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Hibiscus anthocyanins can selectively modulate estrogen receptor activity with favorable toxicology: A computational analysis.

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Hibiscus anthocyanins can selectively modulate estrogen receptor activity with favorable toxicology: A computational analysis.

Abstract:

The estrogen hormone receptor (ER) mediated gene expression mainly regulate the development and physiology of primary and secondary reproductive system alongside bone-forming, metabolism and behaviour. Over-expressed ER is associated with several pathological conditions and play a key role in breast cancer occurrence, progression and metastasis. *Hibiscus sabdariffa* L. or roselle is a rich source of naturally occurring polyphenolic compounds including anthocyanins and reportedly have strong estrogenic activity. To validate these findings, we have investigated the estrogen receptor binding affinity and safety of some previously recorded polyphenols using a suite of computational methods. Our investigation showed the estrogen-receptor binding potential of Pelargonidin, Delphinidin, Cyanidin, and Hibiscetin are more efficient than popular breast cancer drugs, Tamoxifen and Raloxifene, with favourable toxicological parameters and low half maximal inhibitory concentration. This is the first report to investigate the phytochemical basis of estrogenic activity of *Hibiscus sabdariffa* L.

Keywords: In silico; Estrogen Receptor; Hibiscus sabdariffa L.; Phytochemical; Anthocyanin; SERM.

1. Introduction:

The mammalian hormone, Estrogen or its biologically active form Estradiol, is the major female steroid hormone that binds to its intracellular receptors known as estrogen receptors (ER) with high affinity and specificity that trigger multiple physiological functions[1][2]. The ER-mediated gene expression mainly regulates the development and physiology of primary and secondary reproductive system alongside bone-homeostasis, metabolism and behaviour[1][2]. The biological activity of estrogen is mainly mediated by its binding and activation of two nuclear receptor superfamily of transcription factors namely ERα and ERβ[1]. Although, ERα and ERβ are characterized by highly conserved DNA- and ligand-binding domains with 97% homology however their activation can produce both unique and overlapping effects[3][4]. Overexpressed estrogen receptor was reported in approximately 75% of the breast tumour which promotes the growth and metastasis of the breast cancer cells[3][5]. Also, mounting evidence demonstrates that overexpressed ER is also associated with lung, ovarian, prostate and endometrial carcinogenesis[3][6][7]. Besides, several ER-responsive genes including Insulin-like growth factor 1 (IGF-1), cyclin D1, c-myc, and the estrogen-responsive finger protein (efp), are important for proliferation and survival of cancer cells[3]. Thus, blocking or modulating estrogen receptor activity is considered as a crucial therapeutic approach for

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chemoprevention and diagnosis of ER-mediated carcinogenesis including mammary carcinogenesis[3]. Several small-molecule inhibitors or modulators of estrogen receptor known as selective estrogen receptor modulators (SERM) are currently used in the treatment of breast cancer[8][9]. Popular breast cancer chemopreventive agents like tamoxifen, raloxifene and toremifene are selective estrogen receptor modulator that binds to ER and acts as agonists or antagonists of estrogen to inhibit its proliferative actions on the mammary epithelium[8][9]. Although this is a favorable strategy to halt cancer progression however, the toxicity associated with the synthetic molecules limits their use in chemotherapy[10][11]. Thus, plant-derived ER agonists or antagonists received considerable attention because of their estrogen-like activity and selective low toxicity[12][13].

The plant, *Hibiscus sabdariffa* L. (HS) or roselle is a rich source of naturally-occurring phenolic compounds like alkaloids, flavonoids, anthocyanins, coumarins and phytosterols[14][15][16][17]. The alcoholic extracts of the plant showed mild to moderate estrogenic effects on the uteri of immature female rats and exhibited significant effects on the circulating levels Follicle-stimulating hormone (FSH), Prolactin, Estradiol and Testosterone in male Wistar rats[18][19][20]. Previous studies reported a strong inhibitory effect of its extracts on the growth and survival of breast cancer cells (MCF-7) and prostate cancer cells (LNCaP) however, the mechanism of inhibition was not well-depicted[21][22]. Although HS-extracts reportedly alters the cellular localization of the estrogen receptor, no significant progress has been made to understand the role of its phytochemicals underlying these activities[23]. Previously, we have recorded all the phytochemicals extracted from *Hibiscus sabdariffa* L. and reviewed their implication in breast cancer chemotherapy[24]. Based on our previous observation, we have selected twelve prominent phenolic compounds from *Hibiscus sabdariffa* L. (HSP) that could be a potential modulator of estrogen receptor activity. We approached computational techniques as it often used to complement experimental findings that help to prioritize the class of compounds to screen and dramatically reduces the quantity of compounds to screen. The experimental compounds include the five major anthocyanins (Delphinidin, Cyanidin, Malvidin, Peonidin, Pelargonidin), five phytosterols (β-sitosterol, Stigmasterol, α-Spinasterol, Campesterol, Ergosterol) and two other phenolic compounds (Protocatechuic acid and Hibiscetin) which were selected to evaluate their affinity and specificity towards binding and/or modulating estrogen receptor activity using computational docking analysis. Based on the result obtained from the protein-ligand docking studies, toxicology and drug-likeliness of the best-docked compounds were analysed and the 2D quantitative structure-activity relation (QSAR) analysis was performed for these compounds to predict their biological activity.

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2. Materials & Methods:

2.1 Preparation of Experimental Receptors (ERα) and Ligands (HSP):

The validated X-ray diffraction structure of human estrogen receptor alpha (ERα) was retrieved from the RCSB Protein Databank (www.rcsb.org) in .pdb format having PDB_ID: 1X7R (2.00 Å resolution). This particular protein structure was selected as it is already co-crystallized with one of the well-known plant-based ER-agonist, Genistein. The 2D/3D structures and the SMILE strings of twelve experimental molecules were retrieved from the NCBI PubChem (www.pubchem.ncbi.nlm.nih.gov) database[25]. The SMILE strings were converted to .MOL files using Open Babel 3.1.1, a freeware used for conversion of chemical formats[26]. Alongside, two already known SERM molecules: tamoxifen and raloxifene, and the biological ligand of ER, 17β-estradiol were considered as the control for this study. The stereochemical clashes of the compounds were removed by energy minimization to get optimized bond distance, bond angles and set dihedrals using Merck molecular force field (MMFF) in VLIfeMDS 4.6 software. The 3D structure of the target protein (ERα) was optimized using YASARA Energy Minimization Server (http://www.yasara.org/minimizationserver.htm)[27].

