



Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

Phytochemical Screening and In-Silico Investigation of Crocin and Safranal, Constituents of Saffron for Their Cytochrome P450 2C9 Enzyme Activity

Majid Ahmad Ganaie*¹, Abdul Samad², Mohd Nazam Ansari¹, Tajdar Husain Khan¹, Pravej Alam³ and Syed Rizwan Ahamad⁴

¹Department of Pharmacology, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Alkharj, Saudi Arabia

²Department of Pharmaceutical Chemistry, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Alkharj, Saudi Arabia

³Department of Biology, College of Science and Humanities, Prince Sattam Bin Abdulaziz University, Alkharj, Saudi Arabia

⁴Central Laboratory, Research Center, College of pharmacy, King Saud University, Riyadh, Saudi Arabia

****Corresponding author:***

Majid Ahmad Ganaie

Email: majidsays@gmail.com

ABSTRACT

*Dietary phytochemicals are important contributors to various diseases prevention, due to their interactions with CYP family enzymes. Safranal and crocin are bioactive compounds present in *Crocus sativus L.*, commonly known as saffron. In present study, we prepared saffron extract and performed its HPLC phytochemical analysis for safranal and crocin content. We have also determined the effect of crocin and safranal on the metabolic activity of CYP2C9 by using in-silico approaches such as 3D-QSAR molecular docking and pharmacophore mapping studies. The extraction method was simple and the content of crocin and safranal was obtained to be 26.45 ± 0.03 and 11.0 ± 0.02 mg/g of saffron respectively. The predictivity of the best 3D-QSAR model developed for flavonoid derivatives was found to be 91.3%. The predicted activities of crocin and safranal for CYP2C9 were found to be 7.805 and 7.120 respectively. Further, docking studies revealed that crocin bonded CYP2C9 protein with binding affinity -9 kcal/mol, whereas, co-crystallized standard flurbiprofen bonded with 8.2 kcal/mol only. The results obtained in the present study furnish primary data for future in vitro and in vivo herb-drug interaction studies involving CYP2C9 enzyme.*

Keywords: *Crocus sativus L.; crocin; safranal; HPLC; CYP2C9.*

INTRODUCTION

The money investment has increased gradually in drug research and development, but the number of new drugs accepted per year by the FDA has remained almost same for the past decade [1][2]. Tremendous advances in genomics, proteomics, structural biology and computational chemistry have generated new tools to identify targets and the compounds that interact with them [3][4]. Nonetheless, a large number of lead drug candidate compounds are trace compounds, which has led to a bottleneck of target-based screening using cells, proteins or enzymes [5][6][7]. In addition, natural products include a vast library of bioactive compounds, and the screening and identification of bioactive compounds in microorganism fermentation liquids are the foundation of the fermentation industry [8]. The complexity and variability of microorganism secondary metabolites present a considerable

challenge to the analysis, separation and identification of their bioactive ingredients. Under these conditions, high-performance liquid chromatography (HPLC) provides a major boost to the separation and identification of mixtures. However, a large amount of a trace compound that is difficult to prepare from the complex matrix cannot be screened and has typically been ignored. In the last decade, structural biology based tools that include X-ray crystallography, computational modeling and virtual screening have been used to aid in the identification of bioactive compounds for some diseases whose targets have been discovered [9]. The convergence of structural information and computational virtual evaluation techniques provides a more sensitive and convenient method for the discovery and development of new medicines [10].

Saffron, the dry stigmas of the plant *Crocus sativus* L., is currently used as a spice and food colorant. Saffron is used in folk medicine for various purposes such as an antispasmodic, nerve sedative, expectorant, eupptic, carminative, stomachic, aphrodisiac and emmenagogue [11].

Chemical analysis of saffron extracts has revealed about 150 different compounds from which the most studied are the carotenoids crocin and crocetin, a bitter glycoside called picrocrocin (monoterpene aldehydes) and the volatile, aromatic substance safranal [12][13][14]. Crocin as a water-soluble carotenoid gives saffron its typical reddish or yellowish color. Safranal is a monoterpene aldehyde responsible for saffron's characteristic odour, and the bitter taste of saffron is attributed to picrocrocin, which is also a precursor of safranal [15][16]. Pharmacological studies have demonstrated antiepileptic, anti-oxidative, anti-inflammatory, neuroprotective, memory improvement and anti-diabetic effects for crocin and safranal [17][18][19][20].

