tab. 2: % of residue falling in the core region of the Ramachandran's plot

% of residue in most favored regions	68.9
% of residue in the additionally allowed zones	30.7
% of residue in the generously regions	0.5
% of residue in disallowed regions	0.0
% of non-glycine and non-proline residues	100.0

Superimposition of 2HYD with P-glycoprotein domain

The structural superimposition of C^{α} trace of template and P-glycoprotein is shown in fig. 5. The weighted root mean square deviation of C^{α} trace between the template and final refined models was 1.62 A^{o}. This final refined model was used for the identification of active site and for docking of the substrate with the protein.

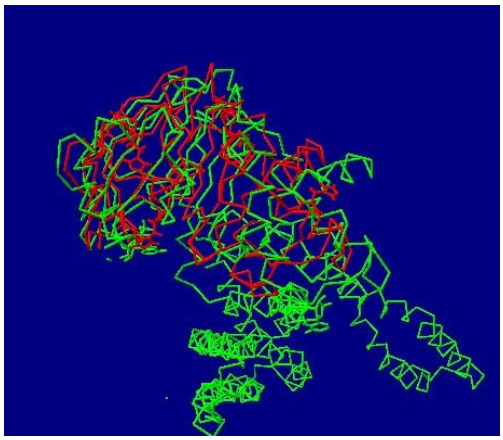

Fig V: Superimposition of C^{α} trace of P-glycoprotein (represented in red color) and 2HYD (represented in green color).

Active site Identification of P-glycoprotein domain

After the final model was built, the possible binding sites of P-glycoprotein was searched based on the structural comparison of template and the model build with CASTP server and was shown in fig. 6. Since, P-glycoprotein and the 2HYD are well conserved in both sequence and structure; their biological function should be identical. Infact from the structure-structure comparison of template, final refined model of P-glycoprotein domain using SPDBV program and was shown in fig. 1. It was found that the residues, GLN- 47, TYR -53, SER -83, ILE- 87 are conserved with the active site of template and was shown in fig. 6. By considering the experimental fact that the active site of 2HYD includes some of the residues as mentioned above. Thus in this study SER -83 and ILE- 87 are chosen as the more favorable sites to dock the substrate, and the other residues are not discussed further. The final stable structure of P-glycoprotein obtained was shown in fig. 2.

Tab. 3: Properties of Flavonoids used for docking studies with P-glycoprotein

Flavonoids	Molecular Formula	Formula Weight	Molar Refractivity cm3	Index of Refraction	Density g/cm3	Polarizability cm3
Daidzein	C15H10O4	254.2375	67.97 ± 0.3	1.698 ± 0.02	1.443 ± 0.06	26.94 ± 0.5 10-24
Genistein	C15H10O5	270.2369	69.85 ± 0.3	1.732 ± 0.02	1.548 ± 0.06	27.69 ± 0.5 10-24
Glycitein	C16H12O5	284.26348	74.64 ± 0.3	1.668 ± 0.02	1.420 ± 0.06	29.59 ± 0.5 10-24
Daidzin	C21H22O9	418.39398	102.19 ± 0.3	1.670 ± 0.02	1.529 ± 0.06	40.51 ± 0.5 10-24
Genistin	C21H20O10	432.3775	103.67 ± 0.3	1.642 ± 0.06	1.642 ± 0.06	41.10 ± 0.5 10-24
Glycitin	C22H22O10	446.40408	108.47 ± 0.3	1.674 ± 0.02	1.545 ± 0.06	43.00 ± 0.5 10-24
Acetyldaidzin	C22H20O9	428.3888	104.77 ± 0.3	1.679 ± 0.02	1.543 ± 0.06	41.53 ± 0.5 10-24
Acetlygenistin	C22H20O10	444.3882	106.65 ± 0.3	1.700 ± 0.02	1.610 ± 0.06	42.28 ± 0.5 10-24
Acetylglycitin	C23H22O10	458.41478	111.45 ± 0.3	1.661 ± 0.02	1.520 ± 0.06	44.18 ± 0.5 10-24
Malonyldaidzin	C23H20O11	472.3983	110.96 ± 0.3	1.695 ± 0.02	1.636 ± 0.06	43.99 ± 0.5 10-24
Malonylgenistin	C23H20O12	488.3977	112.85 ± 0.3	1.715 ± 0.02	1.701 ± 0.06	44.73 ± 0.5 10-24
Malonylglycitin	C24H22O12	502.42428	117.64 ± 0.3	1.676 ± 0.02	1.607 ± 0.06	46.63 ± 0.5 10-24
Biochanin A	C16H12O5	284.26348	74.64 ± 0.3	1.668 ± 0.02	1.420 ± 0.06	29.59 ± 0.5 10-24

Docking of inhibitors with the active site of P08183

A series of 13 Flavonoids were designed by using chem sketch and the properties are identified and shown in Tab. 3. Docking of the Flavonoids with P-glycoprotein was performed using FRED v 2.1, which is based on Rigid Body Shape-Fitting (Open Eye Scientific Software, Santa Fe, NM).

This program generates an ensemble of different rigid body orientations (poses) for each compound conformer within the binding pocket and then passes each molecule against a negative image of the binding site. Poses clashing with this 'bump map' are eliminated. Poses surviving the bump test are then scored and ranked with a Gaussian shape function. We defined the binding pocket using the ligand-free protein structure and a box enclosing the binding site. This box was defined by extending the size of a cocrystalized ligand by 4 Å (addbox parameter of FRED). This dimension was considered here appropriate to allow, for instance, compounds larger than the cocrystallized ones to fit into the binding site. One unique pose for each of the best-scored compounds was saved for the subsequent steps. The compounds which showed best docking was converted in 3D with OMEGA (same protocol as above) (Open Eye Scientific Software, Santa Fe, NM). To this set, the substrate (generation of multiconformer with Omega) corresponding to the modeled protein were added. The docking scores of the best docked Flavonoids were shown in the Tab.4. The active site of the protein along with the best docked Flavonoid (Acetylgenistin), shown in the fig. 7 and the binding energy values are highlighted in the Tab. 4 (red colour).