2.2 Molecular Docking Analysis:

The receptor-ligand molecular docking is a dynamic tool for visualizing and understanding the interaction between a receptor protein and its ligand. The molecular docking analysis was performed using the licensed version of the FlexX™ v2.1.6 included in LeadIT™ package software[28]. The active pocket was defined using the binding site of the known cocrystallized reference ligand by including the amino acid residues within a radius of 6.50Å using the protein preparation wizard of the FlexX LeadIT. Further, the reference ligand and crystal water molecules in the binding pocket were removed from the target protein, as evidently they did not play any role in the ligand-binding ability of ERα. The experimental ligands and the control ligands were then docked with the target proteins which is followed by an analysis of hydrogen bonding parameters. FlexX primarily uses incremental construction algorithm that considers the physicochemical features and variable flexibility of compounds while fitting it in the active site of the rigid target protein[29]. To cross-validate the FlexX results, re-docking was performed using a different docking algorithm in the SwissDock web-server (http://www.swissdock.ch/) that uses CHARMM-based software EADock-DSS[30]. The strength of the predicted crystallographic binding orientations is expressed in term of the energy function, where the lowest energy score is considered as the most stable conformation of the protein-ligand interaction[29]. For re-docking, energy-minimized ERα protein structure was loaded in the swiss-dock server and the ligands were selected from the ZINC database[31]. The SwissDock generates almost 500 docking poses for the protein-ligand complex, among which best-docked pose with lowest energy score was selected from the ViewDock plugin of UCSF Chimera[32].

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2.3 Prediction of ADME/Tox profile & Bioavailability:

The ADME (Adsorption, Distribution, Metabolism and Excretion) and toxicity profiles of the best four hits were performed using PreADMET (https://preadmet.bmdrc.kr), a web server from Yonsei University, Republic of Korea[33]. The PreADMET server uses *in vitro* results and considers several parameters for evaluating the intestinal absorption of potential drug candidates. Among them, Caco2 (human colon adenocarcinoma cells) model and MDCK (Madin-Darby canine kidney) cell model has been recommended as a reliable *in vitro* model for the prediction of oral drug absorption[34]. Besides, HIA (human intestinal absorption) data and skin permeability data can be useful in predicting and selecting between oral and/or transdermal drug delivery[35]. Moreover, skin permeability is a risk assessment parameter for all chemicals that may accidentally come into contact with skin[35]. The Plasma Protein Binding (PPB) data and Blood-Brain Barrier Penetration (BBB) data is useful to predict the availability of drug molecule to interact with its pharmacological target after diffusion or transport across cell membranes and the probability of interfering with the central nervous system[36]. PreADMET server uses a genetic functional approximation to select relevant 2D descriptors, followed by Resilient back-propagation (Rprop) neural network analysis to predict ADME data.

Besides several small molecules fails to attain a good drug-like property after confirming a promising ADME/Tox profile because of low bioavailability and poor pharmacokinetics. Thus, pharmacokinetic parameters like gastrointestinal absorption and brain access play a key role in determining bioavailability and brain-toxicity of molecules under investigation. The Brain or IntestinaL EstimateD permeation method (BOILED-Egg) is one such powerful predictive model developed by Daina A et al.[37] in 2016 which counts on the lipophilicity (n-octanol/water partition coefficient or WLOGP) and polarity (Topological Polar Surface Area or TPSA) of small molecules to accurately predict their passive gastrointestinal absorption and brain penetration. The BOILED-Egg predictive analysis was performed using SwissADME server (http://www.swissadme.ch/)[38]of the Swiss Institute of Bioinformatics. The required physicochemical descriptors were generated by providing the SMILE strings of the experimental molecules that translated into a graphical output of the model.

2.4 2D Quantitative Structure-Activity Relation (QSAR) studies:

Statistically-based Quantitative Structure-Activity Relation (QSAR) study is a powerful computational tool used in medicinal chemistry that helps to predict the biological activity of chemical compounds based on their structural features. For QSAR studies, 50 already known inhibitors of Er-α subtype and their corresponding inhibitory concentrations (IC_{50}) values (**Supplementary, Table 2**) were collected from BindingDB database (www.bindingdb.org)[39] managed by the University of California, USA. Compounds that showed ERα inhibition/modulation in cell-based assays involving ER+ human ductal

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breast cancer cells (MCF7) with plant product-inspired scaffolds and two to five fused ring skeletons were considered for QSAR model generation and validation. The IC_{50} values were converted to their respective negative logarithmic values to reduce their numerical difference, which were further used as the dependent variable for QSAR model development. The whole dataset was divided randomly into 'training set' molecules for model generation and 'test set' molecules for model validation (compounds marked in **Supplementary Table 2**). The random division of training set and test set molecules was based on a trial and error method that generated 50 2D-QSAR models and the process was repeated until a satisfactory r^2 value was attained for statistical significance. The 2D-QSAR analysis was performed using VLifeMDS 4.6[40] software suite that has multiple variable selection options and regressions methods developed by NovaLead Pharma. The builder module of the VLifeMDS drew the 3D structures of the 50 known molecules which were then energy minimized using the Merck molecular force field (MMFF). To create a 2D-QSAR model, 705 physicochemical and topological descriptors of the molecules were generated. After discarding invariable descriptors (values that are constant for all compounds), 125 physicochemical and 240 topological descriptors were considered. The multiple regression analysis was performed considering Log10(IC_{50}) as the dependable variable and the molecular descriptors as independent variables in a stepwise forward-backward variable selection protocol. Following the model building, the fitness plot for training and test set molecules along with the contribution of the individual descriptor for activity was analysed. The bioactivity of the experimental molecules was predicted based on the QSAR model using the Generic Prediction module of VLifeMDS.

3. Results:

3.1 Data set:

The target protein, estrogen receptor alpha (ERα) is one of the major therapeutic targets for breast cancer. The three-dimensional structure of the protein availed from the protein data bank and was energy-minimized using YASARA server. The initial energy of 630912 $kJMol^{-1}$ of ERα structure was optimized to -151332 $kJMol^{-1}$, which enhanced the post-docking results and the reliability of the LeadIT docking protocol. The stereochemical clashes of the twelve experimental ligands (**Table *1***) were minimized using the Merck molecular force field (MMFF) to get validated results especially in further studies like molecular docking simulation, toxicology prediction and 2D-QSAR analysis.