It was described that the metabolic activity of cytochrome P450, which is one of the most important enzymatic systems for xenobiotic biotransformation, is influenced by a large number of natural substances, including carotenoids. The modulation of CYP metabolic activity could lead to clinically relevant changes in plasma concentrations of concurrently administered drugs, and thus also to changes in their pharmacological properties and drug interactions. The ability of systemic administration of safranal and crocin to increase the total protein and the total CYP content in rat liver microsomes (RLM) and to change the metabolic activity of different CYP enzymes has already been reported [21]. The aim of the present study was to perform HPLC phytochemical analysis of saffron for safranal and crocin and to determine the their effect on the metabolic activity of CYP2C9 by *in silico* approaches

MATERIALS AND METHODS

Extraction of crocin and safranal

Saffron stigmata (25 mg) used for extraction of these metabolites were suspended in 10 ml -methanol–water solution (50:50, v/v) and magnetically stirred for 24 hr at room temperature in the dark. The samples were then filtered through filter membrane (0.25 µm pore size; Milipore, USA) and stored at 4°C for further use.

HPLC method validation and quantitative determination

The method used to evaluate the content of crocin and safranal extracted from *C. sativus* stigma was as reported [22]. The crocin and safranal quantitative analysis was studied on the basis of molecular coefficient absorbance of each peak found at the maximum absorbance (wavelength) of the crocin and safranal as previously reported. They are expressed in milligrams per gram of saffron stigmata on dry weight basis.

HPLC analysis was performed in a multi-solvent Agilent1260-Infinity Quaternary LC system consist quaternary pump (G131B) with autosampler (G1367E) and thermostat coupled with Diode Array detector (DAD, noise levels of $\leq \pm 0.6 \mu\text{AU}/\text{cm}$ the revolutionary 6 cm flow cell gives up to 10 times higher sensitivity than other instruments) assembled with computer system (*Dell Computer system*). Agilent Open LAB ChemStation version C.01.05 (Agilent, USA) was used for data acquirement and processing of the chromatogram.

A RP-C18 column (Agilent eclipse Plus, 4.6mm X 100mm, with a pore diameter of 95 Å, 1.8 µm particle sizes) was used for all analyses. A linear gradient of methanol (50%) in water and acetonitrile (90%) was used as a mobile phase with a flow-rate of 1.5 ml/min for a maximum elution time of 5 min at room temperature. The sample volume of 25 µl was injected to the HPLC for the test run. Solvents were pre-filtered before use by a Millipore filtration unit (USA). The analyses were carried out in triplicate for each sample. Concentrations of crocin and safranal are expressed in milligrams per gram (mg/g) of saffron stigmata.

In Silico studies

Data set

A series of flavonoid derivatives reported to be CYP2C9 inhibitors were taken from the literature as shown in Table 1 [23]. The compounds were subjected for the development of 3D-QSAR models for prediction of CYP2C9 affinity. The biological activity values [pIC50 (nM)] were used as the dependent variable. Selected representative constituents of saffron were further subjected to docking studies in the active domain of CYP2C9.

All the compounds were built on 2D drawing workspace of molecular modeling software VLife MDS 3.5 (VLife Sciences Technologies Pvt. Ltd. Pune, India). The software was installed on HP workstation having i5 processor and windows 8.1 as operating system. All the molecules were batch optimized for minimization of energies using Monte Carlo conformational search with 10000 cycles [24] and Merck Molecular Force Field (MMFF) fields. All the molecules of the series were aligned (Fig. 1A) using the template based alignment method by choosing the most effective one as the 'Reference Molecule' (Fig. 1B) and a minimum common structure as the 'Template' (Fig. 1C). The goal was to obtain optimal alignment between the molecular structures necessary for alignment of compounds [25].

3D-QSAR Methodology

For 3D-QSAR studies the aligned molecules of the series were exported to 3D-QSAR module worksheet. The activity of the molecules was fed in their respective columns. This was followed by the field computation of various electrostatic and steric descriptors. The optimal training and test sets were generated using the sphere exclusion algorithm. A training set of 11 molecules, test set of 3 molecules and external validation set of 2 molecules were generated. This data was then applied to one of the modest statistical treatment method i.e. kNN-MFA (k-Nearest Neighbor Molecular Field Analysis). In this methodology the CYP2C9 inhibitory activity of the compounds was taken as dependent variable and rest of the columns were considered as independent variables.