Table 4: The total docking energies of Flavonoids.

Flavonoids	chemgausses	chemscore	plp	screenscore	shapegausses	total
Daidzein	-51.18	-16.97	-34.03	-106.76	-308.74	-517.68
Genistein	-53.32	-17.15	-36.13	-119.67	-317.53	-543.8
Glycitein	-53.39	-21.46	-39.21	-116.16	-316.44	-546.66
Daidzin	-54.99	-18.7	-35.82	-116.08	-390.43	-616.02
Genistin	-63.93	-18.81	-32.45	-110.9	-442.99	-669.08
Glycitin	-66.9	-23.32	-27.91	-122.15	-428.75	-669.03
Acetyldaidzin	-65.96	-22.99	-49.3	-153.5	-441.55	-733.3
Acetlygenistin	-63.18	-20.78	-51.56	-164.16	-448.77	-748.45
Acetylglycitin	-56.63	-17.02	-45.49	-141.52	-430.99	-691.65
Malonyldaidzin	-71.81	-13.77	-30.36	-121.63	-465.05	-702.62
Malonylgenistin	-74.97	-15.61	-26.31	-121.44	-442.52	-680.85
Malonylglycitin	-79.11	-1.29	-14.74	-102.32	-506.18	-703.64
Biochanin A	-56.2	-10.51	-37.3	-118.21	-324.11	-546.33

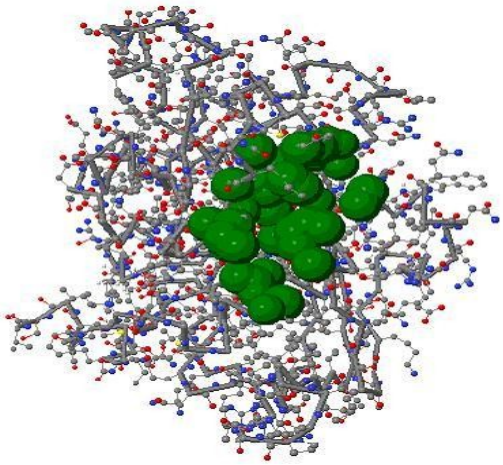

Fig. VI: Possible binding site of P-glycoprotein from Homo sapiens

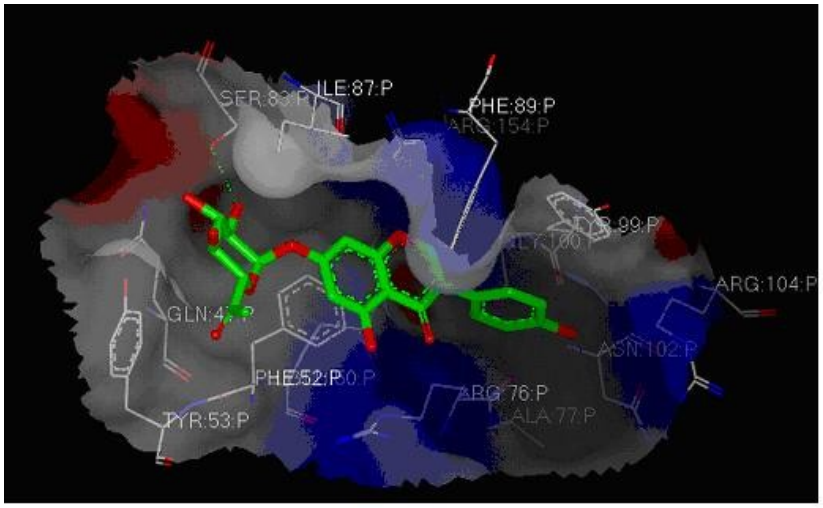

Fig. VII: Binding of the Acetylgenistin, showing best docking energy with the active site of P-glycoprotein.

DISCUSSION

P-glycoprotein (P-gp) is a plasma membrane protein encoded by the human MDR1 and MDR3 genes. P-gp belongs to the ATP-binding cassette transporter superfamily and is responsible for the efflux of a broad array of hydrophobic compounds, including a number of important Cancer chemotherapeutic agents such as vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxol. In this work, we have constructed a 3D model of P-glycoprotein domain, from Human using the MODELLER software and obtained a refined model after energy minimization.

The final refined model was further assessed by ERRAT and PROCHECK program, and the results show that this model is reliable. The stable structure is further used for docking of substrate with the derivatives of Flavonoids. Docking results indicate that conserved amino-acid residues in P-glycoprotein play an important role in maintaining a functional conformation and are directly involved in donor substrate binding. The interaction between the domain and the inhibitors proposed in this study are useful for understanding the potential mechanism of domain and the inhibitor binding. As is well known, hydrogen bonds play important role for the structure and function of biological molecules. In this study it was found that GLN- 47, TYR -53, SER -83, ILE- 87, GLY -100, ARG -154 of P08183 are important for strong hydrogen bonding interaction with the inhibitors. To the best of our knowledge SER -83 and ILE- 87 are conserved in this domain and may be important for structural integrity or maintaining the hydrophobicity of the inhibitor-binding pocket. Among the 13 Flavonoids, Acetylgenistin showed best docking result with P-glycoprotein.

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