3.2 Molecular Docking Analysis:

Recently, a few reports[41][42][43] on the discovery of novel ERα antagonist/agonist using FlexX docking program have been published that reflects the efficiency of FlexX docking algorithm in studying the hormone-receptor interaction. The molecular docking simulation revealed that most of the experimental

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ligands exhibiting strong affinity towards the core cavity of ERα active site like estradiol with functional residues. In the top four evaluations, three anthocyanins (Pelargonidin, Delphinidin, Cyanidin) and Hibiscetin exhibited the most stable conformation (**Figure 2**) with ERα with ΔG binding energies -25.9934, -24.2893, -23.5567, -20.7123 kcal.mol^{-1} respectively. The energy values of these compounds are much lower than the control ligands Tamoxifen and Raloxifene (-16.4166 and -21.0154 kcal/mol^{-1} respectively), suggesting energy favourable bonding and higher stability. All these compounds framed three or more hydrogen bonds with the core cavity residues of ERα active site alongside the several weak hydrophobic interactions (Van der Waals forces). The total binding affinity score is an energy function which is expressed as a sum of the energy contributed by all matched interacting groups, the lipophilic contact area, ambiguous contact area, clash penalty, and the conformational entropy score of the ligand. The total ERα-binding energy scores of the best-matched poses and the contribution of different energy parameters are given in **Table *2***. A comparative 2D interaction map of best-matched compounds with the key active site residues of ERα is provided in the **Figure 3**. Among the anthocyanin ligands, Pelargonidin showed the most stable pose, forming three hydrogen bonds with active site residues Glu353, Leu387 and His524, with bond energies ranging from -4.7 to -3.9 Kcal.mol^{-1}. Besides, Pelargonidin also exhibited several weak Van der Waal interactions with active site residues. All the anthocyanins exhibited strong binding affinities towards ERα, which was more favourable than the control ligands in term of the energy values. A detailed hydrogen-bonding pattern and weak interactions of other best-matched anthocyanins are provided in **Table *3***. Among the flavonoid compounds, Hibiscetin expressed the most promising results by forming five H-bonds with Pro324, Glu353, Ile386 and, Trp393 residues of ERα active site, with bond energies ranging from -4.7 to -3.2 Kcal.mol^{-1}. The total energy score of Hibiscetin was higher than Raloxifen but lower than Tamoxifen, thus Hibiscetin was considered for further analysis. Although ERα is a steroid hormone receptor, surprisingly the five studied phytosterols (Campesterol, Ergosterol, Stigmasterol, α-Spinasterol, β-Sitosterol) did not show favourable interaction with ERα (**Supplementary, Table 1**). In the validatory re-docking step using SwissDock, similar results (-8.60, -8.58 and -8.8 Kcal.mol^{-1} respectively for pelargonidin, delphinidin and cyanidin) were observed as the anthocyanins exhibited much lower binding energy values than control ligands (-7.93 Kcal.mol^{-1} for Tamoxifen and -7.54 Kcal.mol^{-1} for Raloxifen)(**Table *3***). The 2D structures of the best matched compounds are shown in **Figure 1**.

3.3 ADME/Tox Profile and Bioavailability:

According to the ADME prediction of the best hits, *Pelargonidin* and *Cyanidin* were predicted to be well-absorbed by Human intestinal cavity with HIA values 83.58% and 72.5% (**Table *4***). However, *Delphinidin* and *Hibiscetin* showed moderate to low intestinal absorbance with HIA ranged between 20-65%. The Blood-Brain Barrier(BBB) Penetration of all four molecules was predicted to be < 1, which signifies CNS

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(Central nervous system) inactivity. These findings were again validated in BOILED-Egg Model for Gastrointestinal absorption and Brain penetration, which ensured similar results (**Figure 4**). However, according to BOILED-Egg Model prediction, *Hibiscetin* was not effluated by the P- glycoprotein pumping of the BBB which suggest the possibility of BBB penetration of the compound. The Caco2 (human colon adenocarcinoma cells) model for oral drug absorption prediction showed low permeability of all four molecules however, MDCK (Madin-Darby canine kidney) cell model indicated moderate oral permeability of the experimental molecules. The studied compounds alongside the control compounds showed high plasma protein binding potential with PPB values >90% (**Table *4***). The rate of skin permeability of the molecules expressed in term of Human skin permeability coefficient ($LogK_p$) whose value generally ranges between -3 to +6 for good absorption via the skin, showed low skin permeability of all the studied molecules.

3.4 Quantitative Structure-Activity Relation (2D-QSAR) Model & Bioactivity prediction:

In the present study, 50 2D-QSAR models were generated for Log10(IC_{50}) or Bioactivity of 50 already known ERα agonist/antagonist compounds involving total 365 physicochemical and alignment independent topological descriptors by randomization of training and test set data. Among these models, one with the best statistical parametres was selected for further analysis.To express the statistical model in a 2D-QSAR equation, most effective descriptors were selected in a stepwise forward-backward multiple linear regression analysis. As we considered only 50 molecules for generating the QSAR model, only 5 descriptors were included in the final equation by following the N/5 rule and the predicted bioactivity was Log10(IC_{50}) expressed in nM. The regression curve and the contribution plot of the different descriptor is shown in **Figure 5** and **Figure 6** respectively. The resulting regression equation and statistics are shown below:

$$\boldsymbol{Log10(IC_{50})} = -10.8097(\pm 1.1508)\,\boldsymbol{ChiV6chain} - 0.6193(\pm 0.0265)\boldsymbol{T\_O\_S\_6} + 0.7425\,(\pm 0.0265)\boldsymbol{T\_O\_O\_7} - 0.1557(\pm 0.0036)\boldsymbol{SaaaCcount} + 0.2434(\pm 0.0885)\boldsymbol{T\_O\_Cl\_5} - 0.0251(\boldsymbol{Constant})$$

Statistics: N=46, Degree of freedom=40, r^2=0.7619, q^2=2.0606, F_test=25.5933, pred_r^2=0.4774

Descriptor Definitions:

ChiV6Chain= Atomic valence connectivity index for six-membered ring.