Feature selection and model development

An integral aspect of any model-building exercise is the selection of an appropriate set of features with low complexity and good predictive accuracy. This process forms the basis of a technique known as feature selection or variable selection [26].

'Simulated annealing' process was chosen as a variable selection method for the suitable model along with cross correlation limit 0.5, variances cut off zero and 'mean centering' as a scaling method for the development of this model. Further, for k-Nearest Neighbor parameters, maximum and minimum number of neighbors was set 9 & 2 respectively, along with 'kNN classification' as the prediction scheme.

Pharmacophore mapping studies

Generating a pharmacophore is usually the first step for understanding the interaction between a receptor and a ligand. Over the years, pharmacophores have been successfully used in lead generation, scaffold hopping, mining small molecule databases, etc. [27] [28].

Pharmacophore modeling was carried out to develop a hypothetical pharmacophore model for the CYP2C9 inhibition aiming to study the fitting of the series of molecules under study. Pharmacophore mapping was carried out through the Molsign package of VLife MDS 3.5 software. The pharmacophore model was developed by

choosing training set of most active Flavonoid derivatives, including compounds acacinin, amentoflavone, apigenin, chrysin etc.

Docking Studies

We have carried out Molecular Docking studies for crocin and safranal into the active domain of CYP2C9. Crystal structures of CYP2C9 having Flurbiprofen (FLP) bound (PDB ID: 1R9O) [29] with resolution 2.0 Å was downloaded from RCSB Protein Data Bank to serve as the docking template. Docking studies were carried out on AutoDock 4.2 [30] [31], running on Linux Ubuntu 10.0, installed on Pentium i3 workstation. Discovery studio 4 [32] was used for visualization purposes of docked conformations.

For each ligand, the partial atomic charges were calculated using Gasteiger-Marsili method [33] and after merging non-polar hydrogens, rotatable bonds were assigned. The grid maps were calculated using AutoGrid [34]. In all dockings, a grid map with 60 x 60 x 60 points, a grid spacing of 0.503 Å were used, and the maps were centered on the ligand binding site.

Crocin and safranal were modeled by positioning them in the FLP (PDB ID: 1R9O) binding site in accordance with the published crystal structure of FLP bound in the domain of CYP2C9. From the comparative docking study of our compounds with standard binding compound (FLP) we could observe how our compounds might bind to the polymer inhibition site, based on the knowledge of the structure of similar active sites. We redocked FLP into the active site of the protein and then we docked with our compounds in order to compare the binding affinity of both ligand and the test compounds.

RESULT AND DISCUSSIONS

Analysis of a crude plant extract

HPLC is the most commonly used separation technique in analytical science [35]. The extraction method by soaking plant material in the solvent was selected due to its simplicity and easy manageability and the content of crocin and safranal was obtained to be 26.45 ± 0.03 and 11.0 ± 0.02 respectively (Table 2).

In-Silico studies

In silico screening for the set of flavonoid Inhibitors was carried out by 3 approaches, viz. molecular docking, 3D-QSAR studies and pharmacophore mapping studies.

3D-QSAR

Training and test set were selected via sphere exclusion method. Training and test set distribution was validated by unicom statistics as shown in table-3. Additionally, the fitness plot also confirms the optimum distribution of the training and test set (Fig. 2).

Sixty 3D-QSAR models were developed in search of suitable predictive model. Various statistical methods were applied for this purpose such as, Multiple Linear Regression (MLR), Principle component regression (PCR), k-Nearest Neighbor Molecular Field Analysis (kNN-MFA). The best model mentioned here in Table 4 was obtained by kNN-MFA Method in conjunction with simulated annealing as variable selection mode (Fig. 3).

The best chosen model amongst the sixty different models has q^2 value = 0.6739 and pred_r^2 value = 0.913 along with eight descriptors, that has indicated the internal predictive power of the model 67.39% and external prediction 91.3%. The model encompasses all the three types of descriptors, i.e. steric (S), electrostatic (E) and hydrophobic (H) descriptors specifying the regions, where variation in the structural features of different compounds in the training set leads to increase or decrease in activities. The numbers accompanied by the descriptors represent its position in the 3D-MFA grid.

This model was used for predicting the activities of crocin and safranal. Similarly, this model can further be used for CYP2C9 activity of other synthetic or semisynthetic flavonoid derivatives. The predictivity of the model shows prediction very close to observed activity (Table 1). Additionally, Floral diagram (Fig. 4) also depicts the accuracy of prediction by the kNN-MFA model for the training and test data set.

When crocin and safranal were subjected for activity prediction by this model, the values were 7.805 and 7.120 respectively.

Pharmacophore Mapping

The generated hypothetical Pharmacophore (Fig. 5) showed three overlapping points with similar chemical properties in the training set. The mapping was based on two aromatic carbons and one H-bond acceptor. The larger tessellated spheres were indicative of the common pharmacophoric features identified in the molecules, the smaller solid features were for the individual molecules. The pharmacophoric features shown by the tessellated spheres were indicative of the necessary groups needed for the optimum activity. Furthermore, it added the support for structural activity relationship by giving the evidence that both the aromatic rings were necessary for activity along with one carbonyl group joining both the phenyl ring. Moreover, the model depicted that the methoxy groups attached to the phenyl rings at the other end was also a necessary part of pharmacophore. The coloring scheme for the various large tessellated spheres was as: Hydrogen bond donor: Magenta color; Hydrogen bond acceptor: Buff color; Hydrophobic: Orange color; Aliphatic: Orange color; Negative ionizable: Green color; Positive ionizable: Violet color.

Docking studies

The docking studies provided us insight for structural relation of CYP2C9 with flavonoid derivatives. Docking method was validated by redocking the co-crystallized ligand with the CYP2C9 protein and the interactions obtained were considered as the standard to compare with the docking of crocin and safranal. The docked structure exactly overlaps the co-crystallized one, thereby, the methodology adopted for docking is confirmed (Fig. 6).

Crocin has exhibited wonderful binding affinity (-9) with CYP2C9 even better than the standard co-crystallized compound FLP (8.2) whereas safranal has exhibited weaker binding affinity (-5.8). The docked structure of crocin in the active domain of CYP2C9 protein is shown in Fig. 7.

CONCLUSION

The obtained results from *in silico* screening models showed that safranal and crocin has significant potential to influence the metabolic activity of CYP2C9 enzyme. It would be imperative to consider the risk of possible interactions with drugs metabolized by the CYP2C9 enzyme. These findings furnish primary data for future *in vitro* and *in vivo* herb-drug interaction studies of safranal and crocin. However, it is worthwhile to note that influence on CYP2C9 enzyme activity *in silico* does not necessarily imply drug interactions *in vitro* and *in vivo*. Further detailed studies will be needed to establish comprehensively, if safranal and crocin can influence the CYP2C9 enzyme activity *in vitro* and *in vivo*.

ACKNOWLEDGEMENTS

This project was supported by Deanship of Scientific Research at Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia under the Research Project No.2015/03/4215).