T_O_S_6 = The count of the number of oxygen atoms (Single, double or triple bonded) separated from a Sulphur atom by 6 bond distance in a molecule.

T_O_O_7 = The count of the number of oxygen atoms (Single, double or triple bonded) separated from other oxygen atoms by 7 bond distance in a molecule.

SaaaCcouunt = The total number of carbon connected with three aromatic bonds.

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T_O_Cl_5 = The count of the number of oxygen atoms (Single, double or triple bonded) separated from chlorine atoms by 5 bond distance in a molecule.

Based on this model, the Log10(IC_{50}) values (**Table *5***) of the studied compounds were generated using the Generic Prediction module of VLifeMDS. The predicted bioactivity data indicated Pelargonidin as the most potent ER-inhibitor with Log10(IC_{50}) value of 0.775 nM. The bioactivity data of the other three compounds Delphinidin, Cyanidin and Hibiscetin were found to be 0.85, 0.81 and 2.39 nM respectively.

4. Discussion:

The medicinal plant, *Hibiscus sabdariffa* Linn. or roselle gained much attention in the recent years for its robust antioxidant activity and disease-healing action against several diseases including many types of human cancers. The medicinal activity of this plant is principally linked to its rice polyphenolic constituents like the anthocyanins, flavonoids, phenolic acids and phytosterols. Beside exerting estrogen-like effects on the uteri of female rats, extracts of *Hibiscus sabdariffa*(HS) exhibited several other therapeutic impressions as an antimicrobial, antihypertensive, antidiabetic and anticancer agent. Among the several compounds of *Hibiscus sabdariffa*, ER-binding affinity of its polyphenols like Daidzein, Genistein, Kaempferol, Myricetin and Quercetin is well-known. However, the anthocyanins remain under-explored for their ER-binding potential although they form a significant portion of its phytochemical constituent. Consequently, the present study was aimed to investigate the ER-binding affinity and selectivity of HS-anthocyanins along with other less-explored polyphenols to identify and optimize new ER-agonist/antagonist, clinically known as SERM molecules/drugs. As the estrogen receptor is a steroid hormone receptor, all the previously reported phytosterols of this plant were also included in this study to examine their ER-binding ability. The ER-alpha model (1X7R) used in this study was employed in similar previous studies[44][45] that investigated plant-based ER agonists/antagonists that imply the efficacy of this model.

Molecular docking simulation (MDS) is a successful method to predict binding characteristics and affinities of small ligands to biologically relevant target proteins. FlexX docking algorithm was employed in the present study to analyse the ER-binding potential of HS-polyphenols, the control ligands (Tamoxifen and Raloxifene) and the natural ligand of ER, 17B-Estradiol. The results were cross-validated using a different docking algorithm, EADock-DSS. MDS studies revealed that ER-binding activity of the three anthocyanins pelargonidin, cyanidin and delphinidin is more energy favourable than that of popular ER-agonist/antagonist drug tamoxifen and raloxifene, with the precision of natural ER-ligand, 17β-Estradiol. The anthocyanins pelargonidin, delphinidin and cyanidin occupied the same position in the active site of ERα as Estradiol and also conserves many key contacts (Glu353 and Gly521) as that of 17β-Estradiol. The binding site of this anthocyanins is also identical to the co-crystallized plant-based ligand, genistein. The

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key residues in the binding pocket included Glu353, Leu387, His524 and Gly521. Importantly, tamoxifen which is chosen as control ligand for this study also exhibited strong hydrogen bonding with Glu353/Arg394 with bond energies -7.6/-0.7 Kcal.mol^{-1} and distance of 2.13/1.91Å respectively. The binding pocket residues Glu353, Arg394 and Gly521 are also essential for ER-binding of 17β-Estradiol. Alongside several hydrophobic interactions of these anthocyanins were also observed with active site residues: Ala350, Phe404, Leu391, Leu387, Gly521, Leu525, Leu384, Ile424, Leu346, and Met388. Beside the anthocyanins, two other compound, Hibiscetin and protocatechuic acid were among the top five observation for its strong affinity towards ER-active site. The cross-validation of the binding energy parameters yielded similar results that signify better ER-binding ability of the anthocyanins when compared to the control ligands. The difference in total energy score of the validation step might be a result of the algorithmic difference between FlexX and EADock-DSS. Thus, molecular docking study revealed a similar binding property of the anthocyanins and the controls with ERα receptor. Finally, the top four observation were subjected to toxicological studies and bioactivity prediction.

Recently; the toxicology prediction server PreADMET was used in several published reports[46][47][48] that suggests the reliability of the server. Beside, these in silico toxicology and bioavaibility analysis methods are now widely accepted by pharmaceutical industries to support various decision-making in drug development process[49]. The toxicological analysis predicted that Pelargonidin and Cyanidin will be well-absorbed by the human intestine (HIA), which is a prerequisite for an orally administered potential drug candidate[49]. Other two experimental ligands Delphinidn and Hibiscetin showed moderate and low intestinal absorption, which may be improved by further structural modifications of the ligands or utilizing a delivery vehicle. The Caco2 cell permeability of all the experimental compounds was predicted to be very low compared to the control ligands suggesting a slow rate of flux of these compound across polarized human colon epithelial cancer cell (Caco-2) monolayers. However, pelargonidin, cyanidin and delphinidin showed moderate rate of P-glycoprotein efflux across Madin-Darby canine kidney (MDCK) cells, which is used as a Caco-2 alternate technique to predict intestinal permeability and oral absorption of investigational compounds. The MDCK parameters of the experimental ligands were similar to the control ligands. In addition, all the compounds and the control ligands were predicted to bind blood plasma protein strongly, which may hinder their efficiency in transport and distribution through blood stream. The high plasma protein binding nature of the molecules can be enhanced by functional group modifications as suitable to improve their effectiveness in transport and distribution. Lastly, pelargonidin, delphinidin and cyanidin were predicted to be CNS-inactive in both PreADMET and BOILED-Egg analysis indicating low risk of affecting normal CNS activity. These results clearly supports the findings of Pojer E at al.(2013)[50], where they documented the encouraging pharmacokinetic property like rapid absorption, distribution, metabolism and excretion of these anthocyanins. Beside several reports

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also claimed that anthocyanins and their glycosides do not show any toxic signs with doses as high as 100 mg/kg body weight in rodent models[51][52]. With acceptable toxicological data, these compounds were subjected to a 2D-QSAR analysis to predict their half maximal inhibitory concentration while binding ERα.