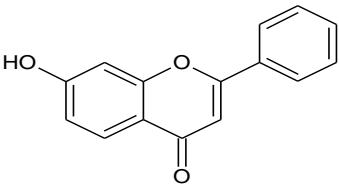
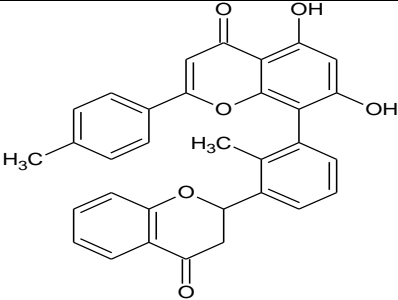
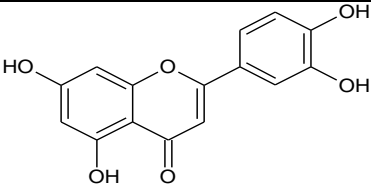
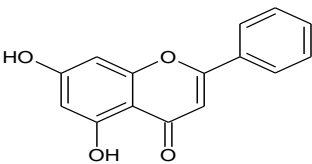
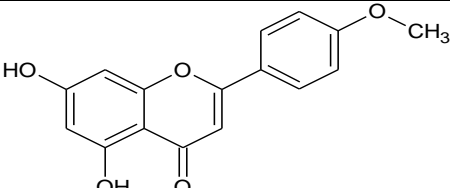
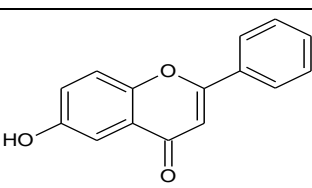
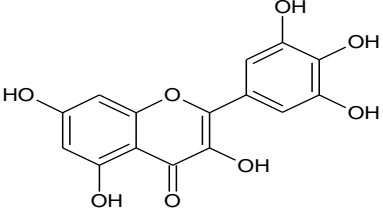
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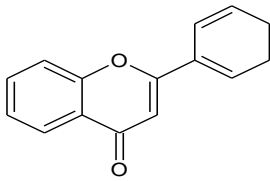
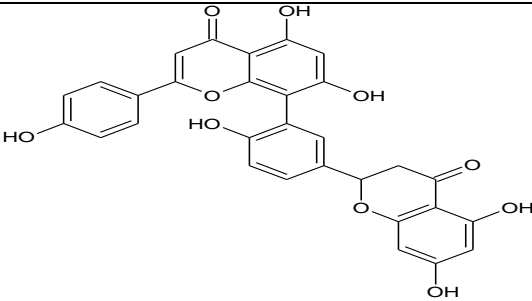
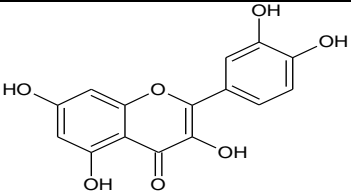
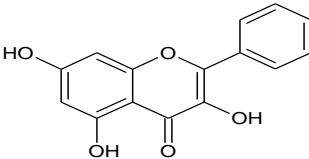
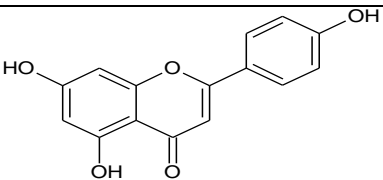
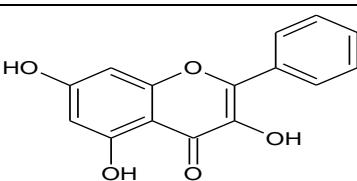
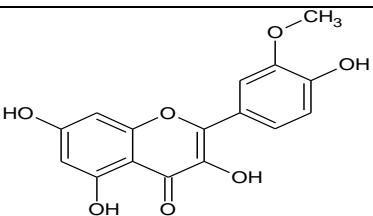
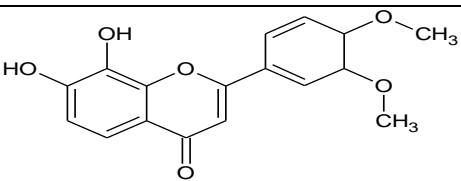
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Table 1 Structure of the compounds under consideration for 3D QSAR model with observed and predicted activities.

S. No.	Molecules	p IC50	Predicted activity	Residual
1.		9.523	8.857	0.666
2.		9.056	8.95	0.106
3.		8.041	8.282	-0.241
4.		8.187	8.5175	-0.3305
5.		8.377	8.4225	-0.0455
6.		8.658	8.7165	-0.0585
7.		7.493	7.854	-0.361

8.		7.854	7.809	0.045
9.		10.523	9.2895	1.2335
10.		7.583	7.809	-0.226
11.		8.125	7.538	0.587
12.		8.194	8.11444	0.07956
13.		9.699	9.28971	0.40929
14.		7.801	7.84789	-0.04689
15.		7.602	8.11036	-0.50836

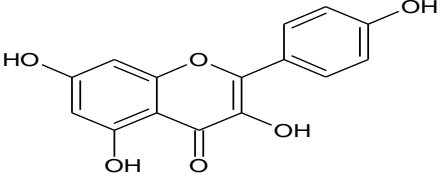
16.		7.9	7.81491	0.08509
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Table 2 Quantitative analyses of crocin and safranal present in samples in stigma of *Crocus sativus* L. analyzed by HPLC (Each value is the mean \pm standard error (n = 3))

	Crocin content mg/g	Safranal content mg/g
Saffron sample	26.45 \pm 0.03	11.0 \pm 0.02

Table 3 Uni-Column Statistics for the training and test set

Column Name	Average	Max	Min	Std Dev	Sum
Training set Columns	8.4927	10.5229	7.4935	0.9059	93.4193
Test set Columns	8.5647	9.6990	7.8013	1.0017	25.6941