The QSAR techniques is based on the popular assumption that structural variation can effect changes in biological activity. The present model was developed using the bioactivity of 50 known ERα inhibitors (dependable variable) and their corresponding physicochemical descriptors (independent variables). The training and test set data were divided randomly untill a statistically satisfactory model was attained. Based on the resultant regression equation, the bioactivities of the experimental ligands were generated. The marginal difference between the actual and the predicted activity (Log10IC50) of the training and test set molecules alongside the acceptable cross-validation (q^2) and coefficient of determination (r^2) value indicates an admissible statistical model. The cross-validation (LOO) regression (q2) of 2.0606 signifies less to moderate statistical noise in the training set compounds. The regression coefficient for the test set (pred_r2) molecules was found to be 0.4774. The high value of 25.5933 for Fisher's test (F_test) signifies the validity of the model. Besides, the even distribution of the molecules around the axis line indicated acceptable model quality. The resulting regression equation shows the five important descriptors that determine the ERα inhibitory or modulatory potential of designated SERM or ERα inhibitory molecules according to this QSAR model. Among the extracted descriptors, *ChiV6Chain* and *SaaaCcouunt* are physicochemical descriptors that contributed inversely to the biological activity which suggests that increasing the value of these descriptors might not be favourable for potent activity. Other descriptors (*T_O_S_6*, *T_O_O_7* and *T_O_Cl_5*) are Alignment Independent (AI) Topological descriptors class, among which *T_O_S_6* showed an inverse relation with the predicted biological activity. The rest topological descriptors (*T_O_O_7* and *T_O_Cl_5*) contributed 24% and 8% to the biological activity Log10(IC_{50}). The predicted IC_{50} values of the experimental molecules suggest that maximum contribution hails from the descriptors *ChiV6Chain* and *T_O_O_7*. The 2D-QSAR analysis revealed that all the three anthocyanins: Pelargonidin, delphinidin and cyanidin could inhibit/limit ERα activity with Log10(IC_{50}) values as low as 0.77, 0.85 and 0.81 nM respectively. Thus, suggesting these compounds must be investigated in proper laboratory setup to validate these findings.

5. Conclusion:

The present study was designed to investigate the ERα binding property of *Hibiscus sabdariffa* polyphenols, largely the anthocyanis and to assess their toxicological risk. Accordingly, molecular docking study was performed to evaluate their potential to bind ERα active site (identical to the binding site of 17β-Estradiol). All the anthocyanins formed comparatively stable conformations with ERα active site when compared to the control ligands, tamoxifen and raloxifen. These results can be further validated using a molecular

dynamic simulation analysis. Further, with favorable toxicological parameters and potent bioactivity, all the anthocyanins exhibited strong affinity towards ERα, which supports the claim of estrogen-like activity of the plant extracts in MCF7 cell lines and in animal models. Among the anthocyanins, pelargonidin was the prominent find that satisfied all the in-silico analysis with promising outcome. These results will help in designing lead for novel ERα-agonists/antagonists as potential anticancer agents. Lastly, the ERα modulatory activity of these compounds must be validated in proper clinical or cell-based studies.

6. Declarations:

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Table 1: Details of the studied compounds from Hibiscus sabdariffa L.

Compound	Class	SMILE string
Campesterol	Phytosterol	CC(C)C(C)CCC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C
Cyanidin	Anthocyanin	C1=CC(=C(C=C1C2=[O+]C3=CC(=CC(=C3C=C2O)O)O)O)O
Delphinidin	Anthocyanin	C1=C(C=C(C(=C1O)O)O)C2=[O+]C3=CC(=CC(=C3C=C2O)O)O.[Cl-]
Ergosterol	Phytosterol	CC(C)C(C)C=CC(C)C1CCC2C1(CCC3C2=CC=C4C3(CCC(C4)O)C)C
Hibiscetin	Hexahydroxyflavone	C1=C(C=C(C(=C1O)O)O)C2=C(C(=O)C3=C(O2)C(=C(C=C3O)O)O)O
Malvidin	Anthocyanin	COC1=CC(=CC(=C1O)OC)C2=[O+]C3=CC(=CC(=C3C=C2O)O)O
Pelargonidin	Anthocyanin	C1=CC(=CC=C1C2=[O+]C3=CC(=CC(=C3C=C2O)O)O)O
Peonidin	Anthocyanin	COC1=C(C=CC(=C1)C2=[O+]C3=CC(=CC(=C3C=C2O)O)O)O
Protocatechuic acid	Phenolic acid	C1=CC(=C(C=C1C(=O)O)O)O
Stigmasterol	Phytosterol	CCC(C=CC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C)C(C)C
α-Spinasterol	Phytosterol	CCC(C=CC(C)C1CCC2C1(CCC3C2=CCC4C3(CCC(C4)O)C)C)C(C)C
β-Sitosterol	Phytosterol	CCC(CCC(C)C1CCC2C1(CCC3C2CC=C4C3(CCC(C4)O)C)C)C(C)C

Table 2: Binding Energy profiles ($KcalMOl^{-1}$) of the best-hit compounds with Estrogen Receptor Alpha (ERα), 1X7R.

Compound	Total Score	Match Score[a]	Lipo[b]	Ambig[c]	Clash Score[d]	Rot Score[e]
Tamoxifen*	-16.4166	-19.4344	-8.9994	-4.9787	4.5960	7.0000
Raloxifene*	-21.0154	-21.1289	-12.2253	-7.5781	6.1169	8.4000
17β-Estradiol*	-16.9891	-15.2275	-10.7578	-3.3068	4.1030	2.8000
Pelargonidin	-25.9934	-22.3911	-11.0262	-6.1717	2.5956	5.6000
Delphinidin	-24.2893	-26.6605	-9.3952	-6.7201	4.6865	8.4000
Cyanidin	-23.5567	-22.0282	-10.9578	-6.4470	3.4764	7.0000
Hibiscetin	-20.7123	-25.0502	-7.2092	-8.8197	5.1669	9.8000
Protocatechuic acid	-17.0909	-17.7002	-5.1839	-5.7688	3.3619	2.8000
Malvidin	-16.0482	-18.8200	-8.5376	-6.5417	4.0511	8.4000
Myricetin	-15.9777	-19.1104	-7.0987	-6.6373	3.0686	8.4000
Peonidin	-15.7434	-18.6875	-6.9084	-6.3393	3.7919	7.0000

*Control Ligands; a= Contribution of the matched interacting groups; b= Contribution of the lipophilic contact area; c= Contribution of the lipophilic-hydrophilic (ambiguous) contact area; d= Contribution of the clash penalty; e= Ligand conformational entropy score.