Table 4 Model Summary of statistically significant 3D QSAR model

Descriptors used in Model	Range of descriptors	Statistics of Model	
E_153	E_153 -0.0040 -0.0010	k Nearest Neighbour	2
S_676	S_676 -0.1640 -0.1500	N	11
S_883	S_883 30.0000 30.0000	Degree_of_freedom	2
H_324	H_324 -0.0350 -0.0220	q2	0.6739
H_1191	H_1191 0.2970 0.3400	q2_se	0.5174
E_719	E_719 0.1650 0.1770	pred_r2	0.9130
H_675	H_675 0.1280 0.1410	pred_r2se	0.2967
E_1142	E_1142 -0.0160 -0.0100	ExternalValidation_r2	0.7679
		ExternalValidation_r2se	0.5154

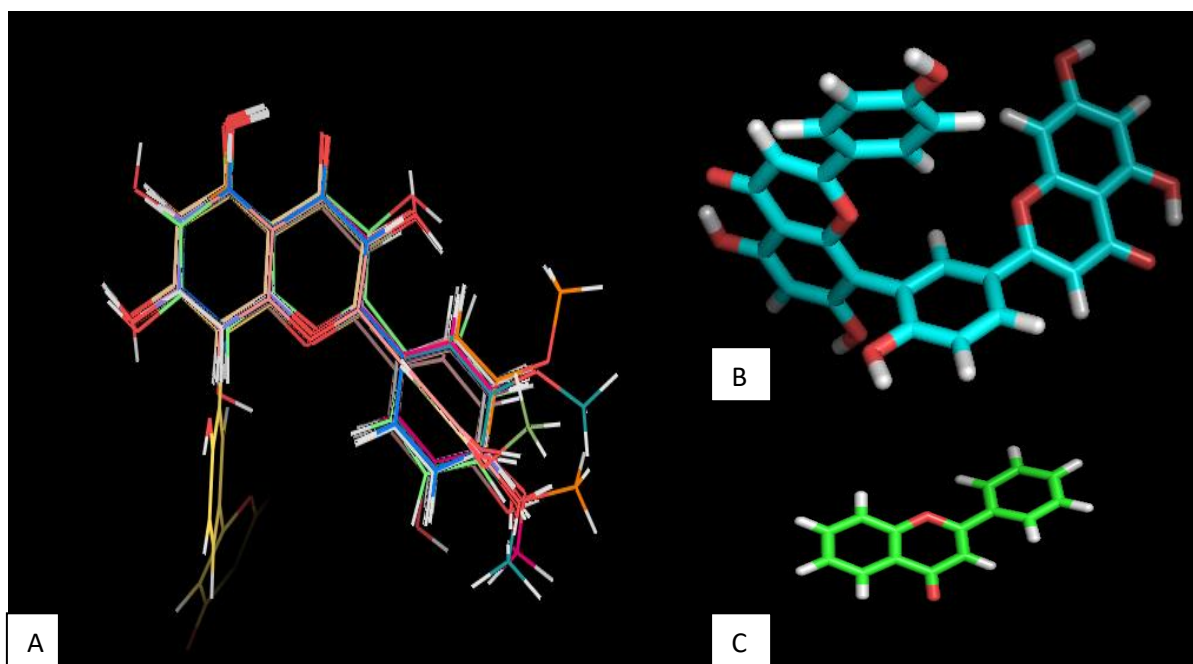


Fig. 1 (A) Aligned molecules of the series; (B) Reference Molecule for the alignment; (C) Template molecule for the alignment

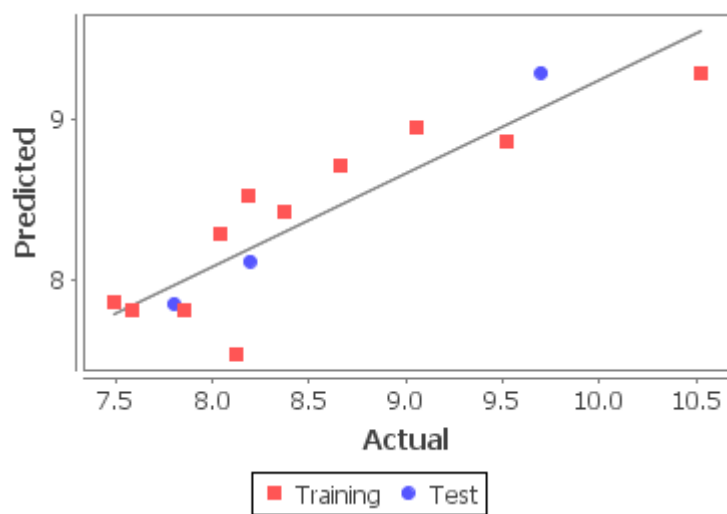


Fig. 2 Fitness plot for the training and test sets

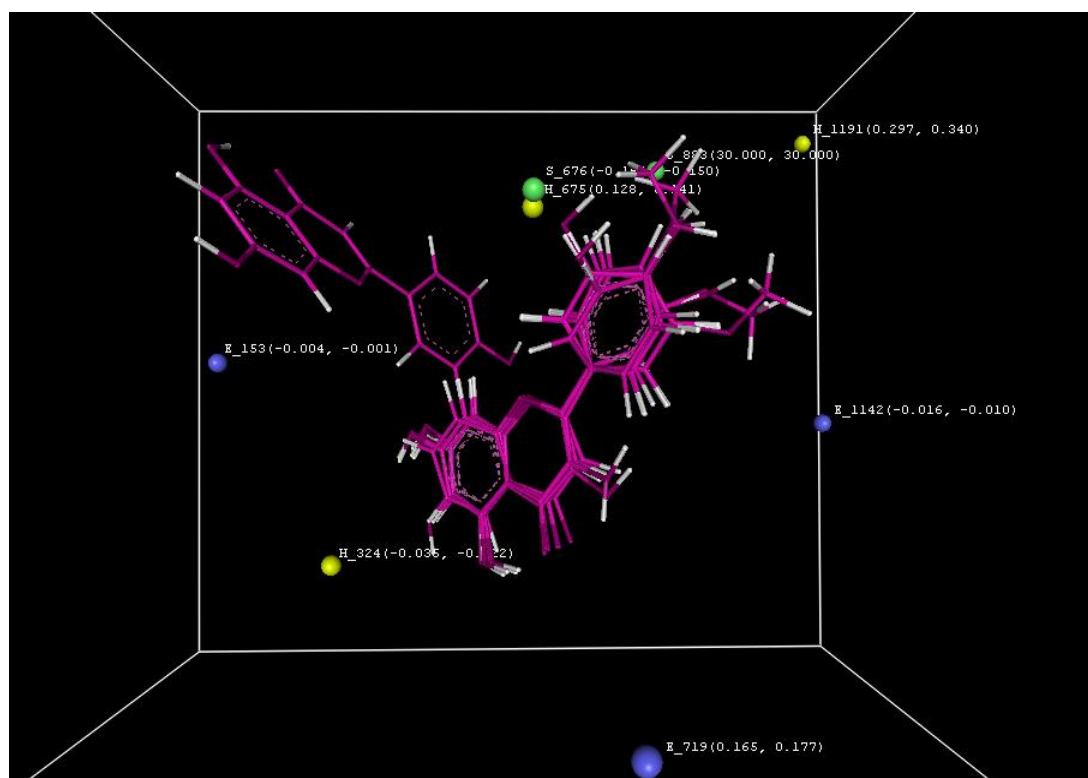


Fig. 3 3D-QSAR model (kNN-MFA plot) indicating relative position of descriptors by solid sphere

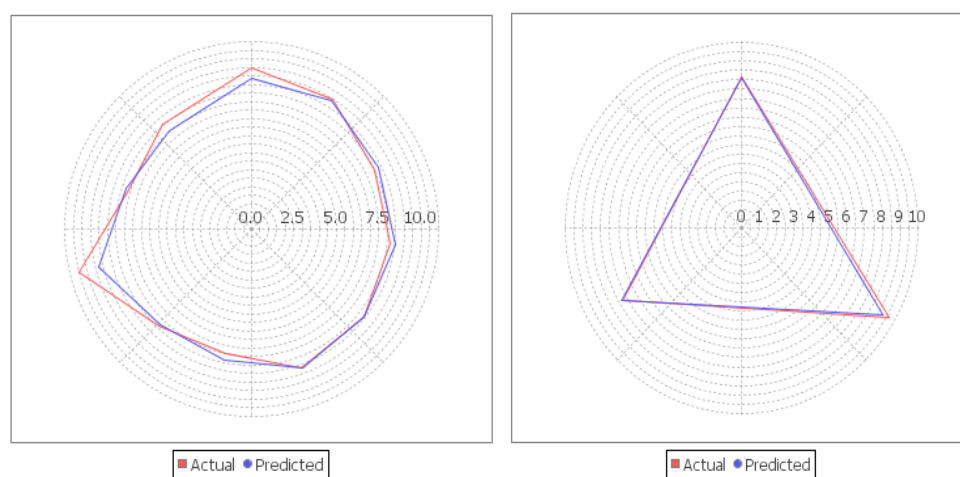


Fig. 4 Comparison of observed versus predicted activity for training & test set compounds according to 3D- QSAR model by kNN-MFA method