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Table 3: Hydrogen bonding patterns and other weak interactions of the best- docked compounds with Estrogen Receptor Alpha(ERα), 1X7R.

Compound	FlexX				SwissDock
	Hydrogen Bonding Pattern			Other weakly interacting residues	Bond Energy (Kcal mol^{-1})
	H-Bonds	Bond Energy (Kcal mol^{-1})	Bond Length (Å)		
***17β-Estradiol**	OE1 GLU-353-A-H44 HH21 ARG-394-A-O20 O GLY-521-A-H36	-4.7 -2.4 -4.4	1.79 2.24 2.22	Ile424, Leu391, Met388, Phe404, Leu384, Met421, Met343, Leu525, Ala350, Thr347, Leu387, Leu346	-6.72
***Tamoxifen**	OE1 GLU-353-A-H58 HH12 ARG-394-A-O17	-7.6 -0.7	2.13 1.91	Glu323, Pro324, Ile326, Trp393, Arg394, Phe445	-7.93
Pelargonidin	OE1 GLU-353-A-H29 O LEU-387-A-H30 ND1 HIS-524-A-H31	-4.7 -4.7 -3.9	1.63 1.61 1.96	Ala350, Phe404, Leu391, Leu387, Gly521, Leu525, Leu384, Ile424, Leu346, Met388	-8.60
Delphinidin	OE1 GLU-353-A-H32 O LEU-387-A-H33 O GLY-521-A-H26 O GLY-521-A-H27 ND1 HIS-524-A-H25	-4.7 -4.7 -4.7 -2.4 -1.9	1.59 1.82 1.61 1.42 2.44	Leu346, Leu391, Met388, Phe404, Leu387, Leu525, Leu384, Ala350	-8.58
Cyanidin	OE1 GLU-353-A-H29 O LEU-387-A-H30 O GLY-521-A-H31 O GLY-521-A-H32	-4.6 -4.6 -2.3 -4.7	1.55 1.59 1.40 2.05	Ala350, Leu346, Leu387, Leu391, Phe404, Leu384, Met388, Leu525	-8.80
Hibiscetin	O PRO-324-A-H34 O GLU-353-A-H31 O ILE-386-A-H32 O ILE-386-A-H33 HE1 TRP-393-A-O8	-4.5 -4.5 -3.2 -4.7 -3.6	1.79 2.21 1.97 2.07 2.16	Trp393, Ile326, Leu387, Pro324, Ile386, Glu353	-7.67

Table 4: Predicted ADME properties of top four compounds.

Compound	HIA (%)	$Caco_2$ (nm/sec)	MDCK (nm/sec)	PPB (%)	BBB (C_{brain}/ C_{blood})	Skin Permeability
Tamoxifen*	100	49.54	69.84	94.74	14.16	-1.42202
Raloxifene*	96.20	32.67	44.13	100	0.71	-2.51994
Pelargonidin	83.58	1.29	59.76	100	0.53	-3.99618
Delphinidin	65.42	2.60	43.45	88.41	0.05	-4.84783
Cyanidin	72.50	0.65	83.10	100	0.31	-4.13468
Hibiscetin	20.97	3.66	2.77	99.32	0.07	-4.59926

* Control Ligands

HIA= Human Intestinal Absorption, Well absorbed compounds 70-100%, Moderate 20-70% and weakly absorbed <20%.

Caco2= Caco2 cell permeability, Highly Permeability >70 nm/sec, Middle 4-70 nm/sec and low permeable < 4 nm/sec

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MDCK= MDCK cell permeability, High Permeability >500 nm/sec, Middle 20-500 nm/sec and low permeability < 25 nm/sec

PPB= Plasma Protein Binding, Strongly bound chemicals >90% and weakly bound chemicals < 90%

BBB= Blood-Brain Barrier Penetration, CNS active compounds BBB >1 and inactive compounds BBB < 1

Skin Permeability= Expressed as $LogK_p$ or Human skin permeability coefficients, values ranging from -3 to +6, which provides the compound with good absorption.

Table 5: Predicted Bioactivity of the studied compounds.

Compounds	**ChiV6chain**	**T_O_S_6**	**T_O_O_7**	**SaaaCcount**	**T_O_Cl_5**	**Predicted Log10(IC_{50})**	**Extrapolation**
Pelargonidin	0.0633425	0	2	0	0	0.77591	0.71606
Delphinidin	0.0563981	0	2	0	0	0.850867	0.641103
Cyanidin	0.059621	0	2	0	0	0.81608	0.67589
Hibiscetin	0.0516333	0	4	0	0	2.3917	2.07916

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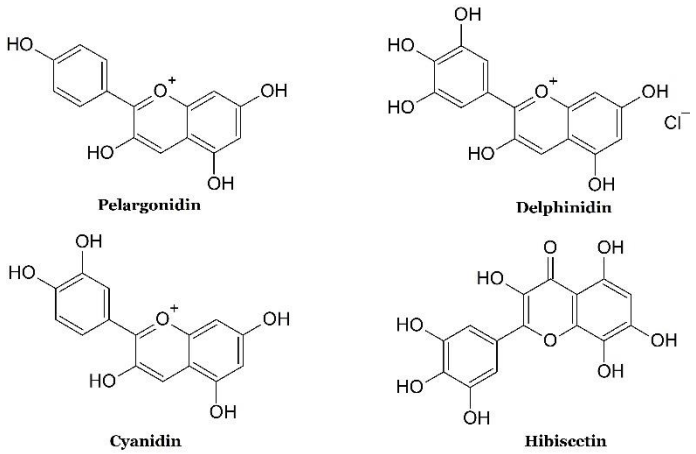

Figure 1: Structure of the four best hit ligands.