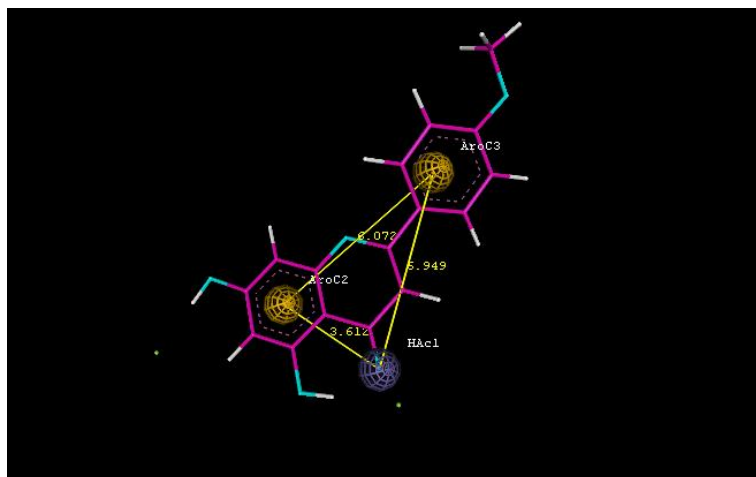


Fig. 5 Pharmacophore established for CYP2C9 for Flavonoid derivatives.

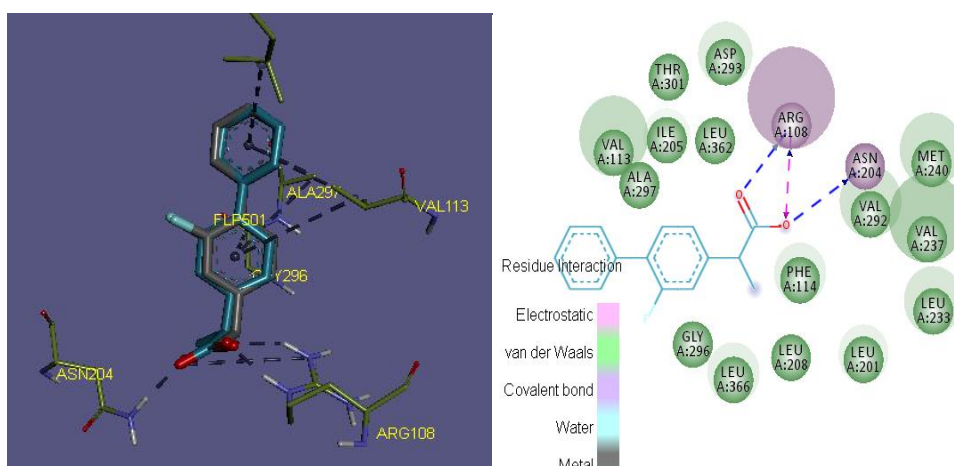


Fig. 6 Redocked structure of FLP (left side) and 2D diagram showing interaction with the surrounding residues of CYP2C9 protein (right side)

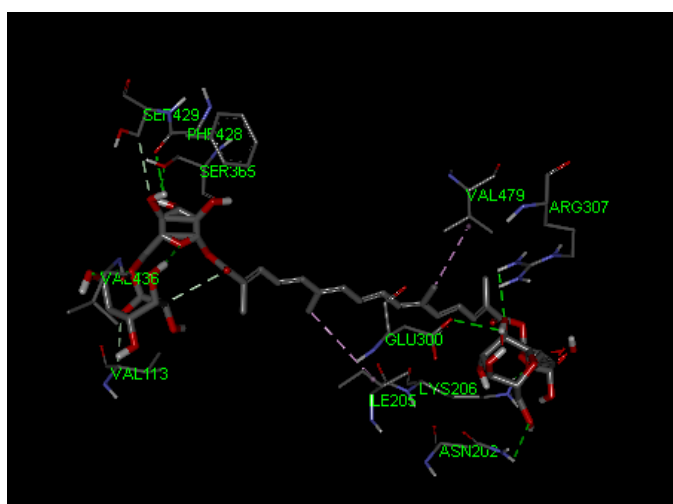


Fig. 7 Crocin docked in the active domain of CYP2C9 protein.