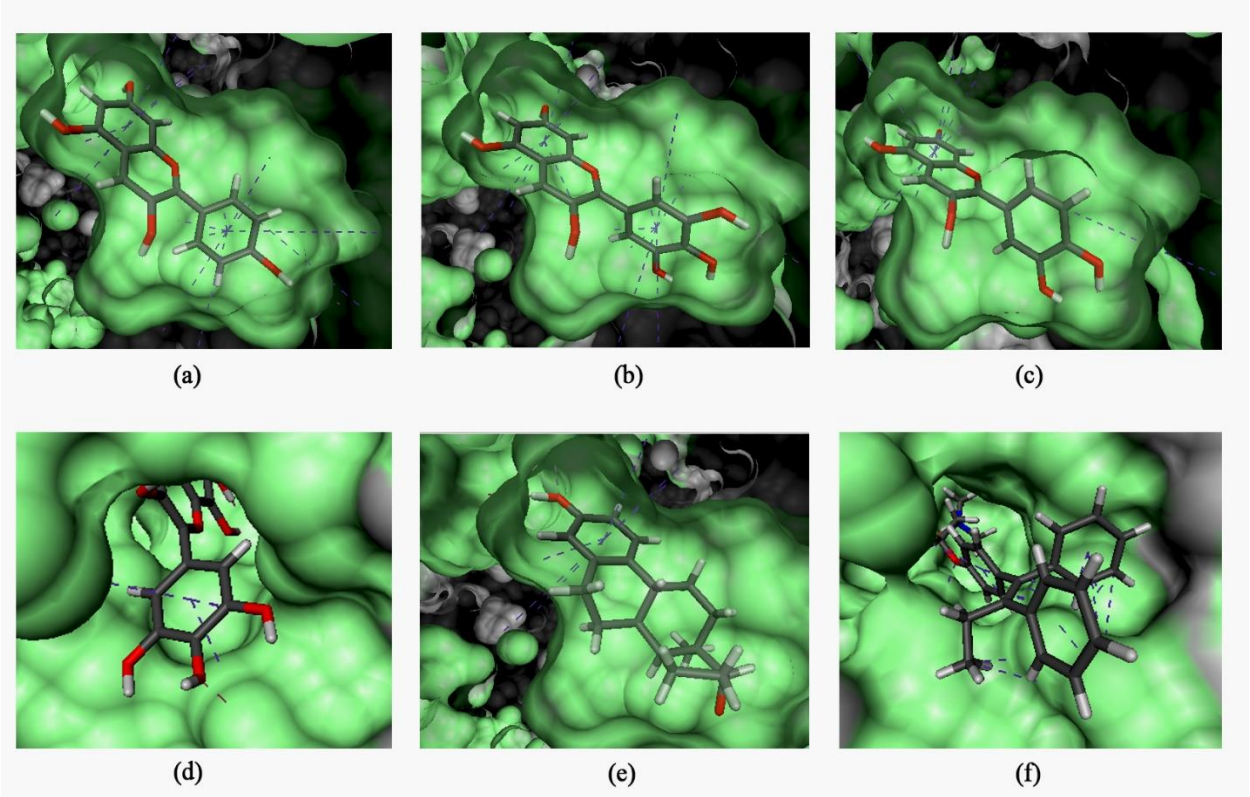

Figure 2: Docking poses of the best-docked compounds and the control in the active site of ERα: (a) Pelargonidin (b) Delphinidin (c) Cyanidin (d) Hibiscetin (e) 17β_Estradiol* (f) Tamoxifen*.

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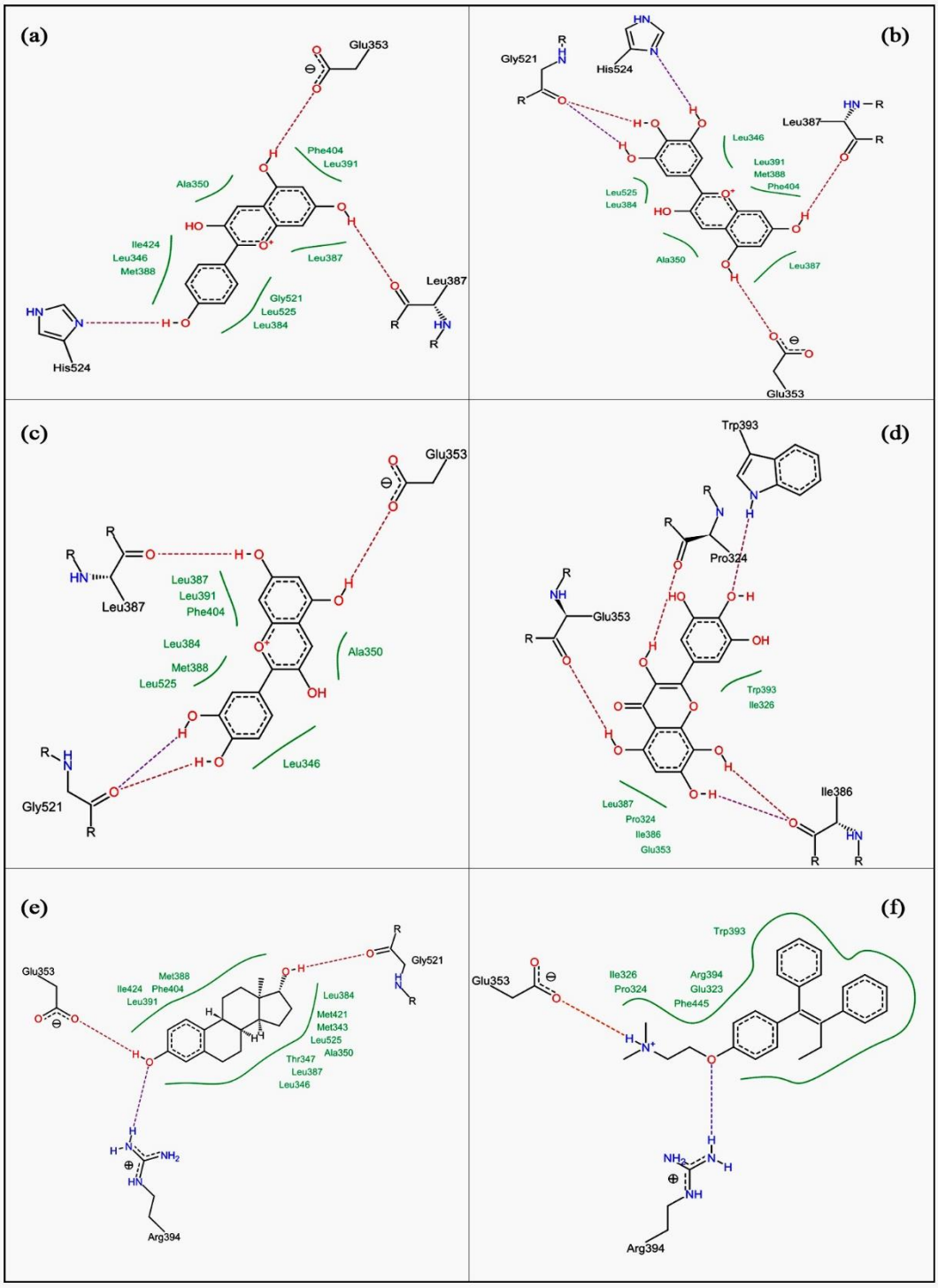

Figure 3: A comparative 2D interaction maps of the dock poses of the best-docked compounds with the active site residues of the ERα : (a) Pelargonidin (b) Delphinidin (c) Cyanidin (d) Hibiscetin (e) 17β_Estradiol* (f) Tamoxifen*.

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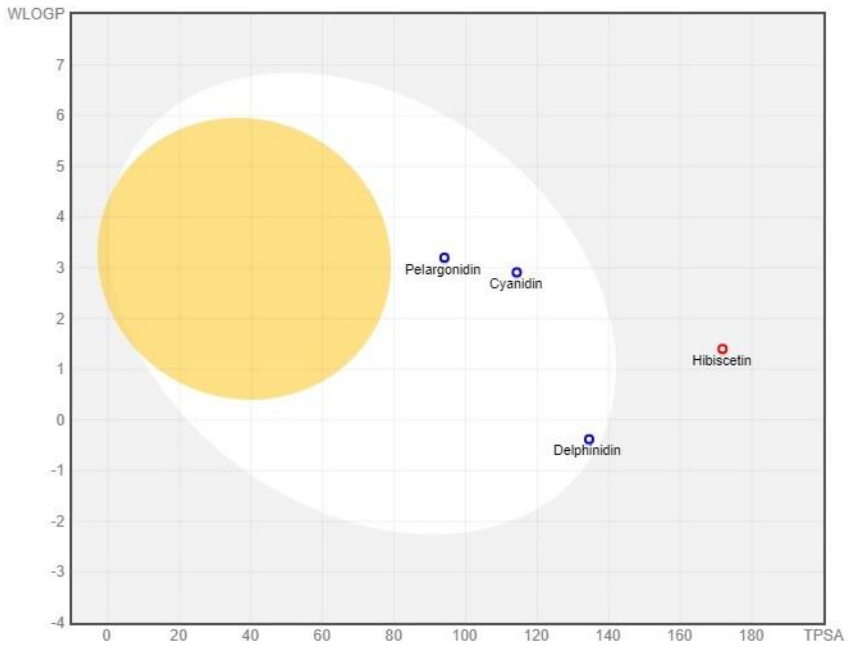

Figure 4: BOILED-Egg Model for Gastrointestinal absorption and Brain penetration: Experimental molecules located in BOILED-Egg's yolk are predicted to passively permeate through the Blood-brain barrier (BBB). Molecules located in the BOILED-Egg's white are predicted to be passively absorbed by the gastrointestinal tract. Blue-dotted molecules are predicted to be effluated from the Central Nervous System (CNS) by the p-glycoproteins and Red-dotted molecules are not effluated.

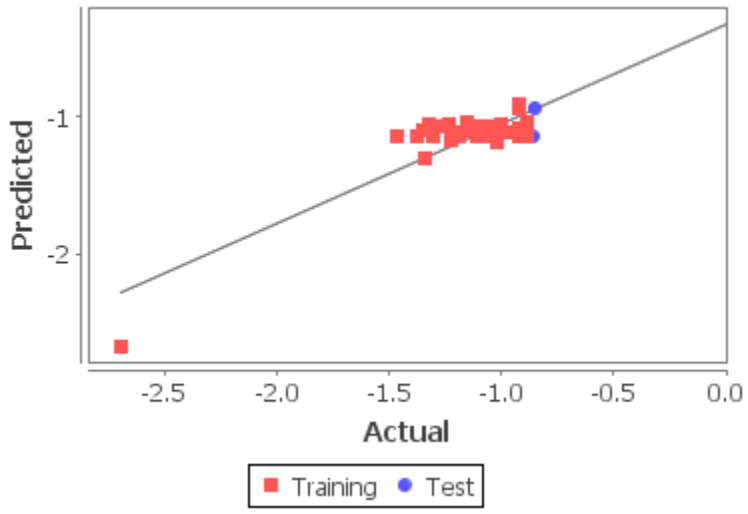

Figure 5: Correlation plot of observed activity and predicted activity of the training and test data set to generate a 2D-QSAR Model.

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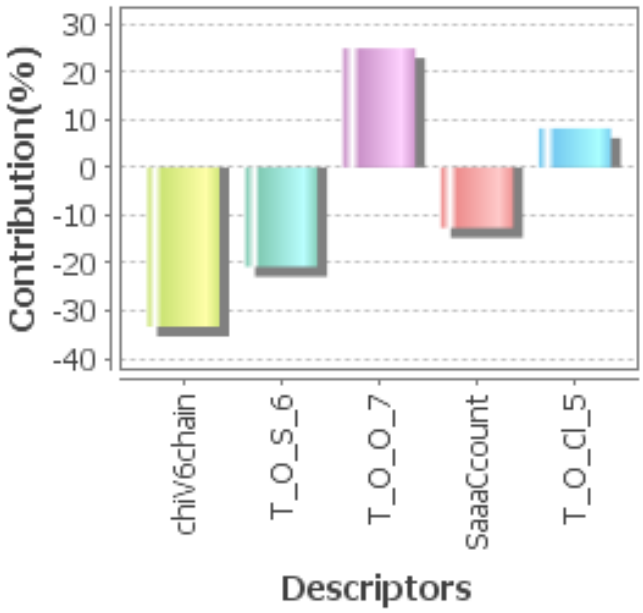

Figure 6: Contribution plot for descriptors in the QSAR equation.